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Selection and characterization of wood decay fungal strains for the development of mycelium-based materials

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To my family

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THESIS ABSTRACT

Fungi, and in particular wood decaying fungi, are being re-considered in the last few years as source for biotechnological and industrial applications. These organisms seem to be particularly suitable for developing myco-materials thanks to their chemical composition, mycelial texture, ease of cultivation, and lack of sporification. In this study 94 different strains of Agaricomycetes, belonging to 75 different species, related to 50 genera (18 families and 5 orders) were isolated using malt extract agar (MEA) medium enriched with hydrogen peroxide. Molecular analysis, extracting and amplifying the ITS region, allowed to confirm the identification of the isolated strains. Among these, twenty-one wood decaying fungal strains were chosen on the base of colour, homogeneity, and consistency of the mycelium. The growth rate of each selected strain was measured and a chemical characterization of all their mycelia obtained in liquid static fermentation was determined through thermogravimetric analysis (TGA). Two different typologies of materials have been produced.

One was obtained developing an exclusive method (newly patented) to improve consistency, structure and thickness of the mycelium mats: among all the strains, *Abortiporus biennis* 064-18, *Fomitopsis iberica* 104-19 and *Irpex lacteus* 076-18 showed to be suitable for a demo production of this material typology. The obtained mats were then analysed throw scanning electron microscopy (SEM) image processing and mechanical tests (tensile strength, elongation at break and Young modulus).

On the other hand, the second kind of material was obtained using fungal strains characterised by their hyphal cell wall chemical composition: highest content of α-glucans, β-glucans or chitin as well as a high growth rate. Five strains out of the twenty-one selected were chosen to evaluate how these differences could influence the mechanical and chemical characteristics of the resulting material.

These five fungal strains were cultivated in liquid submerged dynamic fermentation (both flasks and bioreactor). Then, chitin and glucans were extracted and crosslinked with acetic acid and plasticized with glycerol in order to obtain flexible sheets. In the end, *Abortiporus biennis* 064-18, *Fomitopsis iberica* 104-19 and *Stereum hirsutum* 073-18 were able to produce this kind of material. Thermogravimetric analysis (TGA) allowed to evaluate the principal chemical components of the materials, providing a semi-quantitative indication on mat composition. The material obtained from each species was also mechanically tested (tear strength, elongation at break, and Young's modulus) showing quite different results.

In conclusion, two typologies of sustainable and 100% biobased pure fungal-based row materials have been produced from different wood decaying fungal strains. Further chemical and physical steps are needed in order to let these materials show their high potential in practical applications. This project represents a valuable and in-depth analysis of alternative suitable wood decaying fungal

strains knowledge and an essential groundwork for any further study on this topic. Future researches have a real opportunity to significantly improve these promising myco-materials.

GENERAL INTRODUCTION

I. Wood decaying fungi

It was suggested that fungal kingdom is constituted by 5.1 million species but up to now only around 100.000 different species have been described to science (Blackwell, 2011).

Fungi are heterotrophic organisms and need to oxidate sugar to produce energy for living. From an ecological point of view they can be roughly divided into saprotrophic and symbionts (comprehending mutualistic symbionts and parasites). Fungi usually are ecologically and trophically related to plant kingdom, but in some cases they can live on animals or other fungi as parasites or saprotrophic.

A group of mutualistic symbionts (belonging to different Division) form peculiar structures with root trees known as mycorrhizae: they use as nutritive substances the sugars extruded from roots (ectomycorrhizae) or they directly feed inside the cell (endomycorrhizae). Parasitic fungi can be divided into necrotrophic and biotrophic. The first ones kill the cells in which they are feeding, whilst the second ones keep them alive. Finally, saprotrophic fungi feed on dead organic matter: mainly ligno-cellulosic tissues including leaves and wood.

This work is centred on wood decaying fungi (WDF), also known as lignicolous fungi or wood-decay fungi: these organisms, in nature, are able to feed and to reproduce on living or dead wood degrading cellulose, hemicelluloses and sometimes lignin too.

Wood is an exceptionally difficult substrate to degrade both from a chemical and a physical point of view. One of the principal reasons is that wood contains very low levels of nitrogen, which is needed to produce the enzymes that degrade the main structural polymers of wood. Even considering the wide variability existing among different plant species, it can be said that cellulose represents about 40-50% of the dry weight of wood (Côté, W.A. 1968), hemicelluloses, that are a group of branching heteropolymers of pentose and hexose sugar monomers, represent 15-32% of the wood cell wall. Hemicelluloses are more amorphous polymers if compared to cellulose, and they are considered to be the link between cellulose and lignin that allows the three polymers to behave as an integrated matrix. Finally, always generally speaking, lignin represents 20-35% of the wood cell wall and it is a heteropolymer consisting of repeating units of phenyl propane with a diverse array of bonds between three variations of the monomer form. This diversity in bonding pattern makes lignin extremely resistant to degradation and few microorganisms other than select wood degrading fungi have been able to unravel this system. Furthermore, the higher levels of lignin in wood compared to other plant materials, and manner in which lignin is intimately integrated with the holocellulose components, are major reasons for wood's resistance to degradation (Goodell et al 2020). In addition to these points,

wood often contains potentially fungitoxic compounds, which are deposited in the heartwood. In broad-leaved trees the toxic compounds are usually tannins, well known for their ability to cross-link proteins, making animal skins resistant to decay. In contrast, conifers contain a range of phenolic compounds such as terpenes, stilbenes, flavonoids and tropolones (Srivastava et al. 2013).

Depending on the degrading strategies, degraded molecules and enzyme typologies, different wood rots are classified even if it was demonstrated from a genetic point of view that they are not so well separated (Riley et al. 2014). By the way, for simplicity, wood rots are classified in 3 typologies: white rot (fibrous rot), brown rot (cubic rot) and soft rot.

The first is caused mainly by basidiomycetes and in some cases by ascomycetes. Wood appears bleached and fibrous. Hyphae of white rot fungi produce enzymes able to depolymerize and metabolize all three cell wall polymers. Often, the result of their action is an advanced decayed bleached fibrous mass with a very high weight loss up to 97% of the initial material. The great majority of white rot fungi, called “simultaneous” white rot, depolymerize and immediately utilize all the three main wood molecules. On the other hand, there are also “selective” white rot fungi that preferentially digest the hemicellulose and lignin components leaving much of the crystalline cellulose relatively undegraded. Both the two types of white rot determine structural losses more slowly than in brown rots, and the enzymatic erosion of the cell wall layers as well as the metabolic process of the fungus are the main direct cause of the cell wall depolymerization. White rot fungi prefer hardwoods, but can also attack softwoods (Krah et al. 2018). Lignin is degraded by white rot fungi thanks to specific enzymes, that are primarily oxidative. Among them, the most important are laccase, lignin peroxidase, manganese peroxidase, versatile peroxidase and the dye-decolorizing peroxidases (Goodell 2020). Many of these enzymes are synthetized almost exclusively by white rot fungi: only some dye-decolorizing peroxidases, for example, can be synthetized by select lignin-degrading bacteria. White rots can degrade wood so effectively thanks to a more complete suite of carbohydrate active enzymes they can synthetize. Firstly, through glycoside hydrolases and lytic polysaccharide monooxygenase enzymes they can deconstruct cellulose both from the ends of the long-chain polymer (exo-glucanases) and centrally along the polymer chain (endo-glucanases). Then white rots dismember the resulting holocellulose polymers through a suite of enzymes that can attack oligosaccharide depolymerization products (Goodell et al. 2020).

Brown rot is caused only by basidiomycetes and the attacked wood becomes more or less dark brown and brittle. Brown rot fungi have less types of enzymes to attack and degrade cellulose, compared to white rot fungi. Since brown rot fungi don’t even synthetize peroxidase enzymes for lignin depolymerization, after the initial colonization stages, they firstly digest the carbohydrate polymers of the wood cell wall, also bypassing lignin obstacle. In fact, brown rot fungi rapidly deconstruct

cellulose thanks to a further non-enzymatic mechanism to depolymerize lignin to allow access to the cellulosic components. Lignin then rapidly repolymerizes but with a modified structure. This led researchers to conclude erroneously for a long time that brown rot fungi had little effect on lignin. This oxygen radical-based chemical process ("chelator-mediated Fenton" - CMF system) is unique and it works before the enzymatic action to deconstruct wood polymers (Zhu et al. 2020). CMF chemistry is more complex than conventional Fenton chemistry and allows brown rot fungi to generate powerful hydroxyl radicals within the wood cell wall rather than next to the fungal hyphae (which would kill the fungus). CMF chemistry causes lignin depolymerization and then repolymerization as small, discrete irregular masses, separate from the cellulose (Goodell et al. 2017; Goodell 2020), so that the wood cell wall becomes more vulnerable.

Finally, soft rot is provoked by mostly by ascomycetes and it is not as intense as the two previously described rots. It remains on the surface and carbohydrates are preferred even if some lignin is degraded too. Even if soft rot damage of wood was first observed in the 1860s, soft rot fungi were not classified as a decay type until the 1950s (Savory 1954). There are two types of soft rot attack. The first creates diamond-shaped cavities following the cellulose microfibril angle within the S-2 cell wall layer, while the second type is a more widespread erosion of the S-2 cell wall layer from the lumen outward (Zabel et Morrell 2020). This last attack is more frequent, but some species can generate both types of damage depending on the kind of wood and the environmental conditions (Daniel et Nilsson 1998; Blanchette et al. 1988). Soft rot fungi are often found in soaked wood where conditions are more extreme and then less suitable for white and brown rot fungi (Goodell 2020). Their damage generally involves only the external few mm of wood surface exposed to the environment, maybe because, in interior wood below ground, oxygen concentration is too low. Soft rot damage shows an interesting mixture of white and brown rot characteristics in fact these fungi degrade both cellulose and hemicellulose, but also lignin as revealed by the cavities and erosion they cause. Furthermore, several soft rot fungi synthetize laccase which is also involved in lignin degradation by white rot fungi (Goodell 2020).

As here above stated, fungal decaying wood process involves the degradation of complex chemical compounds, primarily cellulose and/or lignin. Cellulose is a polysaccharide composed of linear chains of glucose molecules. This chemical compound is the primary cell wall component (around 50% of wood in weight) and it is the most common organic compound on Earth. Lignin is a very complex polymer of phenolic units making it very resistant to decay. This amorphous and insoluble polymer is not susceptible to hydrolytic attack, in contrast to cellulose. Although lignin is a formidable substrate, its degradation by certain fungi was recognized and described nearly 125 years ago. A few

Agaricomycotina (Basidiomycota) are the organisms able to depolymerize and mineralize lignin in the most efficient way (Srivastava et al. 2013).

Wood decay fungi are deeply studied in various application fields because of their heterogeneous possible uses.

In natural sciences they are considered of primary importance in relation to carbon and nitrogen dynamics of the forest floor (Gadd 2006), and more specifically they are fundamental actors in forest ecosystems preparing wood for other living organisms, complete carbon cycle and selecting the strongest trees by killing the weakest. Moreover, some particularly demanding species of wood decay fungi that are good examples of old-growth forests (e.g. *Pycnoporellus fulgens*, *Ganoderma carnosum*, *Laricifomes officinalis*, *Podofomes trogii*) are of high ecological value and could be considered as bioindicators of well-preserved forests (Saitta et al 2011; Bernicchia et Gorjòn 2020) and their presence could for instance be a further reason for protecting a specific area.

Wood decay fungi and their interactions with trees (development and prognosis) are also studied in urban environment for people safety (Schwarze et al. 2000).

In the last years they are also used for many different applications: from building of high performance musical instruments (Schwarze et al. 2008; Schwarze et al. 2011), to medicine (Angelini et al. 2018; Badalyan et al. 2015; Badalyan et al. 2019; Saltarelli et al. 2018), nutraceutics, scientific research as model organisms and finally also for bio-materials in design and fashion sectors.

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II. Myco-materials

Bio-fabricated materials are attracting an increasing attention due to a general environmental concern. Mycelium of a few species belonging to Agaricomycotina is now being explored for the production of sustainable materials that can for instance replace traditional ones or even provide novel materials thanks to the natural structure of hyphae organized in the mycelium network (Holt et al. 2012; Pelletier et al. 2013: Jones et al. 2017; Girometta et al 2019; Nawawi et al. 2019).

Many wood decay fungi can be grown on agricultural and forestry residues giving them new life and transforming them into cost-effective, environmentally sustainable, and highly versatile materials (Schmideder et al. 2019; Sun et al. 2019; Złotko et al. 2019; Antinori et al. 2020; Bustillos et al. 2020; Koc et al. 2020; Narayanan et al. 2020; Srubar 2020). The use of by-products or wastes as feedstocks, coupled with the low required energy need for fungal growth and the biodegradability of the biomaterial at the end of its service life together with the broad range of materials that myco-materials could potentially replace, make them industrially and economically convenient (Gandia et al. 2021).

Up to now two main big families of myco-materials exist: bio-composites on the one hand and pure materials on the other hand.

a) Bio-composite materials

Mycelium is able to bind organic matter through a network of hyphal micro-filaments producing both low-value materials like packaging and higher-value composite materials using agricultural and industrial wastes with little or no commercial value (Holt et al. 2012; Pelletier et al. 2013; Haneef et al. 2017; Islam et al. 2017; Jones et al. 2017). Mycelium based materials present many advantages over traditional synthetic materials including their low cost, density and energy consumption in

addition to their biodegradability and low environmental impact and carbon footprint (Arifin et Yusuf 2013; Haneef et al. 2017; Abhijith et al. 2018).

Coupling the wide variety of different substrates with controlled processes techniques like the growth environment and hot pressing, bio-composite mycelium materials can obtain specific structural and functional requirements including thermal and acoustic insulation and fire resistance (Holt et al. 2012; Pelletier et al. 2013; Haneef et al. 2017; Islam et al. 2017; Jones et al. 2017). This not only allows their use as waste-derived environmentally friendly alternatives to synthetic planar materials or synthetic foams and plastics, but also as semi-structural materials (Jiang et al. 2016; Haneef et al. 2017; Islam et al. 2017; Jiang et al. 2017). However, since many factors limit the current application and usage of bio-composite mycelium-based materials like quite low mechanical properties and high water absorption, further research for the development of these materials is necessary (Jones et al. 2020).

b) Pure materials

Fungal pure materials, thanks to their flexible nature, can not only replace traditional products such as bovine leather and its substitutes, synthetic foams for packaging and textiles, but also represent novel materials like high-performance paper-like sheets for new applications (Gandia et al. 2021). These peculiar materials are now gaining consideration both from the academic community and the industrial innovation all over the world. In recent years they have been investigated for many different applications ranging from bioprinting to wound treatment and water purification as well as in fashion and automotive sectors (Jones et al. 2019; Janesch et al. 2020; Attias et al. 2020; Jones et al. 2020; Nawawi et al. 2020; Silverman et al. 2020; Yousefi et al. 2020; Adamatzky et al. 2021; Jones et al. 2021).

Pure fungal materials can be formed directly by mycelium sheets or by an extraction and concentration of specific bio-polymers constituting the hyphal cell wall (Gandia et al. 2021; Jones et al. 2021). In the first case they are the result of complete degradation of the substrate or are obtained by removing the fungal skin from the surface of a substrate constituted by aerial hyphae. In the second they are re-shaped as for paper production starting from an amorphous but homogeneus mycelial biomass. The properties of the final material depend on the substrate that give balanced nutrients, the type of fungus concerning hyphal structure organization and chemical cell wall composition, the process used and finally the growth conditions as well as the post processing.

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AIM OF THE PHD PROJECT

The general aim of this PhD project consists in improving sustainable materials made of mycelium.

In order to achieve this goal, different steps are required:

I) Research in field for sampling basidiomes of wood decay fungi (WDF) in order to isolate and then test as many strains as possible to develope myco-materials;

II) Evaluate the mycelial characteristics, also related to different cultural conditions, to select the most suitable strains for the development of fungi-based materials;

III) Propose a new myco-material based on the selected species, characterized by both chemical and mechanical properties.

The whole process is schematised in Fig. 1.

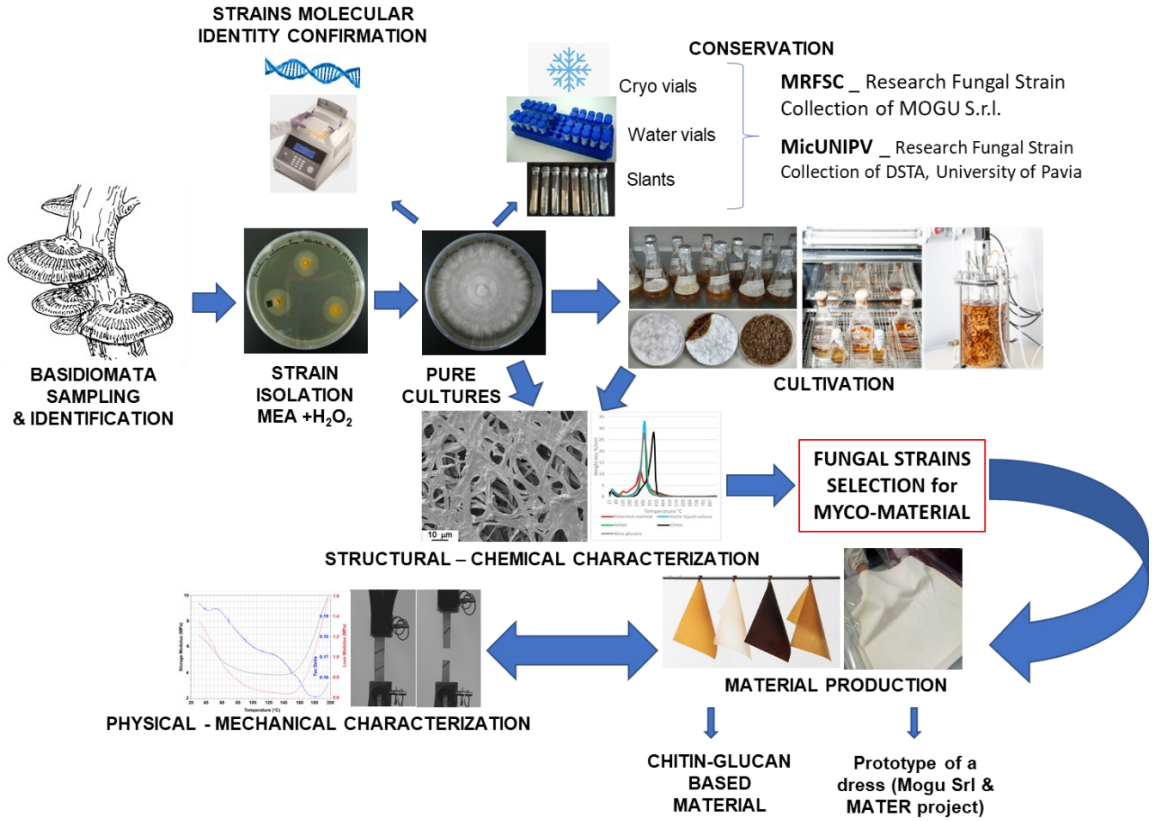

Fig. 1: Graphical abstract of my PhD project.

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Planning of the activities in the three years of my PhD project.

1st year main objects:

- exploration of environments rich in woody fungi ⇒isolation of fungal strains ⇒ molecular analysis to certify the strains identity ⇒ inclusion in research culture collection and their maintenance;
- optimization of cultural conditions;
- first choice of more suitable strains based on mycelium morphological characteristics (thickness, colour, …) and growth rate in Petri dishes.

Actually, isolation of new strains and their molecular identification continued throughout the three years of the doctorate.

2nd year main focus:

- development of fungal mats through a patented method (PM) by MOGU S.r.l.
- characterization of mycelia and the above mentioned fungal mats by thermogravimetric analysis (TGA) and scanning electron microscopy (SEM) in order to select the best performing fungal strains producing valuable and competitive prototypes.

The subsequent steps (mechanical characterization) and improvement of the productive process have been carried on by other researchers (MOGU S.r.l. and MATER project partners).

3rd year main goal:

- selection, based on TGA data (α-glucan, β-glucan and chitin content), of few strains to be tested for chitin-glucan material prototypes;
- comparison of fungal biomass production by cultivation in liquid shacking culture or in bioreactor;
- production of glucan-chitin mats;
- mechanical tests on these mats prototypes;
- TGA on these mats prototypes to evaluate the correspondence of α-glucan, β-glucan and chitin content.

The results regarding the collected WDF strains are reported in chapter 1; the chemical analyses that allowed strains selection are in chapter 2 and the prototype production and characterization of a new material using fungal species never tested before is reported in chapter 3.

Chapter 1. From field to culture collection of wood decay fungal strains for bio-based materials: basidiomes sampling and fungal strains isolation

The basis of this PhD project was to implement the collection of WDF strains already existing at the University of Pavia and constitute a private fungal strain collection inside MOGU S.r.l. in order to have a wide choice for further applications in materials.

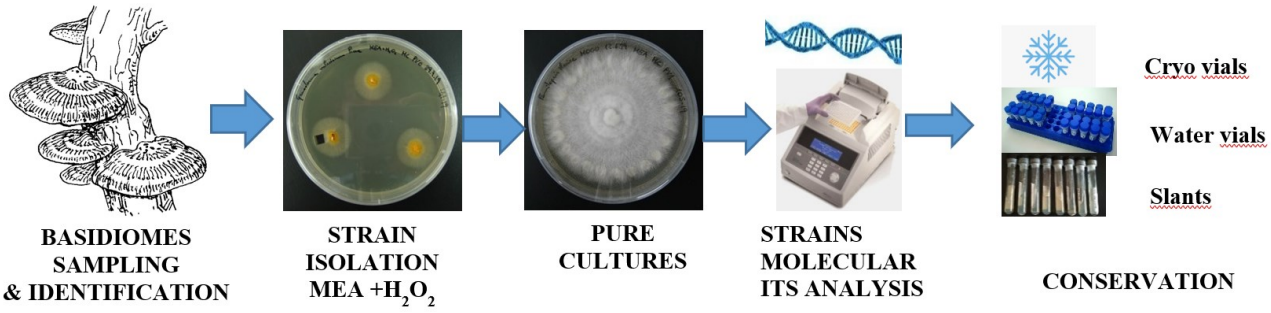

Fig. 2: Activities involved in the first part of my PhD project.

Firstly it was necessary to identify environments particularly rich in trees or wood residues.
Many different environmental typologies were investigated following both literature and previous personal experience. Forests and natural parks, but also urban parks or gardens, have been particularly investigated. The environments were explored without any aim of acquiring ecological information on the distribution of the fungal species.

Province of Varese (Lombardy, Italy) was the most explored area because:
- despite the small size, in the province of Varese there are many different environments, thanks to the simultaneous presence of temperate and thermophilic climate areas;
- it was extensively sampled by the local Mycological Group which provided very useful information. In fact, Varese Mycological Group (AMB) for many years constantly mapped the presence of mushrooms on the territory. In this case you can have all the information needed where to exactly find a specific species.
- it is close to the home and workplace of the author of the present work;

Besides, knowing the unique relationship between host plant and fungal species, in a few cases some environments were sampled in a specific way. E.g. if you are looking for *Truncospora atlantica*, you have to go in a Mediterranean forest of *Quercus ilex* in late autumn and carefully check dead branches preferably if still attached to the tree. On the contrary if you are looking for *Laricifomes officinalis*, you have to look for very old *Larix decidua*, better if hit by lightning or with broken branches. This fungal species is perennial, and its basidiomes are vital for decades but if you want to have more possibilities to isolate its fresh mycelium, you need to take a little piece when it is actively growing, in this case generally from the end of June to early September (depending on the specific season).

As soon as you find a specimen you are interested in, the best practise is to take few pictures that can show its characteristics, fundamental for its morphological identification. Then it could be also important to note down other elements like smell and taste or colour changing.

The collected sample needs to be protected and touched as less as possible in order to maintain their surface clean, to limit contaminations during the subsequent isolation phase. The sample can be stored into an aluminium foil or in a little clean plastic box and keep it stable and fixed.

Once in the lab it can be useful to keep all the collected specimens as they are, protected, at 10-15 °C in order to let the mycelium grow but avoiding or at least limiting the growth of contaminations like *Penicillium* or *Trichoderma* and the germination of their spores.

The last step for a successful isolation consists in using a proper medium, with the fundamental addition of H_2O_2 as suggested in bibliography.

To have the absolute confirmation to have isolated the target mycelium, macro- and microscopical characters are helpful, but not enough and a molecular ID is needed.

Finally, in order to keep cultures available in the long term, conservation of the strains is indispensable.
The best way is to combine different methods and to store the cultures in different places to minimize the risk of losing the strains. For this project, all the cultures are constituting the Mogu Research Fungal Strain Collection (MRFSC) and they were also added to the Mycotheque of the University of Pavia (MicUNIPV). The strains were stored in slants at 25 °C as well as in Petri dishes at 4 °C, in water vial tubes in submerged colonized filter paper discs and in cryo vials at -80 °C.

The search for the WDF basidiomes from which to isolate the strains was focused at the beginning of the PhD (autumn 2018) and it continued till the end of 2021. The molecular identification of the strains ended with the third year, for this reason the most complete and detailed publication on this part of the project has just been submitted to the journal "Diversity" (MDPI). In this publication details about the environments explored, the methods used and the list of isolated and identified strains are reported. Some taxonomic criticalities remain and further studies will be needed to define the specific identity of some strains such as *Antrodia cf. alpina*, *Antrodia cf. favescens*, *Antrodiella cf. faginea* and *Fibroporia cf. albicans*.
The first publication on this topic in this doctorate, Girometta et al. (2020), reports the initial data of the strains collection, and it is restricted to those isolated up to that moment in the Mediterranean environment.

A total of 94 strains, belonging to 75 species and 50 genera of Agaricomycotina was successfully isolated and sequenced. Some are from rare to very rare strains (*Dichomitus squalens*, *Fomitopsis spraguei*, *Polyporus corylinus* and *Sarcoporia polyspora*) for the Italian territory; others can be considered vulnerable or endangered (*Laricifomes officinalis, Ganoderma carnosum*, *Ganoderma valesiacum*); others, if confirmed, represent the first record and isolation in Europe (*Antrodia favescens*, *Fibroporia albicans*). *Fomitopsis iberica* was considered a rare species in Italy, but now its presence is increasing year after year (Bernicchia et Gorjòn 2020).

The maintenance of all these strains must be considered in the context of ex-situ conservation, useful for future studies too.

This part of work is included in the scientific paper submitted in March 2022. The submitted draft is hereafter presented.

Article

Isolation of lignicolous fungal strains useful for studies and applications, including myco-materials production

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Abstract: Fungi, and in particular lignicolous fungi, are being re-considered in the last few years as source for biotechnological and industrial applications. Lignicolous basidiomycetes are the most effective in degrading wood and in particular cellulose, hemicelluloses and lignin, which are among the most resistant biopolymers. This study aims to constitute a research collection of lignicolous fungal strains useful for further studies and applications in different production fields, above all myco-materials one. The basidiomata, used to isolate the strains in pure culture, were firstly identified through macroscopic and microscopic characteristics integrated with ecological data. In order to obtain pure cultures of dikaryotic mycelia, 94 different strains of Agaricomycetes, belonging to 75 different species, related to 50 genera (18 families and 5 orders) were isolated using malt extract agar (MEA) medium enriched with hydrogen peroxide. The identity of the isolated strains was then confirmed by molecular analysis extracting and amplifying the ITS region with ITS 1 and ITS 4 primers. Among all the isolated strains, the most promising belong to *Fomitopsidaceae*, *Hymenochaetaceae*, *Irpicaceae*, *Meruliaceae*, *Phanaerochaetaceae*, *Polyporaceae* and *Stereaceae*. Finally, 21 strains were chosen for further studies in material sciences to develop myco-materials.

Keywords: Fungal strain isolation; Lignicolous fungi; Research fungal strain collection; Myco-materials

1. Introduction

Fungi are relatively understudied, but they are an essential, fascinating and a useful group of organisms with biotechnological potential for pure and applied research as well as for industrial exploitation [1].

Actually, both scientific research and industrial applications look not only for stable and constant material but also for simple and fixed conditions in order to have under control all the variables that can influence the result of the research and to understand which and how each considered parameter modifies it. Being able to reproduce the same experiment is a key scientific principle in research; besides, it is also fundamental from the industrial point of view to have a standard production process to constantly obtain a product with exactly the same characteristics.

This is why basidiomycetes are propagated agamically in artificial conditions, through subculturing vegetative mycelium from Petri dish to Petri dish or to spawn, or to liquid medium in flasks or bioreactors and to complex substrates depending on the scope or application.

The used or selected genetics need to be preserved in order not to lose them. That's why certified collections have been constituted. Furthermore, it's important to consider that the cost in terms of time and resources, both human and financial, needed to create and maintain collections is far below the cost of individual new research of species in the natural environment.

Culture collections are a fundamental source for researchers at international level since exchange and availability of quality-guaranteed and authenticated pure cultures are increasingly needed [2-4].

In addition to official collections in conformity to international guidelines provided by the World Federation for Fungal Collections [5] many research centres all over the world have their own culture collection [6-8]. Strains maintained in many universities and little research centres can be considered as a very important source of biological and genetic material, because are geographically widespread and their contribution could be significantly representative of the biodiversity of local ecosystems [9,10]. They represent initial collections that could merge into an official collection at disposal for scientific community in the future.

Moreover, comparisons among different species or different strains belonging to the same species isolated from different environments or geographical areas, are very often required because biochemical differences among them could be relevant [11-14]. In particular, for myco-materials, mechanical properties of the mycelium as well as their aesthetic appearance depend on substrate, growth conditions but especially on the considered species and strains.

Furthermore, fungal collections have the fundamental role of preserving biodiversity, particularly endangered species, with the possible aim to increase their presence in nature if required. They are also of primary importance in order to deepen taxonomic studies, species distribution, officinal properties or to investigate potential applications based on fungi [15].

Filamentous fungi have been used for more than a century as versatile and highly productive organisms. Nowadays, fungi, and especially basidiomycetes, can be used in many different applications: from medicinal field as strategies against human diseases (antibacterial antibiotics, antifungals, antiviral agents, anti-cancer agents, anti-diabetes, controllers of cardio-vascular diseases, etc.), to agriculture as strategies against plant diseases (against fungi, insects, nematodes, weeds etc.), to the enhancement of crops and forestry, or as low-impact-food and proteins source and finally even for commodities like cosmetics, preservatives, enzymes producers and textile dyes [1].

Bio-fabricated materials are nowadays attracting increasing attention. This is due to a general major environmental concern and material sciences are being increasingly attracted by fungi and mycelium.

Filamentous fungi, thanks to their capacity to grow on organic material, such as plant waste, are now also being investigated for the production of sustainable and fully biodegradable materials that can for instance replace petroleum-based products like plastics in packaging thanks to the natural structure of their tissues formed by hyphae organised in the mycelium [16-18]. Fungi already demonstrated their potential also in the fashion business as they can origin a sustainable material able to substitute leather and leather-like materials that are currently made of polyvinyl chloride (PVC) and polyurethane (PU) [19] or sustainable design objects even with functional characteristics as

acoustic comfort and thermal insulation [20]. Renewable mycelium-based materials could for instance even provide novel materials.

Myco-materials are a newly explored field including two main types: bio-composites and pure material. Not many potentially useful species have been tested yet [19,21-25]. Up to now, the main species of lignicolous fungi that have been tested for bio-fabricated materials are *Ganoderma lucidum*, *Pleurotus ostreatus*, *Schizophyllum commune* and *Trametes versicolor* even if hundreds of species of potentially useful lignicolous fungi grow in the world. In particular, Italy is a country with a high biodiversity including woody fungal species [26,27]. Furthermore, many of the potentially useful species cannot be easily found in the existing local research culture collections.

The aim of this work was to sample as much lignicolous species belonging to Basidiomycota as possible in different environments in order to isolate fungal strains useful for further studies and applications in different production fields, especially for myco-materials.

2. Materials and methods

2.1. Basidiomata sampling

The fieldwork was carried out in different geographic areas belonging to the north and centre of Italy (Piemonte, Lombardia, Liguria and Lazio regions). In order to collect as many species of lignicolous basidiomata as possible, different environments were investigated:

1) coniferous mixed forests constituted by *Pinus sylvestris*, *Quercus robur* and *Castanea sativa*;
2) thermophilous broadleaf forest of *Quercus pubescens*, *Cornus mas* and *Ostrya carpinifolia*;
3) fresh broadleaved forests formed by *Fagus sylvatica*, *Fraxinus excelsior*, *Carpinus betulus*, *Quercus robur* and *Robinia pseudoacacia*;
4) mountain forest dominated by *Picea abies*, *Abies alba* and *Larix decidua* with *Salix* spp., *Sorbus montanus* and *Alnus alnobetula*;
5) Mediterranean scrub formed by *Quercus ilex*, *Myrtus communis* and *Pistacia lentiscus*;
6) urban and suburban environments (tree rows, parks, private and public gardens).

Furthermore, in order to find peculiar and particular species, some areas were more intensively investigated than others and the efforts to collect and isolate strains were not the same for all of them [28].

2.2. Fungal strains isolation

Only actively growing basidiomata were collected. If possible, the cleanest ones were completely or partially harvested (depending on their size and rarity) using a knife and touching them as little as possible.

The collected portion was placed into aluminium foil to keep it clean until laboratory operations.

In order to avoid destroying the basidiomata of *Laricifomes officinalis*, a rare and protected species, its mycelium was directly isolated in the field using a sterilised scalpel and the flame of a lighter. To establish a humid environment where the mycelium could regrow for a couple of days, the harvested basidiomata were put at 10 °C in the dark in little plastic boxes (humid chambers) on some pieces of soaked paper.

The protocol generally used to isolate mycelium from wild basidiomata [29-31] was slightly modified according to Rush Wayn (2001) [32]. To isolate the fungal strains, Petri dishes 90mm Ø were prepared using 2% malt extract agar (MEA) addictioned with 6 ml/l of a solution of 3% hydrogen peroxide in order

to reduce the spore germination of contaminants. Fresh mycelium developed in the above-mentioned humid chambers was transferred in sterile conditions under a laminar flow hood into the Petri dishes.

2.3. Basidiomata and fungal strains identification

The identification of the collected basidiomata was carried out by macro- and micro-morphological identification through dichotomous keys [33-39]. Microscopy was executed using a Paralux monocular microscope with plan-achromatic objectives 4x, 10×, 40×, 100× oil immersion.

Furthermore, microscopic characteristics of the isolated mycelia were checked [29,40].

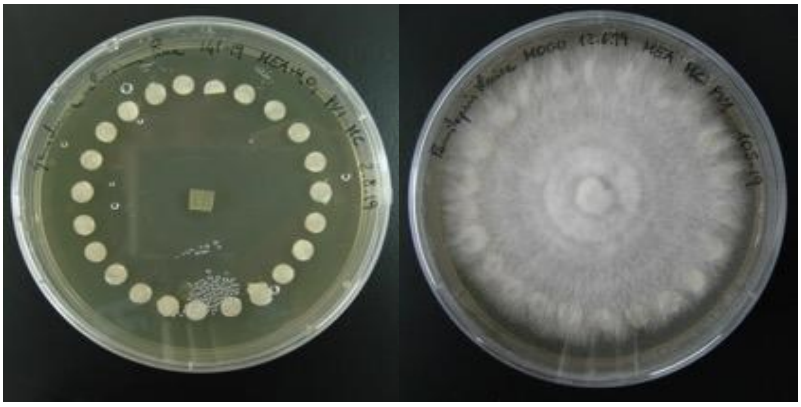
Besides morphological investigations, molecular identification of isolates was needed to confirm the strains identity. At first, to produce a sufficient amount of dry biomass, each strain was put into a 200 ml Erlenmeyer flask containing 50 ml of 2% malt extract broth (MEB) solution and grown for 10 days at 25 °C in the dark in static. The biomass was then collected with forceps in sterility, placed in glass tubes at -18 °C and freeze-dried. DNA was extracted following NucleoSpin Plant II protocol, then DNA was amplified by Polymerase Chain Reaction (PCR) using DreamTaq PCR Green Master Mix and the primer pair ITS1 (19bp) and ITS4 (20bp). Furthermore, the PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sent to Macrogen (The Netherlands) for sequencing. The obtained sequences were assembled, corrected and subsequently analyzed by BLAST searches using the GenBank (NCBI) [41] and Mycobank (CBS) [42] databases. Taxonomic assignments were based on similarity to reference sequences of these databases; afterward genotypic identification was compared with phenotype identification.

Mycobank [42]and Index Fungorum [43] (public databases officially accepted from the scientific community) were taken as reference for taxonomy and systematic. In some cases, the same species is accepted with a different name therefore, in these cases, the most recent publication choices were taken into consideration.

2.4. Fungal strains conservation

The isolated strains were stored in different ways: from the most classic such as storage in Petri dishes or tubes with 2% MEA at 4 °C, to colonised filter paper discs submerged in sterilised demineralized water in water vials at 4 °C [44] and cryopreservation at -80 °C.

As disc preservation regards, 20 paper filter discs of 5 mm Ø were cut, sterilized and disposed circularly into a 2% MEA Petri dish. A piece of actively growing mycelium was put in the Petri dish centre directly in contact with the agar and left to grow until complete colonisation of the discs: generally, depending on the strain, after 7-15 days the mycelium reached simultaneously all the paper discs (figure 1 A & B).



(**a**) (**b**)

Figure 1. (a) Petri dish with paper discs just inoculated; **(b)** Petri dish with colonised paper discs that can be transferred to vials

After the paper discs colonisation, 4 of them were put in each sterilised 1,8 ml autoclavable polypropylene (PP) vial tube together with 1 ml of demineralized and sterilised water. A minimum of 5 tubes for each strain was stored in the dark at 4 °C. To verify vitality of the strains, after 18 months colonised discs were taken out from water under sterile conditions and placed in MEA Petri dishes at 25 °C in the dark.

The protocol followed, planned to inoculate the strain in a flask containing liquid medium (ME 2%) and after 7 days, or when the strain has grown, it could be stored. The mycelium, maintaining sterility, was withdrawn from the flask and placed in a 10 ml tube containing a 15% solution of glycerol. The solution is homogenised through vortexing for 30 seconds at 3000 rpm. For the fungal strains, this step is made in a tube containing a 15% solution of glycerol and broken cover slides previously autoclaved to obtain a propagule suspension. Then, 1 ml of the suspension is placed in 1,5 ml sterile cryotubes. For each fungal strain 4 copies are stored at -80 °C.

All the strains are maintained both in Mogu S.r.l research fungal collection (MRFC) and in the mycotheque of the University of Pavia (MicUNIPV).

3. Results

3.1 Basidiomata sampling

The principal typologies of environment constituting the landscapes of the north of Italy, mainly the ones present in the province of Varese, were investigated during specific sampling campaigns. Fresh broadleaf forests are the most represented habitat and 37 strains (39,4%) were isolated from species collected there. On the other hand also urban and suburban environments presented many lignicolous species allowing the isolation of 27 strains (28,7%) (Figure 2).

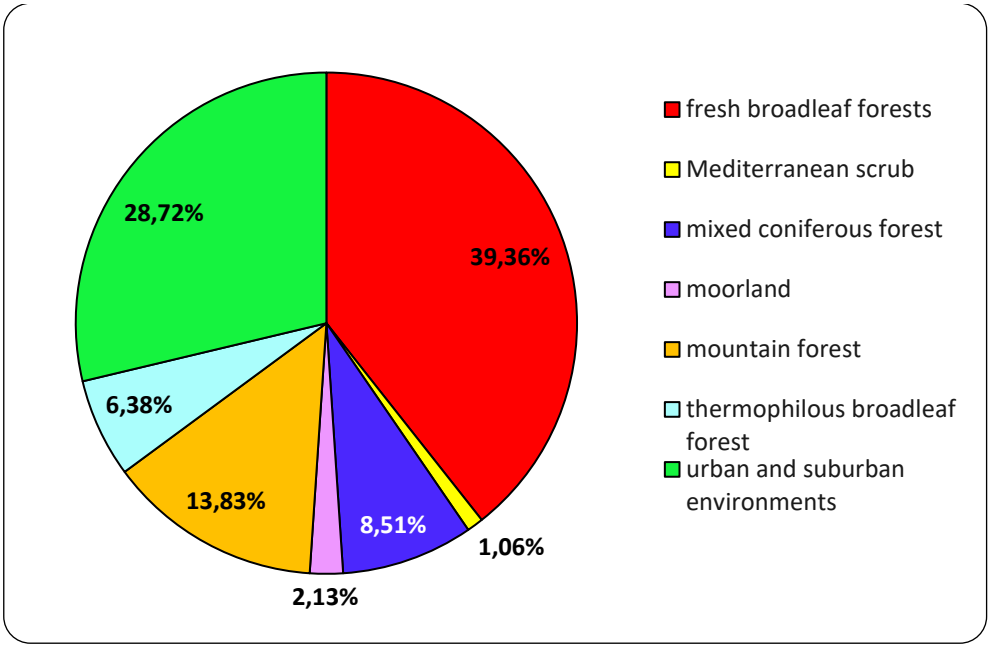


Figure 2. Investigated habitats where basidiomata were collected.

26 genera of trees on which the fungal species were growing could be identified (figure 3). In particular the great majority of collected basidiomata were growing on *Quercus* spp. (12%).

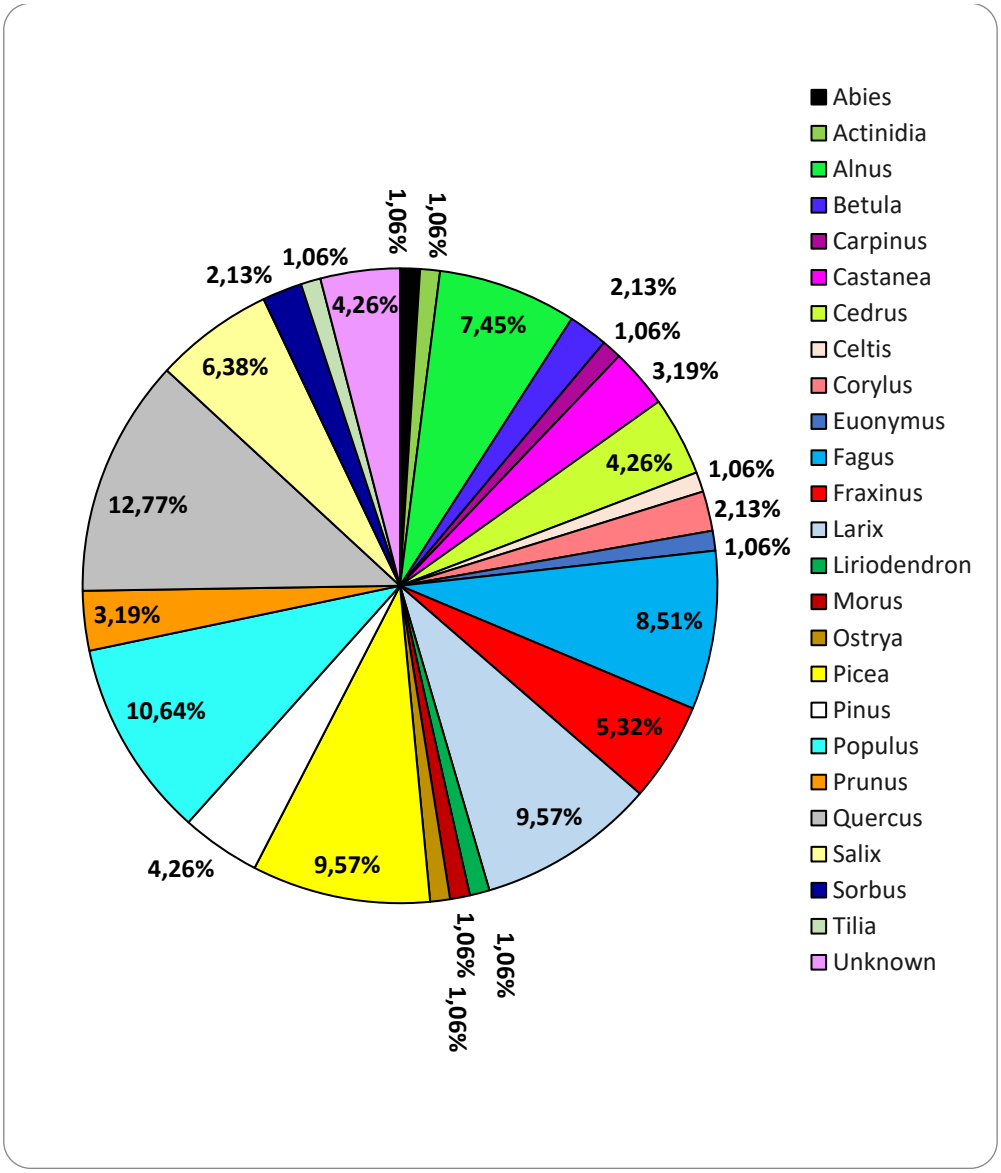


Figure 3. Host trees genera where basidiomata were collected

3.2. Fungal strains collection

Mycelium in pure culture has been successfully isolated from 94 out of 103 basidiomata collected (91,3%). All of them are *Agaricomycetes*, belonging to 75 different species related to 15 genera, 18 families and 5 orders (*Agaricales*, *Gloeophyllales*, *Hymenochaetales*, *Polyporales* and *Russulales*) (Tab. 1).

Table 1. Isolated strains

Order	Family	species	MRFC code
Agaricales	*Mycenaceae*	*Panellus stipticus* (Bull.) P. Karst.	183-21
	Strophariaceae	*Cyclocybe cylindracea* (DC.) Vizzini & Angelini	187-21
Gloeophyllales	*Gloeophyllaceae*	*Gloeophyllum odoratum* (Wulfen) Imazeki	077-18
		Neolentinus lepideus (Fr.) Redhead & Ginns	132-19

Hymenochaetales	*Hymenochaetaceae*	*Fomitiporia mediterranea* M. Fisch.	079-18
		Fomitiporia mediterranea M. Fisch.	082-19
		Fuscoporia contigua (Pers.) G. Cunn.	085-19
		Fuscoporia contigua (Pers.) G. Cunn.	130-19
		Fuscoporia torulosa (Pers.) T. Wagner & M. Fisch.	063-18
		Inonotus radiatus (Sowerby) P. Karst.	053-18
		Phylloporia ribis (Schumach.) Ryvarden	049-18
Polyporales	*Dacryobolaceae*	*Postia alni* Niemelä & Vampola	071-18
		Postia tephroleuca (Fr.) Julich	211-21
	Fomitopsidaceae	*Antrodia sp.*	074-18
		Antrodia cfr. alpina (Litsch.) Gilb. & Ryvarden	134-19
		Antrodia pulvinascens (Pilát) Niemelä	193-21
		Daedalea quercina (L.) Pers.	089-19
		Fomitopsis betulina (Bull.) B.K. Cui, M.L. Han & Y.C. Dai	042-18
		Fomitopsis iberica Melo & Ryvarden	004-18
		Fomitopsis iberica Melo & Ryvarden	104-19
		Fomitopsis pinicola (Sw.) P. Karst.	087-19
		Fomitopsis pinicola (Sw.) P. Karst.	117-19
		Fomitopsis pinicola (Sw.) P. Karst.	124-19
		Fomitopsis spraguei (Berk. & M.A. Curtis) Gilb. & Ryvarden	156-19
		Neoantrodia serialis (Fr.) Audet	111-19
		Osteina obducta (Berk.) Donk	147-19
		Osteina undosa (Peck) B.K. Cui, L.L. Shen & Y.C. Dai	162-19
	Grifolaceae	*Grifola frondosa* (Dicks.) Grey.	212-21

	Incrustoporiaceae	*Skeletocutis amorpha* (Fr.) Kotl. & Pouzar	171-19
		Tyromyces chioneus (Fr.) P. Karst.	158-19
	Irpicaceae	*Irpex lacteus* (Fr.) Fr.	076-18
		Irpex lacteus (Fr.) Fr.	160-19
		Irpex latemarginatus (Durieu & Mont.) C.C. Chen & Sheng H. Wu	109-19
	Ischnodermataceae	*Iscnoderma benzoinum* (Wahlenb.) P. Karst.	195-21
	Laetiporaceae	*Laetiporus sulphureus* (Bull.) Murrill	188-21
		Phaeolus schweinitzii (Fr.) Pat.	136-19
	Meruliaceae	*Abortiporus biennis* (Bull.) Singer	064-18
		Bjerkandera adusta (Willd.) P. Karst.	101-19
		Gloeoporus dichrous (Fr.) Bres.	083-19
		Phlebia rufa (Pers.) M.P. Christ.	186-21
	Phanerochaetaceae	*Antrodiella faginea* Vampola & Pouzar	169-19
		Porostereum spadiceum (Pers.) Hjortstam & Ryvarden	102-19
		Terana caerulea (Schrad. ex Lam.) Kuntze	177-19
	Polyporaceae	*Cerrena unicolor* (Bull.) Murrill	145-19
		Coriolopsis gallica (Fr.) Ryvarden	086-19
		Coriolopsis trogii (Berk.) Domanski	027-18
		Daedaleopsis confragosa (Bolton) J. Schröt.	155-19
		Daedaleopsis tricolor (Bull.) Bondartsev & Singer	148-19
		Daedaleopsis tricolor (Bull.) Bondartsev & Singer	028-18
		Dichomitus campestris (Quél.) Domanski & Orlicz	168-19
		Dichomitus squalens (P. Karst.) D.A. Reid	012-18

		Fomes fomentarius (L.) Fr.	066-18
		Fomes fomentarius (L.) Fr.	091-19
		Fomes fomentarius (L.) Fr.	179-19
		Ganoderma adspersum (Schulzer) Donk	106-19
		Ganoderma adspersum (Schulzer) Donk	007-18
		Ganoderma adspersum (Schulzer) Donk	036-18
		Ganoderma adspersum (Schulzer) Donk	097-19
		Ganoderma adspersum (Schulzer) Donk	112-19
		Ganoderma applanatum (Pers.) Pat.	045-18
		Ganoderma carnosum Pat.	161-19
		Ganoderma carnosum Pat.	191-21
		Ganoderma lucidum (Curtis) P. Karst.	037-19
		Ganoderma lucidum (Curtis) P. Karst.	137-19
		Ganoderma resinaceum Boud.	046-18
		Ganoderma resinaceum Boud.	120-19
		Ganoderma resinaceum Boud.	209-21
		Ganoderma valesiacum Boud.	196-21
		Irpiciporus pachyodon (Pers.) Kotl. & Pouzar	175-19
		Laricifomes officinalis (Vill.) Kotl. & Pouzar	143-19
		Lenzites betulinus (L.) Fr.	088-19
		Perenniporia fraxinea (Bull.) Ryvarden	122-19
		Picipes melanopus (Pers.) Zmitr. & Kovalenko	159-19
		Polyporus alveolaris (DC.) Bondartsev & Singer	096-19
		Polyporus badius (Pers.) Schwein.	093-19
		Polyporus corylinus Mauri	192-21
		Polyporus squamosus (Huds.) Fr.	094-19

		Pycnoporus cinnabarinus (Jacq.) P. Karst.	174-19
		Sarcoporia polyspora P. Karst.	172-19
		Trametes gibbosa (Pers.) Fr.	054-18
		Trametes hirsuta (Wulfen) Pilát	067-18
		Trametes hirsuta (Wulfen) Pilát	144-19
		Trametes suaveolens (L.) Fr.	061-18
		Trametes suaveolens (L.) Fr.	070-18
		Trametes versicolor (L.) Lloyd	139-19
		Trichaptum abietinum (Pers. ex J.F. Gmel.) Ryvarden	133-19
		Truncospora atlantica Spirin & Vlasák	078-18
		Yuchengia narymica (Pilát) B.K. Cui, C.L. Zhao & Steffen	176-19
Russulales	*Bondarzewiaceae*	*Heterobasidion abietinum* Niemelä & Korhonen	069-18
		Heterobasidion annosum (Fr.) Bref.	065-18
	Hericiaceae	*Laxitextum bicolor* (Pers.) Lentz	166-19
	Peniophoraceae	*Peniophora quercina* (Pers.) Cooke	090-19
	Stereaceae	*Stereum hirsutum* (Willd.) Pers.	073-18
		Stereum sanguinolentum (Alb. & Schwein.) Fr.	127-19

It should be noted that, since there was no uniformity between the two main fungal databases (Index fungorum and Mycobank), it was chosen the name *Phylloporia ribis* (Schumach.) Ryvarden (and not *Phellinus ribis* (Schumach.) Quél), according to Wagner & Fischer 2001 [45], *Polyporus alveolaris* (DC.) Bondartsev & Singer instead of *Neofavolus alveolaris* (DC.) Sotome & T. Hatt., *Polyporus badius* (Pers.) Schwein. instead of *Picipes badius* (Pers.) Zmitr. & Kovalenko and *Polyporus squamosus* (Huds.) Fr. instead of *Cerioporus squamosus* (Huds.) Quél according to Mycobank and Bernicchia & Gorjon 2020 [35].

The family of *Polyporaceae* is the most represented in the collection: almost 50% of the isolated species belong to it (Figure 4). As for orders, *Polyporales* is by far the most represented one: 55,7% of the isolated families belong to it (Figure. 5).

Among all species listed in table 1, 80% are considered white rot agents, while 20% are brown rot.

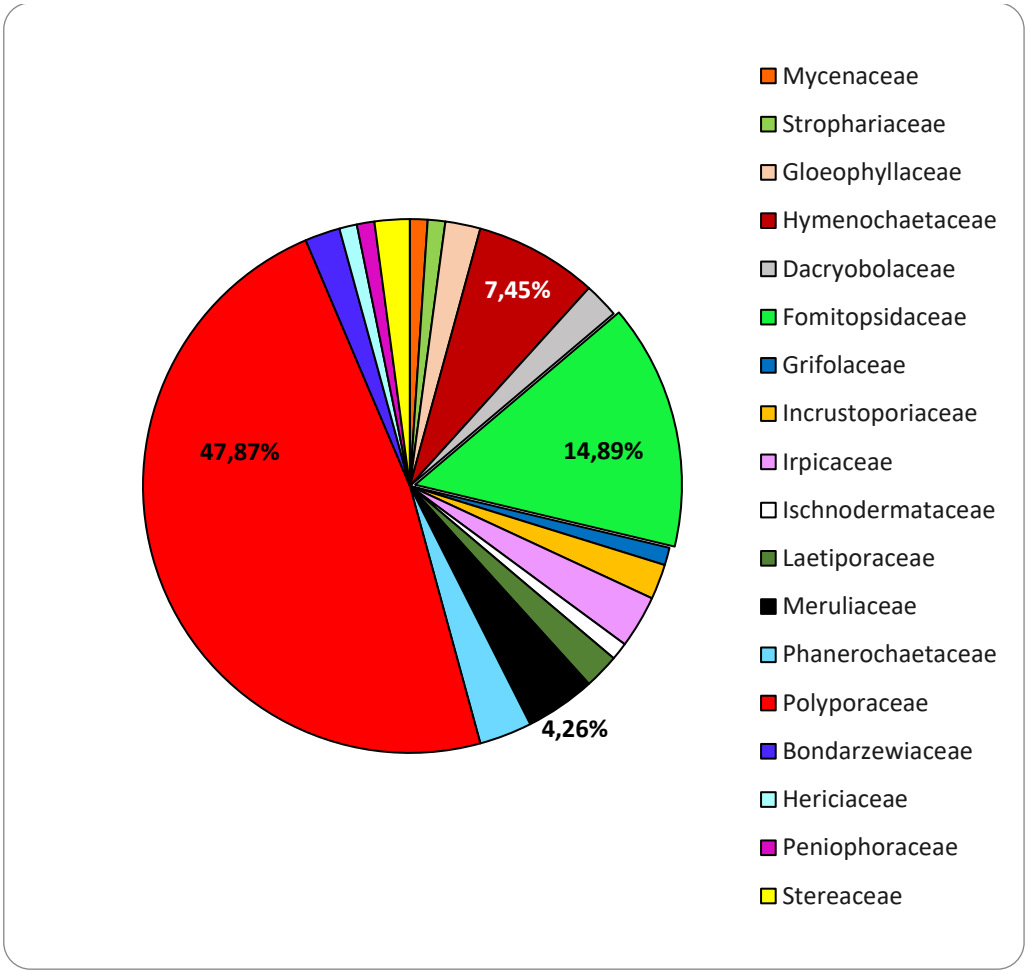


Figure 4. Basidiomycota families represented in the culture collection.

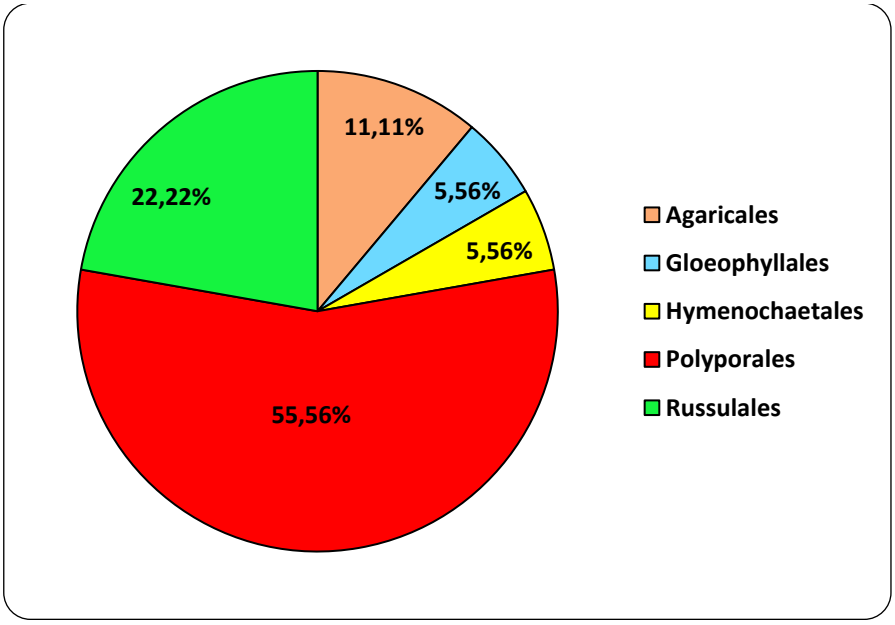


Figure 5. Basidiomycota orders represented in the culture collection.

3.3. Basidiomata and fungal strains identification

The molecular identification of strains generally confirmed the micro-macroscopic identification of the collected basidiomata. Above all it has been fundamental when basidiomata sampled were too young and didn't show all the characteristics indispensable for their identification.

Two species (*Antrodia sp.* and *Antrodia cfr. alpina*) belong to a difficult taxonomical group and still could not be unequivocally identified. In particular, the basidioma macrocharacteristics and host tree (e.g. red teaction to KOH and growth on *Larix decidua*) would suggest *A. alpina*, but molecular analysis doesn't exclude *A. xantha*. Therefore the strain 134-19 is referred to as *Antrodia cfr. alpina*. Further investigations are needed to univocally identify these strain.

The genus Ganoderma is one of the most difficult taxonomical groups to be identified [33]; nevertheless, the reported strains in table 1 had been univocally identified. In particular, the morphological discrimination between *Ganoderma applanatum* and *Ganoderma adspersum* basidiomata could be difficult if the collected specimens are too young: *G. adspersum* in fact could be identified from mycelium layers among tube strates, but if the basidioma has less than two years, this feature can't be observed. In this case, molecular analyses are always needful to be sure of their exact identification. *Ganoderma resinaceum,* when grown on *Salix* or *Alnus* close to water, is sometimes long stipitate and presents a thinner context if compared to the specimens usually growing on *Quercus* spp. This makes it resembling *Ganoderma lucidum* from a morphological point of view. These two species have been well separated by molecular analysis.

Finally, *Cyclocybe cylindracea* taxonomic situation is still uncertain: as shown in Vizzini et al. 2014 [46], two well supported clades exist. Furthermore two names are accepted: *Cyclocybe aegerita* (V. Brig.) Vizzini and *Cyclocybe cylindracea* (DC.) Angelini & Vizzini, but no typus is assigned to them (Vizzini personal comunication). It will be probable that the two accepted names will be assigned to the two existing clades of this collective species.

As it can be observed in table 1, only a little number of corticioid strains have been isolated even if in nature they are abundant and frequent. Actually, it is particularly difficult to isolate them properly since they not only have a very thin context but generally they are hardly found clean and actively growing.

3.4. Fungal strains conservation

All the isolated strains could be maintained vital thanks to the application of combined methods of storage. In particular, it could be demonstrated the ability of all the isolated strains to remain vital for at least 18 months in water vials on paper-filter discs at 4 °C.

4. Discussion

4.1. Basidiomata sampling

More than a blind scanning of all kinds of environments, the real purpose was to precisely find certain species known to grow in a specific habitat or in a specific place thanks to the long experience of the local mycological group carrying out a census of mushroom species growing in Varese province (Italy) since 1990 [47].

Even if urban areas have fewer trees than natural environments, many species of lignicolous basidiomycetes grow in public parks, private gardens and road trees due to an older age of the trees and their poor general health due to the growing condition, over pruning wounds caused by cars, root cutting for excavations etc.. Furthermore, in these places, species of trees that usually belong to completely different environments can coexist.

Among the isolated strains, *Dichomitus squalens, Fomitopsis iberica, Fomitopsis spraguei, Ganoderma carnosum, Ganoderma valesiacum, Laricifomes officinalis, Polyporus corylinus* and *Sarcoporia polyspora* are considered uncommon or rare species, scattered distributed at least in Italy as reported in the Checklist of Italian fungi – Basidiomycota [48] and in Bernicchia & Gorjon 2020 [35].

Some species confirmed to be host-specific like *Ganoderma valesiacum* and *Laricifomes officinalis,* strictly associated with *Larix decidua.* On the contrary, other stains even if not common showed a very large spectrum of hosts: in particular, *Fomitopsis iberica* could grow both on angiosperms and on gymnosperms. Notably this species was found exclusively in urban parks. Other species that grow preferably in urban areas are *Fomitiporia mediterranea, Perenniporia fraxinea, Ganoderma adspersum* and *Ganoderma resinaceum. Ganoderma carnosum,* usually growing in *Abies alba* forests, was found in two different public parks on decayed coniferous stumps.

4.2. Fungal strains isolation

Thanks to the precise choice to isolate mycelium from fresh and actively growing basidiomata, only few collected samples couldn't be isolated: *Fistulina hepatica, Pleurotus dryinus, Serpula himantioides, Meruliopsis taxicola, Dendropolyporus umbellatus*, *Physisporinus sanguinolentus, Lentinus suavissimus*, *Favolaschia calocera and Creolophus cirrhatus* because moulds or bacterial contaminations were always present overgrowing the target mycelium, even with the addition of hydrogen peroxide.

As regards *Terana caerulea* and *Porostereum spadiceum,* their basidiomata are quite common in nature but, as other corticioid species, mycelia are difficult to be isolated in pure culture (basidiomata are thin and close to the ground) so the strains are not so common. Besides, it is quite difficult to maintain these two species on artificial media. The storage through paper-filter disks at 4 °C has proved to be effective as the two species showed the ability to regrow after 18 months of storage.

Regarding mycelium characteristics, all strains related to *Ganoderma* genus had a very compact but thin-layered mycelium. On the other hand, *Agaricales* have a fluffy and inconsistent mycelium if compared to the one of *Polyporales.* Strains belonging to *Hymenochaetaceae* (*Fuscoporia, Phylloporia, Fomitiporia, Inonotus*) presented a coloured mycelium in Petri dish: *Fuscoporia* and *Fomitiporia* show a brownish thick mycelium, *Inonotus* and *Phylloporia* present a thin yellowish mycelium and extruding in agar dark-brown compounds. *Phylloporia* in particular, also showed thin but up to 1cm long crystals. The nature of these peculiar structures was never reported in literature and it could be worthy to be investigated in further specific studies (Figure 6). They could be related to halogenated hydroquinone metabolites already observed in *Phylloporia bordo* [49].

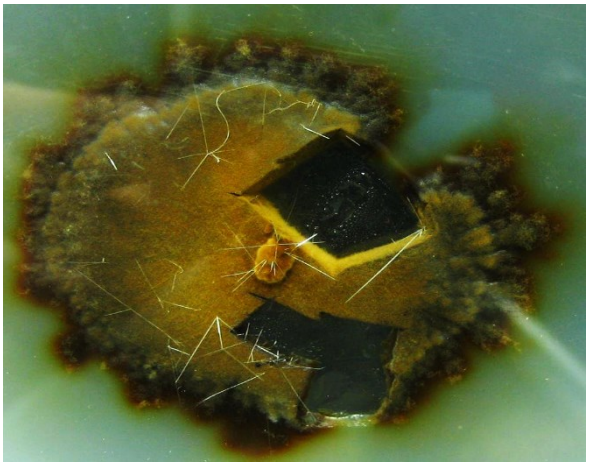
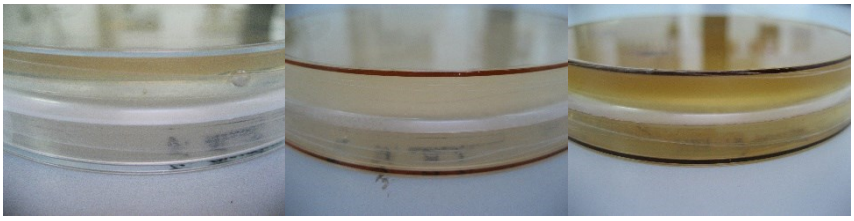


Figure 6. Pure culture of *Phylloporia ribis*, showing white 1cm-long crystals.

Other few strains present a coloured mycelium. *Antrodia cfr. alpina* has a sulphur-yellow mycelium, *Stereum hirsutum* mycelium is light orange, while *Terana caerulea* mycelium is white at the beginning and then it turns to intense blue. Finally, *Pycnoporus cinnabarinus* has an orange-reddish mycelium reflecting the colour of its basidiomata.

Moreover, *Abortiporus biennis* and *Peniophora quercina* produce dark-reddish exudates, while *Heterobasidion abietinum* and *Antrodiella faginea* produce substances that make the plastic of the Petri dish intensely yellow (Figure 7). Notably, nothing similar happens concerning *Heterobasidion annosum* cultures. Further studies will point out the nature of the molecule responsible for the changing colour of plastic and if this phenomenon is due to a partial degradation of the polymer or something different.



(a) **(b)** **(c)**

Figure 7. Changing of colour of Petri's plastic. **(a)** sterile MEA Petri dish; **(b)** and **(c)** *Heterobasidion abietinum* different strains.

Some species make the colour of the medium darker. They are both brown rot wood decay basidiomycetes, like *Gloeophyllum odoratum*, *Neolentinus lepideus*, *Laricifomes officinalis*, *Antrodia cfr. alpina* and *Fomitopsis iberica* and white rot agents like *Fuscoporia contigua* and *Polyporus squamosus* have this ability.

Neolentinus lepideus pure culture has a strong and pleasant anisate smell, similar to the one of its basidiomata.

Important characteristics for biotechnological applications and in particular for the development of myco-materials are the consistency of the mycelium and the growth rate in culture. A few strains presented thin or transparent, inconsistent mycelium (*Irpex latemarginatus, Polyporus squamosus, Stereum sanguinolentum, Terana caerulea*). Others showed a very slow growth (*Antrodia cfr. alpina, Laricifomes officinalis, Osteina obducta* and *Postia alni* among brown rot agents while *Fuscoporia torulosa, Inonotus radiatus, Phylloporia ribis* and *Skeletocutis amorpha* among white rot agents).

The cultures of *Laetiporus sulphureus* and *Laricifomes officinalis* have a dusty surface, because of the production of asexual spores that made them definitely unsuitable for applications in the fungal-material sector.

Some other species like *Abortiporus biennis, Coriolopsis gallica and C. trogii, Daedaleopsis confragosa, Fomes fomentarius, Fomitopsis iberica and F. pinicola, Ganoderma carnosum* and *G. lucidum, Irpex lacteus, Irpiciporus pachyodon, Lenzites betulinus, Polyporus alveolaris, Stereum hirsutum, Trametes gibbosa, T. hirsuta, T. suaveolens p*resented a fast-growing and homogeneous tough colony.

Some species are more represented than others in the collection because they seemed to be the most suitable for further studies for material applications.

4.3. Basidiomata and fungal strains identification

In general molecular identification confirmed the basidiomata morphological identification. In one case molecular analysis confirmed the taxonomic complexity of the species *Ganoderma carnosum*: in fact this European species, even if it is quite simple to macroscopically identify in the field thanks to its dark-red violaceous colours, the laccate surface and the growth on conifers, it revealed to be conspecific with *Ganoderma valesiacum* (presenting a white context, and a growth strictly related to *Larix decidua*), *Ganoderma oregonense* and *Ganoderma tsugae*, (two North American species) [50,51]. By the way, *G. valesiacum* and *G. carnosum* present a quite different mycelium on MEA: the first one pigments quite soon and if forms a thin layer of hyphae, on the other hand the second has a white, thicker and faster mycelium. Further studies are ongoing in order to clarify if they are only one single species or if they are confirmed to be different entities.

Regarding *Fomitiporia mediterranea*, it is possible to distinguish it from *Fomitiporia punctata* only from ITS sequences since all the micro- and macroscopical elements are the same.

Mycobank reported *Laetiporus* genus in *Fomitopsidaceae* because *Laetiporaceae* is considered invalid. Nevertheless, *Phaeolus* is placed in *Laetiporaceae*. We decided to follow Justo et al. (2017) [52] and consider *Laetiporaceae* Julic 1989 [39] valid, so *Laetiporus* could be included in it together with *Phaeolus*.

4.4. Fungal strains conservation

All the strains showed the ability to remain vital in submerged paper discs at 4 °C, but not all regrew immediately when transferred to a new MEA Petri dish: peculiar is the case of *Osteina undosa* 162-19. Colonized filter paper discs, after 18-month storage in water vials at 4 °C, were put on MEA Petri dish for the strain refreshment. Mycelium started to grow again only after 7 months of total inactivity at 25 °C.

5. Conclusions

The successful isolation ratio was very high thanks to the developed method. In total, only 9 out of 103 strains couldn't be isolated (less than 10 %).

Mycenaceae, Strophariaceae, Dacryobolaceae, Laetiporaceae and *Bondartzewiaceae* confirmed their inadequacy for myco-materials as their colonies on artificial medium are thin, slow-growing and formed of an inconsistent mycelium. Strains belonging to *Fomitopsidaceae*, *Hymenochaetaceae*, *Irpicaceae*, *Meruliaceae*, *Phanaerochaetaceae*, *Polyporaceae* and *Stereaceae* fungal families seem to be the most interesting thanks to the high growth rates, homogeneity and stiffness of their mycelial colony.

Taking into consideration that the strains were isolated to study later on the development of myco-materials, among all the isolated fungal strains, after the

evaluation of their mycelial characteristics and their growth rate, 21 different strains belonging to 20 species were selected for the next experimental campaign to obtain pure fungal materials (Cartabia et al. 2021).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, ES, MC; methodology, MC, RMB, SB, CEG; validation, ARB; investigation, MC, SB, RMB; resources, SBa, ES; data curation, MC, CEG; writing—original draft preparation, MC, RMB, SB; writing—review and editing, CEG, ES; supervision, AB, SBa, ES; project administration, ES, RMB; funding acquisition, SBa, ES.

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Data Availability Statement: data are available in the article.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Article

An Italian Research Culture Collection of Wood Decay Fungi

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Abstract: One of the main aims of the University of Pavia mycology laboratory was to collect wood decay fungal (WDF) strains in order to deepen taxonomic studies, species distribution, officinal properties or to investigate potential applications such as biocomposite material production based on fungi. The Italian Alps, Apennines and wood plains were investigated to collect Basidiomycota basidiomata from living or dead trees. The purpose of this study was to investigate the wood decay strains of the Mediterranean area, selecting sampling sites in North and Central Italy, including forests near the Ligurian and Adriatic seas, or near the Lombardy lakes. The isolation of mycelia in pure culture was performed according to the current methodology and the identity of the strains was confirmed by molecular analyses. The strains are maintained in the Research Culture Collection MicUNIPV of Pavia University (Italy). Among the 500 WDF strains in the collection, the most interesting isolates from the Mediterranean area are: *Dichomitus squalens* (basidioma collected from *Pinus pinea*), *Hericium erinaceus* (medicinal mushroom), *Inocutis tamaricis* (white-rot agent on *Tamarix* trees), *Perenniporia meridionalis* (wood degrader through Mn peroxidase) and *P. ochroleuca*. In addition, strains of species related to the Mediterranean climate (e.g., *Fomitiporia mediterranea* and *Cellulariella warnieri*) were obtained from sites with a continental-temperate climate.

Keywords: wood decay fungi (WDF); culture collection; fungal strain; host; Italy; morphological and molecular identification

1. Introduction

Wood decay fungi provide an extraordinary model both for pure and applied research, as well as a food or medicinal mushroom resource.

From an ecological point of view, wood decay fungi have a fundamental role, since they are important degraders of lignocelluloses. Heterogeneity in degradation strategies consists in different enzymatic pools, conditions for secretion and catalysis, alternative non-enzymatic pathways and strategy-switch depending

on environmental conditions [1,2]. Consistently, wood decay fungi shift from necrotrophism to pure saprotrophism, sometimes at an intraspecific level.

Systematic revisions based on multi-locus or genomic approach have revealed an extremely complex scenario concerning both biochemical features and morphology. Similar degradation strategies and similar morphologies are widespread, even among phylogenetically distant taxa, whereas the same taxonomic group may include species displaying different strategies and different morphologies. As a whole, wood decayers appear to be a pivotal model in the study of the evolutionary relationships of both Dikarya and extra-Dikarya taxa [3–5]. Consistently, the incipient molecular-based biogeography of wood decayers seems to display distribution patterns strongly affected by preferred host species (trees or shrubs), which are apparently followed throughout [6].

Wood decay fungi include several edible species as well as species reported to be the source of bioactive compounds, related to either primary (e.g., β-glucans) or secondary metabolism (terpenoids, phenolics, acids, superior alcohols, etc.). Structural diversity, occurrence and distribution among taxa, synthesis stimulation factors and correlation to growth stage, bioactivity pathways, standardization of products and crude extracts are the main current topics under investigation [7–9].

Due to their relatively easy reproduction in culture, several other applications are being developed on wood decay fungi: degradation of organic pollutants and bioremediation [10]; pretreatment of biomasses for production of sugars and bioethanol [11,12]; production of enzymes for industrial purposes, namely Mn peroxidases, laccases, cellulases and hemicellulases [13]; and bioadsorption and bioaccumulation of metal ions either in living or dead biomass [14–16].

Last but not least, necrotrophic wood decay means a loss of harvest in forestry and woody cultures, whereas in public and private green areas it means destabilization and consequently risk for people and objects [17].

Culture collections are an important reference, since availability and exchange of authenticated, quality-guaranteed pure cultures are increasingly needed by researchers at an international level [18,19]. Above all, tests on different species and strains (at intraspecific level) are required since biochemical differences are often not negligible [20–22].

Actually, only a few research centers can afford to structure their culture collection in conformity to international guidelines provided by the World Federation for Culture Collections (WFCC) [23,24]. The strains maintained in many universities or research centers can be considered an important source of experimental material, even without a WFCC certification. Since small uncertified collectors are geographically widespread, their contribution may be significantly representative of local ecosystems and biodiversity [25].

The Mycology Laboratory at the Botanical Garden of Pavia University (Italy) has a long tradition of the isolation, identification and preservation of fungal strains in various areas of mycology. This is supported by the numerous publications from the middle of the last century [26] up to now. Currently, the fungal strains collection is

named MicUNIPV and each working group preserves and enriches the collection.

Although the definition of ecotypes is usually hard, a remarkable intraspecific variability is well documented in several fungal species and it may be particularly true for rare species, whose populations are supposed to be more isolated [27]. This highlights the value of Italian territory for fungal biodiversity and the great potentiality for research [28].

Italy has a wide variety of climates and morphologies, both due to its remarkable latitudinal range (about 13°) and structural-topographic complexity, including the presence of four different seas and two main mountain chains. According to the official maps of MATTM (Ministero dell'Ambiente e della Tutela del Territorio e del Mare), 28 different phytoclimatic classes are recognized, five of which are specifically referred to as Mediterranean [29], also taking into account the biogeographic reference map suggested by Rivas-Martínez [30]. Nevertheless, the pluri-millennial stratification of human impact has made it difficult to distinguish between actual and potential ecosystem features. As a consequence, the classification and mapping of either Italian ecoregions or phytoclimates provide a tool for the comprehension of biodiversity instead of a strict map of biodiversity itself [31].

The present article reports the results obtained by the researchers of the Laboratory of Mycology in DSTA-University of Pavia (Italy) who continuously collect new cultures of wood decay fungi, focusing on fungal biodiversity of species related to the Mediterranean area and climates.

2. Materials and Methods

2.1. Sampling Sites and Field Work

Basidiomata were mostly (but not exclusively) collected in North and Central Italy. Sampling stratification was selectively applied, i.e., specific areas have been more frequently and strictly examined than others and sampling effort was not equal among different species [32]. The different environments examined are resumed hence:

a) highly-fragmented marginal woodlands and shrublands placed into an agricultural landscape, particularly referring to vegetation surrounding the hydrographic network, including major lakes;
b) mountain continuous woodlands and shrublands, both managed and unmanaged;
c) woody cultures (e.g., poplar plantations and vineyards), tree rows and hedges in agricultural landscape;
d) urban and suburban environments (tree rows, parks, private and public gardens).

Environments a, c, and d are mostly related to basal altitudinal belt and upper hill altitudinal belt in Po Plain, Apennines and Prealps (lower mountain thermal belt), as well as Adriatic, Tirrenian and Ligurian coasts; Environment b is mostly related to the lower and upper montane belt in the North and Central Apennines.

The basidiomata were completely or partially harvested by knife, gently brushed to eliminate debris and stored in paper bags until laboratory operations. The collecting sites were geolocalized, and the host species and general features were detected.

2.2. Experimental Procedures

Basidiomata identification was carried out by macro and micro-morphological analysis [6,33,34]; stereo and light microscopy were performed by Zeiss Axioplan and Zeiss Stemi 2000-C.

According to Stalpers [35] and Gams et al. [36], as well as Stamets [37], isolation of mycelia in pure culture was obtained in sterile conditions by inoculating small portions of the basidioma context into Petri dishes containing MEA medium and antibiotic (malt extract 2% + agar 1.5% + cloramphenicol 50 ppm). The incubation was carried out at 24 °C in the dark and each strain growth was checked constantly for a month. Based on the above, all the mentioned strains are to be regarded as dikaryotic.

Besides the morphological checks, molecular identifications of isolates were carried out on mycelia cultured in liquid medium (malt extract 2%). DNA was extracted from lyophilized mycelia by Nucleospin Plant II kit (Macherey-Nagel). Amplification by Polymerase Chain Reaction (PCR) used the primer pair ITS1 (19bp) and ITS4 (20bp)—that is, Internal Transcribed Spacer of ribosomal DNA; this region has been widely used for different fungal taxa [38,39]. PCR protocol exploited Dream Taq Mastermix (Promega) and was performed in a thermocycler, as reported in Table 1.

Table 1. Thermocycling protocol for PCR.

Step	Aim	T (°C)	Duration	Cycle repetitions
I	Denaturation	95	5 min	
	Denaturation	95	30 s	
II	Annealing	50	45 s	35
	Elongation	72	1 min	
III	Final elongation	72	10 min	

The qualitative checking of DNA (5 μL/sample) was performed both after extraction and amplification by DNA run (30 min, 100 V) on electrophoretic gel (1% agarose). SYBR Safe-DNA Gel Stain (Invitrogen) was used as an intercalant; GeneRuler 1kb (Thermo Scientific, Waltham - USA) was used as a ladder; BlueJuice (Invitrogen) was used as a gel loading buffer. The imaging was performed by Gel Doc (Biorad, Berkeley - USA).

ExoSAP-IT (Applied Biosystems, Foster City - USA) was used for the purification of amplification products. According to the suggested protocol, the sample/ExoSAP ratio was 5:2 μL; the reaction was carried out in a thermocycler in two steps—15 min at 37 °C and 15 min at 80 °C.

The sequencing was ordered to Macrogen (The Netherlands). Sequence analysis was performed by Sequencher 5.0 Demo. The sequences were finally matched with the ones available in the molecular identification facility of Mycobank [40].

Strains in pure culture were stored by different methods:

a) on malt extract agar (MEA) in a aPetri plate at 3 °C;
b) in a glass tube corked with cotton at room temperature;

c) colonized paper discs in demineralized water at 4 °C;
d) at −80 °C in glycerol (selected strains only).

Periodic checking and refreshment of cultures was performed to avoid contamination and devitalization.

The strains are maintained in the Fungal Research Culture Collection (MicUNIPV) of Department of Earth and Environmental Sciences of University of Pavia (Italy); each strain is included in a private database with all the information regarding sampling sites, data of collection and ecological notes.

3. Results and Discussion

MicUNIPV includes species related to plant pathology, soil, extreme environments, fresh and marine water, monuments and cultural heritage. As previously mentioned, different working groups within the Laboratory of Mycology (DSTA-University of Pavia) are engaged in the management, preservation and improvement of each MicUNIPV section. The section regarding wood decay species has up to now achieved 500 strains belonging to 110 different species [41–43]. The broad focus on wood decay led us to include in this section species related to different applications such as nutraceuticals, forest pathology, wood degradation and biocomposite materials.

The distribution of most species exceeds the Mediterranean area; nevertheless, several of them also display wide spatial gaps among stations and clear heterogeneity in host preference depending on the geographic location of the population.

Here, we present the species that have a distribution strongly related to the Mediterranean region and/or Southern Europe and/or warm climates, according to Ryvarden and Melo [6] and Bernicchia [33,34,44]. The species related to the Mediterranean diversity are reported in Table 2 and the most peculiar are discussed below.

Table 2. Selected Italian strains from MicUNIPV related to the Mediterranean area. Phytoclimate class as in [29].

Mic UNIPV ID	Species	Authors	Locality	Municipality	Host	Phytoclimate Class
D.con.1	*Daedaleopsis confragosa*	(Bolton) J. Schröt.	Dormelletto	Dormelletto (NO)	*Unidentified broadleaf*	mesotemperate/humid supratemperate
D.con.2	*Daedaleopsis confragosa*	(Bolton) J. Schröt.	Pian Porcino	Bagno di Romagna (FC)	*Unidentified broadleaf*	hyperhumid supratemperate/ultrahyperhumid
D.q.1	*Daedalea quercina*	(L.) Pers.	R.N. Bosco Giuseppe Negri	Pavia (PV)	*Quercus robur*	humid supratemperate/subhumid
D.q.2	*Daedalea quercina*	(L.) Pers.	Cono di Volo Malpensa	Gallarate (VA)	*Quercus rubra*	mesotemperate/humid supratemperate
D.q.3	*Daedalea quercina*	(L.) Pers.	Fosso dell'Oca	Rovescala (PV)	*Quercus petraea*	humid supratemperate/subhumid
D.sq.1	*Dichomitus squalens*	(P. Karst.) D.A. Reid	Pineta di San Vitale	Ravenna (RA)	*Pinus pinea*	supratemperate/humid-subhumid mesotemperate
D.sq.2	*Dichomitus squalens*	(P. Karst.) D.A. Reid	Ispra, Lungolago	Ispra (VA)	*Cedrus sp.*	mesotemperate/humid supratemperate
D.tric.1	*Daedaleopsis tricolor*	(Bull.) Bondartsev and Singer	Rio Bardonezza	Santa Maria della Versa (PV)	*Prunus avium*	humid supratemperate/subhumid
Des.t.1	*Desarmillaria tabescens*	(Scop.) R.A. Koch and Aime	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate/subhumid
Fm.i.1	*Fomitopsis iberica*	Melo and Ryvarden	Via Montello	Varese (VA)	*Corylus avellana*	humid supratemperate/hyperhumid
Fm.i.2	*Fomitopsis iberica*	Melo and Ryvarden	Villa Baragiola	Varese (VA)	*Abies alba*	humid supratemperate/hyperhumid
Fm.i.3	*Fomitopsis iberica*	Melo and Ryvarden	Via Tasso	Varese (VA)	*Cedrus deodara*	humid supratemperate/hyperhumid
Fm.i.4	*Fomitopsis iberica*	Melo and Ryvarden	Via S. Francesco	Inarzo (VA)	*Betula pendula*	humid supratemperate/hyperhumid
Fm.i.5	*Fomitopsis iberica*	Melo and Ryvarden	Villa Toeplitz	Varese (VA)	*Fagus sylvatica*	humid supratemperate/hyperhumid
Fm.i.6	*Fomitopsis iberica*	Melo and Ryvarden	Villa Mylius	Varese (VA)	*Fagus sylvatica*	humid supratemperate/hyperhumid

Fm.m.1	*Fomitiporia mediterranea*	M. Fisch.	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate/subhumid
Fm.m.2	*Fomitiporia mediterranea*	M. Fisch.	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate/subhumid
Fm.m.3	*Fomitiporia mediterranea*	M. Fisch.	RNIS Bosco Siro Negri	Zerbolò (PV)	*Hedera helix*	humid supratemperate/subhumid
Fm.m.4	*Fomitiporia mediterranea*	M. Fisch.	Rio Bardonezza	Santa Maria della Versa (PV)	*Robinia pseudoacacia*	humid supratemperate/subhumid
Fm.m.5	*Fomitiporia mediterranea*	M. Fisch.	Comiso	Ragusa (RG)	*Cistus sp.*	mesomediterranean/subhumid-dry thermomediterranean
Fm.m.6	*Fomitiporia mediterranea*	M. Fisch.	Cono di Volo Malpensa	Gallarate (VA)	*Quercus rubra*	mesotemperate-humid supratemperate
Fm.m.7	*Fomitiporia mediterranea*	M. Fisch.	R.N. Torbiere del Sebino	Provaglio d'Iseo (BS)	*Corylus avellana*	mesotemperate-humid supratemperate
Fm.m.8	*Fomitiporia mediterranea*	M. Fisch.	Villa Augusta	Varese (VA)	*Fagus sylvatica*	humid supratemperate - hyperhumid
Fm.m.9	*Fomitiporia mediterranea*	M. Fisch.	Olgiate Comasco	Olgiate Comasco (CO)	*Actinidia chinensis*	mesotemperate-humid supratemperate
Fm.m.10	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.11	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.12	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.13	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.14	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.15	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.16	*Fomitiporia mediterranea*	M. Fisch.	Pradone nord	Rovescala (PV)	*Vitis vinifera*	humid supratemperate - subhumid
Fm.m.17	*Fomitiporia mediterranea*	M. Fisch.	Unipv_polo scientifico via Ferrata	Pavia (PV)	*Salix alba*	humid supratemperate - subhumid

G.adsp.1	*Ganoderma adspersum*	(Schulzer) Donk	Orto Botanico	Pavia (PV)	*Quercus sp.*	humid supratemperate - subhumid
G.adsp.2	*Ganoderma adspersum*	(Schulzer) Donk	R.N. Bosco Giuseppe Negri	Pavia (PV)	*Populus nigra*	humid supratemperate - subhumid
G.adsp.3	*Ganoderma adspersum*	(Schulzer) Donk	R.N. Bosco Giuseppe Negri	Pavia (PV)	*Unidentified broadleaf*	humid supratemperate - subhumid
G.adsp.4	*Ganoderma adspersum*	(Schulzer) Donk	Parco della Vernavola	Pavia (PV)	*Alnus glutinosa*	humid supratemperate - subhumid
G.adsp.5	*Ganoderma adspersum*	(Schulzer) Donk	RNIS Bosco Siro Negri	Zerbolò (PV)	*Unidentified broadleaf*	humid supratemperate - subhumid
G.adsp.6	*Ganoderma adspersum*	(Schulzer) Donk	Rio Bardonezza	Rovescala (PV)	*Quercus robur*	humid supratemperate - subhumid
G.adsp.7	*Ganoderma adspersum*	(Schulzer) Donk	Madonna del Bocco	Santa Margherita Staffora (PV)	*Quercus cerris*	supratemperate - humid mesotemperate
G.adsp.8	*Ganoderma adspersum*	(Schulzer) Donk	Morina	Rovescala	*Quercus petraea*	humid supratemperate - subhumid
G.adsp.9	*Ganoderma adspersum*	(Schulzer) Donk	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
G.adsp.10	*Ganoderma adspersum*	(Schulzer) Donk	RNIS Bosco Siro Negri	Zerbolò (PV)	*Populus nigra*	humid supratemperate - subhumid
G.adsp.11	*Ganoderma adspersum*	(Schulzer) Donk	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
G.adsp.12	*Ganoderma adspersum*	(Schulzer) Donk	Ca' del Bosco	Nibbiano Val Tidone (PC)	*Quercus cerris*	humid supratemperate - hyperhumid
G.adsp.13	*Ganoderma adspersum*	(Schulzer) Donk	Montebolone	Pavia (PV)	*Cedrus atlantica*	humid supratemperate - subhumid
G.adsp.14	*Ganoderma adspersum*	(Schulzer) Donk	Cascina Scova	Pavia (PV)	*Unidentified broadleaf*	humid supratemperate - subhumid
G.adsp.15	*Ganoderma adspersum*	(Schulzer) Donk	Ticino	Torre d'Isola (PV)	*Quercus sp.*	humid supratemperate - subhumid
G.adsp.17	*Ganoderma adspersum*	(Schulzer) Donk	unknown	Bologna (BO)	*Unidentified broadleaf*	supratemperate/humid mesotemperate-subhumid
G.car.1	*Ganoderma carnosum*	Pat.	Foreste Casentinesi	Poppi (AR)	*Abies alba*	hyperhumid supratemperate/ultrahyperhumid
G.pf.1	*Ganoderma pfeifferi*	Bres.	Prati di Tivo	Pietracamela (TE)	*Fagus sylvatica*	supratemperate/hyperhumid mesotemperate/humid

H.e.1	*Hericium erinaceus*	(Bull.) Pers.	Colle Ciupi	Siena (SI)	*Quercus ilex*	subhumid mesotemperate/humid
H.e.2	*Hericium erinaceus*	(Bull.) Pers.	Castello di Belcaro	Siena (SI)	*Quercus ilex*	subhumid mesotemperate/humid
H.e.3	*Hericium erinaceus*	(Bull.) Pers.	Strada per Castello di Belcaro	Siena (SI)	*Quercus ilex*	subhumid mesotemperate/humid
H.e.4	*Hericium erinaceus*	(Bull.) Pers.	Strada per Castello di Belcaro	Siena (SI)	*Quercus ilex*	subhumid mesotemperate/humid
H.e.5	*Hericium erinaceus*	(Bull.) Pers.	Colle Val d'Elsa	Colle Val d'Elsa (SI)	*Quercus ilex*	subhumid mesotemperate/humid
I.t.1	*Inocutis tamaricis*	(Pat.) Fiasson and Niemelä	Apani	Brindisi (BR)	*Tamarix gallica*	thermomediterranean/mesomediterranean/dry inframediterranean/subhumid
I.t.2	*Inocutis tamaricis*	(Pat.) Fiasson and Niemelä	Ostia Lido	Roma (RM)	*Tamarix gallica*	subhumid mesomediterranean
L.s.1	*Laetiporus sulphureus*	(Bull.) Murrill	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
L.s.2	*Laetiporus sulphureus*	(Bull.) Murrill	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
L.s.3	*Laetiporus sulphureus*	(Bull.) Murrill	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
L.s.4	*Laetiporus sulphureus*	(Bull.) Murrill	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
L.s.5	*Laetiporus sulphureus*	(Bull.) Murrill	Pietragavina	Varzi (PV)	*Castanea sativa*	supratemperate - hyperhumid mesotemperate - humid
L.s.6	*Laetiporus sulphureus*	(Bull.) Murrill	Cono di Volo Malpensa	Gallarate (VA)	*Quercus sp.*	mesotemperate-humid supratemperate
L.w.1	*Cellulariella warnieri*	(Durieu and Mont.) Zmitr. and V. Malysheva	R.N. Bosco Giuseppe Negri	Pavia (PV)	*Quercus robur*	humid supratemperate - subhumid
L.w.2	*Cellulariella warnieri*	(Durieu and Mont.) Zmitr. and V. Malysheva	Bosco del Cecco	Santa Maria della Versa (PV)	*Ulmus minor*	humid supratemperate - subhumid
L.w.3	*Cellulariella warnieri*	(Durieu and Mont.) Zmitr.	Rio Marsinola-Fracion	Rovescala (PV)	*Populus nigra*	humid supratemperate - subhumid

		and V. Malysheva				
L.w.4	*Cellulariella warnieri*	(Durieu and Mont.) Zmitr. and V. Malysheva	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
L.w.5	*Cellulariella warnieri*	(Durieu and Mont.) Zmitr. and V. Malysheva	RNIS Bosco Siro Negri	Zerbolò (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
L.w.6	*Cellulariella warnieri*	(Durieu and Mont.) Zmitr. and V. Malysheva	Bosco Sforza nord-Rio Bardonezza	Ziano Piacentino (PC)	*Ulmus minor*	humid supratemperate - subhumid
P.f.1	*Perenniporia fraxinea*	(Bull.) Ryv.	R.N. Bosco Giuseppe Negri	Pavia (PV)	*Populus nigra*	humid supratemperate - subhumid
P.f.2	*Perenniporia fraxinea*	(Bull.) Ryv.	Via Scala	Pavia (PV)	*Celtis australis*	humid supratemperate - subhumid
P.f.3	*Perenniporia fraxinea*	(Bull.) Ryv.	Rio Bardonezza	Rovescala (PV)	*Salix alba*	humid supratemperate - subhumid
P.f.4	*Perenniporia fraxinea*	(Bull.) Ryv.	Via Ubaldo degli Ubaldi	Pavia (PV)	*Unidentified broadleaf*	humid supratemperate - subhumid
P.f.5	*Perenniporia fraxinea*	(Bull.) Ryv.	Via Borgo Calvenzano	Pavia (PV)	*Platanus x hispanica*	humid supratemperate - subhumid
P.f.6	*Perenniporia fraxinea*	(Bull.) Ryv.	Via Borgo Calvenzano	Pavia (PV)	*Platanus x hispanica*	humid supratemperate - subhumid
P.f.7	*Perenniporia fraxinea*	(Bull.) Ryv.	Bosco Giuseppe Negri	Pavia (PV)	*Populus nigra*	humid supratemperate - subhumid
P.f.8	*Perenniporia fraxinea*	(Bull.) Ryv.	Bosco Giuseppe Negri	Pavia (PV)	*Populus nigra*	humid supratemperate - subhumid
P.f.9	*Perenniporia fraxinea*	(Bull.) Ryv.	Cascina Venara	Zerbolò (PV)	*Populus alba*	humid supratemperate - subhumid
P.f.10	*Perenniporia fraxinea*	(Bull.) Ryv.	Pizzofreddo	Santa Maria della Versa (PV)	*Unidentified broadleaf*	mesomediterranean - humid thermotemperate - subhumid
P.f.11	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid

P.f.12	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.13	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.14	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.15	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.16	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.17	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.18	*Perenniporia fraxinea*	(Bull.) Ryv.	Parco della Vernavola	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.19	*Perenniporia fraxinea*	(Bull.) Ryv.	Via D. Alighieri 25	Illasi (VR)	*Olea europaea*	humid supratemperate - subhumid
P.f.20	*Perenniporia fraxinea*	(Bull.) Ryv.	via Mascherpa	Castelvetro Piacentino (PC)	*Populus alba*	humid supratemperate - subhumid
P.f.21	*Perenniporia fraxinea*	(Bull.) Ryv.	Lungolago	Mergozzo (VCO)	*Robinia pseudoacacia*	humid supratemperate/hyperhumid
P.f.22	*Perenniporia fraxinea*	(Bull.) Ryv.	Cascina Scova	Pavia (PV)	*Robinia pseudoacacia*	humid supratemperate - subhumid
P.f.23	*Perenniporia fraxinea*	(Bull.) Ryv.	RNIS Bosco Siro Negri	Zerbolò (PV)	*Quercus robur*	humid supratemperate - subhumid
P.f.24	*Perenniporia fraxinea*	(Bull.) Ryv.	RNIS Bosco Siro Negri	Zerbolò (PV)	*Populus nigra*	humid supratemperate - subhumid
P.f.25	*Perenniporia fraxinea*	(Bull.) Ryv.	Santa Sofia	Torre d'Isola (PV)	*Quercus robur*	humid supratemperate - subhumid
P.f.26	*Perenniporia fraxinea*	(Bull.) Ryv.	Cono di Volo Malpensa	Gallarate (VA)	*Unidentified broadleaf*	mesotemperate - humid supratemperate
P.f.27	*Perenniporia fraxinea*	(Bull.) Ryv.	Viale Gorizia - Mura Spagnole	Pavia (PV)	*Celtis australis*	humid supratemperate - subhumid
P.f.28	*Perenniporia fraxinea*	(Bull.) Ryv.	Fossone - Bosco della Fame	Rovescala (PV)	*Populus nigra*	humid supratemperate - subhumid

P.m.1	*Perenniporia meridionalis*	Decock and Stalpers	R.N. Regionale Piramidi di Zone	Zone (BS)	*Quercus robur*	hyperhumid supratemperate/humid
P.m.2	*Perenniporia meridionalis*	Decock and Stalpers	Sormano	Castellina in Chianti (SI)	*Olea europaea*	supratemperate/humid mesotemperate/hyperhumid
P.och.1	*Perenniporia ochroleuca*	(Berk.) Ryvarden	Belcaro	Siena (SI)	*Quercus ilex*	subhumid mesotemperate/humid
P.och.2	*Perenniporia ochroleuca*	(Berk.) Ryvarden	Le Manie	Savona (SV)	*Quercus ilex*	mesomediterranean/dry-subhumid thermomediterranean
Ph.c.1	*Phellinus contiguus*	(Pers.) Pat.	Rio Bardonezza	Ziano Piacentino (PC)	*Robinia pseudoacacia*	humid supratemperate - subhumid
Ph.tor.1	*Phellinus torulosus*	(Pers.) Bourdot and Galzin	Ticino	Torre d'Isola (PV)	*Prunus avium*	humid supratemperate - subhumid
Pl.e.1	*Pleurotus eryngii*	(DC.) Quél.	Aidomaggio re	Aidomaggiore (OR)	*unidentified*	subhumid mesomediterranean
Pl.e.2	*Pleurotus eryngii*	(DC.) Quél.	Spadafora	Spadafora (ME)	*unidentified*	thermomediterranean/subhumid mesomediterranean
Punc.s.1	*Punctularia strigosozonata*	(Schwein.) P.H.B. Talbot	Bosco di Bauli'	Palazzolo Acreide (SR)	*Quercus sp.*	mesomediterranean/dry thermomediterranean-subhumid
Sp.p.1	*Spongipellis pachyodon*	(Pers.) Kotl. and Pouzar	Rio Marsinola-Fracion	Rovescala (PV)	*Prunus avium*	humid supratemperate - subhumid
Sp.p.2	*Spongipellis pachyodon*	(Pers.) Kotl. and Pouzar	Civezza	Imperia (IM)	*Quercus pubescens*	mesomediterranean/dry thermomediterranean-subhumid

The species reported in Table 2 represent about one in five of the comprehensive collection of wood decay species in MicUNIPV. The temperate region and Mediterranean region in Italy are reciprocally intersected and several phytoclimates are represented based on both thermal–pluviometrical parameters and floristic–vegetational ones. According to our field observations, this has the consequence that several species can be found in different phytoclimates and on different hosts, whereas a minor fraction is strictly related to one or few hosts.

Daedaleopsis confragosa and *D. tricolor* are easily distinguished by morphology; nevertheless, ITS sequences are important to discriminate the species strain. *D. tricolor* seems more common in Central and Southern Europe; in Italy, it has been reported in seven out of 20 regions [45]. Our strain (MicUNIPV D.tric.1) comes from the lower Apennines in Pavia Province; other field observations suggest that *Prunus avium* is the favourite host of *D. tricolor* in North and Central Italy. The strain has not been characterized yet, although pharmacological effects have been reported [46].

Daedalea quercina has been reported in 11/20 Italian regions [45]; as expected, all MicUNIPV strains were isolated from *Quercus* spp. Nevertheless, strain MicUNIPV D.q.1 efficiently colonized poplar wood chips and confirmed that this species is a typical brown rot agent [47].

Despite being apparently cosmopolitan, *Desarmillaria tabescens* is strictly related to *Quercus* in warm climates, where it behaves as a secondary pathogen [48]. *D. tabescens* has been reported in 16/20 Italian Regions [45]. Accordingly, our strain was isolated from roots of *Q. robur* in RNIS Bosco Siro Negri (Pavia, Italy), which is a significant, unmanaged residue of typical forest of the western Po Plain.

Fomitopsis iberica is a rare species, reported in three Italian regions [45]. All the strains in MicUNIPV were isolated close to Varese lakes, either on broadleaves or conifers.

Ganoderma is represented in MicUNIPV by seven species: *G. adspersum*, *G. applanatum*, *G. carnosum*, *G. pfeifferi*, *G. lucidum*, *G. resinaceum* and *G. valesiacum*. This genus has been intensely studied due to its wide range of secondary metabolites, including several bioactive compounds [49]. According to Ryvarden and Melo [6], *Ganoderma* is one of the most difficult genera to identify at species level. As reported in Table 2, we obtained strains of *G. adspersum*, confirming that is a southern species in Europe [50]. *G. carnosum* is usually located in the *G. lucidum* complex due to its morphological similarity, despite it showing clear differences in host relationship. Our strain was isolated from its type-locality in Italy, i.e., a forest of *Abies alba*, that is likely to be its preferred host in South Europe [33]. Molecular identification by ITS region met difficulties in discriminating *G. carnosum* from *G. tsugae* and *G. oregonensis*; this topic presents questions about the real interspecific diversity within this conifer-related species in *Ganoderma*. Strains in pure culture will thus help us to investigate both the molecular and morphological nested diversity in this complex. An analogous problem concerns *G. pfeifferi,* as it partially shares its trophic niche with *G. lucidum* and *G. resinaceum*.

L. sulphureus is particularly related to *Quercus* according to our observations in North Italy, although *Castanea sativa* is also represented. Interestingly, *C. sativa* is also one of the favourite hosts of *Phellinus torulosus,* according to field observations, despite strain MicUNIPV Ph.tor.1 being isolated from *P. avium*.

Genus *Perenniporia* is represented in MicUNIPV by *P. fraxinea*, *P. meridionalis* and *P. ochroleuca*. According to our field observation, *P. fraxinea* is more common and widespread than expected, particularly in urban areas. We have focused our attention in indentifying strains, which, to date, number 27.

Some of the isolated strains have been used for population studies and tests on heavy metal bioaccumulation [16,51].

Pleurotus eryngii is a typically Mediterranean species as well as its herbaceous hosts in *Apiaceae*. Consistently, the MicUNIPV Pl.e.1 and Pl.e.2 strains were isolated in properly Mediterranean areas (Sardinia and Sicily).

Punctularia strigosozonata is a rare, poorly studied species, typically related to the Mediterranean area; its resupinate morphology increases the difficulty in achieving pure isolates.

Spongipellis pachyodon has a mainly central–southern distribution in Europe; according to Onofri et al. [45], in Italy it is known in five out of 20 Regions, not including either Lombardy or Liguria. This species is reported as uncommon but locally abundant; regarding this, our field observations suggest that the population in the Pavia-Piacenza Apennines is particularly related to *P. avium*.

Further species listed in Table 2 are reported below in more detail owing to their taxonomic controversy or potential applications.

3.1. Cellulariella warnieri (Durieu and Mont.) Zmitr. and V. Malysheva

3.1.1. Background

As detailed in Table 2, the basidiomata of some species were collected in the Mediterranean area and others that are known to prefer warm environment, even if they were collected in continental or temperate zones. An example is *Cellulariella warnieri*, a poorly investigated species related to warm climates, according to Bernicchia and Gorjón [44]; despite not strictly being related to the Mediterranean region, Ryvarden and Melo [6] reported it as a southern and rare species.

The notable scarcity of data about this species has probably contributed to its uncertain systematic and taxonomic status. Currently it is reported as: *Lenzites warnieri* Durieu and Mont. by Mycobank [40], *C. warnieri* by Index Fungorum [52] and *Trametes warnieri* (Durieu and Mont.) Zmitr., Wasser and Ezhov by Ryvarden and Melo [6]. The latter indication is suggested also by Justo and Hibbett [53] based on a five marker-based phylogenetic classification of *Trametes*. Significantly, only 108 records for this species have been reported by the GBIF (Global Biodiversity Information Facility) [54] and only 10 sequences are available in GenBank, almost half of them being critical as they are reported from South East Asia [55]. Further analyses on a more representative number of strains are thus needed to clarify the position of *C. warnieri*.

Strain MicUNIPV L.w.1 was tested for the evaluation of lignocellulolytic activity and resulted in a very low production of Mn peroxydase and lack of lignin peroxydase, whereas cellulase and hemicellulase had the highest presentation among the species under examination [47]. This was also confirmed when testing the effect of its colonization on *Medicago sativa* for pre-treatment, as cellulose and hemicellulose were preferentially removed [56].

3.1.2. MicUNIPV WDF Strains Results

According to our field observations, localities are distributed as small local clusters which are very scattered in turn. Thus, the strains MicUNIPV L.w.1, L.w.4 and L.w.5 were collected from Po plain areas (Pavia and RNIS Bosco Siro Negri), whereas strains the MicUNIPV L.w.2, L.w.3 and L.w.6 were collected from hill area (Oltrepo Pavese).

The six strains of MicUNIPV were collected from *Quercus*, *Ulmus*, *Populus* and *Robinia*; interestingly, our field observations pointed out some preference for *Ulmus*, which was not previously reported as a host in Italy. As expected, all the strains were isolated from individuals behaving as saprotrophs. It should be noted that, since *C. warnieri* develops basidiomata in late autumn but it releases spores in spring, the basidioma itself remains vital even at low temperatures and under the snow.

3.2. Dichomitus squalens (P. Karst.) D.A. Reid

3.2.1. Background

Dichomitus squalens is a model species for studies about the selectivity of white rot and its enzymatic basis [57–60]. Despite being reported all throughout the boreal emisphere, it appears scattered and is commonly found in the northern parts of Europe, North America and Asia [61]; the GBIF [54] places the wide majority of records in the Fennoscandian region. The host relationship is apparently controversial and surprising: Ryvarden and Melo [6] assumed *Pinus* as the only European host species, whereas Bernicchia and Gorjón [44] recorded *Picea abies* for the Italian sample and Niemelä [62] assigned most samples to *Pinus* and a smaller fraction to *Picea abies* in Białowieża Forest (Poland / Belarus). Nevertheless, it should be noted that American samples have been reported on six different genera in *Pinaceae*. Furthermore, young basidiomata of *D. squalens* are easily misidentified due to the close morphological resemblance with *Neoantrodia serialis* (Fr.) Audet and related species. Consistently, a remarkable intraspecific variability in growth and enzyme profiles was revealed by testing different monokarya strains [61]. This is also consistent with the numerous mating types deriving from tetrapolarity [61].

3.2.2. MicUNIPV WDF Strains Results

Strains MicUNIPV D.sq.1 and MicUNIPV D.sq.2 were recovered from *Pinus pinea* and *Cedrus* sp. respectively near the Adriatic Sea and Varese lake. The Italian strains have not yet been investigated for their enzymatic properties, so they may provide an additional tool to explore the diversity in degradation potential of this selective decayer.

3.3. Hericium erinaceus (Bull.) Pers.

3.3.1. Background

Hericium erinaceus (Bull.) Pers. is one of the most famous cultivated medicinal species in the world; a wide range of peculiar compounds, both related to primary (e.g., β-glucans) and secondary metabolism (e.g., erinacines and hericenones) have been up to now characterized and screened for bioactivity [7,63–66]. GBIF [54] places the wide majority of *H. erinaceus* sites in Europe, North America and North Eastern Asia. According to the phylogenetic study by Cesaroni et al. [67], a subclade containing European and American ITS sequences is well distinguished from the Asian clade. Despite relying on ITS region only, these data suggest the possibility to differentiate *H. erinaceus* strains also by the phylogeographic structure. Notwithstanding the scarcity of available data for Asian samples, *H. erinaceus* apparently has a quite broad trophic niche including several host species in *Fagaceae,* and *Aceraceae* to lesser extent, and particularly showing a preference for *Quercus* all throughout its distribution area [37]. Consistently, European samples have mainly been recovered from *Quercus* and *Fagus*, the former likely being the exclusive host in Italy and the only known host in North Africa [34,68]. Strain MicUNIPV H.e.2 was analyzed for the production of erinacine A and hericenes (presumably A, B, C, D). Thus, a complete quali-quantitative comparison of these selected metabolites was provided throughout different growth stages but within the same strain, which is a powerful tool for the standardization of bioactive products [69].

Strain MicUNIPV H.e.1 was selected to test the effect of oral supplementation on mice memory. The results indicate an improvement in recognition memory and induction of hyppocampal and cerebellar neurogenesis during aging. This strain has therefore contributed to pointing out which areas are directly involved in the neuroactivity of *H. erinaceus* compounds, highlighting which type of memory is increased [70].

3.3.2. MicUNIPV WDF Strains Results

Accordingly, all four strains in the MicUNIPV collection were recovered in the municipality of Siena from *Q. ilex*, that is, a featuring species in the flora of Mediterranean area often forming homogeneous woodlands. It is noteworthy that the Mediterranean basin hosts a great variety of *Quercus* species, whose phylogenetic and systematic relationships are still controversial, with particular concern to the *Q. ilex* group [71–73].

3.4. Inocutis tamaricis (Pat.) Fiasson and Niemelä

3.4.1. Background

The relationship of *Inocutis tamaricis* with *Tamarix* is apparently so strict to be regarded as a discriminant character in identification [6,33,44]. Although the genus *Tamarix* consists of 72 accepted species in Europe, Asia and Africa [74], *I. tamaricis* is restricted to the Mediterranean basin and Macaronesia. Here, it grows on different *Tamarix* species according to their availability but shows a preference for *T. gallica* [75,76]. Consistently, strains MicUNIPV I.t.1 and I.t.2 were both isolated from *T. gallica.* As a whole, the intra-familiar phylogeny of *Hymenochaetaceae* is still to be clarified; multiple revisions have tried to point out nested diversity within polyphyletic taxa, such as *Inonotus* [77,78]. Thus, the genus *Inocutis* is nowadays accepted to be distinct from *Inonotus* itself, as formerly suggested by Fiasson and Niemelä [790]. Interestingly, the type-species for *Inocutis* is *I. rheades* (Pers.) Fiasson and Niemelä, which is morphologically very similar to *I. tamaricis* and is mostly distinguished by host and distribution [6]. Thus, *I. tamaricis* may be regarded as the Mediterranean counterpart of *I. rheades*. As a whole, only 12 sequences have been up to now deposited in GenBank [55] as belonging to this species, some of which are lacking data to assess their effective reliability. Further sequences from the Mediterranean area, equipped with information about host and geographic origin, are needed to support studies about intrageneric diversity in *Inocutis* in the light of biogeographic patterns.

3.4.2. MicUNIPV WDF Strains Results

Strains MicUNIPV I.t.1 and I.t.2 were both isolated from *T. gallica*, forming in both cases ornamental rows along the sea coast.

3.5. Fomitiporia mediterranea M. Fisch.

3.5.1. Background

As mentioned for *Inonotus*, the genus *Phellinus* is increasingly revealing its hidden diversity; recognized as being polyphyletic, several species have been distributed into other genera, such as *Fomitiporia* Murrill. *Fomitiporia mediterranea* is a peculiar example due to its morphology, being actually indistinguishable from *P. punctatus*. According to Fischer [80], these two species also show differences in growth rate at selected temperatures and mating behaviour. The same study provides strong evidence for dichotomy in host selection by *F. mediterranea* depending on biogeography, i.e., this species grows on several tree species in Italy [81,82], whereas north of the Alps it apparently grows on *Vitis vinifera* exclusively [83,84,].

It should be considered that misidentification with *P. punctatus* has probably led to the underestimation of *F. mediterranea* in the Mediterranean area [85]. Analogously, Polemis et al. [86] suggested that the relationship with *P. pseudopunctatus* A. David, Dequatre and Fiasson should be reconsidered as well, enclosing the latter in *F. mediterranea* clade. It may be observed that the *P. pseudopunctatus* is apparently more related to the South Mediterranean region and climates [87,88]. Further analyses on strains from different geographic origins and hosts are thus needed to clarify both phylogenetic relationships and biogeographic patterns. As a whole, this species complex is characterized by intense necrotrophic white rot; *F. mediterranea* in particular is regarded as one of

the main agents responsible for wood rot in *V. vinifera*, *Corylus avellana* and *Olea europaea* [82,83,85].

3.5.2. MicUNIPV WDF Strains Results

All of the 17 Italian strains up to now attained by the Laboratory of Mycology DSTA–University of Pavia were assigned to *F. mediterranea* instead of *P. punctatus* and recovered from different substrates in North Italy. Even within one province (Pavia), *F. mediterranea* was located on five hosts, namely *Q. robur, Hedera helix* (State Natural Strict Reserve Bosco Siro Negri), *Salix alba* (University of Pavia courtyard), *R. pseudocacacia* and *V. vinifera* (Oltrepo Pavese hills).

The identified strains thus provide a tool to deepen pathology dynamics and different susceptibility depending on host species and cultivar.

3.6. Perenniporia meridionalis Decock and Stalpers

3.6.1. Background

Genus *Perenniporia* Murrill sensu lato is large, cosmopolitan and supposed to be polyphyletic, and thus is in need of further phylogenetic analysis based on sequences from different species [6]. In turn, an example of intrageneric complexity is provided by *P. meridionalis*, within *P. medulla-panis* (Jacq.) Donk group. Actually, the complete revision by Decock and Stalpers [89] arose the doubt that several records, as well as specimens in herbaria, are to be referred to *P. meridionalis* instead of *P. medulla-panis* (or closely related species), particularly when coming from the Mediterranean area. Up to now, only a partial and fragmentary investigation into the intra-generic diversity in *Perenniporia* has been carried out from a molecular and phylogenetic point of view [90]. It is noteworthy that no sequences at all are available in GenBank by the name of *P. meridionalis*, whereas 40 sequences are referred to *P. medulla-panis* [55]. Strains from culture collections are thus needed as basic material for this purpose. According to the indications by both Bernicchia and Gorjón [44], as well as Ryvarden and Melo [6], *P. meridionalis* is particularly related to *Quercus* (more than *P. medulla-panis*), the holotype having been isolated from *Q. ilex* in Sardinia [62,89].

3.6.2. MicUNIPV WDF Strains Results

Strains MicUNIPV P.m.1 and P.m.2 strains were respectively isolated from *Q. robur* (North Italy, near a lake) and *Q. ilex* (Central Italy). Besides considerations on biodiversity, *P. meridionalis* has a great applicative potential. MicUNIPV P.m.1 showed remarkable selectivity as a white rot agent and versatility when inoculated onto unusual substrates such as grass. The selective removal of lignin by this species contemporarily relies on high activity for Mn peroxidases and very low for one cellulase; the final delignification in the substrate is clear both in thermogravimetric analysis and FTIR spectroscopy [12,47].

3.7. Perenniporia ochroleuca (Berk.) Ryvarden

3.7.1. Background

Perenniporia ochroleuca is another example of the unsolved intra-generic diversity within *Perenniporia*. This species is suspected to hide a complex, and transfer to *Truncospora* Pilát ex Pilát has thus been suggested [91]. According to the same authors, the Iberian/Macaronesian clade gives *T. atlantica* Spirin and Vlasák, whereas the status of Australian samples is more uncertain, which would mainly belong to *T. ochroleuca*. Nevertheless, the new taxonomy has not yet been fully accepted, neither by Mycobank [40] nor by Index Fungorum [52]. These hypotheses therefore need to be supported by entering further sequences into the phylogenetic analyses from an exhaustive geographic range. *P. ochroleuca* was reported by Bernicchia and Gorjón [44] and Ryvarden and Melo

[6] as tropical and growing on several hosts, whereas in Europe it is particularly related to the Mediterranean area. Nevertheless, Bernicchia and Gorjón [44] report a range of typically Mediterranean hosts, whereas Ryvarden and Melo [6] also include host plants whose distribution exceeds the Mediterranean area to include samples from the coasts of South England and Wales. Further phylogenetic analyses focused on the Mediterranean region versus the adjacent Atlantic ones are needed to test the monophyly of the proposed *T. atlantica*.

3.7.2. MicUNIPV WDF Strains Results

Both strains MicUNIPV P.och.1 and P.och.2 were isolated from *Q. ilex* in Central Italy and the Ligurian west coast, respectively.

4. Conclusions

At the moment, MicUNIPV, the fungal research culture collection of University of Pavia (Italy), maintains 500 strains from wood decay species. Examples particularly correlated to the Mediterranean area were discussed and their roles in accomplished research were mentioned in this study.

Culture collections of wood decay fungi are an important tool both for systematic and applied studies. Strains in pure culture are more easily and reliably identified and analyzed for metabolic activities and competitivity. The environmental features of the strain origin place have often been underestimated; nevertheless, the diversity of wood decay fungi strongly depends on biogeography and is related to host distribution. This also highlights the need for an investigation including a wider concept of the Mediterranean region than one strictly limited by climate or phytoclimate classification, i.e., even continental regions surrounding the Mediterranean area contribute to the explanation of Mediterranean diversity.

The Laboratory of Mycology DSTA–University of Pavia (Italy) has up to now successfully collaborated with both researchers from other universities and amateurs in order to increase the diversity richness and geographic origin range of strains, as well as to enter these strains in original pure and applied research such as MATER and CE4WE (grants from Cariplo Foundation and Regione Lombardia).

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Chapter 2. Characterization and selection of most suitable fungal strains not yet investigated to produce myco-materials

Starting from the second PhD year, it was possible to make a first selection based on the mycelium texture (evaluated through SEM) and on the main components of the cell wall (evaluated through TGA).

Characterization is recommended in order to find guidelines and indications to correlate strain or species characteristic features to the corresponding resulting materials.

Furthermore, the aim was also to identify one or more mycelial or cultural characteristics to be used as indicators for future applications so that the production of myco-materials does not take place by trial and error but following certain criteria.

Hereafter the part of the graphical abstract that represents this phase of the work reported also in Cartabia et al. (2021) are schematized in Fig. 3.

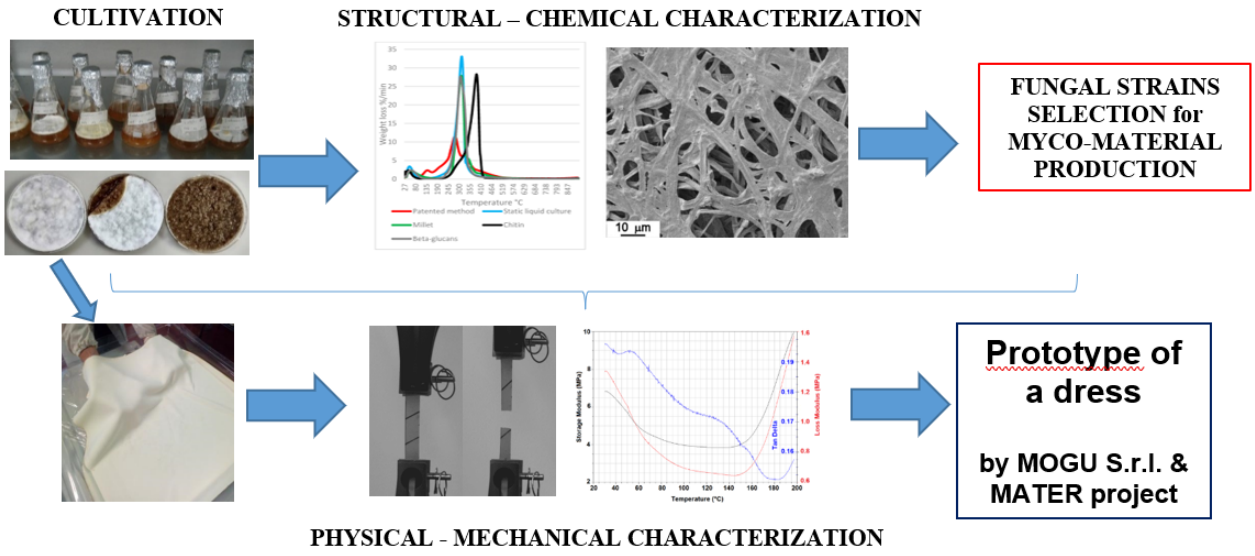

Fig. 3: Activities involved in the second part of my PhD project.

At first, growth rates (Petri dishes with Malt extract agar) and mycelium colour, stiffness and homogeneity as well, were the criteria used to make a first choice within the strains to be tested. Twenty-one best performing strains (belonging to twenty different species) were chosen.

As shown in the graphical abstract, two cultural method were compared: liquid cultures (malt extract, dextrose and yeast extract) and a method patented by MOGU that can be considered a modification of the spawn production. This method allows to produce mats which can then be treated to produce

leather-like fungal materials. Mechanical tests proved essential to improve the quality of the material to be produced as a prototype. Actually, this phase was followed by researchers within MOGU company and by the partner engineers of the MATER project.

Afterwards, different aspects had been considered and evaluated: mycelium chemical composition and mycelial structure regarding both hyphal dimensions and hyphal organization in space. The analysis techniques used for these preliminary investigations were Thermal-Gravimetric Analysis (TGA) for chemical composition and Scanning Electron Microscopy (SEM) for mycelial texture. These tools were also useful to deeply understand the nature of the resulting materials after treatment steps both from a chemical and a structural point of view.
TGA and SEM laid the groundwork for the next material production steps and they allowed to select the most adapt strains for mycelium mats and the most differentiated strains in terms of α-glucan, β-glucan and chitin content.

Despite this, it was not possible to define precise criteria valid for all strains and for the cultural conditions tested.

Besides, three fungal strains (*Abortiporus biennis* 064-18, *Irpex lacteus* 076-18 and *Fomitopsis iberica* 104-19) were selected out of this list of 21 strains with regard to the highest productivity yields (50-92 g/m^2) and the best mechanical performances compared to leather-like materials. In fact, the pure mycelium materials obtained with those strains endured a tensile strength of 0,195-0,373 MPa, presented a Young's modulus of 6,366-10,813 MPa and an elongation at break of 2,25-10,88 % (Fig. 4). Their mechanical properties could be improved in the future by different physical (e.g. pressing, coupling) and chemical post treatment steps (e.g. cross-linking) and further research is being done in this direction.

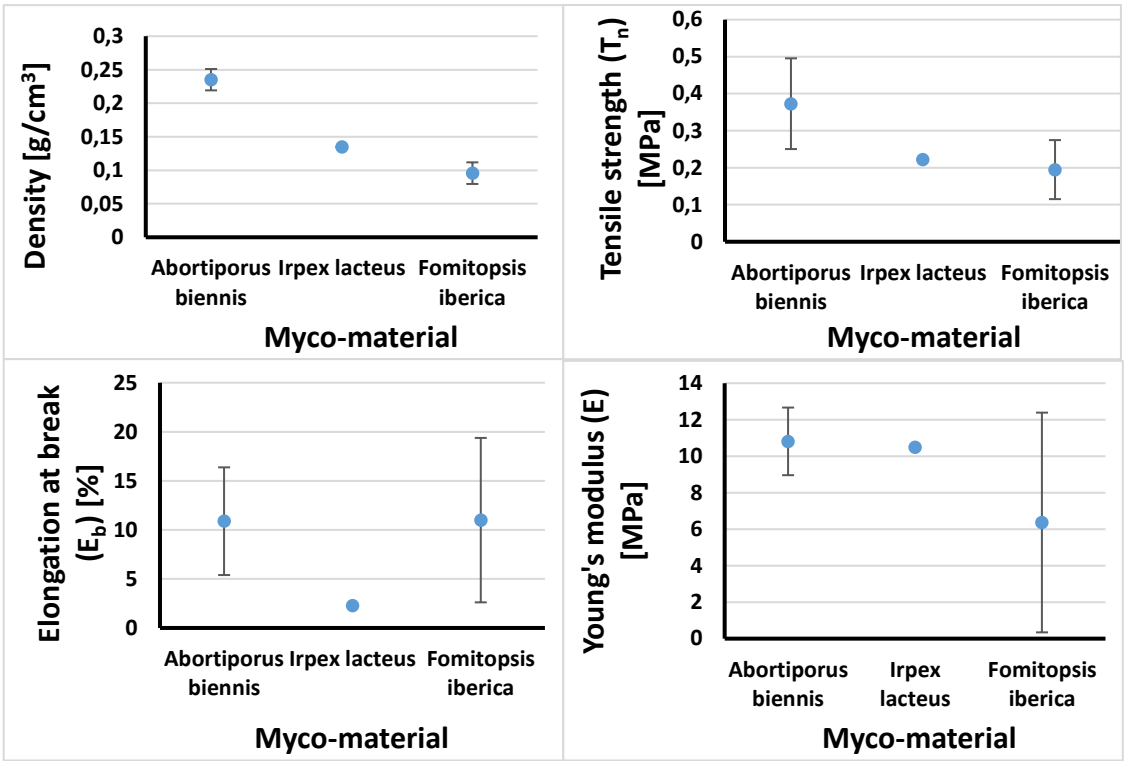

Fig. 4: Average ± standard deviation of density (g/cm^3), tensile strength (MPa), Elongation at break (%) and Young's modulus (MPa) of the selected and tested strains.

In particular, the brittleness of *Irpex lacteus* 076-18 material mats made it impossible to produce more than one single specimen for testing. For the other two typologies, six samples each were tested. Specimens have rectangular shape (10 x 110 mm) (Fig. 5) and were cut out of mycelium mats of 170 x 170 mm (Fig. 6). Before testing, all the specimens were measured with a digital calliper and weighted in order to calculate density.

Tensile strength test showed that *Abortiporus biennis* 064-18 produced the most dense and the most resistant mycelium mats.

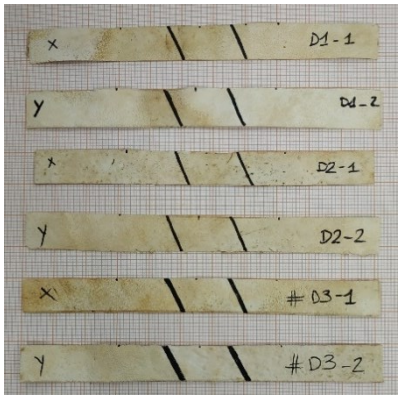

Fig. 5: Cut specimens from mycelium mats of *A. biennis* 064-18.

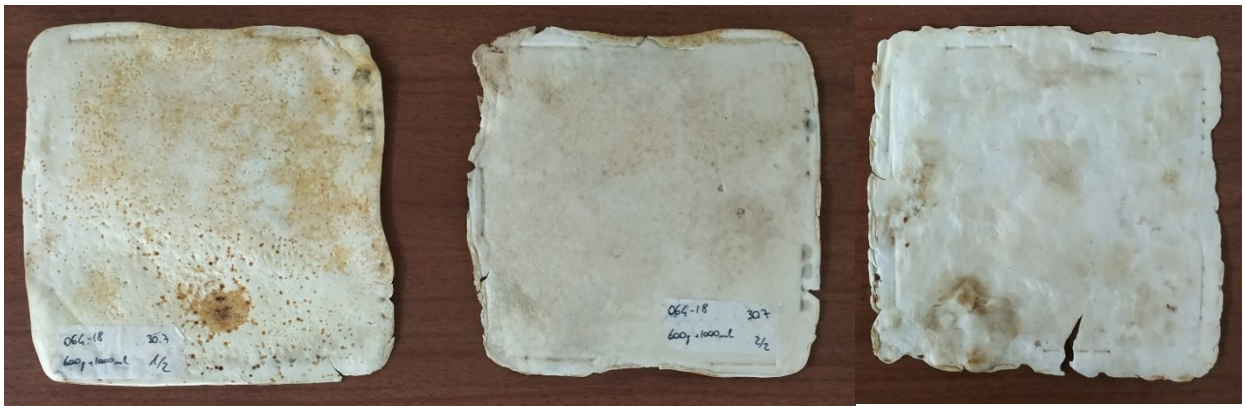
Fig. 6: Mycelium mats of *A. biennis* 064-18 obtained

This preliminary study confirmed the great potential of fungi-derived leather-like materials as an eco-friendly alternative to current leathers and lays the groundwork to further application and research in myco-materials.

These data were the object of a specific oral presentation entitled “Isolation of lignicolous fungal strains and selection for myco-material production” held by the author of this doctorate, into the ISMS e-congress 2021.

At the end, a prototype of a dress (Fig. 7) was developed by MOGU S.r.l. and researcher engineers participating in the MATER project, but this part is outside the present PhD work.


Fig. 7: Dress prototype produced.

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Collection and Characterization of Wood Decay Fungal Strains for Developing Pure Mycelium Mats

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Abstract: Wood decay fungi (WDF) seem to be particularly suitable for developing myco-materials due to their mycelial texture, ease of cultivation, and lack of sporification. This study focused on a collection of WDF strains that were later used to develop mycelium mats of leather-like materials. Twenty-one WDF strains were chosen based on the color, homogeneity, and consistency of the mycelia. The growth rate of each strain was measured. To improve the consistency and thickness of the mats, an exclusive method (newly patented) was developed. The obtained materials and the corresponding pure mycelia grown in liquid culture were analyzed by both thermogravimetric analysis (TGA) and scanning electron microscopy (SEM) to evaluate the principal components and texture. TGA provided a semi-quantitative indication on the mycelia and mat composition, but it was hardly able to discriminate differences in the production process (liquid culture versus patented method). SEM provided keen insight on the mycelial microstructure as well as that of the mat without considering the composition; however, it was able to determine the hyphae and porosity dimensions. Although not exhaustive, TGA and SEM are complementary methods that can be used to characterize fungal strains based on their desirable features for various applications in bio-based materials. Taking all of the results into account, the *Fomitopsis iberica* strain seems to be the most suitable for the development of leather-like materials.

Keywords: wood decay fungi (WDF); mycelium-based material; fungal strain collection; scanning electron microscopy (SEM); thermogravimetric analysis (TGA)

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1. Introduction

Wood decay fungi (WDF) have the fundamental role of degrading the components of plant cell wall (cellulose, hemicellulose, and lignin), displaying different selectivity towards each one depending on the species, developmental stage, and environmental conditions [1,2]. Wood decay species in Basidiomycota (WDB) report the greatest spectrum of degradation patterns, which are either enzyme-based or just enzyme-primed. Moreover, many WDB are easily isolated

in their dykariotic stage, which enables the mycelium to develop certain morphological features similar to those of the basidiome in culture [3,4].

More and more WDF are being studied to evaluate their potential applications in different fields: pharmacology, medicine and nutraceutics, enzyme production, cosmetics, and the biosorption and biodegradation of persistent organic pollutants [5–10]. Another emerging application that involves the use of WDF concerns bio-based materials. Increasing attention has been paid to this specific aspect of WDF all around the world in recent decades. In fact, plastic pollution is quickly becoming one of the most critical global problems in the modern world [11–13]. Plastic polymers are very versatile materials that cannot be totally eliminated. On the other hand, research into alternative sources that are able to create similar materials is a complementary strategy for the replacement of traditional plastics in cases where they are not indispensable.

Fungi are promising candidates thanks to the natural structure of their network, which is formed by the hyphae that are organized in the mycelium [14–17]. Mycelium technology has been attracting increasing attention in material sciences and in the design sector because fungal materials can be used for thermal/acoustic insulation and packaging, and in the design sector, they can replace already existing and polluting materials or can provide novel materials.

Currently, two main families of mycelium-based materials (myco-materials) are available: bio-composites and pure materials. In any case, the properties of myco-materials depend on the substrate, fungal species, and growth conditions. Up until to now, only few species of WDF have been tested as bio-fabricated materials, but hundreds of potentially suitable species grow in the world as well as in Italy [18,19]. Most of them have not been tested yet [16].

Mycelium-based composites result from the growth of filamentous fungi on organic materials such as agricultural waste streams. These novel bio-based materials represent a promising alternative for product design and manufacturing both in terms of sustainable processes and circular lifespan [20,21].

Pure fungal materials mainly consist of mycelium [20]. These materials are either the result of the complete degradation of the substrate or can be obtained by removing the fungal mat from the surface of a liquid or solid substrate [22].

The properties of myco-materials depend on the substrate, fungal species (i.e., fungal strategy), and growth conditions.

In recent years, the textile and fashion design industries have placed a great deal of emphasis on the development of new sustainable and compostable materials that are able to be obtained using environmentally friendly, non-pollutant processes. The production of fungal mats for textile applications promises more sustainable and compostable alternatives to the soft materials that are currently on the market, especially those from the leather sector [22,23].

Leather production has been increasingly raising ethical issues about animal welfare as well as the negative impact of leather production on the environment. The leather industry consumes substantial amounts of natural resources and uses toxic, persistent chemicals that are used for the processing of the skins [24,25]. Leather-like materials are currently made of polyvinyl chloride (PVC) and polyurethane (PU), but they are of environmental concern, at least in terms of their production/degradation processes. To produce animal-free and sustainable soft materials with leather-like properties, materials based on renewable feedstock have been proposed as alternatives to cotton or petroleum-based fibres; examples include pineapple cellulose (Piñatex), grape pomace (Vegeatex), orange fibre, and palm leaf products.

The aim of this study is to collect wood decay fungal strains that are suitable for the development of mycelium mats that are able to be used as a leather-like fashion design prototype. In the present research, differences in the texture and

cell wall components of different mycelium species have been investigated through scanning electron microscopy (SEM) and thermogravimetric analysis (TGA) in order to identify certain possible functional characteristics of fungal strains that can be used as myco-materials.

Both SEM and TGA are increasingly being applied in various contexts and have proven to be useful tools that can be used to understand t the morphology and the essential components of a material, regardless of whether that material is artificial or biogenic [26,27]. Thermogravimetric analysis is a characterization technique that also allows the investigation of plant biomass constituents. It consists of a pyrolysis or combustion process that records mass losses as a function of temperature [28,29]. Pyrolysis enables the molecular decomposition of an organic material by heating and breaking chemical bonds, leading to the formation of simpler molecules. [30]. According to a temperature gradient, heat is applied up to a maximum of 700–900 °C in the presence of an inert gas [31]. Popescu et al. (2010) [32] used this technique to study the physical properties of wood by obtaining information on the interaction between cellulose, lignin, and hemicellulose. TGA has already been used to characterize the major constituents of the fungal cell wall (in particular β-glucans and chitin) [26].

2. Material and Methods

2.1. Collection of Wood Decay Fungal Strains

Many wooded areas in the north of Italy (Lombardia and Piemonte Regions) have been examined for samples that are suitable for the isolation of WDF strains to be taken. Different habitats (moorland, thermophilous broadleaf forest, riparian forest, mixed coniferous forest, ancient larch forest, and Mediterranean scrub) were explored and only basidiome that were actively growing were collected.

Basidiome identification was performed through dichotomous keys based on macro and micro-morphology [33,34]. The taxonomy check relied on Mycobank (www.mycobank.org; accessed 13/11/2021). Whenever possible, specimens were added to the *Herbarium Universitatis Ticinensis* (DSTA, University of Pavia, Italy) or to the *herbarium* of Associazione Micologica Bresadola (AMB)—Varese section (Italy).

The standard protocol for mycelium isolation from the wild basidiomes [3,4,35] was slightly modified to the following procedure: To avoid breaking the basidiomes, only little pieces were collected from each sample. When the basidiome was too thin or small or compromised, pieces of wood that had been colonized by the fungus were collected. Subsequently, they were put in an aluminium sheet and were closed in a plastic box to allow a slightly humid environment suitable for the mycelium regrowth was established for few days. Then, three to six little portions of fresh and growing mycelium were taken from the basidiome under sterile conditions and were placed into a Petri plate containing 2% malt extract agar (MEA) (Biokar diagnostics, Allonne, France and VWR Chemicals, Milano, Italy) + 6 mL/L H_2O_2 (3%) [36]. When the colony reached a proper size (around 2 cm of diameter), a piece was transferred to an MEA 2% plate.

To maintain each strain in pure culture, 20 paper filter discs (5 mm diameter) were cut, sterilized, and disposed circularly into a 2% MEA plate. A plug of actively growing mycelium was put in the center, and after 7–15 days, the mycelium had spread to reach all of the paper discs [37]. For each strain, four discs were put in a sterilized 1.8 mL autoclavable polypropylene (PP) vial tube together with 1 mL of demineralized and sterilized water, and the tube was then sealed with parafilm. A minimum of five tubes per strain were stored in the dark at 4 °C, forming the MOGU S.r.l. (MOGU's Fungal Strain Collection—MFSC)

fungal research collection. Moreover, each WDF strain was maintained through various means, including storage at −80 °C in MicUNIPV, the Research Culture Collection of University of Pavia (Italy). The protocol for cryopreservation proposed by Homolka et al. 2014 was followed, with a few slight modifications [38]. After reaching optimal growth in liquid culture (2% ME), each mycelium was transferred to a 10 mL tube containing a 15% glycerol solution. The solution was homogenized through vortexing for 30 s at 3000 rpm. Then, 1 mL of the suspension was placed in 1.5 mL sterile cryotubes. For each fungal strain, four copies were stored at −80 °C.

2.2. Molecular Identification

Before including the strains in a database, their specific identification was confirmed by sequencing the ITS rDNA, which is generally recognized to be the major barcoding region for fungi [39,40]. Fungal strains were grown in flasks with 50 mL 2% malt extract and were incubated at 24 °C for 15 days in the dark in static. The mycelium was harvested and lyophilized in order to favor sample homogenization, as indicated by the kit manufacturer instructions for DNA extraction. DNA extraction was performed using a Macherey-Nagel Nucleospin plant II extraction kit. The ITS region was amplified using ITS1-ITS4 primers and a Dream Taq Mastermix (Promega, Milano, Italy).

In order to verify the DNA extraction and DNA amplification results, an electrophoretic run (75 V for 5 min, 100 V for 25 min) was performed on 1% agarose gel. SYBR Safe-DNA Gel Stain (Invitrogen, Waltham, MA, USA) was used as an intercalant, and BlueJuice (Invitrogen) was used as a gel loading buffer. Electrophoresis images were generated by Gel Doc (Biorad, Berkeley, CA, USA).

The purification of the post-PCR products was performed by adding ExoSAP-IT™ (Applied Biosystems, Foster City, CA, USA) in the following ratio: 5 μL of a post-PCR reaction product/2 μL of ExoSAP-IT™. The thermal protocol (Thermofisher, Waltham, MA, USA) included 15 min at 37 °C to degrade the remaining primers and nucleotides followed by 15 min at 80 °C to deactivate the ExoSAP-IT™ reagent.

DNA sequencing was performed by Macrogen Europe B.V. (Amsterdam, The Netherlands). The sequences were analyzed with the Sequencher 5.0 Demo software, and they were then matched with the sequences selected in different open repositories by means of MycoBank Molecular ID [41] and NCBI Nucleotide BLAST [42].

2.3. Choice of Fungal Strains

Among the strains that were successfully isolated, 21 strains (belonging to 20 species) were chosen based on the characteristics of each mycelium (Table 1). Most strains are well known for enzyme production and were less known for their application in bio-based materials. Species such as *Fomitopsis iberica*, *Neofavolus alveolaris*, and *Terana caerulea* not known for either enzyme production or for use in bio-based materials. The culture characteristics that were observed for every species were the color (white to cream or yellowish to brown or multicolor), the colony reverse (unchanged, bleached, darker), the general aspects of the mycelia (consistency of the mat), the behavior of the aerial and submerged hyphae, the presence of primordia or exudates (if any), and any smell in the event that it significantly characterized the mat production process.

Methods and terminology for the observation and description of mycelial features were retrieved from the literature and are reported in Table 1.

Table 1. WDF chosen for developing mycelium mats.

Strain Code	Fungal Species	Mycelium Characteristics
1	*Abortiporus biennis* (Bull.) Singer	Colony white to cream, some felty parts; aerial hyphae often resembling skeletal one, submerged hyphae up to 7.5 µm wide. Primordia of sporophores (none of which reaches maturity) are easily formed [3].
2	*Bjerkandera adusta* (Willd.) P. Karst.	Mat white; at first silky becoming cottony-woolly, woolly floccose; advancing zone raised, reaching the lid of the Petri dish [3,43].
3	*Coriolopsis gallica* (Fr.) Ryvarden	Mat white mat at the beginning of its growth, then becomes olive-brownish and thin and crusty after 4–5 days. Aereal hyphae pigmented. Reverse creamed. Appressed to the margin, farinaceous to felty [3].
4	*Coriolopsis gallica* (Fr.) Ryvarden	
5	*Coriolopsis trogii* (Berk.) Domanski	Colony white to cream; marginal hyphae appressed, mat downy to felty; thin and almost transparent. It becomes fluffy and inconsistent [3].
6	*Daedaleopsis confragosa* (Bolton) J. Schröt.	Colony white darkening with age, presence of crustose areas hazel, "pinkish cinnamon" or "snuff brown"; mat downy to fine woolly, becoming felty; reverse darkened [3,43,44].
7	*Daedaleopsis tricolor* (Bull.) Bondartsev & Singer	Colony white, dense, downy—felty, homogeneous when young. After a few weeks of incubation, several primordia of sporophores (none of which reaches maturity) are formed [44].
8	*Fomes fomentarius* (L.) Fr.	Colony wthite to cream to chamois, reverse darker. Advancing zone raised with aerial mycelium uniform. Mat downy to cottony or woolly, then appressed and felty, relatively homogeneous. When it reaches maturity, the mycelium forms brown crusty leathery areas [3,43,44]
9	*Fomitiporia mediterranea* M. Fisch.	Mycelial cultures are cottony to woolly, with aerial hyphae that are yellowish to brownish; mat characterized by a sparse development of aerial hyphae that easily reach the lid of the Petri dish [45].
10	*Fomitopsis iberica* Melo & Ryvarden	References not available; see results 3.1
11	*Fomitopsis pinicola* (Sw.) P. Karst.	Mat white, heterogeneous, at first raised, cottony and woolly; usually uniform in appearance, sometimes forming scattered dots of more compact mycelium [3,43,44].
12	*Ganoderma carnosum* Pat.	References not available; see results 3.1
13	*Ganoderma lucidum* (Curtis) P. Karst.	Mat white with darker (brownish) zones, concentrically arranged, appressed, and powdery near the point of inoculation. Distal zone is ± homogeneous and felty, with small hyphal clusters [46]
14	*Irpex lacteus* (Fr.) Fr.	Mat white, downy to cottony and woolly floccose. Reverse bleached. Advanced zone raised. Some aerial hyphae with thickened walls [3].
15	*Irpiciporus pachyodon* (Pers.) Kotl. & Pouzar	Mat white, downy to cottony to felty with sometimes scattered dots of more compact mycelium. Aerial hyphae easily reaching the lid of the Petri dish [3].
16	*Lenzites betulinus* (L.) Pilát	Mat at first floccose and woolly, beoming patchy, with some areas raised, felty-woolly;intervening areas appressed, thin felty [47]
17	*Neofavolus alveolaris* (DC.) Sotome & T. Hatt.	Colony white to cream. Mat thick, dense and homogeneous at the beginning, then fluffy and woolly; also develops on the edges of the plate. Skeletal hyphae [3,44]

18	*Stereum hirsutum* (Willd.) Pers.	Cream to yellow to light brown with dark brown areas; mycelium heterogeneous, downy to felty areas alternating with translucent ones. The advancing zone is irregular, sinuous [3,44]
19	*Terana caerulea* (Schrad. ex Lam.) Kuntze	References not available; see results 3.1
20	*Trametes hirsuta* (Wulfen) Lloyd	Mat cottony to cottony and woolly and becoming (sub)felty and sometimes farinaceous, white to cream. Advancing zone appressed to raised. Reverse bleached. Most of skeletal hyphae are branched [3].
21	*Trametes suaveolens* (L.) Fr.	Mat white at first, later cream with orange tinges. Downy floccose, becoming woolly to subfelty. Advancing zone appressed to slightly raised. Odor strong, sweet [3,43].

2.4. Fungal Growth under Different Conditions

Fungal growth and consistency are among the most influencing parameters for the production of a bio-based material [47]. In order to evaluate growth rate and mycelium consistency, two different procedures were conducted:

a) Fungal strains growth rate

Even if many parameters can influence fungal growth, a standard approach using Petri plates (diameter 9 cm) on 2% MEA was adopted to evaluate the growth rate of the 21 strains [3]. In order to maximize the space available for radial growth, a mycelium plug (upper surface about 1 cm^2) drawn from actively growing colonies (10 days old) was inoculated at the edge of the Petri plate (with the mycelium in direct contact with the MEA surface) and was incubated at 25 °C in the dark until the full colonization of the plate itself. The radius of the mycelium on the MEA medium was measured day by day for all of the strains by using a ruler until the edge of the Petri plate was reached, i.e., total coverage of the plate. The experiment was conducted in triplicate for each strain. The growth rate (mm day^{-1}) of each strain was calculated at day 7 after inoculation as an average on three replicates.

b) Method of producing fungal mats and materials made therefrom (patented)

In order to increase consistency and strength of the mycelium mat and the thickness of the hyphae, the method described hereafter has been developed and patented by MOGU S.r.l.: patent 102018000010869 issued by MISE (Ministero dello Sviluppo Economico) on 6 June 2020 [48] https://patents.google.com/patent/WO2020115690A1/en?oq=IT201800010869A1 [accessed 13/11/2021].

At first, sterilized millet grains were used as a substrate in spawn bags with a microfilter to allow mycelium colonization for 28 days. Then, the resulting fungus–substrate complex (colonized millet) was blended, and the right amount of water was added in order to produce a creamy mixture that was poured into flat molds (9 cm diameter Petri plates) for 21 days to re-start the colonization process. Finally, the mycelial mat that had developed on the upper surface was removed (Figure 1). The dry weight (50 °C, 24 h) was recorded from the final mat after washing (Figure 2).

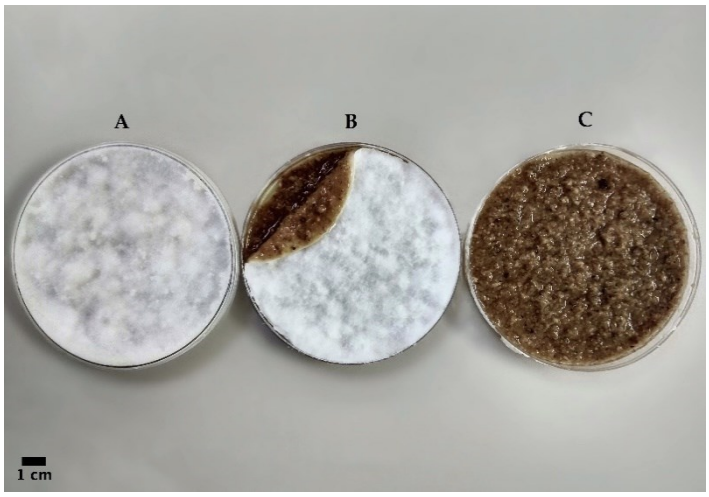

Figure 1. Fresh mycelial mat of *Fomes fomentarius* (8) obtained through patented method after 3 weeks growth in Petri plates: (**A**) the Petri after the 3-week growing phase; (**B**) a Petri where the removable mycelium mat that regrows on top of the slurry is visible; and (**C**) the remaining substrate after the removal of the mycelium mat.

Figure 2. Dry mycelial mats obtained by Mogu's patented method after 3 weeks of growth in Petri plates; five repetitions were conducted per strain: (**A**) *Fomitopsis iberica* (10), (**B**) *Daedaleopsis confragosa* (6), (**C**) *Coriolopsis gallica* (3), and (**D**) *Terana caerulea* (19).

2.5. Thermogravimetric Analysis (TGA) on Mycelium Mats of the 21 Selected Strains Grown on Liquid Culture and Using Mogu's Patented Method

The mycelium mats, which were obtained through the use of the slurry method, and the corresponding pure mycelia grown in liquid culture were analyzed by both thermogravimetric analysis (TGA) and scanning electron microscopy (SEM) in order to determine the functional characteristics in fungal strains that were suitable for their possible use in the context of myco-materials. All of the 21 selected strains in the present study were examined.

Mycelium disks were obtained in 100 mL flasks containing 50 mL of 2% ME broth and were incubated in static at 25 °C in the dark for 21 days, and we assumed that growth had ceased for all of the strains after this period of time. Floating mycelial disks were rinsed with water to eliminate any remaining broth residues. The disks were then dried at 35 °C for 24 h to obtain the dry biomass since TGA requires the sample weight to be standardized and is highly biased by moisture [26].

TGA measurements were conducted using a TGA Q5000 instrument (TA Instruments, New Castle, DE, USA). An amount of up to 5 mg of each sample was weighted. The samples were heated from 25 °C to 900 °C at a scanning rate of 20 °C min^{-1} under nitrogen flux (25 mL min^{-1}). The standards for β-glucan and chitin (both from Sigma Aldrich, St. Louis, MO, USA) as well as those for millet seeds were analyzed in the same conditions, allowing them to be compared with the fungal mass loss at different temperatures.

The thermogravimetric measurements are shown together with their first derivatives (DTGA) in order to better understand the decomposition temperature of the peaks (relative maximum of DTGA) and to locate the starting/ending of the decomposition (zeroing or flattening of DTGA).

2.6. Scanning Electron Microscopy (SEM) on Mycelium Mats Grown on Liquid Culture and Using Mogu's Patented Method

Mycelium mats grown in liquid culture and through the described patented method were prepared as for TGA, as above mentioned.

SEM images were taken from a Zeiss EVO MA10 (Carl Zeiss, Oberkochen, Germany) scanning electron microscope equipped with a LaB_6 emitter. Observations were performed in high vacuum mode through an Everhart–Thornley secondary electron detector at an accelerating voltage of 5 kV with a working distance between 8 and 9 mm. Dry samples were mounted onto aluminum stubs using double sided carbon adhesive tape and were then made to be electrically conductive through being coated in vacuum with a thin layer of Au.

In order to estimate the colony density from the SEM images, grayscale pictures were binarized, with white and black pixels being obtained on a bidimensional representation. The threshold tool Maximum Entropy from the ImageJ 1.53k software was used following the approach of Kapur et al. 1985 [49]. Maximum Entropy automatically defines an image-based threshold in order to optimize its contrast. Once the image had been binarized, the percentage of black pixels and white pixels was computed by the software itself. By convention and good proxy, the white pixels represented mycelium, while the black pixels represented inter-hyphae spaces (pores of the mycelial net). For each cultivation method that was used (static liquid culture and the patented method), three representative pictures per strain were selected, creating a total of 126 images. The picture selection criteria aimed to avoid any substrate or medium residue, thus maximizing the aerial hyphae area; furthermore, the pictures that were taken at the lowest and highest magnifications were discarded.

The hyphal thickness was determined using the measuring tool from the GNU Image Manipulation Program (GIMP) software through a proportion between length in pixels and the scale in μm, which is indicated on the photos that were obtained by SEM. For each strain, 15 measurements were taken.

Data from static liquid culture and patented method were compared using Wilcoxon test in R 4.0.3.

3. Results and Discussion

3.1. Wood Decay Fungal Strains

The field samplings successfully isolated 145 strains belonging to 55 genera and 87 species: we chose 21 strains (belonging to 20 species) to be examined for their potential in developing myco-materials.

In Table 2, the data regarding basidiome collection and strain code are reported.

Table 2. Data about basidiome and strain code of the WDF chosen for developing mycelium mats.

Code	Species	Basidiomes Sample Sites (Italy)	Date of Basidiomes Collection	Host Species	Basidiomes *Legit* and *Determinavit*	MFSC Code
1	*Abortiporus biennis*	Varese (VA)	24 November 2018	*Tilia cordata*	M. Cartabia	064-2018
2	*Bjerkandera adusta*	Albizzate (VA)	29 May 2019	*Populus alba*	M. Cartabia	101-2019
3	*Coriolopsis gallica*	Varese (VA)	30 March 2019	*Fraxinus excelsior*	M. Cartabia	086-2019
4	*Coriolopsis gallica*	Varese (VA)	23 June 2019	*Fagus sylvatica*	M. Cartabia	121-2019
5	*Coriolopsis trogii*	Cazzago Brabbia (VA)	12 June 2018	*Populus tremula*	M. Cartabia	027-2018
6	*Daedaleopsis confragosa*	Inarzo (VA)	29 August 2019	*Fraxinus excelsior*	M. Cartabia	155-2019
7	*Daedaleopsis tricolor*	Baceno (VB)	3 August 2019	*Prunus avium*	M. Cartabia	148-2019
8	*Fomes fomentarius*	Viterbo (VT)	2 December 2019	*Fagus sylvatica*	M. Cartabia	179-2019
9	*Fomitiporia mediterranea*	Varese (VA)	17 December 2018	*Fagus sylvatica*	M. Cartabia	079-2018
10	*Fomitopsis iberica*	Varese (VA)	8 June 2019	*Cedrus deodara*	M. Cartabia	104-2019
11	*Fomitopsis pinicola*	Valganna (VA)	22 June 2019	*Picea abies*	M. Cartabia	117-2019
12	*Ganoderma carnosum*	Varese (VA)	6 September 2019	*Picea abies*	M. Cartabia	161-2019
13	*Ganoderma lucidum*	Varese, (VA)	16 July 2019	*Quercus pubescens*	M. Cartabia	137-2019
14	*Irpex lacteus*	Cazzago Brabbia (VA)	7 December 2018	*Populus tremula*	M. Cartabia	076-2018
15	*Irpiciporus pachyodon*	Imperia (IM)	9 November 2019	*Quercus pubescens*	M. Cartabia	175-2019
16	*Lenzites betulinus*	Cittiglio (VA)	8 April 2019	*Betula pendula*	M. Cartabia	088-2019
17	*Neofavolus alveolaris*	Varese (VA)	3 May 2019	*Populus alba*	M. Cartabia	096-2019
18	*Stereum hirsutum*	Inarzo (VA)	5 December 2018	*Corylus avellana*	M. Cartabia	073-2018
19	*Terana caerulea*	Varese (VA)	2 November 2019	*Ostrya carpinifolia*	M. Cartabia	177-2019
20	*Trametes hirsuta*	Varese (VA)	25 November 2018	*Fagus sylvatica*	M. Cartabia	067-2018
21	*Trametes suaveolens*	Cazzago Brabbia (VA)	4 December 2018	*Salix alba*	M. Cartabia	070-2018

The mycelium characteristics based on references are reported in Table 1. Concerning the three species for which no literature was available, *F. iberica* showed a mat that was white, downy to dense, thick, and quite homogeneous; notably, the colony grew from the edges of the Petri dish and continued to grow on the lid. *G. carnosum* has a mycelium that is thin, whitish when young and that is then olive-brown in the central part of the colony; it is also sometimes brown

spotted; when the mycelium is transfered to other Petri dishes, it remains white for a longer amount of time and is homogeneous; it then becomes thicker and denser. The mat of *T. caerulea* appears to be white at first and then appears to be pigmented and dark blue and also appears to be quite homogeneous but thin and inconsistent; the pigmentation only occurs after storage at a low temperature (4 °C); otherwise it remains white.

Concerning the substrata, *Fomes fomentarius* (8), *Fomitiporia mediterranea* (9), and *Irpiciporus pachyodon* (15) grew on living trees, while all of the other taxa grew on dead wood. Despite such a categorization being increasingly resized and diminished, all of the examined species can be considered to be white rot agents, with the exception of *Fomitopsis iberica* and *Fomitopsis pinicola,* which are brown rot agents.

The identities of all of the strains under examination were successfully confirmed by sequencing the ITS region, the method of which is reported in the Supplementary Materials. Particularly, the use of molecular analysis was required to confirm the cryptic species *Fomitiporia mediterranea*, which was otherwise indistinguishable from *Fomitiporia punctata sensu stricto* [45,50]. On the other hand, molecular identification must be critically addressed for species that are hardly able to be discriminated by the ITS region despite being quite easily discriminated by morphology and/or ecology, such as *D. confragosa* versus *D. tricolor* [51,52].

3.2. Fungal Strains Growth Rate

The recorded growth rates are reported in Table 3; the uncertainty of the random error (absolute uncertainties of the individual terms) was calculated according to Harris (2010) [53]. Despite the vast amount of literature that is devoted to WDF, true rational monographies that have focused on growth parameters in standard culture media are scarce [44,46]; thus, the main references concerning this specific aspect of WDF are Nobles (1948) [43] and Stalpers (1978) [3].

Table 3. Average growth rate calculated at day 7 after inoculation on the three replicates (random error ±2 mm).

Code	Species	MFSC Code	Average Growth Rate (mm day^{-1})
1	*Abortiporus biennis*	064-2018	9
2	*Bjerkandera adusta*	101-2019	11
3	*Coriolopsis gallica*	086-2019	8
4	*Coriolopsis gallica*	121-2019	8
5	*Coriolopsis trogii*	027-2018	6
6	*Daedaleopsis confragosa*	155-2019	4
7	*Daedaleopsis tricolor*	148-2019	7
8	*Fomes fomentarius*	179-2019	7
9	*Fomitiporia mediterranea*	079-2018	5
10	*Fomitopsis iberica*	104-2019	7
11	*Fomitopsis pinicola*	117-2019	6
12	*Ganoderma carnosum*	161-2019	6
13	*Ganoderma lucidum*	137-2019	2
14	*Irpex lacteus*	076-2018	10
15	*Irpiciporus pachyodon*	175-2019	5

16	*Lenzites betulinus*	088-2019	7
17	*Neofavolus alveolaris*	096-2019	7
18	*Stereum hirsutum*	073-2018	11
19	*Terana caerulea*	177-2019	4
20	*Trametes hirsuta*	067-2018	6
21	*Trametes suaveolens*	070-2018	7

In this study, *B. adusta* (2) and *S. hirsutum* (18) showed a very high growth rate (11 mm day^{-1}), confirming the literature data [3,42,43,53]. *A. biennis* (1) and *I. lacteus* (14) also showed high growth rate (>9 mm day^{-1}). The strains of *C. gallica* (3–4) were similar and were reported to be 8 mm day^{-1}.

On the contrary, *G. lucidum* (13) reported the lowest growth rate; this is surprising since *G. lucidum* is usually considered to be a fast-growing species [54]. However, as highlighted by Dresch et al. (2015) [47], the variability among strains is not negligible. *T. caerulea* (19) and *D. confragosa* (6) also reported very low growth rates. *D. confragosa* and *D. tricolor*, whose basidiomes are easily discriminated by morphology, record different growth rates even though the two species are hardly recognized by standard molecular markers (e.g., ITS). This is why some authors propose incorporating *D. tricolor* into *D. confragosa*, resulting in them not being able to be distinguished from each other [51,52]. Marković et al. (2013) [55] actually dealt with *D. tricolor* and reported a higher growth rate than the one in the present study under the same cultural conditions.

A low growth rate was recorded *F. mediterranea* (9); this species was only proposed to be an independent taxon in 2002, as its growth rates can be hardly compared with references to *F. punctata*, with the exception of literature clearly referring to *F. mediterranea* itself [56].

3.3. Strains Cultivation Using Patented Method

Among the 21 strains that were tested, some were unable to re-grow homogenous mats in the Petri plates. All of the obtained mats were dried at 50 °C for 24 h and were weighed (Figure 3).

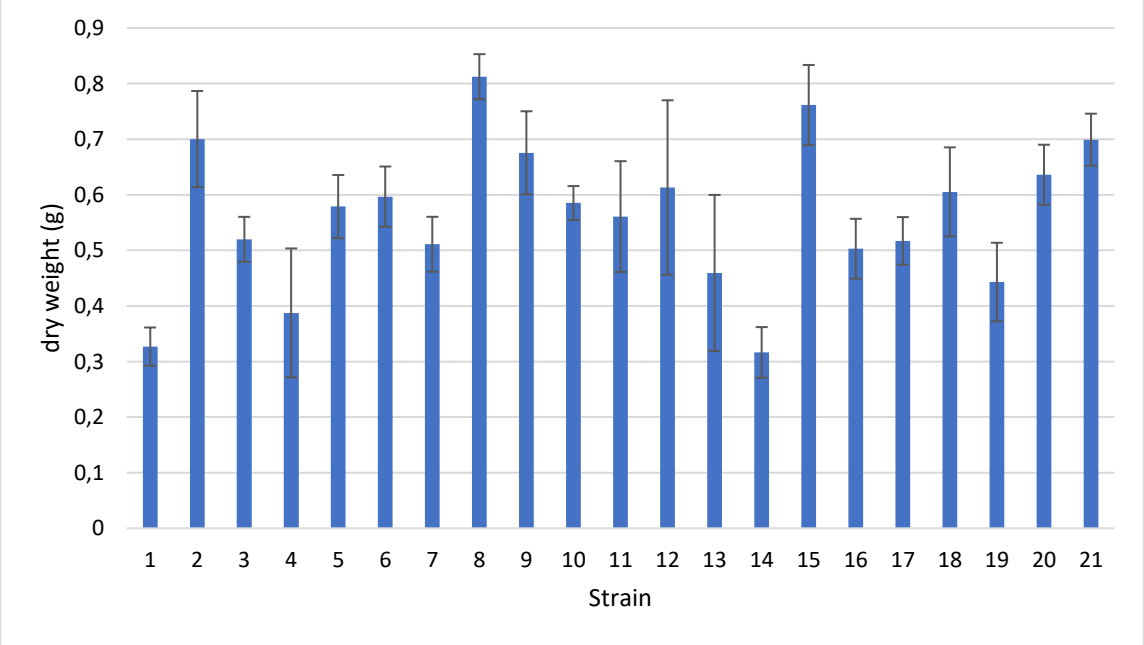

Figure 3. Average dry weight of mycelial mats obtained using the patented method (three repetitions each). The bars represent the standard error.

F. fomentarius (8) and *I. pachyodon* (15) recorded the first and second highest dry weights, respectively, which is consistent with their compact mats. *B. adusta* (2) recorded the third highest dry weight despite the mat appearing fluffy and inconsistent.

All of the mats formed by the *Trametes* species were very fragile and inconsistent. *G. carnosum* (12) and *G. lucidum* (13) were unable to form a uniform mat.

F. pinicola (11) formed a really dense mat but brittle and powdery.

A. biennis (1), *C. gallica* (3 and 4), *C. trogii* (5), and *T. hirsuta* (20) formed a primordia of basidiomes on the upper surface of the mat after a few days, but the mat itself had not yet reached an acceptable consistency. However, a lack of primordia was observed in *A. biennis* (1) because the oxygen exchange was reduced due to the Petri plate being sealed; these conditions provided homogenous mats, similar to the one created by *F. fomentarius* (8). Thus, the mats from *A. biennis* (1) interestingly displayed an appreciable consistency despite its relatively low weight, as did the *I. lacteus* (14) mats.

F. iberica (10) and *F. mediterranea* (9) produced consistent and homogeneous mats that often also showed aerial mycelium; however, the latter species was very slow growing.

As a whole, the dry weight of the mycelial mats was variably affected by the substrate residues (slurry residues) that were embedded in the mats themselves, and therefore, the mats were impossible to remove. This bias was less negligible when the mat was more consistent, i.e., in *F. fomentarius* (8) and *I. pachyodon* (15).

3.4. TGA on Mycelium Mats

TGA profiles reflect the different compositions of each mycelium (see Supplementary Material).

In order to better assess the differences between samples, the first derivatives of the TGA curves (DTGA) are considered in the discussion. The samples are compared to a growing material (millet) and with the main constituents of mycelium (chitin and beta glucans).

It is noteworthy that the mycelia that were obtained in the liquid culture using the patented method show profiles that are not comparable to each other in certain cases(e.g., strain 10), whereas in others, the respective profiles are similar (e.g., strain 16) (Figures 4 and 5).

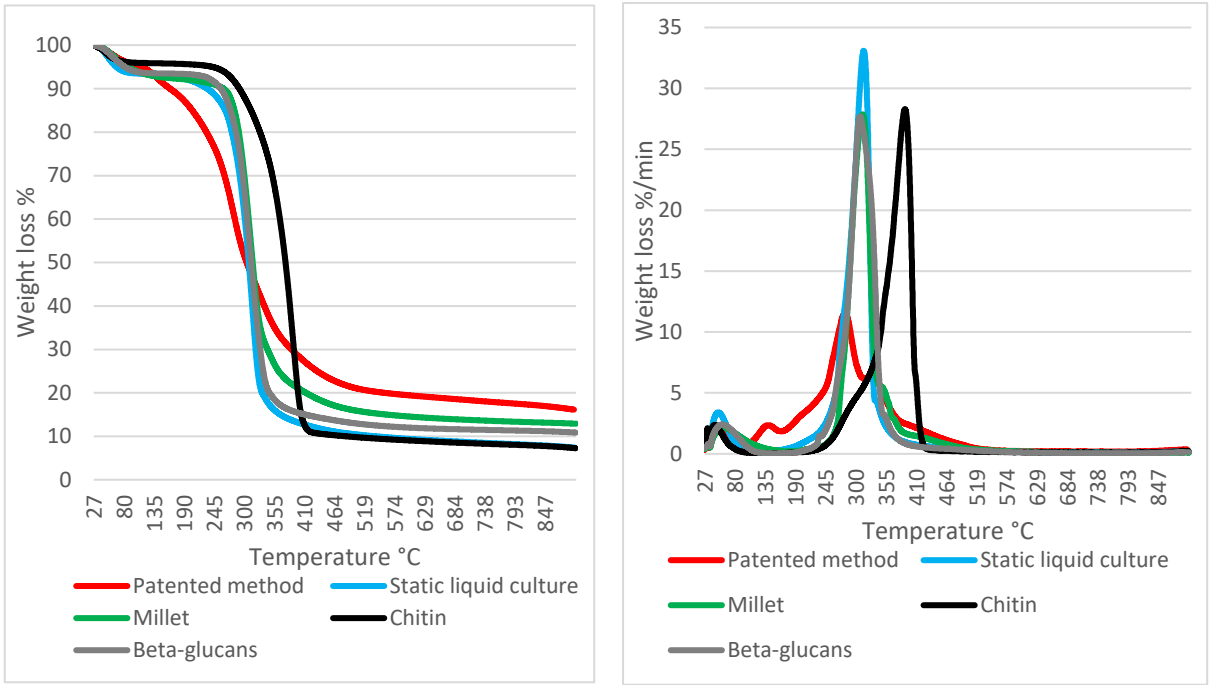

Figure 4. TGA (left) and DTGA (right) profiles of *Fomitopsis iberica* (10) (pure mycelium grown in liquid and mat grown on slurry) compared to reference materials.

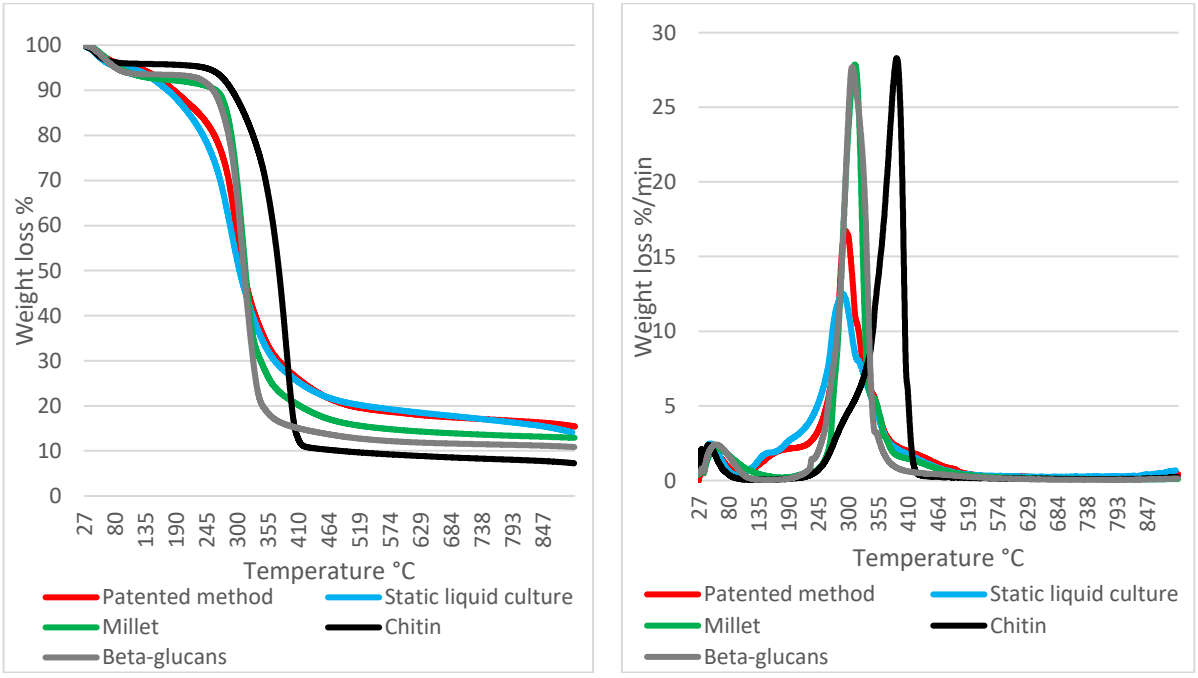

Figure 5. TGA (left) and DTGA (right) profiles of *Lenzites betulinus* (16) (pure mycelium grown in liquid and mat grown on slurry) compared to reference materials.

Taking the abovementioned strains as an example, the pure mycelium (strain 16) profile in Figure 5 almost overlaps with the slurry mat profile; the mass loss behaviors of both strains are both visibly driven by the component decomposition of β-glucan. However, the major difference between strain 16 and strain 10 is that the β-glucans in pure mycelium are much more developed in the latter (10), whereas the slurry mat is less variable in terms of its weight loss percentage.

β-glucans are also the major determinant in the millet profile since cereals are also rich in β-glucans.

As expected, chitin is a minority component at this growth stage, but it is clearly shown by tailing when the temperature is over 350 °C, as was also the case in previous studies on wood decay mycelia [26]. Interestingly, the profiles of strain 16 and strain 10 suggest different behaviors: strain 16 displays a consistent β-glucan component in both the mycelia and slurry mat followed by moderate but overlapping tailing in the chitin degradation range; in strain 10, the pure mycelium is sharply focused on the β-glucan component, whereas the slurry mat developed less β-glucans but displayed a clear chitin tailing instead. Again, similar behaviors were suggested by the profiles discussed in Girometta et al. (2020) [26]. Notably, neither strain 16 nor 10 melanized; thus, the melanin variable can be excluded from the drivers of thermal behavior. Figures 6–8 generalize the comments above to the whole strain spectrum that is under examination.

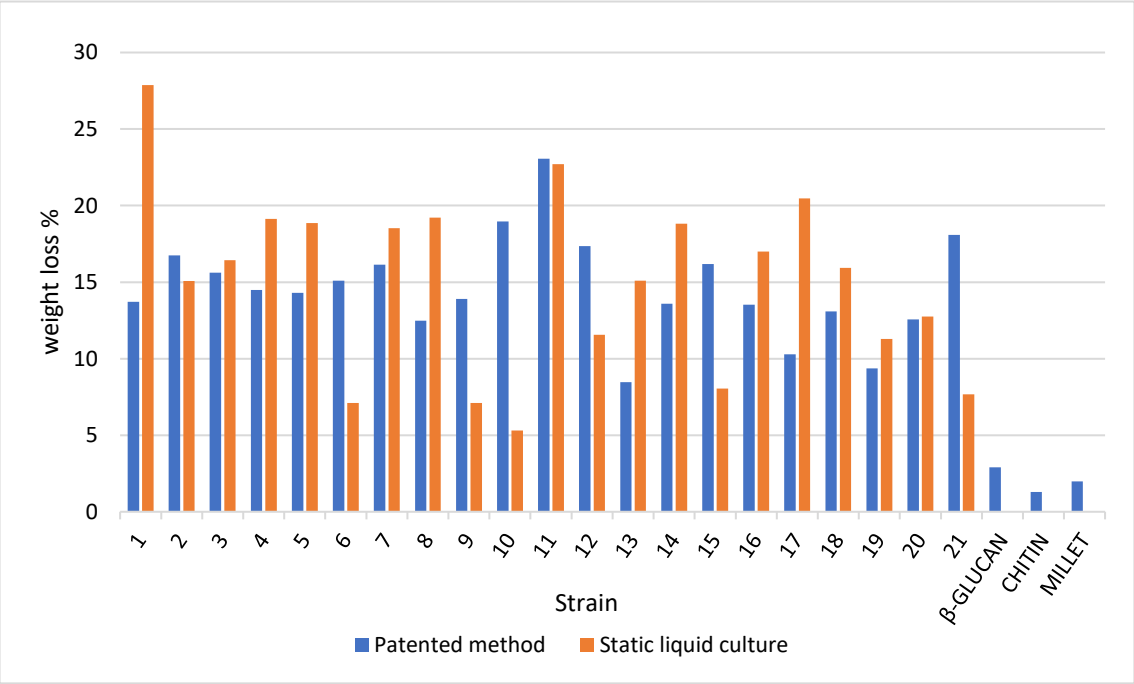

Figure 6. Weight loss percentage for the dry samples in the 125–250 °C temperature range (TGA output) in the mycelium (pure mycelium and slurry mat) and reference materials.

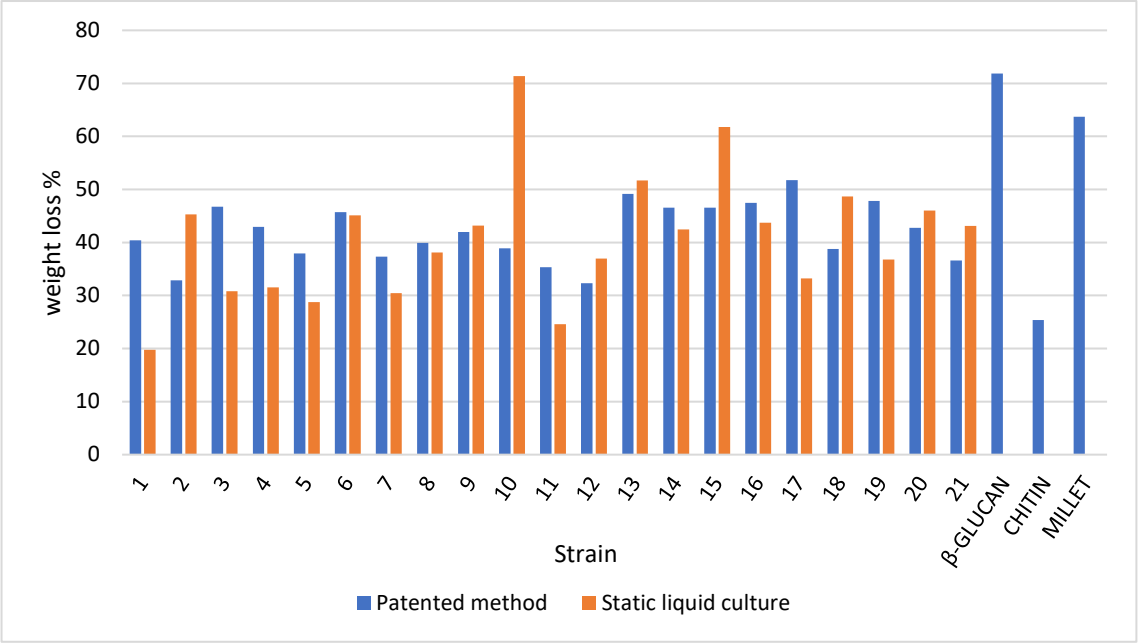

Figure 7. Weight loss percentage for the dry samples in the 250–350 °C temperature range (TGA output) in the mycelium (pure mycelium and slurry mat) and reference materials.

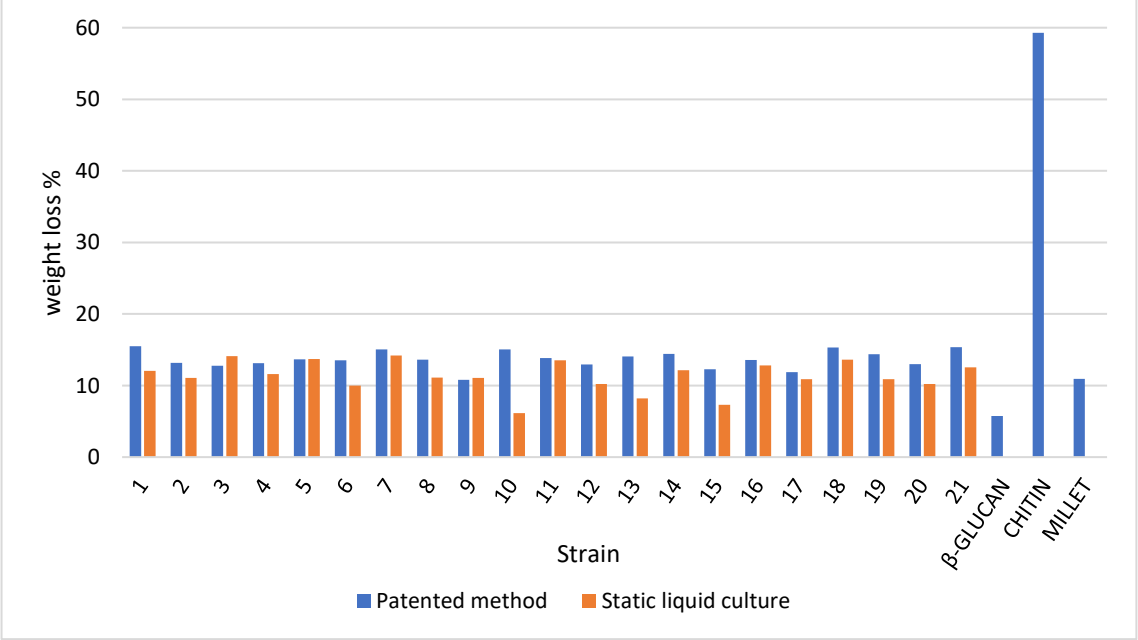

Figure 8. Weight loss percentage for the dry samples in the 350–500 °C temperature range (TGA output) in the mycelium (pure mycelium and slurry mat) and reference materials.

As clearly shown in Figure 6, a weight loss percentage in the range of 125–250 °C is different in terms of both the mycelium and slurry mat for most strains. More than 50% of the strains recorded higher losses in pure mycelium. Therefore, the slurry appears to be more focused on the production of β-glucans, with notable exceptions, such as strain 10, which was discussed above. This confirms that each species (as well strain, at intra-specific level) must be characterized before its application in biomaterials. The differences between pure mycelium and slurry mat behavior are attenuated when the percentage weight loss in the range of 250–350 °C as well as in the range of 350–500 °C is considered, with a few notable exceptions (e.g., strain 10).

3.5. SEM on Mycelium Mats Grown in Liquid Medium and Following Mogu's Patented Method

The mycelia of all the 21 strains were observed by SEM. Microscopy was performed on both the mycelia grown in liquid culture and that had been grown using the patented method.

Concerning the images that were obtained from the pure mycelium, they show that the micro-structure is species-specific, i.e., each species has its own specific microstructure, and therefore, each texture is consequently very variable, forming loose to dense mycelium, as shown in Figure 9, where four examples are reported.

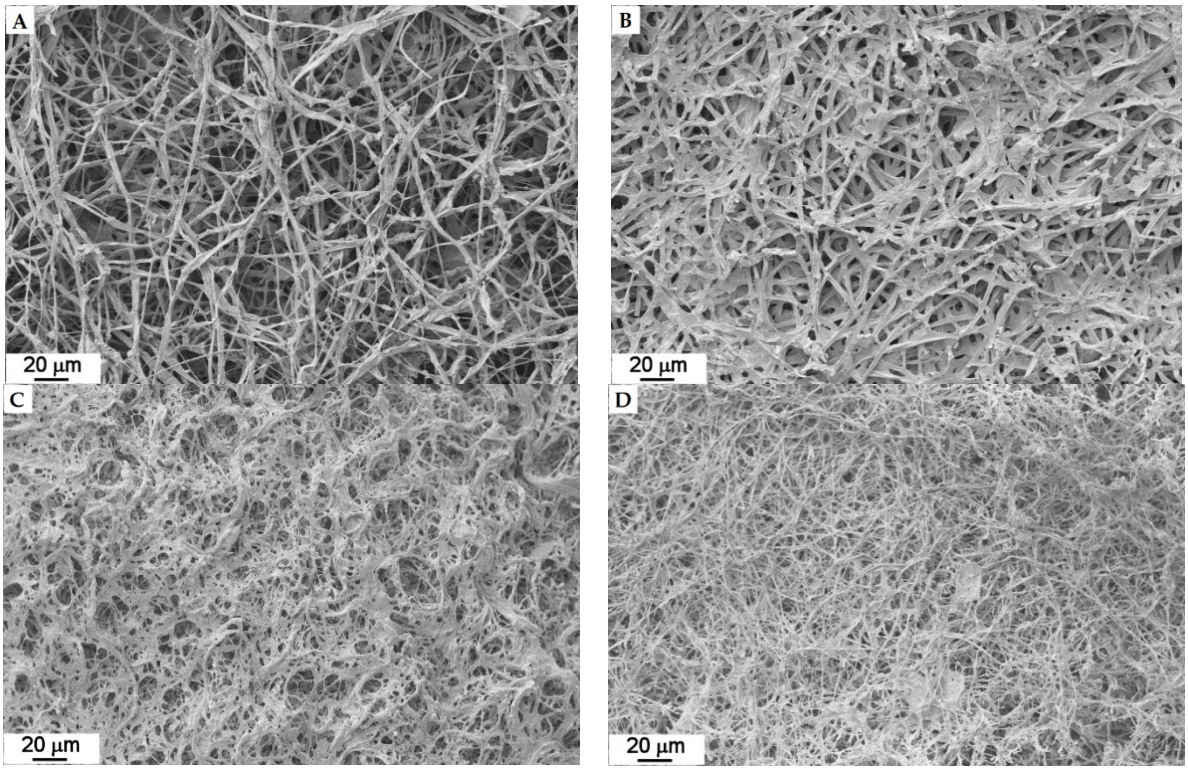

Figure 9. Different kinds of mycelia in strains: (**A**) *Coriolopsis trogii* (5), (**B**) *Irpex lacteus* (14), (**C**) *Daedaleopsis confragosa* (6), and (**D**) *Fomitopsis iberica* (10).

For each strain, the images of the mats that were obtained using the newly patented method are very similar to the ones that were produced by the mycelia obtained in liquid culture. The substrate residues are more evident in some strains than in others. The upper face (front) is always composed of a hairy (aerial) hyphae, while the bottom one (back) is always more compact and is flattened since it was in contact with the substrate (Figures 10 and 11).

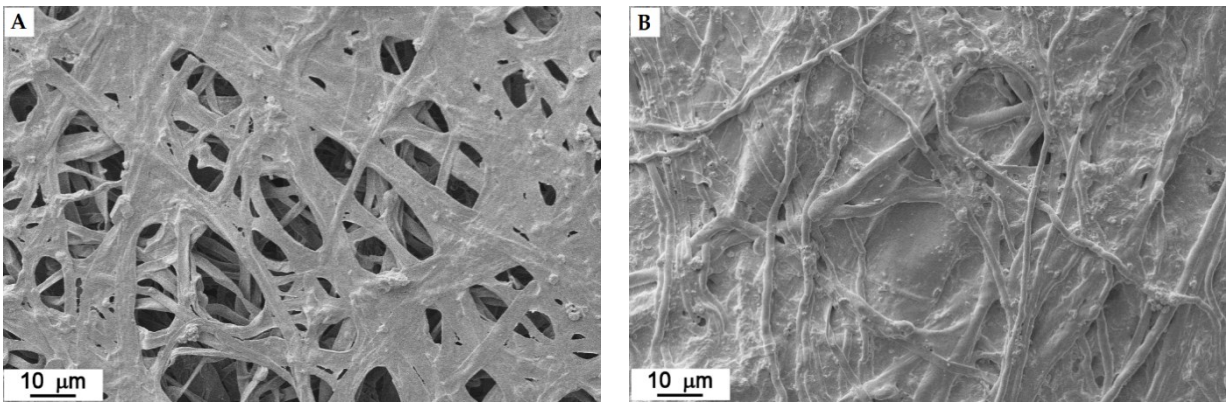

Figure 10. *Fomitopsis iberica* (10). Front (**A**) and back (**B**) of the mat obtained by patented method.

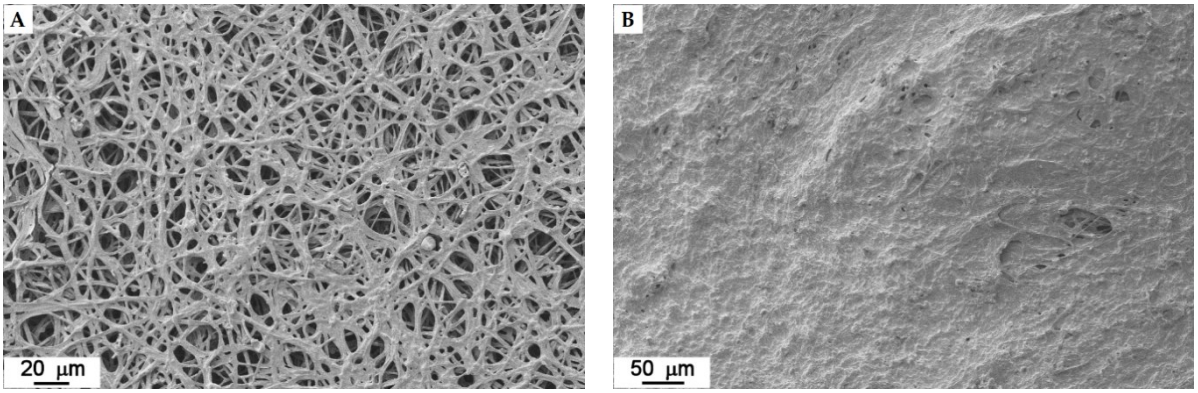

Figure 11. *Irpiciporus pachyodon* (15). Front (**A**) and back (**B**) of the mat obtained by patented method.

The binarization of the SEM images (Figure 12) was computed in order to quantify the concept of "dense" versus "loose" mycelium, i.e., a more or less compact mat. For each strain, the hyphal fraction (the projected area occupied by hyphae) was computed as the percentage of white pixels in the image versus the percentage of black pixels (i.e., the empty spaces). Results are shown in Figure 13.

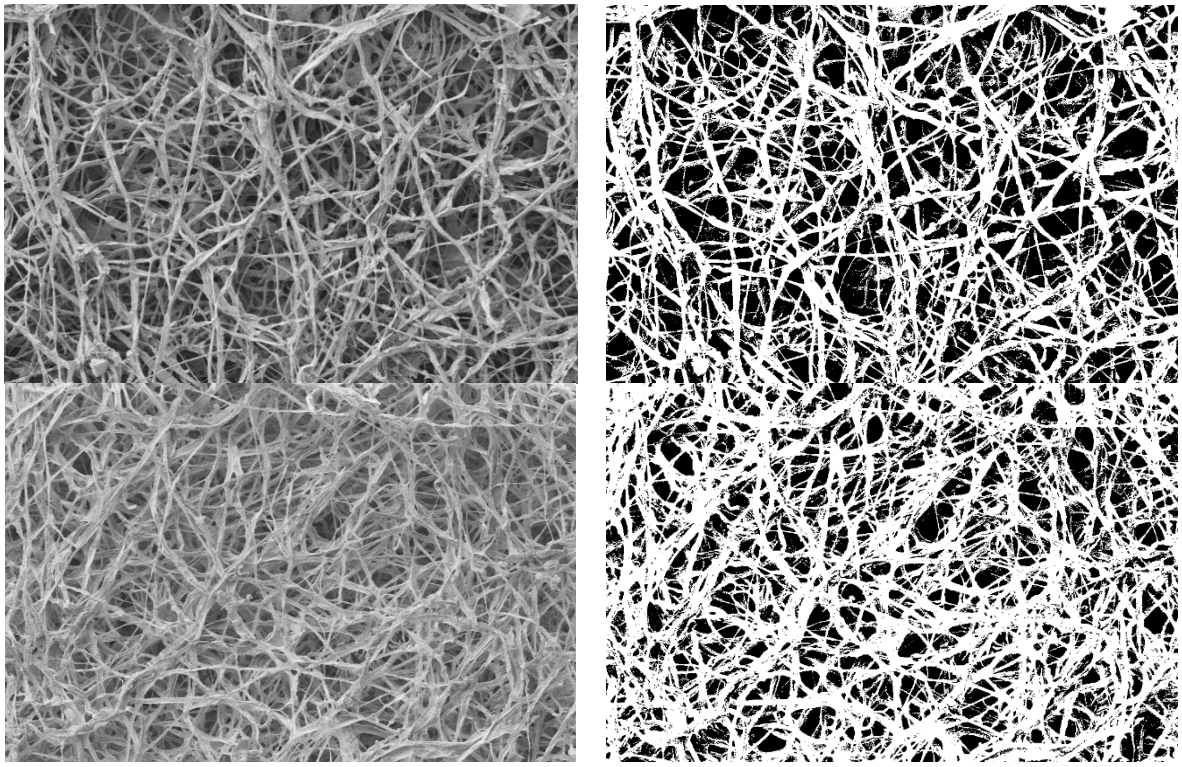

Figure 12. Examples of SEM images before (left) and after (right) binarization.

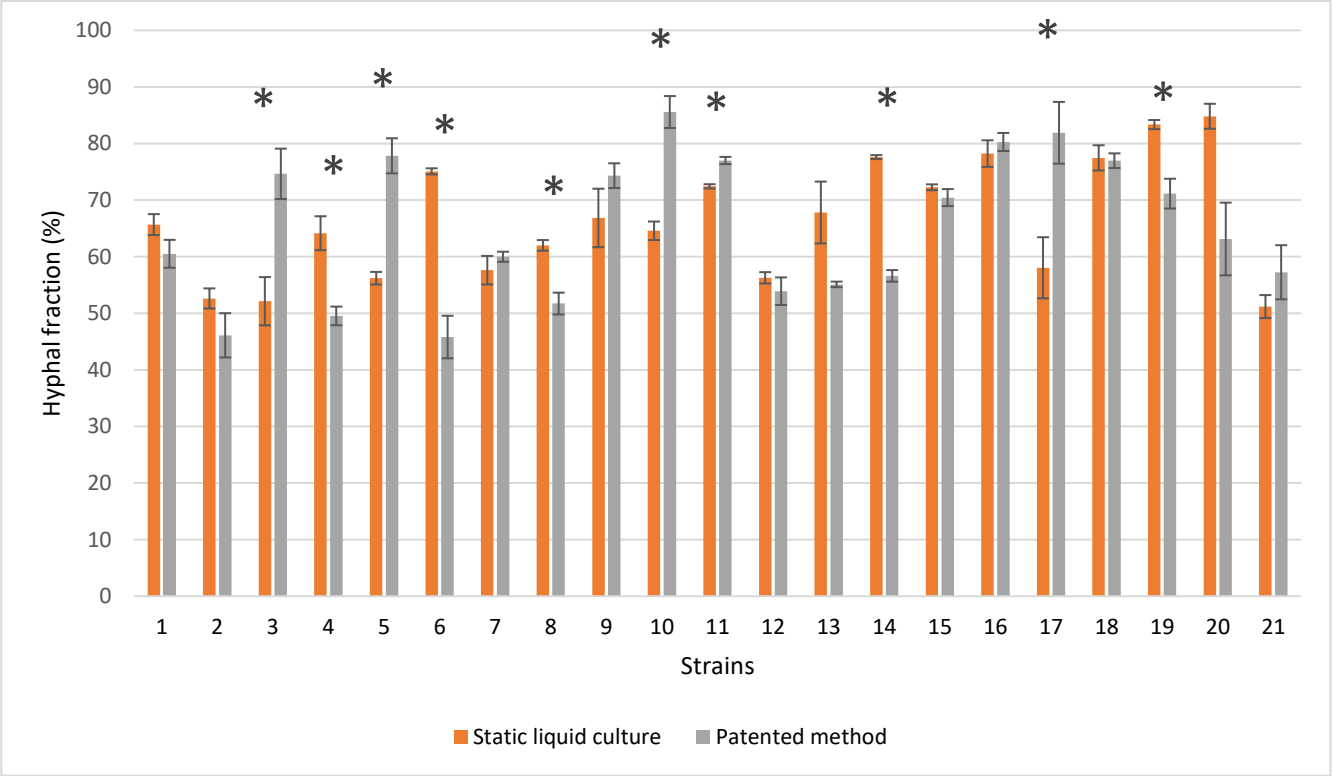

Figure 13. Average ± standard error of hyphal fraction obtained in liquid static culture and by patented method. Significantly different hyphal fractions are marked by * based on Wilcoxon's test (* p-value < 0.05).

The higher hyphal fraction was reported by *T. caerulea* (19) and *T. hirsuta* (20) in the static liquid culture, whereas it was reported by *F. iberica* (10), *L. betulinus* (16), and *N. alveolaris* (17) when the patented method was used. Strain by strain comparisons that were determined using Wilcoxon's test revealed that 10 out of 21 strains developed a significantly different hyphal fraction when the cultivation method was changed (Figure 13): the hyphal fraction was particularly improved in the static liquid culture in *C. gallica* (4), *D. confragosa* (6), and *I. lacteus* (14), whereas the hyphal fraction was better in *C. gallica* (3), *C. trogii* (5), *F. iberica* (10), and *N. alveolaris* (17) when the patented method was used.

As a whole, the mycelium obtained by the patented method does not seem to produce a statistically different hyphal fraction than the ones obtained in a static liquid culture (Wilcoxon test: p-value > 0.05).

Moreover, the mean diameter of the hyphae was measured based on the SEM images, i.e., on a two-dimensional projection. The results are shown in Figure 14.

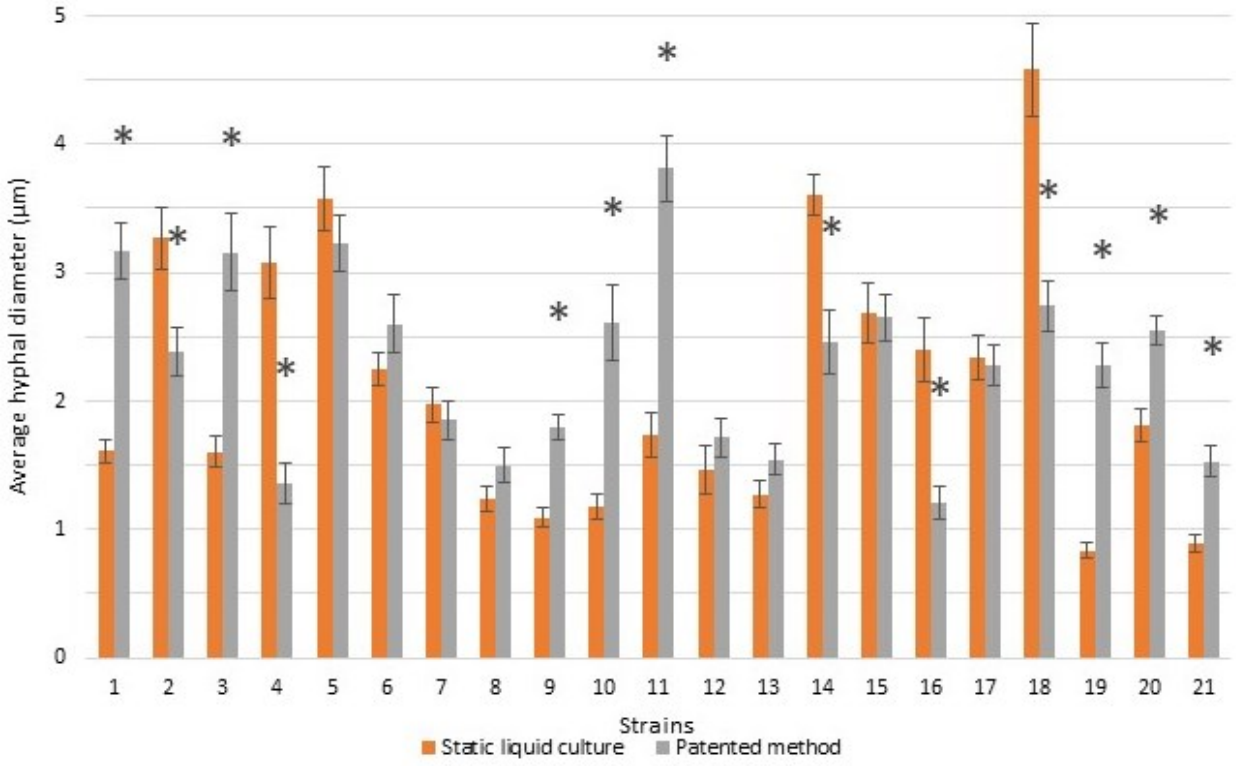

Figure 14. Average ± standard error of mycelium hyphal diameter obtained in liquid static culture and by patented method. Significantly different diameters are marked by * based on Wilcoxon's test (* p-value < 0.05).

In static liquid culture, the higher hyphal diameter was reported by *C. trogii* (5), *I. lacteus* (14), and *S. hirustum* (18), but with patented method, it was reported by *A. biennis* (1), *C. trogii* (5), and *F. pinicola* (11).

Strain by strain tests revealed 13 out of 21 strains developed hyphae with a significantly different width when the mycelia were obtained from the liquid culture versus when obtained following the patented method (Figure 14): t *C. gallica* (4), and *S. hirustum* (18) showed significant improvements in the hyphal diameter when the static liquid culture was used, whereas with the patented method, hyphal diameter improvement was seen in *A. biennis* (1), *F. iberica* (10), and *F. pinicola* (11).

As a whole, the mycelium that was obtained by following the patented method seems to produce statistically different hyphae rather than those obtained in static liquid culture (Wilcoxon test: p-value < 0.05).

C. gallica was the only species to have two trains tested. The two strains both grew rapidly but behaved in opposite ways depending on whether they were grown in the static liquid culture or using the newly patented method. This indicates how important strain selection is when considering the production of bio-based materials.

In the end, there are other additional components that could have affected the consistency and structure of the produced mats, e.g., the oxalate production observed in the *F. fomentarius* (8) and *L. betulinus* (16) mycelia (Figure 15). It is possible that the oxalates could cause the mat to be less uniform.

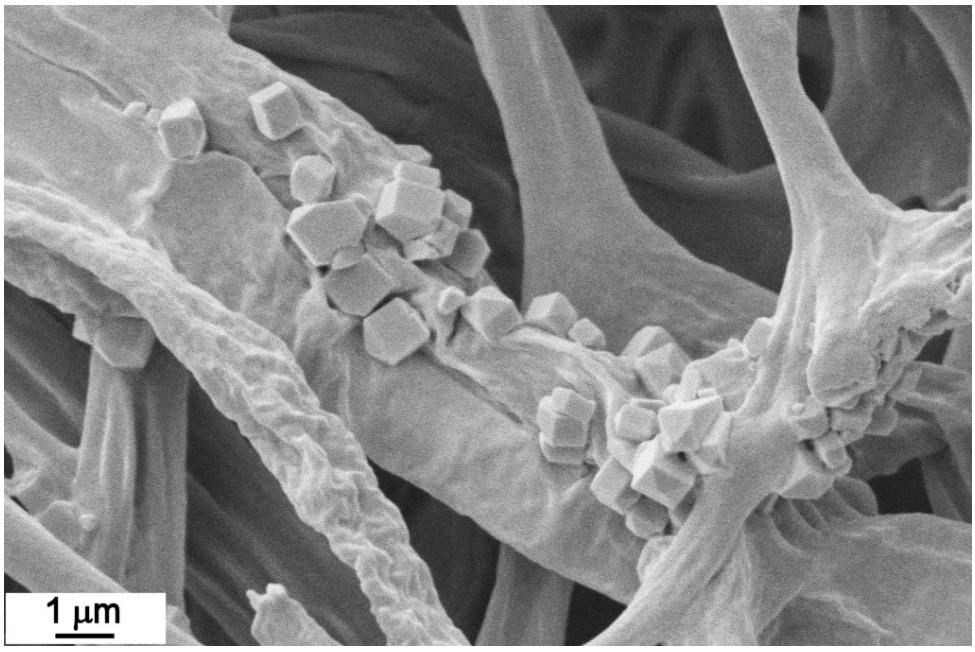

Figure 15. Typical oxalate crystals in *Fomes fomentarius* (8).

4. Conclusions

The structure and texture of each mycelium are highly variable due to species-specific and strain-specific features as well as to growth conditions. Consequently, mycelium characterization in material science requires an integrated, multi-focus approach in order to improve knowledge for next-process tuning.

The aim of the present work was to compare different species of wood decay fungi using two growth cultural methods. In particular, the characteristics that were determined for each mycelium were considered in the context of their use for the development of compact and resistant mats in the future.

Among the 21 strains that were examined, *A. biennis* (1), *B. adusta* (2), *I. lacteus* (14), and *S. hirsutum* (18) could be good choices for the development of myco-materials due to their high growth rate, but the mats that were obtained were light and fragile, meaning that they may not be suitable for the application of developing leather-like materials.

TGA provides a good approximation on the composition of the cell wall; this enables each strain to be characterized shows the significant differences among the different strains. From a compositional perspective, the present study has also documented that TGA can point out differences depending on cultivation condition and the production process that is used to make the mat (namely static liquid culture versus the newly patented method). Composition is not the only determinant of mycelium mat features. This study remarks that SEM imaging is an excellent tool that is able to point out structural details and differences based on species and the mat production process.

SEM image processing revealed that *F. iberica* (10), *T. caerulea (19), and T. hirsuta* (20) produced mats with higher hyphal fractions, while *C. trogii* (5), *F. pinicola* (11), and *S. hirsutum* (18) showed the highest values in terms of their hyphal diameters.

The newly patented method improved the *F. iberica* (10) mats: TGA not only showed a different composition among the principal constituents of the hyphae (higher beta glucans fraction), but the analysis of the SEM images showed an improvement both in terms of the hyphal fraction and hyphal diameter.

In conclusion, TGA and SEM are complementary methods; despite SEM being more time-consuming and expensive and even though it requires

remarkable personal skills, TGA is not capable of selecting strains that are appropriate for bio-based material applications on its own.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: TGA (left) and DTGA (right) profiles of strain 1 (pure mycelium and slurry mat) compared to reference materials, Figure S2: TGA (left) and DTGA (right) profiles of strain 2 (pure mycelium and slurry mat) compared to reference materials, Figure S3: TGA (left) and DTGA (right) profiles of strain 3 (pure mycelium and slurry mat) compared to reference materials, Figure S4: TGA (left) and DTGA (right) profiles of strain 4 (pure mycelium and slurry mat) compared to reference materials, Figure S5: TGA (left) and DTGA (right) profiles of strain 5 (pure mycelium and slurry mat) compared to reference materials, Figure S6: TGA (left) and DTGA (right) profiles of strain 6 (pure mycelium and slurry mat) compared to reference materials, Figure S7: TGA (left) and DTGA (right) profiles of strain 7 (pure mycelium and slurry mat) compared to reference materials, Figure S8: TGA (left) and DTGA (right) profiles of strain 8 (pure mycelium and slurry mat) compared to reference materials, Figure S9: TGA (left) and DTGA (right) profiles of strain 9 (pure mycelium and slurry mat) compared to reference materials, Figure S10: TGA (left) and DTGA (right) profiles of strain 10 (pure mycelium and slurry mat) compared to reference materials, Figure S11: TGA (left) and DTGA (right) profiles of strain 11 (pure mycelium and slurry mat) compared to reference materials, Figure S12: TGA (left) and DTGA (right) profiles of strain 12 (pure mycelium and slurry mat) compared to reference materials, Figure S13 TGA (left) and DTGA (right) profiles of strain 13 (pure mycelium and slurry mat) compared to reference materials, Figure S14: TGA (left) and DTGA (right) profiles of strain 14 (pure mycelium and slurry mat) compared to reference materials, Figure S15: TGA (left) and DTGA (right) profiles of strain 15 (pure mycelium and slurry mat) compared to reference materials, Figure S16: TGA (left) and DTGA (right) profiles of strain 16 (pure mycelium and slurry mat) compared to reference materials, Figure S17: TGA (left) and DTGA (right) profiles of strain 17 (pure mycelium and slurry mat) compared to reference materials, Figure S18: TGA (left) and DTGA (right) profiles of strain 18 (pure mycelium and slurry mat) compared to reference materials, Figure S19: TGA (left) and DTGA (right) profiles of strain 19 (pure mycelium and slurry mat) compared to reference materials, Figure S20: TGA (left) and DTGA (right) profiles of strain 20 (pure mycelium and slurry mat) compared to reference materials, Figure S21: TGA (left) and DTGA (right) profiles of strain 21 (pure mycelium and slurry mat) compared to reference materials, Table S1. ITS sequences of the strains and main parameters on Mycobank Molecular ID and NCBI.

Author Contributions: Conceptualization, M.C., Stefano Babbini, E.S. and D.D.; Data curation, C.E.G. and C.M.; Formal analysis, Simone Buratti; Funding acquisition, Stefano Babbini, E.S. and D.D.; Investigation, M.C., C.M., R.M.B., Simone Buratti, D.S.B. and A.G.; Methodology, C.M., D.V., A.G., E.S. and D.D.; Project administration, D.D.; Resources, C.M., A.G., Stefano Babbini, E.S. and D.D.; Supervision, C.M., E.S. and D.D.; Writing – original draft, M.C.; Writing – review & editing, C.E.G. and E.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement:

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Chapter 3. Optimization of protocols and mechanical tests to obtain material production (prototyping)

The final phase of the PhD project led to the realization of a second material typology and all the involved activities are represented in Fig. 8.

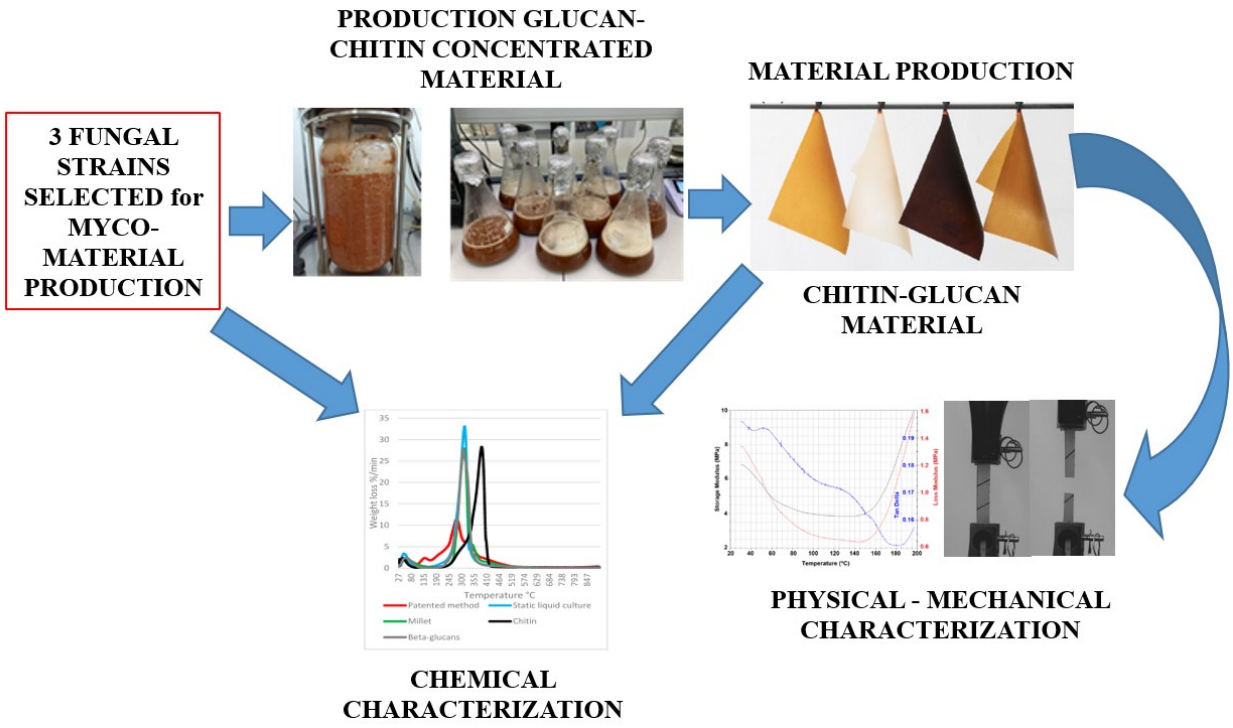

Fig. 8: Activities involved in the third part of my PhD project.

Through the analyses reported in Cartabia et al. (2021), three species were selected to develop a glucan-chitin concentrated material based on their different content in alpha-glucans, beta-glucans or chitin. Therefore, the following strains was tested to develop this kind of material:

- *Abortiporus biennis* 064-18 with a high content of alpha-glucans;
- *Fomitopsis iberica* 104-19 with a high content of beta-glucans;
- *Stereum hirsutum* 073-18 with a high content of chitin.

These strains were cultivated in liquid shacking cultures, both in flasks and in bioreactor to evaluate the best practice to produce highest quantity of biomass with a high content of fungal cell wall polymers.

The produced materials (Fig. 9) were evaluated through chemical analysis (TGA) and mechanical tests (tensile strength).

The process resulted in the production of flexible chitin-glucan based materials, with a rubber-like texture.

Fig. 9: Flexible chitin-glucan based materials produced.

TGA and mechanical tests showed that the materials obtained from these strains had different properties, consistent with the presence of the different components of each mycelium.

This part of the work is in preparation to be submitted to a peer review “ACS Sustainable Chemistry & Engineering”: the draft is reported here after.

Innovative chitin-glucan based material obtained from mycelium of three wood decay fungal strains

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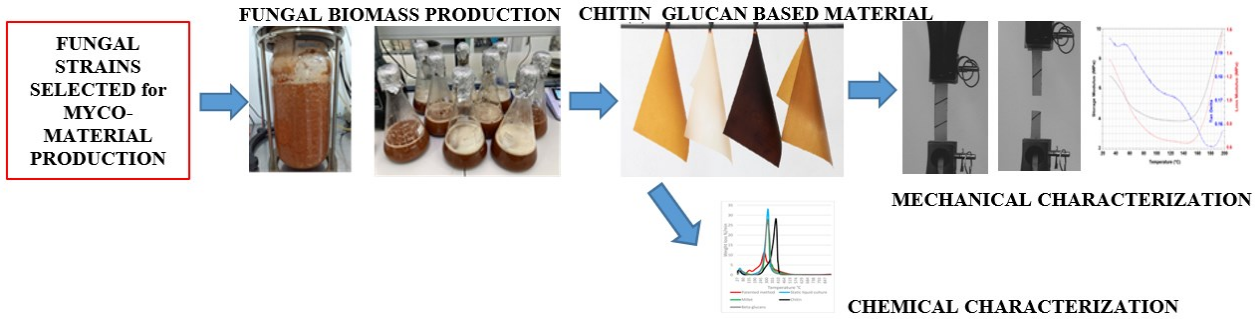
Abstract
Fungi can represent an unconventional alternative, animal-based free source of chitin. In fungi, chitin fibrils are strongly interconnected and bound with glucans, making properties of this matrix absolutely unique; therefore, a number of applications alternative to common polymers have been devised.
The aim of the present study was to extract chitin and glucans from mycelia of a number of wood decay fungal strains in order to obtain flexible materials and to check correlations between chitin content and mechanical properties of these materials.
Initially, five strains were chosen in consideration of their different cell wall chemical composition (high content of α-glucans, β-glucans or chitin) to evaluate how these differences could influence the mechanical and chemical characteristics of the material.
The fungal strains were cultivated in liquid submerged dynamic fermentation (both flasks and bioreactor). Later on, chitin and glucans were crosslinked with acetic acid and plasticized with glycerol in order to obtain flexible sheets. *Abortiporus biennis*, *Fomitopsis iberica* and *Stereum hirsutum* strains were found adapt to produce material with adequate flexibility. Thermogravimetric analysis (TGA) allowed to evaluate the principal chemical components, providing a semi-quantitative indication on mat composition. The material obtained from each species was mechanically tested in terms of tear strength, elongation at break, and Young's modulus.
Taking all the results into account, there was evidence of a correlation between chitin content and material mechanical response.

Keywords
myco-materials, wood decay fungi, flexible materials, chitin-glucan-materials, nanofiber papers

Graphical abstract



Introduction

Global environmental concern pushes research to find bio-based alternative solutions to current polluting approaches. Both process and resulting product need to be the less energy consuming and the less polluting as possible to be sustainable. In material science, the development of new bio-based and bio-degradable products and more in general the search of novel eco-friendly alternatives to fossil based materials is recognized as a main way to improve sustainability (Nawawi et al. 2020).

Chitin is the second most abundant natural polymer globally available, second only to cellulose. Every year, 10^{10}-10^{11} tons of chitin are naturally produced. Even if this polymer is useful for many applications, most of it is thrown away as waste (Liao & Huang 2020; Muzzarelli 2012). Chitin is synthesised by arthropods and by fungi for structural support; more in particular, chitin is located in crustacean shells, insect exoskeletons and in the hyphal cell wall.

Up to now, chitin has been employed as functional bio-based material in food (Wang et al. 2016), agriculture (Sun et al. 2018), medicine (Singh et al. 2018), drug delivery (Huang et al. 2012) and water purification (Cao et al. 2018).

The principal chitin commercial sources are wastes from shellfish industry like shrimp, crab and lobster shells where chitin content ranges between 8% and 40% (Nair et al. 2003; Charoenvuttitham et al. 2006; Kurita 2006).

At present, there are also unconventional chitin sources, like insects and fungi, that are getting more and more interesting (Majtán et al 2007; Draczynski, 2008). Fungi are being deeply investigated for industrial applications, and in particular as bio-based materials, only in the last few years. They cover a wide range of applications, as they can replace plastics, foams, timber, door cores, panelling, flooring, and other furnishings. Since these materials present low thermal conductivity, high acoustic absorption and fire safety properties outperforming traditional construction materials, such as synthetic foams and engineered woods, they are promising as thermal and acoustic insulation means (Nawawi et al.2020).

In particular, the extraction of chitin from non-animal sources as fungi, is possible (Kleekayai et Suntornsuk, 2011; Cheng et al. 2014; Di Mario et al. 2008) and it can be particularly attractive because it has no impact on animals (Hassainia et al. 2018).

Few cultivated edible species of fungi were already tested as chitin sources, in particular with the aim of recycling waste parts: Wu et al. (2004) extracted a fungal chitin from *Agaricus bisporus*, and its degree of acetylation (DA) was ranged from 75,8 to 87,6%, which is similar to commercial chitin products. Shiitake (*Lentinula edodes*) was also used to prepare fungal chitin (Yen & Mau 2007).

In some cases, fungal chitin produced better results if compared to traditional animal chitin: Chien et al. (2016) found out that the chitin extracted from shiitake showed better inhibitory effect on bacterial growth than the one extracted from crab shells. Above all, in the most updated debates about plastic waste plastic production, thanks to the intrinsic bindings between fungal-chitin and glucans, this material has been recently taken into consideration as a novel bio-based nanofiber material (Nawawi et al. 2020).

The extraction of chitin from mushrooms has many advantages. Fungal chitin doesn't contain allergens like tropomyosin, myosin light chain and arginine kinase that are usually present in crustacean cuticles (Muñoz et al. 2015). Moreover, the extraction conditions of chitin from mushrooms are milder than that from crustaceans, because mushrooms contain lower mineral impurities associated with chitin and so the acidic extraction step to remove them is not required (Hassainia et al. 2018; Nawawi et al 2020). Also, the physic-chemical properties of fungal chitin could be well controlled, because they can vary according to the fungal species, fungal strain, the harvesting period (Wu et al. 2004) and because mushrooms can be easily cultivated all year long with no seasonal fluctuations, without need of sunlight or any specific geographical restrictions. Finally, fungi generally include a substantial amount of β-glucans (Manzi, et al. 2001), which may be advantageous for the subsequent produced materials. It was in fact recently shown that chitin based materials from fungal sources have superior tensile properties compared with their crustacean equivalents (Nawawi et al. 2019).

On the other hand, fungal chitin may have some disadvantages: fungal biomass has low chitin content and, as already said before, fungi does not synthesise pure chitin but it is associated with glucans. This makes it more difficult its characterization, but this could represent a great and unique opportunity of selecting desired functionalities not only for material sciences speculations. The impurity of fungal chitin can be seen as an opportunity because glucan presence offers a completely different structured and novel materials with new possibilities of application can be explored (Nawawi et al. 2016). Furthermore, extraction process of fungal chitin needs to be scaled up to industrial level and there is limited literature on this topic for myco-materials production (Nawawi et al. 2020).
In the past, it was proposed as the first application of this kind of material to use it as a skin substitute (Su et al. 1997; Su et al. 1999). In recent years, further prospected applications of fungi based materials, some of which have already reached the market, include paper (Nawawi et al. 2020), packaging and construction materials (Jones et al. 2020) or materials alternative to animal leathers (Jones et al. 2021, Nam et al. 2019).
Araki et al. (2012) prepared nanocomposite gels containing chitosan as a network polymer and chitin nanowhiskers as reinforcing fillers, reaching Young's modulus of 169 kPa, and stress at break of 135 kPa. There are few studies in the literature concerning the mechanical characterization of chitin-based materials (Araki et al. 2012, Jones et al. 2020, Appels et al. 2018, Elsacker et al. 2019). It is extremely important to collect experimental results, both because of the few scientific works present, and because it is not expected that a biomaterial has the same reproducibility compared to synthetic materials, as regards the mechanical parameters.
The present study aims to develop and characterise the mechanical performances of fungal chitin-glucan based materials obtained from fungal strains with different cell wall composition. The goal is also to evaluate, for each tested strain, the correspondence of the tested mycelium cell wall chemical composition to the final physic-mechanical properties of the obtained material. With this aim, an experimental campaign, involving tensile mechanical tests, thermal and environmental analyses was carried out to explore the possible attainable performances. The presented results are fundamental in identifying the properties of chitin-based myco-materials, in order to start with the design of targeted industrial products for selected applications. Homogeneous, compact materials with rubber-like characteristics were obtained. They can be considered as viable alternatives to synthetic rubbers, for example in packaging, flooring or sound/mechanical damping applications.

Material and methods

Fungal strain selection on cell wall polysaccharides content basis

Twenty one fungal strains were characterised concerning cell wall composition through thermogravimetric analysis (TGA); these were selected out of all the fungal strains conserved in the Mogu Research Fungal Strain Collection (MRFSC), isolated from wood decay fungi (Cartabia et al. 2021). On the basis of the obtained results, 5 strains were then chosen for the present work as reported in Tab. 1. *Abortiporus biennis* (064-18) and *Fomitopsis pinicola* (117-19) strains are distinguished by high α-glucan content, *Fomitopsis iberica* (104-19) by high β-glucan content and *Coriolopsis gallica* (086-19) and *Stereum hirsutum* (073-18) were chosen within a group with high chitin content because of their higher growth rates.

Tab. 1: Fungal strains selected, based on cell wall polysaccharides content

Fungal species	**MRFSC code**	**Cell wall polysaccharides content (Cartabia et al, 2021)**
Abortiporus biennis (Bull.) Singer	064-18	rich in α-glucans
Coriolopsis gallica (Fr.) Ryvarden	086-19	rich in chitin
Fomitopsis iberica Melo & Ryvarden	104-19	rich in β-glucans
Fomitopsis pinicola (Sw.) P. Karst.	117-19	rich in α-glucans
Stereum hirsutum (Willd.)Pers.	073-18	rich in chitin

Fungal biomass cultivation

All the five tested strains were cultivated following the same procedure,, in flasks and in bioreactor. For each strain, four plugs (0,5 cm^2) were cut from actively grown cultures in 2% malt extract agar (MEA - Biokar Diagnostic) and transferred into a flask containing 100 ml of sterilised liquid broth of malt (2%), dextrose (0,5%), yeast extract (0,2%).

Then, after 7 days in a shaker incubator (181 rpm) at 25 °C in darkness, the biomass and the spent broth were blended in a sterilised metal blender and poured in a larger flask, containing 500 ml (for "flasks cultivation") or 1000 ml (for "cultivation in bioreactor") solution of the same broth. After three days, all the content of the flask was blended again in a sterile metal blender and, in case of "flasks cultivation", 33 ml of the resulted cream were poured in each of 9 flasks containing 1,2 l of the same liquid broth and then they were placed in a shaker incubator (181 rpm) at 25 °C for 7 days. For the "cultivation in bioreactor", all the resulted cream was poured in a 10 l bioreactor containing 10 l solution of the same broth. In this case, 2 ml of sunflower oil were added to limit the formation of foam. The bioreactor was set up at 25 °C and run for 7 days with saturation of oxygen fixed at 50%: this condition was maintained changing aeration 5-20 l/min and agitation of 80-150 rpm.

Alkali-insoluble molecules extraction and production of chitin-glucan based mats

The protocol used by Nawawi et al. 2016 and Jones et al. 2019 was followed with a slight modification: once the biomass was obtained, it was washed with water to remove residues of the medium and extracellular compounds produced by the fungus and filtered through nylon filtration bag. After washing, the fungal biomass was blended and cooked in hot water at 90 °C for 1 h to remove water soluble compounds. After partial cooling down, the cooked biomass was filtered and washed again several times with nylon filtration bags to obtain a clean biomass. At the end of the last washing cycle, in order to recover it from the bag, the biomass was drained as much as possible and then resuspended in 1,5 l of water in a blender to be homogenised again. After that, 3,5 % w/w of pure NaOH was added and incubated for 2 h at 65 °C in order to eliminate proteins and alkali-soluble carbohydrates. Then, once cooled down, the biomass was filtered again with nylon filtration bags. Several washing steps with water were necessary, until the pH was restored to neutral. The recovered drained biomass of alkali-insoluble molecules was resuspended in water. It forms the alkali-insoluble material (AIM slurry).

The last step consists in crosslinking the fibres. In 3 l of final volume 1% of acetic acid and 10 % of glycerol were added to the AIM slurry. The mixture was blended to avoid the coagulation of the biomass and to ensure a homogeneous reaction. Then, the resulting cream was incubated in a water bath at 60 °C for 4 h.

After the crosslinking, the slurry was poured into a vacuum table (A4 dimension) prepared with a filtration system. Once there was no more water dropping, vacuum was disconnected and the solid cake was transferred to a plastic foil; filter paper was removed and the cake was left for drying for at least 48 h, until the piece was finally dry and self-supporting.

Thermogravimetric Analysis (TGA) of the obtained material sheets

TGA measurements were carried out by a TGA Q5000 instrument (TA Instruments, New Castle, DE, USA). Before testing, parts of the obtained material sheets were reduced in little particles in order to obtain a proper homogenization. Up to 5 mg were weighted per sample. Samples were heated from 25 °C to 900 °C at a scanning rate of 20 °C min^{-1} under nitrogen flux (25 ml min^{-1}). Standards of β-glucan and chitin (both from Sigma Aldrich) were analysed in the same conditions, allowing the comparison with the fungal mass loss at different temperatures.

Thermogravimetric measurements were analysed together with their first derivative (DTGA) in order to better estimate the temperature of the decomposition peaks (relative maximum of DTGA) and to locate the start/end of the decomposition (zeroing or flattening of DTGA).

Mechanical characterization of chitin-glucan based materials

No standards are currently available for the mechanical characterization of mycelium-based materials, so a standard referring to animal leathers was selected as a reference for conducting the tests. In particular, EN ISO 3376 was adopted as reference standard.

A MTS Insight Electromechanical Testing Systems 10 kN (MTS System Corporation), equipped with a 250 N load cell, was used to conduct the tests. Tests were carried out at normal environmental conditions and under displacement control. The pneumatic grips were set 50 mm apart from each other. Dog-bone specimens indicated in the standard EN ISO 3376 are unsuitable for this type of materials. In fact, cracks can form at the curved parts of the sample during cutting. For this reason, rectangular specimens (110 mm × 10 mm) were used. A uniform speed of separation of the jaws of 2 mm/min was adopted, to ensure quasi-static conditions. For each material, six samples were tested until failure (Fig. 1).

Before testing, each sample was measured with a digital calliper and weighted in order to estimate its density. The data were recorded with TestWorks version 4 software and subsequently subjected to a post-processing. Fig. 2 shows a representative uniaxial tension, stress/strain curve.

(a) (b)

Fig. 1: Clamped sample (a) before, and (b) after the test. The images concern a specimen of the material obtained from 073-19.

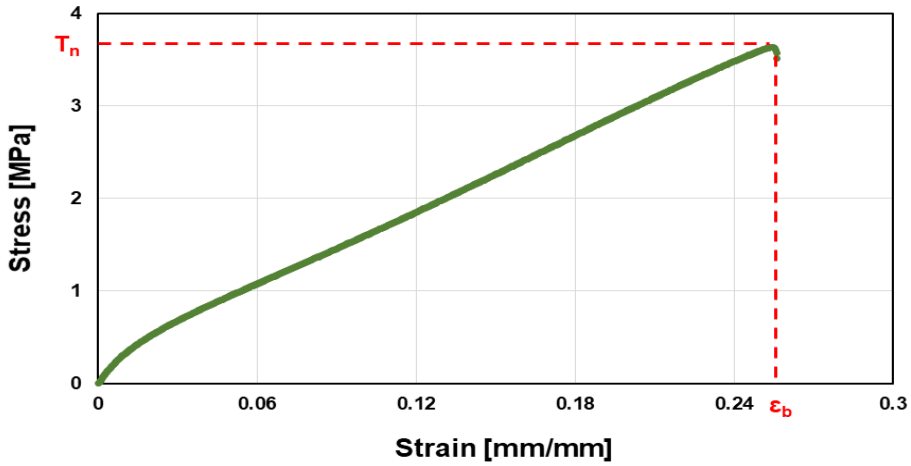


Fig. 2: Representative stress/strain tensile test curve of the myco-materials.

The tensile strength Tn (in MPa), i.e. the maximum stress a material can withstand during elongation before breaking, is defined as follows:

$$Tn = \frac{F}{w\,t}$$

where F is the highest force recorded during the test (in N), w is the specimen width (in mm), and t is the specimen thickness (in mm).
The percentage elongation εp to a specified load value is defined as:

$$\varepsilon p = \frac{L-L0}{L0}\,100$$

where L is the separation of the jaws and L0 is the initial separation of the jaws.
The elongation at break (εb) is the elongation in correspondence of Tn. Young's modulus E was calculated with the method of the secant modulus (Ifuku et al 2013), considering the deformation range between 0 and 0.15% of elongation with respect to the initial length of the specimen.

Elastomeric material obtained from *A. biennis* 064-18 fungal biomass in bioreactor allowed to produce specimens with higher thickness and were tested by Dynamic Mechanical Analysis (DMA) (tensile and shear) tests. Specimens with 0,8 mm thickness were produced and employed in the tests. DMA allowed to characterise the viscoelastic response of the material; in particular, the stiffness variation and the mechanical dissipation capacity as function of temperature were measured.
DMA tests in tensile and in shear mode (plate-plate geometry) were performed by a TA 2980 Dynamic-mechanical analyser and by an AR 2000ex Rheometer (TA Instruments) at 1 Hz frequency. Storage (E', G') and loss (E", G") moduli were measured as function of temperature in the 20 °C/200 °C range; the mechanical damping factor tan δ was also measured.
Differential Scanning Calorimetry (DSC) analysis was carried out on the same material by a DSC 2010 (TA Instruments) at 10 °C/min heating/cooling rate; repeated heating/cooling cycles were applied in the tests to investigate the nature of the recorded thermal events.

Results

Fungal biomass cultivation

As shown in Tab. 2, biomass production (g/l) in bioreactor is always higher than in flasks. In particular, more than two times in the case of *A. biennis* 064-18 and *S. hirsutum* 073-18. The explanation may be attributed to the fact that cultivation in bioreactor allows to keep monitored different parameters as pH, dissolved oxygen in the growing medium, aeration and agitation, thus creating the optimal conditions for growth.

Tab. 2: Fungal biomass production through two different procedures

Content	Strain	Cultivation	Biomass production		
			g/l	g/l*d	dry biomass (g)
High α-glucans	064-18	Bioreactor	8,56	1,43	73,3
		Flasks	3,63	0,61	38,4
	117-19	Bioreactor	3,7	0,62	37
		Flasks	2,96	0,49	31,9
High β-glucans	104-19	Bioreactor	5,13	0,70	51,3
		Flasks	4,19	1,86	44,9
High chitin	073-18	Bioreactor	11,17	2,79	111,7
		Flasks	5,58	1,40	60
	086-19	Bioreactor	2,44	0,41	24,4
		Flasks	2,33	0,18	25

Alkali-insoluble molecules extraction and production of chitin-glucan based mats

The results obtained in the production of mats from the different sources/processes are reported in Table 3. During the treatment of the biomass obtained in liquid culture, problems occurred for *C. gallica* 086-19 and *F. pinicola* 117-19. This was due to the mycelium behaviour that made the filtration and the washing steps impossible. It was observed that, regarding *S. hirsutum* 073-18, there was a higher solid reduction concerning the biomass obtained in bioreactor while for *A. biennis* 064-18 there was a higher solid reduction concerning the biomass obtained in flasks.

Tab. 3: Material production

Content	Strain	Cultivation	Percentage of solids reduction after cooking & NaOH	thickness ± st. dev. (mm)	grammage (g/m^2)
High chitin	073-18	Bioreactor	82	0,3±0,012	388,5
		Flasks	66	0,27±0,01	328,5
	086-19	Bioreactor	nd	nd	nd
		Flasks	nd	nd	nd
High α-glucans	064-18	Bioreactor	59,4	0,793±0,051	1048,2
		Flasks	68,25	0,265±0,13	308,5
	117-19	Bioreactor	nd	nd	nd
		Flasks	nd	nd	nd
High β-glucans	104-19	Bioreactor	43,02	0,298±0,015	388,7
		Flasks	nd	nd	nd

Thermogravimetric Analysis (TGA) of the obtained material sheets

Each material obtained from the strains 073-18, 064-18 and 104-19 was analysed by thermogravimetric analysis (TGA) in order to compare the initial composition of each mycelium (grown in liquid culture) and the corresponding resulting material after all the processing.

Strain diversity in the chemical composition of the materials was generally confirmed after the biomass processing. In particular, *S. hirsutum* 073-19 confirmed the highest chitin amount and *A. biennis* 064-18 confirmed the higher α-glucan amount (Fig. 3).

The addition of glycerol could influence the TGA results, since its degradation temperature (236 °C) is close to β-glucan's one. As expected, the concentration of alkali insoluble polymers generally increased in the material, demostrating the effectiveness of the process. From the TGA curves, it could be clearly seen that the materials obtained from *A. biennis* 064-18 degrade at lower temperatures and are well differentiated from the others (Fig. 4, Fig. 5).

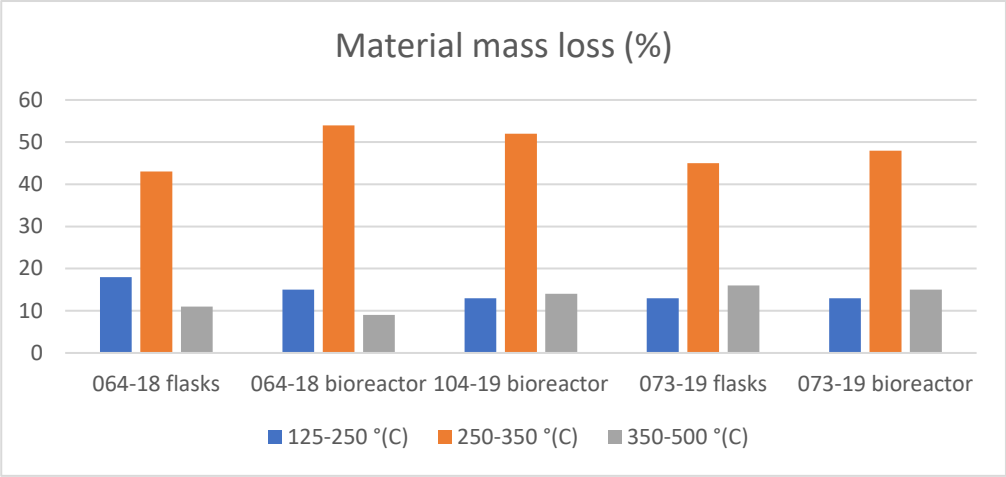


Fig. 3: Percentage of samples mass loss divided in different thermal ranges

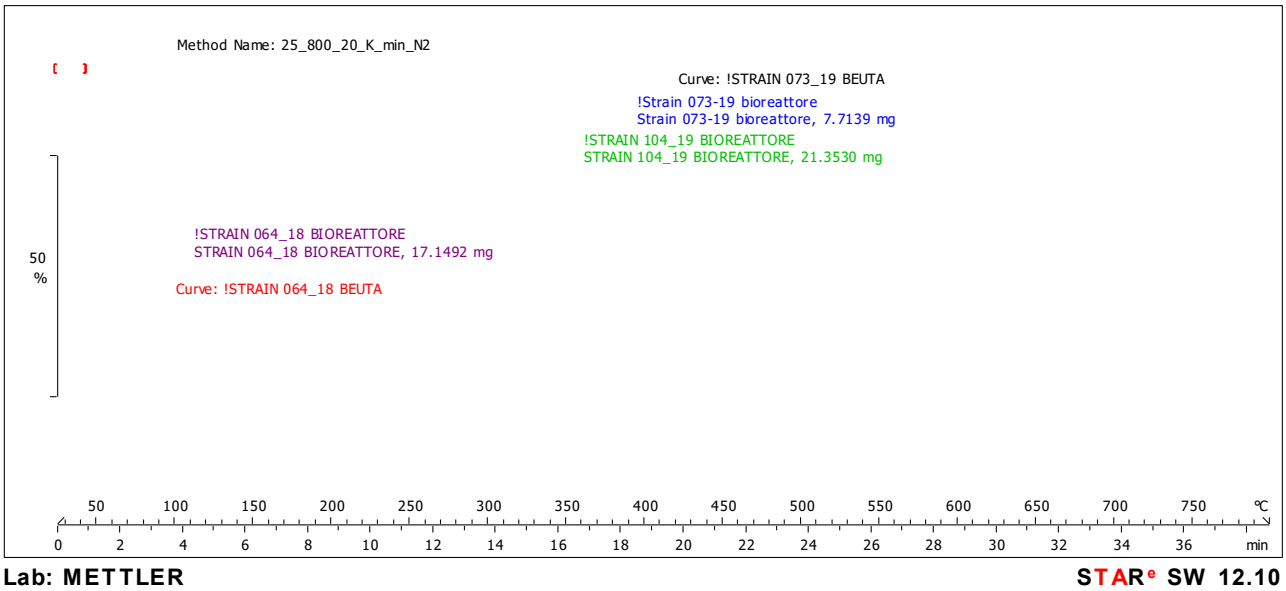


Fig. 4: Obtained TGA curves showing differences of temperature degradations among the materials

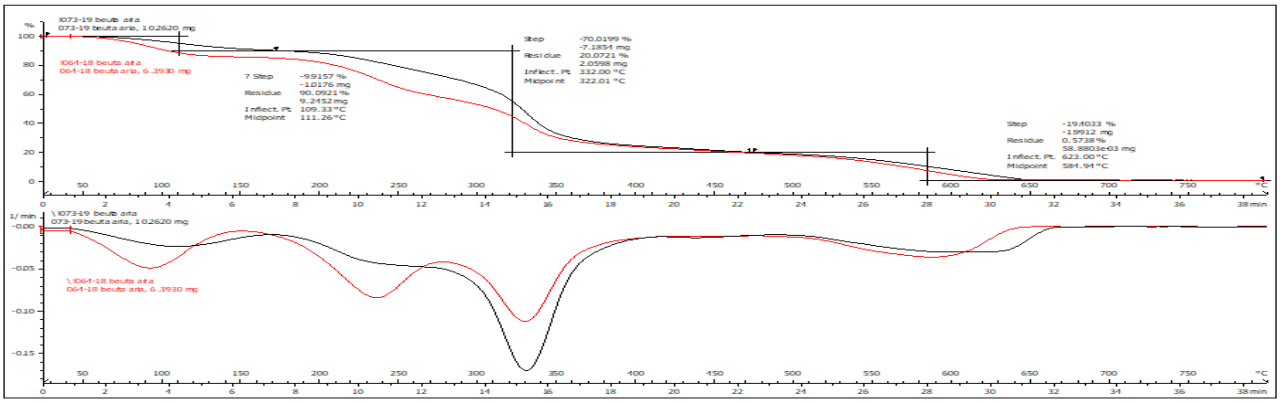


Fig. 5: TGA obtained curve and related primary derivative of materials obtained with *Abortiporus biennis* 064-18 strain

Mechanical tests

The values of T_n, ε_b, and E, obtained for each material group, are reported in Fig. 6, Fig. 7 and Fig. 8. The stress/strain curves were well reproducible for all the myco-materials.
The average tensile strength varied from 1,3 to 8,48 MPa (Fig. 6). The results show that the use of flasks instead of bioreactor increases the tensile strength by an amount of 1,3 MPa for the material obtained from *A. biennis* 064-18 and by an amount of 2,21 MPa for the material obtained from *S. hirsutum* 073-2019. Among all, the highest tensile strength was achieved for *S. hirsutum* 073-18 grown in flasks, while *A. biennis* 064-18 cultivated both in bioreactor and flasks showed the lowest tensile strength.
The average elongation at break ε_b varies from 19,54 to 52,96 % (Fig. 7). Considering *A. biennis* 064-20, an increment of 5% in the elongation at break is observed for the material obtained from mycelium cultivated in bioreactor compared to the material obtained from mycelium grown in flasks. Regarding *S. hirsutum* 073-18, a decrement of 11% in the ε_b is observed for the material obtained from mycelium cultivated in bioreactor compared to the material obtained from mycelium grown in flasks.
In general, the material that stretches the most before breaking was obtained from *S. hirsutum* 073-18 biomass cultivated in bioreactor.
The average Young's modulus varies from 16,21 to 87,47 MPa (Fig. 8). The material obtained from *S. hirsutum* 073-18 biomass cultivated in flasks was the stiffer and it showed a high variability among the six tested samples.
The experimental setup confirmed to be suitable and efficient for conducting the tensile tests on these types of material. Moreover, it was also confirmed that the fungal species, as well as the cultivating procedure, influence the mechanical properties; in particular, the materials produced with flasks appear more resistant to tensile tests.

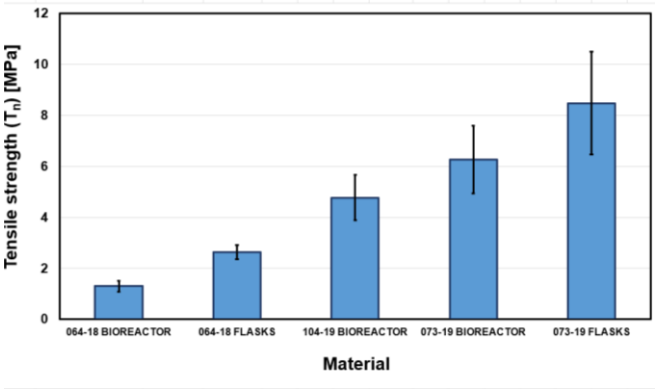


Fig. 6: Maximal average tensile strength reported by chitin-glucan myco-materials.

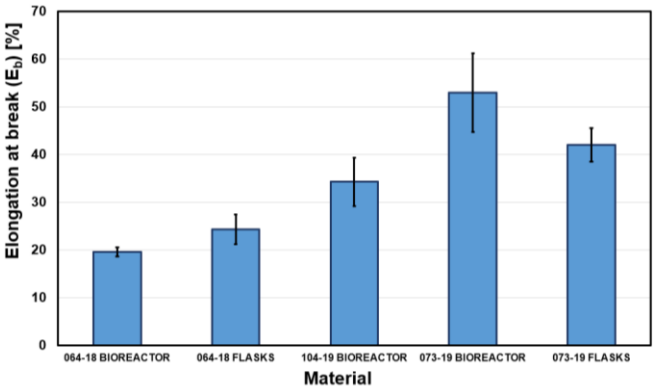


Fig. 7: Maximal average elongation at break reported by chitin-glucan myco-materials.

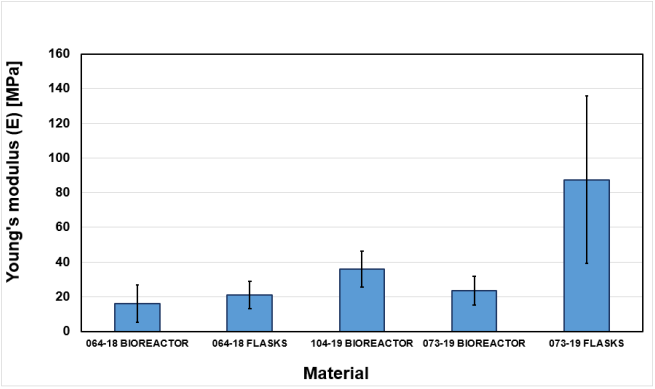


Fig. 8: Maximal average Young's modulus reported by chitin-glucan myco-materials

DMA results of *A. biennis* 064-18 as function of temperature showed that the tested material maintained a soft, elastomeric character from room temperature up to above 140-150 °C (Fig. 9), when an incipient stiffening effect occurs. This is possibly the consequence of absorbed humidity loss; as a matter of facts, a weight reduction of about 45% was measured after desiccation at 150 °C. Loss of glycerol plasticizer may also occur in the high temperature range. A similar behaviour was observed also in shear mode which, as expected, confirmed the persistence of higher stiffness also on cooling after a first heating ramp (Fig. 10). It is interesting to observe that the damping factor tan δ is steadily in the range 0,15-0,20 which is consistent with the values usually presented by synthetic rubbers above Tg.

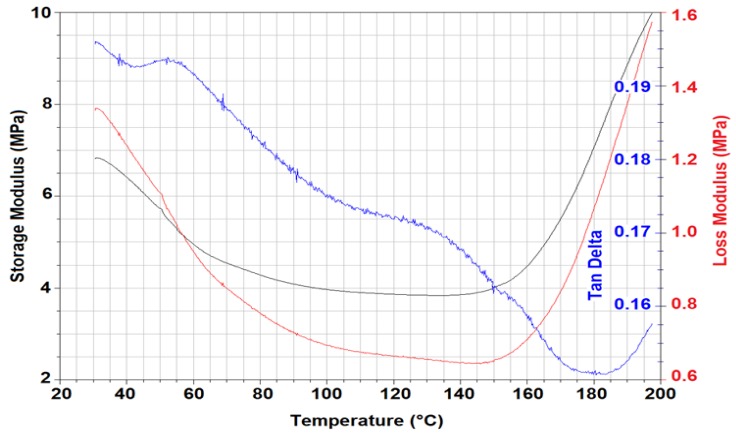


Fig. 9: Elastomeric material from chitin extraction (*A. biennis* 064-2018 cultivated in bioreactor). Dynamic mechanical properties (storage and loss moduli, tan δ) as function of temperature – tensile geometry

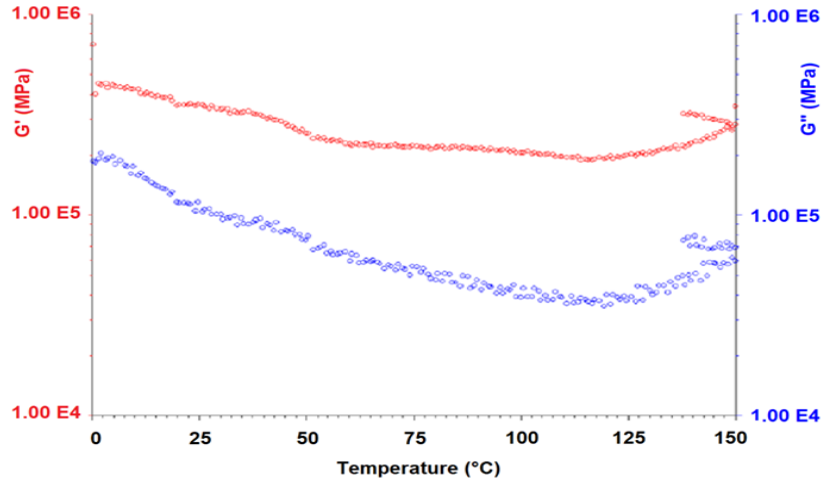


Fig. 10: Elastomeric material from chitin extraction (*A. biennis* 064-18 cultivated in bioreactor). Dynamic mechanical properties (storage and loss shear moduli, tan δ) as function of temperature – plate-plate geometry. The upper points between 150 °C and 140 °C show the cooling results after heating.

DSC tests showed an endothermal peak at 140 °C in the first heating run, which is no more present in subsequent cycles (Fig. 11). This is indication of absorbed water loss, supporting the previous observations of DMA results. However, the relatively high temperature of this loss, well above 100 °C, suggests that water molecules are possibly bonded to polar sites of chitin-glucan molecular structure and that the material can preserve a stable elastomeric character over a wide temperature range.

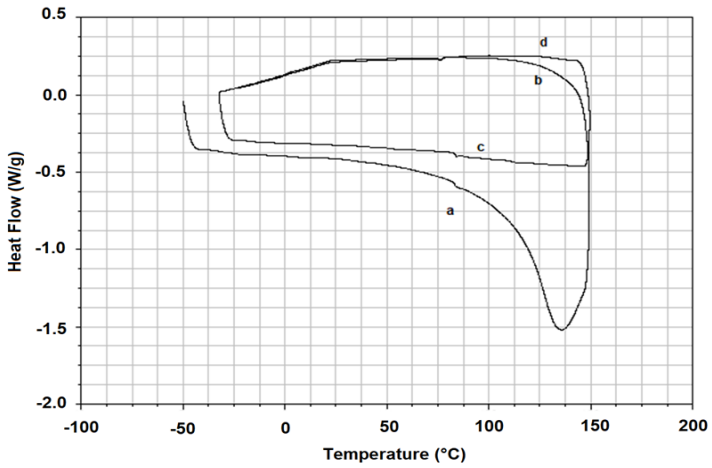


Fig. 11: DSC tests of elastomeric material from chitin extraction (*A. biennis* 064-18 cultivated in bioreactor). (a) and (c): 1st and 2nd heating runs; (b) and (d) 1st and 2nd cooling runs (after heating).

Conclusions

For future applications on a larger scale, regarding fungal strain selection it is necessary to take into account not only the high content of target bio-polymers but also the fungal strain growth rates, biomass production in liquid shacking culture and the biomass reduction after the chitin-glucan extraction treatment. Also, due to unsatisfactory growth rates and physical impossibility to complete all the steps for the bio-polymer extraction, only *A. biennis* 064-18, *F: iberica* 104-19 and *S. hirsutum*

073-19 produced material sheet samples. The use of flasks instead of the bioreactor could modify the growth modalities of the mycelium, changing the content of the hyphal cell walls. Thus, in order to confirm this observation, it could be useful to analyse directly mycelia grown both in flasks and in bioreactor.
The thermal characterisation of the material (TGA) generally allowed the evaluation of the concentration of the respective target bio-polymers after processing even if glycerol could influence the results; moreover, the results provided by this method (TGA) better correlate to the material mechanical performances.
Tensile tests showed that the different fungal species affects the material mechanical characteristics and that, within the same strain, as far as we could verify in our tests, mycelium grown in flasks produces a more resistant material than the one cultivated in bioreactor.
Mechanical testing campaign showed that the materials obtained from *S. hirsutum* 073-18 mycelial biomass are the most resistant (till 8,48 MPa before breaking) but also the most elastic (42-53% stretching), while the materials obtained from *A. biennis* 064-18 mycelial biomass, with the highest α-glucan content and the lowest chitin content, are the less resistant (1,3-2,6 MPa). These two fungal strains together with *F. iberica* 104-19 have actually to be considered as promising candidates for the production of chitin-glucan myco-materials.
In literature there are few works concerning the mechanical characterization of myco-materials. The study of their mechanical resistance is fundamental to investigate possible applications in which they can be used. For this reason, the present work is very relevant in this area. The results obtained enrich the knowledge and consequently the potential of this class of materials.

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Conclusions

The purpose of my PhD was to produce sustainable fungal-based materials testing different strains. These strains were selected because characterized by different specific features in order to find out guidelines aimed to correlate chemical fungal cell wall composition and mycelium structure, to mechanical properties of the resulting material.

Almost all conclusions have been stated in each specific chapter. The following here reported are the most relevant:

- Constitution of the Research Fungal Strain Collection of Mogu S.r.l. (MRFSC) formed by the 94 isolated and identified different strains of wood decay basidiomycetes.
- Development of a specific different isolation protocol modifying already existing techniques and mashing up suggestions from different previous public studies. This method allowed a very high isolation success rate (91,3%).
- Concerning fungal strains identification, *Ganoderma carnosum*, *Ganoderma lucidum*, and *Ganoderma valesiacum* confirmed to be cryptic species and, even if they could be recognised through micro- and macromorphological characters, ITS seemed not to be sufficient and a multigenic analysis is recommended to better define the lineage of these species. Besides, deeper investigation is needed for other strains, as *Antrodia cfr. favescens* 074-18, *Antrodia cfr. alpina* 134-19 and *Fibroporia cfr. albicans* 128-19, not all yet included in the already published/submitted papers. Further studies will be carried out to better understand their correct systematic placement.
- About mycelial structure of the selected strains, it is confirmed that the growing method influences both hyphal diameter dimensions and the mycelial porosity.

  In particular, Mogu's patented method at small scale (growth in sterile condition in 9 cm Petri dishes) improves both parameters of *Coriolopsis gallica* 086-18, *Fomitopsis iberica* 104-19 and *Fomitopsis pinicola* 117-19, while porosity is reduced for *Coriolopsis trogii* 027-18 and *Polyporus alveolaris* 096-19, and hyphal diameter is increased for *Abortiporus biennis* 064-18, *Fomes fomentarius* 079-18, *Terana caerulea* 177-19, *Trametes hirsuta* 067-18 and *Trametes suaveolens* 070-18.

  In parallel, at a bigger scale and in non-sterile conditions (closer to industrial/prototyping processes), *Abortiporus biennis* 064-18 and *Fomitopsis iberica* 104-19 generated a satisfying prototype. Then these fungal strains have actually to be considered as promising candidates for the production of myco-materials. More specifically, *Abortiporus biennis* 064-18 resulted to be more resistant.

- Concerning chemical composition, among the selected wood decaying fungal strains, *Abortiporus biennis* 064-18 followed by *Fomitopsis pinicola* 117-19 showed the highest α-glucan content (28% and 23% respectively); *Fomitopsis iberica* 104-19 and *Irpiciporus pachyodon* 175-19 showed the greater β-glucan content (71% and 62% respectively); *Coriolopsis gallica* 086-19, *Coriolopsis trogii* 027-18, *Daedaleopsis tricolor* 148-19 and *Stereum hirsutum* 073-19 showed the greatest chitin content (all 14%).
- As regards the selection of the most adapt strains for glucan-chitin materials *Abortiporus biennis* 064-18, *Fomitopsis iberica* 104-19 and *Stereum hirsutum* 073-19 were able to produce material sheet specimens, due to their satisfactory growth rates and physical possibility to complete all the steps of the used process. In fact it is necessary to take into account not only the high content of target bio-polymers but also the fungal strain growth rates and biomass production in liquid shacking culture for future applications on a larger scale.
- *Stereum hirsutum* 073-18, *Abortiporus biennis* 064-18 together with *Fomitopsis iberica* 104-19 have actually to be considered as promising candidates for the production of myco-materials also for the material typology based on mycelium chemical composition. In fact tensile test proved that fungal strain affects the material mechanical characteristics and that, within the same strain, as far as we could verify in our tests, mycelium grown in flasks produce a more resistant material than the one cultivated in bioreactor.

  Mechanical testing campaign on these three produced materials showed that the one obtained from *Stereum hirsutum* 073-18 mycelial biomass is the most resistant (up to more than 8 MPa before breaking) but also the most elastic (40-50% stretching), while the one obtained from *Abortiporus biennis* 064-18 mycelial biomass is the less resistant (up to around 2 MPa).
- Comparing the two kind of pure mycelium flexible materials:
    - It is interesting to note that *Abortiporus biennis* 064-18 and *Fomitopsis iberica* 104-19 have actually to be considered promising candidates for the production of both the two myco-material typologies.
    - regarding tensile strength, the material based on chemical composition always resulted to be more resistant: from 2 to 16 times than the other one.

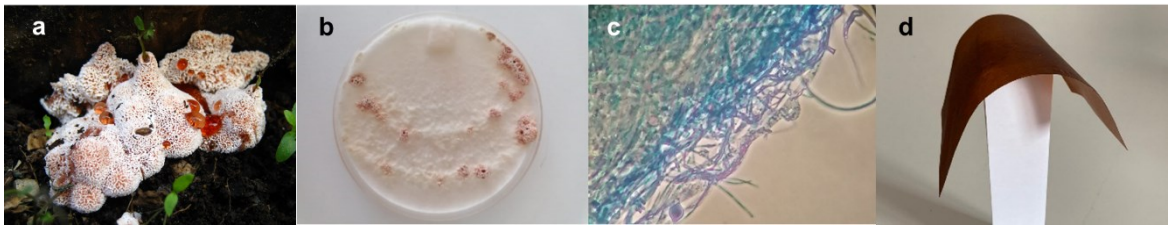

Fig. 10: *Abortiporus biennis* basidiome (a), mycelium in MEA Petri dish (b, c), final material (d)

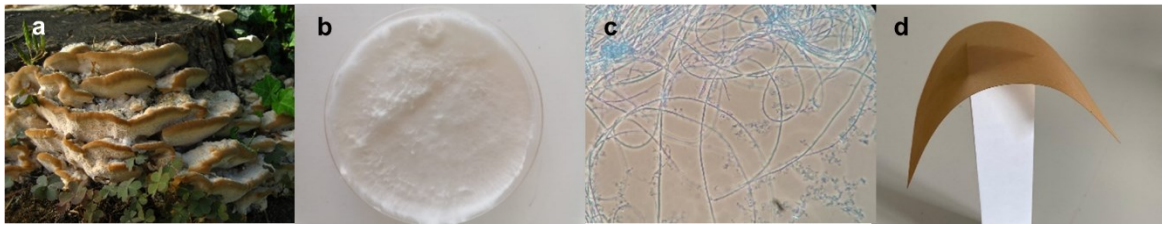

Fig. 11: *Fomitopsis iberica* basidiome (a), mycelium in MEA Petri dish (b, c), final material (d)

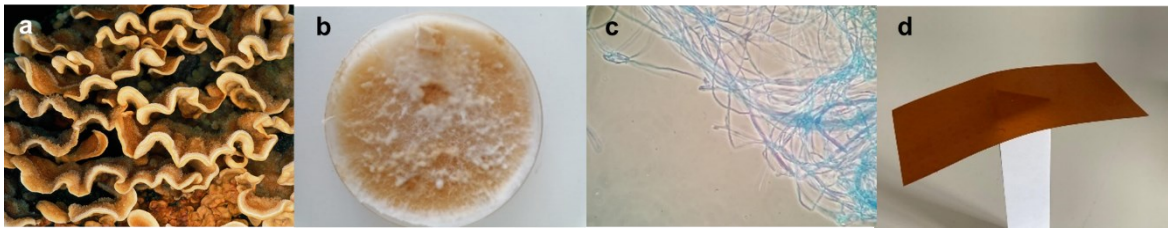

Fig. 12: *Stereum hirsutum* basidiome (a), mycelium in MEA Petri dish (b, c), final material (d)

As general further considerations for possible future research steps, it must be taken into account that the two obtained material typologies still cannot be used as they are, but they need further chemical treatments in order to be applied in practical applications. Furthermore, material durability is another important aspect and it must be considered after the mentioned final further treatments. Actually, biodegradability is a double edged weapon: from an environmental point of view it should be the most rapid as possible, but of course the material life shouldn't be too short and the material has not to be altered by biotic or abiotic agents (insects, moulds, temperature, humidity…) at least in normal dry environmental conditions. So, a compromise has to be found between these two needs.

In the end it can be stated that the row materials produced can be actually considered sustainable. In fact, if they are compared to natural leather, the required production time and the global volume of water needed are far less. Whilst, if they are compared to synthetic leather, the process is longer but eco-leather, because it is synthetic and not bio-degradable, is not eco-friendly at all. Furthermore, eco-leather durability is anyway often much shorter than natural leather.

Many other research activities could actually be developed in the future to improve existing myco-material typologies, but I think that this project represents a valuable and in-depth analysis of alternative suitable wood decaying fungal strains knowledge and a useful basis for any further study on this topic.

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