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

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de Toulouse

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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 C₂H₂ 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 *PeΔbrlA* strain was generated by homologous recombination. In vivo, the suppression of *brlA* completely blocked the development of conidiophores but not the formation of conidia/synnemata. The analysis of the *PeΔbrlA* 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 *PeΔveA* and *PeΔbrlA*, 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 *PeΔbrlA* 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, communines

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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 moisissure 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 le complexe 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔveA* et *PeΔbrlA*, 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 sécrétés 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 *PeΔbrlA* a révélé une grande diversité de chaetoglobosines. 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*, conidiogénèse, synnémates, spores, métabolisme secondaire, microarray, métabolomique, patuline, citrinine, chaetoglobosines, communésines

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List of publications

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2. **Secondary metabolism of *Penicillium spp.* and its regulation**
El Hajj Assaf, C.; Zetina-Serrano, C.; Tahtah, N.; Khoury, A.E.; Atoui, A.; Oswald, I.P.; Puel, O.; Lorber, S. Regulation of Secondary Metabolism in the *Penicillium* Genus. *Int. J. Mol. Sci.* **2020**, *21*, 9462. <https://doi.org/10.3390/ijms21249462>
3. **Deciphering of *Penicillium expansum* metabolome**
Chrystian Zetina-Serrano, Christelle El Hajj Assaf, Robin Constantino, Ophélie Rocher, Selma P Snini, Emilien L. Jamin, Jean-François Martin, Isabelle P. Oswald, Olivier Puel, Sophie Lorber. (Article_In preparation).

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List of abreviations

| | |
|------------------------|---|
| °C | Degrees Celcius |
| λ max | Maximum wavelength |
| μM | Micromoles |
| μg/mL | Micrograms /millitres |
| μg/kg | micrograms/kilograms |
| <i>6-msas</i> | 6-methylsalicylic synthase |
| ABC | ATP-binding cassette |
| APAM | Apple Puree Agar Medium |
| AREs | AbaA Response Elements |
| <i>a_w</i> | 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 |
| <i>Idh</i> | isoeoxydon dehydrogenase |
| LaeA | loss of aflR expression A |
| LBG | Locus Bean Gum |
| LC-HRMS | Liquid Chromatography-High Resolution Mass Spectrometry |
| <i>L_{hgu}</i> | 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/meleagrins |
| 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 (FB1, FB2), 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 *Penicillium*. **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].

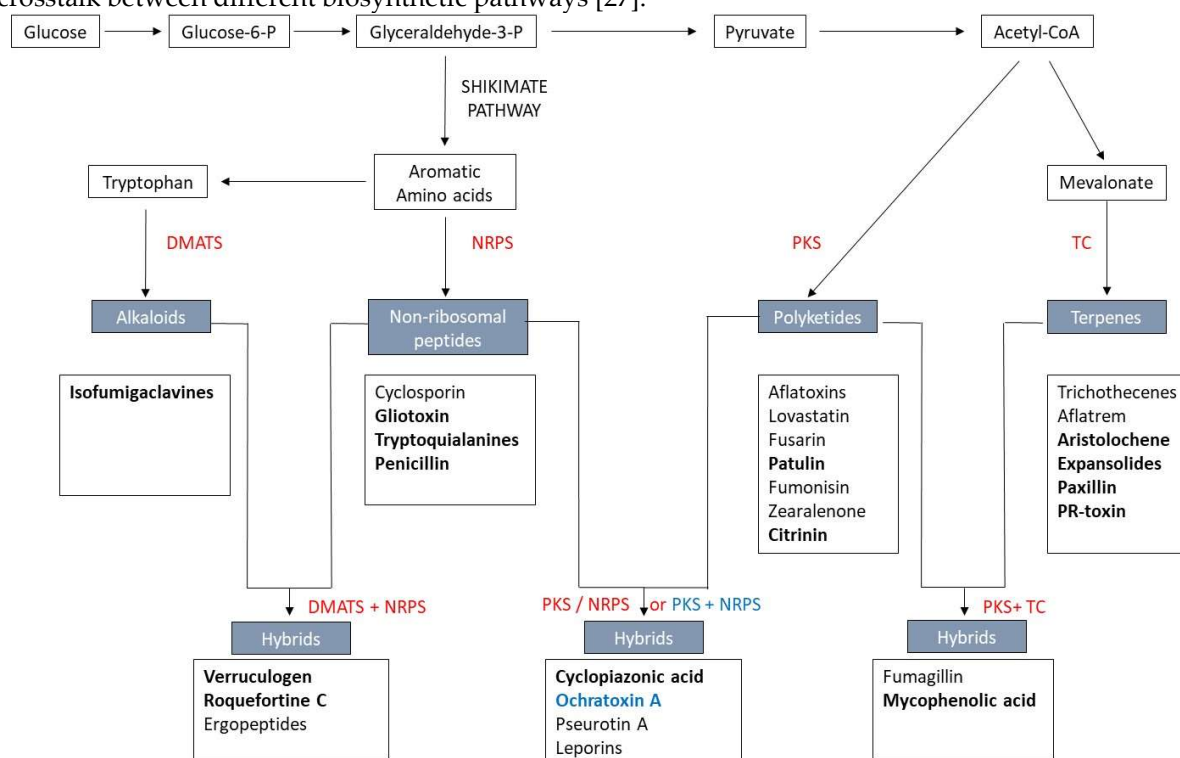


Figure 1. Biosynthetic pathways of secondary metabolites. In grey boxes, the typical backbone of secondary metabolites. In grey, the main mycotoxins produced by these pathways. In red, the enzymes associated with each pathway. In blue, separate PKS and NRPS are involved in ochratoxin A (OTA) biosynthesis; 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.

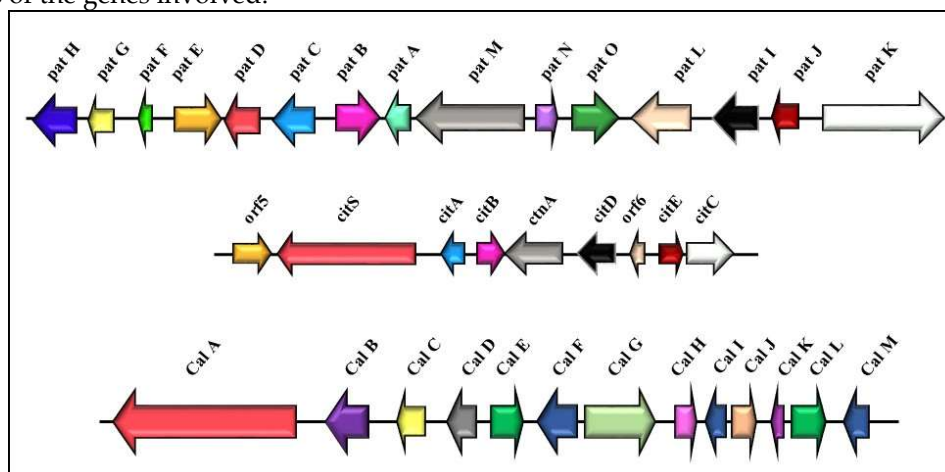


Figure 2. Gene clusters of the patulin biosynthesis pathway (the first one at the top) (15 genes, 40 kb) [39] and the citrinin biosynthesis pathway (the middle group) (nine genes, 22 kb) [40,41] in *Penicillium expansum*; cluster of the calbistrin biosynthesis pathway (the third one at the bottom) (13 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 (Cys₂His₂, Cys₄, or Zn(II)₂Cys₆) [43–45]. Proteins of the Zn(II)₂Cys₆ family are found exclusively in

fungi and yeasts [46], and the C₆ type zinc finger DNA binding protein motif (Cys₆) is frequently encountered in TFs. Cys₆ 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)₂Cys₆ family has increased significantly due to the number of fungal genomes that have since been sequenced. Numerous examples of Zn(II)₂Cys₆ 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 AfIR (aflatoxins), Bik5 (bikaverin), and CtnA (citrinin) for *Aspergillus*, *Fusarium*, and *Monascus*, respectively (Table 1).

Table 1. Examples of identified Zn(II)₂Cys₆ 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 |
|---------------|----------------------------|--------------------------------------|---|------------|
| AfIR | Aflatoxin/Sterigmatocystin | Zn(II) ₂ Cys ₆ | <i>Aspergillus flavus</i> <i>Aspergillus nidulans</i> <i>Aspergillus parasiticus</i> | [51–55] |
| AsaR | Aspergillic Acid | Zn(II) ₂ Cys ₆ | <i>Aspergillus flavus</i> | [56] |
| GliZ | Gliotoxin | Zn(II) ₂ Cys ₆ | <i>Aspergillus fumigatus</i> <i>Penicillium lilacinoechinulatum</i> | [57,58] |
| XanC | Xanthocillin | Basic Leucine zipper | <i>Aspergillus fumigatus</i> | [59] |
| FapR | Fumagillin/Pseurotin | Zn(II) ₂ Cys ₆ | <i>Aspergillus fumigatus</i> | [60] |
| ZEB2 | Zearalenone | Basic Leucine zipper | <i>Fusarium graminearum</i> | [61] |
| SimL | Cyclosporine | Basic Leucine Zipper | <i>Tolyocladium inflatum</i> | [62] |
| OtaR1 | Ochratoxin A | Basic Leucine zipper | <i>Aspergillus carbonarius</i> <i>Aspergillus ochraceus</i> <i>Aspergillus westerdijkiae</i> <i>Penicillium nordicum</i> | [63] |
| SirZ | Sirodesmin PL | Zn(II) ₂ Cys ₆ | <i>Leptosphaeria maculans</i> | [58] |
| MlcR | Compactin | Zn(II) ₂ Cys ₆ | <i>Penicillium citrinum</i> | [64] |
| Bik5 | Bikaverin | Zn(II) ₂ Cys ₆ | <i>Fusarium fujikuroi</i> | [65] |
| DEP6 | Depudecin | Zn(II) ₂ Cys ₆ | <i>Alternaria brassicicola</i> | [66] |
| ZFR1 FUM21 | Fumonisin | Zn(II) ₂ Cys ₆ | <i>Fusarium verticillioides</i> | [67,68] |
| CTB8 | Cercosporin | Zn(II) ₂ Cys ₆ | <i>Cercospora nicotianae</i> | [69] |
| GIP2 | Aurofusarin | Zn(II) ₂ Cys ₆ | <i>Gibberella zeae</i> | [70] |
| CtnA | Citrinin | Zn(II) ₂ Cys ₆ | <i>Monascus purpureus</i> <i>Monascus ruber</i> <i>Penicillium expansum</i> | [40,41,71] |
| LovE | Lovastatin | Zn(II) ₂ Cys ₆ | <i>Aspergillus terreus</i> | [72,73] |
| ApdR | Aspyridone | Zn(II) ₂ Cys ₆ | <i>Aspergillus nidulans</i> | [74] |
| CtnR | Asperfurane | Zn(II) ₂ Cys ₆ | <i>Aspergillus nidulans</i> | [75] |

| | | | | |
|------------------------------|-------------------------------------|--------------------------------------|---|---------|
| MdpE | Monodictyphenone/ Emodin Analogs | Zn(II) ₂ Cys ₆ | <i>Aspergillus nidulans</i> | [76] |
| Cmr1p | Melanin | Zn(II) ₂ Cys ₆ | <i>Colletotrichum lagenarium</i> | [77] |
| Pig1p | Melanin | Zn(II) ₂ Cys ₆ | <i>Magnaporthe grisea</i> | [77] |
| GsfR1 | Griseofulvin | Zn(II) ₂ Cys ₆ | <i>Penicillium griseofulvum</i> | [78] |
| MokH | Monacolin K | Zn(II) ₂ Cys ₆ | <i>Monascus pilosus</i> | [79] |
| CalC | Calbistrin | Zn(II) ₂ Cys ₆ | <i>Penicillium decumbens</i> | [42] |
| CnsN | Communesins | Zn(II) ₂ Cys ₆ | <i>Penicillium expansum</i> | [80] |
| Orf2 | Varicidin A and B | Zn(II) ₂ Cys ₆ | <i>Penicillium variable</i> | [81] |
| Orf10 | PR-Toxin | Zn(II) ₂ Cys ₆ | <i>Penicillium chrysogenum</i> <i>Penicillium roqueforti</i> | [82,83] |
| MacR | Macrosporin | Zn(II) ₂ Cys ₆ | <i>Penicillium terrestris</i> | [84] |
| PatL | Patulin | Zn(II) ₂ Cys ₆ | <i>Penicillium expansum</i> | [85] |
| SorR1 SorR2 | Sorbicillin | Zn(II) ₂ Cys ₆ | <i>Penicillium chrysogenum</i> | [86] |
| TqaK | Tryptoquialanines | Basic leucine zipper | <i>Penicillium aethiopicum</i> <i>Penicillium digitatum</i> | [87,88] |
| Sol4 | Solanapyrone | Zn(II) ₂ Cys ₆ | <i>Ascochyta rabiei</i> | [89] |
| RoIP | Leucinostatin | Zn(II) ₂ Cys ₆ | <i>Paecilomyces lilacinus</i> | [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)₂Cys₆ 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 *mclR* gene encoding a putative 50.2-kDa protein characterized by a Zn(II)₂Cys₆ 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 Cys₆ 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)₂Cys₆ 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.

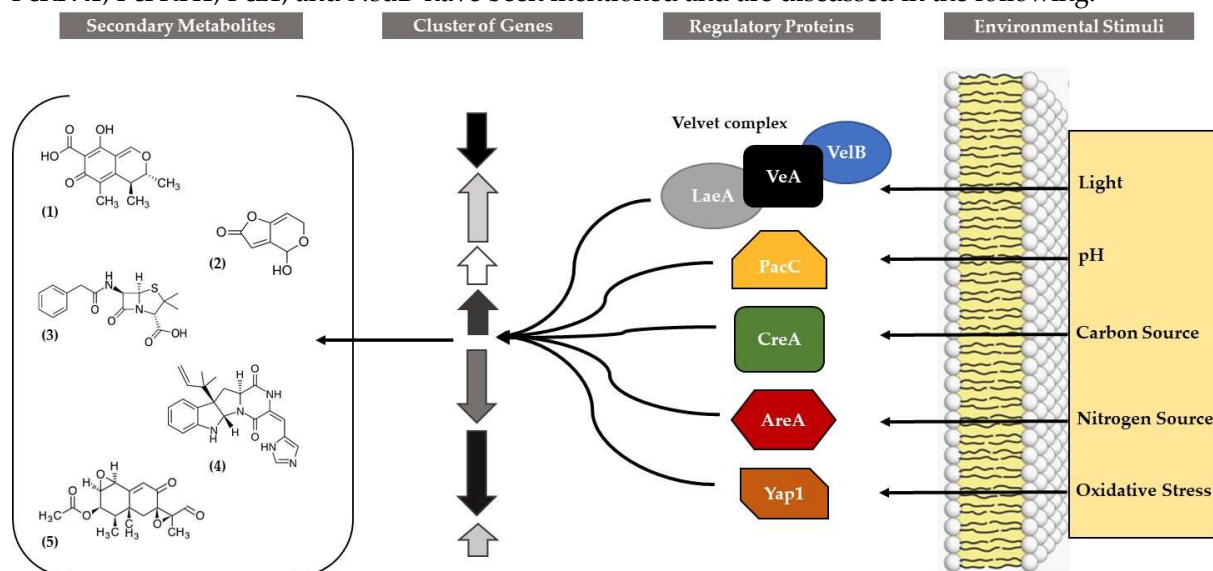


Figure 3. Global regulatory proteins involved in the regulation of gene clusters involved in the production of various secondary metabolites in *Penicillium* (1) citrinin, (2) patulin, (3) penicillin G, (4) roquefortine C, and (5) PR-toxin, adapted from Brakhage [45].

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:His₂ 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 isoeopoxydon 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

Cys₂Cys₂-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 Cys₂His₂ 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 *PeΔpacC* 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*) *marneffeii*. 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 *Afskn7* 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 *Δyap1* 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. marneffeii*, the mutant *ΔyapA*, a *yap1* orthologous gene, was found to be sensitive to oxidative chemicals such as H₂O₂ 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. marneffeii* 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.

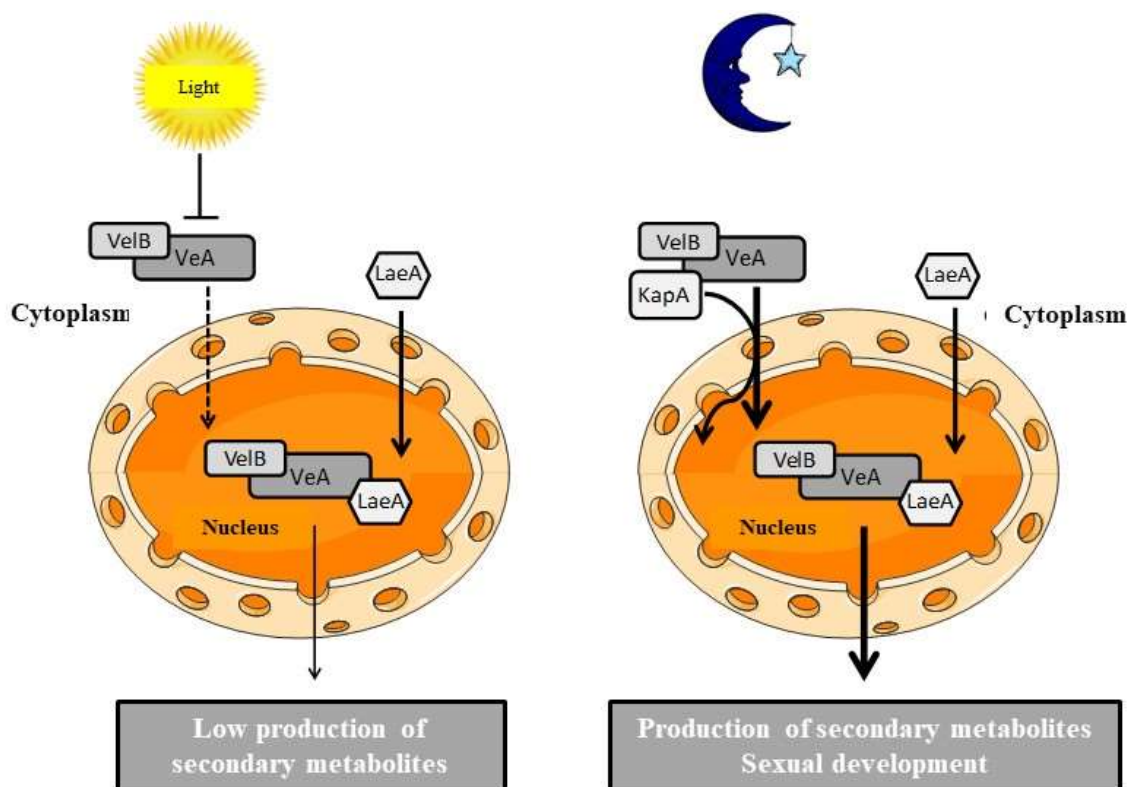


Figure 4. Operating model of the velvet complex in *Aspergillus nidulans* adapted from Bayram et al. [127]. In the presence of light, VeA is retained in the cytoplasm (-----), and LaeA has low activity. In the dark, VeA coupled to VelB is transported in the nucleus by the importin α KapA (→) and the 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 ($\Delta laeA$ vs. wild type (WT)) for the *rpt* gene involved in the second step of the roquefortine biosynthetic pathway suggests that *PclaeA* 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/ meleagrins/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 *PdlaeA* resulted in decreased expression of various secondary metabolite gene clusters, including the *Tq* cluster involved in tryptoguanine 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 *Pd $\Delta laeA$* strains revealed increased sensitivity of the null mutant strain under alkaline conditions. The loss of *PdlaeA* 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 C₂H₂-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, *brlA α* and *brlA β* [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/meleagrins/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 *Pcrfx1* 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 *PoxnsdD* 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 Δ nsdD* strain, the 230 differentially expressed genes identified covered 69 putative BGCs. Among them, 11 were predicted to produce aspyridone, emericellin, citrinin, leucinostatins, roquefortine C/meleagrins, 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 $G\alpha$ proteins belonging to classes I, II, or III [166]. $G\alpha$ 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 $G\alpha$ subunits of classes II and III. The deletion of genes encoding class II $G\alpha$ 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. marneffeii* [173]. The *pga1* gene, encoding subunits of subgroup I $G\alpha$ 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 subtelomerically 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 meleagrins/roquefortine C biosynthesis gene cluster, accompanied by higher meleagrins 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 variable* 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 μ M 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: eupenicinicol 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 μ M of suberoyl bis-hydroxamic acid, a Zn(II)-type or NAD⁺-dependent HDAC inhibitor, and 100 μ M 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 Δ hmbB* strain. *PoxhmbB* 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 CO₂ 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).

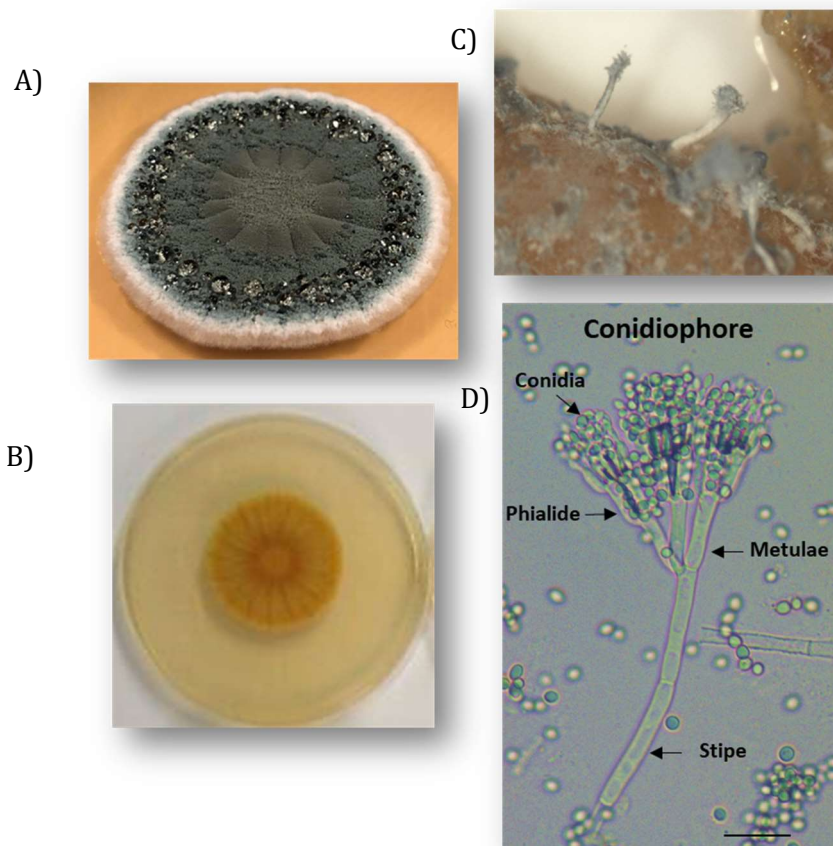


Figure 1. Macroscopic morphology of *Penicillium expansum* A) Recto B) Verso. C) Coremia, observation under stereomicroscope ($\times 12$). D) Microscopic appearance ($\times 400$). The strains were grown on MEA at 25 °C in the dark. Black scale bars represent 100 μm .

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 $-6\text{ }^{\circ}\text{C}$ to $-2\text{ }^{\circ}\text{C}$, according to the studies), its optimal growth is registered about $25\text{ }^{\circ}\text{C}$ and the maximal growth temperature is at $35\text{ }^{\circ}\text{C}$. 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 | | $C_{12}H_{14}NO_2$ | Serotonin 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 ₄ O 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 ₄ O ₂ | Cytotoxic, insecticidal activity | Hayashi et al. 2004; Lin et al. 2015 |
| COMMUNESIN D | Nonribosomal Peptides | C ₃₂ H ₃₄ N ₄ O ₃ | Insecticidal activity | Hayashi et al. 2004 |
| COMMUNESIN E | Nonribosomal Peptides | C ₂₇ H ₃₀ N ₄ O ₂ | Insecticidal activity | Hayashi et al. 2004 |
| COMMUNESIN F | Nonribosomal Peptides | C ₂₈ H ₃₂ N ₄ O | Insecticidal activity | Lin et al. 2015; Kerzaon et al. 2009 |
| COMMUNESIN I | Nonribosomal Peptides | C ₂₆ H ₃₀ N ₄ O | 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 ₄ O ₃ | | Kerzaon et al. 2009 |
| COM570 | Nonribosomal Peptides | C ₃₃ H ₃₈ N ₄ O ₅ | | Kerzaon et al. 2009 |
| COM622 | Nonribosomal Peptides | C ₃₇ H ₄₂ N ₄ O 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 ₇ H ₉ NO ₅ | Antibiotic | Birkinshaw et al. 1942 |
| GENTISYL ALCOHOL | | C ₇ H ₈ O ₃ | | Andersen et al. 2004 |
| GEOSMIN | Terpenes | C ₁₂ H ₂₂ O | Relatively non-toxic | Mattheis and Roberts, 1992 |
| PATULIN | Polyketide | C ₇ H ₆ O 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 ₅ O ₂ | Weak neurotoxin, low toxicity | Andersen et al. 2004; Frivad and Filtenborg 1983 |
| ROQUEFORTINE D | Nonribosomal Peptides | C ₂₂ H ₂₅ N ₅ O 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 C₇H₆O₄ 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 $\mu\text{g}/\text{kg}$, 25 $\mu\text{g}/\text{kg}$ for solid apple products and 10 $\mu\text{g}/\text{kg}$ 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).

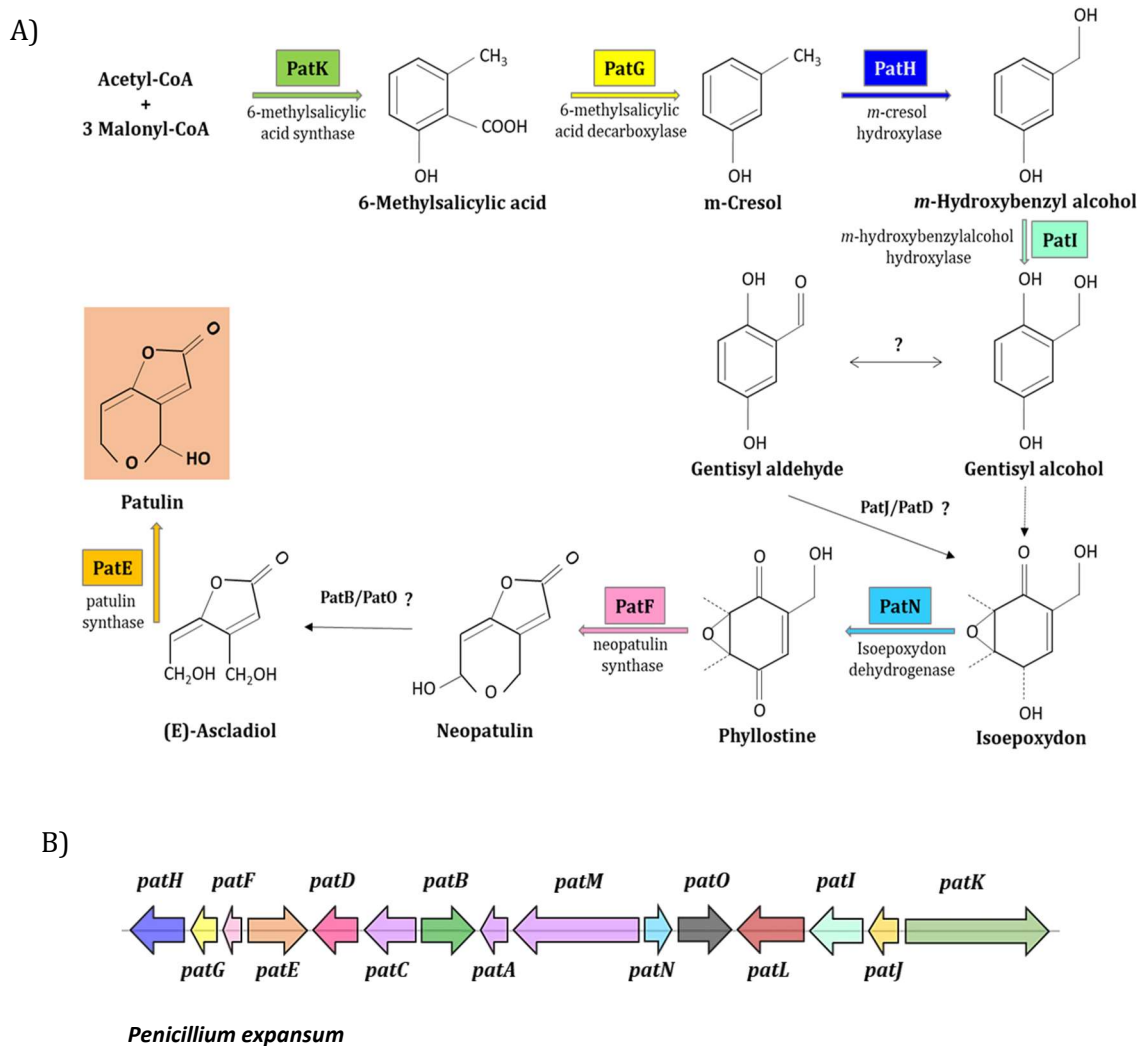


Figure 3. A) Patulin biosynthesis pathway (Adapted from Tannous et al. 2018). B) Schematic representation of the patulin gene cluster (15 genes, 40 kb) in *P. expansum*. The direction of transcription is indicated by arrowheads (Tannous et al. 2014).

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.

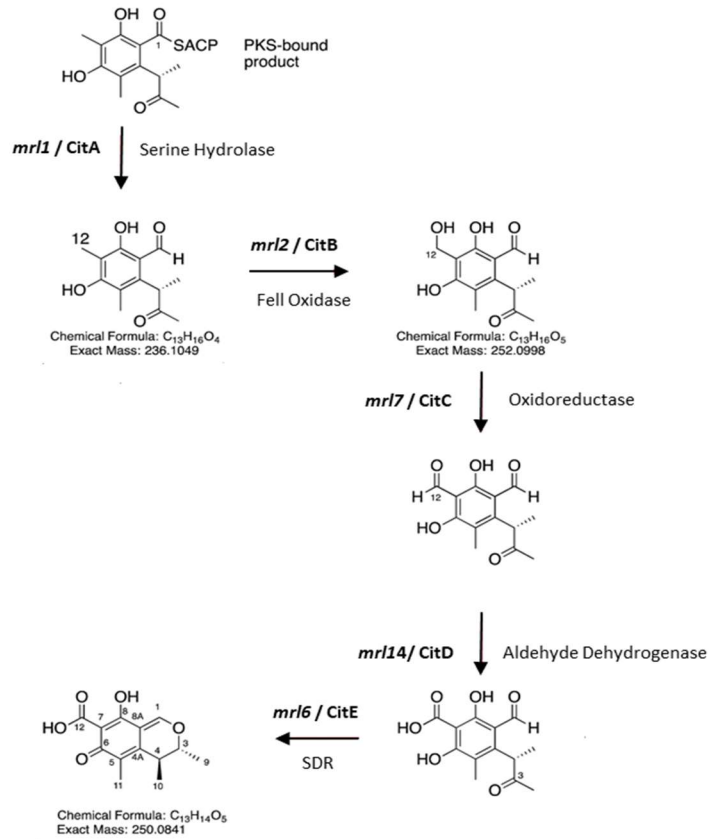
2.2.2 Citrinin

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)

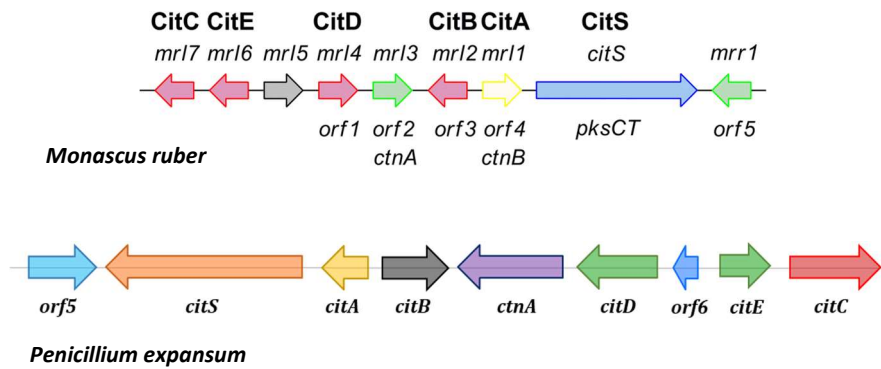


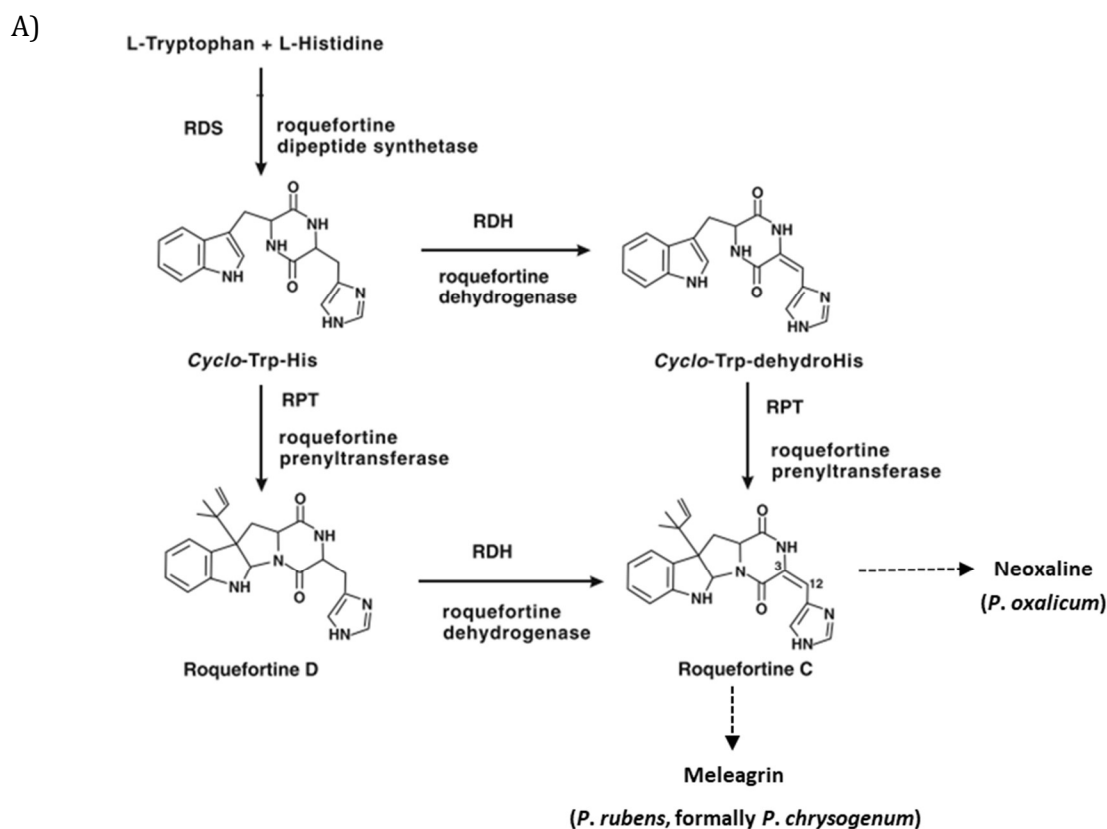
Figure 4. A) Citrinin biosynthesis pathway (He and Cox, 2016). B) Schematic representation of the citrinin gene cluster (nine genes, 22 kb) in *Monascus ruber* (He and Cox, 2016) and in *P. expansum* (Ballester et al. 2015). Arrowheads indicate the direction of transcription.

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)



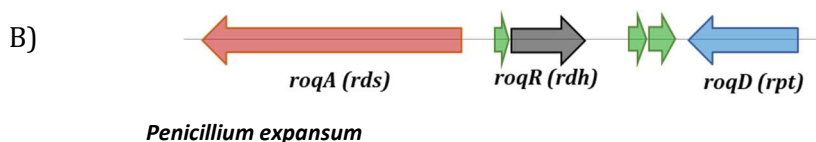
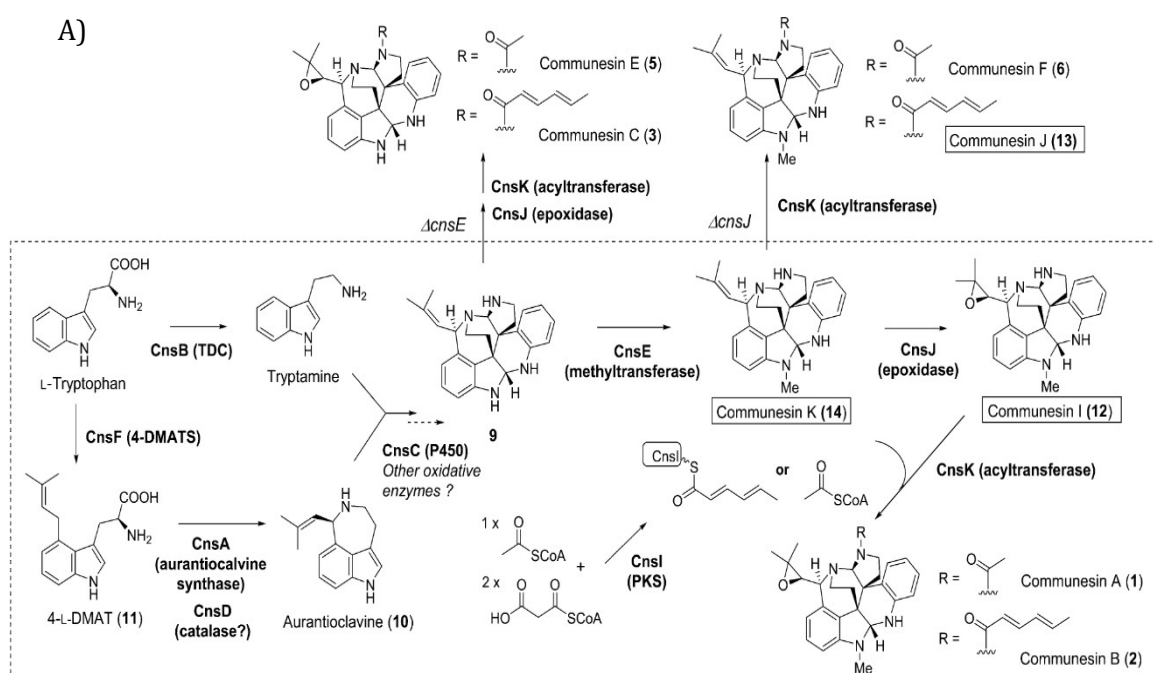


Figure 5. A) Roquefortine C biosynthesis pathway (Martin and Liras, 2015). B) Schematic representation of the roquefortine gene cluster in *P. expansum* (Banani et al., 2016; Martín and Liras, 2016). Arrowheads indicate the direction of transcription.

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).



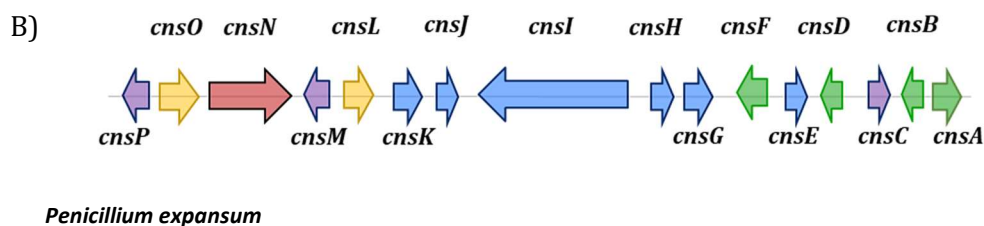


Figure 6. A) Biosynthetic pathway of communesins. The pathway in the dashed box is the main pathway leading to communesins A and B in *P. expansum*. B) Schematic representation of the communesin gene cluster in *P. expansum* (Lin et al. 2015). Arrowheads indicate the direction of transcription.

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, nematocidal, 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).

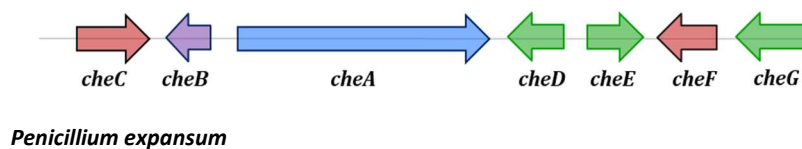


Figure 7. Schematic representation of the chaetoglobosin gene cluster in *P. expansum* described by (Schümann and Hertweck, 2007).

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).

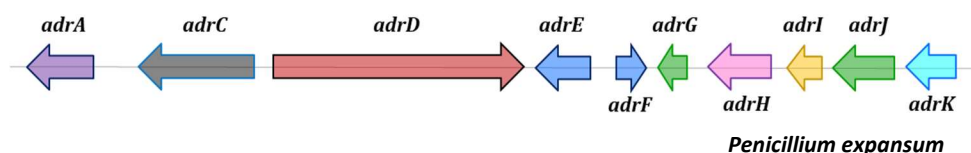


Figure 8. Schematic representation of the andrastin gene cluster in *P. expansum* (Matsuda et al. 2016). Arrowheads indicate the direction of transcription.

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 $\Delta patL$ 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 $Pe\Delta veA$. 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 $Pe\Delta patL$ 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* secretes 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 PeSte12 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 (*PeLysM1*, *PeLysM2*, *PeLysM3*, *PeLysM4*) 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 *PeΔLysM12* 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 *Peprt* 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 Δalp2* deficient mutant. The *alp2* gene is the orthologous *Peprt* 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.

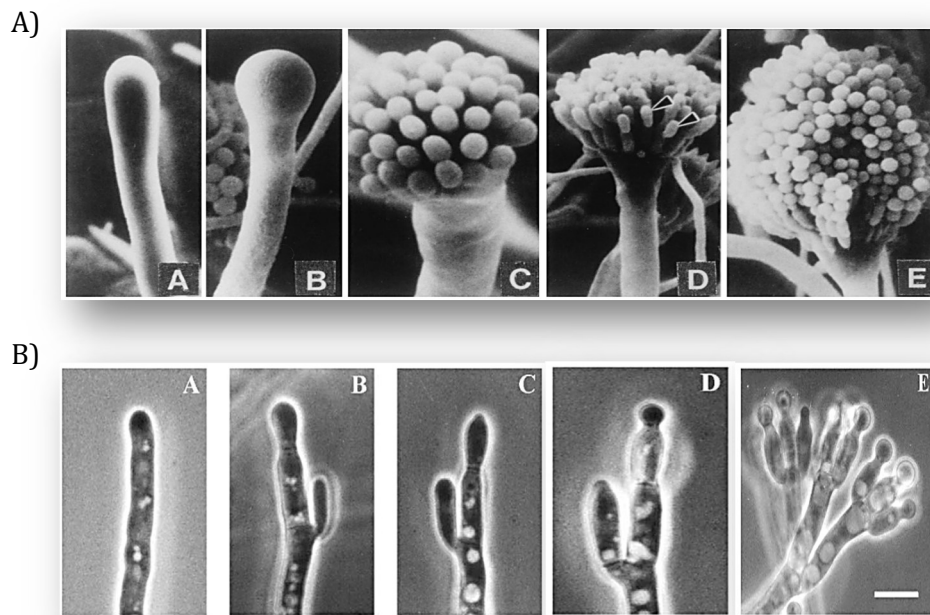


Figure 1. Stages of morphological changes during conidiation A) Development in *A. nidulans* (Adams et al. 1998); (A) Early conidiophore stalk. (B) Vesicle formation from the tip of the stalk. (C) Developing metulae. (D) Developing phialides. (E) Mature conidiophores bearing chains of conidia. B) Development 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 $\Delta rodA$ 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-dihydroxyphenylalanine (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, Ayl1, 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-phosphate (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).

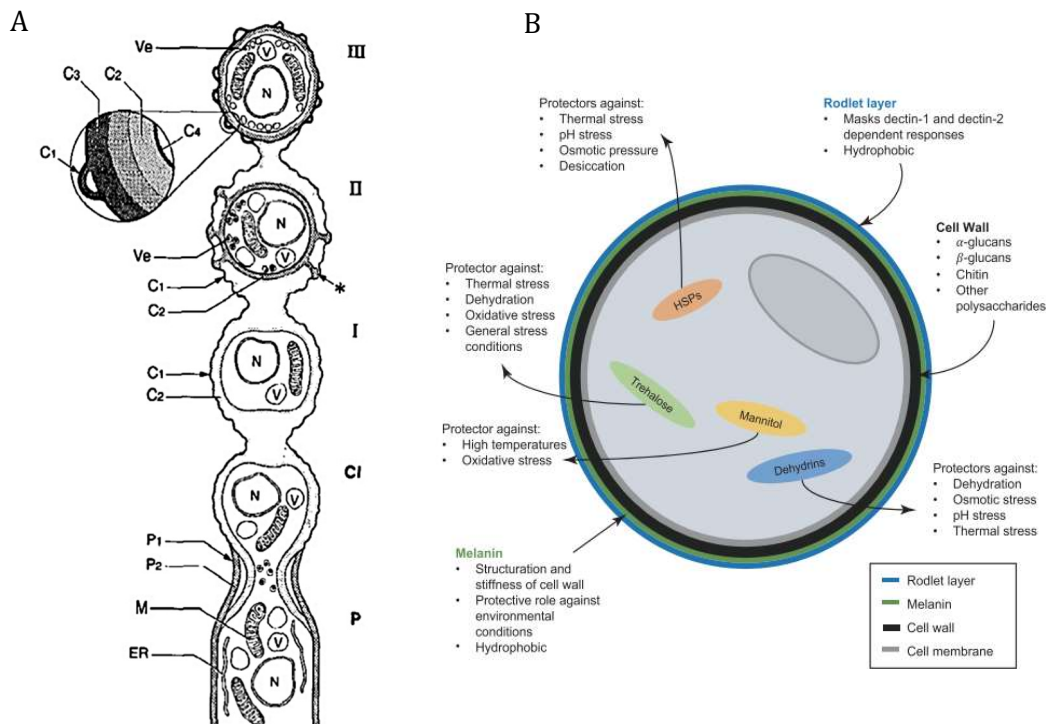


Figure 2. A) Phases of conidium maturation. Each conidium and phialide (P) contains a nucleus (N), mitochondria (M), endoplasmic reticulum (ER), vacuoles (V) and vesicles (Ve). Phialide contains two layers of cell wall: P1 and P2. The conidium in the first stage also contains two cell wall layers: C1 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).

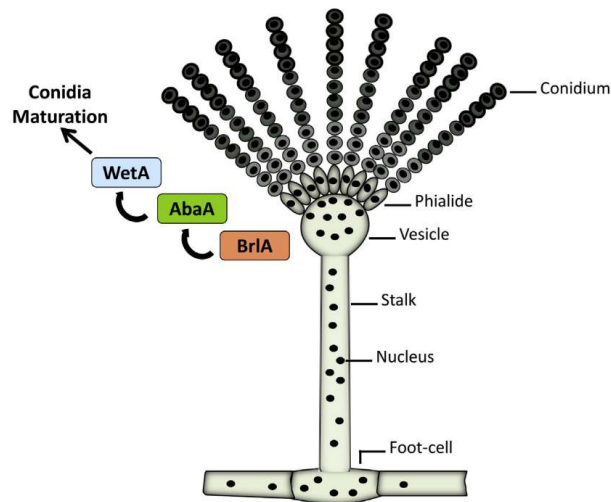


Figure 3. Scheme of the regulatory pathway for conidiation in *A. fumigatus*. BrIA is required for the formation of vesicles, AbaA is required for the differentiation of phialides and conidia and WetA is required for the maturation and integrity of the spore cell wall (Alkhayyat et al. 2015).

3.3.1 *BrlA*

The bristle (*brlA*) gene encodes a C₂H₂-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, *brlA α* and *brlA β* (Han and Adams, 2001). The *brlA α* (2.1 kb) is regulated at the transcriptional level and *brlA β* (2.5 kb) is regulated at both the transcriptional and translational levels. The *brlA β* transcript encodes two open reading frames (ORFs), the first starting with a short ascending ORF (μ ORF) and the second a descending ORF encoding the same polypeptide as *brlA α* , 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 *brlA β* mRNA is transcribed in vegetative cells before developmental induction, but the translation of μ ORF represses translation of BrIA. The removing of the μ ORF initiation codes led to the deregulation of *brlA* expression and consequently to an inappropriate development. (Han et al. 1993) Previous studies revealed that BrIA has protein binding sites also known as BrIA 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).

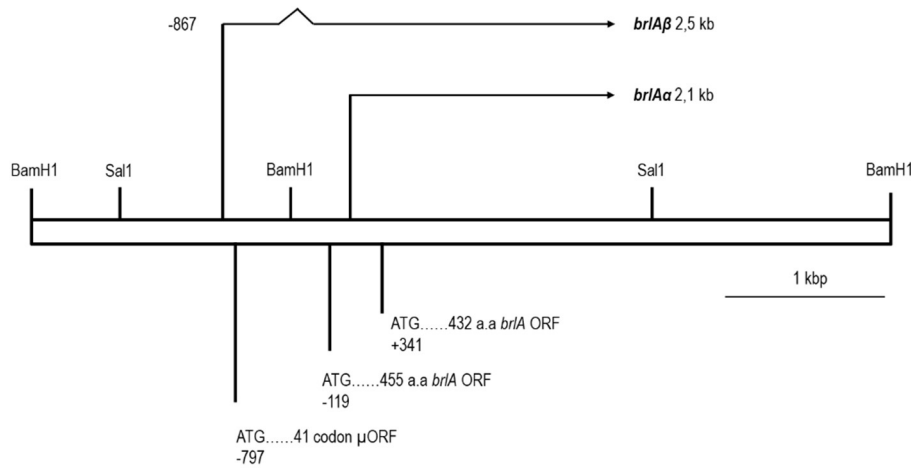


Figure 4. Organization of the *brlA* locus. The *brlAα* and *brlAβ* transcripts are indicated by the arrows (Han and Adams, 2001).

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).

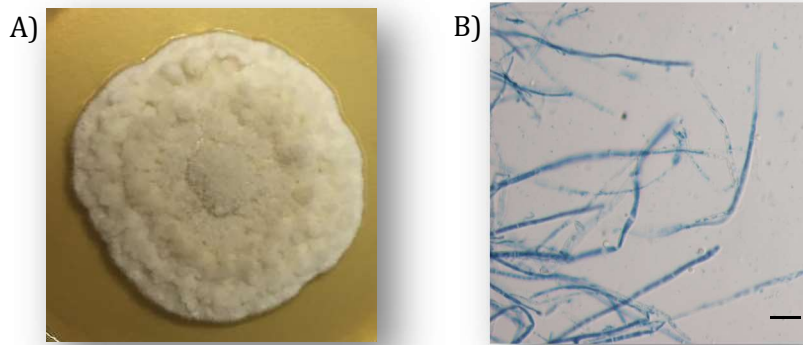


Figure 5. Morphological appearance of the null mutant *PeΔbrlA* strain. A) Macroscopic appearance. B) Microscopic appearance ($\times 400$). The strains were grown on MEA for seven days at 25 °C in the dark. Black scale bars represent 10 μm .

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'-CATTTCY-3' called AbaA Response Elements (AREs) where Y is a pyrimidine. In addition to BREs, multiple ARE elements have been discovered in the promoter regions of developmental genes, including *brlA*, *wetA*, *yA*, *rodA*, and even *abaA* itself (Adams et al. 1998). The phenotype of the null mutants $\Delta abaA$ 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 $\Delta abaA$ and $\Delta brlA$ strains. Furthermore, low levels of GT were detected in the null mutant

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

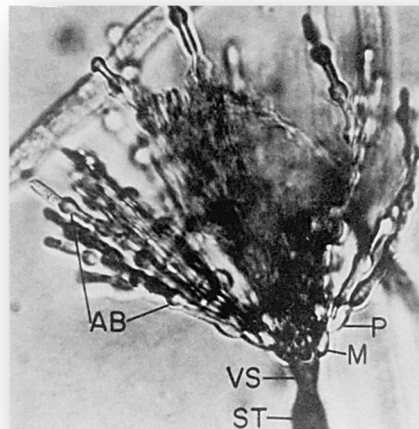


Figure 6. Morphological appearance of the null mutant $\Delta abaA$ strain. The null mutant $\Delta abaA$ strain produces normal conidiophore stalks (ST) and vesicles (VS), but metulae (M) and phialides (P) are abnormal and form abacus (AB) structures instead of conidia (C) (Adams et al. 1998).

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 $Pd\Delta wetA$ 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).

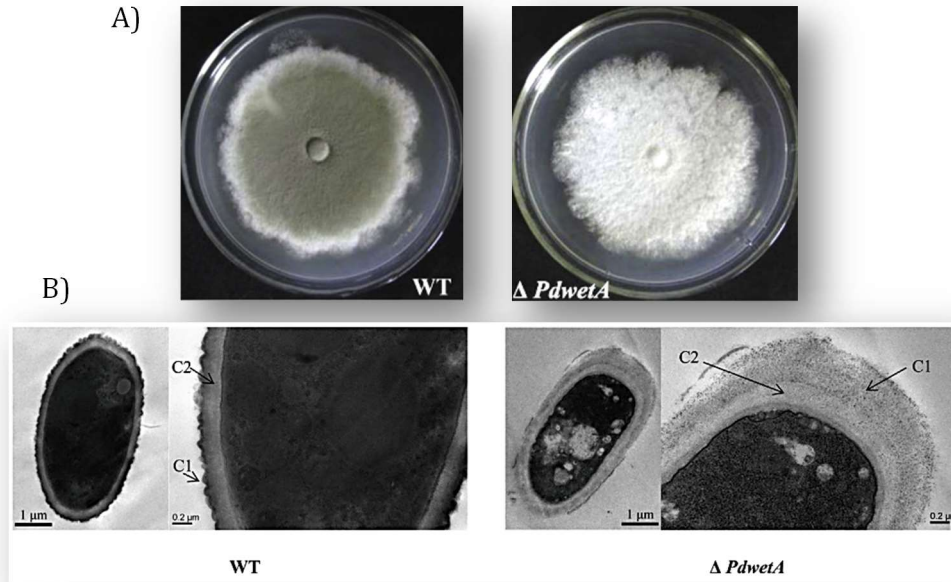


Figure 7. Morphological appearance of the null mutant $\Delta wetA$ strain. Vegetative growth of the wild-type and null mutant $Pd\Delta wetA$ strains on PDA at 25 °C for six days. (B) TEM images of the cell wall of conidia in WT and $Pd\Delta wetA$. Bar, 1 mm or 0.2 mm (Wang et al. 2015).

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

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

| | | | |
|-------------|---------------------|------------|---|
| <i>dlpA</i> | <i>A. nidulans</i> | AN5324 | Encode a dehydrin-like protein which plays a role osmotic and thermal stresses. |
| <i>catA</i> | <i>A. fumigatus</i> | Afu6g03890 | Conidium-specific catalase CatA. |
| <i>scf1</i> | <i>A. fumigatus</i> | Afu1g17370 | Encodes a putative heat shock protein enriched in dormant conidia. |
| <i>conj</i> | <i>A. fumigatus</i> | Afu6g03210 | Putative conidiation gene; deletion of <i>conF</i> and <i>conj</i> results in delayed germination and decreased desiccation resistance. |
| <i>mpkC</i> | <i>A. fumigatus</i> | Afu5g09100 | Putative MAPK involved in the oxidative stress response; transcript abundance increases in response to carbon source and oxidative stress. |
| <i>pbs2</i> | <i>A. fumigatus</i> | Afu1g15950 | Mitogen MAPKK of the HOG signaling pathway that regulates osmotic stress response. |
| <i>pbsA</i> | <i>A. nidulans</i> | AN0931 | |
| <i>sakA</i> | <i>A. fumigatus</i> | Afu1g12940 | Putative MAPK with predicted roles in the osmotic and oxidative stress responses. |
| <i>osrA</i> | <i>A. fumigatus</i> | | Hypothetical osmotic stress regulator. |
| <i>sskB</i> | <i>A. nidulans</i> | AN10153 | MAPKKK of the HOG signaling pathway that regulates the osmotic stress response. |
| <i>sskA</i> | <i>A. nidulans</i> | AN7697 | Response regulator; part of a two-component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response. |
| <i>tcsB</i> | <i>A. nidulans</i> | AN1800 | Transmembrane histidine kinase; part of a two component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response. |
| <i>ypdA</i> | <i>A. nidulans</i> | 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 C₂H₂ 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 $\Delta flbC$ 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).

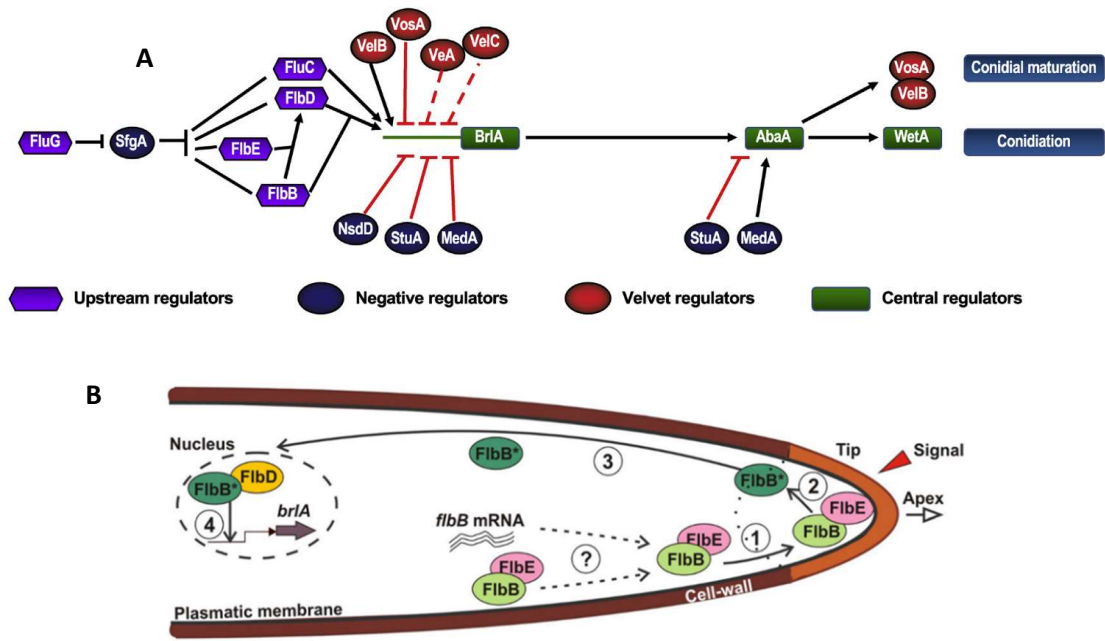


Figure 8. Upstream developmental activators, fluffy genes. A) A genetic model of the regulation of conidiophore development in *A. nidulans* (Ojeda-López et al. 2018). B) FlbB-FlbE and FlbB-FlbD dynamics and transcriptional activity in vegetative hyphae (Oiartzabal-Arano et al. 2016)

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 (Sarıkaya-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 ΔveA 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 (Sarıkaya-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* (Sarıkaya-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 *brlA β* (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.

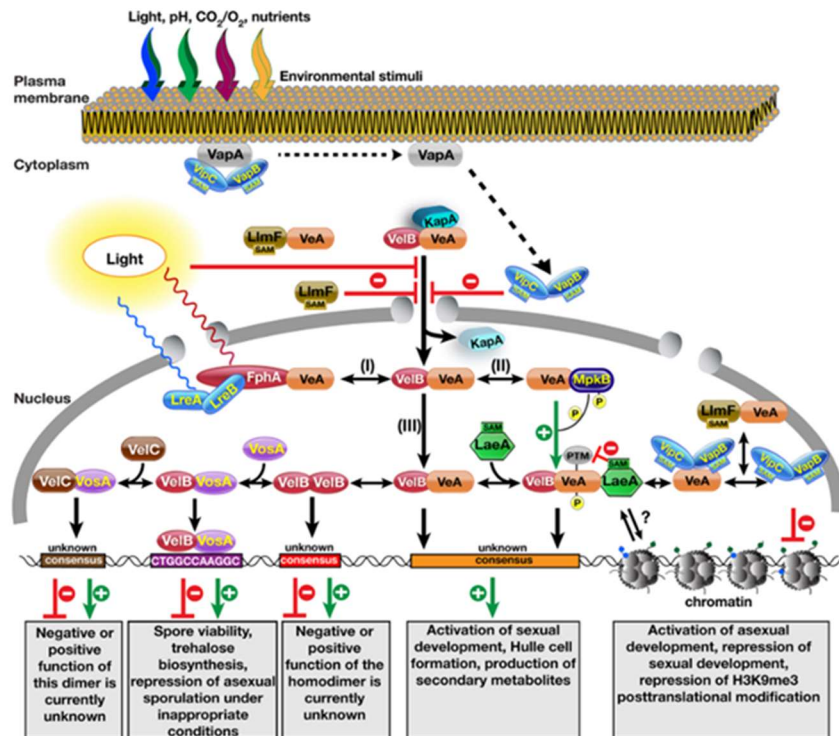


Figure 9. Molecular complexes formed by the velvet family proteins and the methyltransferases on the control of fungal development and secondary metabolite production (Sarikaya-Bayram et al. 2015).

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 *PcΔveA*, *PcΔvelB*, *PcΔvelC*, and *PcΔvosA* 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 *PcΔvelC* and *PcΔveA* strains, penicillin biosynthesis was activated, while in the null mutant *PcΔvelB* strain, penicillin production was repressed. Conidiation was promoted in the null mutant *PcΔvelB* and *PcΔvosA* strains, while it was inhibited in the null mutant *PcΔvelC*.

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 $\Delta nsdD$ 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 $\Delta nsdD\Delta vosA$ 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* (*Itf1*) 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, *stuA α* (3335 nucleotides) and *stuA β* (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 $\Delta stuA$ 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 *brlA* β 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 $\Delta medA$ 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 $\Delta medA$ strains form Hülle cells but do not produce mature cleistothecia or ascospores, whereas null mutant $\Delta stuA$ 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, a low molecular weight organic molecule, that allows a protein to absorb light (Rodríguez-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).

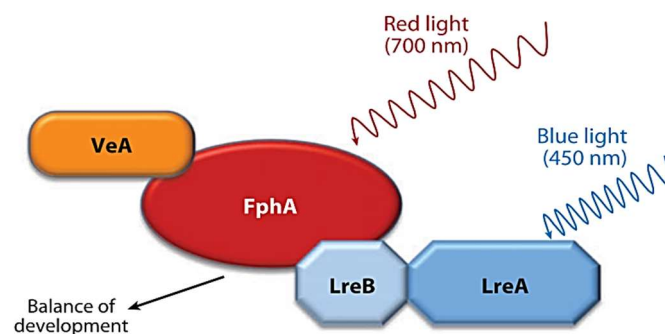


Figure 10. Light regulator complex (Rodriguez-Romero et al. 2010).

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 $\Delta lreA$ and $\Delta fphA$ strains and the double mutant $\Delta lreA\Delta fphA$ 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 $Pe\Delta brlA$ strain in *Penicillium expansum*

Article- International Journal of Molecular Science

(2020, 21: 6660. doi: 10.3390/ijms21186660)



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 $\Delta brlA$ strain in *P. expansum* (*Pe* $\Delta brlA$). 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 *PeΔbrlA* 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*.



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 C₂H₂-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* PeΔ*brlA* 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 PeΔ*brlA* 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 PeΔ*brlA* 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 C₂H₂-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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* strain produced longer stalks but with no conidiophores, whose development was stopped before branches, metulae, and phialides were formed (Figure 1Bd).

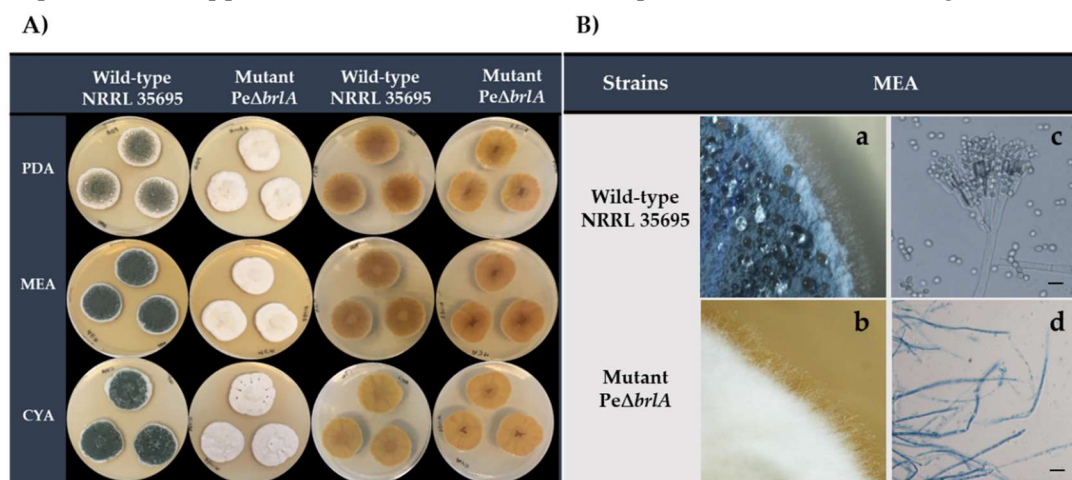


Figure 1. Morphological appearance of wild type *Penicillium expansum* and the null mutant *PeΔbrlA* strains. **A)** Macroscopic appearance of the colonies (recto-verso). The strains were grown on Malt

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 ($\times 12$) after 10 days of incubation (a) wild type strain; (b) null mutant strain *Pe Δ brlA*. Microscopic appearance ($\times 400$): (c) wild type conidiophores; (d) null mutant strain *Pe Δ brlA* stalks after seven days of incubation. The strains were grown on MEA at 25 °C in the dark. Black scale bars represent 10 μ m.

2.2. Effect of *brlA* Deletion on Apple Colonization

2.2.1. Effect of *brlA* Deletion on Pathogenicity

To check if the null mutant *Pe Δ 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 *Pe Δ brlA* strain from the seventh day and significant differences between the *Pe Δ 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 *Pe Δ 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 *Pe Δ 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 *Pe Δ brlA* strain had a significantly higher rot volume than apples infected with the WT strain, 24.14 ± 1.31 cm³, and 17.95 ± 1.53 cm³ (p -value $7.2E-3$), respectively (Figure 2C).

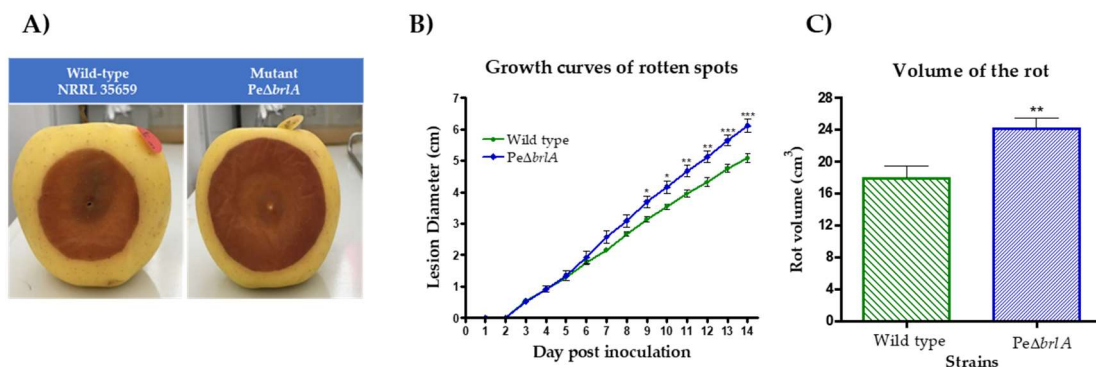


Figure 2. Golden Delicious apples infected with wild type *Penicillium expansum* or the null mutant *Pe Δ brlA* strains, incubated at 25 °C for 14 days in the dark. **A**) Spots of rot 11 days after infection. **B**) Growth curves of spots. The diameter of the lesions was measured daily. **C**) The volume 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 \pm standard error of the mean (SEM) from nine biological replicates and the significant differences between the wild type and the null mutant *Pe Δ 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 *PeΔbrlA* strain not only retained the ability to produce patulin, but produced four times the concentration produced by the WT strain, with $14.11 \pm 8.3 \mu\text{g/g}$ and $58.5 \pm 12.5 \mu\text{g/g}$ (p -value $9.3\text{E-}3$) fresh weight of apples, respectively (Figure 3).

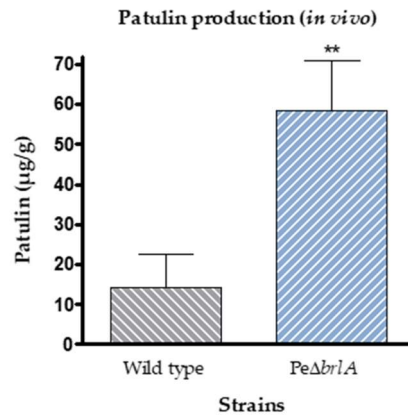


Figure 3. Patulin production in Golden Delicious apples infected with wild type *Penicillium expansum* or the null mutant *PeΔbrlA* strains at 14 days of incubation as previously described by Snini et al. [22]. Detection and quantification were performed by High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis at 277 nm and based on a standard curve, respectively. The graphs show the mean \pm 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 *PeΔbrlA* 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 *PeΔbrlA* strain, the conidiogenesis process was interrupted before the formation of metulae and only rigid, white, and sporeless hyphal structures (synnemata) developed (Figure 4b,d).

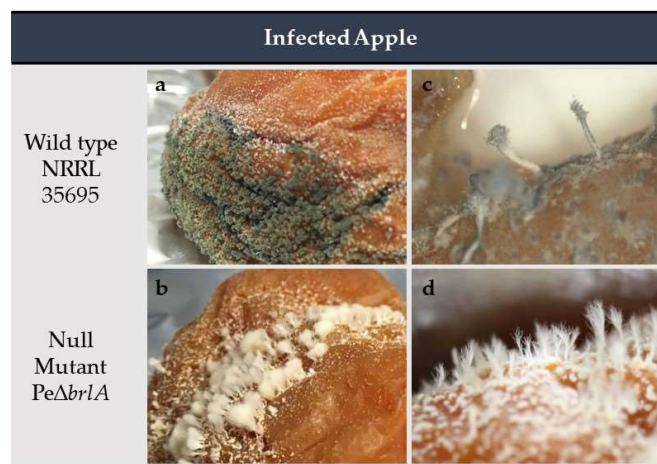


Figure 4. Apples infected with *Penicillium expansum* after 30 days of incubation at 25 °C in the dark. (a) Wild type strain; (b) Null mutant *PeΔbrlA* strain. Stereomicroscope observation ($\times 12$): (c)

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

2.3. Growth Profile in Different Carbon Sources

The null mutant *PeΔbrlA* 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 *PeΔbrlA* strains developed on different substrates.

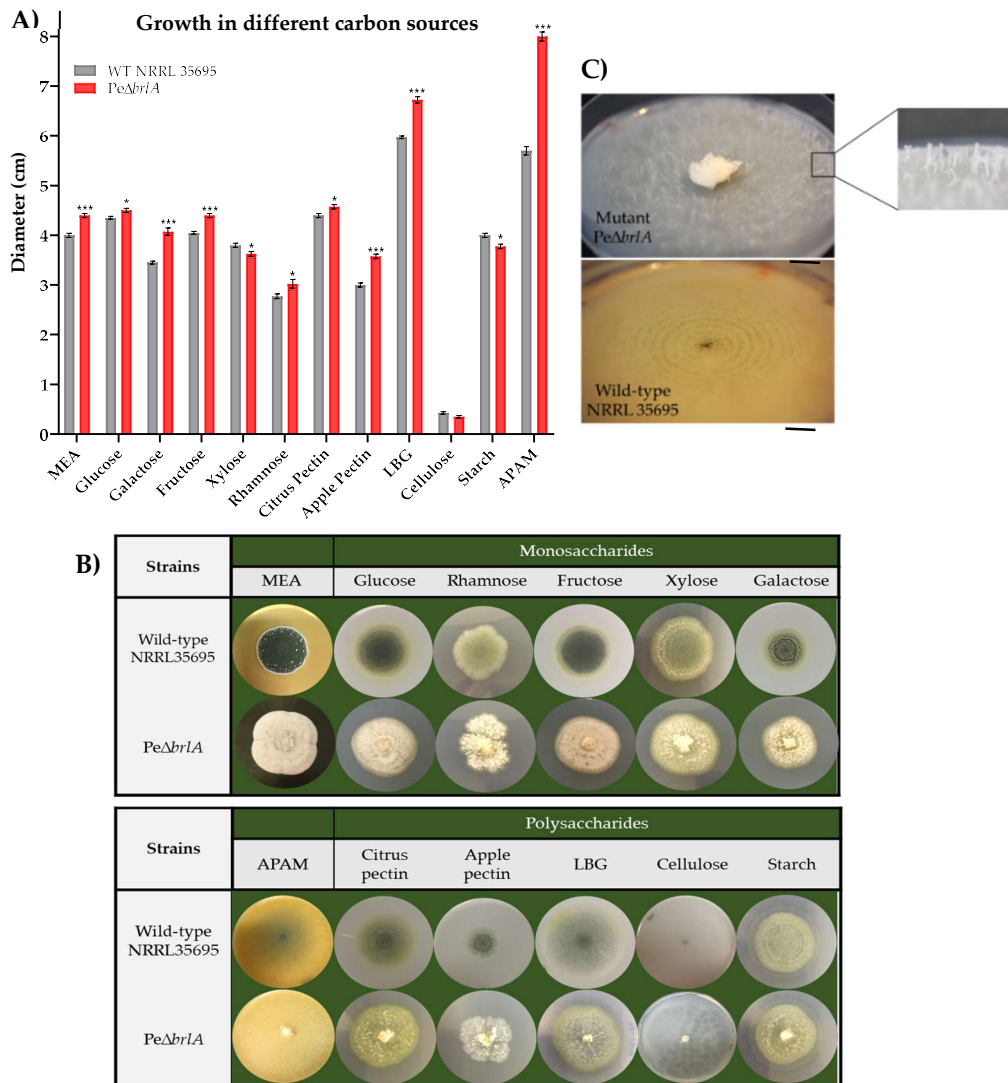


Figure 5. Wild type *Penicillium expansum* and the null mutant *PeΔbrlA* strains were grown in a minimal medium supplemented with different carbon sources for seven days at 25 °C in the dark. **A)** Average diameter (cm) of the colonies and statistical analysis of the wild type and null mutant *PeΔbrlA* strains, developed in the different substrates. The graphs show the mean \pm standard error of the mean (SEM) from three biological replicates and the significant differences between the wild type and the null mutant *PeΔbrlA* 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 *PeΔbrlA* strains, respectively. The difference in strain diameters

was also apparent in the fructose- and galactose-enriched media where the diameters of the null mutant *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* strains after culture on labeled wheat grains.

| Molecular Formula | ¹² C m/z (Da) | ^a R _T (min) | Proposed Identification | WT | <i>PeΔbrlA</i> | Molecular Formula | ¹² C m/z (Da) | ^a R _T (min) | Proposed Identification | WT | <i>PeΔbrlA</i> |
|--|--------------------------|-----------------------------------|--------------------------------------|----|----------------|---|--------------------------|-----------------------------------|---|----|----------------|
| C ₇ H ₆ O ₄ | 153.01919 | 3.59 | Patulin [†] | + | + | C ₂₈ H ₃₂ N ₄ O | 441.26589 | 17.14 | Communesin F ^d | + | ND |
| C ₇ H ₈ O | 109.06509 | 7.11 | m-Cresol [†] | + | + | C ₂₈ H ₃₂ N ₄ O ₂ | 457.26116 | 23.65 | Communesin A ^{ft} | + | + |
| C ₇ H ₈ O ₂ | 125.05998 | 6.79 | m-Hydroxybenzyl alcohol [†] | + | + | C ₂₈ H ₃₂ N ₄ O ₃ | 473.25566 | 15.50 | | + | ND |
| C ₇ H ₈ O ₃ | 141.05493 | 3.80 | Gentisyl alcohol [†] | + | + | C ₂₈ H ₃₆ N ₄ O ₄ | 493.27971 | 31.47 | Fungisporin A or cyclo(VFVF) | + | + |
| C ₇ H ₈ O ₄ | 157.04990 | 2.67 | Ascladiol [†] | + | + | C ₂₈ H ₃₈ N ₄ O ₅ | 511.29099 | 18.01 | VAL-PHE-VAL-PHE | + | + |
| C ₇ H ₁₀ O ₃ | 143.07061 | 4.52 | | + | + | C ₂₈ H ₃₈ N ₄ O ₆ | 527.28755 | 12.46 | VAL-PHE-VAL-TYR | + | + |
| C ₁₀ H ₁₇ NO ₅ | 232.11872 | 8.83 | | + | + | C₂₈H₃₈O₇ | 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 [†] | + | + | C₂₈H₃₈O₈ | 501.24792 | 27.70 | | + | + |
| C ₁₅ H ₁₈ N ₂ | 227.15514 | 6.44 | Aurantioclavine [†] | + | ND | C₂₈H₃₈O₈ | 501.24792 | 28.44 | | + | ND |
| C ₁₅ H ₁₉ NO ₆ | 310.12939 | 12.78 | | + | + | C₂₈H₃₈O₈ | 501.24786 | 29.57 | | + | + |
| C ₁₅ H ₁₉ NO ₆ | 310.12964 | 14.80 | | + | + | C₂₈H₄₀O₆ | 471.27434 | 39.48 | Andrastin C | + | + |
| C ₁₅ H ₂₀ O ₄ | 265.14412 | 15.91 | Expansolide C/ D | + | + | C₂₈H₄₀O₇ | 487.26898 | 30.58 | Andrastin B | + | + |
| C ₁₅ H ₂₀ O ₄ | 265.14410 | 16.91 | | + | ND | C ₂₉ H ₂₇ N ₅ O ₅ | 526.20693 | 15.70 | | ND | + |
| C ₁₅ H ₂₀ O ₄ | 265.14415 | 18.49 | Expansolide C/ D | + | + | C ₂₉ H ₂₇ N ₅ O ₅ | 526.20689 | 17.61 | | ND | + |
| C ₁₅ H ₂₀ O ₄ | 265.14414 | 19.35 | | + | + | C ₂₉ H ₃₁ N ₅ O ₅ | 530.23837 | 14.83 | | ND | + |
| C ₁₆ H ₁₈ N ₂ O ₂ | 271.14496 | 7.62 | Clavicipitic acid [†] | + | + | C ₂₉ H ₃₁ N ₅ O ₅ | 530.23812 | 15.85 | | ND | + |
| C ₁₆ H ₁₈ N ₂ O ₂ | 271.14467 | 8.45 | Clavicipitic acid [†] | + | + | C ₂₉ H ₃₁ N ₅ O ₅ | 530.23840 | 16.68 | | ND | + |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | 375.14202 | 22.02 | | + | + | C ₂₉ H ₃₃ N ₅ O ₆ | 548.24883 | 11.36 | | ND | + |
| C ₁₇ H ₂₂ O ₅ | 307.15471 | 27.39 | Expansolide A/B | + | + | C ₂₉ H ₃₃ N ₅ O ₆ | 548.24860 | 12.14 | | ND | + |
| C ₁₇ H ₂₂ O ₅ | 307.15504 | 30.19 | Expansolide A/B | + | + | C ₂₉ H ₃₃ N ₅ O ₆ | 548.25180 | 15.42 | | + | ND |
| C ₁₇ H ₂₃ N ₃ O ₃ | 318.17943 | 3.12 | | ND | + | C ₃₁ H ₃₆ N ₄ O ₂ | 497.29061 | 31.66 | Putative new undetermined communesin | + | ND |
| C ₁₇ H ₂₃ N ₃ O ₃ | 318.18043 | 25.05 | | ND | + | C ₃₂ H ₃₄ N ₄ O ₃ | 523.27152 | 31.84 | Communesin D ^g | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | 293.12911 | 13.58 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₄ | 513.27635 | 39.10 | Chaetoglobosin J or Prochaetoglobosin III | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | 293.12915 | 17.74 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26776 | 20.92 | Chaetoglobosin B/G | + | + |
| C ₁₈ H ₁₆ N ₂ O ₃ | 309.12421 | 14.76 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26807 | 23.19 | Chaetoglobosin B/G | ND | + |
| C ₁₈ H ₁₈ N ₂ O ₂ | 295.14479 | 14.61 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27058 | 25.21 | Chaetoglobosin B/G | + | + |
| C ₁₈ H ₃₁ NO ₇ | 374.21663 | 23.72 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27029 | 26.71 | Chaetoglobosin B/G | + | + |
| C ₁₈ H ₃₅ N ₃ O ₄ | 358.26901 | 3.54 | | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26757 | 29.56 | Chaetoglobosin B/G | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | 305.12911 | 32.50 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27077 | 30.51 | Chaetoglobosin B/G | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | 305.12933 | 33.17 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26807 | 32.43 | Chaetoglobosin B/G | ND | + |
| C ₁₉ H ₁₆ N ₂ O ₄ | 337.11909 | 13.73 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27067 | 33.36 | Chaetoglobosin A [†] | + | + |
| C ₁₉ H ₁₆ N ₂ O ₄ | 337.11916 | 15.20 | | + | ND | C ₃₂ H ₃₆ N ₂ O 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 ₂ O ₅ | 529.26769 | 35.49 | Chaetoglobosin B/G | ND | + |
| C ₁₉ H ₂₁ NO ₇ | 376.13901 | 17.45 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27063 | 36.41 | Chaetoglobosin C [†] | + | + |
| C ₁₉ H ₂₁ NO ₇ | 376.13904 | 18.65 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27056 | 37.41 | Chaetoglobosin B/G | + | + |
| C₁₉H₃₈O₆ | 361.25871 | 37.23 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26814 | 38.29 | Chaetoglobosin B/G | ND | + |
| C₁₉H₃₈O₆ | 361.25819 | 38.19 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26332 | 27.60 | Putative cytochalasan | + | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | 319.14588 | 35.48 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26241 | 29.23 | Putative cytochalasan | ND | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | 319.14502 | 36.44 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26288 | 30.57 | Putative cytochalasan | + | + |
| C ₂₀ H ₂₁ NO ₉ | 420.12857 | 19.29 | | + | + | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26281 | 31.50 | Putative cytochalasan | + | + |
| C ₂₀ H ₂₁ NO ₉ | 420.12858 | 22.19 | | ND | + | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26294 | 32.48 | Putative cytochalasan | ND | + |
| C ₂₀ H ₂₆ O ₈ | 395.17142 | 13.61 | | + | + | C₃₂H₃₆N₂O₆ | 543.24836 | 34.05 | Putative cytochalasan | + | + |
| C ₂₂ H ₂₃ N ₅ O ₂ | 390.19390 | 15.09 | Roquefortine C [†] | + | + | C ₃₂ H ₃₆ N ₄ O ₂ | 509.29257 | 34.95 | Communesin B [‡] | + | + |
| C ₂₂ H ₂₅ N ₅ O ₂ | 392.20913 | 9.99 | Roquefortine D | + | + | C ₃₂ H ₃₈ N ₂ O ₄ | 515.28895 | 35.59 | Putative cytochalasan | + | + |
| C ₂₃ H ₂₄ N ₂ O ₆ | 425.17179 | 26.92 | | + | + | C ₃₂ H ₃₈ N ₂ O ₄ | 515.28890 | 37.93 | Putative cytochalasan | + | + |
| C₂₄H₂₆N₂O₆ | 437.17091 | 33.79 | | + | ND | C ₃₂ H ₃₈ N ₂ O 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 ₂ O ₅ | 531.28384 | 21.75 | Putative cytochalasan | ND | + |
| C ₂₆ H ₃₀ N ₄ O | 415.25034 | 14.62 | Communesin I ^c | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28990 | 22.76 | Putative cytochalasan | + | + |
| C ₂₆ H ₃₀ N ₄ O | 415.25033 | 18.43 | Communesin I ^c | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28431 | 23.91 | Putative cytochalasan | ND | + |
| C ₂₆ H ₃₂ O ₈ | 473.21511 | 9.67 | | ND | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28360 | 27.38 | Putative cytochalasan | + | + |
| C ₂₆ H ₃₂ O ₈ | 473.21552 | 10.64 | | ND | + | C ₃₂ H ₃₈ N ₂ O 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 ₂ O ₅ | 531.28340 | 28.52 | Putative cytochalasan | + | + |
| C ₂₆ H ₄₀ O ₆ | 449.28939 | 29.93 | | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28338 | 31.56 | Penochalasin F ^h | ND | + |
| C ₂₇ H ₂₉ N ₅ O ₄ | 488.22783 | 10.80 | | ND | + | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27864 | 23.19 | Putative cytochalasan | ND | + |
| C ₂₇ H ₂₉ N ₅ O ₄ | 488.22766 | 11.03 | | ND | + | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27841 | 25.47 | Putative cytochalasan | ND | + |
| C ₂₇ H ₃₀ N ₄ O ₂ | 443.24564 | 15.03 | Communesin E ^d | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27842 | 27.09 | Putative cytochalasan | ND | + |
| C ₂₈ H ₃₀ N ₄ O ₃ | 471.23997 | 19.56 | Com470 ^e | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27851 | 28.27 | Putative cytochalasan | ND | + |
| C ₂₈ H ₃₁ N ₅ O ₅ | 518.24108 | 16.36 | | + | + | C ₃₃ H ₃₈ N ₄ O ₅ | 571.29324 | 18.22 | Com570 ^e | + | ND |
| C ₂₈ H ₃₁ N ₅ O ₅ | 518.24092 | 17.21 | | + | + | C ₃₇ H ₄₂ N ₄ O ₅ | 623.32511 | 29.52 | Com622 ^e | + | ND |

Compounds detected by negative electrospray ionization (ESI-) are in **bold**. ^a R₁= 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 *PeΔbrlA* 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 *PeΔbrlA* strain. Expansolides and andrastins A, B, and C were detected in both strains. Interestingly, the most common compounds found in the *PeΔbrlA* 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 $C_{32}H_{38}N_2O_4$ ($R_T = 35.59, 37.93, \text{ and } 38.15$), $C_{32}H_{38}N_2O_5$ ($R_T = 21.75, 22.76, 23.91, 27.38, \text{ and } 28.52$), $C_{32}H_{36}N_2O_6$ ($R_T = 27.60, 29.23, 30.57, 31.50, 32.48, \text{ and } 34.05$), and $C_{32}H_{38}N_2O_6$ ($R_T = 23.19, 25.47, 27.09, \text{ and } 28.27$). 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 $C_{17}H_{23}N_3O_3$ ($R_T = 3.12 \text{ and } 25.05$), $C_{18}H_{35}N_3O_4$ ($R_T = 3.54$), $C_{19}H_{20}O_5$ ($R_T = 33.29$), $C_{20}H_{21}NO_9$ ($R_T = 22.19$), $C_{26}H_{32}O_8$ ($R_T = 9.67, 10.64, \text{ and } 34.14$), $C_{27}H_{29}N_5O_4$ ($R_T = 10.80 \text{ and } 11.03$), $C_{29}H_{27}N_5O_5$ ($R_T = 15.70 \text{ and } 17.61$), $C_{29}H_{31}N_5O_5$ ($R_T = 14.83, 15.85, \text{ and } 16.68$), and $C_{29}H_{33}N_5O_6$ ($R_T = 11.36 \text{ and } 12.14$) were found only in the null mutant *PeΔbrlA* strain. Unknown compounds (17) with chemical formulae $C_{10}H_{17}NO_5$ ($R_T = 9.65$), $C_{15}H_{20}O_4$ ($R_T = 16.91$), $C_{18}H_{16}N_2O_2$ ($R_T = 13.58 \text{ and } 17.74$), $C_{19}H_{16}N_2O_2$ ($R_T = 32.50 \text{ and } 33.17$), $C_{18}H_{16}N_2O_3$ ($R_T = 14.76$), $C_{20}H_{18}N_2O_2$ ($R_T = 35.48 \text{ and } 36.44$), $C_{19}H_{16}N_2O_4$ ($R_T = 13.73 \text{ and } 15.20$), $C_{19}H_{38}O_6$ ($R_T = 37.23 \text{ and } 38.19$), $C_{24}H_{26}N_2O_6$ ($R_T = 33.79$), $C_{28}H_{32}N_4O_3$ ($R_T = 15.50$), $C_{28}H_{38}O_8$ ($R_T = 28.44$), 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 *PeΔbrlA* 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 *PeΔbrlA* strain (30 dpi).

| Molecular formula | ¹² C <i>m/z</i> (Da) | R _T (min) ^a | Proposed Identification | Molecular formula | ¹² C <i>m/z</i> (Da) | R _T (min) ^a | Proposed Identification |
|--|---------------------------------|-----------------------------------|-------------------------|---|---------------------------------|-----------------------------------|---|
| C₇H₆O₄ | 153.01919 | 3.59 | Patulin | C ₂₉ H ₂₇ N ₅ O ₅ | 526.20689 | 17.61 | Chaetoglobosin J or Prochaetoglobosin III |
| C ₇ H ₈ O ₃ | 141.05493 | 3.80 | Gentisyl alcohol | C ₃₂ H ₃₆ N ₂ O ₄ | 513.27635 | 39.10 | Chaetoglobosin B/G |
| C ₇ H ₈ O ₄ | 157.04990 | 2.67 | Ascladiol | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26776 | 20.92 | Chaetoglobosin B/G |
| C ₁₀ H ₁₇ NO ₅ | 232.11872 | 8.83 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26807 | 23.19 | Chaetoglobosin B/G |
| C ₁₃ H ₁₄ O ₅ | 251.09108 | 21.70 | Citrinin | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27029 | 26.71 | Chaetoglobosin B/G |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | 375.14202 | 22.02 | | C ₃₂ H ₃₆ N ₂ O 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 ₂ O 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 ₂ O ₅ | 529.26807 | 32.43 | Chaetoglobosin B/G |
| C ₁₉ H ₂₁ NO ₇ | 376.13901 | 17.45 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26769 | 35.49 | Chaetoglobosin B/G |
| C ₁₉ H ₂₁ NO ₇ | 376.13904 | 18.65 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27056 | 37.41 | Chaetoglobosin B/G |
| C ₂₂ H ₂₃ N ₅ O ₂ | 390.19390 | 15.09 | Roquefortine C | C ₃₂ H ₃₆ N ₄ O ₂ | 509.29257 | 36.01 | Communesin B |
| C ₂₃ H ₂₄ N ₂ O ₆ | 425.17179 | 26.92 | | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28384 | 21.75 | Putative cytochalasan |
| C₂₈H₃₈O₇ | 485.25405 | 35.32 | | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28431 | 23.91 | Putative cytochalasan |
| C ₂₈ H ₃₈ O ₇ | 487.27063 | 36.21 | Andrastin A | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27864 | 23.19 | Putative cytochalasan |
| C₂₈H₃₈O₈ | 501.24792 | 28.44 | | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27841 | 25.47 | Putative cytochalasan |
| C₂₈H₃₈O₈ | 501.24786 | 29.57 | | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27842 | 27.09 | Putative cytochalasan |
| C₂₈H₄₀O₇ | 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 *PeΔbrlA* 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 C₁₆H₂₆N₂O₄S₂ (R_T= 22.02) C₁₈H₁₆N₂O₂ (R_T = 17.74), C₁₈H₁₈N₂O₂ (R_T = 14.61), C₁₉H₃₈O₆ (R_T = 38.19), C₂₂H₂₀N₃O (R_T = 38.56 and 39.29), C₂₃H₂₄N₂O₆ (R_T = 26.92), C₂₄H₂₆N₂O₆ (R_T = 33.79), C₂₆H₄₀O₆ (R_T = 29.93), C₂₈H₃₈O₇ (R_T = 35.32), C₂₈H₃₈O₈ (R_T = 27.70, 28.44, and 29.57), C₂₉H₃₁N₅O₆ (R_T = 14.66) were found in the synnemata of the null mutant *PeΔbrlA* strain.

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

| Molecular Formula | ¹² C <i>m/z</i> (Da) | ^a R _T (min) | Proposed Identification | Molecular Formula | ¹² C <i>m/z</i> (Da) | ^a R _T (min) | Proposed Identification |
|--|---------------------------------|-----------------------------------|-------------------------|---|---------------------------------|-----------------------------------|-------------------------|
| C ₁₅ H ₂₀ O ₄ | 265.14412 | 15.91 | Expansolide C/ D | C₂₈H₃₈O₈ | 501.24786 | 29.57 | |
| C ₁₅ H ₂₀ O ₄ | 265.14415 | 18.49 | Expansolide C/ D | C₂₈H₄₀O₆ | 471.27434 | 39.48 | Andrastin C |
| C ₁₆ H ₁₈ N ₂ O ₂ | 271.14496 | 7.62 | Clavicipitic acid | C₂₈H₄₀O₇ | 487.26898 | 30.58 | Andrastin B |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | 375.14202 | 22.02 | | C ₂₉ H ₃₃ N ₅ O ₆ | 548.25180 | 14.66 | |
| C ₁₇ H ₂₂ O ₅ | 307.15471 | 27.39 | Expansolide A/B | C ₃₂ H ₃₄ N ₄ O 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 ₂ O ₄ | 513.27635 | 39.10 | Chaetoglobosin J or Prochaetoglobosin III |
| C ₁₈ H ₁₆ N ₂ O ₂ | 293.12915 | 17.74 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27029 | 26.79 | Chaetoglobosin B/G |
| C ₁₈ H ₁₈ N ₂ O ₂ | 295.14479 | 14.61 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26757 | 29.56 | Chaetoglobosin B/G |
| C₁₉H₃₈O₆ | 361.25819 | 38.19 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27077 | 30.51 | Chaetoglobosin B/G |
| C₂₂H₂₀N₃O | 341.15380 | 38.56 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27067 | 33.36 | Chaetoglobosin A |
| C₂₂H₂₀N₃O | 341.15368 | 39.29 | | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26769 | 35.49 | Chaetoglobosin B/G |
| C ₂₂ H ₂₃ N ₅ O ₂ | 390.19390 | 15.09 | Roquefortine C | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27063 | 36.74 | Chaetoglobosin C |
| C ₂₂ H ₂₅ N ₅ O ₂ | 392.20913 | 9.99 | Roquefortine D | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27056 | 37.41 | Chaetoglobosin B/G |
| C ₂₃ H ₂₄ N ₂ O ₆ | 425.17179 | 26.92 | | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26332 | 27.60 | Putative cytochalasan |
| C₂₄H₂₆N₂O₆ | 437.17091 | 33.79 | | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26241 | 29.23 | Putative cytochalasan |
| C ₂₆ H ₃₀ N ₄ | 399.25568 | 19.94 | Communesin K | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26288 | 30.57 | Putative cytochalasan |
| C ₂₆ H ₃₀ N ₄ O | 415.25034 | 14.62 | Communesin I | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26294 | 32.48 | Putative cytochalasan |
| C ₂₆ H ₃₀ N ₄ O | 415.25033 | 18.43 | Communesin I | C ₃₂ H ₃₆ N ₄ O ₂ | 509.29257 | 34.95 | Communesin B |
| C ₂₆ H ₄₀ O ₆ | 449.28939 | 29.93 | | C ₃₂ H ₃₈ N ₂ O ₄ | 515.28895 | 35.59 | Putative cytochalasan |
| C ₂₇ H ₃₀ N ₄ O ₂ | 443.24564 | 15.65 | Communesin E | C ₃₂ H ₃₈ N ₂ O ₄ | 515.28890 | 37.93 | Putative cytochalasan |
| C ₂₈ H ₃₀ N ₄ O ₃ | 471.23997 | 19.56 | Com470 | C ₃₂ H ₃₈ N ₂ O ₄ | 515.28909 | 38.15 | Putative cytochalasan |
| C ₂₈ H ₃₂ N ₄ O | 441.26589 | 17.14 | Communesin F | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28990 | 22.76 | Putative cytochalasan |
| C ₂₈ H ₃₂ N ₄ O ₂ | 457.26116 | 23.65 | Communesin A | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28431 | 23.91 | Putative cytochalasan |
| C₂₈H₃₈O₇ | 485.25405 | 35.32 | | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28354 | 28.02 | Chaetoglobosin E |
| C ₂₈ H ₃₈ O ₇ | 487.27063 | 36.21 | Andrastin A | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28338 | 31.56 | Penochalasin |
| C₂₈H₃₈O₈ | 501.24792 | 27.70 | | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27864 | 23.19 | Putative cytochalasan |
| C₂₈H₃₈O₈ | 501.24792 | 28.44 | | C ₃₂ H ₃₈ N ₂ O 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 *PeΔbrlA* 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 Log₂-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 *PeΔbrlA* strain were related to conidiation (Table 4).

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

| | <i>Penicillium expansum</i> d1 Strain Gene ID | Protein Name | Log ₂ Fold Change <i>PeΔbrlA</i> vs WT | Adjusted <i>p</i> - 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 family chaperone |
| | PEXP_064110 | MedA | 1.13 | 3.51E-05 | Temporal modifier of developmental <i>psi</i> -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 | |
| Trehalose Biosynthesis Kinase | PEXP_097110 | Abr1 | -6.57 | 1.58E-12 | Multicopper oxidase |
| | PEXP_050560 | Ccg-9 | -5.04 | 2.47E-06 | Clock-controlled gene 9 |
| Velvet Protein Family | PEXP_066390 | Gin4 | -5.00 | 2.02E-12 | Localization and function of septins |
| | 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 *dhjA* 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 *PeΔbrlA* strain. The DEGs were considered when the Log₂-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 (Log₂-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 *PeΔbrlA* strain coding for backbone enzymes involved in secondary metabolite biosynthesis.

| | <i>Penicillium expansum</i> Strain d1 Gene ID | Biosynthetic Gene Cluster | Log ₂ Fold Change PeΔbrlA vs WT | Adjusted <i>p</i> - 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).

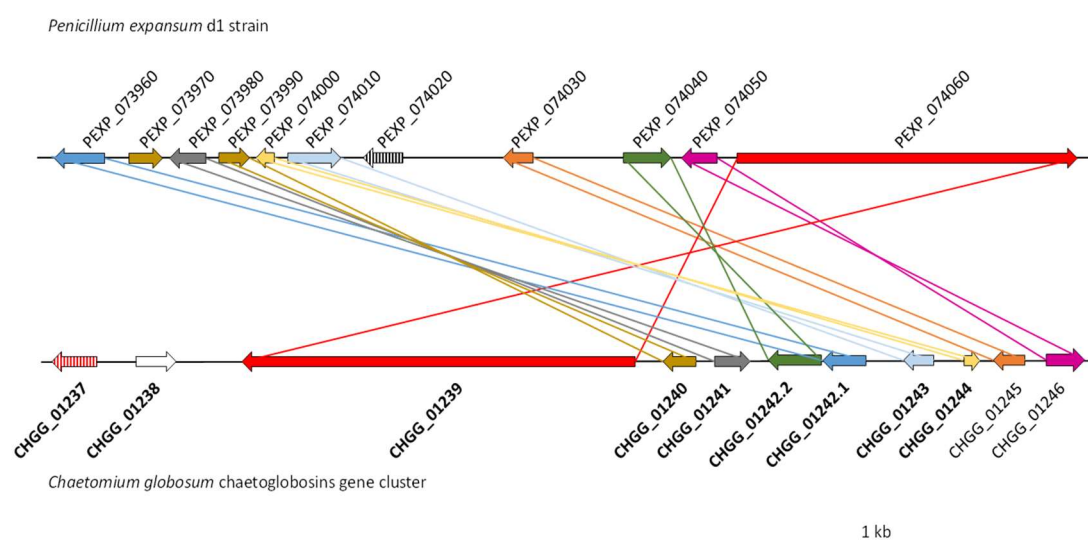


Figure 6. Comparison of the chaetoglobosin gene cluster in *Penicillium expansum* d1 strain and *Chaetomium globosum* strain. In bold, the cluster as described in Ishiuchi et al. [66]

Table 6. Putative chaetoglobosin gene cluster.

| <i>Penicillium expansum</i> Strain d1 | <i>Chaetomium globosum</i> Strain CBS 148.51 | % Identity / similarity | Log ₂ Fold Change PeΔbrlA 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 $\Delta brlA$ 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 C₂H₂-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 $Pe\Delta brlA$ 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 $Pe\Delta brlA$ 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 $Pe\Delta brlA$ 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 hyphal 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*, *abr1*, *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 $\Delta vosA$ 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 $\Delta brlA$ mutant of *P. rubens* [49] and *P. digitatum* [38], the lists of down- and up-regulated genes in the respective *Pe* $\Delta brlA$ strains were compared. Of the 106 genes whose expression decreased with a Log₂FC < -3 at *Pd* $\Delta brlA$, 101 have an orthologous gene in *P. expansum* and 79.2% of these were also significantly down-regulated (Log₂FC < -1, adj. *p*-value < 0.05) in the mutant *Pe* $\Delta brlA$ (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 *Pd* $\Delta brlA$ with a Log₂FC > 2 showed an increase in their expression in *Pe* $\Delta brlA$ (Table S5).

Sigl et al. [49] identified 38 genes regulated in a similar way to *wetA* in *P. rubens* $\Delta brlA$ (*Pr* $\Delta brlA$). Eighteen of these genes were also regulated in the same way in the *Pe* $\Delta brlA$ 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 $\Delta wetA$ 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* ΔtfA strain [78]. In the same study, the authors also identified 93 genes regulated in a similar way to *abaA* in *Pr* $\Delta brlA$, of which 59 orthologous genes were down-regulated in *Pr* $\Delta brlA$ and *Pe* $\Delta brlA$ 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 *Pd* $\Delta brlA$ (Log₂FC = -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 *Pe* $\Delta brlA$ strain in some media. The inability of *Pe* $\Delta brlA$ 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 *Pe* $\Delta brlA$, *Pd* $\Delta brlA$ [38], and *Pr* $\Delta brlA$ [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 *Pe* $\Delta brlA$ 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 *PeΔveA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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. *PeΔbrlA* 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 *PeΔveA*. 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 *PeΔpatL* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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, tryptacidin, and its two precursors (monomethylsulochrin and 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 *PeΔbrlA* 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 *PeΔbrlA*.

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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* strain was generated. Amplification of 5' and 3' locus *brlA/hph* junctions in *PeΔbrlA* 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 *PeΔbrlA* strain. This observation was confirmed in the microarray analysis (Log₂FC = -6.21; adjusted *p*-value 1.07E-14).

4.3. Macroscopic and Microscopic Morphology

The WT and *PeΔbrlA* 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 NaNO₃, 5 g/L MgSO₄ 7 H₂O, 0.1 g/L FeSO₄, 5 g/L KCl), 1 mg/L K₂HPO₄, 5 g/L yeast extract, 30 g/L saccharose, 15 g/L agar] media were inoculated centrally with 10 µl of a 10⁶ spores/mL suspension of the WT strain or 5 mm² of mycelium from the mutant *PeΔbrlA* 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 *PeΔbrlA* 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 (Branson 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 *PeΔbrlA* strains were grown in Petri dishes containing minimal medium (MM) [6.0 g/L NaNO₃, 1.5 g/L KH₂PO₄, 0.5 g/L KCl, 0.5 g/L MgSO₄, 200 μL/L trace elements (10 g/L EDTA, 4.4 g/L ZnSO₄ · 7H₂O, 1.01 g/L MnCl₂ · 4H₂O, 0.32 g/L CoCl₂ · 6H₂O, 0.315 g/L CuSO₄ · 5H₂O, 0.22 g/L (NH₄)₆Mo₇O₂₄ · 4H₂O, 1.47 g/L CaCl₂ · 2H₂O, 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 10⁶ spores/mL suspension of the WT strain or 5 mm² of mycelium from the mutant *PeΔbrlA* 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% ¹³C enriched wheat (¹³C wheat) and 53.4% ¹³C and 96.8% ¹⁵N wheat (¹³C/¹⁵N wheat); 99% ¹²C (¹²C 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 10⁵ spores/mL suspension of the WT strain or three fragments (5 mm²) of mycelium from the mutant *PeΔbrlA* strain. The cultures were incubated at 25 °C for 14 days in the dark; a Petri dish containing uninfected ¹²C 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 *PeΔbrlA* strain. The infected apples were incubated for 30 days at 25 °C in the dark. At the end of incubation, the synnemata (*PeΔbrlA*) 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 (N₂): 30 arbitrary units (a.u.), auxiliary gas

flow rate: 10 a.u. (N₂), 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 (N₂): 55 arbitrary units (a.u.), auxiliary gas flow rate: 10 a.u. (N₂), 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 ¹²C, ¹³C and ¹³C/¹⁵N 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 *PeΔbrlA* 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 10⁶ spores/mL suspension of the WT strain or 5 mm² 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 Pe Δ *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 Pd Δ *brlA* [38] and Pr Δ *brlA* [49] mutants, the microarray results were compared to those obtained in these previous studies. For *P. digitatum*, the differentially expressed genes in Pd Δ *brlA* (with Log₂FC < -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 Pd Δ *brlA* were listed in Table S4 and those homologous to genes significantly up-regulated in Pd Δ *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 Pr Δ *brlA*. The *P. expansum* homologous genes to genes similarly regulated to *wetA* and *abaA* in Pr Δ *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 Pe Δ *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 *PeΔbrlA* strains, Figure S2: Rot growth rates obtained from Golden Delicious apples infected with *Penicillium expansum* wild type NRRL 35695 and the null mutant *PeΔbrlA* strains, Figure S3: Morphological aspect of A) Null mutant *PeΔbrlA* 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 *PeΔbrlA* strains, Figure S5: Double-joint PCR reaction, Figure S6: PCR amplification of *Penicillium expansum* wild type NRRL 35695 (WT) and null mutant *PeΔbrlA* 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 *PeΔbrlA* 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* PdΔbrlA strain [38], Table S5: *Penicillium expansum* genes orthologous to genes significantly up-regulated (Log2 fold change > 2) in *Penicillium digitatum* PdΔbrlA strain [38], Table S6: Eighteen orthologous genes similarly regulated to *wetA* in *Penicillium rubens* ΔbrlA [49] and *Penicillium expansum* ΔbrlA, 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 *PeΔbrlA* 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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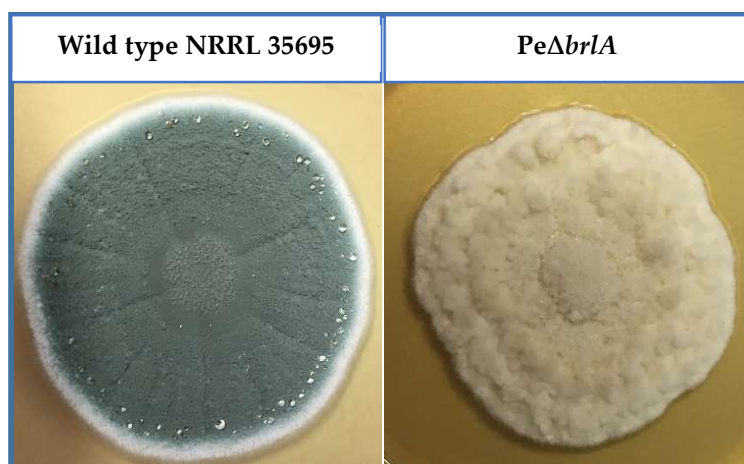
Supplementary Materials

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

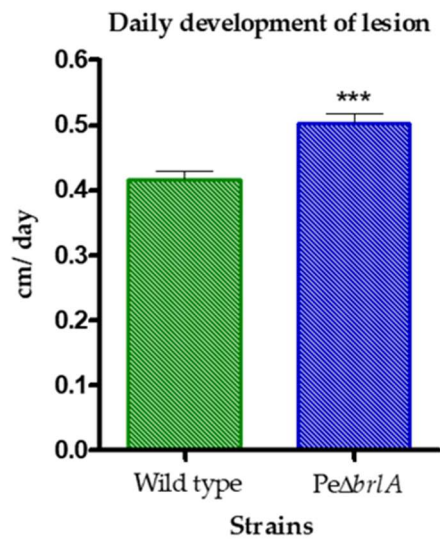
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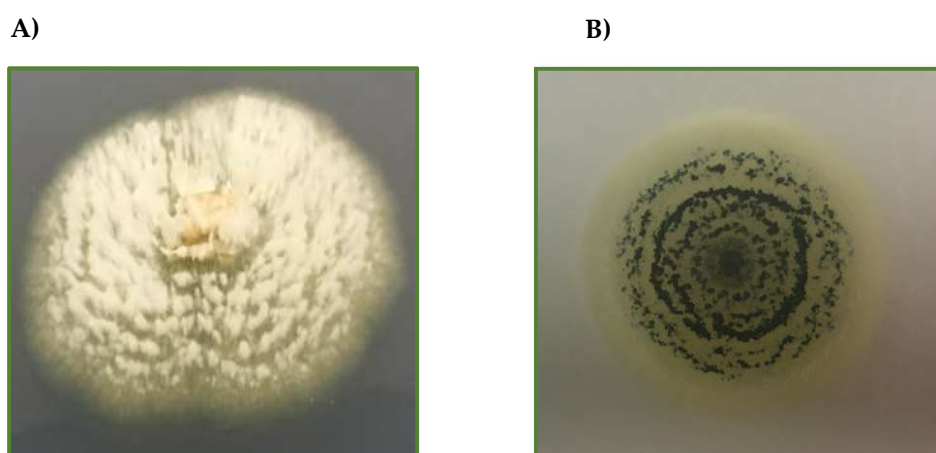
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Supplementary Figure 1. Morphological aspect of *Penicillium expansum* wild type NRRL 35695 and the null mutant Pe Δ brlA strains. The strains were grown on MEA at 25 °C in the dark for seven days.



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



Supplementary Figure 3. Morphological aspect of A) Null mutant *PeΔbrlA* 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 <i>m/z</i> (Da) | ¹³ C <i>m/z</i> (Da) | ¹³ C/ ¹⁵ N <i>m/z</i> (Da) | <i>m/z</i> of major fragment ions |
|--|--------------------------|---------------------------------|---------------------------------|--|---|
| C₇H₆O₄ | Pexp_153.019_3.59 | 153.01919 | 160.04256 | 156.02997 | 153→ 125, 109(100), 97 |
| C ₇ H ₈ O | Pexp_109.065_7.11 | 109.06509 | 116.08844 | 112.07483 | - |
| C ₇ H ₈ O ₂ | Pexp_125.060_6.79 | 125.05998 | 132.08346 | 129.07332 | 125→ 106(100), 97, 95, 83, 81, 79, 69 |
| C ₇ H ₈ O ₃ | Pexp_141.054_3.80 | 141.05493 | 148.07869 | 144.06492 | 141→ 123, 113(100), 110, 95, 79, 69, 55 |
| C ₇ H ₈ O ₄ | Pexp_157.049_2.67 | 157.04990 | 164.07348 | 161.06366 | 157→ 139(100), 111, 97, 83 |
| C ₇ H 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 ₂ O ₂ | Pexp_271.144_7.62 | 271.14496 | 287.19861 | 281.16599 | 271→ 254(100), 225, 215, 168 |
| C ₁₆ H ₁₈ N ₂ O ₂ | Pexp_271.144_8.45 | 271.14467 | 287.19873 | 281.16576 | 271→ 254(100), 225, 215, 168 |
| C ₁₆ H ₂₆ N ₂ O ₄ S 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 ₂ O ₂ | Pexp_293.129_13.58 | 293.12911 | 311.18978 | 305.15587 | 293→ 265(100), 248, 237, 222, 120 |
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_17.74 | 293.12915 | 311.18970 | 305.15668 | 293→ 265(100), 248, 237, 222, 120 |
| C ₁₈ H ₁₆ N ₂ O ₃ | Pexp_309.124_14.76 | 309.12421 | 327.18455 | 320.14798 | 309→ 292 (100), 267, 249, 231 |
| C ₁₈ H ₁₈ N ₂ O 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 ₂ O ₂ | Pexp_305.129_32.50 | 305.12911 | 324.19310 | 316.15289 | 305→ 277(100), 264, 262, 205, 187 |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_33.17 | 305.12933 | 324.19301 | 317.15701 | 305→ 277(100), 264, 262, 205, 187 |

| | | | | | |
|---|---------------------------|------------------|------------------|------------------|--|
| C ₁₉ H ₁₆ N ₂ O 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 ₂ O 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 |
| C₁₉H₃₈O₆ | Pexp_361.258_37.23 | 361.25871 | 380.32251 | 371.29242 | 361→ 343, 325, 283, 273, 257(100), 157 |
| C₁₉H₃₈O₆ | Pexp_361.258_38.19 | 361.25819 | 380.32253 | 371.29252 | 361→ 343, 325, 299, 283, 257(100), 157 |
| C ₂₀ H ₁₈ N ₂ O 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 ₂ O 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 ₅ O ₂ | Pexp_390.193_15.09 | 390.19390 | 412.26737 | 406.21627 | 390→ 334, 322(100), 198, 193 |
| C ₂₂ H ₂₅ N ₅ O ₂ | Pexp_392.209_9.99 | 392.20913 | 414.28326 | 408.22845 | 392→ 336, 324(100), 198, 195 |
| C ₂₃ H ₂₄ N ₂ O ₆ | Pexp_425.171_26.92 | 425.17179 | 448.24874 | 439.20611 | 425→ 407, 383, 365, 288(100), 260 |
| C₂₄H₂₆N₂O₆ | 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 ₄ O | 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 ₄ O | 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 ₄ O 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 ₄ O 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 ₅ O ₅ | Pexp_518.241_16.36 | 518.24108 | 546.33497 | 538.27672 | 518→ 500, 462, 450(100), 321 |
| C ₂₈ H ₃₁ N ₅ O 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 ₄ O | 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 ₄ O 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 ₄ O ₂ | Pexp_457.260_14.97 | 457.26095 | 485.35394 | 476.29996 | 457→ 439, 385(100), 239, 185 |
| C ₂₈ H ₃₂ N ₄ O 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 ₄ O 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 ₄ O ₄ | Pexp_493.279_31.47 | 493.27971 | 521.37363 | 511.31495 | - |
| C ₂₈ H ₃₈ N ₄ O 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 ₄ O ₆ | Pexp_527.287_12.46 | 527.28755 | 555.38184 | 545.32412 | 527→ 281(100), 509, 481, 346, 301, 265, 247, 219, 182 |

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|---|---------------------------|------------------|------------------|------------------|---|
| C₂₈H₃₈O₇ | 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 |
| C₂₈H₃₈O₈ | Pexp_501.247_27.70 | 501.24792 | 529.34203 | 516.29846 | 501→ 457, 441(100), 425, 410, 397, 373, 365, |
| C₂₈H₃₈O₈ | Pexp_501.247_28.44 | 501.24792 | 529.34203 | 516.29846 | 501→ 457, 441(100), 425, 410, 397, 373, 365, |
| C₂₈H₃₈O₈ | Pexp_501.247_29.57 | 501.24786 | 529.34194 | 516.29974 | 501→ 486, 469, 457, 441(100), 425, 397, 373, 366 |
| C₂₈H₄₀O₆ | Pexp_471.274_39.48 | 471.27434 | 499.36727 | 486.32419 | 471→ 456, 439, 411(100) |
| C₂₈H₄₀O₇ | Pexp_487.268_30.58 | 487.26898 | 515.36247 | 502.31903 | 487→ 455, 427(100), 411, 395, 351,336 |
| C ₂₉ H ₃₃ N ₅ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O ₅ | Pexp_529.270_37.41 | 529.27056 | 561.37807 | 548.32181 | 529→511 (100), 493, 453, 380, 327, 185 |
| C₃₂H₃₆N₂O₆ | 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 ₄ O ₂ | Pexp_509.292_36.01 | 509.29257 | 541.39875 | 530.3376 | 509→ 437(100), 451, 185 |
| C ₃₂ H ₃₆ N ₄ O 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 ₄ O ₃ | Pexp_525.287_27.91 | 525.28705 | 557.39461 | 546.33215 | 525→ 508(100), 271, 273, 199 |
| C ₃₂ H ₃₈ N ₄ O ₂ | Pexp_511.306_36.13 | 511.30684 | 543.41590 | 532.35205 | 511→ 439(100), 453, 185, 144 |
| C ₃₂ H ₃₈ N ₄ O 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 ₄ O ₄ | Pexp_543.297_19.36 | 543.29789 | 575.40330 | 564.34345 | 543→ 525, 485, 471(100), 185, 168 |
| C ₃₂ H ₄₀ N ₄ O ₂ | Pexp_513.322_36.57 | 513.32227 | 545.43031 | 533.36211 | 513→ 495, 455, 441(100), 257, 185 |
| C ₃₃ H ₃₈ N ₄ O 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 ₄ O 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 *PeΔbrlA* strain after culture on labeled wheat grains.

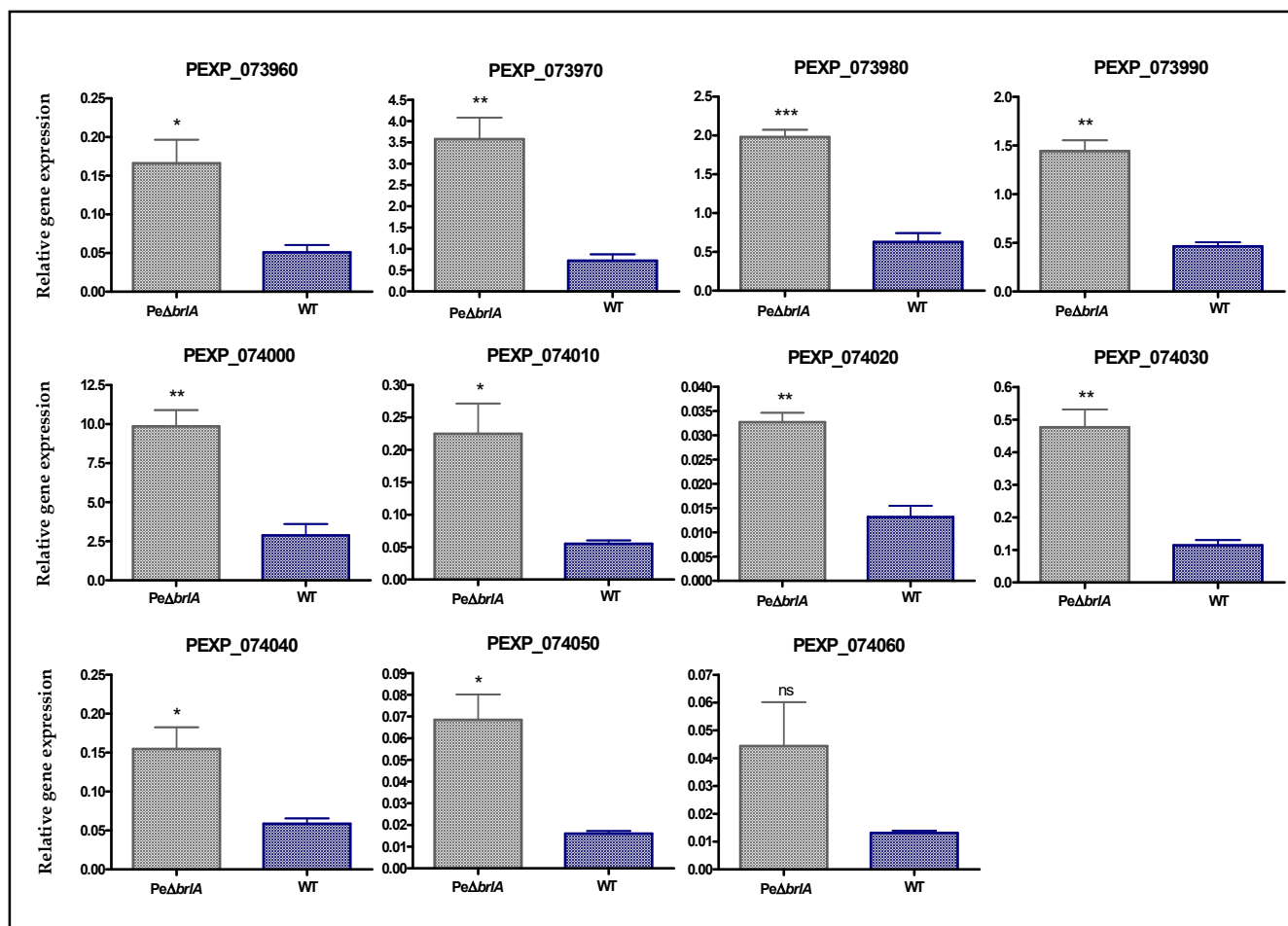
| Molecular formula | Identifier ^a | ¹² C <i>m/z</i> (Da) | ¹³ C <i>m/z</i> (Da) | ¹³ C/ ¹⁵ N <i>m/z</i> (Da) | <i>m/z</i> of major fragment ions |
|---|-------------------------|---------------------------------|---------------------------------|--|---|
| C ₁₇ H ₂₃ N ₃ O ₃ | Pexp_318.179_03.12 | 318.17943 | 335.23746 | 330.20149 | 318→ 301(100), 272, 258, 205, 188, 178, 170, 159, 132 |
| C ₁₇ H ₂₃ N ₃ O 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 ₃ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O ₆ | Pexp_545.262_29.23 | 545.26241 | 577.37065 | 564.31731 | - |
| C ₃₂ H ₃₆ N ₂ O 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 ₂ O ₆ | Pexp_545.262_31.50 | 545.26281 | 577.36975 | 564.31459 | - |
| C ₃₂ H ₃₆ N ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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_Rt). m/z = m/z ratio and Rt= 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 | GCGAGATGTTTGCACCTT | |
| cheF_F2 | AACTGCTGAGAAGTTCA | 900/900 and 900/300 |
| cheF_R2 | GTTGCGTATCTCATTACACC | |
| 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 | GAGGTAGAAGTGGGTTTCAT | |
| PEXP_073980_F1 | GAACAACCAGCCATTGAG | 900/300 |
| PEXP_073980_R1 | GTTAGGATCGCGGTAGAA | |
| PEXP_073990_F | GAGACGGTCTCGTCTTTGG | 300/300 |
| PEXP_073990_R | ACACGATGGCCGAGAAG | |
| PEXP_074000_F | CCAACGTTTCGCTATCCA | 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 | TGAAGTCTCGAACCAA | 300/900 |
| PEXP_074030_R | TTTGCTTGTTTCAGGATGG | |
| PEXP_074040_F1 | TCCTGTCGCCTCATTAT | 900/300 |
| PEXP_074040_R1 | CACCGCTGTCTTGATAG | |
| 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 *PeΔbrlA* strains. The graphs show the mean \pm standard error of the mean (SEM) from three biological replicates and the significant differences between the wild type and the null mutant *PeΔbrlA* 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 Δ brlA strain [38].

| <i>Penicillium digitatum</i> PHI26 strain Gene ID | <i>Penicillium digitatum</i> GenBank Accession number | Log_2 Fold Change Pd Δ brlA Vs WT ^{1*} | % Positives | <i>Penicillium expansum</i> strain d1 Gene ID | Log_2 fold change Pe Δ brlA Vs WT ² | Adjusted <i>p</i> -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 |

¹Pd Δ brlA = *Penicillium digitatum* null mutant Δ brlA strain. WT = wild type *Penicillium digitatum* strain

²Pe Δ 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 (Log₂ fold change > 2) in *Penicillium digitatum* PdΔ*brlA* strain [38].

| <i>Penicillium digitatum</i> PHI26 strain Gene ID | <i>Penicillium digitatum</i> GenBank Accession number | Log ₂ Fold Change PdΔ <i>brlA</i> vs WT ¹ * | % Positives | <i>Penicillium expansum</i> strain d1 Gene ID | Log ₂ fold change PeΔ <i>brlA</i> vs WT ² | Adjusted <i>p</i> -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 | - |

¹Pd Δ brlA = *Penicillium digitatum* null mutant Δ brlA strain, WT = wild type *Penicillium digitatum* strain

²Pe Δ 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* $\Delta brlA$ [49] and *Penicillium expansum* $\Delta brlA$.

| <i>Penicillium expansum</i> strain d1 gene ID | <i>Penicillium rubens</i> strain * Wisconsin 54-1255 gene ID | % Identity | Log ₂ fold change Pe $\Delta brlA$ vs WT | Adjusted <i>p</i> -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]
 $\Delta brlA$ and *Penicillium expansum* $\Delta brlA$.

| <i>Penicillium expansum</i> strain d1 gene ID | <i>Penicillium rubens</i> strain * Wisconsin 54-1255 gene ID | % Identity | Log ₂ fold change Pe $\Delta brlA$ vs WT | Adjusted <i>p</i> -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 | 1.58E-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 *PeΔbrlA* strain

Creation of a Linear Transformation Cassette using Double-Joint PCR

For the creation of the null mutant *PeΔbrlA* strain, a strategy was applied to eliminate the *brlA* gene (gene coding for a master transcript 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].

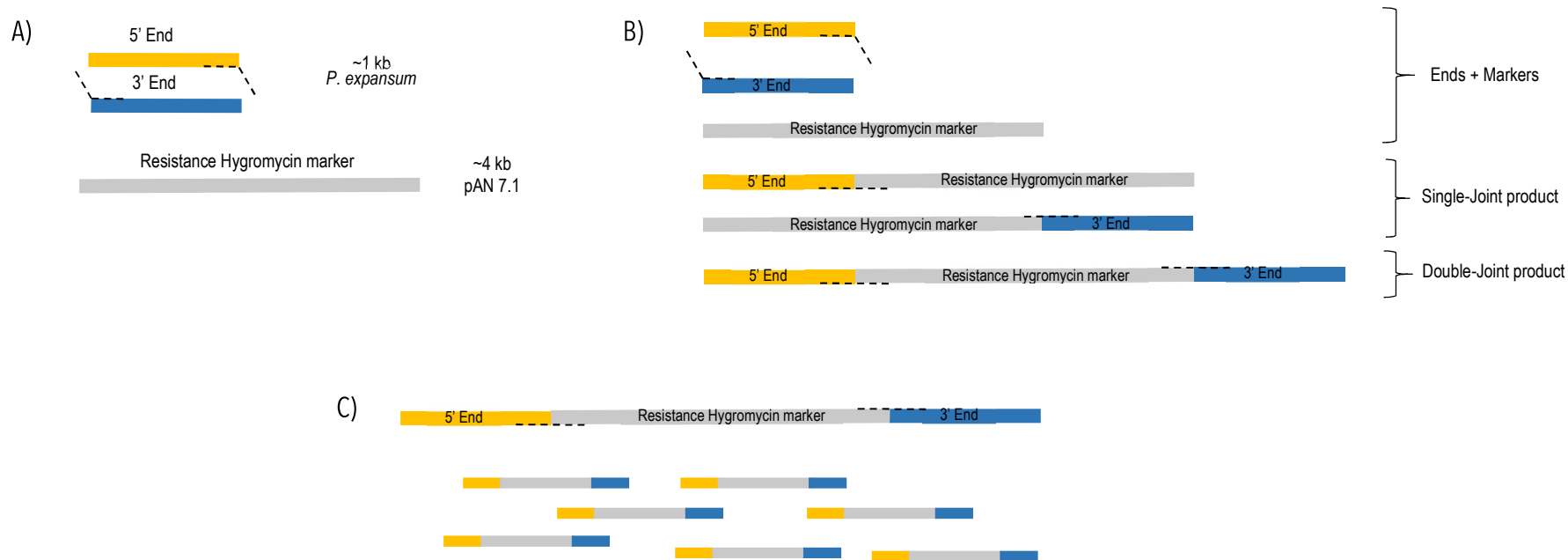
Supplementary Table 8. Primers used in the construction and the validation of the null mutant *PeΔbrlA* strain.

| Primer | Sequence 5' → 3' |
|--------------------------------|---|
| 5fdebBrlAPex | GTAACGCCAGGGTTTTCCAGTCACGACG |
| DebBrlAPex1Rhygro ¹ | GAGCCTGTGTGTAGAGATACAAGGGAATTC <u>TGACCCTTGGCTGTAAAGACTGGTAGG</u> |
| FinBrlAPex1Fhygro ¹ | GTGTAAGCGCCCACTCCACATCTCCACTCGCGTGGCCTTCATTGGCCTTTATTGATAC |
| 3rfinBrlAPex | GCGGATAACAATTTACACAGGAAACAGC |
| hphF1 | GAATTCCTTGTATCTCTACACACAGGCTC |
| hphR1 | CGAGTGGAGATGTGGAGTGGGCG |
| dBrlA-NestedF | GTTGCGGCCGGAACCTTGAACC |
| dBrlA-NestedR | CCAGAGATGTCTGAGCTCCAATCGC |
| dBrlA-geneF | GGCTTCCCCTGGGTGGCAC |
| dBrlA-geneR | GCCATCGACGCTCAGCTCGC |
| Hyg5'1 R | CTTGACACCGCTCCGTCCTCC |
| Hyg3'1 F | GGGTTTACCTCTTCCAGATACAGCTC |
| dBrlA-5'F | GGAGTGTGGGAGATCAGGGTCCG |
| 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 |

¹The 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.

²Primers used in the validation by genome walking.

³Primers used in the validation by qPCR.



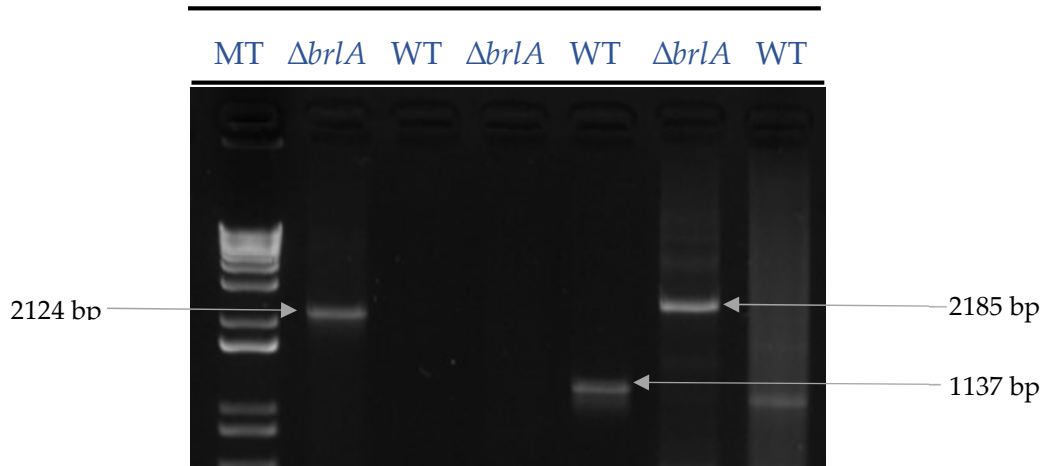
Supplementary Figure 5. Double-joint PCR reaction [97]. A) PCR reaction for amplification of flanking regions 5' upstream and 3' downstream of the *brlA* gene from *Penicillium expansum* and amplification of the hygromycin marker from the pAN7.1 plasmid. B) Primerless PCR reaction using the three previously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

Validation of the Null Mutant Pe Δ brlA strain

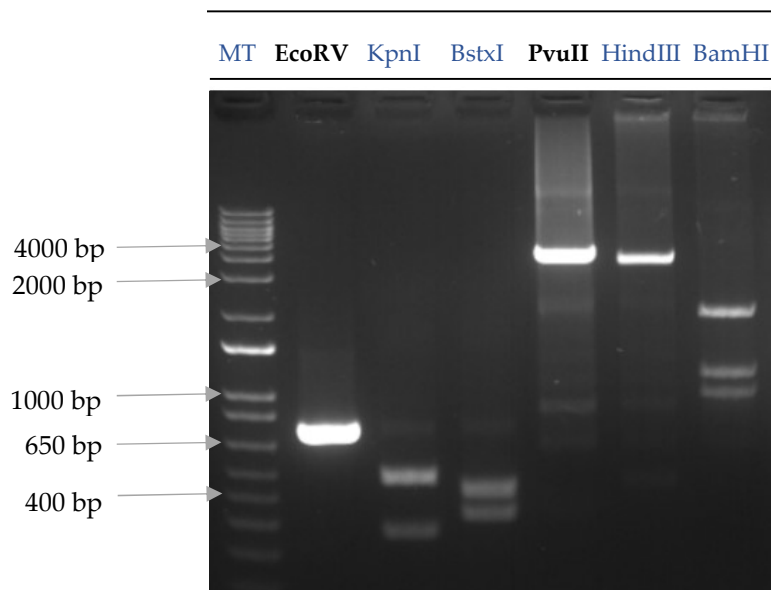
Polymerase Chain Reaction (PCR) and Genome Walking

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 μ g 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 reactions 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 $\Delta brlA$ strains. Primers, lane 1 and 2: dBr1A-5'F / Hyg5'1 R, lane 3 and 4: dBr1A-geneF / dBr1A-geneR and lane 5 and 6: Hyg3'1 F / dBr1A -3'R. The presence of the disruption cassette $\Delta brlA$ 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 dBr1A-5'F / Hyg5'1 R and Hyg3'1 F / dBr1A -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 $\Delta brlA$ strains. Lane 1 and 4: PCR products with the primers AP2-GWBr1A3'2 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

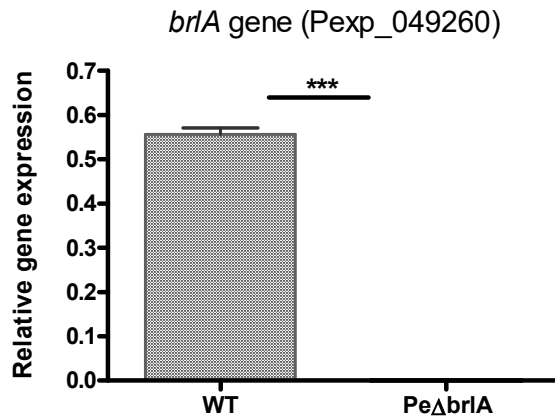
Quantitative real-time PCR Validation

To investigate the gene expression of each transformant strain, a qPCR analysis was performed. The WT and null mutant *PeΔbrlA* 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 10⁶ spores/mL solution for the WT strain, 5 mm² 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 *PeΔbrlA* 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'-GCTGTCAACGATACGCTATAACGGCATGACAGTGT TTT TTT TTT TTT TTT-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^{-ΔΔCT} 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 Pe Δ *brlA* strains. The Student's t-test was performed using GraphPad Software. The graph shows the mean \pm 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 ΔveA strains were grown in three types of wheat; natural wheat ^{12}C and two different isotopic enrichment, ^{13}C labeled wheat and $^{13}\text{C}/^{15}\text{N}$ 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 aggressiveness 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 $^{13}\text{C}/^{15}\text{N}$ 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, *Pe Δ 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 $\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}_2$ and $\text{C}_{20}\text{H}_{18}\text{N}_2\text{O}_2$.

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 *PeΔveA* and *PeΔveA: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 ¹²C, ¹³C and ¹³C/¹⁵N 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% ¹³C enriched wheat (referred to as ¹³C wheat) and the second of 53.4% ¹³C and 96.8% ¹⁵N wheat (referred to as ¹³C/¹⁵N 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 ¹³C wheat, one dish with ¹³C/¹⁵N wheat and two dishes with ¹²C 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 (10⁵ spores/mL), quantified using a Malassez cell (Adjovi et al. 2014), of the WT and null mutant *PeΔveA* 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 ¹²C 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 *PeΔveA* 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 *PeΔveA* 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 10⁶ 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 *PeΔveA*. 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 (10⁴ spores/mL) of the WT and null mutant *PeΔveA* strains. Infected apples were incubated for 14 days at 25 °C in the dark. At the end of the incubation, the spores were 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 (N₂): 55 arbitrary units (a.u.), auxiliary gas flow rate (N₂): 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 (N₂): 30 arbitrary units (a.u.), auxiliary gas flow rate (N₂): 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 ¹²C, ¹³C and ¹³C/¹⁵N 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 ¹²C wheat grains, ¹³C, and ¹³C¹⁵N 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 C₃₁H₃₆N₄O₂. 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 *gliC*, *gliG* and *gliT* 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 Pe Δ veA produce 78 SMs in common, 17 compounds are produced only by the WT, while nine SMs are produced only by the null mutant Pe Δ 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. Pe Δ veA is able of producing all communesin derivatives with the exception of the New DME2 communesin (C₂₈H₃₂N₄O₂, 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 Pe Δ veA producing mainly chaetoglobosin A and C and six of ten putative cytochalasan derivatives. Seven unknown compounds with molecular formula C₁₀H₁₇NO₅ (R_T= 2.42), C₁₇H₁₇NO₇ (R_T=11.75, 12.65 and 15.90), C₂₇H₃₀N₄O (R_T= 10.19) and C₃₀H₃₉N₅O₅ (R_T= 18.25 and 19.14) were only detected in the null mutant strain. While the unknown metabolite with formula C₁₅H₂₀O₄ (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 PeΔveA strains after culture on labeled wheat grains.

| Molecular formula | Identifier ^a | ¹² C m/z (Da) | Proposed identification | WT | PeΔveA | Molecular formula | Identifier ^a | ¹² C m/z (Da) | Proposed identification | WT | PeΔveA |
|--|-------------------------|--------------------------|-------------------------|----|--------|---|---------------------------|--------------------------|--|----|--------|
| C ₇ H ₆ O ₄ | Pexp_153.019_3.59 | 153.01919 | patulin | + | ND | C ₂₈ H ₃₁ N ₅ O ₅ | Pexp_518.241_16.36 | 518.24108 | | + | + |
| C ₇ H ₈ O | Pexp_109.065_7.11 | 109.06509 | m-Cresol | + | ND | C ₂₈ H ₃₁ N ₅ O ₅ | Pexp_518.240_17.21 | 518.24092 | | + | + |
| C ₇ H ₈ O ₂ | Pexp_125.060_6.79 | 125.05998 | m-Hydroxybenzyl alcohol | + | ND | C ₂₈ H ₃₂ N ₄ O | Pexp_441.265_17.14 | 441.26589 | Communesin F ^d | + | + |
| C ₇ H ₈ O ₃ | Pexp_141.054_3.8 | 141.05493 | Gentisyl alcohol | + | ND | C ₂₈ H ₃₂ N ₄ O ₂ | Pexp_457.260_14.27 | 457.26097 | New DMV ¹ communesin-1 | + | + |
| C ₇ H ₈ O ₄ | Pexp_157.049_2.67 | 157.04990 | Ascladiol | + | ND | C ₂₈ H ₃₂ N ₄ O ₂ | Pexp_457.260_14.97 | 457.26095 | New DME ² communesin-1 | + | ND |
| C ₇ H ₁₀ O ₃ | Pexp_143.070_4.52 | 143.07061 | | + | + | C ₂₈ H ₃₂ N ₄ O 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 ₄ O 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 ₄ O 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 ₄ O ₅ | Pexp_511.290_18.01 | 511.29099 | VAL-PHE-VAL-PHE | + | + |
| C ₁₃ H ₁₈ N ₂ | Pexp_227.155_6.44 | 227.15514 | Aurantioclavine | + | + | C ₂₈ H ₃₈ N ₄ O 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 | | + | + | C₂₈H₃₈O₇ | 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 | + | + | C₂₈H₃₈O₈ | Pexp_501.247_27.70 | 501.24792 | | + | + |
| C ₁₅ H ₂₀ O ₄ | Pexp_265.144_16.91 | 265.14410 | | + | ND | C₂₈H₃₈O₈ | Pexp_501.247_28.44 | 501.24792 | | + | + |
| C ₁₅ H ₂₀ O ₄ | Pexp_265.144_18.49 | 265.14415 | Expansolide C/ D | + | + | C₂₈H₃₈O₈ | Pexp_501.247_29.57 | 501.24786 | | + | + |
| C ₁₅ H ₂₀ O ₄ | Pexp_265.144_19.35 | 265.14414 | | + | ND | C₂₈H₄₀O₆ | Pexp_471.274_39.84 | 471.27434 | Andrastin C | + | + |
| C ₁₆ H ₁₈ N ₂ O ₂ | Pexp_271.144_7.62 | 271.14496 | Clavicipitic acid | + | + | C₂₈H₄₀O₇ | Pexp_487.268_30.58 | 487.26898 | Andrastin B | + | + |
| C ₁₆ H ₁₈ N ₂ O ₂ | Pexp_271.144_8.45 | 271.14467 | Clavicipitic acid | + | + | C ₂₉ H ₃₃ N ₅ O ₆ | Pexp_548.251_15.42 | 548.25180 | | + | + |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | Pexp_375.142_22.02 | 375.14202 | | + | + | C ₃₀ H ₃₆ N ₄ O 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 ₅ O 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 ₅ O 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 ₄ O 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 ₄ O ₃ | Pexp_523.271_31.84 | 523.27152 | Communesin D ^e | + | + |
| C ₁₇ H ₂₂ O ₅ | Pexp_307.155_30.19 | 307.15504 | Expansolide A/B | + | + | C ₃₂ H ₃₆ N ₂ O ₄ | Pexp_513.276_39.10 | 513.27635 | Chaetoglobosin J or Prochaetoglobosin III | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_13.58 | 293.12911 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_20.92 | 529.26776 | Chaetoglobosin B/G | + | ND |

| | | | | | | | | | | | |
|---|---------------------------|------------------|---------------------------|----|---|---|---------------------------|------------------|--------------------------------------|----|----|
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_17.74 | 293.12915 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_25.21 | 529.27058 | Chaetoglobosin B/G | + | ND |
| C ₁₈ H ₁₆ N ₂ O ₃ | Pexp_309.124_14.76 | 309.12421 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_26.71 | 529.27029 | Chaetoglobosin B/G | + | + |
| C ₁₈ H ₁₈ N ₂ O ₂ | Pexp_295.144_14.61 | 295.14479 | | + | + | C ₃₂ H ₃₆ N ₂ O 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 ₂ O ₅ | Pexp_529.270_30.51 | 529.27077 | Chaetoglobosin B/G | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_32.50 | 305.12911 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_33.98 | 529.27067 | Chaetoglobosin A | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_33.17 | 305.12933 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_36.68 | 529.27063 | Chaetoglobosin C | + | + |
| C ₁₉ H ₁₆ N ₂ O ₄ | Pexp_337.119_13.73 | 337.11909 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_37.41 | 529.27056 | Chaetoglobosin B/G | + | ND |
| C ₁₉ H ₁₆ N ₂ O ₄ | Pexp_337.119_15.20 | 337.11916 | | + | + | C ₃₂ H ₃₆ N ₂ O 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 ₂ O 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 ₂ O ₆ | Pexp_545.262_31.50 | 545.26281 | Putative cytochalasan | + | ND |
| C₁₉H₃₈O₆ | Pexp_361.258_37.23 | 361.25871 | | + | + | C₃₂H₃₆N₂O₆ | Pexp_543.248_34.05 | 543.24836 | Putative cytochalasin | + | ND |
| C₁₉H₃₈O₆ | Pexp_361.258_38.19 | 361.25819 | | + | + | C ₃₂ H ₃₆ N ₄ O ₂ | Pexp_509.292_36.01 | 509.29257 | Communesin B ^e | + | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | Pexp_319.145_35.48 | 319.14588 | | + | + | C ₃₂ H ₃₆ N ₄ O ₄ | Pexp_541.280_07.78 | 541.28053 | Com540 ^b | ND | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | Pexp_319.145_36.44 | 319.14502 | | + | + | C ₃₂ H ₃₈ N ₂ O 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 ₂ O 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 ₂ O ₆ | Pexp_515.289_38.15 | 515.28909 | Putative cytochalasan | + | + |
| C ₂₂ H ₂₃ N ₅ O ₂ | Pexp_390.193_15.09 | 390.19390 | Roquefortine C | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_531.289_22.76 | 531.28990 | Putative cytochalasan | + | ND |
| C ₂₂ H ₂₅ N ₅ O ₂ | Pexp_392.209_9.99 | 392.20913 | Roquefortine D | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_531.283_27.38 | 531.28360 | Putative cytochalasan | + | + |
| C ₂₃ H ₂₄ N ₂ O ₆ | Pexp_425.171_26.92 | 425.17179 | | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_531.283_28.52 | 531.28340 | Putative cytochalasan | + | + |
| C₂₄H₂₆N₂O₆ | Pexp_437.170_33.79 | 437.17091 | | + | + | C ₃₂ H ₃₆ N ₄ O 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 ₄ O ₃ | Pexp_525.287_27.91 | 525.28705 | New DMV ¹ communesin-3 | + | + |
| C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_14.62 | 415.25034 | Communesin I ^c | + | + | C ₃₂ H ₃₈ N ₄ O ₂ | Pexp_511.306_36.13 | 511.30684 | New DME ² communesin-3 | + | + |
| C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_18.43 | 415.25033 | Communesin I ^c | + | + | C ₃₂ H ₃₈ N ₄ O 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 ₄ O ₄ | Pexp_543.297_19.36 | 543.29789 | New DME ² communesin-5 | + | + |
| C ₂₇ H ₃₀ N ₄ O | Pexp_427.249_10.19 | 427.24923 | | ND | + | C ₃₂ H ₄₀ N ₄ O ₂ | Pexp_513.322_36.57 | 513.32227 | New DME ² communesin-6 | + | + |
| C ₂₇ H ₃₀ N ₄ O ₂ | Pexp_443.245_15.03 | 443.24564 | Communesin E ^d | + | + | C ₃₃ H ₃₈ N ₄ O ₅ | Pexp_571.293_18.22 | 571.29324 | Com570 ^e | + | + |
| C ₂₈ H ₃₀ N ₄ O ₃ | Pexp_471.239_19.56 | 471.23997 | Com470 ^e | + | + | C ₃₇ H ₄₂ N ₄ O ₅ | Pexp_623.325_29.52 | 623.32511 | Com622 ^e | + | + |

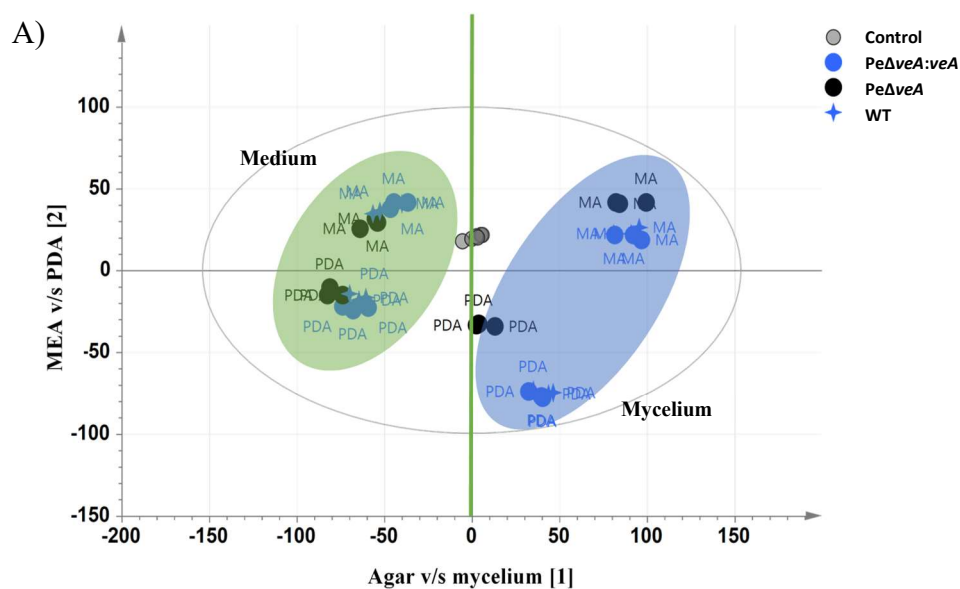
CHAPTER 3: RESULTS

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), ²Dimethylepoxyde (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 *PeΔveA* 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, *PeΔveA* and *PeΔveA: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.



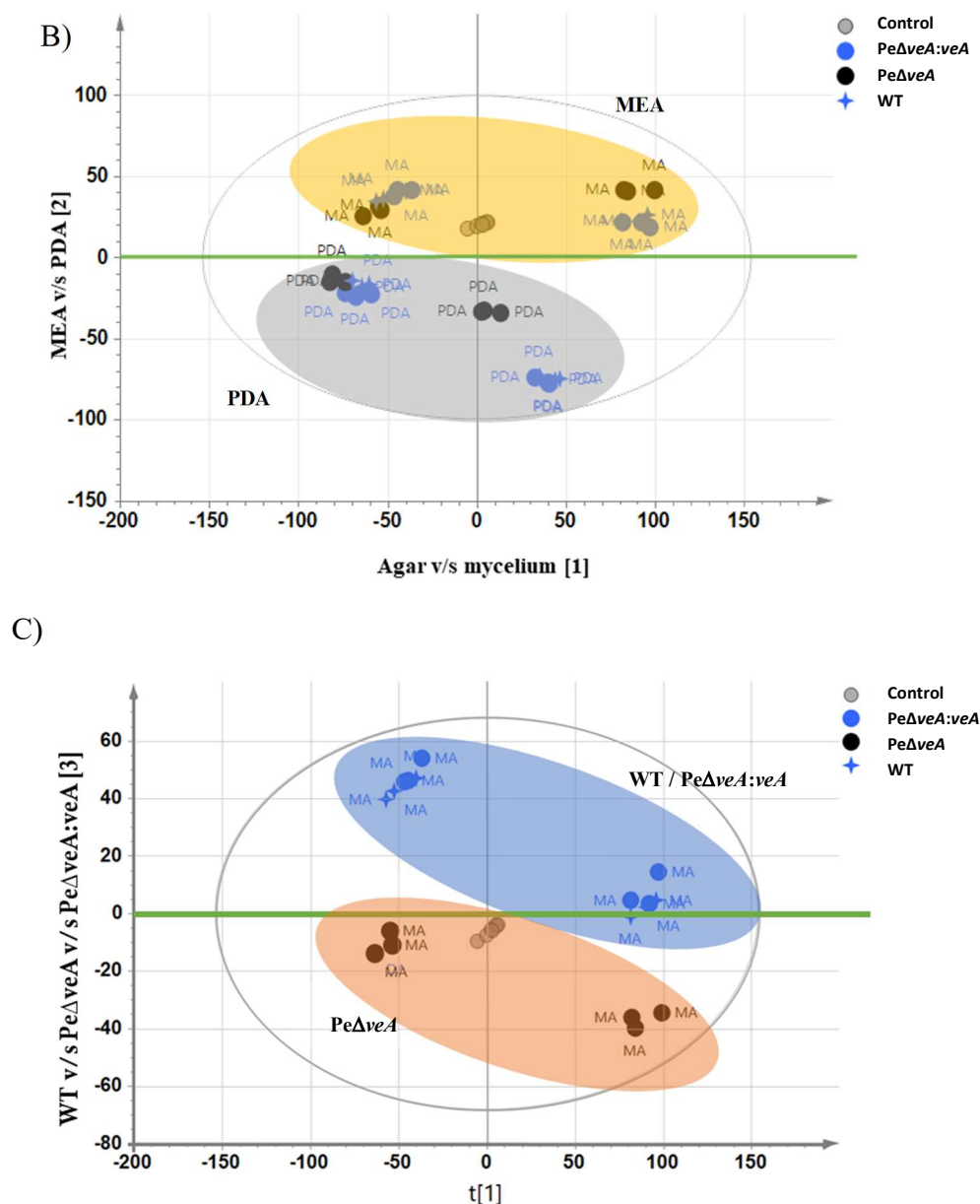


Figure 1. Principal Component Analysis (PCA) of the data obtained from the mass spectra. A) Effect of the location of SM production, agar medium versus fungal extract. B) Substrate effect, MEA medium versus PDA medium. C) Deletion of *veA* effect, PeΔveA versus WT / PeΔveA:veA (MEA medium). The WT results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

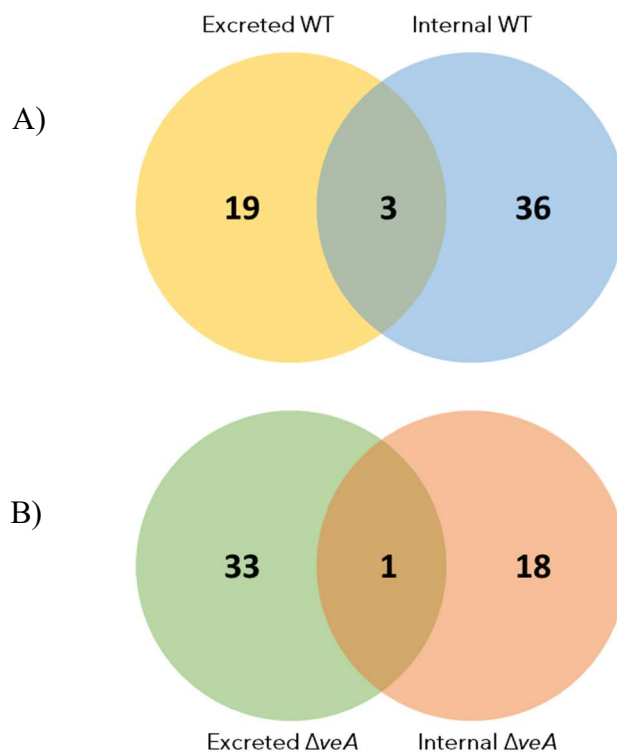


Figure 2. Venn diagram of the compounds detected in the WT and *Pe Δ veA* strains. A) Metabolites detected in the medium (excreted) and in the fungal extract of WT (internal). B) Metabolites detected in the medium (excreted) and in the fungal extract of *Pe Δ veA* (internal). The strains grew in MEA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

| WT | | | | PeΔveA | | | |
|---|--|---|---|--|---|---|---|
| Excreted | Internal | | Both | Excreted | Internal | | Both |
| Ascladiol | Andrastin A | C ₁₉ H ₃₈ O ₆ | C ₁₈ H ₁₆ N ₂ O ₂ | Andrastin A | C ₁₈ H ₁₆ N ₂ O ₂ | Chaetoglobosin A | C ₃₂ H ₃₆ N ₂ O ₄ |
| Andrastin B | Aurantioclavine | C ₁₉ H ₁₆ N ₂ O ₂ | C ₂₈ H ₃₈ O ₇ | Andrastin B | C ₁₈ H ₁₆ N ₂ O ₂ | Communesin B | |
| Clavicipitic Acid | Chaetoglobosin A | C ₁₉ H ₁₆ N ₂ O ₂ | C ₃₂ H ₃₆ N ₂ O ₄ | Ascladiol | C ₁₈ H ₁₈ N ₂ O ₂ | Communesin D | |
| Expansolides A/B | Clavicipitic Acid | C ₁₉ H ₁₆ N ₂ O ₄ | | Aurantioclavine | C ₁₈ H ₃₁ NO ₇ | Communesin F | |
| Expansolides C/D | Communesin A | C ₁₉ H ₁₆ N ₂ O ₄ | | Clavicipitic Acid | C ₁₉ H ₁₆ N ₂ O ₄ | Communesin I | |
| Patulin | Communesin B | C ₂₀ H ₁₈ N ₂ O ₂ | | Clavicipitic Acid | C ₁₉ H ₁₆ N ₂ O ₄ | Com496-1 | |
| C ₁₀ H ₁₇ NO ₅ | Communesin D | C ₂₀ H ₁₈ N ₂ O ₂ | | Communesin A | C ₁₉ H ₂₁ NO ₇ | Roquefortine C | |
| C ₁₅ H ₁₉ NO ₆ | Communesin F | C ₂₄ H ₂₆ N ₂ O ₆ | | Communesin I | C ₂₃ H ₂₄ N ₂ O ₆ | C ₁₉ H ₃₈ O ₆ | |
| C ₁₈ H ₁₈ N ₂ O ₂ | Communesin I | C ₂₈ H ₃₀ N ₄ O | | 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 ₄ O ₂ | | Expansolides A/B | C ₂₈ H ₃₂ N ₄ O ₂ | C ₂₀ H ₁₈ N ₂ O ₂ | |
| C ₁₉ H ₂₁ NO ₇ | Communesin K | C ₂₈ H ₃₂ N ₄ O ₂ | | Expansolides C/D | C ₂₈ H ₃₂ N ₄ O ₂ | C ₂₀ H ₁₈ N ₂ O ₂ | |
| C ₁₉ H ₂₁ NO ₇ | Com622 | C ₂₈ H ₃₂ N ₄ O ₃ | | Roquefortine D | C ₂₈ H ₃₈ O ₇ | C ₂₄ H ₂₆ N ₂ O ₆ | |
| C ₂₀ H ₂₆ O ₈ | Roquefortine C | C ₃₂ H ₃₆ N ₂ O ₅ | | C ₁₀ H ₁₇ NO ₅ | C ₃₂ H ₃₆ N ₂ O ₅ | C ₂₈ H ₃₀ N ₄ O | |
| C ₂₃ H ₂₄ N ₂ O ₆ | Roquefortine D | C ₃₂ H ₃₆ N ₄ O ₃ | | C ₁₅ H ₁₉ NO ₆ | C ₃₂ H ₃₆ N ₂ O ₅ | C ₂₈ H ₃₂ N ₄ O ₃ | |
| C ₂₄ H ₄₀ O ₆ | C ₁₅ H ₁₉ NO ₆ | C ₃₂ H ₃₈ N ₄ O ₃ | | C ₁₅ H ₁₉ NO ₆ | | C ₃₂ H ₃₆ N ₂ O ₅ | |
| C ₂₉ H ₂₇ N ₅ O ₅ | C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | C ₃₂ H ₄₀ N ₄ O ₂ | | C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | | C ₃₂ H ₃₆ N ₄ O ₃ | |
| C ₂₉ H ₂₇ N ₅ O ₅ | C ₁₈ H ₁₆ N ₂ O ₂ | C ₃₇ H ₄₂ N ₄ O ₅ | | C ₁₇ H ₁₇ NO ₇ | | C ₃₂ H ₃₈ N ₄ O ₃ | |
| C ₃₂ H ₃₆ N ₂ O ₅ | C ₁₈ H ₁₆ N ₂ O ₃ | | | C ₁₇ H ₁₇ NO ₇ | | C ₃₂ H ₄₀ N ₄ O ₂ | |
| C ₃₂ H ₃₆ N ₂ O 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 PeΔveA 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.

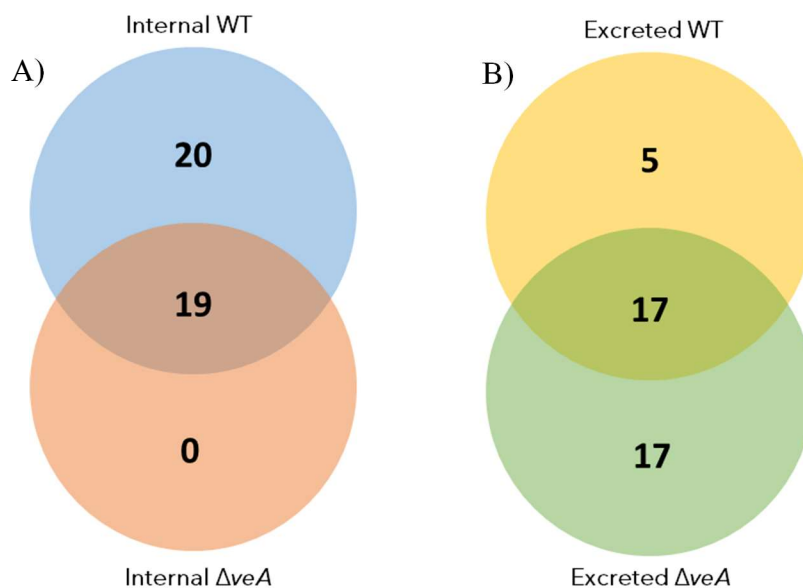


Figure 3. Venn diagram. Comparison between the metabolites produced by the WT and $Pe\Delta veA$ strains. A) Compounds detected in the fungal extract and B) Compounds excreted into the medium. The strains grew in MEA medium at 25°C in the dark for seven days.

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 $Pe\Delta veA$ 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 $Pe\Delta veA$ 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 $Pe\Delta veA$ 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 $Pe\Delta veA$ 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 $Pe\Delta veA$ 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.

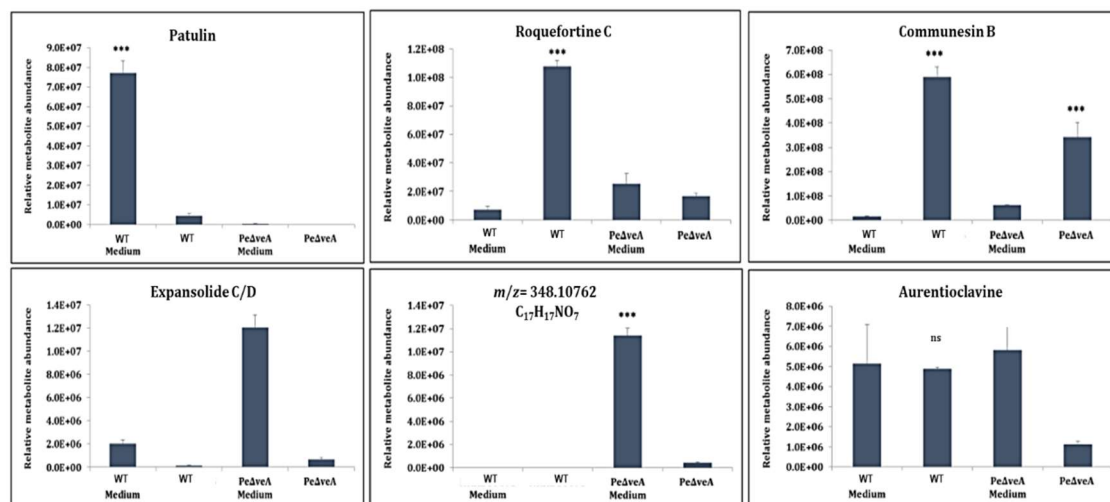
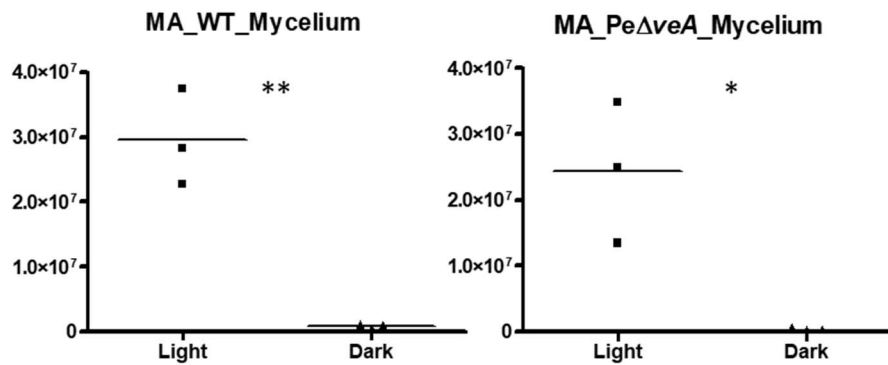


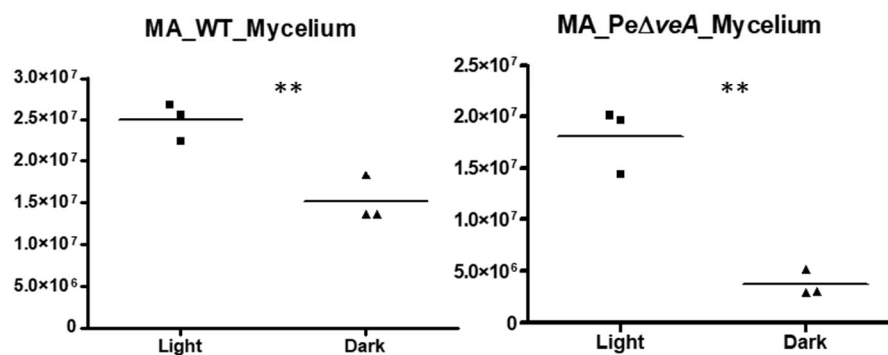
Figure 4. Relative metabolite abundance of the data obtained from the mass spectra. The graphs show the relative abundance of metabolites produced by the WT and the null mutant *PeΔveA* strains. Cultures were grown for five days in the dark at 25 °C. Graphs show the mean ± standard error of the mean (SEM) of three replicates and the significant differences between WT and null mutant *PeΔveA* 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/*PeΔveA*) 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 *PeΔveA* 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 *PeΔveA* strain grown on MEA medium and in the fungal extract of both strains on PDA medium (Figure S5).

A) Roquefortine C



B) Communesin A



C) Andrastin B

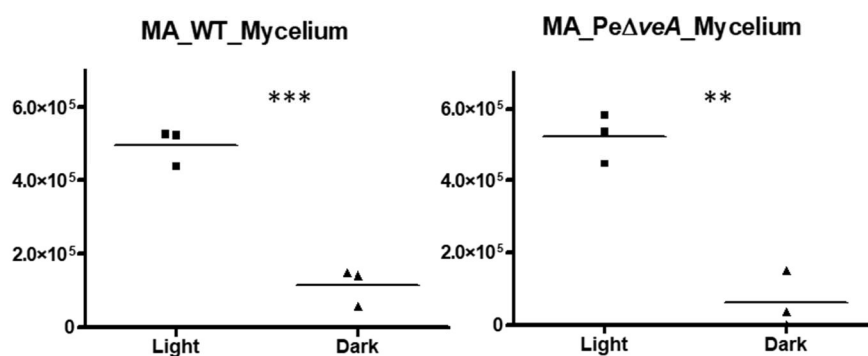


Figure 5. Effect of light. The graphs show the comparison of the relative abundance of the metabolites produced under light or dark conditions by the WT and the null mutant PeΔveA strains. A) Roquefortine C, B) Communesin A, C) Andrastin B. Cultures were grown on MEA medium for five days at 25 °C. Graphs show the mean \pm 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).

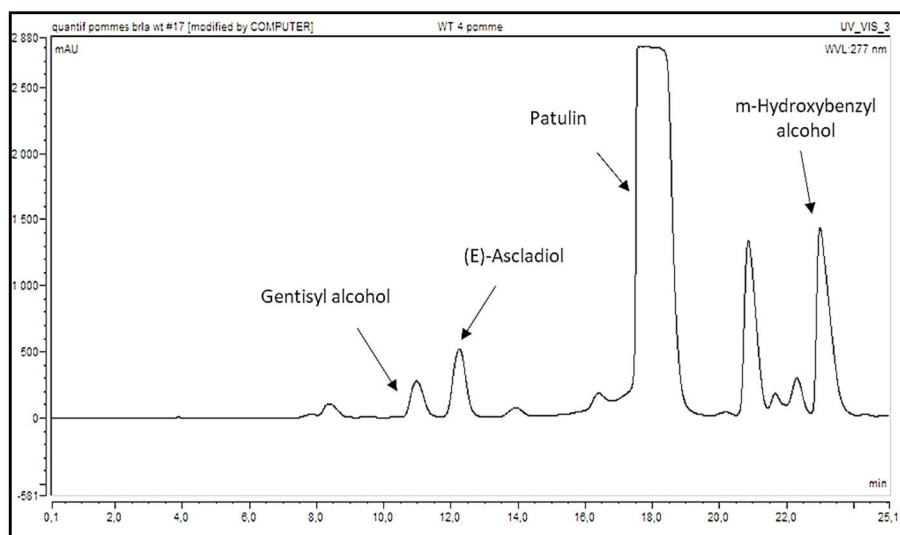


Figure 6. HPLC chromatogram of Golden Delicious apples infected by the *Penicillium expansum* wild type strain.

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 *PeΔveA* strains. Twelve SMs were detected in the null mutant *PeΔveA* 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 *PeΔveA* strain. Communesins A and B, and andrastins A and B were secreted by both the WT and *PeΔveA* strains after 14 dpi. Eight communesins were found in apples treated with WT and *PeΔveA* 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 *PeΔveA* 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 *PeΔveA* 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 *PeΔveA*. 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 *PeΔveA* strains

| Molecular formula | R _T (min) ^a | ¹² C <i>m/z</i> (Da) | Proposed identification | 14 dpi <i>PeΔveA</i> | 14 dpi WT | 30 dpi WT | Molecular formula | R _T (min) ^a | ¹² C <i>m/z</i> (Da) | Proposed identification | 14 dpi <i>PeΔveA</i> | 14 dpi WT | 30 dpi WT |
|--|-----------------------------------|---------------------------------|-------------------------|----------------------|-----------|-----------|---|-----------------------------------|---------------------------------|-----------------------------------|----------------------|-----------|-----------|
| C₇H₆O₄ | 3.59 | 153.01919 | Patulin | ND | + | + | C ₂₆ H ₄₀ O ₆ | 29.93 | 449.28939 | | + | + | ND |
| C ₇ H ₈ O ₂ | 6.79 | 125.05998 | m-Hydroxybenzyl alcohol | ND | + | - | C ₂₈ H ₃₂ N ₄ O | 17.14 | 441.26589 | Communesin F | ND | ND | + |
| C ₇ H ₈ O ₄ | 2.67 | 157.04990 | Ascladiol | ND | + | + | C ₂₈ H ₃₂ N ₄ O 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 ₄ O ₂ | 23.65 | 457.26116 | Communesin A | + | + | + |
| C ₁₃ H ₁₄ O ₅ | 21.70 | 251.09108 | Citrinin | ND | ND | + | C₂₈H₃₈O₇ | 35.32 | 485.25405 | | + | + | + |
| C ₁₆ H ₂₆ N ₂ O ₄ S 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 | + | C₂₈H₃₈O₈ | 28.44 | 501.24792 | | ND | ND | + |
| C ₁₇ H ₂₂ O ₅ | 30.19 | 307.15504 | Expansolide A/B | ND | ND | + | C₂₈H₃₈O₈ | 29.57 | 501.24786 | | + | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | 17.74 | 293.12915 | | ND | ND | + | C₂₈H₄₀O₇ | 30.58 | 487.26898 | Andrastin B | + | + | + |
| C ₁₈ H ₁₈ N ₂ O ₂ | 14.61 | 295.14479 | | + | + | + | C ₃₂ H ₃₄ N ₄ O ₃ | 31.84 | 523.27152 | Communesin D | + | ND | ND |
| C ₁₉ H ₁₆ N ₂ O ₂ | 33.17 | 305.12933 | | ND | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | 26.71 | 529.27029 | Chaetoglobosin B/G | ND | ND | + |
| C₁₉H₃₈O₆ | 38.19 | 361.25819 | | ND | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | 29.54 | 529.26898 | Chaetoglobosin B/G | ND | ND | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | 36.44 | 319.14502 | | ND | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | 30.13 | 529.27539 | Chaetoglobosin B/G | ND | + | + |
| C ₂₂ H ₂₃ N ₅ O ₂ | 15.09 | 390.19390 | Roquefortine C | ND | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | 35.67 | 529.26822 | Chaetoglobosin B/G | ND | ND | + |
| C ₂₃ H ₂₄ N ₂ O ₆ | 26.92 | 425.17179 | | + | ND | + | C ₃₂ H ₃₆ N ₄ O ₂ | 36.01 | 509.29257 | Communesin B | + | + | + |
| C₂₄H₂₆N₂O₆ | 33.79 | 437.17091 | | ND | ND | + | C ₃₂ H ₃₈ N ₄ O 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.

CHAPTER 3: RESULTS

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 *PeΔveA* strain could not be performed, since the formation of coremia was missing in the null *PeΔveA* 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 <i>m/z</i> (Da) | Proposed identification | Molecular formula | Identifier ^a | <i>C m/z</i> (Da) | Proposed identification |
|--|---------------------------|---------------------------------|-------------------------|---|---------------------------|-------------------|---|
| C ₁₆ H ₁₈ N ₂ O ₂ | Pexp_271.154_7.62 | 271.14496 | Clavicipitic acid | C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_18.43 | 415.25033 | Communesin I |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | Pexp_375.142_22.02 | 375.14202 | | C ₂₈ H ₃₀ N ₄ O 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 ₄ O | 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 ₄ O ₂ | Pexp_457.260_14.27 | 457.26097 | New DMV communesin-1 |
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_13.58 | 293.12911 | | C ₂₈ H ₃₂ N ₄ O ₂ | Pexp_457.261_23.65 | 457.26116 | Communesin A |
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_17.74 | 293.12915 | | C₂₈H₃₈O₇ | Pexp_485.254_35.32 | 485.25405 | |
| C ₁₈ H ₁₈ N ₂ O 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 ₂ O ₂ | Pexp_305.129_32.50 | 305.12911 | | C₂₈H₃₈O₈ | Pexp_501.247_28.44 | 501.24792 | |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_33.17 | 305.12933 | | C₂₈H₃₈O₈ | Pexp_501.247_29.57 | 501.24786 | |
| C₁₉H₃₈O₆ | Pexp_361.258_37.17 | 361.25871 | | C₂₈H₄₀O₆ | Pexp_471.274_39.84 | 471.27434 | Andrastin C |
| C₁₉H₃₈O₆ | Pexp_361.258_38.19 | 361.25819 | | C₂₈H₄₀O₇ | Pexp_487.268_30.58 | 487.26898 | Andrastin B |
| C ₂₀ H ₁₈ N ₂ O ₂ | Pexp_319.145_35.48 | 319.14588 | | C ₃₂ H ₃₄ N ₄ O ₃ | Pexp_523.271_31.84 | 523.27152 | Communesin D |
| C ₂₀ H ₁₈ N ₂ O ₂ | Pexp_319.145_36.44 | 319.14502 | | C ₃₂ H ₃₆ N ₂ O ₄ | Pexp_513.276_39.10 | 513.27635 | Chaetoglobosin J or Prochaetoglobosin III |
| C ₂₂ H ₂₃ N ₅ O ₂ | Pexp_390.193_15.09 | 390.19390 | Roquefortine C | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_26.79 | 529.27029 | Chaetoglobosin B/G |
| C ₂₂ H ₂₅ N ₅ O ₂ | Pexp_392.209_9.99 | 392.20913 | Roquefortine D | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_30.51 | 529.27077 | Chaetoglobosin B/G |
| C ₂₃ H ₂₄ N ₂ O ₆ | Pexp_425.171_26.92 | 425.17179 | | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_33.98 | 529.27067 | Chaetoglobosin A |
| C₂₄H₂₆N₂O₆ | Pexp_437.170_33.79 | 437.17091 | | C ₃₂ H ₃₆ N ₂ O 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 ₄ O ₂ | Pexp_509.292_36.01 | 509.29257 | Communesin B |
| C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_14.62 | 415.25034 | Communesin I | C ₃₂ H ₃₈ N ₄ O ₃ | Pexp_527.303_21.41 | 527.30304 | New DME communesin-4 |
| C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_18.43 | 415.25033 | Communesin I | C ₃₂ H ₄₀ N ₄ O 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 *Pencillium 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 *PeΔveA* 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), melegrin (Kozlovskii et al. 2002), ochratoxin A (Paterson et al. 1987), ochrephilon (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.

Meleagrins share a part of its biosynthetic pathway with roquefortine C. Roquefortine C is an intermediate of the meleagrins biosynthesis pathway. RoqN has been identified as responsible for the O-methylation of glandicoline B to yield meleagrins (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 conidia. 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 ΔveA , 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 difference between the WT and the null mutant strain such as 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 *PeΔveA* 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 $Pe\Delta veA$ 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 $Pe\Delta veA$ strain, came third. A lower number of metabolites was excreted in the medium by the WT strain compared to the null mutant $Pe\Delta veA$ 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 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 |
|--|--------------------------|--------------------------|--------------------------|---|---|-------------------------|
| C₇H₆O₄ | Pexp_153.019_3.59 | 153.01919 | 160.04256 | 156.02997 | 153, 125, 109(100), 97 | patulin |
| C ₇ H ₈ O | Pexp_109.065_7.11 | 109.06509 | 116.08844 | 112.07483 | | m-Cresol |
| C ₇ H ₈ O 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 ₇ H ₈ O ₃ | Pexp_141.054_3.80 | 141.05493 | 148.07869 | 144.06492 | 141, 123, 113(100), 110, 95, 79, 69, 55 | Gentisyl alcohol |
| C ₇ H ₈ O ₄ | Pexp_157.049_2.67 | 157.04990 | 164.07348 | 161.06366 | 157, 139(100), 111, 97, 83 | Ascladiol |
| C ₇ H 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 ₂ O 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 ₂ O 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 ₂ O ₄ S 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 ₂ O ₂ | Pexp_293.129_13.58 | 293.12911 | 311.18978 | 305.15587 | 293, 265(100), 248, 237, 222, 120 | |
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_17.74 | 293.12915 | 311.18970 | 305.15668 | 293, 265(100), 248, 237, 222, 120 | |
| C ₁₈ H ₁₆ N ₂ O ₃ | Pexp_309.124_14.76 | 309.12421 | 327.18455 | 320.14798 | 309, 292 (100), 267, 249, 231 | |
| C ₁₈ H ₁₈ N ₂ O 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 ₂ O ₂ | Pexp_305.129_32.50 | 305.12911 | 324.19310 | 316.15289 | 305, 277(100), 264, 262, 205, 187 | |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_33.17 | 305.12933 | 324.19301 | 317.15701 | 305, 277(100), 264, 262, 205, 187 | |
| C ₁₉ H ₁₆ N ₂ O 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 ₂ O 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 | |
| C₁₉H₃₈O₆ | Pexp_361.258_37.23 | 361.25871 | 380.32251 | 371.29242 | 361, 343, 325, 283, 273, 257(100), 157 | |
| C₁₉H₃₈O₆ | Pexp_361.258_38.19 | 361.25819 | 380.32253 | 371.29252 | 361, 343, 325, 299, 283, 257(100), 157 | |
| C ₂₀ H ₁₈ N ₂ O 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 ₂ O 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 ₅ O 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 ₅ O 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 ₂ O ₆ | Pexp_425.171_26.92 | 425.17179 | 448.24874 | 439.20611 | 425, 407, 383, 365, 288(100), 260 | |
| C₂₄H₂₆N₂O₆ | 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 ₄ O | 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 ₄ O | 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 ₄ O 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 ₄ O 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 ₅ O ₅ | Pexp_518.241_16.36 | 518.24108 | 546.33497 | 538.27672 | 518, 500, 462, 450(100), 321 | |
| C ₂₈ H ₃₁ N ₅ O 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 ₄ O | 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O ₄ | Pexp_493.279_31.47 | 493.27971 | 521.37363 | 511.31495 | | Fungisporin A or cyclo(VFVF) |
| C ₂₈ H ₃₈ N ₄ O 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 ₄ O 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 |
| C₂₈H₃₈O₇ | 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 |
| C₂₈H₃₈O₈ | Pexp_501.247_27.70 | 501.24792 | 529.34203 | 516.29846 | 501, 457, 441(100), 425, 410, 397, 373, 365, | |
| C₂₈H₃₈O₈ | Pexp_501.247_28.44 | 501.24792 | 529.34203 | 516.29846 | 501, 457, 441(100), 425, 410, 397, 373, 365, | |

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| | | | | | | |
|---|---------------------------|------------------|------------------|------------------|--|--|
| C₂₈H₃₈O₈ | Pexp_501.247_29.57 | 501.24786 | 529.34194 | 516.29974 | 501, 486, 469, 457, 441(100), 425, 397, 373, 366 | |
| C₂₈H₄₀O₆ | Pexp_471.274_39.48 | 471.27434 | 499.36727 | 486.32419 | 471, 456, 439, 411(100) | Andrastin C |
| C₂₈H₄₀O₇ | 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 ₅ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O 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 ₂ O ₅ | Pexp_529.270_37.41 | 529.27056 | 561.37807 | 548.32181 | 529, 511 (100), 493, 453, 380, 327, 185 | Chaetoglobosin B/G |
| C₃₂H₃₆N₂O₆ | 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 ₄ O 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 | PeDveA | PeΔveA: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 ₂ O ₄ S 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 ₂ O ₂ | 1,745 | 0,307 | 1,867 | 13,50 | 293,13 |
| C ₁₈ H ₁₆ N ₂ O ₂ | 1,018 | 0,764 | 0,987 | 17,60 | 293,13 |
| C ₁₈ H ₁₆ N ₂ O ₃ | 1,683 | nd | 1,776 | 15,24 | 309,12 |
| C ₁₈ H ₁₈ N ₂ O 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 ₂ O ₂ | 207,866 | nd | 313,619 | 32,40 | 305,13 |
| C ₁₉ H ₁₆ N ₂ O ₂ | 79,639 | nd | 91,573 | 33,01 | 305,13 |
| C ₁₉ H ₁₆ N ₂ O ₄ | 2,371 | 0,511 | 2,206 | 14,00 | 337,12 |
| C ₁₉ H ₁₆ N ₂ O 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 ₂ O ₂ | 295,238 | 8,333 | 185,306 | 35,89 | 319,14 |
| C ₂₀ H ₁₈ N ₂ O 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 ₂ O ₆ | 0,816 | 0,315 | 0,770 | 26,70 | 425,17 |
| C ₂₄ H ₂₆ N ₂ O 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 ₄ O | 18,301 | 1,122 | 6,572 | 19,96 | 471,24 |
| C ₂₈ H ₃₂ N ₄ O ₂ | 1,649 | 0,089 | 1,711 | 13,56 | 457,26 |
| C ₂₈ H ₃₂ N ₄ O ₂ | 1,841 | 0,173 | 1,416 | 14,32 | 457,26 |
| C ₂₈ H ₃₂ N ₄ O 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 |

CHAPTER 3: RESULTS

| | | | | | |
|---|---------|--------|---------|-------|--------|
| C ₂₉ H ₂₇ N ₅ O ₅ | 0,163 | nd | 0,072 | 17,84 | 526,21 |
| C ₂₉ H ₂₇ N ₅ O ₅ | 0,372 | nd | 0,177 | 18,56 | 526,21 |
| C ₃₂ H ₃₆ N ₂ O ₄ | 58,394 | 46,305 | 26,099 | 39,21 | 513,27 |
| C ₃₂ H ₃₆ N ₂ O ₅ | 0,726 | 0,160 | 0,807 | 26,87 | 529,27 |
| C ₃₂ H ₃₆ N ₂ O ₅ | 0,176 | 0,092 | 0,276 | 29,65 | 529,27 |
| C ₃₂ H ₃₆ N ₂ O ₅ | 320,220 | 93,288 | 309,543 | 36,68 | 529,27 |
| C ₃₂ H ₃₆ N ₄ O ₃ | 64,481 | 21,169 | 46,193 | 28,19 | 525,29 |
| C ₃₂ H ₃₈ N ₄ O ₃ | 30,545 | 1,266 | 29,948 | 20,17 | 527,30 |
| C ₃₂ H ₄₀ N ₄ O ₂ | 127,245 | 11,801 | 69,811 | 37,01 | 513,32 |
| C ₃₇ H ₄₂ N ₄ O ₅ | 44,129 | nd | 19,806 | 29,99 | 623,33 |

*SM < 1 = EXCRETED (GREEN)

SM > 1 = INTERNAL (YELLOW)

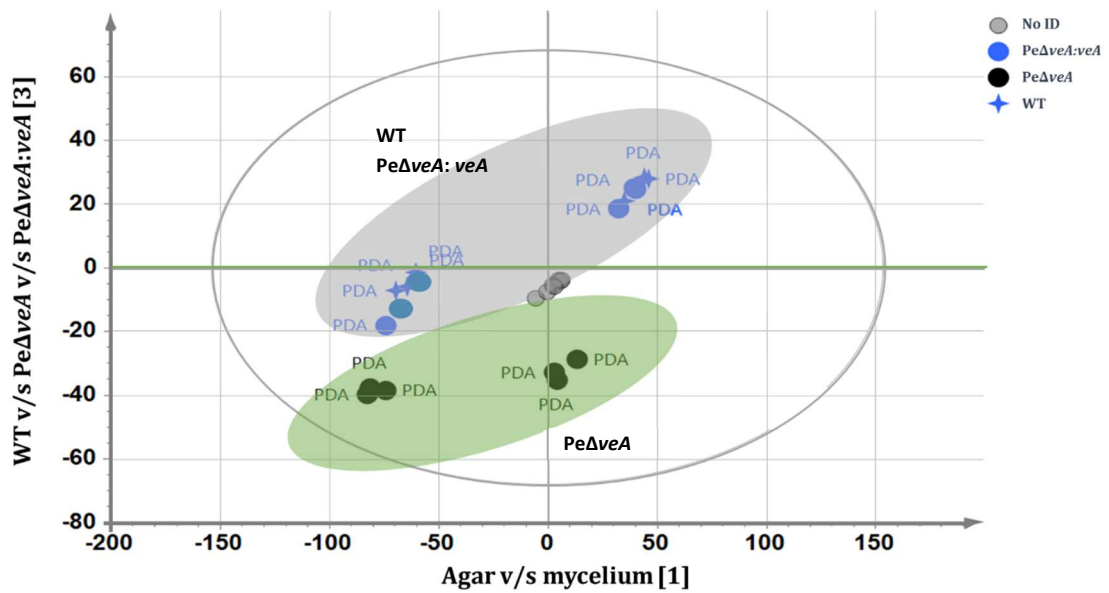


Figure S1. Principal Component Analysis (PCA) of the data obtained from the mass spectra. The strain effect. The strains showed a significant effect when comparing the null mutant PeΔveA strain versus WT or the complemented strain. The strains grew in PDA medium.

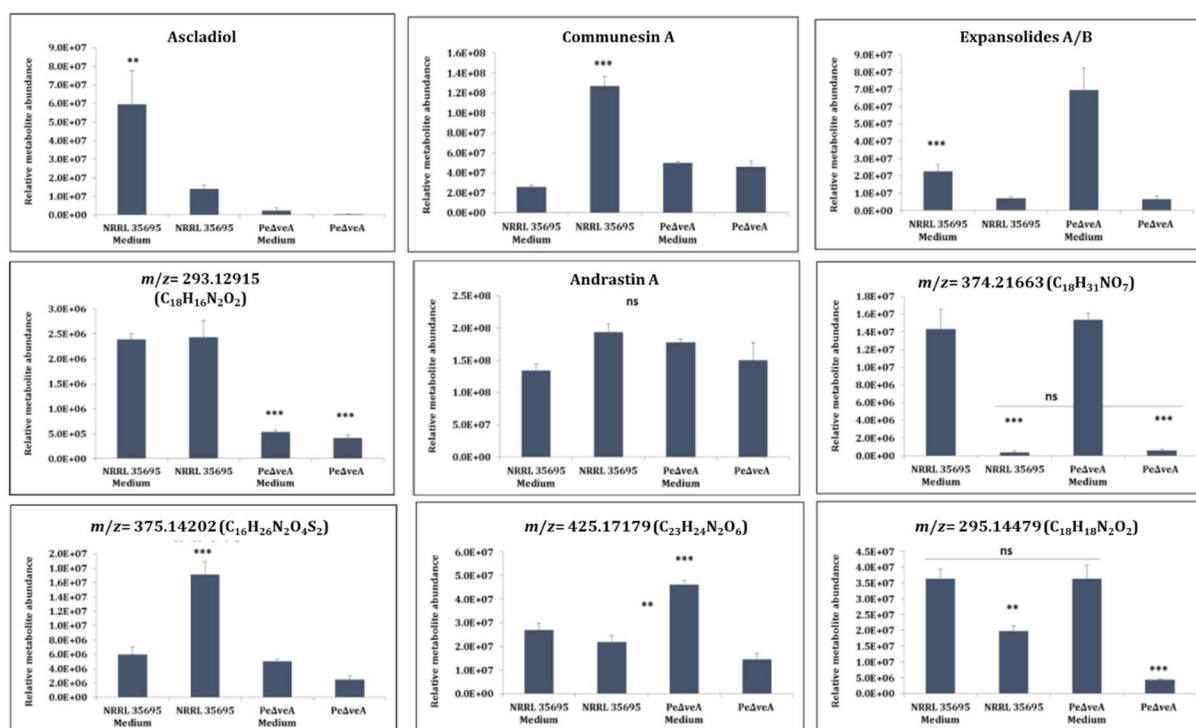


Figure S2. Relative metabolite abundance of the data obtained from the mass spectra. Comparison between the compounds produced by the null mutant strain and WT. Cultures were grown for 5 days in the dark at 25 °C. Graphs show the mean \pm standard error of the mean (SEM) of three replicates and the significant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

MEA Medium (RoqC)

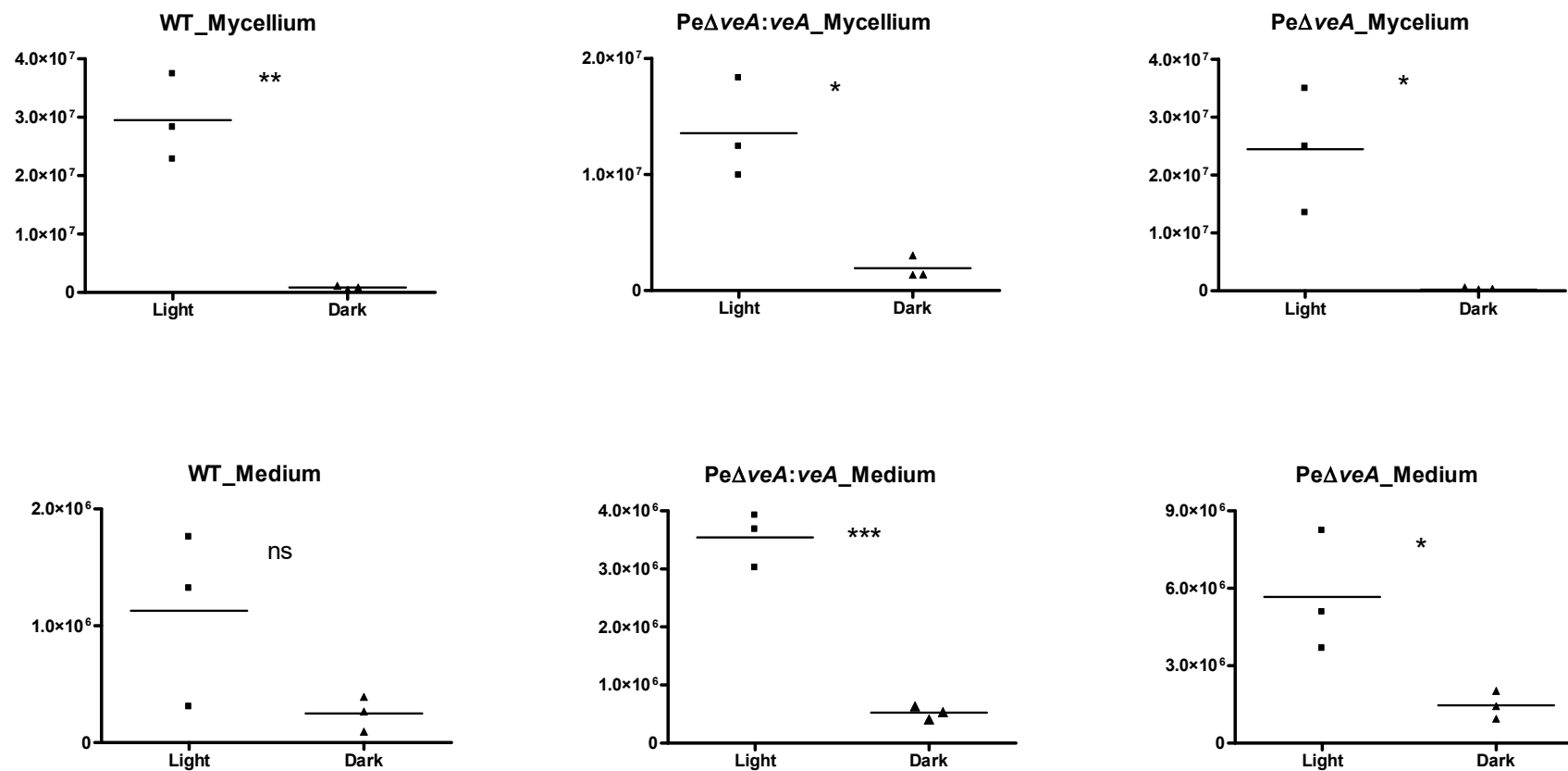


Figure S3a. Effect light. The graphs show the comparison of the relative abundance of roquefortin C produced under light or dark conditions by the WT strain and the null mutant strain *PeΔveA* in MEA for 5 days at 25 °C.

PDA Medium (RoqC)

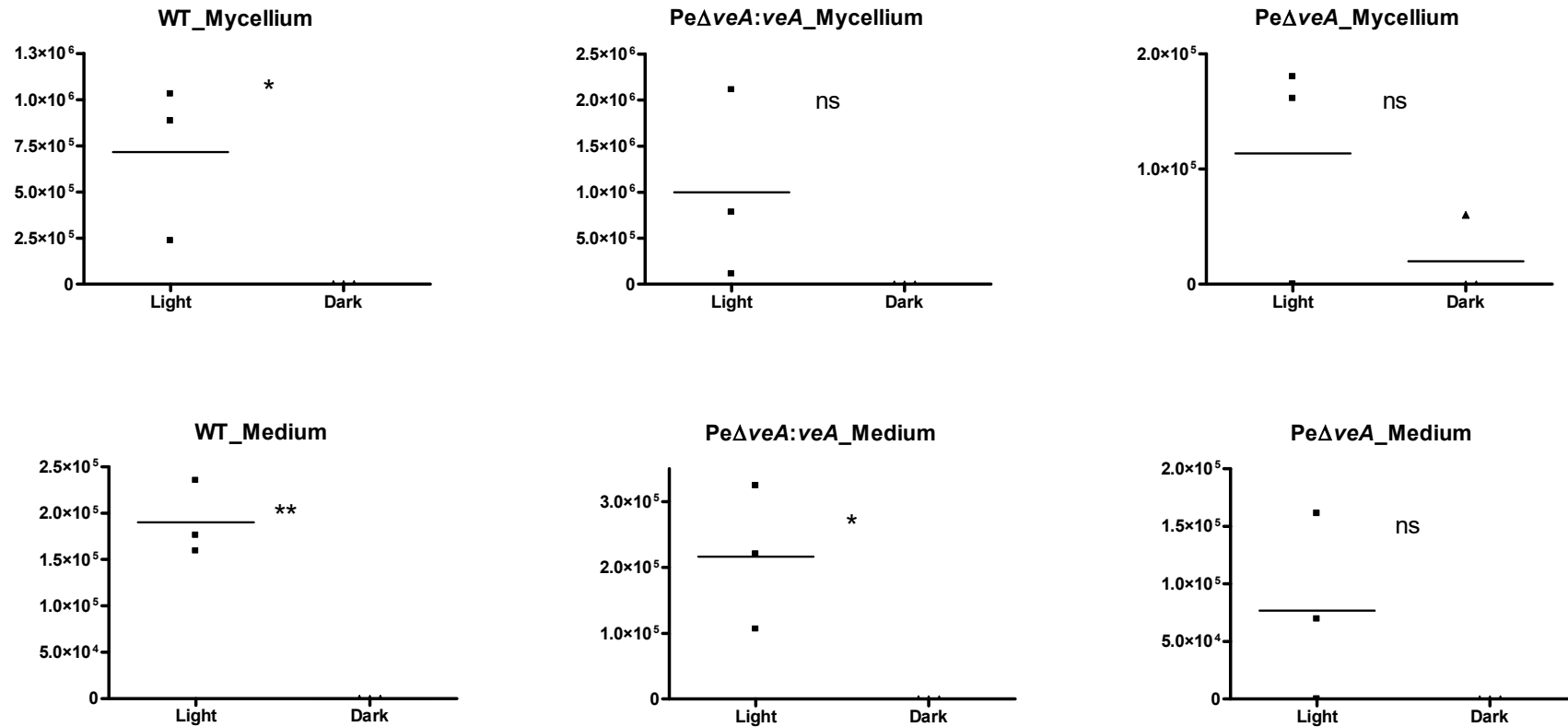


Figure S3a. Effect light. The graphs show the comparison of the relative abundance of roquefortin C produced under light or dark conditions by the WT strain and the null mutant strain *PeΔveA* in PDA for 5 days at 25 °C.

MEA Medium (Clavicipitic acid)

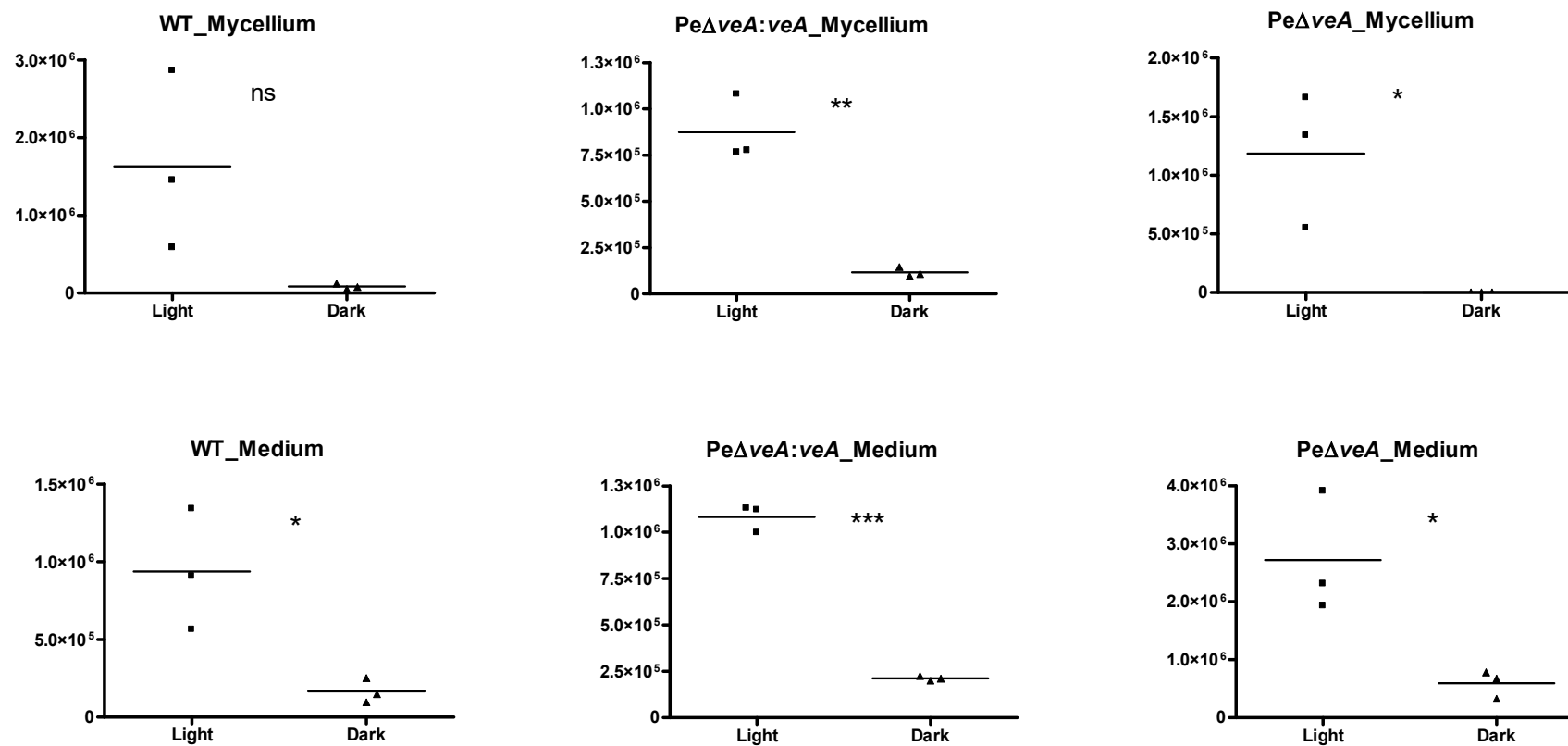


Figure S3b. Effect light. The graphs show the comparison of the relative abundance of clavicipitic acid produced under light or dark conditions by the WT strain and the null mutant strain Pe Δ veA in MEA for 5 days at 25 °C.

PDA Medium (Clavicipitic acid)

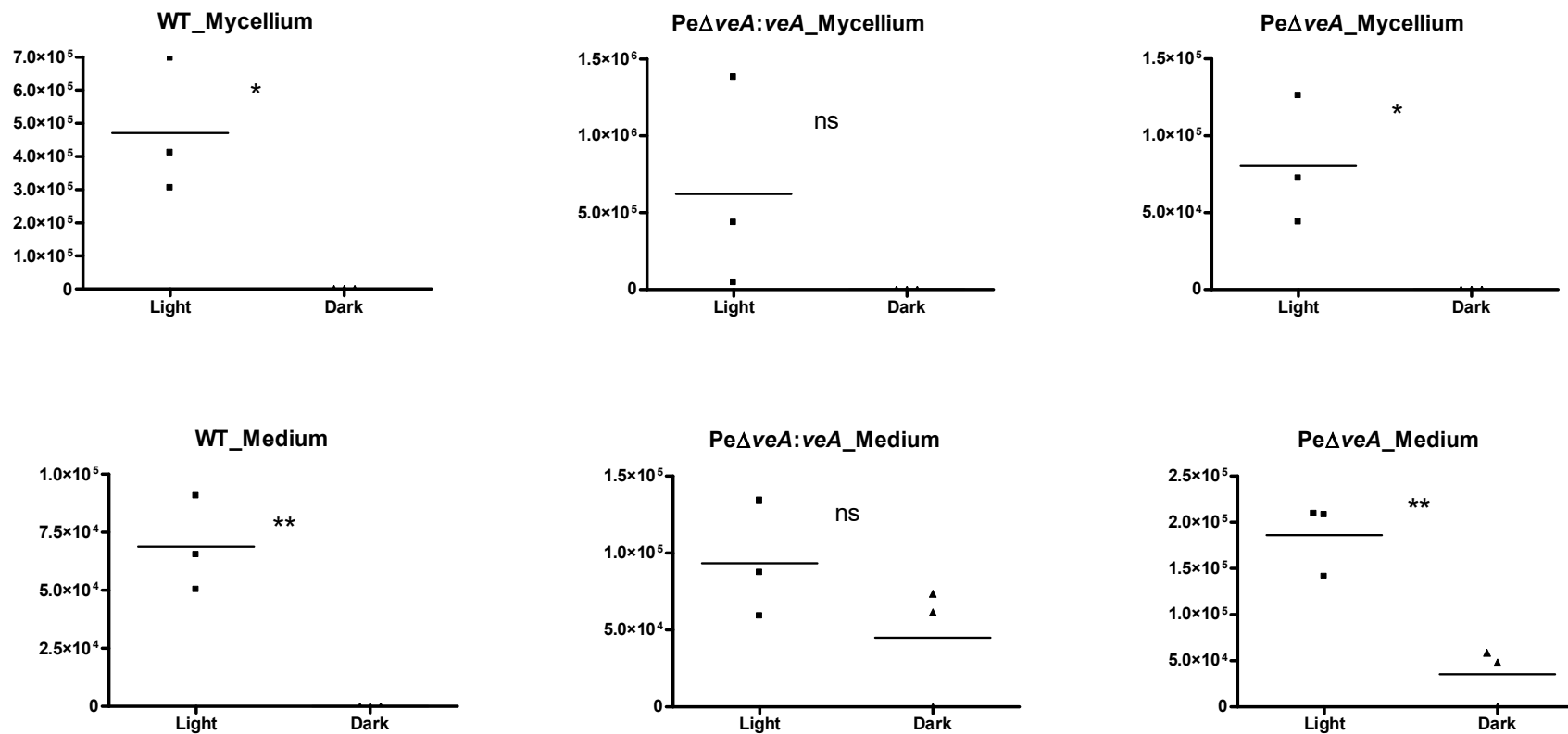
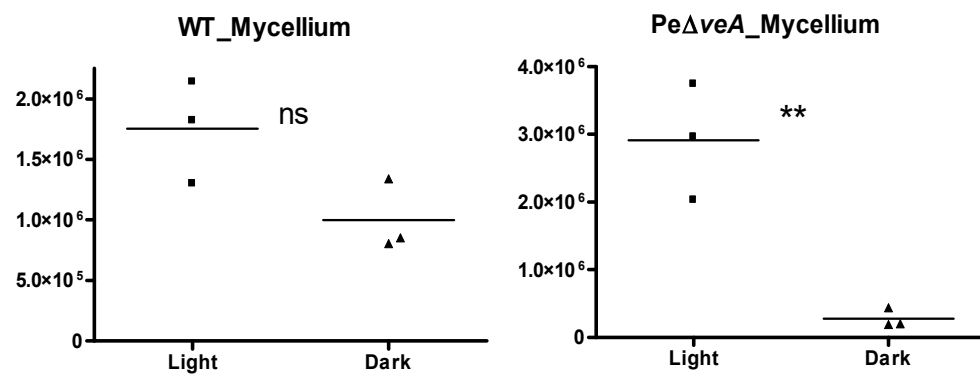


Figure S3b. Effect light. The graphs show the comparison of the relative abundance of clavicipitic acid produced under light or dark conditions by the WT strain and the null mutant strain *PeΔveA* in PDA for 5 days at 25 °C.

MEA Medium (com F)



PDA Medium (com F)

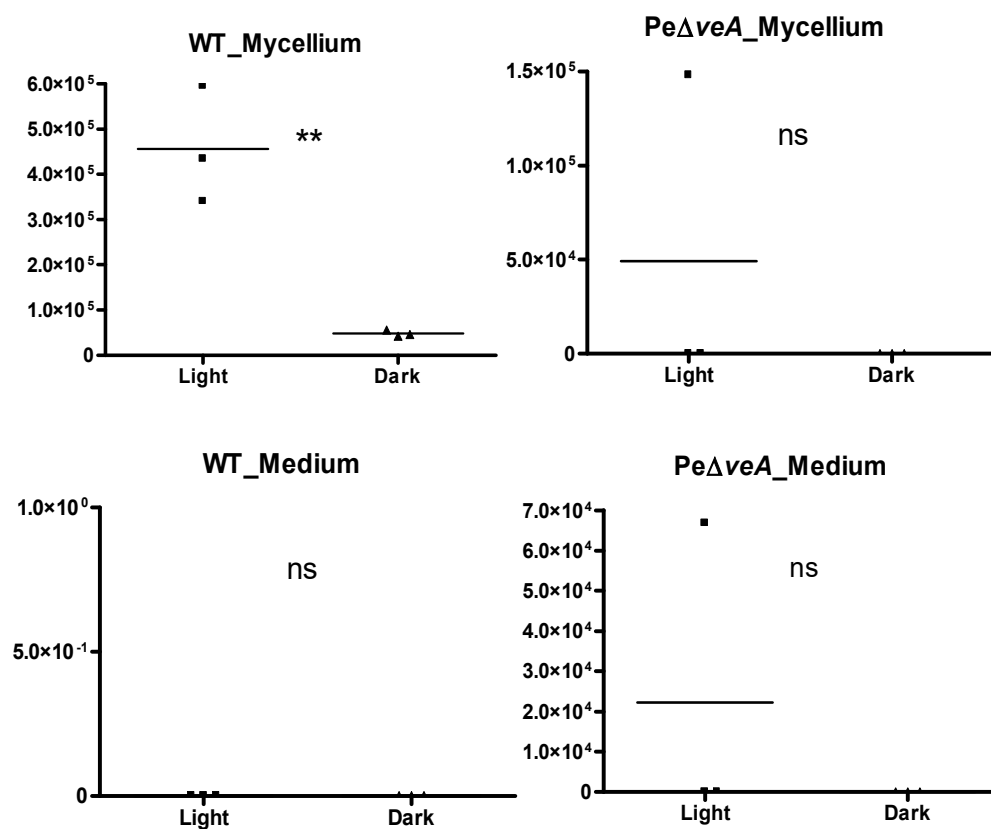


Figure S4. Effect light. The graphs show the comparison of the relative abundance of Communesin F produced under light or dark conditions by the WT strain and the null mutant strain *PeΔveA* in A) MEA and B) PDA for 5 days at 25 °C.

MEA Medium (com 526)

PDA Medium (com 526)

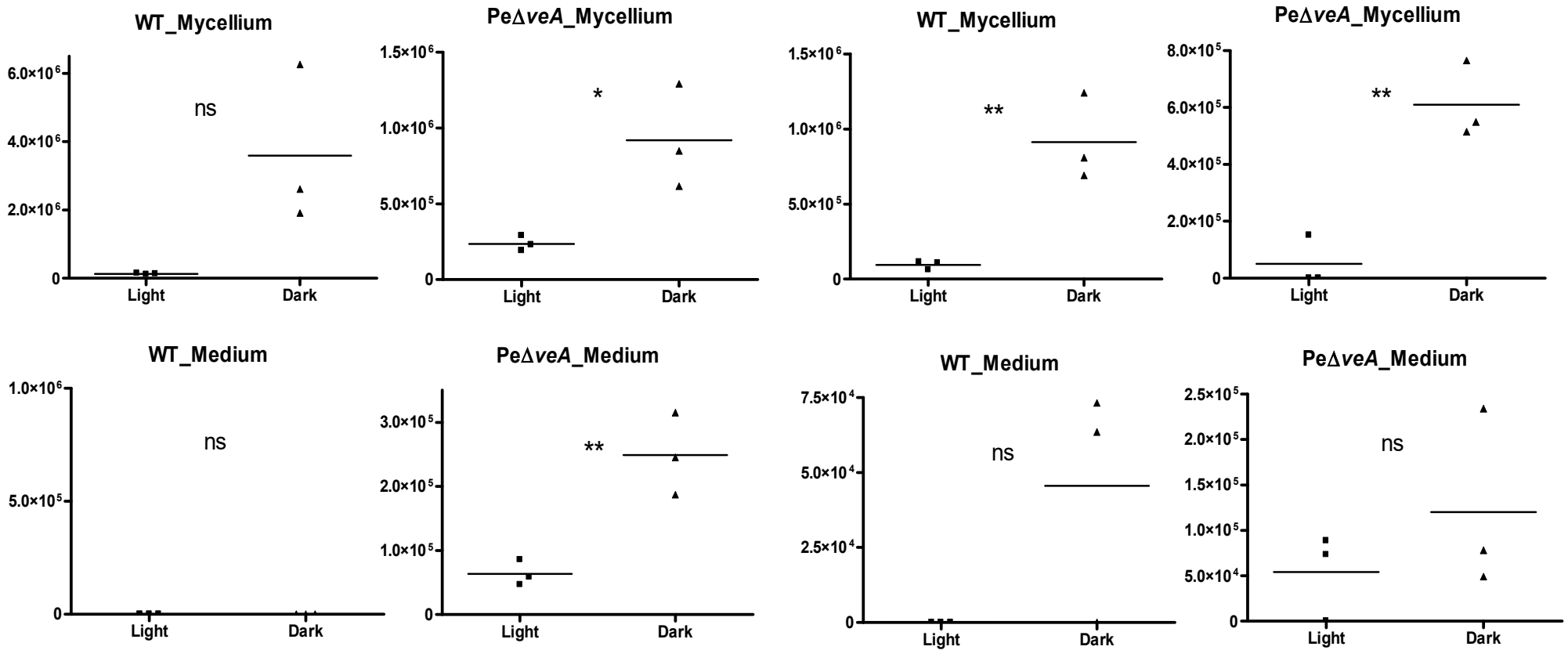


Figure S5. Effect light. The graphs show the comparison of the relative abundance of Com 526 produced under light or dark conditions by the WT strain and the null mutant strain *PeΔveA* in A) MEA and B) PDA for 5 days at 25 °C

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 $Pe\Delta veA$ (previous work carried out in the laboratory) and $Pe\Delta brlA$ (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 an innovative technique for the detection and identification of secondary metabolites. The combination of isotopic labeling of the fungal substrate (^{13}C , $^{13}\text{C}/^{15}\text{N}$) 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 which 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 *PeΔveA* and *PeΔbrlA* 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 *PeΔveA* 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-

GENERAL DISCUSSION AND PERSPECTIVES

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 $\Delta laeA$ and $\Delta velB$ strains and even a strain $\Delta veA:\Delta laeA:\Delta velB$ 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 $Pe\Delta brlA$ 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 $Pe\Delta brlA$ strain, suggesting that, contrary to what was observed in the null mutant $Pe\Delta veA$ 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 $Pe\Delta veA$ and $Pe\Delta brlA$ 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 *PeΔbrlA* 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 *PeΔveA* 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* is 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.

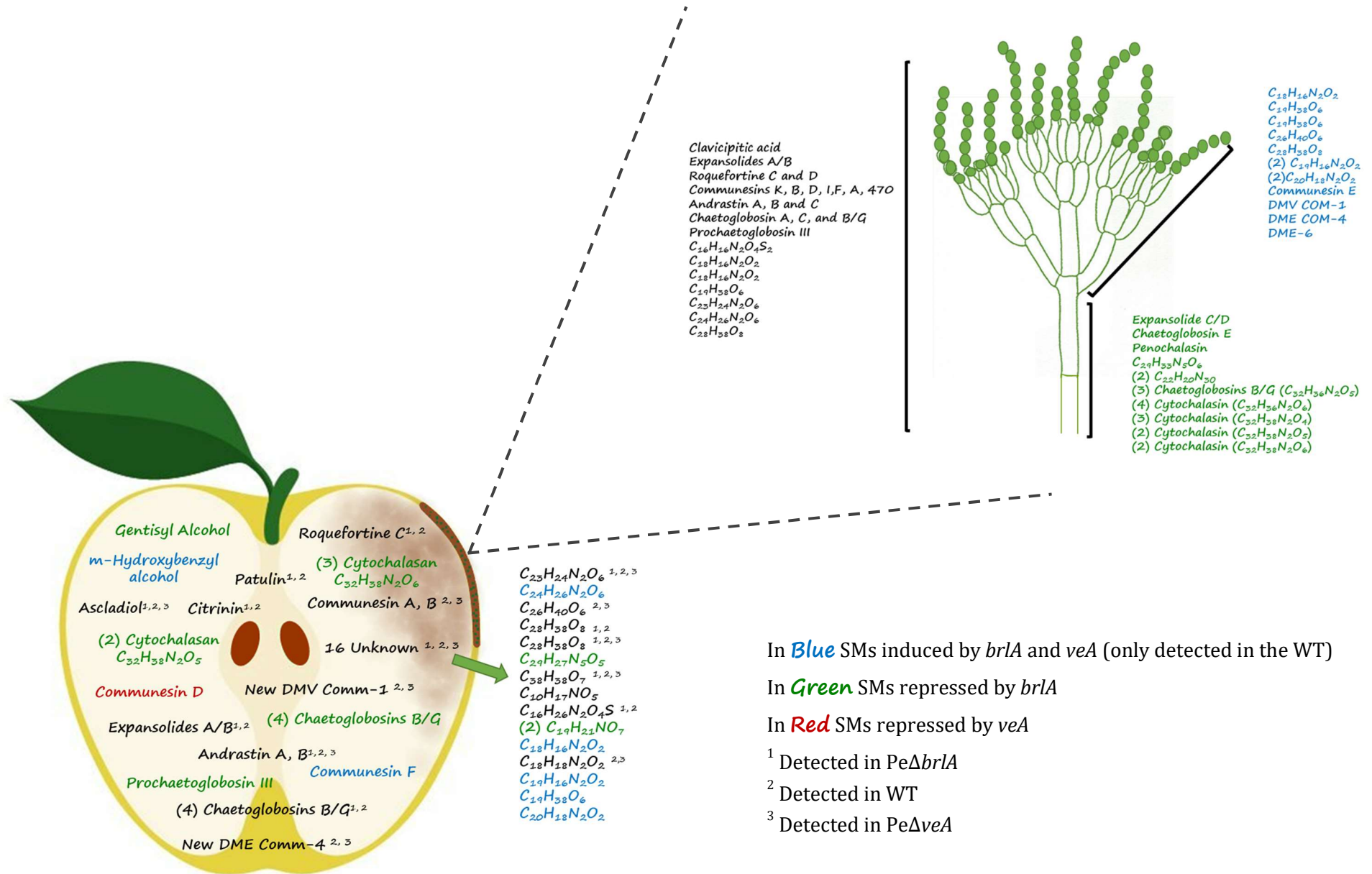


Figure 1. Scheme of spatial location of secondary metabolites produced by *P. expansum* at 30 dpi.

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 ΔveA 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 ΔveA 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 ΔveA 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* (Log₂ Fold Change= 0.89). This result was not observed for the other members of the velvet complex, where on the contrary *velB* (Log₂ Fold Change= -0.53) and *laeA* (Log₂ 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 *PeΔveA* and *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔbrlA* strain was observed. These results could be supported by the increase of patulin detected in the null mutant *PeΔbrlA* 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 *PeΔbrlA* strain came from an up-regulation of the patulin biosynthetic cluster.

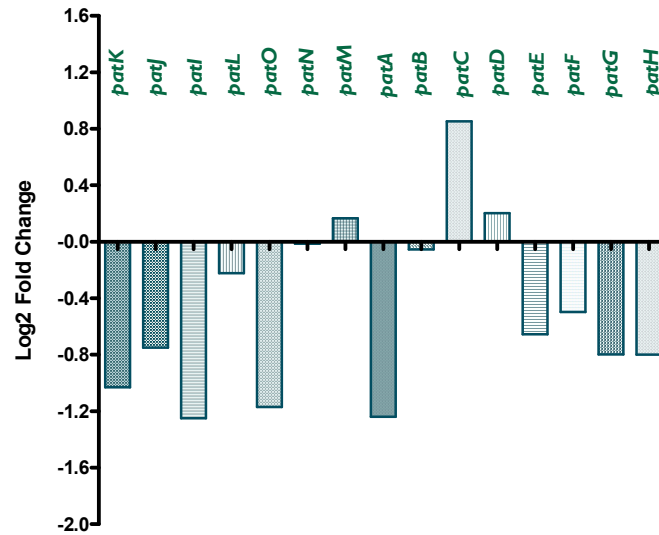


Figure 2. Fold Change expression of the 15 genes involved in the patulin biosynthesis pathway in the null mutant *PeΔbrlA* strain compared to the wild type strain.

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.

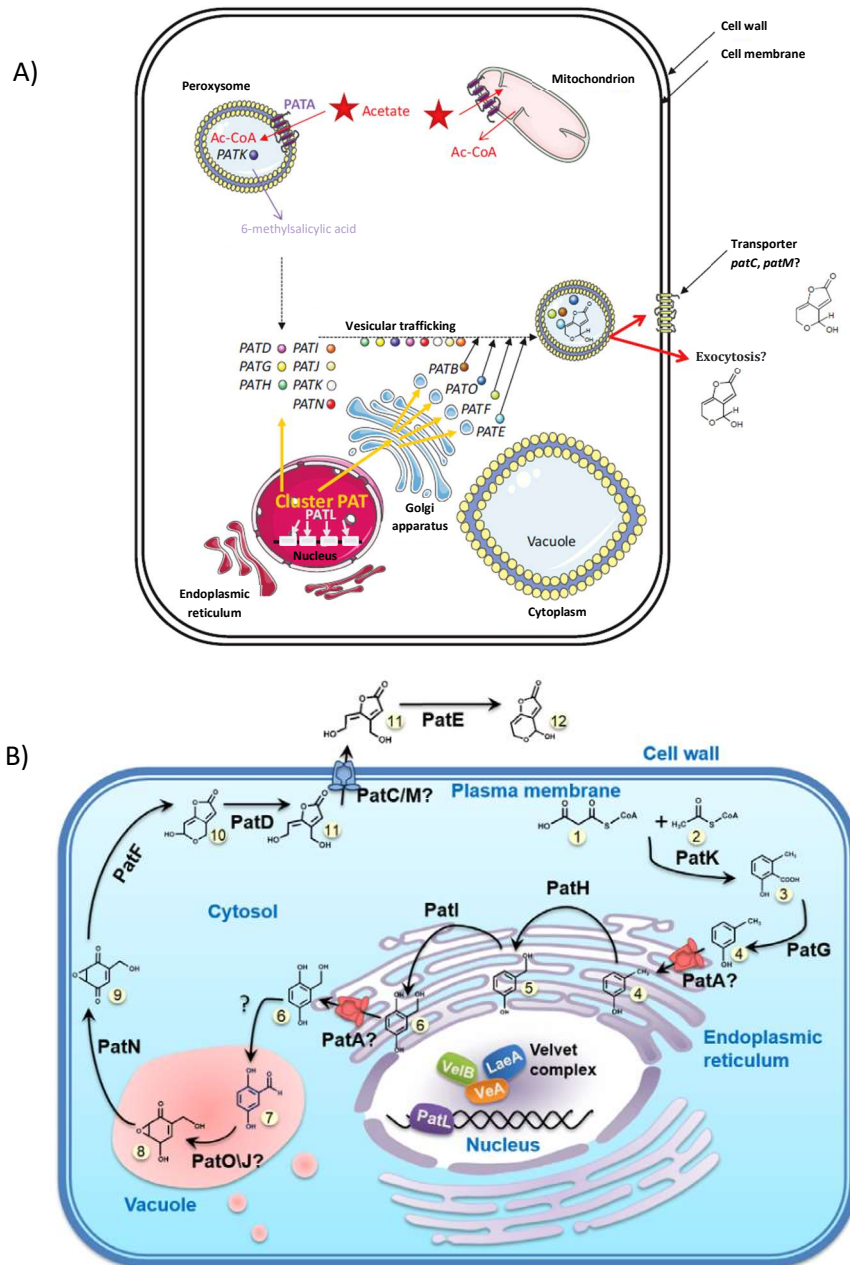
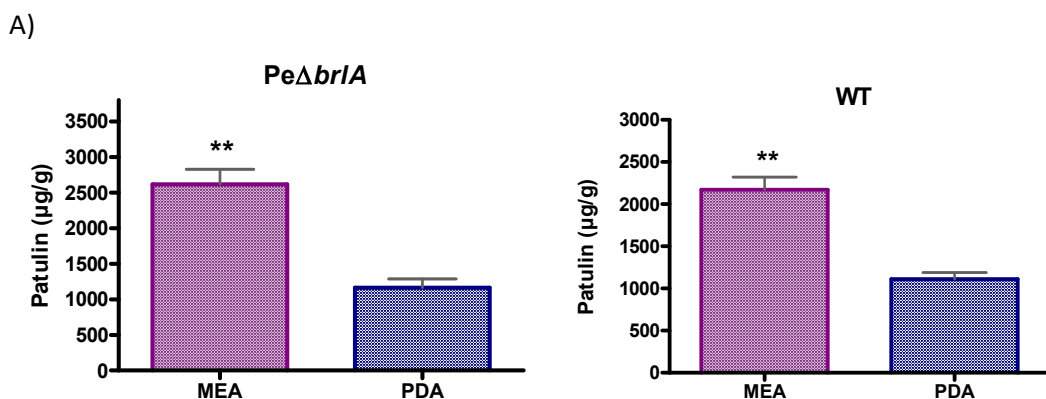


Figure 3. Hypothetical scheme of the spatial organization of the patulin biosynthesis pathway in fungal cells A) by Snini (2014) and B) Li et al. (2019).

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 *PeΔbrlA* 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, *PeΔbrlA* 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 *PeΔbrlA* 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.



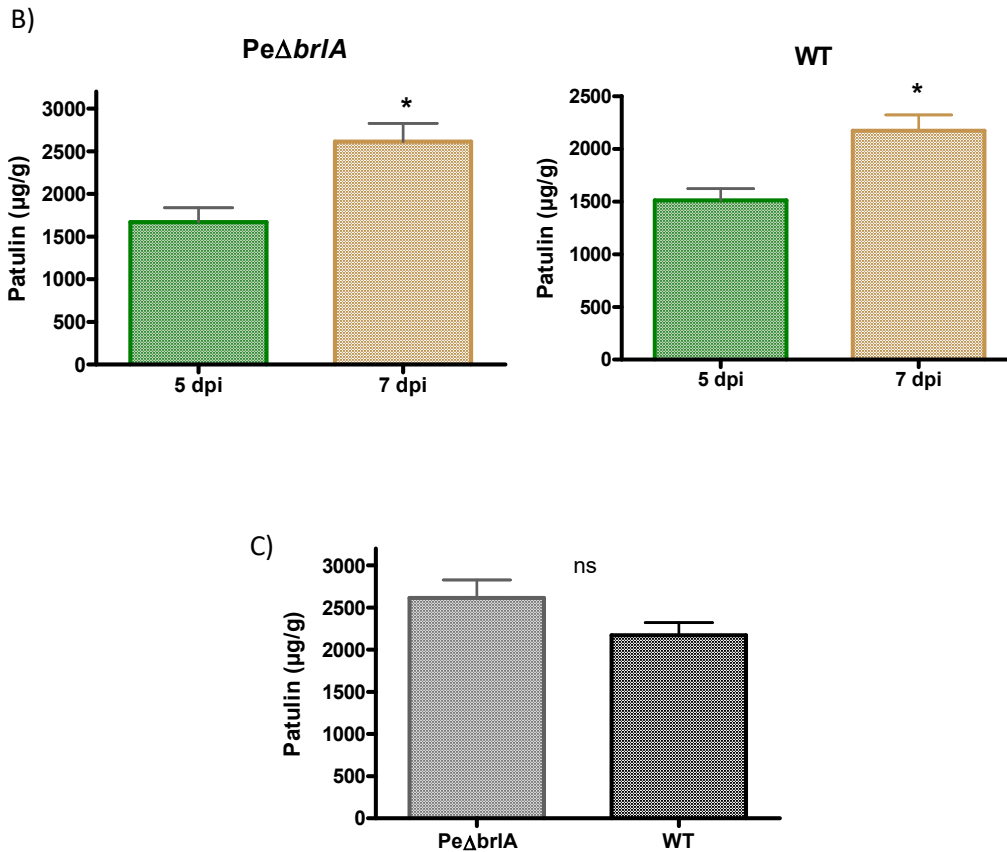


Figure 4. Effect of medium and incubation period on patulin production. A) MEA medium vs PDA medium, B) 5 dpi vs 7 dpi in MEA medium C) *PeΔbrlA* vs WT in MEA medium at 7dpi. dpi= days post-inoculation.

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*.

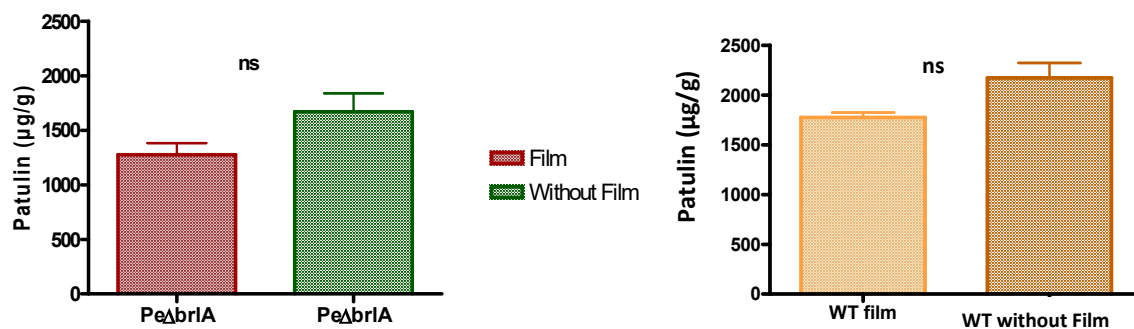


Figure 5. Detection and quantification of patulin production in the mutant Pe Δ brlA and WT strain grown in MEA medium with and without sterile cellophane sheets.

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* (Log₂Fold Change= 0.89, *p*-value= 1.05 x 10⁻⁵) in the null mutant Pe Δ 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 Pe Δ 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).

GENERAL DISCUSSION AND PERSPECTIVES

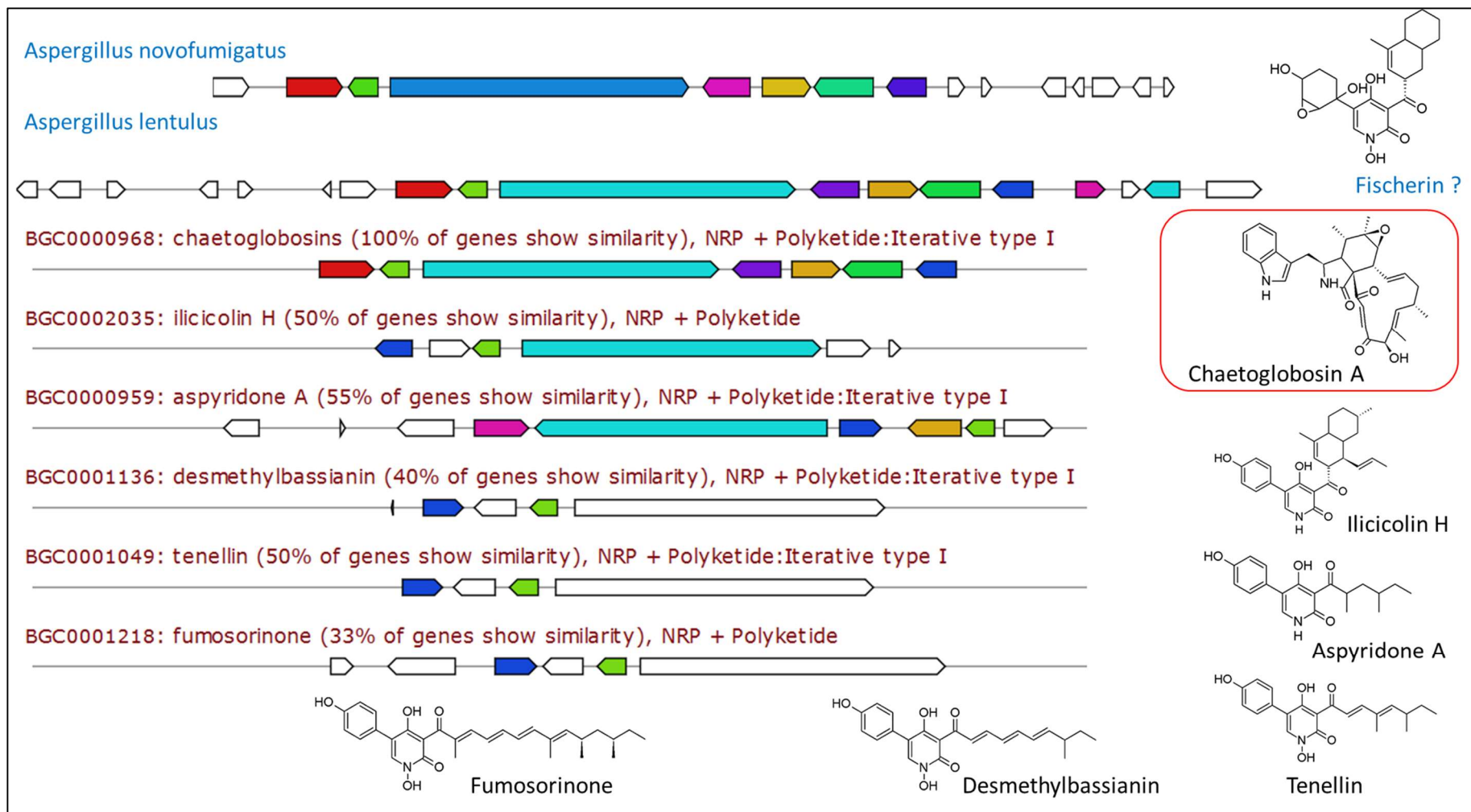


Figure6. Cluster of genes homologous to *che* genes in *Aspergillus novofumigatus*, *A. lentulus* and *A. fumigatiaffinis*.

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 results 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 *PeΔbrlA* 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 *PeΔbrlA* 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 *PeΔveA* 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 *PeΔbrlA* 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 *PeΔveA* and *PeΔbrlA* strains after culture on labeled wheat grains

| Molecular formula | Identifier ^a | Proposed identification | WT | <i>PeΔveA</i> | <i>PeΔbrlA</i> | Molecular formula | Identifier ^a | Proposed identification | WT | <i>PeΔveA</i> | <i>PeΔbrlA</i> |
|--|--------------------------|-------------------------|----|---------------|----------------|---|---------------------------|--------------------------------------|----|---------------|----------------|
| C₇H₆O₄ | Pexp_153.019_3.59 | patulin | + | ND | + | C ₂₈ H ₃₈ N ₄ O ₆ | Pexp_527.287_12.46 | VAL-PHE-VAL-TYR | + | + | + |
| C ₇ H ₈ O | Pexp_109.065_7.11 | m-Cresol | + | ND | + | C₂₈H₃₈O₇ | Pexp_485.254_35.32 | | + | + | + |
| C ₇ H ₈ O ₂ | Pexp_125.060_6.79 | m-Hydroxybenzyl alcohol | + | ND | + | C ₂₈ H ₃₈ O ₇ | Pexp_487.270_36.21 | Andrastin A | + | + | + |
| C ₇ H ₈ O ₃ | Pexp_141.054_3.8 | Gentisyl alcohol | + | ND | + | C ₂₈ H ₃₈ O ₈ | Pexp_501.247_27.70 | | + | + | + |
| C ₇ H ₈ O ₄ | Pexp_157.049_2.67 | Ascladiol | + | ND | + | C₂₈H₃₈O₈ | Pexp_501.247_28.44 | | + | + | ND |
| C ₇ H ₁₀ O ₃ | Pexp_143.070_4.52 | | + | + | + | C₂₈H₃₈O₈ | Pexp_501.247_29.57 | | + | + | + |
| C ₁₀ H ₁₇ NO ₅ | Pexp_232.117_2.42 | | ND | + | ND | C₂₈H₄₀O₆ | Pexp_471.274_39.48 | Andrastin C | + | + | + |
| C ₁₀ H ₁₇ NO ₅ | Pexp_232.118_8.83 | | + | + | ND | C₂₈H₄₀O₇ | 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 ₅ O 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 ₅ O ₅ | Pexp_526.206_17.61 | | ND | ND | + |
| C ₁₅ H ₁₉ NO ₆ | Pexp_310.129_12.78 | | + | + | + | C ₂₉ H ₃₁ N ₅ O ₅ | Pexp_530.238_14.83 | | ND | ND | + |
| C ₁₅ H ₁₉ NO ₆ | Pexp_310.129_14.80 | | + | + | + | C ₂₉ H ₃₁ N ₅ O 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 ₅ O 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 ₅ O 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 ₅ O 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 ₅ O ₆ | Pexp_548.251_15.42 | | + | + | ND |
| C ₁₆ H ₁₈ N ₂ O ₂ | Pexp_271.144_7.62 | Clavicipitic acid | + | + | + | C ₃₀ H ₃₆ N ₄ O ₂ | Pexp_485.292_16.36 | New DMV ¹ communesin-2 | + | + | ND |
| C ₁₆ H ₁₈ N ₂ O ₂ | Pexp_271.144_8.45 | Clavicipitic acid | + | + | + | C ₃₀ H ₃₉ N ₅ O ₅ | Pexp_550.302_18.25 | | ND | + | ND |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | Pexp_375.142_22.02 | | + | + | + | C ₃₀ H ₃₉ N ₅ O ₅ | Pexp_550.302_19.14 | | ND | + | ND |

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|---|---------------------------|-----------------|----|----|----|---|---------------------------|---|----|----|----|
| C ₁₇ H ₁₇ NO ₇ | Pexp_348.108_11.75 | | ND | + | ND | C ₃₁ H ₃₆ N ₄ O 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 ₄ O 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 ₂ O 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 ₂ O 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 ₂ O ₅ | Pexp_529.268_23.19 | Chaetoglobosin B/G | ND | ND | + |
| C ₁₇ H ₂₃ N ₃ O ₃ | Pexp_318.179_3.12 | | ND | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_25.21 | Chaetoglobosin B/G | + | ND | + |
| C ₁₇ H ₂₃ N ₃ O ₃ | Pexp_318.180_25.05 | | ND | ND | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_26.71 | Chaetoglobosin B/G | + | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | Pexp_293.129_13.58 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_29.56 | Chaetoglobosin B/G | + | ND | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_32.50 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_30.51 | Chaetoglobosin B/G | + | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | Pexp_305.129_33.17 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.268_32.43 | Chaetoglobosin B/G | ND | ND | + |
| C ₁₉ H ₁₆ N ₂ O ₄ | Pexp_337.119_13.73 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_33.98 | Chaetoglobosin A | + | + | + |
| C ₁₉ H ₁₆ N ₂ O ₄ | Pexp_337.119_15.20 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O 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 ₂ O 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 ₂ O ₅ | Pexp_529.270_36.68 | Chaetoglobosin C | + | + | + |
| C ₁₉ H ₂₁ NO ₇ | Pexp_376.139_18.65 | | + | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.270_37.41 | Chaetoglobosin B/G | + | ND | + |
| C₁₉H₃₈O₆ | Pexp_361.258_37.23 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | Pexp_529.268_38.29 | Chaetoglobosin B/G | ND | ND | + |
| C₁₉H₃₈O₆ | Pexp_361.258_38.19 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₆ | Pexp_545.263_27.60 | Putative cytochalasan | + | ND | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | Pexp_319.145_35.48 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₆ | Pexp_545.262_29.23 | Putative cytochalasan | ND | ND | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | Pexp_319.145_36.44 | | + | + | ND | C ₃₂ H ₃₆ N ₂ O 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 ₂ O 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 ₂ O ₆ | Pexp_545.262_32.48 | Putative cytochalasan | ND | ND | + |
| C ₂₂ H ₂₃ N ₅ O ₂ | Pexp_390.193_15.09 | Roquefortine C | + | + | + | C₃₂H₃₆N₂O₆ | Pexp_543.248_34.05 | Putative cytochalasin | + | ND | + |
| C ₂₂ H ₂₅ N ₅ O ₂ | Pexp_392.209_9.99 | Roquefortine D | + | + | + | C ₃₂ H ₃₆ N ₄ O ₂ | Pexp_509.292_36.01 | Communesin B ^g | + | + | + |
| C ₂₃ H ₂₄ N ₂ O ₆ | Pexp_425.171_26.92 | | + | + | + | C ₃₂ H ₃₆ N ₄ O ₄ | Pexp_541.280_07.78 | Com540 ^b | ND | + | ND |

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|---|--------------------|--|----|----|----|---|--------------------|--------------------------------------|----|----|----|
| C ₂₄ H ₂₆ N ₂ O ₆ | Pexp_437.170_33.79 | | + | + | ND | C ₃₂ H ₃₈ N ₂ O 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 ₂ O ₆ | Pexp_515.288_37.93 | Putative cytochalasan | + | + | + |
| C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_14.62 | Communesin I ^c | + | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_515.289_38.15 | Putative cytochalasan | + | + | + |
| C ₂₆ H ₃₀ N ₄ O | Pexp_415.250_18.43 | Communesin I ^c | + | + | + | C ₃₂ H ₃₈ N ₂ O 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 ₂ O 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 ₂ O 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 ₂ O ₆ | Pexp_531.283_27.38 | Putative cytochalasan | + | + | + |
| C ₂₆ H ₄₀ O ₆ | Pexp_449.289_29.93 | | + | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_531.283_28.52 | Putative cytochalasan | + | + | + |
| C ₂₇ H ₂₉ N ₅ O ₄ | Pexp_488.227_10.80 | | ND | ND | + | C ₃₂ H ₃₆ N ₄ O ₃ | Pexp_525.287_13.45 | New DME ² communesin-2 | + | + | ND |
| C ₂₇ H ₂₉ N ₅ O ₄ | Pexp_488.227_11.03 | | ND | ND | + | C ₃₂ H ₃₆ N ₄ O ₃ | Pexp_525.287_27.91 | New DMV ¹ communesin-3 | + | + | ND |
| C ₂₇ H ₃₀ N ₄ O | Pexp_427.249_10.19 | | ND | + | + | C ₃₂ H ₃₈ N ₄ O ₂ | Pexp_511.306_36.13 | New DME ² communesin-3 | + | + | ND |
| C ₂₇ H ₃₀ N ₄ O ₂ | Pexp_443.245_15.03 | Communesin E ^d | + | + | + | C ₃₂ H ₃₈ N ₄ O ₃ | Pexp_527.303_21.41 | New DME ² communesin-4 | + | + | ND |
| C ₂₈ H ₃₀ N ₄ O ₃ | Pexp_471.239_19.56 | Com470 ^e | + | + | ND | C ₃₂ H ₃₈ N ₄ O ₄ | Pexp_543.297_19.36 | New DME ² communesin-5 | + | + | ND |
| C ₂₈ H ₃₁ N ₅ O ₅ | Pexp_518.241_16.36 | | + | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | Pexp_531.283_28.02 | Chaetoglobosin E ^h | ND | ND | + |
| C ₂₈ H ₃₁ N ₅ O ₅ | Pexp_518.240_17.21 | | + | + | + | C ₃₂ H ₄₀ N ₄ O ₂ | Pexp_513.322_36.57 | New DME ² communesin-6 | + | + | ND |
| C ₂₈ H ₃₂ N ₄ O | Pexp_441.265_17.14 | Communesin F ^d | + | + | ND | C ₃₂ H ₃₈ N ₂ O ₅ | Pexp_531.283_31.56 | Penochalasin F ^h | ND | + | + |
| C ₂₈ H ₃₂ N ₄ O ₂ | Pexp_457.260_14.27 | New DMV ¹ communesin-1 | + | + | ND | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_547.278_23.19 | Putative cytochalasan | ND | ND | + |
| C ₂₈ H ₃₂ N ₄ O ₂ | Pexp_457.260_14.97 | New DME ² communesin-1 | + | ND | ND | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_547.278_25.47 | Putative cytochalasan | ND | + | + |
| C ₂₈ H ₃₂ N ₄ O ₂ | Pexp_457.261_23.65 | Communesin A ^f | + | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_547.278_27.09 | Putative cytochalasan | ND | + | + |
| C ₂₈ H ₃₂ N ₄ O ₃ | Pexp_473.255_15.50 | | + | + | ND | C ₃₂ H ₃₈ N ₂ O ₆ | Pexp_547.278_28.27 | Putative cytochalasan | ND | ND | + |
| C ₂₈ H ₃₆ N ₄ O ₄ | Pexp_493.279_31.47 | Fungisporin A or cyclo(VFVF) VAL-PHE-VAL- PHE | + | + | + | C ₃₃ H ₃₈ N ₄ O ₅ | Pexp_571.293_18.22 | Com570 ^e | + | + | ND |
| C ₂₈ H ₃₈ N ₄ O ₅ | Pexp_511.290_18.01 | | + | + | + | C ₃₇ H ₄₂ N ₄ O 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 *PeΔbrlA*, *PeΔveA* and WT strain.

| Molecular formula | R _T (min) ^a | ¹² C <i>m/z</i> (Da) | Proposed identification | 14 dpi <i>PeΔveA</i> | 14 dpi WT | 30 dpi WT | 30 dpi <i>PeΔbrlA</i> | Molecular formula | R _T (min) ^a | ¹² C <i>m/z</i> (Da) | Proposed identification | 14 dpi <i>PeΔveA</i> | 14 dpi WT | 30 dpi WT | 30 dpi <i>PeΔbrlA</i> |
|--|-----------------------------------|---------------------------------|-------------------------|----------------------|-----------|-----------|-----------------------|---|-----------------------------------|---------------------------------|---|----------------------|-----------|-----------|-----------------------|
| C₇H₆O₄ | 3.59 | 153.01919 | Patulin | ND | + | + | + | C ₂₆ H ₄₀ O ₆ | 29.93 | 449.28939 | | + | + | ND | ND |
| C ₇ H ₈ O ₃ | 3.80 | 141.05493 | Gentisyl alcohol | ND | ND | ND | + | C ₂₈ H ₃₈ O ₇ | 36.21 | 487.27063 | Andrastin A | + | + | + | + |
| C ₇ H ₈ O ₂ | 6.79 | 125.05998 | m-Hydroxybenzyl alcohol | ND | + | ND | ND | C₂₈H₃₈O₈ | 28.44 | 501.24792 | | ND | ND | + | + |
| C ₇ H ₈ O ₄ | 2.67 | 157.04990 | Ascladiol | ND | + | + | + | C₂₈H₃₈O₈ | 29.57 | 501.24786 | | + | + | + | + |
| C ₁₀ H ₁₇ NO ₅ | 8.83 | 232.11872 | | ND | ND | + | + | C₂₈H₄₀O₇ | 30.58 | 487.26898 | Andrastin B | + | + | + | + |
| C ₁₃ H ₁₄ O ₅ | 21.70 | 251.09108 | Citrinin | ND | ND | + | + | C ₂₉ H ₂₇ N ₅ O ₅ | 17.61 | 526.20689 | | ND | ND | ND | + |
| C ₁₆ H ₂₆ N ₂ O ₄ S ₂ | 22.02 | 375.14202 | | ND | ND | + | + | C ₃₂ H ₃₆ N ₂ O 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 ₂ O 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 ₂ O 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 ₄ O 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 ₂ O ₅ | 26.71 | 529.27029 | Chaetoglobosin B/G | ND | ND | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | 17.74 | 293.12915 | | ND | ND | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 29.54 | 529.26898 | Chaetoglobosin B/G | ND | ND | + | + |
| C ₁₈ H ₁₈ N ₂ O ₂ | 14.61 | 295.14479 | | + | + | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 30.13 | 529.27539 | Chaetoglobosin B/G | ND | + | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | 33.17 | 305.12933 | | ND | ND | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 32.43 | 529.26807 | Chaetoglobosin B/G | ND | ND | ND | + |
| C₁₉H₃₈O₆ | 38.19 | 361.25819 | | ND | ND | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 35.67 | 529.26822 | Chaetoglobosin B/G | ND | ND | + | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | 36.44 | 319.14502 | | ND | ND | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 37.41 | 529.27056 | Chaetoglobosin B/G | ND | ND | ND | + |
| C ₂₂ H ₂₃ N ₅ O ₂ | 15.09 | 390.19390 | Roquefortine C | ND | ND | + | + | C ₃₂ H ₃₆ N ₄ O ₂ | 36.01 | 509.29257 | Communesin B | + | + | + | ND |
| C ₂₃ H ₂₄ N ₂ O ₆ | 26.92 | 425.17179 | | + | ND | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | 21.75 | 531.28384 | Putative cytochalasan | ND | ND | ND | + |
| C₂₄H₂₆N₂O₆ | 33.79 | 437.17091 | | ND | ND | + | ND | C ₃₂ H ₃₈ N ₂ O ₅ | 23.91 | 531.28431 | Putative cytochalasan | ND | ND | ND | + |
| C ₂₈ H ₃₂ N ₄ O | 17.14 | 441.26589 | Communesin F | ND | ND | + | ND | C ₃₂ H ₃₈ N ₂ O ₆ | 23.19 | 547.27864 | Putative cytochalasan | ND | ND | ND | + |

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|-------------------------------------|--------------|------------------|--------------------------------------|---|---|---|----|----------------------|-------|-----------|--------------------------------------|----|----|----|----|
| $C_{28}H_{32}N_4O_2$ | 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 *PeΔbrlA* 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 ₂ O ₂ | 271.14496 | 7.62 | Clavicipitic acid | + | + | C ₂₈ H ₃₈ O ₈ | 501.24786 | 29.57 | | + | + |
| C ₁₆ H ₂₆ N ₂ O ₄ S 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 ₅ O ₆ | 548.25180 | 14.66 | | ND | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | 293.12915 | 13.58 | | + | ND | C ₃₂ H ₃₄ N ₄ O ₃ | 523.27152 | 31.84 | Communesin D | + | + |
| C ₁₈ H ₁₆ N ₂ O ₂ | 293.12915 | 17.74 | | + | + | C ₃₂ H ₃₆ N ₂ O ₄ | 513.27635 | 39.10 | Chaetoglobosin J or Prochaetoglobosin III | + | + |
| C ₁₈ H ₁₈ N ₂ O ₂ | 295.14479 | 14.61 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27029 | 26.79 | Chaetoglobosin B/G | + | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | 305.12911 | 32.50 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26757 | 29.56 | Chaetoglobosin B/G | ND | + |
| C ₁₉ H ₁₆ N ₂ O ₂ | 305.12933 | 33.17 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27077 | 30.51 | Chaetoglobosin B/G | + | + |
| C ₁₉ H ₃₈ O ₆ | 361.25871 | 37.17 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27067 | 33.36 | Chaetoglobosin A | + | + |
| C ₁₉ H ₃₈ O ₆ | 361.25819 | 38.19 | | + | + | C ₃₂ H ₃₆ N ₂ O ₅ | 529.26769 | 35.49 | Chaetoglobosin B/G | ND | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | 319.14588 | 35.48 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27063 | 36.74 | Chaetoglobosin C | + | + |
| C ₂₀ H ₁₈ N ₂ O ₂ | 319.14502 | 36.44 | | + | ND | C ₃₂ H ₃₆ N ₂ O ₅ | 529.27056 | 37.41 | Chaetoglobosin B/G | ND | + |
| C₂₂H₂₀N₅O | 341.15380 | 38.56 | | ND | + | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26332 | 27.60 | Putative cytochalasan | ND | + |
| C₂₂H₂₀N₅O | 341.15368 | 39.29 | | ND | + | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26241 | 29.23 | Putative cytochalasan | ND | + |
| C ₂₂ H ₂₃ N ₅ O ₂ | 390.19390 | 15.09 | Roquefortine C | + | + | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26288 | 30.57 | Putative cytochalasan | ND | + |
| C ₂₂ H ₂₅ N ₅ O ₂ | 392.20913 | 9.99 | Roquefortine D | + | + | C ₃₂ H ₃₆ N ₂ O ₆ | 545.26294 | 32.48 | Putative cytochalasan | ND | + |
| C ₂₃ H ₂₄ N ₂ O ₆ | 425.17179 | 26.92 | | + | + | C ₃₂ H ₃₆ N ₄ O ₂ | 509.29257 | 34.95 | Communesin B | + | + |
| C₂₄H₂₆N₂O₆ | 437.17091 | 33.79 | | + | + | C ₃₂ H ₃₈ N ₂ O 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 ₂ O ₄ | 515.28890 | 37.93 | Putative cytochalasan | ND | + |

| | | | | | | | | | | | |
|---|------------------|--------------|-----------------------------------|----|----|---|-----------|-------|--------------------------|----|----|
| C ₂₆ H ₃₀ N ₄ O | 415.25034 | 14.62 | Communesin I | + | + | C ₃₂ H ₃₈ N ₂ O ₄ | 515.28909 | 38.15 | Putative cytochalasan | ND | + |
| C ₂₆ H ₃₀ N ₄ O | 415.25033 | 18.43 | Communesin I | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28990 | 22.76 | Putative cytochalasan | ND | + |
| C ₂₆ H ₄₀ O ₆ | 449.28939 | 29.93 | | ND | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28431 | 23.91 | Putative cytochalasan | ND | + |
| C ₂₇ H ₃₀ N ₄ O ₂ | 443.24564 | 15.65 | Communesin E | ND | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28354 | 28.02 | Chaetoglobosin E | ND | + |
| C ₂₈ H ₃₀ N ₄ O ₃ | 471.23997 | 19.56 | Com470 | + | + | C ₃₂ H ₃₈ N ₂ O ₅ | 531.28338 | 31.56 | Penochalasin | ND | + |
| C ₂₈ H ₃₂ N ₄ O | 441.26589 | 17.14 | Communesin F | + | + | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27864 | 23.19 | Putative cytochalasan | ND | + |
| C ₂₈ H ₃₂ N ₄ O ₂ | 457.26097 | 14.27 | New DMV ¹ communesin-1 | + | ND | C ₃₂ H ₃₈ N ₂ O ₆ | 547.27841 | 25.47 | Putative cytochalasan | ND | + |
| C ₂₈ H ₃₂ N ₄ O ₂ | 457.26116 | 23.65 | Communesin A | + | + | C ₃₂ H ₃₈ N ₄ O ₃ | 527.30304 | 21.41 | New DME2 communesin-4 | + | ND |
| C₂₈H₃₈O₇ | 485.25405 | 35.32 | | + | + | C ₃₂ H ₄₀ N ₄ O 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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