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"Research is to see what everybody has seen and think what nobody has thought."

Albert Szent-Györgyi (1893-1986)

Received the Nobel Prize for discovering vitamin C

I dedicate the fruit of these long years of study

To my parents,

The reason of what I turned into today.

Thank you for your great support and continuous care.

To my brother and sister,

I am really grateful to both of you.

You have been my soul mates and my inspiration.

I love you all dearly!

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Christelle EL HAJJ ASSAF

List of Publications and Scientific Communications

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- **El Hajj Assaf, C.,** De Clercq, N., Vlaemynck, G., Van Coillie, E., Puel, O., Van Pamel, E. (XXXX) Fate of patulin in apple juices with special emphasis on the impact of ascorbic acid. Mycotoxin research journal (Submitted review)

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Oral communications

- **Christelle El Hajj Assaf**, Nikki De Clercq, Geertrui Vlaemynck, Els Van Coillie, Els Van Pamel. Influence of ascorbic acid on patulin concentration in cloudy apple juice. **40th Mycotoxin Workshop**, June 11th-13th 2018, Munich, Germany. (Oral)

- **Christelle El Hajj Assaf**, Sylviane Bailly, Isabelle Oswald, Sophie Lorber, Olivier Puel, Els Van Pamel, Els Van Coillie, Geertrui Vlaemynck. Patulin, main mycotoxin in apple products: Elucidation of mechanisms involved in its biosynthetic pathway and development of preventive approaches. **PhD challenge**. ILVO (Melle, Belgium), November 21st, 2017. (Oral)

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Poster

- **Christelle El Hajj Assaf**, Nikki De Clercq, Christof Van Poucke, Els Van Pamel. Optimization of the methodology for the detection/quantification of patulin and breakdown products. **40th Mycotoxin Workshop**, June 11th-13th 2018, Munich, Germany.

List of abreviations

A	
a*	CIELAB (1976) colour space coordinate representing the red/green component
A. flavus	*Aspergillus flavus*
AA	Ascorbic acid
AcA	Acetic acid
ACN	Acetonitrile
AcOEt	Ethyl acetate
AFB1, AFB2, AFG1, AFG2 and AFM1	Aflatoxins
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
APAM	Apple-based apple puree agar medium
ASC-E	Ascladiol-E
ASC-E/Z	Ascladiol-(E/Z)
ASC-Z	Ascladiol-Z
ATA	Trichothecene Toxic Food Aleucie
ATP	Adenosine triphosphate
a_w	Water activity
α	Alpha
B	
b*	CIELAB (1976) colour space coordinate representing the yellow/blue component
b-Tubulin	beta-Tubulin
bw/day	Body weight per day
β	Beta
C	
CE	Capillary Electrophoresis
°C	Celsius degree
C*	CIELCH colour space coordinate representing the chroma or colour purity

C.E.H.A.	Christelle El Hajj Assaf
$C_6H_8O_6$	Ascorbic acid (vitamin C)
CAJ	Cloudy apple juice
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCP	Critical control points
cDNA	Complementary Deoxyribonucleic acid
CGA	Chlorogenic acid
CGA	Czapek glucose agar
CIT	Citrinin
cm/day	centimetre per day
Co-60	Cobalt-60
cond (1, 2, 3)	Condition number
CY	Czapek yeast extract liquid medium
CYA	Czapek yeast extract agar
CzA	Czapek dox agar
D	
DHA	dehydroascorbic acid
DKGA	2,3-diketogulonic acid
DMATS	Dimethyl-allyltryptophan synthase
dNTP	Deoxynucleoside triphosphate
DON	Deoxynivalenol
DPA	Desoxypatulinic acid
dpi	Days post-inoculation
Δa*	Difference in redness
Δb*	Difference in yellowness
ΔC*	Chroma difference
ΔE*	Extent of colour difference
ΔL*	Difference in lightness
E	
e.g.	For example (*exempli gratia*)
EC	European Commission

EFSA	European Food Safety Authority
ESI	Electrospray ionization
et al.	*Et alii*
etc.	*Et cetera*
ETPs	Epipolythiodioxopiperazines
EU	European Union
F	
FAO	Food and Agriculture Organization
FB1, FB2	Fumonisins
Fig.	Figure
G	
GAP	Good agricultural practices
GC-MS	Gas Chromatography Mass Spectrometry
GMP	Good manufacturing practices
GLA	D-Gluconic acid
GOX2	Glucose oxidase
γ	Gamma
H	
HHP	High hydrostatic pressure
HACCP	Hazard Analysis Critical Control Points system
HMF	5-hydroxymethylfurfural
HOG	High osmolarity glycerol
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High-performance liquid chromatography with a diode array detector
HPLC-MS	Liquid chromatography coupled with mass spectrometry
HPLC-MS/MS	Tandem Mass Spectrometry
HPLC-UV	High Performance Liquid Chromatography coupled to UltraViolet detection
I	
IARC	International Agency for Research on Cancer
i.e.	*Id est*

ILVO	Flanders Research Institute for Agricultural, Fisheries and Food
J	
JECFA	Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives
K	
KCl	Potassium chloride
L	
L*	CIELAB (1976) colour space coordinate representing the luminance component
L-AA	L-ascorbic acid
LAB	Lactic acid bacteria
LC-MS	Liquid Chromatography - MASS Spectrometry
M	
mg/kg	Milligrams to kilograms
µg/kg	Microgram per kilogram
1-MCP	1-methylcyclopropene
MAPK	Mitogen-activated protein kinase
MC	Moisture concentration
MEA	Malt extract agar
min	Minute
MIP	Molecularly imprinted polymer
mM	Millimolar
N	
NPS	Nano-porous silicon
n	Number of replicates
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
nd	Refractometer index
ND	Not detectable
NMR	Nuclear magnetic resonance

NOEL	No-Observed Effect Level
NRPS	Non-ribosomal peptide synthases
NRPSs	Non-ribosomal peptide synthetases
NTU	Nephelometric Turbidity Units
O	
O_2	Oxygen
O_3	Ozone
OTA	Ochratoxin A
Ox –	Anaerobic conditions
Ox +	Aerobic conditions
P	
P. citrinum	*Penicillium citrinum*
P. expansum	*Penicillium expansum*
PAT	Patulin
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PE	Polyethylene
PeΔ*veA*	Mutated in the *veA* gene
PeΔ*veA*:*veA*	Complemented in the *veA* gene
pH	Potential of hydrogen
p-HHP	pulsed-HHP
PIT	Photonics Immobilization Technique
PKA	Protein kinase A
PKS	Polyketide synthases
PMTDI	Provisional Maximum Tolerable Daily Intake
ppb	Parts per billion
ppm	Parts per million
PPO	Polyphenoloxidase
PTWI	Provisional Tolerable Weekly Intake
p-values	Probability values

Q	
QCM	Quartz crystal microbalance
qPCR	Quantitative real-time PCR
R	
RF	Radio frequency
RM 40	Refractometry
RNA	Ribonucleic acid
RNA_i	Ribonucleic acid interference
ROS	Reactive oxygen species
RP-HPLC method	Reversed-Phase High-Performance Liquid Chromatography
rpm	Revolutions per minute
rt	Retention time
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative reverse transcription-polymerase chain reaction
S	
S (1, 2, 3, 4)	Sampling step number
S. cerevisiae	Saccharomyces cerevisiae
SEM	Standard error of the mean
SM	Secondary metabolites
SO_2	Sulphur dioxide
SOD	Superoxide dismutase
SPE-LC-MS	Solid-phase extraction-liquid chromatography-mass spectrometry
T	
TFs	Transcription Factors
TLC	Thin-Layer Chromatography
TOR	Target of Rapamycin
TS	Terpene synthases
TSS	Total soluble solids
TTO	Tea tree oil
TUV	Tunable UV

U	
UHPLC	Ultra-high-performance liquid chromatography
ULO	Ultra-low oxygen atmospheric condition
UV	Ultraviolet
UV-VIS detector	Ultraviolet/Visible HPLC Detectors
V	
v/v	Volume/volume
W	
w/v	Weight/volume
WHO	World Health Organization
WT	Wild type
Y	
YPD	Yeast extract peptone dextrose
Z	
ZEA	Zearalenone
Zn_2Cys_6	Zinc finger

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Chapter 1 Part 1: Characterization of *veA* gene: study of its impact on development, aggressiveness and metabolome of *Penicillium expansum*

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Introduction

STUDY CONTEXT AND OBJECTIVES

Food-related health concerns are and have been of major importance all around the world for ages. Their repercussions on the global economy are huge and most likely even underestimated. They can be caused by various agents, particularly pathogenic microorganisms.

In addition to viruses and pathogenic bacteria, toxigenic fungi are considered a real threat to humans and animals health either due to their presence or to their secretion of highly toxic substances called mycotoxins. The latter are widespread at all stages of the food and feed chains. As a consequence of their toxicity, regulations have been set to protect consumers from the damaging effects of mycotoxins that can contaminate foodstuffs and to ensure the appliance of correct practices in the food trade. Levels of mycotoxins harmful for health should not be exceeded. While some mycotoxins (e.g. aflatoxins) have been studied intensively since the beginning of their discovery, others (e.g. patulin (PAT)) have remained in the shade for a while but have been fully studied and recently regulated. For mycotoxins such as HT-2 and T-2, the recommendations apply while others are not yet regulated (e.g. *Alternaria* toxins).

PAT, the mycotoxin of interest in this study, was first discovered by Waksman et al. (1942) and was considered as a promising antibiotic, effective against nasal congestion and colds. It was only few years after that it was proven not only toxic to fungi but also to animals and higher plants (Moake et al. 2005). From then one, many researchers were particularly interested in PAT to determine its physicochemical properties, elucidate its biosynthetic pathway, isolate and characterize the fungal producing species, evaluate its toxic potential based on *in vitro* and *in vivo* studies. Apple juices as well as other food products are sometimes heavily contaminated by PAT, exceeding the maximum regulatory limits. Thus, more studies and efforts are needed to minimize the contamination of food products by this mycotoxin.

However, controlling the occurrence of PAT in food products require a better understanding of *Penicillium expansum*, the main producer of this toxin. In addition, elucidation of the biochemical and molecular mechanisms that induce PAT production could help regulate its biosynthesis. The elucidation of the biosynthetic cluster of PAT by Tannous et al. in 2014 evidenced specific factors that regulate its biosynthesis.

The full understanding of the mechanisms involved in the toxin biosynthesis is complicated and the big economic losses that occur due to the food products already contaminated by PAT should be taken into account. In this context, many questions arose.

- What are the factors that affect PAT biosynthesis outside the genes of its cluster and more generally, what is the impact of global regulators on the secondary metabolism of *P. expansum*?

- What are the processing factors that could have an effect on PAT concentration in contaminated food products? Are there any degradation products generated and if so, are they less toxic than PAT?

This multidisciplinary research aims to understand the role of the *veA* gene involved in PAT biosynthesis and to determine the role of the chemical agent ascorbic acid (AA), which affects PAT **(Figure 1)**. The first objective focuses on the elucidation of the roles of *veA* and the study of its impact on the development, aggressiveness, dissemination and secondary metabolism of *P. expansum*, with emphasis on PAT biosynthesis. A null mutant PeΔ*veA* strain and a complemented PeΔ*veA*:*veA* strain were generated using the wild type strain of *P. expansum* (NRRL 35695). A morphological study was conducted and pathogenicity on apples was studied. Expression of genes belonging to the two corresponding clusters of PAT and citrinin, another mycotoxin produced by the fungus, as well as genes that encode backbone enzymes of secondary metabolites found in the genome of *P. expansum* was analysed.

The second objective of this thesis aims to better understand the fate of PAT during the production of cloudy apple juice, more specifically during pressing of apples (using the new spiral filter press technique characterized by low-oxygen pressing conditions) and storage. The influence of AA, known as vitamin C, was examined throughout the process. At first, optimal conditions of action of AA were identified in the presence/absence of oxygen and different storage temperatures. This experiment was performed both on pure PAT standard in aqueous solution and on PAT-contaminated cloudy apple juice (CAJ). An in-house HPLC methodology was optimized leading to a good separation and detection of PAT and degradation and reaction products. Then, the evaluation of the effect of the antioxidant AA on PAT concentration during cloudy apple juice processing and storage was examined (using a VaculIQ 1000 machine). Four conditions of processing CAJ were tested. The effect of AA, oxygen and storage on PAT in the CAJ produced were evaluated.

To conclude, the results obtained in this study will result in a deeper knowledge of the regulation of the secondary metabolism of *P. expansum* by *veA* with a special emphasis on PAT and its fate in CAJ when AA is added. They will allow investigating whether *veA* and AA affect the biosynthesis and degradation of this toxin, respectively, leading to possible processing factors affecting the presence of PAT in apples and by-products.

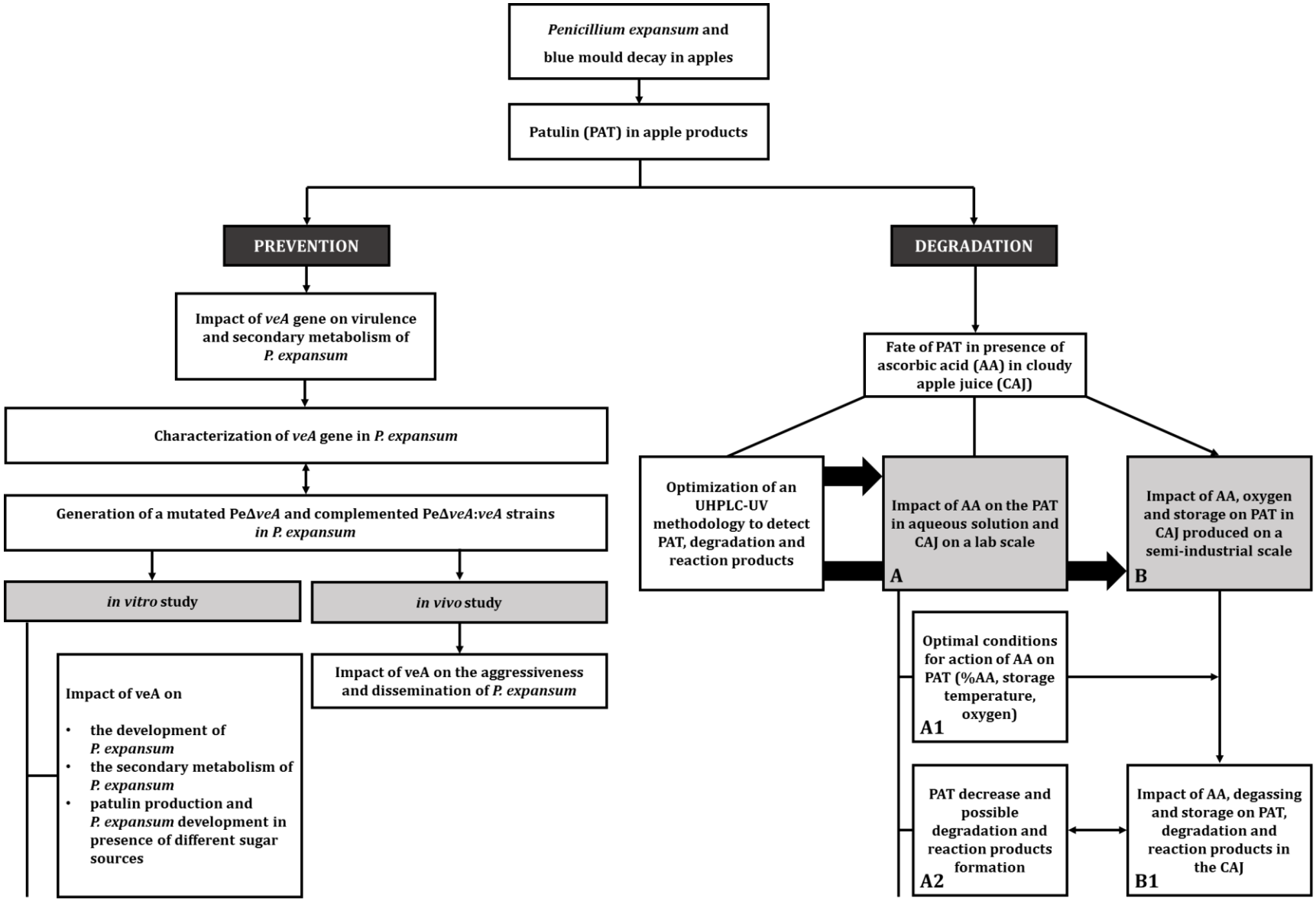

Figure 1 General overview of the PhD thesis

BIBLIOGRAPHIC REVIEW

The bibliographic study carried out during this thesis concerns a critical analysis of the literature and highlights the main issues dealt with later on in this manuscript. This bibliographic review is structured in three main parts. First, it will consist of an overview of the main filamentous fungi and their secondary metabolites with particular interest in mycotoxins and their impact on human health. The second and third part addresses the different levels of regulation of secondary metabolites in *Penicillium sp.* with a special focus on *P. expansum*. In addition, it discusses how the secondary metabolites of *P. expansum* contribute to its virulence and pathogenicity. Finally, it exposes the different control treatments applied for *P. expansum* in order to inhibit its growth, decrease diseases incidence and mycotoxin production. In the last part of this bibliographical summary, an overview on patulin (PAT) mitigation research performed so far is given. It summarizes the current knowledge on PAT detoxification techniques in apple products with a special emphasis on ascorbic acid (AA), its stability and how it may influence PAT concentration and phenolic compounds, especially in apple juice.

A. Moulds and mycotoxins

Nowadays, food security/safety has become a major concern **(Figure 2)** particularly since numerous contaminants are well known and detected in our environment such as dioxins, mycotoxins, heavy metals, pesticides, polycyclic aromatic hydrocarbons, drugs and hormones (Barreira et al. 2010). Among them, mycotoxins present in a large part of the food supply pose an important health problem. As a result of the increasing interest of thousands of researchers around the world, these toxins and their origins, toxicities, stabilities, biosynthetic pathways, control strategies, etc. are the subject of intensive studies. In this chapter, a general introduction on microscopic fungi, mycotoxins and their impact on human health is presented. More detailed information on the filamentous fungus *Penicillium expansum* and its secondary metabolites (e.g. the mycotoxin patulin), will be discussed in the second part of this introduction.

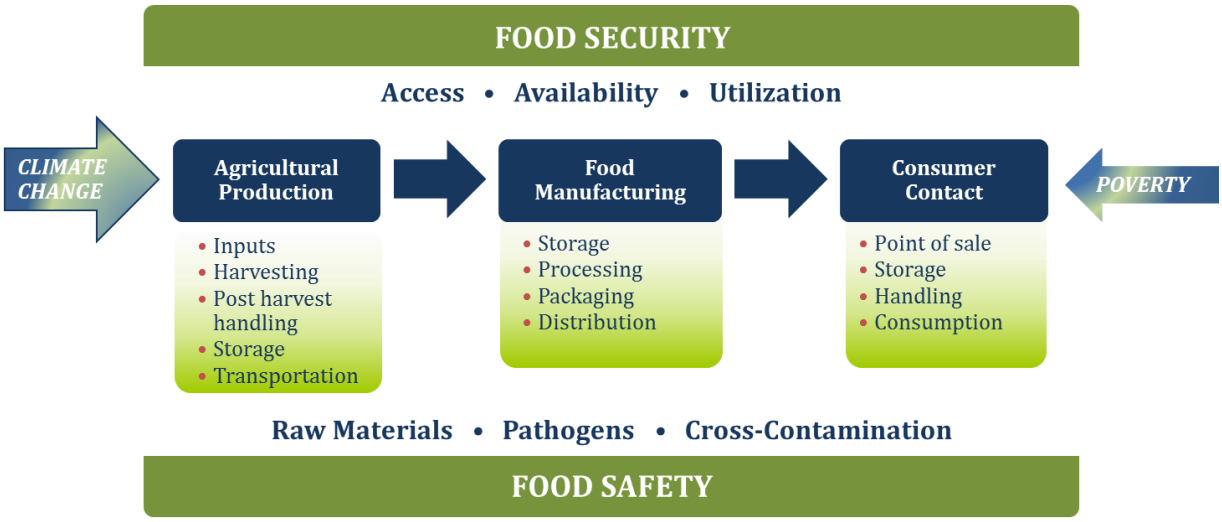

Figure 2 Risks impacting food safety and security throughout the business supply chain

A.1 Fungi: microscopic filamentous fungi

The general term fungi comprise organisms known as mushrooms, moulds and yeasts. Fungi are eukaryotic organisms that traditionally were classified in the plant kingdom. Due to their cell wall containing cellulose or glucans (true fungi) and chitin, also found in the animal kingdom (Méheust 2012), and the absence of chlorophyll (Berger and Guss 2005), these organisms were differentiated from plants, and nowadays constitute a separate kingdom, namely that of the "fungi".

Fungi are one of the largest kingdoms of organisms on earth and play a key role in many ecosystems (Mueller and Schmit 2007). The kingdom is characterized by a large diversity estimated at more than 1.5 million species, although to date, only about 70,000 species have been identified (Paterson 2006). This kingdom includes macroscopic species (macromycetes) and

microscopic ones (micromycetes). The latter are not perceptible by the eye unless their development becomes pronounced (Tabuc 2007). They are ubiquitous organisms able to proliferate in the indoor and outdoor environment (Muro-Cacho et al. 2003) when physico-chemical conditions such as humidity, temperature, and the presence of organic and inorganic substrates allow it (Aydogdu and Gucer 2009). The fungal kingdom is composed of saprophytic fungi that decompose the ecosystem and play an essential role in carbon and nitrogen recycling, and of others affecting plants, animals and humans, causing plant diseases and mycoses (Lutzoni et al. 2004). We also distinguish fungi living symbiotically (*e.g.*, mycorrhizal fungi, lichens, endophytes) (Gargas et al. 1995).

Fungi have a sexual and/or asexual reproduction. The spores, ensuring asexual multiplication, may have a role in their dispersion once produced, but may also play a role in the survival of the organism when environmental conditions become unfavourable (Madelin 1994). Fungi are classified into two broad categories: the unicellular yeast form and the mycelial multicellular form formed of hyphae (Redecker 2002). The mycelial form allows the fungus to have significant radial growth and to rapidly colonize a medium. In fact, hyphae that consist of heterocaryotic (Ascomycota and Basidiomycota) or coenocytic (Zygomycota and Glomeromycota) cells ensure the growth of mycelial fungi. Their extension is restricted to the apex. After division, the newly formed apical part can separate from the rest of the mycelium by a septum (septal mycelium in Ascomycota and Basidiomycota) or not (mycelium siphoned in Zygomycota) (Jennings and Lysek 1996). This mycelial form thus provides a maximum contact surface and allows search for nutrients in all three dimensions (Carlile and Watkinson 1994; Jennings and Lysek 1996).

A.2 Filamentous fungi and their secondary metabolites

Filamentous fungi are of importance in the human environment, in a beneficial or harmful way and have economic consequences. They are involved in different sectors such as the (agri-) food, pharmaceutical and cosmetic, as well as in the medical sector.

In the food industry, certain moulds are used to produce cheeses such as roquefort (*Penicillium roqueforti*) or camembert (*Penicillium camembertii*) (Ropars et al. 2012). They can also be used for the synthesis of organic acids such as citric acid or gluconic acid (*Aspergillus niger*), used as food additives (Karaffa et al. 2001). Some moulds are used for the synthesis/production of enzymes such as maltase and dextrinase converting maltose and starch into alcohol (*Rhizopus oryzae*). This process of alcoholic fermentation is of relevance for manufacturing rice alcohol in Asia (Lv et al. 2012). In the pharmaceutical industry, certain moulds are used for the synthesis of drugs, including antibiotics such as penicillin (*Penicillium chrysogenum*) or cephalosporins (*Cephalosporium acremonium*).

Filamentous fungi are therefore of industrial interest. Nevertheless, they represent a threat for the feed and food sector due to contamination of feed and foodstuffs. The proliferation of moulds, whether pathogenic or not, deteriorates food and feed (i.e. spoilage) and leads to unfavourable changes in dietary and organoleptic characteristics (appearance, texture, smell and flavour). This leads to heavy economic losses. Food commodities losses due to mycotoxin contamination represent above 25% of all food spoiled (Egmond et al. 2004).

In addition, the proliferation of moulds may lead to reduced food safety and increased health risks for the consumer. The production of toxic secondary metabolites such as mycotoxins (see the paragraph **1.3**) represents a major risk for human and animal health. A given species of microscopic fungus can generate one or several mycotoxins, and the same mycotoxin can be produced by several mould species. They can be secreted both at pre- and postharvest stages.

A.3 Mycotoxins and their impact on human health

a. Historical overview

The term "mycotoxin" comes from the combination of the Greek word "mycos" referring to mushroom, and the Latin word "toxicum" meaning poison (Jouany et al. 2009; Rai et al. 2012). The first outbreaks of mycotoxicosis, described in antiquity, were ergotism **(Figure 3A)** that were later named "St. Anthony's fire" **(Figure 3B)** because of the burning sensation felt by the victims (Bennett and Klich 2003; Gravesen 1979). This was at the origin of major epidemics that raged across the Old Continent during the Middle Ages, killing hundreds of thousands of people. The disease was caused by the consumption of rye flour produced from rye contaminated with fungi of the genus *Claviceps* **(Figure 3C)**. It was only in the 19th century that scientists managed to isolate the alkaloids responsible for ergotism and to study their toxicological characteristics. Other mycotoxicoses have been described later, such as Trichothecene Toxic Food Aleucie (ATA), which occurred in Russia in the 1930s, or the Turkey "X" disease, which occurred in Great Britain in 1960. The latter caused an unusual veterinary crisis in England, in which nearly 100,000 turkeys were decimated with severe hepatic necrosis and biliary hyperplasia (Bennett and Klich 2003; Yiannikouris and Jouany 2002; Nesbitt et al. 1962). The cause of this disease has been attributed to aflatoxins, secondary metabolites produced by the fungus *Aspergillus flavus* that have proliferated on peanuts contained in the food of these birds (Medeiros et al. 2012; Nesbitt et al. 1962). After this event, a real "gold rush" of mycotoxins was born and lasted for about fifteen years (1960-1975), during which many scientists joined well-funded research programs (Bennett and Klich 2003).



Figure 3 Art as evidence of mycotoxin contamination. **A:** Paint exemplifying an ergotism outbreak; **B:** "Saint Anthony's hallucinations" by Mathias Grünewald (effects of hallucinations associated to ergotism); C: rye ear contaminated by *Claviceps purpurea*.

b. Definition and characteristics

Mycotoxins are low molecular weight secondary metabolites (<1,000 Dalton) produced by filamentous fungi (Marin et al. 2013). These metabolites are non-essential to the fungus life cycle, which means that they are not associated with fungal cell growth and development (Moss 1991). Nevertheless, once produced, they may confer some competitive advantages (Fox and Howlett 2008b).

To date, 300 to 400 compounds have been recognized as mycotoxins with *Aspergillus*, *Fusarium* and *Penicillium* as the main producers, while many remain unknown (CAST 2003; Bryden 2016). Other genera such as *Alternaria*, *Byssochlamys* and *Claviceps* are known to produce mycotoxigenic compounds as well. The most commonly encountered mycotoxins are aflatoxins produced by *Aspergillus* species, ochratoxins produced by some species of *Aspergillus* and *Penicillium*, fumonisins, trichothecenes and zearalenone produced by *Fusarium* species, and patulin produced by several species of *Aspergillus*, *Penicillium* and *Paecilomyces* (Marin et al. 2013) **(Figure 4)**. Patulin, the mycotoxin of interest in this thesis, is very toxic if ingested by humans and is present in fruits especially apples and pears (Moss 2008). Its toxicity and characteristics are described in

the third section of the introduction. Other mycotoxins have gained interest nowadays like for example citrinin, enniatins, etc. More studies should be done regarding them to be able to properly assess their toxicity and characteristics.

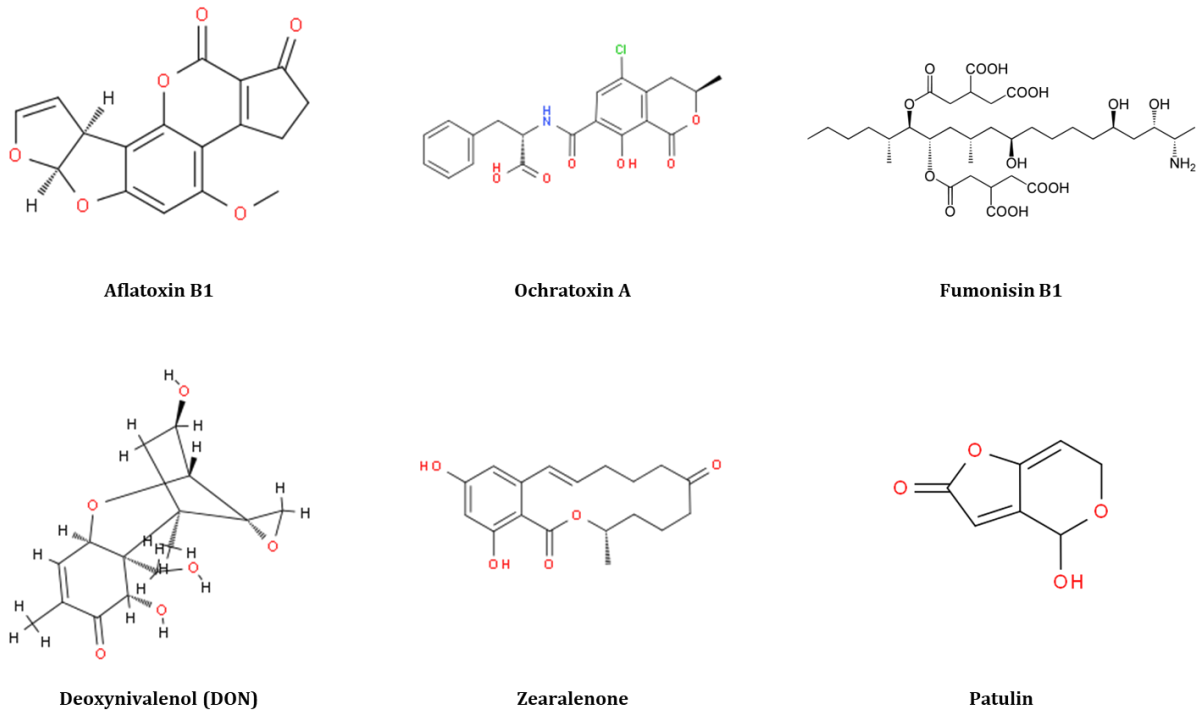

Figure 4 The chemical structures of major mycotoxins

Their enormous structural diversity, their countless biological effects and their different fungal producers make it hard to classify mycotoxins (Bennet and Klich 2003). The mycotoxins were classified based on their clinical effect, *i.e.*, hepatotoxic, nephrotoxic, neurotoxic, immunotoxic and others, while cell biologists classify them as teratogens, mutagens, carcinogens and allergens. In order to prevent these risks, the International Agency for Research on Cancer (IARC) performed carcinogenic hazard assessment of some mycotoxins in humans and categorized them (*e.g.* patulin has an IARC of 3, aflatoxins (AFB1, AFB2, AFG1, AFG2 and AFM1) of 1, etc.). Their chemical structure (*e.g.*, lactones), biosynthetic origin (*e.g.*, amino acid-derived), illness they cause (*e.g.*, aflatoxicosis) or fungal origin (*e.g.*, *Penicillium* mycotoxins) could also be characteristics based on which mycotoxins could be classified (Bennet and Klich 2003). Based on the time point at which fungal invasion and mycotoxin formation take place, a distinction can be made between pre-harvest and post-harvest mycotoxins, referring to those produced by moulds growing on the field (*e.g.*, *Fusarium*) and those produced by moulds at later stages such as harvest, transport, storage, processing, etc... However, this distinction is not always clear-out since post-harvest mycotoxins moulds can occur at pre-harvest stages as well.

c. Health effects

When mycotoxins are ingested, or sometimes inhaled, various toxic effects can be caused depending on the toxin (combination) and structures, the dose and duration of exposure and the person himself as well. They can induce toxicity in different organs and have various cellular and molecular mechanisms of action. Mycotoxins can cause acute or chronic toxicity. Even though chronic exposure to mycotoxins is considered as the main human and veterinary health burden (e.g. kidney toxicity, cancer induction, immune suppression), the best-known mycotoxin incidents were due to profound acute effects (e.g., human ergotism, Turkey X disease, stachybotryotoxicosis) (Bennet and Klich 2003; Cano et al. 2016).

The main problem we are facing is that several filamentous fungi capable of producing one or numerous mycotoxins could contaminate every raw material. In addition, most of the time, a food and feed item is composed of several raw materials. As a result, humans and animals are mostly not exposed to only one mycotoxin but more likely to a mixture of mycotoxins. Streit et al. (2013) showed that out of 17,316 food samples tested, 38% were contaminated with several mycotoxins simultaneously. Several studies indicated that interactions between mycotoxins present at the same time in a product might result in significantly higher toxicity than the individual toxicity of each mycotoxin (Smith et al. 1997; Speijers and Speijers 2004). In fact, as per Alassane-Kpembi et al. (2017), simultaneous exposure could result in synergistic (i.e., interaction resulting in greater effect than expected), additive (no interaction, i.e., as expected) or antagonistic (i.e., interaction leading to lesser effect than expected) effects. For example, a binary or ternary mixtures of type B trichothecenes (DON, NIV, and their acetylated derivatives) revealed synergistic cytotoxicity at low mycotoxin concentrations and additive or nearly additive effect at higher concentrations (Alassane-Kpembi et al. 2013). More studies on the interactions between mycotoxins are needed.

d. Regulations

Regulations exist and maximum levels of contamination by certain mycotoxins in food items have been set, but these legislations do not consider multi-contamination situations. In addition, of all the mycotoxins described, only thirty have proven toxic effects and less than a third of them are regulated. Maximum levels for aflatoxins (AFB1, AFB2, AFG1, AFG2 and AFM1), ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (FB_1, FB_2), T-2 toxin, HT-2 toxin, zearalenone (ZEA) and PAT are set by European commission (EC) Regulation No 1881/2006 and amendments. A limited number of mycotoxins (namely aflatoxins, deoxynivalenol, zearalenone, fumonisins and ochratoxin A) are regularly tested in the feed manufacturing process and are subject to regulations/guidance set by Commission Recommendation (EC) 576/2006 and Commission

Regulation (EU) No 165/2010. Mycotoxins are a very important problem of public health, quality and food safety and further research on this topic is still needed.

In the following sections (part B, C and D) of this introduction, we will be focusing on *P. expansum* and its secondary metabolites/mycotoxins with mean focus on PAT.

B. Secondary metabolism of *Penicillium spp.* and its regulation (review 1)

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Abstract

Penicillium, one of the most common fungi occurring in diverse range of habitats, has a worldwide distribution and a large economic impact on human health. Hundreds of the species belonging to this genus cause disastrous decays of food crops and are able to produce a varied range of secondary metabolites (SM), from which we can distinguish the harmful mycotoxins. Some Penicillium species are considered as important producers of patulin and ochratoxin A, two well-known mycotoxins. The production of these mycotoxins as well as other SM is controlled and regulated by different mechanisms. The aim of this review is to point out the different levels of regulation of SM in *Penicillium sp.* focusing on *P. expansum*. *P. expansum*, principal cause of spoilage of pome fruits throughout the past centuries, is ubiquitous and can infest a varied range of agricultural products during harvest, in storage, or even during processing. Besides, we discuss how the SM of the latter contribute to its virulence and pathogenicity and we end with an exposure of the different control treatments applied on *P. expansum* in order to inhibit its growth, decrease disease incidence and toxin accumulation.

Keywords: *Penicillium spp*, secondary metabolism, regulation, virulence, control of gene expression; transcription factors

B.1 Introduction

Studies have estimated the existence of, at least, 1.5 million fungal species on earth from which only about 10% have been isolated and described (Hawksworth 1991; 2001). *Penicillium*, one of the most common fungi in a various range of habitats, has a worldwide distribution and a large economic impact on human life. This genus is of a great importance in numerous and divert fields such as food spoilage, biotechnology, plant pathology and medicine (Cho et al. 2005; Bazioli et al. 2017) and contains currently 354 accepted species (Visagie et al. 2014). Hundreds of these species, classified as pre- and postharvest pathogens, could cause catastrophic decays of food crops as described by Frisvad and Samson (2004), Pitt and Hocking (2009) and Samson et al. (2010). They could also produce a varied range of secondary metabolites, including several harmful mycotoxins (Frisvad et al. 2004), antibacterial (Fleming 1929, Chain et al. 1940, Abraham et al. 1941, Thom 1945; Rancic et al. 2006; Lucas et al. 2007) and antifungal compounds (Nicoletti et al. 2007), immunosuppressant and cholesterol-lowering agents (Göhrt et al. 1992; Rho et al. 2002; Kwon et al. 2002). The biosynthesis of several secondary metabolites such as mycotoxins depends on several environmental cues like substrate, pH, temperature and water activity and on the interactions of these different factors in the natural environment (Geisen 2004; Schmidt-Heydt and Geisen 2007; Schmidt-Heydt et al. 2008). For example, the effect of the composition of the growth medium on mycotoxin biosynthesis is also influenced by the pH of the medium (Calvo et al. 2002).

Mycotoxins are the final products of enzymatic cascades starting when enzymes such as polyketide synthases (PKS), non-ribosomal peptide synthases (NRPS), terpene cyclase (TC) and dimethyl allyl transferase (DMAT) catalyse respectively the rearrangement or the condensation of simple primary metabolites such as acetyl-CoA, amino acids or terpenes resulting in more complex secondary metabolites (Khan et al. 2014). Different metabolic pathways can lead to their formation. Mycotoxins are classified into five categories according to their structure and their precursor: polyketides (PKS), cyclic terpenes (CT), non-ribosomal cyclic peptides (NRPS), indoles alkaloids (DMATS) and hybrids (PKS / NRPS) (Keller et al. 2005) **(Figure 5)**. Other enzymes are also needed and interfere in the catalysis of subsequent reactions in the biosynthetic pathways of the mycotoxins. In fact, enzymes are activated at the same time and the new synthesized intermediate products are consecutively metabolized by the next enzymes. This phenomenon is made possible thanks to the cluster organization of genes, in the same chromosomal region, encoding for enzymes involved in the biosynthesis. These genes are often co-activated by a specific transcription factor located inside clusters (Osbourn 2010). The structural diversity of mycotoxins results from the variety of chemical reactions (cyclization, aromatization,

glycosylation, hydroxylation and epoxidation) involved in their biosynthesis (Boettger and Hertweck 2012) and lead to their broad spectrum of activities and functions.

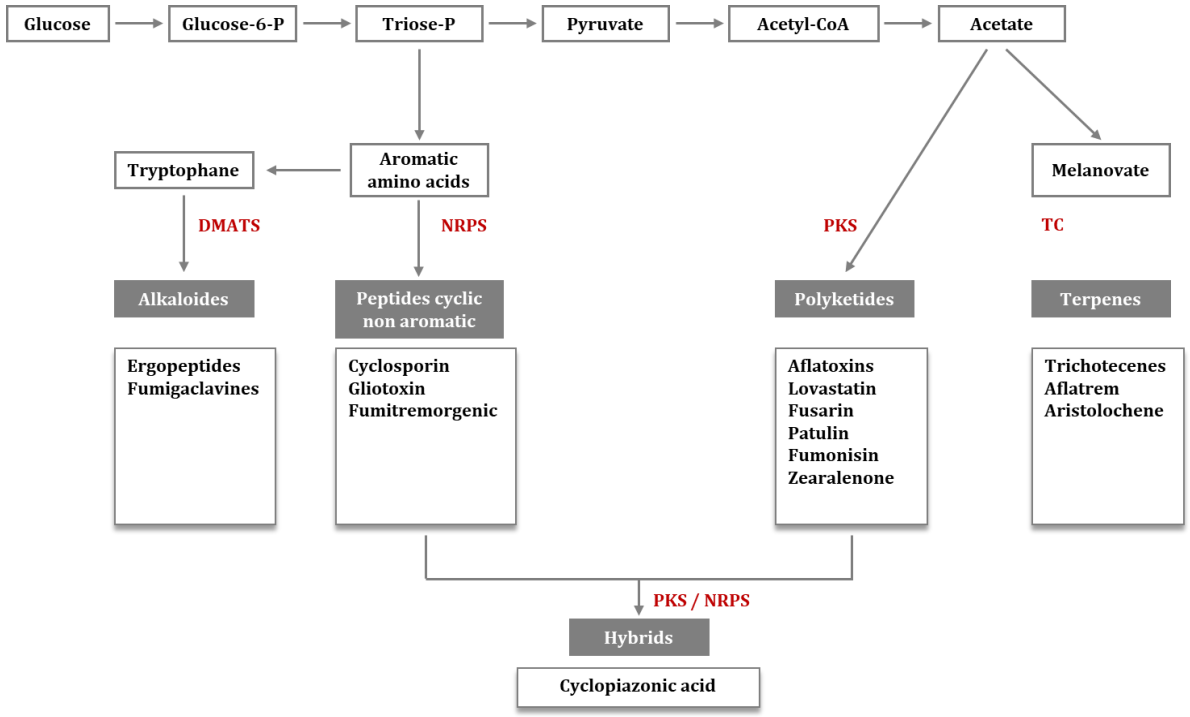

Figure 5 Biosynthetic pathways of secondary metabolites. In blue the groups of secondary metabolites. In grey boxes, the main mycotoxins produced by these pathways. In red the enzymes associated with each pathway; NRPS: non-ribosomal peptide synthetase, PKS: polyketide synthetase, TC: terpene cyclase, DMAT: dimethyl allyl transferase.

Based on bioinformatics analysis and other studies, it was proven that fungal genomes exhibit different and numerous predicted secondary metabolite clusters. In filamentous fungi, the activation of specific transcription factor and by consequence the production of fungal secondary metabolites is controlled, at a higher hierarchical level, by transcription global factors. Understanding the mechanisms underlying the biosynthesis of mycotoxins contributes to defining/identifying strategies or mechanisms to regulate them and reduce their production (Reverberi et al. 2010).

Numerous studies focused on regulators impacting the formation of mycotoxins like patulin or citrinin in *P. expansum* (Snini et al. 2015; El Hajj Assaf et al. 2018) and on their biosynthesis pathways (Tannous et al. 2014) and few reviews (Macheleidt et al. 2016) exposed the complex and multi layered regulation of fungal secondary metabolism. Our review aims to deepen the understanding of the regulation of the secondary metabolism of *Penicillium* and expose all the regulation mechanisms that could occur.

B.2 Regulation of secondary metabolism

As for the synthesis of any secondary metabolite, the regulation of its cluster involves several factors acting for its activation or repression. This regulation occurs on different levels. Most secondary metabolite clusters have Transcription Factors (TFs) that act directly on all other genes located inside cluster. The expression of these **internal regulators** also depends on other more **global TFs**, encoded by genes unrelated to the biosynthetic gene clusters, and themselves under the control of different physiological and/or environmental stimuli. An adaptation to a specific environment may also result in the biosynthesis of a certain secondary metabolite. This biosynthesis is connected and regulated by different **signalling transduction pathways**. And finally, the **epigenetic regulation**, including modification of chromatin and nucleosome structure, can conduct transcriptional control and impact SM synthesis extensively. In the following section, the different regulatory systems studied in *Penicillium* will be discussed.

a. Specific transcription factors / cluster specific regulators

Gene clusters involved in mycotoxin biosynthesis often include a gene encoding a transcription factor that specifically acts and modulates the expression of the other genes in that cluster (*e.g. patL* and *ctnA* in PAT and citrinin biosynthetic pathways, respectively). It has a switching role within the cluster **(Figure 6)**. The transcription factors regulate gene expression by binding specifically to the promoters of the genes involved.

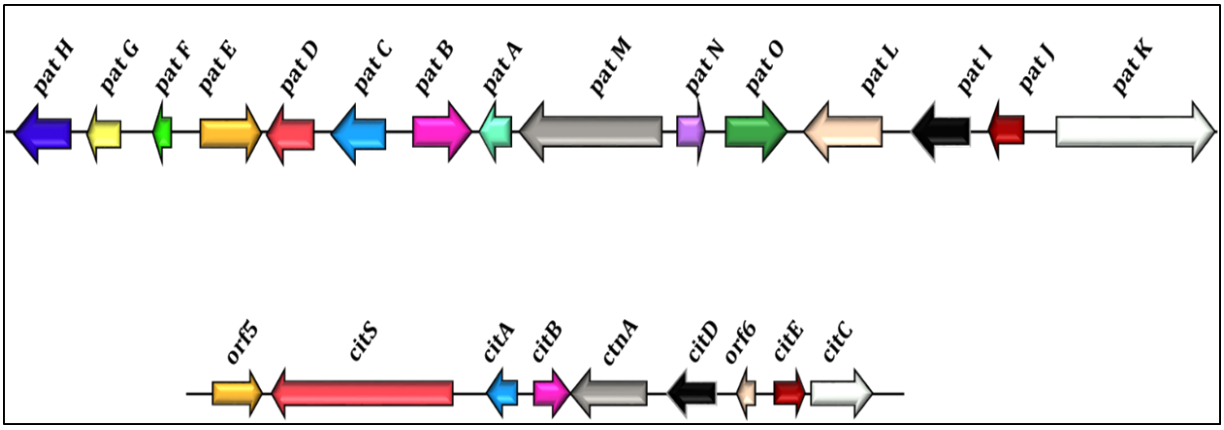

Figure 6 Gene clusters of the patulin biosynthesis pathway (the first at the top) (15 genes, 40 kb (Tannous et al. 2014)) and the citrinin biosynthesis pathway (the second at the bottom) (9 genes, 22 kb (Ballester et al. 2015; He and Cox. 2016) in *P. expansum*.

Several studies comparing sequences of transcription factors showed that the latter can be classified in different families based on the similarities in their protein sequences. We distinguish zinc finger proteins, proteins called helix-turn-helix, and leucine zippers (Todd and Andrianopoulos 1997). Nevertheless, almost 90% of the potential gene clusters involved in the

synthesis of fungal polyketides belong to the family of zinc finger transcription factors (Cys_2His_2, Cys_4 ou Cys_6) (Todd and Andrianopoulos 1997; Brakhage 2013). Proteins of the Zn_2Cys_6 family are found exclusively in fungi and yeasts (Yin and Keller 2011) and C_6 type zinc finger DNA binding protein motif (Cys_6) is frequently encountered in transcription factors. Since 1997, Cys_6 has been identified on more than 80 proteins found mainly in fungi (Todd and Andrianopoulos 1997) and generally considered as transcriptional activators **(Table 1)**. Only in *Saccharomyces cerevisiae*, the zinc finger proteins (ARGR2, LEU3 and UME6) were activators and repressors (Bechet et al. al 1970, Messenguy and Dubois 1988, Strich et al 1994, Rubin-Bejerano et al 1996). Since then, the number of proteins belonging to the Zn_2Cys_6 family has increased significantly due to the number of fungal genomes that have since been sequenced.

Protein	Regulated gene cluster	Regulatory mode	Species	References
AflR	AF/ST	Positive	*A. nidulans*, *A. flavus* *A. parasiticus,*	(Brown et al. 1996; Chang et al. 1995b; Ehrlich et al. 1999; Fernandes et al. 1998; Yu et al. 1996)
GliZ	gliotoxin	Positive	*A. fumigatus*	(Bok et al. 2006)
SirZ	sirodesmin PL	Positive	*Leptosphaeria maculans*	(Fox et al. 2008a)
MlcR	compactin	Positive	*Penicillium citrinum*	(Abe et al. 2002b)
Bik5	bikaverin	Positive	*Fusarium fujikuroi*	(Wiemann et al. 2009)
DEP6	depudecin	Positive	*Alternaria brassicicola*	(Wight et al. 2009)
ZFR1 FUM21	fumonisin	Positive	*Fusarium verticillioides*	(Brown et al. 2007; Flaherty and Woloshuk 2004)
CTB8	cercosporin	Positive	*Cercospora nicotianae*	(Chen et al. 2007)
GIP2	aurofusarin	Positive	*Gibberella zeae*	(Kim et al. 2006)
CtnA	citrinin	Positive	*Monascus purpureus*	(Shimizu et al. 2007)
LovE	lovastatin	Positive	*A. terreus*	(Huang and Li 2009; Kennedy et al. 1999)
ApdR	aspyridone	Positive	*A. nidulans*	(Bergmann et al. 2007)
CtnR	asperfuranone	Positive	*A. nidulans*	(Chiang et al. 2009)
MdpE	monodictyphenone/ emodin analogs	Positive	*A. nidulans*	(Chiang et al. 2010)

Cmr1p	melanin	Positive	*Colletotrichum lagenarium*	(Tsuji et al. 2000)
Pig1p	melanin	Positive	*Magnaporthe grisea*	(Tsuji et al. 2000)
MokH	monacolin K	Positive	*Monascus pilosus*	(Chen et al. 2010)
PatL	patulin	Positive	*P. expansum*	(Snini et al. 2016)
CtnA	citrinin	Positive	*P. citrinum*	(He and Cox 2016)

Table 1 Examples of identified $Zn(II)_2Cys_6$ transcription factors (TFs) involvement in secondary metabolism in fungi adapted and updated from Yin and Keller (2011).

Gliotoxin, a secondary fungal metabolite, belonging to the class of epipolythiodioxopiperazines (ETPs) and characterized by the presence of a sulphur-bridged dioxopiperazine ring (Gardiner et al. 2005), is produced by some of *Aspergillus* and *Penicillium* species (such as *Penicillium lilacinoechinulatum*) (Waring et al. 1987). Within its cluster, a Zn_2Cys_6 finger transcription regulator, GliZ has been identified and was responsible for gliotoxin induction and regulation. A mutation of *gliZ* (Δ*gliZ*) gene in *A. fumigatus* resulted in loss of gliotoxin production. Over-expression of gliZ increased the production of gliotoxin (Bok et al. 2006; Cramer et al. 2006; Kwon-Chung et al. 2009; Scharf et al. 2012; Schoberle et al. 2014). The mlcR gene encoding a putative 50.2-kDa protein characterized by a $Zn(II)_{(2)}Cys_{(6)}$ DNA-binding domain, has been shown to be involved in the regulation and biosynthesis of ML-236B (compactin) in *Penicillium citrinum* (Abe et al. 2002a). Numerous examples of identified $Zn(II)_2Cys_6$ transcription factors (TFs) involved in the secondary metabolism of fungi genus other than *Penicillium* have been largely described in literature such as AflR (aflatoxins), Bik5 (bikaverin), CtnA (citrinin) in *Aspergillus*, *Fusarium* and *Monascus*, respectively (**Table 1**). Another gene coding for a transcription factor in *Penicillium expansum* patL has been shown to affect patulin production (Snini et al. 2016). The protein encoded by this gene had two conserved domains, one of which encoded a Cys_6 DNA binding site and the other was found in transcription factors of the superfamily of zinc finger transcription factors. Orthologous genes of *patL* involved in the patulin metabolic pathway were found in other filamentous fungi genomes such as *P. griseofulvum*, *P. paneum*, *P. vulpinum*, *P. carneum*, *P. antarcticum* (Nielsen et al. 2017) and *A. clavatus* (Artigot et al. 2009). Snini et al. (2016) showed that a disruption of this gene caused the inability of the fungus to produce patulin

with an important decrease of *pat* genes. The reincorporation of the gene in the mutated strain restored the production of patulin.

b. Global regulators

In the past paragraph, we have reviewed specific transcription factors described in *Penicillium* and that are cluster specific. However, numerous regulatory elements affected by environmental cues, affect the expression of fungal secondary metabolites clusters and do not reside within the cluster itself. They are considered as global regulators **(Figure 7)**. Among them, CreA, AreA, Nmc, PacC, Skn7, VeA, LaeA, BrlA, PcRFX1, PcFKH1, pcz1, and NsdD are mentioned and discussed in the following paragraphs.

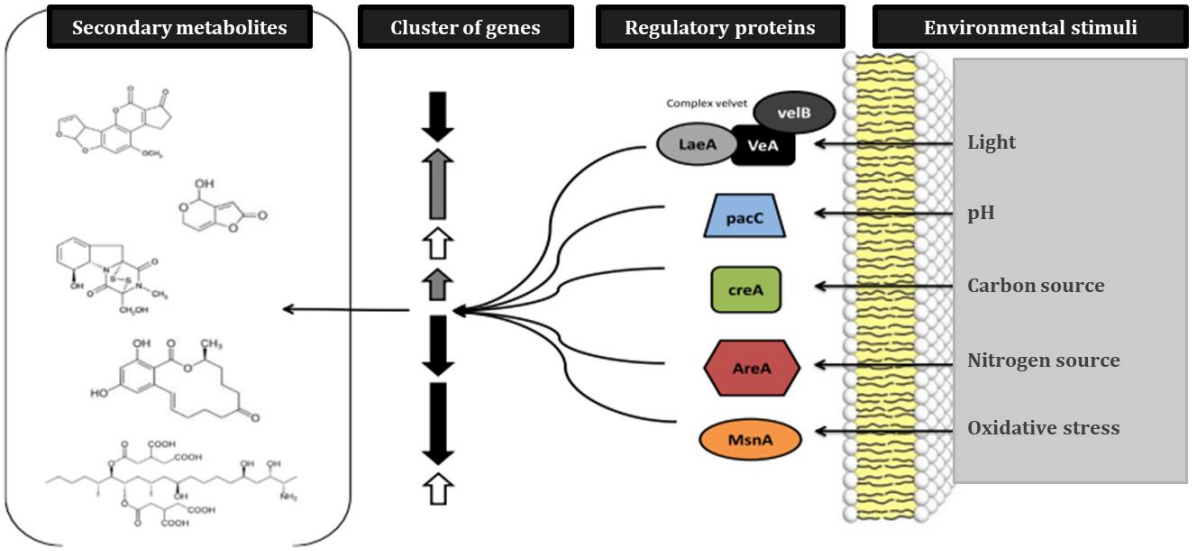

Figure 7 Global regulatory proteins involved in the regulation of gene clusters involved in the production of various fungal secondary metabolites, adapted scheme from Brakhage (2013).

Many of these TFs have been identified so far in *Penicillium* species. For example, the biosynthesis of penicillin in *P. chrysogenum* was shown to be largely regulated by glucose and sucrose and to a lower extent by other sugars (maltose, fructose, and galactose). Lactose did not exert, in *Penicillium chrysogenum* (Martin 2000). Cepeda-García et al. (2014) showed a clear evidence of the involvement of the **CreA** factor in the catabolic repression of penicillin biosynthesis suppression of penicillin biosynthesis (Revilla et al. 1984; Martin 2000). Penicillin production was positively regulated by the transcription factor CreA, a global carbon catabolite regulator and the expression of the *pcbAB* gene, encoding the first enzyme of the pathway in *Penicillium chrysogenum*. They conducted an RNAi strategy attenuating *creA* gene expression. Transformants expressing small interfering RNAs for *creA* showed greater production of penicillin. A recent study showed that the deletion of *creA* gene in *P. expansum* strains did not

allow the production of patulin in apples (Tannous et al. 2018). This increase in production was even more evident when glucose was used as a carbon source. Among the other regulatory proteins, **AreA**, a regulatory factor in response to nitrogen mediates regulation of penicillin biosynthesis in *P. chrysogenum* (Martin 2000). A concentration of ammonium above 40mM caused a repression in the expression of *uidA*, a promoterless gene for beta-glucuronidase of *Escherichia coli*, when fused to the *pcbAB* and *pcbC* promoters in *P. chrysogenum* (Feng et al. 1994). The production of patulin in *P. griseofulvum* was also affected when ammonium ions were added to the culture medium (Ellis 1996). On the other hand, the presence of 30 mM ammonium chloride results in the significant decrease of *isoepoxydon dehydrogenase* (*idh*) and *6-methylsalicylic synthase* (*6-msas*) transcripts, key genes in the pathways of patulin biosynthesis (Fedeshko 1992). The latter interact with the *nrfA* gene, ortholog of the *areA* gene in *P. griseofulvum*, through their several putative GATA sites. The ***nmc*** gene, encoding a global nitrogen regulator, has been characterized in *Penicillium roqueforti*. Nmc has a zinc finger DNA binding domain that is at least 94% identical to that of the homologous fungal proteins (such as *areA* in *Aspergillus*) (Gente et al. 1999). They showed that *nmc* expression is induced and up-regulated by nitrogen starvation. **PacC** is another transcriptional factor with three putative Cys_2His_2 zinc fingers (Tilburn et al. 1995). It seems to conduct a pH regulation of gene expression in *Penicillium chrysogenum* (Suárez and Peñalva 1996). In the same study, it was shown that *Penicillium pacC* transcript levels were higher under alkaline than under acidic growth conditions and elevate at late stages of growth. The effect of pH on regulation of penicillin biosynthesis was also observed by Martin (2000) who explained that PacC negatively regulates penicillin production in *Penicillium chrysogenum*. Barad et al. (2016) also studied the link between ammonia accumulation, activation of *pacC* and synthesis of patulin in *P. expansum*. They concluded that an accumulation of ammonia during nutritional limitation in *P. expansum* could lead to a modification of the ambient environmental pH, a signal for the activation of *pacC* as well as other alkaline induced genes leading to an accumulation of secondary metabolites such as patulin. Oxidative stress is considered as another environmental cue to which filamentous fungi should respond to survive. Most of the knowledge comes from the yeast *Saccharomyces cerevisiae* and the fungal genus *Aspergillus*. **Skn7**, a transcription factor involved in the osmotic and oxidative stress responses in *S. cerevisiae* (Morgan et al. 1997) has been also identified in *Penicillium marneffei*. The gene *skn7* from the latter was used to complement a *skn7*-disrupted strain of *S. cerevisiae* and seemed to be involved in the oxidative stress response in the yeast (Cao et al. 2009). This result indicates the highly conserved nature of *skn7* between the two organisms. Montibus et al. (2013) suggested that *skn7* could be involved in the regulation of the fungal secondary metabolism. The development of filamentous fungi and their ability to produce secondary metabolites is largely influenced by light as well. A velvet

complex has been described in *Aspergillus nidulans* and the **VeA** (velvet A) factor has been largely studied and many proteins seem to interact with it such as VelB (velvet-like B), VosA (viability of spores A), VelC (velvet-like C) and the non-velvet protein **LaeA** (loss of aflR expression A), a methyltransferase involved in chromatin remodelling (Bayram and Braus 2012). Depending on fungal species, VeA is involved in different physiological processes such as development, asexual and sexual reproduction, secondary metabolism and virulence. The regulation mediated by this factor depends particularly on the light. VeA was first characterized in *Aspergillus nidulans* and the *veA* gene encodes a protein of 573 amino acids with a conserved domain at the N-terminus (Bayram et al. 2008a) and a nuclear localization sequence (NLS) (Stinnett et al 2007). At its C-terminus, a PEST domain (rich in proline, glutamic acid, serine and threonine) is present (Kim et al. 2002). This PEST domain is also found in VeA orthologous proteins in *Aspergillus parasiticus*, *Aspergillus fumigatus* and *Neurospora crassa* (Bayram et al. 2008b).

Stinnett et al. (2007) studied the intracellular localization of VeA. This study demonstrates that this location is dependent on light. In the dark, VeA is mainly located in the nucleus whereas in the presence of light, VeA is mainly found in the cytoplasm. In the *veA1* mutant (Käfer 1965), VeA is mostly found in the cytoplasm independently of light. In this mutant, the presence of a mutation on the transcription initiation codon led to a truncated protein where the first 36 amino acids were missing and therefore did not have a functional nuclear localization sequence (NLS), thus explaining the cytoplasmic localization of VeA. In the same study, it is demonstrated that the transfer of VeA into the nucleus depends on the importin α KapA and that a functional NLS is essential, which allows the interaction of these two proteins.

In order to identify the proteins interacting with VeA, Bayram et al. (2008a) used the Tandem Affinity Purification (TAP) technique from a strain of *Aspergillus nidulans* expressing a VeA protein coupled to a tag TAP-tag at the C-terminus. In the dark, the proteins VelB, LaeA, and importin α KapA interact with VeA. Conversely, only VelB interacts with VeA in the presence of light. Using the yeast two-hybrid technique, the analyses confirm the VeA-VelB and VeA-LaeA interactions; however, no interaction is demonstrated between LaeA and VelB suggesting that VeA acts as a bridge between these two proteins. In addition, fluorescence assays showed that the VeA-LaeA interaction occurs in the nucleus, while VeA and VelB interact in the nucleus and the cytoplasm. LaeA is located in the nucleus, its interaction with VeA is nuclear, VelB must therefore be able to enter the nucleus despite the absence of NLS in its sequence. Bayram et al. (2008a) demonstrated that VeA assists VelB to enter the nucleus to form the Velvet complex.

The results obtained in the various studies allowed Bayram et al. (2008a) to propose a mechanism **(Figure 8)** that coordinates the regulation of sexual development and the production of secondary metabolites. In the dark, the VeB \ VeA \ LaeA complex controls and induces the epigenetic activity

of LaeA which consequently controls the expression of the genes of the clusters responsible for the synthesis of the secondary metabolites. In the presence of light, this interaction decreases because VeA is retained in the cytoplasm and LaeA has low activity.

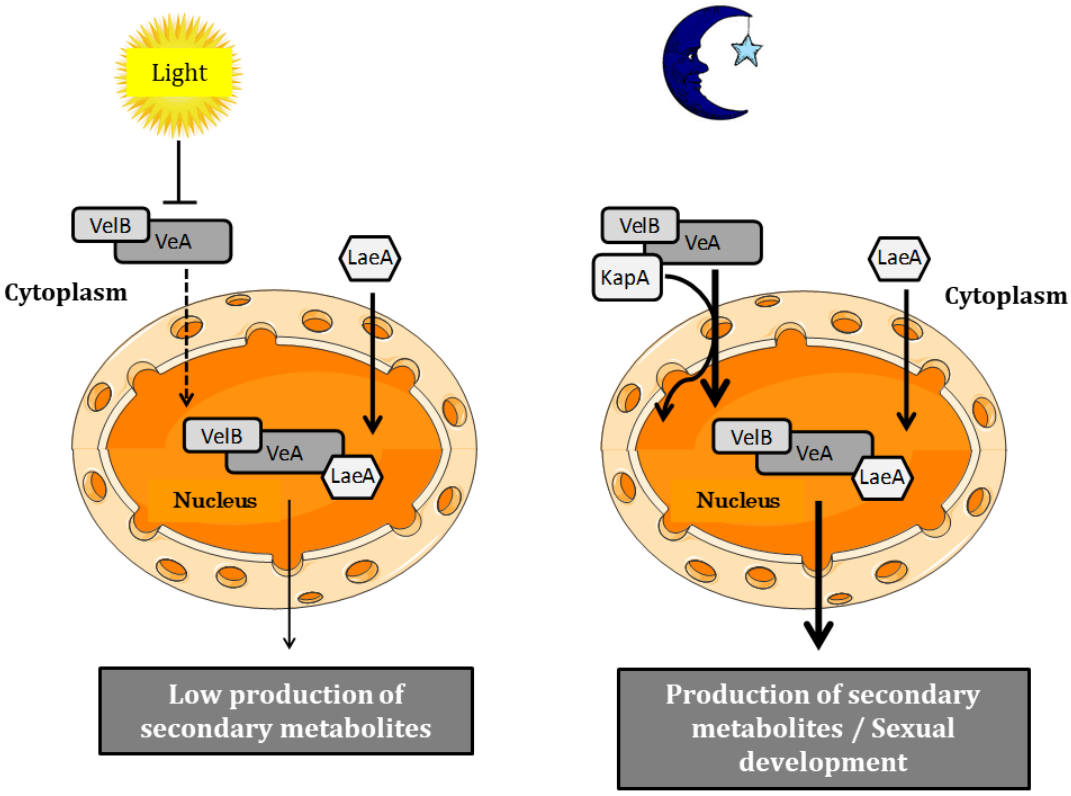

Figure 8 Operating model of the velvet complex in *Aspergillus nidulans* adapted from Bayram et al. (2008a). In the presence of light, VeA is retained in the cytoplasm and LaeA has low activity. In the dark, VeA coupled to VelB is transported into the nucleus by the importin α KapA and the velvet complex is formed with LaeA to activate the production of secondary metabolites and sexual development

Despite its strong conservation among different fungal species, VeA has different roles, reflecting the diversity of fungi development patterns.

Therefore, *veA* has a role in the regulation of secondary metabolism. The expression of genes involved in the synthesis of secondary metabolites is affected by VeA (Kato et al. 2003, Duran et al. 2007, Calvo 2008, Cary and Calvo 2008). Kato et al. (2003) demonstrated that in *Aspergillus nidulans*, VeA regulates the expression of genes involved in sterigmatocystin synthesis. Indeed, it is necessary for the synthesis of AflR, the transcription factor specific of the biosynthetic pathway of this mycotoxin (Chang et al. 1993, Payne et al 1993, Flaherty and Payne 1997). Similarly, in *Aspergillus parasiticus* and *Aspergillus flavus*, the *veA* gene is required for the transcription of *aflR* and *aflJ*, another transcription factor widely recognized in *Aspergillus flavus* (Meyers et al. 1998, Du et al. 2007). Other studies reveal that *veA* is needed for the synthesis of other secondary metabolites such as cyclopiazonic acid and aflatrem in *Aspergillus flavus* (Duran

et al. 2007), penicillin in *Aspergillus nidulans* (Kato et al. 2003) or *Penicillium chrysogenum* (Hoff et al. 2010) or trichothecenes in *Fusarium graminearum* (Merhej et al. 2012). In this last study, FgVe1 was shown to be a positive regulator of the virulence of *Fusarium graminearum*. In *Fusarium verticillioides*, FvVE1 is necessary not only for the production of fumonisins but also for the infection of corn plants by the fungus (Myung et al. 2012). Recently, it was shown that the disruption of *veA* in *P. expansum* caused a quasi-inability to produce patulin and citrinin on synthetic media (MEA and PDA) with a drastic decrease in the expression of patulin (*patA*-*patO*) and citrinin (Pexp_005510-Pexp_005590) genes (El Hajj Assaf et al. 2018). Moreover, the null mutant was not able to produce patulin when it grew in apples. This study was also extended to the whole *P. expansum* secondary metabolism by the evaluation of the impact on VeA on the expression of the backbone genes of secondary metabolites found in the genome of the *P. expansum* d1 strain including PKSs, NRPSs, terpene synthases and DMATSs genes and by a non-targeted LC-MS analysis. Metabolomic analysis displayed a global impact on secondary metabolism whilst gene expression analysis showed a positive or negative regulation of 15/35 backbone genes and support the hypothesis that *P. expansum* secondary metabolism is modulated by the transcriptional regulator factor VeA. Baba et al. (2012) also showed that a disruption of *veA* and/or *laeA* in *P. citrinum* played critical roles in ML-236B production by controlling expression of *mlcR*, the pathway-specific activator gene for ML-236B biosynthesis.

It was also shown that different components of the velvet complex may have opposite roles in the regulation of secondary metabolism. In *Penicillium chrysogenum*, PcVelC, together with the velvet PcVelA (ortholog of VeA in *P. chrysogenum*), and the methyltransferase PcLaeA, induced penicillin production, and in contract, PcVelB acted as a repressor (Kopke et al. 2013). Wolfers et al. (2015) also examined genes regulated by light and by PcLaeA and PcVelA. They found that on 148 regulated genes, six encoded typical fungal transcription factor domains. PcAtfA, a bZIP transcription factor, was the only one with characterized homologs in other fungi and was shown to control spore germination. Under the conditions tested by Kosalkova et al. (2009), Pc*laeA* gene in *P. chrysogenum* controlled some secondary metabolism gene clusters. Its overexpression increased penicillin biosynthesis by 25% and its deletion reduced drastically the expression of penicillin biosynthesis genes. However, PcLaeA did not regulate the biosynthesis of roquefortine C. The regulation of secondary metabolism of *P. expansum* by *laeA* was performed on two culture media (Kumar et al. 2017). Of the 54 backbone genes examined, many appeared to be positively regulated by *laeA* such as those involved in the biosynthesis of roquefortine, an ETP-like metabolite and patulin. The difference observed between these two studies regarding *laeA* regulation of the gene involved in the biosynthesis of roquefortine C could be due to the species used and the medium tested. In *P. oxalicum*, it has been shown that putative methyltransferase

LaeA and the transcription factor CreA control, among other things, the expression of secondary metabolic gene clusters (Zhang et al. 2016). Silenced clusters in Δ*laeA*, specifically those located toward subtelomeric regions of the chromosome, were not observed in Δ*laeA*Δ*creA*. Lack of *creA* activated silent or poorly expressed gene clusters and thus remediated the repression of gene cluster in Δ*laeA* background.

According to Calvo (2008), *veA* is responsible for the activation or repression of several genes such as *brlA*, *rosA*, etc. The transcription factor RosA (Vienken et al. 2005) and the protein encoded by the *lsdA* gene (Lee et al. 2011) involved in the regulation of sexual development in *Aspergillus nidulans* are under the control of VeA. The gene *esdC*, dependent on VeA, is expressed during the early stages of sexual development in *Aspergillus nidulans* (Han et al. 2008). Finally, VeA is required for the expression of *ppo* encoding endogenous oxylipins called "psi factors" involved in the balance between sexual and asexual development (Tsitsigiannis et al. 2004). Kato et al. (2003) demonstrated that VeA plays a role in the expression of the *brlA* gene encoding a transcription factor involved in the asexual development of *Aspergillus nidulans*.

Moreover, the deletion of *laeA* reduced the conidiation in *P. oxalicum*, and down-regulated the expression of *brlA* (Zhang et al. 2016). Ahmed et al. (2013) showed that the expression of **brlA**, a C_2H_2-type zinc finger transcription factor that plays the role of a primary regulatory gene in asexual development, is controlled by the velvet complex. Its expression was studied in *Penicillium decumbens* by Qin et al. (2013) and the expression level of 7/28 gene clusters of secondary metabolites were regulated in a *brlA* deletion strain. The clusters involved in the biosynthesis of the mycotoxins roquefortine C and meleagrin were down-regulated and the expression levels of cellulase genes (catalysing the hydrolysis of the β-(1,4) glycosidic bonds of cellulose and encoding endoglucanase I and II, cellobiohydrolase I and II, β-glucosidase and amylase) were up-regulated in this same strain. In *Penicillium chrysogenum*, the inactivation of the transcription factor StuA involved in the regulation of conidiation, but not BrlA, caused a drastic downregulation of the expression of the penicillin biosynthetic gene cluster. In fact, in a *brlA* deficient strain, the production of penicillin V was not affected but a reduction of almost 99% was assessed by HPLC analysis and observed during liquid-submerged growth in a *stuA* deficient strain (Sigl et al. 2011).

Finally, **PcRFX1** is a new TF that has been characterized in *Penicillium chrysogenum* by Domínguez-Santos et al. (2012). It is the ortholog of the regulatory proteins CPCR1 and RFxA in *Acremonium chrysogenum* and *Penicillium marneffei* respectively. Knock-down and overexpression techniques of the *Pcrfx1* gene have proven that *PcRFX1* regulated *pcbAB*, *pcbC* and *penDE* transcription and thereby controls penicillin biosynthesis. *PcRFX1* was also suggested to be involved in the control of pathways of primary metabolism. **PcFKH1**, another transcription factor of the winged-helix family, also positively regulates penicillin biosynthesis in

P. chrysogenum by binding to the *pcbC* promoter, interacting with the promoter region of the *penDE* gene, and controlling other genes such as *phlA* and *ppt* encoding phenylacetyl CoA ligase and phosphopantetheinyl transferase (Domínguez-Santos et al. 2015). The ***pcz1*** gene (*Penicillium* C_6 zinc domain protein 1) encoding a $Zn(II)_2Cys_6$ protein and controling growth and development processes of the fungus has also been described in *P. roqueforti* and has been suggested to participate in physiological processes in this fungus and play a key role in regulating its secondary metabolism (Gil-Durán et al. 2015; Rojas-Aedo et al. 2018). The silencing of *pcz1* in *P. roqueforti* resulted in the downregulation of the genes of the central conidiation pathway *brlA*, *abaA* and *wetA* (Gil-Durán et al. 2015). In *pcz1* down-regulated strains, the production of the metabolites roquefortine C, andrastin A and mycophenolic acid were severely reduced; however, when *pcz1* was overexpressed, only mycophenolic acid was overproduced and levels of roquefortine C and andrastin A were decreased (Rojas-Aedo et al. 2018).

Finally, the *PoxNsdD* gene of *P. oxalicum* was characterized by He et al. (2018a). It is an ortholog of the ***nsdD*** gene encoding a GATA-type zinc finger TF that was proven to be involved in the production of secondary metabolites. In the Δ*PoxNsdD* strain, the 230 differentially expressed genes identified covered 69 putative biosynthetic gene clusters 11 of which were predicted to produce aspyridone, emericellin, citrinin, leucinostatins, roquefortine C/meleagrin, beauvericin, cytochalasin, malbrancheamide, and viridicatumtoxin.

c. Signal transduction pathways

In general, fungi present very dynamic and a structured cell wall. During the cell cycle, organisms need to adapt quickly to changes under environmental conditions and imposed stresses and thus regulate the composition and structural organization of their cell wall (Klis et al. 2006; Ruiz-Herrera et al. 2006; Munro et al. 2007). All this influences the biosynthesis of SM in the fungus. Numerous signalling pathways activate and regulate the growth and differentiation of filamentous fungi and initiate SM biosynthesis under specific conditions. These signalling pathways sense and transduce signals external to TFs that, in turn, activate the expression of genes that could be involved in the biosynthesis of certain SM. The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), the calcineurin/calmodulin, TOR, and mitogen-activated protein kinase (MAPK) are considered as the most studied pathways (Macheleidt et al. 2016). The production of many secondary metabolites was associated and linked to one of these transduction signals and to specific active molecules. Among the different signalling pathways listed, we will focus on those that affect only the secondary metabolism of *Penicillium* and start with the *Calcium pathway.*

Calcium salts have been reported to inhibit the postharvest decay of apples caused by *P. expansum* (Wisniewski et al. 1995). In the presence of 25 mM of $CaCl_2$, germination of *P. expansum* spores

was reduced by half and to 10-30% when $CaCl_2$ concentration greater than 50 mM was added. The addition of 25 to 175 mM of $MgCl_2$ did not affect the germination of the fungal pathogen spores.

cAMP pathways. Heterotrimeric G proteins are important components of these signal transduction pathways. They can integrate a variety of signals and then transduce them to downstream signalling cascades. Most of the filamentous fungi have three Gα proteins belonging to classes I, II or III (Bölker 1998). Gα subunits belonging to class I have been involved in many aspects related not only to the development of the fungus or its pathogenicity but also its secondary metabolism, which is not the case for Gα subunits of classes II and III. The deletion of class II Gα proteins showed negligible effects on fungal metabolism (Liu and Dean 1997; Gronover et al. 2001); and those of class III have been involved in fungal development and pathogenicity (Chang et al. 2004; Doehlemann et al. 2006; Hu et al. 2013). Alterations have been observed in the secondary metabolism of different fungi, including *Penicillium chrysogenum* (García-Rico et al. 2008) and *P. marneffei* (Zuber et al. 2002). The *pga1* gene, encoding Gα subunits protein in *Penicillium chrysogenum* showed to affect the production of three SM: penicillin, chrysogenin and roquefortine. The deletion of *pga1* induces a decrease in the production of roquefortine and penicillin by regulating the expression of *pcbAB*, *pcbC* and *penDE*, the three structural biosynthetic genes of the penicillin cluster. Chrysogenin biosynthesis is enhanced and roquefortine and penicillin biosynthesis is up-regulated by the presence of a dominant activating *pga1* (G42R) allele or a constitutively active Pga1 (García-Rico et al. 2008). Carrasco‐Navarro et al. (2016) suggested, based on a proteomic analysis, that Pga1 signalling affects penicillin biosynthesis by acting on primary metabolism pathways that are also involved in cysteine, ATP and NADPH biosynthesis. They also propose a model for the Pga1‐mediated signal transduction pathway.

The osmostress response pathway. Usually, inhibition of the HOG (high osmolarity glycerol) signalling pathway negatively affects the production of metabolites, or in other words, challenging osmotic conditions activate the cascade of HOG MAP kinase signal, thereby activating several osmo-regulated genes or downstream TFs by phosphorylation. In their study, Stoll et al. (2013) showed that NaCl induced production of ochratoxin A in correlation with the phosphorylation status of the HOG MAP kinase in *P. nordicum* and *P. verrucosum*. The activation of HOG phosphorylation and ochratoxin A biosynthesis suggests a link between the two processes and that this regulation be mediated by the HOG MAP kinase signal transduction pathway. This was confirmed by inactivating the *hog* gene in *P. verrucosum*, making the fungus unable to produce ochratoxin A under high NaCl conditions. The biosynthesis of citrinin, another *P. verrucosum* toxin, was not affected. This could be explained by the subsequent work of Schmidt-Heydt et al. (2015), which showed the impact of high oxidative stress conditions on citrinin biosynthesis. Indeed, by increasing Cu^{2+}concentrations in a growth medium, *P. verrucosum* shifts the biosynthesis of its

secondary metabolism from ochratoxin A to citrinin. Increasing amounts of external cAMP reduce citrinin biosynthesis depending on the concentration chosen and suggest that citrinin biosynthesis is regulated by a cAMP/PKA signalling pathway.

Although most of the studies on signal transduction pathways were performed on *Aspergillus*, some were performed on *Penicillium* and revealed that many pathways are involved in the SM biosynthesis. There is still work to be done on other *Penicillium* species that will reveal new information regarding known or unknown SM.

d. Epigenetic regulation

As previously discussed, *Penicillium* species, like other fungi, produce a set of bioactive SM that are not essential to their survival. Genes for biosynthesis and regulation of SM in fungi are not evenly distributed over the genomes and tend to be sub-telomerically located (Strauss and Reyes-Dominguez 2011). Global epigenetic regulators have contributed to the study of many unknown SM and many histone modifications have been associated with the regulation of secondary metabolism gene clusters (Bok and Keller 2004; Shwab et al. 2007). Epigenetic phenomena that could occur are reversible and many changes in the fungus' gene expression levels do not alter the DNA sequence and can occur throughout the fungus' life cycle. Fungal epigenetic regulation involves mainly histone modifications such as methylation, acetylation and sumoylation. Histone proteins are the primary protein components of chromatin and through histone modifications, regulation could be limited to a specific region of the chromosome and therefore affect some genes. This supports the advantage of having secondary metabolism genes grouped into clusters. In *Penicillium chrysogenum*, *hdaA*, an ortholog of the histone deacetylase *hdaA1* gene of *S. cerevisiae* appears to be a key regulator of the secondary metabolism of the fungus. Deletion of *hgaA* induced a significant effect on the expression of numerous polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) encoding genes. A downregulation of NRPS encoded gene associated with the biosynthetic gene cluster of chrysogine was observed. In parallel, a transcriptional activation of the biosynthetic gene cluster of sorbicillinoids occurs, associated with the detection of a new compound produced only under these conditions. These results obtained by Guzman-Chavez et al. (2018) suggest a crosstalk between biosynthetic gene clusters. By culturing *Penicillium variabile* on a maltose medium in the presence of 5-azacytidine (a DNA methyltransferase inhibitor), varitatin A synthesis was induced (He et al. 2015). And by growing it on a potato-based medium in the presence of suberoylanilide hydroxamic acid (SAHA, a histone deacetylase inhibitor), seven polyketides were induced, including three known wortmannilactones: E, F and H and new varilactones (A–B) and wortmannilactones (M–N) (He et al. 2018b). In cultures treated with 50 µM of 5-azacytidine (another DNA methyltransferase

inhibitor), *Penicillium citreonigrum* formed exudates, which are droplets rich in primary and secondary metabolites, inorganic substances, and proteins/enzymes and known as guttates, veryrich in different compounds compared to the control. In fact, 5-azacytidine induced the formation of six azaphilones (fungal metabolites with diverse biological activities), pencolide, and two new meroterpenes (Wang et al. 2010). The addition of 5-azacytidine to the culture medium of *Penicillium funiculosum* also altered the metabolic profiles of this fungus (Liu et al. 2014). Two new prenyleudesmane diterpenoids were extracted from the culture and exhibited cytotoxic and antibacterial activities. *Eupenicillium sp.* LG41, an endophytic fungus, was exposed to an epigenetic modulation using nicotinamide, a NAD^+-dependent histone deacetylase (HDAC) inhibitor (Li et al. 2017a). This led to the production of many compounds: eupenicinicols C and D along with eujavanicol A and eupenicinicol A. El-Hawary et al. (2018) showed that cultures of *Penicillium brevicompactum*, a marine-derived fungus, exposed to nicotinamide and sodium butyrate resulted in an induction of the production of phenolic metabolites. In the presence of nicotinamide, many compounds (*e.g.* p-anisic acid, benzyl anisate, syringic acid, sinapic acid, etc.) were isolated and identified. Sodium butyrate also enhanced the production of anthranilic acid and ergosterol peroxide.

The addition of 500 µM of suberoyl bis-hydroxamic acid (SBHA), a Zn(II)-type or NAD^+-dependent HDAC inhibitor, and 100 µM of nicotinamide (an NAD^+-dependent HDAC inhibitor) to a culture of *Penicillium sp.* isolated from leaves of *Catharanthus roseus* improved the production of citreoviripyrone A and citreomontanin. In addition, nicotinamide enhanced the production of (-)-citreoviridin (Asai et al. 2013). Xiong et al. (2018) explored the role of High-Mobility Group (HMG)-box protein, PoxHmbB, involved in chromatin organization and identified in *Penicillium oxalicum*. They saw that in a mutant strain ΔPox*hmbB*, conidiation and hyphae growth were delayed. The expression of genes encoding plant biomass degrading enzymes and others involved in conidiation were regulated by Pox*hmbB*.

In conclusion, chromatin regulation of small molecule gene clusters allows specific control of secondary metabolism gene clusters and allows filamentous fungi to modify chemical diversity and successfully exploit environmental resources. Epigenetic regulation is considered a promising strategy for investigating unknown SM clusters, particularly because, under certain laboratory culture conditions, many clusters could remain silent, making it difficult to elucidate their function and regulatory mechanisms (Van Den Berg et al. 2008)

B.3Conclusion

The fungal secondary metabolism is very broad, and this review focused on that regulated in *Penicillium* genus. Given the diversity of SM, their key roles as virulence and pathogenicity factors

and their great in the medical and agricultural interest, further research should be conducted on them. This review points out how the production of these SM is controlled and regulated. It discusses the different levels of regulation of SM, including specific regulators, global TFs, transduction signalling pathways and epigenetic regulation as well as the combination of many different parameters affecting the biosynthesis pathways of metabolites. Many TFs that affect expression of genes involved in the secondary metabolism seem to belong to the zing-binding proteins category. LaeA and the velvet complex proteins are considered as global regulators and are able to control many clusters at the same time. Although much is known about these global transcription factors and their regulatory proteins, more research is needed to explore the details that link them to the transcription of genes involved in SM biosynthetic pathways. This would help to better understand the molecular mechanism underlying this complex regulatory network.
In addition, the study of the regulation of SM biosynthesis in *Penicillium* is much less advanced than in *Aspergillus* and some orthologous genes already studied in *Aspergillus* (*e.g. rtfA*, *cpsA*, *rmtA*, *mtfA*, etc.) should be investigated in *Penicillium*.

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C. Study of the virulence and control of *P. expansum*

The generic type *Penicillium expansum*, introduced first by Link (1809), is a devastating pathogen in fruit and is considered as the principal agent of the blue mould disease, leading to considerable economical losses during post-harvest handling and storage. *P. expansum* belongs to the subgenus *Penicillium* and the *Penicillium* section (Frisvad and Samson 2004). Morphologically, this species is characterized by terterticillate conidiophores (with phialides on metulae carried by branchs), usually grouped in coremia. Under the microscope, this species is characterized by smooth straight stalk **(Figure 9 A, C)**, bulbous to cylindrical elongated phialides, and dull green conidia with ellipsoidal shape **(Figure 9B)** (Frisvad and Samson 2004; Pitt and Hocking 2009). *P. expansum*, like the majority of species belonging to subgenus *Penicillium*, does not present sclerotia (Frisvad and Samson 2004).

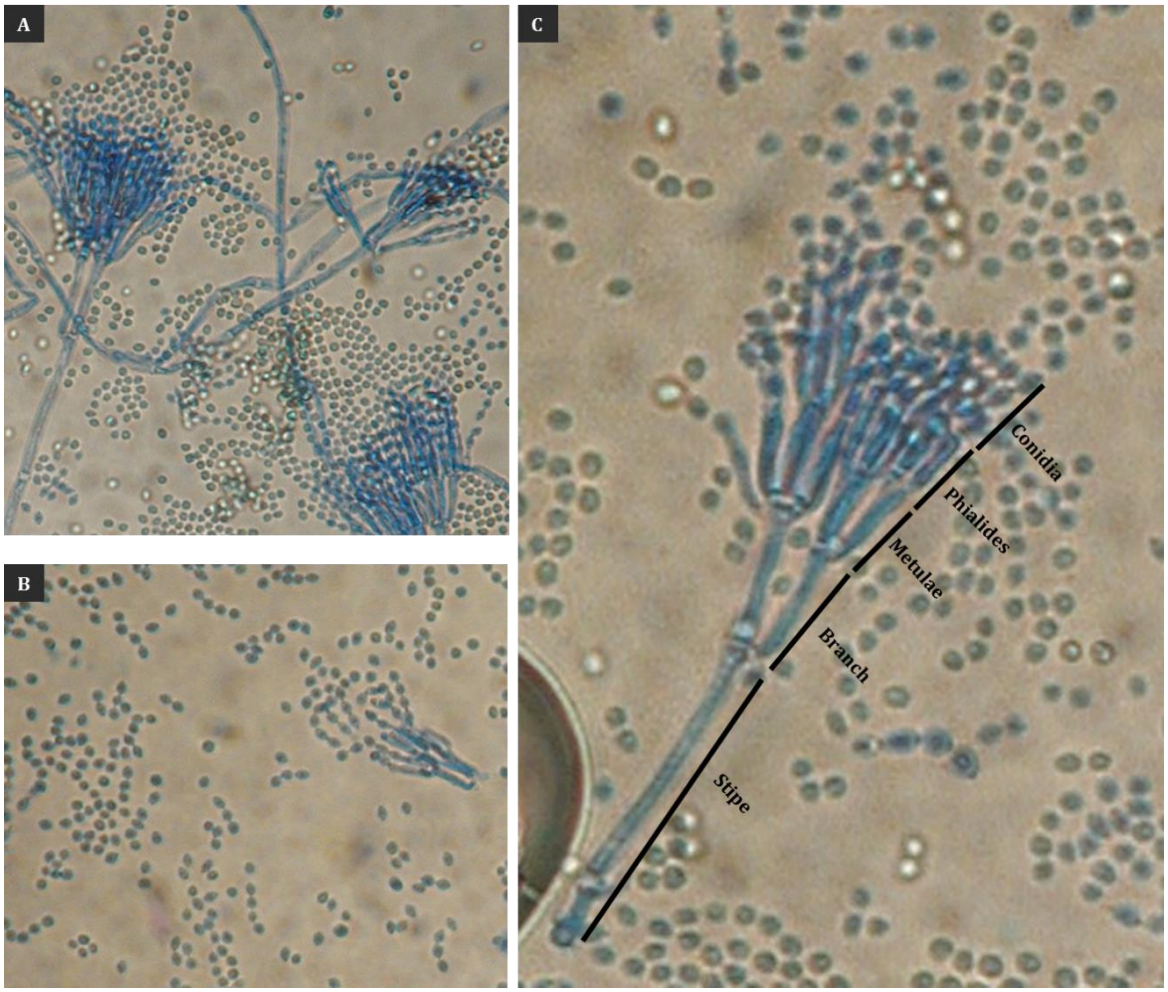
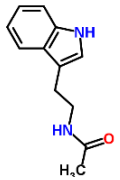
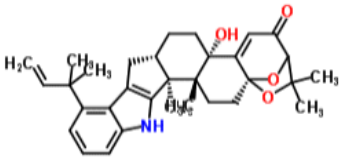
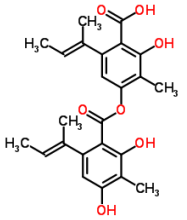
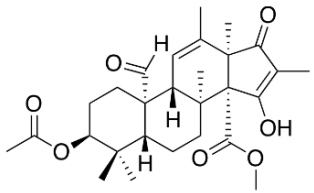
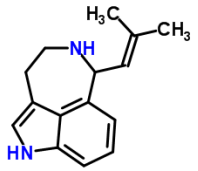
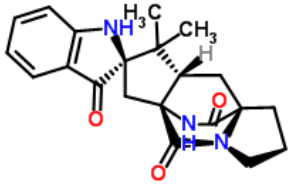
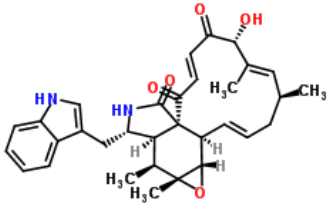
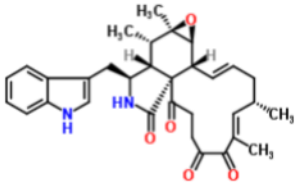
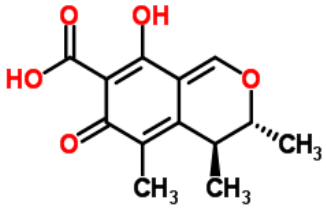


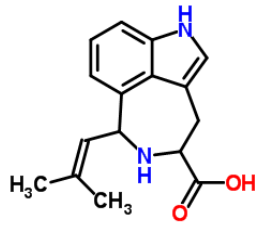
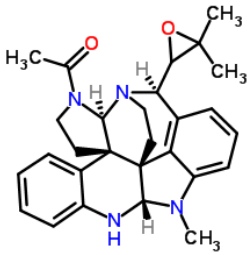
Figure 9 Microscopic appearance of conidiophores **(A, C)** and conidia **(B)** of *Penicillium expansum* (400 ×) after 7 days of cultures at 25°C in the on **(A-B)** Potato Dextrose Agar (PDA) and **(C)** Malt Extract Agar (MEA) inoculated with10^4 spores. (Photos taken under an optical microscope (x 400) during our study)

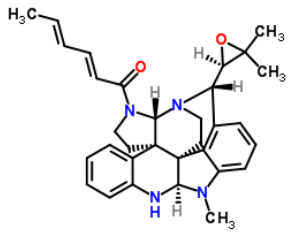
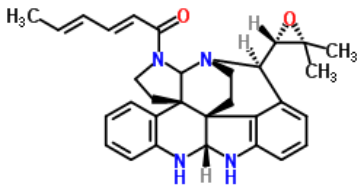
Although most of the studies on *P. expansum* have focused on patulin, its genome is rich in predicted secondary metabolite clusters **(Table2)** that contribute to its aggressiveness and pathogenicity. It should be noted that the metabolites listed in this table were collected based on a literature study. After sequencing three genomes of *P. expansum*, it is now known that *P. expansum* is unable to produce cyclopiazonic acid, mycophenolic acid, aflatrem and ochratoxin A. In the following, a brief overview of how SM contribute to the virulence, aggressiveness and development of *P. expansum* and how we can control the fungus and its toxins is given.

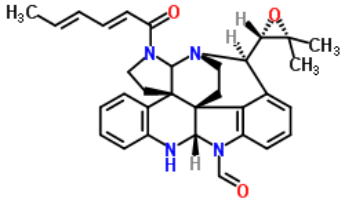
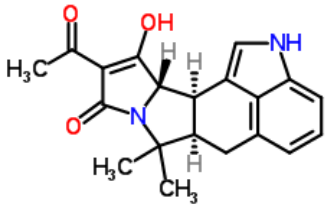
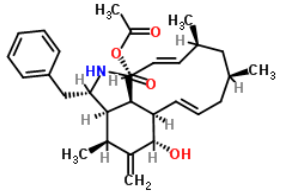
Compounds or secondary metabolites	Chemical structure	Molecular formula	Effects or functionalities	Occurrence	IARC group	References
N-acetyltryptamine		$C_{12}H_{14}NO_2$	Serotinin inhibitor		---	Kozlovsky et al. 2002
Aflatrem		$C_{32}H_{39}NO_4$	Tremorgene		---	Russell et al. 1989
Agonodepside B		$C_{24}H_{26}O_7$			---	Kim et al. 2016
AndrastinsA/B/C		$C_{28}H_{38}O_7$	Antitumoral compound	Blue cheese	---	Kim et al. 2012; Rojas-Aedo et al. 2017

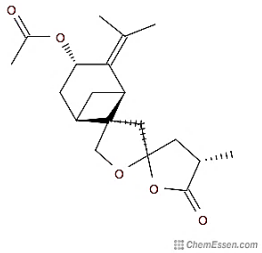
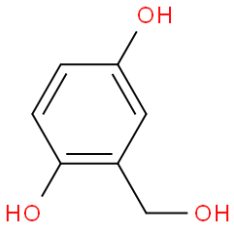
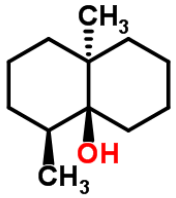
Aurantioclavine		$C_{15}H_{18}N_2$			---	King et al. 1977; Frisvad and Samson 2004
Brevianamide A		$C_{21}H_{23}N_3O_3$	Cytotoxic, teratogenic, insecticide		---	Paterson et al. 1987; Bridge et al. 1989; Russell et al. 1989
Chaetoglobosin A		$C_{32}H_{36}N_2O_5$	Orally toxic to 2-days-old cockerels, toxic to embryonic chickens and toxic and teratogenic to mice.	Apples, pears, quince, cherries, prune, bananas, peaches, apricots, grapes, cereals, silages, Blackcurrant, strawberry, walnuts, roots,	---	Springer et al. 1976; Bridge et al. 1989; Frisvad and Filtenborg 1989; Frisvad 1992; Vesely et al. 1995; Larsen et al. 1998; Andersen et al.

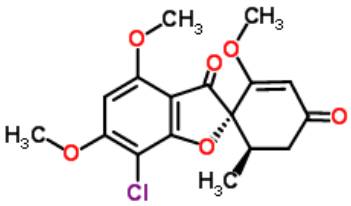
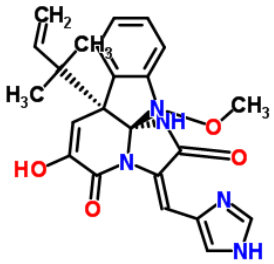
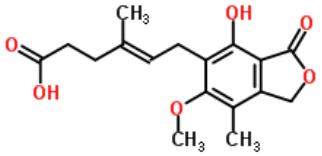
				brussels, carrots and tomatoes.		2004; Tannous et al. 2017a
Chaetoglobosin C		$C_{32}H_{36}N_2O_5$				
Citrinin		$C_{13}H_{14}O_5$	Kidney lesions	Peanuts, rice, grains, corn, barley, wheat	Group 3: Not classifiable as to its carcinogenicity to humans	Harwig et al. 1973; Frisvad and Filtenborg 1983; Paterson et al. 1987; Abou-Zeid 2012; Puel 2007; Andersen et al. 2004; Tannous et al. 2017a

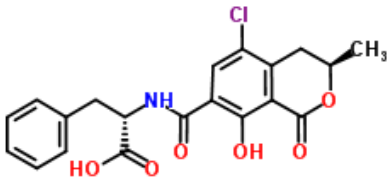
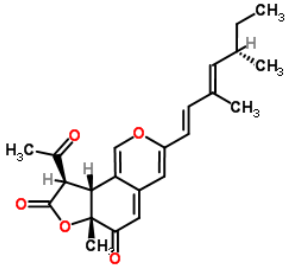
Clavicipitic acid		$C_{16}H_{18}N_2O_2$			---	King et al. 1977; Frisvad et al. 2004
Communesin A		$C_{28}H_{32}N_4O_2$	Cytotoxic	Apples, pears, quince, cherries, prune, bananas, peaches, apricots, grapes, cereals, silages, Blackcurrant, strawberry, walnuts, roots, brussels, carrots, tomatoes and marine alga.	---	Andersen et al. 2004; Samson et al. 2004

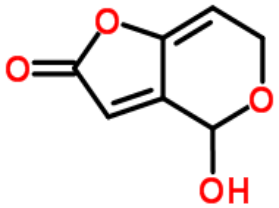
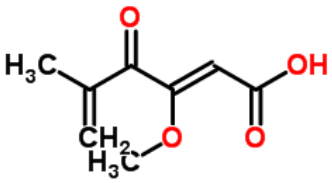
Communesin B		$C_{32}H_{36}N_4O_2$	Neurotoxic, cytotoxic to lymphocytic leukaemia cells	Apples, pears, quince, cherries, prune, bananas, peaches, apricots, grapes, cereals, silages, Blackcurrant, strawberry, walnuts, roots, brussel, carrots, tomatoes and marine alga.	---	Numata et al. 1993; Andersen et al. 2004; Samson et al. 2004; Tannous et al. 2017a
Communesin C		$C_{31}H_{34}N_4O_2$	Cytotoxic	Apples, pears, quince, cherries, prune, bananas, peaches, apricots, grapes, cereals, silages, Blackcurrant, strawberry, walnuts, roots, brussel, carrots,	---	Larsen et al. 1998; Andersen et al. 2004; Hayashi et al. 2004; Kerzaon et al. 2009

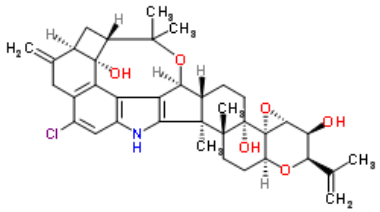
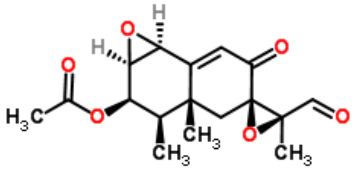
				tomatoes and marine alga.		
Communesin D		$C_{32}H_{34}N_4O_3$			---	
Cyclopiazonic acid		$C_{20}H_{20}N_2O_3$	health risk very minimal, but in high concentrations acutely toxic	cheese, corn, ground nut meal, peanuts	---	Bridge et al. 1989
Cytochalasin		$C_{30}H_{39}NO_4$	Cytotoxic		---	Lugauskas 2005
N-ethylaurantioclavine	Structure not available				---	King et al. 1977

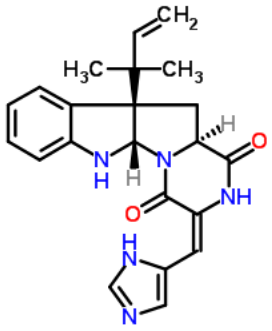
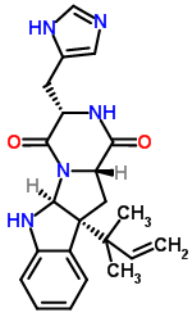
Expansolides A/B		$C_{17}H_{22}O_5$	Growth inhibition of wheat grain coleoptile	Blackcurrant and cherry juice	---	Massias et al. 1990; Larsen et al. 1998; Andersen et al. 2004; Tannous et al. 2017a
Fumaryl-d,l-alanine	Structure not available	$C_7H_9NO_5$	Antibiotic		---	Birkinshaw et al. 1942
Gentisyl alcohol		$C_7H_8O_3$			---	Bridge et al. 1989; Andersen et al. 2004
Geosmin		$C_{12}H_{22}O$			---	Mattheis and Roberts 1992

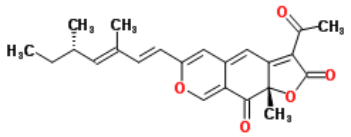
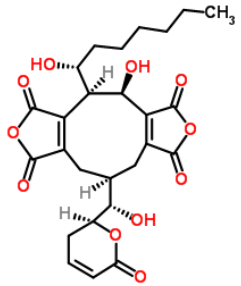
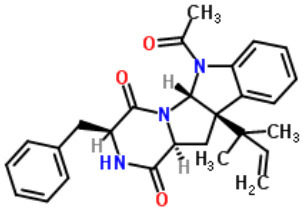
Griseofulvin		$C_{17}H_{17}ClO_6$	Antifungal; Teratogenic; antiproliferative and antimitotic activities towards cancer cells		2B	Bridge et al. 1989; Panda et al. 2005
Isorugulosuvine	Structure not available	$C_{20}H_{19}N_3O_2$			---	Kozlovsky et al. 2002
Meleagrin		$C_{23}H_{23}N_5O_4$	Cytotoxic effects against a number of tumour cell lines		---	Kozlovsky et al. 2002; Wang et al. 2015
Mycophenolic acid		$C_{17}H_{20}O_6$	antibacterial, antifungal, antiviral, antitumor activity and immunosuppressive	Silage	---	Bridge et al. 1989

Ochratoxin A		$C_{20}H_{18}ClNO_6$	immunosuppressive, nephrotoxic, possibly (human) carcinogenic, potent teratogenic; indications for role in Balkan endemic nephropathy (BEN)	barley, cereals, coffee beans, corn, dried fruits, rye, wheat, wine	2B	Krivobok et al. 1987; Paterson et al. 1987
Ochrephilone		$C_{23}H_{26}O_5$			---	Kim et al. 2016

Patulin		$C_7H_6O_4$	Pulmonary haemorrhages Degeneration of cerebral cortex neurons. antimicrobial, antiprotozoal, antiviral, genotoxic, immunological and neurological gastro-intestinal effects, toxic to both animal and plant	Apples, pears, cherries, bananas, peaches, apricots, grapes, cereals, silages	Group 3: Not classifiable as to its carcinogenicity to humans	Frisvad and Filtenborg 1983; Paterson et al. 1987; Andersen et al. 2004; Puel 2007; Fisch 2013; Tannous et al. 2017a; Frisvad et al. 2018
Penicillic acid		$C_8H_{10}O_4$	Antibiotic; Antiviral; Antitumor; Cytotoxic		3	Mintzlaf et al. 1972; Leistner and Pitt 1977

Penitrem A		$C_{37}H_{44}ClNO_6$	Cause tremors or similar nervous signs when administered to or ingested by animals	Cheese, peanuts, soil	---	Richard et al. 1986; Bridge et al. 1989
PR-toxin		$C_{17}H_{20}O_6$	Great toxicological concern, but unstable: converted into less toxic derivatives such as PR imine or PR amide and/or PR acid	Cereal, maize, forages/grass silages, cheese	---	Russell et al. 1989; O'Brien et al. 2006; Fernández-Bodega et al. 2009; Hymery et al. 2014
Raistrick phenols	Structure not available			Grains such as wheat, rice...	---	Frisvad and Filtenborg 1989

Roquefortine C		$C_{22}H_{23}N_5O_2$	bacteriostatic against Gram-positive bacteria, neurotoxic (paralytic) properties	Roquefort (blue cheeses), Wilted grass and whole-crop maize silages, apples, pears, quince, cherries, prune	---	Ohmomo et al. 1980; Frisvad and Filtenborg 1983; Bridge et al. 1989; Haggblom 1990; Auerbach et al. 1997; Andersen et al. 2004; Tannous et al. 2017a
Roquefortine D		$C_{22}H_{25}N_5O_2$				

Rotiorin		$C_{23}H_{24}O_5$			---	kim et al. 2016
Rubratoxin B		$C_{26}H_{30}O_{11}$	Lesions liver and kidney	Corn, animals feed	---	Richmond et al. 1980; Paterson et al. 1987
Rugulosuvine B		$C_{27}H_{29}N_3O_3$	Antibiotic and antitumoral activities		---	Kozlovsky et al. 2002

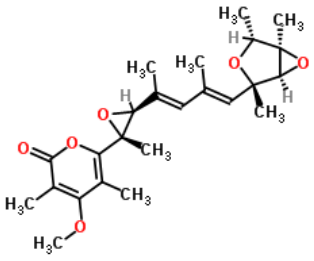
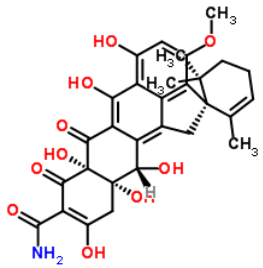
Verrucosidin		$C_{24}H_{36}O6$	Tremorgenic; mycotoxicosis in animals	Cereals	---	kim et al. 2016
Viridicatumtoxin		$C_{30}H_{31}NO_{10}$	Nephrotoxic		---	Hutchison et al. 1973; De Jesus et al. 1982

Table 2 Secondary metabolites of *P. expansum* reported in the literature

C.1 Effect of SM on virulence of *P. expansum*

The role of mycotoxins for their producing fungi is not yet fully elucidated. In order to get a better idea of this and to investigate the role of mycotoxins, the gene disruption technique has been used in many studies. It consists of blocking the biosynthetic pathway at a certain stage to accumulate intermediates (Proctor et al. 1995) or preventing it from the outset so that no intermediates or toxins can be formed (Tsai et al. 1998). Many studies have suggested the involvement of mycotoxins in the pathogenicity and virulence processes of the fungus against their natural substrates. It is important to distinguish pathogenicity from virulence. According to Yoder (1980), the pathogenicity of the fungus suggests its ability to induce disease and is considered a qualitative term; however, virulence is a more quantitative term and indicates the degree of disease caused by the fungus **(Figure 10)**. Virulence factors facilitate the growth of the fungus within the host organism and its spread (Hof 2008). Some studies have confirmed the importance of mycotoxins in the plant pathogenesis. Patulin, for example, has a role in the aggressiveness of *Penicillium expansum*. Sanzani et al. (2012) have shown that patulin plays a role in the development of blue mould decay on apples and may be involved in the virulence of *P. expansum* **(Figure 10)**.

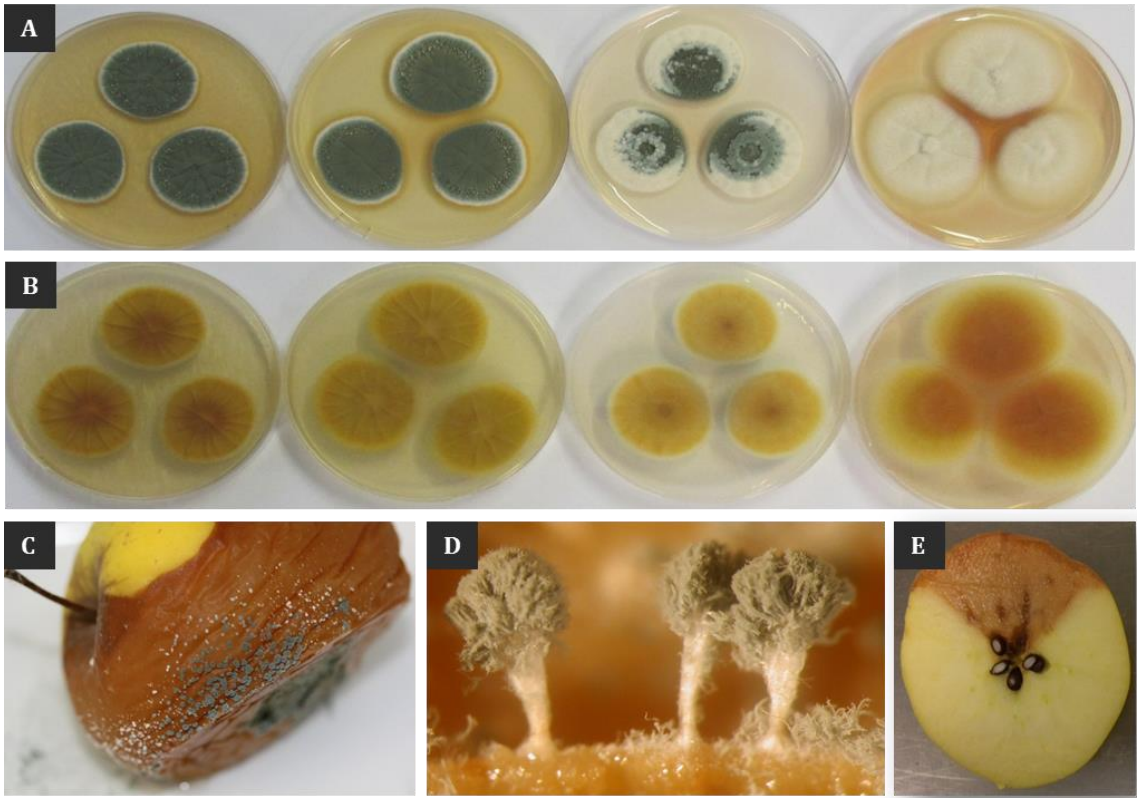


Figure 10 Macroscopic observations of *Penicillium expansum* and its rot. **A:** Colonies of *P. expansum* aggregated in fascicles **B:** Reverse cultures of *P. expansum* when 10^3 spores were grown at 25°C in the dark for 7 days on Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Czapek Dox Agar medium (CzA) and Czapek Glucose Agar medium (CGA) (from left to right); **C:**

Aspect of apple contaminated by *P. expanusm*; **D:** Coremia on the surface of an apple lesion caused by *P. expansum*; **E:** Transversal cut of a rotten apple (photos taken during our study)

Disruption of the gene encoding the enzyme 6-methyl salicylic acid synthase (Pepks) involved in the patulin biosynthetic pathway in a mutant strain resulted in a reduction in patulin production compared to a wild-type strain (WT) in an *in vitro* experiment. On apples artificially inoculated with the mutants, the incidence and severity of the disease were lower than those inoculated with the wild-type strains. The ability to cause disease was restored when patulin was applied exogenously and the phenotype of the WT was restored. This work suggests patulin acts as a virulence factor. Barad et al. (2014) used RNAi (ribonucleic acid interference) to generate *idh*-RNAi mutants, causing a down regulation of the *idh* gene, thus reducing patulin production. A decrease of the disease incidence and its severity were observed in this study. We could conclude from these studies that patulin acts a virulence factor but not as a pathogenicity factor since mutants were always able to produce it, even in smaller quantities. Unlike the two previous studies, Ballester et al. (2015) discussed the fact that neither patulin nor citrinin were directly involved in the pathogenic mechanism of *P. expansum* on apples. They suggested that nine predicted metabolic clusters, specifically expressed during infection, would be involved in the pathogenesis of *P. expansum*. Snini et al. (2016) generated a mutated strain of *P. expansum* deficient in *patL*, a specific regulatory factor for the patulin biosynthetic pathway, which has completely lost its ability to synthesize patulin. *In vivo* experiments showed that mutant strain PeΔ*patL* could still grow on apples even though patulin was no longer produced and that its growth depended on the apple varieties. This also confirmed that patulin acts as a virulence factor. Touhami et al. (2018) focused on the importance of citrinin in apple colonization. Mutants of *P. expansum* in which the *pks*CT gene, encoding the citrinin polyketide synthase, was inactivated, showed a drastic decrease in citrinin production and a reduction in the ability to colonize apples. The addition of exogenous citrinin restored the capacity of mutants to colonize apples in a manner similar to that of the wild type. The pks *patK* and *pks*CT genes, belonging respectively to the patulin and citrinin gene clusters, were both strongly expressed in the first phase of the apple colonization process.

VeA, the global regulator belonging to the velvet family is involved in the regulation of several cellular processes such as the secondary metabolism and sometimes the virulence of the fungus (Rauscher et al. 2016). LaeA is a virulence factor of the fungus in apple (Kumar et al. 2017). Δ*laeA* mutants were unable to fully accumulate patulin and colonize apples as WT strains of *P. expansum*. CreA, the global carbon catabolite regulator, has also an impact on virulence and patulin synthesis. C*reA* strains lose their capacity to produce patulin in apples and are almost avirulent (Tannous et al. 2018).

In addition to the impact of global regulators on the virulence of *P. expansum*, some studies showed that the acidity of the medium also plays in role (Prusky et al. 2004; Hadas et al. 2007). During colonization of apples, *Penicillium expansum* produces and secretes organic acids such as gluconic (GLA), citric, fumaric and oxalicacids, with gluconic acid (GLA) being the main acid produced by *P. expansum*, *P. digitatum* and *P. italicum* and the main cause of the decrease in the pH of colonized apples. Prusky et al. (2004) have shown that the citric and gluconic acids production of *P. expansum* in the decayed tissue reduced the host pH from 0.5 to 1.0 units and promoted the development of brown lesions in Fuji apples. Fumaric acid (Vilanova et al. 2014) and polygalacturonase (Jurick et al. 2010) have been reported for their contribution to the pathogenicity and virulence of the fungus. In fact, the accumulation of GLA, catalysed by glucose oxidase (GOX2), has made the host environment more acidic (Barad et al. 2012). In the same study, the authors showed that a downregulation of *gox2* affected GLA production and thus reduced acidification of the medium, apple colonization and patulin production. Barad et al. (2016) have shown that the accumulation of ammonia in the host environment due to the pathogen may lead to pH acidification and accumulation of SM such as patulin. In addition to the pH of fruits, other factors affect the production of patulin by pathogens, such as fruit cultivar and storage conditions, etc. (Damoglou and Campbell 1986; Morales et al. 2008; Sant'Ana et al. 2008). Beside SM, there are other cues that favour the establishment of the fungus. Some studies describe the effect of reactive oxygen species (ROS) on the virulence of *P. expansum* (Torres et al. 2003; Cerioni et al. 2013; Buron-Moles et al. 2015). These studies suggested that an increased H_2O_2 production by the infected host is correlated with greater resistance to pathogenic infection and reduces the progression of the fungus' growth. Recently, the mutants ΔPd*SUT1* and ΔPd*Slt2*, generated by elimination of *P. digitatum* genes, showed a reduction in fungal virulence during citrus infection. Pd*SUT1* appears to influence susceptibility to fungicides (Ramón-Carbonell and Sánchez-Torres 2017a) and Pd*Slt2* regulates different genes essential for infection and asexual reproduction (de Ramón-Carbonell and Sánchez-Torres 2017b). PeΔ*Ste12* mutants have also shown a significant decrease in the virulence of *P. expansum* (Sanchez-Torres et al. 2018).

To conclude this section, it has been shown that fungal toxins and SM of many mycotoxigenic genera act as mediators of pathogenicity or virulence infecting many foods and causing important diseases. Although we have focused on *Penicillium* species, many other fungi have similar virulence regulation systems. In order to know whether mycotoxins contribute directly to the pathogenic or virulent state of the fungus, the exploration of their molecular regulation remains a very important and interesting question with potential economic benefits.

C.2 Controls of *P. expansum*

During post-harvest storage of fruits, chemical treatments, cold storage or modified atmospheres could help control fungal diseases. However, spoilage fungi appear to be resistant to fungicides in some cases and do not provide satisfactory results. For example, *P. expansum* has developed resistance to antifungal compounds such as thiabendazol, fudioxonil, or pyrimethanil that are currently used after harvest (Errampalli et al. 2006; Li and Xiao 2008; Caiazzo et al. 2014). The control of patulin in apples and derived products is therefore not yet achieved. Although new post-harvest fungicides are being developed, the global trend is to reduce the use of fungicides in fruits and vegetables. The high and growing demand for healthy food, free of moulds and mycotoxins, has stimulated the search for alternative control techniques. Biological control using microbial antagonists to control post-harvest diseases has been extensively investigated. Different yeasts and bacteria (the microbiome) have been reported as antagonists and were naturally present on the surface of fruits (Droby et al. 2003; Droby 2006). Some of them showed an antagonistic activity against *P. expansum* (Chand-Goyal and Spotts 1997). Recent studies have highlighted the role played by *Cryptoccoccus laurentii* and *Rhodotorula glutinis* yeasts as agents for the control of *P. expansum* during apple storage and have highlighted the role of ROS during infection (Castoria et al. 1997; Castoria et al. 2001; Castoria et al. 2003; Castoria et al. 2005). Apple wounds are characterized by the presence of ROS and the production of enzymes such as peroxidases and lipoxygenase. The latter strengthen the cell wall of the wounded apples and protect it from infections by *P. expansum* or other pathogens. However, an excess of ROS during this period may favour fungal infection and patulin production (Tolaini et al. 2010). Co-infection of *P. expansum* with yeasts such as *C. laurentii LS28* and *R. glutinis* has been shown to reduce apple colonization and limit fungal growth. These yeasts, capable of producing high concentrations of superoxide dismutase (SOD) and catalase (CAT), can grow in such aggressive environments and could be considered as biocontrol agents for post-harvest pathogens (Castoria et al. 2003; Castoria et al. 2005). Some studies discussed the inability of *C. laurentii* to provide satisfactory levels of decay control when used alone. Tolaini et al. (2010) studied the combined effect of *C. laurentii* on an extract from the basidiomycete *Lentinula edodes* to improve the biocontrol activity of *C. laurentii*. *L. edodes* culture filtrates had a positive impact on the growth of *C. laurentii* and its catalase, superoxide dismutase and glutathione peroxidase activities, *in vitro*. The combination of these two microorganisms improved the inhibition of *P. expansum* growth and patulin production in wounded apples compared to *C. laurentii* alone. These results show promising microbial antagonistic activities. Recently, Sun et al. (2018) studied the role of the cell wall of *Rhodosporidium paludigenum* yeast in inducing disease resistance against *P. expansum* in pears. Germination of the latter was reduced *in vitro* and *in fruit* wounds accompanied by an increase in

defence enzyme activities and gene expression of pathogenesis-related (PR) proteins (produced by plants and induced as part of acquired systemic resistance). The biocontrol potential of *Bacillus subtilis*, *Agrobacterium tumefaciens* and *Rhodobacter sphaeroides* against *P. expansum* was also evaluated and the ability of these agents to inhibit fungal growth, PAT accumulation in apples and removal of PAT by absorption was investigated (Wang et al. 2016). These antagonists reduced rot diameter by 38%, 27.6% and 23.7%, respectively, and eliminated PAT production by 98.5%, 95.0% and 93.7%, respectively. *R. glutinis*, *Rhodotorula mucilaginosa*, and two *Candida oleophila* strains (L-06 and L-07) have also been tested for their potential antifungal properties on Golden Delicious apples (Guerrero-Prieto et al. 2013). Native strains of *C. oleophila* (L-06 and L-07) caused a 77 and 69% reduction in the severity of lesions caused by *P. expansum*, respectively and also showed great potential for biological control of *P. expansum* on post-harvest apples. Zheng et al. (2017) showed a similar impact on disease incidence caused by *P. expansum* and patulin accumulation using *R. mucilaginosa* and *Rhodotorula kratochvilovae* yeasts.

In addition to the use of yeasts and bacteria, many studies have focused on compounds that could control *P. expansum* and the incidence of blue mould disease. Sanzani et al. (2009) reported the effect of several phenolic compounds on the growth of *P. expansum* and its patulin production. Quercetin and umbelliferone appear to be the most effective against attack by this pathogen but no correlation has been demonstrated between fungal growth and patulin accumulation. It would be interesting to investigate in more detail the effect of phenolic compounds on the patulin biosynthesis pathway **(Figure 11)**.

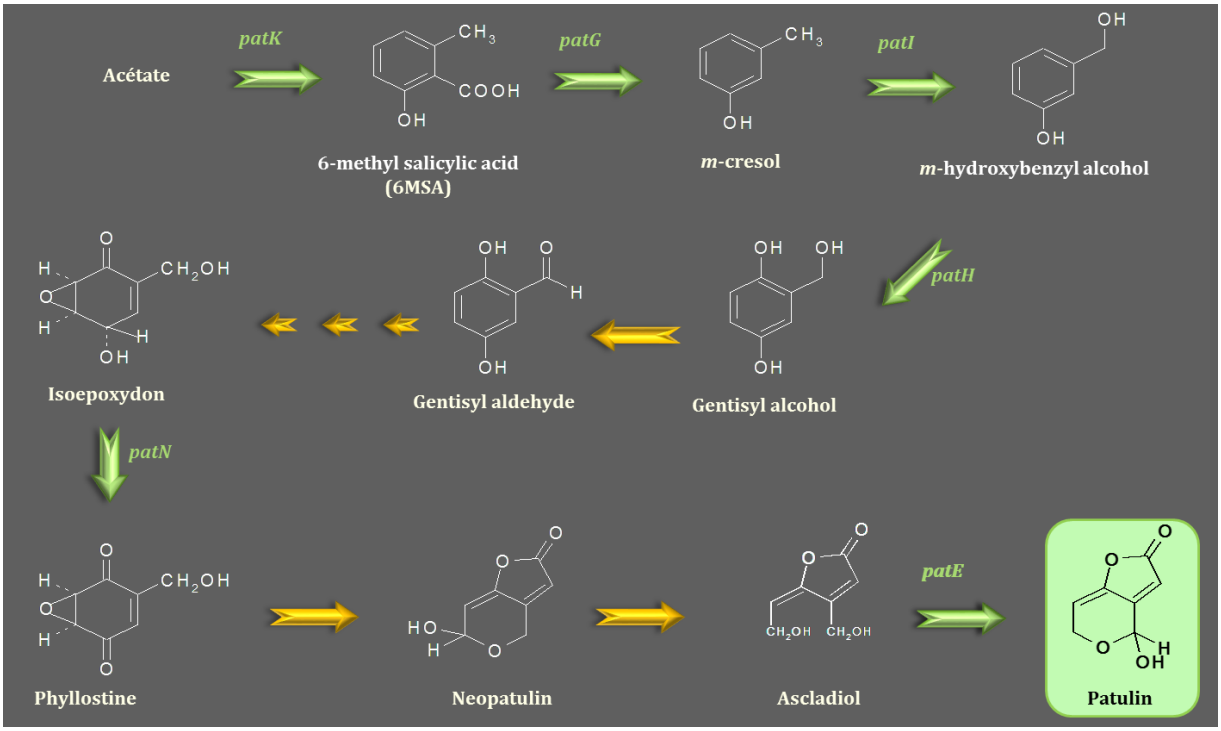


Figure 11 Patulin biosynthesis pathway in *P. expansum* (Tannous et al. 2014). The names in white correspond to the intermediates involved in the patulin biosynthesis pathway. Green names indicate the genes characterized for their involvement in either stage of biosynthesis.

Treatment with chlorogenic acid (CGA), an important phenolic compound that reduced the diameter of peach fruit lesions after infection with *P. expansum* (Jiao et al. 2018). CGA induces resistance against the fungus by activating the signalling pathway of salicylic acid and activating enzymes related to the main defence. Phosphites (Phi), elicitors of resistance to disease of abiotic origin, was also evaluated for their activity 2011). The incidence of blue mould has been reduced by about three times compared to that observed when Phi was applied on fresh unwounded apples. When applied to wounded apples, the incidence of *P. expansum* disease decreased significantly. Treatment with ***ε***-Poly-L-lysine, used against *P. expansum*, *in vitro*, and for their efficacy against blue mould infections on apple fruits (Amiri and Bompeix in food preservation, improved resistance to *P. expansum* in apple fruits by activating the ROS metabolism, the phenylpropanoid pathway and the accumulation of antifungal compounds (Ge et al. 2018).

Ribes et al. (2016) tested emulsions to preserve and extend the shelf life of strawberry jam. Essential oils of cinnamon leaf, clove, mandarin and lemon can reduce, inhibit or retard the growth of *P. expansum*. The antifungal activity of cloves and cinnamon leaf in oil and water emulsions against *P. expansum* was also proved in *in vitro* and *in vivo* tests. Cloves and cinnamon leaf, given their antifungal properties, should be considered as promising food additives for food preservation. The antifungal effect of cinnamon oils has been confirmed by Niu et al. (2018) who have shown that the encapsulation of cinnamon essential oil has antimicrobial activity and has a high efficacy against *Aspergillus niger* and *Penicillium sp.* The combination of cinnamaldehyde and citral (Cin/Cit) affected growth, oxidative damage and patulin biosynthesis in *P. expansum* in a dose-dependent manner (Wang et al. 2018). It inhibited its mycelial growth and spore germination, induced production of ROS, and down-regulated the genes included in the biosynthetic pathway of patulin. Cinnamon and cinnamaldehyde could be considered as potential candidates to control *P. expansum*. The mechanism of tea tree oil (TTO) was also investigated against *P. expansum* and *Botrytis cinerea* (Li et al. 2017b). Treatment with TTO and TTO vapours showed a lower impact on *P. expansum* compared to *B. cinerea* and this was due to the reduced disruption of the cell membrane caused in this organism. Chitosan, a polymer of N-acetyl-glucosamine in shellfish, has been studied in apples against *P. expansum* and its practical use to control blue mould has been evaluated (Yu et al. 2007; Darolt et al. 2016). Chitosan did not work in a curative way but was able to control and prevent the disease when applied at early stages, directly to fruit wounds. Improvements in the physico-chemical properties of this compound are needed. Applications of radiofrequency (RF) treatments for pasteurization and gamma irradiation

combined with fumigation have been tested to also replace the use of fungicides (Cheon et al. 2016; Hou et al. 2018). The first treatment affected the development and growth of *P. expansum*, and the second treatment reduced the incidence of blue mould disease.

Finally, the composition of the atmosphere also has a decisive influence on patulin production. Controlled atmosphere conditioning of 3% CO_2 and 2% O_2 inhibited patulin production at 25°C, but the latter was restored under a modified atmosphere of 2% CO_2 and 10-20% O_2 (Paster et al 1995). Likewise, no patulin production was reported in apples stored 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. This production was restored after subsequent storage of apples at 20°C (Morales et al. 2007).

Although different strategies have been discussed in this section and different compounds have shown good control of *P. expansum* growth and patulin accumulation, biological control remains one of the most promising alternatives for the control of blue mould on apples after harvest and is considered a useful strategy for the control of postharvest disease in other fruits.

C.3 Conclusion

In conclusion, this section focused on the virulence of *P. expansum* and how to control it. Many mycotoxins appear to contribute directly to the pathogenic or virulent state of the fungus, but exploring their molecular basis remains a major issue. Among the control approaches studied so far, biological approaches are a very promising tool to increase disease resistance and inhibit fungal development. But despite all these preventive measures and control approaches, *P. expansum* continues to cause serious damage, patulin production and economic losses. In the following section, we will present research on patulin detoxification and mitigation techniques in apple products and focus on ascorbic acid and it influence on patulin concentration.

D. Fate of patulin in apple juices with special emphasis on the impact of ascorbic acid (review 2)

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Abstract

Patulin (PAT) contamination, most commonly encountered in products derived from fruit processing, especially apples is mainly caused by *Penicillium expansum*. This toxin is of a major concern and is considered as an important hazard due to its toxicity. Its presence in food is regulated and maximum levels of 50 µg/kg for fruit juices, 25 µg/kg for apple purees and compotes and 10 µg/kg for food intended for babies and young children have been set by the European Union (EU). Still, PAT is found in apple-derived products, sometimes exceeding the maximum regulatory limits. This review gives an overview on PAT mitigation research performed, more specific, of how the PAT concentration can be affected by factors like storage, handling, transport, pasteurization, filtration, as well as other non-thermal processing steps. It summarizes knowledge on PAT detoxification techniques in apple products with a special emphasis on ascorbic acid (AA), its stability and how AA may influence the PAT concentration and phenolic compounds. The data presented in this review sheds a light on a compound that could have a major role in PAT degradation in apple products.

Keywords: ascorbic acid, patulin, degradation techniques, cloudy apple juice, ascladiol

D.1 Introduction

The quality and safety of numerous agri-food products has always been threatened by many natural toxins, including mycotoxins, or metabolites of low molecular weight (<1000 g/mol) produced by filamentous fungi, particularly those belonging to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps* (Marin et al. 2013).

Patulin (PAT) is a mycotoxin produced mainly by the species *Penicillium*, *Aspergillus*, *Byssochlamys* and *Paecilomyces* (Sommer et al. 1974; Puel et al. 2010) and is the main mycotoxin produced by *Penicillium expansum* (Andersen et al. 2004; Tannous et al. 2017a).

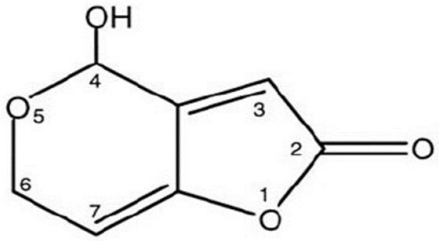

Figure 12 Chemical structure of patulin

PAT **(Figure 12)** is an unsaturated heterocyclic lactone $C_7H_6O_4$ with a molecular weight of 154.121 g/mol, characterized by a melting point of 111°C and a maximal UV absorption at 275 nm. It is soluble in water and in most organic solvents. This mycotoxin can be produced at all temperatures allowing the growth of the fungus being, between 0 and 30°C (Sommer et al. 1974). PAT production was proven to be negatively correlated with pH value. PAT is stable in acid medium but becomes unstable at high pH and loses its activity in alkaline conditions due to the opening of the lactone ring (Cox and Cole 1981; Morales et al. 2008; McCallum et al. 2002). It remains unclear whether low pH favours the production of PAT or high pH results in its degradation. Its stability at high temperatures implies that food preservation processes such as pasteurization between 62 and 80°C are not sufficient to destroy it (Sommer et al. 1974). Alcoholic beverages present less problems related to PAT contamination due to the alcoholic fermentation process (e.g. apple cider) (Stinson et al. 1978; White et al. 2006). During the fermentation process, the mycotoxin is largely degraded into ascladiol by yeasts (Moss and Long 2002), considered to be a non-toxic compound (Tannous et al. 2017b; Maidana et al. 2016). The apple variety also influences the production of PAT by *Penicillium expansum* (McCallum et al. 2002). A study of Zong et al. (2015) focusing on carbon and nitrogen sources showed that glucose-containing sugars, complex N-sources, and acidic conditions favour the production of PAT. Thus, the authors suggest a certain link between PAT production/accumulation and carbon sources. However, more research needs to be done in order to elucidate this possible linkage. We also have to mention that

the infection by *P. expansum* and thus the production of PAT are largely favoured by injuries of the fruit.

In recent years, several surveys on the level of contamination of apples and by-products by PAT have been conducted in various countries around the world. The overall incidence of PAT in Belgian apple juice during 2001 was 79%, compared to 86% for apple juice imported from France, Germany and Switzerland, with an average contamination of apple juice of 10.3 µg/kg and 6.4 µg/kg respectively (Tangni et al. 2003).

Exposure of humans or animals to PAT is associated with immunological, genotoxic and neurological disorders (Puel et al. 2010; Assunção et al. 2016; Tannous et al. 2017a). Several in vitro studies have shown that PAT inhibits the functions of macrophages and presents immunotoxic effects on many immune system cells (Sorenson et al. 1986; Bourdiol et al. 1990). De Melo et al. (2012) observed a dose-dependent increase in damage index (DI) in the brain, liver and kidney of mice exposed to 2.5 and 3.75 mg/kg of PAT. According to Devaraj and Devaraj (1987), repeated doses of PAT cause neurotoxicity leading to tremors and convulsions, as well as inhibition of many enzymes in the intestine and brain.

In 1995, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) re-evaluated the Provisional Tolerable Weekly Intake (PTWI) for PAT and changed it into a Provisional Maximum Tolerable Daily Intake (PMTDI). This assessment considered that the majority of PAT ingested by rats was eliminated within 48 hours and 98% within the 7 following days. According to that, they established a PMTDI of 0.4 µg/kg bodyweight (bw)/day based on a No-Observed Effect Level (NOEL) of 43 µg/kg bw/day and a safety factor of 100 recalculated from the rat study. Maximum intake levels for children and adults are even below the PMTDI, i.e. 0.2 µg/kg bw/day and 0.1 µg/kg bw/day, respectively.

Considering the cytotoxicity, genotoxicity and immunosuppressive properties of PAT, the European Union (EU) has established a specific regulation to protect consumer health: PAT is set at a maximum level of 50 µg/kg for fruit juices and derived products, 25 µg/kg for apple purees and compotes and a maximum level of 10 µg/kg in food intended for babies and young children (EC Commission Regulation, 2006).

Apple juices as well as other food products are sometimes heavily contaminated by PAT **(Table 3; Table 4)**. Therefore, more studies and efforts are needed to minimize the contamination of food products by this mycotoxin.

Numerous review papers on PAT have provided information on its biosynthesis, toxicity, prevention, detection and degradation (Moake et al. 2005; Silva et al. 2007; Sant'Ana et al. 2008; Puel et al. 2010; Ioi et al. 2017). However, none of these discussed in detail the mitigation of this mycotoxin by ascorbic acid (AA) and the factors that affect its stability in the food matrix. The

present review aims at giving an overview of PAT mitigation research. More specifically, the review summarizes knowledge on PAT detoxification techniques in apple products with special focuses on AA, its detection methodology, its stability, and the influence of AA on PAT and phenolic (apples) compounds.

D.2 Determination of PAT in apple products

The high global production and consumption of apples and apple-based commodities led to an enormous improvement in the monitoring, controlling, detection and/or quantification of unwanted compounds (e.g. PAT) that might be present in these products. PAT and other fungal secondary metabolites are not detected by smell or taste. Only physicochemical analysis can reveal their presence.

a. Chromatographic methods

Over the years, different analytical methods have been developed to allow the detection of PAT: Thin-Layer Chromatography (TLC), Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography coupled to UltraViolet detection (HPLC-UV) or tandem Mass Spectrometry (HPLC-MS/MS), Capillary Electrophoresis (CE), etc. Each analytical approach has its (dis)advantages. These numerous methods cited above were described into detail in the literature in the past (Shephard and Leggott 2000; Li et al. 2017).

Based on a study performed by Scott in 1974, a TLC method for PAT analysis was officially approved by the Association of Analytical Communities (AOAC). It consisted of an extraction using ethyl acetate, followed by a separation on a chromatographic column with silica gel. The detection was conducted by a pulverization of 3-methyl-2-benzothiazolinone hydrazone. This method proved to be very efficient and effective for PAT analysis and was used in several studies regarding PAT quantification in food (Martins et al. 2002; Welke et al. 2009b). Later, the application of TLC methods for PAT determination in apple juice has been proven to be very limited in terms of sensitivity and not used frequently anymore nowadays.

Gas chromatography was also used for the analysis of PAT. The first gas chromatographic/ mass spectrometric (GC/MS) method for PAT detection in apple juice goes back to 1977 for PAT acetate (Ralls et al. 1977). Since then, numerous GC-MS analyses with electron impact ionization, using silylated PAT have been reported (Moukas et al. 2008). This technique also allowed the determination of non-derived PAT in apple juice in the negative chemical ionization mode (Roach et al. 2000).

The GC-MS analytical method cited above is still used on a regular base in some laboratories for the determination of PAT. But the progress in research led to the appearance of High Performance

Liquid Chromatography (HPLC) and many publications followed describing HPLC procedures with more complicated clean-up protocols (Stray 1978).

Reversed phase HPLC coupled to UV detection (absorption of PAT around 276 nm) was considered as the most proper method for PAT analysis having a high sensitivity, especially because PAT strongly absorbs UV. Macdonald et al. (2000) validated the effectiveness of an HPLC-UV procedure determining PAT in clear and cloudy apple juice as well as in apple puree. Ethyl acetate extraction was applied for clear apple juice, while cloudy apple juice and apple puree were first treated with a pectinase enzyme before extraction. This method showed acceptable precision and has been considered as an AOAC Official Method. It has become by far the most appropriate and frequently used technique for the detection and quantification of PAT in food matrices (Murillo-Arbizu et al. 2008, Barreira et al. 2010).

In later stages, De Clercq et al. (2016) reported a more sensitive HPLC-UV method, based on the Association of Analytical Communities (AOAC) Official method 2000. They validated the analysis of PAT on apple puree agar medium (APAM) (Baert et al. 2007), cloudy apple juice, and apple puree. This method was characterized by a detection limit of 3 – 4 µg/kg and a quantification limit of 5 – 8 µg/kg for APAM, apple juice, and puree.

Interfering compounds such as 5-hydroxy-methylfurfural (HMF), a product generated during pasteurization of apple juice and presenting similar chromatographic properties as PAT, make it difficult to properly determine the amount of PAT in apple juice due to the overlapping peaks. Liquid chromatography coupled with mass spectrometry (HPLC-MS) has been described and used in some other studies and opposed to this problem (Bandoh et al. 2009; Christensen et al. 2009). LC-MS methods have proven to be more robust and reproducible than the GC-MS methods mentioned above. They can be coupled to different ion sources from which we can cite the electrospray ionization (ESI) source, often used. In numerous studies concerning the determination of PAT in apple juices by means of a solid-phase extraction-liquid chromatography-mass spectrometry (SPE-LC-MS), a limit of detection that is almost 10 times higher compared to an HPLC with a UV detector was attained (Ito et al. 2004). The latter remains the most used methodology nowadays for PAT detection and a methodology, resolving the interfering compounds problem, has been optimized (see CHAPTER 2_Part 1).

b. Other methods

In addition to chromatography-based methodologies other innovative approaches, mostly methods based on specific (bio) sensors (e.g. immune-sensors based on antibody recognition), have been developed and allow determination of PAT.

Unlike other mycotoxins, the low molecular weight and electrophilic properties of PAT are two factors that make it hard to develop polyclonal antibodies directed against this molecule (Boonzaaijer et al. 2005). Nevertheless, a nano-porous silicon (NPS) based immuno-sensor was developed in 2013 by Starodub and Slishek to detect PAT. An antibody against PAT is immobilized on the NPS and reacts with the toxin. However, the effectiveness of the signal decreased with the increase of the PAT concentration making this method only effective for screening purposes. Recently, a new biosensor, based on urease inhibition by PAT, was proposed by Soldatkin et al. (2017). In fact, in this system, PAT interacts with sulfhydryl groups of the active site of the enzyme and inhibits the biological activity. The PAT concentration is quantified by determining the inhibitory potency of the enzymatic reaction. This method is characterized by a high sensitivity, selectivity and reproducibility. Guo et al. (2017) used, for the first time, the molecularly imprinted polymer (MIP) technology, an alternative to antibody-based sorbents and a replacement to immunoassays, combined with carbon dots, chitosan and Au (gold) nanoparticles modification for an electrochemical detection of PAT.

Quartz crystal microbalance (QCM) technology was also used for PAT analysis (Funari et al. 2015). This technique consisted on tethering the antibodies side-up by means of the Photonics Immobilization Technique (PIT). A limit of detection of 140 nM was achieved for PAT using this method and the tests done on extract of apple puree showed an excellent specificity for PAT. Recently, Bagheri et al. (2018) have developed an Ag (silver) nanoparticle/flake-like Zn-based metal organic framework (MOF) nanocomposite (AgNPs@ZnMOF) allowing a selective determination of PAT and its measurement with no important interfering effects from other similar compounds.

Further achievements have been made for PAT determination and detection. However, very useful methods for quantifying PAT in apple juice are not necessarily good methods in terms of sensitivity or specificity in other matrices such as fruit leathers. Hence optimization of methods allowing faster screening, higher sensitivity and selectivity in different food items remains needed.

D.3 Techniques decreasing PAT concentration and its degradation products

PAT is mainly found in mouldy (rotten) apples, although the presence of moulds does not necessarily confirm the presence of PAT, it only indicates the risk. PAT can be found in fruit showing traces of bruising after harvest or during storage in a controlled atmosphere and exposure to ambient conditions, with or without the development of deep rot. Rinsing the fruit or removing mouldy tissue prior to pressing does not necessarily remove all the PAT present in the fruit as it may have spread to visibly healthy tissue. Internal mould growth may also occur because

of intense invasion of apples by insects, which consequently may lead to PAT production in an apparently intact fruit.

Due to the broad spectrum of PAT activities, its toxicity demonstrated by in vivo and in vitro studies, and the significant human exposure, different control strategies, that could be applied all along the apple juice processing steps have been developed (Figure2).

A distinction can be made between (1) the strategies applied at early stages, i.e. control of pathogen development during harvest or storage of apples, and (2) strategies applied during further processing of apple juice.

a. Strategies preventing PAT contamination of the raw material for apple juice

The presence of PAT can be controlled and reduced not to exceed processing acceptable levels in food. Before processing, measures can be applied in the field and this by applying good agricultural practices, using insecticides / fungicides, at harvest or during apple storage. In fact, the harvest method influences the quality of apples and is the first stage in controlling PAT levels. The harvested fruit should be handled as gently as possible and efforts should be made to reduce physical damage at all stages of harvesting and transport procedures. Cider produced from seven varieties of apples harvested from the tree were PAT free, whereas cider produced from four varieties of apples picked up from the ground contained PAT concentrations ranging from 40.2 to 374 µg/kg (Jackson et al. 2003). Washing and sorting have been considered effective post-harvest treatments in controlling fungal contamination and PAT production. Acar et al. (1998) have shown that a high-pressure fruit washing technique, eliminating the rotten parts of these fruits, significantly reduced the levels of PAT found in apple juice up to 54%. Storage at low temperatures combined with controlled atmosphere was shown to be an effective preventive strategy for the growth of *P. expansum* (Sitton and Patterson 1992).

Chemical control of PAT-producing fungi that can contaminate fruit has been applied for ages. Currently, many fungicides have several levels of efficacy. Benzimidazole gives satisfactory results in reducing apple rot at a post-harvest stage and thus reducing PAT production. However, the massive non-recommended use has led to the emergence of a resistant strain of *P. expansum* (Rosenberger et al. 1991).

Fludioxonil is another conventional fungicide that has been shown to have an impact on the different stages of the life cycle of *P. expansum*. The study of its activity on decay formation in Empire and Gala apple cultivars suggests that this compound could be a good alternative to other fungicides in post-harvest control of blue mould of apple (Errampalli 2004). The use of antifungal agents of natural origin has given satisfactory results as well. Propolis, a natural honeybee product, can be used as a natural antifungal agent for *P. expansum*. Silici and Karaman (2014) have

shown that this natural product can inhibit the growth of the fungus, therefore preventing PAT production.

b. Strategies degrading PAT in apple juice

The methods described above help to prevent rot development in stored apples. They require intensive work and are often prone to failure. In addition, a lot of 1,000 apples containing a single apple contaminated with *P. expansum* may be enough to have a PAT concentration exceeding the maximum acceptable level (Salomão et al. 2009). The quality of the harvested apples affects the overall quality of finished apple products. In this chapter, physical, chemical and biological practices that may affect the PAT concentration in apple juice have been categorized. However, we should notice that most of these practices are indications from research studies and that treatments can have an impact on the nutritional and organoleptic quality of the final product as well.

i. Physical treatments

Clarification or filtration techniques are frequently used in the fruit juice manufacturing industries, initially applied to remove solid particles (Acar et al. 1998). However, these also potentially reduce the PAT level in liquid apple products. Adsorption on activated charcoal followed by filtration is the oldest physical treatment and is known to reduce PAT concentration (Huebner et al. 2000). Sands et al. (1976) have shown a reduction of PAT concentration (30 µg/ml) that was up to 91.36% in naturally contaminated cider by the addition of 5 mg/mL of activated charcoal. Even though this treatment is effective, its use is limited due to its negative effects on the organoleptic and physico-chemical properties of the apple juice (color, fumaric acid concentration, pH and brix) (Kadakal and Nas 2002). In addition, it remains an expensive process that can have a strong impact on the environment and regulatory aspects should be taken into account as well.

The effect of γ-irradiation carried out by Cobalt-60 (Co-60), on the PAT concentration and on the chemical composition of apple juice showed a reduction of the mycotoxin in relation to the absorbed dose; 0.35 kilogray of γ-radiation resulted in a 50% reduction of the PAT concentration. However, irradiation of apple juice samples induced acceleration of non-enzymatic browning during storage (Zegota et al. 1988). Other studies aimed to unify methods capable of reducing PAT load while maintaining product quality. Germicidal UV irradiation has shown to have some efficacy in reducing PAT levels in clarified apple juice (Dong et al. 2010, Assatarakul et al. 2012, Zhu et al. 2013, Tikekar et al. 2014). However, it is not practical for unfiltered cider because of the high levels of suspended particles (initial turbidity level of 950 Nephelometric Turbidity Units or

NTU in unfiltered cider compared with 3 NTU in clear apple juice) that absorb or block the radiation before reaching the PAT molecules (Tikekar et al. 2014). UV radiation appeared to be effective without significant alterations in the chemical composition (i.e., pH, Brix, and total acids) and organoleptic properties of the final product (Dong et al. 2010).

Other non-thermal methods, e.g. pulsed light and high hydrostatic pressure (HHP) treatments, have also been proposed for PAT degradation in food products. PAT reduction by pulsed light in McIlvaine buffer (0.1 M citric acid and 0.2 M Na_2HPO_4; pH 3.5), apple juice and apple puree was up to 85-95%, 78% and 51%, respectively (Funes et al. 2013). The degradation level depended on the pulsed light dose and the matrix evaluated. HPP showed to have a great potential in producing high-quality foods that were free from additives, had a fresh taste, were microbiologically safe and for which an extended shelf-life could be obtained (Patterson 2005). Hao et al. (2016) have evaluated the degradation of PAT in different juices that were exposed to HHP treatment. The PAT degradation level depended on the pressure applied as well as on the processing time and was positively correlated with the sulfhydryl group (such as proteins, enzymes and other components of cells) concentration of the juice. HHP is considered as a risk management tool controlling PAT in apple-based beverages, but the processing conditions and compositions of juice remain the two factors that control the amount of mycotoxin degradation. Avsaroglu et al. (2015) have studied the effect of pulsed-HHP (p-HHP) compared to HHP on the PAT concentration (5, 50 and 100 µg/kg) in apple juice. They found that p-HHP was more effective at lower initial PAT concentrations, while HHP was more effective at higher initial PAT concentrations.

ii. Chemical treatments

Some chemical methods have also been found to be effective for PAT detoxification, on a laboratory level.

The degradation of PAT by ammonisation and oxidation by potassium permanganate in acidic and basic environment/media were studied by Frémy et al. (1995). In an aqueous standard solution, treatments led to a 99.9% degradation of PAT. However, in acidic conditions/media, mutagenic residues were generated by the treatment.

Sulphur compounds have also been used to detoxify food products due to the instability of PAT in the presence of these components. A study performed by Ough and Corison (1980) reported a 50% reduction in PAT levels only 15 minutes after the application of 100 mg/kg of sulphur dioxide (SO_2.) In contrast, Burroughs (1977) has shown that the affinity of PAT for SO_2 is not very significant. A concentration of 200 mg/kg of sulphur dioxide (maximum allowed in apple juice and cider production) decreased the PAT level by only 12% in 24 hours. Such a contradiction between the two studies could be due to the difference between the samples and conditions.

Ascorbate (salt of AA) has been shown to accelerate the degradation of PAT in apple juice (Brackett and Marth 1979). The addition of AA or vitamin C (5%) to a contaminated apple juice (300 µg/kg PAT) reduced the level of contamination close to 80% after 15 days of storage at 4°C (Brackett and Marth 1979). More information about AA and its impact on PAT will be elaborated in the last part of this review.

In addition to AA, Yazici and Velioglu (2002) have shown a significant reduction in PAT levels in apple juice concentrates treated with pyridoxine hydrochloride, thiamine hydrochloride and calcium D-pantothenate. Applying 2,500 mg/kg calcium-D-pantothenate resulted in a 94% reduction in PAT level with a good preservation of the quality parameters (such as turbidity, colour and clarity) of the product after 6 months at 22°C (Yazici and Velioglu 2002). In contrast, only 36% of PAT reduction was observed in the control sample of apple juice concentrates stored for 6 months at 4°C. Even if the results seem promising and satisfactory, the long incubation period may not be transferable to an industrial level.

In addition, another chemical detoxification process involves the use of ozone (O_3). According to McKenzie et al. (1997), the toxicity of PAT, cyclopiazonic acid, ochratoxin A, secalonic acid and zearalenone significantly decreased in aqueous equimolar (32 µM) solutions contaminated by this toxin following a treatment with O_3 after 15 seconds, with no by-products detectable by high performance liquid chromatography (HPLC).

To conclude, the toxicity of all these products cited above has not been fully evaluated and without this information their use cannot be considered.

iii. Biological treatments

Despite the results of the different chemical treatments discussed above, the global trend is now to reduce the use of chemicals and to develop alternative methods. A biocontrol step in apple juice, using microorganisms such as the yeast *Saccharomyces cerevisiae* (Guo et al. 2012a; Moss and Long 2002) or the gram-negative bacterium *Gluconobacter oxydans* (Ricelli et al. 2007) is one of these alternative methods. These methods generate degradation products such as E-ascladiol (ASC-E), Z-ascladiol (ASC-Z) and desoxypatulinic acid (DPA) that are less toxic than the mycotoxin itself or even non-toxic (Tannous et al. 2017b; Zhu et al. 2015).

Although biological methods are effective in detoxifying PAT, their use is limited to fermented products. During alcoholic fermentation of apple juice, PAT was reduced by 99% compared to only 10% of reduction in unfermented apple juice (Stinson et al. 1978). In another study conducted by Moss and Long (2002) considering three commercial cider strains of *S. cerevisiae*, 51.4% of PAT was metabolized during active fermentative growth compared to 16% in the control, however

only under aerobic conditions. Polar degradation products were generated and corresponded to the isomers ASC-E and ASC-Z.

In addition to microorganisms known to degrade PAT, others (e.g. *Rhodosporidium paludigenum*) have been described for their ability to adsorb this mycotoxin through their cell walls without degrading it (Hatab et al. 2012b; Zhu et al. 2015).

Besides yeasts, bacteria can also affect the PAT concentration in juices. The bacterium *Gluconobacter oxydans*, originating from rotted apples containing PAT, caused up to 96% degradation of PAT in apple juices contaminated with 800 µg/mL of PAT (Ricelli et al. 2007).

Some of the lactic acid bacteria (LAB) strains used in food processing are known to degrade PAT. These strains are especially favoured as they are considered as food-grade microorganisms. Heat-inactivated LAB cells were shown to have a role in the adsorption of PAT (Wang et al. 2014; Hatab et al. 2012a; Fuchs et al. 2008). Of six LAB strains (*Lactobacillus curvatus* 21019, *Lactobacillus rhamnosus* 6133, *L. rhamnosus* 6224, *Lactobacillus brevis* 20023, *Enterococcus faecium* 20420 and *E. faecium* 21605) used in the study, the highest cell wall volume and specific surface area was attributed to *L. brevis* 20023 (LB-20023), which had the highest ability to adsorb PAT from aqueous solution (Wang et al. 2014). This adsorption capacity is linked to the polysaccharides and/ or protein functional compounds (C–O/ OH/ NH functional groups) of cell walls (Wang et al. 2014; Guo et al. 2012b; Dalié et al. 2010). *Bifidobacterium animalis* VM 12 caused an 80% PAT reduction in a liquid medium (Fuchs et al. 2008) whereas a PAT reduction of 80.4 and 64.5% in apple juice was obtained in the presence of *L. rhamnosus* 6224 and *E. faecium* 21605, respectively (Hatab et al. 2012a).

c. Degradation products of PAT

Numerous studies observed that PAT is degraded into compounds that are potentially less toxic (Castoria et al. 2011; Moss and Long 2002; Tannous et al. 2017b). Biodegradation products of mycotoxins are known for many years and they are generally recognized as less toxic than the original molecule, or even non-toxic. However, toxicity data are very limited compared to data on the toxin itself and hence, more research is needed.

ASC-E is considered the predominant degradation product and a direct precursor of PAT as well (Tannous et al. 2017b). It was considered a mycotoxin because it preserves a quarter of the toxicity of PAT (Suzuki et al. 1971). ASC-Z is an isomer of ASC-E that is non-enzymatically catalysed by sulfhydryl compounds (Castoria et al. 2011; Sekiguchi et al. 1983).

Tannous et al. (2017b) investigated the cytotoxic potential of ASC (E and Z isomers) in vitro for four human cell lines. Their analysis showed no evidence of the cytotoxicity of ASC at concentrations up to 100 µM. On the contrary, PAT tested under the same conditions and with the

same concentration altered negatively the proliferation of human cells. Another study done by Maidana et al. (2016), showed that high levels of PAT can induce mild toxic effects on intestinal mucosa while ASC seemed non-toxic to intestinal tissue.

Besides ASC, DPA was also considered a degradation product of PAT. DPA was found to be slightly toxic for human lymphocytes at concentrations of 50 µM and 100 µM and over a 24-hour incubation period. However, 1 µM of PAT concentration was already enough to cause a decrease of approximately 30% of viable cells after 24 hours of incubation. The low toxicity of DPA is proposed to be a consequence of the hydrolysis of the lactone ring, enabling the molecule to interact with thiol groups (Castoria et al. 2011). Although ASC-E was shown to be the most stable isomer (Sekiguchi et al. 1983), this cannot be generalized. *Sporobolomyces sp.* IAM13481 converted PAT to DPA and ASC. In this case, ASC-E was the transient metabolite, while DPA and ASC-Z were the final degradation products (Ianiri et al. 2013; Chen et al. 2017).

In another study, DPA was found to be the major degradation product and ASC-E/Z were early transient ones (Castoria et al. 2011).

Therefore, it appears that different mechanisms are involved in PAT degradation and that the degradation pathway is dependent on the organism used and conditions tested.

D.4 PAT control steps during apple juice processing

Ready-to-drink pasteurized apple juice can be obtained either by directly processing the fruits or by reformulating and diluting a concentrate of the juice to the desired soluble solid contents. **Figure 13** adapted from Sant'Ana et al. (2008) illustrates the production of both types of apples juices. The diagram represents the general process, so some steps may be slightly different from one industry to another. The main purpose of this illustration is to be able to identify the possible hazards occurring at each step and to specify the critical control points (CCP). CCPs are specific steps in the food manufacturing which should be controlled in order to prevent, reduce, or eliminate the occurrence of a food safety hazard. Consequently, by defining these CCPs, control actions and preventive measures can be established. Some of the control points can be applied before industrial processing (e.g. in the field, at harvest, etc.) and are considered as preventive actions (see previous paragraph entitled “Strategies degrading PAT in apple juice”); others can be applied during or after processing and are considered more control actions as per the Hazard Analysis Critical Control Points (HACCP) system. In this section, the latter is specifically addressed. The main focus will be on the hazards related to the contamination of PAT.

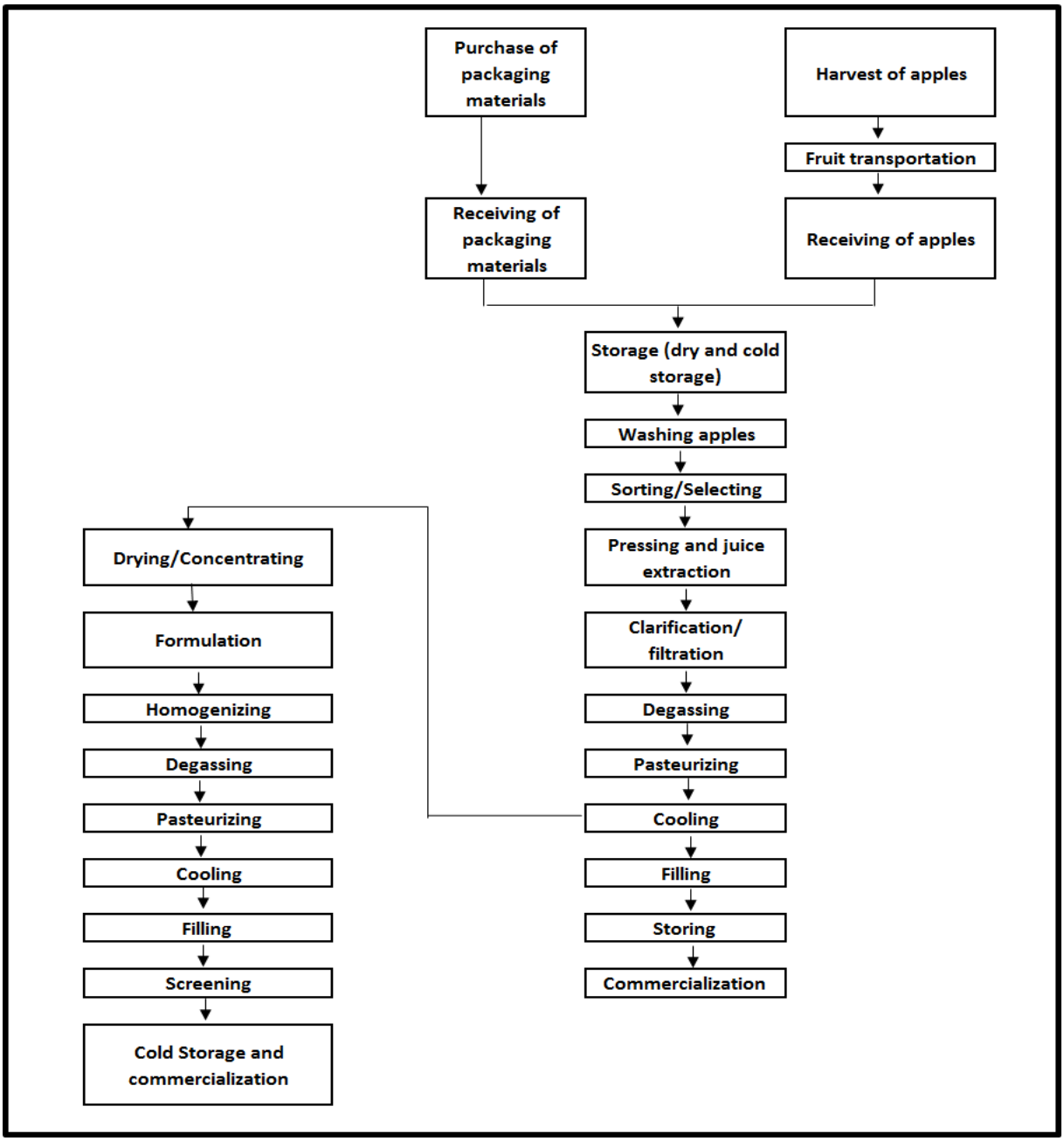

Figure 13 Apple juice processing steps adapted from Sant'Ana et al. (2008)

Based on the flow diagram **(Figure 13)**, five steps are considered as CCPs related to fungal contamination and for PAT hazards, during the apple juice production.

- A first CCP is the post-harvest step in between apple picking and apple transportation. As described in the Codex (2003), apples should be properly stored maximum 18 h after harvest. During transportation and reception, the risk of increasing PAT contamination remains low especially for short distances, but injured apples can be more susceptible to fungal development (FAO 2003). A study of Sydenham et al. in 1977, demonstrated that PAT levels increased from 90 to 2,445 ppb in apples stored in an open area for an extended

period of 33 days before processing. A more recent study of Baert et al. (2012) developing a quantitative risk assessment model and evaluating different strategies for the reduction of PAT in contaminated apple products, distinguished between the first storage step cited and the deck storage step occurring between delivery of apples to the apple juice producer and the processing of apples. It indicated that the duration of deck storage should be considered as another CCP since different extrinsic and intrinsic parameters (e.g. temperature, a_w, pH, etc.) affect the growth of moulds and PAT production at this step.

- A second CCP is the step-in which fruits are selected, sorted and debris is removed. The selection step consists of excluding or discarding damaged or rotten apples. Sydenham et al. (1995) showed that washing and removal of rotten and damaged fruit by hand before processing significantly reduced the concentration of PAT in the juice from 920 to 55 ppb, while PAT levels in the rotten/damaged fruit fractions were up to 2,335 ppb. Washing the fruits and sorting them reduced the levels of PAT from 90 to 75 and 55 ppb respectively.
  Trimming up to 2 cm around the rotten area is believed to be enough to remove the toxin (Rychlik and Schieberle 2001; Marin et al. 2006a; Baert et al. 2012). Still the trimming depends on the depth of the rotten area and the size of the apple.
- A third CCP is filtration. It is a step that consists of the removal of fine, PAT-rich particles that are present in suspension in the juice. As described by Acar et al. (1998), conventional clarification by means of a rotary vacuum pre-coat filter and ultrafiltration using membrane filters resulted in a PAT loss of 39% and 25%, respectively. More studies need to be done in order to see if this step could be applicable in the production of cloudy apple juices.
- Pasteurization is considered as another CCP in the apple juice production chain especially because it could inhibit fungal growth and destroy spores. Heat treatment is mostly applied for the preservation of food (Fellows 2009). As per the FDA, it consists on heating the apple juice to a certain temperature (at least 71.1°C) and for a length of time (at least 6 seconds) allowing the destruction of most of the organisms that might develop. Flash pasteurization is a rapid heating technique at a temperature higher than 88°C for 25 to 30 sec. Heat treatments techniques for apple juices have been described many times before (Sant'Ana et al. 2008; Ioi et al. 2017).
  The use of thermal treatments to reduce the risk of PAT has been questioned due to its resistance to heat (Ioi et al. 2017). The stability of PAT in pasteurized apple juice was evaluated by several researchers (Scott el al. 1968; Kadakal et al. 2002). Results showed a PAT reduction that goes up to 50% when spiked clear apple juice was pasteurized for 20 min at 80°C and a reduction of 13.4% of PAT after pasteurization of clear juice for 10

sec at 90°C. Kadakal et al. (2003) showed that the concentration of PAT in the apple juice samples decreased when the heating temperature increased. Heating at 90 and 100°C for 20 min resulted in reductions in PAT concentration of 19 and 26%, respectively. However, a study of Kubacki (1986) reported that PAT was stable at 80°C for 30 min in apple juice, and a more severe treatment of 120°C for 30 min was required to observe PAT reduction. Heat treatment may result in the reduction of the PAT concentration, but it does not completely eliminate its presence in contaminated juices. Different parameters affect the efficiency of the treatment applied and differ from one study to another. At first, the origin of PAT added (e.g. pure PAT standard vs. PAT-contaminated apple mash obtained by inoculation by *Penicillium expansum*) and tested in each research work should be considered. Then other parameters such as the temperature applied and time of the treatment, the concentration of sugars and pH of the apples, as well as the characteristics of the apple product (clear apple juice, cloudy one, apple cider, etc.) could affect the results obtained. Some of the molecules present in the apples can interact with PAT and either make it unstable or reinforce its stability (Welke et al. 2009a).

- Cooling and cold storage of apple juices are also considered as CCPs when applied, even though they do not seem to have an important effect on the PAT concentration but rather on the fungal growth. After pasteurization, some industries tend to cool the juice before filling while others go through a hot filling process followed by cold storage. Cross-contamination by PAT or moulds should be avoided during these filling step. If the cooling step is applied, it should be carefully monitored. This step should be performed under sterile aseptic conditions and as fast as possible to reduce deterioration by microbial growth. No studies show that PAT concentration is affected during this step. In freshly extracted, unpasteurized apple juice, cooling should be applied within 18 h at a temperature varying between 0 and 4.4°C in order to reduce the possibility of fermentation (WFLO, 2008). In pasteurized apple juice, cooling should be performed between 2°C and 8°C. When it comes to the storage step, juices should be stored at the right temperature (not exceeding 5°C). Even though at this stage the product must be sterile, i.e. free of microorganisms, one cannot neglect the fact that heat-resistant fungi capable of producing PAT (or other toxins), may have survived the pasteurization (e.g. *Byssochlamys fulva* capable of resisting at temperature of 87°C for 30 min in fruit juices are luckily not able to produce PAT (Puel 2007), but other metabolites have been reported to be produced by *B. fulva* such as byssochlamic acid and byssotoxin A (Tournas 1994; Sant'Ana et al. 2010b). However, ascospores of *Byssochlamys nivea*, a PAT producing species, are inactivated by temperatures above 60°C (Dombrink-Kurtzman and Engberg

2006). When storing pasteurized juices that are ready to be commercialized at room temperature, these conditions allow fungus reactivation and multiplication (Sant'Ana et al. 2008).

There are other studies that showed the necessity of proper storage of apple juices and apple juice concentrates. Buys et al. (2013) found that the storage time affected the stability of PAT in clear apple juice to which PAT was added exogenously showing a gradual decrease in the PAT concentration as the storage period progressed. After 28 days of storage, PAT appeared to be more stable at 4°C than at ambient or accelerated shelf-life temperatures. In another study on apple juice concentrates, PAT was reduced within ranges of 45-64% and 66-86%, after 1 month of storage at 22°C and 30°C, respectively, and levels of PAT were below detectable limits after 4 months of storage at both temperatures (Koca et al. 2005). The PAT reduction depends on storage time and temperature, and on its initial concentration in the product (Koca et al. 2005; Buys et al. 2013).

To conclude, different steps in the production chain may contribute to the reduction of PAT in apple juice. However, the reduction is not always enough to completely eliminate the toxin. This means that the selection of raw materials for apple juice production is very critical and should happen in a proper way in order to obtain apple juices with PAT levels below the detection limit. Each step of the HACCP approach should be respected and monitored to obtain the desired food item at the end.

D.5 Ascorbic acid

In the following, focus is put on AA and its effect on PAT because of its presence in fruits and vegetables and this compound is already used on an industrial level.

a. Identity of AA

AA also known as vitamin C **(Figure 14)** is represented by the molecular formula $C_6H_8O_6$ and a molecular weight of 176.124 g/mol. The most commonly used names assigned to it are as follows: vitamin C, L-AA, 3-keto-L-gulofuranolactone, 3-oxo-L-gulofuranolactone and L-threo-hex-2-enoic acid gamma lactone. Sodium L-ascorbate and calcium L-ascorbate are, respectively, the sodium and calcium salts of L-AA. AA is soluble in water and has the capacity to help the human body suppress bacterial infections and form collagen in teeth, bones, etc. This vitamin is found in fruits (for example citrus fruits, banana, apples, etc.) and vegetables (such as beetroot, spinach, cabbage,

etc.), but it cannot be produced nor stored in the human body. AA and its conjugated base (ascorbate anion) have long been known for their antioxidant properties (Stadtman 1991).

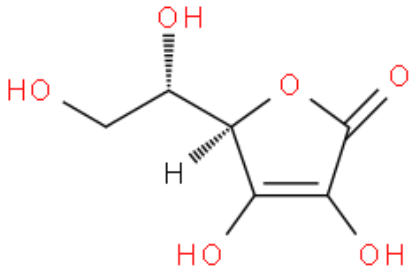

Figure 14 Chemical structure of ascorbic acid

To date, AA and its salts are authorized as food additives in the European Union except for processed cereal-based foods and baby foods for infants and young children (EFSA NDA Panel, 2013).

The FAO/WHO (2004) concluded that 1 g/day of vitamin C is the upper limit of a healthy consumption, and higher doses may cause some negative gastrointestinal effects.

Numerous studies have linked the consumption of large quantities (3 to 4 g/day) of AA with adverse health effects e.g. metabolic acidosis, kidney and renal stones, hyperglycemia, gastrointestinal disturbances (diarrhea, nausea and abdominal cramps), conditioned need, prothrombin, disturbances in serum cholesterol, destruction of vitamin B12 and sterility (Barness 1975). Nevertheless, all these data cannot deny the need for a sufficient amount of vitamin C in a balanced diet to improve human health.

To conclude, AA, sodium ascorbate and calcium ascorbate are authorized EU food additives (Regulation (EC) No 1333/2008), evaluated by JECFA in 1981 (a and b). No indication of genotoxic and/or carcinogenic risks was observed for the majority of its degradation products either (EFSA, 2015).

b. Methods for the detection and determination of AA

Since 1985, numerous studies have been conducted to determine AA in food or pharmaceutical products (Pachla et al. 1985). Most of the methods applied were based on HPLC. A dual detection system, after HPLC separation, with direct detection of AA and indirect fluorometric detection of dehydroascorbic acid (DHA) after a post-column O-phenylenediamine derivatization, was used to assess the amount of vitamin C in food and plasma samples (Kall and Andersen 1999). Later, an innovative analytical HPLC technique coupled to a fluorometric detector and determining AA, DHA, isoascorbic acid and isodehydroascorbic acid in foods was developed (Bognár and Daood 2000). Fontannaz et al. (2006) described a HPLC-UV method that quantifies total AA (AA and its

oxidized form, DHA) and checks the presence of isoascorbic acid in fortified food products, premixes and duomixes. Ullah et al. (2012) have developed and validated a simple and rapid RP-HPLC method with a UV-VIS detector L-2420 that estimates the actual amount of vitamin C present in packaged juices. Their method appeared to be accurate and useful for a variety of beverages.

c. Factors impacting the stability of AA

Studies have shown that AA can undergo degradation in aqueous environments and that many factors, e.g. pH, temperature, light, concentration and matrix composition, are involved in this process. Various ways by which we handle fruits or vegetables could also have an impact on the concentration of this vitamin: peeling an apple might result in the loss of 8 to 25% of its AA concentration and cutting food into pieces could exposure them to air, decreasing by that AA retention (Allen et al. 2006; Gil et al. 2006).

In the presence of oxygen, many degradation products, e.g. threonic, oxalic, glyceric and glyoxylic acids, as well as threose, could be identified by Gas Chromatography Mass Spectrometry (GC-MS) (Shin and Feather 1990; López and Feather 1992).

Bode et al. (1990) used HPLC to assess the decomposition of the oxidized form of vitamin C, DHA. They showed that the decay of DHA to 2,3-diketogulonic acid was higher at high pH values (7-8) than at low ones (3-5), and that it increased with a temperature between 0 to 45°C.

No indication of genotoxic and/or carcinogenic risk from the scientific literature has been described for any of the degradation products.

i. Effect of heating

The stability of AA has proven to be inversely proportional to temperature. It decreases with increasing temperature (Jeney-Nagymate and Fodor 2008) thus presenting a serious problem since a big quantity of this vitamin is lost during processing, storage and preparation (Davey et al. 2000; Jeney-Nagymate and Fodor 2008).

After heating AA in a model system (filtrate solution obtained from the extraction and homogenization of apples), its concentration decreased with increasing temperature. After 5 hours of heating, AA losses were 7, 83 and 100% at 20°C, 45°C and 60°C, respectively (Chow et al. 2011).

Devic et al. (2010) subjected cubed samples of apples to osmotic dehydration and studied the effect of temperature on the different components present in it. They found that the ascorbic losses were temperature dependent: the loss was 30% after 15 minutes of osmotic dehydration of the apple cubes at 45°C, but reached 100% at 60°C.

Significant reduction of AA was observed by Radziejewska-Kubzdela and Biegańska-Marecik (2015) after heating blended beverages of apple juices and red cabbage (83% apple juice + 17% frozen red cabbage/or 17% red cabbage puree; and 98% apple juice + 2% freeze-dried red cabbage) at 90°C for 5 minutes. For apple juices to which frozen or freeze-dried red cabbage were added, 70 to 75% losses of AA occurred. For the juices and beverages to which red cabbage puree was added, the losses ranged from 30 to 40%.

ii. Effect of storage

Storage temperature, atmosphere and matrix have been shown to be the most important factors affecting vitamin C stability in foods and beverages. According to Steskova et al. (2006), higher vitamin C preservation was ensured at lower storage temperatures and degradation rate constants depended on the matrix. In liquid matrices and at comparable storage temperatures, higher degradation rates were found in milk than in fruit juices and drinks. Ajibola et al. (2009) also found that AA decreased gradually during storage, especially for temperature above 0°C.

After storing apples at 1°C, at 1°C under ultra-low oxygen atmospheric conditions (ULO: 0.9% O_2; 1.2% CO_2) and at 1°C under ULO after pre-treatment with 1-methylcyclopropene (1-MCP), a strong decrease in AA in apples was measured (loss up to ± 80%) (Kevers et al. 2011). In another study, Fawbush et al. (2009) showed a decrease in the ascorbic concentration in apples that were stored in air for a period between 4.5 and 9 months but no change of AA concentration was observed in apples stored under ULO. Fresh apples stored at 20°C, in a normal atmosphere simulating domestic storage, showed an ascorbate decrease of 75% after 7 weeks of incubation (Kevers et al. 2011).

Oxygen also has been shown to affect the stability of AA. It is an important reaction partner in the intermediate temperature range. AA degradation decelerates in absence of a headspace oxygen (Verbeyst et al. 2013). However, the degradation of AA appears to be mainly anaerobic and no impact was observed when the oxygen concentration increased from 0.41 to 3.74 mg/L at 36°C (Robertson and Samaniego 1986).

Rojas and Gerschenson (2001) studied the influence of many non-electrolytes (fructose, xylitol, glucose/mannitol mixture) and electrolytes (NaCl, KCl) on anaerobic L-AA degradation in an aqueous model system. D-fructose, which promotes a fast destruction of L-AA at processing temperatures (70, 80, 90°C) seems to enhance its stability at a temperature range of 24-45°C. Moreover, the addition of a humectant in the solution leads to a decrease in water activity and to a higher stability of vitamin C at storage temperatures.

Oey et al. (2006) studied the impact of concentration and concluded that vitamin C stability increases with a higher concentration of the vitamin and declines when increasing oxygen concentrations. The initial oxygen concentration is an important parameter to determine the proportion of aerobic degradation of vitamin C.

d. PAT degradation in presence of AA

AA has been proven to have an impact on PAT degradation and few studies have shown accelerated PAT degradation in the presence of AA (Fremy et al. 1995; Canas and Aranda 1996; Alves et al. 2000; Drusch et al. 2007).

The study of Frémy et al. (1995) showed that no complete disappearance of PAT could be achieved after 92 hours of incubation with AA, although in other studies, AA concentrations of up to 100 mg/kg appeared to reduce the PAT concentration of apple juice to non-detectable level (Canas and Aranda 1996). AA (482 mg/kg) was also able to reduce PAT concentration by 70% in an aqueous juice-like model system, compared to only 29-32% PAT reduction in samples not treated with AA (Drusch et al. 2007).

Kokkinidou et al. (2014) confirmed previous results on PAT degradation by AA using a mathematical model system, namely a Weibull model. It not only described PAT degradation in the absence of AA, but also predicted its degradation when AA was added. Further research is needed using this model to validate its possible application to apple juice. Thermal processing/heat treatment schemes and post-processing/treatment storage conditions could be determined based on it, which would minimize PAT levels in apple juice.

D.6 Conclusion

PAT, a mycotoxin contaminating many food products, poses a food safety problem and represents a danger for consumer health. Different treatments, legally allowed to be applied within the food processing industry and capable of reducing PAT in food products, should be considered in the processing chain in order to increase safety of apple-based products.

Among several strategies that have been developed so far, post-harvest treatments showed to control fungal contamination and PAT production, but these require intensive and laborious work. Furthermore, the massive (non-recommended) use of fungicides led to the emergence of resistant *P. expansum* strains.

Therefore physical, chemical and biological treatments to degrade PAT in apple juice were studied. Some of the strategies proved to be very effective but affect the organoleptic and/or physico-chemical properties of the juice or negatively impact the environment, such as adsorption on activated charcoal. On the other hand, biological control methods using microorganisms

showed no significant impact on the juice quality characteristics and may be considered as alternative methods for PAT degradation, only that their use is limited to products that are fermented.

The use of chemical agents exclusively for mycotoxin reduction, more specifically PAT reduction in apple products, is not allowed; even if some of them showed great impact on PAT degradation. Some studies using biological or chemical treatments shed the light on some degradation products that have been identified but some remain unknown yet. More data on their toxicity is needed.

Finally, different steps in the production chain may contribute to the reduction of PAT in apple juice and several methods appeared to affect the fungal contamination and the PAT concentration. However, the reduction is not always sufficient to completely eliminate the toxin or prevent the PAT contamination from the beginning. This means that more control measures and safety checks should be applied starting from the field culture, to the harvest step, then to the storage step and arriving to the processing and transformation phase.

More research is needed especially focusing on techniques already applied to decrease PAT concentration in food products to an acceptable level. In this respect, the application of AA already naturally present in apple juice seems to have particular future and should be evaluated.

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Conflict of interest

None

Country	Amount of samples positive/total	Patulin levels (range) in positive samples (µg/kg)	References
Australia	-/-	-	Sommer et al. 1974
	-/-	5 - 646	Reddy et al. 2010
Austria	-/-	-	Moake et al. 2005
	35/43	3 - 39	Tangni et al. 2003
Belgium	22/177	6 - 123	Baert et al. 2006
	32/49	>50	Gillard et al. 2009
	1/30	17	de Sylos and Rodriguez-Amaya 1999
Brazil	4/100	3 - 7	Iha and Sabino 2008
	-/16	15 - 46	Welke et al. 2009b
	-/-	-	Scott et al. 1972
Canada	-/-	-	Moake et al. 2005
	2/25	34 - 39	Zhou et al. 2012
China	5/6	4 - 28	Vaclavikova et al. 2015
Czech Republic	21/84	30 - 16400	Lindroth and Niskanen 1978
Finland	-/-	-	Sommer et al. 1974
France	27/27	<610	Barkai-Golan 2008
	12/12	6 - 26	Rychlik and Schieberle 1999
Germany	29/29	1 - 12	Moukas et al. 2008
Greece	1/36	>25	Boonzaaijer et al. 2005
Holland	10/40	24 - 1839	Saxena et al. 2008
India	47/65	15 - 285	Cheraghali et al. 2005
Iran	21/65	ND-285	Cheraghali et al. 2005
	54/58	11 - 122	Karimi et al. 2008
	64/72	15 - 151	Forouzan and Madadlou 2014
	17/64	11 - 191	Rahimi and Jeiran 2015
	6/15	1 - 56	Ritieni 2003
Italy	28/57	1 - 69	Piemontese et al. 2005
	25/53	<48	Spadaro et al. 2007

Country	Amount of samples positive/total	Patulin levels (range) in positive samples (µg/kg)	References
Italy	12/22	1 - 22	Versari et al. 2007
	-/-	0 - 1150	Beretta et al. 2000
	15/76	1 - 46	Ito et al. 2004
Japan	9/188	6 - 15	Watanabe and Shimizu 2005
	9/30	1 - 15	Kataoka et al. 2009
	1/13	27	Lee et al. 2014
Malaysia	28/68	<42	Barreira et al. 2010
Portugal	5/9	1860 to 45470	Cunha et al. 2014
	41/50	<102	Oroian et al. 2014
Romania	-/120	57 - 104	Gashlan 2009
	12/50	0.7–101.9	Oroian et al. 2014
Saudi Arabia	-/-	-	Brown and Shephard 1999
Serbia	65/148	<10	Torović et al. 2017
South Africa	4/17	5 - 45	Legott and Shephard 2001
	5/22	10 - 45	Moake et al. 2005
	3/24	3 - 9	Cho et al. 2010
South Korea	82/100	<170	Prieta et al. 1994
Spain	5/17	2 - 51	Gonzàlez-Osnaya et al. 2007
	30/71	<25	Cano-Sancho et al. 2009
	66/100	1 - 119	Murillo-Arbizu et al. 2009
	2/28	3 - 6	Marín et al. 2011
	21/47	<37	Piqué et al. 2013
	4/12	2 - 25	Marsol-Vall et al. 2014
	5/39	<50	Thuvander et al. 2001
Sweden	-/-	-	Moake et al. 2005
	12/105	15 - 40	Lai et al. 2000
Taiwan	11/30	0 - 167	Zaied et al. 2013
Thailand	ND/40	<maximum residue limit EU	Poapolathep et al. 2017
Tunisia	12/42	4 - 122	Zouaoui et al. 2015

Country	Amount of samples positive/total	Patulin levels (range) in positive samples (µg/kg)	References
Tunisia	215/215	7 - 376	Gökmen and Acar 1998
Turkey	-/482	<376	Gökmen and Acar 1998
	27/45	19 - 733	Yurdun et al. 2001
	12/30	3.2 - 106.9	Demirci et al. 2003
	23/40	10 - 350	Brackett and Marth 1979
USA	8/13	44 - 309	Moake et al. 2005
	-/-	8.8-2700.4	Harris et al. 2009

Table 3 Reports regarding worldwide contamination of apple juice by patulin (- = not specified)

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
Apple	England	-/-	-	Brian et al. 1956
	New Zealand	-/-	-	Walker 1969
	Canada	-/-	-	Harwig et al. 1973
	Canada	45.9	154 - 136154 µg/kg	Harwig et al. 1973
	United States (Washington/ California/ Michigan)	-/-	7-100 mg/kg (*in vivo*); 140-800 mg/kg (*in vitro*)	Sommer et al. 1974
	Canada	-/-	80 mg/kg (*in vivo*); 660 mg/kg (*in vitro*)	Sommer et al. 1974
	Australia	-/-	7 mg/kg (*in vivo*); 660 mg/kg (*in vitro*)	Sommer et al. 1974
	United States	-/-	-	Ware et al. 1974
	Brazil	-/-	150 - 267 µg/kg	de Sylos and Rodriguez-Amaya 1999
	Egypt	100.0	150000 - 1000000 µg/kg	Hasan 2000
	Italy	47.6	1 - 44572 µg/kg	Piemontese et al. 2005
	Spain	-/-	0	Marin et al. 2006
	Brazil	91.4	1010 - 120400 µg/kg	Celli et al. 2009
	United States	0.4	8.8-417.6 µg/kg	Harris et al. 2009
	Italy	-/-	-	Beretta et al. 2000

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
	Portugal	-/-	1-70.6 µg/kg	Cunha et al. 2014
	Czech Republic	33.3	415 µg/kg	Vaclavikova et al. 2015
Apple cider	Burlington, Vermont	9.0	<45000 µg/kg	Wilson and Nuovo 1973
	Georgia	-/-	244 - 3993 µg/kg	Wheeler et al. 1987
	South Africa	25.0	5 - 10 µg/kg	Leggott and Shephard 2001
	Belgium	42.9	3 - 6 µg/kg	Tangni et al. 2003
		-/-	-	Moake et al. 2005
	Italy	25.0	1 - 4 µg/kg	Piemontese et al. 2005
	Michigan	18.7	-	Harris et al. 2009
	Czech Republic	100.0	12 - 48 µg/kg	Vaclavikova et al. 2015
Apple jam	Finland	100.0	-	Lindroth and Niskanen 1978
	Argentina	23.1	17 - 39 µg/kg	Funes and Resnik 2009
	Tunisia	33.3	5 - 554 µg/kg	Zouaoui et al. 2015
Apple juice				**see Table 3**
Apple leather	Iran	100.0	7 - 2559 µg/kg	Montaseri et al. 2014
Apple puree	South Africa	35.0	5-20 µg/kg	Legott and Shephard 2001
	Italy	50.0	16 - 74 µg/kg	Ritieni 2003
	Valencia, Spain	33.3	8 - 28 µg/kg	Gonzàlez-Osnaya et al. 2007
	Argentina	50.0	22 - 221 µg/kg	Funes and Resnik 2009

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
	Portugal	7.0	1.2-5.7 µg/kg	Barreira et al. 2010
	Catalonia, Spain	13.0	6 - 50 µg/kg	Piqué et al. 2013
	Tunisia	20.0	2 - 77 µg/kg	Zouaoui et al. 2015
	Italy	-/-	1.92 µg/kg	Sarubbi et al. 2016
	Serbia	16.7	<10 µg/kg	Torović et al. 2017
Apricot	United States (California)	-/-	ND-18 mg/kg (*in vivo*); <10-610 mg/kg (*in vitro*)	Sommer et al. 1974
Apricot juice (concentrate)	Greece	100.0	12 - 15 µg/kg	Moukas et al. 2008
	Italy	25.9	2 - 32 µg/kg	Spadaro et al. 2008
	Thailand	ND/40	<maximum residue limit EU	Poapolathep et al. 2017
Baby food	Spain	0.0	-	Prieta et al. 1994
	South Africa	37.5	5 - 20 µg/kg	Leggott and Shephard 2001
	Italy	20.0	13 - 18 µg/kg	Ritieni 2003
	Italy	25.0	1 - 13 µg/kg	Piemontese et al. 2005
	Greece	100.0	4 - 7 µg/kg	Moukas et al. 2008
	Catalonia, Spain	33.9	<10 µg/kg	Cano-Sancho et al. 2009
	Portugal	6.6	3 - 6 µg/kg	Barreira et al. 2010
	Italy	-/-	-	Beretta et al. 2000

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
	Italy	65.8	3 - 9 µg/kg	Bonerba et al. 2010
	Tunisia	28.0	0 - 165 µg/kg	Zaied et al. 2013
	Czech Republic	8.3	2 - 5 µg/kg	Vaclavikova et al. 2015
	Italy	-/-	-	Sarubbi et al. 2016
Beer with 35% apple concentrate	Spain	33.3	10 µg/kg	Marsol-Vall et al. 2014
Bell peppers	Belgium	11.4	-	Van de Perre et al. 2014
Blackcurrant jam		-/-	-	Moake et al. 2005
Blackcurrant juice	Germany	100.0	<1 µg/kg	Rychlik and Schieberle 1999
Black mulberry	Turkey	70.0	6.8–157.4 µg/kg	Demirci et al. 2003
Blueberry jam		-/-	-	Moake et al. 2005
Bread		-/-	-	Moake et al. 2005
Cereal based food	Portugal	75.0	0-4.5 µg/kg	Assuncao et al. 2016
Semi hard cheese	Italy	28.0	15-460 µg/kg	Pattono et al. 2013
Cherry	Turkey	90.0	5.6–113.3 µg/kg	Demirci et al. 2003
Cherry juice	Germany	100.0	<1 µg/kg	Rychlik and Schieberle 1999
		-/-	-	Moake et al. 2005
Corn		-/-	-	Moake et al. 2005

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
Dairy products (apple-based)	Valencia, Spain	50.0	4 - 15 µg/kg	Gonzàlez-Osnaya et al. 2007
Dried figs	Turkey	-/-	5 - 152 µg/kg	Karaca and Nas 2006
Dried fruits	China	0.5	-	Wei et al. 2017
Figs	Turkey	-/-	39.3-151.6 µg/kg	Karaca and Nas 2006
Fruit juices	Germany	100.0	1 µg/kg	Rychlik and Schieberle 1999
	South Africa	33.3	5 µg/kg	Leggott and Shephard 2001
	Italy	26.8	2 - 55 µg/kg	Spadaro et al. 2007
	Greece	100.0	3 - 11 µg/kg	Moukas et al. 2008
	Italy	31.0	2 - 25 µg/kg	Spadaro et al. 2008
	Tunisia	40.0	0 - 125 µg/kg	Zaied et al. 2013
	Iran	2.5	5- 190.7 µg/kg	Rahimi and Jeiran 2015
	Tunisia	50.0	10 - 56 µg/kg	Zouaoui et al. 2015
Fruit leather (apple- or pear-based)	USA & China	38.9	4 - 58 µg/kg	Maragos et al. 2015
Fruit salad	Czech Republic	100.0	14 µg/kg	Vaclavikova et al. 2015
Grape	United States (California)	-/-	ND-80 mg/kg (*in vivo*); 65-560 mg/kg (*in vitro*)	Sommer et al. 1974

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
Grape juice	Germany	38.2	-	Altmayer et al. 1982
	Germany	100.0	5 µg/kg	Rychlik and Schieberle 1999
		-/-	-	Moake et al. 2005
	South Korea	16.7	5 - 16 µg/kg	Cho et al. 2010
	Iran	15.0	5 - 17 µg/kg	Rahimi and Jeiran 2015
	Thailand	ND/40	<maximum residue limit EU	Poapolathep et al. 2017
Hazelnuts	Turkey	-/-	16.6-92.4 µg/kg	Ekinci et al. 2014
Hawthorn beverages	China	14.0	20 - 207 µg/kg	Li et al. 2007
Hawthorn juice	China	7.7	12 µg/kg	Zhou et al. 2012
Kiwi fruits	China	42.9	-	Wang et al. 2017
Lychee juice	Malaysia	16.7	13 µg/kg	Lee et al. 2014
Orange juice	Germany	100.0	<1 µg/kg	Rychlik and Schieberle 1999
	Greece	100.0	3 - 11 µg/kg	Moukas et al. 2008
	South Korea	8.3	10 - 31 µg/kg	Cho et al. 2010
Passion fruit juice		-/-	-	Moake et al. 2005
Peach	Brazil	-/-	92 - 174 µg/kg	de Sylos and Rodriguez-Amaya 1999
	Turkey	44.0	4.3–93.2 µg/kg	Demirci et al. 2003

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
Peach juice (concentrate)	Italy	6.7	2 - 5 µg/kg	Spadaro et al. 2008
	Spain	26.7	9 - 21 µg/kg	Marín et al. 2011
	Iran	10.0	5 - 35 µg/kg	Rahimi and Jeiran 2015
	Thailand	ND/40	<maximum residue limit EU	Poapolathep et al. 2017
Pear	United States (California)	-/-	ND-80 mg/kg (*in vivo*); 30-800 mg/kg (*in vitro*)	Sommer et al. 1974
	Brazil	-/-	134 - 245 µg/kg	de Sylos and Rodriguez-Amaya 1999
	Czech Republic	33.3	42 µg/kg	Vaclavikova et al. 2015
Pear products	Argentina	16.7	25 µg/kg	Funes and Resnik 2009
	Tunisia	43.8	17 - 325 µg/kg	Zouaoui et al. 2015
	Italy	-/-	0.79 µg/kg	Sarubbi et al. 2016
Pear juice (concentrate)		-/-	-	Moake et al. 2005
	Italy	33.3	1 - 61 µg/kg	Piemontese et al. 2005
	Italy	64.1	2 - 33 µg/kg	Spadaro et al. 2008
	Spain	50.0	67 - 74 µg/kg	Marín et al. 2011
	Iran	13.3	5 - 31 µg/kg	Rahimi and Jeiran 2015
	Czech Republic	66.7	12 - 39 µg/kg	Vaclavikova et al. 2015

Fresh or processed food products	Country	Amount of positive samples (%)	Patulin levels (range) in positive samples	References
Pineapple juice (concentrate)	Tunisia	47.6	5 - 231 µg/kg	Zouaoui et al. 2015
		-/-	-	Moake et al. 2005
	Greece	100.0	8 µg/kg	Moukas et al. 2008
	Malaysia	16.7	33 µg/kg	Lee et al. 2014
	Thailand	ND/40	<maximum residue limit EU	Poapolathep et al. 2017
Pomegranate juice	Iran	8.3	8 µg/kg	Rahimi and Jeiran 2015
Raspberry	Turkey	60.0	48–746 µg/kg	Demirci et al. 2003
Red fruits (soft)	Belgium	8.0	-	Van de Perre et al. 2014
Strawberry	Turkey	80.0	3.2–572 µg/kg	Demirci et al. 2003
Strawberry jam		-/-	-	Moake et al. 2005
Sweet bell pepper	Belgium	11.4	-	Van de Perre et al. 2014
Tomato	Belgium	10.8	-	Van de Perre et al. 2014
Tomato products	Portugal	35.7	3.22 to 47.72 µg/kg	Cunha et al. 2014
	Italy	-/-	7.15 µg/kg	Sarubbi et al. 2016
White mulberry	Turkey	66.7	32–426 µg/kg	Demirci et al. 2003

Table 4 Reports regarding patulin occurrence in fresh and processed food products (- = not specified)

Experimental Work

Chapter 1

Characterization of *veA* gene

Chapter 1_Part 1

Characterization of *veA* gene: study of its impact on development, aggressiveness and metabolome of *Penicillium expansum*

Summary of the study

As described in the second section of the introduction, *Penicillium expansum*, the principal agent of the blue mould disease, is a destructive phytopathogen causing decay in apples during post-harvest handling and storage. It is considered a major concern due to its wide spread occurrence and capacity to produce patulin (PAT), a well-known mycotoxin. As other major mycotoxins, PAT is the final product of enzymatic cascades where enzymes are activated at the same time and the newly synthesized products are metabolized consecutively by the next enzymes. This phenomenon is made possible thanks to the cluster organization of genes encoding for enzymes involved in the biosynthesis. These genes are often co-activated by a specific transcription factor located inside clusters. Although most of the studies on *P. expansum* have focused on PAT, the genome of this fungus exhibits other predicted secondary metabolite clusters (andrastin, communesin, chaetoglobosin, etc.), based on bioinformatics analysis and other specific studies. In filamentous fungi, the activation of specific transcription factors and by consequence the production of fungal secondary metabolites is controlled at a higher hierarchical level by transcription global factors encoded by genes not located in gene clusters. These genes regulate numerous physiological processes and generally respond to several environmental cues. Among them, the VeA factor is a component of the "velvet complex", a protein complex responding to abiotic factors, and more particularly to light. Depending on fungal species, VeA is involved in different physiological processes such as development, asexual and sexual reproduction, secondary metabolism and virulence.

How *veA* affects the virulence of *P. expansum* on apples and the secondary metabolism with special emphasis on the production of important secondary metabolites (PAT and citrinin) is still unknown.

The first part of this experimental work focused on the *in vivo* and *in vitro* study and characterization of a global transcription factor veA. A null PeΔ*veA* mutant and a complemented PeΔ*veA:veA* strain were generated in *P. expansum*. A morphological study was done first indicating how veA could affect the development of the fungus. Then pathology studies performed on Golden Delicious cultivar indicated the implication of this gene in the virulence of the fungus. An expression of genes study also showed how *veA* could affect the secondary metabolism of *P. expansum*.

To conclude, our findings support the hypothesis that *P. expansum* secondary metabolism is modulated by the transcriptional regulator factor VeA contributing in part to the pathogenicity of the fungus.

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Impact of *veA* on the development, aggressiveness, dissemination and secondary metabolism of *Penicillium expansum*

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SUMMARY

Penicillium expansum, the causal agent of blue mould disease, produces the mycotoxins patulin and citrinin amongst other secondary metabolites. Secondary metabolism is associated with fungal development, which responds to numerous biotic and abiotic external triggers. The global transcription factor VeA plays a key role in the coordination of secondary metabolism and differentiation processes in many fungal species. The specific role of VeA in *P. expansum* remains unknown. A null mutant PeΔ*veA* strain and a complemented PeΔ*veA*:*veA* strain were generated in *P. expansum* and their pathogenicity on apples was studied. Like the wild-type and the complemented strains, the null mutant PeΔ*veA* strain was still able to sporulate and to colonize apples, but at a lower rate. However, it could not form coremia either *in vitro* or *in vivo*, thus limiting its dissemination from natural substrates. The impact of *veA* on the expression of genes encoding proteins involved in the production of patulin, citrinin and other secondary metabolites was evaluated. The disruption of *veA* drastically reduced the production of patulin and citrinin on synthetic media, associated with a marked down-regulation of all genes involved in the biosynthesis of the two mycotoxins. Moreover, the null mutant PeΔ*veA* strain was unable to produce patulin on apples. The analysis of gene expression revealed a global impact on secondary metabolism, as 15 of 35 backbone genes showed differential regulation on two different media. These findings support the hypothesis that VeA contributes to the pathogenicity of *P. expansum* and modulates its secondary metabolism.

Keywords: apples, citrinin, pathogenicity, patulin, *Penicillium expansum*, secondary metabolism, veA.

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INTRODUCTION

Penicillium expansum is a ubiquitous fungus of soil origin that can be found on the peel of pome and stone fruits. During post-harvest processing and storage, this phytopathogen develops on wounds inside the fruit provoking maceration and spoilage. *Penicillium expansum* is the primary cause of blue mould and decay of apples, making them unfit for consumption and causing serious economic losses (Nunes, 2012). In addition, *P. expansum* produces a variety of toxic secondary metabolites, known as mycotoxins. Patulin, one of the main products of the secondary metabolism of *P. expansum*, is detected not only in fruits, but also in apple-based products. Patulin has several deleterious effects on human health (Assunçao *et al*., 2016; Puel *et al*., 2010; Zouaoui *et al*., 2016). As a result of its toxicity, the maximum permitted level of patulin is regulated in most European countries (EC Commission Regulation, 2006).

In addition to patulin, *P. expansum* produces numerous secondary metabolites and mycotoxins *in vitro*, such as citrinin, which has also been found in fruits (Martins *et al*., 2002). Citrinin has nephrotoxic and teratogenic effects on mammals and chickens, respectively (Ciegler *et al*., 1977). In addition to other secondary metabolites, *P. expansum* is able to synthesize roquefortine C (Andersen *et al*., 2004), chaetoglobosins (Andersen *et al*., 2004), expansolides (Massias *et al*., 1990), fumaryl-DL-alanine (Birkinshaw *et al*., 1942), andrastin (Kim *et al*., 2012) and communesins (Andersen *et al*., 2004). Although the ecological roles of secondary metabolites often remain unclear, some are involved in virulence during infection, defence against other microorganisms and communication (Macheleidt *et al*., 2016). For instance, patulin plays a role in pathogenicity during apple infection as a cultivar-dependent aggressiveness factor that promotes the colonization of apples (Snini *et al*., 2016). Genes encoding enzymes and proteins involved in the biosynthesis of secondary metabolites are often grouPeΔ into clusters. The patulin cluster comprises 15 genes (*patA*–*patO*) (Li *et al*., 2015; Tannous *et al*., 2014) and the putative cluster of citrinin includes nine genes (Pexp_005510–Pexp_005590) (Ballester *et al*., 2015; He and Cox, 2016). A gene encoding a specific transcription factor, often located inside the cluster, activates all the genes in each cluster: *patL* (*P. expansum*) (Ballester *et al*., 2015; Snini *et al*., 2016) and *mrl3*/*ctnA*

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(*P. citrinum/P. expansum*) (He and Cox, 2016; Li *et al*., 2017) for patulin and citrinin clusters, respectively. Bioinformatics analysis has shown that the genome of *P. expansum* contains other identified or predicted clusters of secondary metabolites (Ballester *et al*., 2015; Nielsen *et al*., 2017).

Growth, morphological development and the production of secondary metabolites are interconnected and controlled by global transcription factors encoded by genes located outside the biosynthesis gene clusters (Calvo *et al*., 2002). These genes respond to numerous environmental stimuli, including pH, oxidative stress, temperature, nitrate, carbon source, iron and light. Among them, VeA is a phosphoprotein that belongs to the Velvet family, which is composed of three other proteins: VelB, VosA and VelC. VeA is involved in the regulation of several cellular processes: morphogenesis, response to oxidative stress, light-dependent control of sexual or cleistothecial/sclerotial formation, asexual development or conidiation and secondary metabolism, but sometimes also virulence (Kim *et al*., 2002; Rauscher *et al*., 2016). In the dark, VeA is transported, together with VelB, to the nucleus, where they form a heterotrimer in association with LaeA, which acts as a positive regulator of sexual development and secondary metabolism (Kumar *et al*., 2017a; Stinett *et al*., 2007). The disruption of the *laeA* gene in *P. expansum* leads to a drastic reduction in patulin (Kumar *et al*., 2017a).

In most cases, VeA acts as a positive regulator of the biosynthesis of secondary metabolites, such as aflatoxin in *Aspergillus parasiticus* and *Aspergillus flavus* (Duran *et al*., 2007), as well as cyclopiazonic acid and aflatrem in *A. flavus* (Calvo and Cary, 2015), and ochratoxin in *Aspergillus carbonarius* (Crespo-Sempere *et al*., 2013).

How *veA* affects the virulence of *P. expansum* in apples and its secondary metabolism remains unknown. In the present study, a null mutant PeΔ*veA* strain and a complemented PeΔ*veA*:*veA* strain were generated in *P. expansum*. Pathogenicity was studied on apples. Special emphasis was placed on patulin and citrinin production *in vitro*. The expression of genes belonging to the two corresponding clusters was analysed. The study was then extended to the expression of all genes encoding backbone enzymes of secondary metabolites found in the genome of *P. expansum*.

RESULTS

A PeΔ*veA* mutant was generated by replacing the *veA* gene with a hygromycin resistance marker (Parts S1 and S2, see Supporting Information). This mutant was used to characterize the role of VeA, to understand how this protein affects the virulence of *P. expansum* and how it influences the production of several secondary metabolites.

Deletion of *veA* affects *in vitro* growth, macroscopic and microscopic morphology

After 7 days of incubation, no difference was observed between the radial growth of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains, with colonies of 42 ± 2, 44 ±3 and 43 ±2 mm in diameter on potato dextrose agar (PDA) and of 39 ±2, 37 ±1 and 38 ±3 mm on Czapek Dox agar (CzA), respectively. The diameters of the colonies of the WT, null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains grown on malt extract agar (MEA) were 37 ± 1, 41 ± 2 and 38 ±1 mm, respectively. The diameter of the null mutant colony was thus slightly larger (10%) on this medium. The diameters of the colonies of the WT, null mutant and complemented strains were 50 ±4, 44 ±3 and 52 ±5 mm, respectively, on Czapek glucose agar (CGA). The growth of the mutant PeΔ*veA* strain decreased by 12%.

The morphological aspects of the WT and complemented strains differed from those of the null mutant strain. Macroscopically, all the colonies displayed the usual *P. expansum* blue–green colour in conidial areas. A marked white margin was observed in both WT and complemented strains, but was lacking in the null mutant strain. A bright yellow ring was observed on the edge of the colony on MEA and CzA (Fig. 1A and Part S3, see Supporting Information). Radial furrows were also more pronounced and complete in the null mutant strain than in the WT strain (Fig. 1A and Part S3). With regard to texture, the colonies of the null mutant strain presented reduced fasciculation compared with the other strains (Fig. 1A and Part S3). The surface of the null mutant colony thus appeared planar. The texture was velutinous with conidiophores arising from submerged hyphae, but no coremia, whereas the surface of the other strains displayed aggregation of conidiophores that gave the surface a granular appearance. In colonies of WT and complemented strains, coremia were present on all media (Fig. 1A and Part S3). One other particularity of the null mutant was its inability to produce exudates whatever the medium used (Fig. 1A and Part S3).

Microscopically, the structures of the strains also presented some differences. The WT strain had terverticillate conidiophores with branches tied to the main axis (Fig. 1B). The null mutant strain frequently presented biverticillated conidiophores with inflated metulae (Fig. 1B). The null mutant also showed abnor- mally larger conidia that resembled ramoconidia (data not shown).

The macroscopic and microscopic characteristics of the complemented strain resembled those of the WT strain (Fig. 1B).

Deletion of *veA* affects colonization, development and dissemination

To assess the impact of *veA* deletion, freshly picked Golden Delicious apples were inoculated with the WT, PeΔ*veA* and

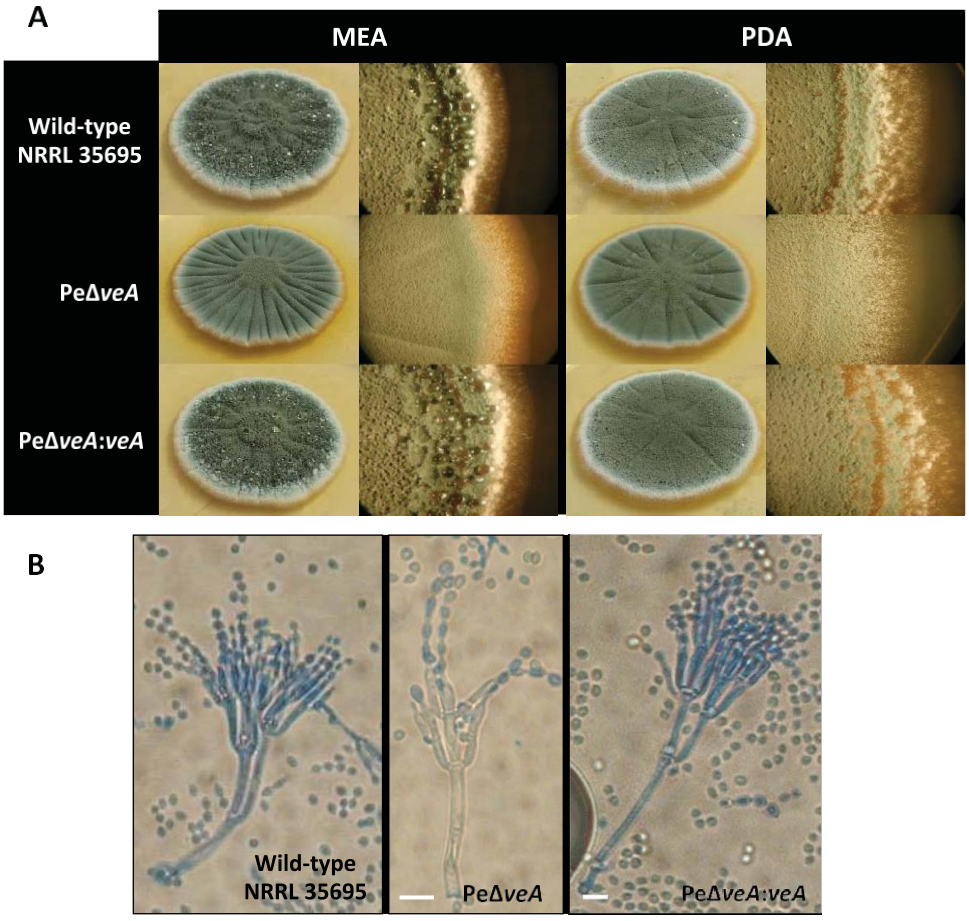

Fig. 1 Morphological appearance of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains. (A) Macroscopic appearance and observations under a stereomicroscope (12×) of colonies after 10 days of culture at 25 °C in the dark on potato dextrose agar (PDA) and malt extract agar (MEA) inoculated with 10^4 spores. (B) Microscopic appearance of conidiophores and conidia (400×) after 7 days of culture at 25 °C in the dark on MEA inoculated with 10^4 spores. The white bars represent 10 mm.

PeΔ*veA*:*veA* strains. During the first 4 days post-inoculation (dpi), the WT and null mutant strains showed the same development pro- file. A decrease in the speed of development of the null mutant strain was observed from 5 dpi. The development profile of the complemented PeΔ*veA*:*veA* strain resembled that of the WT strain (Fig. 2A). Growth rates calculated from growth curves (Fig. 2B) showed that the null mutant PeΔ*veA* strain grew at a rate of 0.15 cm/day, whereas the WT strain grew at a rate of 0.28 cm/day. No significant differences in growth rate were observed between the WT and complemented strains. At the end of the incubation period, the rot volume was calculated as described previously by Baert *et al*. (2007). The rot volume of apples infected with the null mutant PeΔ*veA* strain was 50% smaller than that of apples infected with either the WT or the complemented strain (Fig. 2C).

The implication of *veA* in the formation of coremia and synnemata was evaluated in the presence or absence of light *in vitro* and *in vivo*. After 7 days on Czapek yeast extract agar (CYA) and MEA, no impact of light or darkness on growth was observed (Part S4, see Supporting Information). On MEA, although PeΔ*veA* did not produce coremia, this strain was able to sporulate (Part S4). Apples were inoculated with spores of the WT and null mutant strains and then incubated for 1 month in the light or in the dark. In the presence of light, *P. expansum* appeared to be unable to pierce the wall of apples or to emerge from the fruit to complete its cycle (Fig. 3A,B). In the dark, the formation of coremia and synnemata was induced in the WT strain (Fig. 3C–E) in contrast with the null mutant strain (Fig. 3F–H). The *veA* gene positively regulates the release of the fungus through the formation of coremia on apple substrate.

Deletion of *veA* depresses mycotoxin production *in vivo* and *in vitro*

The production of patulin was measured in Golden Delicious apples at 14 dpi. Patulin concentrations were 99 ± 20 and 70 ± 21 mg/g fresh weight in apples infected with the WT and complemented strains, respectively (Fig. 4). By contrast, no patulin was detected in apples infected with the null mutant strain.

The capacity of the different strains to produce patulin and citrinin was assessed *in vitro* on two different culture media (Fig. 5). After 5 days, large amounts of patulin were detected in both the WT (24.3 ± 2 mg/g on MEA and 31.1 ± 2.5 mg/g on PDA) and the complemented (29.2 ± 2.4 mg/g on MEA and 22.2 ± 1.6 mg/g on PDA) strains (Fig. 5A). Citrinin was also

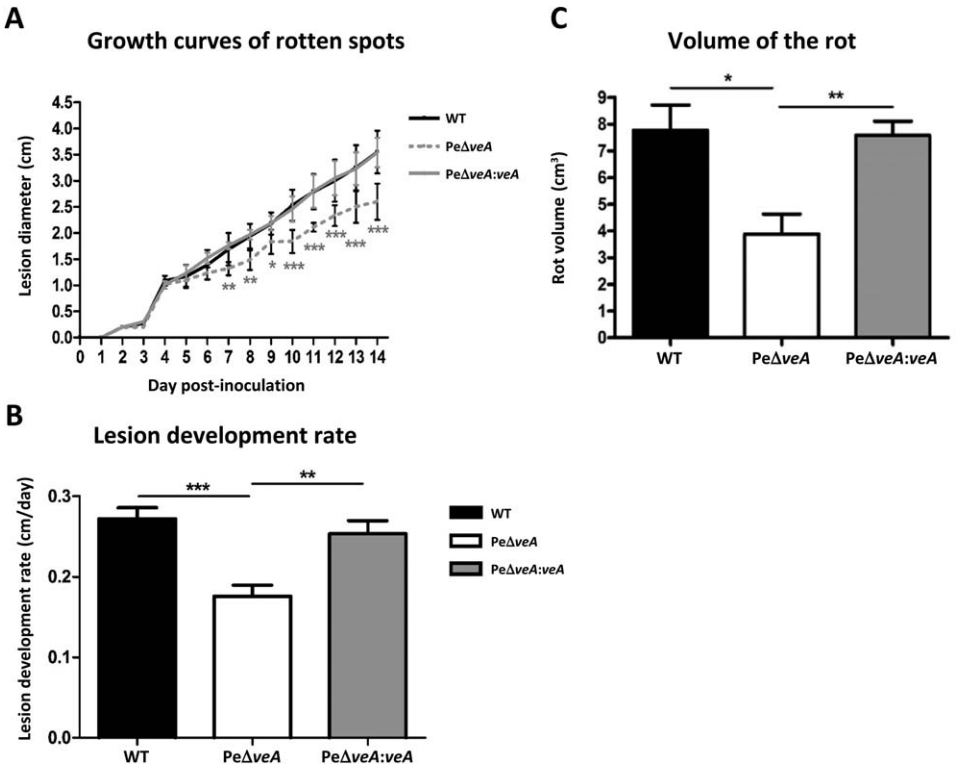

Fig. 2 Growth curves of the rotten spots and rot volume in Golden Delicious apples. (A) Diameters of the rotten spots measured every day of the incubation period. (B) Lesion development rates based on measurements of the rotten spot diameters on Golden Delicious apples. Measurements were taken every day for 14 days. (C) At 14 days post-incubation, rot volumes were calculated using the method described by Baert *et al*. (2007). Apples were inoculated with 10 mL of a 10^4 conidia/mL suspension of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* or complemented PeΔ*veA*:*veA* strain. Apples were kept at 25 °C in the dark. Graphs show the mean ±standard error of the mean (SEM) from six replicates. Asterisks denote significant differences between the WT, null mutant and complemented strains.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

detected, but to a lesser extent in both the WT (3.7 ± 0.25 mg/g on MEA and 0.6 ± 0.2 mg/g on PDA) and the complemented (4.4 ± 0.3 mg/g on MEA and 0.2 ± 0.1 mg/g on PDA) strains (Fig. 5B). Neither patulin nor citrinin was detected in PeΔ*veA* cultures.

The expression of the genes involved in the biosynthesis of patulin (15 genes) and citrinin (nine genes) was assessed on two media (MEA and PDA) and compared in the null mutant, WT and complemented strains. The null mutant strain showed marked down-regulation of all genes compared with the WT strain (Fig. 6). The expression of all genes was restored in the complemented strain (Part S5, see Supporting Information). Amongst the genes that were down-regulated in the null mutant strain, the lowest expression was observed on MEA for *patK*, *patJ*, *patI*, *patO*, *patN*, *patM*, *patA*, *patB* and *patC* in the patulin biosynthesis cluster (Fig. 6A). In the citrinin biosynthesis cluster, *citS* (*Pexp_005520*), *citA* (*Pexp_005530*), *citB* (*Pexp_005540*), *orf6* (*Pexp_005570*) and *citE* (*Pexp_005580*) were the genes most down-regulated on MEA (Fig. 6B). However, *patL* and *ctnA* genes, encoding the transcription factors of the patulin and citrinin clusters, respectively, were only repressed by 40%. The results of the analysis of gene expression on PDA were similar (Part S5).

veA modulates secondary metabolite gene expression

According to the results of bioinformatics analysis and other specific studies, the genome of *P. expansum* exhibits other gene clusters of predicted secondary metabolites. In addition to patulin and citrinin, *P. expansum* produces several secondary metabolites. We analysed the effect of *veA* on the expression of the genes involved in the biosynthesis of these metabolites. We assessed the expression of 35 gene clusters of the 63 that were identified by the antiSMASH 3.0 program from *P. expansum* T01 (GenBank: AYHP00000000.1) and d1 (GenBank: JQFY01000069.1) strain genomes. Backbone genes included polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) and dimethylallyl tryptophan synthases (DMATSs), as categorized by antiSMASH. The expression of backbone genes was measured in the WT, null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains on two media, MEA and PDA.

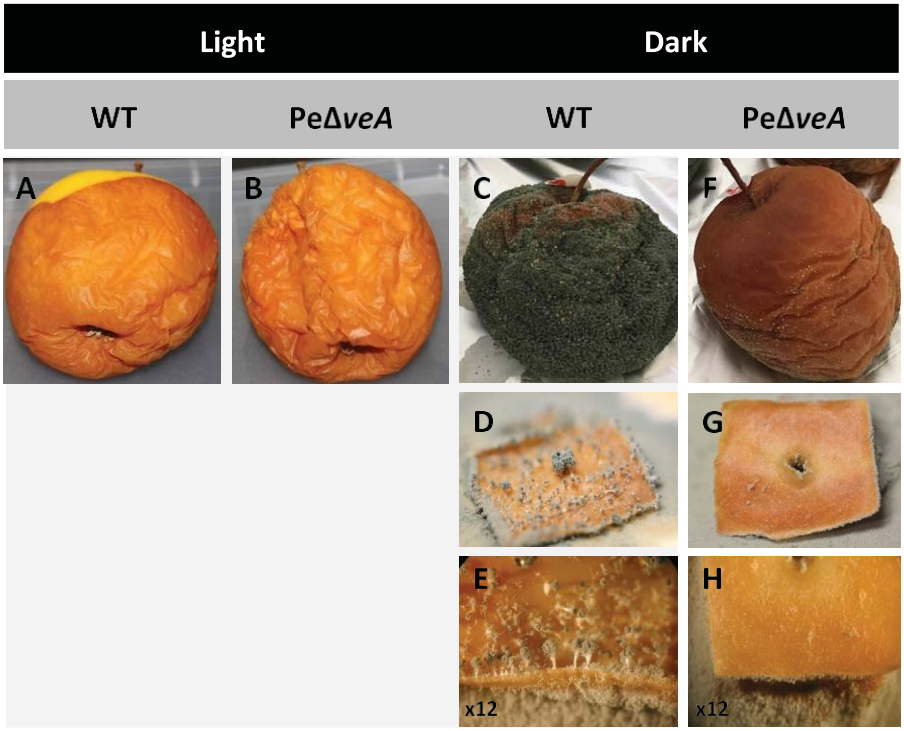

Fig. 3 Implication of *veA* in the development and dissemination of *Penicillium expansum* on Golden Delicious apples. Apples inoculated with the wild-type NRRL 35695 (WT) strain (A) and the null mutant PeΔ*veA* strain (B) stored at 25 °C in the light for 1 month. (C) An apple inoculated with the WT strain and stored at 25 °C in the dark for 1 month. (D) A piece of inoculated apple with the WT strain placed on potato dextrose agar (PDA) and stored for 5 days at 25 °C in the dark. (E) Same sample as in (D) taken under a stereomicroscope (×12). (F) An apple inoculated with the null mutant PeΔ*veA* strain and stored at 25 °C in the dark for 1 month. (G) A piece of apple inoculated with the null mutant PeΔ*veA* strain placed on PDA and stored for 5 days at 25 °C in the dark. (H) Same sample as in (G) taken under a stereomicroscope (×12). Golden Delicious apples were inoculated with 10 mL of a 10^4 conidia/mL suspension of each strain.

After quantitative reverse transcription-polymerase chain reac- tion (RT-qPCR) analysis, 35 backbone genes were expressed in either one medium or in both media, and therefore expressed by at least one of the strains used. The expression of seven backbone genes, Pexp_000130, *citS* (Pexp_005520), Pexp_008740, Pexp_015170, Pexp_028920, Pexp_093210 and *patK* (Pexp_094460), was altered by the deletion of *veA* on both media (Fig. 7 and Part S6, see Supporting Information). Conversely, seven backbone genes, Pexp_000410, Pexp_013580, *cnsI* (Pexp_030540), Pexp_037250, Pexp_078820, Pexp_086670 and Pexp_094810, were up-regulated in the null mutant PeΔ*veA* strain on both media. Pexp_094810 and Pexp_000410 were shown to be the two most down-regulated genes by VeA (Fig. 7 and Part S6).

Four of these backbone genes, *citS* (Pexp_005520), *cnsI* (Pexp_030540), Pexp_093210 and *patK* (Pexp_094460), are involved in the biosynthesis of citrinin, communesin, an uncharacterized monodictyphenone-like compound and patulin, respectively (Kumar *et al*., 2017a; Tannous *et al*., 2018). The identity of the other clusters remains unknown. The expression of *patK* (Pexp_094460) was somewhat higher on PDA than on MEA, in agreement with the patulin level measured in the two media.

The backbone genes *roqA* (Pexp_030090), Pexp_047050, Pexp_076580, Pexp_095540 and Pexp_096630 showed no difference in their expression between the WT and null mutant strains regardless of the medium used. Pexp_030090 belongs to the cluster involved in the biosynthesis of roquefortine, which was thus not regulated by *veA* on either of the media tested.

Interestingly, regulation by *veA* appeared to be medium dependent in some of the gene clusters. The backbone gene Pexp_074060 was not expressed on PDA, whereas it was down-regulated in the null mutant strain on MEA. Moreover, the

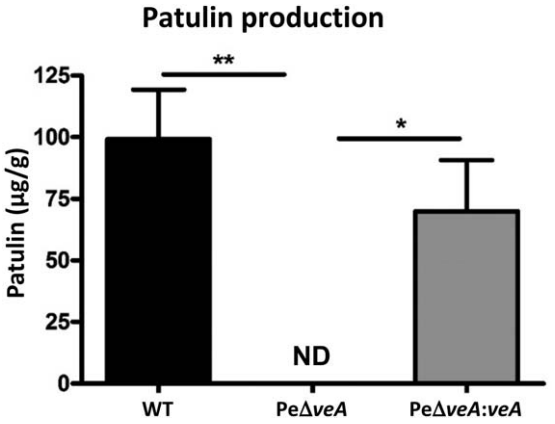

Fig. 4 Patulin production on Golden Delicious apples. Patulin production was measured by high-performance liquid chromatography with a diode array detector (HPLC-DAD) at 277 nm, 14 days post-inoculation on Golden Delicious apples as described previously (Snini *et al*., 2016). Apples were inoculated with 10 mL of a 10^4 conidia/mL suspension of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* or complemented PeΔ*veA*:*veA* strain. Apples were kept at 25 °C in the dark. Graphs show the mean ±standard error of the mean (SEM) of eight replicates. Asterisks denote significant differences between the NRRL35695 (WT), null mutant and complemented strains. *$P < 0.05$; **$P < 0.01$; ND, not detectable.

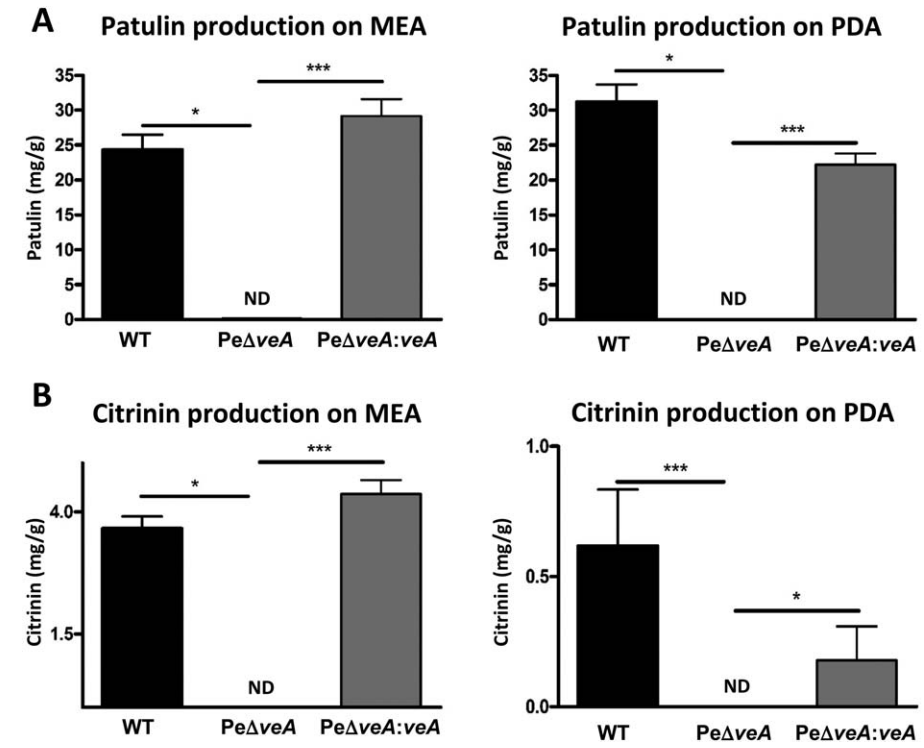

Fig. 5 Patulin and citrinin production on synthetic media by the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains. (A) Production of patulin on malt extract agar (MEA) and potato dextrose agar (PDA).
(B) Production of citrinin on malt extract agar (MEA) and potato dextrose agar (PDA). Patulin and citrinin production was measured by high-performance liquid chromatography by a diode array detector (HPLC-DAD) at 277 and 327 nm, respectively, at 5 days post-inoculation at 25 °C in the dark (10^4 spores). Graphs show the mean ± standard error of the mean (SEM) of eight replicates. **P* < 0.05; ****P* < 0.001; ND, not detectable.

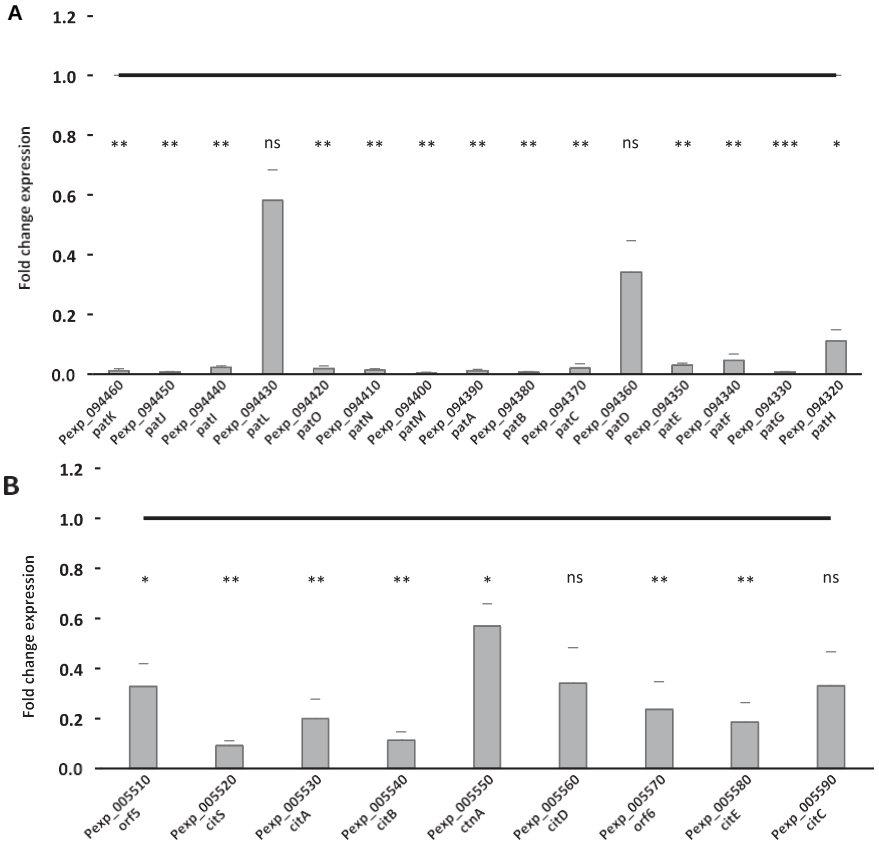

Fig 6. Fold change expression of genes belonging to the clusters involved in citrinin and patulin biosynthesis in the wild-type NRRL 35695 (WT) and null mutant PeΔ*veA* strains. (A) Patulin cluster (adapted from Tannous *et al*., 2014). (B) Citrinin cluster (adapted from He and Cox, 2016). The black line represents the expression level of genes in the WT strain. Strains were grown for 5 days at 25 °C in the dark on malt extract agar (MEA). Values were normalized to *b*-tubulin as housekeeping gene. Graphs show the mean ± standard error of the mean (SEM) from eight replicates. Asterisks denote significant differences between the WT and null mutant PeΔ*veA* strains. ns, no significant changes; *P < 0.05; **P < 0.01; ***P < 0.001.

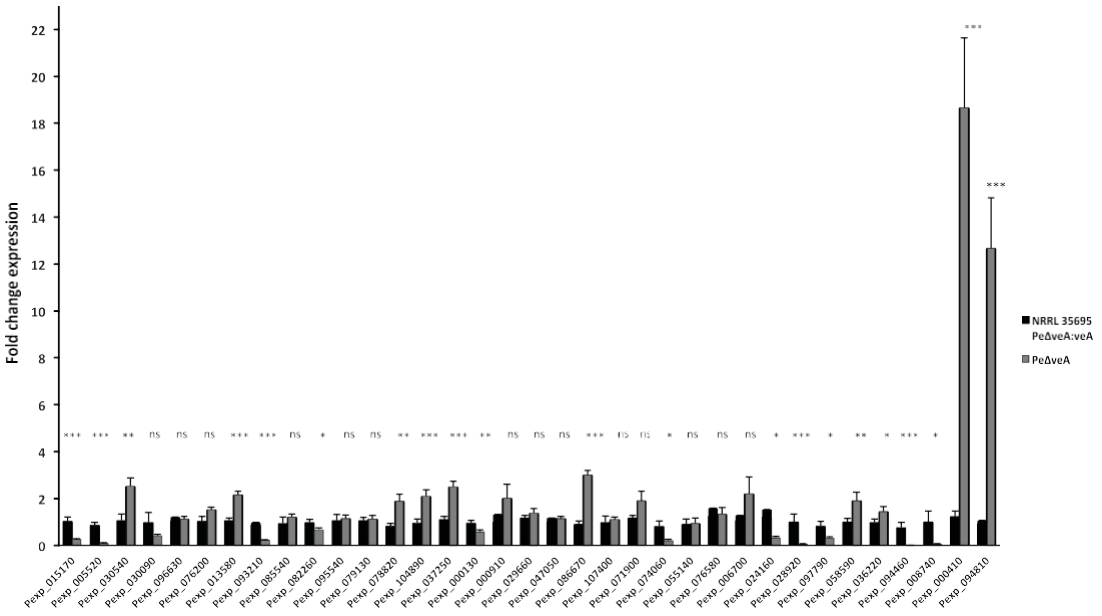

Fig. 7 Relative expression of 35 genes encoding backbone enzymes involved in secondary metabolite biosynthesis. Relative expression of polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) and dimethylallyl tryptophan synthases (DMATs) identified on the sequenced genomes of *Penicillium expansum* T01 and d1 strains grown on malt extract agar (MEA). Gene expression was evaluated in the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains. Primers were designed using Primer Express 2.0 software and tests were carried out using a ViiA7 Real-Time PCR System. Black bars represent the combined expression level of genes in the WT and complemented PeΔ*veA*:*veA* strains after culture in the dark for 5 days at 25 °C. Grey bars represent the level of gene expression in the null mutant PeΔ*veA* strain grown under the same conditions. Graphs show the mean ± standard error of the mean (SEM) of eight replicates. Asterisks denote significant differences between the WT and null mutant PeΔ*veA* strains. ns, no significant changes; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

backbone genes Pexp_071900, Pexp_076200 and Pexp_079130 were up-regulated by VeA on PDA, but not regulated on MEA in the WT and null mutant strains. Moreover, four backbone genes were up-regulated on MEA, but down-regulated (Pexp_082260), not expressed (Pexp_074060) or not regulated (Pexp_024160, Pexp_097790) on PDA.

Pexp_029660, encoding the NRPS of the loline-like biosyn- thetic cluster (Ballester *et al*., 2015), Pexp_055140, Pexp_082260, Pexp_085540 and Pexp_107400 were up-regulated in the PeΔ*veA* strain on PDA, but not regulated on MEA, except Pexp_082260, which was up-regulated on MEA. Likewise, Pexp_036220, Pexp_058590 and Pexp_104890, which encodes the backbone gene belonging to a siderophore-like cluster (Ballester *et al*., 2015), were down-regulated by VeA on MEA, but not regulated by VeA on PDA. The genes in the complemented strain were regulated in the same way as those in the WT strain.

LaeA and *velB* were expressed in the WT, null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains on the two media tested. Expression of *velB* was not affected by the deletion of *veA* on both media. Expression of *laeA* was not significantly different in the null mutant PeΔveA relative to the WT and complemented strains on both media, but increased slightly on MEA (Part S7, see Supporting Information).

DISCUSSION

The global regulation factor VeA positively or negatively controls a large number of secondary metabolite pathways in fungi, includ- ing *A. nidulans* (Bayram and Braus, 2012; Calvo 2008; Rauscher *et al*., 2016) and *A. flavus* (Cary *et al*., 2015). VeA positively affects the production of fumigaclavine C, fumagillin and gliotoxin in the opportunistic human pathogen *Aspergillus fumigatus* (Dhingra *et al*., 2013). In *Fusarium* species, VeA orthologues are required for the synthesis of fumonisins and fusarins in *F. verticillioides* (Myung *et al*., 2012), trichothecenes in *F. graminearum* (Merhej *et al*., 2012) and gibberellins, fumonisins and fusarin C in *F. fujikuroi* (Wiemann *et al*., 2010). Conversely, VeA represses the production of the dark-coloured pigment bika- verin in *F. fujikuroi* (Wiemann *et al*., 2010). In *Penicillium* species, VeA activates the production of penicillin in *Penicillium chrysoge- num* (Kopke *et al*., 2013) and represses the production of ML-236B (compactin) in *Penicillium citrinum* (Baba *et al*., 2012). Its role in *P. expansum* remains unknown. In this study, the positive regulation of patulin and citrinin biosynthesis by VeA was demon- strated. Both toxins have been reported to be positively regulated by LaeA, another member of the trimeric Velvet complex in *P. expansum* and *Monascus ruber*, respectively (Kumar *et al*.,

2017a; Liu *et al*., 2016). The lack of *veA* activity is not caused by *laeA* or *velB* expression changes. VeA acts at several levels including the Velvet complex. Although *veA* did not affect *laeA* and *velB* expression, we cannot rule out *veA* activity via the Velvet complex on patulin biosynthesis. Given the impact of VeA and LaeA on this biosynthesis pathway, the evaluation of the D*velB* mutant with regard to the expression of *pat* genes will strengthen the hypothesis that a functional Velvet complex is necessary for the production of this toxin.

In addition to patulin and citrinin, *P. expansum* produces sev- eral secondary metabolites with different toxic effects (Tannous *et al*., 2018). With regard to other known *P. expansum* secondary metabolites, backbone genes that belong to the clusters of roquefortine and a gliotoxin-like compound showed no difference in expression between the WT and null mutant strains, suggesting that *veA* does not regulate the synthesis of these metabolites, at least under the conditions of the present study.

Kumar *et al*. (2017a) compared the expression of *P. expansum* backbone genes when D*laeA* and WT strains were grown on Czapek yeast extract liquid medium (CY) and the apple-based apple puree agar medium (APAM). Among these genes, 32 and 29 genes that were expressed on MEA and PDA, respectively, were identified in the null mutant *veA* and WT strains. Among these common genes, 11 were up-regulated by LaeA, five on both media, but only five were also up-regulated by VeA: Pexp_028920 and *patK* (Pexp_094460) were expressed on both MEA and PDA, Pexp_074060 was expressed only on MEA, and Pexp_071900 and Pexp_079130 were only expressed on PDA. Pexp_071900 is the backbone gene of a biosynthetic cluster of a putative epipolythiodioxopiperazine-like (ETP-like) compound (Ballester *et al*., 2015). Pexp_107400, which encodes a PKS, was down-regulated by VeA on PDA and by LaeA on all media tested (Kumar *et al*., 2017a). Our results showed that VeA regulates Pexp_015170 positively on both media, whereas LaeA does not regulate it. Pexp_015170 encodes an NRPS displaying 91% identity with HcpA, an NRPS responsible for fungisporin synthesis (Ali *et al*., 2014). Although an HcpA orthologous gene exists in the genomes of all *Penicillium* and some *Aspergillus* species (Klitgaard *et al*., 2015; Nielsen *et al*., 2017), no physiological role of this peptide is known. Pexp_000410 was highly down-regulated by the *veA* gene on both media used, whereas it was up-regulated by LaeA on CY. In addition, Pexp_094810, which was also strongly down-regulated by VeA on both media, was not expressed in either the WT or D*laeA* strains whatever the medium (Kumar *et al*., 2017a). Among the genes down-regulated by LaeA, Pexp_076200, whose expression showed no significant difference between the null mutant PeΔ*veA* and the WT strains on MEA, was up-regulated by VeA on PDA. Contrary to the observations with LaeA, Pexp_008740 was up-regulated by VeA on both media. Pexp_029660, Pexp_055140 and Pexp_085540 were down-regulated by VeA, but up-regulated by LaeA (Kumar *et al*., 2017a).

The backbone gene of the citrinin (*citS*/Pexp_005520) cluster was down-regulated in the PeΔ*veA* strain, and genes of the communesin (*cnsI*/Pexp_030540) cluster and another ETP-like (Pexp_058590) cluster were up-regulated in the PeΔ*veA* strain on at least one medium tested, whereas none of the three were regulated by LaeA (Kumar *et al*., 2017a). Pexp_093210, corresponding to the cluster of the monodictyphenone-like compound, was up-regulated by VeA on both media, but was not expressed in the D*laea* strain or the WT strain on either medium. Pexp_104890, the backbone gene involved in siderophore-like biosynthesis (Gründlinger *et al*., 2013), was not regulated by LaeA on either medium or by VeA on MEA, but was down-regulated by VeA on PDA. The expression of most genes differed between the PeΔ*veA* and D*laeA* strains, but we cannot exclude the possibility that the discrepancies originated in the media used. VeA and LaeA, which sometimes interact (complex with VelB), also appeared to act independently, regulating some secondary metabolite clusters differently.

The null mutant PeΔ*veA* strain develoPeΔ like the WT strain in Golden Delicious apples up to 4 dpi, but the rot progression rate decreased over the following 10 days. The same behaviour has been reported in a PeΔ*patL* strain, which was unable to produce patulin (Snini *et al*., 2016). In the previous study, patulin was only detected from 4 dpi with the WT strain. In the present study, the absence of *veA* completely halted the production of patulin *in vivo* and drastically affected *patL* expression *in vitro*. These results reinforce the hypothesis that patulin is not indispensable for the initiation of the disease (Ballester *et al*., 2015; Li *et al*., 2015), but plays a role as a cultivar-dependent aggressiveness factor (Kumar *et al*., 2017b).

Based on the behaviour of the null mutant strain in apple, *veA* is not required for the initiation of the disease. Knowledge of the pathogenicity determinants that allow the penetration and colonization of the fungus in apple pericarp is very limited. The toxins constitute only some weapons of the *P. expansum* arsenal. Some secreted elements, such as D-gluconic acid (Hadas *et al*., 2007; Prusky *et al*., 2004), fumaric acid (Vilanova *et al*., 2014) and polygalacturonase (Jurick *et al*., 2010), have been reported to contribute to the pathogenicity of *P. expansum*. Recently, it has been found that PeΔSte12 mutants show a significant decrease in *P. expansum* virulence (Sánchez-Torres *et al*., 2018). A possible modulation of one of these virulence factors by the VeA factor has yet to be demonstrated. The importance of *veA* in the virulence of some fungal pathogens has already been demonstrated. For example, BcVEL1 or BcVeA, orthologous to *veA* in *Botrytis cinerea*, is required for the full virulence of this species on bean leaves and grape berries (Schumacher *et al*., 2015; Yang *et al*., 2013). Maize plants inoculated at the seed stage with a null mutant D*veA* strain

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of *F. verticilloides* showed no disease symptoms, whereas, under the same experimental conditions, plants infected by a WT strain exhibited symptoms (Myung *et al*., 2012).

VeA is involved in sexual development and is also known to be a negative regulator of asexual reproduction. Deletion of the corresponding gene was thus expected to stimulate the production of conidia. However, in most species, reduced conidiation was observed in the D*veA* strain compared with the WT strain on synthetic media under both light and dark conditions (Calvo *et al*., 2004; Crespo-Sempere *et al*., 2013; Duran *et al*., 2007; Merhej *et al*., 2012; Wiemann *et al*., 2010). Increased sporulation of the D*veA* strain compared with the WT strain has been reported less frequently (Jiang *et al*., 2011; Park *et al*., 2012; Rauscher *et al*., 2016) and sometimes no difference has been observed between the WT strain and the Δ*veA* strain (Estiarte *et al*., 2016). The different findings, even in the same species, could be partly linked to the different media used. For instance, Kato *et al*. (2003) reported a medium-dependent role of *veA* in conidiation.

Here, conidiophore structure was affected by *veA* in *P. expansum* grown on various synthetic media. The biggest difference was the absence of coremia in the null mutant strain, both *in vitro* and *in vivo*. The null mutant PeΔ*veA* strain, grown on apples in the dark, was still able to sporulate *in vitro* but, because of a lack of coremia formation, was unable to pierce the peel of apples and to emerge from the fruit again to complete its life cycle, in contrast with the WT and complemented strains. The storage of apples, which is usually in the dark and in ventilated cold rooms, favours the development and propagation of fungal species. Coremia are relatively rigid structures made up of conidiophores grouped into small columns that are fertile apically. *Penicillium expansum* has the ability to produce coremia when the fungus is grown on standard laboratory media, but also on natural substrates such as apples. The fresh isolates sometimes also produce synnemata, another fasciculate structure, which consist of appressed fertile hyphae along their entire length. *Penicillium expansum* usually produces conspicuous synnemata in its natural habitats, but tends to lose this ability when grown on synthetic media (Raper *et al*., 1968). Little is known about the mechanisms that lead to the formation of coremia. Tinnel *et al*. (1977) showed that the presence of manganese is very important for coremia formation in *Penicillium vulpinum* (syn. *Penicillium claviforme*) and *Penicillium clavigerum*, both patulin-producing species. A specific manganese requirement for patulin biosynthesis has already been demonstrated. Of eight metal ions, only manganese strongly influenced patulin production in *Penicillium griseofulvum* (syn. *Penicillium urticae*) (Scott *et al*., 1986a). This observation was confirmed in a more recent work (Dombrink-Kurtzman and Blackburn, 2005). Through the use of actinomycin D and cycloheximide, respectively, a transcription and translation inhibitor, manganese, has been suggested to have an effect on patulin biosynthesis at the transcription level (Scott *et al*., 1986b). The similarity between coremia formation and patulin biosynthesis suggests a common regulation. The fact that neither PeΔ*patL* nor PeΔ*veA* produces patulin or coremia renders the hypothesis of a manganese-dependent signalling pathway shared by patulin biosynthesis and coremia formation plausible. To our knowledge, no relationship between manganese and the Velvet complex has been reported to date. This hypothesis deserves further attention in future work. Generally, secondary metabolism and morphological development are intimately associated in fungi, particularly in *Aspergillus* and *Penicillium* species (Calvo *et al*., 2002). VeA is a major component of the global regulatory mechanisms involved in the formation of cleistothecia in homothallic *Aspergillus* species, such as *A. nidulans* (Bayram *et al*., 2008), but also in sclerotial morphogenesis in some heterothallic *Aspergillus* species, such as *A. flavus* (Calvo and Cary, 2015). Sclerotia are considered to be vestiges of cleistothecia that have lost their ability to generate ascospores. As cleistothecia/sclerotia and coremia/synnemata are linked to sexual reproduction and asexual reproduction, respectively, except for VeA, there is no evidence to support a common regulatory mechanism.

In conclusion, our results shed light on the role of VeA in *P. expansum* and the *veA* gene. VeA upregulates the production of patulin and citrinin. Because of the different media used, our results also highlighted the induction or repression of the expression of several backbone genes involved in the biosynthesis of other secondary metabolites with known or still unknown functions. From a physiological and ecological point of view, our results show that VeA is important in the final step of fungal cycle development on natural substrates, enabling the spread of spores and hence the colonization of new substrates.

EXPERIMENTAL PROCEDURES

Strains, media and culture conditions

The *P. expansum* wild-type strain NRRL 35695 (WT) used in this study was originally isolated from grape berries in the Languedoc Roussillon region in France. The compositions of the media are detailed in Part S8. Unless otherwise specified, the strains used in this study were cultured on PDA (Sigma-Aldrich, Saint Quentin Fallavier, France), MEA (Biokar Diagnostics, Allonne, France) and CYA (Sigma-Aldrich) for 5 days at 25 °C in the dark.

For the morphological study, strains were plated on MEA, PDA, CzA (Oxoid, Dardilly, France) and CGA (Sigma-Aldrich), and grown for 7 days at 25 °C in the dark.

For the preparation of protoplasts, we used 250 mL of yeast extract peptone dextrose (YPD) medium (Sigma-Aldrich) inoculated with a 3 × 10^7 conidial culture. The culture was grown on an orbital shaker (150 rpm) set at 25 °C for 12 h. The PeΔ*veA* gene disruption cassette was obtained using *Saccharomyces cerevisiae* FY1679: MATa/MATa ura3–52/ura3–52; trp1D 63/TRP1; leu2D 1/LEU2; his3D 200/HIS3; GAL2/GAL2 by

homologous recombination. This strain was grown on solid YPD medium at 30 °C for 5 days.

Construction and molecular characterization of the null mutant Pe*ΔveA* and complemented Pe*ΔveA:veA* strains

In order to characterize the role of the *veA* gene, to understand how it affects the virulence of *P. expansum* and how it influences the production of a wide diversity of important secondary metabolites, a gene deletion strategy was applied by replacing the coding region with the hygromycin selection marker, as described previously (Snini *et al*., 2016). A more detailed description is available in Part S1. The veA deletion was validated by PCR screening and Southern blot as described in Part S2.

In vitro growth, macroscopic and microscopic morphology

WT, null mutant and complemented strains were plated on PDA. After 7 days of incubation, a spore suspension of each strain was prepared according to Adjovi *et al*. (2014). The concentrations of spore suspensions were quantified using a Malassez cell. An inoculum of 10^4 spores was plated centrally on MEA, PDA, CzA and CGA, and the plates were incubated at 25 °C in the dark for 10 days. The experiment was performed in triplicate for each strain. After 7 days, colony diameters were measured and microscopic characteristics (conidiophore branching pattern, phialide and conidia shape, etc.) were observed under an optical microscope [Olympus CX41 (*3*400 and *3*1000, Rungis, France]. After 10 days, macroscopic features (colour of conidial areas, colony margin, colony texture, pigmentation, exudate, reverse colour, etc.) were studied using a stereomicroscope (Olympus SZX9, *3*12–120).

Analysis of patulin production *in vivo*

To assess the production of blue mould and patulin on apples in the WT, null mutant and complemented strains, an *in vivo* study was performed using Golden Delicious apples. The apples were purchased from a supermarket in Toulouse (Carrefour, France), and were free of defects or injuries. The apples were surface sterilized in a 2% sodium hypochlorite solution and rinsed with water, as described previously by Sanzani *et al*. (2012). The apples were then wounded with a sterile toothpick. For each strain, 10 mL of a suspension at a concentration of 10^4 conidia/mL were deposited into the wounds of apples. Contaminated apples were then incubated at 25 °C in the dark for 14 days. The diameters of the rotten spots were measured every day during the incubation period to determine the growth rate, and the rot volume was calculated using the method described previously by Baert *et al*. (2007). After the incubation period, apples were ground using a blender until a homogeneous apple puree was obtained, as described previously by MacDonald *et al*. (2000) and detailed by Snini *et al*. (2016). Patulin production was determined by high-performance liquid chromatography with a diode array detector (HPLC-DAD) based on a standard calibration curve. Eight biological replicates were performed for each strain.

Analysis of secondary metabolism

The WT, null mutant and complemented strains were grown on two different media: MEA and PDA. A spore suspension of each isolate was prepared from a previous culture on PDA and incubated at 25 °C in the dark for 7 days. Petri dishes containing the medium were overlaid with a sterile cellophane layer and inoculated with 10 μL of a 10^6 spores/mL solution of each isolate.

Extraction of secondary metabolites

After a 5-day incubation period, mycelium was separated from the cellophane layer and split into two equal parts. The first half of the mycelium was used for RNA extraction. The second half of the mycelium and the agar medium were macerated separately in 50 mL of ethyl acetate on a horizontal shaking table at 160 rpm at room temperature for 6 days. The organic phase was then filtered through a Whatman filter paper and evaporated to dryness using a Zymark TurboVap (McKinley Scientific, Sparta, NJ, USA). The dried residue was dissolved in 400 μL of acetonitrile–water (50 : 50, v/v) and filtered through a 0.45-μm syringe filter into a clean 2-mL vial. Eight biological replicates were performed for each strain.

HPLC-DAD analysis of patulin and citrinin

The chromatography device used for the quantification of patulin and citrinin was an Ultimate 3000 HPLC system (Dionex/ThermoScientific, Courtaboeuf, France). Patulin was analysed as described by Snini *et al*. (2016). Citrinin was quantified using a 150 mm ± 2 mm, Luna® 5 mm, C18 column (Phenomenex, Le Pecq, France) at 30 °C at a flow rate of 0.2 mL/min. Eluent A was water acidified with 0.05% formic acid and eluent B was acetonitrile acidified with 0.05% formic acid. The column was equilibrated with a mixture of 80% solvent A and 20% solvent B. Elution conditions were as follows: a 30-min linear increase in solvent B from 20% to 50%, a 5-min linear increase of solvent B from 50% to 90%, 90% solvent B for 10 min, a 5-min linear decrease of solvent B from 90% to 20%, 20% solvent B for 10 min. The presence of citrinin was monitored at a wavelength of 327 nm and confirmed by its retention time (min) according to the standard. Patulin and citrinin standards were purchased from Sigma-Aldrich.

Isolation of fungal RNA and RT-PCR

At the end of the incubation period, mycelia were separated from the cellophane, RNAs were extracted and cDNAs were synthesized as described by Tannous *et al*. (2014). The quality of the extracted RNA was verified by gel electrophoresis (1.2% agarose) and the concentrations were measured using a Dropsense apparatus (Trinean, Proteigene, Saint Marcel, France).

Design and validation of qPCR primers

Primer pairs corresponding to the different genes of the patulin cluster described previously by Tannous *et al*. (2014) were used. Primer pairs corresponding to the different genes of the citrinin cluster were designed (Part S5). Primer pairs corresponding to *laeA* and *velB* were designed (Part S7).

The Antibiotics-Secondary Metabolite Analysis SHell (antiSMASH) pro- gram (Weber *et al*., 2015) was used to identify the different clusters of secondary metabolites on the *P. expansum* genome using the T01 and d1 sequenced strains (GenBank: AYHP00000000.1 and GenBank: JQFY01000069.1, respectively). Sixty-three clusters were identified. The primer pairs of the different PKSs, NRPSs and DMATSs of these clusters were designed. Thirty-five backbone genes were expressed under our conditions (Part S6). The primers were designed and validated as described by Caceres *et al*. (2016). At least two biological replicates of *P. expansum* NRRL 35695 were used to validate the amplification specificity. Negative controls (without reverse transcriptase enzyme) were added to control for the absence of contamination.

Analysis of the expression of the genes linked to the secondary metabolites in *P. expansum*

Real-time PCR was used to quantitatively evaluate the expression of the gene clusters of patulin and citrinin and to measure the expression of the different secondary metabolite genes in each strain.

Experiments were carried out as detailed previously (Caceres *et al*., 2016). *b*-Tubulin was used as housekeeping gene. Changes in gene expression of qPCR experiments were then analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Two separate experiments were performed, each including at least eight biological replicates of each condition.

Data analysis

Non-parametric Kruskal–Wallis and Mann–Whitney tests were used to analyse the differences in the WT, null mutant and complemented strains for each of the analyses of patulin production *in vivo*, and the gene expression of secondary metabolites using the RT-PCR technique. The differences were considered to be statistically significant when the *P* value was below 0.05. The statistical analysis of the data was carried out using GraphPad 4 software (GraphPad Software, La Jolla, CA, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Part S1 Construction of *veA* and *veA:veA* deletion cassette.

Part S2 Molecular characterization of the null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains

Part S3 Macroscopic morphology of the null mutant PeΔ*veA*.

Part S4 Dissemination of *Penicillium expansum*.

Part S5 Expression analysis of gene clusters involved in patulin and citrinin biosynthesis.

Part S6 Expression analysis of genes encoding backbone enzymes involved in secondary metabolite biosynthesis.

Part S7 Expression analysis of *laeA* and *velB*.

Part S8 Composition of the culture media

PART 1. Construction of Δ*veA* and Δ*veA:veA* deletion cassette

In order to characterize the role of the *veA* gene, a PeΔ*veA* mutant was generated by the replacement of *veA* with a hygromycin resistance marker in the wild type NRRL 35695 (WT) strain of *P. expansum*. Construction of the null mutant PeΔ*veA* is shown in Fig. S1. Specific primers of *veA* (Pexp_092360) were designed from the *P. expansum* d1 strain sequence deposited in GenBank (JQFY01000122). The primers are listed in Table S1. The cassette Δ*veA*, carrying an hygromycin resistant cassette, and flanked by DNA sequences corresponding to the sequences located at 5' (left junction) and 3' (right junction) ends of the *veA* gene was constructed using the homologous recombination strategy in *S. cerevisiae* as previously described by Merhej *et al.* (2011) modified by Snini *et al.* (2016) for an application on *P. expansum*. Fragments required for the gene disruption cassette were amplified by PCR, then gathered and assembled into the pRS426 plasmid in *S. cerevisiae*. For the complementation, primers dveAF3/dveAR3 were used to generate the DNA fragment from the WT strain to transform protoplasts of PeΔ*veA* strain.

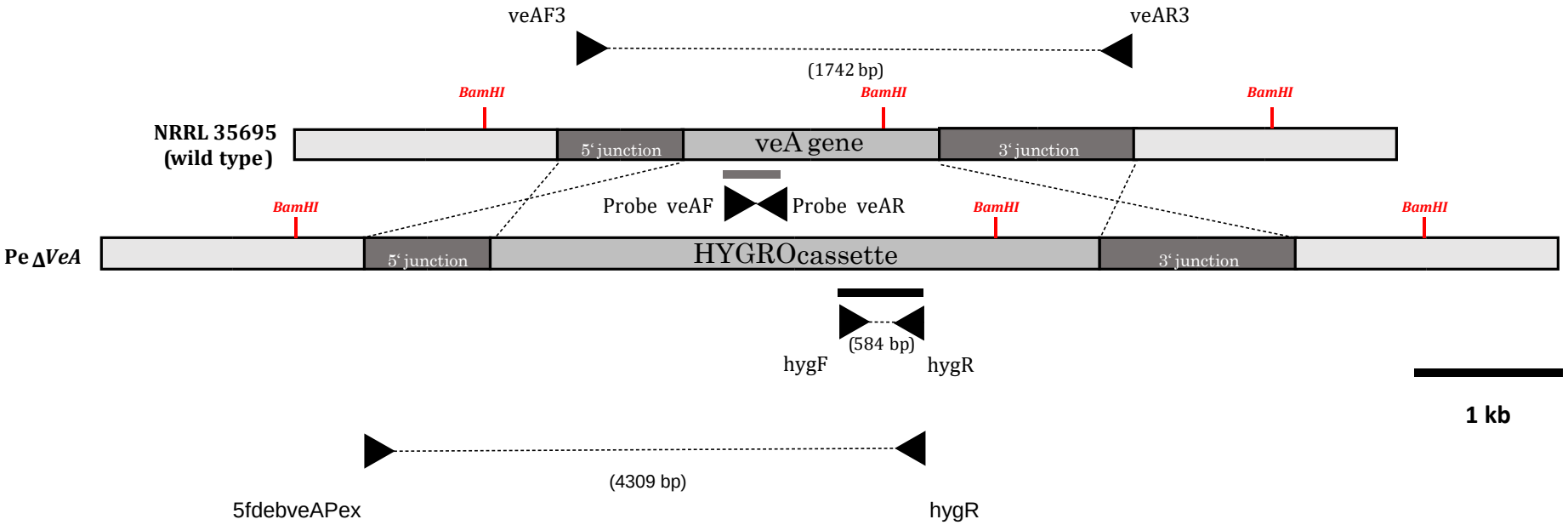

Fig. S1. Construction of the null mutant PeΔ*veA*. Map of the veA wild-type (WT) locus and the replacement construct ΔveA containing a hygromycin resistance cassette. The primers used to design a polymerase chain reaction (PCR) strategy to distinguish between WT and locus integrated transformants are indicated by arrows. The black bar shows the position of the VeA DNA and the grey bar the Hygromycin (hyg) DNA probes used in the Southern blot analysis. BamHI sites are in red.

Table S1. Primers used in the construction and the validation of null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA*.

PRIMER	SEQUENCE 5'→ 3'
5fdebveAPex[1]	GTAACGCCAGGGTTTTCCCAGTCACGACGGGGCAACTCCTGCCAGAAACC
debveAPex1Rhygro[2]	GAGCCTGTGTGTAGAGATACAAGGGAATTCTTTCGCGGATTGATGTTATTATTCCCC
finveAPex1Fhygro[2]	GTGTAAGCGCCCACTCCACATCTCCACTCGTAGAATCTGAAATGGCCTCGAACATCTTGC
3rfinveAPex[1]	GCGGATAACAATTTCACACAGGAAACAGCGTAGATACTGGGCGGTTTCTCGC
hphF1	GAATTCCCTTGTATCTCTACACACAGGCTC
hphR1	CGAGTGGAGATGTGGAGTGGGCG
dveA-NestedF	GTTGCGGCCGGAACCTTGGAACC
dveA-NestedR	CCAGAGATGTCTGAGCTCCAATCGC
dveAF3	GAGAACACGCGGTATCGAAGGGTG
dveAR3	CCACTCACGCTGCGACTCCC
hygF	TCGGAGGGCGAAGAATCTCG
hygR	CCGAACATCGCCTCGCTC
ProbePeveAF	GTTGACCCACCGCCTGTGGTTG
ProbePeveAR	GGTGCTGGTCGCTAGGCCAG

[1]The underlined sequences were added at the 5' end and were identical to the extremities of the pRS426 cloning vector.

[2]The underlined sequences were added at the 5' end and were identical to the extremities of the PCR product generated by primers hphF1/hphR1.

PART 2. Molecular characterization of the null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains

The potential PeΔ*veA* mutants were first screened by polymerase chain reaction (PCR) and the mutants that possibly carried a disrupted *veA* gene were selected. On these mutants, the incorporation of the hygromycin cassette in their genome was confirmed by PCR. The expected size of the amplicon (584 bp) was obtained (Fig. S2A). The hygF and hygR primers, specific to the hygromycin sequence, were used for this purpose. Subsequently, in order to confirm the presence of this disruption cassette Δ*veA* at the *veA* locus, the primers 5fdebveAPex and hygR were used on sort to anneal outside and inside the disruption cassette, respectively and a PCR was performed. The expected size of the amplicon (4309 bp) was obtained (Fig. S2B).

In the following tests, this PeΔ*veA* mutant was the only kept and used. To verify that the phenotype exhibited by the mutant strain resulted from the deletion of *veA*, a complemented transformant PeΔ*veA:veA* was generated by reintroducing the *veA* encoding sequence into the *veA* deleted strain. As expected, PCR and sequencing confirmed that the WT gene was detected, but not the hygromycin sequence.

For a subsequent southern blot confirmation, an enzymatic digestion with BamHI was performed on the WT, the null mutant and the complemented strains to confirm the presence of the disruption cassette at the *veA* locus in the mutant strain, and its replacement by the WT copy of this gene in the complemented one. Southern blot analysis was performed as previously described (Snini *et al.*, 2016). The hygromycin (hyg) DNA probe and veA DNA probe were generated using the primer pairs hygF/hygR and probe Pe*veA*F/probe Pe*veA*R, respectively. Southern blot analysis confirmed the insertion of only one copy of the disruption cassette at the *veA* locus in the PeΔ*veA* mutant and one copy of the *veA* gene in the PeΔ*veA:veA*. Both WT and complemented strains did not present the hygromycin disruption cassette in their genome (Fig. S3A and B).

The PeΔ*veA* deletion mutant showed a null expression of *veA* compared with the WT and complemented strain PeΔ*veA:veA* when grown on Malt Extract Agar medium (MEA) and Potato Dextrose Agar medium (PDA) (Fig. S4).

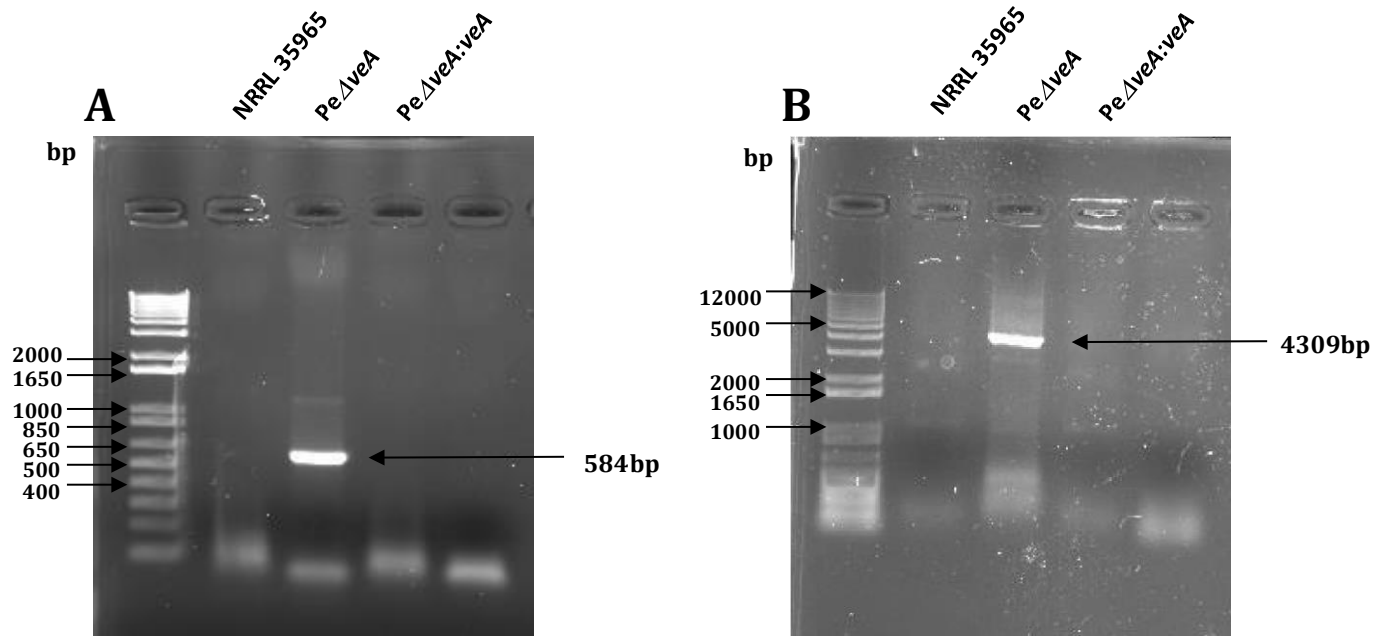

Fig. S2. Polymerase chain reaction (PCR) analyses of genomic DNA of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains. A) PCR amplification with hygF and hygR primers. The hygromycin cassette was incorporated in the genome of the null mutant PeΔ*veA* strain with a predicted PCR product of 584 kb. No amplification was observed in the WT or complemented strains. B) PCR amplification with 5fdebveAPex and hygR primers. The presence of the disruption cassette Δ*veA* at the *veA* locus was confirmed with a predicted PCR product of 4309 kb. No amplification was observed in the WT or complemented strains.

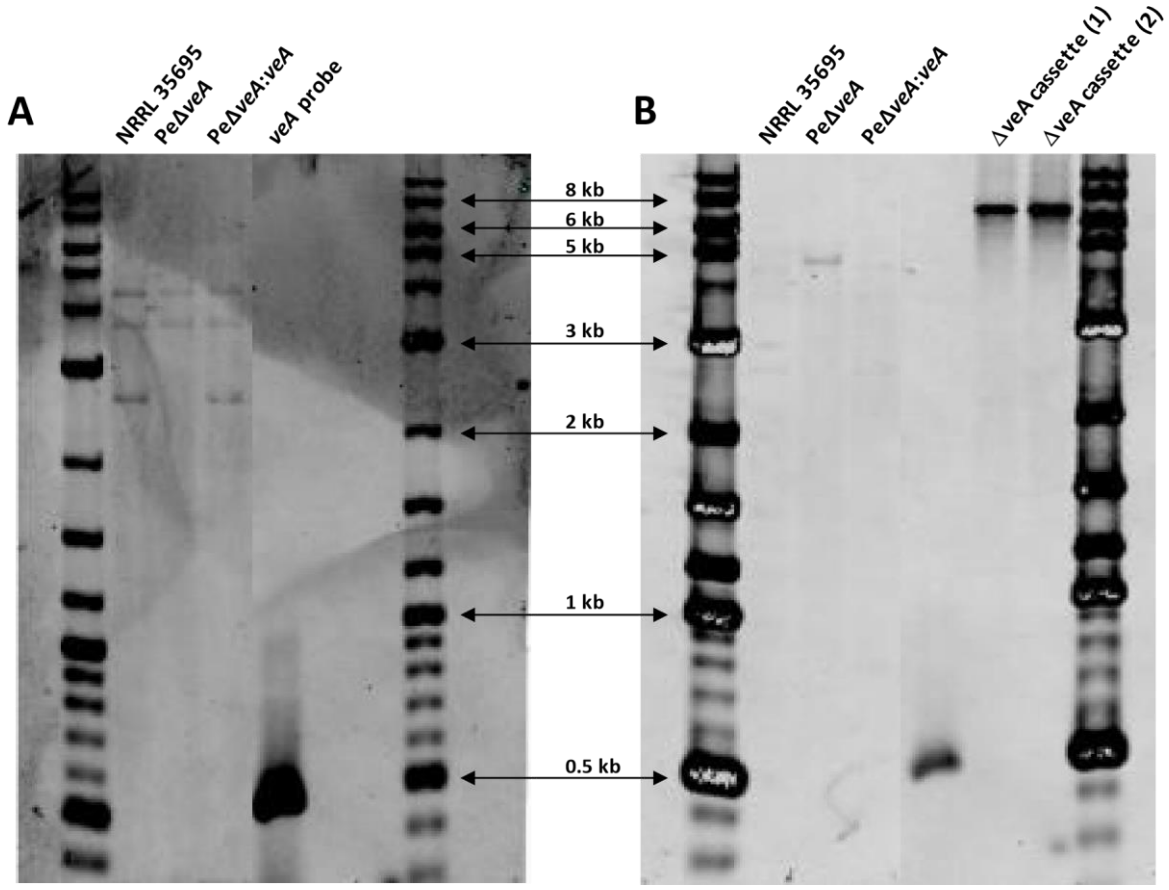

Fig. S3. Southern blot analyses of genomic DNA of the wild-type NRRL 35695 (WT), null mutant Pe*ΔveA* and complemented Pe*ΔveA:veA* strains. A) Southern blot analysis of DNA digested with *BamH*I and hybridized with the VeA probe. The complemented and the WT strains showed a fragment of 2.7 kb absent in Pe*ΔveA*:*veA* strain and which corresponds to the *veA* gene. B) Southern blot analysis of the hybridization of DNA with the hygromycin (hyg) probe after digestion with *BamH*I. Only the null mutant Pe*ΔveA* strain exhibited a unique fragment of 4.7 kb consistent with the replacement of the *veA* gene by the Δ*veA* cassette. No hybridization was observed in either the WT or complemented strains, due to the absence of the Δ*veA* cassette.

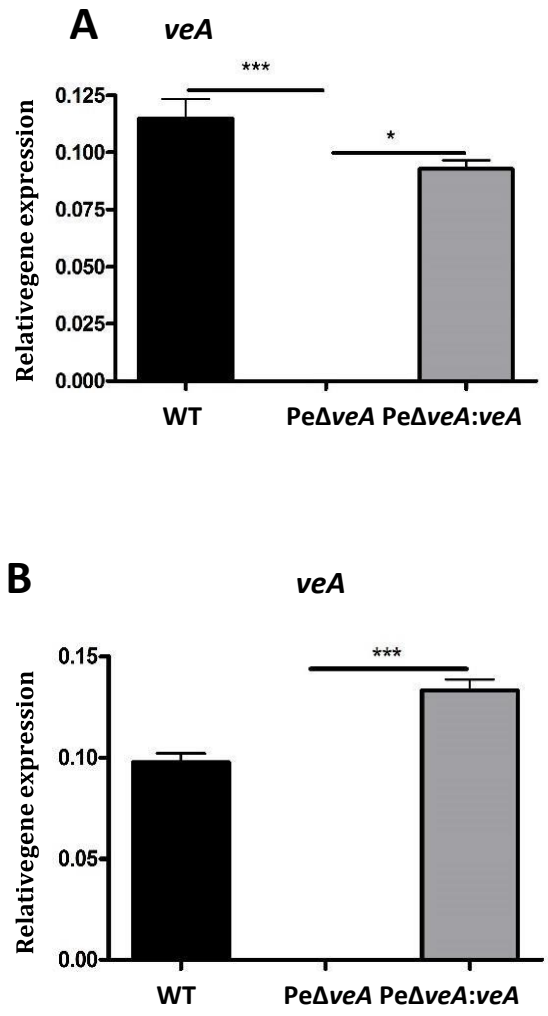

Fig. S4. Expression of the *veA* gene on Malt Extract Agar medium (MEA) and Potato Dextrose Agar medium (PDA). Expression of the *veA* gene was evaluated in the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains on A) MEA and B) PDA media. Graphs show the mean ± standard error of the mean (SEM) of 8 replicates. Asterisks denote significant differences between the WT strain and the null mutant PeΔ*veA* strain. * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001.

PART 3. Macroscopic morphology of the null mutant PeΔveA

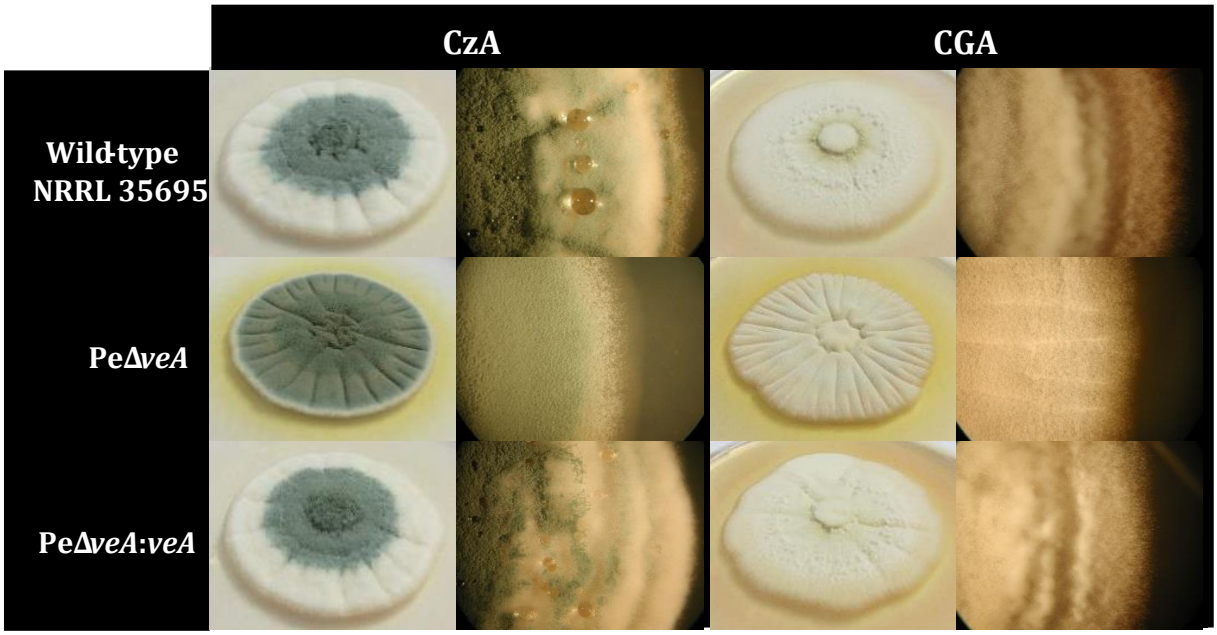

Fig. S5. Morphological appearance of the wild-type NRRL 35695 (WT), null mutant Pe*ΔveA* and complemented Pe*ΔveA:veA* strains. Strains were grown at 25°C in the dark for 10 days on Czapek Dox Agar medium (CzA) and Czapek Glucose Agar medium (CGA) inoculated with 10^4 spores. Macroscopic appearance of the colonies and observations under a stereomicroscope (×12).

PART 4. Dissemination of *P. expansum*

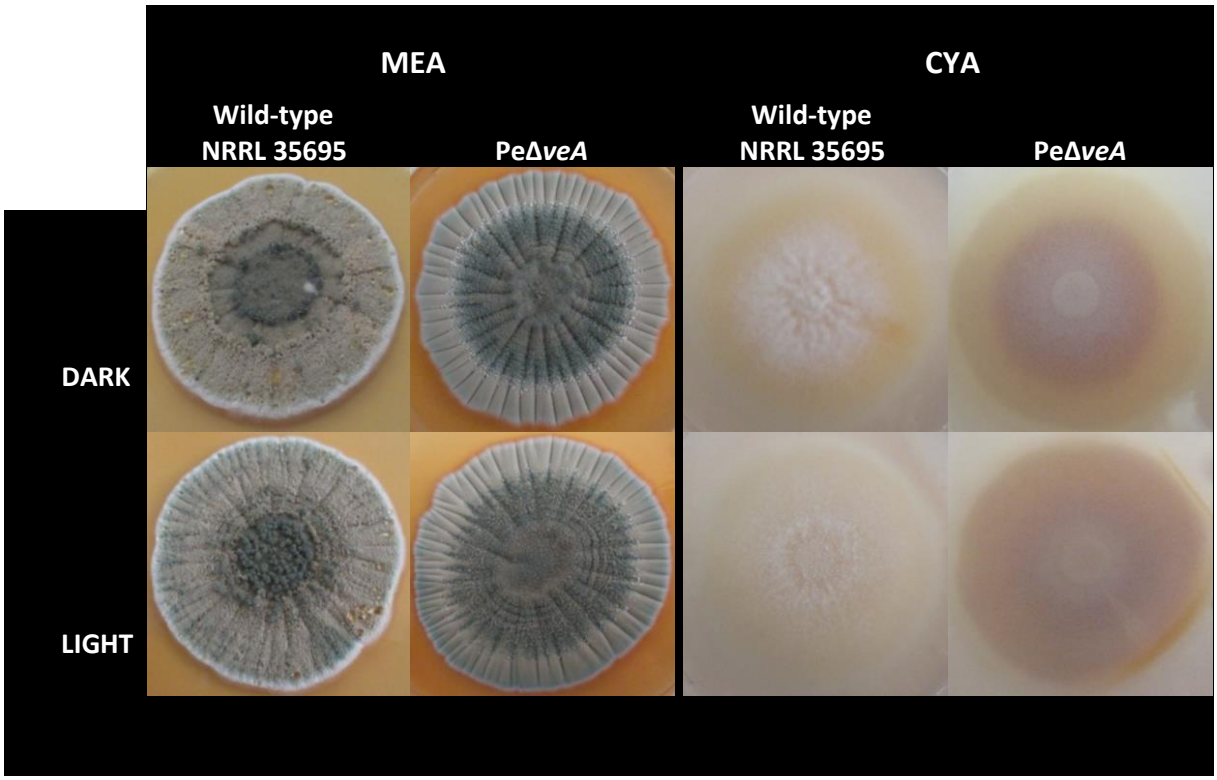

Fig. S6. Influence of light on the morphological appearance of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains. Strains were grown at 25°C for 7 days on Czapek Yeast Extract Agar medium (CYA) and Malt Extract Agar medium (MEA) inoculated with 10^4 spores.

Table S2. Primers used to analyze the expression of the citrinin cluster.

gene	PRIMER	SEQUENCE 5' → 3'
orf5	**Pexp_005510_F1** **Pexp_005510_R1**	GACCACGGTCACTTCCAAAGAG AGTGGTCCCACTGCAAAGCC
citS	**Pexp_005520_F1** **Pexp_005520_R1**	ATATGATCCAGAAAGAAAGTCAACGAG AACAAGAACGGAAACGTATGCATC
citA	**Pexp_005530_F1** **Pexp_005530_R1**	CGCGCCTTGGCTAAATCC ACGCAAAGCGCCAAGATG
citB	**Pexp_005540_F1** **Pexp_005540_R1**	AAATCATCCTTCTATTTACCGGCTG CATCGTTGATTACTTTGCGTGC
ctnA	**Pexp_005550_F1** **Pexp_005550_R1**	GTCAACGAGGGAGCATTAGTGTTAG GATGGGCATGAGAGGATGGAC
citD	**Pexp_005560_F1** **Pexp_005560_R1**	GACTCCATTCCCTCAAGGTGC GTTGTATTGGTCAACAACTACTTTCGTG
orf6	**Pexp_005570_F1** **Pexp_005570_R1**	GTACCCCCGTAGGGCTTGAG CCATGGTGGAAGTAGCAAAGGTC
citE	**Pexp_005580_F1** **Pexp_005580_R1**	GACCAGATGTAAGCAGCATGGAC CACCGGCTTGAGCGAATG
citC	**Pexp_005590_F1** **Pexp_005590_R1**	TGAGAGCATCCCACAGGCTG TGGCCGGTTATAAGACATAAAGTTG

PART 5. Expression analysis of gene clusters involved in patulin and citrinin biosynthesis

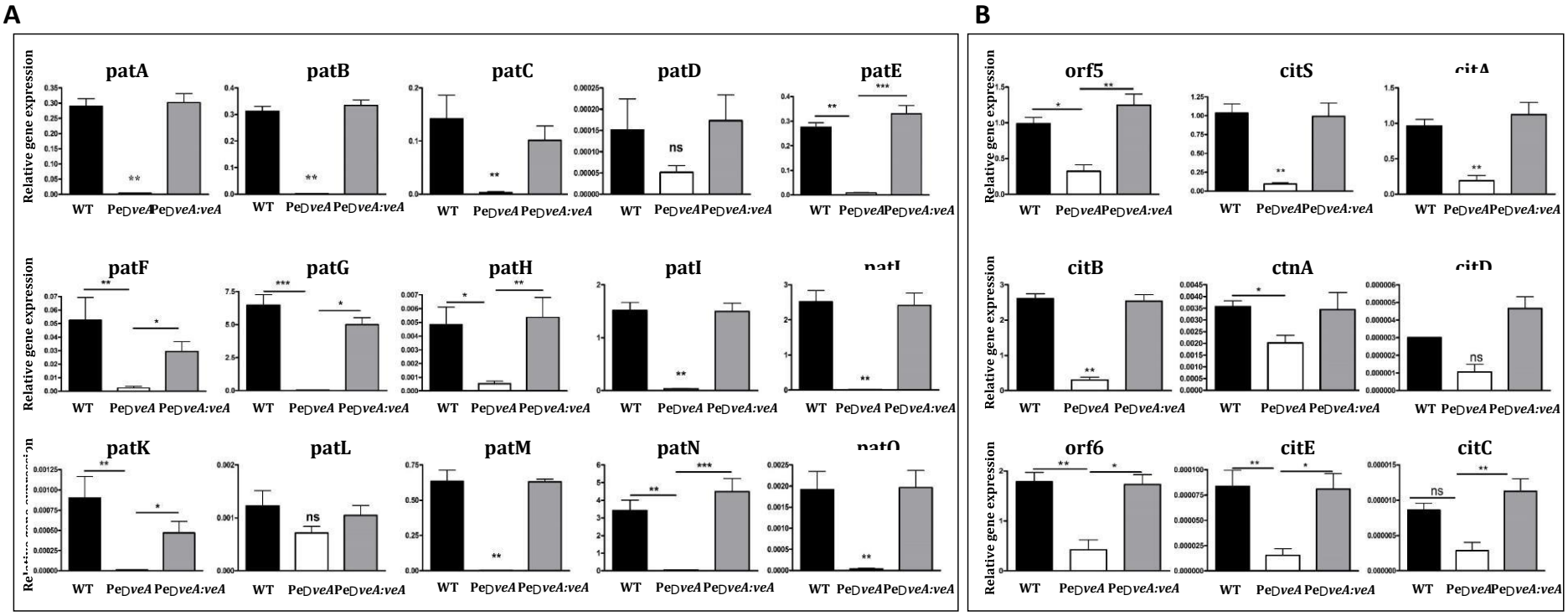

Fig. S7. Relative gene expression of the A) patulin and B) citrinin biosynthesis genes on Malt Extract Agar medium (MEA). Relative expression values are the averages of 8 replicates for the wild-type NRRL 35695 (WT), null mutant Pe*Δveā* and complemented Pe*Δveā:veā* strains with standard deviations shown as error bars. Asterisks denote significant differences between the WT, null mutant Pe*Δveā* and complemented Pe*Δveā:veā* strains. * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001.

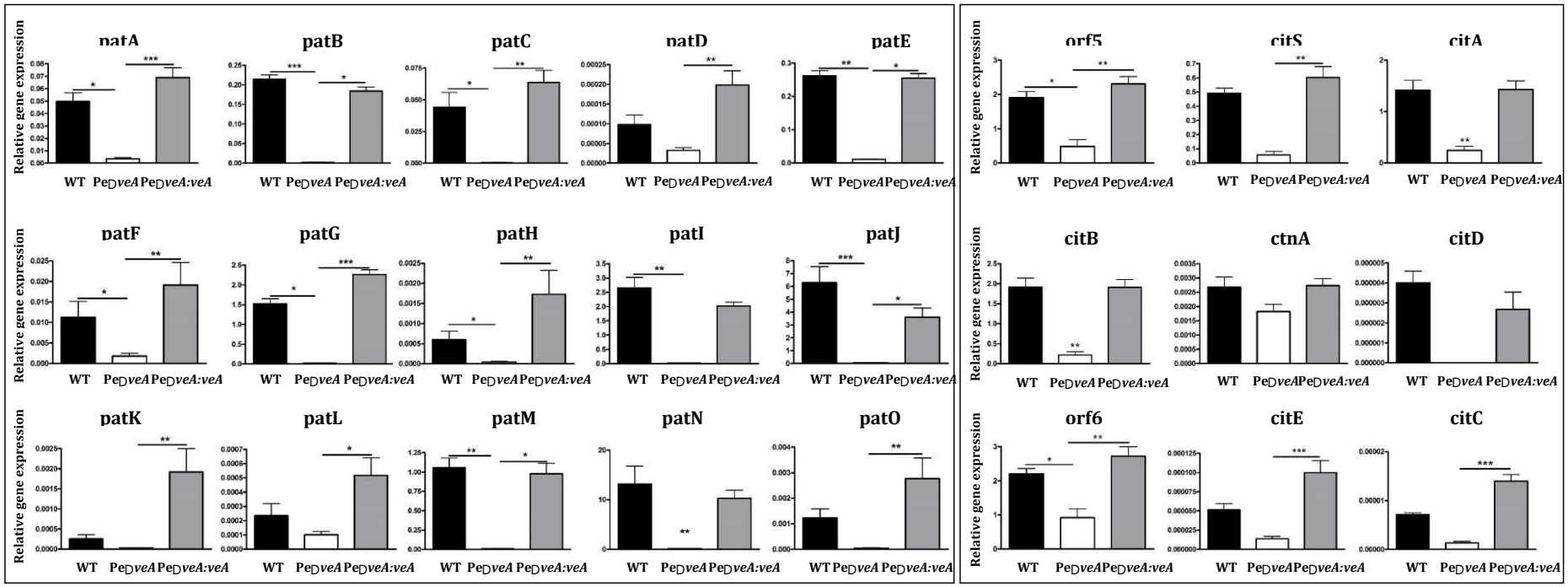

Fig. S8. Relative gene expression of the A) patulin and B) citrinin biosynthesis genes on Potato Dextrose Agar medium (PDA). Relative expression values are the averages of 8 replicates for the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains with standard deviations shown as error bars. Asterisks denote significant differences between the WT, null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

PART 6. Expression analysis of genes encoding backbone enzymes involved in secondary metabolite biosynthesis

Table S3. Primers used to analyze the expression of other backbone enzyme genes.

gene	PRIMER	SEQUENCE 5' → 3'
	Pexp_015170_F1	ACTATCTTCATGCTGCGCACATAC
	Pexp_015170_R1	CATGAAGACGGACTTGGCACTC
cnsI	**Pexp_030540_F2**	ATGTGCGACAGTTCTCAAGGATG
	Pexp_030540_R2	ATAAAGTGATAAAGAAATCCAAGTCTACGTC
	Pexp_095540_F1	CCTGGAACGTCAGTCAACAATACAC
	Pexp_095540_R1	CCACGGTTGTACTGCTTTATATTCTG
	Pexp_030090_F2	GTTTAACACGGTTCTCACAGTGCAG
	Pexp_030090_R2	AATAGCGACGCAAAGGTCATACTC
	Pexp_076200_F1	CTGGTGATCCAATTGAGTCAAGC
	Pexp_076200_R1	ACCACTAGCACCCTCTGTATGGC
	Pexp_013580_F1	CCTCTTACTCAGGGTGCCTGTATC
	Pexp_013580_R1	TCAGTTTGAGCCAATTCGCATC
	Pexp_093210_F1	GGCCCTGGCCGTATCAAC
	Pexp_093210_R1	GGCTTGGCAGGCGACC
	Pexp_058440_F1	CGCTTCATCACGCGCTCTAC
	Pexp_058440_R1	GTACTGGATAAACGGTTTGAACTCG
	Pexp_082260_F2	GCGGCACGGTAACCGAC
	Pexp_082260_R2	AAGCATGAGCGGCCATCTG
	Pexp_096630_F1	GGTGCCTGTCTTACTCCATCGTC
	Pexp_096630_R1	TTCGCAATAAGTGGCTTAGGGAC
	Pexp_094810_F1	GAGGCGCTCGAAAATGCTG
	Pexp_094810_R1	CTCGGATGATTCCCTCTTGGTAG
	Pexp_079130_F1	AAGCAGCCTTGCCATGGAG
	Pexp_079130_R1	GAAAATATCGGCCAACAGGCTC
	Pexp_078820_F1	GATCTCGCATATCTGATCTACACTTCTG
	Pexp_078220_R1	TGATTTATCCCATGGCTTGCTG
	Pexp_104890_F2	ATCTGAAGAAGCGAAAGATGAAGG
	Pexp_104890_R2	CCTCGACATGATGATTCACTGTG
	Pexp_037250_F1	CCCTGCGTGTTCGCTACATC
	Pexp_037250_R1	ACAGGCACTGTGTATAAGAACCGAC
	Pexp_000130_F1	ACTGTTCGAGTCTTTCCCAACG
	Pexp_000130_R1	GCACCCTGTGCAACACCAG
	Pexp_000910_F2	GACAATGAAAAAGGATATCGGCAC
	Pexp_000910_R2	CCAATTTCAACATGTCGGCATC
	Pexp_029660_F1	CGCCCGGCGATTCTTC
	Pexp_029660_R1	CAGGCCTCCTTGAGTCGTCTG
	Pexp_047050_F1	CGTGTCTTCAAGGATCAGGTGC
	Pexp_047050_R1	TCGATGCTAGTCATCATGATTGAGTC

	Pexp_086670_F1 **Pexp_086670_R1**	GTTCGATGGTGCTTCGTGATATG AAATCTAGCGGGGAATTTATCAGC
	Pexp_107400_F1 **Pexp_107400_R1**	CAATTTGATTTTCAGCCCGAAC CTGGGACTTGCCCGTGG
	Pexp_071900_F4 **Pexp_071900_R4**	ATCTACGCATGGTTTATGCCACAG GTGTGAAGGATGTGCAGATAGAACAC
	Pexp_055140_F2 **Pexp_055140_R2**	AGATATTCTCACCCAGCCTTTGC GCCGCTGGTGAATACCACG
	Pexp_006700_F1 **Pexp_006700_R1**	AGCTTTTCAAAGAGGGTATCCCG CAGGGTGTCAATGTGGAAGTCTG
	Pexp_024160_F2 **Pexp_024160_R2**	AGCCAGCATGCGATATTTCTTC ATGCGTGGGCATGACCAG
	Pexp_028920_F1 **Pexp_028920_R1**	GAATTGATTCATCTACGACCACCG GATAGAGGATGCCTTCAGCTATCTCAG
	Pexp_000410_F1 **Pexp_000410_R1**	TAGCATATGAAGGTCTTGAAAATGCTG CTGCAGAAAAAGACCCTACAAAGC
	Pexp_097790_F2 **Pexp_097790_R2**	CCACCAAGTTGGTCTTTGAAAGAG CGATTTGAATGGCGGTCG
	Pexp_008740_F1 **Pexp_008740_R1**	TCTCGGCCACATACATTTCAGG TCTTCGAGTATGATCGCTGTGC
	Pexp_074060_F1 **Pexp_074060_R1**	GTACGTTGCCAGTGGCACG GTCAATGCTGTCAGGGTGACTG
	Pexp_076580_F2 **Pexp_076580_R2**	GGAATGTAGTTGTCAACGTGTTTAGC AGACTTTTTAGAGATGTGGGATCATGG
	Pexp_058590_F1 **Pexp_058590_R1**	GGTATTGTGGAATGCCTTGTTCTC CCGGAAGGTTCTTCGATCTCTG
	Pexp_036220_F1 **Pexp_036220_R1**	CCTCTCGGGTCACGTTGAAG CTACCTCGAATACACTGGAAAAGTGG
citS	**Pexp_005520_F1** **Pexp_005520_R1**	ATATGATCCAGAAAGAAAGTCAACGAG AACAAGAACGGAAACGTATGCATC
patK	**Pexp_094460_F1** **Pexp_094460_R1**	ACTCCTGGTACTGAGTACAGTGAATATGAA CTCTGGAATCTCACCCACTGC

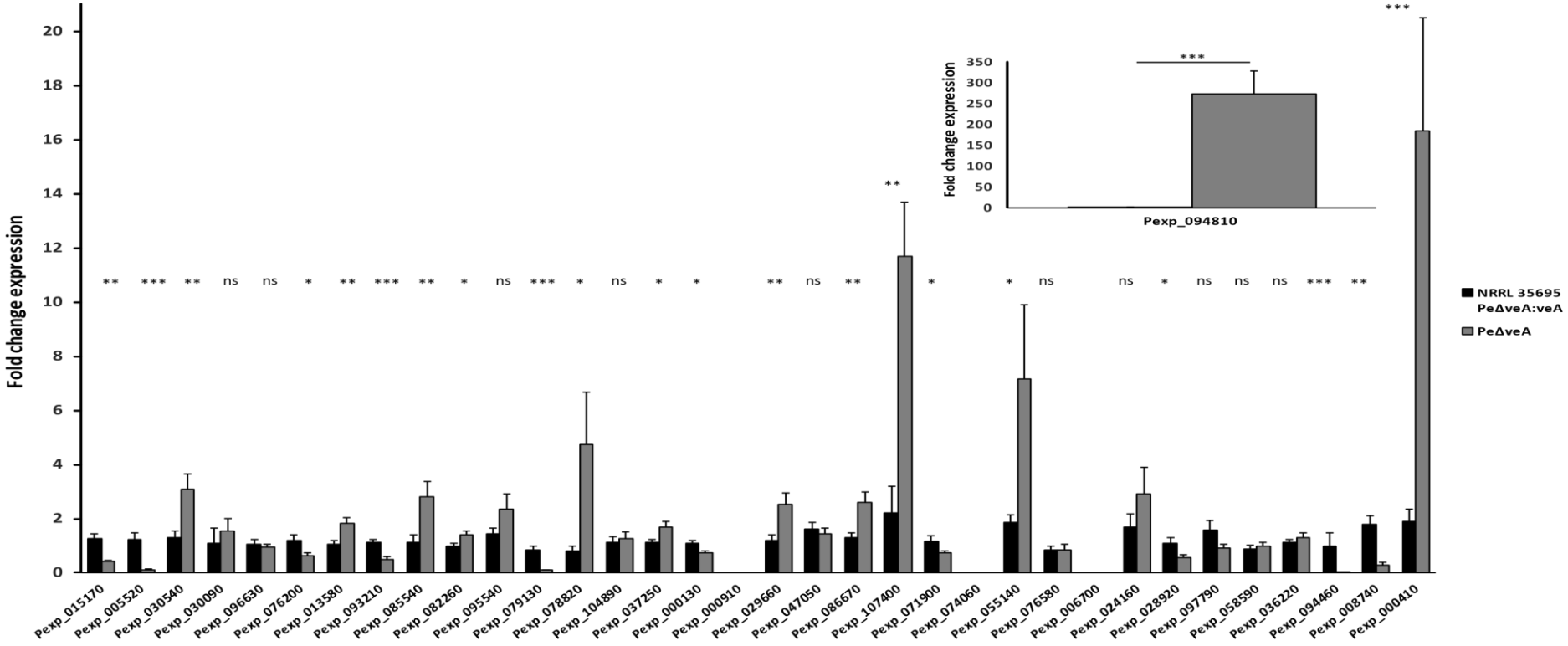

Fig. S9. Relative expression of 35 genes encoding backbone enzymes involved in secondary metabolite biosynthesis. Relative expression of polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) and dimethylallyl tryptophan synthases (DMATs) identified on the sequenced genome of *Penicillium expansum* T01 and d1 strains grown on Potato Dextrose Agar (PDA). Gene expression was evaluated in the wildtype NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains. Primers were designed using PrimerExpress 2.0 software and tests were carried out using a ViiA7 Real-Time PCR System. Black bars represent combined expression level of genes in the WT and complemented strains after culture at 25°C in the dark for 5 days. Grey bars represent gene expression levels in the null mutant strain grown under the same conditions. Graphs show the mean ± standard error of the mean (SEM) of 8 replicates. Asterisks denote significant differences between the WT and the null mutant PeΔ*veA* strains. Inset, fold change expression of Pexp_094810. nd = no difference detected, ns = no significant changes. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

PART 7. Expression analysis of *laeA* and *velB*

Table S4. Primers used to analyze the expression of the *laeA* and *velB* genes.

laeA	**Pexp_laeA_F1**	**CTCCGATTATAATTTCCGATCGCAAGATAC**
	Pexp_laeA_R1	**GTGAACAGTTTGTGGAATATGTCGAGAC**
velB	**Pexp_velB_F1**	**TCGGGGCCCAACTCGCTAC**
	Pexp_velB_R1	**GGGAGTAATGGGTCTCCGGTCC**

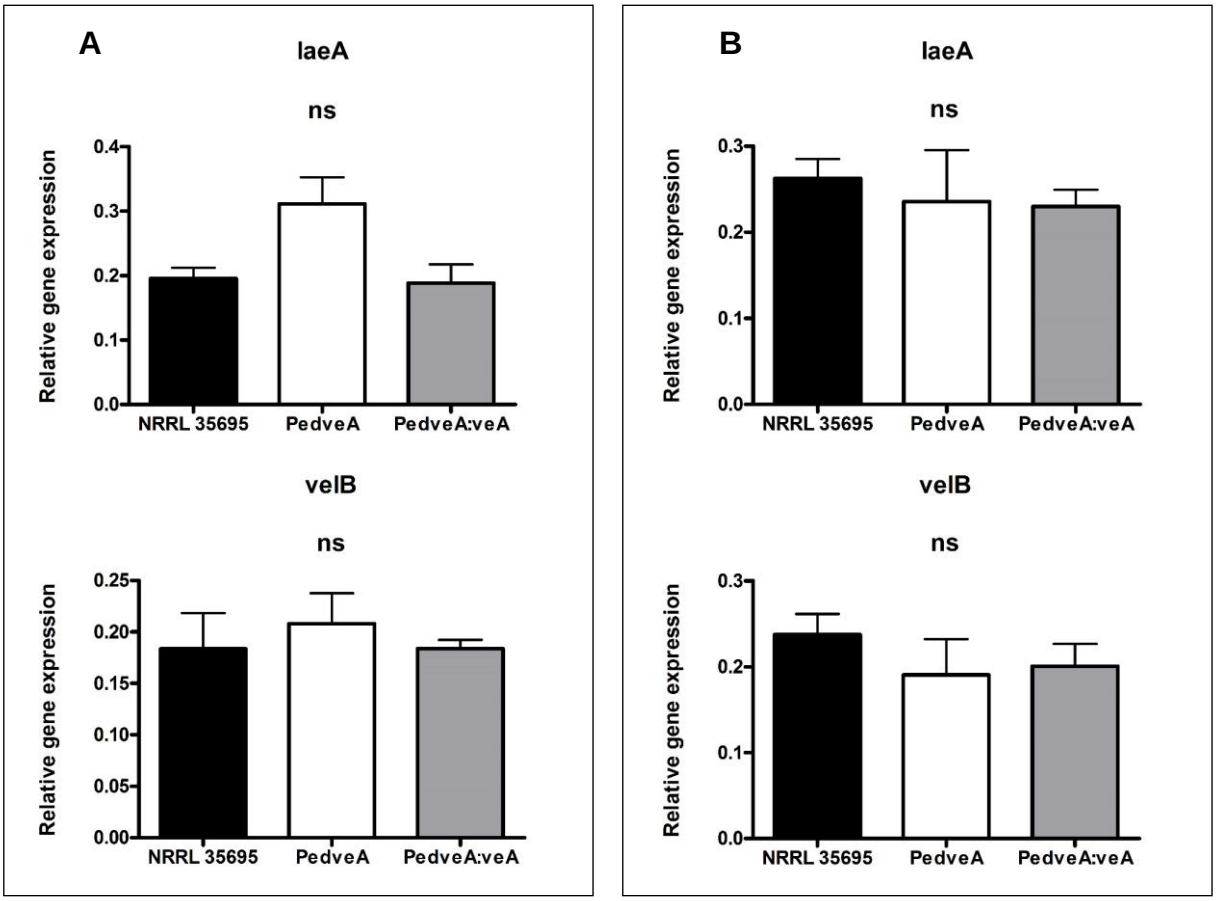

Fig. S10. Relative expression of the *laeA* and *velB* genes on synthetic media.

Relative expression of A) *laeA* and B) *velB* genes on Malt Extract Agar medium (MEA) and Potato Dextrose Agar (PDA). Relative expression values are the averages of replicates for the wild-type NRRL 35965 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains with standard deviations shown as error bars.

PART 8. Composition of the culture media

Culture of the strains used in the study was done on Potato Dextrose Agar medium (PDA) (Sigma-Aldrich, Saint Quentin Fallavier, France), on Malt Extract Agar medium (MEA) (Biokar Diagnostics, Allonne, France) (30 g/L malt extract, 15 g/L agar) and on Czapek Yeast extract Agar medium (CYA) (10 mL/L concentrated Czapek (30 g/L $NaNO_3$, 5 g/L $MgSO_4$ 7 H_2O, 0.1 g/L $FeSO_4$, 5 g/L KCl), 1mg/L K_2HPO_4, 5 g/L yeast extract, 30 g/L saccharose, 15 g/L agar) for 5 days at 25°C in the dark unless otherwise specified.

For the morphological study, strains were plated on MEA, PDA, Czapek Dox Agar medium (CzA) (Oxoïd, Dardilly, France) (2 g/L sodium nitrate; 0.5 g/L potassium chloride; 0.5 g/L magnesium glycerophosphate; 0.01 g/L ferrous sulphate; 0.35 g/L potassium sulphate; 30 g/L sucrose; 12 g/L agar), and Czapek Glucose Agar Medium (CGA) (35 g Czapek Dox broth (Sigma-Aldrich); 8 g/L glucose, 15 g/L agar), and grown for 7 days at 25°C in the dark.

For the protoplast preparation, 250 mL of Yeast extract Peptone Dextrose medium (YPD) (Sigma-Aldrich) (20 g/L dextrose, 10 g/L yeast extract, and 20 g/L bactopeptone) inoculated by a $3x10^7$ conidia culture was used. The culture was grown on an orbital shaker (150 rpm) set at 25°C for 12 hours. The *Saccharomyces cerevisiae* strain was grown on solid YPD medium (15 g/L agar) at 30°C for 5 days.

References

Merhej, J., Urban, M., Dufresne, M., Hammond-Kosack, K.E., Richard-Forget, F. and Barreau, C. (2012) The velvet gene, *FgVe1*, affects fungal development and positively regulates trichothecene biosynthesis and pathogenicity in *Fusarium graminearum*. *Mol. Plant Pathol.* **13**, 363-374.

Snini, S.P., Tannous, J., Heuillard, P., Bailly, S., Lippi, Y., Zehraoui, E., Barreau, C., Oswald, I.O. and Puel, O. (2016) The patulin is a cultivar-dependent aggressiveness factor favoring the colonization of apples by *Penicillium expansum*. *Mol. Plant Pathol.* **17**, 920-9

Chapter 1_Part 2

Impact of *veA* on the metabolome of *Penicillium expansum*

Effect of *veA* on the metabolome of *Penicillium expansum*

As we have seen in the previous sections, *Penicillium expansum* is the causal agent of a common post-harvest disease known as blue mould rot. Fruit products, apples specifically, are the most affected by this pathogen and are considered as the main cause of entry of patulin into the food chain.

Besides patulin, the main toxin produced by *P. expansum*, numerous secondary metabolites remain unknown and it would be interesting to identify them and to study their toxicity.

In order to detect new *P. expansum* compounds and determine their chemical formulas, the fungus was grown on labelled wheat grains with stable isotopes (natural grains with 99% ^{12}C; grains enriched with 97% of ^{13}C; and grains enriched with 53% of ^{13}C and 97% of ^{15}N) followed by an untargeted metabolomic approach. High-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC–HRMS) analysis allowed the detection of fungal metabolites and the characterization of chemical formulas.

Then, secondary metabolites produced by the WT strain of *P. expansum* and its null mutant on a synthetic medium were compared to the metabolites produced by the fungus on labelled wheats. New compounds were highlighted. This research shed the light on the characterization of *veA* and its involvement in the regulation of secondary metabolites in *P. expansum*. In addition, they showed the difference between what is secreted by the fungus in the medium or apple at earlier stages of colonization of his territory and what is secreted in the spores during asexual reproduction. It also highlighted the metabolites produced by the WT, *veA* null mutant and complemented strains in infected apples.

This section provides data on new compounds produced by *P. expansum* and could serve as a basis for further studies on toxins that are potentially more toxic than patulin or on the contrary, with an application in the medical or agronomic field.

1.2.1. Background

The phytopathogenous fungus *Penicillium expansum* is the causal agent of the blue mould of apples and some other fruits, pears, cherries, peaches, and plums, leading to important economic losses in orchards worldwide (Morales et al. 2010; Nunes 2012). *P. expansum* is a psychrophilic and necrotrophic fungus that develops during harvesting and post-harvest processes and storage through injuries (bruises, puncture wounds) on the surface of the fruit, causing tissue decay. Spores or conidia enter the fruit, germinate and then grow as hyphae forming the mycelium (basal

metabolism), which gives rise to thousands of spores (airborne metabolism) during asexual reproduction.

P. expansum produces the mycotoxin patulin, the level of which in apple-derived food products is regulated in many European countries (EC Commission Regulation 2006) due its toxicity (Puel et al. 2010; Glaser and Stopper 2012). Patulin acts as a cultivar-dependent aggressiveness factor (Snini et al. 2016) concomitantly with tissue acidification (Prusky et al. 2016). In addition, *P. expansum* is able to produce several other secondary metabolites of very diverse structure (Tannous et al. 2017). Citrinin has a polyketide (PK) structure like patulin and has antibiotic properties but is nephrotoxic. It may have accessory supportive functions during colonization of apples (Touhami et al. 2018). The indole alkaloids roquefortin C, weakly neurotoxic, and communesins, which have antiinsectan activity, are non-ribosomal peptides (NRPs). The cytotoxic chaetoglobosins A and C, and cytochalasins (potential growth tumour inhibitor) are PK-NRP hybrids. Terpenes were also identified: geosmin, which is associated with the earthy flavour of the species, expansolides A and B, and the meroterpenoids andrastins A, B and C, putative anticancer drugs. Secondary metabolites play ecological roles such as intra- and inter-species communication signals and virulence factors for human and animal infections (Brakhage 2013; Macheleidt et al. 2016). Fungal metabolites have also been developed for medical, agrochemical and cosmetic applications (Bills and Gloer 2017).

The genes involved in the biosynthesis of secondary metabolites are often grouped into clusters, not all of which are expressed under standard conditions. Potential new compounds that are synthesized by enzymes encoded by genes of these cryptic clusters remain thus to be discovered (Ballester et al. 2015; Nielsen et al. 2017). In addition to specific transcription factors involved in secondary metabolites biosynthesis, located within the cluster and modulating the expression of the other genes in this cluster, there are global transcription factors that are regulated by internal and external cues such as pH, light, carbon, nitrogen. VeA is a global transcription factor involved in the regulation of many interconnected cellular processes including growth, development, light-dependent secondary metabolism, sexual and asexual development, sclerotia formation and conidiogenesis (Bayram et al. 2008; Calvo 2008). VeA is the founding member of the velvet family, which includes three other proteins, VelB, VosA and VelC. In light, VeA is found mainly in the cytoplasm and sexual reproduction as well as secondary metabolism are inhibited. In the dark, VeA migrates to the nucleus in association with VelB, where they form a heterotrimeric complex with LaeA, the velvet complex, which controls development and secondary metabolism. In general, VeA increases the production of secondary metabolites, such as penicillin in *Penicillium chrysogenum* (Kopke et al. 2013) or gliotoxin in

Aspergillus fumigatus (Dhingra et al. 2013). Conversely, the biosynthesis of the dark-coloured pigment bikaverin in *Fusarium fujikoroi* (Wiemann et al. 2009) and compactin in *Penicillium citrinum* (Baba et al. 2012) is altered. In *P. expansum*, VeA affects aggressiveness, conidiation and secondary metabolism, for example by upregulating the production of patulin and citrinin or by downregulating that of communesins (El Hajj Assaf et al. 2018).

Except for patulin and citrinin, little information is available on the impact of VeA on the metabolome of *P. expansum*. First, a non-targeted metabolomic study using high-performance liquid chromatography coupled with high-resolution mass spectrometry was performed to investigate the structure of *P. expansum* secondary metabolites using carbon 13 (^{13}C) and nitrogen 15 (^{15}N) labelled wheats and to detect unknown metabolites produced by the fungus during its development and colonization cycle. The production of secondary metabolites was then compared between the wild type strain and the null mutant strain *PeΔveA* strain previously generated in our laboratory on a malt extract agar (MEA) synthetic medium and *in vivo*, on infected apples.

1.2.2. Material and methods

1.2.2.1. Production of ^{13}C- and $^{13}C/^{15}N$ labeled wheats

Triticum aestivum (cv. caphorn) was used to produce isotopic enrichments as previously described (Péan et al. 2007) and as detailed (Hautbergue et al. 2017). Briefly, two different enrichments were realized with ^{13}C-labeled CO_2 and ^{15}N-labeled nitrate and ammonium salts in hermetically sealed chambers (750L) for 6 months: the first one consisted on nearly 100% ^{13}C enriched wheat (referred to as ^{13}C wheat) and the second one on nearly 50% ^{13}C and 100% ^{15}N wheat (referred to as $^{13}C/^{15}N$ wheat). The isotopic enrichments were then measured with a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Illkirch, France).

1.2.2.2. Fungal strains and growth conditions

Three strains of *Penicillium expansum* were used in this study. The wild-type one NRRL 35695 (WT) was isolated from grape berries in the Languedoc Roussillon region in France. The null mutant PeΔ*veA* and PeΔ*veA*:*veA* complemented strains were generated as previously described (El Hajj Assaf et al. 2018). Culture of the strains used in the study was performed in the dark on a Malt Extract Agar medium (MEA) (Biokar Diagnostics, Allonne, France) (30 g/L malt extract, 15 g/L agar) for 5 days at 25°C.

As for the natural and labelled wheat grains, they were treated and inoculated as described by Hautbergue et al. (2017). Succinctly, wheat grains were soaked in water and autoclaved twice. The water activity (a_w) of the grains was measured and sterile water was added until obtaining an a_w of 0.98 before inoculation. A total of 4 culture media were prepared into sterile 140 mm diameter Petri dishes (Sarstedt, Marnay, France) and contained thirty grams of sterilized labelled ^{13}C wheat grains, $^{13}C/^{15}N$ wheat grains, and ^{12}C wheat grains (2 replicates of this culture). Each type of the labelled wheat was inoculated with 250 µL of a spore solution (with a concentration of 10^5 spore/mL) of *P. expansum* from a preculture of the fungus on Potato Dextrose Agar (PDA; Biokar, Allonne, France) (7 days at 25°C). The second Petri plate containing ^{12}C labelled wheat grains served as a control and was not inoculated with the fungus. The four cultures were incubated for 14 days, at 25°C and in the dark.

1.2.2.3. Analysis of secondary metabolite production *in vivo*

To assess the production of secondary metabolites on apples by each of our strains, *in vivo* studies were performed on Golden Delicious apples. The latter were purchased from a supermarket in Toulouse (Carrefour, France) and were surface sterilized as described previously by Sanzani et al. (2012). Apples were then wounded with a sterile toothpick. A first batch of them was inoculated with 10 µL of a suspension at concentration of 10^4 conidia/mL and incubated at 25°C in the dark for 14 days. A second batch of the same apples was inoculated with 20 µL containing 500 spores of each of the strains, and were incubated at 25°C for 30 days, in the dark.

After the incubation period, the apples from the first batch, were grounded as previously described by MacDonald et al. (2000) and secondary metabolites were extracted as detailed by Snini et al. (2016).

As with apples in lot 2, spores were recovered using a pump on 0.45 µm membrane filters uptidisc nylon 47mm (Interchim, France). The membranes were extracted in 50 mL ethyl acetate on a horizontal shaking table at 160 rpm at room temperature. The metabolites were extracted as analysed in previous work (El Hajj Assaf et al. 2018). Six biological replicates were performed for each strain for each of the experiments.

1.2.2.4. Analysis of secondary metabolite production *in vitro*

MEA and PDA were used for the growth of the WT, null mutant and complemented strains and for the secondary metabolites analysis. Precultures done on PDA for each of the strains, incubated for 7

days at 25°C in the dark, were used to prepare the spore suspension. Petri dishes containing the medium and covered with a sterile cellophane film were inoculated with 10 µL containing 10^6 spores of each isolate and were incubated for 5 days at 25°C in the dark. The cellophane film on which the fungi has developed was separated from the agar medium and the metabolites were extracted as described previously (El Hajj Assaf et al. 2018).

1.2.2.5. Secondary metabolome analysis

Secondary metabolites were screened by liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS). The chromatographic system consisted in a RSLC3000 UHPLC (Thermo Scientific, Les Ulis, France) operating with a 125 × 2 mm Luna® C_{18} 5 µm column (Thermo Scientific, Les Ulis, France). A gradient program of (A) water acidified with 0.05% formic acid and (B) acetonitrile was used at 30°C and a flow rate of 0.2 mL min^{-1} as follows: 0 min 20% B, 30 min 50% B, 35 min 90% B, from 35 to 45 min 90% B; 50 min 20% B, from 50 to 60 min 20% B. A volume of 10 µL of each sample diluted twice with mobile phase A was injected. The mass spectrometer corresponded to a LTQ Orbitrap XL (Thermo Scientific, Les Ulis, France) fitted with an Electrospray Ionization Source (ESI). ESI parameters for the negative mode were set as follows: spray voltage: 3.7kV, sheath gas flow rate (N_2): 30 arbitrary units (a.u.), auxiliary gas flow rate (N_2): 10 a.u., capillary temperature: 350°C, capillary voltage: -34V and tube lens offset: -180V. ESI parameters for the positive mode were set as follows: spray voltage: 4kV, sheath gas flow rate (N_2): 55 arbitrary units (a.u.), auxiliary gas flow rate (N_2): 10 a.u., capillary temperature: 300°C, capillary voltage: 25V and tube lens offset: -100V.High resolution mass spectra were acquired between m/z 80 and 800 at a resolution of 30000. The calibration of mass spectrometer was achieved using the calibration solution of Thermo Scientific in agreement with their protocol. MS/MS spectra were obtained with the collision induced dissociation (CID) mode of the ion trap analyser at low resolution and a normalized collision energy of 35%. Three biological replicates were performed for each strain.

1.2.2.6. Identification of secondary metabolites

A mass compare program developed at INRA (Toulouse, Axiom Platform) was used to compare the proposed ^{12}C, ^{13}C and $^{13}C^{15}N$ formulas using a measurement accuracy of 5 ppm. Identified metabolites displayed a comparable MS/MS spectrum to the bibliography and were identified as described by Hautbergue et al. (2017).

1.2.2.7. Metabolome data analysis

Extraction of data from mass spectrometry acquisition files was carried out using xcms software (Smith et al. 2006) with centwave algorithm (Tautenhahn et al. 2008). To avoid variation among samples data were then normalized using Probabilistic Quotient Normalization (Dieterle et al. 2006). On the resulting normalized data, univariate analysis of variance was used to study the effect of deletion of *veA* on the different molecules intensities. All these data analysis steps were performed using workflow4metabolomics, a collaborative portal dedicated to metabolomics data processing (Giacomoni et al. 2015). HPLC-HRMS data were analysed with a methodology previously developed for exposomics studies (Jamin et al. 2014).

1.2.2.8. Data analysis

One-way anova coupled to a Bonferroni test was used to analyse the differences between the secondary metabolites produced by the WT and null mutant strains *in vitro*, in the medium tested and in the fungal extract after running the samples on a LC-MS. It was also used to compare the abundance of metabolites between the different strains in apples. The differences were considered to be statistically significant when the p-value was lower than 0.05. The statistical analysis of the data was carried out using GraphPad 4 software (GraphPad Software, La Jolla, USA).

1.2.3. Results and discussion

A PeΔ*veA* mutant was generated by replacing the *veA* gene with a hygromycin resistance marker in a wild type strain of *Penicillium expansum*. The transformation was described in Chapter 1_Part 1. The null mutant was then used to study the influence of VeA on the production of several secondary metabolites and to differentiate between the metabolites produced in the medium, on which the fungus grew, in the fungus and in the spores.

1.2.3.1. Secondary metabolite production during the fungal cycle

A huge variety of compounds is produced by countless microorganisms by means of specialized biosynthetic pathways. Although secondary metabolites are not crucial for growth and reproduction, they could present a bioactive role to the organism that produce them (Keller et al., 2005). They can increase the organism's chances of survival in an hostile environment by adapting them to extracellular conditions, ensuring defence, competition, interactions between interspecies, etc. (Brachmann et al. 2013; Martinez et al. 2017).

P. expansum strains were found to be able to produce, besides patulin, a huge variety of active secondary metabolites and mycotoxins such as citrinin (Ciegler et al. 1977; Paterson et al.1987), roquefortin C (Andersen et al. 2004), chaetoglobosins (Andersen et al. 2004), expansolides (Massias et al. 1990), fumaryl-dl-alanine (Birkinshaw et al. 1942), and communesins (Andersen et al. 2004). Among the eight communesins (A–H) identified, communesin B was reported to be the most biologically active (Dalsgaard et al. 2005).

The global regulation factor VeA has been described previously as a positive or negative regulator of secondary metabolite biosynthesis in *Aspergillus nidulans* (Bayram and Braus 2012; Rauscher et al. 2016), *A. flavus* (Cary et al. 2015) and *Penicillium expansum* (EL Hajj Assaf et al. 2018). However, none of the studies differentiated between metabolites excreted in the medium and those secreted by the fungus. In our experiment, after 5 days of incubation of the WT and its *veA* null mutant strains on malt extract agar (MEA) medium in the dark at 25°C, the film on which the fungus grew was separated from the agar medium. The metabolites were extracted separately from the medium and fungi (without film) and analysed by HPLC-HRMS.

First, data generated from the LC-MS spectra were illustrated by Venn diagrams in **figure 1**, showing the number of significant ions obtained that differentiate the null mutant strain from each of the WT and Δ*veA*:*veA* complemented strains. No significant differences were observed between the WT and complemented strains. Differences observed between the WT or complemented strains and the null mutant strain were due to the *veA* disruption, confirming what we have seen in the previous parts of chapter 1. There are also differences between the medium, in which the metabolites are excreted, and the fungus. These findings show that not all fungal metabolites are released during substrate infection, but throughout its development.

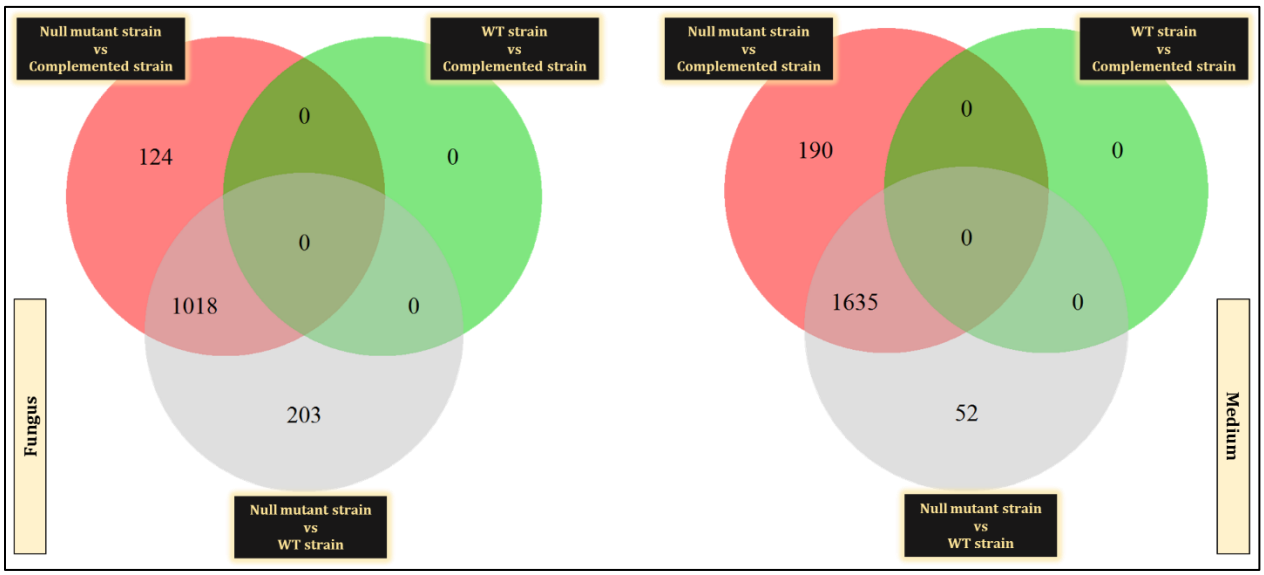

Figure 1 Venn diagrams showing the number of significant ions differentiating the null mutant strain from each of the wild type and complemented strains in the medium where the fungus grew and in the fungus. Cultures were grown in the dark for 5 days at 25°C. Numbers were obtained after screening the samples by LC-MS. Univariate analyses (Wilcoxon and Kruskal and Wallis) were conducted and significant ions were considered when p value < 0.05.

In order to identify secondary metabolites of *P. expansum* and its null mutant strain present in the samples and since many natural products are unavailable as reference compounds to validate their identification, an HPLC-HRMS analysis of the three wheat cultures was performed.

Based on the protocol previously developed and described by Cano et al. (2013), secondary metabolites of *P. expansum* were characterized. The WT strain was grown on ^{13}C wheat and $^{13}C^{15}N$ labelled wheat grains under the conditions described above and a control sample of wheat not infected by the fungus was also placed in the same environment. After extraction of the secondary metabolites from the samples, they were analysed by HPLC-HRMS. Isotopic patterns were detected by positive (ESI+) and negative (ESI−) electrospray ionization modes and a list of secondary metabolites is reported in **Table 1**. For further identification of the detected metabolites, a determination of their possible chemical formulas was performed, based on the m/z ratios measured in ^{12}C, ^{13}C and $^{13}C^{15}N$ extracts for each of the detected secondary metabolites, and then MassCompare software provided a unique final formula. Numerous of the metabolites present in the different samples extracted and analysed after were identified by comparing them to the listed metabolites in **Table 1**.

Identifier[a]	Molecular formula[b]	Parent ion	R_t (min)	^{12}C m/z (Da)	Proposed identification
Pexp_157.049_2.67	$C_7H_8O_4$	[M+H]+	2.67	157.0499	Ascladiol
Pexp_153.019_3.59	$C_7H_6O_4$	[M-H]-	3.59	153.01919	Patulin
Pexp_141.054_3.8	$C_7H_8O_3$	[M+H]+	3.80	141.05493	Gentisyl alcohol
Pexp_143.070_4.52	$C_7H_{10}O_3$	[M+H]+	4.52	143.07061	
Pexp_227.155_6.44	$C_{15}H_{18}N_2$	[M+H]+	6.44	227.15514	Aurantioclavine
Pexp_125.060_6.79	$C_7H_8O_2$	[M+H]+	6.79	125.05998	m-Hydroxybenzyl alcohol
Pexp_109.065_7.11	C_7H_8O	[M+H]+	7.11	109.06509	m-Cresol
Pexp_271.154_7.62	$C_{16}H_{18}N_2O_2$	[M+H]+	7.62	271.14496	Clavicipitic acid
Pexp_293.129_13.58	$C_{18}H_{16}N_2O_2$	[M+H]+	13.58	293.12911	
Pexp_295.144_14.61	$C_{18}H_{18}N_2O_2$	[M+H]+	14.61	295.14479	
Pexp_390.193_15.09	$C_{22}H_{23}N_5O_2$	[M+H]+	15.09	390.1939	Roquefortine C
Pexp_265.144_15.91	$C_{15}H_{20}O_4$	[M+H]+	15.91	265.14412	Expansolide C/ D
Pexp_293.129_17.74	$C_{18}H_{16}N_2O_2$	[M+H]+	17.74	293.12915	
Pexp_265.144_18.49	$C_{15}H_{20}O_4$	[M+H]+	18.49	265.14415	Expansolide C/ D
Pexp_375.142_22.02	$C_{16}H_{26}N_2O_4S_2$	[M+H]+	22.02	375.14202	
Pexp_457.261_23.65	$C_{28}H_{32}N_4O_2$	[M+H]+	23.65	457.26116	Communesin A
Pexp_423.154_27.01	$C_{23}H_{24}N_2O_6$	[M-H]-	27.01	423.15486	
Pexp_425.171_26.05	$C_{23}H_{24}N_2O_6$	[M+H]+	26.05	425.17179	
Pexp_447.153_26.05	$C_{23}H_{24}N_2O_6$+Na	[M+H]+	26.05	447.15351	
Pexp_501.247_27.70	$C_{28}H_{38}O_8$	[M-H]-	27.70	501.24792	
Pexp_307.154_27.39	$C_{17}H_{22}O_5$	[M+H]+	27.39	307.15471	Expansolide A/B
Pexp_307.155_30.19	$C_{17}H_{22}O_5$	[M+H]+	30.19	307.15504	Expansolide A/B
Pexp_529.271_30.53	$C_{32}H_{36}N_2O_5$	[M+H]+	30.53	529.27111	Chaetoglobosin A/B/G
Pexp_501.247_28.44	$C_{28}H_{38}O_8$	[M-H]-	28.44	501.24792	
Pexp_501.247_29.57	$C_{28}H_{38}O_8$	[M-H]-	29.57	501.24786	
Pexp_487.269_30.58	$C_{28}H_{40}O_7$	[M-H]-	30.58	487.26898	Andrastin B
Pexp_523.271_31.84	$C_{32}H_{34}N_4O_3$	[M+H]+	31.84	523.27152	Communesin D
Pexp_305.129_32.50	$C_{19}H_{16}N_2O_2$	[M+H]+	32.50	305.12911	
Pexp_305.129_33.17	$C_{19}H_{16}N_2O_2$	[M+H]+	33.17	305.12933	
Pexp_437.170_33.79	$C_{24}H_{26}N_2O_6$	[M-H]-	33.79	437.17091	

Pexp_529.270_34.05	$C_{32}H_{36}N_2O_5$	[M+H]+	34.05	529.27063	Chaetoglobosin A/B/G
Pexp_543.248_34.05	$C_{32}H_{36}N_2O_6$	[M-H]-	34.05	543.24836	
Pexp_485.254_35.32	$C_{28}H_{38}O_7$	[M-H]-	35.32	485.25405	
Pexp_509.292_36.01	$C_{32}H_{36}N_4O_2$	[M+H]+	36.01	509.29257	Communesin B
Pexp_485.253_36.26	$C_{28}H_{38}O_7$	[M-H]-	36.26	485.25328	Andrastin A
Pexp_487.270_36.21	$C_{28}H_{38}O_7$	[M+H]+	36.21	487.27063	Andrastin A
Pexp_319.145_36.44	$C_{20}H_{18}N_2O_2$	[M+H]+	36.44	319.14502	
Pexp_361.2582_38.19	$C_{19}H_{38}O_6$	[M-H]-	38.19	361.25819	

Table 1 Secondary metabolites detected from *Penicillium expansum* after culture on wheat grains and their MS/MS fragmentation in higher energy collision dissociation (HCD) mode. [a]Secondary metabolites identifier (Pexp_m/z_RT); [b]The chemical formulas were calculated after measuring the m/z ratios obtained from the culture of *P. expansum* in ^{12}C wheat, 97%^{13}C wheat and 53%^{13}C/96%^{15}N wheat.

The total ion current chromatograms obtained after HPLC-HRMS in our extracts are shown in **figure 2**. The chromatograms and mass spectra obtained showed different profiles for the extracts of MEA medium on which the WT and PeΔ*veA* strains grew **(Figure 2 A/C)** and of the WT and PeΔ*veA* strains **(Figure 2 B/D)**.

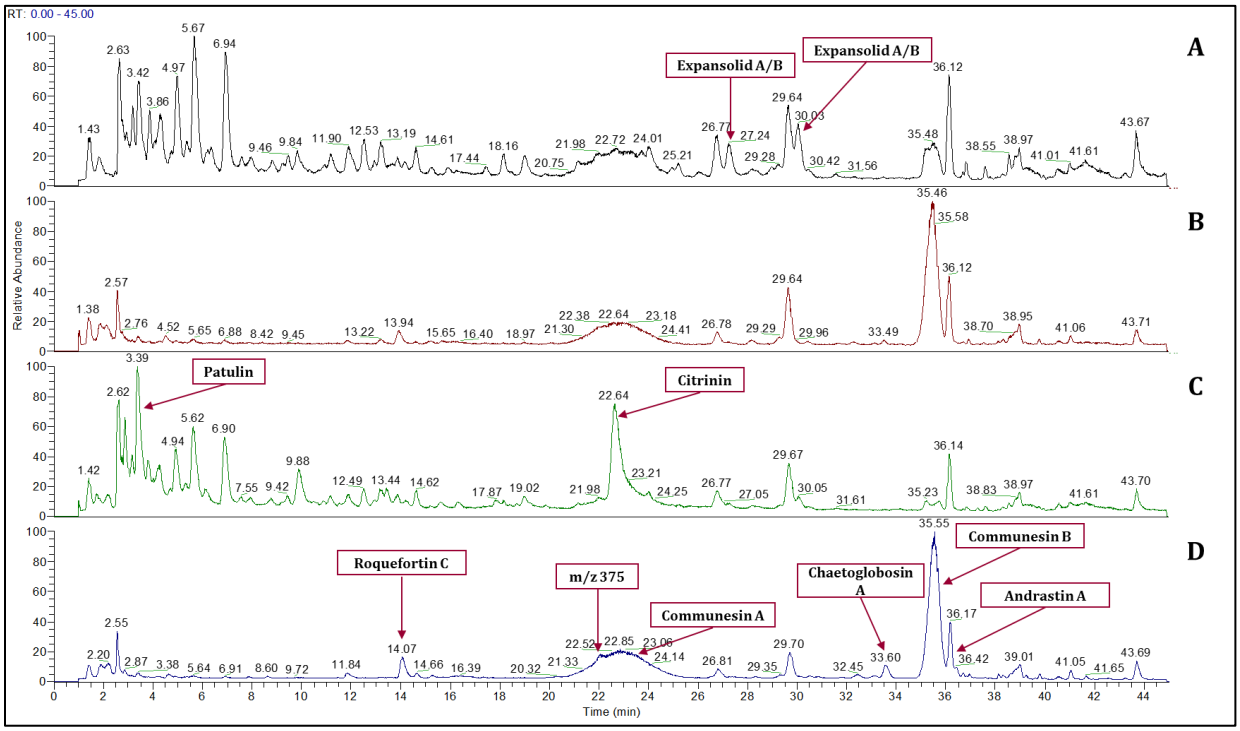

Figure 2 Total ion current chromatograms obtained from the extracts of malt extract agar (MEA) medium on which grew the PeΔ*veA* (A) and WT (C) strains and PeΔ*veA* (B) and WT (D) strains.

Among the various secreted metabolites, some of them were down-regulated in the null mutant strain and detected most in the WT strain such as citrinin, patulin, ascladiol, roquefortin, and communesins. Others were up-regulated in the PeΔ*veA* strain such as expansolides (A, B, C and D). Some metabolites showed no significant difference between the WT and null mutant strains such as andrastin A. Basically, these results confirmed what was measured by HPLC, quantified by Real-Time PCR and discussed in the previous section (El Hajj Assaf et al. 2018).

However, surprisingly, the metabolites found in the medium were not always the same as those found in the fungus. According to Calvo et al. (2002), most of the secondary metabolites produced by the fungus are secreted once the fungus has completed its initial growth phase. They are produced at a stage of development represented by the formation of spores. Secondary metabolites are associated into three broad categories) those activating sporulation (*e.g.* the linoleic acid-derived compounds produced by *A. nidulans*) (Champe and el-Zayat 1989; Mazur et al. 1991; Calvo et al. 2001), ii) those required for sporulation structures (*e.g.* melanins essential for the formation of both sexual and asexual spores) (Kawamura et al. 1999), and iii) toxic metabolites produced by growing colonies at the time of near sporulation (*e.g.* biosynthesis of mycotoxins) (Trail et al. 1995; Hicks et al. 1997).

As shown in **figure 3**, citrinin and patulin secreted by the WT strain were detected in the agar medium. Patulin was also detected in the fungus but at very low concentrations, 17 times lower than in the medium. Ascladiol was measured in small amounts in the fungus but was mainly found in the medium. On the contrary, roquefortin and communesin A and B, up-regulated by *veA*, were detected in higher amounts in the WT strain. This indicates that during its cycle, the fungus does not release all its metabolites at once. It begins by producing metabolites that will allow it to conquer the medium and most likely inhibit the development of other microorganisms in the medium. Then during its aerial metabolism, other metabolites are released that may be more essential for spore formation and dissemination. Expansolides (**table 1**) appeared to be down-regulated by *veA* and found in large amounts in the medium on which the null mutant strain developed. The deletion of the *veA* gene influenced the production of aurantioclavine in the fungus but its production in the medium was not affected. It can be assumed that the production of aurantioclavine, the precursor of communesins (Siengalewicz et al. 2008), follows different pathways in the medium and the fungus. Other natural products were identified according to their molecular formula, m/z ratios and retention time R_t. The metabolite with an m/z ratio of 348 was down-regulated by *veA*. It is only secreted by the null mutant strain and in the medium even if a small amount has been detected in the fungus. Unfortunately, since this metabolite was not secreted by the WT strain, we could not assign it a chemical formula.

However, this metabolite is of interested because it is the most produced by the null mutant strain and in addition, two genes involved in the biosynthesis of secondary metabolites encoding PKSs: Pexp_000410 and Pexp_094810 were highly down-regulated by *veA* on MEA and PDA (Chapter 1_Part1). Pexp_000410 was up-regulated by *laeA* on Czapek yeast extract liquid medium (CY) and Pexp_094810 was not expressed in either the WT or Δ*laeA* strains whatever the medium (Kumar et al. 2017a). By combining the results obtained by LC-MS and RT-PCR, a potential PKS could be attributed to the unknown metabolite, which would allow the gene clusters of a new secondary metabolite to be identified. Patulin and citrinin, the main products of the secondary metabolism of *P. expansum* (Woodhead and Walker 1975), are also two polyketides derived from acetate and whose genes are down-regulated by *veA* on MEA and PDA (El Hajj Assaf et al. 2018).

The metabolite with an m/z ratio of 374 was medium-specific and its production was not regulated by *veA*. The metabolites with m/z ratios of 425/ 375 and 293 were down-regulated and up-regulated by *veA*, respectively. The metabolite with m/z of 375 is mainly produced in the WT fungus; the one with m/z of 293 is mainly produced by the WT strain (in the medium and fungus). Secondary metabolites are obviously linked to fungal developmental programs that respond to various external abiotic or biotic triggers and VeA, a regulatory protein of the velvet family, plays a key role in coordinating this secondary metabolism.

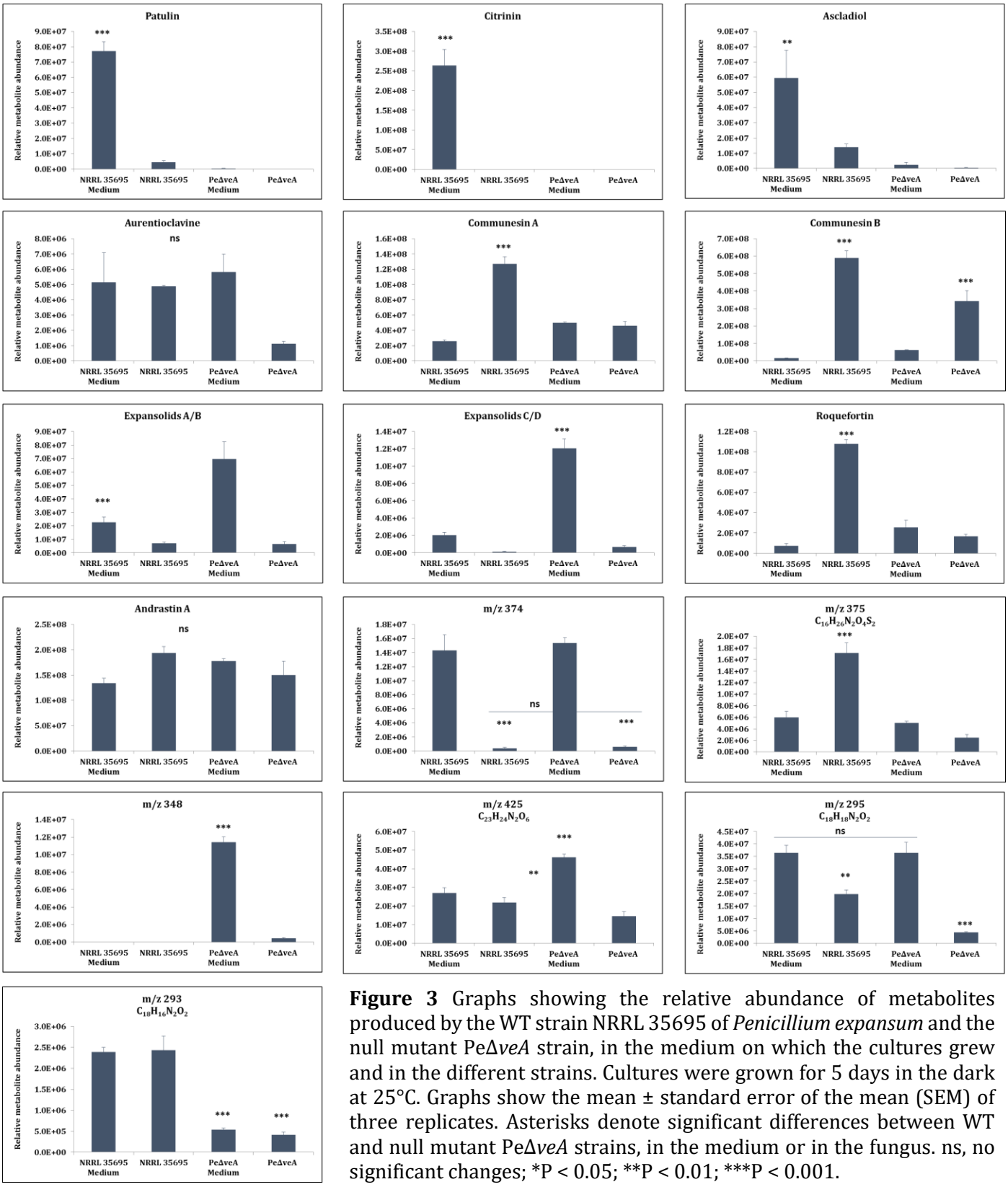

Figure 3 Graphs showing the relative abundance of metabolites produced by the WT strain NRRL 35695 of *Penicillium expansum* and the null mutant PeΔ*veA* strain, in the medium on which the cultures grew and in the different strains. Cultures were grown for 5 days in the dark at 25°C. Graphs show the mean ± standard error of the mean (SEM) of three replicates. Asterisks denote significant differences between WT and null mutant PeΔ*veA* strains, in the medium or in the fungus. ns, no significant changes; *P < 0.05; **P < 0.01; ***P < 0.001.

1.2.3.2. Production of secondary metabolites *in vivo* and *in vitro* growth

After differentiating the metabolites produced in the medium from those produced in the fungus during *in vitro* culture on MEA, we compared the production of metabolites in an *in vivo* study. Secondary metabolites secreted by the WT, null mutant and complemented strains of *P. expansum* in apples inoculated and incubated for 14 days at 25°C in the dark, were analysed and compared with the metabolites secreted by *P. expansum* grown on labelled wheat. In addition to patulin secreted by the WT strain in apples, all the results of which were presented in the first part of this chapter (El Hajj Assaf et al. 2018), other compounds were identified such as communesin A/B, andrastin A **(Figure 4)**. Molecules with m/z ratios of 425 and 447showed very significant differences in abundance between the three strains tested. They are down-regulated by *veA*. The molecule with m/z ratio of 425 was also found in the *in vitro* study and was assigned the chemical formula $C_{23}H_{24}N_2O_6$. On a synthetic medium, the WT strain was able to produce this compound; however, it was not detected in apples. The metabolite with an m/z ratio of 447 was not found in the *in vitro* study, when the fungus was grown on MEA, and same goes for andrastin B. These metabolites could be substrate specific. On the contrary, communesins A, B and andrastin A were secreted by the fungus grown on synthetic medium with communesins being mainly in the fungus and not secreted in the agar medium. From these results, we can conclude that what is observed on a synthetic medium does not always reflect what occurs *in vivo*. Some compounds are substrate specific and many metabolites outside patulin are secreted in infected apples. *veA* has an impact on the biosynthesis pathway of secondary metabolites present in apples and need to further investigation. After measuring the abundance of metabolites produced in the contaminated apples, the ones in the spores were analysed.

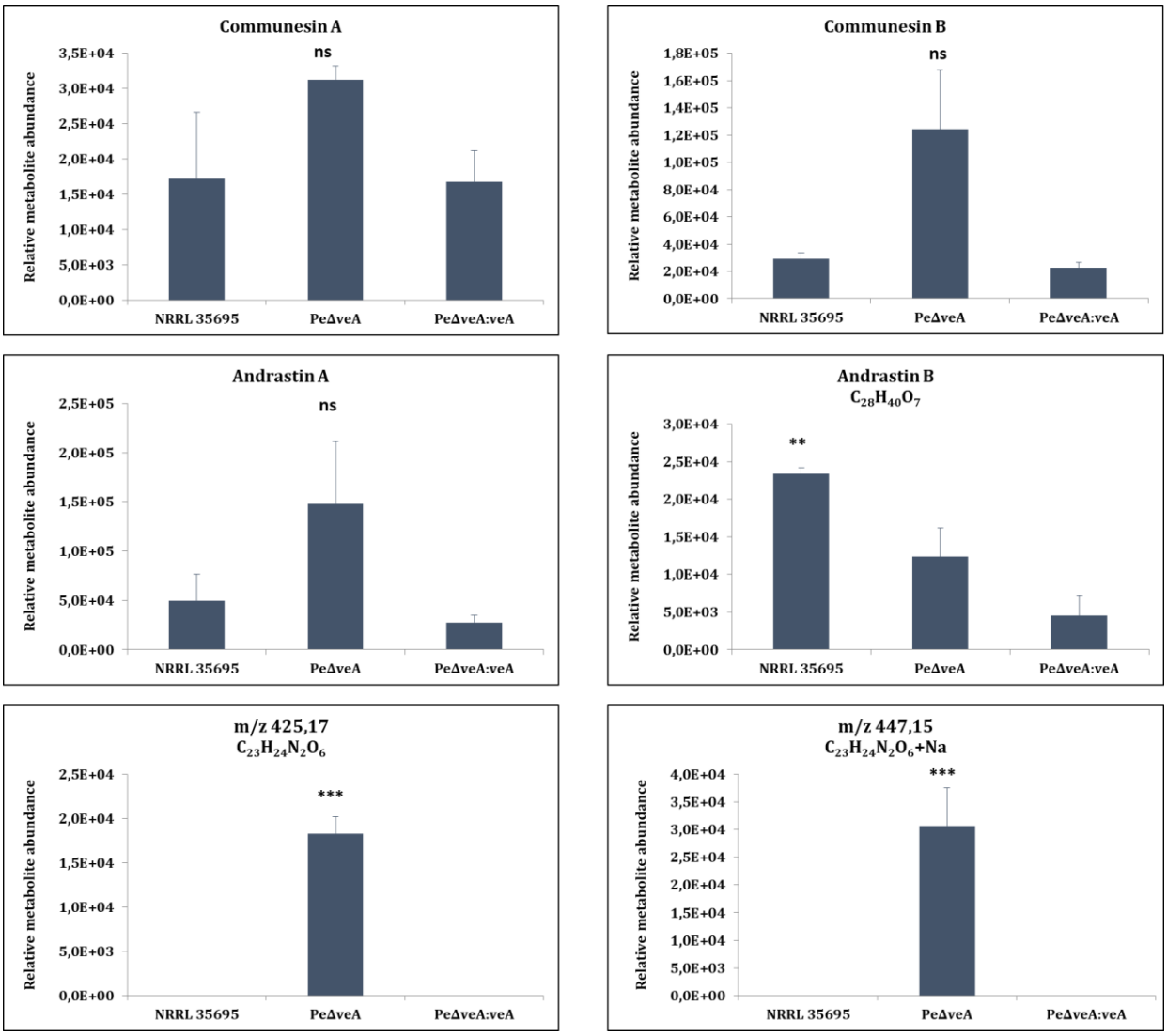

Figure 4 Graphs showing the relative abundance of metabolites produced by the WT strain NRRL 35695 of *Penicillium expansum*, the null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains, in apples inoculated with 10 µL of a suspension at 10^4 conidia/mL and incubated at 25°C in the dark for 14 days. The graphs show the mean ± standard error of the mean (SEM) of three replicates. The asterisks denote significant differences in metabolite production between the three strains used. ns, no significant changes; *P < 0.05; **P < 0.01; ***P < 0.001.

After inoculating Golden Delicious apples with 10^4 conidia/mL of the WT strain and incubating them at 25°C in the dark for 30 days, spores were recovered and their metabolites were extracted. Several studies have shown that compounds excreted by fungi can induce asexual and sexual sporulation in other fungi (Hadley et al. 1958; Park and Robinson 1969). In most cases, these compounds have not yet been identified but they are expected to be produced as the mycelia ages (Calvo et al. 2002). The formation of coremia was missing in the null mutant strain grown *in vivo* and *in vitro* In addition, it

was unable to pierce the apple peel and emerge from the fruits to complete its life cycle (El Hajj Assaf et al. 2018). This explains why the spores recuperated on the apple surface were those of a WT strain. In order to identify the molecules present in the spores, we compared them to the molecules secreted during the culture of *P. expansum* on labelled wheat. More molecules were detected in the spores than in the apples. Some of them were already known (communesins A/B, clavicipitic acid, expansolides A/B and andrastins A/B/C); others were characterized by a molecular formula (for example $C_{18}H_{16}N_2O_2$and an identifier was assigned to them (Pexp_293_13.58)) **(Table 2)**.

Identifier[a]	**Molecular formula[b]**	**Parent ion**	**R_t (min)**	**Proposed identification**
Pexp_271_7.62	$C_{16}H_{18}N_2O_2$	$[M+H]^+$	7.62	Clavicipitic acid
Pexp_293_13.58	$C_{18}H_{16}N_2O_2$	$[M+H]^+$	13.58	
Pexp_295_14.61	$C_{18}H_{18}N_2O_2$	$[M+H]^+$	14.61	
Pexp_293_17.74	$C_{18}H_{16}N_2O_2$	$[M+H]^+$	17.74	
Pexp_375_22.02	$C_{16}H_{26}N_2O_4S_2$	$[M+H]^+$	22.02	
Pexp_457_23.65	$C_{28}H_{32}N_4O_2$	$[M+H]^+$	23.65	Communesin A
Pexp_423_27.01	$C_{23}H_{24}N_2O_6$	$[M-H]^-$	27.01	
Pexp_425_26.92	$C_{23}H_{24}N_2O_6$	$[M+H]^+$	26.92	
Pexp_447_26.92	$C_{23}H_{24}N_2O_6$+Na	$[M+H]^+$	26.92	
Pexp_307_27.39	$C_{17}H_{22}O_5$	$[M+H]^+$	27.39	Expansolide A/B
Pexp_307_30.19	$C_{17}H_{22}O_5$	$[M+H]^+$	30.19	Expansolide A/B
Pexp_501_28.44	$C_{28}H_{38}O_8$	$[M-H]^-$	28.44	
Pexp_501_29.57	$C_{28}H_{38}O_8$	$[M-H]^-$	29.57	
Pexp_487_30.58	$C_{28}H_{40}O_7$	$[M-H]^-$	30.58	Andrastin B
Pexp_305_32.50	$C_{19}H_{16}N_2O_2$	$[M+H]^+$	32.50	
Pexp_303_32.46	$C_{19}H_{16}N_2O_2$	$[M-H]^-$	32.46	
Pexp_305_33.17	$C_{19}H_{16}N_2O_2$	$[M+H]^+$	33.17	
Pexp_303_33.13	$C_{19}H_{16}N_2O_2$	$[M-H]^-$	33.13	
Pexp_437_33.79	$C_{24}H_{26}N_2O_6$	$[M-H]^-$	33.79	
Pexp_485_35.32	$C_{28}H_{38}O_7$	$[M-H]^-$	35.32	
Pexp_509_36.01	$C_{32}H_{36}N_4O_2$	$[M+H]^+$	36.01	Communesin B
Pexp_485_36.26	$C_{28}H_{38}O_7$	$[M-H]^-$	36.26	Andrastin A
Pexp_487_36.21	$C_{28}H_{38}O_7$	$[M+H]^+$	36.21	Andrastin A

Pexp_319_36.44	$C_{20}H_{18}N_2O_2$	$[M+H]^+$	36.44	
Pexp_361_38.19	$C_{19}H_{38}O_6$	$[M-H]^-$	38.19	
Pexp_471_39.84	$C_{28}H_{40}O_6$	$[M-H]^-$	39.84	Andrastin C?

Table 2 List of the secondary metabolites detected in spores of *Penicillium expansum*, recovered on infected apples. [a] Secondary metabolites identifier (Pexp_m/z_RT); [b] The chemical formulas were calculated after comparison of the m/z ratios obtained from the culture of *Penicillium expansum* in ^{12}C wheat, 97%^{13}C wheat and 53%^{13}C/96%^{15}N wheat to the m/z ratios of the metabolites found in the spores of *P. expansum* after growing on apples.

The metabolites present in the spores in the *in vivo* study were expected to be the same as those found in the fungus in the *in vitro* study, unless they are substrate specific. By comparing spores and metabolites isolated from fungi, we find that communesins A and B as well as the two molecules with m/z ratios equal to 425 and 375 and molecular formulae of $C_{23}H_{24}N_2O_6$ and $C_{16}H_{26}N_2O_4S_2$, respectively, were found in both studies and are secreted during asexual reproduction. Aurantioclavine and roquefortin were only detected in the fungus in the *in vitro* study and andrastins B and C as well as other molecules listed in **table 2** were only detected in the spores in the *in vivo* study. Expansolides were detected in spores and not in apples, but the *in vitro* experiment has shown that these metabolites are mainly present in the medium and are secreted by the basal mycelium of the fungus unlike communesins and roquefortin mainly secreted in the fungus (*in vitro* study).

Different environmental signals such as availability of nutrients, fungal pheromones, stress conditions, oxygen supply, etc. contribute to reaching the developmental competence phase (Bayram and Braus 2012). All these signals lead to fungal differentiation of hyphae (including transition to asexual spore formation and to sexual fruiting bodies) and changes in secondary metabolism of the fungus. This would explain the difference between secondary metabolites that are produced in our synthetic medium or in the spores/and fungi.

1.2.4. Conclusion

Finally, our results highlight the impact of VeA on the metabolome of *P. expansum* and differentiate between secondary metabolites secreted throughout the fungus' life cycle. Patulin and citrinin were specific to the phase when the fungus colonized its medium; roquefortin and communesins A and B were present in the fungus. Due to differences between the culture of the fungus on a synthetic medium or on apples, some metabolites appeared to be substrate specific and were not detected in *in vitro* and *in vivo* studies. New metabolites have been identified and raw formulae have been

assigned to them. Our results show that VeA affects the secondary metabolism of *P. expansum* and that the release of secondary metabolites does not occur randomly in a fungus but depends on the substrate and life cycle. The nature of the signalling involved in the different phases of development of the fungal life cycle, the chemical characteristics and possible regulatory effect of the substances produced remain unclear and further research should be carried out.

Chapter 1_Part 3

Impact of *veA* gene on *Penicillium expansum* development and patulin production in presence of different sugar sources

In addition to the work carried out in the first part of this chapter on the characterization of the *veA* gene in *P. expansum*, other experiments were conducted as part of this thesis to improve our knowledge of this fungus and its regulation system.

Since apples are sugar-rich sources (mainly glucose, sucrose and fructose), which affect the secondary metabolism of fungal species, in this section, we tested the effect of different concentrations of these three sugars on the wild-type strain of *P. expansum* NRRL 35695, a null mutant PeΔ*veA* strain and a complemented PeΔ*veA*:*veA* strain. The increase in *in vitro* sugar content favoured fungal development, but this was not correlated with the production of PAT. The three fungal strains grown on a medium supplemented/enriched with more than 3% sugar showed a significant decrease in their PAT production. The null mutant was almost unable to produce PAT, regardless of the percentage of sugar added. These findings support the hypothesis that sugar sources affect the secondary metabolism of *P. expansum* and that VeA contributes to the pathogenicity of this fungus. They also suggest that apple varieties and their degree of maturity could influence PAT production and apple colonization.

1.3.1. Background

The phytopathogenous fungus *Penicillium expansum*, causal agent of blue mould disease on apples and some other fruits (*e.g.* pears, cherries, peaches, and plums), leads to important economic losses in orchards worldwide (Morales et al. 2010; Nunes 2012). It is a psychrophilic and necrotrophic fungus that develops on the surface of the fruit during harvesting, post-harvest handling and storage through injuries (bruises, puncture wounds), thus causing tissue decays. Spores or conidia enter the fruit, germinate and then grow as hyphae forming the mycelium (basal metabolism), which gives rise to thousands of spores (airborne metabolism) during asexual reproduction, after they leave the fruit.

P. expansum is generally regarded as the major producer of patulin (PAT), a potent mycotoxin that can cause serious health concerns. Apple juices and other derived products produced from apples infected by this fungus are the major dietary source of PAT. Due to its broad spectrum of toxicity (Puel et al. 2010; Glaser and Stopper 2012), PAT levels in apple-derived food products are regulated in many European countries (EC Commission Regulation 2006). However, it is present in food products on the market (Ioi et al. 2017; Tannous et al. 2017) and research studies are therefore necessary to prevent accumulation of the toxin in fruits and to minimize the risk of contamination of final products with PAT.

Numerous environmental conditions such as pH, temperature, water activity, nitrogen (N) and carbon (C) sources regulate the biosynthesis of mycotoxins in several filamentous fungi (Schmidt-Heydt et al. 2008; Georgianna and Payne 2009). Among those abiotic factors, the composition of substrates and their nutritional conditions have a significant effect on mycotoxin production. Sucrose stimulates the production of aflatoxins and trichothecenes in *Aspergillus parasiticus* and *Fusarium graminearum*, respectively (Davis and Diener 1968; Jiao et al. 2008; Georgianna and Payne 2009). The biosynthesis of

fumonisin B1 in *Gibberella fujikuroi* and the expression of fumonisin biosynthesis genes in *Fusarium proliferatum* were regulated by a mechanism involving nitrogen metabolite (Shim and Woloshuk 1999; Kohut et al. 2009). Zong et al. (2015) investigated the effects of different C and N sources on the production of PAT in *P. expansum*. They have shown that a concentration of 10 g/L maltose, glucose, fructose, mannose, sucrose, and starch was a favourable source of C for PAT biosynthesis. In the same study, when citrus pectin, lactose, malic acid and cellulose were used as C sources in the defined media, very low levels of PAT were detected.

Apples are rich in sugars. The main ones are glucose, sucrose, fructose and sorbitol. All cultivars contain more fructose and less glucose (Hecke et al. 2006; Wu et al. 2007). During fruit storage, maturation and ripening, the sucrose content decreases and the glucose and fructose contents increase (Chardonnet et al. 2003). Despite the importance of sugar content in apple cultivars and for fruit maturity, few studies have discussed its relationship with the mechanism activation of PAT biosynthesis.

The gene cluster responsible for PAT biosynthesis in *P. expansum* has been recently characterized (Tannous et al. 2014; Ballester et al. 2015). *PatL*, a gene located within the cluster and encoding a specific transcription factor, is responsible for the activation of all genes in the cluster. Its expression was positively associated with the production of PAT (Snini et al. 2016). Global transcription factors appear to be also involved in the regulation of secondary metabolite biosynthesis in fungal species (Kumar et al. 2017; El Hajj Assaf et al. 2018). In the previous study, we showed that VeA acts as a positive regulator of the biosynthesis of different secondary metabolites such as PAT and citrinin (El Hajj Assaf et al. 2018). The disruption of the *laeA* gene in *P. expansum* has also led to a strong reduction in PAT production (Kumar et al. 2017). In the same study, the regulation of *laeA* expression by sugars was also reported. The increase in sucrose content has a negative impact on *laeA* expression and PAT synthesis, but a positive impact on *creA* gene expression, which is a transcription factor that acts as a global carbon catabolite regulator, *in vitro*. No information is available on the impact of VeA on the development of *P. expansum* and PAT biosynthesis in presence of different sugar sources.

Although some work was carried out and contributed to a better understanding of the factors modulating PAT production and accumulation (Tannous et al. 2015; Ballester et al. 2015; Kumar et al. 2017; El Hajj Assaf et al. 2018), more studies should be conducted taking into account host factors (sugar contents, acids, pH, etc.). Studies on the mechanism of sugar metabolism by *Penicillia* are scarce. In this study, we showed the impact of the main sugars of apples on the development and PAT production of *P. expansum* wild type and *veA* null mutant strains. We suggest that apple cultivars and the degree of maturity are involved in the production of PAT and fungal development on colonized fruits.

1.3.2. Material and methods

1.2.2.1. Fungal strains and growth conditions

The NRRL 35695 wild-type strain (WT) of *Penicillium expansum* used in this study was isolated from grape berries in the Languedoc Roussillon region in France. The null mutant and complemented strains PeΔ*veA* and PeΔ*veA*:*veA*, respectively, were generated as previously described (El Hajj Assaf et al. 2018). The strains were grown on a Potato Dextrose Agar medium (PDA) (Biokar, Allonne, France), in the dark for 7 days at 25°C and then on a basic medium enriched with different concentrations of glucose, sucrose and fructose provided by Merck.

1.3.2.2. *In vitro* growth

After 7 days of incubation of the WT, null mutant and complemented strains on PDA, a spore suspension of each of the three strains was prepared (Adjovi et al. 2014). The concentrations of spore suspensions were then quantified using a Malassez cell. A basic medium (15 g/L agar (Merck); 2 g/L yeast extract (Fluka); 10 mL/L concentrated Czapek (3 g/L $NaNO_3$, 5 g/L $MgSO_4$ 7 H_2O, 0.1 g/L $FeSO_4$, 5 g/L KCl)) was first prepared; as well as 70% concentrated solutions of glucose (≥99.5%; Merck), sucrose (≥99.0%; Fluka) and fructose (≥99.0%; Merck). The sugar solutions were added to the basic medium, separately, to obtain media with sugar concentrations that vary between 0% and 8%.

An inoculum of 10^6 spores/30µL was plated centrally on the basic media complemented by sugar, and the plates were incubated at 25°C in the dark for six days. Four biological replicates were done for each strain and each sugar concentration; and each experiment (using glucose, sucrose or fructose) was repeated three times. After 7 days, colony diameters were measured.

1.3.2.3. Extraction of PAT

After a 6-day incubation period at 25°C, the agar medium on which the fungus has developed was macerated in 50 mL of ethyl acetate, separately, on a horizontal shaking table at 160 rpm at room temperature for eight days. The organic phase was filtered through a Whatman filter paper, and then evaporated to dryness using the Zymark TurboVap (McKinley Scientific, Sparta, NJ, USA). The dried residue was dissolved in 400 µL of acetonitrile:water (50:50, v/v) and filtered through a 0.45 µm syringe filter into a clean vial of two mL. Four biological replicates were done for each of the strains used and the three different sugars concentrations tested.

1.3.2.4. HPLC-DAD analysis of PAT

An Ultimate 3000 HPLC system (Dionex/ThermoScientific, Courtaboeuf, France) was the chromatography device used for PAT quantification. PAT analysis were performed using a 250 × 4.60 mm Gemini 5 µm C_6-Phenyl column (Phenomenex, Torrance, CA, USA) at 30°C at a flow rate of 0.9 mL/min.

Eluent A was 0.1% acetic acid and eluent B was methanol. Elution conditions were as follows: 0 min 100% A, 10 min 90% A, 30 min 50% B, 45 min 90% B, from 45 to 50 min 90% B, from 60 to 65 min 0% B.
The presence of PAT was monitored at a wavelength of 277 nm and was confirmed by comparing it the retention time (min) of a pure standard. PAT was quantified by measuring peak area according to the standard curve.

1.3.2.5. Data analysis

Statistical analysis of the data obtained was carried out using the Graphpad 4 software (GraphPad Software, La Jolla, CA, USA). A two-way ANOVA followed by a post-hoc comparison Bonferroni test was conducted to analyse the differences between the WT, null mutant and complemented strains for each of the analyses of fungal growth and PAT production *in vitro*, in presence of different sugars. The differences were considered to be statistically significant when the *P* value was below 0.05.

1.3.3. Results and discussion

1.3.3.1. Addition of sugars affects fungal growth *in vitro*

In fruits, whose sweet taste differs significantly, the main soluble sugars are sucrose, fructose, and glucose whilst malic, citric, and tartaric acids are the primary organic acids (Ma et al. 2015). Given the high sugar content of apples in particular and the fact that sucrose modulates the accumulation of PAT as a function of dose (Barad et al. 2016; Kumar et al. 2016), we wanted to determine the relationships between different concentrations of main apple sugars, fungal development, PAT production and effect of the *veA* gene.
The *P. expansum* NRL35695 WT, null mutant and complemented strains were grown on basic media with increasing concentrations of sugars: glucose (16–389 mM,), sucrose (8–205 mM) and fructose (0–443 mM) representing 0.3–7% for glucose and sucrose, and 0–8% for fructose. After 6 days of incubation at 25°C, fungal radial growth were measured and PAT was extracted **(Figure 1)**. All carbon substrates supported the growth of the fungus, but in varying proportions. The increase in sugar percentages favoured fungal development but not in a linear way. In fact, by adding up to 4% glucose **(Figure 1 A–C)** or fructose **(Figure 1 G–I)**, there is a slight increase in fungal growth. When adding up to 4% sucrose **(Figure 1 D–F)**, a decrease in the radial growth was observed. However, when the percentage of the three sugars exceeded 4%, the development of the fungus was faster and the radial growth increased. No significant differences were observed between the WT, null mutant and complemented strains grown 6 days on a basic medium to which 7% glucose, sucrose or fructose were added. Radial growths of 4.88 ± 0.22 cm, 4.86 ± 0.31 cm and 4.88 ± 0.23 cm were measured, respectively **(Figure S1 A, B, C)**. The only difference observed between sugars was when 3% to 5% sucrose was added to the medium. A decrease in the radial growth of the WT and complemented strains (4.08 ± 0.11 cm) is then observed, followed immediately after by an increase when the sucrose content has again increased in the medium (to reach

7%). In fact, in the Golden Delicious cultivar, for example, 54% of total sugars are fructose, 29% sucrose and the rest glucose (Hecke et al. 2006). A medium enriched with 0.7% sucrose (equivalent to this 29% in apple) would have been sufficient to mimic the apple growth medium. Although the carbon catabolite repression (CCR) regulating mechanism ensures the use of sugars such as D-glucose and sucrose, which are rapidly metabolized compared to other less favourable carbon sources (Dowzer and Kelly 1991; Tannous et al. 2018), the fungus has to first adapt to the amount of carbon it receives and this could be the cause of the observed radial growth decrease by adding 3% to 5% sucrose. In addition, sucrose, which is richer in carbon sources than glucose and fructose, is expected to positively regulate the expression of the *creA* gene. Recently, Kumar et al. (2016) have shown that the increase in sucrose content has a positive impact on *creA* transcription and a negative impact on *laeA* expression and PAT biosynthesis. In our study, it would have been interesting to measure the dry weight of the mycelium and see how it evolves, then it would have been interesting to analyse the expression of the *creA* gene, especially since in fungi, this regulator is activated in media with a high sugar content (Bi et al. 2015). The decrease and further increase in radial fungal growth could be correlated with the expression of this global carbon catabolite regulator.

1.3.3.2. Addition of sugars affects patulin production

Added sugars affect the virulence of the fungus. After 6 days of incubation of the inoculated plates, PAT levels were measured **(Figure 1)**. Similar profiles were obtained for each of the strains when different sugars were added: when adding up to 4% sugar (glucose, sucrose or fructose) to the basic medium, the fungal development was accompanied by an increase in PAT production. The amount of PAT produced was similar for the WT and complemented strains and ranged from 0.15 ± 0.00 mg/mL to 22.29 ± 1.85 mg/mL, 0.22 ± 0.01 mg/mL to 33.01 ± 4.35 mg/mL, 0.10 ± 0.08 mg/mL to 19.11 ± 4.28 mg/mL when grown on enriched medium with 0.3% and 4% glucose, fructose and sucrose, respectively **(Figure S2 A, C)**.

The addition of up to 4% sugar also favoured the production of PAT by the null mutant strain, but in lower amounts. The maximum of PAT produced by the null mutant strain was 4.91 ± 1.13 mg/mL **(Figure 1 B, E, H; Figure S2 B)**.

Surprisingly, the amount of PAT produced by the three strains decreased with increasing sugar concentrations and did not correlate with the radial fungal growth. It reached a minimum of 18.45 ± 5.15 mg/mL, 28.68 ± 0.27 mg/mL and 6.47 ± 1.56 mg/mL in the medium inoculated by the WT and complemented strains and supplemented with 7% glucose, fructose and sucrose, respectively, and a minimum production of 2.50 ± 1.21 mg/mL for the null mutant strain. In fact, the greatest decrease in PAT concentration was observed when 7% sucrose (or 205 mM) was added to the medium; there was 66% and 50% less PAT produced in the medium by the WT and null mutant strains, respectively, than

when the latter were grown in medium with 4% sucrose. These results of PAT production are similar to those observed by Kumar et al. (2016), *i.e.* a decrease in production at 175 mM for the three sugars tested. However, in our experiment, sucrose was the sugar that supported PAT production less than glucose and fructose. This discrepancy could be due to differences in the base medium used and enriched with sugars. Usually, sugars such as sucrose, glucose and fructose are supposed to suppress secondary metabolism. However, Abdollahi and Buchanan (1981) have shown that a glucose-containing medium resulted in aflatoxin production by *Aspergillus parasiticus*. They hypothesized that glucose or a product of its metabolism, induces one or more of the enzymes responsible for aflatoxin synthesis, at the transcriptional level. Kumar et al. (2016) have proven that an increase in *in vitro* sucrose content was correlated with a decrease in PAT production. The novelty of this work is that it showed that an increase in the main sugars in apples caused a decrease in PAT production. It would be interesting to measure the level of expression of the genes belonging to the PAT cluster and see at what level the sources of sugar act. In addition, the analysis of the effect of each sugar separately on fungal development and PAT production should be completed by the analysis of the combined effect of all sugars. The base medium should contain all three sugars at the same time with percentages reflecting the reality of an apple medium. After all, it is true that a significant decrease in PAT concentration was observed when 7% glucose was added to the medium, but this does not mean that all apple varieties contain 7% glucose. The high presence of acids in apples and their contribution to the sweetness of the fruit make it interesting to see the effect of increasing concentration of malic, citric, and tartaric acids on fungal development and PAT production. One last thing we should also point out is that in the presence of increasing concentrations of sugars, fungal strains have decreased their production of PAT, but not their growth. Thus, the addition of sugar to the medium could reduce the pathogenicity of the fungus.

1.3.3.3. Association of sugar content with PAT synthesis and *veA* expression

We noted that the fungal development of the three strains tested was induced by the addition of sugars to the medium and that PAT production increased on the sugar-enriched medium by up to 4% and then decreased thereafter. However we have still highlighted the significant differences observed between the WT and complemented strains on one hand and the null mutant strain on the other hand **(Figure 2)**. In fact, up to 7% glucose in the medium, the null mutant strain developed but failed to reach the radial growth of the WT strain. The radial growth of the WT and null mutant strains was significantly different, 4.95 ± 0.08 cm and 4.50 ± 0.10 cm, respectively **(Figure 2A)**. In presence of fructose, the null mutant strain developed more slowly than the WT strain at first; but once the amount of fructose in the medium reached 3%, the null mutant strain could catch up with the WT strain and no significant difference in their growth was observed after. The mutant strains reached a radial growth of 5.06 ± 0.10 cm, close to that reached by the WT strain on the sugar-enriched medium **(Figure 2B)**. Remarkably, when less than

3% sucrose was added to the base medium, we noticed that the growth of the null mutant strain was lower than that of the WT and complemented strains, but above 3% in the medium, the growth of the null mutant strain was higher than that of the WT strain. The radial growth of the null mutant strain reached 5.06 ± 0.05 cm and 4.64 ± 0.07 cm for the WT strain **(Figure 2C)**. Although the different sugars affected differently the growth of each of the strains tested and favoured the development of the null mutant strain, the situation was different for production of PAT. In fact, whatever the type and percentage of sugar added to the base medium, the null mutant strain barely synthesized PAT and the amount produced was always significantly lower than that produced by the WT and complemented strains. After adding 7% glucose, sucrose and fructose, the null mutant strain produced 82%, 77% and 87% less PAT than the WT strain, respectively **(Figure 2 D, E, F)**.

These results pointed out the inability of the null mutant strain to produce PAT and that its development was not correlated to the amount of PAT produced.

Kumar et al. (2016) have shown that the increase in sucrose content *in vitro* decreased *laeA* expression, therefore they conclude that suppression of PAT production in presence of sucrose is mediated through LaeA in *P. expansum*. Here, we showed that in a *veA* null mutant strain, only very small amounts of PAT were synthesized when increasing sugar concentrations *in vitro*. And of course, even at optimal sugar concentrations (about 3 to 4%), the fungal strain from which *veA* gene was disrupted could not produce the toxin. This made us think about the possibility that *veA* could induce the expression of genes that allow the use of sugar and carbon sources as well as the production of PAT, and the disruption of the *veA* gene does not allow this phenomenon to occur. We also noted that the WT strain could not produce as much PAT when 7% sugar was added to the medium.

1.3.4. Conclusion

Although PAT is a major mycotoxin produced by *P. expansum*, the genome of this fungus is rich and exhibits other predicted secondary metabolite clusters, based on bioinformatics analysis and other specific studies (Ballester et al. 2015). It would be interesting to study the impact of sugars at various concentrations on the expression of all the backbone genes in the genome of the *P. expansum*. Moreover, it would be interesting to measure the level of expression of the *veA* and *creA* genes since increasing sucrose concentration affects *laeA* expression.

In conclusion, our results highlighted the impact of sugar sources, naturally present in apples, on fungal development and PAT production. They also showed the involvement of the *veA* gene in synthesis of PAT and pathogenicity of the fungus. There is still much to study and develop.

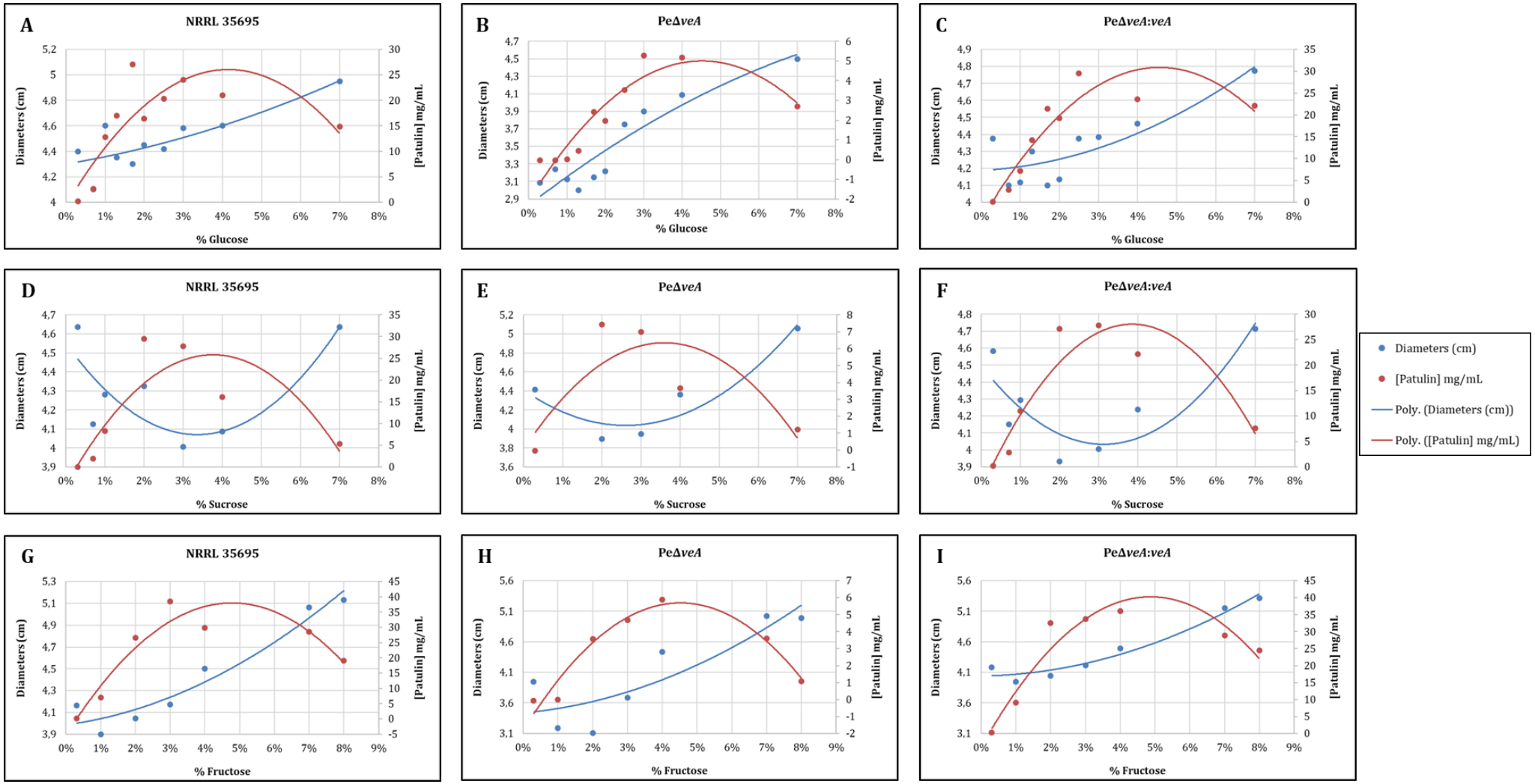

Figure 1 Impact of different sugar concentrations on fungal development and PAT production of the WT, null mutant and complemented strains. The blue line corresponds to the fungal radial development on the base medium enriched with sugar and incubated for 6 days at 25°C. The red line corresponds to the production of PAT by these different strains. Four biological replicates were performed for each strain and each sugar concentration.

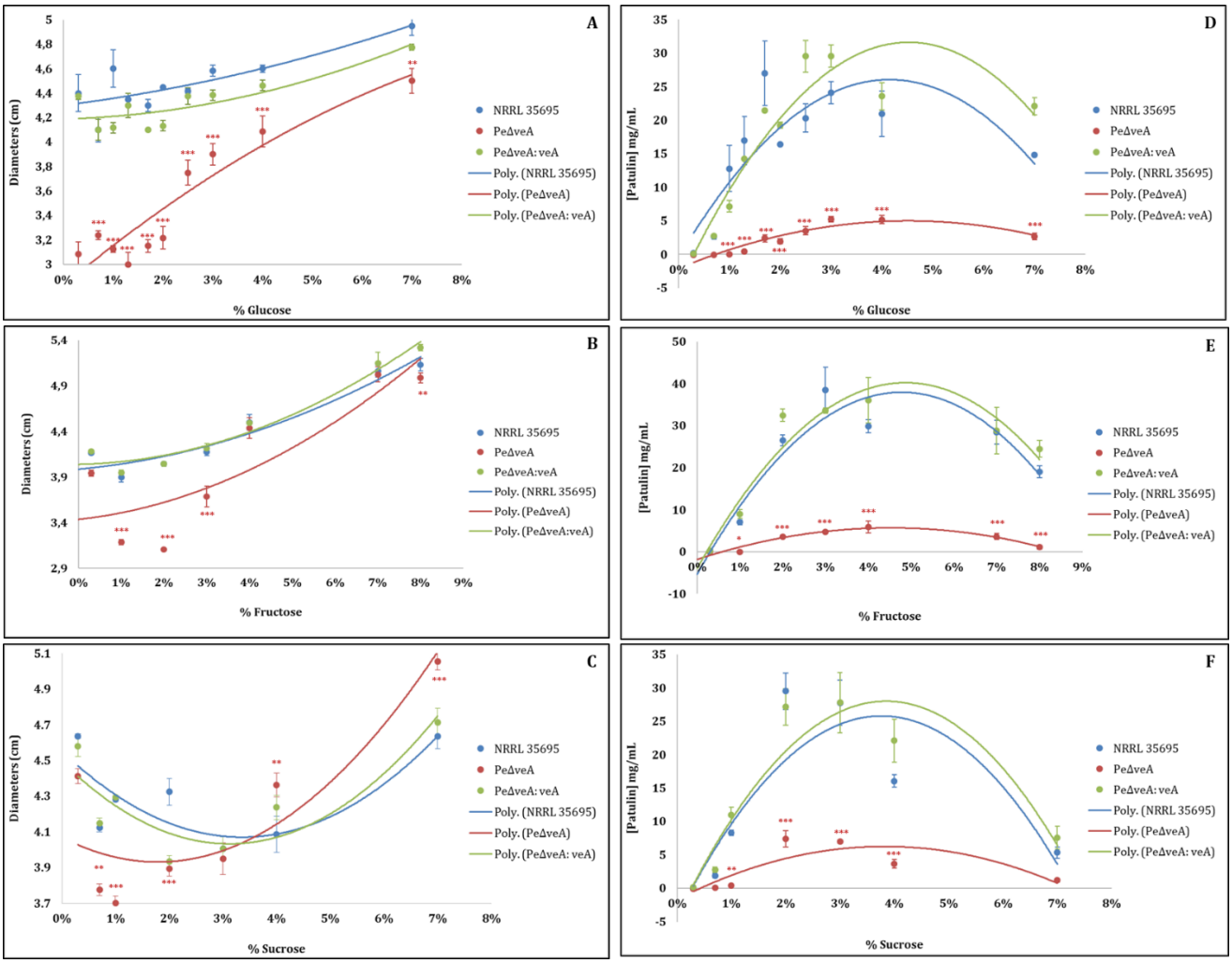

Figure 2 Comparison of the development (A, B, C) and production of PAT (D, E, F) of the WT (NRRL 35695), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains when grown on medium supplemented with various percentages of (A, D) glucose, (C, F) fructose and (B, E) sucrose , for 6 days at 25°C in the dark. Graphs show the mean ± standard error of the mean (SEM) from four replicates. Asterisks denote significant differences between the WT, null mutant and complemented strains.*P < 0.05; **P < 0.01; ***P < 0.001.

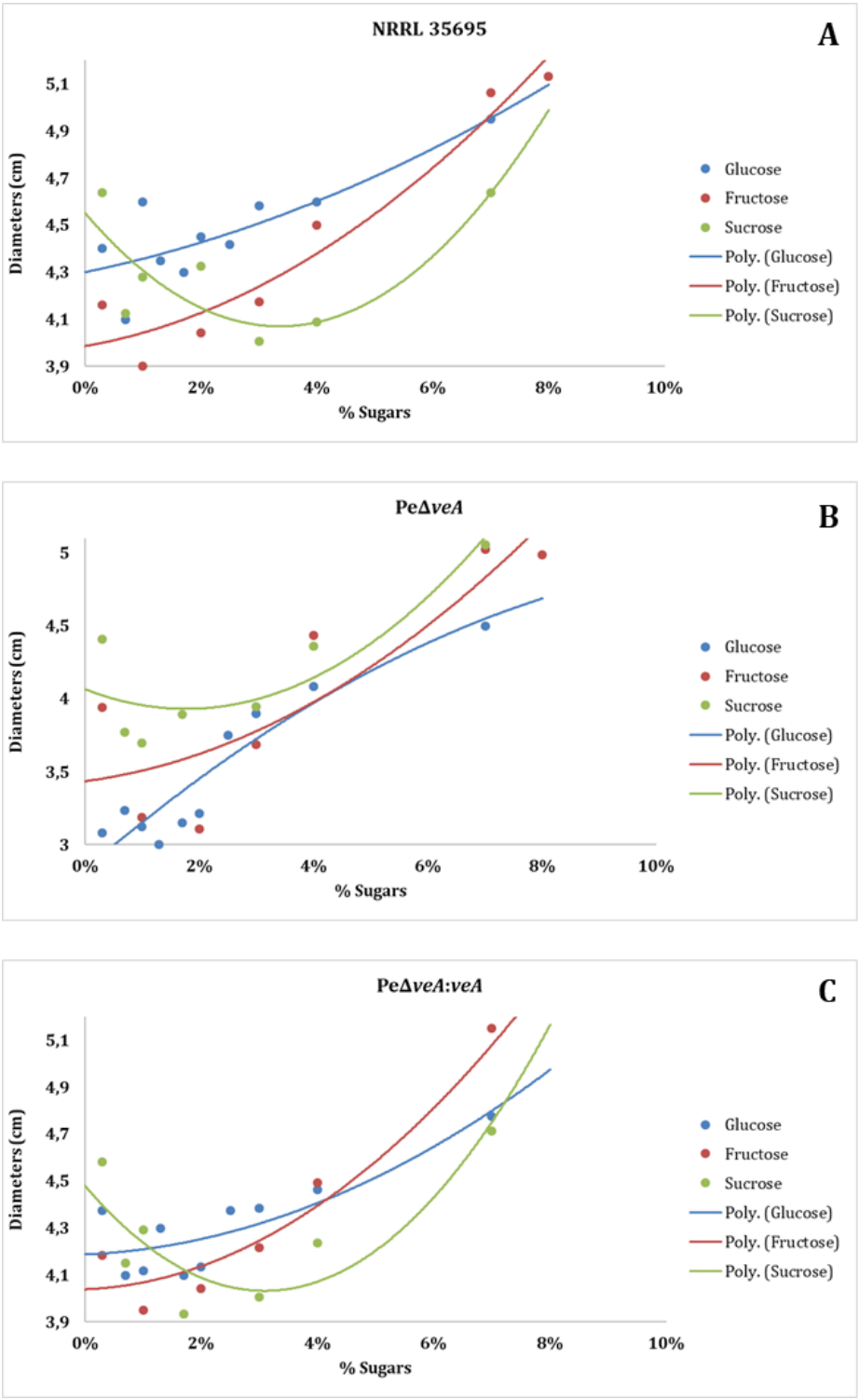

Figure S1 Development of the WT (NRRL 35695), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains grown for 6 days at 25°C in the dark, on base medium supplemented with various percentages of glucose, fructose and sucrose.

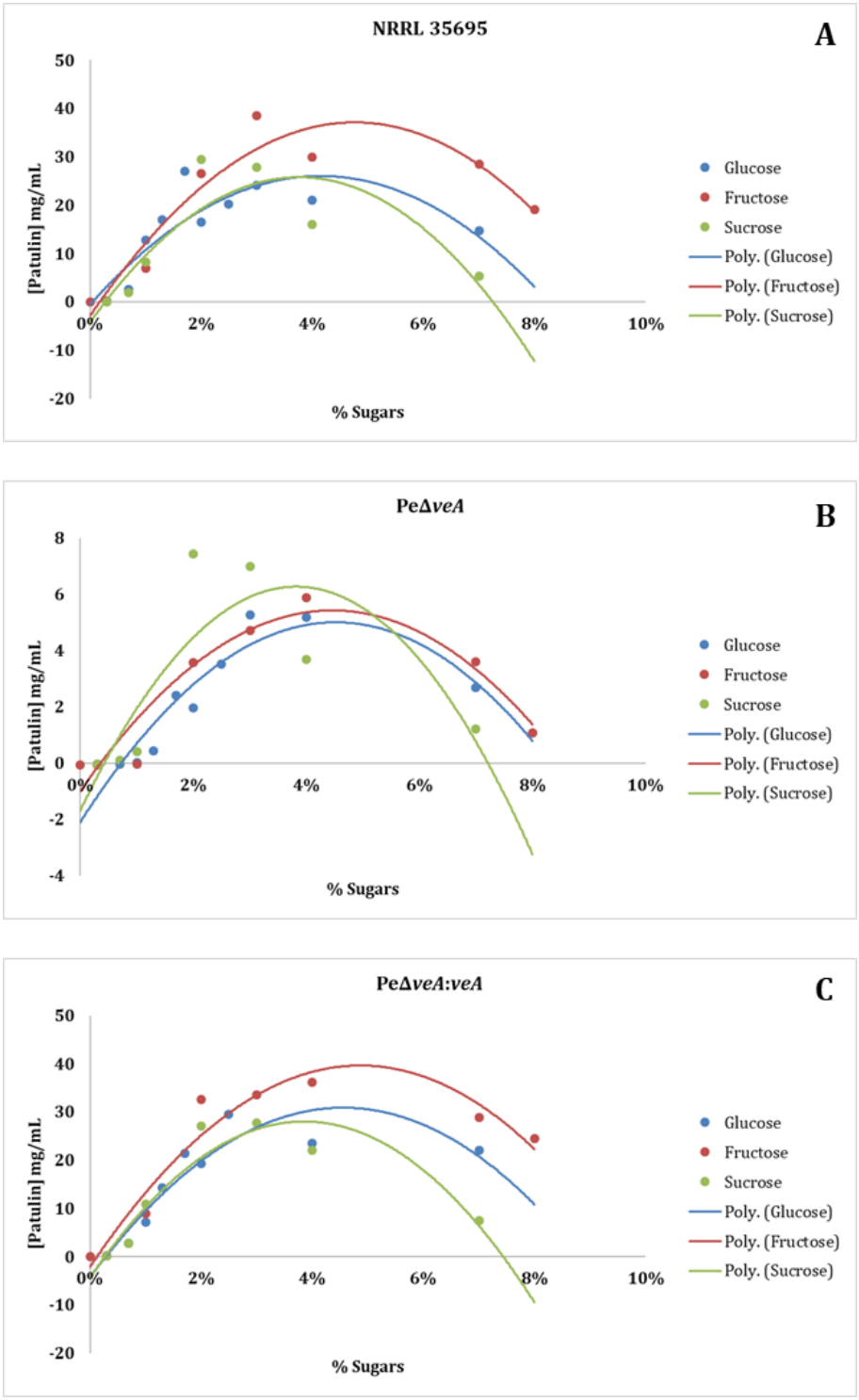

Figure S2 Concentration of PAT (mg/mL) produced by the WT (NRRL 35695), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains when grown for 6 days at 25°C in the dark, on base medium supplemented with various percentages of glucose, fructose and sucrose.

Chapter 2

Impact of ascorbic acid on the patulin concentration on a laboratory and semi-industrial scales

Chapter 2_Part 1

Impact of ascorbic acid on the patulin concentration in aqueous solution and cloudy apple juice

Summary of the study (Article 1_ILVO)

Access to healthy food is a primary prerequisite for public health. In this respect, risk reduction is a crucial element in managing food safety and security, although a total elimination of risk remains a chimerical goal. Fungal growth and/or toxin biosynthesis are serious threats to food quality and safety occurring at different stages of food production. In recent years, different treatments have been extensively studied to reduce the risk of contamination by moulds and avoid toxin presence in products designed for humans or animals.

Patulin (PAT), the mycotoxin of interest within this dissertation, found in apple-derived products could be mitigated. Its concentration is affected by different factors like storage, handling, transport, pasteurization, filtration, as well as other processing steps. Different techniques affecting PAT in apple products and categorised as physical, chemical and biological practices, have been investigated. The generation of (less toxic) breakdown products, such as (E/Z)-ascladiol (ASC-E/Z) and desoxypatulinic acid (DPA) was described in some cases.

The purpose of this research was to see whether ascorbic acid, also known as vitamin C, affects the PAT concentration in cloudy apple juice. Ascorbic acid, naturally present in fruits and vegetables is already used on an industrial level due to its antioxidant properties and for preventing browning reactions. Even though some studies have shown that ascorbic acid accelerates the degradation of PAT in model systems and clear apple juice, optimal conditions of action of the latter have not been elucidated for cloudy apple juice yet.

We started by optimizing an in-house analytical methodology needed to separate and detect the secondary metabolite PAT, and its degradation and reaction products. Then, a study of the impact of ascorbic acid on PAT was performed both in aqueous solution artificially contaminated with a pure PAT standard and in cloudy apple juice contaminated with PAT-containing apple mash. Different concentrations of ascorbic acid were evaluated under different storage temperatures, in presence or absence of oxygen. The formation of reaction (HMF, DHA) and degradation (ASC-E/Z, DPA) products was examined qualitatively.

To conclude, the results of this study confirm that ascorbic acid is capable of degrading PAT and allow the identification of optimal conditions of action of ascorbic acid on a laboratory scale. These data also suggest that ascorbic acid, a food additive improving numerous product qualities, also has an effect on PAT thereby generating less toxic compounds.

Impact of ascorbic acid on the patulin concentration in aqueous solution and cloudy apple juice

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Abstract

Patulin (PAT), a mycotoxin produced by several fungal genera including *Aspergillus*, *Penicillium*, *Byssochlamys* and *Paecilomyces*, is an important contaminant in apples and apple-derived products. Due to its toxicity, it implies important consequences for human and animal health. Chemical, physical and biological treatments described in literature, can cause degradation of this toxin and the generation of (less toxic) breakdown products, such as (E/Z)-ascladiol (ASC-E/Z) and desoxypatulinic acid (DPA). Our study focused on the chemical degradation of PAT in presence of ascorbic acid (AA). In order to do so, an in-house methodology allowing a good separation of PAT from its reaction and breakdown products was optimized first. This methodology was subsequently used to evaluate the influence of AA on PAT. Within this framework, different concentrations of AA were evaluated, as well as the presence/absence of oxygen and different storage temperatures. The experiment was performed both on pure PAT standard in aqueous solution and on PAT-contaminated cloudy apple juice (CAJ) (obtained via addition of apple mash produced from apples inoculated with *P. expansum*). The analysis showed that the highest PAT reduction (60%) in CAJ was achieved after 6 days of incubation at 22°C, in presence of oxygen, with an initial PAT concentration of 100 µg/kg and 0.25% (w/v) AA. It was also found that the treatment by AA resulted in the generation of degradation products less toxic than PAT (such as (E/Z)-ASC). In conclusion, AA approved by European Commission and described as a food additive improving numerous product quality aspects (e.g. colour (less browning), nutritional values, etc.), was shown to have an effect on PAT degradation generating less toxic compounds in presence of oxygen.

Keywords: ascorbic acid, patulin, cloudy apple juice, ascladiol

Introduction

Patulin (PAT) is a mycotoxin produced by several *Penicillium*, *Aspergillus* and *Byssochlamys* mould species. The main producer is *Penicillium expansum* causing blue mould decay on apples and pears (Moake et al. 2005; Morales et al. 2010). Damaged and bruised apples are the most susceptible to contamination by PAT-producing moulds. PAT is most commonly encountered in products derived from fruit processing, especially apples (Chen et al. 2004). Many studies report the natural presence of PAT in various fruit-based processed products such as juices (Özdemir et al. 2009; Reddy et al. 2010), apple compotes or jams (Funes and Resnik 2009). Considering its cytotoxic (Liu et al. 2007; Riley and Showker 1991), genotoxic (Alves et al. 2000; Liu et al. 2003), teratogenic (Ciegler et al. 1976) and immunosuppressive properties (Escoula et al. 1988; Puel et al. 2010), PAT in food is regulated and maximum levels of 50 µg/kg for fruit juices, 25 µg/kg for apple purees and compotes and 10 µg/kg for food intended for babies and young children have been set by the European Union (EU) (EC Regulation 1881/2006). However, PAT can still be found in commercial food and/or beverage products, sometimes exceeding the maximum limits (Ioi et al. 2017; Tannous et al. 2017a; El Hajj Assaf et al. submitted review). Along the food processing chain, PAT concentration may be affected by factors like storage (temperature, modified atmosphere) (Johnsonn et al. 1993; Paster et al. 1995; Baert et al. 2007a; Morales et al. 2007; Sant'Ana et al. 2008), handling (physical removal of fungi and infected tissue, washing, etc.) (Acar et al. 1998; Cole et al. 2003), transport, heat treatment, filtration (Kadakal and Nas 2003; Gökmen et al. 2001), fermentation to cider, etc. Additional non-thermal processing techniques like ultraviolet (UV) radiation (Dong et al. 2010; Assatarakul et al. 2012; Zhu et al. 2013; Zhu et al. 2014; Tikekar et al. 2014; Koutchma et al. 2016), pulsed light (Funes et al. 2013), high hydrostatic pressure (Hao et al. 2016), etc. have shown to have an effect on the PAT concentration as well. Methods decreasing or mitigating PAT after its production are categorized as chemical, physical and biological practices and mostly concern research studies. Some of these treatments can have an impact on the nutritional and organoleptic quality of the end product as well. Although biological control methods using microorganisms were considered as alternative methods for chemical and physical PAT degradation and showed no significant impact on the juice quality characteristics, their use is limited to products that can be fermented. As for chemical treatments, their use exclusively for mycotoxin decrease is not allowed in the EU. However, some chemicals have shown to have a high impact on PAT degradation. For example, ascorbic acid (AA) often used in the food industry and naturally present in fruits and vegetables, is a powerful antioxidant (Stadtman 1991) resulting in an inhibition of browning reactions caused by oxidation of polyphenols. It is used

to improve product quality (e.g. colour, palatability) and restore possible nutritional losses that occur during processing (Bauernfeind 1982). In addition, AA has been described to act as a nucleophile, increasing PAT degradation (Fremy et al. 1995; Canas and Aranda 1996; Alves et al. 2000; Drusch et al. 2007). Different studies focused, not only on the degradation of this toxin, but also on the generation of breakdown products that are less toxic, such as (E/Z)-ascladiol (ASC-E/Z) and desoxypatulinic acid (DPA). ASC-E is the direct precursor in the biosynthetic pathway of PAT by the fungus (Sekiguchi et al. 1983; Tannous et al. 2016). ASC-E is also considered as a predominant product formed during degradation of PAT by microorganisms such as *Saccharomyces cerevisiae* (Moss and Long 2002) and *Gluconobacter oxydans* (Ricelli et al. 2007). In another study, DPA and ASC-Z were the final degradation products of PAT in presence of *Sporobolomyces sp.* IAM13481 (Ianiri et al. 2016). So, depending on the microorganism or chemical, the degradation of PAT would vary and the breakdown products generated could be different. As for AA, the breakdown products that could be formed when added to a solution containing PAT have yet to be defined.

The purpose of this research was to investigate whether AA could reduce the concentration of PAT in cloudy apple juice (CAJ) and if there is an influence of oxygen and temperature. First, an in-house methodology needed optimization in order to allow the detection and quantification of the secondary metabolite PAT as well as its degradation and reaction products. Indeed, the UHPLC-UV signal of dehydroascorbic acid (DHA) resulting from the oxidation of AA interfered with the one of ASC-E (Bode et al. 1990), whereas the one of 5-hydroxymethylfurfural (HMF) formed from sugars during thermal processing of apple juice interfered with the signal of PAT (Bandoh et al. 2009; Christensen et al. 2009). Subsequently, this optimized methodology was used to study the impact of AA on pure PAT standard in aqueous solution on the one hand and on PAT-contaminated apple mash in CAJ on the other hand. The addition of different concentrations of AA was evaluated under different storage temperatures, and in presence or absence of oxygen. In addition, the formation of reaction (HMF, DHA) and degradation (ASC-E/Z, DPA) products was determined qualitatively.

Material and methods

Chemicals and reagents

PAT standard (≥ 98% purity), HMF (≥99% purity), and DHA were purchased from Sigma-Aldrich (St. Louis, MO, France). AA was purchased from Solina group (Eke-Nazareth, Belgium). DPA was provided by Prof. Raffaello Castoria (Department of Agricultural, Environmental and Food Sciences, University of Molise, Italy) and Prof. Rosa María Durán Patrón (Department of Organic Chemistry, University of

Cádiz, Spain); ASC-E/Z standards were provided by Dr. Olivier Puel (Toxalim laboratory, INRA Toulouse, France). Analytical grade solvents used for extraction and high-performance liquid chromatography (HPLC) were ethyl acetate (AcOEt, Pesti-S), hexane (Pesti-S), acetonitrile (ACN, LC-grade) and acetic acid (AcA, 99.99%), all supplied by Biosolve BV (Valkenswaard, The Netherlands). HPLC-grade water was generated by a Milli-Q-grade purification system (Millipore, Darmstadt, Germany) and adjusted to pH 4 with acetic acid.

Strains, media and culture conditions

The *Penicillium expansum* wild type strain NRRL 35695 (WT) used in this study was provided by Dr. Olivier Puel (Toxalim lab, INRA, France). This strain was cultured on a Potato Dextrose Agar (PDA) medium (Biokar, Allonne, France) for 5 days at 25°C in the dark. A spore suspension of the *P. expansum* wild type strain was prepared, according to Adjovi et al. (2014), and quantified using a Malassez cell. A concentration of 100 conidia/ml was used to inoculate Golden Delicious apples.

Preparation of PAT-contaminated apple mash

To assess the PAT degradation level in CAJ in presence of AA, PAT-contaminated starting material needed to artificially contaminate blank juices was produced. To do so, Golden Delicious apples free of defects or injuries were used. They were surface-sterilized in 2% sodium hypochlorite solution and then rinsed with water, as described by Sanzani et al. (2012). Apples were first wounded with a sterile toothpick, after which they were inoculated by 10 µl of the spore suspension at concentration of 100 conidia/ml, and incubated at 25°C in the dark for 14 days.

After the incubation period, contaminated apples were heat treated at 88°C for 2 min to inactivate the mould and then grounded to obtain a homogenized apple puree (MacDonald et al. 2000). The concentration of PAT in the apple mash, serving to inoculate our juice samples, was assessed (35 mg/kg) using the protocol of De Clercq et al. (2016) (n=5). Contaminated apple mash was stored at -20°C.

Identification of optimal conditions of action of AA

PAT in aqueous solution

Ten mL of sterilized water, acidified with acetic acid (pH=4), was inoculated with 100 µg/kg of a pure PAT standard. Solutions of 0% and 0.25% AA were filter-sterilized using a 0.22 µm filter unit (Millex GS by Merck Millipore, Darmstadt, Germany) and added to the mix. Samples were prepared either on

the bench in aerobic conditions or in anaerobic conditions using a Whitley A35 anaerobic workstation (Labconsult; Brussels, Belgium) and anaerobic jars to which AnaeroGenTM 3.5L sachets (Oxoid N.V.; Aalst, Belgium) were added. Samples were then stored at 4°C or at 22°C for up to 24 days, in the dark. After 0, 3, 6, 12, 18 and 24 days of storage, metabolite extraction was conducted as described by De Clercq et al. (2016), and the obtained extracts were analysed by means of ultra-high performance liquid chromatography (UHPLC) coupled to an UV detector (UHPLC-UV) (see below).

Artificially PAT-contaminated CAJ

Ten mL of CAJ was inoculated with 100 µg/kg of PAT by addition of contaminated apple mash prepared as described above. Different concentrated solutions of AA (0%, 0.25% and 4%) were prepared and filtered the same way as cited above and added to the juice mix. The samples were prepared either in presence or absence of oxygen and incubated for 6 days at 4°C or 22°C, in dark conditions. The metabolite extraction and UHPLC-UV analysis was conducted on samples stored for 0, 3 and 6 days.

UHPLC-UV analysis of PAT, and PAT degradation and reaction products

The chromatography device used concerns an ACQUITY UPLC® H-Class System equipped with a Waters® ACQUITY™ UPLC™ Tunable UV (TUV) detector set at 276 nm. Separation of the compounds considered was achieved on an Acquity UPLC® BEH C_{18} 1.7 µm (2.1x150mm) column at 30°C at a flow rate of 0.3 mL/min Eluent A was HPLC-grade water acidified with 0.1% acetic acid and eluent B was ACN. The column was cleaned with a mixture of 100% solvent B and 0% solvent A. An original in-house methodology (De Clercq *et al.* 2016) was optimized based on the chemical properties of our compounds of interest in order to improve their separation. Elution conditions were as follows: a 2 min isocratic passage of solvent B from 0 to 2%, a 7 min gradient increase of solvent B from 2 to 5%, a 1 min gradient increase of solvent B from 5 to 100%, 100% solvent B for 6 min, a 1 min direct decrease in solvent B from 100 to 0%, and re-equilibration at 0% solvent B for 3 min. The presence of PAT was monitored and quantified at a wavelength of 276 nm. For the degradation products (ASC-E/Z; DPA) and reaction products (DHA; HMF), their presence/absence was verified and compared relatively between samples based on the response (area) obtained.

Data analysis

Statistical analysis of the data obtained was carried out using the Statistical Analysis System software (SAS, version 9.4, SAS institute Inc., Cary, NC, USA). A linear regression model was applied with PAT, DHA, ASC-Z and compound X as dependent variables and day, temperature, oxygen and ascorbic acid as independent ones. Appropriate two and three-way interactions were tested for and removed when non-significant (P>0.05). Post-hoc comparison was performed with a Tukey test.

Results

Optimization of metabolite detection methodology

For the extraction and detection of PAT in cloudy apple juice, the method described by De Clercq et al. (2016) was applied. The suitability of this method was explored for the simultaneous detection of PAT, its degradation products ascladiol (E and Z isomers) and DPA, and the reaction products DHA and HMF.

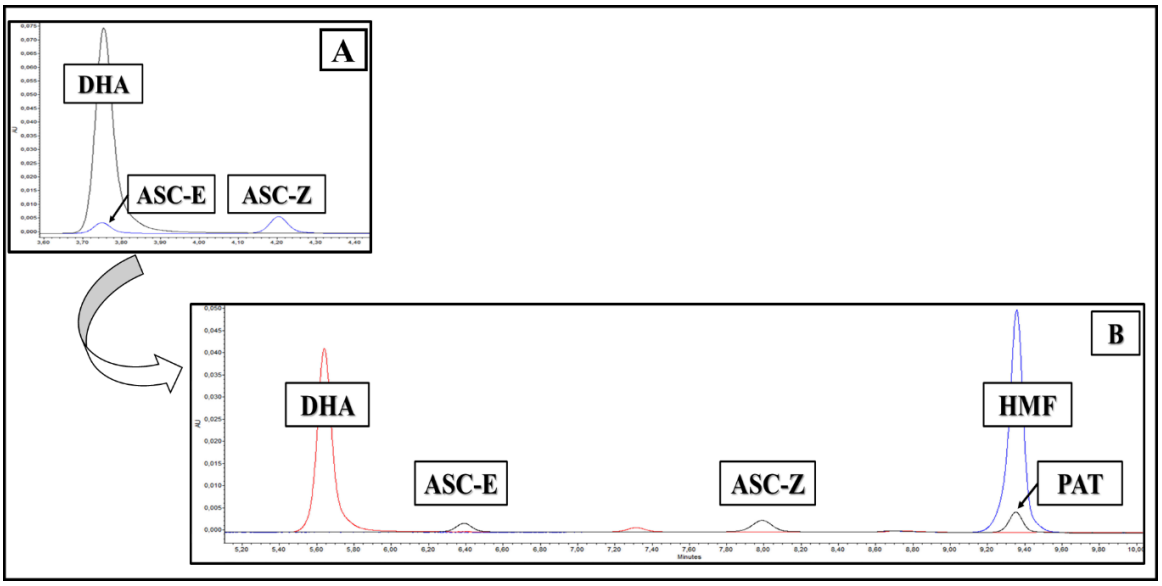

Figure 1 UHPLC chromatograms showing the interference of the UHPLC-UV signal of the secondary metabolites, degradation and reaction products following (A) a gradient method (2% B => 5% B), and (B) an isocratic method (0% B). PAT, patulin; DHA, dehydroascorbic acid; HMF, 5-hydroxymethylfurfural; ASC-E, E-ascladiol; ASC-Z, Z-ascladiol.

However, preliminary UHPLC-UV analysis showed that some of these compounds suffered from peak interference, not allowing correct identification of each of the compounds considered: the signal of DHA, which results from the oxidation of AA, interfered with the one of (E)-ASC causing an overestimation of the latter **(Figure 1A)**. Since these interferences could lead to misidentification

and/or overestimation of the signal of PAT and (E)-ASC, the initial methodology of De Clercq *et al.* (2016) consisting on a gradient passage of solvents was further optimized using pure standard of DHA, (E/Z)-ASC, HMF, PAT and DPA in order to allow the analysis of these compounds in CAJ. This was done by testing an isocratic chromatographic run. However, when applying this method, the chromatographic peak of HMF, which appears in heat-treated juices of apple, interfered with the one of PAT causing an overestimation of the concentration of the latter **(Figure 1B)**. Based on the chemical properties of the metabolites, the UHPLC conditions were further optimized. Therefore, an isocratic elution was combined with a gradient elution using water acidified with 0.1% (v/v) acetic acid as mobile phase A and ACN as mobile phase B (see material and methods section for the final method). This adjusted methodology allowed a good separation of all the compounds mentioned above **(Figure 2)** and was used in all further experiments.

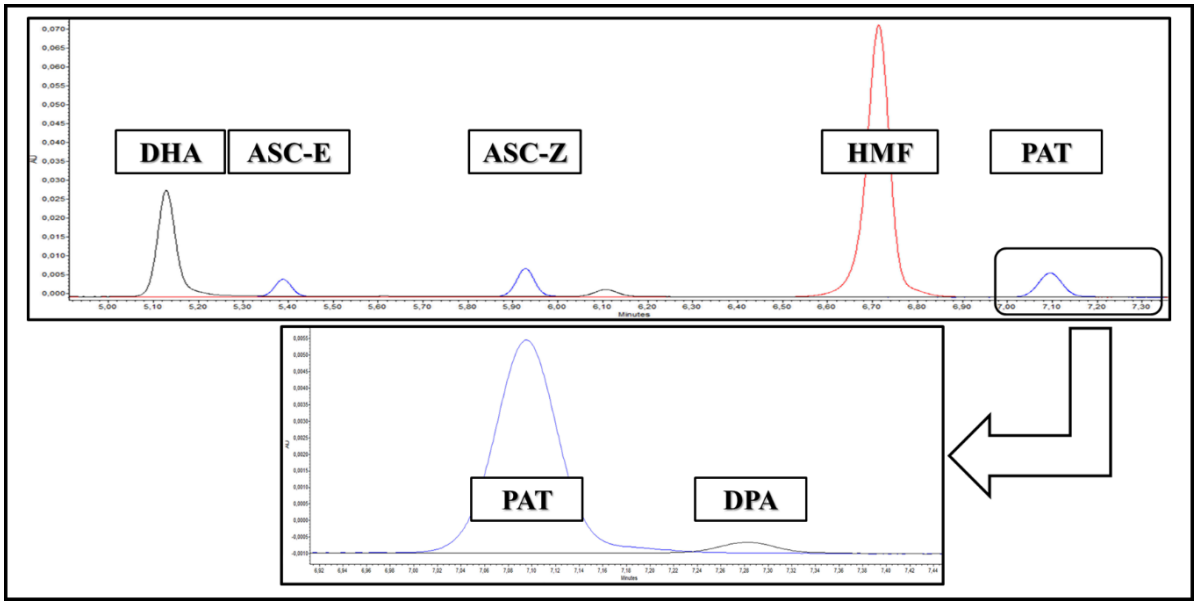

Figure 2 UHPLC chromatogram of mixture of PAT, its breakdown products (ASC-E/Z; DPA) and reaction products (DHA; HMF). PAT, patulin; DHA, dehydroascorbic acid; HMF, 5-hydroxymethylfurfural; DPA, desoxypatulinic acid; ASC-E, E-ascladiol; ASC-Z, Z-ascladiol.

Identification of optimal conditions of action of AA

To study the impact of AA on PAT, the addition of different concentrations of AA was evaluated, in the presence or absence of oxygen, at different storage temperatures. The experiment was performed both with pure PAT standard in aqueous solution and in artificially contaminated CAJ.

Impact of different concentrations of AA and of storage temperature

Different concentrations of AA (0%, 0.25% and 4%) were added to CAJ containing 100 µg/kg of PAT, in the dark and in presence of oxygen, at 4°C and 22°C. The results are shown in **Figure 3.** When no AA was added, no significant difference in the concentration of PAT could be observed within 6 days of incubation at 4°C, but a statistically significant decrease in PAT concentration was observed at 22°C between day 0 and day 6 (P<0.0002). On the other hand, the addition of 0.25% or 4% of AA significantly decreased the PAT concentration at both temperatures tested with a significant difference in PAT concentration observed on day 3 and day 6, when samples were stored at 4°C and 22°C. It was noticed that whether 0.25 or 4% AA was added, a similar decrease in the PAT concentration with no significant differences or the two percentages applied, was observed: 53.2 ± 5.4, 28.3 ± 1, 19.9 ± 1.6 µg/kg when 0.25% AA was added and 43.2 ± 2.5, 27.1 ± 1.3, 12.0 ± 1.5 µg/kg for 4% AA, at 22°C for an incubation period of 0, 3 and 6 days, respectively. It seems that a percentage of 0.25% AA seems sufficient for PAT degradation for the conditions tested **(Figure 3)**.

When comparing the PAT concentration measured for the samples stored at 4°C to those stored at 22°C, in general a higher degradation of PAT at 22°C was observed. Our analyses showed that a lower decrease of PAT (68 and 56%) was reached after 6 days of incubation at 4°C, when respectively 0.25% and 4% (w/v) of AA was added, compared to at 22°C (63 and 78%). The most significant difference in PAT concentration between 4°C and 22°C was observed on day 3 when 0.25% AA was added (P< 0.0321). It was also noticed, that when 0.25% or 4% AA was added, the most significant decreases in PAT concentration occurred between day 3 and day 6 at 4°C (P<0.0001) and between day 0 and day 3 at 22°C (P<0.0001; P<0.0341, respectively).

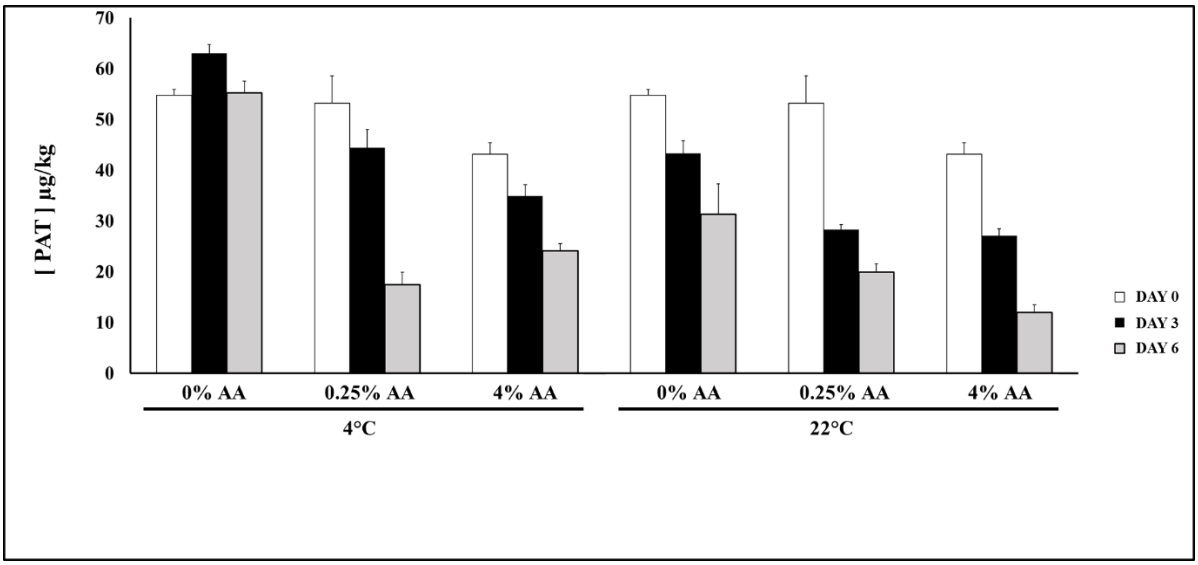

Figure 3 Patulin (PAT) concentration of PAT-contaminated (100 µg/kg) cloudy apple juice (CAJ) within 6 days of incubation with 0, 0.25 and 4% ascorbic acid (AA) at 4°C and 22°C, in presence of oxygen (Ox+), and in the dark. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Impact of oxygen on AA action

To identify whether oxygen is required for AA to have an effect on PAT in apple juice, CAJ containing 100 µg/kg of PAT was separately incubated in presence and absence of oxygen. We noticed in samples with 0.25% AA added that in absence of oxygen **(Figure 4A)**, no significant degradation of PAT occurred whether the samples were incubated at 4°C or at 22°C (56.3 ± 3.3 µg/kg and 55.4 ± 6.4 µg/kg of PAT at 4°C on day 0 and 6, respectively, and 56.3 ± 3.3 µg/kg and 59.0 ± 0.8 µg/kg of PAT at 22°C, respectively, on day 0 and 6). But when comparing samples incubated in presence of oxygen to those incubated in absence of oxygen for 6 days, we noticed that the PAT concentration was significantly lower when 0% or 0.25% AA was added (P<0.0003 and P<0.0001, respectively). In addition, in presence of oxygen, 6 days post-incubation in presence of 0.25% AA, a significant decrease in the PAT concentration was observed, namely a shift from 57.0 ± 5.7 µg/kg to 32.6 ± 1.1 and 20.5 ± 1.8 µg/kg, at 4°C and 22°C respectively (P<0.0001) **(Figure 4B)**. These data also confirm that the highest decrease in PAT concentration in CAJ occurred at room temperature (22°C) compared to refrigeration temperature (4°C). More specifically, a 42% decrease could be observed compared to a 63% decrease in PAT concentration when the samples were stored at 4°C and 22°C, respectively.

In conclusion, the highest degradation of PAT was observed in presence of AA (0.25%) and oxygen after 6 days of incubation at 22°C in the dark.

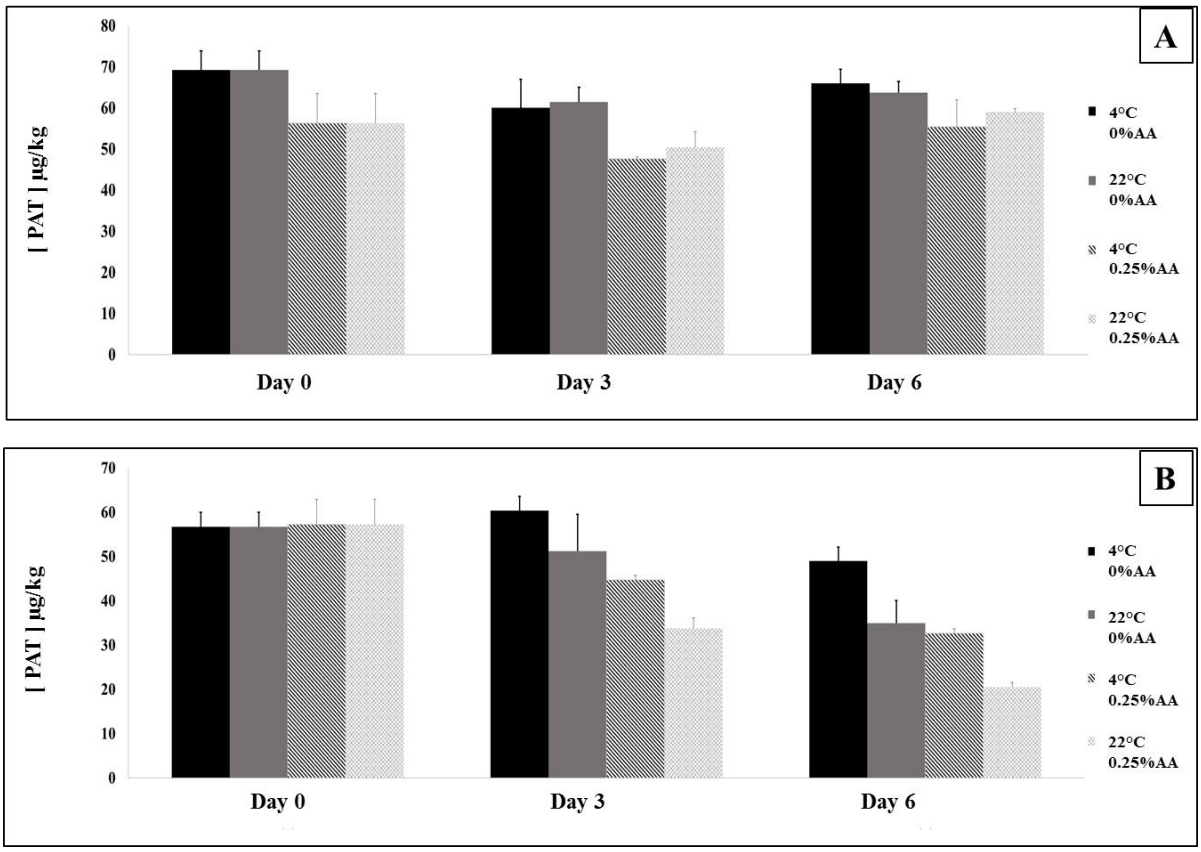

Figure 4 Patulin (PAT) concentration in absence (0%) or presence (0.25%) of ascorbic acid (AA) in PAT-contaminated (100 µg/kg) cloudy apple juice (CAJ), stored in the dark, at 4°C and 22°C in anaerobic (Ox+) (A) and aerobic (Ox-) (B) conditions. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Degradation products' formation

To identify possible degradation products, the experiment was performed in an acidified aqueous solution where a decrease in PAT concentration was observed when AA was added, in function of time and oxygen. Data obtained from experiments performed in both aqueous solution and in CAJ, a more complex matrix, allowed a complemented follow up on the degradation products obtained due to the addition to AA.

The decrease in PAT concentration in the contaminated CAJ or aqueous solution was accompanied by the formation of additional compounds. A compound X was formed when 0.25% AA was added to an aqueous solution (pH 4), spiked at 100 µg/kg pure PAT standard and in presence and absence of oxygen **(Figure 5)**. When 0% AA was added, the peak corresponding to compound X was not detectable. Its formation, was studied semi-quantitatively based on chromatographic peak area but

not quantitatively like in the case of PAT. On day 0, when 0.25% AA was added, this compound was completely absent in our samples incubated under aerobic or anaerobic conditions. Our statistical analysis showed that oxygen does not significantly affect the formation of the compound X; and what we observe is rather due to a time-temperature effect. Starting from day 3 post-incubation, the presence of this compound was observed, with a significantly higher amount at 22°C (1.7 ± 0.0 and 2.2 ± 0.2 mV in presence and absence of oxygen, respectively) than at 4°C (0.2 ± 0.0 and 0.3 ± 0.0 mV in presence and absence of oxygen, respectively). The formation of this compound increased gradually during 24 days of incubation. After 24 days at 4°C, the formation of the compound X reached a not significantly different chromatographic response of maximum of 0.7 ± 0.0 mV and 1.4 ± 0.0 mV in presence and absence of oxygen, respectively (P>0.05). In contrast, during storage at 22°C, a chromatographic response for the metabolite X was obtained (40.9 ± 0.3 mV and 38.6 ± 0.5 mV) in presence and absence of oxygen, respectively (P<0.0001). The peak corresponding to the compound X was almost 58 and 28 times higher when samples were incubated for 24 days at 22°C then those left at 4°C, with or without oxygen respectively. This formation started to be significantly different between 4°C and 22°C from day 6 on (P<0.0001). When comparing this compound to the pure standards (see material and methods section), we noticed that compound X shared the same retention time as the DPA standard. More analytical studies need to be done in order to confirm the identity of this compound. We could conclude that this compound formation is not significantly dependent of the presence of oxygen but its presence is rather affected by the presence of AA, the incubation temperature and time.

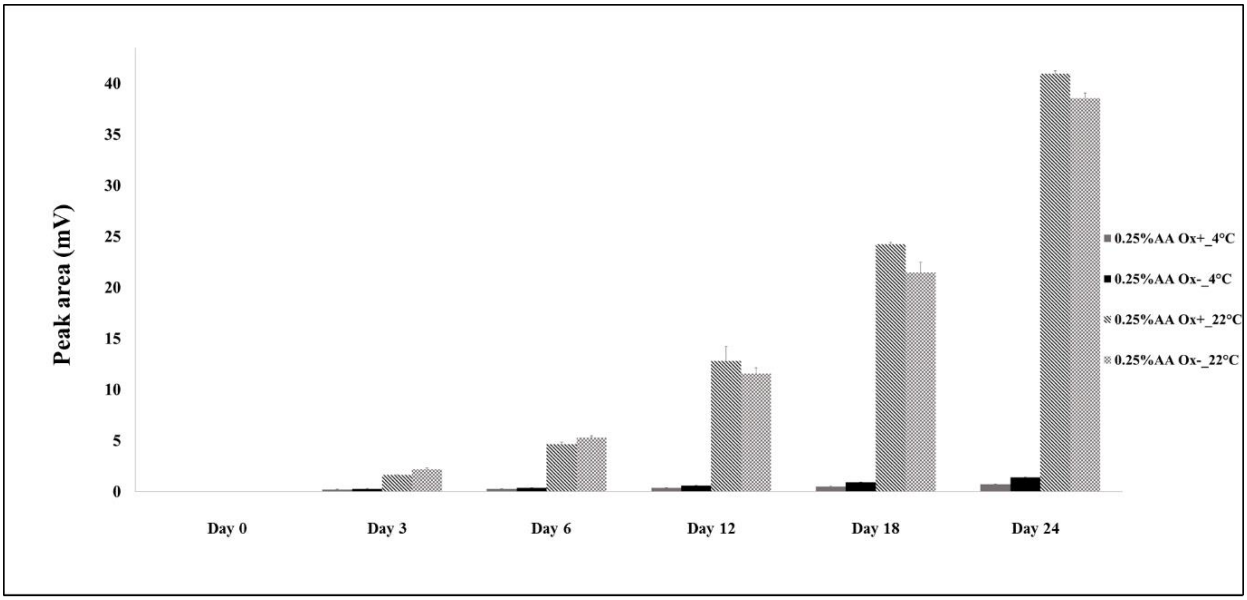

Figure 5 Compound X formation expressed as peak area (mV) in an aqueous solution (pH 4) artificially contaminated with pure patulin (PAT) standard (100 µg/kg). Samples were stored in the dark, at 4°C and 22°C, in aerobic (Ox+) and anaerobic (Ox-) conditions. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

One of the compounds found in the contaminated CAJ was identified as ASC-Z. ASC-Z, one of the breakdown products of PAT previously described in the literature as being less toxic than the toxin itself, appeared to be formed at the highest level 6 days post-incubation at 22°C in presence of 0.25% AA and oxygen **(Figure 6)**. At 4°C, minor peak signals corresponding to ASC-Z were identified and by that no significant increase of this metabolite concentration was observed within the days of incubation (P>1.000). However, at 22°C and in presence of 0.25% AA, a significant difference in chromatographic response was observed between day 0 (0.4 ± 0.1 mV) and day 6 (14.3 ± 1.8 mV) (P<0.0001). As per the statistical analysis, the formation of this compound and the difference detected within the 6 days was due to the time of incubation and temperature. As for ASC-E, the formation of this isomer could not be observed in any of the conditions tested.

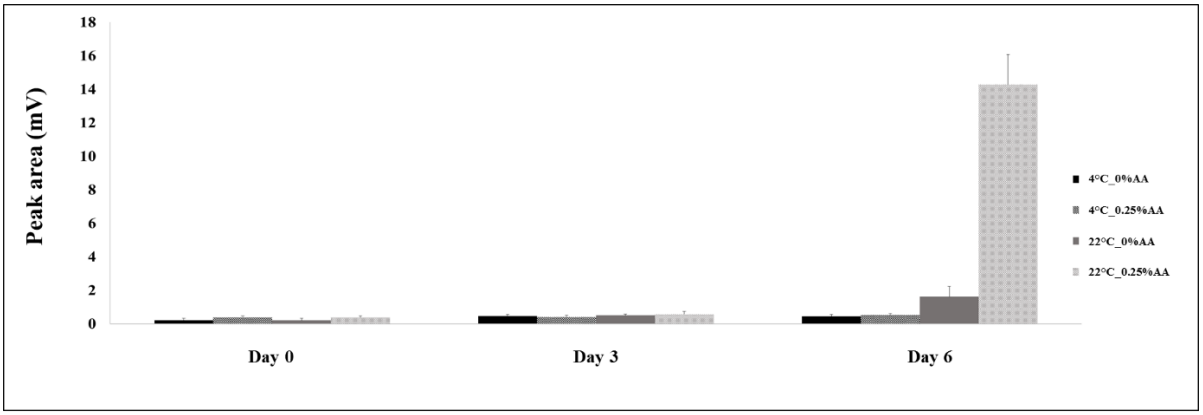

Figure 6 Ascladiol-Z (ASC-Z) formation in a patulin-contaminated (100 µg/kg) cloudy apple juice (CAJ) expressed as peak area (mV). Samples of juice were stored in presence of oxygen (Ox+) and in the dark, at 4 and 22°C, with or without addition AA. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Our study showed that when 0.25% AA was added to a contaminated acidified aqueous solution or CAJ, at 22°C, a significant decrease in the PAT concentration followed by a formation of compound X and ASC-Z, respectively, was observed.

Reaction products' formation

DHA, the oxidized form of AA, is a compound that appeared to be formed during the experiments conducted. Its formation was studied qualitatively in the aqueous solution (pH 4) at different

temperatures (4°C and 22°C). Statistical analysis showed that formation of DHA is affected by time, temperature and oxygen. When no AA was added to the solution, this compound was absent (data not shown). In fact, DHA could only be detected in case 0.25% AA was added to the solution **(Figure 7)**. Still, at 4°C, its presence was barely detectable (whether in Ox + or Ox – conditions) and no significant difference in its presence was detected within the 24 days of incubation (P<1.000). The highest and most significant increase in the concentration of DHA was between day 3 and day 24, at 22°C and in presence of oxygen. In fact, the increase started to be significantly different after 6 days of incubation at 22°C, in presence of oxygen (P<0.0001). We identified peaks that were 29 and 836 times higher on day 24 than on day 0 in absence and presence of oxygen, respectively.

When it comes to HMF, it was only detected in the CAJ with no significant increase during 6 days of incubation.

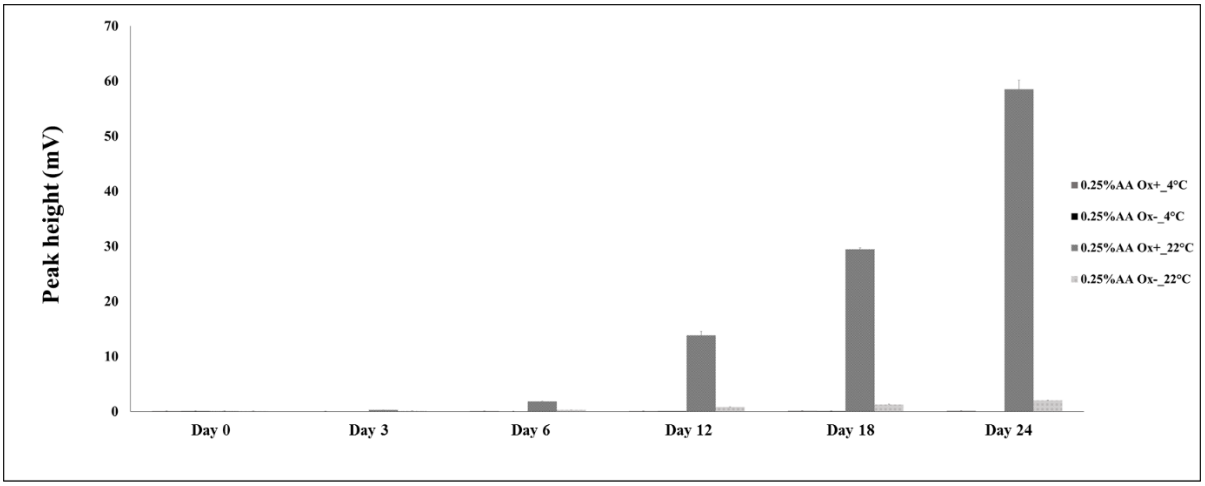

Figure 7 Dehydroascorbic acid (DHA) formation in an aqueous solution (pH 4) artificially contaminated with pure patulin (PAT) standard (100 µg/kg). Samples were stored in in the dark, at 4°C and 22°C, in aerobic (Ox^+) and anaerobic (Ox^-) conditions. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Discussion

In our study, different variables were tested (time, temperature, oxygen, AA). Statistical analysis proved that the degradation and formation of compounds were the result of interactions occurring between these variables.

It was shown that the addition of 0.25% or 4% AA has an impact on the PAT concentration (100 µg/kg) either in cloudy apple juice or in aqueous solution. The degree of PAT breakdown increased when 0.25% or 4% AA were added and was higher when compared to control samples. This is in agreement with other studies on AA and PAT performed for clear apple juices and different model

systems (Brackett and Marth 1979; Fremy et al. 1995; Alves et al. 2000; Drusch et al. 2007). The novelty of our work is that it elucidates optimal conditions of action of AA on PAT in CAJ, and describes the reaction products obtained as well as the biodegradation products detected.

Even though increasing concentrations of AA (from 0% to 3%) seemed to accelerate the rate of disappearance of PAT (5,000 µg/kg) in buffer solutions (Brackett and Marth 1979), our results showed that the addition of 0.25% or 4% AA caused a similar decrease in the PAT concentration in CAJ contaminated by 100 µg/kg of this toxin. This doesn't contradict what has been published before, it only indicates that the quantity of AA needed is directly linked to the initial PAT concentration present in the contaminated juices or buffers. For 100 µg/kg of PAT, which is twice the maximum regulatory limit allowed in Europe, addition of 0.25% AA was sufficient.

How PAT is specifically affected by AA is a mechanism that remains unknown. Earlier studies done by Fliege and Metzler (2000a, b) and Ciegler et al. (1976) focusing on the effects of sulfhydryl compounds, showed that the electrophilic properties of PAT make it a suitable target for nucleophilic attack. Later on, Kokkinidou et al. (2014) hypothesized that AA increases the rate of PAT degradation by acting as a nucleophile using a similar complex mechanism. On the other hand, another mechanism already described in literature seems applicable for our results obtained. In fact, in presence of oxygen and during the incubation period, the oxidation of AA and formation of DHA could have generated singlet oxygen attacking the double bonds of PAT and causing the opening of the lactone ring and making PAT unstable (Brackett and Marth 1979; Drusch et al. 2007). The same phenomenon was described by Aurand et al. (1977) when explaining the role of singlet oxygen in lipid oxidation inhibition. According to Babaali et al. (2017), when AA was completely oxidized by oxygen, no more free radicals would be produced and PAT destruction would decrease. The two mechanisms described previously and cited above are plausible. Still the presence of AA and oxygen in our solutions could have favoured one over the other. This could also be proven by the results presented in **Figure 4** showing no significant decrease of PAT in CAJ when AA was added to the contaminated apple juice, in absence of oxygen. Oxygen turns out to be an important factor in affecting the stability of PAT in presence of AA. Other than the addition of AA, the reaction temperature significantly ($P < 0.001$) affected the PAT concentration in CAJ as well. Disappearance of AA and the formation of other compounds occurred more profound at 22°C compared to 4°C. Many studies done previously discussed the impact of different factors on the decrease in PAT concentration in presence of AA such as pH, oxygen, light, clarity of the solution, etc. (Fremy et al. 1995; Canas and Aranda 1996; Alves et al. 2000; Drusch et al. 2007; Kokkinidou et al. 2014), but none

discussed the two storing temperatures mentioned in our study. In fact, 4°C represented chilled storage and 22°C represented the most popular storage temperature of apple juices in industries or in the supermarkets. This could imply a positive impact in terms of a lower PAT concentration for stored apple juices fortified by AA.

Another thing noticed was that the disappearance of PAT was not directly correlated to a detectable formation of breakdown products. When AA was added, an immediate decrease of PAT was observed within the three first days of incubation with 47% PAT loss and 48% PAT loss when 0.25% and 4% AA was added, respectively. However, the appearance of ASC-Z only occurred afterwards (between day three and six) and no significant difference was observed between day zero and day three. This could be related to the fact that, when the singlet oxygen attacked the bonds of PAT, this latter became unstable and inactive in the solution, but it's just between day 3 and 6 that ASC-Z started to be formed and detected. A possible further explanation could be, that in CAJ, the stability of PAT is not easily affected and the suspended particles and phenolic compounds present in the juice prevent the contact between PAT and AA molecules and make the detection of formed compounds more difficult (Baert et al. 2007b). This same phenomenon occurred between PAT molecules and UV waves (Tikekar et al. 2014). The compound X, identified and presenting a similar retention time as DPA, was formed at later stages of incubation in acidified aqueous solution contaminated by a pure standard of PAT. In fact, this compound was not identified when CAJ was contaminated by PAT standard and incubated during14 days. It could be that PAT is more stable in cloudy juice than in a buffer (Brackett and Marth 1979) and that it might need more time to be formed in such a complex matrix. It could also be that it was formed in small quantities, not detectable by HPLC or it was bounded to other molecules not detected.

When it comes to chemical degradation, information regarding breakdown and reaction products is still lacking. Breakdown products generated during degradation of PAT in presence of AA are not yet elucidated. In our study, two compounds were detected. They were considered as possible degradation products of PAT in presence of AA. One of them was ASC-Z in CAJ and the second one, compound X in aqueous solution, shared the same retention time as DPA pure standard. ASC-Z was expected since it's the direct precursor in the biosynthetic pathway of PAT but DPA was only detected before in case where microorganisms were in contact with PAT. *Rhodosporidium kratochvilovae* lead to the disappearance of PAT and the formation of DPA (Castoria et al. 2011). In fact Moss and Long (2002), reported the biological degradation of PAT into ASC-E by *Saccharomyces cerevisiae*. Ricelli et al. (2007) were the first who reported the bacterial degradation of PAT into ASC-E by

Gluconobacter oxydans. *Candida guilliermondii*, an ascomycetous yeast, showed to degrade PAT into E-ASC (Chen et al. 2017).

Toxicity studies on the breakdown products of PAT, namely DPA and ASC have been performed previously (Suzuki et al. 1971; Tannous et al. 2017b; Castoria et al. 2011). ASC, isolated from a culture of *Aspergillus clavatus* during an attempt to purify PAT, showed an acute toxicity that was four times lower than that of PAT (Suzuki et al. 1971). In a more recent study, it was found a non-toxic compound (Tannous et al. 2017b). ASC-E has been reported to be a direct precursor of PAT, and ASC-Z was the product of a non-enzymatic transformation of ASC-E catalysed by sulfhydryl compounds (homocysteine, cysteine, and glutathione or dithiothreitol) (Sekiguchi et al. 1983). DPA was much less toxic than PAT to human lymphocytes (Castoria et al. 2011).

To conclude, CAJ artificially contaminated with 100 µg/kg PAT and to which 0.25% AA was added showed a significantly lower PAT concentration after 14 days of incubation at 4°C or 22°C in the presence of oxygen, in the dark. These findings indicate that AA has an impact on PAT, and that this antioxidant could be of a value in terms of practical applications in food and agro-businesses. However, before considering the use of this chemical on an industrial level for detoxification purposes, more studies should be performed in order to confirm the structural identities of the degradation products by NMR spectroscopy or isotopic carbon module labelling techniques. Additionally, the non-reversibility of this reaction between PAT and breakdown products should be also studied as well as possible bound forms.

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Conflict of interest

None

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Chapter 2_Part 2

Effect of ascorbic acid, oxygen and storage on patulin in cloudy apple juice produced on a semi-industrial scale

Summary of the study (Article 2_ILVO)

After optimizing the UHPLC-UV methodology for detection and quantification of PAT and the detection of its degradation and reaction products by carrying out preliminary laboratory experiments defining optimal conditions of action of ascorbic acid on PAT, an experiment pressing cloudy apple juice was performed on a semi-industrial scale.

The aim of this study was to evaluate the effect of the antioxidant ascorbic acid on the PAT concentration in artificially contaminated cloudy apple juice produced using a novel spiral-filter press technology avoiding juice oxidation, called VaculIQ 1000. PAT-contaminated cloudy apple juices were also produced under different conditions, flash-pasteurized, packed in γ-radiated bags and stored at 20°C in the dark for 14 days.

Several qualitative parameters (brix, pH, colour, moisture content) of the different juices produced were determined. The decrease in PAT concentration was evaluated quantitatively and possible reaction (DHA, HMF) and degradation (ASC-E/Z and DPA) products generated were determined qualitatively.

To conclude, in this part of the thesis, we demonstrated that the addition of ascorbic acid to PAT-contaminated cloudy apple juice, produced on a semi-industrial scale using the VaculIQ, decreases the PAT concentration without affecting the qualitative parameters of the juice measured. Even though chemical agents are not allowed to be used in industries for detoxification purposes, ascorbic acid which already is applied for its antioxidant properties and capacity to prevent browning reactions, allowed PAT reduction on a semi-industrial scale and should be considered given its impact on patulin.

Effect of ascorbic acid, oxygen and storage on patulin in cloudy apple juice produced on a semi-industrial scale

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Abstract

Patulin (PAT), a mycotoxin mainly produced by *Penicillium expansum*, is of high concern regarding food safety and human health. Even though its presence in apple-derived products is regulated and maximum levels have been set by the European Union (EU), PAT continues to be found at levels exceeding the maximum regulatory limits. The aim of this study was to examine the stability of PAT in artificially contaminated cloudy apple juice (CAJ) produced using a VaculIQ 1000 machine, an innovative technology allowing degassing and pressing under low oxygen conditions. In the experiment performed on a semi-industrial scale, the effect of adding 1.1 g ascorbic acid (AA)/kg apples and of degassing during the production on the PAT concentration was studied as well as possible degradation and reaction products formed. Furthermore, the physicochemical quality of the final juice was investigated as well. The highest degradation of PAT (50%) was observed for flash pasteurized juice, degassed and packed in γ-radiated bags, in the presence of AA after 14 days of storage at 20°C in the dark. Juices produced showed no significant differences in the quality parameters measured (pH, brix, colour, moisture content). This work demonstrates that the addition of AA to PAT contaminated CAJ, on a semi-industrial scale, decreased the PAT concentration without affecting the qualitative parameters of the juice.

Keywords: ascorbic acid, patulin, cloudy apple juice, decontamination, semi-industrial scale

Introduction

In recent years, consumers demand for high quality and minimally processed natural products, resulted in an increased production and consumption of cloudy apple juice (CAJ) (Beveridge 2002; Baron et al. 2006). CAJ is considered as a more valuable source of fibre, natural antioxidants and bioactive substances (such as polyphenols) in comparison to clear juice (Gui et al. 2006; Hubert et al. 2007; Oszmiański et al. 2007; Will et al. 2008; Markowski et al. 2012; Tetik et al. 2013; Teleszko et al. 2016).

However, the possible presence of patulin (PAT), a mycotoxin mainly present in rotten/moulded apples and apple-based products, implies a food safety issue for apple-based products. In fact, apple juice represents the largest source of PAT-intake by humans (Moake et al. 2005). In 2001 in Belgium, 79% of Belgian apple juices and 86% French/German/Swiss imported apple juices were contaminated by PAT with an average contamination ranging between 2.5 and 38.8 µg/kg (µg/kg) Tangni et al. (2003). PAT has a broad toxicity spectrum in animals; it was proven to be carcinogenic, genotoxic, teratogenic and mutagenic (Dickens and Jones 1961; Mayer and Legator 1969; Ciegler 1977; Taniwaki et al. 1992; Alves et al. 2000; Liu et al. 2003). Even though the European Commission (EC, 2006) has started to monitor the presence of this toxin in apple products and protect public health by setting maximum levels of 50 µg/kg in apple juice, 25 µg/kg for apple purees and compotes and 10 µg/kg for food intended for babies and young children, PAT continued to be found in food products present on the market (Ioi et al. 2017; Tannous et al. 2017; El Hajj Assaf et al. **submitted review**). Control strategies like good agricultural practices (GAP) and good manufacturing practices (GMP) are therefore necessary to minimize the risk of contamination of final products by PAT. Biological, chemical and physical treatments have shown to particularly affect this toxin and its corresponding concentration (Ioi et al. 2017; Babaali et al. 2017; El Hajj Assaf et al. **submitted review**).

Biologically based methods using inactive yeast for example were able to reduce or even eliminate PAT in food products without affecting the quality characteristics (Yue et al. 2011), but their use is limited to fermented products. Some of the physically based methods appeared to be very effective for PAT such as charcoal (Sands et al. 1976), but their use was limited because of their strong impact on the environment and negative effects on the organoleptic and physicochemical properties of the juice (colour, pH and brix) (Kadakal and Nas 2002). The use of chemical agents for mycotoxin elimination as such is not allowed. Ascorbic acid (AA), commonly known as vitamin C, is already used in the food industry for different purposes. It is an essential nutrient for human beings and a natural

constituent of apples. AA is present in a range of 3 to 30 mg per 100 g of fresh fruit depending, among other parameters, on the apple variety and the storage conditions (Rucker et al. 1998). It has antioxidant properties and prevents browning reactions mainly due to the action of polyphenoloxidase (PPO) on polyphenols producing quinones, responsible for the developed brown colour (Sapers et al. 1989; Stadtman 1991; Rojas-Graü et al. 2006; Ali et al. 2015). In addition, on a laboratory scale AA was shown to increase the degradation of PAT whether in buffer solutions, in juice-like aqueous model systems or in clear and cloudy apple juices (Brackett and Marth 1979; Fremy et al. 1995; Canas and Aranda 1996; Alves et al. 2000; Drusch et al. 2007; El Hajj Assaf et al. **submitted article**). Only limited studies elucidated the breakdown products or the reaction products from the degradation of PAT when interacting with AA. In our previous work (El Hajj Assaf et al. 2018, **submitted article**), we defined optimal conditions for action of AA on PAT in CAJ on a laboratory scale. In addition, we studied the reaction products (dehydroascorbic acid (DHA) and 5-hydroxymethylfurfural (HMF)), and the degradation products (Z-ascladiol (ASC-Z), E-ascladiol (ASC-E) and desoxypatulinic acid (DPA)) generated when AA interacts with PAT, both in an acidified aqueous solution and in CAJ. The analysis showed that 60% of PAT reduction was obtained for CAJ contaminated with 100 µg/kg of PAT, when adding 0.25% (w/v) AA and incubating the samples for 6 days at 22°C, in the dark and in presence of oxygen. It also showed that the degradation products formed were ASC-Z and a compound sharing the same retention time with DPA in CAJ and in acidified aqueous solution, respectively.

The current study was performed on a semi-industrial scale with the aim to evaluate the effect of the antioxidant AA on the PAT concentration during CAJ processing and storage. At first, artificially contaminated CAJs were produced under different conditions, using a novel spiral-filter press technology avoiding juice oxidation (Siewert et al. 2013; De Paepe et al. 2015a). Flash pasteurized juices were packed in γ-radiated bags and stored at 20°C in the dark. In addition to the effect on PAT, the effect of AA on the qualitative parameters (brix, pH, colour, moisture concentration) of the juice was assessed. Furthermore, the reaction (DHA, HMF) and degradation (ASC-E/Z and DPA) products generated were determined qualitatively as well.

Material and methods

Chemicals and reagents

Standards of PAT (≥ 98% purity), 5-hydroxymethylfurfural (HMF, ≥99% purity), and DHA were purchased from Sigma-Aldrich (St. Louis, MO, France). DPA standard was provided by Prof. Raffaello

Castoria (Department of Agricultural, Environmental and Food Sciences, University of Molise, Italy) and Prof. Rosa María Durán Patrón (Department of Organic Chemistry, University of Cádiz, Spain), and ASC-E/Z standards were provided by Dr. Olivier Puel (Toxalim laboratory, INRA Toulouse, France) respectively. AA was obtained from Solina group (Eke-Nazareth, Belgium). Ethyl acetate (AcOEt, Pesti-S), hexane (Pesti-S), acetonitrile (ACN, LC-grade) and acetic acid (AcA, 99.99%) solvents used for metabolite extraction and ultra-high-performance liquid chromatography (UHPLC) analysis were purchased from Biosolve BV (Valkenswaard, The Netherlands). HPLC-grade water generated by a Milli-Q-grade purification system (Millipore, Darmstadt, Germany) was adjusted to pH 4.0 using acetic acid.

PAT-contaminated apple mash production

The wild type (WT) strain of *Penicillium expansum* NRRL 35695 used in this study was provided by Dr. Olivier Puel (Toxalim lab, INRA, France). It was used to produce PAT-contaminated starting material to artificially contaminate apple juice. After its culturing for 5 days at 25°C in the dark on a Potato Dextrose Agar (PDA) medium (Biokar, Allonne, France), a spore suspension with a concentration of 100 conidia/ml of this *P. expansum* strain was prepared, as previously described by Adjovi *et al.* (2014) and using a Malassez cell. Golden Delicious apples free of any defect were surface-sterilized in a 2% sodium hypochlorite solution and then rinsed with water, as described previously by Sanzani et al. (2012). Apples were then wounded with a sterile toothpick, inoculated by 10 µl of the spore suspension and incubated at 25°C in the dark for 14 days. After the incubation period, the contaminated apples were heat treated at 88°C for 2 min to inactivate the mould and were grounded to obtain a homogenized apple puree, as previously described by MacDonald et al. (2000). PAT was quantified in this contaminated apple mash (n=5) using the protocol of De Clercq et al. (2016). Contaminated apple mash was stored at -20°C.

Experimental setup and pilot scale production of cloudy apple juice

Apple fruits belonging to the Golden Delicious cultivar were purchased from the Belgian market and stored in a cold room (0°C) at normal atmosphere until use. Before CAJ production, apples were sorted manually and defected, injured or rotten ones were discarded, after which they were washed with high pressure cold water. Apples were pressed by means of a spiral-filter pressed machine (VaculIQ 1000, VaculIQ, Hamminkeln, Germany). This innovative technology allows to apply a degassing step that consists of pressing under low oxygen levels. The CAJ was prepared as described

by De Paepe et al. (2015a). Immediately after preparation, the juices were flash pasteurized for 10 seconds at 85°C and aliquots of 0.7L of juice were packed in γ-radiated bags of 2L. Packed juices were stored at 20°C for 14 days in the dark.

Conditions tested	PAT concentration in CAJ (µg/kg)	AA in g / kg of apples	Degassing step
Blank	-	-	+
Cond 1	100	-	+
Cond 2	100	1.1	+
Cond 3	100	1.1	-

Table 1 Specification of the four conditions of cloudy apple juice (CAJ) tested. Indication of the final patulin (PAT) concentration in the juices and the quantity of ascorbic acid (AA) added (in grams) per kilogram of apples. +: applied; -: not applied (degassing) or added (PAT, AA)

Four conditions of CAJ were considered **(Table 1)**. The first one (blank) concerns a control without addition of PAT or AA. Its preparation followed the normal procedure described above with application of a degassing step. For the other three conditions, PAT-contaminated starting material was added to the vessel containing the clean mashed apples in a way to theoretically obtain 100 µg/kg in the final product. In condition 1 (cond 1) and 2 (cond 2), the juice was also pressed under low oxygen conditions (i.e. a degassing step was applied). In order to obtain about 0.3% AA in the final products, as in industrial settings, 1.1 g AA/kg apples was added at the same time as PAT in cond 2. The juice of condition 3 (cond 3) was obtained under same conditions as cond 2, but without applying the degassing step. All four apple juices were produced starting from the same batch of apples and on the same day to avoid inter-batch and inter-day variations.

Sampling

The first one (S1) consisted of sampling from the apple mash vessel after adding PAT-contaminated starting material and/or AA. The apple mash corresponds to the mix of juice, pulp, seeds and peel, etc. The second step (S2) consisted of sampling from the apple pomace obtained after pressing the mash and consists on the dry residue from apples. The third sampling step (S3) consisted of sampling just before pasteurization and the last sampling step (S4) concerns the pasteurized and packed apple juice. At each sampling step and for every test, three samples were taken and pooled. Brix, pH, colour and AA assessments were performed only for the samples of S4 on day 0. Metabolite extraction and moisture content measurement were performed for the samples taken from the different sampling steps of day 0. Juice samples after 3, 6 and 14 days of storage at 20°C were also analysed. Samples for analysing metabolites were stored for maximum four weeks at -20°C before extraction.

Analysis of juice quality parameters

Total soluble solids (TSS) content (°Brix) was measured using a digital refractometry (RM 40). The pH was measured using a digital potentiometry (SevenCompact, Mettler Toledo, Greifensee, Switzerland). Moisture concentration (MC) of apple mash, pomace and cloudy juice was determined by means of a halogen moisture analyser (HB43-S, Mettler Toledo, Schwerzenbach, Switzerland). A UV-Vis spectrophotometer (Sensing Unveils CM-5, Konica Minolta Sensing, Osaka, Japan) in reflectance (topport) served to measure the CIELAB (1976) colour space coordinates L*, a*, and b* in 40 ml of the CAJ produced. L* ranging from 0 to 100 corresponds to the luminance component (L* = 100 for perfect white); a* and b* values varying from −120 to +120 are colour coordinates that are related to the red/green and yellow/blue spectral ranges respectively. C* represents the chroma or colour purity and was calculated as follows: $C^* = (a^{*2} + b^{*2})^{1/2}$; ΔC* referred to the chroma difference between the blank and each of the conditions. ΔE* represents the extent of colour difference between two samples and was calculated using the following equation: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. The AA concentration was determined using a high-performance liquid chromatography method (HPLC) (detection limit of 10 mg/kg). All juice quality parameters were performed in (technical) triplicate.

Extraction of PAT and other metabolites

Patulin and metabolites from mash/pomace and juices samples were extracted following a modified protocol of De Clercq et al. (2016) and El Hajj Assaf et al. (submitted article 2018). Patulin and metabolites were subsequently analysed by means of ultra-high-performance liquid chromatography (UHPLC) coupled to an UV detector (UHPLC-UV).

UHPLC-UV metabolite analysis

An ACQUITY UPLC® H-Class System equipped with a Waters® ACQUITY™ UPLC™ Tunable UV (TUV) detector and an Acquity UPLC® BEH C_{18} 1.7 µm (2.1x150mm) column set at 30°C, was used for metabolite detection at a wavelength of 276 nm. Eluent A was HPLC-grade water acidified with 0.1% acetic acid and eluent B was ACN. The column was equilibrated with a mixture of 100% solvent B and 0% solvent A. The flow rate applied was of 0.3 $mL.min^{-1}$. Elution conditions were as follows: a 2 min isocratic passage in solvent B from 0 to 2%, a 7 min gradient increase of solvent B from 2 to 5%, a 1 min gradient increase of solvent B from 5 to 100%, 100% solvent B for 6 min, a 1 min direct decrease to 0% solvent B and re-equilibration at 0% solvent B for 3 min. PAT was quantified using a matrix-matched standard curve; the formation of biodegradation products (ASC-E/Z; DPA) and reaction

products (DHA; HMF) was verified and compared qualitatively between samples based on the peak area obtained.

Data analysis

Statistical analysis of the data obtained was carried out using the Statistical Analysis System software (SAS, version 9.4, SAS institute Inc., Cary, NC, USA). A linear regression model was applied with PAT, DHA and HMF as dependent variables and day, condition and sampling step as independent ones. Appropriate two and three-way interactions were tested for and removed when non-significant (P>0.05). Post-hoc comparison was performed with a Tukey test.

Results and discussion

In our study, different variables were tested (day, condition and sampling step). Statistical analysis proved that the degradation of PAT and formation of other compounds were the result of interactions occurring between these variables.

Effect of AA on basic juice quality parameters

CAJs are complex matrices containing pulp particles dispersed in a serum constituted by pectins and proteins mostly, and dissolved colloidally in a solution of low-molecular weight compounds such as sugars, organic acids, etc. (Wucherpfennig et al. 1987; Mollov et al. 2006). For all four juices produced, data of basic analytical juice parameters were obtained **(Table 2)**.

The TSS parameter reflecting the dissolved solid content of the apple juice is a good estimation of the sugar concentration especially since sugars make up between 90 and 95% of it. Brix values varied between 12.91 ± 0.25 (cond 1) and 13.36 ± 0.14 (cond 3) but were not significantly different. The pH of the CAJs was not affected by the addition of AA. No significant differences between the pH of the four juices were found. These results indicate that the concentration of AA added did not affect these quality parameters of the juices. The values obtained ranged between 3.6 and 3.7. TSS and pH seem mainly determined by properties like maturity, storage type, and cultivar of the apple varieties used (Markowski et al. 2012).

Colour is an important quality attribute of CAJs. In the four types of juices produced, the a*, b* and L* values did not show any significant differences. A slight difference in colour could be observed visually between the juices from cond 2 and 3 and those of cond 1 and the blank, which was confirmed

by calculating the colour parameters. In fact, all the juices had a light green colour ($\Delta L^* > 0$ and $\Delta a^* > 0$). But the juice of cond 1 was more yellow ($\Delta b^* > 0$) compared to those of cond 2 and cond 3 ($\Delta b^* < 0$). This difference could be attributed to the addition of AA to the latter juices. The yellowish colour characterizes the freshness of the product (Will et al. 2008). Furthermore, based on the ΔC^* values, juice of cond 1 (0.54 ± 0.33) could be described as brighter than juices of cond 2 and 3 (-0.75 ± 0.17 and -0.78 ± 0.18). Based on the study of Mokrzycki and Tatol (2012), ΔE^* of each juice was calculated. These values were all below 2 for the juices of cond 1, 2 and 3 and presented no statistical significant differences. According to Mokrzycki and Tatol (2012), a ΔE^* below 2 indicates that only experienced observer can notice the difference. We could conclude that addition of AA did not seem to affect conditions colours but only slightly the brightness of the juice.

Addition of AA is a common safe and cheap approach for restricting enzymatic browning in CAJ (Komthong et al. 2007). The AA concentration of the blank and cond 1 juices, obtained from Golden Delicious cultivar to which no extra AA was added, were 49.40 ± 13.50 and 53.20 ± 12.30 mg/L, respectively. The AA concentration of the cond 2 and cond 3 juices, to which AA was added, were 1376.70 ± 225.00 and 1343.30 ± 281.10 mg/L, respectively. Fruit and vegetables have long been regarded as a key source of vitamin C in the daily diet (Cao et al. 1998; Chebrolu et al. 2012). The natural concentration of AA in CAJs is variable and rather low, i.e. 8.5 to 46.6 mg/100 ml (Hao et al. 2016). The variation is attributed to apple variety, sun exposure, climate, harvest, pre- and postharvest processing, storage conditions, addition of AA minimizing non-enzymatic browning, etc. (Kolniak-Ostek et al. 2013). Even though we added AA enough to obtain 0.3% in the final product, we ended up by having the half. This could be explained by the fact that vitamin C concentration may be reduced by processing and storage which is probably due to catalytic destruction by enzymes. The low concentration of AA naturally present in apple juices indicates addition of vitamin C is favourable. The moisture content values of each of the juices did not differ significantly. CAJs, apple mash and apple pomace had MC values around 86%, 84% and 70%, respectively. The high moisture content in the (semi-)industrial implementation of apple pomace processing is often between 70 and 80% and agrees with the findings of Dhillon et al. (2013) and Gassara et al. (2010).

	Cloudy apple juice			
	Blank	**Cond 1**	**Cond 2**	**Cond 3**
TSS (°Brix)	13.08 ± 0.18	12.91 ± 0.25	13.23 ± 0.25	13.36 ± 0.14
pH	3.69 ± 0.08	3.69 ± 0.11	3.64 ± 0.09	3.62 ± 0.04
MC apple mash	83.95 ± 0.57	84.34 ± 0.17	83.91 ± 0.35	83.89 ± 0.25
MC apple pomace	71.16 ± 0.46	71.25 ± 1.10	71.08 ± 0.21	74.08 ± 4.44
MC non-pasteurized apple juice	86.54 ± 0.22	86.89 ± 0.25	86.64 ± 0.31	86.62 ± 0.10
MC pasteurized apple juice	86.84 ± 0.21	87.01 ± 0.34	86.85 ± 0.25	86.67 ± 0.21
Ascorbic acid (mg/L; ppm)	49.40 ± 13.50	53.20 ± 12.25	1376.70 ± 225.00	1343.30 ± 281.10
ΔL*		1.06 ± 0.29	0.14 ± 0.06	0.12 ± 0.07
Δa*		0.10 ± 0.10	0.02 ± 0.05	0.00 ± 0.14
Δb*		0.55 ± 0.52	-0.97 ± 0.01	-0.78 ± 0.39
ΔC*		0.54 ± 0.33	-0.75 ± 0.17	-0.78 ± 0.18
ΔE*		1.23 ± 0.49	0.98 ± 0.00	0.80 ± 0.39

Table 2 Juice quality parameters of the four cloudy apple juices (CAJ) produced. Abbreviations: TSS: total soluble solids content; MC: moisture content; L*, luminance CIELAB (1976) colour system coordinate; a*, red/green CIELAB (1976) colour system coordinate; b*, yellow/blue CIELAB (1976) colour system coordinate; Δ, difference between value of condition 1/2 or 3 and blank; ΔL*, difference in lightness; Δa*, the difference in redness; Δb*, the difference in yellowness; ΔC* the difference in colour purity; ΔE* extent of colour difference

We could conclude that the addition of 1.1 g AA/kg apples to the CAJ process had no significant impact on the brix nor on the pH of the juice. The colour purity and brightness of the juice was slightly affected, and the addition of AA made the juice less yellow but, on the other hand, no significant colour difference was noticeable and the nutritional value of juices increased especially that apple juices are not considered as a big source of vitamin C.

Effect of AA on formation of reaction and PAT degradation products

During the production process of cloudy apply juices, with/without AA and/or PAT added to the apple mash, possible presence of reaction products such as DHA and HMF and biodegradation products such as DPA and ASC-E/Z were studied.

Effect of AA on PAT concentration

PAT was measured at all stages of production (from S1 till S4) and analysed till 14 days of storage at 20°C in the dark **(Figure 1; Figure 2)**. When comparing the PAT-concentration of the final juice samples from day 0 (S4), it was noticed that it is significantly higher in cond 1, where no extra AA was added, compared to cond 2 (P<0.0001) and cond 3 (P = 0.0002) where AA was added to the apple mash. In fact, the addition of AA (cond 2 and cond 3) caused a small but significant and direct decrease in the PAT concentration on day 0.

Significant differences in PAT concentrations were detected between all sampling steps when comparing the blank to any of the other conditions (1, 2 or 3) (P<0.0001). In the blank juice, PAT was not detected. This was expected because the apples used were sorted and of high quality without visual rot while in the three other conditions the non-contaminated starting material was artificially contaminated. In PAT-contaminated apple mash, PAT varied between 75.0 ± 0.5, 59.6 ± 2.4 and 56.4 ± 17.9 µg/kg for cond 1, 2 and 3, respectively. In pomace low concentrations of PAT ranging between 7.7 ± 0.3 and 9.8 ± 0.1 µg/kg were found, due to the water-soluble character of PAT (Ciegler 1977). The PAT concentration at S4 were of 94.7 ± 9.1, 66.2 ± 1.0 and 72.6 ± 0.5 µg/kg compared to 73.3 ± 0.3, 39.2 ± 0.1 and 55.4 ± 0.5 at S3 for juices from cond 1, cond 2 and cond 3, respectively. Although the PAT concentration in the pasteurized apple juice samples (S4) were slightly higher than the ones from S3 (non-pasteurized apple juices), this was not found to be significantly different.

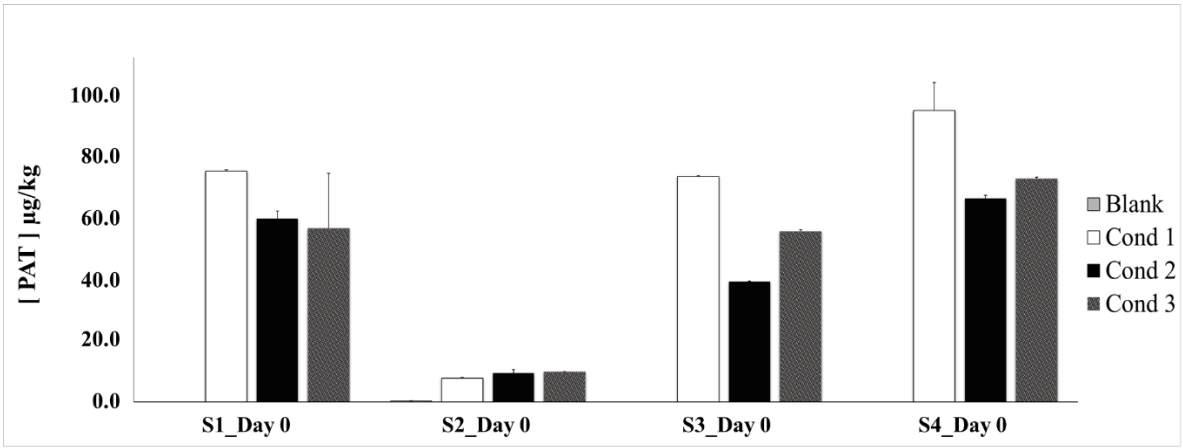

Figure 1 Monitoring of patulin (PAT) concentration in samples taken at different sampling steps along the cloudy apple juice (CAJ) production process, for four conditions considered. S1 for apple mash samples; S2 for apple pomace samples; S3 for non-pasteurized cloudy apple juice samples; S4 for pasteurized CAJ samples from day 0. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

This slight but non-significant increase in PAT concentration could be due to the flash pasteurization process (85°C for 10 seconds) applied. These results contrast with some data already present in the literature (Ioi et al. 2017) describing a PAT degradation during heat treatment of liquid food products. However, the matrix used, processing temperature and time applied were different than the one used in this study. PAT is reduced by 13.4% and 19% at 90°C for 10 seconds in apple juice and apple cider, respectively (Kadakal et al. 2002; Wheeler et al. 1987). The flash pasteurization applied in our study could probably have contributed to the liberation of PAT molecules from solids present in the artificially contaminated CAJ.

The PAT concentration was also monitored during day 0, 3, 6 and 14 of storage of the juices at 20°C in the dark **(Figure 2)**. In fact, in cond 1, where no extra AA was added, 20% of PAT decrease occurred within the 14 days of storage (going from 94.7 ± 9.1 on day 0 to 75.6 ± 3.2 µg/kg on day 14) and was considered as significant between the different days of incubation ($0.0001 \leq P < 0.05$). This 20% decrease could be due to the naturally present (low) concentration of AA in the CAJ. As for cond 2 and 3, where AA was exogenously added to the apple mash during production, a higher PAT decrease occurred. A reduction of 48% and 34% was observed in the juices from cond 2 and cond 3, respectively, after 14 days of incubation. The PAT content of apple juice to which AA was added decreased by 70% compared to 30% in an aqueous juice-like model system without added AA after 34 days of storage (temperature was not indicated) (Drusch et al. 2007). In fact, the PAT disappearance was not significant between day 3 and day 6 but increased significantly between day 6 and day 14 ($P = 0.0032$ and $P = 0.008$ for cond 2 and 3, respectively). The spiral-filter press technology producing CAJ avoids oxidative degradation and that by applying the degassing step and extracting the juice in a low-oxygen extraction cell under vacuum (De Paepe et al. 2015a, 2015b). The final concentration of PAT in the CAJs bags of cond 2 and cond 3 where of 34.4 ± 3.5 and 47.3 ± 2.4 µg/kg. Even though cond 3 consisted of not applying the degassing step when pressing the juice, the highest decrease in PAT concentration was not observed in this condition. Instead, when 1.1 g of AA/ kg of apples was added to the apple mash during CAJ processing and with the presence of low concentration of oxygen (cond2), the PAT degradation was the highest. Statistical analysis indeed showed a significant difference in the PAT concentration on day 14 between cond 1 and each of cond 2 and 3 ($P < 0.0001$) but did not show any significant difference in the PAT concentration between cond 2 and 3 on the same day ($P = 0.0126$). Thus, suggesting that the quantity of oxygen present when pressing the juice did not really influence the PAT degradation rate in a very significant way.

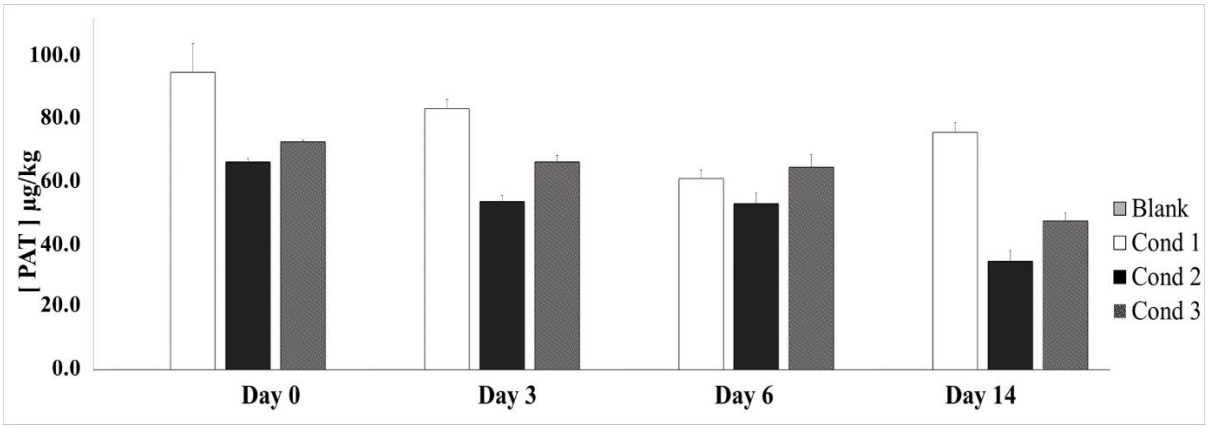

Figure 2 Monitoring of patulin (PAT) concentration in γ-radiated bags containing cloudy apple juices (CAJ) produced under four processing conditions, within 14 days of storage at 20°C in the dark. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Many mechanisms have been described to clarify the process by which AA impacts the PAT concentration. The electrophilic properties of PAT would made it suitable for nucleophilic attack (e.g. AA acting as nucleophile increased PAT degradation). Another hypothesis is that oxidative degradation of AA leads to the formation of reactive free radicals attacking the lactone structure of PAT and making it unstable (Ciegler et al. 1976; Fliege and Metzier 2000a, 2000b; Kokkinidou et al. 2014). AA or more correctly DHA can degrade PAT in the presence of oxygen and ferric ions (Brackett and Marth 1979; Drusch et al. 2007; Hao et al. 2016; El Hajj Assaf et al. **submitted article**). In our study, cond 2 and 3 were found to decrease the PAT concentration in CAJ, with cond 2 leading to an even lower concentration of PAT. The incubation temperature had a significant impact on AA action and PAT degradation as well. In fact, the temperature of storage chosen in this study was based on previous studies showing that the activity of AA against PAT seems to be highly temperature-dependent and is low or negligible at refrigerated storage. The highest degradation of PAT was obtained at an incubation temperature that is around 20°C representing the most relevant industrial storage temperature of apple juices (Amparo et al. 2012; Tikekar et al. 2014; El Hajj Assaf et al. **submitted article**). Important to keep in mind is the fact that in our experiments, CAJ was used and not clear juice. Other studies have shown that PAT could be destroyed more easily in clear fruit juice than cloudy ones due to suspended particles and phenolic compounds in cloudy juices that interact with PAT molecules preventing the contact between PAT molecules and UV waves for example and thus their detection (Tikekar et al. 2014). A similar phenomenon could occur in our case slowing

down the contact between AA or rather the free radicals from the oxidation of AA with PAT molecules.

Formation of degradation products

The decrease in PAT concentration overlaps with the formation of degradation products. However, in our experiment performed on a semi-industrial scale and considering up till day 14 of storage at 20°C in the dark, it was not possible to make solid conclusions regarding this. In fact, ASC-E, ASC-Z and DPA could not be detected in the juices on day 0 but did appear in the samples of cond 1, 2 and 3 after 3, 6 or 14 days of incubation (data not shown). Till day 14, no statistically significant increase of these metabolites was however observed. ASC-E, oxidized to PAT in a one-step enzymatic reaction in PAT biosynthesis (Sekiguchi et al. 1983) was supposed to make a reverse enzymatic reaction leading to the formation of ASC-E from PAT. In our previous work on a lab scale experiment, the conversion of PAT to ASC-Z was observed after 6 day of incubation at 22°C in case AA was added to PAT-contaminated CAJ (El Hajj Assaf et al. **submitted article**). This same study also showed that DPA, could be another degradation product. DPA could be formed through the hydrolysis of the α,β-unsaturated γ-lactone ring and subsequent modifying reaction involving more than a single enzymatic step (Ianiri et al. 2013; unpublished data Duran-Patron). The differences between the results observed on lab scale and semi-industrial scale emphasises the need for validation on (semi-)industrial level where the process and products are more complex.

Formation of reaction products

In addition to the extraction of possible PAT metabolites from the juices stored in γ-radiated bags for 0, 3, 6 and 14 days, we managed to identify the presence of DHA resulting from the oxidation of AA. Its presence was compared relatively to each other. We noticed that after storage of the juice bags for 6 days at 20°C in the dark, the DHA peaks on day 0, 3 and 6 were slightly detectable in the four conditions tested and no significant difference between conditions and day of storage was detected **(Figure 3)**. Between day 6 and 14, DHA started to be formed most explicitly in juices of cond 2 and cond 3 and the corresponding peaks were 5 and 3 times higher on day 14 than on day 6, respectively. In fact, the biggest formation of DHA and degradation of PAT occurred on the same time, between day 6 and 14. A significant difference in the DHA quantity was observed between day 6 and 14 in cond 2 and between cond 2 and all other conditions on these same days as well ($P < 0.0001$). In fact, for the juices obtained in cond 2 with a degassing step, the formation of the highest amount of DHA

could be observed. The peak was 8 time higher than the one measured in cond 3 ($P < 0.0001$). In the juices from the blank and cond 1, where no extra AA was added to the apple mash, only a minor non-significant peak corresponding to DHA was detected all along the 14 days of storage. This could be due to the low initial concentration of AA in the juice.

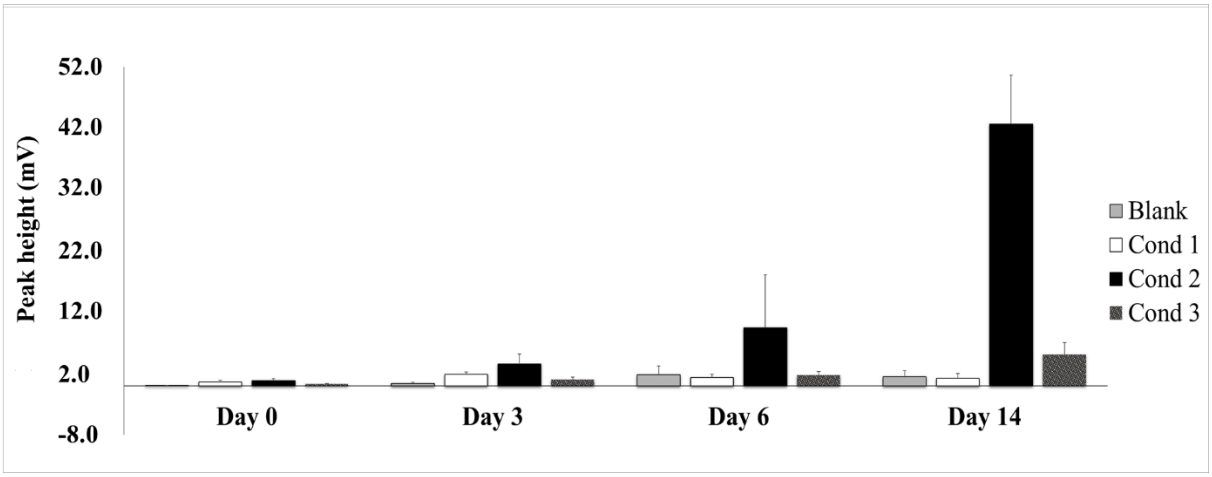

Figure 3 Dehydroascorbic acid (DHA) formation in γ-radiated bags containing cloudy apple juices (CAJ) produced under four processing conditions, within 14 days of storage at 20°C in the dark. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Studies have shown that AA gradually decreases during storage, especially at temperatures above 0°C (Ajibola et al. 2009). Kevers et al. (2011) measured a decrease of 75% of AA after 7 weeks of incubation of fresh apples at 20°C. In our earliest findings (El Hajj Assaf et al. **submitted article**), at low temperatures (4°C), AA decreased less than at higher temperature (22°C). Based on these studies and on the fact that 20°C correspond to the shelf storage temperature, in the current study, this latter temperature was the one applied during storage period. AA catabolism is increased in stressful conditions storage and its rate of decrease is accelerated (cond 2 and 3). The percentage of oxygen needed to oxidize AA and generate DHA is definitely related to the initial concentration of AA in the juice. In our previous study, in complete anaerobic conditions, no oxidation of AA occurred but in presence of oxygen and 4% or 0.25% of AA, DHA was formed and the PAT concentration was reduced (El Hajj Assaf et al. **submitted article**). In our conditions tested, the necessity of oxygen was confirmed but a low concentration appeared to be enough and induced the highest formation of DHA (cond 2) and by that the PAT degradation **(Figure 2; Figure 3)**.

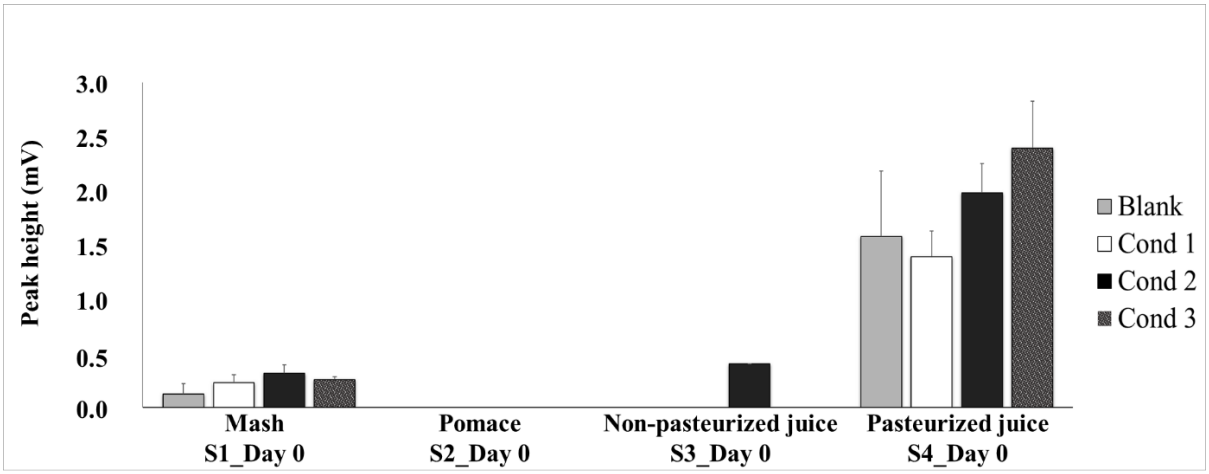

Figure 4 5-hydroxymethylfurfural (HMF) formation in samples taken from different sampling steps all along the cloudy apple juice (CAJ) process, for four conditions tested. S1 for apple mash samples; S2 for apple pomace samples; S3 for non-pasteurized CAJ samples; S4 for pasteurized CAJ samples from day 0. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Another reaction product formed was HMF. Its formation was independent of the condition tested but was more related to the sampling step. As we can see in **Figure 4**, HMF was almost absent in the sampling steps S1, S2 and S3 and was formed when the juice was flash pasteurized (S4). This was expected since HMF is absent in fresh food and present in heated products (Babsky et al. 1986; Matić et al. 2009). HMF is formed from sugars during thermal processing and is a characteristic flavour compound formed during the Maillard reaction (Lee and Nagy 1988; Ramirez-Jimenez et al. 2000; Rada-Mendoza et al. 2002).

The concentration of HMF increases not only during heating but also during storage processes (Lansalot-Matras and Moreau 2003; Karatas and Akkaya 2017). It is used as an indicator of apple juice quality, since its presence is considered as an indication of quality deterioration and the low pH of apple juices seems to be a characteristic of the formation of this compound (Matić et al. 2009). HMF formation increased significantly (3 to 5 times) within the 14 days of storage ($P < 0.0001$) **(Figure 5)** in the juice of pH 3.6.

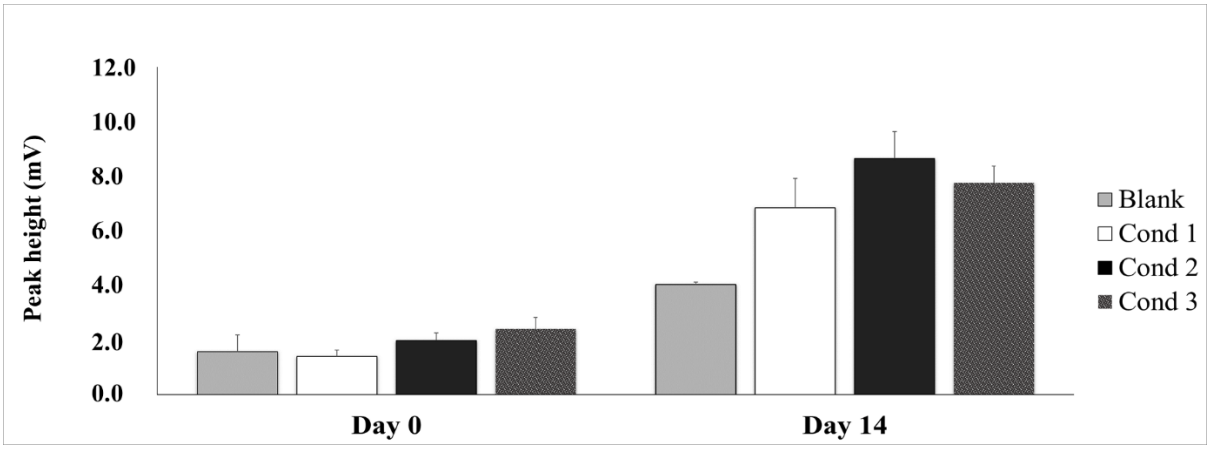

Figure 5 5-hydroxymethylfurfural (HMF) formation containing cloudy apple juices (CAJ) produced under four processing conditions, within 14 days of storage in γ-radiated bags at 20°C in the dark. Graphs show the mean ± standard error of the mean (SEM) from three replicates.

Conclusion

Even though chemical detoxification of mycotoxin-contaminated matrices intended for commodities for human food, such as PAT in apple juices is not allowed within EU, the addition of AA to low PAT-contaminated cloudy apple juice in presence of oxygen decreased PAT concentration by half after 14 days of storage at 20°C. When applying a degassing step with the VacUliq, a more pronounced decrease in PAT concentration was observed then when pressing apple juices in presence of high concentrations of oxygen. Differences observed when working on CAJ on a lab scale and (semi-)industrial scale could be related to production process that is more complex for the latter. Other factors such as storage temperature and days of storage also affected PAT decrease. Packing material used could also influence PAT concentration in CAJ. These studies show promising results but more investigation on (semi-)industrial scale is needed for CAJ.

Acknowledgments

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General discussion

General discussion and perspectives

Mycotoxins are compounds resulting from the secondary metabolism of moulds and present toxic potential regarding humans and animals. Fungal toxins are found as natural contaminants in many foods and feeds such as grains, fruits, vegetables, and dairy products. The mycotoxins most studied from a food and health point of view are aflatoxins, fumonisins, ochratoxins, ergot alkaloids, zearalenone, patulin (PAT) and trichothecenes. These toxins are mainly produced by five genera of fungi: *Fusarium*, *Aspergillus*, *Claviceps*, *Alternaria* and *Penicillium*.

The health and economic consequences of mycotoxin-contaminated food have prompted many researchers to try to clarify and understand the molecular mechanisms of mycotoxin production by filamentous fungi. Indeed, the production of secondary metabolites by moulds can be very variable and depends on several biotic and abiotic parameters. The utmost understanding of these mechanisms is needed to develop relevant preventive and/or control strategies against these contaminants and their producers.

Among the mycotoxins of high concern cited above, PAT (mainly produced by *P. expansum*) contaminates apples in the field and during storage. Its high stability allows it to be found in products resulting from the transformation of contaminated raw material. Biochemical and molecular studies conducted over the years have resulted in the identification of the PAT biosynthesis pathway steps as well as the identification of the complete gene cluster in *P. expansum* (Tannous et al. 2014). Once elucidated, researchers characterized genes implicated in the PAT biosynthesis pathway as well as other genes encoding global regulators (Snini et al. 2015; Kumar et al. 2017).

In this thesis, we wanted to address the problem of contamination of apples and processed products by PAT from a fundamental point of view and also through applied research on the fate of PAT in the presence of ascorbic acid (AA). Thus, we focused in the first part on the characterization of the *veA* gene encoding a global transcription factor located outside the PAT biosynthesis gene cluster in *P. expansum*. We gathered more knowledge about virulence and secondary metabolism (with a special emphasis on PAT and citrinin (CIT) mycotoxin biosynthesis pathways) of the fungus and its regulation when the gene encoding this global transcription factor is disrupted. The impact of different sugar sources on the development of *P. expansum* and PAT production was assessed as well. In the second part, we focused on the fate of PAT in cloudy apple juice (CAJ) in presence of AA.

This discussion is essentially based on assumptions or hypotheses made and formulated on the basis of the work presented in this manuscript and in relation to literature data. Some of the topics already addressed in previous chapters will not be included in this section.

How does the *veA* gene affect the biosynthesis pathways of PAT and CIT?

Currently, few studies describe an interconnection between two mycotoxin biosynthetic pathways. Schmidt-Heydt et al. (2012b) have shown that *P. verrucosum* is able to direct its secondary metabolism towards the production of a mycotoxin at the expense of a second, depending on the culture conditions tested. A medium with a high concentration of NaCl promotes ochratoxin A (OTA) production at the expense of CIT production. However, in presence of oxidative stress (medium rich in Cu^{2+} or fungus exposed to blue light), *P. verrucosum* directs its secondary metabolism towards the production of CIT (Schmidt-Heydt et al. 2011, 2014; Stoll et al. 2015). This interconnection is explained by the fact that these two mycotoxins are polyketides sharing the same precursor, acetyl-CoA (Larsen et al. 2001). The production of one of them in very large quantities under specific conditions depletes the cell stock of acetyl-CoA, thus limiting the production of the other one.

Hidalgo et al. (2014) have described the interconnection between the biosynthetic pathways of mycophenolic acid and PR-toxin in *P. roqueforti*. They have shown that mutated strains of *P. roqueforti*, unable to synthesize PR-toxin, produce mycophenolic acid in large quantities. The fact that mycophenolic acid and PR-toxin belong to different biosynthetic groups (polyketides (Regueira et al. 2011) and sesquiterpenes (Wei et al. 1975), respectively) suggest that the pathways of these mycotoxins are probably not related. However, the authors hypothesized that the biosynthetic pathway of PR-toxin regulates the biosynthetic pathway of mycophenolic acid by using the five-carbon isopentenyl unit essential for their biosynthesis.

In *P. expansum*, cultures of the mutant *veA* strain did not contain the mycotoxins PAT and CIT. On contrast, the wild type strain produced PAT and favoured its biosynthesis in a very large quantity at the expense of CIT, produced in smaller amounts on the culture media tested (Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA)). It is possible that the synthesis of PAT may be inhibited once the medium becomes very toxic and this is when the synthesis of CIT is triggered (Touhami et al. 2018).

The PAT concentration is six to seven times higher than that of CIT on MEA and fifty times higher on PDA. Similar observations have been made by Snini et al. (2015) on an apple based medium (APAM). CIT and PAT, which share the same precursor, acetyl-CoA, can most likely have interconnected

biosynthetic pathways. In fact, since PAT is produced in very large amounts in the wild strain, the acetyl-CoA cell stock can be decreased at the expense of CIT production (Larsen et al 2001). After comparing the level of expression of the genes encoding each of the polyketide synthases, *patK* and *citS*, involved in the first step of each of the biosynthetic pathways of PAT and CIT, respectively, in the wild type and *veA* null mutant strains, no difference of expression was measured (see Chapter 1_Part 1: Figure 7 and Figure S9). This could be explained by a higher enzymatic efficiency of 6-methylsalicylic acid synthase (first step in PAT biosynthesis pathway **(Introduction - Figure11)**) compared to the polyketide synthase involved in the CIT biosynthetic pathway.

The global regulation factor VeA positively or negatively controls a large number of secondary metabolite pathways in fungi such as fumigaclavine C, fumagillin and gliotoxin in *Aspergillus fumigatus* (Dhingra et al. 2013), fumonisins and fusarins in *Fusarium verticillioides* (Myung et al. 2012), trichothecenes in *F. graminearum* (Merhej et al. 2012), gibberellins, fumonisins and fusarin C in *F. fujikuroi* (Wiemann et al. 2010), penicillin in *P. chrysogenum* (Kopke et al. 2013) and compactin in *P. citrinum* (Baba et al. 2012). Here, we elucidated its role in PAT production by *P. expansum*. Mutation of the *veA* gene in the wild type strain of *P. expansum* positively regulates, among other metabolites, the biosynthesis of PAT and CIT. It leads to the extinction or strong decrease of the expression of a majority of the genes belonging to the biosynthetic clusters of PAT and CIT **(Introduction - Figure 6)**. The expression of the *patA* gene encoding an acetate transporter was reduced in the mutant strain on the culture media tested. The absence of this carrier was accompanied by a high decrease in the expression of the *patK* gene responsible for the transformation of acetyl-CoA into 6-methylsalicylic acid. The latter may justify the absence of PAT in the medium and lead us to hypothesize that the transport of acetate for PAT biosynthesis in the peroxisome did not occur from the start **(Figure 1)**.

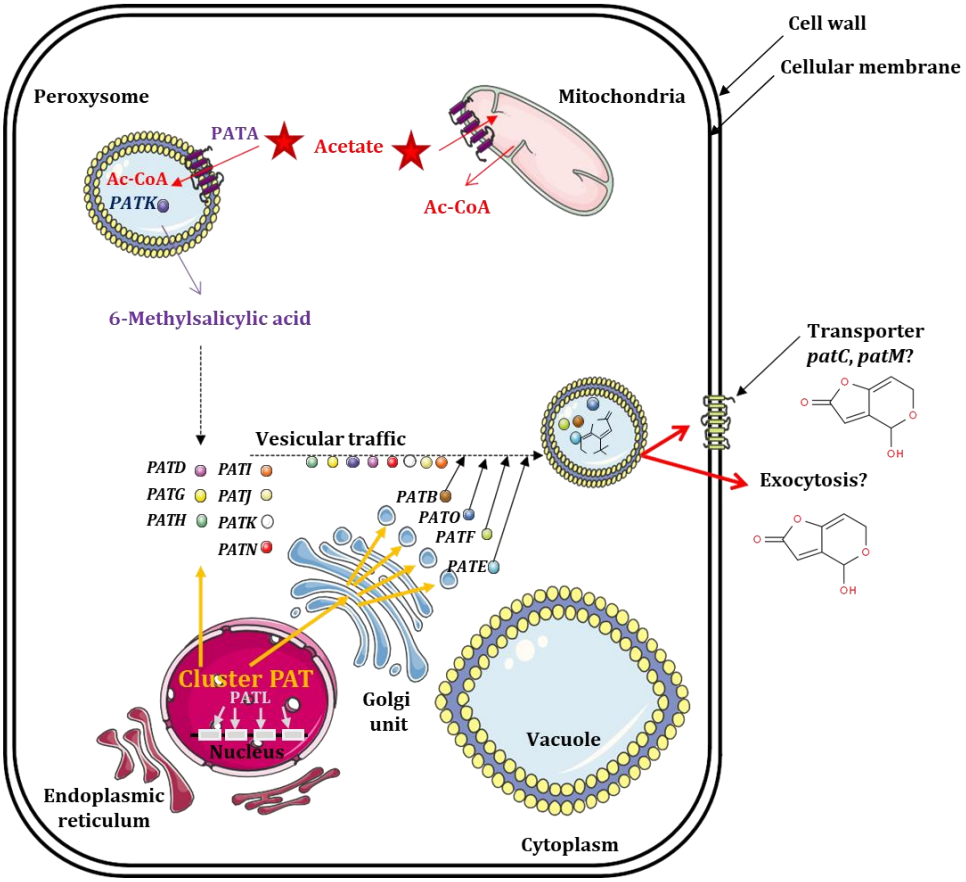

Figure 1 Hypothetical diagram of the spatial organization of the patulin biosynthesis pathway within the fungal cell (adapted from Snini 2014).

The acetate present in the cell is taken over by the acetate carrier PATA present in the peroxisome membrane and then transformed into acetyl-CoA inside. 6-Methylsalicylic acid synthase supports acetyl-CoA to synthesize 6-methylsalicylic acid which is then released into the cytoplasm. It is supported by the other enzymes of the patulin biosynthesis pathway. Enzymes without signal peptides are certainly localized in the cytoplasm and are active in it. The last steps in patulin synthesis appear to be performed in vesicles and by enzymes that present a signal peptide (PATB, PATO, PATF and PATE). Patulin is then excreted in the external environment.

In such case, we think that the stocks of acetate may remain available for CIT biosynthesis in the cytoplasm except that CIT was also absent in the media where the *veA* mutated strain was grown **(Figure 2)**. This makes us believe that the biosynthesis of another secondary metabolite, belonging to the same family of polyketides, is maybe interconnected with those of PAT and CIT and is down-regulated by *veA*. The disruption of *veA* gene induced the production of five other genes encoding polyketide synthases (Pexp_030540; Pexp_037250; Pexp_086670; Pexp_000410 and Pexp_094810).

This production could be at the expense of that of PAT and CIT. This was also confirmed by the HPLC peaks of certain secondary metabolites produced by the null mutant strain but not by the WT strain (see Chapter 1_Part 1).

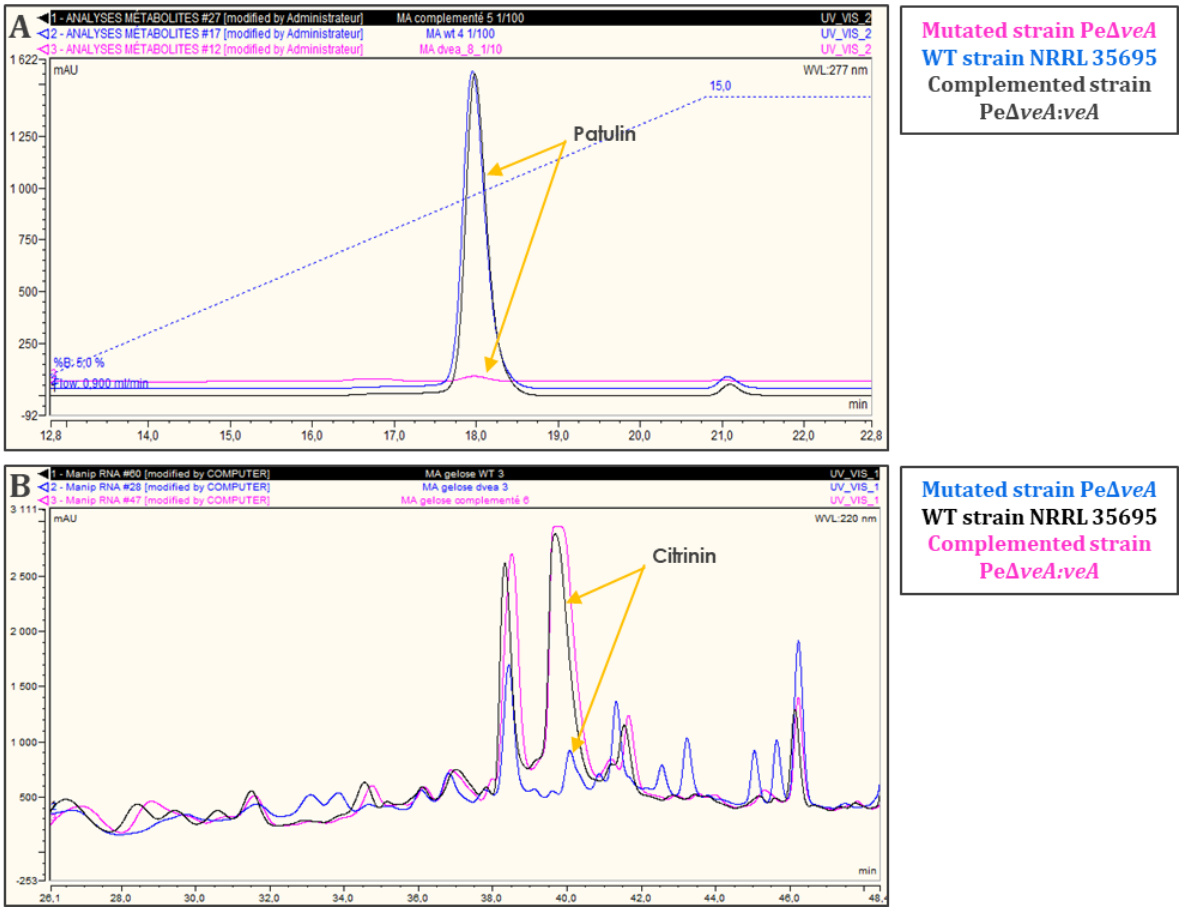

Figure 2 Chromatograms illustrating the production of patulin **(A)** and citrinin **(B)** in a wild type strain of *Penicillium expansum* (NRRL 35695), a null mutant (PeΔ*veA*) and complemented (PeΔ*veA*:*veA*) strains. Cultures were grown on malt extract agar (MEA) medium for 5 days at 25°C in the dark.

A further characterization of the genes down-regulated by *veA* should be established to study the possible interconnection with the PAT and CIT biosynthesis pathways. The corresponding metabolites should be identified. It is by identifying them and their biosynthetic pathways that their interconnection with other mycotoxins could be studied.

In addition, PAT and CIT have been reported to be also positively regulated by LaeA, another member of the trimeric Velvet complex in both *P. expansum* and *Monascus ruber*, respectively (Kumar et al. 2017; Liu et al. 2016). Characterizing the *velB* gene, the third core member belonging to the Velvet family, would be interesting. The evaluation of a Δ*velB* mutant and quantification of the expression

of PAT and CIT genes will strengthen the hypothesis that a functional Velvet complex is necessary for the production of these toxins.

VeA regulates the secondary metabolism

As mentioned in the section above, VeA regulates the secondary metabolism of many fungus such as *A. nidulans* (Calvo et al. 2008; Bayram et al. 2012; Rauscher et al. 2016), *A. flavus* (Cary et al. 2015), etc. Our results shed the light on its role in *P. expansum*. Beside PAT and CIT, *P. expansum* is a consistent producer of many secondary metabolites. Therefore, PAT-negative samples do not always correspond to fungal metabolite-free sample (Andersen et al. 2004).

We noticed that different backbone genes were up-regulated by the *veA* gene like those of CIT and PAT while others were down-regulated. Many genes were regulated similarly by *veA* and *laeA* (Kumar et al. 2017; El Hajj Assaf et al. 2018) but one of them (Pexp_008740) was up-regulated by *veA* in our studies on the media tested and down-regulated by *laeA*. It would be interesting to focus on this backbone gene and investigate whether its expression is not regulated by the velvet complex. To do this, the study of the impact of *velB* on the expression of this gene (Pexp_008740) and the production of the corresponding metabolite must first be evaluated.

We also showed that VeA positively regulated some metabolites that were secreted by the basal mycelium in the media, such as PAT and CIT, and negatively other metabolites such as communesins. These last metabolites were produced in the aerial parts of the fungi during the so-called competence phase (passage to the asexual reproduction). These results suggest that the fungus does not release all toxins at the same time during its cycle. It starts by secreting metabolites such as PAT, probably toxic to other microorganisms or facilitating colonization of substrates, which allows its development. Then, it secretes other toxins for different purposes such as formation of aerial mycelium, surviving extrinsic and intrinsic factors in the environment, etc. It could also be interesting to perform some toxicity studies on these mycotoxins (e.g. expansolides, communesins, etc.) and compare them to the toxicity of PAT.

Link between *veA* gene and other regulators

An *in silico* analysis of the promoter regions of the patulin cluster genes in *P. expansum* revealed the presence of potential binding sites for known transcription factors such as AbaA, BrlA, PacC and NrfA (Tannous 2015). The regulatory factors AbaA and BrlA are both implicated in the sporulation. BrlA

controls the expression of AbaA, which itself controls the differentiation of phialides (Sewall et al. 1990).

In our experiments, we showed that the infection of an apple by *P. expansum* (or the null mutant strain) and its storage in light, inhibited the formation of coremia. However, in the dark, the disruption of *veA* allowed the fungus to sporulate *in vitro* but it was unable to form coremia, pierce the peel of the apple and get out to complete its life cycle, unlike what was observed with the wild type (WT) and complemented strains. Kim et al (2002) showed that *veA* is a negative regulator of asexual development in a light-dependent way in *A. nidulans*, and Rauscher et al. (2016) have suggested that VeA regulates the asexual transcription factor BrlA, whose gene is known for promoting the asexual program in *A. nidulans* (Bayram et al. 2014). The question that arises is the following: is BrlA essential for the formation of coremia and piercing the apple peel or is it VeA? The previous studies made us hypothesize that in *P. expansum* grown in the dark, the loss of *veA* affects the transcription of *brlA* negatively and thus prevents the fungus to complete its cycle and form coremia and synnemata essential for its dissemination. In order to answer these questions, a disruption of the *brlA* gene in a WT strain of *P. expansum* should be performed. This null mutant strain should be used to inoculate apples and store them under light and dark conditions. If in the dark, the fungus still can not form its coremia and pierce the apple peel, then we can conclude that BrlA is responsible of this phenomenon. In addition, it would also be interesting to produce a mutant strain of *P. expansum* whose *veA* and *brlA* genes are both disrupted. This could give us an idea of the regulation of *brlA* by *veA* as well.

In addition, *brlA* was also shown to act on biosynthetis pathways. Its deletion in *A. fumigatus* affects the production of trypacidine, fumiquinazoline C (Gauthier et al. 2012; Lim et al. 2014) and ergot alkaloids (Coyle et al. 2007). Twumasi-Boateng et al. (2009) also proved by a transcriptomic study that the cluster, encoding the genes required for the synthesis of the ergot alkaloid fumigaclavine in *A. fumigatus*, is dependent on BrlA for the expression of its genes. Another study revealed that the level of expression of 7 gene clusters related to secondary metabolism, including that involved in the synthesis of roquefortin C, was strongly regulated by BrlA in *Penicillium oxalicum* (Qin et al. 2013). On the other hand, in aflatoxin producing fungal species, BrlA is involved in the regulation of the *pksA* gene promoter, encoding the polyketide synthase. The latter is involved in the first step of the biosynthesis of this mycotoxin (Ehrlich et al. 2002). The disruption of *brlA* in a WT strain of *P. expansum* would also show enlarge our knowledge on the impact of the BrlA factor on the secondary metabolism in this fungus.

Fungal development and toxin production

An increase in the production of secondary metabolites in fungi has been attributed to the presence of glucose and other carbohydrates. Wu et al. (2016) have shown that fructose can reprogram the metabolome of a *P. citrinum* strain by enhancing the growth and antifungal activity of this organism at once. This exogenously added sugar affects fungal hyphae thickness, biomass production, gene and protein expression, enzyme secretion and production of secondary metabolites (Pessoni et al. 2015). In *P. chrysogenum*, glucose and sucrose largely regulated the biosynthesis of penicillin. Other sugars such as maltose, fructose and galactose also showed an impact, although more weakly (Revilla et al. 1984; Martin 2000).

Our preliminary results showed that the addition of different types of sugars, glucose, sucrose and fructose, to *in vitro* culture media favoured the fungal development not only of the WT strain but also of the *veA* mutant. Fructose contributed to the largest radial development of the fungi. Still, the development of the fungi (WT and null mutant strains) was not correlated to the secretion of PAT in the media. The WT strain developed better and produced more PAT on the media till a certain percentage of fructose added. The null mutant strain of *veA* was able to develop but almost no PAT was secreted in the media, regardless of the percentage of fructose added.

We can conclude that on different apple varieties, which contain different percentages of sugars, a null mutant strain could develop but no PAT production could occur. This strain could be evaluated as a potential biological agent competing with the WT strain producing the toxin. A better idea of the other toxins that the mutant could produce is still needed because the observance of disease incidence on apples is not related to the presence or absence of toxins. These results were also in agreement with those of other studies (Ballester et al. 2015; Li et al. 2015), which state that the infection of Golden delicious apples by *P. expansum* does not require the presence of PAT. The disruption of the *veA* gene decreased the severity of apple disease and was essential to obtain a less aggressive strain (Van der Plank 1965).

It is also important to keep in mind that most of these studies were conducted *in vitro*, on different media. It would also be interesting to evaluate the expression of backbone genes and the production of metabolites *in vivo*, on apples for example. The complexity of the matrices and the accompanying flora could affect the development and the aggressiveness of the fungus differently.

Effect of ascorbic acid on patulin in cloudy apple juice

Different strategies controlling *P. expansum*, responsible for blue mould decay on/in apples and causing many economic losses, have been developed. The use of chemical treatments such as fungicides as main method of control has led to satisfying results. However, these treatments are applied at specific moments before harvest, making sure they are present on the products during their storage period. The reason behind this is to prevent mould growth at storage. The massive use of these fungicides, such as thiabendazole, has led to the development of resistant *P. expansum* strains (Baraldi et al. 2003). However, seen the negative impact of fungicides on both the environment and human health, consumers concern about the presence of poisonous chemical residues in fruits and vegetables has increased. Their demand for healthy food that is free from chemical residues but also of toxic fungi and mycotoxins has encouraged the (re)search for other alternative control strategies that are safer and consumer-friendly to deal with the issue. But even though fungal species can be eliminated by some treatments, their absence does not necessarily imply the absence of toxins. However, European and global regulations have been implemented, and a big improvement in understanding the toxic effects and modes of action of mycotoxins has been achieved so far. All this help controlling the mycotoxins presence and fungi proliferation. Indeed, nowadays, mycotoxins presence can be controlled and reduced to a certain extend. Levels that are harmful for health should be avoided in food products but unfortunately, mycotoxins cannot be completely eliminated from the food chain.

Our research focused on PAT in CAJ. As discussed before, the danger caused by this toxin necessitates its control. Techniques and treatments favoured by food industries and agri-food sectors are those that are easily applicable, with high efficiency and of low cost. That's why this study focused on the fate of PAT in presence of AA. The problem lies in the fact that, sometimes, part of the harvested apples is not suitable for fresh market because of their poor quality. These apples are then designated for the production of transformed products such as juices. Most of the time, apples are kept in open deck storage for a considerable period of time before industrial processing begins thus in some situations accumulating more PAT (Welke et al. 2009).

AA is generally present in low amounts in apple juices. Therefore, apple juices are often fortified with AA because of its antioxidant properties and in order to prevent browning reactions. Its stability is affected by oxygen, temperature, humidity and light (Rojas and Gerschenson 2001). Heat treatments such as pasteurisation were proven to degrade AA, with higher losses at higher temperatures. In acidic products such as fruit juices AA is stable (Robertson and Samaniego-Esguerra 1990). AA

minimizes the decreases in organoleptic qualities (such as colour and flavour) of food products that may occur during processing. It has been described by Brackett and Marth (1979) for being an accelerator of the degradation of PAT in apple juices. However, to the best of our knowledge no studies concerning AA in CAJ have been described yet and its mechanism of action is not fully understood nor clarified in literature.

Mechanism of action of ascorbic acid on patulin

Within the framework of this thesis, the possible need of AA for oxygen to cause PAT degradation was investigated on a laboratory and semi-pilot scale. Our results show that in presence of oxygen, DHA is formed and PAT concentration is reduced. Based on our own and on previous studies, we hypothesize that oxidised AA leads to the formation of DHA and generation of singlet oxygen attacking the double bonds of PAT and causing the opening of the lactone ring (Brackett and Marth 1979; Drusch et al. 2007). Still the (possible) reversibility of this phenomenon needs to be studied further. It was shown that DHA could be reduced back to AA in model solutions, under oxidizing and reducing conditions (Serpen and Gökmen 2007); nevertheless, in most food systems, DHA is irreversibly hydrolysed to 2,3-diketogulonic acid (DKGA) (Davey et al. 2000; Arrigoni and Tullio 2001; Rojas and Gerschenson 2001). The mechanism of action of DKGA on PAT in CAJ is still unknown and it would be interesting to test this and see if it's formed in CAJ produced with the technology used in this PhD study.

As for incubation or storage temperatures, Kevers et al. (2011) found that apples incubated for 7 weeks at 20°C presented a decrease of 75% of their natural concentration of AA. Our findings showed a disappearance of AA (naturally present and added) and a formation of other compounds that is more important at 22°C compared to 4°C in CAJ. This decrease in AA content at 22°C was accompanied by an increase in the reduction of the concentration of PAT in CAJ. These results seem promising since 22°C represents the most popular ambient storage temperature of apple juices in distribution.

When it comes to the concentration of AA that should be added when producing apple juices, different parameters should be considered, like the organoleptic and qualitative ones and the effect on PAT concentration. The amount added in our studies did not seem to have significant effects on pH, colour or brix of the juice but still managed to reduce the PAT concentration to 50% within two weeks of storage. Still, when adding AA, one should always take the quantity of PAT present, the quantity of oxygen needed and the nature of the matrix into consideration. For example, the action

of AA on PAT in clear apple juices will probably differ from the one in CAJ. Solid present in the CAJ can bind to PAT and/or AA and negatively affect the action of AA on PAT. A similar phenomenon was observed when UV light was applied. Reduction of PAT in clear fruit juices was easier than in cloudy ones due to the suspended particles and phenolic compounds in cloudy juices preventing the contact between PAT molecules and UV waves for example (Tikekar et al. 2014).

In addition, the semi-industrial experiment performed in our study consisted on packing the CAJ produced in γ-radiated bags not allowing the light to pass. Another factor that should be considered is light and its impact on AA and PAT degradation. Other packaging materials such as polyethylene (PE) and glass bottles that are translucent should be tested especially that most of artisanal and fresh ones are stored in such bottles.

Degradation products formed

When talking about degrading PAT, degradation products should be analysed in order to ensure the safety of the product. Toxicity data regarding degradation products are very limited compared to those related to the toxin itself. Numerous studies observed that PAT is degraded into compounds potentially less toxic (Castoria et al. 2011; Moss and Long 2002; Tannous et al. 2017b). ASC-E, considered as the predominant degradation product as well as the direct precursor of PAT was considered a mycotoxin itself since it preserves a quarter of the toxic potential of PAT (Suzuki et al. 1971). However, in their recent work, Tannous et al. (2017b) examined the cytotoxic potential of ASC (E and Z isomers) *in vitro* for four human cell lines and showed no evidence for cytotoxicity of ASC till certain concentrations. DPA, another degradation product of PAT was found to be less toxic than PAT (Castoria et al. 2011). These degradation products (ASC-E/Z and DPA) were obtained when PAT is converted by different yeasts. Still, no research describes till today identifying the degradation products generated when AA was added in presence of PAT. In our study, on a laboratory scale, we identified ASC-Z and a compound sharing the same retention as DPA in our extracts. However, when passing to a semi-industrial scale, these degradation products were not detected in PAT-contaminated cloudy apple juice. These differences observed between the two experiments highlight the need for validation of lab-scale results on (semi-)industrial level. Plus, even though the formation of the degradation products was not observed at the same time of the degradation of PAT, it would be interesting to follow this decrease on a longer period. It could be that the compounds formed are binding to other particles in the CAJ and thus not allowing their detection. According to Berthiller et al. (2013), bound mycotoxins, not directly accessible, must be liberated from the matrix before any

chemical analysis and that by applying chemical or enzymatic treatments. The differences occurring between model systems in labs versus complex CAJ matrixes obtained on both semi- and industrial scales show the need for validation of models under real life conditions. The pure standard of PAT and the PAT-contaminated apple mash used to inoculate an acidified aqueous solution and CAJ, respectively, could affect the degrading pathway of PAT as well. It would also be interesting to repeat the same experiment, in the same conditions, on clear apple juice and follow the formation of degradation products.

Analytical hurdles and possible pitfalls

The interference between HMF and PAT has been described previously (Murillo et al. 2008; Ping Lee et al. 2014) and many techniques have been developed in order to separate these two compounds and correctly quantify PAT (review chapter C of the introduction). (However, none of the studies describes a possible signal interference during analysis that could occur between DHA (the oxidized form of AA) and ASC-E, a degradation product of PAT.)

In our research, an in-house methodology separating PAT from the reaction and degradation products resulting from the reaction of AA with PAT in CAJ was optimized in order to allow its detection and/or quantification. Extraction of metabolites from CAJ was performed using the protocol for cloudy apple juice validated by De Clercq et al. (2016). However, PAT is able to bind to the solid particles of the juice, particularly the proteins or small peptides containing cysteine, lysine or histidine residues (Fliege and Metzler 2000) causing a decrease in PAT recovery and an underestimation of the PAT concentration (Baert et al. 2007). That agreed with the research of Bissessur et al. (2001) stating that a clarification of PAT-contaminated CAJ could reduce the occurrence of the toxin in the juice and lead to solid residues that are enriched with the toxin. In our result, we noticed that after applying a flash pasteurisation on the CAJ contaminated by PAT, the concentration of PAT increased in the juice. It could be that the heat treatment favoured the dissociation of bound molecules and liberated the toxin. Additionally, an analysis for the parent toxin and the whole "modified" mycotoxin by means of LC-HRMS could help screen PAT and non-target compounds in our extracts and identify novel compounds (Righetti et al. 2016).

This makes us think about all the mycotoxin-contaminated commodities or modified mycotoxins, generated by fungi, food processing or infected host and that could coexist with their free mycotoxins. Researchers suggested that these masked toxins could be hydrolysed into their free toxins during digestion and threaten animal and human health. Some of them could also contribute to a higher

toxicity (De Boevre et al. 2015; Freire and Sant'Ana 2018). Toxicity data for bound PAT are not available yet, the metabolic conjugation of the electrophilic group is commonly considered as an inactivation reaction and may lead to a loss of toxicity or biological activity until it is released again (Righetti et al. 2016). This still needs to be confirmed.

Beyond mycotoxins …

After observing how AA impacts the PAT concentration in apple juices, it could also be interesting to study its impact in other matrices such as apple puree and apple leather. It could also be useful to see if AA could impact the stability of other mycotoxins occurring in food products especially that it is a compound largely used in industrial food processing. Shalaby in 2009 studied the opposing effect of AA against OTA in the fish *Nile tilapia*. He evaluated the ability of a minimum level (500 mg/ kg diet) of vitamin C to counteract the toxicity of two levels of OTA (400 and 600 µg/kg diet) and showed that AA could normalize the total protein content and aspartate aminotransferase activity in the liver, which are usually disrupted by the toxin, making them similar to those of the control group where no OTA was added.

Apples are considered as a rich source of phenolic constituents distributed in the peel, core and pulp. Their content and composition depend on the apple variety, the area of cultivation, the time and the year of harvest (De Paepe et al. 2015; Krajka-Kuèniak et al. 2015). Polyphenolic compounds include hydroxycinnamic acids, dihydrochalcones, flavonols, catechins, oligomeric procyanidins, and anthocyanins in red apples. Their concentrations are lower in the flesh compared to the peel, except for chlorogenic acid (Candrawinata et al. 2012) and a recent study suggests the presence of high amounts of polyphenolic compounds in apple seeds (Schieber et al. 2003). CAJ is considered as a more valuable source of fibre, natural antioxidants and polyphenolic compounds (Will et al. 2008; Teleszko et al. 2016). In our semi-industrial scale experiment, CAJ was obtained and since its rich in all these compounds, it would be interesting to analyse the polyphenol content in the juice produced, all along the storage period and see how it varies with the decrease of PAT. Polyphenols, just like AA, might impact the PAT concentration and favour its reduction. This could also give us an idea on the storage conditions that are known to affect quality, polyphenolic compounds and antioxidant capacity of CAJ (Kolniak-Ostek et al. 2013). On the other hand, some of the polyphenolic compounds (e.g. esculetin, ferulic acid, quercetin, resveratrol, scopoletin, scoparone, and umbelliferone) seemed to control the development of *P. expansum* on Golden Delicious and Granny Smith apples (Ribes et al. 2017). Dipping apples in quercetin and umbelliferone prevented apples from decaying by 86–92%

compared to the control samples. Clear apple juices which undergo clarification, tend to lose their phenolic compounds.

The apple pomace (waste stream) resulting from clear or CAJ processing, supposed to be rich in antioxidants and polyphenols. PAT is a water-soluble molecule and will probably pass into the juice as observed in our results. The pomace could be used for other purposes such as animals feed and PAT should be evaluated in it. They could serve the need to recycle, reuse and recover energy and valuable chemicals just like winery waste that are used in order to produce platform chemicals, biofuels, heat and energy (Zacharof 2017). Recently, De Gelder et al. (2018) studied the valorisation of mycotoxin contaminated waste streams through anaerobic digestion. They showed that this organic waste contaminated by mycotoxins can be safely valorised into methane and digestate serving as compost. This makes us think of the possibility of undergoing a fermentation on the PAT-contaminated apple juice waste, test them for toxins residues and reuse them after.

Other than its impact on PAT, AA appears to have an impact on pesticides as well. Khan and Sinha (1994) assessed the modulatory effect of higher doses of vitamin C on the genotoxicity of three pesticides (endosulfan, phosphamidon, and mancozeb) in mice. We should note that these pesticides may be used on apples. The vitamin administrated in a dose of 20 mg/kg bw/day simultaneously with each of the three pesticides, was effective as an anti-mutagen. Later on, the possible attenuation of Lambda-cyhalothrin, a synthetic pyrethroid insecticide used worldwide in agriculture (apples included), by vitamin C was studied (Fetoui et al. 2010). Co-administration of vitamin C with lambda-cyhalothrin in rats ameliorated the increase in enzymatic activities and vitamin C was suggested to significantly contribute to numerous beneficial effects seen its antioxidant property. These results suggest the essential global impact of AA on different factors that could be present in food items and harmful to consumers, like the possible effect of AA on pesticide residues in apple-derived products or waste products. More investigations are necessary in order to better characterize the role of AA.

Finally, even though chemical detoxification of mycotoxin-contaminated matrices is not allowed within EU for commodities for human food, addition of AA to apple juice could have an additional positive effect besides organoleptic and quality effects and this due to its effect on the PAT concentration in contaminated juice. After all, consumers would prefer juices PAT-free to those containing PAT below regulatory limit.

More actions towards fungal distribution and toxin contamination are urgently needed. Global warming is a widely acknowledged fact. Climate change factors interacting with each other impact fungal growth and mycotoxin production (Medina et al. 2017a). It risks affecting not only food

security but food and feed safety as well (Medina et al. 2017b). It may also affect the geographical distribution of mycotoxigenic fungi. More research on regulated and non-regulated mycotoxins are needed and the impact of global warming on mycotoxins worldwide should be studied further.

Conclusions …

To conclude, the fungal secondary metabolism is very diversified and is regulated by different factors. In addition, the diversity of secondary metabolites provides them with several roles either positive (e.g. in the medical and agricultural sectors) or negative (e.g. virulence and pathogenicity of fungi), so it is essential to study them further. Apples are among the most consumed and exported fruits worldwide. They are used in several food products designated for infants and young children and can be contaminated by PAT secreted by *P. expansum*.

In this thesis, we addressed the problem of contamination of apples and apple products with PAT from both a fundamental and applied perspective and measures to control this contaminant were discussed. We started by focusing on the characterization of a global transcription factor, VeA, and studied its impact on the secondary metabolism and metabolome of the fungus on different media. Then, we studied the effect of AA on PAT in CAJ on a laboratory and semi-industrial scale and we tested some parameters that could affect this action. Within this framework, it should be considered that the outcome of these experiments cannot be extrapolated to the (semi-)industrial scale. More research and experiments should be performed.

Mycotoxins and other contaminants have long been a major economic burden and pose severe public health risks. Unfortunately, their presence and problems are likely to increase even more with climate change. This indicates that the research challenge will also evolve, hence the importance of conducting multidisciplinary research (molecular, physiological, toxicological, chemical, etc.) and continuing to work on the characterization of (new) stimuli and molecular regulatory mechanisms involved in contamination by moulds and mycotoxins. This will help meet consumer demand for mycotoxin-free apple juice and agri-food to produce safe and high-quality food.

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Patulin, main mycotoxin of the apple industry: regulation of its biosynthetic pathway and influence of processing factors in cloudy apple juice production

Directed by Dr. Olivier PUEL **Co-directed by** Dr. Els VAN PAMEL

Among diseases affecting apples, blue mould caused by *Penicillium expansum* is a major concern. It causes yield and quality losses, as well as food safety issues due to the production of mycotoxins such as patulin (PAT) and citrinin (CIT). PAT is the most worrying one and has cytotoxic, genotoxic and immunosuppressive properties. The European Union (EU) has established specific regulations to protect the consumer's health and maximum levels of PAT of 50 µg/kg is set for fruit juices and derived products, 25 µg/kg for apple purees and compotes and 10 µg/kg for food intended for babies and young children. However, PAT is still found in commercial food and/or beverage products, sometimes exceeding the maximum limits and more research is needed to minimize contamination of food products by this mycotoxin and its fungus. Even though most studies on *P. expansum* have focused on PAT itself, the genome of this fungus exhibits other predicted secondary metabolite (SM) clusters, some of which may be associated with potentially toxic metabolites. In order to control the synthesis of SMs, the study of global transcription factors regulating their production is essential. In a first part, the *veA* gene, belonging to the velvet family, was characterised and its impact on the development of the fungus, its virulence and its secondary metabolism was elucidated. The disruption of this gene led to the failure in PAT and CIT production and a decrease in the expression of their gene cluster. It also revealed a global impact on the secondary metabolism, as 15 of 35 backbone genes showed differential regulation on the media tested. In a second part, the influence of ascorbic acid (AA) on the concentration of PAT in cloudy apple juice was studied on both lab and semi-industrial scale. An analytical methodology separating PAT and other compounds generated during the reaction was optimized. Optimal conditions of action of AA on PAT were studied. In addition, degradation products less toxic than PAT and resulting from AA treatment were identified. To conclude, this thesis is part of the risk management of PAT in the fruit sector; it provides significant improvements at both fundamental and practical levels. These advances are mainly characterized by the description of a mutated strain of *P. expansum* that is less toxic than that naturally occurring in nature, and the description of a food additive that improves numerous products qualities and affects PAT concentration, thus generating less toxic compounds.

Keywords: *Penicillium expansum*, patulin, ascorbic acid, veA, secondary metabolism, cloudy apple juice, degradation products

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Patuline, principale mycotoxine dans les produits à base de pomme: régulation de sa voie de biosynthèse et influence des facteurs de transformation dans la production de jus de pomme trouble

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Parmi les maladies affectant les pommes, la moisissure bleue causée par *Penicillium expansum* est une préoccupation majeure. Elle cause des pertes de rendement et de qualité dues également à la production de mycotoxines telles que la patuline (PAT) et la citrinine (CIT). La PAT est la plus alarmante en raison de ses propriétés cytotoxiques, génotoxiques et immunosuppressives. L'Union européenne (UE) a établi des réglementations spécifiques pour protéger la santé des consommateurs et des niveaux maximaux de 50 µg / kg sont fixés pour les jus de fruits et les produits dérivés, 25 µg / kg pour les purées de pommes et les compotes et 10 µg / kg pour les aliments destinés aux bébés et aux jeunes enfants. En dépit de ces mesures, la PAT continue à être présente dans les aliments et / ou les boissons commerciaux, dépassant parfois les limites maximales. Des recherches supplémentaires sont par conséquent nécessaires pour minimiser la contamination des produits alimentaires par cette mycotoxine et son champignon producteur. Bien que la plupart des études sur *P. expansum* soient essentiellement centrées sur la PAT, le génome de ce champignon présente d'autres clusters de métabolites secondaires (SM) prédits dont certains peuvent être associés à des métabolites potentiellement toxiques. Afin de contrôler la synthèse de SM, l'étude des facteurs de transcription globaux régulant leur production est essentielle. Dans une première partie, le gène *veA*, appartenant à la famille des protéines du complexe velvet, a été caractérisé et son impact sur le développement du champignon, sa virulence et son métabolisme secondaire a été élucidé. La délétion de ce gène a conduit à une réduction de la production de PAT et de CIT et à une diminution de l'expression de leurs clusters de gènes. *VeA* a également un impact global sur le métabolisme secondaire, puisque 15 des 35 gènes de structure présentent une régulation différentielle sur les milieux testés. Dans une deuxième partie, l'influence de l'acide ascorbique (AA) sur la concentration de PAT dans le jus de pomme trouble a été étudiée à la fois en laboratoire et en milieu semi-industriel. Une méthodologie analytique séparant la PAT et d'autres composés générés au cours de la réaction a été optimisée. Les conditions optimales d'action de l'AA sur la PAT ont été analysées. De plus, nous avons identifié des produits de dégradation moins toxiques que la PAT et résultant du traitement par l'AA. Pour conclure, cette thèse se rattache à la gestion des risques de la PAT dans le secteur des fruits ; elle apporte des connaissances et des améliorations significatives tant sur le plan fondamental que sur le plan pratique. Ces avancées résident principalement dans la description d'une souche mutée de *P. expansum* moins toxique que celle naturellement retrouvée dans la nature, et décrivant un additif alimentaire améliorant les qualités de nombreux produits transformés et diminuant la concentration de PAT en générant des composés moins toxiques.

Mots clés: *Penicillium expansum,* patuline, acide ascorbique, veA, métabolisme secondaire, jus de pommes trouble, produits de dégradation

Spécialité : Pathologie, Toxicologie, Génétique et Nutrition

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