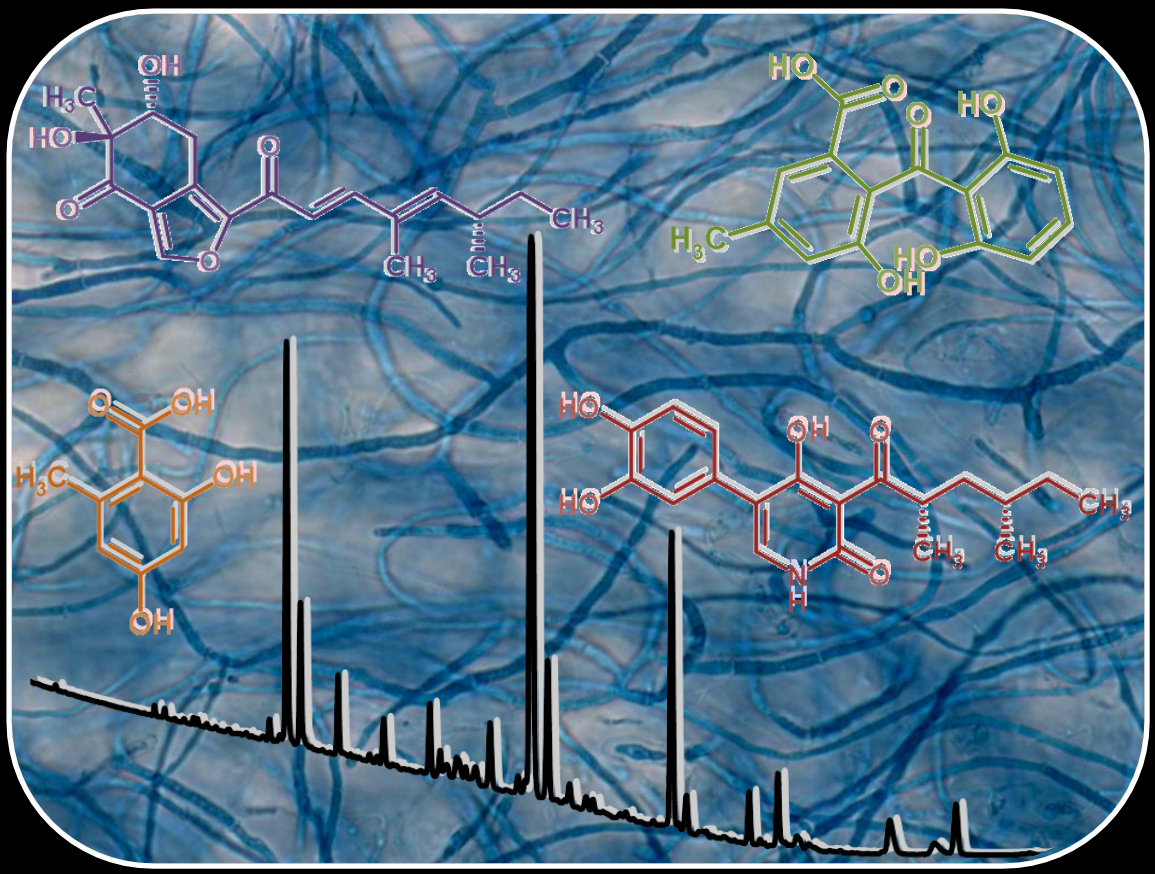
Triggering secondary metabolite biosynthesis: exploring the effects of ionic liquids in fungal metabolism

Paula Cristina Alves



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
April, 2016

UNIVERSIDADE NOVA DE LISBOA

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I declare that the work presented in this thesis, except where otherwise stated, is based on my own research. It was supervised by Doctor Cristina Silva Pereira (ITQB) and Doctor Jörg D. Becker (IGC). The work was mainly performed in *Instituto de Tecnologia Química e Biológica António Xavier*, *Universidade Nova de Lisboa*, between February 2010 and September 2015.

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À minha Mãe,

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Summary

Filamentous fungi are able to synthesise an array of small molecules (secondary metabolites), which are usually not essential for fungal growth but confer competitiveness. As a consequence, numerous secondary metabolites remain cryptic at the artificial conditions of cultivation in a research laboratory. Even in *Aspergillus nidulans*, one of the most well studied fungi, numerous metabolites remain unseen. Several strategies have been used to solve this knowledge gap, some of which require prior knowledge of genomic sequences, relying on manipulation of targeted genes encoding components of either secondary metabolism or regulatory pathways. Other approaches may be applied also in less well characterised strains, such as cultivation with other species/organisms or modification of the growth media composition. The ability of certain ionic liquids to increase the metabolic diversity in the footprint of several environmental fungal strains has been demonstrated. Inspired by these early observations, this thesis analysed in great detail how ionic liquids impact fungal metabolism particularly aiming to understand their ability to trigger production of secondary metabolites. Two model filamentous fungi, namely *A. nidulans* and *Neurospora crassa* were used in this thesis. Both are important species in fungal biology studies and their genomes have been sequenced. Moreover, *A. nidulans* is a talented secondary metabolite producer and a halotolerant species, whereas *N. crassa* capacity to synthesise secondary metabolites is quite humble and is a non-halotolerant species. In addition, two distinct ionic liquids were used as media supplements: cholinium chloride (choline) and 1-ethyl-3-methylimidazolium chloride. These chemicals belong to the most studied families of ionic liquids. The cholinium cation is non-toxic, biocompatible and biodegradable and the resulting ionic liquids can show interesting properties, for example the cholinium alkanoates can solubilise and catalyse suberin depolymerisation. In contrast, the imidazolium cations are usually toxic and non-degradable. Numerous ionic liquids carrying imidazolium-based cations are commercially available; most display high chemical stability, low melting points and viscosities hence extensively studied for many applications including the

dissolution, swelling and processing of lignocellulosic materials. The general alterations provoked by either ionic liquid supplement in the fungal cultures, namely stress response and primary metabolism were accessed using differential proteomics (Chapter II). Either ionic liquid induced, in both fungi, up-accumulation of several stress-responsive proteins. They affected critical biological processes and pathways, as well as developmental programmes. Moreover, they stimulated production of osmolytes, which may play key roles in multiple stress responses. Particularly, either ionic liquid increased the levels of proteins likely involved in the biosynthesis of rare amino acids in *N. crassa*. Whole genome profiling (transcriptomics) was used to evaluate not only the general impact of these chemicals in the metabolism of *A. nidulans* (complementing the proteomic study) but also to analyse in greater detail the expression of genes coding in secondary metabolism, either biosynthetic or regulatory genes (Chapter III). Transcriptome data were complemented by *q*RT-PCR, microscopy and metabolome analysis (liquid chromatography coupled with high resolution mass spectrometry). In general, primary metabolism was up-regulated by choline but down-regulated by 1-ethyl-3-methylimidazolium chloride. Choline could be used as a source of carbon and nitrogen probably via the glycine, serine and threonine metabolic pathway, and incorporated into the central carbon metabolism; whereas 1-ethyl-3-methylimidazolium chloride induced the use of cellular reserves and autophagy. Both ionic liquids induced detoxification mechanisms probably to eliminate toxic cations or intermediates. The biosynthesis of secondary metabolites involves a multi-domain enzyme which is encoded by a backbone gene. The ionic liquid supplements led to up-regulation of twenty one backbone genes (out of sixty six predicted in the genome of this fungus) some of which uncharacterised yet. In agreement, either ionic liquid supplement increased the diversity of compounds in the culture footprints compared to control conditions. Six compounds (out of forty ion masses differentially detected) could be identified using the corresponding pure standards, including monodictyphenone and orsellinic acid, which are usually silent under standard growth media, and gentisic acid and caffeic acid, for the first time identified in *A. nidulans*. Finally, *N. crassa* capacity to produce peptide metabolites under an ionic liquid stress, initially suggested by the

differential proteome data (Chapter II) was analysed in greater detail (Chapter IV). Several methods were used to isolate and characterise the peptide metabolites, namely liquid chromatography, gel electrophoresis, nuclear magnetic resonance spectroscopy, mass spectrometry and amino acid analyses, as well as bioactivity assays. 1-Ethyl-3-methylimidazolium chloride supplement induced the biosynthesis of antimicrobial peptide metabolites that also display potential anti-cancer activity. They contain as structural units 1-aminocyclopropane-1-carboxylic acid and/or α-aminoisobutyric acid. Importantly, the ionic liquid supplement failed to induce production of antimicrobial peptide metabolites in cultures of *N. crassa* mutants impaired in the capacity to synthesise 1-aminocyclopropane-1-carboxylic acid. More studies are required to fully resolve the structure of the produced peptaibiotics but the proof-of-concept of the capacity of certain ionic liquids to trigger production of novel biological active peptides has been established. In the last section of this thesis (Chapter V) an integrated discussion of data from previous chapters, as well as perspectives for future work are presented. It also includes additional data not discussed in detail in this thesis. Overall, the work presented in this thesis supports the use of ionic liquids as triggers to activate unexpected metabolic and developmental changes in fungi, particularly to induce production of otherwise silent fungal secondary metabolites. There should be no doubts that this immense family of compounds may lead discovery of a hidden array of fungal secondary metabolites, some of which enclosing valuable biological activity.

Sumário

Os fungos filamentosos têm a capacidade de sintetizar uma diversidade de pequenas moléculas denominadas de metabolitos secundários que, apesar de normalmente não serem consideradas essenciais para o crescimento do fungo, conferem competitividade ao organismo que as sintetiza. Consequentemente, inúmeros metabolitos secundários permanecem crípticos em condições artificiais de cultura em laboratório. Em *Aspergillus nidulans*, um dos fungos mais estudados, também existem vários metabolitos por descobrir. Têm sido aplicadas várias estratégias para resolver esta lacuna no conhecimento, algumas das quais requerem o conhecimento prévio do genoma para posterior manipulação de genes específicos codificando componentes quer do metabolismo secundário quer de vias de regulação. No caso de estirpes cujo genoma não está sequenciado, podem aplicar-se outras abordagens, tais como o crescimento destas na presença de outros organismos ou a própria modificação da composição do meio de crescimento. Alguns líquidos iónicos têm demonstrado capacidade de aumentar a diversidade molecular de variadas espécies de fungos ambientais. Estes factos inspiraram os estudos apresentados nesta tese nos quais se pretendia desvendar o impacto de líquidos iónicos no metabolismo dos fungos, com o objetivo principal de entender a sua habilidade para induzir a produção de metabolitos secundários. Para a execução destes estudos foram seleccionados dois fungos filamentosos modelo de elevada relevância para o estudo da biologia de fungos cujos genomas estão sequenciados - *A. nidulans* e *Neurospora crassa*. Enquanto *A. nidulans* é um fungo halotolerante conhecido pela sua elevada produção de metabolitos secundários, *N. crassa* é uma espécie não-halotolerante com reduzida capacidade para sintetizar metabolitos secundários. Do mesmo modo, foram escolhidos dois líquidos iónicos distintos para serem adicionados como suplemento aos meios de cultura dos fungos a testar – cloreto de colina e cloreto de 1-etil-3-metilimidazólio. Estes compostos pertencem às famílias de líquidos iónicos mais estudadas até à data. O catião colínio não é tóxico, é biocompatível e biodegradável, e a sua presença na formulação de diversos líquidos iónicos confere-lhes propriedades interessantes, tal como os alcanoatos de colínio que permitem a

solubilização e a catálise da despolimerização de suberina. Por outro lado, apesar de o catião imidazólio ser normalmente considerado tóxico e não-biodegradável, existem vários líquidos iónicos disponíveis comercialmente contendo este catião na sua composição. Na sua maioria apresentam elevada estabilidade química, baixos pontos de fusão e baixa viscosidade, características que potenciam o seu extenso estudo para várias aplicações, como a dissolução e o processamento de materiais lignocelulósicos. As principais alterações provocadas pela suplementação de cada um dos líquidos iónicos escolhidos ao meio de crescimento de cada fungo, nomeadamente a acumulação de proteínas relacionadas tanto com a resposta ao stress como ao metabolismo primário, foram avaliadas por análise diferencial de proteomas (Capítulo II). Os líquidos iónicos também alteraram importantes vias e processos biológicos, tal como o ciclo de desenvolvimento do fungo e estimularam a produção de osmólitos, que podem participar na resposta a vários stresses. Em particular, foi observado o aumento de proteínas envolvidas na biossíntese de aminoácidos raros por ambos os líquidos iónicos em culturas de *N. crassa*. A análise do transcriptoma de *A. nidulans* foi usada não só para avaliar o impacto destes compostos no metabolismo deste fungo (complementando o estudo do seu proteoma), mas também para analisar em maior detalhe a expressão de genes envolvidos quer na biossíntese de metabolitos secundários quer na regulação do metabolismo secundário (Capítulo III). Os resultados obtidos por transcriptómica foram complementados com dados de *q*RT-PCR, microscopia e análise do metaboloma (por cromatografia líquida acoplada a espectrometria de massa de alta resolução). Em termos gerais, o metabolismo primário foi activado pelo cloreto de colínio, que pode ter sido usado como fonte de carbono e nitrogénio pela via metabólica da glicina, serina e treonina, e posteriormente incorporado no metabolismo central do carbono. Pelo contrário, o metabolismo primário foi regulado negativamente pelo cloreto de 1-etil-3-metilimidazólio, que induziu a utilização de reservas celulares e autofagia. Ambos os líquidos iónicos activaram mecanismos de desintoxicação provavelmente para eliminar catiões e intermediários tóxicos. A biossíntese de metabolitos secundários envolve a acção de complexos multi-enzimáticos codificados por um gene designado de *"backbone gene"*. A

adição de líquidos iónicos aumentou a expressão de vinte e um *"backbone genes"* (num total de sessenta e seis *"backbone genes"* previstos no genoma de *A. nidulans*) alguns dos quais ainda não estão caracterizados. Em concordância com os dados anteriores, estes compostos aumentaram a diversidade molecular nas culturas contendo cada um dos líquidos iónicos em comparação com condições controlo. Dos quarenta valores de massa detectados diferencialmente via espectrometria de massa de alta resolução foi possível identificar seis compostos usando os correspondentes compostos padrão, incluindo monodictyphenone e o ácido orselínico, compostos considerados crípticos; e os ácidos gentísico e cafeico, identificados pela primeira vez em *A. nidulans*. O Capítulo IV apresenta o estudo desenvolvido para a análise da capacidade de *N. crassa* produzir metabolitos peptídicos na presença de líquidos iónicos, conforme sugerido pelos dados de proteómica no Capítulo II. Para este fim foram usados vários métodos para isolar e caracterizar os metabolitos peptídicos, como por exemplo cromatografia líquida, electroforese em gel, espectroscopia de ressonância magnética nuclear, espectrometria de massa, análise de aminoácidos e ensaios para testar a sua actividade biológica. O suplemento de cloreto de 1-etil-3-metilimidazólio induziu a biossíntese de metabolitos peptídicos contendo unidades estruturais de ácido 1-carboxílico-1-aminociclopropano e/ou ácido α-aminoisobutírico apresentando actividade antimicrobiana e potencial actividade anticancerígena. Importa realçar que a aplicação das mesmas condições de cultura ao mutante de *N. crassa* com delecção no gene codificando para a enzima envolvida na produção do ácido 1-carboxílico-1-aminociclopropano não permitiu a produção deste tipo de metabolitos peptídicos. Apesar de serem necessários estudos adicionais para a completa elucidação da estrutura destes metabolitos peptídicos, este estudo demonstra a capacidade de determinados líquidos iónicos para induzir a produção de novos metabolitos peptídicos biologicamente activos. A última secção desta tese (Capítulo V) consiste não só numa discussão integrada dos dados apresentados nos capítulos anteriores, mas também na apresentação de dados adicionais não discutidos detalhadamente ao longo da tese e na sugestão de trabalho futuro. De uma maneira geral, o trabalho apresentado nesta tese sustenta o uso de líquidos iónicos como indutores de alterações metabólicas e do desenvolvimento em fungos, em

especial induzindo a produção de metabolitos secundários crípticos. O uso de líquidos iónicos visando a descoberta de novos metabolitos fúngicos com potencial actividade biológica revela-se como uma estratégia alternativa e muito promissora.

Thesis publications

Martins I, Hartmann DO, **Alves PC**, Planchon S, Renaut J, Leitão MC, Rebelo LPN, Silva Pereira C. Proteomic alterations induced by ionic liquids in *Aspergillus nidulans* and *Neurospora crassa*. *Journal of Proteomics*, 2013, 94, 262-278, DOI: 10.1016/j.jprot.2013.09.015.

Alves PC, Hartmann DO, Martins I, Garcia H, Núñez O, Gomes TL, Leitão MC, Galceran MT, Hampson R, Becker JD, Silva Pereira C. Transcriptomic and metabolomic profiling of ionic liquid stimuli unveil enhanced secondary metabolism in *Aspergillus nidulans*. *BMC Genomics*, 2016, 17, 284, DOI: 10.1186/s12864-016-2577-6.

Alves PC, Martins I, Martins C, Núñez O, Gomes TL, Leitão MC, Galceran MT, Hampson R, Becker JD, Silva Pereira C. Investigating peptide metabolites production by *Neurospora crassa* under ionic liquids stimuli. *manuscript in preparation*.

Additional publications

Martins I, Hartmann DO, **Alves PC**, Martins C, Garcia H, Leclercq CC, Ferreira R, He J, Renaut J, Becker JD, Silva Pereira C. Elucidating how the saprophytic fungus *Aspergillus nidulans* uses the plant polyester suberin as carbon source. *BMC Genomics*, 2014, 15, 613, DOI: 10.1186/1471-2164-15-613.

List of acronyms

2DE	Bidimensional gel electrophoresis
Acc	1-Aminocyclopropane-1-carboxylic acid
ACN	Acetonitrile
Aib	α-Aminoisobutyric acid
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
a_w	Water activity
BSA	Bovine serum albumine
[C_2mim]Cl	1-Ethyl-3-methylimidazolium chloride
cDNA	Complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCA	A-Cyano-4-hydroxycinnamic acid
CoA	Coenzyme A
DAD	Diode array detector
DAPI	2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride
DG18	Dichloran glycerol agar base
DIC	Differential interference contrast
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ESI	Electrospray ionisation
FC	Fold-change
FGSC	Fungal genetics stock centre
FLR	Fluorescence detector

GC	Gas chromatography
GEO	Gene expression omnibus
GPI	Glycophosphatidylinositol
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IC50	Inhibitory concentration for 50% of the population
IPG	Immobilised pH gradient
JGI	Joint genome institute
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionisation
MAP	Mitogen-activated protein
MFC	Minimal fungicidal concentration
MFS	Major facilitator superfamily
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
MIPS	Munich information center for protein sequences
MM	Minimal media
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NcΔAcc	*Neurospora crassa* Acc deaminase deletion strain
NCBI	National center for biotechnology information
NMR	Nuclear magnetic resonance spectroscopy
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthetase
PCA	Principal component analysis

PCR	Polymerase chain reaction
PDA	Photodiode array detector
PI	Propidium iodide
PK	Polyetide
PKS	Polyketide synthase
***q*RT-PCR**	Quantitative real-time polymerase chain reaction
Q-TOF	Quadrupole time-of-flight
REACH	Registration, evaluation, authorisation and restriction of chemicals
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
Rt	Retention time
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SM	Secondary metabolite
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
theLiTE™	Thelial – live targeted epithelia
TIC	Total ion chromatogram
TOF	Time-of-flight
tRNA	Transfer ribonucleic acid
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet light

CHAPTER I

Introduction

This introductory chapter contains essential information for the understanding of the subsequent chapters enclosed in this thesis. However, it does not aim to provide a comprehensive revision of all the subjects herein presented.

1.1. Fungi

This section intends to briefly revise general information on fungal biology, ecology and biochemistry. Ascomycota *fungi, especially the model filamentous fungi* Aspergillus nidulans *and* Neurospora crassa, *will be addressed in more detail.*

General introductory aspects

Fungi are eukaryote organisms widespread in nature [1-3]. They represent *ca.* 75% of the soil microbial biomass and have the ability to spread their spores in order to circumvent severe environmental conditions. These organisms are able to obtain nutrients from several sources, due to their heterotrophic (obtaining energy from external organic material) and absorptive (secreting extracellular enzymes to get simpler units from complex substrates) nutrition [3]. The referred characteristics, together with high tolerance to pH, temperature and water availability [4], allow these organisms to be easily spread and to thrive in distinct environmental conditions [3]. Although there are about 100,000 characterised fungal species, the total number of species is estimated to be, at least, one order of magnitude higher [5, 6]. Among the well described fungal species there are saprophytes (*e.g. Aspergillus* spp. [7] and *Neurospora* spp. [8]), pathogens (*e.g. Aspergillus fumigatus* [9]), parasites (*e.g. Tolypocladium ophioglossoides* [10]) or symbionts (*e.g.* mycorrhizas [11]). Fungi impact greatly in human life-style, beneficially as part of human nutrition (*e.g.* mushrooms) and health (*e.g.* the antibiotic penicillin [12]), but also negatively acting as opportunistic pathogens and provoking serious health problems, such as dermatomycosis [13] and invasive fungal infections [14], which are difficult to diagnose and lead to high mortality rates, especially in the increasing immunocompromised population. Fungal metabolites have also an important role in our society. On one hand, fungi are known to produce mycotoxins (*e.g.* aflatoxins, ochratoxins, trichothecenes, fumonisins and patulin) which are toxic substances that cause disease to plants, animals and humans [15, 16]. On the other hand, there are fungal compounds with important biological activity [17], such as terrequinone A (antitumor agent [18]), lovastatin (cholesterol-lowering drug [19]), cephalosporin (antimicrobial agent [20]) and cyclosporin (immunosuppressant [21]); as well as

with biotechnological potential, such as food additives (carotenoids [22] and kojic acid [23]), detergent additives [24], and pulp and paper industry [25]. The above mentioned aspects emphasise the great importance of fungal organisms in several areas, justifying the increasing number of fungal organisms with sequenced genomes [26] and their relevance as model organisms for distinct research topics. For example, the yeast *Saccharomyces cerevisiae* is an eukaryote model organism in molecular and cell biology [27], which genome was the first to be sequenced [28]; *Neurospora crassa* [29] is a model filamentous fungus in cell biology studies more specifically in genetic and phenotypic analysis of the circadian clock [30]; and *Aspergillus nidulans* [31], also a model filamentous fungus in cell biology, biochemistry and eukaryotic genetics [32, 33] and one of the most well studied regarding the biosynthesis of secondary metabolites [34].

A glimpse at the fungal taxonomy

Fungal organisms constitute the unique kingdom *Fungi*, since the proposal of five kingdoms to classify the living beings in 1969 by Whittaker [35]. The classification of organisms has started with Linnaeus in 1735 with the division of life in two distinct kingdoms: animals and plants, the latter of which included fungi [36]. This initial division was rather limited; therefore several division systems have been suggested over time. In 1866, Haeckel defined a third kingdom to enclose the unicellular organisms [37] and, in 1938, Copeland divided the unicellular organisms' kingdom in two, resulting in a classification system of four kingdoms [38]. The five kingdoms classification proposed by Whittaker was not revised until RNA molecular characterisation demonstrated the need to implement a rank above the kingdom level [39, 40]. Consequently, three domains (*Bacteria*, *Archaea* and *Eukarya*) were proposed by Woese in 1990 for a complete natural classification of life [40]. Later in 1998, Cavalier-Smith reinforces the classification system by adding a sixth kingdom (*Chromista*), mainly to include oomycetes and algae [41, 42].

Regarding the kingdom *Fungi*, fungal organisms have been mainly divided in four phyla: *Chytridiomycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota* [2, 43]. However, in 2007, a new higher-level classification for the kingdom *Fungi* was proposed, based on molecular phylogenetic studies [44]. This classification considers one subkingdom (*Dikarya*), seven phyla and ten subphyla [44]. *Ascomycota* and *Basidiomycota* are the main representatives of fungi among these seven phyla (Fig. 1.1).

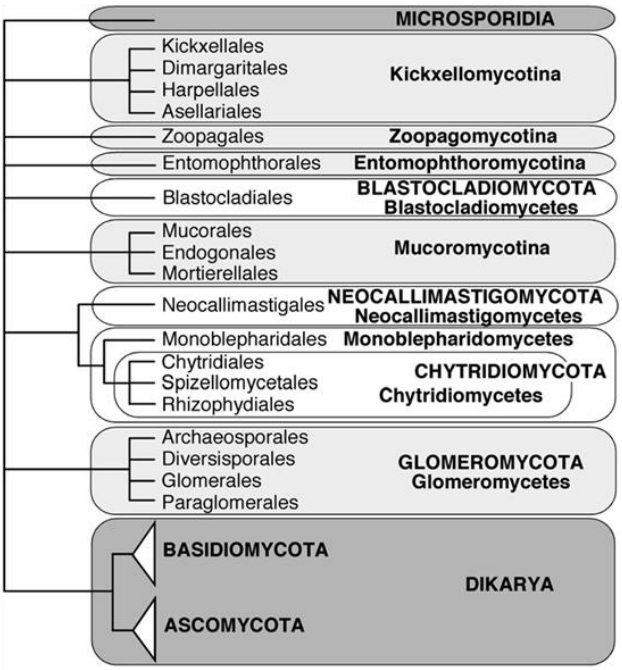


Fig. 1.1. Phylogeny and classification of fungi [44]. *N.b.* the branch lengths do not aim to be proportional to genetic distances.

Ascomycota fungi

Approximately 65% of all known fungal species belong to the very diverse phylum *Ascomycota* [45], which encloses three main subphyla: *Taphrinomycotina*, *Saccharomycotina*, *Pezizomycotina* [44]. Briefly, *Taphrinomycotina* includes the fission yeast *Schizosaccharomyces pombe* [46]; the budding yeast *Saccharomyces cerevisiae* [27] and the important human pathogen *Candida albicans* [47] are classified in the *Saccharomycotina* subphylum; and *Pezizomycotina* contains the

diverse filamentous *Ascomycota*, such as the blue cheese mould *Penicillium roqueforti* [48], the Fleming's penicillin producing strain *Penicillium rubens* [49, 50], the plant pathogen *Fusarium graminearum* [51], the human pathogen *Aspergillus fumigatus* [52], and the orange bread mould and important model organism *Neurospora crassa* [29, 53], just to mention a few.

The basic structure of fungal cells is very similar to other eukaryotic cells [43]. Therefore both contain membrane-bound organelles, such as nucleus, vacuoles, endoplasmic reticulum, Golgi apparatus and mitochondria [43, 54] (Fig. 1.2). However, some characteristics are specific to fungal cells, such as the cell wall and their growth as polarised filaments [43]. Although the cell wall structure is also present in plant cells, in which this structure is mainly composed of cellulose, it differs from the fungal cell wall, a dynamic structure mainly constituted by chitin, glucans and mannoproteins, in *Ascomycota* fungi [43, 55]. This structure which constitutes 15-30% of the cell dry weight [56], offers protection against physical damage and is flexible to cope with osmotic pressure alterations without losing the cell shape [55]. The polarised filaments present a tubular form and constitute the characteristic cell shape of filamentous fungi, also known as hyphae [43]. Hyphae are multinucleated and have incomplete septa (cross-walls) in order to permit the communication of cytoplasm and organelles between the compartments [2] (Fig. 1.2).

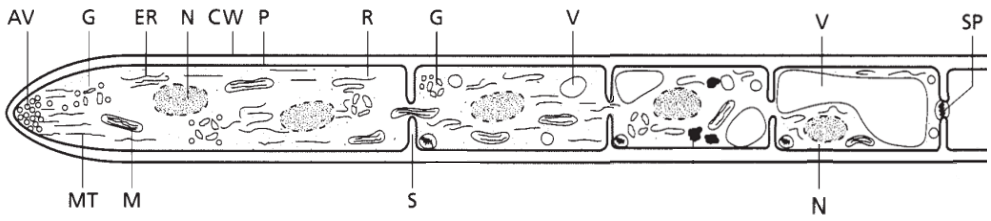


Fig. 1.2. Schematic representation of a hyphal tip. Legend: AV, apical vesicle; CW, cell wall; ER, endoplasmic reticulum; G, Golgi apparatus; L, lipid; M, mitochondria; MT, microtubules; N, nucleus; P, plasma membrane; R, ribosomes; S, septum; SP, septal pore and V, vacuole. Adapted from [2].

Filamentous fungi display a complex network of hyphal cells, forming the mycelium, which allow fungi to expand to great areas in order to obtain nutrients [57]. The mycelial structure results from the extension of hyphae units that branch sub-apically but also from hyphal anastomosis, which consists on the fusion of hyphal branches usually occurring in older mycelium [58, 59]. The polarised growth of hyphae [60] results from the presence of an apical body (*i.e.* the Spitzenkörper) at the hyphal tip (Fig. 1.2), which is considered responsible for the hyphal growth direction [2].

In controlled environments, fungi can present different morphologies depending on the imposed growth conditions. For example, in liquid media fungi can grow as aggregate of hyphae or as pellets (micro-colonies) each exhibiting different metabolisms [61]. Moreover, several fungal strains, such as the pathogenic fungi *Aspergillus fumigatus* or *Candida albicans* can also build biofilms, an exopolysaccharide matrix in which fungi are enclosed in [62]. Hyphal growth might be suspended in several occasions, such as entering asexual or sexual developmental programs, during chemical stresses or in starvation conditions [63]. This last situation might be overcome not only through important nutrient recycling processes like autophagy (*i.e.* self-digestion of aged hyphae) [64] but also through the production of dormant resistant structures (*i.e.* spores) for propagation under adverse conditions. Sexual spores, which are obtained through meiosis, are known to be extremely resistant and to present higher genetic variability compared to the parental cells, which turns out to be a survival strategy in harsh environmental conditions [43, 65]. The moment environmental conditions become favourable, these asexual or sexual spores (*i.e.* conidia or ascospores, respectively) can germinate and a new life cycle is ready to start [61]. As above mentioned, *Ascomycota* fungi life cycle comprises both sexual and asexual reproduction (Fig. 1.3). The name of this phylum derives from *asci*, which are specialised cells where the sexual spores are produced [43]. The sexual reproduction starts with the fusion (plasmogamy) of two haploid sexual hyphae (antheridium and ascogonium) (1) forming dikaryotic ascogenous hyphae (2). These dikaryotic cells constitute the ascocarp (3) which is the fruiting body containing the asci (4). Each ascus results from the fusion of the nuclei

(karyogamy) (5), which undergoes meiosis (6) and subsequent mitosis (7) to obtain mature, binucleate ascospores [61]. These ascospores are then released from the asci, on the surface of the ascocarp (8), and will originate haploid sexual mycelia (9) to allow the cycle to start again. Regarding the asexual reproduction (10), the haploid mycelium is able to produce asexual spores (conidia) [2].

In the end, the obtained resistant sexual and asexual spores undergo the dormancy state [61]. In the presence of favourable conditions for germination, the dormancy state is interrupted and a new haploid mycelial network is formed [61].

The following lines will focus in greater detail the two *Ascomycota* species selected for the studies presented in this thesis, namely *Aspergillus nidulans* and *Neurospora crassa*.

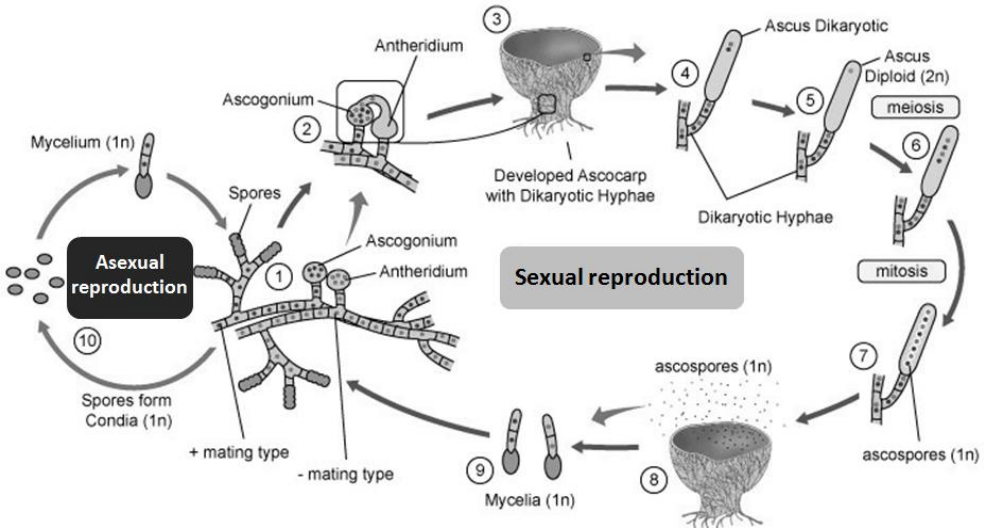


Fig. 1.3. General scheme of the *Ascomycota* life cycle. Adapted from the Department of Biology of Penn State (https://wikispaces.psu.edu/display/110Master/Home).

1.1.1. *Aspergillus nidulans*

The genus *Aspergillus* encloses approximately 250 known species [66] sharing a common asexual spore forming structure, the aspergillum [67]. The name of this structure was suggested by Micheli in 1729 due to the similarity between the structure containing the spores and the aspergillum which was a liturgical implement

used by the Roman Catholic clergy to sprinkle holy water during the *Asperges* (*i.e.* a part of the liturgy) [68] (Fig. 1.4).

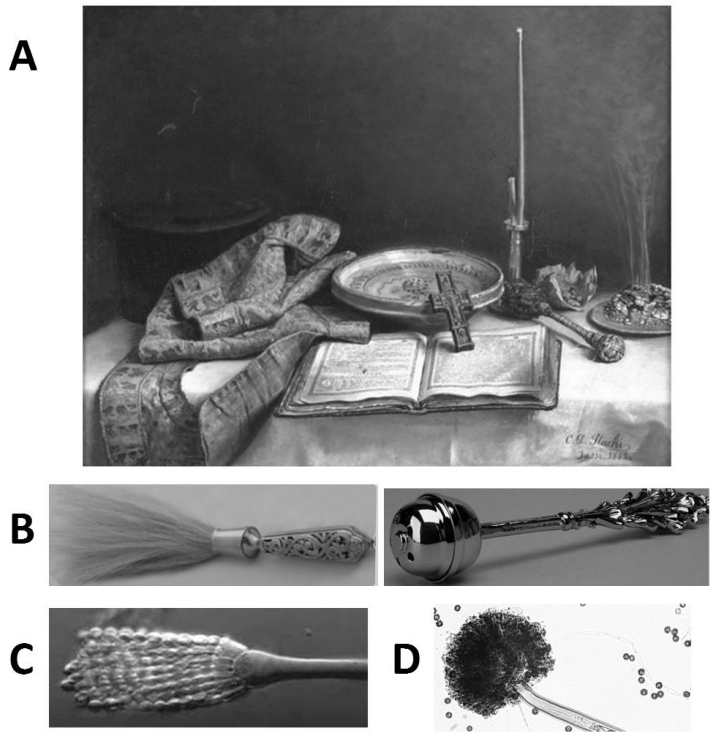


Fig. 1.4. Aspergillum - the origin of the *Aspergillus* conidiophore name. (A) Holy Water, painting by Constantin Daniel Stahi (1882) showing the tools used in the blessing of holy water. On the right of the blessing cross is a candle, the aspergillum made of basil branches and incense (http://www.mnar.arts.ro); (B) aspergillum types; (C) *Aspergillus restrictus* (www.aspergillus.org.uk); (D) *Aspergillus niger* (https://www.inspq.qc.ca).

Among the *Aspergillus* genus there are highly relevant species in diverse fields which genome is sequenced and available [33], such as the opportunistic pathogens *A. fumigatus*[1] [52, 69] and *A. terreus*[2] [70], the latter also known as the lovastatin producer [71]; the plant pathogen and aflatoxin producer *A. flavus*[3] [72,

The genome sequences of the mentioned *Aspergillus* species are available in GenBank:
[1] *Aspergillus fumigatus* Af293 (http://www.ncbi.nlm.nih.gov/genome/18).
[2] *Aspergillus terreus* NIH2624 (http://www.ncbi.nlm.nih.gov/genome/53).
[3] *Aspergillus flavus* NRRL3357 (http://www.ncbi.nlm.nih.gov/genome/360).

73]; the industrial source of citric acid and enzymes *A. niger*[4] [74, 75]; the kojic acid producer *A. oryzae*[5] [23, 76] and the model filamentous fungus *A. nidulans*[6] [31-33] also one of the most well studied fungi regarding secondary metabolism [34] (for further details see section 1.2).

Initially, fungal species were named according to the presence or absence of sexual phase, teleomorph (*e.g. Emericella*) or anamorph (*e.g. Aspergillus*), respectively. However, after the International *Aspergillus* workshop in 2007, it was established that a single culture should be given only one name [77]. Accordingly, along this thesis, *Aspergillus nidulans* will be the name used, notwithstanding this fungus can undergo both sexual and asexual reproduction (Fig. 1.5).

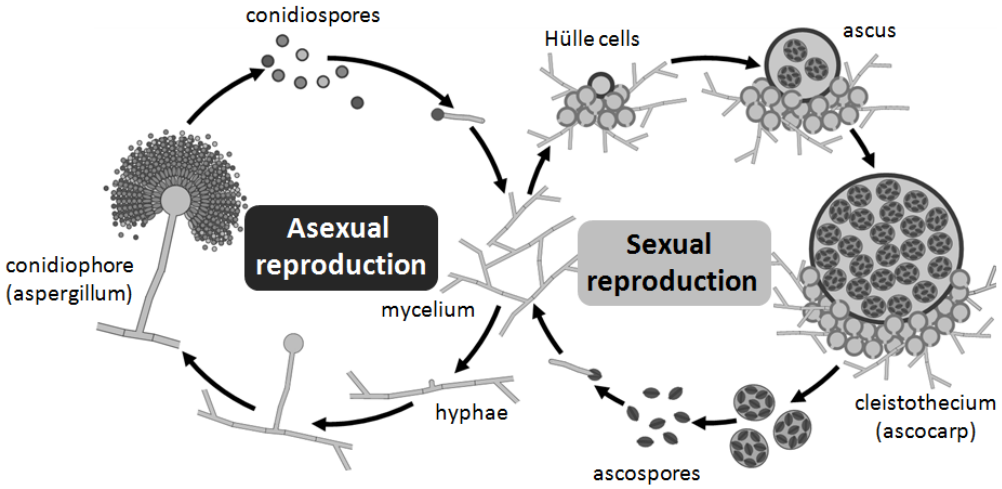


Fig. 1.5. *Aspergillus nidulans* life cycle. Adapted from [78].

Aspergillus nidulans is a homothallic species, meaning that it can enter the sexual stage directly through the mating of any two strains (*i.e.* self-fertile species). This fungus is normally haploid, but it can also grow as a heterokaryon or a vegetative diploid. Additionally, it produces both asexual spores (conidia) and sexual spores (ascospores), which are usually released from the conidiophore or the ascocarp (cleistothecium), respectively (Fig. 1.5).

[4] *Aspergillus niger* (http://www.ncbi.nlm.nih.gov/genome/429).
[5] *Aspergillus oryzae* 3.042 (http://www.ncbi.nlm.nih.gov/genome/526).
[6] *Aspergillus nidulans* FGSC A4 (http://www.ncbi.nlm.nih.gov/genome/17).

The asexual reproduction starts with the germination of a conidiospore that becomes a multinucleate aerial hypha forming the stalk (*i.e.* the first stage represented in Fig. 1.6). The next step involves the swelling of the hyphal tip creating the vesicle, on the top of which sequential layer of cells (metulae and phialides) will be formed [2, 61] (Fig. 1.6).

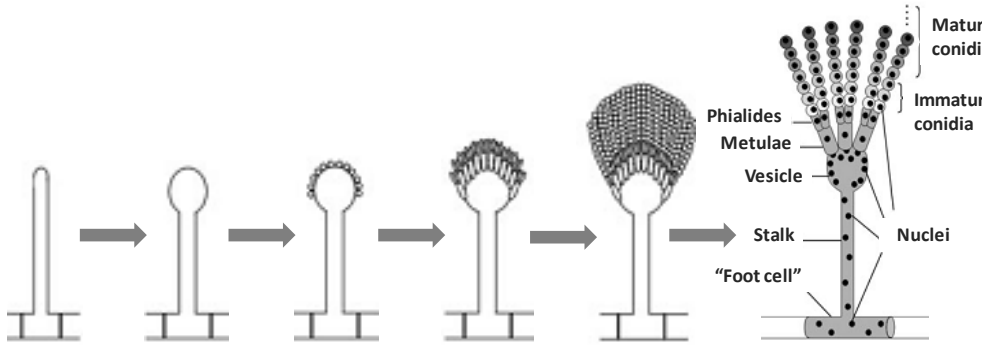


Fig. 1.6. Schematic representation of conidiophore development in *Aspergillus nidulans*. Adapted from [79].

At this stage, the nuclei in the phialides divide mitotically and originate new asexual spores (mature conidia) that can be dispersed in the environment, germinate in different substrates, grow as vegetative hyphae and promote the continuity of the life cycle [80]. The following phase might be the sexual reproduction. In this case, hyphae fuse to produce dikaryotic hyphae, which further develop into the cleistothecia (*i.e.* the fruiting body). Usually, Hülle cells (*i.e.* thick-walled cells) surround the developing cleistothecia in order to 'nurse' this structure during its development providing nutrients and protection [61, 65]. The dikaryotic fertile hyphae within the cleistothecium, undergo differentiation and allow the formation of developing asci, containing ascospores [80]. The obtained ascospores are released upon physical rupture of cleistothecia, which enclose several asci [2, 43]. Although the production of sexual spores involves high metabolic costs, their genetic variation allows the production of genotypes with faster adaptation and higher survival rate of species to adverse environmental conditions than asexual spores [43, 65]. Several factors regulate the balance between sexual and asexual development, including nutrient availability (*e.g.* carbon and nitrogen), light, pH of the growth medium,

atmospheric composition (*e.g.* levels of CO_2 or O_2), humidity and temperature [65] as well as major gene regulators, such as VeA [81] and LaeA [82], constituents of the Velvet complex that link development and secondary metabolism in *Aspergillus nidulans* [83].

General aspects regarding the coordination of fungal development and secondary metabolism (in *Aspergillus nidulans*) will be approached latter during this introductory part (see section 1.2).

1.1.2. *Neurospora crassa*

Neurospora crassa is an important model organism in genetics, biochemistry and molecular biology [84] with a long history in the scientific literature [30, 85, 86]. *Neurospora* species were initially reported in 1843 in Paris [29, 53] when bakeries and bakery products were found massively covered with an orange mould, the so called "*champignons rouges du pain*" [85]. In 1927, Dodge assigned fungal isolates to the new genus *Neurospora* on the basis of their ascospores [87]. Some years before, he was the first to discover that heat could activate ascospores (in 1912) and to describe mating types in *Ascomycota* (1920) [30, 85]. Latter (in 1941), a central *Neurospora* paper [88] established not only the relationship between genes and proteins, known as the 'one-gene-one-enzyme' hypothesis, but also recognised biochemical genetics as an experimental science [29, 84]. Since then, the filamentous fungus *N. crassa* has been intensively used in many laboratories [30, 85] and has become an important model organism for higher eukaryotes in modern genetics, molecular biology and eukaryotic biology [8, 29]. The central role of this species led it to be the first filamentous fungus with its genome fully sequenced[*] [29].

Neurospora species (*e.g. N. crassa, N. sitophila, N. intermedia, N. tetrasperma*, and *N. discrete*) are detectable in nature because of their distinctive orange colour, rapid growth and abundant production of conidia. These organisms

[*] The genome sequence of *Neurospora crassa* is available in GenBank (http://www.ncbi.nlm.nih.gov/genome/19).

are typically found in humid (sub)tropical climates but also occur on burned vegetation (as well as in bakeries), due to the activation of dormant ascospores by heat [85, 89]. Although the natural substrate of *Neurospora* is burned vegetation, it has never been observed to invade living plant tissue or to cause disease in a plant or to produce dangerous secondary metabolites (mycotoxins) [89].

Similarly to other filamentous fungi, *N. crassa* can undergo vegetative growth, asexual and sexual reproduction cycles. The mycelial colonies are constituted by filaments obtained through hyphal tip growth (Fig. 1.2), branching and fusion. These filaments (*i.e.* hypha) are multinucleate and present septa pores, which allow the movement of organelles between the compartments (Fig. 1.2) [90].

The production of asexual spores is usually triggered by circadian rhythms (*i.e.* internal clock mechanisms) [80, 90, 91]. In such conditions, the vegetative hyphae can differentiate into microconidia (*i.e.* uninucleate asexual spores with low viability and scarce, that can also emerge from microconidiophores) or conidiophores, which in turn originate chains of macroconidia (*i.e.* multinucleate asexual spores) to be dispersed (Fig. 1.7 and 1.8) [8].

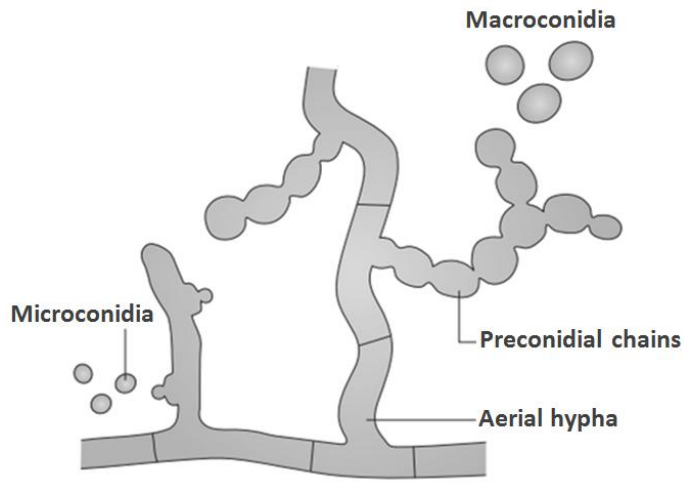


Fig. 1.7. Schematic representation of *Neurospora crassa* asexual conidiophore and spores. Adapted from [92].

The germination of conidia in filamentous fungi is highly regulated by environmental stimuli; nevertheless the morphological evidence of conidial germination is the formation of the germ tube, which requires polarity establishment [90].

In *N. crassa*, the sexual cycle is induced by nitrogen starvation or changes in temperature or light. As an example, the generation of multicellular female sexual organs (protoperithecia) results from the limitation of nitrogen [8]. In this heterothallic fungus, sexual reproduction only occurs in the presence of compatible partners. In such case, the trichogyne (*i.e.* a polarised structure from the protoperithecium) of one mating-type fuses with the male conidium of the opposite mating-type (Fig. 1.8). The conidium moves through the trichogyne into the ascogonial cell within the protoperithecium, which later becomes the perithecium (ascocarp) [80, 93].

The two haploid nuclei undergo repeated divisions within a developing dikaryotic ascogenous hyphal structure. After the growth of ascogenous hyphae and enlargement of the perithecial wall, croziers (where nuclear fusion occurs) are formed and further differentiated into developing asci [93] (Fig. 1.8). At this stage, the perithecium contains ascus with eight ordered sexual spores (ascospores) each that will be shot from the ostiole of the perithecium and dispersed until favourable conditions are set for the ascospore to germinate and restart the sexual cycle [8, 80].

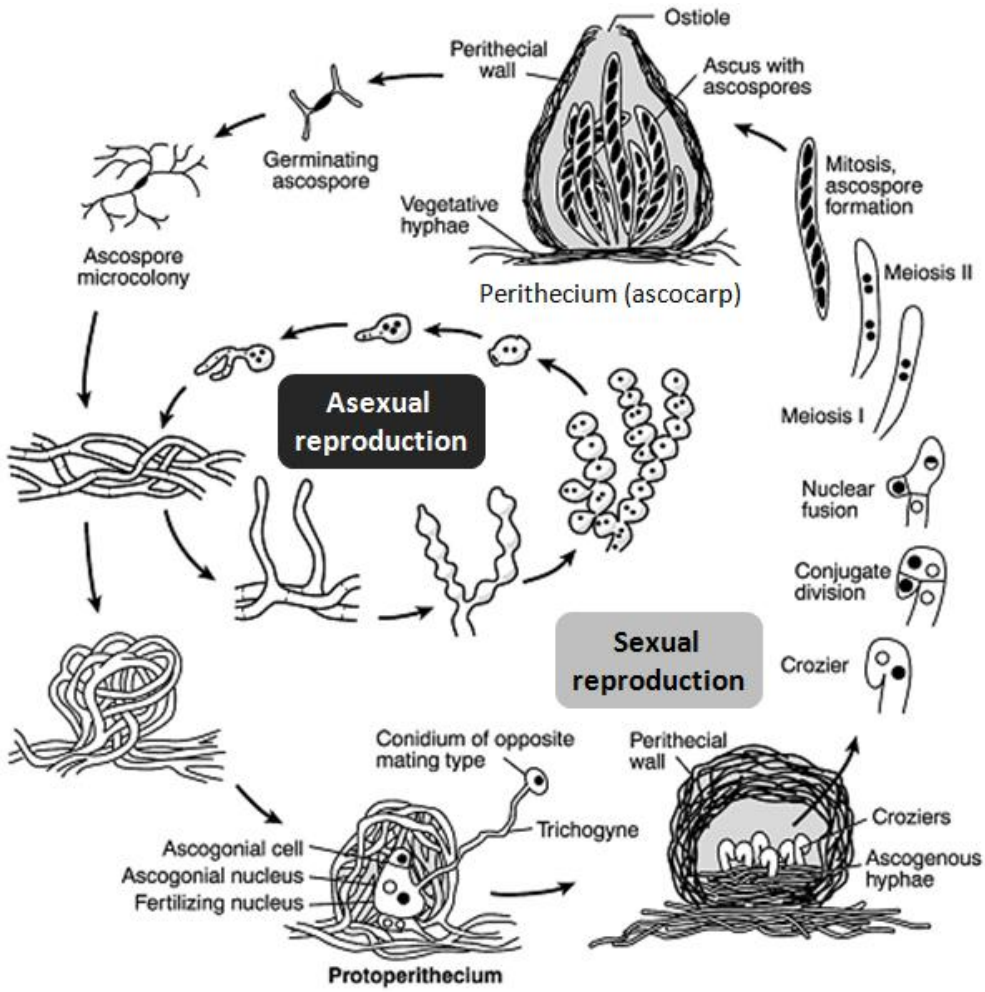


Fig. 1.8. *Neurospora crassa* life cycle. Adapted from [93]. *N.b*. the asexual reproduction includes the formation of macroconidia but not of microconidia.

Considering the description of these two model fungus, it can be noticed that *Aspergillus nidulans* and *Neurospora crassa* present interesting dissimilarities, which push forward the choice of these model species for my doctoral studies. For instance, in comparison with *A. nidulans*, *N. crassa* is less halotolerant and is a rather limited producer of secondary metabolites (although its genetic potential is not fully disclosed). Additional aspects regarding secondary metabolites biosynthesis will be presented afterwards (see section 1.2).

1.2. Fungal secondary metabolites

The following section will focus on secondary metabolites from fungal origin, mainly from Ascomycota. Different families of secondary metabolites will be mentioned and briefly characterised, as well as important aspects related to the regulation of their biosynthesis.

Secondary metabolites (SMs) cover several classes of low-molecular weight compounds that are thought to provide survival functions and competitive advantages to the producing organism in its natural environment, such as differentiation effectors, self-protection and defence against predators, inhibition of competing microorganisms, communication and establishment of interactions with their biotic environment [94-97].

The first fungal SM to be reported was a crystalline compound (later identified as mycophenolic acid [98, 99]) purified from *Penicillium brevicompactum* culture filtrate by Bartolomeo Gosio in 1893, which presented antibiotic activity against anthrax bacteria [100]. During the 1920s and 1930s, more than hundreds of new substances were isolated from fungi, emphasising their capacity for producing a vast diversity of SMs [101]. However, only with the discovery of penicillin, isolated from *Penicillium rubens* by Sir Alexander Fleming in 1928 [50], and characterised by Howard Florey* in 1940 [102], the intensive search of new bioactive microbial metabolites with medical and industrial potential began.

The majority of the known bioactive microbial metabolites have fungal origin (~ 45%) [103]. Filamentous fungi, especially those belonging to *Ascomycota* phylum, can produce numerous and diverse fungal SMs displaying relevant biological activities (*e.g.* the antitumor terrequinone A [18]) and biotechnological potential (*e.g.* the industrially produced citric acid [75]). Major constraints hampering discovery of novel fungal bioactive SMs are the low number of

* Howard Walter Florey (Baron Florey) was an Australian pharmacologist and pathologist who shared the Nobel Prize in Physiology or Medicine in 1945 with Sir Ernst Boris Chain and Sir Alexander Fleming for his role in the development of penicillin. Fleming discovered penicillin and Florey carried out the first ever clinical trials in 1941 of penicillin at the Radcliffe Infirmary in Oxford on the first patient (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/florey-facts.html).

cultivable fungal species (*ca.* 5%) [104] and the fact that SMs are usually cryptic in laboratory conditions [105] (*i.e.* their biosynthetic genes remain silent in artificial conditions and prevent the production of the respective metabolites). These constraints have driven the search of optimal culture conditions, which enable biosynthesis of SMs in high titres, as well as characterisation of genes encoding for either biosynthetic or regulatory enzymes [106]. This scientific endeavour is still extremely active. Despite the discovery of several thousands of substances and the vast knowledge on their biosynthesis, a multitude of metabolites awaits discovery [97].

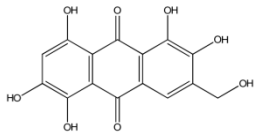
1.2.1. Families of fungal secondary metabolites

Fungal secondary metabolites comprise distinct classes of compounds, namely polyketides (PKs) [107], non-ribosomal peptides (NRPs) [108], indole alkaloids [109] and terpenes [110], as well as hybrid products composed of moieties from different classes (*e.g.* NRP-PKs [111] and meroterpenoids, which result from the fusion between PKs and terpenes [112]).

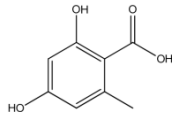
Polyketides

The family of polyketides contain highly diverse natural compounds, usually biosynthesised by polyketide synthases using acyl-CoA building blocks (*e.g.* acetyl-CoA and malonyl-CoA) [113]. For the producing organism, these metabolites may play defensive functions against competing organisms (*e.g.* citrinin [114]) or self-protection from UV radiation (*e.g.* the dark green pigment of *A. nidulans* spores deriving from naphthopyrone YWA1 [115, 116]). Numerous polyketides display remarkable biological activities, for example as antibiotics (*e.g.* griseofulvin [117]), immunosuppressants (*e.g.* mycophenolic acid [99]), cholesterol-lowering (*e.g.* lovastatin [19]) and antitumoral agents (*e.g.* emodin [118]). Although various polyketide compounds are usually cryptic under standard cultivation conditions, several were already successfully identified and the corresponding biosynthetic gene

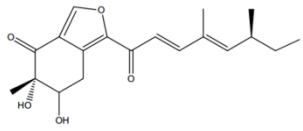
cluster characterised, *e.g.* monodictyphenone [119], asperthecin [120], asperfuranone [121] and orsellinic acid [122] (Fig. 1.9).



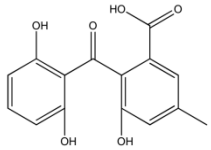
Asperthecin [120]



Orsellinic acid [122]



Asperfuranone [121]



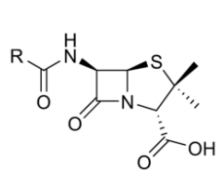
Monodictyphenone [119]

Fig. 1.9. Examples of polyketide metabolites associated to well-characterised biosynthetic gene clusters.

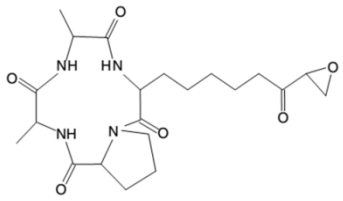
Non-ribosomal peptides

The non-ribosomal peptides are biosynthesised by non-ribosomal peptide synthetases (NRPSs) and constitute one of the major families of structurally diverse and bioactive SMs. Some of these compounds are important for the producing organism, such as the siderophores (*e.g.* ferricrocin [123]); yet the vast majority has been screened for its biotechnological potential, including antibiotics (*e.g.* penicillin [124], echinocandin B [125]), immunosuppressants (*e.g.* cyclosporin A [126]) and toxins (*e.g.* HC-toxin [127]) (Fig. 1.10).

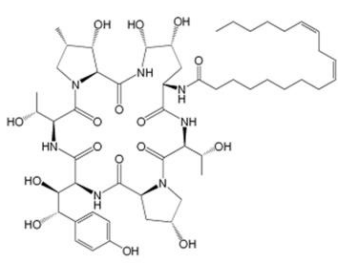
The family of non-ribosomal peptides also encloses peptaibiotics, which are fungal peptides presenting broad antibiotic activity [128, 129], usually due to their ability to form pores in bilayer lipid membranes (*e.g.* lipopeptaibol antibiotics [130]). Previous reports demonstrated that some of these compounds are able to uncouple oxidative phosphorylation, induce the formation fungal pigments, inhibit platelet aggregation and the mitochondrial ATPase, present immunosuppressive and neuroleptic effects [131-133].



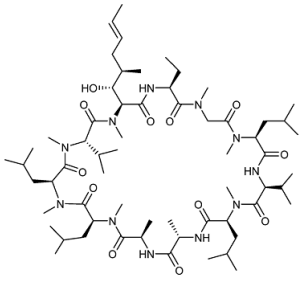
Penicillin [124]



HC-toxin [127]



Echinocandin B [125]

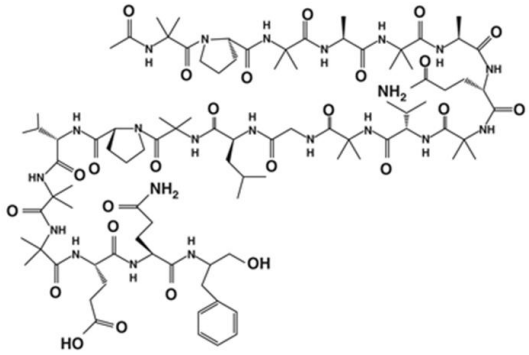


Cyclosporin A [126]

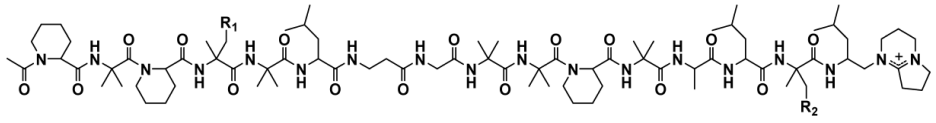
Fig. 1.10. Examples of non-ribosomal peptide metabolites associated to well-characterised biosynthetic gene clusters.

The molecular weight of peptaibiotics usually ranges from 0.5 to 2.2 kDa, comprising five to twenty one amino acid residues, including several moieties of the characteristic α-aminoisobutyric acid (Aib), and generally presenting their *N*-terminus acylated (*i.e.* blocked) [133]. According to additional characteristics, this class of compounds can be divided into three different groups (Fig. 1.11): peptaibols (which contain an alcohol group in the *C*–terminus of the peptide sequence), efrapeptins (containing pipecolic acid and a cationic bicycle amine group at the *C*–terminus) and neoefrapeptins (which also contain the rare amino acids 1-aminocyclopropane-1-carboxylic acid and some also 3-methylproline). In general, peptaibols (*e.g.* alamethicin [134]) have been reported to be synthesised by *Trichoderma* spp [135], efrapeptins by *Tolypocladium* spp [136], and neoefrapeptins by *Geotrichum candidum* [137]. Moreover, peptaibols are suggested to be potential suppressors of tumour cells [138], whereas efrapeptins and neoefrapeptins are inhibitors of mitochondrial ATPase with promising antitumor, antimalarial, and insecticidal activity [137, 139]. As a curiosity, the sum of all peptaibiotics being

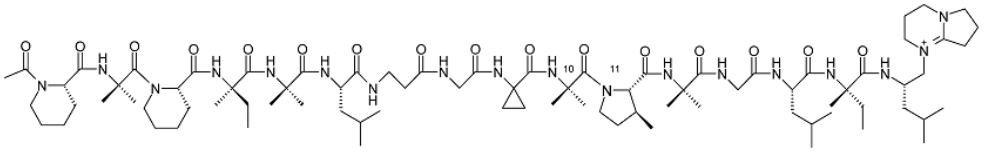
produced by a fungus is described as the peptaibiome; accordingly, peptaibiomics is the analytical methodology for the characterisation of all these bioactive peptides [129].



Alamethecin (peptaibol) [134]



Efrapeptins [140]



Neoefrapeptin F [137]

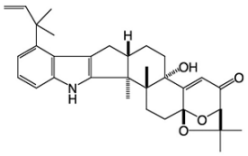
Fig. 1.11. Examples of known peptaibiotics.

Terpenes

Terpenes constitute a large class of natural products, which are more commonly associated with plants; however, fungi are also known to produce indole-diterpenes [141], aristolochenes [142], carotenoids [22], gibberellins [143] and trichothecenes [144] (Fig. 1.12). Isoprene units (derived from the mevalonate pathway) constitute the building blocks used by the terpene cyclases in the biosynthesis of terpenes. The class of terpene cyclases includes sesquiterpene and diterpene cyclases, which mediate production of complex cyclic terpenes, as well as prenyltransferases that synthesise indole-diterpenes, and phytoene synthases that are involved in the

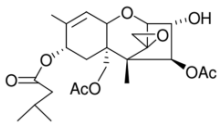
formation of carotenoids [97, 145]. Carotenoids are the SMs more frequently associated to the *Ascomycota Neurospora crassa* [146], *e.g.* neurosporaxanthin which was first discovered and characterised in this fungus. The biosynthetic genes leading to neurosporaxanthin formation are located in a small cluster [146] and encode four enzymes: AL-2 (a bifunctional protein with both phytoene synthase and cyclase activities), AL-1 (desaturase, *i.e.* creates a double bond (C=C) by removing two hydrogen atoms), CAO-2 (carotenoid oxygenase) and YLO-1 (aldehyde dehydrogenase) [22, 147]. Carotenoids are responsible for the pigmentation of the producing organism (*e.g. N. crassa*) and in some animals their carotenoid rich diets leads to pigmentation, such as birds (*e.g.* flamingo), fishes (*e.g.* salmon) or crustaceans [22]. Carotenoids consumption is thought to produce beneficial effects on human health, including protection against oxidative stress, cancer, sight degeneration syndromes and cardiovascular diseases [146, 148].

Indole-diterpene



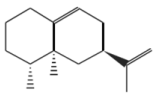
Aflatrem [141]

Trichothecene



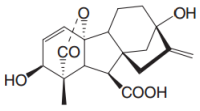
T-2 toxin [144]

Aristolochene



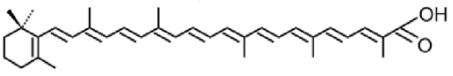
Aristolochene [142]

Gibberellin



GA_3 [143]

Carotenoid



Neurosporaxantin [22]

Fig. 1.12. Examples of well-known terpene metabolites.

Indole alkaloids

Indole alkaloids comprise one of the largest classes of nitrogen-containing SMs displaying potent biological activities and are usually found in plants, bacteria, animals and fungi, especially *Ascomycota* [109]. They are biosynthesised as complex mixtures of derivates of the aromatic amino acid tryptophan (the most abundant indole-containing compound derived from the shikimic acid pathway) and of dimethlyallyl pyrophosphate (from the mevalonate pathway) [97]. Indole alkaloids contain an indole moiety linked to several other different families of compounds, for example, peptide indole alkaloids synthesised by NRPS (*e.g.* roquefortine C [149]), hybrid indole alkaloids synthesised by PKS-NRPS (*e.g.* chaetoglobosin A [109]), indole diterpenes (see above) and other types that use different building blocks, such as dihydroxybenzoquinone core and prenylated indole-pyruvate (*e.g.* terrequinone A [150]) (Fig. 1.13).

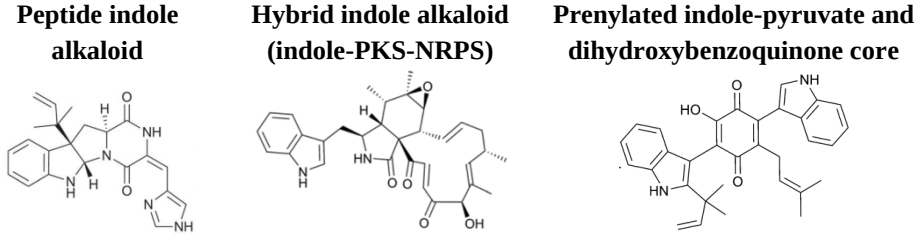


Fig. 1.13. Examples of well-known indole alkaloids.

Hybrid metabolites

The combination of moieties from different classes, as well as the involvement of different types of synthases (*e.g.* activity of PKS and NRPS or only a hybrid enzyme such as a fusion PKS-NRPS) in the biosynthesis of SMs, highly increases the molecular diversity and complexity of these compounds [116]. Among these we can find combination of PKs-NRPs, NRPs-terpenes or meroterpenoids (*i.e.* fusions between PKs and terpenes). As examples of these hybrid metabolites there are some cryptic SMs that have been comprehensively characterised, such as aspyridone A and B [151], nidulanin A [152] and austinol [95] (Fig. 1.14).

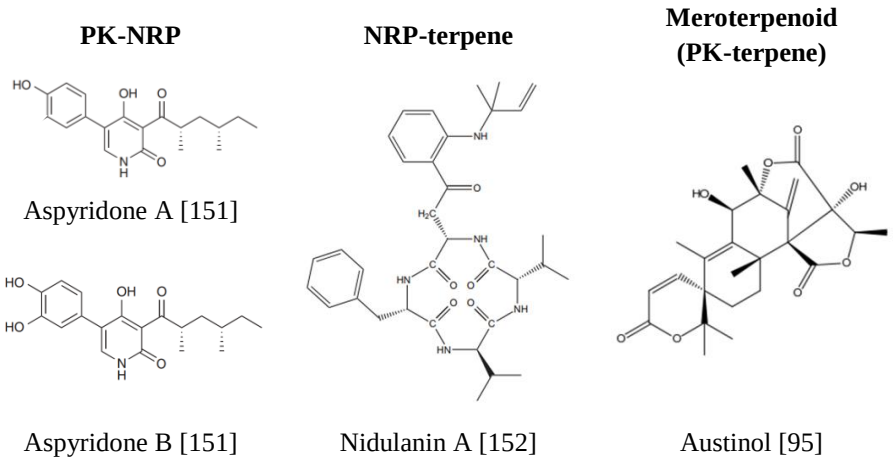


Fig. 1.14. Examples of hybrid metabolites associated to well-characterised biosynthetic gene clusters.

1.2.2. Biosynthesis of fungal secondary metabolites

The production of the majority of secondary metabolites is induced under very specific conditions which are usually difficult to reproduce in standard laboratory conditions [108].

In order to trigger the biosynthesis of SMs several strategies have been explored [153-155]. Some of these require previous knowledge of the genome sequences in order to manipulate genes encoding pathway-specific activators [151] or global regulators [156], alter the chromatin structure [157-159] and the post-translational modification of proteins [120, 160], or screen (*in silico*) specific SM biosynthetic genes by genome mining [161-163] or to heterologous express orphan biosynthetic genes [154, 164]. Other approaches can also be applied in less well-characterised strains, such as co-cultivation methods [165, 166] or the "one strain-many compounds" approach [167], which consists of growing microorganisms in different cultivation conditions by changing *e.g.* composition of the growth media [95], pH [168], temperature [169], aeration rate [170], oxygen availability [171] or by adding exogenous chemicals (*e.g.* sodium citrate [172], suberoylanilide hydroxamic acid [173] or ionic liquids [174]), just to mention a few examples.

Genes involved in biosynthetic pathways of secondary metabolism are, in general, clustered (*i.e.* adjacent to each other) and co-regulated [175, 176] (Fig. 1.15). However, cross-talk between gene clusters located in different chromosomes may be required for the production of metabolites, *e.g.* prenyl xanthones [177] and austinol [178]. The biosynthetic gene clusters comprise multi-domain enzymes, such as non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), hybrid NRPS-PKS enzymes, prenyltransferases or terpene cyclases, and additional tailoring enzymes that modify the SM core structure [179, 180]. The multi-domain enzymes are also designated as backbone enzymes (encoded by backbone genes) because they are responsible for the first step in the biosynthesis of a SM [180].

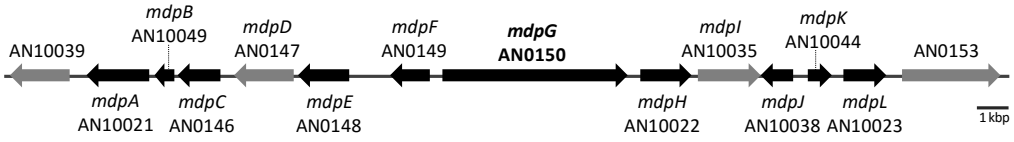


Fig. 1.15. Representation of the biosynthetic gene cluster of monodictyphenone in *Aspergillus nidulans*. The backbone gene is marked in bold (*mdpG*). Essential and non-essential genes are shown in black and grey (arrows), respectively. Adapted from [119].

Accordingly, the genetic potential for the biosynthesis of SMs can be predicted by the presence of various SM backbone genes in fungal genomes. In fact, the genome sequencing projects have revealed many biosynthetic gene clusters for the production of yet unknown SMs [146, 179]. For example, *Neurospora crassa* presents limited capacity for production of SMs with only ten predicted biosynthetic gene clusters [179], while other fungal species enclose a much higher number of predicted biosynthetic gene clusters (number depicted in brackets), such as *Aspergillus nidulans* (71), *A. fumigatus* (39), *A. niger* (81), *A. oryzae* (75) and *Fusarium graminearum* (67) [181, 182].

As above mentioned, several SMs produced by *Aspergillus nidulans* have already been linked to the corresponding biosynthetic gene cluster, including sterigmatocystin [183], penicillin [184], terrequinone A [18, 161], aspyridone A/B

[151], emericellamides [185], asperthecin [120], asperfuranone [121], orsellinic acid and F9775 A/B [122], monodictyphenone and prenyl xanthones [119, 177], microperfuranone [186], cichorine [187], austinol and dehydroaustinol [178], aspernidine A [188], nidulanin A [152] and aspercryptin [189] (*n.b. the respective intermediates are not being mentioned here*). On the contrary, only carotenoids and melanin have been, so far, characterised in *Neurospora crassa* [29]. The biosynthetic SM gene clusters of this fungus have not been systematically studied, notwithstanding one small cluster has been associated to the production of neurosporaxanthin [22, 146, 147] (see above) and another one (by homology with *A. nidulans* genes) to the siderophore ferricrocin [123]. The former metabolite is involved in fungal pigmentation, while the latter is a chelator molecule necessary for iron homeostasis; essential for efficient conidiation in *N. crassa* [123, 190].

The increasing number of fungal genomes being sequenced reveals that the capacity of fungi for producing SMs has been largely underestimated, as demonstrated by the quantity of predicted orphan SM biosynthetic gene clusters awaiting discovery [153]. Several bioinformatics tools[*] [191] (*e.g.* SMURF [179], antiSMASH [192] and FunGeneClusterS [193]) have been created to predict the biosynthetic SM gene clusters and the core chemical structure of the produced SM. The latter presents limited potential because the activity of additional tailoring enzymes in the SM gene clusters can dramatically modify the core structure of SMs [95, 180]. Moreover, the possibility of cross-talk between genes and clusters located in different chromosomes increases the unpredictability of the formed SMs [177, 178].

Recently, the research community proposed the Minimum Information concerning Biosynthetic Gene cluster (MIBiG)[**] data standard [194]. This project aims to provide a comprehensive and standardised specification of biosynthetic gene clusters annotations and associated metadata, allowing their systematic deposition in databases. This way, the information about clusters, pathways and metabolites,

[*] SMURF: http://jcvi.org/smurf/index.php; antiSMASH: http://antismash.secondarymetabolites.org; FunGeneClusterS: https://fungiminions.shinyapps.io/FunGeneClusterS.
[**] MIBiG: http://mibig.secondarymetabolites.org.

which is currently dispersed in the literature, would be concentrated and systematised in a single location and, consequently, promoting further discovery of novel natural products [192, 194].

1.2.3. Regulation of secondary metabolism in fungi

The regulation of secondary metabolism in fungi is extremely complex and involves several interconnecting networks, from cluster specific to global regulators.

The significance of fungal SMs in several areas (*e.g.* health, industry, biotechnology) has been promoting the study of regulatory networks behind their biosynthesis [155, 195, 196]. As emphasised before, the majority of the SM biosynthetic clusters is silent under standard laboratory conditions, in other words, their production requires specific induction [105, 154]. The activation of the biosynthetic SM gene clusters is dependent on global [156] or pathway specific regulators [146], broad range of environmental factors [146, 195] and also on the developmental stage of the producing organisms [83, 146].

The following subsections will focus regulatory mechanisms associated to the synthesis of SMs in fungi, mainly using *Aspergillus nidulans* as the model species used for these studies.

Fungal development and the biosynthesis of secondary metabolites

Fungal development and secondary metabolism are tightly connected, as it is beneficial for the fungus to produce certain SMs in specific stages of development [83, 196-198]. Generally, the production of SMs starts in late stationary growth phase and is usually associated with sporulation processes, where the biosynthesised SMs can activate sporulation, add pigments to the sporulation structures or even protect the colonies in the sporulation phase [199].

Among other processes, sporulation, SMs production, fungal development, stress response and pathogenicity are regulated by G-protein signalling pathways [200, 201]. In *A. nidulans*, the FadA G-protein signalling pathway is constituted by the G-protein, FadA, and the protein kinase, PkaA [200]. Several reports

demonstrate that mutations in genes involved in this pathway affect growth, conidiation, sexual development and SM production [202]; FadA/PkaA regulate SM synthesis and conidiation in *Aspergillus* spp. [203]; in *A. nidulans* FadA regulates negatively and positively the synthesis of sterigmatocystin [204] and penicillin, respectively [205], while promoting vegetative growth [196]; overexpression of *pkaA* represses asexual development and the production of sterigmatocystin [206]; and the deletion of the α-subunit of a heterotrimeric G-protein in *Cryphonectria parasitica* decreased its growth rate, sporulation, pigmentation and virulence [207].

Oxylipins (*i.e.* hormone-like signalling molecules encoded by *ppo* genes) are involved in the sexual and asexual spore development balance, as well as in natural product synthesis in *A. nidulans* [83, 208]. Mutations in *ppo* genes, which expression is regulated by VeA, have been previously reported to alter the asexual to sexual spore ratio and also the production of SMs in *A. nidulans* [208].

The developmental regulator VeA, which is known to interact with LaeA [209] also establishes a direct link between asexual and sexual sporulation and secondary metabolism [146, 209] (see details below).

Global transcriptional regulators

The nuclear protein LaeA (loss of *aflR* expression A) is considered the major global regulator of secondary metabolism in *Aspergillus* spp. [156] and has been reported to control clusters positioned in distinct regions of the chromosomes, such as the heterochromatin regions of the telomeres [210]. These regions are transcriptionally silent; therefore LaeA (a methyltransferase-domain nuclear protein) is thought to activate transcription in these locations through epigenetic control [210]. Several reports demonstrate the impact of LaeA in the expression levels of SM biosynthetic gene clusters. Accordingly, it has been shown to be required for the synthesis of sterigmatocystin and penicillin in *A. nidulans* [156], of gliotoxin and other SMs in *A. fumigatus* [145, 211] and also of gibberellins and bikaverin in *Fusarium fujikuroi* [212].

LaeA participates in the Velvet complex, a multi-protein (complex) constituted of LaeA, VeA and VelB that regulates development and secondary metabolism in response to light [209]. Additionally, this global regulator of secondary metabolism is important to control the formation of the VelB complexes (VosA-VelB and VeA-VelB), which are required for the transition from filamentous cells to Hülle cells, and for the control of fungal development by light (in the presence of VeA) [82, 83]. In *Ascomycota* species, VeA (velvet A) is associated to both development (*i.e* positive and negative regulator of sexual and asexual development, respectively [81, 202, 209]) and production of SMs (*e.g.* sterigmatocystin and penicillin [197], carotenoids [209] and aflatoxin [198, 213]). The other component of the Velvet complex, VelB (velvet-like B), is a light-dependent regulator of fungal development and secondary metabolism that interacts with VeA [209]. In the presence of light, the levels of VeA are low, asexual development is induced by VelB and sterigmatocystin biosynthetic genes remain silent (Fig. 1.16). In the dark, the cytoplasmatic levels of VeA increase, leading to its interaction with VelB; the resulting heterodimer is transported to the nucleus via KapA (importin α) where it is required for sexual development [209, 214]. Then, the heterotrimeric complex with LaeA is built to induce both sexual development and the production of SMs (*e.g.* sterigmatocystin) [209, 215] (Fig. 1.16). In addition, VeA can also build a nuclear complex with light-responsive proteins (*e.g.* FphA, red phytochrome-like protein, and LreA-LreB, blue sensing proteins) that also modulate SM production and fungal development [216, 217]. VelB can additionally recruit VosA (viability of spores A), which plays an essential role in long-term survival of both asexual and sexual spores via coupling sporogenesis and trehalose biogenesis [218], and together this heterodimer regulates asexual development, spore maturation and germination in *A. nidulans* [82, 215] (Fig. 1.16).

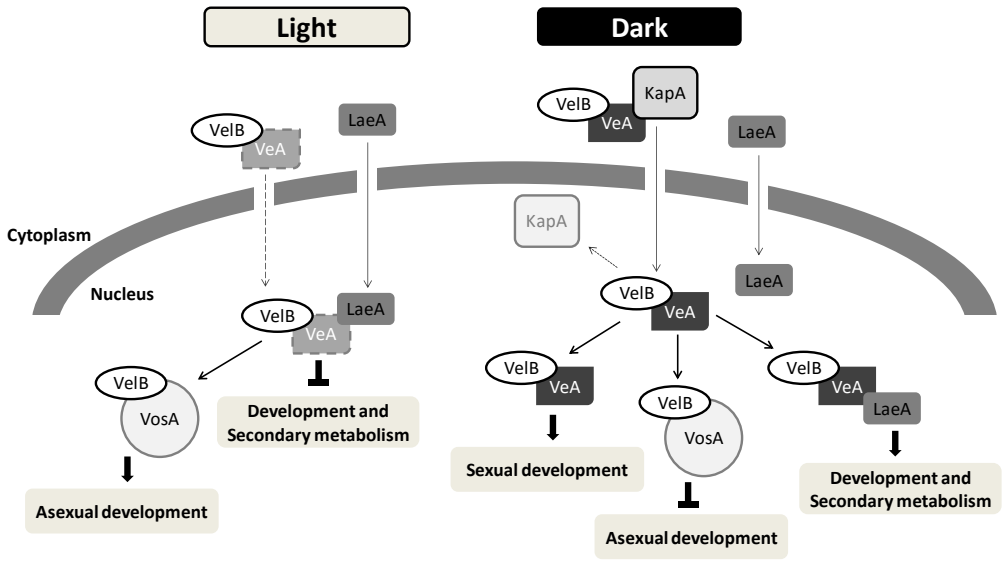


Fig. 1.16. The influence of light in the formation of the Velvet complexes in *Aspergillus nidulans*. Light decreases the cellular levels of VeA, VosA and VelB, which prevents the formation of the Velvet complex with LaeA; therefore repressing the coordination of development and secondary metabolism. In these conditions, asexual sporulation and trehalose biogenesis occur. In the dark, the α-importin KapA transports VeA-VelB dimer to the nucleus, where it is required for sexual development and can interact with LaeA, forming the heterotrimeric Velvet complex that coordinates the regulation of secondary metabolism and development. VelB can also form the heterodimer VosA-VelB, which represses asexual development and is required for the viability of spores by activating trehalose biogenesis. Adapted from [82, 209, 219].

Recently, MtfA has been identified has a new master transcription factor, in *Ascomycota*. In *A. nidulans*, it has been reported to impact the synthesis of *e.g.* terrequinone A, penicillin and sterigmatocystin, and to regulate asexual and sexual morphological development [217]. In *A. fumigatus* it was shown to impact fungal development, gliotoxin production and virulence [220]. MtfA remediates the biosynthesis of sterigmatocystin intermediates in the absence of VeA [217]. Similar function has been attributed to RsmA (remediation of secondary metabolism A), a basic leucine zipper-type (bZIP) transcription factor able to restore SM biosynthesis in *A. nidulans laeA* deletant mutant [221].

Transcriptional regulation – Broad domain factors

The response to environmental conditions is controlled by Cys_2His_2 zinc-finger proteins, like CreA, AreA and PacC for the signalling of carbon, nitrogen and pH, respectively [196], which also influence the production of SM [146, 195]. The use of glucose as carbon source induces the carbon catabolite repression mediated by CreA, *i.e.* the genes required for metabolising other carbon sources are repressed [222]. For example, the production of penicillin is negatively regulated by CreA when *A. nidulans* is grown in glucose based medium [223, 224]. The highly conserved global transcription factor AreA is responsible for repression of nitrogen metabolism in the presence of glutamine or ammonium. In the maize pathogen *Fusarium verticillioides* it acts as a positive regulator for the production of the toxic fumonisin B1 [225]. Alkaline environments are favourable for the positive regulation of PacC, which controls the biosynthesis of metabolites with increased stability and toxicity in a pH environment presenting higher microbial competition [106]. In *A. nidulans*, increased penicillin production was obtained in alkaline environments, under the positive regulation of PacC [200, 226], probably as a defence mechanism. On the contrary, the production of sterigmatocystin is repressed by alkaline pH, thus negatively regulated by PacC [224, 227].

Transcriptional regulation – Pathway specific regulators

Pathway specific regulators can be found within or outside the biosynthetic gene clusters. The genetic manipulation of pathway specific transcription factors inside the clusters allows the concerted induction of the respective silent cluster and the biosynthesis of the respective hidden metabolite [196]. For example, the $Zn(II)_2Cys_6$ zinc binuclear proteins as AflR in *A. nidulans* sterigmatocystin cluster [183] and in *A. flavus* aflatoxin cluster [228]; AfoA [121], ApdR [151] and MdpE [119] required for asperfuranone, aspyridone and monodictyphenone gene clusters expression, respectively, in *A. nidulans*; and the Cys_2His_2 zinc-finger protein Tri6 in *Fusarium sporotrichiodes* biosynthetic trichothecene cluster [229] are pathway specific regulators found within the clusters. In *A. nidulans*, among the known

pathway specific regulators located outside the biosynthetic gene clusters, there is the Cys_2His_2 transcription factor ScpR that was shown to activate the biosynthesis of asperfuranone [230] and the transcriptional complex AnCF, formerly known as PENR1, composed of Hap-like proteins, which is required for penicillin biosynthesis [124, 231].

Chromatin-based regulation/Epigenetic regulation

The eukaryotic genetic material located in the nucleus is packed in the chromatin, which subunits are composed of DNA wraps around histones – the nucleosomes [232, 233]. The condensation state of chromatin may vary from a tightly packed form (*i.e.* heterochromatin) in which the genetic information is inaccessible for transcription, to a less packed form (*i.e.* euchromatin), which allows transcription to occur [232-234]. The remodelling of chromatin landscape can be attained by post-translational modification of histones (H1, H2, H3 and H4) through *e.g.* acetylation, methylation and sumoylation [233-235], also known as epigenetic* processes [236]. Generally, histone acetylation (*e.g.* lysine 4 of H3 and H4 (H3K4, H4K4) and lysine 9 of H3 (H3K9)) tends to be related with euchromatin and gene activation, sumoylation associated with transcriptional repression (*i.e.* heterochromatin) and methylation might be associated to both chromatin states, depending on the methylated residue (*e.g.* trimethylation of H3K4 or H3K9 occurs in euchromatin or heterochromatin states, respectively) [233, 237, 238].

Chemical epigenetics, which relies on the use of small molecules (*e.g.* 5-azacytidine and suberoylanilide hydroxamic acid) to manipulate gene activity without changing the DNA sequence, has been a strategy applied to induce transcription of otherwise silent genes, such as those belonging to SM biosynthetic gene clusters (usually located in heterochromatin state regions of the chromosomes) [157].

In *Aspergillus nidulans*, histone acetyltransferases have been reported to influence the activation of SM biosynthetic gene clusters through acetylation of

* The term "*epigenetic*" literally means "*in addition to changes in genetic sequence*" [237].

H4K12 by EsaA [239] or even the acetylation of H3K9 by GcnE, a histone acetyltransferase component of the SAGA/ADA complex [240]. The disruption of histone deacetylase activity (Δ*hda*) activated sterigmatocystin and penicillin biosynthetic gene clusters, in *A. nidulans* [237]. Similar observations were attained with *A. fumigatus* [241], *Alternaria alternate* and *Penicillium expansum*, suggesting that inhibition of histone deacetylase activity could increase the production of SMs in several fungal genera [157, 237]. Additionally, the use of the histone deacetylase inhibitor, suberoylanilide hydroxamic acid, allowed discovery of a metabolite never reported before in *Aspergillus niger*, nygerone A [173].

Regarding the methylation process, it is more complex to determine its effect on transcription because it depends on the residue, position of the residue being methylated and type of methylation (*e.g.* di-, trimethylation) [232]. For example, trimethylation of lysine 4 of histone 3 (H3K4me3) can be associated to both activating and repressing events [232]. In a previous report, the disruption of *A. nidulans cclA* (*i.e.* gene coding one of the eight members of COMPASS involved in H3K4 methylation [242]) led to the activation of silent clusters and to the consequent biosynthesis of emodin and derivate compounds, monodictyphenone and the cathepsin K inhibitors F9775A and F9775B [158]. There is also a heterochromatin protein-1 (HepA in *A. nidulans* and Hpo *in N. crassa*) which high levels are associated with silent transcription. This transcriptional repressor recognizes methylated H3K9 and binds directly to it, maintaining the heterochromatin structure. The deletion of HepA activates the biosynthesis of sterigmatocystin, penicillin and terrequinone A. For example, during *A. nidulans* active growth phase, the sterigmatocystin biosynthetic gene cluster is marked by trimethylation of H3K9 (by ClrD, the H3K9-specific methylase) and contains high levels of HepA [238, 243].

Sumoylation is a cellular process involved in cell cycle progression, genome integrity, DNA repair, chromatin structure, signal transduction and transcriptional regulation characterised by the post-translational addition of SUMO (*i.e.* small ubiquitin-related modifier family proteins) to many proteins including histones [244, 245]. The histone sumoylation is an epigenetic process associated with

transcriptional repression, therefore it could be involved in the regulation of SM biosynthesis [244, 246, 247]. In fact, the deletion of *sumO* from the genome of *A. nidulans* was shown to significantly impact SM production, which included the detection of the cryptic SM asperthecin [120, 159]. Recently, the connection between SUMO network and the Velvet complex has been identified in *A. nidulans* [248].

Final comments

The activation of SM biosynthetic gene clusters is a complex process that requires the cooperation of several agents. This process can be favoured not only by the attenuation of the heterochromatin (silencing) marks (*e.g.* using Δ*cclA* and Δ*hdaA* strains) but also by the overexpression of genes encoding broad-domain (*e.g. pacC*), global (*e.g. laeA*) and/or pathway specific regulators (*e.g. aflR*) [232]. An activated SM biosynthetic gene cluster can communicate with other clusters located in different chromosomes or chromosome regions leading to the production of an unpredicted multitude of SMs. Despite all the mentioned strategies to manipulate the biosynthesis of cryptic SMs (*i.e.* silent under the cultivation conditions used in laboratory), the capacity of fungi to produce SMs remains largely unseen [106]. The existing gap between the inherent vast genetic potential and the limited observed diversity of fungal SMs produced constitutes therefore a major challenge [105].

1.3. Ionic liquids

The following section aims to present general information on ionic liquids characteristics, including examples of their biological applications, with emphasis on the ionic liquids used in my doctoral studies.

The synthesis and use of substances with reduced potential risk for humans and environment is of most importance, and is the core idea of green (sustainable) chemistry [249]. In this context, ionic liquids have emerged as interesting substitutes of the traditional volatile organic solvents. They constitute a class of organic salts usually labelled as "green solvents" [250, 251] due to their negligible vapour pressure, general non-flammability, high solvability and melting points below 100 ºC [252]. The usual asymmetry and delocalisation of charges of their constituting organic ions prevent their units to pack properly and, contrary to inorganic salts (*e.g.* sodium chloride, solid at room temperature), they are usually liquid below 100 ºC [253, 254]. Molecular simulation studies demonstrated that ionic liquids are nanostructured [255] and present segregated polar and non-polar domains [256]; characteristics that contribute to the different viscosities and solvation capacities to these chemicals.

Ionic liquids have been basically synthesised in two steps. Normally, the first step is the production of the cation, for example by protonation of an amine or phospane (in the case of ammonium or phosphonium cations, respectively) or by quaternisation of the amine with a functional haloalkane [257, 258]. Usually, the second step consists on the exchange of the anion, in case the previous ionic combination is not the designed final chemical [257]. This step might be an acid-base neutralisation reaction, which involves the transfer of a proton from a Brønsted acid to a Brønsted base without by-products [259], or an anion metathesis reaction, such as the production of imidazolium tetrafluoroborate using an imidazolium halide and a tetrafluoroborate salt as starting materials [254]. There are millions of possible ionic liquids' formulations originated by the modification of the cationic or anionic components of these molten salts (Fig. 1.17). Therefore, it is possible to design compounds with different physical and chemical properties (*e.g.* melting

point, viscosity, hydrophobicity, chemical polarity or hydrogen bonding ability) [252, 260].

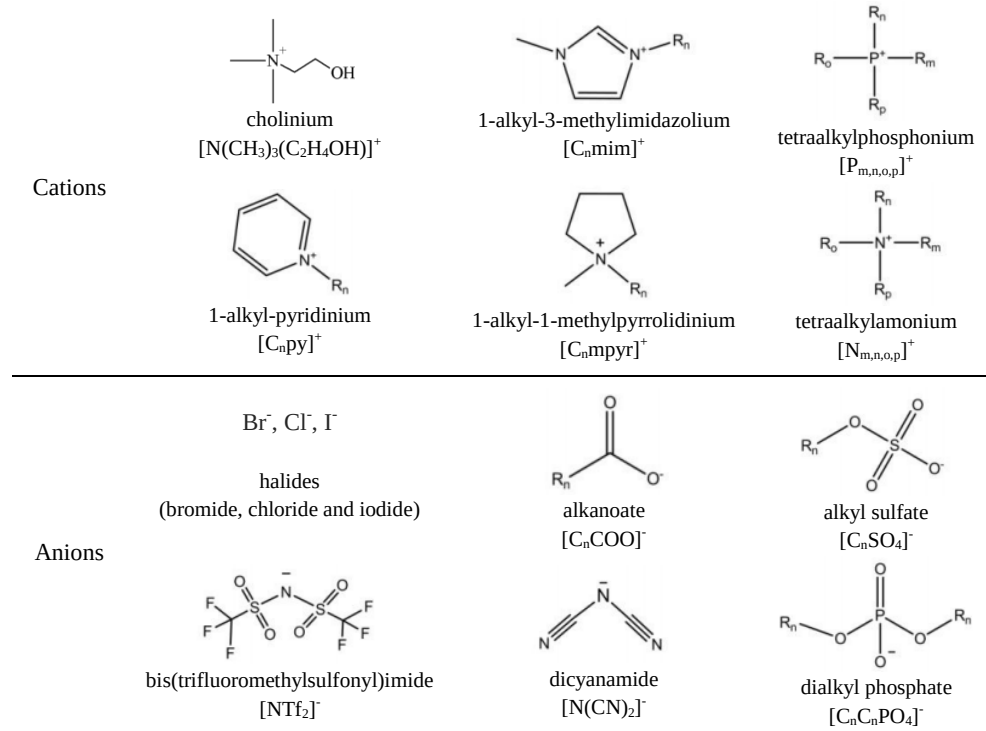


Fig. 1.17. Examples of cations and anions most commonly present in ionic liquids formulations.

The possibility of changing their characteristics according to a specific aim makes them alternative solvents to a diverse set of applications in chemical reactions [261, 262], catalysis [263], electrochemistry [264, 265], material sciences [266], biomass dissolution [267, 268] and also in enzymatic catalysis [269]. Despite all these applications, ionic liquids may also be considerably toxic to several organisms [270]. Their toxicity has been investigated using different parameters such as growth inhibition potential [174, 271], antibiofilm activity [272], induction of apoptosis [273, 274] or autolysis [275] in eukaryotic cells, and enzymatic inhibition [276, 277]. These toxic effects have been attributed to the intrinsic characteristics of the composing ions, for example, aromatic cations (*e.g.* imidazolium-based ionic

liquids) are considered more toxic than non-aromatic ones (*e.g.* quaternary ammonium-based ionic liquids) [278, 279], and long alkyl chain substituents are known to increase lipophilicity, which might explain their higher toxicity, most likely due to interactions with biological membranes [280, 281]. However, the toxic effect of the long alkyl substituent depends on its location. For example, the elongation of a substituent chain length in the cation (*e.g.* alkyltributylphosphonium [271] or 1-alkyl-3-methylimidazolium [281]) can provoke permeabilisation of the plasma membrane, leading to cell death, whereas the increased lipophilicity of long chain anions (*e.g.* alkanoates [281]) is unable to display the same permeabilising effect due to the repulsion of the negatives charges from both the anion and the plasma membrane [281].

One of the initial studies on ionic liquids' toxicity was performed by Pernak and co-authors [282] and inspired the evaluation of the new chemicals being synthesised, such as the imidazolium-based compounds [283-285]. The family of imidazolium-based ionic liquids constitute one of the most studied ones, not only due to their chemical stability, low melting points and viscosities, but also because they are commercially available. Despite that chemicals containing an imidazolium cation are usually toxic and non-degradable [174, 270, 279, 286], this family of ionic liquids have been investigated not only for the dissolution and processing of lignocellulosic materials [268] but also for the treatment of malaria [287] and Chagas disease [288]. Ionic liquids containing the cholinium cation have been also extensively studied. They are usually considered non-toxic, biocompatible and biodegradable [279, 289-291], which emphasise their importance in numerous potential applications, such as the capacity of cholinium alkanoates to play the role of solvent and catalyst in the depolymerisation of suberin [292].

The research field of ionic liquids is far from being completely explored and a multitude of different perspectives remains to be fully understood.

The collected information on their toxicity [279, 291] and more particularly on their mode of action [271, 293] reinforces the need for continuous evaluation of the potential risks of these organic salts in order to consciously develop new ionic liquids following the principles of green chemistry [279]. More and more, these

chemicals are being tested for new biological applications, such as drugs for important etiological agents [287, 288], pharmaceutics development [294], as well as triggers of metabolic alterations and stress responses in living organisms [275, 295], some of which translating into higher metabolic diversity in the footprint of filamentous fungi [174] (different of that induced by common salts, such as sodium chloride [275]). Recently, the idea of these chemicals to be exclusively man-made was challenged by the evidence of the production of an ionic liquid during the confrontation of two ant species, as a defence mechanism [296] and by the formation of viscous liquids (*i.e.* ionic liquids) when mixing plant metabolites produced in high titres [297]. Surely, further research on ionic liquids, especially in the interface of biology, are required to fully uncover their potential and utility.

1.4. Holistic approaches to study fungal secondary metabolism

The following section presents only general information on the "omics" levels relevant for this thesis.

Despite the increasing number of strategies used to activate the biosynthesis of secondary metabolites, as well as the rising number of sequenced fungal genomes, the full capacity of fungi to produce SMs remains largely unseen [106]. The integration of data from different levels, such as transcriptomics, proteomics or metabolomics on fungal systems may help to fill the knowledge gap between the genetically encoded capacity of fungi to produce diverse SMs and the actual disclosed diversity [105]. These methodologies provide efficient measurement of the effects of specific conditions in biological assays, often using robotics, imaging and computation to increase the scale and speed of assays [298]. Consequently, a high quantity of raw data is created, which requires normalisation and statistic analysis for the generation of statistically relevant data from which biological significance may be inferred [299, 300]. Together, these high-throughput approaches are valuable methodologies for revealing a snap-shot view of alterations provoked by a specific condition (Fig. 1.18).

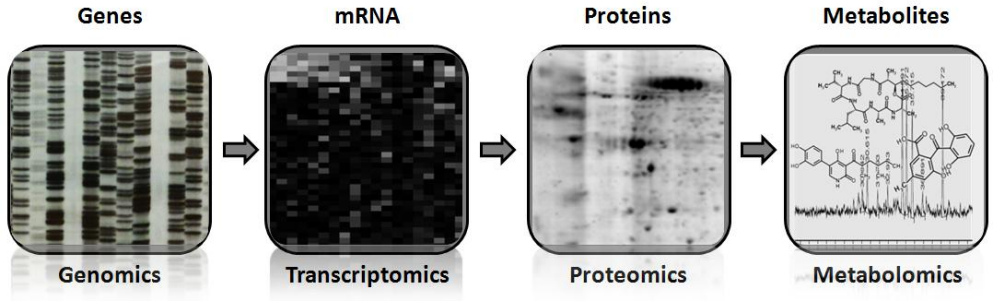


Fig. 1.18. The different levels of "Omics" approaches.

Transcriptomics

Transcriptomics investigates the transcriptome, *i.e.* the complete set of transcripts produced by the cell in certain conditions, in order to determine how transcript

levels change *e.g.* in specific tissues, stages of development or in response to chemicals and environmental factors [301]. Transcriptomics is a powerful technique that can be used for *e.g.* transcriptional profiling and alternative RNA splicing analysis [302, 303]. The doctoral studies included in this thesis used microarray based transcriptomics for fungal transcriptional profiling; therefore the following lines will focus on this topic.

Before the implementation of microarray technology, which allows the global analysis of cellular activity, studies were limited to the investigation of individual biological functions of a few related genes, proteins or pathways (using *e.g.* Northern blots or quantitative PCR) [304, 305]. Differential transcriptome analyses, using DNA microarray techniques to screen the full genome for messenger RNA (mRNA) abundance, allow the identification of differentially expressed genes in distinct conditions [299]. This was the strategy used for the analysis of the ionic liquids effects in the primary and secondary metabolisms of *Aspergillus nidulans* (Chapter 3) and also to determine the complex degrading enzymatic machinery used by this fungus to degrade suberin [7].

There are different microarray platforms, including the now obsolete spotted cDNA microarrays and high-density oligonucleotide microarrays (also known as genechips) [306, 307]. The first type of array contains cDNA probes (0.5-1 Kbp), derived from amplification of genomic sequences, which are then immobilised in specific locations of a glass modified surface, and despite the advantage of its low cost, low specificity with high background noise led to their gradual disappearance [307]. The high-density oligonucleotide microarrays contain *in situ* synthesised probes, which chemical synthesis occurs directly on the coated quartz surface of the array, and are manufactured by *e.g.* Affymetrix, Roche NimbleGen and Agilent Technologies [308-310]. This very high feature density enables the possibility to increase the information on the array by reducing the size occupied by each probe, reducing both the hybridisation volume and the amount of biological sample needed, creating a high number of controls and representing single genes by 11 to 15 probe pairs [306, 307]. Therefore, each transcript is measured 11 times while in spotted cDNA microarrays there is, in general, only one measurement being done [307].

Additionally, there are also one- or two-channelled platforms. The two-channel platform allow the hybridisation of two samples in the same array, this way each sample is labelled with one of the fluorescent dyes (usually *cyanin Cy3* and *Cy5*), and the obtained fluorescent image is translated into the relative expression of one sample over the other [304, 311]. In the single-channel array (*e.g.* Affymetrix arrays) only one sample is hybridised and the analysis is generally more straightforward than with the two-channel microarray [300, 311] (Fig. 1.19). The principal advantages of Genechip technology are the low variability among the manufacture process of the arrays and the high standardisation of protocols and reagents.

Several filamentous fungi microarray studies have already been successfully performed [7, 312]. Some microarrays might contain transcripts of more than one species (relying on cross-species hybridisation), such as GeneChip® Yeast Genome 2.0 Array that allows transcriptome analysis of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the one used for system-wide studies of *A. nidulans*, *A. niger* and *A. oryzae* [313], or even our custom designed array (FungiANC) successfully used to elucidate the machinery used by *A. nidulans* to degrade suberin [7].

Transcriptional profiling can also be achieved by sequence-based approaches that can directly determine cDNA sequences, such as SAGE (serial analysis of gene expression) or CAGE (CAP gene expression analysis), which derive from Sanger sequencing [301, 304]. These tag-based techniques present some disadvantages, for instance the impossibility to fully map short tags to the reference genome. Recently, high-throughput DNA sequencing methods *e.g.* RNA-Seq (*i.e.* RNA sequencing) are expected to promote substantial progress on transcriptomics studies [301]. Transcriptional profiling experiments of the same samples using both RNA-Seq and Affymetrix platforms demonstrated high correlation between the obtained gene expression profiles [314]. RNA-Seq is superior regarding the detection of low and high abundance transcripts, the capacity to differentiate biologically critical isoforms and to identify genetic variants or even non-annotated genes. The fact that RNA-Seq is independent of pre-designed

complement sequence detection probes and is not limited to a specific number of individual probes avoids non-specific and cross-hybridisations, and there is no probe redundancy and annotation that simplifies data analysis. Despite all these advantages, computational analysis of RNA-Seq data is still complex [301, 314].

Proteomics

Proteomics provides a global view of proteins being expressed in a specific biological state (*i.e.* proteome), which allows understanding the events occurring at the molecular level in a specific organism [315]. Proteomic analyses of an organism in a particular biological state allow protein identification, quantification and cellular localisation, as well as the detection of protein posttranslational modifications [315, 316], which discloses important information on metabolic reactions and regulatory cascades taking place [315].

Fungal proteomics research, particularly of filamentous fungi, has improved dramatically due to the increasing number of fungal genomes sequenced, the next-generation nucleic acid sequencing [317] and the development of powerful technologies, especially tandem liquid chromatography-mass spectrometry (LC-MS) [318]. Altogether, these advances facilitate high-throughput protein identification and functional assignment [319, 320].

Comparative proteomics enables the detection and the identification of differentially expressed proteins under specific conditions, such as a developmental stage (*e.g.* early development [321]), saline stress (*e.g.* osmoadaptation [322]) or a chemical (*e.g.* caspofungin [323]). It is possible to analyse subsets of proteins, such as those specific to an organelle (*i.e.* subproteomes [315]), secreted and involved in the secretion (*i.e.* secretome) [324] or responsive to a toxic (*i.e.* toxicoproteome [325]). In particular, the study of the fungal secretome is extremely relevant because, as absorptive organisms, fungi use extracellular enzymes to digest diverse substrates and take up the necessary nutrients [315, 326]. Several of these extracellular proteins play major roles in critical metabolic processes [327], such as in the degradation of lignocellulosic materials [328] and in pathogenicity [329].

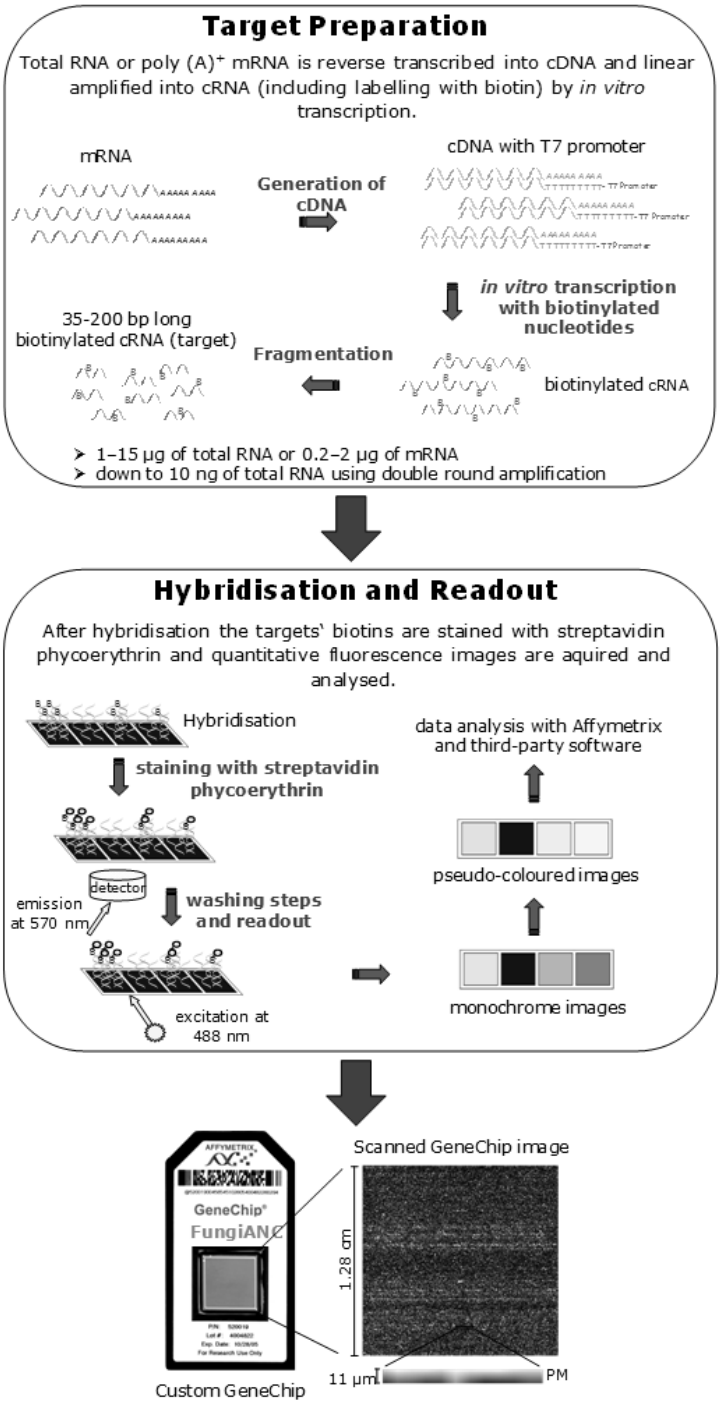


Fig. 1.19. Schematic representation of the preparation of target samples, hybridisation to Genechips and resulting scanned image.

The strategies commonly used for proteomic analysis are the classical gel-based techniques coupled to MS/MS-based identification of polypeptides or shotgun proteomics based on LC-MS/MS, which is gel-independent [330, 331].

The two-dimensional gel electrophoresis (2DE), developed by O'Farrell [332], enables the analysis of complex polypeptide mixtures. In two steps, this approach allows the separation of hundreds of polypeptides in a single gel: the first step is the isoelectric focusing (IEF), which is the separation of polypeptides by their isoelectric point (*pI*) and the second step (in an orthogonal plan) which separates them by their molecular weight in a gel matrix. After this separation, the proteins in the gels are stained, scanned and the images used for bioinformatic statistical analyses. After the selection of the relevant polypeptides in the 2DE map, the respective spots are excised from the gel, digested with proteolytic enzymes (*e.g.* trypsin) and the obtained samples subjected to MS-based techniques for analysis. The obtained peptide mass fingerprints are matched against a protein sequence database in order to identify the polypeptides [326, 333]. The major limitations of the gel dependent proteomic analyses include weak-resolution of the polypeptides with very high and very low *pI* and/or molecular weight, the loss of information of low copy number proteins masked by the highly abundant proteins and the low separation power that results in the overlapping of various polypeptides in a single spot hampering correct protein identification and quantification [326, 330]. The reproducibility of 2DE gels of distinct biological and/or technical replicates is crucial for a reliable quantitative analysis of the proteome. 2DE differential gel electrophoresis (DIGE) [334] is based on the covalent labelling of proteins from different proteomes with different fluorescent cyanine dyes before the electrophoretic separation. Therefore, a single gel is required to reproducibly detect differences between (at least) two protein samples. DIGE technology eliminates technical variability (by reducing the number of gels to be analysed and matched) and increases the reliability of quantification [333].

Advances in analytical technology, as well as in MS and bioinformatic techniques, have reduced substantially the limitations of 2DE gel analyses and provided new gel-free methods for proteomics assays, including multidirectional

protein identification technology (MudPIT) [335], protein microarrays [336], yeast two-hybrid systems [337], isotope coded affinity tags (ICAT) [338], stable isotopic labelling by amino acids in cell culture (SILAC) [339], isobaric tags for relative and absolute quantification (iTRAQ) [340] and activity-based probes (ABPs) [341].

Analysis of gel-based or gel-independent techniques efficacy revealed that in solution digestion followed by LC-MS/MS (*i.e.* gel-independent techniques) is more suitable for in depth proteomic assays. Nonetheless, gel-based proteomics are increasingly leading to the successful protein identification as a consequence of the higher availability of sequenced genomes and major advances in high-throughput LC-MS instrumentation [320], critical information on iso-enzymes and posttranscriptional modifications may be lost and protein quantification is still weak. It is important to note that mass spectrometry is not a quantitative method because the ionisation of analytes strongly depends on their structure, hence requiring *e.g.* the isotopic labelling of proteins (SILAC and ICPL, isotope-coded protein labels [342]) or peptides (ICAT and iTRAQ), which are expensive and complex [333].

As a final note, it is important to mention that the multidomain enzymes enclosed in the SM biosynthetic gene clusters usually present molecular weight higher than 200 kDa, which are difficult to detect using traditional proteomics approaches [343]. New proteomic platforms, such as the orthogonal active site identification system (OASIS) and the proteomic investigation of secondary metabolism (PrISM) have been developed to identify and quantify natural product synthase enzymes. These tools allow the discovery and analysis of SM biosynthetic pathways using proteomics, successfully complementing genetic-based approaches [344]. OASIS combines labelled proteins and analysis using MudPIT. PrISM, allows the targeted detection of non-ribosomal peptides and polyketides, using Fourier transform-ion cyclotron resonance (FT-ICR) MS (methods displaying high accuracy and resolution), and the identification of the gene cluster responsible for synthesis of the natural product [343]. The main difference between these tools is the MS equipment used. While, OASIS MS/MS analyses are conducted in a conventional linear ion trap, PrISM utilizes a hybrid instrument containing a FT-ICR and a linear ion trap mass analyzer in parallel, which allows the high accurate

determination of the precursor ion mass (FT-ICR) followed by unit resolution of MS/MS data [344].

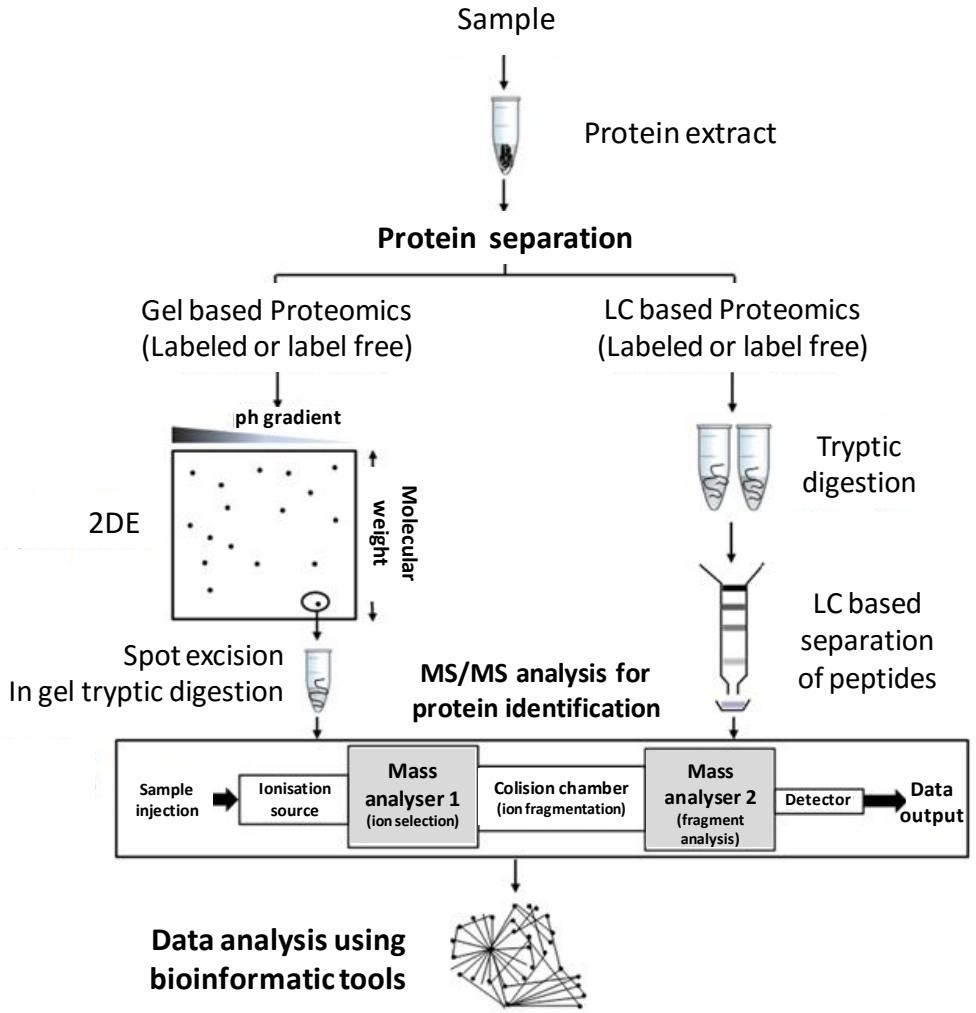


Fig. 1.20. Scheme of a general workflow of comparative proteomic experiments. Adapted from [345].

Metabolomics

Metabolomics studies the complete set of low-molecular-weight metabolites synthesised by a biological system under specific conditions - the metabolome [346, 347]. It is considered the most recent "omics" technique to be included in functional genomics, complementing the existing techniques and providing additional data on general metabolic regulation and secondary metabolism [348-350]. Comparative

metabolic profiling* in wild-type and defined mutant strains, either in control or testing conditions, has been widely used to correlate specific genes or conditions that induce biosynthesis of specific SMs [351]. Changes in the metabolome are usually amplified relatively to alterations in the transcriptome or proteome, impacting directly the phenotype [348].

One obvious advantage of metabolite analysis is the use of generic technology regardless of the biological system contrary to transcripts or proteins that differ from organism to organism and may require prior knowledge of genomic protein sequences and species databases. Metabolomics can be targeted to the search or quantification of a specific metabolite relying on adequate optimised extraction methods and instrumentation, such as the monitoring of a toxin in food products (*e.g.* patulin in fruit-based products by UHPLC-MS/MS [352]). It can also be non-targeted, aiming at the determination of the relative concentration of as many metabolites as possible in a specific sample. The strategy of choice for metabolite analysis should consider the physico-chemical properties of the sample [347], with mass spectrometry (MS) as the most commonly used technique either by direct flow injection or coupled to other techniques, such as separation techniques [353]. In the direct injection mode the insertion of a sample into the ionisation source of the mass spectrometer is done without any previous chromatographic separation, frequently using atmospheric pressure ionisation techniques, *e.g.* electrospray ionisation (ESI) [354]. The direct injection is a high-throughput approach that processes individual samples very fast (in a few minutes); this increases the reproducibility and the accuracy of subsequent cluster analysis. Fungal crude extracts have already been successfully analysed by this technique [355, 356]. Its major drawback is the difficulty to distinguish between compounds with the same nominal mass, a problem that may be overcome using high-resolution mass spectrometry (HRMS) instead. High mass resolution can also make accurate mass measurements of molecular ion signals and calculate the empirical formulas. Direct injection HRMS has been performed using time-of-flight mass spectrometers (TOF-MS) [357] and Fourier

* Metabolic profiling analyses a group of metabolites either related to a specific metabolic pathway or a class of compounds [352].

transform ion cyclotron mass spectrometers (FT-ICR-MS) [358], the latter displaying very high resolution, high mass accuracy and very low detection limits comparing to TOF-MS. These characteristics make FT-ICR-MS the ideal technique for metabolic fingerprinting investigations, so far only hindered due to its high instrument costs that limit its application to be wide distributed in metabolomic research. One of its disadvantages is the inability to distinguish chemical isomers (as they have the same exact mass) that would need to be chromatographically separated and/or further fragmented [353].

Mass spectrometry-based metabolomics allow quantitative analyses with high selectivity and sensitivity aiming at metabolites identification [353]. Coupling MS with separation techniques reduces the sample complexity, enhances resolution and provides additional information on the physico-chemical properties of the metabolites. A brief description of the commonly used MS-coupled techniques will be presented in the following lines. Gas chromatography-mass spectrometry (GC-MS) is a versatile technique suitable to non-targeted metabolite profiling of volatile and thermally stable polar and non-polar metabolites, such as sugars, amino acids, phosphorylated metabolites, organic acids, lipids and amines [353, 359]. Frequently, the majority of these compounds are non-volatile; therefore sample preparation requires complex derivatisations to confer the necessary volatility and thermal stability for analysis [347]. Metabolomic analysis using GC-MS rely mainly on quadrupole instruments [360] or time-of-flight (TOF) mass analysers [361], being TOF-MS mainly used for fast analyte detection rather than for high mass resolution.

Liquid chromatography-mass spectrometry (LC-MS) is another commonly used technique in metabolomics. Sample preparation is simple and compounds that are not suitable for GC-MS analysis (*e.g.* large, thermally unstable and non-volatile) can be detected with this technique [347]. This strategy requires the use of large amounts of MS-grade solvents, which are expensive to purchase and to dispose of, as well as of suitable columns for efficient separation of the class of metabolites of interest (similar to GC-MS). The technical reproducibility of LC-MS is sometimes hampered because of shifts in the retention time; however LC-MS has been often

used to investigate fungal SMs [354, 362-364], contrary to GC-MS because the derivatised SMs are too large to be analysed through this technique [347]. The equipment used for LC-MS analysis is usually constituted by a liquid chromatographer, coupled to a diode array (DAD) and either an ion trap mass spectrometer [364], an high-resolution TOF-MS [362] or quadrupole mass spectrometer [363]. Regarding the LC systems, there are high performance (HPLC) and ultra-high performance (UHPLC) liquid chromatographers [365]. The latter belongs to a new generation of liquid chromatographers working at higher pressure and with lower sample injection volumes than the traditional HPLCs. Although these instruments can work with standard HPLC columns, a new generation of short UHPLC columns with reduced particle size has also emerged. As a consequence, these UHPLC systems display higher chromatographic resolution and sensitivity, reduced time of analysis and robust performance compared to traditional HPLC systems [365].

Nuclear magnetic resonance (NMR) is a spectroscopic and powerful analytical technique that allows the investigation of the structure, dynamics and kinetics of a wide range of biological systems [347]. This technique uses the magnetic properties of atomic nuclei by exploiting their excited states under an external magnetic field and measurement of the energy state transitions using radiofrequency electromagnetic radiation [366, 367]. Although it is very different from the MS-based methods already mentioned here, NMR-based metabolic fingerprinting[**] has marked the beginning of a new metabolomics approach, which is valuable as a biochemistry tool as well as a very powerful screening tool for a variety of signature patterns. NMR is a non-selective and non-destructive detector of molecules in solution that requires minimal sample preparation avoiding its loss or the introduction of artefacts [368]. Although only certain nuclei can be detected (*e.g.* ^{1}H, ^{13}C, ^{2}H, ^{14}N, ^{15}N, ^{17}O, ^{19}F, ^{23}Na, ^{29}Si, ^{31}P and ^{35}Cl), all biomolecules are well covered. Sensitivity is, however, its major drawback and only medium to high

[**] Metabolic fingerprinting analyses all the detectable analytes in a given sample with subsequent classification of samples, based on metabolite patterns, and identification of discriminating metabolites [352].

abundance metabolites are detected. Moreover, the identification of individual metabolites, in complex mixtures, based on chemical shift signals is still a challenge [347, 353, 367]. In order to increase NMR sensitivity and allow the elucidation of molecular structures, hyphenated techniques such as LC-NMR-MS have been developed [350, 368, 369]. Parallel on-line NMR and MS detection efficiently provides complementary data and minimizes ambiguities between LC-MS and LC-NMR systems. Initial problems regarding the physical location of the LC and MS close to the NMR magnet have been overcome. It is now possible to place a benchtop ion trap MS less than one meter far from an ultrashielded spectrometer. This distance limits LC peak broadening, which is translated into enhanced NMR sensitivity. This on-line system is highly versatile and can be used during longer NMR experiments; however solving complex samples requiring two-dimensional NMR experiments and ^{13}C NMR data is still a challenge [368].

1.5. Aim of the thesis

The supplementation of ionic liquids to the culture media of several environmental fungal strains has been previously reported to increase the diversity of compounds in their culture footprints [174]. Stimulated by these results, the present thesis aims to better understand the impact of ionic liquids on fungal metabolism, specially focussing on their ability to induce the production of secondary metabolites.

The fungal organisms selected for this study were the model filamentous fungi, *Aspergillus nidulans* and *Neurospora crassa*. Both are important species in fungal biology studies and their genomes are sequenced and publicly available. *Aspergillus nidulans* is a talented secondary metabolite producer and a halotolerant species, while *N. crassa* capacity to synthesise secondary metabolites is quite humble and is a non-halotolerant species.

The chemicals selected for the studies included in this thesis belong to the most studied families of ionic liquids and they were: cholinium chloride and 1-ethyl-3-methylimidazolium chloride. On the one hand, the cholinium-based ionic liquids can present interesting properties, mainly because their cation is considered non-toxic, biocompatible and biodegradable. As an example, cholinium alkanoates were reported to solubilise and catalyse suberin depolymerisation. On the other hand, several ionic liquids containing imidazolium-based cations are commercially available and have been studied for diverse applications including the dissolution, swelling and processing of lignocellulosic materials. Despite the usual toxic and non-degradable nature of the imidazolium cations, most of the formulations including this cation display high chemical stability, low melting points and low viscosities.

The general stress response and primary metabolism alterations provoked by the presence of either ionic liquid in the culture media of both fungi was accessed by differential proteomics (Chapter II). Transcriptomics was used not only to evaluate the general impact of ionic liquids in the metabolism of *A. nidulans* (complementing the proteomic study) but also to analyse in detail the expression of genes coding in secondary metabolism, either biosynthesis or regulation (Chapter III). Additionally,

*q*RT-PCR, microscopy and metabolome analysis (liquid chromatography coupled with high resolution mass spectrometry) were used to complement these data.

The capacity of *N. crassa* to synthesise peptide metabolites under an ionic liquid stress was initially suggested by the differential proteome data (Chapter II) and further analysed in Chapter IV. In the latter, liquid chromatography, gel electrophoresis, nuclear magnetic resonance spectroscopy, mass spectrometry, amino acid analyses and bioactivity assays were used to attempt the isolation and characterisation of the produced peptide metabolites.

In the last section of this thesis (Chapter V) an integrated discussion of data from previous chapters and perspectives for future work are presented. Additional data (*e.g.* design and validation of an Affymetrix custom microarray, FungiANC [7]) not discussed in detail in this thesis is also included.

In the end, this thesis supports the use of ionic liquids as triggers to activate metabolic and development chances in fungi, in particular to induce production of otherwise silent fungal secondary metabolites. The millions of possible ionic liquids formulations may lead discovery of a hidden array of fungal secondary metabolites, some of which enclosing valuable biological activity.

1.6. References

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CHAPTER II

Proteomic alterations induced by ionic liquids in *Aspergillus nidulans* and *Neurospora crassa*

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Proteomic alterations induced by ionic liquids in *Aspergillus nidulans* and *Neurospora crassa*

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2.1. Abstract

This study constitutes the first attempt to understand at the proteomic level the fungal response to ionic liquid stress. *Ascomycota* are able to grow in media supplemented with high concentrations of an ionic liquid, which, in turn, lead to major alterations in the fungal metabolic footprint. Herein, we analysed the differential accumulation of mycelial proteins in *Aspergillus nidulans* and *Neurospora crassa* after their exposure to two of the most commonly used ionic liquids: 1-ethyl-3-methylimidazolium chloride or cholinium chloride. Data obtained showed that numerous stress-responsive proteins (*e.g.* anti-ROS defence proteins) as well as several critical biological processes and/or pathways were affected by either ionic liquid. Amongst other changes, these compounds altered developmental programmes in both fungi (*e.g.* promoting the development of Hülle cells or conidiation) and led to accumulation of osmolytes, some of which may play an important role in multiple stress responses. In particular, in *N. crassa*, both ionic liquids increased the levels of proteins which are likely involved in the biosynthesises of unusual metabolites. These data potentially open new perspectives on ionic liquid research, furthering their conscious design and their use to trigger production of targeted metabolites.

2.2. Introduction

Ionic liquids, *i.e.* organic salts with a melting temperature < 100 °C, in general exhibit negligible vapour pressure, leading them to be classed as alternative green solvents [1]. They constitute a disparate class of chemicals; any generalisation of properties apt to be misleading [2, 3]. Despite their "greenness", many of these compounds are highly toxic and recalcitrant to biodegradation [2, 3]. It is widely accepted that, regardless of their safety to the atmosphere, ionic liquids can potentially contaminate soil and water [3]. Some ionic liquids are presently under evaluation by the European Union Regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals, REACH), created to safeguard human health and environment from chemical risks. It is important to bear in mind the continually growing use of ionic liquids in industrial applications [4], especially processes that require production levels exceeding one ton *per annum* (*e.g.* 1-ethyl-3-methylimidazolium chloride [5]).

Toxicity of ionic liquids towards a broad range of organisms, from bacteria to invertebrates, is increasingly being investigated [2, 3]. It has been hypothesised that, in some cases (*i.e.* ionic liquids with long alkyl chain substituents), their mode of toxicity is related to necrosis, an energy-independent process which leads to loss of membrane integrity and/or release of cellular constituents [5, 6]. Increase in membrane permeability and loss of cell wall integrity have been observed also in filamentous fungi, namely in *Aspergillus nidulans* [6]. After exposure to sub-inhibitory concentrations of alkyltributylphosphonium chlorides, several genes involved in plasma membrane biosynthesis showed an altered expression profile, suggestive of membrane damage [7]. Some studies have also demonstrated that ionic liquids provoke a highly regulated programmed-cell-death response, a caspase-dependent apoptotic response [8]. Certain ionic liquids might lead to major metabolic alterations, *e.g.* imidazolium-based ionic liquids increase the activity of antioxidant defence enzymes, such as superoxide dismutase, catalase and glutathione S-transferase, in *Daphnia magna* [9]. It was suggested that oxidative stress is also

involved in toxicity of the tested ionic liquids. In addition, filamentous fungi, namely *Ascomycota*, display, in general, high tolerance towards ionic liquids [10-12], yet when exposed to sub-lethal concentrations of these organic salts, fungal metabolic footprint is significantly altered [2, 10]. This has been preliminary associated to the altered expression of genes coding for the biosynthesis of unusual secondary metabolites [13]. Furthermore, filamentous fungi ubiquity, diversity, catabolic potential and extended mycelial networks make them key players in the mitigation of any environmental chemical pollutant.

Knowledge of cellular and biochemical responses to exposure to ionic liquids can give fresh perspectives on how to employ these compounds to manipulate proteins or pathways of biotechnological value. High-throughput analyses, such as transcriptomics or proteomics, are valuable methodologies for revealing a snap-shot view of alterations provoked by a specific condition. Illumina RNA deep sequencing technology has been recently employed to gain insight into the molecular mechanisms altered by exposure to an ionic liquid in *Enterobacter lignolyticus* [14]. Genes encoding compatible solute transporters, efflux pumps, porins, and lipid biosynthetic enzymes were amongst those differentially expressed in the presence of 0.375 M of 1-ethyl-3-methylimidazolium chloride. Even though proteomic surveys, in comparison to transcriptome analysis, are still under-represented in fungal research [15], they can provide direct information on protein species identity, localisation and posttranslational modifications. Gel-based proteomics, which combines the resolution of 2DE with MS/MS sensitivity, can detect and identify hundreds of differentially accumulated proteins [16]. It has provided meaningful insight into fungal proteomes associated with *e.g.* specific developmental stages [10, 17] and response to toxicity and stress stimuli [17-20].

To gain further insight into ionic liquid response in fungi, a comparative analysis of the fungal mycelial proteome after growth in media with or without an ionic liquid was performed here. Two ionic liquids, belonging to the ionic liquid families currently attracting most interest, either academic or industrial, were considered. Specifically, we selected cholinium chloride (choline, non-toxic and

readily biodegradable [21-24]) and 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl, toxic and recalcitrant [25, 26]). They display very distinct antifungal activities (very low and moderate/high, respectively) [10]. In addition, herein two model *Ascomycota* species were selected, namely *Aspergillus nidulans* and *Neurospora crassa*. Both are prime model fungal systems for genetic, cellular, and biochemical research and are dissimilar when accounting for their halo-tolerance and secondary metabolite producing capacity.

This study constitutes the first attempt to understand, at the proteomic level, the response of *Ascomycota* to ionic liquid stress. We aim to advance beyond mere identification of stress responsive proteins and identify at a species-specific level the critical biological processes or pathways affected by each ionic liquid. The data obtained make apparent the capacity of ionic liquids to specifically alter developmental programmes in both fungal species and lead to accumulation of several proteins involved in, amongst other, ROS detoxification and osmolytes biosynthesis. These findings may open new perspectives on ionic liquids research, namely their conscious design and their potential use to trigger specific metabolic processes, in particular the biosynthesis of fungal metabolites.

2.3. Materials and Methods

2.3.1. Chemicals

Cholinium chloride, hereafter referred to solely as choline, and 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl), purchased from Solchemar, were dried *in vacuo* (40-70 °C, 24-48 h, *ca.* 0.01 mbar) prior to use. Compounds used in preparation of minimal media, with the exception of NaCl (Panreac, 99.5%), were purchased from Sigma-Aldrich (D(+)-glucose, K_2HPO_4, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $NaNO_3$), as well as TCA, CHCA and lactophenol blue. DTT, CHAPS, IPG strips (13 cm nonlinear; pH 3-10), IPG buffer (pH 3-10), thiourea and urea were obtained from GE Healthcare, polyvinylpolypyrrolidone from Merck, 30% acrylamide:bisacrylamide solution, 3.3%C and Bradford reagent from Bio-Rad and Trypsin Gold (MS grade) from Promega. All HPLC and MS solvents used were of the highest analytical grade and water was obtained from a Milli-Q system (Millipore).

2.3.2. Fungal strains

Aspergillus nidulans (FGSC A4) and *Neurospora crassa* (FGSC 2489) were obtained from the Fungal Genetics Stock Centre (FGSC) and conserved as a suspension of conidia in saline solution (NaCl, 0.85% w/v) containing glycerol (10% v/v) at -80 °C. Fungal cultures were cultivated in minimal media, supplemented or not with the testing compounds, inoculated with 10^5 conidia *per* mL and incubated in the dark, under orbital agitation (90 rpm) at 27 °C. Minimal media, adjusted to pH 6, contained, *per* litre of water, 1 g of glucose; 1 g K_2HPO_4; 3 g $NaNO_3$; 10 mg $ZnSO_4 \cdot 7H_2O$; 5 mg $CuSO_4 \cdot 5H_2O$; 0.5 g $MgSO_4 \cdot 7H_2O$; 10 mg $FeSO_4 \cdot 7H_2O$ and 0.5 g KCl.

2.3.3. Ionic liquids minimal inhibitory and fungicidal concentrations (MIC and MFC)

MIC and MFC were determined as previously described [11, 27]. Fungal growth was determined by daily measuring absorbance (600 nm). All tests were performed in triplicate. Water activity (a_w) of minimal media supplemented with the MIC of either ionic liquid was determined with a portable water activity indicator (HydroPalm AW1) following the manufacturer's instructions, at 25 °C with resting periods of 10 min (triplicates).

2.3.4. Culture conditions

Minimal media (70 mL) supplemented with either ionic liquid at half the MIC of 1-ethyl-3-methylimidazolium chloride (*i.e.* 0.7 M and 0.2 M for *A. nidulans* and *N. crassa*, respectively, see Results section) was inoculated and incubated as described above for fifteen days (quadruplicate cultures). At the end of incubation, mycelia were recovered using vacuum assisted filtration with glass fibre pre-filters (Millipore) and washed twice with a solution of NaCl (0.85% w/v) containing Tween 80 (0.1% v/v). Both recovered fractions (mycelia and culture filtrate) were immediately frozen in liquid nitrogen and conserved at -80 °C until further analyses.

2.3.5. Microscopy

Mycelia were recovered from fungal cultures at defined incubation time points (5 mL; inoculated and incubated as described above, triplicate samples), mounted on glass slides and stained with lactophenol blue for enhanced contrast. Visualisation of cultures was performed in a DM5500 B microscope (Leica) with 40× or 63× magnification objectives and images captured with a DFC420 C camera (Leica).

2.3.6. Chromatographic analyses

The culture metabolic organic extracts (prepared as previously described [10]), were chromatographically separated using a Waters (Waters Corporation) Acquity chromatographer with Photodiode Array (PDA) detector, cooling auto-sampler and

column oven. A Symmetry® C18 column (250 x 4.6 mm), packed with end-capped particles (5 µm, pore size 100 Å) (Waters Corporation) was used at 26 °C. Data were acquired using Empower 2 software (Waters Corporation, 2006). Samples were injected using a 10 µL loop operated in full-loop mode. The mobile phase, at a flow rate of 0.9 mL/min, consisted of a solution of 0.1% TFA (solvent A) and ACN (solvent B), set to a linear gradient of 99.5% to 0% of solvent A during 30 min, followed by 100% of solvent B for 10 min, 2 min to return to the initial conditions, and additional 10 min to re-equilibrate the column.

Mycelial xylitol and glycerol (extracted as previously reported [22, 28], respectively) were analysed using a standard HPLC methodology for quantification of sugars, acids and alcohols in aqueous solutions as reported before [29]. Quantification detection limits were 0.01 g/L. Betaine detection in the mycelial extracts (prepared as previously reported [22, 28]) was done chromatographically (see equipment above mentioned,) using a Symmetry® C18 column (2.1 x 150 mm, 5 µm particle size, Waters Corporation), set at 30 ºC, and a mobile phase composed of a solution of 50 mM KH_2PO_4 in acetonitrile (1:1, v/v) operated at a flow rate of 0.8 mL/min. Betaine, which was detected at 195 nm, eluted at 16.7 min. Quantification of mycelial betaine (injections of 20 µL) was done using an external standard method, within the quantification limits of 1-10 g/L. The residual concentration of each cation in culture filtrate at the end of the incubation was estimated by liquid chromatography using the equipment and software described above fitted with a Synergi Polar-RP column (150 x 4.6 mm) packed with polar end-capped particles (4 mm, pore size 80 Å) (Phenomenex), at 26 °C as previously described [12].

2.3.7. Mycelial protein extraction

Mycelial proteins were extracted as previously described [30], comprising a set of homogenisation, protein precipitation (acetone with 10% (w/v) TCA and 60 mM DTT) and finally protein resuspension (1% v/v IPG buffer, 4% w/v CHAPS, 2 M thiourea, 7 M urea, 1% w/v Triton X-100, and 60 mM DTT). Protein was quantified

using the Bradford protocol modified by Ramagli [31], with BSA as protein standard.

2.3.8. Electrophoresis and image acquisition

Mycelial protein samples (180 µg) were loaded in precast 13 cm nonlinear IPG strips pH 3-10. Protein isoelectric focusing and electrophoresis were performed as previously described [30]. Three biological samples were analysed. A further gel was run for spot excision. Gels stained with colloidal Coomassie were scanned using a LabScan (GE Healthcare), controlled by the LabScan software v5.0. Calibration used a Kodak set table nº 2 (set in transparent mode, 600 dpi, and a green filter).

2.3.9. Image and statistical analyses

Images were analysed using Progenesis SameSpots v2.0 (Nonlinear Dynamics), according to manufacturer's instructions. After geometric alignment of the pixel levels, spot measurement, background subtraction and subsequent normalization were automatically generated. Protein spots with areas lower than 1500 were excluded from the analysis and the list of *p*-values (ANOVA) for the individual spots was generated. After correction of possible mismatches in individual spots, those showing a p-value < 0.05 (and power > 0.7) were considered to constitute significant alterations. PCA analyses were done according to Valledor *et al.* [32], using SameSpots software (Table S1 and Fig. S1, in Additional file 1[*]).

2.3.10. Protein identification

Selected protein spots were excised from gels manually, and processed using the Ettan Digester robot of the Ettan Spot Handling Workstation (GE Healthcare) as previously reported [30]. Samples (0.7 µL) were then spotted on MALDI-TOF target plates (Applied Biosystems), before the deposit of 0.7 µL CHCA (5 mg/mL in solution A). Peptide mass determinations were carried out using the Applied

[*] *available in the thesis CD.*

Biosystems 4800 Proteomics Analyzer (Applied Biosystems). Both peptide mass fingerprinting and tandem MS in reflectron mode analyses were performed. The eight most intense peaks were selected automatically and used for MS/MS. Calibration was carried out with the peptide mass calibration kit for 4700 (Applied Biosystems).

Proteins were identified by searching against NCBI (restricted to fungi) on an in-house MASCOT server (Version 2.1, Matrix Science, www.matrixscience.com, London, UK) using AGPS platform (Version 3.6, Applied Biosystems). All searches were executed allowing for a mass window of 50 ppm for the precursor mass and 0.75 Da for fragment ion masses. Search parameters allowed for carboxyamidomethylation of cysteine as fixed modification. Oxidation of methionine and tryptophan (single oxidation, double oxidation and kynurenin), methylation of glutamic and aspartic acids and pyrrolidone carboxylic acid (N-terminal glutamine and glutamic acid) were set as variable modifications, as previously described [33, 34]. Homology identification was retained with probability set at 95%. All identifications were confirmed manually (Additional files 2* and 3*). The existence of the most common posttranslational modifications such as phosphorylation, glycosylation and signal peptide presence, were predicted using web-based tools, namely Calculate Molecular Weight and Isoelectric Point (http://scansite.mit.edu/calc_mw_pi.html), NetPhos 2.0 (http://cbs.dtu.dk/services/NetPhos/); NetOGlyc 1.0 (http:// cbs.dtu.dk/services/NetOGlyc/) and signalP (http:// cbs.dtu.dk/services/SignalP/).

* *available in the thesis CD.*

2.4. Results and Discussion

Aspergillus nidulans and *Neurospora crassa* were exposed to sub-lethal concentrations of choline and 1-ethyl-3-methylimidazolium chloride. The metabolic response of the cultures will be defined by both organic and saline nature of these compounds. Control cultures were also analysed, *viz.* minimal medium (hereafter referred as control) and minimal medium supplemented with sodium chloride. The ionic liquids MIC and MFC values for each fungus are listed in Table 2.1. Values should not be interpreted as absolute, but rather an indication of the upper inhibitory and fungicidal concentration limits. Importantly, a_w values of the growth media at MIC (Table 2.1) were substantially higher than those described as growth limiting for *A. nidulans* and *N. crassa* (0.8 and 0.9, respectively) [35]. In strong agreement with previous studies, choline and 1-ethyl-3-methylimidazolium chloride showed very low and moderate toxicity against fungi, respectively [10, 11]. Even though choline is considered non-toxic, at molar concentration it led to growth inhibition. Sodium chloride MIC and MFC values revealed, as anticipated, that the strains display very dissimilar osmotolerance levels. Only *A. nidulans* was able to grow in media containing a concentration of sodium chloride > 1.7 M and is thus classified as halo-tolerant [36]. Ionic liquids MIC values for *A. nidulans* were more than two-fold higher than those determined for *N. crassa*. The halo-tolerance might underlie the capacity of this fungus to withstand higher ionic liquids concentrations.

The growth media were supplemented with half the MIC of 1-ethyl-3-methylimidazolium chloride, in an attempt to homogenise the stress imposed to the cultures. Upon completing incubation, fungal cultures were harvested and the exhausted cultivation media used to evaluate residual concentration of each ionic liquid by chromatographic quantification of the corresponding cations. The concentration of 1-ethyl-3-methylimidazolium cation was unaltered until the end of the incubation period. Its recalcitrance to biodegradation has been often reported [2, 10], even if the bacterial degradation of a related cation has been proposed [37, 38]. The cholinium cation was partially degraded (9.4% ± 3.9% and 19.3% ± 3.2% for

A. nidulans and *N. crassa*, respectively). Similar degradation yields have been reported previously for other filamentous fungi [11, 12].

Table 2.1. Minimal inhibitory and fungicidal concentrations (MIC and MFC) of [C_2mim]Cl, choline and sodium chloride for *Aspergillus nidulans* and *Neurospora crassa*. Water availability (a_w) values at the MICs are also presented.

Compounds		*N. crassa*	*A. nidulans*
[C_2mim]Cl/M	MIC	0.4	1.4
	MFC	0.4	1.4
	a_w	1.0	0.97
Choline/M	MIC	1.2	2.2
	MFC	5.0	2.8
	a_w	0.95	0.88
NaCl/M	MIC	1.4	3.3
	MFC	4.2	4.4
	a_w	0.92	0.81

The metabolic footprint (*i.e.* pool of metabolites produced at a given point under certain culture conditions) of *A. nidulans* and *N. crassa* culture extracts were investigated by liquid chromatography. In general, compound diversity was greater in *A. nidulans* cultures grown in media supplemented with the tested chlorides than the control (Fig. S2 in Additional file 1[*]). In *N. crassa* only ionic liquids led to greater compound diversity (data not shown). These observations corroborate previous findings that ionic liquids significantly alter fungal metabolic footprints of cultures at the stationary phase of growth [10]. This effect is unrelated to that provoked by the inorganic salt. Very small changes in culture conditions (*e.g.* carbon and nitrogen sources and availability [39]) have been seen to shift metabolic profile of various microorganisms. Using systematic alteration of cultivation parameters, including media composition, it was possible to isolate over 100 compounds from only six microorganisms [40].

2.4.1. Overall mycelial proteome analysis

Proteomic analyses of the fungal mycelia were undertaken to better understand the effect of ionic liquids. The protein yield of the method employed for protein

[*] *available in the thesis CD.*

extraction was reasonably similar in all samples. These data, as well as the maximum and minimal number of spots detected after the 2DE separation of the protein extracts (three biological replicates) is depicted in Table 2.2.

Table 2.2. Protein extraction yield and number of detected spots in the different mycelia samples used for proteomic analysis. The symbols ↑ and ↓ stand for acumulated or decreased levels, respectively.

Sample		Protein yield [a]	Gels		Statistically altered spots			
			Max/min number	Average number	Common to both ionic liquids		Specific to each ionic liquid	
					↑	↓	↑	↓
A. nidulans	Control	7.93±0.32	657/620	636±19	-	-	-	-
	[C_2mim]Cl	5.72±1.02	656/610	639±25	5 (12%)	11 (24%)	11 (24%)	3 (7%)
	Choline	9.68±0.25	661/621	638±21			13 (31%)	1 (2%)
N. crassa	Control	7.18±0.19	725/687	696±25	-	-	-	-
	[C_2mim]Cl	6.42±0.11	724/678	702±23	1 (3%)	8 (21%)	7 (14%)	12 (32%)
	Choline	7.56±0.19	728/663	699±32			12 (29%)	3 (1%)

[a.] mg protein/g fresh weight.

Mycelial 2D maps showed 680 and 750 protein spots for *A. nidulans* and *N. crassa*, respectively, accounting for samples (ionic liquids media) and control (Fig. 2.1). The number of statistically altered proteins spots was 42 and 41 each, of which 40 and 38 were manually excised and identified. Identified proteins are listed in Tables 2.3 and 2.4 and grouped according to their biological functions (Fig. 2.2). Some of the spots in Tables 2.3 and 2.4 have different experimental p*I* and molecular masses than the predicted polypeptides. This may be due to posttranslational modification, alternative splicing, or degradation. The difference between observed and expected may imply differences in their regulation [41], sub-cellular localisation [42] and/or multifunctional activity [43]. The presence of the most common posttranslational modifications such as phosphorylation, glycosylation and signal peptide is also indicated in Tables 2.3 and 2.4. An overview of the functional classification of the differentially accumulated mycelial proteins is shown in Fig. 2.2.

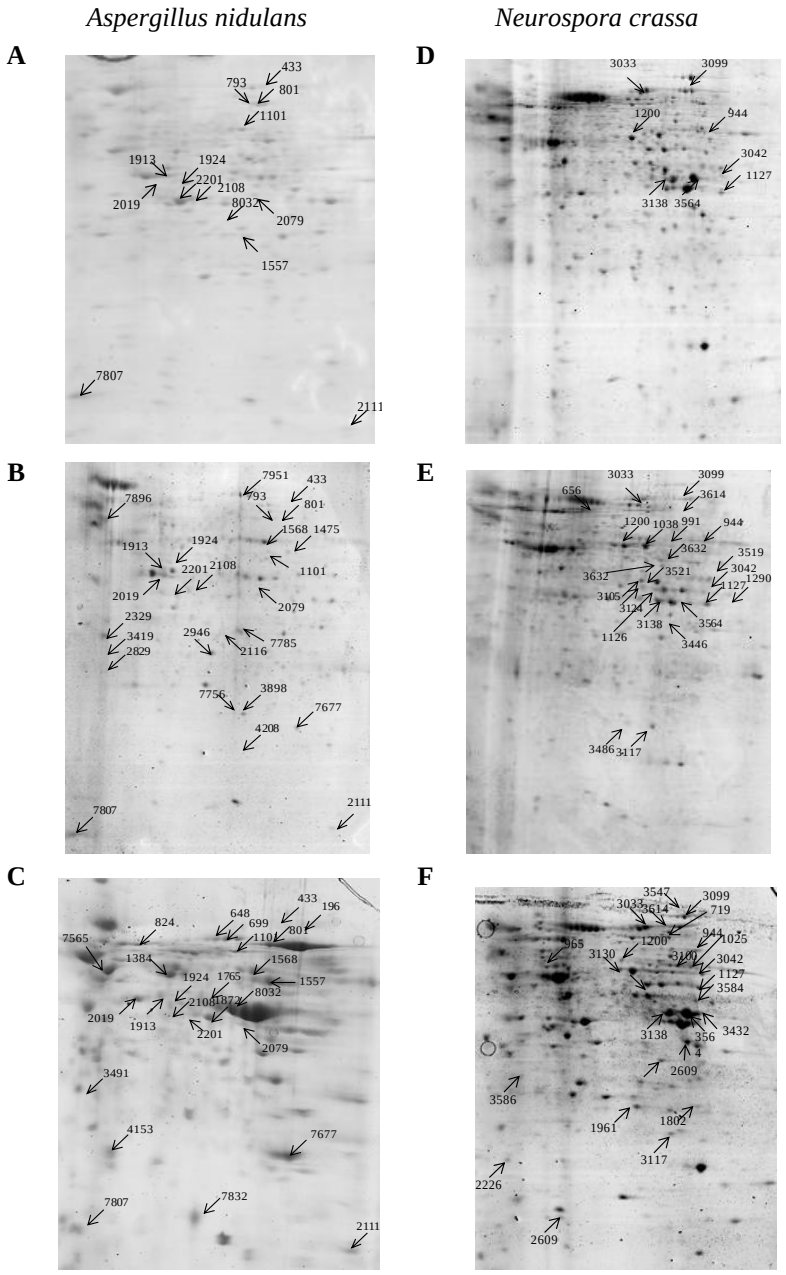

Fig. 2.1. 2DE protein reference maps of mycelial proteome of *Aspergillus nidulans* and *Neurospora crassa* grown in media without (A, D) or with [C_2mim]Cl (B, E) or choline (C, F). Differentially accumulated protein spots were automatically numbered and the respective identification is shown in Tables 2.3 and 2.4.

Table 2.3. Proteins differentially accumulated in *Aspergillus nidulans* cultures after exposure to [C_2mim]Cl or choline. Protein species are grouped by their functional classification.

Gene code	Protein (EC number)	Fold Change		pI/MW (kDa)		*p*-values[a]	#Spot
		[C_2mim]Cl	Choline	Theo.	Exp.		
	Cell fate						
AN0472	β-1,3-endoglucanase (3.2.1.39)	2.1	1.9	5.7/101[b]	6.2/98	0.031	7951
AN4871	Chitinase (3.2.1.14)	1.3	-	5.3/44	5.7/41	0.005	7811
AN4871	Chitinase (3.2.1.14)	1.3	-	5.3/44	5.8/41	0.05	686
AN2903	Vacuolar asparagine protease	1.8	-	4.8/43[c]	4.7/65	0.020	7896
	Carbohydrate metabolism						
	Glycolysis						
AN5746	Enolase (4.2.1.11)	-	2.4	5.4/47[c]	5.6/60	0.017	1384[e]
AN5746	Enolase (4.2.1.11)	1.9	2.1	5.4/47[c]	6.2/55	0.006	1242
AN2875	Fructose-bisphosphate aldolase (4.1.2.13)	0.2	0.2	5.7/39[c]	5.6/44	0.000014	2201
AN2875	Fructose-bisphosphate aldolase (4.1.2.13)	0.4	0.5	5.7/39[c]	5.5/44	0.008	2019
	TCA cycle						
AN1003	Isocitrate dehydrogenase (1.1.1.41)	0.2	0.2	8.5/39	8.46/45	0.0003	2108[e]
	Glyoxylate and dicarboxylate metabolism						
AN6525	Formate dehydrogenase (1.2.1.2)	-	2.4	6.1/40[c]	5.0/59	0.017	1384[e]
AN6525	Formate dehydrogenase (1.2.1.2)	0.3	0.3	6.1/40[c]	5.9/55	0.0003	1557
AN6525	Formate dehydrogenase (1.2.1.2)	-	2.3	6.1/40[c]	5.8/45	0.006	1872
AN6525	Formate dehydrogenase (1.2.1.2)	0.2	0.2	6.1/40	8.46/45	0.0003	2108[e]
AN8099	Oxalate decarboxylase (4.1.1.2)	-	1.6	4.8/52[bd]	7.6/23	0.0008	3491
	Pentoses phosphate pathway						
AN0285	6-phophosgluconolonase (3.1.1.31)	3.2	-	9.2/41[bc]	5.7/32	0.014	2946
AN0688	Transketolase (2.2.1.1)	0.3	-	5.8/76[b]	6.0/80	0.023	801
AN0688	Transketolase (2.2.1.1)	0.4	-	5.8/76[b]	6.0/83	0.028	793
	Translation						
AN8824	Eukaryotic translation initiation factor 6	3.9	-	4.7/26	4.8/27	0.003	3419
AN4015	Eukaryotic translation initiation factor 5A-2	-	1.8	5.0/18[b]	5.0/20	0.005	4153
	Cytoskeleton						
AN6542	Actin	0.4	0.3	5.4/42[b]	5.6/46	0.0002	1924

Gene code	Protein (EC number)	Fold Change		pI/MW (kDa)		*p*-values[a]	#Spot
		[C_2mim]Cl	Choline	Theo.	Exp.		
Stress response							
AN8605	Cyclophilin (5.2.1.8)	0.6	0.4	8.9/18	8.8/10	0.001	5698
AN11227	Heat shock protein 70	-	3.2	5.3/67	5.5/82	0.026	824
AN7388	Peroxidase/catalase 2 (1.11.1.6)	-	3.0	5.9/82	5.8/90	0.020	6697
AN7388	Peroxidase/catalase 2 (1.11.1.6)	-	3.0	5.9/82	5.8/82	0.003	648
Energy metabolism							
AN1523	ATP synthase subunit alpha (3.6.3.14)	2.0	-	9.1/62	4.8/57	0.023	1475
AN1534	ATP synthase subunit 4	2.3	-	9.2/26	7.8/20	0.015	7677
AN7331	Cyanase (4.2.1.104)	-	3.6	5.9/18	5.8/12	0.046	5350
AN11015	S-hydroxymethyl glutathione-dependent formaldehyde-activating enzyme (4.4.1.22)	3.5	-	7.6/21	6.2/25	0.003	3898
AN4905	Glutathione-S-transferase (2.5.1.18)	2.1	-	9.0/34	6.4/35	0.006	7756
Amino acid metabolism							
AN0567	Choline dehydrogenase activity	-	1.4	4.8/70	4.9/68	0.007	7565
AN4443	5-ethyltetrahydropteroyl triglutamate-homocysteine methyltransferase (2.1.1.14)	-	2.5	6.3/86	6.2/90	0.023	196
AN4376	NADP-linked glutamate dehydrogenase (1.4.1.4)	-	1.8	6.2/50	5.9/58	0.016	1765
AN7387	Pyrroline-5-carboxylate reductase (1.5.1.2)	0.8	-	5.4/29	5.6/35	0.047	8032
AN8654	N,N-dimethylglycine oxidase (1.5.8.4)	0.5	0.3	9.1/104	6.4/103	0.003	433
Signalling							
AN4163	Guanine nucleotide-binding protein beta subunit-like protein (CpcB)	0.5	0.5	6.4/35	5.6/39	0.025	2079
Uncharacterised/poorly characterised							
AN0297	NADH-quinone oxidoreductase	2.1	1.8	5.85/22	6.0/19	0.002	4280
AN12222	MFS transmembrane transporter domain	3.5	-	8.84/63	5.0/59	0.017	2329
AN8043	BYS1 domain protein	0.3	0.7	4.6/17	4.5/12	0.008	5245
AN7484	Unknown function protein	2.4	-	5.01/22	5.0/23	0.005	2829
AN8829	Unknown function protein	-	0.6	4.31/14	4.0/19	0.002	7813
AN3739	Unknown function protein	0.3	0.3	9.61/56	5.5/48	0.01	1913

[a.] *p*-value obtained from the ANOVA analyses; [b.] Predicted glycosylation site(s); [c.] Predicted phosphorylation site(s); [d.] Probably a protein degradation product; [e.] Spots where 2 different proteins were identified, fold change refers to the protein spot.

Table 2.4. Proteins differentially accumulated in *Neurospora crassa* cultures after exposure to [C_2mim]Cl or choline. Protein species are grouped by their functional classification.

Gene code	Protein (EC number)	Fold change		pI/MW (KDa)		*p*-values[a]	#Spot
		[C_2mim]Cl	Choline	Theo.	Exp.		
	Cell fate						
NCU05850	Rubredoxin-NAD^+ reductase (1.18.1.1)	0.5	0.7	8.65/66	7.8/64	0.014	949
	Carbohydrate metabolism						
	Glycolysis/Gluconeogenesis						
NCU06075	Pyruvate kinase (2.7.1.40)	2.2	-	6.36/58[c]	9/68	0.012	863
NCU03415	Aldehyde dehydrogenase (1.2.1.3)	3.0	-	5.94/54	6.5/59	0.023	1038
NCU03415	Aldehyde dehydrogenase (1.2.1.3)	-	1.5	5.94/54	6.5/63	0.003	965
NCU01754	Alcohol dehydrogenase (1.1.1.1)	0.4	0.4	6.32/37	6.8/43	0.008	3138
NCU01528	Glyceraldehyde 3-phosphate dehydrogenase (1.2.1.12)	0.8	0.5	6.33/36[bc]	7.6/91	0.005	3099
NCU01528	Glyceraldehyde 3-phosphate dehydrogenase (1.2.1.12)	0.6	0.6	6.33/36[bc]	7.8/43	0.024	3564
NCU07914	Phosphoglycerate kinase (2.7.2.3)	0.8	-	6.17/45[c]	6.6/54	0.002	3124
NCU06836	Acetyl-coenzyme A synthetase (6.2.1.1)	0.5	0.7	6.19/74	7.8/82	0.042	3042
	TCA cycle						
NCU06211	Malate dehydrogenase (1.1.1.37)	0.8	0.6	5.56/34	6.7/31	0.028	1576[d]
NCU01692	Citrate synthase (2.3.3.1)	-	1.8	6.73/48	8.0/53	0.002	1127
	Pentose phosphate pathway						
NCU07281	Glucose-6-phosphate isomerase (5.3.1.9)	2.2	-	6.03/61	5.9/61	0.0004	991
NCU01328	Transketolase (2.2.1.1)	0.4	-	5.87/75[b]	6.6/70	0.022	3105
NCU03100	6-phosphogluconate dehydrogenase (1.1.1.44)	1.5	-	6.3/57	6.7/55	0.026	1126
	Pentoses and glucoronate interconvertions						
NCU04657	1-aminocyclopropane-1-carboxylate deaminase (3.5.99.7)	2.2	1.3	5.78/39	6.4/26	0.013	1200
NCU09041	L-xylulose reductase (1.1.1.10)	-	1.4	6.34/29	6.3/30	0.009	1961
NCU02797	UTP-glucose-1-phosphate uridylyltransferase (2.7.7.9)	-	1.7	6.59/58[c]	7.1/68	0.014	3100
NCU02797	UTP-glucose-1-phosphate uridylyltransferase (2.7.7.9)	-	1.3	6.59/58[c]	7.6/48	0.028	3113
	Glyoxylate and dicarboxylate metabolism						
NCU03813	Formate dehydrogenase (1.2.1.2)	0.6	-	5.93/41[c]	7.1/48	0.016	1206
	Amino acid metabolism						
NCU08771	Acetolactate synthase (2.2.1.6)	0.7	-	6.39/66[c]	6.5/70	0.013	3073
NCU08998	4-aminobutyrate aminotransferase (2.6.1.19)	0.8	0.6	6.21/52[c]	6.7/37	0.028	1576[d]

Gene code	Protein (EC number)	Fold change		pI/MW (KDa)		*p*-values[a]	#Spot
		[C_2mim]Cl	Choline	Theo.	Exp.		
NCU01853	Choline dehydrogenase (1.1.99.1)	-	1.4	8.73/66	6.3/80	0.004	3033
NCU02274	Serine hydroxymethyltransferase (2.1.2.1)	-	1.5	6.93/53	8.1/54	0.016	1025
NCU06512	5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase (2.1.1.14)	-	1.8	6.28/86	7.0/87	0.006	3547
NCU06512	5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase (2.1.1.14)	-	1.3	6.28/86	6.6/81	0.015	578
	Stress response						
NCU00355	Catalase-3 (1.11.1.6)	-	1.3	5.69/76[c]	5.9/76	0.030	656
NCU05770	Catalase-2 (1.11.1.21)	0.7	-	5.97/83[c]	6.2/74	0.006	719
NCU09560	Superoxide dismutase (1.15.1.1)	2.2	-	8.67/25	8.0/16	0.006	2220
NCU02133	Superoxide dismutase (1.15.1.1)	1.6	-	5.74/16	8.5/25	0.002	2425
	Signalling molecules and interactions						
NCU04202	Nucleoside diphosphate kinase (2.7.4.6)	-	1.3	7.82/17[c]	7.6/43	0.028	1288
NCU05810	Guanine nucleotide-binding protein subunit beta-like protein (Cpc-2)	0.4	-	6.79/35	7.6/35	0.016	3446
NCU05810	Guanine nucleotide-binding protein subunit beta-like protein (Cpc-2)	0.4	-	6.79/35	7.5/33	0.021	1481
NCU01484	GTP-binding protein RHO-1 protein	-	0.4	7.0/22[c]	7.6/25	0.018	1802
	Translation						
NCU03826	Elongation factor 1-gamma	-	1.4	5.87/46	7.2/49	0.009	3130
NCU04331	60S ribosomal protein L5	0.7	-	7.75/34	7.8/44	0.012	3039
NCU09269	GTP-binding nuclear protein GSP1/Ran	2.0	-	6.44/24	6.4/26	0.021	1998
	Energy metabolism						
NCU06695	Cytochrome *c* oxidase subunit 6	-	0.6	5.8/17	5.0/13	0.003	2274
NCU01169	NADH-ubiquinone oxidoreductase 24 kDa subunit (1.6.5.3/1.6.99.3)	-	0.5	5.04/24	5.1/23	0.030	1810
	Uncharacterised						
NCU04510	Putative uncharacterized protein	0.5	-	6.0/33.1	7.3/29	0.0008	1604

[a.] *p*-value obtained from the ANOVA analyses; [b.] Predicted glycosylation site(s); [c.] Predicted phosphorylation site(s); [d.] Spots where 2 different proteins were identified, fold change refers to the protein spot.

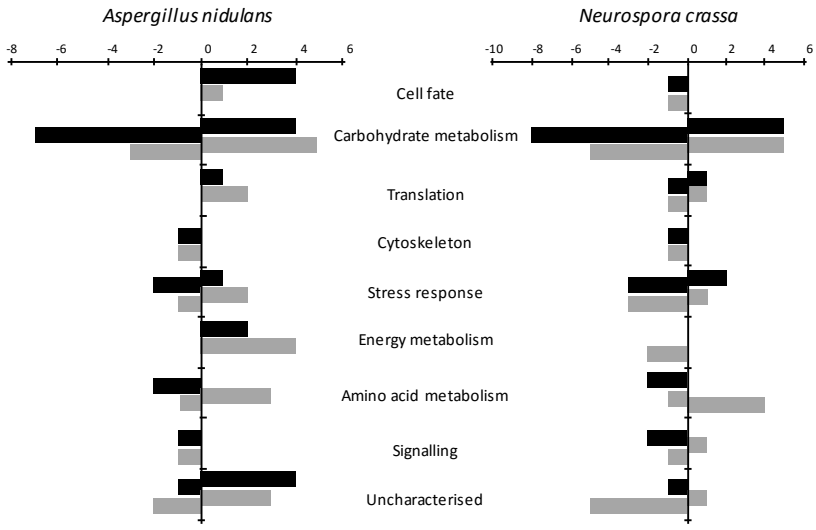

Fig. 2.2. Functional classification of the mycelial protein species identified in the differentially accumulated protein spots in *Aspergillus nidulans* and *Neurospora crassa* cultures after exposure to $[C_2mim]Cl$ or choline. Black and grey stands for $[C_2mim]Cl$ and choline, respectively.

2.4.2. Fungal developmental stage after ionic liquid exposure

Microscopic analysis of the cultures at the end of the incubation period showed that the mycelia and hyphae of both *A. nidulans* and *N. crassa* grown in choline were more robust than those grown in 1-ethyl-3-methylimidazolium chloride or in the control (Fig. 2.3 and 2.4). The altered levels of initiation (eIF6, AN8824 [44] and 5A-2, AN4015 [45]) and elongation (1-gamma, NCU03826 [46]) factors and of a structural 60S ribosomal protein (L5, NCU04331) also emphasise differences in vegetative growth. Accordingly, 1-ethyl-3-methylimidazolium chloride and choline probably led to a decrease and an increase in protein biosynthesis, respectively.

Rho1 GTPase plays a key role in cellular morphogenesis [47]. In *A. nidulans*, its homolog RhoA is responsible for establishment and maintenance of polarity, correct branching and cell wall synthesis [48]. In *N. crassa*, Rho-1 (NCU01484) is also involved in cell wall integrity and hyphal polarisation [47]. Supplementation of the growth media with choline led to decreased Rho-1 in

N. crassa. Even if choline supplementation has been reported to induce morphological alterations in filamentous fungi, reducing branching without altering hyphal extension [21], no obvious alterations were herein detected in hyphal polarisation and branching patterns in *N. crassa* (Fig. 2.4). A compensatory mechanism, that needs further investigation, could justify the decrease in Rho-1. In addition, nucleoside diphosphate kinase (NDK-1, NCU04202) was also increased in choline-supplemented cultures. NDKs are involved in signal transduction from development to stress response [49]; in particular NDK-1 is thought to participate in the control of cellular differentiation, such as branching, aerial hyphae formation, and conidiation [50].

Apoptosis and autolysis have specific cellular and molecular features, which can be interchangeable and difficult to distinguish [51-55]. It can generally be accepted that autolytic events (including cell death), although regulated independently, are inseparable and closely balanced. More than 90% of extracellular chitinase activity measured in *A. nidulans* autolysing cultures was attributed to ChiB (AN4871) [56, 57]. Expression of ChiB was recently shown to be highly coordinated with expression of the β-1,3-endoglucanase EngA (AN0472); both play an essential role in degradation of empty hyphae [6, 58]. ChiB and EngA accumulation, together with higher levels of a proteinase (AN2903, previously detected in autolysing cultures [54]), might imply that 1-ethyl-3-methylimidazolium chloride induced autolysis in *A. nidulans*. 1-Ethyl-3-methylimidazolium chloride exposure also increased putative ATP synthase subunit 4 (AN1534) and ATP synthase alpha chain (AN1523). Mitochondrial alterations have previously been related with increased intracellular ROS levels during apoptosis [59]. Some alkylmethylimidazolium-based ionic liquids have been previously shown to induce apoptosis in eukaryotic cells, apparently in a caspase-dependent manner [8, 60]. This contradicts the prevailing idea that the toxicity of these compounds is associated with necrotic cell death [2]. Fungal genomes contain only type I metacaspases, which have been shown to be involved in cell death regulation [61, 62]. Metacaspases were not detected here amongst proteins responsive to 1-ethyl-3-

methylimidazolium chloride, yet they might have been hidden by more abundant proteins. Supplementation with choline increased EngA but not ChiB in *A. nidulans* cultures. A similar effect has been detected upon exposure to camphotericin; however, the EngA encoding gene was not directly implicated in the response to that toxic [63]. In *N. crassa*, ionic liquids exposure decreased rubredoxin-NAD^+ reductase (a homologue of the apoptosis inducing factor AIF, NCU05850), that has been recently demonstrated to mediate caspase-independent apoptosis in *N. crassa* [64]. In fact, the deletion of AIF encoding gene has increased the resistance of the mutant to phytosphingosine and hydrogen peroxide stimuli for apoptosis-like cell death [65]. In *N. crassa*, AIF has been demonstrated to be localised principally in the cytoplasm, though it is also detected in mitochondria [64, 66]. The authors suggested that AIF may display a fungi-specific role [64]. AIF deficiency in *N. crassa* compromises neither assembly nor function of the mitochondrial respiratory chain [64]. Despite our finding that choline decreased specific polypeptide subunits in two complexes of the respiratory chain (NCU01169, NCU06695, Table 2.4) this is not likely related to AIF decrease.

Autolysis and conidiogenesis seem to be tightly interconnected in *A. nidulans* [58]. The mycelium normally remains entirely undifferentiated in submerged cultures [67], yet there are numerous indications that it can also undergo conidiation [68, 69]. ROS are widely accepted to control cell differentiation in the fungal developmental cycle [70]. In *A. nidulans*, four catalase encoding genes exist, which are differentially regulated along the fungal developmental cycle [67]. Amongst these, only the peroxidase-catalase CatD (AN7388), which is expressed in Hülle cells during sexual development [70, 71], increased upon choline exposure. This observation was validated by microscopic observations of the submerged mycelial mat (Fig. 2.3). By the end of the incubation period, Hülle cells were detected in the cultures in the choline supplemented medium (Fig. 2.3A). Sexual development in submerged cultures has been more often observed in mutants [72], although it was previously reported in common laboratory strains of *A. nidulans* grown under different conditions [73]. These observations require further

investigation, and potentially bring new tools to aid a more in-depth understanding of sexual development in filamentous fungi in long-term cultures.

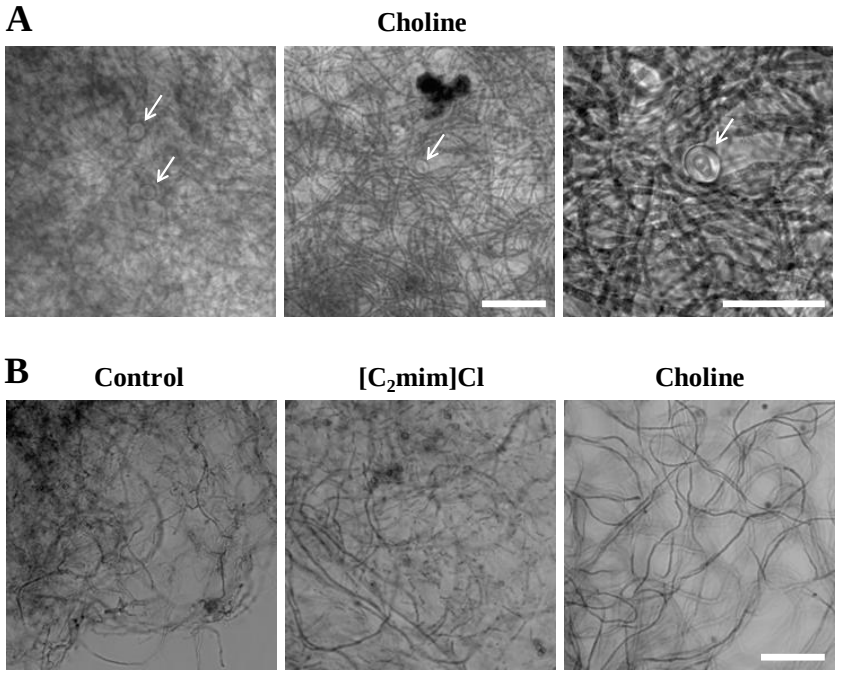

Fig. 2.3. Microscopic images of *Aspergillus nidulans* grown in minimal media (control), minimal media supplemented with $[C_2mim]Cl$ or with choline for fifteen days. Mycelia exposed to choline (A, B), $[C_2mim]Cl$ (B) and control (B). Hülle cells in the choline supplemented media are labelled with white arrows (A). Scale bars: 50 µm.

Microscopic analysis of *N. crassa* cultures along time, showed that supplementation of the growth media with either ionic liquid induced conidiation within two days (Fig. 2.4). In the control culture, conidiation was observed only by the seventh day of incubation. These observations corroborate the differential accumulation of developmentally regulated enzymes. Whilst present throughout the asexual life cycle, the catalases of *N. crassa* are differentially expressed [70]. Cat-1 (NCU08791) accumulates in conidia and during the stationary growth phase; Cat-2 (NCU05770) is associated with cell lysis during conidiation and late stationary phase and Cat-3 (NCU00355) is associated with vegetative growth and the onset of

conidiation. Cat-2 and Cat-3 are both related to early stages of conidiation. Cat-3 increased and Cat-2 decreased in medium supplemented with choline and 1-ethyl-3-methylimidazolium chloride, respectively.

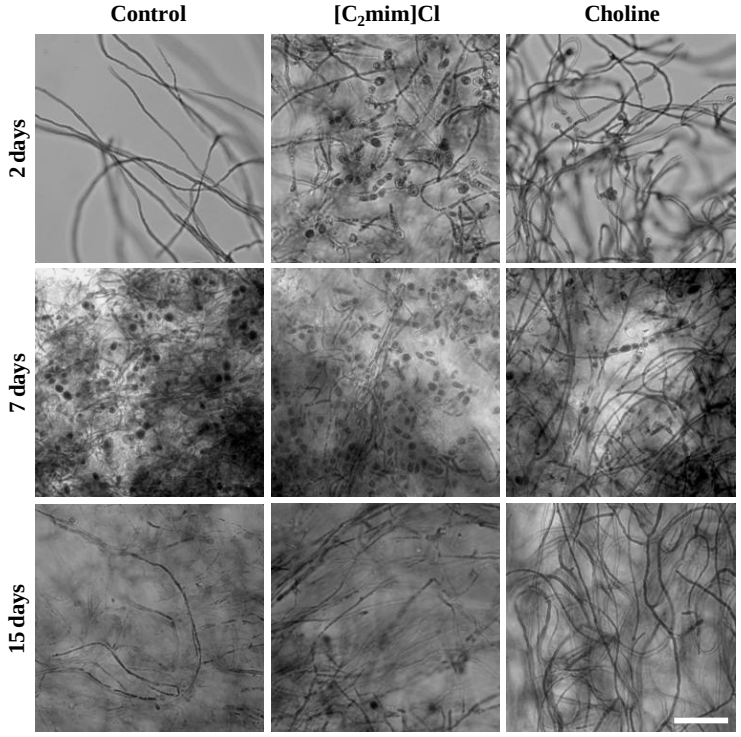

Fig. 2.4. Microscopic images of *Neurospora crassa* grown in minimal media (control), minimal media supplemented with $[C_2mim]Cl$ or with choline for two, seven and fifteen days. Cultures grown in $[C_2mim]Cl$ and choline started conidiating two days after incubation, while in the control, conidiation was only detected after the seventh day. Conidia were observed in all media by the end of the incubation period (not shown). Scale bar: 50 µm.

As aforementioned, mycelia and hyphae of *N. crassa* were seen to be more robust in choline than in 1-ethyl-3-methylimidazolium chloride (Fig. 2.4). While new chains of conidia could still be formed in choline, active conidiation had most probably ceased in 1-ethyl-3-methylimidazolium chloride by the end of the incubation period. Accumulation of formate dehydrogenase (NCU03813) was also

decreased in this medium. Expression of the NCU03813 encoding gene is also developmentally regulated [74]. It is usually detected during conidiation, but not during vegetative growth. Both formate dehydrogenase and Cat-1 have been previously shown to accumulate in the conidial proteome [75, 76]. Absence of Cat-1 in 1-ethyl-3-methylimidazolium chloride was probably due to the fact that the extraction method used here did not efficiently disrupt conidia (data not shown). Despite that formate dehydrogenase typically accumulates in *N. crassa* conidia, its encoding gene presents altered expression levels in various conditions, *e.g.* after growth in media containing plant stem powder [77] or exogenous phytosphingosine [51].

2.4.3. Major alterations in carbohydrate and energy metabolism after ionic liquid exposure

The ionic liquids altered the carbohydrate metabolism in both fungi (Tables 2.3 and 2.4 and Fig. 2.5). Proteins differentially accumulated after 1-ethyl-3-methylimidazolium chloride exposure were comparable in both fungi, with some exceptions. Most glycolytic enzymes decreased, *e.g.* fructose-bisphosphate aldolase (AN2875), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NCU01528) and phosphoglycerate kinase (NCU07914). Downstream pathways, namely TCA cycle, were also affected (isocitrate dehydrogenase, AN1003 and malate dehydrogenase, NCU06211). In previous studies *A. nidulans* cultures exposed to a saline stress have shown altered expression patterns of glycolytic enzymes [78], and increased levels of GAPDH mRNA [79]. Even though such observations suggest glycerol production, none of the *A. nidulans* cultures herein analysed showed similar behaviour. On the contrary, in *N. crassa* glycerol production from glyceraldehyde is suggested in both 1-ethyl-3-methylimidazolium chloride and choline media, by the differential accumulation of aldehyde dehydrogenase (NCU03415) and alcohol dehydrogenase (NCU01754) [78] (Fig. 2.5). These enzymes are also involved in the pyruvate-acetaldehyde-acetate pathway, which is reportedly not the major source of

cytoplasmic acetyl-CoA in phylogenetically related fungi during growth on glucose [80]. Concomitant decrease in acetyl-CoA synthetase (NCU06836) emphasises that glycerol and not acetate was preferentially produced by *N. crassa*. In fact, intracellular glycerol accumulation was detected (see below).

In both fungi 1-ethyl-3-methylimidazolium chloride affected the pentose phosphate pathway, most probably to generate higher NADPH levels, which plays important roles in antioxidant defence [81]. 1-Ethyl-3-methylimidazolium chloride increased some enzymes in the oxidative phase (NCU03100, NCU07281; AN0285) and decreased others in the non-oxidative phase (NCU01328 and AN0688). This was particularly evident in *N. crassa* since 1-ethyl-3-methylimidazolium chloride increased 6-phosphogluconate dehydrogenase (NCU03100). This enzyme, along with glucose 6-phosphate dehydrogenase, reduces $NADP^+$; both are believed to be the main source of NADPH regeneration in the cell [82]. Most filamentous fungi, including *A. nidulans*, exploit glucose primarily through respiration with very low levels of fermentation; *N. crassa* has been reported to ferment glucose to ethanol at an appreciable rate in aerobic culture [83]. Due to this Crabtree-positive effect, the pentose phosphate pathway in *N. crassa* is predominantly used for NADPH production but not catabolic reactions. Similar findings *viz.* inhibition of energy production and catabolism, and stimulation of NADPH regeneration have been reported in proteomic analyses of fungal responses to several toxic compounds, *e.g.* azole [18, 84], farnesol [85], and associated to several stresses (*e.g.* high levels of ROS [20]). Overall, these observations reinforce the high toxicity towards fungi of 1-ethyl-3-methylimidazolium chloride.

Most glycolytic enzymes decreased in the presence of 1-ethyl-3-methylimidazolium chloride; however, two were found to increase, pyruvate kinase (NCU06075) in *N. crassa* and enolase (AN5746) in *A. nidulans*. Vesicular transport of these enzymes has been shown in several *Ascomycota* [86, 87], which suggests that they might be multifunctional [88-90]. Enolase (AN5746) experimental MW and p*I* differ from the predicted (Table 2.3), corroborating the idea of a different sub-cellular localisation [42]. Enolase differential accumulation after farnesol-induced

apoptosis in *A. nidulans* has been recently reported [85], which could further explain its accumulation after 1-ethyl-3-methylimidazolium chloride exposure.

Apart from direct effects in enzymes in the main carbohydrate metabolism pathways, both 1-ethyl-3-methylimidazolium chloride and choline also increased 1-aminocyclopropane-1-carboxylate deaminase (NCU04657) in *N. crassa*. This enzyme mediates the formation of 1-aminocyclopropane-1-carboxylate (Acc). In fungi, as in higher plants, this amino acid can be involved in ethylene production. However, none of the enzymes putatively involved in this pathway, namely Acc oxidase, 2-oxoglutarate-dependent ethylene/succinate-forming enzyme or aromatic aminotransferase [91], were differentially accumulated in cultures here investigated. In some fungi, Acc has been linked to neofrapeptins and acretocins [92, 93], compounds yet to be identified in *N. crassa*. This potentially opens new perspectives regarding the isolation of such fungal metabolites. Preliminary MS analysis showed extracellular compounds with molecular mass values > 1500 Da (data not shown). This is the typical mass range of peptaibiotics. Further amino acid analysis, targeting rare amino acids, would complement the tentative identification of these compounds.

Choline, which is usually biosynthesised endogenously, can also be directly taken-up from the media by some filamentous fungi [21]. Choline is a precursor of phosphatidylcholine, glycine betaine and choline-*O*-sulphate [21, 94]. Exogenous addition of choline to the growth media did not significantly affect glycolysis and downstream pathways in either fungus. In the present study, in *A. nidulans*, four spots were identified as formate dehydrogenase (AN6525), presenting similar molecular weight but p*I* values ranging from 5.8 to 6.0, due to different predicted phosphorylation patterns (Table 2.3). 1-Ethyl-3-methylimidazolium chloride reduced AN6525, especially those showing lower phosphorylation levels. Thus agreeing with the observation that the phosphorylation level of this enzyme decreases with increasing concentrations of NAD^+ [95]. In addition, formate dehydrogenase was also found in decreased levels after exposure to menadione [20] and its mRNA levels decreased upon farnesol exposure [96].

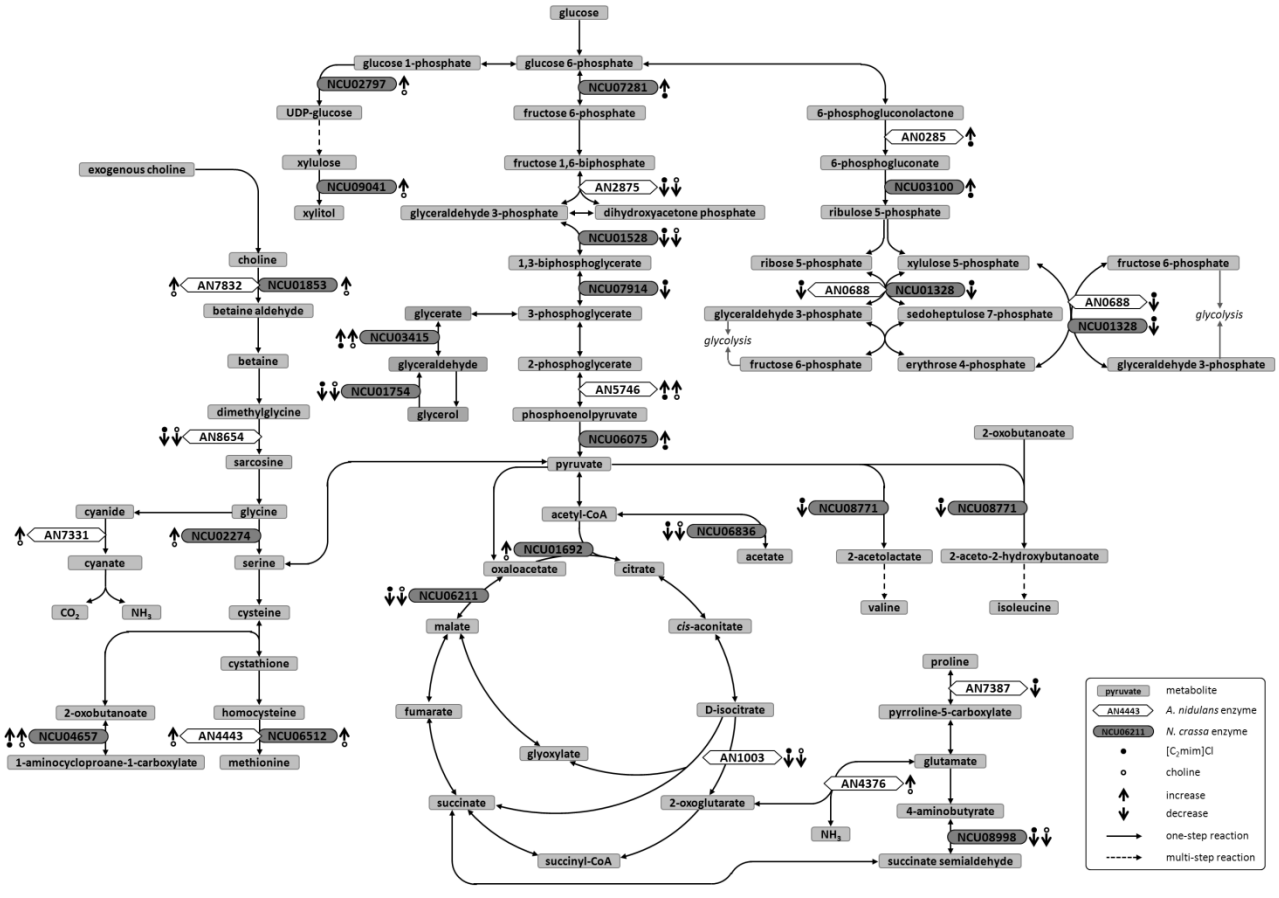

Fig. 2.5. Schematic view of the alterations in carbohydrate and amino acid metabolisms in *Aspergillus nidulans* and *Neurospora crassa* after exposure to [C_2mim]Cl and choline. Many steps and compounds are omitted for simplification (*nb. figure with high resolution available in the thesis CD*).

In *N. crassa*, choline apparently induced production of glycerol (increased and decreased NCU03415 and NCU01754, respectively), and xylitol (increase of UTP-glucose-1-phosphate uridylyltransferase, NCU02797 and L-xylulose reductase, NCU09041) [97, 98]. Based on proteomic data it is probable that glycerol production took place in 1-ethyl-3-methylimidazolium chloride supplemented media. Intracellular levels of glycerol in *N. crassa* upon exposure to 1-ethyl-3-methylimidazolium chloride and choline were 8.1 ± 1.0 and 12.0 ± 2.1 μg/g fresh weight, respectively. These values were higher than the glycerol amounts detected in the control (7.0 ± 0.9 μg/g fresh weight). In all cultures, the values obtained were below those typically detected in a saline stress [99]. Intracellular xylitol was however not detected. Considering high production yields of phylogenetically close species [100], the biomass here obtained (0.33 g ± 0.13 g) would render production of xylitol below the detection limit.

2.4.4. Major alterations in amino acid metabolism after ionic liquid exposure

In both fungi some proteins involved in the synthesis of amino acids and their derivatives decreased after 1-ethyl-3-methylimidazolium chloride exposure (Fig. 2.5). These include acetolactate synthase (NCU08771) and 4-aminobutyrate aminotransferase (NCU08998) in *N. crassa* and pyrroline-5-carboxylate reductase (AN7387) and N,N-dimethylglycine oxidase (AN8654) in *A. nidulans*. Decrease of NCU08771, AN7387 and NCU08998 was probably a direct consequence of glycolysis and TCA cycle repression by 1-ethyl-3-methylimidazolium chloride, reducing isoleucine and valine biosynthesis from pyruvate and proline and glutamate from 2-oxoglutarate. The differential accumulation of AN8654 suggests the accumulation of dimethylglycine - a degradation product of betaine. Based on the differential accumulation of a putative choline oxidase in *A. nidulans* after menadione exposure, it has been suggested that the biosynthesis of betaine was induced [20]. This was corroborated by intracellular accumulation of betaine detected here after exposure to 1-ethyl-3-methylimidazolium chloride (55.27 ± 0.41 mg/g fresh weight), when compared to levels found in the control (6.12 ± 1.86 mg/g fresh weight). The accumulation of betaine (a well-known

osmolyte) has been previously detected as part of the saline stress response in fungi [22, 101]. Addition of betaine to the growth medium supplemented with 0.375 M of 1-ethyl-3-methylimidazolium chloride of *Enterobacter lignolyticus*, resulted in higher biomass yields [14]. Differential accumulation of N,N-dimethylglycine oxidase after exposure to 1-ethyl-3-methylimidazolium chloride further emphasises that betaine might play an important role in multiple stress responses.

As expected, exogenous choline altered several proteins putatively associated with glycine metabolism, including choline dehydrogenases (AN7832, AN0567 and NCU01853), serine hydroxymethyltransferase (NCU02274) and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferases (NCU06512 and AN4443). Increased cyanase (AN7331) suggests that cyanide (product of glycine metabolism) was produced in *A. nidulans* cultures grown in the presence of choline. This enzyme is involved in detoxification of cyanide through conversion to the less toxic cyanate, which is further mineralised to CO_2 and ammonia [102]. In addition, glutamate dehydrogenase (AN4376, NADP-GDH), which is involved in the assimilation of ammonia and is up-regulated by its presence [103], was also increased in medium supplemented with choline.

One response to amino acid limitation is the triggering of a compensatory regulatory network, cross-pathway control [104]. In *A. nidulans*, expression of CpcA, usually in amino acid starvation conditions, activates transcription of more than 50 genes related to amino acid and purine biosynthesis as well as tRNA charging. CpcB (AN4163) acts as a repressor of cross-pathway control under non starvation conditions. It has been shown that deletion of *cpcB* in *A. nidulans* constitutively induces this regulatory network [104]. 1-Ethyl-3-methylimidazolium chloride effected an overall reduction in amino acids biosynthesis, which can justify the observed decrease in CpcB. In *N. crassa*, the homolog Cpc-2 (NCU05810) also had decreased levels in 1-ethyl-3-methylimidazolium chloride. In *A. nidulans*, amino acid starvation and sexual development appear linked by the cross-pathway control. Deletion of *cpcB* leads to a block in sexual development at the microcleistothecial stage, even though auxiliary structures such as Hülle cells are still produced [104]. Choline medium allowed the formation of Hülle cells, but no

other sexual structures were detected (Fig. 2.3A). This phenotype may correlate to the decrease of CpcB in this medium.

2.4.5. Stress response provoked by ionic liquid exposure

Typically fungi respond to various environmental stresses by altering expression levels of numerous genes which lead to major alteration in levels of proteins associated with carbohydrate metabolism or ROS detoxification, signalling, chaperonins, *etc* [52, 105, 106]. In filamentous fungi the major regulators of environmental stress response are yet to be identified [52, 106]. ROS are known to play important roles as signalling molecules in several stress responses [70], and morphological transitions [70], being produced principally in the mitochondrial electron-transport chain. Their intracellular levels are under tight regulation by scavenging and elimination processes. Enzymatic elimination involves superoxide dismutase (SOD), catalases and peroxidases. 1-Ethyl-3-methylimidazolium chloride increased SOD (NCU09560) in *N. crassa* and glutathione S-transferase (AN4905) in *A. nidulans*. ROS production after exposure to alkylmethylimidazolium-based ionic liquids has been reported in eukaryotic cell lines [60, 64] and in *D. magna* [9]; the latter also presented increased levels of glutathione S-transferase. This enzyme promotes ROS detoxification, catalysing the conjugation of glutathione to numerous toxic compounds, *e.g.* menadione exposure has been observed to increase expression levels of AN4905 [107]. The imidazolium cation is not liable to be oxidised; therefore the differential accumulation of glutathione S-transferase is unlikely to be directly correlated with the conjugation of glutathione to the imidazolium cation.

Choline apparently affected the mitochondrial respiratory chain of *N. crassa*, decreasing the NADH-ubiquinone oxidoreductase 24 kDa subunit (NCU01169) and cytochrome *c* oxidase subunit 6 (NCU06695), which belong to complexes I and IV, respectively. Complexes I and IV contain > 35 and > 10 polypeptide subunits, respectively [108-110]. Both the 24 kDa subunit of complex I [111] and subunit 6 of complex IV are required for stability and function of the corresponding multimeric enzymes [110, 112]. Although *N. crassa* has alternative NADH dehydrogenases and cyanide insensitive oxidases [113, 114], choline did not lead to

their differential accumulation. Subunit 6 of complex IV is regulated by several environmental factors [110, 115], in particular osmotic stress [116]. The choline effect on the respiratory chain of *N. crassa* needs further investigation.

Heat shock proteins (HSP), or chaperonins are usually induced under a wide range of stress conditions, *e.g.* temperature and UV [106] and ROS [117]. The requirement of various molecular chaperones is conceivably related to generation of ROS during morphogenesis [70], possibly explaining the accumulation of a heat shock protein 70 (AN11227) in *A. nidulans* in media supplemented with choline. Amongst the uncharacterised proteins a putative major facilitator super-family transmembrane transporter domain (AN12222) was found to increase after 1-ethyl-3-methylimidazolium chloride exposure. This class of proteins has previously been associated with fungal azole resistance [118, 119]. The encoding genes were largely represented among those significantly altered in *E. lignolyticus* after exposure to 0.375 M 1-ethyl-3-methylimidazolium chloride [14]. Finally, although the function of AN7484 protein is unknown; it was detected after exposure to both ionic liquids, and has been previously detected in *A. fumigatus* after exposure *e.g.* to amphotericin B [120] and in *A. nidulans* response to osmotic stress [78].

2.5. Conclusions

To gain further insight into ionic liquid response in *Ascomycota* we identified the differential accumulated mycelial proteins under such conditions. Two very distinct ionic liquids, 1-ethyl-3-methylimidazolium chloride and choline, were selected for this study. These chemicals, regarding toxicity and recalcitrance, are located at opposite extremes. The present study highlighted the higher toxicity of 1-ethyl-3-methylimidazolium chloride in fungi. Data obtained showed that numerous stress-responsive proteins, as well as several critical biological processes and/or pathways, were affected by either ionic liquid. These liquid salts also altered developmental programmes in fungi. In particular, choline and 1-ethyl-3-methylimidazolium chloride promoted, respectively, the development of Hülle cells and of autolysis related proteins in *A. nidulans*, while both prompted conidiation in *N. crassa*. Accumulation of osmolytes, namely glycerol in *N. crassa* and betaine in *A. nidulans*, was also induced. This observation reinforces that betaine may play an important role in multiple stress responses. Exposure to either ionic liquid also decreased AIF levels in *N. crassa*, which might be associated with increased resistance to apoptotic stimuli [65], and further suggests that AIF may display a fungi-specific role. Choline affected two essential subunits for stability and function of complex I and complex IV of the respiratory chain of *N. crassa* [110, 112], but not the levels of alternative NADH dehydrogenases or oxidases. The significance of these observations needs further investigation. Both 1-ethyl-3-methylimidazolium chloride and choline have apparently increased Acc levels in *N. crassa*, which in some fungi has been linked to neoefrapeptins and acretocins [92, 93], compounds yet to be identified in this fungus. Ionic liquid stimuli have been shown to induce major alterations in the fungal metabolic footprint [10]. Recently, we have collected evidence that they alter the expression levels of genes encoding for secondary metabolite biosynthetic enzymes [6]. These data potentially open new perspectives regarding the potential use of ionic liquids to trigger the production of unusual fungal metabolites. The development of novel greener processes for the production of bio-fuel from renewable feedstocks, as well as for production of small molecules

and fine chemicals, is a priority worldwide. Certainly, ionic liquids application in feedstock pre-treatment will further advance [4]. This is also emphasised by the demonstration by our team that cholinium-based ionic liquids are well adapted for the extraction of suberin in high purity from natural composites [27, 121, 122]. As recently proposed by Khudyakov *et al*., the efficient use of ionic liquids in bio-refinery requires better understanding of the mechanisms used by microorganisms to tolerate these "novel" compounds [14]. The biotechnological add-value of fungi is unquestionable, especially due to their ubiquity, enzymatic diversity and resilience [35, 123].

2.6. Acknowledgements

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CHAPTER III

Transcriptomic and metabolomic profiling of ionic liquid stimuli unveils enhanced secondary metabolism in *Aspergillus nidulans*

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Transcriptomic and metabolomic profiling of ionic liquid stimuli unveils enhanced secondary metabolism in *Aspergillus nidulans*

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3.1. Abstract

The inherent potential of filamentous fungi, especially of *Ascomycota*, for producing diverse bioactive metabolites remains largely silent under standard laboratory culture conditions. Innumerable strategies have been described to trigger their production, one of the simplest being manipulation of the growth media composition. Supplementing media with ionic liquids surprisingly enhanced the diversity of extracellular metabolites generated by penicillia. This finding led us to evaluate the impact of ionic liquids' stimuli on the fungal metabolism in *Aspergillus nidulans* and how it reflects on the biosynthesis of secondary metabolites (SMs).

Whole transcriptional profiling showed that exposure to 0.7 M cholinium chloride or 1-ethyl-3-methylimidazolium chloride dramatically affected expression of genes encoding both primary and secondary metabolism. Both ionic liquids apparently induced stress responses and detoxification mechanisms but response profiles to each stimulus were unique. Primary metabolism was up-regulated by cholinium chloride, but down-regulated by 1-ethyl-3-methylimidazolium chloride; both stimulated production of acetyl-CoA (key precursor to numerous SMs) and non proteinogenic amino acids (building blocks of bioactive classes of SMs). In total, twenty one of the sixty six described backbone genes underwent up-regulation. Accordingly, differential analysis of the fungal metabolome showed that supplementing growth media with ionic liquids resulted in *ca.* 40 differentially accumulated ion masses compared to control conditions. In particular, it stimulated production of monodictyphenone and orsellinic acid, otherwise cryptic. Expression levels of genes encoding corresponding polyketide biosynthetic enzymes (*i.e.* backbone genes) increased compared to control conditions. The corresponding metabolite extracts showed increased cell polarity modulation potential in an *ex vivo* whole tissue assay (*The*lial Live Targeted Epithelia; *the*LiTE™).

Ionic liquids, a diverse class of chemicals composed solely of ions, can provide an unexpected means to further resolve the diversity of natural compounds, guiding discovery of fungal metabolites with clinical potential.

3.2. Introduction

Multiple and diverse fungal secondary metabolites (SMs) are already in clinical usage, *e.g.* the antibiotic penicillin and the antitumor terrequinone A [1]. The inherent SM biosynthetic capacity of fungi remains largely unseen because the majority of these pathways are largely silent (cryptic) under culture conditions used in the laboratory [2]. The presence of various SM backbone genes (encoding non-ribosomal peptide synthetases, polyketide synthases, hybrid enzymes, prenyltransferases or terpene cyclases) in fungal genomes hints at the presence of an array of uncharacterised SMs. For example, model fungal species *Aspergillus nidulans* has sixty six predicted backbone genes [3]; approximately one third of these clusters have been linked to the full range of produced SMs, including monodictyphenone and prenyl xanthones [4, 5], asperfuranone [6], emericellamides [7], aspyridone A/B [8], asperthecin [9] and terrequinone A [1, 10], aspernidine A [11], sterigmatocystin [12], penicillin [13], nidulanin A [14], microperfuranone [15], cichorine [16], orsellinic acid and F9775 A/B [17], austinol and dehydroaustinol [18] and aspercryptin [19].

Closing the gap between genetic potential and the observed diversity of fungal SMs produced constitutes a major challenge [20], further complicated by low production titres and the need for specific stimuli to trigger synthesis [21]. Several strategies have been described to stimulate production of particular SMs; some require prior knowledge of genomic sequences, relying on manipulation of targeted genes encoding components of either secondary metabolism [22] (*e.g.* aspoquinolones A-D [23]) or regulatory pathways (*e.g.* monodictyphenone [24] and asperthecin [9]). Other approaches may be applied also in less well characterised strains, such as co-cultivation methods (*e.g.* culturing together *Emericella* spp. and *Salinispora arenicola* triggers production of two cyclic depsipeptides [25]) or modification of the growth media composition (*e.g.* addition of sodium citrate or suberoylanilide hydroxamic acid increased the production of terrein in *A. terreus* [26] and nygerone A in *A. niger*, respectively [27]). We have surprisingly observed

that supplementation of growth media with ionic liquids can significantly increase diversity of compounds in the metabolic footprint of penicillia [28]. Ionic liquids comprise a diverse class of chemicals, composed solely by ions and are commonly classified as alternative green solvents (despite many having pronounced toxic effects) [29]. They are also referred to as task-designed solvents, because their properties can be tuned through simple modification of the structure of either ion [30]. More than 10^9 different formulations are theoretically possible, with thousands already available commercially. Recently we analysed the major cellular responses of *A. nidulans* to either cholinium chloride or 1-ethyl-3-methylimidazolium chloride exposure [31]. Selected chemical stimuli are representative of the most studied families of ionic liquids and represent opposite ends of the spectrum regarding toxicity and recalcitrance. Both compounds increase numerous mycelial stress-responsive proteins (*e.g.* drug transporter proteins) and induce particular developmental changes and production of certain osmolytes [31]. Extracellular compound diversity was apparently greater in *A. nidulans* grown in media supplemented with either ionic liquid than in control cultures. In summary the possibility of ionic liquids being able to activate cryptic SM biosynthetic pathways in fungi deserves further investigation. Here we analyse how cholinium chloride and 1-ethyl-3-methylimidazolium chloride impact on both primary and secondary metabolism in *A. nidulans*. Differential analyses of the fungal metabolome were combined with targeted gene expression analysis and transcriptional profiling (custom Affymetrix microarray [32]). Data highlight ionic liquid's capacity to impact both on primary and secondary metabolism, stimulating SM biosynthesis (*e.g.* the cryptic SM monodictyphenone). The diversity of differentially formed metabolites apparently comprised also unknown compounds with cell polarity modulation potential (*the*LiTE™). This study sheds first light on the vast potential of ionic liquids to reveal the diversity of natural compound biosynthesis potential in fungi.

3.3. Materials and Methods

3.3.1. Chemicals

All standard chemicals (toluhydroquinone, orcinol, epoxysuccinic acid, phenoxyacetic acid, 2,5-dihydroxybenzoic acid (gentisic acid), fusaric acid, 3-(3,4-dihydroxyphenyl)-2-propenoic acid (caffeic acid), propyl-3,4,5-trihydroxybenzoate, jasmonic acid, sterigmatocystin, penicillin G, physcion and riboflavin) and chromatographic solvents were of highest analytical grade and purchased from either Sigma Aldrich or Fisher Scientific, except ethyl acetate (Acros Organics), orsellinic acid (Alfa Aesar) and chrysophanol (Acros Organics). Water was obtained from a Milli-Q system (Millipore). Cholinium chloride (>98%, Sigma Aldrich), hereafter referred to solely as choline, and 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl, >98%, Iolitec) were dried *in vacuo* (40-70 °C, 24-48 h, *ca.* 0.01 mbar) prior to use. Monodictyphenone was kindly provided by Prof. Thomas J. Simpson (University of Bristol, UK) [33].

3.3.2. Fungal strain

Aspergillus nidulans strain FGSC A4 was cultivated on dichloran-glycerol (DG18) agar (Oxoid), and suspensions of fungal conidia, prepared as previously described [31], were stored at -80 °C in cryoprotective solution containing 0.85% w/v NaCl and 10% v/v glycerol.

3.3.3. Experimental conditions

Fungal cultures (5 mL or 50 mL) were initiated from conidia (10^5 conidia *per* mL) in a 0.1% glucose mineral growth media [31] alone (control) or containing 0.7 M [C_2mim]Cl or choline (dosage equivalent to 50% of the minimal inhibitory concentration of [C_2mim]Cl [31]). Liquid cultures (triplicates) were incubated in the dark at 27 °C with orbital agitation (90 rpm), for defined periods of time (2, 5, 7, 10 or 15 days). At the end of incubation, fungal mycelia (mostly submerged) were

recovered by filtration (glass fibre pre-filters) and both mycelia and filtrate were immediately frozen in liquid nitrogen and stored at -80 °C, until further analysis.

3.3.4. Microscopic analysis

Mycelia were recovered from fungal cultures after fifteen days of incubation (50 mL; inoculated and incubated as described above, triplicate samples), mounted on glass slides and stained with lactophenol blue to enhance contrast. Visualisation of cultures was performed using a DM5500 B microscope (Leica) with 40× or 63× magnification objectives and images were captured with a DFC420 C camera (Leica).

3.3.5. Total RNA extraction and cDNA synthesis

Total RNA was isolated from mycelia (previously ground to a powder using mortar and pestle in liquid nitrogen) using the RNeasy Plant Mini Kit (QIAGEN) and further purified following standard ethanol precipitation. Quantity and quality of RNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and RNA integrity assessed by using an Agilent 2100 Bioanalyser with a RNA 6000 Nano Assay (Agilent Technologies). cRNA was fragmented and biotinylated according to GeneChip 3' IVT Express Kit protocols. Briefly, 100 ng total RNA were used for cDNA synthesis, which was *in vitro* transcribed to generate labelled cRNA. After purification and fragmentation, the size distribution of cRNA and fragmented cRNA was assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay.

3.3.6. DNA microarray processing

The custom DNA microarray FungiANC (Affymetrix) was used in this study [32]. The chip contains a total of 20,012 transcripts derived from the genetic information of *A. nidulans* and *Neurospora crassa* (Broad Institute Database, www.broadinstitute.org) and is based on a Perfect Match-only design with 11 micron feature size. Each transcript is represented by 11 oligonucleotides of 25-*mer*

(detailed description in Additional File 1*). The array was processed following Affymetrix GeneChip protocols, in biological triplicates. A total of 200 µL hybridisation mixture containing 10 µg of fragmented cRNA was hybridised to arrays for 16 hours at 45 °C. Standard post-hybridisation washes and double-stain protocols (FS450_0001) were used on an Affymetrix GeneChip Fluidics Station 450, in conjunction with the GeneChip Hybridisation Wash and Stain Kit (Affymetrix). Arrays were scanned on an Affymetrix GeneChip Scanner 3000 7G. All array quality parameters were analysed by Expression Console Software (Affymetrix) for Robust Multiarray Averaging (summarised data) and confirmed to be in the recommended range. The data herein presented have been deposited in NCBI's Gene Expression Omnibus [34] and are accessible through GEO Series accession number GSE65946 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65946).

3.3.7. Microarray data analysis

Microarray data analysis was performed using DNA-Chip Analyzer (dChip) software (www.dchip.org, 2010), applying a probeset mask file considering only *A. nidulans* probes (9674 transcripts). Arrays were normalised to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method [35, 36]. Normalised CEL intensities of the nine arrays were used to obtain model-based gene expression indices based on a Perfect Match-only model [35, 36]. Log2 expression data produced by dChip was imported into R v2.13.0 and differential gene expression analysed with the Bioconductor LIMMA package (www.bioconductor.org) [37]. Principal component analysis and volcano plots were obtained to validate biological replicates and visualise distribution of statistically significant data from each biological condition. Differentially expressed genes (adjusted p-value ≤ 0.05, $|FC| \geq 1.5$), identified using pair-wise comparison between each condition and control (grown on glucose) (Additional File 2*), were analysed with Venn diagrams (Venny, http://bioinfogp.cnb.csic.es/tools/venny/index.html).

* *available in the thesis CD.*

3.3.8. Functional annotation

Annotation of all genes represented on the DNA microarray was obtained from the Broad Institute Database and the *Aspergillus* Genome Database (www.aspgd.org). Full details are given in Additional File 2[*]). Differentially expressed genes for each condition were classified using the FungiFun web annotation tool (https://sbi.hki-jena.de/FungiFun) [38]. Significant hits (p-value ≤ 0.05) were defined using the identities present on the chip as background.

3.3.9. Quantitative real-time PCR analysis

All quantitative real-time PCR (*q*RT-PCR) oligonucleotide pairs – based on *A. nidulans* gene sequences (*Aspergillus* Genome Database, www.aspgd.org) – were designed using the GeneFisher2 web tool (http://bibiserv.techfak.uni-bielefeld.de/genefisher2) and produced by Thermo Fisher Scientific (Table 3.1). *q*RT-PCR analyses were performed in a CFX96 Thermal Cycler (Bio-Rad), using the SsoFast EvaGreen Supermix (Bio-Rad), 250 nM of each oligonucleotide and cDNA template equivalent to 1 ng total RNA, in a final volume of 10 µL *per* well (three technical and three biological replicates). PCR conditions were: enzyme activation at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 59 °C for 30 s; and melting curve obtained from 65 °C to 95 °C, consisting of 0.5 °C increments every 5 s. Data analysis was performed using the CFX Manager Software v3.0 (Bio-Rad). Expression of each gene was calculated relative to control. Expression of all target genes was normalised to the expression of the histone H3 gene, used as internal control.

[*] *available in the thesis CD.*

Table 3.1. List of the designed *q*RT-PCR oligonucleotides (forward and reverse) used in this study for the validation of the microarray data. The selected genes are involved in secondary metabolism regulation and biosynthesis of *Aspergillus nidulans*. Due to its constant expression in all conditions, the histone protein H3 gene (AN0733) was selected as internal control.

Gene (Code)	Forward oligonucleotide (5′ to 3′)	Reverse oligonucleotide (5′ to 3′)
veA (AN1052)	TCAGAGCTCCCATCGACCA	GCCGGTCATCATGACCGAA
velB (AN0363)	CCCAAGCCCAGTACTACCAA	CGGTATGCACTGGCACTCA
laeA (AN0807)	ATGGGTTGCGGTAGTGTCA	CAATGCCGTTCCATCTAGTGA
rsmA (AN4562)	CCTCCGCGCTACAACACA	AGTCCGTAGGAGAGTAGACCA
fluG (AN4819)	AGAACCGTGAGGCGCCTA	GTATATCCCGCAGCCAGGAA
hdaA (AN8042)	CATCCAGGGAAGGACTTAGTGA	TTGGGAGCGGCCTTGGTA
ausA (AN8383)	TACAACCCTCCACCCAGCA	TCGGGCCCTGTAGTTGCA
hepA (AN1905)	GGCGAAGATAAAGGAAAGCGTA	CTTCTTGCCGTTATTCCAGTGA
gcnE (AN3621)	AATGGATCTGTCGACGATGGA	GTTCGCGCTCTTGGCGTA
easB (AN2547)	ATCGCCTCCGACGACGAA	GTGTTGCCGCTGATAGGAGA
mdpG (AN0150)	CGTCAAGATGATTCAGACCGAA	GCCGTGAAGAACCTATTAAGCA
orsA (AN7909)	CACCATTTTCCTGCGCACA	GAGGATGATAAGGGCCTGGA
sidC (AN0607)	CATTCAGAGATCCCTCGCAGA	AAGTGCTGTCCTGCATCCAA
H3 (AN0733)	TGAGATCCGTCGCTACCAGA	AACGGACTCCTGGAGAGCA

3.3.10. Metabolite extraction and analyses

Lyophilised culture filtrates were homogenised in Milli-Q water and extracted three times with ethyl acetate (1:1), dried under soft nitrogen flow and resuspended in a minimal volume of methanol. Preliminary mass spectrometry analyses of the extracts showed much higher abundance of differential *m/z* in negative mode compared to positive mode (data now shown). For that reason, ultra-high performance liquid chromatography-electrospray ionisation-high resolution mass spectrometry (UHPLC-ESI-HRMS) analyses of metabolite extracts were performed in negative mode using a Q-Exactive Orbitrap MS system (Thermo Fisher Scientific) equipped with a heated electrospray ionisation source (HESI-II) [39]. Chromatographic separation was carried out in an UHPLC system (Accela; ThermoFisher Scientific) using an Ascentix Express C18 (150 × 2.1 mm, 2.7 μm particle size) column from Supelco (USA). The mobile phase, at a flow rate of 300 μL/min, consisted of a solution of 0.1% formic acid (solvent A) and a solution of acetonitrile containing 0.1% formic acid (solvent B), set as follows: 10% B in 1 min,

followed by a liner gradient of 10–95% B in 4.7 min, 1.3 min to reach 100% B, 3 min of 100% B, 0.5 min to return to the initial conditions, and 5.5 min to re-equilibrate the column. HESI-II was operated in negative ionisation mode. Nitrogen was used as a sheath gas, sweep gas and auxiliary gas at flow rates of 60, 0 and 10 a.u. (arbitrary units), respectively. Heater temperature was set at 350 °C. Capillary temperature was set at 320 °C and electrospray voltage at − 2.5 kV. An S-Lens RF level of 50 V was used. Q-Exactive Orbitrap MS system was tuned and calibrated using ThermoFisher calibration solution once a week. The HRMS instrument was operated in full MS scan with a *m/z* range from 50 to 600, and the mass resolution tuned into 70,000 full width half maximum (FWHM) at *m/z* 200, with an automatic gain control (AGC) target (the number of ions to fill C-Trap) of 5.0E5 with a maximum injection time (IT) of 200 ms. The full MS scan was followed by a data-dependent scan operated in All Ion Fragmentation (AIF) mode with a fragmentation energy applied of 30 eV into the high-energy collision dissociation (HCD) cell. At this stage, mass resolution was set at 17,500 FWHM at *m/z* 200, AGC target at 5.0E5, maximum IT at 200 ms, and the scan range also from *m/z* 50 to 600. MS data were processed by ExactFinder™ v2.0 software (Thermo Fisher) by applying a user target database list, comprising nearly one thousand four hundred SMs previously described in *Ascomycota* (Additional File 3[*]). Parameters including retention time, accurate mass errors and isotopic pattern matches were used in preliminary manual compound identification. Analytical standards of monodictyphenone, chrysophanol, orsellinic acid, toluhydroquinone, orcinol, epoxysuccinic acid, phenoxyacetic acid, 2,5-dihydroxybenzoic acid (gentisic acid), fusaric acid, 3-(3,4-dihydroxyphenyl)-2-propenoic acid (caffeic acid), propyl-3,4,5-trihydroxybenzoate, jasmonic acid, sterigmatocystin, physcion and riboflavin were used for compound identity validation, applying following criteria: $\Delta RT \leq 0.2$ minutes and $\Delta(m/z) \leq 5$ ppm. Mycelial accumulation of betaine was quantified as previously reported [31].

[*] *available in the thesis CD.*

3.3.11. *Ex vivo* assay for anti-cancer activity

Cell polarity is central to onset and progression of diseases including carcinoma; validated assay *the*LiTE™ measures polarity modulating activity of compounds in live *Drosophila* tissues. All tests were conducted as disclosed in published US patent application 20130136694. Briefly, egg chambers are extracted from female *Drosophila* less than 7 days old and exposed to metabolites (pure compounds and both the crude metabolite extracts and their polar fractions [40]) at standardised concentrations in Schneider's culture medium under controlled standard atmospheric conditions for up to 6h. Egg chambers are observed using standard light and fluorescence microscopy and scored for presence/absence of polarity marker protein Par6. Each assay is done in triplicate. Controls for these assays included pure compounds: *the*-103 (a functional equivalent of aurothiomalate [41], which displays 100% activity in *the*LiTE™), monodictyphenone, orsellinic acid and either ionic liquid, as well as the blank (0.6% v/v DMSO) and metabolite extracts of the control cultures and the non-inoculated media.

3.4. Results and Discussion

3.4.1. *Aspergillus nidulans* differential metabolic footprints under ionic liquids stimuli

The diversity of compounds in the metabolic footprint of fungi depends on growth media composition (*e.g.* carbon and nitrogen sources availability) [42]. Simple, systematic compositional alterations allow discovery of multiple SMs in a single producing organism [43]. This strategy, usually known as the "one strain-many compounds approach", provides high flexibility for screening poorly characterised strains. Adding sub-lethal concentrations of an ionic liquid to the growth media of *Penicillium* spp. [28] or *A. nidulans* [31] augmented the diversity of compounds in the culture footprints. High resolution spectrometric analyses of *A. nidulans* metabolite extracts were performed to expand our initial findings. Compared to control conditions ionic liquids' stimuli altered the fungal metabolic footprint, increasing the diversity of metabolites (Fig. 3.1).

By applying a user target list of Ascomycota SMs, a list of differential ion masses (*m/z*) detected under an ionic liquid stimulus, when compared to the control, could be produced (Additional File 3[*]). There were *ca.* 40 differential ion masses detected in either ionic liquid medium when compared to the control, with 24 and 18 specific to choline or 1-ethyl-3-methylimidazolium chloride supplemented media, respectively (Additional File 3[*]).

Most ion masses matched compounds present in our user target list but only six compound identifications could be validated with standards (Table 3.2). These included four known metabolites of *Aspergillus*: orcinol [44, 45], phenoxyacetic acid [46], orsellinic acid [17] and monodictyphenone [24], as well as gentisic acid and caffeic acid which, to the best of our knowledge, are reported here for the first time in *A. nidulans*. Gentisic acid has previously been detected in *Penicillium griseofulvum* [47], whereas caffeic acid was reported only once in a fungal metabolite screening [48]. None of the remaining putative identifications could be

[*] *available in the thesis CD.*

validated with the corresponding standards, *e.g.* chrysophanol and sterigmatocystin. None of the ion masses identified here matched penicillin, confirming its absence.

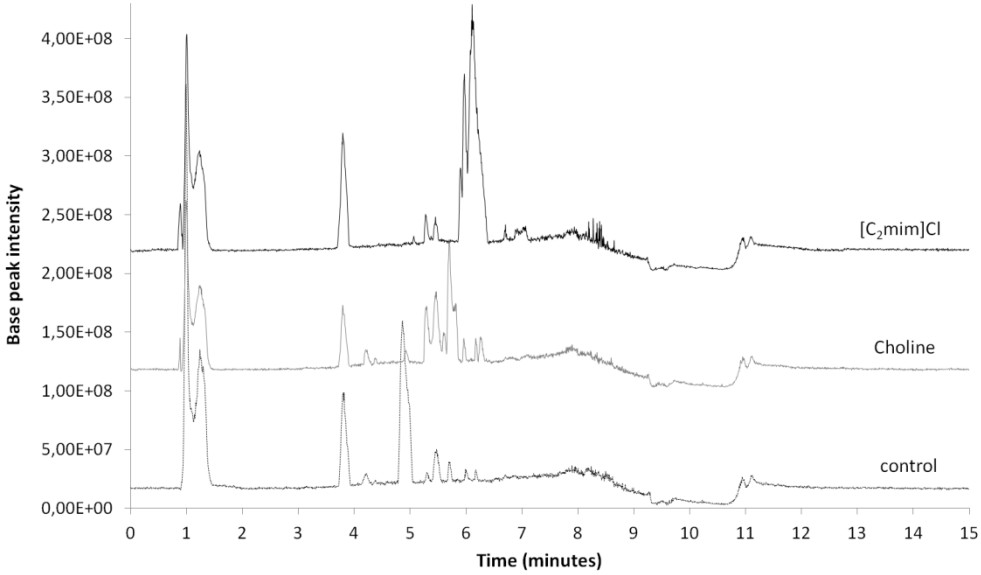

Fig. 3.1. Chromatographic analyses of the metabolic footprint of *Aspergillus nidulans* under ionic liquid stimuli. The base peak intensity chromatograms of the culture extracts after fifteen days of incubation in either choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media revealed higher diversity of metabolites when compared to the control.

Among identified compounds we found two already characterised cryptic SMs (Table 3.2). Monodictyphenone, exclusively found in choline supplemented media, and orsellinic acid, found with both ionic liquid supplements (Fig. 3.2). Monodictyphenone is a product of the monodictyphenone biosynthetic pathway in *A. nidulans* [4, 5, 24, 33], initially characterised in a Δ*cclA* strain (*n.b. cclA* encodes a methyltransferase known to impact secondary metabolism) [24]. Orsellinic acid has been detected in *A. nidulans* during co-cultivation with *Streptomyces hygroscopicus* [49] and, more recently, also in sucrose supplemented media [17]. The production of the otherwise cryptic derivatives of the monodictyphenone cluster

(*e.g.* emodin and chrysophanol), and orsellinic acid, was stimulated in *A. nidulans* grown in continuous fermentation under nutrient limited conditions [50].

Table 3.2. Metabolites differentially produced under ionic liquids stimuli. UHPLC-ESI-HRMS differential analyses of putative compounds (ion masses) detected in the metabolite extracts of either choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media, compared to the control. Compound identifications were validated by the corresponding standards.

m/z	Choline	[C_2mim]Cl	Molecular formula	Compound identification	Reference
123.0450	[a]	✓	C7H8O2	orcinol	[34, 35]
151.0402		✓	C8H8O3	phenoxyacetic acid	[36]
153.0195	✓		C7H6O4	gentisic acid	[38, 39]
167.0348	✓ [b, c]	✓ [b, c]	C8H8O4	orsellinic acid	[37, 39]
179.0352	✓	✓	C9H8O4	caffeic acid	[39]
287.0566	✓ [c]		C15H12O6	monodictyphenone	[14]

a, a compound reported equal *m/z* value but was not validated as orcinol; b, vestigial amounts also found in the control; c, also detected in early time-points: monodictyphenone was detected after 5 and 10 days of incubation in choline media; and orsellinic acid was detected after 5 days of incubation in choline medium and after 10 days of incubation in 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) medium.

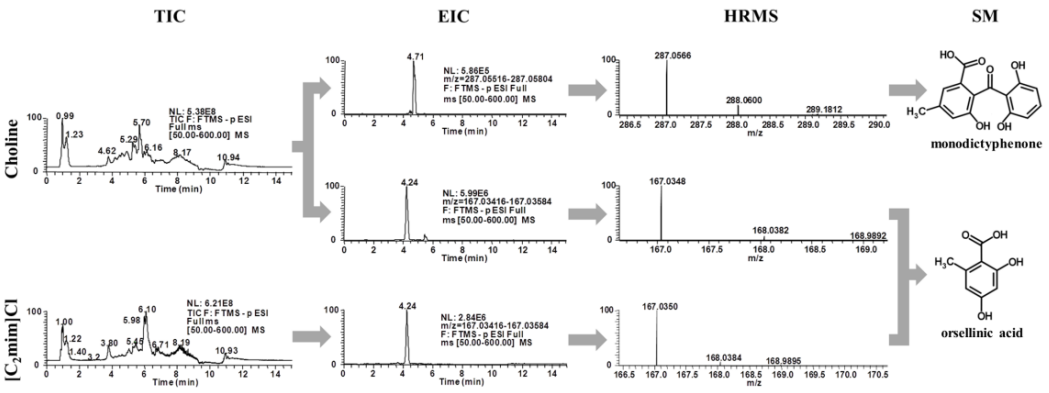

Fig. 3.2. Total ion chromatogram (TIC) of *Aspergillus nidulans* metabolite extracts under ionic liquid stimuli. TIC derived from fifteen day cultures in choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media. Extracted ion chromatograms (EIC) and respective HRMS spectra for peaks corresponding to monodictyphenone and orsellinic acid are also presented.

3.4.2. Transcriptome profiling of *Aspergillus nidulans* during exposure to an ionic liquid

To shed light on the impact of ionic liquids on the *A. nidulans* transcriptome, including genes encoding components involved in secondary metabolism – either biosynthesis or regulation – whole-genome profiling analysis (FungiANC) [32] was performed. Raw signal intensities for all genes are given in Additional File 2*. Principal component analysis showed that the biological replicates of each condition were clustered together (implying a very low degree of replicate variation in transcript levels) and dissociated from the remaining clusters (Fig. 3.3A). Volcano plots clearly show a great number of transcripts with highly significant differential expression (Fig. 3.3B). Pair-wise comparison was used to identify genes expressed differentially between the control and cultures grown in ionic liquid media for fifteen days (adjusted p-value ≤ 0.05 and $|FC| \geq 1.5$). In either ionic liquid media *ca.* 35% of transcripts (total of 9674) showed altered expression levels, but only *ca.* 6% were altered by both (Fig. 3.3C). Choline supplementation led to up- and down-regulation of 1252 and 811 transcripts, respectively. 1-Ethyl-3-methylimidazolium chloride supplement, up-regulated 1207 transcripts and down-regulated 1271 transcripts. According to the functional categories database of the Munich Information Center for Protein Sequences (MIPS), the differentially expressed genes were enriched in the categories of metabolism (MIPS 01), energy (MIPS 02), transcription (MIPS 11) and protein synthesis (MIPS 12). In general, most of these functional categories were up-regulated in choline medium but down-regulated in 1-ethyl-3-methylimidazolium chloride medium (Fig 3.4). These findings match those of our previous proteomic profiling study [31], which reflect the distinct biodegradability and toxicity of these ionic liquids.

* *available in the thesis CD.*

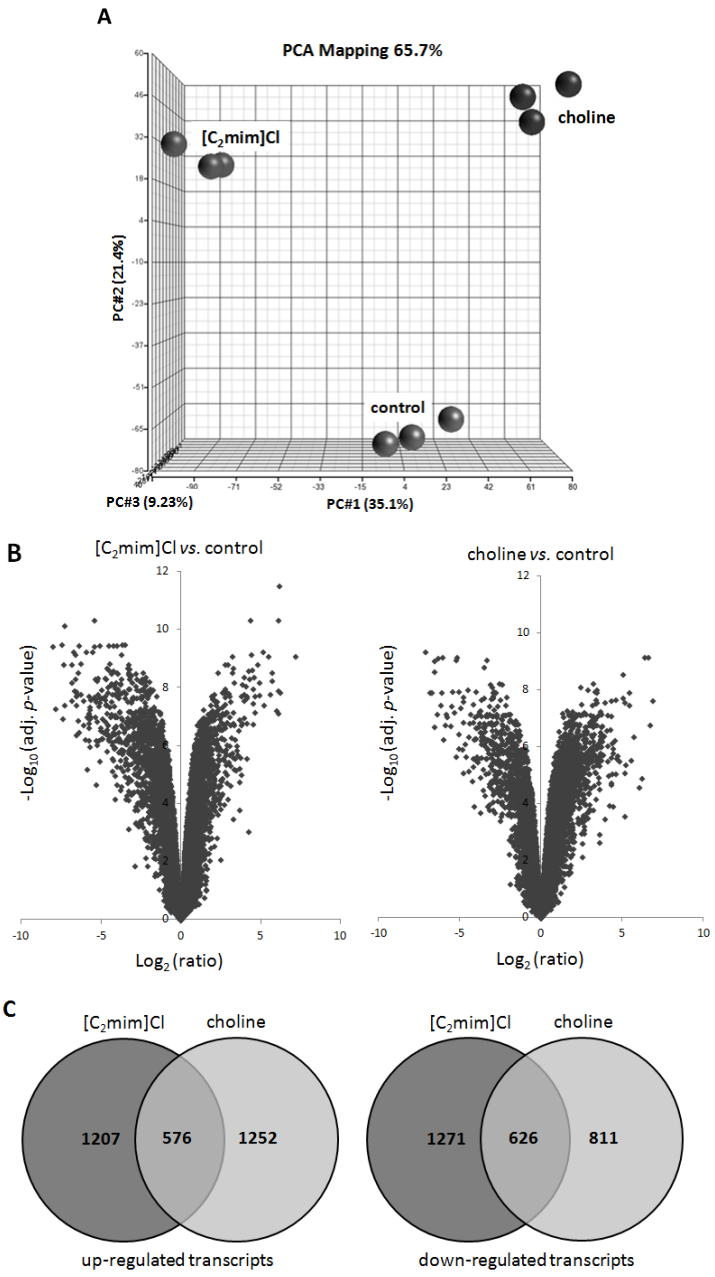

Fig. 3.3. Overview of *Aspergillus nidulans* microarray data. A) Principal component analysis. B) Volcano plots: plotting the statistical significance of the microarray data ($-\log_{10}$ adjusted *p*-value) against the fold-change ($\log_2$ ratio) for each different tested media. Significant genes have positive ($-\log_{10}$) adjusted *p*-values and they can show different levels of fold-changes. C) Venn diagrams: number of differentially expressed genes in the different tested media versus the control.

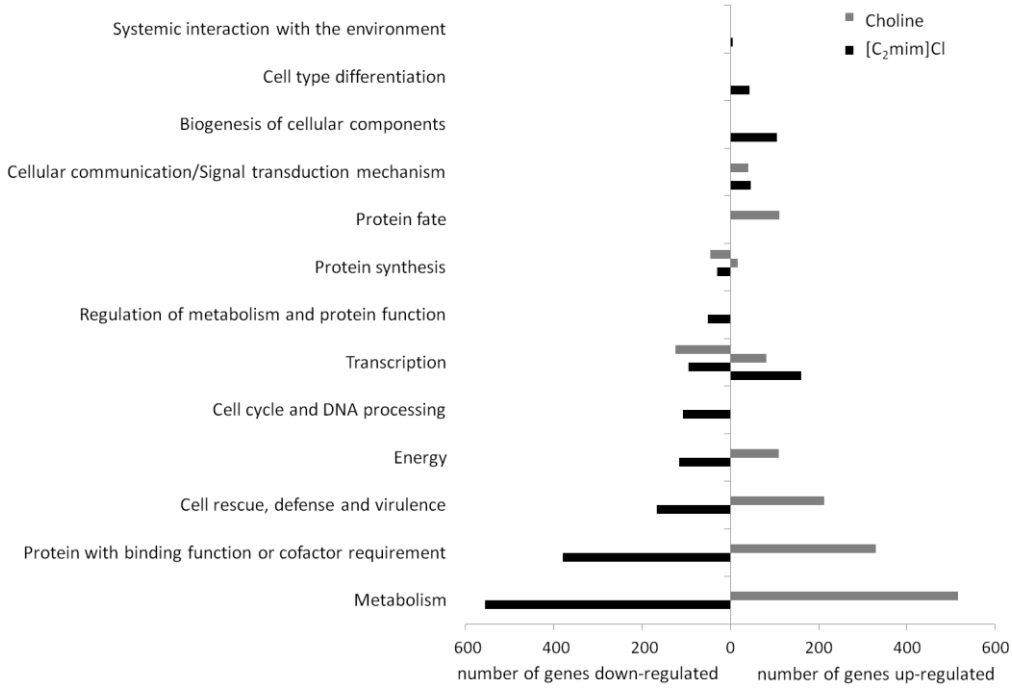

Fig. 3.4. Functional Categories altered in *Aspergillus nidulans* after exposure to choline (grey bars) and 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl, black bars).

3.4.3. Ionic liquids' impact in *Aspergillus nidulans* stress response and primary metabolism

Filamentous fungi respond to very diverse environmental stresses by activating different signalling transduction cascades [51-54]. Extracellular signals are usually sensed and transmitted to response regulators [55] that also impact other processes, ranging from asexual development and cell wall integrity to fungicide sensitivity [53]. *Aspergillus nidulans* can tolerate saline concentrations considerably higher than those used here [56]. Both ionic liquids up-regulated the response regulator of the high osmolarity glycerol (HOG) pathway – *sskA* (AN7697) (Table 3.3), but none of the downstream elements (*pbsB*, AN0931; *hogA*, AN1017; *sskB*, AN10153) nor any transcription factors regulated by this pathway (*e.g. atfA*, AN2911 and *srrA*, AN3688) [57]. In 1-ethyl-3-methylimidazolium chloride supplemented medium, *sskA* up-regulation occurred together with genes coding for catalase A (*catA*,

AN8637), glycerol-3-phosphate dehydrogenase (*gfdB*, AN6792), trehalose-6-phosphate phosphatase (*orlA*, AN3441), neutral trehalase (*treB*, AN5635) and NADP(+)-dependent glycerol dehydrogenase (*gldB*, AN5563) (Table 3.3), strongly suggesting the formation of stress-tolerant conidia [53, 58]. In fact, conidia formation was observed in the floating mycelia at the surface of the liquid media in both cultivation conditions.

It has previously been suggested that 1-ethyl-3-methylimidazolium chloride can induce autolysis in *A. nidulans* [31], a process of self-digestion of aged hyphae [59]. Proteome profiling showed the increase of two autolysis hallmark proteins, β-1,3-endoglucanase (EngA, AN0472) and chitinase B (ChiB, AN4871), during growth in ionic liquid supplemented media [31]. The corresponding transcripts were not found to be up-regulated here. Up-regulation of AN10213 (autophagy protein Apg6) and AN6360 (homolog of ATG17) suggests autophagy was occurring (Table 3.3). This process is related to nutrient recycling during starvation and has been shown to precede autolysis [60]. Microscopic analysis showed that by incubation day fifteen, mycelia and hyphae of *A. nidulans* grown in choline supplemented medium were more robust than those grown in 1-ethyl-3-methylimidazolium chloride supplemented medium, which in turn were similar to the control culture (Fig. 3.5A). The formation of Hülle cells was detected in the choline medium (Fig. 3.5B), as also seen in our previous study [31]. This is consistent with the observed up-regulation of the catalase D gene (*cpeA*, AN7388) (Table 3.3), induced in these specialised cells during sexual development [61].

Aspergillus nidulans is able to take up choline as a source of carbon and nitrogen [62]. In choline supplemented medium a great number of genes involved in the central carbon and amino acids metabolisms were up-regulated (Fig. 3.6, Table 3.4), suggesting activation of primary metabolism. Choline can be phosphorylated and incorporated into phosphatidylcholine, a principal constituent of cellular membranes [63]. Excess of phosphatidylcholine is counterbalanced by its degradation into for example 1,2-diacylglycerol, as suggested by the up-regulation of

the phospholipase C gene (*plcB*, AN7691) (Additional File 2[*]). In our previous proteomic study [31], we observed that choline was most likely taken up and metabolised via the glycine, serine and threonine metabolic pathway, and incorporated into the central carbon metabolism. The transcriptomic data reported here further confirm these observations. Up-regulation of betaine aldehyde dehydrogenase (AN1430) and dimethylglycine oxidase (AN8654) supports the hypothesis that choline enters the primary metabolism through formation of betaine aldehyde and betaine, which is further converted to glycine (Fig. 3.6). The three-fold accumulation of betaine in the mycelia under choline supplementation when compared to the control (39.43±5.29 mg/mL and 12.25±6.42 mg/mL, respectively) further supports this hypothesis. Downstream steps indicate the formation of serine from glycine (up-regulation of AN1198, AN1342 and AN10745) and its conversion into pyruvate, as evidenced by the major up-regulation of the serine dehydratase gene (AN3866), which reached almost 119-fold (Table 3.4). As a consequence of this influx of carbohydrates, most genes involved in the tricarboxylic acid (TCA) cycle and glyoxylate shunt were up-regulated (Fig. 3.6). Some genes involved in glycolysis/gluconeogenesis were also up-regulated (*e.g.* fructose-bisphosphate aldolase, AN2334; triosephosphate isomerase *tpiB*, AN5908; and glyceraldehyde-3-phosphate dehydrogenase *gpdC*, AN2583), suggesting the incorporation of these carbon sources into other metabolic pathways. For example, the activation of the non-oxidative phase of the pentose phosphate pathway (involved in the biosynthesis of precursors of nucleotides and some amino acids) is supported by the up-regulation of ribose 5-phosphate isomerase (AN5907) and deoxyribose-phosphate aldolase (AN4772). Most metabolic pathways of amino acids were affected by choline supplementation, up-regulating genes involved in the metabolism of cysteine and methionine; aspartate, alanine and asparagine; branched and aromatic amino acids; glutamate, glutamine and proline, among others (Fig. 3.6, Table 3.4). Excessive supplementation with choline induced accumulation of cyanase [31]; consistent with

[*] *available in the thesis CD.*

the up-regulation of the encoding gene (AN7331) observed here (Fig. 3.6, Table 3.4). Since cyanide mineralisation is mediated by this enzyme [64], the accumulation of this toxic compound may partially explain how excess choline may result in growth inhibition and activation of stress response in *A. nidulans*.

Major down-regulation on the primary metabolism of *A. nidulans* was provoked by 1-ethyl-3-methylimidazolium chloride, as previously suggested by our proteomic study [31]. Genes involved in glycolysis and TCA cycle were mostly down-regulated, probably a consequence of nutrient limitation (Fig. 3.6, Table 3.4). This is also strongly sustained by the down-regulation of the majority of the genes involved in the biosynthesis of amino acids. A few exceptions were observed, such as the up-regulation of genes involved in alanine, aspartate and asparagine metabolism, namely asparaginases (*ahtA*, AN0300; AN1891; and AN9195), asparagine synthetase (AN4401) and aspartate aminotransferase (AN1993). This is suggestive of degradation of asparagine into aspartate, followed by incorporation into the central carbon metabolism via oxaloacetate. Up-regulation of genes coding for glycogen debranching enzyme (AN10060) and β-glucosidase (AN10124), involved in the degradation of glycogen and glucans, respectively, points to the use of cellular reserves. The data suggest that carbohydrates originated from these reserves enter glycolysis/gluconeogenesis and are channelled to the pentose phosphate pathway (Fig. 3.6). 1-Ethyl-3-methylimidazolium chloride induced also the up-regulation of genes involved in the oxidative phase of the pentose phosphate pathway, 6-phosphogluconolactonase (AN0285), 6-phosphogluconate dehydrogenases (AN6135 and AN10233) and ribulose-phosphate 3-epimerase (AN7588) (Fig. 3.6). A similar effect was noticed before, although more evident in *N. crassa* than in *A. nidulans* [31]. Activation of this pathway leads probably to higher NADPH levels, which plays important roles in antioxidant defence [65].

Both ionic liquids apparently stimulated glutathione biosynthesis, known to play a key role in the stress response in filamentous fungi. This assumption is supported by the up-regulation of its biosynthetic genes, namely gamma-glutamyltranspeptidases genes (*ggtA*, AN10444; and AN5658) and gamma-cysteine

synthetase regulatory subunit gene (AN2514) (Fig. 3.6, Table 3.3). *Aspergillus nidulans* up-accumulated a glutathione S-transferase in media supplemented with 1-ethyl-3-methylimidazolium chloride [31]. Glutathione S-conjugates are probably formed in the presence of either ionic liquid, shown here by the up-regulation of genes encoding glutathione S-transferases (AN3299 and AN6158) (Fig. 3.6, Table 3.3). Accordingly, we also observed that genes coding for efflux pumps (multidrug transporters) belonging to the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS) were up-regulated (Table 3.3). MFS multidrug transporters have been reported to participate in detoxification of 1-ethyl-3-methylimidazolium chloride in *Enterobacter lignolyticus* [66]. Activation of both conjugation reactions and efflux pumps is most likely involved in the ionic liquids' detoxification processes in *A. nidulans*.

Both ionic liquids have possibly triggered the production of acetyl-CoA through pyruvate metabolism (pyruvate decarboxylase *pdcB,* AN8396; aldehyde dehydrogenase *aldA,* AN0554; and acetyl-CoA synthetase *facA,* AN5626) or the degradation of amino acids, such as branched amino acids (Fig. 3.6). Apart from being channelled to central metabolic pathways, acetyl-CoA is also a key precursor in the synthesis of numerous SMs, *e.g.* elongation of polyketide chains [67]. Also supporting production of SMs, we observed here the up-regulation of 1-aminocyclopropane-1-carboxylate deaminase gene (AN8899) in either ionic liquid media. The encoded enzyme was not found in the mycelial proteome of *A. nidulans* cultures exposed to an ionic liquid, notwithstanding 1-aminocyclopropane-1-carboxylate deaminase accumulated in *N. crassa* grown in similar conditions [31]. Importantly, this enzyme mediates formation of the rare amino acid 1-aminocyclopropane-1-carboxylic acid found in bioactive SMs classes such as neoefrapeptins and acretocins [68, 69].

Table 3.3. Stress response genes differentially expressed after ionic liquids stimuli. Microarray analyses (fold-change, FC) in choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media in pair-wise comparisons with the control. Values highlighted in bold are statistically significant (|FC| ≥ 1.5 and *p*-value ≤ 0.05).

		Transcriptional profile*		
GeneID	**Gene**	**Choline**	**[C_2mim]Cl**	**Description**
AN7697	*sskA*	**1.81**	**1.49**	response regulator
AN0931	*pbsB*	**-1.67**	1.30	HOG signaling pathway MAPKK
AN1017	*hogA*	**-1.73**	1.01	osmotic stress-activated kinase
AN8637	*catA*	**-2.32**	**1.94**	catalase A
AN9339	*catB*	**2.66**	1.21	catalase B
AN5918	*catC*	**2.34**	**-4.09**	catalase C
AN7388	*cpeA*	**2.76**	**-2.79**	catalase D, catalase-peroxidase
AN5523	*tpsA*	**-1.85**	1.37	trehalose-6-phosphate synthase subunit 1
AN3441	*orlA*	**-1.74**	**2.62**	trehalose-6-phosphate phosphatase
AN5635	*treB*	1.39	**2.34**	neutral trehalase
AN6792	*gfdB*	**-3.56**	**2.69**	glycerol-3-phosphate dehydrogenase
AN5563	*gldB*	-1.28	**2.74**	NADP(+)-dependent glycerol dehydrogenase
		Glutathione metabolism		
AN2846	*gpxA*	1.23	**-2.65**	glutathione peroxidase
AN4905	*gstA*	**-2.04**	-1.29	theta class glutathione S-transferase
AN3299		**3.58**	**2.11**	glutathione S-transferase
AN6158		**1.89**	**3.15**	glutathione S-transferase
AN10444	*ggtA*	**2.30**	1.48	gamma-glutamyltranspeptidase
AN5658		**2.58**	**-1.93**	gamma-glutamyltranspeptidase
AN3459		**1.98**	**-2.09**	glutamate carboxypeptidase
AN2514		-1.16	**2.81**	gamma-cysteine synthetase regulatory subunit
		Multidrug transporters		
AN0015		**2.01**	**1.68**	ABC multidrug transporter
AN2349		**-4.55**	1.02	ABC multidrug transporter
AN6443		**2.14**	-1.28	ABC multidrug transporter
AN8150		1.48	**3.80**	ABC multidrug transporter
AN8489		**7.89**	**-1.64**	ABC multidrug transporter
AN8892		**2.10**	**73.69**	ABC multidrug transporter
AN9342		**2.11**	**2.60**	ABC multidrug transporter
AN0732		**4.78**	**2.94**	MFS multidrug transporter
AN1243		1.03	**2.17**	MFS multidrug transporter
AN1691		1.04	**2.19**	MFS multidrug transporter
AN2531		**-3.69**	**2.22**	MFS multidrug transporter
AN3301		1.36	**7.78**	MFS multidrug transporter
AN6477		**6.42**	1.48	MFS multidrug transporter
AN6942		**8.86**	**77.17**	MFS multidrug transporter
AN7295		**3.12**	1.25	MFS multidrug transporter
AN7466		**1.62**	**2.52**	MFS multidrug transporter
AN8089		**3.70**	-1.47	MFS multidrug transporter
AN8610		**3.04**	1.05	MFS multidrug transporter
AN8621		**-5.58**	**2.92**	MFS multidrug transporter

GeneID	Gene	Transcriptional profile*		Description
		Choline	[C_2mim]Cl	
			Autolysis/autophagy	
AN0472	*engA*	**-3.46**	**-3.39**	β-1,3-endoglucanase
AN4871	*chiB*	**-2.41**	**-5.07**	chitinase B
AN1760		**-1.81**	1.28	autophagy protein Apg12
AN3734		**-1.69**	-1.26	autophagy protein Apg9
AN5876		**-1.94**	-1.30	autophagy protein Atg22
AN6360		1.01	**1.50**	autophagy protein, ATG17 homolog
AN10213		**2.00**	**4.30**	autophagy protein Apg6

*****values highlighted in bold have $|FC| \geq 1.5$ and p-value ≤ 0.05 in the microarray data.

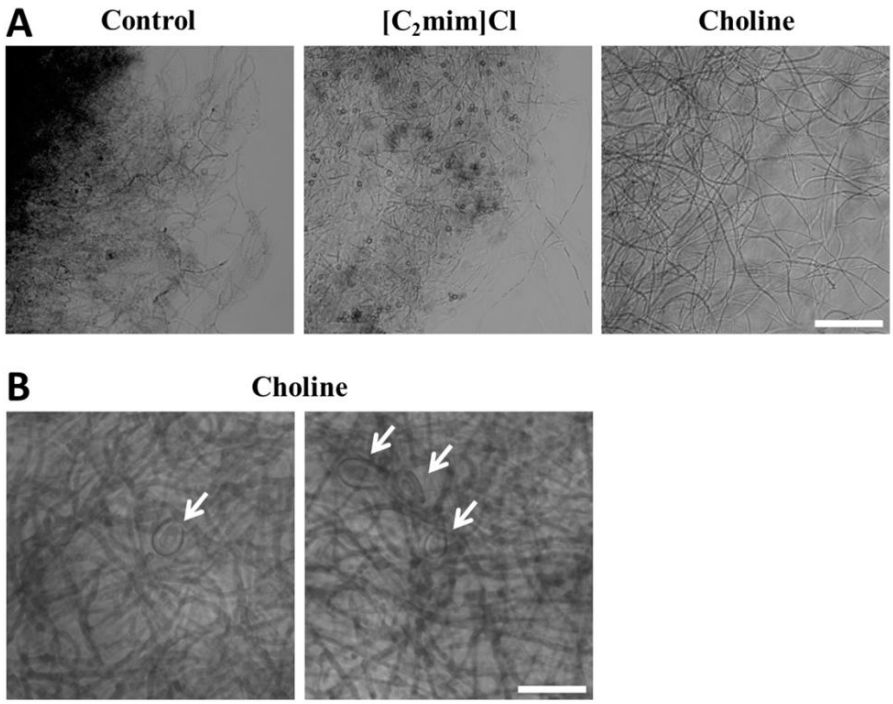

Fig. 3.5. Microscope images of *Aspergillus nidulans* mycelia under ionic liquids stimuli. Images were collected at incubation day fifteen in the control medium or in either choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media. Scale bar: 50 μm.

Fig. 3.6. Main alterations in *Aspergillus nidulans* primary metabolism and stress response under ionic liquids stimuli. Schematic view of the main alterations in the carbohydrate and amino acid metabolisms, and stress response of *A. nidulans* after fifteen days of incubation in the control medium or in either choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media. Many steps and compounds are omitted for simplification (*nb. figure with high resolution available in the thesis CD*).

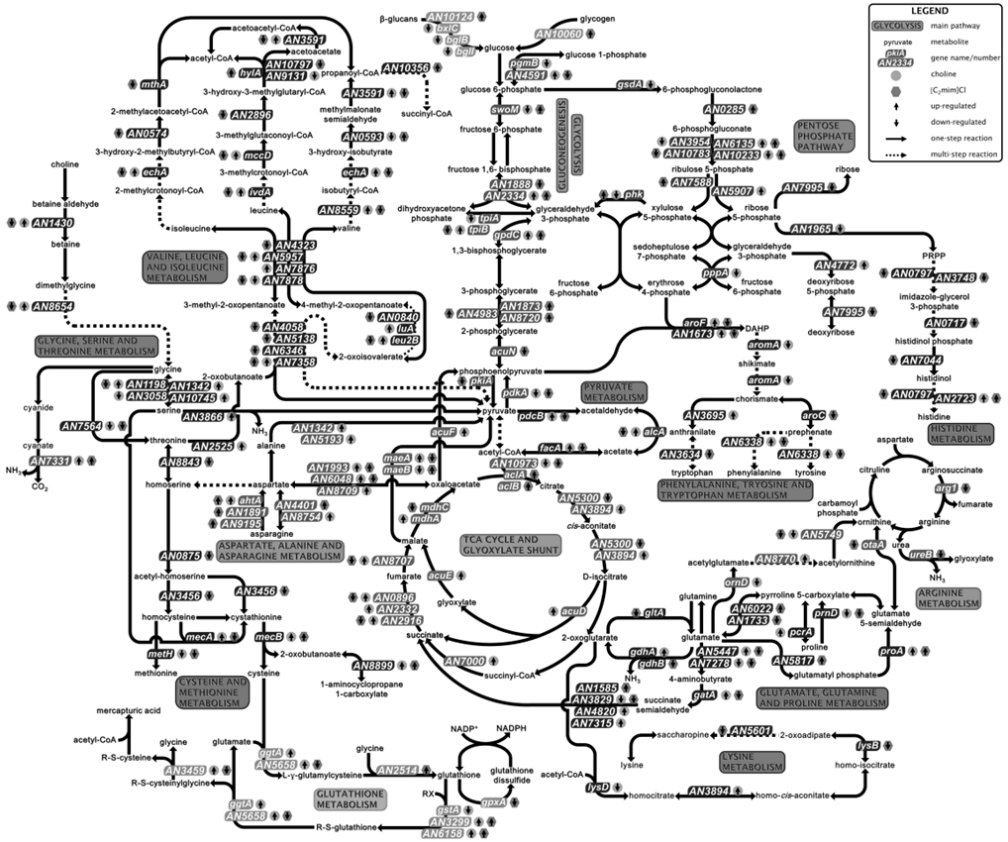

Table 3.4. Primary metabolism genes differentially expressed after ionic liquids stimuli. Microarray analyses (fold-change, FC) in choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media in pair-wise comparisons with the control. Values highlighted in bold are statistically significant ($|FC| \geq 1.5$ and p-value ≤ 0.05).

		Transcriptional profile*		
GeneID	**Gene**	**Choline**	**[C_2mim]Cl**	**Description**
		CARBOHYDRATE METABOLISM		
		Glycolysis and gluconeogenesis		
AN2867	*pgmB*	**-1.82**	1.19	phosphoglucomutase
AN4591		**1.95**	**-1.61**	phosphoglucomutase
AN6037	*swoM*	**-1.70**	**1.53**	glucose 6-phosphate isomerase
AN1888		-1.03	**-1.70**	fructose-bisphosphate aldolase
AN2334		**3.39**	**1.81**	fructose-bisphosphate aldolase
AN6900	*tpiA*	**-1.78**	1.14	triosephosphate isomerase
AN5908	*tpiB*	**1.73**	**-2.08**	triosephosphate isomerase
AN2583	*gpdC*	**3.32**	**7.66**	glyceraldehyde-3-phosphate dehydrogenase
AN1873		1.57	**1.81**	phosphoglycerate mutase
AN4983		-1.23	**2.15**	phosphoglycerate mutase
AN8720		1.06	**2.35**	phosphoglycerate mutase
AN5746	*acuN*	1.43	**-1.56**	enolase
AN5210	*pkiA*	-1.39	**-1.68**	pyruvate kinase
AN5843	*pdkA*	**-5.51**	**-10.15**	phosphoenolpyruvate synthase
		Pyruvate metabolism		
AN8396	*pdcB*	**2.68**	**-2.65**	pyruvate decarboxylase
AN0554	*aldA*	**1.53**	**-2.21**	aldehyde dehydrogenase
AN5626	*facA*	**1.55**	**-3.26**	acetyl-CoA synthetase
		TCA cycle and glyoxylate shunt		
AN2435	*aclA*	1.35	**2.16**	ATP citrate synthase
AN2436	*aclB*	1.19	**1.81**	ATP citrate synthase
AN10973		**-9.47**	**-8.30**	citrate synthase
AN5300		-1.29	**-1.67**	aconitate hydratase
AN3894		**2.17**	1.35	aconitate hydratase
AN7000		**1.73**	1.11	succinate-CoA ligase
AN0896		**1.97**	**-1.93**	succinate dehydrogenase
AN2332		**1.68**	1.13	succinate dehydrogenase
AN2916		**1.86**	**-2.41**	succinate dehydrogenase
AN8707		**1.71**	**-3.89**	fumarate hydratase
AN5634	*acuD*	**2.49**	-1.42	isocitrate lyase
AN6653	*acuE*	**2.30**	-1.00	malate synthase
AN6717	*mdhA*	**1.91**	-1.44	malate dehydrogenase
AN6499	*mdhC*	1.45	**-2.38**	malate dehydrogenase
AN6168	*maeA*	**11.90**	**-3.11**	malate dehydrogenase
AN6933	*maeB*	**-2.04**	**2.39**	malate dehydrogenase
AN1918	*acuF*	**1.85**	-1.11	phosphoenolpyruvate carboxykinase
		Pentose phosphate pathway		
AN2981	*gsdA*	**-1.56**	1.30	glucose 6-phosphate 1-dehydrogenase
AN0285		1.24	**1.82**	6-phosphogluconolactonase
AN3954		**-1.54**	1.19	6-phosphogluconate dehydrogenase
AN6135		**2.57**	**1.61**	6-phosphogluconate dehydrogenase
AN10233		**-4.89**	**4.99**	6-phosphogluconate dehydrogenase
AN10783		1.30	**-2.69**	6-phosphogluconate dehydrogenase
AN5907		**2.31**	-1.23	ribose 5-phosphate isomerase
AN7588		-1.02	**2.01**	ribulose-phosphate 3-epimerase
AN4913	*phk*	**-2.63**	**3.45**	phosphoketolase

		Transcriptional profile*		
GeneID	**Gene**	**Choline**	**[C_2mim]Cl**	**Description**
AN0240	*pppA*	**-1.53**	1.30	transaldolase
AN1965		**-1.64**	1.31	phosphoribosyl diphosphate synthase
AN7995		1.03	**-2.29**	ribokinase
AN4772		**4.69**	-1.19	deoxyribose-phosphate aldolase
			Others	
AN10060		-1.14	**2.07**	glycogen debranching enzyme, alpha-amylase
AN10124		1.48	**2.34**	β-glucosidase
AN0712	*bglB*	**-1.55**	-1.42	β-glucosidase
AN2217	*bxlC*	**-1.74**	1.34	β-glucosidase
AN2227	*bglI*	**-2.67**	1.22	β-glucosidase
			AMINO ACIDS METABOLISM	
			Glutamate, glutamine and proline metabolism	
AN4376	*gdhA*	**2.15**	1.42	NADP-linked glutamate dehydrogenase
AN7451	*gdhB*	1.03	**-2.25**	NAD dependent glutamate dehydrogenase
AN5134	*gltA*	1.00	**-1.54**	glutamate synthase
AN5447		**6.71**	**-2.48**	glutamate decarboxylase
AN7278		**-9.96**	**-17.03**	glutamate decarboxylase
AN2248	*gatA*	**1.98**	**-2.32**	gamma-amino-n-butyrate transaminase
AN1585		1.12	**1.87**	succinate-semialdehyde dehydrogenase
AN3829		**-2.58**	**-1.58**	succinate-semialdehyde dehydrogenase
AN4820		**2.28**	-1.48	succinate-semialdehyde dehydrogenase
AN7315		**1.50**	-1.20	succinate semialdehyde dehydrogenase
AN1733		-1.13	**-2.40**	delta-1-pyrroline-5-carboxylate dehydrogenase
AN6022		-1.08	**1.60**	delta-1-pyrroline-5-carboxylate dehydrogenase
AN7387	*pcrA*	**3.33**	-1.26	pyrroline-5-carboxylate reductase
AN1731	*prnD*	**-4.96**	**-2.88**	proline oxidase
AN5817		1.00	**1.58**	glutamate 5-kinase
AN5799	*proA*	**1.73**	**2.25**	glutamate semialdehyde dehydrogenase
			Arginine metabolism	
AN1810	*otaA*	-1.12	**-1.63**	ornithine aminotransferase
AN5867	*ornD*	**-2.08**	-1.06	acetylglutamate synthase
AN8770		**2.41**	-1.33	acetylglutamate kinase
AN5749		**-1.60**	**-1.90**	peptidase
AN2914	*arg1*	-1.47	**-1.86**	argininosuccinate lyase
AN10079	*ureB*	1.08	**-1.70**	urease
			Lysine metabolism	
AN1990	*lysD*	**-1.57**	1.27	homocitrate synthase
AN3894		**2.17**	1.35	aconitate hydratase
AN5206	*lysB*	1.49	**-1.50**	isocitrate dehydrogenase
AN5601		1.13	**-1.62**	saccharopine dehydrogenase
			Histidine metabolism	
AN3748		1.02	**-1.78**	ATP phosphoribosyltransferase
AN0797		-1.21	**-1.90**	phosphoribosyl-AMP cyclohydrolase
AN0717		-1.19	**-1.94**	histidinol-phosphate aminotransferase
AN7044		1.47	**-2.05**	histidinol phosphatase
AN2723		**3.54**	**1.71**	histidinol dehydrogenase
			Tyrosine, phenylalanine and tryptophan	
AN1673		**2.50**	**-2.00**	phospho-2-dehydro-3-deoxyheptonate aldolase
AN5701	*aroF*	**1.81**	**-1.72**	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
AN0708	*aromA*	**-1.56**	1.11	pentafunctional AROM polypeptide
AN3695		**1.60**	-1.35	anthranilate synthase
AN3634		1.17	**-2.89**	anthranilate phosphoribosyltransferase
AN6866	*aroC*	-1.48	**-1.51**	chorismate mutase
AN6338		**1.69**	**-2.78**	aromatic aminotransferase

GeneID	Gene	Transcriptional profile* Choline	Transcriptional profile* [C_2mim]Cl	Description
Glycine, serine and threonine metabolism				
AN1430		**2.51**	**-1.72**	betaine aldehyde dehydrogenase
AN8654		**20.35**	**-5.15**	dimethylglycine oxidase
AN1342		**4.99**	-1.31	aminotransferase
AN10745		**2.46**	-1.22	serine hydroxymethyltransferase
AN3058		**-2.31**	-1.19	serine hydroxymethyltransferase
AN1198		**5.00**	**-2.15**	glycine cleavage system T protein
AN3866		**118.88**	1.23	L-serine dehydratase
AN7564		**-1.87**	**-3.13**	L-allothreonine aldolase
AN8843		-1.45	**-1.96**	homoserine kinase
AN2525		**1.54**	-1.34	pyridoxal-phosphate dependent enzyme
Cysteine and methionine metabolism				
AN0875		-1.15	**-25.05**	homoserine acetyltransferase
AN5820	*mecA*	**1.97**	**-4.91**	cystathionine beta-synthase
AN3456		-1.30	**-1.56**	cystathionine gamma-synthase
AN1446	*mecB*	**2.34**	**2.40**	cystathionine gamma-lyase
AN4443	*metH*	**-1.72**	**-1.83**	methionine synthase
Aspartate and asparagine and alanine metabolism				
AN1993		**2.87**	**1.66**	aspartate aminotransferase
AN6048		**3.90**	**-1.50**	aspartate transaminase
AN8709		**2.32**	-1.11	aspartate transaminase
AN0300	*ahtA*	**1.94**	**1.52**	L-asparaginase
AN1891		-1.16	**4.04**	asparaginase
AN9195		1.46	**2.01**	L-asparaginase II
AN4401		1.02	**1.62**	asparagine synthetase
AN8754		**2.07**	-1.02	asparagine synthase
AN1342		**4.99**	-1.31	aminotransferase
AN5193		**1.64**	1.16	aspartate aminotransferase
Valine, leucine and isoleucine metabolism				
AN4058		-1.43	**-7.94**	dihydroxy-acid dehydratase
AN5138		1.38	**-1.77**	dihydroxy-acid dehydratase
AN6346		-1.04	**-2.07**	dihydroxy-acid dehydratase
AN7358		**1.92**	**4.11**	dihydroxy-acid dehydratase
AN0840		1.19	**-1.51**	2-isopropylmalate synthase
AN5886	*luA*	**1.59**	1.41	3-isopropylmalate dehydratase
AN2793	*leu2B*	**1.85**	**-1.64**	3-isopropylmalate dehydrogenase
AN4323		1.46	**-2.13**	branched-chain amino acid aminotransferase
AN5957		**1.55**	**-1.57**	branched-chain amino acid aminotransferase
AN7876		**2.46**	-1.04	branched-chain amino acid aminotransferase
AN7878		**-1.61**	**-1.83**	branched-chain amino acid aminotransferase
AN5916	*echA*	**1.56**	**-2.91**	enoyl-CoA hydratase/isomerase
AN0574		1.44	**-2.59**	3-oxoacyl-(acyl-carrier-protein) reductase
AN10512	*mthA*	1.43	**-2.71**	acetyl-CoA-acetyltransferase
AN4688	*ivdA*	**2.24**	**-4.73**	isovaleryl-coenzyme A dehydrogenase
AN4687	*mccD*	**1.95**	**-3.48**	3-methylcrotonyl-CoA carboxylase
AN2896		1.46	**-1.89**	mitochondrial methylglutaconyl-CoA hydratase
AN5273	*hlyA*	-1.15	**1.86**	hydroxymethylglutaryl-CoA lyase
AN9131		**-1.68**	-1.19	hydroxymethylglutaryl-CoA lyase
AN10797		1.39	**-2.42**	hydroxymethylglutaryl-CoA lyase
AN5669		**2.50**	**-2.81**	succinyl-CoA:3-ketoacid-CoA transferase
AN8559		**1.83**	**-3.23**	2-oxoisovalerate dehydrogenase
AN0593		**-2.61**	**2.01**	3-hydroxyisobutyrate dehydrogenase
AN3591		**3.36**	**-3.37**	methylmalonate-semialdehyde dehydrogenase
AN10656		-1.19	**2.77**	propionyl-CoA carboxylase

		Transcriptional profile*		
GeneID	**Gene**	**Choline**	**[C_2mim]Cl**	**Description**
			Other	
AN8899		**9.30**	**1.67**	1-aminocyclopropane-1-carboxylate deaminase

*****values highlighted in bold have |FC| ≥ 1.5 and *p*-value ≤ 0.05 in the microarray data.

3.4.4. Ionic liquids' impact in *Aspergillus nidulans* secondary metabolism - backbone genes

Genes involved in biosynthesis of a particular SM are usually clustered and comprise the backbone gene responsible for biosynthesis of the metabolite core structure and genes coding for additional tailoring enzymes [12, 70]. The backbone gene alone can be responsible for the production of a specific SM (*e.g.* microperfuranone [15] and orsellinic acid [17]), sometimes even when the expression of other clustered genes remains unaltered (*e.g.* penicillin [71]). Overexpression of backbone genes can also result in high production titres of certain SMs (*e.g.* alternariol [72]).

Consistent with the greater diversity of putative compounds found in cultures exposed to either ionic liquid (Table 3.2, Additional File 3[*]), in total twenty one of the sixty six predicted backbone genes [3] were found up-regulated compared to the control (Table 3.5, Additional File 2[*]). Fifteen and nine backbone genes were found up-regulated in choline and 1-ethyl-3-methylimidazolium chloride supplemented media, respectively. This includes *mdpG* in the choline medium and *orsA* in both media. Only three genes coincided between the two conditions, suggesting each ionic liquid induces a specific stimulus. In addition to *mdpG*, the monodictyphenone cluster includes *mdpA* (AN10021) and *mdpE* (AN0148), none of which underwent differential expression in choline media (Fig. 3.7A). These genes encode transcription factors required for full activation of the monodictyphenone cluster; their deletion usually decreases production titres of monodictyphenone, emodin and derivatives [4]. In addition, *mdpC* and *mdpL* were up-regulated, consistent with monodictyphenone formation. The microarray data support the ability of choline supplement to stimulate formation of monodictyphenone, probably

[*] *available in the thesis CD.*

due to up-regulation of *mdpG*, especially since *cclA* expression levels remained unaltered (Additional File 2*). De-repression of *mdpG* increased significantly over time in choline supplemented media compared to control (Table 3.6), probably explaining monodictyphenone identification also on the fifth and tenth days of incubation (Table 3.2).

Only *orsA* out of the three genes of the orsellinic acid cluster is necessary for the production of this metabolite [17]. Orsellinic acid detection is consistent with up-regulation of *orsA* in both ionic liquid supplemented media (Table 3.5), regardless of *orsB* and *orsC* expression values (Fig. 3.7B). *orsA* underwent major up-regulation during exposure to either ionic liquid, although its expression was transient (Table 3.6). In the choline supplemented medium, the accumulation profile over time of orsellinic acid parallels *orsA* expression (Table 3.2). In 1-ethyl-3-methylimidazolium chloride medium, despite *orsA* being more prominently up-regulated on the fifth day of incubation, this SM was detected only at longer incubation times (Table 3.2). The activities of the histone acetyltransferases EsaA (AN10956) [73] and GcnE (AN3621) [74] were reported to activate expression of penicillin, terrequinone A and sterigmatocystin gene clusters, whereas GcnE influences also *orsA* expression. None of the ionic liquids led to differential expression of *esaA* and *gcnE*, or the backbone genes of penicillin, terrequinone A and sterigmatocystin (Additional File 2*). In agreement, penicillin and sterigmatocystin were confirmed to be absent in the culture media (Additional File 3*). Putative identification of terrequinone A only occurred in control cultures (data not shown), which may be explained by the major down-regulation of its backbone gene in both ionic liquid media (Additional File 2*). None of the identified ion masses matched compounds assigned to the remaining up-regulated backbone genes (Table 3.5), probably because their abundance is below the detection limit or accumulated intracellularly, or other yet uncharacterised metabolites from the cluster were produced instead.

* *available in the thesis CD.*

Table 3.5. Secondary metabolite synthase genes up-regulated upon ionic liquid stimulus. Microarray analyses of the backbone genes up-regulated (fold-change, FC) in choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media in pair-wise comparison with the control. Values highlighted in bold are statistically significant ($|FC| \geq 1.5$ and p-value ≤ 0.05).

		Transcriptional profile*			
GeneID	**Gene**	**Choline**	**[C_2mim]Cl**	**Enzyme**	**Secondary metabolite**
AN0150	*mdpG*	**1.90**	-1.18	PKS	monodictyphenone; emodin derivatives [4]
AN0607	*sidC*	**4.78**	**-2.28**	NRPS	ferricrocin (siderophore) [75, 76]
AN1594		1.08	**1.75**	DTS	*ent*-pimara-8(14),15-diene [77]
AN10486		**1.52**	-1.19	NRPS-like	
AN11080	*nptA*	**6.84**	**-12.35**	DMATS	nidulanin A [14]
AN11191		**1.83**	-1.27	PKS	
AN11820		-1.39	**2.00**	NRPS-like	
AN1680		1.20	**1.72**	NRPS-like	
AN2064		1.16	**2.05**	NRPS-like	
AN2547	*easB*	**2.80**	**-2.67**	PKS	emericellamide [7]
AN3230	*pkfA*	1.02	**1.78**	PKS	aspernidine A [11]
AN3396	*micA*	**1.80**	-1.04	NRPS-like	microperfuranone [15]
AN4827		**1.60**	1.13	NRPS-like	
AN5318		**2.39**	**3.20**	NRPS	
AN6236	*sidD*	**1.52**	**-2.80**	NRPS	triacetylfusarinine C (siderophore) [76]
AN6784	*xptA*	-1.53	**1.88**	DMATS	prenyl xanthones [5]
AN6791		**1.67**	1.13	PKS	
AN7071	*pkgA*	**2.37**	**1.89**	PKS	alternariol; isocoumarins [72]
AN12331		**1.86**	**1.56**	PKS-like	
AN7909	*orsA*	**1.61**	1.35	PKS	orsellinic acid; F9775A/B [17]; violaceols [43]
AN9005		**6.81**	**-2.28**	PKS	

*****values highlighted in bold have $|FC| \geq 1.5$ and p-value ≤ 0.05 in the microarray data. NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; DMATS, dimethylallyl tryptophan synthase (prenyltransferase); DTS, diterpene synthase.

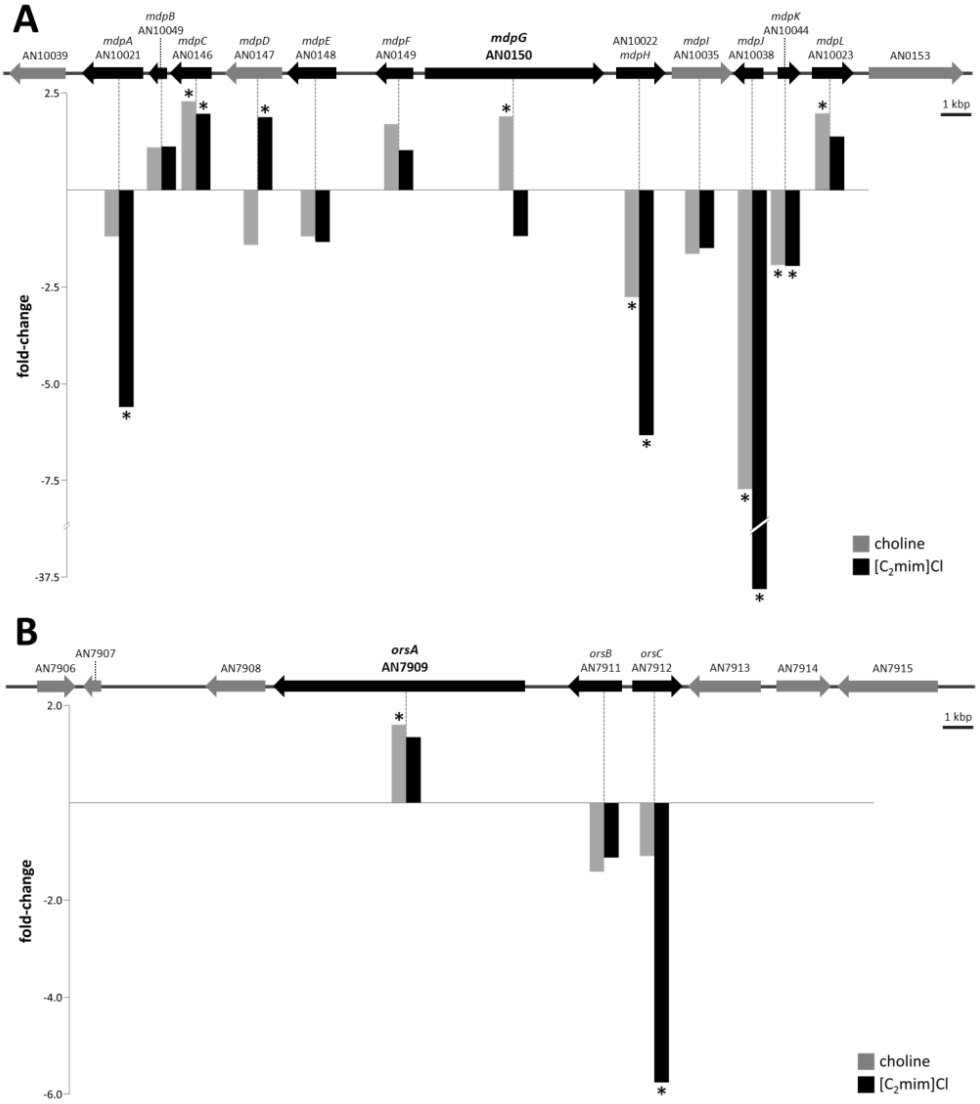

Fig. 3.7. Expression profile of monodictyphenone and orsellinic acid biosynthetic gene clusters under ionic liquids stimuli. Representation of the gene clusters involved in the biosynthesis of monodictyphenone (A) and orsellinic acid (B) in *Aspergillus nidulans*. Essential and non-essential genes are shown in black and grey (arrows), respectively (adapted from [4, 37]). Below each gene the measured fold-change (FC) in either choline (grey bars) or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl, black bars) supplemented media are depicted (microarray data) and, if statistically significant ($|FC| \geq 1.5$ and *p*-value ≤ 0.05), indicated with an asterisk (*).

Table 3.6. Time-course analysis of gene expression of monodictyphenone and orsellinic acid synthases and major regulators. *q*RT-PCR analysis of the expression of *orsA* and *mdpG* (backbone genes of orsellinic acid and monodictyphenone, respectively), and *laeA*, *veA* and *rsmA* (secondary metabolism regulatory genes) along the incubation time in choline or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) supplemented media. Values represent relative gene expression at each culture time in pair-wise comparisons with the control. Expression of each gene was normalised to the expression of the histone protein H3 gene AN0733.

			q*RT-PCR		
Media	**GeneID**	**Gene**	**5d**	**10d**	**15d**
Choline	AN7909	*orsA*	2.04	-3.62	1.49
	AN0150	*mdpG*	-57.33	-44.94	-2.66
	AN0807	*laeA*	-10.09	-4.32	-10.00
	AN4562	*rsmA*	-1.64	1.43	2.30
	AN1052	*veA*	-2.91	-1.77	-1.00
[C_2mim]Cl	AN7909	*orsA*	24.65	1.29	3.42
	AN0150	*mdpG*	-5.54	-74.07	-39.50
	AN0807	*laeA*	-6.93	-13.87	-31.40
	AN4562	*rsmA*	1.94	-2.02	1.09
	AN1052	*veA*	-4.14	-6.85	1.65

*Fold changes (FCs) in pair-wise comparison with the control.

3.4.5. Ionic liquids' impact in *Aspergillus nidulans* secondary metabolism - regulatory genes

A complex regulatory network governs the co-regulation of the clustered SM biosynthetic genes [78], involving hierarchical levels of transcriptional regulatory elements. These can be either pathway-specific (*e.g.* AflR in the sterigmatocystin/aflatoxin gene cluster [79]) or broad domain transcription factors (*e.g.* the Velvet complex), and proteins responsive to general environmental factors that are also implicated in cluster activation (*e.g.* CreA, PacC and AreA) [80]. Global regulators of secondary metabolism in *Aspergillus* spp. include the well-studied VelB/VeA/LaeA transcriptional complex (*i.e.* Velvet complex) that links secondary metabolism with fungal development [78, 81]. In the dark – similar to culture conditions used here – the Velvet complex controls the activity of LaeA (methyltransferase-domain nuclear protein), which in turn controls the expression of

several SM gene clusters [82, 83]. The impact of LaeA on the regulation of secondary metabolism has been well studied in *A. fumigatus* (the *laeA* deletion mutant shows repression of 13 of 22 SM biosynthetic clusters) [84], but is also known to impact other fungi (*e.g. Fusarium verticillioides* [85]). It controls, in general, gene clusters positioned at the telomere proximal region of the chromosomes [86]. Among the backbone genes found up-regulated here only few are located proximal to the telomere, consistent with studies implicating other secondary metabolism regulatory elements apart from LaeA [15].

LaeA (AN0807) is required for biosynthesis of sterigmatocystin and penicillin [81]. However, sterigmatocystin biosynthesis can be restored in *laeA* or *veA* deletion strains by the up-regulation of *rsmA* (*r*emediation of *s*econdary *m*etabolism *A*, AN4562) [87] or the deletion of *mtfA* (*m*aster *t*ranscription *f*actor *A*, AN8741) [88], respectively. Our microarray data showed that both ionic liquids led to down-regulation of *laeA*, whereas *veA* (AN1052) was down-regulated only in 1-ethyl-3-methylimidazolium chloride supplemented medium and *velB* (AN0363) was not differentially expressed in either condition (Table 3.7). *q*RT-PCR analyses of expression levels of *laeA* and *veA* over the incubation period (up to fifteen days) confirmed their down-regulation (Table 3.6). In choline supplemented medium, despite the up-regulation of *rsmA* over time (Table 3.6) sterigmatocystin accumulation was not validated (Additional File 3[*]). In addition, up-regulation of *mtfA* in 1-ethyl-3-methylimidazolium chloride medium likely led to the down-regulation of sterigmatocystin and terrequinone A backbone genes (Additional File 2[*]), hence the absence of these SMs in the cultures [88]. Penicillin accumulation in the culture was not observed, consistent with the down-regulation of *laeA*, which is required for its biosynthesis [81].

Additional genes involved in histone modifications can also impact SM production. Deletion of the histone deacetylase gene *hdaA* (AN8042), for example, induces the production of sterigmatocystin and penicillin [89], and high levels of the heterochromatin protein HepA (AN1905) provoke opposite effects [90]. *hdaA* was

[*] *available in the thesis CD.*

not differentially expressed but *hepA* was up-regulated in 1-ethyl-3-methylimidazolium chloride supplemented medium (Table 3.7), probably contributing to the repression of sterigmatocystin and penicillin biosynthesis in this medium.

A set of thirteen genes involved in secondary metabolism, either coding for SM synthases (*mdpG*, *orsA*, *sidC*, *easB* and *ausA*), chromatin remodelling enzymes (*hepA*, *hdaA* and *gcnE*) or regulatory proteins, including those discussed above (*laeA*, *rsmA*, *veA* and *velB*), as well as *fluG*, were selected to validate the microarray data by *q*RT-PCR (Table 3.7). With few exceptions the majority of the analysed genes displayed an expression profile similar to that detected by microarray analysis.

Table 3.7. *q*RT-PCR analysis of selected genes encoding secondary metabolism components, either biosynthesis or regulation. Values represent the relative expression of selected genes in pair-wise comparisons with the control, after fifteen days of incubation. Expression of each gene was normalised to the expression of the histone protein H3 gene (AN0733). Corresponding microarray data are shown for comparison.

		Choline		**[C_2mim]Cl**	
GeneID	**Gene**	***q*RT-PCR**	**Microarray***	***q*RT-PCR**	**Microarray***
Backbone genes					
AN7909	*orsA*	1.67	**1.61**	2.57	1.35
AN0150	*mdpG*	-3.62	**1.90**	-12.50	-1.18
AN8383	*ausA*	-6.00	-1.30	-3.04	**-2.26**
AN2547	*easB*	2.08	**2.80**	-4.92	**-2.67**
AN0607	*sidC*	3.06	**4.78**	-4.57	**-2.28**
Transcriptional regulators					
AN0807	*laeA*	-6.75	**-6.71**	-25.32	**-17.55**
AN1052	*veA*	-1.08	-1.23	-1.23	**-1.52**
AN0363	*velB*	-1.91	-1.16	1.43	1.45
AN4819	*fluG*	-1.26	-1.12	-1.89	-1.45
AN4562	*rsmA*	-1.21	1.12	-1.66	-1.18
AN1905	*hepA*	-1.06	1.18	1.23	**1.70**
AN8042	*hdaA*	1.39	1.04	1.14	-1.18
AN3621	*gcnE*	1.14	-1.34	1.26	-1.30

*values highlighted in bold have $|FC| \geq 1.5$ and p-value ≤ 0.05 in the microarray data.

3.4.6. Analysis of the biological activity of *Aspergillus nidulans* extracts after an ionic liquid stimulus

To screen the presence of compounds displaying anti-carcinoma potential in the metabolite extracts of *A. nidulans* an *ex vivo* polarity modulation assay (*the*LiTE™) was used. The positive control (30 µM of *the*-103 compound) affected 100% of the *Drosophila* eggs, whereas the blank (*i.e.* 0.6% v/v DMSO which is the solution used to solubilise the metabolite extracts) is devoid of any significant activity (Fig. 3.8A-B). The capacity of a pure compound to impair the fluorescent ring of the polarity marker protein Par6 in more than *ca.* 50% of the eggs is defined as the empirical cut off for a positive result. When dealing with complex mixtures, much weaker activities should be regarded as significant, especially if a de-replicated fraction shows substantially higher activity. Under standard cultivation conditions (control), metabolite extracts (crude and its corresponding polar fraction) were virtually devoid of activity (Fig. 3.8C); both ionic liquids induced the biosynthesis of compounds carrying anti-carcinoma potential. Similar activities were, in general, measured for the metabolite extracts derived in either ionic liquid media at the seventh or the fifteenth day of incubation, which were much stronger that those measured at the second day (data not shown). For example, crude metabolite extracts (24 µg/mL) from 1-ethyl-3-methylimidazolium chloride and choline supplemented media, at the seventh day of incubation, showed high to moderate activity (67% and 31%, respectively); their polar fractions (de-replicated metabolite extracts, dME) showed similar or much greater activity (57% and 81%, respectively) (Fig. 3.8C). The detected activities cannot be related to any vestigial amounts of either ionic liquid, because these compounds *per se* lack activity (data not shown). We verified also that 24 µg/mL of monodictyphenone (83 µM) or orsellinic acid (129 µM) affected 75% or 62% of the eggs, respectively (Fig. 3.8B). Both SMs, primarily monodictyphenone, may contribute to the polarity modulating activity of the metabolite extracts (either crude or de-replicated); additional active components probably remain obscured. The future challenge is to identify, among the compounds specific to each ionic liquid, those that carry intrinsic anti-carcinoma activity.

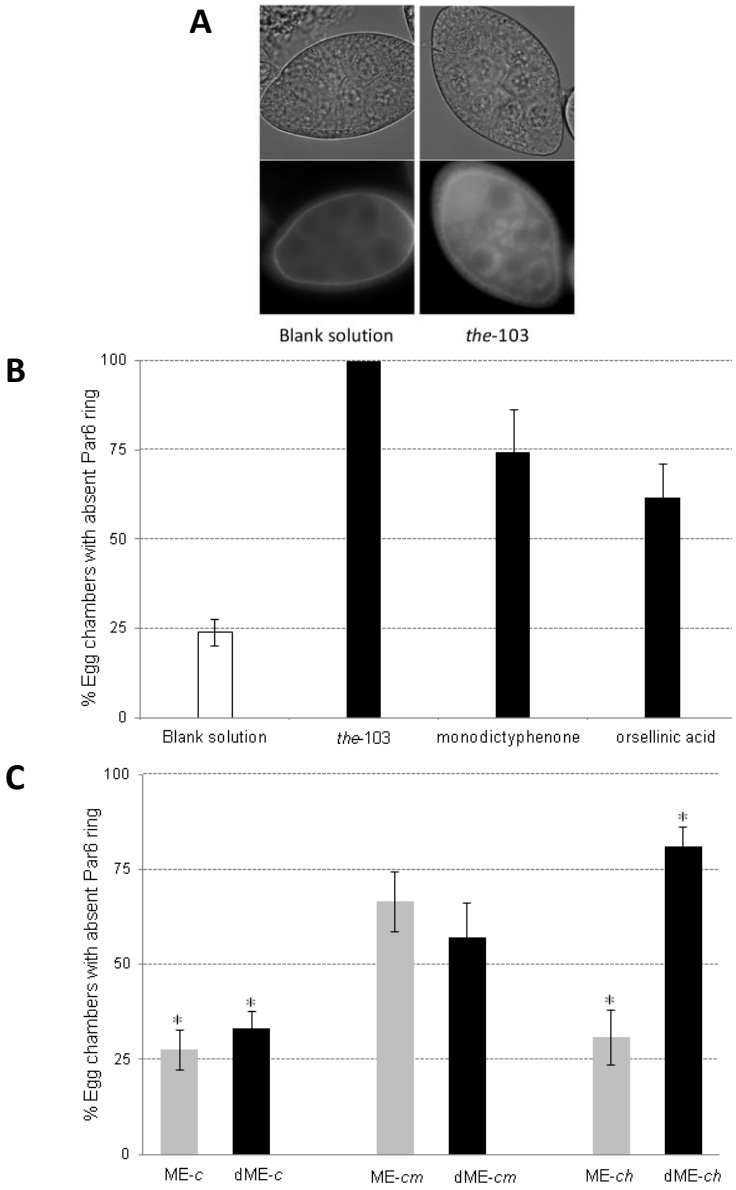

Fig. 3.8. *Ex vivo the*LiTE™ assay of *Aspergillus nidulans* metabolite extracts from ionic liquid supplemented media. Microscope image of *Drosophila* egg chambers showing presence/absence of the polarity marker protein Par6 when exposed to solvent only solution (0.6% DMSO, *i.e*. blank) and *the*-103, respectively (A); % of egg chambers showing Par6 ring impairment in the presence of 24 µg/mL monodictyphenone, orsellinic acid, *the*-103 (the blank is also shown) (B) or metabolite extracts collected at incubation day seven in the control medium (ME-*c*) or either choline (ME-*ch*) or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) (ME-*cm*) supplemented media, before (grey bars) or after dereplication of its polar fraction (dME) (black bars) (C). The asterisk (*) denotes a significant difference (*p*-value ≤ 0.05) of each treatment compared to the control.

3.5. Conclusions

Transcriptional profiling was used for the first time to evaluate ionic liquids broad impacts on both primary and secondary metabolisms of *A. nidulans*. Primary metabolism was up-regulated by choline, but down-regulated by 1-ethyl-3-methylimidazolium chloride. Choline could be used as a source of carbon and nitrogen probably via the glycine, serine and threonine metabolic pathway, and incorporated into the central carbon metabolism. On the contrary, the recalcitrant 1-ethyl-3-methylimidazolium chloride induced the use of cellular reserves and autophagy. Both ionic liquids induced detoxification mechanisms (*viz*. multidrug transporters and glutathione S-conjugates) probably to eliminate the toxic cations or toxic intermediates (*e.g.* cyanide). Despite strongly contrasting effects on primary metabolism either ionic liquid apparently stimulated production of acetyl-CoA, a key precursor to numerous SMs, as well as the production of non proteinogenic amino acids known as building blocks of bioactive classes of SMs. Differential analyses of the fungal metabolome allowed discovery of numerous putative SMs, including gentisic acid and caffeic acid here reported for the first time in *A. nidulans*. Concurrently with numerous differentially formed compounds, multiple genes encoding SM biosynthetic enzymes were up-regulated. Each ionic liquid stimulus activated a specific set of backbone genes, including uncharacterised ones. In addition, growth media supplementation with choline led, for the first time, to monodictyphenone accumulation in a wild type strain of *A. nidulans*. Importantly, we observed here that the complex mixtures of the formed metabolites camouflage compounds with anti-carcinoma potential. This study should inspire the search of novel bioactive fungal SMs biosynthesised under an ionic liquid stimulus.

Ionic liquids' impact on eukaryotic organisms constitutes a fundamental cue for conscious development of this field. This study constitutes a step forward to prior proteomic analyses of ionic liquids' impacts in primary metabolism, providing a more detailed analysis and expanding initial findings to secondary metabolism. We believe it illustrates the potential of ionic liquids to induce metabolic alterations and stress responses in eukaryotic organisms. In particular, we showed that they can

be used to further resolve the diversity of natural compounds, guiding discovery of fungal metabolites with clinical potential. Further studies are necessary to elucidate the capacity of the ionic components to specifically modulate defined response regulators of metabolism and development in fungi. Ionic liquids formation during the confrontation between two ant species has been recently demonstrated, probably as a defence mechanism [91]. The likelihood of natural ionic liquids creates a new paradigm – they are not exclusively man-made chemicals – and supplies a new boost of interest in their research. Unforeseen possibilities have been revealed and future exploration promises to be exciting.

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CHAPTER IV

Investigating peptide metabolites production by *Neurospora crassa* under ionic liquids stimuli

This chapter contains part of ongoing work and includes unpublished data:

Paula C. Alves, Isabel Martins, Celso Martins, Maria Cristina Leitão, Teresa L. Gomes, Richard Hampson, Jörg D. Becker and Cristina Silva Pereira. Investigating peptide metabolites production by *Neurospora crassa* under ionic liquid stimuli. *manuscript in preparation.*

PC Alves declares to have actively contributed for the experimental design, fungal cultivation and sampling, metabolites extraction, samples preparation for HPLC analyses, amino acid hydrolysis, data interpretation and drafting of the manuscript.

Investigating peptide metabolites production by *Neurospora crassa* under ionic liquids stimuli

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4.1. Abstract

Non-ribosomal peptides comprise a large and heterogeneous class of fungal secondary metabolites that includes valuable peptide antibiotics. Previous data demonstrated that supplementation of ionic liquids to the growth media increases metabolite diversity in the fungal footprint compared to control conditions. Moreover, 1-aminocyclopropane-1-carboxylate deaminase – an enzyme involved in the production of 1-aminocyclopropane-1-carboxylic acid, accumulated in the proteome of *Neurospora crassa* when exposed to either 1-ethyl-3-methylimidazolium chloride or cholinium chloride. This observation inspires the hypothesis of production of peptide antibiotics by the fungus under ionic liquids' stimuli. In the present study, we have analysed the nature of the differential peptides produced in *N. crassa* cultures when grown in media containing an ionic liquid supplement compared to control. Peptide production titters were higher in media containing 1-ethyl-3-methylimidazolium chloride compared to cholinium chloride and the first was selected for the following studies. In the presence of 1-ethyl-3-methylimidazolium chloride supplement the peptide metabolites synthesised by the fungus contained the rare amino acids α-aminoisobutyric acid and 1-aminocyclopropane-1-carboxylic acid. Their production was not observed in a mutant devoid of 1-aminocyclopropane-1-carboxylate deaminase. These observations constitute the proof-of-concept of the capacity of certain ionic liquids' cations to induce the biosynthesis of peptide metabolites in *N. crassa*, including peptaibiotics, as well as classes of peptides carrying 1-aminocyclopropane-1-carboxylic acid as a structural unit. The ensuing peptide fractions display strong antibacterial activity, as well as anti-cancer potential. This study constitutes a first attempt to deeply characterise *N. crassa* peptide metabolites, highlighting the yet unexplored capacity of this fungus to synthesise, under specific chemical stimuli, new classes of peptide metabolites carrying interesting pharmacological properties.

4.2. Introduction

Fungal secondary metabolites (SMs) have been described to present an array of applications ranging from pharmaceutical drugs (*e.g.* penicillin [1] and lovastatin [2]) to food additives (*e.g.* kojic acid [3]) but they can also exhibit deleterious effects (*e.g.* mycotoxins such as aflatoxin [4]). These compounds can be divided into several classes according to the multi-domain enzyme participating in the biosynthesis, such as polyketides (PKs), non-ribosomal peptides (NRPs), hybrid NRP-PKs, indole alkaloids and terpenes [5, 6]. Numerous SMs metabolites belong to the class of non-ribosomal peptides [5, 6], which are biosynthesised by a NRP synthetase, either alone or involving an additional set of tailoring enzymes [7].

The genetic potential for the biosynthesis of SMs can be predicted through analysis of the genome sequence [6]. *Neurospora crassa* – a model filamentous fungus important in biology and circadian rhythm studies which genome is fully sequenced; has only ten predicted secondary metabolite biosynthetic gene clusters. Despite its limited SM biosynthetic potential, the biosynthetic gene clusters of this fungus have not been systematically solved. There is one small cluster associated to the production of neurosporaxanthin [5, 8, 9] and a second one that (by homology with *A. nidulans* genes) can be associated to the production of the siderophore ferricrocin [10]. The first metabolite has a role in fungal pigmentation and ferricrocin is a chelator molecule necessary for iron homeostasis that has been also reported to be essential for efficient conidiation in *N. crassa* [10, 11].

The vast majority of fungal SMs remains silent under standard cultivation conditions. Their biosynthesis requires specific stimulus, for example the presence of a competitor organism (co-culture) [12], the alteration of the medium composition (temperature, pH, different concentrations of the medium components) [13] or the addition of exogenous chemicals [14].

Previous studies by our team have demonstrated that supplementation of ionic liquids to the growth media alters the metabolite footprint of filamentous fungi [15, 16]. Ionic liquids, which are solely composed by ions, are usually considered as

alternative green solvents (even if many provoke toxic effects) [17], and their properties can be changed by simple modification of the structure of either ion [18].

1-Aminocyclopropane-1-carboxylate deaminase accumulated in the mycelial proteome of *N. crassa* when an ionic liquid supplement was added to the cultures, namely 1-ethyl-3-methylimidazolium chloride or cholinium chloride [16]. This enzyme is involved in the biosynthesis of 1-aminocyclopropane-1-carboxylic acid (Acc) [16] - a rare amino acid found in neoefrapeptins, a specific subclass of peptaibiotics. The present study aims to investigate peptaibiotics production by *N. crassa* under ionic liquids' stimuli. A combination of methods was used to identify, amongst the differential produced peptide metabolites, those fulfilling the characteristics of peptaibiotics, including chromatographic analyses, functional assays with mutants and biological activity tests.

Further studies are necessary to confirm the production of peptaibiotics by *N. crassa* during growth in 1-ethyl-3-methylimidazolium chloride supplemented media. Nonetheless, the ionic liquid supplement led to accumulation of peptide metabolites, which contain Acc as well as α-aminoisobutyric acid (Aib), displaying strong biological activity. They were absent in cultures of *N. crassa* mutant where Acc production was impaired. Future studies will rely on the use of mutants carrying deletions in specific NRP synthetases as to pin point the gene cluster responsible for the biosynthesis of these peptide metabolites. Finally, their production need to be scaled-up to complete their chemical characterisation.

4.3. Materials and Methods

4.3.1. Chemicals

The standard chemicals (α-aminoisobutyric acid (Aib), 1-aminocyclopropane-1-carboxylic acid (Acc)) and chromatographic solvents were of the highest analytical grade and purchased from either Sigma Aldrich or Fisher Scientific, except ethyl acetate (Acros Organics). Water was obtained from a Milli-Q system (Millipore). Cholinium chloride (>98%, Sigma Aldrich), hereafter referred to solely as choline, and 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl, >98%, Iolitec) were dried *in vacuo* (40-70°C, 24-48 h, *ca.* 0.01 mbar) prior to use.

4.3.2. Fungal strains

Neurospora crassa wild-type strain FGSC 2489 (74-OR23-1VA) and the 1-aminocyclopropane-1-carboxylate deaminase deletion mutant FGSC 16712 (hereafter referred to NcΔAcc) were cultivated on dichloran-glycerol (DG18) agar (Oxoid), and suspensions of fungal conidia, prepared as previously described [16], were stored at - 80 °C in cryoprotective solution containing 0.85% w/v NaCl and 10% v/v glycerol.

4.3.3. Experimental conditions

The toxicity of the ionic liquids to be tested here towards *Neurospora crassa* deletion strain (NcΔAcc) was evaluated by determining its minimal inhibitory (MIC) and fungicidal concentrations (MFC), as previously described [19]. Fungal growth (or lack thereof) was followed daily by measuring the absorbance (600 nm). All tests were done in triplicate.

Fungal cultures (5 mL or 50 mL) were initiated from conidia (10^5 conidia *per* mL) in a 0.1% glucose mineral growth media [16] alone (control) or containing 0.2 M of 1-ethyl-3-methylimidazolium chloride or choline (dosage equivalent to 50% of the MIC of 1-ethyl-3-methylimidazolium chloride [16]). Liquid cultures were incubated in the dark, at 27 °C, under orbital agitation (90 rpm), for defined periods of time (2, 5, 10 or 15 days). At the end of incubation, mycelia were

recovered by filtration (glass fibre pre-filters) and both mycelia and filtrate were immediately frozen in liquid nitrogen and stored at -80 °C, until further analyses.

4.3.4. Metabolite extraction and analyses

The extracellular metabolites present in the fungal cultures of the wild-type and deletion strain were recovered. The lyophilised culture filtrates were solubilised in 10 mL Milli-Q water and extracted with ethyl acetate (1:1, three times). The following step was the collection of the organic phase obtained (hereafter referred to crude extract) and the evaporation of the solvent under soft nitrogen flow. The respective methanolic fractions (hereafter referred as peptide fractions) were obtained using the Sep-Pak plus C18 cartridge (Waters), as previously described [20].

NMR analysis

^{1}H NMR spectroscopic analyses of the crude extracts were carried out by Dr. Pedro Lamosa. Spectra were recorded on a Bruker 500 MHz spectrometer from CERMAX – Centro de Ressonância Magnética António Xavier at ITQB-UNL, Oeiras, Portugal.

Electrophoresis

The crude extracts from cultures of *Neurospora crassa* (200 mL) after 15 days of incubation in minimal media alone (control) or supplemented with cholinium chloride (choline) or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl) were electrophoretic separated by SDS-PAGE according to Schaegger [21]. Briefly, the fraction was resuspended in Tris-HCl buffer, pH 6.8, 25 mM containing 7.5% (v/v) glycerol, 1% (w/v) SDS, 25 mM DTT and traces of Serva Blue. The extracts were boiled for 10 minutes and then they were loaded in Mini-protean® Tris-Tricine precast gels, 10-20%T (BioRad) as well as the protein standards (protein marker < 10 kDa and Serva blue as a low molecular weight marker of 0.8 kDa). The separation conditions were 35 mA for 10 minutes, followed by 60 mA until the dye reached the front of the gel; 100 mM of Tris-HCl, pH 8.9 was used as the anode

buffer while the cathode buffer contained 100 mM Tris-HCl, pH 8.25, 100 mM of tricine and 0.1% (w/v) of SDS. After the electrophoretic separation, the gel was stained with Flamingo dye (BioRad) following the manufacturer's instructions and scanned using a Gel Doc system (BioRad).

Chromatographic analysis

Chromatographic profiles of both crude extracts and their corresponding peptide fractions, were acquired at the wavelength of 205 nm using the column, chromatographic equipment and gradient conditions previously described [16]. Semi-purified compounds (*i.e.* displaying a single chromatographic peak in the chromatogram of the peptide fractions) were recovered manually for further analysis (*ca.* 40 in each condition).

Mass spectrometry analysis

Preliminary mass spectrometry (MS) analysis of the crude extracts was performed in Lund University by Dr. Einar Nilsson using a Q-TOF micro mass spectrometer (Waters) applying electrospray ionisation technique (ESI, positive mode). The semi-purified peptides were sent for MS analysis (MALDI-TOF) on the Proteomics Facility of the University of Barcelona.

4.3.5. Amino acid hydrolysis and analysis

The total hydrolysis of peptides/proteins present in the peptide fractions or in the semi-purified peptides was performed using 6 N HCl for 1 h at 150ºC under nitrogen atmosphere. These hydrolysed samples were further analysed using the AccQ•Tag Ultra Amino Acid Analysis Method™ (eluent concentrates, derivatisation kit and standard mixture of amino acid hydrolysates, Waters) [22, 23]. Briefly, the hydrolysed samples, the standard amino acids (Aib and Acc) and the standard mixture of amino acid hydrolysates were derivatised following the manufacturer's instructions. The obtained derivatives were separated on an AccQ•Tag Ultra column (100 mm x 2.1 mm, 1.7 μm) by reversed phase ultra performance liquid chromatography (UPLC), and detected by absorbance (photodiode array detector,

PDA) and fluorescence (fluorescence detector, FLR), according to the following details. The column heater was set at 55 °C, and the mobile phase flow rate was maintained at 0.7 mL/min. Eluent A was 5% AccQ•Tag Ultra concentrate solvent A and eluent B was 100% AccQ•Tag Ultra solvent B. The separation gradient was 0-0.54 min (99.9% A), 5.74 min (90.9% A), 7.74 min (78.8% A), 8.04 min (40.4% A), 8.05-8.64 min (10.0% A) and 8.73-9.50 min (99.9% A). One microliter (1 μL) of sample was injected for analysis using a 10 μL loop. The PDA detector was set at 260 nm and the FLR detector was set at 266 nm and 473 nm of excitation and emission wavelengths, respectively. Data were acquired using Empower 2 software, 2006 (Waters).

Edman sequencing

Edman sequencing of a semi-purified peptide before and after selective trifluoroacetolysis (*i.e.* a partial hydrolysis using trifluoroacetic acid, TFA) was performed. After the adsorption of the samples to glass fibre, Edman sequencing was executed by Paula Chicau (ITQB) using Procise®, a Protein Sequencing System (model 491) of Applied Biosystems (ABI). Chemicals and sequencing protocols of ABI were used.

4.3.6. Antibacterial assay

The antibacterial assays were executed following the standard methodology implemented by the Clinical and Laboratory Standards Institute [24] using dilutions from 2.4 to 0.006 mg/mL of each peptide fraction in Mueller-Hinton Broth (MHB) medium. A control was analysed containing each bacteria grown solely on MHB. All the tests were done in triplicate. The Inhibitory Concentration of 50% of the population (IC50) was established using a logistic model constructed in Microsoft Excel and then tested using XL-STAT (Addinsoft). To evaluate bacterial viability after incubation with the peptide fraction, aliquots of the culture (including the control) were collected and the cells labelled with propidium iodide (PI), 15 minutes at room temperature with agitation. Bacterial cells were observed by contrast phase and fluorescence microscopy with a DM5500 B fluorescence microscope (Leica)

using a 49 DAPI and N21 filter sets, a 100× magnification objective, and images captured with an Andor Luca R EMCCD camera.

4.3.7. *Ex vivo* assay for anti-cancer activity

All tests were conducted as disclosed in published US patent application 20130136694. Briefly, egg chambers were extracted from female *Drosophila* less than 7 days old and exposed to metabolites (both the crude metabolite extracts and the corresponding peptide fractions) at standardised concentrations in Schneider's culture medium under controlled standard atmospheric conditions for up to 6 h. Egg chambers were observed using standard light and fluorescence microscopy and scored for presence/absence of polarity marker protein Par6. Each assay was done in triplicate. Controls for these assays included pure compounds: *the*-103 (a functional equivalent of aurothiomalate [25], which displays 100% activity in *the*LiTE™), each tested ionic liquid, the blank (0.6% v/v DMSO) and metabolite extracts of the control cultures and the non-inoculated media.

4.4. Results and Discussion

Neurospora crassa is a limited SM producer, since its genome is predicted to carry only ten biosynthetic SM gene clusters [6], which have not yet been systematically characterised.

Previous data reported by our team, showed that ionic liquid supplements in the growth media alter the metabolite footprint of filamentous fungi [15]. Moreover, the accumulation of 1-aminocyclopropane-1-carboxylate deaminase – a protein involved in the production of Acc [16], was noticed in *N. crassa* cultures exposed to an ionic liquid. This observation is consistent with the production of neoefrapeptins [26] or acretocins [27, 28] under such conditions. The likelihood of ionic liquid supplements to stimulate production of *N. crassa* peptide metabolites carrying Acc as building block is here investigated. Peptaibiotics (small fungal peptides biosynthesised via NRP synthetases) are usually found in the culture broth (*i.e.* extracellular) and reported to present antibiotic activity [28]. Their molecular weight usually ranges from 0.5 to 2.2 kDa, comprising 5 to 21 amino acids residues, including Aib, and their *N*-terminus is generally acylated (*i.e.* blocked) [28]. Additional characteristics allow this class to be divided into three different groups: peptaibols (which contain an alcohol group in the *C*–terminus of the peptide sequence), efrapeptins (containing pipecolic acid and a cationic bicycle amine group at the *C*–terminus) and neoefrapeptins (which contain Acc and some also 3-methylproline). In general, peptaibols are synthesised by *Trichoderma* spp [29], efrapeptins by *Tolypocladium* spp [30, 31], and neoefrapeptins by *Geotrichum candidum* [26].

To confirm the hypothesis of peptaibiotics' production by *N. crassa* in media supplemented with an ionic liquid, the crude extracts were first analysed by mass spectrometry, gel electrophoresis and NMR. The mass spectrometry data showed a very weak signal presenting *m/z* value of 1591, consistent with the general characteristics of peptaibiotics (molecular weight range 0.5-2.2 kDa). A possible molecular formula was also predicted (Fig. 4.1). The peptides in the crude extract were separated by electrophoresis and visualised using the Flamingo stain as

depicted in Fig. 4.2. The crude extract from cultures grown in media supplemented with 1-ethyl-3-methylimidazolium chloride, contained high amounts of a small peptide, which was virtually absent in extracts from both control and choline media. The molecular weight of this peptide is estimated in gel to range between 0.8 and 6.5 kDa (Fig. 4.2), therefore in the range of small peptides.

The crude extract from cultures exposed to 1-ethyl-3-methylimidazolium chloride was also analysed by NMR (Fig. 4.3) and the spectrum shows some signals between 5.5 and 8.5 ppm (Fig. 4.3), which are associated to the presence of peptide bonds (R-CO-NHR'). All preliminary results show characteristics consistent with those of peptide metabolites, particularly of peptaibiotics.

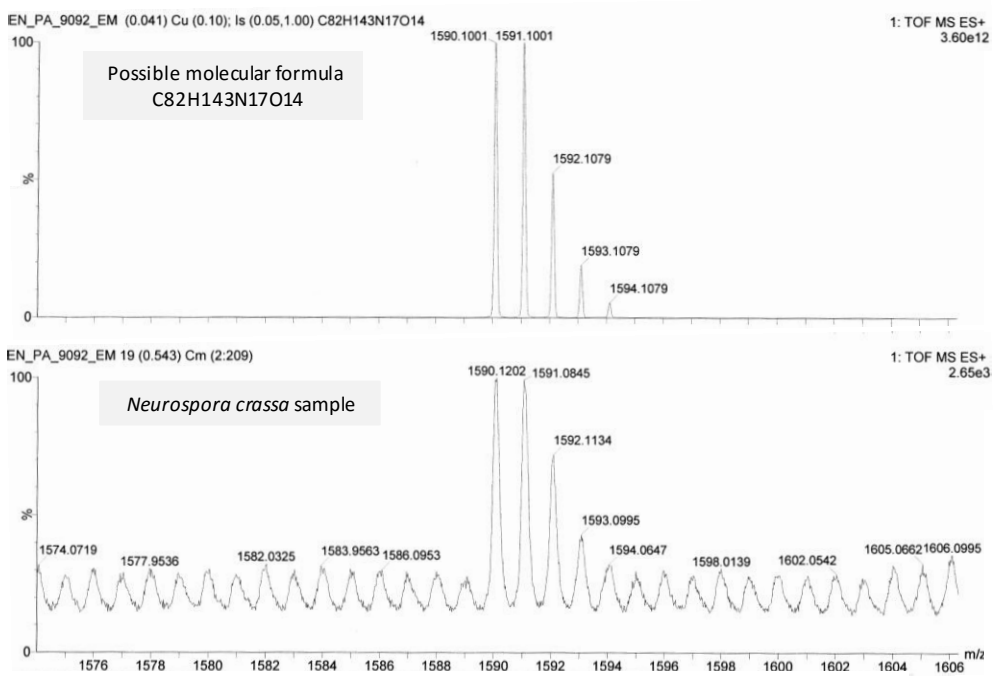

Fig. 4.1. Preliminary mass spectrometry (TOF MS) analysis of metabolite extracts from cultures of *Neurospora crassa* (50 mL) after 15 days of incubation in an ionic liquid supplemented medium. It is presented a prediction of a possible molecular formula for the detected *m/z* value of 1591, which is in the range of the peptaibiotics family (*i.e.* 0.5-2.2 kDa).

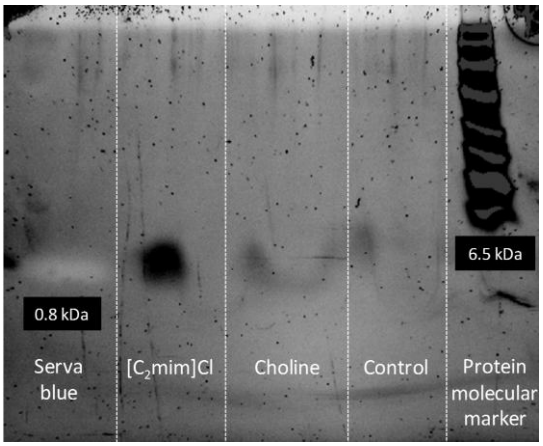

Fig. 4.2. Gel image of the electrophoretic separation of the crude extracts from cultures of *Neurospora crassa* (200 mL) after 15 days of incubation in minimal media alone (control) or supplemented with cholinium chloride (choline) or 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl).

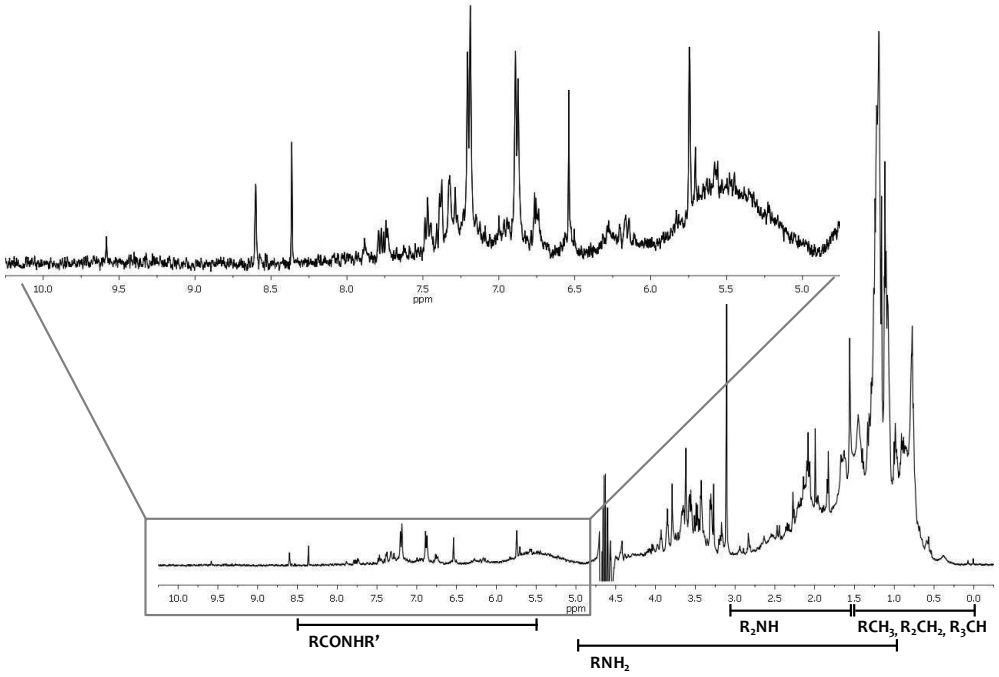

Fig. 4.3. NMR spectra of the metabolite extracts from cultures of *Neurospora crassa* (200 mL) after 15 days of incubation in minimal media supplemented with cholinium chloride.

4.4.1. Optimisation of culture conditions and extraction procedures

Peptide enrichment of* Neurospora crassa *metabolite extracts

The chromatographic profiles at 205 nm of the crude extracts (*i.e.* ethyl acetate extraction) of *N. crassa* cultures grown in the absence (control) or presence of an ionic liquid were virtually similar (Fig. 4.4A). Only after peptide enrichment [20] (*i.e*. peptide fraction) could significant differences be found in the chromatograms (Fig. 4.4B). Either ionic liquid induced formation a higher diversity of compounds as shown in the chromatograms at 205 nm, particularly of compounds eluting between 20 and 30 minutes (Fig. 4.4B). Interestingly, these compounds appeared to be produced in either ionic liquid media (retention time (Rt) of 23, 23.5 and 27.5 minutes) but a slightly higher amount accumulated in the cultures exposed to 1-ethyl-3-methylimidazolium chloride compared to cholinium chloride (Fig. 4.4B).

Time-course analysis of the* Neurospora crassa *metabolic footprints under ionic liquids stimuli

To determine how the production yields of the peptide metabolites evolved along time, peptide fractions were obtained from *N. crassa* cultures after 2, 5, 10 and 15 days of incubation in minimal media alone or supplemented with either ionic liquid. The profile of the corresponding chromatograms is depicted in Fig. 4.5. Significant accumulation of the major differential peak (Rt = 23 minutes) could only be detected after 15 days of cultivation (Fig. 4.5). Slight differences in the chromatograms may be explained by the cultivation volume; nonetheless the data reproduces the data shown in Fig. 4.4B.

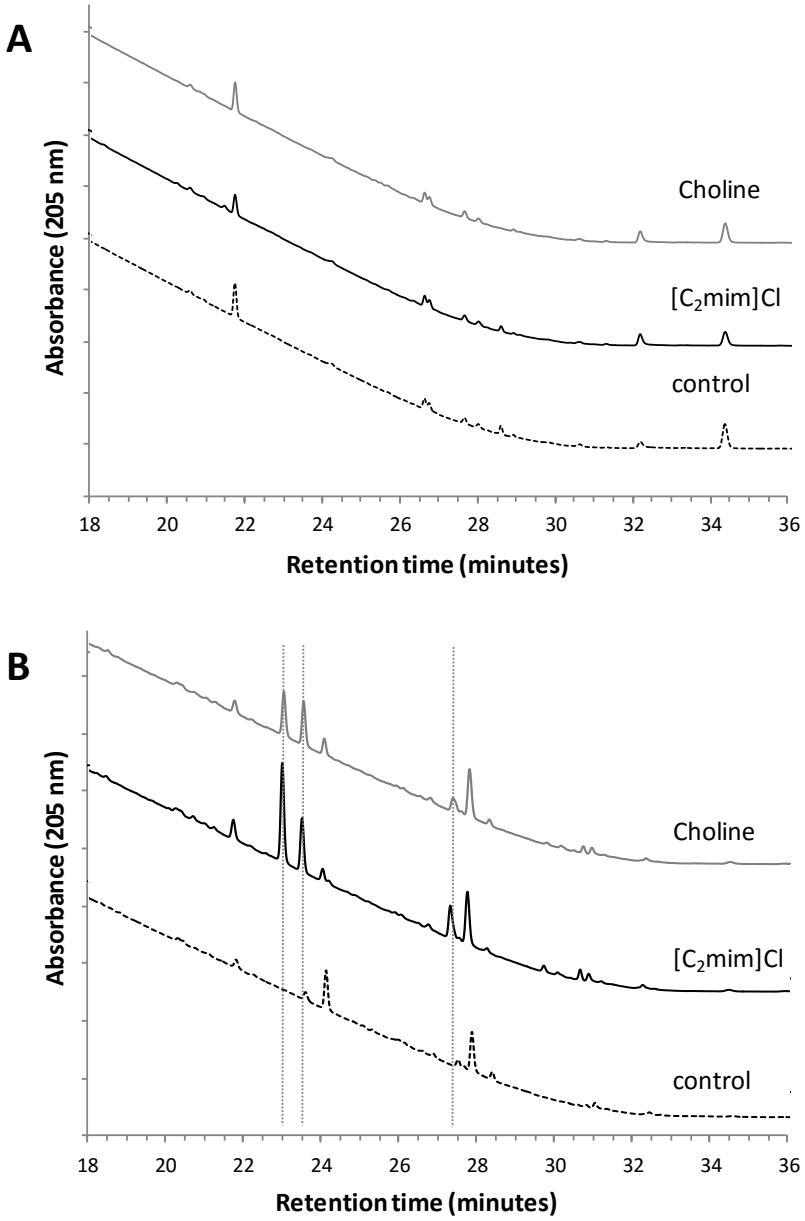

Fig. 4.4. Aligned chromatographic spectra (205 nm) of the crude extracts (A) and the peptide fractions (B) of *Neurospora crassa* cultures (50 mL) after 15 days of incubation in minimal media alone (control, dashed line) or supplemented with either ionic liquid (choline, grey line, or 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl, black line). The vertical dashed lines highlight the most interesting differential peaks at retention times of 23, 23.5 and 27.5 minutes.

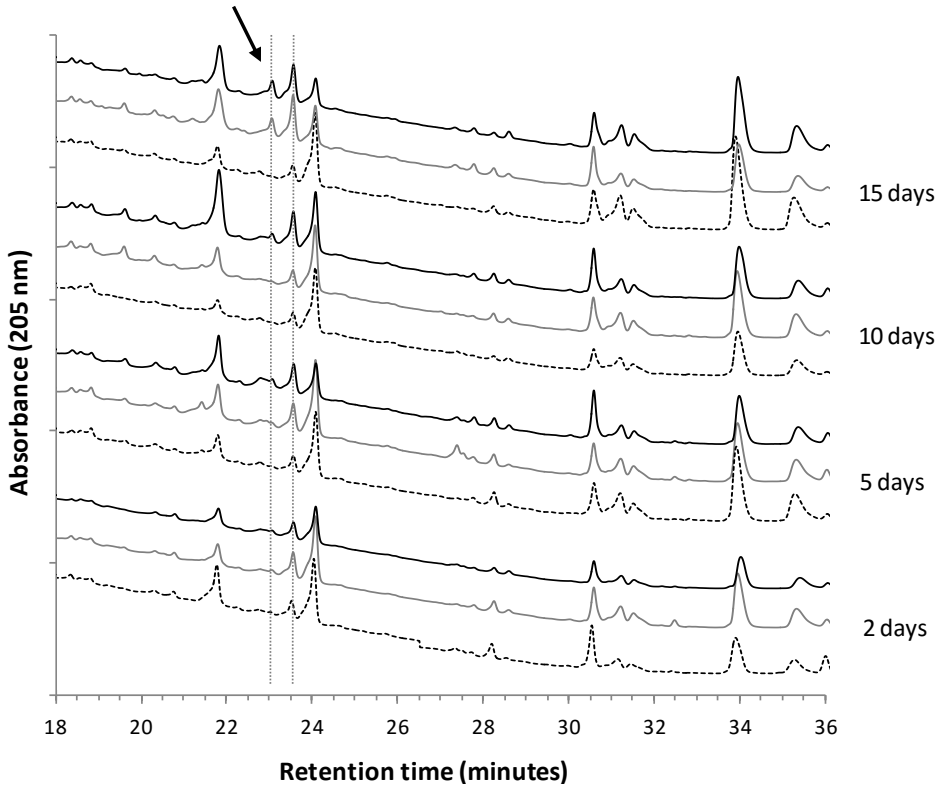

Fig. 4.5. Aligned chromatographic spectra (205 nm) of the peptide fractions of *Neurospora crassa* cultures (15 mL) after 2, 5, 10 or 15 days of incubation in minimal media alone (control, dashed line) or supplemented with either ionic liquid (choline, grey line or 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl, black line). The vertical dashed lines highlight the most interesting differential peaks accumulating in the ionic liquids supplemented media (retention time of 23 and 23.5 minutes). The arrow indicates major differences comparing ionic liquids supplemented media with control, after 15 days of incubation.

4.4.2. Metabolome profiling of *Neurospora crassa* wild-type and mutant strains during exposure to 1-ethyl-3-methylimidazolium chloride

To further strength the hypothesis of 1-ethyl-3-methylimidazolium chloride capacity to trigger the synthesis of peptaibiotics in *N. crassa* we performed additional functional analysis in a *N. crassa* deletion mutant lacking the 1-aminocyclopropane-1-carboxylate deaminase gene, (FGSC 16712, NcΔAcc). The MIC and MFC values of 1-ethyl-3-methylimidazolium chloride against the deletion mutant NcΔAcc were similar to those of the wild type (data not shown). Accordingly, both strains

(FGSC 2489 and FGSC 16712) were exposed to 0.2 M of 1-ethyl-3-methylimidazolium chloride (half of the MIC for this chemical).

As expected, the peptide fraction recovered from the wild-type strain exposed to the ionic liquid shows major differential peptides eluting at 23 and 27.5 minutes compared to control. Deletion of 1-aminocyclopropane-1-carboxylate deaminase gene eliminates the formation of compounds eluting between retention time of 23 and 30.5 minutes, even when cultures were stressed by the ionic liquid (Fig. 4.6). These observations further strength that the differential peptides may be Acc containing peptides. The chromatographic profile of the mutant strain mainly presents signals common to all conditions (*viz.* compounds eluting at 21.75 and 30.5 minutes) (Fig. 4.6).

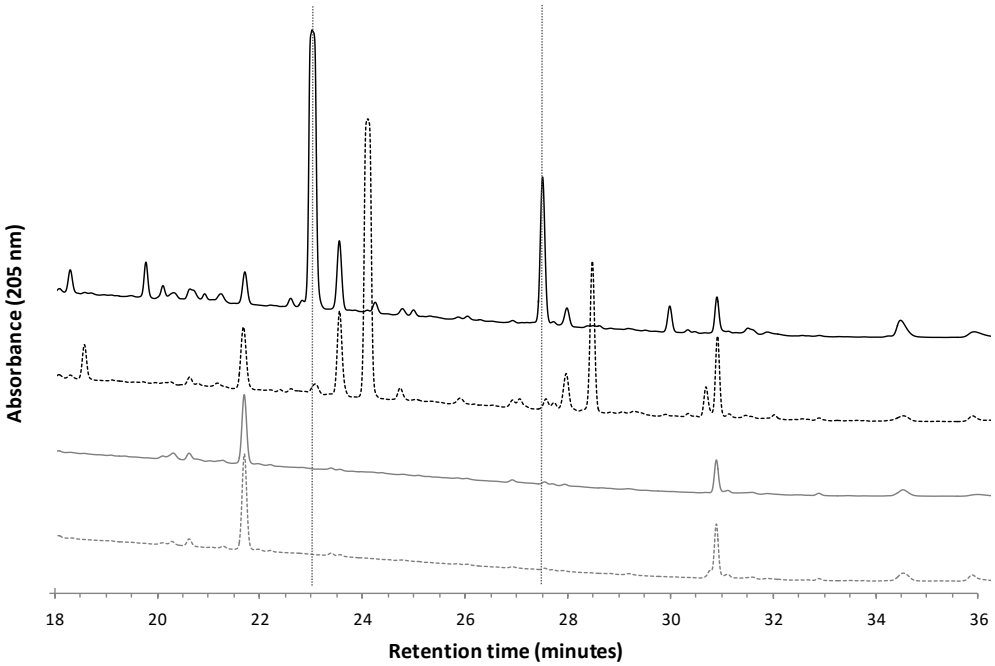

Fig. 4.6. Chromatographic profiles of the peptide fractions recovered from *Neurospora crassa* wild-type (FGSC 2489, black) and NcΔAcc (FGSC 16712, grey) cultures (100 mL) grown for 15 days of in control conditions (dashed lines) or in 1-ethyl-3-methylimidazolium chloride supplemented medium (continuous lines). The vertical dashed lines highlight the differential peaks accumulating in 1-ethyl-3-methylimidazolium chloride supplemented medium (retention time of 23 and 27.5 minutes).

Analysis of the peptide fractions – a scaled up experiment

Based on the time-course analysis (Fig. 4.5), fifteen days of incubation were chosen for producing new cultures of *N. crassa* in media containing 1-ethyl-3-methylimidazolium chloride (ten cultures of 50 mL were pooled together and analysed as bulk). Higher diversity of compounds could be detected in the chromatographic profiles of the peptide fraction corresponding to 500 mL culture (Fig. 4.7) (concentration factor of ten compared to the 50 mL ones depicted in Fig. 4.4B). In the peptide fraction corresponding to 500 mL culture in 1-ethyl-3-methylimidazolium chloride media, nearly 40 differential compounds could be detected (Fig. 4.7), obviously including the differential compounds eluting at 23, 23.5 and 27.5 minutes. All the differential peptides were manually collected for further analysis.

Analysis of the semi-purified peptides

One of the main features of peptaibiotics is the high content of Aib [28]. Accordingly, we evaluated the presence of Aib, as well as of Acc, in the hydrolysates of the peptide fractions of *N. crassa* crude extracts. The hydrolysates of the peptide fractions and of their corresponding semi-purified peptides, recovered from cultures grown in control conditions or exposed to 1-ethyl-3-methylimidazolium chloride were separated using the simple and sensitive AccQ•Tag Ultra Amino Acid Analysis Method™ [22]. Aib could only be detected in the hydrolysate of the peptide fraction from cultures grown under the ionic liquid stress (Fig. 4.8A, C). This observation strengthens the hypothesis of 1-ethyl-3-methylimidazolium chloride capacity to trigger production of peptaibiotics in *N. crassa*. On the other hand, Acc could only be detected in the semi-purified peptide eluting at 23 minutes recovered from cultures exposed to the ionic liquid (Fig. 4.8B). This semi-purified peptide apparently does not contain Aib (Fig. 4.8) or Aib may be below the detection limit of the chromatographic method. Besides fungal peptaibiotics containing Acc as a building block module, such as neoefrapeptins [26] and acretocins [27, 28], the existence of other small peptides containing Acc but lacking Aib has already been reported [32].

To determine the complete amino acid sequence of the semi-purified peptide eluting at 23 minutes, which contains Acc, we have relied on the use of Edman degradation. Peptaibiotics usually have the *N*-terminal blocked - a feature that hampers sequencing by Edman degradation, which was also observed here. The analysis was repeated after selective trifluoroacetolysis cleavage of the semi-purified peptide but also failed most likely due to the presence of interfering contaminants.

Initial MALDI-TOF analyses of the peptide fraction and the corresponding semi-purified peptide eluting at 23 minutes recovered from cultures exposed to the ionic liquid were inconclusive. The signals from small peptides (0.5-2.2 kDa) were overlapping the matrix signals (data not shown).

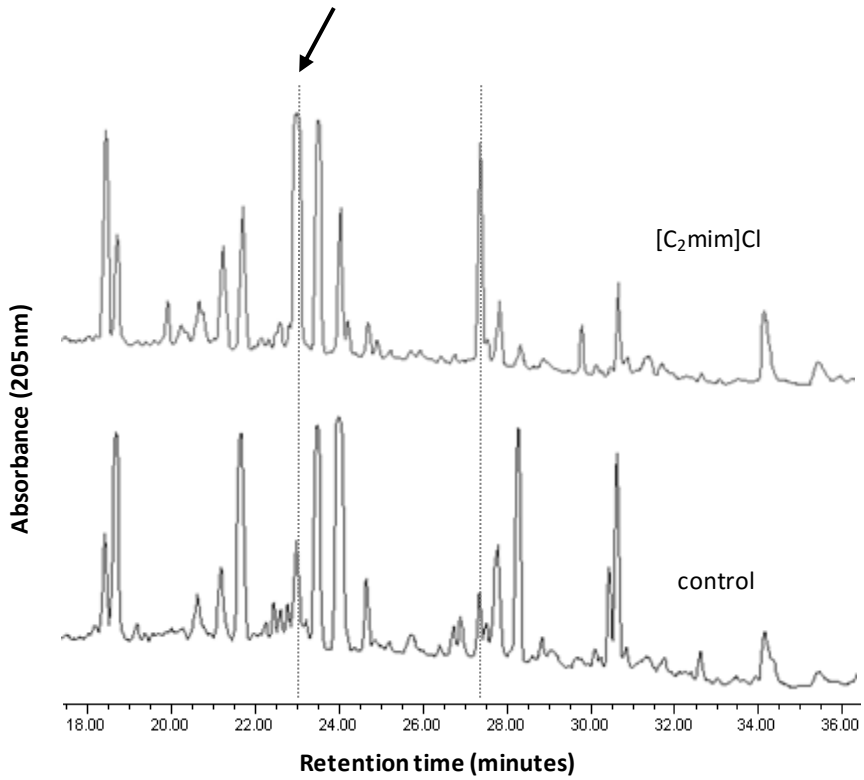

Fig. 4.7. Aligned chromatographic spectra (205 nm) of the peptide fraction of *Neurospora crassa* cultures (500 mL) after 15 days of incubation in minimal media alone (control) or supplemented with 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl). The vertical dashed lines highlight the more intense peaks in 1-ethyl-3-methylimidazolium chloride supplemented media (retention time of 23 and 27.5 minutes). The arrow at retention time of 23 minutes evidences one of the differential peaks detected that was further analysed.

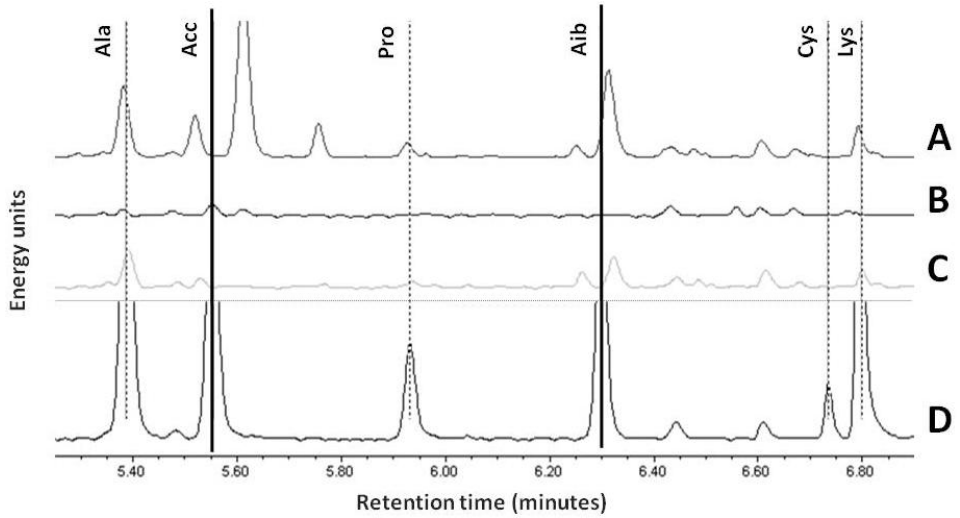

Fig. 4.8. Chromatographic separation of the hydrolysate samples: A) total peptide fraction recovered from *Neurospora crassa* cultures grown in 1-ethyl-3-methylimidazolium chloride supplemented medium; B) semi-purified peptide eluting at 23 minutes from cultures exposed to 1-ethyl-3-methylimidazolium chloride; C) total peptide fraction from *N. crassa* control culture; and D) standard amino acid mixture containing α-aminoisobutyric acid (Aib) and 1-aminocyclopropane-1-carboxylic acid (Acc). The complete profile of the standard amino acid mixture is depicted in Fig. S1 (Additional file 1[*]).

4.4.3. Analysis of the biological activity of *Neurospora crassa* extracts after 1-ethyl-3-methylimidazolium chloride stimulus

The peptide antibiotics are widely known for their biological activities [28]. In addition, neoefrapeptins, which contain Acc as building block, have been described to present insecticidal activity [26].

Antibacterial assays

The IC50 value of the peptide fraction recovered from *N. crassa* cultures exposed to 1-ethyl-3-methylimidazolium chloride against *Escherichia coli* or *Staphylococcus aureus* were 0.480 mg/mL and 0.586 mg/mL, respectively. Much lower activity was found in the peptide fraction recovered from control cultures, which displayed IC50 values of 1.394 mg/mL and 1.171 mg/mL against *E. coli* and *S. aureus*, respectively. This means the peptide metabolites are responsible for an increase of *ca.* 65% and

[*] *available in the thesis CD.*

50% of antibacterial activity; consistent with the capacity of 1-ethyl-3-methylimidazolium chloride to stimulate production of antimicrobial peptides (AMPs). Mixtures of AMPs, such as those probably analysed here, often reach antibacterial activities at concentrations above 1 mg/mL [33], hence in the range of compounds produced in control conditions.

To validate these observations, microscopic assays were undertaken in aliquots of bacterial cultures exposed or not to the peptide fractions. The micrographs depicted in Fig. 4.9 clearly show that the number of dead bacteria (on the right) is substantially higher in the presence of peptide extracts recovered from cultures exposed to the ionic liquid than from control cultures.

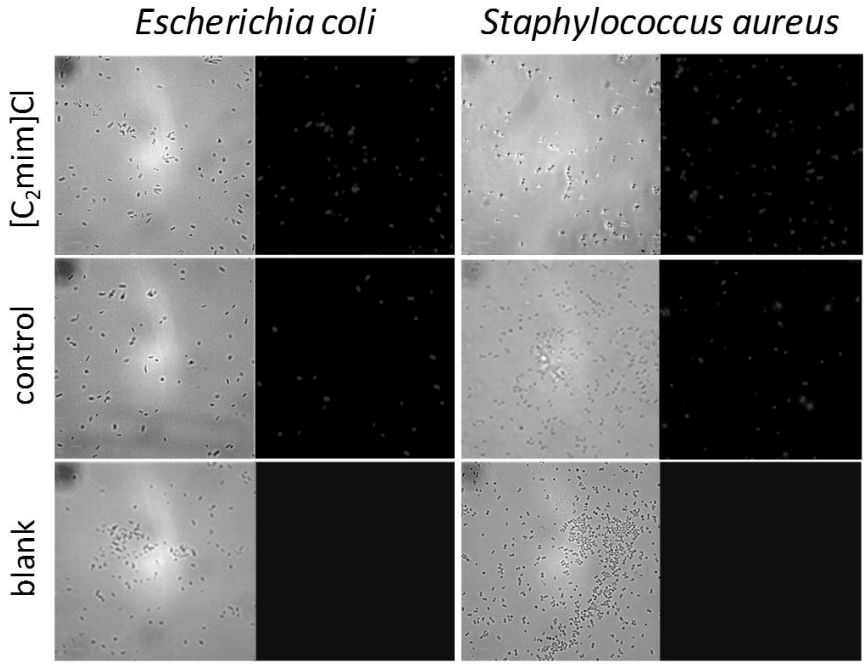

Fig. 4.9. Micrographs of the total population (left, contrast phase bright field) and the dead population (right, fluorescence) of *Escherichia coli* and *Staphylococcus aureus* in standard medium (blank) or expose to peptide fractions recovered from *N. crassa* cultures in control conditions or exposed to 1-ethyl-3-methylimidazolium chloride (*nb. figure with high resolution is available in the thesis CD*).

Propidium iodide labels cells with a disrupted cellular membrane. However, membrane damage can either be the primary mode of action of the antimicrobial

peptides or a consequence of other more complex killing mechanism. Some studies have analysed the mechanism of action of pure AMPs [34-36] but the majority have focussed on synthetic antimicrobial peptides [37]. Peptaibiotics display a wide range of activities, such as antibacterial, antifungal, antiviral and antiparasitic [28], but also ability to form pores in bilayer lipid membranes (*e.g.* lipopeptaibol antibiotics [38]). These activities are achieved in several different ways, such as the ability to inhibit the mitochondrial ATPase, uncouple oxidative phosphorylation, induce fungal morphogenesis, inhibit platelet aggregation and present immunosuppressive and neuroleptic effects, as previously reported and reviewed [39]. As an example, the ability of efrapeptins to inhibit the mitochondrial ATPase justifies their antifungal and insecticidal activities [40].

Anti-cancer activity

Cell polarity is central to onset and progression of diseases including carcinoma. *the*LiTE™, a validated *ex vivo* assay which measures the polarity modulating activity of compounds in live *Drosophila* tissues, was used here to screen the presence of compounds with anti-carcinoma potential in the peptide fractions of *N. crassa* culture extracts (Fig. 4.10). The positive control (*i.e.* 30 µM of *the*-103 compound) affects 100% of the *Drosophila* eggs. On the contrary the blank (*i.e.* 0.6% v/v DMSO) which is the solution used to solubilise the extracts presents no significant activity (Fig. 4.10A). The cut off for a positive result in this assay is 50%, *i.e.* if a pure compound is able to impair the fluorescent ring of the polarity marker protein Par6 in more than half of the analysed eggs, it presents anti-carcinoma activity. The assessment of this potential in mixtures of compounds is more complex and the cut off in these cases is usually lower. All the tested peptide fractions recovered from *N. crassa* cultures either in control conditions or exposed to the ionic liquid showed activity higher than 50% (Fig. 4.10B). The detected activities cannot be related to any vestigial amounts of ionic liquid because it lacks activity (data not shown). Although the peptide fraction from cultures exposed to the chemical stress showed lower activity than that from control cultures, the semi-purified peptide showed virtually the same activity (Fig. 4.10B). The mix of peptide

compounds is higher in cultures exposed to the ionic liquid compared to control conditions; the effect of the active peptide may be diluted by the non-active ones. Nonetheless, peptide metabolites produced by *N. crassa* in either conditions display interesting properties. The possibility of exploring ionic liquids as inducers of the biosynthesis of specific anti-carcinoma peptides has not been fully demonstrated here, but these initial observations should inspire further investigation.

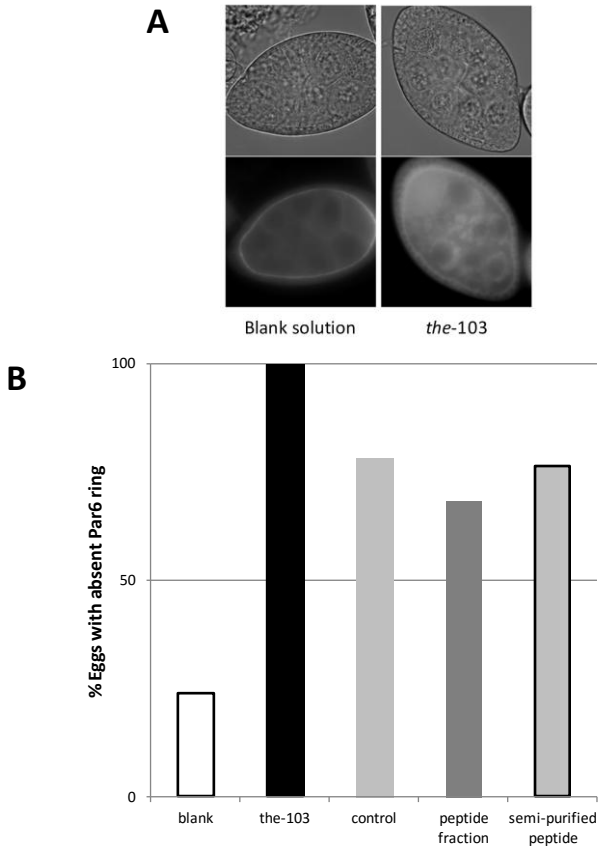

Fig. 4.10. *Ex vivo the*LiTE™ assay of peptide fractions recovered from *Neurospora crassa* control cultures or exposed to 1-ethyl-3-methylimidazolium chloride. A) Microscope image of *Drosophila* egg chambers showing presence and absence of the polarity marker protein Par6 when exposed to the blank (*i.e*. 0.6% DMSO) and *the*-103, respectively. B) % of egg chambers showing Par6 ring impairment in the presence of the blank (white bar) and of *the*-103 (black bar), or in the presence of the peptide fractions (24 µg/mL) recovered after 15 days of incubation from control cultures (light grey bar), cultures exposed to 1-ethyl-3-methylimidazolium chloride (dark grey bar); or in the presence of the semi-purified peptide eluting at 23 minutes from cultures exposed to 1-ethyl-3-methylimidazolium chloride (light grey bar with black line).

4.5. Future perspectives

The obtained data are consistent with the initial hypothesis of 1-ethyl-3-methylimidazolium chloride capacity to trigger production of antimicrobial peptide metabolites containing Acc and/or Aib as structural units in *N. crassa*. Therefore, this work constitutes the first evidence of *N. crassa* capacity to produce such peptide metabolites. Different peptide metabolites have been detected in the culture extracts of other fungi, *e.g. Trichoderma* spp (peptaibols), *Tolypocladium niveum* (efrapeptins) or *Geotrichum candidum* (neoefrapeptins). In addition, *Penicillium roquefortii* [41] and *P. nalgiovense* [42] were reported to be able to produce Aib-containing compounds.

Only in the presence of 1-ethyl-3-methylimidazolium chloride could *N. crassa* synthesise compounds carrying Aib (Fig. 4.8), which is consistent with the presence of peptaibiotics in these culture extracts. Moreover, only the hydrolysate of the semi-purified peptide eluting at 23 minutes, which was recovered from the peptide fraction containing Aib, revealed the presence of Acc. The sequence of this specific peptide remains unresolved but the composing amino acids have been detected (Fig. S1 and S2 in Additional file 1[*]) and quantified (Table S1 in Additional file 1[*]). Nevertheless information on its molecular weight is still lacking (Fig. 4.1); essential to estimate the ratio of each amino acid in the peptide structure. Alternative methods to the ones used here (TOF MS, MALDI-TOF and electrophoresis gel detection) are required to define the molecular weight of this peptide, as well as of the other differential peptides from which general information is still largely lacking. In the peptide metabolite eluting at 23 minutes, even if Aib amounts are lower than the detection limit, the presence of Acc in its structure, supports that it is not a classic peptaibiotic. Previous studies have shown the existence of fungal peptides containing only Acc in its structure, such as BZR-cotoxin II, which is produced by *Ascomycota* [43], and serinoticyns [32].

[*] *available in the thesis CD.*

To conclude this study further analyses are necessary, namely to solve the nature, the sequence and the biological activity of each of the differential peptide metabolites produced under an ionic liquid stress in *N. crassa* cultures. Accordingly, much higher amounts of peptide fractions and of the semi-purified compounds are required. Nonetheless this study has established most of the research strategy and serves as a proof-of-concept of the capacity of 1-ethyl-3-methylimidazolium chloride to trigger production of peptides carrying Acc and/or Aib. This opens the possibility of identifying novel biological active peptides of broad pharmacological interest. Ultimately, data made clear that the model filamentous fungus *N. crassa* still hides valuable secrets, including cryptic secondary metabolites.

4.6. Acknowledgements

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CHAPTER V

Final discussion

This discussion contains parts of the following publication of the author:
Isabel Martins, Diego O. Hartmann, **Paula C. Alves**, Celso Martins, Helga Garcia, Céline C Leclercq, Rui Ferreira, Ji He, Jenny Renaut, Jörg D. Becker and Cristina Silva Pereira. Elucidating how the saprophytic fungus *Aspergillus nidulans* uses the plant polyester suberin as carbon source. *BMC Genomics*, 2014, 15, 613.
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5.1. Final discussion

The following section presents a critical and integrated discussion of data presented in the previous chapters, as well as future perspectives.

The increasing use of ionic liquids, either at laboratory or industrial scales, raises questions about their safeness, especially in case of accidental leakage. Moreover, we have witnessed a series of advances towards the applications of ionic liquids in life sciences, *e.g.* as active pharmacological ingredients [1] that further strength the need to attain fundamental understandings of structure-activity relationships and on their eco-toxicity. The rule of thumb on ionic liquid's mechanism of toxicity is that, in either of the composing ions, the length of the alkyl chain is directly correlated with lipophilicity and permeabilisation of biological membranes, leading to cell death [2]. This simplistic view is only valid for lipophilic cations as recently demonstrated in our team [3], and has a series of exceptions. In this thesis I have been focussing on the effect of ionic liquids supplements in filamentous fungi, hence monitoring the effect of the ions. Even so, it may be excessive to generalise that the diluted ions will act as the neutral molecule, one good example of this is the fact that by synthesising a common antifungal drug as an anion (in combination with different cations) completely alters the mode of action in some cases increasing the drug potency against pathogenic fungi [4]. So far, most data on the toxicity of ionic liquids have been established based essentially on poorly systematised measures of inhibitory or lethal activity against numerous model organisms by using a disparate set of methodologies. Our team decided to address this topic in a different way, in part motivated by the search of some expected outcomes. The first step was to screen their toxicity towards filamentous fungi as model organisms, focussing on several fungal strains that commonly colonise soils (major receptor environmental compartment of any contamination leak). The obtained data revealed that these organisms tolerate very high concentrations (*ca.* 0.05 M) of a wide range of structurally different ionic liquids (all of which were chlorides) compared to other tested model organisms [2]. Additionally, the presence of either organic cation supplements augmented the metabolic diversity of the fungal footprints compared to

controls [5]. These initial results increased our interest in the study of the impact of these chemicals in the general metabolism of filamentous fungi. In order to achieve a broader view we decided to use high-throughput approaches and focus the study on two relevant model strains namely *Aspergillus nidulans* and *Neurospora crassa*. Importantly, these model fungi display very distinct halotolerance, which we hypothesised to be translated into very distinct resistances to ionic liquids.

At that time, RNA-Seq was still in its infancy; therefore we designed a microarray containing the genetic information of these two phylogenetically close related *Ascomycota*. This strategy was not completely novel, since other multi-species microarrays have been established but not containing the two species considered in our array [6]. In fact, the microarray FungiANC was validated in a study focussing on the elucidation of suberin utilisation by *A. nidulans* [7]. This constitutes a relevant output of this PhD because the microarray data provided meaningful information on the potential antifungal effect of this plant polyester.

Proteomics was our first option of a high-throughput approach for analysing the effect of ionic liquids on the general metabolism of the selected model fungi (Chapter II). Cholinium chloride (biocompatible and biodegradable) and 1-ethyl-3-methylimidazolium chloride (toxic and recalcitrant) were chosen as representative ionic liquids, since they display distinct biodegradability and toxicity, and belong to the most studied families of ionic liquids. In the proteomics study we observed that, in both fungi, either ionic liquid induced the accumulation of numerous stress-responsive proteins, the production of osmolytes and altered their developmental programmes. 1-Ethyl-3-methylimidazolium chloride displayed higher toxicity but excessive exogenous supplementations of the cholinium cation also led to growth inhibition probably due to the production of toxic sub-products, notwithstanding this cation is generally assumed as biocompatible.

Of significance is the fact that 1-ethyl-3-methylimidazolium chloride induced the accumulation of multidrug transporters, which were also observed to be involved in the mechanism of resistance of *Enterobacter lignolyticus* to similar ionic liquids [8]. Overall, the obtained data revealed the main alterations induced by the presence of either ionic liquid supplement in the primary metabolism. As expected,

the technique was not suitable to resolve the multi-domain enzymes involved in the biosynthesis of fungal SMs, notwithstanding parallel chromatographic analyses of the cultures extracts showed a differential profile, with significant higher diversity of metabolites in these conditions compared to controls [5]. Aiming to complement the proteomics study, we used transcriptomics yet keeping our conceptual approach: measures of the effects of "representative" ionic liquid supplements in the metabolism of fungi, covering both primary and secondary metabolism. The transcriptomes of both fungi were analysed using the designed microarray, but so far only the data corresponding to *A. nidulans* were fully processed because this fungus is a better model for studies focussing on secondary metabolism, specifically as an exceptional SM producer with a series of already characterised SM biosynthetic gene clusters, some of which defined as cryptic metabolites and only seen through the use of molecular genetics approaches [9] (Chapter III). Data on *A. nidulans* emphasised the higher toxicity of 1-ethyl-3-methylimidazolium chloride, which down-regulated primary metabolism, induced the use of cellular reserves and autophagy. In contrast, cholinium chloride could be used as a source of carbon and nitrogen that incorporated the central carbon metabolism, and consequently up-regulated primary metabolism. Importantly, several SM backbone genes were up-regulated by either ionic liquid supplement and numerous differentially formed compounds (*n.b.* absent in control conditions) could be detected in the metabolome analyses. Among the detected metabolites we could identify monodictyphenone, gentisic acid and caffeic acid; seen for the first time in *A. nidulans* wild-type strain. The expression level of monodictyphenone backbone gene could be related to the production of this SM. In addition, several other uncharacterised backbone genes also underwent significant up-regulation (*e.g.* AN9005, AN12331 and AN5318) constituting now excellent candidates to disclose new SM biosynthetic gene clusters preferentially by taking advantage of mutants carrying deletions in genes coding for its biosynthetic enzymes combined with their functional characterisation in the presence of an ionic liquid supplement. To implement this we will rely on an established method for gene replacement already routinely used in our team [10].

The complex mixture of metabolites formed in the presence of either ionic liquid supplement showed anti-carcinoma potential. This emphasises the potential of this family of chemicals to induce production of metabolites with possible clinical applications. Future studies will require the scale-up of fungal cultivation; probably through the use of established fermentation procedures. This is essential to increase metabolite titres for further and wider biological testing, as well as for NMR analyses to solve their chemical structure. As a curiosity, in a series of published studies on fungal SMs, the fermentation capacity used to conclude on their identity was of dozens of litters [11]; this was here an impediment but on the other hand the effort required to establish optimal fermentation conditions for SM production would likely be very time consuming.

Interestingly, the proteomics study (Chapter II) also revealed that both 1-ethyl-3-methylimidazolium chloride and cholinium chloride have apparently induced the accumulation of 1-aminocyclopropane-1-carboxylate deaminase in *N. crassa*. This enzyme is involved in the production of 1-aminocyclopropane-1-carboxylic acid (Acc), which has been linked to the biosynthesis of fungal peptide antibiotics. This exciting observation created *per se* a great opportunity to undertake a study focussing specifically on peptaibiotics production by *N. crassa* (Chapter IV). The metabolite extracts were first enriched in the peptide fraction and then the differential peptides (*i.e.* produced under an ionic liquid stress compared to control) were spot in UHPLC analyses. This way, all the differential peptides could be collected and their composition in amino acids resolved. Aib was present only in the total hydrolysate of the peptide fraction from cultures grown in an ionic liquid media, whereas Acc (but not Aib) was present in one of the semi-purified peptides of the latter fraction. Importantly, the ionic liquid supplement could not activate the biosynthesis of any differential peptide metabolite in a *N. crassa* mutant strain devoid of 1-aminocyclopropane-1-carboxylate deaminase. Both observations strengthened the hypothesis of 1-ethyl-3-methylimidazolium chloride supplement capacity to trigger production of peptaibiotics in *N. crassa*, constituting the first evidence of the ability of this fungus to produce such peptide metabolites. None of the techniques tested so far (*e.g.* Edman degradation and MALDI-TOF) provided

conclusive data regarding the peptide sequence. Once again, scale-up of the cultures might be essential to increase the peptides production titres, allowing simpler purification and characterisation. The peptide metabolites produced in the ionic liquid media (or mixture of them) displayed very interesting antibacterial activity which may stimulate further analyses. These peptide metabolites belong to the non-ribosomal peptide family of SMs; thus a possible future strategy for their elucidation is to analyse the ensuing diversity of peptides in *N. crassa* NRPS deletion mutants (available in the FGSC) in cultures supplemented or not with 1-ethyl-3-methylimidazolium chloride. Some tests with these mutants were already initiated, including the determination of the minimal inhibitory concentrations of the ionic liquid for each strain, and the design of primers for each of the backbone genes for advancing for RT-PCR analyses (*e.g.* NCU08441, a NRPS that was up-regulated in either ionic liquid, according to *N. crassa* microarray data).

Besides knowing that the production of peptaibiotics requires long incubation periods, information on their regulation and their biological function is still scarce. In general, the supplementation of (rare) amino acids, especially of Aib, induces the production of peptide metabolites containing Aib in their structure. Moreover, the *so called* global regulator of fungal metabolism, LaeA, is also known to impact the expression of certain NRPS involved in the biosynthesis of peptaibiotics. Conditions have been now raised to fill key knowledge gaps on the regulation and biological function of peptide metabolites in *N. crassa*, as well as of multiple SMs in *A. nidulans*; surely I will witness soon further discoveries on these aspects in follow up studies to be undertaken by other colleagues in the team.

The use of high-throughput techniques to obtain a general overview of the impact of ionic liquids in the metabolism of fungi was a profitable strategy. Using this concept several “hot” topics could be identified (some not even disclosed here), creating unexpected opportunities for subsequent detailed analyses. At the same time, this conceptual approach created some technical impediments and challenges, including the efficient extraction of protein and/or RNA from fungal cultures in late stationary phase, the continuous improvement of genome annotations that altered the match between microarray probes and genes, the time-consuming and extremely

complex analysis of both transcriptome and metabolome data, all of which were solved throughout the PhD but hinder rapid progress. Now, to dig further the topic of fungal SMs I would perform time-course experiments and analyse simultaneously gene expression levels and proteins (using "gel-free" proteomics, which is now also in place in the laboratory), obviously ensuring the production of the SMs at larger scale (litter level), if possible using controlled fermentation conditions.

Either way, studying fungal SMs is an exciting and challenging topic. I am amazed by their unseen diversity and complex regulation. Equally amazed by the capacity of ionic liquids supplements (in particular the organic cations) to stimulate production of otherwise cryptic SMs through activation of their backbone genes. The activation of SM biosynthetic gene clusters is a complex process that depends on the concerted action of several agents, such as broad-domain factors (*e.g.* PacC), global (*e.g.* LaeA) and/or pathway specific regulators (*e.g.* AflR), chromatin remodelling enzymes and communication between clusters located in different chromosomes. Not analysed in detail, is the fact that the ionic liquid supplements also influence the expression of a series of genes coding chromatin remodelling enzymes, some of which may play more important roles in the control of secondary metabolism than that recognised so far.

In conclusion, despite the immensity of studies on secondary metabolism in fungi, consequently the established knowledge regarding their biosynthesis and regulation, my thesis added significant and original information, and opens opportunities of unexpected paths to explore in the future; emphasising that there is an avenue of knowledge on secondary metabolism awaiting discovery.

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