Department for Innovation in Biological, Agrofood
and Forest systems (DIBAF)

Ph.D. Program in
**Science, Technology and Biotechnology for Sustainability**
**Curriculum biological/bioindustries systems**
**XXXIII cycle**

# Secondary metabolites from filamentous fungi: potential of a novel *Aspergillus* species

BIO/19

PhD Coordinator
**Prof. Andrea Vannini**

PhD Supervisor
**Prof. Silvia Crognale**

PhD Student
**Martina Felli**

**A.Y. 2019/20**

# INDEX

**GENERAL ABSTRACT** I

**CHAPTER 1 1**

**INTRODUCTION 1**

- 1.1. FILAMENTOUS FUNGI AS A SOURCE OF BIOACTIVE SMs **2**
  - 1.1.1. Extremophilic fungi: marine-derived fungi and bioactive SMs production 3
- 1.2. BIOCHEMISTRY AND ENZYMOLOGY OF FUNGAL SECONDARY METABOLITES 4
  - 1.2.1. Classes of fungal secondary metabolites and their biosynthesis 5
  - 1.3.2. Genomics-based approaches 12

**CHAPTER 2 16**

**BIOLOGICAL ACTIVITIES FROM HALOTOLERANT FUNGI UNDER INCREASING OSMOTIC STRESS 16**

- 2.1. ABSTRACT 16
- 2.2. INTRODUCTION 16
- 2.3. MATERIALS AND METHODS 18
  - 2.3.1 Microbial strains 18
  - 2.3.2. Halotolerance test 20
  - 2.3.3. Extract preparation 20
  - 2.3.4. Antimicrobial Assay 20
  - 2.3.5. Anti-Quorum Sensing assay 22
  - 2.3.6. Antioxidant assay 23
  - 2.3.7. LC-HRMS analysis of extracts 23
  - 2.3.8. Statistical analysis 24
- 2.4. RESULTS 24
  - 2.4.1. Effect of increasing osmotic stress on fungal growth 24
  - 2.4.2. Biological activities 26
  - 2.4.3. LC-HRMS analysis of extracts 32
- 2.5. DISCUSSION 34

**CHAPTER 3 42**

***ASPERGILLUS OLIVIMURIAE* SP. NOV., A HALOTOLERANT SPECIES ISOLATED FROM OLIVE BRINE 42**

- 3.1. ABSTRACT 42
- 3.2. INTRODUCTION 42
- 3.3. MATERIALS AND METHODS 44
  - 3.3.1. Isolation 44
  - 3.3.2. Morphology 44

3.3.3. Light microscopy ........ 45
3.3.4. SEM microscopy ........ 45
3.3.5. DNA techniques ........ 45
3.3.6. Phylogenetic tree ........ 46
3.3.7. Genome sequencing ........ 46
3.4. RESULTS ........ 47
3.4.1. Description of *Aspergillus olivimuriae* Crognale & Peterson sp. nov. ........ 47
3.5. DISCUSSION ........ 51
**CHAPTER 4 ........ 57**
**SECONDARY METABOLITE PROFILE OF THE NOVEL HALOTOLERANT SPECIE *ASPERGILLUS OLIVIMURIAE* ........ 57**
4.1. ABSTRACT ........ 57
4.2. INTRODUCTION ........ 57
4.3. MATERIALS AND METHODS ........ 60
4.3.1. Fungal strain ........ 60
4.3.2. Extract preparation ........ 60
4.3.3. Extrolites analysis ........ 60
4.3.4. Statistical analysis ........ 61
4.3.5. Bioinformatics analysis for whole-genome orthologous genes comparison and secondary metabolites gene clusters prediction ........ 61
4.4. RESULTS ........ 62
4.4.1. Whole-genome orthologous genes comparison ........ 62
4.4.2. Secondary metabolites gene clusters prediction ........ 64
4.4.3. *Aspergillus olivimuriae* secondary metabolites profile ........ 71
4.4.4. Statistical analysis ........ 76
4.5. DISCUSSION ........ 77
**CHAPTER 5 ........ 86**
**DISCUSSION ........ 86**
References ........ 92
Acknowledgments ........ 102
List of Papers ........ 103

# GENERAL ABSTRACT

Global resistance to drugs for existing diseases and new infections that have been reported in recent times pushes towards need to find new natural bioactive compounds. Secondary metabolites (SMs), low-molecular-mass organic compounds not directly involved in growth, development or reproduction of the producing organism, can be investigated for this intent. Among SMs many natural fungal products have biotechnological potential such as antibiotics, antifungal or anticancer agents representing a major source of approved drugs and still play an important role in supplying chemical diversity. Fungi isolated from extreme environments are an excellent potential source of natural products with novel and/or unusual chemical structures. Life in a stressful habitat, such as high salinity, high radiation, extreme temperatures and pressures and variable acidity, pushes towards the production of compounds useful for survival and adaptation to unfavorable living conditions. Fungal biosynthetic capacity of SMs resides into sections of microbial genome named “biosynthetic gene clusters” (BGCs) consist of repeating units or modules typically controlling the incorporation of one molecular entity into the final structure. Often, many BGCs are silent under standard cultivation conditions so they can be induced with the use of different approaches to stimulate their expression with the consequent production of bioactive molecules.

In this contest, this PhD thesis was aimed at researching secondary bioactive compounds from microbial sources to find possible used in the pharmaceutical field. Microbial source have the advantage of feasible and sustainable production of large quantities of anti-infective natural products by large-scale cultivation at reasonable costs.

In particular, the first aim was to use osmotic stress in halotolernat fungal species in order to induce the production of new bioactive compounds that are not expressed in standard growth conditions. It is already known that the presence of sodium chloride has the potential to influence the production of known compounds or the synthesis of new biologically active

secondary metabolites not yet known. So, in this PhD thesis project facultative marine derived fungi are used for discovery and exploitation of new bioactive molecules, due to the adaptation to stressful growth conditions and the development of specific biosynthetic pathways.

Furthermore, the study of SMs produced by a novel microbial species increases chances to identify biologically active natural compounds not yet known to be used in pharmaceuticals industry. So, another important aim was the investigation of a new species of *Aspergillus* in order to know the biosynthetic potential of secondary bioactive metabolites and to research new compounds to be applied in the biotechnological/pharmaceutical field.

Besides CHAPTER 1, where a general state of the art on fungal secondary metabolites has been described, in CHAPTERS 2-5 experimental procedures and designes have bee reported.

In CHAPTER 2 filamentous fungi are investigated for biological activity of secondary metabolites under different conditions of osmotic stress. For the discovery of secondary molecules, OSMAC (One-Strain-Many-Compounds) approach was employed, using different cultivation conditions using sodium chloride to regulate the transcription of genes linked to different secondary metabolites. The results showed that fungal species belonging to the genera *Penicillium*, *Aspergillus*, *Alternaria*, *Trichoderma* and *Arthrinium* were able to produce extracts characterized by biological activity. *Aspergillus* 6C2, furtherly identified as as new specie *A. olivimuriae,* stand out for its bioactivity, showing antimicrobial activity against gram + bacteria, strong antioxidants and anti Quorum Sensing capabilities.

CHAPTER 3 deals with the morphological and molecular characterization for the taxonomic location of the new species of *Aspergillus*. *Aspergillus* strain NRRL 66783 stood out for its bioactivity, isolated from the olive brine, was identified and characterized at the macroscopic and microscopic morphological level and at the molecular level. It has been identified with molecular methods sequencing the identification markers rDNA internal transcribed spacer region (ITS1-5.8S-ITS2), calmodulin (*CaM*), β-tubulin (*BenA*) and second largest subunit of RNA polymerase II (RPB2). Based on the source of isolation, the new species has been named *Aspergillus olivimuriae* and it was located as the most basal species in sect. *Flavipedes*. It was found tolerant to high concentrations of NaCl (15 %) or sucrose (60 %) and it exhibits substantial growth under these conditions. Although the new species grew profusely at 37 °C, no growth was observed at 40 °C, conidia *en masse* were avellaneous on all media. The whole genome has been sequenced.

Final step of this PhD thesis project has been treated in CHAPTER 4 and concerns the study of *Aspergillus olivimuriae* sp. nov. secondary metabolome. The study was conducted both on a genomic scale and through the OSMAC approach. In order to predict the productive potential of secondary metabolites, a bioinformatic study of biosynthetic gene clusters was carried out using two prediction programs, AntiSMASH and SMURF; analysis revealed the presence of biosynthetic gene clusters of secondary metabolites known to be associated with toxins, antitumor compounds and antimicrobials. Parallel to bioinformatics study *Aspergillus olivimuriae* was preliminarily investigated for the productive capacities of secondary metabolites. Through the use of HPLC-DAD analysis the inductive effect for the production of exometabolites due to the addition of salt, enzyme inhibitors and sucrose was studied. It has been observed that the growth of the fungus on salt is the condition that most modifies the metabolic profile of *A. olivimuriae*, clearly decreasing the production of compounds.

# CHAPTER 1

# INTRODUCTION

Metabolites are the intermediates and products of metabolism and are usually restricted to small molecules (Brakhage, 2013). Secondary metabolites (SMs) or natural products are a heterogeneous group of natural metabolic compounds that, unlike primary metabolites, are not directly involved in growth, development or reproduction of the producing organism. Their main features concern the production by narrow taxonomic groups of organisms in a phase subsequent to growth, having unusual chemical structures, being often a mixture of different chemical compounds (Demain and Fang, 2000) and biologically active (Demain, 1999). Due to their bioactive characteristics, these natural products are increasingly of industrial interest as a source of compounds that can be employed in the production of drugs (Newman and Cragg, 2016). In particular, the constant increase in resistance to drugs pushes towards need to find new bioactive compounds for countering, in addition to the known diseases, also new infections that increasingly arise in the world.

Secondary metabolites are produced by different organisms as plants, some marine organisms (sponges, tunicates...), prokaryotic and eukaryotic microorganisms. By searching for new potential sources of novel bioactive molecules special attention has been given to microbial sources that have been very prolific producers of more than 23,000 bioactive metabolites (Demain, 2014). More than half of all antibiotics are produced by actinomycetes, 10%–15% by non-filamentous bacteria and about 20% by filamentous fungi (Demain, 2014). In addition, microorganisms have the advantage of feasible and sustainable production of large quantities of SMs with reasonable cost, by large-scale cultivation and fermentation of the source organisms (Debbab et al., 2010).

## 1.1. FILAMENTOUS FUNGI AS A SOURCE OF BIOACTIVE SMs

Filamentous fungi are well known producers of secondary metabolites (Hoffmeister and Keller, 2007) which intervene in various processes related to development and intercellular communication. These microorganisms are able to colonize and live in complex ecosystems in which the extreme conditions of life (Selbmann et al., 2013) or the coexistence/competition with other microorganisms (Bertrand et al., 2014), drive a loss or overproduction of secondary metabolites. These compounds are characterized by their wide heterogeneity and often by bioactivity, characteristics that make them interesting for application in various industrial sectors, such as pharmaceuticals or food. Since the discovery of penicillin from *Penicillium notatum* by Fleming in 1928, many bioactive molecules produced by fungi have been described.

It has been observed how some of these molecules intervene in the protection from UV radiation; studies conducted on melanin found in spores and hyphae of filamentous fungi, in addition to highlighting its role in protecting from oxidative radiation (Shukla et al., 2016), have shown their importance in driving the virulence of some fungi (Jacobson, 2000). The antioxidant capacity of fungal products has been well observed in fungi of the genus Aspergillus; studies conducted on *A. fumigatus* show its ability to produce compounds with high scavenging effect (89.8% under optimal cultural condition), highlighting how the strain is able to modulate antioxidant compounds production based on the nitrogen and carbon source used (Arora and Chandra, 2011).

The continuous search for bioactive fungal products has led to the discovery of many antimicrobial compounds which are used in the pharmaceutical field. Filamentous fungi including *Aspergillus*, *Penicillium*, *Talaromyces* genus are well known for the production of antimicrobial SMs (Newman and Cragg, 2012; Nicoletti and Trincone, 2016; Al-Fakih and Almaqtri, 2019).

Lovastatin isolated from *Aspergillus terreus* is an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and in addition to being used as a blood cholesterol-lowering drug (Alberts et al., 1980), it can be used in the treatment of chronic diseases such as cancer, Alzheimer's disease and Parkinson's disease (Zhang et al., 2019; Eskandary et al., 2018; Lin et al., 2016).

### 1.1.1. Extremophilic fungi: marine-derived fungi and bioactive SMs production

Fungi isolated from extreme environments such as those characterized by high salinity, high radiation, extreme temperatures and pressures and variable acidity are an excellent potential source of natural products with novel and/or unusual chemical structures (Chavez et al., 2015). Extreme living conditions push them to implement survival strategies that involve the synthesis of specific compounds to be used for adaptation (Gunde-Cimerman and Zalar, 2014; Timling and Taylor, 2012). These characteristics, therefore, make them a source of bioactive natural compounds not yet detected. In the particular case of fungi from extreme environments, the use of conditions very different from those native hinders the prospects of finding new compounds from extreme environment fungi.

The interest in fungi from the marine environment has significantly expanded the catalog of fungal products including many novel chemical scaffolds. Particular attention is paid to the study of marine-derived fungal species. Their classification is not yet well defined but marine fungi related to terrestrial environment are more often associated with this group (Overy et al., 2017). These fungi are not necessarily only salt tolerant, but rather osmotolerant (Pitt & Hocking, 1997). Such fungi are adapted to grow in low-water-activity habitats and would grow well in elevated concentrations of sugars or glycerol (Jin et al., 2005; Gal-Hemed et al., 2011). *Aspergillus* and *Penicillium* are certainly the most studied marine derived fungi for the discovery of new bioactive compounds (Jin et al., 2016; Kaleem et al., 2019; Liu et al., 2019; Zhang et al., 2019). Over the past twenty years, many studies targeting marine derived species have allowed the isolation of new compounds, such as three new indole alkaloids from *Penicillium janthinellum* (Smetanina et al., 2007), three new polyketides penicitrinol G, H and 2,11-dihydroxy-1-methoxycarbonyl-9-carboxylxanthone from marine-derived fungus *Penicillium citrinum* SCSGAF 0167 (Sun et al., 2014) and three new compounds with antimicrobial activity from *Aspergillus terreus* (Wang et al., 2011a).

## 1.2. BIOCHEMISTRY AND ENZYMOLOGY OF FUNGAL SECONDARY METABOLITES

Fungal secondary metabolites can be classified according to their vast diversity in structure, function, and biosynthesis. The main chemical classes of fungal secondary metabolites are terpenes, polyketides, derived from acyl-CoAs, and non-ribosomal peptides derived from amino acids (Figure 1 Keller, 2019). Biosynthesis of this compounds are located in clusters coding by different families of multidomain enzymes (Brakage, 2013) like terpene synthases and terpene cyclases (TSs and TCs), polyketide synthases (PKSs) and non- ribosomal peptide synthetases (NRPSs). Some secondary metabolites are hybrids that are synthesized from two synthases such as fumagillin (PKS–TC hybrid) or echinocandin (PKS–NRPS hybrid). Fungal secondary metabolites that are not generated by the synthases include the ribosomally derived peptide ustiloxin, fatty-acid-derived oxylipins and isocyanide xanthocillin, which require an isocyanide synthase (Umemura et al., 2014; Pettit, 2011; Lim et al., 2018). The most studied secondary metabolites, also for the chemistry involved in their synthesis, are derived from either non-ribosomal peptides (NRPs) or polyketides.

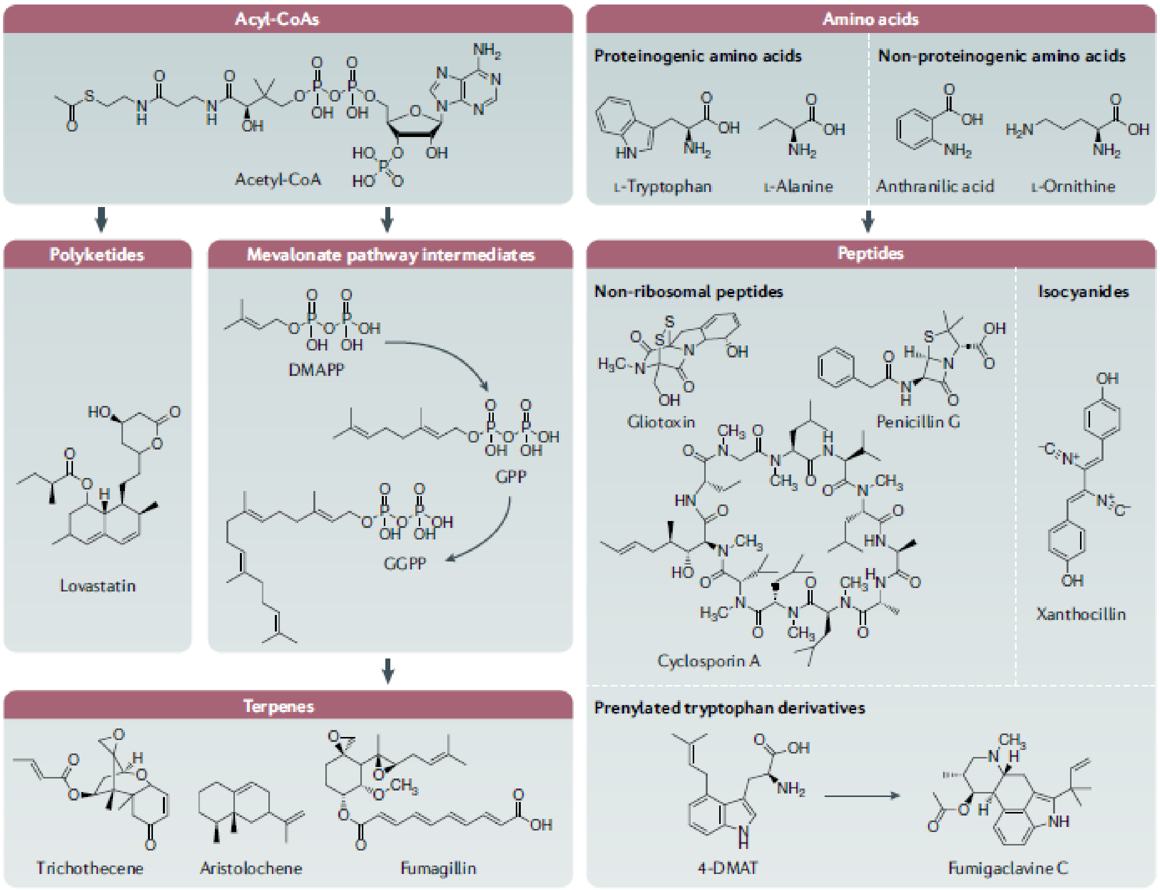


**Figure 1.** The typical building blocks of secondary metabolites and a schematic overview of a biosynthetic gene cluster.

### 1.2.1. Classes of fungal secondary metabolites and their biosynthesis

#### *Polyketides*

Polyketides are the most representative group of fungal secondary metabolites. They are synthesized by polyketide synthases (PKSs), a family of multidomain enzymes, which catalyzes the construction of the poly-β-keto chain formed by condensation of mainly acetyl-CoA (starter unit) and malonyl-CoA (extender unit) (Figure 2 Miyanaga, 2017). These multidomain enzyme systems are organized in modules responsible for starting, elongation and termination chain. A typical PKS elongation module consists of three necessary domains: an acyltransferase (AT) domain for extender unit selection and transfer; an acyl-carrier protein (ACP) for extender unit loading; and a ketoacyl synthase

(KS) domain for decarboxylative condensation of the extender unit with an acyl thioester (Figure 3 Miyanaga, 2017).

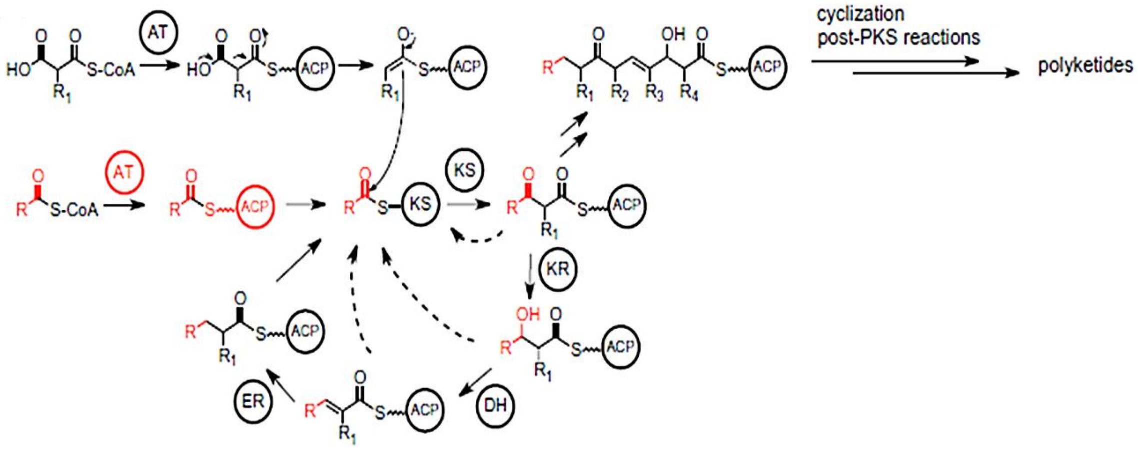


**Figure 2.** The starter units and loading modules are shown in red. General reaction scheme of polyketide chain elongation by PKS. The reaction of the AT-ACP didomain-type modular PKS is shown.

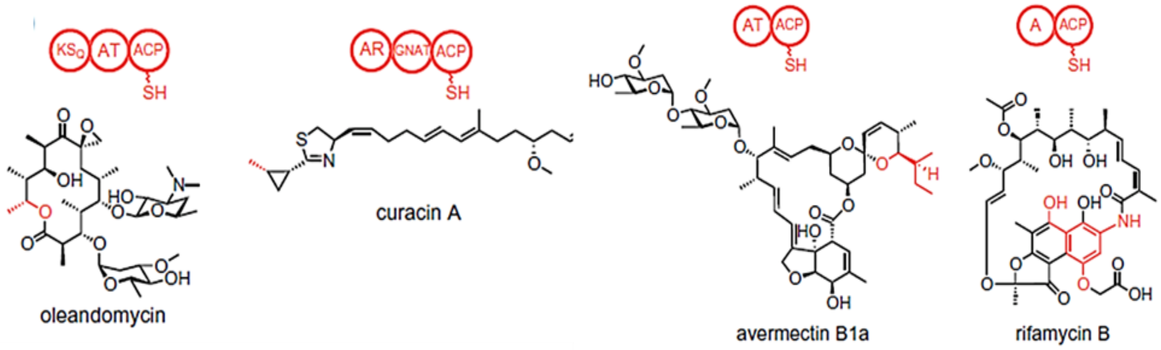


**Figure 3.** Structures of representative polyketides synthesized by modular PKSs

The resulting β-ketothioester may subsequently be processed by β-ketoacyl reductase domains (KR), dehydratase domains (DH) and enoyl reductase domains (ER) (Miyanaga, 2017). (Figure 4 Brakhage, 2013).

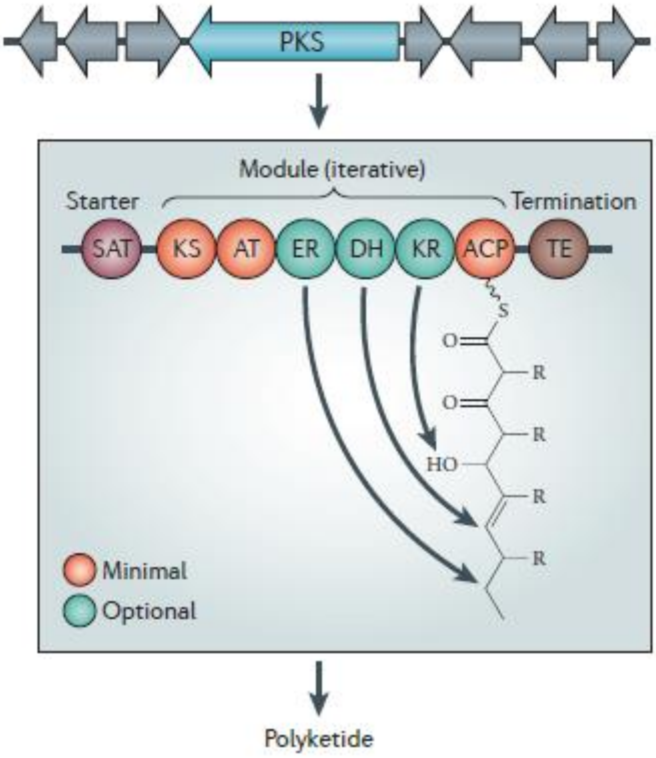


**Figure 4.** Polyketide synthases (PKSs)

Three classes of PKS are distinguished. PKS type I consist of multifunctional enzyme which can be constituted of several domains who catalyze different steps of polyketide chain elongation (modular type I PKS) (Fujii et al., 2005) or a single module with linear organization of active site domains (iterative type I PKS); Type II PKS consist of a complex of subunits such as KS and ACP and Type III PKSs consist of a single keto-synthase (KS) domain (Yu et al., 2012; Navarro-Muñoz and Collemare, 2020).

PKS performs particular reduction and dehydration reactions on each resulting β-keto carbon and catalyzes the intramolecular cyclization of the resulting polyketide chain to generate a monocyclic or polycyclic product.

Fungal polyketides are classified into two groups: aromatic (often multicyclic) and aliphatic (cyclic or acyclic) compounds. Aromatic polyketides are polycyclic compounds with at least one aromatic ring (Hertweck et al., 2007) and they are important for antibacterial, anticancer and antiviral bioactivities. *Aspergillus* species are known producers of these compounds as *A. flavus* and *A. parasticus* producers of aflatoxin B1 (Gizachew et al., 2019).

### *Nonribosomal peptides*

Nonribosomal peptides, together with polyketides, represent the most widespread class of fungal secondary metabolites. They are synthesized by non-ribosomal peptide synthetase (NRPS) multidomain enzyme typically constituted of three domains: an adenylation domain (A) for amino acid activation; a peptidyl carrier protein (PCP) (thiolation domain), which binds the cofactor 4′-phosphopantetheine (4′PP), to which the activated amino acid is covalently attached; and a condensation domain (C), which catalyzes peptide bond formation (Miller and Gulick, 2016) Figure 5. In addition, a range of optional domains (for example, methyltransferase and epimerization domains) have been described (Cane and Walsh, 1999) Figure 6.

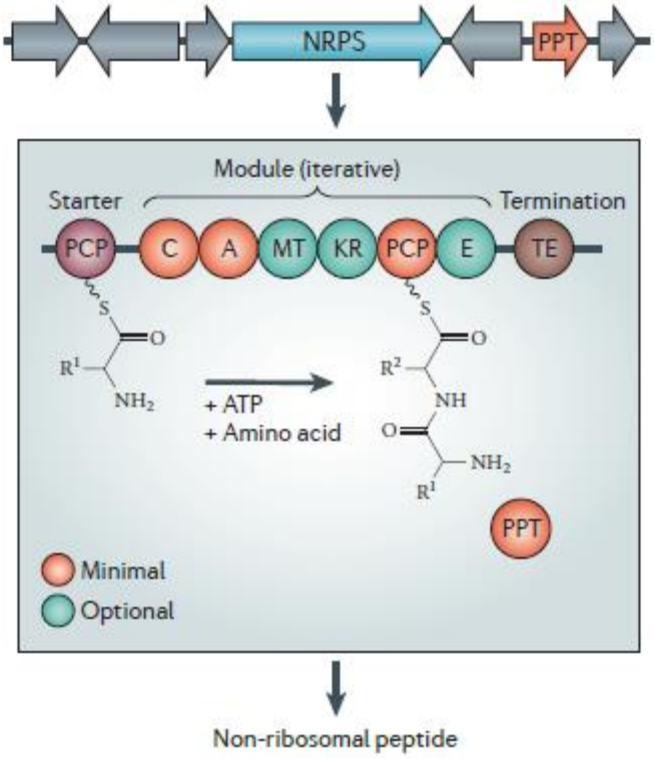


**Figure 5.** Non-ribosomal peptide synthetase (NRPS) gene clusters

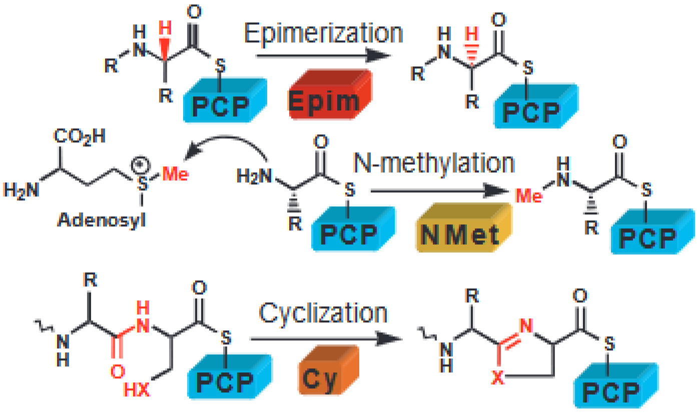


**Figure 6.** Auxiliary NRPS domains mediating epimerization (Epim), peptide N-methylation (NMet), and heterocyclization (Cy) of cysteine or serine residues. (Cane and Walsh 1999).

The N-terminal of NPRs are often modified by fatty acids, heterocyclic compounds, glycosylated or phosphorylated structures (Xue et al., 2012).  NRPs are divided into linear (L-NRPs) and cyclic NRPs (C-NRPs).

These peptides have a structural features such as contain amino acids like ornithine or amino acids, and their structures are macrocyclic, branched macrocyclic, dimers or trimers of identical structural elements (Singh et al., 2012). This structural diversity of nonribosomal peptides also determines their broad spectrum of biological activities utilized in pharmaceutical research.  For example they have diverse properties as toxins, siderophores, pigments, antibiotics, cytostatics, immunosuppressants or anticancer agents (Wang et al., 2014). Penicillin, the first recognized antibiotic, is an NRP produced by the fungus *Penicillium chrysogenum*.

## 1.3 APPROACHES OF STUDY FOR FUNGAL SECONDARY METABOLITES DISCOVERY

### 1.3.1 Activation of cryptic genes clusters

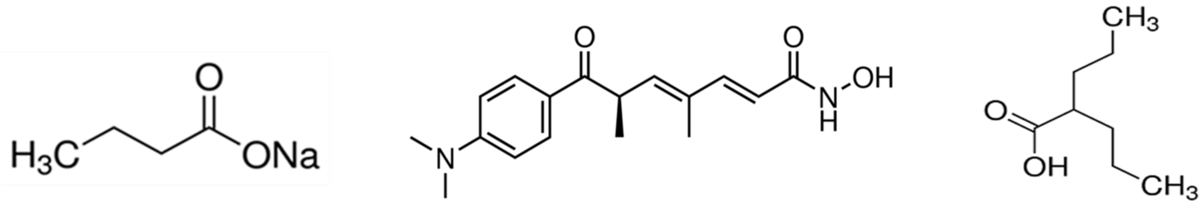
Sequencing data of the fungal genomes has revealed that several gene clusters are not associated with chemically characterized molecules (Romano et al., 2018). This clusters results silent under standard laboratory culture conditions (Rutledge and Challis, 2015). Different strategies for activation of silent clusters can be used, but the most effective and simplest strategy is represented by use of OSMAC (One Strain Many Compounds) approach for metabolite mining. This method provides variation of medium (Frisvad, 2012), changing cultivation condition, co-cultivation with other strain(s) (Boruta et al., 2019) and adding chemical epigenetic modulation (Wiemann and Keller, 2014). Zeeck and co-workers have shown how the variation of parameters such as temperature, salinity and aeration induce production of metabolites in *Aspergillus ochraceus* which in standard cultivation conditions does not produce (Bode et al., 2002).

High osmotic pressure makes cells dehydrated and affects microbial biochemical reactions (Wang et al., 2011b). One mangrove-derived endophyte *Wallemia sebi* PXP-89 cultivated in 10% NaCl broth produced a new cyclopentanol pyridine alkaloid, which was not detected in normal medium (Peng et al., 2011).

The use of different cultivation media is also an excellent strategy to induce the production of different compounds (Frisvad, 2012). For example lactate or starch as the sole carbon source only resulted in low production of secondary metabolites in *Aspergillus niger*, but the combination of the two gave a synergistic effect, so much larger amounts of fumonisins, ochratoxins, kotanins, and naphtho- g -pyrones were produced (Sørensen et al., 2009).

In natural habitats the coexistence between different fungi species and bacteria is usual. Microbial interspecies competition can have effects on metabolites production, which were excreted to defend the habitat or as chemical signals (Adnani et al., 2015). For example the production of pestalone, an antibiotic against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium*, was obtained in the co-culture of a marine-derived gram-negative bacterium *Thalassopia* sp. (CNJ-328) and the marine fungus *Pestalotia* (Cueto et al., 2001). *Aspergillus fumigatus*, well known for the large amount of potentially bioactive SMs (Frisvad et al., 2009), co-cultured with *Streptomyces bullii* produced ergosterol and numerous new metabolites (Rateb et al., 2013). Other studies by Zhang have shown how co-cultivation of the fungal endophyte T*richoderma* sp. 307 and *Acinetobacter johnsonii* B2 led to the isolation of two new sesquiterpenes, two new de-O-methyllasiodiplodins, one new natural product, along with twelve known molecules (Zhang et al., 2017).

Recent studies revealed that tickling the genome of fungi using epigenetic modifiers like enzyme inhibitors bring changes in gene expression without any alteration in DNA sequence (Xiao et al. 2013). The most commonly used class of inhibitors is that of histone deacetylase (HDAC). The switch between transcriptional silenced heterochromatin and active euchromatin is trained by reversible chemical modifications of histones (Wu and Grunstein, 2000; Hayes and Hansen, 2001). Potent HDAC inhibitors able to modulate the “cryptic” expression profile of fungal secondary metabolites are valproic acid (VPA) (Shwab et al. 2007; Sharma et al., 2018), sodium butyrate (Davie, 2003) and trichostatin A (TSA) (Shwab et al., 2007; Cole, 2008) Figure 7.



Sodium butyrate Trichostatin A Valproic acid

**Figure 7** Three class of histone deacetylase (HDAC) inhibitors.

*Cladosporium cladosporioides* when treated with HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), led to the isolation of new cladochromes F, cladochromes G and calphostin B (Williams et al., 2008). Studies conducted by Magotra (2017) have shown how the endophyte *Aspergillus fumigatus* (GA-L7) increases the production of fumiquinazoline C in presence of valproic acid and upregulating the expression of all the genes involved in the biosynthesis of fumiquinazoline C.

### 1.3.2. Genomics-based approaches

In recent years, in parallel with use of experimental strategies related to fungal cultivation, genomic-scale approaches are increasingly used (Shi-Kunne et al., 2019). This approach allows to know fungal biosynthetic potential of secondary metabolites only due to the study of genome sequence data. The increase of whole-genome sequencing projects have shown how metabolic capabilities of natural resources are severely underestimated (Corre and Challis, 2009). Most used strategies concern bioinformatic analysis follow by gene manipulation techniques to drive transcription, translation, and eventual synthesis of the corresponding natural product (Figure 8).

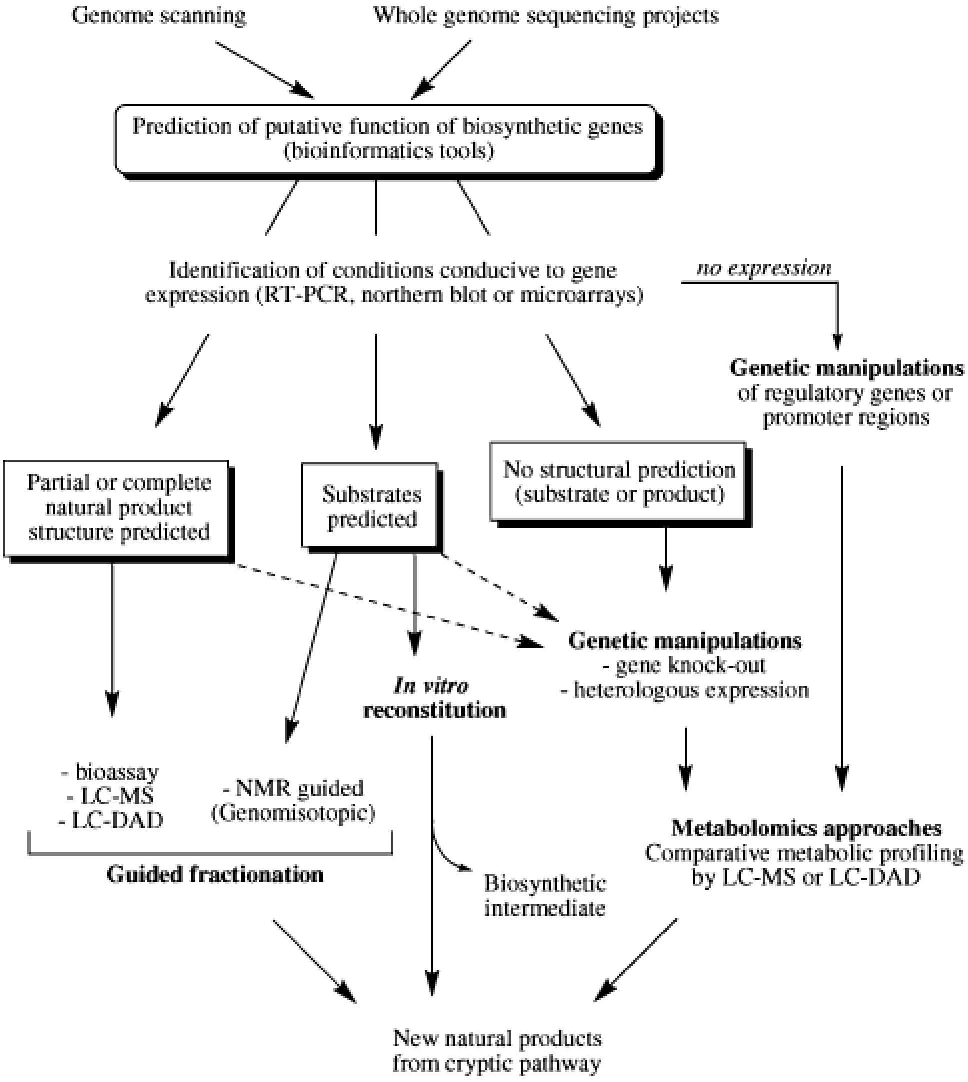


**Figure 8.** Strategies for discovery of novel natural products by genome mining (Corre and Challis 2009).

*Bioinformatics tools*

The first step for studying the fungal biosynthetic potential of secondary metabolites regards bioinformatics analysis from sequenced genomes. For this purpose, computational tools like “Secondary Metabolite Unknown Regions Finder” (SMURF) (Khaldi et al., 2010) and “Antibiotics & Secondary Metabolite Analysis Shell” (antiSMASH) (Medema and Fischbach, 2015; Weber et al., 2015) have been optimized. These tools are complementary to chemical and genetic approaches and have proven very useful in searching for biosynthesis pathways of novel compounds. The distinctive traits of gene clusters (e.g. gene distance) and the conserved signatures of core genes (e.g. conserved domains) can be exploited to identify putative loci involved in secondary metabolites production (Figure 9 Medema et al., 2011). There are though some cluster types difficult to detect by the prediction algorithms such as the terpenoid-based clusters since the amino acid sequences of terpene synthases are not as conserved as PKSs and NRPSs (Keller et al., 2005, Khaldi et al., 2010). Other algorithms identifying these clusters include ClusterFinder (Cimermancic et al., 2014)

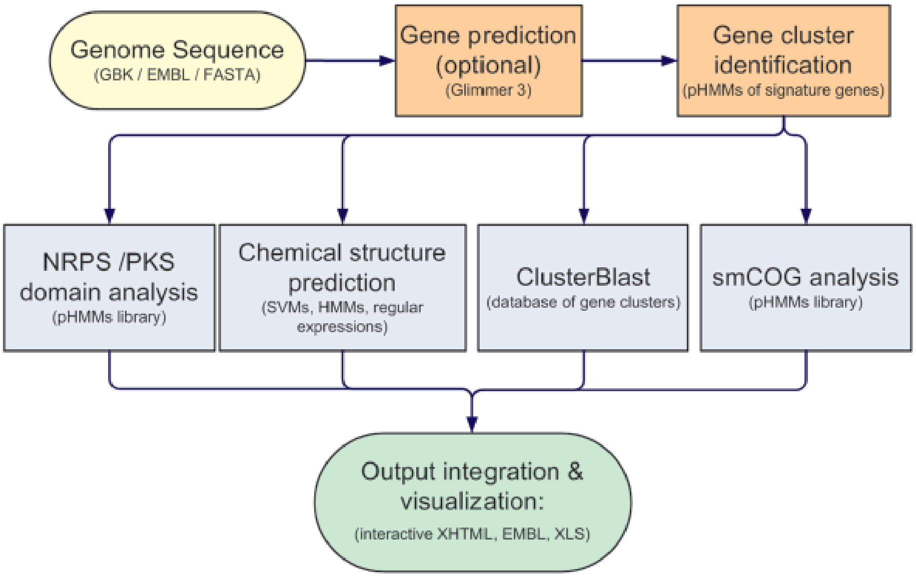


**Figure 9.** Outline of the pipeline for genomic analysis of secondary metabolites.

*Gene manipulation techniques*

The ability to know the gene clusters of specific secondary metabolites increases the possibility to identify natural compounds not yet detected. Genetic manipulation through gene deletion-based strategy in the native host follow by comparative metabolomic profiling, allowed for example to identify gene cluster of phytotoxin cichorine from *A. nidulans*, (Sanchez et al., 2012). Gene manipulation techniques can also be used to increase the expression of genes not expressed under standard culture conditions trough promotor use. The method was first applied by Bergmann et al. (2007) in *A. nidulans* where the cluster-specific transcription factors were integrated under the control of an inducible promoter. This led to the elucidation of the novel SMs aspyridones A and B and identification of the PKS-NRPS hybrid BGC responsible for the biosynthesis. This technique allowed to identify also azaphilone in *A. niger* (Zabala et al., 2012) and four new secondary metabolites, fujikurins A-D, in *Fusarium fujikuroi* (Janevska and Tudzynski, 2018; Von Bargen et al., 2015).

Many fungal species are difficult to cultivate on laboratory and to manipulate at the genetic level so heterologous expression strategies can be applied for study single gene or entire gene clusters.  Several studies applied this strategy for characterize gene clusters including pyripyropene from *A. fumigatus* expressed in *A. oryzae* (Itoh et al., 2010) and a silent asperfuranone pathway from Aspergillus terreus in *A. nidulans* (Chiang et al., 2013).

# CHAPTER 2

## Biological activities from halotolerant fungi under increasing osmotic stress

This chapter is submitted to Folia Microbiologica as:

Felli M., Russo C., Varga M., Gallo A.M., D' Annibale A., Petruccioli M., Crognale S. Biological activities of halotolerant fungi under increasing osmotic stress.

### 2.1. ABSTRACT

The ever-increasing demand for natural bioactive compounds to contrast drug resistant, pushes research towards microbial sources. Marine derived fungi are new promising resource of a huge number of biologically active products since they are able to synthesize specific bioactive metabolites under conditions that represent stress for non-adapted species. For the discovery of secondary molecules, OSMAC (One-Strain-Many-Compounds) approach was employed, using different cultivation conditions with salt to stimulate the transcription of genes linked to different secondary metabolites. The results showed that fungal species belonging to the genera *Penicillium*, *Aspergillus*, *Alternaria*, *Trichoderma* and *Arthrinium* were able to produce extracts characterized by biological activity. *Aspergillus olivimuriae* stand out for its bioactivity, showing antimicrobial activity against gram + bacteria, strong antioxidants and anti Quorum Sensing capabilities.

### 2.2. INTRODUCTION

Despite significant progress in the drug discovery has provided treatment for some major ailments, minor infections, and epidemics; new bioactive compounds are required to combat global resistance to drugs for existing diseases and new infections that have been reported in recent times. Bioactive natural products represent a major source of approved drugs and still play an important role in supplying chemical diversity. In contrast to macro-organisms, microorganisms represent promising natural product sources having the advantage of feasible and sustainable production of large quantities of secondary metabolites with reasonable cost, by large-scale cultivation and fermentation of the source organisms (Debbab et al., 2010).

Marine fungi are important source of bioactive secondary metabolites useful for the drug discovery purposes. Substantial efforts have also been devoted to exploring marine microbial strains as producers of bioactive molecules (Fenical and Jensen, 2006). In recent years, a considerable number of structurally unique metabolites with biological and pharmacological activities have been isolated from the marine-derived fungi, such as polyketides (Rateb and Ebel, 2011; Fouillaud et al., 2016), alkaloids (Wang et al., 2015a), peptides, lactones, terpenoids and steroids. Some of these compounds have anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, antibiotic and cytotoxic properties (Jin et al., 2016).

Although in the last two decades have seen a rapid increase in the discovery of new natural products from fungi isolated from substrata originating in marine habitats, the definition of “marine derived fungus” is still debated (Overy et al., 2014). Mycological community considers that the majority of marine-derived fungi reported in the natural products literature likely come from terrestrial habitats where dormant propagules (i.e., spores) are randomly transported to the sea by air and water, where they persist until they are discovered (Overy et al., 2014). In order to clarify the term “marine derived”, the definition has been diversified in ‘facultative marine’ encompassing fungi from terrestrial and freshwater environments that have also been observed to grow in the marine environment and ‘marine (*sensu strictu*)’ or obligate “marine” to define those fungi that exclusively carry out their life cycles in marine or estuarine habitat, that forms symbiotic relationships with other marine organisms (Pang et al., 2016). Consequently, may be affirmed that the majority of most frequently cited marine-derived taxa are associated with ruderal substratum relationships, belonging to well-known osmotolerant terrestrial genera (Overy et al., 2017).

Experimental methods are available to test the hypothesis that secondary pathways might be differentially activated in a terrestrial isolate grown under saline conditions. The addition of sea salt or any other solute is likely to profoundly affect secondary metabolism because of the interconnectivity of the osmotic stress regulatory system and fungal development (Duran et al., 2010). A link between secondary metabolite production as a response to salt stress is evident from comparative studies where differential expression in secondary metabolite production was observed from a strain of *Aspergillus terreus* (isolated from a saltern) and a marine-derived strain of *Mariannaea elegans* (as *Spicaria elegans*) due to increasing salinity of the cultivation medium compared to water controls (Wang et al., 2011a). Huang et al. (2011) observed that NaCl promoted growth and antimicrobial activity of the 91.5% and 14.9% respectively among 47 marine filamentous fungi tested strains. Similar findings have been obtained for the production of bioactive metabolites in halophilic and halotolerant fungi (Sepcic et al., 2011) as well as for the production of pigments from the marine-derived fungal strain *Talaromyces albobiverticillius* 30548 (Venkatachalam et al., 2019).

During a research program aimed at exploring promising bioactive secondary metabolites from natural sources (acronym: PRONAT), the effects of salinity on growth and biological activities of halotolerant fungal strains have been evaluated. In this study crude extracts produced from fungi cultivated under different osmotic stressed conditions were screened for antimicrobial, antioxidant and anti-quorum sensing activities.

## 2.3. MATERIALS AND METHODS

### 2.3.1 Microbial strains

All microbial strains used in the present study, both bacterial strains used as test microorganisms in antimicrobial assay and halotolerant fungi used for extracts production, were obtained from the culture collection of the Department of Innovation in Biological, Agro-Food and Forest Systems (University of Tuscia, Viterbo).

In particular, among 11 fungal strains used for extracts production,10 fungal strains were selected on the basis of literature for their classification as “facultative marine derived fungi” and simultaneous capability to produce biological active compounds Table 1, one more halotolerant strain recently described (Crognale et al., 2019) and named *A. olivimuriae* was screened and used for bioprospecting.

**Table 1** Terrestrial fungal strains used in the present study classified by literature as "facultative marine derived fungi" producing biological active compounds.

| **Strain** | **Terrestrial isolation source** | **Fungal specie** | **Marine-derived isolation source** | **Reported bioactivities** | **References** |
|---|---|---|---|---|---|
| E4 | Plant biomass | *Alternaria* sp. | Root of a marine semi-mangrove plant | Antioxidant, antifungal | Wang et al. 2015b |
| E6 | Plant biomass | *Arthrinium arundinis* | Sponge | Cytotoxic | Wang et al. 2015a |
| AP4 | Plant biomass | *Trichoderma longibrachiatum* | Mussels | Antibacterial, antifungal, cytotoxic | Mohamed-Benkada et al. 2016 |
| Col G | Arsenic contaminated soil | *Chaetomium* sp. | Marine fish | Cytotoxic | Yamada et al. 2009 |
| Col O | Arsenic contaminated soil | *Penicillium canescens* | Marine sediments | Neurotoxicity | Vansteelandt et al. 2012 |
| AP1 | Plant biomass | *Penicillium citrinum* | Soft coral | Antimicrobial, cytotoxic, Enzyme-inhibitory | Sun et al. 2014; Yurchenko et al. 2013 |
| Col I | Arsenic contaminated soil | *Penicillium janthinellum* | Mangrove rhizosphere soil, marine sediments | Antimicrobial, cytotoxic | Chen et al. 2017; Smetanina et al. 2007 |
| Col L | Arsenic contaminated soil | *Penicillium janthinellum* | Mangrove rhizosphere soil, marine sediments | Antimicrobial, cytotoxic | Chen et al. 2017; Smetanina et al. 2007 |
| Col C | Plant biomass | *Penicillium* sp. | Marine sediments, fish, sponge, coral, mangrove | Antibacterial, cytotoxic, antiproliferative, proapoptotic | Nicoletti e Trincone 2016 |
| E1 | Plant biomass | *Aspergillus tubingensis* | Sponge, crab | Xylanase, antimicrobial | dos Santos et al. 2016; Guo et al. 2016 |
| 6C2 | Olive brine | *Aspergillus olivimuriae* | - | - | Crognale et al. 2012; Crognale et al. 2019 |

All strains were stored by cryopreservation at -80 ºC. An aliquot of each stock culture was transferred aseptically to potato dextrose agar (PDA, Difco TM) plates, incubated at 28 °C for 5 days and maintained at 4 °C.

### 2.3.2. Halotolerance test

Fungal mycelium plugs (Ø 8 mm), were used to inoculate PDA plates containing different concentrations of NaCl (0, 3, 6, 10 e 15% w/vol), incubated for 20 days at 28 °C. Diametral growth was measured daily.

### 2.3.3. Extract preparation

20 days old cultures were used for crude extracts preparation, using the extraction protocol described by Santiago et al. (2012), revised as below. In order to remove salt from the final extract, agarized cultures have been previously lyophilized (VirTis, AdVantage) for 24 h, then the extraction was carried out on dried cultures with media, with 35 ml of ethanol for 48 h at 4 °C. Water removal was necessary to avoid formation of hydroalcoholic solution which it would limit salt removal. Alcoholic suspension was filtrated using Whatman no.1 filter paper, to remove mycelium, agar and salt. The extracts have been evaporated to dryness in a rotary evaporator system (BUCHI, Switzerland) at 35 °C and 43 mbar to obtain a crude extract. The crude extract was suspended in DMSO 20%, to obtain concentrations ranging from 5 to 20 mg ml$^{-1}$ sterilized by filtration (0.2 μm) and stored at -20 °C.

### 2.3.4. Antimicrobial Assay

Ethanol extracts were tested against the yeast *Candida boidinii*, two Gram positive bacteria, *Bacillus pumilus* and *Bacillus cereus*, two Gram negative bacteria, *Pseudomonas stutzeri* and *Escherichia coli*, a fungus *Fusarium culmorum* and a oomycetes *Phytophthora* sp. The bacterial strains were cultured in plate count agar (PCA), the yeast and fungi were cultured in malt extract agar (MEA) media.

### *Evaluation of antimicrobial activity by agar diffusion assay*

All bacterial and yeast strains were inoculated in physiological solution (0.9 % NaCl) and the suspension was adjusted to $1.5\times10^8$ colony forming units (CFU)/ml corresponding to 0.5 McFarland scale (Valgas et al., 2007). The antimicrobial activities were evaluated using agar diffusion assay. The bacterial and yeast culture suspensions were evenly spread out, with the help of sterile L-shaped spreaders, on plate count agar (PCA) and malt extract agar (MEA), respectively. Mycelium plugs (Ø 8 mm) of *Fusarium* and *Phytophthora* sp. were placed in the center of the plates containing malt extract agar (MEA) and carrot agar, respectively.

Fungal crude ethanol extracts of fungi were diluted to a concentration of 1 mg/ml and 2 mg/ml; streptomycin sulfate and cycloheximide were used as positive controls for bacteria and for eukaryotic microbial strains, respectively; solution of DMSO/$H_2O$ (20%) was used as negative control. After inoculation of test microorganism, wells (Ø 6 mm) were created under sterile conditions in the agar to dispense extract and positive and negative controls (20 µl for each well). Plates were incubated at 28 °C for 24 h for bacteria and yeast and for 72 h for fungi and oomycetes. Finally, the diameters of inhibition zones were measured and the activity index (AI) calculated by comparison with positive control and extracts (AI = Inhibition zone of extract/Inhibition zone of positive control).

### *Minimum inhibitory concentration (MIC)*

Determination of the minimum inhibitory concentration (MIC) of the crude ethanol extracts was determined by using broth microdilution method (CLSI 2012) in 96 wells polystyrene microplates (Greiner Bio-OneTM CELLSTARTM), towards those test microorganisms that were found to be inhibited in the antimicrobial agar diffusion assay.  Streptomycin sulfate was used as positive control while Mueller Hinton Broth (MHB) (OxoidTM, Basingstoke, United Kingdom), with DMSO 1% as negative control. Ethanol extracts were diluted (0.8-0.001 mg

$ml^{-1}$) in MHB and 100 µl of each dilution was dispensed into each well. The inoculum suspension was adjusted to achieve final $5 \times 10^5$ CFU $ml^{-1}$ in each well. The plates were incubated at 28 °C for 24 h. Absorbance was measured at 595 nm using Microplate reader (LabTech 4000).

MIC was also detected by adding 10 µl/well of TTC (2,3,5-triphenyl tetrazolium chloride at 2 mg $mL^{-1}$) and incubated for 30 min. The lowest concentration at which color change is not visually observed was taken as the MIC value.

### 2.3.5. Anti-Quorum Sensing assay

The assay was performed in a 96-wells plate using *C. violaceum* CV026 (CECT5999), the violacein release of which is dependent upon the external addition of N-hexanoylhomoserine lactone (HHL). The strain was grown overnight at 27 °C in Luria broth (LB) supplemented with kanamycin (50 µg $ml^{-1}$) and inoculated to a final optical density ($\lambda$= 600 nm) of 0.5. Two batches of plates were prepared, the first one to evaluate the effect of the fungal extracts and DMSO on bacterial growth, with the second one to evaluate the effect of fungal extract and DMSO on violacein production. In the first batch, wells containing LB were supplemented with fungal extract at different concentrations (range 0-1.0 mg $ml^{-1}$) or with DMSO at concentration corresponding to that present in the fungal extract. To the second batch, wells containing LB were prepared as above but in the presence of HHL (15 µg $ml^{-1}$). The final volume in the well was 100 µl. The plate was incubated at 27 °C 150 rpm for 24 h in a shaking incubator. After 24 h the first batch was used to measure the growth using Microplate reader at 595 nm (LabTech 4000), while the second batch was used for violacein extraction. In this last case the plate has been centrifuged at 3000 rpm for 15 min to precipitate the insoluble violacein. The culture supernatant was discarded and 100 µl of DMSO was added to the pellet to solubilize the violacein. The plate was centrifuged at 3000 rpm for 15 min to remove the cells and violacein-containing supernatant was transferred in a new well. The absorbance was read with a Microplate reader (LabTech 4000) at a wavelength of 595 nm. Quorum sensing inhibition was expressed as % of violacein production inhibition.

### 2.3.6. Antioxidant assay

Ferric Reducing Antioxidant Power (FRAP) assay, was based upon the methodology of Benzie and Strain's (1996). The working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 20 mM FeCl3_6$H_2O$ in a 10:1:1 ratio just before use. In each well of 96-wells polystyrene microplates (Greiner Bio-One$^{TM}$ CELLSTAR$^{TM}$) the reaction mixture was composed by 80 µl of working FRAP reagent, 15 µl of $H_2O$ and 5 µl of test extract sample (2 mg/ml) or sample solvent (for negative control). Trolox was used as standard. After incubation of 30 minutes at 37 °C, absorbance was measured at 593 nm using Microplate reader (LabTech 4000) and the absorbance values of control wells were subtracted from the final reading of the sample. Standard curve was prepared using Trolox at concentrations ranging 80-600 µg ml$^{-1}$ (320-2400 µM) and it was then used to determine the Trolox equivalent (TE) for each sample. Each sample was run in triplicate.

### 2.3.7. LC-HRMS analysis of extracts

Extracts were analyzed with Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) technique (Homa et al., 2019). Aliquots of each extract were diluted with acetonitrile and water in 8:2 ratio for having about 0.05% DMSO in the final samples. For the quantification, 5 µl of each sample was applied on a Gemini NX C18, 150 × 2 mm, 3 µm (Phenomenex, United States) column built in a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific, United States) system provided with a degasser, a binary pump and an autosampler. A Gemini NX C18, 20 × 2 mm (Phenomenex, United States) guard column was installed between the injector and the analytical column. For the detection, a Q ExactiveTM Plus Hybrid Quadrupole-OrbitrapTM (Thermo Fisher Scientific, United States) mass spectrometer was connected to the HPLC fitted with a heated electrospray ionization (HESI) source. The separation was carried out at 25 °C at a flow rate of 0.2 ml/min in a total of 25 min using gradient elution from water + 0.1% formic acid / methanol + 0,1% formic acid (80/20, v/v) to 95% of methanol + 0.1% formic acid in 12 min time, then maintaining this percentage for 5 min and at last decreased to the starting value. The detection was in positive and negative ionization mode. The HPLC-HRMS instrument was controlled with the XCaliburTM v. 2.2.1 software (Thermo Fisher Scientific, United States), while the TraceFinder v. 3.3 and Compound Discoverer v. 2.1 software (Thermo Fisher Scientific, United States) were applied for data acquisition and evaluation.

### 2.3.8. Statistical analysis

Values are expressed as arithmetic means. One-way analysis of variance (ANOVA) conducted by Tukey's test ($P \leq 0.05$) using SigmaStat Software package version 3.5 (Jandel Scientific, Germany) was performed to compare the data obtained from the growth of strains, from antioxidant and anti-quorum sensing tests. Dunnett’s test was used for comparison of difference between fungal extracts and negative control (contain only extract solvent) in antioxidant assay and anti-quorum sensing assay.

In order to have an overall view on results a multivariate treatment of data based on principal component analysis (PCA) was performed using the SIMCA software package v. 13 (Umetrics, Umea, Sweden).

## 2.4. RESULTS

### 2.4.1. Effect of increasing osmotic stress on fungal growth

The halotolerance test evaluate strains growth response under increasing osmotic stress due to the addition of NaCl in the culture medium. For each growth condition, maximum specific growth rate ($\mu_{max}$) and growth diameter ($Ø_{max}$) obtained after 20 days of incubation were calculated (Table 2). Besides *Chaetomium* sp. which was not able to growth at salt concentration higher than 3%, all the other strains showed a halotolerant behavior being able to growth at NaCl ≥ of 10%.

**Table 2** Effect of increasing osmotic stress on fungal growth expressed as constant of maximum growth speed ($\mu_{max}$) and maximum growth diameter ($Ø_{max}$) after 20 days of incubation.

| **Strains** | | **Culture conditions*** | | | | |
|---|---|---|---|---|---|---|
| | | **0** | **3** | **6** | **10** | **15** |
| *Alternaria* sp. | $Ø_{max}$** | 5.0±0.1$^{d}$ | 8.1±0.1$^{b}$ | **8.5±0.0$^{a}$** | **8.5±0.0$^{a}$** | 7.3±0.1$^{c}$ |
| | $\mu_{max}$*** | 0.04±0.02$^{c}$ | 0,13±0.02$^{ab}$ | **0,18±0.03$^{a}$** | **0,13±0.02$^{ab}$** | 0,09±0.02$^{bc}$ |
| *Arthrinium arundinis* | $Ø_{max}$ | 8.5±0.0$^{a}$ | **8.5±0.0$^{a}$** | 8.5±0.0$^{a}$ | 8.5±0.0$^{a}$ | 4.0±0.1$^{b}$ |
| | $\mu_{max}$ | 0.26±0.02$^{b}$ | **0.37±0.00$^{a}$** | 0.25±0.01$^{b}$ | 0.15±0.02$^{c}$ | 0.03±0.01$^{d}$ |
| *Aspergillus olivimuriae* | $Ø_{max}$ | 5.4±0.2$^{b}$ | **6.0±0.2$^{a}$** | 3.7±0.1$^{c}$ | 2.7±0.1$^{d}$ | 2.3±0.1$^{e}$ |
| | $\mu_{max}$ | 0.04±0.02$^{ab}$ | **0.06±0.02$^{a}$** | 0.03±0.01$^{ab}$ | 0.02±0.01$^{ab}$ | 0.01±0.01$^{b}$ |
| *Penicillium* sp. | $Ø_{max}$ | 4.2±0.1$^{e}$ | **6.0±0.0$^{a}$** | 5.0±0.0$^{c}$ | 4.6±0.1$^{d}$ | 5.7±0.1$^{b}$ |
| | $\mu_{max}$ | 0.04±0.01$^{b}$ | **0.08±0.01$^{a}$** | 0.06±0.01$^{ab}$ | 0.06±0.01$^{ab}$ | 0.05±0.01$^{ab}$ |
| *Penicillium canescens* | $Ø_{max}$ | 3.9±0.1$^{c}$ | 6.4±0.2$^{b}$ | 6.4±0.2$^{b}$ | **6.9±0.1$^{a}$** | 3.7±0.1$^{c}$ |
| | $\mu_{max}$ | 0.02±0.01$^{b}$ | 0.07±0.02$^{a}$ | 0.08±0.02$^{a}$ | **0.07±0.01$^{a}$** | 0.02±0.01$^{b}$ |
| *Penicillium citrinum* | $Ø_{max}$ | 2.4±0.0$^{d}$ | 3.1±0.0$^{c}$ | 3.9±0.1$^{b}$ | **5.4±0.0$^{a}$** | 3.9±0.1$^{b}$ |
| | $\mu_{max}$ | 0.02±0.01$^{b}$ | 0.03±0.01$^{b}$ | 0.03±0.00$^{b}$ | **0.08±0.01$^{a}$** | 0.02±0.01$^{b}$ |
| *Penicillium janthinellum (Col I)* | $Ø_{max}$ | 8.2±0.1$^{a}$ | **8.0±0.0$^{a}$** | 4.9±0.1$^{b}$ | 3.8±0.1$^{c}$ | 2.3±0.1$^{d}$ |
| | $\mu_{max}$ | 0.10±0.01$^{b}$ | **0.19±0.02$^{a}$** | 0.04±0.02$^{c}$ | 0.02±0.00$^{c}$ | 0.01±0.00$^{c}$ |
| *Aspergillus tubingensis* | $Ø_{max}$ | 3.4±0.1$^{c}$ | **8.5±0.0$^{a}$** | **8.5±0.0$^{a}$** | 7.4±0.1$^{b}$ | 3.2±0.2$^{c}$ |
| | $\mu_{max}$ | 0.03±0.01$^{c}$ | **0.19±0.04$^{a}$** | **0.16±0.01$^{ab}$** | 0.10±0.02$^{b}$ | 0.03±0.01$^{c}$ |
| *Trichoderma longibrachiatum* | $Ø_{max}$ | **8.5±0.0$^{a}$** | **8.5±0.0$^{a}$** | 5.6±0.1$^{b}$ | 4.3±0.1$^{c}$ | n.g$^{§}$ |
| | $\mu_{max}$ | **0.50±0.03$^{a}$** | **0.48±0.20$^{a}$** | 0.04±0.01$^{b}$ | 0.04±0.01$^{b}$ | n.g |
| *Penicillium janthinellum (Col L)* | $Ø_{max}$ | 3.5±0.0$^{c}$ | **5.5±0.0$^{a}$** | 1.3±0.1$^{e}$ | 4.0±0.0$^{b}$ | 2.0±0.0$^{d}$ |
| | $\mu_{max}$ | 0.03±0.01$^{b}$ | **0.07±0.01$^{a}$** | 0.01±0.01$^{c}$ | 0.04±0.01$^{b}$ | 0.01±0.01$^{c}$ |
| *Chaetomium* sp. | $Ø_{max}$ | **8.2±0.0$^{a}$** | 6.5±0.1$^{b}$ | n.g | n.g | n.g |
| | $\mu_{max}$ | **0.12±0.02$^{a}$** | 0.06±0.02$^{b}$ | n.g | n.g | n.g |

* Culture conditions of fungal strains on PDA medium without and with addition of NaCl (0%, 3%, 6%, 10%, 15%). ** $Ø_{max}$ maximum growth diameter expressed on cm and ***$\mu_{max}$ maximum growth speed expressed on $h^{-1}$. §not growth. The bold results indicate the optimum growth condition for each strain.

*Arthrinium arundinis* was able to tolerate high salt concentrations very well, showing only a growth slowdown in 15% NaCl culture condition (4.0 cm). High salt tolerance is also observed in *Alternaria* sp. that grew very well up to 15% of NaCl (7.3 cm – 0.089 $h^{-1}$)

seeming instead to not prefer the absence of salt in the culture medium, with slight decrease both growth diameter (5.0 cm) and rate (0.036 $h^{-1}$).

Among the *Penicillium* strains, although growing slowly, most were not affected by the presence of salt such as *Penicillium* sp, *P. citrinum* and *P. janthinellum* COL L; on the other hand, a weak decrease in growth under 0% and 15% NaCl conditions was observed in *P. canescens*. Different behavior was found in *P. janthinellum* COL I, which instead was affected by the presence of salt already at 6% NaCl; this strain showed greater tolerance at low salt concentrations with faster growth rate which achieved 8 cm in diameter at 0 and 3% NaCl.

Among the *Aspergillus* strains, *A. tubingensis* was less affected by the presence of salt than *A. olivimuriae*, which instead showed a in growth slowdown at NaCl ≥ 6%.

### 2.4.2. Biological activities

*Antimicrobial activity*

During the first screening phase, agar diffusion assay allowed to identify positive extracts with growth inhibition capacity of test microorganisms at concentration of 2 mg/ml, while during second phase MIC was determined for those extracts resulted positive to the previous screening step. All extracts were negative to agar diffusion assay except the extracts obtained from the *Aspergillus olivimuriae* strain which exerted antimicrobial activity towards Gram positive bacteria which resulted to be significantly inhibited by the presence of the extracts. The activity index was calculated based on comparison with positive control (Streptomycin sulfate) and extracts. The highest activity against *Bacillus pumilus* was observed for the extracts produced by high NaCl concentration (15%) condition (AI-0.55).

Minimal inhibitory concentration (MIC) was determined for gram-positive bacterium *B. pumilus* that were significantly inhibited in agar diffusion assay. *Aspergillus olivimuriae* extract produced from 3% NaCl culture condition was the most active at lower concentration

(MIC 200 µg ml$^{-1}$). All extracts, even those produced by high concentrations of NaCl, maintained the antimicrobial capacity showing the same value of MIC of extracts produced at low osmotic stress conditions; in particular the 15% NaCl extract was the most active at the lowest concentration together with 3% NaCl extract (MIC 200 µg ml$^{-1}$) (Table 3).

**Table 3** Antimicrobial activity of *Aspergillus olivimuriae* extracts against the gram positive bacterium *Bacillus pumilus* expressed as Activity Index at 2 mg ml$^{-1}$ (AI = Inhibition zone of extract / Inhibition zone of positive control) and Minimal Inhibitory Concentration (MIC).

| **Extracts*** | **AI** | **MIC (µg/ml)** |
|---|---|---|
| 0 | 0.33 | 300 |
| 3 | 0.44 | 200 |
| 6 | 0.33 | 400 |
| 10 | 0.44 | 300 |
| 15 | 0.55 | 200 |

*Extracts obtained from *Aspergillus olivimuriae* cultures grown on PDA medium without and with addition of NaCl (0%, 3%, 6%, 10%, 15%).

*Anti-Quorum Sensing activity*

All fungal extracts were evaluated for their ability to interfere in bacterial communication. The results of anti QS assay indicate that 6 among tested fungi produce extracts contain metabolites which are able to interrupt the lactone-mediate communication system in *Chromobacterium violaceum* CV026. All extracts were tested at different concentrations (range 0 - 1.0 mg ml$^{-1}$) (supplementary) but for the evaluation of anti QS activity only violacein inhibition values obtained from the maximum concentration tested (1 mg ml$^{-1}$) were taken in consideration. Among all strains, *Alternaria* sp., *Penicillium janthinellum*-Col I, *Penicillium janthinellum*-Col L, *Penicillium* sp. and *Chaetomium* sp. showed no anti QS activity while instead an inhibition of violacein production was observed in the extracts of the other strains, two of the *Penicillium* genus, those of the *Aspergillus* genus, *Trichoderma longibrachiatum* and *Arthrinium arundinis* (Figure 1).

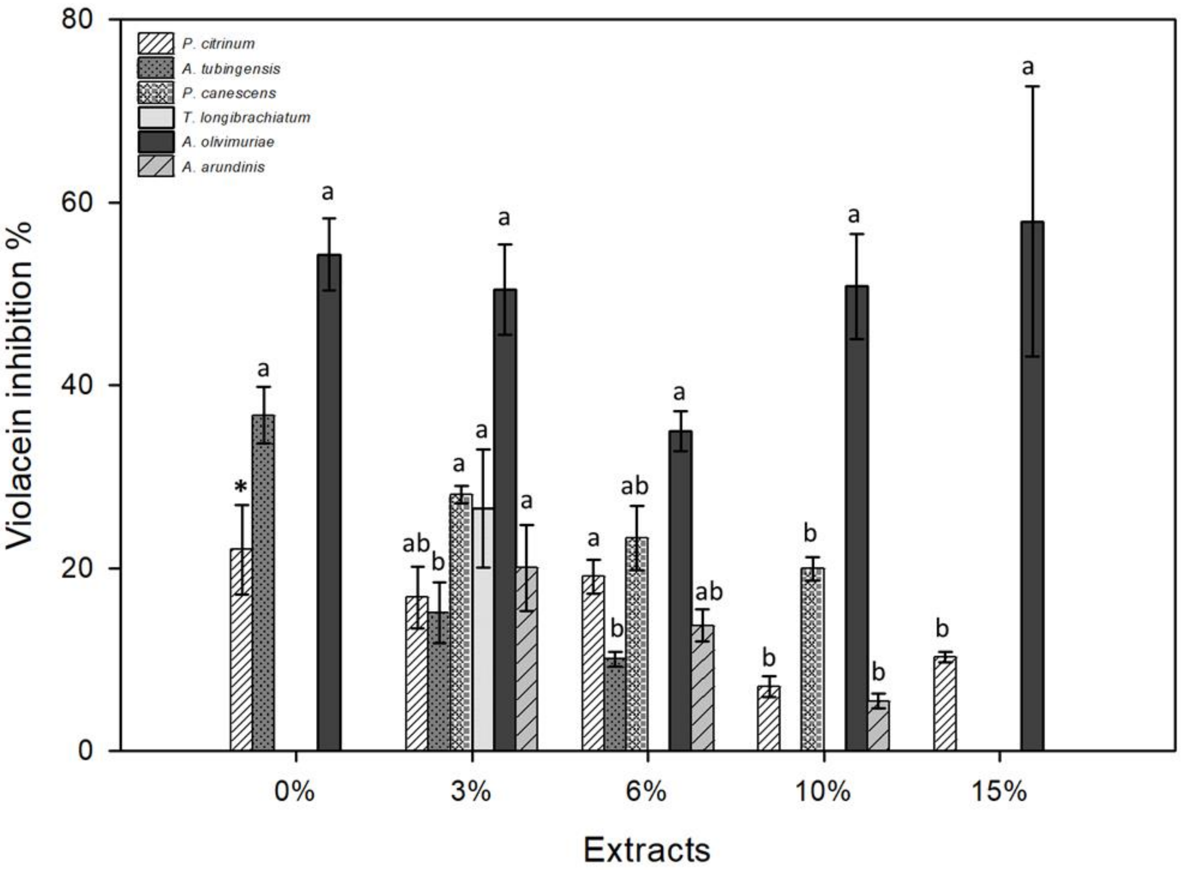


**Figure. 1** Antiquorum sensing assay using Chromobacterium violeceum CV026 as bacterial test. Violacein inhibition percentage observed in the extracts (1 mg ml-1) of active fungal strains obtained from cultures grown on PDA medium without and with addition of NaCl (0%, 3%, 6%, 10%, 15%). Different letters within the same strain indicate statistically significant differences ($P \leq 0.05$). * The asterisk denotes an associated inhibitory effect (data not shown) a on growth which underestimates the anti-QS activity.

The most active extracts of each strain show inhibition rates ranging from 20 to 58 percent; in all cases, greater activity was observed in the extracts deriving from non-optimal growth conditions of each strain, particular in the extracts produced by concentration of NaCl lower than the optimum of growth condition. Some strains require the presence of salt to produce anti-QS molecules including *Penicillium canescens* (no anti-QS activity in 0% NaCl extracts) which showed greater anti-QS activity in the extracts produced by the non-optimal growth conditions (3% NaCl), with 28% of violacein inhibition at concentration 1 mg ml$^{-1}$. A similar response is observed in *Arthrinium arundinis*, which requires salt to show anti-QS activity expressed mostly at 3% salt (20% violacein inhibition). Among the strains that express activity only at salt presence, also find *Trichoderma longibrachiatum*, which produces anti-QS compounds only at the optimal growth condition, 3% NaCl.

*Aspergillus olivimuriae* and *Penicillium citrinum* expressed anti-QS activity in all growth conditions, but for *P. citrinum* the anti-QS capacity for the salt-free condition (22% violacein inhibition) was underestimated as a growth effect at the highest concentration tested was observed.

In *Aspergillus olivimuriae*, found to be the strain with highest anti QS activity at concentration 1 mg ml$^{-1}$ (58% violacein inhibition), the most active extracts are those of the growth conditions with 0% and 15% of NaCl, i.e., those of greatest growth stress for the strain. Same case was observed for *Aspergillus tubingensis*, with the most active extract produced by the condition without salt (0% NaCl) with inhibition percentage of 37% at conc. 1 mg ml$^{-1}$.

### *Antioxidant activity*

Antioxidant activity was tested with the use of FRAP (Ferric Reducing Antioxidant Power) assay through detection of reduction of the ferric-tripyridyltriazine to the ferrous complex. The results are reported as µmol of Trolox equivalent/g of tested extract. Table 4 summarized the results of each fungal strain. Strong antioxidant potential was observed in the extracts obtained from free NaCl conditions of the strains *Aspergillus tubingensis* (493.35±7.42 µmol TE/g), *Penicillium janthinellum* Col L (192.32±14,48 µmol TE/g), *Aspergillus olivimuriae* (181.86±8.47 µmol TE/g), *Alternaria sp* (137.01±12.52 µmol TE/g) and *Penicillium citrinum* (130.92±1.79 µmol TE/g). In all cases the antioxidant potential decreased with NaCl concentration increment. *Aspergillus tubingensis* showed the strongest activity. However, its antioxidant capacity decreased considerably in conditions with the addition of NaCl with percentages of decrease between 86% (3% NaCl extract) and 95% (15% NaCl). Similar behavior was also observed in *Penicillium janthinellum* Col L and *Aspergillus olivimuriae* which, however, showed lower percentages of decrease reaching about 70%. *Alternaria* sp., while showing a decrease in antioxidant activity in response to the increase in NaCl, seems to be less affected by the effect of salt than the other strains, showing percentages of decrease in activity reaching 48% in the condition with 15% of NaCl.

**Table 4** Antioxidant activity determined by FRAP assay of fungal extracts obtained from cultures without and with addition of NaCl (0%, 3%, 6%, 10% and 15%).

| **Strains** | **Antioxidant activity (μmol TE /g)** | | | | |
|---|---|---|---|---|---|
| | **0%** | **3%** | **6%** | **10%** | **15%** |
| *Alternaria* sp. | 137.01±12.52$^{a}$ | 90.39±5.76$^{b}$ | 100.83±5.66$^{b}$ | 93.45±4.14$^{b}$ | 71.31±1.49$^{c}$ |
| *Arthrinium arundinis* | 67.27±2.03$^{a}$ | 45.57±2.40$^{b}$ | 51.11±0.55$^{b}$ | 51.07±2.85$^{b}$ | 38,65±2.00$^{c}$ |
| *Aspergillus olivimuriae* | 181.86±8.47$^{a}$ | 77.32±2.67$^{b}$ | 55.21±0.73$^{c}$ | 41.89±1.19$^{d}$ | 51.43±2.28$^{cd}$ |
| *Penicillium* sp. | 37.02±0.69$^{b}$ | 36.31±2.68$^{b}$ | 30.52±0.39$^{b}$ | 29.91±0.61$^{b}$ | 52.55±3.56$^{a}$ |
| *Penicillium canescens* | 67.68±1.19$^{a}$ | 67.25±3.05$^{a}$ | 60.34±3.49$^{a}$ | 26.58±0.44$^{b}$ | 23.66±1.47$^{b,*}$ |
| *Penicillium citrinum* | 130.92±1.79$^{a}$ | 64.03±3.25$^{b}$ | 71.77±3.03$^{c}$ | 39.41±0.65$^{d}$ | 38.33±2.71$^{d}$ |
| *Penicillium janthinellum* Col I | 34.46±1.53$^{a}$ | 24.67±0.66$^{c}$ | 28.70±0.45$^{b}$ | 24.02±1.10$^{cd}$ | 22.00±0.72$^{d,*}$ |
| *Chaetomium* sp. | 37.90±1.42$^{a}$ | 30.48±3.80$^{b}$ | - | - | - |
| *Aspergillus tubingensis* | 493.35±7.42$^{a}$ | 67.87±3.62$^{b}$ | 30.35±0.16$^{c}$ | 26.36±1.00$^{c}$ | 28.45±1.35$^{c}$ |
| *Trichoderma longibrachiatum* | 44.50±2.63$^{a}$ | 34.03±1.67$^{b}$ | 26.90±0.90$^{c}$ | 24.20±0.27$^{c}$ | - |
| *Penicillium janthinellum* Col L | 192.32±14.48$^{a}$ | 37.48±0.45$^{b}$ | 24.05±1.10$^{b,*}$ | 25.03±1.03$^{b,*}$ | 25.64±0.60$^{b,*}$ |

Antioxidant activity expressed as μmol Trolox equivalent/g of tested extract. Different letters (a–d) indicated significant difference ($P \leq 0.05$) within the same row. No significant differences from the negative control (contain only extract solvent) are denoted by the asterisk by Dunne’t test.

*Statistical analysis*

To get an overall view on results, a multivariate treatment of data based on principal component analysis was performed. Only two principal components were extracted since they satisfied the Kaiser's criterion with their respective eigenvalues being equal to 1.78 and 1.26 and the model thus obtained explained 76.1% of total variance. However, a strong outlier represented by the *A. tubingensis* control cultures was evident since its scores located outside of the Hotelling's $T^2$ confidence ellipse (Figure 2) and this was due to their unusually high antioxidant activity. Diametric growth (RG, p1= 0.681) and maximum growth rate (MGR, p1=0.673) were the variables that drove separation along the first principal component whereas antioxidant (FRAP, p2=0.658) and anti-quorum sensing (AQS, p2=0.701) activities along the second one.  The values of $R^2$VX, a parameter that quantifies the model's ability to explain the variability of a given descriptor, for the variables RG, MGR, FRAP and AQS were 0.867, 0.872, 0.642 and 0.664, respectively.

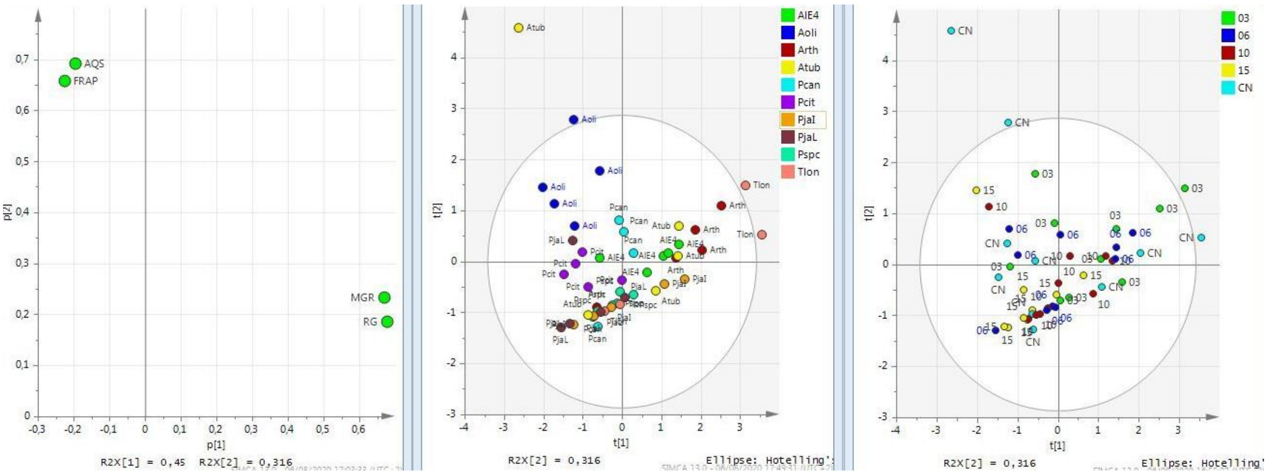


**Figure 2**. Loadings plot and scores plot labeled according to salinity level in the growth medium and species name.

Irrespective of the salinity level in the growth medium, all the scores of *A. olivimuriae* cultures located in the upper-left quadrant, correlating strongly with FRAP and, to a higher extent, with the AQS variable. A similar behavior was found for *A. arundinis* cultures the scores of which, instead, located in the upper right quadrant thus strongly correlating with the RG and MGR variables. In practical terms salinity levels affected the biological activities of *A. olivimuriae* and the growth behavior of *A. arundinis* cultures to a lesser extent than in other strains. *P. citrinum* cultures located in the lower left quadrant being inversely correlated with the RG and MGR variables and thus showed a lower dependence on salinity levels than other strains.

### 2.4.3. LC-HRMS analysis of extracts

The LC-HRMS analysis revealed the presence of several mycotoxins. In *Alternaria* sp. extracts there were two biogenetically interrelated nonribosomal cyclodipeptides, roquefortine C (Figure 3a) and meleagrin, compounds mainly isolated from *Penicillium* species with neuro-, cyto-toxic and antibacterial activities (Du et al., 2010; Martín and Liras, 2015); both decreased with increasing salt concentration. Roquefortine C was never described from Alternaria extracts but, given the results obtained and considering the identification of the strain only at genus level, further molecular analysis can be applied to investigate the taxonomy of the strain.

Polyketide mycotoxin sterigmatocystin was found in the extracts of 4 isolates which are *Alternaria* sp., *A. arundinis, A. tubingensis* and *T. longibrachiatum* (Figure 3b). Salt concentration seemed not affect the molecule amount, except in *T. longibrachiatum* extracts which had a higher production in 3% NaCl condition and a lower production in 10% NaCl condition. In *P. citrinum* and *P. janthinellum* COL I extracts there was citrinin which increased in *P. citrinum* extracts but decreased in *P. janthinellum* extracts with increasing salt concentration. Hexaketide sorbicillin was found in the extracts of 4 isolates which are

*Alternaria* sp., *A. tubingensis*, *P. citrinum* and *T. longibrachiatum* (Figure 3c). Sorbicillin increased in *P. citrinum* extracts but decreased in *Alternaria* sp. extracts with increasing salt concentration; in *A. tubingensis* extracts we found the lower production at 6% NaCl while in *T. longibrachiatum* extrcts we found the higher production at 3% NaCl.

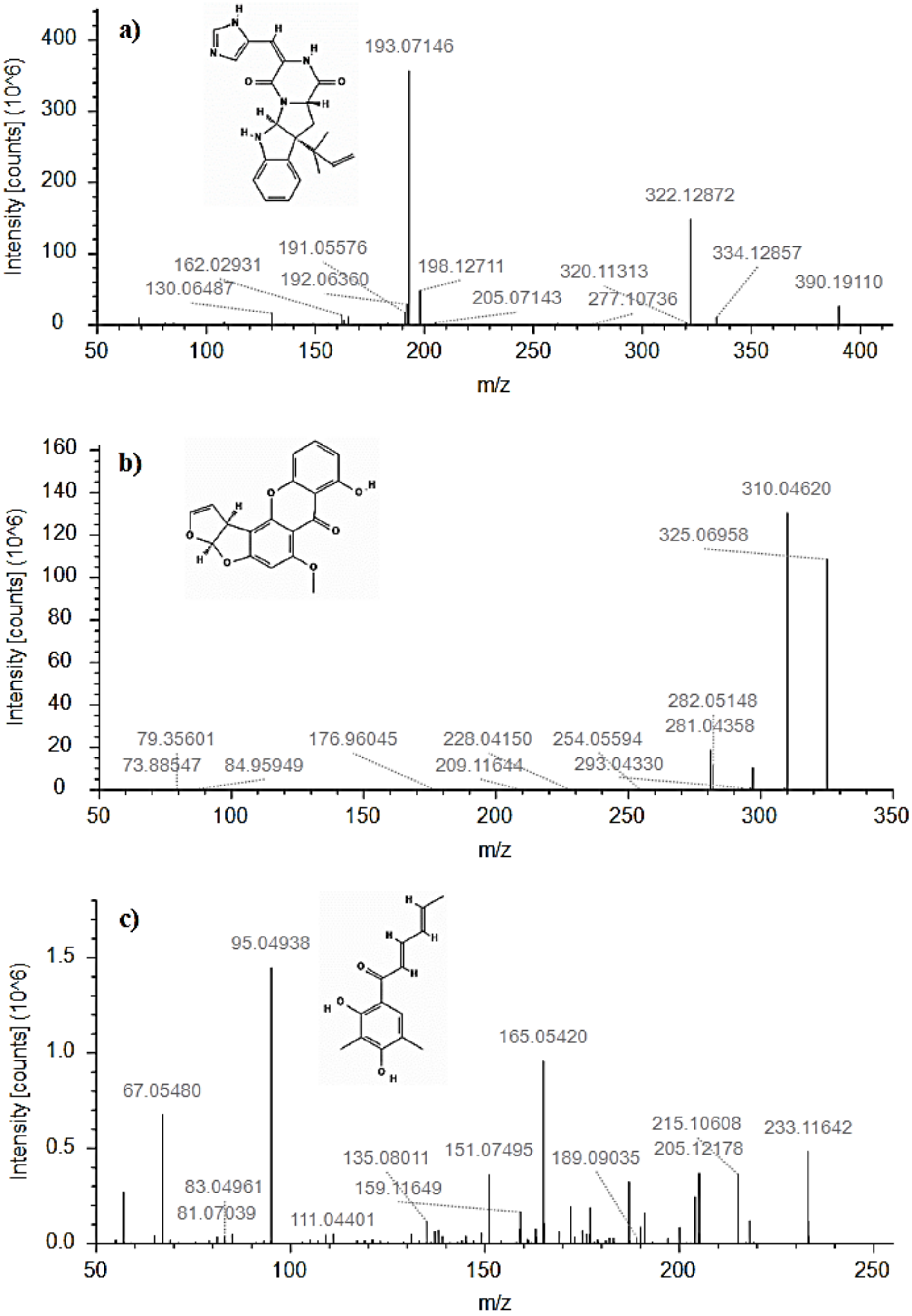


**Figure 3 a)** Roquefortin C from *Alternaria* sp. extracts; **b)** Sterigmatocystin from *T. longibrachiatum* extracts; **c)** Sorbicillin from *T. longibrachiatum* extracts.

In *P. canescens* extracts was found chlorine-containing pentaketide griseofulvin and the higher production was found in 10% NaCl condition.

In *A. olivimuriae* extracts was found geodin and its precursor (iso)sulochrin, both co-metabolites of lovastatin and originally isolated from A. terreus (Kiriyama et al., 1977); Comparing the different conditions, geodin amount was lower in absence of salt while (iso)sulochrin amount was higher. Another compound found in these extracts was 3-(3,3-dichloro-2-hydroxypropyl)-6,8-dimethoxy-1H-isochromen-1-one, an isocoumarins derivate; Also in this case, the compound content was stable in salt presence, but was lower in no salt condition.

## 2.5. DISCUSSION

In this study the effects of salinity on growth and biological activities of halotolerant fungal strains were investigated using five salt levels (0%, 3%, 6%, 10% and 15 NaCl).

The potential for the discovery of novel metabolites in new fungal species is huge, and acquires importance considering the increasing levels of drug resistance in microorganisms and the emergence of new fungal pathogens. The identification of alternative antibacterial drug targets and the subsequent development of new treatment strategies are urgently required. Among strains tested in this work only one, *Aspergillus olivimuriae,* was shown to produce secondary metabolites with restricted antibiotic spectrum since its effect was exerted only towards Gram positive bacteria. Many studies conducted on *Aspergillus* spp. have highlighted the ability of this genus to produce metabolites and extracts with antimicrobial activity. Dihydrogeodin, isolated from *A. terreus*, showed antimicrobial activity against the gram-positive bacterium *B. subtilis* (Inamori et al., 1983). A study conducted by Wang showed that *A. terreus* PT06-2 is able to produce under high salt stress conditions (10% salinity) three new compounds, terremides A, B and terrelactone A showing antibacterial activity (Wang et al., 2011b).

Targeting QS is a promising strategy to inhibit undesirable bacterial traits, in particular biofilm formation or virulence activation. This strategy, referred to as quorum quenching, involves the disruption of the bacterial QS systems with the aid of naturally occurring QS inhibitory (QSI) molecules. Certain fungi have been shown to release QSI compounds in their micro-environment to inhibit competing bacteria from production of antifungal compounds, thus in this paper the effect of salt on potential production of quenching, in particular by mechanisms of reducing the activity of HHL (hexanoylhomoserine lactone) cognate receptor protein or of HHL degradation, has been investigated. Species showing the highest anti-QS activity belong to the *Penicillium* and *Aspergillus* genera. Well-studied examples are patulin and penicillic acid, produced by *Penicillium* spp. (Rasmussen et al., 2005; Vansteelandt et al., 2012), and terrein from *Aspergillus terreus* (Kim et al., 2018) able to block gene expression for biofilm formation and virulence in *P. aeruginosa*.

Free radicals can induce damage to many biomolecules (DNA, membrane lipids, proteins) causing various human diseases such as cancer, atherosclerosis, cardiovascular and inflammatory diseases (Halliwell, 1996). Antioxidants play an important role to protect organisms from oxidative stress-caused damage and this drive a strong interest in the discovery of natural antioxidants such as those produced by filamentous fungi (Smith et al 2015). Studies conducted on *Aspergillus* species have already highlighted the antioxidant compounds production such as p-Coumaric acid, cinnamic acid, gallic acid, and ascorbic acid in *Aspergillus awamori* MTCC 548 (Salar et al., 2017) and have shown the effect of different carbon and nitrogen sources on the production of antioxidant compounds in *Aspergillus fumigatus* (Arora and Chandra, 2011). There are few studies relating to the ratio of fungal antioxidant activity and increase in salt concentration, but it is known that the increase in salt in the growth environment causes a ROS increase in eukaryotes (Tanaka et al., 2006). Contrary to other studies conducted on a halophilic fungus of marine origin, *Phialosimplex* sp., able to increase the antioxidants production in response to high salt concentrations

(Ravindran et al., 2012), in this study antioxidant activity decreased with an increase NaCl concentration.

Among the mycotoxins revealed with the LC-HRMS analysis there are several compounds known for their bioactivity. Citrinin, a pentaketide mycotoxin, is already known in literature to be produced by marine-derived *Penicillium* sp. strains with cytotoxic and antimicrobial activity (Chen et al., 2011; Wang et al., 2013). Another pentaketide, griseofulvin, it is used as antifungal drug (Chooi et al., 2010). Most of naturally occurring isocoumarins derivates are reported from Aspergillus and Penicillium genera and they show several biological activities such antimicrobial, cytotoxic, antioxidant and anti-inflammatory activities (Noor et al., 2020). Sterigmatocystin is a precursor of aflatoxin $B_1$ (Delgado-Virgen et al., 2009) and is a carcinogenic compound that affects several species of crops and experimental animals (Díaz Nieto et al., 2018). Derivates of yellow-colored compound hexaketide sorbicillin have showed antiviral, anti-inflammatory and antimicrobial activities (Salo et al., 2016). Geodin has antiviral and antimicrobial activities (Rønnest et al., 2011) while (iso)sulochrin exhibits cytotoxic activity versus mouse lymphoma cells and breast cancer cells (El-Kashef et al., 2021).

In conclusion, this study has shown that fungal species belonging to the genera *Penicillium*, *Aspergillus*, *Alternaria*, *Trichoderma* and *Arthrinium* coming from different environments, are able to produce extracts characterized by biological activity. In particular, the *Aspergillus olivimuriae* strain has emerged for its antimicrobial activity against gram+ bacteria, antioxidants and anti QS capabilities which makes it of promising use for the production of alternative antimicrobial compounds. Furthermore, the *A. olivimuriae* strain, recently classified as a new species (Crognale et al., 2019) is still to be investigated and is therefore of promising use for the search for new bioactive compounds.

## REFERENCES

Arora DS and Chandra P (2011) In vitro antioxidant potential of some soil fungi: screening of functional compounds and their purification from *Penicillium citrinum*. *Appl Biochem Biotechnol* **165(2)**: 639-51.

Benzie I and Strain J (1996) The Ferric Reducing Ability of Plasma (FRAP) as a Measure of “Antioxidant Power”: The FRAP Assay. *Anal. Biochem.* **239**: 70-76;

Chen L, Liu W, Hu X, Huang K, Wu J-L, & Zhang Q-Q (2011) “Citrinin Derivatives from the Marine-Derived Fungus *Penicillium citrinum*”. *Chem. Pharm. Bull.* **59(4)**: 515-517

Chen M, Shen, NX, Chen ZQ, Zhang FM, Chen Y (2017) Penicilones A–D, Anti-MRSA Azaphilones from the Marine-Derived Fungus *Penicillium janthinellum* HK1-6. J. *Nat. Prod.*, **80(4)**: 1081–1086;

Chooi YH, Cacho R, Tang Y (2010) Identification of the Viridicatumtoxin and Griseofulvin Gene Clusters from *Penicillium aethiopicum*” *Chem Biol.* **17(5)**: 483–494.

CLSI (2012) Methods for Dilution Antimicrobial Susceptibility Test for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

Crognale S, Pesciaroli L, Felli M, Petruccioli M, D’Annibale A, Bresciani A and Peterson SW (2019) *Aspergillus olivimuriae* sp. nov., a halotolerant species isolated from olive brine. *Int J Syst Evol Microbiol* **69(9)**: 2899-2906.

Debbab A, Aly AH, Lin WH, Proksch P (2010) Bioactive Compounds from Marine Bacteria and Fungi. *Microb Biotechnol* **3(5)**: 544–563.

Delgado-Virgen F, Guzman-de-Peña D, (2009) Mechanism of sterigmatocystin biosynthesis regulation by pH in *Aspergillus nidulans*. *Braz J Microbiol* **40**: 933–942

Díaz Nieto CH, Granero AM, Zon MA, & Fernández H (2018) Sterigmatocystin: A mycotoxin to be seriously considered. *Food and Chemical Toxicology* **118**: 460–470.

Dos Santos JA, Vieira JMF, Videira A, Meirelles LA, Rodrigues A, Taniwaki MH, Sette LD (2016) Marine derived fungus *Aspergillus* cf. *tubingensis* LAMAI 31: a new genetic resource for xylanase production. *AMB Express* **6(1)**: 25

Du L, Feng T, Zhao B, Li D, Cai S, Zhu T, Wang F, Xiao X, Gu Q. (2010) Alkaloids from a deep ocean sediment-derived fungus *Penicillium* sp. and their antitumor activities. *J Antibiot (Tokyo)* **63(4)**: 165-70.

Duran R, Cary JW, Calvo AM (2010) Role of the Osmotic Stress Regulatory Pathway in Morphogenesis and Secondary Metabolism in Filamentous Fungi. *Toxins* **2:** 367-381.

El-Kashef DH, Youssef FS, Reimche I, Teusch N, Müller WE, Lin W, ... & Proksch P. (2021). Polyketides from the marine-derived fungus aspergillus falconensis: in silico and in vitro cytotoxicity studies. *Bioorganic & Medicinal Chemistry* **29**: 115883.

Fenical W, Jensen P. (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat Chem Biol*, **2(12)**: 666–673.

Fouillaud M, Venkatachalam M, Girard-Valenciennes E, Caro Y, & Dufossé L (2016) Anthraquinones and Derivatives from Marine-Derived Fungi: Structural Diversity and Selected Biological Activities. *Marine drugs*, **14(4)**: 64.

Guo L, Wang C, Zhu W, Xu F (2016) Bioassay-guidedfractionation and identification of active substances from the fungus *Aspergillus tubingensis* against *Vibrio anguillarum*. *Biotechnol. Biotechnol. Equip*. **30(3)**: 602-606;

Halliwell B. (1996) Antioxidants in human health and disease. *Annu Rev Nutr.***16**: 33-50.

Homa M, Manikandan P, Szekeres A, Kiss N, Kocsubé S, Kredics L, Alshehri B, Dukhyil A, Revathi R, Narendran V, Vágvölgyi C, Shobana CS & Papp T. (2019) Characterization of *Aspergillus tamarii* Strains From Human Keratomycoses: Molecular Identification, Antifungal Susceptibility Patterns and Cyclopiazonic Acid Producing Abilities. *Front. Microbiol*. **10**: 2249

Huang J, Lu C, Qian X et al. (2011) Effect of salinity on the growth, biological activity and secondary metabolites of some marine fungi. *Acta Oceanol. Sin.* **30**: 118

Inamori Y, Kato Y, Kubo M, Kamiki T, Takemoto T, Nomoto K. (1983) Studies on metabolites produced by *Aspergillus terreus* var. *aureus*. I. Chemical structures and antimicrobial activities of metabolites isolated from culture broth. *Chem Pharm Bull.* **31(12)**: 4543–4548.

Jin L, Quan C, Hou X, Fan S (2016) Potential Pharmacological Resources: Natural Bioactive Compounds from Marine-Derived Fungi. *Mar. Drugs* **14**: 76

Kim B, Park J-S, Choi H-Y, Yoon SS & Kim W-G (2018) Terrein is an inhibitor of quorum sensing and c-di-GMP in *Pseudomonas aeruginosa*: a connection between quorum sensing and c-di-GMP. *Sci. Rep.* **8**: 8617

Kiriyama N, Nitta K, Sakaguchi Y, Taguchi Y, Yamamoto Y (1977) Studies in the metabolic products of *Aspergillus terreus*. III. Metabolites of strain IFO 8835. *Chem. Pharm. Bull.* **25**: 2593–2601

Martín JF & Liras P (2016) Evolutionary formation of gene clusters by reorganization: the meleagrin/roquefortine paradigm in different fungi *Appl Microbiol Biotechnol* **100**: 1579–1587

Martín JF, Liras P (2015) Novel antimicrobial and other bioactive metabolites obtained fromsilent gene clusters. In: Demain AL, Sánchez S (eds) Antibiotics: current innovations and futuretrends. *Horizon Scientific Press and Caister Academic Press*, Norfolk

Mohamed-Benkada M, François Pouchus Y, Vérité P, Pagniez F, Caroff N, Ruiz N (2016) Identification and Biological Activities of Long-Chain Peptaibols Produced by a Marine-Derived Strain of *Trichoderma longibrachiatum*. *Chem. Biodivers.* **13(5)**: 521–530;

Nicoletti R & Trincone A (2016) Bioactive Compounds Produced by Strains of *Penicillium* and *Talaromyces* of Marine Origin. *Mar. Drugs* **14(2)**: 37.

Noor AO, Almasri DM, Bagalagel AA, Abdallah HM, Mohamed SGA, Mohamed GA, Ibrahim SRM. (2020) Naturally Occurring Isocoumarins Derivatives from Endophytic Fungi: Sources, Isolation, Structural Characterization, Biosynthesis, and Biological Activities. *Molecules*. **25(2)**: 395.

Overy DP, Bayman P, Kerr RG, Bills GF. (2014) An assessment of natural product discovery from marine (*sensu strictu*) and marine-derived fungi. *Mycology* **5(3)**: 145-167.

Overy DP, Correa H, Roullier C, Chi WC, Pang KL, Rateb M, Ebel R, Shang Z, Capon R, Bills G, Kerr R **(**2017) Does Osmotic Stress Affect Natural Product Expression in Fungi? *Mar Drugs*, ***15*****(8)**: 254.

Pang KL, Overy DP, Jones EBG, da Luz Calado M et al (2016) Marine fungi' and 'marine-derived fungi' in natural product chemistry research: toward a new consensual definition. *Fungal Biol Rev* **30**: 163–175

Rasmussen T, Skindersoe M, Bjarnsholt T, Phipps R, Christensen K, Jensen P, Andersen J, Koch B, Larsen T, Hentzer M, Eberl L, Hoiby N & Givskov M (2005) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. *Microbiol.* **151**, 1325– 1340

Rateb ME, Ebel R (2011) Secondary metabolites of fungi from marine habitats. *Nat Prod Rep* **28**: 290–344.

Ravindran C, Naveenan T, Varatharajan GR, Rajasabapathy R, Meena RM (2012) Antioxidants in mangrove plants and endophytic fungal associations. *Bot Mar* **55**: 269–27

Rønnest MH, Nielsen MT; Leber B, Mortensen UH; Krämer A, Clausen, MH, Larsen TO, Harris P (2011) (+)-Geodin from *Aspergillus terreus*. Acta Crystallographica. Section C: Crystal Structure Communications, **67**(Pt3): o125-o128.

Salar RK, Purewal SS, Sandhu KS. (2017) Bioactive profile, free-radical scavenging potential, DNA damage protection activity, and mycochemicals in *Aspergillus awamori* (MTCC 548) extracts: a novel report on filamentous fungi. *3 Biotech*. **7**(3):164.

Salo O, Guzmán-Chávez F, Ries MI, Lankhorst PP, Bovenberg RAL, Vreeken RJ, Driessen AJM. (2016) Identification of a polyketide synthase involved in sorbicillin biosynthesis by *Penicillium chrysogenum*. *Appl Environ Microbiol* **82**: 3971–3978.

Santiago IF, Alves TMA, Rabello A, Sales PAJ, Romanha AJ, Zani CL, Rosa CA, Rosa LH (2012) Leishmanicidal and antitumoral activities of endophytic fungi associated with the Antarctic angiosperms *Deschampsia antarctica* Desv. and *Colobanthus quitensis* (Kunth) *Bartl. Extremophiles* **16**: 95–103

Sepcic K, Zalar P, Gunde-Cimerman N (2011) Low water activity induces the production of bioactive metabolites in halophilic and halotolerant fungi. *Mar Drugs* **9(1)**: 43-58.

Smetanina OF, Kalinovsky AI, Khudyakova YV, Pivkin MV, Dmitrenok PS, Fedorov SN, Ji H, Kwak J-Y, Kuznetsova TA (2007) Indole Alkaloids Produced by a Marine Fungus Isolate of *Penicillium janthinellum* Biourge. *J. Nat. Prod.* **70**: 906–909

Smith H, Doyle S, Murphy R, (2015) Filamentous fungi as a source of natural antioxidants, *Food Chemistry* **185**: 389-397

Sun YL, Zhang XY, Zheng ZH, Xu XY, Qi SH (2014) Three new polyketides from marine-derived fungus *Penicillium citrinum* SCSGAF 0167. *Nat. Prod. Res.* **28**: 239–244;

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B. (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell.* **18(4)**: 1052-66.

Valgas C, Souza SM, Smânia EFA, Smânia JrA (2007) Screening methods to determine antibacterial activity of natural products. *Braz. J. Microbiol.* **38(2)**: 369-380.

Vansteelandt M, Kerzaon I, Blanchet E, Fossi Tankoua O, Robiou Du Pont T, Joubert Y, Monteau F, Le Bizec B, Frisvad JC, Pouchus YF, Grovel O. (2012) Patulin and secondary metabolite production by marine-derived *Penicillium* strains. *Fungal Biol.* **116(9)**: 954-61.

Venkatachalam M, Gérard L, Milhau C, Vinale F, Dufossé L, & Fouillaud M (2019) Salinity and Temperature Influence Growth and Pigment Production in the Marine-Derived Fungal Strain *Talaromyces albobiverticillius* 30548. *Microorganisms*, **7(1)**: 10.

Wang J, Ding W, Wang R, Du Y, Liu H, Kong X, Li C (2015b) Identification and Bioactivity of Compounds from the Mangrove Endophytic Fungus *Alternaria* sp. *Mar. Drugs* **13**: 4492-4504

Wang J, Wang Z, Ju ZR, Wan JT, Liao SR, Lin XP, Zhang TY, Zhou XF, Chen H, Tu ZC, et al. (2015a) Cytotoxic cytochalasins from marine-derived fungus *Arthrinium arundinis*. *Planta Med* **81**: 160–166

Wang M, Lu C, Xu Q, Song S, Hu, Z., & Zheng, Z. (2013). “Four New Citrinin Derivatives from a Marine-Derived *Penicillium* sp. Fungal Strain”. Molecules, **18(5)**: 5723–5735.

Wang Y, Lu Z, Sun K and Zhu W (2011a) Effects of high salt stress on secondary metabolite production in the marine-derived fungus *Spicaria elegans*. *Mar. Drugs* **9**: 535–542.

Wang Y, Zheng J, Liu P, Wang W, Zhu W (2011b) Three New Compounds from *Aspergillus terreus* PT06-2 Grown in a High Salt Medium. *Mar Drugs* **9**(8): 1368-1378.

Yamada T, Muroga Y, Tanak R (2009) New Azaphilones, Seco-Chaetomugilins A and D, Produced by a Marine-Fish-Derived *Chaetomium globosum*. *Marine Drugs* **7**: 249–257

Yurchenko AN, Smetanina OF, Kalinovskii AI, Kirichuk NN, Yurchenko EA, Afiyatullov SS (2013) Biologically active metabolites of the facultative marine fungus *Penicillium citrinum. Chem Nat Comp* **48**: 996–998.

# CHAPTER 3

## *Aspergillus olivimuriae* sp. nov., a halotolerant species isolated from olive brine

A slightly modified version of this chapter has been published as:

Crognale S, Pesciaroli L, Felli M, Petruccioli M, D'Annibale A, Bresciani A and Peterson SW (2019) *Aspergillus olivimuriae* sp. nov., a halotolerant species isolated from olive brine. *International journal of systematic and evolutionary microbiology*, 69(9) 2899-2906, DOI: 10.1099/ijsem.0.003575

### 3.1. ABSTRACT

A facultative halo-tolerant *Aspergillus* strain was isolated from olive brine waste, the effluent from the debittering process of table olives. Phenotypic and molecular characteristics showed clearly that the isolate represents a novel species. Based on the source of isolation, the new species has been named *Aspergillus olivimuriae*. It was found tolerant to high concentrations of NaCl (15 %) or sucrose (60 %) and it exhibits substantial growth under these conditions. Although the new species grew profusely at 37 _C, no growth was observed at 40 _C, conidia *en masse* were avellaneous on all media. The description of the new species *Aspergillus olivimuriae* brings the total species of *Aspergillus* sect. *Flavipedes* to 15. The type strain of *A. olivimuriae* sp. nov. is NRRL 66783 (CCF 6208), its whole genome has been deposited as PRJNA498048.

### 3.2. INTRODUCTION

Search for new fungal species is always of great interest and extreme environments represent an important source of unknown microorganisms (Sayed et al., 2020). Fungi are well known as able to colonize hostile to life environments thanks also to the use of different survival strategies (Magan, 2007). They can survive in non-optimal ecosystem for living species survival such as Antarctic (Onofri et al., 2007; Durán et al., 2019), heavy metals contaminated sites (Lin et al., 2020), arid (Santiago et al., 2018) and saline environments with low water activity (Gunde-Cimerman and Zalar, 2014; Stevenson et al., 2017).

Unknown fungal species can provide information about the mechanisms used for survival in unusual environments (Hyde et al., 2019), furthermore response to stress induced by extreme living conditions can bring fungi to produce a lot of variety of secondary metabolites usually not expressed (Wang et al., 2011; Chavez et al., 2015; Sayed et al., 2020). The ever increasing demand for natural compounds to be used in production of drugs pushes research towards the study of new species (Bull and Goodfellow, 2019). The approach aimed at studying strains not yet explored represents a good alternative to work around the problem of a continuous rediscovery of known compounds (Genilloud et al., 2011).

The isolate DIBAF_OBW 6C2 (now deposited in a public culture collections as NRRL 66783 and CCF 6208) was isolated from olive brine water (OBW), commonly hosting lactic acid-producing bacteria and several yeast species (Hurtado et al., 2012), that are tolerant of high salinity. Initial microscopic examination of NRRL 66783 indicated that it was an Aspergillus species belonging to sect. Flavipedes.

Many species in section Flavipedes are adapted to reduced water activity conditions and are able to grow in naturally dry habitats, tolerating relatively high concentrations of osmotically active solutes in media (Tresner and Hayes, 1971; Moustafa and Al-Musallam, 1975). In particular, they were isolated from natural habitats with high NaCl concentration including salterns (Moustafa, 1975; Butinar et al., 2011; Cantrell et al., 2006), brackish water (Pawar and Thirumalachar, 1966) or coastal sand of the Dead Sea (Grishkan et al., 2003) and hypersaline soil from Lake Urmia Park in Iran (Arzanlou et al., 2016). Members of section Flavipedes are rich producers of bioactive secondary metabolites, some of which possess biotechnological and pharmacological relevance (Hubka et al., 2015). Section Flavipedes was most recently monographed by Hubka (Hubka et al., 2015) with additional species described by Visagie (Visagie et al., 2014), Sequeira et al. (Siqueira, 2017; Siqueira et al., 2018) and Arzanlou (Arzanlou et al., 2016).

Aim of the present work was to determine the status of isolate NRRL 66783 which does not match any species described to date. Morphology was characterized and four genes, BenA, Cam, ITS and RPB2, were sequenced, phylogenetic trees were analyzed to show that this was a novel species.

For a better understanding of the genetics and for further genome mining of this new species, the whole genome has been sequenced.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Isolation

NRRL 66783 was isolated from OBW, which exhibited these characteristics: pH 3.7; 1.51 g $l^{-1}$ total phenols; 55 g $l^{-1}$ chemical oxygen demand; 120 meq $l^{-1}$ total acidity; 30 mg $l^{-1}$ ammonium nitrogen; 2.1 g $l^{-1}$ total sugars; 0.4 g $l^{-1}$ reducing sugars. OBW was serially diluted and spread into Petri dishes containing rose Bengal agar (Oxoid) supplemented with 0.1 g $l^{-1}$ chloramphenicol and 100 g $l^{-1}$ NaCl and incubated at 28 °C for 5 or 10 days. Pure cultures were transferred from isolation plates to malt extract agar (MEA) medium for maintenance (Crognale et al 2012). Another fungal strain, RSK-23W, was isolated in July 2018 from a surface-sterilized maize seed that was incubated on MEA with antibiotics as detailed by Peterson and Jurjevic (Peterson and Jurjevic 2017).

### 3.3.2. Morphology

Morphological characterization was performed based on Samson et al. (2014). A spore suspension (0.05% Tween 80 solution) was prepared and inoculated in a three-point pattern (0.5–1 µl per spot) in Petri dishes containing MEA, supplemented with 20, 40 or 60% sucrose or 15% NaCl, potato dextrose agar (PDA), Czapek's agar (CZ) and Czapek yeast autolysate agar (CYA) and incubated in the dark for 7 days at 25 °C. Additional CYA cultures were incubated at 30, 37 and 40 °C. The culture was also grown on other descriptive media according to Samson et al. (2014). Reference colors were from Ridgway (Ridgway et al., 1912) and were designated with R- and the plate number.

### 3.3.3. Light microscopy

A small bit of mycelium and conidia was scraped from a culture and teased apart in a drop of 85% lactic acid, covered with a glass slip and viewed on a Zeiss Axioskope fitted with a Nikon D7100 digital camera. The images thus obtained were assembled into composite figures using Photoshop elements 10.

### 3.3.4. SEM microscopy

Mycelium and conidia were recovered by scrapings from a 7 day old MEA plate and were immediately prefixed for 30 min at 4 °C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 0.075% ruthenium red (w/v) and 0.075 M lysine acetate. After washing with 0.1 M cacodylate buffer (pH 7.3) containing 0.075% ruthenium red (buffer A; three changes for 10 min, each at 4 °C), specimens were fixed in 0.1 M cacodylate buffer (pH 7.3) containing 2.5% glutaraldehyde and 0.075% ruthenium red (w/v) for 2 h at 4 °C. The washing step with buffer A was repeated as described above, and specimens were then post-fixed with 2% osmium tetroxide in buffer A for 2 h at 4 °C, washed in the same buffer (three changes for 15 min each at 4 °C), and then dehydrated in a graded ethanol series. Samples were dried by the critical point method using CO2 in a Balzers Union CPD 020 (Vaduz). The samples were attached to aluminium stubs using carbon tape and sputter-coated with gold in a Balzers MED 010 unit. The observation was made by a JSM 6010 LA analytical SEM featuring integrated energy dispersive spectroscopy (JEOL).

### 3.3.5. DNA techniques

The isolate NRRL 66783 was grown in liquid malt extract medium at 180 r.p.m. at 30 °C. Mycelia were recovered after 4 days by centrifugation and DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories). The following identification markers were used: (ITS), (CaM), (BenA) and (RPB2), using primers and PCR conditions reported by Peterson (2008).

### 3.3.6. Phylogenetic tree

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011). A p-distance based evolutionary tree was inferred by using the neighbour-joining algorithm. The bootstrap test was conducted to infer the reliability of branch order, with a round of 1000 reassemblies.

In addition, homologous DNA sequences were aligned by using MAFFT 7.271 (Katoh and Standley, 2013). The aligned data sets were analysed using IQTREE 1.3.11.1 (Nguyen et al., 2015) to calculate the maximum-likelihood tree. Statistical support for branches was established using the SH-alrt (1000 reps) (Guindon et al., 2010) and ultrafast bootstrap (1000 reps) tests in IQTREE (Hoang et al., 2018). Interpreting these values is different from the parametric bootstrap. Ultrafast bootstrap values of >95%support a particular branching and SH-like approximate likelihood ratio test values >85% provide support for branches. Trees were redrawn for publication using TreeView (Page 1996) and CorelDraw.

### 3.3.7. Genome sequencing

Extracted DNA (see above) was quantified using the QuantiT Qubit dsDNA HS Assay Kit (Invitrogen) and diluted to a final concentration of 1 ng $\mu l^{-1}$ for library preparation with the Illumina Nextera XT DNA Library Prep Kit, following manufacturer’s instructions for 2×300 bp MiSeq runs. Before analysis, sequencing reads were trimmed to remove adaptors, low quality ($Q<20$) bases and ambiguous nucleotides, and reads were filtered to eliminate bacterial and human DNA contaminants. Sequences were assembled in the CLC Genomics Workbench version 9.5.2 (Qiagen). The genome is deposited in GenBank as biosample SAMN10279383 and bioproject PRJNA498048.

## 3.4. RESULTS

Phylogenetic analysis of the combined data from four loci are presented as a rooted tree (Figure 1). The new species *Aspergillus olivimuriae* is situated as the most basal species in sect. Flavipedes with support for inclusion in the section from the SH-alrt statistic (85 %) (Guindon et al., 2010) but lacking support in the ultrafast bootstrap (72 %). The individual loci are variable in their treatment of *A. olivimuriae*. The BenA data place *A. olivimuriae* in an undefined basal clade with *Aspergillus luppii*, *Aspergillus movilensis*, *Aspergillus spelaeus* and *Aspergillus polyporicola* and provides low support. The CaM data places *A. olivimuriae* in the basal position with low statistical support. Similarly to BenA, the ITS data places *A. olivimuriae* in a basal clade with *A. luppii*, *A. movilensis*, *A. spelaeus* and *A. polyporicola* with low statistical support.

The RPB2 data place *A. olivimuriae* as a distinct clade, basal in the tree.

Morphologically, *A. olivimuriae*'s spathulate to subglobose vesicles argue for inclusion in sect. Flavipedes, while the colour of the colonies might make placement in sect. Terrei possible.

The unrooted tree based on the four combined loci explains the question best with *A. olivimuriae* on a long branch with weakly positive support for inclusion in section Flavipedes and a large distance between sects. Terrei and Flavipedes.

### 3.4.1. DESCRIPTION OF ASPERGILLUS OLIVIMURIAE CROGNALE & PETERSON SP. NOV.

Mycobank no.: MB826866.

**Etymology**

The species is named Aspergillus olivimuriae for the saline wash used to process table olives, olivimuriae (o.li.vi.mu'ri.ae. L. fem.n. oliva an olive; L. fem. n. muria brine; N.L. gen. n. olivimuriae of olive brine).

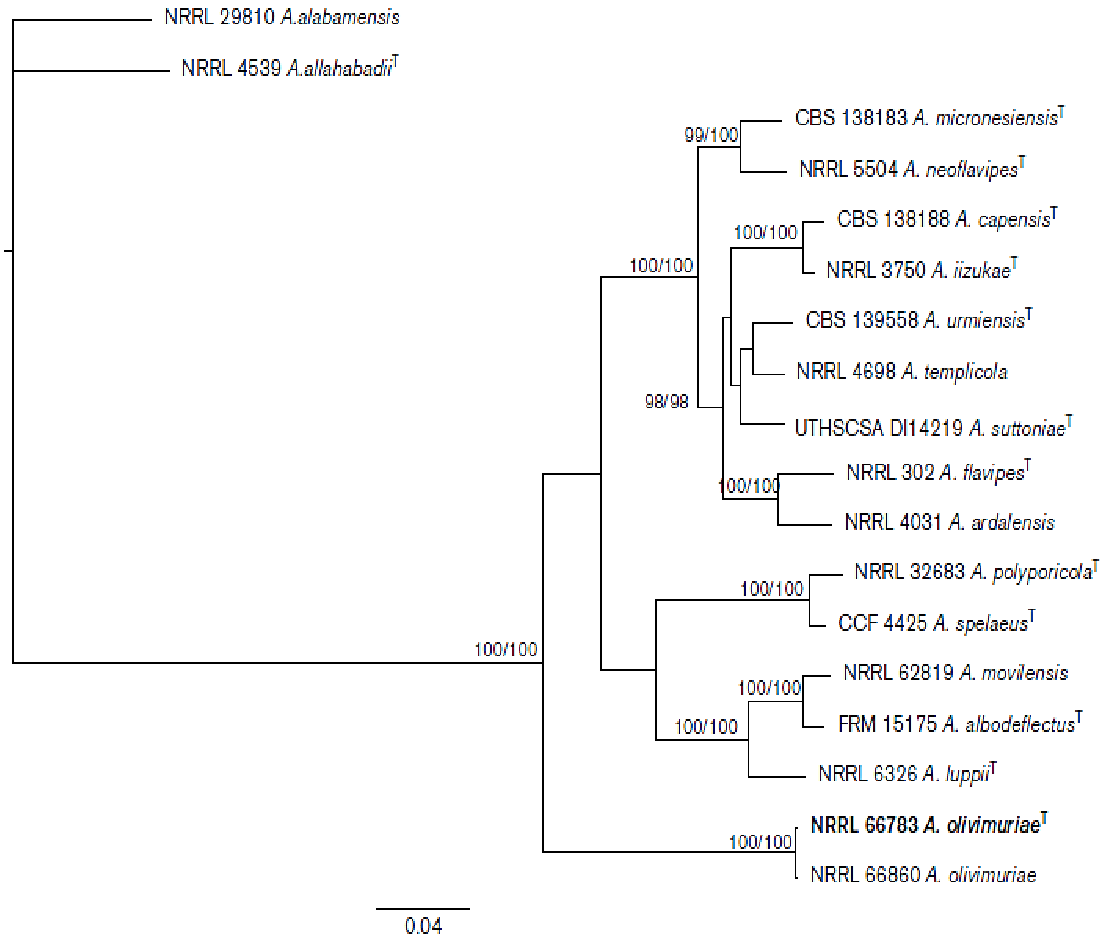


**Figure. 1.** Phylogenetic tree calculated from combined BenA, CaM, ITS and RPB2 sequence data. Numbers on the tree are SH-alrt and ultrafast bootstrap based on 1000 repetitions. Critical values for confidence are >85%and >95%and lower values are not listed. rooted with two species from Aspergillus sect. Terrei.

## Typification

Italy, Viterbo, isolated from olive curing brine, by Crognale et al. (2012); holotype BPI 910647 is a dried colony of NRRL 66783, deposited in the US National Herbarium, Beltsville, MD 20705–2350, USA. Ex type isolate NRRL 66783. Barcode ITS, MH298877. Alternative markers: BenA, MH492010; CaM, MH492011; RPB2, MH492012.

### Description

Cultures were incubated for 7 days in the dark at 25 °C. CYA colonies (Figure 2a, d) are low, sulcate, with good sporulation and conidial colour en masse avellaneous (R-40); they produce exudate in small brown droplets and a yellow soluble pigment, while reverse is coloured amber brown (R-3); no sclerotia or Hülle cells were observed. On Blakeslee's MEA (Figure 2b, e) colonies are velutinous, low, sporulation is good, avellaneous (R-40) in colour, no exudate, no sclerotia, no Hülle cells, no soluble pigments, reverse is amber brown (R-3) centrally to buff yellow (R-4) peripherally. YES colonies (Figure 2c, f) are sulcate radially and concentrically, buckled, sporulation good producing conidia in an avellaneous (R-40) colour, no soluble pigment, no sclerotia or Hülle cells observed, reverse is coloured amber brown (R-3) centrally to apricot buff (R-14) near the edges. CYAS colonies (Figure 2g) are low, sulcate, sporulation good, conidia avellaneous in colour (R-40), no soluble pigments, reverse amber brown (R-40). OA colonies (Figure 2h) are low, velutinous, sporulation good, conidia avellaneous (R-40) in colour, exudate in clear sparse droplets. CREA colonies (Figure 2i) grow thin, plane, sporulation moderate, no acid production, no exudate, conidial heads are maize yellow to yellow buff (R4) in colour, avellaneous en masse.

### Microscopic morphology

Conidiophores (Figure 2j) 100–150×5–6 µm, biseriate, vesicles 8–10 (–15) µm, hyaline, spathulate to subglobose (Figure 2k), metulae covering one half to entire vesicle surface. Metulae 4–6×2–3 µm, supporting one or two phialides 6–8×2.0–2.5 µm. Conidia (Figures 2 and 3) are globose to subglobose, 2.0–2.5 µm, with delicate surface roughness created by shallow grooves. Conidia, observed by SEM, appear to be linked to each other (Figure 3), commonly remaining in chains.

Medium-dependent growth characteristics assessed as colony diameter after 7 days growth at 25 °C on: CYA, 31–35 mm; MEA, 24–27 mm; CZ, 23–28 mm; YES, 43–46 mm; CYAS, 31–33 mm; OA, 23–25 mm; CREA, 23–24 mm; PDA, 27–28mm.

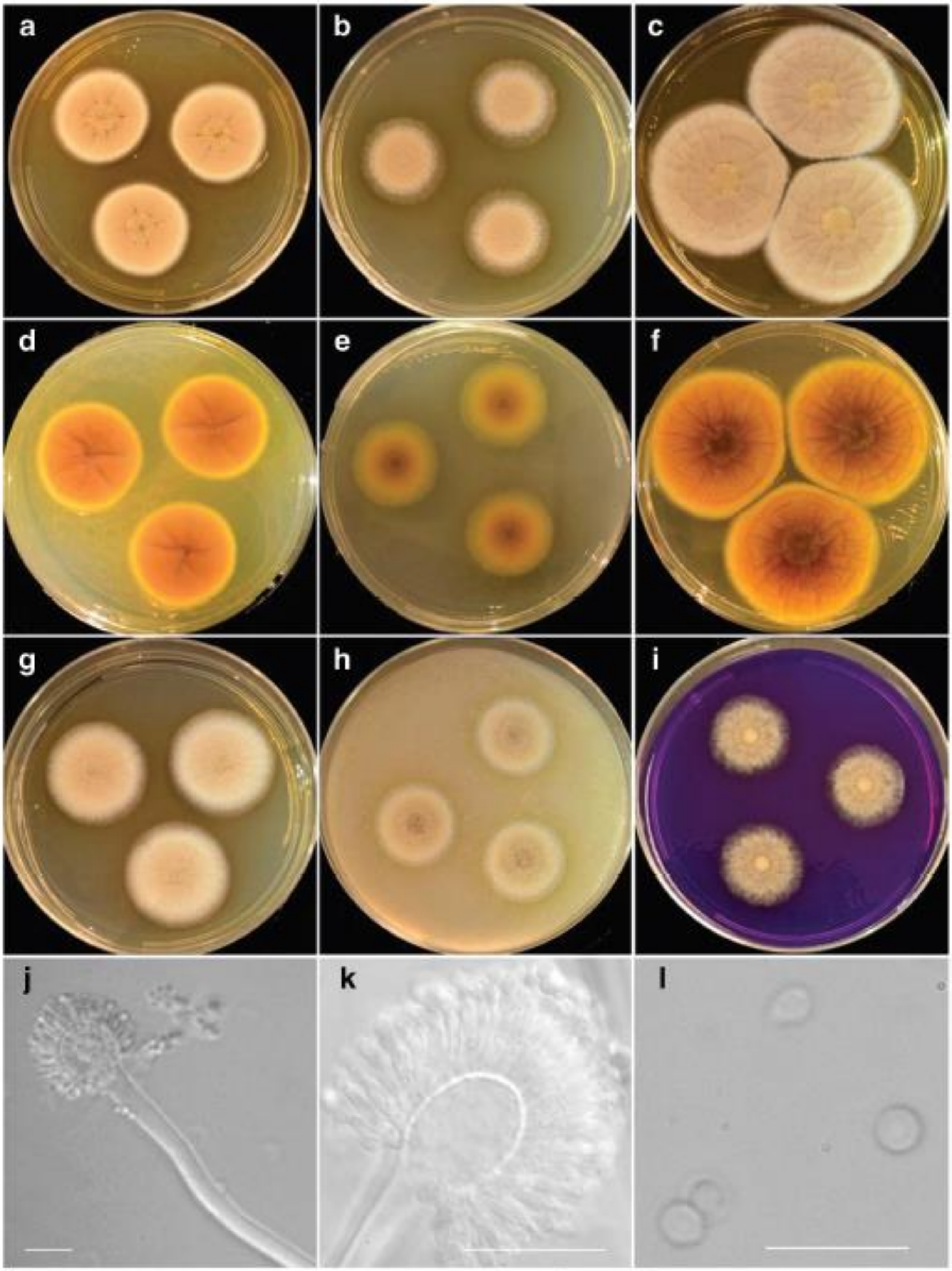


**Figure. 2.** *Aspergillus olivimuriae* NRRL 66783, all cultures were grown for 7 days at 25 _C. (a) CYA obverse. (b) MEA obverse. (c) YES obverse. (d) CYA reverse. (e) MEA reverse. (f) YES reverse. (g) CYAS obverse. (h) OA obverse. (i) CREA obverse. (j) Conidiophore and vesicle. (k) Vesicle, metulae and phialides. (l) Spherical conidia. Bar, 10μm for all figures.

On osmophilic media containing sucrose, growth is: MEA20S, 47–48 mm; MEA40S, 33–36 mm; MEA60S, 13–15 mm. In the case of NaCl, MEA15AS colony size is 17–18mm. Temperature-dependent growth assessed after 7 days growth on CYA at 30 °C is 30–31 mm, at 37 °C is 24–25mm and no growth is observed at 40 °C. On CZ or CYA (Figure 2d) media after 7 days of incubation at 25 °C, an abundant diffusible pastel yellow to light yellow soluble pigmented is visible. The yellow haloes produced around colonies seem to be correlated with the production of neoaspergillic acids (Visagie et al., 2014).

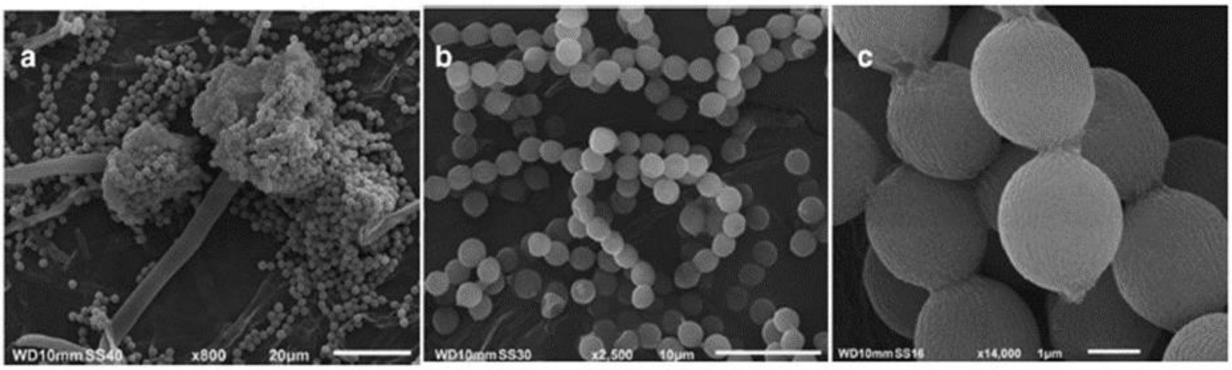


**Figure. 3.** Scanning electron microscope images showing Aspergillus olivimuriae conidiophores (a) and conidia at ×2500 (b) and ×14000 magnification (c).

### Other isolates examined

Strain RKS-23W was isolated from a surface-sterilized maize seed harvested in Sedgewick County, Kansas (37.603318, –97.189075) from cultivar Pioneer 589 corn for dryland farming. The DNA sequences are very similar or identical to the DNA sequences from the ex-type isolate and it conforms to the morphological description of *A. olivimuriae*.

## 3.5. DISCUSSION

Several sections of Aspergillus are known for their xerophilic life styles. In particular, the section Restricti contains obligate and facultative xerophiles such as A. vitricola and A. restrictus (Sklenar et al., 2017), while sections Versicolores and Flavipedes include only facultative xerophiles (Hubka et al., 2015; Jurjevic et al., 2012). Member of the Aspergillus sect. are uniseriate while those belonging to Versicolores and Flavipedes sections are biseriate. The section Versicolores generally encompasses species producing green-coloured conidia, while those produced by member of sect. Flavipedes generally are some shade of white, yellow or brown.

There is a large and statistically valid phylogenetic difference between the sections in Aspergillus (Peterson et al., 2017; Houbraken and Samson, 2011; Kocsube et al., 2016). The deciding factor in placement of species in sections is not the micro- or macromorphology but the phylogeny of isolates (Jurjevic et al., 2012), thus Aspergillus olivimuriae was first placed in sect. Flavipedes mainly on the basis of phylogeny and then on morphological criteria. This trend in mycology is also evident in other genera, such as Talaromyces (Yilmaz et al., 2014), Geosmithia (Houbraken et al., 2012) and Acremonium (Summerbell et al., 2011).

The colony colour of *A. olivimuriae*, on all the media tested, is a light brownish shade (avellaneous) and this feature distinguished it from all other species in sect. Flavipedes. Some species of sect. Flavipedes form brown-, white- or yellow-coloured colonies on CYA, colours which are distinct from the avellaneous. Most species of sect. Flavipedes, such as *A. urmiensis* and *A. flavipes*, form a specialized conidial form, referred to as the accessory conidium (Hubka et al., 2015) when cultured under natural light and this characteristic is also extended to species belonging to the sister section Terrei, such as for instance, *A. terreus* a human pathogenic species. However, and regardless of growth media and culture conditions, these specialized structures were not found in *A. olivimuriae*. Hubka et al. (2015) have pointed out that accessory conidia are present in the pathogenic species *Aspergillus micronesiensis* (syn. *A. frequens*), and also in all members of the sect. Flavipedes.

Another feature shared by several members of the sect. Flavipedes is the occurrence of Hülle cells which are mostly found when the cultures are grown in the dark. Even in this case, Hülle cells were not found in *A. olivimuriae* which, forthis reason, can be associated with other Flavipedes members, such as *A. flavipes*, *A. urmiensis* and *A. polyporicola*.

*A. flavipes, A. polyporicola, A. urmiensis* and *A. olivimuriae* produce no Hülle cells on common media. The conidiophores of *A. olivimuriae* are 100–150×5–6µm while only *A. luppii* has similarly short conidiophores. Other species have conidiophores about 1000 µm long. Conidia of *A. olivimuriae* are globose 2–2.5 µm, while *A. luppii* conidia are 2.7–3.4 µm and globose, distinguishing the two species.

Although *A. olivimuriae* was isolated from OBW, it is possible that the habitat of the fungus might be quite distinct from that of the isolation matrix, mostly populated by lactic acid bacteria and a few species of yeasts. In fact, filamentous fungi have been shown to be less represented than bacteria and yeasts in OBW and *A. olivimuriae* is a facultative halophile.

A second isolate of the fungus was obtained from a surface sterilize maize seed in Kansas (*A. olivimuriae* NRRL66860). We describe *A. olivimuriae* as a rarely encountered new species in *Aspergillus* presumably sect. Flavipedes.

## REFERENCES

Arzanlou M, Samadi R, Frisvad JC, Houbraken J, Ghosta Y (2016) Two novel *Aspergillus* species from hypersaline soils of The National Park of Lake Urmia, Iran. *Mycol Prog* **15**: 1081–1092.

Bull AT and Goodfellow M (2019) Dark, rare and inspirational microbial matter in the extremobiosphere: 16 000 m of bioprospecting campaigns. *Microbiol (Reading)*. **165(12)**: 1252-1264.

Butinar L, Frisvad JC, Gunde-Cimerman N. (2011) Hypersaline waters - a potential source of foodborne toxigenic aspergilli and penicillia. *FEMS Microbiol Ecol* **77**: 186–199.

Cantrell SA, Casillas-Martínez L, Molina M (2006) Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. *Mycol Res* **110**: 962–970.

Chávez R, Fierro F, García-Rico RO, Vaca I (2015) Filamentous fungi from extreme environments as a promising source of novel bioactive secondary metabolites. *Front Microbiol*. **6**: 903.

Crognale S, Pesciaroli L, Petruccioli M, D’Annibale A. (2012) Phenoloxidase-producing halotolerant fungi from olive brine wastewater. *Process Biochem* **47**: 1433–1437.

Durán P, Barra PJ, Jorquera MA, Viscardi S, Fernandez C, Paz C, Mora ML & Bol R (2019) Occurrence of Soil Fungi in Antarctic Pristine Environments. *Frontiers in bioengineering and biotechnology* **7**: 28

Genilloud O, González I, Salazar O, Martín J, Tormo JR and Vicente F. (2011) Current approaches to exploit actinomycetes as a source of novel natural products. *J Ind Microbiol Biotechnol* **38**: 375–389.

Grishkan I, Nevo E, Wasser SP (2003) Soil micromycete diversity in the hypersaline Dead Sea coastal area, Israel. *Mycol Prog* **2**: 19–28.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W et al. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* **59**: 307–321.

Gunde-Cimerman N and Zalar P (2014) Fungi in Solar Salterns. *Food Technol. Biotechnol*. **52(2)**: 170–179

Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS (2018) UFBoot2: Improving the Ultrafast Bootstrap Approximation. *Mol Biol Evol* **35**: 518–522.

Houbraken J, Samson RA (2011) Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. *Stud Mycol* **70**: 1–51.

Houbraken J, Spierenburg H, Frisvad JC (2012) Rasamsonia, a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species. *Antonie van Leeuwenhoek* **101**: 403–421.

Hubka V, Nováková A, Kolařík M, Jurjević Ž, Peterson SW (2015) Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect. nov. *Mycologia* **107**: 169–208.

Hurtado A, Reguant C, Bordons A, Rozes N. (2012) Lactic acid bacteria from fermented table olives. *Food Microbiol* **31**: 1–8.

Hyde KD, Xu J, Rapior S *et al.* (2019) The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Diversity* **97**: 1–136

Jurjevic Z, Peterson SW, Horn BW (2012) *Aspergillus* section *Versicolores*: nine new species and multilocus DNA sequence based phylogeny. *IMA Fungus* **3**: 59–79.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**: 772–780.

Kocsube S, Perrone G, Magist_a D, Houbraken J, Varga J et al. (2016) *Aspergillus* is monophyletic: evidence from multiple gene phylogeniesand extrolites profiles. *Stud Mycol* **85**: 199–213.

Lin Y, Xiao W, Ye Y *et al*. (2020) Adaptation of soil fungi to heavy metal contamination in paddy fields—a case study in eastern China. *Environ Sci Pollut Res* **27**: 27819–27830

Magan (2007) Fungi in Extreme Environments. In: Kubicek C., Druzhinina I. (eds) Environmental and Microbial Relationships. The Mycota, vol 4. Springer, Berlin, Heidelberg.

Moustafa AF, Al-Musallam AA (1975) Contribution to the fungal flora of Kuwait. *Transactions of the British Mycological Society* **65**: 547–553.

Moustafa AF (1975) Osmophilous fungi in the salt marshes of Kuwait. *Can J Microbiol* **21**: 1573–1580.

Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* **32**: 268–274.

Onofri S, Selbmann L, De Hoog GS, Grube M, Barreca D, Ruisi S and Zucconi L (2007) Evolution and adaptation of fungi at boundaries of life. *Adv Space Res* **40**: 1657–1664.

Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**: 357–358.

Pawar V, Thirumalachar M (1966) Studies on halophilic soil fungi from Bombay. *Nova Hedwigia* **12**: 497–508.

Peterson SW, Jurjević Ž (2017) New species of *Talaromyces* isolated from maize, indoor air, and other substrates. *Mycologia* **109**: 537–556.

Peterson SW. (2008) Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* **100**: 205–226.

Ridgway R. (1912) *Color Standards and Color Nomenclature*. Washington;. Published by the author.

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V et al. (2014) Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol* **78**: 141–173.

Santiago IF, Goncalves VN, Gomez-Silva B, Galetovic A and Rosa LH (2018) Fungal diversity in the Atacama Desert. *Antonie Van Leeuwenhoek* **111**: 1345–1360.

Sayed A, Hassan M, Alhadrami H, Hassan H, Goodfellow M and Rateb M (2020) Extreme environments: microbiology leading to specialized metabolites. *J Appl Microbiol*, **128**: 630-657.

Siqueira JPZ (2017) Clinical and environmental *Aspergillus*: morphological and molecular characterization, phylogeny, and antifungal susceptibility profile [dissertation]. *Reus: Universitat Rovira i Virgili*.

Siqueira JPZ, Wiederhold N, Gen_e J, García D, Almeida MTG et al. (2018) Cryptic *Aspergillus* from clinical samples in the USA and description of a new species in section *Flavipedes*. *Mycoses* **61**: 814–825.

Sklenar F, Jurjević Ž, Zalar P, Frisvad JC, Visagie CM et al. (2017) Phylogeny of xerophilic aspergilli (subgenus *Aspergillus*) and taxonomic revision of section *Restricti*. *Stud Mycol* **88**: 161–236.

Stevenson A, Hamill PG, O'kane CJ, Kminek G, Rummel JD, Voytek MA, Dijksterhuis J and Hallsworth JE (2017) Aspergillus penicillioides differentiation and cell division at 0.585 water activity. *Environ Microbiol* **19**: 687–697.

Summerbell RC, Gueidan C, Schroers HJ, de Hoog GS, Starink M et al. (2011) *Acremonium* phylogenetic overview and revision of *Gliomastix*, *Sarocladium*, and *Trichothecium*. *Stud Mycol* **68**: 139–162.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**: 2731–2739.

Tresner HD, Hayes JA (1971) Sodium chloride tolerance of terrestrial fungi. *Appl Microbiol* **22**: 210–213.

Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K et al. (2014) *Aspergillus*, *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. *Stud Mycol* **78**: 63–139.

Wang Y, Coleman-Derr D, Chen G, Gu YQ (2015) OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res* **43**: W78–W84.

Wang Y, Zheng J, Liu P, Wang W, Zhu W (2011) Three new compounds from Aspergillus terreus PT06–2 grown in a high salt medium. *Mar Drugs* **9**: 1368–1378.

Yilmaz N, Visagie CM, Houbraken J, Frisvad JC, Samson RA. (2014) Polyphasic taxonomy of the genus Talaromyces. *Stud Mycol* **78**: 175–341.

# CHAPTER 4

# Secondary metabolite profile of the novel halotolerant specie *Aspergillus olivimuriae*

This chapter is a draft in preparation for publication as:

Felli M., Frisvad JC and Crognale S. Secondary metabolite profile of the novel halotolerant specie *Aspergillus olivimuriae.*

## 4.1. ABSTRACT

The novel halotolerant species *Aspergillus olivimuriae* NRRL 66783, recently described and taxonomically assigned to sect. Flavipeds is still totally to be investigated. The information of its secondary metabolites and the genetic basis for their biosynthesis are unknown. Here, a preliminary investigation for the productive capacities of secondary metabolites was conducted and the inductive effect for the production of exometabolites due to the addition of 10% NaCl, enzymatic inhibitors of histone deacetylase (HADC) (sodium butyrate and valproic acid) o and 40 % sucrose was studied. At the same time the productive potential of secondary metabolites, trough bioinformatic study of biosynthetic gene clusters, was carried out using two prediction programs, AntiSMASH and SMURF. Analysis revealed the presence of biosynthetic gene clusters of secondary metabolites known to be associated with toxins, antitumor compounds and antimicrobials. Furthermore the results showed how the growth on salt is the condition that most modifies the metabolic profile of *A. olivimuriae*, decreasing the production of compounds.

## 4.2. INTRODUCTION

For centuries Aspergillus species have been exploited, as cell factories, for biotechnological purposes because they are able to produce a large number of metabolites. Their secondary metabolites (SMs) include e a wide variety of small bioactive molecules that contribute to their survival, fitness, and pathogenicity. SMs have enormous importance to humankind and big potential in drug discovery (Schueffler and Anke, 2014), they display a broad range of useful antibiotic and pharmaceutical activities (Fu et al., 2015) as well as less desirable immunosuppressant and toxic activities (Yu and Keller, 2005).

Secondary metabolite profiling have been used, also, in “chemophylogeny” (Frisvad et al., 2007; Frisvad, 2015), supporting morphological and DNA-based studies in taxonomical assignation of isolates. Each fungal species has its own chemotype, and many SMs are specific for different sections of Aspergillus (Frisvad and Larsen, 2015), consequently they have been largely used in species descriptions (Hubka et al., 2015; Samson et al., 2011; Varga et al., 2007).

The genes encoding secondary metabolites are contiguously arranged in compact biosynthetic gene clusters (BGCs), however they are commonly silent under growth conditions routinely used in laboratories, but they are expressed under biotic and abiotic stimuli.

The experimental strategy of one strain many compounds (OSMAC) which covers changing medium composition (Frisvad, 2012) and cultivation status, co-cultivation with other strain(s) (Boruta et al., 2019) or adding enzyme inhibitor(s) (Sharma et al., 2018; Shwab et al., 2007; Magotra et al., 2017) has been shown as a simple and powerful tool to decipher the biochemical potential of filamentous fungi permitting the activation many silent biogenetic gene clusters involved in expression of secondary metabolites (Pan et al., 2019).

In the last decade, several studies have been focused on the effect of salinity and osmotic stress on fungal exometabolites production (Sepcic et al., 2011, Lee et al., 2013; Overy et al., 2017).

Some authors suggest that the addition of salt in the culture medium exerts a selective physiological bias on the metabolic output of the fungus, as stress regulatory systems and fungal development are interconnected and directly affect the regulation of secondary metabolism (Overy et al., 2017).

Differential expression in secondary metabolite production was observed in halotolerant strains such as *Aspergillus terreus* (Wang et al., 2011a), *Mariannaea elegans* (as Spicaria elegans) (Wang et al., 2011b) and *Wallemia sebi* PXP-89 (Peng et al., 2011) due to increasing salinity of the cultivation medium compared to water controls.

In recent years, in parallel with use of experimental strategies related to fungal cultivation, genomic-scale approaches are increasingly used (Shi-Kunne et al., 2019). Fungal genomes analysis has shown how SMs potential production is greater than the number of metabolites isolated and identified with classical cultivation approaches. The growing possibility obtaining complete genomic assemblies has led to the optimization of computational tools that allow to identify biosynthetic genes clusters starting from the nucleotide sequences. This approach allows to quickly know the biosynthetic potential of interest microorganism (Medema and Fischbach, 2015).

Although many secondary metabolites have already been structurally elucidated, chemotaxonomic studies have shown that many new secondary metabolites have yet to be characterized, thus fungi from extreme environments seem good potential candidates for the isolation of new bioactive compounds (Chavez et al., 2015).

General aim this work was to explore the chemical diversity of *Aspergillus olivimuriae* NRRL 66783 (CCF 6208) secondary metabolome. This novel, recently described, halotolerant species has been taxonomically assigned to Sec. Flavipeds on the basis of morphological features and multilocus analysis (BenA, Cam, ITS and RPB2), (Crognale et al., 2019), however, until now, no information is present on its secondary metabolites.

In this paper, a battery of solid agar media culture (YES, CYA, MEA), supplemented differently with NaCl, sucrose and two different epigenetic modifiers such valproic acid and sodium butyrate has been used to produce plugs extracts, finally analyzed with ultra-high performance liquid chromatography-diode array detection (UHPLC-DAD).

## 4.3. MATERIALS AND METHODS

### 4.3.1. Fungal strain

*Aspergillus olivimuriae* NRRL 66783 (CCF 6208) originally isolated from olive brine (Crognale et al., 2012) and described by Crognale et al. (2019) was used.

### 4.3.2. Extract preparation

*Aspergillus olivimuriae* was grown 10 days at 25 °C on Malt Extract Agar (MEA-Oxoid formula, malt extract agar 50 g/L, ZnSO 4 $\cdot 7H_2O$ 0.01 g/L, $CuSO_4 \cdot 5H_2O$ 0.005 g/L), Yeast Extract Sucrose agar (YES, Sucrose 150 g/L, yeast extract 20 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $ZnSO_4 \cdot 7H_2O$ 0.001 g/L, $CuSO_4 \cdot 5H_2O$ 0.005 g/L and agar 20 g/L) and Czapek Yeast Autolysate agar (CYA, yeast extract 5 g/L, sucrose 30 g/L, $K_2HPO_4 \cdot 3H_2O$ 1 g/L, $ZnSO_4 \cdot 7H_2O$ 0.01 g/L, $CuSO_4 \cdot 5H_2O$ 0.005 g/L, Czapek concentrate 10 mL/L ($NaNO_3$ 300 g/L, KCl 50 g/L, $MgSO_4 \cdot 7H_2O$ 50 g/L, $FeSO_4 \cdot 5H_2O$ 1 g/L), and agar 20 g/L). Each medium was supplemented differently with sodium butyrate (250 µM, Sigma Aldrich), valproic Acid (250 µM, Sigma Aldrich), NaCl (10 % w/vol) or Sucrose (40 % w/vol). Every culture was used for crude extracts preparation according to the agar plug extraction method of Smedsgaard et al. (1997) as modified by Frisvad et al. (2019). Five plugs of 6 mm diameter were cut from the center of colony and transferred to a vial with 1 ml of the solvent mixture ethylacetate / isopropanol (3:1, vol/vol), with 1 % formic acid. The plugs were extracted ultrasonically for 60 min. The extract was transferred to a clean vial and the organic phase was evaporated to dryness under a stream of nitrogen. The dry was re-dissolved ultrasonically for 10 min in 300 µl methanol and the residues were removed by centrifugation.

### 4.3.3. Extrolites analysis

The extracts were analyzed using HPLC-UV/vis diode array detection (DAD) and fluorescence detection (FLD) which allowed to make a qualitative analysis of secondary metabolome of *A. olivimuriae* by comparison of the UV-Visible DAD spectra and retention times with similar UV spectra of known compounds (Frisvad and Thrane, 1993; Hansen et al., 2005; Nielsen et al., 2011).

Separation was at 40 °C on a 50 × 2 mm i.d., 3 µm, Luna C18 II column (Phenomenex, Torrance, CA, USA) equipped with a Security Guard precolumn. A linear water–MeCN gradient of 15% MeCN–water to 100% MeCN in 20 min was applied at a constant flow of 0.3 mL/min; then 100% MeCN was maintained for 5 min before returning to the starting conditions in 2 min and equilibrating for 5 min. Formic acid was added to all solvents to 20 mM. UV spectra were collected by a DAD every 0.4 s from 200 to 700 nm with a resolution of 2 nm.

### 4.3.4. Statistical analysis

In order to assess similarity relationships between secondary metabolites profiles of 15 media obtained in the present study, principal component analysis (PCA) was performed by using UNSCRAMBLER 10.X software.

### 4.3.5. Bioinformatics analysis for whole-genome orthologous genes comparison and secondary metabolites gene clusters prediction

Protein coding genes were identified using AUGUSTUS with the default parameters and the gene set of *Aspergillus terreus* was used as the training data (Stanke et al., 2004). Genome wide comparison of orthologous genes across multiple *Aspergillus* species was performed using OrthoVenn (Wang et al., 2015), the *Aspergillus flavipes* genome was retrieved from US Department of Energy Joint Genome Institute (www.jgi.doe.gov/).

The Antibiotics-Secondary Metabolite Analysis Shell (anti-SMASH) and the Secondary Metabolite Unique Regions Finder (SMURF) programs were used to predict secondary metabolites (SM) clusters in *A. olivimuriae* (Medema et al., 2011; Khaldi et al., 2010). For

SMURF analysis the protein coding genes prediction of AUGUSTUS was used as input file. Default parameters were used.

## 4.4. RESULTS

### 4.4.1. Whole-genome orthologous genes comparison

Phylogenetic analysis of the combined data from four loci *BenA*, *CaM*, *ITS* and *RPB*2 has already allowed to place *Aspergillus olivimuriae* within the Flavipedes section (Crognale et al., 2019) and Olivimuriarum series (Houbraken & Frisvad nov. MycoBank MB835555) (Houbraken et al., 2020) of which it is the only representative. Further investigations related to the study of orthologous genes represent a way for describing differences and similarities in the composition of genomes from different species. In fact, characteristic of orthologous genes is the derivation from an ancestral gene that was present in a common ancestor of the compared species and which therefore diverges following a speciation event.

The genome of *A. olivimuriae* is approximately 30.5 Mbp and contains 10 083 ORFs. Whole-genome orthologous genes were comprehensively investigated within *A. flavus*, *A. terreus*, *A. flavipes* and *A. fumigatus*. The overall comparison analysis result was displayed as Venn diagram in Figure 1.a. Total 5929 orthologous genes were shared among all the compared species, 36 of which were shared only between *A. olivimuriae* and *A. fumigatus*, 66 between *A. olivimuriae* and *A. flavus,* 88 between *A. olivimuriae* and *A. terreus* and 419 between *A. olivimurae* and *A. flavipes*.

*A. olvimuriae* shows a greatest overlapped conserved genes with *A. flavipes* which is observed in greater conservation of biological processes and molecular functions (Figure 1.b-c). Percentages of overlap of 29% concern the oxidoreductase and 21% transferase activities. A good percentage of homology is also observed for the hydrolase activity (11%).

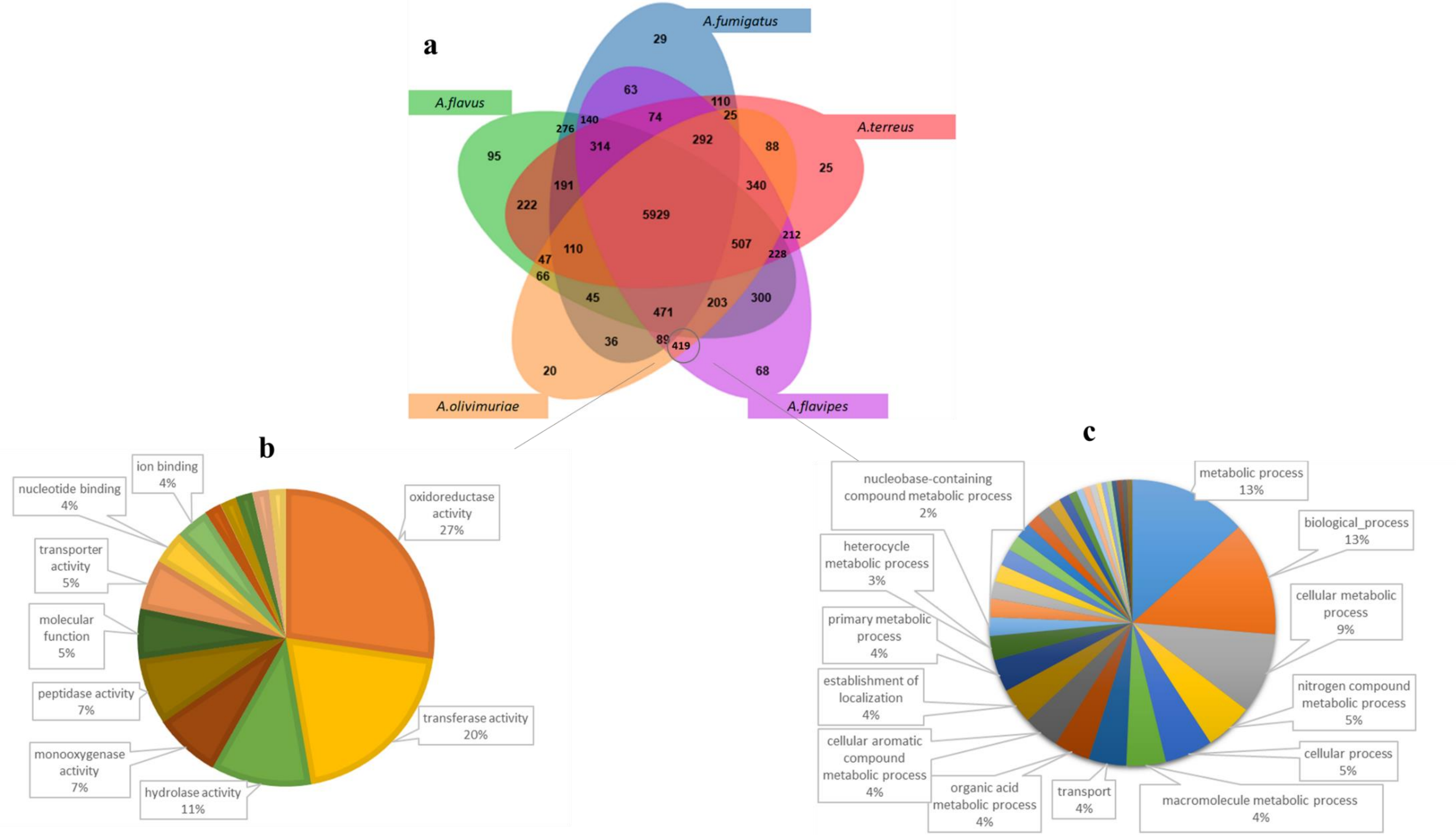


**Figure 1.** a) Venn diagram of whole-genome orthologous genes in other *Aspergillus* species. The numbers in the diagram indicate overlapped conserved genes or un-overlapped specific genes in every single species. Pie charts show the percentage of overlapped molecular function (b) and biological process (c) between *A. olivimuriae* and *A. flavipes.*

### 4.4.2. Secondary metabolites gene clusters prediction

For studying the fungal biosynthetic potential of secondary metabolites at predictive level SMURF and antiSMASH computational tools were used. These algorithms are based on the identification of backbone enzymes like polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), hybrid PKS-NRPS for both and NRPS/PKS-like enzyme or dimethylallyl tryptophan synthase (DMATS) for SMURF and Indole or Terpene for AntiSMASH. Adjacent genes are then scanned for the presence of common secondary metabolite gene domains and boundaries are predicted for each cluster. This genomic-scale approach, also defined bottom-up approach (Luo et al., 2014), allows to know fungal biosynthetic potential of secondary metabolites only due to the study of genome sequence data.

Secondary metabolites gene cluster analysis (Table 1) shows differences in the number of clusters found from the two software. Although antiSMASH provides a greater number of clusters (51 total clusters vs 43 total clusters of SMURF), SMURF prediction, also considering “-like” backbone genes, predicts a larger number of NRPS clusters compared to antiSMASH (25 for SMURF vs 13 for antiSMASH). However, “Other” antiSMASH clusters coincide with the NRPS-like ones identified by SMURF. The diverse total counts are probably due to different algorithms used by the programs: antiSMASH is able to recognize more types of gene clusters (Weber et al., 2015) than SMURF which instead is able to predict only five backbone enzymes type (Khaldi et al., 2010).

The antiSMASH algorithm also makes a comparison analysis between the entire clusters identified in the prediction with those already known stored in the database (based on MIBiG, Minimum Information about a Biosynthetic Gene cluster (Medema et al., 2015) (Table 2). In *A. olivimuriae* 8 clusters are associated with known biosynthetic gene clusters and the similarity percentages are mostly between 12% and 37% and a single cluster shows very high similarity percentages (83%). Six of this known clusters are associated to the PKS class, aflatrem (BGC0000629_c2) (37%), chaetoglobosin (BGC0000968_c1) (28%), terric acid

(BGC0000160_c1) (22%), endocrocin (BGC0001118_c1) (22%), trypacidin (BGC0001403_c1) (21%) and fujikurin (BGC0001305_c1) (83%); one to the PKS-indole class, mycophenolic (BGC0000104_c1) (25%) and one to the terpenes class, azaphilone (BGC0000027_c1) (12%).

**Table 1.** Summarized SMURF and Anti-SMASH results for *Aspergillus olivimuriae*.

| | **Anti-SMASH** | **SMURF** |
|---|---|---|
| **Number of identified secondary metabolite clusters** | **51** | **43** |
| PKS[a] | 15 | 10 |
| NRPS[b] | 13 | 11 |
| Hybrid (PKS-NRPS) | 2 | 2 |
| Indole | 2 | - |
| Terpene | 6 | - |
| Indole–NRPS | 1 | - |
| Indole–T1 PKS | 1 | - |
| Other[c] | 11 | - |
| PKS-like | - | 2 |
| NRPS-like | - | 14 |
| DMAT[d] | - | 1 |
| DMAT-NRPS | - | 1 |
| DMAT-PKS | - | 1 |
| PKS-NRPS-like | - | 1 |

[a] Polyketide synthase cluster; [b] Nonribosomal peptide synthase cluster; [c] Cluster containing a secondary metabolite-related protein that does not fit into any other category; [d] Tryptophan dimethylallyl transferase cluster

**Table 2.** Anti-SMASH prediction of *A. olivimuriae* secondary metabolites gene clusters and BLASTp analysis of cluster backbone enzymes protein sequence

| *Cluster* | *Backbone enzymes* | *Contig* | *Position* | *Most similar known cluster* | *BLASTp (Basic Local Alignment Search Tool) analysis of cluster backbone enzymes protein sequence* |
|---|---|---|---|---|---|
| *Cluster 1* | T1 PKS | 8 | 1-30608 | | 58% polyketide synthase [Aspergillus sclerotioniger CBS 115572] Sequence ID: PWY90345.1 |
| *Cluster 2* | NRPS | 31 | 1-76664 | | 65% hypothetical protein ASPVEDRAFT_187857, partial [Aspergillus versicolor CBS 583.65] Sequence ID: OJI99592.1 |
| *Cluster 3* | OTHER | 72 | 1-21792 | | 72% nonribosomal peptide synthase, putative [Aspergillus fischeri NRRL 181] Sequence ID: XP_001263979.1 |
| *Cluster 4* | T1 PKS | 83 | 5383-55176 | Aflatrem biosynthetic gene cluster (37% of genes show similarity) | 47% polyketide synthase module [Aspergillus oryzae 3.042] Sequence ID: EIT73118.1 |
| *Cluster 5* | T1 PKS | 87 | 6577-49702 | | 53% ketoacyl-synt-domain-containing protein [Aspergillus steynii IBT 23096] Sequence ID: XP_024709463.1 |
| *Cluster 6* | T1 PKS | 89 | 1-42878 | | 81% Beta-ketoacyl synthase [Aspergillus oryzae] Sequence ID: OOO06230.1 |
| *Cluster 7* | OTHER | 107 | 1-28061 | | 79% conserved hypothetical protein [Aspergillus terreus NIH2624] Sequence ID: XP_001209684.1 |
| *Cluster 8* | OTHER | 110 | 1-45668 | | 92%L-aminoadipate-semialdehyde dehydrogenase large subunit [Aspergillus terreus NIH2624] Sequence ID:XP_001213135.1; 87%non-ribosomal peptide synthetase/alpha-aminoadipate reductase [Aspergillus steynii IBT 23096] Sequence ID:XP_024710341.1 |
| *Cluster 9* | NRPS | 156 | 1-44605 | | 89% predicted protein [Aspergillus terreus NIH2624] Sequence ID: XP_001217069.1 |
| *Cluster 10* | T1PKS | 172 | 1-73674 | | 81% polyketide synthase [Aspergillus ustus] Sequence ID: KIA75420.1 |
| *Cluster 11* | T1PKS-NRPS | 186 | 1-54177 | | 71% polyketide synthase [Aspergillus niger] Sequence ID: GAQ42521.1 42% nonribosomal peptide synthetase [Aspergillus sp.] Sequence ID: AVV62216.1; 35% nonribosomal peptide synthase [Aspergillus fumigatus Z5] Sequence ID: KMK63509.1 |
| *Cluster 12* | T1PKS | 226 | 1-29117 | Terreic acid biosynthetic gene cluster (22% of genes show similarity) | 88% polyketide synthase [Aspergillus terreus] Sequence ID: AAC49814.1; 88% 6-methylsalicylic acid synthase [Aspergillus terreus] Sequence ID: BAA20102.2 |

| | | | | | |
|---|---|---|---|---|---|
| *Cluster 13* | T1PKS | 228 | 1-36295 | Chaetoglobosin biosynthetic gene cluster (28% of genes show similarity) | 58% hypothetical protein ASPACDRAFT_53248 [Aspergillus aculeatus ATCC 16872] Sequence ID: XP_020054643.1 |
| *Cluster 14* | NRPS | 230 | 1-35267 | | 38% nonribosomal peptide synthetase [Aspergillus kawachii IFO 4308] Sequence ID: GAA82422.1; |
| *Cluster 15* | OTHER | 256 | 1-14056 | | 64% hypothetical protein CDV55_03348 [Aspergillus turcosus] Sequence ID: OXN36533.1;<br>58% amino acid adenylation domain protein [Aspergillus niger] Sequence ID: OWW30626.1;<br>58% non-ribosomal peptide synthetase, partial [Aspergillus niger ATCC 1015] Sequence ID: EHA20666.1 |
| *Cluster 16* | INDOLE-NRPS | 298 | 12451-57303 | | 47% non-ribosomal peptide synthetase module [Aspergillus oryzae 3.042] Sequence ID: EIT81213.1;<br>93% conserved hypothetical protein [Aspergillus terreus NIH2624] Sequence ID: XP_001214507.1;<br>92% short-chain dehydrogenase [Aspergillus bombycis] Sequence ID: XP_022387261.1 |
| *Cluster 17* | TERPENE | 307 | 1-6326 | | |
| *Cluster 18* | T1PKS | 312 | 2163-46685 | Fujikurins biosynthetic gene cluster (83% of genes show similarity) | 65% putative polyketide synthase [Fusarium sp. NRRL 52700] Sequence ID: ALQ32997.1 |
| *Cluster 19* | TERPENE | 315 | 9874-45268 | | |
| *Cluster 20* | NRPS | 324 | 26147-71051 | | 43% hypothetical protein ABOM_001685 [Aspergillus bombycis] Sequence ID: XP_022393255.1; 38% non-ribosomal peptide synthetase [Aspergillus steynii] Sequence ID: AHZ61901.1 |
| *Cluster 21* | INDOLE-T1PKS | 359 | 1-26543 | Mycophenolic acid biosynthetic gene cluster (25% of genes show similarity) | 75% hypothetical protein CDV55_05548 [Aspergillus turcosus] Sequence ID: OXN35570.1 |
| *Cluster 22* | TERPENE | 382 | 1-21968 | | |
| *Cluster 23* | NRPS | 399 | 1-23844 | | 55% nonribosomal peptide synthetase 1 [Aspergillus lentulus] Sequence ID: GAQ10892.1;<br>62%nonribosomal peptide synthase Pes1 [Aspergillus flavus NRRL3357] Sequence ID: XP_002380492.1 |
| *Cluster 24* | T1PKS-NRPS | 417 | 1-30421 | | 66% beta-ketoacyl synthase domain-containing protein [Aspergillus niger] Sequence ID: GAQ42507.1 |

| | | | | | |
|---|---|---|---|---|---|
| *Cluster 25* | NRPS | 437 | 1-37259 | | 61% hypothetical protein P170DRAFT_464604 [Aspergillus steynii IBT 23096] Sequence ID: XP_024704356.1;<br>37% nonribosomal peptide synthase [Aspergillus arachidicola] Sequence ID: PIG81003.1 |
| *Cluster 26* | T1PKS | 453 | 1-11814 | | 55% polyketide synthase, putative [Aspergillus fischeri NRRL 181] Sequence ID: XP_001261656.1 |
| *Cluster 27* | NRPS | 463 | 13792-93539 | | 77% predicted protein [Aspergillus terreus NIH2624] Sequence ID: XP_001217686.1;<br>36% nonribosomal peptide synthase, putative [Aspergillus clavatus NRRL 1] Sequence ID: XP_001270326.1<br>78% predicted protein [Aspergillus terreus NIH2624] Sequence ID: XP_001217690.1; |
| *Cluster 28* | OTHER | 482 | 1-36782 | | 53% predicted protein [Aspergillus terreus NIH2624] Sequence ID: XP_001216515.1; 46% acetyl-CoA synthetase-like protein [Aspergillus steynii IBT 23096] Sequence ID: XP_024706755.1<br>75% hypothetical protein ATEG_07892 [Aspergillus terreus NIH2624] Sequence ID: XP_001216513.1 |
| *Cluster 29* | NRPS | 498 | 1-38507 | | 34% nonribosomal peptide synthase, putative [Aspergillus clavatus NRRL 1] Sequence ID: XP_001270091.1 |
| *Cluster 30* | TERPENE | 504 | 1-24056 | | |
| *Cluster 31* | NRPS | 520 | 6927-80742 | | 81% predicted protein [Aspergillus terreus NIH2624] Sequence ID: XP_001216109.1 |
| *Cluster 32* | OTHER | 524 | 601-52146 | | 74% NRPS-like enzyme, putative [Aspergillus flavus NRRL3357] Sequence ID: XP_002376391.1 |
| *Cluster 33* | T1PKS | 525 | 12634-37605 | Trypacidin biosynthetic gene cluster (21% of genes show similarity) | 86% hypothetical protein ATEG_08451 [Aspergillus terreus NIH2624] Sequence ID: XP_001217072.1;<br>70%polyketide synthase, putative [Aspergillus fumigatus Af293] Sequence ID: XP_751377.1 |
| *Cluster 34* | NRPS | 556 | 1-33832 | | 47% nonribosomal peptide synthetase 12 [Aspergillus udagawae] Sequence ID: GAO87324.1 52%nonribosomal peptide synthetase 12 [Aspergillus udagawae] Sequence ID: GAO87324.1 |
| *Cluster 35* | NRPS | 600 | 1-30118 | | 37% nonribosomal peptide synthetase [Aspergillus steynii IBT 23096] Sequence ID: XP_024706778.1 |

| | | | | | |
|---|---|---|---|---|---|
| *Cluster 36* | TERPENE | 608 | 1-79202 | Azaphilone biosynthetic gene cluster (12% of genes show similarity) | 60% Beta-ketoacyl synthase C-terminal domain protein [Aspergillus parasiticus SU-1] Sequence ID: KJK67551.1; 66% conserved hypothetical protein [Aspergillus terreus NIH2624] Sequence ID: XP_001217254.1<br>84% Beta-ketoacyl synthase N-terminal domain protein [Aspergillus parasiticus SU-1] Sequence ID: KJK67566.1 |
| *Cluster 37* | OTHER | 735 | 1285-50715 | | 88% hypothetical protein ATEG_02403 [Aspergillus terreus NIH2624] Sequence ID: XP_001211581.1;<br>80% hybrid NRPS/PKS enzyme [Aspergillus fumigatus var. RP-2014] Sequence ID: KEY83021.1<br>92% L-xylulose reductase [Aspergillus terreus NIH2624] Sequence ID: XP_001211583.1 |
| *Cluster 38* | INDOLE | 748 | 4648-26078 | | 81% endoglucanase EG-1 precursor [Aspergillus terreus NIH2624] Sequence ID: XP_001217286.1 |
| *Cluster 39* | T1PKS | 832 | 14343-66941 | | 70% polyketide synthase [Aspergillus niger CBS 513.88] Sequence ID: XP_001394423.2 |
| *Cluster 40* | OTHER | 919 | 1-28027 | | 72% conserved hypothetical protein [Aspergillus terreus NIH2624] Sequence ID: XP_001214973.1;<br>65% NRPS-like enzyme [Aspergillus arachidicola] Sequence ID: PIG69483.1 |
| *Cluster 41* | NRPS | 931 | 1-64495 | | |
| *Cluster 42* | OTHER | 938 | 1-22509 | | 88% aspulvinone E synthetase [Aspergillus terreus] Sequence ID: AND66115.1 |
| *Cluster 43* | T1PKS | 971 | 13872-54940 | | 73% hypothetical protein ATEG_09100 [Aspergillus terreus NIH2624] Sequence ID: XP_001217722.1;<br>69% putative polyketide synthase [Aspergillus flavus AF70] Sequence ID: KOC07781.1 |
| *Cluster 44* | INDOLE | 974 | 928-26261 | | 75% hypothetical protein CDV56_07857 [Aspergillus thermomutatus] Sequence ID: OXS05470.1<br>61% predicted protein [Aspergillus terreus NIH2624] Sequence ID: XP_001211993.1 |
| *Cluster 45* | NRPS | 998 | 1-39907 | | 67% non-ribosomal peptide synthetase/alpha-aminoadipate reductase [Aspergillus oryzae 3.042] Sequence ID: EIT75205.1<br>90% hypothetical protein ATEG_05074 [Aspergillus terreus NIH2624] Sequence ID: XP_001214252.1;<br>86%long-chain-fatty-acid-CoA ligase [Aspergillus steynii IBT 23096] Sequence ID: XP_024699818.1 |

| | | | | | |
|---|---|---|---|---|---|
| *Cluster 46* | T1PKS | 1083 | 1-28639 | | 82% hypothetical protein BDW42DRAFT_199691 [Aspergillus taichungensis] Sequence ID: PLN84151.1<br>76% hypothetical protein P168DRAFT_310784 [Aspergillus campestris IBT 28561] Sequence ID: XP_024692690.1 |
| *Cluster 47* | T1PKS | 1234 | 1-32945 | Endocrocin biosynthetic gene cluster (22% of genes show similarity) | 69% polyketide synthase, putative [Aspergillus fischeri NRRL 181] Sequence ID: XP_001267621.1 |
| *Cluster 48* | T1PKS | 1281 | 1-28529 | | 61% hypothetical protein HK57_00218 [Aspergillus ustus] Sequence ID: KIA75975. |
| *Cluster 49* | OTHER | 1283 | 1-20828 | | 65% aspulvinone E synthetase [Aspergillus terreus] Sequence ID: AND66115.1 |
| *Cluster 50* | OTHER | 1404 | 1-14102 | | 84% non-ribosomal peptide synthetase [Aspergillus niger ATCC 1015] Sequence ID: EHA20401.1 |
| *Cluster 51* | TERPENE | 1824 | 1-6982 | | |

### 4.4.3. Aspergillus olivimuriae secondary metabolites profile

The predicted biosynthetic potential of secondary metabolites for *A. olivimuriae* requires further studies in order to know the real production capacity of compounds. Top-down approach (Luo et al., 2014), in which the strain was subjected to different growth conditions, was therefore used for this purpose. To ensure the maximum expression potential of secondary metabolites it is necessary to use more than a single medium under standardized growth conditions. Media has a very strong influence of secondary metabolites production. The first observation of chromatograms obtained from HPLC analysis of plug extracts from *A. olivimuriae* cultivated on YES (A), MEA (B) and CYA (C) (Figure 2), show how the growth condition on CYA causes a decrease in the number of secondary metabolites produced.

An overview of families extrolites produced by *Aspergillus olivimuriae* is given in Table 3. The largest representation in the produced families of compounds is given by Citreoisocoumarins (7 in YES medium), Trypacidins (7 in MEA), Geodins (4 in YES, Benzomalvins (7 in CYA) and Asterric Acid (5 in MEA) but also the production of Cycloaspeptide, Erdin, Antraquinone, Asperflavin, Asterriquinone, Physcion, Linoleic acid, Ergosterol and Unknown compounds. Among the media, the largest number of compounds produced is observed in YES (31). Furthermore this medium stimulates the production of compounds that are not expressed in the other two media such as Antraquinone (2), Asperflavin (1), Asterriquinone (1) and Physcion (1) and unknown compounds (4).

Synthesis of the most represented families of compounds is linked to PKS biosynthetic gene clusters class which also represent the main clusters observed in the prediction analysis. The PKS cluster of Geodin was studied in *A. terreus* (Nielsen et al., 2013) as well as that of the Citreoisocoumarin, which however has been well studied in Fusarium (Sørensen et al., 2012). Instead in *A. terreus* the Benzomalvins are produced by a cluster encoding two separate NRPS enzymes (Clevenger, 2018).

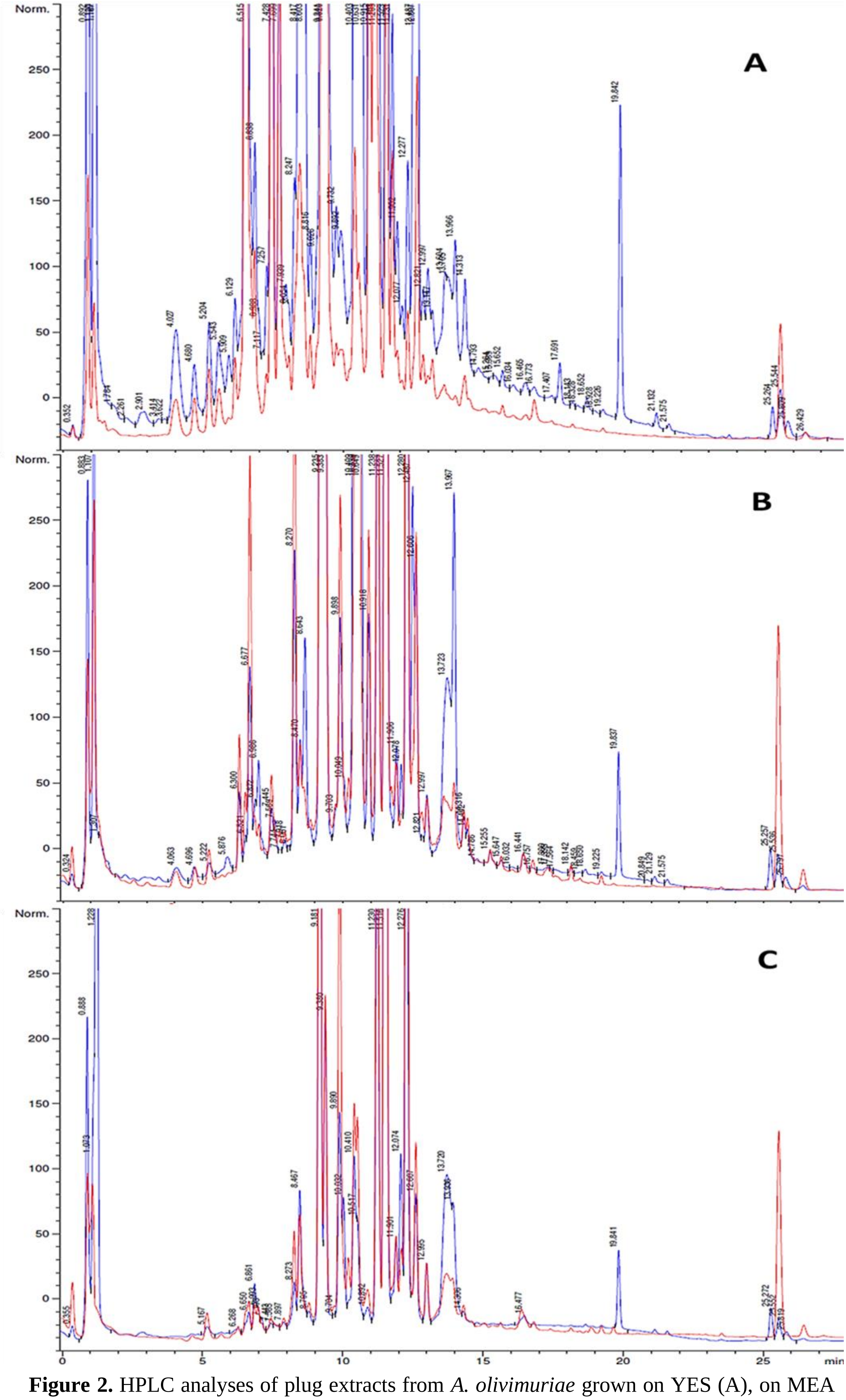
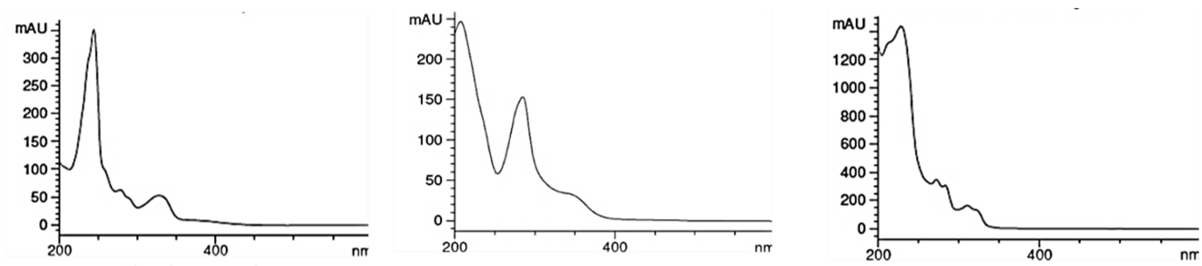


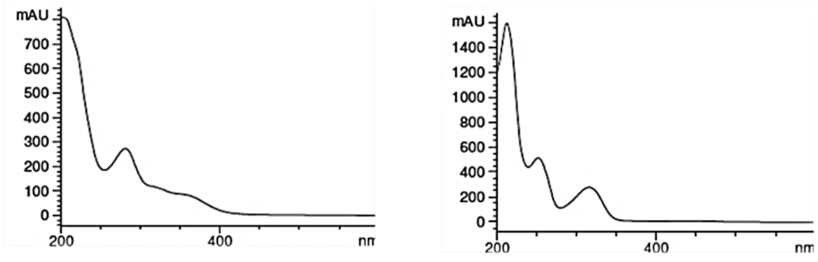
**Figure 2.** HPLC analyses of plug extracts from *A. olivimuriae* grown on YES (A), on MEA (B) and on CYA (C) with the 210 nm trace as a blue line and the 280 nm trace as a red line showing significant differences.

**Table 3.** Families of compounds produced by *A. olivimuriae* on YES, MEA and CYA media

| **Compounds families** | **MEA** | **YES** | **CYA** |
|---:|:---:|:---:|:---:|
| Citroisocoumarin | 3 | 7 | 1 |
| Trypacidins | 7 | 1 | 4 |
| Geodins | 2 | 4 | 1 |
| Geodins-benzomalvin | 1 | 0 | 0 |
| Benzomalvins | 4 | 4 | 7 |
| Asterric acid | 5 | 2 | 1 |
| Cycloaspeptide | 1 | 0 | 1 |
| Erdin | 0 | 0 | 2 |
| Antraquinone | 0 | 2 | 0 |
| Asperflavin | 0 | 1 | 0 |
| Asterriquinone | 0 | 1 | 0 |
| Physcion | 0 | 1 | 0 |
| Linoleic acid | 1 | 1 | 1 |
| Ergosterol | 1 | 3 | 1 |
| Unknown | 0 | 4 | 0 |
| Total Compounds | 25 | 31 | 19 |



Citreoisocoumarin Trypacidins Benzomalvin



Geodin Asterric acid

**Figure 3.** UV-spectra of the most represented families of compounds produced by *A. olivimuriae* in YES, MEA and CYA media.

In addition to the use of different cultivation media, subjecting the fungus to stressful growth conditions can expand the production of secondary metabolites even not yet known. Figure 4 shows the effects on compounds production after the addition on medium of sodium butyrate, valproic acid, NaCl and Sucrose. The addition of salt affects the production of compounds in MEA and YES media but does not show effects in CYA medium probably due to its salt-rich composition. The presence of salt stimulates the production of Shamixanthene and Erdin in MEA and YES furthermore, it allows the expression of Cycloaspeptide in the YES medium. In these media the salinity also determines an increase in the production of compounds belonging to the Benzomalvins family but strongly decreases (YES) to the point of inhibiting (MEA) the expression of compounds of the Citreoisocoumarins family (Figure 4).

The addition of the histone deacetylase (HADC) inhibitors, valproic acid and sodium butyrate, does not particularly affect the production of compounds. The number/type of metabolites produced under this growth condition is very similar to that produced under the standard growth condition without added stress.

The sucrose supplement strongly decreases the production of citreoisocoumarin in CYA and to a lesser extent also in YES, up to completely inhibit its production in MEA. Except this inhibitory effect on the production of citreoisocoumarin, in most cases it induces the production of compounds, in particular by increasing their number. For example, on CYA medium (Figure 4-C) it increases the production of trypacidin and benzomalvin; it also induces indolealkaloid production both on CYA and MEA medium. On YES medium, already rich in sucrose, it does not show a particular effect (Figures 4-B).

As already highlighted, growth on the YES cultural medium determines the greatest number of compounds produced but also the greatest variety (Figure 4-C). Its use in order to evaluate the production of secondary metabolites in *A. olivimuriae* is important mostly because it also allows the expression of unknown compounds that could open further study perspectives.

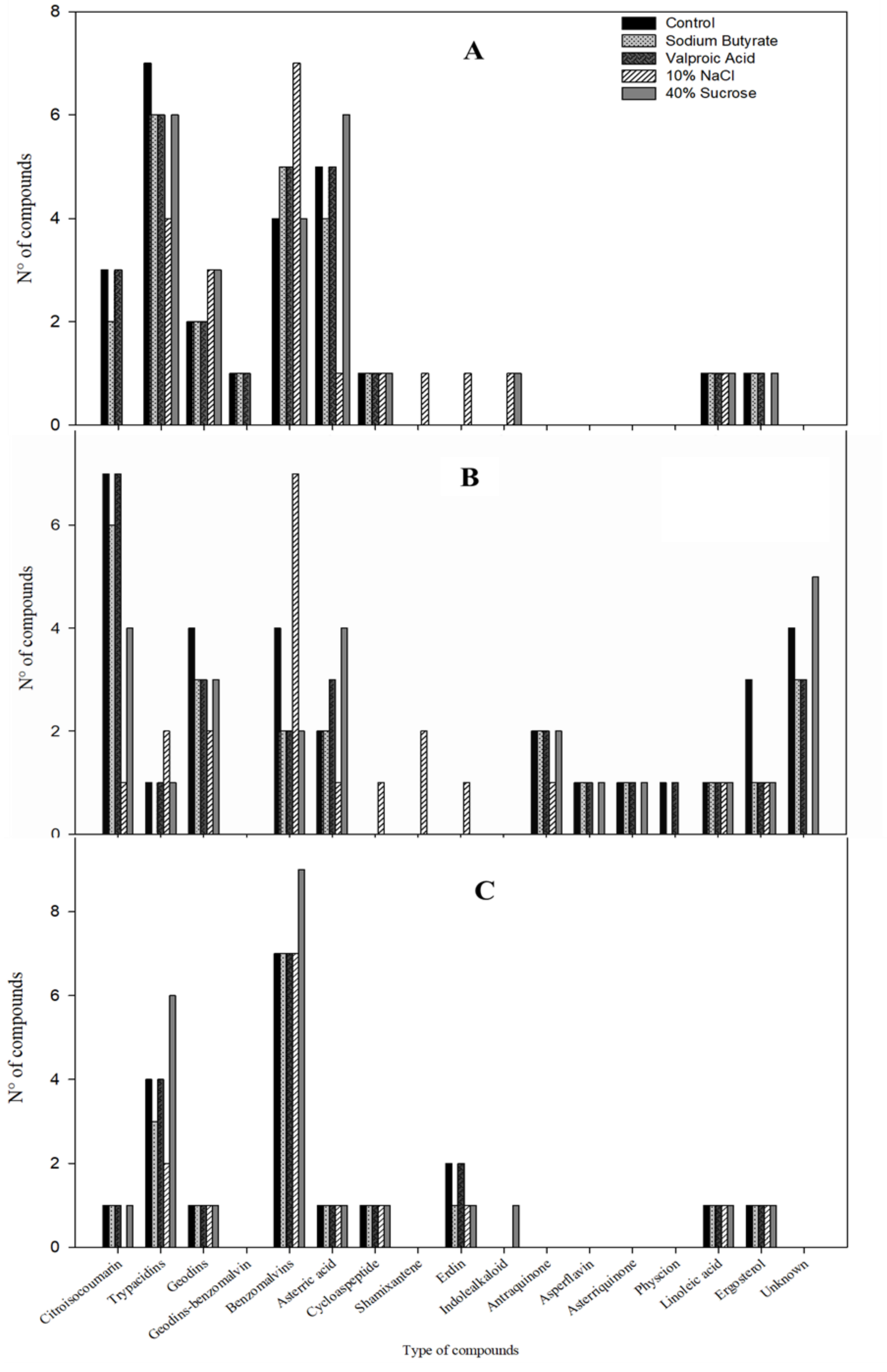


**Figure 4.** Influence of different cultivation condition on the number/type of compounds produced by *A. olivimuriae* using three different media, MEA (**A**), YES (**B**) and CYA (**C**) with addition of two HDAC inhibitors, Sodium butyrate and Valproic acid (250 µM), 10% of Sodium chloride and 40% of Sucrose.

### 4.4.4. Statistical analysis

In order to better understand the influence of cultural condition on production of compounds, multivariate treatment of data based on principal component analysis was performed. The analysis highlighted a strong correlation between media used and variability of compounds produced (33%) (Figure 5).

The presence of salt in the growth medium influences the production of compounds: the extracts from the media with addition of NaCl show a different profile from the others of the same media. In contrast to salt, HDAC inhibitors do not show much deviation from controlas. They don’t seem to have such interesting effects.

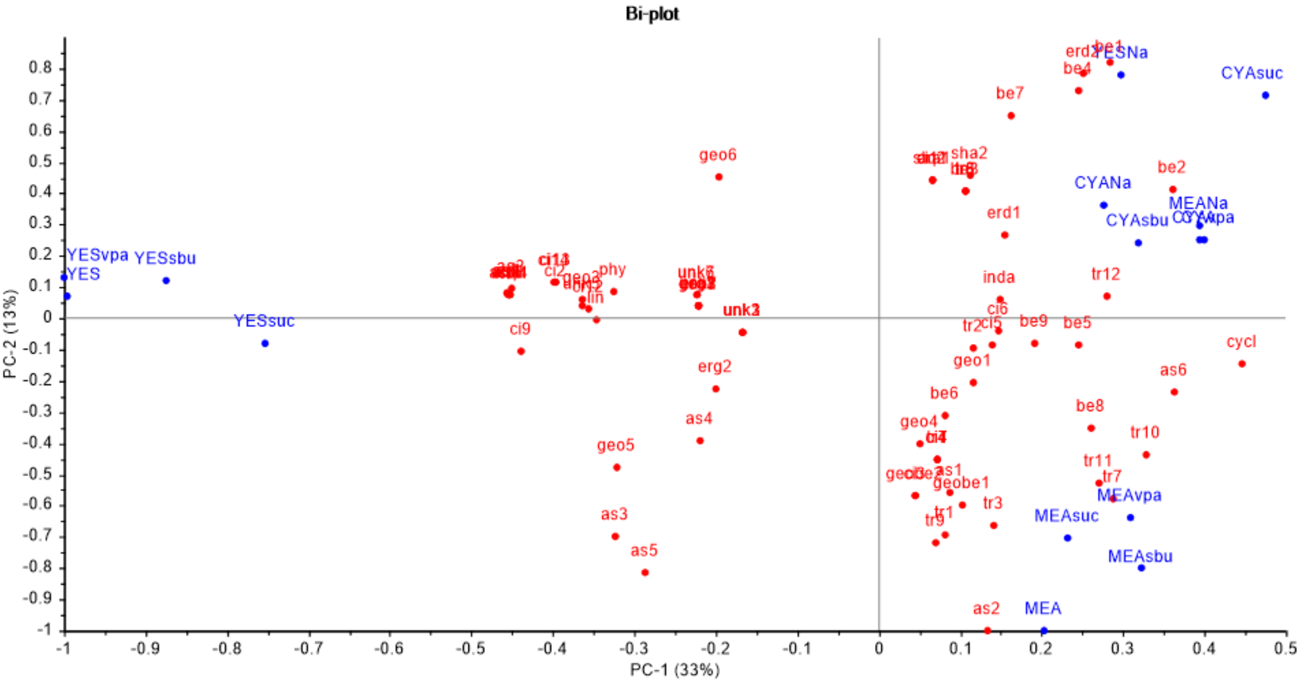


**Figure 5.** Principal component analysis biplot of 15 cultivation conditions (MEA, YES and CYA with addition of two HDAC inhibitors, Sodium butyrate and Valproic acid (250 µM), 10% of Sodium chloride and 40% of Sucrose) and 73 secondary metabolites of *A. olivimuriae.*

## 4.5. DISCUSSION

In this work the production of secondary metabolites of *Aspergillus olivimuriae* NRRL 66783 was investigated. The information regarding the chemical diversity of secondary metabolites produced by this species is still unknown. Two different study approaches allowed to know both the biosynthetic potential and the real ability to produce secondary metabolites. The bottom-up approach made it possible to carry out a genomic-scale study regarding the prediction of metabolites production capacity, thanks to the availability of the whole sequenced genome and of bioinformatic computational tools. AntiSMASH and SMURF are widely used to compare the productive capacities of secondary metabolites of closely related species (Kjærbølling et al., 2017; Pi et al., 2015; Moore et al., 2015), to characterize the genetic basis of SM biosynthesis (Pi et al., 2015) and to study the total biosynthetic capacity of secondary metabolites by detecting the presence of potentially productive silent genes.

The antiSMASH analysis has identified several clusters in A. *olivimriae* that show similarity with already known. Among these, cluster 18 showed very high similarity percentages with fujikuroi cluster, identified in *Fusarium fujikuroi* (Wiemannet et al., 2013), a pathogenic fungus that causes "bakanae" desease in rice, and its products (fujikurins A-D) described by Von Berger and colleagues (2014). Another cluster identified in several fungal genera, such as *Chaetomium, Aspergillus, Zygosporium, Penicillium, Metarhizium* and *Rosellinia* (Guo et al., 2019) is that associated with the production of chaetoglobosin, belonging to the cytochalasin family (Schumann and Hertweck, 2007) a class of products characterized by different biological activities (Scherlach et al., 2010); an example is chaetoglobosin A which is known for its strong cytotoxicity in tumor cells (Takanezawa et al., 2017).

Among the known clusters originally identified in *Aspergillus* species, one associated with the production of aflatrem, a potent mammalian termorgenic toxin belonging to the indole-diterpene class, is observed; it was identified in *A. flavus and A. oryzae* (Gallagher and Wilson, 1980; Nicholson et al., 2009). The trypacidin biosynthetic cluster originally identified in *Aspergillus fumigatus* Af293 (Mattern et al., 2015) shows 21% similarity with cluster 23 of *A. olivimuriae*. Its product is the metabolite trypacidin (Namec et al., 1963) which has been shown to be a potent toxin to lung cells (Gauthier et al., 2012). Other products from *Aspergillus* species are associated with some genes of this cluster such as monodictyphenone (GENE ID: AN0150/mdpG) (Chiang et al., 2010) and asperthecin (Gene ID: AN6000/aptA) (Szewczyk et al., 2008) biosynthesis in *A. nidulans*, endocrocin (GENE ID: Afu4g00210/encA) (Lim et al., 2012) and fumicycline (GENE ID: Afu7g00160/fccA) (König et al., 2013; Chooi et al., 2013) biosynthesis in *A. fumigatus*, and geodin (GENE ID: ATEG_08451/GedC) (Nielsen et al., 2013) from *A. terreus.*

Given the productive potential of secondary metabolites predicted in the analysis with bioinformatics tools, we wanted to investigate the real production capacities under different cultivation conditions. For this purpose, the top-down approach allowed to know the real production of secondary compounds by *A. olivimuriae*.

All extrolite biosynthetic families identified by HPLC-UV-DAD analysis, are recognized to be normally produced within *Aspergillus* genus (Frisvad and Larsen, 2015). However, production differences can be observed with species belonging section Flavipedes to which *A. olivimuriae* belongs: for example, the production of Citreoisocoumarins and Benzomalvins are interesting because usually they are not produced from Flavipedes species; furthermore, benzomalvins production is highly observed in *Penicillium* spp where was originally isolated (Sun et al., 1994). Among the recognized compounds also Geodin is usually produced by *Aspergillus* species belonging to different sections such as *A. fumigatus* and *A. terreus* (Nielsen et al., 2013) but also from species belonging to the closely related flavipedes section (Houbraken et al. 2020). Production of asterric acid was observed in *A. terreus* ATCC 20542 (Boruta and Bizukojc, 2016) and in *A. iizukae* and *A. capiensis,* two species close to *A. olivimuriae*.

The submission of *Aspergillus olivimuriae* to different culture methods allowed to detect families of compounds that were not expressed under growth conditions on standard culture media. In particular, the addition of 10% of NaCl to the growing medium represented the stress that most influenced the metabolome of the fungus.

Extreme fungi able to survive in hypersaline environments are of particular interest for the study of secondary compounds as they are able to implement unique metabolic mechanisms (Peng et al., 2011). Several studies conducted using salt as inducer in the culture medium, have made it possible to isolate and identify new secondary compounds such as indole alkaloids from *Penicillium janthinellum* (Smetanina et al., 2007), polyketides from *Penicillium citrinum* SCSGAF 0167 (Sun et al., 2014) and cyclopentanopyridine alkaloid from *Wallemia sebi* PXP-89 (Peng et al., 2011). On the other hand, however, comparisons between halophilic and halotolerant species of *Penicillium* and *Aspergillus* with less halotolerant species, they have shown how the number of compounds produced decreases in environments with NaCl concentrations higher than 5% (Frisvad, 2005).

Contrary to the effect induced by salt addition, the use of HDAC inhibitors as inducers for the production of secondary compounds did not evidently modify the secondary metabolic profile of *A. olivimuriae*. Other studies instead, have highlighted how the use of HDAC inhibitors upregulated the expression of all the genes involved in the biosynthesis of fumiquinazoline C in *Aspergillus fumigatus* (Magotra et al., 2017) and led to the isolation of new compounds from *Cladosporium cladosporioides* (Williams et al., 2008).

In conclusion, the present study preliminarily investigated the productive capacities of secondary metabolites of *Aspergillus olivimuriae*. The inductive effect for the production of exometabolites due to the addition of salt, enzyme inhibitors and sucrose was studied. It has been observed that the growth of the fungus on salt is the condition that most modifies the metabolic profile of *A. olivimuriae*, clearly decreasing the production of compounds. Furthermore, this study, thanks to a genomic-scale approach, has highlighted the productive potential of secondary compounds through the study of biosynthetic clusters. Bioinformatics analysis revealed biosynthetic clusters of secondary metabolites known to be associated with toxins, antitumor compounds and antimicrobials.

## REFERENCES

Boruta T, Bizukojc M. (2016) Induction of secondary metabolism of Aspergillus terreus ATCC 20542 in the batch bioreactor cultures. *Appl Microbiol Biotechnol.* **100(7)**: 3009-22.

Boruta T, Milczarek I & Bizukojc M (2019) Evaluating the outcomes of submerged co-cultivation: production of lovastatin and other secondary metabolites by *Aspergillus terreus* in fungal co-cultures. *Appl Microbiol Biotechnol* **103**: 5593–5605.

Chiang YM, Szewczyk E, Davidson AD, Entwistle R, Keller NP, Wang CC, Oakley BR (2010) Characterization of the Aspergillus nidulans monodictyphenone gene cluster. *Appl Environ Microbiol* **76(7)**: 2067–2074

Chooi YH, Fang J, Liu H, Filler SG, Wang P, Tang Y (2013) Genome mining of a prenylated and immunosuppressive polyketide from pathogenic fungi. *Org Lett* **15(4)**: 780–783

Clevenger KD, Ye R, Bok JW, Thomas PM, Islam MN, Miley GP, Robey MT, Chen C, Yang K, Swyers M, Wu E, Gao P, Wu CC, Keller NP, Kelleher NL. (2018) Interrogation of Benzomalvin Biosynthesis Using Fungal Artificial Chromosomes with Metabolomic Scoring (FAC-MS): Discovery of a Benzodiazepine Synthase Activity. *Biochemistry.* **57(23)**: 3237-3243.

Crognale S, Pesciaroli L, Felli M, Petruccioli M, D'Annibale A, Bresciani A and Peterson SW (2019) *Aspergillus olivimuriae* sp. nov., a halotolerant species isolated from olive brine. *International journal of systematic and evolutionary microbiology* **69(9)**: 2899-2906

Crognale S, Pesciaroli L, Petruccioli M, D'Annibale A. (2012) Phenoloxidase-producing halotolerant fungi from olive brine wastewater. *Process Biochem* **47**: 1433–1437.

Frisvad J (2005) Halotolerant and halophilic fungi and their extrolite production. In: Gunde-Cimerman N, Oren A, PlemenitasÏ A, editors. Adaptation to life at high salt concentrations in archaea, bacteria, and eukarya. Cellular origin, life in extreme habitats and astrobiology Dordrecht, The Netherlands: Springer;. p. 425±39.

Frisvad JC (2012) Media and growth conditions for induction of secondary metabolite production. *Methods Mol Biol.* **944**: 47-58.

Frisvad JC, Hubka V, Ezekiel CN, Hong SB, Nováková A, Chen AJ, Arzanlou M, Larsen TO, Sklenář F, Mahakarnchanakul W, Samson RA, Houbraken J. (2019) Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Stud Mycol.* **93**: 1-63.

Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabañes FJ, Ehrlich K, Samson RA. (2007) Secondary metabolite profiling, growth profiles and other tools for species recognition and important Aspergillus mycotoxins. *Stud Mycol.* **59**: 31-7.

Frisvad JC, Larsen TO. (2015) Chemodiversity in the genus Aspergillus. *Appl Microbiol Biotechnol.* **99(19)**: 7859-77.

Frisvad JC, Thrane U (1993) Liquid column chromatography of mycotoxins. In: Betina V (ed) Chromatography of mycotoxins: techniques and applications. Elsevier, Amsterdam, pp 253–372

Frisvad JC. (2015) Taxonomy, chemodiversity, and chemoconsistency of Aspergillus, Penicillium, and Talaromyces species. *Front Microbiol.* **5**: 773.

Gallagher RT, Clardy J and Wilson BJ (1980) Aflatrem, a tremorgenic toxin from *Aspergillus flavus*. *Tetrahedron Lett.* **21**: 239–242.

Gauthier T, Wang X, Sifuentes Dos Santos J, Fysikopoulos A, Tadrist S, Canlet C, Artigot MP, Loiseau N, Oswald IP, Puel O (2012) Trypacidin, a spore-borne toxin from Aspergillus fumigatus, is cytotoxic to lung cells. *PLoS One*. **7(2)**: e29906.

Guo QF, Yin ZH, Zhang JJ, Kang WY, Wang XW, Ding G, Chen L (2019) Chaetomadrasins A and B, two new cytotoxic cytochalasans from desert soilderived fungus Chaetomium madrasense 375. *Molecules* **24**: 3240.

Hansen ME, Smedsgaard J and Larsen TO (2005) X-Hitting: an algorithm for novelty detection and dereplication by UV spectra of complex mixtures of natural products. *Anal. Chem.* **77**: 6805–6817.

Houbraken J, Kocsube S, Visagie CM, Yilmaz N, Wang XC, Meijer M, Kraak B, Hubka V, Bensch K, Samson RA, Frisvad JC (2020) Classification of Aspergillus, Penicillium, Talaromyces and related genera (Eurotiales): An overview of families, genera, subgenera, sections, series and species *Studies in Mycology* **95**: 5-169.

Hubka V, Nováková A, Kolařík M, Jurjević Ž, Peterson SW (2015) Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect. nov. *Mycologia* **107**: 169–208.

Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH, Fedorova ND. (2010) SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet Biol* **47(9)**: 736-41.

Kjærbølling I, Vesth TC, Frisvad JC, Nybo JL, Theobald S, Kuo A, Bowyer P, Matsuda Y, Mondo S, Lyhne EK, Kogle ME, Clum A, Lipzen A et al. (2018) Linking secondary metabolites to gene clusters through genome sequencing of six diverse *Aspergillus* species. *Proc Natl Acad Sci U S A.* **115(4)**: E753-E761.

König CC, Scherlach K, Schroeckh V, Horn F, Nietzsche S, Brakhage AA, Hertweck C (2013) Bacterium induces cryptic meroterpenoid pathway in the pathogenic fungus Aspergillus fumigatus. *Chem biochem* **14(8)**: 938–942

Lee YM, Kim MJ, Li H, Zhang P, Bao B, Lee KJ, Jung JH. (2013) Marine-derived Aspergillus species as a source of bioactive secondary metabolites. *Mar Biotechnol (NY).* **15(5)**: 499-519.

Lim FY, Hou Y, Chen Y, Oh JH, Lee I, Bugni TS, Keller NP (2012) Genome based cluster deletion reveals an endocrocin biosynthetic pathway in Aspergillus fumigatus. *Appl Environ Microbiol* **78(12)**: 4117–4125

Magotra A, Kumar M, Kushwaha M, Awasthi P, Raina C, Gupta AP, Shah BA, Gandhi SG, Chaubey A. (2017) Epigenetic modifier induced enhancement of fumiquinazoline C production in Aspergillus fumigatus (GA-L7): an endophytic fungus from Grewia asiatica L. *AMB Express*. 7(1): 43.

Mattern DJ, Schoeler H, Weber J, Novohradská S, Kraibooj K, Dahse HM, Hillmann F, Valiante V, Figge MT, Brakhage AA. (2015) Identification of the antiphagocytic trypacidin gene cluster in the human-pathogenic fungus Aspergillus fumigatus. *Appl Microbiol Biotechnol.* **99(23)**: 10151-61.

Medema M, Kottmann R, Yilmaz P et al. (2015) Minimum Information about a Biosynthetic Gene cluster. *Nat Chem Biol* **11**: 625–631

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res*. **39**: W339-46.

Medema MH, Fischbach MA. (2015) Computational approaches to natural product discovery. *Nat Chem Biol* **11(9)**: 639-48.

Moore GG, Mack BM & Beltz SB. (2015) Genomic sequence of the aflatoxigenic filamentous fungus *Aspergillus nomius . BMC Genomics* **16**: 551

Nicholson MJ, Koulman A, Monahan BJ, Pritchard BL, Payne GA, Scott B. (2009) Identification of two aflatrem biosynthesis gene loci in Aspergillus flavus and metabolic engineering of Penicillium paxilli to elucidate their function. *Appl Environ Microbiol.* **75(23)**: 7469-81.

Nielsen KF, Månsson M, Rank C, Frisvad JC, Larsen TO (2011) Dereplication of microbial natural products by LC-DAD-TOFMS. *J Nat Prod* **74(11)**: 2338–2348

Nielsen MT, Nielsen JB, Anyaogu DC, Holm DK, Nielsen KF, Larsen TO, Mortensen UH (2013) Heterologous reconstitution of the intact geodin gene cluster in Aspergillus nidulans through a simple and versatile PCR based approach. *PLoS One* **8(8)**: e72871

Overy D, Correa H, Roullier C, Chi WC, Pang KL, Rateb M, Ebel R, Shang Z, Capon R, Bills G, Kerr R (2017) Does Osmotic Stress Affect Natural Product Expression in Fungi? *Mar Drugs*. **15**(8): 254.

Pan Y Zheng L, Mao Y, Wang J, Lin LS, Su YQ, Li Y (2019) The antibacterial activity and mechanism analysis of piscidin 5 like from Larimichthys crocea, *Developmental & Comparative Immunology* **92**: 43-49

Peng XP, Wang Y, Liu PP, Hong K, Chen H, Yin X, et al. (2011) Aromatic compounds from the halotolerant fungal strain of *Wallemia sebi* PXP-89 in a hypersaline medium. *Arch Pharm Res* **34(6)**: 907±12.

Pi B, Yu D, Dai F, Song X, Zhu C, Li H, et al. (2015) A Genomics Based Discovery of Secondary Metabolite Biosynthetic Gene Clusters in Aspergillus ustus. *PLoS ONE* **10(2)**: e0116089

Rinderknecht H, Ward JL, Bergel F, Morrison AL (1947) Studies on antibiotics: 2. Bacteriological activity and possible mode of action of certain non-nitrogenous natural and synthetic antibiotics. *Biochem J* **41**: 463–469

Samson RA, Yilmaz N, Houbraken J, Spierenburg H, Seifert KA, Peterson SW, Varga J, Frisvad JC (2011) Phylogeny and nomenclature of the genus Talaromyces and taxa accommodated in Penicillium subgenus Biverticillium. *Studies in Mycology* **70**: 159-183

Scherlach K, Boettger D, Remme N, Hertweck C, (2010) The chemistry and biology of cytochalasans. *Nat. Prod. Rep*. **27**: 869e886.

Scherlach, K., Boettger, D., Remme, N., Hertweck, C., 2010. The chemistry and biology of cytochalasans. *Nat. Prod. Rep*. **27**: 869e886.

Schueffler A, Anke T. (2014) Fungal natural products in research and development. *Nat Prod Rep.* **31(10)**: 1425-48.

Schumann J and Hertweck C (2007) Molecular basis of cytochalasan biosynthesis in fungi: gene cluster analysis and evidence for the involvement of a PKS-NRPS hybrid synthase by RNA silencing. *J. Am. Chem. Soc*. **129**: 9564-9565.

Sepcic K, Zalar P, Gunde-Cimerman N (2011) Low water activity induces the production of bioactive metabolites in halophilic and halotolerant fungi. *Mar Drugs* **9(1)**: 43-58.

Sharma V, Singamaneni V, Sharma N, Kumar A, Arora A, Kushwaha M, Bhushan S, Jaglan S, Gupta P (2018) Valproic acid induces three novel cytotoxic secondary metabolites in Diaporthe sp., an endophytic fungus from Datura inoxia Mill. *Bioorg Med Chem Lett* **28(12)**: 2217-2221.

Shi-Kunne X, Jové RP, Depotter JRL, Ebert MK, Seidl MF, Thomma BPHJ (2019) *In silico* prediction and characterisation of secondary metabolite clusters in the plant pathogenic fungus *Verticillium dahliae*, *FEMS Microbiology Letters* **366(7)**.

Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S and Keller NP (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryot.Cell* **6**: 1656–1664.

Smedsgaard J (1997) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J Chromatogr A*. **760(2)**: 264-70.

Smetanina OF, Kalinovsky AI, Khudyakova YV, Pivkin MV, Dmitrenok PS, Fedorov SN, Ji H, Kwak J-Y, Kuznetsova TA (2007) Indole Alkaloids Produced by a Marine Fungus Isolate of *Penicillium janthinellum* Biourge. *J. Nat. Prod.* **70**: 906–909

Sørensen JL, Nielsen KF & Sondergaard T E (2012). Redirection of pigment biosynthesis to isocoumarins in Fusarium. *Fungal Genetics and Biology*, **49(8)**: 613-618.

Stanke M, Morgenstern B (2005) AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. *Nucleic Acids Res* **33**: W465–W467.

Stanke M, Schöffmann O, Morgenstern B, Waack S. (2006) Gene prediction in eukaryotes with a generalized hidden Markov model that uses hints from external sources. *BMC Bioinformatics* **7**: 62.

Stanke M, Steinkamp R, Waack S, Morgenstern B. (2004) AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Res* **32**: W309–W312.

Sun YL, Zhang XY, Zheng ZH, Xu XY, Qi SH (2014) Three new polyketides from marine-derived fungus Penicillium citrinum SCSGAF 0167. *Nat Prod Res* **28**: 239–244.

Szewczyk E, Chiang YM, Oakley CE, Davidson AD, Wang CCC, Oakley BR (2008) Identification and characterization of the asperthecin gene cluster of Aspergillus nidulans. *Appl Environ Microbiol* **74(24)**: 7607–7612

Takanezawa Y, Nakamura R, Sone Y, Uraguchi S, Kobayashi K, Tomoda H (2017) Variation in the activity of distinct cytochalasins as autophagy inhibitiors in human lung A549 cells. Biochem. Biophys. *Res. Co.* **494**: 641e647.

Wang Y, Coleman-Derr D, Chen G, Gu YQ. (2015) OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res* **43**: W78–W84.

Wang Y, Lu Z, Sun K and Zhu W (2011b) Effects of high salt stress on secondary metabolite production in the marine-derived fungus *Spicaria elegans*. *Mar. Drugs* **9**: 535–542.

Wang Y, Zheng J, Liu P, Wang W, Zhu W (2011a) Three new compounds from Aspergillus terreus PT06–2 grown in a high salt medium. *Mar Drugs* **9**: 1368–1378.

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. (2015) antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res* 43(W1): W237-43.

Wiemann P, Keller NP (2014) Strategies for mining fungal natural products. *J Ind Microbiol Biotechnol* **41**(2): 301-13.

Wiemann P, Sieber CM, von Bargen KW, Studt L, Niehaus EM, Espino JJ, Huß K, Michielse CB, Albermann S, Wagner D, Bergner SV, Connolly LR, Fischer A et al (2013) Deciphering the cryptic genome: genome-wide analyses of the rice pathogen Fusarium fujikuroi reveal complex regulation of secondary metabolism and novel metabolites. *PLoS Pathog.* **9(6)**: e1003475.

Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH (2008) Epigenetic remodeling of the fungal secondary metabolome. *Org Biomol Chem* **6(11)**: 1895–1897

Yu JH, Keller N (2005) Regulation of secondary metabolism in filamentous fungi. *Annu Rev Phytopathol*. **43**: 437-58.

# CHAPTER 5

# DISCUSSION

The search for new compounds characterized by biological activity for application in the pharmaceutical and biotechnological fields is increasingly of interest (Newman and Cragg, 2016), moreover considering the expanding resistance to the antibiotics, it is necessary to identify new compounds of natural origin. Secondary microbial metabolites, in particular from fungi, are good candidates for this purpose, especially for those microorganisms inhabiting extreme environments. The particular living conditions of these environments push them to synthesize substances useful for their survival (Gunde-Cimerman and Zalar, 2014). Several studies conducted on fungi isolated from extreme environments have shown to develop metabolic pathways for the synthesis of specialized molecules characterized by biological activity (Rateb, 2018). Moreover, extreme environments are also a source of unknown microorganisms, which may represent an unexplored reservoir new bioactive compounds (Chavez et al., 2015).

Cultivation under standard conditions does not always allow the discovery of new compounds or in any case limits the expression of their entire biosynthetic potential. For this reason, two approaches can be used to maximize microbial secondary metabolites discovery: top-down and bottom-up (Luo et al., 2011).

Top-down approaches begin at the organism level, utilizing systems-level perturbations to elicit production of new natural products without prior knowledge of the genes and enzymes involved in their biosynthesis. One of the most applied top-down approaches is OSMAC (One Strain Many Compounds), in which fungal species are subjected to variation of medium, changing cultivation condition, co-cultivation with other strain(s), adding epigenetic modifier(s) or biosynthetic precursor(s) (Bode et al., 2002). Application of the OSMAC approach can induce and maximize the production of these molecules going to stimulate and activate large portion of microbial biosynthetic gene clusters silenced under standard cultivation conditions (Bode et al., 2002).

According to OSMAC approach, halotolerant fungal strains, isolated from different terrestrial sources such as contaminated soils, plant biomass and hypersaline waste but identified in the literature as “facultative marine derived fungi”, were subjected to increasing osmotic stress using five NaCl levels added to the growing medium (0%, 3%, 6%, 10% and 15% NaCl) and were investigated for bioactive potential. It is known how salinity governs physical characteristics like the osmotic pressure and enzymes involved in microbial growth and metabolism (Blunt et al., 2015).

The results obtained from biological activity analysis have shown how different species belonging to genera *Penicillium*, *Aspergillus*, *Alternaria*, *Arthrinium*, *Trichoderma* and *Chaetomium* were able to produce extracts with bioactive characteristics. On the other hand, the bioactivity of these extracts did not seem to be influenced by the presence of salt in the culture medium which did not show an induction effect common to all species except for the strain *Aspergillus* 6C2. This strain stood out for its strong antioxidant, antimicrobial against gram+ bacteria and anti-quorum sensing properties.

In *Aspergillus* 6C2 the production of compounds belonging to the Shamixantene family is induced only by the presence of salt in the culture medium. Studies conducted on *A. terreus* PT06-2 show how the growth of the fungus under 10% salinity conditions affects the quantity and profile of secondary metabolites allowing the isolation of three new compounds (Wang et al., 2011b). As opposed to studies in which secondary metabolites increased as a response to NaCl concentration, suggesting a role in the adaptation to hypersaline conditions (Jančič et al., 2016; Huang et al., 2011), the halotolerant species *Aspergillus* 6C2 decreases the production of metabolites in 10% NaCl growth condition. This response was highlighted by comparisons between halophilic and halotolerant species of *Penicillium* and *Aspergillus* with less halotolerant species.  It showed how the number of compounds produced decreases in environments with NaCl concentrations higher than 5% (Frisvad, 2005).

Considering the promising results obtained from the study of biological activities, it was interesting to assign the *Aspergillus* 6C2 strain taxonomically. Many studies conducted on *Aspergillus* spp. have highlighted the ability of this genus to produce metabolites and extracts with biological activity. *Aspergillus niger* is able to produce malformin C, a bicyclic pentapeptide used as anti-cancer drug (Wang et al., 2015) and characterized by antibacterial properties, particularly against *Bacillus* species (Kobbe et al., 1977). *Aspergillus terreus* is a producer of lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase used as a blood cholesterol-lowering drug (Alberts et al., 1980) and in the treatment of cancer, Alzheimer’s disease and Parkinson’s disease (Zhang et al., 2019; Eskandary et al., 2018; Lin et al., 2016). *Aspergillus terreus* produces terrain able to block gene expression for biofilm formation and virulence in *P. aeruginosa* (Kim et al., 2018). *Aspergillus* species are also of particular interest as producers of important mycotoxins (Frisvad et al., 2019) such as aflatoxins, 3-nitropropionic acid, tenuazonic acid and cyclopiazonic acid (Varga et al., 2011).

Thanks to molecular identification through sequencing the identification markers rDNA internal transcribed spacer region (ITS1-5.8S-ITS2), calmodulin (*CaM*), β-tubulin (*BenA*) and second largest subunit of RNA polymerase II (RPB2), *Aspergillus* 6C2 strain, named *Aspergillus olivimuriae*, turned out to be a new species (Crognale et al., 2019) and therefore its properties make it a potential source of new bioactive secondary metabolites.

Using some OSMAC approaches on *A. olivimuriae* allowed to preliminary investigate its production capacity of secondary metabolites. It is well known that media composition can have a great impact on the production of microbial products (Pan et al., 2019).

The cultivation of *A. olivimuriae* on different culture media for the exploration of chemical diversity (Frisvad, 2012) led to the production of a series of exometabolites that are ordered into different biosynthetic families (Ma et al., 2009). *A. olivimuriae* shows to product primarily citreoisocoumarins, trypacidins, geodins, benzomalvins, and asterric acid but also cycloaspeptide, erdin, antraquinone, asperflavin, asterriquinone, physcion, linoleic acid, ergosterol and unknown compounds recognized to be normally produced within *Aspergillus* genus (Frisvad and Larsen, 2015). However, production differences can be observed with species belonging section *Flavipedes* to which *A. olivimuriae* belongs: for example, Citreoisocoumarins and Benzomalvins production usually are not produced from *Flavipedes* species. It is known as the type of carbon and nitrogen sources used affect microbial secondary metabolism (Ruiz et al., 2009; Singh et al., 2017). In *Aspergillus niger* a low production of secondary metabolites is linked to the presence of lactate or starch as the only source of carbon; on the contrary, the combination of the two induced the production of much greater quantities of fumonisins, ochratoxins, kotanins and naphtho-pyrones (Sørensen et al., 2009). Overy and colleagues (2005) demonstrated how production of novel naphthalopyran compound in *Penicillium hordei* was detected when it grown on plant tissue agar such as macerated tulip and yellow onion, oatmeal and red onion, while no production was observed when using defined laboratory media for fungal culture suche as MEA, YES, and CYA. In *A. olivimuriae* yeast extract sucrose (YES) agar was very efficient for production of different exometabolites increasing the number of compounds expressed and stimulating the production of metabolites not expressed in the other used media such as antraquinone, asperflavin, asterriquinone and physcion and unknown compounds.

Another OSMAC approach involves epigenetic modifiers which bring changes in gene expression without any alteration in DNA sequence of fungi (Xiao et al. 2013). Tickling fungal genome using epigenetic modifiers can perturb the production of metabolites leading to induction of silent biosynthetic pathways expression that were not produced under normal growth conditions. Recent studies in several species of fungi have identified regulation of chromatin-based secondary metabolism gene clusters. Activation of these clusters has been demonstrated to be associated with greater acetylation of histones H3 and H4 and, consequently, inhibition of histone de-acetylase (HDAC) activity also leads to a greater production of secondary metabolites (Strauss and Reyes -Dominguez 2011). The inductive potential of histone deacetylase (HDAC) inhibitors has been highlighted (Shwab et al., 2007), which stimulate, for example, the production of new compounds, xylarolide A, diportharine A and xylarolide B in *Diaporthe* sp, (Sharma et al., 2018) but also of "cryptic" antimicrobial compound in *Doratomyces microspores* (Zutz et al., 2016). Contrary to the effect induced by salt addition, the use of HDAC inhibitors as inducers for the production of secondary compounds did not evidently modify secondary metabolic profile of *A. olivimuriae*.

Bottom-up approaches is on genomic-scale and allow to know the productive potential of secondary metabolites of a species regardless of the real expression, first identifying secondary metabolites gene clusters and then utilizing various gene manipulation techniques to drive transcription, translation, and eventual synthesis of the corresponding natural product (Luo et al. 2014). In the last decade, alongside the OSMAC approaches many studies have focused precisely on the research and investigation of secondary metabolites biosynthetic gene clusters thanks also to the availability of whole sequenced genomes. In this context, the development of algorithms able to recognize the conserved genes of the clusters (Medema et al., 2011; Khaldi et al., 2010) has shown that the production capacity of secondary metabolites is much greater than that expressed (Romano et al., 2018) confirming the presence of cryptic (silent) genes that are not always produced.

The use of this genomic approach in study of *A. olivimuriae* allowed to identify putative biosynthetic clusters of secondary metabolites in order to know their genetic basis. Bioinformatic analysis has identified several clusters in *A. olivimriae* that show similarity with already known. Clusters potentially associated with compounds characterized by cytotoxic activity, such as ketoglobosin belonging to the cytochalasin family (Takanezawa et al., 2017) and anti-inflammatory and antitumor activities such as endocrocin (Hirayama et al., 1980; Gautam et al., 2010) are identified. Clusters potentially associated with toxins have also been detected such as aflatrem, originally identified in *Aspergillus* species, and trypacidin, a potent toxin to lung cells (Gauthier et al., 2012) but also other compounds such as asperthecin (Szewczyk et al., 2008), fumicycline (König et al., 2013; Chooi et al., 2013) and geodin (Nielsen et al., 2013). Based on this genetic information, further studies are necessary in order to associate the gene cluster with the metabolite. Genetic approaches of gene deletion, over-expression and heterologous expression, have allowed for example to identify the cluster responsible for the biosynthesis of cyclic tetrapeptide apicidin F (APF) in *F. fujikuroi* (Niehaus et al., 2014) and to describe all 13 genes of the geodin gene cluster of *A. terreus* (Nielsen et al., 2013).

This study could be useful for the public health as it identifies natural compounds to be used in pharmaceuticals to counteract the ever-increasing drugs resistance.

This work opens up future study perspectives related to the identification of secondary compounds produced by *A. olivimuriae* through extensive studies of mass that can also be applied to the analysis of unknown compounds produced by the species.

Furthermore, further studies related to gene clusters and their induction by different cultivation conditions can be deepened with the study of metabolic pathways that intervene in the regulation of silent gene clusters.

# References

Adnani N, Vazquez-Rivera E, Adibhatla SN, Ellis GA, Braun DR, Bugni TS (2015) Investigation of interspecies interactions within marine micromonosporaceae using an improved co-culture approach. *Mar Drugs* **13**: 6082–6098.

Alberts AW, Chen J, Kuron G (1980) Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proceedings of the National Academy of Sciences of the United States of America*. **77(7)**: 3957–3961.

Al-Fakih AA & Almaqtri W (2019) Overview on antibacterial metabolites from terrestrial *Aspergillus* spp. *Mycology*, **10**(4): 191–209.

Arora DS and Chandra P (2011) In vitro antioxidant potential of some soil fungi: screening of functional compounds and their purification from Penicillium citrinum. *Appl Biochem Biotechnol* **165(2)**: 639-51.

Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. (2007) Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat Chem Biol*. **3**: 213–217.

Bertrand S, Bohni N, Schnee S, Schumpp O, Gindro K and Wolfender J (2014) Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. *Biotechnol. Adv*. **32**: 1180–1204.

Blunt JW, Copp BR, Keyzers RA, Munro MHG and Prinsep MR (2015) Marine natural products. *Nat. Prod. Rep*. **32**: 116–211.

Bode HB, Bethe B, Höfs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. *Chembiochem* **3**(7): 619-27.

Boruta T, Milczarek I & Bizukojc M (2019) Evaluating the outcomes of submerged co-cultivation: production of lovastatin and other secondary metabolites by *Aspergillus terreus* in fungal co-cultures. *Appl Microbiol Biotechnol* **103**: 5593–5605.

Brakhage AA (2013) Regulation of fungal secondary metabolism. *Nat Rev Microbiol* **11**: 21-32

Bromann K, Toivari M, Viljanen K, Vuoristo A, Ruohonen L, Nakari-Setälä T and Henrique Goldman G (2012) Identification and characterization of a novel diterpene gene cluster in Aspergillus nidulans. *PLoS ONE*, **7**: e35450.

Cane DE, Walsh CT (1999) The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. *Chem Biol* **6**(12): R319-25.

Chávez R, Fierro F, García-Rico RO, Vaca I. (2015) Filamentous fungi from extreme environments as a promising source of novel bioactive secondary metabolites. *Front Microbiol*. **6**: 903.

Chiang YM, Oakley CE, Ahuja M, Entwistle R, Schultz A, Chang SL, Sung CT, Wang CC, Oakley BR (2013) An efficient system for heterologous expression of secondary metabolite genes in Aspergillus nidulans. *J Am Chem Soc* **135**: 7720-7731.

Chooi YH, Fang J, Liu H, Filler SG, Wang P, Tang Y (2013) Genome mining of a prenylated and immunosuppressive polyketide from pathogenic fungi. *Org Lett* **15(4)**: 780–783

Cimermancic P, Medema MH, Claesen J, Kurita K, Wieland Brown LC, Mavrommatis K, Pati A, Godfrey PA, Koehrsen M, Clardy J, Birren BW, Takano E, Sali A, Linington RG, Fischbach MA. (2014) Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters. *Cell* **158**(2): 412-421.

Cole PA (2008) Chemical probes for histone-modifying enzymes. *Nat Chem Biol* **4**: 590–597.

Corre C, Challis GL. (2009) New natural product biosynthetic chemistry discovered by genome mining. *Nat Prod Rep* **26**(8): 977-86.

Crognale S, Pesciaroli L, Felli M, Petruccioli M, D'Annibale A, Bresciani A and Peterson SW (2019) *Aspergillus olivimuriae* sp. nov., a halotolerant species isolated from olive brine. *International journal of systematic and evolutionary microbiology*, **69(9)**: 2899-2906.

Cueto M, Jensen PR, Kauffman C, Fenical W, Lobkovsky E and Clardy J (2001) Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. *J Nat Prod* **64**: 1444–1446.

Davie JR. (2003) Inhibition of histone deacetylase activity by butyrate. *J Nutr* **133**(7 Suppl): 2485S-2493S.

Debbab A, Aly AH, Lin WH, Proksch P (2010) Bioactive compounds from marine bacteria and fungi. *Microb Biotechnol* **3(5)**: 544-63.

Demain AL (1999) Pharmaceutically active secondary metabolites of microorganisms. *Appl Microbiol Biotechnol* **52(4)**: 455-63.

Demain AL (2014) Importance of microbial natural products and the need to revitalize their discovery. *J Ind Microbiol Biotechnol*. **41**(2): 185-201.

Demain AL and Fang A (2000) The Natural Functions of Secondary Metabolites. In Fiechter A (eds) History of Modern Biotechnology I. Advances in Biochemical Engineering/Biotechnology. *Springer, Berlin, Heidelberg* **69**: 1-39.

Eskandary A, Moazedi AA, Najaph zade varzi H and Akhond MR (2018) Combined Effects of Donepezil and Lovastatin on Cognition Deficit Induced by Bilateral Lesion of the *Nucl. Basalis Magnocellularis* in a Rat Model of Alzheimer's Disease. *Neurophysiology* **50**: 99–107

Franke J, Ishida K, Hertweck C (2012) Genomics-driven discovery of burkholderic acid, a noncanonical, cryptic polyketide from human pathogenic Burkholderia species. *Angew Chem Int Ed Engl* **51**: 11611-11615.

Frisvad JC (2005) Halotolerant and halophilic fungi and their extrolite production. In: Gunde-Cimerman N, Oren A, PlemenitasÏ A, editors. Adaptation to life at high salt concentrations in archaea, bacteria, and eukarya. Cellular origin, life in extreme habitats and astrobiology Dordrecht, The Netherlands: Springer;. p. 425±39.

Frisvad JC (2012) Media and growth conditions for induction of secondary metabolite production. *Methods Mol Biol.* **944**: 47-58.

Frisvad JC, Hubka V, Ezekiel CN, Hong SB, Nováková A, Chen AJ, Arzanlou M, Larsen TO, Sklenář F, Mahakarnchanakul W, Samson RA, Houbraken J. (2019) Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Stud Mycol.* **93**: 1-63

Frisvad JC, Larsen TO. (2015) Chemodiversity in the genus Aspergillus. *Appl Microbiol Biotechnol.* **99(19)**: 7859-77.

Frisvad JC, Rank C, Nielsen KF and Larsen TO (2009) Metabolomics of Aspergillus fumigatus. *Med Mycol* **47**: S53–S71.

Fujii I, Yoshida N, Shimomaki S, Oikawa H, Ebizuka Y (2005) An iterative type I polyketide synthase PKSN catalyzes synthesis of the decaketide alternapyrone with regio-specific octa-methylation. *Chem Biol* **12**(12): 1301-9.

Gal-Hemed I, Atanasova L, Komon-Zelazowska M, Druzhinina IS, Viterbo A, Yarden O (2011) Marine isolates of Trichoderma spp. as potential halotolerant agents of biological control for arid-zone agriculture. *Appl Environ Microbiol* **77**(15): 5100-9.

Gautam R, Karkhile KV, Bhutani KK, Jachak SM (2010) Anti-inflammatory, cyclooxygenase (COX)-2, COX-1 inhibitory, and free radical scavenging effects of *Rumex nepalensis*. *Planta Med.* **76**: 1564–1569.

Gauthier T, Wang X, Sifuentes Dos Santos J, Fysikopoulos A, Tadrist S, Canlet C, Artigot MP, Loiseau N, Oswald IP, Puel O (2012) Trypacidin, a spore-borne toxin from *Aspergillus fumigatus*, is cytotoxic to lung cells. *PLoS One*. **7(2)**: e29906.

Gizachew D, Chang C, Szonyi B, Torre SD & Ting WE (2019) Aflatoxin B1 (AFB1) production by Aspergillus flavus and Aspergillus parasiticus on ground Nyjer seeds: The effect of water activity and temperature. *Int J  Food Microbiol* **296**: 8-13 .

Gunde-Cimerman N and Zalar P (2014) Fungi in Solar Salterns. *Food Technol. Biotechnol*. **52**(2): 170–179

Hayes JJ and Hansen JC (2001) Nucleosome sand the chromatin fiber. *Curr Opin Genet Dev* **11**: 124–129.

Hertweck C, Luzhetskyy A, Rebets Y, Bechthold A. (2007) Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. *Nat Prod Rep.* **24**: 162–90.

Hirayama T, Fujikawa F, Kasahara T, Otsuka M, Nishida N, Mizuno D (1980) Anti-tumor activities of some lichen products and their degradation products. *Yakugaku Zasshi* **100**: 755–759.

Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat Prod Rep* **24**: 393–416

Huang J, Lu C, Qian X et al. (2011) Effect of salinity on the growth, biological activity and secondary metabolites of some marine fungi. *Acta Oceanol. Sin.* **30**: 118

Itoh T, Tokunaga K, Matsuda Y, Fujii I, Abe I, Ebizuka Y and Kushiro T (2010)  Reconstitution of a fungal meroterpenoid biosynthesis reveals the involvement of a novel family of terpene cyclases. *Nat Chem*, **2**: 858-864.

Jacobson ES (2000) Pathogenic roles for fungal melanins. *Clin. Microbiol. Rev.* **13**: 708–717

Jančič S, Frisvad JC, Kocev D, Gostinčar C, Džeroski S, Gunde-Cimerman N. (2016) Production of Secondary Metabolites in Extreme Environments: Food- and Airborne *Wallemia* spp. Produce Toxic Metabolites at Hypersaline Conditions. *PLoS One*. **11(12)**: e0169116.

Janevska S and Tudzynski B (2018) Secondary metabolism in *Fusarium fujikuroi*: strategies to unravel the function of biosynthetic pathways. *Appl Microbiol Biotechnol* **102**: 615-630.

Jin L, Quan C, Hou X, Fan S (2016) Potential Pharmacological Resources: Natural Bioactive Compounds from Marine-Derived Fungi. *Mar Drugs* **14**: 76

Jin Y, Weining S, Nevo E (2005) A MAPK gene from Dead Sea fungus confers stress tolerance to lithium salt and freezing–thawing: Prospects for saline agriculture. *Proc Natl Acad Sci USA*. **102**: 18992–18997.

Kaleem S, Ge H, Yi W, Zhang Z & Wu B (2019) Isolation, structural elucidation, and antimicrobial evaluation of the metabolites from a marine-derived fungus *Penicillium* sp. ZZ1283. *Nat Prod Res* **23**: 1-9

Keller NP (2019) Fungal secondary metabolism: regulation, function and drug discovery. *Nat Rev Microbiol* **17:** 167–180

Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism – from biochemistry to genomics. *Nat. Rev. Microbiol*. **3**: 937–947.

Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH, Fedorova ND. (2010) SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet Biol* **47**(9): 736-41.

Kim B, Park JS, Choi HY. et al. (2018) Terrein is an inhibitor of quorum sensing and c-di-GMP in *Pseudomonas aeruginosa*: a connection between quorum sensing and c-di-GMP. *Sci Rep* **8**: 8617.

Kim, B., Park, J-S, Choi, H-Y, Yoon, S. S. & Kim, W-G. Terrein is an inhibitor of quorum sensing and c-di-GMP in *Pseudomonas aeruginosa*: a connection between quorum sensing and c-di-GMP. *Sci. Rep.* **8**: 8617

Kobbe B, Cushman M, Wogan GN, et al. (1977) Production and antibacterial activity of malforming C, a toxic metabolite of *Aspergillus niger*. Appl. Environ. Microbiol. **33(4)**: 996-997.

König CC, Scherlach K, Schroeckh V, Horn F, Nietzsche S, Brakhage AA, Hertweck C (2013) Bacterium induces cryptic meroterpenoid pathway in the pathogenic fungus *Aspergillus fumigatus*. *Chem biochem* **14(8)**: 938–942

Lim FY, Won TH, Raffa N, Baccile JA, Wisecaver J, Rokas A, Schroeder FC, Keller NP (2018) Fungal isocyanide synthases and xanthocillin biosynthesis in *Aspergillus fumigatus*. *mBio* **9**: e00785–18

Lin CH, Lin HI, Chen ML, Lai TT, Cao LP, Farrer MJ, Wu RM, Chien CT (2016) Lovastatin protects neurite degeneration in LRRK2-G2019S parkinsonism through activating the Akt/Nrf pathway and inhibiting GSK3β activity. *Hum Mol Genet* **25**(10): 1965-1978.

Liu CC, Zhang ZZ, Feng YY, Gu QQ, Li DH & Zhu TJ (2019) Secondary metabolites from Antarctic marine-derived fungus *Penicillium crustosum* HDN153086. *Nat Prod Res* **3**: 414-419.

Ma L, Xing D, Wang H, Wang X, and Xue D (2009) Effect of culture conditions on cell growth and lipid accumulation of oleaginous microorganism. *Chin. J. Biotechnol.* **25**: 55–59.

Magotra A, Kumar M, Kushwaha M, Awasthi P, Raina C, Gupta AP, Shah BA, Gandhi SG, Chaubey A. (2017) Epigenetic modifier induced enhancement of fumiquinazoline C production in Aspergillus fumigatus (GA-L7): an endophytic fungus from Grewia asiatica L. *AMB Express*. **7**(1): 43.

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res*. **39**: W339-46.

Medema MH, Fischbach MA. (2015) Computational approaches to natural product discovery. *Nat Chem Biol* **11**(9): 639-48.

Miller BR, Gulick AM (2016) Structural Biology of Nonribosomal Peptide Synthetases. *Methods Mol Biol* **1401**: 3-29.

Miyanaga A (2017) Structure and function of polyketide biosynthetic enzymes: various strategies for production of structurally diverse polyketides. *Biosci, Biotechnol, and Biochem* **12**: 2227-2236.

Navarro-Muñoz JC and Collemare J (2020) Evolutionary Histories of Type III Polyketide Synthases in Fungi. *Front Microbiol* **10**: 3018.

Newman DJ, Cragg GM (2012) Natural Products as Sources of New Drugs over the 30 Years from 1981 to 2010. *J Nat Prod* **75**: 311-335.

Newman DJ, Cragg GM. (2016) Natural Products as Sources of New Drugs from 1981 to 2014. *J Nat Prod.* **79**(3): 629-61.

Nicoletti R & Trincone A (2016) Bioactive Compounds Produced by Strains of Penicillium and Talaromyces of Marine Origin. *Mar Drugs* **14**(2): 37

Niehaus EM, Janevska S, von Bargen KW, Sieber CM, Harrer H, Humpf HU, Tudzynski B. (2014) Apicidin F: characterization and genetic manipulation of a new secondary metabolite gene cluster in the rice pathogen Fusarium fujikuroi. *PLoS One*. **9(7)**: e103336.

Nielsen MT, Nielsen JB, Anyaogu DC, Holm DK, Nielsen KF, Larsen TO, Mortensen UH (2013) Heterologous reconstitution of the intact geodin gene cluster in *Aspergillus nidulans* through a simple and versatile PCR based approach. *PLoS One* **8(8)**: e72871

Overy D, Correa H, Roullier C, Chi WC, Pang KL, Rateb M, Ebel R, Shang Z, Capon R, Bills G, Kerr R (2017) Does Osmotic Stress Affect Natural Product Expression in Fungi? *Mar Drugs*. **15(8)**: 254.

Overy DP, Zidorn C, Petersen BO, Duus JØ, Dalsgaard PW, Larsen TO, et al. (2005). Medium dependant production of corymbiferone a novel product from *Penicillium hordei* cultured on plant tissue agar. *Tetrahedron Lett.* **46**: 3225–3228.

Pan Y Zheng L, Mao Y, Wang J, Lin LS, Su YQ, Li Y (2019) The antibacterial activity and mechanism analysis of piscidin 5 like from Larimichthys crocea, *Developmental & Comparative Immunology* **92**: 43-49

Peng XP, Wang Y, Liu PP, Hong K, Chen H, Yin X, Zhu WM (2011) Aromatic compounds from the halotolerant fungal strain of *Wallemia sebi* PXP-89 in a hypersaline medium. *Arch J Pharm Re.* **34**: 907–912.

Pettit RK (2011) Small-molecule elicitation of microbial secondary metabolites. *Microb Biotechnol* **4**: 471–478

Pitt JI and Hocking AD (1997) Fungi and Food Spoilage, 2nd ed, *Springer, Boston, MA.*

Rateb ME, Ebel R (2011) Secondary metabolites of fungi from marine habitats. *Nat Prod Rep* **28**: 290–344.

Rateb, M.E., Ebel, R. and Jaspars, M. (2018) Natural product diversity of actinobacteria in the Atacama Desert. *Antonie Van Leeuwenhoek* **111**: 1467–1477.

Romano S, Jackson SA, Patry S, Dobson ADW. (2018) Extending the "One Strain Many Compounds" (OSMAC) Principle to Marine Microorganisms. *Mar Drugs*. 23;16(7):244.

Ruiz B, Chavez A, Forero A, Garca-Huante Y, Romero A, Sanchez M, et al. (2009). Production of microbial secondary metabolites: regulation by the carbon source. *Crit. Rev. Miocrbiol.* **36**: 146–167.

Rutledge, P.J.; Challis, G.L. (2015) Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat Rev Microbiol* **13**: 509–523.

Sanchez JF, Entwistle R, Corcoran D, Oakley BR, Wang CC (2012) Identification and molecular genetic analysis of the cichorine gene cluster in Aspergillus nidulans. *Med Chem Comm* **3**: 997-1002.

Selbmann L, Egidi E, Isola D, Onofri S, Zucconi L, G. Sybren de Hoog, Chinaglia S, Testa L, Tosi S, Balestrazzi A, Lantieri A, Compagno R, Tigini V & Varese GC (2013) Biodiversity, evolution and adaptation of fungi in extreme environments, *Plant Biosystems – Int J Dealing with all Aspects of Plant Biol* 147:1, 237-246.

Sharma V, Singamaneni V, Sharma N, Kumar A, Arora A, Kushwaha M, Bhushan S, Jaglan S, Gupta P (2018) Valproic acid induces three novel cytotoxic secondary metabolites in Diaporthe sp., an endophytic fungus from Datura inoxia Mill. *Bioorg Med Chem Lett* **28**(12): 2217-2221.

Shi-Kunne X, Jové RP, Depotter JRL, Ebert MK, Seidl MF, Thomma BPHJ (2019) *In silico* prediction and characterisation of secondary metabolite clusters in the plant pathogenic fungus *Verticillium dahliae*, *FEMS Microbiology Letters* **366** (7).

Shukla S, Park J, Kim D-H, Hong S-Y, Lee JS & Kim M (2016) Total phenolic content, antioxidant, tyrosinase and α-glucosidase inhibitory activities of water soluble extracts of noble starter culture Doenjang, a Korean fermented soybean sauce variety. *Food Control,* **59**: 854–861.

Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S and Keller NP (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryot.Cell* **6**: 1656–1664.

Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S and Keller NP (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryot.Cell* **6**: 1656–1664.

Singh RK, Singh P, Mohapatra TM (2012). Nonribosomal peptide synthesis in microbes. In Recent advances in microbiology. *Nova Science Publishers, Inc, New York*

Singh V, Haque S, Niwas R, Srivastava A, Pasupuleti M and Tripathi CKM (2017). Strategies for fermentation medium optimization: an indepth review. *Front. Microbiol.* **7**: 2087.

Smetanina OF, Kalinovsky AI, Khudyakova YV, Pivkin MV, Dmitrenok PS, Fedorov SN, Ji H, Kwak J-Y, Kuznetsova TA (2007) Indole Alkaloids Produced by a Marine Fungus Isolate of Penicillium janthinellum Biourge. *J Nat Prod* **70**: 906–909.

Sørensen LM, Lametsch R, Andersen MR, Nielsen PV, Frisvad JC (2009) Proteome analysis of *Aspergillus niger*: Lactate added in starch-containing medium can increase production of the mycotoxin fumonisin B 2 by modifying acetyl-CoA metabolism. *BMC Microbiol* **9**: 255

Strauss J and Reyes-Domínguez Y (2011). Regulation of secondary metabolism by chromatin structure and epigenetic codes. *Fungal Genet. Biol.* **48**: 62–69.

Sun YL, Zhang XY, Zheng ZH, Xu XY, Qi SH (2014) Three new polyketides from marine-derived fungus Penicillium citrinum SCSGAF 0167. *Nat Prod Res* **28**: 239–244.

Szewczyk E, Chiang YM, Oakley CE, Davidson AD, Wang CCC, Oakley BR (2008) Identification and characterization of the asperthecin gene cluster of *Aspergillus nidulans*. *Appl Environ Microbiol* **74(24)**: 7607–7612

Takanezawa Y, Nakamura R, Sone Y, Uraguchi S, Kobayashi K, Tomoda H (2017) Variation in the activity of distinct cytochalasins as autophagy inhibitiors in human lung A549 cells. *Biochem. Biophys. Res. Co.* **494**: 641e647.

Timling I and Taylor DL (2012) Peeking through a frosty window: molecular insights into the ecology of *Arctic soil* fungi. *Fungal Ecol* **5**: 419–429.

Umemura M, Nagano N, Koike H, Kawano J, Ishii T, Miyamura Y, Kikuchi M, Tamano K, Yu J, Shin-ya K, Machida M. (2014) Characterization of the biosynthetic gene cluster for the ribosomally synthesized cyclic peptide ustiloxin B in *Aspergillus flavus*. *Fungal Genet Biol* **68**: 23–30

Varga J, Frisvad JC, Samson RA (2011). Two new aflatoxin producing species and an overview of Aspergillus section Flavi. *Studies in Mycology* **69**: 57–80.

Von Bargen KW, Niehaus E-M, Krug I, Bergander K, Wü E-U, Tudzynski B and Humpf H-U (2015) Isolation and structure elucidation of fujikurins AD: products of the PKS19 gene cluster in *Fusarium fujikuroi*. *J Nat Prod* **78**: 1809-1815.

Wang H, Fewer DP, Holm L, Rouhiainen L, Sivonen K (2014) Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. *Proc Natl Acad Sci* **111(25)**: 9259–9264

Wang J, Jiang Z, Lam W, et al. (2015) Study of malformin C, a fungal source cyclic pentapeptide, as an anti-cancer drug. *PLoS One* **10(11)**: e0140069.

Wang Y, Lu Z, Sun K and Zhu W (2011b) Effects of high salt stress on secondary metabolite production in the marine-derived fungus *Spicaria elegans*. *Mar. Drugs* **9**: 535–542.

Wang Y, Zheng J, Liu P, Wang W, Zhu W (2011a) Three New Compounds from *Aspergillus terreus* PT06-2 Grown in a High Salt Medium. *Mar Drugs* **9**(8): 1368-1378.

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. (2015) antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res* **43**(W1): W237-43.

Wiemann P, Keller NP (2014) Strategies for mining fungal natural products. *J Ind Microbiol Biotechnol* **41**(2): 301-13.

Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH (2008) Epigenetic remodeling of the fungal secondary metabolome. *Org Biomol Chem* **6**(11): 1895–1897

Wu J and Grunstein M (2000) 25 years after the nucleosome model: chromatin modifications. *Trends Biochem Sci* **25**: 619–623.

Xiao L, Yin Y, Sun W, Zhang F, Li Z (2013) Enhanced production of (+)-terrein by *Aspergillus terreus* strain PF26 with epigenetic modifier suberoylanilide hydroxamic acid. *Process Biochem* **48**: 1635–1639

Xue G, Stuart WH, Brian DA, Peng W, Linda PV, Christopher TW, Yi T (2012) Cyclization of fungal non-ribosomal peptides by a terminal condensation-like domain. *Nat Chem Biol* **8**: 823–830.

Yu D, Xu F, Zeng J and Zhan J (2012) Type III polyketide synthases in natural product biosynthesis. *IUBMB Life* **64**: 285–295.

Zabala AO, Xu W, Chooi YH and Tang Y (2012) Characterization of a silent azaphilone gene cluster from *Aspergillus niger* ATCC 1015 reveals a hydroxylation-mediated pyran-ring formation. *Chem Biol* **19**: 1049-1059.

Zhang L, Kang W, Lu X, Ma S, Dong L, Zou B (2019) Weighted gene co-expression network analysis and connectivity map identifies lovastatin as a treatment option of gastric cancer by inhibiting HDAC2. *Gene*. **681**: 15-25.

Zhang L, Niaz SI, Khan D, Wang Z, Zhu Y, Zhou H, Lin Y, Li J & Liu L (2017) Induction of Diverse Bioactive Secondary Metabolites from the Mangrove Endophytic Fungus Trichoderma sp. (Strain 307) by Co-Cultivation with *Acinetobacter johnsonii* (Strain B2). *Mar Drugs*, **15**(2): 35.

Zhang YH, Hou XM, Yu ML and Wang CY (2019) Secondary Metabolites and their Bioactivities from the Gorgonian-Derived Fungus *Aspergillus versicolor*. *Chem Nat Compd* **55**: 327–330.

Zutz C, Bacher M, Parich A, Kluger B, Gacek-Matthews A, Schuhmacher R, Wagner M, Rychli K, Strauss J. (2016) Valproic Acid Induces Antimicrobial Compound Production in *Doratomyces microspores*. *Front Microbiol.* **7**: 510.

## Acknowledgments

*At the end of this work I want to dedicate this space to the people who have contributed, with their support, to the realization of this project.*

*First, I want to thank my supervisor, Prof. Silvia Crognale, the guide who supporting and motiving me both from a scientific, technical and moral point of view.*

*I also want to thank prof Maurizio Petruccioli, for having welcomed me in his laboratory and prof. Alessandro D'Annibale, both always available to offer scientific support.*

*My gratitude goes to prof. Jens Christian Frisvad from University of Denmark for allowing to enter his laboratories by privileging me and sharing his profound knowledge of the subject.*

*A special thanks goes to the whole team, first of all Anna Maria Gallo, the tireless technical support who accompanied, supported and helps me throughout the journey, encouraging me in the many moments of discouragement; but also to Dr. Eleonora Carota and Cristina Russo, supporters of work, experience and advice.*

*Then I want to thank the colleagues/friends who have enriched and lightened the work routine, Barbara, Serena, Enrica, Rosita and Anna Rita, but also all the students who have listened to me, to whom hope I have transmitted curiosity towards my work.*

*I always thank my parents for the support, encouragement and trust they always place in my abilities.*

*An infinite thanks to my husband, Stefano, who despite being far from my working reality has always supported me in moments of discouragement, always believing in me. It was and always will be my peaceful haven.*

*Finally, as always, I thank myself for the commitment, strength and courage I give... Determination is always the engine of our skills!*

Martina

## List of Papers

Scientific Publications produced during the PhD course

- Cocarta DM, Dumitru DM, Pesciaroli L, Felli M, Raduly B & Crognale S (2019) Cultivable Hydrocarbonoclastic Microbial Community from Historically Polluted Soil: Tests for Consortium Development. *Soil and Sediment Contamination: An International Journal*, **28(3)**: 334-345, DOI: 10.1080/15320383.2019.1578335
- Crognale S, Pesciaroli L, Felli M, Petruccioli M, D'Annibale A, Bresciani A and Peterson SW (2019) *Aspergillus olivimuriae* sp. nov., a halotolerant species isolated from olive brine. *International journal of systematic and evolutionary microbiology*, **69(9)**: 2899-2906, DOI: 10.1099/ijsem.0.003575
- Della Pelle G, Perà G, Belardinelli MC, Gerdol M, Felli M, Crognale S, Scapigliati G, Ceccacci F, Buonocore F & Porcelli F (2020). Trematocine, a Novel Antimicrobial Peptide from the Antarctic Fish *Trematomus bernacchii*: Identification and Biological Activity. *Antibiotics (Basel, Switzerland),* **9(2)**: 66. DOI: 10.3390/antibiotics9020066
- Felli M., Russo C., Varga M., Gallo A.M., D' Annibale A., Petruccioli M., Crognale S. Biological activities of halotolerant fungi under increasing osmotic stress. Submitted to *Folia Microbiologica*

Poster Communications

- Felli M, Russo C, Bresciani A, Gennari N, D'Annibale A, Petruccioli M, Peterson S, Crognale S. Biotechnological investigations of the halotolerant species *Aspergillus oleamuriae* sp. Nov. *International Symposium on the Genetics of Industrial Microorganisms (GIM 2019), Pisa, Italy 8-11 September 2019.*
- Buonocore F, Scapigliati G, Perà G, Belardinelli MC, Della Pelle G, Gerdol M, Felli M, Crognale S, Porcelli F. Identification and functional characterization of an antimicrobial peptide from the Antarctic fish Trematomus bernacchii. *Congress of the Italian Society of Biochemistry and Molecular Biology (SIB 2019), Lecce 18-20 September 2019.*