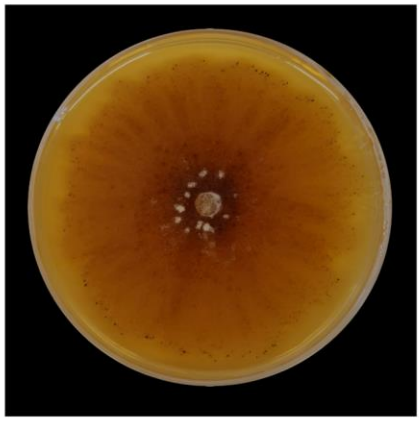
SAPIENZA
UNIVERSITÀ DI ROMA

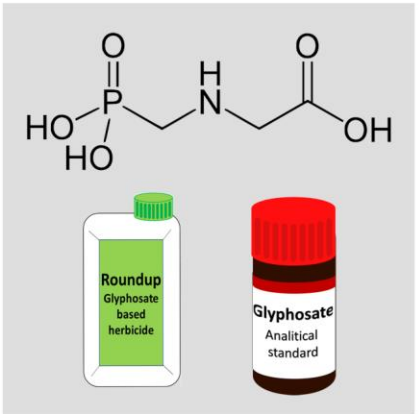
PhD in Environmental and Evolutionary Biology
34th Cycle – Curriculum Botany

Bioresources for a sustainable agriculture: potentialities of *Minimedusa polyspora* and *Chaetomium globosum* as plant growth promoting fungi

Veronica Spinelli

Supervisor: Prof. Anna Maria Persiani





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"LIFE WOULD NOT LONG REMAIN POSSIBLE IN THE ABSENCE OF MICROBES"

Louis Pasteur

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Summary

Feeding a constantly growing global population, while facing global change, without further impairing the environment is probably the greatest challenge our society is facing nowadays.

Modern agriculture mostly depends on the use of agrochemicals, including chemical fertilizers, pesticides and herbicides, due to their determinant role in enhancing efficiently and economically crop production, to meet the constantly increasing food demand. However, modern agriculture pressures determine major detrimental impacts on the environment at different spatial and temporal scales, on all the natural matrices: air, soil, and water. Consequently, mitigating agriculture's impacts on the environment represents an urgent need and a key strategy towards sustainability. Furthermore, this challenge is also concomitant with two other major challenges: increasing food production up to 60% by 2050 due to the world population growth, and adapting to a rapidly evolving climate change. In fact, due to climate change effects, plants are already more frequently subjected to severe abiotic (e.g. drought, flooding, extreme temperature) and biotic (e.g. pathogens and pest outbreaks) stresses, while future scenarios foresee these phenomena to become even more severe.

In this context, plant growth promotion represents an interesting sustainable solution that may play a key role in increasing crop resilience and productivity in adverse environmental conditions, minimizing agrochemicals applications and tackling climate change effects. Indeed, in healthy ecosystems soil microorganisms, through the wide array of ecosystem services they provide, express a multifunctionality that support soil productivity and plant growth. In particular, microbial strains with high soil colonization potential and multiple plant growth promoting traits — such as the ability to stimulate the plant, increase nutrient availability, exert biocontrol activity against detrimental microorganisms, and biodegrade organic pollutant and detoxifying inorganic pollutants — present a higher efficacy due to their multipurpose applicability. In this context, fungi as multifunctional microorganisms, perfectly adapted to soil microhabitats, thanks to their functional traits, metabolic plasticity and highly potent and relatively non-specific enzymes, represent valuable and effective potential bioresources.

This thesis aimed to characterize *Minimedusa polyspora* (Hotson) Weresub & P. M. LeClair and *Chaetomium globosum* Kunze, two strains of soil saprotrophic fungi, for multiple activities — including biostimulation, biocontrol and bioremediation — that may contribute to plant growth promotion, to assess their potential as multifunctional bioresources for biotechnological application aimed at promoting a more sustainable agriculture. Therefore, following a general introduction and literature review on the

topic, three chapters, each one addressing these species characterization for a specific activity that may contribute to plant growth promotion, are reported.

The first study presented in this thesis focused on assessing the efficacy of *M. polyspora* and *C. globosum* culture filtrates as biostimulant for the cultivation *Cichorium intybus* (L.), a plant of agricultural and medicinal interest. In a pot experiment set up in walk-in chambers, chicory plants, one month after the transfer of the seedlings in pots, were stimulated by soil drenching with 8 ml/pot (30 ml/kg of soil) of the culture filtrates obtained by a 14-days incubation of the fungal strains in Malt Extract Broth (MEB), or the same amount of uninoculated MEB in the control group. Fourteen days after the stimulation, plant biomasses were recovered to estimate several growth parameters and analyze the metabolomic variations occurred in roots and leaves through ^{1}H-NMR 600 MHz.

We observed for the first time that *M. polyspora* culture filtrate promotes an increase of biomass, both in shoots and roots, and of the leaf area, while no increase was observed in plants treated with *C. globosum* culture filtrate. Based on ^{1}H-NMR metabolomics data, differential metabolites and their related metabolic pathways were highlighted. A common response in *C. intybus* roots involving the synthesis of 3-OH-butyrate through the decrease of the synthesis of fatty acids and sterols, as a mechanism balancing the NADPH/NADP^{+} ratio, was observed in both the treatments with *C. globosum* and *M. polyspora* culture filtrates. The phenylpropanoid pathway was differently triggered by the fungal culture filtrates. *C. globosum* culture filtrate increased phenylalanine and chicoric acid in the roots. Chicoric acid, whose biosynthetic pathway in chicory plant is putative and still not well known, is a very promising natural compound playing an important role in plant defense. Instead, *M. polyspora* culture filtrate interestingly stimulated an increase of 4-OH benzoate, being benzoic acids precursors for a wide variety of essential compounds playing crucial roles in plant fitness and defense response activation. Therefore, both *C. globosum* and *M. polyspora* culture filtrates affected *C. intybus* metabolome and, based on the findings of this study, could be considered as promising fungal bioresources for further studies aimed the development of new biostimulants.

Moving on, in the second study presented in this thesis, *M. polyspora* and *C. globosum* biocontrol potential against the phytopathogenic fungi *Alternaria alternata* (Fr.) Keissl., *Berkeleyomyces basicola* (Berk. & Broome) W.J. Nel, Z.W. de Beer, T.A. Duong & M.J. Wingf. and *Botrytis cinerea* Pers. was investigated.

Plant diseases, resulting in an annual estimated loss of 10–15% of world's major crops, represent a major threat to global crops production and social and political stability of nations. 70–80% of these diseases are caused by pathogenic fungi, numbers that are expected to increase in future years due to the effect of climate change on plant-pathogens interactions. In the effort to transition to a more sustainable and resilient agriculture, the application of biological control agents and their secondary metabolites

represent a promising option to support the achievement of food security, without further compromise ecosystems' health. Therefore, it is important deepening the potential of known fungal biocontrol agents against the existing fungal pathogens, shedding further light on their action mechanisms and discovering new efficient fungal strains suitable for biotechnological applications. I*n vitro* screenings, despite presenting several limitations, constitute valuable methods for the identification of potential biocontrol agents. Therefore, through an array of *in vitro* plate assays *M. polyspora* and *C. globosum* were assessed for their ability to inhibit *A. alternata*, *B. basicola* and *B. cinerea* growth, aiming also to gain insight on possible antimicriobial mechanism/s involved in their biological control action. More specifically, a dual culture assay, a dual culture for volatile antimicrobial compounds (performed in two different conditions), and a culture filtrate antifungal activity assay were designed to try to discriminate the impact of direct and indirect biological control mechanisms. This study results show that both *M. polyspora* and *C. globosum* were able to inhibit, to a different extent, all the pathogens' growth in the dual culture assay, suggesting a mechanism of biocontrol involving competition for nutrients and space. *M. polyspora,* based on the culture filtrate antifungal activity assay, was found to exert its inhibition on all the pathogens thanks also to an antibiosis mechanism through the release of diffusible compounds. Moreover, *M. polyspora* culture filtrate resulted to be particularly effective especially against *B. basicola* whose growth was completely inhibited; furthermore, its high inhibition effect against this species was also observed in the dual culture for volatile antimicrobial compounds assay, suggesting that *M. polyspora* antagonism against *B. basico*la occurs through multiple or mixed mechanisms. Therefore, based on this preliminary study's results M. *polyspora and C. globosum* are promising biocontrol agents of three fungal phytopathogens of economical and agronomical relevance, and consequently species of interest for further studies in this area aimed at validating their potential as antagonists in *in vivo* conditions.

Finally, the last study focused on evaluating *M. polyspora* and *C. globosum* bioremediation potentialities towards glyphosate. Addressing, in particular, their ability to tolerate and utilize glyphosate as a nutritional source and eventually degrade it. Indeed, glyphosate is the most commonly used herbicide worldwide. Its improper use during recent decades has resulted in glyphosate contamination of soils and waters. Fungal bioremediation is an environmentally friendly, cost effective, and feasible solution to glyphosate contamination in soils. In this study, *M. polyspora* and *C. globosum* together with other 16 saprotrophic fungal strains were screened *in vitro* for their ability to tolerate and eventually utilize Roundup at two different concentrations (1 mM and 10 mM) in different cultural conditions as a nutritional source. *M. polyspora* and *C. globosum* were found to be tolerant to RoundUp, a glyphosate-based herbicide, only at the concentration of 1 mM, while a concentration of 10 mM completely inhibited

their growth. Moreover, *Purpureocillium lilacinum* was further screened to evaluate the ability to break down and utilize glyphosate as a P source in a liquid medium. The dose-response effect for Roundup, and the difference in toxicity between pure glyphosate and Roundup were also studied. This study's results highlight the ability of several strains to tolerate 1 mM and 10 mM Roundup and to utilize it as a nutritional source. *P. lilacinum* was reported for the first time for its ability to degrade glyphosate to a considerable extent (80%) and to utilize it as a P source, without showing dose-dependent negative effects on growth. Pure glyphosate was found to be more toxic than Roundup for *P. lilacinum*. Our results showed that pure glyphosate toxicity can be only partially addressed by the pH decrease determined in the culture medium. In conclusion, despite the strains studied in this thesis were not able to degrade glyphosate, experimental results emphasized the *in vitro* noteworthy potential in glyphosate degradation of *P. lilacinum,* another fungal strain of biotechnological interest.

In conclusion, based on this thesis' results *M. polyspora* and *C. globosum* showed promising potentialities as plant growth promoting fungi and should be further studied as bioresources for eventual biotechnological applications towards a sustainable agriculture.

This thesis, in addition to the studies addressing its aim, includes also an additional section composed of three published papers dealing with topics regarding fungal species conservation applying IUCN red-listing criteria and the biotechnological potentialities of strains preserved in the culture collection of the Fungal Biodiversity Laboratory of the Department of Environmental Biology, Sapienza University of Rome.

CHAPTER 1: GENERAL INTRODUCTION

1.1 AGRICULTURE AND THE ENVIRONMENT: PAST, PRESENT, AND FUTURE CHALLENGES

Feeding a constantly growing global population, while facing global change, without further impairing the environment is probably the greatest challenge our society is facing nowadays.

Population growth and agriculture have been intrinsically linked since the advent of the latter. Indeed, starting from that moment and until the industrial revolution in the XVIII century, each period of rapid human population growth in history was related to agricultural innovations (Tian et al., 2021; Tilman et al., 2002). During the industrial revolution new technologies and production systems in agriculture were developed, and these determined an acceleration in farmland expansion. The new technologies such as irrigation systems, together with a wider application of fertilizers and pesticides determined a significant increase in yields (Tian et al., 2021). The process of intensification of agriculture was completed with the "Green revolution" in 1960s, when these high inputs of water and agrochemicals applied on a large scale were complemented by the selection of new high-yielding cereal varieties. This allowed in following decades/ 50 years to triple the global cereal production with only a 30% increase in cultivated land, determining an increase in the daily food supply per person with a consequent reduction of hunger and an increase in well-being (Pingali, 2012; Tian et al., 2021; Trivedi et al., 2021).

Therefore, modern agriculture mostly depends on the use of agrochemicals including chemical fertilizers, pesticides and herbicides, having a determinant role in enhancing efficiently and economically crop production, to meet the constantly increasing food demand (Mandal et al., 2020; Tian et al., 2021).

Despite intensification preserved new land from being converted to agricultural soils while assuring the enhancement of yields and productivity, it also determined a wide range of detrimental impacts on the environment (Maitra et al., 2022; Pingali, 2012).

Indeed, modern agriculture pressures determine major intrinsically linked to each other negative impacts on the environment at different spatial and temporal scales, on all the natural matrices: air, soil, and water. On the basis of spatial scale, agriculture's impacts can be divided in two groups: those whose effects are at the global scale, and those happening in discrete locations that, despite being present in most countries all over the globe, at present show no significant impact at the global scale (FAO, 2021).

Agriculture has heavy impacts on air pollution, first of all due to its large contribution to GHGs emissions. It has been estimated that greenhouse gas emissions (GHG)

emissions resulting from the 'Agriculture, Forestry and Other Land Use' sector, account for 21% of total global GHG emissions and energy inputs for the agrifood chain contribute an additional 10% (Calicioglu et al., 2019; FAO, 2017). Agriculture constitutes the largest source of global methane and nitrous oxide emissions. Enteric fermentation of ruminant animals together with rice cultivation are the main sources of agricultural methane emissions. Instead, nitrous oxide emissions mainly originate from the application of nitrogen-based fertilizers and animal manure management. Moreover, should also be kept in account the GHGs emitted during the production process of N chemical fertilizers, that at the current global rate of utilization account for 300 Tg of CO_2 per annum (FAO, 2003, 2017; Maitra et al., 2022). Finally, Agriculture contributes to air pollution with ammonia emissions from mineral fertilizer volatilization and livestock production, and with particulate matter and GHGs from land clearance by fire and the burning of rice residues (FAO, 2003).

Chemical fertilizers, herbicides and pesticides pollution together with overexploitation of water represent the main pressures of agriculture on this resource. Extensive leaching of nitrate and/or phosphate from soils into surface water and groundwater are common issues in almost all industrial countries. These leachates from diffuse sources, arising from mineral fertilizer and manure use on both crops and grasslands, pose a risk to human health and contribute to eutrophication of rivers, lakes and coastal waters (FAO, 2003; Mandal et al., 2020).

Pesticides pollution has heavy impacts both on water and soil quality, besides raising concerns on human health risks. Indeed, pesticides fate in terms of persistence, behavior and mobility is quite various (Figure 1.1). Pesticides can be transported to surface waters and groundwater through runoff and infiltration, causing pollution to water bodies and thereby reducing the usability of water resources. So, their over-application in past decades, has led to serious water pollution of surface water and groundwater in countries all over the world (Carvalho, 2017; Dhananjayan et al., 2020; FAO, 2003, 2021; Rodríguez-Eugenio et al., 2018).

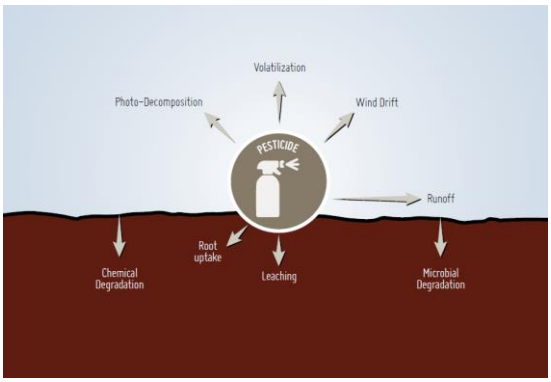

Figure 1.1 Behavior of pesticides in the environment (Rodríguez-Eugenio et al., 2018).

Most commercialized pesticides have been associated with health and environmental issues, leading also in some cases to the ban of certain pesticides for agricultural use. Exposure to pesticides can be through contact with the skin, ingestion, or inhalation, and the possible health outcome depends on the type of pesticide, the duration and route of exposure, and the individual health status (Carvalho, 2017; Nicolopoulou-Stamati et al., 2016). Moreover, since several pesticides are persistent organic pollutants that persist in the environment and bioaccumulate through the food chain, pesticides residues are increasingly found in foods and beverages of daily consumption, posing a major threat to human health to be continuously exposed to low levels over a lifetime (Carvalho, 2017; Dhananjayan et al., 2020; Nicolopoulou-Stamati et al., 2016; Rodríguez-Eugenio et al., 2018).

The problem of pesticide pollution happening worldwide is even worsened by the fact that different active ingredients show additive effects posing concrete risks in agricultural lands (Figure 1.2). Furthermore, pesticide mixtures can elicit synergistic toxicity in non-target organisms under both acute and chronic exposures, this constitutes an emerging global issue (Tang et al., 2021).

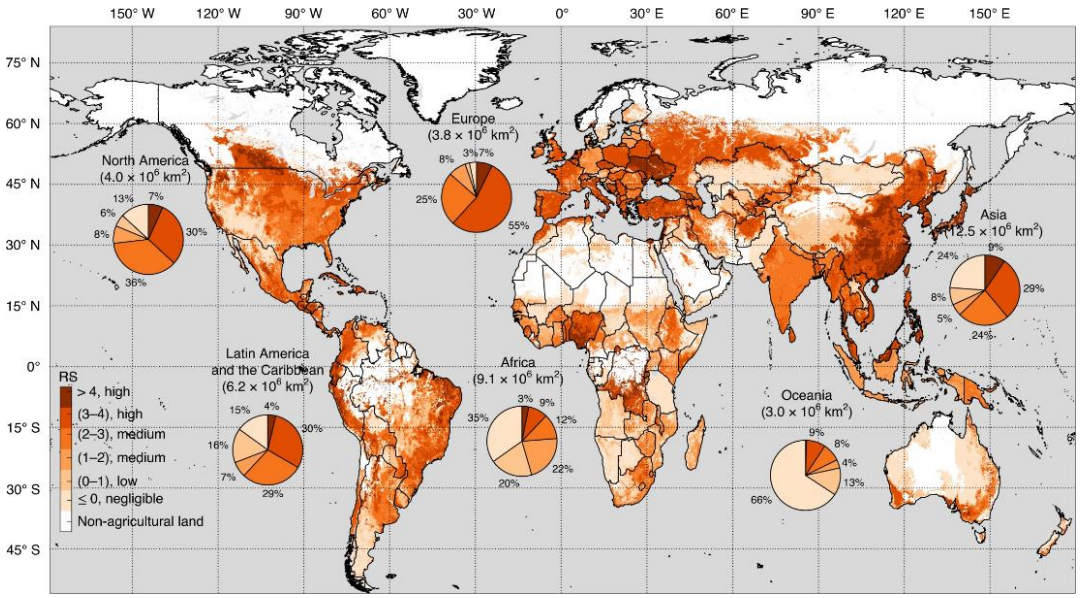

Figure 1.2 Global map of pesticide risk score. The map has a spatial resolution of 5 arcmin, which is approximately 10 km × 10 km at the Equator. The pie charts represent the fraction of agricultural land classed under different RS in each region, and the values in parentheses above the pie charts denote the total agricultural land in that region (Tang et al., 2021).

Pesticides of first generation being environmentally persistent, remained for long times in soils and sediments and accumulating in soil organisms. So, despite in 2002 persistent and bio-accumulative chemical compounds were banned by the Stockholm

Convention, due to their past massive application, these pesticides are still present in soils, in sediments, and in the biosphere.

Since their mechanism of action is not specific for target pests, their toxicity affects also on nontarget organisms causing negative effects on soil biodiversity and ecosystems health (Carvalho, 2017; Dhananjayan et al., 2020; FAO and UNEP, 2021; Mandal et al., 2020).

Finally, additional impacts on the environment due to pesticides are related to stocks of obsolete, unwanted, and banned pesticides that continue to represent a threat to human health, the environment and the sustainable development of the regions in which they are found (Rodríguez-Eugenio et al., 2018).

Besides pesticides, agricultural sources of soil pollutants (Figure 1.3) include fertilizers, manure, trace metals and radionuclides from agrochemicals, excess N and heavy metals. Moreover, even irrigation water may also represent a source of pollution when a poor-quality water, such as wastewater and that from urban sewage, is used (FAO and UNEP, 2021; Rodríguez-Eugenio et al., 2018; Tian et al., 2021).

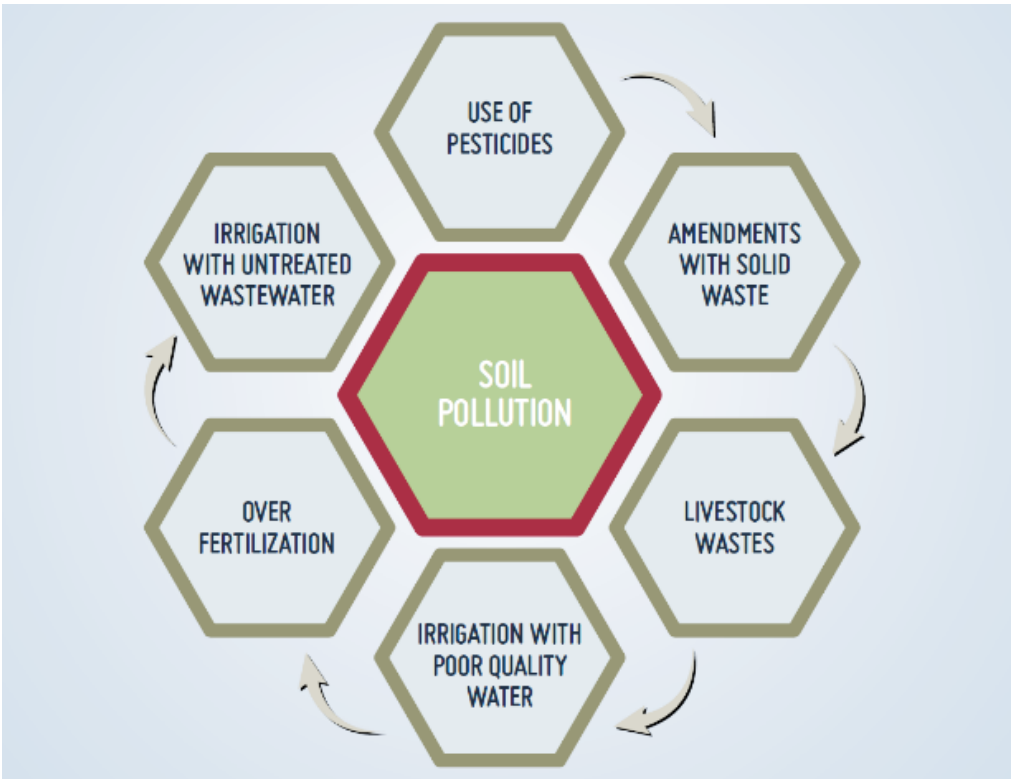

Figure 1.3 Agricultural sources of soil pollution (Rodríguez-Eugenio et al., 2018).

The main contributors to environmental issues linked to agriculture are the over-application of fertilizers and manure and the inefficient use of their main nutrients: N

and P. These two nutrients, in addition to their above-mentioned effects on air and water pollution, constitute a source of diffuse soil pollution. Moreover, nitrogen pollution affects microbial community composition and activities, therefore influencing soil organic matter decomposition, as well as soil acidity and salinity. Excess N in soil constitutes the main cause of soil acidification and salinization through nitrification and other N-transformation processes (Bach et al., 2020; Rodríguez-Eugenio et al., 2018).

Fertilizers have also been reported as a source of soil pollution concerning their content of many heavy metals including As, Cd, Cr, Hg, Pb, and Zn, and radionuclides such as ^{40}K, ^{238}U, ^{232}Th, ^{90}Sr and ^{137}Cs. So, fertilizers repeated applications can lead to a significant accumulation of potentially toxic elements into the soil (FAO and UNEP, 2021; Rodríguez-Eugenio et al., 2018).

Lastly, synthetic fertilizers have also been estimated to be one of the major drivers of land degradation.

According to Stocking (Stocking, 2001), land degradation is defined as the "temporary or permanent decline in the productive capacity of the land, and the diminution of the productive potential, including its major land uses (e.g. rain-fed arable, irrigation, forests), its farming systems (e.g., smallholder subsistence), and its value as an economic resource".

It has been reported that globally more than 33-34% of the land resources is already degraded due to various direct and indirect drivers of changes including also among the others: pollution due to rapid agriculturalization; unsustainable land use practices, deforestation, salinization, desertification, soil erosion, overexploitation of groundwater and other critical natural resources, and climate change (Abhilash, 2021; FAO, 2021). More specifically, concerning agricultural lands it has been estimated that globally almost 52% are degraded to different extent, and are thereby affecting the livelihood of almost 2.6 billion people, who directly depend on agriculture for their sustenance (Abhilash, 2021). However, since the assessment of land degradation is greatly hindered by lack of knowledge on the real status of several areas, these percentages are probably underestimated (Abhilash, 2021; FAO, 2021).

Land degradation represents one of the major but hardly predictable impact of agriculture. Indeed, since land degradation is very variable over small areas, impacts are highly site specific and can also be time-specific. Multiple are the factor determining different impact including soil type, topography, crop type and management practice, climate conditions, precipitation and further on. Additionally, while phenomena like forest clearance for cultivation, and cropping intensification of existing cultivated slopes both leading to soil erosions are easily identified, several other forms of land degradation, such as soil compaction, acidification and reduced biological activity, are difficult to address since they are not readily visible (Abhilash, 2021; FAO, 2003, 2017, 2021).

Land degradation, altering soil properties, also strictly affects soil multifunctionality including the soil's ability to store and cycle nutrients and therefore affects their biogeochemical cycles. Moreover, Agriculture in general plays a significant role in the anthropogenic perturbation of several biogeochemical cycles, especially the cycles of carbon, nitrogen, phosphate and sulphur (FAO, 2003, 2021; Kopittke et al., 2022). For example in the N cycle, agriculture determines considerable emissions of ammonia and nitrous oxide, but also the perturbation of N fixation, since the N fertilizers production, fossil fuels combustion and cultivation of leguminous crops have resulted in anthropogenic N fixation (FAO, 2003).

Phosphorus cycle is also heavily affected by its agricultural application. P application and its leachate to water represent a mechanism by which P gets removed by its cycle. Indeed, the phosphorus present in soil is leached and ends up, both as particulate and in the dissolved form, in the rivers that transport it to the sea (Filippelli, 2008). Most of the phosphorus, in the form of particles, present in rivers is retained within mineral networks and therefore does not participate in the active biogenic cycle. Therefore, much of the phosphorus eroded by the continents reaches the oceans relatively unaltered, where it hardly undergoes further alterations, since the dissolution rates in sea water are extremely low due to the high pH and the strong buffering capacity of bicarbonate, carbonate and hydroxide ions. The phosphorus, therefore, is sedimented on the continental margins and in the deep sea, thus being excluded from the cycle until the sediments are brought back to the surface, through geological phenomena of subduction or tectonic uplift, and altered. P removal from its active biogeochemical cycle represents a major threat since the world's known reserves of phosphate rock are being depleted, and it is estimated that global demand for phosphorus will exceed its supply by 2035 (Cordell et al., 2009; Filippelli, 2008).

Moreover, biogeochemical cycles are closely linked to each other, therefore the perturbation of one can have influences on the others. This is the case for example of the N, P and S that are deeply linked to C soil cycle through soil organic carbon (SOC). Indeed, land use change from natural to agricultural ecosystems, decreasing organic matter and nutrient return to the soil, accelerating SOC decomposition rates through tillage, and also increasing nutrient losses through runoff, erosion, volatilization and leaching, as well as through product removal, can alter or even disrupt nutrient cycling. Further detrimental effects on nutrient cycling determined by change to agricultural use can be cause by the reduction of organic materials diversity entering the soil, which consequently reduces food web diversity and thus nutrient cycling (Figure 1.4) (Geisen et al., 2019; Kopittke et al., 2022; Tsiafouli et al., 2015).

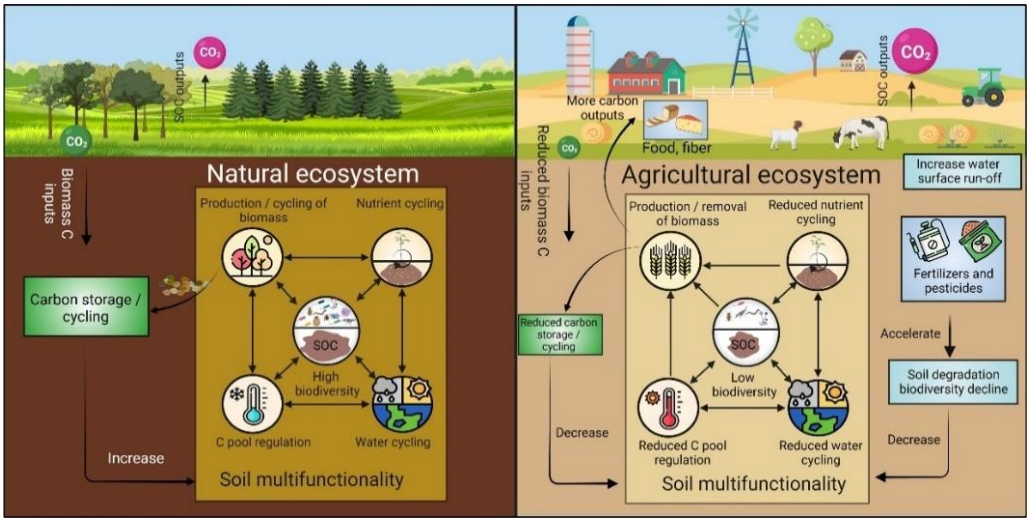

Figure 1.4 The effect of land-use change on soil multifunctionality. Note that in the natural system, the cycling of biomass contributes soil organic carbon (SOC) which increases multifunctionality. In contrast, in the agricultural system where much of the biomass is removed and inputs are decreased, the decrease in SOC stocks contributes to a decrease in soil multifunctionality (Kopittke et al., 2022).

All the above-mentioned agriculture's pressures on soil contribute to determine, and at the same time are influenced by, one of the most relevant impacts: soil biodiversity loss. Soil formation and maintenance are inextricably linked to its biodiversity, and so are the soil quality and the range of ecosystem services that it provides (Bach et al., 2020; FAO, 2020). Indeed, soil organisms, through their activity and complex interactions, play crucial roles in many ecosystem functions, including nutrient cycling, pathogen control, water infiltration, foundations to food webs, and supporting agroecosystems (Figure 1.5) (Adhikari and Hartemink, 2016; Bach et al., 2020; FAO, 2020; FAO et al., 2020; Geisen et al., 2019). Consequently, a loss of soil biodiversity may lead to a reduction of soil multifunctionality. Soil biodiversity structure is intrinsically determined by physical and chemical soil properties, as well as by interactions with other soil and aboveground biota. On local, regional, and global scales, the structure of soil biodiversity is predominantly determined by soil type, pH, carbon and nutrient contents, and soil moisture (Geisen et al., 2019). Therefore, soil pollution and its effects on soil characteristics have been widely reported to have negative impacts on soil biodiversity (FAO and UNEP, 2021). Moreover, effects of land-use change, including the use of soils for agricultural production, results in a marked decrease in soil organic carbon and soil biodiversity, and has been also reported that intensive agricultural production determines a great reduction in the complexity of soil food webs and mass of soil fauna (Geisen et al., 2019; Kopittke et al., 2022).

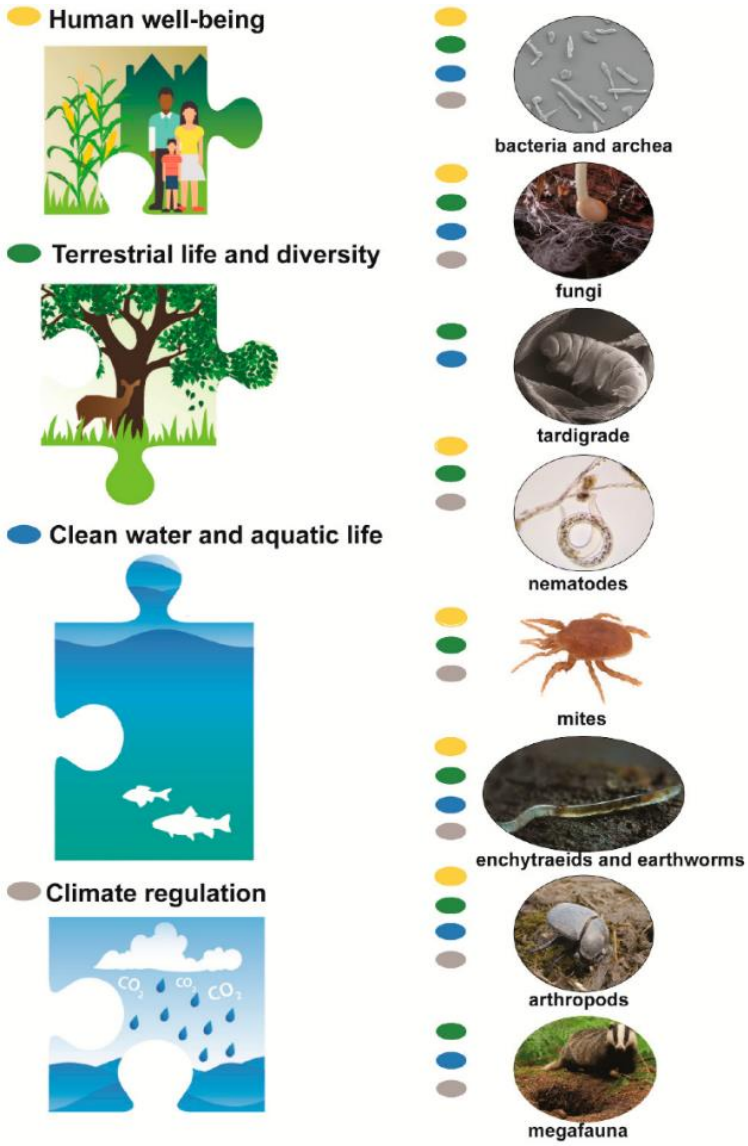

Figure 1.5 Soil organisms support multiple ecosystem services, which underpin global sustainability agendas. The colored circles next to each organism type represent which of the four overarching parts of sustainable development the organism contributes to directly (Bach et al., 2020).

All these agriculture's pressure on the soil determining its loss of diversity and multifunctionality, represent a major threat to the hospitability and survivability of our planet, aggravating the existential challenges that humanity is facing (Bach et al., 2020; FAO, 2021; Kopittke et al., 2022). Healthy soil, providing functions, supports the global existential cornerstones for the sustainable development of humanity: food security, water security, energy security, climate change abatement, biodiversity protection, and ecosystem service delivery (Figure 1.6).

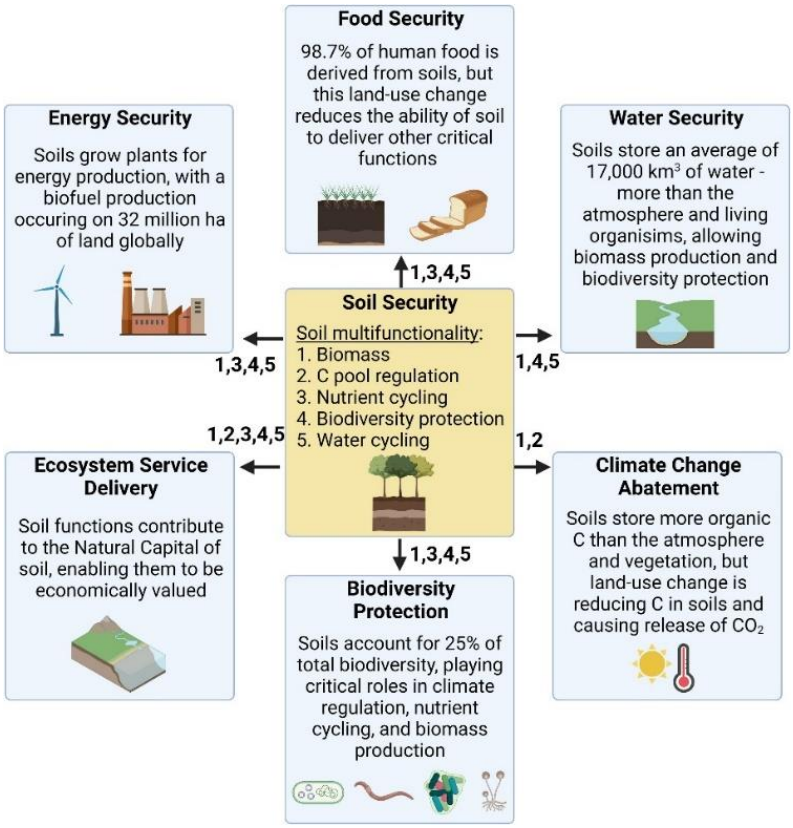

Figure 1.6 Alignment of soil functions to Soil Security and their direct impact on the existential challenges facing society (Kopittke et al., 2022).

However, global land-use change to agricultural use, is decreasing the ability of soils to provide functions since soils managed to increase delivery of one function show substantially reduced capacity to deliver other functions (Kopittke et al., 2022; Vazquez et al., 2021). Considering that estimates report that 98.7% of the daily calories consumed by humans have their origins in soils (2,895 kcal per capita), with only 1.3% from aquatic systems (38 kcal per capita) it is clear that only healthy soils, providing their functions and the necessary ecosystem services, can secure survivability for human societies (Kopittke et al., 2022; Rodríguez-Eugenio et al., 2018). Maintaining the vitality of soil is essential for environmental sustainability and therefore also for the realization of various global goals, since soils contribute to at least 12 out of the 17 United Nations Sustainable Development Goals affirmed in the 2030 Agenda (Figure 1.7)(Kopittke et al., 2022; United Nations, 2015). Assuring soil security and a timely restoration of degraded land are keys actions to improve ecosystem resilience, and ensure future food security of the growing global population (Abhilash, 2021; Kopittke et al., 2022).

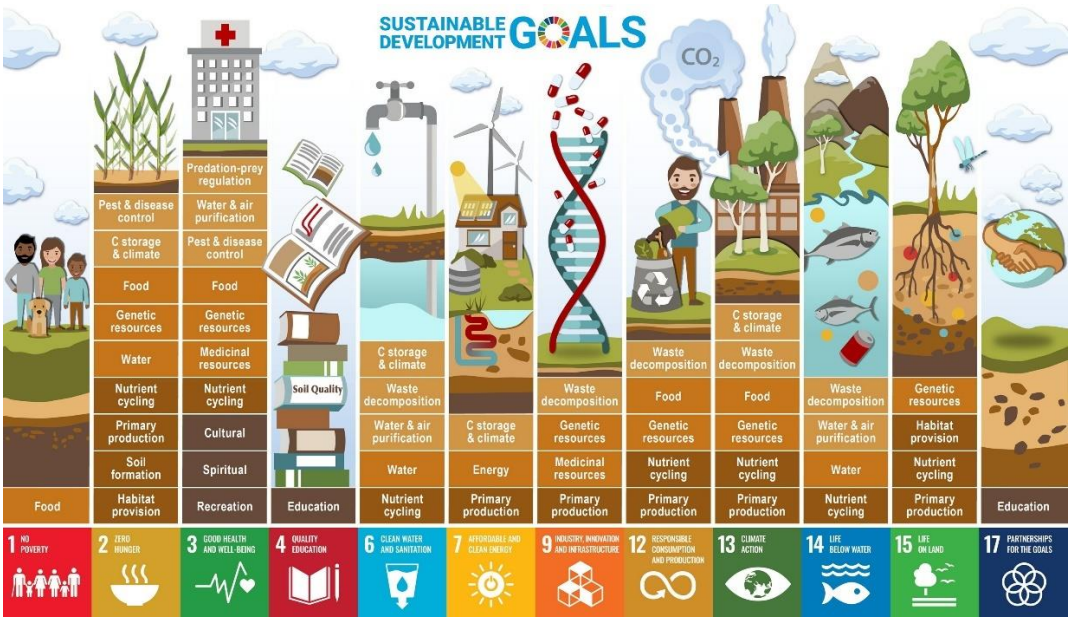

Figure 1.7 The role of soil functions in contributing to at least 12 of the 17 sustainable development goals (SDGs) of the United Nations: (i) biomass production (Food, Primary production, Energy), (ii) C pool regulation (C storage & climate, Waste decomposition), (iii) nutrient cycling (Nutrient cycling, Waste decomposition), (iv) Biodiversity and habitat provision (Genetic resources, Medical resources, Predator-prey regulations, Pest and Disease, Soil formation, Habitat provision), and (v) water cycling (Water, Water & air purification). Cultural aspects of soil are not discussed here (Kopittke et al., 2022).

Therefore, in the report "The state of the world's land and water resources for food and agriculture (SOLAW 2021)" (FAO, 2021) recently released by FAO, has been highlighted that current patterns of agricultural intensification are not sustainable and that agriculture's pressure on land and water resources, have built to the point where productivity of key agricultural systems is compromised and livelihoods are threatened. Indeed, high levels of pollution and greenhouse gas emissions are stretching the productive capacity to the limit and severely degrading land and environmental services (FAO, 2021).

Consequently, it is clear that mitigating agriculture's impacts on the environment represent an urgent need and a key strategy towards sustainability. Reducing land degradation, habitat loss and GHG emissions, and preventing further pollution and loss of ecosystem services while sustaining high production levels represents a central challenge for agriculture (Castiglione et al., 2021; FAO, 2021; Fedoroff et al., 2010; Tian et al., 2021).

However, world population is expected to growth reaching nearly 9.7 billion by 2050, with the consequent need to increase up to 60% food production, percentage that increases taking into account also feed, fiber and fuel crops production.

This challenge to increase food production while minimizing impacts on the environment is also concomitant with the major challenge of adapting to a rapidly evolving climate change. In fact, due to climate change effects, plants are already more frequently subjected to severe abiotic (e.g. drought, flooding, extreme temperature) and biotic (e.g. pathogens and pest outbreaks) stresses, while future scenarios foreseen these phenomena to become even more severe (Nephali et al., 2020; Sangiorgio et al., 2020; Tian et al., 2021; Velásquez et al., 2018).

Therefore, considering this complex picture full of hurdles, agriculture's challenges will require unprecedented efforts to find environmentally friendly and climate-resilient agronomic solutions to increase food production while addressing modern agriculture's issues (FAO, 2021; United Nations et al., 2019).

This need for a sustainable turning point in agriculture is also reaffirmed in the UN 2030 Agenda sustainable development goal 2 to "End hunger, achieve food security and improved nutrition and promote sustainable agriculture" put the focus on the necessity to "ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, that help maintain ecosystems, that strengthen capacity for adaptation to climate change, extreme weather, drought, flooding and other disasters and that progressively improve land and soil quality"(sdgs.un.org)(United Nations, 2015).

Moreover, sustainable increases in agricultural productivity are critical to also address multiple other Sustainable Development Goals (SDGs) such as no poverty (SDG 1) and good health and well-being (SDG 3). Increased productivity can also significantly contribute to various other SDGs including SDG 6 (clean water and sanitation), SDG 9 (industry, innovation and infrastructure), SDG 13 (climate Action) and SDG 15 (life on land) (Trivedi et al., 2021).

In this context, plant growth promotion represents an interesting sustainable solution that may play a key role in increasing crop resilience and productivity in adverse environmental conditions, minimizing agrochemicals applications and tackling climate change effects.

1.2 PLANT GROWTH PROMOTION A FEASIBLE SOLUTION TOWARDS A SUSTAINABLE AGRICULTURE

In healthy ecosystems, soil microorganisms, through a wide array of ecosystem services they provide, express a multifunctionality that support soil productivity and plant growth. Plants in nature are strictly associated with a vast number of microorganisms in their tissues and their rhizosphere, which constitute the plant microbiota (Ahmad et al., 2018; Pozo et al., 2021; Shah et al., 2021). The greatest microbial diversity can be found in the plant-influenced areas: rhizosphere, rhizoplane, and endosphere, the soil near the roots, the root surfaces, and the spaces between plant cells. In fact, the host plant directly influences the abundance and behaviour of the microbiome through root exudates, including organic acids, sugar, vitamins, and other molecules. Reciprocally, the microbiome signal compounds affect the host plant growth. The beneficial microorganisms, present in the phytomicrobiome contribute to increase the plant growth and fitness, not only improving nutrient uptake but also enhancing the resistance to biotic and/or abiotic stresses (Maitra et al., 2022; Shah et al., 2021). In fact, plants depend on soil microorganisms to utilize soils as a growth medium, since nutrients such as P, potassium (K), sulfur (S), calcium (Ca), and magnesium (Mg) cannot be utilized by plants unless mineralized and made available for the uptake.

In addition to the improvement of nutrient acquisition, soil microorganisms influence plant growth through several direct and indirect effects (Figure 1.8) (Ahmad et al., 2018; Owen et al., 2015; Trivedi et al., 2021).

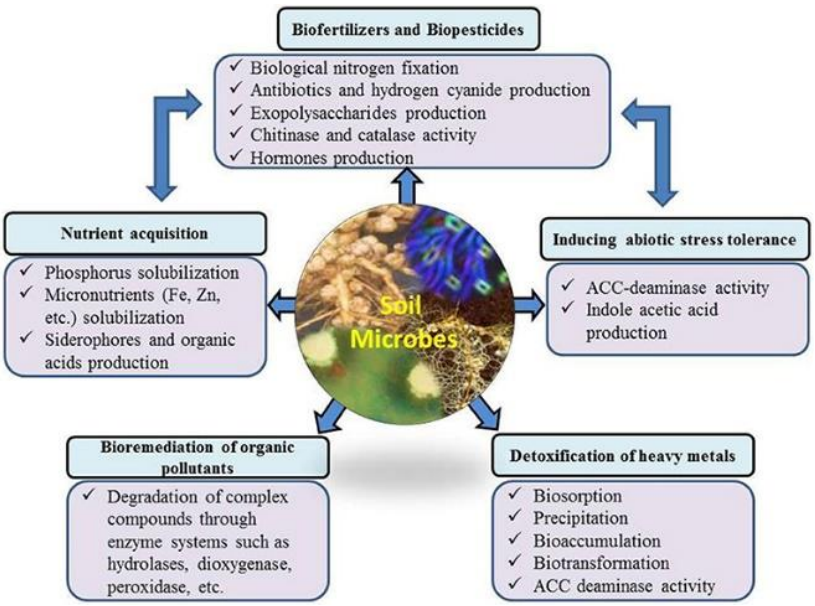

Figure 1.8 Importance of the microbial community for environmental health and possible mechanisms of action (Ahmad et al., 2018).

Some plant beneficial effects are very common among culturable microorganisms, however others may be typical of specific species and/or strains. Microorganisms' beneficial effects may even be related to environmental conditions which determining large fluctuations in microbial communities in the rhizosphere, which influenced by plant species, soil moisture and temperature regimes, environmental conditions and soil physiochemical conditions, determine also microorganisms' activities (Ahmad et al., 2018; Maitra et al., 2022).

The most common direct effects include enhanced availability of nutrients through solubilization/mobilization, phytohormone production, and siderophore production. Indirect effects include biological control of phytopathogens, amelioration of abiotic stresses, and bioremediation of polluted soils.

Among microorganisms' direct effects on plant growth promotion the enhancement of nutrient availability is crucial. Plant growth require about 16 micro- and macro-nutrients and the deficiency of any of these nutrients could lead to an impairment of growth. Beneficial microorganisms can increase nutrients levels, influencing plant metabolism, thus altering the composition of root exudates, which in turn affect the solubility and availability of other nutrients and increase the interactions with other soil microbes. Soil microorganisms exert their action of nutrient availability enhancement, both through organic matter decomposition, and solubilization/mobilization of immobilized nutrients such as phosphorus (P), zinc (Zn), potassium (K), and iron (Fe). The main mechanism of the latter process is the lowering of pH from the production of organic acids. Together with acidolysis, oxidoreduction, chelation or the secretion of compounds like oxalate, gluconate, citrate, catechol, lactate and pseudobactin are also crucial to this activity. Phosphorus solubilization, in particular, depend on the secretion of organic acids as well as of acidic or alkaline phosphatases. Through these processes soil bacteria and fungi are able to restore soil fertility through nitrogen fixation, solubilization of P, K, and Fe, and soil structure enhancing (Rashid et al., 2016), granting better growth conditions to the influenced plants. A particular case is that of Fe, which under aerobic conditions is available as an oxidized ion, i.e. ferric Fe (Fe^{3+}), whilst plant and microbes require iron in its ferrous (Fe^{2+}) form. Also, ferric iron is a highly reactive species, quickly combining into hydroxides and oxyhydroxides, which makes iron biologically unavailable (Kumar et al., 2022). To counteract the natural tendency toward oxidized forms, soil microorganisms can secrete a group of low molecular mass compound which are able to chelate Fe^{3+} and reduce it to Fe^{2+} thanks to the presence of reducer elements. Such compounds are called siderophores, literally "iron carriers", and through the secretion of siderophores microbes increase the bioavailability of iron in soil for both plants and microorganisms.

A final, yet fundamental effect of microorganisms on plant growth is obtained through the synthesis of phytormones and other plant regulating compounds. While plants naturally produce these complex compounds to control different aspects of metabolism and life cycle, it was discovered that microbes are able to secrete these regulators, or alter their levels, significantly enough to cause changes to plant growth and production. (Ahmad et al., 2018). While the production of cytokinins, auxins and ethylene is pretty common among PGP microbes able to secrete gibberllins are rarer to find. It has been widely reported that plants exposed to microbial phytohormones show to be less affected by stress and higher production rates, making microbial phytohormones a sustainable yet effective application of microbial potentialities.

In addition to the above-mentioned beneficial effects on plants, microbial inoculants might provide further indirect benefits through the modulation of native soil and plant-associated microbiomes, consequently also affecting animal and human health, lessening the impact of abiotic stress and by reducing the stress related to contaminants potentially present in the environment (Malusà et al., 2021).

In fact, microorganisms have seen a rising interests in their application in a series of sustainable biotechnologies, thanks to their potential among the others in biofertilization, biocontrol, and bioremediation of hazardous chemicals, representing a key asset to achieving several SDGs (Figure 1.9). Therefore, the application of single strains or consortia of microorganisms is considered a valuable and effective solution to improve agriculture sustainability, increasing yields while restoring soil functionality and quality, and reducing agrochemicals application, natural resources consumption and the other agriculture's detrimental effects on the environment (Ahmad et al., 2018; Akinsemolu, 2018; Kaur et al., 2021; Malusà et al., 2021; Trivedi et al., 2017).

In particular, the ability of soil microbiota to detoxify soil, restoring its quality, has been copiously reported. The ability to remediate organic and inorganic contaminations is a fundamental asset that makes soil microbes key resources for biotechnological applications. While the bulk of the studies is aimed towards the use of soil bacteria, this approach can actually limit the efficacy of treatments by excluding an important player in organic matter degradation: fungi. Fungal enzymes - such as laccases, manganese peroxidases and lignin peroxidases -have been reported to be highly non-specific and able to degrade a wide range of organic pollutants, including pesticides, PAHs and explosives. Moreover, fungi can play a role in the compartmentalization and chelation of heavy metals in soil, lessening the stress caused to plant by those chemical species. Therefore, a rising consensus is that a better approach is to evaluate the potential of consortia of soil microbes, especially when organic and inorganic contaminations coexist, to maximize the benefits of both bacteria and fungi (Anastasi et al., 2010; Banitz et al., 2013; Harms

et al., 2011; Ławniczak et al., 2020; Odukkathil and Vasudevan, 2013; Ramarajan and Manohar, 2017; Spina et al., 2018).

It is clear that microbial strains with a high soil colonization potential, and multiple plant growth promoting traits, such as the ability to stimulate the plant, increase nutrient availability, exert biocontrol activity against detrimental microorganisms, and biodegrade organic pollutant and detoxifying inorganic pollutants, would be most effective due to their multipurpose applicability (Ahmad et al., 2018). In this context, fungi as multifunctional microorganisms, perfectly adapted to soil microhabitats, thanks to their functional traits, metabolic plasticity and highly potent and relatively non-specific enzymes represent valuable potential bioresources.

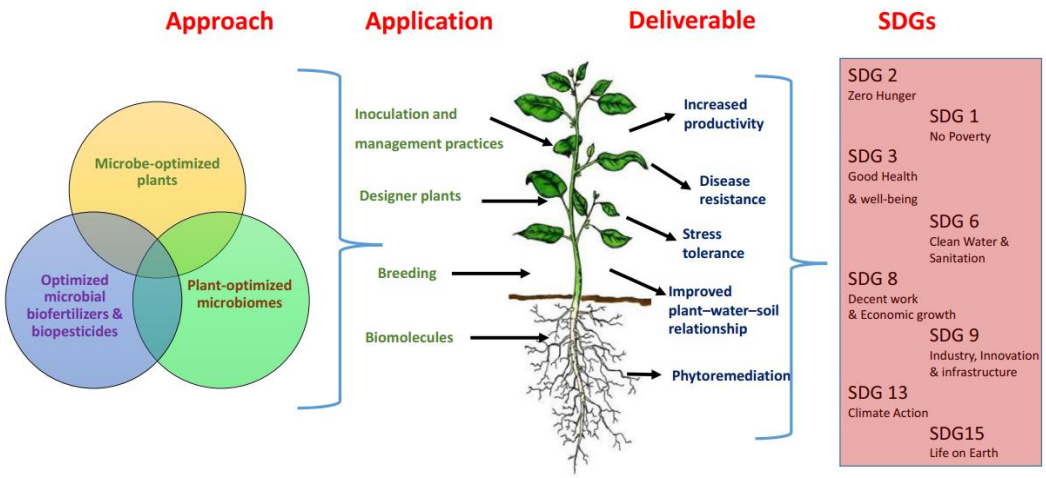

Figure 1.9 Sustainable increase in farm productivity by harnessing microbial technologies is critical for delivery of multiple Sustainable Development Goals (SDGs). It will primary contribute to SDG 2 (by increasing farm productivity) and 1 (by increasing farm profitability) but will also significantly contribute to SDGs 3, 6, 13 and 15 by improving nutrient quality, reducing environmental chemical pollutions, reducing greenhouse gas emission and promoting soil biodiversity respectively. SDGs 8 and 9 will benefit from creating of new industry for the production of microbial products and formulation (Trivedi et al., 2017).

1.3 FUNGI: ECOLOGICAL ROLES AND PLANT GROWTH PROMOTION

Fungi are the largest group in term of biomass of soil microbiota and are the primary agent in soil responsible for a variety of soil ecosystem services, linked to their multiple roles in soil (Orgiazzi et al., 2016). The hyphal growth model, which enables the bridging of air gaps in soil and the translocation of nutrient, while facilitating the colonization of substrates by soil bacteria – the so called "fungal highways" -, resulted in fungi claiming the niche of soil decomposers of organic matter, from simple to the most complex and recalcitrant compounds (De Boer et al., 2005; Kohlmeier et al., 2005; Yadav et al., 2020).

The European Joint Research Center (JRC) in 2016 linked the 4 main soil ecosystem functions (decomposition, nutrient cycling, soil structure, biological population regulation) to the soil biota and fungi were found to affect all 4 the functions and the quality of their related ecosystem services which human population benefit from (Orgiazzi et al., 2016). In fact, fungi as geoactive microorganisms are responsible for maintaining the nutrient and carbon cycle by releasing essential macro and micronutrients through the decomposition of organic matter, making fungal biodiversity a fundamental aspect of soil fertility – and ultimately of production quality and output (Cui and Holden, 2015; Köberl et al., 2020; Yadav et al., 2020). In particular, fungal networks play a key role in carbon, nitrogen and phosphorus cycles by increasing the bioavailability of otherwise immobilized fundamental nutrients for autotrophs and, by extension, for all living beings (Orgiazzi et al., 2016; Whitfield, 2007).

Another role directly linked to fungal growth in soil is the effect on soil structure. Soil structure is an indicator of soil health and quality (Cui et al., 2014) and fungal growth affect and is affected by it (Cavael et al., 2020). Soil fungi habitat formation and maintaining are directly linked to soil structure, making soil managing techniques fundamental to soil biodiversity (Cui and Holden, 2015). In turn, fungal growth is responsible for loose soil binding and reduced water permeability, which helps giving more stability and reducing the loss of nutrients (European Commission, Joint Research Centre, Institute for Environment and Sustainability, 2010; Miller and Jastrow, 2000; Orgiazzi et al., 2016).

A last but pivotal role of soil fungi is the interaction with plants. The most widespread type of interaction between land plants and fungi are the mycorrhizas, the symbiosis which single-handedly allowed the successful colonization of land (De Boer et al., 2005). Mycorrhizas are such a fundamental aspect of plant growth that about 80% of land plants are associated with a vast network of soil fungi, measuring about 100 meters of mychorrhizal hyphae per gram of soil (Whitfield, 2007), establishing a mutually advantageous exchange of nutrients. The mycorrhizal association has profound effects on the soil surrounding growing roots due to the presence of root and hyphal exudates, so much that this soil – called "rhizosphere" – shows different gradients of physical and

chemical characteristics when compared to non-mycorrhizal soil (Helliwell et al., 2019; Miller and Jastrow, 2000; Whalley et al., 2005).

Plant-fungi interactions, however, are not limited to mycorrhizas: aside from plant pathogens, there is another group of fungi interacting with plants outside of the mycorrhizal symbiosis, which is of growing interest for their possible biotechnological application, the plan growth-promoting fungi (PGPF) (Naziya et al., 2020a; Yadav et al., 2020).

PGPF are a heterogeneous group of nonsymbiotic nonpathogenic saprotrophic fungi that live freely in the root surface or the interior of the root itself or the rhizosphere, and mediate improvements in plant growth and health. The belonging to the PGPF group does not imply specific biological characteristics nor in taxonomy, in habitats, in physiology, and in their interaction with plants. PGPF may have a specific effect upon the growth of one plant, therefore a promoting action on one species does not imply the same effect on another plant species. Furthermore, it has been observed that the promotion effect may also vary under different environmental conditions (Hossain et al., 2017; Yadav et al., 2020).

Species of PGPF are ubiquitous saprobes and mostly origin either in the soil or in plant host roots, but may also be endophytic fungi.

As early mentioned, PGPF show diverse taxonomy, however the majority of fungi characterized as PGPF reported in literature primarily belongs to the phylum Ascomycota, while few of them belongs to Basidiomycota and Zygomycota. Among the FPGP reported in literature there are strains mainly belonging among the others to genera such as *Alternaria*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Metharizium*, *Mucor*, *Purpureocillium*, *Penicillium*, *Serendipita*, *Phoma*, *Rhizopus*, *Talaromyces*, and *Trichoderma* (Bagde et al., 2011; Baron et al., 2020; Bilal et al., 2018; Hamayun et al., 2009; Haruma et al., 2018; Hyde et al., 2019; Jahagirdar et al., 2019; López-Bucio et al., 2015; Moya et al., 2020, 2020; Murali and Amruthesh, 2015; Naziya et al., 2020b; Ozimek and Hanaka, 2021; Salas-Marina et al., 2011; Varma et al., 1999; Zhai et al., 2018; Zhang et al., 2016; Zhou et al., 2018).

It is important to note that also several strains lacking virulence belonging to pathogenic species such as *Alternaria alternata*, *Fusarium oxysporum*, *Colletotrichum*, and *Rhizoctonia*, have been reported for plant growth promoting activities.

Being fungi multifunctional microorganisms, perfectly adapted to soil microhabitats, thanks to their functional traits, metabolic plasticity and highly potent and relatively non-specific enzymes, PGPF have been reported to be able to exert all the plant growth promotion activities reported in the previous section, being able to increase nutrient availability, stimulate the plant, provide bioprotection against pathogens and remediate

soils (Figure 1.10) (Owen et al., 2015). In fact, PGPF have been proven to determine improvements in seed germination rate, seedling vigor, root development and morphogenesis, shoot growth, yield, photosynthetic efficiency, flowering, and plant Composition.

Since PGPF also exerts their action through diffusible substances such as phytohormones, enzymes, aminoacids, and siderophores, application of their culture filtrates and fungal metabolites as biostimulants have been widely reported (Hossain et al., 2017; Singh et al., 2019). Culture filtrate have been consistently reported in several studies to be effective in promoting plant growth by enhancing seed germination, biomass production, and metabolites production (Bagde et al., 2011, 2013, 2013; Baroja-Fernández et al., 2021; Bilal et al., 2018; Hamayun et al., 2009, 2010; Hwang et al., 2011; Khalmuratova et al., 2021; Khan et al., 2015, 2008; Murali and Amruthesh, 2015; Rahman et al., 2012; Singh et al., 2003; Sung et al., 2011, 2011; Varma et al., 1999; You, 2012).

Finally, the global diversity of fungi has been estimated between 2.2 and 3.8 million species, therefore it represents an unparalleled pool of unexplored and untapped bio-resources, that may play a key role in ensuring a sustainable future for humanity (Hawksworth and Lücking, 2017).

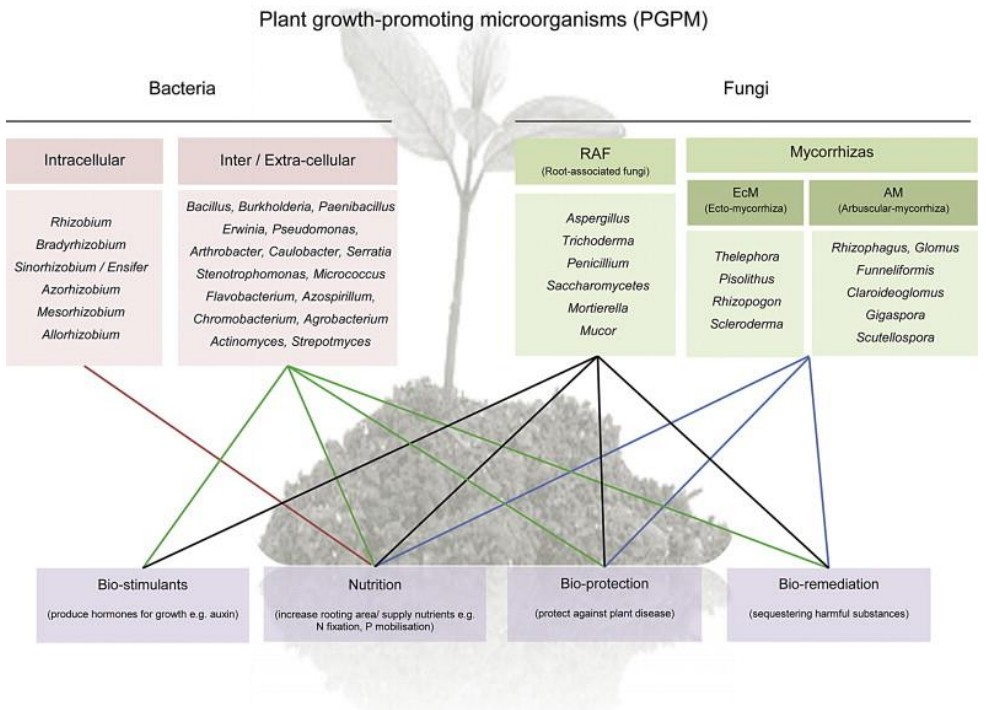

Figure 1.10 Main plant growth-promoting groups (genera in italics) used in commercial bio-inocula and the various mechanisms each employ to promote plant growth (Owen et al., 2015).

1.4 FUNGAL SPECIES TESTED IN THIS PROJECT FOR THEIR POTENTIALITIES AS PLANT GROWTH PROMOTER

In this thesis two fungal strains, isolated in previous studies and currently preserved at the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), *Chaetomium globosum* Kunze FBL 205 and *Minimedusa polyspora* (Hotson) Weresub & P. M. LeClair FBL 503, were studied to assess their potentialities as plant growth promoting fungi.

In the following pages a brief description of the species and the state of the art of the knowledge on their potentialities as plant growth promoters, are reported for each of the two strains.

CHAETOMIUM GLOBOSUM KUNZE

Chaetomium globosum is a filamentous species belonging to the Ascomycota phylum. It presents a homotallic mycelium and ascomata superficial, ostiolate, greenish olivaceous or slightly dark olivaceous buff to grey in reflected light owing to ascomatal hairs, globose, ellipsoid, ovate or obovate, 160–300 µm high, 135–250 µm diam (Figure 1.11). Ascospores are olivaceous brown when mature, limoniform, usually biapiculate, bilaterally flattened, (8–)8.5–10.5(–11) × 7–8(–8.5) × 5.5–6.5(–7) µm, with an apical germ pore (Domsch et al., 2007; Wang et al., 2016).

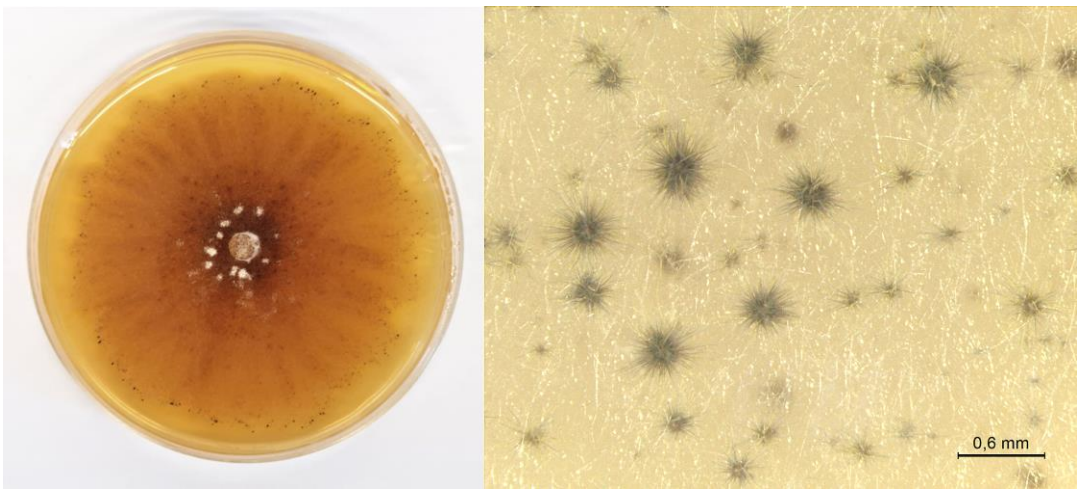

Figure 1.11 *Chaetomium globosum* FBL 205: A. Colony on malt extract agar (MEA) after 10 days of incubation; B. Ascomata on MEA, stereoscopic microscope view.

C. globosum presents a cosmopolitan distribution and has been reported both a saprotrophic and plant endophyte with a wide range of host plants (Figure 1.12 and Figure 1.13) (Linkies et al., 2021; Větrovský et al., 2020). It presents a great adaptability

inhabiting various environments including extreme habitats such as deserts or salt lakes, and is commonly isolated in agricultural soils (Abdel-Azeem, 2020; Linkies et al., 2021; Větrovský et al., 2020).

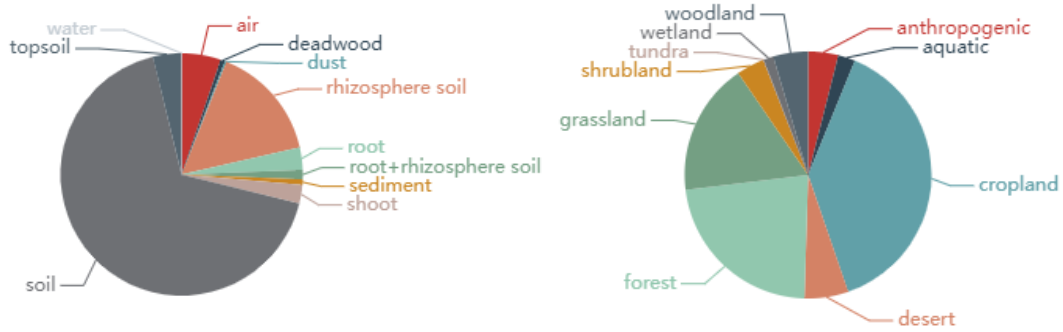

Figure 1.12 A sample type breakdown of the entries of *C. globosum* on the global fungi database; B biome breakdown of the entries of *C. globosum* on the global fungi database from https://globalfungi.com/ (Větrovský et al., 2020).

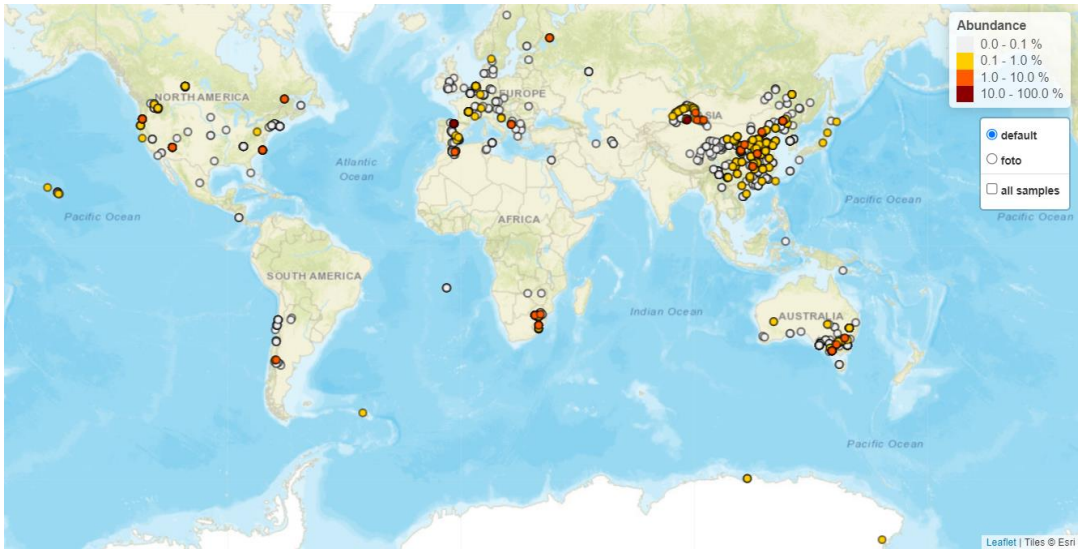

Figure 1.13 *Chaetomium globosum* distribution and abundance from https://globalfungi.com/ (Větrovský et al., 2020).

C. globosum is a widely studied species and several studies highlighted the production of a wide array of bioactive metabolites. *C. globosum* has been reported to produce among the other alkaloids, xanthones, flavonoids, sterols, chaetoglobosins, tetramic acid, terpenes, polyketones, ergosterol, orsellides, diketopiperazines, cytochalasins (Abdel-

Azeem, 2020; Bashyal et al., 2005; Jiao et al., 2004; Kumar et al., 2021; Pothiraj et al., 2021; Qin et al., 2009; Yang et al., 2007).

C. globosum has been consistently reported as a biocontrol agent effective against a large number of soil, seed, and airborne fungal pathogens including *Bipolaris sorokiniana*, *Bipolaris sorokiniana*, *Botrytis cinerea*, *Cochliobolus sativus*, *Fusarium oxysporum*, *Fusarium Sporotrichioides*, *Magnaporthe grisea*, *Phytophthora infestans*, *Phytophthora nicotianae*, *Puccinia recondite*, *Puccinia triticina*, *Pyrenophora graminea*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Setosphaeria turcica,* causing plant diseases as spot blotch of wheat, rice blast, root rot of citrus, potato dry rot, and late blight of potato (Aggarwal et al., 2004, 2016; Hung et al., 2015; Jiang et al., 2017; Kumar et al., 2021; Linkies et al., 2021; Moya et al., 2020; Park et al., 2005; Shanthiyaa et al., 2013; Singh et al., 2021; Yan et al., 2018; Zhang et al., 2013). This biocontrol activity toward phytopathogens is exerted by *C. globosum* through the competition for nutrients and substrates, mycoparasitism, antibiosis, or their combinations, but also inducing in plants induced systemic resistance (Biswas et al., 2012; Istifadah and McGee, 2006; Pothiraj et al., 2021; Singh et al., 2021). Moreover, *C. globosum* and its metabolites showed also inhibition properties towards root-knot nematode (*Meloidogyne incognita*, *Meloidogyne javanica*), cotton aphids (*Aphis gossypii*) and beet armyworms (*Spodoptera exigua*) (Hu et al., 2013; Khan et al., 2019; Zhou et al., 2016).

C. globosum has also been reported as effective plant growth promoter of *Capsicum annuum* L., *Brassica juncea*, *Solanum lycopersicum*, *Pennisetum americanum* L., *Zea mays*. In all the above-mentioned species an increase in biomass production was recorded and as appropriate *C. globosum* caused an increase also in various morphological and physiological parameters of growth and development such as shoot growth, plant height, root length, leaf area, chlorophyll content, stomatal conductance, transpiration rate, seed germination, and nutrient uptake (Abou Alhamed and Shebany, 2012; Khan et al., 2012; Kumar et al., 2021; Singh et al., 2021; Tarafdar and Gharu, 2006).

Finally, *C. globosum* has already been studied also for its potentialities in bioremediation (Maamar et al., 2020; Sowmya et al., 2014; Stoleru et al., 2020).

Tiedje and Hagedorn (Tiedje and Hagedorn, 1975) reported *C. globosum* to rapidly degrade Alachlor [2-chloro-2',6'-diethyl-N-(methoxymethyl)acetanilide], a preemergence herbicide historically utilized which has for weed control in corn and soybeans. Moreover, *C. globosum* has been especially tested in the degradation of plastic materials showing the ability of degrading low molecular weight synthetic polymers such as polyesteracetals (PEA), polyphthalamide (PPA) and polybutylacrilate (PBA) by hydrolizing the compound through the release of broad-spectrum lipases (Benedict et al., 1983; Kim and Rhee, 2003). Instead in the case of poly(ε-caprolactone) (PCL), a biodegradable polymer used in biomedical applications such as drug delivery and

dentistry, *C. globosum* was able to degrade it only up to a certain molecular weight of the polymer, since increased molecular weight of the polyester reduced its degradability (Benedict et al., 1983). Moreover, Vivi et al (2019) showed how *C. globosum* can use PCL and polyvinyl chloride (PVC) as nutrient, and that the degradation efficiency in starving conditions are higher than those in optimal growth condition.

Another biotechnological application for which *C. globosum* has been characterized is the decolorization of textile dyes in industrial wastewater. *C. globosum* was shown to be a potential bioresource for this kind of application due to its enzymatic array that incudes laccases, manganese peroxidases and lignin peroxidase (Manai et al., 2016). Finally, *C. globosum* has been studied for its effect on maize growth under copper stress, showing the ability improve seedling performance and reduce the toxic effects of copper, therefore showing the ability of this fungal strain to tolerate copper (Abou Alhamed and Shebany, 2012).

MINIMEDUSA POLYSPORA (HOTSON) WERESUB & P.M. LE CLAIR

Minimedusa polyspora is a saprotrophic filamentous anamorphic species belonging to the Basidiomycota phylum (Figure 1.14). This species has a hyaline, procumbent mycelium and is mainly characterized from the development of 2-4 µm wide bulbils, originating from the aggregation of lateral aerial hyphae, eventually forming a dense powdery mass, globose smooth, reddish brown with diameters of 90-130 µm.

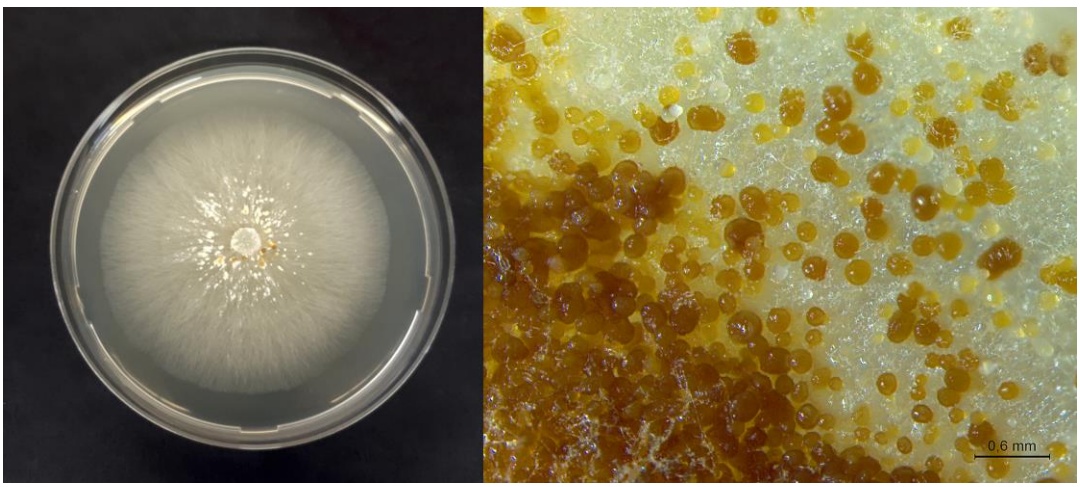

Figure 1.14 *Minimedusa polyspora* FBL 503: A. Colony on malt extract agar (MEA) after 7 days of incubation; B. Masses of bulbils on MEA, stereoscopic microscope view.

M. polyspora predominantly found in soils of forests, grasslands, and croplands (Figure 1.15), has been widely isolated in agricultural soils worldwide (Figure 1.16) and

found to be overrepresented in the tilled soil (Klaubauf et al., 2010; Panelli et al., 2017; Longley et al., 2020; Větrovský et al., 2020; Orrù et al., 2021; Lucadamo).

Figure 1.15 A sample type breakdown of the entries of *M. polyspora* on the global fungi database; B biome breakdown of the entries of *M. polyspora* on the global fungi database from https://globalfungi.com/ (Větrovský et al., 2020).

Figure 1.16 *Minimedusa polyspora* distribution and abundance from https://globalfungi.com/ (Větrovský et al., 2020).

This species presents several traits such as rapid growth, little requirement of nitrogen, ability of concentrating important biogenic microelements (N, P, S, K and Ca) and translocating nutrients, metabolic plasticity, and the production of antibiotic and

antifungal compounds which make it an efficient pioneer colonizer (Beale and Pitt, 1995; Pinzari et al., 2014).

The metabolic plasticity of *M. polyspora*, has also been documented through the Phenotype MicroArray method. Pinzari et al. (2014) found that the fungus possesses a preference for polysaccharides at the initial phases of its growth, and that it prefers hexoses and then oligosaccharides in the later phases of its development. A further peculiarity of this strain is the relatively scarce utilization of the N-containing compounds such as peptides, L-aminoacids and amines.

Several of the above-mentioned characteristics make *M. polyspora* also suitable to work as a networking hub in a microbial community, how was reported in the microbial community of a wastewater treatment plants (Assress et al., 2019).

Furthermore, *M. polyspora* has already been suggested to be a PGPF given its ability to solubilize inorganic phosphorous and to antagonize *Fusarium oxysporum* f.sp. *narcissi* (Beale and Pitt, 1990, 1995; Ceci et al., 2018). More specifically, Beale and Pitt (Beale and Pitt, 1995, 1990, 1992) found that *M. polyspora* was able to overgrow a *F. oxysporum* species by rapid disintegration of its cell walls and revealed that *M. polyspora* culture filtrates contain five antifungal triene compounds providing evidence for antibiosis as the primary mechanism of antagonism in this species.

Despite little is known on the potentialities of *M. polyspora* in bioremediation, this species has been isolated from a variety of contaminated sites, mainly due to organic compounds, ranging from wood-treating facilities to wastewater plants (Assress et al., 2019; Czaplicki et al., 2020; Jones and Hodges, 1974; Siczek et al., 2020). The very few studies on its potentialities in bioremediation are focused mainly on the degradation of organic pollutants, such as pesticides and fluorene (Garon et al., 2004; Jones and Hodges, 1974). In one of the very few studies on *M. polyspora* on bioremediation, Garon et al (Garon et al., 2004), reported *M. polyspora* ability to degrade a 0.005 g l^{-1} fluorene solution by 40%, while Jones and Hodges (Jones and Hodges, 1974) reported its ability to recover 99.9% of labeled carbon from mirex [Dodecachlorooctahydro-1H-1,3,4-(epimethanetriyl)cyclobuta[cd]pentalene] in soil.

Despite it has not yet been extensively studied *M. polyspora* has shown potentiality for its use in the bioremediation of soil, also considering its wide distribution.

1.5 REFERENCES

Abdel-Azeem, A. M. (2020). Recent Developments on Genus *Chaetomium* (Cham, CH: Springer International Publishing).

Abhilash, P.C. (2021). Restoring the Unrestored: Strategies for Restoring Global Land during the UN Decade on Ecosystem Restoration (UN-DER). Land *10*, 201.

Abou Alhamed, M.F., and Shebany, Y.M. (2012). Endophytic *Chaetomium globosum* enhances maize seedling copper stress tolerance. Plant Biology *14*, 859–863.

Adhikari, K., and Hartemink, A.E. (2016). Linking soils to ecosystem services — A global review. Geoderma *262*, 101–111.

Aggarwal, R., Tewari, A.K., Srivastava, K.D., and Singh, D.V. (2004). Role of antibiosis in the biological control of spot blotch (*Cochliobolus sativus*) of wheat by *Chaetomium globosum*. Mycopathologia *157*, 369–377.

Aggarwal, R., Sharma, S., Gupta, S., Singh, K.B.M., and Shanmugam, V. (2016). Role of defense enzymes in biocontrol of spot blotch and leaf rust of wheat (*Triticum* sp. L.) by *Chaetomium globosum*. Journal of Pure and Applied Microbiology *10*, 2071–2079.

Ahmad, M., Pataczek, L., Hilger, T.H., Zahir, Z.A., Hussain, A., Rasche, F., Schafleitner, R., and Solberg, S.Ø. (2018). Perspectives of Microbial Inoculation for Sustainable Development and Environmental Management. Frontiers in Microbiology *9*, 2992.

Akinsemolu, A.A. (2018). The role of microorganisms in achieving the sustainable development goals. Journal of Cleaner Production *182*, 139–155.

Anastasi, A., Spina, F., Prigione, V., Tigini, V., Giansanti, P., and Varese, G.C. (2010). Scale-up of a bioprocess for textile wastewater treatment using *Bjerkandera adusta*. Bioresource Technology *101*, 3067–3075.

Assress, H.A., Selvarajan, R., Nyoni, H., Ntushelo, K., Mamba, B.B., and Msagati, T.A.M. (2019). Diversity, Co-occurrence and Implications of Fungal Communities in Wastewater Treatment Plants. Scientific Reports *9*, 14056.

Bach, E.M., Ramirez, K.S., Fraser, T.D., and Wall, D.H. (2020). Soil Biodiversity Integrates Solutions for a Sustainable Future. Sustainability *12*, 2662.

Bagde, U.S., Prasad, R., and Varma, A. (2011). Influence of culture filtrate of *Piriformospora indica* on growth and yield of seed oil in *Helianthus annus*. Symbiosis *53*, 83–88.

Bagde, U.S., Prasad, R., and Varma, A. (2013). Impact of Culture Filtrate of *Piriformospora indica* on Biomass and Biosynthesis of Active Ingredient Aristolochic Acid in *Aristolochia elegans* Mart. International Journal of Biosciences *6*, p29.

Banitz, T., Johst, K., Wick, L.Y., Schamfuß, S., Harms, H., and Frank, K. (2013). Highways versus pipelines: contributions of two fungal transport mechanisms to efficient bioremediation: Fungal transport mechanisms enhance bioremediation. Environmental Microbiology Reports *5*, 211–218.

Baroja-Fernández, E., Almagro, G., Sánchez-López, Á.M., Bahaji, A., Gámez-Arcas, S., De Diego, N., Dolezal, K., Muñoz, F.J., Climent Sanz, E., and Pozueta-Romero, J. (2021). Enhanced Yield of Pepper Plants Promoted by Soil Application of Volatiles From Cell-Free Fungal Culture Filtrates Is Associated With Activation of the Beneficial Soil Microbiota. Frontiers in Plant Science *12*, 2396.

Baron, N.C., Souza Pollo, A. de, and Rigobelo, E.C. (2020). *Purpureocillium lilacinum* and *Metarhizium marquandii* as plant growth-promoting fungi. PeerJ *8*, e9005.

Bashyal, B.P., Wijeratne, E.M.K., Faeth, S.H., and Gunatilaka, A.A.L. (2005). Globosumones A−C, Cytotoxic Orsellinic Acid Esters from the Sonoran Desert Endophytic Fungus *Chaetomium globosum*. Journal of Natural Products *68*, 724–728.

Beale, R., and Pitt, D. (1995). The antifungal properties of *Minimedusa polyspora*. Mycological Research *99*, 337–342.

Beale, R.E., and Pitt, D. (1990). Biological and integrated control of *Fusarium* basal rot of Narcissus using *Minimedusa polyspora* and other micro-organisms. Plant Pathology *39*, 477–488.

Beale, R.E., and Pitt, D. (1992). Studies on *Minimedusa Polyspora*, a Biological Control Agent of Soilborne Plant Pathogens. In Biological Control of Plant Diseases: Progress and Challenges for the Future, E.C. Tjamos, G.C. Papavizas, and R.J. Cook, eds. (Boston, MA: Springer US), pp. 249–253.

Benedict, C.V., Cook, W.J., Jarrett, P., Cameron, J.A., Huang, S.J., and Bell, J.P. (1983). Fungal degradation of polycaprolactones. Journal of Applied Polymer Science *28*, 327–334.

Bilal, L., Asaf, S., Hamayun, M., Gul, H., Iqbal, A., Ullah, I., Lee, I.-J., and Hussain, A. (2018). Plant growth promoting endophytic fungi *Aspergillus fumigatus* TS1 and *Fusarium proliferatum* BRL1 produce gibberellins and regulates plant endogenous hormones. Symbiosis *76*, 117–127.

Biswas, S.K., Aggarwal, R., Srivastava, K.D., Gupta, S., and Dureja, P. (2012). Characterization of Antifungal Metabolites of *Chaetomium globosum* Kunze and their Antagonism against Fungal Plant Pathogens. Journal of Biological Control *26*, 70–74.

de Boer, W. de, Folman, L.B., Summerbell, R.C., and Boddy, L. (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews *29*, 795–811.

Calicioglu, O., Flammini, A., Bracco, S., Bellù, L., and Sims, R. (2019). The Future Challenges of Food and Agriculture: An Integrated Analysis of Trends and Solutions. Sustainability *11*, 222.

Carvalho, F.P. (2017). Pesticides, environment, and food safety. Food & Energy Security *6*, 48–60.

Castiglione, A.M., Mannino, G., Contartese, V., Bertea, C.M., and Ertani, A. (2021). Microbial Biostimulants as Response to Modern Agriculture Needs: Composition, Role and Application of These Innovative Products. Plants *10*, 1533.

Cavael, U., Tost, P., Diehl, K., Büks, F., and Lentzsch, P. (2020). Correlations of Soil Fungi, Soil Structure and Tree Vigour on an Apple Orchard with Replant Soil. Soil Systems *4*, 70.

Cordell, D., Drangert, J.-O., and White, S. (2009). The story of phosphorus: Global food security and food for thought. Global Environmental Change *19*, 292–305.

Cui, J., and Holden, N.M. (2015). The relationship between soil microbial activity and microbial biomass, soil structure and grassland management. Soil and Tillage Research *146*, 32–38.

Cui, J., Askari, M.S., and Holden, N.M. (2014). Visual Evaluation of Soil Structure Under Grassland management. Soil Use and Management *30*, 129–138.

Czaplicki, L.M., Redfern, L.K., Cooper, E.M., Ferguson, P.L., Vilgalys, R., and Gunsch, C.K. (2020). Investigating the mycobiome of the Holcomb Creosote Superfund Site. Chemosphere *252*, 126208.

Dhananjayan, V., Jayanthi, P., Jayakumar, S., and Ravichandran, B. (2020). Agrochemicals Impact on Ecosystem and Biomonitoring. In Resources Use Efficiency in Agriculture, S. Kumar, R.S. Meena, and M.K. Jhariya, eds. (Singapore: Springer), pp. 349–388.

Domsch, K.H., Gams, W., and Anderson, T.-H. (2007). Compendium of soil fungi (Eching, Germany: IHW).

European Commission. Joint Research Centre. Institute for Environment and Sustainability. (2010). European atlas of soil biodiversity. (LU: Publications Office).

FAO (2003). World agriculture: towards 2015/2030: an FAO perspective (London, UK: Earthscan Publications).

FAO (2017). The future of food and agriculture: trends and challenges (Rome, Italy: Food and Agriculture Organization of the United Nations).

FAO (2020). Biodiversity for food and agriculture and ecosystem services: Thematic Study for The State of the World's Biodiversity for Food and Agriculture (Rome, Italy: FAO).

FAO (2021). The State of the World's Land and Water Resources for Food and Agriculture – Systems at breaking point (SOLAW 2021): Synthesis report 2021 (Rome, Italy: FAO).

FAO and UNEP (2021). Global assessment of soil pollution: Report (Rome, Italy: FAO and UNEP).

FAO, ITPS, GSBI, CBD, and EC (2020). State of knowledge of soil biodiversity - Status, challenges and potentialities, Report 2020 (Rome, Italy: FAO, ITPS, GSBI, CBD and EC.).

Fedoroff, N.V., Battisti, D.S., Beachy, R.N., Cooper, P.J.M., Fischhoff, D.A., Hodges, C.N., Knauf, V.C., Lobell, D., Mazur, B.J., Molden, D., et al. (2010). Radically Rethinking Agriculture for the 21st Century. Science *327*, 833–834.

Filippelli, G.M. (2008). The Global Phosphorus Cycle: Past, Present, and Future. Elements *4*, 89–95.

Garon, D., Sage, L., and Seigle-Murandi, F. (2004). Effects of fungal bioaugmentation and cyclodextrin amendment on fluorene degradation in soil slurry. Biodegradation *15*, 1–8.

Geisen, S., Wall, D.H., and Putten, W.H. van der (2019). Challenges and Opportunities for Soil Biodiversity in the Anthropocene. Current Biology *29*, R1036–R1044.

Hamayun, M., Khan, S.A., Khan, A.L., Rehman, G., Sohn, E.-Y., Shah, A.A., Kim, S.-K., Joo, G.-J., and Lee, I.-J. (2009). *Phoma herbarum* as a New Gibberellin-Producing and Plant Growth-Promoting Fungus. Journal of Microbiology and Biotechnology *19*, 1244–1249.

Hamayun, M., Khan, S.A., Khan, A.L., Rehman, G., Kim, Y.-H., Iqbal, I., Hussain, J., Sohn, E.-Y., and Lee, I.-J. (2010). Gibberellin production and plant growth promotion from pure cultures of *Cladosporium* sp. MH-6 isolated from cucumber (*Cucumis sativus* L.). Mycologia *102*, 989–995.

Harms, H., Schlosser, D., and Wick, L.Y. (2011). Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nature Reviews Microbiology *9*, 177–192.

Haruma, T., Yamaji, K., Masuya, H., and Hanyu, K. (2018). Root endophytic *Chaetomium cupreum* promotes plant growth and detoxifies aluminum in *Miscanthus sinensis* Andersson growing at the acidic mine site. Plant Species Biology 33, 109–122.

Hawksworth, D.L., and Lücking, R. (2017). Fungal Diversity Revisited: 2.2 to 3.8 Million Species. Microbiology Spectrum *5*, 4.

Helliwell, J.R., Sturrock, C.J., Miller, A.J., Whalley, W.R., and Mooney, S.J. (2019). The role of plant species and soil condition in the structural development of the rhizosphere. Plant, Cell & Environment 42, 1974–1986.

Hossain, Md.M., Sultana, F., and Islam, S. (2017). Plant Growth-Promoting Fungi (PGPF): Phytostimulation and Induced Systemic Resistance. In Plant-Microbe Interactions in Agro-Ecological Perspectives, D.P. Singh, H.B. Singh, and R. Prabha, eds. (Singapore, Singapore: Springer Singapore), pp. 135–191.

Hu, Y., Zhang, W., Zhang, P., Ruan, W., and Zhu, X. (2013). Nematicidal Activity of Chaetoglobosin A Poduced by *Chaetomium globosum* NK102 against Meloidogyne incognita. Journal of Agricultural and Food Chemistry *61*, 41–46.

Hung, P.M., Wattanachai, P., Kasem, S., and Poeaim, S. (2015). Efficacy of *Chaetomium* Species as Biological Control Agents against *Phytophthora nicotianae* Root Rot in Citrus. Mycobiology *43*, 288–296.

Hwang, J.-S., You, Y.-H., Bae, J.-J., Khan, S.A., Kim, J.-G., and Choo, Y.-S. (2011). Effects of Endophytic Fungal Secondary Metabolites on the Growth and Physiological Response of Carex kobomugi Ohwi. Journal of Coastal Research *27*, 544–548.

Hyde, K.D., Xu, J., Rapior, S., Jeewon, R., Lumyong, S., Niego, A.G.T., Abeywickrama, P.D., Aluthmuhandiram, J.V.S., Brahamanage, R.S., Brooks, S., et al. (2019). The amazing potential of fungi: 50 ways we can exploit fungi industrially. Fungal Diversity *97*, 1–136.

Istifadah, N., and McGee, P.A. (2006). Endophytic *Chaetomium globosum* reduces development of tan spot in wheat caused by *Pyrenophora tritici-repentis*. Australasian Plant Pathology *35*, 411–418.

Jahagirdar, S., Kambrekar, D.N., Navi, S.S., and Kunta, M. (2019). Plant Growth-Promoting Fungi: Diversity and Classification. In Bioactive Molecules in Plant Defense: Signaling in Growth and Stress, S. Jogaiah, and M. Abdelrahman, eds. (Cham, CH: Springer International Publishing), pp. 25–34.

Jiang, C., Song, J., Zhang, J., and Yang, Q. (2017). Identification and characterization of the major antifungal substance against *Fusarium sporotrichioides* from *Chaetomium globosum*. World Journal of Microbiology and Biotechnology *33*, 108.

Jiao, W., Feng, Y., Blunt, J.W., Cole, A.L.J., and Munro, M.H.G. (2004). Chaetoglobosins Q, R, and T, Three Further New Metabolites from *Chaetomium globosum*. Journal of Natural Products *67*, 1722–1725.

Jones, A.Stevens., and Hodges, C.S. (1974). Persistence of mirex and its effects on soil microorganisms. Journal of Agricultural and Food Chemistry *22*, 435–439.

Kaur, T., Devi, R., Kour, D., Yadav, A., Yadav, A.N., Dikilitas, M., Abdel-Azeem, A.M., Ahluwalia, A.S., and Saxena, A.K. (2021). Plant growth promoting soil microbiomes and their potential implications for agricultural and environmental sustainability. Biologia *76*, 2687–2709.

Khalmuratova, I., Choi, D.-H., Kim, J.-G., and Lee, and I.-S. (2021). Endophytic Fungi of Salt-Tolerant Plants: Diversity and Ability to Promote Plant Growth. *31*, 1526–1532.

Khan, A., Shinwari, Z., Kim, Y.-H., Waqas, M., Hamayun, M., Kamran, M., and Lee, I.-J. (2012). Role of endophyte *Chaetomium globosum* lk4 in growth of *Capsicum annuum* by producion of gibberellins and indole acetic acid. Pakistan Journal of Botany *44*, 1601–1607.

Khan, A.R., Ullah, I., Waqas, M., Shahzad, R., Hong, S.-J., Park, G.-S., Jung, B.K., Lee, I.-J., and Shin, J.-H. (2015). Plant growth-promoting potential of endophytic fungi isolated from Solanum nigrum leaves. World Journal of Microbiology and Biotechnology *31*, 1461–1466.

Khan, B., Yan, W., Wei, S., Wang, Z., Zhao, S., Cao, L., Rajput, N.A., and Ye, Y. (2019). Nematicidal metabolites from endophytic fungus *Chaetomium globosum* YSC5. FEMS Microbiology Letters *366*, fnz169.

Khan, S.A., Hamayun, M., Yoon, H., Kim, H.-Y., Suh, S.-J., Hwang, S.-K., Kim, J.-M., Lee, I.-J., Choo, Y.-S., Yoon, U.-H., et al. (2008). Plant growth promotion and *Penicillium citrinum*. BMC Microbiology *8*, 231.

Kim, D.Y., and Rhee, Y.H. (2003). Biodegradation of microbial and synthetic polyesters by fungi. Applied Microbiology and Biotechnology *61*, 300–308.

Köberl, M., Wagner, P., Müller, H., Matzer, R., Unterfrauner, H., Cernava, T., and Berg, G. (2020). Unraveling the Complexity of Soil Microbiomes in a Large-Scale Study Subjected to Different Agricultural Management in Styria. Frontiers in Microbiology *11*.

Kohlmeier, S., Smits, T.H.M., Ford, R.M., Keel, C., Harms, H., and Wick, L.Y. (2005). Taking the Fungal Highway: Mobilization of Pollutant-Degrading Bacteria by Fungi. Environmental Science & Technology *39*, 4640–4646.

Kopittke, P.M., Berhe, A.A., Carrillo, Y., Cavagnaro, T.R., Chen, D., Chen, Q.-L., Román Dobarco, M., Dijkstra, F.A., Field, D.J., Grundy, M.J., et al. (2022). Ensuring

planetary survival: the centrality of organic carbon in balancing the multifunctional nature of soils. Critical Reviews in Environmental Science and Technology *0*, 1–17.

Kumar, R., Kundu, A., Dutta, A., Saha, S., Das, A., and Bhowmik, A. (2021). Chemo-profiling of bioactive metabolites from *Chaetomium globosum* for biocontrol of *Sclerotinia* rot and plant growth promotion. Fungal Biology *125*, 167–176.

Kumar, S., Diksha, Sindhu, S.S., and Kumar, R. (2022). Biofertilizers: An ecofriendly technology for nutrient recycling and environmental sustainability. Current Research in Microbial Sciences *3*, 100094.

Ławniczak, Ł., Woźniak-Karczewska, M., Loibner, A.P., Heipieper, H.J., and Chrzanowski, Ł. (2020). Microbial degradation of hydrocarbons—basic principles for bioremediation: A review. Molecules *25*.

Linkies, A., Jacob, S., Zink, P., Maschemer, M., Maier, W., and Koch, E. (2021). Characterization of cultural traits and fungicidal activity of strains belonging to the fungal genus *Chaetomium*. Journal of Applied Microbiology *131*, 375–391.

López-Bucio, J., Pelagio-Flores, R., and Herrera-Estrella, A. (2015). *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. Scientia Horticulturae *196*, 109–123.

Maamar, A., Lucchesi, M.-E., Debaets, S., Nguyen van Long, N., Quemener, M., Coton, E., Bouderbala, M., Burgaud, G., and Matallah-Boutiba, A. (2020). Highlighting the Crude Oil Bioremediation Potential of Marine Fungi Isolated from the Port of Oran (Algeria). Diversity *12*, 196.

Maitra, S., Brestic, M., Bhadra, P., Shankar, T., Praharaj, S., Palai, J.B., Shah, M.M.R., Barek, V., Ondrisik, P., Skalický, M., et al. (2022). Bioinoculants—Natural Biological Resources for Sustainable Plant Production. Microorganisms *10*, 51.

Malusà, E., Berg, G., Biere, A., Bohr, A., Canfora, L., Jungblut, A., Kepka, W., Kienzle, J., Kusstatscher, P., Masquelier, S., et al. (2021). A Holistic Approach for Enhancing the Efficacy of Soil Microbial Inoculants in Agriculture: From Lab to Field Scale. Global Journal of Agricultural Innovation, Research & Development *8*.

Manai, I., Miladi, B., El Mselmi, A., Smaali, I., Ben Hassen, A., Hamdi, M., and Bouallagui, H. (2016). Industrial textile effluent decolourization in stirred and static batch cultures of a new fungal strain *Chaetomium globosum* IMA1 KJ472923. Journal of Environmental Management *170*, 8–14.

Mandal, A., Sarkar, B., Mandal, S., Vithanage, M., Patra, A.K., and Manna, M.C. (2020). Chapter 7 - Impact of agrochemicals on soil health. In Agrochemicals Detection, Treatment and Remediation, M.N.V. Prasad, ed. (Butterworth-Heinemann), pp. 161–187.

Miller, R.M., and Jastrow, J.D. (2000). Mycorrhizal Fungi Influence Soil Structure. In Arbuscular Mycorrhizas: Physiology and Function, Y. Kapulnik, and D.D. Douds, eds. (Dordrecht, Netherlands: Springer Netherlands), pp. 3–18.

Moya, P., Cipollone, J., and Sisterna, M. (2020). The Fungal Genus *Chaetomium* and Its Agricultural Applications. In Plant Defence: Biological Control, J.-M. Mérillon, and K.G. Ramawat, eds. (Cham, CH: Springer International Publishing), pp. 289–308.

Murali, M., and Amruthesh, K.N. (2015). Plant Growth-promoting Fungus *Penicillium oxalicum* Enhances Plant Growth and Induces Resistance in Pearl Millet Against Downy Mildew Disease. Journal of Phytopathology *163*, 743–754.

Naziya, B., Murali, M., and Amruthesh, K.N. (2020a). Plant Growth-Promoting Fungi (PGPF) Instigate Plant Growth and Induce Disease Resistance in *Capsicum annuum* L. upon Infection with *Colletotrichum capsici* (Syd.) Butler & Bisby. Biomolecules *10*, 41.

Nephali, L., Piater, L.A., Dubery, I.A., Patterson, V., Huyser, J., Burgess, K., and Tugizimana, F. (2020). Biostimulants for Plant Growth and Mitigation of Abiotic Stresses: A Metabolomics Perspective. Metabolites *10*, 505.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., and Hens, L. (2016). Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. Frontiers in Public Health *4*.

Odukkathil, G., and Vasudevan, N. (2013). Toxicity and bioremediation of pesticides in agricultural soil. Reviews in Environmental Science and Bio/Technology *12*, 421–444.

Orgiazzi, A., Bardgett, R.D., and Barrios, E. (2016). Global soil biodiversity atlas. (Luxembourg: European Commission, Publications Office of the European Union) 176 pp.

Owen, D., Williams, A.P., Griffith, G.W., and Withers, P.J.A. (2015). Use of commercial bio-inoculants to increase agricultural production through improved phosphorus acquisition. Applied Soil Ecology *86*, 41–54.

Ozimek, E., and Hanaka, A. (2021). Mortierella Species as the Plant Growth-Promoting Fungi Present in the Agricultural Soils. Agriculture *11*, 7.

Park, J.-H., Choi, G.J., Jang, K.S., Lim, H.K., Kim, H.T., Cho, K.Y., and Kim, J.-C. (2005). Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. FEMS Microbiology Letters *252*, 309–313.

Pingali, P.L. (2012). Green Revolution: Impacts, limits, and the path ahead. PNAS *109*, 12302–12308.

Pinzari, F., Reverberi, M., Piñar, G., Maggi, O., and Persiani, A.M. (2014). Metabolic profiling of *Minimedusa polyspora* (Hotson) Weresub & P.M. LeClair, a cellulolytic fungus isolated from Mediterranean maquis, in southern Italy. Plant Biosystems*148*, 333–341.

Pothiraj, G., Hussain, Z., Singh, A.K., Solanke, A.U., Aggarwal, R., Ramesh, R., and Shanmugam, V. (2021). Characterization of Fusarium Spp. Inciting Vascular Wilt of Tomato and Its Management by a Chaetomium-Based Biocontrol Consortium. Frontiers in Plant Science *12*, 2544.

Pozo, M.J., Zabalgogeazcoa, I., Vazquez de Aldana, B.R., and Martinez-Medina, A. (2021). Untapping the potential of plant mycobiomes for applications in agriculture. Current Opinion in Plant Biology *60*, 102034.

Qin, J.-C., Zhang, Y.-M., Gao, J.-M., Bai, M.-S., Yang, S.-X., Laatsch, H., and Zhang, A.-L. (2009). Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from Ginkgo biloba. Bioorganic & Medicinal Chemistry Letters *19*, 1572–1574.

Rahman, M.A., Sultana, R., Begum, M.F., and Alam, M.F. (2012). Effect of culture filtrates of *Trichoderma* on seed germination and seedling growth in chili. International Journal of Biosciences *2*, 46–55.

Ramarajan, R., and Manohar, C.S. (2017). Biological pretreatment and bioconversion of agricultural wastes, using ligninolytic and cellulolytic fungal consortia. Bioremediation Journal *21*, 89–99.

Rashid, M.I., Mujawar, L.H., Shahzad, T., Almeelbi, T., Ismail, I.M.I., and Oves, M. (2016). Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. Microbiological Research *183*, 26–41.

Rodríguez-Eugenio, N., McLaughlin, M., and Pennock, D. (2018). Soil pollution: a hidden reality (Rome, Italy: FAO).

Salas-Marina, M.A., Silva-Flores, M.A., Cervantes-Badillo, M.G., Rosales-Saavedra, M.T., and Casas-Flores, M.A.I.-O. and S. (2011). The Plant Growth-Promoting Fungus *Aspergillus ustus* Promotes Growth and Induces Resistance Against Different Lifestyle Pathogens in *Arabidopsis thaliana*. Journal of Microbiology and Biotechnology *21*, 686–696.

Sangiorgio, D., Cellini, A., Donati, I., Pastore, C., Onofrietti, C., and Spinelli, F. (2020). Facing Climate Change: Application of Microbial Biostimulants to Mitigate Stress in Horticultural Crops. Agronomy *10*, 794.

Shah, A., Nazari, M., Antar, M., Msimbira, L.A., Naamala, J., Lyu, D., Rabileh, M., Zajonc, J., and Smith, D.L. (2021). PGPR in Agriculture: A Sustainable Approach to Increasing Climate Change Resilience. Frontiers in Sustainable Food Systems *5*.

Shanthiyaa, V., Saravanakumar, D., Rajendran, L., Karthikeyan, G., Prabakar, K., and Raguchander, T. (2013). Use of *Chaetomium globosum* for biocontrol of potato late blight disease. Crop Protection *52*, 33–38.

Siczek, A., Frąc, M., Gryta, A., Kalembasa, S., and Kalembasa, D. (2020). Variation in soil microbial population and enzyme activities under faba bean as affected by pentachlorophenol. Applied Soil Ecology *150*, 103466.

Singh, J., Aggarwal, R., Bashyal, B.M., Darshan, K., Parmar, P., Saharan, M.S., Hussain, Z., and Solanke, A.U. (2021). Transcriptome Reprogramming of Tomato Orchestrate the Hormone Signaling Network of Systemic Resistance Induced by *Chaetomium globosum*. Frontiers in Plant Science *12*, 1935.

Singh, H. B., Keswani, C., Reddy, M. S., Sansinenea, E., and García-Estrada, C. (2019). Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms: Discovery and Applications (Singapore, Singapore: Springer Singapore).

Singh, U.P., Sarma, B.K., and Singh, D.P. (2003). Effect of Plant Growth-Promoting Rhizobacteria and Culture Filtrate of *Sclerotium rolfsii* on Phenolic and Salicylic Acid Contents in Chickpea (*Cicer arietinum*). Current Microbiology *46*, 131–140.

Sowmya, H.V., Bellibatlu, R., M, K., and B, T. (2014). Low density polyethylene degrading fungi isolated from local dumpsite of shivamogga district. International Journal of Biological Research *2*, 39–43.

Spina, F., Cecchi, G., Landinez-Torres, A., Pecoraro, L., Russo, F., Wu, B., Cai, L., Liu, X.Z., Tosi, S., Varese, G.C., et al. (2018). Fungi as a toolbox for sustainable bioremediation of pesticides in soil and water. Plant Biosystems *152*, 474–488.

Stocking, M.A. (2001). Land Degradation. In International Encyclopedia of the Social & Behavioral Sciences, N.J. Smelser, and P.B. Baltes, eds. (UK, Oxford: Pergamon), pp. 8242–8247.

Stoleru, E., Vasile, C., Oprică, L., and Yilmaz, O. (2020). Influence of the Chitosan and Rosemary Extract on Fungal Biodegradation of Some Plasticized PLA-Based Materials. Polymers *12*, 469.

Sung, G.-H., Shrestha, B., Park, K.-B., Han, S.-K., and Sung, J.-M. (2011). Enhancing Effect of *Shimizuomyces paradoxus* on Seed Germination and Seedling Growth of Canola, Plant Growth of Cucumber, and Harvest of Tomato. Mycobiology *39*, 7–11.

Tang, F.H.M., Lenzen, M., McBratney, A., and Maggi, F. (2021). Risk of pesticide pollution at the global scale. Nature Geoscience *14*, 206–210.

Tarafdar, J.C., and Gharu, A. (2006). Mobilization of organic and poorly soluble phosphates by *Chaetomium globosum*. Applied Soil Ecology *32*, 273–283.

Tian, Z., Wang, J., Li, J., and Han, B. (2021). Designing future crops: challenges and strategies for sustainable agriculture. Plant Journal *105*, 1165–1178.

Tiedje, J.M., and Hagedorn, M.L. (1975). Degradation of alachlor by a soil fungus, *Chaetomium globosum*. Journal of Agricultural and Food Chemistry *23*, 77–81.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. Nature *418*, 671–677.

Trivedi, P., Schenk, P.M., Wallenstein, M.D., and Singh, B.K. (2017). Tiny Microbes, Big Yields: enhancing food crop production with biological solutions. Microbial Biotechnology *10*, 999–1003.

Trivedi, P., Mattupalli, C., Eversole, K., and Leach, J.E. (2021). Enabling sustainable agriculture through understanding and enhancement of microbiomes. New Phytologist *230*, 2129–2147.

Tsiafouli, M.A., Thébault, E., Sgardelis, S.P., de Ruiter, P.C., van der Putten, W.H., Birkhofer, K., Hemerik, L., de Vries, F.T., Bardgett, R.D., Brady, M.V., et al. (2015). Intensive agriculture reduces soil biodiversity across Europe. Global Change Biology *21*, 973–985.

United Nations (2015). Transforming Our World: The 2030 Agenda for Sustainable Development (No. A/70/L.1), New York: United Nations, http://www.un.org/ga/search/view_doc.asp?symbol=A/70/L.1&referer=/english/&Lang=E (accessed on 1 December 2021).

United Nations, Department of Economic and Social Affairs, and Population Division (2019). World population prospects Highlights, 2019 revision.

Varma, A., Savita Verma, Sudha, Sahay, N., Bütehorn, B., and Franken, P. (1999). *Piriformospora indica* a Cultivable Plant-Growth-Promoting Root Endophyte. Applied and Environmental Microbiology *65*, 2741.

Vazquez, C., de Goede, R.G.M., Rutgers, M., de Koeijer, T.J., and Creamer, R.E. (2021). Assessing multifunctionality of agricultural soils: Reducing the biodiversity trade-off. European Journal of Soil Science *72*, 1624–1639.

Velásquez, A.C., Castroverde, C.D.M., and He, S.Y. (2018). Plant–Pathogen Warfare under Changing Climate Conditions. Current Biology *28*, R619–R634.

Větrovský, T., Morais, D., Kohout, P., Lepinay, C., Algora, C., Awokunle Hollá, S., Bahnmann, B.D., Bílohnědá, K., Brabcová, V., D'Alò, F., et al. (2020). GlobalFungi, a global database of fungal occurrences from high-throughput-sequencing metabarcoding studies. Scientific Data *7*, 228.

Vivi, V.K., Martins-Franchetti, S.M., and Attili-Angelis, D. (2019). Biodegradation of PCL and PVC: *Chaetomium globosum* (ATCC 16021) activity. Folia Microbiologica *64*, 1–7.

Wang, X.W., Lombard, L., Groenewald, J.Z., Li, J., Videira, S.I.R., Samson, R.A., Liu, X.Z., and Crous, P.W. (2016). Phylogenetic reassessment of the *Chaetomium globosum* species complex. Persoonia *36*, 83–133.

Whalley, W.R., Riseley, B., Leeds-Harrison, P.B., Bird, N.R.A., Leech, P.K., and Adderley, W.P. (2005). Structural differences between bulk and rhizosphere soil. European Journal of Soil Science *56*, 353–360.

Whitfield, J. (2007). Underground networking. Nature *449*, 136–138.

Yadav, A.N., Kour, D., Kaur, T., Devi, R., and Yadav, N. (2020). Agriculturally Important Fungi for Crop Productivity: Current Research and Future Challenges. In Agriculturally Important Fungi for Sustainable Agriculture: Volume 1: Perspective for Diversity and Crop Productivity, A.N. Yadav, S. Mishra, D. Kour, N. Yadav, and A. Kumar, eds. (Cham, CH: Springer International Publishing), pp. 275–286.

Yan, W., Cao, L.-L., Zhang, Y.-Y., Zhao, R., Zhao, S.-S., Khan, B., and Ye, Y.-H. (2018). New Metabolites from Endophytic Fungus *Chaetomium globosum* CDW7. Molecules *23*, 2873.

Yang, S.-W., Mierzwa, R., Terracciano, J., Patel, M., Gullo, V., Wagner, N., Baroudy, B., Puar, M., Chan, T.-M., and Chu, M. (2007). Sch 213766, A Novel Chemokine Receptor CCR-5 Inhibitor from *Chaetomium globosum*. The Journal of Antibiotics *60*, 524–528.

You, Y.-H. (2012). Fungal Diversity and Plant Growth Promotion of Endophytic Fungi from Six Halophytes in Suncheon Bay. Journal of Microbiology and Biotechnology *22*, 1549–1556.

Zhai, X., Luo, D., Li, X., Han, T., Jia, M., Kong, Z., Ji, J., Rahman, K., Qin, L., and Zheng, C. (2018). Endophyte *Chaetomium globosum* D38 Promotes Bioactive Constituents Accumulation and Root Production in *Salvia miltiorrhiza*. Frontiers in Microbiology *8*, 2694.

Zhang, G., Wang, F., Qin, J., Wang, D., Zhang, J., Zhang, Y., Zhang, S., and Pan, H. (2013). Efficacy assessment of antifungal metabolites from *Chaetomium globosum* No.05, a new biocontrol agent, against *Setosphaeria turcica*. Biological Control *64*, 90–98.

Zhang, S., Gan, Y., and Xu, B. (2016). Application of Plant-Growth-Promoting Fungi *Trichoderma longibrachiatum* T6 Enhances Tolerance of Wheat to Salt Stress through Improvement of Antioxidative Defense System and Gene Expression. Frontiers in Plant Science *7*, 1405.

Zhou, L.S., Tang, K., and Guo, S.X. (2018). The Plant Growth-Promoting Fungus (PGPF) *Alternaria* sp. A13 Markedly Enhances *Salvia miltiorrhiza* Root Growth and Active Ingredient Accumulation under Greenhouse and Field Conditions. International Journal of Molecular Sciences *19*, 270.

Zhou, W., Starr, J.L., Krumm, J.L., and Sword, G.A. (2016). The fungal endophyte *Chaetomium globosum* negatively affects both above- and belowground herbivores in cotton. FEMS Microbiology Ecology *92*, fiw158.

CHAPTER 2: AIM AND OBJECTIVES

The overall aim of this thesis was:

to characterize *Minimedusa polyspora* and *Chaetomium globosum*, two strains of soil saprotrophic fungi, for multiple activities - including biostimulation, biocontrol and bioremediation- that may contribute to plant growth promotion, to assess their potential as multifunctional bioresources for biotechnological application aimed at promoting a more sustainable agriculture.

The specific objectives to fulfil the overall aim were to:

- Assess the efficacy of *M. polyspora* and *C. globosum* culture filtrates as biostimulant for the cultivation *Cichorium intybus*, a plant of agricultural and medicinal interest. Both effects on growth parameters and on the metabolome variation of the plant following the biostimulation, were addressed.

- Investigate *M. polyspora* and *C. globosum* biocontrol potential against the phytopathogenic fungi *Alternaria alternata*, *Berkeleyomyces basicola* and *Botrytis cinerea*.

- Evaluate these strains' bioremediation potentialities towards glyphosate. In particular, it has been evaluated the ability of *M. polyspora* and *C. globosum*, within a wider pool of species, to tolerate and utilize glyphosate as a nutritional source and eventually degrade it.

CHAPTER 3: BIOSTIMULANT EFFECTS OF *CHAETOMIUM GLOBOSUM* AND *MINIMEDUSA POLYSPORA* CULTURE FILTRATES ON *CICHORIUM INTYBUS* PLANT: GROWTH PERFORMANCE AND METABOLOMIC TRAITS

frontiers | Frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 May 2022
doi: 10.3389/fpls.2022.879076

Biostimulant Effects of *Chaetomium globosum* and *Minimedusa polyspora* Culture Filtrates on *Cichorium intybus* Plant: Growth Performance and Metabolomic Traits

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Frontiers in Plant Science 2022, 13:879076; doi: 10.3389/fpls.2022.879076

ABSTRACT

In this study, we investigated the biostimulant effect of fungal culture filtrates obtained from *Chaetomium globosum* and *Minimedusa polyspora* on growth performance and metabolomic traits of chicory (*Cichorium intybus*) plants. For the first time, we showed that *M. polyspora* culture filtrate exerts a direct plant-growth promoting effect, through an increase of biomass, both in shoots and roots, and of the leaf area. Conversely, no significant effect on morphological traits and biomass yield was observed in *C. intybus* plants treated with *C. globosum* culture filtrate. Based on ^{1}H-NMR metabolomics data, differential metabolites and their related metabolic pathways were highlighted. The treatment with *C. globosum* and *M. polyspora* culture filtrates stimulated a common response in *C. intybus* roots involving the synthesis of 3-OH-butyrate through the decrease in the synthesis of fatty acids and sterols, as a mechanism balancing the NADPH/$NADP^{+}$ ratio. The fungal culture filtrates differently triggered the phenylpropanoid pathway in *C. intybus* plants: *C. globosum* culture filtrate increased phenylalanine and chicoric acid in the roots, whereas *M. polyspora* culture filtrate stimulated an increase of 4-OH benzoate. Chicoric acid, whose biosynthetic pathway in

the chicory plant is putative and still not well known, is a very promising natural compound playing an important role in plant defense. On the contrary, benzoic acids serve as precursors for a wide variety of essential compounds playing crucial roles in plant fitness and defence response activation. To the best of our knowledge this is the first study that shows the biostimulant effect of *C. globosum* and *M. polyspora* culture filtrates on *C. intybus* growth and metabolome, increasing the knowledge on fungal bioresources for the development of biostimulants.

KEYWORDS

Chaetomium globosum, *Minimedusa polyspora*, fungal culture filtrates, fungi, biostimulants, plant growth promotion, *Cichorium intybus*, ^{1}H-NMR based metabolomics

3.1 INTRODUCTION

Modern agriculture is currently facing the major challenge of adapting to a rapidly evolving climate change while searching for new strategies to increase food production. Indeed, by 2050, the world population is expected to grow reaching nearly 9.7 billion requiring an increase in food production by up to 60%, while due to climate change plants already are more frequently subjected to severe abiotic and biotic stresses (Nephali et al., 2020; Sangiorgio et al., 2020; Tian et al., 2021; United Nations Department of EconomicSocial Affairs, Population Division, 2019; Velásquez et al., 2018). This challenge is also concomitant with the need to reduce agriculture's impact on the environment, since more than 33% of soils worldwide are already degraded (Abhilash, 2021). In fact, the over-application of agrochemicals, on which agriculture heavily relied to meet the food demand, impaired the environment, determining phenomena such as eutrophication, ecosystem simplification, loss of ecosystem services, and loss of biodiversity and of soil quality (Spinelli et al., 2021; Tian et al., 2021; Tilman et al., 2002).

Therefore, it is necessary to promote a more sustainable and resilient agriculture, based on environmentally friendly strategies and solutions, capable of preserving and restoring ecosystems and natural resources, as also affirmed in the UN 2030 Agenda Sustainable Development Goals (SDG 2.4) (United Nations, 2015). In this context, biostimulants represent an interesting sustainable solution that may play a key role in increasing crop resilience and productivity in adverse environmental conditions, minimizing agrochemicals applications and tackling climate change effects (Castiglione et al., 2021; Del Buono, 2021; Ganugi et al., 2021). Moreover, microbial biostimulants present advantageous characteristics such as not accumulating in the long term, a lower toxicity, and a scarce tendency to select resistant strains of pests and pathogens compared to agrochemicals (Sangiorgio et al., 2020).

The acknowledgment of the significant role of biostimulants in the picture of a more sustainable agriculture is also reflected by their introduction as fertilizing products in the EU regulation 2019/1009 (EU, 2019). According to the new regulation a plant biostimulant "shall be an EU fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency, (b) tolerance to abiotic stress, (c) quality traits, or (d) availability of confined nutrients in the soil or rhizosphere". Concerning microbial biostimulants, the new legislation considers *in vivo*, dead or empty-cell microorganisms, and non-harmful residual elements of the media on which they were produced, but unfortunately lists only *Azospirillum* spp., *Azotobacter* spp., *Rhizobium* spp. and mycorrhizal fungi as suitable microorganisms.

Despite still not being included in the current legislation, several non-mycorrhizal fungal strains have already been reported in scientific literature as effective plant growth-promoting fungi (PGPF), and some of these have also been employed in the formulation of commercialized products (Hyde et al., 2019). Furthermore, the new legislation, to avoid regulatory conflicts with phytochemicals, does not consider the bioprotection effects associated with some biostimulants. In fact, biostimulants may also provide protection from biotic stresses, both eliciting the production of secondary metabolites inducing systemic resistance, and exerting a direct activity against pest and/or pathogens (Ganugi et al., 2021; Sangiorgio et al., 2020).

Since PGPF exert their action also through diffusible substances such as phytohormones, enzymes, amino acids, and siderophores, research focused also on the application of culture filtrates as biostimulants. Culture filtrates have been consistently reported in several studies to be effective in promoting plant growth by enhancing seed germination, biomass production, and metabolites production (Bagde et al., 2011, 2013; Baroja-Fernández et al., 2021; Bilal et al., 2018; Hamayun et al., 2009, 2010; Hwang et al., 2011; Khalmuratova et al., 2021; Khan et al., 2015, 2008; Murali and Amruthesh, 2015; Rahman et al., 2012; Singh et al., 2003; Sung et al., 2011; Varma et al., 1999; You, 2012).

Chaetomium globosum Kunze is a filamentous species belonging to the Ascomycota phylum. *C. globosum* presents a cosmopolitan distribution and has been reported as both a saprotroph and a plant endophyte with a wide range of host plants (Linkies et al., 2021; Větrovský et al., 2020). It presents great adaptability by inhabiting various environments including extreme habitats such as deserts or salt lakes, and is commonly isolated in agricultural soils (Abdel-Azeem, 2020; Linkies et al., 2021; Větrovský et al., 2020).

C. globosum is a widely studied species and several studies highlighted the production of a wide array of bioactive metabolites such as hydrocarbons, phenols, terpenoids and

sulfur compounds including 4-methyl- (1,5-dimethyl-4-hexenyl)-benzene, tetradecane, dodecane, hexadecane, β-bisabolene and dimethyl-propyl-disulfide that were identified as major components, and chlorinated azaphilone derivatives such as chaetomugilins and chaetoglobosins with antifungal activity (Abdel-Azeem, 2020; Kumar et al., 2020, 2021; Qin et al., 2009). Moreover, it has already been reported as a plant growth promoter species and as an effective biocontrol agent against a large number of fungal pathogens and nematodes (Abdel-Azeem, 2020; Abou Alhamed and Shebany, 2012; Aggarwal et al., 2004, 2016; Biswas et al., 2012; Hu et al., 2013; Khan et al., 2012, 2019; Kumar et al., 2021; Linkies et al., 2021; Moya et al., 2020; Singh et al., 2021; Tarafdar and Gharu, 2006; Yan et al., 2018). Definitely less studied is *Minimedusa polyspora* (Hotson) Weresub & P.M. Le Clair, a filamentous anamorphic species belonging to the Basidiomycota phylum. *M. polyspora* has been widely isolated in agricultural soils worldwide and found to be overrepresented in the tilled soil (Klaubauf et al., 2010; Longley et al., 2020; Lucadamo, 2018; Orrù et al., 2021; Panelli et al., 2017; Větrovský et al., 2020). This species presents several traits such as rapid growth, low requirement of nitrogen, ability to concentrate important biogenic macroelements (N, P, S, K and Ca) and translocate nutrients, metabolic plasticity, and the production of antibiotic and antifungal compounds (triene compounds) which make it an efficient pioneer colonizer (Beale and Pitt, 1995; Pinzari et al., 2014). Furthermore, *M. polyspora* has already been suggested to be a PGPF given its ability to solubilize inorganic phosphorous and to antagonize *Fusarium oxysporum* f.sp. *narcissi* (Beale and Pitt, 1990, 1995; Ceci et al., articolo in revisione), nevertheless, to the best of our knowledge, no further studies with a wider focus than *Fusarium* biocontrol have yet been reported on its direct effect on plant growth promotion.

Cichorium intybus L., commonly known as chicory, is a perennial herb belonging to the *Asteraceae* family. Native to Europe, Northern Africa and Mid-Asia, *C. intybus* has been introduced also in Northern America, Southern America, India, Asia, Australia, and New Zealand reaching nowadays a cosmopolitan distribution (Al-Snafi, 2016; Kew Science, 2021; Wang and Cui, 2011).

Chicory, cultivated since the III century before Christ for several purposes, has also been historically reported as a medicinal plant commonly utilized in the traditional medicine of several countries in Europe, Africa and Asia (Puhlmann and de Vos, 2020; Street et al., 2013; Wang and Cui, 2011). *C. intybus* extracts have been reported in recent years to possess analgesic, anti-inflammatory, antimalarial, antimicrobial, anticancer, antineurotoxic, antiviral, hypotensive, hepatoprotective, and anti-protozoal and antiparasitic properties (Al-Snafi, 2016; Bischoff et al., 2004; Janda et al., 2021; Street et al., 2013).

These multiple functions of chicory are deeply related to its rich and complex phytochemical profile including a great number of bioactive substances. Despite being

more abundant in the roots, important phytochemicals are reported to be distributed throughout the whole plant including sesquiterpene lactones, caffeic acid derivatives, organic acids, inulin, flavonoids, polyphenols, alkaloids, steroids, fats, proteins, hydroxycoumarins, terpenoids, oils, glycosides, volatile compounds, vitamins, β-carotene, zeaxanthin, β-sitosterol, tannins and minerals (Bais and Ravishankar, 2001; Janda et al., 2021; Perović et al., 2021; Puhlmann and de Vos, 2020; Street et al., 2013). This rich profile presents multiple nutritionally important compounds, including among the most important ones: carbohydrates, phenolic compounds, flavonoids, amino acids and proteins, fatty acids, sesquiterpene lactones, vitamins, minerals and micronutrients (Perović et al., 2021). Thanks to its valuable nutritional values and its health-promoting characteristics, chicory is commonly used as a vegetable, coffee substitute, forage, and functional ingredient in commercial food products (Janda et al., 2021; Perović et al., 2021; Puhlmann and de Vos, 2020). A wide range of chicory cultivars, selected on the basis of their suitability for each purpose (e.g. leafy salad, root production, and forage), are currently available (Wang and Cui, 2011).

In this study, we aimed at evaluating the effect of the application of fungal culture filtrates of *M. polyspora* and *C. globosum*, as biostimulants, on the growth and metabolism of *C. intybus* plants. Our study has been structured to address: a) the evaluation of the effectiveness of culture filtrates in promoting plant growth, through the assessment of morphological and physiological parameters of growth; b) the evaluation of metabolism modification after culture filtrate application, addressing possible mechanisms of action of biostimulation. To the best of our knowledge, these fungal species have not been previously studied to promote *C. intybus* growth, and we, therefore, aimed at increasing the knowledge on fungal bioresources for the development of biostimulants, applicable for a sustainable cultivation of this species that is of agronomic and medicinal interest.

3.2 MATERIALS AND METHODS

3.2.1 FUNGAL STRAINS AND CULTURE FILTRATES PRODUCTION

Two fungal strains, isolated in previous studies and currently preserved at the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), *C. globosum* Kunze FBL 205 and *M. polyspora* (Hotson) Weresub & P. M. LeClair FBL 503, were studied to assess the ability of their culture filtrate to promote plant growth. Prior to the experiment, the strains were reactivated and maintained on malt extract agar (MEA) at 25°C in the dark. MEA was prepared according to the following composition (g/L in distilled water): malt extract, 20; peptone, 1; dextrose, 20; and bacto agar, 20. All components were purchased from Becton Dickinson (Sparks, MD, USA).

Each strain's culture filtrate was prepared by inoculating four 4-mm diameter plugs of mycelium, taken from the actively growing margin of a 10-day-old stock culture using a sterile cork borer, in 150 mL Erlenmeyer flask containing 60 mL of malt extract broth (MEB). MEB was prepared with the same abovementioned composition of MEA excluding bacto agar. Five replicates were set up for each strain and 5 Erlenmeyer flasks were left uninoculated for the control treatment. The flasks were incubated at 25°C, on an orbital shaker (ASAL 711/D) at 100 rpm, for 14 days.

At the end of the incubation period, the culture medium was recovered and filtered using sterile syringe filters with a 0.45 µm pore size made of mixed cellulose esters (ClearLine®, Dominique Dutscher SAS, Brumath, France). Culture filtrates from different biological replicates of the same fungal strain were pooled together, and a sample was recovered to perform ^{1}H-NMR-based metabolomics analysis to identify the metabolites released by the fungus.

3.2.2 PLANT MATERIAL

Wild chicory (*Cichorium intybus*) seeds (Fratelli Ingegnoli Spa, Milano, Italy) were surface sterilized for 20 minutes in a 20% ethanol solution of 15% sodium hypochlorite, followed by 5 rinses in sterile distilled water. Sterilized seeds were plated on Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) supplemented with 30 g/L of sucrose (Carlo Erba) and 5 g/L of bacto agar (Becton Dickinson, Sparks, MD, USA). Ten chicory seeds were placed in each petri dish. Petri dishes were incubated at 25°C under a photoperiod of 16/8 h (light/dark) to promote seed germination. After 15 days seedlings were transferred to disposable plastic pots of diameter 13 cm and height 10 cm, containing approximately 265 g of autoclaved universal potting soil (COMPO SANA® Universal potting soil, COMPO Italia Srl, Cesano Maderno, Italy). Pots were incubated in a walk-in chamber at 18/22°C under a photoperiod of 15/9 h (light/dark) and watered every 3 days.

3.2.3 APPLICATION OF THE FUNGAL CULTURE FILTRATES

One month after the seedlings were transferred into the pots, 8 mL of the culture filtrate was directly added to the soil of each pot (concentration 30 mL/kg) in 8 different points positioned at two depths according to the scheme reported in **Figure 3.1**. In addition to the two treatments with *M. polyspora* (503) and with *C. globosum* (205) culture filtrates, two control groups treated with a corresponding volume of distilled water (control water) or with a corresponding volume of uninoculated MEB (MEB) were set up. On the treatment day, plants were watered in the morning and let draining excess for 5/6 hours prior to the culture filtrate inoculation. Ten plants were randomly picked and assigned to each treatment.

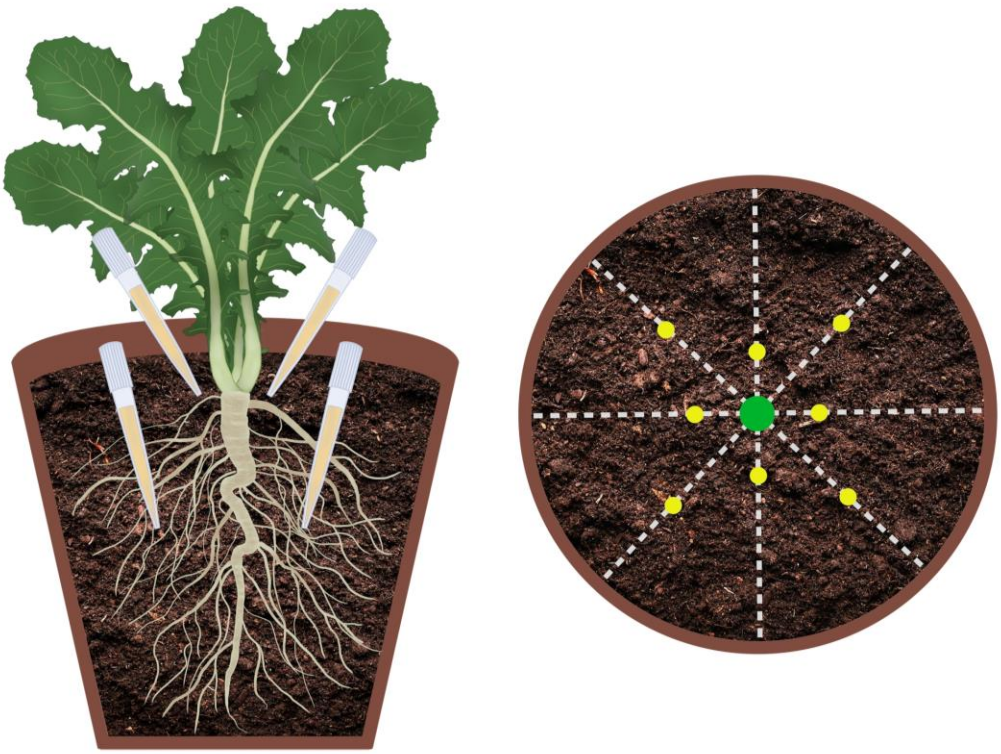


Figure 3.1 Graphical representation of culture filtrate application. Left: Virtual vertical section of the pot; Right: Top view of the pot.

3.2.4 ASSESSMENT OF GROWTH OF *CICHORIUM INTYBUS* PLANTS

Fourteen days after the culture filtrate addition, several parameters were measured to assess the plant growth. Growth parameters of five replicates randomly picked for each treatment were evaluated. Plants were removed from the pots and carefully washed to remove soil assuring to avoid root biomass loss. The root biomass was separated into taproot and secondary roots, and then dried at 70°C for 48 h in the oven and weighted. The number of leaves per plant was recorded. The leaves were cut at the base, wrapped in a moist paper towel, and stored in containers overnight at 4°C. To determine the total leaf area per pot, water-saturated leaves were blotting dried and images of them were acquired and later analyzed using the software ImageJ (version 1.53c, Wayne Rasband, National Institutes of Health, Bethesda, USA. Available online: https://imagej.nih.gov/ij). Following the image acquisition shoot biomass was oven-dried

at 70°C for 48 h to determine the dry weight. Mean specific leaf area (SLA) per pot (total leaf area divided by total dry weight) and root /shoot ratio were also calculated.

3.2.5 SAMPLE PREPARATION, ^{1}H-NMR SPECTRA ACQUISITION AND PROCESSING

Fourteen days after the culture filtrate addition, five replicates randomly picked were recovered for untargeted metabolomic analysis by ^{1}H-NMR for each of the treatments: MEB control (Ctrl), *C. globosum* (205) and *M. polyspora* (503). Plants were removed from the pots, washed from the soil and separated into root and shoot biomass. Both the fractions were immediately frozen with liquid nitrogen to ensure the metabolic quenching and stored at −80°C. For both leaves and roots, 1.5 g of biomass grounded in liquid nitrogen was extracted according to a modified Bligh-Dyer protocol (Giampaoli et al., 2021) using a mixture of methanol, chloroform, and distilled water (3:3:1.2 mL). After overnight incubation at 4°C, samples were centrifuged at 10,000 g for 25 minutes at 4°C on an Itettich Zentrifugen centrifuge (Germany) and hydroalcoholic and chloroformic fractions were separated and dried under N_2 flow. The dried phases were stored at −80°C until the NMR analysis. The dried residue of the hydro-alcoholic phase was dissolved in 0.6 mL CD_3OD/D_2O (1:2 v/v ratio) containing 3-(trimethylsilyl)-propionic-2,2,3,3,-d_4 acid sodium salt (TSP, 2 mM) as internal standard (chemical shift reference). The dried residue of the chloroformic phase was dissolved in 0.6 mL $CDCl_3$, (Cambridge Isotope Laboratories, Inc.), (99.8%) containing 1,1,3,3,5,5-hexamethylcyclo-trisiloxane (HMS) (Sigma-Aldrich, USA) as internal standard (2 mM). All spectra were recorded at 298 K on a Jeol JNM-ECZ 600R spectrometer operating at the proton frequency of 600 MHz and equipped with a multinuclear z-gradient inverse probe head.

Hydroalcoholic ^{1}H spectra were acquired employing the *presat* pulse sequence for solvent suppression with 128 transients, a spectral width of 9013.7 Hz, and 64 k data points for an acquisition time of 7.3 s. The recycle delay was set to 7.7 s in order to achieve complete resonance relaxation between successive scansions.

Chloroform ^{1}H spectra were acquired employing a single-pulse sequence with 128 transients, a spectral width of 9013.7 Hz and 64 k data points for an acquisition time of 7.3 s. The recycle delay was set to 7.7 s in order to achieve complete resonance relaxation between successive scansions.

Resonance assignment was carried out on the basis of 2D ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC experiments. ^{1}H-^{1}H TOCSY spectra were acquired with 64 scans, a spectral width of 9013.7 Hz in both dimensions, a data matrix of 8 k × 256 data points, a recycle delay of 3 s, and a mixing time of 90 ms; ^{1}H-^{13}C HSQC spectra were acquired with 128 scans, a spectral width of 9013.7 Hz and 30,000 Hz for hydrogen and carbon dimension respectively, a data matrix of 8k × 256 data points, a recycle delay of 3 s and a direct constant of 145 Hz.

Fungal culture filtrate analysis was carried out by adding to 0.35 mL of filtrate an amount of 0.35 mL of D_2O containing 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP, 2 mM) as internal chemical shift and concentration standard, and the sample was analyzed by ^{1}H-NMR employing the *presat* pulse sequence.

3.2.6 STATISTICAL ANALYSIS

All statistical analyses on growth parameters were carried out using the statistical software R (version 4.1.0) under the R-studio environment (version 1.4.1106). For all parameters belonging to the morphological dataset, the normality and homoscedasticity of the data were tested using Shapiro-Wilk and, as appropriate, Bartlett or Levene test, respectively (packages lawstat and stats). Hereafter, differences between groups were tested using the Mann-Whitney U Test, performed through the "Wilcoxon rank sum exact test" function (package stats).

Multivariate PCA and PLS-DA were performed on the data matrix using the Unscrambler ver. 10.5 software (Camo Software AS, Oslo, Norway). Univariate t-test and Pearson's correlation coefficients were calculated with SigmaPlot 14.0 software (Systat Software Inc., San Jose, CA, USA).

3.3 RESULTS

3.3.1 EFFECTS OF THE APPLICATION OF FUNGAL CULTURE FILTRATE ON *CICHORIUM INTYBUS* GROWTH

The values of growth parameters, evaluated 14 days after culture filtrate application, are reported in **Table 3.1**. Plant growth promotion effect, in terms of increase of biomass production, has been observed only in plants treated with *M. polyspora* culture filtrate. In fact, a statically significant difference ($p < 0.05$) in the total dry weight of the plant has been observed solely by comparing *M. polyspora* treatment with both water and MEB control, and the same significant result has been observed considering the dry weight of the shoot. Regarding the dry weights of the total root system and of the lateral roots, the only significant difference ($p < 0.05$) observed is between *M. polyspora* treatment and the MEB control, while no statistically significant differences among all treatments have been recorded in the taproot dry weight.

Focusing on shoot parameters, while no statistically significant differences have been observed in the numbers of leaves, a statically significant difference ($p < 0.05$) in leaf area has been observed comparing *M. polyspora* treatment with both MEB control and *C. globosum* treatment. It is interesting to note that the leaf area values of the plants treated

with *C. globosum* culture filtrate resulted to be lower than controls, although it did not result to be statistically significant.

Finally, no significant differences were observed among the treatments concerning the indexes of root/shoot ratio and SLA.

Table 3.1 Values of evaluated growth parameters. The data are expressed as the mean ± standard error of independent replicates (n=5). * Statistically significant compared to Water control; • Statistically significant compared to MEB control; ▲ Statistically significant compared to the other culture filtrate treatment (U Mann – Whitney, $p < 0.05$).

Parameter	Treatment			
	Water Control	**MEB Control**	***C. globosum***	***M. polyspora***
Dry weight total plant (g/plant)	2.90 ± 0.12	2.96 ± 0.03	3.00 ± 0.28	3.59 ± 0.19 *,•
Dry weight shoot (g/plant)	2.23 ± 0.04	2.38 ± 0.06	2.30 ± 0.23	2.83 ± 0.14 *,•
Dry weight total root system (g/plant)	0.66 ± 0.10	0.58 ± 0.03	0.70 ± 0.08	0.76 ± 0.06 •
Dry weight taproot (g/plant)	0.19 ± 0.03	0.18 ± 0.02	0.20 ± 0.03	0.16 ± 0.02
Dry weight lateral roots (g/plant)	0.47 ± 0.07	0.41 ± 0.04	0.50 ± 0.7	0.60 ± 0.07 •
Number of leaves	28.80 ± 1.59	33.40 ± 4.01	31.60 ± 2.01	42.40 ± 5.72
Leaf area (cm^2)	823.86 ± 31.61	789.50 ± 25.02	740.79 ± 42.83 ▲	996.20 ± 66.39 ▲,•
Specific leaf area index (m^2/kg)	36.90 ± 1.17	33.35 ± 1.72	33.36 ± 3.49	35.07 ± 0.95
Root/shoot ratio	0.30 ± 0.04	0.25 ± 0.02	0.31 ± 0.03	0.27 ± 0.01

3.3.2 ^{1}H-NMR ANALYSIS OF CULTURE FILTRATES

The filtrate composition is reported in **Supplementary Table 3.2**. ^{1}H-NMR analysis of *C. globosum* culture filtrate showed a decrease in all amino acids including leucine, isoleucine, valine, threonine, lysine, tyrosine, phenylalanine, glutamate, and acetate that resulted to be consumed by the fungus and an increase of peptone, ethanol and fumarate that were released. A slight decrease in glucose, maltose and fructose was also observed.

Conversely, in *M. polyspora* culture filtrate, a high increase in glucose associated with a decrease in maltose was observed. No variation in amino acids except for alanine and lysine was observed. A production of adenosine and guanosine phosphates nucleotides (AXP and GXP, respectively) was also detected.

3.3.3 ^{1}H-NMR-BASED METABOLOMICS OF *CICHORIUM INTYBUS* LEAVES AND ROOTS

The inspection of the 600 MHz ^{1}H-NMR spectra obtained from hydroalcoholic and chloroformic extracts of chicory leaves and roots revealed the presence of 49 molecules univocally identified. A total of 46 metabolites including amino acids, organic acids, sugars, organic compounds, fatty acids, secondary metabolites and other compounds were integrated. Only the molecules univocally identified were considered for the study, and their quantification was performed by integration of their NMR signals. Due to the overcrowding of ^{1}H-NMR spectra, only those signals that did not overlap with other resonances were considered for integration. Comparing the spectra obtained from leaves and roots, it was possible to observe both qualitative and quantitative differences, while the spectral comparison among the control group and the treatments showed only quantitative differences. Examples of ^{1}H-NMR spectra are reported in **Supplementary Figure 3.11Supplementary Figure 3.14**, and the table of resonance assignment is reported in Supplementary Table 3.3. Quantitative analysis of the phytochemical composition of *C. intybus* roots and leaves are reported in Supplementary Table 3.4 and **Supplementary Table 3.5**, respectively.

To examine an overview of the whole NMR data set, a preliminary unsupervised PCA was performed in leaves and roots separately. In leaves, PC1 and PC2 components explained 42% of the total variation, and PCA score plot revealed a clustering of the samples according to the treatment with the fungal filtrate (**Supplementary Figure 3.15**). In roots, 41% of the total variation was explained by the two main components, with the first and second contributing 24 and 17%, respectively (**Supplementary Figure 3.16**).

With the aim of refining the sample grouping observed in the unsupervised PCA model, PLS-DA discriminant analyses were performed to identify the most important metabolites that discriminated the treatments with *C. globosum* and *M. polyspora* separately in leaves and roots.

3.3.4 EFFECTS OF *CHAETOMIUM GLOBOSUM* CULTURE FILTRATE ON METABOLOME OF *CICHORIUM INTYBUS* LEAVES AND ROOTS

In leaves treated with *C. globosum* filtrate, PLS-DA analysis provided a model (R2 =0.96; Q2 =0.62) with three discriminant components explaining 34, 76 and 15% of the variance **(Figure 3.2)**. The corresponding PLS-DA score plot revealed a clear separation of the leaves treated with *C. globosum* filtrate (205) from control samples.

The levels of campesterol, chlorophyll *a* and *b*, β-sitosterol, valine, oleic acid and isoleucine decreased in *C. globosum* group compared with controls (**Figure 3.3**).

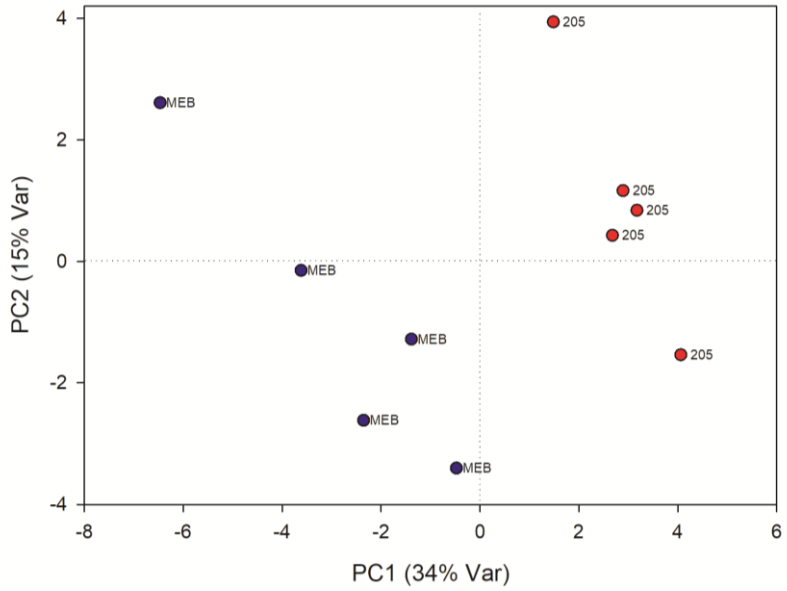


Figure 3.2 PLS-DA score plot of NMR metabolomics data of chicory leaves extracts of MEB (Control) and *C. globosum* (205) groups. Blue dots indicate MEB (control) group and red dots *C. globosum* group.

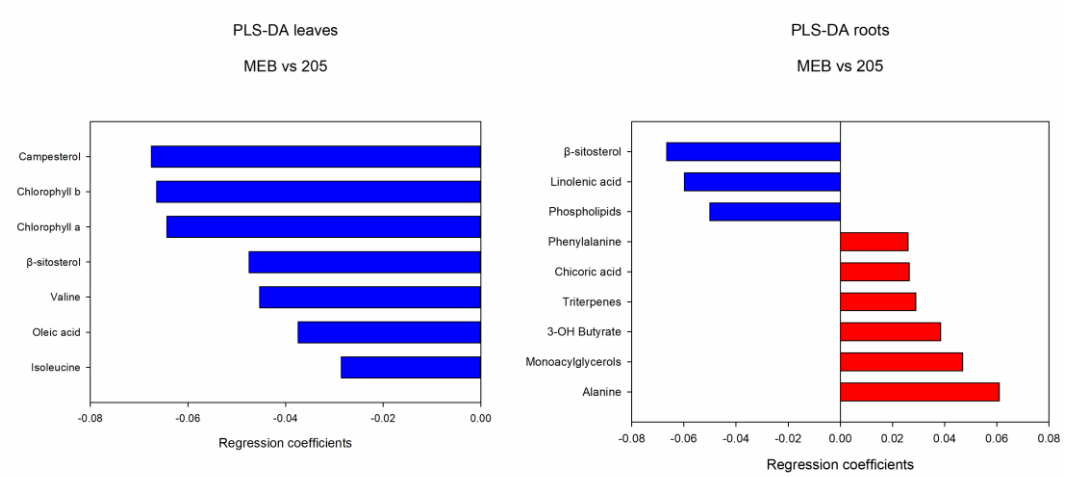


Figure 3.3 PLS-DA regression coefficients of significantly different metabolites in chicory leaf and root extracts of MEB (control) and *C. globosum* (205).

In roots treated with *C. globosum* filtrate, the PLS-DA model showed a clear separation of samples with good descriptive parameters (R2 =0.97; Q2 =0.75) (**Figure 3.4**). A significant decrease of β –sitosterol, linolenic acid (ω-3) and phospholipids, and a significant increase in phenylalanine, chicoric acid, triterpenes, 3-OH-butyrate, monoacylglycerols and alanine were observed in *C. globosum* compared with control samples (**Figure 3.3**).

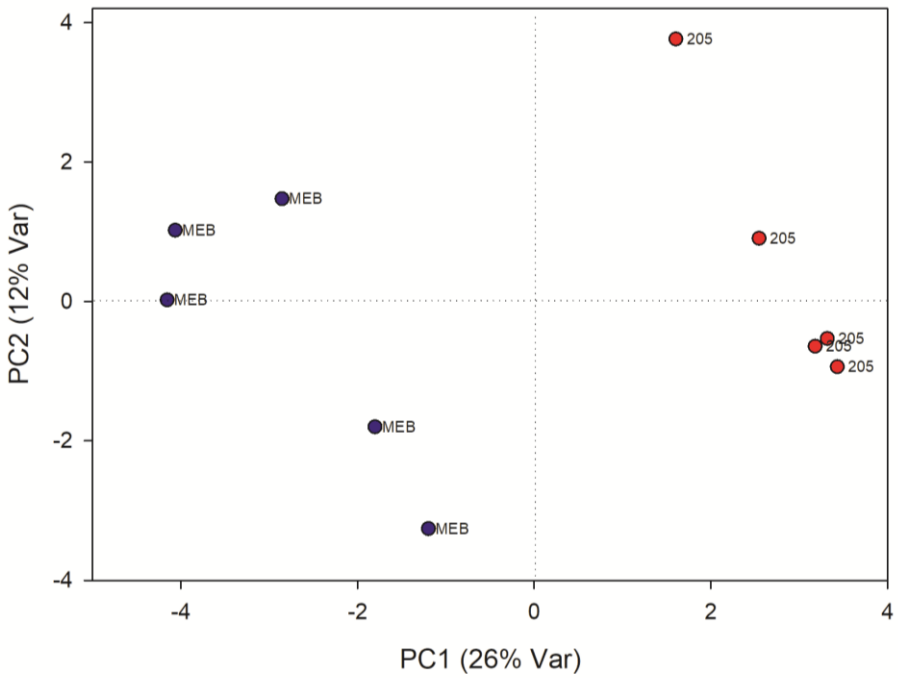


Figure 3.4 PLS-DA score plot of NMR metabolomics data of chicory roots extracts of MEB (control) and *C. globosum* (205) groups. Blue dots indicate MEB (control) group and red dots *C. globosum* group.

3.3.5 EFFECTS OF *MINIMEDUSA POLYSPORA* CULTURE FILTRATE ON METABOLOME OF *CICHORIUM INTYBUS* LEAVES AND ROOTS

The PLS-DA analysis performed to highlight metabolic differences related to *M. polyspora* treatment in *C. intybus* leaves showed an excellent sample separation (R2 =0.99; Q2 =0.62) (**Figure 3.5**). Similar to *C. globosum* treatment, a significant decrease in chlorophyll *a* and *b*, campesterol, acetate, and β-sitosterol and a significant increase in 4-OH-benzoate were observed in leaves of plants treated with *M. polyspora* filtrate compared with control samples (**Figure 3.6**).

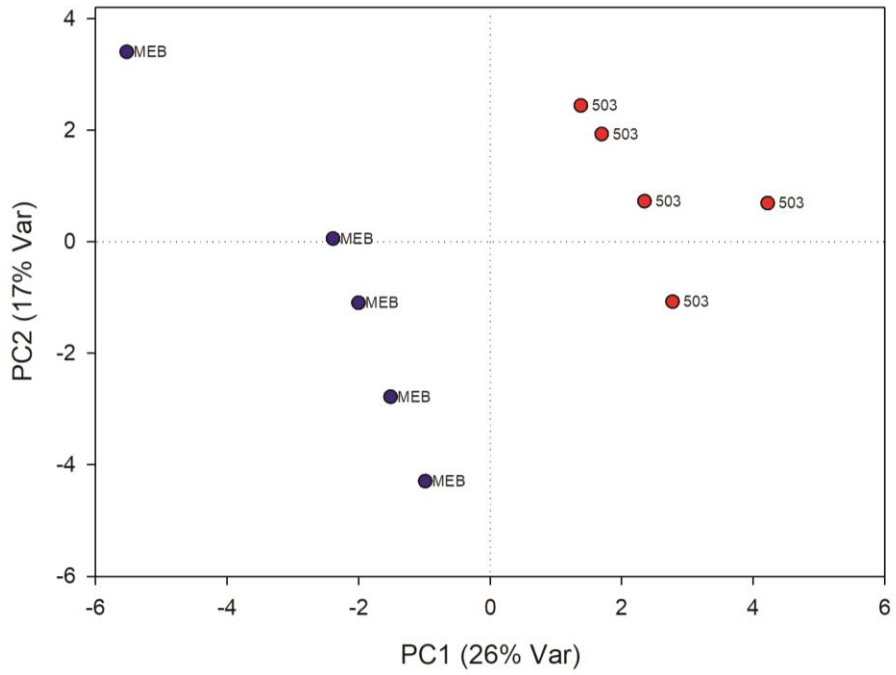


Figure 3.5 PLS-DA score plot of NMR metabolomics data of chicory leaves extracts of MEB (control) and *M. polyspora* (503) groups. Blue dots indicate MEB (control) group and red dots *M. polyspora* group.

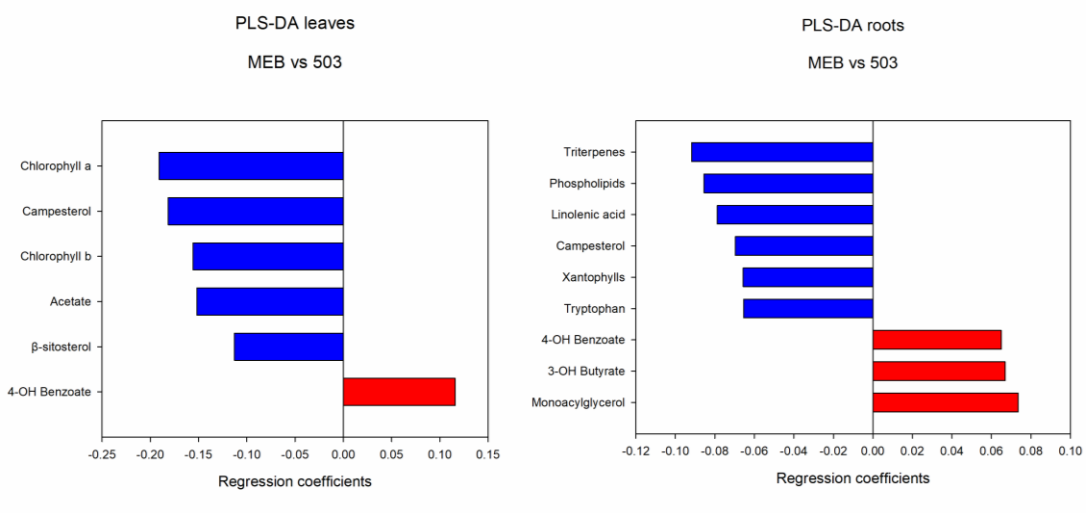


Figure 3.6 PLS-DA regression coefficients of significantly different metabolites in chicory leaf and root extracts of MEB (control) and *M. polyspora* (503).

In the PLS-DA model (R2 =0.77; Q2 =0.52) of root samples (**Figure 3.7**), discriminant variables such as were triterpenes, phospholipids, linolenic acid (ω-3), campesterol, xantophylls and tryptophan were significantly decreased while 4-OH-benzoate, 3-OH-butyrate and monoacylglycerol were significantly increased in roots treated with *M. polyspora* filtrate compared with controls (**Figure 3.6**).

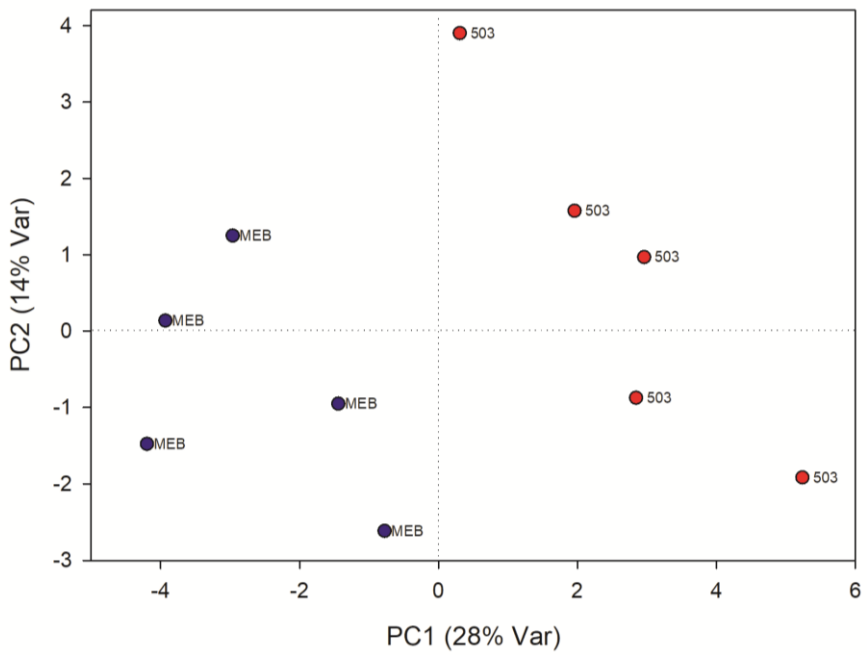


Figure 3.7 PLS-DA score plot of NMR metabolomics data of chicory roots extracts of MEB (control) and *M. polyspora* (503) groups. Blue dots indicate MEB (control) group and red dots *M. polyspora* group.

3.4 DISCUSSION

3.4.1 EFFECTS OF *CHAETOMIUM GLOBOSUM* AND *MINIMEDUSA POLYSPORA* CULTURE FILTRATES ON *CICHORIUM INTYBUS* GROWTH PARAMETERS

Fungal culture filtrates have consistently been reported as effective biostimulants. In the environment, fungi due to a complex extracellular metabolism exert their influence on plants also through the release of diffusible metabolites in soils. Therefore, learning from nature, the metabolites released in the culture medium during growth in controlled

conditions may be used to simulate these interactions and effectively stimulate plant growth.

In this study, we showed that soil application of *M. polyspora* culture filtrate promotes biomass production, both in shoot and root, and leaf area extension in *C. intybus* plants (**Table 3.1**). Considering that no statistically significant differences in the number of leaves were observed, the increase in biomass production seems to be related to the significant increase in leaf area observed. The significant increase in root biomass more specifically occurred in the lateral roots biomass since the taproot biomass did not show differences among the treatments. An increase in lateral roots may have resulted in a more effective exploration of the soil and therefore an increased efficiency in nutrient uptake leading to a higher growth.

In this study, *M. polyspora* is reported for the first time to have a direct plant-growth promoting effect. This effect is in line with what is reported in previous studies on the fungal culture filtrate biostimulant effect. For example, the culture filtrate of *Serendipita indica*, which like *M. polyspora* is an anamorphic species belonging to the Basidiomycota phylum, has been widely reported to increase the biomass production and other growth parameters including plant height, leaves length and width, root length and number, and fruit and seeds production in *Helianthus annus* (common sunflower), *C. intybus* (chicory), *Zea mays* (maize), *Bacopa monniera* (water hyssop) and *Nicotiana tabacum* (tobacco) plants (Bagde et al., 2011, 2013; Rashnoo et al., 2020; Varma et al., 1999).

Several other fungal strains, including *Alternaria alternata*, *Aspergillus fumigatus, Byssochlamys spectabilis, C. globosum, Cladosporium* sp., *Fusarium tricinctum, Fusarium proliferatum*, *Gibberella* spp., *Penicillium melinii, Penicillium citrinum, Penicillium oxalicum, Penicillium* sp., *Phoma herbarum, Sclerotium rolfsii, Shimizuomyces paradoxus, Trichoderma virens, Trichoderma pseudokoningii, Trichoderma harzianum* have been reported for their culture filtrates biostimulant effect determining an increase in growth parameters in several plant species including rice, cucumber, chickpeas, wheat, canola, tobacco and pearl millet (Baron et al., 2020; Bilal et al., 2018; Hamayun et al., 2009, 2010; Haruma et al., 2018; Hwang et al., 2011; Jahagirdar et al., 2019; Khalmuratova et al., 2021; Khan et al., 2015, 2008; López-Bucio et al., 2015; Murali and Amruthesh, 2015; Naziya et al., 2019; Ozimek and Hanaka, 2020; Rahman et al., 2012; Singh et al., 2003; Sung et al., 2011; Tarroum et al., 2021; Zhai et al., 2018; Zhou et al., 2018).

Baroja-Fernández et al. (2021) recently reported the efficacy of *T. harzianum*, *A. alternata* and *Penicillium aurantiogriseum* culture filtrates in promoting plant growth of *Capsicum annuum* (peppers). Moreover, the culture filtrates of the three fungal species were found to contain considerable concentrations of glucose and fructose, and a complex mixture of amino acids, some of which were previously reported to be involved in signaling mechanisms for environmental changes. Similarly, in *M. polyspora* culture filtrate we detected essential amino acids (e.g., alanine, valine, lysine, and leucine),

adenosine and guanosine phosphates nucleotides (AXP and GXP), and glucose at higher concentrations than in MEB (**Supplementary Table 3.2**). The higher concentrations of these metabolites may explain the significant variations of growth parameters in treated chicory plants compared with both the controls. Specifically, in *M. polyspora* filtrate alanine and lysine were higher than the control, while other amino acids (leucine, valine, threonine, glutamate, tyrosine, phenylalanine) occurred in quite similar concentrations. Indeed, fungi can synthesize lysine *de novo* through the so-called α-aminoadipate pathway with α-ketoglutarate as the precursor, while alanine can be formed by transamination from glutamate and pyruvate by glutamate-pyruvate aminotransferase (Smith and Berry, 1976; Zabriskie and Jackson, 2000). Amino acids along with other growth factors present in culture filtrates of some *Fusarium* species have been reported to promote the growth of *Oryza sativa*'s (rice) roots (Ram, 1959). Moreover, the presence of essential amino acids in culture filtrates can confer stress resistance to plants, such as alanine in hypoxic conditions in plants (Baroja-Fernández et al., 2021; Podlešáková et al., 2019). Lysine has been reported to be involved in plant growth and stress resistance, as well. In fact, roots can take up several amino acids, including lysine, and directly incorporate them into new cell biomass and also utilize them for respiration (Owen and Jones, 2001). Moreover, foliar application of iron conjugated lysine on *Brassica napus* (rapeseed) has been reported to increase plant growth, biomass production, chlorophyll content, and essential micronutrients uptake and to reduce oxidative stress enhancing antioxidant enzyme activities in response to chromium stress condition (Zaheer et al., 2020).

Furthermore, the presence of glucose and adenosine and guanosine phosphates nucleotides, as energy sources, can exert a plant growth promotion activity in chicory. In particular, this higher level of glucose compared with MEB may be explained by the extracellular hydrolysis of maltose, leading to the formation of two molecules of glucose, as reported in *Aspergillus niger* through the maltase enzyme (Hamad et al., 2015; Yuan et al., 2008). Indeed, maltose in *M. polyspora* filtrate was almost completely depleted, and it is reasonable to think that this was determined by the release of hydrolytic enzymes, as this cellulolytic strain in different development stages can metabolize polysaccharides, hexoses, and oligosaccharides (Pinzari et al., 2014). Conversely, *C. globosum* culture filtrate in our study did not exert any effect in increasing the biomass production and the other morphological parameter's values. Nevertheless, *C. globosum* and its metabolites have been previously reported as an effective plant growth promoter of *C. annuum* (pepper), *Brassica juncea* (mustard), *Solanum lycopersicum* (tomato), *Pennisetum americanum* (pearl millet), *Z. mays* (maize) and *N. tabacum* (tobacco). In all the abovementioned species, an increase in biomass production was recorded and, as appropriate, *C. globosum* caused an increase also in various morphological and physiological parameters of growth and development such as shoot growth, plant height,

root length, leaf area, chlorophyll content, stomatal conductance, transpiration rate, seed germination, and nutrient uptake (Abou Alhamed and Shebany, 2012; Khan et al., 2012; Singh et al., 2021, 2021; Tarafdar and Gharu, 2006; Tarroum et al., 2021). Unfortunately, no other studies addressing the effect of *C. globosum* on *C. intybus* are available in the literature, and therefore, it is not possible to determine whether its inefficiency is related to the test conditions in this study or to the specific plant-fungal strain interaction. In fact, a PGPF that results effective in promoting the growth of a given plant species, may be less effective or even not present the same beneficial effect at all upon another plant species. Moreover, environmental conditions may also affect the beneficial effect of a PGPF (Hossain et al., 2017).

3.4.2 IMPACT OF SOIL APPLICATION OF *CHAETOMIUM GLOBOSUM* AND *MINIMEDUSA POLYSPORA* CULTURE FILTRATES ON *CICHORIUM INTYBUS* METABOLOME: COMMON THREADS IN LEAVES AND ROOT METABOLISM

Beneficial microorganisms are known to release diffusible substances that promote plant growth. Consistently, soil application of fungal culture filtrates can affect plant metabolism, growth and yield (Baroja-Fernández et al., 2021).

However, how this treatment acts in plants is largely unknown. In this work, we characterized the responses of chicory (*C. intybus*) plants cultured under greenhouse conditions to soil application of culture filtrates obtained from *C. globosum* (205) and *M. polyspora* (503).

^{1}H-NMR-based metabolomics analysis of *C. intybus* roots after treatment with *C. globosum* and *M. polyspora* culture filtrates revealed common metabolic threads involving the increase of 3-OH butyrate and monoacylglycerols associated with the decrease of unsaturated fatty acids (UFAs) such as linolenic acid, sterols including campesterol and β-sitosterol, and phospholipids. We outlined the metabolic network involving 3-OH butyrate, phospholipids, sterols, and fatty acids occurring in *C. intybus* roots after treatment with *C. globosum* (205) and *M. polyspora* (503), (**Figure 3.8**).

Differently from animals, in which 3-OH-butyrate is an intermediate metabolite of fatty acids, the physiological importance and the metabolism of 3-OH-butyrate in plants are not fully understood and characterized. Mierziak et al. (2020) demonstrated that 3-OH-butyrate occurs naturally in flax and could act as a regulatory molecule that most likely influences the expression of genes involved in DNA methylation, thereby altering DNA methylation levels. Recent studies showed that plants contain enzymes such as β-ketothiolase (EC 2.3.1.9) and acetoacetyl-CoA-reductase (EC 1.1.1.36), which are involved in the synthesis of 3-OH butyrate as in bacteria and animals (Beaudoin et al.,

2009; Jin et al., 2012; Mierziak et al., 2021; Tsuda et al., 2016; Xu et al., 1997). In plants, biosynthesis of fatty acids and biosynthesis of sterols, which takes place via mevalonate pathway in plastids and endoplasmic reticulum, occur from acetyl-CoA and acetoacetyl-CoA through NADPH/NADP+ recycling and reductase/synthase enzyme activities. The treatment with *C. globosum* (205) and *M. polyspora* (503) culture filtrates stimulates a common response in *C. intybus* roots involving the synthesis of 3-OH-butyrate through the decrease of the synthesis of fatty acids and sterols, as a mechanism balancing the NADPH/NADP+ ratio.

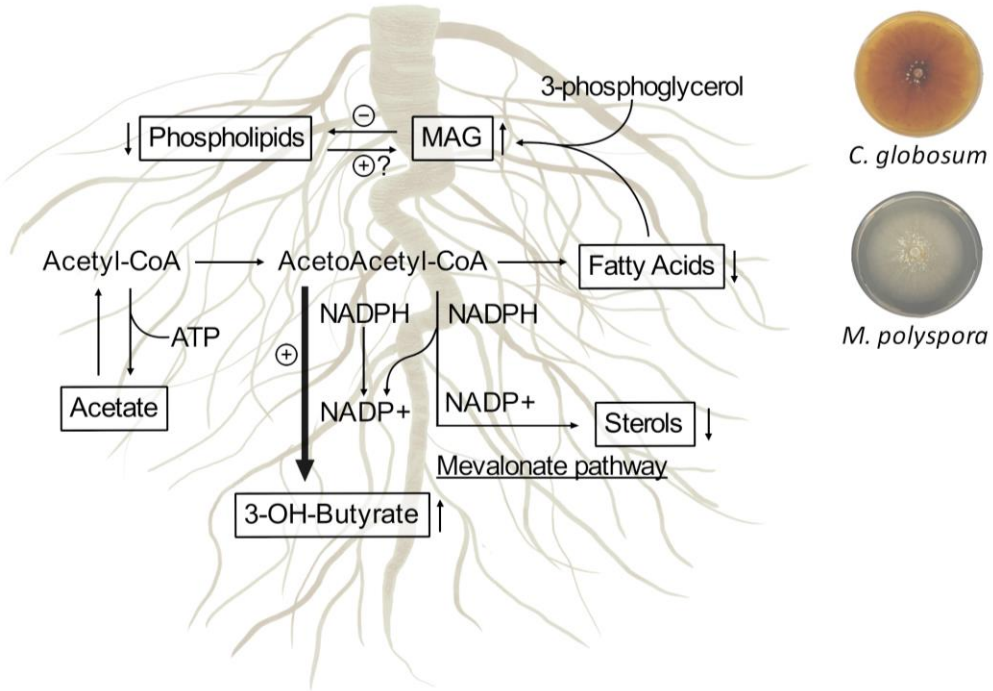


Figure 3.8 Metabolic network involving 3-OH-butyrate, phospholipids, sterols, and fatty acids occuring in *C. intybus* roots after treatment with *C. globosum* (205) and *M. polyspora* (503) culture filtrates. The identified metabolites and their variations are reported in squares.

In most plants, the predominant unsaturated fatty acids (UFAs) are three 18-carbon (C18) species, i.e., 18:1 (oleate), 18:2 (linoleate), and 18:3 (α-linolenate) (He et al., 2020). UFAs compounds play multiple crucial roles and are deeply associated with both abiotic and biotic stresses. Besides being membrane ingredients and modulators in glycerolipids, as well as carbon and energy reserve in triacylglycerols (TAGs), C18 UFAs serve as intrinsic antioxidants, precursors of various bioactive molecules [typically the stress hormone jasmonic acid (JA)], and stocks of extracellular barrier constituents such as

cutin and suberin (He et al., 2020). The predominant sterols in plants, such as β-sitosterol, campesterol, and stigmasterol, are precursors of a group of plant hormones the brassinosteroids, such as gibberellins and abscisic acid, which regulate plant growth and development (Valitova et al., 2016). Plant lipids, released from the roots into the rhizosphere, facilitate signaling and actively shape the microbiome inhabiting the rhizosphere and the subsequent colonization of their root tissues. Lipids play essential roles as the "chemical language" that facilitates the exchange of resources and modulates the cell responses by inhibiting pathogen attack or enhancing microbial symbiosis. The recruitment of the rhizobiome into the plant vicinity is mediated by rhizodeposits. The release of rhizodeposits comes with a wide variety of substances, such as sugars, amino acids, organic acids, enzymes, growth factors and vitamins, flavanones and purines/nucleotides, and miscellaneous substances. In our study we observed a decrease in root phospholipids, fatty acids, and sterols that are probably released from roots into the rhizosphere as chemical signals and/or chemotactic attractors to facilitate the recruitment, nutrition, shaping, and tuning of the microbial communities. The perception of these compounds could lead to the stimulation of regulatory or signaling cascades that cause various responses in the microbes. We also observed an increase in monoacylglycerols (MAG) levels. MAG comprise the bulk of oil storage in plant tissues and are involved in many regulatory processes such as cell signaling and intracellular trafficking (Macabuhay et al., 2022).

^{1}H-NMR-based metabolomics analysis of *C. intybus* leaves after treatment with *C. globosum* and *M. polyspora* culture filtrates showed the reduction in chlorophylls *a* and *b* content not associated with leaf greening decrease during the plant development and/or treatment with fungal culture filtrates. Dynamic control of chlorophyll level determined by the relative rates of chlorophyll anabolism and catabolism processes, that largely occur in chloroplasts, ensures optimal photosynthesis and plant fitness. The accumulation of adequate amounts of chlorophyll is therefore vital for plants to establish photosynthetically active chloroplasts during leaf greening. Furthermore, optimized chlorophyll degradation is not only essential for the detoxification of free chlorophyll released but also indispensable for the remobilization of nutrients during leaf development and senescence. Thus, efficient photosynthesis, plant fitness and yield are critically dependent on the dynamic regulation of chlorophyll levels in response to various developmental and environmental cues (Wang et al., 2020).

3.4.3 *CHAETOMIUM GLOBOSUM* CULTURE FILTRATE TRIGGERS PHENYLPROPANOID PATHWAY IN *CICHORIUM INTYBUS* ROOTS: BIOSYNTHESIS OF CHICORIC ACID

In our study, the treatment of *C. intybus* plants with *C. globosum* culture filtrate triggered the phenylpropanoid pathway through an increase of phenylalanine and

chicoric acid in roots. The biosynthetic pathway of chicoric acid in plants is putative and still not well known, although it is generally understood to form via shikimic acid/phenylpropanoid pathway as other phenolics, analogous to the conjugation of caffeic acid derivatives of rosmarinic acid or chlorogenic acid (Legrand et al., 2016; Peng et al., 2019). Putative metabolic pathway involved in chicoric acid biosynthesis in *C. intybus* roots is shown in **Figure 3.9**.

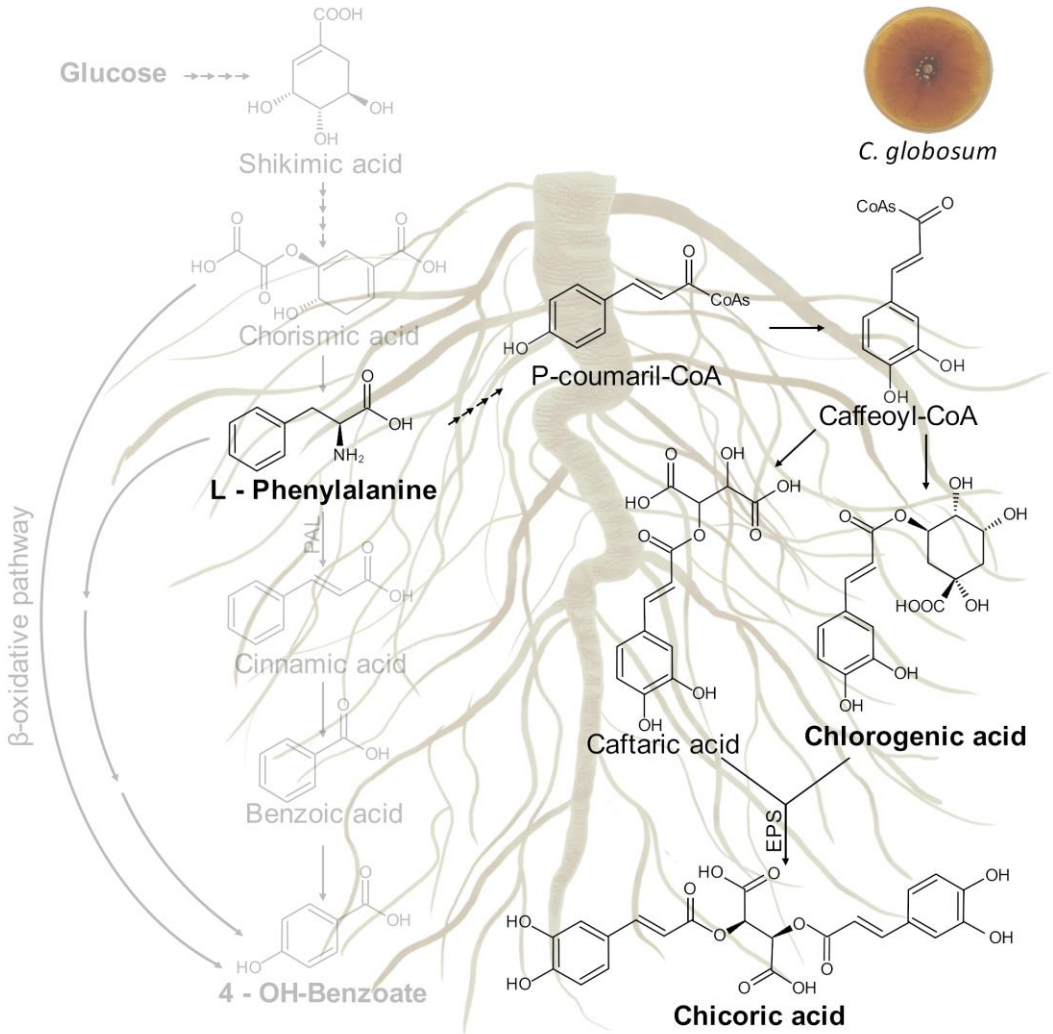


Figure 3.9 Putative metabolic pathway involved in chicoric acid biosynthesis in *C. intybus* roots. In bold are reported the identified metabolites. EPS: acyltransferase.

The entry point is the aromatic amino acid phenylalanine (Phe) arising from the shikimate pathway. Deamination of Phe by Phe ammonia lyase (PAL) leads to cinnamic acid. Cinnamate-4-hydroxylase and 4-coumarate coenzyme A (CoA) ligase (4CL) generate p-coumaroyl-CoA from cinnamic acid. Thereafter, hydroxycinnamoyl transferases (HCTs) convert the CoA-thioester to coumaroyl quinate or coumaroyl shikimate which is subsequently hydroxylated by p-coumarate-30-hydroxylase to form the caffeoyl derivatives. A recent study carried out on purple coneflower highlighted two types of acyltransferases distributed in distinct subcellular compartments and involved in the biosynthesis of chicoric acid. In the cytosol, the BAHD acyltransferase family including a tartaric acid hydroxycinnamoyl transferases (HTT) and a quinate hydroxycinnamoyl transferases (HQT) use caffeoyl CoA from phenylpropanoid metabolism as an acyl donor to synthesize caftaric acid and chlorogenic acid, respectively (D'Auria, 2006). Both products are then transported into the vacuole where CAS, a specialized serine carboxypeptidase-like (SCPL) acyltransferase, uses chlorogenic acid as its acyl donor and transfers the caffeoyl group to caftaric acid to generate chicoric acid (Fu et al., 2021).

For millennia, humans have used plant specialized metabolites as herbal medicines. Chicoric acid is a very promising natural compound, which occurs in a variety of plant species such as *C. intybus*, *Echinacea purpurea* L. (purple coneflower), *Ocimum basilicum* L. (basil), *Lactuca sativa* L. (lettuce), *Taraxacum officinale* (dandelion), *Cucurbita pepo* L. (squash), and *Borago officinalis* L. (borage) (Lee and Scagel, 2013). According to the literature data, chicoric acid plays an important role in plant defense against different diseases caused by viruses, bacteria, fungi, nematodes, and insects (Cheynier et al., 2013; Nishimura and Satoh, 2006).

3.4.4 *MINIMEDUSA POLYSPORA* CULTURE FILTRATE TRIGGERS PHENYLPROPANOID PATHWAY IN *CICHORIUM INTYBUS* LEAVES AND ROOTS: BIOSYNTHESIS OF 4-OH-BENZOATE

As reported above, the treatment of *C. intybus* plants with *M. polyspora* culture filtrate determined significant variations of growth parameters in chicory plants including the dry weight of the total plant, shoot, total roots, lateral roots and leaf area that increased compared with control plants. A relationship between growth parameters and metabolic changes was observed. In particular, *M. polyspora* culture filtrate triggered the phenylpropanoid pathway through an increase of 4-OH-benzoate, which is generated from aromatic amino acids produced via the shikimate pathway (**Figure 3.10**).

The shikimic acid is converted into L-phenylalanine through a chorismic acid intermediate. Thus, the L-phenylalanine is converted into p-coumaric, salicylic, and p-hydroxybenzoic acids, which serve as precursors for other derivatives of phenolic acids. It is thought that hydroxybenzoic acids can be produced from structurally analogous

hydroxycinnamic acids in coenzyme A (CoA)-dependant (β-oxidative) or CoA-independent (non-β-oxidative) pathways or the combination of both of them, which have been determined as occurring in peroxisomes and mitochondria (Widhalm and Dudareva, 2015). Benzoic acids serve as precursors for a wide variety of essential compounds and natural products playing crucial roles in plant fitness and in defense response activation. In this context, 4-OH-benzoate showed *in vitro* antifungal effects on *Eutypa lata* growth (Amborabé et al., 2002).

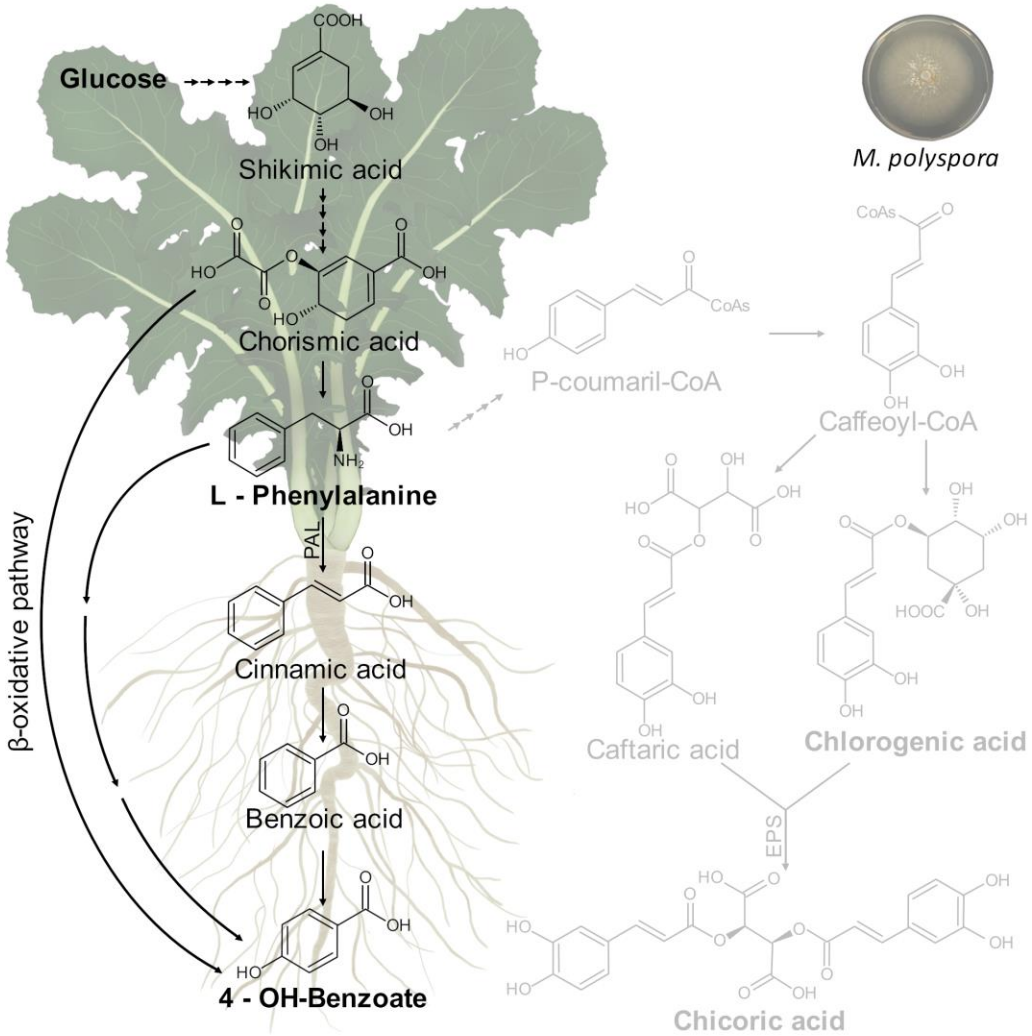


Figure 3.10 Putative metabolic pathway involved in 4-OH-benzoate biosynthesis in *C. intybus* plant (root and shoot) after treatment with *M. polyspora* (503) culture filtrate.

3.5 FINAL REMARKS

In this study we investigated the biostimulant effect of fungal culture filtrates obtained from *M. polyspora* and *C. globosum* on *C. intybus* growth and metabolism.

For the first time, we showed that *M. polyspora* filtrate exerted a direct plant-growth promoting effect, since the application in soil promoted an increase of biomass, both in shoot and root, and of the leaf area in *C. intybus* plants. Conversely, no significant effect on morphological traits and biomass yield was observed in *C. intybus* plants treated with *C. globosum* culture filtrate.

Based on ^{1}H-NMR metabolomics data, differential metabolites and their related metabolic pathways were described. We highlighted that the treatment with *C. globosum* and *M. polyspora* culture filtrates stimulated a common response in *C. intybus* roots involving the synthesis of 3-OH-butyrate through the decrease of the synthesis of fatty acids and sterols, as a mechanism balancing the NADPH/$NADP^{+}$ ratio. Interestingly, the two fungal culture filtrates differently affected the phenylpropanoid pathway in *C. intybus* plants: the treatment with *C. globosum* culture filtrate triggered the phenylpropanoid pathway through an increase of phenylalanine and chicoric acid in roots, whereas *M. polyspora* culture stimulated an increase of 4-OH benzoate. Chicoric acid, whose biosynthetic pathway in chicory plant is putative and still not well known, is a very promising natural compound playing an important role in plant defense. On the contrary, benzoic acids serve as precursors for a wide variety of essential compounds playing crucial roles in plant fitness and defense response activation. To the best of our knowledge, this is the first study that shows the biostimulant effect of *C. globosum* and *M. polyspora* culture filtrates on *C. intybus* growth and metabolome, increasing the knowledge on fungal bioresources for the development of biostimulants.

3.6 REFERENCES

Abdel-Azeem, A.M. (2020). Recent Developments on Genus *Chaetomium* (Cham, CH: Springer International Publishing).

Abhilash, P.C. (2021). Restoring the Unrestored: Strategies for Restoring Global Land during the UN Decade on Ecosystem Restoration (UN-DER). Land *10*, 201.

Abou Alhamed, M.F., and Shebany, Y.M. (2012). Endophytic *Chaetomium globosum* enhances maize seedling copper stress tolerance: *Chaetomium globosum* and copper stress tolerance. Plant Biology *14*, 859–863.

Aggarwal, R., Tewari, A.K., Srivastava, K.D., and Singh, D.V. (2004). Role of antibiosis in the biological control of spot blotch (*Cochliobolus sativus*) of wheat by *Chaetomium globosum*. Mycopathologia *157*, 369–377.

Aggarwal, R., Sharma, S., Gupta, S., Singh, K.B.M., and Shanmugam, V. (2016). Role of Defense Enzymes in Biocontrol of Spot Blotch and Leaf Rust of Wheat (*Triticum* sp. L.) by *Chaetomium globosum*. Journal of Pure and Applied Microbiology *10*, 2071–2078.

Al-Snafi, A. (2016). Medical importance of *Cichorium intybus* – A review. IOSR journal of pharmacy *6*, 41–56.

Amborabé, B.-E., Fleurat-Lessard, P., Chollet, J.-F., and Roblin, G. (2002). Antifungal effects of salicylic acid and other benzoic acid derivatives towards *Eutypa lata*: structure–activity relationship. Plant Physiology and Biochemistry *40*, 1051–1060.

Bagde, U.S., Prasad, R., and Varma, A. (2011). Influence of culture filtrate of *Piriformospora indica* on growth and yield of seed oil in *Helianthus annus*. Symbiosis *53*, 83–88.

Bagde, U.S., Prasad, R., and Varma, A. (2013). Impact of Culture Filtrate of *Piriformospora indica* on Biomass and Biosynthesis of Active Ingredient Aristolochic Acid in *Aristolochia elegans* Mart. International journal of biology *6*, p29.

Bais, H.P., and Ravishankar, G. (2001). *Cichorium intybus* L – cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. Journal of the Science of Food and Agriculture *81*, 467–484.

Baroja-Fernández, E., Almagro, G., Sánchez-López, Á.M., Bahaji, A., Gámez-Arcas, S., De Diego, N., Dolezal, K., Muñoz, F.J., Climent Sanz, E., and Pozueta-Romero, J. (2021). Enhanced Yield of Pepper Plants Promoted by Soil Application of Volatiles From Cell-Free Fungal Culture Filtrates Is Associated With Activation of the Beneficial Soil Microbiota. Frontiers in Plant Science *12*, 752653.

Baron, N.C., Souza Pollo, A. de, and Rigobelo, E.C. (2020). *Purpureocillium lilacinum* and *Metarhizium marquandii* as plant growth-promoting fungi. PeerJ *8*, e9005.

Beale, R.E., and Pitt, D. (1990). Biological and integrated control of *Fusarium* basal rot of *Narcissus* using *Minimedusa polyspora* and other micro-organisms. Plant Pathology *39*, 477–488.

Beale, R.E., and Pitt, D. (1995). The antifungal properties of *Minimedusa polyspora*. Mycological Research *99*, 337–342.

Beaudoin, F., Wu, X., Li, F., Haslam, R.P., Markham, J.E., Zheng, H., Napier, J.A., and Kunst, L. (2009). Functional Characterization of the *Arabidopsis* β-Ketoacyl-Coenzyme A Reductase Candidates of the Fatty Acid Elongase. Plant Physiology *150*, 1174–1191.

Bilal, L., Asaf, S., Hamayun, M., Gul, H., Iqbal, A., Ullah, I., Lee, I.-J., and Hussain, A. (2018). Plant growth promoting endophytic fungi *Aspergillus fumigatus* TS1 and *Fusarium proliferatum* BRL1 produce gibberellins and regulates plant endogenous hormones. Symbiosis *76*, 117–127.

Bischoff, T.A., Kelley, C.J., Karchesy, Y., Laurantos, M., Nguyen-Dinh, P., and Arefi, A.G. (2004). Antimalarial activity of Lactucin and Lactucopicrin: sesquiterpene lactones isolated from *Cichorium intybus* L. Journal of Ethnopharmacology *95*, 455–457.

Biswas, S., Aggarwal, R., Srivastava, K., Gupta, S., and Dureja, P. (2012). Characterization of antifungal metabolites of *Chaetomium globosum* Kunze and their antagonism against fungal plant pathogens. Journal of Biological Control *26*, 70–74.

Castiglione, A.M., Mannino, G., Contartese, V., Bertea, C.M., and Ertani, A. (2021). Microbial Biostimulants as Response to Modern Agriculture Needs: Composition, Role and Application of These Innovative Products. Plants *10*, 1533.

Ceci, A., Pinzari, F., Russo, F., Persiani, A.M., and Maggi, O. (articolo in revisione). Saprotrophic soil fungi skills to improve phosphorus solubilisation and release. Ambio *IPW8 Special Issue "Handling the phosphorus paradox in agriculture and natural ecosystems: Scarcity, necessity and burden of P".*

Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V., and Martens, S. (2013). Plant phenolics: Recent advances on their biosynthesis, genetics, and ecophysiology. Plant Physiology and Biochemistry *72*, 1–20.

D'Auria, J.C. (2006). Acyltransferases in plants: a good time to be BAHD. Current Opinion in Plant Biology 9, 331–340.

Del Buono, D. (2021). Can biostimulants be used to mitigate the effect of anthropogenic climate change on agriculture? It is time to respond. Science of the Total Environment *751*, 141763.

EU (2019). Regulation of the european parliament and of the council laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:L:2019:170:TOC.

Fu, R., Zhang, P., Jin, G., Wang, L., Qi, S., Cao, Y., Martin, C., and Zhang, Y. (2021). Versatility in acyltransferase activity completes chicoric acid biosynthesis in purple coneflower. Nature Communications *12*, 1563.

Ganugi, P., Martinelli, E., and Lucini, L. (2021). Microbial biostimulants as a sustainable approach to improve the functional quality in plant-based foods: a review. Current Opinion in Food Science *41*, 217–223.

Giampaoli, O., Sciubba, F., Conta, G., Capuani, G., Tomassini, A., Giorgi, G., Brasili, E., Aureli, W., and Miccheli, A. (2021). Red Beetroot's NMR-Based Metabolomics: Phytochemical Profile Related to Development Time and Production Year. Foods *10*, 1887.

Hamad, H.O., Alma, M.H., Ismael, H.M., and Göçerī, A. (2015). The Effect of Some Sugars on the Growth of *Aspergillus niger*. KSU Journal of natural science *17*, 7–11.

Hamayun, M., Khan, S.A., Khan, A.L., Rehman, G., Sohn, E.-Y., Shah, A.A., Kim, S.-K., Joo, G.-J., and Lee, I.-J. (2009). *Phoma herbarum* as a new gibberellin-producing and plant growth-promoting fungus. Journal of Microbiology and Biotechnology *19*, 1244–1249.

Hamayun, M., Khan, S.A., Khan, A.L., Rehman, G., Kim, Y.-H., Iqbal, I., Hussain, J., Sohn, E.-Y., and Lee, I.-J. (2010). Gibberellin production and plant growth promotion from pure cultures of *Cladosporium* sp. MH-6 isolated from cucumber (*Cucumis sativus* L.). Mycologia *102*, 989–995.

Haruma, T., Yamaji, K., Masuya, H., and Hanyu, K. (2018). Root endophytic *Chaetomium cupreum* promotes plant growth and detoxifies aluminum in *Miscanthus sinensis* Andersson growing at the acidic mine site. Plant Species Biology 33, 109–122.

He, M., Qin, C.-X., Wang, X., and Ding, N.-Z. (2020). Plant Unsaturated Fatty Acids: Biosynthesis and Regulation. Frontiers in Plant Science *11*, 390.

Hossain, Md.M., Sultana, F., and Islam, S. (2017). Plant Growth-Promoting Fungi (PGPF): Phytostimulation and Induced Systemic Resistance. In Plant-Microbe Interactions in Agro-Ecological Perspectives, D.P. Singh, H.B. Singh, and R. Prabha, eds. (Singapore, SG: Springer Singapore), pp. 135–191.

Hu, Y., Zhang, W., Zhang, P., Ruan, W., and Zhu, X. (2013). Nematicidal Activity of Chaetoglobosin A Poduced by *Chaetomium globosum* NK102 against Meloidogyne incognita. Journal of Agricultural and Food Chemistry *61*, 41–46.

Hwang, J.-S., You, Y.-H., Bae, J.-J., Khan, S.A., Kim, J.-G., and Choo, Y.-S. (2011). Effects of Endophytic Fungal Secondary Metabolites on the Growth and Physiological Response of *Carex kobomugi* Ohwi. Journal of coastal research *27*, 544–548.

Hyde, K.D., Xu, J., Rapior, S., Jeewon, R., Lumyong, S., Niego, A.G.T., Abeywickrama, P.D., Aluthmuhandiram, J.V.S., Brahamanage, R.S., Brooks, S., et al. (2019). The amazing potential of fungi: 50 ways we can exploit fungi industrially. Fungal Divers. *97*, 1–136.

Jahagirdar, S., Kambrekar, D.N., Navi, S.S., and Kunta, M. (2019). Plant Growth-Promoting Fungi: Diversity and Classification. In Bioactive Molecules in Plant Defense: Signaling in Growth and Stress, S. Jogaiah, and M. Abdelrahman, eds. (Cham, CH: Springer International Publishing), pp. 25–34.

Janda, K., Gutowska, I., Geszke-Moritz, M., and Jakubczyk, K. (2021). The Common Cichory (*Cichorium intybus* L.) as a Source of Extracts with Health-Promoting Properties—A Review. Molecules *26*, 1814.

Jin, H., Song, Z., and Nikolau, B.J. (2012). Reverse genetic characterization of two paralogous acetoacetyl CoA thiolase genes in *Arabidopsis* reveals their importance in plant growth and development. Plant Journal *70*, 1015–1032.

Kew Science (2021). *Cichorium intybus* L. — Plants of the World Online. Available online at: http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:194533-1 (accessed November 17, 2021).

Khalmuratova, I., Choi, D.-H., Kim, J.-G., and Lee, I.-S. (2021). Endophytic Fungi of Salt-Tolerant Plants: Diversity and Ability to Promote Plant Growth. Journal of Microbiology and Biotechnology*31*, 1526–1532.

Khan, A., Shinwari, Z., Kim, Y.-H., Waqas, M., Hamayun, M., Kamran, M., and Lee, I.-J. (2012). Role of endophyte *Chaetomium globosum* lk4 in growth of *Capsicum annuum* by production of gibberellins and indole acetic acid. Pakistan Journal of Botany *44*, 1601–1607.

Khan, A.R., Ullah, I., Waqas, M., Shahzad, R., Hong, S.-J., Park, G.-S., Jung, B.K., Lee, I.-J., and Shin, J.-H. (2015). Plant growth-promoting potential of endophytic fungi isolated from *Solanum nigrum* leaves. World Journal of Microbiology and Biotechnology *31*, 1461–1466.

Khan, B., Yan, W., Wei, S., Wang, Z., Zhao, S., Cao, L., Rajput, N.A., and Ye, Y. (2019). Nematicidal metabolites from endophytic fungus *Chaetomium globosum* YSC5. FEMS Microbiology Letters 366, fnz169.

Khan, S.A., Hamayun, M., Yoon, H., Kim, H.-Y., Suh, S.-J., Hwang, S.-K., Kim, J.-M., Lee, I.-J., Choo, Y.-S., Yoon, U.-H., et al. (2008). Plant growth promotion and *Penicillium citrinum*. BMC Microbiology *8*, 231.

Klaubauf, S., Inselsbacher, E., Zechmeister-Boltenstern, S., Wanek, W., Gottsberger, R., Strauss, J., and Gorfer, M. (2010). Molecular diversity of fungal communities in agricultural soils from Lower Austria. Fungal Divers. *44*, 65–75.

Kumar, R., Kundu, A., Dutta, A., and Saha, S. (2020). Profiling of volatile secondary metabolites of *Chaetomium globosum* for potential antifungal activity against soil borne fungi. 6.

Kumar, R., Kundu, A., Dutta, A., Saha, S., Das, A., and Bhowmik, A. (2021). Chemo-profiling of bioactive metabolites from *Chaetomium globosum* for biocontrol of *Sclerotinia* rot and plant growth promotion. Fungal Biology *125*, 167–176.

Lee, J., and Scagel, C.F. (2013). Chicoric acid: chemistry, distribution, and production. Frontiers in Chemistry *1*.

Legrand, G., Delporte, M., Khelifi, C., Harant, A., Vuylsteker, C., Mörchen, M., Hance, P., Hilbert, J.-L., and Gagneul, D. (2016). Identification and Characterization of Five BAHD

Acyltransferases Involved in Hydroxycinnamoyl Ester Metabolism in Chicory. Frontiers in Plant Science7.

Linkies, A., Jacob, S., Zink, P., Maschemer, M., Maier, W., and Koch, E. (2021). Characterization of cultural traits and fungicidal activity of strains belonging to the fungal genus *Chaetomium*. Journal of applied microbiology *131*, 375–391.

Longley, R., Noel, Z.A., Benucci, G.M.N., Chilvers, M.I., Trail, F., and Bonito, G. (2020). Crop Management Impacts the Soybean (*Glycine max*) Microbiome. Frontiers in Microbiology *11*, 1116.

López-Bucio, J., Pelagio-Flores, R., and Herrera-Estrella, A. (2015). *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. Scientia horticulturae *196*, 109–123.

Lucadamo, E.E.K. (2018). Legacy effects of cover crop monocultures and mixtures on soil inorganic nitrogen, total phenolic content, and microbial communities on two organic farms in Illinois. [Master of Science's thesis]. University of Illinois, Urbana (IL), United States. Available online at: http://hdl. handle.net/2142/101553 (accessed January 21, 2022).

Macabuhay, A., Arsova, B., Walker, R., Johnson, A., Watt, M., and Roessner, U. (2022). Modulators or facilitators? Roles of lipids in plant root–microbe interactions. Trends in Plant Science *27*, 180–190.

Mierziak, J., Wojtasik, W., Kulma, A., Dziadas, M., Kostyn, K., Dymińska, L., Hanuza, J., Żuk, M., and Szopa, J. (2020). 3-Hydroxybutyrate Is Active Compound in Flax that Upregulates Genes Involved in DNA Methylation. International Journal of Molecular Sciences *21*, 2887.

Mierziak, J., Burgberger, M., and Wojtasik, W. (2021). 3-Hydroxybutyrate as a Metabolite and a Signal Molecule Regulating Processes of Living Organisms. Biomolecules *11*, 402.

Moya, P., Cipollone, J., and Sisterna, M. (2020). The Fungal Genus *Chaetomium* and Its Agricultural Applications. In Plant Defence: Biological Control, J.-M. Mérillon, and K.G. Ramawat, eds. (Cham, CH: Springer International Publishing), pp. 289–308.

Murali, M., and Amruthesh, K.N. (2015). Plant Growth-promoting Fungus *Penicillium oxalicum* Enhances Plant Growth and Induces Resistance in Pearl Millet Against Downy Mildew Disease. Journal of Phytopathology *163*, 743–754.

Naziya, B., Murali, M., and Amruthesh, K.N. (2019). Plant Growth-Promoting Fungi (PGPF) Instigate Plant Growth and Induce Disease Resistance in *Capsicum annuum* L. upon Infection with *Colletotrichum capsici* (Syd.) Butler & Bisby. Biomolecules *10*, 41.

Nephali, L., Piater, L.A., Dubery, I.A., Patterson, V., Huyser, J., Burgess, K., and Tugizimana, F. (2020). Biostimulants for Plant Growth and Mitigation of Abiotic Stresses: A Metabolomics Perspective. Metabolites *10*, 505.

Nishimura, H., and Satoh, A. (2006). Antimicrobial and nematicidal substances from the root of Chicory (*Cichorium intybus*). In Allelochemicals: Biological Control of Plant Pathogens and Diseases, Inderjit, and K.G. Mukerji, eds. (Dordrecht, NL: Springer Netherlands), pp. 177–180.

Orrù, L., Canfora, L., Trinchera, A., Migliore, M., Pennelli, B., Marcucci, A., Farina, R., and Pinzari, F. (2021). How Tillage and Crop Rotation Change the Distribution Pattern of Fungi. Frontiers in Microbiology *12*, 634325.

Owen, A.G., and Jones, D.L. (2001). Competition for amino acids between wheat roots and rhizosphere microorganisms and the role of amino acids in plant N acquisition. Soil Biology and Biochemistry *33*, 651–657.

Ozimek, E., and Hanaka, A. (2020). *Mortierella* Species as the Plant Growth-Promoting Fungi Present in the Agricultural Soils. Agriculture *11*, 7.

Panelli, S., Capelli, E., Comandatore, F., Landinez-Torres, A., Granata, M.U., Tosi, S., and Picco, A.M. (2017). A metagenomic-based, cross-seasonal picture of fungal consortia associated with Italian soils subjected to different agricultural managements. Fungal Ecology *30*, 1–9.

Peng, Y., Sun, Q., and Park, Y. (2019). The Bioactive Effects of Chicoric Acid As a Functional Food Ingredient. Journal of Medicinal Food *22*, 645–652.

Perović, J., Tumbas Šaponjac, V., Kojić, J., Krulj, J., Moreno, D.A., García-Viguera, C., Bodroža-Solarov, M., and Ilić, N. (2021). Chicory (*Cichorium intybus* L.) as a food ingredient – Nutritional composition, bioactivity, safety, and health claims: A review. Food Chemistry *336*, 127676.

Pinzari, F., Reverberi, M., Piñar, G., Maggi, O., and Persiani, A.M. (2014). Metabolic profiling of *Minimedusa polyspora* (Hotson) Weresub & P.M. LeClair, a cellulolytic fungus isolated from Mediterranean maquis, in southern Italy. Plant Biosystems *148*, 333–341.

Podlešáková, K., Ugena, L., Spíchal, L., Doležal, K., and De Diego, N. (2019). Phytohormones and polyamines regulate plant stress responses by altering GABA pathway. New Biotechnology *48*, 53–65.

Puhlmann, M.-L., and de Vos, W.M. (2020). Back to the Roots: Revisiting the Use of the Fiber-Rich *Cichorium intybus* L. Taproots. Advances in Nutrition *11*, 878–889.

Qin, J.-C., Zhang, Y.-M., Gao, J.-M., Bai, M.-S., Yang, S.-X., Laatsch, H., and Zhang, A.-L. (2009). Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from *Ginkgo biloba*. Bioorganic and Medicinal Chemistry Letters *19*, 1572–1574.

Rahman, M.A., Sultana, R., Begum, M.F., and Alam, M.F. (2012). Effect of culture filtrates of *Trichoderma* on seed germination and seedling growth in chili. International Journal of Biosciences *2*, 46–55.

Ram, C.S.V. (1959). Production of growth-promoting substances by fusaria and their action on root elongation in *Oryza sativa* L. P. Proceedings of the Indian Academy of Sciences *49*, 167–182.

Rashnoo, A., Movahedi, Z., Rostami, M., and Ghabooli, M. (2020). Piriformospora indica Culture Filtrate and Biofertilizer (Nitrokara) Promote Chicory (*Cichorium intybus* L.) Growth and Morpho-physiological Traits in an Aeroponic System and Soil Culture. International Journal of Horticultural Science and Technology *7*, 353-363.

Sangiorgio, D., Cellini, A., Donati, I., Pastore, C., Onofrietti, C., and Spinelli, F. (2020). Facing Climate Change: Application of Microbial Biostimulants to Mitigate Stress in Horticultural Crops. Agronomy *10*, 794.

Singh, J., Aggarwal, R., Bashyal, B.M., Darshan, K., Parmar, P., Saharan, M.S., Hussain, Z., and Solanke, A.U. (2021). Transcriptome Reprogramming of Tomato Orchestrate the Hormone Signaling Network of Systemic Resistance Induced by *Chaetomium globosum*. Frontiers in Plant Science *12*, 721193.

Singh, U.P., Sarma, B.K., and Singh, D.P. (2003). Effect of Plant Growth-Promoting Rhizobacteria and Culture Filtrate of *Sclerotium rolfsii* on Phenolic and Salicylic Acid Contents in Chickpea (*Cicer arietinum*). Current Microbiology *46*, 131–140.

Smith, J.E., and Berry, D.R. (1976). The Filamentous Fungi Volume II : Biosynthesis and Metabolism (London, UK: Edward Arnold Ltd).

Spinelli, V., Ceci, A., Dal Bosco, C., Gentili, A., and Persiani, A.M. (2021). Glyphosate-Eating Fungi: Study on Fungal Saprotrophic Strains' Ability to Tolerate and Utilise Glyphosate as a Nutritional Source and on the Ability of *Purpureocillium lilacinum* to Degrade It. Microorganisms *9*, 2179.

Street, R.A., Sidana, J., and Prinsloo, G. (2013). *Cichorium intybus* : Traditional Uses, Phytochemistry, Pharmacology, and Toxicology. Evidence-Based Complementary and Alternative Medicine *2013*, 1–13.

Sung, G.-H., Shrestha, B., Park, K.-B., Han, S.-K., and Sung, J.-M. (2011). Enhancing Effect of *Shimizuomyces paradoxus* on Seed Germination and Seedling Growth of Canola, Plant Growth of Cucumber, and Harvest of Tomato. Mycobiology *39*, 7–11.

Tarafdar, J.C., and Gharu, A. (2006). Mobilization of organic and poorly soluble phosphates by *Chaetomium globosum*. Applied Soil Ecology *32*, 273–283.

Tarroum, M., Ben Romdhane, W., Ali, A.A.M., Al-Qurainy, F., Al-Doss, A., Fki, L., and Hassairi, A. (2021). Harnessing the Rhizosphere of the Halophyte Grass *Aeluropus littoralis* for Halophilic Plant-Growth-Promoting Fungi and Evaluation of Their Biostimulant Activities. Plants *10*, 784.

Tian, Z., Wang, J., Li, J., and Han, B. (2021). Designing future crops: challenges and strategies for sustainable agriculture. Plant Journal *105*, 1165–1178.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. Nature *418*, 671–677.

Tsuda, H., Shiraki, M., Inoue, E., and Saito, T. (2016). Generation of poly-β-hydroxybutyrate from acetate in higher plants: Detection of acetoacetyl CoA reductase- and PHB synthase- activities in rice. Journal of Plant Physiology *201*, 9–16.

United Nations (2015). Transforming Our World: The 2030 Agenda for Sustainable Development (No. A/70/L.1), New York: United Nations, http://www.un.org/ga/search/view_doc.asp?symbol=A/70/L.1&referer=/english/&Lang=E (accessed December 30, 2021).

United Nations, and Department of Economic and Social Affairs, Population Division (2019). World Population Prospects 2019: Highlights (ST/ESA/SER.A/423), New York: United Nations Available online at: https://population.un.org/wpp/Publications/Files/WPP2019_Highlights.pdf (accessed December 30, 2021).

Valitova, J.N., Sulkarnayeva, A.G., and Minibayeva, F.V. (2016). Plant sterols: Diversity, biosynthesis, and physiological functions. Biochemistry Moscow *81*, 819–834.

Varma, A., Savita Verma, Sudha, Sahay, N., Bütehorn, B., and Franken, P. (1999). *Piriformospora indica*, a Cultivable Plant-Growth-Promoting Root Endophyte. Applied and Environmental Microbiology *65*, 2741–2744.

Velásquez, A.C., Castroverde, C.D.M., and He, S.Y. (2018). Plant–Pathogen Warfare under Changing Climate Conditions. Current Biology *28*, R619–R634.

Větrovský, T., Morais, D., Kohout, P., Lepinay, C., Algora, C., Awokunle Hollá, S., Bahnmann, B.D., Bílohnědá, K., Brabcová, V., D'Alò, F., et al. (2020). GlobalFungi, a global database of fungal occurrences from high-throughput-sequencing metabarcoding studies. Scientific Data *7*, 228.

Wang, Q., and Cui, J. (2011). Perspectives and utilization technologies of chicory (*Cichorium intybus* L.): A review. African Journal of Biotechnology *10*, 1966–1977.

Wang, P., Richter, A.S., Kleeberg, J.R.W., Geimer, S., and Grimm, B. (2020). Post-translational coordination of chlorophyll biosynthesis and breakdown by BCMs maintains chlorophyll homeostasis during leaf development. Nature Communications *11*, 1254.

Widhalm, J.R., and Dudareva, N. (2015). A Familiar Ring to It: Biosynthesis of Plant Benzoic Acids. Molecular Plant *8*, 83–97.

Xu, X., Dietrich, C.R., Delledonne, M., Xia, Y., Wen, T.J., Robertson, D.S., Nikolau, B.J., and Schnable, P.S. (1997). Sequence Analysis of the Cloned glossy8 Gene of Maize Suggests That It May Code for a β-Ketoacyl Reductase Required for the Biosynthesis of Cuticular Waxes. Plant Physiology *115*, 501–510.

Yan, W., Cao, L.-L., Zhang, Y.-Y., Zhao, R., Zhao, S.-S., Khan, B., and Ye, Y.-H. (2018). New Metabolites from Endophytic Fungus *Chaetomium globosum* CDW7. Molecules *23*, 2873. https://doi.org/10.3390/molecules23112873.

You, Y.-H. (2012). Fungal Diversity and Plant Growth Promotion of Endophytic Fungi from Six Halophytes in Suncheon Bay. Journal of Microbiology and Biotechnology *22*, 1549–1556.

Yuan, X.-L., van der Kaaij, R.M., van den Hondel, C.A.M.J.J., Punt, P.J., van der Maarel, M.J.E.C., Dijkhuizen, L., and Ram, A.F.J. (2008). *Aspergillus niger* genome-wide analysis reveals a large number of novel alpha-glucan acting enzymes with unexpected expression profiles. Molecular Genetics and Genomics *279*, 545–561.

Zabriskie, T.M., and Jackson, M.D. (2000). Lysine biosynthesis and metabolism in fungi. Natural Product Reports *17*, 85–97.

Zaheer, I.E., Ali, S., Saleem, M.H., Imran, M., Alnusairi, G.S.H., Alharbi, B.M., Riaz, M., Abbas, Z., Rizwan, M., and Soliman, M.H. (2020). Role of iron–lysine on morpho-physiological traits and combating chromium toxicity in rapeseed (*Brassica napus* L.) plants irrigated with different levels of tannery wastewater. Plant Physiology and Biochemistry *155*, 70–84.

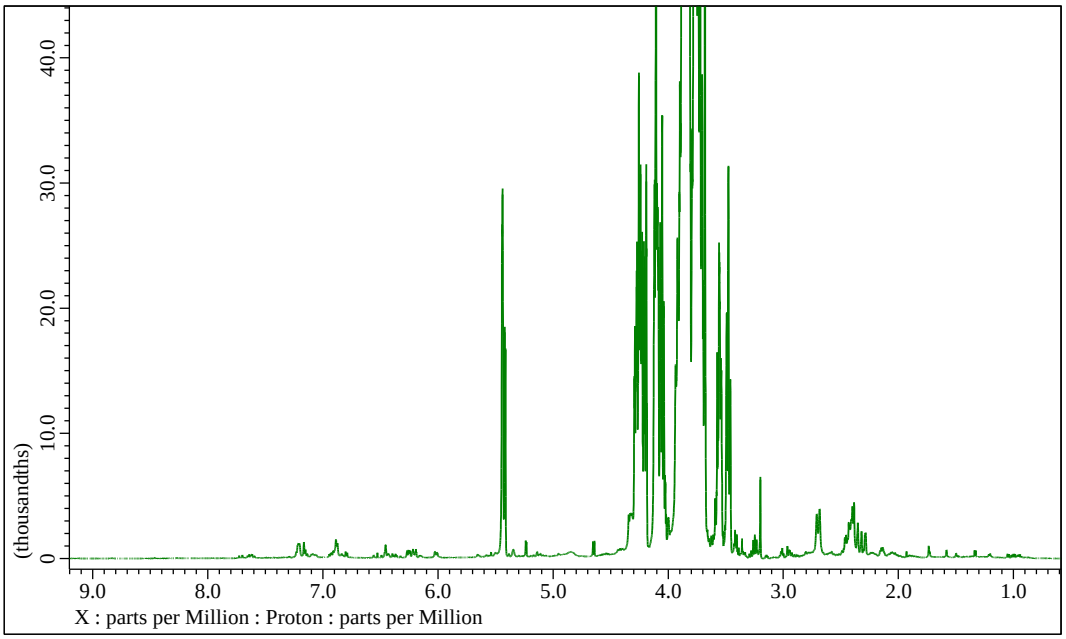
Zhai, X., Luo, D., Li, X., Han, T., Jia, M., Kong, Z., Ji, J., Rahman, K., Qin, L., and Zheng, C. (2018). Endophyte *Chaetomium globosum* D38 Promotes Bioactive Constituents Accumulation and Root Production in *Salvia miltiorrhiza*. Frontiers in Microbiology *8*, 2694.

Zhou, L., Tang, K., and Guo, S. (2018). The Plant Growth-Promoting Fungus (PGPF) *Alternaria* sp. A13 Markedly Enhances *Salvia miltiorrhiza* Root Growth and Active Ingredient Accumulation under Greenhouse and Field Conditions. International Journal of Molecular Sciences *19*, 270.

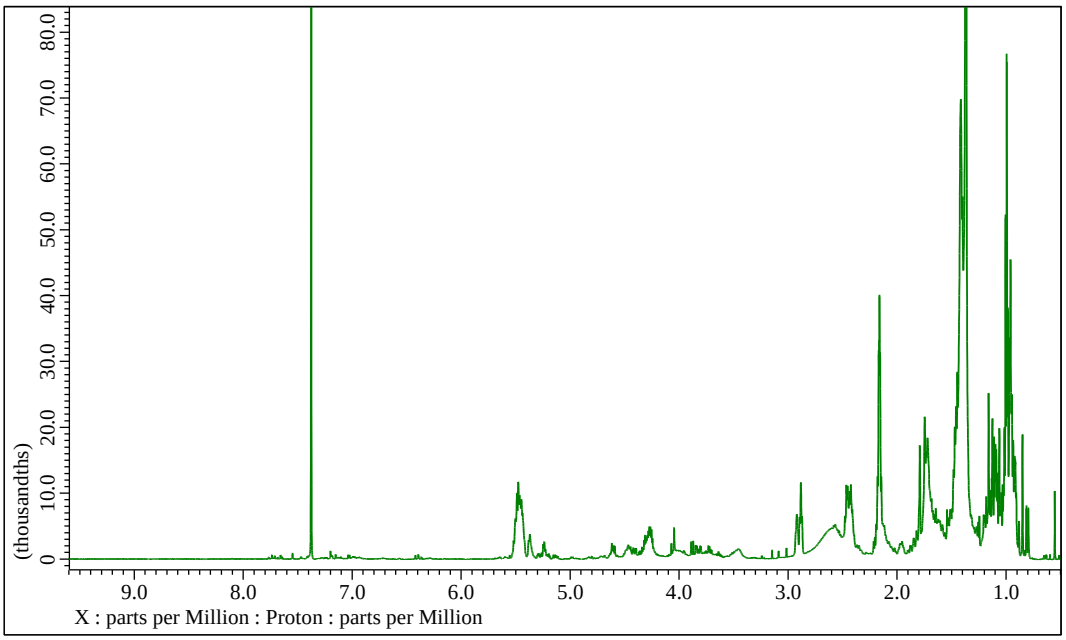
3.7 SUPPLEMENTARY MATERIAL

Supplementary Table 3.2 Filtrate composition assessed by NMR spectroscopy

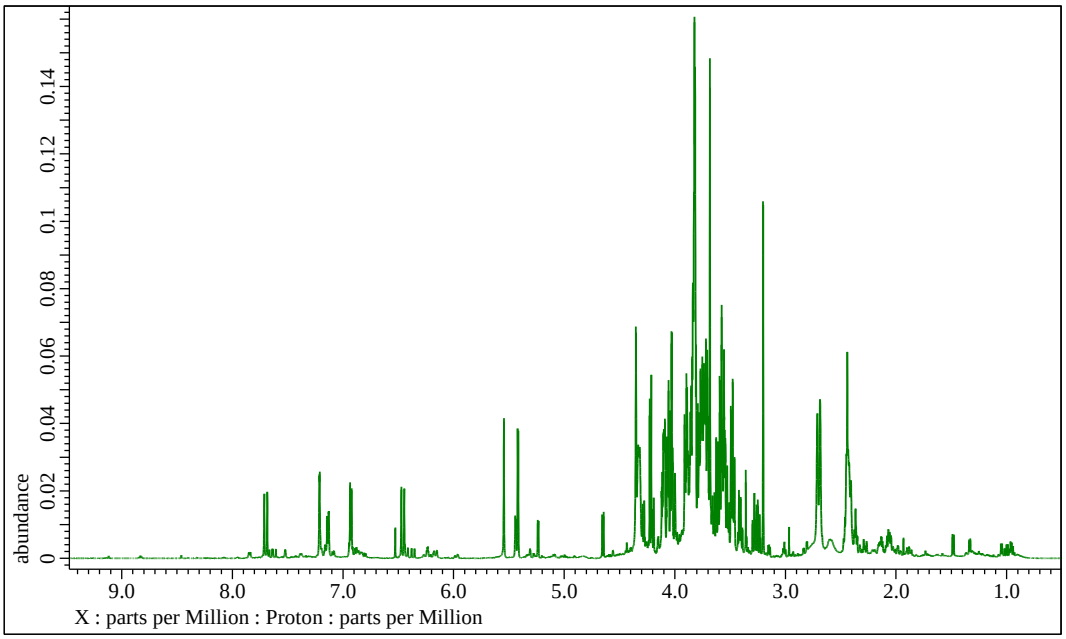
Molecule	Amount (mg/100 ml)		
	MEB Control	*Chaetomium globosum* 205 filtrate	*Minimedusa polyspora* 503 filtrate
Leucine	1.01	0.00	1.34
Isoleucine	0.84	0.00	0.65
Valine	1.00	0.00	1.16
Threonine	1.72	0.00	1.61
Peptone (eq. Lys)	5.99	12.58	0.93
Alanine	1.90	1.80	4.27
Glutamate	0.77	0.00	0.47
GABA	1.29	0.00	0.98
Lysine	2.93	0.00	5.16
Tyrosine	0.72	0.00	0.61
Phenylalanine	1.68	0.00	1.89
Acetate	0.84	0.00	0.04
Fumarate	0.00	1.33	0.00
Formate	0.35	0.00	0.00
Maltose	1266.79	771.54	6.60
Glucose	149.65	101.69	1040.87
Fructose	2.77	1.63	2.13
Ethanol	0.98	17.20	6.26
Choline	0.40	0.00	0.57
AXP	0.00	0.00	1.88
GXP	0.00	0.00	1.56



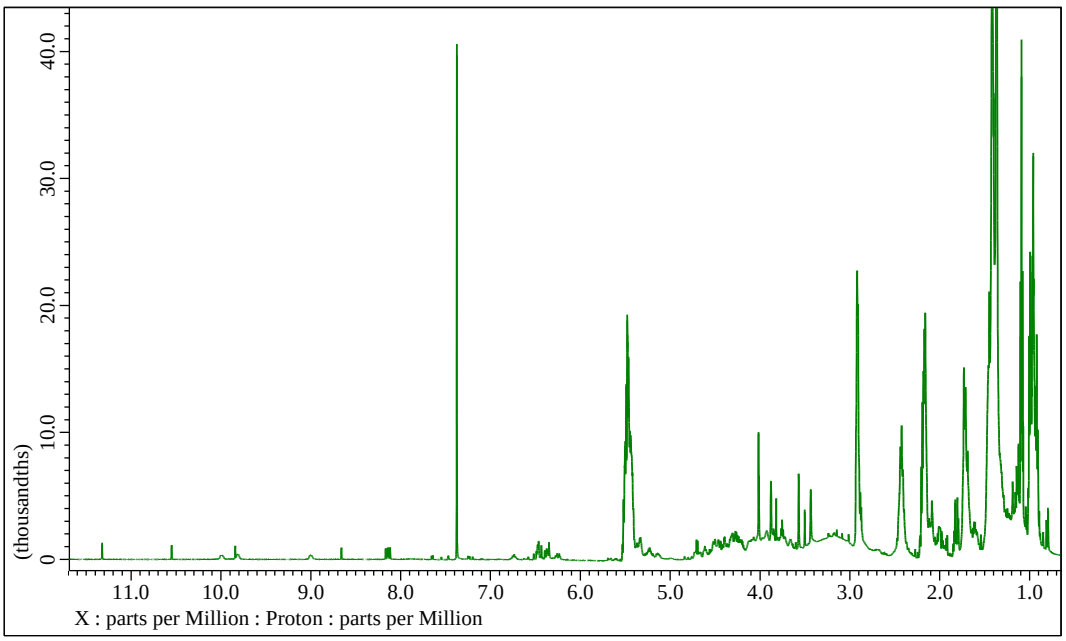
Supplementary Figure 3.11 ^{1}H spectrum of hydroalcoholic extract of chicory roots.



Supplementary Figure 3.12 ^{1}H spectrum of chloroform extract of chicory roots.



Supplementary Figure 3.13 ^{1}H spectrum of hydroalcoholic extract of chicory leaves.



Supplementary Figure 3.14 ^{1}H spectrum of chloroform extract of chicory leaves.

Supplementary Table 3.3 Table of resonance assignment

Compound	Assignment	^{1}H δ (ppm)	Multiplicity	^{13}C δ (ppm)	Portion
Organic acids					
Acetic acid (AA)	**CH_3**	**1.92**	**s**	25.98	L, R
3-hydroxybutyric acid (3HBA)	γ−CH_3	1.19	d	24.5	R
	α,α'-CH	2.30-2.40	dd	49.12	
	β−CH	4.15	m	69.01	
Ascorbic acid (AscA)	CH_2-2'	3.77	m	63.6	L
	CH-1'	3.98	m	69.41	
	CH-5	**4.49**	**d**	77.12	
Caffeic Acid (CafA)	CH-1	7.19	d	117.15	L
	CH-2	7.07	d	124.122	
	CH-3	6.96	dd	118.85	
	CH-4	7.31	d	143.34	
	CH-5	**6.27**	**d**	124.32	
Chicoric Acid (ChA)	CH-1', 2'	5.54	s	54.23	L, R
	CH-8	6.50	d	115.95	
	CH-7	**7.72**	**d**	117.56	
	CH-2	7.22	d	118.90	
	CH-5	6.96	dd	117.81	
	CH-6	6.15	d	149.59	
Citric acid (CA)	**α,γ-CH**	**2.67**	**d**	44.77	L
	α',γ'-CH	2.71	d	44.77	
Chlorogenic acid (CGA)	CH_2-2'	2.02,2.17	m	40.11	L, R
	CH-3'	5.33	m	40.14	
	CH-4'	3.88	dd	75.43	
	CH-5'	4.23	m	72.93	

	CH-8 CH-7 CH-2 CH-5 CH-6	**6.39** 6.94 7.12 7.19 7.65	**d** d dd d d	73.82 117.56 118.90 117.81 149.59	
Formic acid (FA)	**CH**	**8.46**	**s**	171.90	L, R
Fumaric acid (FumA)	**CH=CH**	**6.51**	**s**	137.94	L, R
Lactic acid (LA)	CH_3 CH	1.32 4.11	d q	22.95 77.04	L, R
Malic acid (MA)	**α- CH** β.β'- CH	**4.31** 2.38,2.69	**dd** dd	69.33 40.86	R
p-Hydroxybenzoate (pHBA)	**CH-2,6** CH-3,5	**7.80** 6.84	**d** d	132.96 116.21	L, R
Succinic acid (SA)	2 CH_2	2.39	s	36.31	L, R
Amino acids					
Alanine (Ala)	**β-CH_3** α- CH	**1.49** 3.80	**d** q	19.05 53.56	L, R
Aspartic acid (Asp)	β'-CH β-CH α-CH	2.68 2.72 3.91	dd dd m	39.31 39.31 55.09	L, R
Asparagine (Asn)	β'-CH **β-CH** α-CH	2.86 **2.89** 4.01	dd **dd** m	37.44 37.44 54.09	L, R
Glutamate (Glu)	γ- CH_2 **β,β-CH_2**	2.07 **2.35**	m **m**	29.25 37.12	L, R

	α-CH	3.78	m	58.01	
Glutamine (Gln)	γ- CH_2	2.11	m	29.31	L, R
	β,β-CH_2	**2.45**	**m**	34.02	
	α-CH	3.81	m	57.19	
Isoleucine (Ile)	δ-CH_3	0.95	t	13.85	L, R
	γ-CH_3	**1.02**	**d**	17.38	
	γ'-CH	1.25	m	27.01	
	γ''-CH	1.49	m	27.01	
	β-CH	1.99	m	38.71	
	α-CH	3.69	m	63.04	
Leucine (Leu)	**δ,δ'- CH_3**	**0.97**	**m**	23.85, 24.59	L, R
	γ-CH	1.72	m	26.81	
	β-CH_2	1.73	m	42.60	
	α-CH	3.74	m	56.21	
Lysine (Lys)	δ- CH_2	1.47	m	28.41	L
	γ- CH_2	1.69	m	30.51	
	β- CH_2	1.95	m	43.27	
	δ- CH_2	**3.03**	**t**	38.12	
	α- CH_2	3.78	t	57.32	
γ-aminobutyric acid (GABA)	**β-CH_2**	**1.95**	**t**	26.38	L, R
	γ-CH_2	2.30	m	37.06	
	α-CH_2	3.01	t	42.21	
Phenylalanine (Phe)	CH-2,6	7.32	d	130.3	L, R
	CH-4	7.38	d	128.6	
	CH-3,5	**7.42**	**d**	130.3	
	β-CH_2	3.27	m	37.1	
	α-CH	3.98	dd	56.8	

Pyroglutamic acid (PyrA)	γ- CH_2 **β,β-CH_2** α-CH	2.01 **2.45** 4.16	m **m** m	33.25 28.32 60.91	R
Threonine (Thr)	**γ-CH_3** α-CH β-CH	**1.33** 3.60 4.27	**d** m m	22.15 63.46 68.94	L, R
Tryptophan (Trp)	CH-5 CH-6 **CH-7** CH-4	7.20 7.27 **7.53** 7.73	t t **d** d	124.9 127.9 114.7 121.2	R
Tyrosine (Tyr)	CH-2,6 **CH-3,5** β-CH_2 α-CH	7.22 **7.08** 3.15 3.93	d **d** dd dd	130.0 117.0 37.1 56.8	L, R
Valine (Val)	γ-CH_3 **γ'-CH_3** β-CH α-CH	0.99 **1.05** 2.29 3.62	d **d** m m	19.41 20.75 31.89 63.36	L, R
<u>Carbohydrates</u>					
Amylose (Amy)	**CH-1** CH-2 CH-3 CH-4 CH-5 CH_2-6	**5.11** 3.33 3.37 3.42 3.66 3.63, 3.70	**d** m m m m m	99.10 72.49 77.84 70.67 73.52 66.97	L, R
α-Glucose (α-G)	**CH-1** CH-2 CH-3	**5.25** 3.55 3.72	**d** m m	93.10 72.49 73.84	L, R

	CH-4 CH-5 CH_2-6	3.42 3.84 3.73, 3.90	m m m	70.67 72.52 96.97	
β-Glucose (β-G)	**CH-1** CH-2 CH-3 CH-4 CH-5 CH_2-6	**4.69** 3.26 3.50 3.42 3.48 3.74, 3.91	**d** m m m m m	96.97 75.17 76.84 70.70 74.57 61.80	L, R
Sucrose (S)	**GLC CH-1** CH-2 CH-3 CH-4 CH-5 CH_2-6 FRU CH_2-1' C-2 CH-3' CH-4' CH-5' CH_2-6	**5.42** 3.59 3.79 3.48 3.85 3.82 3.69 \ 4.22 4.06 3.90 3.82	**d** m m m m m m \ m m m m	93.22 72.11 73.54 70.26 73.38 61.18 62.44 104.85 77.45 75.04 82.44 63.38	L, R
Lipids & Sterols					
Linoleic acid (n-6 FA)	CH_3 n-CH_2 CH_2-CH=CH CH=CH **=CH-CH_2-CH=**	0.86 1.36 2.04 5.37 **2.76** 2.06	t m m m **t** m	14.06 29.37 29.45 130.29; 128.45 25.68	L, R

	$C\underline{H}_2$-CH_2-CO_2^- CH_2-CO_2^-	2.31	t	24.75 34.05	
Linolenic acid (n-3 FA)	CH_3 n-CH_2 $C\underline{H}_2$-CH=CH CH=CH **=CH-$C\underline{H}_2$-CH=** $C\underline{H}_2$-CH_2-CO_2^- CH_2-CO_2^-	0.96 1.36 2.04 5.37 **2.78** 2.06 2.31	t m m m **m** m t	14.14 29.37 29.45 130.29; 128.45 25.83 24.75 34.05	L, R
β-Sitosterol (β-ST)	CH_2-1 CH_2-2 CHOH-3 CH_2-4 CH-6 CH_2-7 CH-8 CH-14 CH_2-15 CH_2-16 **CH_3-18** CH_3-25	1.08, 1.85 1.51, 1.84 3.52 2.28 5.34 1.52, 1.98 1.46 0.99 1.57 1.26, 1.85 **0.68** 1.01	m m m m m m m m m m **s** s	37.19 31.50 71.81 42.37 121.79 31.98 31.78 56.74 24.25 28.37 12.20 19.12	L, R
Campsterol (Camp)	CH_2-1 CH_2-2 CHOH-3 CH_2-4	1.08, 1.85 1.51, 1.84	m m m m	37.19 31.50 71.81 42.37	L, R

	CH-6 CH_2-7 CH-8 CH-14 CH_2-15 CH_2-16 **CH_3-18** CH_3-25	3.52 2.28 5.34 1.52, 1.98 1.46 0.99 1.57 1.26, 1.85 **0.70** 1.01	m m m m m m **s** s	121.79 31.98 31.78 56.74 24.25 28.37 12.21 19.12	
Miscellaneous Metabolites					
Adenosine phosphate (AXP)	CH-17' CH-17 CH-5 CH-4 **CH-2** CH-7	4.18 4.24 4.36 4.58 **5.92** 8.12	ddd ddd m dd **d** s	67.40 67.40 73.10 76.35 89.33 140.39	L, R
Choline (Chn)	**$N(CH_3)_3$**	**3.20**	**S**	56.70	L, R
7-Hydroxycoumarin (HCou)	CH-3 CH-4 CH-5 CH-6 CH-8	6.34 **7.67** 7.36 7.09 7.13	d **d** d dd d	115.45 143.21 127.55 125.24 117.03	R
Trigonelline (Trg)	N-CH_3 CH4 CH3,5 **CH1**	4.42 8.07 8.82 **9.11**	s m m **s**	51.1 130.4 148.5 148.1	L, R
Uracile (Ur)	**CH-5**	**5.92**	**d**	103.7	L, R

	CH-6	7.85	d	146.3	
Monoacylglycerol (MAG)	**CH_2** CH_2 CH	**3.65-3,55** 4.05-4.15 3.82	**dd** dd m	65.45 70.32 75.12	L, R
Phospholipids (PP)	CH **$2CH_2$**	**5.13-5.21** 4.15-4.29	bm dd	77.45 68.23	L, R
Triglycerids (TG)	CH **$2CH_2$**	**5.33** 4.15-4.29	m dd	74.64 65.93	A
Triterpenes (TP)	CH-5 CH-9 CH_2-11 CH-12 **CH-18** CH_2-22	0.87 1.65 1.94 5.49 **2.54** 1.97	m t m t **d** t	55.19 48.34 23.9 125.71 53.22 37.43	L, R
Carotenoids (Crt)	CH_2-2,2' CH_2-3,3' CH_2-4,4' CH-7,7' CH-8,8' CH-10,10' **CH-11,11'** CH-12,12' CH-14,14' CH-15,15' CH_3-16,16',17,17' CH_3-18,18' CH_3-19,19'	1.47 1.62 2.02 6.15 6.14 6.14 **6.68** 6.35 6.25 6.63 1.03 1.72 1.97	m m m d d d **m** d d m s s s	39.62 19.27 33.18 126.68 137.78 130.88 125.04 137.26 132.45 130.02 29.01 21.77 12.81	L

Xanthophyl (Xant)	CH-2,2’ CH-3,3’ CH-4 CH-4’ CH-7,7’ CH-8,8’ CH-10,10’ CH-11,11’ CH-12,12’ CH-14,14’ **CH-15,15’** CH_3-16,16’,17,17’ CH_3-18,18’ CH_3-19,19’ CH_3-20,20’	1.47 1.62 2.02 5.45 6.15 6.14 6.14 6.68 6.35 6.25 **6.83** 1.03 1.72 1.97 1.96	m m m bs d d d m d d **m** s s s s	39.62 19.27 33.18 123.18 126.68 137.78 130.88 125.04 137.26 132.45 130.02 29.05 21.77 12.81 12.84	R
Chlorofyll a (Chl A)	CH-5 CH-10 **CH-20**	9.57 9.22 **8.41**	s s **s**	137.6 108.2 93.4	L
Chlorofyll b (Chl B)	**CHO-7** CH-10	**11.22** 9.72	**s** s	178.2 109.4	L

In bold are evidenced the resonances chosen for metabolite quantification; s: singlet, d: doublet, t: triplet, q: quadruplet, dd: doublet of doublets, m: multiplet, bm: broad multiplet; L: leaves, R: roots

Supplementary Table 3.4 Phytochemical composition of chicory roots.

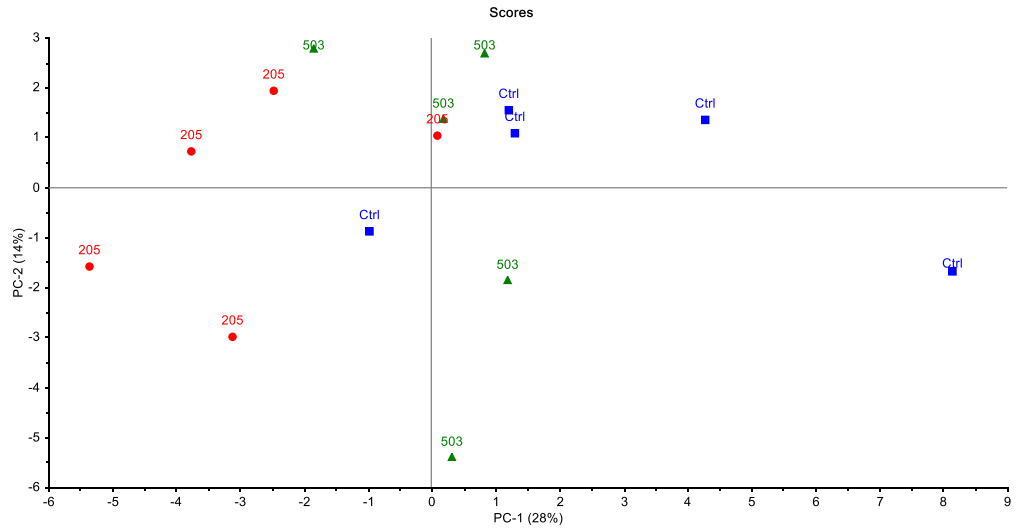
Molecule	Amount (mg / 100 g)		
	MEB Control	*Chaetomium globosum* 205	*Minimedusa polyspora* 503
Leucine	0.19 ± 0.01	0.21 ± 0.03	0.23 ± 0.03
Isoleucine	0.4 ± 0.05	0.43 ± 0.08	0.45 ± 0.07
Valine	0.48 ± 0.06	0.64 ± 0.14	0.62 ± 0.09
Threonine	0.74 ± 0.11	1.03 ± 0.21	1.1 ± 0.25
Alanine	0.06 ± 0.05	0.36 ± 0.07	0.15 ± 0.09
Glutamate	5.64 ± 0.79	6.96 ± 0.93	7.21 ± 1.14
Glutamine	8.17 ± 1.64	12.33 ± 2.55	10.97 ± 2.20
Pyroglutamate	51.9 ± 6.69	58.38 ± 10.62	52.42 ± 8.45
Asparagine	1.46 ± 0.38	6.11 ± 3.42	3.14 ± 1.28
GABA	4.43 ± 0.56	4.17 ± 0.63	4.43 ± 0.5
Tyrosine	0.84 ± 0.44	0.93 ± 0.35	0.73 ± 0.17
Phenylalanine	0.51 ± 0.06	0.68 ± 0.06	0.63 ± 0.08
Tryptophan	0.36 ± 0.11	0.19 ± 0.07	0.11 ± 0.06
3-OHButyrate	0.41 ± 0.03	0.54 ± 0.04	0.55 ± 0.05
Acetate	0.18 ± 0.04	0.16 ± 0.02	0.16 ± 0.02
Malate	2.93 ± 1.58	1.72 ± 0.49	1.76 ± 0.33
Chicoric acid	7.91 ± 3.18	16.74 ± 2.16	12.82 ± 2.62
Chlorogenic acid	2.33 ± 0.92	4.31 ± 0.40	3.59 ± 0.99
Fumarate	0.61 ± 0.20	0.51 ± 0.15	0.42 ± 0.16

4-OH-Benzoate	0.26 ± 0.02	0.37 ± 0.06	0.4 ± 0.07
Formate	0.15 ± 0.02	0.12 ± 0.02	0.13 ± 0.02
Glucose	26.06 ± 3.47	25.26 ± 2.07	22.31 ± 4.84
Sucrose	221.65 ± 48.06	248.54 ± 45.89	226.44 ± 27.73
Amylose	325.09 ± 75.62	268.99 ± 49.84	336.92 ± 52.54
β-Sitosterol	3.39 ± 0.14	2.35 ± 0.12	2.64 ± 0.49
Campesterol	3.44 ± 0.24	2.9 ± 0.17	2.55 ± 0.29
Oleic acid	20.8 ± 2.34	20.82 ± 1.5	23.08 ± 4.87
Linoleic acid	18.29 ± 0.63	16.1 ± 0.93	15.7 ± 2.53
Linolenic acid	5.41 ± 0.21	3.99 ± 0.2	3.63 ± 0.54
Triterpenes (eq. Oleanoic acid)	3.97 ± 0.59	4.48 ± 3.3	0.9 ± 0.11
Glycerol of monoacylglycerols	0.24 ± 0.04	0.41 ± 0.05	0.37 ± 0.03
Glycerol of phospholipids	4.52 ± 0.52	2.14 ± 0.19	1.76 ± 0.49
Choline	2.81 ± 0.23	3.15 ± 0.36	3.4 ± 0.43
Uracil	1.35 ± 0.88	0.66 ± 0.14	0.86 ± 0.14
Adenosine-phospate	0.58 ± 0.09	0.64 ± 0.13	0.78 ± 0.19
Trigonelline	0.11 ± 0.03	0.08 ± 0.01	0.07 ± 0.01
Xantophyl (eq. Luteolin)	0.28 ± 0.02	0.23 ± 0.02	0.2 ± 0.03
7-OH-Coumarin	0.39 ± 0.06	0.31 ± 0.08	0.34 ± 0.06
Aldehyde (eq. Hexanal)	0.1 ± 0.01	0.09 ± 0.01	0.09 ± 0.01

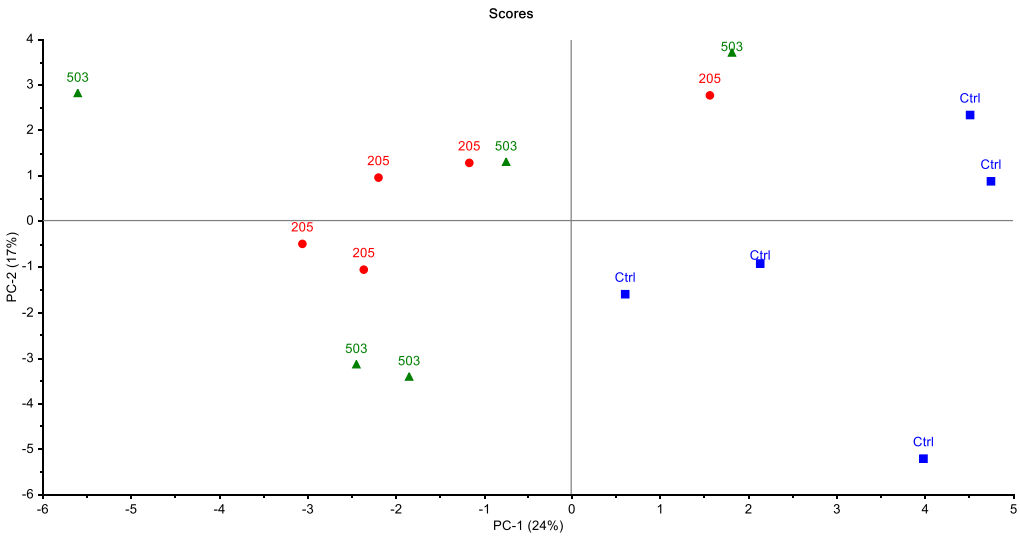
Supplementary Table 3.5 Phytochemical composition of chicory leaves.

Molecule	Amount (mg / 100 g)		
	MEB Control	*Chaetomium globosum* 205	*Minimedusa polyspora* 503
Leucine	0.46 ± 0.03	0.32 ± 0.07	0.42 ± 0.04
Isoleucine	0.54 ± 0.05	0.34 ± 0.08	0.47 ± 0.03
Valine	0.7 ± 0.04	0.45 ± 0.06	0.64 ± 0.03
Threonine	0.84 ± 0.06	0.71 ± 0.04	0.85 ± 0.09
Alanine	1.18 ± 0.19	0.94 ± 0.11	1.01 ± 0.10
Glutamate	4.13 ± 0.99	3.77 ± 1.15	4.36 ± 0.96
Asparagine	3.74 ± 0.54	3.75 ± 0.35	4.67 ± 0.79
GABA	2.6 ± 1.42	1.84 ± 0.83	1.75 ± 0.65
Lysine	2.55 ± 0.26	2.46 ± 0.3	3.25 ± 0.76
Tyrosine	0.73 ± 0.18	0.93 ± 0.17	0.56 ± 0.22
Phenylalanine	0.54 ± 0.1	0.4 ± 0.09	0.7 ± 0.24
Acetate	0.25 ± 0.03	0.17 ± 0.04	0.13 ± 0.03
Citrate	181.88 ± 35.51	128.03 ± 13.23	140.6 ± 13.03
Ascorbate	6.30 ± 1.42	10.1 ± 2.49	8.61 ± 3.52
Caffeic acid	2.26 ± 0.63	3.12 ± 1.70	1.26 ± 0.64
Chlorogenic acid	45.62 ± 7.09	50.51 ± 10.78	46.07 ± 19.3
Fumarate	1.97 ± 0.38	1.37 ± 0.15	1.95 ± 0.21
Chicoric acid	11.56 ± 3.72	12.94 ± 5.98	12.42 ± 4.58
4-OH-Benzoate	0.39 ± 0.04	1.27 ± 0.49	0.64 ± 0.09

Formate	0.23 ± 0.03	0.2 ± 0.02	0.17 ± 0.04
Glucose	13.09 ± 2.8	16.63 ± 4.41	13.57 ± 4.64
Sucrose	73.07 ± 7.63	112.66 ± 22.12	79.2 ± 11.77
Amylose	8.92 ± 3.05	24.64 ± 15.42	6.43 ± 1.33
β-Sitosterol	3.62 ± 0.23	2.7 ± 0.27	2.82 ± 0.19
Campesterol	1.91 ± 0.13	1.02 ± 0.16	0.93 ± 0.11
Oleic acid	26.16 ± 2.76	18.81 ± 1.38	23.25 ± 1.22
Linoleic acid	4.1 ± 0.59	4.11 ± 0.49	5.39 ± 0.66
Linolenic acid	64.91 ± 7.4	54.51 ± 4.54	66.56 ± 1.71
Triterpenes (eq. Oleanoic acid)	7.36 ± 4.08	0.75 ± 0.4	3.06 ± 0.79
Glycerol of monoacylglicerol	1.14 ± 0.48	2.49 ± 0.56	1.54 ± 0.39
Glycerol of phospholipids	2.01 ± 0.73	1.02 ± 0.46	2.01 ± 0.11
Choline	5.08 ± 0.5	4.78 ± 0.61	4.80 ± 0.20
Uracil	0.42 ± 0.19	0.17 ± 0.05	0.11 ± 0.07
Adenosine-phospate	0.64 ± 0.16	0.52 ± 0.09	0.72 ± 0.16
Trigonelline	0.5 ± 0.11	0.42 ± 0.06	0.35 ± 0.07
Xantophyl (eq. Luteolin)	3.58 ± 1.16	3.26 ± 0.34	4.15 ± 0.23
Carotenoids	3.12 ± 0.46	3.03 ± 0.29	3.53 ± 0.07
Chlorophyll *a*	40.98 ± 5.14	3.79 ± 0.67	7.07 ± 0.47
Chlorophyll *b*	9.96 ± 1.21	0.05 ± 0.02	5.18 ± 0.39



Supplementary Figure 3.15 PCA on the whole NMR data set obtained from leaf extracts



Supplementary Figure 3.16 PCA on the whole NMR data set obtained from root extracts

CHAPTER 4: THE GOOD FIGHT: *MINIMEDUSA POLYSPORA* AND *CHAETOMIUM GLOBOSUM* BIOCONTROL POTENTIAL OF PHYTOPATHOGENIC FUNGI

Veronica Spinelli, Andrea Ceci, Roberto Giovannini and Anna Maria Persiani

4.1 INTRODUCTION

Plant diseases, resulting in an annual estimated loss of 10–15% of the world's major crops, represent a major threat to global crops production and social and political stability of nations (Ristaino et al., 2021). About 70–80% of these diseases are caused by pathogenic fungi, which may also represent an associated risk of mycotoxin contamination of processed food and feed (Peng et al., 2021; Sarrocco and Vannacci, 2018).

Based on current climatic trends, and plant and pathogen responses to major climatic factors, concerns are rising on the effect of climate change on plant-pathogens interactions, and emerging plant diseases have already become more frequent. Future scenarios foreseen suboptimal growth conditions for many crops, shifts in the geographic distributions of pests and pathogens in response to climate change, and therefore exacerbation of major plant diseases and emergence of new diseases in critical food-producing regions (Ristaino et al., 2021; Velásquez et al., 2018).

To control phytopathogenic fungi and fulfil crop yield requirements, agriculture for many decades has relied on synthetic agrochemicals, including fungicides. However, this extensive use of pesticides has determined a gradual loss of protection efficiency, determining a continuous increase in the applied dosage. This inappropriate and excessive use of antimicrobials, through a naturally occurring process of microbial adaptation to the environment, led many pests to develop antimicrobial resistance, as exemplified by fungi insensitive to broad-spectrum fungicides (FAO, 2016; Raymaekers et al., 2020). These new resistances acquired by pests, together with the observation of fungicide-induced hormesis phenomena in phytopathogenic fungi, are rising concerns. Furthermore, the use of pesticides determined heavy environmental pollution and has generated serious risk for human and animal health due to their translocation along the food chain (Castaldi et al., 2021; Nicolopoulou-Stamati et al., 2016). While this intensive application of agrochemicals supported crop yields and therefore human wellbeing, being very harmful for the plant-associated microbial community, it heavily impacted soil quality and the ecosystem services that soil and its biodiversity provides. The loss of soil functionality is a process that need to be arrested to meet food demand in future years. Hence, it is necessary to search for environmentally friendly alternatives to improve crop

yield and resilience, and to meet legislation requirements limiting the use of agrochemicals (Directive 335 2009/128/EC and Regulation (EC) No 1107/2009 of the European Parliament and of the Council).

In this effort to transition to a more sustainable and resilient agriculture, solution inspired by nature acquire a great value. In particular, the application of biological control agents and their secondary metabolites represent a promising option to support the achievement of food security enhancing the yield and quality of agricultural products, without further compromise ecosystems' health (Khan et al., 2020). In nature microorganisms such as bacteria, fungi, viruses, yeasts, and protozoans provide beneficial outcomes to plant growth and health through a direct or indirect action of plant diseases control. Direct actions of plant protection mainly occur through antagonistic effect on the pathogen through phenomena of parasitism, antibiosis, or competition for nutrients, space, or infection sites. Indirectly, they may act against pathogens priming plants or inducing resistance against infections. Finally, a broader definition of biocontrol also includes the application of non-living agents of biological origin such as microbial-derived bioactive compounds (Köhl et al., 2019; Raymaekers et al., 2020).

Fungi, thanks to a great adaptability and ability to produce secondary metabolites, hydrolytic enzymes, and proteins, represent very promising candidate for biocontrol applications. Indeed, fungi have already been extensively reported as effective biocontrol agents of phytopathogenic fungi and present a greater potential than bacteria thanks to their great adaptability, the ability to synthesize a broad array of metabolites and to spread through hyphal growth in both soil and rhizosphere (Whipps, 2001).

It is important deepening the potential of known fungal biocontrol agents against the existing fungal pathogens, shedding further light on their action mechanisms and discovering new efficient fungal strains suitable for biotechnological applications. For this purpose, in this study we have investigated two fungal species, namely *Minimedusa polyspora* and *Chaetomium globosum*, for their efficiency as biocontrol agents against three fungal plant pathogens, namely *Alternaria alternata*, *Berkeleyomyces basicola*, *Botritys cinerea*.

Minimedusa polyspora (Hotson) Weresub & P.M. LeClair and *Chaetomium globosum* Kunze have been previously reported for their potential as biocontrol agents of fungal pathogens. Despite only few studies have been carried out on this fungal species, *M. polyspora*, a bulbils-bearing anamorph fungus belonging to Basiodiomycota phylum, showed to control the basal rot in *Narcissus* caused by *Fusarium oxysporum* f. sp. *narcissi* and the vascular wilt of *Dianthus caryophyllus* caused by *Fusarium oxysporum* f. sp. *dianthi*, and to suppress damping-off in carrots due to *Pythium violae* (Beale and Pitt, 1990, 1992, 1995). On the other side, *C. globosum*, the type species of the genus

Chaetomium belonging to Ascomycota phylum, has been reported to control seedborne and soilborne phytopathogens, including *Pyrenophora tritici-repentis* and *Bipolaris sorokiniana* in wheat, *Macrophomina phaseolina* and *Diaporthe phaseolorum* f. sp. *meridionalis* in soybean, *Alternaria brassicicola* and *Alternaria raphani* on radish and *Pythium ultimum* on sugar beet (Hamed et al., 2020; Madbouly and Abdel-Wareth, 2020; Moya et al., 2020).

These two species have been tested in the presence of three of the most important fungal pathogens at global level, *Alternaria alternata* (Fr.) Keissl., *Berkeleyomyces basicola* (Berk. & Broome) W.J. Nel, Z.W. de Beer, T.A. Duong & M.J. Wingf. and *Botrytis cinerea* Pers.. Despite their differences, these pathogens share common ecological and biological features, that make them extremely severe pathogens of economical, agronomical and medicinal importance. All of them have a global distribution, great environmental adaptability and high reproductive efficiency and can attack a wide range of agricultural plants and varieties, including chicory, and foodstuff, causing severe economic damages (Cheung et al., 2020; Domsch et al., 2007; Fedele et al., 2020; Nel et al., 2018). *A. alternata* is a very common species complex, play different ecological roles ranging from saprotrophs to endophytes to pathogens, and is characterized by a strong tolerance to extreme environmental conditions (temperature, humidity, UV light), wide range of substrates' utilization and multifunctional roles in soils, including soil aggregate stabilization (Chung, 2012; Domsch et al., 2007; Troncoso-Rojas and Tiznado-Hernández, 2014). Also, *B. cinerea* is the commonest species of the genus, grows as a parasite or saprotroph on more than 200 plant hosts, and is generally an air-borne or seed-borne fungus, occurring in soil only as decomposer of plant residues (Cheung et al., 2020; Domsch et al., 2007). *A. alternata* is an efficient generalist phytopathogen, able to attack more than 100 plant species, displaying morphological adaptations with variants and "pathotypes" or *formae speciales*, and producing both non-host-specific and host-specific phytotoxins (Chung, 2012; Thomma, 2003). *A. alternata* is also a post-harvest pathogen causing rots and diseases to several economically important crops and foodstuffs and mycotoxin contaminations in cereals and other plants of agronomic importance (Domsch et al., 2007; Tralamazza et al., 2018; Troncoso-Rojas and Tiznado-Hernández, 2014). *B. cinerea* is a species complex, able to attack non-healthy leaves, flowers and fruits of plants of agronomical importance, e.g. grape, acting as necrotrophic pathogen (Cheung et al., 2020; De Simone et al., 2020; Domsch et al., 2007). *B. basicola* is a soilborne hemibiotrophic ascomycetes, causing the black root rot disease in many agricultural and ornamental plants worldwide (Domsch et al., 2007; Nel et al., 2018). The life cycle of *B. basicola*, described for tobacco, pansy and cotton, is complex and is divided in two main phases, biotrophic and necrotrophic, and six different steps in plant hosts (Martino et al., 2021). *B. basicola* produces two main kind of spores, endoconidia (also

known as phialospores) and chlamydospores (or aleuriospores), that are important for its survival in soils (Domsch et al., 2007; Martino et al., 2021).

Despite *in vitro* screenings present several limitations, they constitute valuable methods for the identification of potential biocontrol agents (Raymaekers et al., 2020). The main fungal biocontrol mechanisms identified against phytopathogenic fungi are mostly related to parasitism, antibiosis and competition. Although most of these mechanisms may be assessed through a dual culture plate assay, especially antibiosis phenomena, which may also be observed as inhibition halos of the pathogen growth, this assay does not allow the correct, unambiguous assessment of the antibiotics action of volatile secondary metabolites (Castaldi et al., 2021). The role of antimicrobial volatile organic compounds (VOC) in the control of plant pathogen has been reported as an important aspect in fungal biocontrol potential (de Boer et al., 2019; Morath et al., 2012). Hence, co-cultivation tests can be performed in Petri dishes with separated compartments, not allowing for direct contact nor non-volatile diffusible metabolites exchange, to evaluate possible VOCs effect on the growth of pathogenic strains.

With this in mind, in this study we aimed at evaluating the inhibition ability of two beneficial fungi against three phytopatogens selected among widespread threats to commercially relevant plant species. Also, as the current knowledge on competition mechanisms adopted by the tested beneficial fungi, especially for *M. polyspora*, lacks data on the effect of VOCs, another aim of the study was to gain insight on possible antimicriobial effects involved in the biological control of pathogenic strains, through the use of a set of dual culture plate assay designed to discriminate the impact of indirect biological control.

4.2 MATERIAL AND METHODS

4.2.1 BENEFICIAL AND PLANT PATHOGENS STRAINS

Two fungal strains, isolated in previous studies and currently preserved in the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), *Chaetomium globosum* Kunze FBL 205 and *Minimedusa polyspora* (Hotson) Weresub & P. M. LeClair FBL 503, were studied to assess their potentialities in biocontrol of phytopathogenic fungi.

In all test conditions these two beneficial antagonistic strains were evaluated against three phytopathogenic fungi: *Alternaria alternata* (Fr.) Keissl. (CBS 117587), *Berkeleyomyces basicola* (Berk. & Broome) W.J. Nel, Z.W. de Beer, T.A. Duong & M.J. Wingf. (CBS 117826), and *Botrytis cinerea* Pers. (DSM 877).

Prior to the experiments the strains were reactivated and maintained on Potato Dextrose Agar (PDA) at 25 °C in the dark. PDA was prepared according to the following composition (g/L in distilled water): potato dextrose broth, 24; and bacto agar, 20. All components were purchased from Becton Dickinson (Sparks, MD, USA).

4.2.2 DUAL CULTURE ASSAY

Dual culture plates were arranged according to the scheme in **Figure 4.1** A. Briefly, four 6-mm diameter plugs of the antagonistic strain's mycelium, taken from the actively growing margin of a 10-day old stock culture using a sterile cork borer, were inoculated in the four cardinal places of the plate close to the margin. Contextually, following the same procedure, one 6-mm diameter plug of the pathogenic strain was inoculated in the center of the plate. Control plates with central inoculum of the phytopathogen were set up, as well as antagonistic strains control plate with only the four marginal inocula. All the treatments were set up in quadruplicates and plates were incubated in the dark at 25°C. Images of the plates were recorded daily for ten days and processed with ImageJ2 to measure the area of the colonies. The inhibition effect was determined using the growth inhibition formula:

$$\%Inhibition = \frac{C - T}{C} \times 100$$

where T is the area of the pathogen's colony minus the starting inoculum plug area when co-cultured with the antagonistic strain, and C is the area of the colony minus the starting inoculum plug area in control plates (El-Sayed and Ali, 2020).

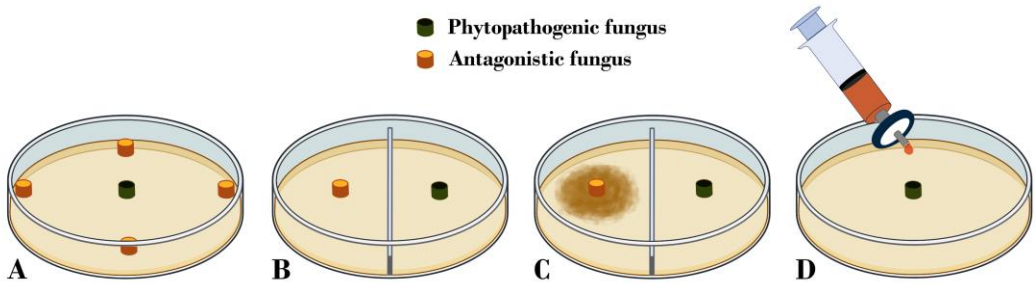


Figure 4.1 A dual culture plate arrangement; B dual culture VOC assay plate arrangement; C dual culture VOC assay plate arrangement with the antagonistic strain inoculum of 5 days in advance; D 10 % of antagonistic culture filtrates added to PDA to assess antifungal activity

4.2.3 DUAL CULTURE ASSAY FOR VOLATILE ANTIMICROBIALS EFFECT

A dual culture assay to assess the inhibition effect of antagonistic strain's volatile antimicrobial metabolites was arranged in petri dishes with two separated compartments, allowing the passage of volatile organic compounds (VOCs) but not direct interaction of the strains. All the treatments were set up in quadruplicates using mycelium plugs taken from the actively growing margin of a 10-day old stock culture using a sterile cork borer, and incubated in the dark at 25°C. Contextually, one 6-mm diameter plug of antagonistic strain's mycelium, was inoculated in one compartment of the plates, while a plug of the pathogen in the other (**Figure 4.1** B). A second experimental group was set up inoculating the antagonistic strain's five days prior the pathogen inoculation (**Figure 4.1** C). All plates were sealed with a double layer of parafilm. Control plates with single inocula of the phytopathogen or the antagonistic strains were set up. Images of the plates were recorded daily for seven days and processed with ImageJ2 to measure the area of the colonies. The inhibition effect was evaluated as inhibition percentage as described above.

4.2.4 CULTURE FILTRATES' ANTIFUNGAL ACTIVITY ASSAY

Each strain's culture filtrate (CF) was prepared inoculating ten 4-mm diameter plugs of mycelium, taken from the actively growing margin of a 10-day old stock culture using a sterile cork borer, in 150 mL Erlenmeyer flask containing 75 mL of Potato Dextrose Broth (PDB) Becton Dickinson (Sparks, MD, USA). Five replicates were set up for each strain and 5 Erlenmeyer flasks were left uninoculated for control treatment. The flasks were incubated at 25°C on an orbital shaker (ASAL 711/D) at 100 rpm for 14 days.

At the end of the incubation period, the culture medium was recovered and filtered using sterile syringe filters with a 0.45 µm pore size made of mixed cellulose esters (ClearLine®, Dominique Dutscher SAS, Brumath, France).

The antifungal activity of culture filtrates was assessed in a plate assay according to Linkies et al. (2020). Fungal culture filtrate was added to molten PDA shortly before solidification to obtain a final concentration of 10%, the same amount of uninoculated PDB was added for control plates. One 6-mm diameter plug of antagonistic strain's mycelium, taken from the actively growing margin of a 10-day old stock culture using a sterile cork borer, was inoculated in the center of the plate (**Figure 4.1** D). All the treatments were set up in quadruplicates and incubated in the dark at 25°C for 14 days. Images of the plates were recorded daily for 14 days and processed with ImageJ2 to measure the area of the colonies. The inhibition effect was evaluated as inhibition percentage as described above.

4.2.5 STATISTICAL ANALYSIS

All statistical analyses were carried out using the statistical software R (version 4.1.0) under the R-studio environment (version 1.4.1106).

Data normality and homogeneity of variance were tested using Shapiro–Wilk test (package stats), followed when appropriate by Bartlett Test (package stats) or Levene Test (package lawstat).

The data of the dual culture assays and of the culture filtrates' antifungal activity assay have been analyzed through Friedman test (package muStat), followed by all pairs comparison post hoc Conover's test (package PMCMRplus). The data of both the dual culture VOC assays were investigated by means of Welch test (package stats), followed by post hoc Duncan's new multiple range test (MRT) (package PMCMRplus).

4.3 RESULTS

4.3.1 DUAL CULTURE ASSAY

The values of colony area and inhibition rates in dual culture assay are shown in **Table 4.1**. At 10 days of growth, all the pathogenic strains' growth were subjected to an inhibition effect by the tested antagonistic fungi. In fact, both *A. alternata* and *B.basicola* showed a statistically significant reduction of their growth ($p < 0.05$) in the presence of both *C. globosum* and *M. polyspora*. *A. alternata* showed inhibition values higher than 70% in both the assays, and *B. basicola* showed inhibition values than 97%, as well. *B. cinerea* also was significantly affected by *C. globosum* ($p < 0.05$), with an 86.4% inhibition of its growth compared to control. Instead, *M. polyspora* inhibition of *B. cinerea* resulted to be more complex. Indeed, *M. polyspora* and *B cinerea* mycelia did not remain apart from each other but an overgrowth of *B. cinerea* was observed; however, this did not inhibit *M. polyspora* growth whose mycelium occupied the whole plate in contrast to *B. cinerea* whose growth stopped before occupying the whole plate. Moreover, these plates were kept after the experiment endpoint and no further growth of *B. cinerea* was observed, while two perpendicular lines of *M. polyspora*'s bulbils were observed in the middle of the plates. Dual culture assay fungal interactions are shown in figure 2.

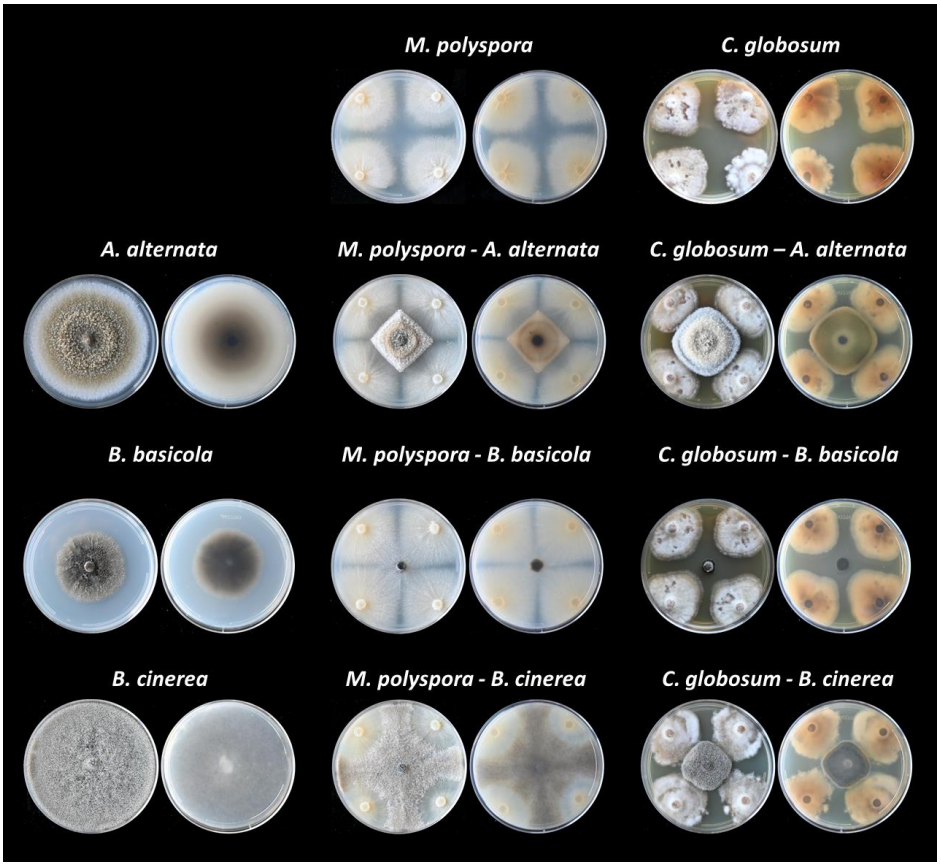


Figure 4.2 Fungal interactions in dual culture assays on tested strains after 10 days of growth on PDA. Front and reverse of the plate are shown for each treatment and control.

Table 4.1 Colony area measurements of the pathogenic fungus after 10 days of growth at 25°C on Potato Dextrose Agar in dual culture plate arrangement with the antagonistic fungus. Data are expressed in mean ± standard error. Asterisks denote a significant difference between the treatment and the respective control (post-hoc Conover test, $p < 0.05$)

Treatment	Colony area (cm²)	% Inhibition
Control *A. alternata*	51.41 ± 1.40	-
M. polyspora – A. alternata	10.93 ± 0.29*	79.4
C. globosum – A. alternata	14.76 ± 0.34*	71.8
Control *B. basicola*	19.40 ± 0.48	-
M. polyspora – B. basicola	0.69 ± 0.12*	98.5
C. globosum – B. basicola	0.95 ± 0.02*	97.1
Control *B. cinerea*	63.60 ± 0.0	-
M. polyspora – B. cinerea	32.30 ± 1.39	49.5
C. globosum – B. cinerea	9.02 ± 0.61*	86.4

4.3.2 DUAL CULTURE ASSAY FOR VOLATILE ANTIMICROBIALS EFFECT

In the dual culture assay for assessing volatiles antimicrobial effect, varied responses among the different treatments were observed in both pathogenic and antagonistic strains at 7 days of growth (Table 4.2). In fact, *A. alternata* was significantly inhibited by *C. globosum*. Instead, *B. basicola* showed a statistically significant reduction of growth in the presence of *M. polyspora* ($p < 0.05$), and a statistically significant stimulation of growth in the treatment with *C. globosum* ($p < 0.05$). None of the antagonistic strains showed an inhibition effect on *B. cinerea*, but in this treatment *M. polyspora* showed a statistically significant increase in the colony area compared to its control ($p < 0.05$), while in contrast *C. globosum* was significantly inhibited. In several treatments a growth stimulation of the antagonistic strains compared to their controls was observed, however in most of the cases it did not result statistically significant.

The results of dual culture VOC assay with advanced inoculum of the antagonistic strains are shown in Table 4.3. *M. polyspora* was observed to inhibit both *A. alternata* and *B. basicola*, however only in the second case the inhibition was statistically significant ($p<0.05$). In contrast, *C. globosum* showed a statistically significant stimulation effect of both pathogens' growth, with *B. basicola* showing a growth increase higher than 80% compared to the control.

In contrast, neither of the antagonistic strain was able to exert a significant effect on *B. cinerea*. Instead, in this treatment, *C. globosum* showed a statistically significant loss of colony area ($p < 0.05$), with a growth inhibition of more than 13%. In addition, *M. polyspora* also showed a slight inhibition due to *B. cinerea* and *B. basicola*, however it was found to be not statistically significant similarly to the slight growth stimulation due to *A. alternata*.

Table 4.2 Colony area measurements after 7 days of growth at 25°C on Potato Dextrose Agar in dual culture VOC plate arrangement. Data are expressed in mean ± standard error. Negative values of inhibition percentage denote a stimulation effect. Asterisks denote a significant difference between the treatment and the strain's respective control (post-hoc Duncan test, $p < 0.05$)

Treatment	Antagonistic strain		Phytopathogenic strain	
	Colony area (cm2)	% Inhibition	Colony area (cm^2)	% Inhibition
Control *M. polyspora*	17.33 ± 0.76	-	-	-
Control *C. globosum*	18.67 ± 0.86	-	-	-
Control *A. alternata*	-	-	21.41 ± 0.38	-
M. polyspora – A. alternata	18.45 ± 0.56	−6.6	21.67 ± 0.44	−1.3
C. globosum – A. alternata	18.83 ± 0.82	−0.8	19.26 ± 0.21*	10.2
Control *B. basicola*	-	-	8.30 ± 0.13	-
M. polyspora – B. basicola	18.54 ± 0.69	−7.1	4.64 ± 0.25*	46.3
C. globosum – B. basicola	18.78 ± 0.59	−0.6	10.04 ± 0.33*	−22.0
Control *B. cinerea*	-	-	27.50 ± 0.00	-
M. polyspora – B. cinerea	19.75 ± 0.52*	−14.3	27.50± 0.00	0.0
C. globosum – B. cinerea	16.27 ± 1.45*	13.1	27.50 ± 0.00	0.0

Table 4.3 Colony area measurements at 25°C on Potato Dextrose Agar in dual culture VOC plate arrangement with advanced inoculum (antagonistic strains: 12 days of growth; Phytopathogens: 7 days of growth). Data are expressed in mean ± standard error. Negative values of inhibition percentage denote a stimulation effect. Asterisks denote a significant difference between the treatments and the strain's respective control (post-hoc Duncan test, $p < 0.05$)

Treatment	Antagonistic strain		Phytopathogenic strain	
	Colony area (cm2)	% Inhibition	Colony area (cm²)	% Inhibition
Control *M. polyspora*	27.50 ± 0.00	-	-	-
Control *C. globosum*	24.38 ± 0.5	-	-	-
Control *A. alternata*	-	-	19.52 ± 1.03	-
M. polyspora – A. alternata	25.89 ± 0.80	5.9	17.53 ± 1.93	10.4
C. globosum – A. alternata	25.23 ± 0.27	−3.9	25.32 ± 0.08*	−30.4
Control *B. basicola*	-	-	9.69 ± 0.14	-
M. polyspora – B. basicola	27.35 ± 0.09	0.6	0.57 ± 0.13*	98.1
C. globosum – B. basicola	25.45 ± 0.10	−4.8	17.86 ± 0.36*	−88.1
Control *B. cinerea*	-	-	27.5 ± 0.00	-
M. polyspora – B. cinerea	25.11 ± 0.41	8.8	26.77 ± 0.72	2.7
C. globosum – B. cinerea	21.13 ± 1.64*	13.3	27.5 ± 0.00	0

4.3.3 CULTURE FILTRATES' ANTIFUNGAL ACTIVITY ASSAY

In the culture filtrates' antifungal activity assay, varied responses among the different treatments were observed.

The values of colony area and inhibition percentages observed in the culture filtrates' antifungal activity assay are shown in **Table 4.4**. The culture filtrate of *M. polyspora* effectively inhibited the growth of all the pathogens, whose colony areas resulted to be significantly reduced compared with the control ($p < 0.05$). In particular, *B. basicola* growth was completely inhibited, showing the absence of mycelium development from the starting inoculum. Conversely *C. globosum* culture filtrate determined only a 24.7% inhibition of *B. basicola* ($p < 0.05$), while it did not exert any inhibitory effect on *A. alternata* and *B. cinerea*.

Table 4.4 Colony area measurements after 14 days of growth at 25°C on Potato Dextrose Agar amended with CF. Data are expressed in mean ± standard error. Asterisks denote a significant difference between the treatment and the respective control (post-hoc Conover test, $p < 0.05$)

Treatment	Colony area (cm^2)	% Inhibition
Control *A. alternata*	56.00 ± 0.00	-
M. polyspora CF – *A. alternata*	46.13 ± 1.06*	17.8
C. globosum CF – *A. alternata*	56.00 ± 0.00	0.0
Control *B. basicola*	33.78 ± 0.66	-
M. polyspora CF – *B. basicola*	0.40± 0.00*	100
C. globosum CF – *B. basicola*	25.54 ± 0.80*	24.7
Control *B. cinerea*	56.00 ± 0.00	-
M. polyspora CF – *B. cinerea*	51.95 ± 1.13*	7.3
C. globosum CF – *B. cinerea*	56.00 ± 0.00	0.0

4.4 DISCUSSION

In this study the *in vitro* ability of two fungal strains to act as biocontrol agents against three common pathogenic fungi of chicory and other plants of agronomic interest was evaluated. The multiple-assay approach allowed to deepen the knowledge on their multifaceted biocontrol mechanism, involving direct competition, diffusible and volatile metabolites.

In the dual culture assay, *C. globosum* and *M. polyspora* were both able to inhibit the growth of all the pathogenic strains, acting as biocontrol agents against the tested phytopathogens. This test results suggest that in all the considered pathogen–antagonistic combination the competition for nutrients and space mechanism is involved. However, in the dual culture test it is not possible to exclude a concomitant effect of one or more additional direct or indirect antagonistic mechanisms as mycoparasitism, modifications of environmental conditions, and antibiosis (Gajera et al., 2013; Madbouly and Abdel-Wareth, 2020; Raymaekers et al., 2020). The competition for nutrients and space can also include the release of specific metabolites, such as siderophores that play an important role in the control of phytopathogens, while antibiosis is related to the excretion of diffusible metabolites that can hinder the phytopathogens' growth (Gajera et al., 2013; Hamed et al., 2020; Moya et al., 2020). More than 200 bioactive metabolites have been described in *Chaetomium* spp., for instance chaetochromones, isolated from *C. indicum*, were successfully tested against different fungal phytopatogens, including *A. alternata* (Lu et al., 2013). In particular, in *C. globosum* several metabolites, including chaetoglobosins, chaetominin, chaetoviridins, cochliodon have been reported to have strong repressive effects on the growth of different fungal phytopathogens, including *B. cinerea* (Hamed et al., 2020; Madbouly and Abdel-Wareth, 2020; Moya et al., 2020). However, in the tested conditions *C. globosum* culture filtrate was only able to determine a 24.7 % inhibition of *B. basicola* growth, while it did not result effective in the inhibition of *A. alternata* and *B. cinerea*. The observed lack of inhibition of *B.cinerea* is not in line with the results obtained by Linkies et al (2020) that, in analogous test conditions, observed a clear inhibitory activity (>50%) of *C. globosum* culture filtrate towards *B. cinerea*. Nonetheless different results may be linked to strain – specific effects. Despite *C. globosum* culture filtrate inefficacy against *A. alternata* and *B. cinerea*, it is not possible to confidently exclude the release of metabolites as possible mechanisms involved in the inhibition effect observed in the dual culture assay. Indeed, studies on fungal strains metabolism showed that coculture conditions, triggering the activation of silent gene clusters involved in the synthesis of secondary metabolites, induce a greater metabolites chemodiversity compared with single strains pure cultures (Bertrand et al., 2013, 2014; Netzker et al., 2015; Zhuang and Zhang, 2021).

Conversely, *M. polyspora* culture filtrate resulted to be effective against all the phytopathogens, especially on *B. basicola* whose growth was completely inhibited preventing any development of the starting inoculum. The observed effect suggests that the release of specific secondary metabolites may be a major component of *M. polyspora* antifungal activities. Indeed, previous studies reported the presence of the acetylenic compound dehydromatricarionol along with an enzyme able to oxidize it, and five trienes, including at least one polyacetylene, in *M. polyspora* culture filtrate (Beale and Pitt, 1995; Domsch et al., 2007; Hodge, 1966). Polyacetylenes, which are bioactive

acetylenic metabolites found in fungi (mainly Basidiomycetes) and in plants, have been reported to possess antibiotic and antifungal activities (Anchel, 1952; Beale and Pitt, 1995; Kuklev et al., 2013; Singh et al., 2020).

VOCs, thanks to the ability of diffusing among soil particles and spreading in the atmosphere over large distances, exerting their effect without requiring a direct or physical contact between the involved organisms, in terrestrial ecosystems play important roles in microbial interactions and communications (Tilocca et al., 2020). These metabolites, by means of antimicrobial activity and other cross-talk interactions, have been found effective in the control of phytopathogenic oomycetes, fungi, and bacteria already at a very low concentration (Schalchli et al., 2015; Tilocca et al., 2020; Weisskopf et al., 2021). Moreover, being VOCs signal molecules, studies reported that strains, due to biological interactions or environmental cues, may specifically release additional volatiles other than those constitutively emitted (Weisskopf et al., 2021). Therefore, two VOC dual culture assays were carried out to gain insight into the production and biocontrol efficacy of VOCs from the tested strains, as a possible metabolic response to the presence of the phytopathogenic strains. In these assays all pathogens were able to stimulate the growth of *M. polyspora* in synchronous inocula conditions, although a growth reduction was observed in advanced inoculum conditions. Instead, *M. polyspora* showed the ability to strongly inhibit the growth of *B. basicola* and slightly that of *A. alternata* in the advanced inoculum condition. In the first case, the VOCs excreted by phytopathogenic strains determined metabolic responses in *M. polyspora*, causing it to grow more rapidly to counteract the perceived threat, while VOCs released by *M. polyspora* may have inhibited the fungal pathogens. As above-mentioned *M. polyspora* is reported to release polyacetylenes that are also related to the production of some VOCs in fungi as reported for instance, in *Serpula lacrymans* through the decarboxylation of an acetylenic compound (Ewen et al., 2004). Hence, in *M. polyspora*, it is possible to hypothesize the involvements of such compounds or other not-yet-described bioactive metabolites that either may diffuse in the medium or spread as VOCs, impairing fungal pathogens' growth according to their specific sensitivity and metabolic responses. In this case, *B. basicola* showed to be more sensitive than *A. alternata*. Indeed, it is known that different fungal species strongly differ in sensitivity to volatiles and, therefore, the type of effect and the extent of inhibition or stimulation depends on the specific strain–strain interaction (Schmidt et al., 2015). In both synchronous and advanced inoculum conditions, *C. globosum* is inhibited by *B. cinerea*, while it inhibited *A. alternaria* only in the synchronous test. In both synchronous and advanced inoculum conditions, *C. globosum* showed the ability to stimulate the growth of *B. basicola* and *A. alternaria*, while *B. basicola* and *A. alternaria* slightly stimulated the antagonistic fungus. In the dual culture arrangement, the effect of *C. globosum* against all fungal strains was inhibitory, instead. This confirms that *C. globosum* uses an extremely varied array of

competitive strategies, including the production of diffusible and volatile compounds. Indeed, in *C. globosum* several VOCs, including alcohols, ketones, alkanols, possessing a strong potential in biocontrol and possibly explaining the inhibition effects on tested fungal pathogenic strains, have been isolated and described (Korpi et al., 1998; Kumar et al., 2021; Sawoszczuk et al., 2015; Tiebe et al., 2010). In this study, despite VOCs released by *C. globosum* may cause eustress in pathogenic strains, stimulating their growth, *C. globosum* was still able to inhibit the phytopathogens in other conditions, resulting in an effective overall biocontrol. Nevertheless, in most of cases, both *C. globosum* and *M. polyspora* were able to hinder pathogens antagonistic actions, including the production of potentially toxic metabolites. In fact, it is worth noting that all tested fungal pathogens are reported to produce toxic metabolites, that could explain the observed inhibition cases in *C. globosum* and *M. polyspora*. For instance, *B. cinerea* is able to produce sesquiterpene botrydial, an antibacteric, fungistatic and phytotoxic metabolite; *A. alternata* can produce several metabolites and VOCs, including antifungal isocoumarin alternariol; *B. basicola* produces VOCs, important in plant pathogenesis, that might have negative effects also on fungi (Choquer et al., 2007; Domsch et al., 2007; Linton and Wright, 1993; Tabachnik and DeVay, 1980; Wang et al., 2014; Weikl et al., 2016). The different inhibition / stimulation effects observed within the same treatment among the synchronous and advanced inoculum VOC assay, may be explained by the fact that the composition of VOCs released by a given species is highly dynamic over time. Therefore, depending on the development stage of the VOCs-producing species, the resulting VOCs composition may determine different effects on cross-talk interactions (Tilocca et al., 2020).

Finally, microbial VOCs, in the past decade, have been gaining increasing attention for their potentialities in biotechnological applications with a particular emphasis on pathogen biocontrol including also their application in the control of post-harvest pathogens during storage (Morath et al., 2012; Tilocca et al., 2020).

This study's results shed further light on the potentialities of *M. polyspora* biocontrol potential which up to now was reported only against *F. oxysporum* f.sp. *narcissi*, *Fusarium oxysporum* f. sp. *Dianthi*, and *Pythium violae*. Conversely, *C. globosum* has already been reported to be effective against a wide range of phytopathogenic fungi, but to the best of our knowledge it is reported here for the first time to be effective against *B. basicola*. In conclusion, *M. polyspora* and *C. globosum*, in *in vitro* conditions, showed to be efficient biocontrol agents of three of the most extremely severe fungal phytopathogens of economical and agronomical relevance. Their biocontrol activities are linked to the activation of different direct and indirect mechanisms, including the release of secondary metabolites, antibiosis, efficient and competitive use of resources, and a strong tolerance to potentially toxic fungal metabolites. Therefore, considering this study results, future research activities will focus on the analyses of fungal extracts and

culture filtrates for their chemical characterization and the evaluation of their efficiency in the biocontrol of fungal phytopathogens. The main value of this study, concerning a broad area of research, consists in the promising results obtained in in-vitro conditions, representing a first step in unraveling the potential biocontrol activities of a target fungal species. However, inoculated microbes or microbial consortia, due to interactions and competition with resident microbes, may fail to establish sufficiently high densities to exert their activities in the soil. Therefore, further studies should be conducted to assess these strains biocontrol potential upscaling to more complex systems to validate the inhibition effects observed *in vitro*.

4.5 REFERENCES

Anchel, M. (1952). Acetylenic Compounds from Fungi. Journal of the American Chemical Society *74*, 1588–1590.

Beale, R.E., and Pitt, D. (1990). Biological and integrated control of Fusarium basal rot of *Narcissus* using *Minimedusa polyspora* and other micro-organisms. Plant Pathology *39*, 477–488.

Beale, R.E., and Pitt, D. (1992). Studies on *Minimedusa Polyspora*, a Biological Control Agent of Soilborne Plant Pathogens. In Biological Control of Plant Diseases, E.C. Tjamos, G.C. Papavizas, and R.J. Cook, eds. (Boston, MA: Springer US), pp. 249–253.

Beale, R.E., and Pitt, D. (1995). The antifungal properties of *Minimedusa polyspora*. Mycological Research *99*, 337–342.

Bertrand, S., Schumpp, O., Bohni, N., Monod, M., Gindro, K., and Wolfender, J.-L. (2013). *De Novo* Production of Metabolites by Fungal Co-culture of *Trichophyton rubrum* and *Bionectria ochroleuca*. Journal of Natural Products *76*, 1157–1165.

Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K., and Wolfender, J.-L. (2014). Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. Biotechnology Advances *32*, 1180–1204. https://doi.org/10.1016/j.biotechadv.2014.03.001.

Castaldi, S., Petrillo, C., Donadio, G., Piaz, F.D., Cimmino, A., Masi, M., Evidente, A., and Isticato, R. (2021). Plant Growth Promotion Function of *Bacillus* sp. Strains Isolated from Salt-Pan Rhizosphere and Their Biocontrol Potential against *Macrophomina phaseolina*. International Journal of Molecular Sciences *22*, 3324.

Cheung, N., Tian, L., Liu, X., and Li, X. (2020). The Destructive Fungal Pathogen *Botrytis cinerea*—Insights from Genes Studied with Mutant Analysis. Pathogens *9*, 923.

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J.-M., Simon, A., and Viaud, M. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. FEMS Microbiology Letters *277*, 1–10.

Chung, K.-R. (2012). Stress Response and Pathogenicity of the Necrotrophic Fungal Pathogen *Alternaria alternata*. Scientifica *2012*, 1–17.

De Simone, N., Pace, B., Grieco, F., Chimienti, M., Tyibilika, V., Santoro, V., Capozzi, V., Colelli, G., Spano, G., and Russo, P. (2020). *Botrytis cinerea* and Table Grapes: A Review of the Main Physical, Chemical, and Bio-Based Control Treatments in Post-Harvest. Foods *9*, 1138.

de Boer, W., Li, X., Meisner, A., and Garbeva, P. (2019). Pathogen suppression by microbial volatile organic compounds in soils. FEMS Microbiology Ecology *95*, fiz105.

Domsch, K.H., Gams, W., and Anderson, T.-H. (2007). Compendium of soil fungi (Eching, Germany: IHW-Verl).

El-Sayed, A.S.A., and Ali, G.S. (2020). *Aspergillus flavipes* is a novel efficient biocontrol agent of Phytophthora parasitica. Biological Control *140*, 104072.

Ewen, R.J., Jones, P.R.H., Ratcliffe, N.M., and Spencer-Phillips, P.T.N. (2004). Identification by gas chromatography-mass spectrometry of the volatile organic compounds emitted from the wood-rotting fungi *Serpula lacrymans* and *Coniophora puteana*, and from Pinus sylvestris timber. Mycological Research *108*, 806–814.

FAO (2016). The FAO Action Plan on Antimicrobial resistance 2016-2020 (Rome, Italy: FAO).

Fedele, G., González-Domínguez, E., and Rossi, V. (2020). Influence of Environment on the Biocontrol of *Botrytis cinerea*: A Systematic Literature Review. In How Research Can Stimulate the Development of Commercial Biological Control Against Plant Diseases, A. De Cal, P. Melgarejo, and N. Magan, eds. (Cham, CH: Springer International Publishing), pp. 61–82.

Gajera, H., Domadiya, R., Patel, S., Kapopara, M., and Golakiya, B. (2013). Molecular mechanism of *Trichoderma* as bio-control agents against phytopathogen system–a review. Current Research in Microbiology and Biotechnology *1*, 133–142.

Hamed, S.R., Abdel-Azeem, A.M., and Dar, P.M. (2020). Recent Advancements on the Role of Biologically Active Secondary Metabolites from *Chaetomium*. In Recent Developments on Genus *Chaetomium*, A.M. Abdel-Azeem, ed. (Cham, CH: Springer International Publishing), pp. 177–204.

Hodge, P. (1966). Natural acetylenes. Part XXIV. The biological oxidation of dehydromatricarianol. Journal of the Chemical Society C: Organic 1617.

Khan, R.A.A., Najeeb, S., Hussain, S., Xie, B., and Li, Y. (2020). Bioactive Secondary Metabolites from *Trichoderma* spp. against Phytopathogenic Fungi. Microorganisms *8*, 817.

Köhl, J., Kolnaar, R., and Ravensberg, W.J. (2019). Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. Frontiers in Plant Science *10*, 845.

Korpi, A., Pasanen, A.-L., and Viitanen, H. (1998). Volatile metabolites of *Serpula lacrymans*, *Coniophora puteana*, *Poria placenta*, *Stachybotrys chartarum* and *Chaetomium globosum*. Building and Environment *34*, 205–211.

Kuklev, D.V., Domb, A.J., and Dembitsky, V.M. (2013). Bioactive acetylenic metabolites. Phytomedicine *20*, 1145–1159.

Kumar, R., Kundu, A., Dutta, A., Saha, S., Das, A., and Bhowmik, A. (2021). Chemo-profiling of bioactive metabolites from *Chaetomium globosum* for biocontrol of *Sclerotinia* rot and plant growth promotion. Fungal Biology *125*, 167–176.

Linton, C.J., and Wright, S.J.L. (1993). Volatile organic compounds: microbiological aspects and some technological implications. Journal of Applied Bacteriology *75*, 1–12.

Lu, K., Zhang, Y., Li, L., Wang, X., and Ding, G. (2013). Chaetochromones A and B, Two New Polyketides from the Fungus *Chaetomium indicum* (CBS.860.68). Molecules *18*, 10944–10952.

Madbouly, A.K., and Abdel-Wareth, M.T.A. (2020). The Use of *Chaetomium* Taxa as Biocontrol Agents. In Recent Developments on Genus *Chaetomium*, A.M. Abdel-Azeem, ed. (Cham, CH: Springer International Publishing), pp. 251–266.

Martino, J.A., Di Feo, L. del V., Paccioretti, M., Contardi, C.A., Sanchez, M.A., Ortega, L.I., and Pastor, S.E. (2021). First report of *Berkeleyomyces basicola* (synonymous: *Thielaviopsis basicola*) on roots of sweet potato (*Ipomoea batatas* (L.) Lam) in Argentina. Revista de la Facultad de Ciencias Agrarias Universidad Nacional de Cuyo *53*, 283–287.

Morath, S.U., Hung, R., and Bennett, J.W. (2012). Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. Fungal Biology Reviews *26*, 73–83.

Moya, P., Cipollone, J., and Sisterna, M. (2020). The Fungal Genus *Chaetomium* and Its Agricultural Applications. In Plant Defence: Biological Control, J.-M. Mérillon, and K.G. Ramawat, eds. (Cham, CH: Springer International Publishing), pp. 289–308.

Nel, W.J., Duong, T.A., Wingfield, B.D., Wingfield, M.J., and de Beer, Z.W. (2018). A new genus and species for the globally important, multihost root pathogen *Thielaviopsis basicola*. Plant Pathology *67*, 871–882.

Netzker, T., Fischer, J., Weber, J., Mattern, D.J., König, C.C., Valiante, V., Schroeckh, V., and Brakhage, A.A. (2015). Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. Frontiers in Microbiology *6*, 299.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., and Hens, L. (2016). Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. Frontiers in Public Health *4*, 148.

Peng, Y., Li, S.J., Yan, J., Tang, Y., Cheng, J.P., Gao, A.J., Yao, X., Ruan, J.J., and Xu, B.L. (2021). Research Progress on Phytopathogenic Fungi and Their Role as Biocontrol Agents. Frontiers in Microbiology *12*, 670135.

Raymaekers, K., Ponet, L., Holtappels, D., Berckmans, B., and Cammue, B.P.A. (2020). Screening for novel biocontrol agents applicable in plant disease management – A review. Biological Control *144*, 104240.

Ristaino, J.B., Anderson, P.K., Bebber, D.P., Brauman, K.A., Cunniffe, N.J., Fedoroff, N.V., Finegold, C., Garrett, K.A., Gilligan, C.A., Jones, C.M., et al. (2021). The persistent threat of emerging plant disease pandemics to global food security. Proceedings of the National Academy of Sciences of the United States of America *118*, 23 e2022239118.

Sarrocco, S., and Vannacci, G. (2018). Preharvest application of beneficial fungi as a strategy to prevent postharvest mycotoxin contamination: A review. Crop Protection *110*, 160–170.

Sawoszczuk, T., Syguła-Cholewińska, J., and del Hoyo-Meléndez, J.M. (2015). Optimization of headspace solid phase microextraction for the analysis of microbial volatile organic compounds emitted by fungi: Application to historical objects. Journal of Chromatography A *1409*, 30–45.

Schalchli, H., Hormazábal, E., Becerra, J., Briceño, G., Hernández, V., Rubilar, O., and Diez, M.C. (2015). Volatiles from white-rot fungi for controlling plant pathogenic fungi. Chemistry and Ecology 31, 754–763.

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., and Garbeva, P. (2015). Volatile affairs in microbial interactions. ISME Journal 9, 2329–2335.

Singh, R., Tiwari, P., Sharma, B., Guerrero-Perilla, C., and Coy-Barrera, E. (2020). Analysis of polyacetylenes. In Recent Advances in Natural Products Analysis, (Amsterdam, Netherlands: Elsevier), pp. 707–722.

Tabachnik, M., and DeVay, J.E. (1980). Black root rot development in cotton roots caused by *Thielaviopsis basicola* and the possible role of methyl acetate in pathogenesis. Physiological Plant Pathology *16*, 109–117.

Thomma, B.P.H.J. (2003). *Alternaria* spp.: from general saprophyte to specific parasite: *Alternaria*. Molecular Plant Pathology *4*, 225–236.

Tiebe, C., Hübert, T., Koch, B., Ritter, U., and Stephan, I. (2010). Investigation of gaseous metabolites from moulds by Ion Mobility Spectrometry (IMS) and Gas Chromatography-Mass Spectrometry (GC-MS). Int. J. Ion Mobil. Spec. *13*, 17–24.

Tilocca, B., Cao, A., and Migheli, Q. (2020). Scent of a Killer: Microbial Volatilome and Its Role in the Biological Control of Plant Pathogens. Frontiers in Microbiology 11.

Tralamazza, S.M., Piacentini, K.C., Iwase, C.H.T., and Rocha, L. de O. (2018). Toxigenic *Alternaria* species: impact in cereals worldwide. Current Opinion in Food Science *23*, 57–63.

Troncoso-Rojas, R., and Tiznado-Hernández, M.E. (2014). *Alternaria alternata* (Black Rot, Black Spot). In Postharvest Decay, (Waltham, MA: Academic Press, Elsevier), pp. 147–187.

Velásquez, A.C., Castroverde, C.D.M., and He, S.Y. (2018). Plant–Pathogen Warfare under Changing Climate Conditions. Current Biology *28*, R619–R634.

Wang, Y., Yang, M.-H., Wang, X.-B., Li, T.-X., and Kong, L.-Y. (2014). Bioactive metabolites from the endophytic fungus *Alternaria alternata*. Fitoterapia *99*, 153–158.

Weikl, F., Ghirardo, A., Schnitzler, J.-P., and Pritsch, K. (2016). Sesquiterpene emissions from *Alternaria alternata* and *Fusarium oxysporum*: Effects of age, nutrient availability and co-cultivation. Scientific Reports *6*, 22152.

Weisskopf, L., Schulz, S., and Garbeva, P. (2021). Microbial volatile organic compounds in intra-kingdom and inter-kingdom interactions. Nature Reviews Microbiology 19, 391–404.

Whipps, J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany *52*, 487–511.

Zhuang, L., and Zhang, H. (2021). Utilizing cross-species co-cultures for discovery of novel natural products. Current Opinion in Biotechnology *69*, 252–262.

CHAPTER 5: GLYPHOSATE-EATING FUNGI: STUDY ON FUNGAL SAPROTROPHIC STRAINS' ABILITY TO TOLERATE AND UTILISE GLYPHOSATE AS A NUTRITIONAL SOURCE AND ON THE ABILITY OF *PURPUREOCILLIUM LILACINUM* TO DEGRADE IT

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Article

Glyphosate-Eating Fungi: Study on Fungal Saprotrophic Strains' Ability to Tolerate and Utilise Glyphosate as a Nutritional Source and on the Ability of *Purpureocillium lilacinum* to Degrade It

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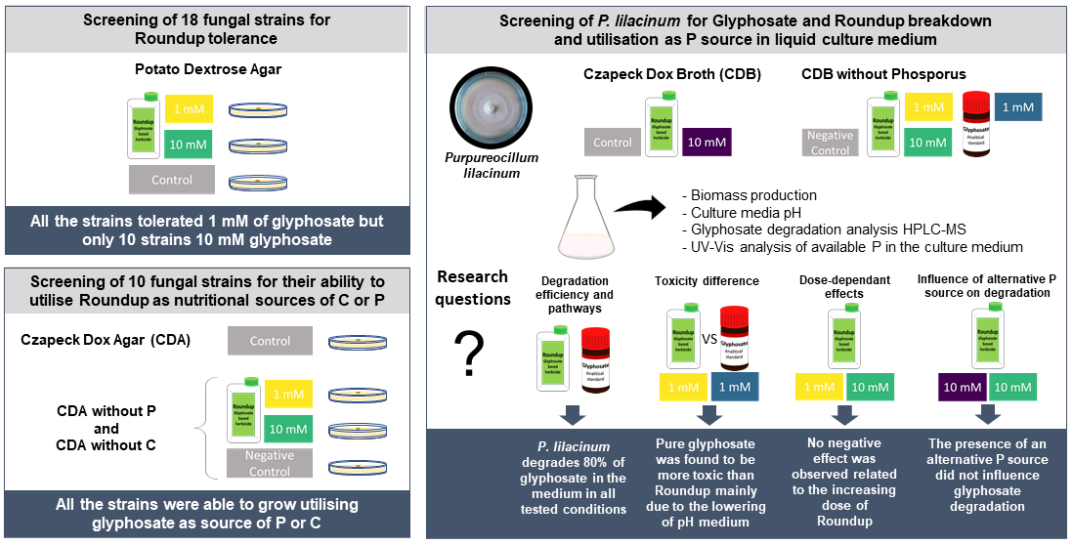
Microorganisms 2021, 9(11), 2179; https://doi.org/10.3390/microorganisms9112179

GRAPHICAL ABSTRACT



ABSTRACT

Glyphosate is the most commonly used herbicide worldwide. Its improper use during recent decades has resulted in glyphosate contamination of soils and waters. Fungal bioremediation is an environmentally friendly, cost effective, and feasible solution to glyphosate contamination in soils. In this study, several saprotrophic fungi isolated from agricultural environments were screened for their ability to tolerate and utilise Roundup in different cultural conditions as a nutritional source. *Purpureocillium lilacinum* was further screened to evaluate the ability to break down and utilise glyphosate as a P source in a liquid medium. The dose–response effect for Roundup, and the difference in toxicity between pure glyphosate and Roundup were also studied. This study reports the ability of several strains to tolerate 1 mM and 10 mM Roundup and to utilise it as nutritional source. *P. lilacinum* was reported for the first time for its ability to degrade glyphosate to a considerable extent (80%) and to utilise it as a P source, without showing dose-dependent negative effects on growth. Pure glyphosate was found to be more toxic than Roundup for *P. lilacinum*. Our results showed that pure glyphosate toxicity can be only partially addressed by the pH decrease determined in the culture medium. In conclusion, our study emphasises the noteworthy potential of *P. lilacinum* in glyphosate degradation.

KEYWORDS

Glyphosate; *Purpureocillium lilacinum*; saprotrophic fungi; bioremediation; Roundup; sarcosine; glyphosate biodegradation pathway; AMPA; tolerance index; biodegradation

5.1 INTRODUCTION

Since the beginning of the Green Revolution, agriculture heavily relied on agrochemicals such as herbicides, pesticides, and chemical fertilisers to support high levels of food production to meet the demand (Tian et al., 2021). However, due to inappropriate and excessive use in the past decades, agrochemicals were determined to have severe detrimental effects on the environment, such as eutrophication, ecosystem simplification, loss of ecosystem services, and loss of biodiversity and of soil quality (Tian et al., 2021; Tilman et al., 2002). Furthermore, the presence of agrochemical residues not only in the environment but also in processed food have raised concerns for their toxic effects on non-target organisms, including humans (FAO and UNEP, 2021; Rodríguez-Eugenio et al., 2018; Tang et al., 2021). These concerns led also to the enactment of legislation requiring the limitation of agrochemical use (Directive 335 2009/128/EC and Regulation (EC) No 1107/2009 of the European Parliament and of the Council).

This recently developed awareness concerns in particular glyphosate, also known as N-(phosphonomethyl)-glycine. Being the active substance in more than 700 available commercial products (Klingelhöfer et al., 2021; Meftaul et al., 2021; Pochron et al., 2020), it is one of the most applied agrochemicals and undoubtedly the most commonly used herbicide worldwide, with an expected use of 740 to 920 thousand tons by 2025 (Clapp, 2021; Maggi et al., 2020; Mesnage et al., 2015; Zhan et al., 2018). Glyphosate as a post-emergent, non-selective systemic herbicide eliminating several weed species at the early growth stage is mainly used in agricultural land. Nevertheless, despite agricultural use accounting for 90% of the total usage, glyphosate is also utilised in non-agricultural land such as ruderal, industrial, or urban areas (Beckie et al., 2020; Espinoza-Montero et al., 2020; Kanissery et al., 2019; Sviridov et al., 2015; Zhan et al., 2018). Its action is based on the inhibition of the 5-enolpyruvyl-shikimate-3-phosphate synthase, an essential enzyme responsible for the synthesis of amino acids in the shikimate pathway in plants and in some microorganisms. More precisely, glyphosate inhibits the production of the essential aromatic amino acids phenylalanine, tyrosine, and tryptophan and, consequently, the production of proteins and secondary metabolites (Kanissery et al., 2019; Sviridov et al., 2015). In this way, glyphosate can generally kill treated plants in a few weeks and provide several other agricultural benefits, including tillage reduction and better crop production (Beckie et al., 2020; Kanissery et al., 2019; Müller, 2021).

Although its toxicity to humans is still under debate, glyphosate residues in humans and animals have been detected and negative effects on animal metabolism, including oxidative stress, have been reported (Myers et al., 2016; Zhan et al., 2018). Residues have been detected also in crop plants and manure fertilisers, and negative effects have been observed on soil microorganisms, and microbes associated with plants and animals (Muola et al., 2021; Sviridov et al., 2015). Among the negative effects due to glyphosate persistence in soil, an increase in bacterial and fungal drug resistance has also been reported. In fact, several microorganisms resistant to glyphosate were found to be resistant also to antibiotics with structural similarities to the herbicide (Curutiu et al., 2017; Raoult et al., 2021; Van Bruggen et al., 2018). Furthermore, glyphosate seems to be linked to plant pathogen increase. In fact, glyphosate causing a reduction in soil biodiversity also alters the key role fulfilled by the latter in avoiding the spread of plant pathogens in the environment. Moreover, a reduction in the resistance to pathogen infections has been observed in plants exposed even to very low concentrations of glyphosate due to residues in soil or water, hence favouring the increase in pathogen spread (Van Bruggen et al., 2018; Zhan et al., 2018).

Despite a great number of commercial formulations being available, relatively little is known on the different toxicity of these compared with pure glyphosate. In this regard, for aquatic animals and mammalian cell lines, glyphosate has been consistently observed to be less toxic than Roundup (Gill et al., 2018). However, Pochron et al. (2020) reported

that glyphosate and not Roundup is toxic to earthworms. The very limited data available suggest that commercial formulations are more toxic than glyphosate for fungi (Morjan et al., 2002).

Due to the widespread use of glyphosate and its environmental persistence, public concerns about the contamination of soils, and surface and underground waters are increasing. For this reason, some countries have started to impose restrictions on its use. In fact, an analysis showed that a globally pervasive low contamination occurs in nearly all croplands where glyphosate is used (Maggi et al., 2020). Furthermore, glyphosate has been reported as a persistent contaminant, at relatively low values, in about 30% of global croplands. Additionally, aminomethylphosphonic acid (AMPA), one of glyphosate's main metabolites, was found to be persistent in about 93% of croplands (Maggi et al., 2020). However, in the case of glyphosate ban, possible alternatives are still limited (Beckie et al., 2020). In 2017, the European Commission relicensed glyphosate for five additional years, and in the same year, 46.5 thousand tons of glyphosate active substances were sold in Europe (Antier et al., 2020; Böcker et al., 2019).

As the world population is expected to grow, reaching nearly 9.7 billion by 2050, requiring an increase by up to 60% in food production, it is clear that a change towards a more sustainable agriculture is necessary (Tian et al., 2021; United Nations et al., 2019). Moreover, meeting the food demand will represent an even harder challenge since more than 33% of soils are already degraded worldwide (Abhilash, 2021). In fact, human activities are overloading the global soil ecosystems as never before, impairing their functionality and stability and causing unreversible degradation and loss.

This need for a change has also been affirmed in the UN 2030 Agenda Sustainable Development Goals (SDG) that put the focus on the necessity to protect and restore this non-renewable resource to face the future global challenges. In fact, in several goals (SDG 2, 3, and 15), the importance of promoting sustainable agriculture that progressively improve land and soil quality and of protecting ecosystems and soil biodiversity that can avoid, reduce, and reverse land degradation is reaffirmed (FAO et al., 2020). Among the threats for soil stability and functioning, contamination is one of the most important (FAO and UNEP, 2021; Rodríguez-Eugenio et al., 2018).

In this context, considering that, in soil (Gill et al., 2018; Zhan et al., 2018), glyphosate can be slowly degraded by microorganisms, bioremediation can provide an environmentally friendly, cost effective, and feasible solution to glyphosate contamination in soils. In particular, fungal bioremediation or mycoremediation employs fungal species as multifunctional microorganisms, perfectly adapted to soil microhabitats (Akhtar and Mannan, 2020; Spina et al., 2018), that can tolerate extreme environmental conditions (Gadd, 2017; Harms et al., 2011, 2017). Thanks to their functional traits, and highly potent and relatively non-specific enzymes, e.g., laccases

and oxidoreductases, fungi can transform natural recalcitrant compounds as well as organic pollutants (Ceci et al., 2019; Harms et al., 2017). In general, co-metabolism of pesticides and other organic persistent pollutants is common in fungi that can transform xenobiotics into less toxic forms. In addition, some pollutants can be completely degraded by fungi, serving as sources of carbon and energy (Harms et al., 2017).

Several fungal strains, mainly belonging to few genera such as *Aspergillus*, *Trichoderma*, *Penicillium*, *Mucor*, and *Fusarium*, have already been reported to tolerate and/or degrade glyphosate. Known strains that tolerate glyphosate as pure molecule include *Penicillium chrysogenum*, which was able to grow in the presence of high concentrations, and *Fusarium solani* and *Fusarium oxysporum*, which showed tolerance to high doses of glyphosate (Klimek et al., 2001; Krzysko-Lupicka and Sudol, 2008). Moreover, these strains were able not only to degrade it but also to utilise it as C or P sources (Krzysko-Lupicka and Sudol, 2008; Krzyśko-Łupicka and Orlik, 1997). Several studies, instead, focused on testing one or more glyphosate-based commercial formulations (e.g., Roundup ControlMax®, Roundup® WG, Tornado plus, etc.), demonstrating that, among others, strains belonging to *Trichoderma* genus, such as *T. viride* and *T. inhamatum*, and to *Aspergillus* genus, such as *A. flavus*, *A. niger*, and *A. oryzae*, were able to tolerate and degrade these products (Arfarita et al., 2016; Carranza et al., 2014, 2017; Kunanbayev et al., 2019). Although in several studies the pure molecule has been compared with one or more commercial formulations, still, little is known about the different toxicities on fungi among them. For instance, Morjan et al. (2002) tested four entomopathogenic fungi in the presence of seven glyphosate formulations, observing that several commercial formulations inhibited more fungal growth than glyphosate.

Despite there being vast literature on fungi and glyphosate, still very limited are studies in this field. For instance, Arfarita et al. (2016) reported that *T. viride* has been able to degrade glyphosate both in vitro and in field conditions; similarly, Kunanbayev et al. (2019) showed that *T. inhamatum* has been successful also in the field. Furthermore, in a study on soil mycoflora, Sailaja and Satyaprasad (2006) observed that there was a predominance of aspergilli, fusaria, penicillia, and *Trichoderma* species and that some *Trichoderma* spp. populations increased in the presence of glyphosate.

Currently, two main glyphosate microbial degradation pathways are known. One involves the C–N bond cleavage leading to the production of AMPA further metabolised to methylamine, and the other involves the C–P bond cleavage releasing sarcosine, which can be processed into glycine and formaldehyde (Sviridov et al., 2015; Zhan et al., 2018).

Some fungal species have been reported to transform glyphosate into AMPA and to utilise it as the sole P source (Kanissery et al., 2019; Sviridov et al., 2015; Zhan et al., 2018). For instance, in *Aspergillus oryzae* A-F02, AMPA is further metabolised to

methylamine, which is then metabolized into other products, suggesting that glyphosate could be used also as N or C sources (Fu et al., 2017). In addition, Correa et al. (2021) observed that the *Penicillium* 4A21 strain produced both AMPA and sarcosine as glyphosate degradation metabolites. Similarly, Adelowo et al. (2014) detected AMPA and sarcosine working on *Trichoderma viridae*, *Aspergillus niger*, and *Fusarium oxysporum*. However, the glyphosate degradation mechanisms in fungi are still widely unknown and other metabolic pathways could be discovered.

In this study, several saprotrophic fungi were tested in the presence of glyphosate commercial formulation in different cultural conditions to select fungal strains able to tolerate and utilise glyphosate as a nutritional source. In this way, new strains representing useful bioresources can enlarge the known pool of candidates suitable for developing a feasible and sustainable strategy for glyphosate bioremediation.

Therefore, this study aimed to evaluate (1) fungal tolerance to glyphosate commercial formulation; (2) the fungal ability to use glyphosate as C or P sources; (3) the dose–response effect to Roundup exposure; (4) the ability of one selected fungal species, *Purpureocillium lilacinum*, to break down and utilise glyphosate as a P source in a liquid medium; and (5) the difference in toxicity between pure glyphosate and commercial formulation for *P. lilacinum*.

5.2 MATERIALS AND METHODS

5.2.1 SCREENING OF FUNGAL STRAINS FOR GLYPHOSATE COMMERCIAL FORMULATION TOLERANCE

Eighteen fungal strains (**Table 5.1**), previously isolated from agricultural environments and currently preserved at the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), were screened to assess their tolerance to Roundup, one of the most common glyphosate commercial formulations. Prior to the experiment the strains have been reactivated and maintained at 25 °C in the dark on Malt Extract Agar (MEA) prepared according to the following composition (g/L in distilled water): malt extract, 20; peptone, 1; dextrose, 20; and bacto agar, 20. All components were purchased from Becton Dickinson (Sparks, MD, USA). Taking into account that, in the field, fungi are exposed to commercial formulations of glyphosate, tolerance tests were performed using Roundup Power 2.0® (Bayer CropScience Srl, Milan, Italy) (RU). This product is a widely utilised glyphosate-based herbicide, nominally containing 360 g/L of pure glyphosate acid as a potassium salt. To evaluate a possible dose-dependent response, tolerance to RU was assessed trough a plate screening at two final concentrations corresponding to 1 mM and 10 mM of glyphosate, based on the reported nominal content, in 25 mL of Potato Dextrose Agar (PDA). The

PDA was prepared according to the following composition (g/L in distilled water): potato dextrose broth, 24, and bacto agar, 20. Both components were purchased from Becton Dickinson (Sparks, MD, USA). Two RU solutions were prepared and filter-sterilised using 0.2 µm Whatman sterile syringe filters. An appropriate amount of RU solution was added in each plate contextually with molten PDA and homogenised before solidification. Control plates without RU were also prepared. One 6 mm diameter plug, taken from the actively growing margin of a 7-day old stock culture using a sterile cork borer, was inoculated in each plate. The assays were carried out in triplicate. The plates were incubated for 14 days in the dark at 25 °C, and diametric measurements of the fungal colonies were recorded daily. The growth responses to RU were evaluated through the R_t:R_c (%) index defined as the ratio of the colony extension growth rates in the presence (R_t) or absence (R_c) of RU: R_t:R_c (%) = (Growth rate of treated mycelium/Growth rate of control mycelium) × 100 (Ceci et al., 2020).

Table 5.1 Fungal strains selected for the study. 1 Fungal strains tested for Roundup (RU) tolerance; 2 fungal strains tested for their ability to use RU as nutritional sources of C or P.

Strain		Phylum	FBL Code
Aspergillus affinis Davolos, Persiani, Pietr., & Maggi	1,2	Ascomycota	535
Aspergillus alliaceus Thom & Church	1,2	Ascomycota	483
Aspergillus flavipes (Bainier & R. Sartory) Thom & Church	1,2	Ascomycota	427
Aspergillus ustus (Bainier) Thom & Church	1,2	Ascomycota	420
Chaetomium globosum Kunze	1	Ascomycota	205
Chaetomium sp.	1	Ascomycota	498
Chaetomium sp.	1	Ascomycota	499
Clonostachys rosea (Link) Schroers, Samuels, Seifert, & W. Gams	1,2	Ascomycota	422
Grifola frondosa (Dicks.) Gray	1	Basidiomycota	450
Metarhizium marquandii (Massee) Kepler, S.A. Rehner & Humber	1,2	Ascomycota	484
Minimedusa polyspora (Hotson) Weresub & P.M. LeClair	1	Basidiomycota	503
Mucor sp.	1,2	Mucoromycota	476
Phycomyces nitens (C. Agardh) Kunze	1,2	Mucoromycota	504
Pleurotus ostreatus (Jacq.) P. Kumm.	1	Basidiomycota	566
Purpureocillium lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones, & Samson	1,2	Ascomycota	478
Trametes hirsuta (Wulfen) Lloyd	1	Basidiomycota	564
Trametes versicolor (L.) Lloyd	1	Basidiomycota	565
Trichoderma sp.	1,2	Ascomycota	650

5.2.2 SCREENING OF FUNGAL STRAINS FOR THEIR ABILITY TO UTILISE GLYPHOSATE AS NUTRITIONAL SOURCES OF C OR P

Ten fungal species (**Table 5.1**) that were able to tolerate RU at 10 mM on PDA were further screened for their ability to utilise RU as nutritional sources of phosphorus (P) and carbon (C). Strains were tested on Czapeck dox agar (CDA) in enrichment conditions either for P (CDA P−) or C (CDA C−), according to the method previously applied in analogous studies (Carranza et al., 2017; Krzysko-Lupicka and Sudol, 2008). CDA was prepared according to the following composition (g/L distilled water): sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; and Bacto agar, 20. CDA P− was prepared following the abovementioned composition without K_2HPO_4, while CDA C− was prepared without sucrose. All chemicals were purchased from Merck (Darmstadt, Germany), except Bacto agar, which was purchased from Becton Dickinson (Sparks, MD, USA). To assess a potential dose-dependent response, the assays were set up at 1 mM and 10 mM of RU concentration. The controls were prepared on non-supplemented CDA, and the negative controls were prepared on CDA P− and CDA C− to assess the potential exploitation of agar and trace residues as P or C sources. Plate preparation and inoculation were the same as described in Section 2.1. The experiments were carried out for 14 days, in the dark at 25 °C. Growth responses and RU utilisation as a nutritional source were investigated by diametric measurements and tolerance index R_t:R_c (%). The tolerance index was calculated utilising the growth rate control on CDA, which represents a growth baseline for the strains in a stress-free condition.

5.2.3 SCREENING OF *PURPUREOCILLIUM LILACINUM* FOR GLYPHOSATE AND ROUNDUP BREAKDOWN AND UTILISATION AS P SOURCE IN LIQUID CULTURE MEDIUM

Based on previous test results and interest in other applications in agricultural biotechnology (Baron et al., 2020; Cavello et al., 2015; Elsherbiny et al., 2021; Hajji et al., 2017), *Purpureocillium lilacinum* was selected for further screening. In order to evaluate the difference in toxicity between the pure molecule of glyphosate (GLY) and the commercial product (RU), the species was tested in a liquid Czapeck dox medium enrichment condition without phosphorus (CDB P−) at a concentration of 1 mM both GLY and RU. Moreover, to evaluate the influence of nutritional stress on the degradation process and possible co-metabolism, a treatment with RU concentration equivalent to 10 mM glyphosate active ingredient was set up in complete CDB and CDB P−. The two treatments in CDB P− at 1 mM and 10 mM were aimed also at evaluating a dose-dependent response. Furthermore, a negative control was set up in CDB P−. CDB was prepared according to the following composition (g/L distilled water): sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; and $FeSO_4 \cdot 7H_2O$, 0.01. CDB P− was

prepared following the abovementioned composition without K_2HPO_4. The experiment was carried out in 100 mL Erlenmeyer flasks containing 50 mL of culture medium which were inoculated with a spore suspension of *P. lilacinum* at the final concentration of 1.55 × 10^7 spores/mL. Prior to utilisation, all of the glassware was washed with 2 M HCl and rinsed multiple times with double distilled water (Millipore).

The experiments were carried out without shaking at 25 °C in the dark for four weeks. A sample of the culture media was recovered weekly, and biomass production was evaluated at the final time. Moreover, to evaluate the inhibition effect of GLY or RU on fungal growth, the inhibition index (%) was calculated: (dry weight of control mycelium − dry weight of treated mycelium)/dry weight of control mycelium × 100 (Eman et al., 2013). The inhibition index was calculated using the CDB control that represents a growth baseline for the strains in a stress-free condition.

Measurements of medium pH were recorded weekly to evaluate the modifications during fungal growth.

5.2.4 SPECTROPHOTOMETRIC DETERMINATION OF P AS A GLYPHOSATE BREAKDOWN PROXY

Unfortunately, due to a strong interference effect of the culture media, applying direct determination methods of glyphosate such as (Bhatt and Iyer, 2021) was not possible. Therefore, the analysis of P released as a proxy of the degradation of glyphosate was carried out (Adelowo et al., 2014; Krzysko-Lupicka and Sudol, 2008). Orthophosphate was determined with a UV-Visible spectrophotometer (Shimadzu UV—1280) at 882 nm, using the ascorbic acid method (Murphy and Riley, 1962). Samples of the culture broth were filtered using a sterile syringe filter with a 0.45 μm pore size made of mixed cellulose esters (ClearLine®, Dominique Dutscher SAS, Brumath, France) and, when necessary, appropriately diluted before analysis. The P release due to glyphosate breakdown was evaluated as the difference between P detected in the inoculated sample and P in the chemical controls. However, in this kind of analysis, P determination is underestimated since the P uptaken during biomass growth is not detectable in the culture broth.

Analytical standards of glyphosate, AMPA, glycine and sarcosine were purchased from Sigma Aldrich/Merck (Milan, Italy). Formic acid (≥98% purity) and RS grade acetonitrile were purchased from the same supplier. Ultrapure water was produced through a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA). The PVDF syringe filters (0.45 μm) were from Sigma-Aldrich.

Stock standard solutions were prepared at concentrations of 1 mg/mL by dissolving 1 mg of the compound (Ohaus DV215CD Discovery semi-micro and analytical balance, 81/210 g capacity, 0.01/0.1 mg readability) in ultrapure water in 1 mL volumetric flasks.

Individual standard solutions at 1 and 10 ng/µL as well as a 2 ng/µL of multi-standard working solution were prepared by diluting the individual standard solutions in ultrapure water.

All of the samples (both the *P. lilacinum* inoculated Czapeck Dox Broth (CDB) media and the relative not inoculated blanks) were 10-fold water-diluted and filtered through 0.45 µm PVDF syringe filters before direct injection (5 µL) into the HPLC–MS system.

The chromatographic analysis was performed by means of a micro HPLC series 200 (PerkinElmer, Norwalk, CT, USA) equipped with a vacuum degasser, an autosampler, and a column oven. The target compounds were separated on a Kinetex F5 column (4.6 × 150 mm, 2.6 µm) from Phenomenex (Torrance, CA, USA) and kept at 298 K under isocratic conditions. The mobile phase was 90% water/10% acetonitrile, with both solvents added with 0.1% formic acid. A mixture of water and acetonitrile in the same proportion was used for washing the autosampler needle. The 1 mL/min flow rate was split by a post-column T-valve so that just 200 µL/min was entirely introduced into a 4000 Qtrap (AB SCIEX, Foster City, CA, USA) mass spectrometer equipped with an electrospray ionisation (ESI) probe on Turbo V source. The chromatograms were acquired in dual polarity (glyphosate and AMPA were detected in negative ion mode, sarcosine, and glycine in positive ion mode), with a needle current (NC) of 3 µA and a probe temperature of 723 K. As both the curtain (40 psi) and collision (4 mTorr) gas high-purity nitrogen were used, while air acted as the nebuliser (55 psi) and make-up (30 psi) gas. The chromatograms were acquired both in full scan (Q1) and in multiple reaction monitoring (MRM) mode by considering two MRM transitions for each analyte. Among these, the peak area of the most intense one (quantifier) was taken for the quantitative analysis. Calibration curves were obtained in solvent, since the matrix effect was minimised by the high dilution factor applied to the samples. The data were acquired and processed by Analyst® 1.6.2 Software (AB Sciex, Foster City, CA, USA).

5.2.5 SCREENING OF PH MEDIUM INFLUENCE ON GLYPHOSATE AND ROUNDUP BREAKDOWN BY *PURPUREOCILLIUM LILACINUM*

Following the results of the abovementioned tests, a further experiment was necessary to determine whether the different biomass production between GLY and RU was either due to a different toxicity of the compounds or to the pH medium decrease caused by the addition of GLY. Therefore, the species was tested in CDB P− with and without tris(hydroxymethyl)aminomethane (TRIS) buffer (Adelowo et al., 2014; Dick and Quinn, 1995) at a concentration of 1 mM GLY and RU. Furthermore, positive and negative controls were set up in CDB and CDB P− with and without TRIS. The experiment was carried out in 100 mL Erlenmeyer flasks containing 50 mL of culture medium which were inoculated with a spore suspension of *P. lilacinum* at the final

concentration of 1.55×10^7 spores/mL. The cultures were incubated without shaking at 25 °C in the dark for 2 weeks. The culture media and fungal biomass were recovered at the final time. The medium's pH was measured to evaluate modifications due to the fungal growth. The culture broth was also analysed for the spectrophotometric determination of P as described in Section 2.4.

5.2.6 STATISTICAL ANALYSIS

All statistical analyses were carried out using the statistical software R (version 4.1.0) under the R-studio environment (version 1.4.1717). The data normality and homogeneity of variance were tested using the Shapiro–Wilk test (package stats), and Bartlett Test (package stats) or Levene Test (package lawstat) (Gastwirth et al., 2020; R Core Team, 2021). For normal homoscedastic data, a one-way analysis of variance (package stats) was performed, followed by the all-pairs comparison post hoc Tukey honest significant differences test (package stats). In some cases, the Welch test (package stats), followed by the all pairs comparison post hoc Dunnet's T3 test (package PMCMRplus), or Friedman test (package muStat), followed by the all-pairs comparison post hoc Conover's test (package PMCMRplus), were applied (Thorsten, 2021; Wittkowski and Tingting, 2012). In Supplementary Material (**Errore. L'origine riferimento non è stata trovata.**), the statistical tests performed on the data of each screening are reported. A two-way ANOVA was performed to examine the effects of TRIS buffer addition and treatments on dry weight values (packages rstatix and emmeans) (Supplementary Material**Supplementary Table 5.10Supplementary Table 5.11**, **Supplementary Figure 5.3**) (Kassambara, 2021; Russell, 2021).

5.3 RESULTS

5.3.1 SCREENING OF FUNGAL STRAINS FOR GLYPHOSATE COMMERCIAL FORMULATION TOLERANCE

The values of diametric growth in fungi incubated on PDA are shown in **Table 5.2**. All tested species were able to tolerate 1 mM RU, while only ten were able to tolerate 10 mM RU. *A. alliaceus* FBL 483 did not show the differences between the control and treatments at any tested concentrations. All of the other species in the 10 mM RU treatment showed significant growth reductions compared with control ($p < 0.05$). Only *A. flavipes* FBL 427 and *P. lilacinum* FBL 478 showed no differences between 1 mM RU and 10 mM RU but showed significant differences between control and 10mM RU. Most of calculated R_t:R_c values for the 1 mM RU treatment showed values higher than 80%,

indicating a strong tolerance of tested species (**Table 5.3**). R_t:R_c values at 10 mM RU showed values higher than 80% only for *Mucor* sp. FBL 476 and *A. alliaceus* FBL 483.

Table 5.2 Diametric values of fungal colonies after fourteen days of growth exposed to Roundup (RU) on Potato Dextrose Agar (PDA) at 25 °C. The data are expressed as the mean ± standard error of independent biological replicates a.

	Diameter (mm)		
Strain	**PDA**	**PDA 1 mM RU**	**PDA 10 mM RU**
Aspergillus affinis FBL 535	77.3 ± 4.3	68.5 ± 1.8	37.2 ± 2.9 * ■
Aspergillus alliaceus FBL 483	86.0 ± 0.0	86.0 ± 0.0	86.0 ± 0.0
Aspergillus flavipes FBL 427	40.0 ± 2.0	26.7 ± 2.3	16.5 ± 2.0 *
Aspergillus ustus FBL 420	77.3 ± 0.4	46.3 ± 3.7 *	8.0 ± 1.0 * ■
Chaetomium globosum FBL 205	51.7 ± 1.4	31.7 ± 1.2	6.0 ± 0.0 * ■
Chaetomium sp. FBL 498	86.0 ± 0.0	84.0 ± 2.0	6.0 ± 0.0 * ■
Chaetomium sp. FBL 499	79.0 ± 5.1	67.3 ± 6.2	6.0 ± 0.0 * ■
Clonostachys rosea FBL 422	85.3 ± 0.7	73.0 ± 0.6 *	23.0 ± 2.1 * ■
Grifola frondosa FBL 450	40.3 ± 2.3	34.5 ± 0.9	6.0 ± 0.0 * ■
Metarhizium marquandii FBL 484	58.5 ± 2.5	32.3 ± 1.3 *	12.2 ± 0.6 * ■
Minimedusa polyspora FBL 503	77.7 ± 5.4	28.3 ± 3.2 *	6.0 ± 0.0 * ■
Mucor sp. FBL 476	86.0 ± 0.0	86.0 ± 0.0	76.0 ± 1.4 * ■
Phycomyces nitens FBL 504	86.0 ± 0.0	86.0 ± 0.0	19.0 ± 4.2 * ■
Pleurotus ostreatus FBL 566	86.0 ± 0.0	81.3 ± 1.2	6.0 ± 0.0 * ■
Purpureocillium lilacinum FBL 478	72.5 ± 0.6	65.0 ± 2.1	35.3 ± 2.3 *
Trametes hirsuta FBL 564	86.0 ± 0.0	77.3 ± 1.5	6.0 ± 0.0 * ■
Trametes versicolor FBL 565	86.0 ± 0.0	82.8 ± 1.4	6.0 ± 0.0 * ■
Trichoderma sp. FBL 650	86.0 ± 0.0	86.0 ± 0.0	61.5 ± 2.0 * ■

[a] Asterisks (*) denote a significant difference between the treatments and the control while squares (■) denote a significant difference between the treatments (post hoc Conover test, $p < 0.05$).

Table 5.3 Tolerance index (Rt:Rc) of the tested species exposed to Roundup on PDA.

R_t:R_c (%) Tolerance Index		
Strain	**1 mM RU**	**10 mM RU**
Aspergillus affinis FBL 535	87.6	43.7
Aspergillus alliaceus FBL 483	100.0	100.0
Aspergillus flavipes FBL 427	60.8	30.9
Aspergillus ustus FBL 420	56.6	2.8
Chaetomium globosum FBL 205	56.2	0.0
Chaetomium sp. FBL 498	97.5	0.0
Chaetomium sp. FBL 499	84.0	0.0
Clonostachys rosea FBL 422	84.5	21.4
Grifola frondosa FBL 450	83.0	0.0
Metarhizium marquandii FBL 484	50.2	11.7
Minimedusa polyspora FBL 503	31.2	0.0
Mucor sp. FBL 476	100.0	87.5
Phycomyces nitens FBL 504	100.0	16.3
Pleurotus ostreatus FBL 566	94.2	0.0
Purpureocillium lilacinum FBL 478	88.7	44.1
Trametes hirsuta FBL 564	89.2	0.0
Trametes versicolor FBL 565	96.0	0.0
Trichoderma sp. FBL 650	100.0	69.4

5.3.2 SCREENING OF FUNGAL STRAINS FOR THEIR ABILITY TO UTILISE GLYPHOSATE AS NUTRITIONAL SOURCE OF C OR P

All tested species were able to grow in all treatments on CDA C− (**Table 5.4** and **Table 5.5**). However, all the tested species were significantly affected by the presence of 10 mM RU in comparison with control ($p < 0.05$). *A. alliaceus* FBL 483, *A. flavipes* FBL 427, *Mucor* sp. FBL 476, *P. nitens* FBL 504, and *Trichoderma* sp. FBL 650 were not significantly affected by the presence of 1 mM RU in comparison with the control ($p < 0.05$). The growth of all tested species in the 10 mM RU treatment was significantly affected in comparison with 1 mM RU ($p < 0.05$). The calculated R_t:R_c values for 1 mM RU were higher than 70% for all tested species with the only exception of *A. affinis* FBL 535 and *C. rosea* FBL 422, disclosing high tolerance.

Table 5.4 Diametric values of fungal colonies after fourteen days of growth at 25 °C on Czapeck dox agar (CDA) in enrichment conditions either for P (CDA P−) or C (CDA C−). The data are expressed as the mean ± standard error of independent biological replicates a.

	Diameter (mm)						
Strain	**CDA**	**CDA P−**	**CDA P− 1 mM RU**	**CDA P− 10 mM RU**	**CDA C−**	**CDA C− 1 mM RU**	**CDA C− 10 mM RU**
Aspergillus affinis FBL 535	52.7 ± 0.3	62.7 ± 0.9	60.0 ± 0.0	33.2 ± 1.6 *• ■	35.8 ± 1.4 *	38.5 ± 3.8 *	21.3 ± 2.2 *• ■
Aspergillus alliaceus FBL 483	81.2 ± 0.2	80.3 ± 0.2	81.3 ± 0.3	61.8 ± 2.3 *• ■	77.0 ± 2.0	72.0 ± 1.0	52.3 ± 0.3 *• ■
Aspergillus flavipes FBL 427	39.7 ± 0.9	32.7 ± 0.3	32.0 ± 0.0	11.7 ± 0.7 *• ■	55.3 ± 2.9 *	34.7 ± 0.7•	6.0 ± 0.0 *• ■
Aspergillus ustus FBL 420	64.2 ± 1.6	46.5 ± 1.3 *	54.8 ± 1.7	12.8 ± 1.6 *• ■	77.7 ± 1.3 *	49.8 ± 0.4 *•	7.2 ± 1.2 *• ■
Clonostachys rosea FBL 422	83.0 ± 0.6	81.3 ± 0.9	78.2 ± 0.6	19.2 ± 0.6 *• ■	82.3 ± 0.9	38.0 ± 0.9 *•	9.5 ± 0.3 *• ■
Metarhizium marquandii FBL 484	71.3 ± 2.7	69.2 ± 1.1	49.5 ± 0.5 *•	13.3 ± 0.2 *• ■	64.7 ± 2.2	52.7 ± 2.9 *	12.0 ± 0.3 *• ■
Mucor sp. FBL 476	86.0 ± 0.0	86.0 ± 0.0	86.0 ± 0.0	28.2 ± 1.8 *• ■	86.0 ± 0.0	86.0 ± 0.0	12.7 ± 0.4 *• ■
Phycomyces nitens FBL 504	86.0 ± 0.0	86.0 ± 0.0	86.0 ± 0.0	7.3 ± 0.3 *• ■	86.0 ± 0.0 *	82.0 ± 2.6 •	6.0 ± 0.0 *• ■
Purpureocillium lilacinum FBL 478	69.7 ± 0.3	70.0 ± 0.0	57.3 ± 0.7 *•	33.8 ± 0.4 *• ■	66.5 ± 0.0	54.3 ± 0.2 *	28.5 ± 0.6 *• ■
Trichoderma sp. FBL 650	86.0 ± 0.0	86.0 ± 0.0	86.0 ± 0.0	39.7 ± 0.3 *• ■	86.0 ± 0.0	86.0 ± 0.0	21.3 ± 0.7 *• ■

[a] Asterisks (*) denote a significant difference between the negative control or RU treatments, and the control; dots (•) denote a significant difference between the RU treatments and the negative control; and squares (■) denote a significant difference between 1 and 10 mM RU treatments in the same cultural medium (post hoc Conover test, $p < 0.05$).

Table 5.5 Tolerance index (Rt:Rc) of the tested species exposed to Roundup as the only source of P or C on enriched CDA.

	CDA P−		CDA C−	
Strain	**1 mM RU**	**10 mM RU**	**1 mM RU**	**10 mM RU**
Aspergillus affinis FBL 535	115.7	58.2	69.6	32.9
Aspergillus alliaceus FBL 483	100.2	74.3	87.8	61.6
Aspergillus flavipes FBL 427	77.2	16.8	96.1	0.0
Aspergillus ustus FBL 420	84.0	11.7	75.6	2.0
Clonostachys rosea FBL 422	94.3	17.2	41.6	4.5
Metarhizium marquandii FBL 484	66.6	11.2	71.4	9.2
Mucor sp. FBL 476	100.0	27.7	100.0	8.3
Phycomyces nitens FBL 504	100.0	1.7	95	0.0
Purpureocillium lilacinum FBL 478	80.6	43.7	75.9	35.3
Trichoderma sp. FBL 650	100.0	42.1	100.0	19.2

All tested species were also able to grow in both RU treatments on CDA P− (**Table 5.4** and **Table 5.5**). Although the diametric growth of all species was significantly reduced in the treatment with 10 mM RU in comparison with the control ($p < 0.05$), no significant differences in fungal growth in the treatment with 1 mM RU in comparison with the control were observed. With the only exception of *M. marquandii* FBL 484, all R_t:R_c values for 1 mM RU were higher than 70%. The growth of all tested species in the 10 mM RU treatment was significantly reduced in comparison with the 1 mM RU treatment ($p < 0.05$). Tolerance indices for *A. affinis* FBL 535 and *A. alliaceus* FBL 483 at 1 mM RU were higher than 100%, suggesting a stimulation effect due to the presence of glyphosate.

In both the negative controls CDA C− and CDA P−, fungi were able to grow, probably exploiting agar as nutritional source (**Table 5.4** and **Table 5.5**, **Figure 5.1**). However, it is worthwhile to note that the fungal mycelia were morphologically different from the control and, in most of cases, explorative (**Figure 5.1**).

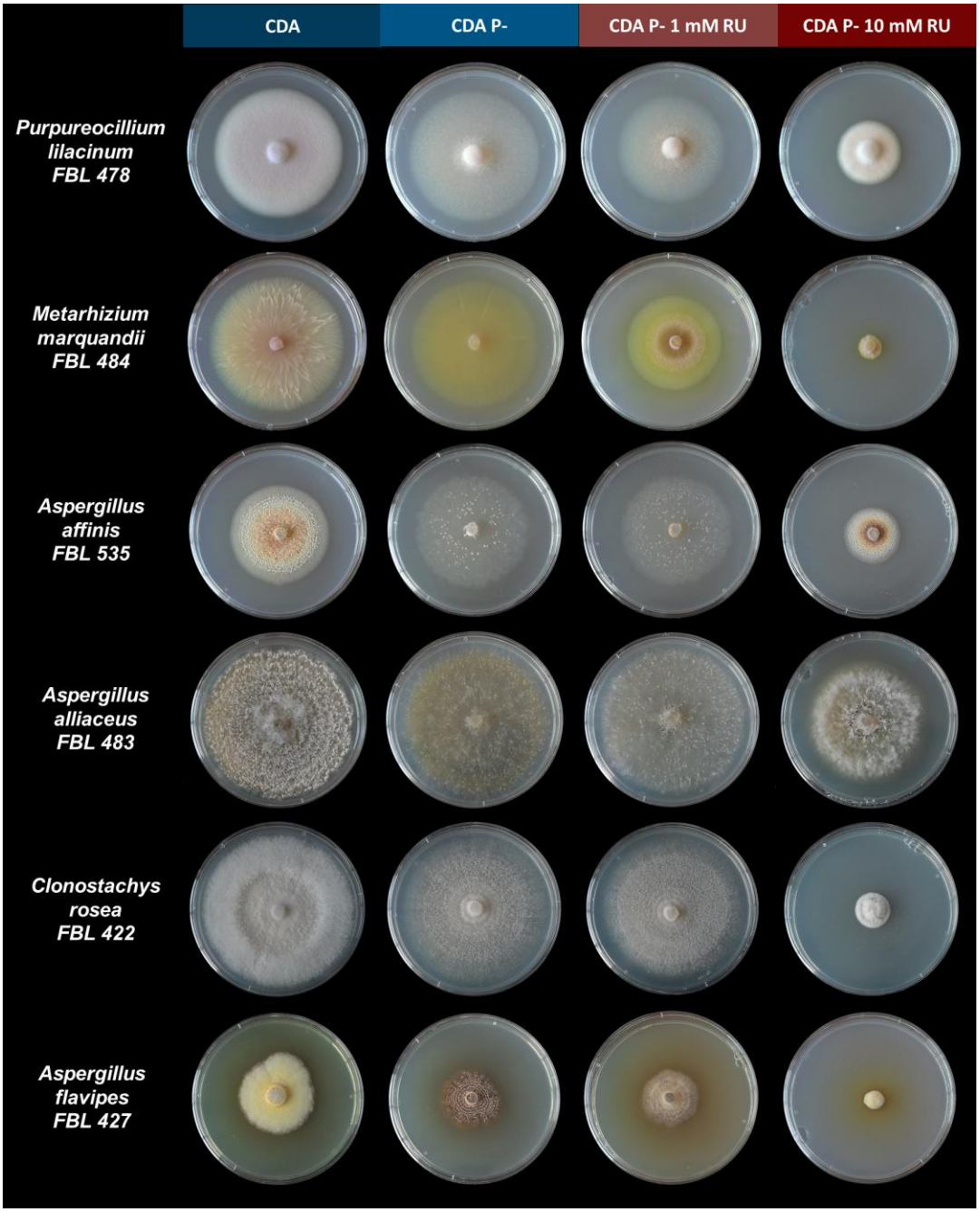


Figure 5.1 Morphological characteristics of selected strain colonies in the controls on Czapek Dox Agar (CDA), negative controls on Czapek Dox Agar without phosphorus (CDA P−), 1 mM RU treatment on Czapek Dox Agar without phosphorus (CDA P− 1 mM RU), and 1 mM RU treatment on Czapek Dox Agar without phosphorus (CDA P− 10 mM RU) at 14 days of incubation.

5.3.3 SCREENING OF *PURPUREOCILLIUM LILACINUM* FOR GLYPHOSATE AND ROUNDUP BREAKDOWN AND UTILISATION AS A P SOURCE IN LIQUID CULTURE MEDIUM

5.3.3.1 EVALUATION OF FUNGAL GROWTH THROUGH BIOMASS PRODUCTION AND INHIBITION INDEX

The dry weight values of *P. lilacinum* FBL 478 after four weeks of growth and the inhibition index (%) values are shown in **Table 5.6**. The data showed that the presence of glyphosate or Roundup significantly reduced biomass production in comparison with the control ($p < 0.05$), inducing an inhibition of fungal growth ranging from 39.3% to 84.1%. While both RU and GLY caused relevant reductions in biomass production at 1 mM concentration, the dry weight in the latter was significantly lower ($p < 0.001$) and the lowest overall for all treatments ($p < 0.05$). By determining an 84.1% inhibition, GLY was way more toxic than RU, which caused a 58.2% inhibition. The dry weights of the CDB P− + 1 mM RU and CDB P− + 10 mM RU treatments were not significantly different; therefore, no dose-depending response in terms of fungal growth was observed for RU. However, CDB P− + 10 mM RU showed higher dry weight values and a lower inhibition index compared with CDB P− + 1 mM RU. Moreover, these treatments' dry weights were higher than those of the negative control ($p < 0.05$), indicating the use of RU as a P source.

Table 5.6 Inhibition index and dry weights of fungal biomass after four weeks of growth at 25 °C in liquid Czapeck dox medium (CDB) enrichment condition without phosphorus (CDB P−) at a concentration of 1mM both pure glyphosate (GLY) and RU. The data for dry weights (g) are expressed as the mean ± standard error of biological independent replicates.

Treatment	Dry Weight (g)	Inhibition Index (%)
CDB 478	0.0241 ± 0.0013 ^{A}	–
CDB P− 478	0.0065 ± 0.0004 ^{B}	–
CDB P− + 1 mM GLY 478	0.0038 ± 0.0004 ^{C}	84.1
CDB P− + 1 mM RU 478	0.0101 ± 0.0002 ^{D}	58.2
CDB P− + 10 mM RU 478	0.0142 ± 0.0016 ^{E}	41.0
CDB + 10 mM RU 478	0.0146 ± 0.0007 ^{E}	39.3

The same superscript letters denote no significant difference between values (post hoc Dunnet T3 test, $p > 0.05$).

Furthermore, no difference between treatment CDB P− + 10 mM RU and CDB + 10 mM RU was observed; therefore, the difference in nutritional condition (complete medium+ RU/RU enrichment) did not affect the biomass production, showing very close inhibition percentages.

In general, all treatments in CDB P− showed lower biomass production than the control (CBD), and 1 mM GLY was even lower than negative control (CBD P−) ($p < 0.05$).

5.3.3.2 EVALUATION OF THE PH OF MEDIUM MODIFICATION DURING 4 WEEKS OF INCUBATION

The culture media pH values during the 4 weeks of incubation are shown in **Table 5.7**. All pH values of the chemical controls remained stable at their initial values during the four weeks.

In the first week, *P. lilacinum* was able to increase the pH of CDB− to 6.6 compared with its chemical control (5.0) ($p < 0.05$), while no variation was observed in the CBD pH. Instead, during the second week, *P. lilacinum* lowered the CDB pH value to 4.7 while increased it to 5.5 and 5.8 during the third and fourth weeks, respectively. In all of the other treatments, *P. lilacinum* increased the pH of the medium during the four weeks of growth. *P. lilacinum* in the CDB P− + 1 mM GLY treatment progressively increased the pH in comparison with its chemical control (3.1) up to pH 4.5 at the fourth week ($p < 0.05$); however, this treatment showed the lowest pH values in all 4 weeks compared with all the other treatments ($p < 0.05$). The low pH of this treatment's medium was caused by glyphosate addition to a medium without phosphate or another buffering agent. The addition of Roundup also acidified the medium of the RU treatments, but the pH values were ≥ 4.5, probably due to the presence of additives with buffering action. In fact, the pH values of the CDB P− + 1 mM RU, CDB P− + 10 mM RU, and CDB + 10 mM RU chemical controls were, respectively, 4.6, 4.5, and 4.7 and were statistically different from their respective treatments inoculated with *P. lilacinum*. In both the treatments at 10 mM RU, *P. lilacinum* progressively increased the pH of the medium up to 5.8 and to 5.7, respectively, in the CDB P− + 10 mM RU and CDB + 10 mM RU treatments. It is interesting to note that, despite almost the same starting pH value, *P. lilacinum* in the CDB P− + 1 mM RU showed a statistically significantly higher increase in the medium's pH compared with the CDB P− + 10 mM RU treatment, reaching a pH value of 7.1 by the fourth week.

At the fourth week, no differences in the medium's pH between CDB− (pH = 7.1) and CDB− with 1 mM RU (pH = 7.2) and between CDB− with 10 mM RU (pH = 5.8) and CDB with 10 mM RU (pH = 5.7) were observed. In all of these cases, *P. lilacinum* increased the pH medium by about one unit in 4 weeks.

Table 5.7 The pH values for the medium after fungal growth for 1, 2, 3, and 4 weeks at 25 °C and the P concentration in the medium. The data are expressed as the mean ± standard error of independent replicates [a].

pH				
Treatment	**7 Days**	**14 Days**	**21 Days**	**28 Days**
CDB 478	5.7 ± 0.0 ^{A}	4.7 ± 0.0 ^{A}	5.5 ± 0.1 ^{A}	5.8 ± 0.0 ^{A}
CDB P− 478	6.6 ± 0.1 ^{B}	6.6 ± 0.0 ^{B}	6.8 ± 0.1 ^{B}	7.2 ± 0.0 ^{B}
CDB P− + 1 mM GLY 478	3.4 ± 0.0 ^{C}	3.6 ± 0.1 ^{C}	4.1 ± 0.3 ^{C}	4.5 ± 0.1 ^{C}
CDB P− + 1 mM RU 478	6.0 ± 0.0 ^{A}	6.4 ± 0.1 ^{B}	6.9 ± 0.1 ^{B}	7.1 ± 0.1 ^{B}
CDB P− + 10 mM RU 478	4.9 ± 0.0 ^{D}	5.1 ± 0.0 ^{D}	5.6 ± 0.0 ^{A}	5.8 ± 0.0 ^{A}
CDB + 10 mM RU 478	4.9 ± 0.0 ^{D}	5.1 ± 0.0 ^{D}	5.5 ± 0.1 ^{A}	5.7 ± 0.1 ^{A}
CDB Control	5.7 ± 0.0 ^{A}	5.7 ± 0.0 ^{E}	5.7 ± 0.0 ^{A}	5.7 ± 0.0 ^{A}
CDB P− Control	5.0 ± 0.1 ^{D}	5.0 ± 0.0 ^{D}	5.1 ± 0.2 ^{D}	4.9 ± 0.0 ^{C}
CDB P− + 1 mM GLY Control	3.1 ± 0.0 ^{E}	3.1 ± 0.0 ^{F}	3.1 ± 0.0 ^{E}	3.1 ± 0.0 ^{D}
CDB P− + 1 mM RU Control	4.6 ± 0.0 ^{FG}	4.5 ± 0.0 ^{G}	4.6 ± 0.0 ^{C}	4.5 ± 0.0 ^{C}
CDB P− + 10 mM RU Control	4.5 ± 0.0 ^{F}	4.5 ± 0.0 ^{G}	4.5 ± 0.0 ^{C}	4.5 ± 0.0 ^{C}
CDB + 10 mM RU Control	4.7 ± 0.0 ^{G}	4.7 ± 0.0 ^{A}	4.7 ± 0.0 ^{C}	4.6 ± 0.0 ^{C}
Liquid Medium P Content (mg/L)				
Treatment	**7 Days**	**14 Days**	**21 Days**	**28 Days**
CDB 478	195.3 ± 1.7 ^{A}	195.4 ± 4.2 ^{A}	195.9 ± 2.8 ^{A}	195.9 ± 6.4 ^{A}
CDB P− 478	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CDB P− + 1 mM GLY 478	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CDB P− + 1 mM RU 478	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CDB P− + 10 mM RU 478	0.5 ± 0.1 ^{B}	0.4 ± 0.0 ^{B}	0.2 ± 0.0 ^{B}	0.0 ± 0.0
CDB + 10 mM RU 478	183.8 ± 3.3 ^{A}	185.4 ± 2.5 ^{A}	186.7 ± 4.2 ^{A}	184.7 ± 4.1 ^{A}
CDB Control	204.0 ± 8.3 ^{A}	207.6 ± 5.4 ^{A}	206.0 ± 8.8 ^{A}	204.3 ± 2.0 ^{A}
CDB P− Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CDB P− + 1 mM GLY Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CDB P− + 1 mM RU Control	0.2 ± 0.0 ^{C}	0.2 ± 0.0 ^{C}	0.2 ± 0.0 ^{B}	0.2 ± 0.0 ^{B}
CDB P− + 10 mM RU Control	0.8 ± 0.0 ^{D}	0.8 ± 0.0 ^{D}	0.9 ± 0.1 ^{C}	0.8 ± 0.1 ^{C}
CDB + 10 mM RU Control	194.0 ± 2.0 ^{A}	198.0 ± 6.6 ^{A}	194.3 ± 11.7 ^{A}	194.6 ± 5.9 ^{A}

[a] The same superscript letters denote no significant difference between values within the same timepoint column (post hoc Dunnet T3 test or Conover test, $p > 0.05$).

5.3.3.3 SPECTROPHOTOMETRIC DETERMINATION OF AVAILABLE P IN THE CULTURE MEDIUM

The phosphorus concentrations in the culture media during the four weeks of incubation are reported in **Table 5.7**. Overall, in none of the biological treatments with *P. lilacinum* was an increase in P concentration observed. In the CDB P− and CDB P− + 1 mM GLY treatments' chemical controls, no P concentration was detected, while negligible P concentrations were detected in the CDB P− + 1 mM RU and CDB P− + 10 mM RU treatments' chemical controls (respectively, 0.2 and 0.8 mg/L), likely due to the P traces present in RU. The P concentrations in treatments' chemical controls remained stable during the 4 weeks of incubation. *P. lilacinum* biological control CDB 478 samples showed lower P concentration (195 mg/L P) in comparison with the chemical control (204 mg/L P). An analogous P concentration reduction of about 10 mg/L was observed in CDB + 10 mM RU 478 (184 mg/L P) compared with the respective chemical control (194 mg/L P); however, in both cases, the differences were not statistically significant. Interestingly, the P concentration detected in these biological treatments compared with those of the chemical control showed a decrease by the first week, and later on, during the following three weeks, no further reduction was observed. No P was detected in the culture media of treatments CDB P−, CDB P− + 1 mM GLY and CDB P− + 1 mM RU inoculated with *P. lilacinum*.

CDB P− + 10 mM RU inoculated with *P. lilacinum*, despite showing very low P concentration, was the only treatment in which a progressive reduction in P concentration occurred during the four weeks of incubation.

5.3.3.4 HPLC–MS GLYPHOSATE DEGRADATION ANALYSIS OF P. LILACINUM SCREENING IN LIQUID CULTURE MEDIUM

The quantitative analysis of CDB media with or without phosphorus, both at 10 mM in commercial glyphosate (Roundup), was conducted on different aliquots at time zero (not inoculated sample) and once a week after the inoculation of *P. lilacinum*. As represented in **Figure 5.2**, the degradation of glyphosate, in which total loss reached 80%, occurred within the first week from the cultural media inoculation and then remained constant until the fourth week. A similar trend was also obtained for the samples at a 1 mM GLY/RU concentration.

Identification of the possible metabolites (sarcosine, AMPA, glycine (Fu et al., 2017)) in real samples was carried out working in HPLC–Q1 full scan; however, it was particularly challenging due to their polarity, low masses, and low concentrations occurring in highly complex matrices. The extracted ion chromatogram at 90.1 m/z showed a chromatographic peak of low intensity, which matched the retention time of

the standard solution of sarcosine; the latter was identified as the only metabolite in the samples starting from the third week after fugal inoculation.

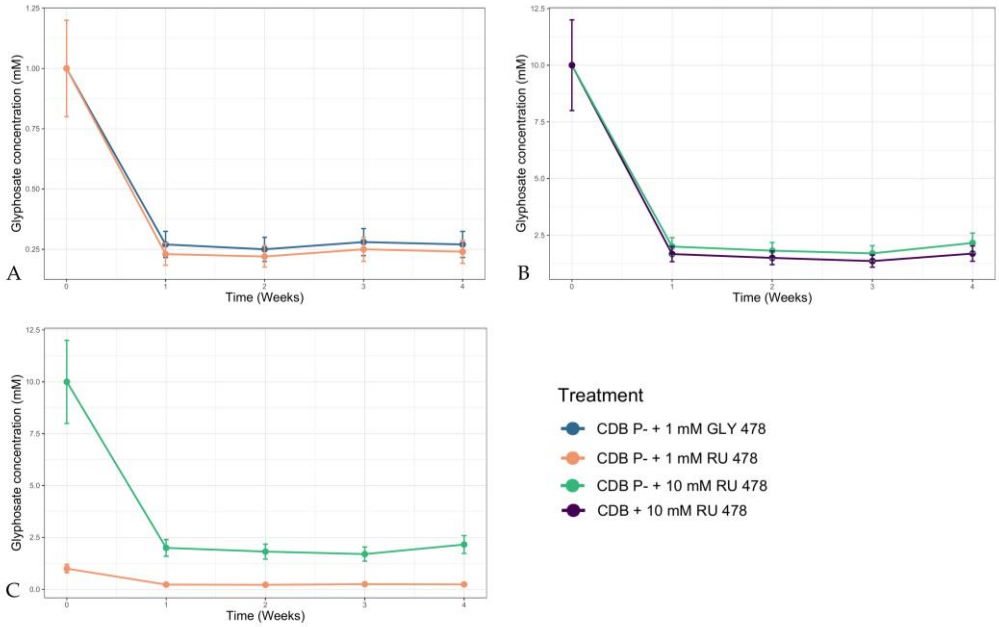


Figure 5.2 Glyphosate concentration (mM) during the four weeks of incubation: (A) glyphosate concentration trend in CDB P− + 1 mM GLY 478 and CDB P− + 1 mM RU 478, (B) glyphosate concentration trend in CDB P− + 10 mM RU 478 and CDB + 10 mM RU 478, and (C) glyphosate concentration trend in CDB P− + 10 mM RU 478 and CDB P− + 1 mM RU 478.

5.3.4 SCREENING OF THE INFLUENCE OF THE MEDIUM'S PH ON GLYPHOSATE AND ROUNDUP BREAKDOWN BY *PURPUREOCILLIUM LILACINUM*

5.3.4.1 EVALUATION OF FUNGAL GROWTH IN BUFFERED MEDIA

The dry weight values of *P. lilacinum* FBL 478 after two weeks of growth are shown in **Table 5.8**. CDB + TRIS showed higher dry weights than CDB ($p < 0.05$). The addition of glyphosate or Roundup affected the fungal growth significantly, causing lower dry weights compared with the control ($p < 0.05$) and therefore a percentage of inhibition ranging from 31.6% to 77.2%. The dry weight in CDB P− + 1 mM GLY + TRIS was found to be significantly higher than in CDB P− + 1 mM GLY ($p < 0.05$), and inhibition in the treatment buffered with TRIS showed lower values (60.6%) than in the treatment without it (77.2%). Even though dry weights in CDB P− + 1 mM RU + TRIS showed slightly higher values than those in CDB P− + 1 mM RU, these two treatments were not statistically different. Moreover, 1 mM RU treatments did not show a statistically

significant difference when compared with CDB P− + 1 mM GLY + TRIS. Finally, no statistically significant differences between CDB P− and CDB P− + TRIS as well as between these treatments and those with 1 mM GLY were observed. These findings were confirmed also by the two-way ANOVA, revealing a statistically significant interaction between the buffer addition and the treatment influencing dry weights in all treatment with the exception of P− + 1mM RU.

5.3.4.2EVALUATION OF THE MODIFICATION OF THE MEDIUM'S PH AFTER TWO WEEKS OF INCUBATION IN BUFFERED MEDIA

The pH values of the medium after two weeks of growth are shown in **Table 5.8**. CDB and CDB + TRIS pH after *P. lilacinum* growth resulted in lower values than their chemical controls ($p < 0.05$). In CDB P−, *P. lilacinum* caused a basification of the medium ($p < 0.05$), while in CDB P− + TRIS, an acidification ($p < 0.05$). It is interesting to note that considering two different starting pH, respectively 4.8 and 7.2, the final pH in both treatments was almost the same (6.7 and 6.8) showing no significant differences. In CDB P− + 1 mM GLY the fungal growth caused a significant decrease in the medium pH when compared with its respective chemical control. Instead, the pH of CDB P− + 1 mM GLY chemical control (3.1) resulted in significantly lower values than those of the respective biological treatment (3.8) ($p < 0.05$). This observed pH value was very close to 3.6, i.e., the pH observed at the second week of growth in the previous experiment (Table 7). All treatments buffered with TRIS, showed pH ≥ 6.8 in chemical controls, despite the addition of GLY or RU. A similar pattern to that observed for GLY was observed also for RU. In fact, in CDB P− + 1 mM RU *P. lilacinum* increased the pH of the medium, while in CDB P− + 1 mM RU+ TRIS, caused an acidification. No statistical difference between the pH of the biological treatments in CDB P− + 1 mM GLY and CDB P− + 1 mM RU was observed.

5.3.4.3 SPECTROPHOTOMETRIC DETERMINATION OF AVAILABLE P IN BUFFERED CULTURE MEDIA

Phosphorus concentration in culture media after two weeks of fungal growth are reported in **Table 5.8**. No P was detected in CDB P− and CDB P− + 1 mM RU + TRIS, while very low P concentrations, probably due to P traces in RU and TRIS, were detected in CDB P− + TRIS, CDB P− + 1 mM GLY, CDB P− + 1 mM GLY + TRIS and CDB P− + 1 mM RU chemical controls. In the abovementioned treatments inoculated with *P. lilacinum* no P was detected. *P. lilacinum* caused a reduction in P concentration in CDB from 194 mg/L in the control to 188 mg/L in the respective treatment and from 195 mg/L to 182 mg/L in CDB + TRIS.

Table 5.8 Values of dry weights, inhibition index, medium pH, and P concentration after fungal growth for 2 weeks at 25 °C in different cultural conditions with or without tris(hydroxymethyl)aminomethane (TRIS) buffer. The data are expressed as the mean ± standard error of independent replicates [a].

	Dry Weight (g)	Inhibition Index (%)	pH	mg/L P
CDB 478	0.0182 ± 0.0010 [A]	–	4.8 ± 0.09 [A]	187.9 ± 3.7 [A]
CDB + TRIS 478	0.0238 ± 0.0009 [B]	–	5.9 ± 0.00 [B]	181.9 ± 3.7 [A]
CDB P− 478	0.0075 ± 0.0017 [CD]	–	6.7 ± 0.02 [C]	0.0 ± 0.0
CDB P− + TRIS 478	0.0029 ± 0.0010 [E]	–	6.8 ± 0.00 [D]	0.0 ± 0.0
CDB P− + 1 mM GLY 478	0.0041 ± 0.0005 [C]	77.2	3.7 ± 0.02 [E]	0.0 ± 0.0
CDB P− + 1 mM GLY + TRIS 478	0.0094 ± 0.0006 [DF]	60.6	6.5 ± 0.00 [F]	0.0 ± 0.0
CDB P− + 1 mM RU 478	0.0124 ± 0.0006 [F]	31.6	6.4 ± 0.14 [F]	0.0 ± 0.0
CDB P− + 1 mM RU + TRIS 478	0.0134 ± 0.0007 [F]	43.8	6.0 ± 0.00 [G]	0.0 ± 0.0
CDB Control	–	–	5.6 ± 0.01 [H]	193.9 ± 2.7 [A]
CDB + TRIS Control	–	–	7.2 ± 0.00 [I]	195.1 ± 3.9 [A]
CDB P− Control	–	–	4.8 ± 0.01 [A]	0.0 ± 0.0
CDB P− + TRIS Control	–	–	7.2 ± 0.00 [I]	< LOD[◊]
CDB P− + 1 mM GLY Control	–	–	3.1 ± 0.00 [L]	0.2 ± 0.0 [B]
CDB P− + 1 mM GLY + TRIS Control	–	–	6.8 ± 0.00 [D]	0.2 ± 0.0 [B]
CDB P− + 1 mM RU Control	–	–	4.5 ± 0.01 [M]	< LOD[◊]
CDB P− + 1 mM RU + TRIS Control	–	–	6.8 ± 0.00 [D]	0.0 ± 0.0

[◊] value below limits of detection (LOD). [a] The same superscript letters denote no significant difference between values within the same parameter column (post hoc Tukey, Conover or Dunnet T3 test, $p > 0.05$).

5.4 DISCUSSION

This study evaluated the in vitro ability of several fungal strains to tolerate and utilise Roundup, one of the most used glyphosate's commercial formulation, as a nutritional source. All 18 tested strains were able to tolerate 1mM RU; however, only ten tolerated the 10 mM concentration, suggesting that, in the optimal culture condition, there is a dose-dependent response.

In this study's test condition, none of the strains belonging to the Basidiomycota phylum (*P. ostreatus*, *T. hirsuta*, *T. versicolor*, *G. frondosa*, and *M. polyspora*) were able to tolerate RU at the highest concentration, while all except *M. polyspora* FBL 503 showed high tolerance at 1 mM concentration. In a previous study on glyphosate bioremediation, *P. ostreatus* was not able to degrade glyphosate and tolerated lower glyphosate concentrations compared with the highest one tested in this study (Mygind et al., 2019). Although there are few studies on basidiomycetes' glyphosate tolerance, the bioremediation potential of ligninolytic enzymes they possess have been studied more thoroughly. Laccase from *Pleurotus* sp. were successfully tested with glyphosate (Do et al., 2017) and laccases and other enzymes from *T. versicolor* were positively tested for glyphosate biodegradation (Pizzul et al., 2009). *G. frondosa* and *T. hirsuta* are known to possess ligninolytic enzymes with a potential in the biodegradation of persistent organic pollutants, even though they have not been studied with glyphosate (Abadulla et al., 2000; Nitheranont et al., 2011).

Most of the tested fungi belonging to the Ascomycota and Mucoromycota phyla were able to tolerate glyphosate at a 1 mM concentration, and in particular, *A. alliaceus* and *Mucor* sp. were the only species with high tolerance at 10 mM RU. Strains belonging to the *Mucor* genus have already been reported to be tolerant to glyphosate and use it as C and P sources (Krzyśko-Łupicka and Orlik, 1997), while *A. alliaceus* has not been previously studied for its tolerance to glyphosate. However, considering our results in addition to previous reports on its ability to degrade high concentrations of atrazine, it is possible to affirm that this strain shows high potential in the bioremediation of organic pollutants (Gajendiran et al., 2017). Interestingly, all three strains belonging to the *Chaetomium* genus, despite showing a good tolerance at 1 mM concentration, were not able to tolerate glyphosate at the highest concentration. Considering that, previously, *C. globosum* was tested up to about 4 mM RU with little effect on growth (Grossbard and Harris, 1977), probably, the inhibition occurs at higher concentrations.

The species that tolerated 10 mM RU when tested in enrichment conditions were able to grow without added P and C even at the 10 mM concentration, with the only exception of *A. flavipes* and *P. nitens*, which both did not grow in CDA C− 10 mM RU. However, in both C− and P− treatments, morphological modifications occurred in the treatments with RU compared with the control and among the two treatments. Other than a

diametric growth reduction, morphological modifications were mainly observed in terms of sporulation, pigmentation, and mycelium density. Interestingly, most of the strains at 10 mM concentration, despite a diametric growth reduction, showed a morphological aspect more similar to the control than those of the 1 mM treatment, in terms of pigmentation and mycelium density. However, all of the strains were able to grow on CDA P− and CDA C−, probably exploiting agar as a nutritional source. This is consistent with several other studies on fungi where negative controls, even in water agar, showed a relevant diametric growth (Carranza et al., 2017; Krzysko-Lupicka and Sudol, 2008; Krzyśko-Łupicka and Orlik, 1997). In our study, however, mycelia in negative controls were mostly thin; explorative; and especially in CDA C−, less pigmented, showing visible differences from the RU treatments. Hence, despite plate assay agar representing an interference, making it difficult to unequivocally evaluate glyphosate utilisation as a nutritional source, this assay may surely represent a first-line screening to select promising species for further tests.

A. alliaceus, *A. affinis*, *A. flavipes*, *A. ustus*, *C. rosea*, *M. marquandii*, *M. polyspora*, *G. frondosa*, *T. hirsuta*, *P. nitens*, and *P. lilacinum* are reported for the first time in this study for their ability to tolerate and utilise RU as a nutritional source.

Among the strains showing a good potential and not previously studied for glyphosate remediation, *P. lilacinum* was selected for further testing because of the wide interest in its biotechnological application. In fact, *P. lilacinum*, being an entomopathogenic species, has garnered wide interest for biocontrol application. Furthermore, it has been studied also for plant growth promoting activities; phytopathogen biocontrol; and bioremediation of potentially toxic elements such as As, Cd, Cr, and Pb (Sharma and Adholeya, 2011; Xia et al., 2015; Xu et al., 2017; Zucconi et al., 2003). Moreover, *P. lilacinum* has already been reported to be tolerant to several other herbicides with active ingredients such as Pendimethalin, Pethoxamid, Clomazone, Chlorotoluron, and Imazamox (Ondráčková et al., 2019).

Our data confidently report the tolerance and ability of *P. lilacinum* to degrade glyphosate, exploiting it as a P nutritional source.

An HPLC–MS glyphosate degradation analysis showed this strain's ability to degrade 80% of the initial concentration of glyphosate as pure molecule or commercial formulation within a week. No quantitative differences in the degraded glyphosate or in the produced metabolites were observed between RU and GLY despite the differences in biomass development being consistent. No dose-dependent negative effect was observed in terms of glyphosate degradation considering the 1 mM and 10 mM RU concentration treatments. In fact, both showed 80% degradation of glyphosate initial concentration, with the 10 mM RU treatment also showing a statistically significant higher biomass production even if it was lower than the control in CDB. Considering that no differences

in biodegradation occurred between CDB P− + 10 mM RU and CDB + 10 mM RU, it is possible to deduce that an alternative source of P in the medium does not affect glyphosate degradation. In fact, in CDB, degradation occurred to the same extent as that in CDB P−, where RU represents the only P source. This finding also suggests that the enzymes probably responsible for the glyphosate degradation in *P. lilacinum* are not induced by P starvation as for other microorganisms (Correa et al., 2021; Dick and Quinn, 1995; Zhan et al., 2018).

Among the known glyphosate degradation metabolites, sarcosine was the only one detected in the samples starting from the third week. This finding may suggest that *P. lilacinum* degrades glyphosate through the pathway that involves a C–P bond cleavage, causing the release of a phosphate group and sarcosine, where the latter may be further degraded upon releasing glycine and formaldehyde (Fu et al., 2017). In our samples, glycine has never been detected probably due to either an incomplete pathway or the uptake of sarcosine and/or released glycine by the biomass, since it can be utilised as nutritional sources (Feng et al., 2020; Griffin, 1994). Uptake in biomass may also explain the reason why, despite glyphosate degradation occurring mainly during the first week, sarcosine is not detected before the third week.

The findings of this study are consistent with the results obtained on other fungal species by Adelowo et al. (2014) (*Trichoderma viridae*, *Aspergillus niger*, and *Fusarium oxysporum*) and Correa et al. (2021) (*Penicillium* sp., *Aspergillus* sp., and *Trichoderma* sp.). However, Adelowo and Correa also detected AMPA, the first metabolite reported in another known glyphosate degradation pathway in fungi, in their samples (Adelowo et al., 2014; Correa et al., 2021; Fu et al., 2017). The AMPA pathway involves the cleavage of the C–N bond of glyphosate releasing AMPA as first step of degradation, which can either be degraded to methylamine and phosphate or to phosphoformaldehyde and, later, to formaldehyde (Feng et al., 2020; Fu et al., 2017; Zhan et al., 2018). Therefore, considering their results, it is possible to hypothesise that the same strain may operate at the same time through different pathways.

Interestingly, in none of *P. lilacinum* samples AMPA was detected. Unfortunately, due to the impossibility to detect methylamine with the applied analytical technique, it is not possible to confidently affirm if glyphosate was not degraded to AMPA at all or, if otherwise, it has been completely degraded. Since AMPA has been reported for its toxicity (Correa et al., 2021), the absence of its residues in *P. lilacinum* glyphosate degradation products makes it a particularly suitable candidate for bioremediation applications.

Considering the great potential showed by this strain, additional experiments aimed at further evaluating and confirming the degradation pathway would be valuable. To be effective for this purpose, future experiments would include both analyses of the culture

filtrate and of the fungal biomass, with much shorter intervals between sampling times during the first week.

Despite the biomass growth values in treatments being significantly higher than in the negative control, indicating that P was obtained from glyphosate, no increases in the available P in the culture media were observed. In fact, all treatments, including the control in CDB, showed a decrease in the concentration of available P in the culture media within the first week. An analogous trend of concentration reduction has also been observed by Adelowo et al. (2014) and Afarita et al. (2013, 2014). The lack of available P increase in the medium may be explained by fungal uptake of the P released from glyphosate. It is interesting to note that the biomass produced in CDB P− + 10 mM RU is the same as that produced in CDB + 10 mM RU and that the decrease of P amount in inoculated CDB and CDB + 10 mM RU compared with their respective chemical controls are analogous. Therefore, it is possible to deduce that the presence of glyphosate did not cause a reduction in P uptake in the fungus. Despite providing valuable information on the fungal growth dynamics and validating the starting absence of P in enrichment culture media, the phosphorus concentration in the culture media was not shown to be a reliable proxy of glyphosate degradation.

Finally, our data indicate that, for *P. lilacinum*, GLY is more toxic than RU. Despite the strain being able to show a reduction of 80% in the concentration of both compounds and to exploit them as nutritional source, RU caused a lower reduction in biomass production than GLY in the four-week experiment. In fact, biomass production was strongly affected in the presence of GLY, being lower than in the medium amended with 10 mM RU. Part of this strong biomass production inhibition in GLY may have been linked to the very low pH, determined by the addition of GLY to the medium. In fact, despite a tolerance range of pH 2–10, *P. lilacinum* has an optimal pH of 6.5 (Domsch et al., 2007). This may also explain the lower biomass production even compared with the negative control, where, despite growth being based on spores' P reservoirs, the pH was 5.0 and the fungus was able to increase it to 6.6 within the first week. In the two-week experiment, biomass production in the buffered medium amended with GLY was significantly higher than that in the unbuffered medium. Nevertheless, it did not show any statistically significant difference compared with biomass production in the buffered and unbuffered media amended with RU, hence pointing out pH involvement in higher GLY toxicity. A significant increase in biomass production in the buffered medium amended with GLY compared with the unbuffered one was observed. Therefore, a higher GLY toxicity is surely related to low pH, but there may also be other drivers of toxicity contributing to the final effect. However, this pH-related effect may be due to the test condition in the liquid medium, considering that, in soil, the pH decrease due to the GLY occurrence is counteracted thanks to adsorption and buffering phenomena. Our finding on the higher toxicity of GLY than RU disagrees with what is reported from Morjan et

al. (2002), according to whom several RU formulations, but not GLY, induced growth inhibition in four entomopathogenic fungi (*Beauveria bassiana*, *Metarhizium anisopliae*, *Nomuraea rileyi*, and *Neozygites floridana*). However, these opposing results may be due to the differences in test conditions, since Morjan et al. tested in a solid medium with the disk diffusion method, which could have prevented the lowering of medium's pH and therefore its relative effects (Morjan et al., 2002).

5.5 CONCLUSIONS

This study reports several strains not previously studied for their ability to tolerate and utilise glyphosate as a nutritional source, contributing to widen the knowledge on fungal strains representing potential bioresources for glyphosate bioremediation.

P. lilacinum is reported for the first time for its ability to degrade glyphosate to a considerable extent (80%) and to utilise it as P source. The *P. lilacinum*'s degradation performance was not affected by the increase in RU concentration; in fact, the higher amount of RU did not cause negative effects and even promoted a higher biomass production. Neither did the presence of alternative P sources cause a reduction in glyphosate degradation differently from what is reported in the literature for other microorganisms. Finally, in this study, it was observed that GLY is more toxic than RU for *P. lilacinum*, even though its toxicity is mainly determined by the pH reduction in culture media.

As pointed out from this study's results, *P. lilacinum* represents a great candidate for the development of biotechnological applications for glyphosate remediation to support sustainable soil restoration in agricultural lands.

5.6 REFERENCES

Abadulla, E., Tzanov, T., Costa, S., Robra, K.-H., Cavaco-Paulo, A., and Gübitz, G.M. (2000). Decolorization and Detoxification of Textile Dyes with a Laccase from *Trametes hirsuta*. Applied and Environmental Microbiology*66*, 3357–3362.

Abhilash, P.C. (2021). Restoring the Unrestored: Strategies for Restoring Global Land during the UN Decade on Ecosystem Restoration (UN-DER). Land *10*, 201.

Adelowo, F.E., Olu-Arotiowa, O.A., and Amuda, O.S. (2014). Biodegradation of Glyphosate by Fungi Species. Advances in Bioscience and Bioengineering *2*.

Akhtar, N., and Mannan, M.A. (2020). Mycoremediation: Expunging environmental pollutants. Biotechnology Reports *26*, e00452.

Antier, C., Andersson, R., Auskalnienė, O., Barić, K., Baret, P., Besenhofer, G., Calha, I., Carrola Dos Santos, S., De Cauwer, B., Chachalis, D., et al. (2020). A survey on the uses of glyphosate in European countries. INRAE. Https://Doi.Org/10.15454/A30K-D531.

Arfarita, N., Imai, T., Kanno, A., Yarimizu, T., Xiaofeng, S., Jie, W., Higuchi, T., and Akada, R. (2013). The Potential use of *Trichoderma Viride* Strain FRP3 in Biodegradation of the Herbicide Glyphosate. Biotechnology & Biotechnological Equipment *27*, 3518–3521.

Arfarita, N., Imai, T., and Prasetya, B. (2014). Potential Use of Soil-born Fungi Isolated From Treated Soil in Indonesia to Degrade Glyphosate Herbicide. Journal of Degraded and Mining Lands Management *1*, 63–68.

Arfarita, N., Djuhari, D., Prasetya, B., and Imai, T. (2016). The application of *Trichoderma viride* strain frp 3 for biodegradation of glyphosate herbicide in contaminated land. AGRIVITA, Journal of Agricultural Science *38*, 275–281.

Baron, N.C., Souza Pollo, A. de, and Rigobelo, E.C. (2020). *Purpureocillium lilacinum* and *Metarhizium marquandii* as plant growth-promoting fungi. PeerJ *8*, e9005.

Beckie, H.J., Flower, K.C., and Ashworth, M.B. (2020). Farming without Glyphosate? Plants *9*, 96.

Bhatt, V.K., and Iyer, B.D. (2021). A new spectrophotometric method for the determination of glyphosate: statistical optimization and application in biodegradation studies. Int. J. Environ. Sci. Technol. *18*, 997–1008.

Böcker, T., Britz, W., Möhring, N., and Finger, R. (2019). An economic and environmental assessment of a glyphosate ban for the example of maize production. European Review of Agricultural Economics. European Review of Agricultural Economics 47, 371–402

Carranza, C.S., Barberis, C.L., Chiacchiera, S.M., and Magnoli, C.E. (2014). Influence of the pesticides glyphosate, chlorpyrifos and atrazine on growth parameters of nonochratoxigenic *Aspergillus* section *Nigri* strains isolated from agricultural soils. Journal of Environmental Science and Health, Part B *49*, 747–755.

Carranza, C.S., Barberis, C.L., Chiacchiera, S.M., and Magnoli, C.E. (2017). Assessment of growth of *Aspergillus* spp. from agricultural soils in the presence of glyphosate. Revista Argentina de Microbiología *49*, 384–393.

Cavello, I.A., Crespo, J.M., García, S.S., Zapiola, J.M., Luna, M.F., and Cavalitto, S.F. (2015). Plant Growth Promotion Activity of Keratinolytic Fungi Growing on a Recalcitrant Waste Known as "Hair Waste." Biotechnology Research International *2015*, 1–10.

Ceci, A., Pinzari, F., Russo, F., Persiani, A.M., and Gadd, G.M. (2019). Roles of saprotrophic fungi in biodegradation or transformation of organic and inorganic pollutants in co-contaminated sites. Applied Microbiology and Biotechnology *103*, 53–68.

Ceci, A., Spinelli, V., Massimi, L., Canepari, S., and Persiani, A.M. (2020). Fungi and Arsenic: Tolerance and Bioaccumulation by Soil Saprotrophic Species. Applied Sciences *10*, 3218.

Clapp, J. (2021). Explaining Growing Glyphosate Use: The Political Economy of Herbicide-Dependent Agriculture. Global Environmental Change *67*, 102239.

Correa, L.O., Bezerra, A.F.M., Honorato, L.R.S., Cortez, A.C.A., Souza, J.V.B., and Souza, E.S. (2021). Amazonian soil fungi are efficient degraders of glyphosate herbicide; novel isolates of *Penicillium*, *Aspergillus*, and *Trichoderma*. Braz. J. Biol. *83*.

Curutiu, C., Lazar, V., and Chifiriuc, M.C. (2017). Pesticides and antimicrobial resistance: from environmental compartments to animal and human infections. In New Pesticides and Soil Sensors, (Cambridge, MA: Academic Press Elsevier), pp. 373–392.

Dick, R.E., and Quinn, J.P. (1995). Glyphosate-degrading isolates from environmental samples: occurrence and pathways of degradation. Applied Microbiology and Biotechnology *43*, 545–550.

Do, L.T.T., Le, P.H.D., Nguyen, Q.M., and Luong, U.B. (2017). Solid-state production of laccase by *Pleurotus* sp. applied in glyphosate degradation. Sci. Tech. Dev. J. *20*, 147–151.

Domsch, K.H., Gams, W., and Anderson, T.-H. (2007). Compendium of soil fungi (Eching, Germany: IHW).

Elsherbiny, E.A., Taher, M.A., Abd El-Aziz, M.H., and Mohamed, S.Y. (2021). Action mechanisms and biocontrol of *Purpureocillium lilacinum* against green mould caused by *Penicillium digitatum* in orange fruit. J Appl Microbiol jam.15016.

Eman, A., Abdel-Megeed, A., Suliman, A., Sadik, M., and Sholkamy, E.N. (2013). Biodegradation of glyphosate by fungal strains isolated from herbicides polluted-soils in Riyadh area. British Journal of Environmental Sciences *1*, 7–29.

Espinoza-Montero, P.J., Vega-Verduga, C., Alulema-Pullupaxi, P., Fernández, L., and Paz, J.L. (2020). Technologies Employed in the Treatment of Water Contaminated with Glyphosate: A Review. Molecules *25*, 5550.

FAO and UNEP (2021). Global assessment of soil pollution: Report (Rome, Italy: FAO and UNEP).

FAO, ITPS, GSBI, CBD, and EC (2020). State of knowledge of soil biodiversity - Status, challenges and potentialities, Report 2020 (Rome, Italy: FAO, ITPS, GSBI, CBD and EC.).

Feng, D., Soric, A., and Boutin, O. (2020). Treatment technologies and degradation pathways of glyphosate: A critical review. Science of The Total Environment *742*, 140559.

Fu, G., Chen, Y., Li, R., Yuan, X., Liu, C., Li, B., and Wan, Y. (2017). Pathway and rate-limiting step of glyphosate degradation by *Aspergillus oryzae* A-F02. Preparative Biochemistry and Biotechnology *47*, 782–788.

Gadd, G.M. (2017). The Geomycology of Elemental Cycling and Transformations in the Environment. Microbiology Spectrum *5*, 1–16.

Gajendiran, A., Vijayavenkatesan, V., and Abraham, J. (2017). Bioremediation of Herbicide Atrazine by Fungal sp. *Aspergillus alliaceus* Strain JAV1 Isolated from Paddy Field Soil in Vellore. AJW *14*, 75–82.

Gastwirth, J.L., Gel, Y.R., Hui, W.L.W., Lyubchich, V., Miao, W., and Noguchi, K. (2020). Package lawstat. lawstat: Tools for Biostatistics, Public Policy, and Law. R package version 3.4. Available online: https://CRAN.R-project.org/package=lawstat (accessed on 1/7/2021).

Gill, J.P.K., Sethi, N., Mohan, A., Datta, S., and Girdhar, M. (2018). Glyphosate toxicity for animals. Environmental Chemistry Letters *16*, 401–426.

Griffin, D.H. (1994). Fungal physiology (New York, USA: Wiley-Liss).

Grossbard, E., and Harris, D. (1977). Selective action of 'Gramoxone W' and 'Roundup' on *Chaetomium globosum* in relation to straw decay. Transactions of the British Mycological Society *69*, 141–146.

Hajji, L., Hlaoua, W., Regaieg, H., and Horrigue-Raouani, N. (2017). Biocontrol Potential of *Verticillium leptobactrum* and *Purpureocillium lilacinum* Against *Meloidogyne javanica* and *Globodera pallida* on Potato (*Solanum tuberosum*). Am. J. Potato Res. *94*, 178–183.

Harms, H., Schlosser, D., and Wick, L.Y. (2011). Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nature Reviews Microbiology *9*, 177–192.

Harms, H., Wick, L.Y., and Schlosser, D. (2017). Chapter 31 The Fungal Community in Organically Polluted Systems. In Mycology, J. Dighton, and J.F. White, eds. (Boca Raton, FL: CRC Press), pp. 459–470.

Kanissery, R., Gairhe, B., Kadyampakeni, D., Batuman, O., and Alferez, F. (2019). Glyphosate: Its Environmental Persistence and Impact on Crop Health and Nutrition. Plants *8*, 499.

Kassambara, A. (2021). rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package version 0.7.0. Available online: https://CRAN.R-project.org/package=rstatix (accessed on 1/7/2021).

Klimek, M., Lejczak, B., Kafarski, P., and Forlani, G. (2001). Metabolism of the phosphonate herbicide glyphosate by a non-nitrate-utilizing strain of *Penicillium chrysogenum*. Pest Management Science: Formerly Pesticide Science *57*, 815–821.

Klingelhöfer, D., Braun, M., Brüggmann, D., and Groneberg, D.A. (2021). Glyphosate: How do ongoing controversies, market characteristics, and funding influence the global research landscape? Science of The Total Environment *765*, 144271.

Krzyśko-Łupicka, T., and Orlik, A. (1997). The use of glyphosate as the sole source of phosphorus or carbon for the selection of soil-borne fungal strains capable to degrade this herbicide. Chemosphere *34*, 2601–2605.

Krzysko-Lupicka, T., and Sudol, T. (2008). Interactions between glyphosate and autochthonous soil fungi surviving in aqueous solution of glyphosate. Chemosphere *71*, 1386–1391.

Kunanbayev, K., Churkina, G., Rukavitsina, I., Filippova, N., and Utebayev, M. (2019). Potential Attractiveness of Soil Fungus *Trichoderma inhamatum* for Biodegradation of the Glyphosate Herbicide. Journal of Ecological Engineering *20*, 240–245.

Maggi, F., la Cecilia, D., Tang, F.H.M., and McBratney, A. (2020). The global environmental hazard of glyphosate use. Science of The Total Environment *717*, 137167.

Meftaul, I.M., Venkateswarlu, K., Annamalai, P., Parven, A., and Megharaj, M. (2021). Glyphosate use in urban landscape soils: Fate, distribution, and potential human and environmental health risks. Journal of Environmental Management *292*, 112786.

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., and Séralini, G.E. (2015). Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food and Chemical Toxicology *84*, 133–153.

Morjan, W., Pedigo, L., and Lewis, L. (2002). Fungicidal effects of glyphosate and glyphosate formulations on four species of entomopathogenic fungi. Environmental Entomology *31*, 1206–1212.

Müller, B. (2021). Glyphosate—A love story. Ordinary thoughtlessness and response-ability in industrial farming. Journal of Agrarian Change *21*, 160–179.

Muola, A., Fuchs, B., Laihonen, M., Rainio, K., Heikkonen, L., Ruuskanen, S., Saikkonen, K., and Helander, M. (2021). Risk in the circular food economy: Glyphosate-based herbicide residues in manure fertilizers decrease crop yield. Science of The Total Environment *750*, 141422.

Murphy, J., and Riley, J.P. (1962). A modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta *27*, 31–36.

Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., et al. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environmental Health *15*, 19.

Mygind, M.R., Rugaard-Morgan, Z.-Z., Wadle, L., and Herzog, S. (2019). Bioremediation of Glyphosate by Lignin-Modifying Fungi *Pleurotus oestreatus*. (Roskilde, Denmark: Roskilde University (RUC)) pp. 1–21. Available online: https://rucforsk.ruc.dk/ws/portalfiles/portal/64967276/Bioremediation_of_Glyphosate_by_Lignin_ModifyingFungiPleurotus_oestreatus.pdf (accessed on 1 July 2021).

Nitheranont, T., Watanabe, A., Suzuki, T., Katayama, T., and Asada, Y. (2011). Decolorization of Synthetic Dyes and Biodegradation of Bisphenol A by Laccase from the Edible Mushroom, *Grifola frondosa*. Bioscience, Biotechnology, and Biochemistry *75*, 1845–1847.

Ondráčková, E., Seidenglanz, M., and Šafář, J. (2019). Effect of seventeen pesticides on mycelial growth of *Akanthomyces*, *Beauveria*, *Cordyceps* and *Purpureocillium* strains. Czech Mycology *71*, 123–135.

Pizzul, L., Castillo, M. del P., and Stenström, J. (2009). Degradation of glyphosate and other pesticides by ligninolytic enzymes. Biodegradation *20*, 751–759.

Pochron, S., Simon, L., Mirza, A., Littleton, A., Sahebzada, F., and Yudell, M. (2020). Glyphosate but not Roundup® harms earthworms (*Eisenia fetida*). Chemosphere *241*, 125017.

R Core Team (2021). Package stats. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online: https://www.R-project.org/ (accessed on 1/7/2021).

Raoult, D., Hadjadj, L., Baron, S.A., and Rolain, J.-M. (2021). Role of glyphosate in the emergence of antimicrobial resistance in bacteria? Journal of Antimicrobial Chemotherapy *76*, 1655–1657.

Rodríguez-Eugenio, N., McLaughlin, M., and Pennock, D. (2018). Soil pollution: a hidden reality (Rome, Italy: FAO).

Russell, V.L. (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.6.3. Available online: https://CRAN.R-project.org/package=emmeans (accessed on 1/7/2021).

Sailaja, K., and Satyaprasad, K. (2006). Degradation of glyphosate in soil and its effect on fungal population. Journal of Environmental Science & Engineering *48*, 189–190.

Sharma, S., and Adholeya, A. (2011). Detoxification and accumulation of chromium from tannery effluent and spent chrome effluent by *Paecilomyces lilacinus* fungi. International Biodeterioration & Biodegradation *65*, 309–317.

Spina, F., Cecchi, G., Landinez-Torres, A., Pecoraro, L., Russo, F., Wu, B., Cai, L., Liu, X.Z., Tosi, S., Varese, G.C., et al. (2018). Fungi as a toolbox for sustainable bioremediation of pesticides in soil and water. Plant Biosystems *152*, 474–488.

Sviridov, A.V., Shushkova, T.V., Ermakova, I.T., Ivanova, E.V., Epiktetov, D.O., and Leontievsky, A.A. (2015). Microbial degradation of glyphosate herbicides (Review). Applied Biochemistry and Microbiology *51*, 188–195.

Tang, F.H.M., Lenzen, M., McBratney, A., and Maggi, F. (2021). Risk of pesticide pollution at the global scale. Nature Geoscience *14*, 206–210.

Thorsten, P. (2021). Package PMCMRplus: PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended. R package version 1.9.0. Available online: https://CRAN.R-project.org/package=PMCMRplus (accessed on 1/7/2021).

Tian, Z., Wang, J., Li, J., and Han, B. (2021). Designing future crops: challenges and strategies for sustainable agriculture. Plant Journal*105*, 1165–1178.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. Nature *418*, 671–677.

United Nations, Department of Economic and Social Affairs, and Population Division (2019). World population prospects Highlights, 2019.

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K.C., Finckh, M.R., and Morris, J.G. (2018). Environmental and health effects of the herbicide glyphosate. Science of The Total Environment *616–617*, 255–268.

Wittkowski, K.M., and Tingting, S. (2012). Package muStat: muStat: Prentice Rank Sum Test and McNemar Test. R package version 1.7.0. Available online: https://CRAN.R-project.org/package=muStat (accessed on 1/7/2021).

Xia, L., Xu, X., Zhu, W., Huang, Q., and Chen, W. (2015). A Comparative Study on the Biosorption of Cd2+ onto *Paecilomyces lilacinus* XLA and Mucoromycote sp. XLC. International Journal of Molecular Sciences *16*, 15670–15687.

Xu, X., Xia, L., Chen, W., and Huang, Q. (2017). Detoxification of hexavalent chromate by growing *Paecilomyces lilacinus* XLA. Environmental Pollution *225*, 47–54.

Zhan, H., Feng, Y., Fan, X., and Chen, S. (2018). Recent advances in glyphosate biodegradation. Applied Microbiology and Biotechnology *102*, 5033–5043.

Zucconi, L., Ripa, C., Alianiello, F., Benedetti, A., and Onofri, S. (2003). Lead resistance, sorption and accumulation in a *Paecilomyces lilacinus* strain. Biology and Fertility of Soils*37*, 17–22.

5.7 SUPPLEMENTARY MATERIAL

Supplementary Material S1

Supplementary Table 5.9 In the table are reported the performed tests on each screenings' data.

Screening	Outcome variable	Performed tests
Screening of fungal strains for glyphosate commercial formulation tolerance	Diameter (mm)	Friedman test followed by post-hoc Conover's test
Screening of fungal Strains for their ability to utilise glyphosate as nutritional source of C or P	Diameter (mm)	Friedman test followed by post-hoc Conover's test
Screening of *Purpureocillium lilacinum* for Glyphosate and Roundup breakdown and utilisation as P source in liquid culture medium	Dry weights (g)	Welch test followed by post-hoc Dunnet's T3 test
	pH	Welch test followed by post-hoc Dunnet's T3 test
	P concentration (mg/L)	Welch test followed by post-hoc Dunnet's T3 test
Screening of pH medium influence on Glyphosate and Round Up breakdown by *Purpureocillium lilacinum*	Dry weights (g)	One-way ANOVA followed by post-hoc Tukey honest significant differences test Two-way ANOVA followed by Simple main effect and Simple pairwise comparisons
	pH	Friedman test followed by post-hoc Conover's test
	P concentration (mg/L)	Welch test followed by post-hoc Dunnet's T3 test

Two-way ANOVA was conducted to examine the Treatments and TRIS buffer addition effects on Biomass production (dry weights) in the Screening of pH medium influence on Glyphosate and Round Up breakdown by *Purpureocillium lilacinum*

The two-way ANOVA assumptions were verified through appropriate tests whose results were:

- Absence of extreme outliers;
- normally distributed residuals (Shapiro-Wilk's normality test $p > 0.05$); Normality assumption was also confirmed for each cell of design ($p > 0.05$);
- homogeneity of variance ($p > 0.05$).

A two-way ANOVA was conducted to examine the Treatments and TRIS buffer addition effects on Biomass production (dry weights). ANOVA showed a statistically significant interaction between the buffer addition and the treatment influencing dry weights, F(3, 17) = 12.52, p = 0.00014, eta2[g] = 0.69.

Supplementary Table 5.10 ANOVA Table

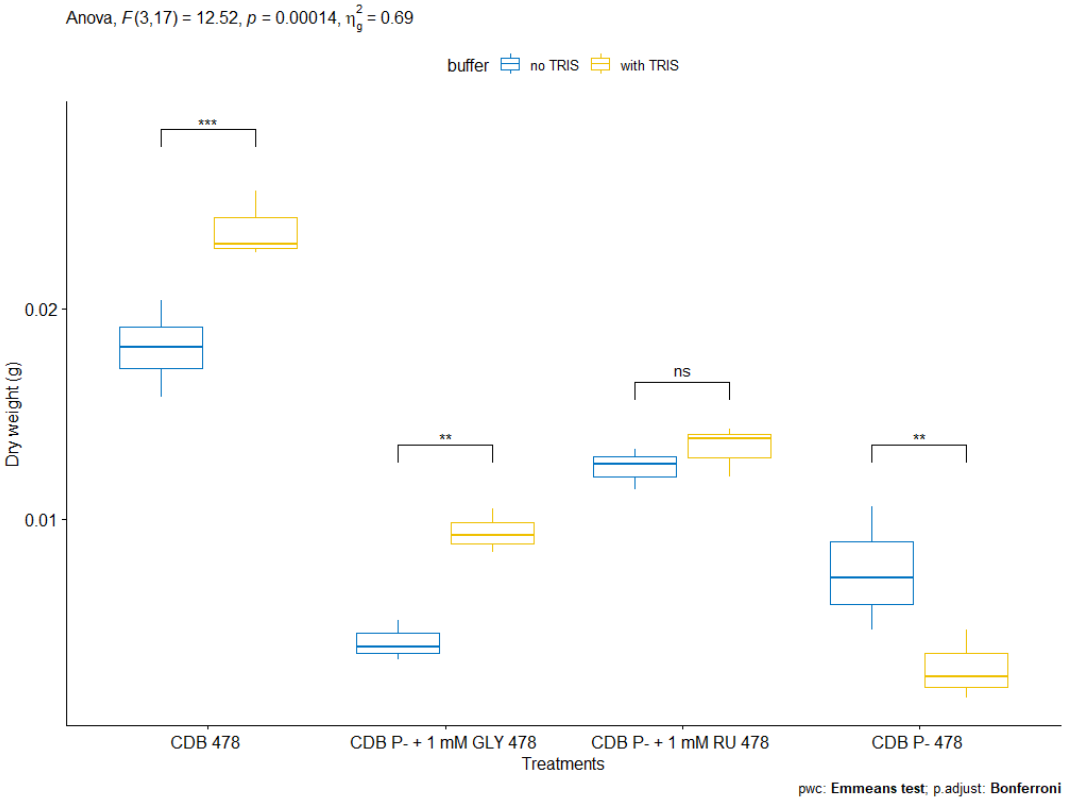
	Effect	DFn	DFd	F	p	p<.05	ges
1	Treatment	3	17	114.950	1.73e-11	*	0.953
2	Buffer	1	17	8.326	1.00e-02	*	0.329
3	Treatment:Buffer	3	17	12.515	1.45e-04	*	0.688

Consequently, an analysis of simple main effects has been performed with statistical significance adjusted with the Bonferroni method. Finally, a simple pairwise comparison analysis was performed. A statistically significant difference in dry weight means between TRIS presence and TRIS absence conditions was observed in all treatments with the exception of CDB P- 1mM RU (p > 0.05).

Supplementary Table 5.11 Table of Simple pairwise comparison

A tibble: 4 x 10

Treatment	term	.y.	group1	group2	df	statistic	p	p.adj	p.adj.signif
<chr>	<chr>	<chr>	<chr>	<chr>	<dbl>	<dbl>	<dbl>	<dbl>	<chr>
CDB	Buffer	dw	no TRIS	with TRIS	17	-4.42	0.000371	0.000371	***
CDB P-	Buffer	dw	no TRIS	with TRIS	17	3.36	0.00376	0.00376	**
CDB P-+ 1 mM GLY	Buffer	dw	no TRIS	with TRIS	17	-3.82	0.00138	0.00138	**
CDB P-+ 1 mM RU	Buffer	dw	no TRIS	with TRIS	17	-0.681	0.505	0.505	ns



Supplementary Figure 5.3 Boxplot of the Simple pairwise comparison output.

CHAPTER 6: CONCLUDING REMARKS BASED ON THE EXPERIMENTAL RESULTS

- *Minimedusa polyspora* was proven able to promote C*ichorium intybus* growth in microcosm, determining significant variations of growth parameters including the dry wight of total plant, shoot, total roots, lateral roots and leaf area.

- *M. polyspora* and *C. globosum* culture filtrates determined a metabolic variation in *C. intybus* roots activating the metabolic pathway involving 3-OH-butyrate, phospholipids, sterols and fatty acids.

- *M. polyspora* culture filtrate activated phenylpropanoid pathway in leaves and roots, while *C. globosum* culture filtrate triggered the phenylpropanoid pathway and chicoric acid biosynthesis in roots.

- *M. polyspora* and *C. globosum* in *in vitro* culture conditions showed to be efficient biocontrol agents of three of the most extremely severe fungal phytopathogens of economical and agronomical relevance: *Alternaria alternata*, *Berkeleyomyces basicola* and *Botrytis cinerea*.

- *M. polyspora* and *C. globosum,* in *in vitro* culture conditions, were found to be tolerant to RoundUp a glyphosate-based herbicide only at the concentration of 1mM, while a concentration of 10mM completely inhibited their growth. However, considering both species have been previously reported for their degradation ability, the observe susceptibility may be related to the origin of these strains that have been isolated from natural uncontaminated environments.

- Despite the strains studied in this thesis were not able to degrade glyphosate, another fungal strain of biotechnological interest *Purpureocillium lilacinum* was found to be able to degrade 80% of glyphosate initial concentration *in vitro* conditions.

CHAPTER 7: SUPPLEMENTARY INVESTIGATIONS ON FUNGAL CONSERVATION AND ITS IMPORTANCE FOR SUSTAINABLE BIOTECHNOLOGIES

7.1 INTRODUCTION

The fungal global diversity is estimated between 2.2 and 3.8 million species, although the number of described fungal species currently is approximately 120,000 (Hawksworth and Lücking, 2017). However, many of these still unknown species may never be described as fungal biodiversity is currently heavily threatened due to anthropogenic activities, pollution phenomena and climate change, which can cause severe ecological alterations. These alterations, determining effects such as loss or fragmentation of the habitats as well as loss of the symbiotic partners, may impair conditions that are fundamental for the survival of many fungal species (Moore et al., 2001). Despite the ecological importance of fungi, that play fundamental ecological and geological roles in ecosystems and provide ecosystem services fundamental for human well-being, the actions for their conservation, especially in the past, resulted to be inadequate or totally absent (Moore et al., 2001). The conservation of mycological biodiversity, indeed, is a multifaced issue that presents several difficulties, including the need to firstly identify the characteristics and conservation status of fungal species to prioritize those that are more sensitive to anthropogenic activities or to rapid climatic and environmental changes. However, in recent years, the situation has been positively changing and international actions for fungal conservation have been carried out, such as red listing of the threatened fungal species, to ensure an adequate protection, and the activation of *in situ* and *ex situ* strategies (Minter, 2011; Moore et al., 2001). Despite the abovementioned improvements, to support and boost fungal biodiversity conservation, further efforts including improving the scientific research, education, and conservation practices, but also by orienting economic and politic actions towards sustainability and decisions inspired by nature, are still needed. These desirable further improvements will be possible only thanks to a larger commitment from all people, not only scientists or mycologists, but also politicians, citizens, farmers (Barron, 2017; Minter, 2011).

Culture collections represent the pillars of fungal biodiversity *ex situ* conservation and, as highlighted also by this thesis' results, represent an inestimable asset of bioresources suitable to sustain human activities and needs. Indeed, the Ph.D. project resulting in this thesis focused on testing two fungal strains, preserved in culture collection of the Fungal Biodiversity Laboratory, for their multifunctional roles and their potential as bioresources for sustainable agriculture applications. In addition, another study highlighting the importance of conservation in mycological collections, of fungal strains isolated from highly contaminated soils, which may be suitable

bioresources for bioremediation, is reported in this chapter. Indeed, simultaneously with the activities strictly addressing the Ph.D. project's aim, I participated in a study, in which four soil saprotrophic fungi *Absidia spinosa*, *Purpureocillium lilacinum*, *Metarhizium marquandii*, and *Cephalotrichum nanum*, isolated from soils with naturally high arsenic concentrations and preserved in the culture collection of the Fungal Biodiversity Laboratory, were successfully tested for their ability to tolerate different sodium arsenite concentrations and accumulate As in different cultural conditions. The results of this study, suggest that the tested fungi are effective potential candidates for the bioremediation of As contaminated soil and worthy of further investigation.

Concerning *in situ* conservation, on the other hand, the IUCN Red List of endangered species certainly represents the most valuable tool available to stimulate and direct the actions to be undertaken by identifying threatened species that must be prioritized and the related threats to be mitigated. In this context, during my Ph.D., I participated in the activities leading to the conservation status assessment of two fungal strains: the coprophilous ascomycete *Poronia punctata* and the ectomycorrhizal basidiomycete *Alessioporus ichnusanus*. Therefore, this chapter finally also includes two publications on the conservation status of these two fungal species, in which valuable and critical aspects addressed during the evaluation process according to the IUCN red-listing criteria are highlighted and discussed.

7.2 REFERENCES

Barron, E.S. (2017). Chapter 21 Who Cares? The Human Perspective on Fungal Conservation. In The Fungal Community. Its Organization and Role in the Ecosystem. Fourth Edition., (Boca Raton, FL: CRC Press), pp. 321–330.

Hawksworth, D.L., and Lücking, R. (2017). Fungal Diversity Revisited: 2.2 to 3.8 Million Species. Microbiology Spectrum *5*, 4.

Minter, D. (2011). Fungal conservation needs help from botanists. Plant Biosystems *145*, 945–949.

Moore, D., Nauta, M.M., Evans, S.E., and Rotheroe, M. (2001). Fungal Conservation: Issues and Solutions (Cambridge, UK: Cambridge University Press).

7.3 PAPER: FUNGI AND ARSENIC: TOLERANCE AND BIOACCUMULATION BY SOIL SAPROTROPHIC SPECIES

applied sciences

MDPI

Article

Fungi and Arsenic: Tolerance and Bioaccumulation by Soil Saprotrophic Species

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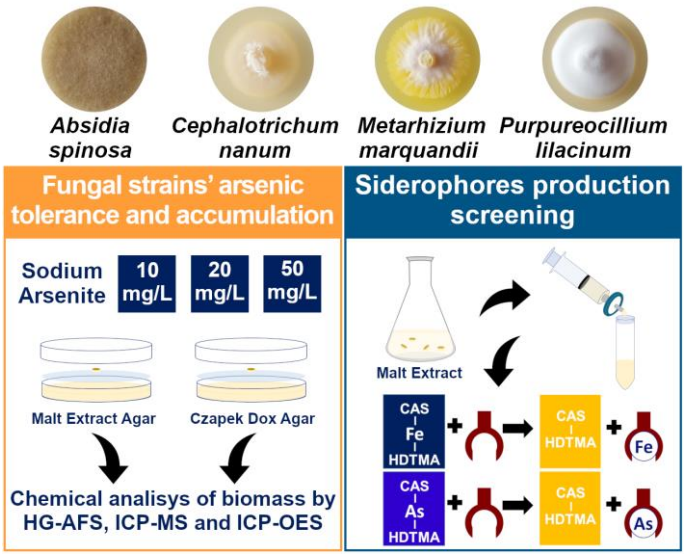
Appl. Sci. 2020, 10(9), 3218; https://doi.org/10.3390/app10093218

GRAPHICAL ABSTRACT



ABSTRACT

Increasing arsenic environmental concentrations are raising worldwide concern for its impacts on human health and ecosystem functionality. In order to cope with arsenic contamination, bioremediation using fungi can represent an efficient, sustainable, and

cost-effective technological solution. Fungi can mitigate arsenic contamination through different mechanisms including bioaccumulation. In this work, four soil saprotrophic fungi *Absidia spinosa*, *Purpureocillium lilacinum*, *Metarhizium marquandii*, and *Cephalotrichum nanum*, isolated from soils with naturally high arsenic concentrations, were tested for their ability to tolerate different sodium arsenite concentrations and accumulate As in different cultural conditions. pH medium after fungal growth was measured to study pH variation and metabolic responses. Arsenic bioaccumulation and its influence on the uptake of other elements were investigated through multi-elemental analysis using hydride generation atomic fluorescence spectrometry (HG-AFS), inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES). Considering the increasing interest in siderophore application for metal bioremediation, the production of siderophores and their affinity for both Fe and As were also evaluated. All species were able to tolerate and accumulate As in their biomass in all of the tested conditions and produced siderophores with different affinities for Fe and As. The results suggest that the tested fungi are attractive potential candidates for the bioremediation of As contaminated soil and worthy of further investigation.

KEYWORDS

Soil fungi; bioaccumulation; arsenic; arsenite tolerance; *Absidia spinosa*; *Purpureocillium lilacinum*; *Metarhizium marquandii*; *Cephalotrichum nanum*; siderophores; multi-elemental analysis

7.3.1 INTRODUCTION

Increasing arsenic (As) environmental concentrations are raising worldwide concern due to the impacts on human health and ecosystem functionality (Singh et al., 2015b; Urík et al., 2007). In fact, chemical species of As can be toxic for living organisms as arsenate can compete with the essential inorganic phosphate, and arsenite can inactivate many enzymes by binding to protein thiols (Ceci et al., 2015a). In humans, As can cause severe toxic effects to integumentary, cardiovascular, reproductive, and neurological systems, leading in extreme cases to the development of malignant tumours and death (Mohammed Abdul et al., 2015).

Arsenic is a naturally occurring metalloid that is widely distributed in the Earth's crust (Ceci et al., 2015a). Primarily associated with igneous and sedimentary rocks, As is naturally found in the environment because of natural processes including weathering of As-enriched minerals, volcanic emissions, and biological activities (Mandal, 2002; Mohammed Abdul et al., 2015; Sharma and Sohn, 2009; Smedley and Kinniburgh, 2002).

In several locations worldwide (e.g., Bangladesh, India, China, USA) high As concentrations in groundwater, drinking water, and soils are associated with natural geologic sources (Mandal, 2002; Sharma and Sohn, 2009; Singh et al., 2015b). In many Italian areas, highly natural As concentrations in lakes, volcanic aquifers, drinking waters, rocks, soils, plants, and food have been reported to be associated with geological and environmental factors (Cinti et al., 2015; Cubadda et al., 2010; Vivona et al., 2007). On the other hand, other important sources contributing to As environmental contamination are anthropogenic activities including, for instance, the use of agricultural pesticides, wood preservatives, and medicines, waste incineration, and mineral ore processing (Adeyemi, 2009; Ceci et al., 2015a; Sharma and Sohn, 2009). Indeed, in some Italian areas, former mining activities have contributed to an increase of As in the environment, leading to its high concentrations (Baroni et al., 2004; Frau and Ardau, 2003).

In order to cope with As contamination, bioremediation and bioaugmentation using microorganisms can represent an efficient, environmentally-friendly, and cost-effective technological solution. In particular, fungi are promising bioresources in the remediation of As pollution (Adeyemi, 2009; Ceci et al., 2015a; Singh et al., 2015b; Urík et al., 2007). Fungi are important geoactive agents, playing very important geological roles in several processes including decomposition, biogeochemical cycling, element biotransformation, metal and mineral transformations, bioweathering, and soil formation (Ceci et al., 2015a, 2019; Gadd, 2017; Liang and Gadd, 2017). They are able to tolerate extreme and very limiting environmental conditions such as highly concentrated mixtures of toxic substances. Some species have been reported to accumulate high concentrations of arsenic and volatilize it via methylation (Ceci et al., 2019; Harms et al., 2017; Singh et al., 2015b; Urík et al., 2007). Several soil fungi isolated by As-contaminated soils have been successfully tested for As tolerance and removal (Mukherjee et al., 2010; Srivastava et al., 2011). Among these, the majority belong to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus*, and *Trichoderma* (Srivastava et al., 2011; Su et al., 2011; Urík et al., 2007; Valix and Loon, 2003). Most of the above-mentioned tested strains were isolated from contaminated sites such as agricultural soils, paddy fields, and mines mainly in China and India. In order to maximize the efficiency in mycoremediation applications, it is very important to focus on saprotrophic fungi isolated from soils with high As concentrations as they can be better equipped to survive and cope with the associated chemical stress (Russo et al., 2019a, 2019b).

In this context, siderophores, which are low-molecular-weight organic compounds, are crucial for many of the above-mentioned roles played by fungi in several processes, especially for soil mineral weathering and biogeochemical cycles (Ahmed and Holmström, 2014). Their main role is to bind extracellular Fe(III), generally not available for organisms, and transport it inside the cell to meet the metabolic

requirements (Renshaw et al., 2002; Retamal-Morales et al., 2018). However, siderophores are remarkably effective in solubilizing and increasing the mobility not only of Fe, but also of a wide range of elements including Cd, Ni, As, and actinides (Ahmed and Holmström, 2014; Mehnert et al., 2017; Renshaw et al., 2002; Retamal-Morales et al., 2018). Consequently, in the last years, there has been an increasing interest in siderophore application for metal bioremediation (Ahmed and Holmström, 2014). Most fungi can produce hydroxamate-type siderophores, but other types have also been observed including rhizoferrin, a polycarboxylate siderophore, in Zygomycetes and phenolate compounds in other fungi (Haas, 2014; Holinsworth and Martin, 2009; Renshaw et al., 2002). Evidence has shown that since microorganisms can also acquire Fe from As-bearing minerals (es. Scorodite), consequently increasing the As mobilization, siderophore production can correlate with increased As resistance, even if a direct link to the siderophores' action binding As was not observed (Retamal-Morales et al., 2018). The potential of siderophores in the bioremediation of potentially toxic elements is very high, as siderophore-producing fungi are abundant in soils, and siderophores can strongly influence speciation, bioavailability, and the fate of metals, metalloids, and radionuclides in the environment (Renshaw et al., 2002). Therefore, since siderophores represent a useful tool, in order to deepen the tested species potential in As bioremediation, siderophore production was taken into account.

In this context, four fungal species, *Absidia spinosa* Lendn., *Cephalotrichum nanum* (Ehrenb.) S. Hughes, *Metarhizium marquandii* (Massee) Kepler, S.A. Rehner & Humber, and *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, previously isolated from Italian soils with naturally high As concentrations, were investigated in the presence of As, as As(III) or arsenite, in two different nutritional conditions. In fact, fungi are strongly influenced by nutritional condition in their growth and ecology and therefore also in their ability to tolerate stresses. Furthermore, medium composition may have an effect on metal uptake (Errasquín and Vázquez, 2003; Sharma and Pandey, 2010). To our best knowledge, these species have not been previously studied for their ability to tolerate and accumulate As, but their potential in the bioremediation of some toxic metals has been reported (Coles et al., 1999; Lukšienė et al., 2012; Sharma and Adholeya, 2011; Słaba and Długoński, 2011; Słaba et al., 2005; Xia et al., 2015; Xu et al., 2017; Zucconi et al., 2003). In particular, Coles et al. (Coles et al., 1999) reported on the tolerance and solubilization of zinc compounds by *A. spinosa*, as fungal species associated with *Thlaspi caerulescens*. *A. spinosa*, and *P. lilacinum* adsorbed plutonium onto mycelium and spores (Lukšienė et al., 2012). *P. lilacinum* was reported to be tolerant to and accumulate Pb, Cd, and Cr (Sharma and Adholeya, 2011; Xia et al., 2015; Xu et al., 2017; Zucconi et al., 2003), while *M. marquandii* resulted tolerant to Zn and Pb and able to uptake them (Słaba and Długoński, 2011; Słaba et al., 2005). No species of the genus *Cephalotrichum* was previously tested with metals.

However, being oligotrophic fungi, these species inhabit extreme habitats with low availability of nutrients, for example, *C. stemonitis* has been isolated from a closed gold mine with high As concentrations (Chlebicki, 2013; Jiang et al., 2017). Hence, these four fungi show a great potential for the development of biotechnological applications for As remediation, and consequently the knowledge of their interactions with arsenic deserves to be deepened. Therefore, to shed further light on this topic, this study was aimed to investigate the (1) fungal tolerance to high As concentrations; (2) arsenic bioaccumulation in fungal biomass; (3) influence of As presence in the medium on the uptake and accumulation of other elements; and (4) the production of siderophores and their affinity for As compared to that for Fe.

7.3.2 MATERIALS AND METHODS

7.3.2.1 SCREENING OF FUNGAL STRAINS FOR ARSENIC TOLERANCE AND ACCUMULATION

Four strains of soil saprotrophic fungi, previously isolated from environments with high natural concentrations of arsenic and currently preserved at the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), were screened to evaluate growth responses and assess As tolerance and As bioaccumulation. The tested strains, belonging to the Mucoromycota and Ascomycota phyla, were *Absidia spinosa* (FBL 69), *Cephalotrichum nanum* (FBL 73), *Metarhizium marquandii* (FBL 484), and *Purpureocillium lilacinum* (FBL 478). *A. spinosa* and *C. nanum* were isolated from different soils of the geothermal area of Travale-Radicondoli, characterized by high concentrations of potentially toxic elements including As (Loppi and Bargagli, 1996), while *P. lilacinum* and *M. marquandii* were isolated from the soils of Latin Valley, which are characterized by high background concentrations of As (Marescotti et al., 2011). The strains were reactivated and maintained at 25 °C in the dark on Malt Extract Agar (MEA), prepared according to the following composition (g/L in distilled water): malt extract, 20; peptone, 1; glucose, 20; agar, 20. All components were purchased from Becton Dickinson (Sparks, MD, USA). Prior to the experiments 7-day old stock cultures of the fungal strains were established. Tolerance screenings to sodium arsenite ($NaAsO_2$) (assay ≥ 90%; antimony (Sb) ≤ 0.5%; Sigma-Aldrich) were carried out in Petri plates containing solid culture medium supplemented with a sodium arsenite solution and mixed to homogenize before solidification. In order to evaluate the influence of nutritional conditions on the strains' ability to tolerate and accumulate arsenic, the plate test was performed on two cultural media, Malt Extract Agar (MEA) and Czapek-Dox Agar (CDA). The composition of CDA was as follows (g/L distilled water): sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; and $FeSO_4 \cdot 7H_2O$, 0.01. All chemicals were purchased from Merck (Darmstadt, Germany). The pH of CDA was adjusted to 5.8

by introducing HCl, while MEA pH was left unmodified (pH 5.4). To assess a possible dose-dependent response, three different concentrations 10 mg/L, 20 mg/L, and 50 mg/L of $NaAsO_2$ were tested, which were equivalent to 5.8 mg/L, 11.5 mg/L, and 28.8 mg/L of As(III), respectively. The concentrations were selected considering the As concentrations tested in previous papers such as Vala et al. (2010) and the environmental As concentrations of the soils from which the fungi were isolated. Controls and chemical blanks were also set up. All assays were carried out in quadruplicate. Growth responses and fungal tolerance to As were investigated by tolerance indexes based on the growth data of diametric extension and dry weights. Two indexes were calculated: Rt:Rc (%) defined as the ratio of the colony extension rates in the presence (Rt) or absence (Rc) of $NaAsO_2$ and the Tolerance Index (T.I.) based on the dry weights (DW) of fungal biomass (T.I. (%) = (DW of treated mycelium/DW of control mycelium) × 100) (Russo et al., 2019a). To facilitate the recovery of the mycelium, sterile cellophane membranes, allowing the passage of nutrients and metabolites between the medium and the colony, were placed on the surface of the culture medium (Russo et al., 2019b). Inoculation was carried out placing at the center of the plate a 6-mm-diameter plug taken from the actively growing margin of the stock cultures using a sterile cork borer. During the seven days of incubation, measures of diametric extension were carried out daily. After incubation for seven days at 25 °C in the dark, using a sterile razor blade, the mycelia were recovered from the membrane and oven dried at 100 °C until reaching a constant weight for at least two days.

Following the biomass removal, the pH of the culture medium was measured at specific intervals across the diameter of the Petri dish using a pH meter HI 99161, fitted with a conical tip FC 202D pH electrode (Hanna Instruments, Woonsocket, RI, USA) to evaluate the pH variation related to metabolic responses.

7.3.2.2 ELEMENTAL CHEMICAL ANALYSIS OF THE TESTED SPECIES' FUNGAL BIOMASS

Concentrations of 18 elements (As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Sn, and Sr) in the fungal biomass of *A. spinosa*, *C. nanum*, *M. marquandii*, and *P. lilacinum* were analyzed to evaluate the different accumulation of the elements in the four tested strains grown on MEA and CDA media (control) and on MEA and CDA media added with 10 mg/L, 20 mg/L, and 50 mg/L of sodium arsenite. All treatments, sample preparation, and chemical analyses were carried out in quadruplicate.

Four samples for each of the four fungal species subjected to the eight different treatments (MEA and CDA media added or not with 10 mg/L, 20 mg/L, and 50 mg/L of sodium arsenite), for a total of 128 samples, were prepared and chemically analyzed in accordance with the following procedure. Each sample was transferred to a quartz vessel

and subjected to microwave assisted acid digestion (Ethos Touch Control with Q20 rotor, Milestone, Bergamo, Italy) for 30 min, using 2 mL of ultrapure concentrated HNO_3 (assay > 67%; residue < 1 mg/L) and 1 mL of H_2O_2 (assay > 30%; residue < 20 mg/L); HNO_3 and H_2O_2 were high purity solvents for trace analysis and were purchased from Promochem, LGC Standards GmbH (Wesel, Germany). The vessel was irradiated with a maximum power of 1000 W and microwave digestion was carried out at maximum temperature (180 °C) and pressures ≤ 40 bar. The digested solution was then diluted to 50 mL with deionized water (Arioso UP 900 Integrate Water Purification System) and filtered through syringe filters (25 mm diameter, 0.45 μm pore size, GVS Filter Technology, Morecambe, UK).

Concentrations of Ca, Fe, K, Mg, Na, P, and S were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Vista MPX CCD Simultaneous; Varian, Victoria, Mulgrave, Australia) in axial view mode and equipped with a cyclonic spray chamber. Determination of Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Sn, and Sr concentrations was performed by quadrupole inductively coupled plasma mass spectrometer (ICP-MS; model 820-MS; Bruker, Bremen, Germany) equipped with a glass nebulizer (0.4 mL/min; Analytik Jena AG, Jena, Germany). Overall, ICP-OES was used for the determination of elements present in higher concentrations, while ICP-MS was used for the analysis of trace and ultra-trace elements. For both ICP-OES and ICP-MS determinations, external standard calibration curves were performed by serially diluting multi-element standard stock solutions (1000 ± 2 mg/L; Exaxol Italia Chemical Manufacturers Srl, Genoa, Italy). To control the nebulizer efficiency, rhodium was set at 5 μg/L as the internal standard for all of the measurements and was prepared from standard stock solutions (1000 ± 2 mg/L; Panreac Química, Barcelona, Spain). For ICP-MS analysis, a standard solution containing 5 μg/L of Ba, Be, Ce, Co, In, Pb, Mg, Tl, and Th was prepared daily in 1% HNO_3 from a multi-standard stock solution (10.00 ± 0.05 mg/L; Spectro Pure, Ricca Chemical Company, Arlington, TX, USA) to select the best ICP-MS performance. Further details about the ICP-OES and ICP-MS conditions are reported in Canepari et al. (2006, 2009) and in Astolfi et al. (2018), respectively. Atomic fluorescence spectrometry with hydride generation (HG-AFS; 8220 Titan HG-AFS, FullTech Instruments, Rome, Italy) was used for the determination of the total As concentration in fungal biomass, since this technique enables avoiding possible interferences in As determination by ICP-MS due to Cl cluster formation and has a higher sensitivity for As with respect to ICP-OES, thus allowing a higher analytical quality of the data to be obtained (Agatemor and Beauchemin, 2011; Canepari et al., 2014). Calibration standard solutions were obtained using certified standard mono-element solution of As (1000 ± 10 mg/L; Merck, Darmstadt, Germany), 5% HCl (Promochem, LGC Standards GmbH, Wesel, Germany) was used as the carrier and 2% $NaBH_4$ (Sigma-Aldrich Chemie GmbH, St. Louis, MO, USA) in 0.5% NaOH (Carlo Erba Reagenti Srl,

Rodano, MI, USA) as the reducing agent. Standards and samples were prepared in 5% HCl + 1% KI (Sigma-Aldrich Chemie GmbH, St. Louis, MO, USA) + 0.5% ascorbic acid (Fluka Analytical, St. Louis, MO, USA).

The values of blanks, subjected to similar sample preparation and analytical procedures, were deducted from all measurements and the limits of detection were set at three times the standard deviation of 10 replicate blank determinations. Finally, the obtained values were divided by the dry weight of each sample to obtain the element concentrations (Massimi et al., 2019; Piacentini et al., 2019; Ristorini et al., 2020) in the fungal biomass of the tested species at the different examined conditions. The obtained element concentrations are reported in Supplementary Materials**Supplementary Table 7.8****Supplementary Table 7.11**. The certified reference materials BCR 482 lichen (IRMM, Geel, Belgium) and NIST 1515 apple leaves (National Institute of Standards and Technology) (Conti et al., 2018; Piacentini et al., 2019) were used to test the accuracy of the measurements and to validate the entire analytical process (Supplementary Materials **Supplementary Table 7.12** and **Supplementary Table 7.13**). Results were in good agreement with certified values (95% confidence level); recovery percentages (≥80%) and standard deviations (≤20%, except for Sn) were satisfactory and indicated a good precision of the measurements.

7.3.2.3 SIDEROPHORE PRODUCTION SCREENING

Siderophore production was screened using the Chrome Azurol CAS Assay. The assay was performed both for arsenic (As-CAS) and iron (Fe-CAS), according to the protocol modified by Mehnert (Mehnert et al., 2017). Cultures of the four fungal strains were inoculated in 100 mL Erlenmeyer flasks, containing 50 mL of Malt Extract Broth (MEB), using four plugs of 6 mm diameter of mycelium taken from seven day old stock cultures on MEA using a cork borer. The cultures, established in triplicate, were incubated in the dark at 25 °C with constant shaking at 100 rpm on a rotatory shaker (ASAL 711/D). Samples of culture medium from the established cultures were collected at 7, 14, and 21 days of growth. To avoid mycelium fragments possibly altering the spectrophotometric measurements, the culture medium was filtered using a 33 mm diameter sterile syringe filter with a 0.45 μm pore size made of mixed cellulose esters (ClearLine). The test was performed in a 96 multi-well plate in order to screen all the biological replicates, performing multiple readings of each one. In each well, culture filtrate and CAS solution were added in a 1:1 ratio, in the amount of 100 μL of each one. A negative control was arranged using sterile culture media and CAS solution, while a positive control was arranged adding 180 mM Ethylenediaminetetraacetic acid (EDTA) from a 500 mM stock (pH = 8) to the MEB and CAS solution. Absorbance was read at 620 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific™) after four hours of incubation.

7.3.2.4 STATISTICAL ANALYSIS

All statistical analyses were carried out using the statistical software R (version 3.5.2) (R Core Team, 2021) under the R-studio environment (version 1.1.463) (RStudio Team, 2016).

The Shapiro–Wilk test was used to evaluate the normality of the data (package stats) (Shapiro and Wilk, 1965). To test data homoskedasticity, as appropriate, the Levene test (package lawstat) (Levene, 1960) and Bartlett test (package stats) (Bartlett, 1937) were used. Hereafter, analysis of variance was performed using the Welch test (package stats) (Welch, 1951), followed by the all-pairs comparison post-hoc test Tamhane's T2 (package PMCMRplus) (Lee and Lee, 2018).

Principal component analysis (PCA) was performed on the element concentrations determined in the fungal biomass of the tested species grown on MEA and CDA media (control) and on MEA and CDA media added with 10 mg/L, 20 mg/L, and 50 mg/L of sodium arsenite (Supplementary Materials**Supplementary Table 7.8****Supplementary Table 7.11**) to cluster the samples (scores) according to the different accumulation of the elements (loadings). The matrix of the data, composed of 120 samples (four samples for each of the four fungal species subjected to the eight different treatments; eight outlier samples were excluded from the multivariate statistical computation) and 18 variables (As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Sn, and Sr), was transformed by performing column mean centering and row and column autoscaling. This allowed correcting variations of the data due to the different scaling and units of the examined variables. Data were then analyzed by using PCA.

7.3.3 RESULTS

7.3.3.1 EVALUATION OF GROWTH AND TOLERANCE OF TESTED FUNGI IN THE PRESENCE OF ARSENIC AND CULTURE MEDIUM PH ANALYSIS

The values of diametric growth, biomass production, and tolerance indexes of the tested species in the presence of different As concentrations are shown in Table 1, Table 2 and Table 3. In all tested conditions, As did not inhibit the growth of the tested fungal species, which showed normal colony development and sporulation. Moreover, growth differences among the tested species, also in relation to the tested media, could be appreciated (**Table 7.1** and **Table 7.2**). In fact, on CDA, *A. spinosa* showed greater growth than other species, while the lowest values of diameter and dry weights in both nutritional conditions were observed in *C. nanum*. *M. marquandii* like *C. nanum* showed greater diametric growth on CDA than on MEA, and biomass production of *C. nanum* on CDA was higher than on MEA.

Table 7.1 Diametric values of fungal colonies after seven days of growth at 25 °C. Data are expressed as the mean ± standard error of independent biological replicates [a].

Diameter (mm)									
Media		**MEA**				**CDA**			
$NaAsO_2$ Concentration		**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	86.0 ± 0.0	76.9 ± 1.4	59.0 ± 0.9 **	45.8 ± 1.5 *	86.0 ± 0.0	84.6 ± 0.8	86.0 ± 0.0	85.5 ± 0.5
	***P. lilacinum* FBL 478**	38.8 ± 0.3	38.0 ± 0.0	38.1 ± 0.4	37.7 ± 0.7	39.1 ± 0.5	38.5 ± 0.3	38.0 ± 0.4	36.1 ± 0.4
	***M. marquandii* FBL 484**	32.0 ± 1.0	30.1 ± 0.7	33.1 ± 0.8	31.0 ± 0.7	34.6 ± 1.6	35.3 ± 0.5	36.9 ± 0.6	34.6 ± 0.4
	***C. nanum* FBL 73**	14.8 ± 0.3	15.4 ± 0.9	15.3 ± 0.1	16.4 ± 1.0	24.0 ± 0.2	24.5 ± 0.6	23.9 ± 0.1	23.4 ± 0.4

[a] Asterisks denote a significant di_erence (post-hoc Tamhane's T2-test) between the treatment and the control (* $p < 0.1$; ** $p < 0.05$).

Table 7.2 Dry weight values of fungal biomass after seven days of growth at 25 °C. Data are expressed as the mean standard error of independent biological replicates [a].

Dry Weight (g)									
Media		**MEA**				**CDA**			
$NaAsO_2$ Concentration		**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	0.0880 ± 0.0011	0.0783 ± 0.0013	0.0550 ± 0.0014 ***	0.0465 ± 0.0027 *	0.1021 ± 0.0042	0.0956 ± 0.0039	0.0966 ± 0.0024	0.1119 ± 0.0039
	***P. lilacinum* FBL 478**	0.0423 ± 0.0011	0.0395 ± 0.0006	0.0421 ± 0.0014	0.0409 ± 0.0004	0.0415 ± 0.0015	0.0437 ± 0.0019	0.0422 ± 0.0015	0.0403 ± 0.0009
	***M. marquandii* FBL 484**	0.0241 ± 0.0014	0.0231 ± 0.0010	0.0274 ± 0.0010	0.0243 ± 0.0029	0.0236 ± 0.0007	0.0241 ± 0.0016	0.0245 ± 0.0014	0.0240 ± 0.0014
	***C. nanum* FBL 73**	0.0105 ± 0.0001	0.0116 ± 0.0007	0.0106 ± 0.0002	0.0117 ± 0.0007	0.0152 ± 0.0022	0.0155 ± 0.0008	0.0160 ± 0.0011	0.0146 ± 0.0006

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$).

Table 7.3 Tolerance indexes of the tested species exposed to arsenic (Rt:Rc and T.I.) on Malt Extract Agar (MEA) and Czapeck Dox Agar (CDA) [a].

MEA							
Tolerance Index		**Rc:Rt (%)**			**T.I. (%)**		
$NaAsO_2$ Concentration		**10 mg/L**	**20 mg/L**	**50 mg/L**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	88.7	66.7	50.3	88.9	62.5	52.8
	***P. lilacinum* FBL 478**	97.8	98.1	96.8	93.4	99.4	96.5
	***M. marquandii* FBL 484**	93.1	104.2	96.3	95.8	113.5	100.7
	***C. nanum* FBL 73**	106.4	105.1	116.7	110.6	101.2	111.4
CDA							
Tolerance Index		**Rc:Rt (%)**			**T.I. (%)**		
$NaAsO_2$ Concentration		**10 mg/L**	**20 mg/L**	**50 mg/L**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	98.3	100	99.4	93.6	94.6	109.6
	***P. lilacinum* FBL 478**	98.2	96.7	91.2	105.5	101.9	97.2
	***M. marquandii* FBL 484**	102.1	107.6	100	102.4	104.2	102.0
	***C. nanum* FBL 73**	102.6	99.3	96.7	101.9	105.4	96.3

[a] Rt:Rc is defined as the ratio of the colony extension rates in the presence (Rt) or absence (Rc) of arsenic. T.I. is defined as the ratio of the dry weight of the fungal biomass in the presence or absence of arsenic.

Most of the calculated values for the tolerance indexes were higher than 80%, disclosing a strong tolerance of the tested strains (**Table 7.3**). *A. spinosa* on MEA with 20 mg/L and 50 mg/L of sodium arsenite represented the only exception, showing both Rt:Rc and T.I. indexes lower than 70%. As the arsenic concentration increased in the medium, the values of both indexes reduced up to 50.3% for Rt:Rc and 52.8% for T.I. This trend was observed only on MEA, as the tolerance indexes for *A. spinosa* on CDA were >90%. It is worth mentioning that in some cases, the tolerance indexes were higher than 110%. In fact, the Rt:Rc for *C. nanum* grown on MEA amended with 50 mg/L sodium arsenite was 116.7%, while the T.I. for *C. nanum* on MEA with 10 mg/L and 50 mg/L were 110.6% and 111.4%, respectively (**Table 7.3**). However, the diametric values and biomass production of *C. nanum* on MEA for all tested As concentrations were not significantly different from that of the control (**Table 7.1** and **Table 7.2**). Regarding *M. marquandii*, the T.I. was 113.5% on MEA with 20 mg/L, even if biomass production on MEA control was not significantly different from that on MEA with 20 mg/L (**Table 7.2** and **Table 7.3**).

The addition of different concentrations of sodium arsenite to both media did not change the medium pH (**Table 7.4**). Regardless of As concentration, *A. spinosa* lowered the pH of both tested media, while *P. lilacinum*, *M. marquandii*, and *C. nanum* increased CDA pH (**Table 7.4**). No variations of MEA pH were observed in *M. marquandii* and *C. nanum*, while *P. lilacinum* was able to slightly acidify MEA (control, 10, and 20 mg/L of sodium arsenite) (**Table 7.4**).

7.3.3.2 EVALUATION OF AS BIOACCUMULATION IN FUNGAL BIOMASS

All of the tested species under all of the tested conditions were able to accumulate As (**Table 7.5**). The highest value of As concentration was observed in *C. nanum* on both MEA with 50 mg/L of sodium arsenite and CDA with 50 mg/L, while *A. spinosa* on CDA and MEA with 50 mg/L showed the highest values of As content. The statistical analysis showed that for all tested species, arsenic concentrations in the samples of all tested conditions were statistically different when compared with those of the control samples (**Table 7.5**). Cultural conditions, at least in some cases, influenced the concentration and content of As in fungi.

Table 7.4 pH values of the medium after growth of the fungal species for seven days at 25 °C. Data are expressed as the mean ± standard error of independent biological replicates [a].

		Medium pH							
Medium		**MEA**				**CDA**			
$NaAsO_2$ Concentration		**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
	Blank	5.4 ± 0.1	5.3 ± 0.0	5.3 ± 0.1	5.6 ± 0.1	5.7 ± 0.0	5.7 ± 0.0	5.6 ± 0.1	5.8 ± 0.1
Species	***A. spinosa* FBL 69**	3.5 ± 0.1 ***	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1***	4.4 ± 0.0*	4.5 ± 0.1	4.6 ± 0.1 **	4.4 ± 0.0 ***
	***P. lilacinum* FBL 478**	4.9 ± 0.2	4.6 ± 0.1	4.8 ± 0.1	5.2 ± 0.1	6.3 ± 0.0***	6.2 ± 0.0	6.0 ± 0.1	6.2 ± 0.0
	***M. marquandii* FBL 484**	5.3 ± 0.0	5.4 ± 0.0	5.3 ± 0.1	5.4 ± 0.1	6.4 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6.1 ± 0.0
	***C. nanum* FBL 73**	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.5 ± 0.1	6.7 ± 0.0**	6.3 ± 0.1	6.5 ± 0.0 **	6.3 ± 0.0

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the blank and the species for the respective treatments (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$).

Table 7.5 As content and As concentration in the tested species' fungal biomass after growth of the fungal species for seven days at 25 °C. Data are expressed as the mean ± standard deviation of independent biological replicates [a].

As content in Fungal Biomass (ng)									
Medium		**MEA**				**CDA**			
$NaAsO_2$ Concentration		**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	11.1 ± 4.5	2066 ± 57 ***	3378 ± 254 ***	5305 ± 209 ***	6.2 ± 5.4	1538 ± 991	3634 ± 222 ***	9996 ± 1135 **
	***P. lilacinum* FBL 478**	4.2 ± 4.7	1264 ± 34 ***	2351 ± 138 ***	5453 ± 474 *	**≤ LOD [b]**	1513 ± 58	2457 ± 522	6360 ± 2501
	***M. marquandii* FBL 484**	1.9 ± 2.9	746 ± 39 ***	1377 ± 347	3103 ± 709 *	5.2 ± 3.6	878 ± 77 ***	1668 ± 382 *	3504 ± 621 **
	***C. nanum* FBL 73**	1.8 ± 3.5	683 ± 24 ***	1278 ± 155 **	2577 ± 1164	2.3 ± 3.6	832 ± 54 ***	1333 ± 584	3170 ± 221 ***
As concentration in Fungal Biomass (µg/g)									
Medium		**MEA**				**CDA**			
$NaAsO_2$ Concentration		**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	0.13 ± 0.0052	26 ± 1.1 ***	62 ± 7.9 **	115 ± 12 ***	0.06 ± 0.05	16 ± 10	38 ± 2 ***	90 ± 13 **
	***P. lilacinum* FBL 478**	0.10 ± 0.11	32 ± 0.91 ***	56 ± 6.6 **	133 ± 9.3 **	**≤ LOD [b]**	35 ± 2	58 ± 11	160 ± 69
	***M. marquandii* FBL 484**	0.11 ± 0.18	32 ± 1.9 ***	51 ± 15	129 ± 8.2 ***	0.22 ± 0.16	37 ± 7.8 *	68 ± 10 **	146 ± 22 **
	***C. nanum* FBL 73**	0.17 ± 0.34	59 ± 5.2 ***	120 ± 11 ***	218 ± 94	0.13 ± 0.21	54 ± 4 ***	81 ± 26	217 ± 14 ***

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$). [b] value below detection limit (LOD).

7.3.3.3 EVALUATION OF ELEMENT CONCENTRATIONS IN FUNGAL BIOMASS

To evaluate the different accumulation of the elements in the fungal biomass of *A. spinosa*, *C. nanum*, *M. marquandii*, and *P. lilacinum* grown on MEA and CDA media (control) and on MEA and CDA media added with 10 mg/L, 20 mg/L and 50 mg/L of sodium arsenite, the concentration variability of As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Sn, and Sr was assessed (Supplementary Materials **Supplementary Table 7.8Supplementary Table 7.11**).

Principal component analysis performed on the obtained data allowed us to cluster the samples (scores) according to the different accumulation of the elements (loadings) in the four tested strains in the different experimental conditions. Five significant components accounting for 71.47% were obtained (the scores and loadings are shown in Supplementary Materials **Supplementary Table 7.14** and **Supplementary Table 7.15**); the variance explained by each component was 22.3%, 18.2%, 15.3%, 8.9%, and 6.7%. The first component (PC1), which explains 22.3% of the total variance, well separated the samples of the fungal species in which higher concentrations of As were accumulated from the others. On the other hand, PC2, which explains 18.2% of the total variance, well clustered the elements, depending on their concentration variability among the samples and separated the samples of the fungal strains grown on MEA medium from those grown on the CDA medium. Therefore, PC1 and PC2, which explain 40.5% of the total variance, are represented in the score plot and loading plot of **Figure 7.1**.

In the score plot in Figure 1, we can observe that all of the samples of the fungal species grown on CDA medium were separately clustered from the samples grown on MEA due to their different variability in element concentrations. In fact, the fungal strains grown on CDA showed higher concentrations (Supplementary Materials **Supplementary Table 7.8Supplementary Table 7.11**) of Fe, K, Mg, Na, P, and S (because of the higher accessibility of nutrients contained in CDA medium; composition is reported in Section 2.1), which are plotted on the left upper part of the corresponding loading plot. Among the four species, *A. spinosa* samples were separately clustered from the others, this can reasonably be ascribed to the different variability in concentrations of Ca, Co, Fe, Mg, P, and Sr, plotted on the left part of the corresponding loading plot. Indeed, as can be seen from Supplementary Materials**Supplementary Table 7.8** and **Supplementary Table 7.11Supplementary Table 7.10**, *A. spinosa* was found to be the strain with the highest concentrations of Ca, Co, Fe, Mg, P, and Sr. On the other hand, *P. lilacinum* appeared to accumulate the highest concentration of K, and *C. nanum* and *M. marquandii* were found to be the species with the highest concentrations of Na and S.

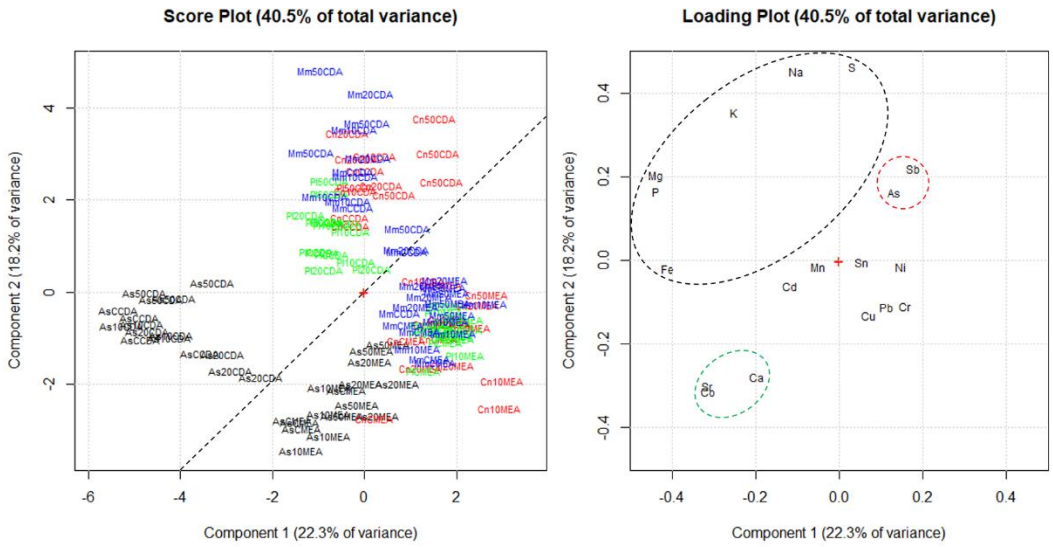


Figure 7.1 Score plot and loading plot of PC1 and PC2 obtained by the Principal Component Analysis (PCA) performed on the element concentrations determined in the fungal biomass of the tested species *A. spinosa* (As), *C. nanum* (Cn), *M. marquandii* (Mm), and *P. lilacinum* (Pl) grown on MEA and CDA media (C) and on MEA and CDA media added with 10 mg/L, 20 mg/L and 50 mg/L of sodium arsenite, respectively. Sample labels: AsCMEA, As10MEA, As20MEA, As50MEA, AsCCDA, As10CDA, As20CDA, As50CDA (black color); MmCMEA, Mm10MEA, Mm20MEA, Mm50MEA, MmCCDA, Mm10CDA, Mm20CDA, Mm50CDA (blue color); PlCMEA, Pl10MEA, Pl20MEA, Pl50MEA, PlCCDA, Pl10CDA, Pl20CDA, Pl50CDA (green color); CnCMEA, Cn10MEA, Cn20MEA, Cn50MEA, CnCCDA, Cn10CDA, Cn20CDA, Cn50CDA (red color).

Cadmium, Cu, Cr, Mn, Ni, Pb, and Sn were plotted on the central part of the loading plot, since their concentration values had poor variability among the samples. Therefore, As addition in both culture media did not appear to have affected the accumulation of these elements by the different fungal species. On the contrary, from the score plot of **Figure 7.1**, we can observe that within each cluster, samples grown on culture media added with 50 mg/L of sodium arsenite, were plotted in the direction of As and Sb on the right upper part of the loading plot, since they showed different concentration variability of these two elements. In fact, from Supplementary Materials **Supplementary Table 7.8Supplementary Table 7.11**, we can observe that these samples accumulated the highest concentrations of As and Sb. The accumulation of As and Sb increased as the addition of sodium arsenite in the culture media increased, thus revealing a dose-dependent response for all of the tested fungal strains.

7.3.3.4 EVALUATION OF SIDEROPHORE ACTIVITY OF TESTED FUNGI IN THE PRESENCE OF ARSENIC AND IRON.

Optical density values, reported in Table 6, show the tested species' siderophore activity for Fe and As. EDTA (180 mM), which showed complete chelation activity and therefore decolorization, was used as a reference. For Fe-CAS, *A. spinosa* and *P. lilacinum* both showed high chelation activity after 14 growth days. *C. nanum* and *M. marquandii*, despite to a lower extent, showed decolorization, reaching the maximum chelation activity at 7 and 21 growth days, respectively (**Table 7.6**). The statistical analysis revealed that the OD values for Fe-CAS of each tested species were significantly lower than that of the control (**Table 7.6**). The tested species generally showed modest Fe-chelating activity, but a very low or absent affinity for As. In fact, for As-CAS, *A. spinosa*, *C. nanum*, and *M. marquandii* showed only a slight absorbance reduction after 21 days. The OD values for As-CAS of *C. nanum* after 21 days were the lowest and statistically different from the control (**Table 7.6**).

Table 7.6 Optical density (OD) measurements for the tested species in the Fe-CAS and As-CAS assays. Data are expressed as the mean ± standard error of independent biological replicates a.

		Optical Density					
		Fe-CAS			As-CAS		
Control		0.523 ± 0.003			0.399 ± 0.006		
180 mM EDTA		0.067 ± 0.000 ***			0.047 ± 0.003 ***		
Days of Fungal Growth		**7 d**	**14 d**	**21 d**	**7 d**	**14 d**	**21 d**
Species	***A. spinosa* FBL 69**	0.221 ± 0.003 ***	0.202 ± 0.003 ***	0.220 ± 0.004 ***	0.386 ± 0.010	0.373 ± 0.010	0.358 ± 0.012
	***P. lilacinum* FBL 478**	0.272 ± 0.006 ***	0.202 ± 0.001 ***	ND	0.390 ± 0.014	0.438 ± 0.011	0.468 ± 0.008 ***
	***M. marquandii* FBL 484**	0.496 ± 0.003 ***	0.479 ± 0.001 ***	0.424 ± 0.019 *	0.414 ± 0.012	0.415 ± 0.009	0.378 ± 0.017
	***C. nanum* FBL 73**	0.380 ± 0.004 ***	0.412 ± 0.005 ***	0.413 ± 0.002 ***	0.330 ± 0.017	0.335 ± 0.025	0.318 ± 0.008 ***

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$).

7.3.4 DISCUSSION

This work evaluated the potential of four saprotrophic fungal species to tolerate high sodium arsenite concentrations, accumulate arsenic in their biomass, and release siderophores with an affinity for the complexation of As and Fe. The tested species, even though they have been previously reported for their potential in the bioremediation of other toxic elements (Coles et al., 1999; Lukšienė et al., 2012; Sharma and Adholeya, 2011; Słaba and Długoński, 2011; Słaba et al., 2005; Xia et al., 2015; Xu et al., 2017; Zucconi et al., 2003), have never been studied before for their tolerance to As.

The tested species were tolerant to all of the tested As concentrations, as pointed out by the tolerance indexes in almost all experimental conditions (**Table 7.3**). In fact, only *A. spinosa* was very sensitive to increasing As concentrations on MEA, despite it growing in all tested conditions (**Table 7.3**). Since this sensitivity was observed only on MEA, this is clearly related to the differences in nutritional conditions. In particular, it may be determined by the influence of one or more interacting factors including increased As bioavailability, reduced nutrient bioavailability, formation of toxic As organic species by interaction of arsenite with MEA components, and inhibition of fundamental enzyme activities due to As(III) reactions to thiols (Hughes, 2002). In fact, malt extract generally possesses a rich composition of carbohydrates, proteins, peptides, amino-acids, and vitamins (Waites et al., 2001; Zimbro et al., 2009), which require an efficient extracellular enzyme system to be metabolized. On the contrary, CDA grants a greater accessibility of nutrients as it is characterized by a composition mainly based on salts including sodium nitrate as the N source and sucrose as the main C and energy source. However, the importance of the nutritional conditions in determining the tolerance to As seems to be species specific. In fact, contrary to *A. spinosa*, *C. nanum* was not affected in tolerance to As by the differences in nutritional conditions, despite, in all conditions including the controls, it showing a greater diametric growth and an higher biomass production on CDA than on MEA (coherently with the higher accumulation of Fe, K, Mg, Na, P, and S) (**Table 7.1** and **Table 7.2**). Different patterns of growth between the different nutritional conditions were observed in *M. marquandii*, which showed a higher diametric growth on CDA than on MEA, but not a significant greater biomass production. However, it was able to tolerate As in both media. Considering the equal biomass production in *M. marquandii* on both media, the greater development of the colony on CDA suggests a greater presence of exploration hyphae with a lower number of branches and, therefore, a lower density. It is also worth mentioning that *M. marquandii* and *C. nanum* were slightly stimulated in the presence of sodium arsenite, as the tolerance indexes were higher than 110% (**Table 7.3**). Given that toxic elements can influence the physiology and morphology of fungal mycelia, resulting interactions can lead to different mycelial growth strategies and hyphal aggregation (Ceci et al., 2018; Fomina et al.,

2003). Adaptative fungal growth patterns, observed at high metal concentrations in different fungi, led to growth exceeding the control and tolerance index higher than 1 (100%) (Valix and Loon, 2003). High fungal tolerance to sodium arsenite is even more noteworthy, if it is considered that chemical species of arsenic (III) are thought to be more toxic for these organisms than arsenic(V) ones (Hughes, 2002; Wang et al., 2011). High fungal tolerance to toxic elements may be related to metabolism-dependent or -independent mechanisms of tolerance/resistance to cope with chemical stress (Ceci et al., 2018; Gadd et al., 2012). Despite there being no previous data on the As tolerance of the tested species, several fungal species have been reported as being tolerant to very high concentrations of As trivalent and pentavalent chemical species (Singh et al., 2015a; Srivastava et al., 2011; Su et al., 2010, 2011; Vala, 2010; Zeng et al., 2010) including As minerals (Adeyemi, 2009; Ceci et al., 2015a). For instance, Vala (2010) reported that *Aspergillus candidus* was very tolerant to 25 and 50 mg/L of sodium arsenite and sodium arsenate, showing a luxuriant growth in all treatments. In another work, Srivastava et al. (2011) reported values of tolerance index around 1 for taxa belonging to different genera including *Aspergillus*, *Penicillium*, *Rhizopus*, and *Trichoderma*, which were tested to high concentrations of sodium arsenate up to 10,000 mg/L.

Other mechanisms that result from interactions between fungi and toxic elements include accumulation of metals and metalloids, and the production of extracellular enzymes and other metabolites such as organic acids and siderophores (Ceci et al., 2018; Gadd, 2010). The dominance of As species in the environment is strictly dependent on pH and redox potential (E_h). Under reducing conditions and pH less than 9.2, the arsenite species H_3AsO_3 predominates, while under oxidizing conditions, the arsenate species $H_2AsO_4^-$ and $HAsO_4^{2-}$ are dominant at low and high pH, respectively (Smedley and Kinniburgh, 2002). Arsenic addition can alter the pH of the medium and its presence, influencing the fungal metabolism, which can indirectly determine pH modification during the fungal growth. In this study, none of the tested species at any concentration showed a significant pH modification compared the control, nor did the addition of sodium arsenite change the pH of the media in the chemical blanks (**Table 7.4**). Conversely, the pH variations due to the fungal growth were strongly related to the tested medium, with the pH values of all tested conditions on MEA generally more acidic than those on CDA. Once again, this may be explained by the composition of the different media. Moreover, the pH was strongly related to the metabolism of the tested species (**Table 7.4**). In fact, *A. spinosa* acidified both media more than the other species. This may be due to the release of organic acids by this species, which can excrete gluconic acid (Takahashi and Asai, 1930). Other species of the *Absidia* genus can produce organic acids such as *A. coerulea* that can release citric, tartaric, succinic, and glutaric acids (Domsch et al., 2007). Fungi can produce and release organic acids (e.g., oxalic, citric, and lactic acids) according to the growth conditions including the C and N sources, the

availability of nutrients, and the presence of trace elements (Ceci et al., 2015a, 2015b; Gadd, 1999; Gadd et al., 2012). The pH is another important factor that can influence organic acid production and can play an important role in the transformations of minerals and elements by fungi, controlling hydrolysis and complexation, mineral dissolution and precipitation, medium acidification or alkalinization (Ceci et al., 2015a, 2015b; Gadd, 2010; Gadd et al., 2012). However, acidification can also be due to other mechanisms including proton excretion through plasma membrane ATPase, uptake of cations in exchange for H^+, and CO_2 production for fungal respiration (Ceci et al., 2018). Unlike the situation for *P. lilacinum*, that on MEA slightly lowered the pH while on CDA it was slightly increased, showing a greater influence of the medium than any other species (**Table 7.4**). In fact, the medium pH around 6 for *P. lilacinum*, *M. marquandii*, and *C. nanum* on CDA (**Table 7.4**) may be related to different factors such as the buffering effects of CDA components (e.g., phosphate) and ammonium and lipase production (Srivastava et al., 2011). As well as the assimilation of nitrate, the N source in CDA, can result in medium alkalinization for the release of OH^-, as observed with *Penicillum radicum* and *P. cyclopium* (Whitelaw et al., 1999). pH modulation by fungi is important in the regulation of several enzymes, as reported, for instance, in the case of pathogenic fungi (Alkan et al., 2013; Vylkova et al., 2011). For example, in the entomopathogenic fungus *Metarhizium anisopliae*, the production of ammonia, increasing the medium pH, allowed the production of subtilisin proteases, which were active only at basic pH (Leger et al., 1999).

All tested species were able to accumulate high concentrations of As in their biomass during the seven days of growth (**Table 7.5**). In *C. nanum*, As concentration was the highest at 50 mg/L sodium arsenite in both media, while *A. spinosa* showed the highest As content. This difference is mainly due to the biomass production, which was higher in *A. spinosa* than in *C. nanum* (**Table 7.2**). In general, the arsenic content and concentration in fungal biomass increased as arsenic concentrations increased in media (**Table 7.5**). Along with As, Sb concentration also increased. It is important to note that Sb showed the same behavior of As because it was present as an impurity (<0.5%) in the sodium arsenite added in the culture media. This behavior is mainly due to the chemical similarities between the two metalloids, both belonging to Group 15 of the periodic table (Wilson et al., 2010), which usually leads them to follow similar accumulation pathways (Fu et al., 2016). However, even if Sb was present at a relatively low concentration, it was highly accumulated by all of the tested species, thus revealing the outstanding Sb accumulation ability of these strains, which merits further investigation. Moreover, increasing As concentrations in both culture media did not affect the accumulation in the fungal biomass of trace and ultra-trace elements (Cd, Cu, Cr, Mn, Ni, Pb, and Sn). Results in Supplementary Materials S1 confirmed that in both culture media, *C. nanum* was the species able to accumulate the highest concentrations of As and Sb, while *A.*

spinosa was found to be the strain with the lowest accumulation ability of both elements, which was also observable in the plots obtained by principal component analysis.

Bioaccumulation of As was reported in several works in the presence of trivalent or pentavalent arsenic with fungi belonging to different genera (e.g., *Aspergillus*, *Penicillium*, and *Trichoderma*) (Adeyemi, 2009; Singh et al., 2015a; Srivastava et al., 2011; Su et al., 2010; Vala, 2010; Zeng et al., 2010). For instance, after five days of growth with 50 mg/L As(V), As bioaccumulation in *Penicillium janthinellum* was 87.0 μg/g (Zeng et al., 2010). The highest As removal in *Aspergillus candidus* were observed after three days of growth in the presence of 25 mg/L or 50 mg/L of trivalent or pentavalent arsenic (Vala, 2010). Iron-coated fungal biomass of a *Paecilomyces* sp. successfully removed As(III) from aqueous solution by biosorption (Acosta Rodríguez et al., 2013). Arsenic can be transported inside fungal cells via specific or non-specific transport processes (e.g., via specific and non-specific phosphate, glycerol, or hexose transporters) and mechanisms involved in As bioaccumulation in fungi can include surface association (e.g., biosorption to cell wall components), vacuolar compartmentalization, chelation, immobilization, and sequestration by metal-binding peptides (e.g., glutathione) (Gadd et al., 2012; Singh et al., 2015a). In *Saccharomyces cerevisiae*, the arsenate, taken up by phosphate transporter, is reduced to arsenite inside the fungal cell, conjugated with glutathione, and transported into vacuoles (Ghosh et al., 1999). According to Cánovas and De Lorenzo (2007), similar mechanisms were also reported in the arsenate-hypertolerant *Aspergillus* sp. P37. In fungi, As can be methylated, resulting in As volatilization, and As efflux can occur by arsenite extrusion through the arsenite carrier protein in the plasma membrane (e.g., Acr3p channel in *S. cerevisiae*) (Gadd et al., 2012; Ghosh et al., 1999; Singh et al., 2015a; Srivastava et al., 2011; Su et al., 2010; Urík et al., 2007). These mechanisms can influence arsenic mobilization in the environment.

Since the mobilization of As from rocks, minerals, soils, and other substrates by fungi and other organisms can also be caused by the production of siderophores (Gadd et al., 2012), they may represent feasible, environmentally-friendly bioresources for bioremediation application (Ahmed and Holmström, 2014; Saha et al., 2016). In this study, the tested species showed the production of siderophores with a high affinity for Fe complexation, while low or no affinity for As (**Table 7.6**). In *A. spinosa* and *C. nanum*, albeit to a very small extent, siderophore activity in chelating As was observed. In contrast, *P. lilacinum* and *M. marquandii* showed OD values greater than the reference value. This may be explained by the presence of secondary metabolites in the culture filtrates, interfering with the assay and by a low affinity of siderophores for As. With reference to the secondary metabolites, some pigments in *P. lilacinum* and *M. marquandii* have been isolated and characterized (Cabrera et al., 2006; Dong et al., 2012; Hong, Jongki et al., 2009) and may have influenced the assay.

Conversely, all the tested species released siderophores with an Fe affinity. *A. spinosa* along with *P. lilacinum* showed the highest affinity for Fe complexation since the 14 days of growth reading, with *A. spinosa* showing a slight reduction of chelation at 21 days. A reduction in the effectiveness of Fe chelation over growth time was also observed in *C. nanum*, which showed the lowest OD values at seven days. The efficacy of *A. spinosa* in chelating Fe agrees with the fact that is reported in literature, which is to produce bacterioferritin (Carrano et al., 1996). This special siderophore belongs to the carboxylate type of siderophores, generally produced by Zygomycetes. Despite this varying according to the species, it has been observed for *Petromyces alliaceus* that its siderophores have a better affinity for Fe than for other tested metals and metalloids (Mehnert et al., 2017).

The highest ability of *A. spinosa* and *P. lilacinum* in Fe complexation was confirmed by the higher Fe accumulation in the fungal biomass of these strains (Supplementary Materials**Supplementary Table 7.8Supplementary Table 7.10**), which were found to be the species with the highest concentrations of Fe, thus confirming their efficacy in chelating Fe. Furthermore, in *A. spinosa* biomass were found the highest concentrations of P, this may be explained by the known ability of Mucorales to accumulate P as polyphosphates, which serves as a phosphate store (Werner et al., 2007) and as chelators for metals and metalloids (De Lima et al., 2003).

In conclusion, the tested fungal species were tolerant to high concentrations of arsenic. In most of the cases, the nutritional conditions did not influence the tolerance to arsenic. Therefore, the tested fungal species are promising novel candidates for future scale-up studies in As mycoremediation. Considering their potential in As bioaccumulation, these species can be new tools in biotechnological applications (e.g., As recovery). Moreover, from our results, it emerged that these species can uptake other potentially toxic elements such as antimony, which have a strategic importance for industrial and high-tech applications. The production of siderophores with high affinity for Fe has been observed. Considering that siderophores may be involved in the process of tolerance and bioaccumulation, further research is needed to evaluate an eventual release of siderophores with As affinity triggered by As exposure. Finally, this study also led to results that have a potential ecological significance with respect to the role of fungi in arsenic geochemistry and its cycle in ecosystems.

7.3.5 REFERENCES

Acosta Rodríguez, I., Martínez-Juárez, V.M., Cárdenas-González, J.F., and Moctezuma-Zárate, M. de G. (2013). Biosorption of Arsenic(III) from aqueous solutions by modified fungal biomass of *Paecilomyces* sp. Bioinorganic Chemistry and Applications *2013*, 1–5.

Adeyemi, A.O. (2009). Bioaccumulation of arsenic by fungi. American Journal of Environmental Sciences *5*, 364–370.

Agatemor, C., and Beauchemin, D. (2011). Matrix effects in inductively coupled plasma mass spectrometry: A review. Analytica Chimica Acta *706*, 66–83.

Ahmed, E., and Holmström, S.J.M. (2014). Siderophores in environmental research: roles and applications. Microbial Biotechnology *7*, 196–208.

Alkan, N., Espeso, E.A., and Prusky, D. (2013). Virulence Regulation of Phytopathogenic Fungi by pH. Antioxidants & Redox Signaling *19*, 1012–1025.

Astolfi, M.L., Marconi, E., Protano, C., Vitali, M., Schiavi, E., Mastromarino, P., and Canepari, S. (2018). Optimization and validation of a fast digestion method for the determination of major and trace elements in breast milk by ICP-MS. Analytica Chimica Acta *1040*, 49–62.

Baroni, F., Boscagli, A., Di Lella, L.A., Protano, G., and Riccobono, F. (2004). Arsenic in soil and vegetation of contaminated areas in southern Tuscany (Italy). Journal of Geochemical Exploration *81*, 1–14.

Bartlett, M.S. (1937). Properties of sufficiency and statistical tests. Proc. R. Soc. A *160*, 268–282.

Cabrera, G.M., Butler, M., Rodriguez, M.A., Godeas, A., Haddad, R., and Eberlin, M.N. (2006). A Sorbicillinoid Urea from an Intertidal *Paecilomyces marquandii*. Journal of Natural Products *69*, 1806–1808.

Canepari, S., Cardarelli, E., Pietrodangelo, A., and Strincone, M. (2006). Determination of metals, metalloids and non-volatile ions in airborne particulate matter by a new two-step sequential leaching procedurePart B: Validation on equivalent real samples. Talanta *69*, 588–595.

Canepari, S., Perrino, C., Astolfi, M., Catrambone, M., and Perret, D. (2009). Determination of soluble ions and elements in ambient air suspended particulate matter: Inter-technique comparison of XRF, IC and ICP for sample-by-sample quality control. Talanta *77*, 1821–1829.

Canepari, S., Astolfi, M., Marconi, E., and Farao, C. Caratterizzazione del refluo prodotto nel processo idrometallurgico per il recupero di elementi pregiati da schede elettroniche (RAEE) sviluppato da ENEA.

Cánovas, D., and De Lorenzo, V. (2007). Osmotic stress limits arsenic hypertolerance in *Aspergillus* sp. P37. FEMS Microbiology Ecology *61*, 258–263.

Carrano, C.J., Böhnke, R., and Matzanke, B.F. (1996). Fungal ferritins: The ferritin from mycelia of *Absidia spinosa* is a bacterioferritin. FEBS Letters *390*, 261–264.

Ceci, A., Kierans, M., Hillier, S., Persiani, A.M., and Gadd, G.M. (2015a). Fungal bioweathering of mimetite and a general geomycological model for lead apatite mineral biotransformations. Applied and Environmental Microbiology *81*, 4955–4964.

Ceci, A., Rhee, Y.J., Kierans, M., Hillier, S., Pendlowski, H., Gray, N., Persiani, A.M., and Gadd, G.M. (2015b). Transformation of vanadinite [$Pb_5(VO_4)_3Cl$] by fungi: Fungal biotransformation of vanadinite. Environmental Microbiology *17*, 2018–2034.

Ceci, A., Pinzari, F., Riccardi, C., Maggi, O., Pierro, L., Petrangeli Papini, M., Gadd, G.M., and Persiani, A.M. (2018). Metabolic synergies in the biotransformation of organic and metallic toxic compounds by a saprotrophic soil fungus. Applied Microbiology and Biotechnology *102*, 1019–1033.

Ceci, A., Pinzari, F., Russo, F., Persiani, A.M., and Gadd, G.M. (2019). Roles of saprotrophic fungi in biodegradation or transformation of organic and inorganic pollutants in co-contaminated sites. Applied Microbiology and Biotechnology *103*, 53–68.

Chlebicki, A. (2013). *Cephalotrichum stemonitis* as a biofilm inhabitant in gold mine in Poland. Acta Mycologica *43*, 67–70.

Cinti, D., Poncia, P.P., Brusca, L., Tassi, F., Quattrocchi, F., and Vaselli, O. (2015). Spatial distribution of arsenic, uranium and vanadium in the volcanic-sedimentary aquifers of the Vicano–Cimino Volcanic District (Central Italy). Journal of Geochemical Exploration *152*, 123–133.

Coles, K.E., David, J.C., Fisher, P.J., Lappin-Scott, H.M., and Macnair, M.R. (1999). Solubilisation of zinc compounds by fungi associated with the hyperaccumulator *Thlaspi caerulescens*. Botanical Journal of Scotland *51*, 237–247.

Conti, M.E., Canepari, S., Finoia, M.G., Mele, G., and Astolfi, M.L. (2018). Characterization of Italian multifloral honeys on the basis of their mineral content and some typical quality parameters. Journal of Food Composition and Analysis *74*, 102–113.

Cubadda, F., Ciardullo, S., D'Amato, M., Raggi, A., Aureli, F., and Carcea, M. (2010). Arsenic Contamination of the Environment−Food Chain: A Survey on Wheat as a Test Plant To Investigate Phytoavailable Arsenic in Italian Agricultural Soils and as a Source of Inorganic Arsenic in the Diet. Journal of Agricultural and Food Chemistry *58*, 10176–10183.

De Lima, M.A.B. de, Nascimento, A.E. do, Souza, W. de, Fukushima, K., and Campos-Takaki, G.M. de (2003). Effects of phosphorus on polyphosphate accumulation by Cunninghamella elegans. Brazilian Journal of Microbiology *34*, 363–372.

Domsch, K.H., Gams, W., and Anderson, T.-H. (2007). Compendium of soil fungi (Eching, Germany: IHW).

Dong, Q., Wang, H., Xing, X., and Ji, S. (2012). Identification and characterization of a special species of *Paecilomyces*. Annals of Microbiology*62*, 1587–1592.

Errasquín, E.L., and Vázquez, C. (2003). Tolerance and uptake of heavy metals by Trichoderma atroviride isolated from sludge. Chemosphere *50*, 137–143.

Fomina, M., Ritz, K., and Gadd, G.M. (2003). Nutritional influence on the ability of fungal mycelia to penetrate toxic metal-containing domains. Mycological Research *107*, 861–871.

Frau, F., and Ardau, C. (2003). Geochemical controls on arsenic distribution in the Baccu Locci stream catchment (Sardinia, Italy) affected by past mining. Applied Geochemistry *18*, 1373–1386.

Fu, Z., Wu, F., Mo, C., Deng, Q., Meng, W., and Giesy, J.P. (2016). Comparison of arsenic and antimony biogeochemical behavior in water, soil and tailings from Xikuangshan, China. Science of The Total Environment *539*, 97–104.

Gadd, G.M. (1999). Fungal Production of Citric and Oxalic Acid: Importance in Metal Speciation, Physiology and Biogeochemical Processes. In Advances in Microbial Physiology *41*, 47–92.

Gadd, G.M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology *156*, 609–643.

Gadd, G.M. (2017). The Geomycology of Elemental Cycling and Transformations in the Environment. Microbiology Spectrum *5*, 1–16.

Gadd, G.M., Rhee, Y.J., Stephenson, K., and Wei, Z. (2012). Geomycology: metals, actinides and biominerals. Environmental microbiology reports *4*, 270–296.

Ghosh, M., Shen, J., and Rosen, B.P. (1999). Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences *96*, 5001–5006.

Haas, H. (2014). Fungal siderophore metabolism with a focus on *Aspergillus fumigatus*. Natural Product Reports *31*, 1266–1276.

Harms, H., Wick, L.Y., and Schlosser, D. (2017). Chapter 31 The Fungal Community in Organically Polluted Systems. In Mycology, J. Dighton, and J.F. White, eds. (Boca Raton, FL: CRC Press), pp. 459–470.

Holinsworth, B., and Martin, J.D. (2009). Siderophore production by marine-derived fungi. BioMetals *22*, 625–632.

Hong, Jongki, Bae, Kyung Sook, Kim, Mi Ae, and Lee, Sang Mong (2009). α-Pyrones and Yellow Pigments from the Sponge-Derived Fungus *Paecilomyces lilacinus*. Bulletin of the Korean Chemical Society *30*, 188–192.

Hughes, M.F. (2002). Arsenic toxicity and potential mechanisms of action. Toxicology Letters *133*, 1–16.

Jiang, J.-R., Cai, L., and Liu, F. (2017). Oligotrophic fungi from a carbonate cave, with three new species of *Cephalotrichum*. Mycology *8*, 164–177.

Lee, S., and Lee, D.K. (2018). What is the proper way to apply the multiple comparison test? Korean Journal of Anesthesiology *71*, 353–360.

Leger, R.J.St., Nelson, J.O., and Screen, S.E. (1999). The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. Microbiology *145*, 2691–2699.

Levene, H. (1960). Robust tests for equality of variances. In Contributions to Probabilityand Statistics: Essays in Honor of Harold Hotelling, (Standford, CA: Stanford University Press), pp. 278–292.

Liang, X., and Gadd, G.M. (2017). Metal and metalloid biorecovery using fungi. Microbial Biotechnology *10*, 1199–1205.

Loppi, S., and Bargagli, R. (1996). Lichen biomonitoring of trace elements in a geothermal area (central Italy). Water, Air, and Soil Pollution *88*, 177–187.

Lukšienė, B., Druteikienė, R., Pečiulytė, D., Baltrūnas, D., Remeikis, V., and Paškevičius, A. (2012). Effect of microorganisms on the plutonium oxidation states. Applied Radiation and Isotopes *70*, 442–449.

Mandal, B. (2002). Arsenic round the world: a review. Talanta *58*, 201–235.

Marescotti, P., E, O., A, V., and G, P. (2011). Mineralogical and chemical investigations for the evaluation of arsenic background values in natural and anthropogenic soils: A case Study from the Colleferro industrial site (Valle del Sacco, Italy).

Massimi, L., Conti, M.E., Mele, G., Ristorini, M., Astolfi, M.L., and Canepari, S. (2019). Lichen transplants as indicators of atmospheric element concentrations: a high spatial

resolution comparison with PM10 samples in a polluted area (Central Italy). Ecological Indicators *101*, 759–769.

Mehnert, M., Retamal-Morales, G., Schwabe, R., Vater, S., Heine, T., Levicán, G.J., Schlömann, M., and Tischler, D. (2017). Revisiting the Chrome Azurol S assay for various metal ions. Solid State Phenomena *262*, 509–512.

Mohammed Abdul, K.S., Jayasinghe, S.S., Chandana, E.P.S., Jayasumana, C., and De Silva, P.M.C.S. (2015). Arsenic and human health effects: A review. Environmental Toxicology and Pharmacology *40*, 828–846.

Mukherjee, A., Das, D., Kumar Mondal, S., Biswas, R., Kumar Das, T., Boujedaini, N., and R. Khuda-Bukhsh, A. (2010). Tolerance of arsenate-induced stress in *Aspergillus niger*, a possible candidate for bioremediation. Ecotoxicology and Environmental Safety *73*, 172–182.

Piacentini, D., Falasca, G., Canepari, S., and Massimi, L. (2019). Potential of PM-selected components to induce oxidative stress and root system alteration in a plant model organism. Environment International *132*, 105094.

R Core Team (2021). Package stats. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online: https://www.R-project.org/ (accessed on 1/7/2021).

Renshaw, J.C., Robson, G.D., Trinci, A.P.J., Wiebe, M.G., Livens, F.R., Collison, D., and Taylor, R.J. (2002). Fungal siderophores: structures, functions and applications. Mycological Research *106*, 1123–1142.

Retamal-Morales, G., Mehnert, M., Schwabe, R., Tischler, D., Zapata, C., Chávez, R., Schlömann, M., and Levicán, G. (2018). Detection of arsenic-binding siderophores in arsenic-tolerating Actinobacteria by a modified CAS assay. Ecotoxicology and Environmental Safety *157*, 176–181.

Ristorini, M., Astolfi, M.L., Frezzini, M.A., Canepari, S., and Massimi, L. (2020). Evaluation of the Efficiency of *Arundo donax* L. Leaves as Biomonitors for Atmospheric Element Concentrations in an Urban and Industrial Area of Central Italy. Atmosphere *11*, 226.

RStudio Team (2016). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Russo, F., Ceci, A., Pinzari, F., Siciliano, A., Guida, M., Malusà, E., Tartanus, M., Miszczak, A., Maggi, O., and Persiani, A.M. (2019a). Bioremediation of DDT-contaminated agricultural soils: the potential of two autochthonous saprotrophic fungal strains. Applied and Environmental Microbiology *85*, e01720-19.

Russo, F., Ceci, A., Maggi, O., Siciliano, A., Guida, M., Petrangeli Papini, M., Černík, M., and Persiani, A.M. (2019b). Understanding fungal potential in the mitigation of contaminated areas in the Czech Republic: tolerance, biotransformation of hexachlorocyclohexane (HCH) and oxidative stress analysis. Environmental Science and Pollution Research *26*, 24445–24461.

Saha, M., Sarkar, S., Sarkar, B., Sharma, B.K., Bhattacharjee, S., and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. Environmental Science and Pollution Research *23*, 3984–3999.

Shapiro, S.S., and Wilk, M.B. (1965). An analysis of variance test for normality (complete samples). Biometrika *52*, 591.

Sharma, G., and Pandey, R. (2010). Influence of culture media on growth, colony character and sporulation of fungi isolated from decaying vegetable wastes. Journal of Yeast and Fungal Research *1*, 157–164.

Sharma, S., and Adholeya, A. (2011). Detoxification and accumulation of chromium from tannery effluent and spent chrome effluent by *Paecilomyces lilacinus* fungi. International Biodeterioration & Biodegradation *65*, 309–317.

Sharma, V.K., and Sohn, M. (2009). Aquatic arsenic: toxicity, speciation, transformations, and remediation. Environment International *35*, 743–759.

Singh, M., Srivastava, P.K., Verma, P.C., Kharwar, R.N., Singh, N., and Tripathi, R.D. (2015a). Soil fungi for mycoremediation of arsenic pollution in agriculture soils. Journal of Applied Microbiology *119*, 1278–1290.

Singh, R., Singh, S., Parihar, P., Singh, V.P., and Prasad, S.M. (2015b). Arsenic contamination, consequences and remediation techniques: A review. Ecotoxicology and Environmental Safety *112*, 247–270.

Słaba, M., and Długoński, J. (2011). Efficient Zn^{2+} and Pb^{2+} uptake by filamentous fungus *Paecilomyces marquandii* with engagement of metal hydrocarbonates precipitation. International Biodeterioration & Biodegradation *65*, 954–960.

Słaba, M., Bizukojć, M., Pałecz, B., and Długoński, J. (2005). Kinetic study of the toxicity of zinc and lead ions to the heavy metals accumulating fungus *Paecilomyces marquandii*. Bioprocess and Biosystems Engineering *28*, 185–197.

Smedley, P.L., and Kinniburgh, D.G. (2002). A review of the source, behaviour and distribution of arsenic in natural waters. Applied Geochemistry *17*, 517–568.

Srivastava, P.K., Vaish, A., Dwivedi, S., Chakrabarty, D., Singh, N., and Tripathi, R.D. (2011). Biological removal of arsenic pollution by soil fungi. Science of The Total Environment *409*, 2430–2442.

Su, S., Zeng, X., Bai, L., Jiang, X., and Li, L. (2010). Bioaccumulation and biovolatilisation of pentavalent arsenic by *Penicillin janthinellum*, *Fusarium oxysporum* and *Trichoderma asperellum* under laboratory conditions. Current Microbiology *61*, 261–266.

Su, S., Zeng, X., Bai, L., Li, L., and Duan, R. (2011). Arsenic biotransformation by arsenic-resistant fungi *Trichoderma asperellum* SM-12F1, *Penicillium janthinellum* SM-12F4, and *Fusarium oxysporum* CZ-8F1. Science of The Total Environment *409*, 5057–5062.

Takahashi, T., and Asai, T. (1930). On Gluconic Fermentation: Part I.[1)] on bacterium *Hoshigaki var rosea* nov. Spec. Proceedings of the Imperial Academy *6*, 348–351.

Urík, M., Čerňanský, S., Ševc, J., Šimonovičová, A., and Littera, P. (2007). Biovolatilization of arsenic by different fungal strains. Water, Air, and Soil Pollution *186*, 337–342.

Vala, A.K. (2010). Tolerance and removal of arsenic by a facultative marine fungus *Aspergillus candidus*. Bioresource Technology *101*, 2565–2567.

Valix, M., and Loon, L.O. (2003). Adaptive tolerance behaviour of fungi in heavy metals. Minerals Engineering *16*, 193–198.

Vivona, R., Preziosi, E., Madé, B., and Giuliano, G. (2007). Occurrence of minor toxic elements in volcanic-sedimentary aquifers: a case study in central Italy. Hydrogeology Journal *15*, 1183–1196.

Vylkova, S., Carman, A.J., Danhof, H.A., Collette, J.R., Zhou, H., and Lorenz, M.C. (2011). The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH. MBio *2*, e00055-11.

Waites, M.J., Morgan, N.L., Rockey, J.S., and Higton, G. (2001). Industrial microbiology: an introduction (Osney Mead, Oxford, UK ; Malden, MA: Blackwell Science).

Wang, Q., He, M., and Wang, Y. (2011). Influence of combined pollution of antimony and arsenic on culturable soil microbial populations and enzyme activities. Ecotoxicology *20*, 9–19.

Welch, B.L. (1951). On the comparison of several mean values: an alternative approach. Biometrika *38*, 933–943.

Werner, T.P., Amrhein, N., and Freimoser, F.M. (2007). Specific localization of inorganic polyphosphate (poly P) in fungal cell walls by selective extraction and immunohistochemistry. Fungal Genetics and Biology *44*, 845–852.

Whitelaw, M.A., Harden, T.J., and Helyar, K.R. (1999). Phosphate solubilisation in solution culture by the soil fungus *Penicillium radicum*. Soil Biology and Biochemistry *31*, 655–665.

Wilson, S.C., Lockwood, P.V., Ashley, P.M., and Tighe, M. (2010). The chemistry and behaviour of antimony in the soil environment with comparisons to arsenic: A critical review. Environmental Pollution *158*, 1169–1181.

Xia, L., Xu, X., Zhu, W., Huang, Q., and Chen, W. (2015). A Comparative Study on the Biosorption of Cd2+ onto *Paecilomyces lilacinus* XLA and Mucoromycote sp. XLC. International Journal of Molecular Sciences *16*, 15670–15687.

Xu, X., Xia, L., Chen, W., and Huang, Q. (2017). Detoxification of hexavalent chromate by growing *Paecilomyces lilacinus* XLA. Environmental Pollution *225*, 47–54.

Zeng, X., Su, S., Jiang, X., Li, L., Bai, L., and Zhang, Y. (2010). Capability of pentavalent arsenic bioaccumulation and biovolatilization of three fungal strains under laboratory conditions. Clean - Soil, Air, Water *38*, 238–241.

Zimbro, M.J., Power, D.A., Sharon, M.M., Wilson, G.E., and Johnson, J.A. (2009). Difco & BBL Manual: manual of microbiological culture media (Sparks, MD: Becton, Dickinson).

Zucconi, L., Ripa, C., Alianiello, F., Benedetti, A., and Onofri, S. (2003). Lead resistance, sorption and accumulation in a *Paecilomyces lilacinus* strain. Biology and Fertility of Soils*37*, 17–22.

7.3.6 SUPPLEMENTARY MATERIALS

7.3.6.1 SUPPLEMENTARY MATERIAL S1: ELEMENT CONCENTRATIONS IN FUNGAL BIOMASS

Supplementary Table 7.7 Detection limits (LOD) of elemental determinations (ng).

As	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Pb	S	Sb
1.2	4424	0.19	1.1	50	12	56	3280	125	3.2	26495	60	2226	1.3	4304	0.18

Supplementary Table 7.8 Element concentrations in fungal biomass of *Absidia spinosa* FBL 69 and *Purpureocillium lilacinum* FBL 478 grown on MEA.

Medium			**MEA**																					
Species			***Absidia spinosa* FBL 69**										***Purpureocillium lilacinum* FBL 478**											
As Concentration			**Control**			**10 mg/L**			**20 mg/L**			**50 mg/L**			**Control**			**10 mg/L**			**20 mg/L**			**50 mg/L**		
Technique	**UoM**	**Element**	**Mean**	±	*SD*	**Mean**	±	*SD*	**Mean**	±	*SD*	**Mean**	±	*SD*	**Mean**	±	*SD*	**Mean**	±	*SD*	**Mean**	±	*SD*	**Mean**	±	*SD*
HG-AFS	mg/Kg	**As**	**0.13**	±	*0.05*	**26**	±	*1*	**62**	±	*8*	**115**	±	*12*	**0.1**	±	*0.1*	**32**	±	*1*	**56**	±	*7*	**133**	±	*9*
ICP-OES	g/Kg	**Ca**	**8.3**	±	*0.7*	**6.6**	±	*0.5*	**5.1**	±	*1.2*	**6.6**	±	*2.4*	**1.2**	±	*1.4*	**0.7**	±	*0.6*	**1.3**	±	*0.4*	**1.3**	±	*1.9*
ICP-MS	µg/Kg	**Cd**	**348**	±	*72*	**341**	±	*48*	**258**	±	*51*	**237**	±	*56*	**255**	±	*8*	**255**	±	*21*	**227**	±	*15*	**242**	±	*53*
ICP-MS	µg/Kg	**Co**	**92**	±	*20*	**104**	±	*23*	**67**	±	*20*	**72**	±	*29*	**50**	±	*11*	**47**	±	*9*	**35**	±	*12*	**42**	±	*12*
ICP-MS	µg/Kg	**Cr**	**418**	±	*117*	**548**	±	*169*	**533**	±	*316*	**383**	±	*111*	**375**	±	*62*	**367**	±	*45*	**382**	±	*136*	**291**	±	*90*
ICP-MS	mg/Kg	**Cu**	**7.4**	±	*1.5*	**8.2**	±	*2.2*	**7.2**	±	*1.9*	**7.5**	±	*2.6*	**5.7**	±	*0.3*	**4.6**	±	*1.1*	**4.3**	±	*1.4*	**3.9**	±	*0.9*
ICP-OES	mg/Kg	**Fe**	**110**	±	*7*	**89**	±	*14*	**75**	±	*19*	**77**	±	*16*	**25**	±	*2*	**34**	±	*10*	**39**	±	*11*	**28**	±	*14*
ICP-OES	g/Kg	**K**	**7.6**	±	*0.9*	**7.7**	±	*0.7*	**8.6**	±	*1.2*	**10**	±	*2*	**7.9**	±	*1.7*	**9.0**	±	*2.0*	**7.3**	±	*2.0*	**7.7**	±	*2.0*
ICP-OES	g/Kg	**Mg**	**1.2**	±	*0.2*	**1.1**	±	*0.1*	**0.88**	±	*0.09*	**0.99**	±	*0.12*	**0.36**	±	*0.04*	**0.38**	±	*0.07*	**0.38**	±	*0.06*	**0.34**	±	*0.14*
ICP-MS	mg/Kg	**Mn**	**16**	±	*7*	**38**	±	*18*	**23**	±	*14*	**36**	±	*22*	**8.5**	±	*4.7*	**15**	±	*10*	**11**	±	*6*	**8.7**	±	*4.0*
ICP-OES	g/Kg	**Na**	**4.4**	±	*0.6*	**4.3**	±	*0.7*	**2.9**	±	*0.7*	**2.2**	±	*0.2*	**2.2**	±	*0.6*	**1.7**	±	*0.3*	**2.0**	±	*1.0*	**0.79**	±	*0.81*
ICP-MS	mg/Kg	**Ni**	**0.64**	±	*0.20*	**0.82**	±	*0.18*	**1.3**	±	*1.0*	**0.83**	±	*0.22*	**1.4**	±	*0.3*	**1.3**	±	*0.1*	**1.3**	±	*0.2*	**1.1**	±	*0.3*
ICP-OES	g/Kg	**P**	**3.3**	±	*0.4*	**3.2**	±	*0.3*	**3.1**	±	*0.4*	**3.5**	±	*0.5*	**2.3**	±	*0.4*	**2.6**	±	*0.5*	**2.3**	±	*0.5*	**2.4**	±	*0.7*
ICP-MS	µg/Kg	**Pb**	**300**	±	*27*	**300**	±	*68*	**217**	±	*77*	**286**	±	*201*	**114**	±	*45*	**500**	±	*805*	**178**	±	*115*	**77**	±	*45*
ICP-OES	g/Kg	**S**	**3.4**	±	*0.4*	**3.0**	±	*0.5*	**2.5**	±	*0.3*	**2.8**	±	*0.3*	**2.3**	±	*0.4*	**2.4**	±	*0.5*	**2.3**	±	*0.6*	**2.0**	±	*1.0*
ICP-MS	µg/Kg	**Sb**	**47**	±	*24*	**112**	±	*24*	**166**	±	*55*	**344**	±	*89*	**163**	±	*91*	**289**	±	*200*	**377**	±	*202*	**289**	±	*56*
ICP-MS	µg/Kg	**Sn**	**89**	±	*26*	**158**	±	*69*	**136**	±	*68*	**139**	±	*72*	**79**	±	*38*	**190**	±	*263*	**71**	±	*47*	**74**	±	*51*
ICP-MS	mg/Kg	**Sr**	**56**	±	*13*	**62**	±	*10*	**43**	±	*4*	**46**	±	*8*	**1.9**	±	*0.2*	**2.2**	±	*0.5*	**2.5**	±	*0.5*	**2.1**	±	*0.5*

Supplementary Table 7.9 Element concentrations in fungal biomass of *Metarhizium marquandii* FBL 484 and *Cephalotrichum nanum* FBL 73 grown on MEA.

Medium			MEA																			
Species			*Metarhizium marquandii* FBL 484										*Cephalotrichum nanum* FBL 73									
As Concentration			Control			10 mg/L			20 mg/L			50 mg/L	Control			10 mg/L			20 mg/L			50 mg/L		
Technique	UoM	Element	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean ± SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
HG-AFS	mg/Kg	As	**0.11**	±	*0.18*	**32**	±	*2*	**51**	±	*15*	**129** ± *8*	**0.17**	±	*0.34*	**59**	±	*5*	**120**	±	*11*	**218**	±	*94*
ICP-OES	g/Kg	Ca	**0.72**	±	*0.33*	**1.7**	±	*1.0*	**0.99**	±	*0.48*	**1.0** ± *1.0*	**5.8**	±	*4.2*	**3.4**	±	*2.5*	**7.8**	±	*4.0*	**2.1**	±	*0.8*
ICP-MS	µg/Kg	Cd	**325**	±	*103*	**333**	±	*85*	**277**	±	*47*	**276** ± *28*	**328**	±	*20*	**298**	±	*81*	**296**	±	*12*	**317**	±	*61*
ICP-MS	µg/Kg	Co	**63**	±	*11*	**41**	±	*23*	**36**	±	*23*	**50** ± *21*	**18**	±	*20*	**37**	±	*35*	**21**	±	*21*	**26**	±	*18*
ICP-MS	µg/Kg	Cr	**596**	±	*151*	**472**	±	*307*	**362**	±	*126*	**455** ± *244*	**440**	±	*316*	**1066**	±	*833*	**725**	±	*156*	**462**	±	*137*
ICP-MS	mg/Kg	Cu	**8.3**	±	*4.4*	**4.3**	±	*0.9*	**3.4**	±	*0.7*	**4.2** ± *0.8*	**9.6**	±	*4.5*	**14**	±	*8*	**4.9**	±	*1.6*	**3.7**	±	*0.3*
ICP-OES	mg/Kg	Fe	**47**	±	*48*	**24**	±	*6*	**33**	±	*10*	**28** ± *2*	**116**	±	*140*	**116**	±	*98*	**94**	±	*104*	**43**	±	*14*
ICP-OES	g/Kg	K	**7.8**	±	*2.0*	**8.4**	±	*2.3*	**9.7**	±	*0.6*	**9.0** ± *1.0*	**5.2**	±	*0.8*	**3.9**	±	*1.7*	**5.0**	±	*1.0*	**6.3**	±	*2.5*
ICP-OES	g/Kg	Mg	**0.57**	±	*0.1*	**0.61**	±	*0.16*	**0.65**	±	*0.02*	**0.57** ± *0.02*	**0.6**	±	*0.1*	**0.41**	±	*0.12*	**0.58**	±	*0.06*	**0.57**	±	*0.24*
ICP-MS	mg/Kg	Mn	**38**	±	*69*	**3.1**	±	*0.8*	**2.3**	±	*0.3*	**2.3** ± *0.4*	**42**	±	*48*	**79**	±	*90*	**9.2**	±	*6.4*	**5.9**	±	*4.3*
ICP-OES	g/Kg	Na	**1.4**	±	*0.5*	**0.58**	±	*0.05*	**2.0**	±	*1.0*	**1.3** ± *0.6*	**2.7**	±	*1.1*	**1.5**	±	*0.5*	**2.5**	±	*0.8*	**2.6**	±	*1.1*
ICP-MS	mg/Kg	Ni	**0.7**	±	*0.2*	**2.6**	±	*2.7*	**0.55**	±	*0.15*	**1.0** ± *0.3*	**1.3**	±	*0.6*	**2.8**	±	*1.6*	**1.1**	±	*0.6*	**0.79**	±	*0.32*
ICP-OES	g/Kg	P	**3.2**	±	*0.9*	**3.3**	±	*0.9*	**3.7**	±	*0.2*	**3.4** ± *0.3*	**4.1**	±	*0.3*	**3.1**	±	*1.0*	**4.0**	±	*0.4*	**4.6**	±	*1.9*
ICP-MS	µg/Kg	Pb	**177**	±	*63*	**133**	±	*81*	**14**	±	*21*	**73** ± *63*	**460**	±	*521*	**653**	±	*495*	**756**	±	*1000*	**410**	±	*320*
ICP-OES	g/Kg	S	**3.8**	±	*0.5*	**3.6**	±	*0.7*	**4.4**	±	*0.2*	**3.9** ± *0.2*	**3.6**	±	*0.2*	**2.4**	±	*0.8*	**3.4**	±	*0.1*	**3.1**	±	*1.3*
ICP-MS	µg/Kg	Sb	**149**	±	*70*	**311**	±	*138*	**316**	±	*108*	**475** ± *230*	**220**	±	*83*	**376**	±	*51*	**691**	±	*279*	**742**	±	*162*
ICP-MS	µg/Kg	Sn	**142**	±	*93*	**108**	±	*60*	**49**	±	*10*	**64** ± *25*	**210**	±	*139*	**230**	±	*104*	**305**	±	*99*	**177**	±	*124*
ICP-MS	mg/Kg	Sr	**2.0**	±	*1.0*	**2.1**	±	*1.2*	**2.1**	±	*0.7*	**2.1** ± *1.5*	**6.8**	±	*1.9*	**5.4**	±	*0.5*	**5.8**	±	*0.7*	**6.1**	±	*0.9*

Supplementary Table 7.10 Element concentrations in fungal biomass of *Absidia spinosa* FBL 69 and *Purpureocillium lilacinum* FBL 478 grown on CDA.

Medium			CDA																				
Species			*Absidia spinosa* FBL 69											*Purpureocillium lilacinum* FBL 478									
As Concentration			Control			10 mg/L			20 mg/L			50 mg/L			Control			10 mg/L			20 mg/L			50 mg/L		
Technique	UoM	Element	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
HG-AFS	mg/Kg	As	**0.06**	±	*0.05*	**16**	±	*10*	**38**	±	*2*	**90**	±	*13*	≤ LOD*			**35**	±	*2*	**58**	±	*11*	**160**	±	*69*
ICP-OES	g/Kg	Ca	**5.1**	±	*1.0*	**5.0**	±	*1.0*	**4.9**	±	*1*	**5.1**	±	*0.3*	**2.5**	±	*2.4*	**0.64**	±	*0.33*	**3.0**	±	*1.0*	**1.2**	±	*1.4*
ICP-MS	µg/Kg	Cd	**371**	±	*17*	**388**	±	*28*	**352**	±	*44*	**284**	±	*41*	**208**	±	*29*	**203**	±	*16*	**186**	±	*17*	**202**	±	*52*
ICP-MS	µg/Kg	Co	**97**	±	*6*	**102**	±	*6*	**108**	±	*5*	**87**	±	*14*	**66**	±	*24*	**53**	±	*12*	**63**	±	*14*	**53**	±	*20*
ICP-MS	µg/Kg	Cr	**281**	±	*40*	**283**	±	*38*	**387**	±	*184*	**267**	±	*31*	**409**	±	*361*	**211**	±	*83*	**278**	±	*45*	**261**	±	*166*
ICP-MS	mg/Kg	Cu	**3.0**	±	*1.0*	**3.3**	±	*0.7*	**3.8**	±	*1.6*	**2.8**	±	*0.8*	**5.7**	±	*5.2*	**3.3**	±	*1.5*	**5.2**	±	*2.7*	**4.1**	±	*1.6*
ICP-OES	mg/Kg	Fe	**343**	±	*65*	**359**	±	*59*	**193**	±	*66*	**356**	±	*73*	**151**	±	*37*	**123**	±	*24*	**147**	±	*32*	**197**	±	*38*
ICP-OES	g/Kg	K	**19**	±	*3*	**20**	±	*2*	**15**	±	*4*	**22**	±	*1*	**27**	±	*4*	**25**	±	*3*	**26**	±	*5*	**34**	±	*7*
ICP-OES	g/Kg	Mg	**3.9**	±	*0.5*	**3.8**	±	*0.5*	**3.2**	±	*0.7*	**3.8**	±	*0.3*	**1.8**	±	*0.2*	**1.7**	±	*0.1*	**1.8**	±	*0.2*	**2.2**	±	*0.5*
ICP-MS	mg/Kg	Mn	**20**	±	*8*	**26**	±	*6*	**30**	±	*20*	**11**	±	*7*	**44**	±	*54*	**19**	±	*13*	**41**	±	*30*	**29**	±	*16*
ICP-OES	g/Kg	Na	**3.6**	±	*0.8*	**3.7**	±	*0.3*	**2.8**	±	*0.8*	**4.1**	±	*0.2*	**7.4**	±	*1*	**6.0**	±	*1.0*	**6.6**	±	*0.9*	**7.9**	±	*1.8*
ICP-MS	mg/Kg	Ni	**0.6**	±	*0.1*	**0.69**	±	*0.04*	**0.94**	±	*0.13*	**0.5**	±	*0.1*	**1.1**	±	*0.6*	**1.1**	±	*0.2*	**1.2**	±	*0.5*	**1.2**	±	*0.2*
ICP-OES	g/Kg	P	**25**	±	*4*	**24**	±	*3*	**20**	±	*4*	**25**	±	*2*	**10**	±	*1*	**10**	±	*1*	**11**	±	*1*	**12**	±	*2*
ICP-MS	µg/Kg	Pb	**110**	±	*26*	**93**	±	*13*	**83**	±	*18*	**76**	±	*53*	**168**	±	*52*	**83**	±	*70*	**210**	±	*239*	**87**	±	*13*
ICP-OES	g/Kg	S	**2.2**	±	*0.5*	**2.1**	±	*0.4*	**1.8**	±	*0.4*	**2.2**	±	*0.1*	**5.2**	±	*0.5*	**4.6**	±	*0.5*	**4.8**	±	*0.6*	**5.9**	±	*1.2*
ICP-MS	µg/Kg	Sb	**47**	±	*26*	**69**	±	*29*	**125**	±	*28*	**247**	±	*34*	**157**	±	*105*	**122**	±	*52*	**176**	±	*41*	**406**	±	*28*
ICP-MS	µg/Kg	Sn	**115**	±	*22*	**126**	±	*25*	**109**	±	*40*	**69**	±	*23*	**165**	±	*168*	**275**	±	*444*	**294**	±	*363*	**95**	±	*19*
ICP-MS	mg/Kg	Sr	**42**	±	*2*	**41**	±	*4*	**46**	±	*6*	**34**	±	*4*	**2.6**	±	*0.3*	**2.5**	±	*0.3*	**3.0**	±	*1.0*	**2.6**	±	*1.0*

*value below detection limits (LOD)

Supplementary Table 7.11 Element concentrations in fungal biomass of *Metarhizium marquandii* FBL 484 and *Cephalotrichum nanum* FBL 73 grown on CDA.

Medium			CDA																				
Species			*Metarhizium marquandii* FBL 484										*Cephalotrichum nanum* FBL 73										
As Concentration			Control			10 mg/L			20 mg/L			50 mg/L			Control			10 mg/L			20 mg/L			50 mg/L		
Technique	UoM	Element	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
HG-AFS	mg/Kg	As	**0.22**	±	*0.16*	**37**	±	*8*	**68**	±	*10*	**146**	±	*22*	**0.13**	±	*0.21*	**54**	±	*4*	**81**	±	*26*	**217**	±	*14*
ICP-OES	g/Kg	Ca	**0.46**	±	*0.14*	**1.6**	±	*1.5*	**1.1**	±	*1.1*	**2.3**	±	*2.0*	**3.6**	±	*2.3*	**1.2**	±	*0.4*	**1.5**	±	*0.9*	**2.6**	±	*2.3*
ICP-MS	µg/Kg	Cd	**335**	±	*55*	**316**	±	*51*	**290**	±	*37*	**393**	±	*398*	**345**	±	*166*	**354**	±	*80*	**285**	±	*55*	**283**	±	*51*
ICP-MS	µg/Kg	Co	**19**	±	*27*	**17**	±	*16*	**8.0**	±	*7.1*	**11**	±	*15*	**32**	±	*33*	**28**	±	*21*	**19**	±	*8*	**37**	±	*25*
ICP-MS	µg/Kg	Cr	**545**	±	*145*	**345**	±	*64*	**335**	±	*74*	**254**	±	*132*	**627**	±	*243*	**494**	±	*61*	**284**	±	*81*	**797**	±	*229*
ICP-MS	mg/Kg	Cu	**4.7**	±	*2.3*	**4.6**	±	*4.3*	**2.3**	±	*0.3*	**2.0**	±	*1.2*	**5.4**	±	*4.9*	**8.4**	±	*4.3*	**9**	±	*13*	**7.1**	±	*8.1*
ICP-OES	mg/Kg	Fe	**45**	±	*29*	**66**	±	*52*	**45**	±	*10*	**48**	±	*19*	**68**	±	*31*	**69**	±	*33*	**159**	±	*90*	**104**	±	*78*
ICP-OES	g/Kg	K	**13**	±	*4*	**15**	±	*2*	**14**	±	*2*	**13**	±	*4*	**33**	±	*14*	**23**	±	*9*	**25**	±	*6*	**24**	±	*4*
ICP-OES	g/Kg	Mg	**2.0**	±	*0.8*	**2.8**	±	*0.3*	**2.8**	±	*0.5*	**3.0**	±	*0.8*	**1.9**	±	*0.7*	**1.4**	±	*0.3*	**1.5**	±	*0.3*	**1.5**	±	*0.2*
ICP-MS	mg/Kg	Mn	**16**	±	*13*	**29**	±	*48*	**5.9**	±	*1.8*	**4.5**	±	*3.2*	**26**	±	*32*	**59**	±	*46*	**42**	±	*58*	**57**	±	*86*
ICP-OES	g/Kg	Na	**8.4**	±	*4.0*	**13**	±	*2*	**12**	±	*3*	**15**	±	*4*	**12**	±	*4*	**7.0**	±	*2.6*	**8.4**	±	*2.9*	**9.8**	±	*1.1*
ICP-MS	mg/Kg	Ni	**1.0**	±	*0.4*	**1.2**	±	*0.7*	**0.44**	±	*0.22*	**0.63**	±	*0.63*	**1.3**	±	*0.7*	**1.0**	±	*0.5*	**1.5**	±	*1.1*	**1.4**	±	*0.9*
ICP-OES	g/Kg	P	**10**	±	*4*	**11**	±	*3*	**9.9**	±	*2.5*	**11**	±	*4*	**13**	±	*5*	**9.2**	±	*2.5*	**10**	±	*3*	**10**	±	*1*
ICP-MS	µg/Kg	Pb	**88**	±	*22*	**116**	±	*82*	**333**	±	*594*	**104**	±	*103*	**347**	±	*322*	**321**	±	*177*	**113**	±	*21*	**439**	±	*519*
ICP-OES	g/Kg	S	**5.4**	±	*1.3*	**7.8**	±	*1.9*	**7.9**	±	*2.8*	**7.4**	±	*2.8*	**8.9**	±	*3.3*	**6.0**	±	*2.3*	**6.9**	±	*2.0*	**6.3**	±	*1.0*
ICP-MS	µg/Kg	Sb	**233**	±	*164*	**185**	±	*39*	**353**	±	*153*	**453**	±	*244*	**146**	±	*51*	**566**	±	*221*	**838**	±	*269*	**1708**	±	*188*
ICP-MS	µg/Kg	Sn	**98**	±	*42*	**80**	±	*23*	**191**	±	*255*	**63**	±	*45*	**212**	±	*182*	**267**	±	*101*	**283**	±	*229*	**340**	±	*246*
ICP-MS	mg/Kg	Sr	**6.8**	±	*2.4*	**7.9**	±	*0.8*	**8.5**	±	*0.9*	**7.1**	±	*3.9*	**6.3**	±	*1.8*	**4.8**	±	*0.8*	**4.0**	±	*0.5*	**4.1**	±	*0.3*

Supplementary Table 7.12 Mean values (mg/Kg d.w.), standard deviations (SD) and recovery (R) percentages of the element concentrations obtained in four replicates of the certified reference material BCR 482 lichen (IRMM, Geel, Belgium).

	BCR 482 lichen Certified Value				**Obtained Value**				
	Mean	±	*SD*	SD (%)	**Mean**	±	*SD*	SD (%)	R (%)
As	**0.85**	±	*0.07*	8	**0.82**	±	*0.05*	6	97
Cd	**0.56**	±	*0.02*	4	**0.57**	±	*0.02*	4	102
Co					**0.3**	±	*0.02*	7	
Cr	**4.1**	±	*0.2*	5	**3.1**	±	*0.2*	7	80
Cu	**7**	±	*0.2*	3	**6.7**	±	*0.2*	3	95
Fe					**791**	±	*7*	1	
Mg					**477**	±	*25*	5	
Mn					**30**	±	*0.3*	1	
Ni	**2.5**	±	*0.1*	4	**2.4**	±	*0.1*	4	97
Pb	**41**	±	*1*	2	**41**	±	*4*	10	99
Sb					**0.29**	±	*0.01*	3	
Sn					**1.8**	±	*1*	56	

Supplementary Table 7.13 Mean values (mg/Kg d.w.), standard deviations (SD) and recovery (R) percentages of the element concentrations obtained in four replicates of the certified reference material NIST 1515 apple leaves (National Institute of Standards and Technology).

	NIST 1515 apple leaves Certified Value				**Obtained Value**				
	Mean	±	*SD*	SD (%)	**Mean**	±	*SD*	SD (%)	R (%)
Ca	**15300**	±	*150*	1	**17100**	±	*98*	1	89
Cd	**0.013**	±	*0.002*	15	**0.012**	±	*0.001*	8	108
Cu	**5.64**	±	*0.24*	4	**5.6**	±	*0.3*	5	101
Fe	**83**	±	*5*	6	**99.6**	±	*0.5*	1	83
K	**16100**	±	*200*	1	**18800**	±	*320*	2	86
Mg	**2710**	±	*80*	3	**3080**	±	*53*	2	88
Mn	**54**	±	*3*	6	**59**	±	*1*	2	92
Na	**24.4**	±	*1.2*	5	**29**	±	*3*	10	84
Ni	**0.91**	±	*0.12*	13	**1.1**	±	*0.1*	9	83
P	**1590**	±	*110*	7	**1310**	±	*30*	2	121
Pb	**0.47**	±	*0.024*	5	**0.46**	±	*0.03*	7	102
Sr	**25**	±	*2*	8	**27.8**	±	*0.2*	1	90

7.3.6.2 SUPPLEMENTARY MATERIAL S2: SCORES AND LOADINGS OF THE FIVE SIGNIfiCANT COMPONENTS OBTAINED BY THE PCA PERFORMED ON THE ELEMENT CONCENTRATIONS IN FUNGAL BIOMASS OF THE TESTED SPECIES.

Supplementary Table 7.14 Scores of the five significant components (accounting for 71.47%) obtained by the PCA performed on the element concentrations in fungal biomass of the tested species.

Sample N°	Sample Label	PC1	PC2	PC3	PC4	PC5	Sample N°	Sample Label	PC1	PC2	PC3	PC4	PC5	Sample N°	Sample Label	PC1	PC2	PC3	PC4	PC5	Sample N°	Sample Label	PC1	PC2	PC3	PC4	PC5
1	AsCMEA	-1.6	-2.8	-0.2	0.8	-2.0	31	Mm50MEA	1.8	-0.2	-1.2	0.0	0.5	61	Cn50MEA	2.3	-0.8	-0.3	0.6	1.4	91	Mm50CDA	-1.2	3.0	-0.1	3.2	-4.7
2	AsCMEA	-1.4	-2.8	0.2	0.5	-1.8	32	Mm50MEA	1.8	0.0	-1.2	0.4	0.8	62	AsCCDA	-4.9	-1.0	0.1	0.4	0.3	92	Mm50CDA	-1.0	4.8	-1.8	0.1	0.0
3	AsCMEA	-1.4	-3.0	0.5	0.1	-1.4	33	PlCMEA	1.5	-0.8	-1.4	-1.5	0.0	63	AsCCDA	-3.6	-1.3	-0.9	0.0	0.0	93	PlCCDA	-1.0	0.9	-0.9	-1.2	0.7
4	AsCMEA	-0.4	-2.1	-0.9	-0.1	-0.4	34	PlCMEA	1.8	-0.8	-1.3	-1.3	0.0	64	AsCCDA	-4.9	-0.6	-0.7	0.3	0.4	94	PlCCDA	-0.7	0.8	-0.9	-0.9	0.5
5	As10MEA	-0.8	-2.7	1.1	0.3	-1.5	35	PlCMEA	1.8	-1.1	-0.9	-1.2	0.1	65	AsCCDA	-5.3	-0.4	0.0	0.2	0.3	95	PlCCDA	-0.9	0.8	3.9	-3.2	0.5
6	As10MEA	-0.8	-2.1	-0.6	0.3	-0.7	36	PlCMEA	1.3	-1.7	-1.3	-1.2	0.1	66	As10CDA	-4.2	-0.9	0.0	0.1	0.3	96	PlCCDA	-0.8	1.5	-0.4	-0.9	-0.5
7	As10MEA	-0.8	-3.1	0.4	0.2	-1.0	37	Pl10MEA	1.7	-0.4	-1.2	-0.4	0.6	67	As10CDA	-4.2	-1.0	-0.5	0.2	0.4	97	Pl10CDA	-0.2	0.7	-0.5	-1.6	0.7
8	As10MEA	-1.4	-3.4	1.8	0.1	-1.6	38	Pl10MEA	2.2	-1.4	2.0	0.4	1.1	68	As10CDA	-4.8	-0.7	-0.4	0.2	0.1	98	Pl10CDA	-0.5	1.5	1.9	-0.7	1.8
9	As20MEA	0.3	-2.7	1.8	0.0	-1.1	39	Pl10MEA	1.4	-0.9	-1.0	-1.1	0.2	69	As10CDA	-5.3	-0.7	0.4	0.3	0.4	99	Pl10CDA	-0.3	1.3	-1.7	-1.2	0.9
10	As20MEA	0.7	-2.0	0.2	-0.7	0.3	40	Pl10MEA	2.0	-1.0	-1.6	-0.9	0.5	70	As20CDA	-2.9	-1.7	-1.1	0.5	-0.1	100	Pl10CDA	-0.7	1.5	-1.5	-1.2	1.0
11	As20MEA	0.1	-1.5	-1.1	0.3	0.1	41	Pl20MEA	1.8	-0.3	-1.3	-0.3	0.7	71	As20CDA	-3.1	-1.4	-0.4	0.1	0.5	101	Pl20CDA	-1.3	1.7	-0.4	-0.8	0.8
12	As20MEA	-0.1	-2.0	-0.5	0.3	0.2	42	Pl20MEA	1.9	-0.7	-0.8	-0.2	0.8	72	As20CDA	-4.7	-0.8	0.0	0.6	0.1	102	Pl20CDA	0.2	0.5	1.7	0.2	2.0
13	As50MEA	0.5	-1.1	0.4	0.2	1.2	43	Pl20MEA	1.9	-1.0	-2.1	-0.7	0.9	73	As20CDA	-2.3	-1.9	0.6	-0.3	0.1	103	Pl20CDA	-0.9	1.5	-0.3	-1.3	1.1
14	As50MEA	-0.5	-2.7	2.3	1.1	0.1	44	Pl20MEA	1.9	-0.9	-0.3	-0.5	0.4	74	As50CDA	-4.1	-0.1	0.0	0.9	1.4	104	Pl20CDA	-0.9	0.5	1.8	-2.2	0.8
15	As50MEA	0.2	-1.3	-1.1	0.9	0.7	45	Pl50MEA	2.2	-0.6	-1.6	-0.1	1.8	75	As50CDA	-4.4	-0.2	-0.6	1.1	1.2	105	Pl50CDA	-0.8	2.1	0.8	-0.8	1.1
16	As50MEA	-0.2	-2.4	0.4	0.2	0.2	46	Pl50MEA	2.1	-0.8	-1.5	-0.1	1.3	76	As50CDA	-3.3	0.2	-1.0	0.4	1.3	106	Pl50CDA	-0.8	2.4	0.2	-0.7	2.0
17	MmCMEA	0.9	-0.7	-1.0	-0.3	-1.1	47	Pl50MEA	1.4	-0.7	-1.1	0.3	0.7	77	As50CDA	-4.8	0.0	-0.3	1.0	1.7	107	Pl50CDA	-0.2	2.3	-0.9	-0.4	1.9
18	MmCMEA	1.4	-1.5	2.1	-3.1	0.1	48	CnCMEA	1.6	-1.0	-0.4	-0.2	-1.4	78	MmCCDA	0.8	-0.5	-0.4	-0.7	-1.5	108	CnCCDA	-0.3	1.4	0.3	0.3	-1.7
19	MmCMEA	1.2	-0.9	-1.5	-0.3	-1.1	49	CnCMEA	1.8	-0.6	-1.0	-0.7	-0.6	79	MmCCDA	0.9	0.9	-1.7	-0.5	-0.4	109	CnCCDA	-0.3	1.6	-1.0	-0.6	-0.3
20	MmCMEA	1.6	-1.5	-0.2	-0.5	-1.0	50	CnCMEA	0.9	-1.1	0.1	-0.5	-0.8	80	MmCCDA	-0.2	1.8	-0.2	-0.8	-2.3	110	CnCCDA	0.0	2.6	-0.8	-1.0	-1.4
21	Mm10MEA	1.8	-0.6	-1.0	0.0	0.1	51	CnCMEA	0.2	-2.8	4.9	-0.8	-0.6	81	MmCCDA	-0.2	2.6	-0.8	-0.2	-1.6	111	Cn10CDA	-0.2	2.2	3.4	-0.5	-1.4
22	Mm10MEA	2.6	-0.3	-0.2	-3.4	0.6	52	Cn10MEA	3.0	-2.5	1.7	2.3	-2.0	82	Mm10CDA	-0.9	2.1	1.9	-2.0	-1.1	112	Cn10CDA	1.3	0.2	-1.0	0.3	-0.2
23	Mm10MEA	1.9	-0.9	-1.3	-0.4	-0.1	53	Cn10MEA	1.5	-0.7	0.9	-1.3	-0.6	83	Mm10CDA	-0.2	2.5	-1.2	0.3	-1.9	113	Cn10CDA	0.2	3.0	1.3	-0.4	-0.4
24	Mm10MEA	1.2	-1.2	-0.2	0.3	-1.5	54	Cn10MEA	3.0	-1.9	2.9	-0.5	0.3	84	Mm10CDA	-0.2	3.5	-0.9	-0.5	-1.9	114	Cn20CDA	-0.4	3.4	0.3	-0.1	0.6
25	Mm20MEA	1.1	-0.3	-1.6	-0.3	-0.1	55	Cn20MEA	1.2	-1.7	0.3	1.6	0.2	85	Mm10CDA	-0.4	2.0	-1.2	-0.1	-1.6	115	Cn20CDA	0.4	2.3	6.3	-3.5	-1.4
26	Mm20MEA	1.2	0.1	-2.1	-0.2	0.0	56	Cn20MEA	1.9	-1.6	3.7	4.1	0.5	86	Mm20CDA	0.1	2.9	-1.3	0.7	-1.4	116	Cn20CDA	-0.2	2.9	0.1	0.6	0.4
27	Mm20MEA	1.4	-0.1	-1.4	-0.1	0.3	57	Cn20MEA	2.5	-0.3	0.9	1.1	0.4	87	Mm20CDA	0.9	0.9	1.0	1.6	-0.3	117	Cn50CDA	1.5	3.8	1.0	2.9	0.3
28	Mm20MEA	1.8	0.3	-1.9	-0.3	0.1	58	Cn20MEA	1.9	-0.7	-0.1	2.3	-0.1	88	Mm20CDA	0.1	4.3	-1.0	0.4	-1.5	118	Cn50CDA	1.6	3.0	3.2	4.2	1.6
29	Mm50MEA	1.9	-0.5	-0.1	1.0	0.7	59	Cn50MEA	2.7	-0.1	0.4	3.1	1.2	89	Mm50CDA	0.1	3.7	-0.5	0.9	-0.7	119	Cn50CDA	0.7	2.1	8.5	-0.4	1.4
30	Mm50MEA	1.7	0.1	-1.8	0.1	0.8	60	Cn50MEA	1.7	0.2	-0.5	2.6	1.4	90	Mm50CDA	0.9	1.4	-1.2	1.7	0.1	120	Cn50CDA	1.7	2.4	1.3	2.6	0.8

Supplementary Table 7.15 Loadings of the five significant components (accounting for 71.47%) obtained by the PCA performed on the element concentrations in fungal biomass of the tested species.

Element	PC1	PC2	PC3	PC4	PC5
As	0.1	0.2	0.1	0.5	0.4
Ca	-0.2	-0.3	0.2	0.3	-0.1
Cd	-0.1	-0.1	0.0	0.3	-0.6
Co	-0.3	-0.3	0.1	0.0	0.1
Cr	0.2	-0.1	0.2	0.2	-0.3
Cu	0.1	-0.1	0.5	-0.3	-0.2
Fe	-0.4	0.0	0.2	0.0	0.2
K	-0.3	0.4	0.1	-0.1	0.2
Mg	-0.4	0.2	0.0	0.1	-0.1
Mn	-0.1	0.0	0.5	-0.3	0.0
Na	-0.1	0.5	0.1	0.1	-0.3
Ni	0.1	0.0	0.2	-0.3	0.0
P	-0.4	0.2	0.0	0.0	0.1
Pb	0.1	-0.1	0.3	0.3	0.0
S	0.0	0.5	0.1	-0.1	-0.3
Sb	0.2	0.2	0.3	0.3	0.2
Sn	0.1	0.0	0.4	0.1	0.2
Sr	-0.3	-0.3	0.0	0.1	-0.1

7.4 PAPER: VALUES AND CHALLENGES IN THE ASSESSMENT OF COPROPHILOUS FUNGI ACCORDING TO THE IUCN RED LIST CRITERIA: THE CASE STUDY OF *PORONIA PUNCTATA* (XYLARIALES, ASCOMYCOTA)

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
2021, VOL. 155, NO. 2, 199–203
https://doi.org/10.1080/11263504.2020.1813833

Taylor & Francis
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BRIEF REPORT

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Values and challenges in the assessment of coprophilous fungi according to the IUCN Red List criteria: the case study of *Poronia punctata* (Xylariales, Ascomycota)

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ABSTRACT

Coprophilous fungi are specialized microorganisms, playing key roles in ecosystems and in several other contexts, whose protection requires more substantial efforts. This paper aims to highlight and discuss valuable and critical aspects faced during the process of the threat status assessment of *Poronia punctata*, providing inspirations for future conservation actions.

KEYWORDS

Fungal biodiversity, coprophilous fungi, *Poronia punctata*, habitat loss, threat status, IUCN Red List, fungal conservation

7.4.1 WHY SHOULD WE PROTECT COPROPHILOUS FUNGI?

Coprophilous fungi are species that live on or are associated with animal dung or dung-amended soil. Most of them have been mainly found on dung of warm-blooded animals, domesticated and wild mammals and birds, while only few were observed on droppings of other vertebrates and invertebrates (Krug et al., 2004). Coprophilous fungi, belonging to all main fungal taxa including Mucoromycota, Ascomycota, Basidiomycota and anamorphic fungi, have been worldwide reported mainly from dungs of herbivores (sometimes also in omnivores and carnivores) (Dix and Webster, 1995; Krug et al., 2004; Misra et al., 2015). Coprophilous fungi represent astonishing examples of specialized microorganisms, perfectly adapted to complex and extreme substrate, like excrements. Coprophilous specialization involves specific mechanisms, including spore discharge, efficient and long-lasting adhesion on herbage, spore survival to digestion, spore activation by passage through animal digestive tract, specialized nutritional requirements and adaptation to specific physicochemical conditions of the dung (Dix and Webster, 1995). Dung represents a complex rich substrate, providing a wide range of nutrients (readily-available carbohydrates, cellulose, lignin, high nitrogen content, water, growth-factors, minerals) as well as micro-habitats (Dix and Webster, 1995). As model systems, coprophilous fungi provide information on the organization and functions of natural fungal communities and on the interactions among decomposer functional groups, including succession, interspecific antagonism and interspecific synergism (Dix and Webster, 1995; Wicklow, 1992).

As decomposers, dung fungi are multifunctional organisms, which are of importance in different contexts, including biodiversity, ecology, paleoecology and biotechnology, and are worth of protection (Fernández et al., 2020). From an ecological point of view, they provide fundamental ecological services, playing pivotal roles in dung decomposition, soil formation and stabilization, biogeochemical cycling of nutrients and elements (Barron, 2017; Dix and Webster, 1995). They represent ideal organisms for both teaching and research in ecology and biodiversity (Krug et al., 2004). Moreover, looking at dung fungi as bioresources they represent a potential source of several bioactive compounds including antibiotics, that they use in nature against dung-competitors, with antifungal properties to be applied against fungal pathogens of humans and animals. Among these bioactive compounds, there are some that activate fungal spores and lignocellulolytic enzymes that could be of interest in fermentation industry (Krug et al., 2004; Wicklow, 1992). Another reason why it is so crucial to protect coprophilous fungi is that they could be useful indicators of both biodiversity and habitat quality, providing information for biodiversity and ecosystem assessment (Fernández et al., 2020; Krug et al., 2004). The presence and distribution of their spores are currently used as proxies in palynological and fossil analyses, providing useful information on population dynamics

of herbivores in paleoecology (Davies, 2019; Davis and Shafer, 2006; Feranec et al., 2011; Graf and Chmura, 2006; Richardson, 2001).

Considering all these aspects, the protection and conservation of coprophilous fungi, as other fungi, is therefore important and requires more substantial efforts even if, fungi are not still completely recognized for their importance (Minter, 2011; Wagensommer et al., 2018). To prioritize conservation planning and make appropriate decisions it is important to know the status and trend analyses of the species, following scientific-based and standardized criteria, documentation sources and databases. Therefore, the red-listing system developed by the International Union for Conservation of Nature (IUCN) is recognized as the most authoritative guide and classification scheme that can be applied to report on the current status of biological diversity for all living species worldwide and, consequently, the most widespread assessment (Dahlberg and Mueller, 2011). The IUCN Red List includes several species that can be associated with dung, however only one is an obligate coprophilous, namely *Poronia punctata* (L.) Fr. The recent publication of the assessment of its threat status in the IUCN Red List (https://www.iucnredlist.org/) represents a relevant contribution to the conservation of fungi. This paper aims to highlight and discuss the valuable and critical aspects that the working group faced during the assessment process of *P. punctata*, in order to provide food for thought that can lead to an improvement for future efforts on fungal conservation.

7.4.2 *PORONIA PUNCTATA*: THE CASE STUDY

Despite the great importance of coprophilous fungi, their threat status assessment may result to be quite complex or not possible due to many critical aspects and in some cases to limited knowledge available (**Figure 7.2**). Main constraints to the species assessment include insufficient taxonomic knowledge of many species, few available biological information and a limited number of world countries investigated (Krug et al., 2004). This could lead the IUCN to designate them with the category "Data Deficient". Instead, for *P. punctata* several records are available since 1753, and many publications on its taxonomy, biology and ecology have been published since 1900 (Dawson, 1900; Doveri, 2004; Granito and Lunghini, 2006; Linnaeus, 1753; Minciarelli, 2013; Minter, 2006; Ravera et al., 2017; Szczepkowski and Obidziński, 2016; Venturella and Saitta, 2009). Belonging to the Xylariacaee family, *P. punctata*, known also as the nail fungus, is an obligate coprophilous ascomycete with stipitate, nail-shape stromata (Rogers, 1970; Szczepkowski and Obidziński, 2016). *P. punctata* is a cosmopolitan species, occurring in 39 nations in Africa, America, Asia, Europe, and Oceania. At least until the end of the 1800, it was common and abundant throughout many European countries, however nowadays it is considered as one of the rarest fungi in Europe (Minter, 2006; Venturella et al., 2011).

Figure 7.2 *Poronia punctata*. (A) Single ascoma showing black ostioles on the stromatic disc surface; (B) stromata and conidiomata arising from the dung; (C) section of an ascoma showing ascomatal cavities and white flesh; (D) section of a conidioma; (E) single conidioma; (F) specimen with original drawings and notes by G. De Notaris showing an ascomatal section with perithecia (RO-Herbarium Generale, ex Agro romano, misit E. Fiorini Mazzanti, 1862, s.n.); (G) mature and immature ascospores showing oil droplets and germinative slits; (H) section through ascoma showing asci and black ascospores. Personal photographs by Dario Lunghini (B-C-D-E-G-H) and Veronica Spinelli (A).

P. punctata is generally associated with natural grasslands, shrublands, heaths, dune areas, meadows, steppes and other natural or semi-natural habitats, including mesotrophic grazed grasslands, often dry pastures (Szczepkowski and Obidziński, 2016). *P. punctata* is almost obligatorily associated with dungs of horses and other equines, such as donkeys, ponies and mules (Szczepkowski and Obidziński, 2016). To a lesser extent, it was also found on dungs of cows (Bignell and King, 2011; Edwards, 2015; Matočec, 2000), sheep, elephants (Szczepkowski and Obidziński, 2016), and occasionally on droppings of rabbits (Mukerji et al., 1969) and pellets of kangaroo (Cribb, 1988; Doveri, 2004). This ascomycete has a complex life cycle, which includes an anamorphic state belonging to the genus *Linquistia* Subram. & Chandrashekara (Granito and Lunghini, 2006; Stiers et al., 1973). It is thought that air-dispersed ascospores, possibly also conidia, can colonize dung directly. However, it is also possible that like for many other dung fungi, the ascospore can only germinate after the passage through the digestive tract of an herbivore (Minter, 2006). In this case, *P. punctata* could be considered a late colonist because of its slow growth due to hierarchical competition. In fact, *P. punctata* appears to outcompete several other fungi (Szczepkowski and Obidziński, 2016) due to its higher efficiency in the degradation of the cellulose compared to the earlier successional species (Angel and Wlcklow, 1983). For *P. punctata*, the long permanence of the dungs in the ecosystems is therefore particularly important for the substrate colonization and to complete the life cycle. *P. punctata*, like most coprophilous fungi, requires the interaction of multiple factors for fruiting, e.g. temperature, nutrients, type and height of vegetation which are responsible for creating favorable microclimatic conditions, such as proper moisture level around the dung, determining fungal growth. Moreover, the presence of insects (especially dung beetles) is also known to improve dung aeration and decay (Bignell and King, 2011; Edwards, 2015; Edwards et al., 2015; Matočec, 2000). In addition, the potential presence of coprophilous fungal competitors is a relevant factor. To cope with interference competition, *P. punctata* produces antibiotics and other metabolites of pharmaceutical importance, such as punctatins and isoepoxydon, to inhibit the growth of other mycelia (Gloer and Truckenbrod, 1988; Poyser et al., 1986; Szczepkowski and Obidziński, 2016; Wicklow and Hirschfield, 1979).

Current knowledge on the biology, ecology and distribution data in space and time of *P. punctata* were essential for the threat assessment. A tricky point considered in the assessment is the limited knowledge of the population trend and its difficult estimation. For instance, the misidentification among species of the same genus (e.g. *P. erici* also present in equine dung and with similar morphological features of *P. punctata*) could result in an overestimation/underestimation of its occurrence (Granito and Lunghini, 2006; Matočec, 2000; Minter, 2006; Szczepkowski and Obidziński, 2016). Another issue for this species' assessment was related to the evaluation of its habitat decline. Being an obligate coprophile, the distribution and abundance of *P. punctata* is indirectly

connected to the presence of domestic herbivores. Therefore, a proper assessment of *P. punctata* population trend requires reliable and updated information on the population dynamics of horses and cows grazing on natural vegetation. To evaluate the number of mature individuals in coprophilous fungi, IUCN recommends to consider the single confined substratum, i.e. dung, as a single mature individual (Dahlberg and Mueller, 2011). According to IUCN, fungi, such as *P. punctata* (and all coprophilous ones) growing on ephemeral substrata, are likely to possess short generation lengths with changes over a 10-year period. For most of the countries, available data are scattered and mainly included in publications and databases older than 10 years, while only for few ones population trends in longer periods are well documented. During the assessment the five IUCN criteria (A-E) used to evaluate if a taxon belongs to an IUCN Red List threatened category were carefully examined for *P. punctata* on the global scale (Dahlberg and Mueller, 2011). Considering *P. punctata*'s cosmopolitan distribution and its peculiarities as coprophilous, it was found that it did not match any of the five criteria. Consequently, *P. punctata* has been included by the IUCN in the Global Red List with the status of Least Concern (Persiani and Ainsworth, 2020). Despite the result of the global assessment, concerns about the threatened status of this species are still present in several nations. In fact, *P. punctata* is included in many National Red Lists of European countries (Szczepkowski and Obidziński, 2016), including Italy (Ravera et al., 2017; Rossi et al., 2013; Siniscalco et al., 2019). In U.K., *P. punctata* is a Priority Species under the Post-2010 UK Biodiversity Framework and a Species of Principal Importance under the Natural Environment and Rural Communities (NERC) Act 2006 (Edwards, 2015; Minter, 2006). Studies suggested that populations of the nail fungus are currently increasing, in fact *P. punctata* in U.K. Red List was downgraded from "Endangered" to "Near Threatened" (Edwards, 2015). A peculiar situation occurred in Poland where *P. punctata* was not reported for more than 100 years (1905 to 2009). The possible reasons of this gap could be the lack of good preserved collections (e.g. specimens lost, erroneous records), political, economic and social changes (e.g. world wars, political borders changes, change in soil use and agriculture practices), the paucity of specialists, the scarcity of interest and the reduced number of studies concerning coprophilous fungi of open areas, meadows and pastures (Szczepkowski and Obidziński, 2016). Threats to *P. punctata* include the loss of traditional free horse grazing due to intense closed animal husbandry, the reduction of populations of equines and other domestic animals, the reduction or loss of typical natural and semi-natural habitats due to intense agriculture activities, the use of agrochemicals and pesticides that impact on vegetation and insects supporting fungal growth, and the use of veterinary additives that impact on fungal development (Fernández et al., 2020; Minter, 2006; Persiani and Ainsworth, 2020).

The importance of this coprophilous fungus as bioresource and its multifunctional roles in ecosystems call for major efforts aimed at its protection and its conservation,

both *in situ* and *ex situ* (e.g. fungal collections) and to move towards environmental sustainability. Therefore, a commitment from all people (scientists, politicians, citizens, farmers, etc.) is important to keep the nail fungus protected, not only by improving the scientific research, education and conservation practices, but also by orienting economic and politic actions towards sustainability and decisions inspired by nature (Minter, 2006). To raise public awareness on its importance and therefore promote its protection, it is crucial to put this species under a spotlight. To reach this goal, citizen scientists, volunteers who are directly involved in scientific research, could play particularly relevant roles, not only collecting data but also working to increase public awareness on environmental and scientific issues, thanks to their actions of community networking and environmental education (Mueller, 2017; Nimis et al., 2019). The inclusion in the IUCN Red List also has the function of promoting on a larger scale the knowledge of the characteristics, usefulness and distribution of a species favoring an increase in its survey and occurrence record that, eventually, leads to more abundant and better detailed data. The share of the data and information, the robust recording and monitoring of the fungal populations in both space and time scales are essential for the correct evaluation of the status of the species and for the threat assessment on local to global scales.

The work that led to the assessment of *P. punctata* was possible only thanks to an Italian collaboration network including academic groups, research institutes and amateur associations joining forces with an international collaboration of scientists and amateur volunteers through the online platform The Global Fungal Red List Initiative (http://iucn.ekoo.se). Therefore, this experience represents not only a great result for fungal conservation but also a proof that what fungal conservation actually needs most is a wider and better collaboration within mycologists from different backgrounds and from all over the world.

7.4.3 ACKNOWLEDGMENTS

We gratefully acknowledge the Herbarium Museum of Sapienza University of Rome (RO) and in particular Dr. Anna Millozza and Dr. Agnese Tilia for the support in the specimen's examination. We also gratefully acknowledge Prof. Dario Lunghini for his precious consultation on the ecology of the species and for providing photographs. We gratefully acknowledge the Mycology Working Group of the Italian Botanical Society, the working group of the project "Progetto Speciale Funghi" of ISPRA and the mycological association Associazione Micologica ed Ecologica Romana. At last but not least we gratefully acknowledge the Italian Botanical Society and in particular the President Maria Consolata Siniscalco for the support to the activities that finally led to this publication.

7.4.4 REFERENCES

Angel, Sr.K., and Wlcklow, D.T. (1983). Coprophilous fungal communities in semiarid to mesic grasslands. Canadian Journal of Botany *61*, 594–602.

Barron, E.S. (2017). Chapter 21 Who Cares? The Human Perspective on Fungal Conservation. In The Fungal Community. Its Organization and Role in the Ecosystem. Fourth Edition., (Boca Raton, FL: CRC Press), pp. 321–330.

Bignell, S., and King, D. (2011). Monitoring Survey for the Nail Fungus, *Poronia punctata*, in the New Forest. Hampshire: Hampshire & Isle of Wight Wildlife Trust working on behalf of the New Forest National Park Authority; [accessed 2020 Feb 1]. http://www.hampshirefungi.org.uk/docs/PoroniaHWT.pdf.

Cribb, A.B. (1988). Fungi on kangaroo dung. In Lake Broadwater. The Natural History of an Inland Lake and Its Environs.(Toowoomba, AU: Darling Downs Institute Press) p. 52–56.

Dahlberg, A., and Mueller, G.M. (2011). Applying IUCN red-listing criteria for assessing and reporting on the conservation status of fungal species. Fungal Ecology *4*, 147–162.

Davies, A.L. (2019). Dung fungi as an indicator of large herbivore dynamics in peatlands. Review of Palaeobotany and Palynology *271*, 104108.

Davis, O.K., and Shafer, D.S. (2006). *Sporormiella* fungal spores, a palynological means of detecting herbivore density. Palaeogeography, Palaeoclimatology, Palaeoecology *237*, 40–50.

Dawson, M. (1900). On the biology of *Poronia punctata* (L.). Annals of Botany *14*, 245–262.

Dix, N.J., and Webster, J. (1995). Fungal ecology (London, UK: Chapman & Hall).

Doveri, F. (2004). Fungi fimicoli italici A guide to the recognition of Basidiomycetes and Ascomycetes living on feacal material. (Trento, IT: Associazione Micologica Bresadola). Italian, Latin and English.

Edwards, N. (2015). Does Breckland vegetation and its management influence abundance of *Poronia punctata*? School of Life and Medical Sciences MSc Environmental Management for Agriculture. University of Hertfordshire; [accessed 2020 Feb 1]. http://rgdoi.net/10.13140/RG.2.1.4965.9761.

Edwards, N., Leech, T., and Warner, D. (2015). Nail Fungus *Poronia punctata* in Norfolk, and possible factors affecting its appearance at Hockwold Heath and Cranwich Camp. Transactions of the Norfolk and Norwich Naturalists Society *48*, 34–39.

Feranec, R.S., Miller, N.G., Lothrop, J.C., and Graham, R.W. (2011). The *Sporormiella* proxy and end-Pleistocene megafaunal extinction: A perspective. Quaternary International *245*, 333–338.

Fernández, A., Sánchez, S., García, P., and Sánchez, J. (2020). Macrofungal diversity in an isolated and fragmented Mediterranean Forest ecosystem. Plant Biosystems *154*, 139–148.

Gloer, J.B., and Truckenbrod, S.M. (1988). Interference Competition among Coprophilous Fungi: Production of (+)-Isoepoxydon by *Poronia punctata*. Applied and Environmental Microbiology *54*, 861.

Graf, M.-T., and Chmura, G.L. (2006). Development of modern analogues for natural, mowed and grazed grasslands using pollen assemblages and coprophilous fungi. Review of Palaeobotany and Palynology *141*, 139–149.

Granito, V.M., and Lunghini, D. (2006). Updated observations on *Poronia punctata*. Micol. e Veget. Medit. *21*, 71–76.

Krug, J.C., Benny, G.R., and Keller, H.W. (2004). Coprophilous fungi. In Biodiversity of Fungi: Inventory and Monitoring Methods. (Burlington, MA: Elsevier Academic Press) p. 467–499.

Linnaeus, C. (1753). Species plantarum (Stockholm , SE: Imprensis Laurentius Salvius.).

Matočec, N. (2000). The endangered European species *Poronia punctata* (Xylariales, Ascomycotina) still alive and well in Croatia. Nat. Croat. *9*, 35–40.

Minciarelli, L. (2013). *Poronia punctata* (L.) FR. (Ascomycota, Xylariales), nei pascoli del Parco Regionale del Monte Cucco (PG-Italia). Boll. AMER *90*, 46–50.

Minter, D. (2011). Fungal conservation needs help from botanists. Plant Biosystems *145*, 945–949.

Minter, D.W. (2006). *Poronia punctata*. IMI Descriptions of Fungi and Bacteria. Set 170 No. 1700. 1–4.

Misra, J.K., Pandey, S., Gupta, A.K., and Deshmukh, S.K. (2015). Coprophilous fungi-a review and selected bibliography. In Fungi from Different Substrates, (New York, NY, USA: CRC Press/Taylor and Francis Group), pp. 170–200.

Mueller, G.M. (2017). Progress in conserving fungi: engagement and red listing. BGJournal *14*, 30–33.

Mukerji, K.G., Bedi, K., Tewari, J.P., and Tewari, I. (1969). Morphology of Indian species of *Xylaria* and *Poronia*. Phytomorphology *19*, 219–224.

Nimis, P.L., Pittao, E., Altobelli, A., De Pascalis, F., Laganis, J., and Martellos, S. (2019). Mapping invasive plants with citizen science. A case study from Trieste (NE Italy). Plant Biosystems *153*, 700–709.

Persiani, A.M., and Ainsworth, A.M. (2020). *Poronia punctata*: The IUCN Red List of Threatened Species 2019: e.T58517228A58517248 (International Union for Conservation of Nature).

Poyser, J.P., Edwards, R.L., Anderson, J.R., Hursthouse, M.B., Walker, N.P.C., Sheldrick, G.M., and Whalley, A.J.S. (1986). Punctatins A,D,E, and F (antibiotics M95464, M167906, M171950, and M189122), isomeric allylic alcohols from the fungus *Poronia punctata*: X-ray crystal structures of D and of E acetonide. The Journal of Antibiotics *39*, 167–169.

Ravera, S., Vizzini, A., Cogoni, A., Aleffi, M., Assini, S., Bergamo Decarli, G., Bonini, I., von Brackel, W., Cheli, F., Darmostuk, V., et al. (2017). Notulae to the Italian flora of algae, bryophytes, fungi and lichens: 4. Italian Botanist *4*, 76–86.

Richardson, M.J. (2001). Diversity and occurrence of coprophilous fungi. Mycological Research *105*, 387–402.

Rogers, J.D. (1970). Cytology of *Poronia oedipus* and *P. punctata*. Canadian Journal of Botany *48*, 1665–1668.

Rossi, G., Montagnani, C., Gargano, D., Peruzzi, L., Abeli, T., Ravera, S., Cogoni, A., Fenu, G., Magrini, S., and Gennai, M. (2013). Lista Rossa della Flora spontanea italiana. 1. Policy Species e altre specie minacciate.

Siniscalco, C., Lavorato, C., Bianco, P.M., Rotella, M., De Marco, V., Floccia, F., and Campana, L. (2019). Contribution of ISPRA "Centers of Excellence" to the development of the Italian Fungal Red List-2019. Publication of the Project" Progetto Speciale Funghi" of ISPRA 2019. Rome (IT); [accessed 2020 Apr 24]. http://www.isprambiente.gov.it/it/temi/biodiversita/lispra-e-la-biodiversita/attivita-e-progetti/progetto-speciale-funghi-1/pdf/Contribution_2019_Italian_Red_List.pdf.

Stiers, D.L., Rogers, J.D., and Russell, D.W. (1973). Conidial state of *Poronia punctata*. Canadian Journal of Botany *51*, 481–484.

Szczepkowski, A., and Obidziński, A. (2016). *Poronia punctata* (L.: Fr.) Rabenh. (Xylariales, Ascomycota) in Poland: a threatened, rare, or overlooked species? Acta Mycologica *51*.

Venturella, G., and Saitta, A. (2009). *Poronia punctata*, a rare ascomycetes from Italy. Bocconea *23*, 277–279.

Venturella, G., Altobelli, E., Bernicchia, A., Di Piazza, S., Donnini, D., Gargano, M.L., Gorjòn, S.P., Granito, V.M., Lantieri, A., Lunghini, D., et al. (2011). Fungal biodiversity and *in situ* conservation in Italy. Plant Biosystems *145*, 950–957.

Wagensommer, R.P., Bistocchi, G., Arcangeli, A., Rubini, A., Perini, C., Venanzoni, R., and Angelini, P. (2018). An assessment of red list data for the Pezizomycotina (Ascomycota): Umbria (Italy) as a test case. Plant Biosystems*152*, 1329–1337.

Wicklow, D.T. (1992). The coprophilous fungal community: an experimental system. In The Fungal Community: Its Organization and Role in the Ecosystem. Second Edition., (George C. Carroll and Donald T. Wicklow), pp. 715–728.

Wicklow, D.T., and Hirschfield, B.J. (1979). Evidence of a competitive hierarchy among coprophilous fungal populations. Canadian Journal of Microbiology *25*, 855–858.

7.5 PAPER: NEW INSIGHTS ON THE OCCURRENCE AND CONSERVATION STATUS IN ITALY OF *ALESSIOPORUS ICHNUSANUS* (BOLETACEAE), AN IUCN RED LISTED MYCORRHIZAL SPECIES

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
2021, VOL. 155, NO. 2, 195–198
https://doi.org/10.1080/11263504.2020.1813832

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BRIEF REPORT

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New insights on the occurrence and conservation status in Italy of *Alessioporus ichnusanus* (Boletaceae), an IUCN red listed mycorrhizal species

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ABSTRACT

The conservation status of *Alessioporus ichnusanus* is discussed considering new insights emerged from the IUCN global assessment. New locations were identified and Italy is confirmed as the center of distribution of this species. According to the IUCN categories and criteria, *A. ichnusanus* is assigned to the category Vulnerable C2a(i).

KEYWORDS

Fungal biodiversity, Boleto Sardo, *Xerocomus ichnusanus*, habitat loss, threat status, IUCN Red List, fungal conservation

7.5.1 INTRODUCTION

In recent years, human activities, fragmentation of habitats and climate change have caused an increase in the number of fungal species at risk of decline (Fernández et al., 2020; UNEP, 2012). Despite their ecological importance for their fundamental roles in ecosystems the actions for fungal conservation were inadequate or totally absent, especially in the past. In recent years, the situation has changed and international actions for their conservation have been carried out, including red listing of the threatened fungal species to ensure an adequate protection (Minter, 2011). Until 2015, the only Italian non-lichenized fungus included in the Global IUCN Red List of Threatened Species was *Pleurotus nebrodensis* (Inzenga) Quél. (Venturella, 2016), an endemic edible mushroom from Sicily (Gargano et al., 2011; Montmollin and Strahm, 2005).

In November 2018, the Mycology Working Group of the Italian Botanical Society (MWG–SBI) organized a workshop on the conservation status of fungi in Italy and started to assess some fungal species according to Red List of Threatened Species criteria (https://www.iucnredlist.org). Thanks to a national collaboration network promoted by MWG–SBI, including also the Italian Institute for Environmental Protection and Research (ISPRA) and Amateur Mycological Associations (AMER, Agaricwatching), *Alessioporus ichnusanus* (Alessio, Galli & Littini) Gelardi, Vizzini & Simonini (formerly *Xerocomus ichnusanus* Alessio, Galli & Littini), was assessed and later published as globally vulnerable by the IUCN working group (Persiani, 2019).

This paper aims to draw attention on *A. ichnusanus* and the need of conservation actions for this species, highlighting the importance of the assessment as a tool to gather knowledge on the biology, distribution, phenology and responses to threats of the species, and to raise public awareness on its importance and therefore promote its protection.

7.5.2 DISCUSSION

The first record of *A. ichnusanus* dates back to the early 1980s in Sardinia (Galli, 1981), while the description of the species under the binomial of *Xerocomus ichnusanus* Alessio, Galli & Littini is reported in Alessio (1984). Then, the species has been reported in many other Italian administrative Regions (Onofri et al., 2005). In 2014, the genus *Alessioporus* Gelardi, Vizzini & Simonini (Boletaceae, Boletales, Agaricomycetes) was proposed as a monotypic genus to accommodate the European species *X. ichnusanus*, based on both morphological features and multigene molecular phylogeny (nrITS, nrLSU and *tef-1α* data sets) (Gelardi et al., 2014). The name of the genus was referred to Carlo Luciano Alessio (1919–2006) who dedicated most of his mycological studies to the investigation of Italian boletes (Gelardi et al., 2014). Nowadays the genus also includes

an American species, *Alessioporus rubriflavus* J.L. Frank, A.R. Bessette & Bessette (Frank et al., 2017).

A. ichnusanus is easily recognizable by the reticulate, rooting stipe and by its commonly caespitose growth. This species differs from the European *Xerocomus* species in its carpogenesis which, most probably, is secondarily angiocarpic; remnants of the connection between stipe and pileus are often observed in the form of a narrow, granulose "ring-zone" in the middle or in the upper half of the stipe (**Figure 7.3**) (Alessio, 1984, 1985; Boccardo et al., 2008; Estades and Lannoy, 2004; Muñoz et al., 2008).

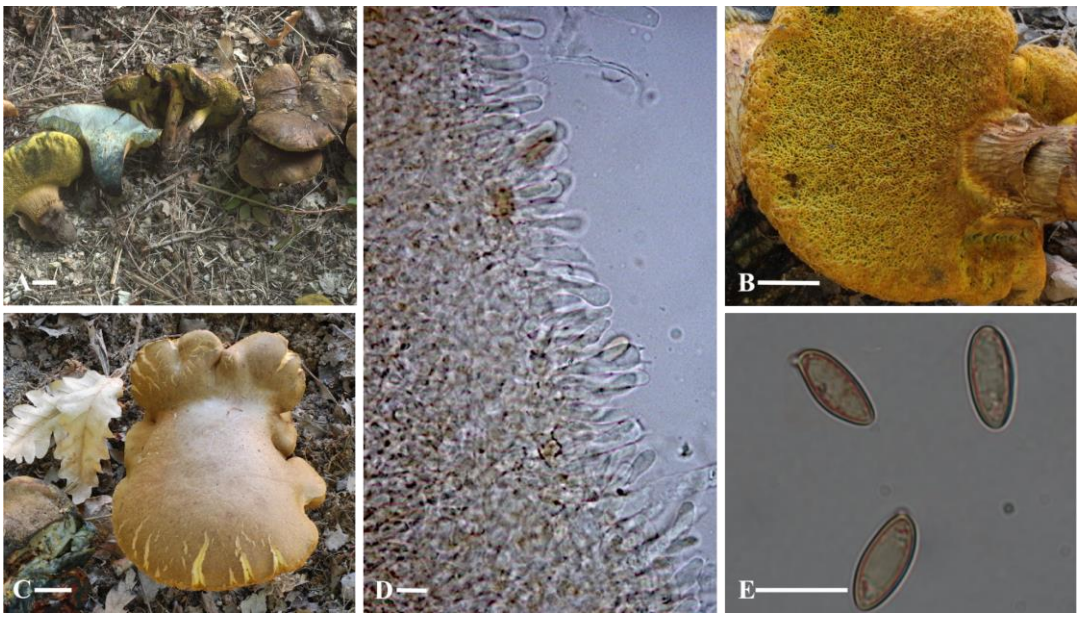

Figure 7.3 *Alessioporus ichnusanus*. (A, B) Basidiocarps with pore and narrow granular pseudo-annular zone in the middle-upper part of the stipe. (C) Pileus ochraceous brown with brownish black fibrils and surface dry and finely tomentose. (D) Basidia and pleurocystidia. (E) Basidiospores. Bars: A–C = 1 cm, D-E = 15 µm.

Ectomycorrhizal, solitary to gregarious or more often caespitose, *A. ichnusanus* occurs in the Mediterranean area associated with *Quercus* sp. pl. [*Quercus ilex* L. subsp. *ilex*, *Q. ilex* L. subsp. *ballota* (Desf.) Samp., *Q. faginea* Lam., *Q. suber* L., *Q. coccifera* L., *Q. cerris* L., *Q. robur* L., *Q. pubescens* Willd., *Q. petraea* (Matt.) Liebl., *Q. pyrenaica* Willd., and *Q. frainetto* Ten.] and, with less frequency, *Cistus* sp. pl., rarely with *Castanea sativa* Mill. and probably also with the non-native *Eucalyptus camaldulensis* Dehnh. (Angelini et al., 2017; Gelardi et al., 2014; Ortega et al., 2010; Salerni and Perini, 2013; Zotti et al., 2008). A recent phylogenetic molecular analysis confirmed the genus *Alessioporus* as ectomycorrhizal (Tedersoo and Smith, 2017). The presence of *A. ichnusanus* in Europe

is mostly associated with low or medium-altitude habitats. Found on dry and calcareous soils (pH 7.6–8), ubiquitous, however known to be infrequent to rare (Angelini et al., 2016; Assyov and Stoykov, 2011; Gelardi et al., 2014; Salerni and Perini, 2013; Zotti et al., 2008).

It has been recorded in several different localities of Sardinia (Galli, 1987), Sicily and mainland Italy and from several European countries, mostly in the Mediterranean area, i.e., France, Spain, Portugal, Bulgaria, North Macedonia, and Greece (including the Cyclades Islands), with a single finding in Austria (Persiani, 2019). Considering this distribution range, the main threats this species is subject to are ecosystem modification due to fire and changes of management, wood harvesting, human activities, livestock farming.

The global conservation status of *A. ichnusanus* was assessed according to the IUCN categories and criteria, using published and unpublished data of field surveys since 1980 (IUCN, 2012a, 2019).

Considering the ecology and the distribution of the species, we aimed to evaluate a link between the distribution sites of this species and the land use/cover of the occurrence sites. In fact, physical factors together with biological factors and their mutual interaction determine the not random biodiversity distribution.

According to the guidelines for fungi given by Dahlberg and Mueller (2011), the Area of Occupancy, Extent of Occurrence, number of mature individuals were calculated.

In addition, trying to link the distribution of this fungal species with the habitats, a georeferenced database of species occurrence records was overlaid to the Corine Land Cover inventory using Google Earth Pro. For Europe the most recent CLC 2018 was used (https://land.copernicus.eu/pan-european/corine-land-cover/clc2018), while for Italy a more detailed analyses for the natural and semi-natural classes at level IV (CLC 2012, http://www.sinanet.isprambiente.it/it/sia-ispra/download-mais/corine-land-cover/corine-land-cover-2012-iv-livello/view) was checked. This more accurate separation of the type of forest, scrublands and grasslands can be useful to support sustainable management and the planning of natural resourches' conservation and use (ISPRA 2010; Siniscalco et al., 2014, 2019).

Analyzing the available data, taking into account the possible unrecorded sites, following the IUCN protocol (IUCN, 2012a, 2012b, 2019), this species showing a decreasing trend of small populations, resulted Vulnerable, VU C2a(i) (Persiani, 2019). Prior to the IUCN assessment, in Italy the species was considered Endangered with a very small population (Rossi et al., 2013), but thanks to the new insights it should be assigned also at the national level to the Vulnerable category.

Taking into consideration land use, according to the nomenclature adopted by the Corine Land Cover 2018, at European scale, it was possible to observe that nearly half of the records of *A. ichnusanus* (46%) occurred in broad-leaved forest (CLC2012 class code 311), 13% in sclerophyllous vegetation (323) and 13% in land predominantly occupied by agriculture with significant areas of natural vegetation (243). To a lesser extent this species results to grow also in green urban areas (7%), complex cultivation patterns (7%) and agro-forestry areas (7%) (**Figure 7.4**). According to the more detailed characterization of natural and semi-natural classes (CLC2012) at Italian scale *A. ichnusanus* is mainly found in deciduous oak forests (code 3112), (11%) (**Table 7.16**). This finding agrees with the certain degree of specificity of this species for *Quercus* sp. pl. as reported in literature. At low and medium altitude, the species occurs mainly in mixed coniferous and broad-leaved forests (code 3131), followed by evergreen oak forests (code 3131), mixed broadleaved and coniferous forests (code 3132), chestnut forests (code 3113). *A. ichnusanus* is less present at higher altitudes, where it is mainly associated with beech forests (code 3115). Interestingly, at low altitudes and in lowlands, this species was recorded in both intensive crops (code 2111) and discontinuous urban fabric (code 112) (**Table 7.16**). Italian ecosystems showing a wide variation in natural vegetation types and, having been influenced by human pressures, in semi-natural ones constitute a wide area with potential presence of this species (Chelli et al., 2019). However it has been observed that *A. ichnusanus*, due to its preference for thermophilus and dry environments, appears to display a narrower distribution in relation to its potential geographic range.

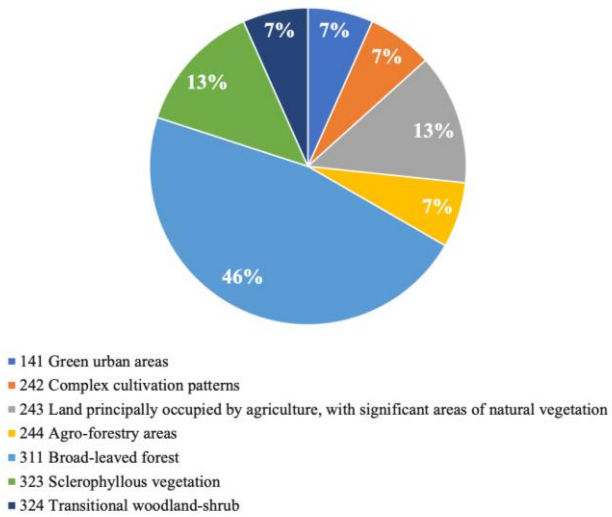

Figure 7.4 Distribution of *Alessioporus ichnusanus* records (%) through the CLC 2018 code, at European scale.

Table 7.16 Distribution of Alessioporus ichnusanus records through the CLC 2012 code, at the Italian scale.

Code CLC12	Land Cover	Records no. (%)
112	Discontinuous urban fabric	12 (9%)
121	Industrial or commercial units	2 (2%)
141	Green urban areas	2 (2%)
142	Sport and leisure facilities	6 (5%)
222	Fruit trees and berry plantations	2 (2%)
223	Olive groves	4 (3%)
231	Pastures	2 (2%)
242	Complex cultivation patterns	6 (5%)
243	Land principally occupied by agriculture, with significant areas of natural vegetation	7 (5%)
244	Agro-forestry areas	3 (2%)
322	Moors and heathland	1 (1%)
324	Transitional woodland-shrub	4 (3%)
2111	Intensive crops	12 (9%)
2112	Extensive crops	2 (2%)
3111	Evergreen oak forests (holm and cork oaks)	8 (6%)
3112	Deciduous oak forests (turkey, downy, Italian, sessile, pedunculate oaks)	15 (11%)
3114	Chestnut forests	5 (4%)
3115	Beech forests	8 (6%)
3116	Hygrophilous forests (willows, poplars, alders)	1 (1%)
3117	Woods and former plantations dominated by exotic broadleaved (black locust and *Ailanthus altissima*)	1 (1%)
3121	Mediterranean pines and cypress forests (pine, maritime pine, Aleppo pine)	2 (2%)
3122	Oro-Mediterranean and mountain pine forests (black pine and larch, Scots pine, Bosnian pine)	1 (1%)
3131	Mixed coniferous and broadleaved forests	11 (8%)
3132	Mixed broadleaved and coniferous forests	7 (5%)
3231	High maquis	4 (3%)
3232	Low maquis and garrigues	5 (4%)

Considering the importance of *A. ichnusanus* and consequently the need of conservation actions to protect it, a broad dissemination and resonance of the assessment would represent a first and crucial step to raise public awareness on its importance and should be therefore ensured as much as possible.

Furthermore, since this ectomycorrhizal species is associated with a certain degree of specificity to several tree species, another important step required for its conservation would be the implementation and dissemination of guidelines for the conservation of its habitats, with particular attention to forests management.

7.5.3 ACKNOWLEDGMENTS

We gratefully acknowledge the Italian Botanical Society and in particular President Maria Consolata Siniscalco for the support to the activities that finally led to this publication.

7.5.4 REFERENCES

Alessio, C. (1984). Un boleto non ancora noto: *Xerocomus ichnusanus* Alessio, Galli et Littini sp. nov. Boll Gruppo Micol G Bresadola. 27:166–170.

Alessio, C. (1985). Fungi Europaei 2: *Boletus* Dill. ex L.(sensu lato) Libreria editrice Biella Giovanna.

Angelini, P., Bistocchi, G., Arcangeli, A., Rubini, A., and Venanzoni, R. (2016). Inventory, diversity and communities of macrofungi in the Collestrada forest (Umbria, central Italy). Plant Biosystems-An International Journal Dealing with All Aspects of Plant Biology *150*, 1096–1105.

Angelini, P., Arcangeli, A., Bistocchi, G., Rubini, A., Venanzoni, R., and Perini, C. (2017). Current knowledge of Umbrian macrofungi (central Italy). Plant Biosystems*151*, 915–923.

Assyov, B., and Stoykov, D.Y. (2011). First record of *Boletus ichnusanus* (Boletaceae) in Bulgaria. Phytologia Balcanica *17*, 269–272.

Boccardo, F., Traverso, M., Vizzini, A., and Zotti, M. (2008). Funghi d'Italia (Zanichelli).

Chelli, S., Marignani, M., Barni, E., Petraglia, A., Puglielli, G., Wellstein, C., Acosta, A.T.R., Bolpagni, R., Bragazza, L., Campetella, G., et al. (2019). Plant–environment interactions through a functional traits perspective: a review of Italian studies. Plant Biosystems*153*, 853–869.

Dahlberg, A., and Mueller, G.M. (2011). Applying IUCN red-listing criteria for assessing and reporting on the conservation status of fungal species. Fungal Ecology *4*, 147–162.

Estades, A., and Lannoy, G. (2004). Les bolets européens (Fédération mycologique Dauphiné-Savoie).

Fernández, A., Sánchez, S., García, P., and Sánchez, J. (2020). Macrofungal diversity in an isolated and fragmented Mediterranean Forest ecosystem. Plant Biosystems*154*, 139–148.

Frank, J., Bessette, A.R., and Bessette, A.E. (2017). *Alessioporus rubriflavus* (Boletaceae), a new species from the eastern United States. North American Fungi *12*, 1–8.

Galli, R. (1981). Uno *Xerocomus* sconosciuto in Sardegna. Natura e Funghi *2*, 28–31.

Galli, R. (1987). I boleti delle nostre regioni: atlante pratico-monografico per la determinazione dei boleti: volume 2 (Edizioni La tipotecnica).

Gargano, M.L., Saitta, A., Zervakis, G.I., and Venturella, G. (2011). Building the jigsaw puzzle of the critically endangered *Pleurotus nebrodensis*: historical collection sites and an emended description. Mycotaxon *115*, 107–114.

Gelardi, M., Simonini, G., Ercole, E., and Vizzini, A. (2014). *Alessioporus* and *Pulchroboletus* (Boletaceae, Boletineae), two novel genera for *Xerocomus ichnusanus* and *X. roseoalbidus* from the European Mediterranean basin: molecular and morphological evidence. Mycologia *106*, 1168–1187.

ISPRA. 2010. La realizzazione in Italia del Progetto Corine Land Cover 2006. ISPRA, RAPPORTI 131/2010; [accessed 2019 Nov 10]. http://www.isprambiente.gov.it/it/pubblicazioni/rapporti/la-realizzazione-in-italia-del-progetto-corine.

IUCN. 2012a. IUCN red list categories and criteria: version 3.1. 2nd ed. Gland: IUCN; [accessed 2019 Nov 10]. http://s3.amazonaws.com/iucnredlistnewcms/staging/public/attachments/3097/redlist_cats_crit_en.pdf.

IUCN. 2012b. Unified classification of direct threats: version 3.2; [accessed 2019 Nov 10]. http://www.iucnredlist.org/technical-documents/.classification-schemes/threats-classification-scheme.

IUCN. 2019. Guidelines for using the IUCN red list categories and criteria. Version 14. Standards and Petitions Subcommittee; [accessed 2019 Nov 10]. http://www.iucnredlist.org/documents/RedListGuidelines.pdf.

Minter, D. (2011). Fungal conservation needs help from botanists. Plant Biosystems*145*, 945–949.

Montmollin, B. de, and Strahm, W. (2005). The Top 50 Mediterranean Island Plants: Wild Plants at the Brink of Extinction, and what is Needed to Save Them (IUCN).

Muñoz, J., Cadiñanos Aguirre, J., and Fidalgo, E. (2008). Contribución al catálogo corológico del género *Xerocomus* en la Peninsula Iberica. Boletin de la Sociedad Micologica de Madrid *32*, 249–277.

Onofri, S., Vizzini, A., Zotti, M., Zucconi, L., Bernicchia, A., Filipello Marchisio, V., Padovan, F., Perini, C., Ripa, C., and Salerni, E. (2005). Checklist dei funghi italiani-Checklist of Italian fungi, Basidiomycetes. (Sassari, Italy: C. Delfino ed.) p. 1–380.

Ortega, A., Lorite, J., and Valle, F. (2010). Mycorrhizal macrofungi diversity (Agaricomycetes) from Mediterranean Quercus forests; a compilation for the Iberian Peninsula (Spain and Portugal). Nova Hedwigia *91*, 1.

Persiani, A.M. (2019). *Alessioporus ichnusanus*: Persiani, A.M.: The IUCN Red List of Threatened Species 2019: e.T147144825A167285525 (International Union for Conservation of Nature). [accessed 2020 Apr 8]. https://dx. doi.org/10.2305/IUCN.UK.2019-3.RLTS.T147144825A167285525.en. Rossi G, Montagnani C, Gargano D, Peruzzi L, Abeli T, Ravera S, C

Rossi, G., Montagnani, C., Gargano, D., Peruzzi, L., Abeli, T., Ravera, S., Cogoni, A., Fenu, G., Magrini, S., and Gennai, M. (2013). Lista Rossa della Flora spontanea italiana. 1. Policy Species e altre specie minacciate. Comitato Italiano IUCN, Ministero dell'Ambiente e della Tutela del Territorio e del Mare. Stamperia Romana, p. 54.

Salerni, E., and Perini, C. (2013). Mycodiversity of nature reserves in Central Italy. Acta Mycologica *42*.

Siniscalco, C., Bianco, P., Parrettini, G., Floccia, F., Campana, L., and Jacomini, C. (2014). Abbinamento dei macromiceti italiani ai sistemi di classificazione degli habitat. Prima correlazione tra specie fungine, habitat e coperture del suolo sul territorio nazionale. ISPRA, Manuali e linee guida n. 119/2014: 1-533.

Siniscalco, C., Lavorato, C., Bianco, P.M., Rotella, M., De Marco, V., Floccia, F., and Campana, L. (2019). Contribution of ISPRA "Centers of Excellence" to the development of the Italian Fungal Red List-2019. Publication of the Project" Progetto Speciale Funghi" of ISPRA 2019. Roma. [accessed 2020 April 10]. http://www.isprambiente.gov.it/it/temi/biodiversita/lispra-e-la-biodiversita/attivita-e-progetti/progetto-speciale-funghi-1/pdf/Contribution_2019_Italian_Red_List.pdf.

Tedersoo, L., and Smith, M.E. (2017). Ectomycorrhizal fungal lineages: detection of four new groups and notes on consistent recognition of ectomycorrhizal taxa in high-

throughput sequencing studies. In Biogeography of Mycorrhizal Symbiosis, (Cham, CH: Springer International Publishing), pp. 125–142.

UNEP (2012). Global Environment Outlook 5: Environment for the future we want. Nairobi. [accessed 2019 Nov 10] https://wedocs.unep.org/bitstream/handle/20.500.11822/8021/GEO5_report_full_en.pdf?isAllowed=y&sequence=5.

Venturella, G. (2016). Pleurotus nebrodensis ssp. nebrodensis: Venturella, G.: The IUCN Red List of Threatened Species 2016: e.T61597A102952148 (International Union for Conservation of Nature).

Zotti, M., Vizzini, A., Traverso, M., Boccardo, F., Pavarino, M., and Mariotti, M.G. (2008). The macrofungal checklist of Liguria (Italy): current survey status. Mycotaxon *105*, 167–170.

PUBLICATIONS

REFERRED PAPERS

- **Spinelli V.**, Brasili E.*, Sciubba F., Ceci A., Giampaoli O., Miccheli A., Pasqua G., Persiani A. M. (2022) Biostimulant effects of *Chaetomium globosum* and *Minimedusa polyspora* culture filtrates on *Cichorium intybus* plant: growth performance and metabolomic traits. *Frontiers in Plant Science*. 13:879076. doi: 10.3389/fpls.2022.879076. (*Corresponding author).

- **Spinelli V.***, Ceci A.*, Dal Bosco C., Gentili A., Persiani A. M. (2021) Glyphosate-eating fungi: study on fungal saprotrophic strains' ability to tolerate and utilise glyphosate as nutritional source and on *Purpureocillium lilacinum* ability to degrade it. *Microorganisms*. 9, 2179. https://doi.org/10.3390/microorganisms9112179. (*Corresponding authors).

- Ceci A.*, Angelini P., Iotti M., Lalli G., Leonardi M., Pacioni G., Perrone L., Pioli S., Siniscalco S., **Spinelli V.**, Venturella G., Wagensommer R. P., Zotti M., Persiani A. M. (2021) Values and challenges in the assessment of coprophilous fungi according to the IUCN red list criteria: The case study of *Poronia punctata* (Xylariales, Ascomycota). *Plant Biosystems*. 155(2), 199–203, doi: 10.1080/11263504.2020.1813833. (*Corresponding author).

- Angelini P., Antonini D., Antonini M., Arcangeli A., Bianco P. M., Bistocchi G., Campana L., Ceci A., Floccia F., Gargano M. L., Gelardi M., Lalli G., Leonardi M., Maneli F., Perini C.*, Perrone L., Salerni E., Segneri G., Siniscalco C., **Spinelli V.**, Vasquez G., Venanzoni R., Venturella G., Wagensommer R. P., Zotti M., Persiani A. M. (2021), New insights on the occurrence and conservation status in Italy of *Alessioporus ichnusanus* (Boletaceae), an IUCN red listed mycorrhizal species. *Plant Biosystems*. 155(2), 195–198 doi: 10.1080/11263504.2020.1813832. (*Corresponding author).

- Ceci A.*, **Spinelli V.***, Massimi L., Canepari S., Persiani A. M. (2020), Fungi and arsenic: tolerance and bioaccumulation by soil saprotrophic species. Published in the Special Issue "Advances in Bioremediation of Wastewaters and Contaminated Soils". *Applied Sciences*. 10(9), 3218; doi:10.3390/app10093218. (*Corresponding authors).

POSTERS

- Spinelli V., Ceci A., Pinzari F., Felici B., Persiani A. M., Fungi handling phosphorus: soil fungi ability to solubilise inorganic phosphate and mediate secondary minerals formation. New Topics in Mineralogy 2: the mineral-microbe interface through time and space, Poster presentation at Mineralogical Society, Online, 2-3 December 2021.

- Spinelli V., Ceci A., Dal Bosco C., Gentili A., Persiani A. M.. Glyphosate-eating Fungi: Potentiality Of Saprotrophic Fungi To Break Down Glyphosate And Utilise It As Nutritional Source, Poster presentation at World Microbe Forum, Online, 20–24 June 2021.

- Spinelli V., Sciubba F., Ceci A., Valletta A., Brasili E., Miccheli A., Pasqua G., Persiani A. M., Boosting plant growth: fungal metabolites as biostimulants for growth promotion of *Hypericum perforatum* (L.), Poster presentation at 15th European Conference on Fungal Genetics (ECFG), Rome, Italy, 17–20 February 2020.

- Malusà E., Tartanus M., Miszczak A., Russo F., Ceci A., Spinelli V., Maggi O., Furmańczyk E., Persiani A. M., Rhizo-bioremediation of DDT-contaminated soils, Poster presentation at *18th Congress of European Mycologists*, Book of Abstract: ISBN 978-83-940504-5-0, Warsaw-Białowieża, Poland, 16–21 September 2019.

- Ceci A., Spinelli V., Massimi L., Canepari S., Persiani A. M., Potential in mycoremediation of soil saprotrophic fungi: arsenic uptake and tolerance in different nutritional conditions, Poster presentation at *18th Congress of European Mycologists*, Book of Abstract: ISBN 978-83-940504-5-0, Warsaw-Białowieża, Poland, 16–21 September 2019.

- Ceci A., Spinelli V., Massimi L., Guadagnino S., Canepari S., Persiani A. M., Fungi and arsenic: tolerance and bioaccumulation by soil, Poster presentation at *114th Congress of Italian Botanical Society - VI International Plant Science Conference*, Book of Abstract: ISBN: 978-88-85915-23-7, Padova, Italy, 4–7 September 2019.

- Ceci A., Pinzari F., Spinelli V., Russo F., Maggi O., Persiani A. M., Study of TCP solubilization by some soil microfungi and evaluation of their potential as efficient plant growth-promoting organisms for *Glycine max*, Poster presentation at *113th Congress of Italian Botanical Society - V International Plant Science Conference*, Book of Abstract: ISBN 978-88-85915-22-0, Universitary Campus, Fisciano, Italy, 12–15 September 2018.
- Ceci A., Pinzari F., Spinelli V., Russo F., Maggi O., Persiani A. M., Evaluation of soil microfungi as efficient plant growth-promoting organisms for P mobilization for *Glycine max*, Poster presentation at 3rd European Sustainable Phosphorus Conference 2018 (ESPC3), Helsinki, Finland, 11–13 June 2018.

ORAL PRESENTATION AND COMMUNICATION

- Spinelli V., Brasili E., Sciubba F., Ceci A., Miccheli A., Pasqua G., Persiani A. M., Fungal bioresources to increase secondary metabolites production: elicitation effect on *Chicorium intybus* hairy roots by *Chaetomium globosum* culture filtrate, *115th Congress of Italian Botanical Society - VII International Plant Science Conference (IPSC)*, Book of Abstract: ISBN: 978-88-85915-24-4, Online, 9–11 September 2020.

- Spinelli V., Ceci A., Pinzari F., Persiani A. M., What it takes to be a plant growth promoter: characterization of two strains of soil saprotrophic fungi *Minimedusa polyspora* and *Chaetomium globosum*, Conference of Young Botanists (CYBO), Genoa, Italy, 6–7 February 2020.

- Pecoraro L., Clark B., Ceci A., Spinelli V., Persiani A. M., Marchisio M. A., Borris R. P., The newly established fungal collection and the research on medicinal mushrooms at the School of Pharmaceutical Science and Technology, Tianjin University, China, The 10th International Medicinal Mushroom Conference, Nantong, China, 19–22 September 2019.

- Ceci A., Spinelli V., Russo F., Maggi O., Persiani A.M., 2019, Biodiversity of fungi as bioresources to face diversity of soil threats, XXXVIII Annual Meeting of European Culture Collections' Organisation (ECCO), Turin, Italy, 12–14 June 2019.

- Spinelli V., Ceci A., Pinzari F., Russo F., Felici B., Maggi O., Persiani A. M., (2018), Verso un'agricoltura sostenibile: potenzialità dei funghi saprotrofi del suolo nella solubilizzazione del tricalcio fosfato, XXII Convegno Nazionale di Micologia[1], Siena, Italy, 6–8 September 2018.

ACKNOWLEDGMENTS

I would like to start with a general thank you to each and every person that crossed my path during these PhD years offering collaboration, help, support, advice, smiles, coffee and whatever else had a positive impact on this amazing journey leading to this thesis.

First of all, I would like to express all my gratitude to my supervisor Prof. Anna Maria Persiani for the constant guidance and support during this PhD. Thank you for having always believed in my potential, and for pushing me to overcomes those I believed to be my limits to grow further as a scientist and as a person.

The second thank you goes to Dr. Andrea Ceci and Roberto Giovannini, thanks for sharing this journey and the lab with me. Thank you for your precious help in difficult times, for the scientific confrontations, and finally for laughs and coffees. A special thanks goes to the former members of the lab Dr. Oriana Maggi and Dr. Fabiana Russo (my Powerpoint soulmate), even from the distance you are still very present. Thanks also to the lab's undergraduate students during these years: Samuele Guadagnino, Alessia Caglioti, Anna Gilia Collalto and Luca Talamona.

I would also like to thank all the amazing persons I had the pleasure to collaborate with during this PhD project. Thanks to: Dr. Elisa Brasili, Dr. Fabio Sciubba, Prof. Alfredo Miccheli, Prof. Gabriella Pasqua, Prof. Silvia Canepari, Dr. Flavia Pinzari and Prof. Lorenzo Pecoraro. Thank you for your contribution to the research work and to my personal scientific growth.

I would like to express my gratitude to Prof Solveig Tosi and Dr. Loredana Canfora for accepting to review this PhD thesis.

To my fellow PhD Giulia, Maria Agostina and Maria, thank you for your friendship and for sharing this crazy journey (luckily, we all attended that course!).

To Dr. Natalie Justine Oram, thank you for being such an inspiring role model! you are the toughest woman and scientist I know. Thank you for your emails that always bring laughs, advice and comfort, especially during dark times, and for sharing my same passion for witty titles. Thank you for your kindness, your honest perspectives on science and life, and finally for everything you taught me, including root washing!

To Giulia Maccaroni, Dear! Life is unfair, today you should have been here and even if you will never read this, I must write it... if I got here, it's also thanks to you. Thanks for your friendship, laughs and support. About ten years ago you gave me the courage to take that botany exam and now I'm getting a Botany PhD!!! To me you are still in a million little things, including this thesis. I promise you that one day I'll start laughing

again when someone mention the Casparian strip without saddening myself anymore. Thank you for everything Dear!!!

A special thanks goes to Dr. Alessandro Sebastiani (Sebba), against all odds we shared this journey till the end… I can't imagine these last years without your friendship, thank you for the constant support and encouragement, thank you for this nine years of sharing joys and difficulties.

To Samsu, you are one of the silver linings of this PhD, thank you for being a great friend and safe place to vent, laugh and freely express my dark humor.

To Chiara, I've chosen you 25 years ago and I couldn't have done better, I cannot imagine my life without our friendship ... thank you for everything. Love you Sorè

Moving on I would like to thank Elena, Gaia, Novi, Xi Yu, Per Hans and all my other friends who, from near or far, have never made me lack their support, love and even their understanding every time I could not be there because of this PhD. You all sparks joy... I'll keep you!

Finally, a huge thank you from the bottom of my heart goes to my parents for the endless love and support that they never made me lack of. You are my guiding lights, and what's good in me I took it from you… together with bad genetics.