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ZETINA-SERRANO C. 2020

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Title: Development and secondary metabolism in *Penicillium expansum*: involvement of global transcription factors VeA and BrlA

Abstract:

Penicillium expansum is a known and widely studied mold of the genus *Penicillium* because it is the causative agent of post-harvest blue mold disease in apples. This filamentous fungus can infest a wide range of fruits primarily during harvest and throughout the storage period. Representing a significant economic problem for the fresh fruit industry, *P. expansum* is also known to produce a wide variety of secondary metabolites with diverse chemical structures including the mycotoxins patulin, citrinin and roquefortine C. Secondary metabolites are the end products of enzyme cascades where the enzymes involved are encoded by genes arranged contiguously as a cluster of biosynthetic genes. These clusters of genes are often transcriptionally co-regulated by a variety of different genetic mechanisms ranging from specific regulation by DNA-linked transcription factors to global regulation by changes in chromosome structure.

BrlA is a global zinc finger type C_2H_2 transcription factor required for the development of conidiophores in the family *Aspergillaceae*. It is involved in the production of secondary metabolites, in particular in the regulation of genes coding for metabolites synthesized during spore formation. VeA is a protein with a velvet domain and forms with VelB (another velvet protein) and methyltransferase LaeA the heterotrimeric VelB-VeA-LaeA complex, which plays an important role in development, sporulation, secondary metabolism and pathogenicity.

The characterization of the role of the *brlA* and *veA* genes (already performed for *veA*) in the regulation of the biosynthetic pathways of certain secondary metabolites in *P. expansum* as well as the determination of the environmental conditions favoring their expression are essential to understand the behavior of this fungus and to predict fungal development in their natural substrate. In this context, one of the objectives of the thesis was the investigation of the roles of the transcription factor BrlA on the virulence, development and secondary metabolism of *P. expansum*. For this purpose, a Pe∆*brlA* strain was generated by homologous recombination. In vivo, the suppression of *brlA* completely blocked the development of conidiophores but not the formation of coremia/synnemata. The analysis of the Pe∆*brlA* transcriptome in vitro revealed the positive regulation of a cluster of genes showing a strong similarity with the chaetoglobosin cluster of *Chaetomium globosum* in addition to the genes involved in conidiation such as *wetA*, *abaA*, hydrophobin or melanin-like pigment coding genes.

A second objective of the thesis was the characterization of the secondary metabolome of *P. expansum*. Metabolomic analysis, carried out in the wild-type and null mutant strains Pe∆*veA* and Pe∆*brlA*, showed that patulin biosynthesis was not related to conidiogenesis. Patulin was not detected in the spores or synnemata, showing that patulin synthesis stopped when the fungus emerged from the apple. The biosynthesis of secondary metabolites is linked to the different stages of fungal development, some of which were secreted by the basal metabolism and were believed to be aggressive factors allowing faster invasion of the substrate, while others were produced by aerial metabolism during dissemination. The analysis of the metabolome of Pe∆*brlA* revealed a great diversity of chaetoglobosins. Chaetoglobosins A and C were only present in synnemata while the other derivatives were found in apple flesh, suggesting a spatial-temporal organization of the chaetoglobosin biosynthesis pathway.

Key-words: *Penicillium expansum*, *brlA*, *veA*, conidiogenesis, synnemata, spores, secondary metabolism, microarray, metabolomics, patulin, citrinin, chaetoglobosins, communesins

Discipline: Pathology, Toxicology, Genetic and Nutrition

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ZETINA-SERRANO C. 2020

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Titre : Développement et métabolisme secondaire chez *Penicillium expansum* : implication des facteurs de transcription globaux VeA et BrlA

Résumé :

Penicillium expansum est une moisissures connue et largement étudiée du genre *Penicillium* car c'est l'agent responsable de la maladie de la moisissure bleue post-récolte des pommes. Ce champignon filamenteux peut infester une large gamme de fruits principalement pendant la récolte et toute la période d'entreposage. Représentant un problème économique important pour l'industrie des fruits frais, *P. expansum* est également connu pour produire une grande variété de métabolites secondaires aux structures chimiques diverses dont les mycotoxines patuline, citrinine et roquefortine C. Les métabolites secondaires sont les produits finaux de cascades enzymatiques où les enzymes impliquées sont codées par des gènes disposés de manière contiguë sous forme de cluster de gènes biosynthétiques. Ces clusters de gènes sont souvent co-régulés de manière transcriptionnelle par une variété de mécanismes génétiques différents allant de la régulation spécifique par des facteurs de transcription liés à l'ADN à la régulation globale par des modifications de la structure du chromosome.

BrlA est un facteur de transcription global en doigt de zinc de type C_2H_2 nécessaire au développement des conidiophores dans la famille des *Aspergillaceae*. Il est impliqué dans la production de métabolites secondaires, en particulier dans la régulation de gènes codant pour des métabolites synthétisés lors de la formation des spores. VeA est une protéine comportant un domaine velvet et forme avec VelB (protéine velvet) et la méthyltransférase LaeA lecomplexe hétérotrimérique VelB-VeA-LaeA, qui joue un rôle important dans le développement, la sporulation, le métabolisme secondaire et la pathogénicité.

La caractérisation du rôle des gènes *brlA* et *veA* (déjà effectuée pour *veA*) dans la régulation des voies de biosynthèse de certains métabolites secondaires chez *P. expansum* ainsi que la détermination des conditions environnementales favorisant leur expression sont essentielles pour comprendre le comportement de ce champignon et pour prédire le développement fongique dans leur substrat naturel. Dans ce contexte, un des objectifs de la thèse a été l'investigation des rôles du facteur de transcription BrlA sur la virulence, le développement et le métabolisme secondaire de *P*. *expansum*. Pour cela, une souche Pe∆*brlA* a été générée par recombinaison homologue. In vivo, la suppression de *brlA* a complètement bloqué le développement des conidiophores mais pas la formation des corémies/synnémates. L'analyse du transcriptome de Pe∆*brlA* in vitro a mis en évidence la régulation positive d'un cluster de gènes présentant une forte similarité avec celui des chaetoglobosines de *Chaetomium globosum* en plus des gènes impliqués dans la conidiation tels que *wetA*, *abaA*, les gènes codant pour les hydrophobines ou pour les pigments de type mélanine.

Un second objectif de la thèse résidait dans la caractérisation du métabolome secondaire de *P. expansum*. L'analyse métabolomique, réalisée chez la souche sauvage et les souches mutantes nulles Pe∆*veA* et Pe∆*brlA*, a montré que la biosynthèse de la patuline n'était pas liée à la conidiogenèse. La patuline n'est détectée ni dans les spores ni dans les synnémates, montrant que la synthèse de patuline s'arrête quand le champignon ressort de la pomme. La biosynthèse des métabolites secondaires est liée aux différents stades du développement fongique, certains sont secrétés par le métabolisme basal et seraient des facteurs d'agressivité permettant d'envahir plus rapidement le substrat tandis que d'autres sont produits par le métabolisme aérien lors de la dissémination. L'analyse du métabolome de Pe∆*brlA* a révélé une grande diversité de chaetoglobosins. Les chaetoglobosines A et C étaient seulement présentes dans les synnémates alors que les autres dérivés étaient retrouvés dans la chair des pommes, suggérant une organisation spatio-temporelle de la voie de biosynthèse des chaetoglobosines.

Mots-clés : *Penicillium expansum*, *brlA*, *veA*, conidiogenèse, synnémates, spores, métabolisme secondaire, microarray, métabolomique, patuline, citrinine, chaetoglobosines, communésines

Discipline : Pathologie, Toxicologie, Génétique et Nutrition

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List of publications

1. **The *brlA* Gene Deletion Reveals That Patulin Biosynthesis Is Not Related to Conidiation in *Penicillium Expansum*.**
   Zetina-Serrano, C.; Rocher, O.; Naylies, C.; Lippi, Y.; Oswald, I.P.; Lorber, S.; Puel, O. The *brlA* Gene Deletion Reveals That Patulin Biosynthesis Is Not Related to Conidiation in *Penicillium expansum*. *Int. J. Mol. Sci*. **2020**, *21*, 6660. https://doi.org/10.3390/ijms211866

2. **Secondary metabolism of *Penicillium spp.* and its regulation**
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3. **Deciphering of *Penicillium expansum* metabolome**
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List of abreviations

°C	Degrees Celcius
λ max	Maximum wavelength
µM	Micromoles
µg/mL	Micrograms /militres
µg/kg	micrograms/kilograms
6-msas	6-methylsalicylic synthase
ABC	ATP-binding cassette
APAM	Apple Puree Agar Medium
AREs	AbaA Response Elements
a_W	Water activity
BLAST	Basic Local Alignment Search Tool
BREs	BrlA Response Elements
bp	base pair
cAMP	cyclic adenosine monophosphate
CRP	Central Regulatory Pathway
cm	Centimeter
Com	Communesin
CYA	Czapek Yeast extract Agar
CWDEs	Cell wall degrading enzymes
DEG	Differential Expressed Genes
DHN	1,8-Dihydroxynaphthalene
DMAT	DiMethyl Allyl Transferase
dpi	day post inoculation
ETP	EpipolyThiodioxoPiperazine
GEO	Gene Expression Omnibus
GLA	D-gluconic acid
HDAC	Histone DeACetylase
HPLC	High Performance Liquid Chromatography
HOG	high osmolarity glicerol
Idh	isoepoxydon dehydrogenase
LaeA	loss of aflR expression A
LBG	Locus Bean Gum
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
L_{hgu}	hyphal growth unit Length
MEA	Malt Extract agar
MFS	major facilitator superfamily
NAA	nicotinamide
NLS	nuclear localization sequence
nm	nanometers
NRPS	Non-Ribosomal Peptide Synthase
OTA	Ochratoxin
ORF	Open reading frames
PDA	PDA Potato Dextrose Agar
pH	Potential of hydrogen
PKS	PolyKetide Synthase
PKS / NRPS	Hybrid polyketide-nonribosomal peptides
qPCR	Quantitative real-time polymerase chain reaction
RDS	Roquefortine dipeptide synthetase
R/M	Roquefortine C/meleagrin
Rt	Retention time, min

SAHA	SuberoylAnilide Hydroxamic Acid
SEM	standard error of the mean
SM	Secondary Metabolite
TC	Terpene Cyclase
TF	Transcription Factor
UDAs	upstream developmental activators
UV light	Ultra-violet light
VeA	Velvet A
VelB	Velvet-like B
VosA	Viability of spores A
VelC	Velvet-like C
WCC	white-collar complex
WT	Wild Type

CHAPTER 1



INTRODUCTION

STUDY CONTEXT AND OBJECTIVES

Filamentous fungi are important to humans because some can be beneficial and some can be harmful, and have economic consequences. They are used in different sectors such as food, pharmaceuticals, cosmetics and medicine. Nevertheless, some filamentous fungi can pose a threat to the feed and food industry due to contamination by bioactive compounds called mycotoxins, which are harmful to humans. Mycotoxins are low molecular weight secondary metabolites (<1,000 Dalton) produced by filamentous fungi (Marin et al. 2013). Food losses due to mycotoxin contamination account for more than 25% of all spoiled foods, resulting in large economic losses worldwide (Egmond et al. 2004).

The term "mycotoxin" comes from the combination of the Greek word "mycos", which refers to a fungus, and the Latin word "toxicum", which means poison (Jouany et al. 2009; Rai et al. 2012). These metabolites are not essential to the life cycle of the fungus, but they may confer some competitive advantages (Moss 1991; Keller, 2019). Among them, 300 to 400 compounds have been recognized as mycotoxins, of which the main producers are *Aspergillus*, *Fusarium*, *Penicillium, Alternaria*, *Byssochlamys* and *Claviceps* species (Bryden 2016). The mycotoxins most commonly studied due to their toxicity 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). The enormous structural diversity of mycotoxins, their countless biological effects and their different fungal producers make the classification of mycotoxins difficult (Bennet and Klich 2003). Mycotoxins can cause various toxic effects depending on the toxin (alone or in combination), structure, dose and duration of exposure. They can induce toxicity in different organs and have various cellular and molecular mechanisms of action. The maximum levels of 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 its amendments.

The main problem we encounter is that filamentous fungi are capable of producing numerous secondary metabolites, among which there could be unknown mycotoxins that could contaminate the raw materials and be even more toxic to humans. For this reason, there is increasing interest in investigating the study of these toxins and their origin, toxicity, stability, biosynthetic pathways, secondary metabolism and control strategies.

Although most studies on *P. expansum* have focused on patulin, the fungus produces many other known SMs and others that remain to be discovered given the number of potential BCGs present in its genome. For this reason, the objective of the research of this thesis was to determine and characterize the secondary metabolism of *P. expansum*, its regulation by key factors and its spatio-temporal location during crucial stages of development.

BIBLIOGRAPHIC REVIEW

The bibliographic review performed in the progress of this thesis work presents an analysis of the literature and highlights the main issues that were addressed during the development of the work. This bibliographic review is structured in three main parts; **first**, it includes a review published and accepted in the **International Journal of Molecular Science**, it consists of a general review of the main regulatory mechanisms in the production of secondary metabolites of the genus *Penicilium*. **In the second part**, the state of the art of P. expansum is presented, focusing on the physiological and morphological characteristics and the principal secondary metabolites produced by this fungus with special attention to the mycotoxin biosynthesis pathways and the organization of the genes encoding the enzymes involved in the cluster form. Finally, in the **third part** of this bibliographic review, information on asexual reproduction (conidiogenesis) in filamentous fungi is presented, emphasizing the central regulatory pathway, the positive and negative regulators that allow carrying out this important survival process in filamentous fungi.

1. Regulation of secondary metabolism in the *Penicillium* genus

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Review

Regulation of Secondary Metabolism in the *Penicillium* Genus

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Abstract: *Penicillium*, one of the most common fungi occurring in a 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 decay in food crops and are able to produce a varied range of secondary metabolites, from which we can distinguish harmful mycotoxins. Some *Penicillium* species are considered to be important producers of patulin and ochratoxin A, two well-known mycotoxins. The production of these mycotoxins and other secondary metabolites is controlled and regulated by different mechanisms. The aim of this review is to highlight the different levels of regulation of secondary metabolites in the *Penicillium* genus.

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

1. Introduction

Studies have estimated the existence of at least 2.2–3.8 million fungal species on Earth, from which only around 10% have been isolated and described [1,2]. *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 great importance in numerous and diverse fields, such as food spoilage, biotechnology, plant pathology, and medicine [3,4], and currently contains 483 accepted species [5]. Several of these species, classified as pre- and post-harvest pathogens, can lead to catastrophic decay in food crops, as described by Frisvad and Samson [6], Pitt and Hocking [7], and Samson et al. [8]. *Penicillium* can also produce a varied range of secondary metabolites, including several harmful mycotoxins [9], antibacterial [10–14] and antifungal compounds [15], immunosuppressants, and cholesterol-lowering agents [16–19]. The most iconic example of a drug of fungal origin is penicillin, the first antibiotic substance in history [20].

The biosynthesis of several secondary metabolites, such as mycotoxins, depends on several environmental cues including the substrate, pH, temperature, water activity, interrelationships with other microorganisms, and the interactions of these different factors in the natural environment [21–23].

Secondary metabolites are products of enzymatic cascades starting when backbone enzymes such as polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), terpene cyclases (TCs),

and dimethylallyl tryptophan synthases (DMATSs) catalyze, respectively, the rearrangement or condensation of simple primary metabolites, such as acetyl-CoA, amino acids, or isoprene units, resulting in more complex secondary metabolites [24]. Different metabolic pathways can lead to their formation (Figure 1). Fungal secondary metabolites are classified into five categories according to their structures and their precursors: polyketides, cyclic terpenes, non-ribosomal peptides, indole alkaloids, and hybrids (Figure 1) [25]. Other enzymes named tailoring enzymes are also needed and interfere in the catalysis of subsequent reactions in the biosynthetic pathways of mycotoxins. The structural diversity of mycotoxins results from the variety of chemical reactions (cyclization, aromatization, glycosylation, hydroxylation, methylation, acetylation, and epoxidation) involved in their biosynthesis [26] and leads to their broad spectrum of biological properties and functions. The combined involvement of backbone enzymes in the same biosynthesis pathway infinitely broadens this structural diversity of secondary metabolites. This diversity is also enriched by the infrequent existence of crosstalk between different biosynthetic pathways [27].

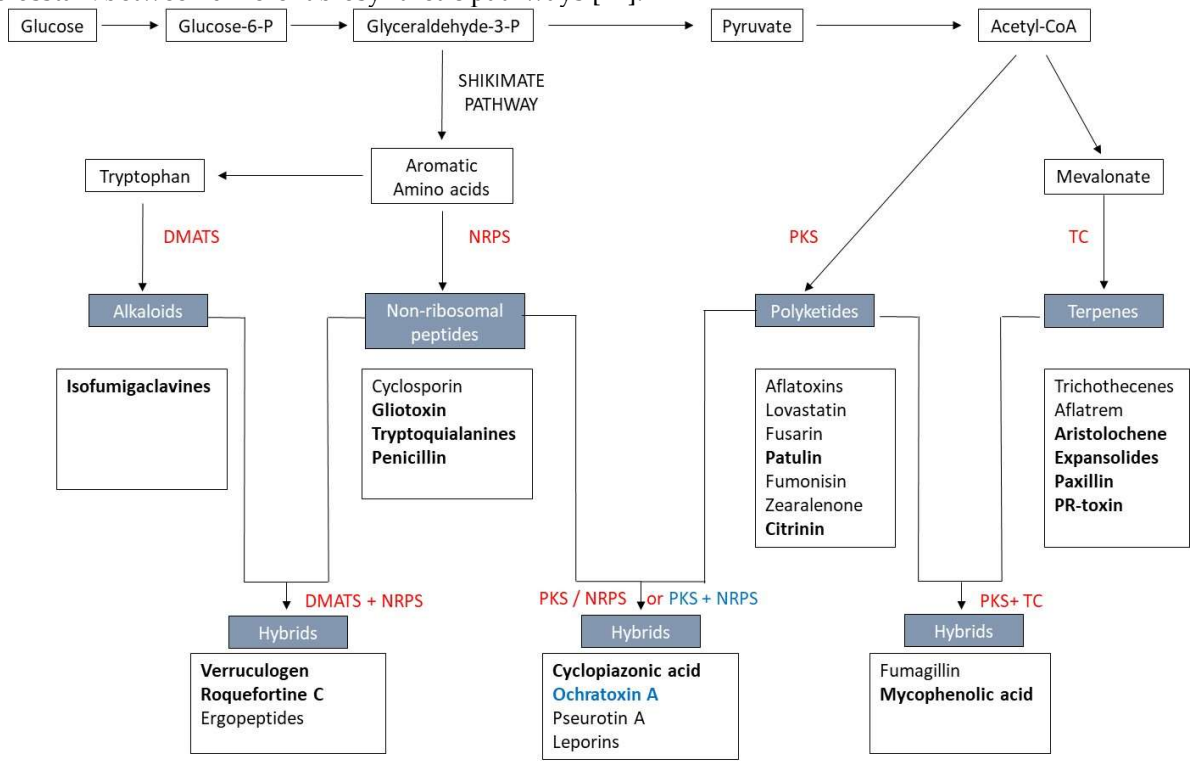


Figure 1. Biosynthetic pathways of secondary metabolites. In grey boxes, the typical backbone of secondary metabolites. In grey, the main mycotoxins produced by these pathways. In red, the enzymes associated with each pathway. In blue, separate PKS and NRPS are involved in ochratoxin A (OTA) biosynthesis; NRPS: non-ribosomal peptide synthetase, PKS: polyketide synthase, TC: terpene cyclase, DMATS: dimethylallyl tryptophan synthase. In **bold**, mycotoxins produced by *Penicillium* species.

Enzymes are activated at the same time, and the newly synthesized intermediates are consecutively metabolized by the following enzymes. This phenomenon is possible due to the cluster organization of the genes encoding the enzymes involved in the biosynthesis of the metabolites in the same chromosomal region. These genes are often co-activated by a specific transcription factor (TF) located within the clusters [28]. Based on bioinformatics analysis and other studies, it was proven that fungal genomes exhibit different and numerous predicted secondary metabolite clusters. A recent review estimated the number of fungal biosynthetic gene clusters (BGCs) at several million [29]. For the two well-known genera of *Aspergillus* and *Penicillium* alone, which contain 446 and 483 species, respectively [5], the number of non-redundant clusters is approximately 25,000. In filamentous fungi, the activation of specific TFs and the resulting production of fungal secondary metabolites is controlled at a higher hierarchical level by global TFs. Understanding the mechanisms underlying mycotoxin biosynthesis

contributes to defining/identifying strategies or mechanisms to regulate them and reduce their production [30].

Numerous studies have focused on regulators impacting the formation of secondary metabolites in *Aspergillus*, *Penicillium*, and *Fusarium*, but few reviews have explored the complex and multi-layered regulation of fungal secondary metabolism [31–34]. While several excellent articles reviewed the different regulatory mechanisms known for *Aspergillus* [29,35–37], this review aims to deepen the understanding of the regulation of secondary metabolism in *Penicillium* and highlight all the regulatory mechanisms that can occur.

2. Regulation of Secondary Metabolism

For the synthesis of any secondary metabolite, the regulation of its cluster involves a number of factors for activation or repression. This regulation occurs at different levels. Most secondary metabolite clusters have genes encoding TFs that act directly on all other genes located within the cluster. Expression of these internal regulators also depends on other, more global TFs encoded by genes unrelated to the BGCs, which are 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 signaling transduction pathways. Finally, epigenetic regulation, including modification of the chromatin and nucleosome structure, can yield transcriptional control and impact secondary metabolite synthesis extensively [38]. In the following section, the different regulatory systems studied in *Penicillium* will be discussed.

2.1. Specific Transcription Factors/Cluster-Specific Regulators

Gene clusters involved in secondary metabolite biosynthesis often include a gene encoding a TF that specifically acts and modulates the expression of the other genes in that cluster (e.g., *patL*, *calC*, and *ctnA* in patulin, calbistrin, and citrinin biosynthetic pathways, respectively). This gene has a switching role within the cluster (Figure 2). The TFs regulate gene expression by binding specifically to the promoters of the genes involved.

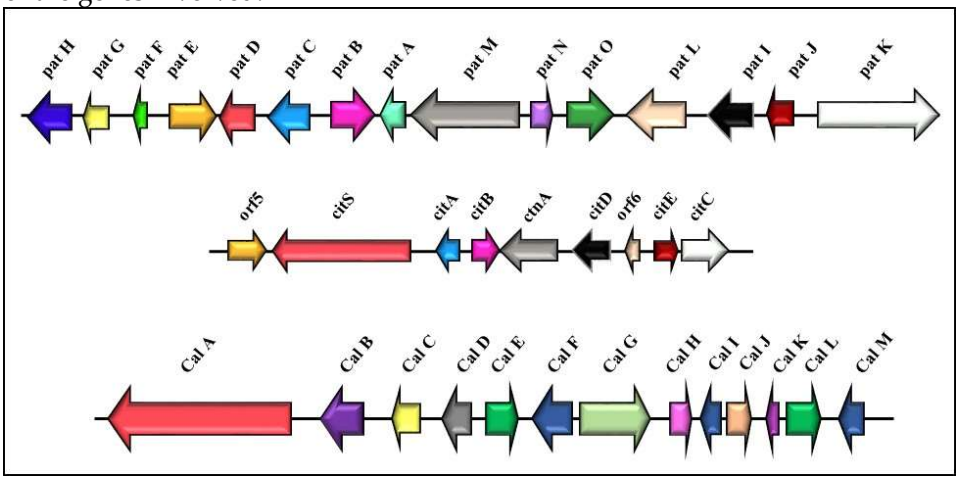


Figure 2. Gene clusters of the patulin biosynthesis pathway (the first one at the top) (15 genes, 40 kb) [39] and the citrinin biosynthesis pathway (the middle group) (nine genes, 22 kb) [40,41] in *Penicillium expansum*; cluster of the calbistrin biosynthesis pathway (the third one at the bottom) (13 genes, 35kb) in *Penicillium decumbens* [42].

Several studies comparing TF sequences have shown that these TFs can be classified into different families based on the similarities in their protein sequences. We can distinguish between zinc finger proteins, proteins called helix-turn-helix, and leucine zippers [43]. Nevertheless, almost 90% of the potential gene clusters involved in the synthesis of fungal polyketides belong to the family of zinc finger TFs (Cys_2His_2, Cys_4, or $Zn(II)_2Cys_6$) [43–45]. Proteins of the $Zn(II)_2Cys_6$ family are found exclusively in

fungi and yeasts [46], and the C_6 type zinc finger DNA binding protein motif (Cys_6) is frequently encountered in TFs. Cys_6 has been identified on more than 80 proteins found mainly in fungi [43] and is generally considered a transcriptional activator (Table 1). Only in *Saccharomyces cerevisiae* are the zinc finger proteins (ARGR2, LEU3, and UME6) activators and repressors [47–50]. Subsequently, the number of proteins belonging to the $Zn(II)_2Cys_6$ family has increased significantly due to the number of fungal genomes that have since been sequenced. Numerous examples of $Zn(II)_2Cys_6$ TFs identified as being involved in the secondary metabolism of fungi genera other than *Penicillium* have been largely described in the literature. As examples, we can quote AflR (aflatoxins), Bik5 (bikaverin), and CtnA (citrinin) for *Aspergillus*, *Fusarium*, and *Monascus*, respectively (Table 1).

Table 1. Examples of identified $Zn(II)_2Cys_6$ and leucine zipper transcription factor (TF) involvement in secondary metabolism in fungi; adapted and updated from Yin and Keller [46].

TF	Biosynthetic Gene Cluster	TF Family	Species	References
AflR	Aflatoxin/Sterigmatocystin	$Zn(II)_2Cys_6$	*Aspergillus flavus* *Aspergillus nidulans* *Aspergillus parasiticus*	[51–55]
AsaR	Aspergillic Acid	$Zn(II)_2Cys_6$	*Aspergillus flavus*	[56]
GliZ	Gliotoxin	$Zn(II)_2Cys_6$	*Aspergillus fumigatus* *Penicillium lilacinoechinulatum*	[57,58]
XanC	Xanthocillin	Basic Leucine zipper	*Aspergillus fumigatus*	[59]
FapR	Fumagillin/Pseurotin	$Zn(II)_2Cys_6$	*Aspergillus fumigatus*	[60]
ZEB2	Zearalenone	Basic Leucine zipper	*Fusarium graminearum*	[61]
SimL	Cyclosporine	Basic Leucine Zipper	*Tolypocladium inflatum*	[62]
OtaR1	Ochratoxin A	Basic Leucine zipper	*Aspergillus carbonarius* *Aspergillus ochraceus* *Aspergillus westerdijkiae* *Penicillium nordicum*	[63]
SirZ	Sirodesmin PL	$Zn(II)_2Cys_6$	*Leptosphaeria maculans*	[58]
MlcR	Compactin	$Zn(II)_2Cys_6$	*Penicillium citrinum*	[64]
Bik5	Bikaverin	$Zn(II)_2Cys_6$	*Fusarium fujikuroi*	[65]
DEP6	Depudecin	$Zn(II)_2Cys_6$	*Alternaria brassicicola*	[66]
ZFR1 **FUM21**	Fumonisin	$Zn(II)_2Cys_6$	*Fusarium verticillioides*	[67,68]
CTB8	Cercosporin	$Zn(II)_2Cys_6$	*Cercospora nicotianae*	[69]
GIP2	Aurofusarin	$Zn(II)_2Cys_6$	*Gibberella zeae*	[70]
CtnA	Citrinin	$Zn(II)_2Cys_6$	*Monascus purpureus* *Monascus ruber* *Penicillium expansum*	[40,41,71]
LovE	Lovastatin	$Zn(II)_2Cys_6$	*Aspergillus terreus*	[72,73]
ApdR	Aspyridone	$Zn(II)_2Cys_6$	*Aspergillus nidulans*	[74]
CtnR	Asperfuranone	$Zn(II)_2Cys_6$	*Aspergillus nidulans*	[75]

MdpE	Monodictyphenone/ Emodin Analogs	$Zn(II)_2Cys_6$	*Aspergillus nidulans*	[76]
Cmr1p	Melanin	$Zn(II)_2Cys_6$	*Colletotrichum lagenarium*	[77]
Pig1p	Melanin	$Zn(II)_2Cys_6$	*Magnaporthe grisea*	[77]
GsfR1	Griseofulvin	$Zn(II)_2Cys_6$	*Penicillium griseofulvum*	[78]
MokH	Monacolin K	$Zn(II)_2Cys_6$	*Monascus pilosus*	[79]
CalC	Calbistrin	$Zn(II)_2Cys_6$	*Penicillium decumbens*	[42]
CnsN	Communesins	$Zn(II)_2Cys_6$	*Penicillium expansum*	[80]
Orf2	Varicidin A and B	$Zn(II)_2Cys_6$	*Penicillium variabile*	[81]
Orf10	PR-Toxin	$Zn(II)_2Cys_6$	*Penicillium chrysogenum* *Penicillium roqueforti*	[82,83]
MacR	Macrophorin	$Zn(II)_2Cys_6$	*Penicillium terrestris*	[84]
PatL	Patulin	$Zn(II)_2Cys_6$	*Penicillium expansum*	[85]
SorR1 **SorR2**	Sorbicillin	$Zn(II)_2Cys_6$	*Penicillium chrysogenum*	[86]
TqaK	Tryptoquialanines	Basic leucine zipper	*Penicillium aethiopicum* *Penicillium digitatum*	[87,88]
Sol4	Solanapyrone	$Zn(II)_2Cys_6$	*Ascochyta rabiei*	[89]
RolP	Leucinostatin	$Zn(II)_2Cys_6$	*Paecilomyces lilacinus*	[90]

Gliotoxin, a secondary fungal metabolite belonging to the class of epipolythiodioxopiperazines (ETPs) and characterized by the presence of a sulfur-bridged dioxopiperazine ring [91], is produced by some *Aspergillus* and *Penicillium* species, such as *Penicillium lilacinoechinulatum* [92], a strain of this species was misidentified as *Penicillium terlikowskii* in a study by Waring et al. [92,93]. Within its cluster, a $Zn(II)_2Cys_6$ finger transcription regulator, GliZ, was identified to be responsible for gliotoxin induction and regulation. A mutation of the *gliZ* (Δ*gliZ*) gene in *Aspergillus fumigatus* resulted in the loss of gliotoxin production, while overexpression of *gliZ* increased the production of gliotoxin [94,95]. In *P. lilacinoechinulatum*, a homologous gene is present in the genome, but the heterologous complementation of the *A. fumigatus* Δ*gliZ* mutant with Pl*gliZ* failed to restore gliotoxin production [58]. The *mlcR* gene encoding a putative 50.2-kDa protein characterized by a $Zn(II)_2Cys_6$ DNA-binding domain was shown to be involved in the regulation and biosynthesis of ML-236B (compactin) in *Penicillium citrinum* [96].

Another gene encoding PatL, a specific TF in *Penicillium expansum*, was shown to affect patulin production [85]. The protein encoded by this gene has two conserved domains, one of which encodes a Cys_6 DNA binding site and the other of which was found in the TFs of the superfamily of zinc finger TFs. Orthologous genes of *patL* involved in the patulin metabolic pathway were found in other filamentous fungi genomes, such as *Penicillium griseofulvum*, *Penicillium paneum*, *Penicillium vulpinum*, *Penicillium carneum*, *Penicillium antarcticum* [97], and *Aspergillus clavatus* [98]. Sometimes, BGCs such as the sorbicillin gene cluster can contain two genes encoding TFs. In this example, SorR1, a $Zn(II)_2Cys_6$ factor, acts as an activator for the expression of all genes located within the cluster. The second zinc finger TF (SorR2) controls the expression of the *sorR1* gene [86]. Few cases of TFs belonging to the basic leucine zipper (bZIP) family have been reported to act as specific TFs of secondary metabolite pathways. These TFs include ZEB2, SimL, and OtaR1. The latter TF is present in the OTA cluster in *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus carbonarius*, and *Penicillium nordicum*. Its inactivation in *A. ochraceus* leads to the complete inhibition of OTA production [63]. SimL regulates the production of the well-known immunosuppressant drug cyclosporine [62]. The transcripts of genes located within the zearalenone gene cluster were not detected when the *zeb2* gene encoding bZIP was deleted [61,99]. *TqaK*, another gene encoding a bZIP protein, was reported to be located inside the tryptoquialanine gene cluster in *Penicillium aethiopicum*. The deletion of *tqaK* led to tryptoquialanine production equal to only one-twentieth that of the parental strain [87]. An orthologous gene is also present in the genome of

Penicillium digitatum, another tryptoquialanine-producing species. Thus far, OtaR1 and TqaK are the only bZIP proteins identified to be directly involved in secondary metabolite biosynthesis in *Penicillium*.

2.2. Environmental Signals and Associated Regulators

In the previous section, we reviewed specific TFs described in *Penicillium* that are cluster-specific. However, numerous regulatory elements affected by environmental cues modulate the expression of fungal secondary metabolite clusters and do not reside within the cluster itself. They are considered to be global regulators (Figure 3). Among them, CreA, AreA, Nmc, PacC, Skn7, Yap1, VeA, LaeA, BrlA, PcRFX1, PcFKH1, Pcz1, and NsdD have been mentioned and are discussed in the following.

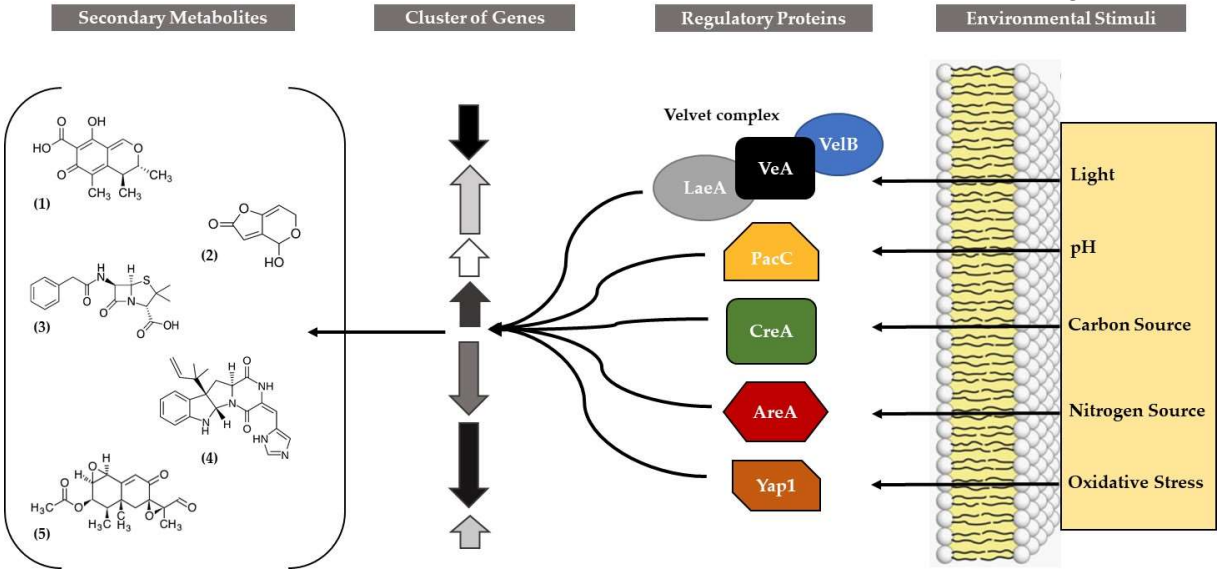


Figure 3. Global regulatory proteins involved in the regulation of gene clusters involved in the production of various secondary metabolites in *Penicillium* (1) citrinin, (2) patulin, (3) penicillin G, (4) roquefortine C, and (5) PR-toxin, adapted from Brakhage [45].

In the fungal kingdom, the synthesis of secondary metabolites is often a response to environmental or ecological changes and is dependent on the developmental stage of the producing species. The activation of a biosynthetic pathway is influenced by the composition of the substrate on which the fungus grows—in particular, the carbon source and the nitrogen source. Glucose and other assimilable sugars can suppress secondary metabolite pathways mediated by **CreA**, a protein displaying two Cys_2His_2 zinc finger domains. For example, the biosynthesis of penicillin in *Penicillium chrysogenum* was shown to be largely regulated by glucose, sucrose and, to a lesser extent, by other sugars (maltose, fructose, and galactose). Cepeda-García et al. [100] showed clear evidence of the involvement of the CreA factor in the catabolic repression of penicillin biosynthesis and the expression of the *pcbAB* gene, encoding the first enzyme of the penicillin pathway in *P. chrysogenum*. The authors applied an RNAi strategy attenuating *creA* gene expression. Transformants expressing small interfering RNAs for *creA* showed greater production of penicillin. By contrast, a recent study showed that the deletion of *creA* in *P. expansum* strains leads to the absence of patulin production in apples [101], although expression of the *pat* genes is increased. Regarding the nitrogen source, a similar regulatory mechanism called nitrogen metabolite repression exists in Ascomycetes. For instance, a concentration of ammonium above 40 mM caused a repression in the expression of *uidA*, a promoterless gene for β-glucuronidase in *Escherichia coli*, when fused to the promoters of *pcbAB* (*acvA*) and *pcbC*, two genes encoding the two first enzymes of the penicillin pathway in *P. chrysogenum* [102]. In *P. griseofulvum* (formerly *P. urticae*), the production of patulin was also affected when ammonium ions were added to the culture medium [103]. On the other hand, the presence of 30 mM ammonium chloride results in a significant decrease in isoepoxydon dehydrogenase (*idh*) and 6-methylsalicylic synthase (*6-msas*) transcripts, key genes in the pathways of patulin biosynthesis [104,105]. This nitrogen metabolite repression is mediated by **AreA**, a

Cys_2Cys_2-type zinc finger TF [31]. This regulatory factor binds to the intergenic region of *acvA-pcbC* [106] in response to nitrogen and mediates the regulation of penicillin biosynthesis in *P. chrysogenum* [107]. The *idh* (*patN*) and *6-msas* (*patK*) genes interact with the NrfA protein, an orthologue of the AreA protein in *P. griseofulvum*, through several putative GATA sites located on their promoter. The *nmc* gene, encoding the AreA orthologous factor, has been characterized in *Penicillium roqueforti*. This protein, which displays at least 94% identity with that of homologous fungal proteins (AreA in *Aspergillus*) [108], is induced and upregulated by nitrogen starvation, but no data regarding its impact on *P. roqueforti* secondary metabolites have already been published.

Another well-known environmental stimulus that induces or represses the secondary metabolism in filamentous fungi is the pH of the substrate. This regulation is mediated by **PacC**, the key factor of pH fungal regulation [109]. This TF displays three putative Cys_2His_2 zinc fingers [110]. In the genus *Aspergillus*, a neutral to alkaline extra-cellular pH is required for the activation of PacC via two proteolytic steps [111]. These steps are mediated by the *pal* (*palA*, *palB*, *palC*, *palF*, and *palI*) pathway [109]. The final mature form of this protein activates the expression of genes expressed under alkaline conditions and, by contrast, represses the transcription of genes expressed under acidic conditions. Many examples of PacC's involvement in the regulation of biosynthetic pathways in *Aspergillus* or *Fusarium* species have been reported in the literature [65,112,113]. Suárez and Peñalva [114] showed that *Penicillium pacC* transcript levels were higher under alkaline than acidic growth conditions and elevated in later stages of growth. The level of the *pcb* transcripts followed the same trend, leading to increased production of penicillin under alkaline pH. Barad et al. [115] also studied the link between ammonia accumulation, the activation of *pacC*, and the synthesis of patulin in *P. expansum*. The authors 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.

Barad et al. [116] analyzed the role of PacC in the regulation of D-gluconic acid (GLA) production and patulin accumulation in *P. expansum*. On the one hand, their results showed that GLA production plays a role in the activation of patulin production. On the other hand, this study, based on the characterization of *pacC*-RNAi mutants of *P. expansum*, concluded that PacC plays a key role in the regulation of GLA accumulation via the transcriptional regulation of *gox2*, the most important gene involved in GLA production in *P. expansum*. This regulation of GLA production through PacC largely affects patulin accumulation in the mutants. A recent publication reported that the production of patulin is completely inhibited in the null mutant PeΔ*pacC* strain when grown at pH > 6.0 [117]. In *P. digitatum*, PacC was reported to regulate the expression of genes encoding polygalacturonase PG2 and pectin lyase PNL1, enzymes both involved in the degradation of the citrus cell wall [118].

Osmotic and oxidative stress are considered to be other environmental cues to which filamentous fungi should respond in order to survive. Most of the relevant knowledge comes from the yeast *S. cerevisiae* and the fungal genus *Aspergillus*. **Skn7**, a TF involved in the osmotic and oxidative stress responses in *S. cerevisiae* [119], has also been identified in *Talaromyces* (formerly *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 [120]. This result indicates the highly conserved nature of *skn7* between the two organisms. Montibus et al. [121] suggested that *skn7* could be involved in the regulation of fungal secondary metabolism. A recent study seemed to confirm this hypothesis since the deletion of Af*skn7* resulted in a drastic decrease in aflatoxin B1 production in *Aspergillus flavus* [122]. **Yap1**, another TF, coordinates the interplay between oxidative stress and secondary metabolism. In *Aspergillus parasiticus*, the deletion mutant Δ*yap1* exhibited an increase in aflatoxin production [123]. The same team later reported that the suppression of the *yap1* orthologous gene led to increased OTA accumulation in *Aspergillus ochraceus* [124]. In *T. marneffei*, the mutant Δ*yapA*, a *yap1* orthologous gene, was found to be sensitive to oxidative chemicals such as H_2O_2 or menadione and featured growth, germination, and conidiation delays [125]. For the genus *Penicillium*, the only works on orthologues Skn7 and Yap1 are mentioned above; their roles in the secondary metabolism of *T. marneffei* have not yet been investigated.

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 widely studied, as well as many proteins that 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 remodeling [126]. Depending on the 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 light. VeA was first characterized in *A. nidulans*, whose gene encodes a protein of 573 amino acids with a conserved domain at the N-terminus [127] and a nuclear localization sequence (NLS) [128]. At its C-terminus, a PEST domain (rich in proline (P), glutamic acid (E), serine (S), and threonine (T)) is present [129]. This PEST domain is also found in VeA orthologous proteins in *A. parasiticus*, *A. fumigatus*, and *Neurospora crassa* [130].

Stinnett et al. [128] studied the intracellular localization of VeA. This study demonstrated that this localization 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 [131], 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 NLS, thus explaining the cytoplasmic localization of VeA. In the same study, it was demonstrated that the transfer of VeA into the nucleus depends on the importin α KapA and that a functional NLS is essential to allow the interaction of these two proteins.

To identify the proteins interacting with VeA, Bayram et al. [127] used the Tandem Affinity Purification (TAP) technique from a strain of *A. nidulans* expressing a VeA protein coupled to a 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, these analyses confirmed the interactions of VeA–VelB and VeA–LaeA; however, no interaction was 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, and 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. [127] demonstrated that VeA helps VelB to enter the nucleus to form the velvet complex.

The results obtained in the various studies allowed Bayram et al. [127] to propose a mechanism (Figure 4) that coordinates the regulation of sexual development and the production of secondary metabolites in *A. nidulans*. In the dark, the VelB/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.

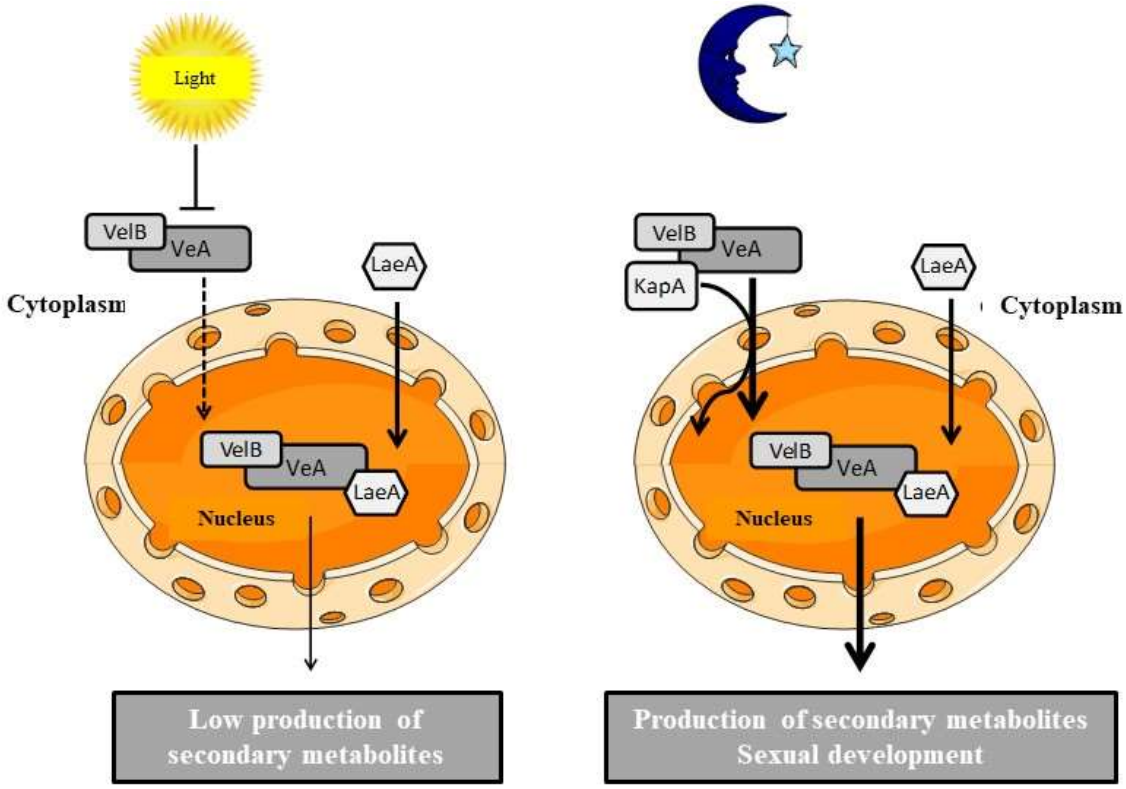


Figure 4. Operating model of the velvet complex in *Aspergillus nidulans* adapted from Bayram et al. [127]. In the presence of light, VeA is retained in the cytoplasm (-----), and LaeA has low activity. In the dark, VeA coupled to VelB is transported in the nucleus by the importin α KapA ( ), and the 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 fungal 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 [132–135]. Kato et al. [132] demonstrated that in *A. nidulans*, VeA regulates the expression of genes involved in sterigmatocystin synthesis. Indeed, VeA is necessary for the expression of *aflR*, which encodes the TF specific to the biosynthetic pathway of this mycotoxin [136]. Similarly, the *veA* gene is required for the transcription of *aflR* and *aflJ*, another gene coding for a TF, also located within the aflatoxin/sterigmatocystin cluster in *A. flavus* [137,138]. Other studies revealed that VeA is needed for the synthesis of other secondary metabolites, such as cyclopiazonic acid and aflatrem in *A. flavus* [133], penicillin in *A. nidulans* [132], or trichothecenes in *Fusarium graminearum* [139]. In this last study, FgVe1 was shown to be a positive regulator of the virulence of *F. 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 [140]. In *P. chrysogenum*, *veA* controls penicillin biosynthesis [141]. Recently, it was shown that the disruption of *veA* in *P. expansum* quasi-totally altered patulin and citrinin production when the fungus was grown on the usual mycological media (Malt Extract Agar and Potato Dextrose Agar). This decrease in production is explained by a drastic decrease in the expression of patulin and citrinin genes [142]. This finding was confirmed in vivo, as no patulin was detected when the null mutant was developed in apples. In the same study, an analysis of the impact of VeA on the expression of all secondary metabolism backbone genes in *P. expansum* was performed from the genome of the d1 strain, including PKS, NRPS, terpene synthase, and DMATS genes. The expression analysis showed a positive or negative regulation of 15/35 backbone genes and supports the hypothesis that *P. expansum*'s secondary metabolism is modulated by the transcriptional regulator factor VeA. In a recent study, Li et al. [143] assessed the involvement of the proteins VeA, VelB, VelC, and VosA, belonging to the velvet

family, in the regulation of patulin biosynthesis in *P. expansum*. The absence of VeA and VelB blocked the production of the mycotoxin, whereas the absence of VelC caused a drastic decrease in patulin production. In contrast, deletion of the *vosA* gene had no effect on the capacity of the fungus to synthesize patulin. These findings suggest the lack of involvement of VosA in the biosynthesis of patulin in *P. expansum* in contrast to the other three proteins (VeA, VelB, and VelC) of the velvet complex.

Baba et al. [144] also showed through gene deletion that *veA* plays critical roles in the production of the hypocholesterolemic lovastatin analogue compactin (ML-236B) in *P. citrinum* by controlling the expression of *mlcR*, the pathway-specific activator gene for compactin biosynthesis.

It was also shown that different components of the velvet complex may play opposite roles in the regulation of secondary metabolism. In *P. chrysogenum*, PcVelC, together with the velvet PcVeA (orthologue of VeA in *P. chrysogenum*) and the methyltransferase PcLaeA, induced penicillin production, and, in contrast, PcVelB acted as a repressor [141,145].

Under the conditions tested by Kosalkova et al. [146], LaeA controlled some secondary metabolism gene clusters in *P. chrysogenum*. Its overexpression resulted in a four-fold increase in *pcbC* and *penDE* expression, leading to a 25% increase in gene expression in penicillin biosynthesis, while its suppression significantly reduced the expression of these genes. In contrast, the absence of an expression level difference (Δ*laeA* vs. wild type (WT)) for the *rpt* gene involved in the second step of the roquefortine biosynthetic pathway suggests that Pc*laeA* does not regulate the biosynthesis of roquefortine C. The regulation of the secondary metabolism of *P. expansum* by *laeA* was investigated from two cultures on different media [147]. Of the 54 backbone genes examined, many appeared to be positively regulated by *laeA*, such as those involved in the biosynthesis of roquefortine C, an unknown ETP-like metabolite, and patulin. In *Penicillium oxalicum*, it has been shown that the putative methyltransferase LaeA controls, among other things, the expression of some secondary metabolic gene clusters [148]. However, the cluster predicted to be involved in roquefortine C/ meleagrin/oxaline biosynthesis was not affected by the suppression of the *laeA* gene in *P. oxalicum*. The difference observed between these studies regarding *laeA* regulation of the genes involved in the biosynthesis of roquefortine C could be due to the species used and the medium tested.

Zhu et al. [149] demonstrated the role of *laeA* in secondary metabolism regulation, conidial production, and stress responses in *P. digitatum*. The deletion of Pd*laeA* resulted in decreased expression of various secondary metabolite gene clusters, including the *Tq* cluster involved in tryptoquialanine biosynthesis. Deletion of this gene also affected the expression of several regulators of conidiation, including BrlA. A comparison between the WT and the null mutant PdΔ*laeA* strains revealed increased sensitivity of the null mutant strain under alkaline conditions. The loss of Pd*laeA* had no significant effect on the virulence of the null mutant strain. This work showed the involvement of *laeA* in the biosynthesis of several secondary metabolites, as well as the development and the adaptation of *P. digitatum* to its environment.

Yu et al. [150] showed that the overexpression of LaeA in the *Penicillium dipodomyis* marine-derived strain YJ-11 leads not only to morphological but also metabolic changes. Overexpression mutants displayed the ability to produce several sorbicillinoids, two of which are new compounds, as well as four known sorbicillin analogues. These results indicate that LaeA plays a key role in the activation of cryptic genes that are silent under normal *laeA* expression.

Kumar et al. [151] showed the effects of the intrinsic factors of apples in modulating patulin accumulation and on *laeA* and *pat* gene expression in apples colonized by *P. expansum*. The authors used two apple varieties, Golden Delicious and Granny Smith, which have similar total soluble solid value profiles at the time of ripening but different pH values and malic acid concentrations. These factors differentially affected the expression of LaeA along with the expression of the patulin cluster genes and, therefore, patulin accumulation. To understand the complexity of these interactions, in vitro studies were performed. These studies proved that sucrose and malic acid concentrations and pH are all involved, in association with chlorogenic acid and epicatechin, in a complex interaction system that modulates the regulation and production of patulin.

Penicillium brocae HDN-12-143 is a fungus isolated from marine sediments that has strong potential for the biosynthesis of secondary metabolites. Wang et al. [152] studied the effect of overexpression of the *laeA* gene on the secondary metabolism of *P. brocae*. This overexpression revealed that four compounds could be isolated, including fumigatin chlorohydrin and a new polyketide compound, iso-fumigatin chlorohydrin. In summary, the results indicate that LaeA can suppress or activate the expression of gene clusters and that its overexpression can induce the production of new secondary metabolites.

In *Aspergillus*, VeA is responsible for the activation or repression of general genes such as *brlA* [134,153]. **BrlA** is a C_2H_2-type zinc finger TF which is part of the central regulatory pathway (CRP) controlling the expression of genes specific to asexual reproduction. The conformation of *brlA* is complicated and comprises two overlapping transcription units, *brlAα* and *brlAβ* [154]. Expression of the *brlA* gene was studied in *P. oxalicum* strains, initially identified as *Penicillium decumbens,* by Qin et al. [155], and the expression levels of 7/28 gene clusters of secondary metabolism were regulated in a Δ*brlA* deletion strain. The cluster involved in the roquefortine C/meleagrin/oxaline biosynthetic pathway was downregulated. In a *P. chrysogenum brlA*-deficient mutant, the production of penicillin V was not affected, whereas a reduction of almost 99% was determined via HPLC analysis accompanied by a drastic downregulation of the expression of penicillin biosynthetic genes in a *stuA*-deficient strain [156]. Moreover, the deletion of *laeA* reduced the conidiation in *P. oxalicum*, and the expression of *brlA* was downregulated [148]. A recent study in *P. expansum* showed that the *brlA* gene not only affects the stage of conidiation of the fungus but also affects the biosynthesis of secondary metabolites. Zetina-Serrano et al. [157] showed that the suppression of *brlA* results in a strain devoid of conidia and that the production of communesins and derivatives was drastically decreased, whereas the production of chaetoglobosins and derivatives increased. Neither patulin nor citrinin production was affected by the suppression of *brlA*.

PcRFX1 is a TF that was characterized in *P. chrysogenum* by Domínguez-Santos et al. [158]. PcRFX1 is the orthologue of the regulatory proteins CPCR1 and RFxA in *Acremonium chrysogenum* and *T. marneffei*, respectively. Knockdown and overexpression techniques of the Pc*rfx1* gene have proven that PcRFX1 regulates *pcbAB*, *pcbC,* and *penDE* transcription and thereby controls penicillin biosynthesis. PcRFX1 was also suggested to be involved in the control of the pathways of primary metabolism.

PcFKH1, another TF 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 [159].

The ***pcz1*** gene (*Penicillium* C6 zinc domain protein 1), encoding a Zn(II)2Cys6 protein and controlling the growth and development processes of the fungus, has also been described in *P. roqueforti*. It was suggested to participate in the physiological processes in this fungus and plays a key role in regulating its secondary metabolism [160,161]. The silencing of *pcz1* in *P. roqueforti* resulted in the downregulation of the *brlA*, *abaA,* and *wetA* genes of the CRP [160]. In *pcz1* downregulated strains, the production of the metabolites roquefortine C, andrastin A, and mycophenolic acid was severely reduced; however, when *pcz1* was overexpressed, only mycophenolic acid was overproduced, and levels of roquefortine C and andrastin A were decreased [161].

Finally, the Pox*nsdD* gene of *P. oxalicum* was characterized by He et al. [162]. This gene is an orthologue of the ***nsdD*** gene (initially isolated in *A. nidulans*) encoding a GATA-type zinc finger TF that was proven to be involved in the production of secondary metabolites. In the Pox*ΔnsdD* strain, the 230 differentially expressed genes identified covered 69 putative BGCs. Among them, 11 were predicted to produce aspyridone, emericellin, citrinin, leucinostatins, roquefortine C/meleagrin, beauvericin, cytochalasin, malbrancheamide, and viridicatumtoxin.

2.3. Signal Transduction Pathways

In general, fungi present a very dynamic and 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 [163–165]. All these factors influence the biosynthesis of secondary metabolites in the fungus. Numerous signaling pathways activate and regulate the growth and differentiation of filamentous fungi and initiate secondary metabolite biosynthesis under specific conditions. These signaling 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 secondary metabolites. The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), calcineurin/calmodulin, TOR, and mitogen-activated protein kinase are the most studied pathways [34]. The production of many secondary metabolites has been associated with one of these transduction signals and specific active molecules. Among the different signaling pathways listed, we focus on those that affect only the secondary metabolism of *Penicillium*, starting with the cAMP pathway.

2.3.1. cAMP Pathways

Heterotrimeric G proteins are considered to be important components of these signal transduction pathways. They can integrate a variety of signals and then transduce them to downstream signaling cascades. Most filamentous fungi have three Gα proteins belonging to classes I, II, or III [166]. Gα subunits belonging to class I are 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 genes encoding class II Gα proteins showed negligible effects on fungal metabolism [167,168], while those of class III are involved in fungal development and pathogenicity [169–171]. Alterations have been observed in the secondary metabolism of different fungi, including *P. chrysogenum* [172] and *T. marneffei* [173]. The *pga1* gene, encoding subunits of subgroup I Gα protein in *P. chrysogenum*, has been shown to affect the production of three secondary metabolites: penicillin, chrysogenin, and roquefortine C. The deletion of *pga1* induces a decrease in the production of roquefortine C 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 upregulated by the presence of a dominant activating *pga1* (G42R) allele or a constitutively active Pga1 [172]. Based on a proteomic analysis, Carrasco-Navarro et al. [174] suggested that Pga1 signaling affects penicillin biosynthesis by acting on the 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.

2.3.2. The Osmostress Response Pathway

Usually, inhibition of the HOG (high osmolarity glycerol) signaling pathway negatively affects the production of metabolites; in other words, challenging osmotic conditions activate the cascade of the HOG MAP kinase signal, thereby activating several osmo-regulated genes or downstream TFs by phosphorylation. In their study, Stoll et al. [175] showed that NaCl induced production of OTA in correlation with the phosphorylation status of the HOG MAP kinase in *P. nordicum* and *Penicillium verrucosum*. The activation of HOG phosphorylation and the concomitant OTA biosynthesis suggest a link between the two processes and that this regulation may 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 OTA 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. [176], 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 OTA 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 signaling pathway.

2.4. Epigenetic Regulation

As previously discussed, *Penicillium* species, including other fungi, produce a set of bioactive secondary metabolites that are not essential to their survival. Genes for biosynthesis and the regulation of secondary metabolites in fungi are not evenly distributed over the genomes and tend to be sub-telomerically located [177]. The manipulation of global epigenetic regulators has contributed to the study of many unknown secondary metabolites, and many histone modifications have been associated with the regulation of secondary metabolism gene clusters [178,179]. The epigenetic phenomena that can occur are reversible, and many changes in the gene expression levels of the fungus 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 their modifications, regulation can be limited to a specific region of the chromosome and, therefore, affect some genes. This supports the advantage of grouping secondary metabolism genes into clusters. The first involvement of the epigenetic regulation of secondary metabolites described in the literature was that of the *A. nidulans* histone deacetylase coded by *hdaA,* an orthologue of the histone deacetylase *hdaA1* gene of *S. cerevisiae*. Deletion of this gene caused the activation of two secondary metabolite gene clusters. In the same paper, treatment of the *P. expansum* culture with trichostatin A, a histone deacetylase (HDAC) inhibitor, resulted in the overproduction of several non-determined metabolites [179].

In *P. chrysogenum*, *hdaA* appears to be a key regulator of the secondary metabolism of the fungus. Deletion of *hdaA* induced a significant effect on the expression of numerous PKS and NRPS-encoding genes. A downregulation of the NRPS encoded gene associated with the BGC of chrysogine was also observed. This observation was confirmed by Ding et al. [180]. In parallel, transcriptional activation of the BGC of sorbicillinoids occurs, which is associated with the detection of a new compound produced only under these conditions. These results obtained by Guzman-Chavez et al. [181] suggest the existence of crosstalk between BGCs. In a recent study, the disruption of *hdaA* led to an upregulation of the meleagrin/roquefortine C biosynthesis gene cluster, accompanied by higher meleagrin production [180].

Akiyama et al. [182] investigated the involvement of *clr3* in *Penicillium brasilianum* physiology. **Clr3** is a homologue of the class 2 histone deacetylase *hda1* in *S. cerevisiae*. On the one hand, the deletion of *clr3* resulted in decreased fungal growth under oxidative stress conditions. In addition, various secondary metabolites, such as austin-related meroterpenoids, brasiliamides, cyclodepsipeptides, and mycotoxins, including verruculogen and penicillic acid, were downregulated in the null mutant Δ*clr3* strain. On the other hand, epigenetic modulation was studied using suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, and nicotinamide. These treatments also resulted in reduced secondary metabolite biosynthesis. Together, these findings suggest that *clr3* plays a key role in the regulation of secondary metabolism in *P. brasilianum*.

By growing *Penicillium variabile* on a maltose medium in the presence of 5-azacytidine (a DNA methyltransferase inhibitor), varitatin A synthesis was induced [183]. In addition, by growing it on a potato-based medium in the presence of SAHA, seven polyketides were induced, including three known wortmannilactones (E, F, and H), as well as new varilactones (A-B) and wortmannilactones (M-N) [184]. In cultures treated with 50 μM of 5-azacytidine, *Penicillium citreonigrum* formed exudates, which are droplets rich in primary and secondary metabolites, inorganic substances, and proteins/enzymes and are known as guttates. These exudates were very rich in different compounds compared to the control. Indeed, 5-azacytidine induced the formation of six azaphilones (fungal metabolites with diverse biological activities), pencolide, and two new meroterpenes [185]. The addition of 5-azacytidine to the culture medium of *Penicillium funiculosum* also altered the metabolic profiles of this fungus [186]. 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 HDAC inhibitor [187]. This led to the production of many compounds: eupenicinicols C and D, along with eujavanicol A and eupenicinicol A. El-Hawary et al. [188] showed that cultures of a marine-derived strain of *Penicillium*

brevicompactum exposed to nicotinamide and sodium butyrate result in the production of phenolic metabolites. In the presence of nicotinamide, many compounds, including *p*-anisic acid, benzyl anisate, syringic acid, and sinapic acid, were isolated and identified. Sodium butyrate also enhanced the production of anthranilic acid and ergosterol peroxide.

In one of the many studies to explore compounds with innovative structures and biological activities from endophytes of ancestral Chinese medicine, Guo et al. [189] used chemical epigenetic manipulation to evaluate the secondary metabolism of the *Penicillium herquei* strain, recovered from the fruiting body of *Cordyceps sinensis*. This latter has been used for thousands of years by the Chinese to boost longevity, endurance, and vitality. The DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine, affected the production of secondary metabolites, purifying three previously unpublished polyketides with a pyran-2-one scaffold.

Ying et al. [190] showed that cultures of *Penicillium* sp. HS-11, isolated from the medicinal plant *Huperzia serrata*, produced two compounds in the presence of SAHA: 4-epipenicillone B and (R)-(+)-chrysogine, which are both absent under normal laboratory conditions.

The addition of 500 μM of suberoyl bis-hydroxamic acid, 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 [191]. Xiong et al. [192] explored the role of the high-mobility group box protein, **PoxHmbB**, involved in chromatin organization and identified in *P. oxalicum*. The authors observed that conidiation and hyphae growth were delayed in a mutant Pox*ΔhmbB* strain. Pox*hmbB* regulated the expression of genes encoding plant biomass-degrading enzymes and other genes involved in conidiation. Although the suppression of the orthologous gene resulted in an absence of sterigmatocystin production in *A. nidulans* [193], the involvement of this protein in the secondary metabolism of *Penicillium* has not yet been investigated.

Tannous et al. [194] evaluated the involvement of the epigenetic reader SntB in the pathogenicity and secondary metabolism of *P. expansum*. Firstly, the results showed that the deletion of *sntB* caused numerous phenotypic changes in the plant pathogen. In the absence of *sntB*, *P. expansum* showed delayed vegetative growth, reduced conidiation, an accelerated germination rate, and decreased virulence in apples. Secondly, the data showed that *sntB* played a key role in regulating secondary metabolism, especially patulin and citrinin biosynthesis. In addition, the role of *sntB* in the positive regulation of three TFs of secondary metabolism and virulence (LaeA, CreA, and PacC) was demonstrated. Finally, this study revealed the downregulation of *sntB* in response to environmental factors such as low temperature and high CO_2 levels, conditions to which apples are subjected during storage. These findings suggest a possible method for integrating these epigenetic control strategies to fight post-harvest fruit rot.

Finally, the chromatin regulation of small molecule gene clusters allowed the specific control of secondary metabolism gene clusters and permitted filamentous fungi to modify chemical diversity and successfully exploit environmental resources. Epigenetic regulation is considered a promising strategy for investigating unknown secondary metabolite clusters, particularly because under certain laboratory culture conditions, many clusters can remain silent, making it difficult to elucidate their functions and regulatory mechanisms [195].

3. Conclusion

Fungal secondary metabolism is very broad, and this review focused on metabolism regulated in the *Penicillium* genus. Given the diversity of secondary metabolites, their key roles as virulence and pathogenicity factors, and their great medical and agricultural interest, further research should be conducted on these metabolites. This review highlighted how the production of these secondary metabolites is controlled and regulated. It discussed the different levels of regulation of secondary metabolites, including specific regulators, global TFs, transduction signaling pathways, and epigenetic regulation, as well as the combination of many different parameters affecting the biosynthesis pathways of metabolites. Many TFs that affect the expression of genes involved in secondary metabolism seem to

belong to the category of zinc-binding proteins. LaeA and the velvet complex proteins are considered to be global regulators and are able to control many clusters at the same time. Although much is known about these global TFs and their regulatory proteins, more research is needed to explore the details that link them to the transcription of genes involved in secondary metabolite biosynthetic pathways. This would help us to better understand the molecular mechanisms underlying this complex regulatory network. The analysis of a large number of works related to secondary metabolism regulation in filamentous fungi revealed great complexity. This complexity is suggested by the observation of inter-species differences in the impact of a given TF gene deletion on the same biosynthetic pathway.

The study of the regulation of secondary metabolite biosynthesis in *Penicillium* is much less advanced than that in *Aspergillus*, and some orthologous genes already studied in *Aspergillus* (including *rtfA*, *cpsA*, *rmtA*, *mtfA*) should be investigated in *Penicillium* sp.

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Abbreviations

BGC	Biosynthetic Gene Cluster
bZIP	Basic leucine zipper
CRP	Central regulatory pathway
DMATS	Dimethylallyl tryptophan synthase
ETP	Epipolythiodioxopiperazine
HDAC	Histone deacetylase
HOG	High osmolarity glycerol
NLS	Nuclear localization sequence
NRPS	Non-ribosomal peptide synthetase
OTA	Ochratoxin A
PKS	Polyketide synthase
SAHA	Suberoylanilide hydroxamic acid
TAP	Tandem Affinity Purification
TC	Terpene cyclase
TF	Transcription factor
WT	Wild Type

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2. The phytopathogenic fungus *Penicillium expansum*, secondary metabolism and regulation

2. *Penicillium expansum*

2.1 Morphological, physiological and biological characteristics

Taxonomically, *Penicillium expansum* belongs to the phylum Ascomycota, class Eurotiomycetes, member of the family Aspergillaceae and genus *Penicillium* (Houbraken et al. 2020; Li et al. 2020). *Penicillium* derives from the Latin "painter's brush", which refers to the morphology of its conidiophores that resemble the brush's bristles.

The phytopathogenic fungus *P. expansum* is a well-known and much studied species. When grown on a rich medium such as Malt Extract Agar (MEA) at 25 °C, colonies are 40-50 mm in diameter, have a velvety and granular texture with a blue-green color in the conidial areas and a white external margin (Figure 1a). They also present shallow radial grooves with small drops of exudate on the surface of the mycelium. The reverse side of the colony is pale or yellowish (Figure 1b). *In vivo*, *P. expansum* mycelium produces simple fused conidiophores (coremia) emerging from the hyphae that pierce the apple epicarp (Figure 1c).

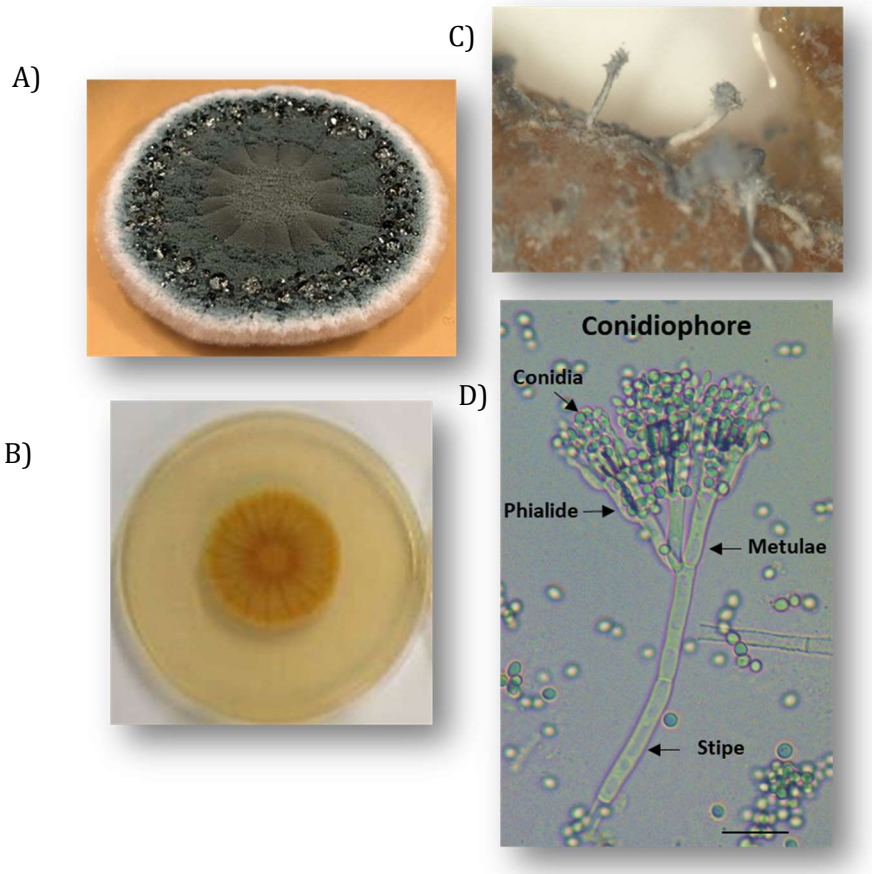


Figure 1. Macroscopic morphology of *Penicillium expansum* A) Recto B) Verso. C) Coremia, observation under stereomicroscope (×12). D) Microscopic appearance (×400). The strains were grown on MEA at 25 °C in the dark. Black scale bars represent 100 µm.

At a microscopic scale, *P. expansum* has aerial hyphae or conidiophores derived from basal hyphae (mycelium). Conidiophores are formed by smooth-walled, 200 to 500 µm long stipes that typically branch into terverticillate (*Penicilli* can be biverticillate, terverticillate and/or quarterverticillate) (Figure 1d) (Frisvad and Samson, 2004). Each branch has metulae from 12 to 15 µm long, phialides in the form of blisters up to almost cylindrical from 8 to 11 µm long, while conidia, packed in long chains, are smooth ellipsoid-circular walls from 3 to 3.5 µm long (Pitt and Hocking, 2009).

P. expansum is a psychrophilic fungus that can grow at low temperatures (the minimal temperature range is from -6 °C to -2 °C, according to the studies), its optimal growth is registered about 25 °C and the maximal growth temperature is at 35 °C. An acidic pH (4.0 to 5.0), low O_2 levels and high relative humidity (a_w 0.98) are required for mycelial growth and spore germination (Li et al. 2020; Pitt and Hocking, 2009).

In addition, the species *P. expansum* is described as a necrotrophic fungus for its ability to aggressively invade fruit tissue by killing host cells to obtain nutrients (Vilanova et al. 2014). It is a pathogenic fungus that develops during harvest, post-harvest processing and storage, causing tissue decay and resulting in up to 10% loss of harvested fruits (Li et al. 2015). *P. expansum* can be found in the natural environment, especially in the air and soil. It infects mainly pomaceous fruits like pears, quince and specially apples. Sometimes it can also be isolated from other commodities such as cherries, peaches, plums, nuts, pecans, hazelnuts, acorns, legumes, cereal grains, including non-consumable substrates such as building materials that contain cellulose (Andersen et al. 2004; Filtenborg et al. 1996; Moss, 2008; Tannous et al. 2018). It is considered the principal agent of blue mold disease in apples (Baert et al. 2007; Tannous et al. 2018).

The fungus invades the fruits through wounds caused by insects, birds or unfavorable weather conditions before harvest (early frost, excessive heat, dryness), but also as a result of mechanical damage caused by rough handling during harvest and fruit transport (Li et al. 2020; Tannous et al. 2018). Infection also occurs via natural openings like lenticels and stem ends (Luciano-Rosario, Keller, & Jurick, 2020; Rosenberger et al. 2006) Spores or conidia enter the fruit through a wound, germinate and then grow as hyphae forming the mycelium that invades the mesocarp. When the fruit is completely colonized and the pulp entirely consumed, the fungus drills the epicarp, giving rise to thousands of spores during asexual reproduction (detailed in the following section). Symptoms of blue mold disease appear as smooth, light brown, concentric circles where lesions spread rapidly over the surface and within the fruit tissue (Figure 2).



Figure 2. Golden Delicious apple infected by *P. expansum* after 5, 14, 20 and 35 days of incubation (from left to right) at 25 °C in the dark.

2.2 Production of secondary metabolites

In foods and plants under appropriate conditions, *P. expansum* is able to produce a large number of bioactive molecules called secondary metabolites (SMs) (Keller, 2019; Tannous et al. 2018). SMs have specific functions, such as virulence or aggressiveness factors, weapons or communication signals during their coexistence with other microorganisms, protection against damage caused by UV light or other abiotic stress. These metabolites have diverse biosynthetic origins leading to a wide structural diversity. This broad diversity could explain their large spectrum of activities and functions. (Bills and Gloer, 2016; Calvo et al. 2002; Keller, 2019; Macheleidt et al. 2016). Table 1 details the SMs produced by *P. expansum* according to the literature.

Table 1. Secondary metabolites produce by *Penicillium expansum* strain.

SECONDARY METABOLITES	BACKGROUND	MOLECULAR FORMULA	ADVERSE TOXIC EFFECTS OR USE	REFERENCE
N-ACETYLTRYPTAMINE		$C_{12}H_{14}NO_2$	Serotinin inhibitor	Kozlovskii et al. 2002
ANDRASTIN A	Terpenes	$C_{28}H_{38}O_7$	Antitumoral compound, antitrypanosomal activity	Kim, et al. 2016
ANDRASTIN B	Terpenes	$C_{28}H_{38}O_7$	Antitumoral compound	Kim, et al. 2016
ANDRASTIN C	Terpenes	$C_{28}H_{38}O_7$	Antitumoral compound	Kim, et al. 2016
AURANTIOCLAVINE		$C_{15}H_{18}N_2$		King et al. 1977
CHAETOGLOBOSIN A	Hybrid polyketide-nonribosomal peptides	$C_{32}H_{36}N_2O_5$	Cytotoxicity	Andersen et al. 2004; Larsen et al. 1998
CHAETOGLOBOSIN A	Hybrid polyketide-nonribosomal peptides	$C_{32}H_{36}N_2O_5$	Cytotoxicity	Andersen et al. 2004
CITRININ	Polyketide	$C_{13}H_{14}O_5$	Genotoxic, immunotoxic, teratogenicity, cytotoxic,	Andersen et al. 2004 ; Harwing et al. 1973
COMMUNESIN A	Nonribosomal Peptides	$C_{28}H_{32}N_4O_2$	Cytotoxic, insecticidal activity	Andersen et al. 2004; Hayashi, et al.

				2004; Lin et al. 2015
COMMUNESIN B	Nonribosomal Peptides	$C_{32}H_{36}N_4O_2$	Cytotoxic, neurotoxic, insecticidal activity	Andersen et al. 2004; Hayashi et al. 2004; Lin et al. 2015
COMMUNESIN C	Nonribosomal Peptides	$C_{31}H_{34}N_4O_2$	Cytotoxic, insecticidal activity	Hayashi et al. 2004; Lin et al. 2015
COMMUNESIN D	Nonribosomal Peptides	$C_{32}H_{34}N_4O_3$	Insecticidal activity	Hayashi et al. 2004
COMMUNESIN E	Nonribosomal Peptides	$C_{27}H_{30}N_4O_2$	Insecticidal activity	Hayashi et al. 2004
COMMUNESIN F	Nonribosomal Peptides	$C_{28}H_{32}N_4O$	Insecticidal activity	Lin et al. 2015; Kerzaon et al. 2009
COMMUNESIN I	Nonribosomal Peptides	$C_{26}H_{30}N_4O$	Insecticidal activity	Lin et al. 2015
COMMUNESIN K	Nonribosomal Peptides	$C_{26}H_{30}N_4$	Insecticidal activity	Kerzaon et al. 2009
COM470	Nonribosomal Peptides	$C_{28}H_{30}N_4O_3$		Kerzaon et al. 2009
COM570	Nonribosomal Peptides	$C_{33}H_{38}N_4O_5$		Kerzaon et al. 2009
COM622	Nonribosomal Peptides	$C_{37}H_{42}N_4O_5$		Kerzaon et al. 2009
EXPANSOLIDES A / B	Terpenes	$C_{17}H_{22}O_5$		Andersen et al. 2004; Larsen et al. 1998 ; Massias et al. 1990
FUMARYL-D,L-ALANINE		$C_7H_9NO_5$	Antibiotic	Birkinshaw et al. 1942
GENTISYL ALCOHOL		$C_7H_8O_3$		Andersen et al. 2004
GEOSMIN	Terpenes	$C_{12}H_{22}O$	Relatively non-toxic	Mattheis and Roberts, 1992
PATULIN	Polyketide	$C_7H_6O_4$	Genotoxic, immunotoxic, teratogenicity, cytotoxic, cardiotoxic	Andersen et al. 2004; Frisvad and Filtenborg, 1983; Harwing et al. 1973; Puel et al. 2010
ROQUEFORTINE C	Nonribosomal Peptides	$C_{22}H_{23}N_5O_2$	Weak neurotoxin, low toxicity	Andersen et al. 2004 ; Frivad and Filtenborg 1983
ROQUEFORTINE D	Nonribosomal Peptides	$C_{22}H_{25}N_5O_2$		Andersen et al. 2004 ; Frivad and Filtenborg 1983

2.2.1 Patulin

Patulin is produced by several species including *Penicillium*, *Aspergillus*, *Paecilomyces*, and *Byssochlamys*, however, *P. expansum* is considered the main producer of patulin (Puel et al. 2010; Tannous et al. 2018). The compound patulin was isolated in the 1940s and due to the co-discovery of the compound by several groups, it was known under various names such as: clavacin, expansine, claviformin, clavatin, gigantic acid, and myocin C (Moake et al. 2005).

Patulin (4-hydroxy-4H-furo [3,2c] pyran-2[6H]-one) is a small toxic unsaturated lactone with the molecular formula $C_7H_6O_4$ and a molecular mass of 154.12 g/mol (Moake et al. 2005). It is soluble in water and organic solvents (ethanol, methanol, acetone, ethyl acetate, ether and

chloroform) but insoluble in pentane and hexane. Patulin has a melting point of about 110 °C and a maximum UV absorption (λ max) of 275 nm (Ciegler, 1977). It is a toxic SM, undetectable by taste and smell, found not only in apple fruits but also in apple-based products (juices, purees). Heat treatments do not affect the overall stability of this mycotoxin and long-term exposure to patulin-contaminated products can cause serious health disorders (Tannous et al. 2018).

Patulin was initially used for medicinal purposes due to its antibiotic properties, but soon after, it was shown that acute consumption of patulin could cause symptoms included agitation, convulsions, pulmonary congestion, edema, ulceration, hyperemia, intestinal inflammation, vomiting, gastrointestinal and kidney damage. Chronic exposure to patulin was associated with neurotoxic, immunosuppressive, cytotoxic, teratogenic and immunotoxic health risks (Glaser and Stopper, 2012; Pitt and Hocking, 2009; Puel et al. 2010). Patulin is also classified in group 3 (not classifiable as to its carcinogenicity to humans) of the IARC group due to its toxicity, maximum levels of patulin in food are regulated in most countries, including the United States, China, and the European Union. The maximum level of patulin in fruit juices is limited to 50 µg/kg, 25 µg/kg for solid apple products and 10 µg/kg for apple-based products for infants (EC Commission Regulation, 1881/2006).

The patulin biosynthetic pathway (Figure 3a) has been studied since the 1950s but is still not fully elucidated. It consists of 10 enzymatic steps at least (Artigot et al., 2009; Li et al. 2019; Puel et al. 2010; Li et al. 2020, Snini et al., 2014; Tannous et al. 2018):

1. One acetyl-CoA unit and three malonyl-CoA units are condensed by the multifunctional 6-methylsalicylic synthase (MSAS) to form 6-methylsalicylic acid.
2. 6-methylsalicylic acid is decarboxylated by the 6-methylsalicylic decarboxylase to form *m*-cresol.
3. Then, the methyl group of *m*-cresol is oxidized by the cytochrome P-450 CYP619C3 (*m*-cresol hydroxylase, PatH) to an aldehyde group to give *m*-hydroxybenzyl alcohol.
4. This step is followed by a hydroxylation reaction catalyzed by another cytochrome P450, CYP619C2 (*m*-hydroxybenzyl hydroxylase, PatI), that leads to gentisyl alcohol formation. CYP619C2 also catalyzes the hydroxylation of *m*-cresol to toluquinol, a branch-product.
5. Putative conversion of gentisaldehyde to gentisyl alcohol.
6. The next step leads to the formation of isoepoxydon.
7. The *patN* gene (idh) encodes an isoepoxydon dehydrogenase that is responsible for the conversion of isoepoxydon to phyllostine.
8. Conversion of phyllostine to neopatulin catalyzed by PatF (neopatulin synthase).
9. Conversion of neopatulin to (E)-ascladiol by a reduction NADPH-dependent.
10. The last step is carried out by PatE, a glucose-methanol-choline (GMC) oxidoreductase, to convert (E)-ascladiol to patulin.

As for many fungal SMs, genes involved in the biosynthesis of patulin are grouped into the same cluster (Ballester et al. 2015; Li et al. 2015; Tannous et al. 2014). The cluster of patulin comprises 15 genes (PEXP_094320-PEXP_094460) (Figure 3b), which are classified into three categories: transporters, transcription factor (TF) and biosynthetic enzymes (Ballester et al. 2015; Li et al. 2015; Tannous et al. 2014).

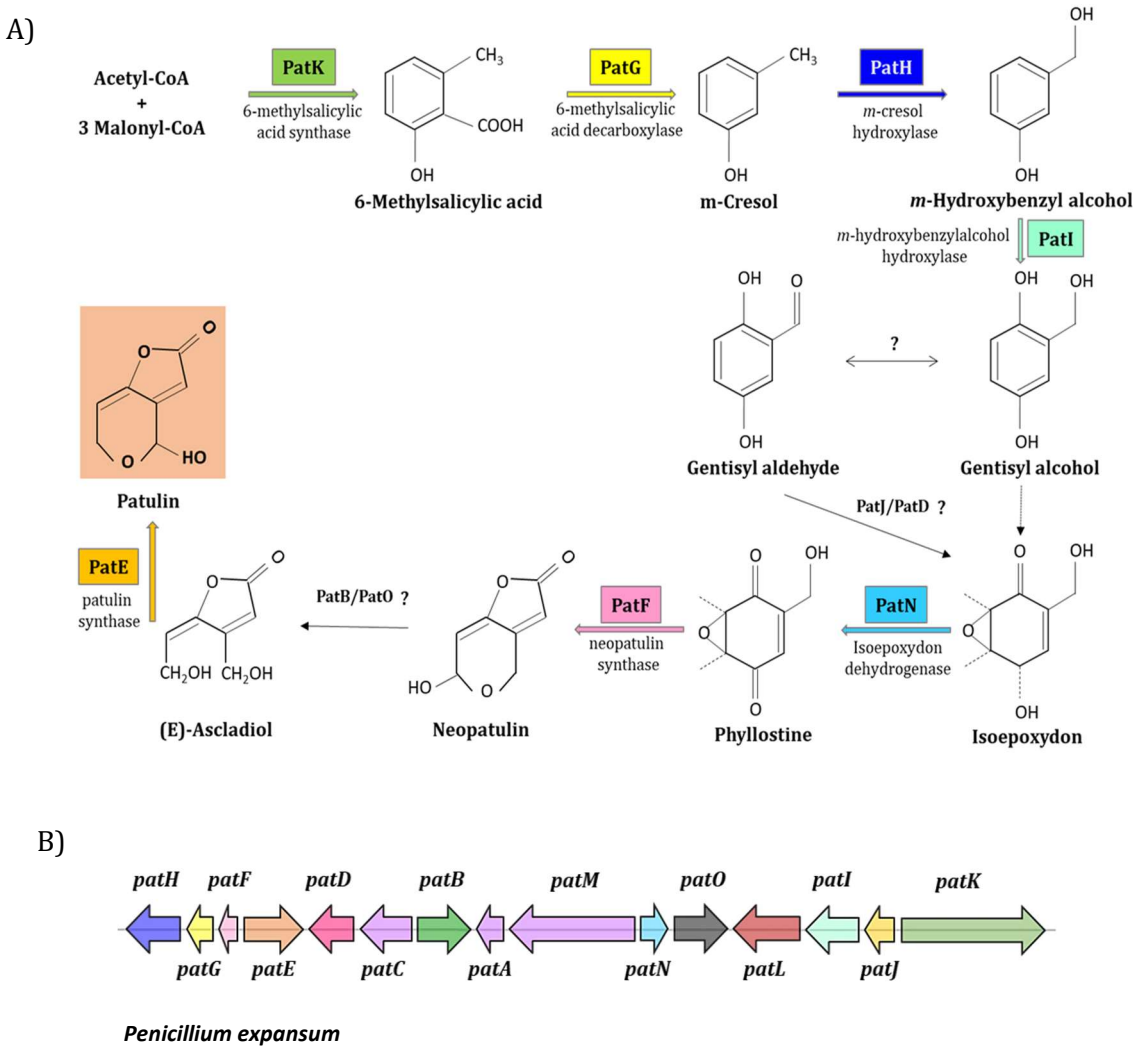


Figure 3. A) Patulin biosynthesis pathway (Adapted from Tannous et al. 2018). B) Schematic representation of the patulin gene cluster (15 genes, 40 kb) in *P. expansum*. The direction of transcription is indicated by arrowheads (Tannous et al. 2014).

Three genes encode transporters, PatA, member of the acetate transporters, PatC, major facilitator superfamily (MFS) transporter, and PatM, ATP-binding cassette (ABC) transporter, respectively. The gene *patL* encodes a putative TF located in the nucleus. Previous research has shown that the patulin biosynthetic gene cluster is regulated and activated specifically by PatL (PEXP_094430) (Ballester et al. 2015; Li et al. 2015; Snini et al. 2016). Eleven genes encode biosynthetic enzymes, PatB, PatD, PatF, PatG, PatK, and PatN, located in the cytosol, PatH and PatI,

located in the endoplasmic reticulum, PatJ and PatO located in the vacuole and PatE in the cell wall (Li et al. 2019). The function of four genes (*patB*, *patD*, *patO* and *patJ*) is still little known (Ballester et al. 2015; Li et al. 2015; Tannous et al. 2014).

Patulin production is also positively regulated by PacC and CreA, two TFs that respond to abiotic stimuli such as pH and carbon source, respectively (Barad et al. 2016; Tannous et al. 2018). LaeA (methyltransferase) and VeA (member of the velvet family), two components of the velvet complex have been reported as positive regulators of the patulin biosynthesis (Kumar et al. 2017; El Hajj Assaf et al. 2018). Tannous et al. (2020) have shown that the deletion of *sntB*, a gene coding for an epigenetic reader, resulted in a decreased patulin production in vitro and in planta.

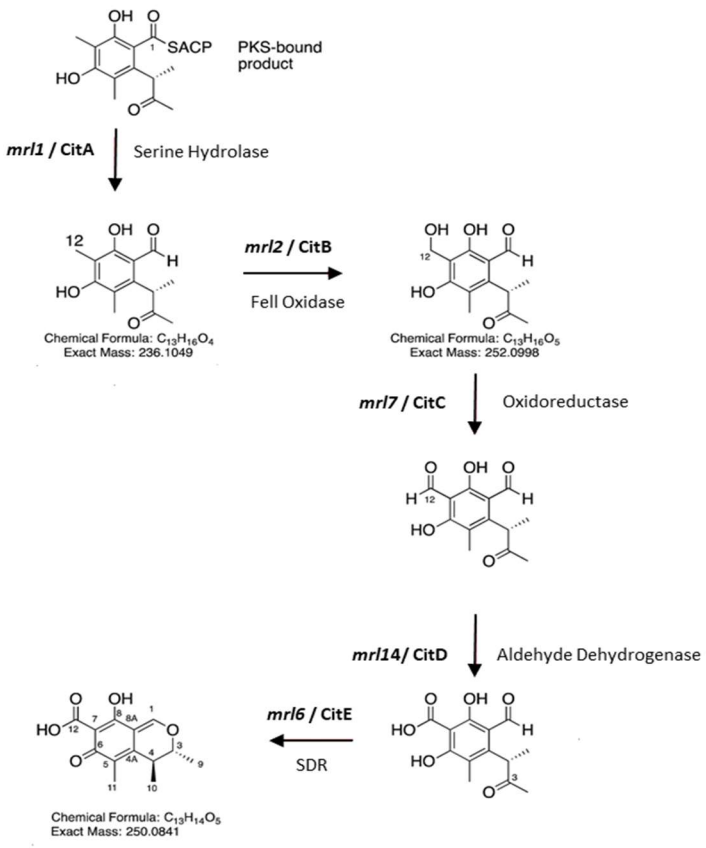
2.2.2 Citrinin

Citrinin was discovered in the 1930's by Hetherington and Raistrick (1931) and its structure was determined years later (Brown et al. 1948). Citrinin is a mycotoxin produced by *Aspergillus*, *Penicillium*, and *Monascus* species that displays a biosynthetic polyketide (PK) origin like patulin. The citrinin biosynthesis pathway (Figure 4a) was recently elucidated in *Monascus ruber* strain and involves the following steps (He and Cox, 2016):

1. The pathway begins with one acetyl-CoA unit and three malonyl-CoA units condensed to produce an unreduced trimethylated pentaketide by a non-reducing polyketide synthase (nrPKS) known as CitS.
2. The molecule involves a hydrolysis step catalyzed by CitA, resulting to a keto-aldehyde as the first intermediary in the biosynthesis pathway.
3. CitB, a non-heme iron oxidase that oxidizes the methyl of C-12 to an alcohol, carries out the next stage.
4. The next step is catalyzed by CitC, an oxidoreductase that oxidizes the alcohol of C-12 to an aldehyde
5. CitD, an aldehyde dehydrogenase, converts the C-12 aldehyde into carboxylic acid.
6. Finally, CitE carries out a reduction of C-3 resulting in the citrinin molecule.

The gene cluster for citrinin was discovered in *M. purpureus* (Shimizu et al. 2007) and recently, similar clusters have been discovered in *M. ruber* (He and Cox, 2016), *M. aurantiacus* (Li et al. 2012), and *P. expansum* (Ballester et al. 2015). The putative gene cluster in *P. expansum* comprises nine genes; *orf5* coding for a MFS transporter, *citS* encoding the PKS , *citA* and *citB* coding for an hydrolase and a dioxygenase, respectively, two genes coding for the dehydrogenases CitE and CitD, *citC* encoding an oxidoreductase , and one gene encoding the TF *ctnA*.

A)



B)

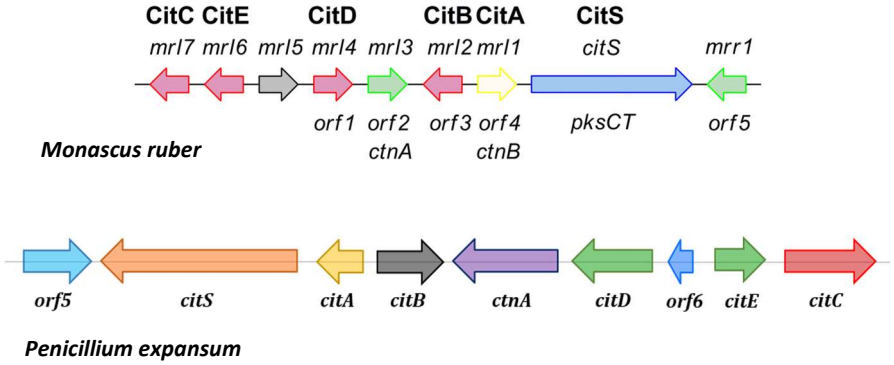


Figure 4. A) Citrinin biosynthesis pathway (He and Cox, 2016). B) Schematic representation of the citrinin gene cluster (nine genes, 22 kb) in *Monascus ruber* (He and Cox, 2016) and in *P. expansum* (Ballester et al. 2015). Arrowheads indicate the direction of transcription.

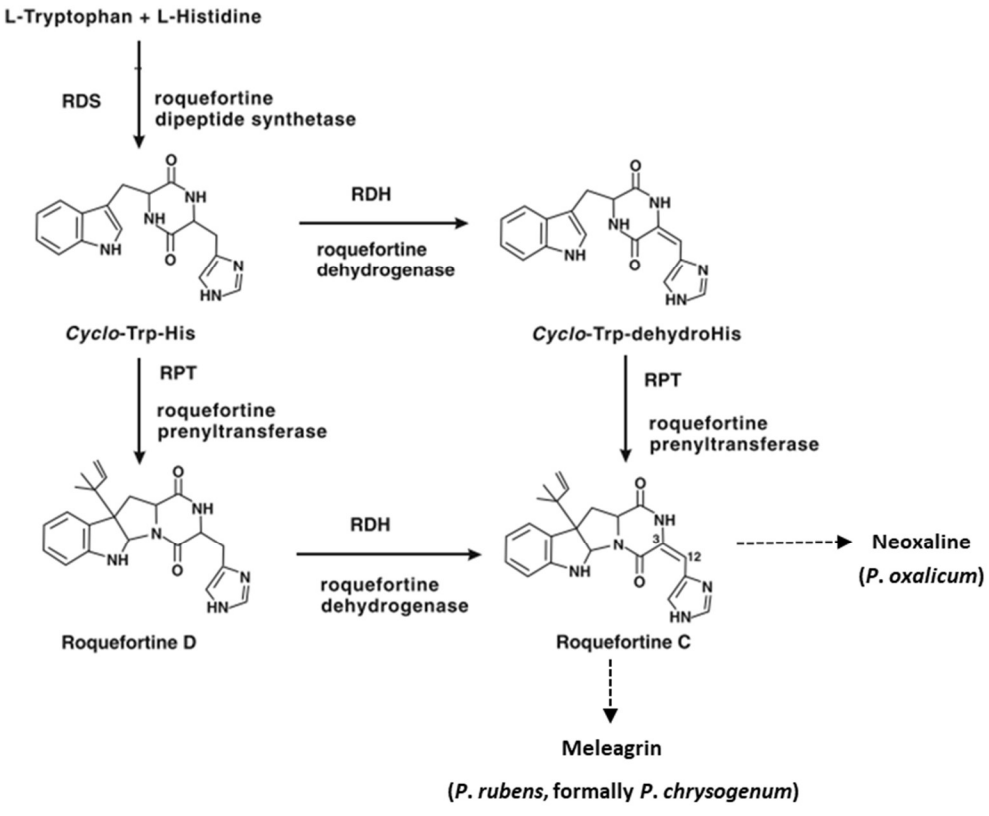
The production of citrinin in *P. expansum* is not necessary for colonization, but could have an auxiliary supportive functions during apple colonization and the formation of aerial mycelium (Touhami et al. 2018). Citrinin has antibacterial, antifungal properties and possible antiandrogenic and neuroprotective effects in vitro, but citrinin is mostly studied for its toxicity toward mammals (de Oliveira Filho et al. 2017; Flajs and Peraica, 2009).

2.2.3 Roquefortine C

Roquefortine C is a mycotoxin member of the indole alkaloid family, produced by several fungi, particularly species of the genus *Penicillium*. It has a Non-Ribosomal Peptides (NRP) origin. Roquefortine C is a weakly neurotoxic compound and has been described to have antibacterial activity, mainly against Gram-positive bacteria (Kopp-Holtwiesche and Rehm, 1990)

The biosynthetic cluster of roquefortine C was characterized in *Penicillium chrysogenum* (Ali et al. 2013), and recently it was identified in *P. expansum* (Banani et al., 2016; Martín and Liras, 2016). However, in the last species the number of genes involved in biosynthesis is lower than in the other species. The biosynthesis of roquefortine C derives from the condensation of the amino acids L-tryptophan and L-histidine to form a tryptophan-histidine cyclodipeptide containing a diketopiperazine ring, catalyzed by the roquefortine dipeptide synthetase (RDS) (Figure 5a) (Martin and Liras, 2015). The late reactions of the pathway convert the roquefortine-type skeleton into a different compound, meleagrin or neoxalin by a carbon skeleton reorganization. However, no meleagrin or neoxalin has been detected in *P. expansum*. The roquefortine C/meleagrin (R/M) gene cluster (Figure 5b) is only partially conserved in *P. expansum,* but it has a short group of four genes containing the three essential genes (*rds*, *rdh*, *rpt*) sufficient for the biosynthesis of roquefortine C and a MFS transporter gene *roqT* (Banani et al. 2016; Martin and Liras, 2015)

A)



B)

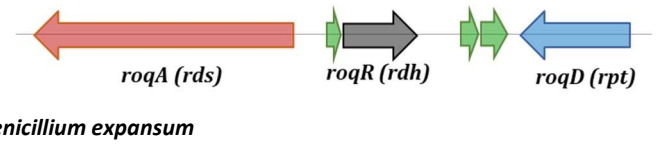


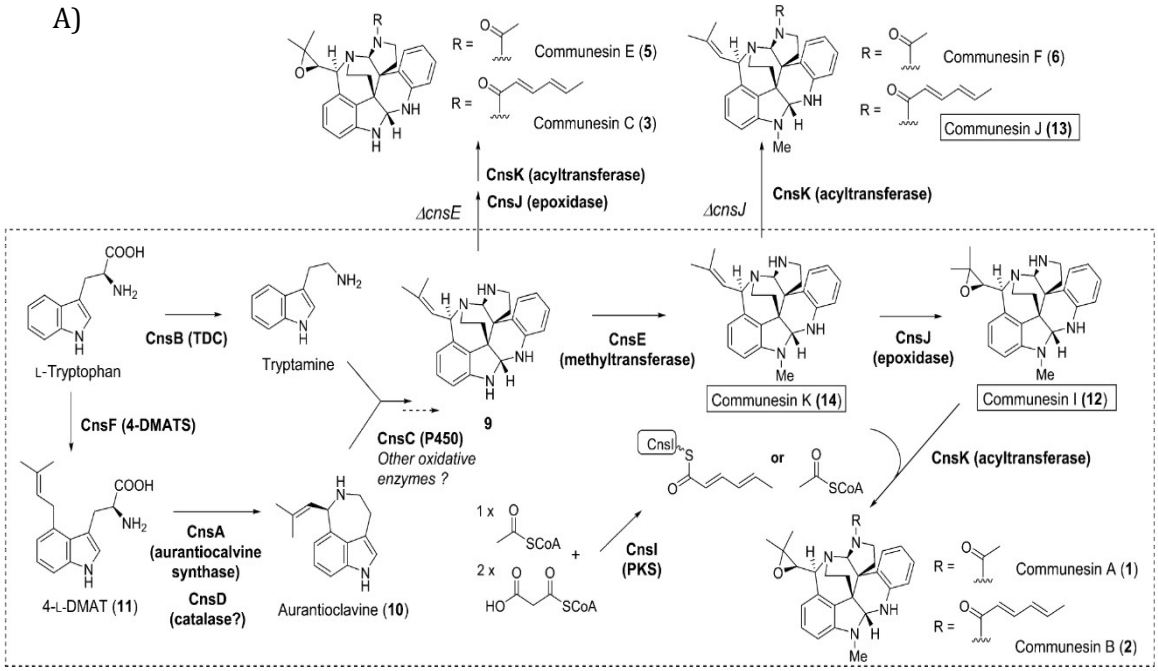
Figure 5. A) Roquefortine C biosynthesis pathway (Martin and Liras, 2015). B) Schematic representation of the roquefortine gene cluster in *P. expansum* (Banani et al., 2016; Martín and Liras, 2016). Arrowheads indicate the direction of transcription.

2.2.4 Communesins

Another SMs produced by *P. expansum* are communesins, which are also members of the indole alkaloid family. Different derivatives of communesins have been detected in *P. expansum*; to date eight communesins (A–H) have been identified, differing from each other by their three substituents R1, R2 and R3. Kerzaon et al. (2009) identified and characterized seven (Com470, Com570, Com524, Com622, Com512, Com644 and Com 522) new derivatives of communesins produced by *P. expansum,* using liquid chromatography analysis coupled to mass spectrometry (LC/ESI-MS/MS) or high resolution mass spectrometry (LC/HRMS/MS). Communesins showed anti-insecticidal activity against silkworms (Hayashi et al. 2004), but it has been reported that communesins A and B are the most biologically active, exhibiting moderate to potent cytotoxic activity in different cell lines (Numata et al. 1993).

The biosynthetic pathway of communesins was described in *P. expansum* (Figure 6a) (Lin et al. 2015). Communesins are biosynthesized by the coupling of tryptamine and aurantioclavine, two building blocks derived from L-tryptophan. The biosynthetic gene cluster comprises 16 genes, among which *cnsN* encodes a specific TF (Figure 6b) (Lin et al. 2015).

A)



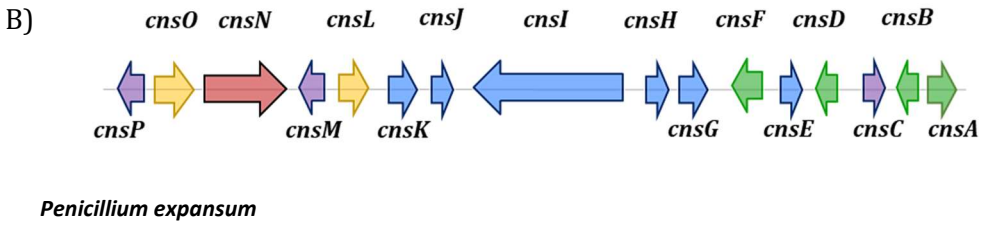


Figure 6. A) Biosynthetic pathway of communesins. The pathway in the dashed box is the main pathway leading to communesins A and B in *P. expansum*. B) Schematic representation of the communesin gene cluster in *P. expansum* (Lin et al. 2015). Arrowheads indicate the direction of transcription.

2.2.5 Chaetoglobosins

Chaetoglobosins are SMs belonging to cytochalasan alkaloids and have a polyketide/non-ribosomal peptide hybrid origin. A wide diversity of chaetoglobosins have been isolated and identified, mainly from the fungus *Chaetomium globosum*. Previous studies have shown that chaetoglobosins have a broad range of biological activities, including antitumor, antifungal, phytotoxic, fibrinolytic, antibacterial, nematicidal, anti-inflammatory, and anti-HIV activities (Chen et al. 2020).

Andersen et al. (2004) analyzed 260 strains of *P. expansum* from different substrates and geographical origins. They showed that *P. expansum* is a consistent producer of chaetoglobosins A and C. Chaetoglobosin A is biosynthesized by a hybrid polyketide synthase–non ribosomal peptide synthetase. Chaetoglobosin analogues have different degrees of oxidation introduced during the biosynthesis pathway (Ishiuchi et al. 2013). The biosynthetic gene cluster (Figure 7) was partially characterized in *P. expansum* and comprises seven genes (*cheA-cheG*) (Schümann and Hertweck, 2007). However, this cluster was not found in any of the *P. expansum* strains sequenced in the study of Ballester et al. (2015).

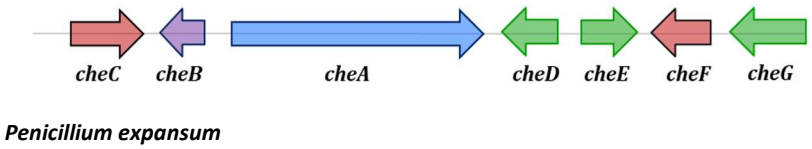


Figure 7. Schematic representation of the chaetoglobosin gene cluster in *P. expansum* described by (Schümann and Hertweck, 2007).

2.2.6 Andrastins

Andrastins are meroterpenoid compounds produced by several fungi of the genus *Penicillium* and are biosynthesized from terpene and polyketide. A wide diversity of andrastins have been isolated. *P. expansum* has been found to produce andrastin A, B, and C (Kim et al. 2016; Kim et al. 2012). Andrastin A has interesting biological activities that make it a promising antitumoral compound. It has been shown to inhibit the farnesyltransferase activity and that this inhibition would alter the membrane localization and activation of oncogenic Ras proteins (Shiomi et al. 1996). The andrastin gene cluster has been identified in diverse species including *Aspergillus oryzae* and *Penicillium chrysogenum* (Matsuda et al. 2013) and both clusters contain 11 genes (*adrA*-*adrK*). The putative andrastin gene cluster (Figure 8) has been proposed for *P. expansum* by Matsuda et al. (2016). The cluster is quite similar in terms of gene organization, but contains only 10 genes, with the *adrB* gene (encoding a protein with unknown function) not present in the cluster. Similar results were found in *Penicillium roqueforti* (Rojas-Aedo et al. 2017).

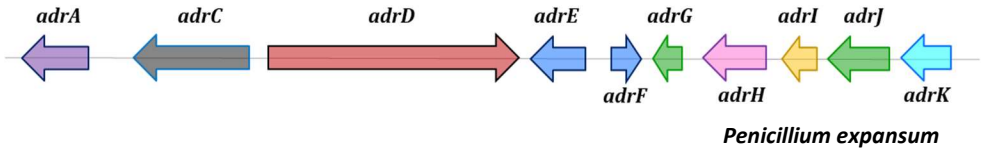


Figure 8. Schematic representation of the andrastin gene cluster in *P. expansum* (Matsuda et al. 2016). Arrowheads indicate the direction of transcription.

2.2.7 Geosmin

Geosmin is a SM member of the terpene family. This compound is mainly responsible for the rancid and earthy odor associated with *P. expansum* (Mattheis and Roberts, 1992). Numerous organisms including cyanobacteria and fungi produce geosmin. The gene cluster of geosmin has not yet been identified in *P. expansum*. Little is known about the biosynthesis of geosmin by *P. expansum*.

A proteomic study revealed the existence of glycolytic enzymes that could explain the different pathways (glycolytic, mevalonate, and methylerythritol phosphate pathways) of geosmin biosynthesis in *P. expansum* (Behr et al. 2014).

2.2.8 Expansolides

Expansolides are bioactive compounds that, like geosmin, belong to the terpene family. It is an SM produced by *P. expansum* (Andersen et al. 2004; Massias et al. 1990). Expansolides have been detected in rotten areas of the apple fruit (Watanabe, 2008) and in cherry juice (Larsen et al. 1998). The biosynthetic gene cluster as well as the toxicity of this metabolite have not yet been clarified.

2.3 Pathogenicity and control

P. expansum is the main agent of blue mold disease, causing severe financial losses worldwide. It produces mycotoxins and secondary metabolites that are linked to different stages of physiological development. Studies have suggested that patulin production may be linked to the pathogenicity and virulence mechanisms of the fungus. Considering that pathogenicity is the ability of an organism to induce host damage and that virulence indicates the degree of disease caused by the organism, some research has focused on studying the role of patulin in the pathogenicity of *P. expansum*.

Snini et al. (2016) created a strain lacking *patL* by homologous recombination and found that the null mutant strain Δ*patL* completely suppressed patulin production in *P. expansum*. The deletion also reduced the fungal virulence in apples inoculated with the null mutant strain. However, when patulin was added exogenously, a normal growth rate was restored, suggesting that patulin plays a role in the development of apple spoilage. This role is not essential since the null mutant is still able to colonize apple but with a lower growth rate. These observations are in agreement with reports that patulin is an important but not essential factor in the pathogenicity of *P. expansum* (Ballester et al. 2015; Sanzani et al. 2012; Snini et al. 2016). These results were also confirmed in Golden delicious apples infected with the null mutant strain PeΔ*veA*. Elimination of *veA*, involved in SM production, also suppressed patulin production and reduced the virulence of *P. expansum* (El Hajj Assaf et al. 2018). Snini et al. (2016) performed pathogenicity studies on 13 apple varieties, showing that both the null mutant PeΔ*patL* and the WT strains were able to infect apples. However, the intensity of the symptoms depended not only on the patulin production capacity but also on the genetic background of the apple, suggesting that patulin is a factor of aggressiveness rather than virulence. The stages of fruit ripening also influence the pathogenicity and virulence of the fungus, because a higher accumulation of patulin in ripe fruits infected by *P. expansum* was reported (Kumar et al. 2017). Several decades ago, conidiogenesis was linked to patulin production when a mutation at an early stage of conidiation caused a notable decrease in patulin production in *Penicillium griseofulvum* (syn=*P. urticae*) (Sekiguchi and Gaucher 1977).

The current information about other pathogenicity determinants are very scarce. Like other post-harvest pathogens, *P. expansum* secrets various virulence factors to kill host cells including cell wall degrading enzymes (CWDEs), proteases, and organic acids. A key factor in the pathogenicity of *P. expansum* is the secretion of pectolytic enzymes (Jurick et al. 2010). The action of one class of these enzymes, polygalacturonases (the most studied CWDEs), results in tissue maceration, accompanied by acidification, mainly caused by the production and secretion of gluconic acid and fumaric acid (Prusky et al. 2004; Vilanova et al. 2014). This acidification of apple tissue activates the transcription of polygalacturonases by the fungus and patulin production.

Other factors playing a role in virulence have been reported. PeSte12, the orthologue of *Saccharomyces cerevisiae* Ste12p was shown to play a role in virulence. The deletion of Pe*Ste12* gene led to lower rate of decay development when apple were stored at 0°C. The apple colonization by the mutant was not impaired when fruits were stored at 20°C (Sanchez-Torres et al. 2018). As much necrotrophic fungal pathogens, *P. expansum* secretes effectors. Among the most studied effector, Levin et al. (2019) focused on NRP1-like proteins. These proteins induce the necrosis and ethylene production by the host. Two NLP genes were found in *P. expansum* genome and were characterized. *Penlp1*, encoding a type 1 NLP, was shown to be mostly expressed during apple infection and its deletion led to decreased in virulence. PeNLP2, a type 3 NLP, does not seem to be involved in the pathogenicity. Its deletion does not provoke any reduction of in vivo growth rate and the highest levels of its expression were measured in the dormant conidia.

The role of Lysin motif (lysM) effectors in pathogenicity and virulence of several fungal pathogens has also been demonstrated (De Jonge et al. 2009; De Jonge et al. 2010). The effectors sequester the chitin oligosaccharides and prevent the pathogen-associated molecular patterns (PAMPs) triggered immunity. Chitin is the major component of fungal cell wall and chitin oligosaccharides, breakdown products of cell wall released during the fungal invasion, act as PAMPs in plant cells. Two independent studies reported 18 and 19 LysM genes in *P. expansum* genomes, respectively (Levin et al. 2017; Chen et al. 2020). Among them, four genes (Pe*LysM1*, Pe*LysM2*, Pe*LysM3*, Pe*LysM4*) were actively transcribed during apple infection (Levin et al. 2017). In order to characterize the function of these four proteins, the knockout mutants were generated. Only the deletion of *PeLysM3* affected the fungal development with a slightly lower growth rate on PDA medium, a lower germination rate and shorter germ tubes observed in the null mutant when compared to the WT strain. No deletion resulted in a significant effect on the in vivo growth rate. In the second study, the PeΔ*LysM12* mutant showed in vivo reduced lesion diameters when compared to the WT strain.

In a recent study, 17 genes were predicted to be potentially involved in *P. expansum* pathogenicity. Among them, the Pe*prt* gene encoding a subtilisin–related peptidase S8 is highly expressed during apple infection at 24 h post-inoculation (Levin et al. 2019). The macro- and microscopical morphologies of knockout mutant were altered with a significant reduction in spore production. The mutant culture showed a much less number of conidiophores accompanied by short chains of conidia when compared to the WT culture. This reduction of conidiation was already observed in *A. fumigatus* Δ*alp2* deficient mutant. The *alp2* gene is the orthologous Pe*prt* gene (Reichard et al. 2000).

A random mutation approach enabled to highlight Blistering1, a protein with a DnaJ domain involved in the secretion of carbohydrate enzymes essential for degrading plant cell walls (Jurick et al. 2020). The loss of the *blistering1* gene resulted in a reduced virulence, a 30 times less patulin

production and prevented the secretion of three enzymes encoded by *pat* genes (*patB*, *patO* and *patE*).

Due to the toxicity of patulin, new technologies both for the control of *P*. *expansum* and/or for the detoxification of patulin-contaminated foods were recently summarized (Li et al. 2020; Moake et al. 2005; Tannous et al. 2018; Zhong et al. 2018). Since apple products are mainly produced from stored apples, pre- and post-harvest strategies are important to mitigate patulin. From storage to processing, mitigation strategies for *P. expansum* have been developed. New control technologies include physical treatments, biological control, antifungal compounds and proteins, induced resistance and natural chemical compounds (Li et al. 2020; Zhong et al. 2018).

Wang et al. (2018) studied the anti-fungal power of the combination of cinnamaldehyde and citral (Cin/Cit) in *P*. *expansum*. They observed that the combination Cin/Cit could slow down or totally inhibit the colony extension, mycelia biomass accumulation and spore germination of *P*. *expansum*. The inhibitory effects are related to the dose and treatment duration. This finding suggests that Cin/Cit could be a potential candidate to control *P*. *expansum*

The best results have been observed for biological control, for example, the efficacy of *Pichia caribbica* in controlling blue mold decay produced by *P*. *expansum* was investigated by Cao et al. (2013). They showed that germination of spores and growth of *P*. *expansum* were inhibited by *P*. *caribbica* under in vitro condition. The in vivo study showed that the incidence of blue mold decay of apples treated by *P*. *caribbica* was significantly reduced and the production of patulin was controlled. In addition, *P*. *caribbica* was able to degrade patulin directly. Other treatments such as the use of salicylic acid or natamycin also reduced the disease caused by *P*. *expansum* by 100% (Li et al. 2020).

In addition to the control on *P. expansum*, new technologies including physical methods, chemical methods and biological methods have also been developed for the detoxification of foods derived from patulin-contaminated fruits (Li et al. 2020; Moake et al. 2005; Tannous et al. 2018; Zhong et al. 2018). Some methods such as absorption, irradiation, detoxification by ozone and the use of biological organisms reduce from 90% to 100% the initial concentration of patulin (Li et al. 2020). However, some physical and chemical methods affect the nutrients and taste of the products (Tannous et al. 2018), therefore new methods to reduce patulin contamination that do not alter the sensory standards of the product are still being investigated.

3. Conidiogenesis in filamentous fungi

3. Conidiogenesis

3.1 Asexual reproduction in filamentous fungi

Sexual and asexual reproduction are mechanisms of evolution and survival used by a large number of species. In filamentous fungi asexual reproduction or conidiation involves the formation of differentiated multicellular structures, called conidiophores, which produce pigmented haploid conidia at precisely programmed times (Mooney and Yager, 1990). The conidiation process begins with the growth of the aerial hyphae, followed by the formation of well-structured conidiophores and ends with the maturation of conidia that disperse easily by air stream (Adams et al. 1998; Ruger-Herreros and Corrochano, 2020).

Conidiation is regulated by several environmental factors including aeration, nutrient limitation, and the circadian clock. Previous studies have shown that conidiation is strongly inhibited in cultures submerged and that it only occurs when the mycelium are exposed to an air interface (Mooney and Yager, 1990). In addition, different regulatory pathways involving regulatory proteins, and signaling pathways that regulate and act in the different stages of conidiation were proposed (Mirabito et al. 1989; Ruger-Herreros and Corrochano, 2020).

Neurospora crassa (Ruger-Herreros and Corrochano, 2020; Ruger-Herreros et al. 2011) and members of the genus *Aspergillus such as A. nidulans, A. fumigatus*, and *A. flavus* have been used for a long time as model organisms to study the asexual development processes (Ojeda-López et al. 2018).

3.2 *Phases in conidiophore germination*

In filamentous fungi, vegetative growth begins with the germination of a spore. The process of development is programmed with accuracy from the germination to the formation of conidiophores, to be regulated at the right time and space of the life cycle of the fungus (Park and Yu, 2012). The presence of nutrients including inorganic salts, sugars and amino acids as well as water and air is required for proper conidial germination (Baltussen et al. 2020). The initiation of asexual reproduction or conidiation can take place in response to different internal (fungal development) or external (nutrient deficiency) stimuli (Baltussen et al. 2020).

In the genus *Aspergillus*, the formation of asexual spore structures called conidiophores occurs in different stages (Figure 1a) (Adams et al. 1998; Park and Yu, 2012, 2016):

I. Under proper conditions, the spore germinates and develops tubular hyphae that grow in a polar form, which branch out to form a network of interconnected cells called mycelium.
II. In older mycelium, vegetative development stops and foot cells begin to form. These cells are attached to the vegetative hyphae and have a wall of two layers, the first or external

layer is connected to the mycelium, while the second or internal layer forms an aerial hyphae called conidiophore stalk.

III. When the extension of the aerial stalks stops, the tip of the stalk begins to swell and forms a multinucleated vesicle. The cell of the foot, the stalk and the vesicle form a complete unit.

IV. On the surface of the vesicle, the metulae are formed and subsequently the phialides. Finally, the phialides undergo repeated asymmetric mitotic division to generate conidial chain.

V. Conidium requires a maturation process involving changes in cell wall structure and chemical composition (Figure 2a) (Ni et al. 2010). In a first step, the conidia are separated from the phialide and form two layers of cell wall, the external layer (C1) and the internal layer (C2). Subsequently the C2 layer is condensed and the C1 layer becomes a crenulated rodlet layer (Figure 6b-WT). Finally, between layers C1 and C2 a third layer of cell wall is formed (C3), while inside the spore a fourth (C4) and last layer is created. The C4 layer gives the mature conidium protection, making it impermeable and inactive under difficult conditions.

The model fungus *Aspergillus nidulans* was used as a basis for deciphering the process of conidiogenesis in the genus *Penicillium*. Different stages in the formation of conidiophores in *Penicillium* have been identified (Figure 1b) (Roncal and Ugalde, 2003):

I. Spore germination and mycelium formation. Vegetative hyphae resulting from apical growth stop their extension and begin the development of the conidiophore.

II. When vegetative growth stops, aerial hyphae begin to develop, delimited by a swelling septum, and in turn form subapical branches.

III. The subapical buds formed begin to differentiate into phialides.

IV. Finally, at the tip of the phialides, a series of conidia sprout, giving rise to the formation of characteristic brush structures called penicilli.

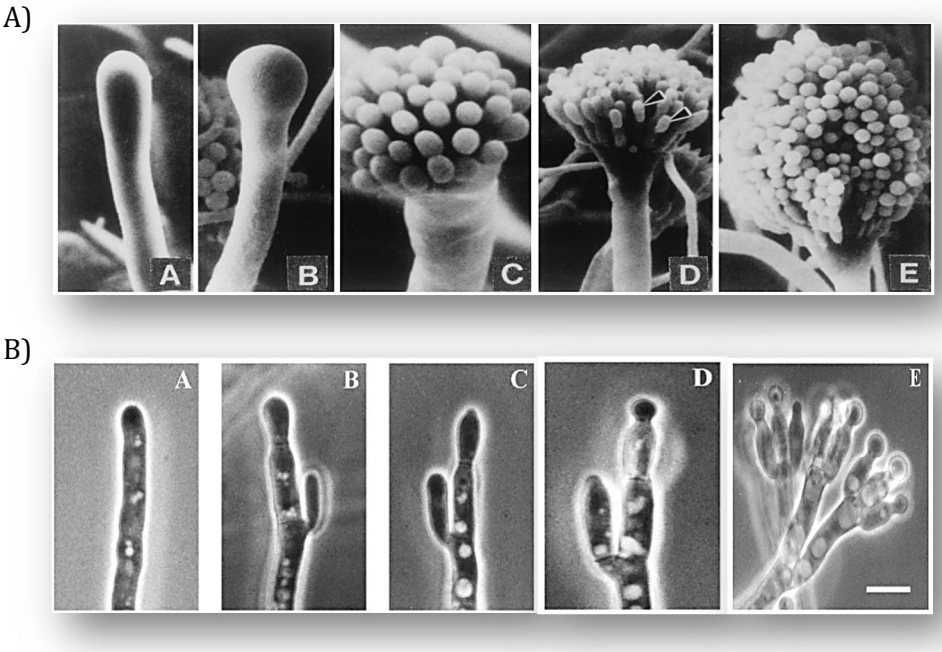


Figure 1. Stages of morphological changes during conidiation A) Development in *A. nidulans* (Adams et al. 1998); (A) Early conidiophore stalk. (B) Vesicle formation from the tip of the stalk. (C) Developing metulae. (D) Developing phialides. (E) Mature conidiophores bearing chains of conidia. B) Development in *P. cyclopium* (Roncal and Ugalde, 2003); (A) Vegetative hypha. (B) Apical cell swelling and subapical branching. (C) Phialide formation. (D) Conidium formation. (E) Penicilli. Scale bar: 10 µm.

The first conidiophores are formed in the center of the colony and their formation extends to the margin of the colony, leaving the older conidiophores in the center and the newly formed conidiophores at the margin of the growing colony (Adams et al. 1998).

Mature conidia are dispersed through air, water and soil remaining in a phase called "dormant conidia" in which the conidia enters a lethargy phase until it finds a favorable substrate and/or the vegetative development phase begins again (Baltussen et al. 2020). As mentioned previously, mature conidia are conformed of multiple layers that provide protection in the dormant phase. The resistance of conidia to desiccation is due in part to the presence of a hydrophobic layer (Figure 2b) called "rodlet layer" composed of proteins from the hydrophobin family that covers the surface of the conidia (Baltussen et al. 2020). In *Aspergillus fumigatus*, genes encoding seven hydrophobins have been identified: *ArodA*, *rodB*, *rodC*, *rodD*, *rodE*, *rodF*, and *rodG*, which are expressed in sporulated cultures. However, only *rodA* has been shown to be responsible for the formation of the structure of the rodlet layer. Suppression of *rodA* led to a loss or decrease in the permeability, hydrophobicity, resistance to physical damage and immune inertia of the cell wall of conidia in the null mutant Δ*rodA* strain in *A. fumigatus* (Paris et al. 2003; Valsecchi et al. 2018). Similar results were found in *A. nidulans*, where six genes coding for hydrophobins, *rodA*,

dewA, *dewB, dewC*, *dewD*, and *dewE* have been characterized. Deletion of *rodA* led to the loss of the rodlet layer structure, while deletion of the hydrophobin genes *dewB*, *dewC*, *dewD* and *dewE* only resulted in the rodlets being less robust (Grünbacher et al. 2014). *Penicillium decumbens* has three hydrophobin coding genes (*rodA*, *rodA*-like, and *rodB*) whose expression is regulated by *brlA*, but the dependence may be mediated by the condition of the culture state (solid or liquid) (Qin et al. 2013). In *Penicillium camemberti*, a protein sequence analysis showed that the hydrophobin RodA has a similar organization to RodA in *A. fumigatus* (Aimanianda et al. 2009; Boualem et al. 2008).

Inactive conidia also have a pigment layer that provides them structure, rigidity in the cell wall, ultraviolet (UV) protection and hydrophobicity (Figure 2b). A biologically important pigment in many fungi is melanin. Two types of pigments are found in fungi, melanin 1,8-dihydroxynaphthalene (DHN) and L-3,4-dihyroxyphenylalanine (L-DOPA) melanin (Baltussen et al. 2020). In *A. fumigatus* genome, a cluster of six genes located in the second chromosome of about 19 kb and coding for DHN-melanin biosynthesis has been identified (Perez-Cuesta et al. 2020). The enzymes of the DHN melanin biosynthetic pathway are classified into two groups: the early biosynthetic enzymes PksP/Alb1, Ayg1, Arp1, and Arp2 located in the cytoplasm and the late melanin biosynthetic enzymes Abr1 and Abr2 located in the endosomes (Perez-Cuesta et al. 2020). However, the genes coding for these enzymes are not conserved in all *Aspergilli* and the biosynthesis of melanin can vary depending on the species studied. In *Aspergillus flavus, A. nidulans*, *Aspergillus niger*, and *Aspergillus terreus*, there is little evidence that the biosynthetic gene cluster of melanin is composed of these six enzymes (Baltussen et al. 2020; Upadhyay et al. 2013). In *P. decumbens*, six orthologue genes (*abrA*, *abrB/yA*, *aygA*, *arpA*, *arpB* and *albA/wA*) and two *arp*-like genes (*arpA*-like and *arpB*-like) involved in the biosynthesis of pigments previously reported in *A. fumigatus* were identified (Qin et al. 2013).

In mature and dormant conidia, large amounts of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) are also detected, which functions as a source of energy and protection for the survival of conidia under adverse conditions, including thermic stress, osmotic pressure, temperature variations, pH, activity water(a_w) and UV (Figure 2b) (Baltussen et al. 2020; Elbein et al. 2003). Svanström et al. (2014) showed that trehalose biosynthesis is encoded by six genes: three potential trehalose-6-phosphate synthase, *tpsA*, *tpsB* and *tpsC* and three putative trehalose phosphate phosphatase, *tppA*, *tppB* and *tppC* in *A. niger*. In *Neurospora crassa*, the clock-controlled gene 9 (ccg-9) encodes a trehalose synthase (TSase), which catalyzes the synthesis of trehalose from D-glucose and α-D-glucose-1-phophate (Shinohara et al. 2002). The deletion of the *tpsA*, *tppA* and *tppB* genes caused a decreased trehalose content. However, deletion of *tpsA* also resulted in a dramatic decrease in sporulation with abnormal conidiophores. Trehalose deficiency in *A. nidulans* caused a dramatic reduction in heat and oxidative stress tolerance and total loss of cytoplasm, organelles and spore viability (Ni et al. 2007).

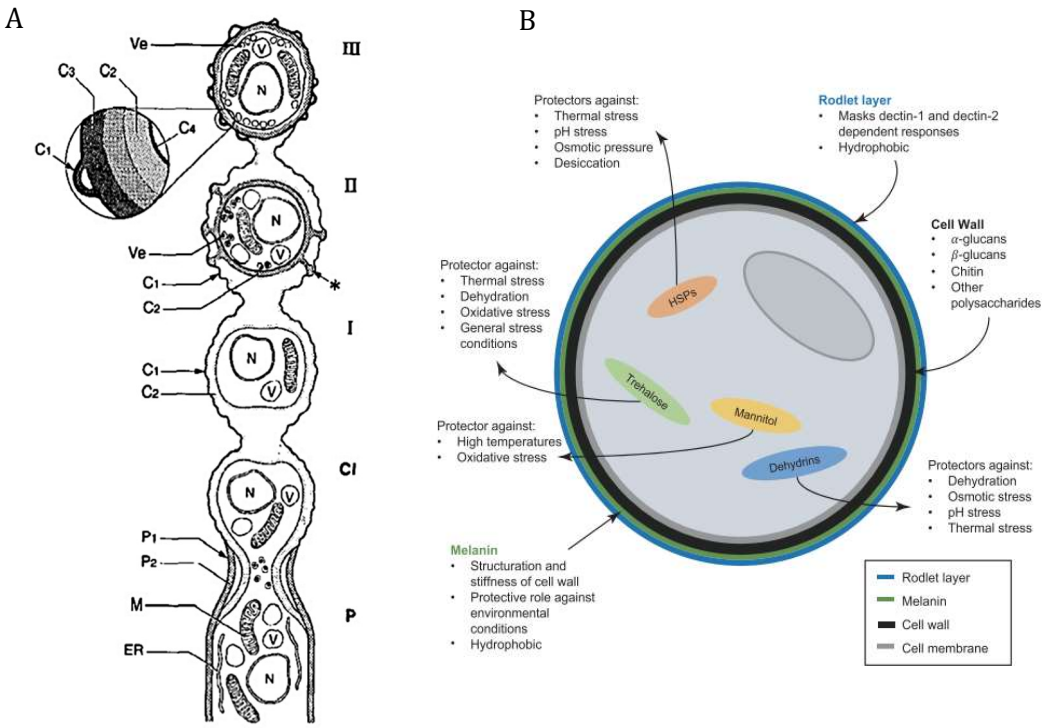


Figure 2. A) Phases of conidium maturation. Each conidium and phialide (P) contains a nucleus (N), mitochondria (M), endoplasmic reticulum (ER), vacuoles (V) and vesicles (Ve). Phialide contains two layers of cell wall: P1 and P2. The conidium in the first stage also contains two cell wall layers: C1 and C2. The C2 layer condenses to form projections (*) in contact with C1. Finally, conidium becomes mature, containing four layers of cells: C1 to C4(Ni et al. 2010). B) Schematic representation of the conidial cell (Baltussen et al. 2020).

3.3 Central Regulatory Pathway

As mentioned above, the process of conidiation takes place in a genetically controlled time and space. Previous studies have shown that asexual reproduction is regulated by specific genes of conidiation that activate in a hierarchical order the development of conidiophores and the formation and maturation of conidia (Zhang et al. 2019). Research focused on *A. nidulans* has suggested that *brlA* (bristle), *abaA* (abacus-like) and *wetA* (wet-white) genes form a central regulatory pathway (CRP) (Boylan et al. 1987, Adams et al. 1988, Mirabito et al. 1989) that controls the expression of conidiation-specific genes (Figure 3), and that are strongly conserved in the genus *Aspergillus* (Alkhayyat et al. 2015; Navarro-Bordonaba and Adams, 1994; Park and Yu, 2016). Boylan et al. (1987) cloned and characterized the *brlA, abaA* and *wetA* genes and determined by RNA accumulation the order of expression of these regulators; *brlA* → *abaA* →*wetA* in *A*. *nidulans*. The RNAs encoded by *brlA* and *abaA* accumulate specifically in the cells of the conidiophores while the RNAs encoded by *wetA* accumulate in the mature conidia. The mutation of the *brlA*, *abaA* and *wetA* genes does not affect vegetative growth (Navarro-Bordonaba and

Adams, 1994). However, the absence of *brlA* completely blocks asexual reproduction, the deletion of *abaA* led to the formation of abnormal phialides that blocked the formation of conidia, while the mutation of *wetA* causes the spores to autolyze during the final stages of differentiation (Adams et al. 1998).

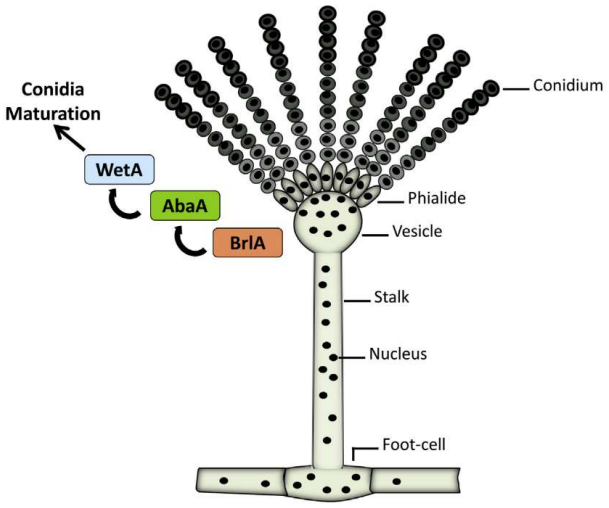


Figure 3. Scheme of the regulatory pathway for conidiation in *A. fumigatus*. BrlA is required for the formation of vesicles, AbaA is required for the differentiation of phialides and conidia and WetA is required for the maturation and integrity of the spore cell wall (Alkhayyat et al. 2015).

3.3.1 BrlA

The bristle *(brlA*) gene encodes a C_2H_2-zinc-finger TF, suggesting that *brlA* is directly involved in the regulation of other genes important for fungal development. The structure of the *brlA* gene is complex and consists of two overlapping transcription units, *brlAα* and *brlAβ* (Han and Adams, 2001). The *brlAα* (2.1 kb) is regulated at the transcriptional level and *brlAβ* (2.5 kb) is regulated at both the transcriptional and translational levels. The *brlAβ* transcript encodes two open reading frames (ORFs), the first starting with a short ascending ORF (μORF) and the second a descending ORF encoding the same polypeptide as *brlAα*, except that it includes 23 additional amino acids at the N-terminal end (Adams et al. 1998) (Figure 4). These two transcription units are individually required for normal development but the products of these transcripts or mRNAs have redundant functions (Prade and Timberlake, 1993). The *brlAβ* mRNA is transcribed in vegetative cells before developmental induction, but the translation of μORF represses translation of BrlA. The removing of the μORF initiation codes led to the deregulation of *brlA* expression and consequently to an inappropriate development. (Han et al. 1993) Previous studies revealed that BrlA has protein binding sites also known as BrlA Response Elements (BREs) 5'- (C/A)(G/A)AGGG(G/A)-3'. Multiple BREs elements have been identified in the promoter regions of developmental regulatory

genes, including *abaA*, *wetA*, *rodA*, *alb1*, and velvet regulator genes (Aramayo and Timberlake, 1993; Alkhayyat et al. 2015; Ni et al. 2010).

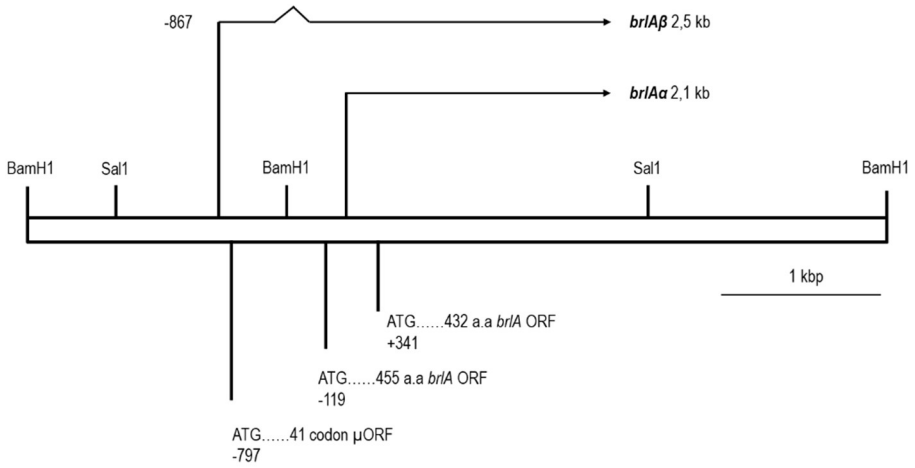


Figure 4. Organization of the *brlA* locus. The *brlAα* and *brlAβ* transcripts are indicated by the arrows (Han and Adams, 2001).

The *brlA* gene is expressed at the early stage of conidiation and in the genus *Aspergillus* , the BrlA protein is mainly found in vesicles, metulae, and phialides but not in hyphae or mature conidia (Ni et al. 2010). Studies in *A. nidulans* have shown that *brlA* is an extremely important gene in the CRP of conidiation since it activates the expression of *abaA* and *wetA*, two other genes in this pathway (Han and Adams, 2001). The *brlA* mutants have a "bristle-like" phenotype (Figure 5) (no conidium formation) because gene deletion blocks the transition of stalks to swollen vesicles and subsequent structures required for the formation of conidia, resulting only in elongated aerial stalks (Adams et al. 1998; Alkhayyat et al. 2015). On the other hand, overexpression of the *brlA* gene in vegetative cells leads to the formation of viable conidia directly from the tips of the hyphae (Adams et al. 1998; Han et al. 2018). In some fungi, including *Aspergillus clavatus* (Han et al. 2018), *A. fumigatus* (Lim et al. 2014; Lind et al. 2018; Shin et al. 2015), and *P. decumbens* (Qin et al. 2013), *brlA* has been shown to have an impact on the vegetative growth and biosynthesis of SMs in addition to a major role in asexual reproduction, suggesting that *brlA* could have many more roles than those identified so far. Orthologues of *brlA* are only present in *Aspergillus* and *Penicillium* species (Ojeda-López et al. 2018), although much less studied in *Penicillium* sp. (Qin et al. 2013; Sigl et al. 2011; Wang et al. 2015).

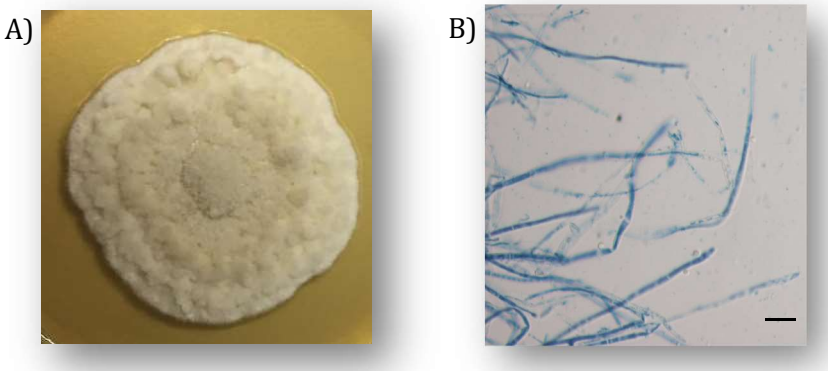


Figure 5. Morphological appearance of the null mutant PeΔ*brlA* strain. A) Macroscopic appearance. B) Microscopic appearance (×400). The strains were grown on MEA for seven days at 25 °C in the dark. Black scale bars represent 10 μm.

3.3.2 AbaA

The *abaA* gene is activated by BrlA during the middle stages of conidiation; it participates in the differentiation and functionality of the metulae and phialides, its expression therefore depends only on *brlA* and not on *wetA* (Tao and Yu, 2011). The expression of *abaA* activates the expression of specific genes in conidium maturation such as *wetA*, but it also influences *brlA*. Suppression of *abaA* leads to overexpression of *brlA*, while overexpression of *abaA* causes activation of *brlA*, suggesting that *brlA* and *abaA* are reciprocal inducers, but that *abaA* regulates both positively and negatively *brlA* (Aguirre 1993). AbaA contains an ATTS/TEA DNA-binding domain and a potential leucine zipper (Park and Yu, 2012). AbaA binds to the consensus sequence 5'-CATTCY-3' called AbaA Response Elements (AREs) where Y is a pyrimidine. In addition to BREs, multiple ARE elements have been discovered in the promotor regions of developmental genes, including *brlA*, *wetA*, *yA*, *rodA*, and even *abaA* itself (Adams et al. 1998). The phenotype of the null mutants Δ*abaA* has been described as "abacus" (Figure 6) since mutant strains form undifferentiated phialides with aconidial conidiophores, resulting in long chains of phialides-conidia that simulate beads on a string as in an abacus (Adams et al. 1998; Sewall et al. 1990). In addition, *abaA* expression has also been shown to influence secondary metabolism and the expression of other developmental genes. Shin et al. (2015) deleted the *abaA* and *brlA* genes in *A. fumigatus* strain and showed that the GliM and GliT proteins of the gliotoxin (GT) biosynthesis pathway and fungal self-protection against GT were significantly down-regulated in the null mutants Δ*abaA* and Δ*brlA* strains. Furthermore, low levels of GT were detected in the null mutant

Δ*abaA* strain, suggesting that the *abaA* gene interferes with more functions than previously studied.

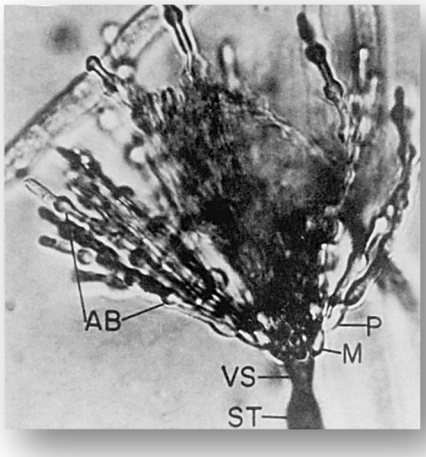


Figure 6. Morphological appearance of the null mutant Δ*abaA* strain. The null mutant Δ*abaA* strain produces normal conidiophore stalks (ST) and vesicles (VS), but metulae (M) and phialides (P) are abnormal and form abacus (AB) structures instead of conidia (C) (Adams et al. 1998).

3.3.3 WetA

The *wetA* gene is activated by *abaA* during the last phase of conidiation and plays a role in the synthesis of a crucial component of the conidial wall, the internal layer C4 (Figure 2a), which makes the conidia impermeable and mature (Tao and Yu, 2011). The *wetA* gene encodes a polypeptide rich in serine (14%), threonine (7%) and proline (10%) that regulates spore-specific gene expression (Adams et al. 1998). The suppression of the *wetA* gene does not influence the development and functionality of the conidiophores and phialides; however, mutants acquire a phenotype like "wet-white" since the mutation totally blocks the pigmentation of the conidia and the colonies acquire a white coloration. (Figure 7a). Results of the *wetA* gene suppression in *P. digitatum* showed that layers C1 and C2 did not condense, resulting in a thinner cell wall in the null mutant PdΔ*wetA* strains. In addition, the border of layer C1 was smooth and did not form the rodlets (Figure 7b). Suppression of the *wetA* gene also caused a delayed germination and a drastic reduction in tolerance to osmotic stress, detergents, heat shock and oxidative stress (menadione). However, the production of trehalose, involved in viability of conidia and stress resistance, was not affected in *Penicillium digitatum* (Wang et al. 2015). By contrast, Tao and Yu (2011) reported that suppression of the *wetA* gene in *A. fumigatus* abolished the production of trehalose, suggesting that *wetA* plays a critical role in the biogenesis of trehalose in conidia, probably affecting the viability and stress tolerance of conidia. These results may suggest that gene expression is regulated differently between the genera *Aspergillus* and *Penicillium* during the last

stage of conidiation. Overexpression of the *wetA* gene in the hyphae inhibited vegetative growth, and caused excessive branching in *Aspergillus nidulans* (Navarro-Bordonaba and Adams, 1994).

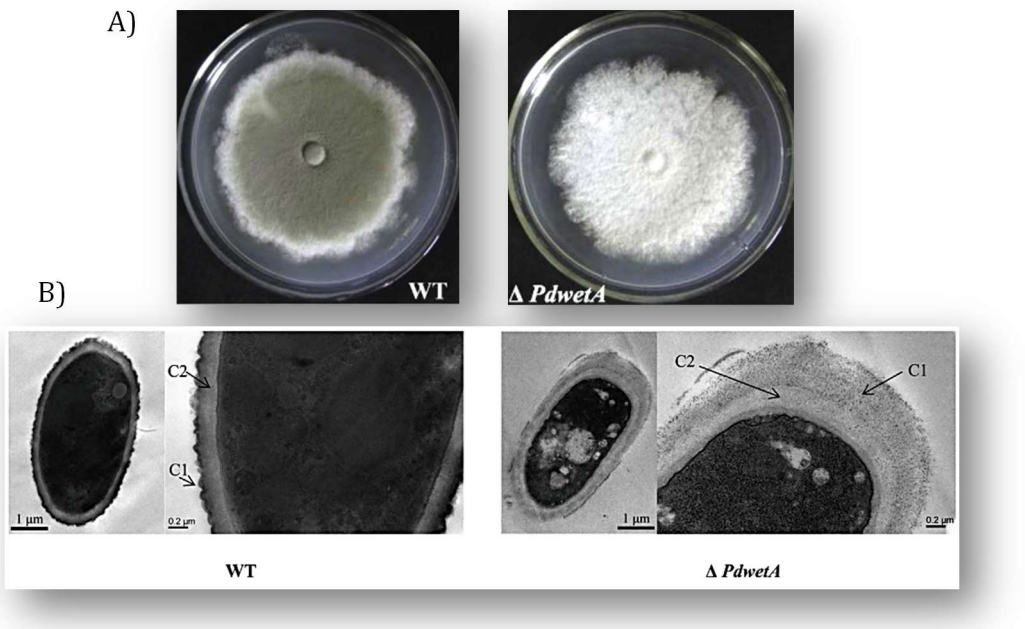


Figure 7. Morphological appearance of the null mutant Δ*wetA* strain. Vegetative growth of the wild-type and null mutant PdΔ*wetA* strains on PDA at 25 °C for six days. (B) TEM images of the cell wall of conidia in WT and PdΔ*wetA*. Bar, 1 mm or 0.2 mm (Wang et al. 2015).

3.4 CRP upstream regulatory proteins

The genes involved in the CRP are fundamental during conidiogenesis. However, other regulatory proteins are also involved upstream and downstream of the process activating or repressing the expression of the CRP genes and during the conidial maturation stage. Table 1 shows the main genes and regulatory proteins involved in asexual reproduction in fungi.

Table 1. List of proteins and genes expressed in asexual reproduction.

Gene	*Aspergillus* species	Systemic name	Description
brlA	*A. niger*[a]		Central regulatory pathway. C^2H^2-zinc-finger transcription factor. Essential for conidiation.
abA	*A. niger*[a]		Central regulatory pathway. Differentiation and functionality of the metulae and phialides.
tpsC	*A. niger*	An14g02180	Trehalose biosynthesis gene. Trehalose is important for conidial survival.
tppB	*A. niger*	An13g00400	Trehalose biosynthesis gene. Trehalose is important for conidial survival.
tppC	*A. niger*	An07g08720	Trehalose biosynthesis gene. Trehalose is important for conidial survival.
tpsA	*A. fumigatus*	Afu6g12950	Trehalose biosynthesis gene. Trehalose is important for conidial survival.
	A. niger	An08g10510	Trehalose biosynthesis gene. Trehalose is important for conidial survival.
tpsB	*A. fumigatus*	Afu2g04010	Trehalose biosynthesis gene. Trehalose is important for conidial survival.
mtdA	*A. niger*	An15g05450	Gene for putative mannitol dehydrogenase; transcripts found abundantly present in dormant conidia. Mannitol is important for conidial survival.
mpdA	*A. niger*	An02g05830	Gene for putative mannitol dehydrogenase; transcripts found abundantly present in dormant conidia. Mannitol is important for conidial survival.
hsp90	*A. fumigatus*	Afu5g04170	Gene for heat shock protein important for spore viability, germination, hyphal growth, and conidiation.
wetA	*A. fumigatus*	Afu4g13230	Essential for trehalose biogenesis in conidia; gene for developmental regulatory protein involved in conidial development.
arp1	*A. fumigatus*	Afu2g17580	Conidial DHN melanin biosynthesis gene.
arp2	*A. fumigatus*	Afu2g17560	Conidial DHN melanin biosynthesis gene.
ayg1	*A. fumigatus*	Afu2g17550	Conidial melanin biosynthesis gene.
mcoC	*A. niger*	An03g03750	Conidial melanin biosynthesis gene.
fwnA	*A. niger*	An09g05730	Conidial melanin biosynthesis gene.
ayg1	*A. niger*	An14g05350	Conidial melanin biosynthesis gene; mutants produce gray conidia and small conidiophores.
dprA	*A. fumigatus*	Afu4g00860	Encodes a dehydrin-like protein, which plays a role in the oxidative stress response.
dprB	*A. fumigatus*	Afu6g12180	Encodes a dehydrin-like protein, which plays a role in osmotic and pH stress responses.
dprC	*A. fumigatus*	afu7g04520	Encodes a dehydrin-like protein, which plays a role in tolerance against freezing.
fluG	*A. nidulans*[b]		Upstream developmental activators to conidiation.

vosA	*A. fumigatus*	Afu4g10860	Encodes a protein involved in conidiation. The VelB-VosA heterodimer is required for spore maturation, trehalose biogenesis, long-term viability of conidia, and resistance to stresses and negatively controls spore germination.
velB	*A. fumigatus*	Afu1g01970	Encodes a protein involved in conidiation. The VelB-VosA heterodimer is required for spore maturation, trehalose biogenesis, long-term viability of conidia, and resistance to stresses and negatively controls spore germination.
veA	*A. fumigatus*	Afu1g12490	Encodes a protein involved in conidiation. The VelB-VeA heterodimer negatively controls spore germination.
laeA	*A. nidulans*	AN0807	Encodes a methyltransferase. The formation of the VelB-VosA heterodimer complex is directed by LaeA.
ganB	*A. nidulans*	AN1016	GanB plays a role in controlling conidial germination by mediating the cAMP/PKA signaling pathway.
mybA	*A. fumigatus*	Afu3g07070	Involved in the control of conidial formation and maturation; plays an essential role in the survival of the conidia.
cspA	*A. fumigatus*	Afu3g08990	CspA is involved in spore viability and cell wall permeability.
atfA	*A. fumigatus*	Afu3g11330	Basic-region leucine zipper transcription factor. Plays a role in the response of conidia to stress.
tslA	*A. fumigatus*	Afu7g03940	Encodes a regulatory protein related to trehalose, which interacts with CsmA chitin synthase. Δ*tslA* mutants have an altered cell wall structure.
csmA/chsE	*A. fumigatus*	Afu2g13440	Class V chitin synthase which interacts with trehaloserelated regulatory protein TslA.
scf1	*A. fumigatus*	Afu1g17370	Encode a putative heat shock protein enriched in dormant conidia.
awh11	*A. fumigatus*	Afu6g12450	Encode a putative heat shock protein enriched in dormant conidia.
bipA	*A. fumigatus*	Afu2g04620	HSP70 chaperone enriched in dormant conidia.
hsp70	*A. fumigatus*	Afu1g07440	Molecular chaperone enriched in dormant conidia.
clxA	*A. fumigatus*	Afu4g12850	Putative calnexin enriched in dormant conidia.
egd2	*A. fumigatus*	Afu6g03820	Predicted role in protein folding. Encodes a protein enriched in dormant conidia.
hsp30	*A. fumigatus*	Afu3g14540	Encodes a putative heat shock protein enriched in dormant conidia.
rodA	*A. fumigatus*	Afu5g09580	Conidial hydrophobin required for formation of the rodlet layer of conidia.
abr2	*A. fumigatus*	Afu2g17530	Encodes a protein involved in conidial melanin biosynthesis.
pep2	*A. fumigatus*	Afu3g11400	Encodes a protein enriched in conidia; cell surface associated.
	A. fumigatus	Afu5g02040	Putative extracellular lipase enriched in conidia.
pdiA	*A. fumigatus*	Afu2g06150	Encodes a protein enriched in conidia; cell surface associated.
	A. fumigatus	Afu7g06750	Encodes a protein enriched in conidia; cell surface associated.
aspf3	*A. fumigatus*	Afu6g02280	Allergen Asp f3; cell surface associated.
cipC	*A. fumigatus*	Afu5g09330	Encodes a protein enriched in conidia; cell surface associated.
wos2	*A. fumigatus*	Afu5g13920	Putative HSP90 binding cochaperone; cell surface associated.
rpp1	*A. fumigatus*	Afu1g06830	Encodes a putative 60S acidic ribosomal protein super family member; cell surface associated.
ccpA	*A. fumigatus*	Afu1g13670	Encodes a conidial surface protein highly abundant in resting conidia; CcpA reduces recognition by the innate immune system.

dlpA	*A. nidulans*	AN5324	Encode a dehydrin-like protein which plays a role osmotic and thermal stresses.
catA	*A. fumigatus*	Afu6g03890	Conidium-specific catalase CatA.
scf1	*A. fumigatus*	Afu1g17370	Encodes a putative heat shock protein enriched in dormant conidia.
conJ	*A. fumigatus*	Afu6g03210	Putative conidiation gene; deletion of conF and conJ results in delayed germination and decreased desiccation resistance.
mpkC	*A. fumigatus*	Afu5g09100	Putative MAPK involved in the oxidative stress response; transcript abundance increases in response to carbon source and oxidative stress.
pbs2	*A. fumigatus*	Afu1g15950	Mitogen MAPKK of the HOG signaling pathway that regulates osmotic stress response.
pbsA	*A. nidulans*	AN0931	
sakA	*A. fumigatus*	Afu1g12940	Putative MAPK with predicted roles in the osmotic and oxidative stress responses.
osrA	*A. fumigatus*		Hypothetical osmotic stress regulator.
sskB	*A. nidulans*	AN10153	MAPKKK of the HOG signaling pathway that regulates the osmotic stress response.
sskA	*A. nidulans*	AN7697	Response regulator; part of a two-component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response.
tcsB	*A. nidulans*	AN1800	Transmembrane histidine kinase; part of a two component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response.
ypdA	*A. nidulans*	AN2005	Encodes a histidine-containing phosphotransfer protein; part of a two-component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response.

Adapted from Baltussen et al. (2020). These data were extracted from [a] Adams et al. (1998), [b] (Rodríguez-Urra et al. 2012).

3.4.1 Upstream regulators

The programmed transition from hyphae growth to conidiophore development occurs with the activation of *brlA*, which requires several upstream regulators. In *Aspergilli*, *brlA* is controlled by upstream developmental activators (UDAs) that consist of six members of the fluffy genes, *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE* (*flb* for fluffy low *brlA* expression). Mutations in all these genes result in reduced *brlA* expression, with aconidial colonies and fluffy phenotypes (Lee and Adams, 1996; Ojeda-López et al. 2018; Wieser et al. 1994).

FluG is an upstream TF that activates the other fluffy genes (Figure 8a). It is responsible for the synthesis of an endogenous diffusible factor (dehydroaustinol) that accumulates in the aerial hyphae and induces conidiation when this factor is in conjunction with diorcinol (Rodríguez-Urra et al. 2012).

The *flbA* gene is another member of the fluffy genes, which has a positive role in activating asexual development in fungi. It promotes conidiation by suppressing the G-protein-mediated signaling pathway, which inhibits conidiation but promotes vegetative growth via *pkaA* (Lee and

Adams, 1996; Li et al. 2017). Lee and Adams (1996) proposed that *flbA* and *fluG* work interdependently to activate accumulation of *brlA* mRNA and induce conidiophore development.

Besides FluG-dependent pathway in *A. nidulans*, the FlbB-FlbE heterodimer is required for the expression of *flbD*, which subsequently leads to the formation of the FlbB-FlbD heterodimer required for the activation of *brlA* (Figure 8b) (Alkhayyat et al. 2015; Oiartzabal-Arano et al. 2016). FlbB may also be a key factor in the transition of metulae to phialides in *A. nidulans* (Ojeda-López et al. 2018). FlbD has a Myb-like DNA-binding domain, which may indicate that this member of the fluffy genes is involved in the remodeling of chromatin at the *brlA* promoter to facilitate its expression (Ojeda-López et al. 2018).

FlbC is a C_2H_2 zinc finger protein involved in binding directly to the promoter of *brlA* and inducing its expression. In some species such as *Fusarium* and *N. crassa*, the null mutants Δ*flbC* resulted in a reduction of aerial hyphae and conidiation (Boni et al. 2018). Both *flbC* and *flbD* encode DNA-binding proteins, indicating that they function as TFs in controlling the activation of specific developmental genes (Lee and Adams, 1996).

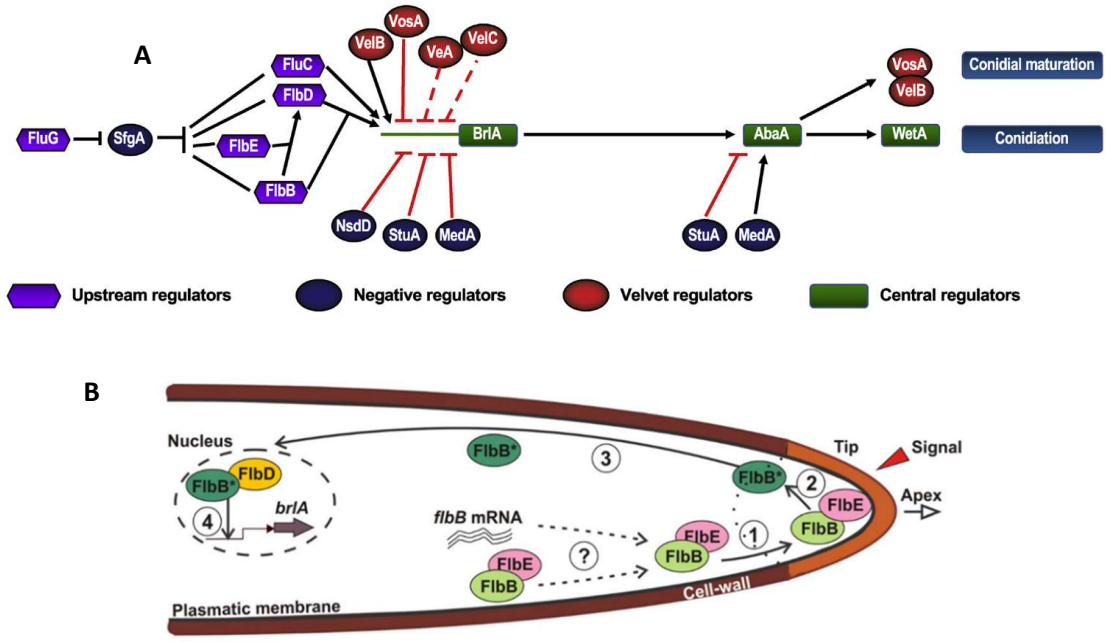


Figure 8. Upstream developmental activators, fluffy genes. A) A genetic model of the regulation of conidiophore development in *A. nidulans* (Ojeda-López et al. 2018). B) FlbB-FlbE and FlbB-FlbD dynamics and transcriptional activity in vegetative hyphae (Oiartzabal-Arano et al. 2016)

3.4.2 Velvet regulators

Velvet regulators are highly conserved in filamentous fungi and form a family of proteins composed of VeA, VelB, VelC and VosA that contain a DNA binding domain (Alkhayyat et al. 2015; Baltussen et al. 2020). The velvet proteins form homo-and/or heterotrimeric complexes (VelB-VeA-LaeA, VosA-VelB, VosA-VelC, and VelB-VelB) (Figure 9) with each other or with the methyltransferase LaeA (non-velvet protein) that regulate several processes in filamentous fungi, including biosynthesis of SMs, sexual and asexual development and spore maturation and viability.

In *A*. *nidulans*, the *veA* gene encodes a protein of 574 amino acids that is regulated by light. It is a founding member of the velvet complex, VeA-VelB-LaeA, required for the coordination of fungal development and biosynthesis of SMs (Sarikaya-Bayram et al. 2015). In addition, this protein interacts with the phytochrome red light receptor FphA, which, together with the blue light receptors LreA-LreB form the light complex (Atoui et al. 2012; Röhrig et al. 2013; Ruger-Herreros et al. 2011). In *A*. *niger*, deletion of the *veA* gene led to a decreased *brlA* expression resulting in a reduction in the number of conidia, while vegetative growth was favored (Zhang et al. 2018). In *A*. *nidulans*, the mutant Δ*veA* strains were unable to produce sterigmatocystin, a precursor of aflatoxin, and no sexual fruiting bodies (perithecia) were formed (Ahmed et al. 2013).

In *A*. *nidulans*, the heterodimeric complex VelB-VosA represses the genes regulating germination, but activates genes required during the final stages of conidial maturation, spore viability, trehalose biogenesis and resistance to heat, oxidative stress and UV (Sarikaya-Bayram et al. 2015;Alkhayyat et al. 2015; Baltussen et al. 2020; Park et al. 2017). The *velB* and *vosA* genes were found to be positively regulated by AbaA (Alkhayyat et al. 2015; Baltussen et al. 2020). The velvet regulator VosA contains a DNA-binding motif that specifically recognizes an 11-nucleotide consensus sequence (CTG-GCCAAGGC) found in the promoters of asexual regulatory genes such as *brlA*, *wetA*, *vosA*, as well as in the biosynthesis genes of trehalose *tpsA* and *treA* (Sarikaya-Bayram et al. 2015; Park et al. 2017). Although VosA governs the maturation of conidia, it also regulates the negative feedback of *brlA* by binding to the VosA-responsive element (VRE) in the promoter of *brlAβ* (Lee et al. 2016). In *A*. *nidulans*, deletion of the *vosA* or *velB* genes results in the down-regulation of genes associated with trehalose biosynthesis (*tpsA*, *tpsC* and *orlA*) leading to a low or null trehalose content in the spores.

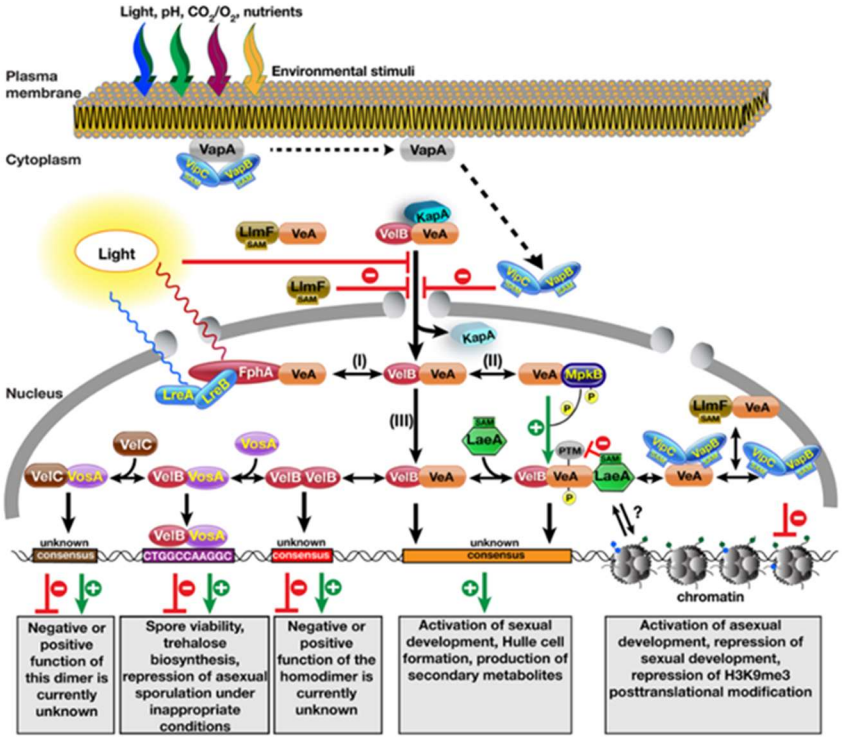


Figure 9. Molecular complexes formed by the velvet family proteins and the methyltransferases on the control of fungal development and secondary metabolite production (Sarikaya-Bayram et al. 2015).

The VelC protein has also been identified and characterized in *A. nidulans*, it was described as a positive regulator of sexual development as VeA and VelB since the suppression of the *velC* gene causes a decrease in the number of cleistothecia and an increase in the number of conidia. The VosA-VelC heterodimer could control sexual development and spore viability by binding to alternative promoter sequences (Sarikaya-Bayram et al. 2015).

In *Penicillium chrysogenum*, homologues of velvet regulatory proteins were also identified and characterized. Kopke et al. (2013) generated and characterized the null mutant PcΔ*veA*, PcΔ*velB*, PcΔ*velC*, and PcΔ*vosA* strains from *P. chrysogenum*. The results showed that the velvet subunits have opposite roles in the regulation of penicillin biosynthesis and conidiation. In the null mutant PcΔ*velC* and PcΔ*veA* strains, penicillin biosynthesis was activated, while in the null mutant PcΔ*velB* strain, penicillin production was repressed. Conidiation was promoted in the null mutant PcΔ*velB* and PcΔ*vosA* strains, while it was inhibited in the null mutant PcΔ*velC*.

3.4.3 Negative regulators

Asexual reproduction is suppressed by factors that promote sexual reproduction. Although there are several proteins and factors that repress conidiogenesis, three negative regulators, SfgA, VosA and NsdD are key factors that act at different control points in the genetic cascade of developmental process.

The NsdD repressor protein is a GATA-type zinc-finger TF, involved in the activation of sexual development in *A. nidulans* (Han et al. 2003). In *Aspergillus flavus*, NsdD is necessary for the production of sexual sclerotia, biosynthesis of aflatoxins and development of conidiophores (Cary et al. 2012). The null mutant Δ*nsdD* strain showed an altered aspect in their morphology, they did not produce sclerotia and a significant decrease in the number of conidia was observed (Cary et al. 2012). However, the deletion of *nsdD* in *A. nidulans* resulted in increased conidiation. The Δ*nsdD*Δ*vosA* double mutant showed a high level *brlA* gene expression suggesting that NsdD and VosA cooperatively repress *brlA* expression (Lee et al. 2016). These results were also observed when the *nsdD* orthologues in *Fusarium fujikuroi* (*csm-1*) and *Botrytis cinerea* (*ltf1*) were deleted (Ojeda-López et al. 2018). In *A. nidulans*, overexpression of *nsdD* resulted in excessive formation of cleistothecia, even in the presence of a sexual development inhibitor (KCL) (Han et al. 2003). Lee et al. (2016) have shown that NsdD acts downstream of UDAs and upstream or at the same level as BrlA.

SfgA is a putative TF with a Gal4-type Zn(II)2Cys6 binuclear DNA-binding domain that inhibits FluG function and can repress the expression of *flbA*, *flbB* and *flbC* by binding to their promoters (Lee et al. 2016). In *A. nidulans*, deletion of the *sfgA* gene leads to excessive conidiation and eliminates the need for *fluG* in conidiation and sterigmatocystin production (Lee et al. 2014).

3.4.4 Regulators Modifiers of Conidiation

In addition to CRP, the conidial process requires developmental modifier genes. These include TFs stunted (*stuA*) and medusa (*medA*), which are called developmental modifiers since they are necessary for the proper development of conidiophores (Busby et al. 1996; Ojeda-López et al. 2018). StuA and MedA have been shown to be necessary for the spatial and temporal organization of gene expression in conidiophore development (Navarro-Bordonaba and Adams, 1994; Adams et al. 1998).

StuA is a DNA-binding protein essential for the morphogenesis, it has been considered to spatially modulate *brlA* expression at the periphery of the conidiophore vesicle, metulae and phialides, while regulating *abaA* expression in the metulae, phialides and immature conidia (Navarro-Bordonaba and Adams, 1994). The *stuA* gene is complex in that two transcripts, *stuAα* (3335 nucleotides) and *stuAβ* (3470 nucleotides) are produced from distinct transcriptional start sites. (Navarro-Bordonaba and Adams, 1994). StuA has response elements that are present upstream of the *brlA* and *abaA* transcription start sites (Busby et al. 1996).

The null mutant Δ*stuA* strains have extremely shortened aerial hyphae that lack metulae and phialides and only differentiate a few conidia directly formed in the vesicle of the

conidiophore (Adams et al. 1998). The suppression of the *stuA* gene leads to the delocalized expression of *abaA* and *brlA* fusions, which could indicate that *stuA* acts by providing the appropriate cellular distribution of the AbaA and BrlA proteins (Navarro-Bordonaba and Adams, 1994). In *Penicillium rubens* (formally *P. chrysogenum*), *stuA* is essential for penicillin biosynthesis and its deletion led to a drastic down-regulation of the roquefortine gene cluster (Sigl et al. 2011).

MedA is a conidiation regulator that acts at different levels of the CRP. Previous studies have suggested that *medA* suppresses the expression of the two *brlA* transcripts (α and β) during the early stages of development and decreases the expression of *brlAβ* during the late stages of conidiophore development. MedA is also used as a coactivator in the expression of *abaA* (Busby et al. 1996). Level of *medA* transcripts is high during vegetative growth and gradually declines following developmental induction (Navarro-Bordonaba and Adams, 1994)

The null mutant Δ*medA* strains exhibit a delayed differentiation of phialides and conidia and show different phenotypes depending on the species, but in all cases, the levels of conidiation are affected. In *A. nidulans*, *medA* suppression produces conidiophores with multi-layer metulae (three or four layers) before differentiation of phialides and conidia (Navarro-Bordonaba and Adams, 1994; Ojeda-López et al. 2018), whereas in *A. fumigatus* the medusoid aspect was not observed, although mutants continued to produce some conidia (Ojeda-López et al. 2018). The delay in the differentiation of metulae and phialides could be due to an insufficient expression of *abaA* (Aguirre 1993).

MedA and StuA are also known as regulators that activate sexual reproduction. In *A. nidulans*, the null mutant Δ*medA* strains form Hülle cells but do not produce mature cleistothecia or ascospores, whereas null mutant Δ*stuA* strains fail to form either cleistothecial primordia or Hülle cells (Navarro-Bordonaba and Adams, 1994). The *medA* and *stuA* genes perform functions independent of those involved in the conidiation (Busby et al. 1996). The *stuA* gene is apparently important for proper spatial distribution of *brlA*, whereas *medA* appears to be required for correct temporal expression of *brlA* (Adams et al. 1998).

3.5 Conidiation and light

In nature, exposure to air and light are two important conditions for the induction of asexual development. In *A. nidulans*, light is a crucial regulator between sexual and asexual development, however excess light can be harmful, especially UV light.(Ruger-Herreros et al. 2011). Fungi use a series of receptors at specific wavelengths. Receptors are photoreceptor proteins that detect light through a chromophore, an low molecular weight organic molecule, that allows a protein to absorb light (Rodriguez-Romero et al. 2010). *N. crassa* has been used to study the mechanisms of action of photoreceptor proteins. Previous studies have shown that *N. crassa*

detects light through the white-collar protein 1 (WC-1), which acts as a photoreceptor for blue light, and the white-collar protein 2 (WC-2). WC-2 interacts with WC-1 to form a heterodimeric complex called the white-collar complex (WCC: WC-1/WC-2) that binds to promoters of light-regulated genes (Röhrig et al. 2013; Ruger-Herreros et al. 2011). Other blue light photoreceptors in *N*. *crassa* are VIVID (VVD) proteins, required for photoadaptation, and the cryptochrome CRY-1. In addition to blue light receptors, a chromoprotein that detects red light and far red light has been identified in *N*. *crassa* phytochromes (Ruger-Herreros et al. 2011). In *A*. *nidulans*, several *N*. *crassa* photoreceptors orthologues have been identified, including the red light receptor, phytochrome FphA, the white-collar complex homologues, LreA (WCC-1) and LreB (WCC-2), and a cryptochrome, CryA (Alkhayyat et al. 2015; Ruger-Herreros et al. 2011). These photoreceptors bind to the VeA protein to form the light regulator complex (Figure 10) (Atoui et al. 2012; Röhrig et al. 2013; Ruger-Herreros et al. 2011). The homologues of the white-collar complex, LreA and LreB, act as positive factors for the sexual cycle repressing conidiation (Alkhayyat et al. 2015; Rodriguez-Romero et al. 2010).

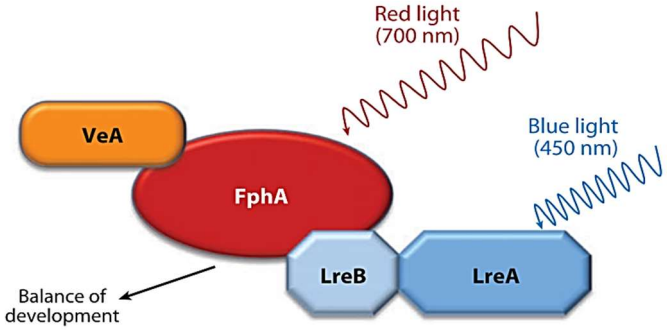


Figure 10. Light regulator complex (Rodriguez-Romero et al. 2010).

Mooney and Yager (1990) showed that light is necessary for the formation of viable conidiophores and conidia in *A*. *nidulans*, as long as the mycelium is in contact with air. Their study showed that red light can trigger the conidial response, but that, other wavelengths, including far red, inhibit asexual development of the fungus. In addition, they found that light dependence is subject to the VeA protein, the deletion of the velvet gene allows conidiation to occur in the absence of light. So, VeA is considered to be a repressor of light-regulated conidiation and an activator of sexual development, while phytochrome FphA induces conidia production and represses sexual development (Alkhayyat et al. 2015; Mooney and Yager 1990; Ruger-Herreros et al. 2011).

Röhrig et al. (2012) studied the light complex in *A*. *nidulans*, showing that germination of conidia is delayed in the presence of blue (450 nm), red (700 nm) and far-red light (740 nm), with far-red light having the most significant effect. Blue light inhibited germination to the same extent

as red light, but the photoreceptors did not have the same function. The deletion of the genes encoding the blue light photoreceptors, LreA and LreB, produced no change in the germination of the mutant strain compared to the wild type strain. When the deletion was performed on the *fphA* gene, germination of the mutant strain increased in the presence of blue, red and far-red light, suggesting that the phytochrome suppresses the germination of spores in the presence of light.

Similar results were found in *A. fumigatus*, where both blue and red light had an inhibitory effect on the germination rate of conidia. Studies on the null mutant Δ*lreA* and Δ*fphA* strains and the double mutant Δ*lreA*Δ*fphA* strain showed conidial levels comparable to the wild type strain, suggesting that LreA and FphA play no significant role in conidiation (Fuller et al. 2013).

Schmidt-Heydt et al. (2011) showed that in *P. verrucosum* the red (627 nm) and blue wavelength (455-470 nm) had inhibitory effects on growth and biosynthesis of ochratoxin A (OTA) by modulating the level of expression of ochratoxin polyketide synthase. Light irradiation has an opposite effect on OTA than on citrinine, two mycotoxins that can be produced simultaneously in *P. verrucosum*. Citrinine was essentially produced under light conditions that inhibited the biosynthesis of OTA. On the other hand, they observed that blue light causes an inactivation effect on the spores. After 24 hours of incubation under blue light almost 97% of the spores were no longer able to germinate.

CHAPTER 2

RESULTS

Creation and characterization of the null mutant PeΔ*brlA* strain in *Penicillium expansum*

Article- International Journal of Molecular Science

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Summary of the study

The production of secondary metabolites in fungi is related to specific functions including protection and defense against other microorganisms, or as virulence and aggressiveness factors. However, their production is also associated with the different stages of growth of the fungus. The development of fungi is regulated by different transcription factors (TFs), including factors related to sexual and asexual reproduction. These include VeA, a global TF that regulates light-dependent sexual reproduction, and BrlA, a TF that regulates asexual reproduction in fungi.

Some studies have shown that TFs regulate the expression of other genes during fungal development. For example, suppression of the velvet regulators *vosA*, *veA*, or *velB* caused excessive conidiation and high accumulation of *brlA* in *A. fumigatus*, suggesting that these factors negatively regulate conidiation (Alkhayyat et al. 2015). In *P. expansum,* the disruption of the *veA* gene completely abolished patulin production in the null mutant strain. In addition, *veA* deletion altered the morphology of the strain, resulting in a strain unable to form coremia (El Hajj Assaf et al. 2018). In *A. niger*, the production of ochratoxin A, the production of conidia, and the expression of the *brlA* gene were significantly reduced in the null mutant strain when the *veA* gene was deleted (Zhang et al. 2018).

In recent decades, conidiation has been the subject of much research because it is one of the main reproduction and diffusion mechanism used by fungi, but also because it is closely linked to the production of secondary metabolites. As BrlA is a key TF in the regulation of asexual reproduction, which is expressed at an early stage of conidiation, we wanted to study in more detail the roles of this gene in *P. expansum*. In particular, we focused on the impact of this gene on the development and production of secondary metabolites.

The aim of the first part of the experimental work was the creation and the characterization of the null mutant Δ*brlA* strain in *P. expansum* (PeΔ*brlA*). For this objective, the *brlA* gene was replaced by the hygromycin resistance marker (*hph*), using the homologous recombination technique. First, a macro- and microscopic morphological study was carried out. Suppression of the *brlA* gene completely blocked the conidial process of the fungus, resulting in a white, devoid of conidia and with a "bristle-like" appearance strain. Pathogenicity studies on Golden Delicious apples revealed that the diameter and volume of rot were significantly higher in the null mutant strain than in the WT strain. The metabolomic study carried out in vitro on a

synthetic medium and in vivo on apples showed the influence of the *brlA* gene on the production of some secondary metabolites including communesins and chaetoglobosins. The diversity of chaetoglobosins was enhanced while the diversity of communesins was decreased in the null mutant PeΔ*brlA* strain. However, the production of the mycotoxins patulin, citrinin and roquefortine were not affected by the deletion of the *brlA* gene. A microarray study showed that *brlA* deletion also affected expression of several genes, including *abaA* and *wetA*, the two other members of the central regulatory pathway of conidiation, *rodA* and *rodB*, which encode hydrophobins, genes involved in the biosynthesis of melanins: *alb1*, *arp1*, *arp2*, *ayg1*, and *abr1*, *vosA*, which codes for a member of the velvet complex, and the *Ccg-9* gene, which is involved in trehalose biosynthesis.

The experimental section, the results and the discussion of this study are detailed in this chapter according to the publication format of the *International Journal of Molecular Science*.

International Journal of Molecular Sciences

MDPI

Article

The *brlA* Gene Deletion Reveals That Patulin Biosynthesis Is Not Related to Conidiation in *Penicillium Expansum*

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Abstract: Dissemination and survival of ascomycetes is through asexual spores. The *brlA* gene encodes a C_2H_2-type zinc-finger transcription factor, which is essential for asexual development. *Penicillium expansum* causes blue mold disease and is the main source of patulin, a mycotoxin that contaminates apple-based food. A *P. expansum* Pe*ΔbrlA* deficient strain was generated by homologous recombination. In vivo, suppression of *brlA* completely blocked the development of conidiophores that takes place after the formation of coremia/synnemata, a required step for the perforation of the apple epicarp. Metabolome analysis displayed that patulin production was enhanced by *brlA* suppression, explaining a higher in vivo aggressiveness compared to the wild type (WT) strain. No patulin was detected in the synnemata, suggesting that patulin biosynthesis stopped when the fungus exited the apple. In vitro transcriptome analysis of Pe*ΔbrlA* unveiled an up-regulated biosynthetic gene cluster (PEXP_073960-PEXP_074060) that shares high similarity with the chaetoglobosin gene cluster of *Chaetomium globosum*. Metabolome analysis of Pe*ΔbrlA* confirmed these observations by unveiling a greater diversity of chaetoglobosin derivatives. We observed that chaetoglobosins A and C were found only in the synnemata, located outside of the apple, whereas other chaetoglobosins were detected in apple flesh, suggesting a spatial-temporal organization of the chaetoglobosin biosynthesis pathway.

Keywords: *Penicillium expansum*; *brlA*; conidiogenesis; synnemata; secondary metabolism; patulin; chaetoglobosins; communesins; metabolomics; microarray

1. Introduction

Penicillium is a well-known genus of filamentous ascomycetous fungi. Taxonomically, it is a member of the *Aspergillaceae* family, in nature it is mainly found in the soil although it has also been detected in decaying organic matter, cereals, seeds, and various food products, and is consequently of great economic importance [1–3]. The genus currently contains 483 accepted species [3], fungi that are very common in the environment and are important in different fields including biotechnology, medical and food industries but also in phytopathology and food spoilage (pre- and postharvest pathogens) [4,5]. The species of this fungal genus are also known to produce biologically active compounds called secondary metabolites (SMs) that can range from potent pharmaceutical drugs to mycotoxins that are harmful to humans and animals [6–10].

Penicillium expansum is a post-harvest pathogen of apples and together with the species *Penicillium italicum* and *Penicillium digitatum* (citrus pathogens), it may cause up to 10% losses of

harvested products [11]. *P. expansum* mainly infects apple fruit, but has also been isolated in other hosts including pears, cherries, peaches, plums, nuts, pecans, hazelnuts, and acorns [12–15].

P. expansum is a psychrophilic and necrotrophic fungus that develops during harvesting, postharvest processing, and storage through injuries that cause maceration and decomposition. It is considered to be the main agent of blue mold disease [16] and the main source of patulin in the human diet. Patulin is a toxic SM, undetectable by taste and smell, found not only in apple fruits but also in apple-based products. Heat treatments do not affect the overall stability of this mycotoxin and long-term exposure to patulin-contaminated products can cause serious health disorders. Patulin has been shown to be mutagenic, neurotoxic, genotoxic, cytotoxic, teratogenic, and immunotoxic to animals [15,17,18]. Due to its toxicity, maximum levels of patulin in food are regulated in most European countries (50, 25, and 10 μg of patulin/kg, in fruit juices, solid apple products, and apple-based products for infants, respectively) [19]. As for many fungal SMs, gene encoding enzymes, transporters and transcription factor (TF) involved in the biosynthesis of patulin are gathered into a cluster [11,20,21]. This cluster comprises 15 genes (PEXP_094320-PEXP_094460). Previous research has shown that this biosynthetic gene cluster is activated specifically by PatL (PEXP_ 094430) [11,21,22]. Patulin production is also positively regulated by PacC and CreA; these two TFs respond to abiotic stimuli such as pH and carbon source, respectively [23,24]. Some components of the velvet complex such as LaeA [25] and VeA [26] have been reported as positive regulators of the patulin biosynthesis. A recent study has shown that the deletion of *sntB*, a gene coding for an epigenetic reader, resulted in a decreased patulin production in vitro and *in planta* [27].

Although most studies on *P. expansum* have focused on patulin, the fungus produces many other SMs including citrinin, roquefortine C, chaetoglobosins A and C, expansolides A and B, andrastins A, B, and C and communesins [13]. The development of the fungus is generally linked to the production of SMs, [28–31] that may have specific ecological functions as virulence or aggressiveness factors, chemical weapons, communication signals, defense against fungivores or against damage [32].

P. expansum has a complex life cycle as its asexual life cycle involves four morphogenetic stages: (stage 1) vegetatively interconnected hyphal cells that form the mycelium, (stage 2) swelling of apical cells and subapical branching, (stage 3) formation of phialides, and (stage 4) formation of conidia [33]. The *brlA* (bristle), *abaA* (abacus-like), and *wetA* (wet-white) genes have been suggested to form a central regulatory pathway (CRP) that controls the expression of conidiation-specific genes [34–36]. The three genes are expressed sequentially and work in coordination to control the formation of conidiophores and conidium maturation [33,37,38]. The *abaA* gene is activated by BrlA in the middle stages of conidiophore development and is believed to be involved in the proper differentiation and functionality of phialides after the formation of metulae [34,36]. The *wetA* gene is activated by the *abaA* gene and is involved in the late stages of conidiation in the synthesis of crucial components (e.g., hydrophobins, melanins, and trehalose) of the cell wall layers that render mature conidia impermeable and resistant [39,40].

The *brlA* gene is expressed in the first stage of conidiation and encodes a C_2H_2-zinc-finger TF, which is considered a master regulator in the development of conidiophores. The structure of the *brlA* gene is complex and consists of two overlapping transcription units, *brlAα* and *brlAβ* [41]. These two transcription units are individually required for normal development but the products of these transcripts or mRNAs have redundant functions [42]. In the genus *Aspergillus*, the BrlA protein is mainly found in vesicles, metulae, and phialides but not in hyphae or mature conidia [37]. Studies of *Aspergillus nidulans* have shown that *brlA* is an extremely important gene in the CRP of conidiation since it activates the expression of *abaA* and *wetA* [41]. The *brlA* mutants have a "bristle-like" phenotype (no formation of conidia) because gene deletion blocks the transition of stalks to swollen vesicles and subsequent structures required for the formation of conidia resulting only in elongated aerial stalks [43,44]. On the other hand, overexpression of the *brlA* gene in vegetative cells leads to the formation of viable conidia directly from the tips of the hyphae [43,45]. In some fungi, including *Aspergillus clavatus* [45], *Aspergillus fumigatus* [31,46,47], and *Penicillium decumbens* [48], *brlA* has been shown to have an impact on the vegetative growth and biosynthesis of SMs, in addition to a major

role in asexual reproduction, suggesting that *brlA* could have many more roles than those identified so far. Orthologs of *brlA* are only present in *Aspergillus* and *Penicillium* species [49–52].

To gain further insight into the functions of the *brlA* gene in *P. expansum*, a PeΔ*brlA* mutant was generated using the homologous recombination strategy. The impacts of the deletion of *brlA* on growth, in vitro macro and microscopic morphology, in vivo pathogenicity in apples, metabolome, and transcriptome (DNA microarray) were evaluated. Taken together, our data revealed that deletion of the *brlA* gene blocked conidiation but not the formation of synnemata formed by aggregation of hyphal mycelia. The ability to form synnemata was boosted when PeΔ*brlA* strain grew on cellulose medium under light-dark cycle compared to the WT strain. The *brlA* deletion impaired the typical *P. expansum* morphology and enhanced the in vivo aggressiveness. The production of the two best-known *P. expansum* mycotoxins patulin and citrinin was not impaired on synthetic media whereas a significantly increased production of patulin was observed in vivo, explaining the higher aggressiveness. The *brlA* deletion resulted in decreased communesin production. On other hand, an increase in chaetoglobosin biosynthesis was observed. The microarray analysis displayed a down-regulation of genes involved in communesin biosynthesis and it unveiled the up-regulation of an 11-gene cluster sharing high similarity with the chaetoglobosin gene cluster characterized in *Chaetomium globosum*.

2. Results

2.1. Effect of brlA Deletion on in Vitro *Macroscopic and Microscopic Morphology*

At the macroscopic level, the WT strain appeared blueish-green in the conidial areas with an external white margin, while the null mutant PeΔ*brlA* strain was entirely white throughout the colony, regardless of the culture media used (Figure 1A). As expected, the WT strain produced simple fused conidiophores (coremia) that emerged from the hyphae. The deletion of the BrlA TF-coding gene led to the complete absence of conidia but elongated hyphae stalks emerged, giving the null mutant strain a "bristle-like" appearance that was completely different from the velvety and granular texture of the WT strain (Figures 1Ba, 1Bb). The WT strain colony also displayed shallow radial furrows on Malt Extract agar (MEA) whereas they were not observed in the null mutant PeΔ*brlA* strain (Figure S1). Both strains produced droplets of exudate on the surface of the mycelium, but the WT strain seemed to produce more exudate than the null mutant PeΔ*brlA* strain. At a microscopic scale, the strains displayed completely different morphology (Figures 1Bc, 1Bd). The WT strain had terverticillate conidiophores, which branched from hyphae; phialides were cylindrical and conidia ellipsoid-circular (Figures 1Bc). Deletion of the *brlA* gene blocked asexual reproduction in *P. expansum*. The null mutant PeΔ*brlA* strain produced longer stalks but with no conidiophores, whose development was stopped before branches, metulae, and phialides were formed (Figure 1Bd).

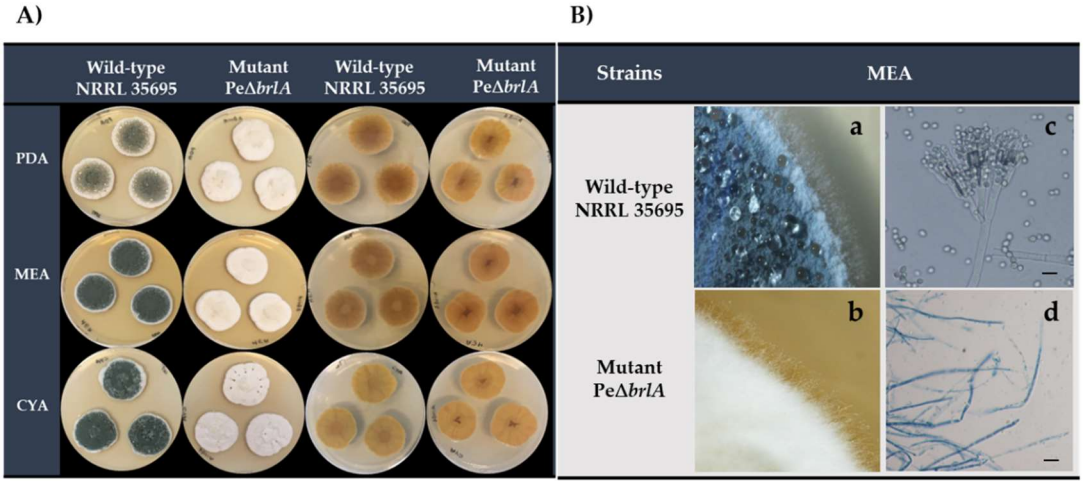


Figure 1. Morphological appearance of wild type *Penicillium expansum* and the null mutant PeΔ*brlA* strains. **A**) Macroscopic appearance of the colonies (recto-verso). The strains were grown on Malt

Extract agar (MEA), Potato Dextrose Agar (PDA), and Czapek Yeast extract Agar (CYA) for seven days at 25 °C in the dark. **B**) Stereomicroscope observation (×12) after 10 days of incubation (a) wild type strain; (b) null mutant strain PeΔ*brlA*. Microscopic appearance (×400): (c) wild type conidiophores; (d) null mutant strain PeΔ*brlA* stalks after seven days of incubation. The strains were grown on MEA at 25 °C in the dark. Black scale bars represent 10 μm.

2.2. Effect of brlA Deletion on Apple Colonization

2.2.1. Effect of *brlA* Deletion on Pathogenicity

To check if the null mutant PeΔ*brlA* strain could also trigger blue mold disease, Golden Delicious apples were infected. Both WT and null mutant strains were able to colonize the apples and showed the same development pattern for the first six days. An increase in rot diameter was observed in the null mutant PeΔ*brlA* strain from the seventh day and significant differences between the PeΔ*brlA* and WT strains were observed from the ninth day (Figures 2A, 2B). At the end of the 14-day incubation period, the diameter of the lesion caused by the PeΔ*brlA* strain was about 20% larger than the WT strain, 6.11 ± 0.14 cm and 5.08 ± 0.19 cm (*p*-value 7E-4), respectively. The growth rates calculated from the growth curves proved that the rotting rate of the PeΔ*brlA* strain (0.50 ± 0.02 cm/day) was significantly higher than the WT strain (0.42 ± 0.01 cm/day) (*p*-value 5E-4) (Figure S2). The rot volume calculated at the end of the incubation period showed that apples infected with the PeΔ*brlA* strain had a significantly higher rot volume than apples infected with the WT strain, 24.14 ± 1.31 cm^3, and 17.95 ± 1.53 cm^3 (*p*-value 7.2E-3), respectively (Figure 2C).

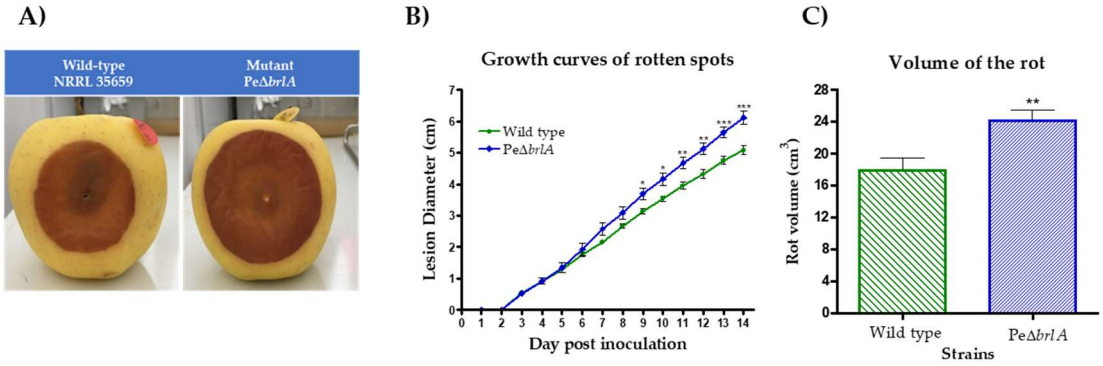


Figure 2. Golden Delicious apples infected with wild type *Penicillium expansum* or the null mutant PeΔ*brlA* strains, incubated at 25 °C for 14 days in the dark. **A**) Spots of rot 11 days after infection. **B**) Growth curves of spots. The diameter of the lesions was measured daily. **C**) The volume of rot measured at the end of the 14-day incubation period using the method described by Baert et al. [16]. The graphs show the mean ± standard error of the mean (SEM) from nine biological replicates and the significant differences between the wild type and the null mutant PeΔ*brlA* strains. *p*-value *< 0.05; **< 0.01; ***< 0.001.

2.2.2. Effect of *brlA* Deletion on in Vivo Patulin Production

Patulin concentrations measured in Golden Delicious apples after 14 days of incubation showed that the null mutant PeΔ*brlA* strain not only retained the ability to produce patulin, but produced four times the concentration produced by the WT strain, with 14.11 ± 8.3 µg/g and 58.5 ± 12.5 µg/g (*p*-value 9.3E-3) fresh weight of apples, respectively (Figure 3).

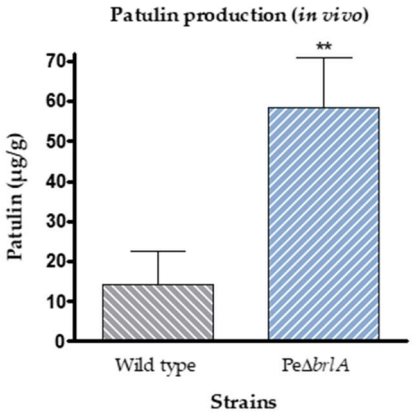


Figure 3. Patulin production in Golden Delicious apples infected with wild type *Penicillium expansum* or the null mutant PeΔ*brlA* strains at 14 days of incubation as previously described by Snini et al. [22]. Detection and quantification were performed by High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis at 277 nm and based on a standard curve, respectively. The graphs show the mean ± standard error of the mean (SEM) from nine biological replicates. *p*-value **< 0.01.

2.2.3. Effect of *brlA* Deletion on in Vivo Macroscopic Morphology

After 30 days of development on Golden Delicious apples, results showed that the WT and PeΔ*brlA* strains completely invaded the apple mesocarp, causing tissue decay. When the fruit was fully colonized, the fungus drilled the apple epicarp and conidiogenesis occurred (Figure 4a). Normal asexual reproduction in the WT strain involves the production of conidiophores clustered in coremia (Figure 4a,c) whereas in the mutant PeΔ*brlA* strain, the conidiogenesis process was interrupted before the formation of metulae and only rigid, white, and sporeless hyphal structures (synnemata) developed (Figure 4b,d).

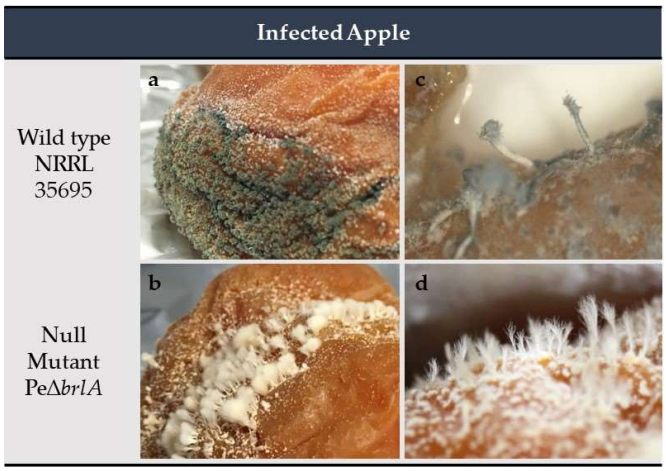


Figure 4. Apples infected with *Penicillium expansum* after 30 days of incubation at 25 °C in the dark. (**a**) Wild type strain; (**b**) Null mutant PeΔ*brlA* strain. Stereomicroscope observation (×12): (**c**)

development of conidiophores in the wild type strain; (**d**) development of only sporeless synnemata in the null mutant PeΔ*brlA* strain. The experiment was carried out with four biological replicates.

2.3. Growth Profile in Different Carbon Sources

The null mutant PeΔ*brlA* strain grew significantly more than the WT strain in 75% of cultures on minimal media supplemented with mono- or polysaccharides. Figure 5A shows the comparison of the fungal growth (colony diameters, in cm) between the WT and PeΔ*brlA* strains developed on different substrates.

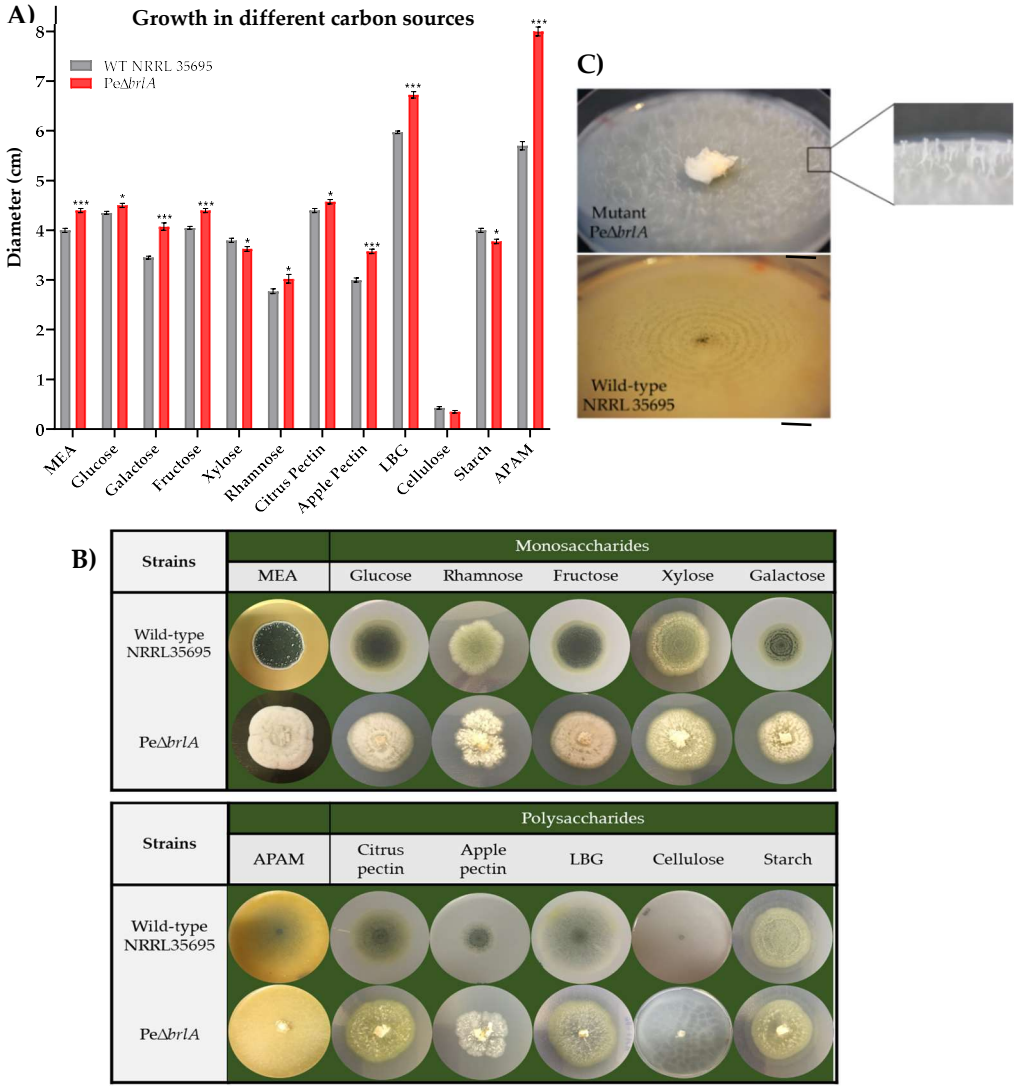


Figure 5. Wild type *Penicillium expansum* and the null mutant PeΔ*brlA* strains were grown in a minimal medium supplemented with different carbon sources for seven days at 25 °C in the dark. **A**) Average diameter (cm) of the colonies and statistical analysis of the wild type and null mutant PeΔ*brlA* strains, developed in the different substrates. The graphs show the mean ± standard error of the mean (SEM) from three biological replicates and the significant differences between the wild type and the null mutant PeΔ*brlA* strains. *p*-value *< 0.05; ***< 0.001. **B**) Photos of the strains cultured in monomeric or polymeric carbon sources. **C**) Strains grown in cellulose-supplemented medium after 15 days of incubation at 25 °C in a 16:8 light/dark cycle (16L8D cycle). Black scale bars represent 10 mm.

Strain development was favored in glucose- and fructose-supplemented media, while rhamnose-enriched media produced the smallest diameters observed with values of 2.70 ± 0.04 cm and 3.05 ± 0.08 cm for the WT and PeΔ*brlA* strains, respectively. The difference in strain diameters

was also apparent in the fructose- and galactose-enriched media where the diameters of the null mutant Pe*ΔbrlA* strain were up to 8.6% and 16% larger than the WT strain, respectively. Furthermore, carbon sources such as glucose, galactose, and fructose favored the growth of aerial mycelium in the null mutant Pe*ΔbrlA* strain (Figure 5B).

The polysaccharide locus bean gum (LBG) significantly favored the development of both strains, in contrast to cellulose in which very poor growth was observed. In starch- and cellulose-supplemented media the diameters of the mutant strain were smaller (−5% and −11.7%, respectively) than those of the WT strain. When the strains developed in the cellulose-supplemented medium using a 15-day incubation period and a 16:8 light/dark cycle (16L8D cycle), the diameter of the WT strain was 4.00 ± 0.02 cm, eight times the mean value obtained in a seven-day incubation period in the dark. The WT strain produced spores that formed concentric circles. Under the same growth conditions, the mutant Pe*ΔbrlA* strain increased its diameter value approximately 20 times (7.4 ± 0.1 cm) to the mean value obtained in a seven-day incubation period in the dark. The null mutant strain produced rigid synnemata that were scattered over the entire surface of the substrate (Figure 5C).

The Pe*ΔbrlA* strain showed significantly higher diameters than the WT strain in LBG and apple pectin-supplemented media with values of 12.5% and 19% higher, respectively. The diameters of both strains were bigger in the citrus pectin-supplemented medium than in the apple pectin-supplemented medium. However, the difference in diameter between the two strains was more pronounced when the strains were grown in apple pectin-enriched medium, the Pe*ΔbrlA* diameters were 19% larger than in the WT strain. The highest colony diameter values were obtained within the apple puree agar medium (APAM) [16], in which the diameter of the mutant Pe*ΔbrlA* strain was 40% larger than in the WT strain, similar to the in vivo results observed in apples.

Morphologically, the WT strain exhibited its characteristic blue-green color in almost all media, except in the rhamnose-, xylose-, and starch-supplement media, where its color was light green (Figure 5B). The mycelium of the null mutant Pe*ΔbrlA* strain was entirely white except in citrus pectin-, LBG- and starch-supplemented media, where the mycelium was slightly yellow in color (the origin of this yellow color was not determined). Glucose, fructose, and galactose favored the development of the aerial mycelium of the Pe*ΔbrlA* strain, giving it a fluffy texture (Figure S3a). Galactose favored the formation of coremia in the WT strain (Figure S3b) whereas in the rest of the substrates studied, this strain appeared to be flatter and smoother. The WT and null mutant strains produced no exudates in any of the media tested. Yellow halos were observed around both strains in the LBG-supplemented medium and in the WT strain in the citrus pectin-supplemented medium.

2.4. BrlA is a Key Factor in the Regulation of Penicillium expansum Secondary Metabolites

2.4.1. Secondary Metabolites Produced in Vitro

BrlA is a key regulator of fungal conidiation, but its role is not limited to asexual development. The ability to produce SMs by the null mutant Pe*ΔbrlA* strain was analyzed using liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS). The metabolites detected were identified after being cultured on labeled wheat and by comparing them with the reference metabolome of the WT strain grown under the same conditions. A total of 120 compounds were detected, of which 67 were identified and 53 remain unknown (Table 1). Some compounds with the same chemical formulae displayed similar MS/MS spectra (Table S1 and Table S2).

Table 1. Comparison of secondary metabolites detected in WT NRRL35695 and PeΔ*brlA* strains after culture on labeled wheat grains.

Molecular Formula	^{12}C m/z (Da)	$^{a}R_T$ (min)	Proposed Identification	WT	PeΔ*brlA*	Molecular Formula	^{12}C m/z (Da)	$^{a}R_T$ (min)	Proposed Identification	WT	PeΔ*brlA*
$C_7H_6O_4$	**153.01919**	**3.59**	**Patulin** †	+	+	$C_{28}H_{32}N_4O$	441.26589	17.14	Communesin F [d]	+	ND
C_7H_8O	109.06509	7.11	m-Cresol †	+	+	$C_{28}H_{32}N_4O_2$	457.26116	23.65	Communesin A [ft]	+	+
$C_7H_8O_2$	125.05998	6.79	m-Hydroxybenzyl alcohol †	+	+	$C_{28}H_{32}N_4O_3$	473.25566	15.50		+	ND
$C_7H_8O_3$	141.05493	3.80	Gentisyl alcohol†	+	+	$C_{28}H_{36}N_4O_4$	493.27971	31.47	Fungisporin A or cyclo(VFVF)	+	+
$C_7H_8O_4$	157.04990	2.67	Ascladiol †	+	+	$C_{28}H_{38}N_4O_5$	511.29099	18.01	VAL-PHE-VAL-PHE	+	+
$C_7H_{10}O_3$	143.07061	4.52		+	+	$C_{28}H_{38}N_4O_6$	527.28755	12.46	VAL-PHE-VAL-TYR	+	+
$C_{10}H_{17}NO_5$	232.11872	8.83		+	+	**$C_{28}H_{38}O_7$**	**485.25405**	**35.32**		+	+
$C_{10}H_{17}NO_5$	232.11872	9.65		+	ND	$C_{28}H_{38}O_7$	487.27063	36.21	Andrastin A †	+	+
$C_{13}H_{14}O_5$	251.09108	22.13	Citrinin †	+	+	**$C_{28}H_{38}O_8$**	**501.24792**	**27.70**		+	+
$C_{15}H_{18}N_2$	227.15514	6.44	Aurantioclavine †	+	ND	**$C_{28}H_{38}O_8$**	**501.24792**	**28.44**		+	ND
$C_{15}H_{19}NO_6$	310.12939	12.78		+	+	**$C_{28}H_{38}O_8$**	**501.24786**	**29.57**		+	+
$C_{15}H_{19}NO_6$	310.12964	14.80		+	+	**$C_{28}H_{40}O_6$**	**471.27434**	**39.48**	**Andrastin C**	+	+
$C_{15}H_{20}O_4$	265.14412	15.91	Expansolide C/ D	+	+	**$C_{28}H_{40}O_7$**	**487.26898**	**30.58**	**Andrastin B**	+	+
$C_{15}H_{20}O_4$	265.14410	16.91		+	ND	$C_{29}H_{27}N_5O_5$	526.20693	15.70		ND	+
$C_{15}H_{20}O_4$	265.14415	18.49	Expansolide C/ D	+	+	$C_{29}H_{27}N_5O_5$	526.20689	17.61		ND	+
$C_{15}H_{20}O_4$	265.14414	19.35		+	+	$C_{29}H_{31}N_5O_5$	530.23837	14.83		ND	+
$C_{16}H_{18}N_2O_2$	271.14496	7.62	Clavicipitic acid †	+	+	$C_{29}H_{31}N_5O_5$	530.23812	15.85		ND	+
$C_{16}H_{18}N_2O_2$	271.14467	8.45	Clavicipitic acid †	+	+	$C_{29}H_{31}N_5O_5$	530.23840	16.68		ND	+
$C_{16}H_{26}N_2O_4S_2$	375.14202	22.02		+	+	$C_{29}H_{33}N_5O_6$	548.24883	11.36		ND	+
$C_{17}H_{22}O_5$	307.15471	27.39	Expansolide A/B	+	+	$C_{29}H_{33}N_5O_6$	548.24860	12.14		ND	+
$C_{17}H_{22}O_5$	307.15504	30.19	Expansolide A/B	+	+	$C_{29}H_{33}N_5O_6$	548.25180	15.42		+	ND
$C_{17}H_{23}N_3O_3$	318.17943	3.12		ND	+	$C_{31}H_{36}N_4O_2$	497.29061	31.66	Putative new undetermined communesin	+	ND
$C_{17}H_{23}N_3O_3$	318.18043	25.05		ND	+	$C_{32}H_{34}N_4O_3$	523.27152	31.84	Communesin D [g]	+	+
$C_{18}H_{16}N_2O_2$	293.12911	13.58		+	ND	$C_{32}H_{36}N_2O_4$	513.27635	39.10	Chaetoglobosin J or Prochaetoglobosin III	+	+
$C_{18}H_{16}N_2O_2$	293.12915	17.74		+	ND	$C_{32}H_{36}N_2O_5$	529.26776	20.92	Chaetoglobosin B/G	+	+
$C_{18}H_{16}N_2O_3$	309.12421	14.76		+	ND	$C_{32}H_{36}N_2O_5$	529.26807	23.19	Chaetoglobosin B/G	ND	+
$C_{18}H_{18}N_2O_2$	295.14479	14.61		+	+	$C_{32}H_{36}N_2O_5$	529.27058	25.21	Chaetoglobosin B/G	+	+
$C_{18}H_{31}NO_7$	374.21663	23.72		+	+	$C_{32}H_{36}N_2O_5$	529.27029	26.71	Chaetoglobosin B/G	+	+
$C_{18}H_{35}N_3O_4$	358.26901	3.54		ND	+	$C_{32}H_{36}N_2O_5$	529.26757	29.56	Chaetoglobosin B/G	+	+
$C_{19}H_{16}N_2O_2$	305.12911	32.50		+	ND	$C_{32}H_{36}N_2O_5$	529.27077	30.51	Chaetoglobosin B/G	+	+
$C_{19}H_{16}N_2O_2$	305.12933	33.17		+	ND	$C_{32}H_{36}N_2O_5$	529.26807	32.43	Chaetoglobosin B/G	ND	+
$C_{19}H_{16}N_2O_4$	337.11909	13.73		+	ND	$C_{32}H_{36}N_2O_5$	529.27067	33.36	Chaetoglobosin A †	+	+
$C_{19}H_{16}N_2O_4$	337.11916	15.20		+	ND	$C_{32}H_{36}N_2O_5$	529.26771	34.25	Chaetoglobosin B/G	ND	+

$C_{19}H_{20}O_5$	329.13738	33.29		ND	+	$C_{32}H_{36}N_2O_5$	529.26769	35.49	Chaetoglobosin B/G	ND	+
$C_{19}H_{21}NO_7$	376.13901	17.45		+	+	$C_{32}H_{36}N_2O_5$	529.27063	36.41	Chaetoglobosin C †	+	+
$C_{19}H_{21}NO_7$	376.13904	18.65		+	+	$C_{32}H_{36}N_2O_5$	529.27056	37.41	Chaetoglobosin B/G	+	+
$C_{19}H_{38}O_6$	**361.25871**	**37.23**		+	ND	$C_{32}H_{36}N_2O_5$	529.26814	38.29	Chaetoglobosin B/G	ND	+
$C_{19}H_{38}O_6$	**361.25819**	**38.19**		+	ND	$C_{32}H_{36}N_2O_6$	545.26332	27.60	Putative cytochalasan	+	+
$C_{20}H_{18}N_2O_2$	319.14588	35.48		+	ND	$C_{32}H_{36}N_2O_6$	545.26241	29.23	Putative cytochalasan	ND	+
$C_{20}H_{18}N_2O_2$	319.14502	36.44		+	ND	$C_{32}H_{36}N_2O_6$	545.26288	30.57	Putative cytochalasan	+	+
$C_{20}H_{21}NO_9$	420.12857	19.29		+	+	$C_{32}H_{36}N_2O_6$	545.26281	31.50	Putative cytochalasan	+	+
$C_{20}H_{21}NO_9$	420.12858	22.19		ND	+	$C_{32}H_{36}N_2O_6$	545.26294	32.48	Putative cytochalasan	ND	+
$C_{20}H_{26}O_8$	395.17142	13.61		+	+	**$C_{32}H_{36}N_2O_6$**	**543.24836**	**34.05**	Putative cytochalasan	+	+
$C_{22}H_{23}N_5O_2$	390.19390	15.09	Roquefortine C †	+	+	$C_{32}H_{36}N_4O_2$	509.29257	34.95	Communesin B g†	+	+
$C_{22}H_{25}N_5O_2$	392.20913	9.99	Roquefortine D	+	+	$C_{32}H_{38}N_2O_4$	515.28895	35.59	Putative cytochalasan	+	+
$C_{23}H_{24}N_2O_6$	425.17179	26.92		+	+	$C_{32}H_{38}N_2O_4$	515.28890	37.93	Putative cytochalasan	+	+
$C_{24}H_{26}N_2O_6$	**437.17091**	**33.79**		+	ND	$C_{32}H_{38}N_2O_4$	515.28909	38.15	Putative cytochalasan	+	+
$C_{26}H_{30}N_4$	399.25568	19.94	Communesin K b	+	ND	$C_{32}H_{38}N_2O_5$	531.28384	21.75	Putative cytochalasan	ND	+
$C_{26}H_{30}N_4O$	415.25034	14.62	Communesin I c	+	+	$C_{32}H_{38}N_2O_5$	531.28990	22.76	Putative cytochalasan	+	+
$C_{26}H_{30}N_4O$	415.25033	18.43	Communesin I c	+	+	$C_{32}H_{38}N_2O_5$	531.28431	23.91	Putative cytochalasan	ND	+
$C_{26}H_{32}O_8$	473.21511	9.67		ND	+	$C_{32}H_{38}N_2O_5$	531.28360	27.38	Putative cytochalasan	+	+
$C_{26}H_{32}O_8$	473.21552	10.64		ND	+	$C_{32}H_{38}N_2O_5$	531.28354	28.02	Chaetoglobosin E h	ND	+
$C_{26}H_{32}O_8$	473.21489	34.14		ND	+	$C_{32}H_{38}N_2O_5$	531.28340	28.52	Putative cytochalasan	+	+
$C_{26}H_{40}O_6$	449.28939	29.93		+	+	$C_{32}H_{38}N_2O_5$	531.28338	31.56	Penochalasin F h	ND	+
$C_{27}H_{29}N_5O_4$	488.22783	10.80		ND	+	$C_{32}H_{38}N_2O_6$	547.27864	23.19	Putative cytochalasan	ND	+
$C_{27}H_{29}N_5O_4$	488.22766	11.03		ND	+	$C_{32}H_{38}N_2O_6$	547.27841	25.47	Putative cytochalasan	ND	+
$C_{27}H_{30}N_4O_2$	443.24564	15.03	Communesin E d	+	+	$C_{32}H_{38}N_2O_6$	547.27842	27.09	Putative cytochalasan	ND	+
$C_{28}H_{30}N_4O_3$	471.23997	19.56	Com470 e	+	+	$C_{32}H_{38}N_2O_6$	547.27851	28.27	Putative cytochalasan	ND	+
$C_{28}H_{31}N_5O_5$	518.24108	16.36		+	+	$C_{33}H_{38}N_4O_5$	571.29324	18.22	Com570 e	+	ND
$C_{28}H_{31}N_5O_5$	518.24092	17.21		+	+	$C_{37}H_{42}N_4O_5$	623.32511	29.52	Com622 e	+	ND

Compounds detected by negative electrospray ionization (ESI-) are in **bold**. [a] R_T= retention time, [b] Communesin K [53], [c] Communesin I [53,54], [d] Communesin E, and Communesin F [55], [e] Com470, Com570 and Com622 [56], [f] Communesin A [57], [g] Communesin D, and Communesin B [58]. [h] Chaetoglobosin E and Penochalasin F [59]. † Identified by standard. + = Detected. ND= Not detected.

The WT and PeΔ*brlA* strains have 65 metabolites (50%) in common while 23 were produced only by the WT strain and 32 are produced only in the null mutant strain. The production of patulin, citrinin, and roquefortines C and D was not affected by the deletion of the *brlA* gene. Intermediate compounds of the patulin biosynthetic pathway such as m-cresol, m-hydroxybenzyl alcohol, gentisyl alcohol, and ascladiol were also detected. The WT strain produced 12 communesin derivatives, whereas there was a drastic reduction in the production of these compounds in the null mutant strain, with only communesins I, E, Com470, A, D, and B represented. Moreover, aurantioclavine, an intermediate in the biosynthesis of communesins, was not detected, suggesting that it was not accumulated in the PeΔ*brlA* strain. Expansolides and andrastins A, B, and C were detected in both strains. Interestingly, the most common compounds found in the PeΔ*brlA* strain were the cytochalasans, of which 15 chaetoglobosins and one penochalasin were detected. In addition, a wide variety of unknown metabolites (18 compounds) were detected in the null mutant strain with chemical formulae $C_{32}H_{38}N_2O_4$ (R_T = 35.59, 37.93, and 38.15), $C_{32}H_{38}N_2O_5$ (R_T = 21.75, 22.76, 23.91, 27.38, and 28.52), $C_{32}H_{36}N_2O_6$ (R_T = 27.60, 29.23, 30.57, 31.50, 32.48, and 34.05), and $C_{32}H_{38}N_2O_6$ (R_T = 23.19, 25.47, 27.09, and 28.27). Comparison of MS/MS spectra with those of chaetoglobosins A and C suggested that the compounds are dehydroxylated and saturated ($C_{32}H_{38}N_2O_4$), saturated ($C_{32}H_{38}N_2O_5$), hydroxylated ($C_{32}H_{36}N_2O_6$), or saturated and hydroxylated ($C_{32}H_{38}N_2O_6$) forms of chaetoglobosin. Hence, there are 34 members of the cytochalasan alkaloid (chaetoglobosins/cytochalasins) family. Unknown metabolites (17) with chemical formulae $C_{17}H_{23}N_3O_3$ (R_T = 3.12 and 25.05), $C_{18}H_{35}N_3O_4$ (R_T = 3.54), $C_{19}H_{20}O_5$ (R_T = 33.29), $C_{20}H_{21}NO_9$ (R_T = 22.19), $C_{26}H_{32}O_8$ (R_T = 9.67, 10.64, and 34.14), $C_{27}H_{29}N_5O_4$ (R_T = 10.80 and 11.03), $C_{29}H_{27}N_5O_5$ (R_T = 15.70 and 17.61), $C_{29}H_{31}N_5O_5$ (R_T = 14.83, 15.85, and 16.68), and $C_{29}H_{33}N_5O_6$ (R_T = 11.36 and 12.14) were found only in the null mutant PeΔ*brlA* strain. Unknown compounds (17) with chemical formulae $C_{10}H_{17}NO_5$ (R_T = 9.65), $C_{15}H_{20}O_4$ (R_T = 16.91), $C_{18}H_{16}N_2O_2$ (R_T = 13.58 and 17.74), $C_{19}H_{16}N_2O_2$ (R_T = 32.50 and 33.17), $C_{18}H_{16}N_2O_3$ (R_T = 14.76), $C_{20}H_{18}N_2O_2$ (R_T = 35.48 and 36.44), $C_{19}H_{16}N_2O_4$ (R_T = 13.73 and 15.20), $C_{19}H_{38}O_6$ (R_T = 37.23 and 38.19), $C_{24}H_{26}N_2O_6$ (R_T = 33.79), $C_{28}H_{32}N_4O_3$ (R_T = 15.50), $C_{28}H_{38}O_8$ (R_T = 28.44), and $C_{29}H_{33}N_5O_6$ (R_T = 15.42) were no longer produced in the null mutant strain.

2.4.2. Secondary Metabolites Produced in Vivo

In vivo results revealed that after 30 days incubation, the null mutant PeΔ*brlA* strain had completely colonized the fruit. Table 2 lists the 33 compounds detected in apple flesh infected with the null mutant strain. Gentisyl alcohol and ascladiol were found in addition to the final product of patulin biosynthesis. Citrinin, expansolides A/B, roquefortine C, andrastins A and B were also identified. Nine chaetoglobosins and five putative members of the cytochalasan family were also detected. However, only one communesin derivative, communesin B, was found.

Table 2. Secondary metabolites detected in Golden Delicious apples infected with the null mutant PeΔ*brlA* strain (30 dpi).

Molecular formula	^{12}C *m/z* (Da)	R_T (min) [a]	Proposed Identification	Molecular formula	^{12}C *m/z* (Da)	R_T (min) [a]	Proposed Identification
$C_7H_6O_4$	**153.01919**	**3.59**	**Patulin**	$C_{29}H_{27}N_5O_5$	526.20689	17.61	
$C_7H_8O_3$	141.05493	3.80	Gentisyl alcohol	$C_{32}H_{36}N_2O_4$	513.27635	39.10	Chaetoglobosin J or Prochaetoglobosin III
$C_7H_8O_4$	157.04990	2.67	Ascladiol	$C_{32}H_{36}N_2O_5$	529.26776	20.92	Chaetoglobosin B/G
$C_{10}H_{17}NO_5$	232.11872	8.83		$C_{32}H_{36}N_2O_5$	529.26807	23.19	Chaetoglobosin B/G
$C_{13}H_{14}O_5$	251.09108	21.70	Citrinin	$C_{32}H_{36}N_2O_5$	529.27029	26.71	Chaetoglobosin B/G
$C_{16}H_{26}N_2O_4S_2$	375.14202	22.02		$C_{32}H_{36}N_2O_5$	529.26898	29.54	Chaetoglobosin B/G
$C_{17}H_{22}O_5$	307.15471	27.39	Expansolide A/B	$C_{32}H_{36}N_2O_5$	529.27539	30.13	Chaetoglobosin B/G
$C_{17}H_{22}O_5$	307.15504	30.19	Expansolide A/B	$C_{32}H_{36}N_2O_5$	529.26807	32.43	Chaetoglobosin B/G
$C_{19}H_{21}NO_7$	376.13901	17.45		$C_{32}H_{36}N_2O_5$	529.26769	35.49	Chaetoglobosin B/G
$C_{19}H_{21}NO_7$	376.13904	18.65		$C_{32}H_{36}N_2O_5$	529.27056	37.41	Chaetoglobosin B/G
$C_{22}H_{23}N_5O_2$	390.19390	15.09	Roquefortine C	$C_{32}H_{36}N_4O_2$	509.29257	36.01	Communesin B
$C_{23}H_{24}N_2O_6$	425.17179	26.92		$C_{32}H_{38}N_2O_5$	531.28384	21.75	Putative cytochalasan
$C_{28}H_{38}O_7$	**485.25405**	**35.32**		$C_{32}H_{38}N_2O_5$	531.28431	23.91	Putative cytochalasan
$C_{28}H_{38}O_7$	487.27063	36.21	Andrastin A	$C_{32}H_{38}N_2O_6$	547.27864	23.19	Putative cytochalasan
$C_{28}H_{38}O_8$	**501.24792**	**28.44**		$C_{32}H_{38}N_2O_6$	547.27841	25.47	Putative cytochalasan
$C_{28}H_{38}O_8$	**501.24786**	**29.57**		$C_{32}H_{38}N_2O_6$	547.27842	27.09	Putative cytochalasan
$C_{28}H_{40}O_7$	**487.26898**	**30.58**	**Andrastin B**				

Compounds detected by negative electrospray ionization (ESI-) are in **bold**. [a] R_T= retention time, dpi= days post inoculation.

In the second step, the synnemata that developed on the apple epicarp were extracted and the SMs present were analyzed. Table 3 lists the 54 SMs detected in the synnemata from the null mutant PeΔ*brlA* strain. Patulin and citrinin were no longer produced in synnemata. However, 21 of the 34 members of the cytochalasan family, including the well-known chaetoglobosins A and C, were detected in the synnemata. Forty of the SMs identified, including clavicipitic acid, expansolides A/B and C/D, roquefortines C and D, andrastins A, B, and C and eight communesins (A, B, D, E, F, I, K, and com470) were also detected in synnemata. Finally, unknown metabolites (14) with chemical formulae $C_{16}H_{26}N_2O_4S_2$ (R_T= 22.02) $C_{18}H_{16}N_2O_2$ (R_T = 17.74), $C_{18}H_{18}N_2O_2$ (R_T = 14.61), $C_{19}H_{38}O_6$ (R_T = 38.19), $C_{22}H_{20}N_3O$ (R_T = 38.56 and 39.29), $C_{23}H_{24}N_2O_6$ (R_T = 26.92), $C_{24}H_{26}N_2O_6$ (R_T = 33.79), $C_{26}H_{40}O_6$ (R_T = 29.93), $C_{28}H_{38}O_7$ (R_T = 35.32), $C_{28}H_{38}O_8$ (R_T = 27.70, 28.44, and 29.57), $C_{29}H_{31}N_5O_6$ (R_T = 14.66) were found in the synnemata of the null mutant PeΔ*brlA* strain.

Table 3. Secondary metabolites detected in synnemata that pierced the epicarp of apples infected with the PeΔ*brlA* strain (30 dpi).

Molecular Formula	^{12}C *m/z* (Da)	[a] R_T (min)	Proposed Identification	Molecular Formula	^{12}C *m/z* (Da)	[a] R_T (min)	Proposed Identification
$C_{15}H_{20}O_4$	265.14412	15.91	Expansolide C/ D	**$C_{28}H_{38}O_8$**	**501.24786**	**29.57**	
$C_{15}H_{20}O_4$	265.14415	18.49	Expansolide C/ D	**$C_{28}H_{40}O_6$**	**471.27434**	**39.48**	**Andrastin C**
$C_{16}H_{18}N_2O_2$	271.14496	7.62	Clavicipitic acid	**$C_{28}H_{40}O_7$**	**487.26898**	**30.58**	**Andrastin B**
$C_{16}H_{26}N_2O_4S_2$	375.14202	22.02		$C_{29}H_{33}N_5O_6$	548.25180	14.66	
$C_{17}H_{22}O_5$	307.15471	27.39	Expansolide A/B	$C_{32}H_{34}N_4O_3$	523.27152	31.84	Communesin D

$C_{17}H_{22}O_5$	307.15504	30.19	Expansolide A/B	$C_{32}H_{36}N_2O_4$	513.27635	39.10	Chaetoglobosin J or Prochaetoglobosin III
$C_{18}H_{16}N_2O_2$	293.12915	17.74		$C_{32}H_{36}N_2O_5$	529.27029	26.79	Chaetoglobosin B/G
$C_{18}H_{18}N_2O_2$	295.14479	14.61		$C_{32}H_{36}N_2O_5$	529.26757	29.56	Chaetoglobosin B/G
$C_{19}H_{38}O_6$	**361.25819**	**38.19**		$C_{32}H_{36}N_2O_5$	529.27077	30.51	Chaetoglobosin B/G
$C_{22}H_{20}N_3O$	**341.15380**	**38.56**		$C_{32}H_{36}N_2O_5$	529.27067	33.36	Chaetoglobosin A
$C_{22}H_{20}N_3O$	**341.15368**	**39.29**		$C_{32}H_{36}N_2O_5$	529.26769	35.49	Chaetoglobosin B/G
$C_{22}H_{23}N_5O_2$	390.19390	15.09	Roquefortine C	$C_{32}H_{36}N_2O_5$	529.27063	36.74	Chaetoglobosin C
$C_{22}H_{25}N_5O_2$	392.20913	9.99	Roquefortine D	$C_{32}H_{36}N_2O_5$	529.27056	37.41	Chaetoglobosin B/G
$C_{23}H_{24}N_2O_6$	425.17179	26.92		$C_{32}H_{36}N_2O_6$	545.26332	27.60	Putative cytochalasan
$C_{24}H_{26}N_2O_6$	**437.17091**	**33.79**		$C_{32}H_{36}N_2O_6$	545.26241	29.23	Putative cytochalasan
$C_{26}H_{30}N_4$	399.25568	19.94	Communesin K	$C_{32}H_{36}N_2O_6$	545.26288	30.57	Putative cytochalasan
$C_{26}H_{30}N_4O$	415.25034	14.62	Communesin I	$C_{32}H_{36}N_2O_6$	545.26294	32.48	Putative cytochalasan
$C_{26}H_{30}N_4O$	415.25033	18.43	Communesin I	$C_{32}H_{36}N_4O_2$	509.29257	34.95	Communesin B
$C_{26}H_{40}O_6$	449.28939	29.93		$C_{32}H_{38}N_2O_4$	515.28895	35.59	Putative cytochalasan
$C_{27}H_{30}N_4O_2$	443.24564	15.65	Communesin E	$C_{32}H_{38}N_2O_4$	515.28890	37.93	Putative cytochalasan
$C_{28}H_{30}N_4O_3$	471.23997	19.56	Com470	$C_{32}H_{38}N_2O_4$	515.28909	38.15	Putative cytochalasan
$C_{28}H_{32}N_4O$	441.26589	17.14	Communesin F	$C_{32}H_{38}N_2O_5$	531.28990	22.76	Putative cytochalasan
$C_{28}H_{32}N_4O_2$	457.26116	23.65	Communesin A	$C_{32}H_{38}N_2O_5$	531.28431	23.91	Putative cytochalasan
$C_{28}H_{38}O_7$	**485.25405**	**35.32**		$C_{32}H_{38}N_2O_5$	531.28354	28.02	Chaetoglobosin E
$C_{28}H_{38}O_7$	487.27063	36.21	Andrastin A	$C_{32}H_{38}N_2O_5$	531.28338	31.56	Penochalasin
$C_{28}H_{38}O_8$	**501.24792**	**27.70**		$C_{32}H_{38}N_2O_6$	547.27864	23.19	Putative cytochalasan
$C_{28}H_{38}O_8$	**501.24792**	**28.44**		$C_{32}H_{38}N_2O_6$	547.27841	25.47	Putative cytochalasan

Compounds detected by negative electrospray ionization (ESI-) are in **bold**.[a] R_T = Retention time.

2.5. Analysis of the Transcriptome of PeΔbrlA

A microarray analysis was performed to evaluate the impact of *brlA* deletion on *P. expansum* transcriptome after five days of growth on MEA. The PeΔ*brlA* strain showed 918 up-expressed genes and 1,398 down-regulated genes compared to WT strain. Genes were considered to be significantly differentially expressed when the Log_2-fold change was < -1 or > 1 with a *p*-value < 0.05. Among these, 365 genes were regulated 10 or more times, with 322 genes down-regulated and 43 genes up-regulated, respectively. As the central genetic regulatory cascade BrlA → AbaA → WetA exists in *Aspergillus* species, we investigated the change in the expression of *abaA* and *wetA* in the null mutant strain. These genes were 12-fold and 14-fold down-regulated, respectively. As expected, a lot of down-regulated genes in the PeΔ*brlA* strain were related to conidiation (Table 4).

Table 4. Differential expressed genes (DEG) involved in fungal development.

	Penicillium expansum d1 Strain Gene ID	Protein Name	Log_2 Fold Change PeΔ*brlA vs* WT	Adjusted *p*-Value	Putative Role
Regulation of Development	PEXP_029020	AbaA	−3.62	4.07E-11	Transcription factor
	PEXP_077410	WetA	−3.82	1.02E-10	DNA-binding transcription factor
	PEXP_085800	Axl2	−2.51	5.36E-11	Phialide morphogenesis regulatory protein
	PEXP_040110	PhiA	1.83	1.73E-05	Phialide development protein
	PEXP_003940	VadA	−2.83	1.37E-09	Spore-specific regulator
	PEXP_102520	DnjA	1.33	2.75E-07	DnaJ familly chaperone
	PEXP_064110	MedA	1.13	3.51E-05	Temporal modifier of developmental
	PEXP_050580	PpoC	1.29	6.05E-06	*psi*-Producing oxygenase
Hydrophobins	PEXP_062290	RodA	−13.00	1.07E-18	Rodlet A, Hydrophobic protein
	PEXP_020490	RodB/ DewB	−11.4	1.43E-16	Rodlet B, Hydrophobic protein
	PEXP_071760	DewC	−0.546	2.14E-01	
	PEXP_043320	DewD	−5.62	5.94E-13	
	PEXP_098360	DewE	−0.906	2.41E-03	
Pigmentation DHN-Melanin Like Pigment	PEXP_096630	Alb1	−12.2	6.41E-17	Putative polyketide synthase
	PEXP_097170	Arp1	−8.64	1.31E-15	Putative protein-Conidial pigmentation
	PEXP_097180	Arp2	−8.34	1.13E-13	HN reductase
	PEXP_097190	Ayg1	−6.57	9.55E-13	
	PEXP_097110	Abr1	−6.57	1.58E-12	Multicopper oxidase
Trehalose Biosynthesis	PEXP_050560	Ccg-9	−5.04	2.47E-06	Clock-controlled gene 9
Kinase	PEXP_066390	Gin4	−5.00	2.02E-12	Localization and function of septins
Velvet Protein Family	PEXP_092360	VeA	0.89	1.05E-05	Global transcription factor
	PEXP_065290	VelB	−0.53	1.01E-03	Velvet-like protein B
	PEXP_009420	VelC	0.43	3.66E-04	Regulator of sexual development
	PEXP_042660	LaeA	−0.44	5.39E-03	Putative methyltransferase
	PEXP_076870	VosA	−0.98	2.35E-04	Multifunctional regulator of development

Firstly, the deletion of *brlA* dramatically affected the expression of *rodA* and *rodB* genes that encode hydrophobins, the latter conferring a hydrophobic character to asexual spores. Except for the gene *abr2*, all the genes involved in 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis (*alb1*, *arp1*, *arp2*, *ayg1* and *abr1*) were strongly under-expressed compared to those in the WT strain. The velvet proteins (VeA, VelB, VelC, VosA) and their partner LaeA play a role in fungal development, more particularly in the balance between asexual and sexual reproduction in *A. nidulans* [60,61]. In *P. expansum*, only a slight decrease in the expression of the *vosA* gene and a slight increase in the expression of the *veA* gene occurred when the *brlA* gene was deleted. Trehalose is associated with conidiation, germination, and survival of asexual spores. Several studies have shown that WetA and VosA govern trehalose biosynthesis [62]. As the deletion of *brlA* affected the normal expression of *wetA* and *vosA* to a lesser extent, we focused on the expression of genes involved in trehalose biosynthesis such as *tpsA*, *orlA*, and *ccg-9* [62]. Only the expression of the latter was significantly reduced in the null mutant strain.

We also observed a decrease in the expression of *vadA*, a recently characterized spore-specific regulator [60], and significant up regulation of the VosA-repressed *dnjA* gene encoding the molecular chaperone [63].

Several examples of SMs specific to the spores have been reported in filamentous fungi [64,65]. Thus, we particularly focused on genes coding for backbone enzymes involved in secondary metabolism. In addition to the *alb1* gene mentioned above, the expression of nine backbone genes, *cnsF* (PEXP_030510), PEXP_018960, PEXP_095510, PEXP_095540, PEXP_072870, PEXP_006700, PEXP_037250, PEXP_029660, and PEXP_043150 was markedly altered in the PeΔ*brlA* strain. The DEGs were considered when the Log_2-fold change was < -2 with a *p*-value < 0.05. One of these genes (*cnsF*) has been reported to be involved in communesin biosynthesis.

By contrast, six genes were significantly up-regulated (Log_2-fold change was > 2 and a *p*-value < 0.05) when *brlA* was deleted, PEXP_074060, PEXP_096300, PEXP_045260, PEXP_028920, PEXP_063170, and PEXP_060620 (Table 5).

Table 5. Differentially expressed genes in PeΔ*brlA* strain coding for backbone enzymes involved in secondary metabolite biosynthesis.

	Penicillium expansum Strain d1 Gene ID	Biosynthetic Gene Cluster	Log_2 Fold Change PeΔ*brlA* vs WT	Adjusted *p*-Value
DMATS (Dimethylallyl tryptophane synthase)	PEXP_030140	Roquefortine C	1.68	1.40E-05
	PEXP_030510	Communesins	−4.64	3.08E-10
	PEXP_058590	-	−1.31	2.72E-08
PKS (Polyketide synthase)	PEXP_006700	-	−4.51	5.61E-15
	PEXP_028920	-	4.51	1.77E-04
	PEXP_030540	Communesins	−1.76	5.50E-07
	PEXP_037250	-	−2.14	4.85E-08
	PEXP_063170	-	2.73	1.29E-02
	PEXP_076200	-	−1.44	4.74E-05
	PEXP_094460	Patulin	−1.03	1.21E-05
	PEXP_094770	-	1.12	3.62E-03
	PEXP_095510	-	−2.89	7.63E-08
	PEXP_096630	Pigment	−12.2	4.72E-20
	PEXP_097790	-	1.52	4.63E-03
	PEXP_099180	-	−1.81	4.23E-10
	PEXP_102410	-	−1.92	3.65E-10
NRPS (Non ribosomal peptide synthetase)	PEXP_012360	-	−1.35	7.72E-08
	PEXP_015170	Fungisporins	1.71	1.34E-07
	PEXP_018960	-	−8.55	2.90E-11
	PEXP_029660	-	−2.97	7.36E-10
	PEXP_030090	Roquefortine C	0.99	2.57E-03
	PEXP_055140	-	1.55	1.17E-08
	PEXP_095540	-	−3.46	7.65E-09
	PEXP_096300	-	2.95	1.96E-11
	PEXP_104890	-	−1.1	2.04E-04

Hybrid PKS/NRPS	PEXP_008740	-	1.02	1.41E-05
	PEXP_074060	Chaetoglobosins	2.17	5.15E-10
NRPS-like	PEXP_045260	-	3.89	8.31E-07
	PEXP_050450	-	1.03	2.53E-07
	PEXP_060620	-	2.11	5.21E-11
	PEXP_072870	-	−2.05	6.25E-08
	PEXP_080590	-	−1.4	1.68E-08
	PEXP_082750	-	1.18	7.81E-09
	PEXP_095480	-	1.43	2.51E-10
Terpene cyclase	PEXP_043150	-	−2.68	1.19E-04

AntiSMASH analysis showed that PEXP_074060 belongs to a putative biosynthetic gene cluster. This cluster is composed of 11 genes that were up-regulated in the null mutant compared to the WT strain (Table 6). To confirm the microarray results, the expression of all genes of the putative cluster were assessed by qPCR (Table S3, Figure S4). AntiSMASH analysis revealed also that this putative cluster shares features with the chaetoglobosin cluster [66]. A more detailed manual BlastP analysis showed that, except two genes, other chaetoglobosin genes have homologous genes located in the putative cluster (Figure 6).

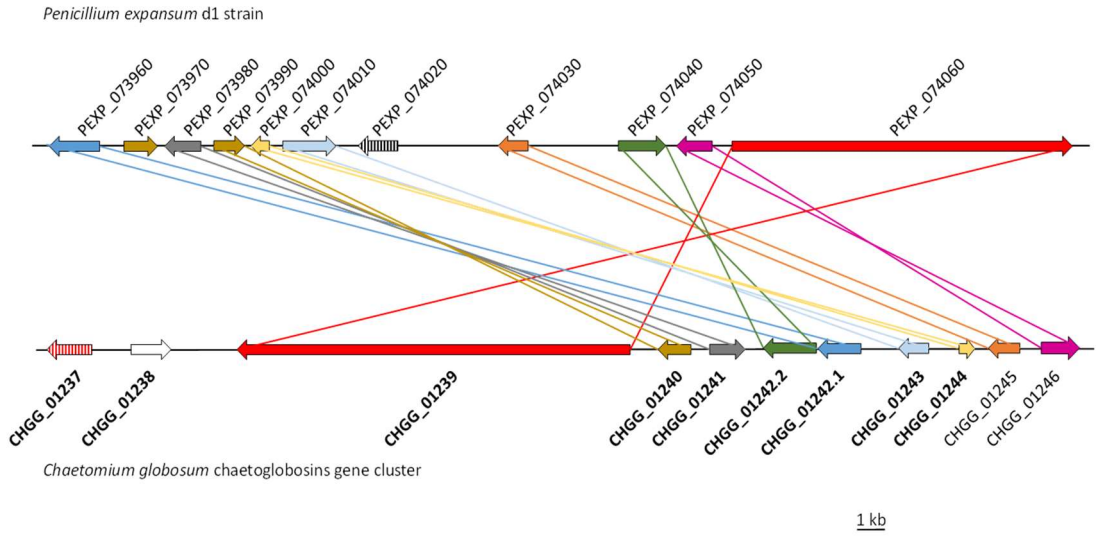


Figure 6. Comparison of the chaetoglobosin gene cluster in *Penicillium expansum* d1 strain and *Chaetomium globosum* strain. In bold, the cluster as described in Ishiuchi et al. [66]

Table 6. Putative chaetoglobosin gene cluster.

***Penicillium expansum* Strain d1**	***Chaetomium globosum* Strain CBS 148.51**	**% Identity / similarity**	**Log_2 Fold Change PeΔ*brlA* vs WT**	**Putative Function**
PEXP_073960	CHGG_01242.1/ **CHGG_05285**	48/64; **53/70**	1.84	CYP450
PEXP_073970	CHGG_01240/ **CHGG_05283**	41/63; **64/79**	2.07	Enoyl reductase
PEXP_073980	CHGG_01241/ **CHGG_05282**	48/64; **65/80**	1.68	Hypothetical protein
PEXP_073990	CHGG_01240/ **CHGG_05283**	47/65; **39/54**	1.89	Enoyl reductase
PEXP_074000	CHGG_01244	41/55;	2.08	Hypothetical protein
PEXP_074010	CHGG_01243/ **CHGG_05281**	47/66; **53/71**	1.82	CYP P450
PEXP_074020	CHGG_05287	31/49;	1.82	Transcription factor *
PEXP_074030	CHGG_01245/ **CHGG_05284**	47/62; **43/59**	1.95	Short-chain dehydrogenase
PEXP_074040	CHGG_01242.2/ **CHGG_05280**	38/53; **48/65**	1.84	FAD-dependent oxidoreductase

PEXP_074050	CHGG_01246/ **CHGG_05287**	54/70; **54/72**	1.97	Alpha/beta hydrolase
PEXP_074060	CHGG_01239/ **CHGG_05286**	43/61; **52/68**	2.17	PKS-NRPS

* Homologous with cytochalasin pathway-specific TF CcsR (ACLA_078640) in *Aspergillus clavatus* [67].
In **bold** a second chaetoglobosin gene cluster is present in *Chaetomium globosum* genome.

BrlA was previously studied in two *Penicillium* subgenus *Penicillium* species: *P. digitatum* [38] and *Penicillium rubens* [49]. As transcriptome data from Δ*brlA* mutants were available for *P. digitatum* and *P. rubens*, data from the three species were compared. Without being exhaustive, Tables S4, S5, S6, and S7 summarize this comparative study and are the subject of part of the discussion.

3. Discussion

Expressed for the earliest step of asexual reproduction in *Aspergillaceae* fungi, the *brlA* gene encodes a C_2H_2-type zinc-finger TF essential for conidiation. [37,68]. Although the *brlA* gene has been extensively studied, previous research focused on the *Aspergillus* genus ignored a wide field of research in other species. Here by creating a null mutant PeΔ*brlA* strain, we demonstrated that the *brlA* gene not only plays a fundamental role in the regulation of asexual development in *P. expansum*, but also influences the biosynthesis of certain SMs. The development of the null mutant PeΔ*brlA* strain on solid media results in a completely different phenotype from that of the WT strain. The *brlA* deletion resulted in a strain devoid of conidia, because in the absence of the *brlA* gene, the conidiogenesis process was stopped before metulae were created, which then formed only elongated aerial hyphae and gave the strain a "bristle-like" appearance. These results have also been reported in *A. niger* [69], *A. clavatus* [45], and *A. nidulans* [34] where inactivation of the *brlA* gene resulted in white aconidial strains resembling the PeΔ*brlA* strain in appearance. In genus *Penicillium*, the deletion of *brlA* in *P. digitatum* resulted also in a complete absence of conidiation [38]. Since the null mutant strain is entirely white, it can consequently be concluded that the *brlA* gene regulates the biosynthesis of conidia in *P. expansum*. In *P. decumbens*, the deletion of *brlA* produced strains that lack conidiophores [48]. During its development, the hyphae in the null mutant strain had more branches than the WT strain, but the hyphae were shorter displaying lower values of hypal growth length (Lhgu), evidence that removing *brlA* led to more frequent branching [48]. Preliminary studies in the *Aspergillus* genus have shown that *brlA* is an extremely important gene in the CRP of conidiation because it activates the expression of *abaA*, which in turn, activates *wetA*, the other two genes in this pathway, resulting in the reproduction and dissemination of the fungus [41,45]. The mutation of the regulation factors, AbaA and WetA, did not interfere in the formation of the vesicles, but eliminating *abaA* led to the formation of abnormal phialides that blocked the formation of conidia, while the mutation of *wetA* causes the spores to autolyze during the final stages of differentiation [34]. In *P. expansum*, *brlA* deletion led to a 12-fold and 14-fold decrease in the expression of the *abaA* and *wetA* genes, respectively.

As expected, the deletion of *brlA* also blocked the expression of other genes involved in conidiation. Among the genes most impacted by the deletion were genes encoding elements of the outer layer of conidia. This layer is composed of hydrophobins encoded by *rodA* and *rodB* and the DHN-melanin pigment is deposited beneath this hydrophobin layer. The induction of the DHN-melanin gene cluster by BrlA has already been identified in *A. fumigatus* [70] and in *P. decumbens* [48]. In *A. fumigatus*, the DHN-melanin pathway is encoded by six genes (*alb1*, *ayg1*, *arp2*, *arp1*, *abr*1, *abr2*) located in a cluster [71]. In *P. expansum*, five of them are scattered throughout the genome, with the exception of a cluster reduced to three or four genes depending on the strain studied. Therefore, there is no presence of an *abr2* orthologous gene in *P. expansum* suggesting that the DHN-melanin pathway stops before the last enzymatic step and leads to the synthesis of 1,8-DHN.

A weaker decrease in *axl2* gene expression was also observed in the null mutant strain. During *A. nidulans* conidiation, this transmembrane protein is localized to phialide-spore junctions. It is required for the septation event that splits the new conidia from their phialides. The *axl2* gene is over-expressed during conidiophore development in response to overexpression of *brlA* or *abaA* [72]. In *A. flavus*, *A. fumigatus* and *A. nidulans*, *vosA* is constantly induced by *wetA* [39]. AbaA is also required for expression

of *vosA* [73]. Here, we observed a slight decrease in *vosA* gene expression in the null-mutant compared to the WT strain.

Efforts have been made for several years to identify target genes of VosA in the model species *A. nidulans*. This work led to the discovery of the proteins DnjA, VadA, and VidA. VosA activates *vadA* [60] and *vidA* [74] whereas it represses *dnjA* in *A. nidulans* [63]. In *P. expansum*, the deletion of *brlA* resulted in a lesser *vadA* and a higher *dnjA* transcripts level but did not affect *vidA* expression. The deletion of *brlA* also led to overexpression of the PEXP_047560 gene, a *zcfA* homolog. Discovered and characterized recently in *A. flavus* and *A. nidulans*, this Zn2Cys6 TF is essential for the balance between sexual and asexual reproduction. The authors showed that its level of transcripts increased in the Δ*vosA* mutant of each species [75].

While the role of BrlA is well documented in the genus *Aspergillus*, data on BrlA in the genus *Penicillium* are limited to three studies on *P. decumbens* [48], *P. rubens* (formerly identified as *P. chrysogenum*) [49], and *P. digitatum* [38]. Although together with *P. expansum, P. rubens*, and *P. digitatum* belong to the *Penicillium* subgenus *Penicillium*, *P. digitatum*, and *P. expansum* are phylogenetically closely related and are classified in the *Penicillium* section [76]. A recent study estimated that the two species diverged only about 15 million years ago (MYA) [77], while the *P. rubens* ancestor separated from the *P. digitatum* and *P. expansum* ancestors about 20 MYA. Following transcriptome analyses of the Δ*brlA* mutant of *P. rubens* [49] and *P. digitatum* [38], the lists of down- and up-regulated genes in the respective PeΔ*brlA* strains were compared. Of the 106 genes whose expression decreased with a $Log_2FC < -3$ at PdΔ*brlA*, 101 have an orthologous gene in *P. expansum* and 79.2% of these were also significantly down-regulated ($Log_2FC < -1$, adj. *p*-value < 0.05) in the mutant PeΔ*brlA* (Table S4). This suggests that the gene network under direct or indirect positive influence of BrlA factor has remained relatively unchanged for 15 million years. Surprisingly, if one considers now the genes under negative BrlA regulation, only 12 genes out of the 39 ortholog genes up-regulated in PdΔ*brlA* with a $Log_2FC > 2$ showed an increase in their expression in PeΔ*brlA* (Table S5).

Sigl et al. [49] identified 38 genes regulated in a similar way to *wetA* in *P. rubens* Δ*brlA* (PrΔ*brlA*). Eighteen of these genes were also regulated in the same way in the PeΔ*brlA* strain (Table S6). Among them, we identified five genes (PEXP_018490, PEXP_030380, PEXP_096550, PEXP_096560, PEXP_037140) that have orthologous genes in *A. flavus*, *A. fumigatus*, and *A. nidulans*, and that were all under-expressed in the Δ*wetA* strain in all three species [39]. The role of these genes is not yet known. The only information available is that, except the PEXP_096560 gene, all orthologous genes were down-regulated at the conidiation stage in *A. fumigatus* Δ*atfA* strain [78]. In the same study, the authors also identified 93 genes regulated in a similar way to *abaA* in PrΔ*brlA*, of which 59 orthologous genes were down-regulated in PrΔ*brlA* and PeΔ*brlA* strains (Table S7). Only a few of these genes have been characterized to date. Among them, particular attention has been paid to *gin4* (PEXP_066390). This gene is also strongly down-regulated in PdΔ*brlA* ($Log_2FC = -4.9$) and encodes a kinase conserved in Ascomycetes. It has been demonstrated that it phosphorylated septins in Saccharomycetales such as *Saccharomyces cerevisiae* [79]. In *A. fumigatus*, the deletion of *gin4* led to an increase in the interseptal distance [80]. Although a link between greater interseptal distance and hyphal radial growth has not been demonstrated, the lengthening of the interseptal distance could explain the higher radial growth observed in the PeΔ*brlA* strain in some media. The inability of PeΔ*brlA* strain to produce conidia led us to (i) the impossibility of generating a complemented mutant to restore the WT phenotype, (ii) the use of two types of inoculum with a risk to introduce a bias in the transcriptomic results. The strong similarities in the transcriptome data between the three mutants PeΔ*brlA*, PdΔ*brlA* [38], and PrΔ*brlA* [49] support the idea that the difference of inoculum had a minor impact and that it was indeed the deletion of *brlA* that caused the changes observed in the PeΔ*brlA* strain.

VeA is a global TF that is a member of the velvet complex involved in the regulation of many cellular processes, including SM biosynthesis and fungal development, which positively regulates sexual reproduction [61,81]. In *A. nidulans* VeA acts upstream of BrlA to inhibit asexual development. However, our results showed that BrlA had a negative effect on the expression of *veA* [60,61]. The slight increase in *veA* expression in the null mutant strain could explain the higher patulin production since

patulin is strongly affected when the *veA* gene is deleted [26]. El Hajj Assaf et al. [26] have shown that, surprisingly, the null mutant PeΔ*veA* strain has lost the ability to create coremia, rigid structures formed by aggregation of conidiophores, both in vitro and in vivo. In the dark, the strain was still able to sporulate on synthetic media, but due to the absence of coremia, the null mutant strain was unable to pierce the epicarp of the apple and emerge from the fruit to complete its life cycle [26]. Our results showed that, although the absence of the *brlA* gene completely blocked the production of conidiophores, in vivo the mutant PeΔ*brlA* strain was able to produce rigid synnemata that allowed it to pierce the epicarp and emerge from the apple. To summarize the sequence of events that take place at the end of apple infection, VeA is essential for the formation of synnemata, which, in turn, is indispensable for perforation of the epicarp. BrlA is required in the second step to enable the formation of the entire fruiting structure, e.g., the conidiophore with all its components (rami, ramuli, metulae, phialides and conidia).

As *P. expansum* is the main cause of blue mold disease in apples and producer of patulin [16], we also analyzed the pathogenicity of the mutant PeΔ*brlA* strain in Golden Delicious apples. First, we observed that the null mutant strain was able to colonize the fruit, thereby inducing the disease, but differently from the WT strain. During the first six days, both strains showed the same development profile. From day nine on, a significant increase in the rot rate was observed in the null mutant strain, resulting in a final lesion diameter 20% larger than that of the WT strain. In addition, we found that the absence of the *brlA* gene in *P. expansum* did not reduce or stop patulin production. PeΔ*brlA* quadrupled compared to the WT strain. This observation is in agreement with reports that patulin is an important but not essential factor in the pathogenicity of *P. expansum*[21,22,82]. When the *patL* gene encoding the specific TF in the patulin biosynthesis pathway was deleted in *P. expansum*, patulin production was completely suppressed. The mutation also reduced virulence in apples inoculated with the null mutant strain. However, when patulin was added exogenously, the ability to cause disease was restored, suggesting that patulin plays a role in the development of apple spoilage [22]. These results were also observed in Golden Delicious apples infected with the null mutant strain PeΔ*veA*. Where the elimination of global FT VeA, involved in MS production, also suppressed patulin production and reduced the virulence of *P. expansum* [26]. Pathogenicity studies in 13 apple varieties showed that both the null mutant PeΔ*patL* and the WT strains were able to infect apples, but the intensity of symptoms depended not only on the capacity to produce patulin but also on the genetic background of the apple, suggesting that patulin is an aggressiveness factor rather than a virulence factor [22]. Several decades ago, conidiogenesis was linked to patulin production when a mutation at an early stage of conidiation caused a notable decrease in patulin production in *Penicillium griseofulvum (*syn = *P. urticae*) [83]. These results are in contradiction with the present results. Unfortunately, the mutant strain in the last study was generated by chemical mutagenesis and the mutation(s) has (have) not been genetically characterized for more in-depth discussion.

The stages of fruit ripening also influence the pathogenicity and virulence of the fungus, leading to increased accumulation of patulin in ripe fruits infected by *P. expansum* [25]. On the other hand, the availability of nutritional sources, such as carbon and nitrogen, is a key factor in the development of fungi and in the biosynthesis of SMs. When *P. griseofulvum* was grown on PDA and MEA media, conidiation and production of griseofulvin were reported to increase in media with higher carbon content (PDA) [84]. As apples are a good source of carbon, rich in glucose, sucrose, and fructose, we studied the growth profiles of the null mutant PeΔ*brlA* and WT strains in minimal media enriched with different carbon sources. The greatest development was observed in the APAM medium, perhaps because it is an apple-based natural medium. Media supplemented with glucose and fructose promoted the growth of the strains compared to the other monosaccharides. Surprisingly, when the strains were grown in a medium containing citrus pectin, their diameters were bigger than when they were grown in apple pectin. In 75% of the media tested, the null mutant strain developed significantly better than the WT strain. Other in vitro studies have also shown that high concentrations of sugars such as glucose and sucrose reduced the production and accumulation of SMs [23,25].

The production of natural metabolites in filamentous fungi is often linked to cell development and differentiation processes, as the environmental conditions required for sporulation and secondary metabolism are similar [28]. The most widely studied compounds are mycotoxins, due to their harmful effects on human and animal health. The relationship between sporulation and mycotoxin production has been assessed in several genera. The influence of several inhibitors of conidiophore maturation has been studied in *Aspergillus parasiticus*. At a concentration of 1 mg/mL of these inhibitors, both sporulation and aflatoxin B production were strongly affected, suggesting conidiogenesis and secondary metabolism are interrelated [85]. Recently, the suppression of early-acting regulators of sexual and asexual reproduction has been shown to be closely correlated with SM biosynthesis. For example, deletion of the *veA* gene in *A. niger* not only reduced ochratoxin A production and tolerance to oxidative stress, but also the production of conidia, as *brlA* gene expression was significantly reduced [86]. Satterlee et al. [87] reported that deletion of the *hbxA* gene that encodes a transcriptional developmental regulator, not only affected the biosynthesis of fumigaclavines, fumiquinazolins and chaetomine, but also reduced production of conidia since deletion resulted in under-expression of *brlA* and the fluffy genes *flbB*, *flbD*, and *fluG* in *A. fumigatus*. The Flb (For Fluffy low *brlA* expression) B and D are BrlA upstream development activators activated by FluG, which is responsible for the biosynthesis of an extracellular diffusible factor [88–90].

Our in vitro results showed that 50% of the SMs are produced by both the null mutant PeΔ*brlA* and the WT strains, meaning that the loss of *brlA* has no impact on the production of these compounds under the conditions tested here. The PeΔ*brlA* strain was unable to produce 32 compounds present in the WT strain, showing that BrlA is required for the production of these compounds. Conversely, BrlA negatively controlled the production of 32 other compounds only present in the null mutant strain. When the null mutant strain grew on dead vegetal biomass (wheat grains), the production of the mycotoxins patulin, citrinin, and roquefortines C and D as well as the bioactive compounds expansolides and andrastins A, B, and C were not inhibited by the suppression of the *brlA* gene. The compounds that were not produced by the null mutant strain were mainly communesins, of which only six of the 20 communesin derivatives produced by the WT strain were detected, in addition to the unknown metabolites of *m/z* 305.129 (Rt = 32.50 and 33.17) and 319.145 (Rt = 35.48 and 36.44), we suggest that these compounds may be related to pigmentation or spore protection. Among the new compounds not produced by the WT strain, we found a wide range of chaetoglobosins as well as compounds that could be members of the cytochalasan alkaloid family. The production of several chaetoglobosins has already been detected in different isolates of *P. expansum* [13] and these compounds have a wide range of biological activities, including antitumor, antifungal, or antibacterial properties [91].

The main compounds detected in the synnemata were chaetoglobosins, with 14 different derivatives (including chaetoglobosins A and C) whereas other derivatives are produced by the hyphae inside the apple. This may mean that biosynthesis of these metabolites takes place when the fungus emerges from the fruit. This observation suggests a spatial organization of this biosynthesis pathway. An example of selective accumulation of a particular secondary metabolite in a specific fungal tissue has already been reported [46]. Lim et al. [46] observed a predominant accumulation of fumiquinazoline C in the conidia of *A. fumigatus* whereas its biosynthetic precursors, fumiquinazolines A and F, were detected at comparable levels at different stages of development (basal hyphae, conidiophores, and conidia). Patulin and citrinin were detected only in apple flesh, not in synnemata. Disruption of the *brlA* gene in *A. fumigatus* not only yielded strains lacking conidiophores but that were also unable to produce the ergot alkaloids (festuclavine and fumigaclavines A, B, and C) fumiquinazoline C, trypacidin, and its two precursors (monomethylsulochrin questin), present in the conidia of the WT strain [64,92]. By contrast, strong production of fumitremorgins and verruculogen was reported [64]. The comparison of metabolome analyses of PeΔ*brlA* and WT cultures on sterilized labeled wheat grains evidenced the disappearance of some compounds and the appearance of other metabolites. Our results indicated that some SMs were specifically regulated by the *brlA* gene in *P. expansum* and confirmed that patulin production was not linked to the conidiogenesis. The in vivo analyses showed that its biosynthesis takes place in the vegetative mycelium inside fruits and stops when the competence phase begins.

A cluster of genes involved in the biosynthesis of chaetoglobosins has been identified in *P. expansum* [93]. It consists of seven genes (*cheA-cheG*). Surprisingly, only *cheF* (PEXP_043620) coding for a regulator is present in the eight *P. expansum* genomes sequenced and available in GenBank. Our transcriptomic analysis showed that this gene was very poorly expressed, and we observed no significant difference between the WT and the null mutant strains. These results were confirmed by qPCR. Additionally, several qPCR attempts using several primer designs were made to detect any expression of the other six *che* genes. All these attempts failed, suggesting that only *cheF* gene subsists in the genome of strain NRRL 35695. However, the production of chaetoglobosins by *P. expansum* is consistent, since another study showed that 100% of the strains originating from different substrates and geographical origins produced chaetoglobosins [13]. Instead, a cluster of 11 genes sharing high similarity with the gene cluster of chaetoglobosins in *Chaetomium globosum* [66] is present in *P. expansum* genomes. Although Ishiuchi et al. [66] delineated the cluster at nine genes, the two genes located directly downstream (CHGG_01245 and CHGG_01246) have a corresponding homolog in *P. expansum* (Figure 6). The difference between the clusters in the two species is the absence in *P. expansum* of a gene homologous to CHGG-01238 coding for a transposase and the absence of CHGG_01237 coding for a regulator. In *P. expansum*, the latter is replaced by another TF homologous to the cytochalasin cluster-specific regulator in *A. clavatus* [67]. The involvement of the transposase in chaetoglobosin biosynthesis has not been demonstrated to date. However, the absence of the gene CHGG-01238 in another chaetoglobosin-producing strain of *C. globosum* suggests that it is not essential for the synthesis of these compounds [94]. Our transcription analyses (microarray and qPCR) showed that all genes of this putative gene cluster were over-expressed in Pe*ΔbrlA.*

In this study, we also showed that the deletion of the *brlA* gene leads to over-production of chaetoglobosins with the appearance of minor compounds that were undetectable in the WT strain. Taken together, these data strongly suggest that this putative cluster is responsible for the biosynthesis of chaetoglobosins in *P. expansum.* To confirm this hypothesis and to determine the exact role of homologous proteins to CHGG_01245 and CHGG_01246, the generation of monogenic null mutants is currently underway.

4. Materials and Methods

4.1. Fungal Strains and PeΔbrlA Mutant Strain Construction

Penicillium expansum NRRL 35695, originally isolated from grape berries in Languedoc-Roussillon (France) was used as a wild type strain (WT). To understand and study the role of the *brlA* gene in *P. expansum*, a gene deletion strategy was applied in the WT *P. expansum* NRRL 35695 strain. Considering that BrlA is conserved in *Aspergillaceae* [50], the sequence for *P. expansum brlA* (PEXP_049260) were obtained from the genomic sequence of *P. expansum* strain d1 [21] after a BlastP analysis using the previously characterized *A. fumigatus* (AFU1G16590) [95] and *P. rubens* (PC06g00470) [49] BrlA proteins. The protein encoded by PEXP_ 049260 shares 95.5% identity with BrlA (PC06g00470) from *P. rubens* and 60% identity with BrlA (AFU1G16590) from *A. fumigatus*, respectively.

The construction of the null mutant strain is detailed in Supplementary Materials (Figure S5). Briefly, using the homologous recombination strategy, the *brlA* gene was replaced by the hygromycin resistance marker (*hph*), flanked by the DNA sequences corresponding to the 5′ upstream and 3′ downstream sequences of the *brlA* coding sequence. The gene disruption cassette was constructed by PCR, whereby the flanking regions 5′ upstream and 3′ downstream were amplified from the genomic DNA of *P. expansum* strain NRRL 35695. The pAN7.1 plasmid was used to generate the amplicon containing the hygromycin resistance gene [96], subsequently all fragments were assembled using double-joint PCR [97,98]. The cassette in which the *brlA* gene was replaced by the hygromycin resistance marker was used to transform the WT strain according to the method described by Snini et al. [22].

Contrary to usual practice, a complemented strain was not generated. To generate a complemented mutant, we have to transform protoplasts prepared 12 h after inoculation of conidia, but the null mutant Pe*ΔbrlA* was no longer able to produce the conidia essential for the formation of protoplasts.

4.2. Validation of PeΔbrlA Mutant Strain

In order to confirm the insertion of the hygromycin marker at the *brlA* locus of *P. expansum* and the deletion of the *brlA* gene, only the transformants that exhibited morphological characteristics different from those of the WT, e.g., the strains were white, with a "bristle-like" appearance and devoid of conidiophores, were molecularly or genetically tested. Figure S6 details the results obtained by PCR screening in the WT and PeΔ*brlA* strains. PCR with primers specific to the *brlA* gene, dBrlA-geneF/dBrlA-geneR (Table S8), generated a 1,137 base pair (bp) fragment for the WT strain, while no fragment for the null mutant PeΔ*brlA* strain was generated. Amplification of 5′ and 3′ locus *brlA*/*hph* junctions in PeΔ*brlA* strain displayed fragments of 2,121 bp and 2,185 bp, confirming the replacement of *brlA* by *hph*.

Validation of the transformants by genome walking confirmed the correct insertion of the selection marker at the *brlA* locus, the EcoRV library generated an amplicon of 623 bp, while the PvuII library generated an amplicon of 4,009 bp (Figure S7). The restriction cutting for the EcoRV library produced fragments of 217 and 483 bp with the enzyme KpnI and of 276 and 424 bp with the enzyme BstxI. For the PvuII library, the enzyme HindIII produced fragments of 298 and 3,702 bp and the enzyme BamHI of 929, 1,159, and 2,004 bp (Figure S7). These results show that there is only one copy of the disruption cassette in *P. expansum* and that it is integrated at the locus *brlA*.

The final validation of the transformants was performed by qPCR analysis. Figure S8 confirms the absence of *brlA* gene expression in the null mutant PeΔ*brlA* strain. This observation was confirmed in the microarray analysis (Log_2FC = -6.21; adjusted *p*-value 1.07E-14).

4.3. Macroscopic and Microscopic Morphology

The WT and PeΔ*brlA* strains were grown in MEA (Biokar diagnostics, Allonne, France; 30 g/L malt extract, 15 g/L agar) Petri dishes for seven days at 25 °C, after which spore suspension was made of the WT strain and its concentration was quantified using a Malassez cell [99]. MEA, PDA (Merck KGaA, Darmstadt, Germany; 30 g/L potato extract, 15 g/L agar), and 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), 1 mg/L K_2HPO_4, 5 g/L yeast extract, 30 g/L saccharose, 15 g/L agar] media were inoculated centrally with 10 µl of a 10^6 spores/mL suspension of the WT strain or 5 mm^2 of mycelium from the mutant PeΔ*brlA* strain and were incubated for 10 days at 25 °C in the dark. Microscopic characteristics were observed using an optical microscope CX41 (×400 and ×1000) (Olympus, Rungis, France) after seven days. Macroscopic characteristics were studied using a stereomicroscope SZX9 (×12–120) (Olympus), after 10 days. The experiment was performed in triplicate.

4.4. Pathogenicity Study and Patulin Production

An in vivo study was performed to investigate the impact of the *brlA* gene mutation on the aggressiveness of the blue mold caused by *P. expansum*. Golden Delicious apples were purchased in a supermarket in Toulouse (Carrefour, Toulouse, France) and wash-sterilized in a 2% sodium hypochlorite solution [82]. To obtain equivalent study conditions, first, a few spores of the WT strain or a fragment of mycelium of the mutant PeΔ*brlA* strain were placed in 50 mL of a liquid yeast extract glucose medium (Merck KGaA; 5 g/L yeast extract, 20 g/L glucose) on an orbital shaker set at 150 rpm at 25 °C for 72 h. Then, 50 mg of mycelium of each strain was weighed and placed in 3 mL of 0.05‰ Tween 80 and sonicated for 5 h in an ultrasonic sonicator (Bransonic 221 Ultrasonic bath, Roucaire, Les Ulis, France). Apples were wounded with a sterile toothpick on one side and 10 µl of the suspension was deposited. Infected apples were incubated for 14 days at 25 °C in the dark. The diameter of the rotten spots was measured daily and the volume of rot was determined at the end of the incubation period [16]. At the end of the incubation period, the whole apples were ground in a blender into puree, and an aliquot (10 g) of each sample was analyzed for patulin production as described by Snini et al. [22] and El Hajj Assaf et al. [26]. Patulin was quantified by HPLC as described previously [22,26]. The experiment was performed with nine biological replicates of each strain.

4.5. Analysis of Growth on Different Carbon Sources

The WT and null mutant Pe*ΔbrlA* strains were grown in Petri dishes containing minimal medium (MM) [6.0 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4$, 200 µl/L trace elements (10 g/L EDTA, 4.4 g/L $ZnSO_4 \cdot 7H_2O$, 1.01 g/L $MnCl_2 \cdot 4H_2O$, 0.32 g/L $CoCl_2 \cdot 6H_2O$, 0.315 g/L $CuSO_4 \cdot 5H_2O$, 0.22 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.47 g/L $CaCl_2 \cdot 2H_2O$, 1.0 g/L FeSO4 • 7H2O), 10g/L glucose, 15g/L agar] [100] supplemented with different carbon sources. Glucose, galactose, fructose, rhamnose, and xylose were used as monosaccharides at a final concentration of 25 mM. The polysaccharides: cellulose, starch, citrus pectin, apple pectin, and LBG were added at a final concentration of 0.5% [101]. The APAM medium, an apple-based substrate permissive for patulin production, was prepared as described by Baert et al. [16]. The media were inoculated centrally with 10 µl of a 10^6 spores/mL suspension of the WT strain or 5 mm^2 of mycelium from the mutant Pe*ΔbrlA* strain and incubated at 25 °C for seven days in the dark. The diameters of the colonies were measured at the end of the incubation period. All the experiments were conducted in triplicate.

4.6. Secondary Metabolism Study

4.6.1. Fungal Growth Conditions on Labeled Wheats

The SMs were analyzed using a non-targeted metabolomic approach combining LC-HRMS. Known and unknown metabolites produced by the fungus were detected and unambiguously characterized by a unique chemical formula. Briefly, wheat grains labeled with stable isotopes were used, 96.8% ^{13}C enriched wheat (^{13}C wheat) and 53.4% ^{13}C and 96.8% ^{15}N wheat ($^{13}C/^{15}N$ wheat); 99% ^{12}C (^{12}C wheat) natural wheat grains were also used. The natural and labeled wheat grains were produced and treated as described previously [102,103]. Ten grams aliquots of each type of sterilized wheat grains were placed in sterile Petri dishes (45 mm diameter) and inoculated 100 µl of a 10^5 spores/mL suspension of the WT strain or three fragments (5 mm^2) of mycelium from the mutant Pe*ΔbrlA* strain. The cultures were incubated at 25 °C for 14 days in the dark; a Petri dish containing uninfected ^{12}C natural wheat grains was used as control. After 14 days, the substrate is fully colonized by each strain, reducing the bias introduced by the use of two types of inoculum. At the end of the incubation period, fungal metabolites were extracted according to the methodology described previously [103].

4.6.2. In Vivo production of Secondary Metabolites

The detection of SMs in vivo was performed on Golden Delicious apples. The fruits were treated as detailed above and wounded with a sterile toothpick. The apples were inoculated with 20 µL of a suspension (as detailed in section 4.4.) from the null mutant Pe*ΔbrlA* strain. The infected apples were incubated for 30 days at 25 °C in the dark. At the end of incubation, the synnemata (Pe*ΔbrlA*) were collected on nylon membrane (UptiDisc nylon membrane, 47 mm diameter; Interchim, Montluçon, France) using a vacuum pump and extracted with 50 mL ethyl acetate for 72 h. Apples were ground [104] and SMs were extracted as detailed in Snini et al. [22]. Four biological replicates were performed.

4.6.3. Analytical Parameters for LC-HRMS

The extracts were analyzed by LC-HRMS. The chromatographic system consisted in an ultimate 3000 HPLC device (Dionex/Thermo Scientific, Courtaboeuf, France). A gradient program of water acidified with 0.05% formic acid (phase A) and acetonitrile acidified with 0.05% formic acid (phase B) was used at 30 °C with a flow rate of 0.2 mL/min 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 10 µL aliquot of each sample diluted twice with the mobile phase A was injected into a reversed-phase Luna® C18 column (125 × 2 mm × 5 µm) (Dionex/Thermo Scientific). The mass spectrometer corresponded to an LTQ Orbitrap XL (Dionex/Thermo Scientific) fitted with an Electrospray Ionization Source (ESI) in the positive and negative modes. For the negative mode, the ionization parameters were set as follows: spray voltage: 3.7kV, capillary temperature: 350 °C, sheath gas flow rate (N_2): 30 arbitrary units (a.u.), auxiliary gas

flow rate: 10 a.u. (N_2), capillary voltage: -34V and tube lens offset: -180V. For the positive mode, the ESI parameters were set as follows: spray voltage: 4kV, capillary temperature: 300 °C, sheath gas flow rate (N_2): 55 arbitrary units (a.u.), auxiliary gas flow rate: 10 a.u. (N_2), capillary voltage: 25V and tube lens offset: -100V. High resolution mass spectra were acquired between *m*/*z* 100 and 800 at a resolution of 7,500. MS/MS spectra were obtained with the collision induced dissociation (CID) mode of the ion trap analyzer at low resolution and a normalized collision energy of 35%. The mass spectrometer was calibrated using the Thermo Fisher Scientific protocol.

4.6.4. Parameters for High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD)

For patulin detection, the chromatography apparatus Ultimate 3,000 HPLC system (Dionex/Thermo Scientific) equipped with a detector DAD was used. The presence of patulin was monitored at a wavelength of 277 nm with a 250 mm × 4.60 mm Gemini® 5 µm C6-Phenyl column (Phenomenex, Torrance, CA, USA) at a flow rate of 0.9 mL/min at 30 °C. Eluent A was water acidified with 0.2% acetic acid and eluent B was HPLC-grade methanol (Thermo Fisher Scientific). The elution conditions were as follows: 0 min 0% B, 8 min 0% B, 20 min 15% B, 25 min 15% B, 35 min 90% B, from 35 to 40 min 90% B, from 45 to 60 min 0% B. The presence of patulin was confirmed by its retention time (min) and UV spectrum according to an authentic standard (Merck KGaA). Patulin concentration was calculated based on a standard curve.

4.6.5. Identification of Fungal Metabolites

Results obtained from ^{12}C, ^{13}C and $^{13}C/^{15}N$ cultures were compared using our in-house MassCompare program to determine the elemental composition of each compound with a mass measurement accuracy of 5 ppm [103,105]. The metabolites were identified based on the MS/MS spectrum, chemical formulae, retention times, the MS/MS fragmentation pattern of the standard compound, AntiBase 2012 database [106], and the literature.

4.7. Identification of Secondary Metabolites Clusters

In order to identify the different SM clusters, an antiSMASH (Antibiotics-Secondary Metabolites Analysis Shell) analysis [107] was performed on *P. expansum* d1 genome [21].

4.8. Microarray Gene Expression Studies

The WT and null mutant PeΔ*brlA* strains were pre-cultured on MEA medium for seven days at 25 °C in the dark, after which a spore suspension was made with the WT strain. The mycelium of the null mutant strain was used as inoculation material as this strain does not produce conidia. Petri dishes containing MEA covered with sterile cellophane sheets were inoculated with 10 µL of a 10^6 spores/mL suspension of the WT strain or 5 mm^2 mycelium of the null mutant strain. The strains were incubated at 25 °C for five days in the dark. Total RNA was isolated at the end of the growth period. The mycelium was transferred to lysing matrix D tubes (1.4 mm ceramic spheres, Thermo Fisher Scientific), to which 760 µL of lysis buffer [10 µL of β-mercaptoethanol (Applied Biosystem, Thermo Fisher Scientific) and 750 µL of RLT buffer (Rneasy mini kit, QIAGEN, Courtaboeuf, France)] were added, and the tubes were placed in liquid nitrogen. The mycelium cells were homogenized in a Precellys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) with three grindings at a speed of 6,500 rpm for 15 s followed by 5 min incubation on ice, at 6,500 rpm for 25 s and 5 min on ice, and a final 6,500 rpm for 15 s. The samples were subsequently centrifuged at 16,000 g at 4 °C for 10 min. The supernatant was recovered in QIAshredder spin columns (QIAGEN) and the total RNA was purified using RNeasy spin minicolumns (QIAGEN) as described by Tannous et al. [20]. RNA quality was checked by electrophoresis using 4,200 TapeStation System (Agilent Technologies, Les Ulis, France) and the concentration was determined using a Dropsense™ 96 UV/VIS droplet reader (Trinean, Ghent, Belgium).

Gene expression profiles were performed at the GeT-TRiX facility (GénoToul, Génopole Toulouse Midi-Pyrénées, France). Briefly, each sample was prepared from 200 ng of total RNA following procedure previously described by Tannous et al. [108] using Agilent Technologies instructions and hybridized on Agilent Sureprint G3 Custom microarrays (8 × 60K, design 085497) of 62,976 spots following the manufacturer's instructions. The expression analysis was performed with five and six biological replicates for PeΔ*brlA* and WT strains, respectively.

Microarray data and experimental details are available in NCBI's Gene Expression Omnibus [109] and are accessible through Gene Expression Omnibus (GEO) Series accession number GSE155057 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155057). The list of DEGs is available as supplementary material (Table S9).

Considering that transcriptome data were available for PdΔ*brlA* [38] and PrΔ*brlA* [49] mutants, the microarray results were compared to those obtained in these previous studies. For *P. digitatum*, the differentially expressed genes in PdΔ*brlA* (with $Log_2FC < -3$ and > 2) were extracted from Table S2 linked to Wang et al. publication [38] and a search of *P. expansum* homologous genes was carried out by BlastP. The *P. expansum* genes homologous to genes significantly down-regulated in PdΔ*brlA* were listed in Table S4 and those homologous to genes significantly up-regulated in PdΔ*brlA* were listed in Table S5.

As the CRP BrlA→ AbaA→ WetA exists in *Penicillium* species, we compared our results with genes identified as similarly regulated to *abaA* (Table S6 from [49] and with those similarly regulated to *wetA* (Table S5 from [49]) in PrΔ*brlA*. The *P. expansum* homologous genes to genes similarly regulated to *wetA* and *abaA* in PrΔ*brlA* were listed in Table S6 and S7, respectively.

4.9. Statistical Analysis of Microarray Data

Microarray data were analyzed using R and Bioconductor packages [110] as described in GEO accession GSE155057. Raw data (median signal intensity) were filtered, log2 transformed, and normalized using smooth quantile normalization (qsmooth) method [111]. A model was fitted using the limma lmFit function [112]. Pair-wise comparison between biological conditions was applied using specific contrast. A correction for multiple testing was applied using the Benjamini-Hochberg (BH) procedure [113] to control for False Discovery Rate (FDR). Probes with FDR ≤ 0.05 were considered to be differentially expressed between conditions.

4.10. Statistical Analysis

A Student's test and one-way analysis of variance (ANOVA) were used to analyze the differences between the WT and the null mutant PeΔ*brlA* strains. Differences were considered to be statistically significant with a p-value ≤ 0.05. Statistical analysis of the data was performed using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA).

5. Conclusions

In conclusion, we showed in this study that *brlA* suppression led to no development of conidiophores but had no impact on synnemata formation. This effect has previously been described in other *Aspergillus* and *Penicillium* species. Transcriptome analysis showed that the gene network under the positive influence of BrlA was relatively conserved in *Penicillium* subgenus *Penicillium* and that many of them were genes involved in conidiation such as *wetA*, *abaA*, hydrophobin encoding genes, and melanin-like pigments encoding genes.

Metabolome and transcriptome analyses showed that *brlA* suppression resulted in altered communesin biosynthesis counterbalanced by enhanced chaetoglobosin production, unveiling a putative chaetoglobosin gene cluster. This study demonstrated that patulin production was not affected by inhibition of conidiation, confirming that there is no link between the biosynthesis of this mycotoxin and conidiogenesis. Furthermore, the absence of patulin in synnemata suggests that patulin was produced by hyphae when the fungus grew in the flesh of the apple and that its production stopped when the fungus was released from the fruit as synnemata.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Morphological aspect of *Penicillium expansum* wild type NRRL 35695 and the null mutant PeΔ*brlA* strains, Figure S2: Rot growth rates obtained from Golden Delicious apples infected with *Penicillium expansum* wild type NRRL 35695 and the null mutant PeΔ*brlA* strains, Figure S3: Morphological aspect of A) Null mutant PeΔ*brlA* strain and B) *Penicillium expansum* wild type NRRL 35695 strain grown in a minimal media supplemented with galactose, Figure S4: Relative gene expression of the putative chaetoglobosin biosynthetic gene cluster (PEXP_073960-PEXP_074060) in *Penicillium expansum* wild type NRRL 35695 and the null mutant PeΔ*brlA* strains, Figure S5: Double-joint PCR reaction, Figure S6: PCR amplification of *Penicillium expansum* wild type NRRL 35695 (WT) and null mutant PeΔ*brlA* strains, Table S1: MS/MS spectra of secondary metabolites detected from *Penicillium expansum* wild type NRRL 35695 after culture on labeled wheat grains, Table S2: MS/MS spectra of the specific secondary metabolites only detected in the null mutant PeΔ*brlA* strain after culture on labeled wheat grains, Table S3: Primers used in qPCR for analysis of putative chaetoglobosin gene clusters, Table S4: *Penicillium expansum* genes orthologous to genes significantly down-regulated (Log2 fold change < -3) in *Penicillium digitatum* PdΔ*brlA* strain [38], Table S5: *Penicillium expansum* genes orthologous to genes significantly up-regulated (Log2 fold change > 2) in *Penicillium digitatum* PdΔ*brlA* strain [38], Table S6: Eighteen orthologous genes similarly regulated to *wetA* in *Penicillium rubens* Δ*brlA* [49] and *Penicillium expansum* Δ*brlA*, Table S7: Fifty-nine orthologous genes similarly down-regulated to *abaA* in *Penicillium rubens,* Table S8: Primers used in the construction and the validation of the null mutant PeΔ*brlA* strain.

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Abbreviations

APAM	Apple Puree Agar Medium
bp	base pair
CRP	Central Regulatory Pathway
Com	Communesin
CYA	Czapek Yeast extract Agar
DEG	Differential Expressed Genes
DHN	1,8-Dihydroxynaphthalene
dpi	day post inoculation
GEO	Gene Expression Omnibus
HPLC	High Performance Liquid Chromatography
LBG	Locus Bean Gum
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
L_{hgu}	hyphal growth unit Length
MEA	Malt Extract agar
PDA	Potato Dextrose Agar
Rt	Retention time, min
SM	Secondary Metabolite
TF	Transcription Factor

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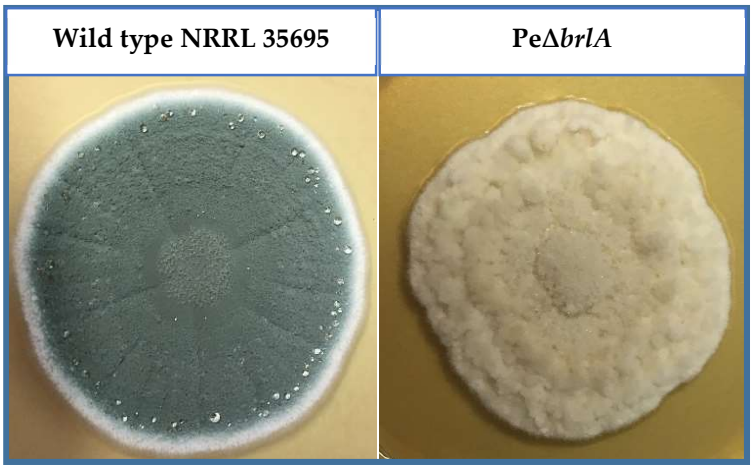
Supplementary Materials

The *brlA* gene deletion reveals that patulin biosynthesis is not related to conidiation in *Penicillium expansum*

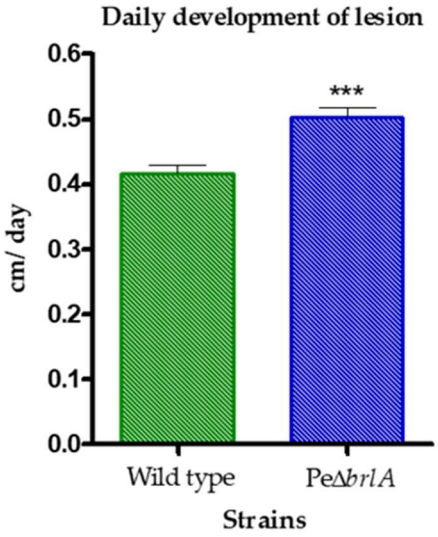
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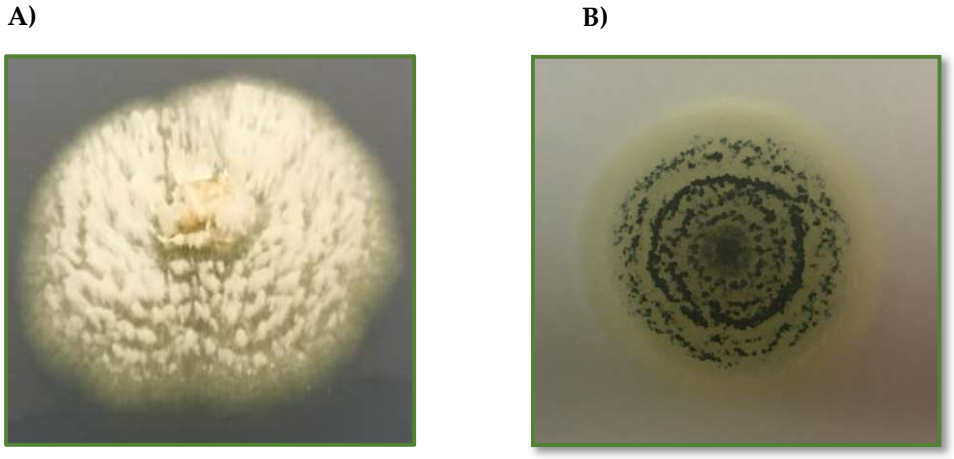
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Supplementary Figure 1. Morphological aspect of *Penicillium expansum* wild type NRRL 35695 and the null mutant PeΔ*brlA* strains. The strains were grown on MEA at 25 °C in the dark for seven days.



Supplementary Figure 2. Rot growth rates obtained from Golden Delicious apples infected with *Penicillium expansum* wild type NRRL 35695 and the null mutant PeΔ*brlA* strains and incubated at 25 °C for 14 days in the dark. The graph shows the mean ± standard error of the mean (SEM) from nine biological replicates. *** p-value < 0.001.



Supplementary Figure 3. Morphological aspect of A) Null mutant PeΔ*brlA* strain and B) *Penicillium expansum* wild type NRRL 35695 strain grown in a minimal media supplemented with galactose and incubated for seven days at 25 °C in the dark. This medium promoted the development of coremia or synnemata.

Supplementary Table 1. MS/MS spectra of secondary metabolites detected from *Penicillium expansum* wild type NRRL 35695 after culture on labeled wheat grains.

Molecular formula	Identifier [a]	^{12}C *m/z* (Da)	^{13}C *m/z* (Da)	$^{13}C/^{15}N$ *m/z* (Da)	*m/z* of major fragment ions
$C_7H_6O_4$	**Pexp_153.019_3.59**	**153.01919**	**160.04256**	**156.02997**	**153→ 125, 109(100), 97**
C_7H_8O	Pexp_109.065_7.11	109.06509	116.08844	112.07483	-
$C_7H_8O_2$	Pexp_125.060_6.79	125.05998	132.08346	129.07332	125→ 106(100), 97, 95, 83, 81, 79, 69
$C_7H_8O_3$	Pexp_141.054_3.80	141.05493	148.07869	144.06492	141→ 123, 113(100), 110, 95, 79, 69, 55
$C_7H_8O_4$	Pexp_157.049_2.67	157.04990	164.07348	161.06366	157→ 139(100), 111, 97, 83
$C_7H_{10}O_3$	Pexp_143.070_4.52	143.07061	150.09415	147.08401	143→ 125, 113(100), 107, 97, 79
$C_{10}H_{17}NO_5$	Pexp_232.118_8.83	232.11872	242.15222	238.13252	232→ 214, 185, 157, 119 (100), 112, 101, 86
$C_{12}H_{14}O_3$	Pexp_207.101_9.65	207.10110	219.14115	213.12114	207→ 189(100), 179, 174, 171, 161, 142, 137
$C_{13}H_{14}O_5$	Pexp_251.091_21.70	251.09108	264.13455	258.11474	251→ 233(100), 215, 198, 167, 143
$C_{15}H_{18}N_2$	Pexp_227.155_6.44	227.15514	242.20521	237.17554	227→ 210(100), 198, 171, 154, 130
$C_{15}H_{19}NO_6$	Pexp_310.129_12.78	310.12939	325.17984	319.15312	310→ 292 (100), 264, 240, 186, 158, 140, 107
$C_{15}H_{19}NO_6$	Pexp_310.129_14.80	310.12964	325.17835	319.14477	310→ 292 (100), 264, 240, 185, 158, 140, 107
$C_{15}H_{20}O_4$	Pexp_265.144_15.91	265.14412	280.19431	273.17072	265→ 247, 229, 219(100), 205, 201, 191, 151, 135, 107
$C_{15}H_{20}O_4$	Pexp_265.144_16.91	265.14410	280.19364	273.17064	265→ 247, 229, 219(100), 205, 201, 191 151, 135, 107
$C_{15}H_{20}O_4$	Pexp_265.144_18.49	265.14415	280.19430	273.17081	265→ 247, 229, 219(100), 205, 201, 191 151, 135, 107
$C_{15}H_{20}O_4$	Pexp_265.144_19.35	265.14414	280.19429	273.17082	265→ 247, 229, 219(100), 205, 201, 191 151, 135, 107
$C_{16}H_{18}N_2O_2$	Pexp_271.144_7.62	271.14496	287.19861	281.16599	271→ 254(100), 225, 215, 168
$C_{16}H_{18}N_2O_2$	Pexp_271.144_8.45	271.14467	287.19873	281.16576	271→ 254(100), 225, 215, 168
$C_{16}H_{26}N_2O_4S_2$	Pexp_375.142_22.02	375.14202	391.19584	385.16297	375→ 357, 347, 222, 178, 154(100),
$C_{17}H_{22}O_5$	Pexp_307.154_27.39	307.15471	324.21155	316.18482	307→ 265(100), 247, 229, 219, 211, 201, 135
$C_{17}H_{22}O_5$	Pexp_307.155_30.19	307.15504	324.21163	316.18515	307→ 265(100), 247, 229, 219, 211, 201, 135
$C_{18}H_{16}N_2O_2$	Pexp_293.129_13.58	293.12911	311.18978	305.15587	293→ 265(100), 248, 237, 222, 120
$C_{18}H_{16}N_2O_2$	Pexp_293.129_17.74	293.12915	311.18970	305.15668	293→ 265(100), 248, 237, 222, 120
$C_{18}H_{16}N_2O_3$	Pexp_309.124_14.76	309.12421	327.18455	320.14798	309→ 292 (100), 267, 249, 231
$C_{18}H_{18}N_2O_2$	Pexp_295.144_14.61	295.14479	313.20540	307.17251	295→ 267(100), 250, 239, 222, 120
$C_{18}H_{31}NO_7$	Pexp_374.216_23.72	374.21663	392.27652	385.24644	374→ 356(100), 204, 186, 158
$C_{19}H_{16}N_2O_2$	Pexp_305.129_32.50	305.12911	324.19310	316.15289	305→ 277(100), 264, 262, 205, 187
$C_{19}H_{16}N_2O_2$	Pexp_305.129_33.17	305.12933	324.19301	317.15701	305→ 277(100), 264, 262, 205, 187

$C_{19}H_{16}N_2O_4$	Pexp_337.119_13.73	337.11909	356.18283	349.14659	337→ 319, 309(100), 296, 294, 280, 252, 237, 203, 121
$C_{19}H_{16}N_2O_4$	Pexp_337.119_15.20	337.11916	356.18294	349.14676	337→ 319, 309(100), 296, 280, 237, 203, 121
$C_{19}H_{21}NO_7$	Pexp_376.139_17.45	376.13901	395.20280	387.16962	376→ 233, 207(100), 189
$C_{19}H_{21}NO_7$	Pexp_376.139_18.65	376.13904	395.20262	387.16966	376→ 233, 207(100), 189
$C_{19}H_{38}O_6$	**Pexp_361.258_37.23**	**361.25871**	**380.32251**	**371.29242**	**361→ 343, 325, 283, 273, 257(100), 157**
$C_{19}H_{38}O_6$	**Pexp_361.258_38.19**	**361.25819**	**380.32253**	**371.29252**	**361→ 343, 325, 299, 283, 257(100), 157**
$C_{20}H_{18}N_2O_2$	Pexp_319.145_35.48	319.14588	339.21182	332.17585	319→ 291(100), 278, 262, 250, 234, 205, 201, 188, 132
$C_{20}H_{18}N_2O_2$	Pexp_319.145_36.44	319.14502	339.21173	332.17596	319→ 291(100), 278, 260, 250, 234, 205, 201, 188, 160, 132
$C_{20}H_{21}NO_9$	Pexp_420.128_18.38	420.12857	440.19571	432.16272	420→ 251(100), 233
$C_{20}H_{21}NO_9$	Pexp_420.128_21.20	420.12858	440.19605	432.16318	420→ 251(100), 233
$C_{20}H_{26}O_8$	Pexp_395.171_13.61	395.17142	415.23827	406.20763	395→ 251(100), 233
$C_{22}H_{23}N_5O_2$	Pexp_390.193_15.09	390.19390	412.26737	406.21627	390→ 334, 322(100), 198, 193
$C_{22}H_{25}N_5O_2$	Pexp_392.209_9.99	392.20913	414.28326	408.22845	392→ 336, 324(100), 198, 195
$C_{23}H_{24}N_2O_6$	Pexp_425.171_26.92	425.17179	448.24874	439.20611	425→ 407, 383, 365, 288(100), 260
$C_{24}H_{26}N_2O_6$	**Pexp_437.170_33.79**	**437.17091**	**461.25116**	**451.20575**	**437→ 395, 377(100), 333, 286**
$C_{26}H_{30}N_4$	Pexp_399.255_19.94	399.25568	425.34268	417.29076	399→ 382, 365, 241(100), 224, 185, 159, 144, 130
$C_{26}H_{30}N_4O$	Pexp_415.250_14.62	415.25034	441.33736	433.28593	415→ 398, 357, 343, 257(100), 240, 199, 185, 159
$C_{26}H_{30}N_4O$	Pexp_415.250_18.43	415.25033	441.33739	433.28576	415→ 397, 257(100), 240, 228, 224, 201, 159, 130
$C_{26}H_{40}O_6$	Pexp_449.289_29.93	449.28939	475.37674	463.33658	449→ 431(100), 386, 384, 221, 211, 203, 179, 166
$C_{27}H_{30}N_4O_2$	Pexp_443.245_15.65	443.24564	470.33554	461.28105	443→ 385, 371(100), 201, 185, 171, 154
$C_{28}H_{30}N_4O_3$	Pexp_471.239_19.56	471.23997	499.33402	490.27887	471→ 399, 371, 201, 199(100), 171, 159, 154
$C_{28}H_{31}N_5O_5$	Pexp_518.241_16.36	518.24108	546.33497	538.27672	518→ 500, 462, 450(100), 321
$C_{28}H_{31}N_5O_5$	Pexp_518.240_17.21	518.24092	546.33488	538.27706	518→ 500, 462, 450(100), 402, 390, 326, 210
$C_{28}H_{32}N_4O$	Pexp_441.265_17.14	441.26589	469.35970	460.30445	441→ 424(100), 385, 365, 239, 224, 185, 183
$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.27	457.26097	485.35452	476.29968	457→ 440(100), 385, 257,255, 240, 210, 201, 185
$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.97	457.26095	485.35394	476.29996	457→ 439, 385(100), 239, 185
$C_{28}H_{32}N_4O_2$	Pexp_457.261_23.65	457.26116	485.35429	476.29971	457→ 399, 385(100), 201, 185, 159, 144, 130
$C_{28}H_{32}N_4O_3$	Pexp_473.255_15.50	473.25566	501.34996	492.29429	473→ 456(100), 401, 397, 273, 271, 253, 244, 201, 199, 171
$C_{28}H_{36}N_4O_4$	Pexp_493.279_31.47	493.27971	521.37363	511.31495	-
$C_{28}H_{38}N_4O_5$	Pexp_511.290_18.01	511.29099	539.38447	529.32631	511→ 493, 465, 394, 346, 301, 265(100), 247, 219, 166
$C_{28}H_{38}N_4O_6$	Pexp_527.287_12.46	527.28755	555.38184	545.32412	527→ 281(100), 509, 481, 346, 301, 265, 247, 219, 182

$C_{28}H_{38}O_7$	**Pexp_485.254_35.32**	**485.25405**	**513.34696**	**500.30389**	**485→ 470, 457, 453(100), 425, 411, 409, 393**
$C_{28}H_{38}O_7$	Pexp_487.270_36.21	487.27063	515.36381	502.32065	487→ 427(100), 409, 395, 377, 243
$C_{28}H_{38}O_8$	**Pexp_501.247_27.70**	**501.24792**	**529.34203**	**516.29846**	**501→ 457, 441(100), 425, 410, 397, 373, 365,**
$C_{28}H_{38}O_8$	**Pexp_501.247_28.44**	**501.24792**	**529.34203**	**516.29846**	**501→ 457, 441(100), 425, 410, 397, 373, 365,**
$C_{28}H_{38}O_8$	**Pexp_501.247_29.57**	**501.24786**	**529.34194**	**516.29974**	**501→ 486, 469, 457, 441(100), 425, 397, 373, 366**
$C_{28}H_{40}O_6$	**Pexp_471.274_39.48**	**471.27434**	**499.36727**	**486.32419**	**471→ 456, 439, 411(100)**
$C_{28}H_{40}O_7$	**Pexp_487.268_30.58**	**487.26898**	**515.36247**	**502.31903**	**487→ 455, 427(100), 411, 395, 351,336**
$C_{29}H_{33}N_5O_6$	Pexp_548.251_15.42	548.25180	577.34902	568.28775	548→ 530, 518, 492, 480(100), 462, 402, 390, 356
$C_{30}H_{36}N_4O_2$	Pexp_485.292_16.36	485.29245	515.39378	505.33425	485→ 468(100), 429, 365, 239, 224. 222, 183, 185, 159, 144
$C_{31}H_{36}N_4O_2$	Pexp_497.290_31.66	497.29061	528.39421	517.33225	497→ 479, 425(100), 439, 257, 241, 185, 168, 159
$C_{32}H_{34}N_4O_3$	Pexp_523.271_31.84	523.27152	555.37917	543.30477	523→ 451, 423(100), 253, 199, 171, 159, 154,
$C_{32}H_{36}N_2O_4$	Pexp_513.276_39.10	513.27635	545.38309	532.32773	513→ 495(100), 477, 455, 430, 412, 384, 366, 250
$C_{32}H_{36}N_2O_5$	Pexp_529.270_25.21	529.27058	561.37837	548.32196	529→ 511(100), 493, 475, 382, 380, 362, 307, 185
$C_{32}H_{36}N_2O_5$	Pexp_529.270_26.71	529.27029	561.37834	548.32343	529→ 511(100), 493, 475, 382, 380, 362, 185
$C_{32}H_{36}N_2O_5$	Pexp_529.270_30.51	529.27077	561.37872	548.32190	529→ 511(100), 493, 469, 475, 483, 457, 451, 359, 333
$C_{32}H_{36}N_2O_5$	Pexp_529.270_33.98	529.27067	561.37843	548.32208	529→ 511(100), 501, 493, 483, 475, 465, 457, 431, 417, 400, 382
$C_{32}H_{36}N_2O_5$	Pexp_529.270_35.67	529.27092	561.37735	548.31076	529→ 511 (100), 493, 469, 382, 359, 331
$C_{32}H_{36}N_2O_5$	Pexp_529.270_36.41	529.27063	561.37820	548.32186	529→ 511(100), 493, 487, 483, 475, 469, 457, 400, 398, 382, 380, 364, 328, 310, 185
$C_{32}H_{36}N_2O_5$	Pexp_529.270_37.41	529.27056	561.37807	548.32181	529→511 (100), 493, 453, 380, 327, 185
$C_{32}H_{36}N_2O_6$	**Pexp_543.248_34.05**	**543.24836**	**575.35597**	**562.29931**	543→ 499, 481, 471, 445(100), 427, 375, 352, 248
$C_{32}H_{36}N_4O_2$	Pexp_509.292_36.01	509.29257	541.39875	530.3376	509→ 437(100), 451, 185
$C_{32}H_{36}N_4O_3$	Pexp_525.287_13.45	525.28735	557.39329	546.33239	525→ 507, 481, 467, 453(100), 409, 343, 340, 257, 240,225, 199, 185, 159
$C_{32}H_{36}N_4O_3$	Pexp_525.287_27.91	525.28705	557.39461	546.33215	525→ 508(100), 271, 273, 199
$C_{32}H_{38}N_4O_2$	Pexp_511.306_36.13	511.30684	543.41590	532.35205	511→ 439(100), 453, 185, 144
$C_{32}H_{38}N_4O_3$	Pexp_527.303_21.41	527.30304	559.41082	548.34834	527→ 509, 469, 453, 437(100), 275, 215
$C_{32}H_{38}N_4O_4$	Pexp_543.297_19.36	543.29789	575.40330	564.34345	543→ 525, 485, 471(100), 185, 168
$C_{32}H_{40}N_4O_2$	Pexp_513.322_36.57	513.32227	545.43031	533.36211	513→ 495, 455, 441(100), 257, 185
$C_{33}H_{38}N_4O_5$	Pexp_571.293_18.22	571.29324	604.40405	592.33802	571→ 553, 513, 499(100), 455, 397, 299
$C_{37}H_{42}N_4O_5$	Pexp_623.325_29.52	623.32511	660.44909	646.37781	623→ 565, 551(100), 507, 449, 437, 371, 354, 299

In bold the compounds detected by negative electrospray ionization (ESI-). a Secondary metabolites identifier (Pexp_*m*/*z*_RT). *m*/*z* = *m*/*z* ratio and RT= retention time.

Supplementary Table 2. MS/MS spectra of the specific secondary metabolites only detected in the null mutant PeΔ*brlA* strain after culture on labeled wheat grains.

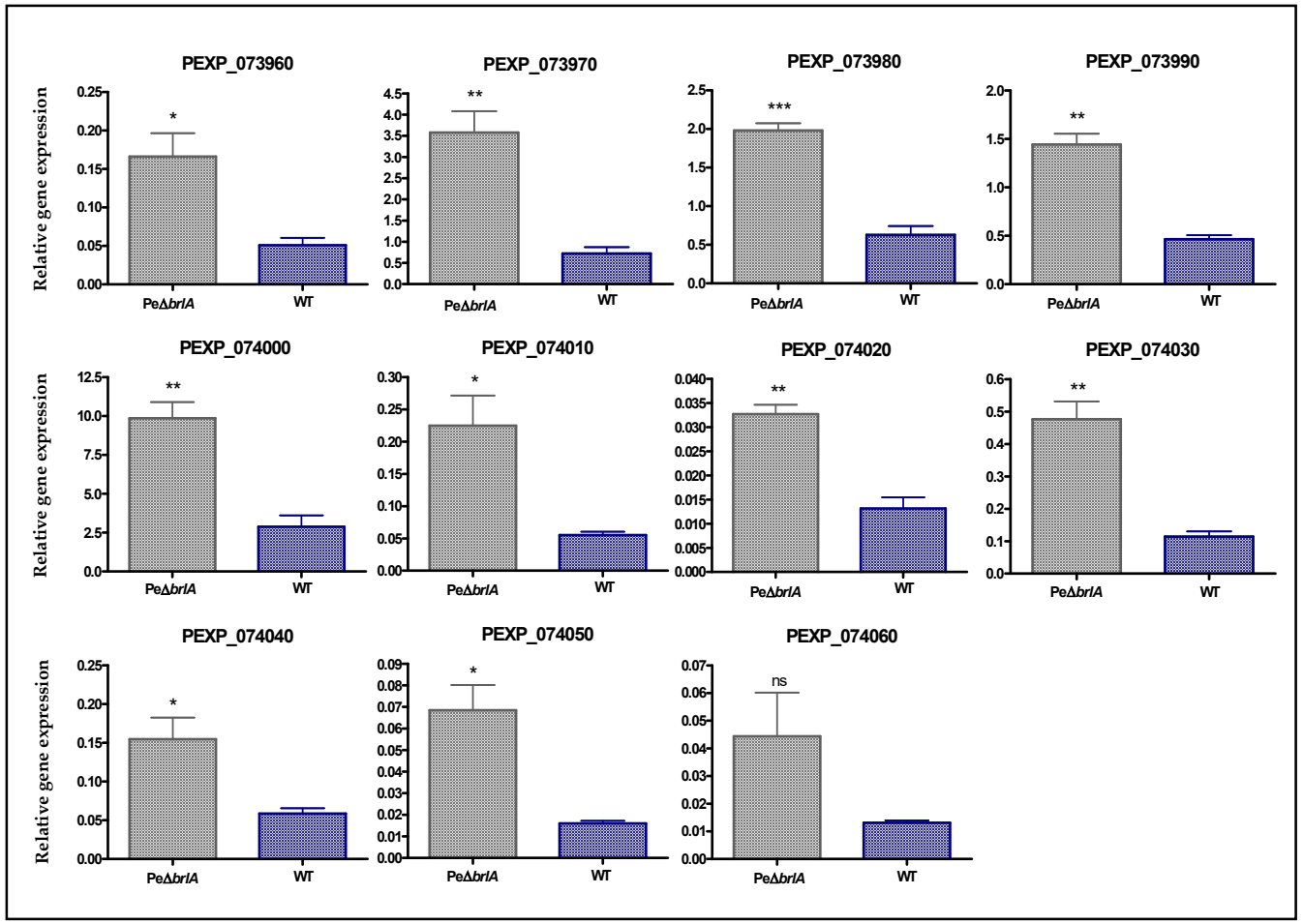
Molecular formula	Identifier [a]	^{12}C *m/z* (Da)	^{13}C *m/z* (Da)	$^{13}C/^{15}N$ *m/z* (Da)	*m/z* of major fragment ions
$C_{17}H_{23}N_3O_3$	Pexp_318.179_03.12	318.17943	335.23746	330.20149	318→ 301(100), 272, 258, 205, 188, 178, 170, 159, 132
$C_{17}H_{23}N_3O_3$	Pexp_318.180_25.05	318.18043	335.23716	330.20173	318→ 300, 272, 205(100), 188, 159, 141, 132
$C_{18}H_{35}N_3O_4$	Pexp_358.269_0.354	358.26901	376.32935	370.29051	358→ 340, 227(100), 199
$C_{19}H_{20}O_5$	Pexp_329.137_33.29	329.13738	348.20079	339.17092	329→ 311(100), 293, 285, 269, 258, 241, 229, 205, 177, 161
$C_{26}H_{32}O_8$	Pexp_473.215_9.67	473.21511	499.30281	487.26381	-
$C_{26}H_{32}O_8$	Pexp_473.215_10.64	473.21552	499.30112	487.26454	-
$C_{26}H_{32}O_8$	Pexp_473.214_34.14	473.21489	499.29541	487.25368	-
$C_{27}H_{29}N_5O_4$	Pexp_488.227_10.8	488.22783	515.30922	507.26058	488→ 470(100), 458, 420, 402, 390, 322
$C_{27}H_{29}N_5O_4$	Pexp_488.227_11.3	488.22766	515.31807	507.26066	488→ 470(100), 458, 420, 402, 390, 375, 322
$C_{29}H_{27}N_5O_5$	Pexp_526.206_15.7	526.20693	555.30405	546.24399	526→ 508, 498, 458(100), 442, 430, 412, 390, 374, 334, 322, 216, 193
$C_{29}H_{27}N_5O_5$	Pexp_526.206_17.61	526.20689	555.29234	546.24884	526→ 508, 498, 480, 470, 458(100), 390, 334, 322, 204, 192
$C_{29}H_{31}N_5O_5$	Pexp_530.238_14.83	530.23837	559.33601	550.27623	530→ 512, 468, 462, 390(100), 338, 322, 276, 252, 208, 193
$C_{29}H_{31}N_5O_5$	Pexp_530.238_15.85	530.23812	559.33514	550.27487	530→ 512, 474, 468, 462, 390(100), 338, 322, 276, 193
$C_{29}H_{31}N_5O_5$	Pexp_530.238_16.68	530.23840	559.33720	550.27338	530→ 512, 474, 462(100), 402, 390, 338, 320, 270, 210, 193
$C_{29}H_{33}N_5O_6$	Pexp_548.248_11.36	548.24883	577.34601	568.28444	548→ 530, 504, 480, 474(100), 390, 356, 322, 263, 193
$C_{29}H_{33}N_5O_6$	Pexp_548.248_12.14	548.24860	577.34575	568.28418	548→ 530, 518, 480, 474(100), 468, 402, 390, 356, 322
$C_{32}H_{36}N_2O_5$	Pexp_529.268_23.19	529.26807	561.37549	548.32110	529→ 511(100), 493, 486, 465, 431, 417, 403, 380, 349, 331, 292, 185, 167, 159
$C_{32}H_{36}N_2O_5$	Pexp_529.268_32.43	529.26807	561.37583	548.3197	529→ 511(100), 501, 493, 483, 475, 429, 411, 398, 382, 362, 327, 185
$C_{32}H_{36}N_2O_5$	Pexp_529.268_35.49	529.26769	561.37490	548.31990	529→ 511(100), 493, 483, 475, 471, 465, 455, 417, 380, 362, 347, 327, 319, 185, 167
$C_{32}H_{36}N_2O_6$	Pexp_545.262_29.23	545.26241	577.37065	564.31731	-
$C_{32}H_{36}N_2O_6$	Pexp_545.262_30.57	545.26288	577.36827	564.31312	545→ 527(100), 509, 491, 481, 463, 449, 429, 411, 396, 360, 185
$C_{32}H_{36}N_2O_6$	Pexp_545.262_31.50	545.26281	577.36975	564.31459	-
$C_{32}H_{36}N_2O_6$	Pexp_545.262_32.48	545.26294	577.36998	564.31425	545→ 527(100), 509, 499, 491, 473, 445, 427, 365, 363, 345, 184
$C_{32}H_{38}N_2O_5$	Pexp_531.283_21.75	531.28384	563.39083	550.33694	531→ 513(100), 495, 477, 459, 429, 411, 397, 384, 313, 295, 267, 185
$C_{32}H_{38}N_2O_5$	Pexp_531.284_23.91	531.28431	563.39246	549.33473	531→ 513(100), 495, 477, 455, 397, 371, 331, 185, 165
$C_{32}H_{38}N_2O_5$	Pexp_531.283_28.02	531.28354	563.39229	550.33654	531→ 513(100), 495, 477, 485, 471, 467, 459, 445, 431, 399, 364, 331, 185
$C_{32}H_{38}N_2O_5$	Pexp_531.283_28.52	531.28340	563.39080	549.32886	531→ 513(100), 495, 477, 449, 413, 397, 371, 331, 309, 185, 165
$C_{32}H_{38}N_2O_5$	Pexp_531.283_31.56	531.28338	563.39064	550.33378	531→ 513(100) ,495, 485, 477, 467, 459, 455, 384, 349, 331, 185

$C_{32}H_{38}N_2O_6$	Pexp_547.278_23.19	547.27864	579.38570	566.32988	547→ 529(100), 511, 493, 483, 465, 349, 185
$C_{32}H_{38}N_2O_6$	Pexp_547.278_25.47	547.27841	579.38548	566.32988	547→ 529(100), 511, 493, 483, 465, 417, 327, 185
$C_{32}H_{38}N_2O_6$	Pexp_547.278_27.09	547.27842	579.38554	566.32965	547→ 529(100), 511, 493, 465, 483, 429, 475, 380, 185

[a] Secondary metabolite identifier (Pexp_*m*/*z*_R_T). *m*/*z* = *m*/*z* ratio and R_T= retention time.

Supplementary Table 3. Primers used in qPCR for analysis of putative chaetoglobosin gene clusters.

Primer name	Sequence	Ratio (F/R)
cheC_F	GACCAACCTTGGCATATA	300/300
cheC_R	TCGTCGATCTGTATGTAGAC	
cheB_F1	TCAAATCCTCACGCTATCT	300/900
cheB_R1	GCATGTGGGAGACTGATA	
cheA_F2	GAAGTGGGTTGTGGTAATG	900/300
cheA_R2	CAGTAGATCCCTCCAATATGTA	
cheD_F1	GAGGAACGCCTTCTGTAA	300/900
cheD_R1	GCGAGATGTTTGCACTTT	
cheF_F2	ACACTGCTGAGAAGTTCA	900/900 and 900/300
cheF_R2	GTTGCGTATCTCATTCACC	
cheG_F2	CTCCAGCGATGGTTCCT	300/900
cheG_R2	GCCTTGGCTGGATGATTG	
PEXP_073960_F2	TCCATTCTCTTAGTCTAGTGAC	900/900
PEXP_073960_R2	TTCAGATGCTGGTTAGGG	
PEXP_073970_F1	GGTGATTGTGCTGGTATTG	900/300
PEXP_073970_R1	GAGGTAGAAGTGGGTTCAT	
PEXP_073980_F1	GAACAACCAGCCATTGAG	900/300
PEXP_073980_R1	GTTAGGATCGCGGTAGAA	
PEXP_073990_F	GAGACGGTTCTCGTCTTTGG	300/300
PEXP_073990_R	ACACGATGGCCGAGAAG	
PEXP_074000_F	CCAACGTTCGCTATCCA	300/300
PEXP_074000_R	TGCGGACCTTGGTAATG	
PEXP_074010_F2	GCCACAAACGACATTGA	300/900
PEXP_074010_R2	CGGAACAACACCAAGAG	
PEXP_074020_F1	GGAAGCCGATCAGGATAA	300/300
PEXP_074020_R1	GCTGGTCAGGACTAGTTG	
PEXP_074030_F	TGAAGTCCTCGAACCAA	300/900
PEXP_074030_R	TTTGCTTGTTCAGGATGG	
PEXP_074040_F1	TCCTGTCGCCTCATTAT	900/300
PEXP_074040_R1	CACCGCTGTTCTTGATAG	
PEXP_074050_F1	CACTGACGAACCAGAAAG	300/300
PEXP_074050_R1	GTTGCACAGAGCGATAG	
PEXP_074060_F2	TAGAGTTGGGTCGTGGT	300/900
PEXP_074060_R2	CTCTCTCACTGTACATCTTGG	



Supplementary Figure 4. Relative gene expression of the putative chaetoglobosin biosynthetic gene cluster (PEXP_073960-PEXP_074060) in *Penicillium expansum* wild type NRRL 35695 and the null mutant PeΔ*brlA* strains. The graphs show the mean ± standard error of the mean (SEM) from three biological replicates and the significant differences between the wild type and the null mutant PeΔ*brlA* strains. *p*-value *< 0.05; **< 0.01; ***< 0.001. ns = no significant.

Supplementary Table 4. *Penicillium expansum* genes orthologous to genes significantly down-regulated (Log_2 fold change < -3) in *Penicillium digitatum* Pd*ΔbrlA* strain [38].

Penicillium digitatum PHI26 strain Gene ID	*Penicillium digitatum* GenBank Accession number	Log_2 Fold Change Pd*ΔbrlA* Vs WT[1*]	% Positives	*Penicillium expansum* strain d1 Gene ID	Log_2 fold change Pe*ΔbrlA* Vs WT[2]	Adjusted *p*-value
GI:425772521	EKV10922.1	-8.5	98.07	PEXP_097180	-8.34	1.13E-13
GI:425776459	EKV14676.1	-8.4	92.95	PEXP_020490	-11.4	1.43E-16
GI:425767953	EKV06503.1	-8.3	81.25	PEX2_067760	ns	-
GI:425772520	EKV10921.1	-8.1	95.19	PEXP_097170	-8.64	1.15E-15
GI:425779884	EKV17912.1	-7.9	88.53	PEXP_044610	-6.44	1.95E-03
GI:425765865	EKV04510.1	-7.6	74.22	PEXP_098690	-10.6	2.53E-18
GI:425772463	EKV10864.1	-7.2	87.2	PEXP_008790	-6.68	2.81E-14
GI:425767228	EKV05802.1	-7.0	ns	-	-	-
GI:425769821	EKV08303.1	-6.9	93.79	PEXP_020490	-11.4	1.43E-16
GI:425781523	EKV19483.1	-6.9	80.16	PEXP_033790	-8.30	8.53E-16
GI:425775975	EKV14214.1	-6.8	86.77	PEXP_062310	-10.9	7.29E-16
GI:425773118	EKV11490.1	-6.8	92.02	PEXP_001060	-9.51	1.43E-16
GI:425776321	EKV14543.1	-6.7	84.95	PEXP_075490	-2	6.55E-08
GI:425770425	EKV08898.1	-6.7	93.33	PEXP_052120	-10.1	8.09E-17
GI:425775531	EKV13796.1	-6.7	98.43	PEXP_049260	-6.21	1.07E-14
GI:425770751	EKV09215.1	-6.6	91.34	PEXP_004280	ns	-
GI:425781932	EKV19866.1	-6.6	95.51	PEXP_018490	-10.7	2.61E-16
GI:425775976	EKV14215.1	-6.5	96.95	PEXP_062290	-13.0	1.07E-18
GI:425774025	EKV12348.1	-6.5	78.85	PEXP_011470	-7.65	1.10E-14
GI:425765801	EKV04449.1	-6.5	87.60	PEXP_074900	-7.92	1.14E-14
GI:425779486	EKV17537.1	-6.4	97.28	PEXP_000200	-5.57	3.94E-13
GI:425774428	EKV12735.1	-6.4	66.67	PEXP_014810	-6.15	8.11E-15
GI:425773468	EKV11821.1	-6.3	62.31	PEXP_108060	-8.35	1.14E-14
GI:425769509	EKV08001.1	-6.3	97.23	PEXP_027310	ns	-

GI:425775526	EKV13791.1	-6.2	96.63	PEXP_049320	-1.18	3.91E-07
GI:425770516	EKV08985.1	-6.2	96.40	PEXP_092210	-4.28	1.47E-13
GI:425781930	EKV19864.1	-6.1	95.29	PEXP_018530	-7.61	1.31E-15
GI:425780307	EKV18317.1	-6.1	46.67	-	-	-
GI:425777761	EKV15917.1	-5.9	86.23	PEXP_104370	-4.56	1.23E-12
GI:425767950	EKV06500.1	-5.8	96.39	PEXP_098120	-3.81	6.07E-10
GI:425780304	EKV18314.1	-5.8	47.35	-	-	-
GI:425770152	EKV08625.1	-5.7	96.02	PEXP_057120	-3.38	9.46E-12
GI:425772498	EKV10899.1	-5.7	86.47	PEXP_096890	-8.24	7.29E-16
GI:425765867	EKV04512.1	-5.6	96.91	PEXP_098720	-1.92	8.61E-07
GI:425777762	EKV15918.1	-5.6	91.55	PEXP_104360	-1.79	1.25E-10
GI:425770173	EKV08646.1	-5.5	97.08	PEXP_056860	-4.96	1.58E-12
GI:425771697	EKV10134.1	-5.5	89.82	PEXP_046360	-7.73	5.74E-14
GI:425770151	EKV08624.1	-5.5	93.64	PEXP_057120	-3.38	9.46E-12
GI:425775516	EKV13783.1	-5.4	96.38	PEXP_049220	-7.25	2.76E-13
GI:425766389	EKV05002.1	-5.4	94.81	PEXP_017260	-6.11	2.85E-14
GI:425780925	EKV18916.1	-5.4	-	-	-	-
GI:425768899	EKV07410.1	-5.3	74.59	PEXP_078920	-8.16	8.83E-16
GI:425781235	EKV19211.1	-5.3	63.40	PEXP_041860	-7.50	7.81E-14
GI:425780308	EKV18318.1	-5.3	89.77	PEXP_014270	-5.36	5.20E-10
GI:425773477	EKV11830.1	-5.2	92.33	PEXP_108160	-5.54	7.73E-14
GI:425774331	EKV12639.1	-5.1	79.23	PEXP_086170	-6.03	2.26E-14
GI:425778756	EKV16861.1	-5.1	88.37	PEXP_041050	-5.32	1.39E-09
GI:425770554	EKV09023.1	-5.1	80.10	PEXP_057440	-4.27	2.05E-12
GI:425772522	EKV10923.1	-5.0	97.00	PEXP_097190	-6.57	9.55E-13
GI:425769933	EKV08411.1	-4.9	85.80	PEXP_052780	-2.45	2.26E-08
GI:425777781	EKV15937.1	-4.9	96.22	PEXP_104170	ns	-
GI:425777125	EKV15315.1	-4.9	93.23	PEXP_066390	-5.00	2.02E-12
GI:425770752	EKV09216.1	-4.9	96.82	PEXP_004290	ns	-

GI:425775661	EKV13918.1	-4.9	91.64	PEXP_005290	-2.08	5.13E-10
GI:425769850	EKV08332.1	-4.9	71.97	PEX1_062950	-1.68	1.46E-08
GI:425772518	EKV10919.1	-4.8	92.96	PEXP_097110	-6.57	1.58E-12
GI:425777151	EKV15335.1	-4.7	87.87	PEXP_053700	ns	-
GI:425781931	EKV19865.1	-4.7	96.23	PEXP_018520	-7.46	1.93E-15
GI:425770818	EKV09278.1	-4.7	77.76	PEXP_031660	-5.95	9.64E-12
GI:425778273	EKV16412.1	-4.7	84.14	PEXP_025000	-8.34	6.10E-16
GI:425781017	EKV18999.1	-4.6	93.04	PEXP_072860	-1.48	3.78E-06
GI:425772484	EKV10885.1	-4.6	96.87	PEXP_096630	-12.2	6.41E-17
GI:425768595	EKV07113.1	-4.4	93.65	PEXP_077410	-3.82	1.02E-10
GI:425769499	EKV07991.1	-4.4	95.13	PEXP_027120	ns	-
GI:425767944	EKV06494.1	-4.4	55.56	PEXP_017960	ns	-
GI:425777763	EKV15919.1	-4.3	93.11	PEXP_104360	-1.79	1.25E-10
GI:425777780	EKV15936.1	-4.3	91.90	PEXP_104180	ns	-
GI:425774443	EKV12750.1	-4.2	75.84	PEXP_015010	-9.46	1.59E-15
GI:425775660	EKV13917.1	-4.2	74.88	PEXP_005280	ns	-
GI:425775572	EKV13831.1	-4.2	89.60	PEXP_109570	2.13	1.18E-08
GI:425779317	EKV17384.1	-4.2	99.16	PEXP_006710	-1.94	1.67E-10
GI:425765863	EKV04508.1	-4.2	98.94	PEXP_098670	-6.02	3.39E-13
GI:425765896	EKV04537.1	-4.1	98.09	PEXP_011080	-1.75	1.53E-09
GI:425769807	EKV08289.1	-4.1	81.42	PEXP_053340	-5.41	6.44E-13
GI:425775463	EKV13732.1	-4.1	93.61	PEXP_101250	-1.89	2.97E-10
GI:425772050	EKV10476.1	-4.0	97.94	PEXP_043320	-5.62	5.94E-13
GI:425770280	EKV08753.1	-4.0	94.29	PEXP_050580	1.29	6.05E-06
GI:425773857	EKV12182.1	-4.0	91.77	PEXP_011880	1.37	3.96E-04
GI:425770281	EKV08754.1	-4.0	94.50	PEXP_050600	1.14	4.60E-07
GI:425766239	EKV04863.1	-4.0	94.21	PEXP_094000	-5.22	4.79E-13
GI:425770817	EKV09277.1	-3.9	92.31	PEXP_031670	-7.8	3.76E-15
GI:425781867	EKV19804.1	-3.8	91.75	PEXP_098500	-1.55	1.81E-06

GI:425768068	EKV06612.1	-3.7	90.29	PEXP_054900	-9.62	7.29E-16
GI:425772896	EKV11276.1	-3.7	91.82	PEXP_029020	-3.62	4.07E-11
GI:425773464	EKV11817.1	-3.6	78.31	PEXP_108000	ns	-
GI:425779318	EKV17385.1	-3.6	95.01	PEXP_006700	-4.51	5.43E-13
GI:425777478	EKV15650.1	-3.6	92.00	PEXP_060200	-2.05	2.27E-08
GI:425769811	EKV08293.1	-3.6	95.65	PEXP_053400	-5.65	7.21E-04
GI:425773376	EKV11732.1	-3.5	92.58	PEXP_108370	-5.87	1.67E-13
GI:425767550	EKV06119.1	-3.5	95.95	PEXP_025390	-7.18	8.81E-05
GI:425775389	EKV13661.1	-3.5	95.48	PEXP_101070	-2.2	1.54E-08
GI:425767941	EKV06491.1	-3.5	96.22	PEXP_017870	ns	-
GI:425775318	EKV13596.1	-3.3	93.79	PEXP_048530	-10.7	6.41E-17
GI:425766258	EKV04882.1	-3.3	93.22	PEXP_103160	-7.8	1.41E-16
GI:425771518	EKV09959.1	-3.3	96.73	PEXP_045340	-2.46	6.74E-10
GI:425774860	EKV13155.1	-3.2	94.79	PEXP_013330	-1.13	4.40E-05
GI:425770183	EKV08656.1	-3.2	94.21	PEXP_056750	-2.05	1.35E-07
GI:425771298	EKV09744.1	-3.2	ns	-	-	-
GI:425765581	EKV04252.1	-3.1	84.34	PEXP_002300	1.01	4.37E-02
GI:425768542	EKV07063.1	-3.1	92.55	PEXP_039690	ns	-
GI:425766255	EKV04879.1	-3.1	96.27	PEXP_103220	-2.74	3.12E-04
GI:425770893	EKV09353.1	-3.0	95.65	PEXP_030880	ns	-
GI:425768940	EKV07451.1	-3.0	91.37	PEXP_078340	-5.64	7.41E-13
GI:425775292	EKV13570.1	-3.0	83.98	PEXP_048770	ns	-
GI:425766211	EKV04835.1	-3.0	96.32	PEXP_094330	ns	-
GI:425772975	EKV11353.1	-3.0	95.79	PEXP_029600	-6.06	1.28E-05

[1]PdΔ*brlA* = *Penicillium digitatum* null mutant Δ*brlA* strain. WT = wild type *Penicillium digitatum* strain

[2]PeΔ*brlA* = *Penicillium expansum* null mutant Δ*brlA* strain. WT = wild type *Penicillium expansum* strain

[*] These data are extracted from Wang *et al*. [38]

Supplementary Table 5. *Penicillium expansum* genes orthologous to genes significantly up-regulated (Log_2 fold change > 2) in *Penicillium digitatum* PdΔ*brlA* strain [38].

Penicillium digitatum PHI26 strain Gene ID	*Penicillium digitatum* GenBank Accession number	Log_2 Fold Change PdΔ*brlA* vs WT[1*]	% Positives	*Penicillium expansum* strain d1 Gene ID	Log_2 fold change PeΔ*brlA* vs WT[2]	Adjusted *p*-value
GI:425767861	EKV06414.1	2.0	96.04	PEXP_023250	ns	-
GI:425770858	EKV09318.1	2.0	89.14	PEXP_031240	ns	-
GI:425766441	EKV05051.1	2.0	79.37	PEXP_016660	ns	-
GI:425774248	EKV12561.1	2.0	96.55	PEXP_041510	ns	-
GI:425776778	EKV14982.1	2.0	95.27	PEXP_105890	1.33	7.88E-05
GI:425781020	EKV19002.1	2.1	ns	-	-	-
GI:425774460	EKV12767.1	2.1	90.71	PEXP_015220	-1.20	4.62E-03
GI:425772900	EKV11280.1	2.1	90.98	PEXP_028970	ns	-
GI:425765571	EKV04242.1	2.1	98.53	PEXP_002430	3.30	4.21E-10
GI:425766130	EKV04757.1	2.2	96.62	PEXP_080820	2.67	1.27E-08
GI:425767714	EKV06280.1	2.2	92.37	PEXP_068990	-2.59	8.77E-09
GI:425777056	EKV15250.1	2.2	95.53	PEXP_066110	ns	-
GI:425770829	EKV09289.1	2.3	86.38	PEXP_031560	1.13	8.19E-05
GI:425768269	EKV06797.1	2.3	80.59	PEXP_062990	2.63	8.79E-10
GI:425767408	EKV05982.1	2.3	42.00	-	-	-
GI:425771110	EKV09564.1	2.3	92.49	PEXP_106040	ns	-
GI:425776352	EKV14572.1	2.4	97.15	PEXP_021430	1.86	3.59E-08
GI:425771668	EKV10105.1	2.4	76.45	PEXP_061820	ns	-
GI:425770387	EKV08860.1	2.5	88.07	PEXP_051690	ns	-
GI:425769485	EKV07977.1	2.5	99.02	PEXP_026950	ns	-
GI:425773099	EKV11471.1	2.6	94.55	PEXP_000520	2.17	1.09E-07
GI:425776932	EKV15129.1	2.6	93.18	PEXP_065730	-1.46	1.78E-06
GI:425773150	EKV11520.1	2.6	85.53	PEXP_099440	ns	-
GI:425765550	EKV04227.1	2.7	43.79	-	-	-
GI:425773151	EKV11521.1	2.7	95.00	PEXP_099450	ns	-
GI:425767405	EKV05979.1	2.7	ns	-	-	-
GI:425776933	EKV15130.1	2.7	97.41	PEXP_065690	1.26	7.42E-06

GI:425779566	EKV17613.1	2.7	95.06	PEXP_054530	ns	-
GI:425772465	EKV10866.1	2.8	89.10	PEXP_096290	-2.51	4.85E-08
GI:425765551	EKV04228.1	2.8	47.10	-	-	-
GI:425771655	EKV10092.1	2.9	94.68	PEXP_061690	ns	-
GI:425771656	EKV10093.1	3.1	92.64	PEXP_061700	2.53	9.23E-08
GI:425777193	EKV15377.1	3.2	94.44	PEXP_069630	1.38	1.89E-04
GI:425765774	EKV04422.1	3.3	87.93	PEXP_099150	1.36	3.45E-08
GI:425777197	EKV15381.1	3.4	97.60	PEXP_070500	ns	-
GI:425767246	EKV05820.1	3.8	84.83	PEXP_067100	1.38	1.94E-05
GI:425776500	EKV14717.1	3.9	89.10	PEXP_021090	ns	-
GI:425781626	EKV19580.1	3.9	52.24	-	-	-
GI:425768677	EKV07195.1	4.3	71.58	PEXP_038390	-2.11	5.06E-05
GI:425781865	EKV19802.1	4.3	92.66	PEXP_098360	0.91	2.41E-03
GI:425777310	EKV15491.1	4.5	97.03	PEXP_037440	ns	-
GI:425780864	EKV18860.1	4.5	95.00	PEXP_071760	ns	-
GI:425779404	EKV17468.1	4.5	82.97	PEXP_019000	ns	-
GI:425775638	EKV13895.1	4.6	67.95	PEXP_102300	ns	-
GI:425770813	EKV09273.1	4.8	96.50	PEXP_031710	ns	-
GI:425781957	EKV19891.1	6.3	95.43	PEXP_018170	ns	-

[1]PdΔ*brlA* = *Penicillium digitatum* null mutant Δ*brlA* strain, WT = wild type *Penicillium digitatum* strain

[2]PeΔ*brlA* = *Penicillium expansum* null mutant Δ*brlA* strain, WT = wild type *Penicillium expansum* strain

[*] These data are extracted from Wang *et al*. [38]

Supplementary Table 6. Eighteen orthologous genes similarly regulated to *wetA* in *Penicillium rubens* Δ*brlA* [49] and *Penicillium expansum* Δ*brlA*.

Penicillium expansum strain d1 gene ID	*Penicillium rubens* strain * Wisconsin 54-1255 gene ID	% Identity	Log_2 fold change PeΔ*brlA* vs WT	Adjusted *p*-value
PEXP_022650	Pc12g00920	94.9	-5.87	6.90E-13
PEXP_052290	Pc12g14760	85.8	-3.79	3.17E-09
PEXP_041930	Pc13g15970	54.0	-3.42	3.41E-08
PEXP_056810	Pc18g01100	73.7	-4.35	2.83E-12
PEXP_084350	Pc19g00410	64.4	-2.12	1.90E-07
PEXP_096560	Pc19g00420	92.5	-7.51	4.58E-09
PEXP_096550	Pc20g06330	88.5	-6.75	3.06E-08
PEXP_037140	Pc21g06360	86.6	-7.61	7.77E-14
PEXP_028230	Pc21g12140	88.0	-3.90	2.87E-13
PEXP_030380	Pc21g15150	83.6	-2.17	6.41E-10
PEXP_096630	Pc21g16000	94.2	-12.20	6.41E-17
PEXP_097170	Pc21g16420	80.0	-8.64	1.31E-15
PEXP_097180	Pc21g16430	95.2	-8.34	1.13E-13
PEXP_097190	Pc21g16440	90.7	-6.57	9.55E-13
PEXP_077410	Pc22g03220	94.0	-3.82	1.02E-10
PEXP_076870	Pc22g06890	86.6	-0.99	2.35E-04
PEXP_091180	Pc22g26950	62.3	-4.38	6.23E-07
PEXP_018490	Pc20g10220	82.6	-10.7	9.61E-16

*The Wisconsin 54-1255 strain was formerly identified as *Penicillium chrysogenum*.

Supplementary Table 7. Fifty-nine orthologous genes similarly down-regulated to *abaA* in *Penicillium rubens* [49] Δ*brlA* and *Penicillium expansum* Δ*brlA*.

Penicillium expansum strain d1 gene ID	*Penicillium rubens* strain * Wisconsin 54-1255 gene ID	% Identity	Log_2 fold change PeΔ*brlA* vs WT	Adjusted *p*-value
PEXP_023320	Pc12g00010	83.2	-5.43	7.80E-14
PEXP_074900	Pc12g03260	74.2	-7.92	1.14E-14
PEXP_070580	Pc12g08750	86.5	-4.10	6.07E-10
PEXP_025850	Pc12g12350	90.8	1.49	9.90E+05
PEXP_084240	Pc12g08800	82.9	-4.15	8.76E-12
PEXP_053400	Pc12g13430	89.4	-5.65	7.21E-14
PEXP_052120	Pc12g14960	78.5	-10.10	6.09E-17
PEXP_104370	Pc13g06200	86.1	-4.56	1.23E-12
PEXP_022150	Pc13g06290	77.7	-3.61	2.72E-12
PEXP_006710	Pc13g08700	81.2	-1.94	1.67E-10
PEXP_025390	Pc13g13450	90.9	-7.18	8.01E-15
PEXP_025000	Pc13g13920	69.1	-8.34	6.10E-16
PEXP_000200	Pc14g00160	95.7	-5.57	3.94E-13
PEXP_074520	Pc15g00470	76.6	-1.68	4.01E-09
PEXP_054900	Pc16g00180	87.7	-9.62	7.29E-16
PEXP_085800	Pc16g02410	88.2	-2.51	5.36E-11
PEXP_086170	Pc16g02800	61.5	-6.03	2.26E-04
PEXP_006020	Pc16g04550	79.9	-7.01	7.37E-14
PEXP_015010	Pc16g04840	61.4	-9.46	1.59E-15
PEXP_020090	Pc16g08460	97.7	-3.17	2.09E-09
PEXP_029020	Pc16g09610	93.0	-3.62	4.07E-11
PEXP_001060	Pc16g10900	84.3	-9.51	1.43E-16
PEXP_000580	Pc16g11310	77.5	-6.21	1.49E-13
PEXP_043320	Pc16g13260	88.7	-5.62	5.94E-13
PEXP_056860	Pc18g01150	92.4	-4.96	1.58R-12
PEXP_017120	Pc18g01410	88.7	-3.38	9.46E-12
PEXP_092210	Pc18g02600	90.2	-4.28	1.47E-13
PEXP_031660	Pc18g04570	66.0	-5.95	9.64E-12
PEXP_031670	Pc18g04580	93.5	-7.80	3.76E-15
PEXP_016810	Pc20g01320	91.6	-5.75	3.58E-14
PEXP_073050	Pc20g02080	89.9	-1.49	6.57E-07
PEXP_072230	Pc20g03190	87.2	-3.22	6.03E-12
PEXP_018550	Pc20g10170	87.3	-4.36	2.05E-12
PEXP_063090	Pc20g10270	87.9	-2.75	4.31E-09
PEXP_011470	Pc20g13710	67.5	-7.65	1.10E-14
PEXP_010960	Pc20g14200	92.1	-1.17	5.06E-06
PEXP_088520	Pc21g02590	72.0	-6.66	1.17E-13
PEXP_109240	Pc21g08640	88.9	-1.39	1.54E-08

PEXP_108160	Pc21g09810	81.9	-5.54	1.73E-14
PEXP_040480	Pc21g13950	86.3	-1.57	1.56E-07
PEXP_096890	Pc21g16130	68.2	-8.24	7.29E-16
PEXP_097110	pc21g16380	79.2	-6.57	1.58E-12
PEXP_020490	Pc21g18350	76.3	-11.40	1.43E-16
PEXP_020680	Pc21g18530	81.7	-2.97	2.24E-11
PEXP_044610	Pc21g20790	70.7	-6.44	1.95E-13
PEXP_078340	Pc22g04080	76.8	-5.64	7.41E-13
PEXP_078920	Pc22g04640	92.6	-8.16	8.83E-16
PEXP_102970	Pc22g07130	93.1	-2.95	3.93E-09
PEXP_103160	Pc22g07470	89.2	-7.80	1.41E-16
PEXP_103170	Pc22g07480	82.1	-1.43	9.53E-08
PEXP_094000	Pc22g07760	83.9	-5.22	4.79E-13
PEXP_095030	Pc22g08820	54.7	-3.13	2.08E-11
PEXP_013250	Pc22g12240	94.2	-1.08	2.29E-05
PEXP_062290	Pc22g14290	87.8	-13.00	1.07E-18
PEXP_062310	Pc22g14300	76.3	-10.90	7.29E-16
PEXP_062520	Pc22g14640	89.8	-6.55	3.32E-14
PEXP_033790	Pc22g17040	61.3	-8.30	8.53E-16
PEXP_065060	Pc22g22050	91.2	-1.31	4.34E-08
PEXP_066390	Pc22g23660	90.3	-5.00	2.02E-12

*The Wisconsin 54-1255 strain was formerly identified as *Penicillium chrysogenum*.

Construction and Characterization of the null mutant Pe∆*brlA* strain

Creation of a Linear Transformation Cassette using Double-Joint PCR

For the creation of the null mutant Pe∆*brlA* strain, a strategy was applied to eliminate the *brlA* gene (gene coding for a master transcriptor factor indispensable to conidiogenesis) by replacing the coding region with the hygromycin selection marker in *Penicillium expansum* wild type NRRL 35695 (WT) strain. Specific primers of the *brlA* gene were designed from the *P. expansum* strain d1, sequence deposited in GenBank (PEXP_049260) and all primers used in this study are listed in Table S8. The Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Illkirch, France) was used for all PCR amplifications.

The 5' upstream and 3' downstream flanking regions served as complementary sequences to allow homologous recombination at the locus of interest. The fragments necessary for the construction of the gene disruption marker were amplified by PCR and assembled using double-joint PCR [96, 97]. Shortly, a 1,147 bp DNA fragment that matches the promoter and the 5' untranslated region of the *brlA* gene and a 1,080 bp fragment that matches the 3' untranslated region of the *brlA* gene were amplified from the genomic DNA of the WT strain using two pairs of primers, 5fdebBrlAPex/ DebBrlAPex1Rhygro and FinBrlAPex1Fhygro/ 3rfinBrlAPex, respectively. The hphF1 / hphR1 primer pair was used to amplify a 4,107 bp fragment of pAN 7.1 plasmid (access number Z32698), which contains the hygromycin (*hph*) resistance gene [97]. The underlined sequences in the DebBrlAPex1Rhygro and FinBrlAPex1Fhygro primers were added at their 5' and 3' ends (Table S8). These sequences are identical to the extremities of the PCR product generated by the hphF1 / hphR1 primers, and served to promote binding between PCR products. The PCR conditions for the amplification of the 5' junction and 3' junction were as follows: denaturation at 98 °C for 45 s, followed by 40 denaturation cycles at 98 °C for 45 s, annealing at 61 °C for 45 s and extension at 72 °C for 1 min and 15 s. A final extension step at 72 °C for 10 min was performed. The PCR conditions for the amplification of the hygromycin resistance marker were the same as those described above, except for the annealing temperature (62 °C) and the cycle extension, which was performed for 2 min. The amplicons were purified using a GenElute™PCR Clean-Up Kit (Merck KGaA, Darmstadt, Germany), as described in the procedure specified by the manufacturer.

The three fragments were assembled by double-joint PCR, using a fragment number ratio of 1:2:1 (flank 5': marker: flank 3') according to the procedure described by Lim *et al*. [97] with slight modifications. Briefly, in the first step of the PCR we used the three amplicons previously obtained as matrices and a primerless PCR reaction is achieved, resulting in the union of the two flanking regions, the selection marker, the single-union product and the double-union product (Figure S5). The amplification conditions for this first step were as follows: denaturation at 98 °C for 5 min, followed by 12 denaturation cycles at 98 °C for 45 s, annealing at 60 °C for 2 min and extension at 64 °C for 3 min. A final extension step at 64 °C for 5 min was performed. For the second step of the procedure, the dBrlA-NestedF/dBrlA-NestedR nested primers were used, amplifying only the double-union product. For the amplification conditions a temperature gradient was followed, starting with denaturation at 98 °C for 5 min, followed by a cycle of denaturation at 98 °C for 45 s, annealing at 65 °C for 30 s and extension at 68 °C for 3 min. The annealing temperature decreased by 0.5 °C/cycle to 62 °C, followed by 24 denaturation cycles at 98 °C for 45 s, annealing at 62 °C for 30 s and extension at 68 °C for 3 min. A final extension step at 64 °C for 5 min was performed.

The final PCR product (6,292 bp), containing the hygromycin resistance marker fused to the flanking regions of the *brlA* gene, was used to transform protoplasts of *P. expansum* according to the methodology described by Snini *et al*. [22].

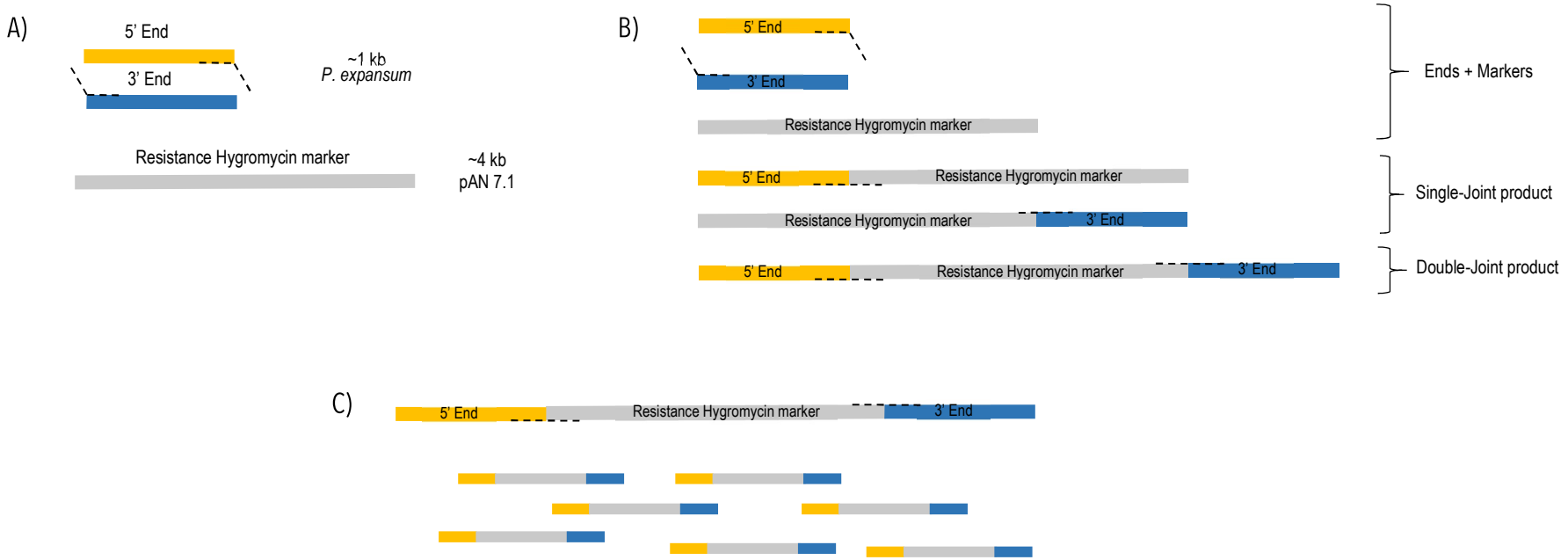
Supplementary Table 8. Primers used in the construction and the validation of the null mutant PeΔ*brlA* strain.

Primer	Sequence 5′ → 3′
5fdebBrlAPex	GTAACGCCAGGGTTTTCCCAGTCACGACG
DebBrlAPex1Rhygro[1]	GAGCCTGTGTGTAGAGATACAAGGGAATTCTGACCCTTGGCTGTAAAGACTGGTAGG
FinBrlAPex1Fhygro[1]	GTGTAAGCGCCCACTCCACATCTCCACTCGCGTGGCCTTCATTGGCCTTTATTGATAC
3rfinBrlAPex	GCGGATAACAATTTCACACAGGAAACAGC
hphF1	GAATTCCCTTGTATCTCTACACACAGGCTC
hphR1	CGAGTGGAGATGTGGAGTGGGCG
dBrlA-NestedF	GTTGCGGCCGGAACCTTGGAACC
dBrlA-NestedR	CCAGAGATGTCTGAGCTCCAATCGC
dBrlA-geneF	GGCTTCCCCTGGGTGGCAC
dBrlA-geneR	GCCATCGACGCTCAGCTCGC
Hyg5′1 R	CTTGACACCGCTCCGTCCTCC
Hyg3′1 F	GGGTTTACCTCTTCCAGATACAGCTC
dBrlA-5′F	GGAGTGTGGGAGATCAGGGTCG
dBrlA -3′R	CGGTGTAGCGGTGGACTCTGG
GWBrlA3′1[2]	CTTCACCGCGGCTATATGTATCGTTCAACC
GWBrlA3′2[2]	GGAACACAGTGCCCCGAATACGCACTG
Pexp_brlA Forward[3]	CCTCGATGCCTCAATACA
Pexp_brlA Reverse[3]	GTAAAGATTGGACGAGACAAG
Pexp_btub Forward[3]	TGAACGTCTACTTCAACCATGCC
Pexp_btub Reverse[3]	CCAAATCGACGAGAACGG

[1]The underlined sequences were added at the 5' end and 3′ end were identical to the extremities of the PCR product generated by primers hphF1/hphR1.

[2]Primers used in the validation by genome walking.

[3]Primers used in the validation by qPCR.



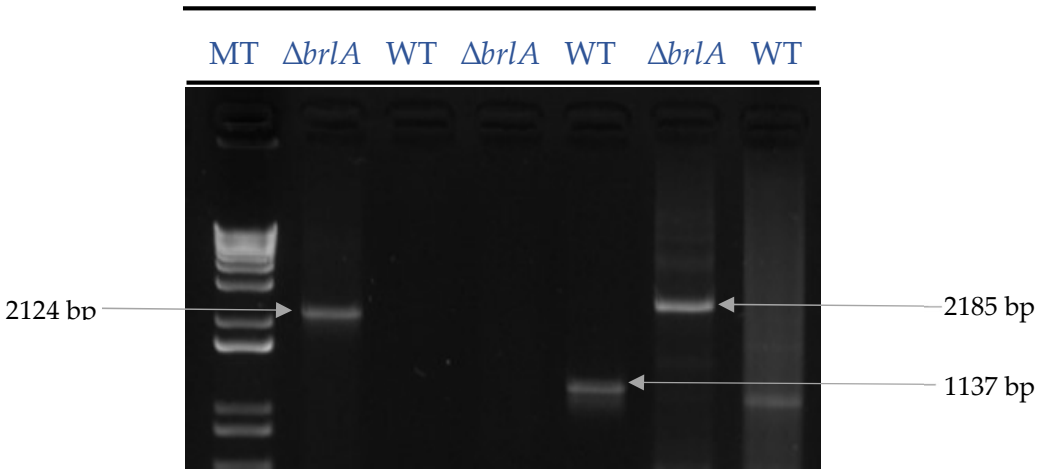
Supplementary Figure 5. Double-joint PCR reaction [97]. A) PCR reaction for amplification of flanking regions 5 'upstream and 3' downstream of the *brlA* gene from *Penicillium expansum* and amplification of the hygromycin marker from the pAN7.1 plasmid. B) Primerless PCR reaction using the three previously obtained amplicons as matrices. C) Nested PCR reaction for amplification only of the double union product.

Validation of the Null Mutant PeΔbrlA strain

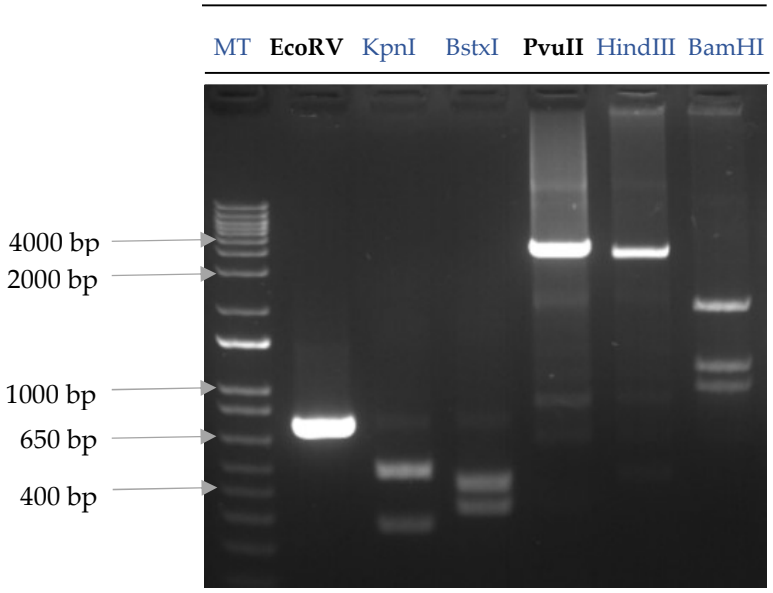
Polymerase Chain Reaction (PCR) and Genome Walking

To confirm the insertion of the hygromycin resistance marker at the *brlA* locus in the transformant strains, a PCR reaction and an enzymatic digestion by genome walking were achieved. First, genomic DNA was extracted according to a previously published method [114] and used to screen the obtained transformants by PCR to detect the replacement between *brlA* and *hph*. The transformants were screened using the dBrlA-geneF/dBrlA-geneR specific primers, according to the PCR conditions: 35 denaturation cycles at 98 °C for 45 s, annealing at 62 °C for 45 s and extension at 72 °C for 1 min and 30 s. A final extension step at 72 °C for 10 min was performed. The *Taq* DNA Polymerase (Thermo Fisher Scientific) was used. Next, PCR amplifications with the dBrlA-5'F/Hyg5'1R and Hyg3'1F/ dBrlA-3'R hygromycin-specific primers were carried out on the selected null mutants. The dBrlA-5'F and dBrlA-3'R primers are located upstream and downstream of the 5' and 3' junctions of the disruption cassette, respectively, and the Hyg5'1R /Hyg3'1F primer pairs are located inside the hygromycin marker. The following PCR conditions were used: 35 denaturation cycles at 98 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 2 min. A final extension step at 72 °C for 10 min was performed. The Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used.

The transformants were also verified by genome walking (GenomeWalker Universal Kit, Takara Bio Europe SAS, Saint-Germain-en-Laye, France), a simple method for finding unknown sequences adjacent to a known genomic DNA sequence. The use of this technique enabled us to verify that the cassette was inserted only once at the *brlA* locus, i.e. that it is present in a single copy in the genome of *P. expansum*. Following the protocol recommended by the manufacturer, the first step consists in constructing groups of uncloned adaptor-ligated genomic DNA fragments, referred to as "libraries". Briefly, aliquots of 2.5 µg of genomic DNA were digested overnight using the restriction enzymes EcoRV and PvuII. Each batch of digested genomic DNA was then ligated to the GenomeWalker adaptors. After the libraries were constructed, amplification was carried out by specific PCR. The first or primary PCR used the adaptor primer (AP1) provided in the kit and an external gene-specific primer (GSP1): GWBrlA3'1, which was designed from the 3' end sequence of the disruption cassette. For PCR conditions, the two-step cycle parameters were as followed: first, seven denaturation cycles at 94 °C for 25 s and annealing/extension at 72 °C for 3 min, then 32 denaturation cycles at 94 °C for 25 s and extension at 67 °C for 3 min. A final step at 67 °C for seven min was performed. The amplification product obtained from this PCR was diluted (1:50) and used as a template of a second (or nested) PCR. Primers, the nested adaptor primer (AP2) and GWBrlA3'2 (GSP2) were used. For PCR conditions, the two-step cycle parameters were as followed: first, five denaturation cycles at 94 °C for 25 s and annealing/extension at 72 °C for 3 min, then 20 denaturation cycles at 94 °C for 25 s and extension at 67 °C for 3 min. A final step at 67 °C for seven min was performed. The Advantage 2 Polymerase Mix (Takara Bio Europe SAS) was used. The sequences of the primers used are shown in Table S8. Subsequently, the fragments generated by the nested PCR were purified using a GenElute™PCR Clean-Up Kit (Merck KGaA) and two sequencing reaction were performed using the AP2 and GWBrlA3'2 primers by the BIOfidal sequencing laboratory (Vaulx-en-Velin, France). In addition, a restriction mapping was generated from genome walking products using the restriction enzymes KpnI and BstxI for the PCR product obtained from the EcoRV library, which produced fragments of 217, 483 bp and 276, 424 bp, respectively. HindIII and BamHI enzymes were used for the PCR product of the PvuII library, which produced fragments of 298, 3,702 bp and 929, 1159, 2004 bp, respectively, in accordance with the DNA genomic sequence deposited in GenBank (JQFY01000187.1).



Supplementary Figure 6. PCR amplification of *Penicillium expansum* wild type NRRL 35695 (WT) and null mutant PeΔ*brlA* strains. Primers, lane 1 and 2: dBrlA-5'F / Hyg5'1 R, lane 3 and 4: dBrlA-geneF / dBrlA-geneR and lane 5 and 6: Hyg3'1 F / dBrlA -3'R. The presence of the disruption cassette Δ*brlA* at the *brlA* locus was confirmed in null mutant strains amplified fragments of 2,124 bp and 2,185 bp, while no amplification was observed in the WT for PCR with the pair of primers dBrlA-5'F / Hyg5'1 R and Hyg3'1 F / dBrlA -3'R. In contrast, the presence of the *brlA* gene was not detected in the null mutant strain, the WT strain produced an amplicon of 1,137 bp. MT Lane: 1 kb plus DNA ladder.



Supplementary Figure 7. Genome walking (GW) analyses of genomic DNA of *Penicillium expansum* wild type NRRL 35695 and null mutant PeΔ*brlA* strains. Lane 1 and 4: PCR products with the primers AP2-GWBrlA3'2 from the EcoRV (623 bp) and PvuII (4,009 bp) libraries. Lane 2 and 3: Enzymatic digestion of the EcoRV library amplicon with the enzymes KpnI (217 and 483 bp) and BstxI (276 and 424 bp) Lane 5 and 6: Enzymatic digestion of the PvuII library amplicon with the enzymes HindIII (298 and 3,702 bp) and BamHI (929 and 1,159 and 2,004 bp). MT Lane: 1 kb plus DNA ladder

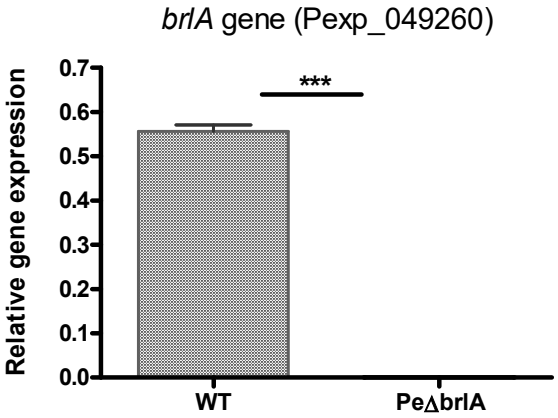
Quantitative real-time PCR Validation

To investigate the gene expression of each transformant strain, a qPCR analysis was performed. The WT and null mutant PeΔ*brlA* strains were pre-cultured on MEA medium. A spore suspension was made with the WT strain and the mycelium of the null mutant strain was used as inoculation material as this strain does not produce conidia. The strains were grown in Petri dishes containing MEA covered with sterile cellophane sheets (10 µL of a 10^6 spores/mL solution for the WT strain, 5 mm^2 mycelium for the null mutant strain) and incubated at 25 °C in the dark for five days. At the end of the growth period, total RNA was isolated. From each strain, 110-120 mg of mycelium were weighed and transferred to lysing matrix D tubes (1.4 mm ceramic spheres, Thermo Fisher Scientific), to which 760 µL of lysis buffer [10 µL of β-mercaptoethanol (Applied Biosystem, Thermo Fisher Scientific) and 750 µL of RLT buffer (Rneasy mini kit, QIAGEN, Courtaboeuf, France)] were added, and were placed in liquid nitrogen. The mycelium cells homogenized in a Precellys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) by three grindings at a speed of: 6,500 rpm during 15 s followed by 5 min incubation on ice, 6,500 rpm for 25 s and 5 min on ice and finally 6,500 rpm for 15 s. The samples were subsequently centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was recovered into QIAshredder spin columns (QIAGEN) and processed according to the Rneasy mini kit (QIAGEN) protocol, detailed by Tannous *et al*. [20]. The total RNA extracted was purified using gDNA-eliminator mini-columns (QIAGEN). The RNA quality was checked by gel electrophoresis (1.2% agarose) and the concentration was determined by a NanoDrop ND1000 (Labtech, Palaiseau, France). The final RNA concentrations were around 153-255 ng/µL ($A_{260/280}$= 2.14) and 304-335 ng/µL ($A_{260-280}$= 2.10) for the PeΔ*brlA* and WT strain respectively.

For the reverse transcription step we used a volume of 10 µl of RNA adjusted to a final concentration of 153 ng/µL and 1 µL of oligo (dT) Bys 3' Primer: (5'-GCTGTCAACGATACGCTATAACGGCATGACAGTGTTTTTTTTT-3') according to the protocol detailed by Caceres *et al*. [115].

The qPCR experiment was performed with a 7300 Real Time PCR System (Applied Biosystems, Thermo Fisher Scientific) using the three-step cycling. The primers Pexp_brlA Forward and Pexp_brlA Reverse (Table S8) were used to amplify 50-100 bp of the *brlA* target gene (PEXP_049260). The β-tubulin gene was used as the reference gene. The primers were designed and validated as described previously [115]. PCR amplification was carried out in a total volume of 25 µL, using 5 µL of cDNA template, 12.5 µL of SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) and 7.5 µL of primers mix (900 nM and 300 nM for the *brlA* forward/reverse primers, respectively, and 300 nM for the reference gene primers). The following PCR program was used: denaturation at 95 °C for 10 min, followed by 40 denaturation cycles at 95 °C for 15 s, annealing at 60 °C for 1 min, and a final extension step at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s was achieved. The melting curves were analyzed with the 7300 System SDS Software (Applied Biosystems, Thermo Fisher Scientific) to confirm the specificity of the amplification. Changes in gene expression of qPCR experiments were analyzed using the $2^{-\Delta\Delta CT}$ method [116]. The Student's t-test was performed as a statistical analysis using Graph Pad 4 software (GraphPad Software, San Diego, CA, USA), a p-value < 0.05 was considered statistically significant. Four biological replicates were performed for each strain.

The RNA extraction and qPCR methodology was also used to confirm the results obtained in the microarray analysis, where the expression of all the genes of the putative chaetoglobosin cluster was evaluated. We used the RNA samples that served for the microarray analysis. The primers used are listed in Table S3.



Supplementary Figure 8. Validation by quantitative real-time PCR analysis. The expression of the *brlA* gene was evaluated in *Penicillium expansum* wild type NRRL 35695 (WT) and in the null mutant PeΔ*brlA* strains. The Student's t-test was performed using GraphPad Software. The graph shows the mean ± standard error of the mean (SEM) from four biological replicates. *p*-value considered statistically significant: * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001.

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CHAPTER 3

RESULTS

Deciphering of secondary metabolome of *Penicillium expansum*



Summary of the study

As described in the second section of the introduction, *P. expansum* is a phytopathogenic fungus, which develops during harvest and post-harvest processes. It is considered the main cause of the blue mould disease and an important producer of patulin, a mycotoxin. From an economic and health point of view, *P. expansum* is one of the most important species among the toxigenic fungi due to its wide dissemination and the capacity to produce toxic secondary metabolites (SMs).
In food and plants under appropriate conditions, *P. expansum* is able to produce a large number of bioactive molecules (Tannous et al. 2018), among which are patulin, citrinin, roquefortines, chaetoglobosins, communesins, andrastins, geosmin and expansolides. The secondary metabolites are the products catalyzed by enzymes encoded by genes arranged contiguously within biosynthetic gene clusters (BGCs). An antiSMASH analysis has predicted 63 putative BGCs from *P. expansum* T01 and d1 strains (El Hajj Assaf et al., 2018; Weber et al. 2015). The production of SMs is controlled at a hierarchical level by specific transcription factors located in the gene cluster that can activate or silence the biosynthesis of a compound. However, SM biosynthesis is also controlled by global transcription factors regulated by abiotic factors such as pH, light, carbon and nitrogen sources.

Given this number of BGCs, the number of reported SM families produced by *P. expansum* seems very low. In this context, a part of the experimental work was aimed at deciphering the metabolome of *P. expansum*. In order to establish a list of SMs produced by *P. expansum*, a non-targeted metabolomic approach combining liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was performed. The *P. expansum* wild-type and null mutant Δ*veA* strains were grown in three types of wheat; natural wheat ^{12}C and two different isotopic enrichment, ^{13}C labeled wheat and $^{13}C/^{15}N$ labeled wheat. Following the extraction of SMs and LC-HRMS analysis, the in-house MassToFormula Compare program was used to obtain the raw formula of each SM detected. Finally, the formulas obtained are compared with reference standards and with AntiBase, a database specialized in natural products from microorganisms, to identify the compounds (Laatsch, 2012). The interest of this first experiment is to unambiguously determine the raw formula of maximum of known and unknown metabolites produced by *P. expansum*. In addition, analyses were carried out in vitro on synthetic media (MEA, PDA), in light and darkness conditions, and in vivo in infected apples to explore the spatial-temporal organization of the metabolome.
This study detected 62 known and 42 unknown metabolites produced by *P. expansum* at different physiological stages of its development, such as substrate colonization and dissemination. We showed that *P. expansum* produces compounds such as patulin and citrinin, which are mainly detected in the medium it invades and others such as chaetoglobosins, communesins A and B, and

roquefortine C, which are mainly detected in the mycelium or reproduction structures as conidiophores. The elimination of the *veA* gene, caused abolition of patulin and citrinin production in the strain but up-regulated the production of other unknown compounds. In vitro results showed that there is a significant difference between the compounds excreted to the environment and those detected in the mycelium of the fungus. In the in vivo study, a greater amount of metabolites was detected when the incubation period was 30 dpi. Forty compounds were detected in the spores, including chaetoglobosins and communesins. No patulin was detected in the spores.

Deciphering of secondary metabolome of *Penicillium expansum*

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Summary

Penicillium expansum, a post-harvest pathogen, is the causative agent of blue mold disease and producer of the mycotoxins patulin and citrinin among other secondary metabolites. Secondary metabolism is associated with fungal development, which responds to numerous external biotic and abiotic factors. An antiSMASH analysis has predicted 63 putative biosynthetic gene clusters (BGCs) of *P. expansum* strains T01 and d1, indicating that *P. expansum* has the ability to produce a large number of secondary metabolite (SM) families.

In order to establish an exhaustive list of the SMs produced by *P. expansum*, a non-targeted metabolomic approach combining stable isotope labeling and liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) was performed. In addition, analyses in vitro on synthetic media, Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA), and in vivo on infected apples to explore the spatial-temporal organization of the metabolome were carried out. Our results showed the detection of 104 SMs in *P. expansum*, of which 62 are known metabolites. New metabolites have been identified and raw formulae have been assigned to them. Patulin and citrinin were specific to the colonization phase of the fungus, whereas roquefortines C and D, and communesins A and B were present mainly in the fungal extract (basal mycelium, aerial mycelium, conidiophore and spores) of the strains. Secondary metabolism of *P. expansum* does not occur randomly in a fungus but depends on environmental conditions, substrate and life cycle.

Key words: Apples, metabolomic analysis, *Penicillium expansum*, secondary metabolism, spores, *veA*.

1. Introduction

The phytopathogenic fungus *Penicillium expansum* is the causal agent of the blue mould disease in apples and some other fruits like pears, cherries, peaches and plums, that leading to important economic losses in orchards worldwide (Luciano-Rosario et al. 2020; Morales et al. 2010; Nunes, 2012). *P. expansum* is a psychrophilic and necrotrophic fungus that develops during harvesting and post-harvest processes as well as during storage. The fungus infects through injuries (bruises, puncture wounds) or natural openings on the surface of the fruit, causing tissue decay. Under favorable conditions such as moderate temperature (25 °C) and high relative humidity (a_w 0.98), *P. expansum* produce a large number of bioactive molecules, called secondary metabolites (SMs) (Tannous et al. 2018).

The production of SMs is usually linked to the development of fungi (Keller, 2019; Macheleidt et al. 2016). SMs could have specific functions, such as virulence or agressiveness factors, chemical weapons or communication signals during their coexistence with other microorganisms and protection against damage caused by UV light or other abiotic stress (Keller, 2019). These metabolites have diverse biosynthetic origins, leading to a wide structural diversity. This broad diversity could explain their large spectrum of activities and functions. (Bills and Gloer, 2016; Calvo et al. 2002; Keller, 2019; Macheleidt et al. 2016).

P. expansum produces a wide variety of SMs, including patulin, the main mycotoxin of this mold. Patulin acts as a cultivar-dependent aggressiveness factor (Snini et al. 2016) in conjunction with tissue acidification due to the conversion of glucose to gluconic acid by glucose oxidase 2 (Chen et al. 2018; Prusky et al. 2016). Due to its toxicity (Glaser and Stopper, 2012; Puel et al. 2010), maximum levels of patulin are regulated in apple-based products in many European countries (EC Commission Regulation, 1881/2006). Citrinin, another SM produced by *P. expansum* displays a biosynthetic polyketide (PK) origin like patulin and exhibits antibiotic properties. It could also have auxiliary supportive functions during apple colonization (Touhami et al. 2018), but citrinin is mostly studied for its nephrotoxicity toward mammals and is also proposed to have teratogenic effects (de Oliveira Filho et al. 2017; Doughari, 2015; Flajs and Peraica, 2009). Non-ribosomal peptides (NRPs), such as roquefortine C, a weakly neurotoxic compound, and communesins that have anti-insect activity, have also been detected. Other compounds produced by *P. expansum* are the NRP hybrids chaetoglobosins A and C, terpenes such as geosmin, expansolides A and B, and andrastins A, B and C, which are supposed anticancer drugs (Andersen et al. 2004; Tannous et al. 2018).

In filamentous fungi, SMs are the products catalyzed by enzymes encoded by genes arranged contiguously within biosynthetic gene clusters (BGCs). An antiSMASH analysis (Blin et al. 2019; Weber et al. 2015) has predicted 63 putative BGCs of *P. expansum* T01 and d1 strains (El

Hajj Assaf et al.). Given this number of BGCs, the number of reported SM families produced by *P. expansum* seems very low. Thus, potential new compounds that are synthesized by enzymes encoded by genes of unknown clusters remain to be discovered (Ballester et al. 2015; Nielsen et al. 2017). BGCs can be activated or suppressed by a global transcription factors (TFs) that are regulated by abiotic factors such as pH, light, carbon or nitrogen sources. Among them, the global TF VeA, a component of the velvet complex, is 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). In general, VeA increases the production of SMs, such as penicillin in *Penicillium chrysogenum* (Kopke et al. 2013) or gliotoxin in *Aspergillus fumigatus* (Dhingra et al. 2013). In *P. expansum*, the disruption of *veA* affected strain aggressiveness, asexual coremia formation and drastically reduced the production of patulin and citrinin on synthetic media (EL Hajj Assaf et al. 2018).

Although there is some information on *P. expansum*, there are still many SMs to be discovered and characterized, as small changes in growth conditions could completely alter the secondary metabolism of the fungus. In this context, the objective of this work was to establish an exhaustive list of the known and unknown SMs produced by *P. expansum* as well as the spatial-temporal organization of the metabolome during the life cycle of the fungus. In order to establish the SMs produced by *P. expansum*, a study with a non-targeted metabolomic approach combining isotopic labeling and liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) was performed. Three types of wheat were used: natural ^{12}C wheat and two different isotopic enrichment, a ^{13}C labeled wheat and $^{13}C/^{15}N$ labeled wheat. To explore the spatial-temporal organization of the metabolome, analyses were carried out in vitro on synthetic media, MEA and PDA, under light and darkness conditions, and in vivo on infected apples. For both studies, we used a wild type (WT) strain and a mutant strain for the global TF VeA, PeΔ*veA*.

This study evidenced 62 known and 42 unknown metabolites produced by the fungus at different physiological stages of its development, such as substrate colonization and dissemination. In addition, we showed that *P. expansum* produced compounds such as patulin and citrinin, that are only detected in the medium it invades and others such as chaetoglobosins, communesins A and B and roquefortine C that are mainly detected in the fungal extract (basal mycelium, aerial mycelium, conidiophore and spores). Light had a significant effect on the biosynthesis of some SMs, but it was the least discriminating factor among the variables studied. In the conidiogenesis stage, *P. expansum* could activate other SM pathways such as the one leading to the biosynthesis of two compounds with the chemical formulas $C_{19}H_{16}N_2O_2$ and $C_{20}H_{18}N_2O_2$.

2. Material and Methods

2.1 Fungal Strains

The WT *Penicillium expansum* strain NRRL 35695 was isolated from grape berries in Languedoc Roussillon (France). The null mutant PeΔ*veA* and PeΔ*veA*:*veA* strains were generated by homologous recombination as described previously by El Hajj Assaf et al. (2018). Briefly, a gene deletion strategy was applied by replacing the coding region with the hygromycin selection marker, following the protocol detailed by Snini et al. (2016).

2.2 Fungal growth condition on ^{12}C, ^{13}C and $^{13}C/^{15}N$ labeled wheats and metabolite extraction

Triticum aestivum (cv. caphorn) was used to produce isotopic enrichments as described previously (Péan et al. 2007) and as detailed by Cano et al. (2013) and Hautbergue et al. (2017). The isotopic enrichments were then measured with a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Illkirch, France). The first consisted of 96.8% ^{13}C enriched wheat (referred to as ^{13}C wheat) and the second of 53.4% ^{13}C and 96.8% ^{15}N wheat (referred to as $^{13}C/^{15}N$ wheat).

The natural and labeled wheat grains were treated and inoculated as described by Hautbergue et al. (2017). Succinctly, wheat grains were drenched in water and autoclaved twice. The water activity (a_w) of the grains was measured and sterile water was added until an a_w of 0.98 was obtained prior to inoculation. Four cultures media containing 30 g of sterilized labeled: one dish with ^{13}C wheat, one dish with $^{13}C/^{15}N$ wheat and two dishes with ^{12}C wheat were prepared in sterile 140 mm diameter Petri dishes (Sarstedt, Marnay, France). Each type of labeled or unlabeled wheat was inoculated with 250 µL of a spore solution (10^5 spores/mL), quantified using a Malassez cell (Adjovi et al. 2014),of the WT and null mutant PeΔ*veA* strains previously grown on Potato Dextrose Agar (PDA) medium (Merck, Darmstadt, Germany; 4 g/L potato extract, 20 g/L dextrose, 15 g/L agar) for seven days at 25 °C. The second Petri dish containing unlabeled ^{12}C wheat grains served as a control and was not inoculated. The cultures were incubated for 14 days at 25 °C in the dark. After incubation, wheat grains and mycelium were extracted together with 500 mL of ethyl acetate for 72 h on a shaking table IKA Labor technik HS501 (IKA, Staufen, Germany) at 140 rpm at room temperature. The extract was then filtered and evaporated to dryness. The extraction was repeated twice. The resulting residue was dissolved in 400 µL of a water:acetonitrile mixture (50:50, v/v) and centrifuged 10 min at 10 000 g before liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) analysis.

2.3 Analysis of secondary metabolite production in vitro

Precultures of the WT and the null mutant PeΔ*veA* strains on Malt Extract Agar (MEA) medium (Biokar Diagnostics, Allonne, France; 30 g/L malt extract, 15 g/L agar) were incubated for seven days at 25 °C in the dark. Then, a spore suspension was made and its concentration quantified as previously (Adjovi et al. 2014). Cultures of the WT and PeΔ*veA* strains were performed on MEA and PDA media, Petri dishes containing the medium were covered with a sterile cellophane film and inoculated with 10 µL of a spore suspension containing 10^6 spores/mL. Cultures were incubated for five days at 25 °C in the dark or in the light. The cellophane film on which the fungus grew was separated from the agar medium and the metabolites were extracted from the mycelium of the fungus and the agar medium separately with 50 mL of ethyl acetate for six days as described previously (EL Hajj Assaf et al. 2018). Experiments were performed in triplicate.

2.4 Analysis of secondary metabolite production in vivo

In vivo studies were carried out on Golden Delicious apples purchased in a supermarket (Carrefour, Toulouse, France). Two strains were used for the study, the WT and the null mutant PeΔ*veA*. Apples were surface sterilized in a 2% sodium hypochlorite solution as described previously by Sanzani et al. (2012). Subsequently, apples were wounded with a sterile toothpick and inoculated with 10 µL of a suspension (10^4 spores/mL) of the WT and null mutant PeΔ*veA* strains. Infected apples were incubated for 14 days at 25 °C in the dark. At the end of the incubation, the spores was collected using a nylon membrane (UptiDisc nylon membrane, 47 mm diameter; Interchim, Montluçon, France) equipped with a vacuum pump. Then, the membranes were extracted with 50 mL ethyl acetate on a horizontal shaking table (IKA) at 160 rpm at room temperature for 72 h. The solvent was evaporated to dryness and the residue was resuspended in 300 µL of a water:acetonitrile mixture (50:50, v/v). Apples were ground as described previously by MacDonald et al. (2000) and SMs were extracted as detailed by Snini et al. (2016). Experiments were performed in triplicate.

2.5 Analytical Parameters for HPLC-DAD

Analyses of SMs were performed on an Ultimate 3000 High Performance Liquid Chromatography (HPLC) (Dionex/Thermo Scientific, Courtaboeuf, France) system equipped with a Diode Array Detector (DAD). For the detection of patulin, a Gemini® 5 µm C6-Phenyl column 110 Å (250 mm x 4.60 mm x) (Phenomenex, Torrance, CA, USA) was used at a flow rate of 0.9 mL/min at 30 °C. Eluent A was water acidified with 0.2% acetic acid and eluent B was HPLC-grade methanol (Thermo Fisher Scientific). The elution was carried out under the following conditions: 0 min 0% B, 8 min 0% B, 20 min 15% B, 25 min 15% B, 35 min 90% B, from 35 to 40 min 90% B,

from 45 to 60 min 0% B. Patulin and some of its precursors were monitored at a wavelength of 277 nm and their presence was confirmed by their retention time (min) and comparison of their UV spectrum with those of authentic standards.

For the other metabolites, a Luna® 5 µm C18 column 100 Å (125 mm x 2 mm) (Phenomenex, Le Pecq, France) was used at a flow rate of 0.2 mL/min at 30 °C. Eluent A was water and eluent B was acetonitrile, both acidified with 0.05% formic acid. The column was equilibrated with a mixture of 80% eluent A and 20% eluent B. The elution was carried out under the following conditions: 30 min linear increase from 20% to 50% B, 5 min from 50% to 90% B, 10 min 90% B, 5 min from 90% to 20% B, finally 10 min 20% B. Three biological replicates were performed for each strain.

2.6 LC-HRMS analysis

The chromatographic system consisted in an ultimate 3000 HPLC device (Dionex/Thermo Scientific) operating with a Luna® 5 µm C18 column 100 Å (125 mm x 2 mm) (Dionex/Thermo Scientific). Eluent A was water acidified with 0.05% formic acid and eluent B was HPLC-grade acetonitrile. A volume of 10 µL of each sample diluted twice with mobile phase A was injected. The flow rate was 0.2 mL/min at 30 °C with a gradient program of: 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. The mass spectrometer corresponded to a LTQ Orbitrap XL (Dionex/Thermo Scientific) fitted with an Electrospray Ionization Source (ESI) in positive and negative modes. 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. 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. High-resolution mass spectra were acquired between m/z 100 and 800 at a resolution of 7500. The calibration of the mass spectrometer was achieved using the calibration solution of Thermo Fisher Scientific in agreement with their protocol. MS/MS spectra were obtained with the collision induced dissociation (CID) mode of the ion trap analyzer at low resolution and a normalized collision energy of 35%. Three biological replicates were analyzed for each strain.

2.7 Identification of secondary metabolites

A mass compare program developed at the French National Research Institute for Agriculture, Food and Environment (INRAE) was used to compare the results obtained of ^{12}C, ^{13}C and $^{13}C/^{15}N$ cultures and determine the elemental composition of each compound with a mass measurement accuracy of 5 ppm (Cano et al. 2013; Hautbergue et al. 2017). The identification of fungal products was determined and confirmed based on their chemical formula, MS/MS

spectrum, retention times, MS/MS fragmentation pattern of the standard compound, data from the literature and AntiBase 2012, a specific database of natural products (Laatsch, 2012).

2.8 Metabolome data analysis

Raw acquisition files from mass spectrometer (blank, quality control pool samples and biological samples) were converted in mzXML format by Proteowizard MSconvert software. Extraction and alignment of data were carried out using xcms software (Smith et al. 2006) with centwave algorithm (Tautenhahn et al. 2008). Features with intensities lower than two times blank intensities were discarded.

Results were first examined using Principal Component Analysis (PCA) in order to verify the validity of the acquisition. Then, Projection to Latent Structure-Discriminant Analysis modeling (PLS-DA) was carried out to find out discriminant features for the different factors of the study. Models with $Q^2 > 0.4$ were considered valid. For valid model, a permutation test was then involved to assess the robustness of models. For valid and robust models, significant features were selected by their variable importance on projection (VIP). A feature with a VIP > 1 was considered significant. In a second step, significant features were analyzed using Kruskal and Wallis non-parametric univariate test. All these steps were carried out using Workflow4metabolomics, a collaborative portal dedicated to metabolomic data processing (Giacomoni et al. 2014).

3. Results

3.1 *P. expansum* is an Important Producer of Bioactive Compounds: Analysis of Labeled Wheats Cultures

The PeΔ*veA* strain was grown on natural ^{12}C wheat grains, ^{13}C, and $^{13}C^{15}N$ labeled wheat grains and the fungal products were analyzed by LC-HRMS. The detected metabolites were identified by comparing them with the reference metabolome of the WT strain grown on labelled wheat under the same conditions (Zetina-Serrano et al. 2020).

Table 1 lists 104 SMs detected in the WT and PeΔ*veA* strains. Of these, 62 have been identified and 42 remain unknown. With the exception of aurantioclavine and communesin K, the proposed structures contain one or more oxygen atoms and 75% of the SMs, 80 out of 104, have one to five nitrogen atoms. Some compounds with the same chemical formulae displayed similar MS/MS spectra (Table S1). Among the compounds identified are patulin, the best known mycotoxin of *P. expansum*, as well as other compounds previously reported to be produced by this fungal species, including citrinin, roquefortine C, fungisporins, andrastins, expansolides, chaetoglobosins and communesins. Several derivatives of certain mycotoxin families were found, such as chaetoglobosins or communesins (Table 1). We have highlighted 22 derivatives of communesins, among which we detected a new one with m/z= 457.26097 (R_T=31.66) and molecular formula $C_{31}H_{36}N_4O_2$. Communesins have been identified based on their calculated molecular formula and MS/MS spectrum, and are classified into two series according to the identification of a dimethylepoxyde (DME) or a dimethylvinyl (DMV) group in their structure. This was determined by observing in the MS/MS spectrum either of the simultaneous neutral losses of -18, -58 and -72 for the DME analogs (Kerzaon et al. 2009), or a neutral loss of -17 for the DMV analogs, which was confirmed by the analysis of a pure standard of communesin F (O. Grovel, personal communication). The first stable intermediate of communesin biosynthetic pathway is aurantioclavine, which was also detected. Nineteen derivatives of cytochalasan family, with among them nine chaetoglobosin were detected. Furthermore, clavicipitic acid was detected for the first time in an extract of *P. expansum.* In addition to the final product, some intermediates of the patulin biosynthesis pathway have been identified including *m*-cresol, m-hydroxybenzyl alcohol, gentisyl alcohol and ascladiol. An unknown metabolite of m/z= 375.14202 with two sulphur atoms was detected at 22.02 min. This metabolite could correspond to an epipolythiodioxopiperazine (ETP) structure, a class of fungal SMs derived from diketopiperazines characterized by the presence of unique di- or polysulfide bridges. This is in accordance with the prediction of SM clusters based on bioinformatics analyses of *P. expansum* (Ballester et al. 2015). The heterologous *gli*C, *gli*G and *gli*T genes responsible for the transfer of sulphur atoms and the closure of the di-sulphide bridge during gliotoxin biosynthesis would indicate the presence of two

potential clusters in the genomes of *P. expansum*. No metabolite with a halogenated heteroatom was detected, as expected regarding what is known about the secondary metabolism of *P. expansum*. A blast search for a halogenase encoding gene was achieved on the different genomes of *P. expansum* available in GenBank, confirming the absence of a gene encoding a halogenase in *P. expansum*.

The WT and PeΔ*veA* produce 78 SMs in common, 17 compounds are produced only by the WT, while nine SMs are produced only by the null mutant PeΔ*veA* strain. The deletion of the *veA* gene in *P. expansum* completely abolished the production of patulin and citrinin in the mutant strain. Also not detected in the null mutant strain: *m*-cresol, *m*-hydroxybenzyl alcohol, gentisyl alcohol and ascladiol, all precursors of the patulin biosynthesis pathway. PeΔ*veA* is able of producing all communesin derivatives with the exception of the New DME2 communesin ($C_{28}H_{32}N_4O_2$, R_T= 14.97). Com540 was only detected in the null mutant strain, that is, the WT is unable to synthesize it. Expansolides A, B, C and D, clavicipitic acid, roquefortine C and D, and andrastins A, B, and C were detected in both strains. A reduction in the production of compounds derived from the cytochalasan alkaloid family was observed in the null mutant strain PeΔ*veA* producing mainly chaetoglobosin A and C and six of ten putative cytochalasan derivatives. Seven unknown compounds with molecular formula $C_{10}H_{17}NO_5$ (R_T= 2.42), $C_{17}H_{17}NO_7$ (R_T=11.75, 12.65 and 15.90), $C_{27}H_{30}N_4O$ (RT= 10.19) and $C_{30}H_{39}N_5O_5$ (R_T= 18.25 and 19.14) were only detected in the null mutant strain. While the unknown metabolite with formula $C_{15}H_{20}O_4$ (R_T= 16.91 and 19.35) is produced only by the WT strain.

Table 1. Comparison of secondary metabolites detected in WT NRRL 35695 and PeΔ*veA* strains after culture on labeled wheat grains.

Molecular formula	Identifier [a]	^{12}C *m/z* (Da)	Proposed identification	WT	PeΔ*veA*	Molecular formula	Identifier [a]	^{12}C *m/z* (Da)	Proposed identification	WT	PeΔ*veA*
$C_7H_6O_4$	**Pexp_153.019_3.59**	**153.01919**	**patulin**	+	ND	$C_{28}H_{31}N_5O_5$	Pexp_518.241_16.36	518.24108		+	+
C_7H_8O	Pexp_109.065_7.11	109.06509	m-Cresol	+	ND	$C_{28}H_{31}N_5O_5$	Pexp_518.240_17.21	518.24092		+	+
$C_7H_8O_2$	Pexp_125.060_6.79	125.05998	m-Hydroxybenzyl alcohol	+	ND	$C_{28}H_{32}N_4O$	Pexp_441.265_17.14	441.26589	Communesin F[d]	+	+
$C_7H_8O_3$	Pexp_141.054_3.8	141.05493	Gentisyl alcohol	+	ND	$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.27	457.26097	New DMV[1] communesin-1	+	+
$C_7H_8O_4$	Pexp_157.049_2.67	157.04990	Ascladiol	+	ND	$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.97	457.26095	New DME[2] communesin-1	+	ND
$C_7H_{10}O_3$	Pexp_143.070_4.52	143.07061		+	+	$C_{28}H_{32}N_4O_2$	Pexp_457.261_23.65	457.26116	Communesin A[f]	+	+
$C_{10}H_{17}NO_5$	Pexp_232.117_2.42	232.11784		ND	+	$C_{28}H_{32}N_4O_3$	Pexp_473.255_15.50	473.25566		+	+
$C_{10}H_{17}NO_5$	Pexp_232.118_8.83	232.11872		+	+	$C_{28}H_{36}N_4O_4$	Pexp_493.279_31.47	493.27971	Fungisporin A or cyclo(VFVF)	+	+
$C_{13}H_{14}O_5$	Pexp_251.091_21.70	251.09108	Citrinin	+	ND	$C_{28}H_{38}N_4O_5$	Pexp_511.290_18.01	511.29099	VAL-PHE-VAL-PHE	+	+
$C_{15}H_{18}N_2$	Pexp_227.155_6.44	227.15514	Aurantioclavine	+	+	$C_{28}H_{38}N_4O_6$	Pexp_527.287_12.46	527.28755	VAL-PHE-VAL-TYR	+	+
$C_{15}H_{19}NO_6$	Pexp_310.129_12.78	310.12939		+	+	**$C_{28}H_{38}O_7$**	**Pexp_485.254_35.32**	**485.25405**		+	+
$C_{15}H_{19}NO_6$	Pexp_310.129_14.80	310.12964		+	+	$C_{28}H_{38}O_7$	Pexp_487.270_36.21	487.27063	Andrastin A	+	+
$C_{15}H_{20}O_4$	Pexp_265.144_15.91	265.14412	Expansolide C/ D	+	+	**$C_{28}H_{38}O_8$**	**Pexp_501.247_27.70**	**501.24792**		+	+
$C_{15}H_{20}O_4$	Pexp_265.144_16.91	265.14410		+	ND	**$C_{28}H_{38}O_8$**	**Pexp_501.247_28.44**	**501.24792**		+	+
$C_{15}H_{20}O_4$	Pexp_265.144_18.49	265.14415	Expansolide C/ D	+	+	**$C_{28}H_{38}O_8$**	**Pexp_501.247_29.57**	**501.24786**		+	+
$C_{15}H_{20}O_4$	Pexp_265.144_19.35	265.14414		+	ND	**$C_{28}H_{40}O_6$**	**Pexp_471.274_39.84**	**471.27434**	**Andrastin C**	+	+
$C_{16}H_{18}N_2O_2$	Pexp_271.144_7.62	271.14496	Clavicipitic acid	+	+	**$C_{28}H_{40}O_7$**	**Pexp_487.268_30.58**	**487.26898**	**Andrastin B**	+	+
$C_{16}H_{18}N_2O_2$	Pexp_271.144_8.45	271.14467	Clavicipitic acid	+	+	$C_{29}H_{33}N_5O_6$	Pexp_548.251_15.42	548.25180		+	+
$C_{16}H_{26}N_2O_4S_2$	Pexp_375.142_22.02	375.14202		+	+	$C_{30}H_{36}N_4O_2$	Pexp_485.292_16.36	485.29245	New DMV[1] communesin-2	+	+
$C_{17}H_{17}NO_7$	Pexp_348.108_11.75	348.10762		ND	+	$C_{30}H_{39}N_5O_5$	Pexp_550.302_18.25	550.30203		ND	+
$C_{17}H_{17}NO_7$	Pexp_348.108_12.65	348.10718		ND	+	$C_{30}H_{39}N_5O_5$	Pexp_550.302_19.14	550.30207		ND	+
$C_{17}H_{17}NO_7$	Pexp_348.108_15.90	348.10696		ND	+	$C_{31}H_{36}N_4O_2$	Pexp_497.290_31.66	497.29061	Putative new undetermined communesin	+	+
$C_{17}H_{22}O_5$	Pexp_307.154_27.39	307.15471	Expansolide A/B	+	+	$C_{32}H_{34}N_4O_3$	Pexp_523.271_31.84	523.27152	Communesin D[g]	+	+
$C_{17}H_{22}O_5$	Pexp_307.155_30.19	307.15504	Expansolide A/B	+	+	$C_{32}H_{36}N_2O_4$	Pexp_513.276_39.10	513.27635	Chaetoglobosin J or Prochaetoglobosin III	+	+
$C_{18}H_{16}N_2O_2$	Pexp_293.129_13.58	293.12911		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_20.92	529.26776	Chaetoglobosin B/G	+	ND

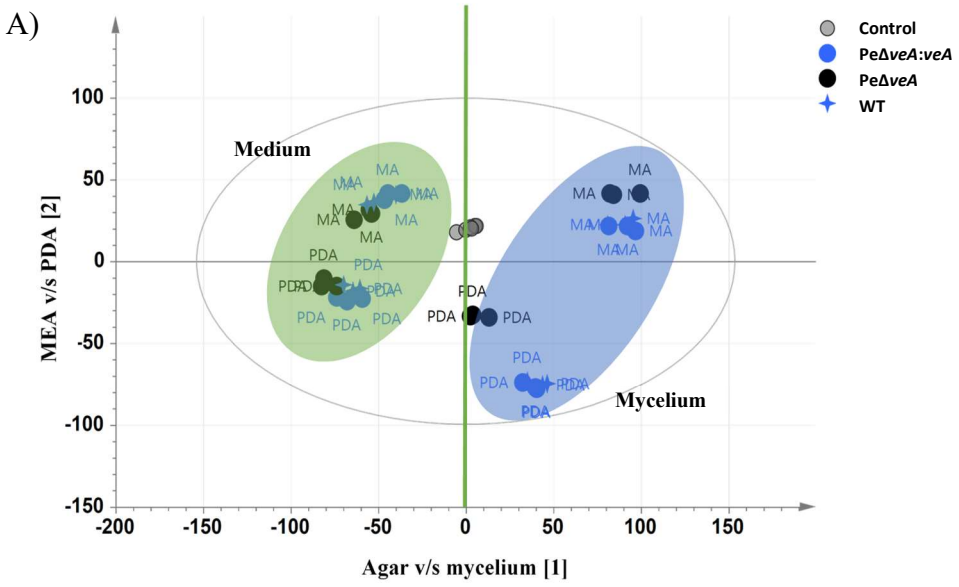
$C_{18}H_{16}N_2O_2$	Pexp_293.129_17.74	293.12915		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_25.21	529.27058	Chaetoglobosin B/G	+	ND
$C_{18}H_{16}N_2O_3$	Pexp_309.124_14.76	309.12421		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_26.71	529.27029	Chaetoglobosin B/G	+	+
$C_{18}H_{18}N_2O_2$	Pexp_295.144_14.61	295.14479		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_29.56	529.26757	Chaetoglobosin B/G	+	ND
$C_{18}H_{31}NO_7$	Pexp_374.216_23.72	374.21663		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_30.51	529.27077	Chaetoglobosin B/G	+	+
$C_{19}H_{16}N_2O_2$	Pexp_305.129_32.50	305.12911		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_33.98	529.27067	Chaetoglobosin A	+	+
$C_{19}H_{16}N_2O_2$	Pexp_305.129_33.17	305.12933		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_36.68	529.27063	Chaetoglobosin C	+	+
$C_{19}H_{16}N_2O_4$	Pexp_337.119_13.73	337.11909		+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_37.41	529.27056	Chaetoglobosin B/G	+	ND
$C_{19}H_{16}N_2O_4$	Pexp_337.119_15.20	337.11916		+	+	$C_{32}H_{36}N_2O_6$	Pexp_545.263_27.60	545.26332	Putative cytochalasan	+	ND
$C_{19}H_{21}NO_7$	Pexp_376.139_17.45	376.13901		+	+	$C_{32}H_{36}N_2O_6$	Pexp_545.262_30.57	545.26288	Putative cytochalasan	ND	+
$C_{19}H_{21}NO_7$	Pexp_376.139_18.65	376.13904		+	+	$C_{32}H_{36}N_2O_6$	Pexp_545.262_31.50	545.26281	Putative cytochalasan	+	ND
$C_{19}H_{38}O_6$	**Pexp_361.258_37.23**	**361.25871**		**+**	+	**$C_{32}H_{36}N_2O_6$**	**Pexp_543.248_34.05**	**543.24836**	Putative cytochalasin	+	ND
$C_{19}H_{38}O_6$	**Pexp_361.258_38.19**	**361.25819**		+	+	$C_{32}H_{36}N_4O_2$	Pexp_509.292_36.01	509.29257	Communesin B[g]	+	+
$C_{20}H_{18}N_2O_2$	Pexp_319.145_35.48	319.14588		+	+	$C_{32}H_{36}N_4O_4$	Pexp_541.280_07.78	541.28053	Com540[b]	ND	+
$C_{20}H_{18}N_2O_2$	Pexp_319.145_36.44	319.14502		+	+	$C_{32}H_{38}N_2O_6$	Pexp_515.288_35.59	515.28895	Putative cytochalasan	+	+
$C_{20}H_{21}NO_9$	Pexp_420.128_19.29	420.12857		+	+	$C_{32}H_{38}N_2O_6$	Pexp_515.288_37.93	515.28890	Putative cytochalasan	+	+
$C_{20}H_{26}O_8$	Pexp_395.171_13.61	395.17142		+	+	$C_{32}H_{38}N_2O_6$	Pexp_515.289_38.15	515.28909	Putative cytochalasan	+	+
$C_{22}H_{23}N_5O_2$	Pexp_390.193_15.09	390.19390	Roquefortine C	+	+	$C_{32}H_{38}N_2O_6$	Pexp_531.289_22.76	531.28990	Putative cytochalasan	+	ND
$C_{22}H_{25}N_5O_2$	Pexp_392.209_9.99	392.20913	Roquefortine D	+	+	$C_{32}H_{38}N_2O_6$	Pexp_531.283_27.38	531.28360	Putative cytochalasan	+	+
$C_{23}H_{24}N_2O_6$	Pexp_425.171_26.92	425.17179		+	+	$C_{32}H_{38}N_2O_6$	Pexp_531.283_28.52	531.28340	Putative cytochalasan	+	+
$C_{24}H_{26}N_2O_6$	**Pexp_437.170_33.79**	**437.17091**		+	+	$C_{32}H_{36}N_4O_3$	Pexp_525.287_13.45	525.28735	New DME[2] communesin-2	+	+
$C_{26}H_{30}N_4$	Pexp_399.255_19.94	399.25568	Communesin K[b]	+	+	$C_{32}H_{36}N_4O_3$	Pexp_525.287_27.91	525.28705	New DMV[1] communesin-3	+	+
$C_{26}H_{30}N_4O$	Pexp_415.250_14.62	415.25034	Communesin I[c]	+	+	$C_{32}H_{38}N_4O_2$	Pexp_511.306_36.13	511.30684	New DME[2] communesin-3	+	+
$C_{26}H_{30}N_4O$	Pexp_415.250_18.43	415.25033	Communesin I[c]	+	+	$C_{32}H_{38}N_4O_3$	Pexp_527.303_21.41	527.30304	New DME[2] communesin-4	+	+
$C_{26}H_{40}O_6$	Pexp_449.289_29.93	449.28939		+	+	$C_{32}H_{38}N_4O_4$	Pexp_543.297_19.36	543.29789	New DME[2] communesin-5	+	+
$C_{27}H_{30}N_4O$	Pexp_427.249_10.19	427.24923		ND	+	$C_{32}H_{40}N_4O_2$	Pexp_513.322_36.57	513.32227	New DME[2] communesin-6	+	+
$C_{27}H_{30}N_4O_2$	Pexp_443.245_15.03	443.24564	Communesin E[d]	+	+	$C_{33}H_{38}N_4O_5$	Pexp_571.293_18.22	571.29324	Com570[e]	+	+
$C_{28}H_{30}N_4O_3$	Pexp_471.239_19.56	471.23997	Com470[e]	+	+	$C_{37}H_{42}N_4O_5$	Pexp_623.325_29.52	623.32511	Com622[e]	+	+

In **bold** the compounds detected by negative electrospray ionization (ESI-).[a] Secondary metabolites identifier (Pexp_*m/z*_R_T). *m/z* = *m/z* ratio and R_T= retention time, [b] Communesin K (Lin et al. 2015), [c] Communesin I (Fan et al. 2015; Lin et al. 2015), [d] Communesin E and Communesin F (Hayashi et al. 2004), [e] Com470, Com570 and Com622 (Kerzaon et al. 2009), [f] Communesin A (Numata et al. 1993), [g] Communesin D and Communesin B (Jadulco et al. 2004). [1] Dimethylvinyl (DMV), [2]Dimethylepoxyde (DME). + = Detected. ND = Not detected.

3.2 Analysis of Secondary Metabolite Production in vitro

To stimulate the production of SMs, the strains were grown on two different media, MEA and PDA, which are known to be rich media for high SM production. The strains were grown in the dark or in the light as this environmental parameter could also influence the production of SMs and could reveal new compounds. Strains grown in the dark would assess SMs produced inside the apple (basal mycelium) and strains grown in the light would assess SMs produced outside the fruit (aerial mycelium).

Data generated from LC-MS spectra were processed using Principal Component Analysis (PCA) to find out discriminant factors. All factors induced significant differences for a large number of ions. Considering the number of significant ions, the most important effect was observed according to the location of SM production, i.e., in the agar medium (excreted SMs) versus in the fungal extract (internal SMs) (PCA axis 1; 4,194 significant ions) (Figure 1A). Effect of the substrate, MEA medium versus PDA medium, is the second effect observed (PCA axis 2; 3,798 significant ions) (Figure 1B). The strain effect is significant when the null mutant PeΔ*veA* strain is compared to the other two strains (WT and complemented strains) (PCA axis 3; 1,292 significant ions) (Figure 1C) regardless of the medium in which they grew. Finally, the effect of light versus dark was the least discriminating factor. For this study we used three strains; the WT, PeΔ*veA* and PeΔ*veA*:*veA* (complemented strains). The WT and complemented strains did not show significantly different ions under the different conditions studied. The differences observed between the WT or complemented strains and the null mutant strain were due to *veA* disruption.



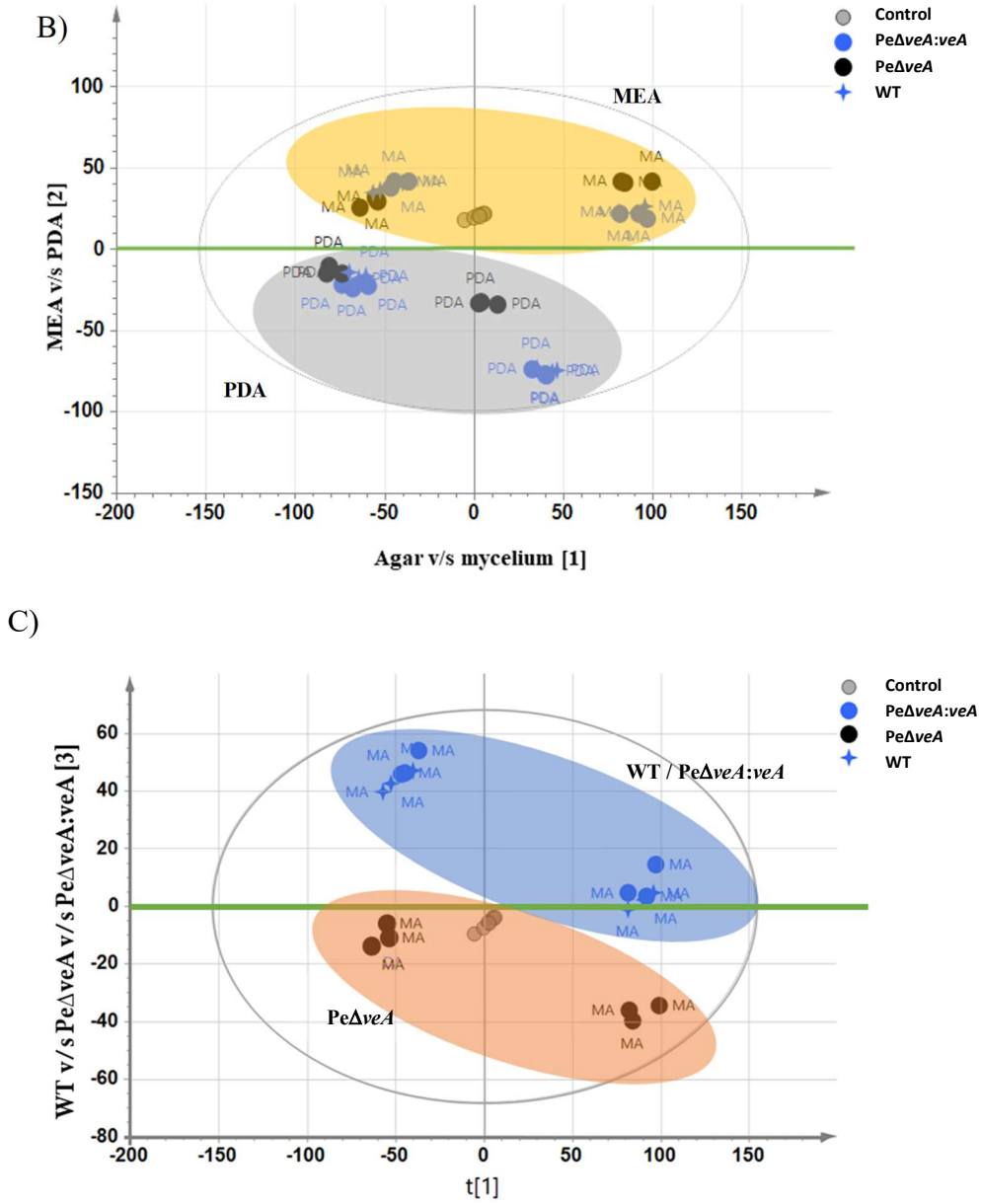


Figure 1. Principal Component Analysis (PCA) of the data obtained from the mass spectra. A) Effect of the location of SM production, agar medium versus fungal extract. B) Substrate effect, MEA medium versus PDA medium. C) Deletion of *veA* effect, PeΔ*veA* versus WT /PeΔ*veA*:*veA* (MEA medium). The WT results are represented by the blue star, the black dots represent the mutant strain PeΔ*veA*, the blue dots the complemented strain PeΔ*veA*:*veA*, while the grey dots represent the control. Experiments were performed in triplicate.

Once established the list of secondary compounds produced by the WT and the null mutant PeΔ*veA* strain, the subsequent objective of this work was to know the space-time organization of the secondary metabolism during the life cycle of the fungus. The analysis of the results obtained from the in vitro study in the different growth conditions previously detailed, allowed identifying the location of the SM of both strains (Table S2).

The Venn diagram (Figure 2a) shows that of the 58 SMs produced by the WT strain in MEA medium, 19 compounds are excreted into the medium, 36 compounds were detected in the fungal extract and only three unknown metabolites ($C_{18}H_{16}N_2O_2$, $C_{28}H_{38}O_7$, and $C_{32}H_{36}N_2O_4$) were identified in both the fungal extract and the invaded medium. The list of the SMs is detailed in Table 2. The null mutant PeΔ*veA* strain, produced 52 compounds (Table 2) in the MEA medium, of which 33 were detected in the agar medium, 18 metabolites were detected in the fungal extract and one unknown metabolite ($C_{32}H_{36}N_2O_4$) was identified in both the medium and fungal extract (Figure 2b).

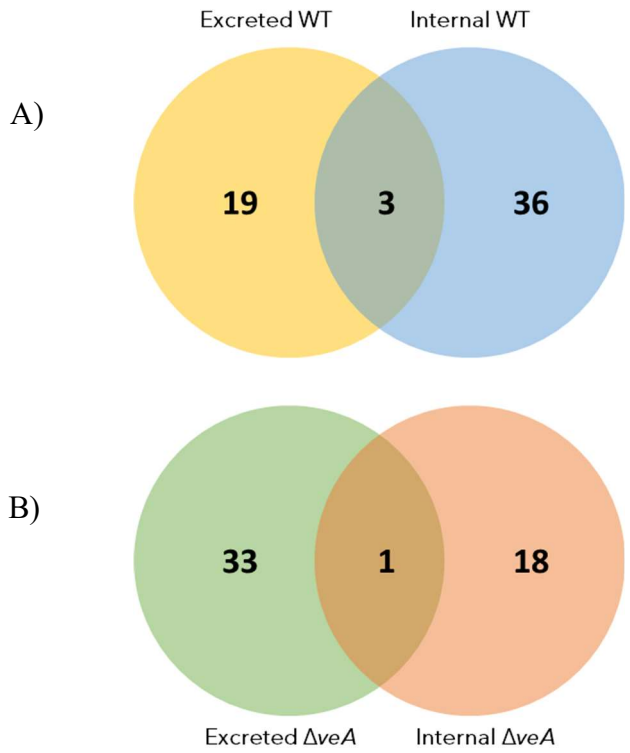


Figure 2. Venn diagram of the compounds detected in the WT and PeΔ*veA* strains. A) Metabolites detected in the medium (excreted) and in the fungal extract of WT (internal). B) Metabolites detected in the medium (excreted) and in the fungal extract of PeΔ*veA* (internal). The strains grew in MEA medium at 25°C in the dark for seven days.

If we compare the compounds detected in the fungal extract of the strains (Figure 3a), it was found that they have 19 compounds in common, among which were identified roquefortine C, communesins I, F, D, B, and com622, chaetoglobosin A and 12 unknown metabolites. Twenty compounds were detected only in the fungal extract of the WT, among which aurantioclavine, clavicipitic acid, roquefortine D, communesin I, K, and A, andrastin A and 13 unknown metabolites were identified.

Table 2. Localization of the secondary metabolites produced by the WT and PeΔ*veA* strains. The strains grew in MEA medium at 25°C in the dark for seven days

WT				PeΔ*veA*			
Excreted	**Internal**		**Both**	**Excreted**		**Internal**	**Both**
Ascladiol	Andrastin A	$C_{19}H_{38}O_6$	$C_{18}H_{16}N_2O_2$	Andrastin A	$C_{18}H_{16}N_2O_2$	Chaetoglobosin A	$C_{32}H_{36}N_2O_4$
Andrastin B	Aurantioclavine	$C_{19}H_{16}N_2O_2$	$C_{28}H_{38}O_7$	Andrastin B	$C_{18}H_{16}N_2O_2$	Communesin B	
Clavicipitic Acid	Chaetoglobosin A	$C_{19}H_{16}N_2O_2$	$C_{32}H_{36}N_2O_4$	Ascladiol	$C_{18}H_{18}N_2O_2$	Communesin D	
Expansolides A/B	Clavicipitic Acid	$C_{19}H_{16}N_2O_4$		Aurantioclavine	$C_{18}H_{31}NO_7$	Communesin F	
Expansolides C/D	Communesin A	$C_{19}H_{16}N_2O_4$		Clavicipitic Acid	$C_{19}H_{16}N_2O_4$	Communesin I	
Patulin	Communesin B	$C_{20}H_{18}N_2O_2$		Clavicipitic Acid	$C_{19}H_{16}N_2O_4$	Com496-1	
$C_{10}H_{17}NO_5$	Communesin D	$C_{20}H_{18}N_2O_2$		Communesin A	$C_{19}H_{21}NO_7$	Roquefortine C	
$C_{15}H_{19}NO_6$	Communesin F	$C_{24}H_{26}N_2O_6$		Communesin I	$C_{23}H_{24}N_2O_6$	$C_{19}H_{38}O_6$	
$C_{18}H_{18}N_2O_2$	Communesin I	$C_{28}H_{30}N_4O$		Communesin K	$C_{24}H_{40}O_6$	$C_{19}H_{38}O_6$	
$C_{18}H_{31}NO_7$	Communesin I	$C_{28}H_{32}N_4O_2$		Expansolides A/B	$C_{28}H_{32}N_4O_2$	$C_{20}H_{18}N_2O_2$	
$C_{19}H_{21}NO_7$	Communesin K	$C_{28}H_{32}N_4O_2$		Expansolides C/D	$C_{28}H_{32}N_4O_2$	$C_{20}H_{18}N_2O_2$	
$C_{19}H_{21}NO_7$	Com622	$C_{28}H_{32}N_4O_3$		Roquefortine D	$C_{28}H_{38}O_7$	$C_{24}H_{26}N_2O_6$	
$C_{20}H_{26}O_8$	Roquefortine C	$C_{32}H_{36}N_2O_5$		$C_{10}H_{17}NO_5$	$C_{32}H_{36}N_2O_5$	$C_{28}H_{30}N_4O$	
$C_{23}H_{24}N_2O_6$	Roquefortine D	$C_{32}H_{36}N_4O_3$		$C_{15}H_{19}NO_6$	$C_{32}H_{36}N_2O_5$	$C_{28}H_{32}N_4O_3$	
$C_{24}H_{40}O_6$	$C_{15}H_{19}NO_6$	$C_{32}H_{38}N_4O_3$		$C_{15}H_{19}NO_6$		$C_{32}H_{36}N_2O_5$	
$C_{29}H_{27}N_5O_5$	$C_{16}H_{26}N_2O_4S_2$	$C_{32}H_{40}N_4O_2$		$C_{16}H_{26}N_2O_4S_2$		$C_{32}H_{36}N_4O_3$	
$C_{29}H_{27}N_5O_5$	$C_{18}H_{16}N_2O_2$	$C_{37}H_{42}N_4O_5$		$C_{17}H_{17}NO_7$		$C_{32}H_{38}N_4O_3$	
$C_{32}H_{36}N_2O_5$	$C_{18}H_{16}N_2O_3$			$C_{17}H_{17}NO_7$		$C_{32}H_{40}N_4O_2$	
$C_{32}H_{36}N_2O_5$	$C_{19}H_{38}O_6$			$C_{17}H_{17}NO_7$			

When comparing the metabolites detected in the medium for the two strains (Figure 3b), we found that there are 17 metabolites in common that are excreted. Contrary to observations in the fungal extract, the mutant PeΔ*veA* strain excreted more metabolites to the medium than the WT strain, 17 compounds including aurantioclavine, clavicipitic acid, roquefortine D, communesin I, K, and A, andrastin A, which are identified mainly in the fungal extract of the WT strain, were identified. Since deletion of the *veA* gene in *P. expansum* results in the abolition of patulin production, five compounds including patulin and four unknown metabolites are only excreted by the WT.

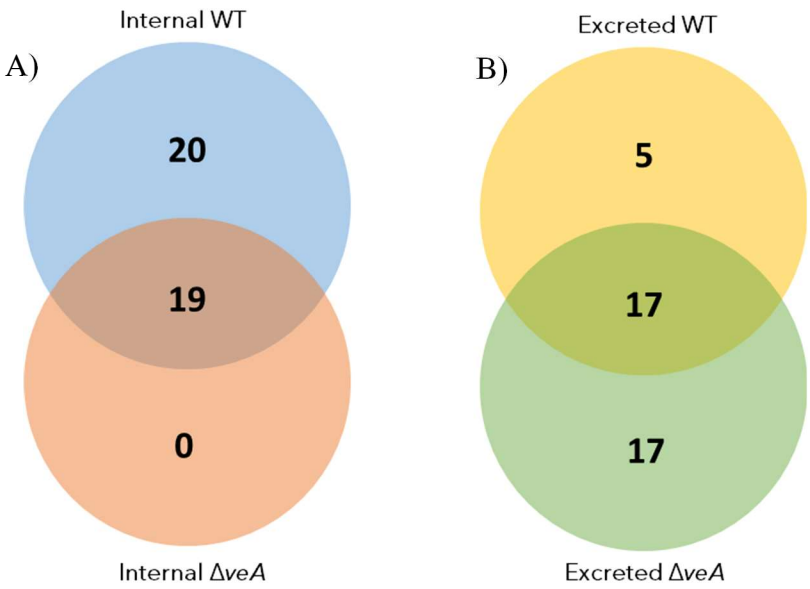


Figure 3. Venn diagram. Comparison between the metabolites produced by the WT and PeΔ*veA* strains. A) Compounds detected in the fungal extract and B) Compounds excreted into the medium. The strains grew in MEA medium at 25°C in the dark for seven days.

Patulin was also detected in the fungal extract, but at concentrations 17 times lower than in the medium (Figure 4). Ascladiol was detected in small amounts in the fungal extract of the WT strain but was mainly found in the agar medium. Roquefortine C, and communesins A and B were produced by both strains, however larger amounts were detected in the fungal extract of the WT strain (Figure 4). The production of expansolides, which were detected mainly in the agar medium, was favored for the null mutant PeΔ*veA* strain. Other natural products were identified according to their molecular formula, *m*/*z* ratio and retention time (R_T). The metabolite with an *m*/*z*= 348.10762 and molecular formula $C_{17}H_{17}NO_7$ (R_T=11.75, 12.65 and 15.90) was only produced by the null mutant PeΔ*veA* strain, mainly in the medium, even if a small amount has been detected in the fungus (Figure 4), this metabolite is of interest because it is the one most produced by the null mutant PeΔ*veA* strain. The metabolites with an *m*/*z*= 374.21663 ($C_{18}H_{31}NO_7$) and *m*/*z*= 295.14479 ($C_{18}H_{18}N_2O_2$) were mainly excreted in the agar medium by both the WT and null mutant PeΔ*veA* strains (Figure S2). The metabolite with *m*/*z*= 375.14202 ($C_{16}H_{26}N_2O_4S_2$) is produced by both strains, but mainly in the WT strain fungal extract. The compound with m/z= 293.12915 ($C_{18}H_{16}N_2O_2$) was detected in the WT strain, equally in the fungal extract and in the agar medium, while the production of this compound was strongly decreased both in the medium and in the fungal extract of the null mutant strain. (Figure S2). Production of the metabolite with *m*/*z*= 425.17179 ($C_{23}H_{24}N_2O_6$) is significantly detected in the agar medium of the PeΔ*veA* strain

(Figure S2). The production of certain metabolites, such as aurantioclavine and andrastin A (Figure S2) showed no significant differences under the conditions studied, they are detected both in the fungal extract and agar medium and they are produced by both strains.

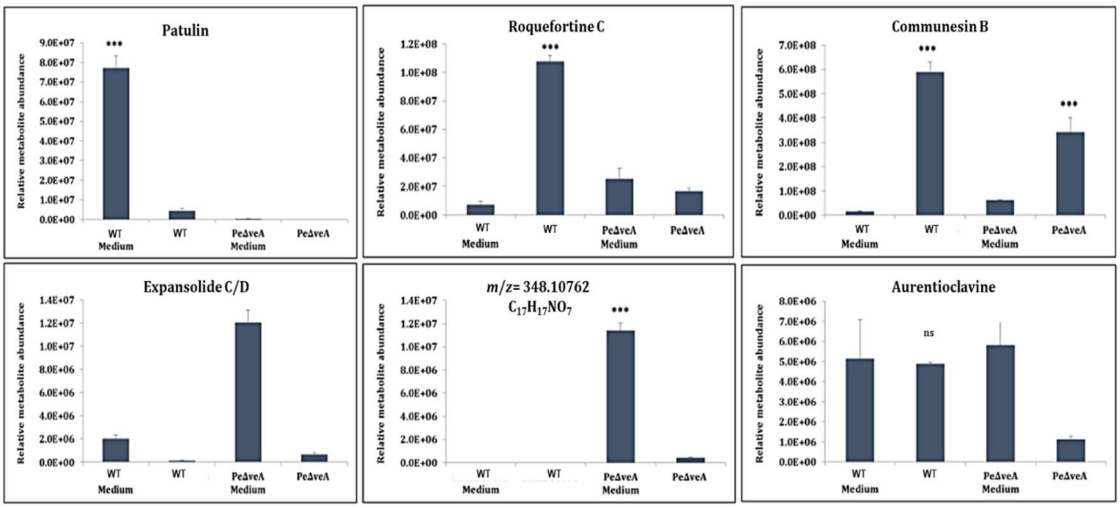


Figure 4. Relative metabolite abundance of the data obtained from the mass spectra. The graphs show the relative abundance of metabolites produced by the WT and the null mutant PeΔ*veA* strains. Cultures were grown for five days in the dark at 25 °C. Graphs show the mean ± standard error of the mean (SEM) of three replicates and the significant differences between WT and null mutant PeΔ*veA* strains, in the medium or in the fungus. *p*-value * < 0.05; ** < 0.01; *** < 0.001, ns= no significant changes.

Light had a positive effect on the production of some metabolites such as roquefortine C (ROQC) (Figure 5a), communesins (Com) A (Figure 5b), F, K, andrastin B (Figure 5c) and clavicipitic acid for both strains in MEA. In the dark, the production of these SMs decreased significantly compared to light, mainly when the metabolites were detected in the fungal extract. For ROQC (Figure S3a) and clavicipitic acid (Figure S3b), the effect of light was significant regardless of the study conditions, there was an increase of the two metabolites in the presence of light in the different substrates (MEA/PDA), strains (WT/PeΔ*veA*) and location (fungal extract/agar medium) used. For COM F, the effect of light was significant on the fungal extract of the WT strain, but only when grown in PDA medium, while for the null mutant PeΔ*veA* strain, it was significant when grown in MEA medium (Figure S4). Conversely, COM526 was detected largely in the dark, especially in the fungal extract and the agar medium of the null mutant PeΔ*veA* strain grown on MEA medium and in the fungal extract of both strains on PDA medium (Figure S5).

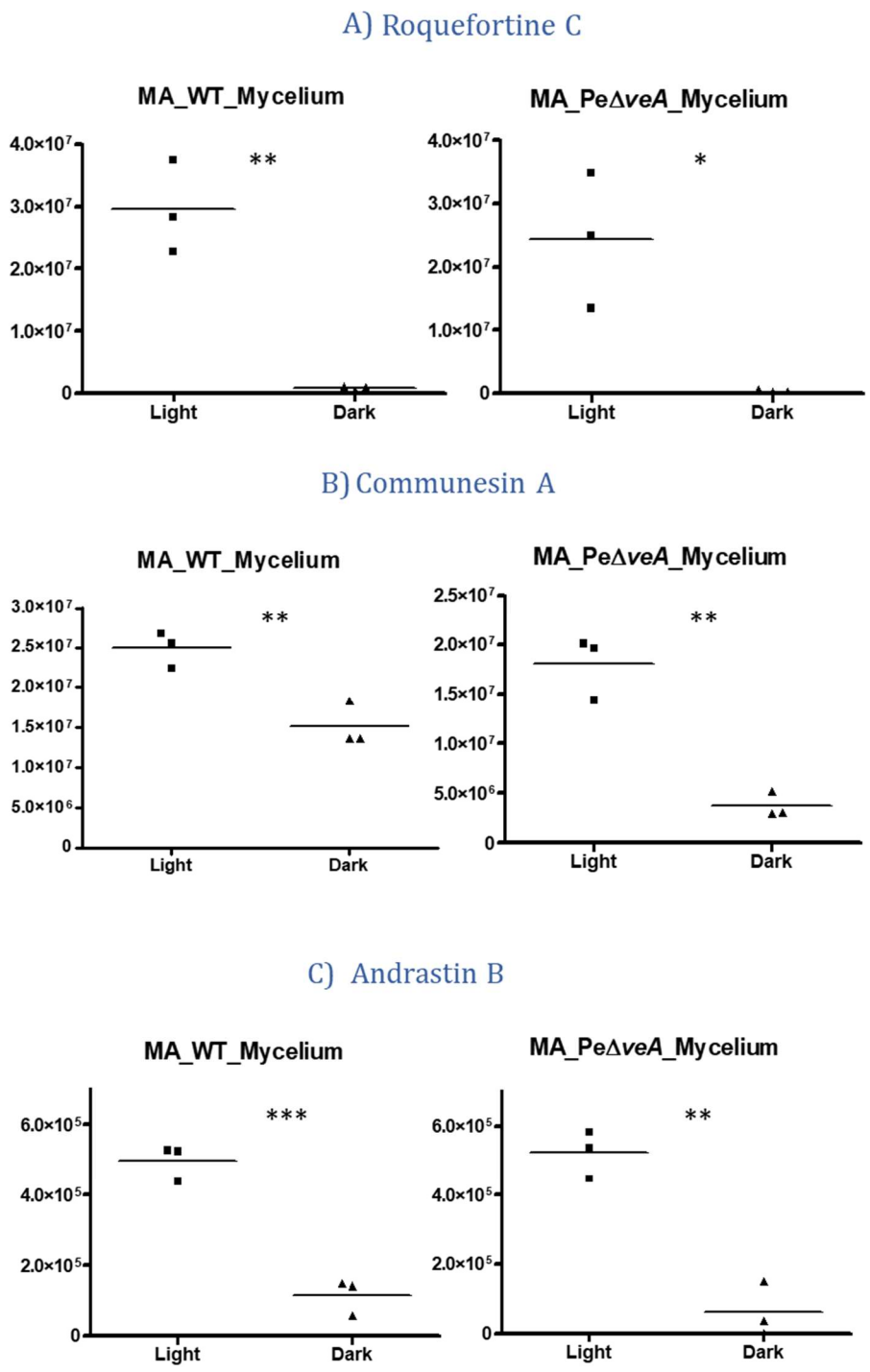


Figure 5. Effect of light**.** The graphs show the comparison of the relative abundance of the metabolites produced under light or dark conditions by the WT and the null mutant PeΔ*veA* strains. A) Roquefortine C, B) Communesin A, C) Andrastin B. Cultures were grown on MEA medium for five days at 25 °C. Graphs show the mean ± standard error of the mean (SEM) of three replicates. *p*-value * < 0.05; ** < 0.01; *** < 0.001, ns= no significant changes.

3.3 Production of Secondary Metabolites in vivo

After discriminating SMs produced in the agar medium from those produced in the fungal extract in vitro on MEA and PDA, the production of SMs was analyzed in vivo in Golden Delicious apples. HPLC results showed that WT-infected apples contained not only patulin but also intermediate metabolites of the patulin biosynthesis pathway including gentisyl alcohol, E-ascladiol, and *m*-hydroxybenzyl alcohol, as well as desoxypatulinic acid (DPA), a patulin derivative (Figure 6).

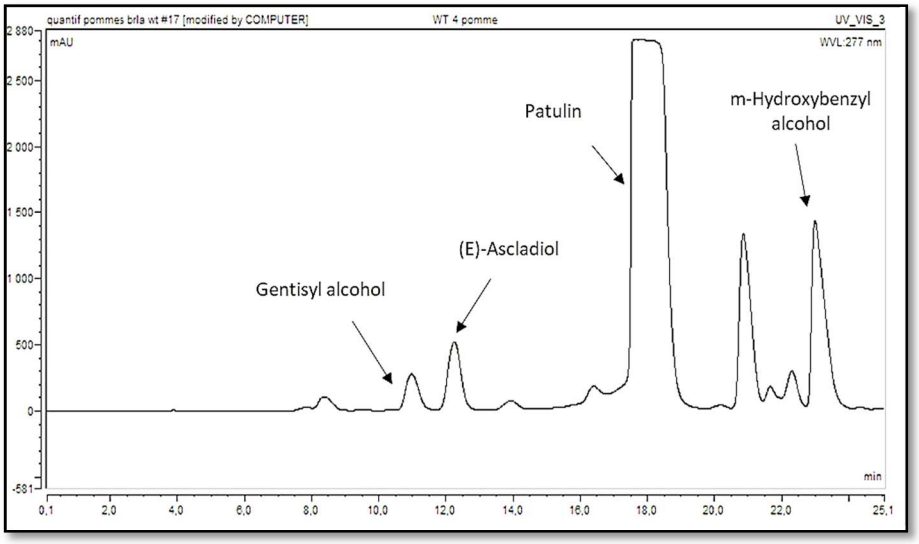


Figure 6. HPLC chromatogram of Golden Delicious apples infected by the *Penicillium expansum* wild type strain.

SMs were analyzed by LC-HRMS and compared with the reference metabolome of *P. expansum* obtained from the labelled wheat culture (Table 1). Table 3 shows the SMs detected in apples in the WT and null mutant PeΔ*veA* strains. Twelve SMs were detected in the null mutant PeΔ*veA* strain, 13 SMs in the WT at 14 days post-inoculation (dpi) and 29 SMs at 30 dpi.

Patulin or intermediates of patulin biosynthesis were no longer produced by the null mutant PeΔ*veA* strain. Communesins A and B, and andrastins A and B were secreted by both the WT and PeΔ*veA* strains after 14 dpi. Eight communesins were found in apples treated with WT and PeΔ*veA* strains, but only comD was found in apples treated with the null mutant strain. The SM with m/z= 449.28939 was only present at 14 dpi in WT and PeΔ*veA* treated apples. The molecule with m/z= 425.17179, also detected in the in vitro study and assigned to the chemical formula $C_{23}H_{24}N_2O_6$, was detected only in apples infected with the null mutant PeΔ*veA* strain and not in WT-infected apples after 14 dpi. In the WT strain, this compound was detected only at 30 dpi suggesting an early production in the null mutant PeΔ*veA*. At 30 dpi, citrinin, roquefortine C, communesin F, and chaetoglobosin B/G were detected in apples infected with the WT strain.

Table 3. Secondary metabolites detected in Golden Delicious apples infected with the WT and null mutant PeΔ*veA* strains

Molecular formula	R_T (min) [a]	^{12}C *m/z* (Da)	Proposed identification	14 dpi PeΔ*veA*	14 dpi WT	30 dpi WT	Molecular formula	R_T (min) [a]	^{12}C *m/z* (Da)	Proposed identification	14 dpi PeΔ*veA*	14 dpi WT	30 dpi WT
$C_7H_6O_4$	**3.59**	**153.01919**	**Patulin**	ND	+	+	$C_{26}H_{40}O_6$	29.93	449.28939		+	+	ND
$C_7H_8O_2$	6.79	125.05998	m-Hydroxybenzyl alcohol	ND	+	-	$C_{28}H_{32}N_4O$	17.14	441.26589	Communesin F	ND	ND	+
$C_7H_8O_4$	2.67	157.04990	Ascladiol	ND	+	+	$C_{28}H_{32}N_4O_2$	14.27	457.26097	New DMV[1] communesin-1	+	+	+
$C_{10}H_{17}NO_5$	8.83	232.11872		ND	ND	+	$C_{28}H_{32}N_4O_2$	23.65	457.26116	Communesin A	+	+	+
$C_{13}H_{14}O_5$	21.70	251.09108	Citrinin	ND	ND	+	**$C_{28}H_{38}O_7$**	**35.32**	**485.25405**		+	+	+
$C_{16}H_{26}N_2O_4S_2$	22.02	375.14202		ND	ND	+	$C_{28}H_{38}O_7$	36.21	487.27063	Andrastin A	+	+	+
$C_{17}H_{22}O_5$	27.39	307.15471	Expansolide A/B	ND	ND	+	**$C_{28}H_{38}O_8$**	**28.44**	**501.24792**		ND	ND	+
$C_{17}H_{22}O_5$	30.19	307.15504	Expansolide A/B	ND	ND	+	**$C_{28}H_{38}O_8$**	**29.57**	**501.24786**		+	+	+
$C_{18}H_{16}N_2O_2$	17.74	293.12915		ND	ND	+	**$C_{28}H_{40}O_7$**	**30.58**	**487.26898**	**Andrastin B**	+	+	+
$C_{18}H_{18}N_2O_2$	14.61	295.14479		+	+	+	$C_{32}H_{34}N_4O_3$	31.84	523.27152	Communesin D	+	ND	ND
$C_{19}H_{16}N_2O_2$	33.17	305.12933		ND	ND	+	$C_{32}H_{36}N_2O_5$	26.71	529.27029	Chaetoglobosin B/G	ND	ND	+
$C_{19}H_{38}O_6$	**38.19**	**361.25819**		ND	ND	+	$C_{32}H_{36}N_2O_5$	29.54	529.26898	Chaetoglobosin B/G	ND	ND	+
$C_{20}H_{18}N_2O_2$	36.44	319.14502		ND	ND	+	$C_{32}H_{36}N_2O_5$	30.13	529.27539	Chaetoglobosin B/G	ND	+	+
$C_{22}H_{23}N_5O_2$	15.09	390.19390	Roquefortine C	ND	ND	+	$C_{32}H_{36}N_2O_5$	35.67	529.26822	Chaetoglobosin B/G	ND	ND	+
$C_{23}H_{24}N_2O_6$	26.92	425.17179		+	ND	+	$C_{32}H_{36}N_4O_2$	36.01	509.29257	Communesin B	+	+	+
$C_{24}H_{26}N_2O_6$	**33.79**	**437.17091**		ND	ND	+	$C_{32}H_{38}N_4O_3$	21.41	527.30304	New DME[2] communesin-4	+	ND	+

In **bold** the compounds detected by negative electrospray ionization (ESI-). dpi = days post inoculation. [a] R_T= retention time. + = Detected. ND = Not detected.

After detecting the SMs produced in infected apples, the spores were analyzed. SMs present in the spores (Table 4) were compared to the molecules secreted by *P. expansum* on labeled wheat (Table 1). Forty SMs were identified in the spores including various known compounds such as communesins (12 derivatives), clavicipitic acid, expansolides A and B, andrastins A, B and C, chaetoglobosins A, B/G and J, and roquefortines C and D. Fifteen unknown metabolites, for which a molecular formula was assigned, $C_{16}H_{26}N_2O_4S_2$ (R_T= 22.02), $C_{18}H_{16}N_2O_2$ (R_T= 13.58 and 17.54), $C_{18}H_{18}N_2O_2$ (R_T= 14.61), $C_{19}H_{16}N_2O_2$ (R_T= 32.50 and 33.17), $C_{19}H_{38}O_6$ (R_T= 37.17 and 38.19), $C_{20}H_{18}N_2O_2$ (R_T= 35.48 and 36.44), $C_{23}H_{24}N_2O_6$ (R_T= 26.92), $C_{24}H_{26}N_2O_6$ (R_T= 33.79), $C_{28}H_{38}O_7$ (R_T= 35.32), and $C_{28}H_{38}O_8$ (R_T= 28.44 and 29.57), were found. No patulin was found in the spores of apples infected with the WT strain.

The analysis of spores in the null mutant PeΔ*veA* strain could not be performed, since the formation of coremia was missing in the null PeΔ*veA* mutant strain grown in vivo and in vitro. Therefore, it was unable to pierce the apple peel and emerge from the fruits to complete the conidiation cycle.

Table 4. Secondary metabolites detected in spores collected from Golden Delicious apples infected with the *Penicillium expansum* wild type strain

Molecular formula	Identifier [a]	^{12}C *m/z* (Da)	Proposed identification	Molecular formula	Identifier [a]	C *m/z* (Da)	Proposed identification
$C_{16}H_{18}N_2O_2$	Pexp_271.154_7.62	271.14496	Clavicipitic acid	$C_{26}H_{30}N_4O$	Pexp_415.250_18.43	415.25033	Communesin I
$C_{16}H_{26}N_2O_4S_2$	Pexp_375.142_22.02	375.14202		$C_{28}H_{30}N_4O_3$	Pexp_471.239_19.56	471.23997	Com470
$C_{17}H_{22}O_5$	Pexp_307.154_27.39	307.15471	Expansolide A/B	$C_{28}H_{32}N_4O$	Pexp_441.265_17.14	441.26589	Communesin F
$C_{17}H_{22}O_5$	Pexp_307.155_30.19	307.15504	Expansolide A/B	$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.27	457.26097	New DMV communesin-1
$C_{18}H_{16}N_2O_2$	Pexp_293.129_13.58	293.12911		$C_{28}H_{32}N_4O_2$	Pexp_457.261_23.65	457.26116	Communesin A
$C_{18}H_{16}N_2O_2$	Pexp_293.129_17.74	293.12915		**$C_{28}H_{38}O_7$**	**Pexp_485.254_35.32**	**485.25405**	
$C_{18}H_{18}N_2O_2$	Pexp_295.144_14.61	295.14479		$C_{28}H_{38}O_7$	Pexp_487.270_36.21	487.27063	Andrastin A
$C_{19}H_{16}N_2O_2$	Pexp_305.129_32.50	305.12911		**$C_{28}H_{38}O_8$**	**Pexp_501.247_28.44**	**501.24792**	
$C_{19}H_{16}N_2O_2$	Pexp_305.129_33.17	305.12933		**$C_{28}H_{38}O_8$**	**Pexp_501.247_29.57**	**501.24786**	
$C_{19}H_{38}O_6$	**Pexp_361.258_37.17**	**361.25871**		**$C_{28}H_{40}O_6$**	**Pexp_471.274_39.84**	**471.27434**	**Andrastin C**
$C_{19}H_{38}O_6$	**Pexp_361.258_38.19**	**361.25819**		**$C_{28}H_{40}O_7$**	**Pexp_487.268_30.58**	**487.26898**	**Andrastin B**
$C_{20}H_{18}N_2O_2$	Pexp_319.145_35.48	319.14588		$C_{32}H_{34}N_4O_3$	Pexp_523.271_31.84	523.27152	Communesin D
$C_{20}H_{18}N_2O_2$	Pexp_319.145_36.44	319.14502		$C_{32}H_{36}N_2O_4$	Pexp_513.276_39.10	513.27635	Chaetoglobosin J or Prochaetoglobsin III
$C_{22}H_{23}N_5O_2$	Pexp_390.193_15.09	390.19390	Roquefortine C	$C_{32}H_{36}N_2O_5$	Pexp_529.270_26.79	529.27029	Chaetoglobosin B/G
$C_{22}H_{25}N_5O_2$	Pexp_392.209_9.99	392.20913	Roquefortine D	$C_{32}H_{36}N_2O_5$	Pexp_529.270_30.51	529.27077	Chaetoglobosin B/G
$C_{23}H_{24}N_2O_6$	Pexp_425.171_26.92	425.17179		$C_{32}H_{36}N_2O_5$	Pexp_529.270_33.98	529.27067	Chaetoglobosin A
$C_{24}H_{26}N_2O_6$	**Pexp_437.170_33.79**	**437.17091**		$C_{32}H_{36}N_2O_5$	Pexp_529.270_36.74	529.27063	Chaetoglobosin C
$C_{26}H_{30}N_4$	Pexp_399.255_19.94	399.25568	Communesin K	$C_{32}H_{36}N_4O_2$	Pexp_509.292_36.01	509.29257	Communesin B
$C_{26}H_{30}N_4O$	Pexp_415.250_14.62	415.25034	Communesin I	$C_{32}H_{38}N_4O_3$	Pexp_527.303_21.41	527.30304	New DME communesin-4
$C_{26}H_{30}N_4O$	Pexp_415.250_18.43	415.25033	Communesin I	$C_{32}H_{40}N_4O_2$	Pexp_513.322_36.72	513.32227	New DME communesin-6

In **bold** the compounds detected by negative electrospray ionization (ESI-). [a] Secondary metabolites identifier (Pexp_*m/z*_R_T). *m/z* = *m/z* ratio and R_T= retention time.

4. Discussion

Filamentous fungi produce a diverse array of SMs and although the metabolites are not critical for growth and reproduction, they may have a bioactive role for the organism that synthesizes them (Keller et al. 2005). They can increase the organism's ability to survive in a hostile environment by adapting them to extracellular conditions, ensuring defense, competition and interspecies interactions, and providing protection against damage caused by ultraviolet light or other abiotic stress (Keller, 2019). Fungal metabolites have beneficial activities and are used in the pharmaceutical or food industry, but also toxic activities that harm humanity by being involved in disease interactions with plants, animals, or humans (Bills and Gloer, 2016; Macheleidt et al. 2016).

Several *Penicillium* species are closely related phylogenetically but differ in the profiles of their SMs although only a few genomes have been sequenced, eight to date (Ballester et al. 2015; Li et al. 2015; Wu et al. 2019; Yang et al. 2014; Yin et al. 2017). In Ascomycetes, the secondary metabolism is very specific unlike the primary metabolism that is widely distributed within taxa (Rokas et al. 2018). In other words, secondary metabolites are often found in a single species or a few associated species within a clade. A previous metabolomic analysis showed that only 20% of the metabolites produced by *Pencilllium nordicum* and *Penicillium verrucosum* were shared by both species (Hautbergue et al. 2019). Penicillium expansum belongs to the *Penicillium* section with two other fruit pathogens, *P. digitatum* and *P. expansum*, as the closest relatives of *P. expansum* (Houbraken et al, 2016). To date, no secondary metabolites common to all three species have been found. Our results showed that *P. expansum* NRRL 35695 strain was able to produce, besides patulin, a huge variety of active SMs. One hundred four SMs were detected in *P. expansum* WT and the null mutant PeΔ*veA* strain when they were grown on dead vegetal biomass (wheat grains), of which 62 have been identified and 42 remain unknown. We detected citrinin, ascladiol, expansolides A, B, C and D, roquefortine C and D, fungisporin A, in addition to andrastins A, B and C. Fungisporin A and andrastins are produced by a multitude of *Penicillium* species, while expansolides are considered specific to the *Penicillium* section (Nielsen et al. 2005; Grijseels et al, 2017). So far, only *P. expansum* and *P. marinum*, a *P. expansum* marine form raised to the status of species recently, have been identified as expansolide-producing species (Houbraken et al. 2016; Frisvad et al. 2014). Among the numerous SMs produced by this species, 22 derivatives of communesins, a class of cytotoxic and insecticidal indole alkaloids, were also identified, among which a new putative communesin was found $C_{31}H_{36}N_4O_2$ (m/z= 497.29061, R_T=331.66). These results are consistent with those of Kerzaon et al. (2009) who identified, using the strategy of electrospray ionization coupled with tandem mass spectrometry (ESI-MS/MS) and high-resolution mass spectrometry (HRMS), five known communesins (A, B, D, E, and F) and elucidated the structure of seven new communesins (Com470, Com570, Com524, Com622, Com512,

Com644, and Com522) in a marine strain of *P. expansum*. Among these new communesins, three (Com470, Com570 and Com622) were identified in our study. The communesins are specific to the *Penicillium* section with only for producing species, *P. expansum* and *P. marinum* (Houbraken et al. 2016; Frisvad et al. 2014). The communesins biosynthesis pathway has been partially elucidated and the BGC has been identified (Li et al. 2015; Li et al. 2016). It turns out that the number of molecules identified as communesins is much higher than the number of potential intermediates. The communesin cluster contains 16 genes coding for 13 enzymes, 2 transporters and a transcription factor. Thus, if the biosynthesis pathway is linear, the number of molecules should not exceed 13 or 14. The large number of molecules suggests that the biosynthesis pathway may be grid type. In this type of pathway, some enzymes are not 100% substrate specific and take in charge other molecules that are often the substrates of other enzymes involved either upstream or downstream of the biosynthesis pathway. Similarly, the other possibility to explain this large number would be the existence of crosstalk between the communesins biosynthesis pathway and other biosynthesis pathways (Tsunematsu et al. 2013). Andersen et al. (2004) conducted a study on 260 isolates of *P. expansum* from different substrates and geographical origins. They reported that *P. expansum* is a consistent producer of chaetoglobosins, with chaetoglobosin A detected in 100% of isolates. Our results are in agreement with theirs, as we showed that *P. expansum* is a great producer of the cytochalasan family compounds, including besides chaetoglobosin A and C, chaetoglobosin E, penochalasin, chaetoglobosin J (or prochaetoglobosin III), six chaetoglobosin B/G derivatives and ten putative cytochalasins.

Although several unknown metabolites were found in *P. expansum* extracts, the compounds aflatrem (Russell et al. 1989), agonodepside B (Kim et al. 2016), brevianamide A (Bridge et al. 1989; Russell et al.1989), cyclopiazonic acid (Bridge et al. 1989), griseofulvin (Bridge et al. 1989), meleagrin (Kozlovskii et al. 2002), ochratoxin A (Paterson et al. 1987), ochrephilone (kim et al. 2016), penicillic acid (Leistner and pitt 1997), penitrem A (Bridge et al. 1989), rotiorin (kim et al. 2016), rubratoxin B (Paterson et al. 1987), rugulosuvine B (Kozlovsky et al. 2002), verrucosidin (kim et al., 2016) and mycophenolic acid (Bridge et al. 1989) described as produced by *P. expansum* in previous works, sometimes due to misidentification of the species, were not detected in our study.

Most of these works dates back to before the democratization of molecular biology and its use to identify with certainty the species studied. It is also frequent that some publications dating from this period and reporting the production of a secondary metabolite are based on a misidentification. So far, clusters of genes involved in the biosynthetic pathways of aflatrem (Zhang et al. 2004), cyclopiazonic acid (Chang et al. 2009), penitrem A (Nicholson et al 2015) and mycophenolic acid (Regueira et al. 2011) have been characterized. As previously mentioned, no gene coding for a halogenase was detected by Blast in the eight *P. expansum* genomes available in

GenBank. Griseofulvin and ochratoxin A contain a chlorine atom. Therefore, it is impossible for *P. expansum* to produce either of these two molecules. The biosynthetic gene clusters involved in their biosynthesis were unveiled (Chooi et al. 2010; Ferrara et al. 2016; Gallo et al. 2012; Gallo et al. 2014). The search of backbone genes located in all above quoted biosynthetic gene clusters showed the absence of homologous genes in *P. expansum* genomes, confirming the inability of *P. expansum* to produce these secondary metabolites.

Meleagrin shares a part of its biosynthetic pathway with roquefortine C. Roquefortine C is an intermediate of the meleagrin biosynthesis pathway. RoqN has been identified as responsible to the O-methylation of glandicoline B to yield meleagrin (Ali et al. 2013; Newmister et al. 2018). A blastP search of RoqN showed that no gene encoding a RoqN homologue is present in *P. expansum* genomes.

The discrimination between metabolites excreted by the basal mycelia in the environment and those remaining on the superior part of mycelium of the fungus was also investigated. In the in vitro study, metabolites excreted in the agar come from the basal mycelia metabolism and reflect what happens in the apple following infection by the fungus. Conversely, what remains in the mycelium represents aerial metabolism, and corresponds to what the fungus produces when it starts the conidiogenesis stage to exit the apple. Interestingly, 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 SMs produced by the fungus are secreted once the fungus has completed its initial growth phase. Among the metabolites detected in the medium (those excreted by the fungus), are patulin, citrinin and ascladiol, while communesins A and B, roquefortine C and chaetoglobosin A were detected mainly in the fungal extract.

The global regulation factor VeA has been described previously as a positive or negative regulator of SM biosynthesis in *Aspergillus nidulans* (Bayram and Braus, 2012; Rauscher et al. 2016), *Aspergillus flavus* (Cary et al. 2015) and *P. expansum* (EL Hajj Assaf et al. 2018). The elimination of the *veA* gene in *P*. *expansum* caused the abolition of patulin and citrinin production in the mutant strain. This result is consistent with that reported by EL Hajj Assaf et al. (2018), where the mutant strain not only lost the ability to produce patulin, but also affected the asexual development of the fungus, decreasing the formation of coremia. These results have also been reported in other species, where VeA acts positively on the biosynthesis of metabolites. The deletion of *veA* in *Aspergillus fumigatus* affected the biosynthesis of fumigaclavine C, fumagillin and gliotoxin (Dhingra et al. 2013). However, other metabolites were up-regulated by the mutant strain PeΔ*veA*, such as the unknown compounds with an m/z= 348.10762 and molecular formula $C_{17}H_{17}NO_7$ (R_T=11.75, 12.65 and 15.90) detected only in the mutant strain. Some metabolites showed no significant different between the WT and de the null mutant strain such andrastin A and aurantioclavine.

We evaluated the production of metabolites in an in vivo study. We found that in apples infected with the WT strain, patulin and also other intermediate metabolites of the patulin biosynthesis pathway were present. No trace of patulin was found in the WT spores analyzed, confirming the results obtained in the in vitro study, where this mycotoxin was essentially found in the agar medium. This result confirms the previously published studies (Zetina et al. 2020). Patulin is absent not only in synnematas but also in the conidiophore. Several studies have shown that excreted compounds are involved in sexual and asexual reproduction in fungi and that certain metabolites are produced during specific stages of fungal development (Calvo et al. 2002; Hadley and Harrold, 1958). Disruption of *brlA*, a sporulation-specific transcription factor, produces a strain unable of producing fumiquinazoline C (Lim et al. 2014), the ergot alkaloids (festuclavine and fumigaclavines A, B, and C) (Coyle et al. 2007), trypacidin and its two precursors (monomethylsulochrin and questin) (Gauthier et al. 2012) in *A*. *fumigatus*, suggesting that these metabolites are not produced in the vegetative mycelium of the fungus but are directly associated with the conidiogenesis process. The formation of coremia was deficient in the null mutant PeΔ*veA* strain grown both in vivo and in vitro. In addition, the mutant strain was unable to pierce the apple peel and exit the fruits to complete its life cycle and did not produce patulin. We have demonstrated that *P. expansum* produces patulin only during its colonization phase in apples when it grows as basal mycelium form inside the fruit and it stops producing patulin when it drills the epicarp and initiates conidiogenesis. These results suggest that patulin biosynthesis is not linked to the conidiogenesis process and that its production is detected only in the flesh. A mutant that lost its ability to produce patulin or patulin biosynthesis intermediates was still able to form spores in amounts equivalent to the wild strain of *Penicillium urticae*, indicating that these compounds are not necessary for the process of conidiogenesis (Sekiguchi and Gaucher, 1977). In the conidiogenesis stage, *P*. *expansum* could activate as yet unknown SM biosynthesis pathways. Unlike patulin, which appears early in apple infection, citrinin was detected later (not at 14 dpi but at 30 dpi). These results are consistent with those of Touhami et al. (2018) who reported that citrinin was found in apples after prolonged storage. Citrinin would not seem to be necessary at the beginning of the colonization process and seems to be more of a settlement factor during this process.

These findings show that not all fungal metabolites are released during the colonization of the substrate, but throughout its development. The fungus begins by producing metabolites that will allow it to conquer the medium (factors of aggressiveness) and most likely inhibit the development of other microorganisms in the medium. Then, other metabolites are released during its aerial metabolism, which may be more essential for the formation and dissemination of spores. Conversely, the production of certain metabolites with no further role in conidiogenesis is stopped.

Conclusion

In conclusion, this work highlighted many known and unknown SMs produced by *P*. *expansum* at different physiological stages of its development, such as substrate colonization and dissemination. A wide range of unknown compounds were identified and raw formulas were assigned to them. In addition, a new communesin with molecular formula $C_{31}H_{36}N_4O_2$ (*m*/*z*= 457.26097, R_T=31.66) was evidenced.The mycotoxins patulin and later citrinin were secreted in the medium and were specifically identified in the colonization phase of the fungus. Patulin is an early factor of aggressiveness while citrinin was produced later and could be a factor in colonization establishment. Chaetoglobosins, communesins A and B, and roquefortines C and D were mainly detected in the fungal extract. The elimination of the *veA* gene altered the metabolism of *P.expansum*. We observed an abolition of patulin and citrinin production in the null mutant strain, however three unknown compounds with the molecular formula $C_{17}H_{17}NO_7$ (*m*/*z*= 348.10762, R_T=11.75, 12.65 and 15.90) was detected only in the null mutant PeΔ*veA* strain.

In vitro results showed that there was a significant difference between the compounds excreted in the medium and those detected in the fungal extract. The substrate, MEA versus PDA medium, was the second most discriminating factor. The strain effect, WT versus null mutant PeΔ*veA* strain, came third. A lower number of metabolites was excreted in the medium by the WT strain compared to the null mutant PeΔ*veA* strain. Light had a significant effect on the biosynthesis of some SMs, but was the least discriminating factor among the factors studied.

In the in vivo study, a greater number of metabolites was detected in apple flesh when the incubation period was 30 dpi. A large number of metabolites including chaetoglobosins A and C, roquefortines C and D, andrastins A, B and C among others compounds were detected in the conidiophore/synnemata. However, no patulin was detected in the these structures. At the conidiogenesis stage, *P*. *expansum* could activate other SM biosynthetic pathways such as the one leading to the biosynthesis of two compounds with chemical formulas $C_{19}H_{16}N_2O_2$ (m/z=305.129) and $C_{20}H_{18}N_2O_2$ (m/z= 319.145). In conclusion, the biosynthesis of SMs in *P. expansum* does not occur randomly, but depends on environmental conditions, substrate and the life cycle of the fungus.

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Author contributions

Isabelle P. Oswald, Olivier Puel, Sophie Lorber conceived, supervised, and designed the experiments. Chrystian Zetina-Serrano and Christelle El Hajj Assaf performed the experiments, analyzed the data and contributed to experiment design. Robin Constantino, Olivier Puel, Emilien Jamin, Thi Phuong Thuy Hoang, Olivier Grovel and Catherine Roullier performed the LC-HRMS analyses. Jean-François Martin performed statistical analyses of the LC-MS data. Chrystian Zetina-Serrano, Christelle, El Hajj Assaf, Sophie Lorber and Olivier Puel wrote the paper.

Supplementary Materials

Deciphering of secondary metabolome of *Penicillium expansum*

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Table S1. Secondary metabolites detected from *Penicillium expansum* after culture on labeled wheat grains.

Molecular formula	Identifier [a]	^{12}C *m/z* (Da)	^{13}C *m/z* (Da)	$^{13}C/^{15}N$ *m/z* (Da)	*m/z* of major fragment ions	proposed identification
$C_7H_6O_4$	**Pexp_153.019_3.59**	**153.01919**	**160.04256**	**156.02997**	**153, 125, 109(100), 97**	**patulin**
C_7H_8O	Pexp_109.065_7.11	109.06509	116.08844	112.07483		m-Cresol
$C_7H_8O_2$	Pexp_125.060_6.79	125.05998	132.08346	129.07332	125, 106(100), 97, 95, 83, 81, 79, 69	m-Hydroxybenzyl alcohol
$C_7H_8O_3$	Pexp_141.054_3.80	141.05493	148.07869	144.06492	141, 123, 113(100), 110, 95, 79, 69, 55	Gentisyl alcohol
$C_7H_8O_4$	Pexp_157.049_2.67	157.04990	164.07348	161.06366	157, 139(100), 111, 97, 83	Ascladiol
$C_7H_{10}O_3$	Pexp_143.070_4.52	143.07061	150.09415	147.08401	143, 125, 113(100), 107, 97, 79	
$C_{10}H_{17}NO_5$	Pexp_232.118_8.83	232.11872	242.15222	238.13252	232, 214, 185, 157, 119 (100), 112, 101, 86	
$C_{12}H_{14}O_3$	Pexp_207.101_9.65	207.10110	219.14115	213.12114	207, 189(100), 179, 174, 171, 161, 142, 137	
$C_{13}H_{14}O_5$	Pexp_251.091_21.70	251.09108	264.13455	258.11474	251, 233(100), 215, 198, 167, 143	Citrinin
$C_{15}H_{18}N_2$	Pexp_227.155_6.44	227.15514	242.20521	237.17554	227, 210(100), 198, 171, 154, 130	Aurantioclavine
$C_{15}H_{19}NO_6$	Pexp_310.129_12.78	310.12939	325.17984	319.15312	310, 292 (100), 264, 240, 186, 158, 140, 107	
$C_{15}H_{19}NO_6$	Pexp_310.129_14.80	310.12964	325.17835	319.14477	310, 292 (100), 264, 240, 185, 158, 140, 107	
$C_{15}H_{20}O_4$	Pexp_265.144_15.91	265.14412	280.19431	273.17072	265, 247, 229, 219(100), 205, 201, 191, 151, 135, 107	Expansolide C/ D
$C_{15}H_{20}O_4$	Pexp_265.144_16.91	265.14410	280.19364	273.17064	265, 247, 229, 219(100), 205, 201, 191 151, 135, 107	
$C_{15}H_{20}O_4$	Pexp_265.144_18.49	265.14415	280.19430	273.17081	265, 247, 229, 219(100), 205, 201, 191 151, 135, 107	Expansolide C/ D
$C_{15}H_{20}O_4$	Pexp_265.144_19.35	265.14414	280.19429	273.17082	265, 247, 229, 219(100), 205, 201, 191 151, 135, 107	
$C_{16}H_{18}N_2O_2$	Pexp_271.144_7.62	271.14496	287.19861	281.16599	271, 254(100), 225, 215, 168	Clavicipitic acid
$C_{16}H_{18}N_2O_2$	Pexp_271.144_8.45	271.14467	287.19873	281.16576	271, 254(100), 225, 215, 168	Clavicipitic acid
$C_{16}H_{26}N_2O_4S_2$	Pexp_375.142_22.02	375.14202	391.19584	385.16297	375, 357, 347, 222, 178, 154(100),	
$C_{17}H_{22}O_5$	Pexp_307.154_27.39	307.15471	324.21155	316.18482	307, 265(100), 247, 229, 219, 211, 201, 135	Expansolide A/B
$C_{17}H_{22}O_5$	Pexp_307.155_30.19	307.15504	324.21163	316.18515	307, 265(100), 247, 229, 219, 211, 201, 135	Expansolide A/B
$C_{18}H_{16}N_2O_2$	Pexp_293.129_13.58	293.12911	311.18978	305.15587	293, 265(100), 248, 237, 222, 120	
$C_{18}H_{16}N_2O_2$	Pexp_293.129_17.74	293.12915	311.18970	305.15668	293, 265(100), 248, 237, 222, 120	
$C_{18}H_{16}N_2O_3$	Pexp_309.124_14.76	309.12421	327.18455	320.14798	309, 292 (100), 267, 249, 231	
$C_{18}H_{18}N_2O_2$	Pexp_295.144_14.61	295.14479	313.20540	307.17251	295, 267(100), 250, 239, 222, 120	
$C_{18}H_{31}NO_7$	Pexp_374.216_23.72	374.21663	392.27652	385.24644	374, 356(100), 204, 186, 158	
$C_{19}H_{16}N_2O_2$	Pexp_305.129_32.50	305.12911	324.19310	316.15289	305, 277(100), 264, 262, 205, 187	
$C_{19}H_{16}N_2O_2$	Pexp_305.129_33.17	305.12933	324.19301	317.15701	305, 277(100), 264, 262, 205, 187	
$C_{19}H_{16}N_2O_4$	Pexp_337.119_13.73	337.11909	356.18283	349.14659	337, 319, 309(100), 296, 294, 280, 252, 237, 203, 121	
$C_{19}H_{16}N_2O_4$	Pexp_337.119_15.20	337.11916	356.18294	349.14676	337, 319, 309(100), 296, 280, 237, 203, 121	

$C_{19}H_{21}NO_7$	Pexp_376.139_17.45	376.13901	395.20280	387.16962	376, 233, 207(100), 189	
$C_{19}H_{21}NO_7$	Pexp_376.139_18.65	376.13904	395.20262	387.16966	376, 233, 207(100), 189	
$C_{19}H_{38}O_6$	**Pexp_361.258_37.23**	**361.25871**	**380.32251**	**371.29242**	**361, 343, 325, 283, 273, 257(100), 157**	
$C_{19}H_{38}O_6$	**Pexp_361.258_38.19**	**361.25819**	**380.32253**	**371.29252**	**361, 343, 325, 299, 283, 257(100), 157**	
$C_{20}H_{18}N_2O_2$	Pexp_319.145_35.48	319.14588	339.21182	332.17585	319, 291(100), 278, 262, 250, 234, 205, 201, 188, 132	
$C_{20}H_{18}N_2O_2$	Pexp_319.145_36.44	319.14502	339.21173	332.17596	319, 291(100), 278, 260, 250, 234, 205, 201, 188, 160, 132	
$C_{20}H_{21}NO_9$	Pexp_420.128_18.38	420.12857	440.19571	432.16272	420, 251(100), 233	
$C_{20}H_{21}NO_9$	Pexp_420.128_21.20	420.12858	440.19605	432.16318	420, 251(100), 233	
$C_{20}H_{26}O_8$	Pexp_395.171_13.61	395.17142	415.23827	406.20763	395, 251(100), 233	
$C_{22}H_{23}N_5O_2$	Pexp_390.193_15.09	390.19390	412.26737	406.21627	390, 334, 322(100), 198, 193	Roquefortine C
$C_{22}H_{25}N_5O_2$	Pexp_392.209_9.99	392.20913	414.28326	408.22845	392, 336, 324(100), 198, 195	Roquefortine D
$C_{23}H_{24}N_2O_6$	Pexp_425.171_26.92	425.17179	448.24874	439.20611	425, 407, 383, 365, 288(100), 260	
$C_{24}H_{26}N_2O_6$	**Pexp_437.170_33.79**	**437.17091**	**461.25116**	**451.20575**	**437, 395, 377(100), 333, 286**	
$C_{26}H_{30}N_4$	Pexp_399.255_19.94	399.25568	425.34268	417.29076	399, 382, 365, 241(100), 224, 185, 159, 144, 130	Communesin K[b]
$C_{26}H_{30}N_4O$	Pexp_415.250_14.62	415.25034	441.33736	433.28593	415, 398, 357, 343, 257(100), 240, 199, 185, 159	Communesin I[c]
$C_{26}H_{30}N_4O$	Pexp_415.250_18.43	415.25033	441.33739	433.28576	415, 397, 257(100), 240, 228, 224, 201, 159, 130	Communesin I[c]
$C_{26}H_{40}O_6$	Pexp_449.289_29.93	449.28939	475.37674	463.33658	449, 431(100), 386, 384, 221, 211, 203, 179, 166	
$C_{27}H_{30}N_4O_2$	Pexp_443.245_15.65	443.24564	470.33554	461.28105	443, 385, 371(100), 201, 185, 171, 154	Communesin E[d]
$C_{28}H_{30}N_4O_3$	Pexp_471.239_19.56	471.23997	499.33402	490.27887	471, 399, 371, 201, 199(100), 171, 159, 154	Com470[e]
$C_{28}H_{31}N_5O_5$	Pexp_518.241_16.36	518.24108	546.33497	538.27672	518, 500, 462, 450(100), 321	
$C_{28}H_{31}N_5O_5$	Pexp_518.240_17.21	518.24092	546.33488	538.27706	518, 500, 462, 450(100), 402, 390, 326, 210	
$C_{28}H_{32}N_4O$	Pexp_441.265_17.14	441.26589	469.35970	460.30445	441, 424(100), 385, 365, 239, 224, 185, 183	Communesin F[d]
$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.27	457.26097	485.35452	476.29968	457, 440(100), 385, 257,255, 240, 210, 201, 185	New DMV[1] communesin-1
$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.97	457.26095	485.35394	476.29996	457, 439, 385(100), 239, 185	New DME[2] communesin-1
$C_{28}H_{32}N_4O_2$	Pexp_457.261_23.65	457.26116	485.35429	476.29971	457, 399, 385(100), 201, 185, 159, 144, 130	Communesin A[f]
$C_{28}H_{32}N_4O_3$	Pexp_473.255_15.50	473.25566	501.34996	492.29429	473, 456(100), 401, 397, 273, 271, 253, 244, 201, 199, 171	
$C_{28}H_{36}N_4O_4$	Pexp_493.279_31.47	493.27971	521.37363	511.31495		Fungisporin A or cyclo(VFVF)
$C_{28}H_{38}N_4O_5$	Pexp_511.290_18.01	511.29099	539.38447	529.32631	511, 493, 465, 394, 346, 301, 265(100), 247, 219, 166	VAL-PHE-VAL-PHE
$C_{28}H_{38}N_4O_6$	Pexp_527.287_12.46	527.28755	555.38184	545.32412	527, 281(100), 509, 481, 346, 301, 265, 247, 219, 182	VAL-PHE-VAL-TYR
$C_{28}H_{38}O_7$	**Pexp_485.254_35.32**	**485.25405**	**513.34696**	**500.30389**	**485, 470, 457, 453(100), 425, 411, 409, 393**	
$C_{28}H_{38}O_7$	Pexp_487.270_36.21	487.27063	515.36381	502.32065	487, 427(100), 409, 395, 377, 243	Andrastin A
$C_{28}H_{38}O_8$	**Pexp_501.247_27.70**	**501.24792**	**529.34203**	**516.29846**	**501, 457, 441(100), 425, 410, 397, 373, 365,**	
$C_{28}H_{38}O_8$	**Pexp_501.247_28.44**	**501.24792**	**529.34203**	**516.29846**	**501, 457, 441(100), 425, 410, 397, 373, 365,**	

$C_{28}H_{38}O_8$	**Pexp_501.247_29.57**	**501.24786**	**529.34194**	**516.29974**	**501, 486, 469, 457, 441(100), 425, 397, 373, 366**	
$C_{28}H_{40}O_6$	**Pexp_471.274_39.48**	**471.27434**	**499.36727**	**486.32419**	**471, 456, 439, 411(100)**	**Andrastin C**
$C_{28}H_{40}O_7$	**Pexp_487.268_30.58**	**487.26898**	**515.36247**	**502.31903**	**487, 455, 427(100), 411, 395, 351,336**	**Andrastin B**
$C_{29}H_{33}N_5O_6$	Pexp_548.251_15.42	548.25180	577.34902	568.28775	548, 530, 518, 492, 480(100), 462, 402, 390, 356	
$C_{30}H_{36}N_4O_2$	Pexp_485.292_16.36	485.29245	515.39378	505.33425	485, 468(100), 429, 365, 239, 224. 222, 183, 185, 159, 144	New DMV[1] communesin-2
$C_{31}H_{36}N_4O_2$	Pexp_497.290_31.66	497.29061	528.39421	517.33225	497, 479, 425(100), 439, 257, 241, 185, 168, 159	Putative new undetermined communesin
$C_{32}H_{34}N_4O_3$	Pexp_523.271_31.84	523.27152	555.37917	543.30477	523, 451, 423(100), 253, 199, 171, 159, 154,	Communesin D[g]
$C_{32}H_{36}N_2O_4$	Pexp_513.276_39.10	513.27635	545.38309	532.32773	513, 495(100), 477, 455, 430, 412, 384, 366, 250	Chaetoglobosin J or Prochaetoglobosin III
$C_{32}H_{36}N_2O_5$	Pexp_529.270_25.21	529.27058	561.37837	548.32196	529, 511(100), 493, 475, 382, 380, 362, 307, 185	Chaetoglobosin B/G
$C_{32}H_{36}N_2O_5$	Pexp_529.270_26.71	529.27029	561.37834	548.32343	529, 511(100), 493, 475, 382, 380, 362, 185	Chaetoglobosin B/G
$C_{32}H_{36}N_2O_5$	Pexp_529.270_30.51	529.27077	561.37872	548.32190	529, 511(100), 493, 469, 475, 483, 457, 451, 359, 333	Chaetoglobosin B/G
$C_{32}H_{36}N_2O_5$	Pexp_529.270_33.98	529.27067	561.37843	548.32208	529, 511(100), 501, 493, 483, 475, 465, 457, 431, 417, 400, 382	Chaetoglobosin A
$C_{32}H_{36}N_2O_5$	Pexp_529.270_35.67	529.27092	561.37735	548.31076	529, 511 (100), 493, 469, 382, 359, 331	Chaetoglobosin B/G
$C_{32}H_{36}N_2O_5$	Pexp_529.270_36.41	529.27063	561.37820	548.32186	529, 511(100), 493, 487, 483, 475, 469, 457, 400, 398, 382, 380, 364, 328, 310, 185	Chaetoglobosin C
$C_{32}H_{36}N_2O_5$	Pexp_529.270_37.41	529.27056	561.37807	548.32181	529, 511 (100), 493, 453, 380, 327, 185	Chaetoglobosin B/G
$C_{32}H_{36}N_2O_6$	**Pexp_543.248_34.05**	**543.24836**	**575.35597**	**562.29931**	543, 499, 481, 471, 445(100), 427, 375, 352, 248	Putative cytochalasin
$C_{32}H_{36}N_4O_2$	Pexp_509.292_36.01	509.29257	541.39875	530.3376	509, 437(100), 451, 185	Communesin B[g]
$C_{32}H_{36}N_4O_3$	Pexp_525.287_13.45	525.28735	557.39329	546.33239	525, 507, 481, 467, 453(100), 409, 343, 340, 257, 240,225, 199, 185, 159	New DME[2] communesin-2
$C_{32}H_{36}N_4O_3$	Pexp_525.287_27.91	525.28705	557.39461	546.33215	525, 508(100), 271, 273, 199	New DMV[1] communesin-3
$C_{32}H_{38}N_4O_2$	Pexp_511.306_36.13	511.30684	543.41590	532.35205	511, 439(100), 453, 185, 144	New DME[2] communesin-3
$C_{32}H_{38}N_4O_3$	Pexp_527.303_21.41	527.30304	559.41082	548.34834	527, 509, 469, 453, 437(100), 275, 215	New DME[2] communesin-4
$C_{32}H_{38}N_4O_4$	Pexp_543.297_19.36	543.29789	575.40330	564.34345	543, 525, 485, 471(100), 185, 168	New DME[2] communesin-5
$C_{32}H_{40}N_4O_2$	Pexp_513.322_36.57	513.32227	545.43031	533.36211	513, 495, 455, 441(100), 257, 185	New DME[2] communesin-6
$C_{33}H_{38}N_4O_5$	Pexp_571.293_18.22	571.29324	604.40405	592.33802	571, 553, 513, 499(100), 455, 397, 299	Com570[e]
$C_{37}H_{42}N_4O_5$	Pexp_623.325_29.52	623.32511	660.44909	646.37781	623, 565, 551(100), 507, 449, 437, 371, 354, 299	Com622[e]

Table S2. Secondary metabolites detected in fungal extracts and MEA medium at 25°C in the dark

DARK/ MEA					
Secondary Metabolites	**WT**	**PeD*veA***	**PeΔ*veA*:*veA***	**Rt**	***m/z***
Andrastin A	1,548	0,862	1,638	36,16	485,25
Andrastin B	0,571	0,374	0,658	30,48	487,27
Ascladiol	0,242	0,714	0,251	2,85	157,05
Aurantioclavine	1,136	0,167	2,562	5,23	227,16
Chaetoglobosin A	37,909	16,207	32,500	33.49	529,27
Clavicipitic Acid	2,073	0,293	3,896	7,85	271,15
Clavicipitic Acid	0,149	0,029	0,262	8,64	271,15
Com496-1	4,366	1,909	4,973	31,89	497,29
Communesin A	4,910	0,926	4,877	23,01	456,26
Communesin B	36,550	5,547	24,429	35,48	509,29
Communesin D	38,839	6,755	41,395	32,37	523,27
Communesin F	37,371	1,585	55,353	16,38	441,26
Communesin I	1,625	0,453	2,178	13,41	415,25
Communesin Ib	85,919	3,423	104,693	17,23	415,25
Communesin K	8,189	0,649	13,660	18,64	399,26
Expansolides A/B	0,331	0,093	0,309	27,20	307,15
Expansolides A/B	0,316	0,096	0,298	29,98	307,15
Patulin	0,057	nd	0,050	3,38	155,03
Roquefortine C	14,092	1,515	18,016	14,08	390,19
Roquefortine D	15,854	0,564	18,993	8,58	392,21
$C_{10}H_{17}NO_5$	0,044	0,018	0,046	8,83	232,12
$C_{15}H_{19}NO_6$	0,041	0,071	0,044	13,41	310,13
$C_{15}H_{19}NO_6$	1,435	0,110	1,729	15,22	310,13
$C_{16}H_{26}N_2O_4S_2$	2,842	0,489	3,050	21,92	375,14
$C_{17}H_{17}NO_7$	nd	0,027	nd	12,03	348,11
$C_{17}H_{17}NO_7$	nd	0,026	nd	12,65	348,11
$C_{18}H_{16}N_2O_2$	1,745	0,307	1,867	13,50	293,13
$C_{18}H_{16}N_2O_2$	1,018	0,764	0,987	17,60	293,13
$C_{18}H_{16}N_2O_3$	1,683	nd	1,776	15,24	309,12
$C_{18}H_{18}N_2O_2$	0,545	0,113	0,581	14,56	295,14
$C_{18}H_{31}NO_7$	0,028	0,037	0,035	23,96	374,22
$C_{19}H_{16}N_2O_2$	207,866	nd	313,619	32,40	305,13
$C_{19}H_{16}N_2O_2$	79,639	nd	91,573	33,01	305,13
$C_{19}H_{16}N_2O_4$	2,371	0,511	2,206	14,00	337,12
$C_{19}H_{16}N_2O_4$	4,542	0,411	5,353	15,47	337,12
$C_{19}H_{21}NO_7$	0,025	nd	0,027	17,55	376,14
$C_{19}H_{21}NO_7$	0,024	0,067	0,028	18,65	376,14
$C_{19}H_{38}O_6$	252,235	44,092	148,051	37,23	361,26
$C_{19}H_{38}O_6$	367,756	117,188	72,209	38,12	361,26
$C_{20}H_{18}N_2O_2$	295,238	8,333	185,306	35,89	319,14
$C_{20}H_{18}N_2O_2$	20,068	9,153	21,228	36,37	319,14
$C_{20}H_{26}O_8$	0,001	nd	0,001	13,61	395,17
$C_{23}H_{24}N_2O_6$	0,816	0,315	0,770	26,70	425,17
$C_{24}H_{26}N_2O_6$	1,924	2,959	1,903	33,54	437,17
$C_{24}H_{40}O_6$	0,390	0,330	0,561	30,05	471,27
$C_{28}H_{30}N_4O$	18,301	1,122	6,572	19,96	471,24
$C_{28}H_{32}N_4O_2$	1,649	0,089	1,711	13,56	457,26
$C_{28}H_{32}N_4O_2$	1,841	0,173	1,416	14,32	457,26
$C_{28}H_{32}N_4O_3$	6,406	1,870	5,140	15,21	473,26
$C_{28}H_{38}O_7$	1,173	0,362	0,989	35,19	485,25

$C_{29}H_{27}N_5O_5$	0,163	nd	0,072	17,84	526,21
$C_{29}H_{27}N_5O_5$	0,372	nd	0,177	18,56	526,21
$C_{32}H_{36}N_2O_4$	58,394	46,305	26,099	39,21	513,27
$C_{32}H_{36}N_2O_5$	0,726	0,160	0,807	26.87	529.27
$C_{32}H_{36}N_2O_5$	0,176	0,092	0,276	29.65	529,27
$C_{32}H_{36}N_2O_5$	320,220	93,288	309,543	36.68	529,27
$C_{32}H_{36}N_4O_3$	64,481	21,169	46,193	28,19	525,29
$C_{32}H_{38}N_4O_3$	30,545	1,266	29,948	20,17	527,30
$C_{32}H_{40}N_4O_2$	127,245	11,801	69,811	37,01	513,32
$C_{37}H_{42}N_4O_5$	44,129	nd	19,806	29,99	623,33

*SM < 1 = EXCRETED (GREEN)
SM > 1 = INTERNAL (YELLOW)

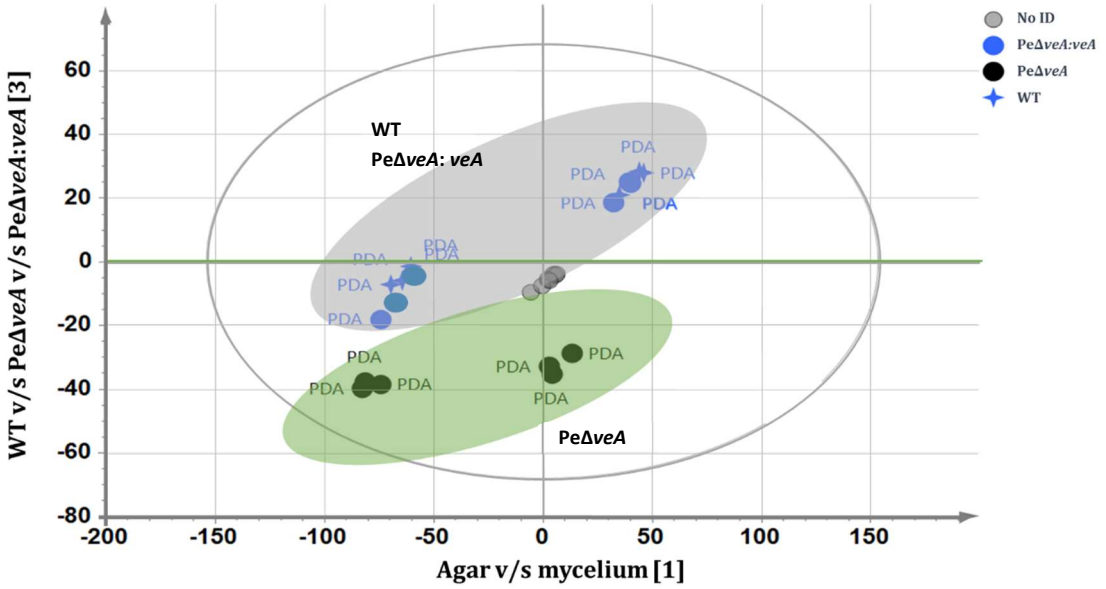


Figure S1. Principal Component Analysis (PCA) of the data obtained from the mass spectra. The strain effect. The strains showed a significant effect when comparing the null mutant PeΔ*veA* strain versus WT or the complemented strain. The strains grew in PDA medium.

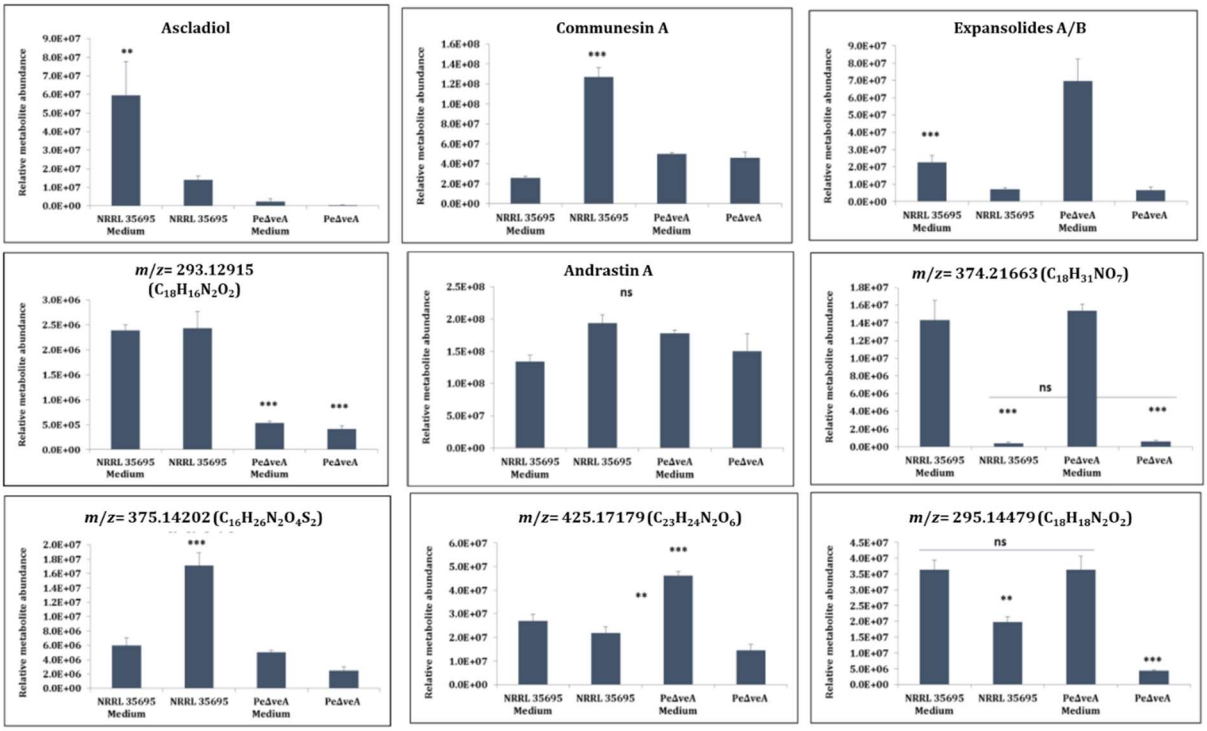


Figure S2. Relative metabolite abundance of the data obtained from the mass spectra. Comparison between the compounds produced by the null mutant strain and WT. Cultures were grown for 5 days in the dark at 25 °C. Graphs show the mean ± standard error of the mean (SEM) of three replicates and the significant differences between WT and null mutant PeΔ*veA* strains, in the medium or in the fungus. *p*-value * < 0.05; ** < 0.01; *** < 0.001, ns= no significant changes.

MEA Medium (RoqC)

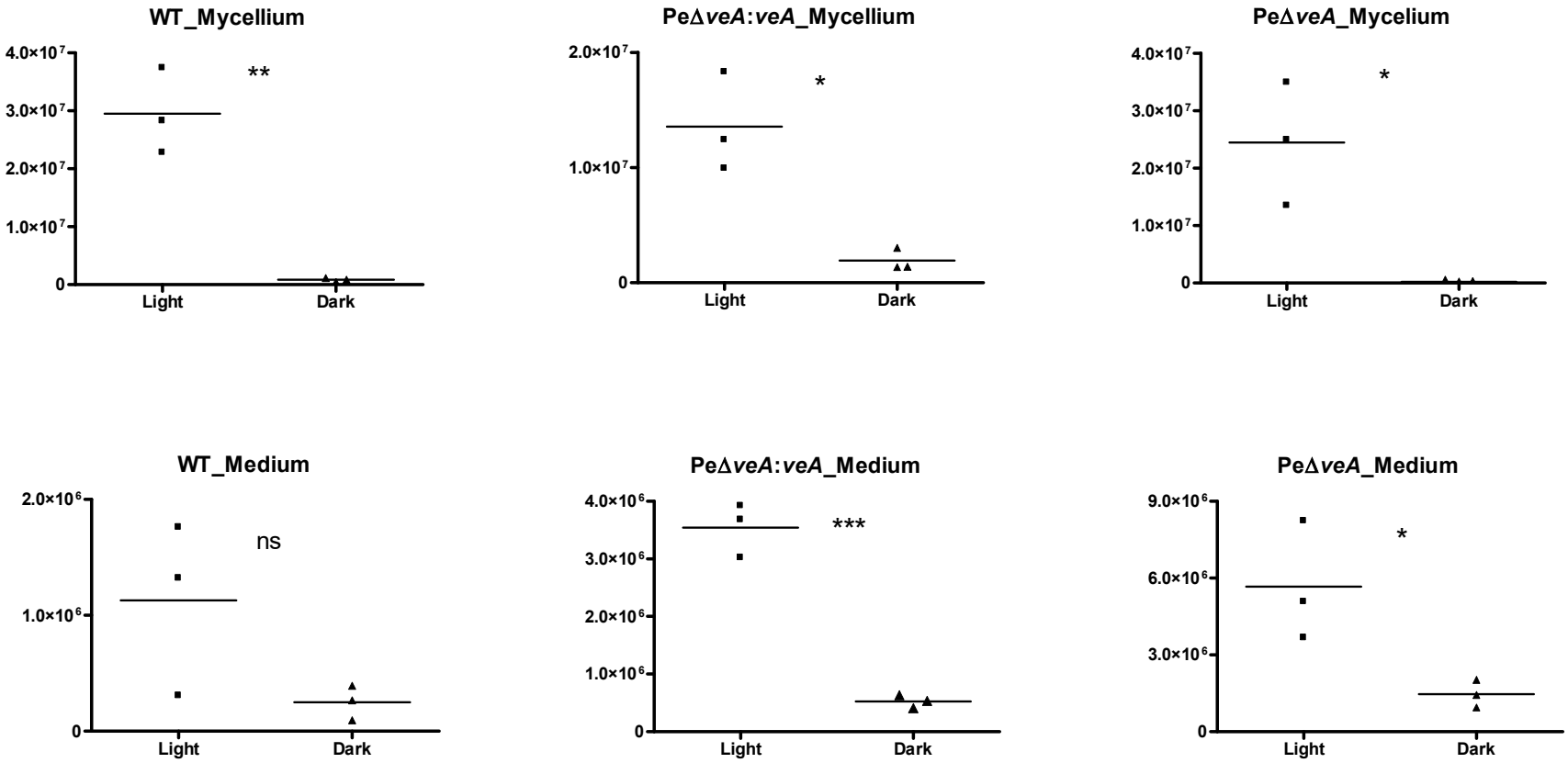


Figure S3a. Effect light. The graphs show the comparison of the relative abundance of roquefortin C produced under light or dark conditions by the WT strain and the null mutant strain PeΔ*veA* in MEA for 5 days at 25 °C.

PDA Medium (RoqC)

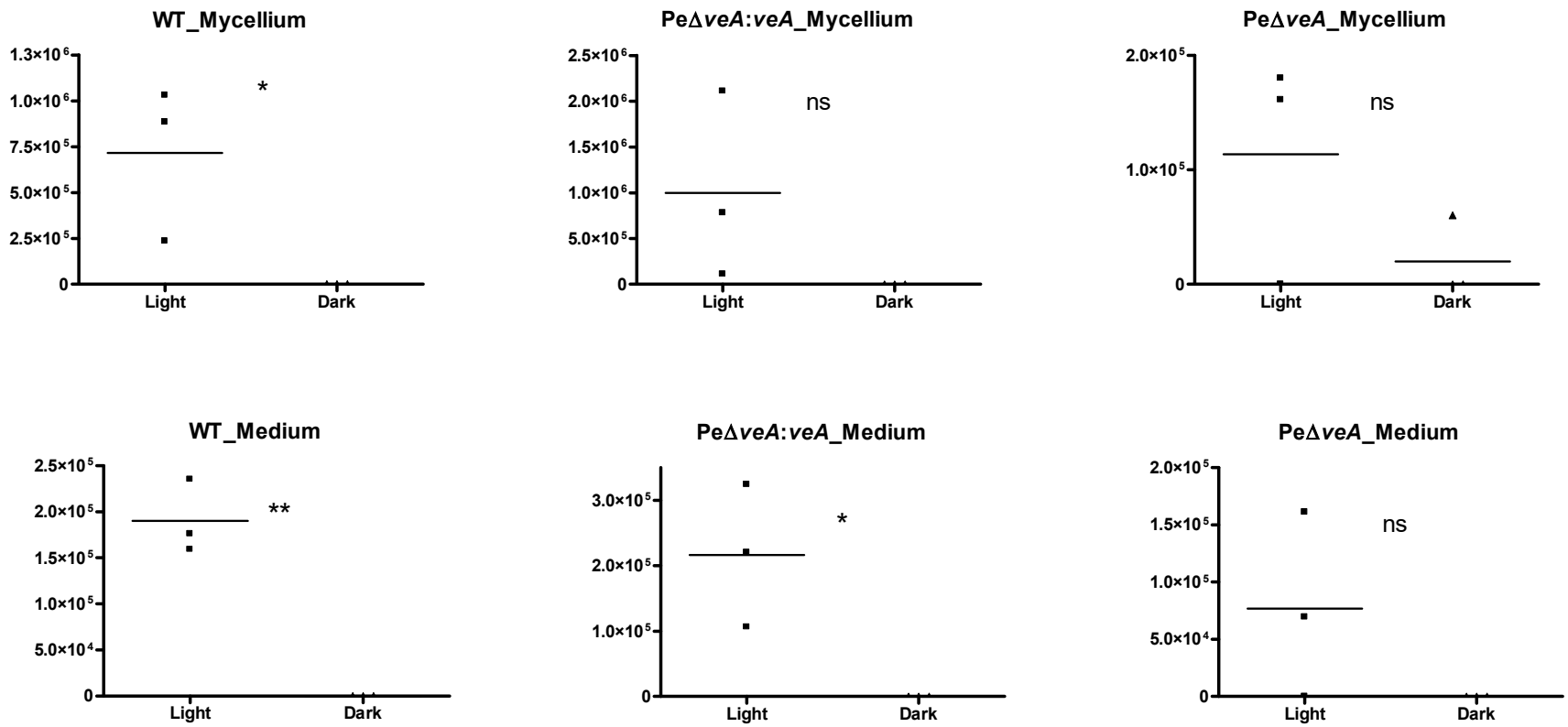


Figure S3a. Effect light. The graphs show the comparison of the relative abundance of roquefortin C produced under light or dark conditions by the WT strain and the null mutant strain PeΔ*veA* in PDA for 5 days at 25 °C.

MEA Medium (Clavicipitic acid)

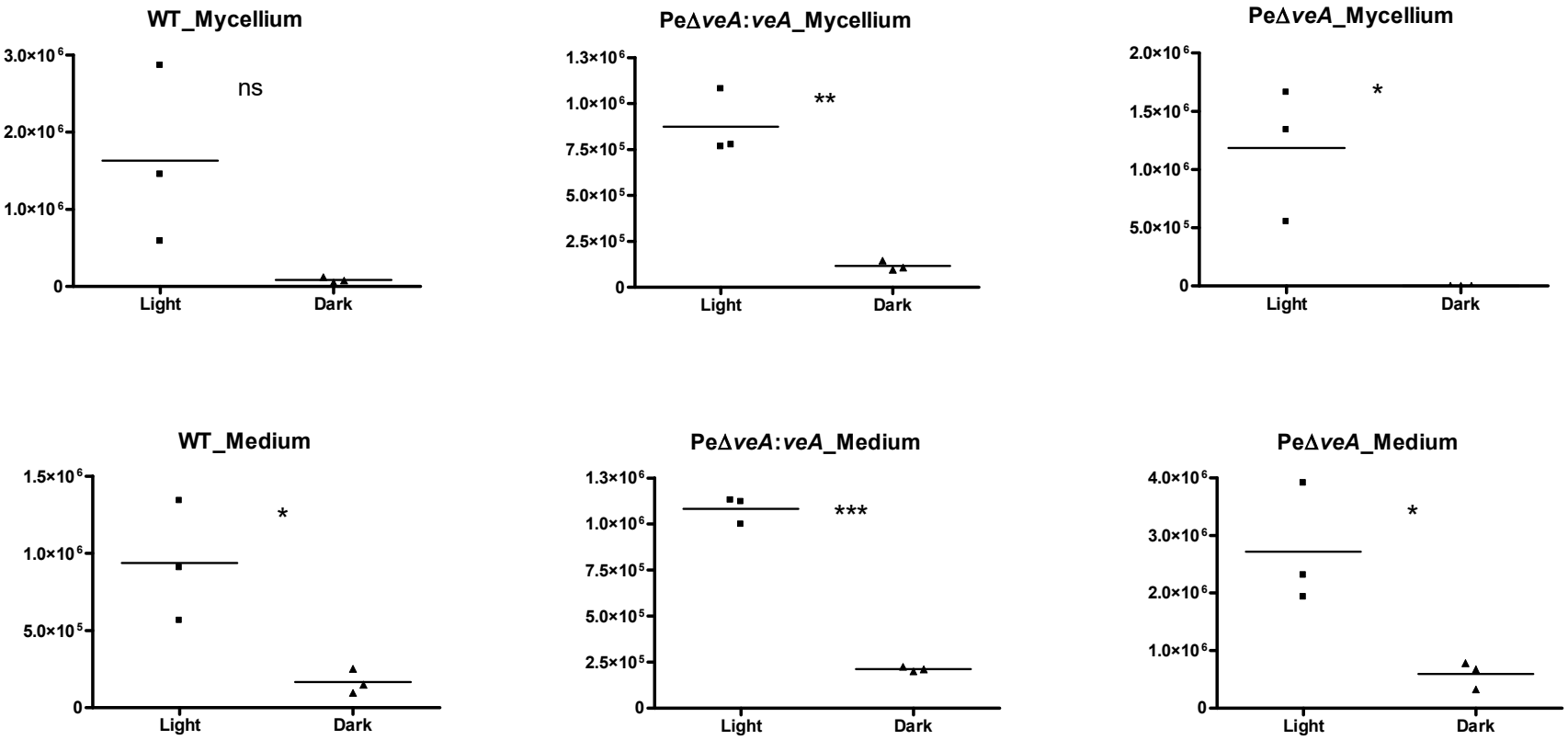


Figure S3b. Effect light. The graphs show the comparison of the relative abundance of clavicipitic acid produced under light or dark conditions by the WT strain and the null mutant strain PeΔ*veA* in MEA for 5 days at 25 °C.

PDA Medium (Clavicipitic acid)

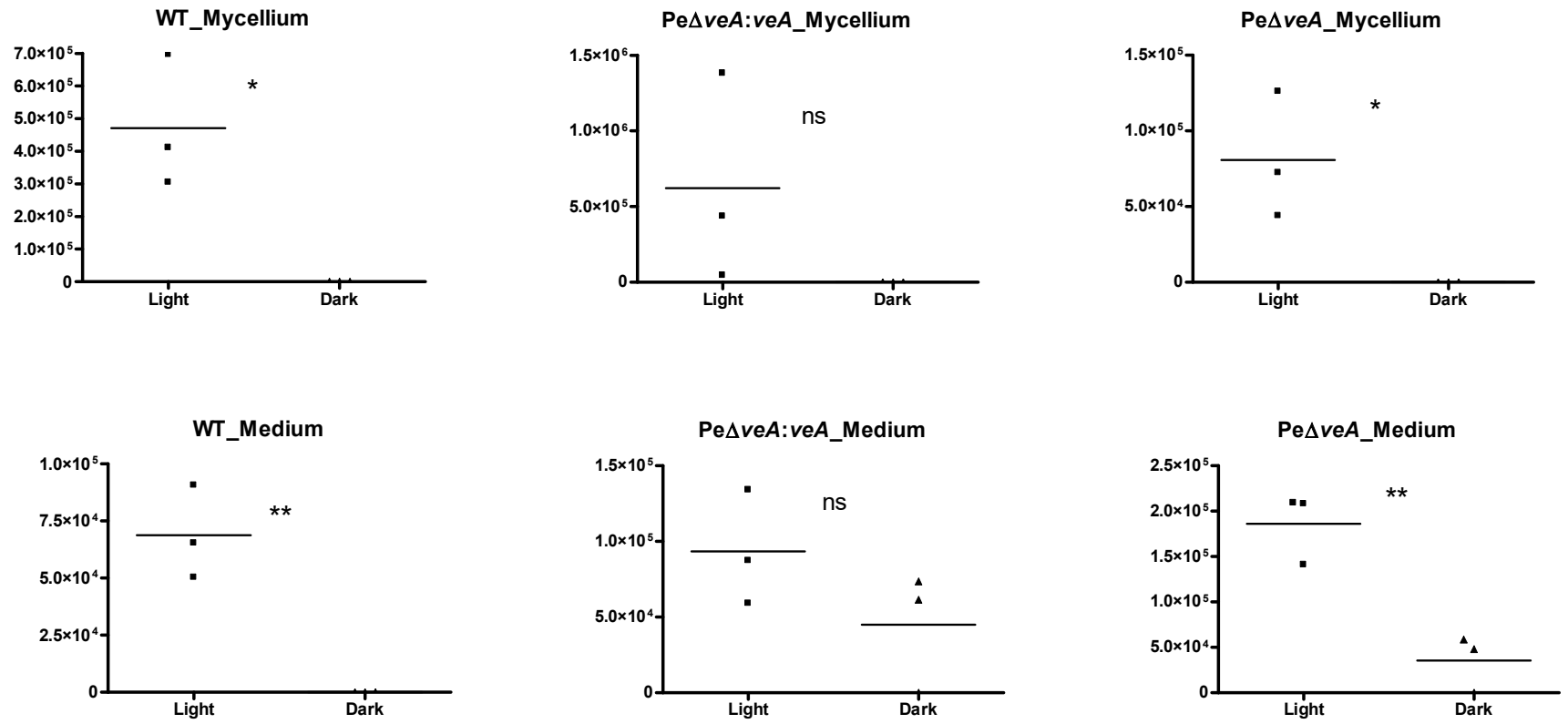


Figure S3b. Effect light. The graphs show the comparison of the relative abundance of clavicipitic acid produced under light or dark conditions by the WT strain and the null mutant strain PeΔ*veA* in PDA for 5 days at 25 °C.

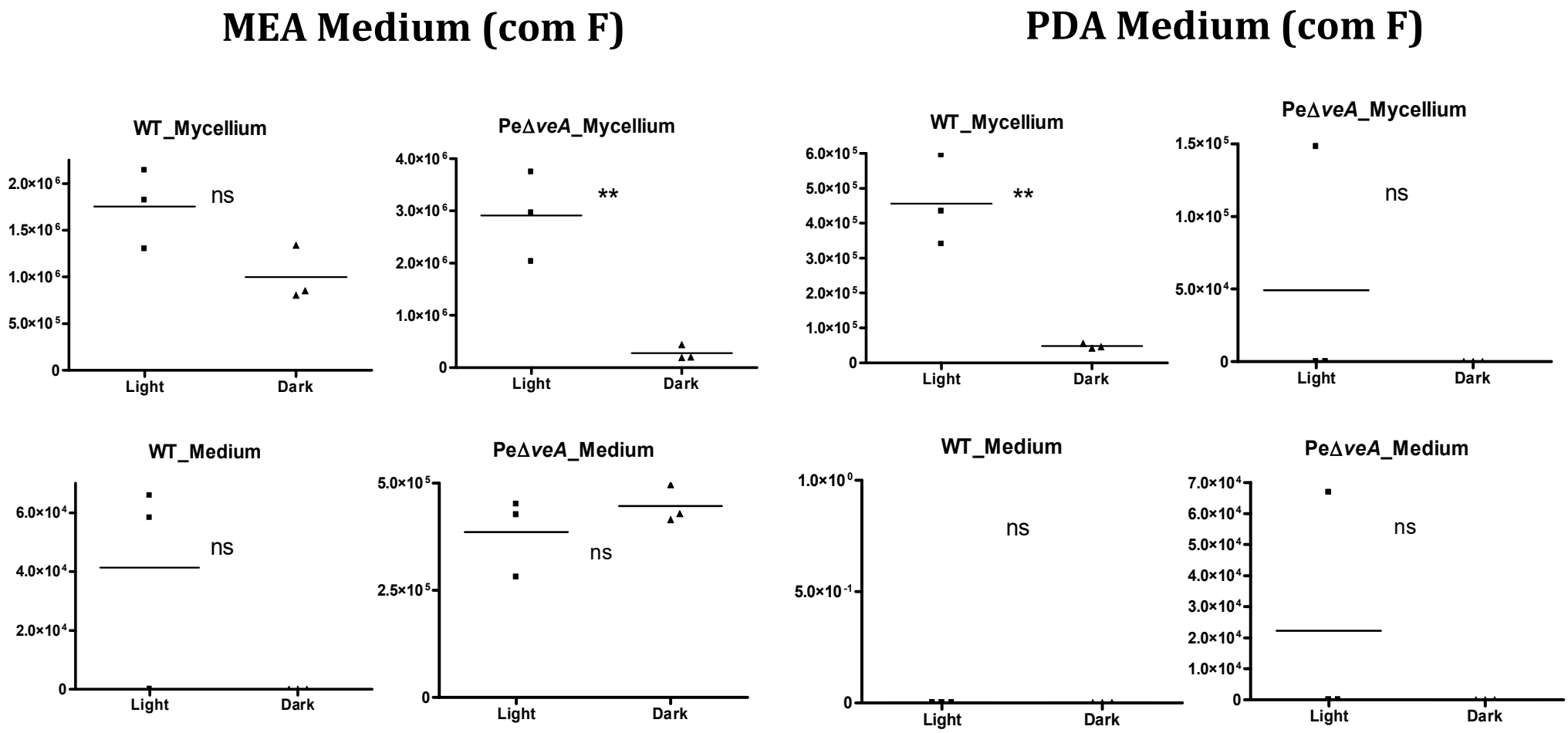


Figure S4. Effect light. The graphs show the comparison of the relative abundance of Communesin F produced under light or dark conditions by the WT strain and the null mutant strain PeΔ*veA* in A) MEA and B) PDA for 5 days at 25 °C.

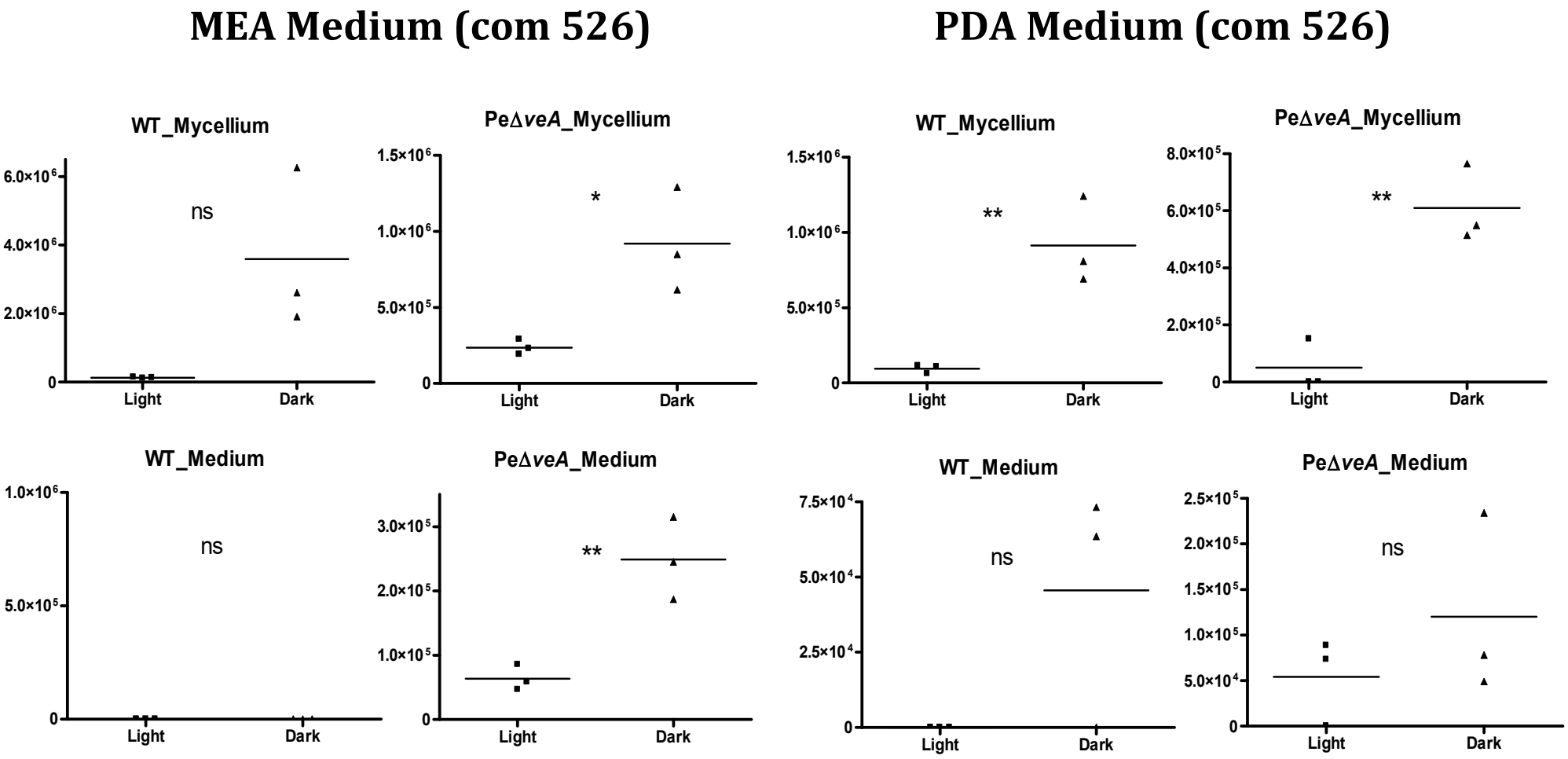


Figure S5. Effect light. The graphs show the comparison of the relative abundance of Com 526 produced under light or dark conditions by the WT strain and the null mutant strain PeΔ*veA* in A) MEA and B) PDA for 5 days at 25 °C

GENERAL DISCUSSION AND PERSPECTIVES



General discussion and perspectives

In this section, a general synthesis of the results presented during this thesis will be addressed, in order to critically discuss our results and compare them with previous works and the literature. This will allow us to establish advances or developments and to advance new hypotheses in the understanding of the mechanisms regulating development and secondary metabolism in the post-harvest pathogenic fungus *Penicillium expansum*.

My work focused on important aspects such as deciphering as thoroughly as possible the secondary metabolome of *P*. *expansum*, enriching knowledge and clarifying uncertainties concerning the identification of compounds supposedly produced by this fungus. The use of a non-target metabolomic approach combining stable isotopic labelling with liquid chromatography coupled to high-resolution mass spectrometry provides great possibilities for the discovery of new bioactive compounds, useful for agriculture, food and pharmaceutical industries or conversely the identification of new mycotoxins.
On the other hand, the creation and characterization of two null mutant PeΔ*veA* (previous work carried out in the laboratory) and PeΔ*brlA* (subject of chapter 2) strains, lacking an essential transcription factor involved in either sexual or asexual reproduction, allowed a better understanding of the impact of these transcription factors on the development and the secondary metabolism of *P*. *expansum* as well as and the relationship between the two.

The results of this work have led us to new issues that we will try to resolve throughout this chapter, as well as to recommendations that could improve and further extend the knowledge obtained throughout this work.

It is important to note that some of the discussion points already presented in the previous chapters (articles) will not be repeated in this section.

Penicillium expansum; a Big Factory of Secondary Metabolites

For the development of this thesis, we used a innovative technique for the detection and identification of secondary metabolites. The combination of isotopic labeling of the fungal substrate (^{13}C, $^{13}C/^{15}N$) with high-resolution mass spectrometry analysis (HRMS) allowed the specific detection of secondary metabolites secreted by a fungus. The double isotopic labeling allowed to detect the number of carbon and nitrogen present in the molecule, thus simplifying the chemical characterization and compound identification. The use of a natural culture (^{12}C) not infected as a control allowed the identification of the secondary metabolites produced only by the fungus by deducting those produced by the substrate.

This technique has already been applied to other strains as a method for characterizing secondary fungal metabolome. In *Aspergillus fumigatus*, 21 SMs were unequivocally identified, in addition to the elucidation of the formula and structure of fumigaclavin D (Cano et al. 2013). In *Penicillium verrucosum*, 98 SMs were detected, of which only 18 compounds were identified and 80 metabolites, whose chemical formula was determined, were completely unknown (Hautbergue et al. 2017). *P. nordicum* produced at least 92 metabolites, 69 of wich were completely unknown (Hautbergue et al. 2019). The interest of this method is that it offers great possibilities for the discovery of potential new bioactive molecules for the pharmaceutical or food industries.

In this thesis work, we focused on the study of the secondary metabolism of the WT strain of *P. expansum* and of the two null mutant PeΔ*veA* and PeΔ*brlA* strains, deficient for VeA or BrlA, TFs both involved in asexual reproduction, as well as in the regulation of BGCs. Considering the metabolism of the three strains studied, we have identified 91 SMs (Annex A, Table 1) in the WT strain of which 60 compounds were identified by MS/MS spectrum, chemical formulae, retention times, MS/MS fragmentation spectra of the standard compound, AntiBase 2012 database (Laatsch, 2012), and literature. Thirty-one compounds remain unknown, but thanks to the methodology studied, the chemical formulas were determined. Several identified metabolites presented the same chemical formula, but were detected with different retention times and could be isomers.

The *veA* gene encodes a protein of 574 amino acid long, which is required for sexual development and SM production in several species (Kim et al. 2002; Sarikaya-Bayram et al. 2015; Stinnett et al. 2007). The elimination of the *veA* gene in *P. expansum* highlighted its role in the secondary metabolism of the fungus. We showed that the null mutant strain PeΔ*veA* produced 88 SMs (Annex A, Table 1), of which 74 compounds were also produced by the WT strain and 14 compounds were specific to the mutant strain. This means that *veA* up-regulates the production of 17 SMs in *P. expansum* among which there are four patulin intermediates (m-cresol, m-

hydroxybenzyl alcohol, gentisyl alcohol and ascladiol), citrinin, eight derivatives of the cytochalasin family, new DME communesin-1 and two unknown compounds with formula $C_{15}H_{20}O_4$ (R_T= 16.91 and 19.35). The regulation of secondary metabolism by VeA has been extensively studied, as VeA has been shown to influence the production of different SMs in a wide variety of species such as patulin and citrinin in *P. expansum* (El Hajj Assaf et al.,2018), ochratoxin A in *Aspergillus niger* (Zhang et al. 2018) and in *Aspergillus carbonarius* (Crespo-Sempere et al. 2013), penicillin and sterigmatocystin in *Aspergillus nidulans* (Kato et al. 2003), aflatoxin B1, and aflatrem in *Aspergillus flavus* (Duran et al. 2007). VeA forms a heterotrimeric protein complex with VelB and the methyltransferase LaeA, called the velvet complex. It would be interesting to create null mutant Δ*laeA* and Δ*velB* strains and even a strain Δ*veA*:Δ*laeA*:Δ*velB* to analyze whether the absence of a velvet complex protein similarly influences the secondary metabolism of *P. expansum* and what other consequences would result from the total elimination of the velvet complex in the fungus.

In addition, *brlA* gene encodes a C_2H_2-type zinc-finger TF, which is essential for asexual development. Some studies have shown that *brlA* is not only involved in the regulation of the conidiogenesis process but also in the regulation of SM biosynthesis (Han et al. 2018; Lim et al. 2014; Qin et al. 2013; Shin et al. 2015). In *P. expansum*, we showed that the deletion of the *brlA* gene generated changes in the metabolism of the strain. The null mutant PeΔ*brlA* strain produced 101 SMs (Annex A, Table 1), of which 71 compounds were also produced by the WT strain and 30 compounds were detected only in the null mutant strain. This means that there are 30 SMs that are down-regulated and 20 SM that are up-regulated by *brlA* in *P. expansum*. Thus, there is a strong connection between *brlA* and the secondary metabolism of *P. expansum*. The mycotoxins patulin and citrinin were detected in the null mutant PeΔ*brlA* strain, suggesting that, contrary to what was observed in the null mutant PeΔ*veA* strain, *brlA* would not be involved in the biosynthesis of these compounds. However, we observed a drastic decrease in communesin diversity and an increase in the diversity of chaetoglobosin derivatives and members of the cytochalasan alkaloid family. These results indicate that these compounds could be closely related to the conidiogenesis process.

An in vivo study was carried out on Golden Delicious apples to identify the compounds produced by the fungus in its natural habitat. The use of the WT strain and the null mutant PeΔ*veA* and PeΔ*brlA* strains, respectively lacking the key factors of sexual and asexual development, gave us an overview of the spatio-temporal location of the secondary metabolism of *P. expansum*.
Forty-six SMs (Appendix A, Table 2) excreted by the fungus from the apple mesocarp were detected. In addition to patulin and citrinin, the expansolides A/B, roquefortine C, communesins F, B, D and A, andrastin A and B, chaetoglobosins B/G, new DMV communesin-1, new DME

communesin-4, and 16 unknown SMs (Figure 1) were identified. A large diversity of chaetoglobosins B/G (nine derivatives) and putative cytochalasans (five derivatives) were also detected in apples infected with the null mutant PeΔ*brlA* strain, of which only four derivatives were identified in apples infected by the WT strain at 30 day-post inoculation (dpi) but not detected at 14 dpi. This could suggest that these metabolites are synthesized at a late stage of fungal development such as conidiogenesis.
In apples infected with the null mutant PeΔ*veA* strain, a lower number of SMs was identified. However, the incubation period was only 14 dpi, so analysis at 30 dpi is strongly recommended to check and compare the absence/presence of SMs produced and excreted by the fungus.

According to Calvo et al. (2002), most of the SMs produced by the fungus are secreted once the fungus has completed its initial growth phase. SMSs are divided into three main categories I) 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).
The spores/synnemata analysis (Annex A, Table 3) allowed us to identify 63 SMs in the conidiophores. We found that clavicipitic acid, expansolides A/B, roquefortines C and D, communesins (A, B, D, F, I, K, com470), andrastin A, B and C, chaetoglobosin A, C, B/G, prochaetoglobosin III and nine unknown SMs are present throughout the structure of the conidiophore/synnemata.

The suppression of the *brlA* gene in *P. expansum* left a strain devoid of conidiophores, but able to form aerial, rigid, white, sporeless structures called synnemata. Compared to spores, a higher number of SMs (24 compounds) was detected in the synnemata, among which we detected expansolides C/D and six unknown compounds, with chemical formula $C_{22}H_{20}N_3O$ (m/z= 341.153, R_T= 38.56 and 39.29), $C_{29}H_{33}N_5O_6$ (m/z= 548.25180, R_T= 14.66), $C_{26}H_{40}O_6$ (m/z= 449.28939, R_T= 29.93) and $C_{28}H_{38}O_8$ (m/z= 501.24792, R_T= 27.70). However, the compounds detected in greater numbers were the members of the cytochalasan alkaloid family, of which chaetoglobosin E, three chaetoglobosin B/G (R_T= 29.56, 35.49 y 37.41), penochalasin, and eleven derivatives of putative cytochalasans. This abundance of chaetoglobosins is explained by the repression of chaetoglobosin BGC by BrlA. When BrlA es deleted the chaetoglobosin production is enhanced. It is interesting to note that in the conidiophores, mainly chaetoglobosin A and C and a lower diversity of cytochalasins were detected. Since the various chaetoglobosin derivatives serve as precursors for the formation of other chaetoglobosins after multiple oxidation steps (Ishiuchi et al. 2013), we suggest that the high diversity of chaetoglobosins, due to the removal of

chaetoglobosin BGC repressor, detected in the synnemata could serve for a better understanding of chaetoglobosin biosynthesis.

In spores/conidiophore/synnemata extracted from apple infected by WT, nine SMs were detected, three new communesins and six unknown SMs with chemical formula $C_{19}H_{16}N_2O_2$ (m/z= 305.129, R_T= 32.50 and 33.17), $C_{19}H_{38}O_6$ (m/z= 361.25871, R_T= 37.17), $C_{20}H_{18}N_2O_2$ (m/z= 319.145, R_T= 35.48 and 36.44), and $C_{18}H_{16}N_2O_2$ (m/z= 293.129, R_T= 13.58). This suggests that these compounds would be synthesized or required for higher structures such as spores. Fungal spores contain SMs that can protect them from a multitude of abiotic and biotic stresses. For example in *A*. *fumigatus*, fumiquinazoline is an additional UV-C-protective molecule and DHN-melanin is a factor of virulence that are required depending on the environmental stress (Blachowicz et al. 2020). In order to better characterize these unknown metabolites (molecular structure, function), a molecular network of the secondary metabolome of *P*. *expansum* created from the targeted MS/MS spectra could be performed to determine their origin and find similarities with other metabolites or known MS groups.

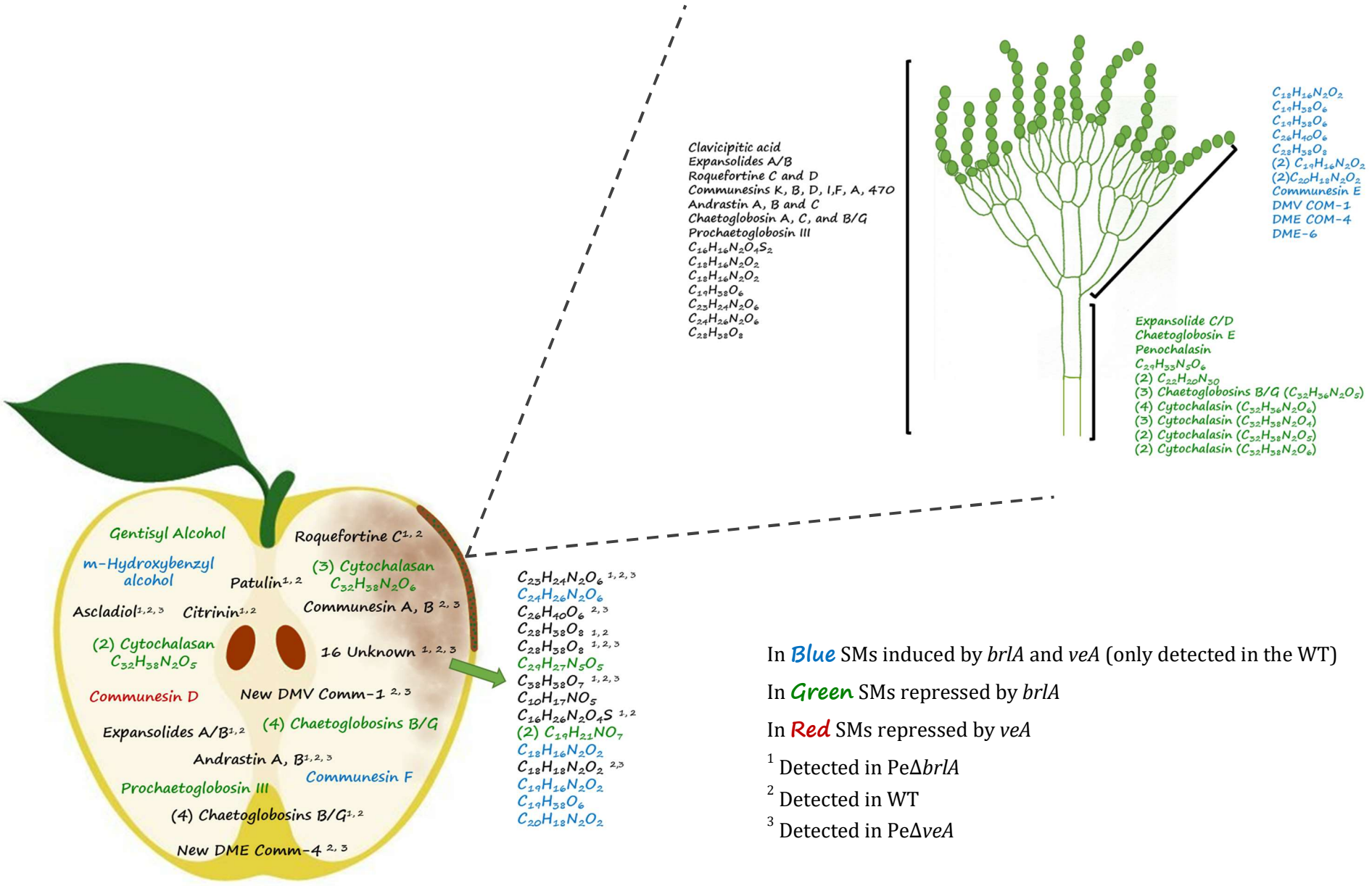


Figure 1. Scheme of spatial location of secondary metabolites produced by *P. expansum* at 30 dpi.

Link between *veA* and *brlA* genes in conidiation

The heterotrimeric complex VeA-VelB-LaeA (velvet complex) coordinates fungal development and SM production. In the velvet complex, formed predominantly in the nucleus when the fungus grows in the dark, VeA is associated with VelB and the dimer VEA-VelB is transported by the KapA α-importin inside the nucleus. Light is an inhibitor of *veA* expression, preventing the formation of the velvet complex (Sarikaya-Bayram et al. 2015). VelB has additional functions with another velvet protein (VosA) in spore viability and trehalose biogenesis, which requires that VelB is inside the nucleus to form the VelB-VosA heterodimer. Given the influence of VeA on secondary metabolism, it would be advisable to evaluate the impact of laeA and VelB suppression on the secondary metabolism of *P. expansum*

Kim et al. (2002) have shown that the mutant strains lacking the *veA* gene in *Aspergillus nidulans* are unable to form sexual structures (Hülle cells and cleistothecia) even under conditions that favored sexual development, thus favoring asexual structures. In contrast, overexpression of *veA* in *A. nidulans* leads to a greater number of sexual structures with a reduced number of conidiophores. These results confirm the role of VeA in the development of the fungus. However, a recent study in *Aspergillus niger* has shown that *veA* acts as a positive regulator of conidial production, since the number of conidia decreases in the null mutant Δ*veA* strain compared to the WT strain, when the strain grows under both light and dark conditions. In addition, expression of the *brlA* gene was significantly reduced (Zhang et al. 2018). In *Aspergillus fumigatus*, results are contradictory; Dhingra et al. (2012) have shown that the deletion of *veA* results in lower conidiation levels, suggesting that a proper VeA signal is required to achieve WT conidiation levels. In contrast, Park et al. 2012 have shown that the deletion of some genes encoding velvet proteins (VosA, VeA or VelB) causes hyperactive conidiation and early and high accumulation of *brlA* during development. In *P. expansum*, the deletion of the *veA* gene resulted in a strain capable of sporulating in the dark but which lost the ability to create coremia, both in vitro and in vivo (El Hajj Assaf et al. 2018).

Relationship between the *veA* and *brlA* genes have been observed in *A. nidulans* and *A. parasiticus*. In *A. nidulans*, loss of *veA* affects the proportion of *brlA* α/β transcripts that control conidiation (Kato et al. 2003). In *A. parasiticus*, *brlA* is expressed in the early stages of growth in the null mutant Δ*veA* strain and then its expression is temporarily decreased, contrary to what is observed in the WT strain. Although the lack of an air interface do not prevent the expression of *brlA* in the Δ*veA* strain growing in submerged cultures, *brlA* expression is not sufficient to initiate conidiation in *A. parasiticus* (Calvo et al. 2004).

In our study, we observed that deletion of the *brlA* gene caused a slight overexpression of *veA* (Log_2 Fold Change= 0.89). This result was not observed for the other members of the velvet complex, where on the contrary *velB* (Log_2 Fold Change= -0.53) and *laeA* (Log_2 Fold Change= -0.44) are down-regulated.

These results suggest that there is a regulation in conidiation that could be linked to a self-regulation between *veA* and *brlA* with as yet unknown and more complex mechanisms, which would explain the different results. A transcriptomic analysis of the null mutant PeΔ*veA* and PeΔ*brlA* strains and of a double mutation could help to better understand the relationship between *veA* and *brlA*, two important genes in the reproduction of fungus and to study more extensively the effect of *veA* in conidiation.

Pathogenicity and Patulin Production; a potential increase detected in the mutant PeΔ*brlA* strain

P. expansum is considered the main cause of the blue mould disease and an important producer of the mycotoxin patulin. Due to the toxicity of patulin, the maximum level of patulin detected in food was regulated in some countries, including the United States, China and the European Union. In order to study if the null mutant PeΔ*brlA* strain could also trigger blue mold disease, Golden Delicious apples were infected. We observed that the first six days of incubation the mutant strain had a similar development profile to the WT strain, however, from the ninth day a significant increase in the rot diameter of the mutant PeΔ*brlA* strain was observed. These results could be supported by the increase of patulin detected in the null mutant PeΔ*brlA* strain, where the concentration was four times higher than that produced by the WT strain in Golden Delicious apples. Since patulin has been considered an important factor of aggressiveness in the pathogenicity of *P. expansum* (Ballester et al. 2015; Sanzani et al. 2012; Snini et al. 2016b), we suggest that a higher production of patulin caused an increase in the rate and rot diameter of the fungus. Similar effects were observed in *P. expansum* when disruption of *patL* (Snini et al. 2016), *patK* (Sanzani et al. 2012) and *veA* (EL Hajj Assaf et al. 2018), three important genes in patulin biosynthesis affected the aggressiveness and virulence in the mutant strains. These disruptions resulted in a decrease or abolition of patulin production in the null mutant strains, but also reduced the rotting diameters in the apples inoculated with the null mutant strains. However, when exogenous patulin was added, the growth rate was restored in all cases, suggesting that patulin plays an aggressive role in facilitating the growth of *P. expansum* in the fruit flesh.
Nevertheless, how is it possible that the removal of the *brlA* gene led to an overproduction of patulin in apples? To answer this question; we analyzed the results obtained from the in vitro

transcriptomic analysis to determine if the elimination of *brlA* had caused an up-regulation of the gene cluster involved in patulin biosynthesis. Surprisingly, we noticed that 12 out of 15 genes in the cluster were down-regulated and only three (*patM*, *patC* and *patD*) were up-regulated (Figure 2). This could contradict the idea that the overproduction of patulin in the null mutant PeΔ*brlA* strain came from an up-regulation of the patulin biosynthetic cluster.

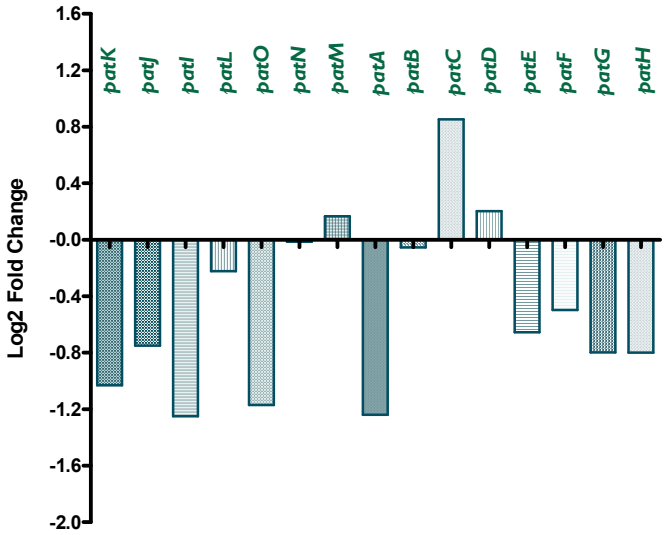


Figure 2. Fold Change expression of the 15 genes involved in the patulin biosynthetis pathway in the null mutant PeΔ*brlA* strain compared to the wild type strain.

Among the genes that are up-regulated, two of the genes code for transporters, *patC*, a major facilitator superfamily transporter, and *patM,* an ATP-binding cassette transporter. Considering the hypothetical schemes of the spatial organization of the patulin biosynthesis pathway in fungal cells described by Snini (2014) and Li et al. (2019) (Figure 3): Acetate of the cell is transported by the acetate carrier (PATA) located on the peroxisome membrane and then transformed into acetyl-CoA within the peroxisome. From acetyl-CoA, the PKS (PATK, 6-methylsalicylic acid synthase) synthesizes 6-methylsalicylic acid, which is then released in the cytoplasm. Patulin biosynthesis continues with the other enzymes of the pathway, with some modifications according to the authors. Finally, patulin then excreted in the external environment (Snini, 2014; Li *et al.*, 2019), probably by transporter(s) (PATC and/or PATM). This leads us to the hypothesis that, although there is a slight down-regulation of several genes involved in the biosynthesis of patulin, patulin would be completely excreted in the invaded medium (without accumulation in the mycelium) due to the overexpression of the transporter genes.

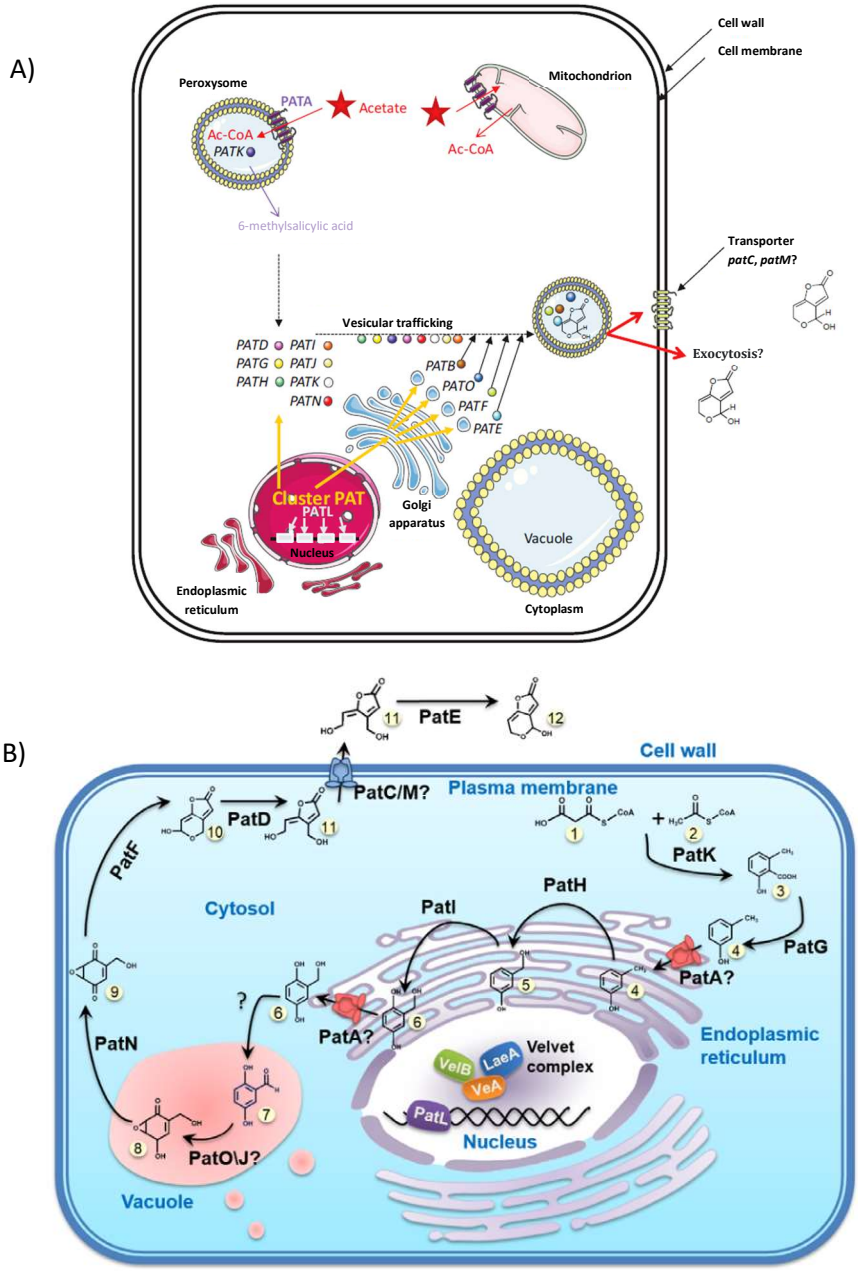
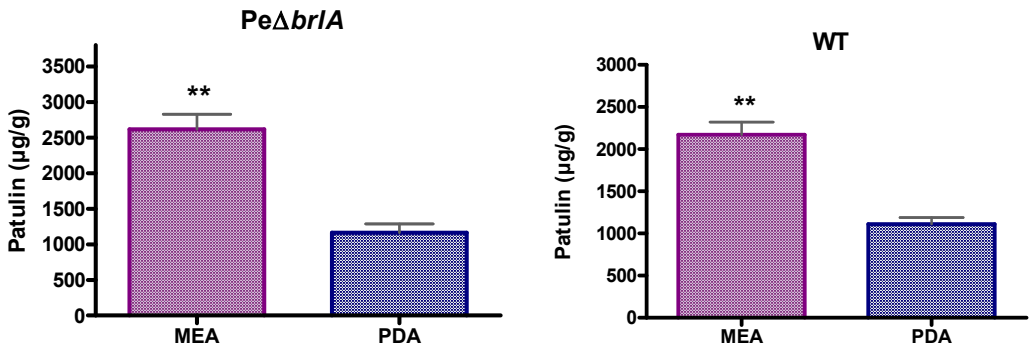


Figure 3. Hypothetical scheme of the spatial organization of the patulin biosynthesis pathway in fungal cells A) by Snini (2014) and B) Li et al. (2019).

Since the results of the transcriptomic analysis did not reflect the observed in vivo on Golden Delicious apples, we suggest that the overproduction of patulin may be related to other factors. For example, light, pH, temperature, and carbon and nitrogen sources often act as important signals for a large number of cellular events, including growth, reproduction, pathogenicity, and biosynthesis of secondary metabolites (Keller 2019; Macheleidt et al. 2016).

Dombrink-Kurtzman and Blackburn (2005) compared six different liquid culture media to determine maximum production of patulin, in five different *Penicillium* species (*Penicillium expansum, Penicillium griseofulvum, Penicillium clavigerum, Penicillium coprobium* and *Penicillium sp*.). They found that the highest production of patulin in *P. expansum* was obtained in a manganese-enriched potato dextrose (PDA) medium at 96 h incubation. In *P. griseofulvum*, favored growth was observed when the strain grew in PDA medium compared to Malt Extract Agar (MEA) medium. Production of patulin and griseofulvin was detected at 5 dpi and especially at 7 dpi (Valente et al. 2020). When we compared the patulin production of the null mutant PeΔ*brlA* and WT strains under different conditions, we observed that both strains produced significantly more patulin when grown in the MEA (Biokar diagnostics, Allonne, France; 30 g/L malt extract, 15 g/L agar) medium compared to the PDA medium (Merck KGaA, Darmstadt, Germany; 30 g/L potato extract, 15 g/L agar) (Figure 4a) contrary to that observed in *P. griseofulvum* (Valente et al. 2020).For the transcriptomic study, we thus used strains grown in (MEA) culture medium and the strains were incubated for five days at 25° C in the dark. Growing time may also be a consideration. In order to determine when *P. expansum* begins to produce patulin Snini et al. (2016) conducted a kinetic study on *P. expansum*-infected Golden Delicious apples. They found that the strain begins to produce patulin from the fourth day of incubation gradually increasing the concentration over the days. The results of our study showed that the two strains,, PeΔ*brlA* and WT, had significantly higher production at seven days post inoculation (dpi) than at five dpi (Figure 4b). Although there is no difference in the concentration of patulin detected in vitro between the PeΔ*brlA* and WT strain at 7 dpi in the MEA medium, a slightly higher production of patulin is observed in the null mutant strain (Figure 4c). Since for the transcriptomic study we used a five-day incubation period to improve the quality of RNAs (beyond this time, the RNAs are not of sufficient quality) in the MEA medium, these factors could justify the different results observed in vivo and in vitro studies. The variability in patulin production is linked to the growth media used and the incubation time.

A)



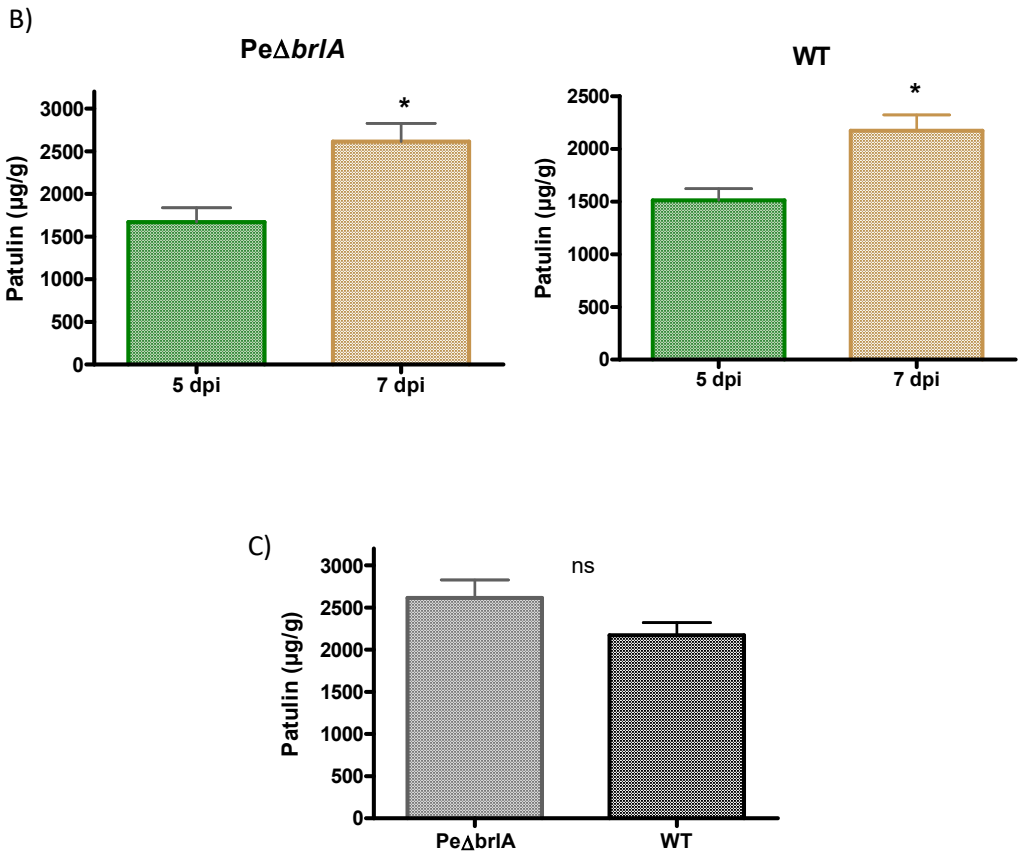


Figure 4. Effect of medium and incubation period on patulin production. A) MEA medium vs PDA medium, B) 5 dpi vs 7 dpi in MEA medium C) Pe*ΔbrlA* vs WT in MEA medium at 7dpi. dpi= days post-inoculation.

The transcriptomic studies were performed with strains grown in MEA medium covered with sterile cellophane sheets that facilitated the separation between mycelium and agar to obtain good quality RNAs. In the Figure 5, we found that patulin production was not compromised under these unfavorable conditions. However, the in vitro conditions do not completely resemble what happened in the natural environment. Although our in vitro RNA extraction methodology is a robust technique, the ideal would be to perform RNA extraction in vivo, i.e. directly from the fruit to actually observe what happens in the fungus when it grows in its natural substrate. RNA extraction of the fungus from fruits is a technique used by several laboratories, each with a different methodology (Ballester et al. 2006; Gasic et al. 2004; López-Pérez et al. 2015; Sánchez-Torres et al. 2018; Touhami et al. 2018; Vilanova et al. 2014).

RNA extraction from apples is a methodology that should be studied, developed and applied in our laboratory for a better understanding of the fungal development and production of secondary metabolites in pathogenic fungi such as *P. expansum*.

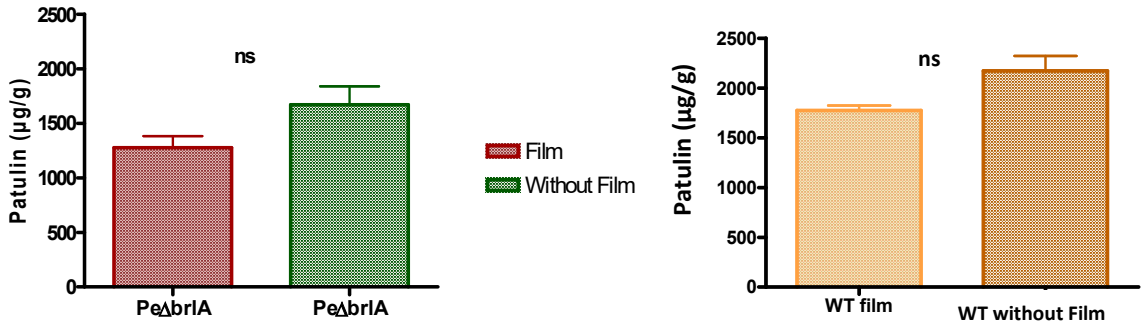


Figure 5. Detection and quantification of patulin production in the mutant PeΔ*brlA* and WT strain grown in MEA medium with and without sterile cellophane sheets.

From another point of view, VeA is a phosphoprotein belonging to the velvet family, which is involved in the regulation of several cellular processes including morphogenesis, response to oxidative stress, sexual development or formation of cleistothecia/sclerotia, asexual development or conidiation and secondary metabolism (Rauscher et al. 2016; Sarikaya-Bayram et al. 2015). It is a founding member of the velvet complex, VelB-VeA-LaeA required for the coordination of fungal development and biosynthesis of secondary metabolites (Sarikaya-Bayram et al. 2015). In *A. nidulans*, VeA it acts as a negative conidial regulator and is located upstream (Figure 7, Part 3 of introduction) of the *brlA* gene (Ojeda-López et al. 2018). Deletion of the *veA* gene in *A. niger* led to a decreased *brlA* expression resulting in a reduction in the number of conidia, while vegetative growth was favored (Zhang et al. 2018). By contrast, elimination of the *brlA* gene in *P. expansum* caused a slight increase in the expression of *veA* (Log_2Fold Change= 0.89, *p*-value= 1.05 x 10^{-5}) in the null mutant PeΔ*brlA* strain, this could suggest that *veA* and *brlA* are autoregulated or that they follow different pathways depending on the species. In addition, the high patulin production detected in vivo could be due to the increased expression of *veA*, since the disruption of *veA* in *P. expansum* caused the abolition of patulin production in vivo and in vitro studies (EL Hajj Assaf et al. 2018).

A putative gene cluster of chaetoglobosins in *P. expansum*

Under appropriate conditions, *P. expansum* is able to produce, in addition to patulin, a wide diversity of metabolites with a broad structural spectrum. As mentioned in previous chapters, the *brlA* gene deletion resulted in increased biosynthesis of chaetoglobosins and derivatives. Metabolomic analysis showed that the null mutant PeΔ*brlA* strain produced 15 chaetoglobosin derivatives including chaetoglobosin A, C and B/G, one penochalasin and 18 compounds that also appear to belong to the cytochalasan alkaloid family (chaetoglobosins/cytochalasins). The

production of several chaetoglobosins has already been detected in different isolates of *P. expansum* (Andersen et al. 2004). Chaetoglobosins have a hybrid polyketide/non-ribosomal peptide origin. In the transcriptomic study, we therefore focused on the analysis of genes encoding backbone enzymes, polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS) involved in the secondary metabolism in *P. expansum*. We observed an up-regulation of two genes, PEXP_008740 and PEXP_074060, that encode an hybrid PKS/NRPS. An AntiSMASH (antibiotics & Secondary Metabolite Analysis Shell) (Medema *et al.*, 2011) analysis showed that PEXP_074060 belongs to a putative biosynthetic gene cluster. This cluster is composed of 11 genes, PEXP_073960-PEXP_074060, which were up-regulated in the null mutant strain compared to the WT strain (Table 6, Chapter 2). Schümann and Hertweck (2007) characterized the chaetoglobosin cluster in *P. expansum* (Figure 7, Chapter 1, Part 2), which consists of seven genes (*cheA*-*cheG*). However, only *cheF* (PEXP_043620), coding for a regulator, is present in all eight *P. expansum* genomes sequenced and available in GenBank. This result is in agreement with that reported by Ballester *et al.* (2015), who did not find this chaetoglobosin gene cluster in any of the sequences of the *P. expansum* strains studied.

A Blast search of each *che* gene reveals a homologue for each gene in the genomes of *Aspergillus novofumigatus*, *A. lentulus* and *A. fumigatiaffinis*. These species are very phylogenetically close. They belong to the *Fumigati* section. An Antismash analysis of the genomes of these three species shows the existence of the same cluster in all three species (Figure 6). This cluster contains all the genes of the Che cluster and displays the same topology with the same orientation of the genes. The analysis also shows similarities with the clusters of ilicicolin H, aspyridone and tennelin (Lin X et al. 2019; Zhang et al. 2019; Eley et al. 2007,
The structures of ilicicolin H and aspyridone do not resemble those of chaetoglobosins. Therefore, the production of chaetoglobosins by these three species has never been reported (Frisvad and Larsen, 2016).

AntiSMASH analysis revealed that our putative cluster of 11 genes shares a strong similarity with the chaetoglobosin gene cluster identified in *Chaetomium globosum* (Ishiuchi et al. 2013). The gene cluster of chaetoglobosins in *Chaetomium globosum* contains only nine genes (CHGG_01237- CHGG_01244). However, the two genes located directly downstream, CHGG_01245 and CHGG_01246, have a corresponding homolog in *P. expansum*, PEXP_074030 and PEXP_074050. Another difference between the clusters of the two species is the absence in *P. expansum* of two homologous genes, CHGG-01238, which codes for a transposase and CHGG_01237, which codes for a regulator. In *P. expansum*, the latter is replaced by another transcription factor homologous to the cytochalasin cluster-specific regulator in *A. clavatus* (Qiao et al. 2011).

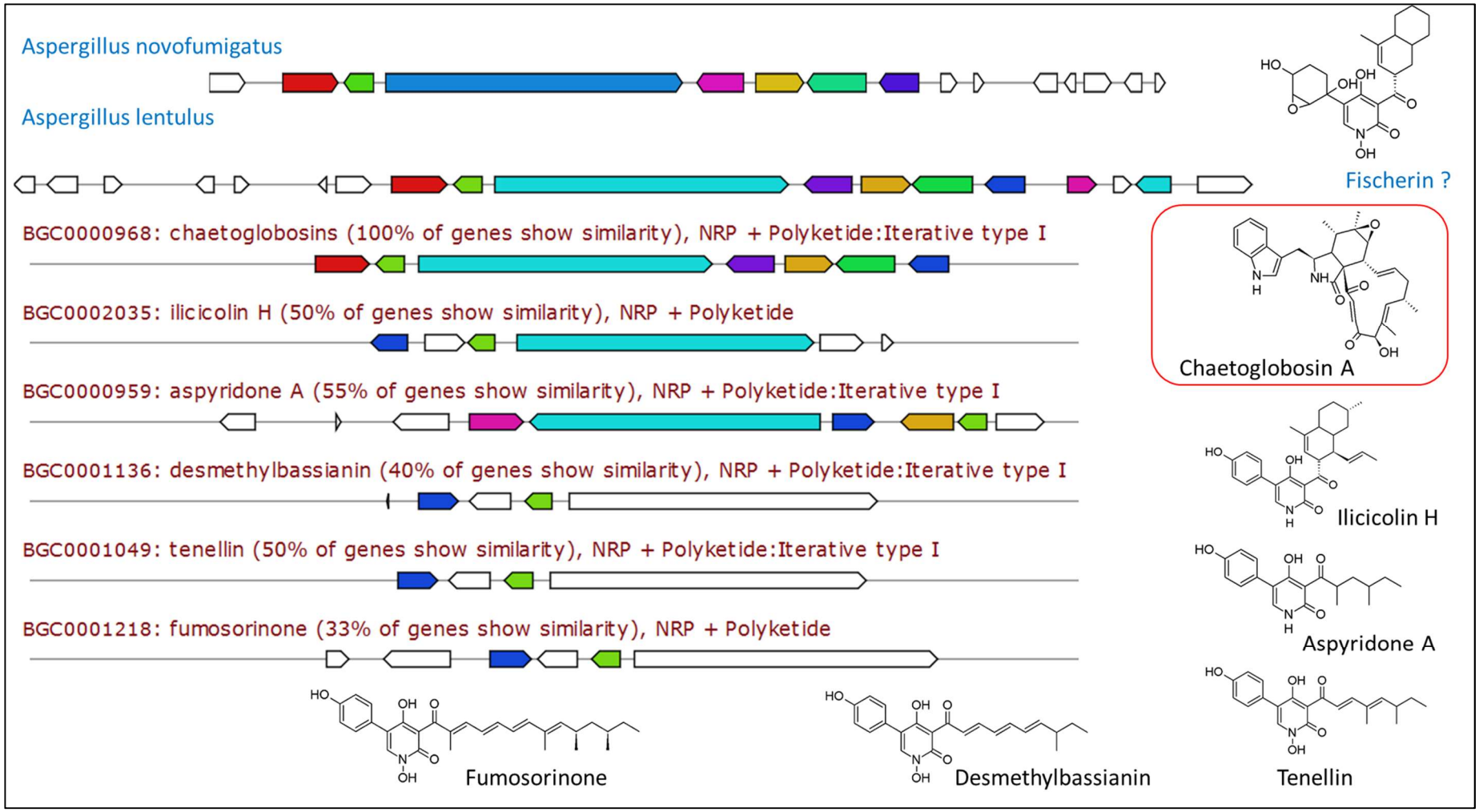


Figure6. Cluster of genes homologous to *che* genes in *Aspergillus novofumigatus*, *A. lentulus* and *A. fumigatiaffinis.*

This suggests that the cluster of chaetoglobosins in *P. expansum* may actually consist of 11 genes, among which the gene PEXP_074020 would code for a transcription factor and the gene PEXP_074060 for a PKS/NRPS. To confirm this hypothesis, it is necessary to validate and characterize the gene cluster in *P. expansum*.

Our laboratory has been involved for several years in the development of techniques to find biosynthetic gene clusters and to investigate the functions of the genes involved in secondary metabolite biosynthesis. Among these works, we can highlight the elucidation of the patulin gene cluster in *P. expansum* (Tannous et al. 2014) –concomitantly with Ballester *et al.* (2015) and Li *et al.* (2015)- and the elucidation of the function of *patL* (Snini et al. 2016), and the cytochrome P450s (*patH* and *patI*) (Artigot et al. 2009) in patulin biosynthesis pathway. These resulst were confirmed by Li *et al.* (2019).

During this work, the identification of homologous genes of the putative cluster was carried out. To confirm the results of the microarray, primers were designed to evaluate the expression of all the genes of the supposed cluster using qPCR (Suppl. Material, Chapter 2). The proposed next step would be the validation of the cluster. For this purpose the genome walking technique can be used, which allows "walking" along the genome in small segments to check the order and direction of each gene involved in the chaetoglobosin cluster.

Further analysis would include characterization of the cluster, which would define the role of each gene within the cluster. For this purpose, the heterologous expression in *Saccharomyces cerevisiae* can be used. However, the disadvantage of this methodology is the commercial availability of the hypothetical substrates necessary for the biochemical reactions. On the other hand, the methodology of creating mutant strains of specific genes by homologous recombination, is increasingly used in our laboratory and would allow to determine the role of each gene within the cluster.

These studies would confirm that the cluster of 11 up-regulated genes in the null mutant PeΔ*brlA* strain is the true cluster of chaetoglobosins in *P. expansum*.

GENERAL CONCLUSION

To conclude, this thesis work allowed us to investigate and analyze the secondary metabolism of *Penicillium expansum*. The results showed that *P. expansum* is a major producer of potentially bioactive compounds. Although most of the secondary metabolites (65%) have been identified or reported to belong to a known SM family, and described as produced by this strain, there is still a wide diversity of compounds to be identified. SMs are closely related to the stages of development of the fungus and although they are not considered essential compounds, they can provide selective advantages such as protection and defense against hostile environmental conditions for survival.

P. expansum is a pathogenic fungus that affects pomaceous fruits, mainly apples. It causes blue mold disease and is the main producer of patulin. However, under appropriate growth conditions, we observed that this fungus was able to produce a wide diversity of metabolites, as demonstrated by the use of a non-targeted metabolomic approach combining stable isotope labeling with LC-HRMS that provides high possibilities for the discovery of new drugs or mycotoxins.

In order to decipher the spatio-temporal organization of secondary metabolism that takes place in *P. expansum* we performed the creation and characterization of the null mutant PeΔ*brlA* strain, lacking a key transcription factor involved in asexual reproduction in *P. expansum*. Metabolomic analyses of this strain as well as of the null mutant PeΔ*veA* strain allowed us to understand the impact of the deletion of these genes on the development and secondary metabolism of *P. expansum* as well as the close relationship between both genes. VeA and BrlA are essential in the last events that occurs at the end of apple infection. VeA is necessary for the epicarp perforation since this TF is involved in the synnemata formation, while BrlA is essential for the conidiophore development at a later stage.

We revealed that there was a significant difference between the compounds excreted in the medium by the basal mycelium and those detected in the fungal extract (aerial mycelium, conidiophores, spores). Detected essentially in medium extract (in vitro) and apple flesh (in vivo), patulin and citrinin were specific to the colonization phase of the fungus and by contrast, they were not detected in the conidiophores. VeA is required for a normal patulin and citrinin production . The PeΔ*brlA* strain did not lose its ability to produce patulin and even the suppression of the *brlA* gene results in overproduction of patulin in vivo. The absence of patulin in synnemata localized outside the fruit confirms definitively that patulin production was not linked to conidiogenesis in *P. expansum*.

Our analyzes showed the presence of other secondary metabolite produced by the fungus when it colonized the apple. It would be important to evaluate whether they play an active role in the *P. expansum* pathogenicity.

In recent years, research on bioactive compounds produced by fungi has been increasing. On one hand, identification of SMs and the knowledge of their roles and regulation are crucial for the understanding of fungal development, and could therefore provide leads for new control strategies. On the other hand, this important source of active molecules could allow the development of new drugs, or highlight new mycotoxins that could represent a health problem for the population.

Annex A

Table 1. Comparison of secondary metabolites detected in WT NRRL35695 PeΔ*veA* and PeΔ*brlA* strains after culture on labeled wheat grains

Molecular formula	Identifier [a]	Proposed identification	WT	PeΔ*veA*	PeΔ*brlA*	Molecular formula	Identifier [a]	Proposed identification	WT	PeΔ*veA*	PeΔ*brlA*
$C_7H_6O_4$	**Pexp_153.019_3.59**	**patulin**	+	ND	+	$C_{28}H_{38}N_4O_6$	Pexp_527.287_12.46	VAL-PHE-VAL-TYR	+	+	+
C_7H_8O	Pexp_109.065_7.11	m-Cresol	+	ND	+	**$C_{28}H_{38}O_7$**	**Pexp_485.254_35.32**		+	+	+
$C_7H_8O_2$	Pexp_125.060_6.79	m-Hydroxybenzyl alcohol	+	ND	+	$C_{28}H_{38}O_7$	Pexp_487.270_36.21	Andrastin A	+	+	+
$C_7H_8O_3$	Pexp_141.054_3.8	Gentisyl alcohol	+	ND	+	$C_{28}H_{38}O_8$	**Pexp_501.247_27.70**		+	+	+
$C_7H_8O_4$	Pexp_157.049_2.67	Ascladiol	+	ND	+	**$C_{28}H_{38}O_8$**	**Pexp_501.247_28.44**		+	+	ND
$C_7H_{10}O_3$	Pexp_143.070_4.52		+	+	+	**$C_{28}H_{38}O_8$**	**Pexp_501.247_29.57**		+	+	+
$C_{10}H_{17}NO_5$	Pexp_232.117_2.42		ND	+	ND	**$C_{28}H_{40}O_6$**	**Pexp_471.274_39.48**	**Andrastin C**	+	+	+
$C_{10}H_{17}NO_5$	Pexp_232.118_8.83		+	+	ND	**$C_{28}H_{40}O_7$**	**Pexp_487.268_30.58**	**Andrastin B**	+	+	+
$C_{13}H_{14}O_5$	Pexp_251.091_21.70	Citrinin	+	ND	+	$C_{29}H_{27}N_5O_5$	Pexp_526.206_15.70		ND	ND	+
$C_{15}H_{18}N_2$	Pexp_227.155_6.44	Aurantioclavine	+	+	ND	$C_{29}H_{27}N_5O_5$	Pexp_526.206_17.61		ND	ND	+
$C_{15}H_{19}NO_6$	Pexp_310.129_12.78		+	+	+	$C_{29}H_{31}N_5O_5$	Pexp_530.238_14.83		ND	ND	+
$C_{15}H_{19}NO_6$	Pexp_310.129_14.80		+	+	+	$C_{29}H_{31}N_5O_5$	Pexp_530.238_15.85		ND	ND	+
$C_{15}H_{20}O_4$	Pexp_265.144_15.91	Expansolide C/ D	+	+	+	$C_{29}H_{31}N_5O_5$	Pexp_530.238_16.68		ND	+	+
$C_{15}H_{20}O_4$	Pexp_265.144_16.91		+	ND	ND	$C_{29}H_{33}N_5O_6$	Pexp_548.248_11.36		ND	ND	+
$C_{15}H_{20}O_4$	Pexp_265.144_18.49	Expansolide C/ D	+	+	+	$C_{29}H_{33}N_5O_6$	Pexp_548.248_12.14		ND	ND	+
$C_{15}H_{20}O_4$	Pexp_265.144_19.35		+	ND	+	$C_{29}H_{33}N_5O_6$	Pexp_548.251_15.42		+	+	ND
$C_{16}H_{18}N_2O_2$	Pexp_271.144_7.62	Clavicipitic acid	+	+	+	$C_{30}H_{36}N_4O_2$	Pexp_485.292_16.36	New DMV[1] communesin-2	+	+	ND
$C_{16}H_{18}N_2O_2$	Pexp_271.144_8.45	Clavicipitic acid	+	+	+	$C_{30}H_{39}N_5O_5$	Pexp_550.302_18.25		ND	+	ND
$C_{16}H_{26}N_2O_4S_2$	Pexp_375.142_22.02		+	+	+	$C_{30}H_{39}N_5O_5$	Pexp_550.302_19.14		ND	+	ND

$C_{17}H_{17}NO_7$	Pexp_348.108_11.75		ND	+	ND	$C_{31}H_{36}N_4O_2$	Pexp_497.290_31.66	Putative new undetermined communesin	+	+	ND
$C_{17}H_{17}NO_7$	Pexp_348.108_12.65		ND	+	ND	$C_{32}H_{34}N_4O_3$	Pexp_523.271_31.84	Communesin D[g]	+	+	+
$C_{17}H_{17}NO_7$	Pexp_348.108_15.90		ND	+	ND	$C_{32}H_{36}N_2O_4$	Pexp_513.276_39.10	Chaetoglobosin J or Prochaetoglobosin III	+	+	+
$C_{17}H_{22}O_5$	Pexp_307.154_27.39	Expansolide A/B	+	+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_20.92	Chaetoglobosin B/G	+	ND	+
$C_{17}H_{22}O_5$	Pexp_307.155_30.19	Expansolide A/B	+	+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.268_23.19	Chaetoglobosin B/G	ND	ND	+
$C_{17}H_{23}N_3O_3$	Pexp_318.179_3.12		ND	ND	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_25.21	Chaetoglobosin B/G	+	ND	+
$C_{17}H_{23}N_3O_3$	Pexp_318.180_25.05		ND	ND	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_26.71	Chaetoglobosin B/G	+	+	+
$C_{18}H_{16}N_2O_2$	Pexp_293.129_13.58		+	+	ND	$C_{32}H_{36}N_2O_5$	Pexp_529.270_29.56	Chaetoglobosin B/G	+	ND	+
$C_{19}H_{16}N_2O_2$	Pexp_305.129_32.50		+	+	ND	$C_{32}H_{36}N_2O_5$	Pexp_529.270_30.51	Chaetoglobosin B/G	+	+	+
$C_{19}H_{16}N_2O_2$	Pexp_305.129_33.17		+	+	ND	$C_{32}H_{36}N_2O_5$	Pexp_529.268_32.43	Chaetoglobosin B/G	ND	ND	+
$C_{19}H_{16}N_2O_4$	Pexp_337.119_13.73		+	+	ND	$C_{32}H_{36}N_2O_5$	Pexp_529.270_33.98	Chaetoglobosin A	+	+	+
$C_{19}H_{16}N_2O_4$	Pexp_337.119_15.20		+	+	ND	$C_{32}H_{36}N_2O_5$	Pexp_529.267_34.25	Chaetoglobosin B/G	ND	ND	+
$C_{19}H_{20}O_5$	Pexp_329.137_33.29		ND	ND	+	$C_{32}H_{36}N_2O_5$	Pexp_529.267_35.49	Chaetoglobosin B/G	ND	ND	+
$C_{19}H_{21}NO_7$	Pexp_376.139_17.45		+	+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_36.68	Chaetoglobosin C	+	+	+
$C_{19}H_{21}NO_7$	Pexp_376.139_18.65		+	+	+	$C_{32}H_{36}N_2O_5$	Pexp_529.270_37.41	Chaetoglobosin B/G	+	ND	+
$C_{19}H_{38}O_6$	**Pexp_361.258_37.23**		+	+	ND	$C_{32}H_{36}N_2O_5$	Pexp_529.268_38.29	Chaetoglobosin B/G	ND	ND	+
$C_{19}H_{38}O_6$	**Pexp_361.258_38.19**		+	+	ND	$C_{32}H_{36}N_2O_6$	Pexp_545.263_27.60	Putative cytochalasan	+	ND	+
$C_{20}H_{18}N_2O_2$	Pexp_319.145_35.48		+	+	ND	$C_{32}H_{36}N_2O_6$	Pexp_545.262_29.23	Putative cytochalasan	ND	ND	+
$C_{20}H_{18}N_2O_2$	Pexp_319.145_36.44		+	+	ND	$C_{32}H_{36}N_2O_6$	Pexp_545.262_30.57	Putative cytochalasan	ND	+	+
$C_{20}H_{21}NO_9$	Pexp_420.128_19.29		+	+	+	$C_{32}H_{36}N_2O_6$	Pexp_545.262_31.50	Putative cytochalasan	+	ND	+
$C_{20}H_{26}O_8$	Pexp_395.171_13.61		+	+	+	$C_{32}H_{36}N_2O_6$	Pexp_545.262_32.48	Putative cytochalasan	ND	ND	+
$C_{22}H_{23}N_5O_2$	Pexp_390.193_15.09	Roquefortine C	+	+	+	**$C_{32}H_{36}N_2O_6$**	**Pexp_543.248_34.05**	Putative cytochalasin	+	ND	+
$C_{22}H_{25}N_5O_2$	Pexp_392.209_9.99	Roquefortine D	+	+	+	$C_{32}H_{36}N_4O_2$	Pexp_509.292_36.01	Communesin B[g]	+	+	+
$C_{23}H_{24}N_2O_6$	Pexp_425.171_26.92		+	+	+	$C_{32}H_{36}N_4O_4$	Pexp_541.280_07.78	Com540[b]	ND	+	ND

$C_{24}H_{26}N_2O_6$	**Pexp_437.170_33.79**		+	+	ND	$C_{32}H_{38}N_2O_6$	Pexp_515.288_35.59	Putative cytochalasan	+	+	+
$C_{26}H_{30}N_4$	Pexp_399.255_19.94	Communesin K[b]	+	+	ND	$C_{32}H_{38}N_2O_6$	Pexp_515.288_37.93	Putative cytochalasan	+	+	+
$C_{26}H_{30}N_4O$	Pexp_415.250_14.62	Communesin I[c]	+	+	+	$C_{32}H_{38}N_2O_6$	Pexp_515.289_38.15	Putative cytochalasan	+	+	+
$C_{26}H_{30}N_4O$	Pexp_415.250_18.43	Communesin I[c]	+	+	+	$C_{32}H_{38}N_2O_5$	Pexp_531.283_21.75	Putative cytochalasan	ND	ND	+
$C_{26}H_{32}O_8$	Pexp_473.215_9.67		ND	ND	+	$C_{32}H_{38}N_2O_6$	Pexp_531.289_22.76	Putative cytochalasan	+	ND	+
$C_{26}H_{32}O_8$	Pexp_473.215_10.64		ND	ND	+	$C_{32}H_{38}N_2O_5$	Pexp_531.284_23.91	Putative cytochalasan	ND	+	+
$C_{26}H_{32}O_8$	Pexp_473.214_34.4		ND	ND	+	$C_{32}H_{38}N_2O_6$	Pexp_531.283_27.38	Putative cytochalasan	+	+	+
$C_{26}H_{40}O_6$	Pexp_449.289_29.93		+	+	+	$C_{32}H_{38}N_2O_6$	Pexp_531.283_28.52	Putative cytochalasan	+	+	+
$C_{27}H_{29}N_5O_4$	Pexp_488.227_10.80		ND	ND	+	$C_{32}H_{36}N_4O_3$	Pexp_525.287_13.45	New DME[2] communesin-2	+	+	ND
$C_{27}H_{29}N_5O4$	Pexp_488.227_11.03		ND	ND	+	$C_{32}H_{36}N_4O_3$	Pexp_525.287_27.91	New DMV[1] communesin-3	+	+	ND
$C_{27}H_{30}N_4O$	Pexp_427.249_10.19		ND	+	+	$C_{32}H_{38}N_4O_2$	Pexp_511.306_36.13	New DME[2] communesin-3	+	+	ND
$C_{27}H_{30}N_4O_2$	Pexp_443.245_15.03	Communesin E[d]	+	+	+	$C_{32}H_{38}N_4O_3$	Pexp_527.303_21.41	New DME[2] communesin-4	+	+	ND
$C_{28}H_{30}N_4O_3$	Pexp_471.239_19.56	Com470[e]	+	+	ND	$C_{32}H_{38}N_4O_4$	Pexp_543.297_19.36	New DME[2] communesin-5	+	+	ND
$C_{28}H_{31}N_5O_5$	Pexp_518.241_16.36		+	+	+	$C_{32}H_{38}N_2O_5$	Pexp_531.283_28.02	Chaetoglobosin E [h]	ND	ND	+
$C_{28}H_{31}N_5O_5$	Pexp_518.240_17.21		+	+	+	$C_{32}H_{40}N_4O_2$	Pexp_513.322_36.57	New DME[2] communesin-6	+	+	ND
$C_{28}H_{32}N_4O$	Pexp_441.265_17.14	Communesin F[d]	+	+	ND	$C_{32}H_{38}N_2O_5$	Pexp_531.283_31.56	Penochalasin F [h]	ND	+	+
$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.27	New DMV[1] communesin-1	+	+	ND	$C_{32}H_{38}N_2O_6$	Pexp_547.278_23.19	Putative cytochalasan	ND	ND	+
$C_{28}H_{32}N_4O_2$	Pexp_457.260_14.97	New DME[2] communesin-1	+	ND	ND	$C_{32}H_{38}N_2O_6$	Pexp_547.278_25.47	Putative cytochalasan	ND	+	+
$C_{28}H_{32}N_4O_2$	Pexp_457.261_23.65	Communesin A[f]	+	+	+	$C_{32}H_{38}N_2O_6$	Pexp_547.278_27.09	Putative cytochalasan	ND	+	+
$C_{28}H_{32}N_4O_3$	Pexp_473.255_15.50		+	+	ND	$C_{32}H_{38}N_2O_6$	Pexp_547.278_28.27	Putative cytochalasan	ND	ND	+
$C_{28}H_{36}N_4O_4$	Pexp_493.279_31.47	Fungisporin A or cyclo(VFVF)	+	+	+	$C_{33}H_{38}N_4O_5$	Pexp_571.293_18.22	Com570[e]	+	+	ND
$C_{28}H_{38}N_4O_5$	Pexp_511.290_18.01	VAL-PHE-VAL-PHE	+	+	+	$C_{37}H_{42}N_4O_5$	Pexp_623.325_29.52	Com622[e]	+	+	ND

Annex A

Table 2. Secondary metabolites detected in Golden Delicious apples infected with the null mutant Pe*ΔbrlA,* Pe*ΔveA* and WT strain.

Molecular formula	R_T (min) [a]	^{12}C *m/z* (Da)	Proposed identification	14 dpi Pe*ΔveA*	14 dpi WT	30 dpi WT	30 dpi Pe*ΔbrlA*	Molecular formula	R_T (min) [a]	^{12}C *m/z* (Da)	Proposed identification	14 dpi Pe*ΔveA*	14 dpi WT	30 dpi WT	30 dpi Pe*ΔbrlA*
$C_7H_6O_4$	**3.59**	**153.01919**	**Patulin**	ND	+	+	+	$C_{26}H_{40}O_6$	29.93	449.28939		+	+	ND	ND
$C_7H_8O_3$	3.80	141.05493	Gentisyl alcohol	ND	ND	ND	+	$C_{28}H_{38}O_7$	36.21	487.27063	Andrastin A	+	+	+	+
$C_7H_8O_2$	6.79	125.05998	m-Hydroxybenzyl alcohol	ND	+	ND	ND	**$C_{28}H_{38}O_8$**	**28.44**	**501.24792**		ND	ND	+	+
$C_7H_8O_4$	2.67	157.04990	Ascladiol	ND	+	+	+	**$C_{28}H_{38}O_8$**	**29.57**	**501.24786**		+	+	+	+
$C_{10}H_{17}NO_5$	8.83	232.11872		ND	ND	+	+	**$C_{28}H_{40}O_7$**	**30.58**	**487.26898**	**Andrastin B**	+	+	+	+
$C_{13}H_{14}O_5$	21.70	251.09108	Citrinin	ND	ND	+	+	$C_{29}H_{27}N_5O_5$	17.61	526.20689		ND	ND	ND	+
$C_{16}H_{26}N_2O_4S_2$	22.02	375.14202		ND	ND	+	+	$C_{32}H_{36}N_2O_4$	39.10	513.27635	Chaetoglobosin J or Prochaetoglobosin III	ND	ND	ND	+
$C_{17}H_{22}O_5$	27.39	307.15471	Expansolide A/B	ND	ND	+	+	$C_{32}H_{36}N_2O_5$	20.92	529.26776	Chaetoglobosin B/G	ND	ND	ND	+
$C_{17}H_{22}O_5$	30.19	307.15504	Expansolide A/B	ND	ND	+	+	$C_{32}H_{36}N_2O_5$	23.19	529.26807	Chaetoglobosin B/G	ND	ND	ND	+
$C_{19}H_{21}NO_7$	17.45	376.13901		ND	ND	ND	+	$C_{32}H_{34}N_4O_3$	31.84	523.27152	Communesin D	+	ND	ND	ND
$C_{19}H_{21}NO_7$	18.65	376.13904		ND	ND	ND	+	$C_{32}H_{36}N_2O_5$	26.71	529.27029	Chaetoglobosin B/G	ND	ND	+	+
$C_{18}H_{16}N_2O_2$	17.74	293.12915		ND	ND	+	ND	$C_{32}H_{36}N_2O_5$	29.54	529.26898	Chaetoglobosin B/G	ND	ND	+	+
$C_{18}H_{18}N_2O_2$	14.61	295.14479		+	+	+	ND	$C_{32}H_{36}N_2O_5$	30.13	529.27539	Chaetoglobosin B/G	ND	+	+	+
$C_{19}H_{16}N_2O_2$	33.17	305.12933		ND	ND	+	ND	$C_{32}H_{36}N_2O_5$	32.43	529.26807	Chaetoglobosin B/G	ND	ND	ND	+
$C_{19}H_{38}O_6$	**38.19**	**361.25819**		ND	ND	+	ND	$C_{32}H_{36}N_2O_5$	35.67	529.26822	Chaetoglobosin B/G	ND	ND	+	+
$C_{20}H_{18}N_2O_2$	36.44	319.14502		ND	ND	+	ND	$C_{32}H_{36}N_2O_5$	37.41	529.27056	Chaetoglobosin B/G	ND	ND	ND	+
$C_{22}H_{23}N_5O_2$	15.09	390.19390	Roquefortine C	ND	ND	+	+	$C_{32}H_{36}N_4O_2$	36.01	509.29257	Communesin B	+	+	+	ND
$C_{23}H_{24}N_2O_6$	26.92	425.17179		+	ND	+	+	$C_{32}H_{38}N_2O_5$	21.75	531.28384	Putative cytochalasan	ND	ND	ND	+
$C_{24}H_{26}N_2O_6$	**33.79**	**437.17091**		ND	ND	+	ND	$C_{32}H_{38}N_2O_5$	23.91	531.28431	Putative cytochalasan	ND	ND	ND	+
$C_{28}H_{32}N_4O$	17.14	441.26589	Communesin F	ND	ND	+	ND	$C_{32}H_{38}N_2O_6$	23.19	547.27864	Putative cytochalasan	ND	ND	ND	+

$C_{28}H_{32}N_4O_2$	14.27	457.26097	New DMV[1] communesin-1	+	+	+	ND	$C_{32}H_{38}N_2O_6$	25.42	547.27841	Putative cytochalasan	ND	ND	ND	+
$C_{28}H_{32}N_4O_2$	23.65	457.26116	Communesin A	+	+	+	ND	$C_{32}H_{38}N_2O_6$	27.09	547.27842	Putative cytochalasan	ND	ND	ND	+
$C_{28}H_{38}O_7$	**35.32**	**485.25405**		+	+	+	+	$C_{32}H_{38}N_4O_3$	21.41	527.30304	New DME[2] communesin-4	+	ND	+	ND

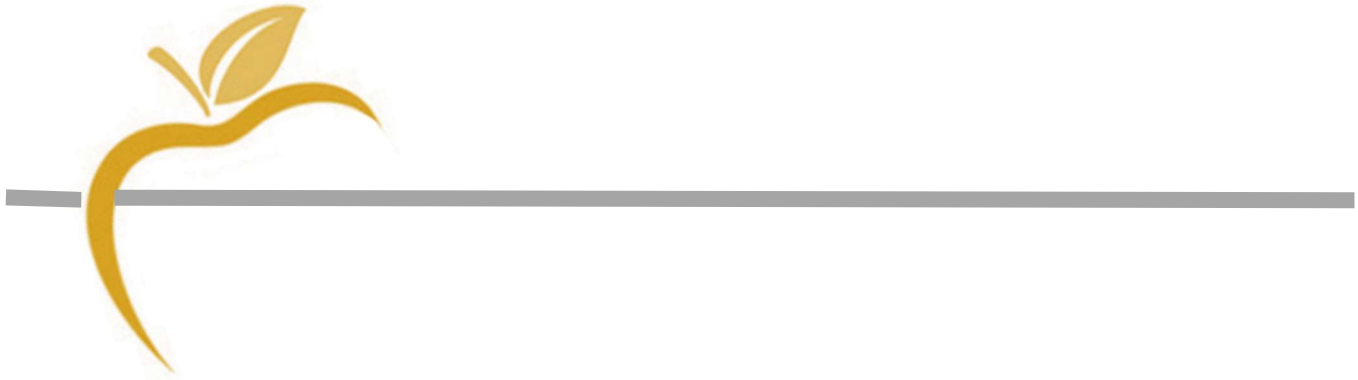
Annex A

Table 3. Secondary metabolites detected in spores and synnemata that pierced the epicarp of apples infected with the WT and PeΔ*brlA* strain (30 dpi)

Molecular Formula	^{12}C *m/z* (Da)	[a] R_T (min)	Proposed Identification	SPO	SYN	Molecular Formula	^{12}C *m/z* (Da)	[a] R_T (min)	Proposed Identification	SPO	SYN
$C_{15}H_{20}O_4$	265.14412	15.91	Expansolide C/ D	ND	+	**$C_{28}H_{38}O_8$**	**501.24792**	**27.70**		ND	+
$C_{15}H_{20}O_4$	265.14415	18.49	Expansolide C/ D	ND	+	**$C_{28}H_{38}O_8$**	**501.24792**	**28.44**		+	+
$C_{16}H_{18}N_2O_2$	271.14496	7.62	Clavicipitic acid	+	+	**$C_{28}H_{38}O_8$**	**501.24786**	**29.57**		+	+
$C_{16}H_{26}N_2O_4S_2$	375.14202	22.02		+	+	**$C_{28}H_{40}O_6$**	**471.27434**	**39.48**	**Andrastin C**	+	+
$C_{17}H_{22}O_5$	307.15471	27.39	Expansolide A/B	+	+	**$C_{28}H_{40}O_7$**	**487.26898**	**30.58**	**Andrastin B**	+	+
$C_{17}H_{22}O_5$	307.15504	30.19	Expansolide A/B	+	+	$C_{29}H_{33}N_5O_6$	548.25180	14.66		ND	+
$C_{18}H_{16}N_2O_2$	293.12915	13.58		+	ND	$C_{32}H_{34}N_4O_3$	523.27152	31.84	Communesin D	+	+
$C_{18}H_{16}N_2O_2$	293.12915	17.74		+	+	$C_{32}H_{36}N_2O_4$	513.27635	39.10	Chaetoglobosin J or Prochaetoglobosin III	+	+
$C_{18}H_{18}N_2O_2$	295.14479	14.61		+	+	$C_{32}H_{36}N_2O_5$	529.27029	26.79	Chaetoglobosin B/G	+	+
$C_{19}H_{16}N_2O_2$	305.12911	32.50		+	ND	$C_{32}H_{36}N_2O_5$	529.26757	29.56	Chaetoglobosin B/G	ND	+
$C_{19}H_{16}N_2O_2$	305.12933	33.17		+	ND	$C_{32}H_{36}N_2O_5$	529.27077	30.51	Chaetoglobosin B/G	+	+
$C_{19}H_{38}O_6$	361.25871	37.17		+	ND	$C_{32}H_{36}N_2O_5$	529.27067	33.36	Chaetoglobosin A	+	+
$C_{19}H_{38}O_6$	361.25819	38.19		+	+	$C_{32}H_{36}N_2O_5$	529.26769	35.49	Chaetoglobosin B/G	ND	+
$C_{20}H_{18}N_2O_2$	319.14588	35.48		+	ND	$C_{32}H_{36}N_2O_5$	529.27063	36.74	Chaetoglobosin C	+	+
$C_{20}H_{18}N_2O_2$	319.14502	36.44		+	ND	$C_{32}H_{36}N_2O_5$	529.27056	37.41	Chaetoglobosin B/G	ND	+
$C_{22}H_{20}N_3O$	**341.15380**	**38.56**		ND	+	$C_{32}H_{36}N_2O_6$	545.26332	27.60	Putative cytochalasan	ND	+
$C_{22}H_{20}N_3O$	**341.15368**	**39.29**		ND	+	$C_{32}H_{36}N_2O_6$	545.26241	29.23	Putative cytochalasan	ND	+
$C_{22}H_{23}N_5O_2$	390.19390	15.09	Roquefortine C	+	+	$C_{32}H_{36}N_2O_6$	545.26288	30.57	Putative cytochalasan	ND	+
$C_{22}H_{25}N_5O_2$	392.20913	9.99	Roquefortine D	+	+	$C_{32}H_{36}N_2O_6$	545.26294	32.48	Putative cytochalasan	ND	+
$C_{23}H_{24}N_2O_6$	425.17179	26.92		+	+	$C_{32}H_{36}N_4O_2$	509.29257	34.95	Communesin B	+	+
$C_{24}H_{26}N_2O_6$	**437.17091**	**33.79**		+	+	$C_{32}H_{38}N_2O_4$	515.28895	35.59	Putative cytochalasan	ND	+
$C_{26}H_{30}N_4$	399.25568	19.94	Communesin K	+	+	$C_{32}H_{38}N_2O_4$	515.28890	37.93	Putative cytochalasan	ND	+

$C_{26}H_{30}N_4O$	415.25034	14.62	Communesin I	+	+	$C_{32}H_{38}N_2O_4$	515.28909	38.15	Putative cytochalasan	ND	+
$C_{26}H_{30}N_4O$	415.25033	18.43	Communesin I	+	+	$C_{32}H_{38}N_2O_5$	531.28990	22.76	Putative cytochalasan	ND	+
$C_{26}H_{40}O_6$	449.28939	29.93		ND	+	$C_{32}H_{38}N_2O_5$	531.28431	23.91	Putative cytochalasan	ND	+
$C_{27}H_{30}N_4O_2$	443.24564	15.65	Communesin E	ND	+	$C_{32}H_{38}N_2O_5$	531.28354	28.02	Chaetoglobosin E	ND	+
$C_{28}H_{30}N_4O_3$	471.23997	19.56	Com470	+	+	$C_{32}H_{38}N_2O_5$	531.28338	31.56	Penochalasin	ND	+
$C_{28}H_{32}N_4O$	441.26589	17.14	Communesin F	+	+	$C_{32}H_{38}N_2O_6$	547.27864	23.19	Putative cytochalasan	ND	+
$C_{28}H_{32}N_4O_2$	457.26097	14.27	New DMV[1] communesin-1	+	ND	$C_{32}H_{38}N_2O_6$	547.27841	25.47	Putative cytochalasan	ND	+
$C_{28}H_{32}N_4O_2$	457.26116	23.65	Communesin A	+	+	$C_{32}H_{38}N_4O_3$	527.30304	21.41	New DME2 communesin-4	+	ND
$C_{28}H_{38}O_7$	**485.25405**	**35.32**		+	+	$C_{32}H_{40}N_4O_2$	513.32227	36.72	New DME2 communesin-6	+	ND
$C_{28}H_{38}O_7$	487.27063	36.21	Andrastin A	+	+						

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