



Universidade do Minho
Escola de Engenharia

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**Quality parameters in a culture collection -
Micoteca da Universidade do Minho**

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degree of Ph.D. in Chemical and Biological Engineering

Work developed under supervision of

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STATEMENT

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(Marta Filipa Jesus de Freitas Simões)

“A verdade não se pensa – sabe-se; o que se pensa é a explicação da verdade.”

António Maria Lisboa, in “Erro Próprio”.

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Whenever one starts a new stage in life, it is like when an artist takes up a white canvas and starts filling it with innumerable colours and patterns. When I first arrived at Braga, it was a new beginning for me. I was welcomed into a new world of knowledge by the group of Applied Mycology. Micoteca da Universidade do Minho (MUM) was a growing culture collection with a well-established background and infra-structure. But after filling me with all the colours and patterns of mycology and culture collections context, I was able to participate and engage in a new era for MUM. It is a gratifying feeling to see a complex and developed work of art, just like it is to realize and be held account for improvements, changes and goals obtained at MUM during the whole time of my Ph.D.

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QUALITY PARAMETERS IN A CULTURE COLLECTION –

MICOTECA DA UNIVERSIDADE DO MINHO

ABSTRACT

The biological diversity is quite important to the world, in a social, industrial, economic and scientific point of view. Nowadays it is mandatory to assure and guarantee biodiversity conservation, its success, sustainable use and the equitable share of benefits arising from the use of genetic resources through: ethical sourcing practices, and collaborations between the different Biological Resource Centres (BRCs). It is now the era of awareness of quality assurance. This work presents ways to contribute and increase the quality, knowledge, information, maintenance and preservation of biological resources, applied to filamentous fungi.

The achievement of quality within the BRCs and culture collections (CC) context is a dynamic process, always evolving as well as the backlog and build-up of biological resources data that increase with each research made. An option to achieve quality is the implementation of a Quality Management System (QMS) based on the standard ISO 9001:2008, like the one here described and explained on a CC of filamentous fungi: Micoteca da Universidade do Minho (MUM). The QMS implemented at MUM in 2011 and the obtained certification, are a continuous improvement process focused on customer satisfaction.

Maintenance of biological resources implies the choice of the best methods and constant search of cheaper, faster and more practical, with assured procedures and validation of preservation success. With this purpose, an assessment of preservation methods was performed, through a polyphasic approach using several techniques of fungal characterisation [macroscopic (photography), microscopic (optical, stereomicroscopy and SEM), mycotoxin screening (HPLC), enzymatic screening (spectrophotometry and specific inducer media), MALDI-TOF MS and molecular biology analysis]. It was found that long-term stored lyophilized samples were not significantly altered making this method one of the most appropriate in the case of filamentous fungi preservation. The minor changes observed were dependent on the strain to preserve.

In the same context of the continuous strive for improvement, a proposal to develop new methods for the preservation of strains, specially delicate and/or recalcitrant strains of fungi was made. Perlite and alginate encapsulation were used in a specific group of selected fungal strains. For the conditions chosen, after morphological and MALDI-TOF MS analysis, it was found that perlite does not allow the viability of samples after preservation whether alginate encapsulation proved to be a better alternative to the common and well known Castellani method, i.e., preservation in water.

QUALITY PARAMETERS IN A CULTURE COLLECTION –

MICOTECA DA UNIVERSIDADE DO MINHO

RESUMO

A diversidade biológica é muito importante do ponto de vista social, industrial, económico e científico. Assegurar e garantir a conservação da diversidade biológica, o seu uso sustentável e a repartição equitativa dos benefícios resultantes da utilização dos recursos genéticos, através de: práticas de abastecimento éticas e colaborações entre os diferentes Centros de Recursos Biológicos (BRCs), é um requisito actual. Este trabalho pretende apresentar formas de contribuir e aumentar a qualidade, conhecimento, informação, manutenção e conservação de fungos filamentosos.

A obtenção de qualidade dentro do contexto dos BRCs e Colecções de Cultura (CCs) é um processo dinâmico, em constante evolução, bem como o aumento crescente e acumulação de dados sobre os recursos biológicos que aumentam a cada pesquisa feita. Uma opção para alcançar a qualidade é a implementação de um Sistema de Gestão da Qualidade (SGQ) baseado na norma ISO 9001:2008, como o aqui descrito e explicado para uma CC de fungos filamentosos: Micoteca da Universidade do Minho (MUM). O SGQ implementado na MUM em 2011 e a certificação obtida, são um processo de melhoria contínua focado na satisfação do cliente. A manutenção dos recursos biológicos implica a escolha dos melhores métodos e a busca constante de outros mais baratos, rápidos, práticos, com procedimentos assegurados e validados. Com esta finalidade, foi realizada uma avaliação de métodos de preservação, através de uma abordagem polifásica com o uso de várias técnicas de caracterização fúngicas [macroscópica (fotografia), microscópica (óptica, esteromicroscópica e SEM), rastreio de micotoxinas, rastreio de produção enzimática, MALDI-TOF MS e análise de biologia molecular]. Verificou-se que as amostras liofilizadas armazenadas por longos períodos não sofrem grandes alterações o que torna este método como um dos mais apropriados na preservação de fungos filamentosos. As poucas alterações observadas são dependentes da estirpe a preservar.

Foi ainda realizada uma proposta de desenvolvimento de novos métodos para a preservação de estirpes fúngicas, delicadas e/ou recalcitrantes. Foram testados: perlite e encapsulamento em alginato. Verificou-se que a perlite não permite a viabilidade das amostras após preservação, mas o encapsulamento em alginato demonstrou, após análise morfológica e por MALDI-TOF, ser uma boa alternativa ao método de preservação em água, ou seja: método Castellani.

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A- front of the colony, B –reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotioniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

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A- front of the colony, B –reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotioniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

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aculeatus MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM

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3. Glossary of Acronyms and Abbreviations

A2LA	The American Association for Laboratory Accreditation
ACOI	<i>Algoteca de Coimbra</i>
AFLA	Aflatoxin
AFNOR	<i>Association Française de Normalisation</i>
AGO	Arocrete Group Co.
AP	Provision
APCER	<i>Associação Portuguesa de Certificação</i>
ATCC	American Type Culture Collection
BCCM	Belgian Coordinated Collections of Micro-organisms
BioCC	<i>Colección de Cultivos Microbianos</i>
BioCen	<i>Centro Nacional de Biopreparados</i>
BOTFCP	Collection of Microorganisms from the Department of Botany of the Faculty of Sciences - <i>Universidade do Porto</i>
BSc	Bachelor of Science
CBD	Convention on Biological Diversity
C.D.B.	Collection of the Department of Biology
CABI	CAB International Genetic Resource Collection
CABRI	Common Access to Biological Resources and Information
CAc	Control of Access
CAs	Corrective Actions
CBS	<i>Centraalbureau voor Schimmelcultures</i>
CC	Culture collection
CCENUTAD	CC Sector Enology - Department of Food and Drink Industries from <i>Universidade de Trás-os-Montes e Alto Douro</i>
CCMA	CC of Food Microorganisms
CCMI	CC of Industrial Microorganisms

CCRC	Culture Collection and Research Centre
CD	Control of Documents
CDS	Criteria for Deposit Of Strains
CEB	Centre of Biological Engineering
CECT	<i>Colección Española de Cultivos Tipo</i>
CHCA	α -cyano-4-hydroxycinnamic Acid
CIP	<i>Collection de l'Institut Pasteur</i>
CMA	Coconut Milk Agar
CMC	Carboxymethyl Cellulose
CMCase	Carboxymethyl Cellulase
CR	Control of Records
CRBIP	Biological Resource Centre - Institut Pasteur
CYA	Czapek Agar With Yeast Extract
CZ	Czapek Agar
DEB	Department of Biological Engineering
IDR	Informatic documents of registries
DHB	Dihydroxybenzoic Acid
DNS	Dinitrosalicylic acid
DSMZ	<i>Deutsche Sammlung von Mikroorganismen und Zellkulturen</i>
ECACC	European Collection of Cell Cultures
ECCO	European Culture Collections' Organisation
ECCCO	European Culture Collections Curators' Organisation
EMbaRC	European Project Consortium Microbiological Resource Centres
ERIC	Enterobacteria Repetitive Intergenic Consensus
ESFRI	European Strategy Forum on Research Infrastructures
EU	European Union
EVN	<i>Estação Vitivinícola Nacional</i>
F	Form
FB2	Fumonisin B2
FIRDI	Food Industry Research and Development Institute
FTIR	Fourier Transform Infrared
FT-NIR	Fourier Transform Near Infrared

FUM	Fumonisin
GBRCN	Global Network of Biological Resource Centres
GLP	Good Laboratory Practice
HPA	Health Protection Agency
HPLC	High-Performance Liquid Chromatography
IA	Internal Audit
IBB	Institute for Biotechnology and Bioengineering
IBET	<i>Instituto de Biologia Experimental e Tecnologia</i>
ICLC	Interlab Cell Line Collection
IGC	<i>Instituto Gulbenkian de Ciência</i>
IHEM	Institute of Hygiene and Epidemiology, Mycology
INETI	<i>Instituto Nacional de Engenharia, Tecnologia e Inovação</i>
INSDB	International Nucleotide Sequence Databases
IPR	Intellectual Property Rights
IQNet	International Certification Network
ISA	Microbiology Laboratory - <i>Instituto Superior de Agronomia</i> – Lisboa, Portugal
ISO	International Standards Organisation
ISO/TC 212	International Organisation for Standardisations's Technical Committee 212
IT	Information Technology
ITS	Internal Transcribed Spacer
IUCN	International Union for Conservation of Nature
LB	Luria Bertani Media
LMECYA (LME)	Cyanobacteria Culture Collection Estela Sousa e Silva, from <i>Laboratório de Microbiologia e Ecotoxicologia - Instituto Nacional de Saúde Dr. Ricardo Jorge</i>
LOD	Limit Of Detection
M.INSA	<i>Micoteca do Instituto Nacional de Saúde</i>
M.L.R.V.A.	<i>Micoteca - Laboratório Regional de Veterinária - Angra do Heroísmo, Açores, Portugal</i>
Man	Galactomannan
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry
MEA	Malt Extract Agar
MEAN	<i>Micoteca da Estação Agronómica Nacional</i>

MEAN/LISE	<i>Micoteca from Estação Agronómica Nacional</i>
MGYP	Malt Extract-Glucose-yeast Extract-peptone
MINE	Microbial Information Network Europe
MIRCEN	Microbiological Resource Centre
MMD	Monitoring and Measurement Devices
MME	Measurement and Monitoring Equipment
MMEM	Monitoring and Measuring Equipment Management
MPP	Material Preservation Process
MRP	Material Reception Process
MS	Recipe of Media and Solutions
MSc	Master of Science (degree)
MSP	Material Supply Process
MTA	Material Transference Agreement
MUCL	<i>Mycothèque de l'Université Catholique de Louvain</i>
MUM	<i>Micoteca da Universidade do Minho</i>
NaAlg	Sodium Alginate
NCIMB	National Collections of. Industrial, Food and Marine Bacteria
NBRC	NITE Biological Resource Centre
NC	Nonconformities
NCCA	Nonconformities and Corrective Actions
NCPV	National Collection of Pathogenic Viruses
NCTC	National Collection of Type Cultures
NCYC	National Collection of Yeast Cultures
NITE	National Institute of Technology and Evaluation
No.	Number
OECD	Organisation for Economic Co-operation and Development
OTA	Ochratoxin A
P	Process
PC	Pectin of <i>Citrus</i> sp.
PCB	Portuguese Collection of Bacteria (Microbiology Lab, University of Coimbra)
PCC	Pasteur culture Collection of Cyanobacteria
PDA	Potato Dextrose Agar

PDCA	Plan-Do-Check-Act
Ph.D.	Doctor of Philosophy (<i>Philosophiae</i> Doctor)
PIA	Preventive and Improvement Actions
PYCC	Portuguese Yeast Culture Collection
QM	Quality Manual
QMan	Quality Manager
QMS	Quality Management System
QP	Quality Policy
R&D	Research and Development
SCBD	Secretariat of the Convention on Biological Diversity
SEM	Scanning electron microscope
SKM	Skim Milk Agarised Medium
SM	System Management
SMEs	Small and Medium-sized Enterprises
SNM	Synthetic Nutrient Medium
SOP	Standard Operating Procedure
ST	Staff Training
UDBN	UK DNA Banking Network
UMIP	Fungi Culture Collection belonging to Biological Resource Centre - Institut Pasteur (CRBIP)
UN	United Nations
UNESCO	United Nations Educational, Scientific and Cultural Organization
WDCM	World Data Centre for Microorganisms
WFCC	World Federation for Culture Collections
WIPO	World Intellectual Property Organization
WX	Wheat Xylan
YES	Yeast Extract Sucrose

1. GENERAL INTRODUCTION

1.1. Biodiversity and Biological Resources

It is currently acknowledged that biodiversity is a global asset of high value to present and future generations. There is broad consensus that it is critically threatened and that large-scale interventions are necessary for its protection (Brechin et al., 2002). Therefore, efforts are being made to help stopping biodiversity loss. One of these efforts was the adoption, in 1992 at Rio de Janeiro, Brazil, of the CBD (CBD, 2013b). This international convention for the conservation of biodiversity, for the sustainable use of the components of biodiversity and for the equitable and fair sharing of the benefits derived from the use of genetic resources (Smith, 2003). The CBD had a near universal participation of 193 parties (CBD, 2013c). Portugal signed this convention in 1993. One year later, on the forest day, 21st of Mach, the Portuguese law-decree No. 21/93 from *Diário da República* No. 143, series I-A, 21st June, approved, for ratification, the CBD. Several protocols were also created: the Cartagena Protocol, created in order to protect the biological diversity from the potential risks posed by living modified organisms (Mackenzie et al., 2003; SCBD, 2013); and the Nagoya Protocol, a new international agreement under the CBD, adopted in 2010, that intends to facilitate the implementation of resolved principles (CBD, 2011). These principles include the fact that research and development (R&D) of biodiversity-based products can only take place with the approval of relevant countries and communities, which must also share in the benefits. For these reasons, public and general involvement, and awareness of biodiversity is a must that should be considered in all the actions taken and to be made in the future (Smith, 2003).

The biological resources of our planet are vital to humanity's economic and social development. According to the Convention on Biological Diversity (CBD), biodiversity is “the variability among living organisms from all sources including, among others, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems”. Also, biological resources differ

from biological diversity and “include genetic resources, organisms or parts thereof, populations, or any other biotic component of ecosystems with actual or potential use or value for humanity” (CBD, 2013a). Biological resources exclude the ecosystem and the ecological complexes of which they are part. The notion of sustainability has become an integral part of all legal instruments addressing biological resources (Cullet, 2001). It has been referred that 40% of the world's economy is based directly and indirectly on the use of biological resources justifying their sustainable use to be turned into a goal (CBD, 2013d).

1.1.1. The importance of filamentous fungi

Microorganisms – bacteria, archaea, viruses, viroids, filamentous fungi, yeast, microalgae and protozoans – comprise the greatest numbers of organisms on Earth (Çaktü & Türkoglu, 2011).

Fungi are a diverse group of unique eukaryotic organisms currently accepted as the Eumycota kingdom. According to Hawksworth (2001), the underestimated number of fungal species is 1.5 million of which only a small number have been identified (ca. 8-10%). Several studies have been conducted concerning the great gap between known and estimated fungal species richness (Hawksworth, 2001; Hawksworth, 2012; Simões et al., 2013a). They are ubiquitous in nature with an extraordinary ability to decompose plant wastes while also causing much spoilage of food and other relevant commodities. Certain species are used directly as food and others in the manufacture of foodstuffs, antibiotics, enzymes, organic acids and alcohols. Still others can infect humans, animals and crops (Simões et al., 2013a). Mycologists have been collecting fungi for scientific purposes for more than 300 years (Abd-Elsalam et al., 2010).

The filamentous fungi have dynamic and variable hyphal structures. Combining direct observation with indirect measures of biochemical screenings, molecular biology characteristics, are attributes of fungi that can be accessed in the study of their taxonomic and phylogenetic information. The filamentous fungi are known to be particularly sensitive to stresses, such as physical disturbance, mineral nitrogen, pesticides, earthworm activity, and heavy metals, when compared with the soil bacteria (Klein & Paschke, 2004).

There is an unquestionable importance on preserving multiple strains of a species, because there is physiological and genetic diversity within microbial species (Uzunova-Doneva & Donev, 2004-2005). This intraspecific diversity results in differences in antibiotic resistance, sterilization temperature, and other properties with important impact for industrial fermentations,

food preservation, and pathology. Researchers can isolate fungi themselves from nature, obtain them from colleagues, or request them from CCs, but they are advised to use CCs when possible. Service collections have the facilities and expertise to assure proper species identification, minimize genetic drift that often occurs with repeated transfer, and assure pure and viable cultures (Boundy-Mills, 2012).

1.2. Culture collections and Biological Resource Centres

For many years, infrastructures and resources, either physical or economical, were very limited. Catalogues, when available, only existed in printed versions and most collections served only national or even local users (Simões et al., 2013a). But, the existent culture collections (CCs) and Biological Resource Centres (BRCs) have been gaining increased acknowledgement on the importance of their existence. In the beginning of the last century scientists recognized the need for the existence of collections of microorganisms to serve scientific research all over the world. Since then other collections started to be developed. And nowadays, the importance of *ex situ* preservation for the use of microbial resources is undeniable (Lima, 2008). The quality assurance in the services and holdings they have and provide, help them becoming professionalised and recognised for the value they hold and provide (McCluskey, 2011). Such data of cultures is usually as valuable as the organism itself and its use saves researchers valuable time and resources, with benefits in industrial, research and teaching settings (Abd-El salam et al., 2010; Boundy-Mills, 2012).

CCs are scattered all over the world, among the five continents and have different types of support. Most have governmental or university support, but there are others with semi-governmental, private or industrial support.

CCs offer multiple services: storage, patent deposits, distribution, identification, training and consultant services (Stackebrandt, 2010; WFCC, 2013). Their holdings can include different kinds of resources: algae, archaea, bacteria, deoxyribonucleic acid (DNA), cell lines (animal and plants), lichens, plasmids, protozoa, vectors, viruses (animal, bacteria and plants) and yeasts (WFCC, 2013). CCs are also responsible for the maintenance, distribution and divulgation of the data associated with the biological materials through free access, yet structures and projects that facilitate that access.

A system of cumulative information has evolved since the origin of CCs. This was needed as new specimens and analyses led to a re-evaluation of older specimens and the original

depositors became unavailable. Preserving the accuracy of data is critical for science and research (Canhos, 2003). As referred by Bidartondo et al. (2008), it is known that there are some sequence errors and some erroneous lineage designations in the data available in public repositories. DNA sequences published in GenBank have been attributed to incorrectly named organisms (Hawksworth, 2004). Gene function annotation in protein sequence databases is error-prone. This becomes inefficient and unsustainable over long-term (Bidartondo et al., 2008). But working together with common goals BRCs can develop strategies to overcome all those obstacles.

Advanced databases are crucial to facilitate access and transfer knowledge (Çaktü & Türkoglu, 2011). The most important web databases for fungal diversity are: MycoBank (www.mycobank.org), Common Access to Biological Resources and Information (CABRI) HyperCatalogue (www.cabri.org), Index Fungorum (www.indexfungorum.org), StrainInfo (www.straininfo.net), GBIF (www.gbif.org) together with the information of nucleotide and protein sequences deposited in GenBank (Simões et al., 2013a).

StrainInfo is a digital platform that integrates information from various CCs and BRCs and is becoming a reference on information and meta-information of microorganisms. It uses Microbial Common Language (MCL), intended to be broadly applicable in situations where microbial material is referenced or used and therefore has been designed to be interoperable with existing and future standards (Verslyppe et al., 2010; www.straininfo.net).

1.2.1. The Culture Collections in Portugal

According to the database project: Resource Identification for a Biological Collection Information Service in Europe (BioCISE), Portugal has been described as having 70 Collections of biological material scattered among the Portuguese Islands and on the mainland (BioCISE, 2002), but only 5 are registered at the World Federation for Culture Collections (WFCC) and World Data Centre for Microorganisms (WDCM). The BioCISE was followed up by a new project Biological Collection Access Service for Europe (BioCASE) which is a transnational network of biological collections of all kinds. BioCASE enables widespread unified access to distributed and heterogeneous European collections and observational databases using open-source, system-independent software and open data standards and protocols (BIOCASE, 2005).

At European level, ECCO was formally established in 1982. With a defined mission of bringing managers of the major public service collections in Europe together, with the intention of

discussing common policy, exchanging technologies and seeking collaborative projects (Smith, 2003; ECCO, 2007). ECCO comprises above 60 members from 22 European countries that hold over 350,000 strains representing filamentous fungi, yeasts, bacteria, archaea, phages and plasmids. ECCO also comprises members from the animal cells field, including human and hybridoma cell lines, as well as animal and plant viruses, plant cells, algae and protozoa. ECCO members have helped to produce practical approaches to international rules and regulation (ECCO, 2007; Simões et al., 2013a). Among these, there are only three Portuguese collections: PYCC (since 1987), MUM (since 2002) and MEAN (since 2007).

From ECCO and with the effort of all members, several actions have been developed. European projects such Microbial Information Network Europe (MINE), CABRI, EMbaRC and MIRRI were established.

Moreover, from a survey conducted by MUM in 2002 and updated afterwards, 17 CCs were referenced (Table 1) (Lima, 2002). But it is known that many others exist in places where the research in microbiology field is leader. However, the vast majority of these collections correspond, to more or less extensive sets of the material necessary to the development of their local research projects.

Table 1 - Culture Collections referenced in Portugal, from a survey performed by Micoteca da Universidade do Minho (Lima, 2002; Lima, 2007).

Acronym	Collection	Location	Starting year
ACOI	<i>Algoteca de Coimbra</i>	Coimbra	1972
BOTFCP	Collection of Microorganisms from the Department of Botany of the Faculty of Sciences of Porto	Porto	1966
C.D.B.	Collection of the Department of Biology	Braga	1993
CCENUTAD	CC Sector Enology - Department of Food and Drink Industries from <i>Universidade de Trás-os-Montes e Alto Douro</i>	Vila Real	1981
CCMA	CC of Food Microorganisms	Lisbon	1966
CCMI	CC of Industrial Microorganisms	Lisbon	1985
EVN	Collection of Microorganisms from <i>Estação Vitivinícola Nacional</i>	Torres Vedras	1973
IBET	<i>Instituto de Biologia Experimental e Tecnologia</i>	Oeiras	1991
ISA	Microbiology Laboratory of the <i>Instituto Superior de Agronomia</i>	Lisbon	1980

Acronym	Collection	Location	Starting year
LMECYA (LME)	Cyanobacteria Culture Collection Estela Sousa e Silva, from <i>Laboratório de Microbiologia e Ecotoxicologia</i> of the <i>Instituto Nacional de Saúde Dr. Ricardo Jorge</i>	Lisbon	1962
M.INSA	<i>Micoteca do Instituto Nacional de Saúde</i>	Lisbon	1989
M.L.R.V.A.	Micoteca of the <i>Laboratório Regional de Veterinária</i> , from Angra do Heroísmo, Açores	Angra do Heroísmo	1995
MEAN/LISE	Micoteca from <i>Estação Agronómica Nacional</i>	Oeiras	1948
MUM	Micoteca da Universidade do Minho, Braga	Braga	1996
PCB	Portuguese Collection of Bacteria (Microbiology Lab, University of Coimbra)	Coimbra	1988
PYCC	Portuguese Yeast CC (Portuguese Collection of Yeast Cultures, <i>Faculdade de Ciências e Tecnologia/ Universidade Nova de Lisboa</i>)	Caparica	1952

The collections mentioned in table 1, are globally responsible for the preservation, in Portugal, of microorganisms such as: algae, bacteria, archaea, filamentous fungi and yeast. Some of these collections are more specialized than others, depending on their size and level of organisation, but 70 % of them have implemented the use of more than one method of preservation to ensure long-term sustainability. The method of choice for preservation is the cryopreservation, at -80 °C. At the time of the survey, it was referred, that the lack of services and their limitations was due to financial and task performance difficulties; also due to the fact that the human resources were mainly on a voluntary basis (Lima, 2002). At the time of that survey it was not available for most collections the existence of metadata or data with informatics availability. The production of online catalogues and material related information was not available for most collections and this scenery has not been altered to the present days. This is mainly due to the lack of human resources and skills in building databases with different relational levels. Provision of the information associated with the biological resources, authentication of data, standardized terminology and format for exchange and data transmission, Internet publishing and data protection, and preservation are of great importance and contribute to the constant development of projects. Associations have been encouraging all CCs to value all data and material which is often the result of the work of several generations of researchers and thus become BRCs.

1.2.2. Biological Resource Centres

The concept of BRC appears for the first time, associated with the program Microbiological Resource Centre (MIRCEN), launched by United Nations Educational, Scientific and Cultural Organization (UNESCO) in 1946 with the aim of establishing microbiological resource centres as guardians of microbial diversity, extremely valuable and threatened by lack of financial resources for less developed countries. It was also the UNESCO that supported the inventory classification of microbial genetic resources in the two decades that preceded the United Nations (UN) Conference on the Human Environment, held in Stockholm, Sweden, in 1972.

Following the Rio Convention and the recognition of the important role of BRCs as essential infrastructures to the growth of R&D in Biology and Biotechnology, Japan, in 1998, took the initiative to call the attention of the Organisation for Economic Co-operation and Development (OECD) for the need to develop policies to support and maintain BRCs. The first step was the creation of a workshop dedicated to scientific and technological infrastructures, in February 1999, from where the conclusions and recommendations developed are contained in the report: Biological Resource Centres - Underpinning the Future of Life Sciences and Biotechnology published in 2001 (OECD, 2001). This report, approved by OECD member states including Portugal, unequivocally points to the need of reinforcement and modification of BRCs in order to incorporate recent scientific developments and to be up to the needs and demands of the present and future days. The report also emphasises the need for the collections to adhere to high standards of quality and competence, demanded by the international community of scientists and industry on the supply of information and biological materials. Finally, the report challenges state-members to establish national BRCs that respect common quality standards of competence and financial stability, guaranteed by international criteria and governmental systems or independent accreditation/certification bodies. It was based on this approach, to encourage international cooperation and economic development that the creation of a Global Network for BRCs was made through the development of the GBRCN and all the continuing projects (GBRCN, 2012; Simões et al., 2013a).

The BRCs are an essential part of the infrastructure that supports the life sciences and biotechnology. They are compelled to demonstrate their ability to:

- (1) provide authenticated biological resources for scientific, industrial, in agriculture, environment, medicine, education and R&D. The microbiological based metrology

- guarantees the quality of the supplied material; mainly the reproducibility, reliability and validation of the obtained results;
- (2) have an active role in R&D, due to increased skills on identification, characterisation and preservation of biological resources;
 - (3) preserve biological and genetic resources *ex situ*, helping to strengthen the CBD, in particular with regard to the sustainability of living systems in the biosphere and sustainable use of biological diversity;
 - (4) deposit biological materials that are protected by agreements on rights of intellectual property and patents. For microorganisms such supply is made in accordance with the Budapest Treaty on the international recognition of deposit of microorganisms for the purposes of patent procedures (WIPO, 2013). Collections will have to ensure the preservation of safe and confidential deposits, with restricted distribution according with the contract made with the depositor;
 - (5) provide knowledge to formulate, informed and reasoned, governmental policies on biological resources and information to ensure the general public. They must be actively connected to the ethical aspects regarding confidentiality and consent the use of materials; biosafety and biosecurity.

It is important to specify the following concepts:

A – Biosafety: concerns the distribution of microorganisms hazardous to man and the environment, referring to the development and implementation of administrative policies, practical working conditions of the premises related to the prevention of transmission of biologic agents to workers, other people, community and environment. In Portugal, the decree-law No. 84/97, ordinance No. 405/98 and ordinance No. 1036/98 establish legislation for this matter.

B – Biosecurity: refers to the control of supply of bio-hazardous materials and relates to the protection of the consequences of using subversive intentional biological agents, toxins, or critical relevant information.

BRCs are considered to be a key element for a sustainable international scientific infrastructure, which is necessary to underpin successful delivery of the benefits of biotechnology, whether within the health sector, the industrial sector among others (OECD, 2007) and they are essential to conserve and manage the provision of the biological resources in order to guarantee

the biodiversity preservation. Their establishment and maintenance depends on the implementation of reliable preservation techniques and appropriate quality assurance to allow them to become effective and efficient, and since this has been recognized as a fact, collections have been complying with and developing legislation in accordance (NP EN 1619, 1999).

The OECD defines BRCs as service providers and repositories of the living cells, genomes of organisms, and information relating to heredity and functions of biological systems. BRCs contain collections of cultivable organisms such as: microorganisms, plants and animal cells; replicable parts of these (for example: genomes, plasmids, virus, DNAs), viable but not cultivable organisms, cells and tissues, as well as database containing molecular, physiological and structural information relevant to these collections and related bioinformatics (OECD, 2001). It has been referred by Boundy-Mills (2012) that approximately 70% of new species description publications come out of CCs. BCRs can be considered the next generation CCs and are the establishment goal for all the collections in order to underpin the biotechnology and drive the bioeconomy (Lima, 2008). The networks between BCRs will allow for collaborative R&D, with shared tasks, and common work criteria and will become an international mark of quality; these will also allow for the access of users to high quality biological materials and associated information, with conformity, continuity in quality and authenticity through the use of common processes and procedures and the following of national and international laws, policies and procedures. BCRs will be technically improved collections with: well established Quality Management Systems (QMS), Material Transference Agreements (MTA), biosafety and biosecurity standards, Intellectual Property Rights (IPR) regulations; accredited or certified to the standards of the International Standards Organisation (ISO); highly trained staff; clear management program and collection strategy in place and sustainable funding mechanisms with governmental support (OECD, 2007).

1.3. Micoteca da Universidade do Minho

Micoteca da Universidade do Minho (MUM) is a filamentous fungal CC established in May 1996 by the *Universidade do Minho* Rectorate (www.micoteca.deb.uminho.pt). It is hosted by the Department of Biological Engineering (DEB) – a centre of excellence integrated in the Institute for Biotechnology and Bioengineering (IBB) at *Universidade do Minho* (www.uminho.pt), a university founded in 1973 at Braga, Portugal. The purpose of MUM is to maintain and provide strains for teaching and research in biotechnology and life sciences, in order to provide the

highest quality services to customers. It aims to collect, maintain and supply fungal strains and their associated data, and so, to act as a centre of expertise, information and training in accordance with international quality standards. This is in conformity with the institutional policy of *Universidade do Minho*, in establishing transversal and integrated services to support research. All the expended work and research developed in underpinning this CC, all the contributions that MUM gives at national and international level, as well as the strong linkage that MUM has developed among academia, industry, services, and other important stakeholders encouraged MUM to embrace the concept of closing the gap between science and society and are a constant stimuli for MUM to exceed upon itself (Paterson et al., 2012).

The researchers working at the laboratory of MUM are part of the Applied Mycology Group (AMG) formed to consolidate the research activity performed in the Centre of Biological Engineering at the University of Minho, in the field of Applied Mycology. The AMG integrates, together with the Centre of Biological Engineering (CEB) of Minho University, part of the Associated Laboratory IBB. AMG includes a group of researchers from different nationalities: Portuguese, Brazilian, Spanish and English. This research group aims to develop new or to improve applications of fungi in Biotechnology and Bioengineering. And, it relies on accurate identifications and proper preservation of the fungal resources used in research. For these reasons, the maintenance of the fungal CC of *Universidade do Minho* – MUM, constitutes part of the activities of this group. The current areas of research of the AMG include: preservation of filamentous fungi; study of the natural occurrence and application of polyphasic approaches to species identification and authentication, and also strain typing of fungi isolated from: environmental, clinical and agro-food processes samples. The members of this research team have either authored or co-authored a large number of publications in the field of Applied Mycology. They are distributed among the three interdisciplinary research areas of CEB: Environmental Biotechnology, Biotechnology in Health and Industrial Biotechnology. The location of MUM in the CEB building is an advantage for all MUM workers and collaborators, since it allows the use of common infrastructures and a direct contact with an international environment focused on research and entrepreneurship with access to the latest findings and developments in the scientific field.

The introduction of more stringent quality parameters in the identification and authentication of biological material preserved by MUM has led to a scientific recognition of this collection and an increase of its competitiveness worldwide. Within a collection of thousands of

fungi, MUM has more than 800 strains in catalogue, covering 184 species belonging to 68 genera, isolated during research activities on biodegradation and biodeterioration, mycotoxins in food commodities and also studies of air quality in industrial and hospital environments. Many of the strains are of industrial importance, others are taxonomic type strains; some are used for assays, testing, teaching, biochemical and genetic research, while others are of general scientific interest. Among its holdings, MUM has enough different species and strains to backup an enormous variety of research work. The activity of MUM is reflected in international publications, support for postgraduate research with production of masters and doctoral theses, contracts with industry and services in national and international projects. Researchers working at the laboratory of MUM have also contributed to science with the description of eight new species: *Aspergillus brasiliensis*, *Aspergillus ibericus*, *Aspergillus uvarum*, *Penicillium astrolabium* and *Penicillium neocrassum*, *Aspergillus sergii*, *Aspergillus mottae* and *Aspergillus transmontanensis*. MUM holds a herbarium for the exsiccate specimens of these species and also their ex-type cultures which are essential on the identification and future species comparisons.

MUM has joined the WFCC (www.wfcc.info) on the 13th September 2001, and is registered with the number 816 in the WDCM (WFCC, 2011), being one of the five current Portuguese collections (Table 2), in a total of 633 worldly scattered Culture Collections (CCs), registered in the WDCM. In 2002, MUM joined the European Culture Collections' Organisation (ECCO).

The WFCC consists of several committees composed by CCs from all over the world (WFCC, 2011), being Asia the continent with the higher number of registered CCs: 219, followed by the European continent with 218; and with Oceania as the continent with the lowest number of registered CCs: 42. From the registered CCs, there are a totally of 2,098,976 microbes from which 559,554 are fungi (WFCC, 2013).

In 1981, several curators from European CCs formed the European Culture Collections Curators' Organisation (ECCCO), currently ECCO and since then the major public service collections in Europe have been discussing together the common policy, exchanging technologies and seeking collaborative projects (Smith, 2003; Uruburu, 2003).

The WDCM maintains and updates the database on biological resources and the CCs registered at WFCC, and hubs the microbial information resources including the international nucleotide sequence databases of DDBJ/EMBL/GenBank (INSD, 2002).

Table 2 - Portuguese Culture Collections registered at the World Data Centre for Microorganisms (Adapted from: WFCC, 2011).

WDCM Number	Acronym	Collection
WDCM 595	PYCC/IGC	Portuguese Yeast CC/ <i>Instituto Gulbenkian de Ciência</i>
WDCM 761	CCMI	CC of Industrial Microorganisms
WDCM 816	MUM	<i>Micoteca da Universidade do Minho</i>
WDCM 881	MEAN	<i>Micoteca da Estação Agronómica Nacional</i>
WDCM 906	ACOI	<i>Algoteca de Coimbra</i>

MUM was involved as a partner, among 17 others distributed worldwide; from November 2008 until November 2011; in the demonstration project of the Global Network of Biological Resource Centres (GBRCN) (www.gbrcn.org) and with 7 partners on the European Project Consortium Microbiological Resource Centres (EMbaRC) (www.embarc.eu) and it is also currently involved in the Microbial Resource Research infrastructure (MIRRI) (www.mirri.org). EMbaRC was a European consortium, created on the 1st February 2009, to establish a self-sustained community of European Microbial Resource Centres representing a large bio-diversity and offering a wide-range of, bio-resources and expert services. These services intended to: enable the development of new partnerships with public institutes in the field of biodiversity and also to facilitate revenue-generating partnerships with the economic sector. The main goals were: to enable all the EMbaRC partners, as well as other European microbial resource centres, to avoid the loss and/or increase the danger of losing endangered collections and to create all necessary infrastructures and funding to create a solid and consistent existence for BRCs. During the occurrence of the project, an extensive training and outreach program was used, allowing the exchange and sharing of experiences and knowledge (EMbaRC, 2013).

The latest, MIRRI, is a new project, a pan-European distributed infrastructure, established on the European Strategy Forum on Research Infrastructures (ESFRI) roadmap. It is a European Commission (EC) funded project with the goal to improve access to the microbial resources and services that are needed to accelerate research and discovery processes. Launched with a kick-off meeting in November 2012, held at Braunschweig, Germany, intends to bring together European collections with stakeholders (their users, policy makers, potential funders and the plethora of microbial research efforts) aiming at improving access to enhanced quality microbial resources in an appropriate legal framework, thus underpinning and driving life sciences research. It will build the European platform within the GBRCN for microorganisms. MIRRI

intends to encourage and facilitate: innovation, research and development, in pharmaceutical, biotechnological, healthcare and agricultural areas. It will help deliver the EU Innovation Union goals, address societal challenges and support EU competitiveness in the knowledge based bio-economy (MIRRI, 2012).

The ESFRI roadmap emphasised the evident need for improved availability of high quality materials and reagents to the study of species. ESFRI supported a coherent and strategy-led approach to policy making on new and existing pan-European and global research infrastructures (European Communities, 2009). MIRRI cross cuts and supports most biotechnological sectors. It will help in the understanding the microbial diversity role in the area of soil fertility, food and agriculture, needed to develop approaches to improve agricultural and food production. Better managed resources will lead to further discovery in all areas of the life sciences including healthcare. MIRRI and the involved partners will provide coherence in the application of quality standards, homogeneity in data storage and management and sharing the work to help the release of the potential of microorganisms (MIRRI, 2012).

In international partnerships, beyond its European partners, MUM collaborates with Australia, Brazil, Canada, Chile, Egypt, Malaysia, Pakistan and USA. Brazil, due to historical and cultural reasons is, and has been for many years, one of the priorities for MUM on bilateral cooperation.

1.4. Quality and Services

Quality is a term that can have several definitions. Within the context of this work and according to Versen and colleagues (2000), “quality is the totality of characteristics of an entity (can be an activity or a process, a product, an organisation, a system, a person or any combination thereof) that bear on its ability to satisfy stated and implied needs”. This implies that quality is: the qualities and characteristics of a product and/or service, which make it suitable to fulfil the demands of a customer, as well as, the totality of all activities of quality management, quality planning, quality control and quality testing in order to verify the conformity of all the operations involved in the product and/or service, to guarantee its suitability for all the established and expected demands (Versen et al., 2000).

The existing and established guidance for microbial and cell CCs was not enough, there was the need to create common quality standards for BRCs. Some countries have developed legislation specific for BRCs. This is the case of France, where *Association Française de*

Normalisation (AFNOR) created a French standard dedicated to quality management and quality control in BRCs, NF S96-900 (FBRCMi, 2013). The French standard is a standard that gives general legislative provisions of a QMS to guarantee the proper management of BRCs under a quality-oriented policy (NF S 96-900, 2008).

Although publications on collection management and methodology give information on protocols and procedures, the QMS must go further and set minimum standards. Additionally, the CABRI electronic catalogue project made available a set of guidelines to aid collections to put in place best practice (CABRI, 2012). These cover critical elements in the handling, storage, characterisation and distribution of microorganisms and cell cultures, and the handling of associated information. There are several standards that can be applied to microbiology laboratories such as Good Laboratory Practice (GLP) (Stevenson & Jong, 1992) and several ISO standards, for example: ISO 17025, ISO Guide 25, ISO 15189 and the ISO 9000 series (Lima, 2007; Smith & Ryan, 2012). There is a group of collections that acknowledged the importance of implementing quality systems based on recognised standards (Table 3) and have voluntarily decided to be certified or accredited.

Table 3 - Collections with certified or accredited QMS by independent third party. (Adapted from: A2LA, 2011; Smith & Ryan, 2012; FBRCMi, 2013).

Country	Collection	System
Australia	IFM-Quality Services Pty Ltd	ISO Guide 34:2009, in combination with the relevant requirements of ISO/IEC 17025:2005
Belgium	Institute of Hygiene and Epidemiology, Mycology (IHEM)	ISO 9000:2000 series
Belgium	Belgian Coordinated Collections of Micro-organisms (BCCM)\LMBP – Plasmid collection	ISO 9000:2000 series
Belgium	Belgian Coordinated Collections of Micro-organisms (BCCM)\LMG – Bacteria Collection, University of Gent	ISO 9000:2000 series
Belgium	Belgian Coordinated Collections of Micro-organisms (BCCM)\ <i>Mycothèque de l'Université catholique de Louvain</i> (MUCL)	ISO 9000:2000 series
Belgium	Belgian Coordinated Collections of Micro-organisms (BCCM)\DCG – Diatoms Collection	ISO 9000:2000 series
Belgium	Belgian Coordinated Collections of Micro-organisms (BCCM)\ITM – Mycobacteria Collection	ISO 9000:2000 series
Belgium	Belgian Coordinated Collections of Micro-organisms (BCCM)\ULC – Polar cyanobacteria Collection	ISO 9000:2000 series

Country	Collection	System
Brazil	Micoteca URM from Mycology Department, Federal University of Pernambuco	ISO 9000:2000 series
Cuba	<i>Colección de Cultivos Microbianos</i> (BioCC) from <i>Centro Nacional de Biopreparados</i> (BioCen)	ISO 9000:2000 series
Czech Republic	Czech Collection of Microorganisms (CCM)	ISO 9000:2000 series
France	<i>Collection de l'Institut Pasteur</i> (CIP), belonging to Biological Resource Centre of the Institut Pasteur (CRBIP)	ISO 9000:2000 series and NF S96-900
France	Pasteur Culture Collection of Cyanobacteria (PCC), belonging to Biological Resource Centre of the Institut Pasteur (CRBIP)	ISO 9000:2000 series and NF S96-900
France	Fungi Culture Collection (UMIP), belonging to Biological Resource Centre of the Institut Pasteur (CRBIP)	ISO 9000:2000 series and NF S96-900
France	ICAReB biobank, belonging to Biological Resource Centre of the Institut Pasteur (CRBIP)	ISO 9000:2000 series and NF S96-900
Germany	<i>Deutsche Sammlung von Mikroorganismen und Zellkulturen</i> (DSMZ)	ISO 9000:2000 series
Italy	Interlab Cell Line Collection (ICLC)	GLP
Japan	NITE Biological Resource Centre (NBRC)- National Institute of Technology and Evaluation (NITE)	ISO 9000:2000 series
Netherlands	<i>Centraalbureau voor Schimmelcultures</i> (CBS)	ISO 9000:2000 series
Portugal	Micoteca da Universidade do Minho (MUM)	ISO 9000:2000 series
Spain	<i>Coleccion Espanola de Cultivos Tipo</i> (CECT)	ISO 9000:2000 series
Taiwan	Arocrete Group Co. (AGO)	ISO 9000:2000 series
Taiwan	Culture Collection and Research Centre (CCRC) from Food Industry Research and Development Institute (FIRDI)	ISO 9000:2000 series
UK	CAB International Genetic Resource Collection (CABI)	Part of services to ISO 17025
UK	European Collection of Cell Cultures (ECACC), from Health Protection Agency (HPA)	ISO 9000:2000 series
UK	National Collections of Industrial, Food and Marine Bacteria (N CIMB)	ISO 9000:2000 series
UK	National Collection of Pathogenic Viruses (NCPV), from Health Protection Agency (HPA)	ISO 9000:2000 series
UK	National Collection of Type Cultures (NCTC), from Health Protection Agency (HPA)	ISO 9000:2000 series
UK	National Collection of Yeast Cultures (NCYC)	ISO 9000:2000 series

Note: NF S96-900 is a French standard dedicated to quality management and quality control in biological resource centres (FBRCMi, 2013).

Though there is an increasing acknowledgment about BRCs and their continuous quality attainment, it is hard to define specific and simple common criteria due to the complex variety of BRCs and their holdings and services.

There are numerous definitions, parameters, points and factors to consider and develop: the organisation (long-term sustainability, management responsibilities, qualifications and formation of staff, and hygiene); construction (specific areas and dedicated, national regulatory compliance on buildings and levels of physical containment, access, maintenance and inspection, external support services and suppliers); use of equipment, calibration, testing, maintenance records and logs; documentation of quality management; Information Technology (IT) (application of computers and telecommunications equipment to store, retrieve, transmit and manipulate authenticated data with standardized terminology and format, internet publishing and data protection; laboratory protocols (preparation of media and reagents); access to deposits of BRCs (reception and handling of biological materials, documentation, quality check); preservation and maintenance (methodology, stock control, storage and validation of methods and procedures); supply and distribution (requests accepted only when verified and according to relevant national and international regulations, associated information about the material to be supplied, packaging, invoices and distribution charges, traceability of materials, management of nonconformities (NC) and complaints, reimbursement, confidentiality); evaluations, reviews and audits (definition of responsibilities, implementation processes, methods and procedures for the verification and validation of quality and audits) (Lima, 2007).

All of the developed work is intended to embed the traditional collections in this new concept: BRC, as well as the requirements and specificities inherent and contribute to the constant and dynamic development of new projects and collaborations with the same goals. History has shown that shared resources are more valuable and the communities that share the most, gained the most (McCluskey, 2011). All of the existent projects and collaborations are proof of the effort being made. The collections have also enhanced their skills regarding management systems for quality, administrative and technical procedures (e.g. NP EN ISO 17025), GLP (Stevenson & Jong, 1992), management processes to provide quality products that meet customer requirements (ISO 9001:2008), and supplying biological reference materials (ISO Guide 34).

ISO 17025 was approved by members of the ISO in December of 1999 and refers to general requirements for the competence of testing and calibration laboratories (Honsa & McIntyre, 2003). Thus, it fails to address some of the key operational requirements of BRCs. Some of these are the compliance with various legal requirements in association with practical tasks like: the handling and shipping of biological materials, the use and preparation of reagents, media, and other supplies, a strategic plan for BRC future sustainability in order to avoid the loss of biological resources, and data management and staff qualifications and competence. However, in some other aspects, it goes too far and its total application would demand that each process, each preservation technique, each authentication method, and the supply of strains, would have to be independently accredited. Despite this, ISO 17025 could be used by a BRC to demonstrate its competence in the preservation and supply of authentic materials (Smith & Ryan, 2012).

GLP are a set of practices for quality system of management controls for research laboratories and organisations to try to ensure the quality and integrity of chemical (including pharmaceuticals) safety and efficacy tests (WHO, 2009; Davis et al., 2012).

ISO Guide 34 refers to general requirements for the competence of reference material producers. It has been recommended by accreditation bodies to be the most suitable for BRCs. However, it was written for reference material producers and used for the calibration of measuring equipment and for the evaluation or validation of measurement procedures such as pharmacopoeia standards and substances differing when applied to living biological material. The guide states that the reference material producer shall use documented procedures based on accepted statistical principles for the assignment of property values and lays down the procedures on which this should be based. Many of these principles cannot be applied directly to living cells, and this must be taken into account in documents that would give guidance on the accreditation procedure for BRCs. The implementation of ISO guide 34 requires the additional implementation of other guides (NP EN ISO/IEC 17025, 2005; Smith & Ryan, 2012).

ISO 15189 is specific for medical laboratories – it refers to particular requirements for quality and competence. The standard was developed by the International Organisation for Standardisations' Technical Committee 212 (ISO/TC 212). ISO/TC 212 assigned ISO 15189 to a working group to prepare the standard based on the details of ISO 17025 and ISO 9001. It is a document that takes into consideration the specific requirements of the clinical environment and the importance of the clinical laboratory for patient care, and helps laboratories in developing their quality management systems and assessing their own competence. It can also be used for

confirming or recognizing the competence of medical laboratories by laboratory customers, regulating authorities and accreditation bodies (NP EN ISO/IEC 17025, 2005; ISO, 2013g).

So far, BRCs have been implementing the existing legislations and adapting to it, but with the continual search for improvement legislations are now adapting to the common needs of all BRCs.

1.4.1. Characterisation and Identification of fungi

Data concerning each fungal strain, morphological and molecular descriptions, including the spectral data (Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and Fourier Transform Infrared (FTIR) Spectroscopy), physiological and biochemical properties, ecological roles, and societal risks or benefits, are key elements in fungal identification. In order to attain a sound fungal identification a polyphasic approach is required while characterising each strain. It is achieved through the integration of all biological traits data (Simões et al., 2013a).

Identifications can be a long and seemingly never-ending processes with frequent revisions of the taxonomic schemes and even more complicated for non-specialised researchers as each taxonomic group has specialised literature, terminology and characters. This occurs to the extent that identifications can only be undertaken by a narrow group of skilled specialised scientists. Data derived from a polyphasic approach (Figure 1) allows to easily get an accurate identification and authentication, in order to structure reliable CCs and to generate accurate, quality and useful data (Passarini et al., 2013; Simões et al., 2013a).

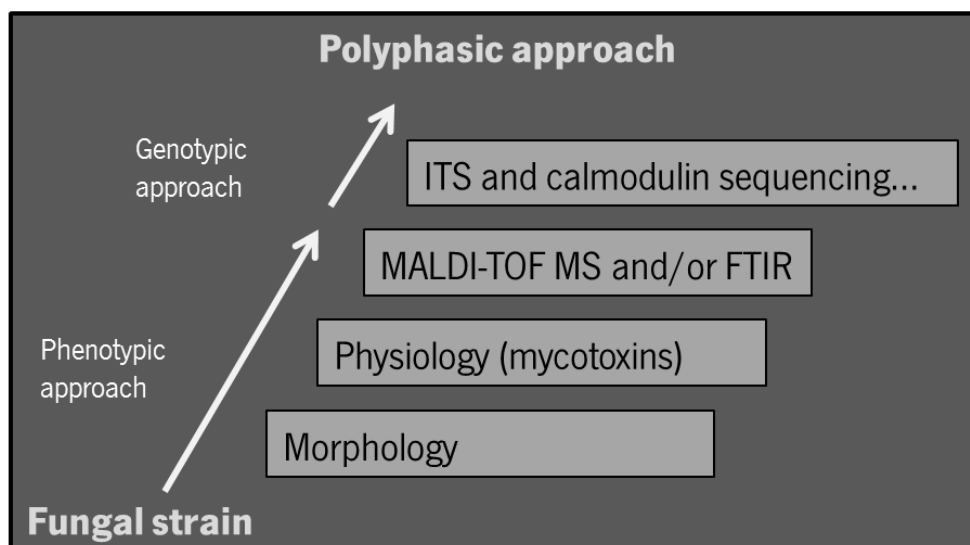


Figure 1 - Polyphasic methodology of filamentous fungi identification (Simões et al., 2013a). Note: ITS means Internal Transcribed Spacer.

The above described polyphasic approach recognises molecular biology as the gold standard technique in the step-by-step identification, but it does not exempt any of the other methodologies (Balajee et al., 2009; Santos et al., 2009; Santos et al., 2010; Simões et al., 2013a).

Along with the rest of biology, the study of filamentous fungi has been revolutionized by molecular techniques. In eukaryotic organisms, particularly the fungi, the non-coding Internal Transcribed Spacer (ITS) regions, ITS1 and ITS2, have been particularly useful at the genus and species levels because of a high degree of sequence variability (Klein & Paschke, 2004). Also β -tubulin and calmodulin genes as well as actin and α -elongation factor are rather effective (Samson et al., 2007; Balajee et al., 2009).

The first methodology commonly used in the polyphasic process is morphology, with micro and macro characterisation, followed by biochemical screenings, spectral analysis and molecular biology. Due to the costs and answer time, molecular biology is normally used as the last methodology in the polyphasic approach proposed at the laboratory of MUM (Simões et al., 2013).

1.4.2. Preservation of fungi

Parallel with the process of isolation, selection, characterisation and identification a need arises for the preservation of strains, their vitality, specificity, activity, immunogenicity and other properties in laboratory conditions. The production standard and quality depend on the right choice of preservation methods for the strains (Uzunova-Doneva & Donev, 2004-2005). A variety of methods are available for strain preservation, which keep their vitality and authenticity.

Maintaining and preserving fungal cultures are essential elements of systematic and biodiversity studies. Since fungi are such a diverse group, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological and genetic integrity of the cultures over time. Though, the cost and convenience of each method are important aspects to be taken into consideration. To help in the decision making of a preservation and storage method, decision-based keys can be used. It is important to consider that no preservation method should be assumed to guarantee total physiological and genetic stability of an isolate; this is why it is recommendable to preserve several replicates of fungi strains and use more than one technique to reduce the chance of strain deterioration and strain

loss (Ryan et al., 2000). Preservation techniques can be differentiated based on several parameters like the storage period, being short- or long-term and costs. The main preservation techniques are shortly described in Annex I. The choice of preservation methods depends on the species of concern, on the objective of the preservation (whether it is for a short-term assay or if it is for perpetuation), the resources available (both human and technological) and the goal of the project. No single technique has been applied successfully to all fungi (Kolkowski & Smith, 1995; Abd-Elsalam et al., 2010). This is why, whenever possible, the fungal strains should be preserved in more than one of the methods to guarantee safe deposits for future supplies.

Small culture collections have many difficulties in using expensive methodologies and have to restrain themselves to simple, inexpensive and sometimes not so successful methods.

The methods used in MUM for preservation are chosen among the following: subculture, silica-gel, mineral oil, water, freezing and lyophilisation.

1.5. Background and outline of thesis

BRCs and CCs are, as already referred, an important base for several areas of knowledge, R&D and for the development and maintenance of several industries and areas of work (biotechnology, clinical and health care, food industry and pharmaceutical among others). Nowadays, this importance has been gaining more acknowledgment which makes Quality the key issue for both BRCs and CCs all over the World. These driving forces have been playing an important role on the prosperity, development and support among all BRCs and CCs.

The main goal that lead to this work was the strive for utmost Quality, specifically in the environment of an academic located CC: Micoteca da Universidade do Minho. Two main subjects were studied and addressed: 1) the implementation of a management system to ease all administrative issues and 2) technical methods and procedures that ensure the preservation of samples collected.

Chapter 1 presents a general introduction to the context and all related subjects on BRCs and CCs, providing background on the subjects covered in this thesis and stating the basis for all the developed research.

Chapter 2 explains the importance of a well-established QMS and describes the implementation and maintenance of the QMS based on ISO 9001, at MUM, that led to certification in 2011.

In Chapter 3 the preservation through lyophilisation was evaluated. The procedure was done by accelerated storage and strains analysis at several different time points through a polyphasic approach. This approach allows the strains characterisation with several different techniques (photography, stereomicroscopy, optical microscopy, scanning electron microscopy, spectrophotometry, HPLC, MALDI-TOF MS and molecular biology analysis).

Chapter 4 shows the results obtained for two new explored methods on the preservation of a group of selected strains with the objective of being used, as an alternative, for the preservation of delicate and/or recalcitrant strains of fungi. These were perlite and alginate encapsulation. Their use has already been researched on some fungal strains but some possibilities were not covered and considered and it was this chapter intention to contribute to the increase of information on these new possibilities.

Chapter 5 states the general conclusions and future perspectives, respectively.

At the end of the thesis, a reference section complies all references cited in the thesis.

2. CERTIFICATION OF THE QUALITY MANAGEMENT SYSTEM OF MUM IN CONFORMITY WITH THE STANDARD NP EN ISO 9001

2.1. Certification – the worldwide impact of the ISO 9000 family

There is a consensus that the relationship between an organisation and its customers is critical to the survival and success of that organisation. Such relationships are especially important and applicable in the case of services (Bendapudi & Berry, 1997). To improve and contribute to these relationships legislation has been created: ISO 9000 family of standards.

ISO is the largest standards developing organisation of the world.

ISO standards are a very important part of a complex system of solutions for the global challenges of our days. They exist to facilitate world trade, clarify the market and competition, and disseminate useful technologies and good business practices. ISO 9001 is nowadays implemented by more than one million organisations in 179 countries. ISO gives the requirements for a QMS that enables organisations to meet the quality requirements of their customer in terms of customer satisfaction, regulatory compliance and continual improvement (Ollila, 2012; ISO, 2013a).

Because the demands placed on ISO in the near future are going to be heavy and complex, ISO will need to build and strengthen its position. It will need: to apply the principle of continual improvement embedded in its ISO 9000 series of standards; to leverage the extensive network it has at its disposal to increase the visibility of International Standards and to promote the ISO system more broadly as an increasingly attractive platform for the development of globally and market-relevant standards (Takeda, 2011; ISO, 2013a).

ISO 9001 was the first universally recognised management system standard and soon after its use, it was realized that the principles underpinning it could be extended to other areas such as environmental, health and safety (ISO, 2013f).

ISO 9001 certification is frequently used to establish confidence between business partners, to select supplies in global supply chains or as a condition to tender for contracts. ISO 9001 is successfully implemented by large and small organisations, both multinationals and

Small and Medium-sized Enterprises (SMEs), in manufacturing and services, in business and public sectors, in industrialised, developing and transitional economies (Poza et al., 2009; ISO, 2013b).

When implemented properly, ISO 9001 ensures that the promises an organisation makes to its customers, will be kept. That implies having an effective management involvement and commitment. It means that key processes are properly understood and sometimes redefined; and, that the continuous improvement cycle, a key characteristic of the QMS is fully comprehensive, having specified sets of actions. Performance of an implemented QMS can be determined, but in order to do that, one needs to have reliable metrics and data from which to make fact base decisions (ISO, 2013f).

It is of high importance to define key processes and their responsible. The key points and indicators for the quality of the implemented system facilitate goals definition, identify risks related to the legal demands, provide a common tool to level everybody's knowledge, motivate to have improved productivity and raise an opportunity of group work. Also, one should make the performance noticeable by monitoring the efficacy and efficiency and there should be specific criteria to identify all necessary resources (Tsim et al., 2002). Corrective Actions (CAs) should be defined, comparing the obtained results with the objectives and analysing the deviation causes. The system should be continuously improved (Cockalo et al., 2011; ISO, 2013e).

Besides all the benefits, ISO 9000 has been regularly criticised and designated as paper-driven and overly bureaucratic, leading to collaborators and employees resistance (Tsim et al., 2002; Ollila, 2012). This can happen when top and middle management lack the skills needed for interpreting and implementing results obtained through QMS (Ollila, 2012).

2.1.1. ISO 9001:2008 - standard requirements, scope and application

The International Standard ISO 9001:2008 (latest version in ISO 9000 series) was officially published on the 15th November 2008.

ISO 9001:2008 has attracted all kind of different disciplines, markets, industries, professions and sectors. This happens because the requirements for QMS are the same, independent from the geographical location and the QMS architecture is generic and applicable to any type and size of organisation (Tsim et al., 2002). This standard has proven to be an effective model for managing an organisational system.

The ISO 9001:2008 is based on eight core quality management principles (Table 4) that are the foundation of all systems.

Table 4 - Core principles of ISO 9001:2008 (Bendapudi & Berry, 1997; Rebull, 2008; Cockalo et al., 2011; ISO, 2013i).

Principle	Description
Continual improvement	This should be a permanent feature of any organisation that really wishes to excel within its marketplace. Whatever the organisation does to improve performance should be subject to a continuing cycle of review, and this may result in the further raising of targets and goals to new levels. This is necessary to keep ahead of the competition.
Customer focus	For any organisation concerned about quality improvement, customers and their needs should dictate much of the strategy of the organisation, because, without customer satisfaction any organisation will ultimately fail. Organisations should understand the current and future needs of their customers and endeavour to meet or even exceed them. Customers can maintain relationship with an organisation either due to constraints or because of dedication. In either the case, it is the best interest of the organisation to offer the most satisfying products and services to comply with that relationship.
Factual approach to decision making	All key strategic decisions should be based on the analysis of data and information.
Involvement of people	All collaborators will provide the front line customer services and follow the vision of the leadership, but they need to be encouraged and involved. Their full involvement is a benefit for the organisation.
Leadership	The strategy, direction and ultimate success of an organisation is largely dependent upon its leadership, along with the environment and even the culture that is encouraged throughout the organisation. A clearly communicated vision and purpose on the part of management is a key in ensuring business improvement. Leaders establish purpose and direction of the organisation.
Mutually beneficial supplier relationships	The relationship between suppliers and customers can be seen as an interdependent partnership that provides mutual benefits to both sides. An organisation and its suppliers have an interdependent and mutually beneficial relationship that enhances the ability of both to create value.
Process approach	It is possible to see virtually all business activities as processes and managing in this way will enable greater efficiencies through a clearer view of what is on-going. The desired results are more efficiently achieved when activities and resources are managed as processes.
System approach to management	Management should view all activities and processes as parts of an integrated system. Identifying, understanding and managing interrelated processes as a system, encourages greater efficiency and effectiveness throughout the organisation.

An implemented QMS, according to the ISO standard, is a group of human resources, organisation, materials and information system, implemented in an entire organisation. It must

respect its Quality Policy (QP), attain the quality objectives and satisfy the customer through the products conformity according to the demands and requirements defined by: law, the customer (contractually) or the company/organisation. Quality guarantee is not the only goal; there are particular quality objectives to be implemented. The ISO 9001 standard specifies requirements for a QMS where an organisation is required to demonstrate its ability to persistently and continuously supply products that meet specific requirements (customer's or regulatory). The requirements of this international standard are generic, appropriate and applicable for all kinds and sizes of organisations, and appropriate and applicable for any kind of product. The organisation must have defined objectives for achieving customer's satisfaction which shall be achieved by the effective implementation of a QMS. When the goals are define, one should consider the actual and future needs of the organisation and the markets it wants to reach, results and conclusions of the meetings on management review, actual performance of the products and processes, satisfaction level of all the interested parties, benchmarking, opportunities of improvement; and all the necessary resources (ISO, 2013i).

The QMS has a hierarchy (Figure 2) which includes continual improvement activities, assurance of conformity to the requirements of the customers and assurance of conformity to regulatory requirements (9001 Quality management knowledge center, 2013).

There are several levels to consider when implementing a QMS (NP EN ISO 9001, 2008):

- Strategic: conformity and guarantee for the customer satisfaction, constant focusing on quality improvement, and guidance based on directed requirements for a management through processes.
- Tactical: increased interaction between quality and global management, progress in people management, and only essential documental exigency.
- Operational: planning of realisation processes, processes directly related to the customer, conception and development of the product, control of the product and services, and Monitoring and Measurement Devices (MMD).

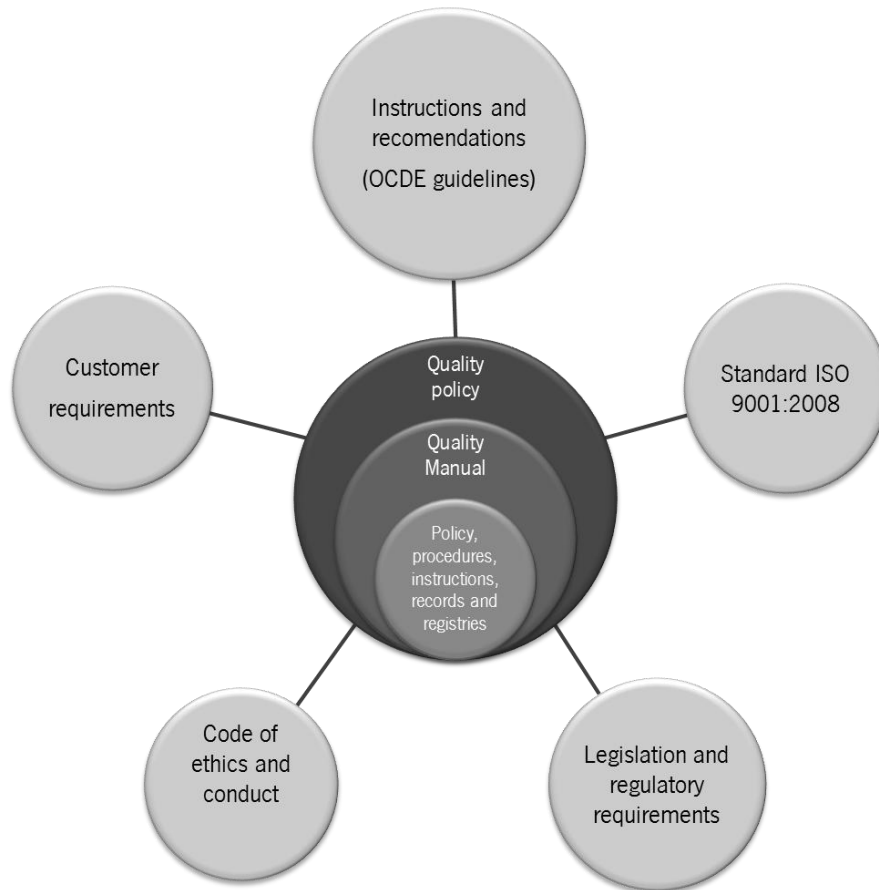


Figure 2 - Hierarchy of the Quality Management System.

Implementing a QMS implies the definition of several concepts (Figure 3): 1) **processes**, which are a group of activities related and dependent among them, which transform entry elements into exit elements and their supervision is a management responsibility. The entering of one process is usually the exit of another; in an organisation, the processes are usually planned and executed under controlled conditions in order to get product realisation and add up value; 2) **objectives**, that are defined and variable according to the conjecture and the organisation situation and result from measurement, analysis and continuous improvement; these allow to verify the fulfilment of the several steps from the QP as well as to evaluate their development; they must be expressed in a way that allows the analysis of the evaluation of the obtained results; 3) **continuous improvement**, which is the group of actions that increase the efficacy (rates of the performed planned activities and achievement of the planned results) and efficiency (relation between the obtained results and the used resources) of the activities and processes with benefits for both the organisation and the clients/customers; 4) **clients/customers satisfaction** that is referred along the entire standard 9001:2008, and creates a need to

identify all the needs of the customers; 5) **continuous improvement** on the efficacy of the processes in which the Plan-Do-Check-Act (PDCA) gets all collaborators to participate and measure the customers satisfaction as a rate of progress (Tsim et al., 2002; NP EN ISO 9001, 2008; ISO, 2013c).

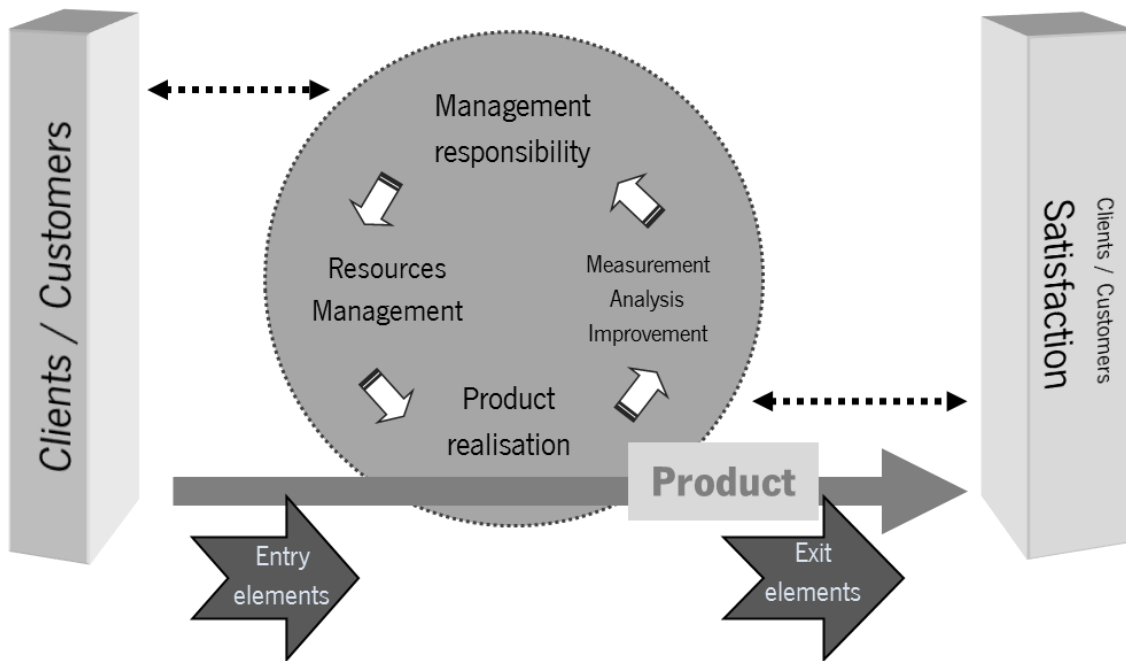


Figure 3 - Continuous improvement of a Quality Management System, through Plan-Do-Check-Act applied in processes based system (NP EN ISO 9001, 2008).

The processes created can be of different kind: a) management processes, processes from direction, that contribute to determine the policy, guide and ensure the consistency of operational or realisation processes and also the support processes; b) operational or realisation processes, these include all the processes that have the interface customer/customer, and contribute directly to the product realisation, detection of the needs of the customers and their satisfaction, they are related to the “life cycle” of the product; and, c) support processes, these are all the internal processes that need to be activated to respond to operational processes, giving them the necessary resources, these do not create direct value and acknowledged value by the customer but are indispensable to the good functioning of the organisation (EN NP ISO 9001, 2008; Cockalo et al., 2011).

After the QMS creation, it is, according to the standard, mandatory for its implementation to identify all the necessary processes to the QMS, and determine their sequence and interaction, as well as the criteria and methodology to assure their efficacy and their control; ensure the

availability of the necessary information for the function and monitor of the processes; measure, analyse and monitor them; implement the necessary actions to improve the attainment of the planned results and their continuous improvement. The documents required for these actions are the Quality Manual (QM), the documental control and records control (EN NP ISO 9001, 2008).

Within the QMS, a document is defined as a communication of information, an evidence of correspondence and must share knowledge. It has to be approved, and its responsible must be defined as well as who will approve it and when. Updating the documentation is another requirement that ensures the continuous improvement and the use of the last edition of the documents. For this, organisations manage a list of editions and updates for documents, to guarantee that the edition in use is the last edition. But to better manage all documents, organisations must document the method on the documents control procedure, recording information such as: date of last update, the reason for the update, the function who demanded the update and who authorized the update (Tsim et al., 2002; EN NP ISO 9001, 2008).

The control of documents should be made by: approving them according to their use before editing, revise them and update them on regular basis, identify the modifications and its actual state (by a given ordering number) all the documents (internal or external) must be identified or numbered and have their availability and distribution defined; make available all the necessary and useful versions of the documents, make sure that all documentation is available to be read and clearly identified, make sure that external documents are identified and their distribution is controlled and prevent the wrong use of obsolete documents. The entire documental base must have evidenced regular backups (NP EN ISO 9001, 2008).

To comply with the requirements of customers and achieve their satisfaction about the processes of the QMS, there are several points to take under consideration (Figure 4). It is the management responsibility to determine and define the QP, goals, identification of legal requirements, do the quality planning and keep the internal communication as outlined in QMS. For these, there are some points to consider, those being: resources provision, competencies, awareness and formation, information control and infrastructure and working environment control (NP EN ISO 9001, 2008).



Figure 4 - Schematic overview of all parameter involved in the Quality Management System implementation.

It is also part of the system to manage resources, these being human resources, infrastructures and working environment; and guaranteeing that all collaborators have formal education and regular formation for their specified job description and have all the equipment, space and conditions to effectively and efficiently perform their jobs.

Measuring and monitoring all the QMS is done by the annual Internal Audits (IA), by analysis of the customer satisfaction, for example through the use of inquiries, and through the evaluation of the performance of processes by defining specific measurable indicators.

The continuous improvement implies a constant evaluation of the implemented QMS and changes whenever they are regarded as necessary by all the collaborators of the organisation. This is done based on suggestions and claims, and all the notified preventive and corrective actions. The QMS is regularly analysed and internal and external audits are performed once a year.

In the contemporary global economy and highly competitive business environment, it is essential for an organisation to be customer-oriented and customer-centred. To have highly satisfied and loyal customers, organisations must strive to produce world class products and services of high quality. The satisfaction of the customers has been considered the key success factor for every profit-oriented organisation as it affects market share and customer retention. In addition, satisfied customers tend to be less influenced by competitors, less price sensitive, and

stay loyal for longer time. Considering that the satisfaction level is a function of the difference between perceived performance and expectation, ISO standard comes to emphasize the need to achieve high satisfaction of the customers and it is of ultimate importance to evaluate the satisfaction, this can be performed by data collection and by having reporting systems, but an easier way to do this is to use survey methodologies or simply have a questionnaire that will allow to quantify and measure the retrieved data (Bauer et al., 2005; Cockalo et al., 2011).

2.1.2. The impact of ISO 9001:2008 on Biological Resource Centres

BRCs aim at collecting, maintaining and distributing microbial strains, and are considered to be a way to preserve microbial diversity *ex situ*. They originated after Koch's school introduced pure culture techniques in bacteriology, and the first CC to provide services was established by Professor Frantisek Král, in 1890, at the German University of Prague, Czech Republic, who published the first catalogue of strains from a CC in 1900 (Canhos, 2003; Uruburu, 2003; Çaktü & Türkoglu, 2011). After the first collection of Král, many others were developed, with MUCL and CBS being two of the oldest working CCs established (Uruburu, 2003; Çaktü & Türkoglu, 2011). Since then, many others have been established, some with general purposes, others specialised in particular groups of microorganisms (Uruburu, 2003). In 1925, the American Type Culture Collection (ATCC) was created in Washington and is now located in Manassas, Virginia. With time, international connections between CCs were started and in 1970 the WFCC was created (Uruburu, 2003).

The ISO 9001:2008 standard requires certification by an external body and this independent external approval provides confidence on the consistency of an organisation. It is not specific to any production or services processes. It allows organisations to choose to include or exclude any given process from their QMS. ISO 9001:2008 allows and encourages quality system management at all levels. Any changes or alterations identified as beneficial can be introduced because it is flexible and dynamic. It requires continual improvement of the QMS through actions which can be derived from inconsistency data analysis. Also, ISO 9001:2008 is harmonized and non-confrontational with other international standards simplifying administration of the quality environment (NP EN ISO 9001, 2008; Davis et al., 2012).

The ISO 9001:2008 helps CCs to be more structured and organised, to achieve better results, consistency of products and services and by that, more benefits for customers. Certified CCs, just like any organisation, qualify for more tenders and have more satisfied customers and,

consequently, more orders and more profits. The ISO 9001:2008 puts the satisfaction of customer and continual improvement at the main objectives of the standard requirements (9001 Quality management knowledge center, 2013).

2.1.3. Micoteca da Universidade do Minho – a future Biological Resource Centre, its needs and goals

The quality assurance concept started to be embedded at MUM since its beginnings. MUM must meet the needs of its customers by being influential, flexible, globally relevant, and attuned to international business and other organisations. These were the main reasons that lead MUM to implement a QMS. The implementation was based on the standard ISO 9001:2008.

In its strategic plan for the next years, MUM has now committed itself to six major objectives: 1) its deliverables meet customer needs; 2) its standards promote innovation and provide solutions to address global challenges; 3) it excels in reaching out to and engaging stakeholders; 4) it fosters partnerships that further increase the value and efficient development of international standards; 5) MUM and its processes are significantly improved; 6) MUM and the value of international standards are clearly understood by customers, stakeholders and the general public.

MUM intends to address global challenges, along with customer needs, and the mission statement reflects this intention. It is action oriented, and to attain its vision and mission, MUM set several practical and achievable objectives which, together with a series of specific actions for their accomplishment, establish the strategic plan of action. The goals of MUM were developed in compliance with recognised best practice and objectives, from other types of standards or specifications like the OECD guidelines for BRCs (OECD, 2007; NP EN ISO 9001, 2008).

ISO certification as a result for MUM, will strengthen cooperation with other relevant CCs and also with, industry consortia, the scientific community and civil society. Considering the continuous improvement, MUM intends to significantly improve its processes, so that they become clearer, more transparent and rigorous to support the development of high quality deliverables.

The implementation of a QMS at MUM is hereby presented as a case-study.

2.2. Methods – the development and implementation of a Quality Management System at MUM

Initially, when considering the implementation of a QMS, preparation is necessary. Studying and understanding the ISO 9001 standard is the first step. Some organisations can appeal to outside organisations or individual entities for consulting services. In the case of MUM, GesQaf was the company hired for consulting services. GesQaf is a company of Consulting, Auditing and Training, created in November of 2008, located at Braga.

The first step was to build a detailed knowledge of the collection and future intentions and to have a structured awareness of what services were performed, the locations of operation and the number of people that work and collaborate with MUM. Being in the DEB building means that MUM has common areas with other laboratories, but has exclusive areas and the common spaces have detailed policies. MUM is located in a laboratory of mycology where daily research occurs, but all of the equipments of MUM are well identified and the ones that are considered critical for the QMS processes have exclusive use for the collection. At the time of certification MUM had only 3 collaborators, but at least 5 functions are defined in the Manual of Functions (MF).

The herbarium of MUM, a requirement of the Botanical Code when describing new species, was not considered for the QMS. It was not considered as in need of management and was therefore excluded of the QMS implementation.

After defining all the details and specifications necessary for the realisation of the final product, a conformity assessment of the available arrangements for quality management was made and all the required actions were listed for which an appropriate timetable was then created.

To implement the QMS, MUM established and followed a set schedule presented in a detailed timeline (Figure 5).

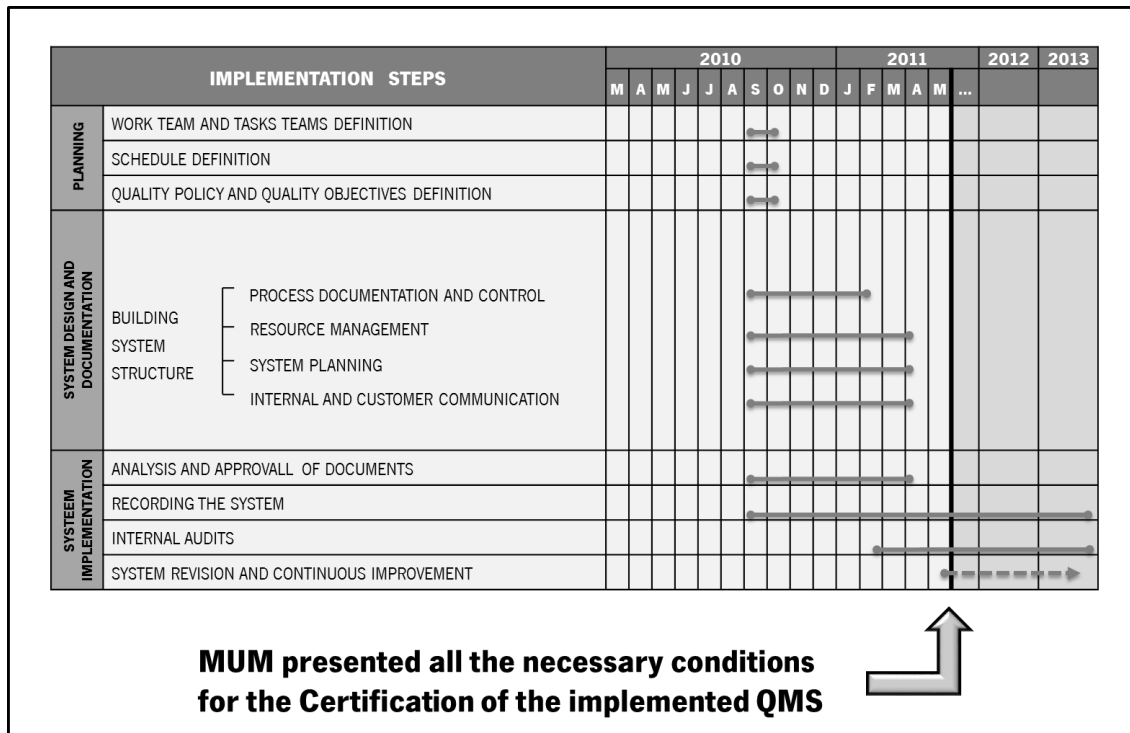


Figure 5 - Timeline for the implementation of the Quality Management System.

The implementation of the QMS occurred for almost one year, during which three main steps were developed.

2.2.1. First step of implementation

The first step was the planning of the QMS, where specific tasks were attributed to the different collaborators, deadlines were established and a QP as well as quality objectives were primarily and generally defined. To define the QP, account was taken for a commitment to quality, the valuing of customer’s needs and the supplying of high quality products and services. A mission was also defined to be included with the QP in the QM. And the processes for the QMS were established.

2.2.2. Second step of implementation

The second step of implementation was the design of the system and the development of the documental structure (Figure 6), with the creation and detailed description of: QP, QM and MF; detailed processes, proceedings and standard operating procedures (SOPs); forms, records and registries. The distribution of the several documents on the pyramid scheme presented in figure 6 was done according to their importance on the QMS.

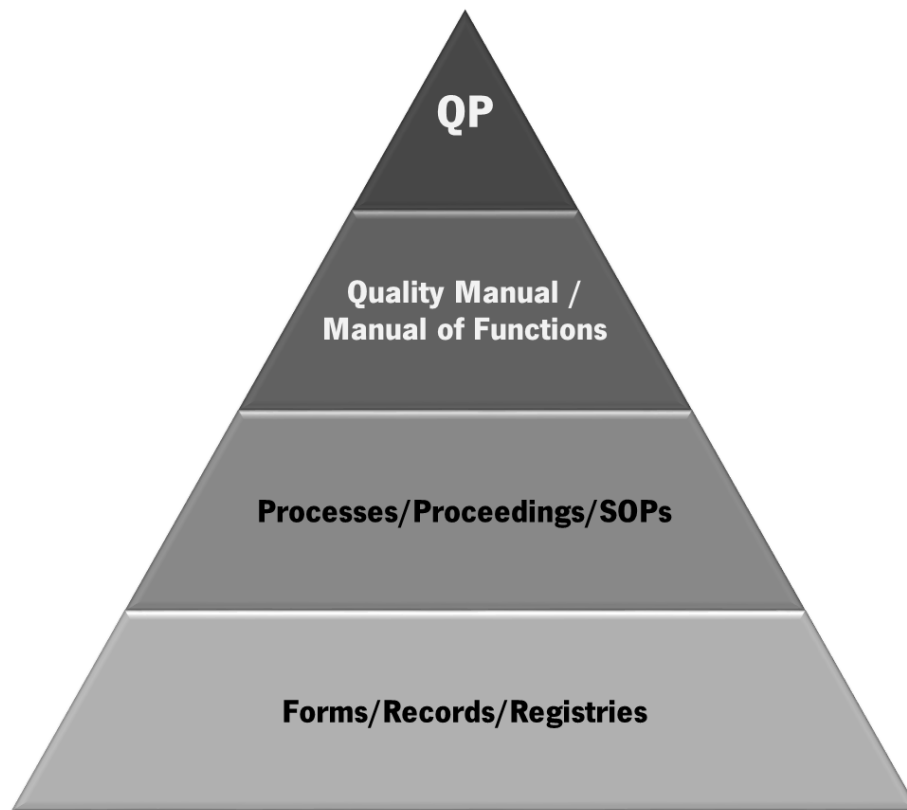


Figure 6 - Documental structure developed for the Quality Management System implementation.

The documental base for all the QMS was developed and included proceedings, SOPs, forms, records and registries. Another standard requirement is the control of documents, having a defined way of checking the last altered document, being able to distinguish different versions and identifying the last version created.

There were several stages on the implementation of the QMS: description and implementation of processes, procedures and SOPs; selection, evaluation and qualification of suppliers (reception of materials, NCs record, CAs request); infra-structure and work environment maintenance; Measurement and Monitoring Equipment (MME) (inventory, equipment charts, Control and Maintenance plan); human resources management (minimal skill, responsibilities and job description) and client's satisfaction evaluation (satisfaction inquiry, complaints evaluation, suggestions, Quality Plan).

In order to develop the management process to continually improve the services according to the defined Plan, quality objectives and goals were determined: strive for complete understanding and meet the standards of customer, continually improve MUM process performance by developing key indicators, to monitor and measure the processes, by

identification of critical success factors, follow the technological and scientific development of the sector by being present on a regular basis at relevant scientific events, continually develop the expertise, professionalism and integrity of MUM collaborators by appropriate training, maintain a dynamic work team, perform an innovative high-quality research and foster a team approach to detect prevention and problem solving. The quality objectives are formulated in accordance with the established QP, and like the key indicators, they are to be stipulated annually.

2.2.3. Third step of implementation

The third and last step was the implementation of the QMS with the achievement of the certification in May 2011. From this date on MUM has been keeping and will keep on performing a QMS constant revision, as well as annual IAs and external annual audits to identify issues and improve compliance and to maintain the pursuit for continuous improvement.

This will be assured by: audits, efficacy analysis, corrective and preventive actions, action planning and review of the entire system performed by all collaborators of MUM. And, records and registries provided evidence of compliance.

Audits, whether internal as external, provide information on compliance to internal documented procedures and to adherence to standard requirements. They initiate change and improvement. As audits continue and the system matures, findings become more detailed. The benefits include the changes made to increase compliance to documented processes by training of collaborators or by changing the processes to make compliance easier (Ollila, 2012).

The real advantages of implementing a quality system begin after the certification process. Proper maintenance of the system is required to uncover issues and constantly improve. It requires the coordination, monitoring and completion of required actions. Records keeping provides information on the adherence to system practices as well as the groundwork for process control measurement. Review of these measurements and responses to observed tendencies provide continuous improvement of the system (ISO, 2013c).

2.3. Results – MUM certification

2.3.1. Planning of the Quality Management System

An effort was made to comply with the timetable originally designed. Following that agenda, covering the most issues and topics during the planning facilitated the compliance.

The QP is a set of intentions and orientations of MUM, related to quality, formally expressed by top management: Director/Quality Director (QD). The QP of MUM explains the commitment to Quality by valuing customer's needs and supplying high quality products and services. To guarantee the QP, a main goal was established: the on-going development of management processes to continually improve the service by. This goal was achieved by: using and developing key performance indicators; creating a dynamic work environment by being innovative and performing high-quality research; following the technological and scientific development of the sector and continuously developing the expertise, professionalism and integrity of the collaborators of MUM.

The QP constitutes the baseline reference of the QMS of MUM defining the general objectives from the perspective of requirements to meet and continual improvement. In order to verify its constant adequacy, it was considered important to establish an annual revision of the statements. It is the Director/QD responsibility to make sure that the QP is reported, understood and internalised by all the collaborators of MUM and that it is disclosed to the exterior. One possible form of disclosure is to post the document on the website of MUM.

MUM defined its mission as to:

“provide the highest quality services to our customers, collecting, maintaining and supplying fungal strains and their associated information for teaching and research in biotechnology and life sciences. MUM intends to be a centre of knowledge, information and training in mycology, operating at a global level and under national and international regulations”.

It also defined three processes (Annex II), those being: Material Reception Process (MRP) (Annex II.A), Material Preservation Process (MPP) (Annex II.B) and Material Supply Process (MSP) (Annex II.C). Each process was fully described and a scheme was made with all the actions related to each process and all the forms and SOPs to be used in each step of the process. MUM also developed a documental base for all the QMS including proceedings, SOPs, forms, documents of informatics registry, as well as quality objectives and goals for a continuous improvement.

2.3.2. System design and documental structure

MUM started to implement the QMS based on international references. Due to this implementation, and together with the fact that MUM is involved in international projects with international partners, it made sense to have all the system in English, this being a final goal to

attain. But, since part of the documental structure was already being used in Portuguese, it was decided to maintain a bilingual system and in time change all the Portuguese documents into English. For this reason, most of the forms created and used exist in two versions, one in English and another in Portuguese, with different attributed code numbers (a list of all the created forms is presented as Annex III). In a total of 98 forms created, 45% are in Portuguese. Part of these forms is used to record data relating to processes and is kept as registries once they are filled.

The internal diffusion of every document was decided to be evidenced by signing a specific form created for the purpose of recording the revisions and distributions of documents (F.015) (Annex III).

Each document has acronyms attributed according to the type of files, followed by a sequential number, a dot and the number for the version in use. Forms – F; Processes – P; SOPs; and Recipes of Media and Solutions – MS.

The acceptance of standards and guidelines to be used in the QMS of MUM was attained by consensus meetings of all collaborators and the consultant. All legislation documents were carefully chosen according to MUM objectives and were maintained in the QMS as external documents (Table 5) to which a control is made through a record of all versions used and update or altered. External documents include recommendations and disclosure documents both legal and scientific (Table 6) and they comprehend the “state of art” of the processes used in the implemented QMS.

Table 5 - List of external documents used in the Quality Management System of Micoteca da Universidade do Minho.

Document (type and nº)	Date	Subject
Decree-law No. 84/97	16 th of April, 1997	Minimal prescriptions for protection of security and health of employees against risks associated with exposure to chemical and biological agents during work.
Ordinance No. 1036/98	15 th of December, 1998	Alteration to the list of classified biological agents, annexed to the ordinance No. 405/98.
Portuguese standard NP EN 1619	May, 1999	General requirements for management and organisation for strain conservation procedures.
Directive 2000/54/CE from European Parliament and Council	18 th of September, 2000	Relative to the protection of workers against risks associated to the exposure of biological agents during work.
Biological resource centres – underpinning the future of life sciences and biotechnology	2001	OECD guidelines.

Document (type and n°)	Date	Subject
Decree-law No. 290/2001	16 th of November, 2001	Rules for the protection of employees against exposing risks to chemical agents.
Interpretive Guide – ISO 9001:2000 – APCER	December, 2003	Interpretive Guide.
WHO laboratory biosafety manual	2004	Recommendations of World Health Organisation (WHO) on good practices and biosecurity in the microbiology laboratory.
Portuguese standard NP EN ISO/IEC 17025:2005	December, 2005	General requirements for the competence of testing and calibration laboratories.
Portuguese standard NP EN ISO 9000:2005	December, 2005	Describes fundamentals of quality management systems, which form the subject of the ISO 9000 family, and defines related terms.
OECD best practices guidelines for biological resources centres	2007	These best practice guidelines are intended to serve as a target for the quality management of biological resource Centre collections.
Internal Emergency Plan – Universidade do Minho	2008	Detailed information about physical infrastructure of Minho University.
WHO guidance on regulations for the transport of infectious substances 2009–2010	2008	Recommendations of World Health Organisation (WHO) on the transport of infectious substances.
WFCC information resource on international postal regulations for shipping biological materials	April, 2008	WFCC recommendations on international postal regulations for shipping biological materials, the current up regulations and their background.
Portuguese standard NP EN ISO 9001:2008	November, 2008	Specifies requirements for a quality management system.
APCER – the brandt	11 th of May, 2011	Explanatory guide.
Decree-law No. 3/2009	5 th of January, 2009	Conditions under which certain harmful organisms, plants, plant products and other materials may be introduced or moved within the community.
Commission directive 2008/61/EC	17 th June, 2008	Establishing conditions under which certain harmful organisms, plants, plant products and other objects may be introduced into or moved within the community.
WFCC guidelines 2010	2010	WFCC recommendations WFCC relating to the implementation and operation of CCs of microorganisms.
Guide of good practices in a mycology laboratory	2012	Added to the external documents after the implementation of the QMS, on the 23 rd of April of 2012.
Security Manual – Department of Biological Engineering	2012	Added to the external documents after the implementation of the QMS, on the 23 rd of April of 2012.

Key indicators for each process to evaluate performance and analyse the quality objectives were defined as the following: 1) the percentage of strains mistakenly identified or with insufficient information; 2) the percentage of requests accepted for deposit; 3) the percentage of

non-viable strains after accepted request for supply; 4). the percentage of complaints; 5). General Index of Customer Satisfaction (mean value obtained from the question of the inquire of satisfaction); 6) Time elapsed between the request and the response there to that request. The key indicators are quantifiable parameter that show if performance is being successful according to the criteria designed and imposed by MUM.

The guidelines and standards were chosen to create all documental structure and to define the several types of documents defined as internal documents (Table 6). These have a sense of duty attached to them. They have an internal obligation within MUM and a binding relevant character that implies compliance to what has been described as essential to the processes.

Table 6 - List of internal documents used in the Quality Management System of Micoteca da Universidade do Minho.

Document:	Code:	
Processes	Material reception process	MRP
	Material preservation process	MPP
	Material supply process	MSP
Procedures	Nonconformities and corrective actions	NCCA
	Control of documents	CD
	Control of records	CR
	Monitoring and measuring equipment management	MMEM
	Preventive and improvement actions	PIA
	System management	SM
	Staff training	ST
	Criteria for deposit of strains	CDS
	Internal audit	IA
	Provision	AP
	Control of access	CAC
Quality Manual	MQ	
Function manual	FM	
Standard operating procedures	Lyophilisation preservation	SOP.001
	Mineral oil preservation	SOP.002
	Silica-gel preservation	SOP.003
	Water preservation - 1	SOP.004

Document:		Code:
	Water preservation - 2 (miniaturised)	SOP.005
	Maintenance at 4 °C	SOP.006
	Cryopreservation (-80 °C)	SOP.008
	Potentiometer maintenance	SOP.009
	Quarantine	SOP.010
	Isolation and purification	SOP.011
	Biological material packaging, transport and shipment	SOP.012
	Surfaces and hands disinfection	SOP.013
	Handling microorganisms in bsl2 laboratory	SOP.014
	Waste and disposal	SOP.028
	Media and solutions	SOP.029
Media and solutions	76 different recipes of media	MS.

The necessary processes to the adequate performance of the activities of MUM, in the context of its QMS, have fully detailed descriptions and include the objectives of the processes and the applicable indicators to ensure the monitoring throughout the different activities and over time. In the description of the processes, are referred indicators identified as likely to be used for monitoring of these processes; however, their effective use is, for each period, dependent on the defined objectives. Equally, other indicators might be identified for the processes. They also mention the sequence of activities and their descriptions; the responsible for the realisation of the activities; the criteria for decision making and the methods that assure the realisation in effective and efficient terms; and, the necessary information for their realisation.

The processes, which sustain the QMS, and their interrelationships are represented/described in the model of key system areas of ISO 9001:2008 designed for MUM (Figure 7).

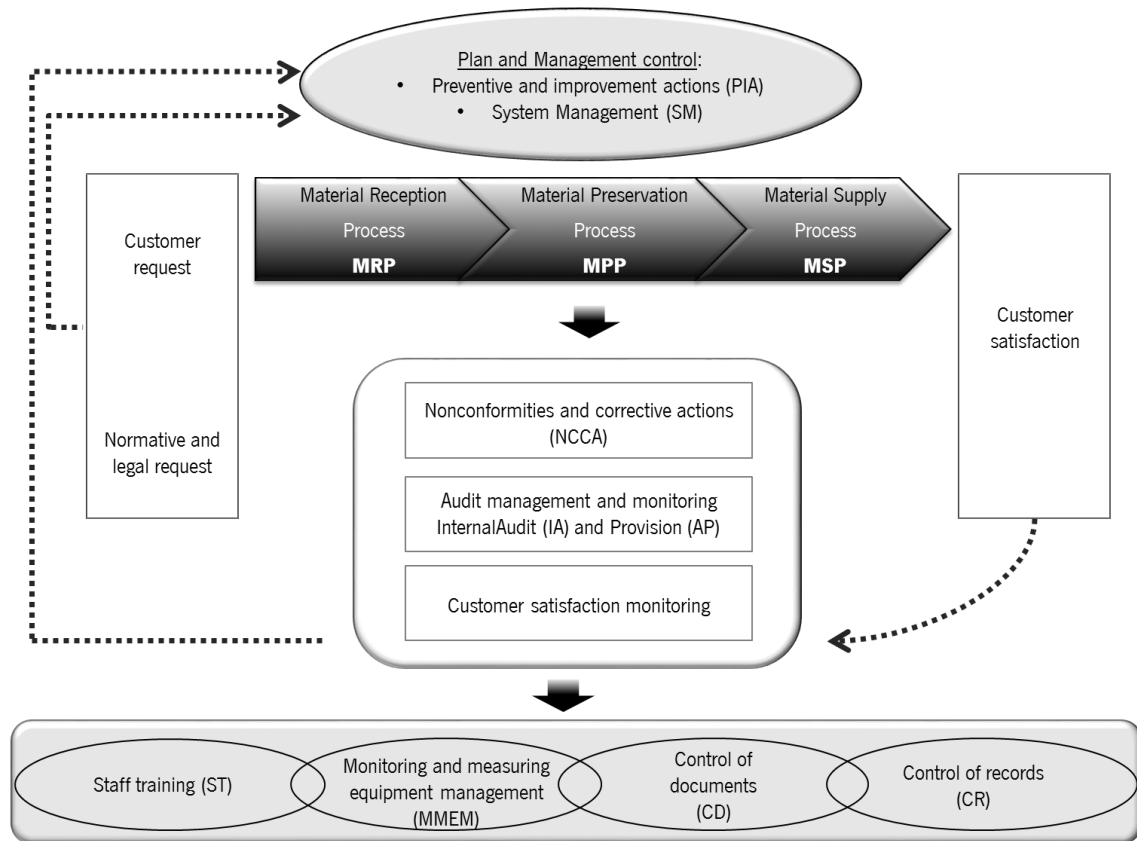


Figure 7 - Model of key system areas of ISO 9001:2008, with all processes and procedures that sustain the Quality Management System and their interrelationships, at Micoteca da Universidade do Minho.

For all the procedures listed in table 6, a set of information was established, defined and recorded. All of the procedures have objectives, scope, responsibilities and detailed information on how to proceed.

2.3.2.1. Procedures description

Preventive and improvement actions (PIA) is the procedure that defines the methodology used to correct and eliminate the cause (or causes) of possible NC and to introduce improvements in the system. It applies to all cases that could lead to a NC. When a potential cause of NC is identified, a preventive action is defined in a specific form (F.006) in order to eliminate it. Any collaborator can register the preventive action but has to ensure that the Quality Manager (QMan) is aware of it. Its analysis is periodically made by the QD together with all the available collaborators. Once identified the cause, the QMan shall define the required actions, its goal, the implementation period, the responsible for the execution and the responsible for monitoring the implementation. Analysis of effectiveness of the implemented preventive action takes place within the period prescribed and is done by verifying the achievement of defined goal,

by QD, or QMan (in his absence). If the preventive actions were not successful (goal not achieved), the causes shall be analysed again and new preventive action is delineated.

System management (SM) is the procedure intended to ensure the management revision from the collection and analysis of data, relating to processes, products and QMS - to identify trends and improvement opportunities - in order to guarantee the adequacy and fulfilment of the QP, the establishment of quality objectives, planning and development of the system and the processes improvement, giving fulfilment to the requirements and thus obtaining customer satisfaction. On an annual basis, despite the possibility of accomplishment of extraordinary "management revisions", a meeting is scheduled by the QD with the goal of reviewing the QMS, planning activities, planning changes and identifying possible needs. At the Management Review Meeting, conducted by the QD, should be reported and analysed the data related to: adequacy of the QP and other system documents as the QM; results of audits and status of preventive and corrective actions; information on the degree of customer satisfaction (customer complaints and suggestions and inquiries results); results of process measurement and monitoring and how quality objectives were achieved; analysis of changes that may affect the QMS, including those resulting from legislation and sector specific applied regulation; verification of the implemented actions from previous management reviews; and, recommendations for improvement.

Nonconformities and corrective actions (NCCA) is the designation of a procedure applicable to all NC identified during the activity of MUM. It defines the methodology used to correct and eliminate the cause/causes of detected NC in order to prevent their recurrence. A NC involving a client (complaint included) must be resolved as soon as possible and, if necessary, a subsequent contact is arranged.

Staff training (ST) is the procedure that describes the methodology established to implement training plans for the collaborators of MUM. Actions of training and the evaluation of its effectiveness are carried out in ensuring that all collaborators possess the awareness, knowledge and appropriate skills to best perform their duties. It was established that during the first quarter of the year, an annual Staff Training Plan (F.018) should be prepared, based on identified training needs established by the QD during management review, through an informal way, by asking each collaborator about the areas where he/she wants to acquire more information and knowledge, and after analysing the

training plans available. Besides this survey, the collaborators have the opportunity, throughout the year, to inform the QD from the training needs felt in the conduct of their working projects. If new training is decided then the annual Staff Training Plan is reviewed. QMan prepares the staff training, by sending the registration form to the training entity.

Monitoring and measuring equipment management (MMEM) is the procedure created to define the management methods of monitoring and measuring equipment in order to ensure their calibration, internal tests, verification and conservation. It must be applied to all equipment performing measurements and determinations with relevance to the quality of product or service yielded from the processes. For this procedure several responsibilities were attributed to the QMan: elaboration and updating of MME inventory, making of the equipment coding, elaboration and execution of an equipment calibration plan and verification of the calibration and internal test results of the equipment considered as critical for the performance of the processes.

Each equipment was identified with a label bearing its code and serial number, as specified in form F.009 and calibration requirements were determined and registered in form F.008. Both labels are placed together in a visible part of the equipment like shown in the example in figure 8. The encoding of the equipments is composed by two letters (XX), representing the type of equipment, and a sequential number (Ox) within the same type of equipment, separated by a dot like shown in figure 8.

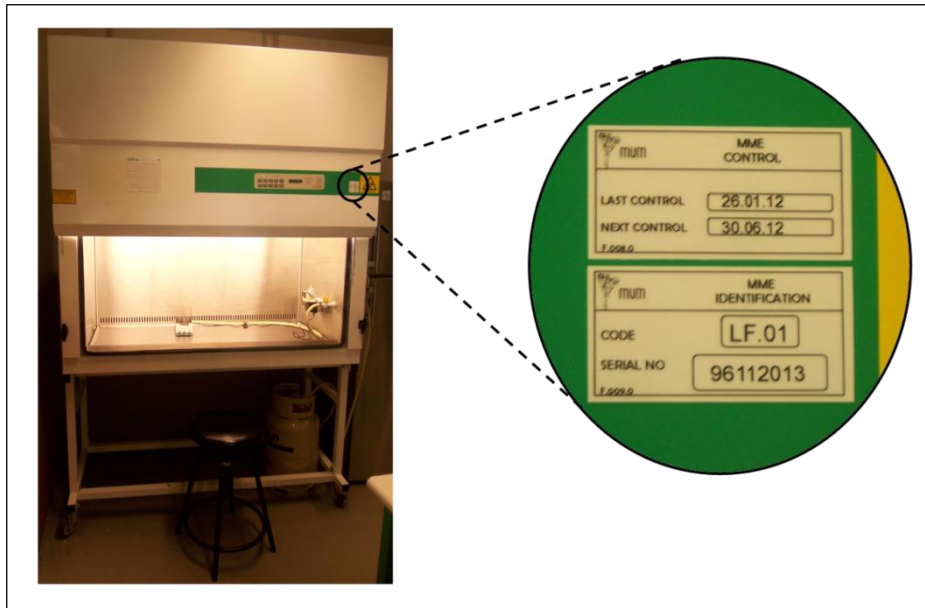


Figure 8 - Example of labels (F.008 and F.009) used on the laminar flow cabinet/biological safety cabinet (LF.01) of Micoteca da Universidade do Minho.

The external calibrations of equipment are carried out through accredited laboratories, and its evidence is made by certificates from the accredited laboratory.

Devices that are not subjected to calibration or other type of control are identified with a label stating "NOT SUBJECT TO CONTROL" (Figure 9), corresponding to form F.038.



Figure 9 - Example of a label (F.038) used on an incubator (IC.01) from Micoteca da Universidade do Minho not subjected to control or calibration, placed together with F.009.

The Control of documents (CD) is the procedure that establishes the methodology used for preparation, modification, codification and control of documents. It applies to all the documents that have an impact on MUM products, services and MUM itself and which are: QM, documented statement of QP, documented processes, procedures and SOPs, Fs, informatics documents of registries (IDR), applicable legislation, FM, reference standards and other applicable regulations, equipment manuals and safety data sheets. Preparation of documents is responsibility of the QMan, however, any collaborator can suggest the establishment or amendment of a document. Documents like: processes, procedures or standard operating procedures should contain an objective, a description on how to proceed and responsibilities. Inputs, outputs and methods for measuring and monitoring processes must, additionally, be defined on processes description or related table. QMS documents shall be approved by QD by signing and recording the date. IDRs are approved by QD by making it available through the computer system and by giving it an access password. While revisions made on forms are recorded on Forms List (and IDR on IDR List), revisions of the remaining documents are recorded on "Record of Revisions and Distribution" (F.015).

F (or IDR) code is incremented in "1" when reviewed. The other documents revision is pointed by the new date. Therefore, any document of QMS highlights when it was created or last updated. Documents are available for consultation by all collaborators, in its original file. Whenever a document is shown or given to a collaborator, the form F.015 has to be signed by that collaborator.

Distribution of documents is managed and controlled as follows:

- Forms: QMan gives a copy to collaborators when they need it.
- QM, Processes description, Procedures description, Standard Operating Procedures: QMan gives a copy to the collaborators who request it and collects the copies when the document in question is revised.
- External documents, approved by the QD, are presented to all collaborators at the meetings or posted on specific locations to disseminate. To highlight the acknowledgement of those documents, collaborators sign them.
- IDRs are made available through password access distributed by QD. Backup plans were developed to grantee the safety of MUM account files.

The Control of records (CR) was the procedure created to establish the methodology used in order to manage the records elaborated under the operation of the QMS: its identification, file mode, retention period and manner of destruction. During the file retention time, which is defined by the QD (Annex III – List of forms created for the QMS of MUM), records in paper format are subjected to proper storage to keep them from deterioration. The computer records are subjected to a regular backup. All records, like contact reports, are filed for, at least, five years and are responsibility of QD. They are stored in files properly identified always containing an index.

Provision (AP) was the procedure created to establish and describe the activities and responsibilities associated with (1) control and supply of products/materials and (2) request services, relevant to the quality of the final product. This procedure specifies a methodology for selecting and evaluating suppliers. It applies to the purchase of chemicals, laboratory supplies and services, such as technical assistance on the equipment, and suppliers of products and services relevant to the quality of the final product. The need to purchase chemicals is identified by warning of reduced stock given by a computer application on a specific IDR: "Stock Management Product". The stock monitoring equipment is made by a different IDR: "Material Management Stock".

The supplier is selected from the list of suppliers detailed on another IDR ("Suppliers List and Evaluation"). And whenever necessary to choose a new supplier, criteria is followed for that purpose, according to the evaluation made that considers the speed of response when contacted, cost and payment terms, image and position in its market area among other that might appear as important at the time of choice.

The list of products, solvents and solutions is maintained on an IDR database ("Chemicals and Suppliers").

The management of all the IDRs related to AP are the responsibility of the Curator. But, any critical and final decision is made by the QD.

Control of access (CAc) is a procedure that aims to establish rules for access and movement of people within the premises of MUM and it applies to all collaborators and outsiders. Visitors are not permitted to enter in the laboratory, unless previously authorized by the Director. Any visitor of MUM must display an identification document (F.022 or F.037), obtained when authorized to visit. The Curator is responsible for receiving requests and ensuring that a registry of entrances is made on an attendance book. The entry for

repairs, revisions, delivery and maintenance of equipment is permitted only upon official request to the Director of MUM and after delivery of the budget in case of technical interventions.

All contacts (fax, telephone, mail or personal) received at MUM are numbered and recorded by the Curator or the Technician in a specific F (F.081). Whenever considered relevant, additional information must be described on a report of contact (F.080).

After-hours access to facilities of MUM also requires previous authorization by the Director. Given keys to access MUM are registered on F "Key Distribution of MUM" (F.070). Access to restricted areas is conditioned by the existence of lock.

Finally, in terms of access, the data for all material preserved are stored in a database with password entry. It is up to the Director to define levels of access to the database.

Criteria for deposit of strains (CDS) is the procedure that sets the rules in order to receive a biological sample for deposit at MUM. A formal request must be made by the use of an official document or a signed form. The deposit can be free or confidential. MUM only accepts for deposit fungi that belong to risk groups 1 or 2 in accordance with Directive 2000/54/EC of the Council on the Protection of Workers risks related to Exposure to Biological Agents at Work. In a free deposit, the most common at MUM, the strain will be preserved and kept in the general collection of MUM and will be available for supply and distribution.

In a confidential deposit, MUM makes the preservation and long-term storage of fungi, and the distribution of strains is restricted, according to the instructions of the depositor/customer. The strains are owned by the depositor and all information concerning the applicant and the nature of the deposited material is strictly confidential. These samples do not appear in the catalogue of MUM, or at any public list of strains. Access to this deposited material is only possible through formal written request made by the depositor/customer. MUM acts merely as a guardian of the deposit and only performs the operations necessary for the preservation, maintenance, rejuvenation and viability check of deposited material.

Internal audit (IA) is the designation of the procedure that defines the actions of planning and execution of IAs and subsequent actions to evaluate the adequacy and effectiveness of the QMS. A program of annual audits (Form F.016) is prepared in the first quarter of every year to guarantee that all QMS processes are audited. And audits are conducted

based on the standard references, documental system and legal requirements. The audit team is selected by the QD, based on curriculum vitae analysis and according to the minimum skills stipulated on the MF. After every audit, a report is prepared and then analysed by all collaborators and actions derived from it are taken.

2.3.2.2. Manuals: Quality Manual and Manual of functions

The QM created describes all the means and procedures adopted by MUM to ensure a QMS adequate to its activities. It is a support document that has the descriptions of the organisational structure, responsibilities, practices, procedures, processes and resources and presents the QP. The QM reflects a set of standards of MUM and the commitment of the Director/QD for the Quality Management.

The QMS of MUM was implemented on a baseline reference to promote the continuous improvement. It is the responsibility of the Director/QD to ensure compliance at all levels, of all the determinations set forth in the QM. The Director/QD has self-appointed representative to the issues related to implementation, monitoring and control of the QMS: the QMan. The QMan also describes the Mission, the QP and the entire QMS, and is to be applied in all the functional structure of MUM.

Functions for the collaborators of MUM were established and an organigramme was defined (Figure 10) and is available for consultation on the QM.

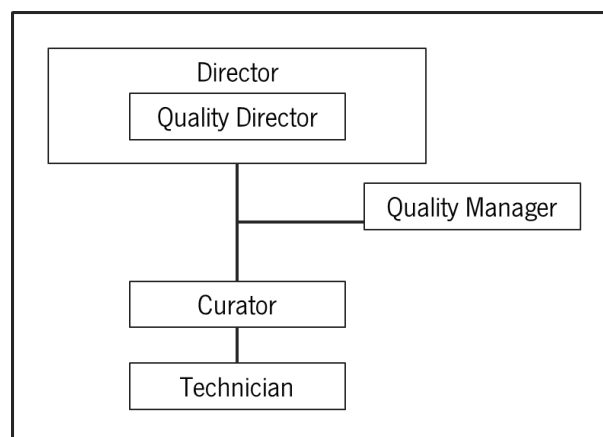


Figure 10 - Functional organigramme of Micoteca da Universidade do Minho.

The minimal competences and skills necessary for each function are object of evaluation in the moment of admission of collaborators. But there is a list of specifications, mandatory for

each function defined on the MF as well as responsibilities, authority and competences associated to each function (Table 7). The Director of MUM, assumes the responsibilities related to Quality Direction, being also the QD. All documents of QMS refer those two functions indistinctly.

Table 7 - Profile and minimal skills required for each function, included in the Manual of function of Micoteca da Universidade do Minho.

Function	Profile And Minimum Skills Required
Director/ Quality Director	Ph.D., with microbiological skills recognised nationally and internationally. High expertise on mycology covered by high number of publications in the field. With <i>h index</i> ≥ 10 .
Quality Manager	Ph.D., with scientific skills on microbial identification and authentication and, experience on fungal CCs. Leadership competences and strong commitment with microbial CC quality management.
Curator	MSc as minimum requirement. High skills in microbial preservation techniques and in daily base quality management.
Technician	BSc, as minimum requirement, with skills to maintain the operational work on the microbial CC.
Internal Auditor	Academic degree in life sciences; training in Auditing practices; minimum experience in QMSs of two years. Auditor of a Certification Body dispenses other requirements except graduation in life sciences.

The Director/QD of MUM takes on the compromise of: making the organisation understand the importance of customer satisfaction through the compliance of all the requisites, needs and expectancies implicit and explicit; establishing the QP, disclosing it and reviewing it whenever necessary, periodically establishing the quality objectives resultant of the performed revisions; making sure that the QMS is analysed and reviewed periodically in order to maintain its adequacy; and assuring the availability of all the necessary resources for the good performance of MUM.

But, the Director/QD, just as all of the other collaborators of MUM, has detailed responsibilities, authority and job description (Table 8).

Table 8 - Responsibilities and authority for each function, included in the Manual of function of Micoteca da Universidade do Minho.

Function	Responsibilities and authority
Director/ Quality Director	Plans, directs and coordinates MUM in all its activities and tasks. Determines the execution/suspension of services; approves/rejects products, processes and services; provides the acquisition of equipment and infrastructure; defines levels of access within the system; defines, adopts and communicates the QP, ensuring that it is understood by all collaborators. Sets annual process indicators and objectives; approves the QM; ensures monitoring and dissemination of all documents relating to applicable legislation; analyses Internal and External Audit Reports, records of complaints and NCs and ensures the effectiveness of corrective actions implemented. Promotes and monitors preventive and improvement actions; approves the Annual Plans of Training, Audits, MME Control and Maintenance of Infrastructure; ensures monitoring and dissemination of all documents relating to legislation; promotes and coordinates the annual review of the QMS to ensure their adequacy to QP and objectives established; approves processes and other documents of the Quality System; identifies and approves the need of resources; approves the number of strains to preserve; performs the database update; manages the acceptance and refusal of deposit requests; manages the acceptance and refusal of material supply; approves shipment contents and packaging of sent material; ensures the MUM's data confidentiality; authorizes the access to MUM in extra-time providing the necessary keys.
Quality Manager	Develops the QM, process description and other documents of the QMS, in collaboration with the QD and other collaborators; gives support in the implementation of the QMS; ensures monitoring and dissemination of all documents and records of the QMS; ensures Annual Plans execution; monitors and resolves system deviations; identifies and records any problems relating to product and QMS. Participates in the definition of corrective and preventive actions as well as their monitoring, implementation and efficacy analysis. Collects and analyses system data; ensures records and documents management and maintenance; analyses and participates in customer complaints resolution; prepares Annual Plans of Training, Audits, MME Control and Maintenance of Infrastructure and proposes its approval to the Director.
Curator	Manages the reception and archive of deposit request, deposit request decisions and notifications to costumers; records and manages all the contacts made/received within the activity of MUM; makes all the necessary contacts with costumers; performs procedures for quarantine and material acceptance/rejection for preservation (after quarantine) and request of new material, when necessary; analyses and approves material identification; performs the attribution of MUM access number for the collection; decides on the choice of the appropriated preservation method; Identifies the material samples to preserve; manages the storage of material samples to preserve; records material preservation date; records material preservation necessary data; decides on the master cell bank use; performs and checks material confirmation of identification, viability and purity. Decides on the disposal of material with non-confirmed identification; takes stock material to supply; prepares shipment of requested material; manages customer satisfaction inquire delivery, collection and data treatment; manages and performs material Identification and characterisation; manages the stock (setting minimum and maximum stock products, order products, product return, product reception and monitoring of products expire date). Manages product storage. Is responsible for forward visitors and ensures all the necessary records. Ensures the compliance of bio-safety, bio-security and laboratory rules of MUM, of all collaborators. Provides to new collaborators the necessary training to attain the needed skills for their function; ensures evaluation and qualification of suppliers; and controls first aid cabinet contents.
Technician	Performs material identification and characterisation; performs material disposal; collects, delivers and proceeds with the material supply requests; receives material orders and verifies their compliance; and, reports any problems found, incidents or accidents.
Internal Auditor	Performs the IAs included in the Annual Audit Plan and for which he/she is nominated. Performs the Audit Report.

The MQ also institutes that MUM must identify the needs of acquisition of physical resources and infra-structures necessary to its proper functioning, and must assure its operability. The infra-structure that supports the processes is adequate to the realisation of the product and the supply of services, consisting of: building; equipment; specific furniture for the storage of material; material and laboratory equipment; air-conditioning system; informatics equipment; application software and communications network. The necessary actions to maintain the infra-structure available include preventive maintenance and corrective maintenance. The preventive maintenance is planned (Form F.013, Infra-structure Maintenance Plan) and registered after being performed.

The working environment is maintained adequate to the activities of MUM, being these: environmental conditions secured by an air conditioning system and systems of incubation, refrigeration and deep-freezing. Given its relevance to the system, the latter are measured and monitored by specific equipment (data logger).

2.3.3. Analysis and approval of documents

MUM assumed and described on its QM, the compromise of assuring that the QMS is object of periodic revision, in order to verify its adequacy. It was established that this revision shall occur during the first trimester of each year, and the Director/QD decides whose presence is mandatory. During the meeting for revision, all data derived from the system must be analysed and properly treated. This meeting must provide the exchange of ideas on the functioning of the system, evaluate the actual performance of processes and define future actions to improve the overall performance. And the review of the system must be recorded.

From the daily routines and the occurrence of the defined processes, registries were recorded on the appropriate created forms. This type of document has a continual use and data is accumulated to generate evidence of the implemented QMS and to guarantee traceability for each steps of each process. From these activities some forms end up being altered to be more adequate for the specified tasks and some are removed from the QMS for demonstrating to be useless and inadequate for the processes. Also, with time, new forms can be created if collaborators find it necessary.

Complying with the established procedure for monitoring of general and associated documents, MUM ensures that the basic documents that support the QMS are defined in terms

of format and content; approved before they are issued; subjected to review and update when necessary, being possible, at any time, to identify their review status; available, in an updated version, in all the locations where they are needed; kept legible and available for use during a period of time appropriate to the needs of MUM and to its commitments with customers and other stakeholders; available in file archives; and, whenever becoming obsolete are promptly removed from the daily use, destroyed or identified as obsolete.

According to the dispositions described on the procedure for the control of records (CR), MUM assures that the records that evidence the functioning of the QMS and the obtained results are controlled for their unambiguous identification; kept in appropriate storage conditions; protected from damage caused by misuse; kept for a period of time compatible with the commitments of MUM with the customers and other stakeholders; destroyed appropriately to prevent improper dissemination of information with confidential character or with exclusive interest for MUM.

The system is regularly analysed and annual IAs are performed. The auditor chosen for IA was contracted for the purpose. During IAs, the effectiveness of the QMS was assessed, and compliance with the requirements of processes and reference standards is evaluated. The first IA was performed on the 18th of March, 2011. From its results, MUM implemented 11 corrective actions.

During IA several points are analysed: possible opinions, complaints and suggestions of the customer, previously defined key indicators, nonconformities, preventive actions, improvement actions, follow-up actions arising from previous revisions, changes that could affect the QMS, analysis of improvements made in the system, list of future improvements to the system. IAs are made in order to analyse: the adequacy of the QP, MQ, quality objectives and other system documentation; previous audit results; the feedback of customers; the results of monitoring and measuring processes, product conformity and quality objectives; the status of corrective and preventive actions; the follow-up actions from previous management reviews; the changes that might affect the QMS, including legislation and regulatory changes; and define recommendations for improvement.

The *Associação Portuguesa de Certificação* (APCER) is a private Portuguese organisation dedicated to the certification of management systems, services, products and people, to ensure quality and foster competitive advantages to organizations, public or private, both national and international. It was the entity chosen for the process of certification of the implemented QMS. In

Portugal, APCER is a market leader, and is the unique Portuguese entity representative of the international certification bodies the International Certification Network (IQNet) which allows immediate international recognition of entities certified by APCER.

The certification audit of concession, first phase, took place on 29th March, 2011. It was performed by an auditor from APCER. All elements of the ISO standard were covered. The auditor looked for evidence of compliance with the ISO standard and with internal documents. This evidence included records of analysis, customer contracts, equipment and calibration, training records, and interviews of personnel. Any nonconformities (NC) required a written corrective action. All of this is reviewed by the auditor on the next audit. On the audit of first phase, the auditor detected 8 opportunities of improvement.

On the audit of second phase, also performed by an auditor from APCER, on the 12th of April, 2011, MUM had to respond to 3 NCs and the auditor suggested 5 opportunities of improvement.

MUM obtained its certification on 4th May, 2011, and received the certificate of registration (Annex IV), proving conformity and the international certificate from IQNet (Annex V). From this date on, MUM started to include the certification logo (Figure 11) on marketing and divulgation documents and emails.



Figure 11 - Logo of certified organisation by *Associação Portuguesa de Certificação* (APCER).

The use of the logo came with some rules that were included on the list of external documents used in the QMS of MUM.

About a year after obtaining certification, during which the implemented QMS was maintained and improved, MUM performed a new IA on 23rd April, 2012. And, a new first follow-up audit was performed, by an auditor from APCER, on the 30th of April, 2012. From this audit,

MUM had to respond to 1 NCs and the auditor suggested 8 opportunities of improvement. But all the system presented evidences of its maintenance and continual improvement.

2.4. Discussion

2.4.1. Assessment of the implemented Quality Management System

The first IA was performed by a third party contracted specifically for that purpose. It could have been performed by a collaborator of MUM but to have total assurance of the system readiness, it was decided to appeal to an unbiased subject.

2.4.2. The impact of ISO 9001:2008 on Micoteca da Universidade do Minho

Collections chose to implement QMS for several different reasons. It needs to be noted that ISO 9001 is mostly a tool, and as such, it can be wrongly or improperly used leading to increased costs and frustration.

Davis and colleagues refer a research infrastructure, UK DNA Banking Network (UDBN), which considered enabling appropriate access to the resources as their primary goal. Just like MUM, they understood that, that access required not only a fair access policy but also a QP implemented via a QMS. They also decided to achieve consistency in sample management by identifying and implementing a suitable QMS with external certification and also chose ISO 9001 (Davis et al., 2012). Just like in the case of MUM, ISO 9001 was selected because of its greater breadth of scope.

Many of ISO 9001:2008 users are in the service sectors. MUM is a particularly interesting example of ISO 9001 in action because it is in the service sector for being a Portuguese filamentous fungi CC inserted in an academic environment.

All requirements of ISO 9001:2008 are generic and are intended to be applicable to all organisations, regardless of type, size and product provided. This generic character is essential because it allows the flexibility that an academic located CC, like MUM, needs to introduce or remove a process and to design their QMS to the specific and changing needs of the collection. Whenever any requirements of ISO 9001:2008 cannot be applied due to the nature of an organisation and its product, this can be considered for exclusion. When exclusions are made, claims of conformity to ISO 9001:2008 are not acceptable unless these exclusions are limited to requirements within Clause 7 of the standard, and such exclusions do not affect the ability of the

organisation, or responsibility, to provide a product that meets customer and applicable statutory and regulatory requirements.

From the NP EN ISO 9001:2008, MUM considered the requirements for "Design and Development" as excluded - clause 7.3 - because they were deemed as not applicable to the activity of MUM. It was considered that the exclusion of this clause does not affect the ability nor the responsibility to provide products and services that satisfy customer requirements, and legal standards. Furthermore, as defined in the QM of MUM, the quality resulting from the individual initiative is essentially based on the qualification of staff, in the supporting documentation, and in the continuous monitoring by the Director of MUM.

But MUM has all the infrastructures to change this exclusion and include it on the implemented QMS, this is one of the advantages of ISO 9001:2008, it allows the continual dynamic evolution of the organisation and its QMS.

There is always the need to improve internal operations, formalize the know-how, and qualify all the involved collaborators. MUM has evolved from an internal quality to a QMS with international recognition. Competitiveness is nowadays a constant, not only from the point of view of supply capacity to the customer, but also in terms of contests/support/finance/attribution of projects and others in terms of finances. The implementation of the QMS and its certification are an evidence of the will and action of organisations regarding the reach to meet the expectations of both customers and stakeholders.

The ISO 9001 allowed MUM and CCs to become part of a socio-economic logic that aims to combine high-level scientific research with the provision of specialized services rigorously managed. This is also the logic behind the concept of BRC that intends for the traditional CCs to evolve as infrastructures of support for science and biotechnology future.

2.4.3. MUM before and after its QMS

MUM is a CC created in the academic environment, inside a department of teaching and research within a university, and fully adapted to that co-existence. Being in a developing and growing environment meant the increasing need for space both for MUM and the department where it is located. But so far, both organisations have managed to compromise and cooperate in terms of space, common services and equipment.

Along time, MUM has been gaining access to equipment (e.g. High-Performance Liquid Chromatography (HPLC), MALDI-TOF, Fourier Transform Near Infrared (FT-NIR) spectroscopy)

and state-of-the art research and methods that has been using. MUM has multiple and regular contacts and collaborations with other national and international CCs and related organisations. MUM belongs to several international organisations and has hosted several international meetings, workshops, courses and conferences. All of these facts imply a constant and dynamic development. Having all the parameters to guarantee a complete satisfaction of all customers it was a logical and natural step to achieve a certification of quality. Therefore, all of the collaborators of MUM contributed to the implementation of a QMS based on the ISO 9001:2008. This led MUM to reach a new step in its existence: it attained all the necessary conditions for the certification of the implemented QMS and obtained the certificate of ISO 9001:2008 for the Deposit, Preservation and Supply of Filamentous Fungi from the independent assessment agency (Portuguese Association for Certification - APCER with the International Certification Network - IQNet). With the certification for its QMS, MUM now provides greater credibility and competitiveness, either national as internationally. Certification was the result of implementing an effective quality system and now allows faster identification and resolution of any steps or parameters regarding methods, collaborators or equipment, improved customer satisfaction, achievement of quality requirements and overall increased quality of services.

Of the 633 CCs from 72 countries registered in the WFCC (WFCC, 2013), MUM is the 1st Portuguese and the 23rd CC in the world to obtain this important qualification.

After having an implemented QMS and keeping the maintenance of all the key elements of the QMS, performing regular IAs to keep on achieving continuous improvements is a beneficial obligation. There are specific advantages from the QMS implementation and certification, these being: improved control and planning, improved efficiency and productivity, consistency in products and services, reduced waste of time and resources, reduced costs and improved collaborators with higher retention capability and higher motivation.

MUM has now a clearly-defined mission and benefits from staff with high international reputations; possesses well preserved and characterised filamentous fungi supported by an excellent database; is a descriptor of species new to science; leads Portuguese studies of filamentous fungi; is recognised internationally as the most well-structured CC in Portugal; is a core member of international initiatives and projects (e.g. OECD, WFCC, ECCO, GBIF, GBRCN, EMbaRC, MIRRI); is well known for very high numbers of international research papers, post-graduate research, and R&D projects; and, has excellent relationships with stakeholders and end-users to sustain its work.

The obtained certificate will allow MUM to have a better international status and closely approach to being a BRC as defined by OECD. MIRRI, the latest engagement of MUM, proposes an European infrastructure for microbial CCs as described in the ESFRI roadmap, which was unanimously approved by the 27 participating EU countries (European Communities, 2009; MIRRI, 2012). All the referred data prove the high level of commitment of MUM to keep on the strive for continuous improvement.

2.4.4. Biosecurity assurance at Micoteca da Universidade do Minho

Best practices defined for BRCs and CCs demand the performance of authentication tests and establishes baseline information for storage, maintenance check and validation after preservation. The OECD best practice guidelines instruct in order to operate a maintenance plan for periodic control of preserved organisms. These guidelines were taken into consideration at MUM and most recommendations are followed. For example, biological material is preserved whenever possible by at least two different preservation methods. Master and working cell banks must be kept to guarantee a minimum number of transfers or generations from the original material. Guidance requires the assurance of biological material stability, detailed inventory control, restocking practices and their documentation, validation of methods and the recording of related data and details. Also, quality audits and revision of procedures are regularly performed at MUM (Smith & Ryan, 2012).

2.5. Conclusion - the future of Micoteca da Universidade do Minho

MUM will continually strive to become better and will focus on building awareness of the importance of collections certification for the recognition of the importance of its activities.

With a clear mission, and well defined objectives and actions, there is no doubt that MUM will achieve its vision and is now an example of excellence for other collections.

Globalisation has made the world smaller, making the challenges of the few into the challenges of the many; and being part of national and international consortia and organisations, with mutual consensus and harmonised action will be a positive contribution to today's challenges.

The obtained certificate has further enhanced the reputation of MUM as a CC that pursues the goals of excellence and leadership implicit in these new and exacting standards. Additionally, MUM has become an even more relevant partner of the EMbaRC project and the

GBRCN. MUM is fully committed to: the OECD best practices guidelines relating to quality management for BRCs, and to the MIRRI project, which is ongoing.

MUM intends to keep on being a centre of knowledge, information and training in mycology, operating at a global level and under national and international regulations, with services of high quality and authenticity, oriented for problem-solving and engaged in the valorisation of the chain of knowledge, investing in research, development and innovation. This is why, in a near future, it is MUM's intention to broaden the scope of the actual certification and include R&D and training as new certified services.

Many CCs offer training workshops to the scientific public. CABI (www.cabi.org) provides training in molecular identification of microorganisms, morphological identification of fungi, microbial techniques, isolation methods for fungi and bacteria and preservation of microorganisms through accredited microbial training courses. CECT (www.cect.org) offers workshops on maintenance and characterization of microbial strains. CBS (www.cbs.knaw.nl) offers courses on fungal biodiversity, food and airborne fungi, and medical mycology (Boundy-Mills, 2012). And MUM has organised in the past, in Portugal and abroad, several meetings and specialized courses. This is an area that can be included in certification of MUM and is under evaluation for that purpose.

Though, it is becoming clear that ISO 9001:2008 is not enough to cover all the operations of MUM; and although it helped put in place a good management system, it does not address the product or the competence to produce it. Often the microorganisms that CCs maintain, authenticate, grow, preserve, and supply are referred to as reference strains. ISO have a number of standards that apply to reference materials, and these have also been adopted and tested by CCs. This is why, to better assure and maintain the search for excellency, MUM is also considering to do its accreditation based on the standard NP 17025 and will be attentive to new standards that might be developed and applied to BRCs. the formal recognition by an independent body, generally known as an accreditation body, that a certification body is capable of carrying out certification. Accreditation, just as certification, is not mandatory but it adds another level of confidence, as 'accredited' means the certification body has been independently checked to make sure it operates according to international standards (ISO, 2013d).

Faster detection and resolution of issues regarding methods, collaborators or equipment, improved customer satisfaction, and overall increased laboratory activities are all the result of implementing an effective quality system which can be assured by complementing the already

existent certification with a future accreditation. MUM would then benefit from more frequent audits by external auditors to keep the system sharp.

3. CONTRIBUTIONS TO THE DEVELOPMENT OF THE MUM PROCESS: MATERIAL PRESERVATION PROCESS: EVALUATION OF PRESERVED *ASPERGILLUS* (SECTION *NIGRI*) STRAINS

High-quality BRCs and global network efficiency is being accomplished by the transition of traditional culture collections to BRCs. In order to support this transition, an international quality management criteria and appropriate preservation technologies and data management are mandatory. A huge demand lies on assuring the authenticity of the preserved strains, and supplying these strains to the users as a certified reference material. As a consequence, there is a need to increase our knowledge and understanding about how to preserve microorganisms and to implement mechanisms to collate, analyse and provide related information. This chapter intends to support these goals.

3.1. The section *Nigri* of *Aspergillus*

Fungi are an extremely vast and diverse group of eukaryotic microorganisms with recognised biotechnological potential. At this stage this kingdom, Mycota, accepts one subkingdom, 10 subphyla, 35 classes, 12 subclasses, and 129 orders. However, fungal classification is very dynamic as shown by recent discovery reported by Jones and co-authors (2011). They referred the new proposed phyla Cryptomycota (fungi isolated from aquatic environments which also include the genus *Rozella* considered one of the earliest diverging lineages of fungi), and showed how much less we know about fungi, even today. The fungal taxonomy evolves continuously with successive redefinitions of the fungal tree of life. From the existing fungi, only a small part has been acknowledged and fewer have been fully characterised (Jones et al., 2011; Simões et al., 2013a).

The first description of *Aspergillus* dates from 1729, when clerical Pier Antonio Micheli described the genus. He gave it this name because the spore-bearing structure characteristic of the genus resembled an *aspergillum*, a liturgical device used by the Catholic Church to sprinkle

holy water (Klich, 2002; Dijksterhuis & Wösten, 2013). The *Aspergillus* genus has a worldwide distribution, being one of the best characterised genus of the filamentous fungi due to its economical relevance in industrial processes (Astoreca et al., 2010). Aspergilli are among the most abundant organisms on earth, they have evolved the capacity to synthesize a myriad of metabolites in order to adapt to the variety of niches they inhabit. Some of these have been exploited by humankind. A number of *Aspergillus*-related patents have been issued for medical compounds. A number of antibiotic, anti-tumoral and antifungal agents have been derived from *Aspergillus* metabolites. Enzymes production by aspergilli, like amylase and citric acids, has had a great positive economic impact on industry. But, aspergilli are also a major cause of degradation of agricultural products. Some species are human and animal pathogens and they can also produce mycotoxins, secondary metabolites harmful to humans and animals (Freire et al., 2000; Abrunhosa et al., 2010; Dijksterhuis & Wösten, 2013).

The genus *Aspergillus* belongs to the Deuteromycetes and is characterised by its shape, a conidiophore stipe terminating in a vesicle. The vesicle has one or two layers of synchronously formed specialized cells and asexually formed spores called conidia. The specialized cells bearing the conidia are called phialides. An *Aspergillus* with phialides formed directly on the vesicle is referred to as uniseriate. If a second layer of specialized cells is present on the vesicle, it is referred to as biseriate. The second layer of cells, located between the phialides and vesicle, are called metulae. The *Aspergillus* stipe is often thick walled and has no or only a few thin septa and a basal part called "foot-cell" (Klich, 2002).

Of the existing and acknowledged fungi, fewer have been fully characterised and *Aspergillus* from section *Nigri* is a group that even being studied by many taxonomists presents great difficulties concerning classification and identification (Perrone et al., 2008).

Species of *Aspergillus* section *Nigri* (known as black aspergilli) have been extensively used for various biotechnological purposes and are among the best studied fungi that cause bio-deterioration of diverse commodities, including food (Abarca et al., 2004). This section has evolved continuously and last revision includes 26 different taxa (Varga et al., 2011). However, due to molecular problems detected on β -tubulin DNA primers by Hubka and Loralik (2012), *A. japonicus* should be treated as a synonym of *A. violaceofuscus* and similarly *A. fijiensis* is reduced to synonymy with *A. brunneoviolaceus*. This means that, currently, 25 different taxa are accepted within the *Nigri* section of *Aspergillus* (Simões et al., 2013b).

3.2. Preservation along time and samples ageing

Storage of preserved microorganisms is affected by the impact of the environment conditions. It has been proved that increased rate of inactivation is generally correlated with increased storage temperature. Water activity (a_w) is another parameter that has been shown to influence the viability of spores. And some solutes appear to have a protective effect against the loss of viability during long-term storage (Yang, 1997).

It is long known within BRCs and CCs, the viability of microorganisms is an essential characteristic. The length of time microorganisms remain viable and keep their characteristics after preservation is the key issue. The time of useful life or shelf-life is defined as the time of storage after preservation at which a given percentage of the original population remains viable and unchanged. It is, therefore, desirable to know for how long the cells will remain viable and how viability will be influenced by the environment. This knowledge can be acquired by creating mathematical models to predict storage longevity or by evaluating preservation methods and testing accelerated storage procedures to extrapolate to storage conditions (Dearmon et al., 1962). It has been described that accelerated storage done by keeping preserved bacterial strains at 37 °C for 14 days can be assumed and comparable to a period of 20 years storage at 4 °C. The process of accelerated storage is very useful to evaluate and predict the viability and strains characteristics and conditions after long-term preservation (Sakane & Kuroshima, 1997). The accelerated storage of lyophilised samples has also been successfully applied to the study of viruses (Yordanova et al., 1996).

Several methods of cultivation and preservation are required to ensure the viability and morphological, physiological and genetic integrity of the cultures over time. Though, the cost and convenience of each method are important aspects to be taken into consideration. To help in the decision making of preservation and storage method, decision-based keys can be used. It is important to consider that choosing a preservation method depends on the strain to preserve and that no method guarantees total physiological and genetic stability of an isolate (Ryan et al. 2000; Simões et al, 2013a).

Among the several techniques used to commonly preserve filamentous fungi: subculturing, mineral oil, in water (Castellani method), silica-gel, freezing, in liquid nitrogen and by lyophilisation; the last method is regarded as one of the most reliable for long-term preservation (Homolka, 2013).

The best way to preserve fungi and assure their stability in long-term is: to find conditions where storage guarantees that after retrieval there hasn't been any growth or reproduction, and where all the structural and functional characteristics are maintained. All the studies and researches aim to minimize the number of generations from the initial isolate and reduce the cellular activity where the metabolic reactions are brought to a minimal. Understanding and controlling all factors affecting strain preservation allows having a better control on achieving Quality on preservation methods. These factors can be: the cultivation method, whether in liquid broth or agar media; temperature regime, incubation temperature; media composition and pH; aeration; age of the preserved samples and their state at the time of preservation, physiological condition and culture concentration (Simões et al., 2013a). Based on the correlations determined by Sakane and Kuroshima (1997) for bacterial strains storage, the same logic was applied to fungal strains after preservation by lyophilisation.

3.3. Fungi characterisation – Polyphasic approach

Fungal strains identification and characterisation are important tasks within the context of BRC. Using a polyphasic approach for identification and characterisation, allows the increment of associated information for every fungal strain (Rodrigues et al., 2008). With this in mind and to harness the experimental based knowledge for scientific research on filamentous fungi, specifically the *Nigri* section of *Aspergillus*, and to evaluate preservation methods several methodologies can be used to characterise a group of selected strains and with those data compare different times points of preservation (Simões et al., 2013a; Simões et al., 2013b).

The identification of species is an important goal in taxonomic microbiology, but it can be a long and tedious process with frequent revisions of the taxonomic schemes. These changes make identifications even more complicated for the non-specialised researchers as each taxonomic group has specialised literature, terminology and characters. This occurs to the extent that identifications can only be undertaken by a narrow group of scientists especially skilled in the “art”. The concept of species is clearly abstract and delimitations are very difficult, and often not consensual (Simões et al, 2013b). Taking this into account, microbial taxonomy (more evident in fungal taxonomy) and their associate data can often be best applied at the moment where the data are used for a specific purpose: A pragmatic definition is “data fit for use”. It is gradually becoming clearer that microbial identification, and authentication require a polyphasic approach to generate quality data which are accurate and useful (Keys et al., 2004). In fact, this shows the

need to combine traditional phenotypic and physiological methods with modern molecular biology techniques. It is assumed that the genotype of the each species is only an indirect indication of its phenotype and ecological adaptation. In other words, microbial species are the smallest aggregation of population with a common lineage that share unique described phenotypic characters (Erhard et al., 1997). Recently, microbial mass spectral analysis has been employed for phenotype typing (Rodrigues et al., 2008).

3.3.1. Morphological characterisation

Analysing morphological features is highly important, both for identification and characterisation of fungi. In fungal taxonomy, when dealing with numerous species in a genus, it is necessary to divide species into groups based on their characteristics (Simões et al., 2013b).

Macromorphology are all the features observed with the naked eye, through colony observation or stereomicroscope. These features include: colony colour, for which the conidial heads are responsible; colony diameter after a certain growth period, this can be influenced by media content and incubation temperature; exudates, droplets of liquid that form on the mycelium surface; reverse colour of the colony, this is often media dependent; sclerotia, firm masses of hyphae that contain no spores; among others.

Micromorphology defined as all the features observed through microscopy techniques. These include: seriation, referring to the series of cell layers between the vesicle wall and the conidia, uniseriate species have only phialides and biseriate have phialides and metulae; vesicle, shape and size; conidia, shape, size and ornaments on their surface; stipe, length, colour and surface texture, among others (Klich, 2002; Simões et al., 2013b).

3.3.2. Assessment of mycotoxins production

Only in the last 30 years has it become clear that commonly occurring fungi growing in foods and feeds may produce toxins, known as mycotoxins. These toxins have caused major human and zoonotic during history. Mycotoxins are secondary metabolites that appear to have no role in the normal metabolism involving growth of fungi. The major potential danger of mycotoxins in the human diet, resides in the inability to detect them biologically (Pitt, 2000; Klich, 2002; Serra et al., 2003; Serra, 2005; Rodrigues et al., 2012).

Mycotoxins have four basic kinds of toxicity: acute, chronic, mutagenic and teratogenic. The symptoms caused by mycotoxicoses are almost as diverse as the chemical structures of the

compounds themselves. Many of the toxigenic fungi are ubiquitous and, in some cases, apparently have a strong ecological link with human food supplies. The natural fungal flora existing in conjunction with food production is dominated by three genera: *Aspergillus*, *Fusarium* and *Penicillium*. *Fusarium* species are destructive pathogens on cereal crops and other commodities, and produce mycotoxins before, or immediately after, harvest. Certain species of *Aspergillus* and *Penicillium* are also plant pathogens or commensals, but these genera are more commonly associated with commodities and foods during drying and storage (Pitt, 2000; Abrunhosa et al., 2001).

Mycotoxins are a concern in human food supplies due to being wide-spread. Excessive detected levels of detected aflatoxin (AFLA) in food and blood samples mean that these toxins are directly related to a significant cause of death in some parts of the world. Detection of ochratoxin A (OTA) in a wider range of foods than was previously supposed, and in the blood of many people, has raised awareness that this toxin is widespread. It is very likely that mycotoxins play a significant and direct role in human health (Pitt, 2000; Abrunhosa et al., 2010).

Some of the species belonging to the *Aspergillus* genus are known as producers of industrial enzymes and metabolites. The main mycotoxins produced by species belonging to *Aspergillus* Genus are: AFLA (B1,B2,G1, G2, M1, M2); sterigmatocystin; cyclopiazonic acid; ochratoxin A (OTA); patulin; penicillic acid; citrinin; cytochasin E; verruculogen and fumitremorgin A and B (Klich, 2002). Ochratoxins, the second mycotoxin group in importance after AFLA, includes at least nine metabolites that are similar in structural terms. Among these, OTA is the most studied metabolite due to its occurrence in food and feed and toxicological significance in human and animal diets. This toxin is known to have a nephrotoxic, immunotoxic, teratogenic and carcinogenic effects on animals. The International Agency for Research of Cancer (IARC) has classified OTA as a group 2B carcinogen based on toxicity studies on rats (Valero et al., 2005; Astoreca et al., 2010; Morales-Valle, 2011).

The section *Nigri* belongs to the subgenus *Circumdati* (Varga et al, 2003) and has a significant impact on the modern society because many of its strains are responsible for food degradation and are also used in the fermentation industry, on the production of hydrolytic enzymes (amylases, lipases, etc. ...) and organic acids (citric acid, gluconic acid, ...).

Besides its economical relevance, the strains from the *Nigri* section represent one of the most important sources of mycotoxins contamination of foods and feeds. The major mycotoxins produced by this group of filamentous fungi are ochratoxin A (OTA); from grapes, dried grapes,

wines, beers, cereals and derivatives meant for humans and animals, oilseeds and derivate products, coffee and cacao; and fumonisins (FUMs) of the B series, in particular FUM B2 (FB2) (Kozakiewicz, 1989; Serra et al., 2003; Astoreca et al., 2010; Storari et al., 2012).

3.3.2.1. Aflatoxin

Aflatoxins (AFLAs) are bifuranocoumarin mycotoxins commonly produced by *A. flavus* and *A. parasiticus*, with AFLA B1 (Figure 12) being the most hepatotoxic, showing mutagenic and carcinogenic and, probably, teratogenic properties in animals. According to the International Agency for Research on Cancer, AFLA B1 is classified as a human carcinogen class 1 (Moreno et al., 1988; Silva et al., 2004).

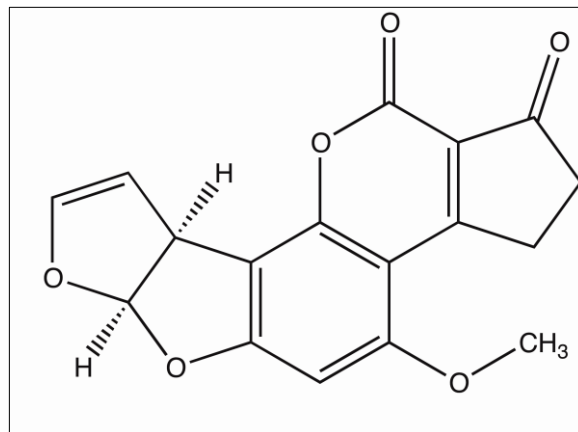


Figure 12 - Aflatoxin B1 (Aflatoxin B2 = 15,16-Dihydro).

It has been long proven that AFLA-production can be detected in 2 to 5 days grown fungal colonies in coconut agar (CAM) medium without the requirement of any additives. This detection can be made under long-wave UV light (365 nm) by blue fluorescence on the reverse side. Davis and colleagues confirmed this relation by verifying through chemical analysis (Davis et al., 1987).

AFLAs are both acutely and chronically toxic to animals and humans. Liver damage, liver cirrhosis, induction of tumours and teratogenic effects are acute toxicity effects. The four major naturally produced are known as AFLAs B₁, B₂, G₁, and G₂. The letters B and G refer to the blue and green fluorescent colours produced by these compounds under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Because of their high toxicity, low acceptable limits for AFLAs in foods and feeds have been established in many countries. Under recent agreements, 15 µg/kg of total

AFLAs is likely to become the maximum level permitted in all food commodities in world trade (Pitt, 2000). Other bio-transformed aflatoxins may occur, for example in milk, such as aflatoxins M₁ and M₂ (Pildain et al., 2008).

The most important production of AFLAs in nature, from a public health point of view, has been described for *Aspergillus flavus* and *A. parasiticus* (Pitt, 2000; Pildain et al., 2008). *A. flavus* is ubiquitous and when described and analysed has been used to name also *A. parasiticus*. Since the discovery of AFLAs, it has become the most widely reported food-borne fungus, reflecting its economic and medical importance and ease of recognition, as well as its universal occurrence. *A. flavus* and *A. parasiticus* have a particular affinity for nuts and oilseeds. Peanuts, maize and cotton seed are the three most important crops affected. Established patterns of local consumption, where substandard nuts and maize may be consumed without any form of control or inspection, mean that AFLA ingestion remains far too high in many countries, especially in rural areas (Pitt, 2000; Soares et al., 2010).

3.3.2.2. Ochratoxin A

OTA (Figure 13) is a mycotoxin naturally found in various food products including green coffee bean, roasted coffee and instant coffee. It is carcinogenic, teratogenic, genotoxic, immuno suppressive and causes potential harm to animals and humans (Noonim et al., 2008; Abrunhosa et al., 2010).

OTA is a serious animal health problem because OTA is fat soluble and not readily excreted, it accumulates in the depot fat of affected animals, like pigs, and from there it is ingested by humans. A second source is bread made from barley or wheat containing the toxin. Although clear evidence of human disease is still elusive, such levels indicate a widespread problem with ochratoxin (Pitt, 2000; Serra, 2005).

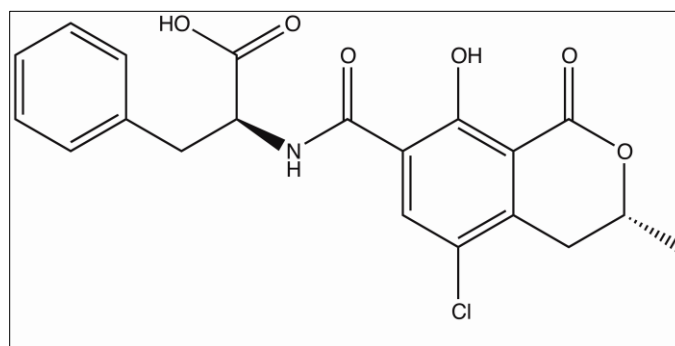


Figure 13 - Ochratoxin A.

FUM B2 (FB2) was found to be produced by *Aspergillus niger* (Frisvad et al., 2007). When grape-derived products were subsequently analysed, FB2 contamination was found in raisins, must and wine. In a study performed by Abrunhosa et al. (2011a), FB2 was not detected in *Aspergillus carbonarius* or *A. ibericus* strains, but it was detected in strains belonging to *A. niger* aggregate.

3.3.3. Enzymatic screening

Enzymatic activity is one of the fungal characteristics that can be analysed and be part of biotechnological potential assessment.

Aspergillus fungi use mainly polysaccharides as a carbon source. These complex molecules need to degrade before being used as substrates. Some of these polysaccharides can be split in three major groups: cellulose, hemicellulose (xylan, galactomannan and xyloglucan) and pectin (Coutinho et al., 2009). For the degradation of the referred plant polysaccharides and according to Coutinho and colleagues (2009), fungi produce a broad range of hydrolytic enzymes with different and complementary catalytic activities which can be screened for each fungal strain. Some of these enzymes have a large industrial potential; and to study their enzymatic profiles and explore the bio-potential of fungal strains, support research on their application (Guimarães et al., 2006).

The methods for determining enzymatic activity vary according the class of reaction and they can be hydrolysis, synthesis or isomerisation. Following incubation in a given substrate solution, under controlled operational conditions, samples are taken periodically and analysed according to different techniques. Those techniques can be spectrophotometric methods, along with high performance liquid chromatography (HPLC) methods, gas-chromatography among others.

Bioconversions to achieve full hydrolysis of carbohydrates, are followed by the formation of reducing sugars through the Nelson-Somogyi method or the dinitrosalicylic acid (DNS) method (Shaffer & Somogyu, 1933; Nelson, 1944; Miller, 1959).

Colorimetric methods for assessing α -amylase and β -glucosidase activity have also been developed and are able to be done in microplates. This strongly contributes for high-throughput processing, like the one developed and implemented at *Institute National de la Recherche Agronomique* (INRA), Marseille (Figure 15); by allowing the simultaneous analyses of multiple

samples, fastening process characterisation and analysis. This approach has also been adapted to the analysis of reducing sugars (Navarro et al., 2010).

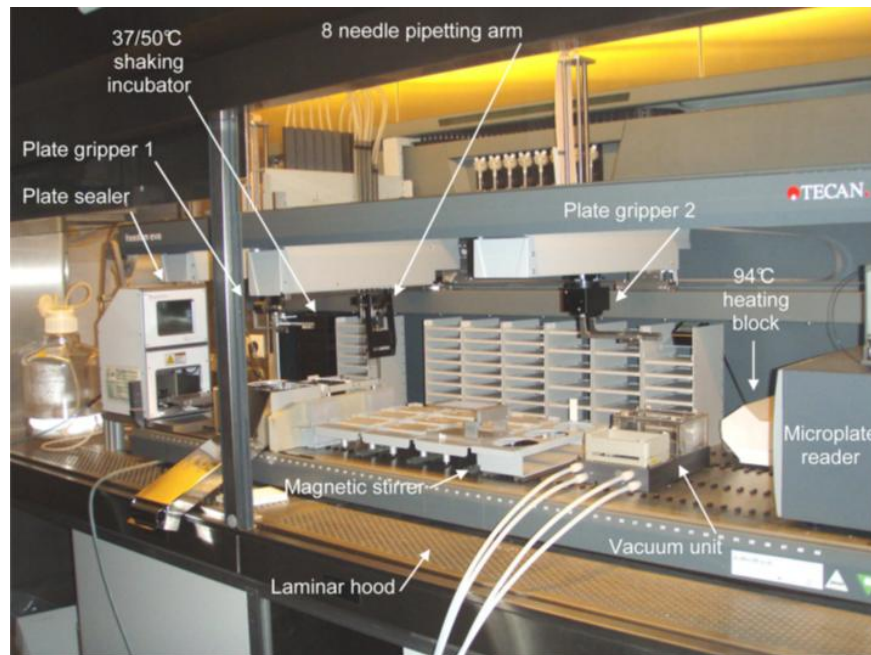


Figure 15 - Highthroughput screening apparatus at INRA, Marseille, for enzymes detection.

Plate screening methods for the detection of enzymes production is another methodology fully described in the literature. These methods provide an array of relatively straightforward and simply applicable tools for specific detection of polysaccharide-degrading strains of fungi.

Most of these methods are based on the complex formation between polysaccharides and dyes, the solubility, the gel-forming properties of polysaccharides, and on the use soluble and insoluble dye-labelled polysaccharides. But, screening of microorganisms with a set of hydrolytic activities or with a specific combination of degrading activities is labour-intensive and time-consuming, especially for large-scale searching programs turning these methods not appropriate for this purpose (Ten et al., 2004).

3.3.4. MALDI-TOF MS

Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) emerged in the late 1980s as a sound technique to investigate the mass spectrometry of molecular high-mass of organic compounds through a soft ionisation of the molecules resulting in minimum fragmentation (Tanaka et al., 1988). Lately, MALDI-TOF MS has been contributing to improve the knowledge on microbial identification and characterisation. MALDI-TOF MS has been

used to generate spectrum of protein masses in a range of 2000 to 20000 Da that is a taxon specific fingerprinting (Kallow et al., 2006).

MALDI-TOF MS involves subjecting a sample covered with an UV-absorbing matrix that functions as an energy mediator, to a pulsed nitrogen laser. Matrices are chemical compounds generally containing aromatic moieties that transfer the absorbed photoenergy from the irradiation source to the surrounding sample molecules, resulting in minimum fragmentation. They are used as solution and their final composition is constituted by chemical compound that is the matrix properly, dissolved in an organic solvent, generally ethanol and/or acetonitrile, and water (Hillenkamp and Peter-Katalinić, 2007; Santos and Lima, 2010). Choosing appropriate matrix for identification of filamentous fungi is crucial. It depends on the laser wavelength used in each MALDI-TOF MS instrument. The use of an appropriate matrix leads to an optimal signal/noise ratio with the narrowest analyte peaks, little signal suppression and therefore the best analytical results (Simões et al., 2013 a).

Currently, the two most used matrices for filamentous fungi identification are 2,5-dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxycinnamic acid (CHCA). Both DHB and CHCA matrices are appropriate for the analysis of molecules with a mass range between 2 and 20 kDa. Constantly expressed and highly abundant proteins, such as ribosomal proteins that appear on this specific mass range can be used as biomarkers. Based on this knowledge, different studies have demonstrated the high potential of this technique for species and strain identification of filamentous fungi (Santos and Lima, 2010; Santos et al. 2010; Dias et al. 2011; Rodrigues et al. 2011).

Bacterial identification by MALDI-TOF MS based on a methodology with previous sample preparation with minimal purification of cell contents, developed by the seminal paper by Cain and colleagues (1994) was the important milestone for the use of MALDI-TOF MS technique in fungal taxonomy. Two years later Holland et al. (1996) described, for the first time, a method for the rapid identification of whole bacteria with simple sample preparation (Simões et al., 2013a).

MALDI-TOF MS for the identification and classification of microorganisms needs dedicated software and database to enable comparisons of the unknown protein with reference molecular masses. Ribosomal proteins are used normally as reference molecular masses as they are the most abundant in the cells. External MALDI-TOF MS calibration is performed by using well characterised proteins from *Escherichia coli* (Ryzhov and Fenselau, 2001). From tens of ribosomal proteins of intact *E. coli* cells 12 well defined proteins are used as MALDI-TOF MS

standard (4,365.4, 5,096.8, 5,381.4, 6,241.4, 6,255.4, 6,316.2, 6,411.6, 6,856.1, 7,158.8, 7,274.5, 7,872.1, 9,742 and 12,227.3 Da).

The cost, simple procedure to growth and obtain fresh *E. coli*, and reliability found in these biomarkers make them the first choice for companies producers of MALDI equipment and software devoted for microbial identification with routine purpose.

The advantages of this approach as a microbial identification, authentication and characterisation method are the simple sample preparation procedure, short time of analysis (few minutes), the high number of samples that can be analysed, reliability of the data and the fact that it is an inexpensive technique involving mainly labour work (Dickinson et al., 2004; Simões et al., 2013a).

There are several types of MALDI-TOF equipment being used nowadays. At MUM the equipment used (Figure 16) follows the same principles of functioning of all this kind of equipment.

As already referred, the sample for MALDI is uniformly mixed in a large quantity of matrix and each sample is applied in a well on a specific plate (Figure 17), until it crystalizes. The matrix absorbs the UV (nitrogen laser light, wavelength 337 nm) and converts it to heat energy. A small part of the matrix heats rapidly and is vaporized, together with the sample. Charged ions of various sizes are then generated.



Figure 16 - MALDI-TOF apparatus being used at Micoteca da Universidade do Minho.



Figure 17 - MALDI-TOF plate.

A potential difference between the sample slide and ground attracts the ions. The velocity of the attracted ions is determined by the law of conservation of energy. As the potential difference is constant with respect to all ions, ions with smaller m/z value (lighter ions) and more highly charged ions move faster through the drift space until they reach the detector. Consequently, the time of ion flight differs according to the mass-to-charge ratio (m/z) value of the ion (Shimadzu Corporation, 2013).

3.3.5. Molecular Biology

Molecular biology tools, in particularly polymerase chain reaction (PCR) assays have formed the basis of many studies on fungal diversity due to the sensibility of this molecular approach. It allows seeing repeated trait evolution patterns and the direct discrimination of artificial groupings that are created by traditional methodologies. Molecular data information allows for a better understanding of the fungal relationships and the progress of a more natural classification (Begerow et al., 2010; Branco, 2011; Simões et al., 2013a).

Bioinformatics tools allowed for the creation of genetic database that can be used as sources for molecular barcodes. DNA barcoding is a technique for an easy characterisation of organisms at species level, using a short DNA sequence from a standard and agreed-upon position in the genome. It is an accurate, rapid, cost-effective, culture-independent, universally accessible and usable by non-experts technique (Frezal & Leblois, 2008; Simões et al. 2013a).

Standardizing methodologies is a hard task, especially when it comes to fungal identification and characterisation. Therefore, it becomes important to pinpoint genes which characterise fungal organisms at different taxonomic levels in a straightforward way. The most frequently studied markers are one or more of the nuclear rRNA genes: small subunit (SSU) rRNA gene, the internal transcribed spacer (ITS), rDNA region including the 5.8S rRNA gene and a part of the large subunit rRNA gene are several of the most used. Though, ITS is the most widely accepted and used DNA fragment for identifying fungi at the species level, mainly because it is a well disclosed and used method, ITS fragment is easily amplified by PCR, it is fast and easy to use, even for low-quality samples (Simões et al., 2013a). Sequence-based strategies are the “gold standard” for species identification for they use target DNA and conserved housekeeping genes. The most common genes used for fungal identification are: calmodulin, β -tubulin and RNA-polymerase.

But there are other possibilities of using molecular biology when studying fungi. For this work, it was taken into account that stability of cultures related to the culture age and subcultures frequency, can be tested by Enterobacteria Repetitive Intergenic Consensus (ERIC)/PCR, to confirm the suitability of long-term preservation. This was done by Gullo and colleagues (2012) that analysed *Acetobacter* species preserved for 9 years by short and long time methods.

There are several steps in this approach that can be problematic. The first one, which can be critical, is the DNA extraction. In this step, several factors can influence the obtained result, those being: sample type, it can be a culture or clinical specimen, hyphae and/or spores,

sterile or non-sterile, from a single-spore culture or a mixed sample, whole blood or serum or plasma; cell wall disruption, that can be enzymatic, chemical or mechanical; and the DNA extraction method, classic protocol or commercial kits. Integrity, purity and concentration are parameters that can change an amplification result on a PCR.

The major problems for PCR methodologies are inexistence of amplification, contamination and low specificity.

The relation of homology between amplified fragments through ERIC primers are a common choice in the study of bacterial genetic diversity. This approach has also been employed in fungi but there is not much information on homology and amplified fragments.

3.4. Experimental procedures description

3.4.1. Samples preparation - Description of preservation techniques used

Twenty-one strains belonging to *Aspergillus* section *Nigri* were selected and supplied by the Micoteca da Universidade do Minho (MUM) fungal culture collection (Table 9).

All the samples were initially grown in malt extract agar (MEA) and incubated in the dark at 25 °C for 7 days, for further processing.

Table 9 - List of strains selected.

Species	Isolate number	Geographical origin	Substrate
<i>A. aculeatus</i>	MUM 03.11 [†] *(NRRL 5094 [†])	Unknown	Tropical soil
<i>A. brasiliensis</i>	MUM 06.179	Portugal	Grapes Cabernet Sauvignon
	MUM 06.180	Portugal	Grapes <i>Tinta Miúda</i>
	MUM 06.181	Portugal	Grapes <i>Tinta Barroca</i>
<i>A. carbonarius</i>	MUM 01.08	Unknown	Unknown
<i>A. ellipticus</i>	MUM 03.12 [†] (NRRL 5120 [†])	Costa Rica	Soil
<i>A. ibericus</i>	MUM 03.49	Portugal	Grapes <i>Periquita</i>
	MUM 04.68	Portugal	Grapes <i>Aragonês</i>
	MUM 04.86	Portugal	Grapes <i>Tinta Barroca</i>
<i>A. japonicus</i>	MUM 03.02	Puerto Rico	Soil
	MUM 98.03 **(DSM 2345)	Unknown	Unknown
<i>A. lacticoffeatus</i>	MUM 06.150 [†] *** (CBS 101883 [†])	Indonesia	Coffee robusta (<i>Rubiaceae</i>)

<i>A. niger</i>	MUM 03.01	E.U.A.	Tannin-gallic acid fermentation
	MUM 05.11	Unknown	Unknown
	MUM 05.13	Portugal	Grapes <i>Aragonés</i>
<i>A. phoenicis</i>	MUM 03.05 (NRRL 365)	Unknown	Unknown
	MUM 03.10 (NRRL 4750)	Unknown	Unknown
<i>A. sclerotium</i>	MUM 06.151 [†] (CBS 115572 [†])	India	Arabic coffee
<i>A. tubingensis</i>	MUM 06.152 [†] (CBS 134.48 [†])	Unknown	Unknown
<i>A. uvarum</i>	MUM 08.01	Portugal	Grapes <i>Tinta Barroca</i>
<i>A. vadensis</i>	MUM 06.153 [†] (CBS 113365 [†])	Unknown	Dead plant tissue

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** *Deutsche Sammlung von Mikroorganismen*

*** *Centraalbureau voor Schimmelcultures*

3.4.2. Preservation by lyophilisation

The preservation through dehydration by lyophilisation was made following the protocol described at SOP.001 developed and implemented at MUM daily proceedings, for the apparatus Edward (Figure 18).



Figure 18 - Lyophiliser at Micoteca da Universidade do Minho.

The ampoules were initially treated with HCl at 2% (v/v) and rinsed two times, first with tap water then with distilled water. After dried, the ampoules were stoppered with carded/brushed cotton and sterilised by autoclave (121 °C for 20 minutes) and let to dry overnight in an incubator above 50 °C.

The procedure involved two fundamental steps.

In a first phase, primary lyophilisation, the conidial suspension (ca 0.2 mL distributed in each ampoule), prepared from healthy grown cultures (grown from 5 to 7 days at 25 °C in slants, with an inclination angle of ca 30° with the horizontal, in MEA (Annex 7.6.1.)) and milk and myo-inositol (Annex 7.6.2.) as suspension fluid, underwent a cooling aided by evaporation caused by depressurisation of the lyophilisation chamber (-44 °C, ca 4 psi), this happened at the same time of centrifugation until it froze. Centrifugation was then interrupted. Negative pressure was maintained on the chamber and the humidity content of the frozen suspension was removed by sublimation. At the end of this step, the lyophilisation chamber was let to reach the atmospheric pressure. The ampoules were then removed from the lyophiliser to proceed to their constriction through heat.

On the second phase of the procedure, secondary lyophilisation, the ampoules were organised in a manifold over a drying agent, phosphorus pentoxide, and negative pressure was applied to the system. The remaining water on the sample is then removed. At the end of the lyophilisation process the ampoules were sealed under vacuum with a torch.

The efficiency of the process was checked with a spark test to confirm the good state of the samples.

The ampoules were opened with the aid of a glass cutter and lyophilised strains re-hydration was made with a few drops of sterile water, without agitation and let to rest for at least 30 minutes time after which it was homogenized by gently aspirating and dispersing with a Pasteur pipette, and some drops of the homogenized suspension were applied in an appropriate solid media and then incubated at the recommended light and temperature conditions.

3.4.3. Ageing of preserved samples

Ageing of the samples was performed through accelerated storage, by keeping the ampoules with the lyophilised samples at 37 °C in the dark for 3 time points: 2 weeks (II), 4 weeks (III) and for 6 weeks (IV).

For the analysis, comparisons were made between the time points II and III with the samples before being preserved (I).

And the last time point (IV) was only analysed for viability of strains in MEA.

3.4.4. Characterisation through polyphasic approach

For the characterisation of the samples, the polyphasic approach was followed using the already referred techniques and methodologies.

3.4.4.1. Macro-morphological evaluation

For the macro-morphological analysis, the selected strains were grown on: MEA, potato dextrose agar (PDA) (OXOID, UK), Czapek agar (CZ – Annex 7.6.3.) and Czapek agar with Yeast extract (CYA – Annex 7.6.4.).

In order to guarantee the same media depth in all of the plates and a standardised growth, 20 ml of each media were poured into standard (90 mm) sterile and disposable Petri dishes. Each plate was inoculated at 3 points, equidistant from the center, and incubated in the dark, at 25 °C, for 7 days. To prevent stray colonies on the plates, inoculation was made from a spore suspension on a solution of 0.2% agar with 0.05 % tween 80.

3.4.4.2. Stereomicroscopic and microscopical evaluation

For microscopic and stereomicroscopic analysis, the strains were grown 3 or 4 days on MEA.

In order to have a clear image comparison of the size of the conidial head, a stereomicroscope (Leica MZ12.5) was used. The fungal characterisation was carried out using a light microscope (Leica DM R) and a scanning electron microscope [NanoSEM - FEI NovaTM 200 (FEG/SEM); EDAX - Pegasus X4M (EDS/EBSD)].

The samples prepared for the light microscopy were mounted on a slide, abundantly washed with alcohol (96%) to remove excessive spores and stained with a lactophenol blue solution.

3.4.4.3. Scanning electron microscopical evaluation

For the scanning electron microscopy (SEM) analysis, a drop of media (MEA) was mounted in a SEM stub in aseptic conditions, which was inoculated and incubated in the dark, at

25 °C. After a visible fungal growth, the sample was directly covered with a mixture of gold and platinum (80/20%) for posterior analysis. Each single image was digitally produced and registered at variable magnifications.

3.4.4.4. Assessment of mycotoxins production

3.4.4.4.1. Rapid screening method for mycotoxins detection

An agar medium containing commercial coconut extract was prepared for a rapid screening of mycotoxin production, previously described for the detection of AFLA, OTA and citrinin (Davies et al., 1987; Lin & Dianese, 1976; Mohamed et al., 2013).

The media was distributed into Petri dishes (with 60 mm of diameter) and after solidification, each plate was inoculated with the selected strains from the different conditions. A non-inoculated plate was used as a negative control to be used as a reference parameter. All strains were incubated at 25 °C in the dark for 4 days.

The presence of metabolites was noted by the presence of fluorescence in the grown colonies in coconut milk agar (CMA) (Annex 7.6.9) medium when exposed to UV light as demonstrated in figure 19.

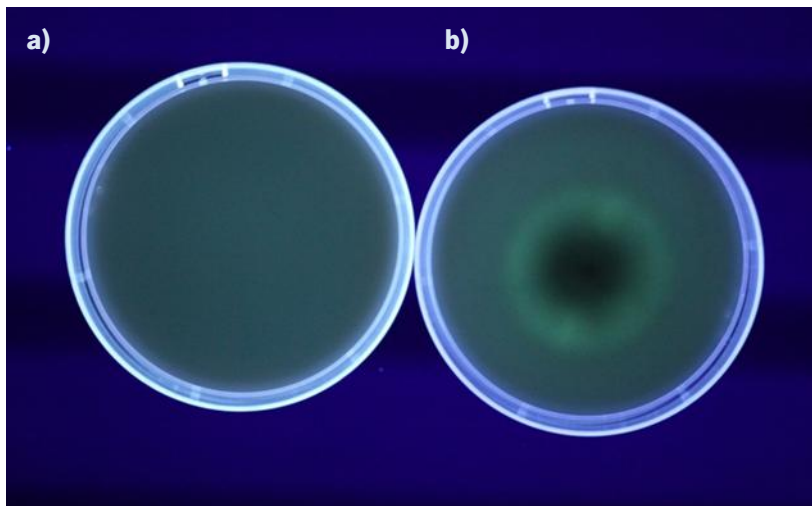


Figure 19 - Example of the production of secondary metabolites; a) negative control, b) positive control.

3.4.4.4.2. Ochratoxin A detection

For the selected strains of *Aspergillus*, secondary metabolites were extracted for posterior analysis by high-performance liquid chromatography method that used fluorescence detection (HPLC-FL) (Bragulat et al., 2001; Serra et al., 2004).

All the *Aspergillus* strains were tested for OTA production in Yeast Extract Sucrose (YES) (Annex 7.6.5.), at the different time points (I, II and III). The strains were inoculated on 90 mm diameter Petri dishes and incubated at 25 °C for 7 days in the dark. Extraction methodology described by Bragulat and colleagues (2001) was employed: briefly, 3 agar plugs were removed from one colony, and placed into a 4 mL vial, where 1 mL of methanol was added. After 60 minutes, the extract was filtered through 25 mm syringe filters with 0.45 µm PFTE membranes (VWR International, Reference No. 514-0071) into new 2 mL vials, evaporated and further dissolved in 1 mL of mobile phase (acetonitrile:methanol, 99:99:2 v/v/v).

Afterwards, detection and quantification of OTA was processed. Samples were analysed by HPLC with a Jasco FP-920 fluorescence detector (333 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 x 4.6 mm, 5 µm) fitted with a precolumn with the same stationary phase. The mobile phase was water: acetonitrile:methanol (99:99:2, v/v) pumped at 0.8 mL/min. The injection volume was 50 µL.

OTA standard was supplied by Biopure (Austria). An initial calibration curve was prepared and quantification was made using Galaxie software (1.9.302.952 version) and extracts with the same retention time as OTA standard were considered to be OTA positive.

3.4.4.4.3. Fumonisin B2 detection

The experimental procedure for the FUM determination was done as described in Abrunhosa et al. (2011b).

Strains were tested for FB2 production in CYA. Strains were inoculated on 90 mm diameter Petri plates and incubated at 25 °C for 7 days in the dark. The used extraction and derivatisation methodology consisted in removing 5 agar plugs from one colony, and placing them into a 4 mL vial, where 1 mL of methanol:water (75:25, v/v) was added. After sonication for 50 minutes, the extract was filtered through 25 mm syringe filters with 0.45 µm PFTE membranes (VWR International, Reference No. 514-0071), evaporated, kept at 4 °C until further derivatisation for FUM B2 detection as described in Abrunhosa et al. (2011b).

Since fumonisins are not fluorescent, a derivatisation procedure must be performed prior to HPLC, with fluorescence, detection analysis.

Briefly, 3 initial solutions were prepared. In each vial with the dry extracts the solutions were added on the following order: 200 µL of methanol, 200 µL of borate buffer (0.05 M) (Annex

7.6.6.), 100 μ L of Sodium cyanide solution (0.13 mg/mL) (Annex 7.6.7.) and 100 μ L of NDA (0.25 mg/mL) (Annex 7.6.8.). The samples were then placed in heat block for 15 minutes at 60 °C and diluted with 1.4 mL of acetonitrile/water (3:2, v/v). Homogenisation was done by vortexing. And the derivatised samples were transferred into new vials and put on an automatic sampler for HPLC.

Samples were analysed by HPLC with a Jasco FP-920 fluorescence detector (420 nm excitation wavelength; 500 nm emission wavelength). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 x 4.6 mm, 5 μ m), fitted with a precolumn with the same stationary phase. The mobile phase was acetonitrile:water:acetic acid (60:40:1, v/v/v) pumped at 1.0 mL/min. The injection volume was 50 μ L.

Fumonisin B2 standard was supplied by Sigma (USA).

An initial calibration curve was prepared and quantification was made using Galaxie software (1.9.302.952 version) and extracts with the same retention time as FUM standard were considered to be FUM positive.

3.4.4.5. Enzymatic screening

The main goal of the enzymatic screening for this work was to complement the polyphasic approach to characterise the selected fungi on the different time points of preservation, adding value to strains data, and to evaluate the preservation method of lyophilisation in order to determine the best practice for the maintenance of metabolic and genetic integrity of the analysed strains.

With this in mind, the selected fungal strains supplied by MUM had their enzymatic profiles determined on the 3 time points, before lyophilisation and after accelerated storage (2 and 4 weeks).

The enzymatic activity of this fungal section was assessed by screening the activity of a set of enzymes. This screening was performed according to the protocols used for high throughput screening of enzymes used at the CIRM-CF collection (International Centre of Microbial Resources dedicated to Filamentous Fungi) from INRA (French National Institute for Agricultural Research) at Marseille, France (Navarro et al., 2010).

3.4.4.6. Culture conditions

The selected strains of *Aspergillus* used in this study were initially grown in MEA and incubated for 7 days at 25 °C in the dark. Spores from the 7day-old cultures were scraped with a sterile scraper and recovered into sterile 50 ml Falcon tubes using sterile NaCl 8.5 g/L solution containing Tween 80 (0.02 %). After dilution (900 µl of sterile NaCl solution + 100 µl of each suspension), if necessary, the spore concentration was determined using a Fuchs-Rosenthal counting chamber.

The fungal cultures were grown in 250 ml-baffled flasks containing 100 ml of malt extract-glucose-yeast extract-peptone (MGYP) (Annex 7.6.10.).

The cultures were inoculated with 2×10^5 spores/mL and incubated at 25 °C in duplicate under shaking at 150 rpm (Certomat rotary shaker) for 7 days.

Aliquots (2 mL) from each 100 mL culture were taken at different days of incubation (1, 2, 3, 5 and 7 days), and filtered with 0.45 µm polyethersulfone membrane (Vivaspin Sartorius). Since all the cultures were prepared in duplicate, to better achieve a representative sample of the cultures, filtered aliquots from duplicates were pooled into a 15 mL falcon tube. All the tubes containing the filtered culture media (enzymatic extracts) were kept at -20 °C until the analyses.

3.4.4.7. Enzyme activities

3.4.4.7.1. Proteolytic activity determination

This assay was done by evaluating the proteolytic activity by the degradation of substrate, observed by the appearance of a transparent halo around the fungi colonies.

Skim milk agarised medium (SKM) (Annex 7.6.12.) was prepared and distributed into Falcon tubes (5 mL per tube), leaving a flat surface on the media. The tubes were inoculated with all the 21 selected strains at the 3 different time points (I, II and III), and were incubated at 25 °C in the dark for 7 days.

On the 7th day registry was made of the halo formed in high from the colony.

3.4.4.7.2. Polysaccharide-hydrolytic enzymes assay

Polysaccharide-degrading enzyme assays were miniaturized using complex substrates, adapted to 96-well plates as described by Navarro et al. (2010). Complex substrates used in this study, were carboxymethyl cellulose (CMC), wheat xylan (WX), pectin of *Citrus* sp. (PC) and

galactomannan (Man), to measure carboxymethyl cellulase (CMCase), xylanase, pectinase and mannanase activities respectively. Solutions (1 % w/v) were prepared in sodium acetate buffer 50 mM (Annex 7.6.11.) at pH= 5.2.

In microtubes, 25 μ L of enzymatic extract were added to 150 μ L of each substrate (200 μ L of final volume per microtube). Afterwards, all microtubes were incubated at 37 °C for 2 hours. Reducing sugars were quantified using the adapted DNS method described by Gonçalves and colleagues (2010). The enzyme activities were expressed as nkat per mL, defined as 1 nmol glucose equivalent released per mL of medium and per second under the assay conditions. Glucose was used to determine a standard curve.

3.4.4.7.3. Total protein assay

To ensure a better evaluation of the enzymatic activities accessed in 3.4.4.6.2, total protein was determined by Bradford method using bovine serum albumin as standard (Bradford, 1976). A miniaturized assay was performed in a 96 well-plate, with a final volume of 200 μ L, using 40 μ L of reagent and 160 μ L of sample. After homogenisation, the absorbance was measured at 595 nm.

3.4.5. MALDI-TOF MS

Samples for spectral analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were grown in MEA and incubated in the dark, at 25 °C, for 4 days. The procedures were followed as described by Rodrigues et al. (2011). Briefly: for the flex target plate preparation, approximately 1 μ g of spores and young mycelium of each species was transferred directly from the culture plate to the 48-well MALDI-TOF plate. Immediately after, 0.5 μ L of matrix solution [7.5% 2,5-dihydroxybenzoic acid in ethanol/water/acetonitrile (1:1:1) with 0.03% trifluoroacetic acid] was added to the samples and mixed gently. The sample mixtures were air dried at room temperature. Each sample was spotted in duplicate to test reproducibility. During the analyses, all solutions were prepared and stored at 4 °C. Data were analysed based on a matrix of pairwise correlation values for spectra after smoothing baseline corrections and peak detections. The peak lists of the selected strains were directly transferred into the SARAMIS® (AnagnosTec, Germany) software.

3.4.6. Molecular Biology

The use of ITS is a common practice in molecular biology studies in fungi. However there are also disadvantages in the choice and use of the ITS region. The most problematic issue, is that there is variability in ITS region, within species, as the different copies within a genome are not exactly identical. Furthermore, the capacity to discriminate at the species level differs considerably across fungal groups that in some case the identification at species level could be ambiguous (Soares et al. 2012b).

To use a different approach from ITS, ERIC was chosen to type the selected fungi on the different timing of preservation by lyophilisation (I, II and III).

3.4.6.1. DNA extraction

Five 8 mm diameter plugs were cut, with a sterile cork borer, from the periphery of a 7 day old colony grown in MEA and inoculated in 250 ml Erlenmeyer flasks filled with 100 ml MGY. These were incubated on a rotary shaker (Certomat) for 5 days at 25 °C and 150 rpm. After fungal growth, mycelium was frozen and later used for genomic DNA extraction as previously described by Raeder and Broda (1985).

3.4.6.2. Typing of subcultures by ERIC/PCR

ERIC/PCR was performed on genomic DNA from grown cultures of the selected strains, on the 3 times points: before lyophilisation (I), 2 weeks after accelerated storage at 37 °C (II) and 4 weeks after accelerated storage at 37 °C (III). Reproducibility was search for through the analysis of two repeated DNA extracts and several independent amplifications were performed using the conditions as following described.

Initially the primers ERIC 1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-CGC TCA CCC CAG TCA CTT ACT T-3') (both supplied by Eurofins MWG Operon) were diluted in purified water to a final concentration of 100 µM.

PCR mix contained, per microtube of reaction, 1 µL of DNA, 10 µL of buffer green (Promega, USA), 5 µL of MgCl₂ (2.5 mM) (Fermentas), 1 µL dNTPs NZymix (10 mM) (NZytech), 2.5 µL of each primer (10 µM) and 0.4 µL of GoTaq Hot startpolymerase (2 U) (Promega), for a final volume of 50 µL in purified water. The parameter for the reaction were the following: 1 cycle of 2 minutes at 95 °C; 35 cycles of: 95 °C for 1 minute, 50 °C for 1 minute and 75 °C for 2

minutes; 1 cycle of 72 °C for 10 minutes and final ending at 4 °C. The reactions were run in a thermocycler MyCicler (Biorad).

Amplification and electrophoresis were performed. A 10 Kb DNA molecular size marker (Fermentas) was loaded. Gels were visualized under UV light and digital image captured using ChemiDoc XRS+ (Biorad) with ImageLab v4.0 (Biorad).

3.5. Results and discussion of characterisation with each method for the samples selected.

3.5.1. Macroscopy – Photography and stereomicroscopy

The results presented here are the images of the grown colonies in MEA, times I, II and III (Figures 20, 21 and 22); PDA, times I, II and III (Figures 23, 24 and 25); CZ, times I, II and III (Figures 26, 27 and 28); and CYA, times I, II and III (Figures 29, 30 and 31). Followed by the images obtained through stereomicroscopy of grown colonies in MEA, times I, II and III (Figures 32, 33 and 34).

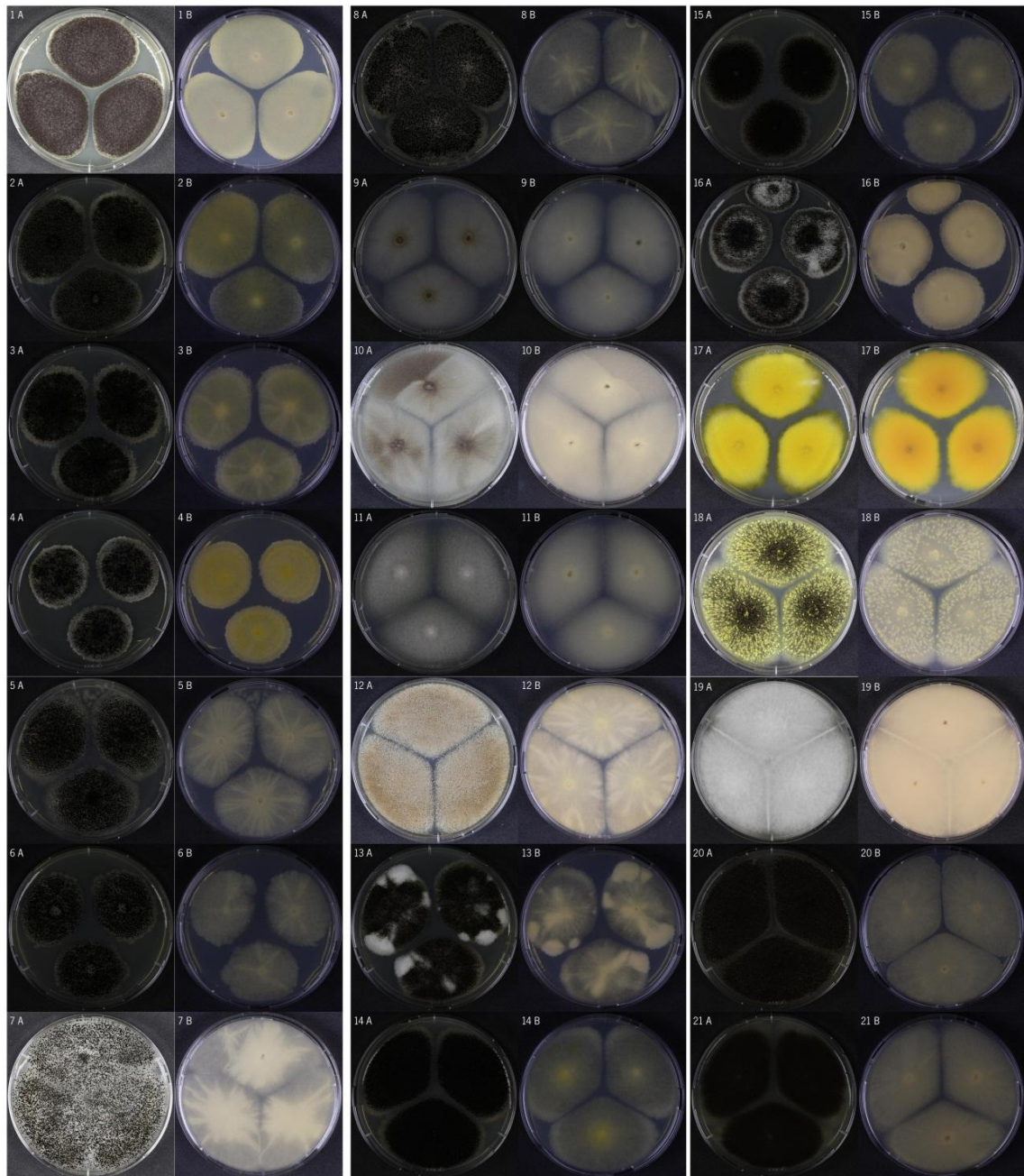


Figure 20 - Fungi colonies, from time point I, grown 7 days in the dark, at 25 °C in MEA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

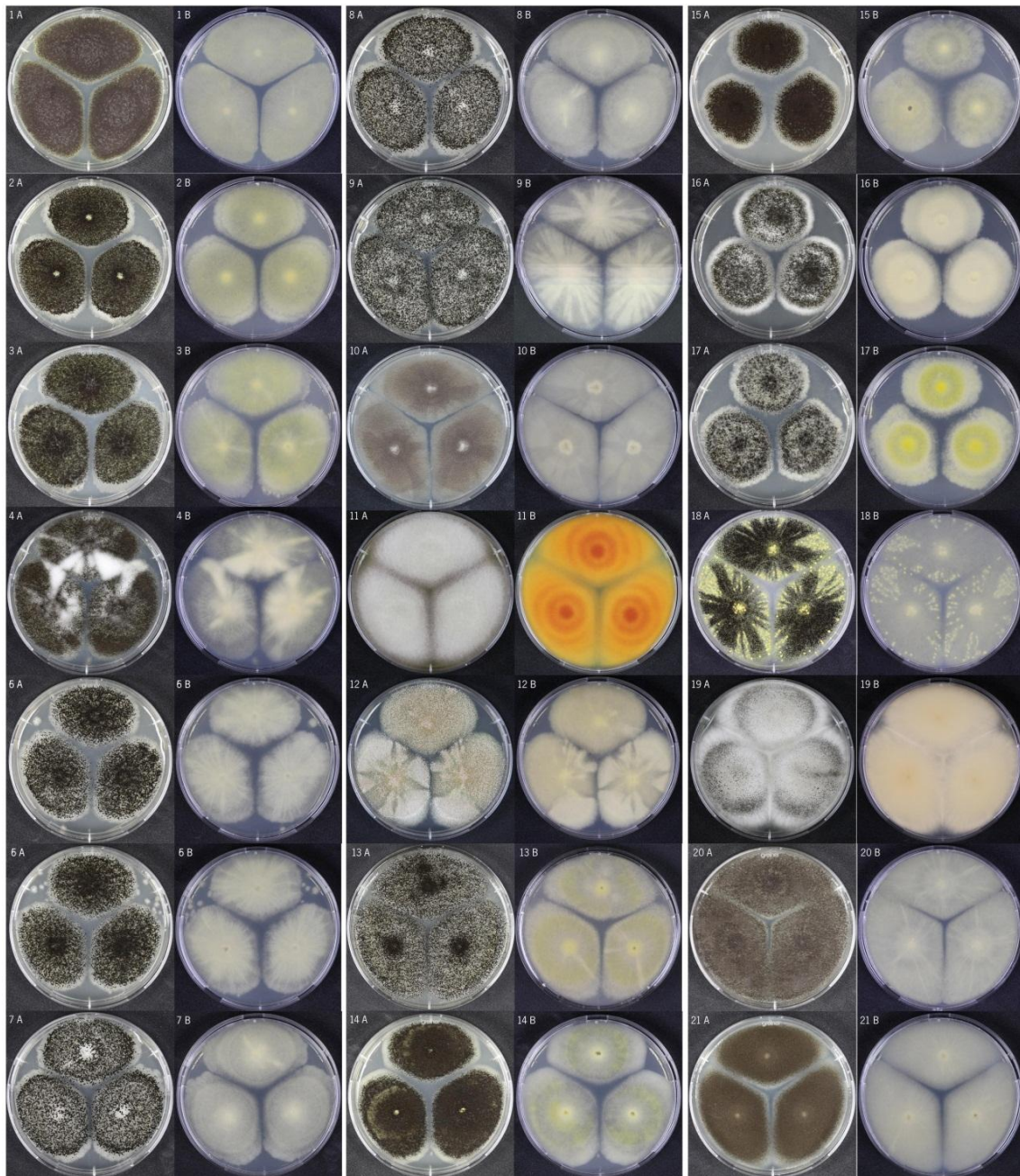


Figure 21 - Fungi colonies, from time point II, grown 7 days in the dark, at 25 °C in MEA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150; 13- *A. niger* MUM 03.01; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151; 19- *A. tubingensis* MUM 06.152; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153.

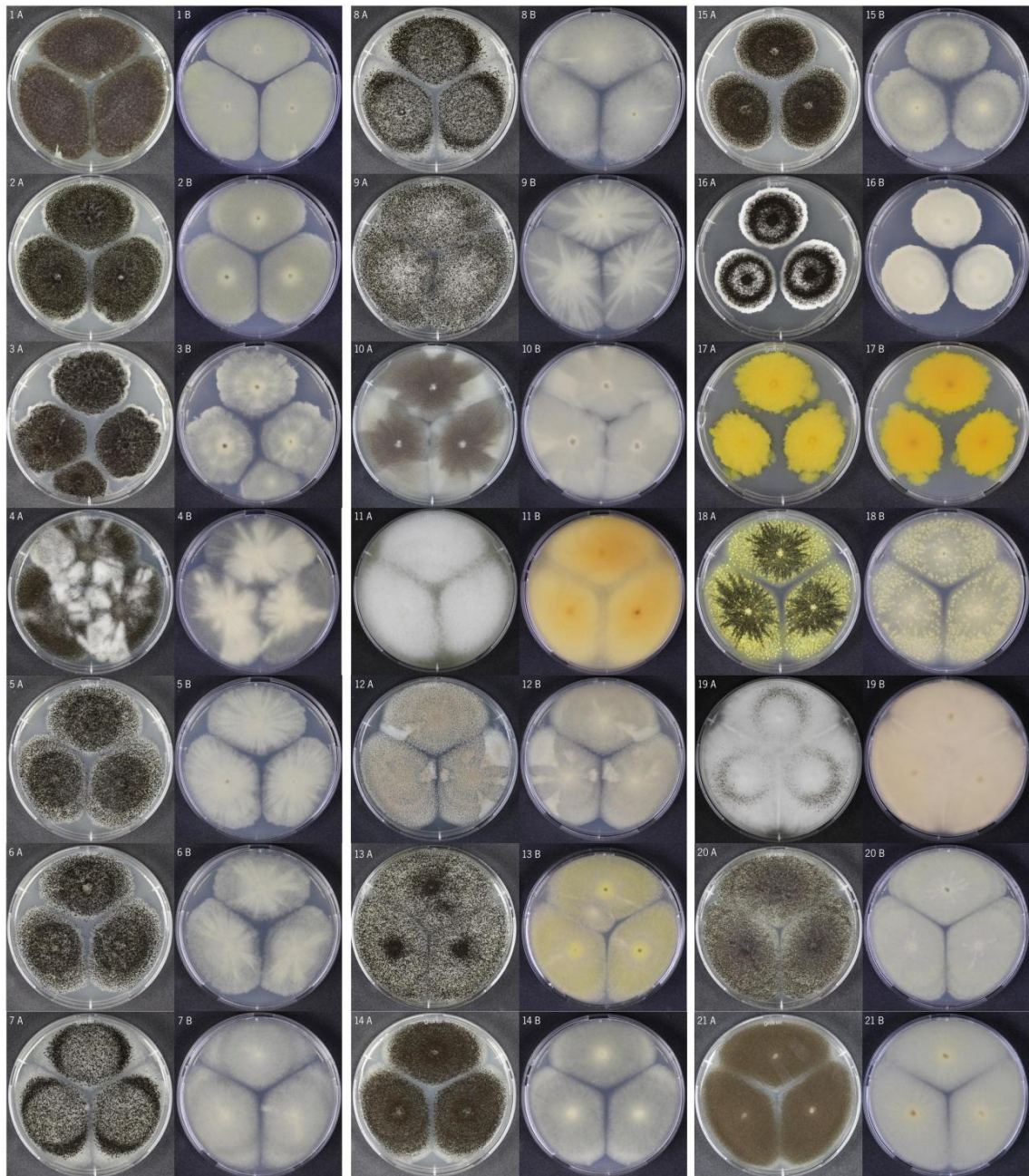


Figure 22 - Fungi colonies, from time point III, grown 7 days in the dark, at 25 °C in MEA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

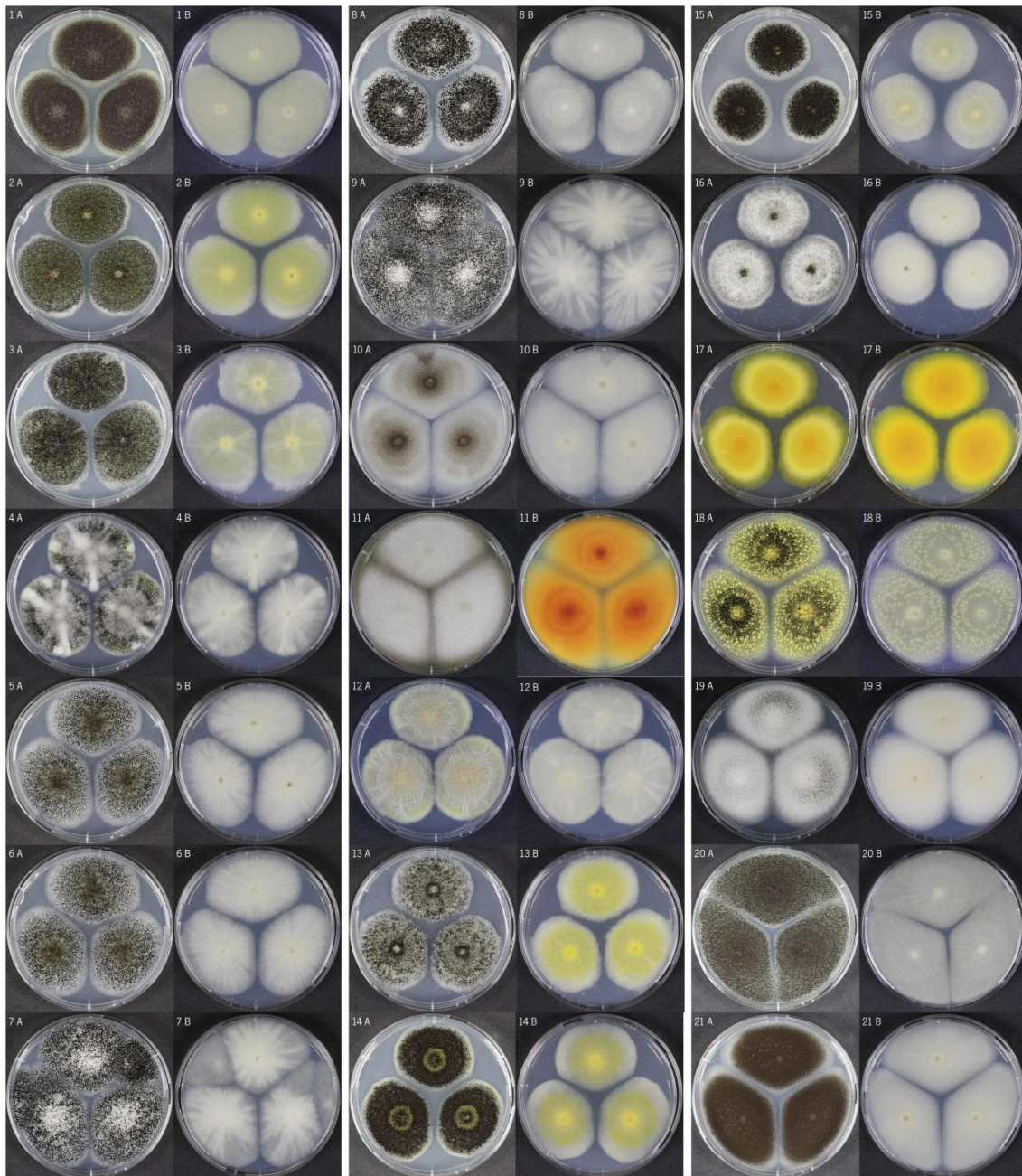


Figure 23 - Fungi colonies, from time point I, grown 7 days in the dark, at 25 °C in PDA. A - front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150; 13- *A. niger* MUM 03.01; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151; 19- *A. tubingensis* MUM 06.152; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153.

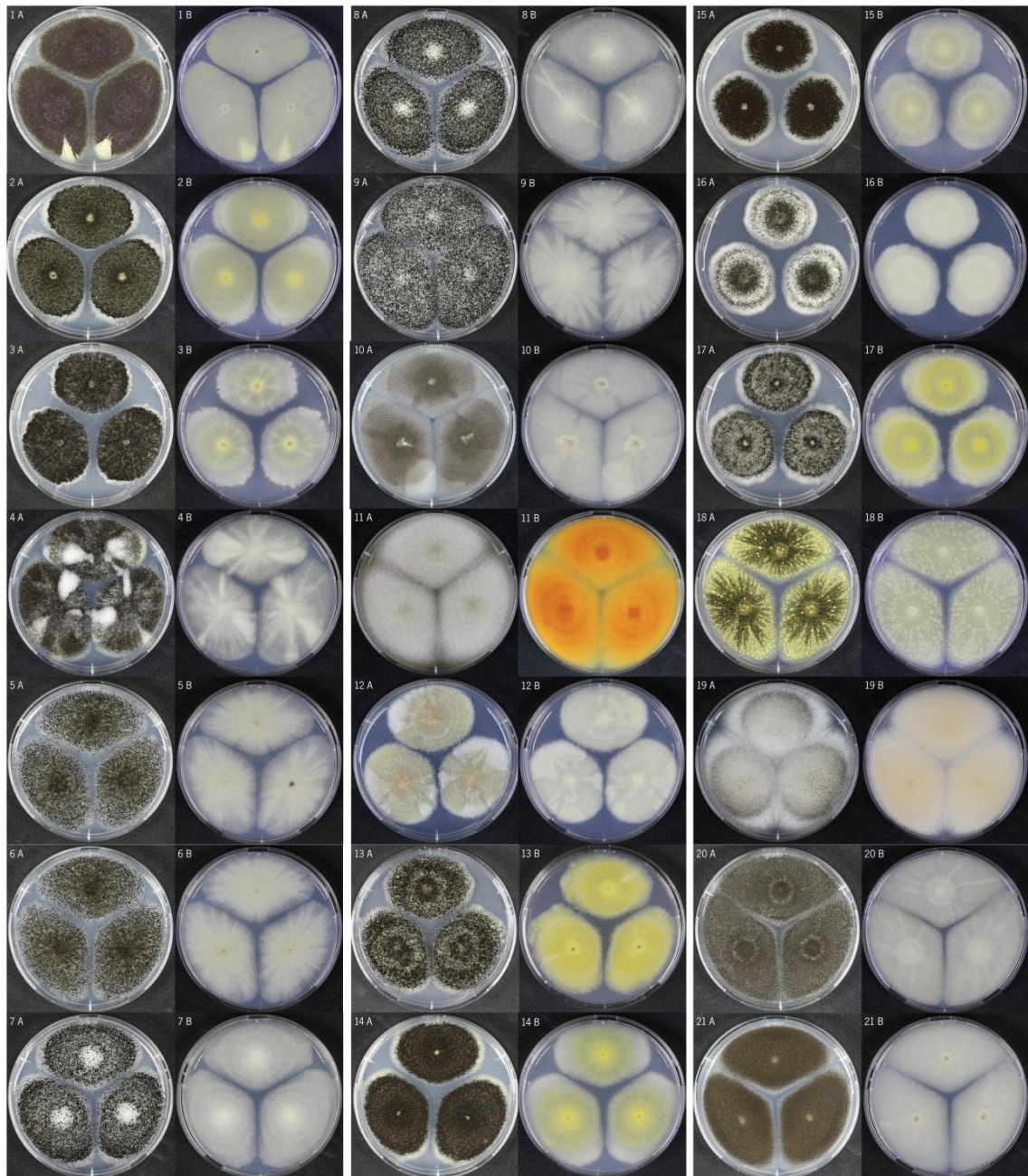


Figure 24 - Fungi colonies, from time point II, grown 7 days in the dark, at 25 °C in PDA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150; 13- *A. niger* MUM 03.01; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151; 19- *A. tubingensis* MUM 06.152; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153.

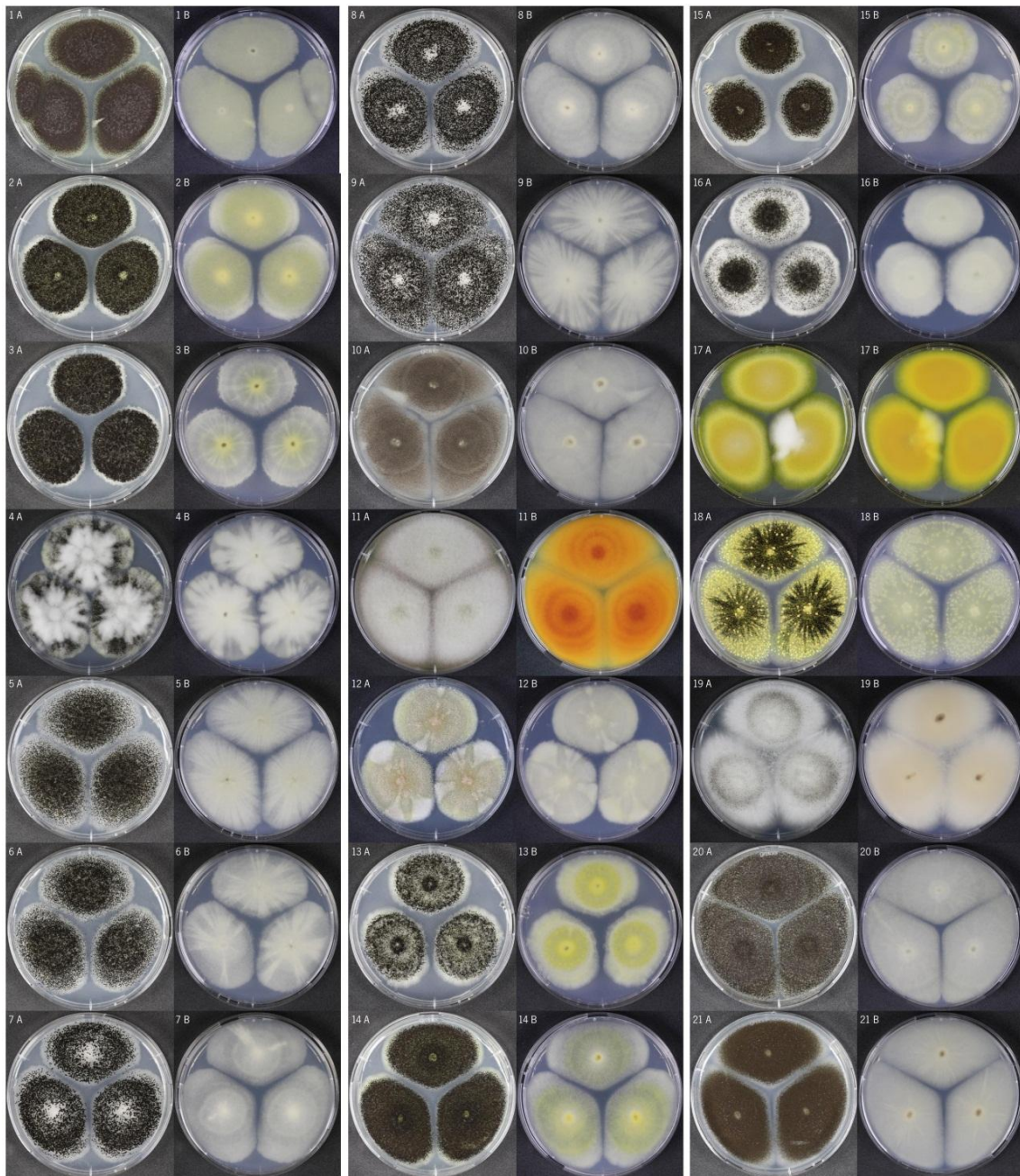


Figure 25 - Fungi colonies, from time point III, grown 7 days in the dark, at 25 °C in PDA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

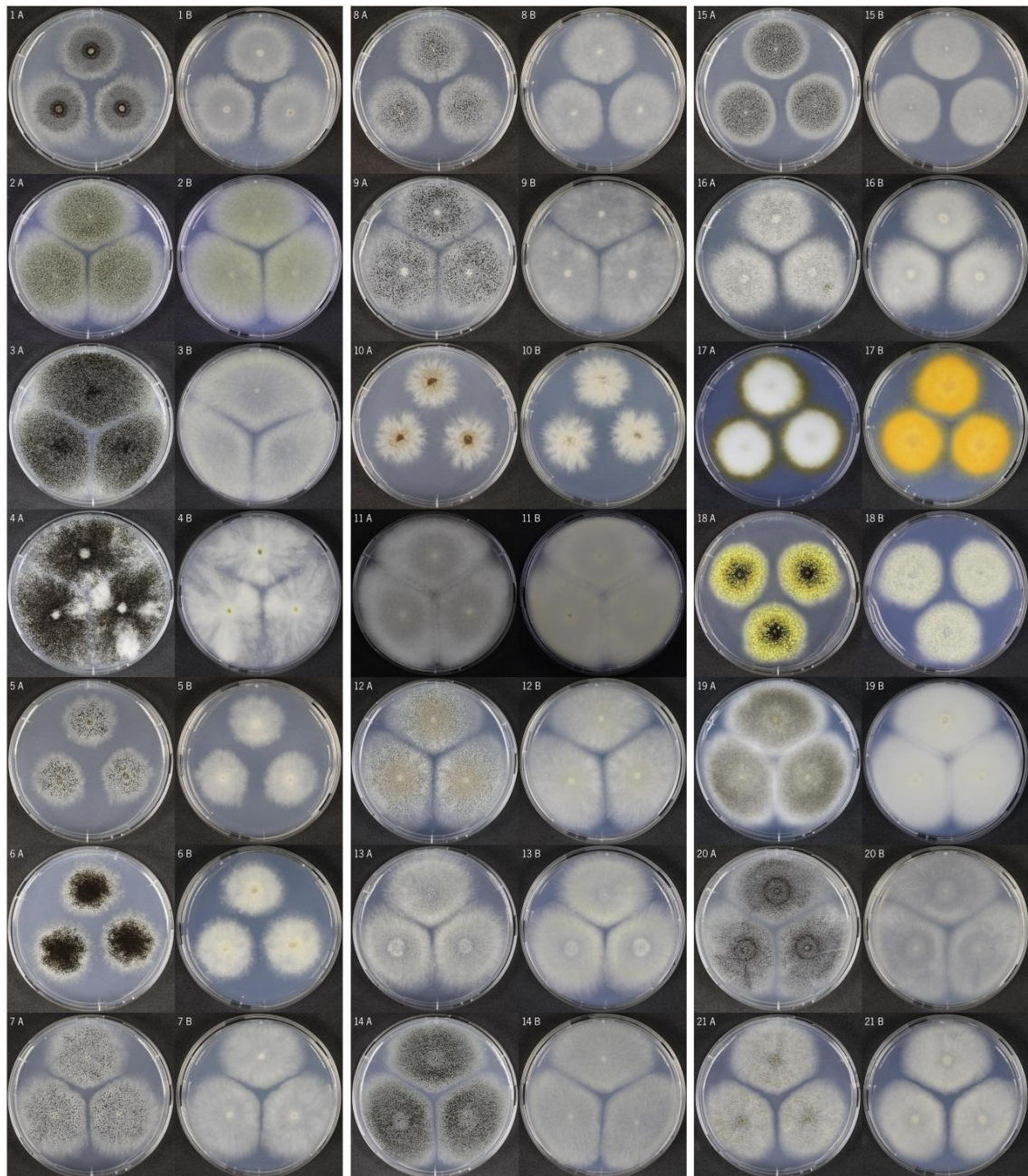


Figure 26 - Fungi colonies, from time point I, grown 7 days in the dark, at 25 °C in CZ. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

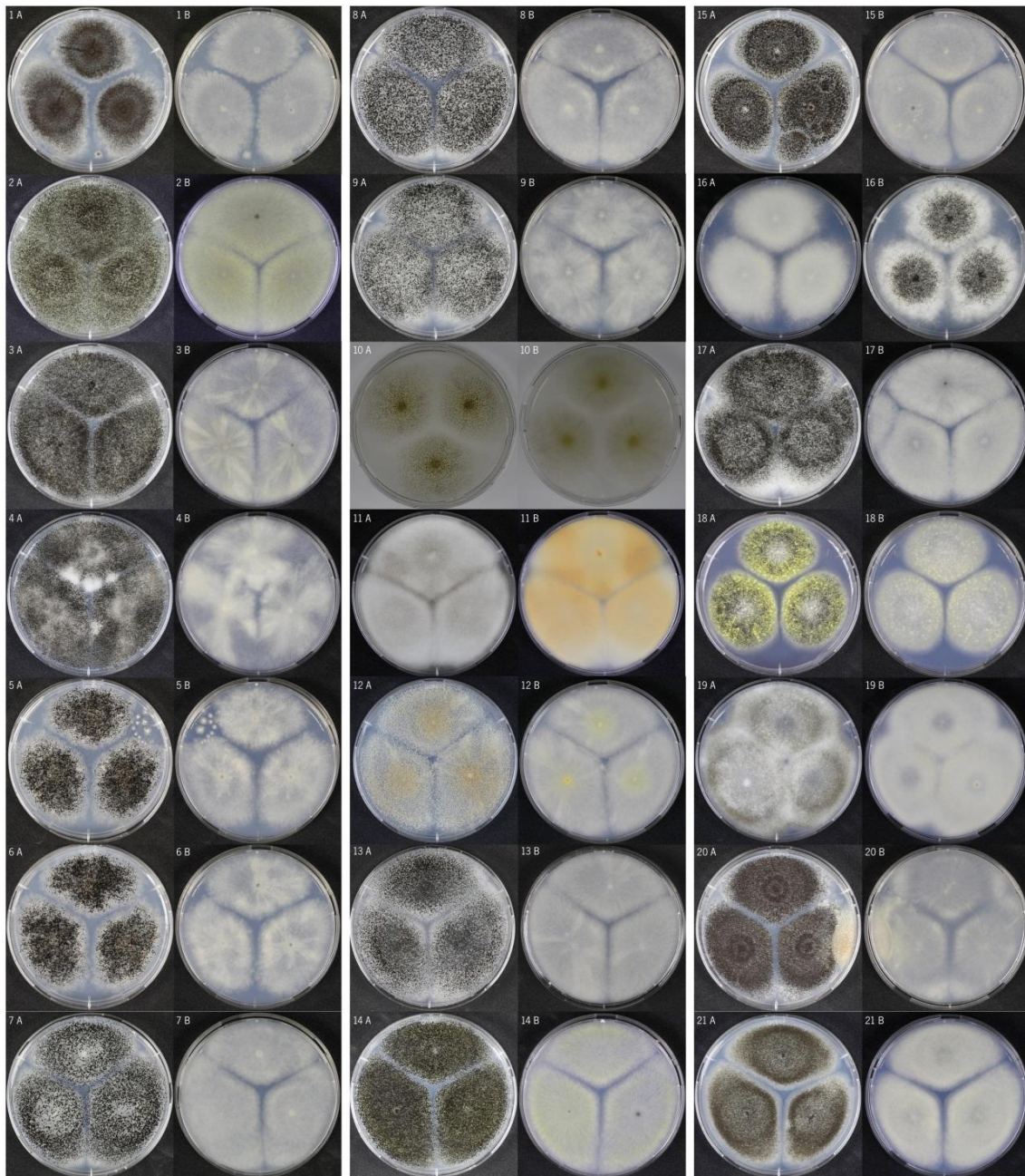


Figure 27 - Fungi colonies, from time point II, grown 7 days in the dark, at 25 °C in CZ. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

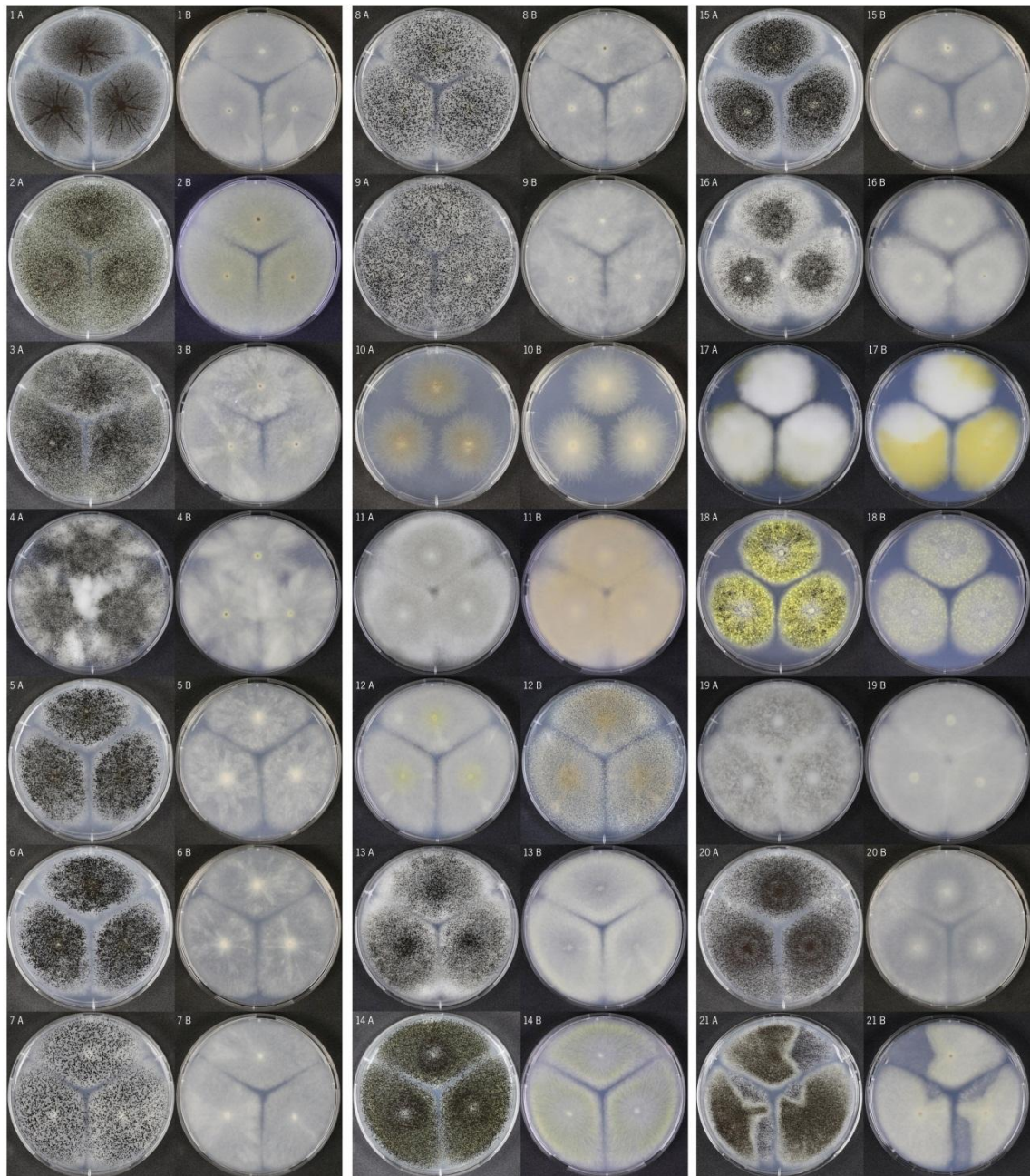


Figure 28 - Fungi colonies, from time point III, grown 7 days in the dark, at 25 °C in CZ. A - front of the colony, B - reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

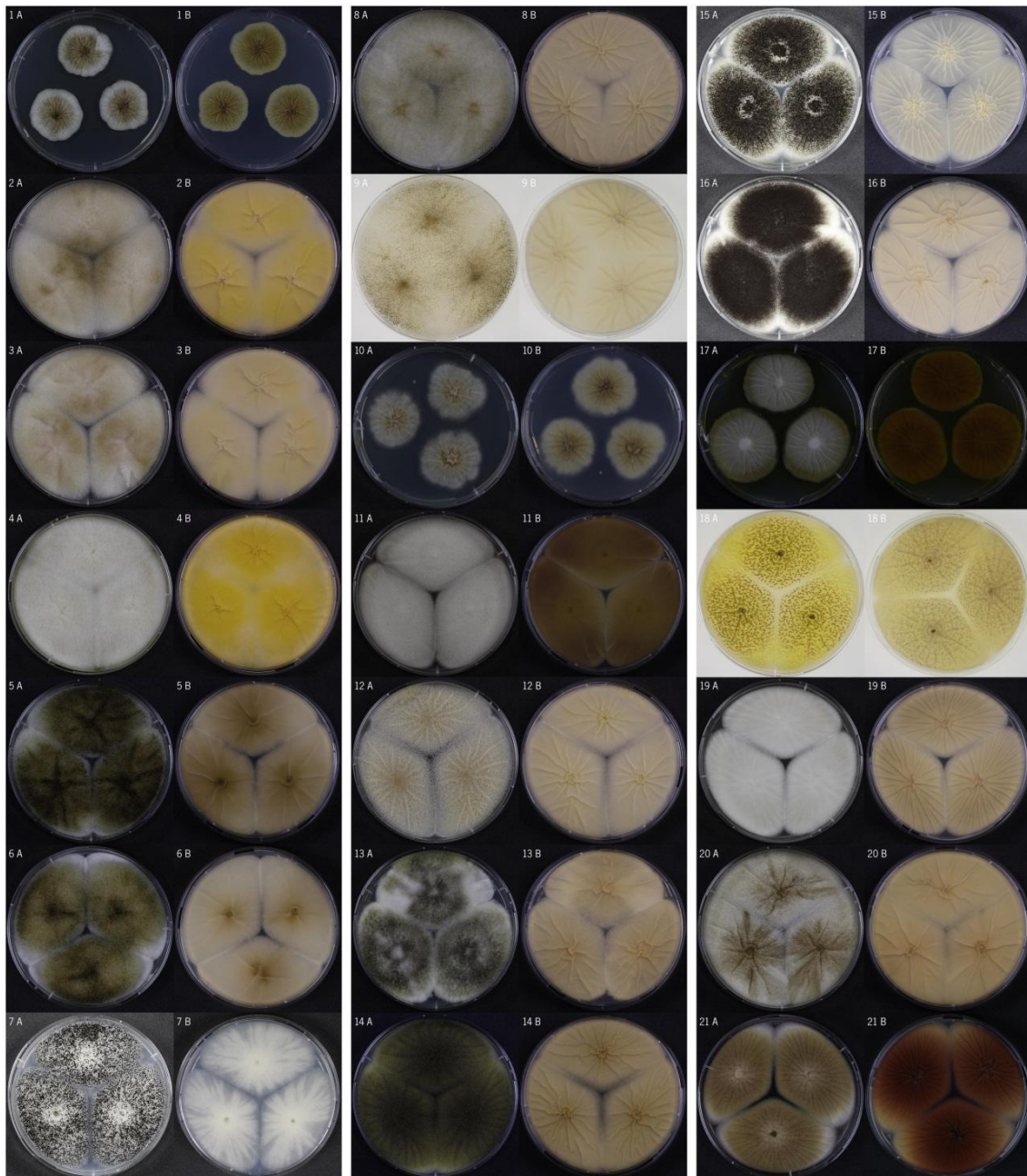


Figure 29 - Fungi colonies, from time point I, grown 7 days in the dark, at 25 °C in CYA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

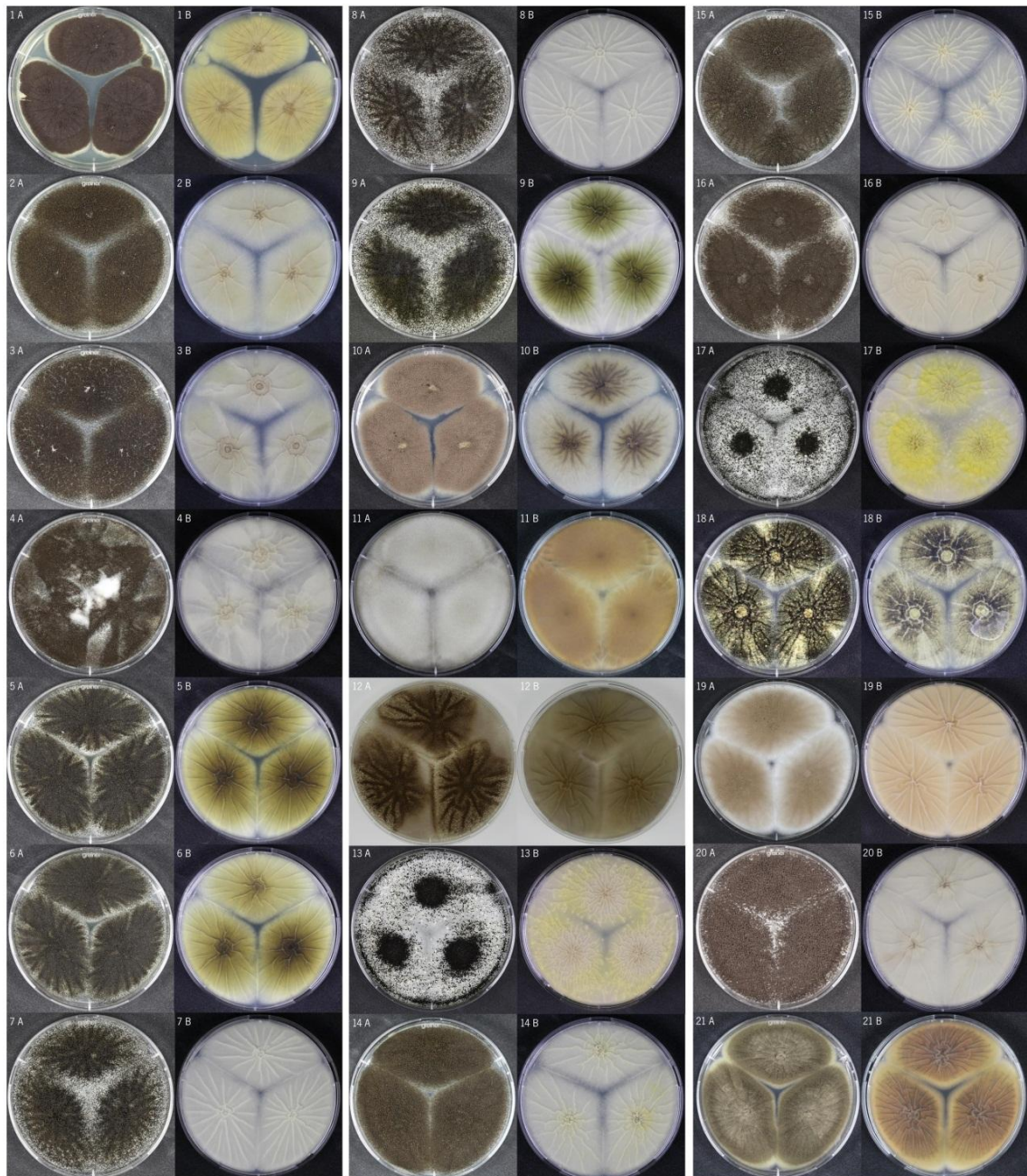


Figure 30 - Fungi colonies, from time point II, grown 7 days in the dark, at 25 °C in CYA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

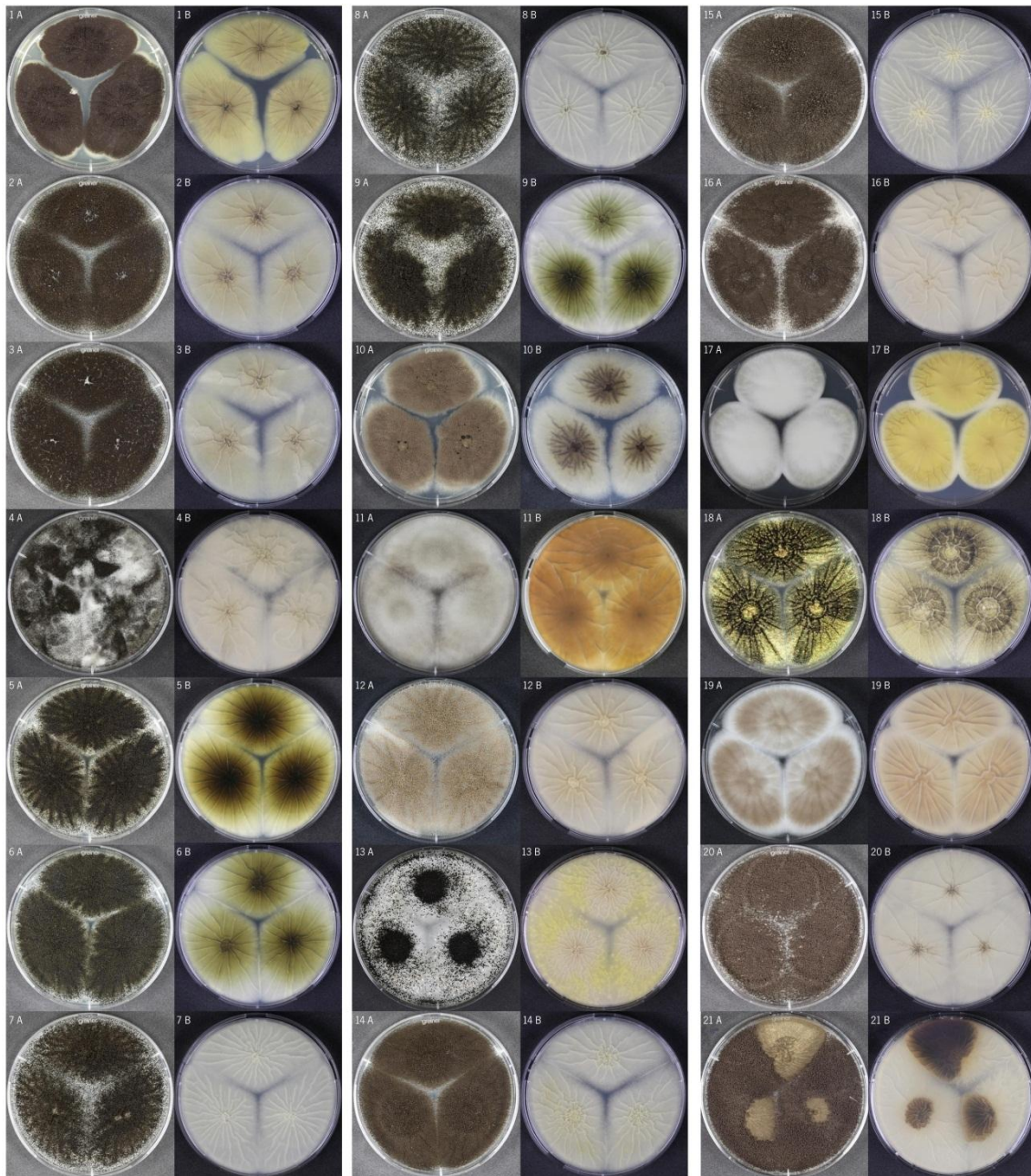


Figure 31 - Fungi colonies, from time point III, grown 7 days in the dark, at 25 °C in CYA. A- front of the colony, B – reverse of the colony. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

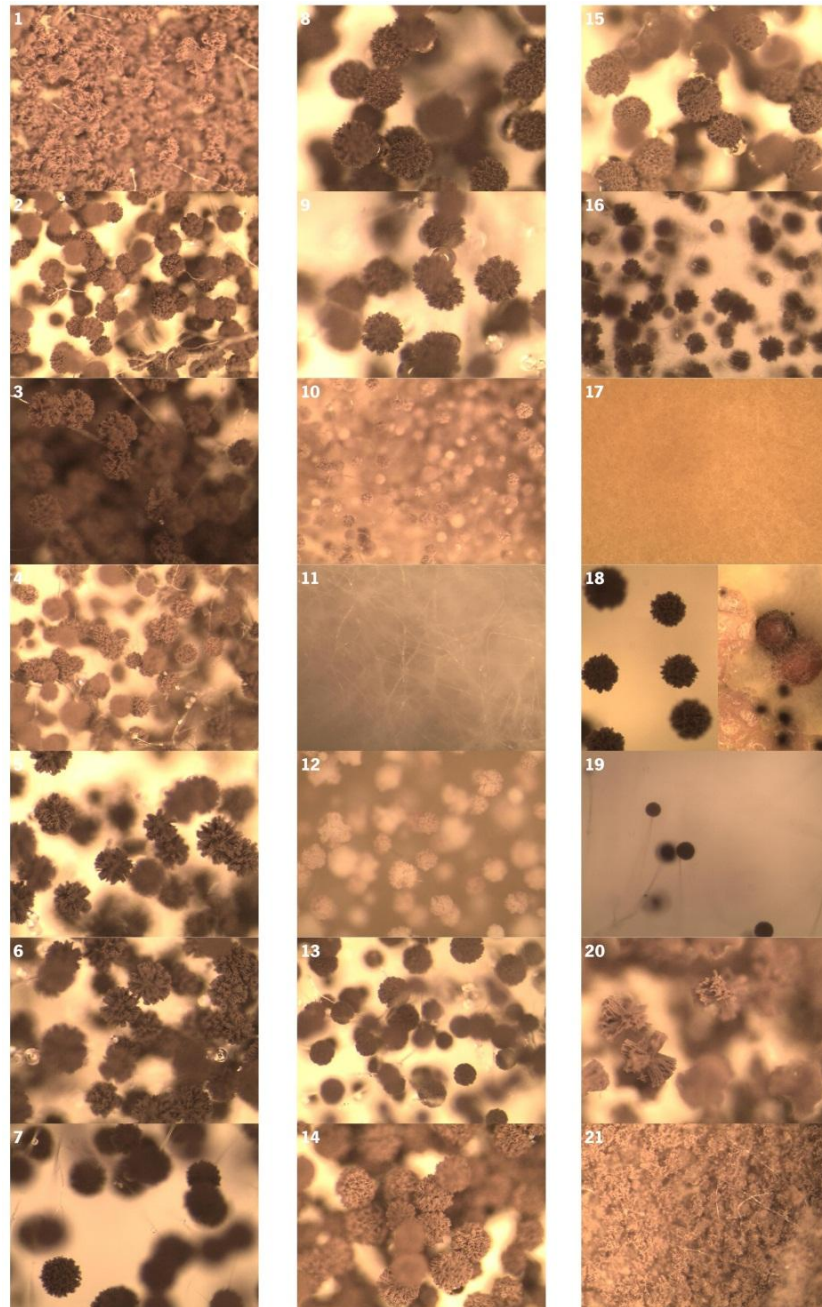


Figure 32 - General colour and morphology characteristics, from time point I. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179[†]; 3- *A. brasiliensis* MUM 06.180[†]; 4- *A. brasiliensis* MUM 06.181[†]; 5- *A. carbonarius* MUM 01.08[†]; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49[†]; 8- *A. ibericus* MUM 04.68[†]; 9- *A. ibericus* MUM 04.86[†]; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03[†]; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11[†]; 15- *A. niger* MUM 05.13[†]; 16- *A. phoenicis* MUM 03.05[†]; 17- *A. phoenicis* MUM 03.10[†]; 18- *A. sclerotiumiger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01[†]; 21- *A. vadensis* MUM 06.153[†]. Total magnification: 32 x.

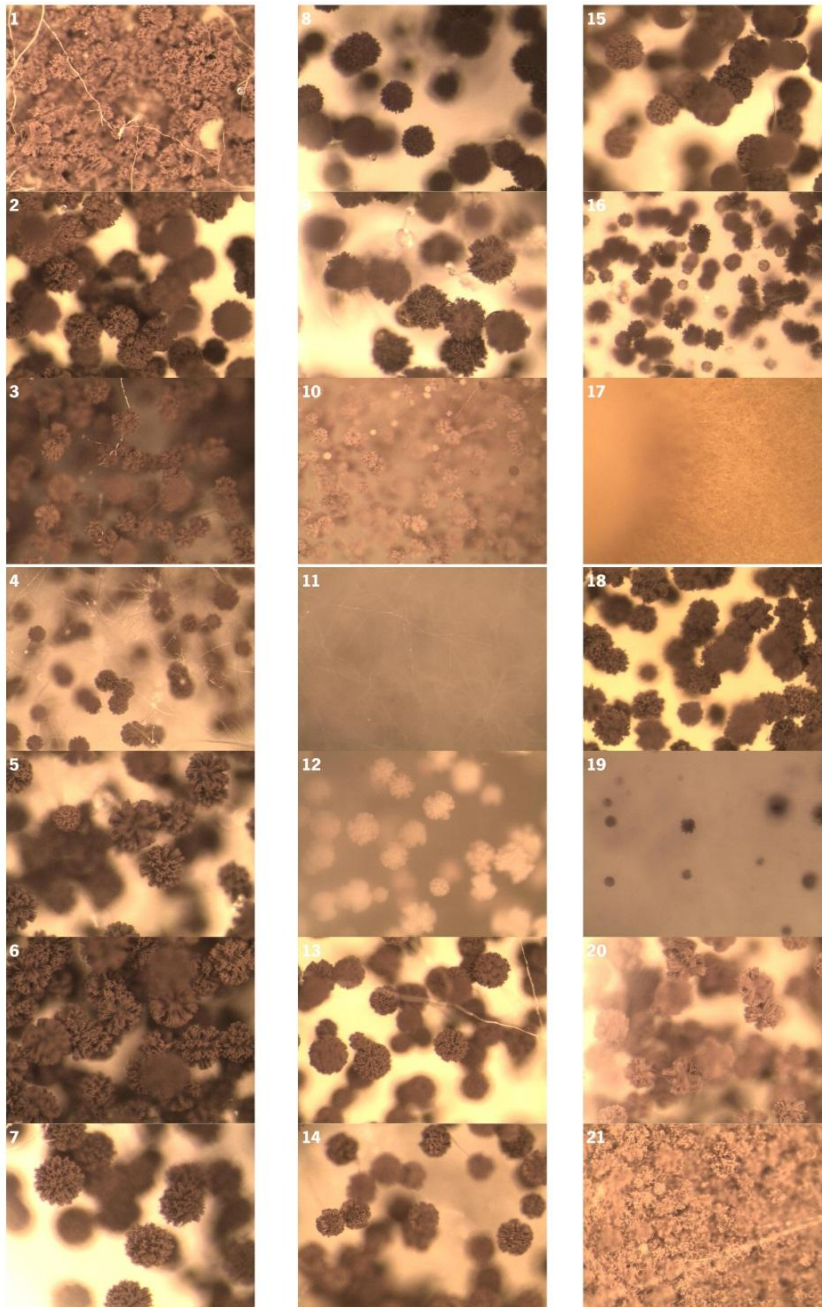


Figure 33 - General colour and morphology characteristics, from time point II. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumiger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†]. Total magnification: 32 x.

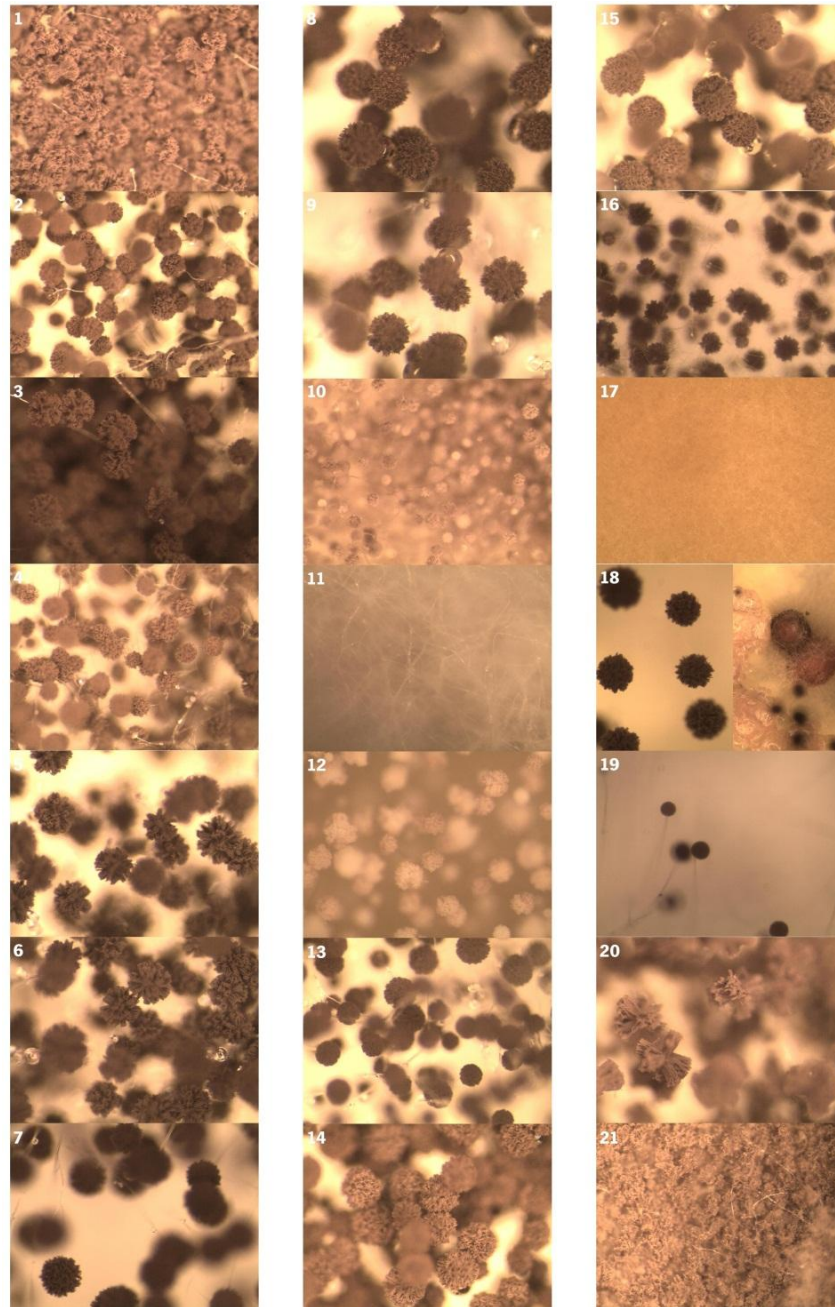


Figure 34 - General colour and morphology characteristics, from time point III. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotioniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†]. Total magnification: 32 x.

3.5.2. Microscopy

On MEA, conidiophores are largely produced allowing an easy retrieval of spores for analysis and characterisation. *Aspergillus aculeatus*, *A. japonicus* and *A. uvarum* were the only uniseriate species presenting only phialides; all the other strains presented metulae and phialides

(biseriate), which is concordant with the existent literature (Klich, 2002; Simões et al., 2013b). These features were common and equal for all the times (I, II and III) analysed (Figures 35, 36 and 37).

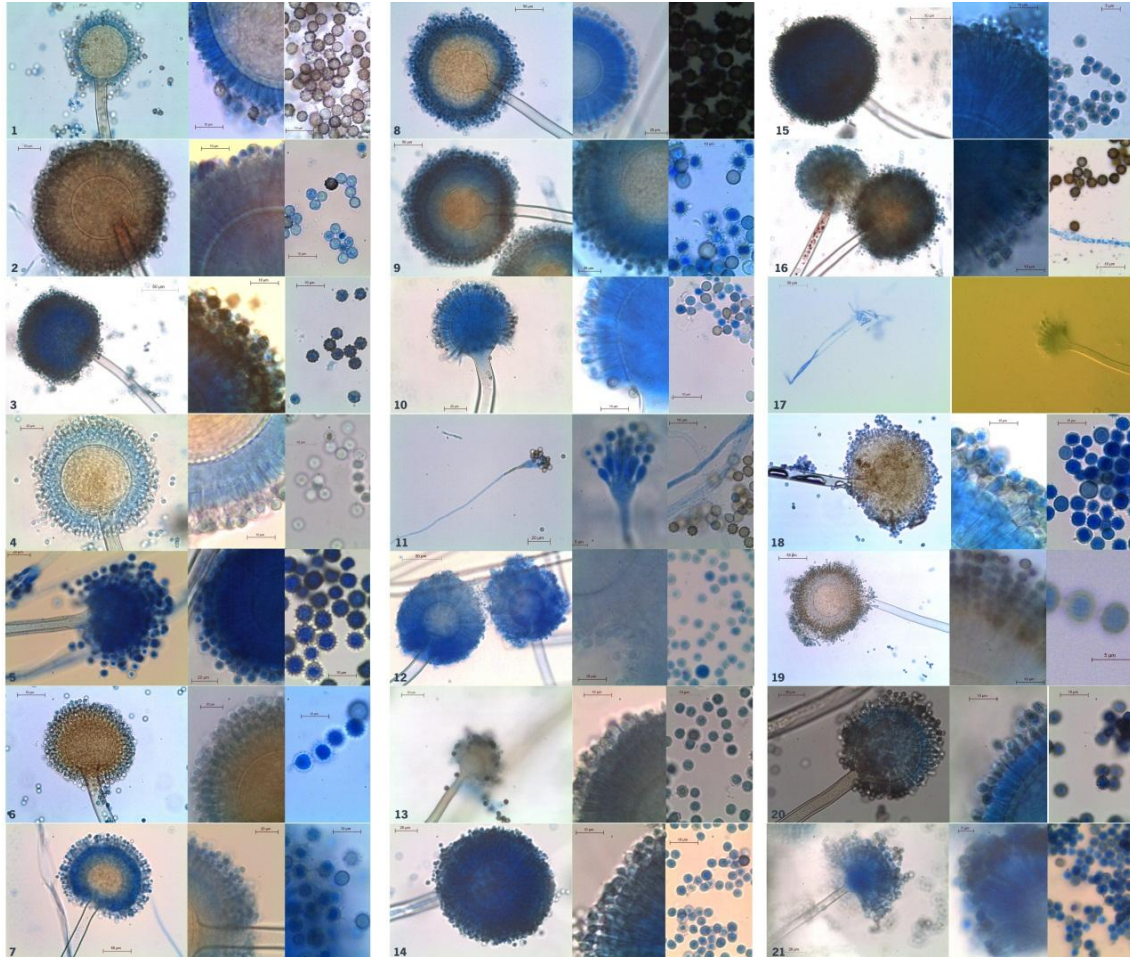


Figure 35 - Spores of the different species under light microscopy, from time point I. 1- *A. aculeatus* MUM 03.11¹; 2- *A. brasiliensis* MUM 06.179¹; 3- *A. brasiliensis* MUM 06.180¹; 4- *A. brasiliensis* MUM 06.181¹; 5- *A. carbonarius* MUM 01.08¹; 6- *A. ellipticus* MUM 03.12¹; 7- *A. ibericus* MUM 03.49¹; 8- *A. ibericus* MUM 04.68¹; 9- *A. ibericus* MUM 04.86¹; 10- *A. japonicus* MUM 03.02¹; 11- *A. japonicus* MUM 98.03¹; 12- *A. lacticofeatus* MUM 06.150¹; 13- *A. niger* MUM 03.01¹; 14- *A. niger* MUM 05.11¹; 15- *A. niger* MUM 05.13¹; 16- *A. phoenicis* MUM 03.05¹; 17- *A. phoenicis* MUM 03.10¹; 18- *A. sclerotium* MUM 06.151¹; 19- *A. tubingensis* MUM 06.152¹; 20- *A. uvarum* MUM 08.01¹; 21- *A. vadensis* MUM 06.153¹.

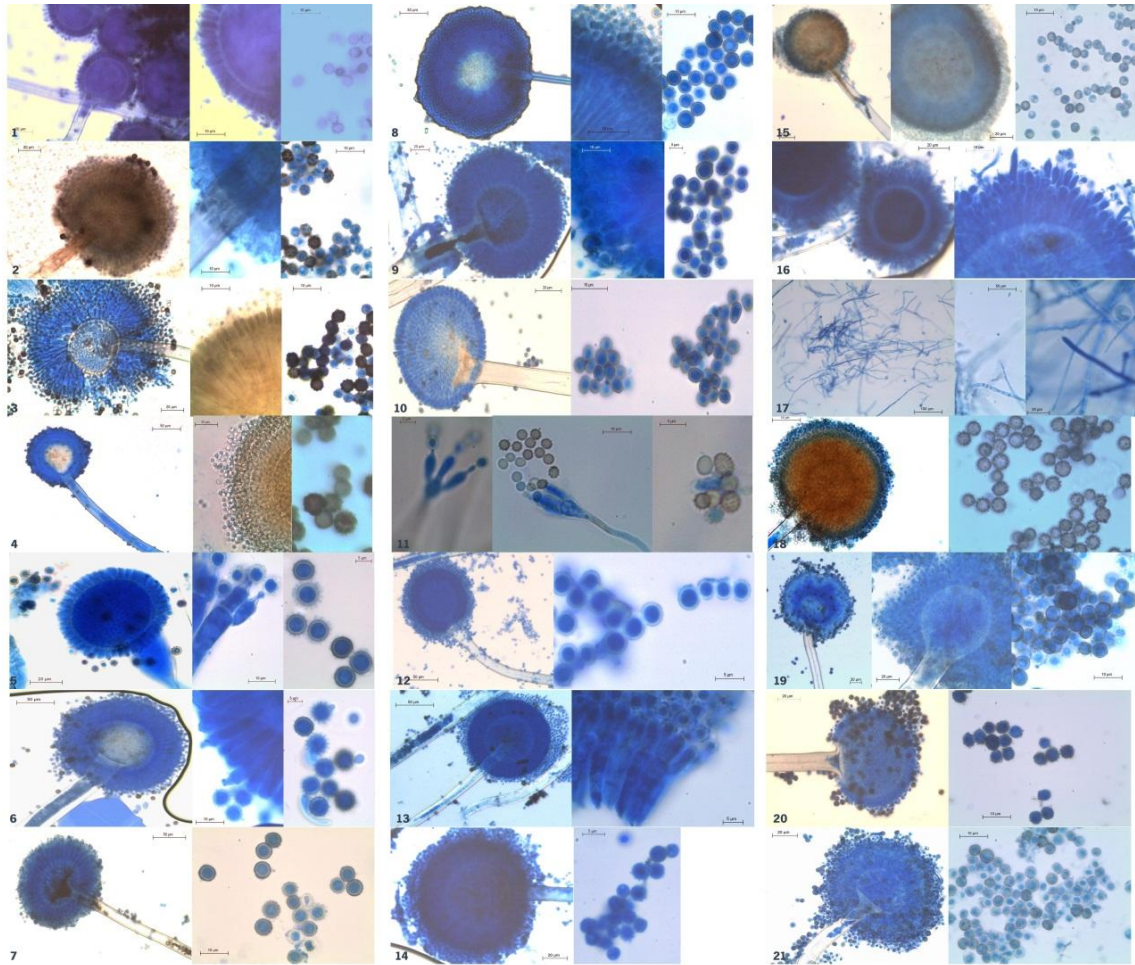


Figure 36 - Spores of the different species under light microscopy, from time point II. 1- *A. aculeatus* MUM 03.11; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150; 13- *A. niger* MUM 03.01; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151; 19- *A. tubingensis* MUM 06.152; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153.

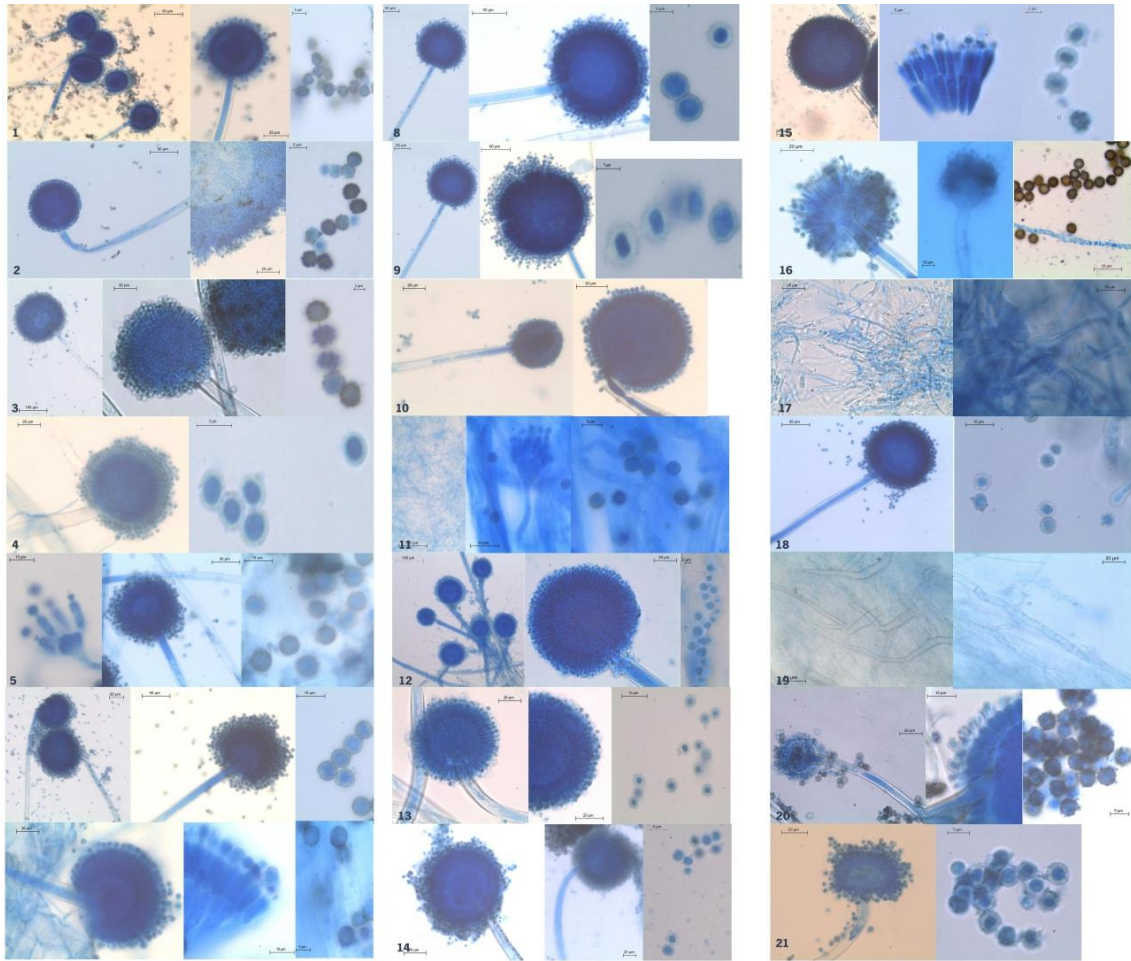


Figure 37 - Spores of the different species under light microscopy, from time point III. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotioniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†].

3.5.3. SEM

Moreover, each species presented spores with different characteristics (Figure 38), either in shape, size as in ornamentation features.

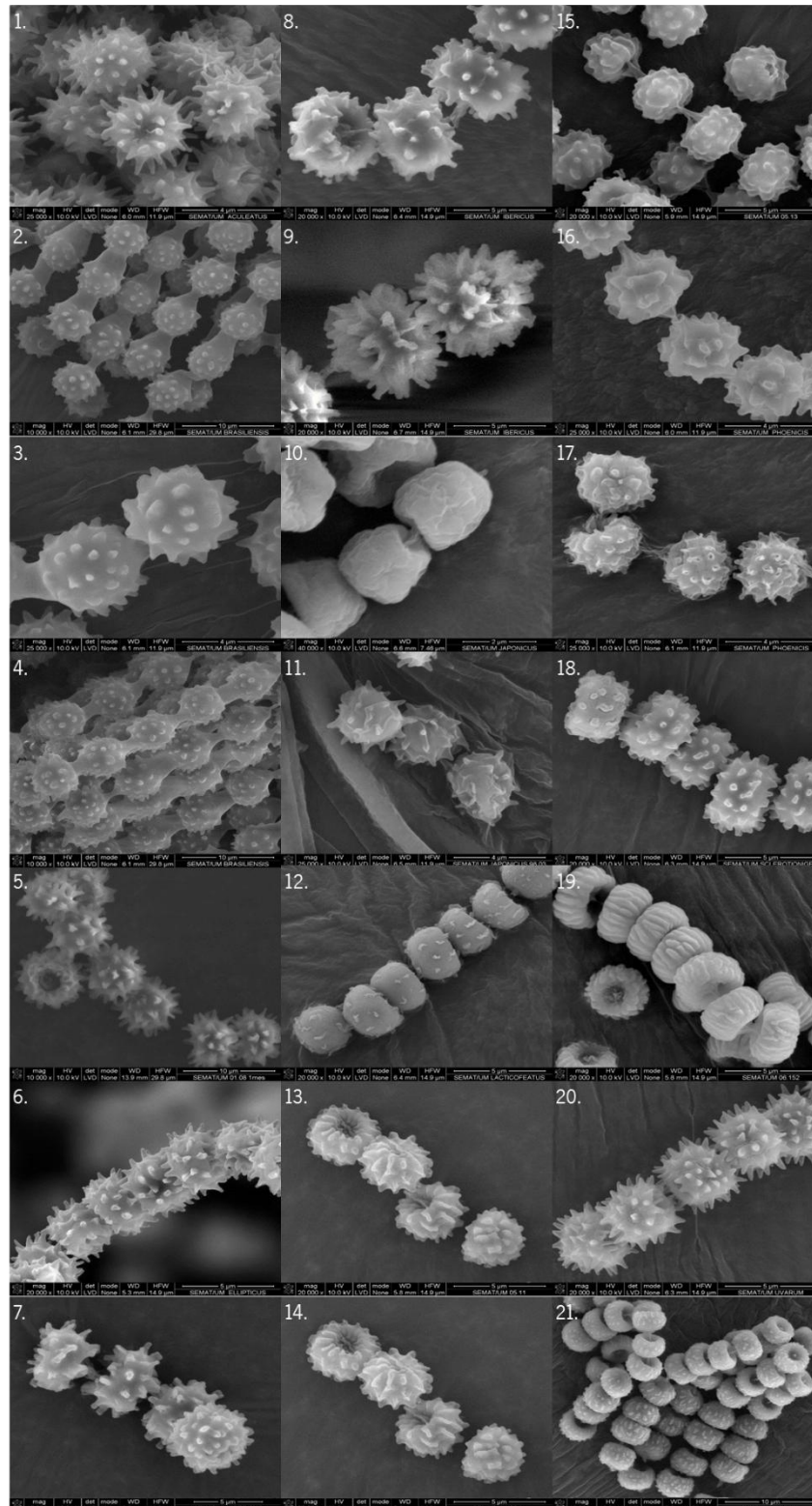


Figure 38 - SEM images of spores of the different species. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotium* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†]. Bars: 2, 4, 5 and 21: 10µm; 6-9, 12-15 and 18-20: 5µm; 1, 3, 11, 16 and 17: 4µm; and 10: 2µm.

3.5.4. Assessment of mycotoxins production

3.5.4.1. Qualitative assessment of mycotoxins production

For the detection of secondary metabolites, the 21 selected strains were analysed for the time points I, II and III (before preservation, after 2 weeks of accelerated storage and after 4 weeks of accelerated storage) and the qualitative assessment is presented on table 10.

Table 10 - Mycotoxins production detected for the selected *Aspergillus* strains for the time points I, II and III.

Strain	MUM N°	Fluorescence in CMA		
		I	II	III
<i>A. aculeatus</i>	03.11	-	-	-
	06.179	-	-	-
<i>A. brasiliensis</i>	06.180	-	+	-
	06.181	-	+	-
<i>A. carbonarius</i>	01.08	-	-	-
<i>A. ellipticus</i>	03.12	-	-	-
	03.49	-	-	-
<i>A. ibericus</i>	04.68	-	-	-
	04.86	-	-	-
<i>A. japonicus</i>	03.02	-	-	-
	98.03	-	-	-
<i>A. lacticoffeatus</i>	06.150	+	+	-
	03.01	-	-	-
<i>A. niger</i>	05.11	-	-	-
	05.13	++	++	++
<i>A. phoenicis</i>	03.05	-	+	+
	03.10	+	+	-
<i>A. sclerotiumniger</i>	06.151	+	+	+
<i>A. tubingensis</i>	06.152	-	-	-
<i>A. uvarum</i>	08.01	-	-	-
<i>A. vadensis</i>	06.153	-	-	-
Negative control (CMA media)		-	-	-

- = absence of fluorescence; + = presence of regular fluorescence; ++ = presence of high fluorescence.

3.5.4.2. Assessment of mycotoxins production (OTA and FB2)

For the detection of OTA a standard curve was prepared. The limit of detection (LOD) considered for this essay was of: 0.72 ng/mL. And the equation achieved for the following calculus was the one presented in figure 39.

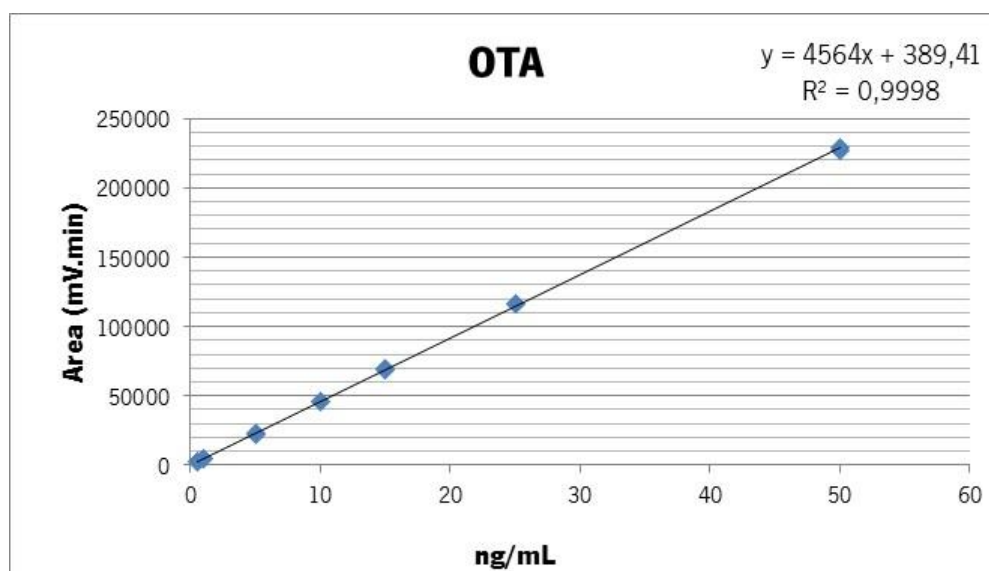


Figure 39 - Standard curve for the OTA determination.

For the detection of fumonisins, the essay was performed using the standard solution of fumonisin B2 (FB2) and the standard curve achieved (Figure 40) was used for the FB2 determination of the evaluated strains.

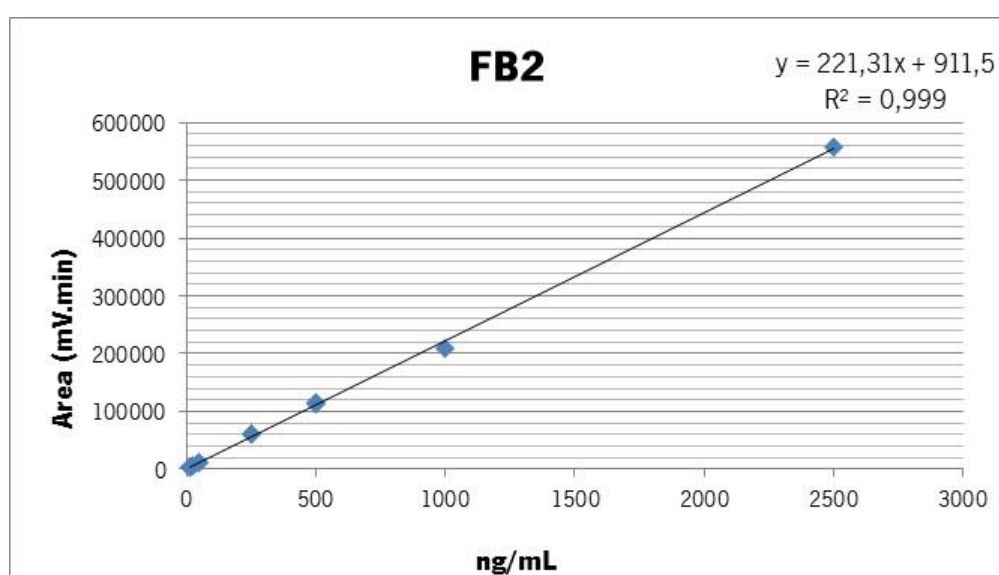


Figure 40 - Standard curve for the FB2 determination.

The LOD considered for the FB2 detection was: 75 ng/mL.

For the assessment of the mycotoxins, OTA and FB2, two different concentrations of a standard solution were used as control while injecting the samples. The values detected for the time points: I – before preservation, II – after 2 weeks of accelerated storage and III – after 4 weeks of accelerated storage; are listed in table 11.

Table 11 - OTA and FB2 concentrations determined for the selected *Aspergillus* strains for the time points I, II and III.

Strain	MUM No.	Mycotoxins					
		OTA ng/mL (Rt*=16.2min)			FB2 ng/mL (Rt*= 23.5 min)		
		I	II	III	I	II	III
<i>A. aculeatus</i>	03.110	-	1.113	-	-	-	-
	06.179	1.8	17.463	26.818	-	-	-
<i>A. brasiliensis</i>	06.180	85.810	10.574	5.662	104.865	-	-
	06.181	-	11.840	9.659	-	276.840	573.180
<i>A. carbonarius</i>	01.08	11.200	22.235	17.168	-	-	-
<i>A. ellipticus</i>	03.12	-	-	12.951	-	-	-
	03.49	-	-	-	-	-	-
<i>A. ibericus</i>	04.68	-	-	-	-	-	-
	04.86	-	-	-	-	-	-
<i>A. japonicus</i>	03.02	-	-	-	-	-	-
	98.03	72.150	≤	-	-	-	-
<i>A. lacticoffeatus</i>	06.150	535.500	2.632	705.114	-	-	-
	03.01	-	≤	83.205	81.000	-	-
<i>A. niger</i>	05.11	2508.000	4858.365	2673.144	-	-	-
	05.13	-	-	-	-	-	-
<i>A. phoenicis</i>	03.05	4.200	13.290	7.219	-	-	-
	03.10	-	-	-	-	-	-
<i>A. sclerotiumniger</i>	06.151	2284.000	1018.994	1513.948	-	-	-
<i>A. tubingensis</i>	06.152	-	-	-	-	-	-
<i>A. uvarum</i>	08.01	-	-	-	-	-	-
<i>A. vadensis</i>	06.153	-	12.253	39.005	-	-	-

*Rt = retention time; - = no detectable production; ≤ = production equal or beneath the cut value considered for detection.

3.5.5. Enzymatic screening results

3.5.5.1. Proteolytic activity determination

Positive results were achieved by the clearing of the medium under the inoculated and grown colony, resulting in a translucent part of the media like shown in figure 41.

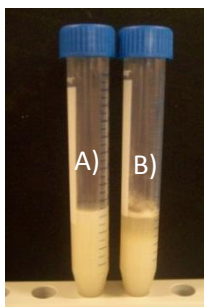


Figure 41 - Representative photograph of a negative (A) and a positive (B) protease producer.

After the 7th day of incubation, all the strains presented proteolytic activity like shown in figure 42.

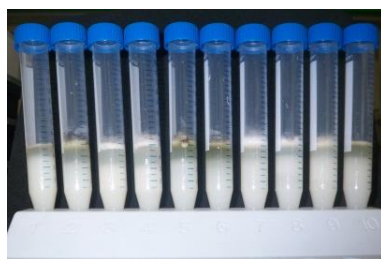


Figure 42 - Representative image of the results obtained for proteolytic activity determination.

It was possible to distinguish higher activities, with clearing distances higher than 14 mm, on some strains. Others presented smaller distances of clearing in the medium, lower than 6 mm, and these were considered to have proteolytic activity, but were not regarded for the quantification analysis. The obtained results for the time points: I – before preservation, II – after 2 weeks of accelerated storage and III – after 4 weeks of accelerated storage, are listed in table 12.

Table 12 - Determined levels of proteolytic activity, for the time points I, II and III (before preservation, after 2 weeks of accelerated storage and after 4 weeks of accelerated storage).

Strain	MUM N°	Deep clearing distance from inoculated colony (mm)		
		I	II	III
<i>A. aculeatus</i>	03.11	< 6	11	11
<i>A. brasiliensis</i>	06.179	11	11	9

Strain	MUM N°	Deep clearing distance from inoculated colony (mm)		
		I	II	III
	06.180	< 6	6	9
	06.181	8	6	11
<i>A. carbonarius</i>	01.08	< 6	11	14
<i>A. ellipticus</i>	03.12	< 6	11	14
	03.49	< 6	16	16
<i>A. ibericus</i>	04.68	15	16	14
	04.86	< 6	11	14
<i>A. japonicus</i>	03.02	< 6	11	9
	98.03	8	6	7
<i>A. lacticoffeatus</i>	06.150	< 6	14	14
	03.01	< 6	11	11
<i>A. niger</i>	05.11	< 6	11	11
	05.13	< 6	11	11
<i>A. phoenicis</i>	03.05	8	6	11
	03.10	< 6	<6	14
<i>A. sclerotiumniger</i>	06.151	8	16	14
<i>A. tubingensis</i>	06.152	< 6	11	11
<i>A. uvarum</i>	08.01	< 6	11	8
<i>A. vadensis</i>	06.153	< 6	<6	<6

All the samples have activity and they do not lose this capacity even after aged by accelerated storage. In fact, when analysing all the results, there seems to be a general increase of the proteolytic activity correspondent to the age increase.

3.5.5.2. Polysaccharide-hydrolytic enzymes assay

The production of polysaccharide-hydrolytic enzymes by the strains of *Aspergillus* grown on maltose medium has been followed for 7 days as described previously. All the 21 selected strains were analysed on the 3 time points, (I, before preservation; II, 2 weeks after accelerated storage and III, 4 weeks of accelerated storage). The maximum activity was detected for each strain, and is presented in nKat/mL, on the table 13.

Table 13 - Maximum enzymatic activity detected for the *Aspergillus* strains studied, for the time points I, II and III.

Strain	MUM No.	Max activity (nkat/mL)				Day of Max Activity				Time point of the analysed strain			
		CMC	Xylan	Pectin	Mannan	CMC	Xylan	Pectin	Mannan	CMC	Xylan	Pectin	Mannan
<i>A. aculeatus</i>	03.110	0.018	0.028	0.056	0.044	7	5	1	7	I	II	III	III
	06.179	0.018	0.026	0.086	0.045	7	5	1	7	III	II	II	II
<i>A. brasiliensis</i>	06.180	0.021	0.017	0.046	0.047	7	7	1	7	III	III	III	III
	06.181	0.020	0.037	0.046	0.045	7	5	1	7	III	II	III	III
<i>A. carbonarius</i>	01.08	0.020	0.019	0.082	0.048	7	5	1	7	III	II	III	III
<i>A. ellipticus</i>	03.12	0.019	0.021	0.078	0.047	7	5	1	7	III	III	III	III
	03.49	0.023	0.012	0.088	0.042	7	3	1	7	I	II	II	I
<i>A. ibericus</i>	04.68	0.019	0.016	0.137	0.034	7	7	1	7	III	III	II	III
	04.86	0.021	0.012	0.079	0.050	7	5	1	7	II	I	II	III
<i>A. japonicus</i>	03.02	0.018	0.042	0.096	0.045	7	7	1	7	III	I	II	III
	98.03	0.019	0.027	0.093	0.046	7	1	1	7	III	III	III	II
<i>A. laticoffeatus</i>	06.150	0.006	0.012	0.109	0.029	7	1	1	7	III	II	III	III
<i>A. niger</i>	03.01	0.020	0.037	0.046	0.045	7	5	1	7	III	II	III	III
	05.11	0.019	0.014	0.097	0.044	7	7	1	7	III	III	II	III
<i>A. phoenicis</i>	05.13	0.018	0.017	0.109	0.043	7	7	1	7	III	III	II	III
	03.05	0.024	0.020	0.046	0.035	3	5	1	7	II	I	I	III
	03.10	0.003	0.005	0.089	0.019	7	2	1	7	III	III	II	III
<i>A. sclerotiumniger</i>	06.151	0.020	0.028	0.058	0.048	7	5	1	7	III	II	III	III
<i>A. tubingensis</i>	06.152	0.025	0.022	0.141	0.046	7	2	1	7	III	III	II	III
<i>A. uvarum</i>	08.01	0.022	0.025	0.119	0.046	7	2	1	7	III	III	III	III
<i>A. vadensis</i>	06.153	0.021	0.023	0.074	0.056	7	2	1	2	III	III	III	III

For the analysed strains, and for all the time points, carboxymethyl cellulose activity was generally detected on the 7th day of the assay. The highest value detected was 0.025 nKat/mL, for the strain *A. tubingensis* MUM 06.152, on the 7th day corresponding to the most aged sample (III).

The maximum activity of xylan was detected, mainly, on the 5th day. Nine from all the fungi present the higher values of activity on this day. The most aged samples, from time point III, are the ones that presented higher values of activity (10 from the 21 strains selected). The higher values of enzymatic activity were detected on the strains: *A. japonicus* MUM 03.02, *A. niger* MUM 03.01 and *A. sclerotiumniger* MUM 06.151. And, even though the peaks of maximum

activity were detected for days 3 and 5, there is not a common profile of xylanase activity for all the strains.

The higher values of pectinolytic activity were detected for the strain *A. tubingensis* MUM 06.152, on the 1st day of the assay, for the most aged sample (time point III). The higher activities were detected, in 100%, for the 1st day of the assay; and, 11 strains in the 21 selected presented higher values of activity for the most aged samples (time point III).

For the mannanase activity, the maximum values were detected on the 7th day of the assay; and 18 strains from the 21 selected had higher enzymatic activity on the most aged samples. The strains that, on the 7th day, presented higher values of activity were *A. ibericus* MUM 04.86 (0.050 nKat/mL) and *A. sclerotium* MUM 06.151 (0.048 Kat/mL).

The chosen medium for the growth of the chosen targeted strains was adequate to determine, evaluate and screen the enzymatic profiles for the four targeted enzymes: CMCase, xylanase, pectinase and mannanase. The enzymes analysed in this screening assay were present in all of the *Aspergillus* strains tested. For all the samples analysed it was possible to observe that older samples, with more time of accelerated storage and therefore more aged, have higher values of enzymatic activity, especially when compared with non-aged samples (time point I). Nevertheless, no common pattern was detected that allowed achieving an enzymatic profile for all the strains analysed on the different time points.

3.5.6. MALDI-TOF MS results

Reproducibility of the spectra is not completely achieved when analysing filamentous fungi through MALDI-TOF MS. There is always a small percentage of differences when comparing spectra that can easily be justified by differences in several parameters. The heterogeneous morphological phenotypes of filamentous fungi translate into heterogeneity in the MALDI-TOF mass spectra, either between different strains of the same species as well as between subcultures of the same strain (Normand et al., 2013). And the sample preparation can have minor changes that will influence the final spectral result. When doing identification, this can be diminished, just like suggested by Normand et al. (2013), by increasing both the number of reference spectra of a given strain included in the reference library and the number of deposits used to generate each reference spectra. But, when using comparison of spectra to understand the differences between different time points of a preservation method, like in this particular case, the main focus has to be the principal peaks obtained in each spectra (Figures 43 and 44).

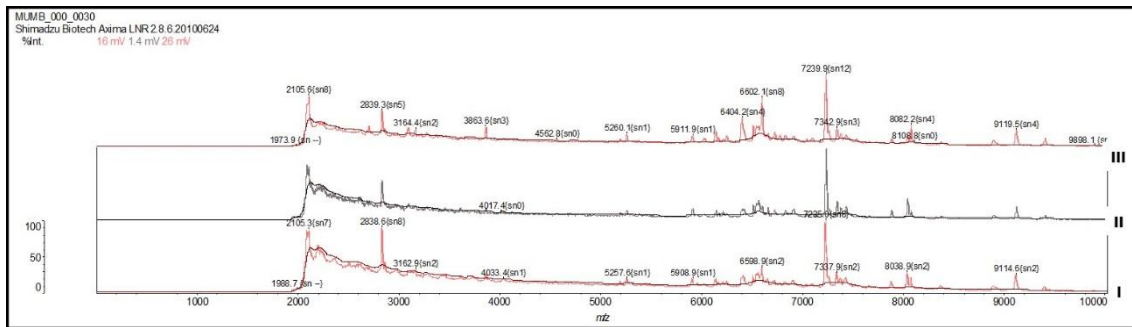


Figure 43 - Representative spectra comparison of the three time points for the strain *A. brasiliensis* MUM 06.180. Time points: I – before preservation, II – after 2 weeks of accelerated storage and III – after 4 weeks of accelerated storage.

It is notable in figure 43, that all spectra present a high similarity. But the last time point (III), 4 weeks of ageing, presents a higher number of peaks within its spectrum.

In figure 44, the most different spectrum, presenting a higher number of peaks, is the one corresponding to time point II, 2 weeks of accelerated storage.

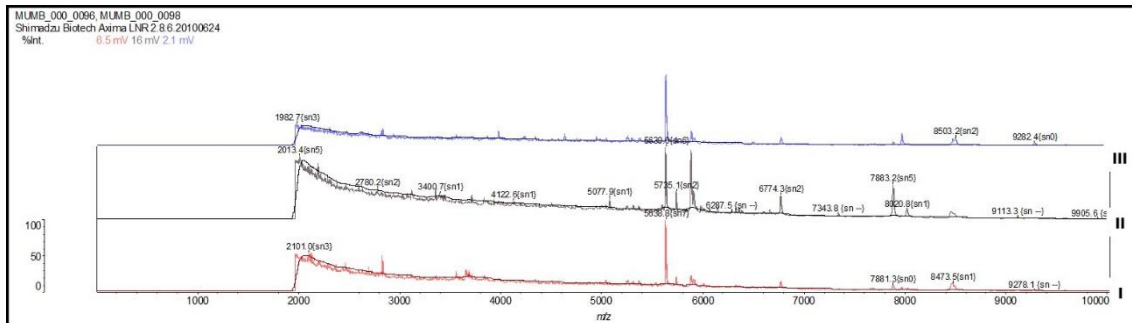


Figure 44 - Representative spectra comparison of the three time points for the strain *A. japonicus* MUM 03.02. Time points: I – before preservation, II – after 2 weeks of accelerated storage and III – after 4 weeks of accelerated storage.

In all the strains analysed, it was possible to see that, after ageing, there were significant differences on the spectral data with an increase, and fewer times a decrease, on the number of peaks (Table 14). But for some, this increase was noted on the time point II and for others on time point III.

Table 14 - Similarity between spectral data for the *Aspergillus* strains for the time points I, II and III.

Strain	MUM N°	Evolution in the number of peaks from spectral data, from time points II and II, compared with I	
		II	III
<i>A. aculeatus</i>	03.11	I	S
<i>A. brasiliensis</i>	06.179	S	S
	06.180	S	I
	06.181	S	S
<i>A. carbonarius</i>	01.08	S	S
<i>A. ellipticus</i>	03.12	S	S
	03.49	I	S
<i>A. ibericus</i>	04.68	S	D
	04.86	I	D
<i>A. japonicus</i>	03.02	I	S
	98.03	I	D
<i>A. lacticoffeatus</i>	06.150	I	S
<i>A. niger</i>	03.01	S	I
	05.11	C	S
	05.13	I	C
<i>A. phoenicis</i>	03.05	D	S
	03.10	D	S
<i>A. sclerotioniger</i>	06.151	D	I
<i>A. tubingensis</i>	06.152	S	I
<i>A. uvarum</i>	08.01	I	I
<i>A. vadensis</i>	06.153	S	S

C = changes in the location of obtained peaks; D = decreased the number of peaks; I = increased the number of peaks; S = similar number of peaks.

In figure 45, it is possible to observe that some of the different strains of *Aspergillus* section *Nigri* group with the 3 time points tested.

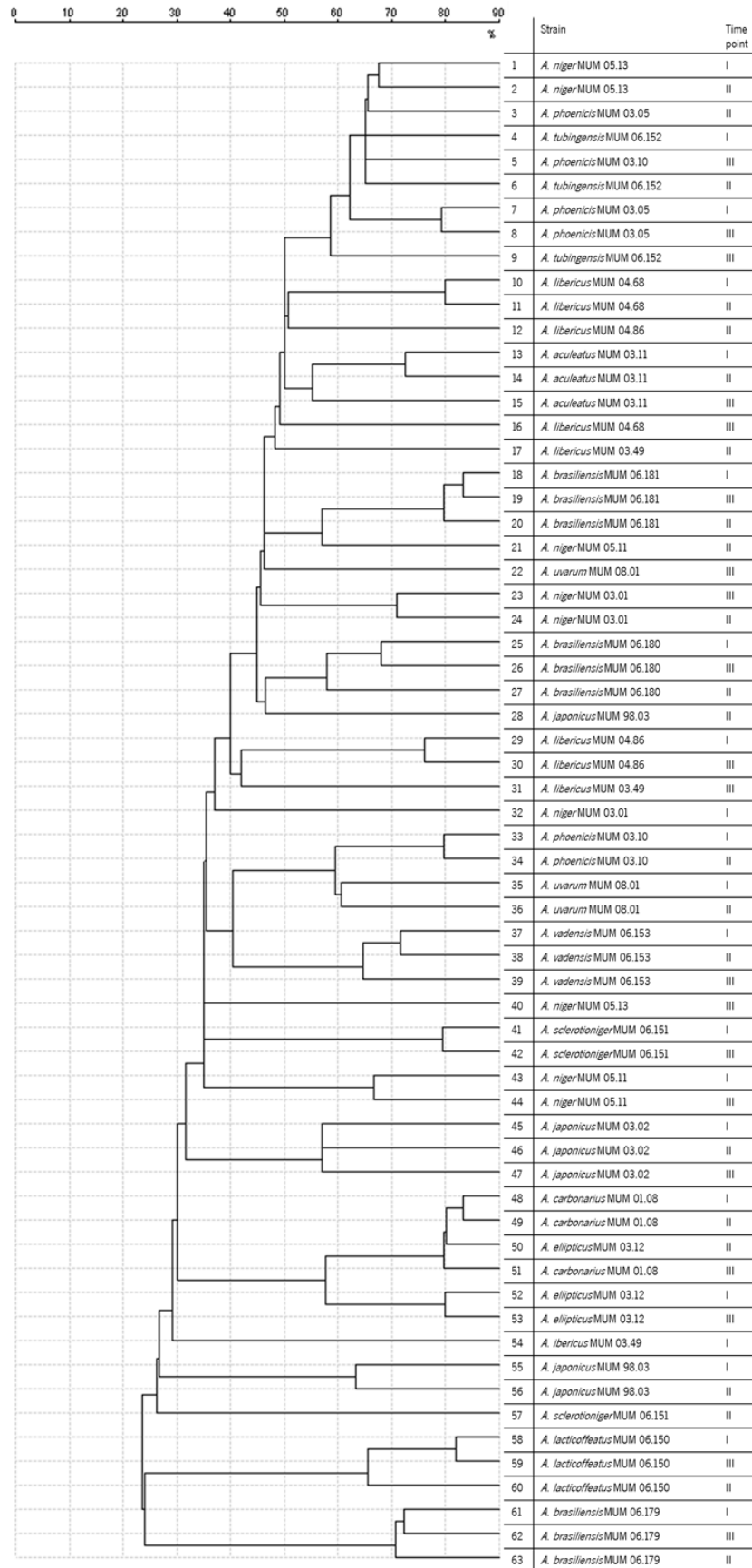


Figure 45 - Dendrogram with all data from the three time points for all the strains tested. Time points: I – before preservation, II – after 2 weeks of accelerated storage and III – after 4 weeks of accelerated storage.

From MALDI-TOF MS analysis, either spectral comparison or dendrogram, it is possible to see that after ageing strains, they suffer some alterations. But in general, no significant differences were observed which allows to conclude that lyophilisation is a good preservation method for *Aspergillus* strains from section *Nigri*.

3.5.7. Molecular biology analysis

The most commonly used approaches to discriminate between different strains within a species, like ITS, do not allow to discriminate some cases. Therefore, it was seen as a good exploration hypothesis the use of other genomic areas.

For this analysis step, the goal was to characterise the amplification products and verify their homology. Though there seemed to be a pattern from the results obtained from the samples before preservation when compared to the results after preservation. It was not possible to standardise the results. The same conditions presented different results not allowing for this technique to be used as a comparison method on the polyphasic approach for the evaluation of the samples in their different times before and after preservation.

3.5.7.1. Quality control of DNA extraction

For all the 21 selected strains, DNA was extracted from grown colonies from the 3 time points (I, II and III).

After DNA extraction the samples were applied in an electrophoresis gel to check for the success on extraction (Figure 46), and the DNA was then quantified by spectrophotometry (NanoDrop 1000, Thermo Scientific).

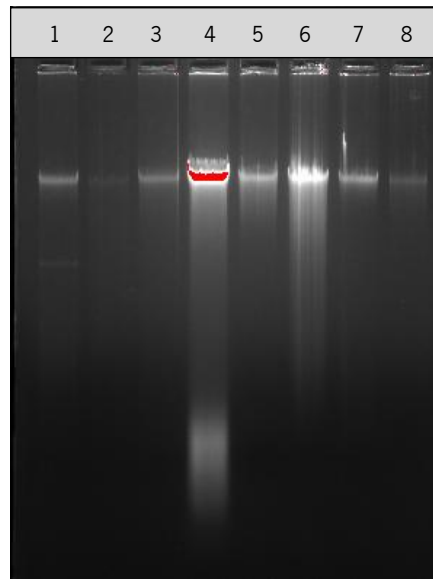


Figure 46 - Representative image of an electrophoresis gel of DNA products from the samples: 1- *A. niger* MUM 03.01†; 2- *A. tubingensis* MUM 06.152†; 3- *A. phoenicis* MUM 03.10; 4- *A. lacticoffeatus* MUM 06.150†; 5- *A. brasiliensis* MUM 06.179; 6- *A. ibericus* MUM 04.86; 7- *A. niger* MUM 05.13 and 8- *A. phoenicis* MUM 03.05.

3.5.7.2. Screening through ERIC/PCR

For the samples from time point I, before being preserved by lyophilisation, an initial screening was made, and the strains obtained were the ones presented on figure 47.

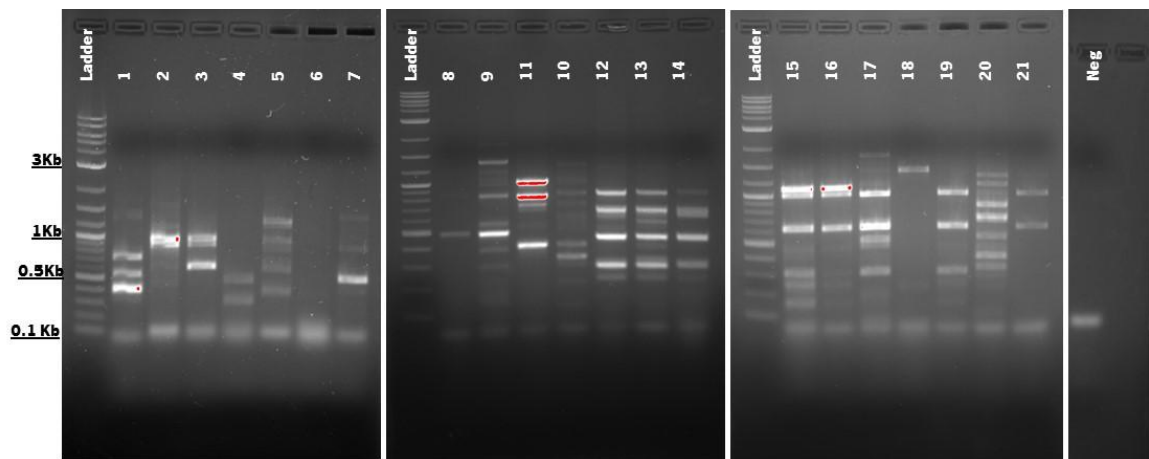


Figure 47 - Amplification results from ERIC/PCR for the strains from time point I. 1- *A. aculeatus* MUM 03.11†; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12†; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02†; 11- *A. japonicus* MUM 98.03; 12- *A. lacticoffeatus* MUM 06.150†; 13- *A. niger* MUM 03.01†; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151†; 19- *A. tubingensis* MUM 06.152†; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153†. Neg is the negative control.

For the time point II, 2 weeks of accelerated storage, the samples were analysed and the results are presented on figure 48.

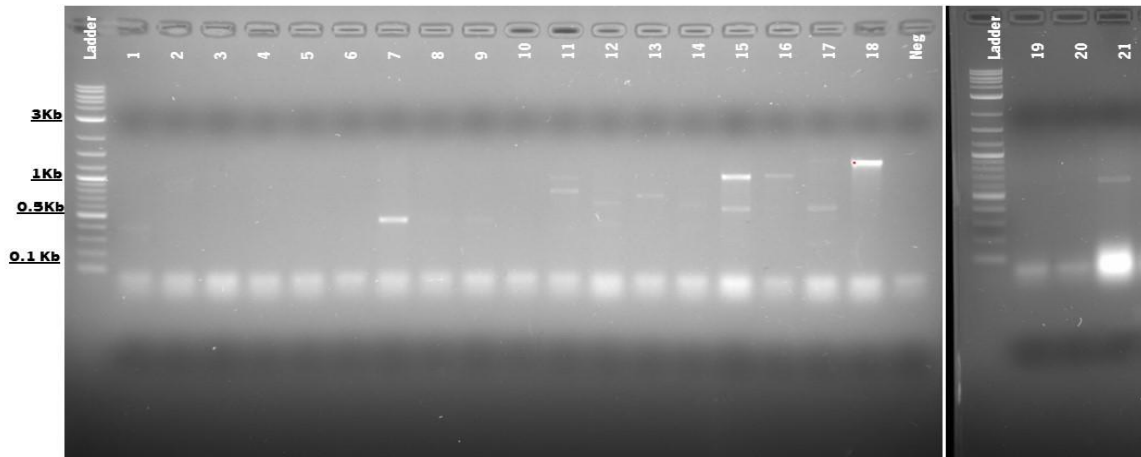


Figure 48 - Amplification results from ERIC/PCR for the strains from time point II. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†]. Neg is the negative control.

For the samples from the time point III, 4 weeks of accelerated storage, the same procedures were applied, and the obtained results are shown in figure 49. For this set of samples, several thermocyclers were used to discard for changes in temperatures on the PCR parameter.

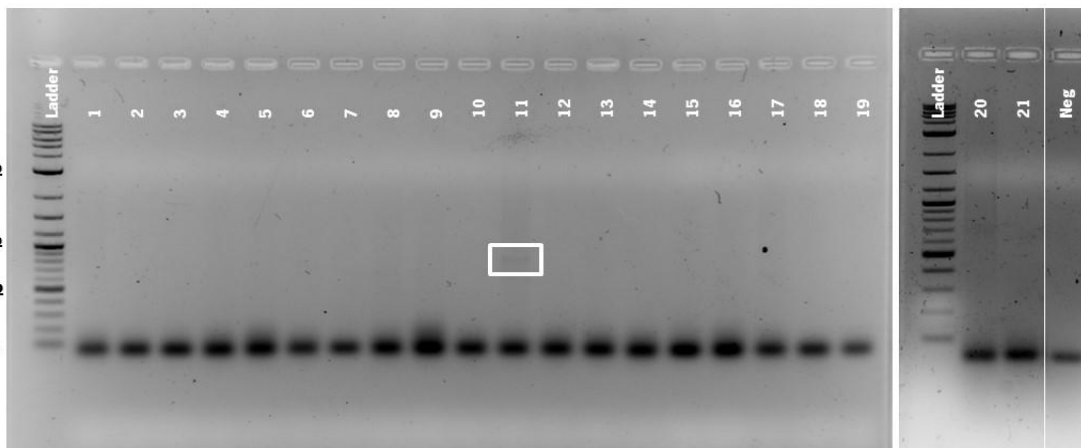


Figure 49 - Amplification results from ERIC/PCR for the strains from time point III. 1- *A. aculeatus* MUM 03.11[†]; 2- *A. brasiliensis* MUM 06.179; 3- *A. brasiliensis* MUM 06.180; 4- *A. brasiliensis* MUM 06.181; 5- *A. carbonarius* MUM 01.08; 6- *A. ellipticus* MUM 03.12[†]; 7- *A. ibericus* MUM 03.49; 8- *A. ibericus* MUM 04.68; 9- *A. ibericus* MUM 04.86; 10- *A. japonicus* MUM 03.02[†]; 11- *A. japonicus* MUM 98.03; 12- *A. lacticofeatus* MUM 06.150[†]; 13- *A. niger* MUM 03.01[†]; 14- *A. niger* MUM 05.11; 15- *A. niger* MUM 05.13; 16- *A. phoenicis* MUM 03.05; 17- *A. phoenicis* MUM 03.10; 18- *A. sclerotiumniger* MUM 06.151[†]; 19- *A. tubingensis* MUM 06.152[†]; 20- *A. uvarum* MUM 08.01; 21- *A. vadensis* MUM 06.153[†]. Neg is the negative control.

The results obtained on the first time point showed a pattern that could be used on a basic screening to group the different species of fungi. But the results did not allow reproducibility, turning the ERIC/PCR approach not useful for comparison of the different time points of each species.

There are several possibilities to explore in the future. One is the use of more specific and different primers. Another possibility is the development of a protocol using touchdown-PCR, where an initial annealing temperature above the projected melting temperature of the primers being used is applied and a progressively transition to a lower, more permissive annealing temperature over the course of successive cycles is made; with potential increase of specificity, sensitivity and yield (Korbie and Mattick, 2008). And several other methods can be explored within molecular biology techniques.

3.5.8. Data analysis

Data analysis for comparison of the preservation for different time points of all the selected strains was performed based on all characteristics on those different time points evaluated (I, II and III). These analysis were performed using SPSS software v.12.0 (SPSS Inc., Chicago, IL, USA). The data used for this analysis were macroscopic results (size of the colonies, their appearance – lanose and/or sectorised and their colour), microscopic results (vesicle shape, vesicle size, seriation, conidial shape, conidial diameter and conidial surface) and mycotoxin production (rapid screening method results, FUM and OTA).

Analysing each set of data for each strain it is possible to conclude that the differences found with some methods are not significant and the several time points of preservation appear in clusters for the same strains.

For time point I, the data set for all strains was organised and as presented in figure 50. This figure presents the phenotypical characteristics used as control in which the dendrogram shows the set point of strains interrelations according to their phenotypic characteristics.

Two major clusters were formed: A with 13 strains, and B with 8 of the selected strains (Figure 50). On cluster A, the populational distances were smaller than the ones on cluster B. The main characteristics responsible for the two main branching on the dendrogram are the phenotypical ones.

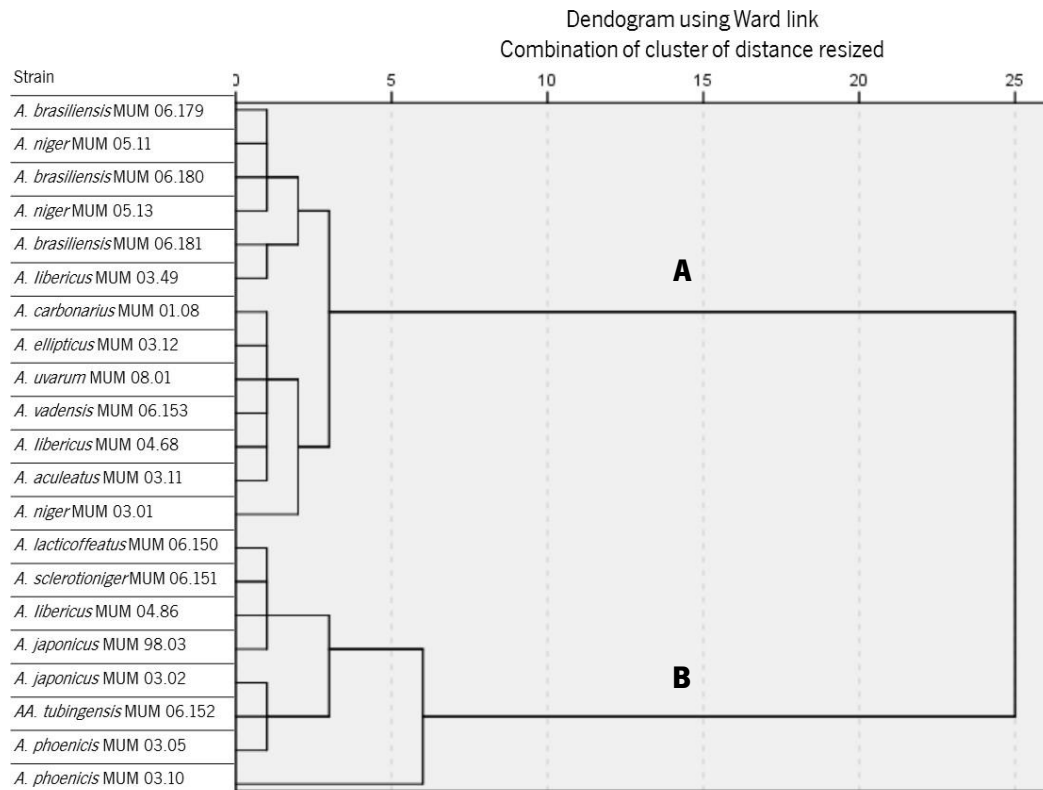


Figure 50 - Dendrogram of all data obtained for time point I (before preservation). Data was processed using squared Euclidian distance and Ward method from SPSS.

For time point II, the data set for all strains was organised on a dendrogram presented on in figure 51. The two main clusters, A and B, obtained for the first time point were maintained. But the strain of *A. ibericus* MUM 04.86 migrated from cluster B to cluster A.

The microscopic characteristics are of most importance to discriminate different species and are constant for each species. These do not present changes along the different time points analysed. But, there are characteristics that discriminate the different strains: macroscopic and mycotoxin production.

The strain *A. ibericus* MUM 04.86 presented colour and size of colonies alteration after aged for two weeks at 37 °C.

It is also notable that after the first time point of accelerated storage of the selected strains, the cluster A showed an increase on the populational distances.

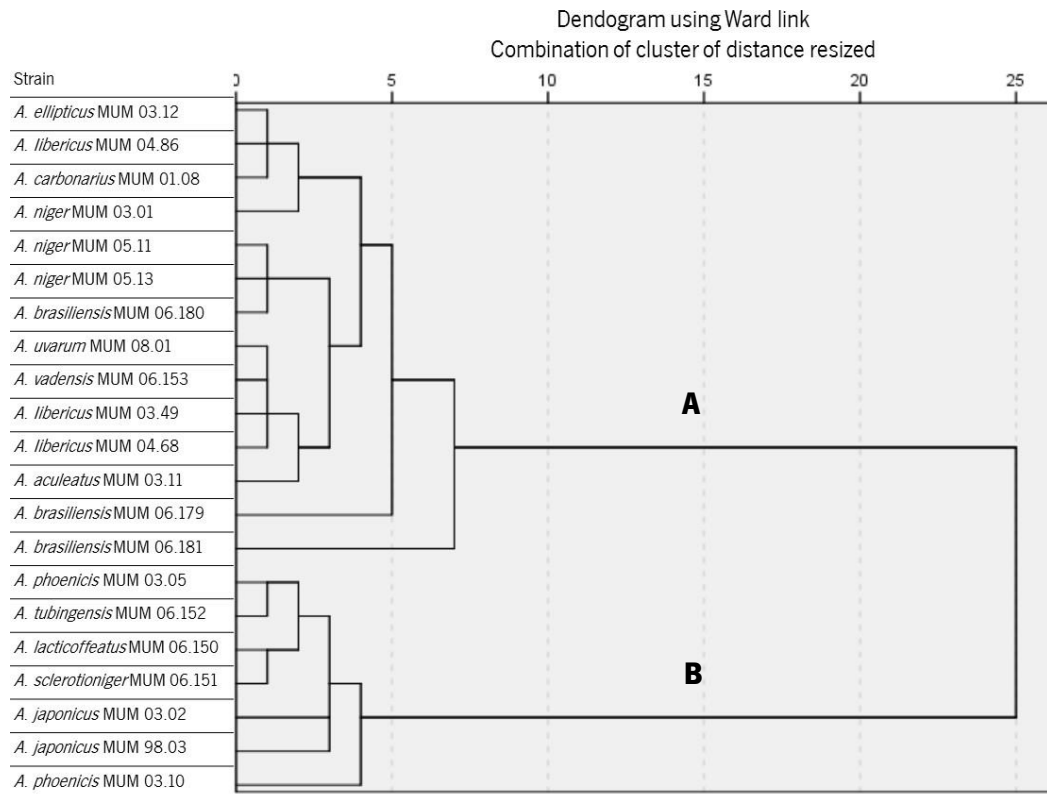


Figure 51 - Dendrogram of all data obtained for time point II (2 weeks of accelerated storage at 37 °C). Data was processed using squared Euclidian distance and Ward method from SPSS.

And, for time point III, the data set for all strains was organised and presented as a dendrogram shown in figure 52.

On this dendrogram the two main clusters, A and B, were maintained. But the strain *A. ibericus* MUM 04.86 that migrated from cluster B to A on the time point – two weeks of ageing (II), still appeared on cluster A.

On this time point (III), another strain migrated from cluster B to A: *A. phoenicis* MUM 03.05. It is also notable that the populational distances are directly related to the increased of time of ageing.

From all the selected strains analysed it is possible to conclude that *A. ibericus* MUM 04.86 and *A. phoenicis* MUM 03.05 are more difficult to maintain phenotypically stable after preservation. Being lyophilisation a more critical method for *A. ibericus* MUM 04.86 than for *A. phoenicis* MUM 03.05.

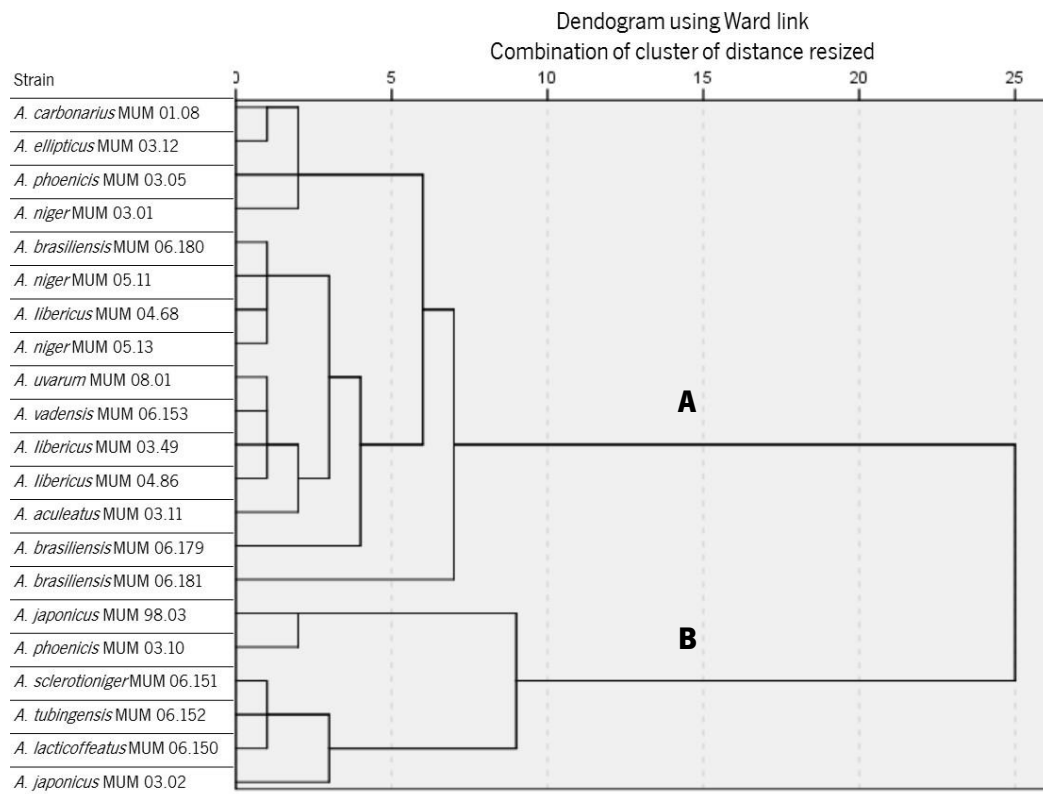


Figure 52 - Dendrogram of all data obtained for time point III (4 weeks of accelerated storage at 37 °C). Data was processed using squared Euclidian distance and Ward method from SPSS.

Overall, it was found that a main pattern of two clusters was maintained along the time after preservation. But some minor changes were noted, initially for one strain and then on time point III, for two strains.

When comparing all data, from the 3 time points analysed (I, II and III), for all the strains, a dendrogram was obtained (Figure 53). The 2 main cluster, A and B, seen for the different time points, were still present. But cluster B only presented 7 strains while cluster A had the remaining 14.

A. ibericus MUM 04.86 was the strain initially identified as being more sensible to lyophilisation and appears on cluster A, on this dendrogram, and has the time point II as the most distant from the other time points.

On this dendrogram the majority of strains group together or close together proving the suitability of the lyophilisation as a preservation method for the *Aspergillus* from section *Nigri*.

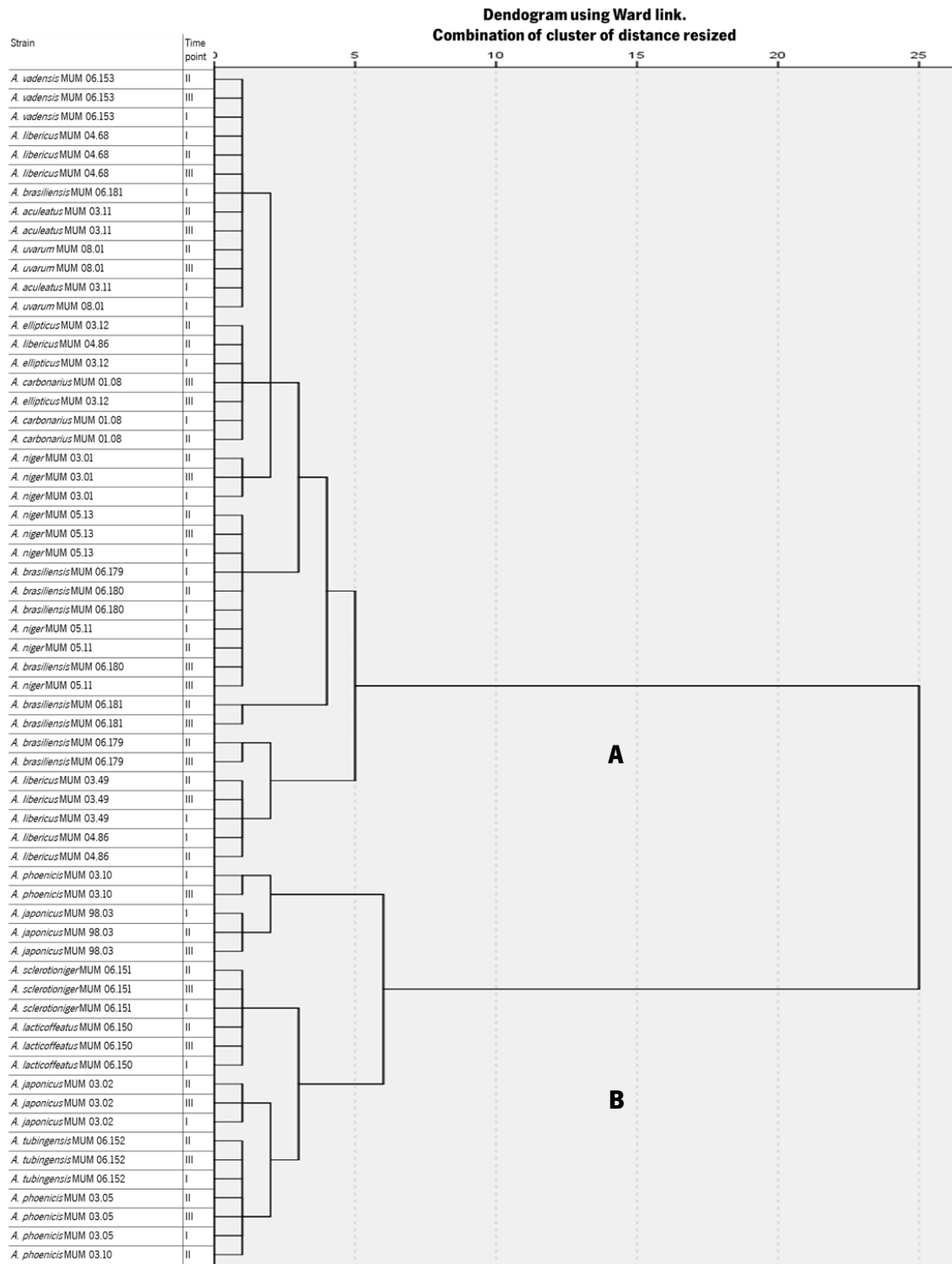


Figure 53 - Dendrogram of all data from time points I, II and III combined, with processing of data using squared Euclidian distance and Ward method from SPSS.

3.6. General insights and conclusions

Evaluating a preservation method is possible through the analysis of preserved samples with different times of preservation. An easy way to obtain a group of samples, preserved with the same method and with equal times of preservation for several time points is ageing the preserved samples by accelerated storage. In this work the 21 selected strains of *Aspergillus* section *Nigri* were preserved by lyophilisation and then aged by accelerated storage done by storage in the dark at 37 °C for specific time points.

The evaluation of all the samples within the several time points was done through a polyphasic approach of characterization techniques.

Some of the methods used showed that with time, the lyophilised samples suffer some alterations: phenotype of the colonies formed, products excreted (enzymes and mycotoxins) and even molecular masses. Some of the methods used were not the most proper to evaluate lyophilisation along time, this was the case of the use of ERIC primers to type all the strains tested.

But, even though some changes were detected for several methodologies, in all of them, the changes were minor and not relevant enough, allowing to conclude that lyophilisation is a good method of preservation for strains of *Aspergillus* section *Nigri*.

4. ALTERNATE METHODS FOR PRESERVATION OF FILAMENTOUS FUNGI STRAINS

4.1. General Introduction

4.1.1. Preservation methods

Standard methods of preservation commonly used in CCs and BRCs have been found to always present some disadvantage and not to be proper for all the samples to preserve (Annex I). Several researches have been developed in order to circumvent the negative aspects of each technique but more studies need to be done to find the most convenient methods for each samples.

4.1.2. Recalcitrant species and difficult to preserve strains

Efficient mycological work requires a reliable source of cultures, with well-defined and taxonomic associated information, to be used as starting material, and this is ensured by safe storage which implies good methodologies with assured Quality. The main requirement of a proper high-quality preservation method is to maintain the fungal strain in a viable and stable state without morphological or genetic change until it is required for further use (Kolkowski & Smith, 1995). Most of the standard preservation methods currently used are not completely reliable (Smith & Onions, 1983; Simões et al., 2013a). But, several strain-specific adaptations have been made to the existent methodologies, and this is a subject that has been receiving increased attention in mycology (Kolkowski & Smith, 1995). One of the reasons is that, even with the implementation of the best practices and more appropriate methods, there are specific strains that under particular conditions do not survive preservation, impairing all mycological work.

Species of filamentous fungi that are preservation-recalcitrant are a group of fungi hard to be preserved, with large difficulties of survival after preservation. Efforts have been made to overcome this problem, either through the development of new preservation techniques, as well as through specific alterations in the already existent methodologies. All the techniques currently

used on preservation have disadvantages. None of the available methods can be successfully applied to all strains of fungi (Ryan, 1999).

4.1.3. *Botrytis cinerea*

The *Botrytis* Pers. genus was first documented in 1729 by Micheli and validated by Persoon in 1801. This genus has held 380 species, but, in a revision of the genus it was reduced to 22 species (Hennebert, 1973). Still, there are species yet to be described and some of the already existent are wrongly characterised (Card, 2005).

One of the most well-known species, within the genus *Botrytis*, is the type species *Botrytis cinerea*, which is the conidial or anamorphic state of *Botryotinia fuckeliana*. The name “botrys”, has a Greek origin and means bunch/cluster of grapes, describing the arrangement of the conidia; and, “cinereus” means grey in Latin, corresponding to the species colour (Card, 2005). *B. cinerea* is a haploid, filamentous, coenocytic, heterothallic fungus and a common phytopathogenic fungus observed in many plants, mainly dicotyledonous and monocotyledonous non graminean (Jarvis, 1977; Delcan et al., 2002; Card, 2005).

Since the variability of the fungus is well documented, a storage method which preserves the characteristics of the isolates is mandatory. The most commonly used methods to preserve *B. cinerea* use cryopreservation, sterile soil and silica gel. Delcan and colleagues (2002) confirmed that when spores are stored dried, the viability rate is very low and sclerotia size and sporulation are reduced after several years of storage, independently of the method used. They found that storage in sand at 4 °C or in glycerol at -20 °C were the best methods for preserving *B. cinerea* isolates, but they presented a decrease on growth rate. In addition, viability and virulence were maintained for 4 years using these methods of storage (Delcan et al., 2001).

Although cryopreservation has been used to successfully store isolates of some fungal species, the survival of *B. cinerea* after freezing is very low. The addition of cryoprotectants (such as glycerol) to the conidia before freezing, results in an enhancement of survival. Cryoprotectants are usually added to spore suspensions of fungi to avoid cell injury during the freezing process (Smith & Onions, 1994). However, in some cases, the addition of cryoprotectants results in harmful effects which is the case described by Holden and Smith (1992) for the preservation of *Puccinia abrupta*, where the addition of the cryoprotectants like glycerol, dimethyl sulphoxide, trehalose or polyvinyl pyrrolidone, prior to freezing, reduced both viability and virulence. And

reduction of sclerotia and/or conidia production caused by storage has been also reported in other fungi (Holden & Smith, 1992).

4.1.4. Perlite

Some fungal strains are difficult to preserve and, for these, a common practice of preservation is serial transference. This method has several disadvantages, mostly related to the frequency of transference that implies the danger of selection, variation, contamination or even infection with mites (Smith & Onions 1983; Smith & Onions 1994; Smith et al. 2001; Nakasone et al. 2004; Simões et al., 2013a).

Perlite is an inert, inorganic, unique volcanic aluminosilicate mineral holding and retaining substantial amounts of water, which can be released as needed, and because of these characteristics, Homolka and colleagues have been studying its use as a solid carrier on preservation for several fungal strains (Miles & Wilcoxson, 1984; Homolka et al., 2001; Homolka et al., 2007a;). It has also been used as a substrate in some media composition to induce the production of conidia (Miles & Wilcoxson, 1984).

In a study made by Homolka and Lisá (2008), perlite was suggested as a good substitute of serial transference used with agar cultures in long-preservation of fungi and more appropriate than oil preservation that presented to be unsuitable for the majority of the cultures studied in their assay (Homolka & Lisá, 2008).

Cryopreservation of basidiomycete species had no success with routinely used procedures, but, for these fungi, perlite was found to be a particular solid carrier to be used in the growth media and a possible new alternate method to be used on cryopreservation (Homolka et al., 2001).

According to Homolka and Lisá (2005), the perlite cryopreservation procedure they developed, with perlite, presents several advantages. They consider it an easy form of cultivation by growing the fungi directly into the cryovials. Also, the transparency of the cryovials makes it possible to check the growth and even possible contamination. Mycelium grows continuously on perlite, not being damaged by excessive handling on agar plates. And, this procedure avoids contamination resulting from excessive handling. They claim that this method is evidently suitable for different fungal strains that require special treatment and describe it as being generally applicable to most fungal cultures (Homolka & Lisá, 2005).

All considerations taken into account lead to conclude that perlite can be a good alternative method of preservation to difficult strains such as *B. cinerea*.

4.1.5. Alginate encapsulation and its uses on preservation

Encapsulation was first employed and developed as a method of cryopreservation applied to a wild potato species: *Solanum phureja* (Bouafia et al., 1996). But, since then, it has been applied to many different types of plant germplasm derived from a diverse species range. It has been particularly important for the conservation of plant systems that are difficult to cryopreserve using traditional methods (Block, 2003; Nair & Reghunath, 2008; Gholami et al., 2013; Soliman, 2013) and also for the preservation of seeds (Engelmann, 2004), and to some algae (Zhang et al., 2008).

Alginate-encapsulation is a commonly used, simple and cost effective method with several applications and immobilisation of microorganisms for biotechnology industry. It has also been used in food processing, medical and pharmaceutical industries, both as a carrier and in the preparation of gels for compounds delivery (Soares et al., 2004; Zahmatkesh et al., 2010; Basha et al., 2011). Alginate has proven to be protective of tissues against physical and environmental damage minimising dehydration (FMC Corporation, 2011). It has also been used in fungi for purposes of a source of viable inoculum (Abdullah et al., 1995; Daigle & Cotty, 1997; Lalaynia et al., 2012) to be used mainly by the biotechnology industry (Ryan, 2001). Its application for the preservation of fungi was intended to present as an alternative for the commonly used preservation methods (Ryan, 2001). In this work this intention was focused especially on recalcitrant fungal strains.

The encapsulation–dehydration with alginate beads procedure is based on the technology developed for the production of artificial seeds. The biological material is encapsulated in alginate beads, pre-grown in liquid medium enriched with high molarity sucrose, partially desiccated to an optimum low moisture content (water content around 20%), which is achieved either in an open sterile air flow or over silica gel before cryopreservation, then frozen rapidly. Survival is high and growth recovery of cryopreserved samples is generally rapid and direct (Block, 2003; Engelmann, 2004).

Encapsulating the biological material is thought to promote a vitrified state in the tissue regardless of the cooling and re-warming rates, thus reducing the damage caused from ice crystal formation. And it is also thought to reduce mechanical stress because the bead protects

the material from damage during handling. The benefits of this method include: avoiding the use of high concentrations of cryoprotectors that can be toxic, and enhancing post-regeneration survival or re-growth after preservation (Kaczmarczyk et al., 2012).

Alginic acid is an anionic polysaccharide, a heteropolymer containing mannuronic acid and guluronic acid groups (Soares et al., 2004; Kim et al., 2011). Its chemical formula is shown in figure 54.

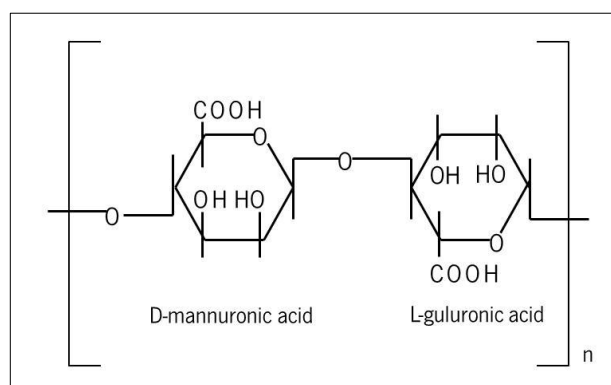


Figure 54 - Chemical structure of alginic acid.

As structural component in marine brown algae, comprising up to 40% of dry weight, and as capsular polysaccharides in soil bacteria, alginates are quite abundant in nature. Nowadays, alginates are widely used as food additives to modify food texture and in tissue engineering (Kim et al., 2011; Sun & Tan, 2013). Alginate is of particular interest for a broad range of applications: as a biomaterial and especially as the supporting matrix or delivery system for tissue repair and regeneration and in a variety of biomedical applications including tissue engineering, drug delivery and in some formulations preventing gastric reflux (Sun & Tan, 2013), and even as microbial carrier in bioprocesses (Brachkova et al., 2010). The numerous applications are due to its properties in terms of biocompatibility, biodegradability, non-antigenicity and chelating ability (Basha et al., 2011; Sun & Tan, 2013).

Both residues can be combined to form homopolymer blocks, 1,4-linked β -D-mannuronic acid (M-block) and α -L-guluronic acid (G-block), or copolymer blocks (MG-block) with several possible polymers (Soares et al., 2004; Kim et al., 2011). The increase on the content of guluronic acid monomer implies an increase on the gel strength (Kim et al., 2011). Alginic acid forms water-soluble salts with monovalent cations but is precipitated upon acidification. Alginates of many bivalent cations, particularly of Ca^{2+} , Sr^{2+} and Ba^{2+} , are insoluble in water, forming a gel,

and can be prepared when sodium ions of sodium alginate (NaAlg) are replaced by di- and trivalent cations (Soares et al., 2004; Kim et al., 2011).

NaAlg, the sodium salt of alginic acid, is a water soluble anionic polymer, a carbohydrate polymer that is a biocompatible material. NaAlg is a block-wise copolymer of G-block and M-block. During the gelation process, G-G and M-G homo- or heteroblocks are electrostatically cross-linked with divalent ions and form a porous matrix. The general formula of this polymer is $(C_6H_7O_6Na)_n$ (Soares et al., 2004).

Encapsulation of healthy grown pellets in a porous biomaterial, like NaAlg, represents an alternative to overcome some of the drawbacks that common preservation methods have on some fungal strains. In the present study, several experiments were conducted to test and adapt the alginate encapsulation for preservation to several filamentous fungi species, *Botrytis* and *Aspergillus* strains, but without drying and freezing to surpass the intracellular ice formation. The main goal is to achieve a medium term preservation method with more advantages than the existing ones.

4.2. Methods

4.2.1. Selected strains

The following fungi strains were selected: *Botrytis cinerea* MUM 10.163, *Botrytis cinerea* MUM 10.165, *Botrytis cinerea* MUM 10.167, *Aspergillus ibericus* MUM 04.68 and *Aspergillus brasiliensis* MUM 06.181. The *Aspergillus* strains were used as positive control strains for this assay. All the strains were initially grown in Potato Dextrose Agar (PDA) (Oxoid, UK) medium.

4.2.1. Perlite

Since the *B. cinerea* were isolated from grapes, two different media were prepared, a media with extract of grape juice (I) and a synthetic nutrient medium (SNM) (II), similar to grape composition (Valero et al., 2005).

- I. An extract of grape juice was prepared according to Santos et al. (2002). Two Kg of dark grapes were left overnight in a 5% bleach solution and were then rinsed with sterile distilled water and macerated; the juice was collected and centrifuged (10 minutes, at 10000 rpm, at 14 °C) for removal of large particles. The supernatant (\pm 500 mL) was lyophilised and kept at -20 °C until further use. The lyophilisate was resuspended in 200 mL of distilled water. This volume was then brought to boiling

for 1 hour to reduce the volume and it was then autoclaved for 15 minutes at 121 °C. It was then splitted into 20 mL aliquots for future use. The prepared extract was then diluted in distilled and sterile water (1:1) and 1 mL was distributed into cryovials half-filled with sterile perlite (sterilised by autoclave at 121 °C for 15 minutes and then dried for 2 days at 50 °C).

- II. The SNM was prepared according to Valero et al (2005) (196 g/L D(+) Glucose, 204 g/L D(-) Fructose and 288.5 g/L glycerol in distilled water. This media was sterilised by autoclave for 15 minutes at 121 °C).

A plug of each fungal strains, initially grown in PDA (Oxoid, UK), was cut and poured directly into the cryovial, with perlite moistened with different growth media (I and II), and left to incubate for 2 weeks at 25 °C, according to the procedure described by Homolka et al. (2001, 2007a). They were then frozen at – 80 °C.

After a 6-month storage period at -80 °C, the samples were retrieved, thawed and checked for viability, purity and changes in growth, morphology and biochemical characteristics. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF), that emerged in the late 1980s as a sound technique to investigate the mass spectrometry of molecular high-mass of organic compounds through a soft ionisation of molecules resulting in minimum fragmentation, was the technique chosen to evaluate strains alterations because of the possibility of analysing the intact fungal cell generating peptides and proteins profiles.

4.2.2. Alginate

From the strains initially grown in PDA (Oxoid, UK), one plug was cut from each grown strain and each plug was transferred into 10 mL of malt-extract-glucose-yeast extract-peptone medium (MGYP – Annex 7.6.10) and left to incubate for 7 days at 30 °C with 150 rpm.

The pellets of grown fungi, dense and spherical pellets of viscous mycelia (2-10 mm of diameter), were encapsulated in alginate by the way described as follow.

The pellets were individually retrieved from the liquid medium, put into a sterile plate with a sodium solution of NaAlg (30 g/L in distilled water, sterilised by autoclave at 121 °C, for 15 minutes) at ± 60 °C, and then passed into a new plate with a solution of calcium chloride (20 g/L of CaCl₂ in distilled water, sterilised by autoclave at 121 °C, for 15 minutes) until solidification of the alginate.

The encapsulated pellets were then retrieved into the final flask for preservation. The pellets were stocked into two different preservation media: (I) - deionised and sterile water and (II) - 10 % glycerol sterile solution (in distilled water). The flasks were kept at 4 °C.

The viability of these strains was studied.

The assessment was made by comparison with the method of Castellani preservation in water (III), using morphologic and MALDI-TOF MS analyses for the evaluation of the 3 preservation methods. For the morphologic analysis one bead or plug from each strain was incubated in PDA (Oxoid, UK) at 25 °C for 3 days. MALDI-TOF MS analyses were carried out as described by Rodrigues and colleagues (2011).

4.3. Results

4.3.1. Perlite results

From all the strains used in this assay not one survived to the preservation regime. There was no viability from the thawed samples.

4.3.2. Alginate results

The encapsulated samples were maintained at 4 °C, in closed flasks, until their assessment (Figure 55).

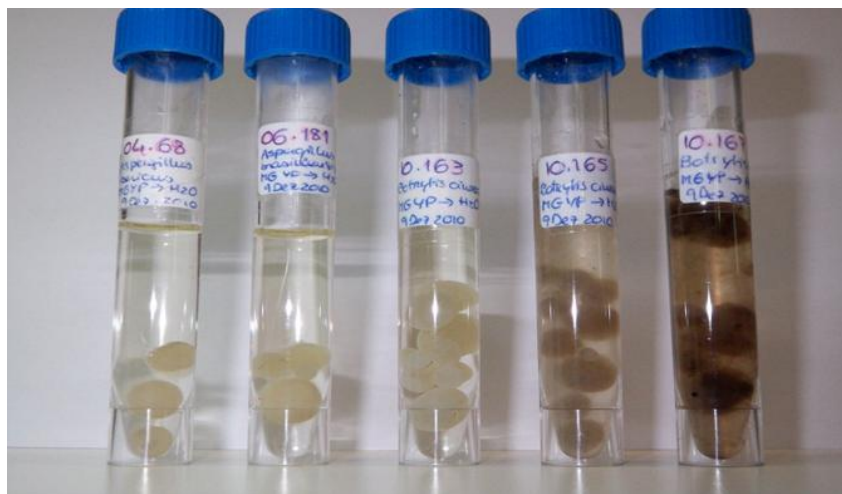


Figure 55 - Different encapsulated fungi, maintained in sterile distilled water, at 4 °C.

The viability of the several strains was compared by the observation of growth in the samples (Figures 56 and 57).

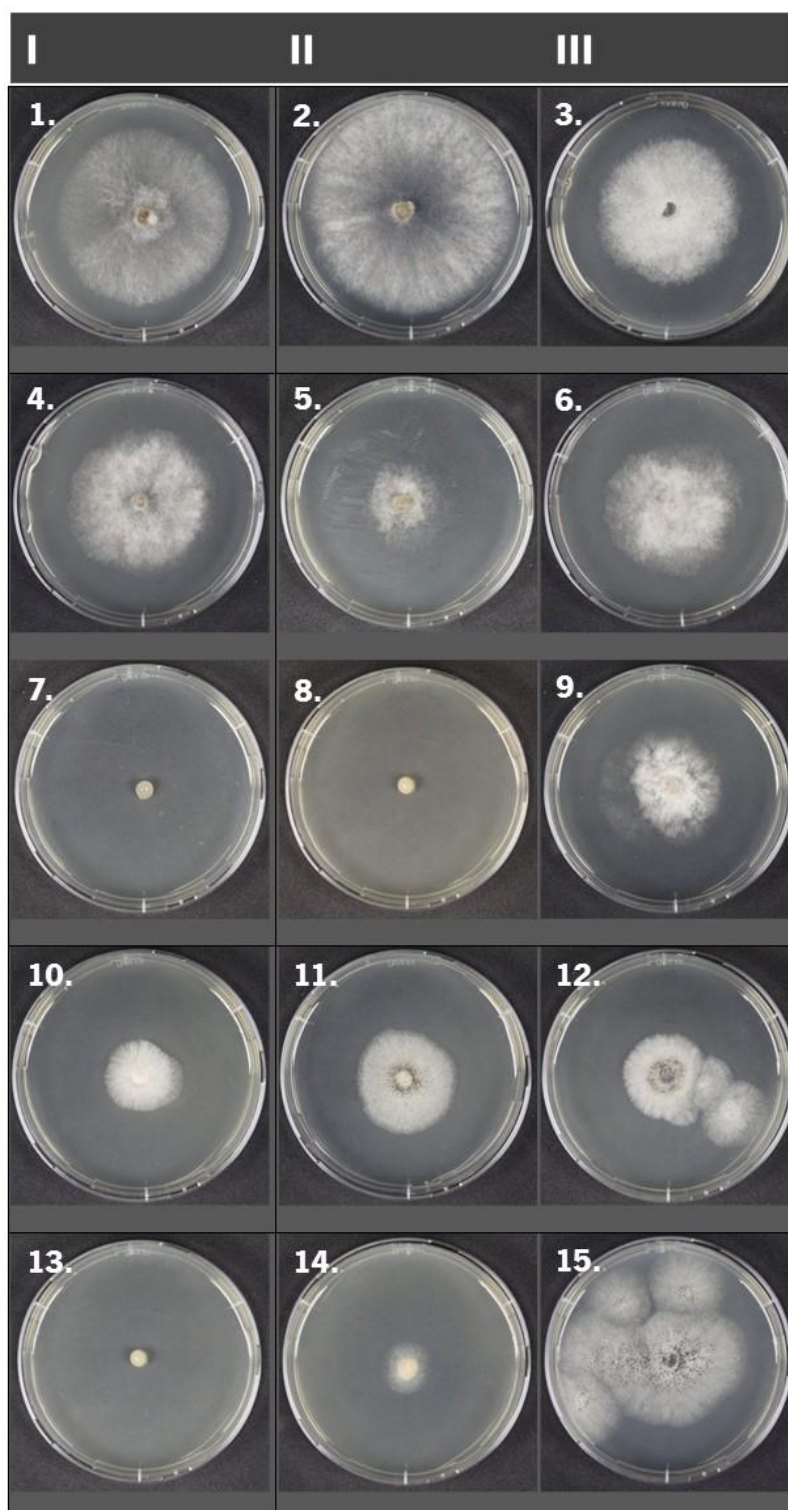


Figure 56 - Three days old colonies (verse, in MEA) from the samples preserved in alginate in water (I), alginate in 10% glycerol (II) and with the Castellani method in water (III). (1. - *B. cinerea* MUM 10.167, (I); 2. - *B. cinerea* MUM 10.167, (II); 3. - *B. cinerea* MUM 10.167 (III); 4. - *B. cinerea* MUM 10.165, (I); 5. - *B. cinerea* MUM 10.165, (II); 6. - *B. cinerea* MUM 10.165, (III); 7. - *B. cinerea* MUM 10.163, (I); 8. - *B. cinerea* MUM 10.163, (II); 9. - *B. cinerea* MUM 10.163, (III); 10. - *A. brasiliensis* MUM 06.181, (I); 11. - *A. brasiliensis* MUM 06.181, (II); 12. - *A. brasiliensis* MUM 06.181, (III); 13. - *A. ibericus* MUM 04.68, (I); 14. - *A. ibericus* MUM 04.68, (II) and 15. - *A. ibericus* MUM 04.68, (III)).

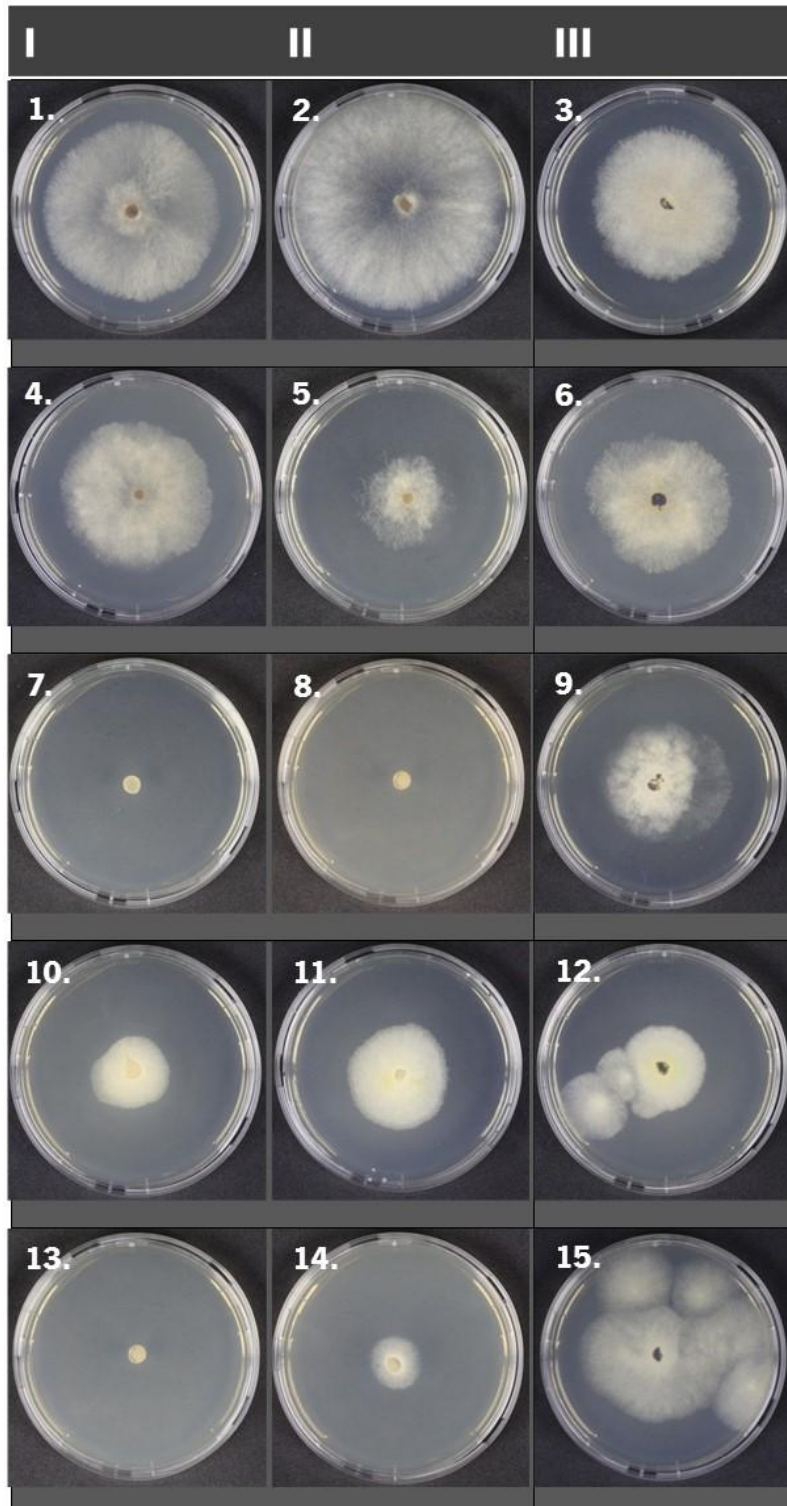


Figure 57 - Three days old colonies (reverse, in MEA) from the samples preserved in alginate in water (I), alginate in 10% glycerol (II) and with the Castellani method in water (III). (1. - *B. cinerea* MUM 10.167, (I); 2. - *B. cinerea* MUM 10.167, (II); 3. - *B. cinerea* MUM 10.167 (III); 4. - *B. cinerea* MUM 10.165, (I); 5. - *B. cinerea* MUM 10.165, (II); 6. - *B. cinerea* MUM 10.165, (III); 7. - *B. cinerea* MUM 10.163, (I); 8. - *B. cinerea* MUM 10.163, (II); 9. - *B. cinerea* MUM 10.163, (III); 10. - *A. brasiliensis* MUM 06.181, (I); 11. - *A. brasiliensis* MUM 06.181, (II); 12. - *A. brasiliensis* MUM 06.181, (III); 13. - *A. ibericus* MUM 04.68, (I); 14. - *A. ibericus* MUM 04.68, (II) and 15. - *A. ibericus* MUM 04.68, (III)).

B. cinerea MUM 10.163 preserved in alginate in water and in 10% glycerol, and *A. ibericus* MUM 04.68 preserved in alginate in water, were not viable after preservation.

The selected strains were then analysed by MALDI-TOF MS. A dendrogram was obtained (Figure 58). And the obtained clusters group the different preservation methods evaluated.

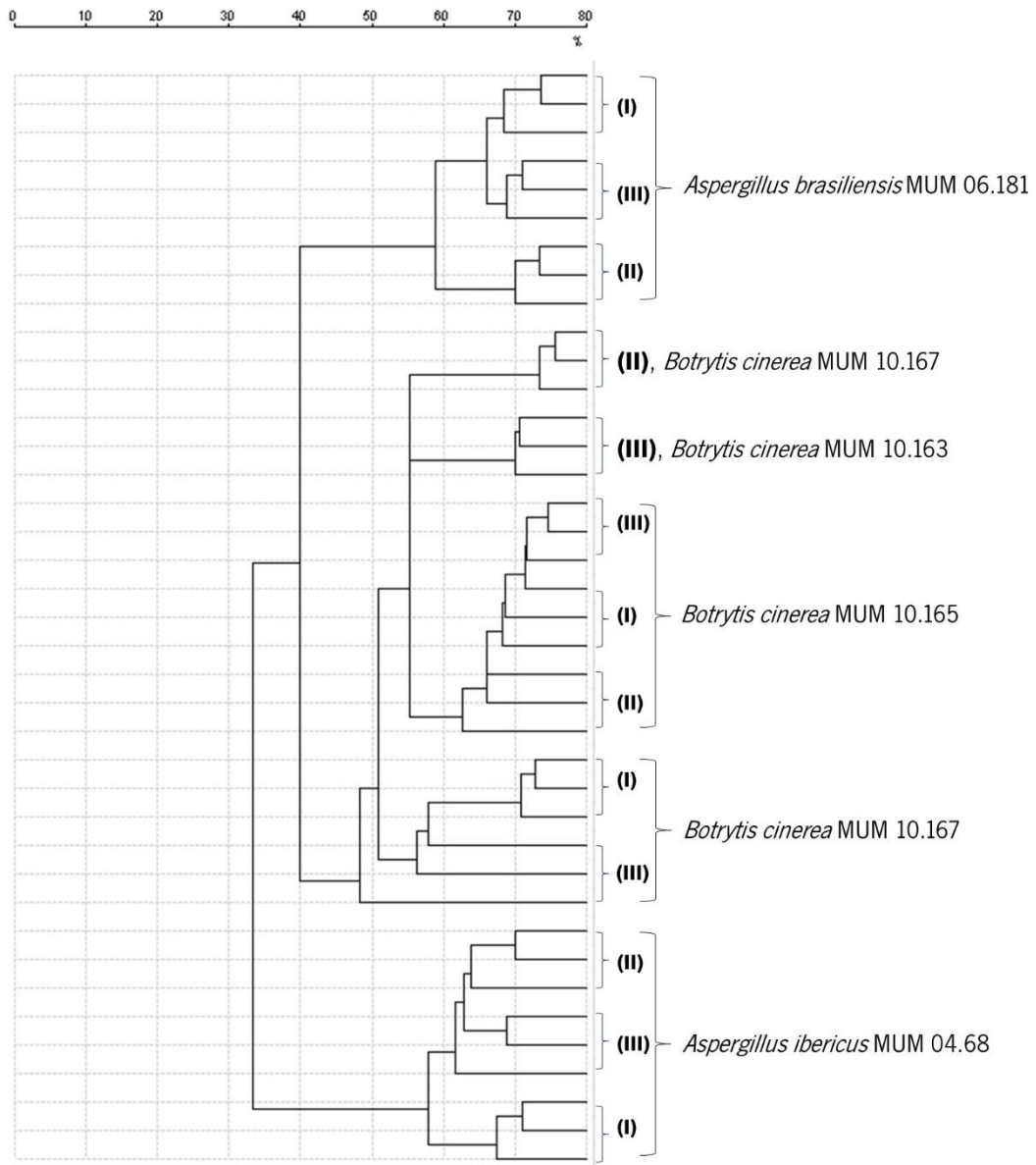


Figure 58 - Dendrogram resulting from single linkage cluster analysis of MALDI-TOF mass spectra of all the samples preserved in alginate in water (I), alginate in 10% glycerol (II) and in the Castellani method in water (III).

Spectral comparison was made for all the preservation methods for each of the selected strains. All the comparisons presented similar results with minor variations between the spectrum for each strain. Figure 59 is a representative comparison of analysis made.

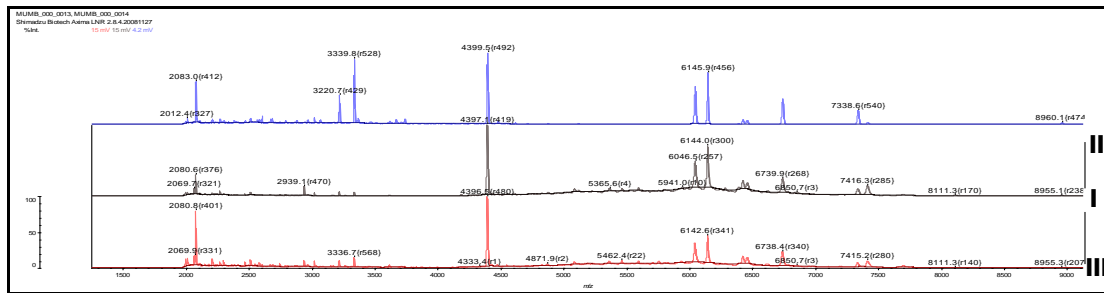


Figure 59 - Representative spectral mass profile of *Botrytis cinerea* MUM 10.165 and comparison between the three types of preservation: III) plug in water, I) alginate in water, II) alginate in 10% glycerol.

4.4. Discussion

4.4.1. Perlite discussion

The lack of capability to survive the preservation methodologies used in this assay is related to the fact that most of the strains used are not able to survive freezing. If perlite was used as a solid carrier on a different methodology successful results could have been obtained after preservation, since Homolka et al. (2001, 2006, 2007a, 2007b) and Homolka and Lisá (2008) have already proven its capability as a good carrier for fungi preservation. Impairment for the success of this technique was the absence of cryoprotectors to avoid the common damage caused by the freezing process. A potentially wide range of adaptations could be made by incorporating additional components to the media, such as glycerol or any other compatible solutes and by preparing the samples by initially growing them in a specific medium using natural substrates as grape juice to induce sporulation and healthy growth.

4.4.2. Alginate discussion

Since it is already known that changes in substrate or environment allow for different patterns of intermingling of the nuclei in *B. cinerea* and hence can alter the morphological expression and therefore changes in conidia or sclerotia production; and, like other fungi, *B. cinerea* easily adapts to dryness and abnormal conditions of osmosis and consequently it may temporarily change its behaviour (Delcan et al., 2002); morphological analysis is not a characteristic to analyse in this study. Other parameters were evaluated: viability, growth and health colony forming. *Aspergillus* strains selected for this evaluation were used for being easy to preserve and to serve as reference for comparison.

The encapsulated samples of the strains preserved in distilled water (I) (Figure 55), presented lower viability than those preserved in 10% glycerol (II). However, when comparing the growth from the samples preserved in water (III) with the ones encapsulated and maintained in 10% glycerol (II), it was evident that the last ones presented a healthier and faster growth (Figures 56 and 57).

From the MALDI-TOF MS analysis, it was observed that the strains were clustered according to species identification (Figure 58), and for *Botrytis cinerea* all presented small differences (< 5%) in the percentages of spectral similarity, except for *B. cinerea* MUM 10.167 (>5%) when preserved in water (III). For each strain, the spectral mass profile was analysed, as shown in the representative spectra (Figure 59) where comparison between the different preservation methods was made. And, all spectral profiles presented to be very similar demonstrating that strains kept their proteomic stability after preservation. From general evaluation it was possible to conclude that the success of the preservation method is strain dependent.

4.5. Conclusions

In this study, the encapsulated samples of the strains preserved in 10% glycerol had a healthier and faster growth presenting as an optional and better method than the Castellani method (Castellani, 1939). MALDI-TOF MS analysis showed that strains cluster according to species identification and the spectral mass profile of each strain demonstrated that strains kept their proteomic stability after preservation. Therefore, alginate encapsulation is a successful method of preservation, especially *Botrytis cinerea* that are difficult to preserve with the most common routine preservation techniques. But, its success will depend on the strain being preserved.

5. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5.1. General conclusions

Micoteca da Universidade do Minho is a CC that engaged on several projects with other CCs and BRCs. From these collaborations and by being partner in the project of EMbaRC, MUM and all the internal collaborators understood the need for differentiation and the need of assured quality. These were part of the motivations for the work developed in this thesis.

In general, this thesis refers to the common operational framework facilitating access and to the key benefits that contribute to innovative solutions for the global challenges and impact on the bioeconomy in the context of BRCs. Common ways of managing, working, doing research and improving between all CCs, will make governments acknowledge the importance of preserving biological resources and associated data as well as understanding the important role of all CCs and BRCs in the several areas where biological resources are needed.

Having all CCs and BRCs with common strategies and methods to have a global assurance of Quality is the main subject to assure the deliverance of their tasks, services and products. This can be achieved by several critical steps that are approached in this thesis. Among those critical steps are the following: harmonisation of quality control and/or quality assurance through the implementation of a QMS with defined and secure protocols for the purpose of certification and/or accreditation; funding through reliable business plans, with support of stakeholders and governments to avoid constraints on the development; human resources with the creation new jobs, and the maintenance of training for taxonomists; training, education and continual professional development (e.g. through new and specific master courses for the area of curating in CCs and BRCs); networking and bioinformatics, with dedicated IT to guarantee the management of the large amount of data within CCs and BRCs and to guarantee the healthy functioning of each CC site and webpage and the many informatics platforms, to be proper data providers; facilities and premises must be adequate to guarantee biosecurity rules (e.g. in some CCs and BRCs there is a dispute of the little existent space with other institutions);

and, relations between BRCs and CCs with the society and their stakeholders should always be encouraged because dissemination of science is and can be a major contribution, that is why closing the gap between science and society is a subject that Micoteca da Universidade do Minho endeavours.

There are several main issues within the context of CCs and BRCs. Quality is a mandatory parameter just as proper and successful preservation methodologies. In this work, it is shown that the implementation of a QMS based on ISO 9001:2008 is a possible and advantageous approach to guarantee Quality in services and products. This approach requires time, team involvement and restructuring of some procedures, but it turns the processes clearer, accurate and allow for better results within the achievement of the product and customer satisfaction keeping improvement as a continuous action. The implementation of the QMS of MUM allowed it to be the first Portuguese CC to obtain certification and to be part of a small group of CCs with this differentiation. This differential allows the customers of MUM to be more satisfied, which is noticeable on the analysed indicators of the QMS. There were more requests and supplies made after the QMS implementation and they have been increasing.

There were several lessons learned. MUM was dependent on specific people, the director and the curator. But with the QMS, MUM has an existence that does not depend on people and can exist besides the existence of specific people. The continuity of MUM is assured. It is not dependent of the will of an individual person. The QMS was designed to guarantee that processes are possible in an academic environment, with shared common spaces and infrastructures, and with all the issues related to this. Due to all the developments made, MUM has been an example and has served as a motivation to other CCs allowing for the transference of “know-how”.

The development and analysis of preservation methods, performed with all the established quality parameters, guarantees their development and evaluation regarding phenotypic and genotypic stability of the samples studied for the matter. In this research the samples used were strains of filamentous fungi held in the collection of MUM. The polyphasic approach, with all the methodologies involved, to evaluate lyophilisation on filamentous fungi strains along time, showed that it is an appropriate method to preserve *Aspergillus* strains. When analysed separately the results of each method do not allow this clear conclusion, but when analysed together and through statistical analysis, it is possible to observe that the strains are well preserved on all the time points analysed.

The use of perlite and alginate for preservation are possibilities that have been discussed before and with some good results. This was the case of the studies made by Homolka and colleagues. Perlite did not present as a possible form of preservation, but with other experimental design it can still be a possibility. For it has several characteristics that can be advantageous on preservation. Alginate, a largely used polysaccharide in many different areas, can be a good alternative to the Castellani method of preservation in water. It presents as a stable, fast and easy to do technique.

Furthermore, the results obtained from the studies on preservation methods allowed complementing the existent data of the used strains within the collection of MUM.

5.2. Future perspectives

Some researchers are not used of going to CCs. They commonly exchange biological samples from laboratory to laboratory. Most do not understand the utility of using well characterised strains or proper material and information. Most publishers do not demand the use of research with referenced strains and even the funding agencies do not require this as a must. But, users are becoming more aware of CCs and BRCs importance. With the current situation, information and data being created might not become useful in the future for the lack or inexistence of the strains used on those researches. This is why CCs and BRCs need to gain ground on science and society.

As far as MUM, much can be done to keep on the task of continuous improvement. A following and sensible step within the quality context will be accreditation, according to the standard ISO 17025. This will make all the processes: reception, preservation and supply of materials more precise, competent and proper.

In the analysis of lyophilisation as a preservation technique, it will be important to do additional assays. Especially after storage and before awakening the strains. The direct analysis of the lyophilisate through techniques such as flow cytometry, Fourier transform infrared spectroscopy or even confocal microscopy can add important information about the capability of the lyophilisation for the preservation of filamentous fungi.

The creation of new alternatives to the commonly used preservation methods is also a continuous improvement that can be achieved by altering and testing different parameters on the existent methodologies, or by developing totally new hypothesis of guaranteeing preservation, which can be difficult for some strains.

If all conditions are gathered, much can be done as a continual development of the work and assays developed in this thesis.

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7. ANNEXES

7.1. Annex I – Main preservation techniques short description

	Methods	References
Serial transference or subculturing	<p><u>Short description:</u> Growth in appropriate media, temperature, light, aeration, pH and water activity. Transfer of an inoculum from the vigorous, healthy and actively growing fungus culture to test tubes or Petri dishes containing an agar medium of choice, in determined time periods, and keeping the samples at room temperature or at 4 °C in-between the transferences that usually occur every two months. After establishing and keeping a culture, it must be checked periodically for contamination and desiccation.</p> <p><u>Advantages:</u> Simple procedure, inexpensive and widely used. Periodic transfer is a good option for small collections with cultures in constant use for short periods, inferior to one year. It does not require specialised equipment and allows an easy retrieval.</p> <p><u>Disadvantages:</u> Short-term preservation technique. Time consuming and labour intensive. Cultures require constant specialist supervision to check for contamination by air-borne spores, mites or other microorganisms and to ensure that the fungus is not replaced by a contaminant or subcultured from an atypical sector. Cultures dry more easily and in addition, there is a danger of variation, the morphology, physiology and even pathogenicity of fungi may change over time; in particular, the ability to sporulate or to infect a host may be lost after repeated subcultures.</p>	Smith & Onions 1983;Smith & Onions 1994;Smith et al. 2001; Nakasone et al. 2004
Preservation with mineral oil	<p><u>Short description:</u> Fungal cultures are grown on agar slants in tubes and afterwards covered with sterilised mineral oil or liquid paraffin. The entire agar surface and fungal culture must be completely submerged in the oil and the tubes are kept in an upright position at room temperature, but if the oil or paraffin is deeper than 10 mm, the fungus may not receive sufficient oxygen and it may die. Additionally, the oil level in the tubes or vials must be checked periodically, and more oil should be added, if necessary. To retrieve a culture from mineral oil, a small amount of the fungal colony must be removed and placed on an appropriate media after as much oil as possible has been drained.</p> <p><u>Advantages:</u> Simple procedure. Low-cost and low-maintenance method. It is a long-term method of fungal preservation where cultures can be kept for several years at room temperature. Appropriate for mycelial or nonsporulating cultures that are not adequate to be preserved by freezing or freeze drying. Oil reduces mite infestations, prevents dehydration and slows down the metabolic activity and growth through reduced oxygen tension. Although, many basidiomycetes can be maintained this way, the growth rates of the cultures slow as storage time increases.</p> <p><u>Disadvantages:</u> Subculturing the colony several times might be necessary in order to get a vigorous oil-free culture. There is a continuous growth of the fungi, allowing the occurrence of the selection for mutants with the capacity to grow under adverse conditions, some cultures after retrieval show sectoring or mutation presenting themselves different from the original. And growth rates slow as storage time increase. Needs enough storage space.</p>	Smith & Onions 1994;Sharma & Smith 1999;Nakasone et al 2004

	Methods	References
Preservation in water	<p><u>Short description:</u> First described by Castellani. Cut disks from growing colony edges are transferred to sterile cotton-plugged or screw-cap test tubes filled with several millilitres of water. Or small and sterile screw-cap cryovials are filled with several discs and topped with sterile distilled water. The tubes are stored at room temperature or at 4 °C. For retrieval, disks are removed aseptically and transferred to fresh agar medium, placing them with the mycelium down to retrieve the cultures. An alternative method for sporulating fungi involves inoculating agar slants of preferred media with fungal cultures and then incubating them at 25 °C for several weeks to induce sporulation. Then, sterile distilled water is added aseptically to the culture, and the surface of the culture is gently scraped with a pipette to produce a spore and mycelia suspension. This suspension is removed with the same pipette and placed in a sterile, vial. The cap is tightened, and the vials are stored at room temperature. To retrieve a culture, a small volume of the suspension is removed from the vial and placed on fresh medium.</p> <p><u>Advantages:</u> Inexpensive and low-maintenance method. It has been described that water suppresses morphological changes in most fungi. Avoids mite contamination.</p> <p><u>Disadvantages:</u> Viability decreases with the increasing of storage time. Needs enough storage space.</p>	Castellani 1967; Capriles et al. 1989; Smith & Onions 1994; Nakasone et al. 2004
Silica gel method	<p><u>Short description:</u> Screw-cap tubes are partially filled with silica without indicator dye, which has been sterilised by dry heat and stored in tightly sealed containers. Spores are suspended in a cooled solution of skimmed milk (5%). The silica gel is also chilled to about 4 °C and placed in an ice-water bath. The spore suspension is added to the silica gel to wet the gel. Tubes are stored with the caps loose at 25 or 30 °C for several days. Viability is checked by shaking and dropping a few crystals onto a suitable medium. If the cultures are viable, caps are tightened, and the tubes are stored in a tightly sealed container at room temperature. On a slight modified protocol, silica gel method can also be used for nonsporulating strains of fungi where aerial hyphae and mycelia are scraped from the agar surface on a grown culture with a sterile blade and transferred to test tube containing a small portion of water. The mixture is grounded to obtain a creamy homogenate which is then gently mixed with sterile skim milk. The obtained suspension is pipetted over the particles of silica gel and mixed in order to distribute the inoculum over the silica, it is then placed in an ice-water bath for several minutes and then left to dry. When the particles appear dry, the tubes are sealed and stored.</p> <p><u>Advantages:</u> Storage at low temperatures can increase the survival period twofold to threefold over storage at room temperature, therefore it can be used as a medium term storage method, especially for sporulating fungi if facilities for freeze-drying or for storage in liquid nitrogen are not available. It prevents all fungal growth and metabolism. It is an inexpensive, rapid and simple technique to use. Revival of cultures is easy and the same storage container can be used for successive sampling, though it is recommended that a stock flask or bottle is kept in case contamination occurs during retrieval. It produces very stable cultures. Penetration by mites is prevented as mites cannot survive in dry conditions. It can also be applied to other microorganisms other than fungi.</p> <p><u>Disadvantages:</u> Some fungi such as <i>Pythium</i>, <i>Phytophthora</i> and some <i>Oomycota</i> species, do not survive this process. It is limited to some fungi with delicate or complex spores. Needs enough storage space.</p>	Perkins 1962; Grivell & Jackson 1969; Smith & Onions 1994; Sharma & Smith 1999; Nakasone et al. 2004
Freezing	<p><u>Short description:</u> Cultures grown on agar slants in bottles or test tubes with screw caps can be placed directly in the freezer. But in a different process, one can grow vigorous cultures in tubes, flood them with a solution containing 10% glycerol in water, scrape the cultures to produce a suspension with spores and mycelium, and then apply this suspension into cryovials with sterile glass beads inside, afterwards frozen at -80 °C, with a cooling rate of -1 °C per minute. Retrieval implies the recovery of one glass bead to be put in an appropriate media.</p> <p><u>Advantages:</u> The retrieval procedure is very simple. It is a long-term process where most fungal cultures frozen at -20 °C to -80 °C remain viable. Most fungi cultures are successfully preserved for up to 5 years. In general, vigorously growing and sporulating cultures survive the freezing process better than less vigorous strains. In this method, one form of improving it is with the addition of protective agents like glycerol or DMSO that are known for protecting eukaryotic cells against freezing damage.</p> <p><u>Disadvantages:</u> Repeated freezing and thawing will significantly reduce the cultures viability and there are several factors that can affect the retrieval viability and effectiveness, these being: species, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell water content, lipid content and composition of the cells, density at freezing, composition of the freezing medium, cooling rate, storage temperature and duration of storage, warming rate, and recovery medium.</p>	Smith & Onions 1994; Baker & Jeffries 2006

	Methods	References
Freezing to -196 °C and preservation in liquid nitrogen or its vapour	<p><u>Short description:</u> For fungal cultures that do not sporulate or that produce mycelia that grow deep into the agar, sterilised screw-cap vials are filled with one quarter to half volume with a 10% glycerol solution. Plugs are cut from vigorously growing cultures and placed in a vial which is placed directly into the vapour phase of a liquid-nitrogen tank. Frozen preparations are retrieved by removing the vials from the freezer and rapidly thawing them in a 37 °C water bath. The thawed agar plugs are placed on appropriate agar plates. To make suspensions of spores or mycelia fragments from cultures growing on the surface of agar slants or plates, the colony surface is flooded with a glycerol or a DMSO solution and gently scraped. The suspension is then distributed into cryovials and placed into the liquid nitrogen tank. For a fungus that grows only in liquid culture, the mycelium must be macerated before it can be pipetted into vials. The broth culture is fragmented in a sterile mini-blender and mixed with a glycerol or a DMSO solution. This fungal mycelial and spore suspension is then aliquoted into sterile cryovials. Filled vials are placed into racks that are then put into the freezing chamber of a programmable freezer and allowed to equilibrate at 4 °C. They are then cooled from 4° to 40 °C at a rate of 1 °C per minute and from -40 to -90 °C at 10 °C per minute. After reaching -90 °C, vials are transferred immediately to liquid nitrogen vapour at -150 to -180 °C. For retrieval, cultures are thawed rapidly by placing the cryovials in a water bath at 37 to 55 °C. Culture samples are then transferred aseptically to appropriate growth media.</p> <p><u>Advantages:</u> Various alternative techniques for liquid-nitrogen storage, such as using plastic straws instead of vials or tubes, have been reported. Procedures used to harvest materials differ depending on whether the fungi sporulate, have mycelia that penetrate below the surface of the agar, or grow only in liquid culture. It is an effective, timesaving with reduced labour requirements method with increased assurance of long-term availability.</p> <p><u>Disadvantages:</u> Samples of human pathogens are scraped from the agar surface, or agar plugs are cut from the cultures. Such samples are never macerated in a mechanical blender because of the hazard of aerosol dispersion of the pathogen. It is an expensive method due to constant maintenance by refilling of liquid nitrogen and high cost apparatus. It needs constant surveillance. Also there space requirements for the refrigeration unit.</p>	Nakasone et al. 2004; Smith and Onions 2004
Lyophilisation or freeze-drying	<p><u>Short description:</u> Protectants are generally added to these systems to reduce damage during the freezing and drying processes. The procedure involves a two-stage process. First, ampoules containing the cultures as suspensions are cooled to -60 °C; air pressure in the ampoules is reduced and water is removed by ablation as the temperature is allowed to rise. Then, the ampoules are placed on a vacuum manifold for secondary drying (until the pressure in the system reaches about 30 milliTorrs), evacuated, and sealed under vacuum. Finished lyophilisation ampoules are stored. The purity and viability of the preparation in one lyophilisation vial should be checked 1-2 weeks after preservation. For the procedure, an agar slant with medium that supports good growth and sporulation is inoculated with the organism, which is allowed to grow until it reaches the resting phase. Lyophilised preparations from cultures much younger or older than resting phase often exhibit very low post-lyophilisation viability.</p> <p><u>Advantages:</u> Long-term storage at room temperature. Low-cost form of permanent preservation. Besides keeping the microorganisms viable for long periods, this method offers convenience of storing and of transport by mail with large-scale production of samples. It is the method of choice for many spore-forming fungi that produce large number of small (10-µm or less in diameter) spores.</p> <p><u>Disadvantages:</u> Not appropriate for all fungi. In fact, the technique is used primarily with species that form numerous, relatively small propagules. Viability can be improved when lyophilisation is done in the presence of trehalose. Large spores tend to collapse during the process, and the structural damage caused is not reversible by hydration. A significant number of the spores of appropriate size are also physically damaged and killed during the freezing process by the formation of ice crystals. Thus, each ampoule initially must contain many viable spores. Rapid freezing and the addition of a solution that dissolves ice crystals minimises growth of ice crystals. The two most common solutions are non-fat dry milk powder (in a 5% or 10% solution) and filter-sterilised bovine serum, although other proteinaceous materials can be used. Needs specialised technicians and equipment. Genetic changes may occur.</p>	Fry and Greaves 1951; Schipper and Holtman 1976; Sharma and Smith 1999; Nakasone et al. 2004; Uzunova-Doneva and Donev 2005; Miyamoto-Shinohara et al. 2006;

7.2. Annex II - Schemes of the processes of MUM

7.2.1. Annex II.A – Material Reception Process (MRP)

RESP	FLOW CHART	HOW TO PROCEED
QD/CURATOR	<pre> graph TD A([MATERIAL DEPOSIT REQUEST]) --> B{REQUEST APPROVED?} B -- Y --> C[NOTIFY DEPOSITOR] B -- N --> D([NOTIFY DEPOSITOR AND END OF PROCESS]) </pre>	<p>Request for deposit of material can be received via mail, post, fax or personally.</p> <p>Form F.052/053 is sent by MUM to notify about request reception and form F.066/067 is sent by MUM to formalize the request. Alternatively requests can be formalized by sending to MUM an institutional letterhead paper and with signature and stamp of the institutional responsible.</p> <p>All requests are analysed and decided by MUM Quality Director, within two weeks maximum (accordingly to CDS – Criteria for Deposit of Strains).</p> <p>Requests decision is recorded (F.066/067). In case of non-acceptance of material deposit F.054/055 is sent. A copy is filed in any case. In case of non-approval of the customer request F.068/069 is sent, and a copy is filed.</p> <p>For the accepted material deposit the customer is asked to fill, and send back to MUM, form F.046/047 (accordingly to CDS).</p> <p>Note: In case of a customer claim, procedure NCCA (Nonconformities and Corrective Actions) should be followed.</p> <p>Note: Records are filed and maintained accordingly to CR (Control of Records) procedure .</p>
CURATOR	<pre> graph TD A[MATERIAL RECEPTION] </pre>	<p>Depositor is notified of material reception (F.048/049).</p> <p>An internal number of order is given upon material reception (F.046/047).</p>
QD/CURATOR	<pre> graph TD A[QUARANTINE] --> B((A)) </pre>	<p>Newly acquired material should be isolated in a quarantine area. Purity and sanitary conditions of the material are verified (SOP.010) and quarantine results are recorded on F.046.</p> <p>If these conditions are not met, material is segregated and disposed (SOP.028). Procedure NCCA is followed for non-conformity event record (F.005). Customer of confidential deposit is notified and records maintained on form F.046/047 (field 25). Depending on the importance of the sample, confidential depositor is asked for the delivery of a new sample of the same biological material using forms (F.072/073).</p>

RESP	FLOW CHART	HOW TO PROCEED
CURATOR	<pre> graph TD A((A)) --> B[IDENTIFICATION AND CHARACTERIZATION] </pre>	<p>Material identification is based on macro and micromorphological features. Results are recorded on F.046/047. Additional identification data is recorded for genera <i>Aspergillus</i> (F.060/061) and <i>Penicillium</i> (F.062/063), respectively.</p>
CURATOR	<pre> graph TD A{ID CONFIRMED?} -- Y --> B[CUSTOMER NOTIFICATION] A -- N --> B B --> C{CUSTOMER CONSENT FOR PRESERVATION?} C -- Y --> D[MUM No ATTRIBUTION] C -- N --> E([MATERIAL DISPOSED]) </pre>	<p>Confirmation of material identification or misidentification is always reported to customer in case of confidential deposit (F.074/075).</p> <p>In case of misidentification, consent of the customer is asked, in order to know about his interest in maintain preservation of material at MUM (F.074/075). If there is no consent, material is disposed (SOP.028) and records are maintained on F.046/047 at the field 28. Procedure NCCA is followed for non-conformity event record (F.005).</p> <p>Free deposit may be maintained by MUM if considered relevant for collection.</p> <p>Note: In case of a customer claim, procedure NCCA should be followed.</p>
QD/CURATOR	<pre> graph TD A[MUM No ATTRIBUTION] --> B([MATERIAL READY TO PRESERVATION]) </pre>	<p>An unique accession number of MUM collection is attributed to material (acronym: 2 last digits of the year; order number per year) – e.g. MUM 10.01. Records are made on form F.046.0/047. MUM collection database is updated. Customer of confidential deposit is notified about MUM number attributed to material deposited (F.064/065).</p> <p>Note: Records are filed and maintained accordingly with CR procedure.</p>

7.2.2. Annex II.B – Material Preservation Process (MPP)

RESP	FLOW CHART	HOW TO PROCEED
CURATOR		Whenever possible or necessary, preservation of material is performed in BCC (Culture Collection Bank) and BCT (Work Culture Bank). BCCs are a bank of cultures from which originate all subsequent culture banks (BCT). Once BCT is created, BCC are stored and should not be used unless running out the stock of BCT.
CURATOR		Two of the five appropriated preservation methodologies are chosen for each material: <ul style="list-style-type: none"> - Mineral oil - Water - Silica-gel - Cryopreservation at – 80 °C - Lyophilisation
CURATOR		For each chosen preservation method, the execution of the method should followed each appropriated SOP: <ul style="list-style-type: none"> - Mineral oil (SOP.002) - Water (SOP.004, SOP.005) - Silica-gel (SOP.003) - Cryopreservation at – 80 °C (SOP.008) - Lyophilisation (SOP.001) Note: Reagents used in MUM in all preservation methods should be acquired following "Aprovisionamento" procedure (AP).
CURATOR		For each material the number of samples to be stored and its location of storage are defined in each SOP; Validity period depends on each type of preservation method and its defined on each SOP. For each strain the preservation date is recorded on the database. All preservation information for each material sample is registered in forms: <ul style="list-style-type: none"> F.043 - Mineral oil F.041 - Water F.040 - Silica-gel F.039 - Cryopreservation at – 80 °C F.043 – Lyophilisation Material is stored in the preservation cabinets 1, 2, 3, or deep-freezer (DF.02) according to each SOP. Note: Deep-freezer (DF.02) is a monitoring and measuring equipment (MME) periodically calibrated following monitoring and measuring equipment management procedure (MMEM).

RESP	FLOWCHART	HOW TO PROCEED
TECH		For verification of viability, purity and identification confirmation procedures are described in SOPs <ul style="list-style-type: none"> - Mineral oil (SOP.002) - Water (SOP.004, SOP.005) - Silica-gel (SOP.003) - Cryopreservation at - 80°C (SOP.008) - Lyophilisation (SOP.001) Periodicity of preservation and verification of viability, purity and identification confirmation depend on the material and the preservation technique defined in each SOP.
TECH		Viability is confirmed by material growth. If the material is no longer viable, it should be disposed (SOP.028); Records are maintained on form F.046. Procedure NCCA is followed for non-conformity event record (F.005). Records are filed and maintained accordingly to CR procedure.
TECH		If multiple growth is observed isolation is performed (SOP.011). If the material is contaminated isolation and purification procedures are performed; If these are not successful the material is disposed (SOP.028). Procedure NCCA is followed for non-conformity event record (F.005); Records are maintained on form F.046. Records are filed and maintained accordingly to CR procedure.
TECH		If identification is not confirmed, material is disposed (SOP.028). Records are maintained on form F.046. Procedure NCCA is followed for non-conformity event record (F.005); Records are maintained on form F.046. Records are filed and maintained accordingly to CR procedure.
TECH		Storage maintenance is performed according to each SOP followed for preservation.

7.2.3. Annex II.C – Material Supply Process (MSP)

RESP	FLOW CHART	HOW TO PROCEED
QD/CUR		<p>MUM only receives official requests, signed by the institutional or legal representative, as intermediate or end-user. A statement is required to report the reason that material is asked for. Following details will be asked: address, contact person phone and fax (F.066/067). Applicant is notified about the acknowledgment of MUM of the requests reception (F.052/053).</p>
QD/CURATOR		<p>Material ordering request from an embargoed country with restrictive measures that is listed on national* and international** documentation is immediately refused. A list of unauthorised recipients is consulted. Acceptance or denial of customer requests are recorded (F.066/067). All requirements are analysed by QD and decision of denial is sent to notify customer (F.068/069). Accepted requests are answered in accordance to their specifications. In case of requests not approved applicant is notified of the decision.</p> <p>* http://www.mne.gov.pt/mne/pt/AutMedidasRestritivas.htm ** Overview CFSP related sanctions</p> <p>Note: In case of a customer complain, the procedure NCCA should be followed (F.056/057 or F.058/059).</p>
TECH		<p>Material request is delivered to the laboratory to provide material.</p> <p>Request response is recorded (F.066/067). Records are filed and maintained accordingly with CR procedure.</p>
CURATOR		<p>Lyophilised material has immediate availability to the recipient and should be delivered up to 15 days. The material preserved by other methods may not have immediate availability. If delayed more than 2 weeks, customer is notified by telephone, fax or e-mail to inform about the estimated supply date and the distribution mode. If is not possible to provide requested material (e.g Because viability loss) customer is notified (F.068/069). Database is updated indicating the number of vials / plates or tubes transferred to customer.</p>
QD/CURATOR		<p>Shipment contents (F.034/35) are prepared and customer satisfaction inquiry/suggestion form (F.023) is provided with each delivered material. If forms F.026/027, F.028/029, and F.030/031 are sent, they should be returned by the customer. Material Transfer Agreement is sent to customer (F.017). This form should be kept by the customer. Term of transfer of material from a confidential deposit is signed by depositor and sent with material (F.032/033). A representative of customer, identified in form F.032/033, in confidential deposit is allowed to pick up the material only upon presentation of identification card or citizen card. Shipment of material is made in proper packaging and labelling in compliance with the applicable laws and shipping regulations for biological material (SOP.012).</p>

7.3. Annex III – List of forms created for the QMS of MUM

Denomination	Code	LAN	Retention time (years)
List of Forms	F.001	EN	NA
Vertical Model Sheet	F.002	EN	NA
Horizontal Model Sheet	F.003	EN	NA
Events Record	F.004	EN	NA
Non Conformities and Corrective Actions	F.005	EN	NA
Preventive and Improvement Actions	F.006	EN	NA
Treatment of Complaints	F.007	EN	NA
MME Control	F.008	EN	NA
MME Identification	F.009	EN	NA
MME Control Plan.Xls	F.010	EN	3
MME Inventory.Xls	F.011	EN	While in use
Equipment Form	F.012	EN	3
Infra-structure Maintenance Plan	F.013	EN	While in use
Cleaning and Maintenance Plan of Working Areas and Emergency Equipment	F.014	EN	While in use
Record of Revisions and Distribution	F.015	EN	During Processes existence
Annual Audit Program	F.016	EN	3
Material Transfer Agreement	F.017	EN	Until new revision
Staff Training Plan	F.018	EN	3
Training Efficacy Analysis	F.019	EN	3
Quality Plan_Quality Objectives	F.020	EN	3
Indicators And Objectives	F.021	EN	3
Badge	F.022	EN	While in use
Costumers Satisfaction Inquire	F.023	PT	3
Verification of Calibration Certificate	F.024	PT	While in use
Media and Solutions Registry MRP, MSP	F.025	EN	1
Declaração de Responsabilidade pelo Material Recebido	F.026	PT	5
Statement of Liability for the Received Material	F.027	EN	5
Declaração de Libertação de Responsabilidade	F.028	PT	5
Statement of Release of Liability (Disclaimer)	F.029	EN	5
Declaração de Responsabilização Pelo Uso de Materiais Potencialmente Patogénicos	F.030	PT	10
Statement of Accountability For The Use of Potentially Pathogenic Materials	F.031	EN	10
Termo de Cedência de Estirpes ao Cliente	F.032	PT	Forever
Term of Biological Material Transfer (To The Client)	F.033	EN	Forever
Lista de Conteúdos (A Enviar Pela MUM)	F.034	PT	NA
List of Contents (To Be Sent By MUM)	F.035	EN	NA
Preçário 2011	F.036	PT	5
Crachá	F.037	PT	While in use
MME Not Subject To Control	F.038	EN	NA
Registo de Conservação_Criopreservação (-80 °C)	F.039	PT	Forever

Denomination	Code	LAN	Retention time (years)
Registo de Conservação - Sílica Gel	F.040	PT	Forever
Registo de Conservação _ Em Água	F.041	PT	Forever
Registo de Conservação _Liofilização	F.042	PT	Forever
Registo de Conservação - Óleo Mineral	F.043	PT	Forever
Ficha de Dados de Segurança	F.044	PT	While in use
Safety Data Sheet	F.045	EN	While in use
Admissão de Culturas	F.046	PT	Forever
Admission of Cultures	F.047	EN	Forever
Notificação de Recepção de Material (Ao Depositário/Cliente)	F.048	PT	NA
Notification (To The Customer) of Material Reception	F.049	EN	NA
Crachá em Branco	F.050	PT	While in use
Declaração de Sigilo	F.051	PT	Until 5 years after leaving MUM
Notificação de Recepção de Pedido de Material/Informação	F.052	PT	NA
Acknowledgement of Reception of Material/Information Request	F.053	EN	NA
Pedido de Depósito Indeferido	F.054	PT	NA
Material Rejection Notification	F.055	EN	NA
Aceitação de Reclamação Relacionada com a Conformidade do Material Biológico	F.056	PT	NA
Acceptance of Complaint Related to Conformity of Biological Material	F.057	EN	NA
Rejeição de Reclamação	F.058	PT	NA
Rejection of The Complaint	F.059	EN	NA
Identificação e/ou Descrição de Estirpes de <i>Aspergillus</i>	F.060	PT	Forever
Identification and/or Description of <i>Aspergillus</i> Strains	F.061	EN	Forever
Identificação e/ou Descrição de Estirpes de <i>Penicillium</i>	F.062	PT	Forever
Identification and/or Description Of <i>Penicillium</i> Strains	F.063	EN	Forever
Atribuição de N° MUM	F.064	PT	NA
(Letter to Inform the Customer About the) MUM Number Attributed	F.065	EN	NA
Pedido de Depósito ou Fornecimento de Material	F.066	PT	Forever
Material Deposit or Supply Request to MUM	F.067	EN	Forever
Notificação de Resposta (de Não Aprovação) do Pedido Realizado à MUM	F.068	PT	3
Notification to the Customer of the Request Non-Approval	F.069	EN	3
Distribuição de Chaves Da MUM	F.070	PT	While in use
Não Utilizar	F.071	PT	NA
Pedido de Novo Material ao Cliente (Por o Anterior Não Estar Conforme)	F.072	PT	NA
New Material Request to the Customer (For Having the Anterior Material Non-Conform)	F.073	EN	NA
Carta para o Cliente (Sobre: Não/Confirmação da ID e Se Não/Está Interessado Em Manter a Amostra na Colecção.)	F.074	PT	NA
Letter to the Customer (on: Whether the ID Is/Not Confirmed and If the Customer Is/Not Still Interested In Deposit The Strain.)	F.075	EN	NA
Papel Timbrado	F.077	PT	While in use
Internal Training Registry	F.078	EN	3
Individual Training Record	F.079	PT	3
Relatório de Contacto	F.080	PT	3

Denomination	Code	LAN	Retention time (years)
Índice de Contactos	F.081	PT	3
Lista de Documentos Externos	F.082	PT	While in use
Instruções de Abertura De Ampolas	F.083	PT	NA
Registo de Localização na Arca A -80 °C	F.084	PT	Forever
Temperature Monitoring	F.085	EN	Forever
List of Internal Documents	F.086	EN	While in use
List of documents of informatics registry	F.087	EN	While in use
Function Responsibilities and Authority	F.088	EN	3
Profile and Minimum Skills Required	F.089	EN	3
Acta de Revisão pela Gestão	F.090	PT	3
Validação Semestral de Competências	F.091	PT	1
Registo de Abertura de Arca	F.092	PT	Forever
Media and Solutions Registry MPP	F.093	EN	Forever
Control of Records Table	F.094	EN	While in use
Índice AC	F.095	PT	While in use
Índice AP	F.096	PT	While in use
Inquire Delivery list	F.097	EN	While in use

*LAN = Language; EN = English; PT = Portuguese; NA = Not applied.

7.4. Annex IV – Certificate of Registration obtained from APCER



7.5. Annex V – International certificate from IQNet



THE INTERNATIONAL CERTIFICATION NETWORK

CERTIFICATE

IQNet and
APCER

hereby certify that the organization

MICOTECA DA UNIVERSIDADE DO MINHO

Centro de Engenharia Biológica, Campus de Gualtar
4710-057 BRAGA - PORTUGAL

for the following field of activities

Deposition, preservation and supply of filamentous fungal strains

has implemented and maintains a

Quality Management System

Which fulfils the requirements of the following standard

ISO 9001:2008

Issued on: 2011-05-04
Validity date: 2014-05-03

Registration Number: PT- 2011/CEP.3911



Michael Drechsel
President of IQNet



José Leitão
APCER CEO



Any additional clarification concerning the scope of this certificate may be obtained by consulting APCER.

IQNet Partners*:

AENOR Spain AFNOR Certification France AIB-Vinçotte International Belgium ANCE Mexico APCER Portugal CCC Cyprus
CISQ Italy CQC China CQM China CQS Czech Republic Cro Cert Croatia DQS Holding GmbH Germany DS Denmark
ELOT Greece FCAV Brazil FONDONORMA Venezuela HKQAA Hong Kong China ICONTEC Colombia IMNC Mexico
Inspecta Certification Finland IRAM Argentina JQA Japan KFQ Korea MSZT Hungary Nemko AS Norway NSAI Ireland
PCBC Poland Quality Austria Austria RR Russia SII Israel SIQ Slovenia SIRIM QAS International Malaysia SQS Switzerland
SRAC Romania TEST St Petersburg Russia TSE Turkey YUQS Serbia
IQNet is represented in the USA by: AFNOR Certification, CISQ, DQS Holding GmbH and NSAI Inc.

* The list of IQNet partners is valid at the time of issue of this certificate. Updated information is available under www.iqnet-certification.com

7.6. Annex VI – Solutions and media composition

7.6.1. Malt extract agar (MEA)

Malt extract	20 g
Mycological peptone	1 g
Agar	20 g
Glucose	20 g
Distilled water	1000 ml

The solution was homogenized and sterilized by autoclave at 121 °C for 15 minutes.

7.6.2. Preparation of cryoprotectant

A solution of skim milk and myo-inositol was prepared in distilled water, in aliquots of 10 mL, in universal glass bottles. This solution was sterilised in autoclave (114 °C for 10 minutes). It is important not to exceed this amount of time, because caramelisation will happen for a sterilisation process longer than 10 minutes.

Solution of milk and myo-inositol:

Skim milk in powder	10 % (p/v)
Myo-inositol	5 % (p/v)

Each batch of this solution was numbered and tested for contaminations by scattering a few drops on a Petri dish on Luria Bertani Media (LB) and Malt extract agar (MEA). The Petri dishes were incubated for 48 hours at 25 or 30 °C. Whenever no growth was observed on the next day, the plates were considered to be sterile and fit for use.

7.6.3. Czapek agar (CZ)

Solution A	50 mL
Solution B	50 mL
Sucrose	30 g
Agar	20 g
Distilled water	900 mL

The solution was homogenized and sterilized by autoclave at 121 °C for 15 minutes.

Solution A:

NaNO ₃	40 g
KCl	10 g
MgSO ₄ ·7H ₂ O	10 g
FeSO ₄ ·7H ₂ O	0.2 g
Distilled water	1000 mL

Solution B:

K ₂ HPO ₄	20 g
Distilled water	1000 mL

7.6.4. Czapek agar with yeast extract (CYA)

Sucrose	30 g
Yeast extract	5 g
NaNO ₃	3 g
KCL	0.5 g
MgSO ₄ ·7H ₂ O	0.5 g
FeSO ₄ ·7H ₂ O	0.01 g
K ₂ HPO ₄	1 g
Agar	20 g
Water	1000 mL

The medium was homogenized and sterilized by autoclave at 121 °C for 15 minutes.

7.6.5. Yeast extract sucrose (YES)

Yeast extract	20 g
Sucrose	150 g
Agar	20 g
Magnesium sulphate	0.5 g
Water	1000 mL

The solution was homogenized and sterilized by autoclave at 121 °C for 15 minutes.

7.6.6. Borate buffer (0.05 M)

Sodium borate decahydrate	3.61 g ($B_4Na_2O_7 \cdot 10H_2O$) (Sigma-Aldrich ®)
Distilled water	100 mL

The solution was stirred until complete dissolution of the solid and pH adjusted to 9.5 with HCl (6N). It was stored at 4 °C.

7.6.7. Sodium cyanide solution (0.13 mg/mL)

Sodium cyanide	0.13 g (Sigma-Aldrich ®)
Distilled water	100 mL

The sodium cyanide was mixed with 100 mL of distilled water to obtain a 1.3 mg/mL solution. The solution was homogenized and 10 mL of this first solution were transferred to a 100 mL volumetric flask. Distilled water was added until final volume of 100 mL. It was then homogenized and stored at 4 °C.

7.6.8. NDA (0.25 mg/mL)

NDA (Sigma-Aldrich)	2 mg
Methanol	8 mL

The solution was homogenized and stored at 4 °C for a maximum of 5 days.

7.6.9. Coconut milk agar (CMA)

Commercial coconut milk	200 mL
Agar	8 g

The solution was homogenized and sterilized by autoclave at 121 °C for 15 minutes.

7.6.10. Malt extract-glucose-yeast extract-peptone (MGYP)

Malt extract	3 g
Glucose	10 g
Yeast extract	3 g
Peptone	5 g
Distilled water	1000 mL

The solution was homogenized and sterilized by autoclave at 121 °C for 15 minutes.

7.6.11. Sodium acetate buffer 50 mM (pH 5.2)

Acetic acid (0.2 M)	7.4 mL
Sodium acetate (0.2M)	17.6 mL

7.6.12. Skim milk agarised medium (SKM)

Glucose	10 g/L
KH ₂ PO ₄	1 g/L
KCl	0.5 g/L
MgSO ₄ .7H ₂ O	0.2 g/L
CaCl ₂ .2H ₂ O	0.1 g/L
Skim milk 22.5 % (w/v)	25 mL/L
Agar	12 g/L

The solution was homogenized and sterilized by autoclave at 110 °C for 15 minutes.