UNIVERSIDADE DE LISBOA
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Developing tools and criteria for sustainable cultivation of an endogenous product – *Terfezia* species

" Documento Definitivo"

Doutoramento em Biologia

Especialidade de Biotecnologia

Inês Isabel da Silva Ferreira Andrade

Tese orientada por:

Professora Doutora Cristina Cruz

Documento especialmente elaborado para a obtenção do grau de doutor

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“The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with weary feet,
Until it joins some larger way,
Where many paths and errands meet.
And whither then? I cannot say.”

J.R.R. Tolkien, The Lord of the Rings

DECLARATION

According to the paragraph 2a) of the Article 26 of the Regulation of the Post-graduate Studies of the University of Lisbon (Diário da República Nº 175 de 2020, 2ª série de 8 de setembro de 2020), this thesis includes a compilation of scientific papers published in collaboration with other co-authors.

This thesis is comprised by the papers corresponding to Chapters 1 to 4, of which the candidate is the leading author, and was responsible for conceptualization, methodology (sample collection and processing, laboratory analytical procedures), formal analysis (data and statistical analysis), and manuscript writing of all the papers. The four papers have been published and submitted in international peer-reviewed journals:

Chapter 1
Sustainable production of ectomycorrhizal fungi in the mediterranean region to support the european green deal
Ferreira, I.; Corrêa, A.; Cruz, C.
Published in *Plants, People, Planet* (2023) *5*, 14–26.
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Chapter 2
First steps in developing a fast, cheap and reliable method to distinguish wild mushroom and truffle species
Ferreira, I.; Dias, T.; Melo, J.; Mouazen, A.M.; Cruz, C.
Submited to *Resources* (2023).

Chapter 3
Using science and technology to unveil the hidden delicacy *Terfezia arenaria*, a desert truffle
Ferreira, I.; Dias, T.; Mouazen, A.M.; Cruz, C.
Published in *Foods* (2023) 12, 3527.
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Chapter 4
The potential of ectomycorrhizal fungi to modulate below and aboveground communities may be mediated by 1-octen-3-ol.
Ferreira, I.; Dias, T.; Cruz, C.
Published in *Journal of Fungi* (2023) 9, 180.
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RESUMO

A cultura de fungos ectomicorrízicos apresenta um futuro promissor na região mediterrânica, oferecendo benefícios económicos, culturais e ambientais. Os recentes avanços biotecnológicos têm permitido o desenvolvimento de métodos e tecnologias de cultivo mais sustentáveis, especialmente para espécies de elevado valor como as trufas do deserto. Os fungos ectomicorrízicos são cada vez mais relevantes para as comunidades rurais, uma vez que a sua exploração abre novas oportunidades de negócio. Além disso, estes fungos silvestres são um recurso alimentar valioso, e simultaneamente desempenham um papel vital nos ecossistemas florestais. No entanto, a diversidade de espécies de fungos ectomicorrízicos e não ectomicorrízicos presentes nas florestas apresenta diversas oportunidades e desafios. A colheita de cogumelos é uma fonte de rendimento significativa para as comunidades rurais. No entanto, os entusiastas mais inexperientes debatem-se frequentemente com a identificação exata das espécies, o que é crucial para uma gestão e cultivo sustentáveis. São por isso necessários métodos eficientes e económicos para distinguir as diversas espécies de fungos, incluindo as potencialmente nocivas.

Esta investigação propõe a combinação de um nariz eletrónico com análise multivariada, nomeadamente análise discriminante, para diferenciar espécies de fungos silvestres e avaliar a sua comestibilidade com base nos seus perfis aromáticos. Embora seja necessária a recolha de um maior número de dados, os resultados iniciais sugerem que esta abordagem pode igualar a precisão das identificações efetuadas por micologistas e especialistas em biologia molecular. Além disso, o nariz eletrónico tem a vantagem de exigir menos formação técnica, oferecendo uma alternativa analítica mais barata e mais rápida de identificação. Numa fase inicial, recomenda-se a utilização centralizada em centros regionais de distribuição, associações micológicas ou serviços oficiais. No futuro, devido à expansão tecnológica, perspetiva-se a possibilidade da sua utilização se tornar mais acessível a toda a população.

Para além da colheita de fungos silvestres, o seu cultivo torna-se cada vez mais importante para as comunidades rurais. O cultivo de trufas do deserto tem-se destacado como um exemplo de sucesso, em particular as trufas do género *Terfezia*. Estas trufas são iguarias tradicionais muito apreciadas na região mediterrânica e têm numerosas aplicações biotecnológicas. As espécies de *Terfezia*, como *T. arenaria*, apresentam uma composição nutricional e química equilibrada e um aroma único dominado por compostos orgânicos

voláteis, como o 1-octen-3-ol. Com um aroma distinto e uma composição nutricional semelhante à da carne, esta trufa do deserto é um excelente candidato para utilização em produtos à base de carne de origem vegetal.

Devido ao seu perfil volátil singular, foi utilizado um nariz eletrónico para identificar *T. arenaria*; sendo possível distingui-la com sucesso de outras espécies de fungos comestíveis. Esta ferramenta pode contribuir significativamente para a produção e comercialização sustentáveis de *T. arenaria*, garantindo a autenticidade e a qualidade destas trufas no mercado alimentar. Para além da indústria alimentar, a utilização do nariz eletrónico pode fazer avançar a investigação sobre estas trufas, incluindo o estudo do seu papel ecológico, apoiar a identificação de novas espécies de *Terfezia* e o desenvolver métodos de deteção precoce em campo.

Os compostos orgânicos voláteis que estes fungos produzem, para além de conferir o seu característico aroma, servem como ferramentas de comunicação entre várias espécies, incluindo fungos ectomicorrízicos, microrganismos, animais e até mesmo seres humanos. Um dos voláteis mais abundantes nos fungos, o 1-octen-3-ol, é responsável pelo seu reconhecível "aroma a cogumelo". Este volátil, que se encontra em concentrações elevadas nos esporocarpos de muitos fungos, desempenha um papel vital nas interações simbióticas, influenciando os ecossistemas acima e abaixo do solo. Este volátil atua como um mediador na alteração destas comunidades. Do ponto de vista mais prático, o 1-octen-3-ol pode servir como um indicador da frutificação de fungos ectomicorrízicos. Durante o desenvolvimento dos esporocarpos, estes fungos produzem 1-octen-3-ol, levando à formação de "áreas queimadas". Nestas áreas a germinação das plantas é inibida ou atrasada, permitindo que o micélio do fungo se expanda e crie espaço para o crescimento dos esporocarpos. Além disso, o 1-octen-3-ol poderá ser detetado por tecnologias como o nariz eletrónico, ajudando na deteção precoce de fungos hipógeos como *T. arenaria*. Esta deteção precoce pode ajudar a monitorizar a produção e a acelerar a colheita, especialmente de espécies de *Terfezia*.

Esta investigação realça a importância das novas tecnologias, como o nariz eletrónico, como uma ferramenta que permite criar soluções inovadoras e sustentáveis para a gestão, cultivo e identificação de espécies de fungos ectomicorrízicos. Além disso, ao combinar abordagens multidisciplinares, esta tese explora e apresenta um recurso endógeno – *T. arenaria*. Esta trufa do deserto é um produto de elevada qualidade nutricional, ainda subestimado, e com um elevado potencial ecológico, económico e social. Ao aprofundar o conhecimento da sua

ecologia e fisiologia, podemos desenvolver práticas sustentáveis que valorizem estes recursos naturais endógenos e contribuam para capacitar as comunidades rurais.

Palavras-chave

Cultivo sustentável, Trufas do deserto, *Terfezia arenaria*, Nariz eletrônico, 1-octen-3-ol

ABSTRACT

Ectomycorrhizal fungi (ECMF) cultivation has a promising future in the Mediterranean region, offering economic, cultural, and environmental benefits. Recent biotechnological advancements have enabled the development of sustainable cultivation methods and technologies, especially for high-value species like desert truffles. ECMF is increasingly relevant for rural communities as it opens new business opportunities. Furthermore, these wild fungi are a valuable food resource and play a vital role in forest ecosystems. However, the diversity of fungal species, both ECMF and non-ECMF, in forests presents opportunities and challenges. Mushroom harvesting is a significant income source for rural communities. However, inexperienced enthusiasts often struggle with accurate species identification, which is crucial for sustainable management and cultivation. Efficient, cost-effective methods for distinguishing fungal species, including potentially harmful ones, are needed.

This research proposes combining an electronic nose with discriminant analysis to differentiate wild fungal species and assess their edibility based on aromatic profiles. While further data expansion is necessary, initial results suggest this approach could match the accuracy of identifications performed by mycologists and molecular biology experts. Moreover, the electronic nose has the benefit of requiring less technical training while offering a cheaper and faster analytical alternative. Initially, centralized usage in distribution centres, mycological associations or official services is recommended, with the potential for broader accessibility in the future.

In addition to wild fungi harvesting, ectomycorrhizal fungi cultivation is becoming increasingly relevant for rural communities. The cultivation of desert truffles, particularly *Terfezia* truffles, stands out as a successful example of ectomycorrhizal fungi cultivation. These truffles are highly valued traditional delicacies in the Mediterranean region and have numerous biotechnological applications. *Terfezia* species, like *T. arenaria*, offer balanced nutritional and chemical composition and a unique aroma dominated by C8 volatile organic compounds, such as 1-octen-3-ol. With a distinct aroma and nutritional composition similar to meat, this desert truffle is an excellent candidate for use in plant-based meat products.

Due to their singular volatile profile, an electronic nose was used to identify *T. arenaria;* due to its unique aroma profile, it was possible to distinguish it from other edible fungi species. This tool can significantly contribute to the sustainable production and commercialization of *T.*

arenaria by ensuring the authenticity and quality of these truffles in the food market. Beyond the food industry, e-nose technology can advance research on these truffles, including studying their ecological role, identifying new *Terfezia* species, and developing early field detection methods.

The volatile organic compounds these fungi produce serve as communication tools among various species, including ectomycorrhizal fungi, microorganisms, animals, and even humans. One of the most abundant volatiles in fungi is, 1-octen-3-ol, is responsible for their recognizable "mushroom aroma." This volatile, found in high concentrations in the sporocarps of many fungi, plays a vital role in ECMF interactions, influencing underground and above-ground ecosystems. It may act as a key mediator in altering these communities. Additionally, it may serve as an indicator of fungal fructification. During sporocarp development, ECMF produces 1-octen-3-ol, which could trigger the formation of "burnt areas". Plant germination is inhibited or delayed in these areas, allowing fungal mycelium to expand and create space for sporocarp growth. Furthermore, 1-octen-3-ol could be detected by technologies like the electronic nose, aiding in the early detection of hypogeous fungi like *T. arenaria*. This early detection can help monitor production and expedite harvesting, particularly *Terfezia* species and other hypogeous fungi.

This research highlights the significance of new technologies, such as the electronic nose, as tools to create innovative and sustainable solutions for managing, cultivating, and identifying ECMF species. Also, by combining multidisciplinary approaches, this thesis explores and showcases an endogenous resource – *T. arenaria* - a highly nutritional quality product, a still underrated product with a high ecologic, economic, and social potential. By deepening our understanding of their ecology and physiology, we can develop sustainable practices that value these endogenous natural resources and empower rural communities.

Keywords

Sustainable cultivation, Desert truffles, *Terfezia arenaria*, Electronic nose, 1-octen-3-ol

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LIST OF ABBREVIATIONS

Abbreviations

AI	Aridity index
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAP	Common Agriculture Policy
C-8	Eight carbon compounds
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DW or dw	Dry weight
ECM	Ectomycorrhizal
ECMF	Ectomycorrhizal fungi
e-nose	Electronic nose
EGD	European Green Deal
EU	European Union
GC–MS	Gas chromatography–mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
LDA	Linear discriminant analysis
MAT	Mating-type idiomorphs
MT	Mushrooms and truffles
MHB	Mycorrhiza helper bacteria
n.a	Data not available
NDVI	Normalised difference vegetation index
PCA	Principal component analysis
PCR	Polymerase chain reactions
PDA	Potato Dextrose Agar

PLS-DA	Partial least square discriminant analysis
rt	Retention time
RT	Room temperature
RL	Root length
RW	Root weight
SDG	Sustainable Development Goals
SL	Shoot length
SPME	Headspace-solid phase microextraction
SW	Shoot weight
SWP	Soil water potential
VIS-NIR	Visible-near infrared spectroscopy
VOC	Volatile organic compound
VPD	Air vapor pressure deficit

Treatments Abbreviations

Chapter 3

40 ℃	Pre-analysis incubation at 40 ℃
RT	Pre-analysis incubation at room temperature
Terf1, Terf2 or Terf3	*Terfezia arenaria* samples

Chapter 4

VOC_low	Low dose of 1-octen-3-ol
VOC_high	High dose of 1octen-3-ol
Ta	*Terfezia arenaria*
Tlep	*Terfezia leptoderma*
Ldel	*Lactarius deliciosus*
CT	Control
Ta	*Terfezia arenaria* inoculum

TaVOC	*Terfezia arenaria* inoculum and 1-octen-3-ol 1 µM
VOC	1-octen-3-ol 1 µM

Units

cm	Centimeters
EUR or €	Euros
h	Hour
ha	Hectare
kg	Kilograms
mg	Miligrams
µg	Micrograms
L	Litres
mL	Mililitre
µL	Microlitre
mM	Milimolar
µM	Micromolar
min	Minutes
N, N° or n	Number
p	P-value
SD	Standard Deviation
t	Tonnes
°C	Degree Celcius
%	Percentage

CHAPTER 1

General Introduction

The general introduction is based in the following article:

Ferreira, I.; Corrêa, A.; Cruz, C. (2023) Sustainable Production of Ectomycorrhizal Fungi in the Mediterranean Region to Support the European Green Deal. *Plants, People, Planet. 5*, 14–26, doi:10.1002/PPP3.10265.

General Introduction

Sustainable Production of Ectomycorrhizal Fungi in the Mediterranean Region to Support the European Green Deal

Abstract

Ectomycorrhizal fungi (ECMF) cultivation is an important economic activity in the Mediterranean region. Sporocarps from ECMF species such as *Terfezia claveryi*, *Tuber melanosporum*, *Tuber aestivum* and *Lactarius delicious* have been successfully cultivated. Due to biotechnological advances, a considerable evolution in ECMF cultivation techniques was observed in the last decade. New technologies and intensified Research and Development allow for a better understanding of the physiology of the plant-fungi symbioses and how climate change affects them. Studying forest management practices is essential to optimise the natural production of ectomycorrhizal sporocarps and help develop sustainable production practices contributing to support the rural.

A successful example of ECMF cultivation is the production of *Terfezia* species, namely *T. claveryi* and *T. boudieri*. *Terfezia* truffles are traditional delicacies with high socioeconomic relevance, especially in the Mediterranean region, and numerous biotechnological applications. Furthermore, these Mediterranean native species are an important tool to develop the bioeconomy in rural areas by creating new production strategies associated with new business models in line with the European Green Deal; the Farm to Fork and the EU Biodiversity strategies for 2030 and the Climate Law. This work reviews ECMF cultivation practices and forest management studies, presenting the case of *Terfezia* cultivation and how the sustainable production of wild and planted ECMF may contribute to achieve the fair transition to a more resilient and carbon neutral Europe.

Keywords

European Green Deal; Ectomycorrhizal fungi; Mediterranean region; Ectomycorrhiza cultivation; Bioeconomy; *Terfezia*

Societal impact statement

The planet faces a climate crisis with severe health, economic and environmental consequences. Political actions such as the European Green Deal aim to mitigate climate change by shifting the production and consumption patterns, and the production of mycorrhizal sporocarps – "the fruiting body of fungi", is no exception. The production of mycorrhizal sporocarps has a high economic, cultural, and environmental impact in the Mediterranean region. With a key role in forest ecosystems, ectomycorrhizal fungi provide services and goods essential to maintain soil quality, ecosystem functions and food, contributing to the achievement of sustainable production and the European Green Deal goals – a climate-neutral Europe.

Introduction

Over the centuries, humans have learned to harness endogenous natural resources such as fungi and their fruiting bodies, a knowledge that passed from generation to generation (Blondel 2006). Unfortunately, much of this knowledge has been lost due to rural abandonment (Comandini and Rinaldi 2020). Simultaneously, these and other natural resources are under pressure due to intensive agriculture (Baccar et al. 2020), abandonment of traditional forestry (Lasanta-Martínez et al. 2005), urban sprawl, pollution and climate changes (European Commission 2019). This resulted in high biodiversity loss affecting ecosystem functions, goods and services (Cardinale et al. 2012). Therefore, new solutions are needed to simultaneously protect biodiversity and make it economically productive (Pérez-Moreno et al. 2021a).

Together with other soil microorganisms, ectomycorrhizal fungi (ECMF) are essential for ecosystem processes, services, and functions (Cohen-Shacham et al. 2016; Bakker et al. 2019). Approximately six thousand fungal species form ectomycorrhizae (ECM) with woody plants (Wang and Qiu 2006). ECM are mutualistic symbioses where the fungal partner receives carbohydrates from the host plant, which are essential for mycelial growth and fruitbody production, and plants receive water and nutrients from ECMF (Agerer 2006). ECM can also have non-nutritional effects that improve host plant fitness (e.g. protection against pathogens, toxic minerals or drought), be agents of environmental change, interact with the soil food web and contribute to soil quality (Strullu-Derrien et al. 2018).

Fungi, including ECMF, are used primarily as food but are also well-known sources of biocompounds such as enzymes, proteins, vitamins, pigments and volatile organic compounds (VOC) (Wong et al. 2010; Culleré et al. 2010; Xu et al. 2011; Erjavec et al. 2012; Kalač 2013a), with many still to be discovered (Antonelli et al. 2020).

In 2013, Peintner et al. listed the edible sporocarps authorised for trade in 27 European countries, including 14 European Union (EU) member states. Only three species are common to all EU countries: *Cantharellus cibarius*, *Boletus edulis* and *Lactarius deliciosus*. These and other 12 ECMF species are authorised for trade-in at least nine EU countries. However, insufficient data makes it challenging to provide accurate information on ECMF production and market prices (Table 1.1). Because national or European official data are scarce there are also few published studies and analyses of sporocarp production and its socioeconomic impacts, with most studies focusing on specific areas or regions (Tahvanainen et al. 2016; Tahvanainen et al., 2019; Bonet et al., 2020) (Table S1.1).

ECMF are traditionally harvested in forests, and their formation is linked to habitat characteristics and climate conditions (Parladé et al. 2014). They are an important food and income source for rural populations (De Román and Boa 2006), and their international trade has increased in recent years (de Frutos 2020). ECMF currently represent up to 25 % of the soil expectation value (Tomao et al. 2017b), confirming their importance as a natural resource at ecological and socioeconomic levels.

However, their natural production has declined over the last century (Yun and Hall 2004), and several species are now in danger of extinction (Arnolds 1991; Egli 2011; Nic Lughadha et al. 2020). Among ECMF, *Terfezia* species, commonly known as desert truffles, are luxury products, being some of the most expensive products in the international market (Milanesi et al. 2020) and among the most studied (Gajos and Hilszczańska 2013; Morte et al. 2017).

This work aims to review the current state of ECMF cultivation, focusing on the production of *Terfezia* truffles, and to discuss how their exploitation can promote sustainable practices in line with the European Green Deal (EGD) strategies and policies.

Table 0.1 - Several edible ectomycorrhizal (ECM) species authorised for trade-in EU countries, and available data of commercialised product and market prices.

ECM species	Nº of EU Countries†	Commercialized product		Market prices		Country	References
		t/year‡	Period	€/kg ‡	Period		
Boletus edulis	14	25,000	2014	12	2017	Spain	Bonet et al., 2020;
		400	1978–2016	7.7	1978–2016	Finland	Baars, 2017 Tahvanainen et al., 2019
Cantharellus cibarius	14	2500	2007	20	2003	Spain	Bonet et al., 2020;
		12.6	1978–2016	13.8	1978–2016	Finland	Román and Boa, 2004 Tahvanainen et al., 2019
Hydnum repandum	12	700	n.a	9.9	2002	Spain	Bonet et al., 2020; Román and Boa, 2004
Lactarius deliciosus	14	6800	1990-1998	13	2002	Spain	Bonet et al., 2020;
		100	1978–2016	4.0	1978–2016	Finland	Román and Boa, 2004 Tahvanainen et al., 2019
Terfezia claveryi	2	670	2001-2015	60	n.a.	Spain	Andrino et al., 2019; Oliach et al, 2020
Tuber aestivum	8	30	2016	50	n.a	Spain	Oliach et al., 2020
Tuber brumale	6	0.5	2015	120	n.a	Spain	Oliach et al., 2020
Tuber melanosporum	9	47	2013-2017	550	2016-2017	Spain	Oliach et al., 2020

† - data from Peintner et al. (2013); ‡ Maximum values registered; t/year – tonnes per year; €/kg – Euros per kilogram; n.a. - data not available

From mycorrhizal plant production to forest management

Several edible species from genera *Tuber*, *Lactarius* and *Terfezia* are currently successfully cultivated (Slama et al. 2010; Karwa et al. 2011; Morte et al. 2012; Donnini et al. 2013), but most commercial species are not. This is mainly because the mechanisms affecting ECMF production, such as mycorrhizal establishment, symbiotic processes, life cycle and ecological drivers, are not fully understood (Domínguez-Núñez et al. 2019), making their cultivation difficult to "master". Furthermore, because of their symbiotic relationships with plants, the fruiting conditions for those currently cultivated are complex. This led to increased

research into new mycosilviculture techniques and management to improve sporocarp production (Tomao et al. 2017b; Jiang and Yanbin 2018). Although several mycorrhizal plant production and management methodologies are well implemented (Figure 1.1), the development and optimisation of techniques can still be observed, often adapted to the target host and ECM species.

ECMF current cultivation practice

ECMF cultivation and sporocarp production require the production of ECM seedlings (Álvarez-Lafuente et al. 2018) (Figure 1.1). Seedlings are usually inoculated in nurseries and later transplanted into forests, but tree inoculation in the field has also been achieved using root traps with spore inoculum (Azul et al. 2014). In addition, host plants can be inoculated *in vivo* using ECMF spores or *in vitro* using ECMF mycelium. The selected method depends on the ECMF species, and the method used to obtain the host plant (seed germination or *in vitro* cloned plants).

The greatest challenge for spore and mycelium inoculation methods is producing high-quality mycorrhizal plants colonised with only the desired ECMF (Murat and Martin 2008). Contamination can happen during mycorrhizal establishment in nurseries or following seedling transplantation into the field, imperilling the persistence and spread of the inoculated ECMF (Domínguez-Núñez et al. 2019). In addition, several aspects of fungal physiology and ecology may affect mycorrhisation and sporocarp production, namely interactions with mycorrhiza helper bacteria (MHB). MHB improve mycorrhisation, plant survival in nursery conditions and sporocarp production (Azul et al. 2014; Navarro-Ródenas et al. 2016), reduce environmental and pathogen impacts on ECM hosts, fix nitrogen and improve nutrient acquisition (Domínguez-Núñez et al. 2019). MHB include *Bacillus, Pseudomonas, Burkholderia* and *Streptomyces* (Mello et al. 2010; Choudhary et al. 2017). Our knowledge of ECMF interactions with other microorganisms is still limited but may be key to improve their commercial production.

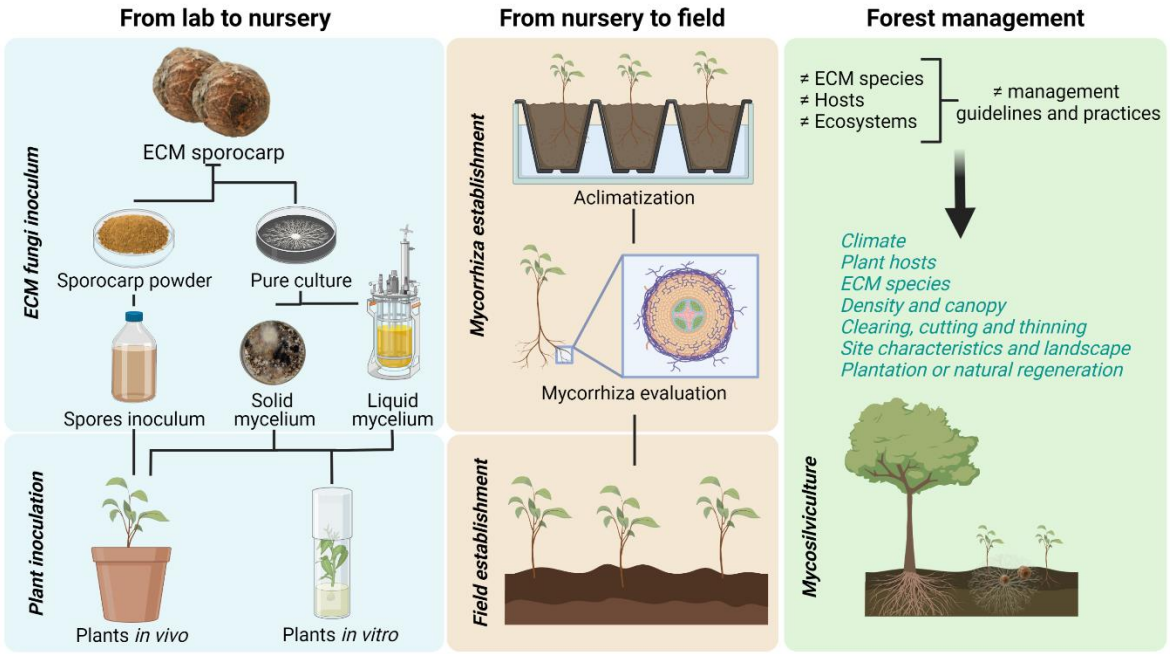

Figure 0.1 - Scheme of current phases for ectomycorrhizal (ECM) plants production and forest management criteria for their field implantation. ECM: Ectomycorrhizal. Created with BioRender.com.

After mycorrhizal establishment and acclimatisation in the nursery, the mycorrhizal plants are transferred to the field. At this stage, it is crucial to select an area with the best possible conditions for plant and mycorrhiza development, such as the landscape, soil proprieties and climatic characteristics (Oliach et al. 2020). These vary with the selected plant host and ECM and the cultivation practices to apply. The cultivation of ECMF is still recent compared with other horticultural practices, and further research and full-scale experiments are needed to develop practices that guarantee and increase ECMF production (Guerin-Laguette 2021). The current cultivation practices for ECMF sporocarp production include maintenance of the plantations by performing weed control and clearing (with or without mechanisation), irrigation systems, among others (Olivera et al. 2014; Oliach et al. 2020; Guerin-Laguette 2021). After the mycorrhizal plants have been established in the field, it is important to monitor the persistence and development of the introduced ECMF species, which is usually performed using microscopy and molecular tools (Guerin-Laguette 2021).

Forest management

Climate conditions have been considered the main factor promoting variability in sporocarp production (Olano et al. 2020). Dry seasons were observed to affect sporocarp production, especially in Mediterranean regions. However, climate cannot be dissociated from

other variables. Because of their relationship with plants, ECMF communities are sensitive to shifts in vegetation (Lauber et al. 2008). Appropriate forest management practices are, therefore, key for both preserving fungal diversity (Tomao et al. 2017b) and increasing fungal productivity.

Recent studies suggest that forest stand structure (including plant species, age and density, landscape, canopy cover and the relationship between tree and sporocarp production) and forest management practices (e.g. understory thinning and clearing, regulation of edible sporocarp harvest and mycorrhizal plant regeneration and use) also play an essential role (Suz et al. 2015; Tomao et al. 2017b) (see Table S1.1). For example, Collado et al. (2019) found that sporocarp yield was correlated with tree growth (seasonal wood production) and mediated by summer and autumn precipitation, indicating that tree growth and sporocarp biomass are sensitive to precipitation events under water-limited conditions. Olano et al. (2020) observed that *Boletus edulis* and *Lactarius deliciosus* sporocarp yields were correlated with previous year normalised difference vegetation index (NDVI), indicating that higher carbon availability favours ECMF development.

Several studies show that ECMF are more abundant in younger stands, which can be related to higher tree growth rates (Bonet et al. 2008; Egli et al. 2010; Martínez-Peña et al. 2012a; Ágreda et al. 2014; Tahvanainen et al. 2016). Martínez-Peña et al. (2012a) also observed a second yield peak of *L. deliciosus* sporocarp production in stands over 70 years old, suggesting a relationship with the more open canopies and more intensive management in older stands. Accordingly, forest management practices such as thinning and clearing have positively affected sporocarp production, with higher sporocarp yields observed immediately after thinning (Bonet et al. 2012; Tahvanainen et al. 2016; Collado et al. 2018). Tree radial growth following thinning has also led to increased ECMF sporocarp production (e.g. *B. edulis*) (Egli et al. 2010).

Predictive models can be developed to facilitate management decisions (Table S1.1). There should be as many variables as possible in order to predict the impacts of management strategies and climate on sporocarp seasonal production (de Frutos et al. 2019a, b). The published models were based on studies conducted in native and planted forests, and many of them address the production of both mycorrhizal and saprotrophic fungi (Table S1.1). Some models focused only on ECMF production, with special attention to the productivity of edible marke(t?) species (Salerni and Perini 2004; Ortega-Martínez et al. 2011; Martínez-Peña et al.

2012a, b; Tahvanainen et al. 2016; Olano et al. 2020). Most models include precipitation, temperature, and forest management as main variables. Still, they do not consider how ECMF production affects soil quality and contributes to improved water-saving and quality, which are main factors for sustainable production and halting climate change. The influence of forest management practices on ECMF sporocarp yields reflects the importance of host trees for fungal biomass production and reinforces the importance of forest management to increase sporocarp production. However, it needs to be better understood and analysed with other variables, such as soil and water quality (Tomao et al. 2017a; Bonet et al. 2020).

New technologies for ECMF safe and sustainable exploitation

ECMFs are a source of revenue for rural populations who cultivate or collect them from their natural habitats (Yun and Hall 2004), and at least fifteen ECMF species are traded in Europe (Peintner et al. 2013). There are 14,000 fungi known to produce sporocarps (mushrooms) (Li et al. 2021). However, only 2,500 fungi produce edible sporocarps, from which 200 ECMF species are found in the north hemisphere (Yun and Hall 2004; Li et al. 2021). Considering these numbers, many ECMF and non-ECMF species are unsafe for human consumption, and misidentification can lead to intoxication (Eren et al. 2010). New methodologies and technologies have been developed to address the lack of knowledge about wild sporocarps (including ECMF species) and contribute to their identification (Wei et al. 2022; Lee et al. 2022; Hodgson et al. 2023).

Morphological identification is the most common way of identifying wild sporocarps (Peintner et al. 2013; Wei et al. 2022). Morphological identification has also been supported by instrumental analysis (e.g. gas chromatography-mass spectrometry – GC-MS) and molecular biology approaches (Peintner et al. 2013; Wei et al. 2022; Lallawmsanga and Carrasco 2022). However, most of the population collecting wild sporocarps are amateurs, have limited knowledge, and have little access to laboratory analysis.

In recent years, new tools have emerged to support the identification of wild fungi sporocarps. Portable visible-near infrared spectroscopy (VIS-NIR) combined with chemometrics has successfully identified the geographic origin, species, year of production, mineral content, and quality of edible sporocarps (Roy et al. 1993; Casale et al. 2016; Meenu and Xu 2019; Segelke et al. 2020; Yan et al. 2023). Currently, new technologies enable the collection of field images using a camera, smartphone, or drone and in real-time image

recognition (Zahan et al. 2021; Zhao et al. 2021; Chaschatzis et al. 2022; Ooro 2022; Picek et al. 2022; Lee et al. 2022; Hodgson et al. 2023) by integrating machine learning for species identification (Wibowo et al. 2018; Jahan Pinky et al. 2019; Chumuang et al. 2020; Kousalya et al. 2022; Rahman et al. 2022) for species identification.

Odour is also an important trait for identifying wild fungi, and many ECMF are widely recognized for their aroma (e.g. truffles and boletus). Odour is commonly detected by instrumental analysis (e.g. GC-MS) and is beginning to be explored using technologies such as the electronic nose (-nose) (Wei et al. 2022). This sensitive device can detect and analyse the volatile compounds in the air, creating a unique "*smellprint*" for each fungal species (Ferreira et al. 2023c). Several studies have shown that the e-nose can accurately distinguish between wild fungi (Zhou et al. 2015; Zhang et al. 2016; Portalo-Calero et al. 2019b, a, 2020; Gómez et al. 2022). This technology has been used for fungi applications in the food industry (Zhou et al. 2015; Chilo et al. 2016; Pei et al. 2016; Song et al. 2019; Gómez et al. 2022; Gholami et al. 2023). The e-nose can potentially be a tool for support in identifying wild sporocarps in the field or distribution centres. However, more research is needed before they can be widely adopted.

A case of success: *Terfezia* species cultivation

Terfezia species, known as desert truffles, are adapted to various soil types and edaphoclimatic conditions throughout the Mediterranean region and are both a source of valuable sporocarps and biocompounds (Díez et al. 2002). Desert truffles have been used since the Bronze Age in the Middle Euphrates (Shavit 2014) and remain very popular due to their high nutritional and gastronomic value and profitable sporocarps (Mandeel and Al-Laith 2007; Khalifa et al. 2019). They are important for local traditions and economy, but native traditions related to desert truffles are disappearing, and their habitats and natural abundance are decreasing due to excessive harvesting, climate changes, rural abandonment and natural disturbances, among other factors (Boa 2004; Morte et al. 2012).

Desert truffles are hypogeous fungi that form *ectendomycorrhizae* with Cistaceae plants (Figure 1.2) and are mainly distributed throughout the Mediterranean basin (Navarro-Ródenas et al. 2012; Sitrit et al. 2014; Marqués-Gálvez et al. 2020a). *Terfezia* truffle production is seasonal and takes place in spring, in arid and semiarid regions (Morte et al. 2012). *Terfezia claveryi* and *T. boudieri* have been successfully cultivated (Morte et al. 2017, 2020), and *T. arenaria* cultivation in acid soils is currently under development (Louro 2020a; Louro et al. 2021). In addition, sixteen other *Terfezia* species were found in the Iberian Peninsula (Table S1.2), opening new possibilities for *Terfezia* cultivation and exploitation. However, much is still unknown about their edibility, properties and associated economic value.

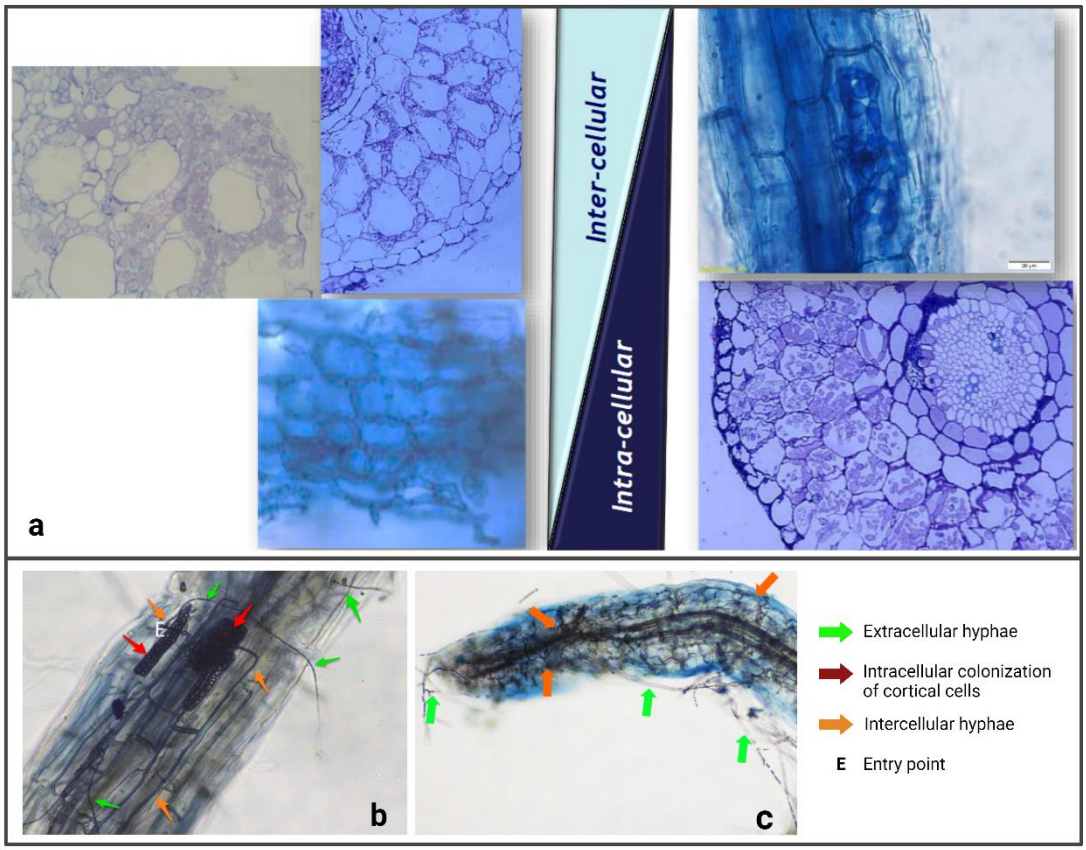

Figure 0.2 - Ectendomycorrhizae of *Terfezia* species with Cistacea plants. a) *T. claveryi* ectendomycorrhizae scheme, adapted from Morte 2023; b) *T. arenaria* × *Tuberaria guttata* mycorrhizae, roots collected from *T. arenaria* ascocarps; c) *T. arenaria* × *C. salviifolius* in initial stages of root colonization (40 ×) - images from unpublished work of the authors. Created with BioRender.com.

Accurately distinguishing between *Terfezia* species is important because misidentification can lead to incorrect conclusions about their ecological roles, distribution, and conservation status. The taxonomic history of the genus is intricated by numerous old species names, many of which are synonyms of earlier species and lack clear diagnostic features, leading to sparse citations (Díez et al. 2002; Louro et al. 2019; Louro et al. 2021). This issue persisted before

molecular methods because species identification relied primarily on ambiguous morphological, anatomical, and chemical features (Bordallo and Rodríguez 2014). Molecular studies have revealed significant intraspecific diversity and the existence of species complexes, including cryptic species, within *Terfezia* (Bordallo et al. 2013; Díez et al. 2002; Kóvacs et al. 2011). High variability in the rDNA internal transcribed spacer (ITS) region was found in *Terfezia leptoderma* and *Terfezia olbiensis*, identifying at least four distinct lineages of *Terfezia* with spiny spores (Kóvacs et al. 2011). Although *T. olbiensis* was previously thought to be synonymous with or an immature form of *T. leptoderma*, it is now considered a distinct species (Bordallo et al. 2013). Moreover, *T. leptoderma* was frequently considered by some authors as synonymous with *Terfezia fanfani* (Chevalier 2014; Venturela 2004); recent phylogenetic studies showed that *T. leptoderma* and *T. fanfani* are synonyms denominating specimens belonging to the same species (Louro et al. 2019). Despite this, the correct name was not yet assigned according to the rules of the International Code of Nomenclature for algae, fungi, and plants, and *T. leptoderma* and *T. fanfani* are not considered synonyms in the nomenclatural indices and repositories- Index Fungorum, Fungal Names and MycoBank.

Terfezia species are an example of successful cultivation of mycorrhizal fungi, achieved through a combination of biotechnology, for optimization of fungal inoculum production, and forest management techniques (Morte and Honrubia 1992; Morte et al. 2009; Morte and Andrino 2014; Louro et al. 2021). Knowledge by local collectors and producers has been fundamental in understanding plant phenology as a key factor affecting desert truffle production, e.g., the coincidence of the production season with flower blooming (Marqués-Gálvez et al. 2020).

Similarly to most ECMF, *Terfezia* breeding programs are based on the plantation of inoculated plants (Morte et al. 2009, 2020; Morte and Andrino 2014; Arenas et al. 2018; Louro et al. 2021). *Terfezia* mycorrhization (*in vitro* and *ex vitro*) and maintenance have been studied (Gutiérrez et al. 2003; Zaretsky et al. 2006; Navarro-Ródenas et al. 2012; Jamali and Banihashemi 2013; Zitouni-Haouar et al. 2014; Turgeman et al. 2016), and one method patented (Andrino et al. 2013).

Management protocols for establishing desert truffle plantations have been developed over the last two decades (Morte et al. 2009, 2017; Andrino et al. 2019). These protocols need to consider that precipitation is critical for desert truffle production (Morte et al. 2012). Therefore, agroclimatic parameters such as evapotranspiration, soil water potential (SWP),

relative air humidity, aridity index (AI) and air vapor pressure deficit (VPD) are essential to understand truffle production. SWP and AI were the main agroclimatic parameters determining annual truffle yields in a 15-year old *Helianthemum almeriense* × *T. claveryi* plantation (Andrino et al. 2019), and can be managed using irrigation during autumn and spring to maximise desert truffle production (Andrino et al. 2019). Marqués-Gálvez et al. (2020a) reported a switch in the phenology of *H. almeriense* × *T. claveryi* mycorrhizal plants during the spring-summer transition, namely a sigmoidal relationship between stomatal conductance and VPD, which was correlated with total truffle production, i.e. truffle yield was observed to decrease in years with early summers and early VPD threshold. This indicates that the VPD – stomatal conductance relationship can be used as a marker for truffle production and, together with VPD control, be a tool for desert truffle production management (Marqués-Gálvez et al. 2020a).

Climate changes are predicted to lead to future increases in temperature and decreases in precipitation and relative humidity in Mediterranean regions (Dubrovsky et al. 2014), which would result in lower VPD (Andrino et al. 2019). Future decreases in truffle production due to climate changes may therefore arise. Climate changes are also predicted to lead to increased atmospheric CO_2 concentrations. Because high CO_2 concentrations induce partial stomatal closure, decreasing water loss by transpiration (Lindner et al. 2010), it is important to understand how this increase can affect ECMF. For example, high atmospheric CO_2 concentrations coupled with drought and high VPD (water stress) were observed to improve net C assimilation and water use efficiency in *H. almeriense* × *T. claveryi* and lead to increased flowering events (Marqués-Gálvez et al. 2020b).

Andrino et al. (2019) showed that the production of *T. claveryi* is conditioned by many climatic factors, leading to production fluctuations that directly affect the final product yields and economic revenue. So, future management practices for desert truffle production should consider these and other questions related to climate change effects.

Desert truffle life strategies may also be important for their cultivation. For example, *Terfezia* species were recently found to be heterothallic, i.e. they have two mating-type idiomorphs (MAT1-1 and MAT1-2), and only strains with differing MAT are sexually compatible (Martin et al. 2010; Marqués-Gálvez et al. 2020a). However, in *T. borchii,* which is also heterothallic, strains with the same matting type were found to produce truffles (Iotti et al.

2016). Therefore, improved knowledge of fungal life cycles and reproduction is needed to master their cultivation.

A better understanding of the effects of soil type and rhizosphere bacteria on plants mycorrhizal with desert truffles can also be essential to develop novel techniques to improve plant fitness and survival and mycorrhizal establishment (Navarro-Ródenas et al. 2016).

Terfezia truffles as functional foods and new applications

Desert truffles are considered functional foods because of their high protein (20 % of dry weight) and carbohydrate contents. They also have high fibre and lipid contents (Ahmed et al. 1981; Kıvrak 2015; Hamza et al. 2016; Tejedor-Calvo et al. 2020). *Terfezia* species are rich in saturated (Tejedor-Calvo et al. 2020), monosaturated and polyunsaturated fatty acids, namely oleic and linoleic acids (Murcia et al. 2003; Tejedor-Calvo et al. 2020). They have an interesting aromatic profile and flavour. Several volatile compounds have been identified (e.g. namely 1-octen-3-ol; hexanal, 2-octenal; Kamle et al. 2017), especially in *Tuber* species (Mauriello et al. 2004; Splivallo et al. 2011).

Among the most interesting properties of *Terfezia* species are their antioxidant (Dundar et al. 2012; Dahham et al. 2018), enzymatic (Pérez-Gilabert et al. 2005, 2014; Benaceur et al. 2020a), antibiotic (Janakat et al. 2004, 2005; Neggaz et al. 2018; Harir et al. 2019a), anti-proliferative and anti-cancer activities (Dahham et al. 2018; Al Obaydi et al. 2020; Tejedor-Calvo et al. 2020) (Table S1.3). Several species of *Terfezia* produce common food antioxidants, such as tocopherol (α and δ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (Martínez-Tomé et al. 2014; Tejedor-Calvo et al. 2020). They also have an advantage over other food products since preservation and freezing do not affect their properties (Murcia et al. 2003; Martínez-Tomé et al. 2014). Their addition can improve food nutritional quality and antioxidant activity, allowing its consumption at any time of the year (Gadallah and Ashoush 2016). Their nutritional and aromatic profile together with their biological activities make them a potential resource for new plant-based meat products, as can be seen by the number of patents involving *Terfezia* species. Their proprieties also make them an important resource for cosmetic and pharmaceutical industries.

Socioeconomic relevance

Terfezia truffles are a natural resource with promising cultural and economic potential. Their cultivation can be a source of revenue for rural populations through truffle

commercialisation or mycotourism (truffle hunting) and other leisure and well-being activities (Honrubia et al. 2014; Serra et al. 2017). Furthermore, their cultivation is sustainable and can be combined with other agroforestry activities (Morte et al. 2009), similarly to true truffle (*Tuber* spp.) production (Sourzat 2020). Because of their ability to grow in dry environments, *Terfezia* species can be potentially cultivated in new areas as climate changes progress, as predicted for *Tuber* species (Čejka et al. 2020). They can also be a solution to rehabilitate unproductive or disturbed lands, since they can decrease soil erosion and promote soil biological activity (Morte et al. 2008).

Consortia of stakeholders with different expertise, namely producers, technological developers, research institutions and restaurateurs, such as the Asociación Española de Turmicultura2017 (https://trufadeldesierto.com/), have started to promote cultivation and consumption of desert truffles. However, to ensure rural development, the ecosystem management must follow three fundamental principles: i) production through the exploitation of endogenous natural resources; ii) conservation by pursuing sustainable criteria; and iii) taking into account local biodiversity and multifunctionality (Honrubia et al. 2014).

Terfezia is therefore an important sustainable crop that profits from the sustainable exploitation of endogenous resources and simultaneously contributes to environmental awareness and climate change mitigation.

Importance of ECMF within European policies

European rural areas have suffered from land abandonment during the latter 20th century, with environmental, socioeconomic and landscape repercussions (Lasanta et al. 2017), such as loss of traditional knowledge and changes in land-use patterns (Hummel and Smith 2017).

Sporocarps have been in the past, and still are in certain European regions, an essential resource in rural areas, providing food, medicines, and substantial income to some rural families and small companies, and are part of an increasing economic and cultural interest in the use of non-timber forest products (Miina et al. 2020). Creating new sustainable methodologies that value these endogenous resources can bring economic value to rural populations and be a strategy for sustainability, ecosystem conservation and decreasing the exodus from rural areas (Serra et al. 2017; Martínez-Ibarra et al. 2019). These activities are supported by national and European policies and financial strategies integrated into the European agricultural fund for

rural development (European Commission 2021), created by the EU as a Common Agriculture Policy (CAP) funding instrument to support rural development strategies and projects. Sporocarp-related economic activities fit into the priorities of this fund because of their environmental, economic, and social aspects. The impacts of *Terfezia* truffle sustainable cultivation (Figure 1.3) illustrate how sporocarps can contribute to achieving the EGD aim of climate neutrality by 2050 (European Commission 2019). Plantations of *T. claveryi* can reach a production of 400 kg/ha per year, while natural areas only produce between 50 to 170 kg/ha (Oliach et al. 2020). The low production rates in natural areas are mainly due to climate change disturbances, such as low precipitation, and anthropogenic factors. The implementation of measures that promote sustainable production is crucial to mitigate climate change and loss of biodiversity and optimising the cultivation of these species and maintaining natural production. Investing in R&D will contribute to the development of nature-based solutions and new sustainable production techniques, which are crucial tools for rural development and the EGD goals.

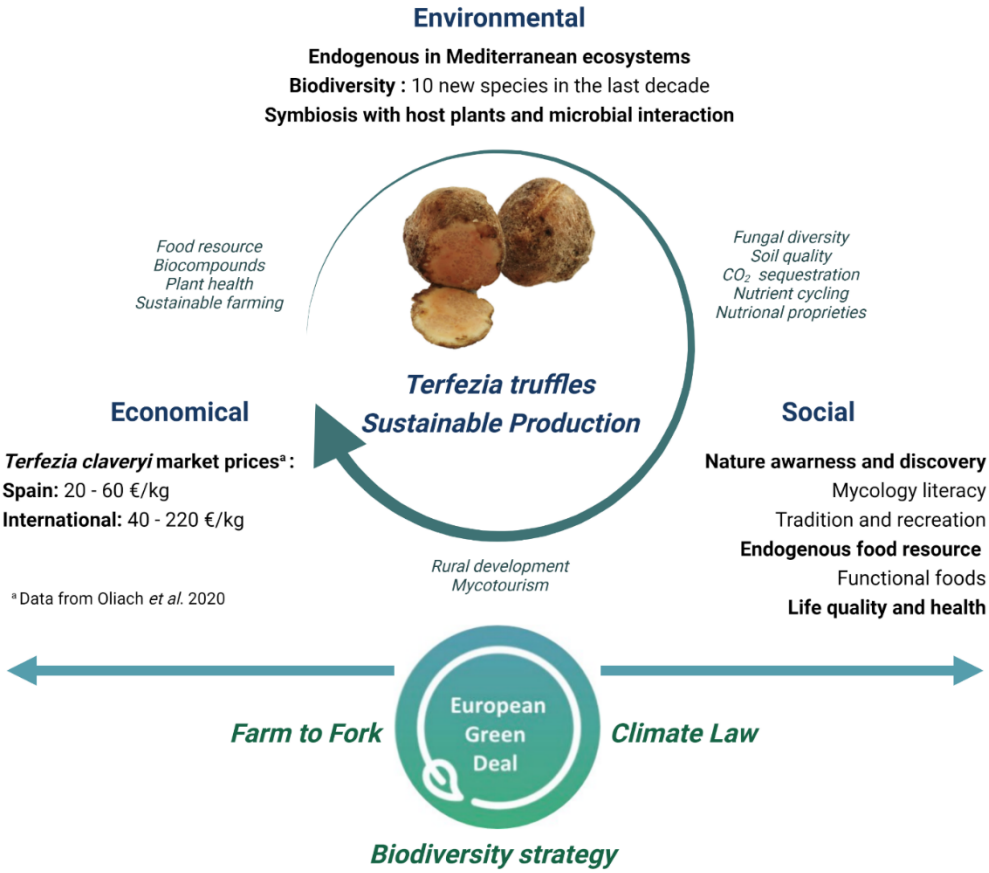

Figure 0.3 - Conceptual model for the sustainable cultivation and exploitation of *Terfezia* truffles, and how this can contribute to the European Green Deal (EGD) goals (green text) and strategies (blue arrows). Created with BioRender.com.

Within European Green Deal strategies

The EU recently developed new efforts to meet the goals defined in the 2015 Paris Agreement (United Nations 2015a) and the United Nations 2030 Agenda for Sustainable Development (United Nations 2015b), adopting a robust agenda, the EGD (European Commission 2019). These measures meet the current public demands derived from increased awareness of environmental questions, mostly those concerning climate change, natural disturbances, biodiversity loss, and human welfare implications (Fisher 2019).

Currently, the legislation within the EU regarding wild and edible ECM sporocarps is still scarce, and only a few EU member countries have lists or guidelines of edible sporocarps authorised for trade (Peintner et al. 2013). Moreover, there is a lack of uniformity between EU and country levels regarding i) legislation for trade and food safety; ii) management and conservation; iii) creation of protected zones and a list of protected species; iv) specific areas and authorisations for collecting and harvesting, among others. Supportive legislation and regulation of activities are essential to support rural development, namely small businesses, farmers, and forest owners. Moreover, understanding how economic activities associated with ECMF cultivation meet the EGD strategies and contribute to sustainable rural development is essential.

The EGD agenda aims to reinforce Europe's resilience by halting biodiversity loss and building a healthy and sustainable food system. To achieve these goals, the EU presented a bold package of measures within the Biodiversity Strategy 2030, the Farm to Fork and the European Climate Law (European Commission 2019), which include actions that directly affect edible ECMF cultivation and conservation. Several aspects of ECMF cultivation and their ecological and socioeconomic importance are mentioned in these documents, showing how ECMF can contribute to the EGD goals. The Farm to Fork and Biodiversity strategies mutually reinforce and bring together nature, farmers, businesses, and consumers to work towards a sustainable future (European Commission 2020a). With the new Biodiversity Strategy, the European Commission aims to bring nature back into our lives (European Commission 2020b). ECMF can play an important role in this awareness. As already stated, several species can adapt to a range of edaphoclimatic environments or locations. Many have high economic value and represent important seasonal goods and services for many rural communities (see Table 1.1 and Figure 1.3), e.g. mycotourism represents an average income of 32 million euros per year in the Spanish region of in Castilla y Léon (Bonet et al. 2014; Tahvanainen et al. 2016; Buntgen et al.

2017; Martínez-Ibarra et al. 2019; Oliach et al. 2020). Their diversity brings various opportunities in terms of different food, pharmaceutical and health applications, and production flexibility and is critical to developing cultural, recreational and mycotourism activities.

Implementing support from European funds to develop *Terfezia* and other ECMF production will improve the quality of life of rural populations in Europe and contribute to their sustainable exploitation. This type of support could be applied to the creation of new business opportunities, such as desert truffle cultivation, the development of new food products (e.g. *Terfezia* as plant-based meat), and the development of new technologies for safe consumption and authentication of these products. The support of research projects that explore the ecological aspects of these fungi could unveil new aspects that contribute to their sustainable exploitation.

On the other hand, ECMF contributes to halting biodiversity loss. Moreover, their role in improving plant health and soil quality, leading to decreased nutrient losses and increased CO_2 sequestration, directly affects the production of healthy and environmentally friendly food, the Farm to Fork strategy (European Commission 2020c).

Their recognised nutritional value and importance as a source of biocompounds with industrial applications make them a strategic product directly impacting rural development by creating new revenue sources. All these contributions meet objectives of the Farm to Fork strategy, such as: i) ensure food security, nutrition and public health; ii) mitigate climate change and adapt to its impacts; iii) preserve the affordability of food while generating fairer economic returns; and iv) ensure food security, nutrition and public health (European Commission 2020c). ECMF cultivation and exploitation will therefore play an important role in future food production (Farm to Fork strategy), environmental protection (EU Biodiversity strategy for 2030) and climate change (Climate Law).

Conclusions

ECMF cultivation evolved in recent decades due to technological development and increased research. Cultivation of species of high economic value, such as truffles, has contributed to this evolution. Understanding the factors that affect their production, such as forest management, is crucial to efficient and sustainable production. The development of research and discovery of new species with productive potential also bring new business opportunities.

ECMF are inextricably linked to forestry and agricultural activities, which are associated with rural areas. Developing new production techniques and business models and creating support to promote ECMF production is increasingly more important for the development of rural communities. Notably, the activities associated with ECM sporocarp production are in line with the guidelines of the EGD and related European policies, which aim at a more sustainable Europe, values its endogenous natural resources, and empower rural communities.

Supplementary Materials

The following supporting information can be consulted in Appendix 1:

Table S1.1 - Studies and predictive models of climatic and forest management variables influencing natural production of macrofungi mushrooms**.**

Table S1.2 - *Terfezia* species identified in the Iberian Peninsula**.**

Table S1.3 - Biological activity of *Terfezia* species**.**

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Objectives and Thesis outline

The general goal of this thesis is to develop novel tools and criteria to promote the sustainable cultivation of an endogenous product - *Terfezia* species. Specifically, this thesis aims to contribute to the safe and sustainable exploitation of ectomycorrhizal fungi such as *T. arenaria*. New technological tools were explored to showcase *T. arenaria* as a highly nutritional quality product, a still underrated product with a high ecologic, economic, and social potential. Accordingly, the specific goals of the current work were:

a) Promote the sustainable production of ECMF – *T. arenaria*, in the Mediterranean region to support the EGD.
b) Develop a fast and reliable method to distinguish wild fungi species, which could be used to ensure the quality and authenticity of ECMF products.
c) Highlight the potential of *T. arenaria* as a valuable food resource and develop a method for rapid identification of this species.
d) Investigate the role of ECMF volatiles in influencing belowground and aboveground communities to better understand ECMF ecology and management.

Thesis outline

The present thesis is divided into five chapters (Figure 1.3). It comprises four scientific papers published or submitted in peer-reviewed international journals, each corresponding to a chapter. Hence, a significant part of this thesis is a compilation of the published work.

Chapter 1, "General Introduction", in their majority, was published in the review: Ferreira, I.; Corrêa, A.; Cruz, C. (2023) Sustainable Production of Ectomycorrhizal Fungi in the Mediterranean Region to Support the European Green Deal. *Plants, People, Planet. 5*, 14–26.

This first chapter overviews the current literature concerning ECMF production, particularly *Terfezia* species, and their relevance in current EU policies. It emphasizes the importance of cultivating ECMFs, discusses recent biotechnological advancements, and highlights their role in sustainable cultivation practices. Moreover, we explore how sustainable wild and cultivated ECMF production can contribute to achieving the EGD objectives and a more resilient Europe.

Chapter 2 explores the development of a fast, cost-effective, and reliable method for distinguishing various wild fungi sporocarps (mushrooms and truffles) using an e-nose. It

emphasizes the need for efficient identification methods for safe and sustainable exploitation of these forest resources. In this chapter, we investigate the potential of electronic nose technology combined with discriminant analysis to identify wild fungi species and assess their edibility based on aroma profiles.

Chapter 3 focuses on *T. arenaria*, a desert truffle native to the Mediterranean Basin, investigates its nutritional and chemical composition and volatile aroma profile and describes the development of a rapid identification method using an e-nose. The chapter emphasizes the uniqueness of *Terfezia arenaria*'s aroma and its potential as a valuable food resource with a nutritional composition similar to meat, making it suitable for plant-based meat products.

Chapter 4 investigates the role of the VOC 1-octen-3-ol in the mechanisms employed by ECMF to influence both belowground and aboveground communities. It discusses the effects of 1-octen-3-ol on the mycelium growth of three ECM fungal species and its impact on seed germination in host Cistaceae species. The findings highlight the sensitivity of different fungal species to 1-octen-3-ol and its influence on seed germination, suggesting its role in mediating changes in belowground and aboveground communities.

Finally, a general conclusion is presented in Chapter 5, where an integrated view of the main outcomes is given, and future perspectives and knowledge gaps for further investigation are pointed out.

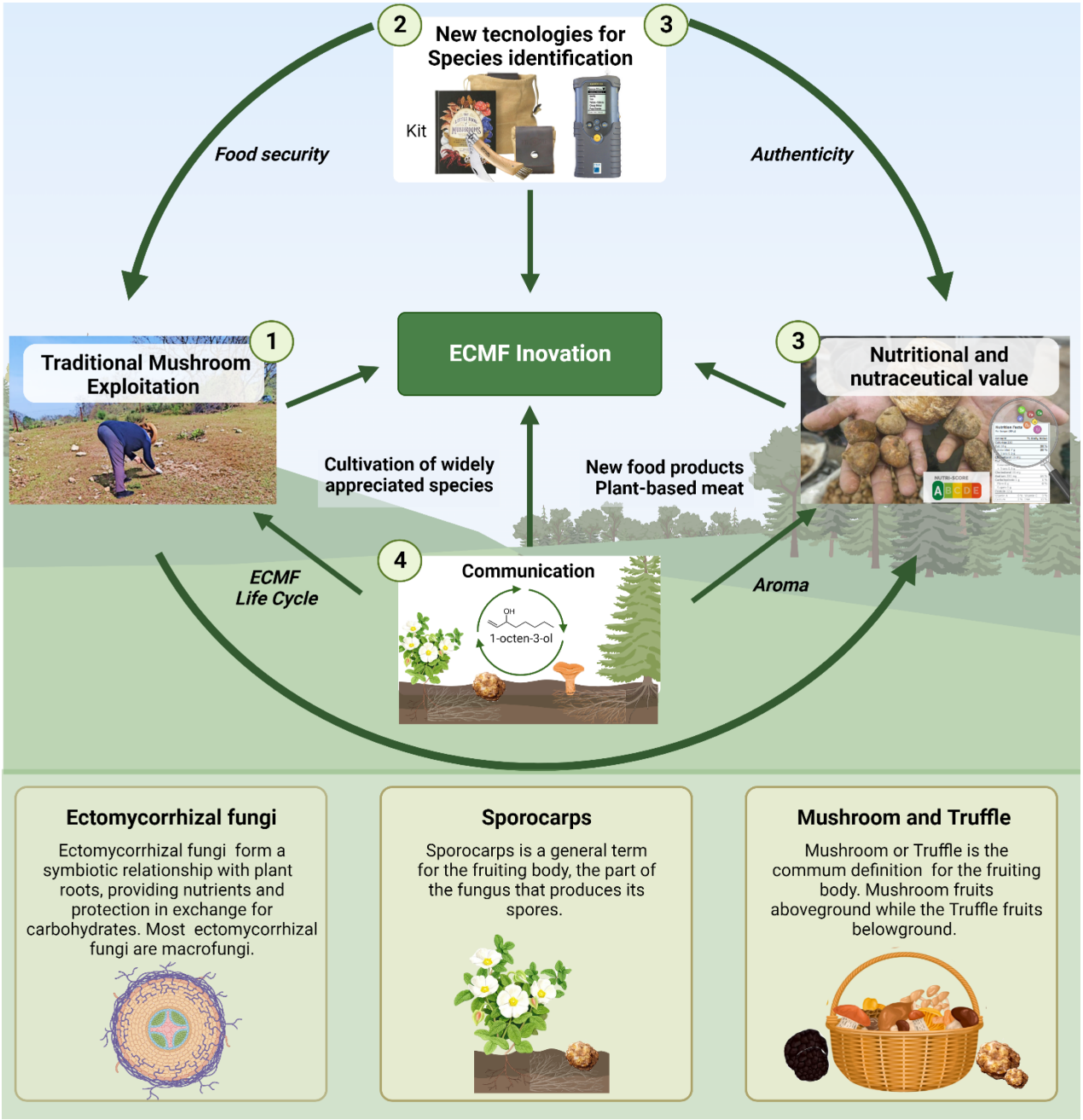

Figure 0.4 - Schematic representation of the thesis outline and glossary of mycological terminology. Created with BioRender.com.

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CHAPTER 2

First Steps in Developing a Fast, Cheap and Reliable Method to Distinguish Wild Mushroom and Truffle Species

This chapter is based in the following article:

Ferreira, I.; Dias, T.; Melo, J.; Mouazen, A.M.; Cruz, C. (2023) First Steps in Developing a Fast, Cheap and Reliable Method to Distinguish Wild Mushroom and Truffle Species. *Resources.* Submitted

First Steps in Developing a Fast, Cheap and Reliable Method to Distinguish Wild Mushroom and Truffle Species

Abstract

Wild mushrooms and truffles (MT) are important resources, which can contribute to the socioeconomic sustainability of forestry ecosystems. However, not all wild MT are edible. Fast, cheap, and reliable methods that distinguish wild MT species (including the deadly ones) can contribute to value these important forest resources. Here, we tested if wild MT species, and their edibility, could be distinguished based on their aroma profiles (i.e. smellprints). For that, we combined the use of the e-nose with classification models (linear discriminant analysis – LDA – and partial least squares discriminant analysis – PLS-DA) to distinguish between 14 wild MT species (including edible and non-edible species) collected in Portugal. The 14 wild MT species could be accurately distinguished using LDA (93 % accuracy), while the edible and non-edible species could be accurately distinguished using both LDA and PLS-DA (97 % and 99 % accuracy, respectively). Our data shows the potential of the combined use of the electronic nose with discriminant analysis to distinguish wild MT species and their edibility based on their aromatic profile. Although a larger dataset will be necessary to develop a quick and reliable identification method. The proposed methodology shows potential to be as accurate as the identification performed by mycologists and molecular biology, yet requiring less technical training, using cheaper and faster analyses.

Keywords

Electronic nose; Forest resources; Identification method; Volatile profile; Wild mushrooms and truffles

Introduction

Approximately 148,000 species of fungi have been identified so far. However, it is believed that more than 90 % of the fungal species remain unknown and the total number of fungal species worldwide could reach 2.2 to 3.8 million species (Antonelli et al. 2020; Cheek et al. 2020). Fungi producing mushrooms and truffles (MT) include the most studied species (Antonelli et al. 2020). MT both consist of the fruiting body of macrofungi, but mushrooms fruit aboveground while truffles fruit belowground (El Enshasy et al. 2013). Indeed, for millennia, humans have included wild MT into their diets, medicinal practices, and ceremonial traditions (Garibay-Orijel et al. 2007). In the contemporary era, these natural wonders have evolved into non-timber forest commodities, embodying a vast genetic reservoir that holds profound ecological, sociocultural, economic, medicinal, and biotechnological importance worldwide (Garibay-Orijel et al. 2009; Frutos et al. 2009; Schulp et al. 2014; Delic and Ibrahimspahic 2017). In the last decades, we have seen a growing interest in MT for their rich composition and bioactive compounds which make them a great resource of exciting ingredients for food and nutraceuticals (Román and Boa 2004; Kalac et al. 2009; Kalač 2013b; López-Hortas et al. 2022; Gopal et al. 2022).

However, not all MT species are safe for human consumption (e.g. *Amanita phalloides*, *Agaricus xanthodermus, Galerina marginata*). From the 14,000 MT species identified so far (Gopal et al. 2022), Li and colleagues (Li et al. 2021) reviewed 2,786 MT species from 99 countries. From that list, most MT species were considered edible, i.e. 79 % of the species were identified as edible, and 72 % were considered safe for human consumption. An additional 7 % of those species required specific pre-treatment measures before they could be considered safe or had been associated with allergic reactions in some instances. Furthermore, 17 % of those species were categorized as of uncertain edibility due to a lack of conclusive evidence of human safe consumption, while 3 % of those species remained unconfirmed due to ongoing debates and differing opinions regarding their edibility and potential toxicity.

Although wild MT constitute a highly esteemed delicacy in specific parts of the world (Ferreira et al. 2023a, c), they are faced with great scepticism in other regions, and therefore they are not valued as an important forest resource. The valorisation of wild MT has the potential to promote the socioeconomic sustainability of rural communities and forests (Bonet et al. 2014a; Brown et al. 2018). Supporting the sustainable harvesting and cultivation of these resources, can generate income for local people, conserve biodiversity, mitigate climate change

(Field et al. 2020; Ferreira et al. 2023a), and contribute to forest ecosystem processes and services (Niego et al. 2023). Wild MT are versatile resources with potential application in medicine, food, cosmetics, and recreation, from developing novel drugs and therapies, to the development of culinary delights and skincare products (Barros et al. 2008; Ghorai et al. 2009; Erjavec et al. 2012; Lu et al. 2020; López-Hortas et al. 2022). Their diverse properties offer opportunities for innovation across various industries (Niego et al. 2023), while highlighting the importance of responsible and ethical use.

Counteracting this scepticism about wild MT in some regions, society has recently and progressively developed a strong interest for wild MT hunting and consumption (Comandini and Rinaldi 2020). The down side of this growing interest for wild MT are the cases of poisoning which often occur as a common outcome of enthusiastic wild MT gathering and consumption by mushroom enthusiasts that are not highly skilled (i.e. people with insufficient training on wild MT species identification) (Peintner et al. 2013). As an example, a retrospective study showed that around 94 % of the reported mushroom poisoning cases resulted from the consumption of wild mushrooms incorrectly identified (Eren et al. 2010). To avoid cases of poisoning, the safe trade of wild MT must rely on the implementation of guidelines and the enactment of legislation that ensures food safety (Peintner et al. 2013). To bridge knowledge gaps and contribute to wild MT species identification, new identification methodologies and technologies (please see below some examples) have been developed, further enhancing our understanding and utilization of these valuable natural resources (Wei et al. 2022; Lee et al. 2022; Hodgson et al. 2023).

The traditional methods for identifying wild MT species include mainly morphological identification, instrumental analysis (e.g. gas chromatography–mass spectrometry – GC–MS) and molecular biology approaches (Wei et al. 2022). However, in the last decade the development of technologies [e.g. image recognition (Zahan et al. 2021; Zhao et al. 2021) integrating machine learning (Wibowo et al. 2018; Jahan Pinky et al. 2019; Chumuang et al. 2020; Kousalya et al. 2022; Rahman et al. 2022)] have been largely applied for wild MT species identification. With the globalization of the internet of things (IoT), methods to identify wild MT species from field-collection images using a smartphone application have been developed (Lee et al. 2022; Hodgson et al. 2023). These smartphone identification applications hold promise to aid clinical toxicologists and the general public in accurately identifying wild MT species, but their low accuracy turns them insufficiently reliable to distinguish edible wild MT from

potentially toxic ones (Hodgson et al. 2023). Similar phenomena are occurring with artificial intelligence generated books for foraging and MT identification, which contain inaccurate information such as “taste and smell” as an identifying feature (Milmo 2023). This could lead amateurs to the incorrect assumption that tasting is an identification method, which can ultimately result in wild MT poisoning. Therefore, it is important to always follow reliable sources, and use highly accurate tools that build greater trust on wild MT species identification.

One of the traits that plays a significant role in identifying wild MT is their odour. In accordance, most field guides for foraging and identifying wild MT include details about the odour alongside the macroscopic description of the flesh. For example, the odour of *Agaricus xanthodermus* is reported as phenolic, that of fresh *Clitocybe odora* has strong anise component, and that of young *Amanita phalloides* is described as very faint (MushroomExpert, 2023). Although, the odour may offer valuable insights into identifying specific wild MT species, these odour descriptions are typically quite vague and generalized. Therefore, such odour identification cannot be regarded as definitive on their own when it comes to wild MT species (Portalo-Calero et al. 2019a, 2020). Nevertheless, this specific trait (i.e. the odour) is beginning to be explored in the identification of wild MT species using technologies such as the electronic nose (or simply e-nose) (Wei et al. 2022).

The e-nose is a sensitive device that can obtain information about odours, the smellprint. Slight changes in volatile compounds' odour, composition, or concentration can result in a different sensor response (Sensigents, 2023). The e-nose has been used to assess the volatile profiles of mushrooms for several applications in food industry (Zhou et al. 2015; Gómez et al. 2022), including quality control during postharvest processing (Pei et al. 2016; Ma et al. 2018; Chen et al. 2021), monitoring of the maturation process (Chilo et al. 2016) and assessment of shelf life and packaging (Song et al. 2019; Gholami et al. 2023). Recently, several studies have investigated the e-nose’s capacity to accurately distinguish different wild MT species by analysing their volatile profiles (Zhou et al. 2015; Zhang et al. 2016; Portalo-Calero et al. 2019b, a, 2020; Gómez et al. 2022; Ferreira et al. 2023c). While the findings obtained using the e-nose are promising, a wild MT species identification based on the e-nose requires further research to expand the datasets, to standardize the methodology, and to explore the integration of machine learning algorithms to maximize its potential in wild MT species identification. Although a few studies have recently focused on this specific question, they covered a small number of wild MT species and genera. Further research is needed to explore a wider range of wild MT species

and genera, including those species with similar morphological features which can easily lead to incomplete or incorrect wild MT species identification.

Therefore, the present study aims to further explore wild MT's odours as an identification trait by using a fast, cheap, and reliable methodology to analyse their aroma profiles. For that, we: i) captured the aroma composition of fourteen wild MT species using the Cyranose 320 e-nose; and ii) applied multivariate analysis techniques (Principal Component Analysis – PCA –, Linear Discriminant Analysis – LDA – and Partial Least Squares-Discriminant Analysis - PLS-DA) to the wild MT aroma profiles to develop classification models able to distinguish the wild MT species, and between edible and non-edible species.

Materials and Methods

Wild mushrooms and truffles (MT)

Fourteen wild-growing MT species were collected during field trips in the South and Centre of Portugal between the autumn of 2022 and the spring of 2023 (Table 1). At least two individuals of each species were collected from each site, adding up to a total of 28 samples. After relevant notes of their morphological and ecological features were taken, each individual was placed in a separate paper bag and brought to the laboratory in a cooler bag. Specimens were freed from substrate debris at the site and further cleaned in the laboratory. The wild MT samples were kept at 4 °C until analysis in the first 48 hours postharvest.

Each wild MT species was identified in the present study by the research team based on morphological features, and using standard reference field identification books (Llamas Frade and Alfonso 2005; Baptista-Ferreira 2013; Henriques 2016; Mehmood et al. 2018). When the morphological features were not enough to have a precise identification of the species, the specimens were sent for molecular analysis to confirm their identification. The identification of six wild MT species was confirmed by molecular analysis. Genomic DNA was extracted from 100 mg of each fresh mushroom/truffle. DNA degradation was performed in microtubes with 200 µL of degradation corrosion (Proteinase K at 0.5 mg/mL in 100 mM Tris-HCl pH 9.0) preheated to 60 °C. The mixture was kept in a dry bath at 60 °C overnight (about 16 hours), and initially (in the first 30 minutes of incubation) they were strongly vortexed for periods of 30 seconds. DNA extraction was followed by amplification using the forward primer (ITS5 F) 5' – GGAAGTAAAAGTCGTAACAAGG – 3' and the reverse primer (ITS4 R) 5' – TCCTCCGCTTATTGATATGC – 3'. Polymerase chain reactions (PCR) were carried out in a final volume of 20 µl. The reaction mixture consisted of 10 µl of MyTaq Red Mix 2x (Bioline, Paris, France), 1 µl of each primer F and R (at 10 µM/each), 1 µl of DNA sample, and 7 µl of ultrapure water. The mixture was placed in Tpersonal cycler (Whatman Biometra, Göttingen, Germany), programmed as follows: initial denaturation at 95 °C for 5 min, then 35 cycles each one consisting in three steps: denaturation at 95 °C for 10 sec, annealing at 43 °C for 10 sec, elongation at 72 °C for 60 sec, and then a final elongation at 72 °C for 7 min. Aliquots of the PCR reactions were resolved on 0.7 % agarose gels stained with ethidium bromide. PCR products were purified by Zymoclean DNA kit (Zymo Research, Irvine, California, USA), following the manufacturers' instructions. Purified PCR products were sequenced in both

directions at StabVida (Caparica, Portugal) using the primers previously cited. The obtained sequences were compared with the sequences available from the National Centre for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov) using the BLAST algorithm to identify our wild MT species.

After the identification, each wild MT species' edibility was attributed based on existing literature. Finally, a literature survey identified other wild MT species that can be morphologically confused with the species we collected.

Exploring wild MT's odours using the e-nose

A Cyranose-320 e-nose (Sensigent, Pasadena, CA, USA) was used to develop a non-destructive, fast, cheap, and reliable method to distinguish between the 14 wild MT species and their edibility. The portable Cyranose-320 e-nose can rapidly detect and identify samples based on their aroma profile. It is equipped with a nanocomposite sensor array of 32 nanosensors, an internal air sampling pump, and advanced pattern recognition algorithms. The sensor array measures the responses of the nanosensors to the chemical vapours in the air. The pattern recognition algorithms then use these responses to create a "smellprint" of the sample, which is a unique signature that can be used to identify it (Sensigents, 2023). Therefore, we specifically used this e-nose to collect volatile profile information of the specimens belonging to the fourteen wild MT species and create a smellprint for each species.

Two fresh samples of each wild MT species were analysed separately. Four grams of each sample were weighed and introduced in a 10 mL vial and were incubated for 1 hour at room temperature (i.e. 24 °C). The Cyranose-320 was mounted on a tripod, which could be adjusted for inserting the e-nose needle into the vials for headspace reading. Five readings per sample were performed, adding up to a total of ten readings per species. The e-nose was coupled to the computer and PCnose software was used to set the list of parameter settings of the Cyranose-320 (see Table S1) and data acquisition of the smellprint.

Smellprints statistical analyses

Distinguishing the 14 wild MT species

To compare each sensor's response between the 14 wild MT species, we used the Kolmogorov-Smirnov test, a nonparametric test. To evaluate the possibility to distinguish the fourteen wild MT species based on their smellprints, a PCA was carried out. This helped to identify patterns in the data, and to compare the smellprints of the different species.

Furthermore, we performed a LDA to classify the smellprints of the different wild MT for each species. The LDA found linear combinations of the features of a sample to classify them into one of several classes (i.e. the 14 wild MT species). Then, LDA detected the directions in which the classes were most separated, i.e. the discriminant functions. Finally, each sample was classified (i.e. identified as a wild MT species) by the discriminant function that gave the highest value. The LDA model performance was evaluated for its accuracy using the following equation:

$$Accuracy\ (\%) = \frac{True\ positive}{Total} x\ 100$$

All analyses were performed using the software Microsoft Excel 2019 and XLSTAT-Premium (Version 2021.4.1, Addinsoft, Inc., Brooklyn, NY, USA).

Distinguishing the 14 wild MT species according to their edibility

To evaluate the possibility of distinguishing edible and non-edible species by their smellprints, two classification models were performed, the LDA and the PLS-DA. These classification methods were used to test if the groups (edible and non-edible), to which the observations belong are distinct and to reveal the properties of these groups using e-nose sensor response as explanatory variables. The LDA was tested as previously described but here the classes were edible and non-edible. In the PLS-DA, the edibility classification was the dependent variable, and the e-nose sensors data were the independent variables. PLS-DA is a classification method that can simultaneously perform dimensionality reduction and discriminant analysis. PLS-DA is more flexible than LDA because it can handle cases where the classes are not linearly separable. The model performance for the LDA and PLS-DA was evaluated for accuracy as described above using equation 1. Multivariate analyses were performed using the software Microsoft Excel 2019 and XLSTAT-Premium (Version 2021.4.1, Addinsoft, Inc., Brooklyn, NY, USA).

Results

Distinguishing the 14 wild MT species

Based on the analysis of the morphological features by our experienced mycologist, we undoubtedly identified the following eight wild mushroom species: *Cantharellus cibarius, Craterellus lutescens, Craterellus tubaeformis, Cyclocybe cylindracea, Hydnum repandum, Lactarius deliciosus, Pisolithus tinctorius, Suillus collinitus* (Table 1). The six species whose identification was confirmed by molecular analysis were the wild mushrooms *Agaricus xanthodermus, Amanita phalloides, Amanita subparvipantherina, Hygrocybe helobia, Lepista nuda* and the wild truffle *Terfezia arenaria*. Most of the wild MT species we collected (nine out of 14) were edible and included the wild truffle *T. arenaria* and the wild mushrooms *C. cibarius, C. lutescens, C. tubaeformis, C. cylindracea, H. repandum, L. deliciosus, L.nuda* and *S. collinitus*. The non-edible wild mushroom species included *A. xanthodermus, A. phalloides, A. subparvipantherina, H. helobia* and *P. tinctorius*. All wild MT species had been reported to be morphologically confused with species from the same genus (e.g. *A. subparvipantherina* can be morphologically confused with *A. citrina* and other *Amanita* spp.) or from other genera (e.g. *A. phalloides* can be morphologically confused with *Agaricus* spp., *Russula* spp., *Thricoloma* spp).

The Cyranose-320 e-nose was able to detect the wild MT smellprints. From the 14 wild MT species analysed, the truffle *T. arenaria* was the species that induced higher responses for most of the 32 sensors (Figure 2.1). The sensors' responses induced by *T. arenaria* were different from those induced by the other 13 wild MT species, except for two sensors (S30 and S31; Table S2.1). Furthermore, four edible wild MT species (mushrooms *C. lutescens, L. deliciosus* and *L. nuda,* and truffle *T. arenaria*) induced sensors' responses for the 32 sensors different from those induced by the non-edible and deadly mushroom *A. phalloides*. However, the sensors' responses to the other non-edible wild mushroom species could not be distinguished from those of the edible wild mushrooms.

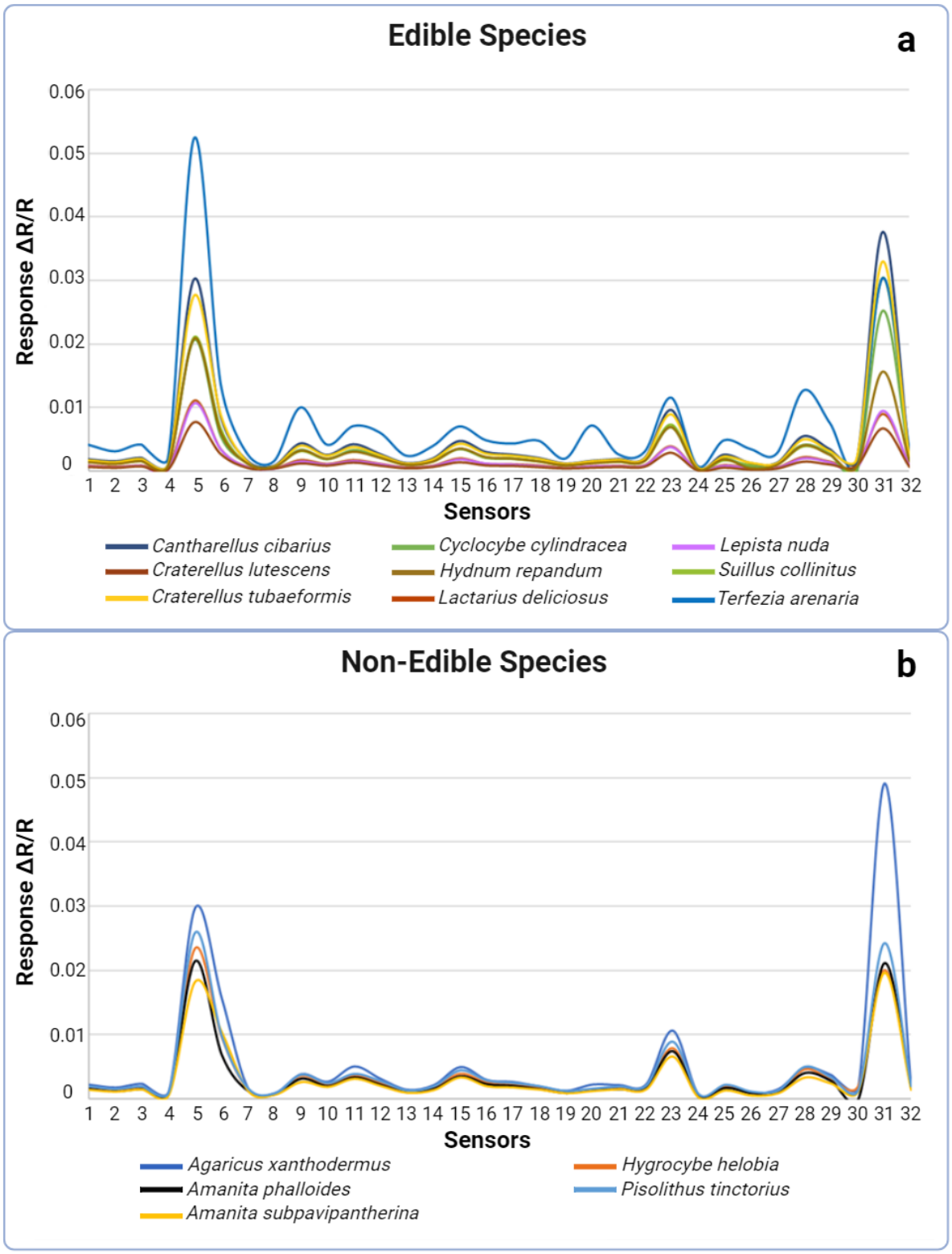

Figure 0.1 - Cyranose-320 e-nose smellprints integrating the response of the 32 sensors for each of the 14 wild **mushrooms and truffle (**MT) species. The wild MT species were grouped according to their edibility: edible (a) and non-edible (b) species. Each peak represents the response of a different sensor for each species. Each line represents the average response per species (n = 10 replicates). Created with Microsoft Excel and BioRender.com.

Table 0.1 - List of the wild mushrooms and truffle (MT) species that were collected for this study, with corresponding class, edibility, location, and species with which it can be morphologically confused.

Class and Species	Identified by	Edibility	Location	Morphologically confused with	Reference
Ascomycetes					
Terfezia arenaria (Moris) Trappe	Molecular biology (100 %) AN: PP782124*	Edible	Alentejo, Crato village, in montado	*Terfezia* spp.*; Choiromyces gangliformis, Choiromyces meandriformis*	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Basidiomycetes					
Agaricus xanthodermus Genev.	Molecular biology (100 %) AN: PP782119*	Not edible	Lisboa, Quinta das Conchas, in urban garden	*Agaricus* spp.	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Amanita phalloides (Vaill. ex Fr.) Link	Molecular biology (99.70 %) AN: PP782121*	Not edible	Sintra, Parques de Sintra, in pine forest	*Agaricus* spp., *Russula* spp., *Tricholoma* spp.	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Amanita subparvipantherina Zhu L. Yang, Q. Cai & Y.Y. Cui	Molecular biology (100 %) AN: PP782123*	Not edible	Leiria, Carreira village, in mixed wood	*Amanita citrina Amanita* spp.	(Bhatt 2017; Mehmood et al. 2018)
Cantharellus cibarius Fr.	Mycologist	Edible	Azambuja, in montado	*Hygrophoropsis aurantiaca, Omphalatus olearius; Omphalatus illudens*	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Craterellus lutescens (Fr.) Fr.	Mycologist	Edible	Leiria, Carreira village, in mixed wood	*Craterellus tubaeformis*	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Craterellus tubaeformis (Fr.) Quél.	Mycologist	Edible	Leiria, Carreira village, in mixed wood	*Craterellus lutescens*	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Cyclocybe cylindracea (DC.) Vizzini & Angelini 2014	Mycologist	Edible	Lisboa, Quinta das Conchas, in urban garden	*Amanita* spp.	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Hydnum repandum L.	Mycologist	Edible	Leiria, Carreira village, in mixed wood	*Hydnum rufescens, Cantharellus cibarius*	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Hygrocybe helobia (Arnolds) Bon	Molecular biology (99.33 %) AN: PP782122*	Not edible	Leiria, Carreira village, in mixed wood	*Hygrocybe* spp.	(Llamas Frade and Alfonso 2005)
Lactarius deliciosus (L.) Gray	Mycologist	Edible	Leiria, Bajouca village, in mixed wood	*Lactarius* spp.	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Lepista nuda (Bull.) Cooke	Molecular biology (100 %) AN: PP782120*	Edible	Lisboa, Quinta das Conchas, in urban garden	*Lepista sordida, Cortinarius* spp.	(Llamas Frade and Alfonso 2005; Silva et al. 2013; Henriques 2016)
Pisolithus tinctorius (Mont.) E. Fisch	Mycologist	Not edible	Leiria, Carreira village, in eucalyptus forest	*Pisolithus* spp.*; Scleroderma* spp.	(Llamas Frade and Alfonso 2005; Henriques 2016)
Suillus collinitus (Fr.) Kuntze	Mycologist	Edible	Leiria, Bajouca village, in mixed wood	*Suillus* spp.*; Boletus* spp.	(Llamas Frade and Alfonso 2005; Henriques 2016)

*GeneBank accession number (AN) of ITS sequences

The first two components of PCA (based on the response of the 32 sensors for each of the 14 wild MT species) explained 96 % of the total variance (PC1 90.3 % and PC2 5.3 %) (Figure 2.2). Despite explaining most of the variance, the PCA model showed overlapping of the smellprints of most wild MT species, with only two species showing a clearly different smellprint from the remaining: *A. xanthodermus* and *T. arenaria*. Finally, the clusters that were formed based on the wild MT species smellprints included edible and non-edible species. Therefore, the PCA was not able to clearly distinguish the 14 wild MT species or their edibility.

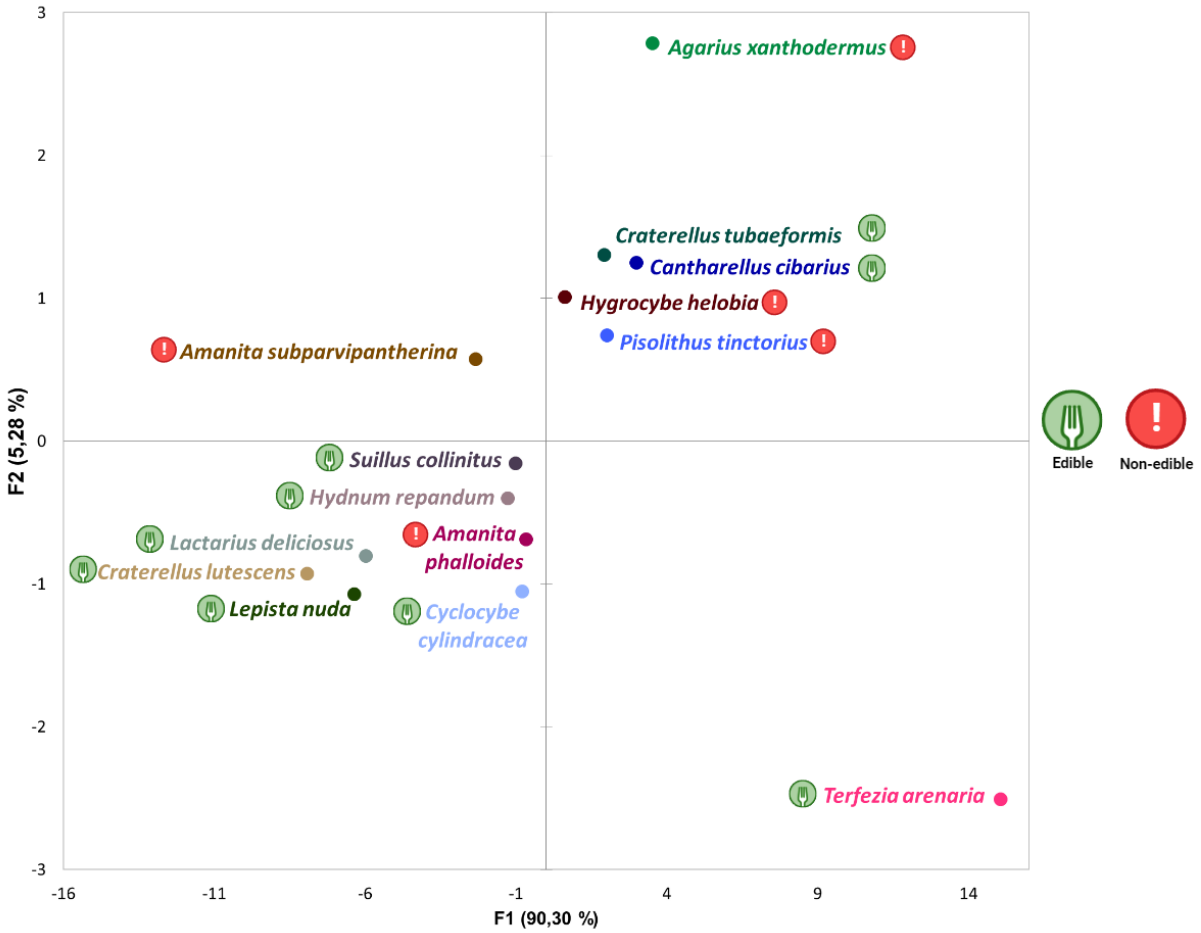

Figure 0.2 - Principal component analysis (PCA) of the smellprints integrating the response of the 32 sensors for each of the 14 wild mushrooms and truffle (MT) species. The wild MT species are further classified as edible or non-edible. Symbols are the mean (n = 10) per species.). Created with XLSTAT and BioRender.com

The LDA classification model presented an overall accuracy of 93 % for distinguishing the 14 wild MT species. This overall accuracy level integrates the cases when species were always identified correctly, and those that were identified incorrectly. The LDA classification model correctly identified nine out of the 14 species analysed, with 100 % accuracy (Figure 2.3). The species correctly identified by the LDA classification model (i.e. the species where

all samples were correctly, with no incorrect identifications) included: i) the edible *C. cylindracea*, *H. repandum*, *S. collinittus*, *T. arenaria*; and ii) the non-edible *A. xanthodermus*, *A. phalloides*, *A. subparvipantherina*, *H. helobia* and *P. tinctorius*. It is important to highlight that all non-edible species that we tested were correctly classified (i.e. 100 % accuracy).

On the other hand, the LDA classification model showed misclassifications (i.e. incorrect identifications) for five wild edible mushroom species, with an accuracy of 70 % to 90 % (Figure 2.3). In a few samples, the LDA classification model misclassified C. *tubaeformis* as *C. cibarius* (and vice versa), *C. lutescens* as *L. nuda*, *L. deliciosus* as *C. lutescens and L. deliciosus* as *L. nuda* (and vice versa). This can be related to the proximity of the smellprints of these species, which was also observed by the overlapping in the PCA (Figure 2.2). However, although *A. phalloides*, *C. cylindracea*, *H. repandum* and *S. collinitus* were also close in the PCA, the LDA analysis correctly identified these species.

Distinguishing the 14 wild MT species edibility

The confusion matrix for the LDA and the PLS-DA models for classifying (i.e., identifying) edible and non-edible species are shown in Figure 2.4. For both classification models (LDA and PLS-DA) we observed a very high percentage of correct identifications. Using the LDA classification model we only observed five incorrect identifications of samples, thus reaching a 97 % accuracy. Using the PLS-DA classification model we only observed one incorrect identification of samples, thus reaching a 99 % accuracy (Figure 2.4). Both classification models (LDA and PLS-DA) were highly accurate in distinguishing the smellprints of edible from those of the non-edible species.

Discussion

By combining the use of the e-nose with discriminant analysis we were able to distinguish 14 wild MT species, and their edibility (i.e. distinguish the edible from the non-edible species), using an accurate, fast, and cheap method. Our study used the highest number of wild MT species in similar studies so far, including wild MT species with similar morphological features which can easily lead to incomplete or incorrect identifications.

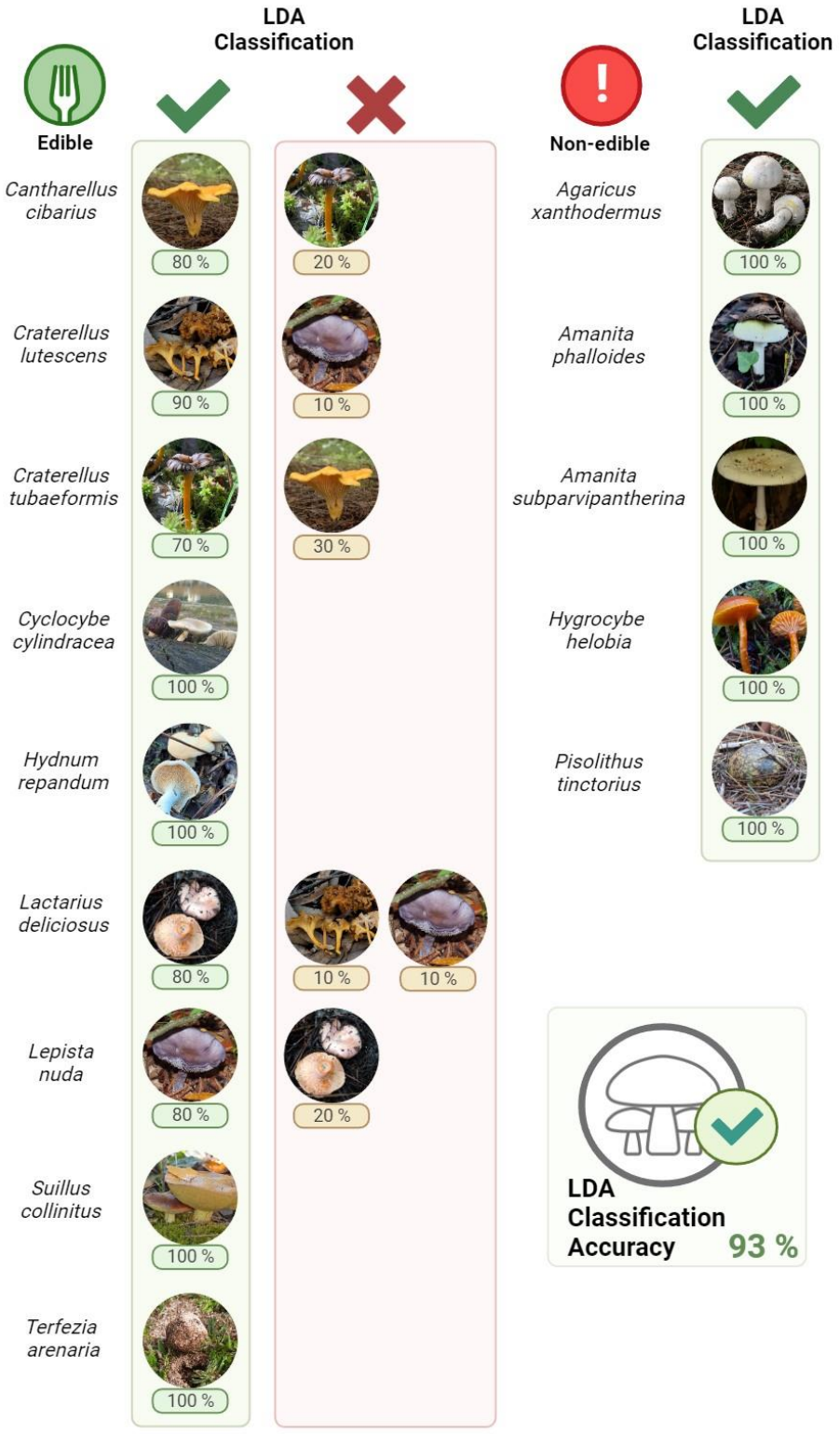

Figure 0.3 - Overall species identification accuracy of the **linear discriminant analysis** (LDA) classification model based on the e-nose smellprints of the 14 wild mushrooms and truffle (MT) species.). Created with BioRender.com.

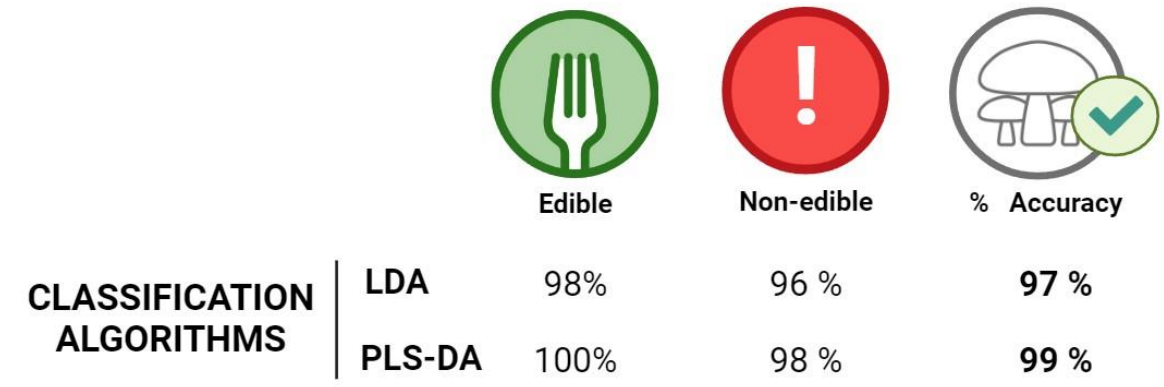

Figure 0.4 - Edibility identification accuracy of the **linear discriminant analysis** (LDA) and **partial least square discriminant analysis** (PLS-DA) classification models based on the e-nose smellprints of the 14 wild **mushrooms and truffle (**MT) species. Created with BioRender.com.

Fast, cheap and reliable method to distinguish wild MT species and their edibility

The smellprint of the truffle *T. arenaria* stood out from those of the other wild mushrooms (Figs 1 and 2). Being a belowground fruiting fungus, *T. arenaria*'s unique aroma, plays an important ecological role by mediating this truffle's communication with below and aboveground communities (Ferreira et al. 2023b), including attracting animals that help disperse the truffle spores (Splivallo et al. 2011). Despite the variability in the aromatic profile, this truffle species can have volatile organic compounds (VOC) that act as a species-specific fingerprint (Splivallo et al. 2011; Ferreira et al. 2023c), which can help explain its distinctive smellprint when compared to that of the wild mushrooms.

Similar phenomena can help explain why the smellprint of the non-edible and lethal wild mushroom *A. phalloides*, was so different from that of the nine edible wild MT species of this study (Figure 2.3). While 15 *Amanita* spp. have been described as lethal worldwide (Cai et al. 2016), two *Amanita* spp. are considered delicacies and with economic interest in the Mediterranean region (*A. caesarea* and *A. ponderosa*) (Batista et al. 2017). The use of the e-nose for successfully distinguishing between *Amanita* mushrooms, was demonstrated by Portalo-Calero and colleagues (Portalo-Calero et al. 2019b, a, 2020). These studies obtained an accuracy of 97.7 % to 99.9 % using multivariate analysis for smellprint classification, and included two lethal species (*A. phalloides* and *A. verna*), and the two edible delicacies *A. caesarea* and *A. ponderosa* (Portalo-Calero et al. 2019b, 2020). The use of electronic devices, such as the e-nose, that accurately distinguish between potentially dangerous wild MT and the safe ones can add an extra layer of safety to wild MT hunting. Improving wild MT safe consumption can

contribute to the socioeconomic sustainability of forest ecosystems since several of the wild edible MT studied here are highly appreciated and represent an important bio-resource of food and income for rural populations (De Román and Boa 2006). For example, *C. cibarius, H. repandum* and *L. deliciosus* are among the wild edible mushroom species authorized for trade and commercialization in at least twelve European Union (EU) countries (Ferreira et al. 2023a), and their international trade has increased in recent years (de Frutos 2020).

In accordance with previous studies (Keshri et al. 2003; Zhou et al. 2015; Gómez et al. 2022), the LDA classification model was able to accurately identify the 14 wild MT species by their smellprint (Fig. 3), and both the LDA and PLS-DA classification models could accurately distinguish between the edible and non-edible species (Fig. 4). These classification models have been largely used for statistical treatment of the volatile compounds of food matrices, and have been showing high accuracy (Gębicki and Szulczyński 2018; Sánchez et al. 2021; Gui et al. 2023). For example, the combined use of the e-nose and PLS-DA contributed for the successful identification of filamentous fungal (Gębicki and Szulczyński 2018) and plant species (Gui et al. 2023), monitoring product quality during and after production processing (Qin et al. 2020; Sánchez et al. 2021, 2022; Zhou et al. 2023), product quality (Chen et al. 2022) and establishing geographic origin (Wu et al. 2022). Moreover, comprehensive datasets encompassing a wide range of wild MT species should be established to enhance the accuracy and robustness of the e-nose's identification capabilities. This would require the collection and analysis of smellprints of a diverse range of wild MT, including rare and lesser-studied species. Additionally, exploring the potential of machine learning algorithms and artificial intelligence combined with e-noses could further enhance the capabilities of this technology. By training algorithms with large datasets of smellprints and corresponding wild MT species, it may be possible to develop automated, real-time identification systems that can identify wild MT species accurately, quickly and at low costs.

Although our approach allowed us to accurately distinguish wild MT species and their edibility based on their smellprints, the number of samples we used was limited. The reduced number of wild MT species and specimens we analysed reflected a reduction in the fruiting of the wild MT due to climate change. Specifically, since these wild MT species require high soil water availability for producing their fruit bodies in arid and semi-arid areas (e.g. most of mainland Portugal) (de Aragon 2007). The severe droughts that affected mainland Portugal during

the sampling period and previous years (IPMA, 2023), negatively impacted the wild MT abundance. Furthermore, the wildfires also resulted in a loss of productive forest areas, and consequently lower wild MT abundance. Despite our efforts to include more wild MT species and specimens, further research is needed to: i) increase the number of species (including rare and lesser-studies ones) and specimens; ii) account for the different phenological phases of the wild MT (e.g. maturation); iii) assess the importance of geographic origin; and iv) test product quality.

Perspectives for wild MT identification

One direct application of our approach (combined use of the e-nose and discriminant analysis) is to support accurate wild MT species identification, which can significantly enhance the identification accuracy of mycologists and wild MT enthusiasts. Traditional methods of wild MT identification typically involve time-consuming processes such as microscopic analysis and chemical reagent tests (Figure 2.5). Although professional and experienced collectors can avoid the harvest of hazardous and non-edible wild MT, the increasing number of amateurs collecting wild MT increases the risk of poisoning (Portalo-Calero et al. 2020). During this study, we faced some difficulties identifying some species based on their morphological characteristics alone, namely *A. xanthodermus* (could also be *A. silvestris*), *A. subparvipantherina* (could also be *A. citrina*) and *H. helobia* (we could only identify the genus, but not the species). That was why we performed molecular identification of these species and another three as control. Therefore, even experienced mycologists may need to use other methodologies to identify (or confirm) wild MT species.

Wild MT identification can include morphological features, instrumental analysis and molecular biology methods (Wei et al. 2022) (Figure 2.5). Morphological identification is a long-established approach, based in fungal taxonomy, requires long training, and greatly benefits from experience. Wild MT species identification by an expert (mycologist) is critical to guarantee safe consumption. For example, in Switzerland, a free service is offered to the population to confirm edibility of self-harvested wild mushrooms to promote the safe and sustainable harvesting of these forest resources (Vapko, 2023). However, sometimes, even expert identification needs support from other methodologies, such as molecular biology. Molecular biology is more expensive than mycologist's identification and is mainly applied to fresh fruiting bodies, primary processed products and deeply processed products (Wei et al. 2022). Both identification techniques (mycologist and molecular biology) have high accuracy but require time-

consuming expert training, some equipment, time and costs for the analysis (especially in the case of molecular biology – Figure 2.5). Using an e-nose has the potential to become a fast, cheap and reliable method for wild MT species identification because: i) it is a technique easy to learn and training can be done in a few weeks; ii) the e-nose previously trained allows a fast identification within a few hours; iii) once the e-nose is purchased, it only requires maintenance costs and therefore analysis will be cheap; and iv) it accurately distinguishes wild MT species and their edibility (Figures 2.3 and 2.4). Therefore, this can be a valuable alternative or complementary approach, especially in cases where the morphological features of wild MT do not provide definitive results.

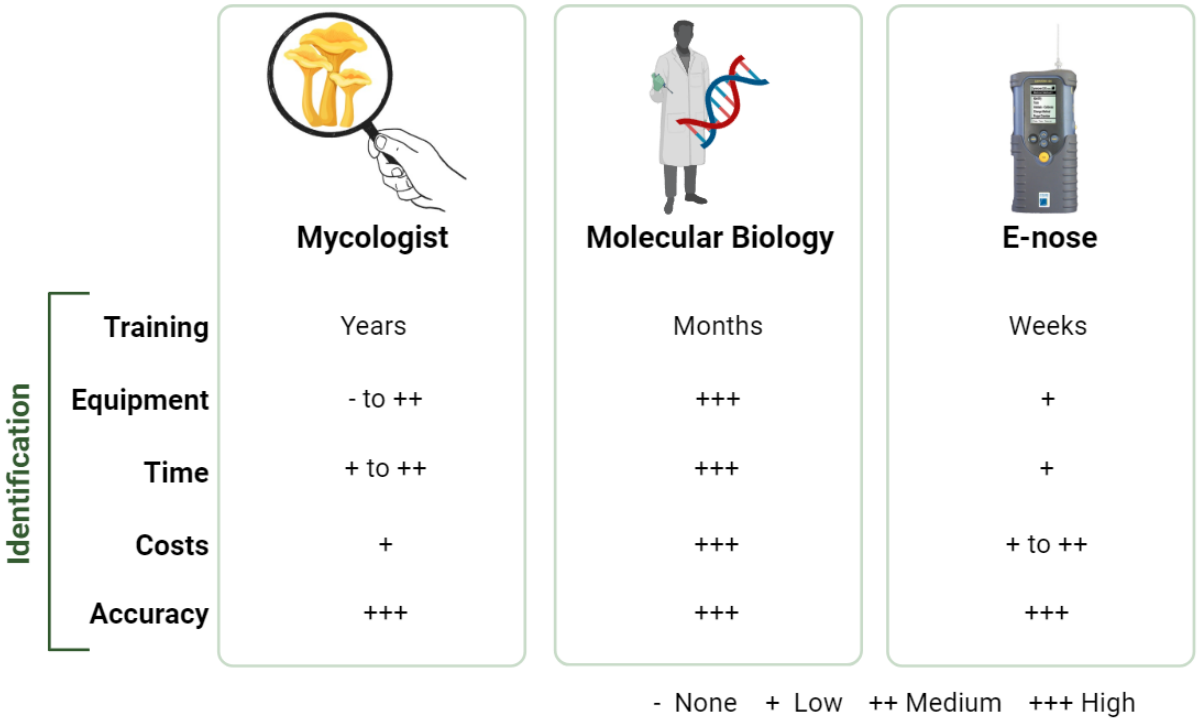

Figure 0.5 - Comparison of methods to identify wild mushrooms and truffle (MT) species based on their training duration, equipment, time, costs, and accuracy involved in the analysis. Created with BioRender.com.

The potential impact of using an e-nose for wild MT identification extends beyond its immediate applications. A better understanding of the volatile profile – smellprint, of different wild MT species can contribute to the knowledge of their biology and ecology. Using tools such as the electronic nose can lead to advancements in mycology, food security, and environmental monitoring while improving the socioeconomic sustainability of forest ecosystems. To validate the use of e-noses to identify wild MT and their edibility, it is essential to develop new meth-

odologies and test new models. E-noses have the potential to add value to this forest bio-resource highlighting the aromatic profile of wild MT that can be of interest to the food industry, and by certifying products and gaining consumer's trust.

Conclusions

The e-nose alone distinguished the smellprints of the edible truffle *T. arenaria* and the non-edible mushroom *A. phalloides* from those of the other wild mushroom species. Furthermore, the combined use of the e-nose and discriminant analysis accurately distinguished between 14 wild MT species and their edibility (i.e. between edible and non-edible species). These results suggest that the e-nose could be a valuable tool for wild MT species identification, for example, in cases when the morphological features of wild MT do not provide definitive results.

Applying the e-nose for wild MT identification can be a fast, cheap, and accurate method to support wild MT species identification, even in the field. This tool could help prevent wild MT poisoning, a serious public health problem. Despite its great potential, using the e-nose for wild MT species identification is still in its early stages.

This study was limited by the small number of wild MT species and specimens analysed. Further research is needed to develop and validate wild MT species identification methods based on the e-nose. Future studies should include a wider variety of wild MT species to confirm the findings of our study. Such future studies should also account for the different phenological phases of the wild MT (e.g. maturation), assess the importance of geographic origin, and test product quality. The e-nose could potentially be used to identify wild MT that are ripe for harvest or to detect spoilage in wild MT, helping to improve the efficiency of wild MT harvesting in the field and later processing. The e-nose showcased potential for mushroom identification, offered high accuracy rates in classifying edible and non-edible mushrooms, and hinted at broader applications in food industry quality control and harvesting efficiency, emphasizing the need for further research to validate its accuracy. This application will add value to this forest's bio-resources and strengthen consumer confidence in this type of commodity.

Supplementary Materials

The following supporting information can be consulted in Appendix 2:

Table S2.1 - Parameters setting of the Cyranose-320;

Table S2.2 - Comparison by Kolmogorov -Smirnov of the 32 sensors' response between the 14 wild species of edible and non-edible mushrooms and truffle.

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CHAPTER 3

Using Science and Technology to Unveil the Hidden Delicacy *Terfezia arenaria*, a Desert Truffle

This chapter is based in the following article:

Ferreira, I.; Dias, T.; Mouazen, A.M.; Cruz, C. (2023) Using Science and Technology to Unveil The Hidden Delicacy *Terfezia arenaria*, a Desert Truffle. *Foods. 12*, 3527, doi:10.3390/foods12193527

Using Science and Technology to Unveil the Hidden Delicacy *Terfezia arenaria*, a Desert Truffle

Abstract

Terfezia arenaria is a desert truffle native to the Mediterranean Basin region, highly appreciated for its nutritional and aromatic properties. Despite the increasing interest in this desert truffle, *T. arenaria* is not listed as an edible truffle authorized for trade in the European Union. Therefore, our objective was to showcase *T. arenaria*'s nutritional and chemical composition and volatile profile. The nutritional analysis showed that *T. arenaria* is a good source of carbohydrates (67 %), proteins (14 %), and dietary fibre (10 %), resulting in a Nutri-Score A. The truffle's volatile profile was dominated by eight-carbon volatile compounds, with 1-octen-3-ol being the most abundant (64 %), and 29 compounds were reported for the first time for *T. arenaria*. *T. arenaria*'s nutritional and chemical compositions were like those of four commercial mushroom and truffle species, while the aromatic profile was not. An electronic nose corroborated that *T. arenaria*'s aromatic profile differs from that of the other four tested mushroom and truffle species. Our data showed that *T. arenaria* is a valuable food resource with a unique aroma and an analogous composition to meat, which makes it an ideal source for plant-based meat products. Our findings could help promote a sustainable future exploitation of *T. arenaria* and ensure the quality and authenticity of this delicacy.

Keywords

Desert truffles; Electronic nose; Mushroom and truffles; Nutritional composition; Plant-based meat; Volatile organic compounds

Introduction

Food production currently faces many challenges (Köberle 2022). One of these challenges is climate change, which causes severe health, economic and environmental problems (Ferreira et al. 2023a). Current political actions, such as the United Nation's Sustainable Development Goals (SDG) (e.g. SDG 12 Sustainable consumption and production, SDG13 Climate action), aim to mitigate climate change by shifting production and consumption patterns, and the production of mycorrhizal sporocarps (i.e. the fruiting body of macrofungi usually known as mushroom or truffle) is no exception (Boa 2004; Ferreira et al. 2023a). With a key role in ecosystems (Ferreira et al. 2023b), ectomycorrhizal fungi provide services and goods essential to maintain soil quality, ecosystem functions and food (some species) (Ferreira et al. 2023a).

The global trade of mushrooms and truffles has grown significantly in the last two decades (de Frutos 2020; Ferreira et al. 2023b); in 2000, the global production of mushrooms and truffles was 8.78 million tons, and by 2021, this number had grown to 44.20 million tons. This represents a growth of over four times. The average producer price of mushrooms and truffles also increased during this period, by 1.5 times. However, a wider investment in mycorrhizal mushrooms and truffles as a food source, with the associated health, environmental and economic benefits (Pérez-Moreno et al. 2021b, a), is still hampered by insufficient science to showcase its benefits as a food source, and technology to boost its sustainable production and ensure its reliable identification. In agreement, although the production of mycorrhizal sporocarps has long had a high economic, cultural and environmental impact in the Mediterranean Basin region (Ferreira et al. 2023a), the list of the edible sporocarps authorized for trade in 27 European countries only includes 12 species.

Furthermore, insufficient data make it challenging to provide accurate information on mycorrhizal mushrooms and truffles production and market prices. Taking the desert truffles (i.e. a family of truffles endemic to arid and semiarid areas of the Mediterranean Basin Region, North Africa and the Middle East, which includes several genera, namely *Terfezia*, *Tirmania* and *Mattirolomyces*) as an example, they are among the wild mushrooms and truffles with higher selling prices (Andrino et al. 2019; Oliach et al. 2020). Due to strong cultural traditions (Bradai et al. 2015), desert truffles have been part of the Mediterranean, North African and Middle Eastern cultures for centuries (Shavit 2014), and are widely consumed in these regions (Chevalier 2014; Shavit 2014). As most of the world's trade of desert truffles occurs in North

African countries (Morte et al. 2021), and most of this regional trade is not official, there is a lack of official data in these countries (Pérez-Moreno et al. 2021b). Therefore, the world's production and annual market of desert truffles is still unknown (Morte et al. 2021). Nevertheless, desert truffle plantation yields can reach approximately 350 kg ha^{-1}, representing an expected average income of 7,000 EUR ha^{-1} (Morte et al. 2021). Besides the economic revenue, desert truffles also constitute an important nourishment source in North African and Arabic countries, being frequently used as a meat substitute (Shavit 2008; Bradai et al. 2015) or as a powder to increment the nutritional quality of bread and biscuits (Gadallah and Ashoush 2016; Najjaa et al. 2021). Despite its great potential as a food source, only one edible desert truffle species is widely traded: *T. claveryi.* All other potentially edible desert truffle species are being ignored. Therefore, a wider investment in desert truffles (and other mycorrhizal sporocarps) as a food source, with the associated health, environmental and economic benefits, is being hampered by insufficient science to showcase its benefits as a food source, and technology to boost its sustainable production and ensure its reliable identification.

T. areanaria (Moris) Trappe is another desert truffle that forms a seasonal edible truffle with important ecological and socio-economic relevance (Khalifa et al. 2019b). Some studies have shown that this species, like other desert truffles, is rich in carbohydrates, proteins, and dietary fibre, making it a suitable addition to a balanced diet (Ahmed et al. 1981; Martínez-Tomé et al. 2014; Kıvrak 2015; Hamza et al. 2016; Al Obaydi et al. 2020a; Tejedor-Calvo et al. 2021). *T. arenaria* has also been reported to have important biological activities, such as antioxidant, antimicrobial and antitumoral (Amara et al. 2017; Harir et al. 2019b; Benaceur et al. 2020b; Tejedor-Calvo et al. 2021). Despite the increasing interest in exploring the nutritional and chemical composition of desert truffles (Martínez-Tomé et al. 2014; Kıvrak 2015; Hamza et al. 2016; Al Obaydi et al. 2020a), *T. arenaria*'s consumption and trade are still limited to small regions where this species is native. Therefore, one of our objectives was to showcase *T. arenaria*'s nutritional value by comparing it with other edible mushrooms and truffles, namely species that are well-known by the consumer and are widely available in the market.

Furthermore, ensuring food security and authentication are vital strategies for the sustainable exploitation of this native resource, preserving its long-term viability and conservation. Desert truffles are hypogeous (i.e. mushroom formation occurs belowground), which makes them difficult to detect (Moreno et al. 2013). However, as *T. arenaria* (and other desert truffle species) are mycorrhizal fungi that are associated with a host plant [most frequently from the Cistaceae

family (Ammarellou et al. 2014); *T. arenaria* associates with the annual plant species *Tuberaria guttata* (Dafri and Beddiar 2018b)], and screening the potential host plants is part of the detection method. Once their potential location is detected, the traditional method of harvesting desert truffles involves a pointed stick to carefully probe the soil (Chevalier 2014). This potentially destructive technique is time-honoured and traditionally passed from generation to generation, predominantly in Mediterranean regions (Shavit 2008; Brenko et al. 2022). However, harvesting desert truffles is a difficult process practiced by specialists (Bradai et al. 2015), which are becoming fewer and fewer among the new generations due to the abandonment of rural areas and traditional forestry (Morte et al. 2021; Ferreira et al. 2023a). Desert truffles' (and wild mushrooms in general) incorrect harvesting, including excessive harvesting, can lead to the destruction of fungal structures, making future productivity unfeasible (Egli et al. 2006). In the case of incorrect species identification, it can cause poisoning, leading to a feeling of insecurity (mycophobia) among consumers (Boa 2004; Peintner et al. 2013). Like the mushrooms and truffles commonly found in supermarkets, it becomes crucial to establish comprehensive knowledge regarding the safe consumption of desert truffles and other wild mushrooms and truffles. Altogether, developing and implementing guidelines that ensure food safety becomes especially significant for the mushroom trade (Peintner et al. 2013). Only by prioritizing the development of sustainable harvesting techniques and tools to assess quality and authenticity can we establish a fair value chain for these endogenous products. These steps are essential to meet consumer's health and nutritional needs while safeguarding the resource and promoting equitable practices in its utilization.

So far, *T. arenaria* and other desert truffle species identification has relied on traditional knowledge and morphological identification by experts. However, besides its nutritional value and potential health benefits, *T. arenaria* has a unique bouquet of volatile organic compounds (VOCs; includes alcohols, aldehydes, ketones, and sulphur compounds (Harki et al. 2010; Kamle et al. 2017; Farag et al. 2021a)), which is perceived by humans as a subtle, sweet and agreeable flavour (Chevalier 2014; Bradai et al. 2015) and contributes to promote its quality and gastronomic value (Shavit 2008; Brenko et al. 2022). Therefore, we consider that *T. arenaria*'s VOCs bouquet could be explored to develop a robust and efficient analysis method to certify the quality and authenticity of this delicacy.

Studies on the VOCs present in desert truffle species are still scarce (Farag et al. 2021a), and only one study included *T. arenaria* (Harki et al. 2010). Currently, the most common identification and quantification methods for VOCs analysis is gas chromatography–mass spectrometry (GC–MS) (Zhu et al. 2022b), and it has been widely applied to truffles and desert truffles (Mustafa et al. 2020; Farag et al. 2021a). GC–MS is a powerful analytical technique with high sensitivity, easy metabolite identification, and has the possibility to couple with separation techniques (Lubes and Goodarzi 2018). However, it can be time consuming to prepare samples as it is a destructive analysis operated by highly qualified technicians and it is very expensive (Lubes and Goodarzi 2018; Zhou et al. 2021). On the other hand, the use of electronic nose (e-nose) technology has gained attention in recent years for the identification and analysis of aroma profiles in mushrooms (Zhou et al. 2015; Guo et al. 2022; Zhu et al. 2022a; Gholami et al. 2023) and other food products (Falasconi et al. 2012; Chilo et al. 2016). This methodology has been frequently combined with GC–MS analysis, as a non-destructive and rapid approach to quality control and product authentication (Zhu et al. 2022b). The e-nose was proven a successful methodology to distinguish between the volatile profile of several filamentous fungi species and/or strains, for health, environmental and food control applications (Mota et al. 2021). In the case of mushrooms, most studies reported the volatile profile in relation with quality analysis in post-harvest processes (Chilo et al. 2016; Pei et al. 2016; Ma et al. 2018; Song et al. 2019; Chen et al. 2021; Gholami et al. 2023). Similarly to what was reported for filamentous fungi, this technology can also be applied for the identification and differentiation of mushroom and truffles species (Keshri et al. 2003; Zhou et al. 2015; Portalo-Calero et al. 2019b, a; Gómez et al. 2022). The use of e-noses in the food industry is widespread, with applications in meat, dairy products, aquatic products, cereals, fruits, and vegetables. Advantages of the e-nose include their rapid response, low cost and a relatively simple operating process (Shi et al. 2017). Therefore, given that *T. arenaria* has a unique bouquet of volatile organic compounds, we tested if the e-nose was capable of distinguishing *T. arenaria* from other edible mushroom and truffle species, and therefore guarantee this desert truffle's authenticity. For that, we used the Cyranose-320 e-nose to analyse *T. arenaria*'s volatile profile, applying two pre-analysis incubation temperatures to understand if the temperature could affect VOCs emissions and compromise the e-nose's identification efficiency: (a) *T. arenaria* samples incubated for one hour at 40 °C and (b) *T. arenaria* samples incubated for one hour at room temperature (RT). In the identification process, four commercial edible species (*Agaricus bisporus*, *Lentinula*

edodes, *Pleurotus ostreatus* and *Tuber melanosporum*) were also tested to confirm the ability of Cyranose-320 to distinguish *T. arenaria* from other edible species.

Our review on *T. arenaria*'s nutritional and health value, and proposal for the first steps in developing a non-destructive and rapid identification method for early detection of *Terfezia* truffles, their growth stages, and quality are crucial for sustainable resource exploitation. This innovation could promote our understanding and management of desert truffle populations, ensuring their preservation and responsible production and use in the long term.

Materials and Methods

Terfezia arenaria Samples

Desert truffles naturally fruit in the spring from February to May. For three weeks of the 2019 spring season, sixty-three *T. arenaria* truffles were harvested in Alentejo (south of Portugal) (see Figure S3.1). The samples were collected in four sampling sites—S1, S2, S3 and S4—with an area of 200 m^2 each; the sampling sites were separated by 1 to 2 km distance. In all the sampling sites, the Cistaceae host plants were abundant (*T. guttata*), but the forest-dominant species differed: *Quercus suber* in sites 1 and 2, *Pinus pinea* in site 3 or mixed in site 4 (Figure S3.1). Specimens were freed from substrate debris at the site and further cleaned in the laboratory and used to analyse *T. arenaria*'s nutritional and chemical composition and volatile profile (using 2 techniques). *T. arenaria* samples were (i) kept at −20 °C until molecular analysis; (ii) dried for nutritional and chemical composition analyses; and (iii) kept at 4 °C until volatile profile analysis in the first 48 h post-harvest.

The specimens were identified by molecular analysis.

For the nutritional and chemical analyses, we used three dry samples of *T. arenaria* truffles collected in four of the sampling sites (sites S1, S2, S3, S4) during the first week of April 2019. Four desert truffle of similar size and appearance -one from each sampling site, were analysed.

For the volatiles profile analysis, we used three fresh samples of *T. arenaria* truffles of similar size and appearance, each collected in one of the three of the sampling sites (sites S2, S3, S4) during the first week of April 2019.

To validate *T. arenaria*'s identification using the e-nose, we used a total of five mushroom and truffle species: *T. arenaria*, *A. bisporus*, *L. edodes*, *P. ostreatus* and *T.*

melanosporum. Mature *T. arenaria* truffles were harvested in Alentejo (south of Portugal) as described, *A. bisporus*, *L. edodes*, and *P. ostreatus* were purchased in a local supermarket, and *T. melanosporum* was purchased at Espora Gourmet, SL. All fresh mushrooms and truffles samples were kept at 4 °C until analysis in the first 48 h post-harvest.

Comparing T. arenaria's Nutritional Value with That of Other Edible Mushrooms and Truffles, and Meat

To showcase its nutritional value, we collected and analysed *T. arenaria*'s samples for their nutritional and mineral composition. Furthermore, *T. arenaria*'s data was compared with that reported in the literature for other edible mushroom and truffle species (*A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum*), and with meat (cow, pig, and chicken). The criteria for selecting the other edible mushroom and truffle species and the types of meat were wide consumption and the easiness of buying and finding in the supermarkets.

Twelve *T. arenaria* composite samples, three for each sampling site, were prepared. The samples were dried at 40 °C for 72 h to determine their moisture content, and the dry material was powdered in a porcelain mortar and kept in brand-new sealed polyethylene bags under dry conditions at RT until analysis. Using the AOAC procedures (AOAC 1990), the dry samples were analysed for their (i) crude protein content (applying the conversion factor of N × 4.38), which was estimated by the macro-Kjeldahl method, (ii) crude fat, which was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus, and (iii) ash concentration, which was determined by incineration at 600 ± 15 °C. A bomb calorimeter (Parr 6200 Isoperibol Calorimeter) was used to estimate the energy of the samples. Total carbohydrates were calculated using the following equation:

$$Carbohydrates\ (\frac{g}{100g}DW) = total\ solids - (protein + lipids + fibre + ash)$$

The chemical elemental analysis was determined by inductively coupled plasma mass spectrometry (ICP-MS; Agilent Technologies, Bellevue, WA, USA) after digestion with concentrated nitric acid (68 % HNO_3), and filtered and diluted 20 times with double distilled water (WP750, PG Instruments, UK) to a total volume of 15 mL. For inductively coupled plasma mass spectrometry (ICP-MS) determinations, external standard calibration curves were performed by serially diluting multi-element standard stock solutions. This protocol was adapted from Mędyk et al. 2016 (Mędyk et al. 2017).

Additionally, we compared the nutritional and mineral composition of *T. arenaria* with that of *A. bisporus*, *L. edodes*, *P. ostreatus* (edible mushrooms) and *T. melanosporum* (edible truffle), and beef, pork, and chicken meat. Data used for the other edible mushrooms and truffles were selected from studies published in international peer-review journals reporting the use of methodologies similar to those we used in our study. Therefore, we used one article for *T. arenaria*, three for *A. bisporus*, five for *L. edodes*, six for *P. ostreatus* and two for *T. melanosporum*. For beef, pork, and chicken meat, one database and one article were consulted for each.

Finally, data on dietary reference intakes of nutrients and elements were compiled from Dietary Reference Intakes Datasets from the USA, Canada (Medicine 2005), and the EU (2009, 2017; SCHER 2012; Turck et al. 2023). The contribution of 100 g of dried and fresh *T. arenaria* to the daily intake of each nutrient and element was calculated considering the dietary reference intakes values previously compiled. Finally, we determined the Nutri-Score for *T. arenaria* based on its nutritional composition per 100 g of dry truffles. We used the nutritional content determined in this study, and complemented it with data on sugars and fatty acids from Tejedor-Calvo et al., 2021 (Tejedor-Calvo et al. 2021). To determine the Nutri-Score we used the recent algorithm made available by Sante Publique France (https://www.santepubliquefrance.fr/en/nutri-score) (Sante Publique France)(Sante Publique France, 2023).

Volatiles Profile by GC–MS

To showcase its unique bouquet of VOCs, we collected (as previously described in Section 2.1.) and analysed *T. arenaria*'s samples for their VOCs profile. Furthermore, *T. arenaria*'s data was compared with that reported in the literature for the same other edible mushroom and truffle species previously described (*A. bisporus*, *L. edodes, P. ostreatus* and *T. melanosporum*).

Analysis of *T. arenaria*'s VOCs profile was performed using Headspace-Solid Phase Microextraction Gas Chromatography–Mass Spectrometry coupled to GC–MS (HS–SPME/GC–MS), adapted from the protocol reported by Splivallo and Ebeler (2015) (Splivallo and Ebeler 2015). The three fresh specimens were ground with a clean knife to small cubes of approximately 125.000 mm^3, and accurately weighed in 1.5 mL tightly sealed glass vials. A pre-extraction was performed in the vial at 60 °C for 10 min, then the SPME fibre (PDMS/DVB65um) was implanted manually, and the volatile compounds were extracted at 60 °C for 30 min. Afterwards, the SPME fibre was removed and placed manually in the injection port of the GC–MS. The analysis of volatile compounds was conducted on an GC–MS-QP2010

(Shimadzu, Japan), with acquisition mode SCAN (35–600 *m/z*) and equipped with a TRB-5 MS column (Teknokroma, Spain). The injector and MS interface temperatures were both held at 250 °C. The analytic conditions were the following: the constant flow of helium in the column was kept at 1.0 mL min^{-1}; the oven temperature was held at 40 °C for 10 min, then raised at a rate of 10 °C min^{-1} to 160 °C, and finally reached 260 °C with a rate of 50 °C min^{-1} and kept for 2 min. Blank GC–MS runs were performed during the analyses.

Finally, we compared the VOCs profile of *T. arenaria* with that of *A. bisporus*, *L. edodes, P. ostreatus* (edible mushrooms) and *T. melanosporum* (edible truffle). Data used for these edible mushrooms and truffle were selected from studies published in international peer-review journals reporting the use of methodologies similar to those we used in our study. Therefore, we used one article per each species.

First Steps in Developing a Non-Destructive and Rapid Identification Method for T. arenaria

To test if *T. arenaria*'s unique VOCs profile could be applied in developing a non-destructive and rapid identification method for the early detection of *T. arenaria*, we used the Cyranose-320 e-nose (Sensigent, Pasadena, CA, USA). The Cyranose-320 is a portable e-nose equipped with a nanocomposite sensor array (32 nanosensors), an internal air sampling pump, and advanced pattern recognition algorithms. These technologies enable rapid detection and identification of substances based on their chemical profile as visualized by the smellprint (Sensigents, 2023). Therefore, we specifically tested if the e-nose was capable of distinguishing *T. arenaria* from other edible mushroom and truffle species, and therefore guarantee this desert truffle's authenticity. This was conducted in the following two phases (Figure 3.1):

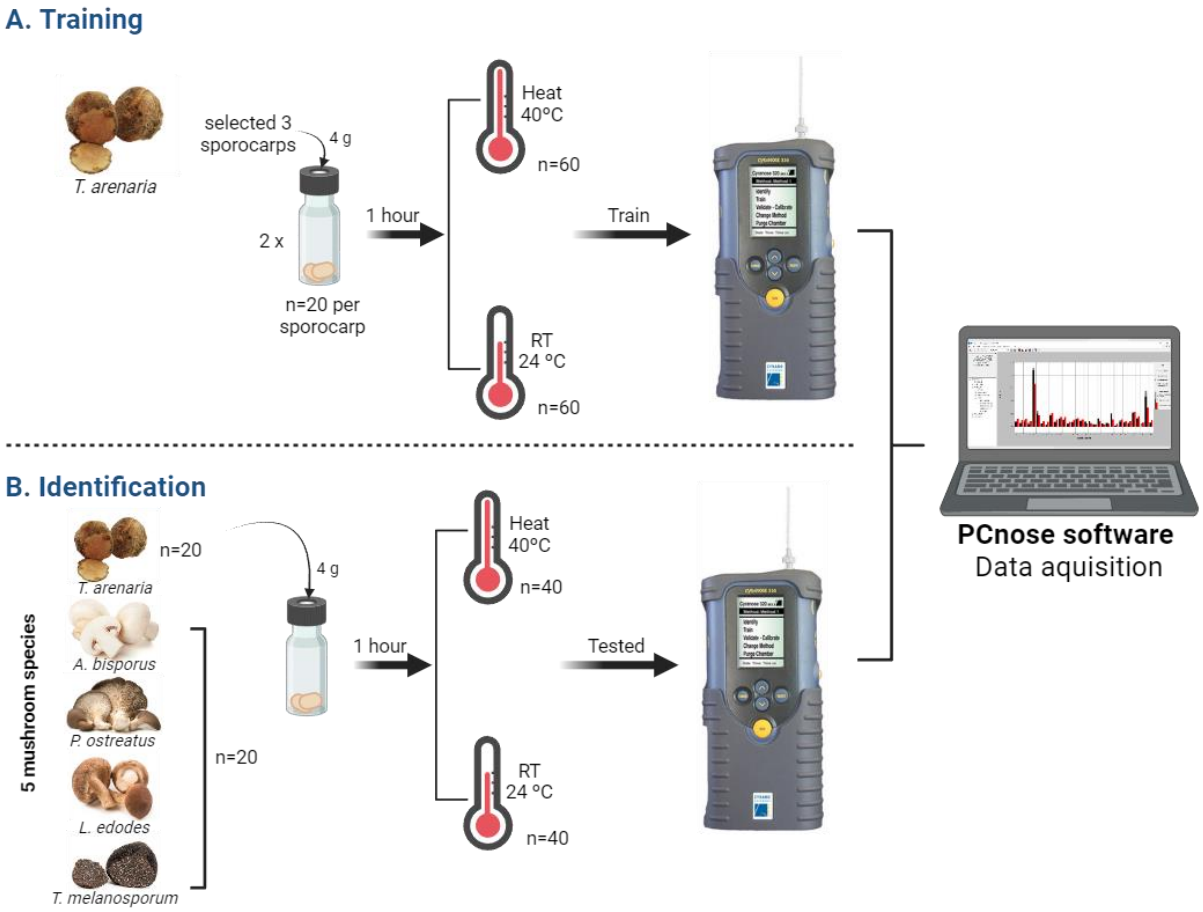

Figure 0.1 - Schematic representation of the two phases of the non-destructive and rapid identification method for *Terfezia arenaria* identification using the Cyranose-320 e-nose. First, the e-nose was trained with *T. arenaria* samples (A), and then mushroom and truffle (MT) samples belonging to five species were tested for an accurate identification of *T. arenaria* (B). Two pre-analysis incubation temperatures were tested in both phases: samples were kept at room temperature (RT) or heated at 40 °C. Created with BioRender.com.

Phase 1: E-Nose Training

For the training process (Figure 3.1), *T. arenaria*'s samples were subjected to one of two pre-analysis incubation temperatures: (a) 40 °C treatment with samples incubated for 1 h at 40 °C; and (b) RT treatment with samples incubated for 1 h at RT (i.e. 24 °C). The samples used for analysing *T. arenaria*'s VOCs profile with the e-nose were clean as previously described, and were kept at 4 °C until analysis in the first 48 h post-harvest. Three fresh *T. arenaria* truffles were analysed separately. Two replicates with 4 g of *T. arenaria* were weighed and introduced in a 10 mL vial for each sporocarp and training method. The *T. arenaria* truffles were identified as: Terf1, Terf2 and Terf3. The Cyranose-320 was mounted on a tripod, which could be adjusted for inserting the e-nose needle into the vials for headspace reading. Ten readings per samples were performed. The e-nose was coupled to the computer and PCnose

software was used to set the list of parameter settings of the Cyranose-320 (see Table S3.1), data acquisition, and analysis. To finish the training phase, an internal data cross-validation was used to assess the accuracy of sample classification in relation to their respective class labels, serving as a measure of effectiveness for the e-nose system (Santos et al. 2004).

Phase 2: E-Nose Identification Accuracy

Similarly to what was conducted in the training phase, four grams of three fresh mushrooms were weighed and introduced in a 10 mL vial, and the two pre-analysis incubation temperatures were applied. Afterwards, each sample headspace was read with Cyranose-320 in the identification mode activated for the respective method trained (40 °C or RT pre-analysis incubation temperatures). Results were displayed in Cyranose-320 and recorded on the PCnose software. The results displayed in the Cyranose-320 are rated with asterisks, between one and five asterisks, accordingly to the identification quality performed. Regarding quality, only samples between three and five asterisks are considered acceptable results (i.e. acceptable, good, and excellent, respectively). When the e-nose does not recognize the tested sample, "Confused" or "Unknown" will be displayed (Table S3.5).

Statistical Analysis

We used a principal component analysis (PCA) to analyse nutritional and mineral composition (based on fresh weight values) for *T. arenaria* determined in this study, and *A. bisporus*, *L. edodes* and *P. ostreatus* and fresh beef, pork, and chicken meat with values from the literature. *T. melanosporum* was not included in the PCA because moisture content was not available on the selected literature and thus, we were unable to express its nutritional and mineral composition for fresh samples. The PCA explored how *T. arenaria*'s nutritional and mineral composition compares to reference values for edible mushrooms and meat.

Pie charts and Venn diagram were performed to compare the VOCs profiles of *T. arenaria* with literature values for *A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum* (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Standardized data from the 32 sensors were analysed blinded to reference standard results using PCA to explore the sensors' response to the two pre-analysis incubation temperatures (40 °C and RT). Differences between 40 °C and RT pre-analysis incubation temperatures were compared using the Kruskal–Wallis one-way analysis of variance. Multiple pairwise comparisons were performed using Dunn's test ($p < 0.05$). All statistical analysis were

performed using Microsoft Excel 2019/XLSTAT-Premium (Version 2021.4.1, Addinsoft, Inc., Brooklyn, NY, USA).

Results and Discussion

Showcasing T. arenaria's Nutritional Value

The diverse array of nutrients found in mushrooms and truffles, including carbohydrates, proteins, lipids, minerals, fibre, and water, contribute to their potential positive effect on the human diet. The average moisture of the *Terfezia arenaria* samples was 77 %, which is within the range reported for other desert truffles (Table 3.1) (MA Murcia 2003; Kıvrak 2015; Hamza et al. 2016). However, *T. arenaria*'s lipid concentration was slightly (2 % to 8 %) lower than that reported for other desert truffles (Ahmed et al. 1981; MA Murcia 2003; Hamza et al. 2016; Tejedor-Calvo et al. 2021), but similar to that reported for the commercial edible mushroom and truffle species *A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum* (Roncero-Ramos et al. 2016; Ekute 2019; Yu et al. 2020; Jacinto-Azevedo et al. 2021; Tejedor-Calvo et al. 2021). Carbohydrates are the major nutrient category in edible mushrooms and truffles (Kalač 2013a). However, the concentrations determined in *T. arenaria* were lower than those reported for other desert truffles (Ahmed et al. 1981; MA Murcia 2003; Hamza et al. 2016). *T. arenaria*'s energy potential (387 kcal per 100 g of dry weight) was similar to that reported for the other edible mushrooms and truffles (Table 3.1) (Kıvrak 2015; Roncero-Ramos et al. 2016; Tejedor-Calvo et al. 2021; Jacinto-Azevedo et al. 2021).

Furthermore, 18 mineral elements were identified in *T. arenaria* samples (Table 3.2), with potassium, phosphorus, sulphur, magnesium, and calcium being the most abundant. Eight trace elements (iron > zinc > copper > manganese > chromium > molybdenum > selenium > nickel) and two nonessential elements (aluminium and lithium) were also identified. These mineral elements are critical for human health, and their intake must be carefully balanced to avoid health problems. Lithium (Li, 37 µg 100 g^{-1} dw) and selenium (Se, 50 µg 100 g^{-1} dw) are of particular importance to human health, due to their proprieties, i.e., antiviral, immunomodulatory, neuroprotective effects, and can be used to treat several mental health conditions (Rybakowski and Ferensztajn-Rochowiak 2022; Turck et al. 2023). Considering the dietary reference intakes (see Table S3.2), a balanced consumption of *T. arenaria* (especially dry) could

contribute to a proper intake of these elements. In the case of lithium, this element is higher in *T. arenaria* than the other edible mushrooms and truffles, which could be an interesting characteristic in the development of new plant-based meat products based on this desert truffle.

Two trace elements with a detrimental health effect were identified in the *T. arenaria*, Arsenic (As, 10 µg 100 g^{-1} dw) and Barium (Ba, 32 µg 100 g^{-1} dw). The As value is similar to the values reported for *A. bisporus*, *L. edodes* and *Pleurotus ostreatus,* while the Ba was lower than that reported for *A. bisporus* and *L. edodes* (Siwulski et al. 2021, 2022) (Table 3.2), and other edible mushroom species (Kalac 2019). Furthermore, according to Siwulski et al. (2021), the estimated daily intakes of these mushrooms, particularly *L. edodes*, are low and do not pose a health risk (Siwulski et al. 2021). Also, considering the established dietary reference intakes for Arsenic (15 µg kg^{-1} body weight per day) and Barium (0.2 mg kg^{-1} body weight per day), the contribution of *T. arenaria* is residual when considering a consumption of 100 g of dry or fresh truffles (Table S3.2).

The nutritional and mineral value of mushrooms is influenced by parameters such as the stage of development, the substrate where they grow, the geographic origins, and their genetic variability intra and interspecies (Manzi et al. 1999). *Terfezia arenaria* samples were harvested from different locations with different dominant forest species and at different times during a three-week harvest season. Although *T. arenaria* has a wider distribution area and fruits for a longer period, both its distribution area and fruiting period have been severely reduced by lower precipitation in autumn and spring [crucial for desert truffle fructification (Andrino et al. 2019)] due to climate change and wildfires that destroy productive areas. However, when considering the nutritional values previously reported for other desert truffles, we consider that it is likely that the *T. arenaria*'s nutritional and chemical composition we report here could represent this species' composition. Nonetheless, *T. arenaria*'s nutritional and mineral composition was similar to the most commercialized and appreciated species of mushrooms and truffles in the world, such as *A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum* (Reyna and Garcia-Barreda 2014; Royse et al. 2017) (Tables 3.2 and 3.3). Despite their similarities, *T. arenaria* have a closer resemblance to meat than the other edible mushrooms (Figure 3.2).

The Nutri-Score is a promising new front-of-pack nutrition labelling system that has the potential to improve population diets (WHO 2013). It is easy to understand, well-accepted by consumers, and can be effective in encouraging healthier food choices (van den Akker et al. 2022). The Nutri-score algorithm showed that 100 g of dry *T. arenaria* has a Nutri-Score of A

(i.e. the A score is the best nutritional score when applying the Nutri-Score), which indicates that this product has a very good nutritional profile. Products with an A score are typically low in calories, saturated fat, and sugar, and high in fibre and protein.

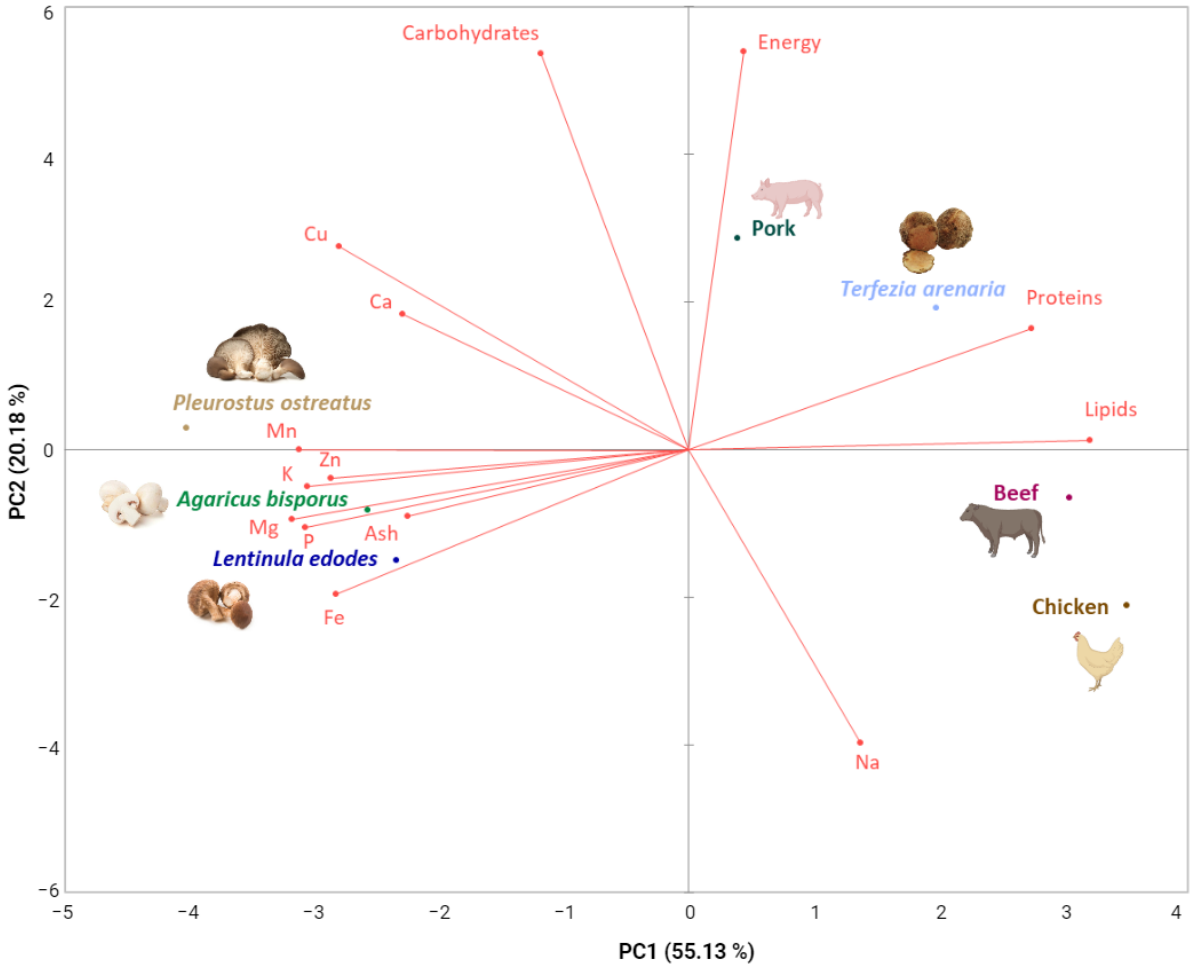

Figure 0.2 - Fresh *Terfezia arenaria*'s nutritional and mineral composition in relation to reference values reported for other fresh edible mushrooms and meat. Plot of the first two principal components of the principal component analysis (PCA) model built with common nutritional and mineral composition values for fresh *T. arenaria* determined in this study, and fresh *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus* and beef, pork, and chicken meat from the literature. Created with XLSTAT and BioRender.com

Our data on *T. arenaria*'s nutritional value can help explain why this desert truffle had, and still has, such an important role in the nourishment of rural populations, often serving as a meat substitute (Shavit 2008; Bradai et al. 2015). Indeed, poor rural populations in North Africa and Arab countries have used mushrooms as meat substitutes for centuries (Shavit 2008, 2014; Bradai et al. 2015). Furthermore, *T. arenaria* shares equally appealing properties with other edible commercial species (Tables 3.1 and 3.2) as *T. arenaria*'s protein can range between 14 and 23 g per 100 g^{-1} dw, its carbohydrates can range between 67 and 77 g/100 dw, and its lipids

can range between 2.2 and 5.1 g/100 dw (Tables 3.1 and 3.2) (Tejedor-Calvo et al. 2021). *T. arenaria*'s protein value is similar to the average values for pork and beef meats (pork 13.2 g; beef 19.9 g), while the carbohydrates are higher (pork 2.4 g; beef 2.0 g) and the lipids are lower (pork 37.0 g; beef 4.2 g) (Wang and Zhao 2023). These characteristics, together with the mineral composition, show that *T. arenaria* is suitable to be employed in new food products as a potential plant-based meat (Figure 3.2).

Table 0.1 - Nutritional composition and energy values for *Terfezia arenaria* and other desert truffles, commercial mushrooms, and meat (pork, beef, and chicken).

		Moisture % fw	**Ash** g/100g	**Proteins** g/100g	**Lipids** g/100g	**Carbohydrates** g/100g	**Fibre** g/100g	**Energy** kcal/100g	**References**
Desert Truffles	*Terfezia arenaria* [a]	77	7.3	14	2.2	67	10	387	This study
		n.a	4.3	23	5.1	77	n.a	394	Tejedor-Calvo et al. 2021
	Terfezia claveryi [a]	73	4.3	16	7.0	65	8	n.a	Murcia 2003
		83	15.3	32	2.8	46	n.a	338	Kıvrak 2015
	Terfezia boudieri [a]	n.a	12.9	17	6.4	60	4	n.a	Ahmed et al. 1981
		78	4.5	26	8.0	62	n.a	n.a	Hamza et al. 2016
	Terfezia olbiensis [a]	80	15.3	36	3.2	48	n.a	366	Kıvrak 2015
Commercial mushrooms	*Agaricus bisporus* [a]	91	12.7	19	2.0	67	10	360	Jacinto-Azevedo et al. 2021
		90	9.4	25	2.3	64	n.a	374	Roncero-Ramos et al. 2016
	Pleurotus ostreatus [a]	91	7.8	18	2.6	71	14	382	Jacinto-Azevedo et al. 2021
		n.a	9.3	9	1.3	70	11	n.a	Ekute 2019
		89	6.7	13	2.5	78	n.a	383	Roncero-Ramos et al. 2016
		n.a	3.8	18	0.9	30	32	264	Yu et al. 2020
	Lentinula edodes [a]	94	6.7	16	1.8	74	15	382	Jacinto-Azevedo et al. 2021
		88	7.4	17	2.1	73	n.a	381	Roncero-Ramos et al. 2016
	Tuber melanosporum [a]	n.a	0.0	22	2.3	75	n.a	411	Tejedor-Calvo et al. 2021
Meat	*Pork* [b]			13	37.0	2.4	n.a	390	Wang and Zhao 2023
		64	0.9	18	17.5	n.a	n.a	228	USDA 2023
	Beef [b]			20	4.2	2.0	n.a	126	Wang and Zhao 2023
		63	0.8	18	19.4	n.a	n.a	243	USDA 2023
	Chicken [b]			19	1.3	9.4	n.a	167	Wang and Zhao 2023
		75	1.0	18	7.2	n.a	n.a	133	USDA 2023

[a] mushroom values presented for dry weight, except for moisture (% fresh weight). [b] meat values presented for fresh weight; n.a—data not available.

Indeed, as awareness on the adverse effects of meat consumption grows (Kwasny et al. 2022; Andreani et al. 2023), there is a notable shift towards incorporating plant-based ingredients, such as mushrooms and truffles, into meat-based dishes (Guinard et al. 2016; Li et al. 2023). This increasing acceptance reflects a growing interest in blending plant-based alternatives with traditional meat-based foods (Lang 2020). By incorporating mushrooms and truffles as blenders in meat products, over 7.5 million L of water can be saved per 10,000 portions of this product (made with 70 % beef and 30 % mushrooms) (Lang 2020). *A. bisporus*, *L. edodes*

and *P. ostreatus* are among the most produced mushrooms species worldwide, and are already often used in these products (Royse et al. 2017; De Cianni et al. 2023).

Table 0.2 - Mineral content [mg kg^{-1} dw] for *Terfezia arenaria* and other desert truffles, commercial mushrooms, and meat (pork, beef and chicken). Values for *T. arenaria* were determined in the present study while values for the other mushrooms, truffles and meat were determined in other studies.

Minerals	***Terfezia Arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***	**Pork**	**Beef**	**Chicken**
Major essential elements								
Ca	26	580	438	730	817	60	70	60
K	3695	38,400	21,700	14,244	7356	3180	2730	3020
Mg	128	1300	1330	2800	241	190	164	205
Na	23	491	144	35	67	540	550	630
P	1407	8210	4080	6204	2678	1730	1440	1660
S	299	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Essential trace elements								
Cr	0.9	7.0	0.3	n.a	n.a	n.a	n.a	n.a
Cu	6.6	34.2	7.1	39	18	0.7	0.6	0.4
Fe	19.6	49.4	35.7	130	12	7.9	19.6	5.9
Mn	1.4	6.6	19.3	14	1	<0.125	<0.125	0.1
Mo	0.6	0.3	0.2	<0.01	n.a	n.a	n.a	n.a
Ni	0.1	0.7	0.1	0.7	n.a	n.a	n.a	n.a
Se	0.5	1.7	1.1	0.3	n.a	n.a	n.a	n.a
Zn	11.0	51.5	76.3	110.4	37	22.3	38.5	11.8
Non-essential elements								
Al	10.7	17.9	5.8	n.a	n.a	n.a	n.a	n.a
Li	0.4	< 0.1	0.1	0.3	n.a	n.a	n.a	n.a
Elements with detrimental health effects								
As	0.1	0.3	0.5	<0.1	n.a	n.a	n.a	n.a
Ba	0.3	2.8	1.7	n.a	n.a	n.a	n.a	n.a
References	This study	Siwulski et al. 2022	Siwulski et al. 2021	Hoa et al. 2018; Koutrotsios et al. 2020; Ahmad 2020 Zakil et al. 2022	Shimokawa et al. 2020	USDA 2023	USDA 2023	USDA 2023

n.a—data not available.

Volatiles Profile by GC–MS

Volatile profiles, particularly in mushrooms and truffles, are crucial in determining their characteristic odours and strongly influence consumers' preferences. *T. arenaria*'s distinct volatile profile serves as a key characteristic and significantly impacts consumers' preferences.

Thirty-two VOCs were identified in *T. arenaria* fresh samples, i.e., eight hydrocarbons, six alcohols, five aldehydes, three ketones, three esters, two terpenes, and five other compounds. Among them, the most abundant were the eight carbon (C-8) compounds and Hexanal, with 1-octen-3-ol being the main volatile (64 %) in *T. arenaria* (see Tables S3.3 and S3.4). 1-Octen-3-ol is generally referred to as the mushroom alcohol and is one of the most abundant VOCs produced by fungi (Combet et al. 2006; Quintana-Rodriguez et al. 2018). This is consistent with the fact that C-8 compounds are the main volatile components found in several edible mushrooms and truffles (Maga 1981; Zhang et al. 2020; Feng et al. 2021; Tagkouli et al.

2021) (Tables S3.3 and S3.4). Despite using similar methodologies, Harki et al. (2010) identified 27 VOCs in *T. arenaria*, but only three compounds were common with our study (nonanal, 3-octanone and 2-octenal) (Harki et al. 2010). Both internal factors [e.g. maturity stage, tissue specificity and postharvest storage (Tasaki et al. 2019)] and external factors [e.g. place of origin, interaction with microorganisms (Splivallo et al. 2011)], result in distinct metabolic processes within the fungi, which alters their VOCs profile. The main volatiles identified in *T. arenaria* were C-8 compounds resulting from the breakdown of fatty acids (such as linoleic acid) by lipoxygenase and related enzymes (Table 3.3), which is in agreement with the evidence that the umami taste, so characteristic of mushrooms, is associated with the fatty acid metabolism (Sun et al. 2020). Lipoxygenases are pivotal in the biosynthesis of leukotrienes, associated with various inflammatory conditions such as cancer, arthritis, asthma, and allergies (Czapski et al. 2012). Given their role in these disease processes, lipoxygenase inhibitors are being explored as potential therapeutic options for preventing and managing these inflammatory disorders (Mashima and Okuyama 2015; Nkadimeng et al. 2021). Notably, certain mushroom extracts have demonstrated the ability to inhibit these enzymes, offering potential health benefits (Nkadimeng et al. 2021; Szydłowska-Tutaj et al. 2023a, b). These extracts have been used to enhance the nutritional value of pasta, contributing to healthier products (Szydłowska-Tutaj et al. 2023a, b). Exploring the potential inhibition of lipoxygenase by *Terfezia* may be necessary for incorporating this product in food formulations.

Table 0.3 - List of the volatile organic compounds (VOCs) detected in *Terfezia arenaria* using gas chromatography–mass spectrometry (GC–MS). The VOCs are listed according to their abundance (i.e. area). The values represent the mean relative peak areas (expressed as % from total peak areas) and retention times (rt). Information on each VOC's classification, metabolic pathway and odor is also presented (n = 3). (*Continue*)

Formula	rt	Area (%)	Name of Compounds	Functional Groups	Pathway	Odor
$C_8H_{16}O$	15.847	57.21	1-Octen-3-ol	Alcohols	Lipoxygenase–linoleic acid	Mushroom like
$C_8H_{16}O$	16.007	12.86	3-Octanone	Ketones	Lipoxygenase–linoleic acid	Green apple-like
$C_6H_{12}O$	7.412	4.29	Hexanal	Aldehydes	Lipoxygenase–linoleic acid	Green, grassy
$C_8H_{16}O$	17.952	3.22	(Z)-2-Octen-1-ol	Alcohols	Lipoxygenase–linoleic acid	Green, citrus
$C_{10}H_{16}$	14.379	1.87	α-Pinene	Terpenes	Monoterpenoid biosynthesis	Woody, resinous
$C_8H_{18}O$	16.256	1.54	3-Octanol	Alcohols	Lipoxygenase–linoleic acid	Floral, fatty
$C_8H_{14}O$	15.658	1.38	(5Z)-Octa-1,5-dien-3-ol	Alcohols	Lipid metabolism	Sweet or floral
$C_8H_{16}O_2$	16.549	0.98	Pentyl propanoate	Ester	n.a	Fruity, sweet
$C_8H_{14}O$	17.735	0.86	I-2-Octenal	Aldehydes	Lipoxygenase–linoleic acid	Fatty, nutty
$C_{21}H_{41}IO_2$	24.933	0.39	Propionic acid, 3-iodo-, octadecyl ester	Ester	n.a	n.a
$C_{10}H_{16}$	17.086	0.35	Limonene	Terpenes	Monoterpenoid biosynthesis	Citrus
C_8H_9N	18.411	0.32	Pyridine, 5-ethenyl-2-methyl-	Other compounds	n.a	Pungent, fish-like
$C_{18}H_{37}ClO_2S$	25.156	0.3	1-Octadecanesulphonyl chloride	Other compounds	n.a	Strong and pungent
$C_{21}H_{42}O_2$	25.127	0.29	Henicosanoic acid	Other compounds	n.a	Odorless
$C_{32}H_{66}$	25.155	0.28	Dotriacontane	Hydrocarbons	n.a	Odourless
C_8H_{16}	17.479	0.26	Caprylene (1-octene)	Hydrocarbons	n.a	Petroleum-like
$C_8H_{15}NO_3$	20.904	0.23	2-Octanone, 1-nitro-	Ketones	n.a	Sweet
$C_{12}H_{24}O_3$	23.046	0.22	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	Ester	n.a	Mild, fruity or sweet
C_8H_8O	17.417	0.21	Benzeneacetaldehyde	Aldehydes	Phenylalanine metabolism	Sweet, floral

n.a—data not available.

Table 0.4 - List of the volatile organic compounds (VOCs) detected in *Terfezia arenaria* using gas chromatography–mass spectrometry (GC–MS). The VOCs are listed according to their abundance (i.e., area). The values represent the mean relative peak areas (expressed as % from total peak areas) and retention times (rt). Information on each VOC's classification, metabolic pathway and odor is also presented (n = 3).

Formula	rt	Area (%)	Name of Compounds	Functional Groups	Pathway	Odor
$C_{14}H_{30}$	24.692	0.21	Tetradecane	Hydrocarbons	n.a	Gasoline-like to odorless
$C_{14}H_{30}$	24.692	0.21	Eicosane-7-hexyl	Hydrocarbons	n.a	n.a
$C_{13}H_{22}O_3Si_2$	18.831	0.19	Benzaldehyde, 2,5-bis[(trimethylsilyl)oxy]	Aldehydes	n.a	n.a
$C_{16}H_{34}$	24.134	0.15	Hexadecane	Hydrocarbons	Fatty acid degradation	Odourless
$C_6H_{13}ClO$	23.201	0.14	Chlorohexanol	Alcohols	n.a	Odorless
$C_{10}H_{22}$	24.654	0.13	3,3,5-Trimethylheptane	Hydrocarbons	n.a	Gasoline-like
$C_7H_7NO_2$	24.979	0.13	Anthranilic acid	Other compounds	L-tryptophan-kynurenine	Odorless
$C_9H_{18}O$	20.273	0.12	Nonanal	Aldehydes	n.a	Fruity, waxy
$C_8H_{10}O_2$	23.461	0.11	Tyrosol	Other compounds	Tyrosine metabolism	Floral, phenolic
$C_{16}H_{32}$	23.812	0.1	1-Dodecanol	Alcohols	n.a	Waxy, fatty
$C_{20}H_{41}Cl$	25.336	0.1	1-chloroeicosane	Hydrocarbons	n.a	n.a
$C_{13}H_{22}O$	23.68	0.09	Geranylacetone	Ketones	Ketone Body Metabolism	Sweet, floral, fruity
$C_{20}H_{42}$	23.201	0.08	Eicosane	Hydrocarbons	n.a	Odourless

n.a—data not available.

Nine VOCs identified in *T. arenaria* (i.e. 1-octen-3-ol, 3-octanol, 3-octanone, 2-octenal, hexanal, nonanal, benzeneacetaldehyde, eicosane, limonene and α-pinene) had been previously reported for the desert truffles *T. boudieri* and *T. claveryi* (Kamle et al. 2017; Darwish et al. 2021; Farag et al. 2021a). These compounds are also prevalent in commercial edible mushrooms and truffles such as *A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum* (Zhang et al. 2020; Choo et al. 2021; Feng et al. 2021; Tagkouli et al. 2021). On the other hand, 18 VOCs detected in *T. arenaria,* had not been reported for other *Terfezia* species or *A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum* (Table S3.4). Additionally, a review of the reported VOCs composition of these species revealed that only three compounds are common between

T. arenaria and these four commercial species (Figure 3.3). These VOCs, which are C-8 compounds (i.e. 1-octen-3-ol, 3-octanol, 3-octanone), are abundant in *A. bisporus*, *L. edodes*, *P. ostreatus* and *T. arenaria* and greatly contribute to their floral and green aromas (Zhu et al. 2022b) (Table 3.2, Table S3.3). They are also present in *T. melanosporum*, but in lower quantities (Splivallo et al. 2011; Khalifa et al. 2019b).

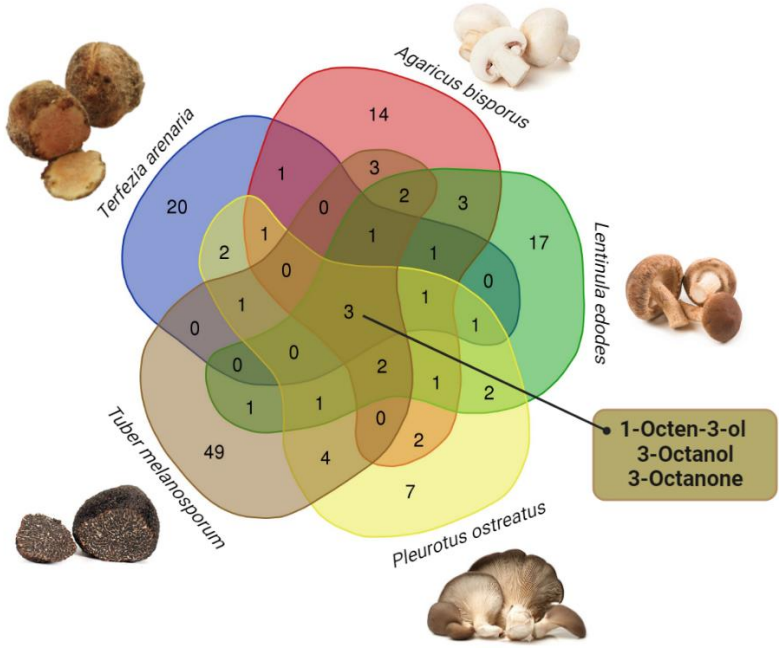

Figure 0.3 - A Venn diagram comparing *Terfezia arenaria*'s volatile organic compounds (VOCs) profile with that reported in the literature for *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus* and *Tuber Melanosporum*.Created with http://bioinformatics.psb.ugent.be/webtools/Venn/ and BioRender.com.

When comparing *T. arenaria*'s aroma profile with that from other edible mushrooms and truffles (*A. bisporus*, *L. edodes*, *P. ostreatus*, and *T. melanosporum*) (Figure 3.3, Table S3.4), *T. arenaria* exhibits a distinct composition. Figure 3.4 highlights potential differences between *T. arenaria* and the other edible mushrooms and truffles in terms of the number and quantity of compounds per main functional group. In *T. arenaria*, there is a higher presence of alcohol compounds compared to *A. bisporus* and *T. melanosporum*, although it is less diverse in terms of the number of compounds. Unlike *L. edodes* and *T. melanosporum*, *T. arenaria* does not contain detectable acids or sulphur compounds, making it stand apart in its aromatic profile. The latter two species have a greater variety of compounds, indicating a more complex volatile profile than *T. arenaria*.

On the other hand, *A. bisporus* and *P. ostreatus* demonstrate simpler volatile profiles, with only five main functional groups identified. Among all the analysed edible mushrooms

and truffles, *A. bisporus* appears to have the most similar aromatic profile to *T.* arenaria. Despite the high similarity between the VOCs profiles of *T. arenaria* and *A. bisporus* (Figure 3.4), the e-nose successfully distinguished between the volatile profiles these fungal species (Keshri et al. 2003; Zhou et al. 2015; Portalo-Calero et al. 2019b, a; Gómez et al. 2022).

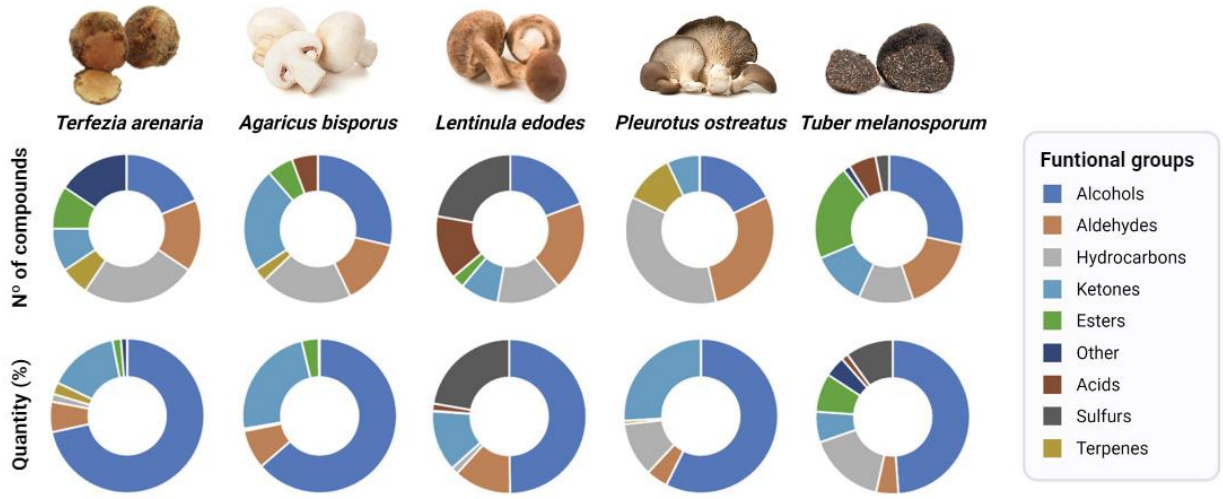

Figure 0.4 - Aromatic profiles of the five edible mushroom and truffle (MT) species as shown by the number and identity of the functional groups of compounds and their relative proportion. The pie charts present the number of identified compounds and their relative proportion (%) per group in each MT species Created with BioRender.com.

First Steps in Developing a Non-Destructive and Rapid Identification Method for T. arenaria

Electronic noses have been coupled with GC–MS to analyse aromas in various food products (Mohd Ali et al. 2020). This technique has gained prominence due to its versatility and speed of response. Although GC–MS is a more precise and accurate technique, it is also more complex, expensive, and time-consuming. For example, in this work, analysing only three samples using GC–MS required one week, including sample preparation, sample reading, and data processing. In contrast, using an e-nose, after the initial equipment training (which takes about 4 h), a quick response can be obtained within one to two hours (including sample preparation, incubation, and equipment reading).

Phase 1: E-Nose Training

As the Cyranose-320 e-nose showed sensitivity to the pre-analysis incubation temperature (40 ℃ or 24 °C), temperature influenced the volatile compounds emitted by *T. arenaria* fresh samples (Figure 3.5). The Cyranose-320 correctly classified 73 % of the *T. areanaria* samples incubated at RT, and 81 % of the *T. areanaria* samples incubated at 40 °C. All 32 sensors of the Cyranose-320 e-nose signalled the

emitted VOCs for pre-analysis incubation temperatures (40 °C and 24 °C). From those, five sensors had major responses in the two pre-analysis incubation temperatures (i.e., S5, S6, S23, S28, and S31), and sensor number 31 showed the highest sensitivity (Figure 3.5a). The pre-analysis incubation temperature influenced these sensors (i.e., S5, S6, S23, S28, and S31; $p < 0.001$), with higher responses at the higher temperature. The first two principal components (PC1 and PC2) explained 95.1 % of the total variance (92.5 % and 2.6 %, respectively), which means that the incubation temperature has a significant effect on the volatile compounds emitted by *T. arenaria* (Figure 3.5b). Similar results were reported for *A. bisporus* (92 % and 99 %) stored at different temperatures (Song et al. 2019); *Tuber indicum* (100 %) under different drying processes (Ma et al. 2018); and *Thricholoma matsutake* (90 %) from different provenience regions. This confirms the importance of the pre-analysis temperature (i.e., storage temperature) and the sensitivity of the e-nose methodology to detect the volatile profile of different mushroom and truffle species. The score plot (Figure 3.5b) showed that the aroma profile of *T. arenaria* subjected to different pre-analysis incubation temperatures can be discriminated and the two clusters were very similar in terms of their volatile compound content (Figure 3.5a).

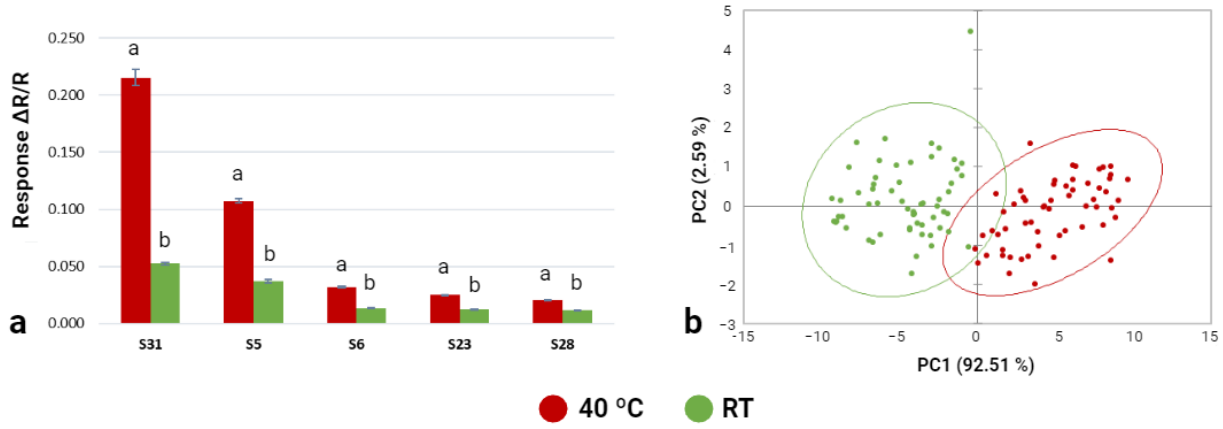

Figure 0.5 - Effect of the pre-analysis incubation temperature (40 °C and RT) on the Cyranose-320 sensor's response to *Terfezia arenaria* fresh samples. (a) Histogram of the 5 sensors showing larger responses to *T. arenaria* samples. Different letters show a significant effect ($p < 0.05$) of the pre-analysis incubation temperature on the sensors responses. (b) Plot of the first two principal components of the principal component analysis (PCA) model built with the Cyranose-320 data related to *T. arenaria* samples at 40 °C and RT. Created with XLSTAT and BioRender.com.

Phase 2: E-Nose Identification Accuracy

Based on their volatile profile, the e-nose Cyranose-320 accurately recognized the *T. arenaria* samples (Terf1, Terf2 or Terf3) and rated their result with stars (Table S3.5). Although all *T. arenaria* samples were identified as one of the trained volatile profiles in both pre-analysis incubation temperatures, the pre-analysis incubation temperature influenced the identification accuracy. So, when the pre-analysis incubation was performed at room temperature, the identification of 80 % of the samples was acceptable or excellent, while when the pre-analysis

incubation was performed at 40 °C, only 45 % of the samples were acceptable or excellent, and considered accurately identified (Table S3.5). Despite this trend, the pre-analysis incubation temperature did not affect the *T. arenaria* samples' identification accuracy ($p < 0.05$) (Figure 3.6). Nevertheless, pre-analysis incubation at room temperature improved the e-nose's capacity to distinguish *T. arenaria* samples from those of the other edible mushrooms and truffles (*A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum*) (Figure 3.6).

Indeed, when the pre-analysis incubation was performed at 40 °C, some samples were misclassified as *T. arenaria* (Figure 3.6), specifically some *A. bisporus* and *L. edodes* samples were identified with a 100 % probability of being *T. arenaria* (5 stars). The pre-analysis incubation temperature affected the e-nose's capacity to distinguish between *T. arenaria* and *A. bisporus* and *L. edodes* but not *P. ostreatus* and *T. melanosporum* ($p < 0.05$).

Most of the studies reporting the use of the e-nose technology on mushrooms and truffles focused on sample quality as influenced by dehydration (Pei et al. 2016; Ma et al. 2018; Chen et al. 2021), shelf life and packaging (Song et al. 2019; Gholami et al. 2023), and maturation process (Chilo et al. 2016). Some studies also explored the differentiation of species by analysing their volatile profiles using the e-nose (Keshri et al. 2003; Zhou et al. 2015; Portalo-Calero et al. 2019b, a; Gómez et al. 2022). By applying the e-nose methodology for a rapid and non-destructive accurate identification of *T. arenaria* samples, we demonstrate our approach's potential for mushrooms and truffles identification during harvest. It is important to keep in mind that *T. arenaria* is traditionally harvested near the host plant (*T. guttata)*, with the collector using a pointed stick to repeatedly explore the soil until a truffle is detected and extracted (Chevalier 2014). Therefore, the development of a tool based on our non-destructive and rapid methodology could contribute to the early detection of *Terfezia* truffles in the field, potentially discriminating between maturity stages and sample quality, which would contribute to the much-needed technology to boost *Terfezia*'s sustainable production and ensure its reliable identification. Furthermore, this could be useful for other truffles whose belowground development makes it difficult to detect them and distinguish maturity stages and quality (Shavit 2008). As the VOCs emitted by mushrooms and truffles are important to the food industry, especially for developing new food products or even for new tools that could contribute to food security, our study contributes to unlock many possibilities for using this delicacy (*T. arenaria*) in the food industry worldwide.

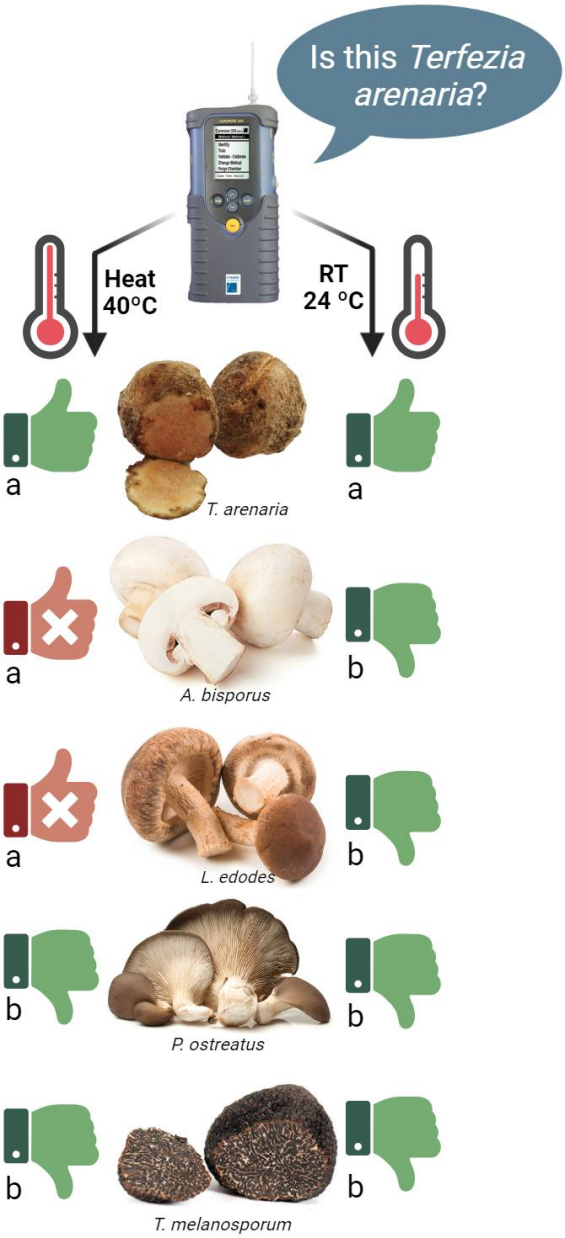

Figure 0.6 - Effect of the pre-analysis incubation temperature (40 °C and RT) on the Cyranose-320 e-nose capacity to accurately identify *Terfezia arenaria*, and distinguish it from *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus* and *Tuber melanosporum*. The "thumbs up" symbol represents the cases when the e-nose identified the sample as being *T. arenaria*, while the "thumbs down" symbol represents the cases when the e-nose identified the sample as not being *T. arenaria*. Green thumbs indicate an accurate identification, while red thumbs indicate an inaccurate identification by the e-nose. Different letters show a significant effect ($p < 0.05$) of the pre-analysis incubation temperature on the identification accuracy for each species. Created with BioRender.com.

Conclusions

From a nutritional standpoint, *T. arenaria* is a well-balanced food, rich in carbohydrates, fibres, and proteins, while containing a low-fat content. It is also a good source of minerals, including lithium, selenium, and iron. Furthermore, it has a unique aroma dominated by the C8-compounds produced in the lipoxygenase pathway. Twenty-nine new VOCs were identified for *T. arenaria*, from which the C8-compounds produced in the lipoxygenase pathway were predominant. Further analysis is required to understand the variations attributed to the specific internal and external factors and how these regulate fatty acid metabolism. In addition to the importance of defining *T. arenaria*'s aromatic profile, this metabolic pathway is also related to the umami taste, essential for the development of plant-based meat.

The Cyranose-320 e-nose accurately identified *T. arenaria* samples (especially when samples were incubated at RT before analysis) and was able to distinguish *T. arenaria* from other edible mushrooms and truffles (*A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum*). The Cyranose-320 e-nose was more accurate when mushroom and truffle samples were pre-incubated at RT than at 40 °C. Our data point the e-nose's great potential as a rapid and non-destructive detection tool for identifying *T. arenaria* and possibly other mushroom and truffle species. However, larger datasets (more samples and in different maturity stages) are necessary to fine-tune the parameter settings of the Cyranose-320 and optimize the identification process.

Altogether, *T. arenaria* is a nutritious and sustainable food that has the potential to be used in a variety of new food products. It is a good source of protein and minerals, and it has a Nutri-Score of A. *T. arenaria* could be used as a meat substitute or as an ingredient in plant-based meat products. Studying the nutritional composition and volatile profile of *T. arenaria* provides valuable insights into its potential as a nutritious food source. The application of the electronic nose technology enhances our ability to identify and authenticate the unique aroma profiles of these desert truffles. Moreover, this study unveils the e-nose's potential for the early detection of *T. arenaria* in the field, which could contribute to the sustainable production of this delicacy. The electronic nose could also be used to distinguish between maturity stages and quality of *T. arenaria*, which would be valuable for ensuring the quality of this food product. This knowledge contributes to advancements in food science and technology, supporting the development of quality control measures and ensuring the authenticity of food products in the market.

Supplementary Materials

The following supporting information can be consulted in Appendix 3:

Figure S3.1 - Map of the sampling areas of *Tefezia arenaria* collected during this study, and forest-dominant species per area.

Table S3.1 - Parameters settings for using the Cyranose-320;

Table S3.2 - Dietary Reference Intakes for nutrients and elements;

Table S3.3 - Comparison of the abundance of the main VOCs identified in *T. arenaria* and in the other edible mushroom and truffle species;

Table S3.4 - Characterization of the VOCs identified in *T. arenaria*, and in the other edible mushroom and truffle species (*A. bisporus*, *L. edodes*, *P. ostreatus* and *T. melanosporum*);

Table S3.5 - Results and rates of Cyranose-320 identification of *T. arenaria*, *A. bisporus*, *L. edodes*, P. ostreatus and *T. melanosporum* with 40 °C and RT pre-analysis incubation temperatures.

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CHAPTER 4

The Potential of Ectomycorrhizal Fungi to Modulate Below and Aboveground Communities may be Mediated by 1-octen-3-ol

This chapter is based in the following article:

Ferreira, I.; Dias, T.; Cruz, C. (2023) The Potential of Ectomycorrhizal Fungi to Modulate below and Aboveground Communities May Be Mediated by 1-Octen-3-Ol. *J. Fungi. 9*, 180, doi:10.3390/JOF9020180/S1

The Potential of Ectomycorrhizal Fungi to Modulate Below and Aboveground Communities may be Mediated by 1-octen-3-ol

Abstract

It is known that ectomycorrhizal (ECM) fungi can modulate below and aboveground communities. They are a key part of belowground communication as they produce a vast array of metabolites, including volatile organic compounds (VOCs), such as 1-octen-3-ol. Here, we tested if the VOC 1-octen-3-ol may be involved in the ECM fungal mechanisms that modulate below and aboveground communities. For that, we conducted three in vitro assays with ECM fungi and the 1-octen-3-ol volatile to (i) explore the effects of mycelium growth of three ECM species, (ii) investigate the impact on the germination of six host Cistaceae species, and (iii) study the impact on host plant traits. The effects of 1-octen-3-ol on mycelium growth of the three ECM species depended on the dose and species: *Boletus reticulatus* was the most sensitive species to the low (VOC) dose, while *Terfezia leptoderma* was the most tolerant. In general, the presence of the ECM fungi resulted in higher seed germination, while 1-octen-3-ol resulted in lower seed germination. The combined application of the ECM fungus and the volatile further inhibited seed germination, possibly due to the accumulation of 1-octen-3-ol above the plant species' threshold. Seed germination and plant development of Cistaceae species were influenced by ECM fungal volatiles, suggesting that 1-octen-3-ol may mediate changes in below and aboveground communities.

Keywords

1-octen-3-ol; C-8 volatiles; Fungal volatiles; Ectomycorrhizal fungi; *Terfezia*; Cistaceae

Introduction

Although it remains unclear when and how ectomycorrhizal (ECM) fungi mediate the direction and strength of feedback in plant communities, it is consensual that they can modulate below and aboveground communities (Nguyen et al. 2016). Several factors can contribute to explain the effects of ECM fungi on communities, namely the mediation of plant–soil feedbacks (Kadowaki et al. 2018) and of plant defences (Quintana-Rodriguez et al. 2018; Moisan et al. 2020), plant facilitation (Montesinos-Navarro et al. 2019) and communication with other soil microorganisms (Deveau et al. 2012; Duc et al. 2022).

Volatile organic compounds (VOCs) are a vital communication strategy among individuals of a species and of different kingdoms (Werner et al. 2016a). Among the most common fungal VOCs there are the eight carbon compounds (C-8), which result from the oxidation of linoleic acid (Combet et al. 2006). These compounds are also known as oxylipins, and play key roles in regulating fungal morphogenesis, plant interactions, and biocontrol (Contreras-Cornejo et al. 2022). Among these, 1-octen-3-ol also known as the "mushroom alcohol" due to its characteristic mushroom flavour (Combet et al. 2006; Xiong et al. 2017), is one of the most abundant VOCs produced by fungi (Quintana-Rodriguez et al. 2018), and is considered a signalling molecule, produced in different fungal structures, conidial masses, sporocarps and hyphae (Chitarra et al. 2004a, 2005).

The C-8 volatile oxylipin 1-octen-3-ol has an important role in fungal interactions, presenting a regulatory effect on the physiology and development of several fungi from different genera (Chitarra et al. 2004b). This compound can inhibit fungal growth and spore germination of *Botrytis cinerea* (Quintana-Rodriguez et al. 2018), *Penicillium paneum* (Chitarra et al. 2005), *Penicillium chrysogenum, Monilinia fructicola* (Wang et al. 2022), *Fusarium tricinctum* and *F. oxysporum* (Xiong et al. 2017). Furthermore, 1-Octen-3-ol also inhibits the growth of several bacterial species (e.g., *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, *S. epidermidis*) (Xiong et al. 2017), while in saprophytic species, such as *Agaricus bisporus*, this volatile is involved in bacterial stimuli for primordium formation (Noble et al. 2017).

Besides being involved in fungi-fungi and fungi-bacteria interactions, 1-octen-3-ol is also involved in fungi-plant interactions. For example, 1-octen-3-ol inhibits the fungal pathogen *Botrytis cinerea*, and contributes to enhance plant resistance by the induction of defence signalling cascades (Combet et al. 2006; Contreras-Cornejo et al. 2022). For instance, treating *Zea*

mays seeds with 100 µL of 1-Octen-3-ol in the form of the patented product (Omega™) resulted in season-long improvements in shoot and root growth, similar to those obtained when applying *Trichoderma* spp. (Harman and Uphoff 2019). Low concentration (0.05 µL/L) of this VOC also showed plant growth-promoting activity in Tomato plants (Kamaruzzaman et al. 2021). Therefore, 1-octen-3-ol can stimulate or inhibit the growth of neighbouring organisms (other fungi, bacteria and plants) in a dose-dependent manner (Schulz-Bohm et al. 2017).

1-Octen-3-ol is considered a biomarker characteristic for symbiotic fungi, being emitted in high doses by these fungi, while a low emission of this compound can be observed in non-symbiotic fungi (Guo et al. 2021). In agreement, and although the 1-octen-3-ol was first identified in ECM fungi in their fruitbodies (Dickschat 2017), mycelium (Splivallo et al. 2007a) and during ectomycorrhiza synthesis (Menotta et al. 2004), its effects on spores germination, mycelium growth and primordia initiation of these fungi have not yet been documented. Furthermore, the study of interactions between ECM fungal VOCs, such as 1-octen-3-ol, and their host plants have been insufficiently studied.

ECM fungi play an important role in the ecology of Mediterranean shrublands by helping plants to survive in such nutrient-poor soils that are common in these ecosystems (Ferreira et al. 2023a). In this biome, ECM fungi have been found to be associated with many plant species such as *Cistus*, *Quercus* and *Pinus* (Azul et al. 2010; Leonardi et al. 2020; Prieto-Rubio et al. 2022). *Cistus* sp. are frequent in Mediterranean Basin plant communities and are well adapted to the harsh conditions of Mediterranean shrublands (Correia and Ascensão 2017). Also, *Cistus* sp. have been found to form mycorrhizal relationships with various ECM fungal species, including those in the genus *Terfezia*, *Lactarius* and *Boletus* (Rinaldi et al. 2006; Águeda et al. 2006; Martins 2016; Hernández-Rodríguez et al. 2017; Albuquerque-Martins et al. 2019). Therefore, the present study aims to evaluate the effects of the fungal volatile 1-octen-3-ol in ECM fungi and their host plants in different stages of their interactions towards the symbiosis establishment. We hypothesized that 1-octen-3-ol has the potential to modulate below and aboveground communities, due to different thresholds of ECM fungal and plant species. For that we performed three *in vitro* assays to (i) explore the effects of 1-octen-3-ol on mycelium growth of three ECM species: *T. leptoderma*, *L. deliciosus* and *B. reticulatus*; (ii) investigate the impact of the 1-octen-3-ol and the two ECM species (*T. leptoderma* and *L. deliciosus)* on the germination of six host Cistaceae species: *Cistus albidus* L., *C. ladanifer* L., *C. salviifolius* L., *C. psilosepalus* Sweet, *Halimium halimifolium* (L.) Willk. and *Tuberaria guttata*

(L.) Fourr.); (iii) study the impact of the 1-octen-3-ol or ECM fungus (*T. arenaria*) on host plant *C. salviifolius* traits along nine months.

Materials and Methods

Fungal material

Mature *T. arenaria*, *T. leptoderma*, *L. deliciosus* and *B. reticulatus* sporocarps were harvested from different locations in Portugal (Alentejo and Leiria regions), during autumn and spring seasons of 2019. Specimens were freed from substrate debris at the site and further cleaned in the laboratory. The sporocarps were identified by their morphological macro- and microscopic characteristics, and following several authors (Bon et al. 1988; Llamas Frade and Alfonso 2005) and online keys (http://www.mycokey.com/) (MycoKey, 2023). In the case of the *Terfezia* species, due to the similarity between species, the samples were further identified by molecular analysis.

The mycelia of three ectomycorrhizal species (*T. leptoderma*, *L. deliciosus* and *B. reticulatus)* were isolated from sporocarps on potato dextrose agar (PDA) (VWR), pH 5.5. Cultures were incubated at 25 °C, transferred onto fresh medium every 3 months and maintained at 4 °C. Thereafter, the sporocarp samples were dried at 35 °C in a forced ventilation oven (Lab Companion, Model OF-11E) until constant mass. Dried fungal materials were powdered in a porcelain mortar and kept in brand-new sealed polyethylene bags under dry conditions. For sporal inoculum preparation, previously powdered, 2.4 g of dried *T. arenaria* ascocarps were added to 400 mL of sterile distilled water and left to shake overnight at 23 °C ± 1˚C in the dark before use. A 100 µL aliquot was taken to count the spore's concentration under the microscope, which was found to be $1x10^8$ of spores mL^{-1}.

Plant material

Seeds from *C. salviifolius* L., *C. psilosepalus* Sweet and *T. guttata* (L.) Fourr. plants growing in Leiria region (Portugal) were collected in September 2018 in a *Pinus pinaster* forest area with natural shrubs understory. The seeds were dried at room temperature and kept in the dark until use. Seeds of *C. albidus* L., *C. ladanifer* L. and *H. halimifolium* (L.) Willk. were purchased from Sementes de Portugal, and kept in the dark until use.

To break seed dormancy, seed scarification was performed by rubbing the seeds over a rough surface, (i.e., sandpaper), and then heating them at 105 °C in an oven (Lab Companion, Model OF-11E) for 10 min and left to cool down until room temperature. The seeds were hydrated for 10 min in sterile distilled water, surface sterilized by immersion in 30 % H_2O_2 for 30 min, washed three times with sterile distilled water, followed by another immersion in a 20 % bleach solution with 3 drops of Tween for 5–10 min. Afterwards, the seeds were washed three times in sterilized water. Seeds were then used in the germination tests.

VOC assays

To understand the potential of 1-octen-3-ol to modulate below and aboveground communities, we conducted three *in vitro* assays to test the effects of 1-octen-3-ol on the development of ECM fungal species and host plants, namely on ECM mycelium growth, Cistaceae species. seeds and in *C. salviifolius* traits.

On the following assays three doses of 1-octen-3-ol were tested: 0 µg (designated as CT); 0.17 µg (designated as VOC_low); and 1280 µg (designated as VOC_high). The doses were selected from the range reported in several studies using 1-octen-3-ol (Beltran-Garcia et al. 1997; Okull et al. 2003; Chitarra et al. 2004a; Splivallo et al. 2007c; Sawahata et al. 2008). The low dose we used took into consideration the positive results obtained in previous works, while the high dose was higher than all the doses reported.

Mycelium development

To evaluate the effect of the 1-octen-3-ol dose on the development of the mycelium of the three ECM fungi (*Terfezia leptoderma*, *Lactarius deliciosus* and *Boletus reticulatus)* we tested three doses per plate: 0 µg (CT); 0.17 µg (VOC_low); and 1280 µg (VOC_high). For that we used PDA (VWR) with pH 5.5 as the culture medium (without antibiotics addition). On the border of the Petri dishes (9 cm diameter) with the PDA medium, we added 10 µL of 1-octen-3-ol (VOC) in different doses.

The solution of 1-octen-3-ol at 1 µM was prepared using 1-octen-3-ol 98 % (Alfa Aesar) diluted in Chloroform 99 % (Merck), and sterilized by filtration (0.2 µm, 47 mm membrane filter, Minisart® NML, Sartorius). The mycelial plugs with 0.25 cm^2 of *T. leptoderma, L. deliciosus and B. reticulatus* were transferred from 1-month pure cultures (in PDA), and placed in the centre of the Petri dishes of each culture medium. Each treatment was replicated 10 times (N = 10 Petri dishes).

The ECM fungal cultures were placed in a growth chamber at 23 °C ± 1 °C in the dark. Colonies' growth was measured every 7 days for 63 days by measuring mycelia radial growth at the bottom of the Petri dish.

Effect of VOC and ECM fungi on Cistaceae species germination

To evaluate the effect of the 1-octen-3-ol (VOC) on the germination of plant hosts to the ECM fungi, we used six Cistaceae species (*C. albidus* L., *C. ladanifer* L., *C. salviifolius* L., *C. psilosepalus* Sweet, *H. halimifolium* (L.) Willk. and *T. guttata* (L.) Fourr.) and two ECM fungal species (*T, leptoderma* and *L. deliciosus*) whose growth was not affected by the low VOC dose. Given that high concentrations of 1-octen-3-ol can damage plants (Guo et al. 2021), we used the lower doses (i.e. 0 µg; 0.17 µg per plate). Four treatments were tested for each Cistaceae species., with five replicates (i.e. 9 cm Ø Petri dishes) for each species and treatment (Figure 4.1).

PDA pH 5.5 culture medium (without antibiotics addition) was used for the germination of Cistaceae seeds with ECM mycelium or the VOC. For all treatments, in each Petri dish we placed 4 seeds previously sterilized, scarified and heated to break dormancy (as previously explained in section 2.2). For the VOC treatment, on the centre of the Petri dish, we added 10 µL of 1 µM 1-octen-3-ol solution (0.17 µg dose per plate). In the CT were added only 10 µL of chloroform. For the ECM fungi treatments, mycelium plugs (0.25 cm^2) of *T. leptoderma* (Tlep) or *L. deliciosus* (Ldel), were placed in the centre of the Petri dishes. In the control (CT) Petri dishes, only the seeds were used (i.e., ECM fungi and VOC were absent). Five replicates of each treatment were prepared. The Petri dishes were placed in a growth chamber at 23 °C ± 1 °C in the dark, with 60 ± 5 % relative humidity and germination was evaluated after 35 days.

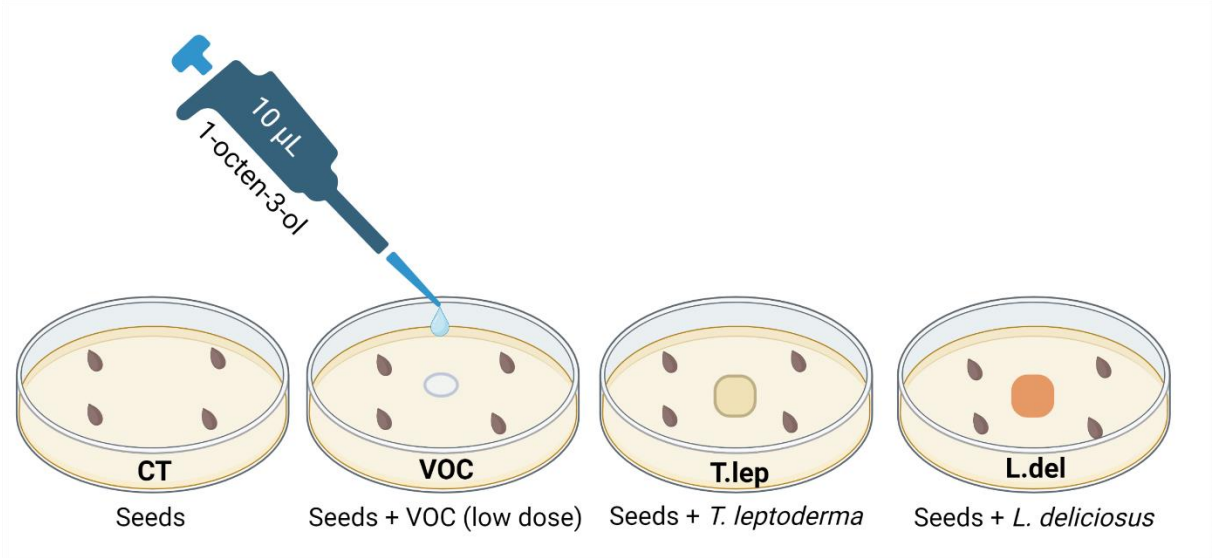

Figure 0.1. Experimental design to evaluate the effect of 1-octen-3-ol and ectomycorrhizal (ECM) mycelium on Cistaceae species germination (N = 5). Created with BioRender.com.

Effect of VOC and ECM on *C. salviifolius* germination and development

Although we used *T. leptoderma* in the previous assays, we did not have a viable spore inoculum to carry out this assay. Since both *T. arenaria* and *T. leptoderma* are characteristic of acid soils and are able to form *in vitro* mycorrhizal associations with *C. salviifolius* (Rinaldi et al. 2006; Louro et al. 2021), we used *T. arenaria* in this third assay; based on the tolerance of *T. leptoderma* and of *C. salviifolius* to the low VOC dose, we used *C. salviifolius* as the plant host and *T. arenaria* as the ECM fungus to evaluate the effect of the 1-octen-3-ol (VOC) during symbiosis establishment on host plant traits. The following four treatments were implemented: CT - Control treatment only with *C. salviifolius* seeds; VOC - Treatment with 10 µL of 1-octen-3-ol low dose (0.17 µg per plate); Ta - Treatment with 10 mL of *T. arenaria* sporal inoculum (10^8 mL^{-1}); TaVOC - Treatment with 10 µL of 1 µM 1-octen-3-ol solution (0.17 µg dose per plate) and 10 mL of *T. arenaria* sporal inoculum (10^8 mL^{-1}) (Figure 4.2).

Germination was performed in polypropylene transparent microboxes (90 mm Ø and 120 mm in height) with a cover without filters. Each box contained 100 mL of sterilized substrate (peat: perlite mixture, 3:1 v/v) and 50 mL of distilled water was autoclaved at 121 °C for 60 min. In a flow chamber, ten previously disinfected seeds (as described above) were placed in each container.

For the treatments with the ECM fungus (Ta and TaVOC), after the ten seeds were distributed in the microboxes, 10 mL of *Terfezia* sporal inoculum was distributed in each microbox,

of the two treatments with Terfezia. In the treatments with the VOC (VOC and TaVOC), 10 µL of 1-octen-3-ol 1 µM was sprayed inside each microbox. As control, we used microboxes only with *C. salviifolius* seeds (CT), and we were added only 10 µL of chloroform.

The microboxes (N = 18 per treatment) were kept in a growth chamber without direct light, and 16h dark/ 8h light photoperiod and 25 °C/ 20 °C (± 2 °C) day/night temperature. The bottom part of the microboxes, containing the substrate with the seeds and the spores, was covered with aluminium foil to decrease light incidence in this area. The number of germinated plants was counted every month for three months. In the third month, root samples from six microboxes per treatment were collected to confirm mycorrhization by microscopic characterization. At 6 and 9 months, six microboxes per treatment were selected randomly and the following plant traits were measured: shoot length, fresh shoot weight, root length, fresh root weight, number of branches per shoot and number of leaves per shoot. The root and shoot fresh weight were measured in and analytical scale (Radwag), with 0.0001 g resolution. The shoot and root length were measured with a ruler, and the number of number of branches and number of leaves were counted per shoot.

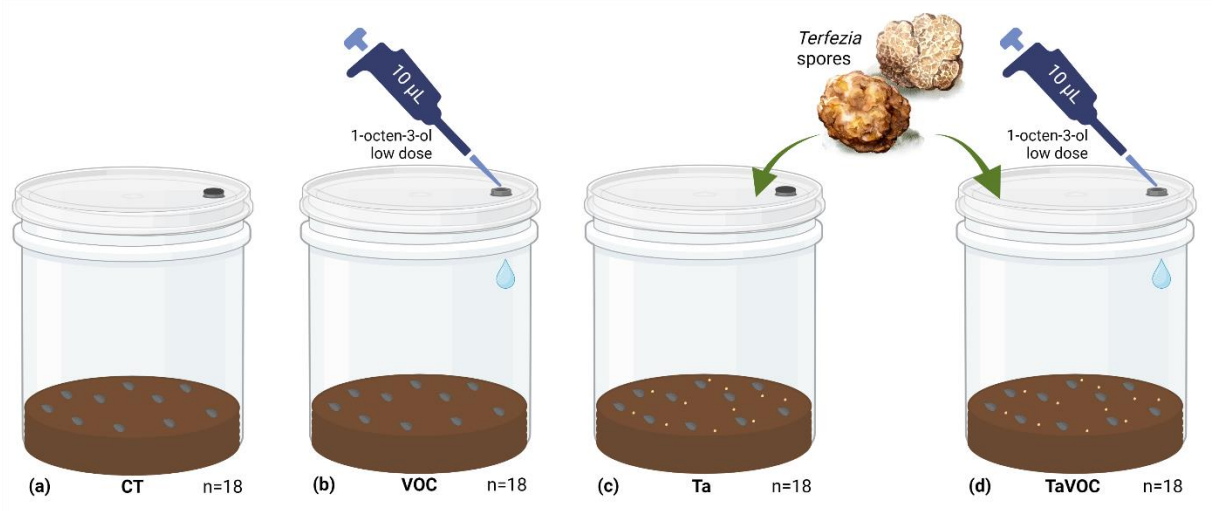

Figure 0.2. Experimental design to evaluate the effect of 1-octen-3-ol and *Terfezia* spores on *Cistus salviifolius* germination and plant traits. (a) Control treatment only with *C. salviifolius* seeds; (b) Treatment with 10 µL of 1-octen-3-ol low dose (c) Treatment with 10 mL of *Terfezia* sporal inoculum (10^8 mL^{-1}) (d) Treatment with 10 µL of 1-octen-3-ol low dose and 10 mL of *Terfezia* sporal inoculum (10^8 mL^{-1}). Ten seeds previously sterilized were distributed in each container (N = 18 per treatment). Created with BioRender.com.

Statistical Analysis

Statistical analysis was conducted using Microsoft Excel 2019/XLSTAT-Premium (Version 2021.4.1, Addinsoft, Inc., Brooklyn, NY, USA). Since our numerical variables did not follow a normal distribution, the Kruskal-Wallis test was selected. Differences between the treatments were compared using the Kruskal- Wallis one-way analysis of variance. Multiple pairwise comparisons were performed using the Dunn test ($p < 0.05$).

Results

Mycelium growth

Although no antibiotics were added to the PDA medium, contaminations were not observed on the pure cultures of the ECM fungi. While the high 1-octen-3-ol dose (VOC_high) fully inhibited the mycelium growth of all three ECM species, the low 1octen-3-ol dose (VOC_low) resulted in different responses in the ECM species (Figure 4.3). When compared to the control, the mycelium growth of *B. reticulatus* was inhibited (Figure 4.3a), that of *L. deliciosus* showed a non-significant tendency for inhibition (Figure 4.3b) and that of *T. leptoderma* was not affected (Figure 4.3c**)**. Therefore, the mycelium growth of these three ECM species showed a sensitivity gradient to the 1-octen-3-ol low dose (VOC_low) ranging from no effect to growth inhibition. From the tested ECM species, *B. reticulatus* was shown to be the most sensitive species to the low VOC dose, while *T. leptoderma* was the most tolerant.

Cistaceae species germination

The germination of the six Cistaceae species (*C. albidus*, *C. ladanifer*, *C. psilosepalus*, *C. salviifolius*, *H. halimifolium* and *T. guttata*) after 35 days in co-culture with *T. leptoderma*, *L. deliciosus* or VOC (lower 1-octen-3-ol dose) showed distinct responses to the volatile and ECM mycelium (Figure 4.4, Table S4.1). We observed seed germination for all Cistaceae species in the four treatments. Germination without the volatile and ECM mycelium (i.e., CT) did not result in higher germination rates for any of the Cistaceae species, and in *T. guttata,* it resulted in the lowest germination rate (40 %).

The low VOC dose had contrasting effects on the Cistaceae species germination. For half of the Cistaceae species tested (*C. albidus*, *C. psilosepalus* and *H. halimifolium*), the presence of the low VOC dose resulted in the lowest germination rates ranging from 20 % in *C.*

albidus and *C. psilosepalus*, to 30 % in *H. halimifolium*. In *C. ladanifer* and *T. guttata*, there was a non-significant tendency to stimulate germination, resulting in high germination rates for these species (60 % and 85 %, respectively). In *C. salviifolius*, germination was similar in all treatments, including the low VOC dose. Altogether, the tested Cistaceae species showed different sensitivities to the low 1-octen-3-ol dose.

Finally, the presence of the ECM fungi never had a negative effect on the Cistaceae species germination; it had no effect (e.g., *C. ladanifer* and *C. salviifolius*) or it stimulated germination (e.g., *C. albidus*, *C. psilosepalus* and *T. guttata* with Ldel, and *H. halimifolium* with Tlep).

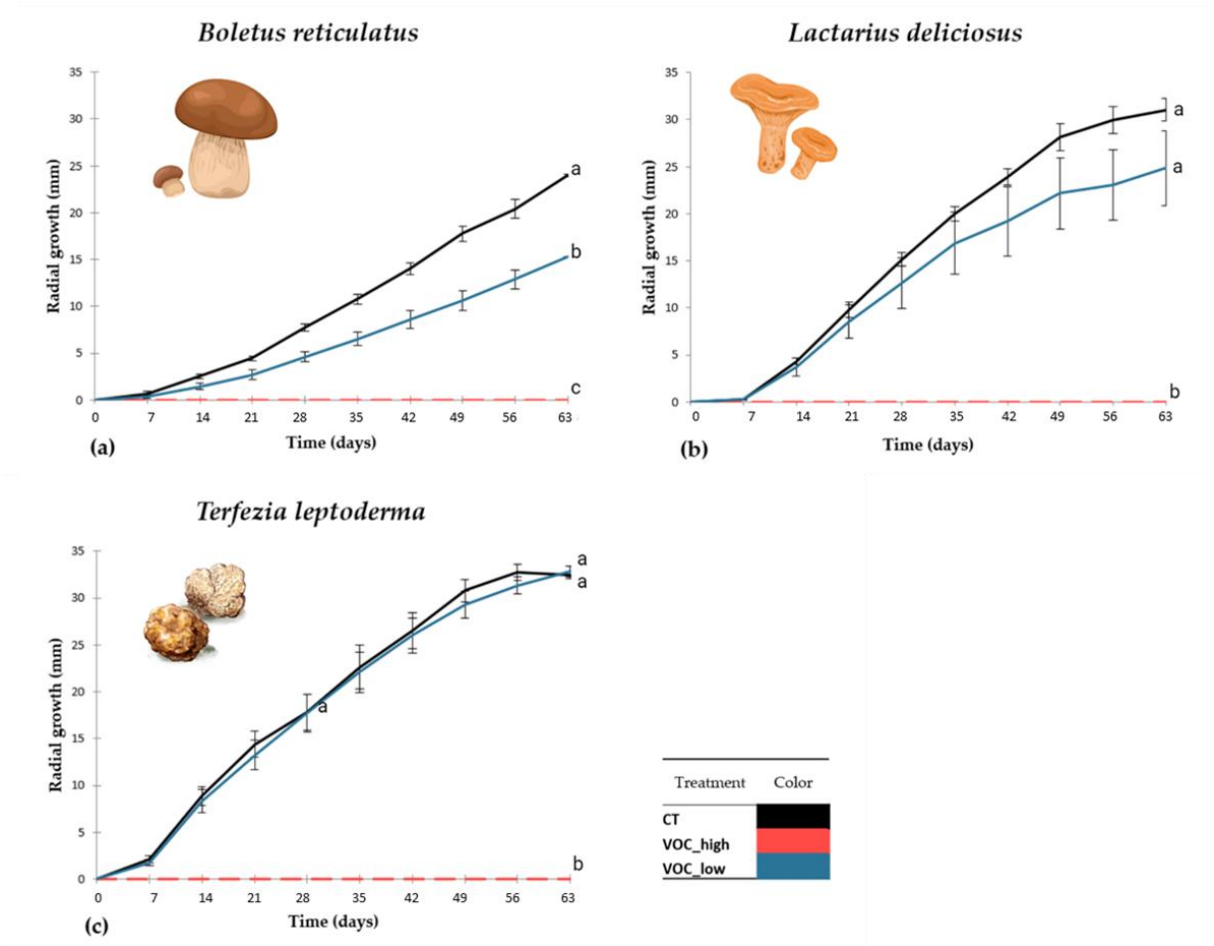

Figure 0.3 - Effects of the 1-octen-3-ol doses on mycelium growth of *Boletus reticulatus*, *Lactarius deliciosus* and *Terfezia leptoderma* (N = 10). (a) Mycelium growth of *B. reticulatus* (b) mycelium growth of *L. deliciosus* (c) mycelium growth of *T. leptoderma*, measured during 63 days. Treatments: CT - black line; VOC_high – red line; VOC_low – Blue line. Data were compared using a Kruskall–Wallis test. Post hoc comparisons were made using a Dunn's test, respectively. Data are mean ± standard error. Different letters show significant differences ($p < 0.05$) between treatments for each ectomycorrhizal (ECM) fungus. Created with XLSAT and BioRender.com.

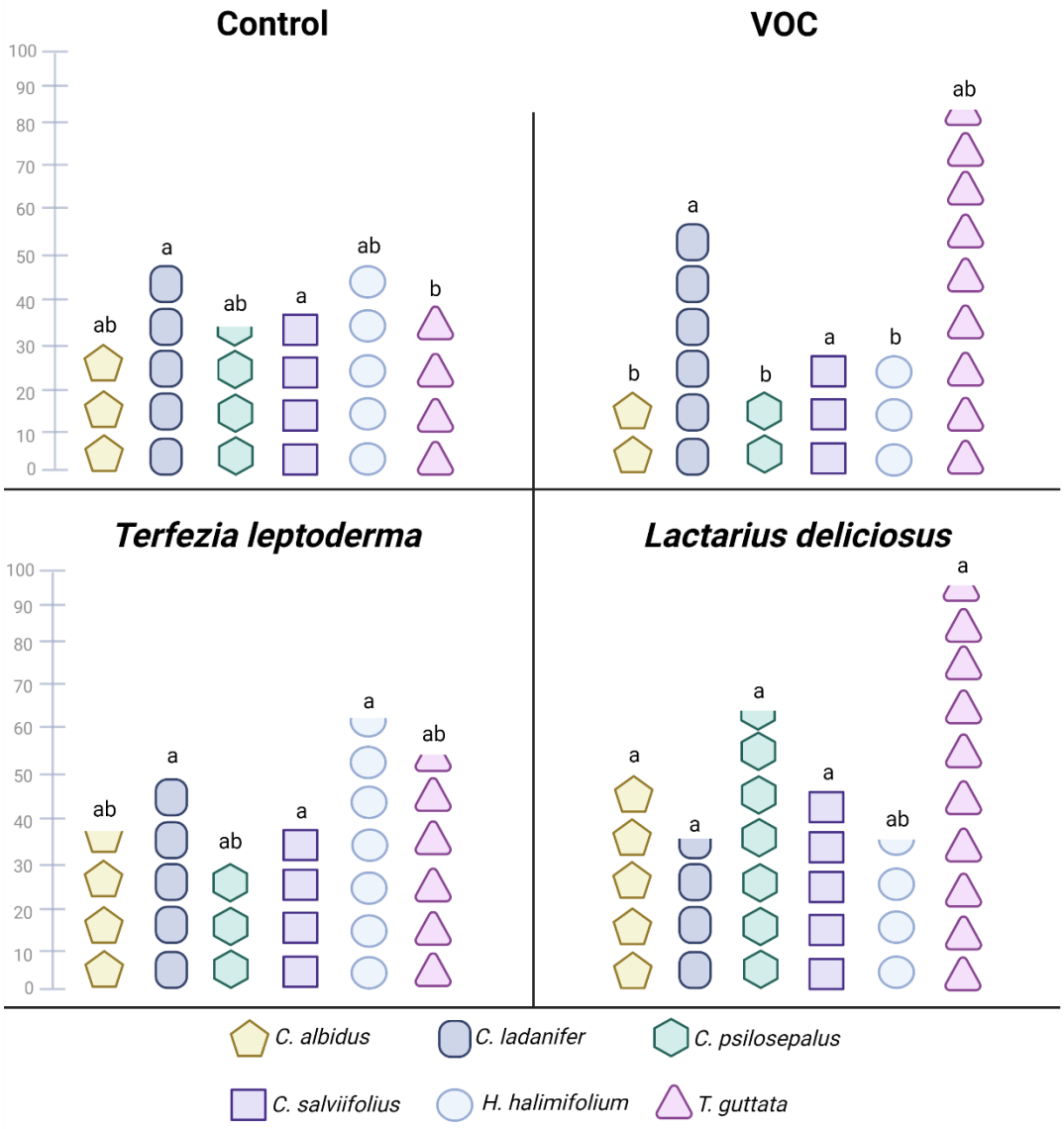

Figure 0.4 - Effects of 1-octen-3-ol 1 µM (VOC) and ectomycorrhizal (ECM) mycelium (*Terfezia leptoderma and L. deliciosus*) on Cistaceae germination rates (%, N = 5). Data were compared using a Kruskall–Wallis test. Post hoc comparisons were made using a Dunn's test, respectively. Data are means. Different letters show significant differences ($p < 0.05$) between treatments, for each Cistaceae species. Created with XLSTAT and BioRender.com.

Cistus salviifolius traits

Although there were no differences in *C. salviifolius* germination between the low 1-octen-3-ol dose (VOC) and the control (CT) after three months, the combined addition of the volatile with the ECM fungus (TaVOC) inhibited the germination by ~ 60 % (in relation to the CT). By contrast, adding the ECM fungus alone (Ta) stimulated *C. salviifolius* germination by ~ 35 % (in relation to the CT) (Figure 4.5).

From the analysis of the root samples in the third month, for the treatments with T. arenaria inoculum, mycorrhizal structures were detected in the initial stage. From the six *C.*

salviifolius traits evaluated six and nine months after the beginning of the assay, only the number of lateral shoots and the number of leaves showed differences between the treatments (Table 4.1). At month six, the combined addition of the volatile with the ECM fungus (TaVOC) stimulated the number of lateral shoots ($p < 0.05$), and no lateral shoots could be observed in the CT plants. Still at month six, the addition of the volatile (VOC) or the ECM fungus (Ta) resulted in lower numbers of lateral shoots, similar to the CT.

Nine months after the beginning of the assay, TaVOC still resulted in the highest number of lateral shoots, and so did Ta, and both were higher than the CT ($p < 0.05$). The number of leaves, which was similar for all treatments at month six, showed differences at month nine: Ta and TaVOC plants had more leaves that the CT plants ($p < 0.05$). Also, for the shoot and root fresh weight, there was a tendency for the TaVOC plants to show higher values.

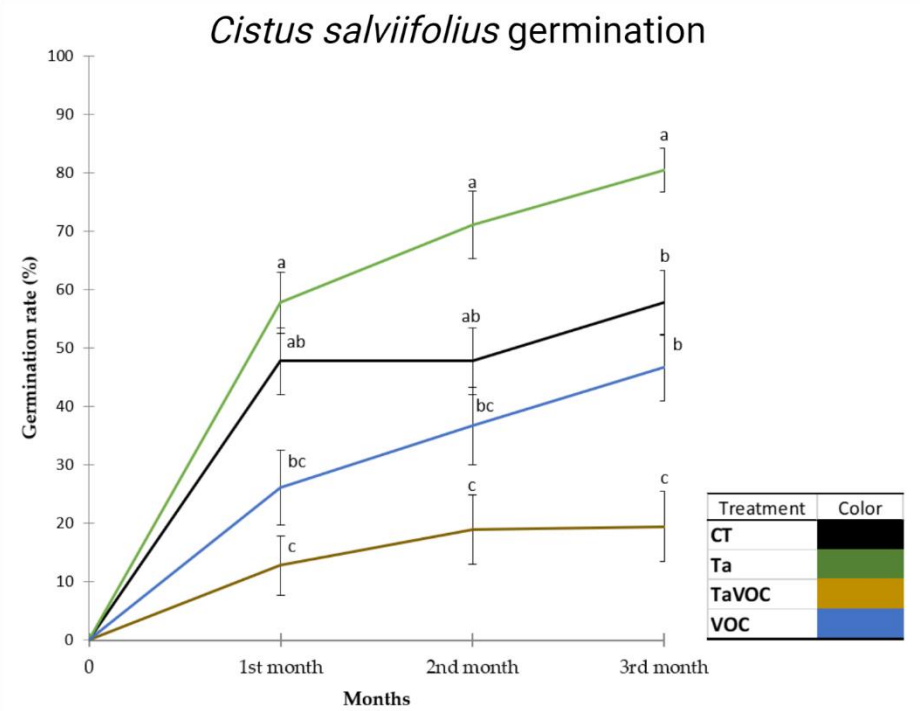

Figure 0.5*Cistus salviifolius* seeds germination at the during three months, showing the effect of *Terfezia arenaria* inoculum and 1-octen-3-ol on *C. salviifolius* (N = 18 per treatment). Treatments: **CT** – control; **VOC** - 1-octen-3-ol 1 µM; **Ta** – *T. arenaria* inoculum; **TaVOC** – *T. arenaria* inoculum and 1-octen-3-ol 1 µM. Data were compared using a Kruskall–Wallis test. Post hoc comparisons were made using a Dunn's test, respectively. Data are mean ± standard error. Different letters show significant differences ($p < 0.05$) between treatments. Created with XLSAT and BioRender.com.

Table 0.1 - Effects of *Terfezia arenaria* inoculum and 1-octen-3-ol on *C. salviifolius* plant traits: shoot length (**SL**), shoot weight (**SW**), root length (**RL**), root weight (**RW**), number of lateral shoots, number of leaves, observed 6 and 9 months after the inoculation. (N = 6 per treatment). Treatments: **CT** – control; **VOC** - 1-octen-3-ol 1 µM; **Ta** – *T. arenaria* inoculum; **TaVOC** – *T. arenaria* inoculum and 1-octen-3-ol 1 µM.

		6 Months			9 Months		
Trait	**Treatment**	**Mean**	**SD**		**Mean**	**SD**	
Shoot length(cm)	CT	4.34	± 3.14		8.91	± 4.09	
	Ta	5.63	± 3.26		10.38	± 4.33	
	TaVOC	4.83	± 3.04		11.40	± 4.69	
	VOC	4.35	± 2.61		7.54	± 4.29	
Root length (cm)	CT	6.11	± 3.31		9.28	± 4.13	
	Ta	4.83	± 2.25		7.23	± 2.88	
	TaVOC	3.81	± 1.74		6.94	± 2.47	
	VOC	4.75	± 3.22		5.94	± 2.20	
Shoot fresh weight (mg)	CT	52.11	± 46.14		99.75	± 74.21	
	Ta	86.35	± 84.16		146.93	± 144.51	
	TaVOC	149.33	± 129.22		309.02	± 245.66	
	VOC	53.80	± 45.01		89.45	± 82.70	
Root fresh weight (mg)	CT	16.84	± 14.76		42.35	± 40.05	
	Ta	21.65	± 20.46		46.16	± 43.21	
	TaVOC	32.35	± 31.13		58.74	± 34.40	
	VOC	12.02	± 13.01		44.85	± 40.45	
Branches (Number per shoott)	CT	0.00	± 0.00	b	0.81	± 0.61	B
	Ta	0.32	± 0.07	b	4.05	± 3.52	A
	TaVOC	1.57	± 0.61	a	5.00	± 4.90	A
	VOC	0.38	± 0.09	b	1.05	± 0.74	a,b
Leaves (Number per shoot)	CT	10.90	± 5.58	a	11.39	± 5.96	B
	Ta	13.71	± 5.05	a	28.10	± 19.35	A
	TaVOC	13.55	± 5.97	a	49.43	± 39.12	A
	VOC	12.24	± 5.64	a	13.24	± 4.88	a,b

Data were compared using a Kruskall–Wallis test. Post hoc comparisons were made using a Dunn's test.
Data are mean ± standard deviation (SD). Different letters show significant differences ($p < 0.05$) between treatments.

Discussion

The lower dose of 1-octen-3-ol inhibited the mycelium growth of *B. reticulatus* but not of *L. delicious* and *T. leptoderma*. This may reflect distinct responses of ECM species to 1-octen-3-ol, where ECM species may differ in their thresholds to the volatile. So, the 1-octen-3-ol produced by soil microorganisms, including ECM fungi, by interfering in fungal growth may modulate the structure and composition of ECM fungal communities belowground. ECM fungi produce 1-octen-3-ol mainly on their fruitbodies, an area with high number of spores, similarly to what occurs in the conidial masses of microfungi (Chitarra et al. 2004a). It is known that 1-octen-3-ol can inhibit fungal growth and spore germination of several microfungi (Chitarra et al. 2004a; Xiong et al. 2017; Quintana-Rodriguez et al. 2018; Wang et al. 2022). For example, in *Penicillium paneum* 1-octen-3-ol is a self-inhibitor volatile that blocks the germination process, and in *Monilinia fructicola* 1-octen-3-ol destroyed the hyphae morphology and cell structure (Wang et al. 2022). It is suggested by Chitarra and colleagues (Chitarra et al. 2004a), that

this C-8 volatile has a common function as an inhibitor of premature spore germination. In microfungi, spores and conidial masses are directly exposed to the air, so a volatile produced in the fruitbody or by conidia could be a more efficient self-inhibitor compound of the germination until more appropriate environmental conditions prevail (Chitarra et al. 2004a). Moreover, in *Agaricus bisporus* it is an effective autoregulator of fruiting body development (Kües et al. 2018).

Our experimental design did not allow to distinguish between a self-inhibitory behaviour or a lower threshold, but clearly showed *B. reticulatus* is more sensitive to 1-octen-3-ol than *L. deliciosus* and *T. leptoderma*. The effects of 1-octen-3-ol on mycorrhizal sporocarps have been studied to a lesser extent compared to other fungal gilds. However, a recent study showed that VOCs can act as an attractant for certain insects which is important for spores' dispersal and spore germination of some mycorrhizal fungi (Vašutová et al. 2019). Also, 1-octen-3-ol was identified during *T. borchii–Tilia americana* symbiosis process (Menotta et al. 2004), showing that this and other VOCs can have a role on symbiosis communication. Three VOCs (1-pentanol, 2,3-dimethyldecane, and *p*-isopropylbenzaldehyde) were found to be involved in pre-symbiotic communication between *Populus* and the ECM fungi *Laccaria bicolor* (Ditengou et al. 2015). Moreover, this ECM fungi produces a sesquiterpene (thujopsene) that increases lateral root formation and root hair length in the pre-symbiotic phase, facilitating mycorrhizal establishment (Ditengou et al. 2015).

The fact that we observed seed germination in all treatments for all Cistaceae species showed that the seeds were viable. In general, the 1-octen-3-ol and the ECM fungi resulted in contrasting responses on Cistaceae plant species germination. Although ECM fungi can produce the volatile, the positive effect of the ECM fungi on Cistaceae species germination goes beyond the production of the 1-octen-3-ol as ECM fungal exudates can also promote seed germination (Leake et al. 2004; Mahmoudi et al. 2021). Regardless of whether there is mycorrhization or not, the activity and growth of ECM fungi in the soil produces exudates, including VOCs. What our data suggest is that they may have different effects. However, it is also worth noting that more research is needed to understand how fungal exudates and VOCs affect host plants.

On the other hand, the negative effect of 1-octen-3-ol on some Cistaceae species germination may reflect different species-specific sensitivities to the volatile. Inhibitory effects on germination and seedling and vegetative development were reported for *Arabidopsis thaliana*

(Splivallo et al. 2007c; Hung et al. 2014a; Lee et al. 2019) and *Cistus incanus* (Splivallo et al. 2007c). In both plant species, the exposure to low concentrations of 1-octen-3-ol reduced seed germination, and in the seedlings led to inhibition of root growth and cotyledon bleaching as a result of H_2O_2 production (Splivallo et al. 2007c; Lee et al. 2019). Furthermore, in *A. thaliana* it was observed that even the seeds that were exposed to low 1-octen-3-ol doses were able to germinate when the volatile was removed (Splivallo et al. 2007c). This shows that the volatile does not damage the seeds permanently, its presence is required to inhibit germination.

Although the application of the volatile or the ECM fungi had no effect on *C. salvifolius'* germination, their combined application inhibited seed germination. This contrasting response may reflect the presence of two sources of 1-octen-3-ol (i.e., the added volatile solution and ECM mycelium) that together exceeded this plant species' threshold. In agreement, inhibitory effects on germination and seedling and vegetative development of *Arabidopsis thaliana* and *C. incanus* were after exposing the plants to both the synthetic volatiles and to 1 g of truffle (Splivallo et al. 2007c).

Our data showed that 1-octen-3-ol, of external or biogenic origin, was able to differently inhibit or stimulate ECM fungi and seed germination, which constitutes evidence for a potential ecological mechanism capable of changing below- and aboveground communities (Figure 4.6). In agreement, *C. incanus* (a truffle host plant of *Tuber* species) can be inhibited by the volatiles produced by its own symbiont. The truffle hyphae produces 1-octen-3-ol and other volatiles (2-phenylethanol, 3-methyl-1-butanol, 1-hexanol, 3-octanol, 3-octanone, and trans-2-octenal) that are considered phytotoxic due their ability to harm plants (Splivallo et al. 2007b, c). They are key to the formation of so called "burnt areas" in truffle orchards, an area with no, or limited vegetation around the mycorrhizal plant or tree (Werner et al. 2016b). These volatile organic compounds were also reported in *Terfezia* species (Farag et al. 2021b).

Low concentrations of this volatile can also have positive effects. For example, at a low concentration (0.05µL/L) 1 octen-3-ol showed plant growth-promoting activity in Tomato plants, by enhancing plant height, basal stem diameter, root number, fresh weight and dry weight (Kamaruzzaman et al. 2021). Also in *A. thaliana*, 1-octen-3-ol improves resistance of mature plants to *Botrytis cinerea* by the activating defence genes (Kishimoto et al. 2007; Hung et al. 2014b).

As discussed, VOCs, namely 1-octen-3-ol, exert several effects, including growth suppression or induction in both plants and fungi (Splivallo et al. 2007b, c), induction of defensive

behaviours (Combet et al. 2006; Contreras-Cornejo et al. 2022), and inhibition of spore (Chitarra et al. 2004a; Xiong et al. 2017; Quintana-Rodriguez et al. 2018; Wang et al. 2022) and seed germination (Splivallo et al. 2007c). The results we obtained suggest that these ECM fungi could modulate below- and aboveground communities. Figure 4.6 integrates the results obtained in the first two assays in a conceptual model about the putative effects of 1-octen-3-ol on the ECM fungi and Cistaceae community (below and above communities) in a Mediterranean shrubland. The application of 1-octen-3-ol influences the relative abundance of ECM fungi and of the host (Cistaceae) species in a species-specific way. Further, from our results we conclude that the interplay between ECM fungi and host plants is much more complex than just a communication based on 1-octen-3-ol. Since the relative abundance of host plants tends to decrease in the presence of the volatile, while it increases in presence of ECM fungi, our results clearly show that 1-octen-3-ol has a role in modulating below and aboveground communities. But we are still at the beginning of the path leading to the understanding of the complete mechanism involved in this co-regulation.

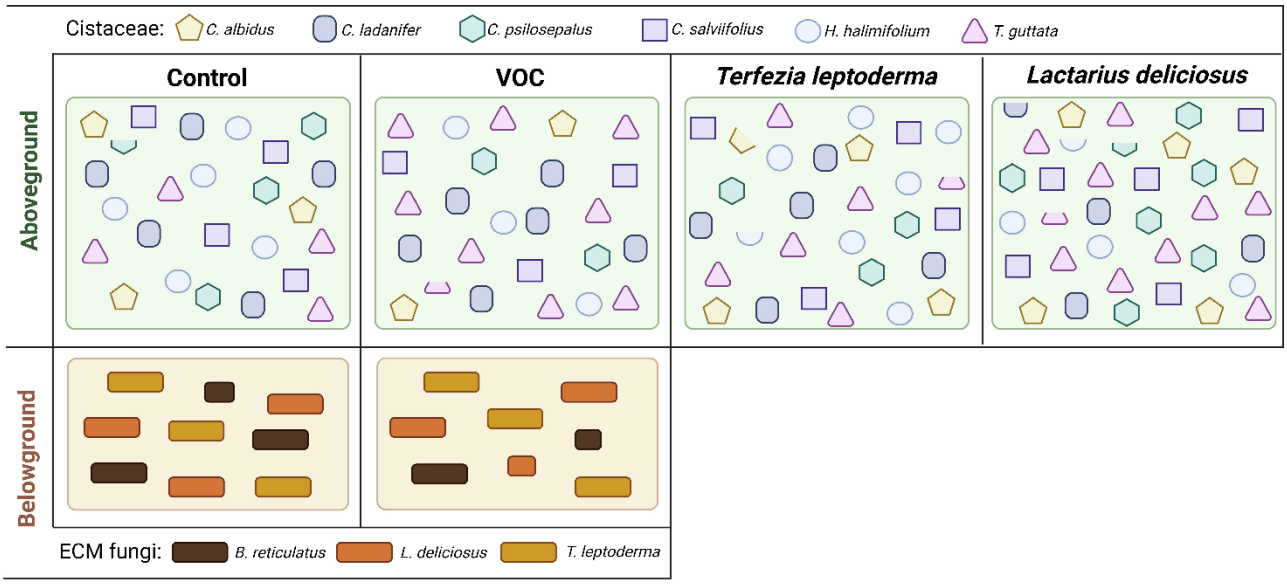

Figure 0.6 - Illustration of the effects of 1-octen-3-ol, of external or biogenic origin, on below- and aboveground communities, namely on the ectomycorrhizal (ECM) mycelial growth and Cistaceae species germination. Created with BioRender.com.

Conclusions

As hypothesized, both the ECM fungal species and the Cistaceae species showed different thresholds to the low 1-octen-3-ol dose. Seed germination and plant development of Cistaceae species were influenced by ECM fungal volatiles, such as 1-octen-3-ol. Like previous studies, we observed that a low dose of this volatile inhibited or delayed seed germination. This inhibition could be part of the ECM fungal life cycle regulation, namely during fruiting season. During the development of the fruitbody the fungus produces volatiles, such as 1-octen-3-ol, that lead to the formation of "burnt areas". In these areas the germination of host and other plants are inhibited or delayed, which allows the mycelium to spread in the soil and creates space for the fructification of the fungus. Further research is needed to understand the mechanisms underlying this phenomenon, and how ECM fungal volatiles influence their plant host development and below- and aboveground communities.

Supplementary Materials

The following supporting information can be consulted in Appendix 4:

Table S4.1 - Effects of 1-octen-3-ol 1 μM (VOC) and ECM mycelium on Cistaceae germination rates (%, n = 5). Treatments: **Tlep** – *T. leptoderma*; **Ldel** – *L. deliciosus*; **VOC** - 1-octen-3-ol 1 μM; **CT** – control.

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CHAPTER 5

Conclusions and Future Perspectives

Conclusions and Future Perspectives

This thesis explored different aspects of ectomycorrhizal fungi (ECMF) research, focusing on the case of the *Terfezia* species, where three main elements were addressed: sustainable production, species identification and ecological role. The conclusions were integrated to maximize the impact of scientific achievements on society, predicting new sustainable strategies for managing forest ecosystems, food products and technological applications (Figure 5.1).

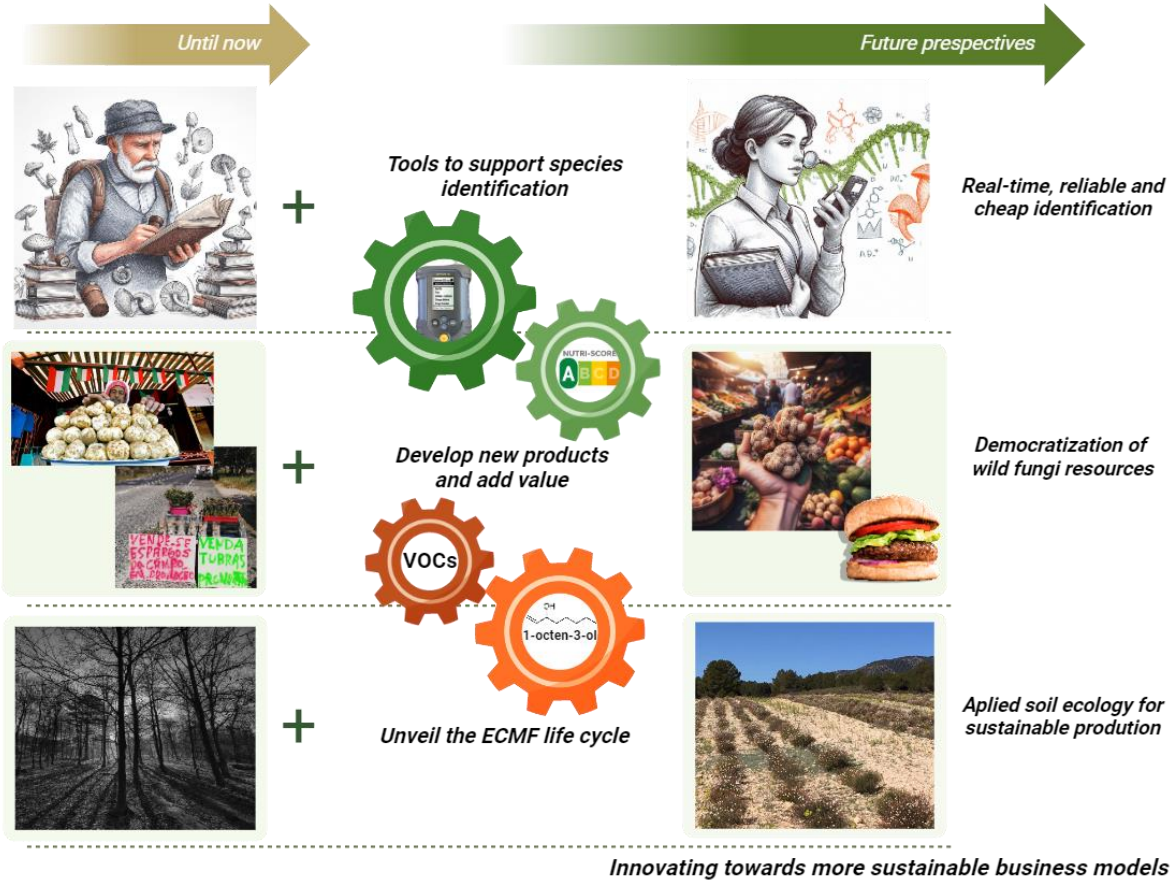

Figure 0.1 - Schematic representation of the main achievements of this thesis and future applications. Created with BioRender.com.

The case of desert truffles, namely *Terfezia* truffles, is a notable example of successful ECMF cultivation. The high number of fungal species, ECFM and non-ECMF, found in the forest can open up a world of opportunities but also bring uncertainty. Some rural communities derive significant seasonal income from mushroom harvesting. However, this activity has become popular among inexperienced enthusiasts who may have difficulty to accurately identify harvested species. Correct identification of ECMF sporocarps and other fungal species

is essential for safe and sustainable management, cultivation, and post-harvest application. However, many fungal species are challenging to identify by sporocarp morphology alone. In this context, the need for efficient and low-cost methods that offer reliability in distinguishing between different species of fungi, including potentially harmful ones, stands out.

While traditional knowledge of wild mushrooms may have diminished over time, our contemporary society benefits from rapid and widespread access to a wealth of online information. Although more information is available, there is still a feeling of insecurity since people are aware of the dangers of incorrectly picking mushrooms.

This research highlights the significant promise of combining an electronic nose with discriminant analysis to distinguish wild fungal species (mushrooms and truffles) and assess their edibility based on their aromatic profiles. Although further expansion of the data set is essential to establish a rapid and reliable identification method, the results indicate that this approach has the potential to match the accuracy of identifications performed by mycologists and molecular biology experts. Crucially, it has the benefit of requiring less technical training while offering a cheaper and faster analytical alternative. The electronic nose has shown the potential for mushroom identification. It can add value to the forest's biological resources and reinforce consumer confidence in this product. However, the electronic nose is still an expensive device and not affordable to the everyday consumer. Initially, its use should be centralized in regional distribution centres, mycological associations, or official control services. Nonetheless, with the rapid technological development, it will be possible for this technology to be within reach of the general population. This would be essential for the global democratisation of mycological resources, making wild fungi identification safer and more accessible. Moreover, it will contribute to access to new food resources and products.

The harvesting and production of ECMF sporocarps has long had a high economic, cultural, and environmental impact in the Mediterranean region. Desert truffles are an example of a traditional delicacy brought into the future by new cultivation technologies. *Terfezia* species are well known in the region; however, some have not yet been evaluated. *T. arenaria* is a truffle, which proves to be a balanced food, rich in carbohydrates, fiber, proteins, and some minerals, but with low-fat content and a unique aroma dominated by C-8 compounds produced in the lipoxygenase pathway. The characteristic volatile profile of *T. arenaria* can contribute to its identification through the aromas produced and the umami flavour, which is essential for developing plant-based meat products.

Plant-based meat alternatives from fungi are usually presented as meat-mushroom blends or mushroom protein meat analogues. They can be produced from the edible sporocarp or mycelium. The sporocarp is more accessible for developing these products due to product texture, taste, and flavour, while mycelium-based products are still far from the real meat characteristics. Also, mycelium needs more technological processes to produce, which is expensive, and additional research is required to optimize this type of product. In the case of wild fungi, the production of sporocarps is seasonal, so it would be interesting to explore the mycelium as an alternative to produce plant-based meat products.

T. arenaria is a valuable food resource with a distinct aroma and a nutritional composition similar to meat, making it an excellent candidate for use in plant-based meat products. Moreover, an electronic nose further confirmed the distinct aroma of *T. arenaria*. These findings enhance our ability to identify and authenticate the unique aroma profiles of these desert truffles. Additionally, this research reveals the potential of using an electronic nose for early field detection of *T. arenaria*, which could contribute to their sustainable production and ensure the maintenance of its quality and authenticity as a delicacy.

Furthermore, the electronic nose exhibits the potential to distinguish between different fungi species. This tool could support sporocarp species identification in cases when the morphological features of wild fungi do not provide definitive results. On the other hand, the electronic nose could also distinguish between maturity stages and the quality of edible fungi, such as *T. arenaria*. The electronic nose, a fast, cheap, and reliable method, can significantly contribute to the progress of mycology, facilitating the establishment of robust quality control protocols and assuring the authenticity of these food products in the market.

ECMFs are not only an important food resource, they also play a vital role in forest ecosystems. These fungi help to promote tree growth, nutrient cycling, plant defence and carbon sequestration. They are indirectly crucial to helping mitigate climate change by helping to improve the resilience of forests to drought and other climate stressors.

Volatile organic compounds function as communication strategy tools among individuals of a species and different kingdoms. They contribute to the communication between ectomycorrhizal fungi and their hosts, other microorganisms in the soil, animals and even humans via their aroma. The 1-octen-3-ol is a signalling molecule and one of the most abundant VOCs produced by fungi. 1-Octen-3-ol is the main compound responsible for the characteristic “mushroom aroma”. Smell and flavour are responsible for consumer product appreciation and

influence a species' social and economic impact (e.g. Boletus, Chanterelles, Desert truffles). This volatile is present with high concentrations in the sporocarps of several fungi, including *T. arenaria*, and is involved in the mechanisms employed by ECMF influencing both belowground and aboveground communities.

1-octen-3-ol may play a pivotal role in mediating alterations in both belowground and aboveground communities in the context of ECMF interactions. It will also be interesting to verify if 1-octen-3-ol can be an indicator of fungal fructification. The inhibition of seed germination and the different thresholds of mycelium development might be part of the ECMF life cycle regulation, particularly during the fruiting season (sporocarp production). During the sporocarp development, the ECMF produces volatiles, such as 1-octen-3-ol, that trigger the formation of "burnt areas". In these areas, plant germination is either inhibited or delayed, allowing the fungal mycelium to spread through the soil and create space for the sporocarp to grow. On the other hand, the presence of 1-octen-3-ol could also act as a chemical indicator that could be detected by technologies such as the electronic nose. Using the electronic nose to detect the presence of this volatile on the soil, hypogeous fungi such as *T. arenaria* could be detected at an early stage. *Terfezia* species are hypogeous until near maturity when they expand and partially emerge from the soil, so these indicators would allow for production to be monitored and expedite harvesting.

Aware that this study is a starting point in developing novel tools and criteria to promote the sustainable cultivation of ectomycorrhizal fungi, namely *Terfezia* species; the development of new technologies to support the identification of wild fungi accurately and understand the mechanisms of how these fungi interact with above and belowground communities is essential to achieve further sustainable management and cultivation practices. Also, exploring these fungi's nutritional and biological proprieties is crucial for creating sustainable nature-based solutions (food and medicine) that contribute to rural communities' development and make these resources accessible to everyone.

APPENDICES

Appendix 1 - Supplementary Material of Chapter 1

Appendix 2 - Supplementary Material of Chapter 2

Appendix 3 - Supplementary Material of Chapter 3

Appendix 4 - Supplementary Material of Chapter 4

Appendix 1

Supplementary Material of Chapter 1

Table S1.1 - Studies and predictive models of climatic and forest management variables influencing natural production of macrofungi mushrooms. (*Continue*)

Country /Region		Forest type	Fungi guild	C	Ld	Age	Pc	DC	rTF	TCC	Cund	Reg	Others	Reference
Finland	Karelia, Savonia	*Picea abies*	Myc	+	-	+	+	+	-	+	-	-	-	Tahvanainen et al. 2016
Finland, Spain, and Switzerland		Mixed stands	Myc +Sap	+	-	-	+	-	+	-	-	-	-	Collado et al. 2019
France	Burgudy	Mixed stands	Myc +Sap	+	+	-	-	-	-	-	-	-	Fertilization; Tree composition	Buée et al. 2011
Italy	Tuscany	*Albies alba* *Picea abies*	Myc	+	-	-	+	+	-	+	+	-	-	Salerni and Perini 2004
Norway	Oslo	Norwegian Mycology Database	Myc + Sap	+	-	-	-	-	-	-	-	-	Time of fruiting	Kauserud et al. 2008
Portugal	Évora	Montado	Myc	-	-	-	-	+	-	-	+	-	-	Santos-Silva and Louro 2016
		Montado	Myc +Sap	-	-	-	-	+	-	-	-	-	-	Santos-Silva et al. 2011
Slovenia	Southern and western	*Fagus sylvatica* *Albies alba* *Picea abies*	Myc	-	-	-	-	-	+	-	-	-	-	de Groot et al. 2016
Spain	Catalonia	Pinus stands	Myc + Sap	+	+	-	+	-	+	-	-	-	-	Primicia et al. 2016
		Pinus stands	Myc + Sap	+	+	+	+	+	+	-	-	-	-	Martínez de Aragón et al. 2007
		Pinus stands	Myc	+	+	+	-	+	-	-	-	-	-	de-Miguel et al. 2014
		Pinus pinaster	Myc + Sap	-	+	-	-	+	-	+	-	-	-	Collado et al. 2020
		Pinus pinaster	Myc + Sap	+	+	-	-	-	+	+	-	-	-	Collado et al. 2018
		Pinus pinaster	Myc	+	+	-	-	-	-	+	-	-	-	Bonet et al. 2012
		Pinus pinaster	Myc + Soil	+	+	-	-	-	-	+	-	-	-	Castaño et al. 2018

Table S1.1 - Studies and predictive models of climatic and forest management variables influencing natural production of macrofungi mushrooms. (*Continue*)

Country /Region		Forest type	Fungi guild	C	Ld	Age	Pc	DC	rTF	TCC	Cund	Reg	Others	Reference
Spain	Castilla y León	*Cistus ladanifer*	Myc + Sap	+	-	+	-	+	-	+	-	-	Fire	Hernández-Rodríguez et al. 2015b
		Cistus ladanifer	Myc + Sap	+	-	-	+	+	-	+	-	-	Fire	Hernández-Rodríguez et al. 2015a
		Pinus pinaster	Myc + Sap	-	-	+	-	+	-	-	-	+	-	Ágreda et al. 2014
		Pinus pinaster	Myc + Sap	+	+	-	-	-	-	-	-	+	Mycorrhizal plants	Taye et al. 2016
		Pinus sylvestris	Myc	+	+	-	+	+	-	-	-	-	-	Martínez-Peña et al. 2012a
		Pinus sylvestris	Myc	-	-	+	-	+	-	-	-	-	-	Martínez-Peña et al. 2012c
		Pinus sylvestris	Myc	-	-	+	-	-	-	-	-	-	-	Ortega-Martínez et al. 2011
		Pinus pinaster *Pinus sylvestris*	Myc + Sap	+	-	-	-	-	+	-	-	-	Remote sensing	Olano et al. 2020
	Central Pyrenees	*Pinus sylvestris*	Myc	-	+	+	-	+	-	-	-	-	-	Bonet et al. 2008
Switzerland	South-western	Mixed stands	Myc	-	-	-	-	-	+	+	-	-	-	Egli et al. 2010

The symbols + and – indicate the variables considered in each study. +: considered; –: not considered. C: Climate; Ld.: Landscape and/or site characteristics; Pc: Plant characteristics; DC: Density and canopy; rTF host tree relation with ECMF; TCC: Thinning, clearing and cutting; Cund: control understory; Reg: Regeneration. Myc: Mycorrhizal; Sap: Saprophytic; Soil: Soil fungal communities.

Table S1.2 - *Terfezia* species identified in the Iberian Peninsula.

Species	Soil	Host species	Geographic distribution	References
Terfezia albida	Alkaline	*Helianthemum spp.*	Spain	Bordallo et al. 2013; Kovács et al. 2011
Terfezia alsheikhii	Acid	*Helianthemum salicifolium* and *Tuberaria lignosa*	Spain	Bordallo et al. 2013; Kovács et al. 2011;
Terfezia arenaria	Acid	*Helianthemum spp.* and *Tuberaria guttata*	Portugal and Spain	Alsheikh 1994; Díez et al. 2002; Kovács et al. 2011
Terfezia boudieri	Alkaline	*Helianthemum spp.*	Spain	Alsheikh 1994; Díez et al. 2002
Terfezia cistophila	Acid	*Cistaceae*	Portugal and Spain	Bordallo et al. 2015; Louro et al. 2020b; Santos-Silva et al. 2021
Terfezia claveryi	Alkaline	*Helianthemum spp.*	Portugal and Spain	Alsheikh 1994; Díez et al. 2002; Kovács et al. 2011; Santos-Silva et al. 2021
Terfezia crassiverrucosa	Alkaline	*Helianthemum spp.*	Spain	Kovács et al. 2011; Zitouni-Haouar et al. 2018
Terfezia dunensis	Acid	*Cistus salvifolius* and *Halimium halimifolium*	Portugal and Spain	Crous et al. 2019; Santos-Silva et al. 2021
Terfezia eliocrocae	Alkaline	*Helianthemum spp.*	Spain	Bordallo et al. 2013;
Terfezia extremadurensis	Acid	*Tuberaria guttata*	Portugal and Spain	Bordallo et al. 2013; Santos-Silva et al. 2021
Terfezia fanfani [a]	Acid	*Tuberaria guttata*	Portugal and Spain	Bordallo et al. 2013; Kovács et al. 2011; Louro et al. 2020b; Santos-Silva et al. 2021
Terfezia grisea	Acid, Alkaline	*Helianthemum spp.; Tuberaria guttata*	Portugal and Spain	Bordallo et al. 2015; Moreno et al. 2019; Santos-Silva et al. 2021
Terfezia honrubiae	Acid	*Tuberaria guttata*	Spain	Crous et al. 2019; Moreno et al. 2019
Terfezia leptoderma [a]	Acid	*Cistus spp. Pinus spp.* and *Tuberaria guttata*	Portugal and Spain	Alsheikh 1994; Díez et al. 2002; Kovács et al. 2011; Bordallo et al. 2015
Terfezia lusitanica	Acid	*Tuberaria guttata*	Portugal and Spain	Bordallo et al. 2018; Santos-Silva et al. 2021
Terfezia morenoi	Alkaline	*Pinus spp. and Quercus ilex*	Spain	Crous et al. 2018
Terfezia olbiensis	Alkaline	*Helianthemum spp., Pinus spp.* and *Quercus spp.*	Portugal and Spain	Kovács et al. 2011;
Terfezia pini	Acid	*Pinus spp., Quercus spp.*	Portugal and Spain	Kovács et al. 2011; Bordallo et al. 2013; Santos-Silva et al., 2021
Terfezia pseudoleptoderma	Acid	*Cistaceae spp.*	Spain	Bordallo et al. 2013
Terfezia solaris-libera	Acid	*Tuberaria guttata*	Portugal	Bordallo et al. 2013; Louro et al. 2020b; Santos-Silva et al. 2021;

[a] *T. fanfani* and *T. leptoderma* are considered separate species in this table. Although they were shown to be synonyms (Louro et al. 2019), they are not yet recognized as such in the nomenclatural indices and repositories.

Table S1.3 - Biological activity of *Terfezia* species. (*Continue*)

Species	Origin	Biological activity	Target	Biocoumpounds	Reference
Terfezia arenaria (Moris) Trappe	Spain	Antiproliferative	Tumour cell lines	n.a.	Tejedor-Calvo et al. 2020
		Antioxidant	n.a.	α and δ – Tocopherol	
		Organic acids	n.a.	Oxalic acid	
		Phenolic compounds	n.a.	Gallic acid, Protocatechuic acid, p-Hydroxybenzoic acid, p-Coumaric acid, Cinnamic acid	
	Algeria	Antibiotic	Aspergillus niger, Penicillium sp., Candida albicans, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa	Methanol and dichloromethane extracts	Harir et al. 2019
		Enzymatic	n.a.	Polyphenol oxidase	Benaceur et al. 2020
Terfezia boudieri Chatin	Turkey	Antioxidant	n.a.	n.a.	Dundar et al. 2012
	Tunisia		Wheat Bread	n.a.	Najjaa et al. 2021
	Jordan	Anticancer and Immunomodulatory	Human epithelial breast cancer, breast adenocarcinoma, colon carcinoma and epitheloid cervix carcinoma cell lines	n.a.	Al Obaydi et al. 2020
	Spain	Enzymatic	n.a.	Phosphatase	Navarro-Ródenas et al. 2009
Terfezia claveryi Chattin	Iran	Antibacterial	Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus	Bio-peptides	Janakat et al. 2004; Saddiq et al. 2016; Farzaneh et al. 2018, 2019
	Jordan			Aqueous extract	
		Antioxidant	n.a	BHA, BHT and propyl gallate	Martínez-Tomé et al. 2014
	Iraq	Antiproliferative	Brain cancer lines		Dahham et al. 2018
		Antiangiogenic	Rat aortic ring		
		Hepatoprotective	Liver function		Janakat et al. 2010
	Algeria	Insecticidal	Sitophilus oryzae (L.) and Rhyzopertha dominica (F.)	n.a.	Neggaz et al. 2020
	Spain			Lipoxygenase	Pérez-Gilabert et al. 2005, 2014; Marqués-Gálvez et al. 2019
				Esterase, alkaline phosphatase, and tyrosinase	
				Catalase (TcCAT-1)	

Table S1.3 - Biological activity of *Terfezia* species.

Species	Origin	Biological activity	Target	Biocoumpounds	Reference
Terfezia leptoderma (Tul. & C. Tul.) Tul. & C. Tul.	Spain	Antiproliferative	Tumour cell lines	n.a.	Tejedor-Calvo et al. 2020
		Antioxidant	n.a.	α and δ – Tocopherol	
		Phenolic compounds	n.a.	Gallic acid, Protocatechuic acid, p-Hydroxybenzoic acid, p-Coumaric acid	
Terfezia magnusii Mattir.	Spain	Antiproliferative	Tumour cell lines	n.a.	Tejedor-Calvo et al. 2020
		Antioxidant	n.a.	α and δ – Tocopherol	
		Organic acids	n.a.	Oxalic acid	
		Phenolic compounds	n.a.	Gallic acid, Protocatechuic acid, p-Hydroxybenzoic acid	

n.a. – not applicable; BHA - Butylated Hydroxyanisole; BHT - Butylated Hydroxytulene; TcCAT-1 - catalase from T. claveryi.

Appendix 2

Supplementary Material of Chapter 2

Table S1.2 - Parameters setting of the Cyranose-320.

Method setting	Parameter setting	Pump speed
Baseline purge	10 sec	Medium
Sample draw	10 sec	Medium
Air intake purge	5 sec	High
Sample gas purge	30 sec	High
Digital filtering	On	
Substrate heater	On: 42°C	
Training repeat count 1	1	
Identifying repeat count 1	1	
Statistical analysis by PCnose		
Algorithm	Canonical	
Pre-processing	Auto-scaling	
Normalization	Normalization 1	
Identification Quality	Medium	

Table S2.2 - Comparison by Kolmogorov-Smirnov of the 32 sensors' response between the 14 wild mushroom and truffle (MT) species. This table shows the p-values summary where values with $p < 0.05$ are presented in bold. (*Continue*)

	Agarcius xanthodermus vs												
Sensor	*Cyclocybe cylindracea*	*Amanita phalloides*	*Amanita subparvipantherina*	*Cantharellus cibarius*	*Craterellus lutescens*	*Craterellus tubaeformis*	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	***0.001***	***<0,0001***	***<0,0001***	***0.015***	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***0.003***	***0.003***	***<0,0001***
S2	***0.001***	***<0,0001***	***<0,0001***	*0.164*	***<0,0001***	*0.055*	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	***0.015***	***0.003***	***<0,0001***
S3	***0.001***	***<0,0001***	***<0,0001***	*0.164*	***<0,0001***	***0.015***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***0.003***	***0.001***	***<0,0001***
S4	***0.003***	***0.003***	***<0,0001***	*0.164*	***<0,0001***	***0.015***	***<0,0001***	***0.015***	***<0,0001***	***<0,0001***	*0.759*	***0.003***	***<0,0001***
S5	***<0,0001***	***<0,0001***	***<0,0001***	*0.759*	***<0,0001***	*0.400*	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	***0.015***	***0.003***	***<0,0001***
S6	***<0,0001***	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	*0.400*
S7	***0.015***	***0.001***	***0.001***	*0.759*	***<0,0001***	*0.400*	***0.001***	*0.055*	***<0,0001***	***<0,0001***	*0.400*	***0.003***	***<0,0001***
S8	*0.055*	***0.015***	***0.001***	*0.759*	***<0,0001***	*0.055*	***0.001***	*0.055*	***<0,0001***	***<0,0001***	*0.988*	***0.015***	***<0,0001***
S9	***0.015***	***0.001***	***<0,0001***	*0.055*	***<0,0001***	*0.988*	***0.003***	*0.164*	***<0,0001***	***<0,0001***	*0.759*	***0.015***	***<0,0001***
S10	***<0,0001***	***<0,0001***	***<0,0001***	*0.759*	***<0,0001***	*0.055*	***<0,0001***	***0.015***	***<0,0001***	***<0,0001***	*0.055*	***0.001***	***<0,0001***
S11	***0.001***	***<0,0001***	***<0,0001***	*0.055*	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***0.001***	***0.001***	***0.001***
S12	***0.003***	***<0,0001***	***<0,0001***	*0.055*	***<0,0001***	***0.015***	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	***0.015***	***0.003***	***<0,0001***
S13	***0.001***	***<0,0001***	***<0,0001***	*0.759*	***<0,0001***	*0.055*	***<0,0001***	***0.015***	***<0,0001***	***<0,0001***	*0.400*	***0.001***	***<0,0001***
S14	***0.003***	***<0,0001***	***<0,0001***	*0.759*	***<0,0001***	*0.055*	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	*0.055*	***0.003***	***<0,0001***
S15	***<0,0001***	***<0,0001***	***<0,0001***	*0.759*	***<0,0001***	*0.055*	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	*0.055*	***0.001***	***<0,0001***
S16	***0.001***	***0.001***	***<0,0001***	*0.164*	***<0,0001***	*0.400*	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	*0.400*	***0.003***	***<0,0001***
S17	***0.001***	***<0,0001***	***<0,0001***	*0.988*	***<0,0001***	*0.400*	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	*0.055*	***0.003***	***<0,0001***
S18	***0.015***	***0.003***	***0.001***	*0.055*	***<0,0001***	*0.759*	***0.001***	*0.055*	***<0,0001***	***<0,0001***	*0.759*	***0.015***	***<0,0001***
S19	***0.001***	***0.001***	***0.001***	*0.988*	***<0,0001***	*0.164*	***0.001***	***0.015***	***<0,0001***	***<0,0001***	*0.400*	***0.003***	***<0,0001***
S20	***<0,0001***	***<0,0001***	***<0,0001***	***0.003***	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***0.001***	***<0,0001***
S21	***<0,0001***	***<0,0001***	***<0,0001***	*0.164*	***<0,0001***	***0.015***	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	***0.015***	***0.001***	***0.001***
S22	***<0,0001***	***<0,0001***	***<0,0001***	*0.164*	***<0,0001***	***0.015***	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	*0.055*	***0.003***	***<0,0001***
S23	***<0,0001***	***<0,0001***	***<0,0001***	*0.164*	***<0,0001***	*0.055*	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***0.003***	***0.003***	***0.003***
S24	***0.015***	*0.164*	***0.003***	*0.400*	***0.003***	*0.759*	*0.055*	*0.400*	***0.003***	***0.003***	*0.055*	*0.164*	***<0,0001***
S25	*0.400*	*0.400*	***<0,0001***	***<0,0001***	***<0,0001***	***0.003***	*0.759*	*0.055*	***<0,0001***	***<0,0001***	***0.003***	*0.164*	***<0,0001***
S26	*0.164*	*0.400*	*0.055*	*0.164*	***0.015***	*0.055*	***0.015***	*0.400*	*0.400*	*0.400*	*0.400*	*0.400*	***<0,0001***
S27	***0.015***	***<0,0001***	***<0,0001***	*0.164*	***<0,0001***	***0.015***	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	***0.015***	***0.003***	***<0,0001***
S28	*0.055*	***0.015***	***<0,0001***	***0.015***	***<0,0001***	*0.988*	***0.001***	*0.400*	***<0,0001***	***<0,0001***	*0.164*	***0.015***	***<0,0001***
S29	***0.001***	***<0,0001***	***<0,0001***	*0.400*	***<0,0001***	*0.055*	***<0,0001***	***0.001***	***<0,0001***	***<0,0001***	***0.015***	***0.003***	***<0,0001***
S30	***<0,0001***	***<0,0001***	*0.164*	*0.759*	***0.001***	*0.400*	***0.003***	*0.164*	***0.001***	***0.001***	*0.400*	***0.001***	*0.400*
S31	***<0,0001***	***<0,0001***	***<0,0001***	*0.055*	***<0,0001***	***0.015***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***<0,0001***	***0.015***	***0.001***
S32	***0.015***	***0.001***	***<0,0001***	*0.164*	***<0,0001***	*0.164*	***<0,0001***	***0.003***	***<0,0001***	***<0,0001***	*0.055*	***0.015***	***<0,0001***

Table S2 - (*Cont.*)

	Cyclocybe cylindracea vs											
Sensor	*Amanita phalloides*	*Amanita subparvipantherina*	*Cantharellus cibarius*	*Craterellus lutescens*	*Craterellus tubaeformis*	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	0.759	**0.001**	0.164	**<0,0001**	0.988	**<0,0001**	0.988	**<0,0001**	**<0,0001**	**0.015**	0.055	**<0,0001**
S2	0.055	**0.003**	**0.001**	**<0,0001**	0.400	**0.003**	0.759	**<0,0001**	**<0,0001**	0.055	0.164	**<0,0001**
S3	0.400	0.055	**0.001**	**<0,0001**	0.164	0.164	0.400	**<0,0001**	**<0,0001**	**0.003**	0.055	**<0,0001**
S4	0.759	0.055	0.164	**<0,0001**	0.055	0.400	0.759	**<0,0001**	**<0,0001**	0.055	0.988	**<0,0001**
S5	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	0.400	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S6	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**
S7	0.988	0.164	**0.001**	**<0,0001**	0.164	0.759	0.164	**<0,0001**	**<0,0001**	**0.001**	0.164	**<0,0001**
S8	0.988	**0.003**	0.164	**<0,0001**	0.988	0.055	0.400	**<0,0001**	**<0,0001**	0.055	0.055	**<0,0001**
S9	0.759	**<0,0001**	**0.001**	**<0,0001**	**0.015**	0.759	**0.015**	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**<0,0001**
S10	0.055	0.988	**<0,0001**	**<0,0001**	**0.001**	0.164	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S11	0.164	0.400	**0.001**	**<0,0001**	0.055	0.164	**0.003**	**<0,0001**	**<0,0001**	**0.001**	0.055	**<0,0001**
S12	0.759	0.055	**0.015**	**<0,0001**	0.759	0.164	**0.015**	**<0,0001**	**<0,0001**	**0.001**	0.055	**<0,0001**
S13	0.988	**0.015**	**0.015**	**<0,0001**	0.164	0.759	0.164	**<0,0001**	**<0,0001**	**0.001**	0.055	**<0,0001**
S14	0.759	**0.001**	**0.003**	**<0,0001**	0.400	**0.015**	**0.015**	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**<0,0001**
S15	0.164	0.400	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S16	0.164	0.164	**<0,0001**	**<0,0001**	**0.001**	0.055	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S17	0.400	0.055	**<0,0001**	**<0,0001**	**0.003**	0.759	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S18	0.759	0.055	**0.001**	**<0,0001**	**0.003**	0.400	**0.015**	**<0,0001**	**<0,0001**	**0.001**	0.400	**<0,0001**
S19	0.988	0.055	**<0,0001**	**<0,0001**	**0.001**	0.759	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S20	0.759	**0.003**	**0.015**	**<0,0001**	0.164	**0.003**	0.164	**<0,0001**	**<0,0001**	**0.003**	0.055	**<0,0001**
S21	0.400	0.988	**0.001**	**<0,0001**	**0.003**	0.759	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S22	0.055	**0.003**	**<0,0001**	**<0,0001**	**0.001**	0.759	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S23	0.055	0.400	**<0,0001**	**<0,0001**	**0.001**	0.164	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S24	0.055	0.055	**<0,0001**	0.055	**0.001**	0.759	**<0,0001**	0.055	0.055	**<0,0001**	0.400	**<0,0001**
S25	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	0.988	0.164	**<0,0001**	**<0,0001**	**0.015**	**0.015**	**<0,0001**
S26	0.164	0.055	**0.001**	**0.015**	**0.001**	0.759	**0.015**	0.164	0.164	**0.003**	0.759	**<0,0001**
S27	**0.015**	**<0,0001**	0.164	**<0,0001**	0.400	**<0,0001**	0.400	**<0,0001**	**<0,0001**	**0.015**	**0.003**	**<0,0001**
S28	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	0.400	**0.015**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S29	0.164	**0.003**	**0.001**	**<0,0001**	**0.015**	0.164	**0.003**	**<0,0001**	**<0,0001**	**0.001**	0.055	**<0,0001**
S30	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S31	**0.001**	**<0,0001**	**0.001**	**<0,0001**	0.400	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.759	0.164	**0.015**
S32	0.400	**<0,0001**	**0.003**	**<0,0001**	0.759	**0.015**	0.988	**<0,0001**	**<0,0001**	0.164	**0.015**	**<0,0001**

Table S2 - (*Cont.*)

	Amanita phalloides vs										
Sensor	*Amanita subparvipantherina*	*Cantharellus cibarius*	*Craterellus lutescens*	*Craterellus tubaeformis*	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	**0.003**	**0.003**	**<0,0001**	0.400	**0.015**	0.759	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S2	0.055	**<0,0001**	**<0,0001**	**0.015**	0.055	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**
S3	0.400	**<0,0001**	**<0,0001**	0.055	0.400	0.164	**<0,0001**	**<0,0001**	**0.003**	0.759	**<0,0001**
S4	0.055	0.164	**<0,0001**	0.400	0.055	0.988	**<0,0001**	**<0,0001**	0.164	0.759	**<0,0001**
S5	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	0.164	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S6	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S7	0.164	**<0,0001**	**<0,0001**	**0.015**	0.400	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S8	**0.015**	0.055	**<0,0001**	0.759	0.055	0.759	**<0,0001**	**<0,0001**	**0.015**	0.164	**<0,0001**
S9	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	0.759	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S10	**0.015**	**<0,0001**	**<0,0001**	**0.015**	0.400	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S11	0.164	**0.001**	**<0,0001**	0.400	**0.003**	**0.015**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S12	**0.015**	**0.001**	**<0,0001**	0.164	0.055	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S13	**0.015**	**0.001**	**<0,0001**	0.164	0.164	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S14	**0.003**	**<0,0001**	**<0,0001**	0.055	**0.015**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S15	0.164	**<0,0001**	**<0,0001**	**0.015**	0.759	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S16	**0.001**	**<0,0001**	**<0,0001**	0.055	0.164	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S17	**0.001**	**<0,0001**	**<0,0001**	**0.003**	0.400	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S18	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.988	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S19	0.055	**<0,0001**	**<0,0001**	**0.003**	0.759	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S20	**0.003**	**0.003**	**<0,0001**	0.400	**0.003**	0.988	**<0,0001**	**<0,0001**	**0.003**	0.055	**<0,0001**
S21	0.055	**<0,0001**	**<0,0001**	0.055	0.164	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S22	**<0,0001**	**<0,0001**	**<0,0001**	0.400	0.055	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S23	**0.003**	**<0,0001**	**<0,0001**	**0.015**	**0.015**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S24	**0.003**	**0.003**	**0.001**	**0.015**	0.400	0.055	**0.001**	**0.003**	**<0,0001**	0.400	**<0,0001**
S25	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S26	**0.003**	**0.001**	**0.003**	**0.015**	0.055	0.759	**0.003**	**0.003**	**0.015**	0.400	**<0,0001**
S27	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**0.015**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S28	**0.001**	**<0,0001**	**<0,0001**	**0.003**	0.400	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S29	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**0.001**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S30	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	0.400	**0.015**
S31	0.164	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	0.759	**<0,0001**	**<0,0001**	**0.015**	0.164	**<0,0001**
S32	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.015**	0.400	**<0,0001**	**<0,0001**	**0.015**	0.055	**<0,0001**

Table S2 - (*Cont.*)

	Amanita subparvipantherina vs									
Sensor	*Cantharellus cibarius*	*Craterellus lutescens*	*Craterellus tubaeformis*	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	**0.001**	**<0,0001**	**0.015**	0.164	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S2	**<0,0001**	**<0,0001**	**0.003**	0.055	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**
S3	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S4	**0.001**	0.055	**0.001**	0.164	**0.015**	0.164	**0.015**	**0.001**	0.400	**<0,0001**
S5	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**
S6	**0.003**	**<0,0001**	0.055	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.055	**0.001**	**0.003**
S7	**<0,0001**	**<0,0001**	**0.015**	0.400	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S8	**0.003**	**0.003**	0.055	0.400	0.400	**0.015**	**0.003**	**<0,0001**	0.759	**<0,0001**
S9	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**
S10	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S11	**<0,0001**	**<0,0001**	**0.015**	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S12	**<0,0001**	**<0,0001**	**0.003**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S13	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S14	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S15	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S16	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S17	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S18	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S19	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S20	**<0,0001**	**<0,0001**	**0.001**	0.759	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S21	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**
S22	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**
S23	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**
S24	**<0,0001**	0.759	**<0,0001**	**0.003**	**<0,0001**	0.055	0.055	**<0,0001**	**0.015**	**<0,0001**
S25	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	**0.001**	**<0,0001**
S26	**0.001**	0.055	**<0,0001**	0.164	**0.015**	0.400	0.400	**0.003**	**0.015**	**<0,0001**
S27	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	0.055	**<0,0001**
S28	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**
S29	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**
S30	0.055	**0.015**	0.055	0.400	**0.015**	0.055	**0.015**	0.164	0.055	0.400
S31	**<0,0001**	**<0,0001**	**0.003**	**0.001**	0.759	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S32	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**

Table S2 - (*Cont.*)

	Cantharellus cibarius vs								
Sensor	*Craterellus lutescens*	*Craterellus tubaeformis*	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	**<0,0001**	0.055	**<0,0001**	0.055	**<0,0001**	**<0,0001**	0.400	**0.015**	**<0,0001**
S2	**<0,0001**	0.055	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S3	**<0,0001**	0.055	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.055	**0.001**	**<0,0001**
S4	**<0,0001**	0.400	**0.015**	0.164	**<0,0001**	**<0,0001**	0.988	0.164	**<0,0001**
S5	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**0.003**	**<0,0001**
S6	**<0,0001**	0.759	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**
S7	**<0,0001**	0.164	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S8	**<0,0001**	0.164	**0.003**	0.164	**<0,0001**	**<0,0001**	0.759	**0.015**	**<0,0001**
S9	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**
S10	**<0,0001**	0.164	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.164	**0.001**	**<0,0001**
S11	**<0,0001**	0.055	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S12	**<0,0001**	0.055	**<0,0001**	0.055	**<0,0001**	**<0,0001**	0.400	**0.003**	**<0,0001**
S13	**<0,0001**	0.164	**0.001**	**0.015**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S14	**<0,0001**	0.055	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S15	**<0,0001**	0.055	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.164	**0.001**	**<0,0001**
S16	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**0.001**	**<0,0001**
S17	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.001**	**<0,0001**
S18	**<0,0001**	0.164	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.055	**0.003**	**<0,0001**
S19	**<0,0001**	0.400	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S20	**<0,0001**	0.400	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.400	**0.003**	**<0,0001**
S21	**<0,0001**	0.055	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S22	**<0,0001**	**0.015**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S23	**<0,0001**	0.055	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	0.400	**0.003**	**0.015**
S24	**<0,0001**	0.988	**0.001**	0.164	**<0,0001**	**<0,0001**	0.400	**0.003**	**<0,0001**
S25	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**
S26	**0.001**	0.400	**0.001**	**0.001**	**0.001**	**0.001**	0.164	**0.015**	**<0,0001**
S27	**<0,0001**	0.164	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.400	**0.003**	**<0,0001**
S28	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.001**	**<0,0001**
S29	**<0,0001**	0.055	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.400	**0.003**	**<0,0001**
S30	**<0,0001**	0.164	**0.003**	0.055	**<0,0001**	**<0,0001**	0.164	**<0,0001**	0.400
S31	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	0.164
S32	**<0,0001**	0.055	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**

Table S2 - (*Cont.*)

	Craterellus lutescens vs							
Sensor	*Craterellus tubaeformis*	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**
S2	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	0.400	**<0,0001**	**<0,0001**	**<0,0001**
S3	**<0,0001**	**<0,0001**	**<0,0001**	0.164	0.400	**<0,0001**	**<0,0001**	**<0,0001**
S4	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	0.759	**<0,0001**	**0.001**	**<0,0001**
S5	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S6	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S7	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**
S8	**<0,0001**	**<0,0001**	**<0,0001**	0.164	0.164	**<0,0001**	**0.001**	**<0,0001**
S9	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S10	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S11	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	0.055	**<0,0001**	**<0,0001**	**<0,0001**
S12	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S13	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**
S14	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**
S15	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S16	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S17	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S18	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S19	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**
S20	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S21	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**
S22	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S23	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S24	**<0,0001**	**0.015**	**<0,0001**	**0.003**	**0.015**	**<0,0001**	**0.001**	**<0,0001**
S25	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S26	**<0,0001**	**0.015**	**0.015**	**0.003**	**0.003**	**0.003**	**0.003**	**<0,0001**
S27	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S28	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S29	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**
S30	**0.001**	0.164	**<0,0001**	0.759	0.055	**0.001**	**0.015**	0.055
S31	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**
S32	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**

Table S2 - (*Cont.*)

	Craterellus tubaeformis vs						
Sensor	*Hydnum repandum*	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	**0.003**	0.759	**<0,0001**	**<0,0001**	**0.015**	0.164	**<0,0001**
S2	**0.003**	0.400	**<0,0001**	**<0,0001**	0.055	0.055	**<0,0001**
S3	**0.003**	0.164	**<0,0001**	**<0,0001**	0.400	**0.015**	**<0,0001**
S4	**0.003**	0.400	**<0,0001**	**<0,0001**	0.164	0.055	**<0,0001**
S5	**<0,0001**	0.164	**<0,0001**	**<0,0001**	0.400	**0.015**	**<0,0001**
S6	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**0.015**	**0.003**	**0.003**
S7	**0.015**	0.164	**<0,0001**	**<0,0001**	0.164	**0.003**	**<0,0001**
S8	0.400	0.988	**<0,0001**	**<0,0001**	0.055	0.400	**<0,0001**
S9	**0.003**	0.164	**<0,0001**	**<0,0001**	0.400	**0.015**	**<0,0001**
S10	**0.001**	0.400	**<0,0001**	**<0,0001**	0.055	**0.003**	**<0,0001**
S11	**0.003**	0.164	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S12	**0.015**	0.400	**<0,0001**	**<0,0001**	**0.015**	**0.015**	**<0,0001**
S13	0.164	0.400	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S14	**0.001**	0.400	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S15	**0.015**	0.400	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S16	**<0,0001**	0.400	**<0,0001**	**<0,0001**	0.164	**0.003**	**<0,0001**
S17	**0.001**	0.164	**<0,0001**	**<0,0001**	0.164	**0.003**	**<0,0001**
S18	**<0,0001**	0.164	**<0,0001**	**<0,0001**	0.400	**0.003**	**<0,0001**
S19	**0.001**	0.400	**<0,0001**	**<0,0001**	0.164	**0.003**	**<0,0001**
S20	**0.003**	0.400	**<0,0001**	**<0,0001**	0.400	0.055	**<0,0001**
S21	**0.001**	0.759	**<0,0001**	**<0,0001**	**0.015**	**0.003**	**0.001**
S22	**0.003**	0.759	**<0,0001**	**<0,0001**	**0.015**	**0.015**	**0.001**
S23	**<0,0001**	0.400	**<0,0001**	**<0,0001**	0.055	**0.015**	**0.003**
S24	**0.003**	0.164	**<0,0001**	**<0,0001**	0.164	**0.015**	**0.001**
S25	**0.001**	0.055	**<0,0001**	**<0,0001**	0.759	**0.003**	**<0,0001**
S26	**0.001**	0.055	**<0,0001**	**<0,0001**	0.400	**0.015**	**<0,0001**
S27	**0.001**	0.400	**<0,0001**	**<0,0001**	**0.015**	0.055	**<0,0001**
S28	**0.001**	0.400	**<0,0001**	**<0,0001**	0.164	**0.015**	**<0,0001**
S29	**<0,0001**	0.759	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S30	**0.003**	0.988	**0.001**	**0.001**	**0.015**	**0.001**	0.164
S31	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	0.164	0.759	0.400
S32	**<0,0001**	0.400	**<0,0001**	**<0,0001**	0.400	**0.015**	**<0,0001**

Table S2 - (*Cont.*)

	Hydnum repandum vs						*Hygrocybe helobia vs*				
Sensor	*Hygrocybe helobia*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*	*Lactarius deliciosus*	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*
S1	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	0.055	**<0,0001**
S2	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	0.055	0.055	**<0,0001**
S3	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**
S4	0.055	**0.001**	**<0,0001**	**0.003**	0.759	**<0,0001**	**<0,0001**	**<0,0001**	0.400	0.400	**<0,0001**
S5	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S6	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**0.001**	**0.001**
S7	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.003**	**<0,0001**
S8	0.400	**<0,0001**	**<0,0001**	**0.001**	0.759	**<0,0001**	**<0,0001**	**<0,0001**	0.055	0.400	**<0,0001**
S9	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**0.015**	**<0,0001**
S10	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**0.003**	**<0,0001**
S11	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**0.015**	**<0,0001**
S12	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.003**	**<0,0001**
S13	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**<0,0001**
S14	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**
S15	**0.003**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S16	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**
S17	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.003**	**<0,0001**
S18	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**<0,0001**
S19	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.003**	**<0,0001**
S20	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**0.015**	**<0,0001**
S21	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**
S22	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S23	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**
S24	**0.003**	**0.015**	0.055	**<0,0001**	0.988	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**
S25	**0.015**	**<0,0001**	**<0,0001**	**0.001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.003**	**<0,0001**
S26	0.055	0.055	0.055	**0.003**	0.055	**<0,0001**	**0.015**	**0.015**	0.164	0.400	**<0,0001**
S27	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**
S28	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.003**	**<0,0001**
S29	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**0.015**	**<0,0001**
S30	**<0,0001**	0.164	0.164	**0.015**	0.400	0.055	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	0.055
S31	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	0.164	**<0,0001**
S32	**0.001**	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**<0,0001**	**<0,0001**	**<0,0001**	0.055	**0.015**	**<0,0001**

Table S2 - (*Cont.*)

	Lactarius deliciosus vs				*Lepista nuda vs*			*Pisolithus tinctorius vs*		*Suillus collinitus vs*
Sensor	*Lepista nuda*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*	*Pisolithus tinctorius*	*Suillus collinitus*	*Terfezia arenaria*	*Suillus collinitus*	*Terfezia arenaria*	*Terfezia arenaria*
S1	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**
S2	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S3	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**
S4	**0.001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	0.164	**<0,0001**	**<0,0001**
S5	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S6	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**0.001**	**0.001**
S7	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S8	0.988	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S9	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S10	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**
S11	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**
S12	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**
S13	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**
S14	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**
S15	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**
S16	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S17	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S18	0.055	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S19	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**
S20	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S21	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S22	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S23	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**0.001**
S24	0.759	**<0,0001**	**0.001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**0.001**	**0.001**	**<0,0001**
S25	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S26	0.400	**0.003**	**0.003**	**<0,0001**	**0.003**	**0.003**	**<0,0001**	0.055	**<0,0001**	**<0,0001**
S27	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S28	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S29	0.164	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.003**	**<0,0001**	**<0,0001**
S30	0.055	**0.001**	**0.015**	0.055	**0.001**	0.164	0.055	**0.001**	0.400	0.055
S31	0.759	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	0.400	**0.003**	0.164
S32	0.400	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**0.015**	**<0,0001**	**<0,0001**

Appendix 3

Supplementary Material of Chapter 3

Figure S3.1 - Map of the sampling areas of *Tefezia arenaria* collected during this study, and forest-dominant species per area. Created with BioRender.com.

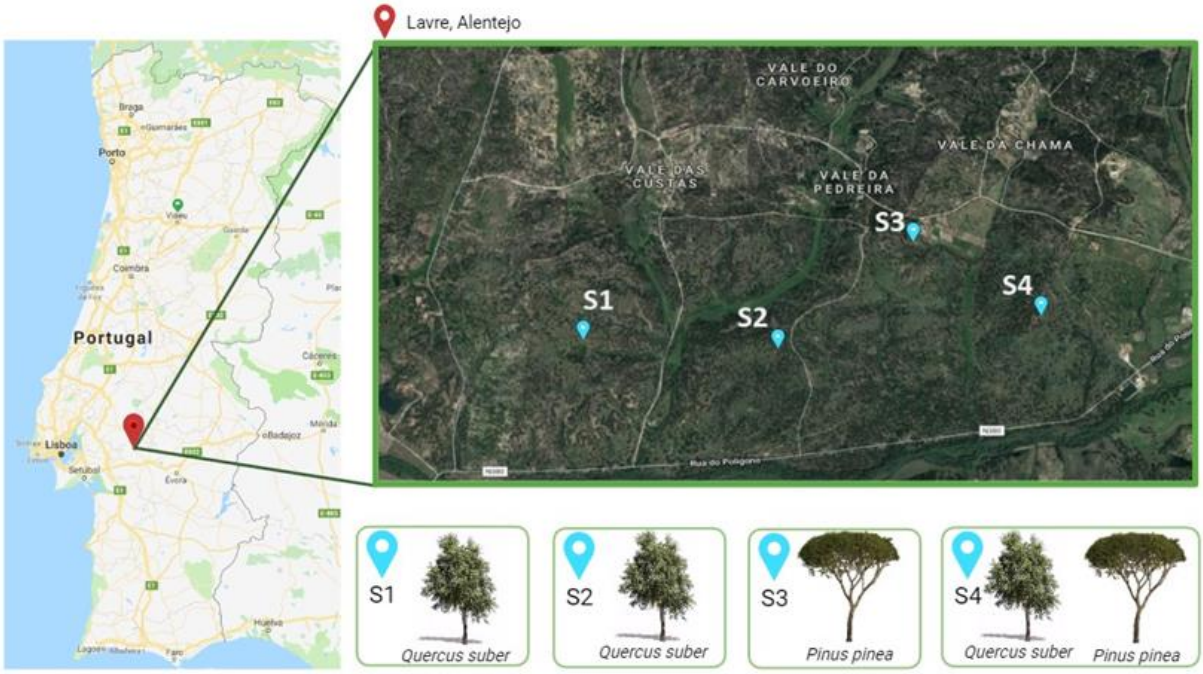

Table S3.1 - Parameters settings for using the Cyranose-320.

Method setting	Parameter setting	Pump speed
Baseline purge	10 sec	Medium
Sample draw	10 sec	Medium
Air intake purge	5 sec	High
Sample gas purge	30 sec	High
Digital filtering	On	
Substrate heater	On: 42°C	
Training repeat count 1	1	
Identifying repeat count 1	1	
Statistical analysis by PCnose		
Algorithm	Canonical	
Pre-processing	Auto-scaling	
Normalization	Normalization 1	
Identification Quality	Medium	

Table S3.2 - Dietary Reference Intakes for nutrients and elements. This table presents the Recommended Dietary Allowances (RDA, shown in bold), Adequate Intakes (AI, identified with *) and Tolerable Upper Intake Level (UL, identified with †). These values were adapted from Dietary Reference Intakes Datasets from the USA, [61], and the EU [62–65]. The two right-most columns show the contribution (in %) of an 100 g intake of dry and fresh *T. arenaria*, to each nutrient and mineral element, considering the RDA or AI values.

			Adults (>18)			***Terfezia arenaria***	
		Unit	**Males**	**Females**	**UL**	**100 g dry**	**100 g fresh**
Nutrients	Carbohydrates	g/day	130	130		55 %	< 1 %
	Total Fiber	g/day	30 - 38*	21 - 25*		48 %	< 1 %
	Protein [a]	g/day	56	46		33 %	< 1 %
	Fat	g/day	ND	ND			
Elements	Cr	(μg/day)	30 - 35*	20 - 25*	ND	> 100%	3 % - 6 %
	Li	(μg/day)	ND	ND	2 [b,d]	> 100%	2 %
	Se	(μg/day)	55	55	255	91%	1 %
	Cu	(μg/day)	900	900	10,000	73%	< 1 %
	P	(mg/day)	700	700	4000	20 %	< 1 %
	Fe	(mg/day)	8	8 - 18	45	11 % - 24 %	< 1 %
	K	(mg/day)	3400*	2600*	ND	10 % - 14 %	< 1 %
	Zn	(mg/day)	11	8	40	10% - 13 %	< 1 %
	Mn	(mg/day)	2.3*	1.8*	11	6 % - 11 %	< 1 %
	Mg	(mg/day)	420	310 - 320	350	3 % - 4 %	< 1 %
	As†	(μg/kg bw per day)	ND	ND	< 15	1 %[b]	0.01% [c]
	Ba†	(mg/kg bw per day)	ND	ND	0.2	< 1 % [b]	< 0.01% [c]
	Ca	(mg/day)	1000 - 1200	1000 - 1200	2500	< 1 %	< 0.01%
	Mo	(μg/day)	45	45	2000	< 1 %	< 0.01%
	Na	(mg/day)	1500*	1500*	2300	< 1 %	< 0.01%
	Ni	(mg/day)	ND	ND	1.0	< 1 % [c]	< 0.01%d

[a] Based on g of protein per kg of body weight for the reference body weight, e.g., for adults 0.8 g kg^{-1} of body weight for the reference body weight; [b] Provisional reference dose; [c] Values considering a person with 60 kg of reference body weight; [d] Was considered the UL value; † Elements with detrimental health effects; ND: Not determined.

Table S3.3 - Comparison of the abundance of the main VOCs identified in *Terfezia arenaria* and in the other edible mushroom and truffle (MT) species. Values for *T. arenaria* were determined in the present study while values for the other mushrooms and truffle were determined in other studies. Abundance: +++ high; ++ medium; + low; - absent.

Compounds		***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Alcohols	1-Octen-3-ol	+++	+++	+++	++	+
	3-Octanol	+	++	+	++	+
	2-Octen-1-ol	+	+	+	-	-
Aldehydes	Benzeneacetaldehyde	+	+	+	-	+
	Hexanal	+	-	-	+	+
	2-Octenal	+	+	+	+	-
	Nonanal	+	-	-	+	-
Hydrocarbons	Tetradecane	+	+	-	-	-
Ketones	3-Octanone	++	++	++	++	+
Terpenes	Limonene	+	+	-	+	-
References		This study	Feng et al. 2021	Zhang et al. 2020	Tagkouli et al. 2021	Choo et al. 2021

Table S3.4 - Characterization of the volatile organic compounds (VOCs) identified in *Terfezia arenaria*, and in the other edible mushroom and truffle (MT) species (*Agaricus bisporus, Lentinula edodes, Pleurotus ostreatus and Tuber melanosporum*). The quantity of each volatile is presented in % of the total VOCs detected. The results for *Terfezia arenaria* were originated from this study, while the other mushroom species data was collected from literature review (Continue).

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Alcohols	1-Octen-3-ol	64.411%	35.919%	35.909%	28.437%	0.226%
	3-Octanol	1.734%	23.040%	5.813%	27.538%	0.192%
	1-Octanol		1.369%	4.139%	0.789%	0.011%
	Phenylethyl Alcohol		0.036%	0.341%		0.802%
	3-methyl-1-Butanol					17.877%
	1-Hexanol				0.507%	0.011%
	2-ethyl-1-hexanol				0.140%	0.011%
	2-butanol					0.440%
	1-Propanol, 2-methyl					3.173%
	1-Butanol					0.011%
	1-Butanol, 2-methyl-					25.793%
	1-Pentanol					0.011%
	1-Butanol, 2-ethyl					0.011%
	1-Pentanol, 4-methyl-					0.011%
	2-Heptanol, 6-methyl-					0.011%
	1-Hexanol, 3-methyl					0.011%
	Ethanol, 2-(methylthio)-					0.090%
	trans-(2-Ethylcyclopentyl)methanol					0.011%
	1-Propanol, 3-(methylthio)-					0.113%
	(Z)-2-Octen-1-ol	3.625%	1.102%	3.187%		
	Benzyl Alcohol		1.920%	0.170%		
	Benzeneethanol, β-methyl-			0.271%		
	1-Nonanol		0.124%			

Table S3.4 - *Cont.*

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Alcohols	3-methyl-1-Butanol		0.071%			
	3-Nonanol		0.053%			
	3-Heptanol		0.036%			
	(5Z)-Octa-1,5-dien-3-ol	1.554%				
	Chlorohexanol	0.158%				
	1-Dodecanol	0.113%				
Sulphurs	Disulfide, dimethyl			0.882%		0.056%
	Dimethylsulfide					9.972%
	Carbon disulfide			8.118%		
	Lenthionine			5.332%		
	Dimethyl trisulfide			4.630%		
	Tetrasulfide, dimethyl			1.303%		
	1,2,4-Trithiolane			1.042%		
	1,2,4,5-Tetrathiane			0.291%		
	Cyclic octaatomic sulfur			0.702%		
Acids	Acetic acid		0.107%	0.431%		0.056%
	Propanoic acid, 2-methyl-					0.068%
	Butanoic acid,4-hydroxy-					0.011%
	Butanoic acid, 2-methyl-					1.299%
	Cystine			0.551%		
	n-Hexadecanoic acid			0.170%		
	Pentadecanoic acid			0.080%		
	Tetradecanoic acid			0.261%		
	Propanoic acid		0.053%			

Table S3.4 - *Cont.*

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Aldehydes	Benzaldehyde		5.566%	0.822%	0.300%	0.045%
	Benzeneacetaldehyde	0.236%	0.249%	0.401%		0.011%
	Hexanal	4.830%			1.438%	0.011%
	Octanal			0.261%	0.407%	0.011%
	2-Methyl-Butanal		0.036%			0.316%
	3-Methyl-Butanal				0.860%	0.474%
	Acetaldehyde					3.320%
	Butanal					0.011%
	2-Butenal					0.440%
	4-Methyl-Hexanal					0.011%
	5-Methyl-Hexanal					0.011%
	(E)-2-Octenal	0.968%	2.009%	0.601%	1.240%	
	Nonanal	0.135%			0.032%	
	trans-2-hexenal / (E)-2-Hexenal				0.127%	
	2,4-nonadienal				0.144%	
	2-Phenylpropenal			9.421%		
	2-Phenylpropionaldehyde			0.231%		
	2-Propenal, 3-phenyl-			0.261%		
	(E, E)-2,4-Octadienal		0.302%			
	Benzaldehyde, 2,5-bis[(trimethylsi-lyl)oxy]	0.214%				
Hydrocarbons	2,4-Dithiapentane					0.023%
	Benzene, 1-methoxy-3-methyl-					8.357%
	Benzene, 1,2-dimethoxy-					0.440%

Table S3.4 - *Cont.*

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Hydrocarbons	Benzene, 1,3-dimethoxy-					0.011%
	Benzene, 1,4-dimethoxy-2-methyl-					0.124%
	Butane, 1-methoxy-2-methyl-					0.124%
	Anisole					6.098%
	Toluene				7.548%	1.016%
	Undecane		0.018%		1.414%	
	Dodecane		0.036%	0.040%	0.305%	
	Nonadecane				0.160%	
	Pentadecane			0.100%	0.478%	
	Heptadecane			0.210%	0.319%	
	Hexadecane	0.169%	0.018%	0.140%	0.393%	
	cis-α-Bisabolene		0.036%		0.360%	
	Octadecane				0.262%	
	Eicosane	0.090%			0.107%	
	2-Methyl-2-phenyl-Oxirane			1.012%		
	2-Methyl-2-phenyl-Oxirane		0.036%			
	Decane		0.036%			
	Tetradecane	0.236%	0.231%			
	Dotriacontane	0.315%				
	Eicosane-7-hexyl	0.236%				
	3,3,5-Trimethylheptane	0.146%				
	1-chloroeicosane	0.113%				
	Caprylene (1-octene)	0.293%				

Table S3.4 - *Cont.*

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Ketones	3-Octanone	14.479%	19.027%	11.736%	25.880%	0.203%
	2-Butanone					3.817%
	2-Pentanone					0.384%
	4-Heptanone					0.011%
	Acetone		0.213%			1.863%
	2-Hexanone, 5-methyl-					0.011%
	2-Heptanone, 6-methyl-					0.011%
	Acetoin					0.113%
	2,3-octanedione				0.101%	
	2-Undecanone		0.036%	0.080%		
	1-Isoindolinone			0.932%		
	2-Octanone		0.302%			
	(E)-6,10-dimethyl-5,9-Undecadien-2-one		0.551%			
	1-Octen-3-one		2.792%			
	3-Nonanone		0.036%			
	3-Cyclohepten-1-one		0.925%			
	2-Octanone, 1-nitro-	0.259%				
	Geranylacetone	0.101%				
Esters	Hexadecanoic acid ethyl ester		0.071%			
	Hexanedioic acid, bis(2-ethylhexyl) ester		3.539%			
	Formic acid,1-methylethyl ester					0.011%
	1-Butanol, 2-methyl-, acetate					0.395%
	Ethyl acetate					0.113%

Table S3.4 - *Cont.*

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melanosporum***
Esters	Ethane, 1,1-diethoxy-					1.852%
	Propanoic acid, 2-methyl-, ethyl ester					0.011%
	Butanoic acid, 2-methyl-ethyl ester					2.428%
	Butanoic acid, 3-methyl-ethyl-ester					0.429%
	Formic acid, 2-methylbutylester					0.892%
	Propanoic acid, 2-methyl-, 2-methylpropyl ester					0.011%
	Propanoic acid ,2-methyl-, 2-methylbutyl ester					0.011%
	Butyl 2-methylbutanoate					0.011%
	Butanoic acid, 2-methyl-, 2-methylbutyl ester					1.739%
	Butanoic acid, 3-methyl-, 2-methylbutyl ester					0.011%
	Pentanoic acid, 2-methylbutyl ester					0.192%
	Methyl palmitate			0.130%		
	Pentyl propanoate	1.103%				
	Propionic acid, 3-iodo-, octadecyl ester	0.439%				
	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	0.248%				
Terpenes	Pristane				0.360%	
	Phytane				0.328%	
	Limonene	0.394%	0.107%		0.025%	
	α-Pinene	2.105%				

Table S3.4 - Characterization of the volatile organic compounds (VOCs) identified in *Terfezia arenaria*, and in the other edible mushroom and truffle species (*Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus and Tuber melanosporum*). The quantity of each volatile is presented in % of the total VOCs detected. The results for *Terfezia arenaria* were originated from this study, while the other mushroom species data was collected from literature review.

	Compounds	***Terfezia arenaria***	***Agaricus bisporus***	***Lentinula edodes***	***Pleurotus ostreatus***	***Tuber melano-sporum***
Other com-pounds	Carbon dioxide					4.337%
	1-Octadecanesulphonyl chloride	0.338%				
	Anthranilic acid	0.146%				
	Tyrosol	0.124%				
	Henicosanoic acid	0.327%				
	Pyridine, 5-ethenyl-2-methyl-	0.360%				
References		This study	Feng et al. 2021	Zhang et al. 2020	Tagkouli et al. 2021	Choo et al. 2021

Table S3.5 - Results and rates of Cyranose-320 identification of *Terfezia arenaria*, *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus* and *Tuber melanosporum* with 40 °C and room temperature (RT) pre-analysis incubation temperatures.

	Pre-analysis 40 °C			**Pre-analysis RT**		
Sample	**Result**	**Rate[a]**		**Result**	**Rate[a]**	
Terfezia arenaria	Terf3	*****	excellent	Terf3	*****	excellent
Terfezia arenaria	Terf3	*****	excellent	Terf2, Terf2		
Terfezia arenaria	Terf3	*****	excellent	Terf3	*****	excellent
Terfezia arenaria	Terf1	*****	excellent	Terf3	***	acceptable
Terfezia arenaria	Terf3	*	not acceptable	Terf3	*	not acceptable
Terfezia arenaria	Terf3, Terf3			Terf2, Terf2		
Terfezia arenaria	Terf2	*	not acceptable	Terf3	*****	excellent
Terfezia arenaria	Terf2	*	not acceptable	Terf3	*****	excellent
Terfezia arenaria	Terf2, Terf2			Terf3	***	acceptable
Terfezia arenaria	Terf3	*	not acceptable	Terf3	*****	excellent
Terfezia arenaria	Terf2	*	not acceptable	Terf2	*****	excellent
Terfezia arenaria	Terf2	*****	excellent	Terf3	*****	excellent
Terfezia arenaria	Terf2	*****	excellent	Terf3	*****	excellent
Terfezia arenaria	Terf2	*****	excellent	Terf3	***	acceptable
Terfezia arenaria	Terf2	*****	excellent	Terf3	*****	excellent
Terfezia arenaria	Terf3	***	acceptable	Terf3	***	acceptable
Terfezia arenaria	Terf2, Terf3			Terf3	*****	excellent
Terfezia arenaria	Terf3	*	not acceptable	Terf3	*	not acceptable
Terfezia arenaria	Terf3	*	not acceptable	Terf3	*****	excellent
Terfezia arenaria	Terf3, Terf3			Terf3	*****	excellent
Agaricus bisporus	Terf2	*****	excellent	Unknown		
Agaricus bisporus	Terf3	*	not acceptable	Confused		
Agaricus bisporus	Terf1	*****	excellent	Confused		
Agaricus bisporus	Terf1	***	acceptable	Terf1	***	acceptable
Agaricus bisporus	Terf2	*	not acceptable	Terf1	*****	excellent
Lentinula edodes	Confused			Terf3	***	acceptable
Lentinula edodes	Terf2	*****	excellent	Unknown		
Lentinula edodes	Terf1	*****	excellent	Unknown		
Lentinula edodes	Terf1	*****	excellent	Unknown		
Lentinula edodes	Confused			Terf3	*****	excellent
Pleurotus ostreatus	Confused			Unknown		
Pleurotus ostreatus	Confused			Terf3	***	acceptable
Pleurotus ostreatus	Terf1	***	acceptable	Terf3	*	not acceptable
Pleurotus ostreatus	Confused			Unknown		
Pleurotus ostreatus	Confused			Unknown		
Tuber melanosporum	Unknown			Unknown		
Tuber melanosporum	Unknown			Terf1	***	acceptable
Tuber melanosporum	Unknown			Terf2	*	not acceptable
Tuber melanosporum	Unknown			Unknown		
Tuber melanosporum	Unknown			Unknown		

[a] Result rate with stars, where:
5 stars (*****) - 100% of probability – excellent
4 stars (****) - 80% of probability - good
3 stars (***) - 60% of probability – acceptable
2 stars (**) - 40% of probability – bad
1 star (*) - 20% of probability – not acceptable

Appendix 4

Supplementary Material of Chapter 4

Table S4.1 Effects of 1-octen-3-ol 1 µM (VOC) and ectomycorrhizal (ECM) mycelium on Cistaceae germination rates (%, n = 5). Treatments: **Tlep** - *Terfezia leptoderma*; **Ldel** - *Lactarius deliciosus*; **VOC** - 1-octen-3-ol 1 µM; **CT** – control. Data were compared using a Kruskall–Wallis test. Post hoc comparisons were made using a Dunn's test, respectively. Data are means and standard deviation (SD). Different letters indicate significance at $p < 0.05$, of each Cistaceae species in each treatment.

	Cistus albidus			*Cistus ladanifer*			*Cistus psilosepalus*		
Treatment	**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
CT	30.00	± 11.18	a,b	50.00	± 17.68	a	35.00	± 13.69	a,b
Tlep	35.00	± 13.69	a,b	50.00	± 17.68	a	30.00	± 11.18	a,b
Ldel	50.00	± 17.68	a	35.00	± 13.69	a	65.00	± 13.69	a
VOC	20.00	± 11.18	b	60.00	± 13.69	a	20.00	± 11.18	b

	Cistus salviifolius			*Halimium halimifolium*			*Tuberaria guttata*		
Treatment	**Mean**	**SD**		**Mean**	SD		**Mean**	**SD**	
CT	40.00	± 13.69	a	50.00	± 17.68	a,b	40.00	± 22.36	b
Tlep	40.00	± 13.69	a	65.00	± 13.69	a	55.00	± 32.60	a,b
Ldel	50.00	± 17.68	a	35.00	± 13.69	a,b	95.00	± 11.18	a
VOC	30.00	± 11.18	a	30.00	± 11.18	b	85.00	± 22.36	a,b