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An investigation into the cognitive ecology and electrophysiology of fungi and plants

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An investigation into the cognitive ecology and electrophysiology of fungi and plants

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by

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Supervised by Prof. Brian John Pickles, Prof. Mark Tibbett, and Dr Francesco Tamagnini

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

André Geremia Parise

Acknowledgements

Writing the acknowledgements is always a tricky task. A doctorate is (in this case) a three-year project that culminated in this document, which sometimes receives the hateful name of a 'monograph.' There could be no more misleading name than monograph, because every doctoral thesis, including those in the social sciences, but especially in the natural sciences, is the result of a collective effort by many people who collaborate with the main author—in this case, the doctor-to-be. And not only collaborators in the present or the past three years. A doctorate is one cycle of a very long process of continuous learning that starts the moment we first open our eyes, and every person that has ever interacted with the doctor-to-be has had a role to play. If I wanted to be corny, this would be the moment to quote Sir Isaac Newton's metaphor, "if I have seen further, it is by standing on the shoulders of giants." The problem with this quote, which presumably was first uttered by the French twelfth-century scholar Bernard of Chartres, is that it is mostly true. And I say mostly because we do not see further only because we stand on the shoulders of giants, but also on the shoulders of very ordinary people who, nevertheless, in one way or another have contributed to the proto-doctor reaching where they have arrived. Therefore, assuming the great risk of committing an injustice and forgetting or ignoring important people (giant or not), I will try to write my acknowledgements.

And I would like to start from the most important: my family, my foundation. I would like to thank my parents, Tania Maria de Carvalho Parise and Luis André Parise, for the love they granted me, and for raising me surrounded by books and creativity, always nurturing my thirst for knowledge. To my brothers, Raffaele Parise and Luís Eduardo Parise, for the companionship throughout my lifetime. To Leonardo Tavares Dias, my brother at heart. To my beloved husband, Emanuel Scherdien da Rosa, who brightens up my life, and whose tenderness, smiles, and patience have been so important in keeping me going, especially during the years when a whole ocean was keeping us apart. To my aunt and uncle, Patricia Jackson and Airton Parise, for all the encouragement throughout my life and support when I arrived in Europe. To all my family, as we are the product of our upbringing and the people who surround us. To all my forebears, because *le radici sono importanti*.[1]

[1] Quoted from *La Grande Bellezza*, a 2013 film by Paolo Sorrentino

I would like to thank all the friends I left behind when I embarked on this adventure in the United Kingdom, but who I nevertheless feel so close to, and whom I have the joy of meeting occasionally. Especially, Ana Luiza Leite Bado, Carlos Eduardo Santos, Daniel Barboza Capella, Fabiola Eckert, Frederico Rottgers Marcineiro, Gabriela Farias Gubert, Guilherme Souza, Karina Farina, Leonardo Kretzer, Luisa Binder, Maria Eduarda de Andrade Borges, Maurício Bueno, Theo Cardozo Bräscher, Tomás Honaiser Rostirolla, and Victor Coelho. You always give me another reason to return to Brazil.

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Similarly, I would like to thank my co-supervisors, Mark Tibbett and Francesco Tamagnini, for coming on board with this project to try to study the cognition of both fungi and plants. My thanks also go to the University of Reading and the Doctoral and Researcher College for placing such trust in me and granting me an International PhD Studentship, which was essential to support me in these three years. To the School of Biological Sciences and the School of Agriculture, Policy, and Development for receiving me with such an open and welcoming environment, and for my Monitoring Committee, Sofia Gripenberg and Martin Lukac for following me in those three years with advice and encouragement.

This doctorate could have been a solitary journey were it not for the people I met during its course and the friendships I made along the way. I would like to thank all my old and new friends who walked this path with me, in particular Ana Carolina Yamakawa, Ashinsa de Silva Wijeyeratne, Bárbara Lima, Carys Cunningham, Chris Pesso, Hannah Rhyannon Hall, Kerry Stewart, Nattanon Meeprom, Renan de Oliveira, Rosy Scholes, Sharon Mandipe, Vinícius Henrique de Oliveira, and all my colleagues and friends in the School of Biological Sciences and the School of Agriculture, Policy, and Development.

My gratitude also goes to all the people and friends with whom I collaborated during the course of this doctorate, including César Marín, Gabriela Niemeyer Reissig, Margherita Bianchi, Michael Marder, Monica Gagliano, Silvia Guerra, Thiago Francisco de Carvalho Oliveira, and Umberto Castiello. I would also like to extend my gratitude to all the administrative and technical staff of both Schools and the University for all their support during the development of this research, which was crucial for its success.

Finally, I would like to acknowledge the V. Kann Rasmussen Foundation for the grant received halfway through the course of this doctorate, which effectively allowed me to embark on a new but related research direction and securing a post-doctoral position at the University of Reading to continue the work commenced with this thesis.

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Impact statement

Of the six chapters in this thesis, three are currently published in peer-reviewed journals:

Parise AG, Marín C, Tamagnini F, Tibbett M, Pickles BJ. (2025). How mycorrhizal fungi could extend plant cognitive processes. Symbiosis, https://doi.org/10.1007/s13199-025-01065-y

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Chapter 5 has been successful as a grant proposal with the V. Kann Rasmussen Foundation.

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"*What we see is not nature itself, but nature exposed to our method of questioning*"

Werner Heisenberg

Abstract

The cognitive ecology of non-neural organisms like plants and fungi is a new and controversial research field that has gained momentum since the turn of the century. Many studies have suggested that plants and fungi perceive and respond plastically to their environment, implement behaviours that maximise their chances of survival, and that they have the ability to store memories, learn and communicate. However, little is known about how these phenomena occur and what underpins it. This is not only a scientific question, but also philosophical, with deep implications for what we understand by cognition. This thesis sought to contribute to this debate by focusing on the symbiotic relationship between mycorrhizal fungi and plants. After a general introduction situating the thesis in the epistemological debate and describing the challenge of establishing methods to study the cognitive ecology of plants and fungi in Chapter 1, Chapter 2 departs from the post-cognitivist tradition to build the hypothesis that the cognitive process of plants can be extended to that of mycorrhizal fungi when they are in symbiosis. Chapter 3 describes a failed attempt to test this hypothesis with the use of Perspex microcosms. Chapter 4 focused on the putative cognition of ectomycorrhizal fungi and how memory could be involved in its foraging behaviour, a hypothesis not supported by the evidence gathered during this study. Chapter 5 describes the successful attempt of using electrophysiological equipment to record the spontaneous and evoked electrical signalling of different fungal species, suggesting that this signalling could have the key to understand, in part, the complex and plastic behaviours these organisms present. The thesis concludes with Chapter 6, a rumination on the philosophical and practical challenges of both traditional and alternative views of cognition in non-neural organisms.

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Chapter 1: General Introduction

1.1 The meeting of opposite paradigms

Thomas Kuhn (1970) wrote that science evolves through the repetition of two periods: there is a period of "normal science", where the work of every scientist in a particular area adds up to a general body of knowledge relatively well established; and then there is the period of "revolution", where science passes through a time of crisis followed by a change in the paradigm that guides the scientific enterprise. Sometimes, one is lucky enough to witness this change with their own eyes.

A paradigm is a body of ideas, practices, and understandings that serves as a framework to one or more branches of a scientific discipline. It not only helps in understanding the world but also provides the pathway to explore what is still not known in this very same world. Ideas that do not belong to the dominant paradigm are, at best, dismissed as inaccurate, insufficient, and wrong. Yet, paradigms are not perfect, and they cannot accommodate all the knowledge generated through the scientific development during the reign of a particular paradigm, the period known as 'normal' (Kuhn 1970). Time and again, evidence that does not fit into the dominant paradigm, or even contradicts it, accumulates, and at a certain point, the paradigm enters into a crisis. This is a period of confusion and great creativity, where many different ideas start to coexist and compete for the acceptance of the majority of the scientific community. Eventually, for reasons that lie beyond the scope of this essay, a new paradigm is accepted by this majority in a particular area of knowledge, and it becomes dominant. The alternative paradigms are pushed to the periphery of science or even disappear completely. Everything settles into a new period of normality, to which the scientific research adds gradually until a new crisis emerges (Kuhn 1970).

This process was eloquently described by Thomas Kuhn in his influential book *The Structure of Scientific Revolutions*, originally published in 1962. He described the crisis and emergence of new paradigms with several examples, from the Copernican revolution to the rise of modern physics and chemistry. Examples of such crises include astronomical observations that contradicted the dominant geocentrism from the 16th century onwards (Kuhn 1970), the data in favour of the Darwinian evolution that challenged the dominant paradigm of creationism in the late 19th century (Kuhn 1970),

and palaeontological evidence that challenged the established catastrophist theory postulated by the French naturalist Georges Cuvier and his followers, also in the 19th century (Faria 2012). In all these cases, the crisis was initiated by 'anomalies' that did not fit the dominant paradigm such as, respectively, the movement of heavenly bodies incompatible with the geocentric theory, fossils showing the gradual evolution of species and the active change of species' anatomy through breeding, demonstrating that species are not immutable; and the findings of human fossils together with extinct animals, suggesting the coexistence of humans with fauna predating the world created by God as described in the Bible (Faria 2012).

Currently, there is a movement occurring in both biological and cognitive sciences which, if not driving a crisis of the dominant paradigm of both disciplines, could be at least seen as challenging it. Despite its roots in the late 19th century (e.g., Darwin and Darwin 1880; Verworn 1889; Binet 1891), the contemporary movement probably commenced somewhere around the turn of this century, and is caused by accumulating evidence from research on non-neural organisms like plants, fungi, bacteria, and slime moulds, showing behaviours compatible with what could be called cognition in these organisms. Research from different groups with different organisms describes phenomena comparable with memory (Casadesús and D'Ari 2002; Reid et al. 2012; Pissolato 2024), learning (Gagliano et al. 2014; Boisseau et al. 2016; Abramson and Chicas-Mosier 2016), problem-solving (Trewavas 2005), attention (Parise et al. 2021; 2022), communication (Miller and Bassler 2001; Karban et al. 2014; Briard 2020), anticipation (Schwartz and Koller 1986; Koller and Levitan 1989; Shemesh et al. 2010), and several other so-called cognitive phenomena. These studies and claims have been met with great scepticism (e.g., Flannery 2002; Firn 2004; Alpi et al. 2007; Rehm and Grandmann 2010), interestingly not only because of a presumed lack of scientific rigour of at least some of these works, but also because—it could be argued—their interpretation of the data does not fit what the dominant paradigm on cognitive sciences and plant sciences predicate, which is, respectively, that organisms without brains cannot be cognitive and, consequently plants and fungi are reactive, biochemical machines (Adams 2018; Lee 2023). However, the surprisingly sophisticated behaviour of non-neural organisms cannot be explained by mere reactions to the environment, and the results obtained by these studies could be considered as an 'anomaly' under the current paradigm.

It is difficult to pinpoint exactly what is the current paradigm of the cognitive sciences and botany, especially because the philosophy and history of science usually deal with events that have already passed, elaborating in hindsight on what occurred. The philosophy of science is an owl that only comes out after dusk to observe what happened in the previous day[1]. Nevertheless, it is safe to say that plant sciences do not contemplate the suggestion of plants and fungi as cognitive organisms (Taiz et al. 2015), and that contemporary cognitive science is heavily influenced by the cognitive revolution of the 1950s. Therefore, cognition is broadly understood as a process of computation where information is processed obeying a set of formal rules to produce outputs (Pylyshyn 1986; Dennett 1991; Lee 2023), usually, behavioural ones, that allow the organism to make sense of the world and act productively in it. Hence, cognition needs a processor—a centralised unit that receives information from the sense organs, processes it, and commands the response to the stimuli received. This powerful processor happens to be the brain, for which its unique and complex structure made of neurons in their on-or-off states—just like computers with their transistors—renders it analogous to a computer. Consequently, the possession of a brain has implicitly become mandatory to ascribe cognition to any system.

Cognition as a phenomenon undeniably encompasses other phenomena such as memory, learning, decision making, agency, attention, and communication, among many others, which are collectively called cognitive capabilities; all of them depending on a brain to happen. Without a brain, these phenomena cannot occur naturally. Hence, plants, fungi, bacteria, and any non-neural organism are *a priori* barred from having any of these capabilities or being cognitive. This was not always the case. During the late 19th century and early 20th century, heavily inspired by the newly established theory of evolution, several scientists believed that the study of basal organisms like protists and plants could provide insights into the origin and evolution of the human mind (Binet 1891; Yerkes 1913; Warden 1928; Castiello 2021)—something many contemporary cognitive scientists might have forgotten. In other words, if the mind exists, and was not created out of nothing by the breath of God[2], then it evolved from something. The study of simpler organisms could provide cues about the building blocks of the mind and its origin from

[1] "The owl of Minerva spreads its wings only with the falling of the dusk" ("*Die Eule der Minerva beginnt erst mit der einbrechenden Dämmerung ihren Flug*.") (Hegel 1911)
[2] "And the LORD God formed man of the dust of the ground, and breathed into his nostrils the breath of life; and man became a living soul." The Bible, King James Version, Genesis 2:7

simpler forms. This is why protists were a popular subject in comparative psychology up until the 1930s (Warden et al. 1935). These studies were ignored when the cognitivist paradigm became dominant somewhere in the second half of the 20th century.

However, around the end of that same century, studies with non-neural organisms like plants and slime moulds started to show that these organisms possess several cognitive abilities comparable to those of brained animals, some of them described above (Kelly 1990; 1992; Nakagaki et al. 2000; Trewavas 2003). These studies clash with the assumption that these organisms are only reactive to their immediate conditions, unable to engender more sophisticated behaviours. This evidence contradicts the dominant paradigm and consequently, risks accusations of being inaccurate, insufficient, and wrong.

The challenge to the cognitivist paradigm, and to traditional plant physiology, was made explicit in 2002 and 2003, when Prof. Anthony Trewavas published a short communication in *Nature* (Trewavas 2002), and then a longer paper in the journal *Annals of Botany* explicitly proposing plants as intelligent organisms (Trewavas 2003). Three years later, Brenner et al. (2006) published another influential article in *Trends in Plant Science* proposing the field of *plant neurobiology* to study the signalling, behaviour, and cognition of plants. At this point, critics of the newly-developing field begun to muster, and a heated exchange of papers was produced both against and for the claims regarding plant cognition, intelligence, and neurobiology (e.g., Flannery 2002; Firn 2004; Alpi et al. 2007; Rehm and Grandmann 2010). An analysis of this conflict could easily yield a whole doctorate on its own, and the arguments pertinent to the present work are addressed in their respective chapters when applicable, so it will not be discussed here.

Nevertheless, a fair amount of this controversy comes from, precisely, the clash of the dominant epistemological tradition with alternative ones that seek to explain the behaviours observed in plants (Adams 2018; Segundo-Ortin and Calvo 2019; Lee 2023). In effect, the impossibility of explaining the extraordinary behaviours found in plants and other non-neural organisms led researchers to adopt alternative approaches (one might say, paradigms), such as frameworks stemming from the post-cognitivist traditions developed from the 1970s onwards, which are more liberal regarding the vehicles of cognition. These approaches involve, for example, non-representational views of cognition, as proposed by James J. Gibson (1966), the autopoiesis framework proposed

by Humberto Maturana and Francisco Varela (Maturana and Varela 1980), Bateson's (1972) ecology of mind; embodied cognition (Shapiro 2019), and enacted cognition (Varela et al. 2016). These approaches to cognition, being more grounded on biology instead of philosophy[3], have been slowly but increasingly accepted, not only in cognitive sciences in general (e.g., Newen 2018; Buzsáki 2019) but also, to a degree, in biology as a response to the facts raised by studies on non-neural cognition (Lyon et al. 2021).

Thomas Kuhn (1970) warned that alternative paradigms may coexist in the periphery of the dominant one, usually kept by a small community of scientists. Once the dominant paradigm enters into a period of crisis, other paradigms compete for acceptance. Although it might be premature to claim that the dominant paradigms of both cognitive science and plant science are near any crisis, the fact remains that nowadays, research into the cognitive abilities of non-neural organisms has reached mainstream science, and is published in mainstream journals (e.g., Nakagaki et al. 2000; Calvo et al. 2020; Lyon et al. 2021). There is a tension between traditional and alternative paradigms, because, if studies on non-neural cognition are made with the necessary scientific rigour and a solid epistemological basis—therefore, accomplishing their necessary role of bearing the burden of proof—, the dominant paradigm is called to explain the remarkable behaviours observed in non-neural organisms. If it fails, it may enter into a crisis. And being alive to witness the friction between antagonistic paradigms is a privilege that few researchers have had. The next decades will tell how this conflict will unfold. What is certain, however, is that the only way of advancing this debate is with more data, and with well-designed experiments to test the pertinent hypotheses about non-neural cognition. The goal of this doctoral research is to contribute to this debate.

1.2 What is this thesis about

The present thesis has no intention to change any paradigm in any way. I would not be so pretentious. Nevertheless, this thesis was not produced in a vacuum, and it undoubtedly finds itself in the context outlined above. Similarly, this is not research with an agenda, but it clearly departs from alternative, post-cognitivist understandings of cognition to

[3] Philosophy heavily influenced the thinking of cognitive sciences. For example, the implicit mind-body dualism, very common in cognitive sciences, finds its roots through Descartes' meditations (Descartes 1874) down to Plato's dualism. A good discussion on this subject can be found in Buzsáki (2019)

develop experiments that could, potentially, test the hypotheses of cognition in plants and fungi. The lack of experimental data is a real issue for advances in the field of non-neural cognition, and in addition to this, the lack of established *methods* to study non-neural cognition in plants and fungi is an important hindrance that needs to be addressed. This kind of research is so new that there are no established methods and protocols to study cognitive phenomena in non-neural organisms with a biology so different from that of animals. Hence, this thesis proposes to develop methods to study some cognitive phenomena of plants and fungi, with the aim of contributing to the debate and to the understanding of what plants and fungi are capable of. Cognition is not something that happens inside an organism isolated from the world, but in interaction with the world. Therefore, to understand what an organism is doing in cognitive terms, it is crucial to study cognition *in context* with the environment presented to the organism. This is why I refer to *cognitive ecology* in the title of the thesis. Cognitive ecology is nothing more than the study of cognitive phenomena in its natural and social context (Hutchins 2010). In this thesis, the cognitive ecology of plants and fungi was studied.

However, like many doctorates, it followed a convoluted path to reach this point. From an initial proposal, it drifted in several directions before stabilising in a completely different and unexpected course. Nevertheless, all this wandering was made under the proposal of working with non-neural cognition.

I had never worked with mycorrhizas before. My previous experience was on the electrophysiology of plants and its relationship to the presumed plant cognition (e.g., Parise et al. 2021; Parise et al. 2022; Parise et al. 2023). However, during my Master's research and the hiatus of almost three years before starting this doctoral research (courtesy of the COVID-19 pandemic), I have been developing with collaborators the concept of Extended Plant Cognition (EPC), a very post-cognitivist proposal of plants as not only cognitive organisms, but whose cognitive process extends beyond their bodies to the environment they influence and shape through volatile organic compounds, root exudates, and the harnessing of soil microbiota, including mycorrhizal fungi (Parise et al. 2020; Parise and Marder 2024). From all these means of extension, mycorrhizal fungi seemed the least explored and the most promising. This doctoral research started with the aim of testing empirically the presumed extended cognition of plants through ectomycorrhizal fungi. Therefore, the next chapter (**Chapter 2**) is a literature review where this hypothesis is articulated in detail. Therein is explained how EPC relates with

alternative views of cognition—in particular the post-cognitivist 4E model of cognition which proposes it as an Embodied, Embedded, Enacted, and sometimes Extended phenomenon, and four case studies are drawn from the literature in support of our argument.

Subsequently, I set out to develop an experiment to test this hypothesis empirically. I tested whether the ectomycorrhizal fungus *Suillus granulatus* would provide its host (seedlings of Scots pine, *Pinus sylvestris*) with information about the structure of the environment around the roots and guiding them in complex environments, thereby extending the plant's perception of the belowground environment (**Chapter 3**)—a cognitive task that likely would require mycorrhizal fungi as an extension of the plant's perceptive system to accomplish. This experiment would implement the same technique of classical ectomycorrhizal experiments to address a contemporary question. Unfortunately, the experiment did not work. Not because the hypothesis was rejected after rigorous experimentation, but because of technical and biological issues that arose during the course of the experiment and that rendered the hypothesis impossible to test. Despite this unsuccessful outcome, it was decided to report the experiment regardless because even these failures could be useful for other scientists trying to employ the same technique. Besides, an important conclusion of this experiment was that the *P. sylvestris*-*S. granulatus* system should be abandoned for this doctorate. The development of *P. sylvestris* seedlings is so slow that it makes it a risky subject for a three-years doctoral research. Repeating this experiment would take at least other four months and, if it were unsuccessful again, I would have been close to the middle of the doctorate without any data.

On the advice of my supervisors, I decided to switch to more rapid experiments of the kind that could yield data in less time, and we focused on fungal behaviour and cognition, an emerging field that started to be explored quite recently (Fukasawa et al. 2020; Aleklett and Boddy 2021; Money et al. 2021; Richter et al. 2024). Furthermore, fungal cognition was less explored than plant cognition, and demonstrating that fungi too are cognitive could strengthen the hypothesis of EPC through mycorrhizas. Inspired by Fukasawa et al. (2020) and their study with the saprotrophic fungus *Phanerochaete velutina*, I devised a method to test whether the ectomycorrhizal fungus *Laccaria bicolor* could present some form of directional memory of a source of nutrients that was present in the past. This fungus was chosen because it grows well and rapidly in artificial

medium, it has a distinct morphology that could be useful to test the hypothesis, and has the additional advantage of having its genome completely sequenced, which would allow deeper investigation should we find promising results. This experiment is described in **Chapter 4**. Differently from Chapter 3, the experiment worked mostly as it should but we could not corroborate our hypothesis. Nevertheless, the methods developed here could also be helpful for scientist interested in fungal behaviour, so again we decided to report this experiment, and it is currently published in the journal *Communicative & Integrative Biology*.

Around the time Chapter 3 was being developed, during a course on epistemology of science, I had a serendipitous encounter with Dr Francesco Tamagnini, a neuroscientist who, on learning about my past research on plant electrophysiology, was very much interested and proposed a collaboration. I explained I was studying EPC through mycorrhizas, and how much I would like to investigate the role of electrical signalling in this symbiosis and in the putative extended cognitive system of plants. To test if this hypothesis would be feasible, Dr Tamagnini and I started some pilot experiments on pure mycelium of different ectomycorrhizal fungal species growing on agar, investigating whether they have a detectable electrical activity and whether they respond to electrical stimulation. At the time, I was also invited by the V. Kann Rasmussen Foundation (New York, NY, USA) to submit a research proposal on the topic of "*Sentience and Cognition in Nature*". Therefore, we submitted our preliminary results with the proposal of studying the electrophysiology of ectomycorrhizas, starting with that of the fungi. To our joy, the project was accepted and we received a grant to buy a sophisticated equipment for studying electrophysiology and funding for one year of postdoctoral research for me after completing the doctorate. The expanded version of the grant submitted is what constitutes **Chapter 5**. I am currently learning to use the equipment and doing the first tests with the machine and hopefully, in the near future, we will have results to share with the scientific community and the society. This is the direction I am pointing to at this moment.

As a final note, to avoid the repetition of the same references, despite this thesis being organised as a collection of publications, all the references are listed together at the end of the document.

Chapter 2: How mycorrhizal fungi could extend plant cognitive processes

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How mycorrhizal fungi could extend plant cognitive processes

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Abstract
Traditionally, mycorrhizas are studied for their role in plant health and nutrition through a mutually beneficial exchange of solutes. Recent research has revealed additional roles for mycorrhizas, including shaping plant communities and enhancing stress resistance. However, a critical aspect for the survival of organisms remains largely ignored in the study of mycorrhizal symbioses: cognition. This review explores the possibility that plants benefit from the cognition and behaviour of mycorrhizal fungi to enhance their own survival. We examine four case studies that are suggestive of plants extending their cognition through mycorrhizal associations: i) foraging complementarity between roots and mycorrhizal fungi; ii) recruitment and abandonment of mycorrhizal fungi depending on the host plant nutritional status; iii) expanded perception of the below-ground environment; and iv) shaping the mycorrhizal community to meet survival needs. Whilst extended plant cognition is implied, direct experimental evidence corroborating this hypothesis is needed, and we propose a delimiting criterion with suggestions of experiments to test this hypothesis.

Keywords Functional complementarity · Plant cognition · Root foraging · Root traits · Functional team selection · Fungal behaviour

1 Introduction

The mycorrhizal symbiosis is one of the most important symbioses in the living world. Plants and fungi developed a partnership so successful that it has lasted over 400 million years (Remy et al. 1994; Bidartondo et al. 2011; Strullu-Derrien et al. 2018) and may have enabled plants to colonise the dry landmasses of the planet, transforming them into prolific habitats for terrestrial lifeforms (Pirozynski and Malloch 1975; Smith and Read 2008). There are several types of mycorrhizas, the four major being arbuscular, ectomycorrhizal, orchid, and ericoid, but the list is growing with the ongoing research on these underground mutualisms (Kariman et al. 2018, 2024; Howard et al. 2022; Furtado et al. 2023; Lutz et al. 2025). Yet, despite their importance, much remains to be discovered about mycorrhizal relationships. For example, the mechanisms by which plants and mycorrhizal fungi communicate to form and secure the symbiotic association are poorly understood (Müller and Harrison 2019; Boyno and Demir 2022).

Early research on how plants benefit from the mycorrhizal symbiosis mainly focused on antibiotics produced by the fungal partner (e.g., Zak 1964; Marx 1966, 1972; Marais and Kotzé 1976) and nutrient exchange between the two partners, specifically carbon transfer to the fungus and plant uptake of phosphate and nitrogen (e.g., Clarkson 1985; Nolan 1991; Koide 1991). Recent research on mycorrhizas has demonstrated the importance of this symbiotic relationship regarding different processes including plant competition, plant-fungal signalling, resistance to stresses, seedling survival, and ecosystem services (e.g., Bingham and Simard 2012; Wagg et al. 2014; Stanescu and Maherali 2017; Yu et al. 2022; Kakouridis et al. 2022). Despite the rich body of

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2.1 Abstract

Traditionally, mycorrhizas are studied for their role in plant health and nutrition through a mutually beneficial exchange of solutes. Recent research has revealed additional roles for mycorrhizas, including shaping plant communities and enhancing stress resistance. However, a critical aspect for the survival of organisms remains largely ignored in the study of mycorrhizal symbioses: cognition. This review explores the possibility that plants benefit from the cognition and behaviour of mycorrhizal fungi to enhance their own survival. We examine four case studies that are suggestive of plants extending their cognition through mycorrhizal associations: i) foraging complementarity between roots and mycorrhizal fungi; ii) recruitment and abandonment of mycorrhizal fungi depending on the host plant nutritional status; iii) expanded perception of the below-ground environment; and iv) shaping the mycorrhizal community to meet survival needs. Whilst extended plant cognition is implied, direct experimental evidence corroborating this hypothesis is needed, and we propose a delimiting criterion with suggestions of experiments to test this hypothesis

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2.2 Introduction

The mycorrhizal symbiosis is one of the most important symbioses in the living world. Plants and fungi developed a partnership so successful that it has lasted over 400 million years (Remy et al. 1994; Bidartondo et al. 2011; Strullu-Derrien et al. 2018) and may have enabled plants to colonise the dry landmasses of the planet, transforming them into prolific habitats for terrestrial lifeforms (Pirozynski and Malloch 1975; Smith and Read 2008). There are several types of mycorrhizas, the four major being arbuscular, ectomycorrhizal, orchid, and ericoid, but the list is growing with the ongoing research on these underground mutualisms (Kariman et al. 2018, 2024; Howard et al. 2022; Furtado et al. 2023; Lutz et al. 2025). Yet, despite their importance, much remains to be discovered about mycorrhizal relationships. For example, the mechanisms by which plants and mycorrhizal fungi communicate to form and secure the symbiotic association are poorly understood (Müller and Harrison 2019; Boyno and Demir 2022).

Early research on how plants benefit from the mycorrhizal symbiosis mainly focused on antibiotics produced by the fungal partner (e.g., Zak 1964; Marx 1966, 1972; Marais and Kotzé 1976) and nutrient exchange between the two partners, specifically carbon transfer to the fungus and plant uptake of phosphate and nitrogen (e.g., Clarkson 1985; Nolan 1991; Koide 1991). Recent research on mycorrhizas has demonstrated the importance of this symbiotic relationship regarding different processes including plant

competition, plant-fungal signalling, resistance to stresses, seedling survival, and ecosystem services (e.g., Bingham and Simard 2012; Wagg et al. 2014; Stanescu and Maherali 2017; Yu et al. 2022; Kakouridis et al. 2022). Despite the rich body of literature on the relationship between plants and mycorrhizal fungi, there are several aspects of these symbioses still to be uncovered. In particular, how mycorrhizas relate to the likely cognition of plants.

2.3 Cognition from brains to biology

Every living organism needs to monitor fluctuations in environmental conditions and rapidly respond to them in order to keep its self-organisation functioning properly (in other words, its homeostasis) (Maturana and Varela 1980). However, it is not adaptive to only react to environmental cues because cues sensed at the present may not reliably indicate future conditions. If an organism were only reactive—that is, capable only of immediate, inflexible responses to stimuli without modulation or anticipation (a common criticism to non-neural cognition), it could not prepare to what is coming next, nor could it improve its current conditions to maximise survival (Okasha 2024). Therefore, organisms need some plasticity to deal with unexpected and unpredictable variations in environmental conditions, especially combining past experiences to improve future responses (Sims 2023). Without the ability to perceive the environment, integrate what is perceived, improve its responses over time, and act with anticipation, it is likely impossible to survive for long. This dynamic relationship between living systems and the environment, paired with the ability to respond to internal processes and to predict, process and to flexibly adapt to ever changing environmental conditions, is what we refer to as cognition (Maturana and Varela 1980; Souza et al. 2018; Bechtel and Bich 2021; Lyon et al. 2021). As we will explore below, this working definition contrasts with classical views of cognition, and aligns with biological, rather than purely computational, models to explain this phenomenon.

Cognition is a complex and contentious concept with no universally accepted definition despite over a century of research on it (Bayne et al. 2019). Neisser (1967) defined cognition as referring to “all the processes by which the sensory input is transformed, reduced, elaborated, stored, recovered, and used. It is concerned with these processes even when they operate in the absence of relevant stimulation”. A similar

definition was adopted over forty years later by Shettleworth (2010) in a very influential book on compared cognition. These views on cognition often implicitly or explicitly exclude non-neural organisms from the realm of the cognitive—largely due to a cognitivist tradition that emerged alongside and was influenced by early developments in symbolic artificial intelligence and computationalism. This tradition characterises cognition as the manipulation of discrete symbolic representations according to formal rules—a view that equates cognition with digital information processing, and mirrors the architecture of early computer systems receiving inputs and providing outputs (Pylyshyn 1986; Miller 2003; Piccini and Scarantino 2011). Such an understanding may obscure alternative models of cognition because it requires a brain to fulfil the role of central processor in this scheme, thereby automatically barring non-neural organisms from being considered cognitive. Despite being influential, this approach to cognition is not unanimous, and less brain-centric alternatives have existed for decades and are gaining traction recently (Gibson 1966; Bateson 1972; Maturana and Varela 1980; Souza et al. 2018; Bechtel and Bich 2021).

Today, it is clear that the brain does not work as a computer processing inputs and providing outputs (Dreyfus 1992; Brette 2019; Buzsáki 2019; Richards and Lillicrap 2022). Rather, the brain is an active element in our cognitive system, actively seeking stimuli and creating information in interaction with our bodies and the environment (Buzsáki 2019). Alternative approaches to the cognitivist programme recognise that cognition emerges from the functioning of the whole body in interaction with the environment, and ground cognition in biology rather than philosophy, where cognitive science has its roots (Buzsáki 2019). Cognition is thus understood as the dynamic process of an organism interacting with the environment and modifying its behaviour to keep its self-organised structure functioning properly (Maturana and Varela 1980; Souza et al. 2018; Lyon et al. 2021). It enables biological systems to flexibly cope with environmental fluctuation depending on both external and internal (i.e., physiological) circumstances, giving rise to complex and adaptive behaviour, eventually leading to the most complex forms of cognition that we are presently aware of, including human intelligence. The focus, then, is not on subjective experiences and how the brain processes information, but rather, on the process that organisms enact to flexibly adjust their homeostasis and behaviour to meet existential needs such as nutrition, growth, and

reproduction. Within this framework, it is perfectly possible for organisms without neurons, like plants, fungi, and bacteria, to be regarded as cognitive systems.

The idea that non-neural organisms can be cognitive is not new. In fact, it can be traced back to the origins of psychology itself. Alfred Binet, the inventor of the IQ test, wrote a whole monograph on "The psychic life of micro-organisms" (Binet 1891), where he described several aspects of protist behaviour. He was not the only one to do so (Verworn 1889; Jennings 1904). Charles Darwin and his son Francis studied the movement of shoot and roots in several plant species, and famously compared the behaviour of roots in particular to that of "one of the lower animals" (Darwin and Darwin 1880). According to the authors, the ability of root tips to respond to the environment and direct the movement of the adjoining root makes them functionally comparable to the anterior body part of organisms like worms when they are foraging in the soil (Darwin and Darwin 1880). Despite these pioneering works, the study of non-neural forms of cognition was never mainstream in science. Nevertheless, in the last couple of decades, there has been a 'renaissance' of these kinds of studies, and knowledge on the cognitive capacities of organisms like bacteria (Shapiro 2007; Bechtel and Bich 2021), slime moulds (Latty and Beekman 2011; Boussard et al. 2021), fungi (Aleklett and Boddy 2021; Fukasawa et al. 2020, 2024; Marín and Suárez 2024), and plants (Trewavas 2003, 2016; Brenner et al. 2006; Gagliano 2015; Souza et al. 2018; Calvo et al. 2020) has undergone a significant development and increasing acceptance.

Lyon et al. (2021) developed the concept of "basal cognition", the most basal form of cognition that is observed in every living organism, and from which all taxa in the tree of life are considered to have evolved their own form of cognition according to the complexity of their bodies and sensorial and enactive apparatuses. Basal cognition comprises sub-phenomena like memory, communication, problem-solving, anticipation, and sensing/perception, among others. The reader is invited to refer to Lyon et al. (2021) for the full list with an explanation of what these sub-phenomena are. This all-inclusive approach to cognition, embraced by many authors (e.g., Cazalis et al. 2017; Bechtel and Bich 2021; Lyon et al. 2021; Shapiro 2021) is the one we adopt here.

Specifically, we adopt the "4E model" of cognition, which considers cognition as an Embodied, Embedded, Enacted, and often Extended process (Calvo Garzón 2007); Dawson 2014; Newen et al. 2018). The first three Es are relatively straightforward:

cognition requires a body (it is Embodied), it is inextricable from the environment (it is Embedded), and it expresses itself through actions in the world (it is Enacted). The fourth E, Extended cognition, is the most controversial idea: that cognition can happen partly outside an organism's body (Clark and Chalmers 1998; Clark 2008; Menary 2010). Despite evidence for this form of cognition in mammals, arthropods, and even non-neural organisms, testing this empirically is challenging (Parise et al. 2023). Kaplan (2012) proposed using Craver's (2007a,b) mutual manipulability criterion to solve this issue. This criterion predicts matched inter-level interventions (Craver 2007a, b; Craver et al. 2021) between the cognitive system (organism) and the object, such that manipulation of the organism causes an alteration of the object, and manipulation of the object causes an alteration in the (cognitive) functioning of the organism. Extended cognition may partly explain how organisms with minimal or no brains perform complex cognitive behaviours. For example, Japyassú and Laland (2017) proposed that a putative extension of spider cognitive process to their spiderwebs could explain the highly complex behaviours of some spiders (considering the size of their brains), and Sims and Kiverstein (2022) argued that secreted slime may be an external element of the memory of slime moulds. Parise et al. (2020) argued that plants could extend their cognition as well, and that extended cognition may be more common in nature than previously imagined (Parise et al. 2023).

Given the discussion on a contemporary understanding of cognition above, we aim to explore the cognition of plants and fungi, and the idea that plants may benefit from the behaviour of mycorrhizal fungi through a process called extended cognition (Clark and Chalmers 1998; Parise et al. 2020). We propose that, due to the close link between plants and mycorrhizal fungi, their cognitive abilities may overlap somewhat, with the fungi becoming part of the system by which plants perceive and act in the world. We analyse four case studies to strengthen our hypothesis, and conclude that extended plant cognition (EPC) through mycorrhizas is a plausible hypothesis, but requires original studies designed to test it specifically and confirm whether it happens in nature and how it mechanistically works.

2.4 Cognition in plants and fungi

Conceptualising plants as cognitive systems has been controversial, sparking much debate since this hypothesis started to feature in mainstream journals (Flannery 2002; Trewavas

2002, 2003, 2004; Firn 2004; Adams 2018; Chamovitz 2018; Segundo-Ortin and Calvo 2019; Calvo et al. 2020). We do not address this debate here but note that the controversy comes mostly from the clash of two radically different epistemological traditions: one that sees cognition as necessarily tied to a brain or central nervous system, and another that sees cognition as a requirement for every living organism, as discussed in the previous section.

Regardless of the definitions adopted, it is undeniable that plants present behaviours usually considered cognitive such as learning and memory (Thellier and Lüttge 2013; Gagliano et al. 2014; Crisp et al. 2016; Galviz et al. 2020), communication between plants and between plants and other organisms (Oldroyd 2013; Karban 2015; Ninkovic et al. 2020; Falik et al. 2023), decision-making (Runyon et al. 2006; Dener et al. 2016; Gagliano et al. 2017; Gruntman et al. 2017; Née et al. 2017; Wang et al. 2023), and speed-accuracy trade-offs (Ceccarini et al. 2020). Plant anticipatory behaviours are particularly relevant because they cannot be fully explained as mere reactions to environmental stimuli. In these cases, plants respond to likely future conditions based on past experiences and present stimuli (Novoplansky 1991; Shemesh et al. 2010; Latzel and Münzbergová 2018; Guerra et al. 2019). With no brains, the cognitive process of plants (and fungi) could be based on the plastic network structure of their bodies—for example on chemical and electrical signalling (de Toledo et al. 2019; Debono and Souza 2019; Adamatzky et al. 2022), on epigenetic regulation (Crisp et al. 2016; Latzel et al. 2016), and on reinforcement and interplay of metabolic pathways (Thellier and Lüttge 2013; Souza et al. 2018).

Fungi, for their part, have a network architecture that presumably allows processing of information (Adamatzky et al. 2022). Their behaviours and cognition are much less studied than that of plants, but this gap in the knowledge has begun to be addressed quite recently (Fukasawa et al. 2020; Aleklett and Boddy 2021; Aleklett et al. 2021; Marín and Suárez 2024). Other fungal studies indirectly show some cognitive abilities like the capacity to integrate environmental information to make decisions (Brown Jr et al. 1999; Hornby et al. 2001; Shareck and Belhumeur 2011; Sudbery 2011), memory (Caudron and Barral 2013; Ben Meriem et al. 2019; Fukasawa et al. 2020), and employing foraging strategies (Fukasawa and Ishii 2023). The likely involvement of electrical signalling in these processes is suggested by the production of electrical signals in response to environmental factors (Olsson and Hansson 1995), which can be

sophisticated enough to guide the steering of a robot in response to light stimulation through a fungus-machine interface (Mishra et al. 2024). These studies give a glimpse of what fungi are capable of. However, further research in fungal cognitive ecology is needed to understand how this phenomenon operates, and which is the extent of their cognitive capabilities.

According to the extended plant cognition (EPC) hypothesis, the cognition of fungi could be complementing that of plants (Parise and Marder 2023). The EPC hypothesis proposes that, since plants possess a rich sensorial apparatus, but no brain nor neurons, extending their cognitive process to the environment could partly explain their complex cognitive behaviours (Parise et al. 2020), i.e., the ones they implement to meet existential needs such as root foraging, fighting herbivores, and communicating. Plants shape their environment both physically (i.e., through root morphology) and chemically through substances released by their organs, and such modifications may encode information, increase their sensory abilities, and be responsible for external information processing (e.g., Falik et al. 2005; Karban et al. 2014; Wheeldon et al. 2021; Vismans et al. 2022). Plants potentially extend their cognition through at least four different channels: volatile organic compounds (VOCs), root exudates, rhizosphere microbiota, and mycorrhizal associations (Parise and Marder 2023).

In the following sections, we examine the possibility of mycorrhizal fungi being part of their host's cognitive system. This is intriguing because fungi are not simply objects in the environment but living organisms with cognitive systems of their own. In this case, our rationale is that: 1) plants are cognitive systems; 2) fungi are cognitive systems; 3) plants and fungi establish mycorrhizas whereby, when working as mutualists, they functionally become a single unit; therefore, 4) fungi are part of plant (extended) cognitive systems. Considering fungi as part of plant cognitive systems could change our perspective on this symbiosis, and adds an extra layer to the importance of soil health for plant development and resilience.

2.5 Extended plant cognition through mycorrhizal fungi

Mycorrhizal fungi establish a tight connection with plants through arbuscules in arbuscular mycorrhizas (AM)—where the fungus penetrate the root cells to establish a surface contact shaped like an arbuscle—, a Hartig net in ectomycorrhizas (ECM)—when

the fungus grows hyphae around the cortical cells of the roots to establish contact, although sometimes this structure is absent (see Furtado et al. 2023)—, or other interfaces. Mycorrhizas often exhibit an impressive contact surface between the cell membranes of both partners, where they exchange nutrients, peptides, miRNAs and hormones (Smith and Read 2008; Müller and Harrison 2019). In a study on *Lotus tenuis* roots, Mendoza and Pagani (1997) found six AM entry points per mm, with 400 cm of colonised roots on average suggesting at least 24,000 fungus-plant interfaces per plant even before considering the intimate fungus-plant interactions of arbuscules. In a study of the colonisation of *Allium cepa* by *Glomus mosseae*, every cm of root had 40.7 mm2 of plant-fungal contact (Cox and Tinker 1976). Similarly, in *Medicago truncultata*, 1 cm of root colonised by *Glomus intraradices* had 1–200+ arbuscules and 1–40 vesicles (Salzer et al. 1999). One plant may have millions of such connections, making it difficult to separate plants and AM fungi. This leads us to ask: i) beyond solutes, do plants and fungi signal each other about environmental conditions and their physiological statuses?, ii) does this symbiosis essentially fuse plant and fungal cognition together?, iii) do plants extend their cognition to mycorrhizal fungi? We think that a likely answer to all these questions is yes, and will try to address them in the following sections. Of course, these inquiries are valid only to the plants that form mycorrhizas. Whereas extended cognition may help plants improve survival, it is likely to be time- and context-dependent, and it is conceivable that plants are not always extending their cognition to mycorrhizal fungi. Likewise, not extending cognition does not necessarily impose fitness disadvantages to non-mycorrhizal plants—they can survive just as well—but overall, natural selection favours mycorrhizal plants in most environments (Maherali et al. 2016).

To explore our hypothesis that plants extend their cognition through association with mycorrhizal fungi, we examine case studies that could be considered plausible evidence. Unfortunately, since none of these studies were designed to test extended cognition, we cannot fully apply the mutual manipulability criterion outlined above, and our interpretation is necessarily limited. However, this does not invalidate the idea, especially considering that, overall, the behaviour of plants is significantly impaired without mycorrhizas, which already partially fulfils the mutual manipulability criterion. We nevertheless emphasise caution and note that future studies exploring this relationship should be specifically designed to meet the mutual manipulability criterion.

2.5.1 Case study 1: foraging complementarity between roots and mycorrhizal fungi

Nutrients are patchy and transient in soil, requiring plants to adjust their root growth dynamically and rapidly to forage efficiently and secure nutrient sources (Giehl and von Wirén 2004; Rajaniemi 2007). Plant roots are not particularly efficient in foraging (van Vuuren et al. 1996), but mycorrhizal fungi help plants immensely in this endeavour. For example, colonisation by AM fungi can increase plant N uptake by 3- to 12-fold (Hestrin et al. 2019). Mycorrhizal associations are so critical that plants may invest 20–30% of assimilated carbon into them (Ek 1997; Leake et al. 2004; Ji and Bever 2016). Most mycorrhizal fungi, especially AM, cannot survive without a plant partner (Smith and Read 2008), making this association obligate for many fungi.

Mycorrhizal fungi dramatically increase the extent and absorbing area of the plant-fungal system, facilitating contact with soil pores and particles and increasing its ability to forage for nutrients and water. Following the reasoning proposed by Leake et al. (2004), approximately 16.66 m of mycorrhizal hyphae provide the same surface area as 0.1 m of root. Yet, just 1 g of soil can harbour 200–600 m of ECM hyphae, and 2–8 m of AM hyphae (Leake et al. 2004). Read (1999) calculated the carbon cost to the host per unit of absorptive area and found that mycorrhizal hyphae were 10 times cheaper than root hairs, and 100 times cheaper than roots. In pot cultures of *Pinus taeda* colonised by *Pisolithus tinctorius*, mycelium accounted for 75% of the absorbing area, but only 5% of the plant-fungal belowground biomass (Rousseau et al. 1994). Essentially, mycorrhizal hyphae are cheaper, go farther, and can be rearranged more easily and rapidly without significant cost to the plant compared to roots. This makes them great candidates for being part of the foraging apparatus of plants and they can be more important than root proliferation in foraging (Tibbett 2000; Eissenstat et al. 2015). Foraging is not only about absorbing nutrients. It also requires finding resources and employing strategies to secure them (Cahill Jr et al. 2010). Foraging behaviour arguably requires cognition because it involves abilities such as decision-making and anticipation (Kelly 1990; 1992; Koch et al. 2004; Runyon et al. 2006; Grüter and Ratnieks 2011; Calhoun et al. 2014; Dener et al. 2016; Sandhu et al. 2018; Billard et al. 2020; Fukasawa and Ishii 2023). Hence, mycorrhizal fungi, being part of the foraging structure of plants, could also be part of the cognitive structure that foraging represents.

Plant species have different absorbing root thicknesses, which impact the precision of foraging. Studies with different tree species have demonstrated that foraging precision typically decreases with increasing root thickness, especially in ECM species (Liu et al. 2015; Chen et al. 2016; Cheng et al. 2016). However, fungal partners can help thick root plants to compensate for lack of foraging precision (Eissenstat et al. 2015; Cheng et al. 2016). This is particularly effective in ECM symbioses because many ECM fungi can extend their hyphae great distances in the soil (Agerer 2001). Hence, trees may delegate their foraging to fungi, especially in the case of ECM trees with thick roots. Since organic nutrients are patchy and ephemeral, thick-rooted trees cannot afford proliferating too many roots to secure these resources and may use mycorrhizal fungi to do the job for them.

Rosling et al. (2004) studied foraging preferences of *Hebeloma crustuliniforme* and *Piloderma fallax* associated with *Pinus sylvestris*. Plants and fungi developed well in microcosms when cultivated in pure *Sphagnum* peat, with the plants spreading their roots uniformly. However, when inoculated seedlings were cultivated in vertically divided microcosms, one half peat and the other a mineral soil, both fungi and roots preferred mineral soil, allocating 14C-label and roots in these substrates (**Figure 2.1**). Fungi may have detected the mineral soil as a better source of nutrients, and sent these nutrients to the plant, which preferentially allocated carbon to fungi in contact with the mineral soil. Hyphal growth and synthesis of enzymes and exudates requires carbon, but in turn makes more nutrients available to the plant. This feedback might stimulate further hyphal growth and guide the roots to follow the hyphal front to the richest area of resources. Ultimately, the plant benefits from more efficient foraging and root placement. Although predictors of plant success and improved fitness such as plant growth and nutrient uptake were not examined by Rosling et al. (2004), root behaviour appears to have been induced by the behaviour of hyphae, which is an interesting example of fungi potentially being part of the perception and action process of plants.

Mycorrhizas can also buffer potentially toxic effects of nutrient excess. In a study with *Eucalyptus marginata* and *Acacia celastrifolia*, both native to soils poor in phosphate (P), Tibbett et al. (2022) demonstrated that P-fertilisation beyond a certain threshold is toxic to *E. marginata*. However, in inoculated plants, AM fungi significantly restrict the amount of P incorporated into plant biomass, an effect not observed in the P-tolerant *A. celastrifolia*. This implies that AM mycorrhizas are required for ensuring the

homeostasis of the whole plant-fungal system in a challenging environment, where AM fungi regulate P-intake to maintain plant health. Such a process requires plant-fungus communication, with the plant using the fungal partner to solve a problem it cannot tackle alone. Hence, fungi appear to be an integral part of the system that perceives the environment and solves problems, implying extended cognition, although more studies are necessary to confirm the mechanisms behind the behaviour observed.

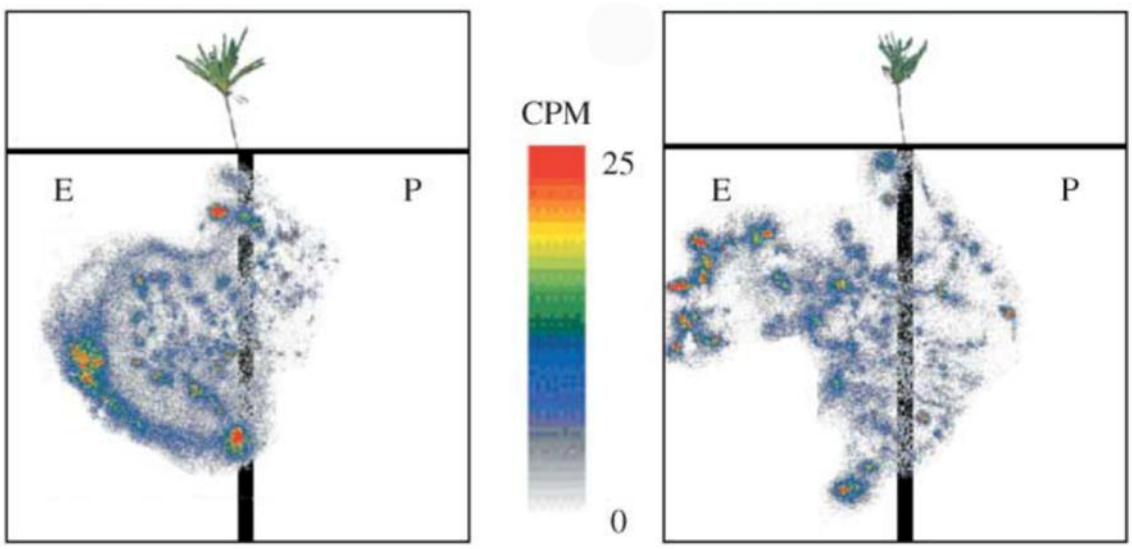

Figure 2.1. Figure from Rosling et al. (2004) showing ectomycorrhizal *Pinus sylvestris* cultivated in microcosms with *Hebeloma crustuliniforme* (left) or *Piloderma fallax* (right). The microcosms were vertically divided, and the left side contained mineral soil (E) and the right side, peat (P). Electronic autoradiography of labelled 14C shows the C allocation of both hyphae and roots, here represented as counts per minute (CPM). There is a clear preference of both partners for the mineral soil, despite the fact that they can grow well in peat only. The mechanisms behind this uneven choice of substrate could point to extended cognitive mechanisms where plants use mycorrhizal roots to find the best nutrient patches and distribute their roots more effectively. Reproduced from Rosling et al. (2004) with permission

2.5.2 Case study 2: plants recruit and abandon mycorrhizal fungi depending on their nutritional status

It is widely known that soil fertilisation or high inorganic nutrient availability inhibits mycorrhizal formation in both ECM (Jones et al. 1990; Nilsson and Wallander 2003; Sun et al. 2010; Corrales et al. 2017) and AM systems (Thingstrup et al. 1998; Ryan et al. 2000; Covacevich et al. 2008; Konvalinková et al. 2017; Zhang et al. 2016; Yazici et al. 2021). Mycorrhizal fungi respond differently to the type of nutrient (organic or inorganic) added to the soil (Allison et al. 2008; Avolio et al. 2009; Corrales et al. 2017; DeForest and Snell 2020), but this response can be mediated by the host (Avolio et al. 2009) and/or the fungal species (Corrales et al. 2017).

Nevertheless, plants seem to be very much in control of the symbiosis. Their roots attract mycorrhizal hyphae by secreting the signalling molecules strigolactones and flavonoids in the soil, which stimulate spore germination, hyphal growth, and branching, helping fungi to find the roots (Akiyama et al. 2005; Yoneyama et al. 2012; Decker et al. 2017; Tian et al. 2021). Strigolactone synthesis is influenced by nutrient starvation (Foo et al. 2013a; Decker et al. 2017). Yet, despite having an important stimulating role, strigolactones do not simply regulate the symbiosis through a linear chain of events (Foo et al. 2013a). This suggests that communication between plants and fungi is more complex than cause-consequence mechanisms and may involve feedback loops. Conversely, when plants experience high phosphate availability, they suppress or decrease mycorrhizal colonisation (Jones et al. 1990; Nilsson and Wallander 2003; Covacevich et al. 2008; Foo et al. 2013a; Eissenstat et al. 2015; Liu et al. 2015; Konvalinková et al. 2017; Zhang et al. 2016; Yazici et al. 2021; Bennett and Groten 2022), indicating that plants can control mycorrhizal colonisation depending on their nutritional needs. The proposed mechanism for suppressing AM colonisation is limiting the supply of carbohydrates, perhaps with the involvement of plant hormones like gibberellic and salicylic acids (Foo et al. 2013b; Yu et al. 2014). The mechanisms that govern this dynamic are not fully understood. Some researchers invoke biological markets where trade of carbon-for-nutrients is regulated by sanctions and rewards (Kiers et al. 2011; Wyatt et al. 2014; Hortal et al. 2017; Noë and Kiers 2018). Others, in turn, suggest that if plants cannot use all the C assimilated through photosynthesis, the surplus of C is sent to fungi, either as a mere surplus disposal (Corrêa et al. 2012; Prescott et al. 2020) or following a stoichiometry of resources, particularly C, P, and N (Johnson 2010). The debate, however, is not yet settled, and both hypotheses need more empirical evidence (Bunn et al., 2024). Nevertheless, the available evidence suggests that plants have some kind of control over the symbiosis which is based in their own physiological status.

Plant nutrient acquisition strategies depend on internal assessment of nutrient status and comparison with environmental nutrient availability. This trade-off can result in suppression of mutualistic partners under high nutrient conditions, or recruitment of mutualists to acquire nutrients when experiencing starvation (Johnson et al. 2010, 2014). Mycorrhizal associations can be facultative in many plants (Moora 2014; Meng et al. 2023), meaning such plants may employ mycorrhizas as a problem-solving strategy. Mycorrhizal fungi are sometimes described as extensions of the roots (Cheng et al. 2016;

Bunn et al. 2024), but beyond an extension of plant nutritional apparatus, mycorrhizal fungi may also be part of plant cognitive systems as an essential element of the plants' problem-solving apparatus. Studies are needed to verify how the nutritional status of plants influences the behaviour of mycorrhizal symbiont partners when foraging in the soil, or the types of mycorrhizal fungi that plants will associate with.

2.5.3 Case study 3: plant communication through mycorrhizal networks

Connection between two or more plants via the mycelium of at least one mycorrhizal fungus creates what is called common mycorrhizal networks (CMN). These networks may or may not involve direct hyphal contact between two or more roots (Rillig et al., 2024) and can facilitate the exchange of solutes, water, and infochemicals between plant roots. In particular, the possibility of roots sharing information through these networks was demonstrated in two laboratory-based experiments (Song et al. 2010; Babikova et al. 2013).

Song et al. (2010) cultivated tomato plants in compartments separated by membranes that either allowed mycelium to connect the roots or not. Donor plants were infected with the leaf pathogen *Alternaria solani* and all plants were enclosed in plastic bags, preventing aboveground communication through VOCs. After 65 h, receiver plants separated from infected donors by mycelium-permissive membranes presented higher activity of defence-related enzymes (peroxidase, polyphenol oxidase, chitinase, β−1,3-glucanase, phenylalanine ammonia-lyase, and lipoxygenase) and higher expression of defence-related genes (Song et al. 2010). Finally, when receiver plants were infected with *A. solani*, those connected to previously infected donor plants exhibited significantly higher disease resistance.

Using *Vicia faba* plants, Babikova et al. (2013) planted four receiver plants around a donor that would be infested with aphids. One receiver could interact with the donor through both roots and hyphae, a second only by hyphae, a third could initially interact through hyphae with connection severed prior to donor infestation, and a fourth receiver was a control, with both root and hyphal contact blocked by a mesh. Each plant was isolated aboveground to avoid VOC communication. Following donor infestation, the production of defence-related VOCs was analysed in all plants. Receiver plants that could interact via roots and/or hyphae after donor infestation presented the best results when it

came to repelling aphids and attracting parasitoid wasps, with the VOC methyl salicylate being a key component modulating the response of both insect species (Babikova et al. 2013).

Both studies potentially show transmission of information between plants (i.e., communication). Unfortunately, to our knowledge, these are the only reliable studies addressing signalling between plants directly through a continuous CMN, and there is currently no published evidence that this phenomenon happens in the field. However, at least in these experimental settings a putative extended cognition through CMN might have happened.

When plants associate with a mycelium, there are two ways in which they could extend their cognition. The first is by linking roots of the same plant. Due to the dendritic architecture of roots and branches, communication between spatially close apexes can be slow if they are physiologically distant. Mycorrhizal hyphae could provide a shortcut belowground in a manner analogous to VOCs aboveground (Frost et al., 2007; Heil and Karban 2010; Parise and Marder 2023). If true, mycorrhizal hyphae would perform a similar role in plant cognition to internal channels of communication, perhaps using hormones, electrical signals, and/ or hydraulic cues. Functionally, for the plant there would be no difference between the cognitive processes resulting from communication through plant tissue or fungal tissue, implying that plant cognition is extended through mycorrhizas (Parise and Marder 2023). The second is by linking roots of different plants. This could potentially open channels of communication with other plants belowground, expanding their sensorial world. Hence, through mycorrhizal hyphae, plants may gain perception of each other's existence. This alternative expands the array of possible interactions, cognitive or otherwise, of plants with a perceptually wider environment.

Song et al. (2010) and Babikova et al. (2013) studies can be analysed in two layers. The first layer suggests communication between plants through mycorrhizal hyphae, even if only in a laboratory setting, and likely via transmission of signalling molecules indicating pathogen or herbivory attack. Thanks to these conduits allowing reliable information transfer, plants prepared themselves for a future stress by upregulating defence-related genes, increasing the activity of defence enzymes, and changing VOC composition. The second layer is that plants increase their perception of other plants and their physiological status through mycorrhizal hyphae. It could well be

the case that plants have other means for perceiving distant plants, some of them not yet characterised (Gagliano et al. 2012; del Stabile et al. 2022). Nevertheless, the parameters analysed in the studies of Song et al. (2010) and Babikova et al. (2013) suggest that the receiver plants could not be informed about the donor plant's physiological status without connection to the CMN. The evidence provided by Song et al. (2010) and Babikova et al. (2013) are a case in favour of plant-plant communication through CMN, but in the future, it will be important to verify whether this happens on the field, particularly, since the occurrence and importance of CMN in situ has been soundly questioned (Karst et al. 2023). However, they show another mechanism by which EPC could be operating at least in laboratory conditions.

2.5.4 Case study 4: plants shape the mycorrhizal communities according to their needs

We have seen earlier that plants can strengthen or reduce their mutualistic behaviour based on internal (e.g., nutrient stoichiometry) and external (e.g., nutrient availability) conditions. There is also evidence that plants can actively select the most beneficial fungi from the pool of species and strains available in the environment (Bever 2015; Chagnon et al. 2015; Werner and Kiers 2015; Bogar et al. 2019). However, recent research has suggested that through time (often, within an individual's lifetime), plants can alter the community of mycorrhizas associated with them (Frew and Aguilar-Trigueros 2024) to adapt to local environments. They could do so by preferentially allocating more resources to the more advantageous fungi depending on the context (Ji and Bever 2016), thus building a community over time that helps them survive in specific environments. This is particularly important in stressful environments, but not so much in benign conditions. For example, by studying *Bouteloua gracilis*, a grass native to North America, Remke et al. (2020) found that the sympatric communities of AM fungi support their hosts better than allopatric communities during drought stress. These results were later confirmed in a three years-long field experiment (Remke et al. 2022), where the origin of mycorrhizal inoculum was the best predictor of plant biomass, specific leaf area, and seed production in plants transplanted to drier and warmer environments. In another field study, Janoušková et al. (2023) analysed the composition of AM fungi associated with transplanted *B. gracilis*, demonstrating that the initial inoculum is the primary determinant of the fungal community, with edaphic and climatic factors playing a

secondary role. While this highlights the importance of abiotic conditions, the findings also suggest an active role of the plant in shaping its associated fungal community to optimise performance in diverse environments.

Together, these studies are quite interesting to the EPC hypothesis because they seem to clearly follow the mutual manipulability criterion outlined in Section 2.3. When challenged with a novel, often stressful environment (a top-down manipulation), plants relied on the mycorrhizal communities they shaped for solving the problems imposed by the environment (a cognitive task). If the inoculum is experimentally changed (bottom-up manipulation), this significantly impairs plant performance, at least until the plant has the chance to rebuild its community. This seems to satisfy the mutual manipulability criterion by establishing relations of constitutive relevance of the fungi to the cognitive process of plants.

2.6 Cognition in plants, fungi, and beyond

Plants rely on mycorrhizas to accomplish processes important to their survival such as nutrition, foraging, problem-solving, and perhaps communication, pointing to the intriguing and unexplored possibility that plants extend their cognition to mycorrhizal fungi. Furthermore, if plants indeed extend their cognition to mycorrhizas, the fitness benefits are evident: it may allow plants to perceive nutrient patches or harmful substances in the soil from a distance, inform plants of where to invest more root growth and make foraging decisions, provide awareness of the space available for root growth, facilitate communication, and shortcut physiological constraints due to the modular architecture of the roots, among others. However, compelling phenomena may not be proof of extended cognition, but simple causal background conditions (Kaplan 2012). Well-constructed experiments can develop our understanding of whether extended cognition through mycorrhizas occurs and where the dynamic boundaries of plant cognition might be. Future research will likely have one or another epistemological flaw because philosophical proposals are not easy to transfer directly to empirical experiments. In effect, ‘perfect’ mycorrhizal experiments that capture the complexity of these symbioses are nearly impossible to achieve (Egger and Hibbett 2004; Jones and Smith 2004). However, we anticipate that a robust empirical framework corroborating (or refuting) extended plant cognition will emerge from the body of studies asking similar

questions; much like the way neuroscience determined the neural components underlying human cognition (Kaplan 2012). Ideally, these studies will employ empirical criteria like Craver's (2007a,b) mutual manipulability to establish these relations. For example, by controlling the plant's ability to deliver carbon (Kiers et al. 2011) or the fungus' capacity to provide nutrients (Whiteside et al. 2019), manipulations of both levels could be achieved. See **Table 2.1** for some suggestions on how to test EPC via mycorrhizas using the mutual manipulability criterion. Results from such experiments could have implications in agricultural, forestry, and restoration practices focused on mycorrhizal fungi, because management practices that diminish EPC via mycorrhizas might negatively impact plant growth, yield, and/or ecosystem functioning.

Immanuel Kant famously said that experience without concepts is blind, while concepts without experience are empty. With this, he was denouncing—indirectly—scientific advances not based on a solid metaphysic foundation, while also denouncing dogmatic metaphysics which at the time was often not based on empirical data or support (Kant 1998). The EPC via mycorrhizas framework proposed here allows to simultaneously adjust established concepts (like extended cognition more broadly) based on new findings, while at the same time suggesting new experiments and methods to validate such concepts (**Table 2.1**). Thus, EPC is different from 'regular' extended cognition, as other examples of extended cognition do not involve the recruitment of other organisms for it (Menary 2010). This requires to theoretically re-evaluate extended cognition and the 4E model when more than one agent (or millions in this case) are interacting simultaneously.

The main idea is to put plants in a condition that requires mycorrhizas to solve a problem or complete a task, and do both bottom-up and top-down manipulations to establish mycorrhizal fungi as constitutively relevant for the completion of that task (see Craver 2007a,b; Kaplan 2012; Japyassú and Laland 2017; Craver et al. 2021). Presumably, plants without mycorrhizas or with disrupted communication with the fungi would perform worse than those with pristine mycorrhizas.

Table 2.1. Suggestion of possible methods that, combined, could be used to test extended plant cognition through mycorrhizas. The main idea is to put plants in a condition that requires mycorrhizas to solve a problem or complete a task, and do both bottom-up and top-down manipulations to establish mycorrhizal fungi as constitutively relevant for the completion of that task (see Craver 2007a,b; Kaplan 2012; Japyassú and Laland 2017; Craver et al. 2021). Presumably, plants without mycorrhizas or with disrupted communication with the fungi would perform worse than those with pristine mycorrhizas.

Experiment: Grow plants in conditions that require mycorrhizas to solve problems	
Top-down manipulations	**Bottom-up manipulations**
Prevent plants from being colonised (mutation, blocking)	Use different species of fungi for solving the same problem
Prevent plants from delivering carbon to the fungi	Prevent fungi from delivering nutrients to the plant
Blocking plant communication with the fungi (e.g., strigolactones)	Alter the fungal community available to the plant
Prevent plants from access certain compartments in the substrate	Prevent communication from the fungus to the plant
	Use competitors, fungicides, or substances repellent to the fungus but not to the plant

An issue in some cases of extended plant cognition is the problem of "cognitive ownership" (Smart 2022). Who 'owns' the cognitive process when it is extended? When the cognitive agent is manipulating inanimate objects, like a person using a calculator, a spider weaving a web, or a plant releasing VOCs in the air, this is obvious. A human is not the extended element of the cognition of a calculator. But things become blurry when two cognitive agents interact. We suggest that either plants extend their cognition to fungi or both become a single cognitive entity, rather than whole plants becoming an extended element of fungal cognition. In fact, beyond a limited ability to mobilise nutrients in the network and connect with different hosts, the evidence does not suggest that fungi extend their cognition to plants or manipulate them in the way plants do to fungi. Plants seem to have much more control over the symbiosis. They can even survive without mycorrhizal fungi, whereas the opposite is not possible. After all, plants ultimately are the primary producers in the relationship; they hold the 'keys of the treasure' (carbohydrates and lipids), and use it to their benefit. They can recruit and abandon fungi according to their needs, and even parasitise the fungi—even without producing carbohydrates themselves, as seen in the case of mycoheterotrophs (Merckx 2013). Plants seem to shape the mycorrhizal community according to their needs, and use it to solve problems, find nutrients, and perhaps, communicate. Plants are the focal point of this extended cognitive system, and without plants, it would disappear. If fungi extend their cognition to plants, the magnitude of this extension is likely to be more localised, for example, around the roots the fungi are colonising. This is an interesting question worth pursuing when more data becomes available.

When studying the presumed cognitive association between plants and mycorrhizal fungi, shifts in environmental conditions and temporal dynamics must be considered. Like the cognitive process, the interactions between plants and its microbiota is flexible, plastic, and context-dependent. EPC through mycorrhizas can be transient, depending on the context and physiological, developmental, and phenological status of fungi and plants. As can be extracted from our case studies, it could be particularly critical for seedlings, herbaceous plants, and plants in early stages of development, for they have limited resources and ability to synthesise carbohydrates. Using fungi to help guiding foraging and root placement seems advantageous not only for the plant but for the fungi that would benefit from the success of its host. However, nothing prevents mature plants from benefitting from EPC as well, especially locally at the roots level.

Plants and symbiotic fungi often collaborate in mutualistic interactions but, like in any holobiont, the relationship between host and symbionts can shift to a parasitism depending on environmental and biotic context (Johnson et al. 1997; Suárez and Stencel 2020; Harrower and Gilbert 2021). Some questions for the future are: are plants extending their cognitive process to mycorrhizal fungi all the time, or only in specific moments when the symbiosis is working as a mutualism? Under which environmental and biotic conditions do the presumed EPC reach its optimal dynamics? What happens when the mycorrhizal symbiosis drifts toward the parasitism end of the mutualism-parasitism continuum?

Another question for the future, if extended cognition through mycorrhizas is confirmed, is: how does the communication between plants and fungi happen to allow this exchange of information? Hormones, small RNAs, and mycorrhiza-induced small secreted proteins (MiSSPs) are obvious candidates, but calcium, reactive oxygen species (ROS) and electrical signalling are also likely to contribute (Kapoor and Singh 2017; Thomas and Cooper 2022). Overall, what happens at the root-fungus interface is still largely unknown and needs to be better studied (Martin et al. 2016).

The hypothesis of extended plant cognition closely aligns with contemporary hypotheses about holobionts that take a holistic approach to study organisms not as separate individuals, but as clusters of several organisms productively interacting among themselves (Vandenkoornhuyse et al. 2015). It resonates, for example, with the Functional Team Selection framework (Johnson and Marín 2025), a framework to study plant adaptation that does not overlook the role of the microbial community for plant adaptation and survival. Who adapts to the environment is not the plant *and* the microbial community, but the plant *with* the microbial community. EPC further contributes to these views by adding the often-neglected cognitive component to these holobionts, helping these “teams” to solve problems, recall past stresses, forage efficiently, and choose the best ways to adapt to new conditions. This perspective is worthwhile, as it will at the very least stimulate scientific questions and original approaches to the study of mycorrhizal symbioses that were never tried before.

Finally, plants are rarely alone in the environment. They are embedded in a rich assemblage of many species and individuals, all of them potentially exchanging nutrients, resources, allelopathic compounds, and information. They may be interconnected in an

underground mycorrhizal network with one or many fungal individuals which are in turn connected to one or many other plants, and this is very different from reductionist experiments that investigate single plants and associated mycorrhizal fungi in laboratory settings (Giovannetti et al. 2004; Beiler et al. 2010; Tedersoo et al. 2020). Hence, whereas it might be possible to delineate the boundaries of the plant's cognitive system in laboratory conditions, the reality in the field may prove to be very different.

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2.8 Declarations

2.8.1 Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

2.8.2 Open Access

Chapter 3: The pitfalls of ectomycorrhizal microcosms: lessons learnt for future success

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The pitfalls of ectomycorrhizal microcosms: lessons learnt for future success

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ABSTRACT

Mycorrhizal fungi are known to support their host plants by facilitating nutrient acquisition and enhancing resistance to biotic and abiotic stress. However, the possibility that they also convey structural information about the soil has not yet been tested. Here, we attempted to investigate whether ectomycorrhizal hyphae could guide root growth in response to physical obstacles by using Scots pine (*Pinus sylvestris*) and *Suillus granulatus* in a microcosm experiment fitted with U-shaped silicone mazes. Despite initial success in achieving ectomycorrhizal colonisation (88% of the inoculated seedlings), the fungi failed to produce the expected hyphal networks. Extensive and unexpected root growth rendered the system unsuitable for testing our hypothesis. Furthermore, structural issues with the microcosms compromised substrate integrity, possibly inhibiting fungal development. While our results were inconclusive, this report highlights challenges associated with replicating classical ectomycorrhizal experiments, underscoring the need for methodological refinement. We provide detailed recommendations and methodological clarifications that may aid future research. Although our initial hypothesis could not be tested, we argue that traditional microcosm experiments retain potential for advancing our understanding of mycorrhizal ecology, provided they are critically revisited and technically improved. Negative results, when well contextualised, are valuable contributions toward more robust and reproducible experimental frameworks.

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Hyphae; maze; microcosm; negative results; *Pinus sylvestris*; seedling; structural information; *suillus granulatus*

Introduction

Over a hundred years of research into mycorrhizal symbioses have elucidated many roles for this interaction between plants and their associated fungi. Several studies have shown that, when in association, mycorrhizas increase plant nutritional status,[1–3] protect plants from pathogens and diseases,[4–6] improve resistance to abiotic stress,[7,8] and potentially help seedling establishment.[9–11] However, many questions remain open, and the full implications of this symbiosis for both plants and fungi are far from being entirely known.

One aspect of the symbiosis that has hitherto been ignored is whether, beyond providing nutrients and water to their host plants, mycorrhizal fungi might also provide the host with information about the structure of the belowground environment. It is known that many trees delegate their foraging behaviour to mycorrhizal fungi.[2,12,13] Instead of growing roots to seek and exploit nutrient patches, they employ the more versatile, dynamic, and carbon-efficient mycelial systems.[14–16] This process is known as foraging complementarity,[13] and seems to be more present in tree species with thick (i.e., ~ > 0.4 mm-wide) absorbing roots.[12,17] However, if fungal hyphae are growing beyond the roots, scouting ahead of them, they may find structural complexities in the soil, like rocks or zones of compaction, and divert away from them, eventually guiding root growth to avoid these obstacles. To our knowledge, this hypothesis has never been explicitly tested.

Here, we carried out an experiment to test the hypothesis that the growth of mycorrhizal hyphae could provide structural information to the host plant about the belowground environment. We used Scots pine (*Pinus sylvestris* L., Pinaceae) and the ectomycorrhizal fungus *Suillus granulatus* (L.) Roussel (1796) (Boletaceae). The fungus was chosen because, like others in its genus, it is easy to grow in axenic culture, it associates easily with hosts under experimental conditions, and it produces large hyphal strands that are visible to the naked eye.[18] The plant species was chosen because it is an ectomycorrhizal host with thick roots (i.e., its root tips are usually 0.47–0.48 mm thick, see [19] and,[20] and therefore likely to depend on its fungal partner to explore the environment. Scots pine is native throughout the mountainous boreal regions of Eurasia, from Scotland to Siberia,[21] where the soil is often rocky and potentially challenging to navigate. A young seedling that has just germinated from a small seed must grow its roots into suitable areas and avoid dead ends and cracks between the rocks. We infer that the metabolic cost for a small seedling to correct this growth is likely to be high. We hypothesised that ectomycorrhizal fungi could help the seedling mitigate its carbon costs, potentially leading to more carbon available for the fungal partner, by guiding its roots to the most suitable soil patches for stability and further growth.

To carry out this study, we attempted to replicate some classical experiments on ectomycorrhizas using *Pinus sylvestris* and *Suillus* spp.[22–25] We compared papers from the literature

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Mycorrhizal fungi are known to support their host plants by facilitating nutrient acquisition and enhancing resistance to biotic and abiotic stress. However, the possibility that they also convey structural information about the soil has not yet been tested. Here, we attempted to investigate whether ectomycorrhizal hyphae could guide root growth in response to physical obstacles by using Scots pine (*Pinus sylvestris*) and *Suillus granulatus* in a microcosm experiment fitted with U-shaped silicone mazes. Despite initial success in achieving ectomycorrhizal colonisation (88% of the inoculated seedlings), the fungi failed to produce the expected hyphal networks. Extensive and unexpected root growth rendered the system unsuitable for testing our hypothesis. Furthermore, structural issues with the microcosms compromised substrate integrity, possibly inhibiting fungal development. While our results were inconclusive, this report highlights challenges associated with replicating classical ectomycorrhizal experiments, underscoring the need for methodological refinement. We provide detailed recommendations and methodological clarifications that may aid future research. Although our initial hypothesis could not be tested, we argue that traditional microcosm experiments retain potential for advancing our understanding of mycorrhizal ecology, provided they are critically revisited and technically improved. Negative results, when well contextualised, are valuable contributions toward more robust and reproducible experimental frameworks.

Keywords: Hyphae · maze · microcosm · negative results · *Pinus sylvestris* · seedling · structural information · *Suillus granulatus*

3.2 Introduction

Over a hundred years of research into mycorrhizal symbioses have elucidated many roles for this interaction between plants and their associated fungi. Several studies have shown that, when in association, mycorrhizas increase plant nutritional status (Nolan 1991; Tibbett and Sanders 2002; Umar et al. 2024), protect plants from pathogens and diseases (Marx 1972; Sikes et al. 2009; Dey and Gosh 2022), improve resistance to abiotic stress (De Oliveira et al. 2020; Marro et al. 2022), and potentially help seedling establishment (van der Heijden and Horton 2009; Teste et al. 2009; Booth and Hoeksema 2010). However, many questions remain open, and the full implications of this symbiosis for both plants and fungi are far from being entirely known.

One aspect of the symbiosis that has hitherto been ignored is whether, beyond providing nutrients and water to their host plants, mycorrhizal fungi might also provide the host with information about the structure of the belowground environment. It is known that many trees delegate their foraging behaviour to mycorrhizal fungi (Tibbett and Sanders 2002; Eissenstat et al. 2015; Cheng et al. 2016). Instead of growing roots to

seek and exploit nutrient patches, they employ the more versatile, dynamic, and carbon-efficient mycelial systems (Rousseau et al. 1994; Read 1999; Leake et al. 2004). This process is known as foraging complementarity (Cheng et al. 2016), and seems to be more present in tree species with thick (i.e., ~ > 0.4 mm-wide) absorbing roots (Eissenstat et al. 2015; Bergmann et al. 2020). However, if fungal hyphae are growing beyond the roots, scouting ahead of them, they may find structural complexities in the soil, like rocks or zones of compaction, and divert away from them, eventually guiding root growth to avoid these obstacles. To our knowledge, this hypothesis has never been explicitly tested.

Here, we carried out an experiment to test the hypothesis that the growth of mycorrhizal hyphae could provide structural information to the host plant about the belowground environment. We used Scots pine (*Pinus sylvestris* L., Pinaceae) and the ectomycorrhizal fungus *Suillus granulatus* (L.) Roussel (1796) (Boletaceae). The fungus was chosen because, like others in its genus, it is easy to grow in axenic culture, it associates easily with hosts under experimental conditions, and it produces large hyphal strands that are visible to the naked eye (Lofgren et al. 2024). The plant species was chosen because it is an ectomycorrhizal host with thick roots (i.e., its root tips are usually 0.47–0.48 mm thick, see Ostonen et al. 2007 and Chen et al. 2016), and therefore likely to depend on its fungal partner to explore the environment. Scots pine is native throughout the mountainous boreal regions of Eurasia, from Scotland to Siberia (Critchfield and Little, Jr 1966), where the soil is often rocky and potentially challenging to navigate. A young seedling that has just germinated from a small seed must grow its roots into suitable areas and avoid dead ends and cracks between the rocks. We infer that the metabolic cost for a small seedling to correct this growth is likely to be high. We hypothesised that ectomycorrhizal fungi could help the seedling mitigate its carbon costs, potentially leading to more carbon available for the fungal partner, by guiding its roots to the most suitable soil patches for stability and further growth.

To carry out this study, we attempted to replicate some classical experiments on ectomycorrhizas using *Pinus sylvestris* and *Suillus* spp. (Duddridge 1986; Finlay and Read 1989; Bending and Read 1994; Rosling et al. 2004). We compared papers from the literature that conducted experiments with pine seedlings to understand the methods used, and tried to follow them. Our intention was to grow inoculated *P. sylvestris* seedlings in thin Perspex microcosms that would allow the observation of root and hyphal development. An obstruction in the soil was simulated by affixing a U-shaped silicone

maze placed below the seedling. We predicted that the fungal hyphae would grow faster than the roots, reach the bottom of the maze, and potentially signal to the plants that an obstacle was present, which would trigger more lateral root formation as a response to avoid the maze. Consequently, we would expect more root mass inside the maze for plants that are not inoculated with *S. granulatus* than for plants that were inoculated, which would have more lateral root development to avoid the maze. This kind of maze was chosen because it was also used in other experiments with slime moulds, organisms with a similar structure and behaviour as fungi (Reid et al. 2012), and to study the behaviour of arbuscular mycorrhizal hyphae (Richter et al. 2024). It was also used in tests with simple robots to test the robots' ability to escape basic traps like a dead end (Zou and Zhu 2003; Luh and Liu 2008).

3.3 Material and Methods

3.3.1 Synthesis of mycorrhizas

The techniques described here were inspired by works like Duddridge (1986), Finlay and Read (1989), Bending and Read (1995), and Rosling et al. (2004). *Pinus sylvestris* seeds were acquired from Chiltern Seeds (Wallingford, UK). The seeds were harvested in plantations in Shropshire and Norfolk (UK) between 2019 and 2020 and had been stored at -4 °C until purchased in March 2023, subsequently being stored at 4 °C until sown. To obtain aseptic seedlings, the seeds were surface sterilised in a laminar flow cabinet by soaking them in H_2O_2 30 % for 15 min in a glass beaker, stirring often to ensure sterilisation. Then, the H_2O_2 was removed with a pipette and the seeds were washed 5 times with autoclaved milli-Q water. After the fifth wash, the seeds were covered with autoclaved milli-Q water, and the beaker was closed with aluminium foil and kept in the dark, refrigerated at 5.5 ± 1 for 72 h. Then, again in the laminar flow cabinet, the seeds were sown in Petri dishes with agar (15 g · L^{-1}) and glucose (2 g · L^{-1}), sealed with Parafilm, and taken to a 2.50 x 1.85 x 2.00 (W x L x H) controlled environment room (Fitotron® SGR – Weiss Technik, Heuchelheim, Germany). The Petri dishes were kept tilted at approximately 45 ° in a 16 h daylight regime (06:00–22:00), 15 °C during the day and 10 °C during the night, humidity constant at 60 %, and photon flux density 170 µmol m^2 · s^{-1} PAR. This procedure was based on information retrieved from the articles cited

above, and a comparison between the methods for synthesising mycorrhizas can be found in Supplementary Material 3.1.

After 20 days, the seedlings were inoculated with *Suillus granulatus* obtained from the University of Reading mycological collection. In a laminar flow cabinet, Petri dishes were prepared by carving a notch in one of the edges with a hot scalpel. They were filled with peat and vermiculite (1:4, v:v) that had been previously sieved through a 2 mm mesh and disinfested by autoclaving at 105 °C for 1 h on two consecutive days. Three seedlings were laid on the peat with the stems protruding outside through the notch (**Figure 3.1**). Two or three agar plugs (⌀ 11 mm) containing the growing edges of a 24-day-old culture of *S. granulatus*, cultured on potato-dextrose-agar (PDA; Thermo Fischer Scientific, Waltham, Massachusetts, USA), were placed onto the root tips. The roots and agar were covered with a layer of the pre-prepared peat and vermiculite and moistened with a liquid Modified Melin-Nokrans nutrient medium (MMN) without a carbon source by spraying c. 28 mL of medium on it with a spray bottle. This was enough to soak the substrate. Then, the Petri dish was closed and sealed with a Parafilm® strip and anhydrous lanolin around the stems. The control plants underwent the same procedure, but without adding the agar plugs. The Petri dishes were wrapped with aluminium foil, taken to the same controlled environment room and conditions described before and kept vertically. After approximately two days of acclimation, the photon flux density was increased to 210.5 μmol m^2 s^{-1}.

Figure 3.1. Example of the inoculation set-up. Three *P. sylvestris* seedlings were positioned with the roots inside a Petri dish filled with peat and vermiculite, inoculated with agar plugs with *S. granulatus*, and sealed with Parafilm® and anhydrous lanolin.

3.3.2 Setting up the microcosms

After 60 days from inoculation, the seedlings were transferred to the microcosms. The microcosms consisted of a pair of 0.6 cm thick 30 x 40 (W x H) Perspex plates separated by 0.3 cm thick and 1 cm wide black silicone spacers. The spacers were glued with the silicone sealant in all the inner edges except for the top of the microcosm. In the middle of the microcosm, a silicone maze shaped like a square U was also glued with silicone sealant (**Figure 3.2**). A plan of the microcosm with the position of the maze and all the measures can be found in Supplementary Material 3.2. Then, the microcosms were filled with the sterile mix of peat and vermiculite, moistened by spraying MMN medium. One seedling was placed on the top of the microcosm. Subsequently, silicone sealant was applied along the maze, and the microcosm was covered with the other Perspex plate. Four 0.41 cm-wide foldback clips were used to hold the plates together. The control and inoculated microcosms were set up alternately by two people to avoid bias between how the experimental groups were set up. Some seedlings had already evident and well-formed mycorrhizal tips before being transferred to the microcosms (**Figure 3.3**a,b).

Finally, all the microcosms were wrapped with aluminium foil and taken to the same growth room. Since the seedlings were now at a higher position (40 cm above the bench), they were exposed to c. 302.1 µmol m^2 s^{-1} PAR. In total, we set up 15 inoculated and 15 control microcosm. The other environmental conditions remained the same as before.

Figure 3.2. Photograph of one of the microcosms prior to being wrapped in aluminium foil and before the interventions to secure it.

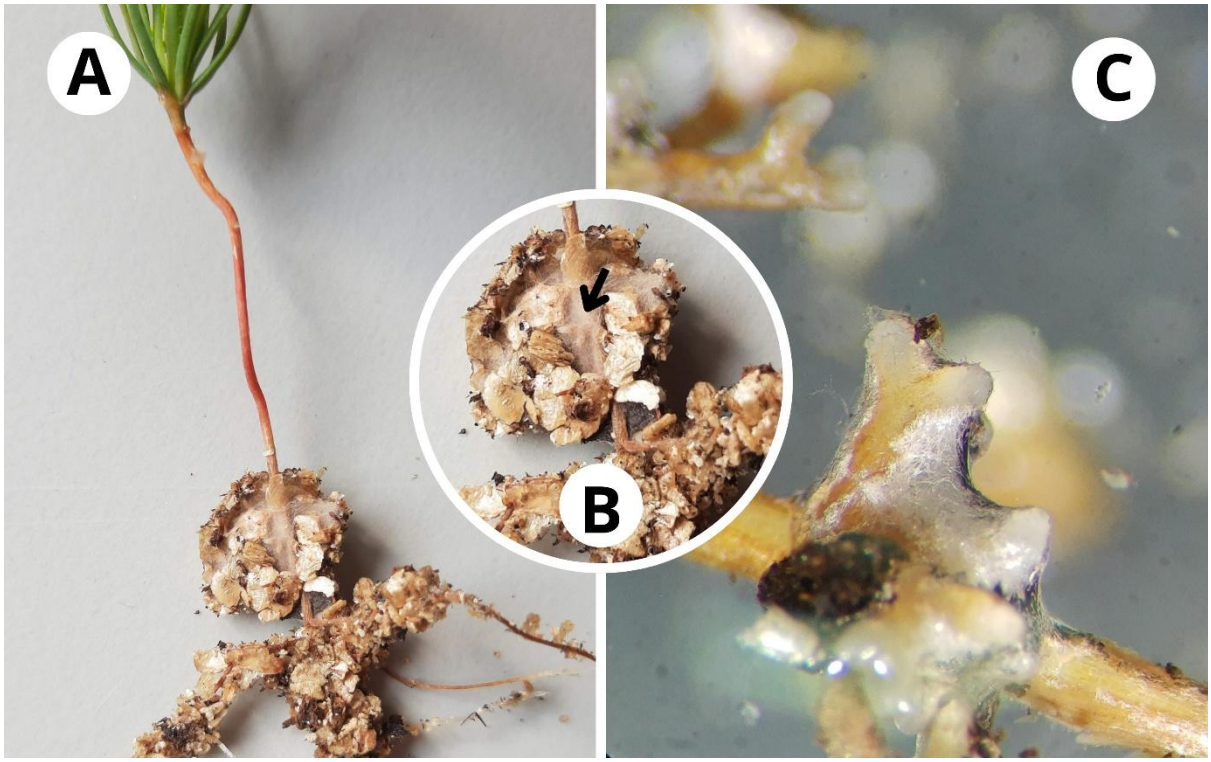


Figure 3.3. Inoculated seedlings. A: After the inoculation period with *Suillus granulatus*, some seedlings of *Pinus sylvestris* had evident ectomycorrhizas, with the fine root tips well shrouded by the hyphal mantle. B: Close-up of the root section of the seedling shown in A exhibiting two root tips (arrow) surrounded by a thick mantle of hyphae. C: Root tip of one seedling not used in the microcosm, under the stereomicroscope, showing the development of tip and mantle, with characteristic hydrophobicity.

Inoculated seedlings that were not used for the microcosms were quickly analysed under a stereo microscope to assess colonisation. We checked for the presence of hyphae and fine roots (**Figure 3.3**c) as a proxy for the presence of ectomycorrhizas. Then, they were wrapped in moist paper-towel and stored in plastic bags in the fridge at 4 °C for morphological subsequent analysis.

3.3.3 Fixing problems with microcosms

After a few days, we noticed that the Perspex plate had bent outwards in the extremities, markedly at the upper side, exposing the substrate and the roots. One by one, they were taken out of the growth room and unwrapped. We added four extra foldback clips of the same kind to the extremities. Then, on each side above the maze an extra 30 × 10 cm Perspex plate (0.6 cm thick) was placed and held by two spring clamps with a 5.0 cm opening (Manufacturer ID: T58200EL7. Irwin Industrial Tools, Huntersville, North Carolina, USA). The new Perspex and spring clamps applied, uniformly, more pressure

on the mazes. After adding those new components to the microcosms, the dry upper layer of substrate was moistened by spraying milli-Q water, and the microcosm was completed with dry substrate added to the top. The substrate was dry to create an air cushion between the moist substrate below and the atmosphere, hence retaining more water in the microcosms.

Finally, the microcosms were wrapped in new aluminium foil and returned to the growth room. All microcosms were adjusted in this way over 1 week.

3.3.4 Plant harvest

After four weeks (31 days) since setting up the microcosms, harvest started. We first checked some plants and noticed that they barely grew into the maze, and no developed hyphae were seen. Therefore, we decided to harvest only eight plants and leave the remaining ones for further two weeks in order to check for development of roots and hyphae.

The choice of the plants to be harvested was made using a random number generator website (https://sorteador.com.br). The microcosms were photographed, the plants were wrapped in moist paper-towel and stored in a fridge at c. 4 °C for later analysis.

3.3.5 Plant morphology analysis

On the day following the harvest, we washed the roots thoroughly to remove as much substrate as possible. Then, we scanned all roots of the seedlings from the experimental microcosms as well as those not used in the microcosms, using the software WinRHIZO^{TM} (Regent Instruments Inc., Ottawa, ON, Canada). Morphological parameters analysed were total root length (cm), total root area (cm^2), total root volume (cm^3), and number of root tips (not necessarily ectomycorrhized root tips).

We took pictures of the microcosm with a Motorola One Action cell phone (Motorola, Inc., Schaumburg, Illinois, USA) and used ImageJ (version 1.54, National Institutes of Health, Bethesda, Maryland, USA) to measure the height of the seedlings. This was done by converting the picture in an 8-bit greyscale image with the command

Image > Type > 8-bit, then setting the scale for each image using the 1 cm edge of the maze as a reference, and finally measuring the length of the stem from the substrate to the basis of the first needle with the *Segmented line* tool.

3.3.6 Statistical analysis

All analyses were carried out with the software XLSTAT®. We transformed the data by $\sqrt{x}$ to obtain normality (Shapiro-Wilk, $p > 0.05$), and homoscedasticity was checked with a Levene test ($p > 0.05$). After parametric assumptions were met, we carried out a preliminary three-way ANOVA to verify if there were any influence of two other factors beyond the treatments: "evidence of ectomycorrhizas prior to transplanting" and "experimenter identity". Since neither factor was significant ($p > 0.05$), a one-way ANOVA was carried out for all variables, to assess differences between Inoculated and Control microcosms, for plants harvested at 4 weeks, and plants harvested at 6 weeks.

3.4 Results

3.4.1 Seedlings before test and synthesis of ectomycorrhizas

After two weeks in the Petri dishes, the needles of a few seedlings started to become chlorotic (**Figure 3.4**). When the Petri dishes were opened, the substrate looked dry, despite visible moisture condensed in the walls of the dishes. Nevertheless, the analysis of the seedlings not used in the microcosm showed that 88% of them ($n = 41$) presented fine root tips surrounded by hyphae, which we used as an indication of ectomycorrhizas partially or completely formed (**Figure 3.3**c).

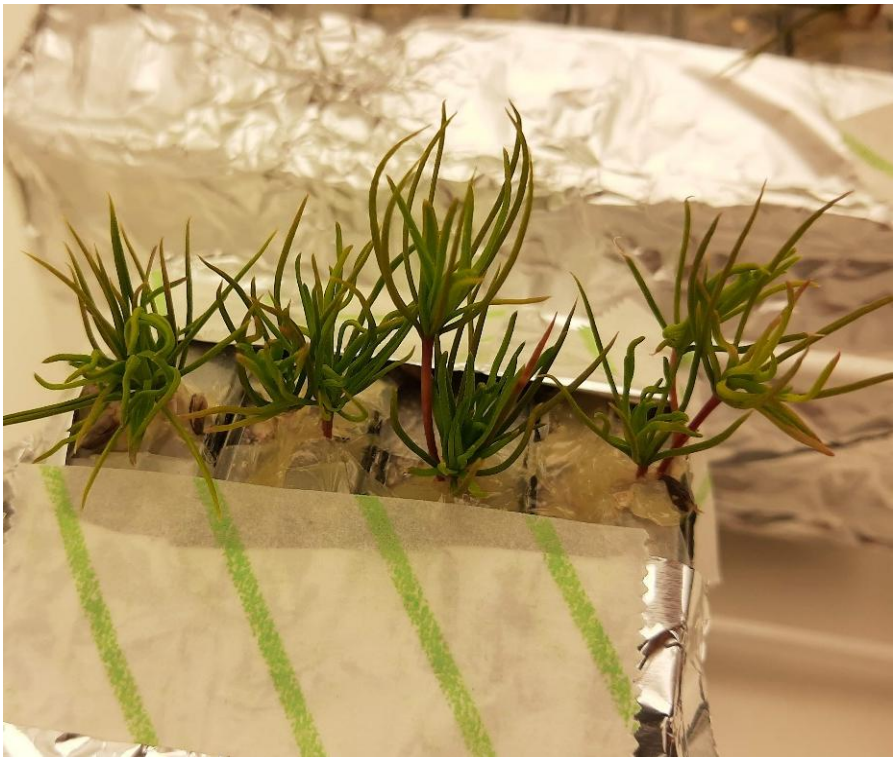


Figure 3.4. Pine seedlings in vertical petri dishes shortly before being harvested and transplanted to the microcosms, showing signs of stress.

After inoculation in the Petri dishes, both inoculated and control seedlings had unexpectedly extensive root growth (**Figure 3.5**). All root morphological features that were evaluated are presented in **Table 3.1**. Seedlings from the inoculated treatment had higher total root length, increased surface area of roots, and higher number of root tips than control plants.

Figure 3.5. Seedlings of *P. sylvestris* 4 weeks after inoculation with *S. granulatus* in Petri dishes with peat and vermiculite, before being used in the microcosms. On the left, a control seedling. On the right, an inoculated seedling. Note the unusually extensive development of the root system. The scale bars represent 5 cm for both seedlings.

Table 3.1. Comparison of mean root features (± standard deviation) between seedlings of P. sylvestris inoculated with S. granulatus (ECM) and control treatments without inoculation (NM). 'Mycorrhizal structures' refer to the absolute number of seedlings with at least one root tip with hyphae, therefore the standard deviation is not applicable.

Root traits	NM (n = 26)	ECM (n = 41)	R^2	F	p
Length (cm)	48. 9 ± 18.6	62.6 ± 22.2	0.10	6.86	0.011
Surface area (cm^2)	6.3 ± 2.6	7.7 ± 2.8	0.06	4.18	0.045
Root volume (cm^3)	0.07 ± 0.03	0.08 ± 0.03	0.03	2.00	0.162
Fine root tips (count)	78.1 ± 30.0	117.7 ± 44.4	0.20	16.0	0.0002
Mycorrhizal structures (count)	—	36	—	—	—

3.4.2 Microcosms

During the course of the experiment (approximately four days after setting up the microcosms), the microcosms started bending outwards (**Figure 3.6**). This exposed the substrate to become dry in its upper layer, which required adjustments during the

experiment, i.e., the inclusion of additional clips and clamps, a refill of substrate, and further water addition.

None of the microcosms exhibited significant hyphal growth like the ones shown in the classical studies we used as a reference (e.g., Duddrige 1986; Finlay and Read 1986; Bending and Read 1995; Rosling et al. 2004). A few roots grew into the mazes but did not touch the bottom. It was impossible to quantify the biomass inside the maze because the roots attached to the Perspex, and the whole root system had to be moved when opening the microcosms. To try a method for solving this problem, we tested freezing two microcosms in a -20 °C cold room before opening them. This held the roots in place, although it broke them as they became brittle. With this method, precision with the WinRHIZO measurements is lost, but at least it presumably allows quantifying the biomass inside and outside of the U-shaped mazes.

Figure 3.6. Gaps opened in the microcosms due to the folding back of the Perspex plates. In some cases, the gaps between the plates were as wide as 0.5 cm.

3.4.3 Growth parameters after 4 and 6 weeks

The results for all the parameters assessed after 4 and 6 weeks after the experiment are presented in **Table 3.2**. No significant difference was observed in any of

the parameters between treatments after 4 or 6 weeks. All the raw data for the seedlings before and after the microcosm tests can be found in the Supplementary Material.

Table 3.2. Root and shoot features of *P. sylvestris* seedlings inoculated with *S. granulatus* (ECM) and control (NM) after 4 and 6 weeks (values are averages ± standard deviation). The one-way ANOVA test did not show statistically significant differences ($p \leq 0.05$) between treatments, regardless of the growth period. Note that root tips means the total number of fine root tips defined by WinRHIZO, and not necessarily colonised root tips. Only the ECM seedlings were inoculated with *S. granulatus*.

Parameter	4 weeks					6 weeks				
	NM (*n* = 8)	**ECM (*n* = 8)**	***R²***	***F***	***p***	**NM (*n* = 7)**	**ECM (*n* = 7)**	***R²***	***F***	***p***
Root length (cm)	140.2 ± 34.9	127.2 ± 23.2	0.04	0.60	0.45	158.3 ± 36.1	177.0 ± 70.9	0.02	0.24	0.63
Root surface area (cm^2)	17.8 ± 5.2	16.0 ± 2.3	0.04	0.56	0.47	18.3 ± 4.7	21.0 ± 9.0	0.02	0.31	0.59
Root total volume (cm^3)	0.2 ± 0.1	0.2 ± 0.0	0.03	0.49	0.50	0.2 ± 0.0	0.2 ± 0.1	0.03	0.37	0.56
Root tips (count)	336.7 ± 135.0	270.6 ± 58.5	0.10	1.58	0.23	380.3 ± 58.2	406.7 ± 147.5	0.01	0.08	0.78
Root mass (g)	0.04 ± 0.01	0.04 ± 0.01	0.02	0.26	0.62	0.05 ± 0.02	0.06 ± 0.02	0.04	0.51	0.49
Shoot mass (g)	0.05 ± 0.02	0.04 ± 0.01	0.02	0.36	0.56	0.05 ± 0.02	0.06 ± 0.02	0.01	0.13	0.72
Total mass (g)	0.09 ± 0.03	0.08 ± 0.02	0.03	0.37	0.55	0.10 ± 0.03	0.11 ± 0.04	0.03	0.32	0.58
Shoot height (cm)	2.0 ± 0.6	1.6 ± 0.7	0.06	0.87	0.37	2.0 ± 0.6	1.6 ± 0.6	0.05	0.62	0.45

3.5 Discussion

In this work, we hypothesised that in a microcosm setting, hyphae of ectomycorrhizal fungi would grow faster than the roots of their host plant and guide the growth of these roots, preventing them from being trapped inside a U-shaped maze placed below the seedlings. The seedlings were harvested after growing for 4 and 6 weeks but, due to technical issues, it was not possible to test this hypothesis.

The experiment was unlikely to succeed when, 8 weeks after inoculating *P. sylvestris* seedlings with the fungus *S. granulatus*, they presented enormous root growth. This was unexpected, as we followed similar protocols to classical works of the past, in particular Duddrige (1986), Finlay and Read (1986), Bending and Read (1995) and Rosling et al. (2004). In these experiments, the initial root growth was minimal, rarely exhibiting more than two lateral roots. This amount of lateral roots would have been ideal for testing our hypothesis in the microcosm experiment.

When we opened the Petri dishes where the seedlings were inoculated, we noticed that the substrate looked dry despite condensation in the walls of the dish. Although drought stress is known to increase root growth in many plant species (Kou et al. 2022), drought-stressed *P. sylvestris* actually reduce root growth (Pálatová 2002; Meng et al. 2023). Therefore, it is unlikely that the lack of growth medium caused the excessive root growth. A possible reason for such growth could be the genetics of the plant. Different genotypes can yield different growth rates, so perhaps the seedlings from this batch of seeds naturally grew longer roots. Additionally, these seeds were collected from two different locations in the United Kingdom (Shropshire and Norfolk), and obtained through open pollination in the plantations (Chiltern Seeds, *personal communication*). Consequently, the varied genetic of the seeds could lead to high variance in the results, which potentially interferes in how easily the results can be reproduced. In this case, when possible, it would be ideal to use clone seedlings, or at least seeds from the same parent tree, so that at least 50% of the genome of the seedlings is identical.

We acknowledge that this study could have benefitted from more preliminary tests to assess root growth or other potential issues prior to the experiment. However, this work was conceived and conducted as an exploratory, proof-of-concept study. We aimed to assess whether the existing methodology, which has been widely used and reported in the literature (e.g., Duddridge 1986; Finlay and Read 1989; Rosling et al. 2004), could be

replicated and adapted to a novel hypothesis. Nonetheless, the unexpectedly vigorous root growth observed here highlights the necessity of such preliminary testing in future implementations of this technique. In this case, a researcher willing to use the same technique must be mindful of the time frame required to do all the tests prior to commencement of the actual experiment, for as we have noted, it takes quite a long time from sowing the seeds to having the seedlings inoculated and ready for experimentation.

Ectomycorrhizas were established at a very good rate (up to 88%), which is a good indicator of the vigour and viability of the inoculum. Ensuring inoculum viability is an important step in mycorrhizal research because some ectomycorrhizal fungi stop forming ectomycorrhizal tips after being kept in culture for a long time. However, in our experiment, most colonisation occurred close to the soil surface rather than on newly forming root tips on lateral roots down the soil profile. As such, their positioning would be ineffective to guide root growth. Extensive hyphal growth, like those shown in the classical studies, which was anticipated and considered critical for testing the hypothesis, was not observed.

Problems with this experiment were further aggravated when the Perspex plates started to bend outwards. This exposed the plant roots, dried the substrate, and likely hindered fungal development. This might explain why they did not develop hyphae like in the classical studies, and likely explains why we did not observe any significant difference between the control and inoculated plants. One positive aspect of the procedure was that we did not observe significant levels of contamination despite all these problems, which is very positive for follow-up tests to be done in the future.

Despite this experiment not yielding the output expected, we feel it is important to report it because the information in the material and methods of older papers is often insufficient to allow an accurate replication of the experiments they describe. Here, we synthesised the methods of several papers together and, with information kindly provided by some of the authors (Roger Finlay and Anna Rosling, *personal communication*), we came up with a methodology that represented an ‘average’ of what was done in the past for the studies we used as a reference. Even if the methods employed here did not work completely, we believe it is a step forward for designing a methodology to conduct these types of studies on mycorrhizas. We hope that researchers willing to do similar experiments can learn from our failures and successes and perfect this method, and we

urge them to report their methodology with as much accuracy as possible. The technique of synthesising mycorrhizas and growing inoculated seedlings in microcosms is old, but can still provide valuable information about the ecology and behaviour of mycorrhizas and their importance for seedling development and root architecture.

We conclude this report with the following recommendations to anyone interested in using this technique:

- Although it was not a concern in our experiment, it is important to ensure when initiating the experiment that both the seeds and fungal inoculum are fresh and active. Fungal strains that have been in culture too long may prove difficult to inoculate onto seedlings.
- When inoculating the seedlings with mycorrhizal fungi, agar plugs without fungi should be included in the substrate of the control plants as well. This will help to control for differences in the growth of the seedlings due to the agar acting as a source of nutrients.
- Ensure that the Perspex plates are firmly held along the entire length to avoid bending and exposure of the substrate, roots, and agar.
- Root growth in our experiment was highly unusual compared to previous microcosm experiments and should be investigated. It may be because of the plant genetics, but we recommend testing seedling growth with and without ectomycorrhizal fungi in different combinations of substrate before initiating further experiments (e.g., different proportions of peat and vermiculite; 1:2, 1:1, 1:0, 2:1, 4:1, 0:1) and also trying different concentrations of MMN medium. For this kind of experiment, ideally, there should be no more than two lateral roots before transplanting to the microcosms.
- Whenever possible, use clones for the seedlings or seeds from the same parent tree to minimise genetic variability and facilitate reproducibility of the results.
- We found that the roots of our seedlings attached to the Perspex plates, making it impossible to open the microcosm without disrupting the position of the roots. Freezing the microcosms at -20 °C before opening them allowed us to recover the biomass inside and outside of the mazes more reliably.
- In our experience, the length of time required to conduct this experiment was too long to allow reasonable adjustments and repetition (>15 weeks before obtaining

the data). We highly recommend experimental examination of alternative substrate mixtures in smaller microcosms over shorter time periods before embarking on experiments on the same scale as ours, unless time is not a limiting factor.

Mycorrhizas are among the most widespread terrestrial symbioses in the world, and so much is still unknown about them. Despite falling considerably out of fashion, classical experiments with microcosms can still provide important information about the ecology and behaviour of plants associated with ectomycorrhizal fungi at a relatively low cost. We hope this report inspires researchers to investigate how ectomycorrhizas may influence host plant root growth by improving upon this technique.

3.6 Acknowledgments

We are indebted to Roger Finlay and Anna Rosling for their generous advice on synthesising the ectomycorrhizal associations used in this study. We thank Ash Dobie for building the microcosms, Liam Doherty for his assistance in managing the growth room, and Cameron Martin for technical support during the microcosm set-up. AGP is supported by an International PhD Studentship from the University of Reading.

3.7 Disclosure statement

The authors report there are no competing interests to declare.

Supplementary Material 3.1

Comparison of methods for mycorrhizal synthesis in different papers

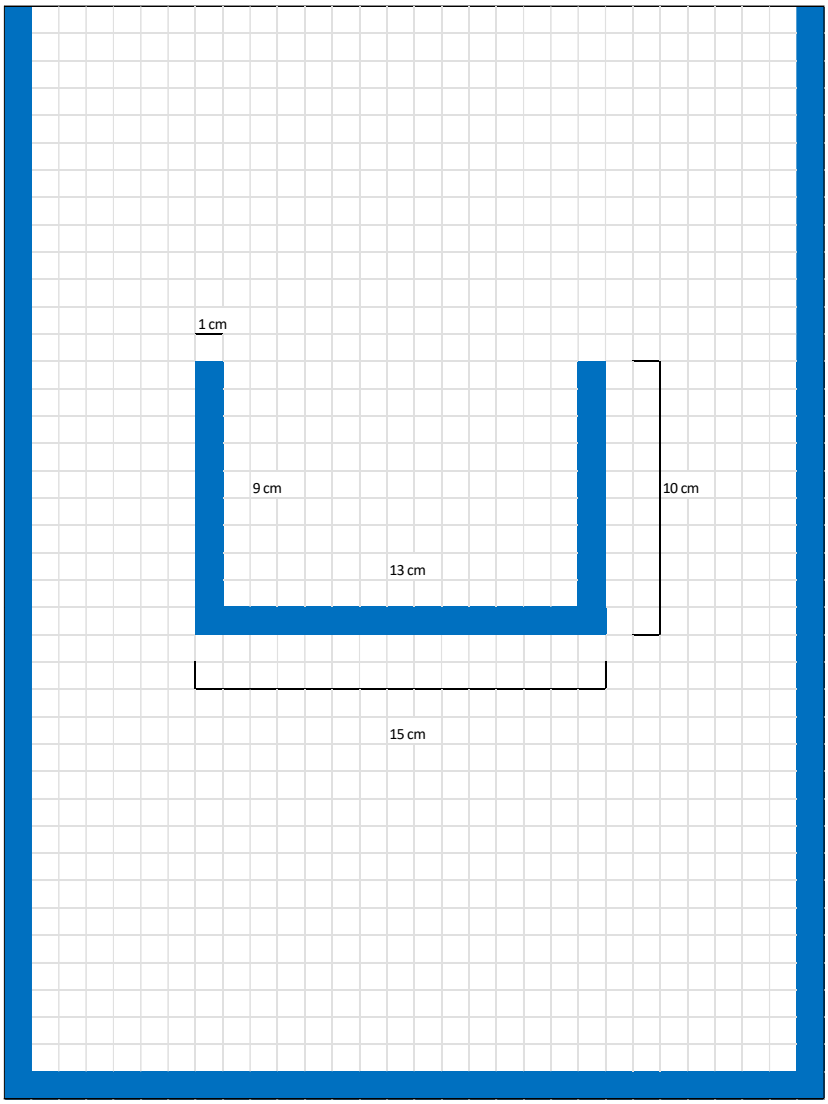
This table summarises how different authors synthesised mycorrhizas on *Pinus sylvestris*. Blank cells means that this information could not be found in the paper.

Reference	Plant species	Peat : vermiculite	Sterilisation	Moistened with	Light day/night	Irradiance	Temperature day/night	Time to form association
Rosling et al. 2004	*Pinus sylvestris*	1 : 4		MMN		300 µmol PAR	14-16/6-8	8 weeks
Finlay and Read 1986	*Pinus sylvestris*	1 : 4	Autoclaved	MMN	16/8	38 W m^{-2}	15/10	8 weeks
Duddridge 1986	*Pinus sylvestris*	1 : 4		1 : 4 MMN no sugar : water	16/8	160 µmol PAR	15/10	
Bending and Read 1995	*Pinus sylvestris*	1 : 3	Autoclaved	2 : 1 MMN : water	16/8	150 µmol PAR	15/10	8 weeks
Finlay 1989	*Pinus sylvestris*			MMN 1.25 g L^{-1} glucose, 5 g L^{-1} malt extract	16/8		20/15	4-9 weeks

Supplementary Material 3.2

Microcosm design

The maze was placed 15.5 cm above the bottom of the microcosm, and 6.5 cm to the sides. Each square has 1 cm side. Blue areas indicate the silicone spacers and the maze.



Chapter 4: An experimental approach to study foraging memory in ectomycorrhizal mycelium

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RESEARCH ARTICLE

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An experimental approach to study foraging memory in ectomycorrhizal mycelium

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ABSTRACT

Behavioral ecology of fungi is an emerging field investigating how fungi respond to environmental stimuli through morphological and physiological changes. Progress requires methodologies suited to fungal biology. Here, we developed an experimental approach to test for memory in the ectomycorrhizal fungus *Laccaria bicolor*. We hypothesized that mycelium exposed to pea cotyledons would retain directional information about the nutrient source. To test this, a portion of the mycelium was transferred to fresh medium, where memory would be assessed by asymmetrical growth toward the former nutrient position. The hypothesis was not supported, but the methods offer a framework for exploring fungal behavior in both ectomycorrhizal and saprotrophic species. Although no evidence of memory was found, this study highlights the value of publishing both positive and negative results and provides tools to advance research on fungal cognition and behavior.

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Cognitive ecology; foraging; fungal behavior; fungal ecology; negative results

Introduction

The behavioral ecology of fungi, also known as fungal ethology [1], is a recent research field that has gained traction in the last few years [2]. Such research has been carried out since at least the 1990s (e.g., [3–5]), but it is only recently that it has emerged as a specific field. Studies on fungal behavior [6,7], memory [8], foraging, and decision-making [9] are now appearing in the scientific literature with more regularity. Some authors indeed proposed them as cognitive or even conscious [1,10]. Demonstrations of fungi with the abilities outlined above can also be glimpsed by other studies that did not focus specifically on these functions. For example, the decision-making process of the pathogenic fungus *Candida albicans* to switch from yeast to filamentous forms, includes the perception and integration of several cues from the environment such as temperature, O_2, CO_2, pH, serum, and signaling molecules from other cells to decide whether to continue as a yeast or switch to the hyphal form [11–14].

Despite these studies, gaps in the knowledge base surrounding fungal behavioral ecology remain vast, and our work aims to contribute to the field by investigating a phenomenon recently identified in fungi: memory. Memory can be described as the capacity to encode information about past experiences and recall them in the future, regardless of the system that manifests it [15,16]. One way of studying it is by observing how past experiences influence the actions of the system under study when the conditions that created the memory are no longer present or appear again after some time. Memory is the basis of learning, an important adaptive phenomenon that optimizes the interactions of the organism with the environment over time [15].

There is some evidence for memory in fungi. For example, *Saccharomyces cerevisiae* seems to store information of past events that helps it adapt to fluctuations of the environment in the future, which can be considered a form of memory and learning. Yeasts that had been submitted to hyperosmotic stress

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4.1 Abstract

Behavioural ecology of fungi is an emerging field investigating how fungi respond to environmental stimuli through morphological and physiological changes. Progress requires methodologies suited to fungal biology. Here, we developed an experimental approach to test for memory in the ectomycorrhizal fungus *Laccaria bicolor*. We hypothesised that mycelium exposed to pea cotyledons would retain directional information about the nutrient source. To test this, a portion of the mycelium was transferred to fresh medium, where memory would be assessed by asymmetrical growth towards the former nutrient position. The hypothesis was not supported, but the methods offer a framework for exploring fungal behaviour in both ectomycorrhizal and saprotrophic species. Although no evidence of memory was found, this study highlights the value of publishing both positive and negative results and provides tools to advance research on fungal cognition and behaviour.

Keywords: fungal ecology · fungal behaviour · cognitive ecology · ectomycorrhizal fungi · reproducibility · negative results · null results · memory · foraging

4.2 Introduction

The behavioural ecology of fungi is a recent research field that has gained traction in the last few years (Aleklett and Boddy 2021). Such research has been carried out since at least the 1990s (e.g., Donnelly and Boddy 1996; Hughes and Boddy 1996; Boddy 1999), but it is only recently that it has emerged as a specific field. Studies on fungal behaviour (Aleklett et al. 2021; Fukasawa et al. 2024), memory (Fukasawa et al. 2020), foraging, and decision-making (Richter et al. 2024) are now appearing in the scientific literature with more regularity. Some authors indeed proposed them as cognitive or even conscious (Money 2021; Reber 2024). Demonstrations of fungi with the abilities outlined above can also be glimpsed by other studies that did not focus specifically on these functions. For example, the decision-making process of the pathogenic fungus *Candida albicans* to switch from yeast to filamentous forms, includes the perception and integration of several cues from the environment such as temperature, O_2, CO_2, pH, serum, and signalling molecules from other cells to decide whether to continue as a yeast or switch to the hyphal form (Brown Jr et al. 1999; Shareck and Belhumeur 2011; Sudbery 2011; Zhao and Rusche 2021).

Despite these studies, gaps in the knowledge base surrounding fungal behavioural ecology remain vast, and our work aims to contribute to the field by investigating a phenomenon recently identified in fungi: memory. Memory can be described as the capacity to encode information about past experiences and recall them in the future, regardless of the system that manifests it (Galviz et al. 2020; Pissolato et al. 2024). A way of studying it is by

observing how past experiences influence the actions of the system under study when the conditions that created the memory are no longer present or appear again after some time. Memory is the basis of learning, an important adaptive phenomenon that optimises the interactions of the organism with the environment over time (Galviz et al. 2020).

There is some evidence for memory in fungi. For example, *Saccharomyces cerevisiae* seems to store information of past events that helps it adapting to fluctuations of the environment in the future, which can be considered a form of memory and learning. Yeasts that had been submitted to hyperosmotic stress decreased the activity of the stress-responsive STL1 promoter, reducing the stress response to a subsequent hyperosmotic event (Ben Meriem et al. 2019). In another study, unsuccessful mating created a memory that, when yeasts were exposed to mating pheromones again, transiently prevented them from budding. However, if they did not reproduce sexually in a short stretch of time, they would resume the asexual reproduction through the formation of buds (Caudron and Barral 2013).

In filamentous fungi, it could be useful to retain memory of the location of nutrient sources so as to find them again after the hyphae are severed. This possibility was demonstrated by Fukasawa et al. (2020) when studying the directional memory of *Phanerochaete velutina*. The authors observed that, if these saprotrophic fungi were allowed to forage on a fresh piece of wood as its nutrient source (bait), then have their inoculum (the wooden block from where the fungus was growing) removed from the experimental setup and placed in a new one, they would grow more hyphae in the direction of where the bait had previously been located. The authors, nonetheless, honestly discuss that their results could be criticised because the directional memory could be explained not only because the fungi encoded the information about the direction of the bait, but simply because there would be more propagules on the side of the inoculum that faced the wooden bait (Fukasawa et al. 2020).

In this work, we took inspiration from Fukasawa et al. (2020) to design an experiment to test directional memory in an ectomycorrhizal fungus, *Laccaria bicolor*, that would: 1) potentially prevent the problem of uneven propagules outlined by Fukasawa et al. (2020), and 2) offer an easier way of testing direction memory in fungi. In our case, instead of using soil trays for observing fungal development, which limits the species that can be used and presents space and time constraints, we would make a similar experiment on potato-dextrose-agar (PDA) medium in Petri dishes. Doing these experiments in Petri dishes has the

advantages of being easier and cheaper to carry out, it requires less space and allows a higher number of experimental replicates.

Furthermore, to our knowledge, this is the first study of this kind using ectomycorrhizal fungi. Memory could be an important ability even to ectomycorrhizal fungi because although they obtain their carbon from their host plant, they still need to uptake nutrients and water from the environment, so the ability to regrow hyphae towards sources of nutrients remains as important as it is to saprotrophic fungi to acquire C. Since the functional mutualism of the mycorrhizal system depends on a compatible exchange of solutes between both partners there would be a selective pressure for fungal nutrient acquisition and consequent mycelial foraging behaviours.

We thus tested whether *L. bicolor* could recall the presence and direction of a past source of nutrients and grow more mycelia towards it. We hypothesised that: 1) *L. bicolor* would encode the direction of a discrete source of organic nutrients and grow more mycelium in that direction after part of the primed mycelium was transferred to a new medium; and 2) this effect would be more pronounced with fungi growing on a nutrient-depleted medium. The experimental set-up we developed aimed to solve the potential problem of more propagules on one side of the inoculum causing a growth bias that does not relate to memory (Fukasawa et al. 2020), enabling unequivocal assessment of directional memory in a fungus.

4.3 Material and methods

4.3.1 'Priming' of the fungi

A step-by-step diagram of the experiment is shown in **Figure 4.1**. Potato-dextrose-agar (PDA; Thermo Fischer Scientific, Waltham, Massachusetts, USA. Lot: 3794083) media were prepared in two concentrations: full concentration (39 g PDA · L^{-1}), or at ⅓ of full concentration (13 g PDA · L^{-1}) with added 10 g · L^{-1} of non-nutritious agar powder (Alfa Aesar-Termo Fisher Scientific, Heysham, UK. Lot: 10231469) for keeping the same consistency as full concentration. These were used for making, respectively, the two experimental conditions: full PDA (Full condition) and PDA diluted at ⅓ of its original concentration (Diluted condition). 25 mL of the media was poured onto standard acrylic 9 cm Petri dishes. Then, with a 0.5 cm-wide cork borer, agar plugs were removed from the growing edge of 50-days old *L. bicolor* kept in a fridge at 4 °C, and inoculated at the centre of the

Petri dishes, which were sealed with Parafilm® (Bemis/Amcor, Zurich, Switzerland) and placed on the top shelf of an incubator (model INCU-270C, SciQuip, Rotherham, UK), internal dimensions (W x D x H): 60 x 60 x 75 cm. The top shelf was 25 cm below the ceiling panel of the incubator. The Petri dishes were kept in darkness at 18 °C. On alternate days, a line was drawn around the edges of the growing colony at the bottom of the Petri dishes, and then the dishes were randomly reshuffled to avoid any influence of the incubator on the direction of hyphal growth.

Ten days after inoculation, when the cultures growing in full PDA had a diameter of approximately 2.9 cm ± 0.1 cm (n = 37) and the ones growing in diluted PDA had a diameter of approx. 3.1 cm ± 0.2 cm (n = 34), the Petri dishes were randomly assigned to different treatments. The treatments consisted of: Test, where one yellow pea (*Pisum sativum* L.) cotyledon (hereafter, just 'pea') was placed 1.5 cm away from the centre of the Petri dish; Control 1, where no pea was included in the Petri dish, and Control 2, where two cotyledons were placed equidistantly 1.5 cm from the centre of the Petri dish in opposite sides. The yellow split peas (Lot: 82670-1-1-1, produced in the UK; ASDA, Leeds, UK) were oven-dried at 40 °C until constant weight was achieved, then weighed in an analytical balance (Mettler AE160, Mettler-Toledo, Leicester, UK). Only peas that weighed exactly between 100 and 105 mg were used in this experiment. This is to minimise any effect of different mass in the growth direction of hyphae.

The peas were previously autoclaved in an open glass Petri dish for 5 minutes at 121 °C—counted from the moment the pressure indicator valve lifted—in a portable steam steriliser (Classic, model 210048, Prestige Medical, Blackburn, UK). After this time, the steriliser was turned off and a fan placed behind it to cool it as quickly as possible.

In a sterile laminar flow cabinet, the Petri dishes were opened, the peas were added, and then the Petri dishes were resealed with Parafilm®. Petri dishes for Control 1 (0 peas) were opened and subsequently closed again. All Petri dishes were taken back to the same

incubator as before, in the same conditions, and incubated for 7 days, being randomly repositioned at alternate days.

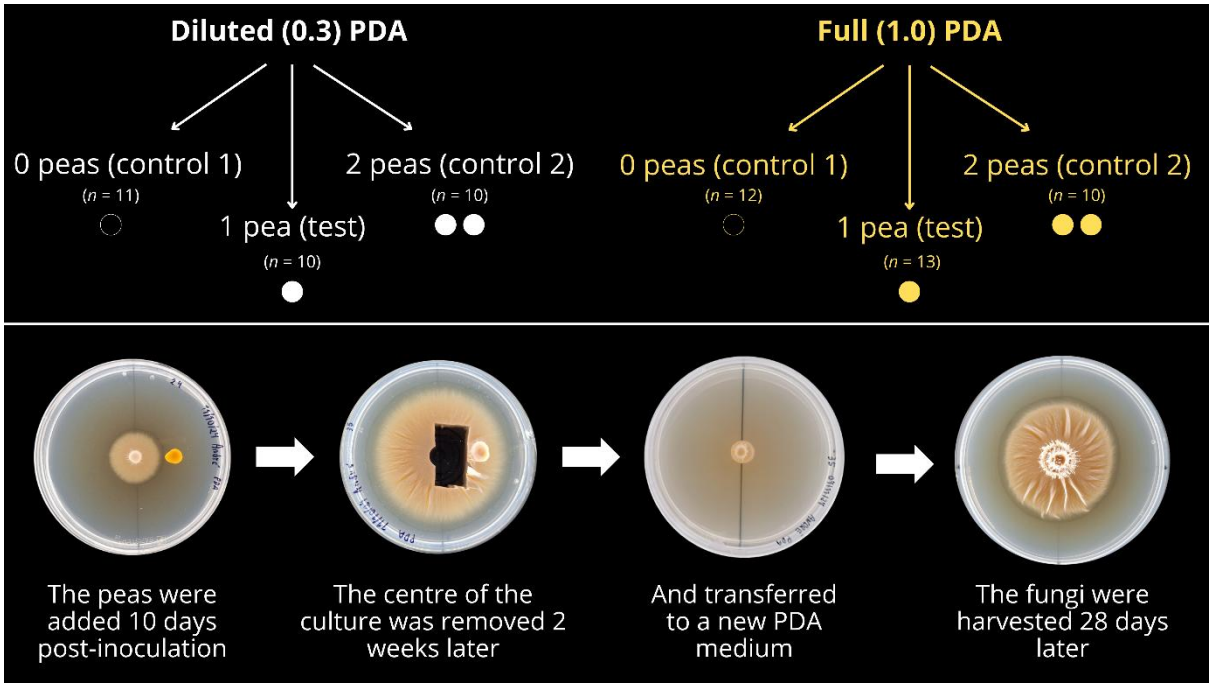

Figure 4.1. Diagram showing the experimental design above, with three treatments (Control 1, Test, and Control 2) for each condition (Diluted and Full PDA). Below, a step-by step guide of the procedure adopted, regardless of the number of peas.

4.3.2 Transfer to a new medium

14 days after including the peas, all the Petri dishes were taken to the laminar flow cabinet again. They were opened and, with a sterile 1.0 cm-wide (internal measurement) cork borer, an agar plug was bored around the 0.5 cm plug that inoculated the plate. Then, sterile pieces of aluminium foil were placed on the edges of the plug where it intersects the line drawn under the Petri dish to mark the position of the plug. The plug was carefully removed with a scalpel and placed on the centre of a new Petri dish with 25 mL of PDA at the corresponding dilution of the treatment (Full or Diluted) and in the same position as they were in the previous Petri dish, but without any peas present. These new Petri dishes had a line drawn at the bottom dividing it in two halves. With this arrangement, hyphae would have to grow down to the bottom of the plug towards the new agar before they started spreading radially, thus minimising any propagule effect.

The new dishes were sealed with Parafilm® and taken to the same incubator as before, under the same conditions. After a few days, we noticed that the Petri dishes in the diluted

PDA condition were contaminated due to a problem with the autoclave, but it did not seem to have affected the growth of the fungi. They just engulfed the bacterial colonies as if they were not there.

The Petri dishes were left undisturbed for 5 days in the incubator to allow the hyphae to penetrate the new agar from the plug, securing it in place. This was indicated by hyphae growing around the plug on the new agar. The Petri dishes were then removed from the incubator, a line was drawn around the edges of the colony at the bottom of the plate, and they were reshuffled before being taken back to the incubator. Drawing the line was always made by the same experimenter, holding the plate c. 30 cm away from the face and wearing an eye patch over the non-dominant eye to avoid distortions in the drawing due to parallax. This ensured the lines to be exactly above the edges of the colony.

4.3.3 Harvest of fungi

28 days after transferring the centre of the cultures to the new Petri dishes, the cultures were photographed with a Samsung Galaxy A54 cell phone (Samsung, Suwon, South Korea) with 50 MP resolution, following Rodrigues et al. (2022) protocol for photographing microbial cultures (using an 11.5 cm high observing tube instead of 23 cm, see Rodrigues et al. 2022). Then, the fungi were stored in a cold room at 4 °C. They were removed one by one from the fridge over the next three days for collecting the biomass. For doing this, we modified the protocol of Karaduman et al. (2012) and De Oliveira and Tibbett (2018). The PDA was removed from the Petri dish and placed in a larger, glass Petri dish with milli-Q water. Then, the fungi were microwaved in a Russell Hobbs microwave (model RHM2087B-TS, Failsworth, UK) at medium high power for c. 07:40 ± 2 minutes for diluted PDA plates, and 05:50 ± 01 for full PDA plates. This was enough to lightly boil the water, effectively dissolving the agar underneath it. The difference in time between the conditions is because we noticed that *L. bicolor* growing on diluted PDA typically grew more hyphae into the agar, requiring more time to properly melt. The mycelium was then removed from the water, bathed in cold milli-Q water for a few seconds, then blotted dry on a paper towel. All the mycelia were dried in a drying oven at 40 °C until constant weight. Their masses were measured with the same Mettler AE160 scale mentioned above. The empty Petri dishes were photographed to show all the lines drawn. The empty Petri dishes with the concentric lines were photographed in the same way described above for analysis.

4.3.4 Analyses

4.3.4.1 Growth and asymmetry

To measure growth rate, we used ImageJ (version 1.54, National Institutes of Health, Bethesda, Maryland, USA) to calculate the area of the mycelium that was in each side of the Petri dish using the polygon tool. The area of the whole mycelium was calculated by adding the area of both sides of the culture. We used a normalised index of asymmetry to check the position of the agar plug in relation to the reference line that divided the halves of the Petri dish (Equation 4.1).

Equation 4.1: $A_i = \frac{\mathrm{A} - \mathrm{B}}{\mathrm{A} + \mathrm{B}}$

Where A_i is the asymmetry index, A is the area of the plug in one half of the Petri dish, and B is the area in the other half. If the A_i of the plug was < -0.05 or > 0.05, we recalculated the centre of the plug with ImageJ, and only then measured the area of the mycelium in both sides of the Petri dish. We used the same equation to calculate the asymmetry of the culture every day, during 12 days. The value of A_i can range from -1, which would indicate 100% of mycelium growth in the side B of the Petri dish (away from the pea, in the case of the test treatment), and 1, where all mycelium would have grown in the side A of the treatment (towards the pea in the case of the test treatment). $A_i = 0$ indicates perfect symmetry of the culture, but we considered only the asymmetry indexes beyond the < -0.05 and > 0.05 range as significant.

4.3.4.2 Morphology

During the experiment, we noticed that several *L. bicolor*, in particular those exposed to the peas, regardless of the number, assumed a distinctive morphology, forming ridges that radiated from the centre of the culture. We used this as a parameter to analyse the effect of the peas on the fungi. With ImageJ, we used the *Circle Tool* to crop the culture. Then, prior to the analysis, we used the *Circle Tool* to remove the centre of the agar plug from the image. The agar plug was above the plane of the mycelium and could interfere with the colour threshold due to the bright white fungal structures it had. To count the number of ridges, we used the command *Image* > *Adjust* > *Colour Threshold*. In the threshold adjustment window, we adjusted the 'brightness' histogram by placing the cursor at the slope on the brighter side (since the ridges appeared brighter in the photos). Using the *Magic Wand Tool*, we selected

all the visible ridges. We manually checked for and removed any false positives before counting the ridges and measuring the area of the image covered by each ridge.

4.3.4.3 Nutrient analyses

To understand whether the results observed were due to fungi exposed to peas being better nourished than the ones not exposed to peas, we analysed the content of nutrients in the mycelia as follows:

4.3.4.4 Nitrogen and carbon determination

We quantified nitrogen (N) and carbon (C) in the mycelia using elemental combustion analysis. Due to the small dry mass of each sample, we pooled at least three mycelia to form one sample, resulting in three samples per treatment per condition. Mycelia from at least three replicates were ground with a pestle and mortar in liquid N_2, then dried at 70 °C for four days. We used 100 mg of ground mycelium to determine C and N content with a Leco CNH 628 analyser (LECO Corporation, St. Joseph, MI, USA).

4.3.4.5 Mineral nutrients determination

For the mineral nutrient determination, we digested 50 mg of ground dried mycelium as described above with 6 mL of a HNO_3 (69%) + 2 mL H_2O_2 solution (3:1 v/v), using an Ethos Easy 44-Max Microwave Digestor (Milestone Srl., Sorisole, Italy), dried plant tissue programme (heat up to 200 °C in 25 minutes and hold at 200 °C for 15 minutes.) Samples were pre-digested in room temperature for 15 minutes before heating. Extracts were then filtered using Whatman 540 (Cytiva, Danaher Corporation, Wilmington, DE, USA) paper filter and diluted with ultra-pure water (UPW) to 50 mL. An aliquot of 2.5 mL was further diluted with 7.5 mL UPW (1:3 v/v) before analysis through inductively coupled plasma optician emission spectroscopy (ICP-OES) (PerkinElmer Avio500, PerkinElmer, Inc., Shelton, Connecticut, USA). Blank samples and the plant certified reference material (IAEA-359 cabbage leaves) were included for quality control. Elements determined were calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulphur (S) and zinc (Zn).

4.3.4.6 Statistical analysis

Data was analysed by one-way ANOVA, followed by Tukey test to discriminate differences between each treatment ($p < 0.05$). Homoscedasticity was determined by the Levene test ($p > 0.05$), and normality by Shapiro-Wilk ($p > 0.05$). Dry mass data was transformed by $log_{(x)}$ to attain normality. For other non-normally distributed data, Kruskal-Wallis tests ($p < 0.05$) were applied. All analyses were carried out using the software XLStat® (version 2019.2.2, Lumivero, Denver, CO, USA).

4.3.5 Control for agar-borne chemicals

During the course of the experiment, we considered the possibility that substances like nutrients or hormones could be leaking from the peas and impregnating the agar which could then potentially be transferred with the fungal plugs depending on their spatial distribution. If true, the presence of these agar-borne chemicals might be the cause of any differences in morphology in the fungi previously exposed to the peas, rather than any internal mechanism for storing information. We also noticed that *L. bicolor* growing in full PDA detached very easily from the medium in comparison to that in the diluted medium. Hence, we carried out an additional experiment to control for the potential presence of chemicals exuded from the peas into the agar. With the exception of the nutrient and asymmetry analyses, the same experiment described above was repeated but, instead of transferring a 1 cm-wide agar plug as described in Section 2.2, we fully detached the mycelium from the agar and only transferred the mycelium to the new medium thereby controlling for any inadvertent transfer of agar-borne chemicals.

4.4 Results

In this study, we tested fungi in two conditions: PDA with ⅓ of the original concentration (Diluted) and PDA with the normal, full concentration (Full). For each condition we used three treatments: Control 1 (no peas), Test (one pea), and Control 2 (two peas), with the hypothesis that the fungi in the one Test treatment would grow asymmetrically towards where the pea was. Therefore, it could be argued that this would be an effect of the memory of the past presence of the peas in the medium, and not a simple physiological response to the presence of nutrients.

Overall, the fungi from the Diluted condition grew more in area, regardless of the treatment, than the fungi in the Full condition. There was no significant difference in any growth parameter between the treatments (**Table 4.1**). Fungi in the Diluted treatment seem to have grown more mycelia inside the agar than those in the Full treatment, which rendered them much more difficult to remove from the agar than those in the Full agar. When microwaving them, it was impossible to separate all the agar from the mycelium and they kept a 'slimy' texture in the mycelium surface that had contact with the agar. Therefore, the data regarding the dry mass, C and N proportions, and the mineral composition of this group is unreliable and cannot be compared to the Full group. Additionally, fungi grown in the Diluted condition were paler and smoother when compared to the ones grown on Full condition, which were heavily ornamented (**Figure 4.2**).

Table 4.1. Average area and dry mass of the fungi in each condition and treatment (indicated as number of peas in previous Petri dish), with standard deviation. Fungi in the Diluted condition grew more in area than those on Full PDA, and there was no significant difference in the growth parameters between the treatments. *n* = number of replicates. 0 Peas = Control 1; 1 Pea = Test; 2 Peas = Control 2. Different letters represent significant differences between treatments after one-way ANOVA, followed by Tukey test ($p < 0.05$).

Condition	*n*	Peas	Area (cm²)	Dry mass (mg)
Diluted	11	0	44.7 ± 4.6 a	114 ± 24 a
	10	1	44.2 ± 3.9 a	111 ± 18 a
	10	2	42.4 ± 2.8 a	115 ± 24 a
Full	12	0	27.4 ± 8.4 b	87 ± 35 b
	13	1	23.7 ± 4.3 b	79 ± 21 b
	11	2	29.4 ± 8.3 b	104 ± 36 b

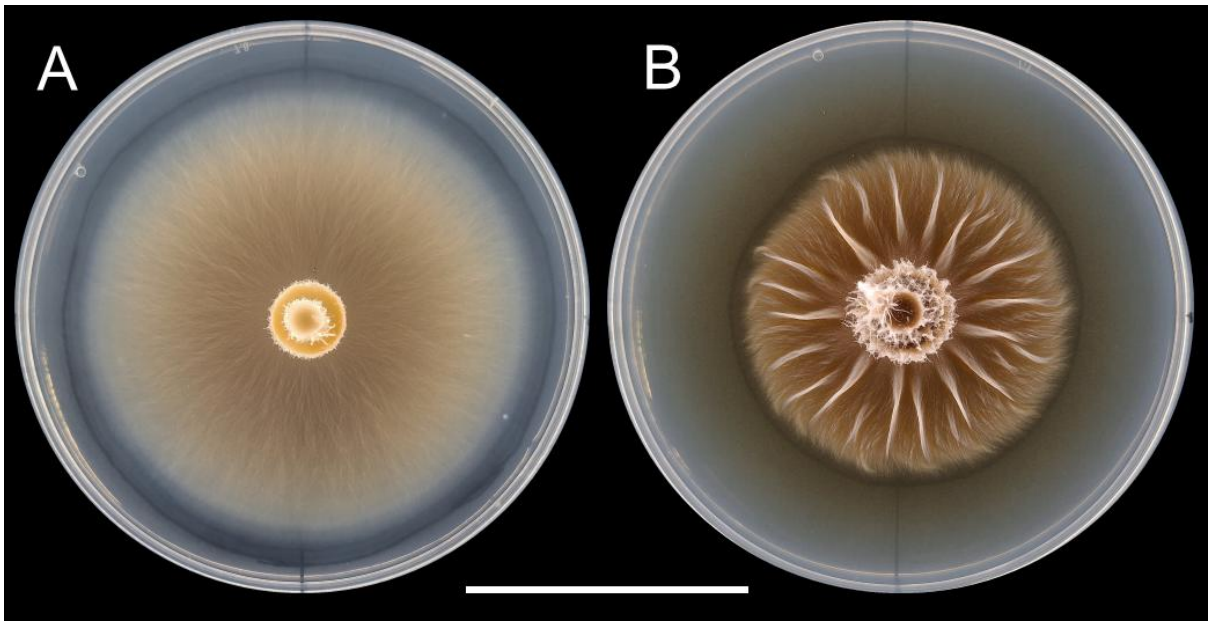

Figure 4.2. Different morphologies caused by the dilution of the PDA medium to *L. bicolor*. A: fungi growing in PDA medium at ⅓ of the original concentration. B: a fungus growing on full PDA. Note the smaller area and presence of thickened ripples as radial ridges in B compared to the diffuse growth pattern in A. The scale bar represents 4 cm.

The presence of one or two peas did not have any significant effect in the concentration of C, N, and several mineral nutrients (**Table 4.2**). The dry mass, C and N percentage, and the mineral content, were essentially the same across all the treatments.

The asymmetry analysis did not show any growth preference for the side where the peas were in any of the days analysed, as shown in **Table 4.3**. The fungi grew consistently in a circular shape. This effect was observed even in the fungi before transfer, when the peas were still present, as confirmed by Kruskal-Wallis ($p > 0.05$).

Table 4.2. Percentage of total C and N in the mycelia, and concentration of mineral nutrients. For each treatment, three mycelia were pooled together. Diluted = fungi grown on PDA diluted at ⅓ of the original concentration. Full = fungi grown on PDA at normal, full concentration. 0P = Control 1; 1P = Test, 2P = Control 2. There were no significant differences among Pea treatments after one-way ANOVA, except for K in the Diluted condition (in bold), where different letters correspond to significant differences after Tukey test ($p < 0.05$).

Condition	Peas	C %	N %	Ca (mg g^{-1})	Cu (µg g^{-1})	Fe (µg g^{-1})	K (mg g^{-1})	Mg (mg g^{-1})	Mn (µg g^{-1})	P (mg g^{-1})	S (mg g^{-1})	Zn (µg g^{-1})
Diluted	0	46.9 ± 0.4	2.0 ± 0.2	0.50 ± 0.04	5.5 ± 1.6	40.3 ± 14.5	**1.6 ± 0.1 ab**	0.40 ± 0.03	3.1 ± 0.3	5.2 ± 0.3	3.0 ± 0.1	12.0 ± 0.6
	1	47.0 ± 0.3	2.0 ± 0.2	0.48 ± 0.03	4.5 ± 0.3	34.5 ± 7.6	**1.3 ± 0.2 b**	0.39 ± 0.06	2.9 ± 0.6	5.2 ± 0.9	2.8 ± 0.1	13.2 ± 0.6
	2	47.0 ± 0.5	2.0 ± 0.3	0.48 ± 0.1	10.7 ± 9.3	26.0 ± 5.9	**1.9 ± 0.2 a**	0.43 ± 0.06	2.9 ± 0.6	5.4 ± 1.3	2.9 ± 0.1	17.1 ± 8.3
Full	0	49.9 ± 0.3	4.4 ± 0.1	0.36 ± 0.1	6.9 ± 0.5	71.0 ± 18.8	0.50 ± 0.1	0.20 ± 0.04	4.5 ± 0.5	4.3 ± 0.2	2.3 ± 0.1	43.6 ± 2.5
	1	50.2 ± 0.2	4.7 ± 0.1	0.44 ± 0.1	8.2 ± 1.0	73.0 ± 10.2	0.72 ± 0.1	0.28 ± 0.03	5.6 ± 0.5	5.4 ± 0.3	2.9 ± 0.2	54.0 ± 4.8
	2	49.8 ± 0.3	4.1 ± 0.1	0.45 ± 0.1	10.7 ± 4.5	91.9 ± 48.0	0.56 ± 0.1	0.19 ± 0.02	4.2 ± 0.3	4.1 ± 0.2	2.5 ± 0.2	45.5 ± 2.6

Table 4.3. Asymmetry index for the fungal cultures at each day of measurement. Positive values indicate more mycelium towards where the pea was, and negative values, more mycelium away from the pea. We considered values between -0.05 and 0.05 as indicating perfect symmetry, i.e., no growth preference for any side. There was no significant difference between the treatments in each condition after one-way ANOVA ($p > 0.05$).

Condition	*n*	Peas	Days after transfer												
			0	5	7	9	11	13	15	17	19	21	23	25	27
Diluted	11	0	-0.01	-0.01	-0.02	-0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
	10	1	-0.01	-0.03	-0.02	-0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	10	2	0.00	-0.01	-0.02	-0.01	0.00	0.00	-0.01	0.00	0.00	0.00	0.00	0.00	0.00
Full	12	0	0.00	0.00	0.00	0.01	-0.01	0.00	0.01	0.00	0.00	0.01	0.00	0.03	0.01
	13	1	0.02	0.00	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00
	11	2	0.00	-0.01	0.00	-0.01	-0.01	0.01	0.00	-0.01	-0.01	0.00	0.00	-0.01	0.00

Table 4.4. Number of ridges and area of ridges in Experiment 1 (fungi transferred with agar plug) and subsequent Experiment 2 (fungi transferred without agar plug). Experiment 1 consisted of 3 treatments with different numbers of peas, and experiment 2 only had 2 treatments (0 and 2 peas). Different letters correspond to significant differences within each experiment, after one-way ANOVA and Tukey test ($p < 0.05$).

Experiment	*n*	Peas	*n* ridges	Area ridges
1	12	0	6.7 ± 6.2 b	0.07 ± 0.05 a
	13	1	13.0 ± 5.3 a	0.07 ± 0.03 a
	10	2	13.5 ± 6.1 a	0.09 ± 0.02 a
2	30	0	1.8 ± 2.7 A	0.04 ± 0.04 A
	22	2	2.7 ± 3.1 A	0.04 ± 0.04 A

In the first test, we noticed that the fungi exposed to the peas, regardless of the number, assumed a different morphology than the fungi not exposed to them. They presented a significant higher number of radial ridges that departed roughly from the centre of the culture (**Figure 4.2**b, **Figure 4.3**a,b). When we controlled for agar-borne substances derived from the peas by transferring only the mycelium without the agar plug with them, this effect disappeared, and their area was significantly larger (**Figure 4.3**, **Table 4.4**).

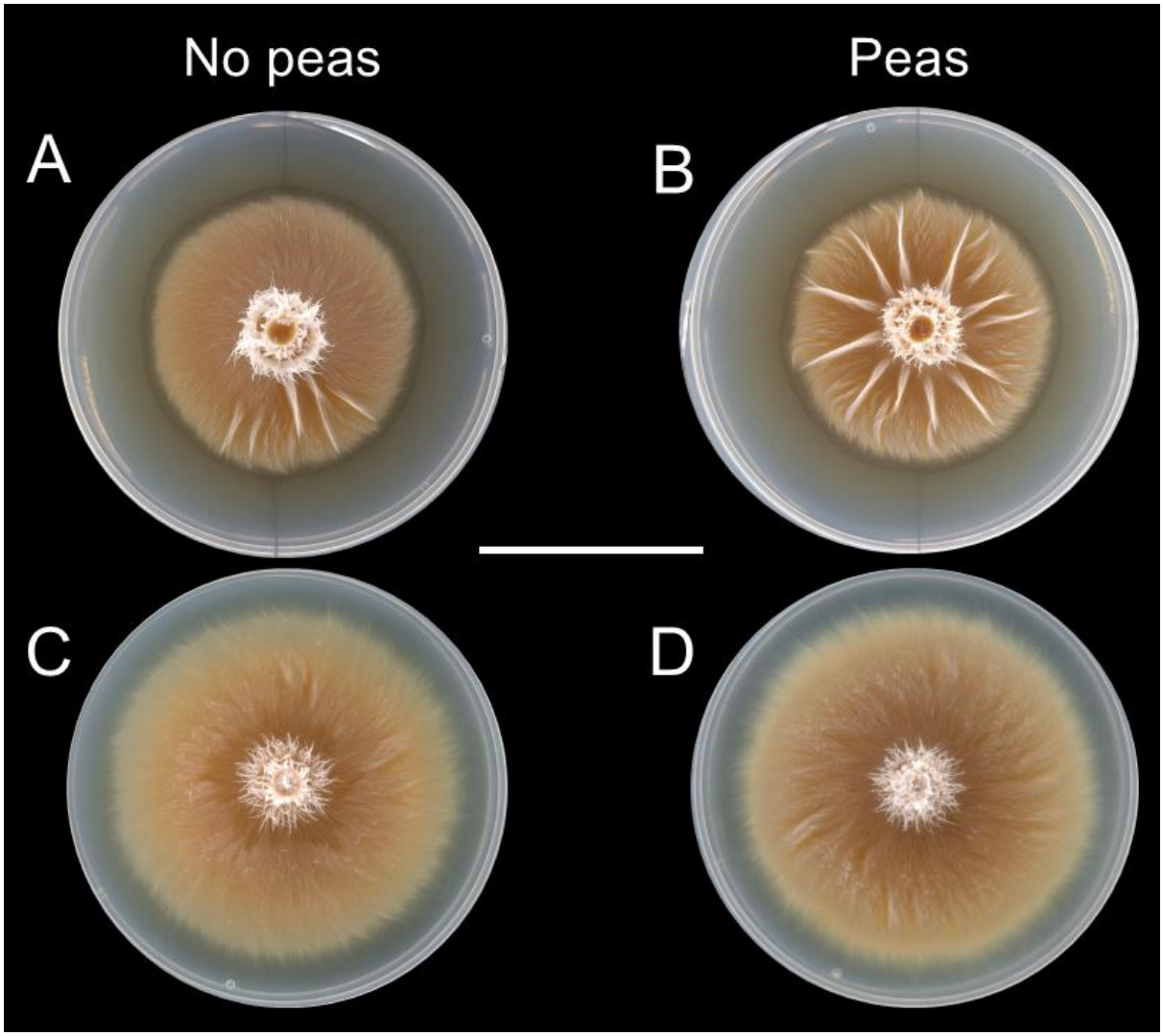

Figure 4.3. Expression of growth variation of *Laccaria bicolor* cultures grown on agar with and without peas, and in the presence or absence of retained agar. Left: fungi previously not exposed to the peas. Right: fungi exposed to the peas. In the Full PDA condition, when the mycelium was transferred with the agar plug to new PDA, the fungi previously not exposed to the peas developed significantly less ridges (A) than those previously exposed to them, regardless of the number of peas (B). When the mycelium was transferred without the agar plug, this effect disappeared, and there was no significant morphological difference between the fungi not exposed to the peas (C) and those exposed to them (D). The scale bar represents 4 cm.

All the raw data for these analyses (dry mass, N% and C%, mineral nutrients, area and asymmetry of the mycelia, and pictures) is available in the Supplementary Material.

4.5 Discussion

In this investigation, we developed a method to study the putative directional memory of fungal mycelium in agar plates instead of soil trays (Fukasawa et al. 2020). Studying the behavioural ecology of fungi in Petri dishes has the advantage of being technically easier, simpler, and quicker than in soil trays. Additionally, it can be performed in simple incubators without the need for any specialised facilities or appliances.

Inspired by Fukasawa et al. (2020), we tested whether the ectomycorrhizal fungus *L. bicolor* would present a directional memory of the past presence of a pea cotyledon in its vicinity as a source of nutrients, particularly N and P. To the best of our knowledge, this is the first study to address memory ability of an ectomycorrhizal fungus. After incubating fungal cultures with none, one pea on one side of the culture, or two peas (one on each side), we transferred the centre of the culture to a new Petri dish with the hypothesis that the fungi incubated with just one pea would asymmetrically grow mycelium preferentially towards the direction where it had contacted the pea in the previous petri dish.

The first observation we made was that the fungi growing in diluted PDA grew over a greater area than the ones in the full concentration. They also seemed to attach more to the agar, which could suggest that the fungi in this condition were exploring for more nutrients. It was not possible to conclusively determine if they grew more or less dense mycelium—which would support this claim—because the attachment to the agar implied that some of the agar was embedded in the mycelium when we measured the dry mass.

We did not observe any significant change in the dry mass, C and N proportion, and mineral content of the mycelia across the treatments. This may suggest that the peas did not have a significant nutritional effect on the fungi (which could explain the negative result) or that the effect was so small that it cannot be detected by these analyses. It is noteworthy that, although we could not measure this quantitatively in our experiment, we observed that the fungi seemed to have at least partly digested the peas. In a preliminary

test, we noticed that the pea ‘dissolves’ almost completely after a few weeks under the mycelium (Supplementary Figure 4.1).

Regarding the main goal of this study, in none of the conditions (Full or Diluted PDA) did the fungi show any growth preference towards or away from the direction of the peas. We did not obtain an asymmetry index greater than 0.05 or smaller than -0.05 in any day of the measurement period, and towards the end of the test, this index was essentially 0.00 in all conditions and treatments. With this result, we can conclude that *L. bicolor* did not show any directional memory in this experiment.

The initial observation of significantly different morphologies (radial ridges) between fungi previously exposed to the peas compared to those not exposed was not found again when instead of transferring an agar plug with the mycelium, we transferred only the mycelium. Therefore, it raises the intriguing possibility of unidentified compounds leaching from the autoclaved peas, impregnating the agar, travelling over 1 cm in less than two weeks towards the centre of the Petri dish, and staying there for several days, active enough in the transferred plug to induce the formation of radial ridges in the following cultures and suppress growth in area.

We did not investigate which compounds these could be, but they would likely be plant hormones, conformationally resistant to autoclaving, that leached from the peas, such as auxins and cytokinins. It has been known for several decades that auxin can stay in agar for long enough to cause growth and morphological changes in plants (Lewis and Muday 2009), and the auxin indole-acetic acid (IAA) can remain stable after autoclaving at 120 °C for 20 minutes (Yamakawa et al. 1979). Similarly, the cytokinins trans-zeatin (tZ), 6-(γ,γ-dimethylallylamino) purine (2iP), kinetin, benzyladenine (BA), and *m*-topolin conserved their stability after autoclaving at 121 °C for 30 minutes (Hart et al. 2016). Both cytokinins and auxins are present in pea seedling extracts (Barba-Espin et al. 2010) and are known to influence the physiology of ectomycorrhizal fungi (Gogala and Pohleven 1976; Župančič and Gogala 1980; Anand et al. 2022). In high quantities, IAA combined with cytokinins inhibited the growth of *Suillus variegatus* (Gogala and Phleven 1976). If they have a similar effect on *L. bicolor*, this could partly explain why the fungi exposed to peas grew over less area (**Figure 4.3**). It is interesting, though, that the effect of this unknown compound was only visible in the full PDA condition, revealing some kind of context-dependency in the fungal response to it. Future studies should try to

identify which substance has such a strong effect on the structure of *L. bicolor* mycelium, as it may prove useful to deepen the understanding of the physiology of this ectomycorrhizal species and give insights into how to manipulate its growth in agricultural and forestry contexts.

In this study, despite promising initial observations, we could not corroborate our hypothesis. Fungi exposed to a single nutrient source did not grow in the direction of a previously contacted source following mycelial transfer to a new medium. One of the possible reasons for this is because *L. bicolor* is an ectomycorrhizal fungus and, in this case, it was growing in axenic conditions, i.e. in PDA medium without a plant host. Despite growing rather well and appearing healthy, the fact it was not in symbiosis with host could have an influence on how it interacts with the environment. For directional memory, if this happens at all, it could be hypothesised that the fungus uses the plant root/s to which it is attached—and from which it receives carbohydrates—as a reference point and navigates outwards from there. When in culture, it would assume the standard radial growth common to many fungi. Evidently, memory could have occurred at metabolic and epigenetic levels, but these were not addressed here, as our initial interest was on the concept of spatial memory and how this would affect fungal growth. Another alternative hypothesis is that the transfer to new medium and subsequent measurements was too stressful for the fungus, and it lost the memory of the pea positioning. However, we note that preferential growth towards the pea was not observed even when the pea was present in the medium, before transfer.

Despite yielding null results in this case, we still believe this methodology can be fruitful for the study of the behavioural ecology of fungi. We have demonstrated that the centre of an ectomycorrhizal mycelium growing on agar can be extracted, transferred to a new growth medium, and continues to grow without difficulty. It would be worthwhile testing this same set-up with other species of ectomycorrhizal fungi and with saprotrophic fungi, to explore whether their response would be different from that of *L. bicolor*. Instead of peas, other, bespoke sources of nutrients could be used to control for hormones or other undesired substances that could affect the results. The study of fungal behavioural ecology is in its early stages, and the development of appropriate methodologies is essential. Although the outcome did not conform to our predictions, this work is another step in the direction of building a framework to study how fungi perceive and interact with the world.

4.6 Acknowledgements

The authors would like to thank Becky Dillingham, Nephele Swann, Richard Casebow, and Yiran Zou for their technical support throughout the course of this study. We are grateful to Hugues Massicotte for insightful discussions about mycelial growth in culture. AGP is supported by a University of Reading International PhD Studentship.

4.7 Conflict of interest statement

The authors declare to have no financial or personal relationships that could be perceived as a conflict of interest with this study.

4.8 Data availability statement

All the raw data relevant for this research is available as supplementary material.

4.10 Supplementary Material Statement

All the raw data relevant to this research are available in:

Parise, André Geremia; De Oliveira, Vinicius Henrique; Tamagnini, Francesco; Tibbett, Mark; Pickles, Brian John (2025), "An experimental approach to study foraging memory in ectomycorrhizal mycelium – Supplementary Material," Mendeley Data, V1, doi: 10.17632/phstb5yttk.1. https://data.mendeley.com/datasets/phstb5yttk/1

Supplementary Figure 4.1

Fungal mycelium after being microwaved, showing the empty space were formerly the pea was. This suggests that the fungus fully digested the pea cotyledon.

Chapter 5: A new application of electrophysiology in ectomycorrhizal research

5.1 Abstract

There is ample evidence for the importance of electrical signalling for the physiology and behaviour of plants, and some evidence that electrical signalling plays a similarly important role in fungi. The role of bioelectricity in mycorrhizas, the symbiotic association between plant roots and fungi, however, remains seldom explored, despite it being perhaps unlikely that plants and fungi cannot sense each other's electrical signals. To address this non-negligible gap in mycorrhizal knowledge, we proposed to adapt techniques routinely used in cellular neuroscience to explore the role of bioelectricity in mycorrhizas, starting with the fungal partner. Here, we employed an electrophysiology rig equipped with one bipolar microelectrode to stimulate the mycelium of three ectomycorrhizal fungal species—*Amanita muscaria*, *Laccaria bicolor*, and *Suillus granulatus*—with short pulses of electrical currents, and recorded their response. This preliminary exploration revealed that these fungi possess unique electrical activity which is possible to evoke and record as variations in electrical potential. Additionally, the response of the fungi showed some plastic properties that are promising for further studies. This work provides evidence that it is possible to study fungi with neuroscience equipment and opens an avenue of research recognised by the V. Kann Rasmussen Foundation (New York, NY, USA), which granted a generous fund for deepening these studies.

Keywords: electrical signalling · fungal electrophysiology · electrophysiology rig · microelectrodes · *Amanita muscaria* · *Laccaria bicolor* · *Suillus granulatus*

5.2 Introduction

> This chapter is adapted from the successful grant proposal submitted to the V. Kann Rasmussen Foundation through the Spring 2024 Call for Proposals *Sentience and Cognition in Nature*.

With the turn of the century, the interest in the interaction between plants and their mycorrhizal symbionts has been expanded beyond the mere improvement of plant yield and production of antibiotics by fungi (Zak 1964; Marx 1966, 1972; Marais and Kotzé 1976; Clarkson 1985; Koide 1991; Nolan 1991). Previous research has shown the importance of mycorrhizas in assisting plants when acquiring nutrients (Tibbett and Sander 2002; Eissenstat et al. 2015; Cheng et al. 2016), withstanding abiotic stresses (Pickles and Simard 2017; Tibbett et al. 2022), potentially communicating between them (Song et al. 2010; Babikova et al. 2013), and stealing carbon from common mycorrhizal networks (CMN) (Bidartondo et al. 2002; Merckx 2011; Rillig et al. 2024). Yet, despite these advancements, there is still much to be elucidated. For example, aspects related to

the pre-mycorrhizal communication between plants and fungi, the exchange of information during the formation of the symbiosis, and a potential ongoing communication between partners after mycorrhizas have been established remain poorly understood (Oldroyd 2013; Martin et al. 2016).

The sustenance of the symbiosis between plants and mycorrhizal fungi depends on communication, for plants need to attract mycorrhizal fungi to their roots and 'know' that the fungi trying to penetrate its epidermis are mutualistic and not pathogenic. Communication is present from the very beginning of the association, when plants release strigolactones that attract arbuscular mycorrhizal fungi (Akiyama et al. 2005; Yoneyama et al. 2012), and the fungi reciprocate with mycorrhizal factors, i.e., chemical substances that inform the plant that they are beneficial symbionts and not pathogens. In ectomycorrhizal hosts a range of root exudates may be responsible for the same type of process (Plett and Martin 2012). Only after this communication will the plant shut down its immune system locally to allow the fungi to enter (Plett et al. 2011; Oldroyd 2013).

Once inside the plant roots, the fungi establish a close interface with the plant cells through which they start to exchange nutrients and signalling molecules like effector proteins (Plett et al. 2011), mycorrhizal-induced small signalling proteins (MiSSP) (Plett et al. 2014a), hormones (Plett et al. 2014b; Pons et al. 2020), and small RNAs (Silvestri et al. 2024). However, what exactly happens at these interfaces is largely unknown (Martin et al. 2016). In a recent review on ectomycorrhizas, Martin et al. (2016) indicated this gap in the knowledge with a simple, unassuming question mark in the first figure of their work (**Figure 5.1**). In addition to the systems of communication mentioned above, there is one that has remained mostly ignored, with little scientific investigation: electrical signalling.

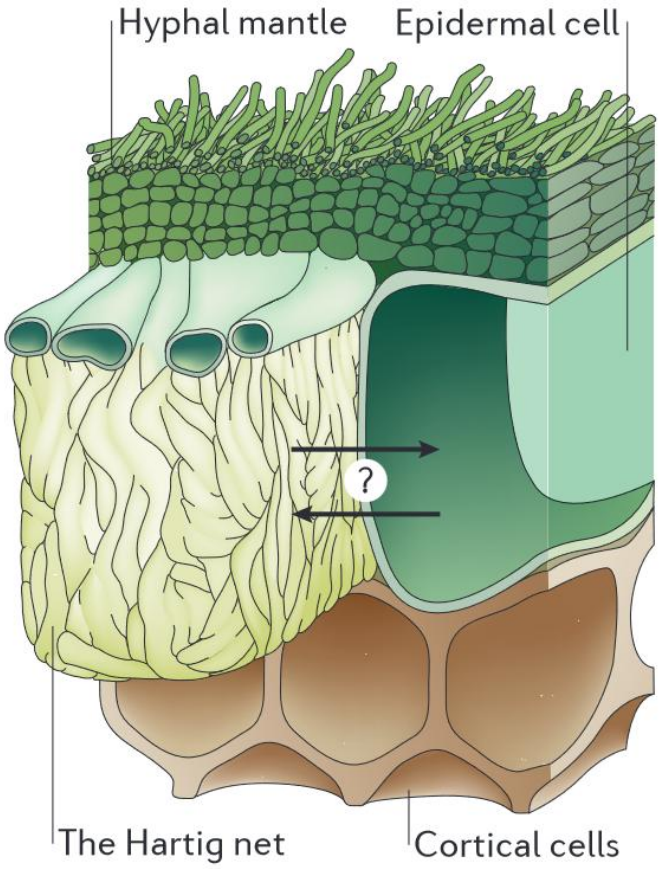

Figure 5.1. Figure extracted from Martin et al. (2016) showing a schematic view of the structure of ectomycorrhizas. The unknown nature of the exchanges between fungi and plants is indicated as a question mark.

Following the pioneering works of eminent scientists like Sir John Scott Burdon-Sanderson (1828–1905) and Sir Jagadish Chandra Bose (1858–1937), it is known that plants produce electrical signals (Burdon-Sanderson 1873; Bose 1926). Over a century later, much is known about this intriguing physiological phenomenon that seems to be related to everything a plant perceives and does. In plants, electrical signals are produced by any cell through the maintenance of an ion gradient between the cytoplasm and the apoplast (Vodeneev et al. 2016; de Toledo et al. 2019). These signals can be triggered by activating ion channels (calcium channels, in the case of plants) with chemicals or mechanical stimulation (Hedrich 2012).

Electrical signals travel fast within plant and fungal tissues—an action potential can travel up to 20 cm s^{-1} or more, but usually not more than 3 cm s^{-1} in plant tissues (Huber and Bauerle 2016)—and are greatly versatile. Action potential-like events have

been previously observed in plants and fungi (Huber and Bauerle 2016; Olsson and Hansson 1995). They have fixed amplitude and duration and are only fired after a threshold has been surpassed. Once fired, they are irreversible (in what is called the *all-or-none principle*, Vodeneev et al. 2016). Other signals, like the slow wave potentials or the systemic potentials, are proportional to the intensity of the stimulus that triggered them and fade with distance (Stahlberg et al. 2006; Zimmermann et al. 2009; de Toledo et al. 2019). The combination of these and several other signals has the potential to be responsible for the encoding and processing of environmental information (de Toledo et al. 2019) and arguably for the acquisition of experience-dependent memory. In fact, specific electrical signals and dynamics seem to be related to virtually everything involved in plant perception and action, from the regulation of photosynthesis to responses to osmotic stress and cold stimuli (Koziolek et al. 2004; Sukhov 2016; Souza et al. 2017), from the detection of other plants nearby to the alarm caused by herbivory (Parise et al. 2021; Reissig et al. 2021; Aratani et al. 2023).

In the case of fungi, there is considerably less information about their electrophysiology. However, some studies have already indicated that they can perceive objects around them and produce electrical signals that are reminiscent of animal action potentials in response. For example, Olsson and Hansson (1995) observed the wood-decaying fungi *Armillaria bulbosa* and *Pleurotus ostreatus* responding to the presence of a wooden bait with trains of spikes in their electrical activity. In natural environments, Fukasawa et al. (2023) recorded changes in the bioelectrical activity of *Laccaria bicolor* basidiomes after a rainfall event. Other fungal species have been shown to produce non-random (i.e., different from pure white noise, with some complexity) electrical signals, like *Schizophyllum commune*, *Omphalotus nidiformis*, *Flammulina velutipes*, *Cordyceps militaris*, and *Pleurotus djamor* (Adamatzky 2018, 2022; Adamatzky et al. 2023). More recently, Mishra et al. (2024) were able to harness the electrical signalling of UV light-stimulated *Pleurotus eryngii* to steer a biohybrid robot for a few metres.

Considering the rich electrical activity of plants and fungi, the hypothesis that plants and mycorrhizal fungi could be mutually sensitive to each other's electrical signalling springs up almost naturally. However, there is virtually no evidence showing this kind of interaction between them. It is known that the communication mediated by chemicals at the presymbiotic stage triggers an influx of calcium (Ca^{2+}) to the cytoplasm and nucleus of the plant cells (Oldroyd 2013), and transient variations in cytosolic Ca^{2+}

are often related to electrical signalling—in plants, electrical signals are usually commenced by the influx of apoplastic Ca^{2+} into the cytoplasm (de Toledo et al. 2019). Berbara et al. (1995) have also demonstrated that both root cells and expanding mycorrhizal hyphae produce electrical currents of ions. In the case of plants, they usually flow outwards at the tip of the root and inwards at more mature tissues (Collings et al. 1992; Berbara et al. 1995). Plant electrical currents could be elicited and sensed by fungal hyphae, which would use inward currents to guide its growth towards the penetration point in the root cell (Berbara et al. 1995). According to these authors:

> "Our studies demonstrate that there is an electrophysiological dimension to the plant fungus interaction and have shown that an early event in the formation of a mycorrhizal symbiosis is the modulation of ion transport in the host cortical membrane. The significance of these changes to the symbiotic partnership are not known." (Berbara et al. 1995, p. 437).

Surprisingly, since Berbara et al.'s (1995) study, little was found about the significance of electrical signalling between plants and mycorrhizal fungi. More recently, Thomas and Cooper (2022) have shown that two plants could transmit endogenous electrical signals between them through arbuscular mycorrhizal fungi. Their study suggests that plants can, potentially, produce electrical signals that are transmitted to the common mycelial network and through it to other plants. The details of how the signal is transferred from the plant to the fungus, and then to the other plant, remain unknown.

Since plant cells and fungal hyphae have an intimate surface contact (**Figure 5.1**), it is not unlikely that they can sense variations in the electrical potential of their symbiont when an electrical signal happens. In other words, an electrical signal commenced by a plant cell in contact with the Hartig net (fungal hyphae that establish contact with root cells) necessarily involves the influx of Ca^{2+} into the plant cell cytoplasm (de Toledo et al. 2019). This implies a transient depletion of Ca^{2+} in the apoplast causing a variation in the electrical potential of the adjoining fungal hyphae, which in turn may trigger voltage-sensitive ion channels in the fungus. The same phenomenon starting with the fungal hyphae is equally possible. This could be accounted for as an indirect form of communication. Another, direct form of communication could be the release of chemicals by one partner that activates Ca^{2+} or other ion channels in the other. There is no scientific evidence for any of these methods of communication, and that is precisely the gap in the knowledge that we intend to address.

We propose to investigate the details of the potential electrical communication between plants and mycorrhizal fungi. We aim to develop a robust protocol to study this using an electrophysiological rig and multielectrode arrays (MEAs), both routinely used to study the functioning of mammalian brain networks (Kandel et al. 2000; Shin et al. 2021). With the protocol established, we will study isolated fungal cultures, then root cuttings, and then mycorrhizal systems (roots colonised by mycorrhizal fungi). The ultimate goal of this research is to address the hypothesis that plants and fungi exchange meaningful electrical signals and that those are used for processing information. This goal, evidently, lies in the medium-term future, very likely beyond this doctoral research.

In this chapter, we describe the development of novel electrophysiological tests on fungal tissue from multiple species, with encouraging results. Previous research on fungal electrophysiology used techniques like vibrating microelectrodes (e.g., Berbara et al. 1995; Olsson and Hansson 1995) or needle electrodes (e.g., Adamatzky et al. 2023; Fukasawa 2024) but the scale of measurement of both techniques is either too small (at the level of hypha) or too large (at the level of several hyphae and tissues). We explored an intermediate option with an electrophysiological rig that provides data on the network properties of small areas of tissue, in the range of a few tens of micrometres. As there was no established methodology, any findings could be new, so we embarked on an iterative methodological development (trial and error) to refine a functional method. Hence, the initial experiments were exploratory in nature. Nevertheless, the results obtained at this early stage already point to a promising direction—so much indeed as to win us a grant to fund two years of research in this area by the V. Kann Rasmussen Foundation. In this chapter, we describe our preliminary results with the electrophysiological rig.

5.3 Materials and methods

5.3.1 Fungal material

We selected three species of ectomycorrhizal fungi from the University of Reading fungal collection to initiate these studies. *Amanita muscaria* and *Laccaria bicolor* are in the same order (Agaricales), but in very distinct clades (Matheny et al. 2006), whereas *Suillus granulatus* belongs to the Boletales order. All of them are very common and important ectomycorrhizal fungi in natural environments. Furthermore, *L. bicolor* has had its genome completely sequenced (Martin et al. 2008), which makes it an interesting

candidate for electrophysiological studies as it is possible to search for specific sequences coding for ion channels in its genome.

Cultures of *A. muscaria*, *S. granulatus*, and *L. bicolor* were prepared on Modified Melin-Nokrans medium (Gafur et al. 2004) in 3 cm wide Petri dishes. They were stored in an incubator at 12 °C, in total darkness. The three cultures (one specimen per species per day) were used for the electrophysiological tests when they were 94 and 101 days old, respectively.

5.3.2 Electrophysiological tests

The tests were made on an electrophysiological rig, at room temperature. Inside a Faraday cage, the fungal culture was submerged in Hank's Buffered Saline Solution (HBSS), containing 0.137 mM of NaCl, 5.4 mM of KCl, 0.25 mM of Na_2HPO_4, 0.1 g of glucose, 0.44 mM of KH_2PO_4, 1.3 mM of $CaCl_2$, and two microelectrodes were placed touching the mycelium (**Figure 5.2**). One of the electrodes is a stimulator, and the other is a glass micropipette with an access resistance of 290 kOhm–5 MOhm, filled with HBSS and mounted over an AgCl recording electrode. Stimulating electrodes consist of 50 µm diameter twisted tungsten wires coated in PTFE. The stimulator applies an electrical current square stimulus of variable duration and intensity (as specified) on the fungus via an isolation box. The stimulation artefact and evoked electrophysiological responses were detected via the recording electrode, connected to a Multiclamp 700A amplifier (Molecular Devices, San José, CA, USA) and digitised using an Axon Digidata 1550B (Molecular Devices, San José, CA, USA). Sweeps were lowpass filtered at 2–10 kHz, amplified with a 1000 gain, and digitised with a sampling frequency of 10–100 kHz. PClamp 10.7 was used to visualise and store sweeps in a computer. For the input-output experiments, we applied stimulations of increasing intensity and/or duration as specified below. The time series were processed on Clampfit (Molecular Devices, San Jose, USA) and analysed in Origin (Malvern Instruments, Malvern, UK). The baseline of the time series was arbitrarily fixed as 0.

We did not follow a specific protocol because there was no information on the appropriate parameters to work from. To our knowledge, this technique has never been used before in fungi, therefore we did not know which would be the best protocol. Consequently, we employed an adaptive experimentation approach, applying electrical

stimuli with different intensities and varying the parameters. The tests were with one individual per species each day. For these tests, we were particularly interested in understanding: 1) if it is possible to study these fungi using equipment usually employed in neuroscience, 2) if the fungi respond to electrical stimulation, and 3) if there are any properties that we can observe in the fungal response.

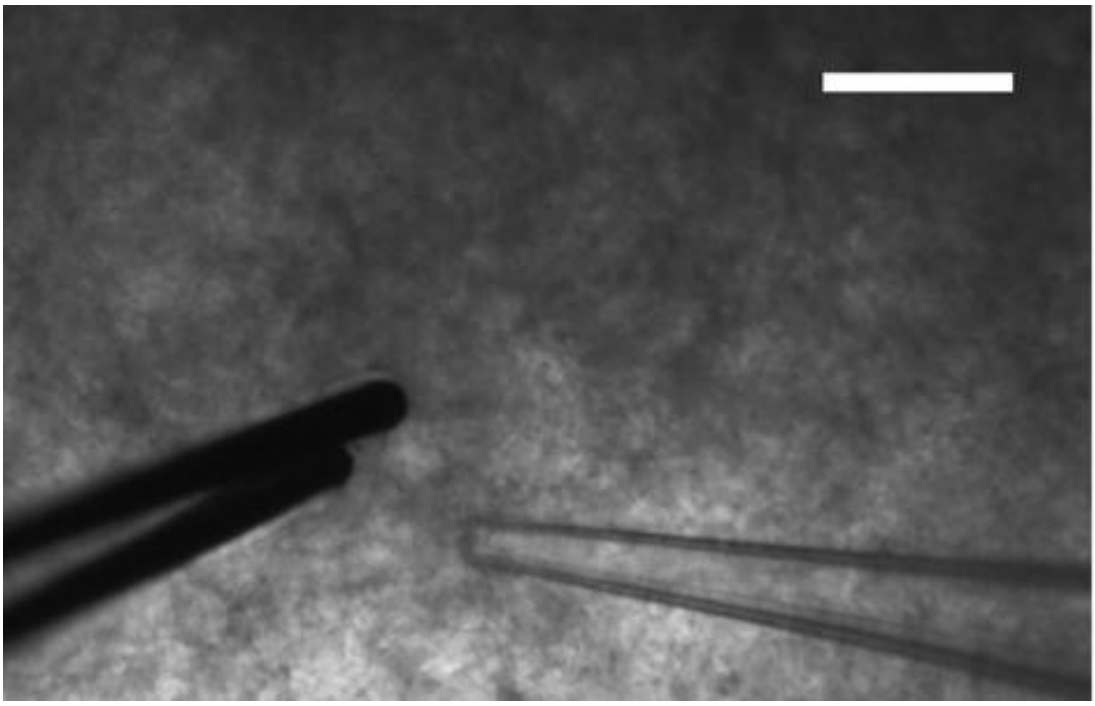

Figure 5.2. Microscopy picture of the mycelium with both electrodes placed. At the left, the stimulation electrode that applies an electrical current. To the right, the pipette containing the recording electrode. The scale bar represents 200 μm.

As a control, for each fungus, we removed the stimulating electrode to observe if the response disappeared. Then, we removed the recording electrode, again observing the lack of response. We then lowered the stimulating electrode back on the mycelium, and finally, repositioned the recording electrode on the mycelium. If we observed the response only when both electrodes were placed, it meant that the signal was biological, and not an artefact. When the fungus was killed with bleach, and the response disappeared, this provided further evidence for the biological origin of the detected responses.

5.4 Results

5.4.1 Investigations on Amanita muscaria

Upon electrical stimulation, *A. muscaria* presented a very stereotyped and consistent response characterised by a square-shaped wave of lower potential in the time series (**Figure 5.3**). This wave was evoked only after a stimulus ≥ 65 µA, and its maximum length was c. 400 ms with just one stimulation. When in this threshold of 65 µA, the fungus seemed to become increasingly sensitive to the stimulus, with the wave getting progressively longer until its maximum length (**Figure 5.4**).

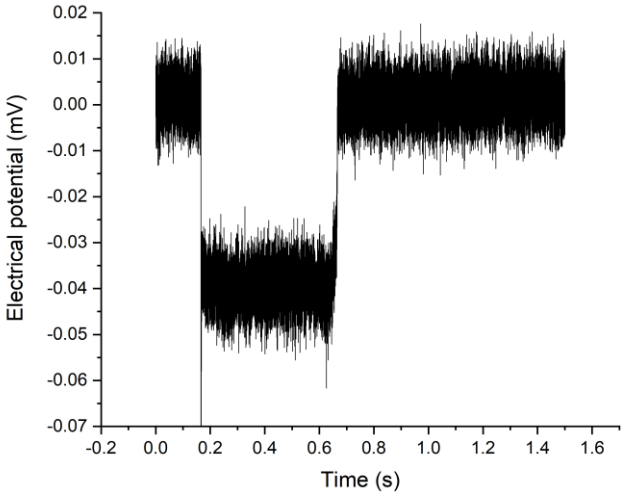

Figure 5.3. Square wave observed in *Amanita muscaria* after electrical stimulation. The vertical bar shortly before 0.2 s is an artefact produced by the stimulus.

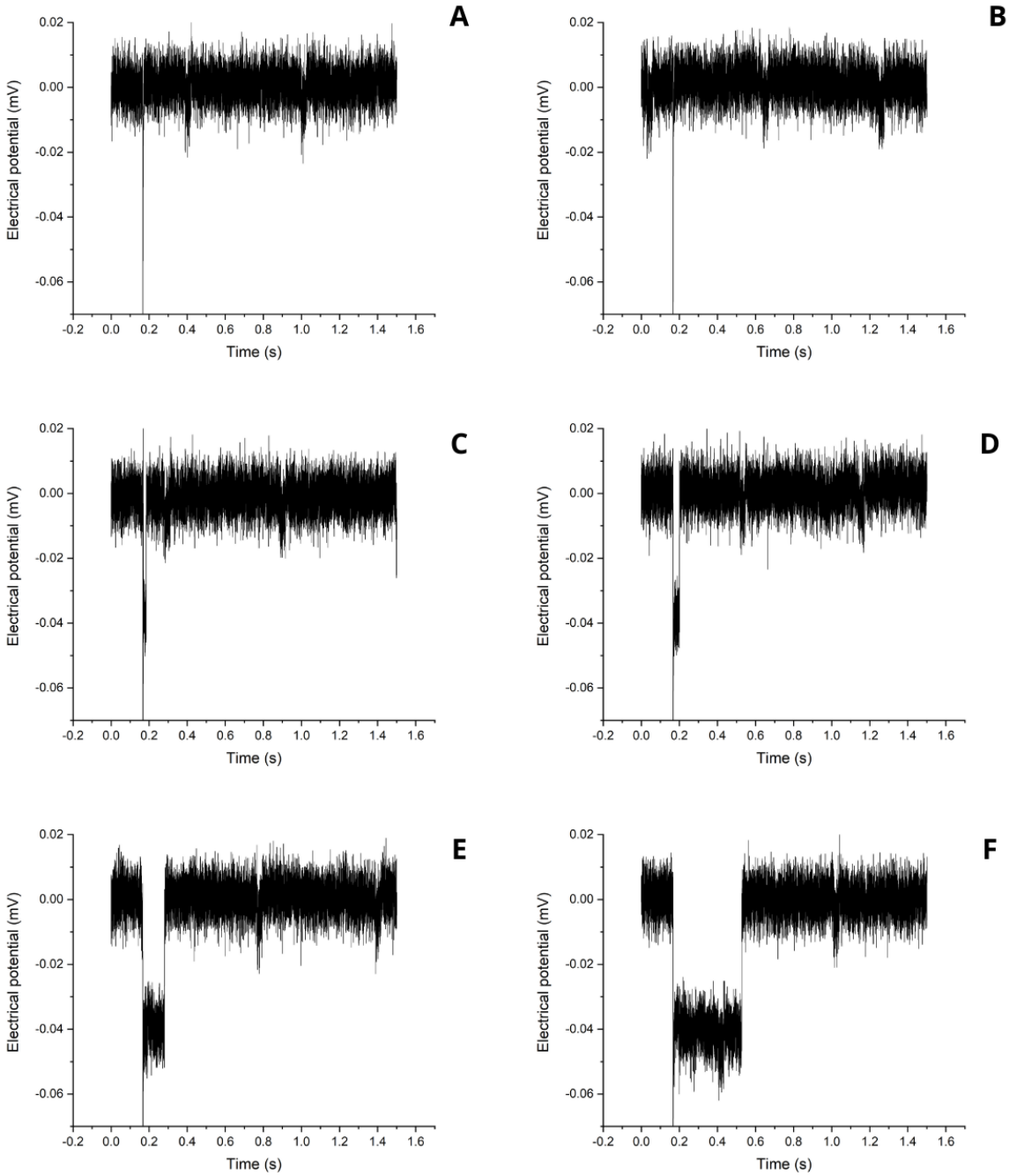

Figure 5.4. Repeated stimulation with a pulse of electrical current at 65 µA every 10 s (A-E) on the mycelium of *A. muscaria*, showing an electrical response as a square wave of increasing length. At F, the current was 67.2 µA. At each stimulus, the response was more intense.

When we compared the response of *A. muscaria* mycelium to increasingly intense stimulation to that of mouse neural cells—which demonstrably have input-specificity plasticity—we noticed a similarity in the overall behaviour of the tissues (**Figure 5.5**).

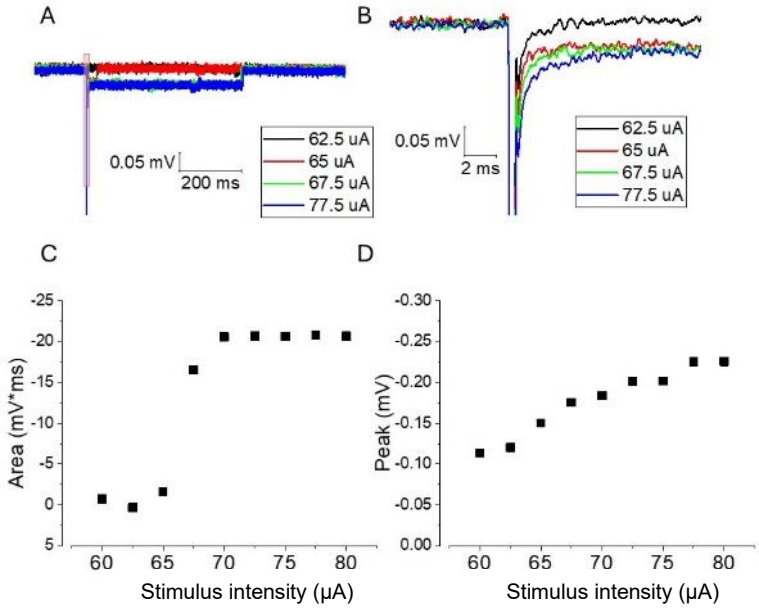

Figure 5.5. Comparison between the responses of *Amanita muscaria* mycelium (A) and mouse brain cells (B) with the same, increasing currents applied as a stimulus. Both systems seem to show input-specificity responses, with the intensity of the response increasing with that of the stimulus. Adapted from the V. Kann Rasmussen grant proposal.

The application of consecutive stimuli at increasing intervals yielded an increase in the length of *A. muscaria*'s square wave up to a limit of c. 0.846 ms (roughly twice the length of the natural response of c. 400 ms), after which it was broken into two separated waves (**Figure 5.6**).

These signals were absent when either the stimulating electrode and/or the recording electrode were removed, or when the fungus was killed with bleach. This means that the signal is not an artefact but was produced by the fungus itself. The meaning of this wave and the mechanisms that underpin it remain to be elucidated.

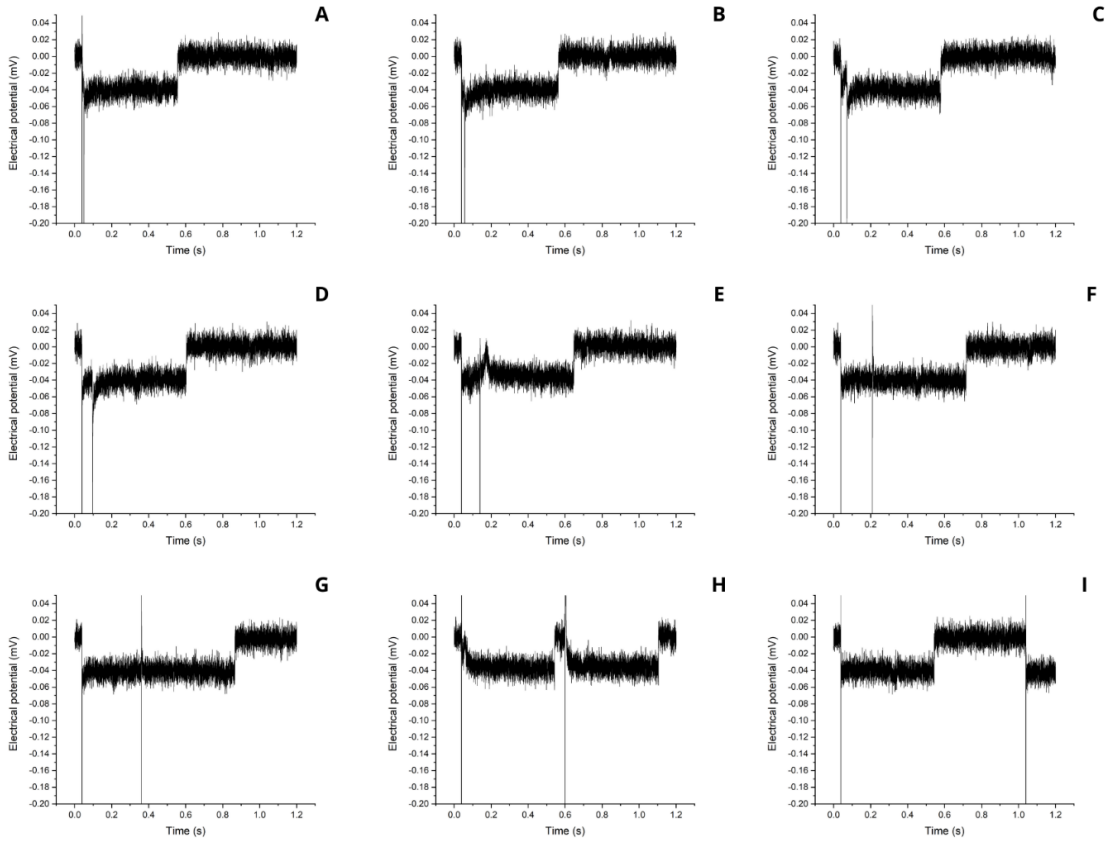

Figure 5.6. Extension of response from *Amanita muscaria* following multiple stimuli. A-G: The square wave can be progressively stretched by increasing the time between the stimuli. G: With an interval of 0.319 s between stimuli, the square wave reaches its maximum length of 0.846 s. H-I: With intervals greater than 0.319 s between stimuli the response splits in two waves.

5.4.2 Investigations on Laccaria bicolor

The behaviour of *L. bicolor* in response to electrical stimulation was distinct from that of *A. muscaria*. It did not present a conspicuous wave with a particular shape, but like *A. muscaria* it did display sensitivity to the stimuli. The most promising result detected in the initial trials was an increased sensitivity to the same stimulus when presented repeatedly, a response that may be indicative of a process comparable to sensitisation (**Figure 5.7**).

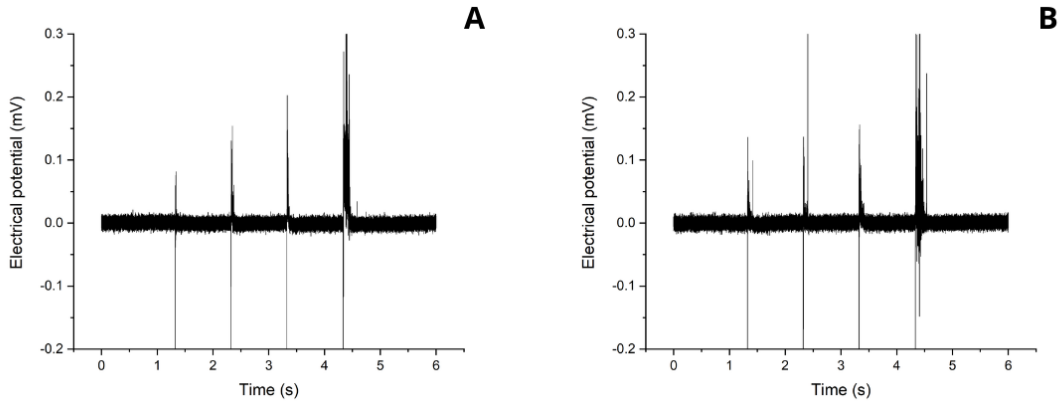

Figure 5.7. *Laccaria bicolor* appears increasingly sensitive to electrical stimulation both at 30 µA (A) and 300 µA (B). The vertical bars touching the X axis in the negative direction of the Y axis are artefacts caused by the electrical stimulus.

5.4.3 Investigations on Suillus granulatus

S. granulatus was the ectomycorrhizal fungus we explored the least in our tests. It did not have any significant response to any of the stimuli, like shown in **Figure 5.8**. Therein, the lack of response of the mycelium to a stimulation of 300 µA is depicted.

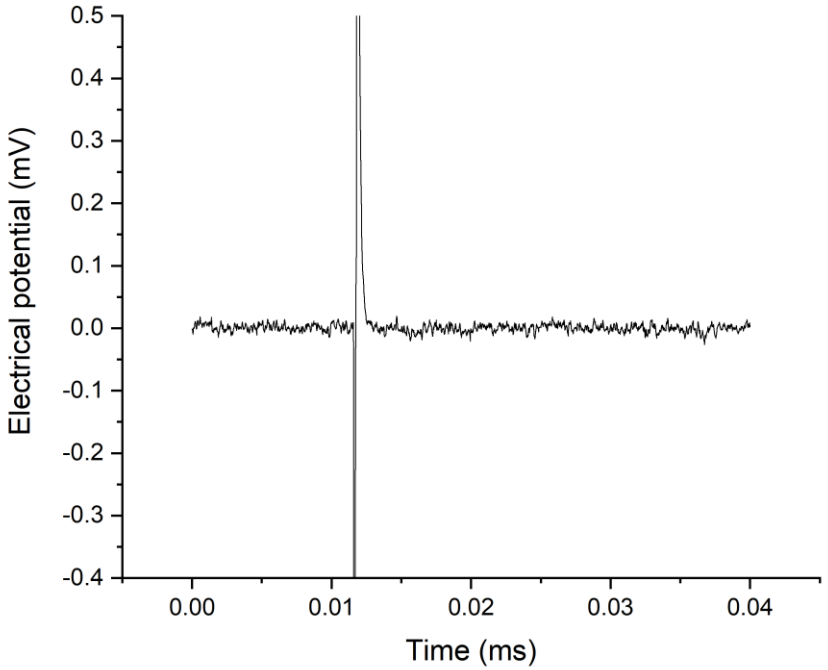

Figure 5.8. Time series of variations of electrical potential in the mycelium of *Suillus granulatus*. The lack of response to a short pulse of stimulation with 300 µA is noticeable after the artefact, when nothing changes in the dynamics of the series.

5.5 Discussion

The first explorations of electrophysiological responses with ectomycorrhizal fungi yielded encouraging results. The principal outcome is that we now know that it is possible to study these fungi using the techniques described above and that we can obtain meaningful results on the network dynamics of the mycelium. This in itself is already a significant achievement. Furthermore, it demonstrates that each fungal species has its own electrophysiological dynamics, the most noticeable so far being the square waves produced by *A. muscaria.*

We made these observations without following an established protocol *a priori* (i.e., there was an element of trial and error and adaptive experimentation) because we did not know what to expect or what we would find. With the encouraging results of these initial experiments, including others not shown here, we can start to build a protocol to investigate the electrophysiology of ectomycorrhizal fungi using these techniques.

The electrophysiological behaviour observed in both *A. muscaria* and *L. bicolor* was intriguing. In *A. muscaria*, when stimulating the mycelium at 65 µA, the length of the square wave increased gradually. In other words, the same stimulus, when repeated, caused a response that become increasingly stronger. This suggests a process analogous to what is observed in short-term synaptic plasticity in neuronal networks (cf. Zucker and Regehr 2002), which is associated with short-term memory encoding, such as short-term sensitisation. This result was observed when repeated stimulation with the same intensity makes the response increasingly strong (Ginsburg and Jablonka 2009). Evidently, this effect must be investigated further with a rigorous protocol. Similarly, *L. bicolor* mycelium stimulated repeatedly with 30 µA or 300 µA showed a corresponding increase in the amplitude of its electrical signals in response, which also appear to be a sensitisation process.

The fact that two species (*A. muscaria* and *L. bicolor*) showed a behaviour compatible with learning by sensitisation is very interesting and points to a plasticity property in the dynamics of their electrical signalling. We were also encouraged by the input-specificity test, which showed that the intensity of the response of *A. muscaria* increases with the intensity of the stimulus (**Figure 5.5**). These results point to some form of bioelectrical plasticity in the mycelium, which could be the basis for processing information.

Indeed, some studies have suggested that fungal mycelium has a network architecture with non-random electrical signals being produced, which presumably allows the formation of Boolean circuits for information-processing (Adamatzky et al. 2022). Below, I provide a speculative suggestion of how fungal and mycorrhizal information-processing could operate through electrical signalling, if this really happens.

Mycelial information-processing could be potentially achieved through altering the dynamics of its electrical signalling by changing the expression of ion channels in the membranes of the hyphae, like plants do (Canales et al. 2018). Additionally, for medium to long-term adjustments, mycelial information processing might work by rearranging fungal hyphae through the formation of new anastomoses (de la Providencia et al. 2005; Putra et al. 2022), providing the equivalent of structural plasticity in neural networks, for the long-term storage of information (Lamprecht and LeDoux 2004). In principle, this can give rise to properties of plasticity like cooperativity (when, presumably, the simultaneous triggering of electrical signals in a group of hyphae strengthens their connection), input specificity (when the response to stimuli is proportional to its intensity), and associativity (when the simultaneous firing of a strong signal in one hypha or group of hyphae and a weak signal in another hypha or group of hyphae makes the weaker signal stronger over time) (Hebb 1949; Bliss and Lømo 1973). Whilst the specific processes are different, these properties are observed in the brain (in the case, regarding synapses) and lead to long-term potentiation, a mechanism of consolidation of synapses that is considered the basis of the brain's capacity to encode memories and learn (Kandel et al. 2000; Hao et al. 2018). If such properties or similar are found in hyphae, this could open an avenue to understand whether fungi are cognitive and how this cognition operates. Given the inextricability of plants and fungi in mycorrhizal systems, this raises the possibility that the cognition of plants and fungi merge through the interaction of their bioelectrical signalling systems.

For example, let us imagine a hypothetical case. A plant root system is connected simultaneously to millions of hyphae, all of them competing for resources provided by the plant (i.e., sugars and lipids). They receive these resources in return for the nutrients retrieved from the soil. But how could a single hypha or group of hyphae be particularly favoured by a stronger connection with the plant, and not rejected by it, when competing with a million others? If it finds nutrients in the soil and sends them to the plant, receiving carbon in return, it will have more energy to invest in the production and maintenance of

ionic gradients across its membranes, being able to produce more electrical signals. If the plant responds to these signals with other electrical signals, perhaps with the involvement of more exchange of resources, the strength of this mycorrhiza could be increased to the detriment of other, less rewarding mycorrhizas. On a broad scale, this will create the pattern of a few hyphae strongly connected and several less connected, with the potential to encode information about, e.g., environmental conditions following a Hebbian-like type of learning, where the intensity and frequency of the afferent stimulation, drives the long-term change in the efficiency of information transfer (Seung 2000; Simard et al. 2018).

The example above is of course highly speculative. At present, there is too little evidence to support such a hypothesis, but it will be less far-fetched if more evidence for electrical communication between plants and fungi is found. The behaviour of plants and fungi is highly plastic (Karban 2015; Trewavas 2015; Aleklett and Boddy 2021) but the mechanisms underlying such plasticity are poorly understood. Electrical signalling, due to its universality, versatility, and variability, can provide a substrate for proposing hypotheses to explain mechanistically these behaviours. It is also, evidently, a possible mechanism for explaining the dynamic electrical interaction between plants and fungi. This is why we propose to study the electrophysiology of mycorrhizas in a systematic, evidence-based manner from the perspectives of internal fungal activity, plant root activity, and at the fungus-root interface (the mycorrhizas themselves). At the very least, anything discovered will be completely new, as the techniques we are employing have never been used before to study fungi nor mycorrhizas. We anticipate that the mere establishment of a functional protocol will prove to be a noteworthy achievement.

Chapter 6: General discussion

This doctoral journey reflects how most scientific enterprises advance, especially when exploring uncharted waters. From one clear objective, we navigated through several failures until finding a different, but suitable and promising course. It was a journey of convoluted twists and turns, changes of direction, decisions and surrenders. Nonetheless, at the same time, we found in this journey unexpected new waters to navigate.

It would have been much easier and safer to do a doctorate using established methods and following a similar path to that others have followed. However, at least for me, the thrill of doing science is not in staying safe, repeating variations of what others have done, but in exploring the unknown. Of being in full contact with mystery.

This is a perilous approach that comes at a high cost. To follow the initial proposal of testing whether plants extend their cognition to ectomycorrhizal fungi (**Chapter 2**), we had to spend quite some time thinking about and developing methods to address our questions. Essentially, we had to conceive methods that seemed acceptable to test the hypotheses we wanted to test. To further complicate things, even methods that seemed fairly well-established in the literature (**Chapter 3**) turned out to be unpredictable and difficult to work with. We then moved on to something presumably smaller, i.e., testing memory in fungi (**Chapter 4**), and for this, we had to do several trials until deciding that *Laccaria bicolor* was the best subject for this experiment. The study ran mostly smoothly, but the hypothesis we wanted to test was not corroborated. Concomitantly, the opportunity for a completely different, albeit related, kind of research appeared. We tested whether it would be possible to use neurophysiology instruments to study the electrical signalling of fungi, and it not only worked (**Chapter 5**) but also secured us a grant to proceed with this research. In a sense, after many turns, I am back to my electrophysiology origins. And I am glad to do so, because there is so much work to do in this direction.

This doctoral thesis illustrates a key issue for the field of non-neural cognitive ecology: if the lack of data to support claims was not sufficiently problematic, establishing methods and standards to test hypotheses is a real challenge. How to test for cognition in organisms so different from animals like plants and fungi? How to obtain reliable data that can serve as common ground for both proponents and sceptics to debate

it? As we can see in this thesis, this is not straightforward. Only a few groups have achieved an experimental system that allows reproducibility in large scale, such as Prof. Lynne Boddy's group at the University of Cardiff, where soil trays are used to study the behaviour of *Phanerochaete velutina*, or Prof. Umberto Castiello's group at the University of Padua, in Italy, which developed a successful method to study the behaviour of climbing pea plants (*Pisum sativum*).

Before discussing the methods, the thesis is also situated in a deeper debate, and which outcome may or may not undermine the interpretation of results—should one have them: a debate on the epistemology of science, which perhaps, is the most important. The debate starts with a long-standing question: what is cognition? Which methods are deemed acceptable to test cognition in a system? Without a definition of this intriguing phenomenon, it is impossible to appropriately propose hypotheses and interpret the results. The question *what is cognition?* is easy to ask, but finding an answer for it is all but easy, even when excluding non-neural organisms from the debate. The Editor-in-Chief of the journal *Current Biology*, Geoffrey North, asked eleven cognitive scientists to define cognition, and amazingly, each of them provided a different version of what they believe cognition is, with broader or narrower scopes (Bayne et al. 2019). Cognition still lacks a universally accepted definition (Akagi et al. 2018). To further complicate things, many definitions of cognition rely on equally vague concepts such as *representation*, *learning*, and, crucially, *information* (e.g., Neisser 1976; Adams and Aizawa 2001; Shettleworth 2010; Rowe et al. 2014). This lack of consensus alone should inspire humility in the sceptics who claim that non-neural organisms are not cognitive because when one says confidently that something is not cognitive (or that something *is* cognitive), this implies that this person (1) knows exactly what cognition is and (2) knows exactly how to determine the boundaries of this flexible and intricate phenomenon (cf. Bianchi 2024).

I do not have these answers either, but from what I have studied so far, I believe it is relatively safe (for now)[1] to heuristically say that cognition is a property of the living matter. This could be a starting point that few would dispute, so one might question: *which kinds* of living matter are cognitive? One can use humans as a starting point (a choice somewhat arbitrary, but historically, this is what has been done), but if cognition is

[1] The debate on whether computers and machines can be cognitive is vast (e.g., Searle 1980; Cuskley et al. 2024; Strachan et al. 2024) and beyond the scope of this thesis.

a property of living matter, it evolved through natural selection from somewhere. We therefore might ascribe cognition to other primates, and to our shared common ancestor. The same goes for other mammals and our common ancestor with them, and so forth. So, where to establish the limit when we start to consider certain forms of living matter not cognitive? One could reasonably say that cognition is defined by the presence of a (central) nervous system, which enables all the cognitive behaviours we observe. This sounds like an elegant Popperian solution because it clearly delimits the boundaries of a scientific theory, providing explanatory simplicity—being restrictive and preventing overextension of cognition to everything; operational clarity, as the constitutive parts of the cognitive system are relatively easily demarcated and measurable (e.g., neurons, synapses, neural activity); and comparative utility, as it provides a neural basis for comparative cognitive studies across species. Nonetheless, this traditional view has a problem: cognition is inextricably associated with phenomena like memory, learning, decision-making, attention, and several others. What would happen if we observed these phenomena in organisms without a nervous system? In the previous chapters, I have referred to several examples of such findings.

If keeping with the traditional view of cognition, there are two possible ways out of this conundrum. One solution is to state that these phenomena are not really cognitive-—they are "cognitive-like", denying the existence of true learning, memory, anticipation, etc., in non-neural organisms. This solution risks being arbitrary and can lead to ambiguity in both scientific and lay communication and unwanted misunderstandings (cf. Leonetti 2025). The other solution is to invent new categories to explain such phenomena (e.g., plant-learning, fungal-communication) which are fundamentally different from their counterparts in neural organisms, even if functionally similar. The risk in this case it to create *ad hoc* classifications to save the mainstream theory (only neural organisms are cognitive) from these challenges, effectively creating double standards. Either way, these solutions stumble on a crucial issue: the definitions of each phenomenon. To decide whether something is or not cognitive, or learning, or memory, we must ask: what is memory? What is learning? What is communication? And so on. As we can see, this is a complicated problem that requires a good deal of philosophy more than science.

Taken together, these problems highlight the need for reconsidering the definition of all these concepts, in particular cognition. As said before, it is reasonable, as a starting point, to assume that cognition is a phenomenon present in *all* living matter, and not only

that with neurons. It is an evolved phenomenon; therefore, it was to some degree present in the earlier forms of life that populated this planet. This interpretation succeeds in explaining the several "cognitive-like" behaviours we see in different organisms from all the kingdoms of life, and allows testable predictions for similar phenomena, regardless of the organism. When assuming that non-neural organisms can be cognitive, we can make falsifiable predictions that would be impossible with the traditional view of cognition.

In line with the above, post-cognitivist approaches to cognition seem well suited to serve as a starting point to make such predictions and elaborating hypotheses without *a priori* excluding anything from being cognitive. It must be noted that the claim of traditional cognitive sciences that "only neural organisms are cognitive" is not scientific because it forbids testing for cognition in non-neural organisms. If one finds evidence for cognition in such organisms, the claim dismisses the evidence from the outset, because only neural organisms are cognitive. This is a circular reasoning, which should not be accepted as scientific. A theory of cognition that is falsifiable and open to challenge is what is needed. However, testing for cognition requires the development of standards and accepted methods to sufficiently test hypotheses regarding non-neural cognition.

In this thesis, particularly in **Chapter 2**, I presented with my coauthors a working definition of cognition as an embodied, embedded, enacted, and sometimes, extended phenomenon, heavily inspired by the Santiago theory of cognition (Maturana and Varela 1980) and by other post-cognitivist authors. Then, I moved on to the challenge of developing methods to test predictions on the assumption that plants and fungi are cognitive (and that their cognition can be extended). One experiment failed for technical reasons (**Chapter 3**), and the experiment that worked the best was the one described in **Chapter 4**, which did not corroborate our hypothesis, illustrating the difficulties in operationalising definitions of memory in fungi. This does not mean the hypothesis that ectomycorrhizal fungi are cognitive is exhausted, because it was just one experiment. The lesson learnt with that experiment is that either the method is inappropriate to test for memory in ectomycorrhizal fungi, or that ectomycorrhizal fungi indeed do not have memory capacity. The distinction between these alternatives will become clear once more experiments are performed with the same or other techniques and methods. Science is a collective effort, and scientific facts will emerge from this collaborative work.

Regarding the electrophysiology studies, electrophysiology is undeniably related to animal cognition (Kandel et al. 2000), and many claim that is related to plant (Calvo Garzón 2007; Souza and Debono 2019; Parise et al. 2022) and fungal cognition as well (Adamatzky 2022; but see Blatt et al. 2025 for a critique of the latter). It is a promising tool to study communication in natural networks, regardless of their kingdom. Therefore, I shall invest in this direction in the hope that we can make more accurate predictions and not only describe behaviours, as we did in **Chapter 5**, but also explain the mechanisms behind it.

Opening the possibility that non-neural organisms are cognitive not only inaugurates whole new research lines but also gives us the opportunity to shed new light on concepts that many have taken for granted, such as memory, learning, decision-making, attention, and so on (see Lyon et al. 2021 for a comprehensive list of cognitive phenomena presumably present in every organism). In fact, there is no simple way of testing cognition in organisms because cognition is a complex, multifaceted phenomenon. Probably, the best way of testing cognition is to select a sub-phenomenon that presumably contributes to the 'higher' phenomenon of cognition and test it in the organisms of interest. With time, from the accumulation of evidence for several cognitive phenomena in non-neural organisms, it will become clear if these beings are cognitive. This approach is similar to the "piecemeal approach" proposed by Lee (2023) for studying plant cognition—in Lee's (2023) case, shifting away from the question of *whether* plants are cognitive to focus on smaller, more easily circumscribable questions about what "cognitive features" similar to undisputable cases of cognition, plants exhibit. Such studies will help us to understand from where and how this fascinating phenomenon evolved and has the potential to redesign our understanding of cognition, our relationship with other organisms, and the ethics of our interactions with them. As Colaço (2022) remarked, the benefits of studying plant (and fungal, I would add) cognition lie in conjecturing and hypothesising about cognition with fresh approaches and novel methodological practices that are valuable to cognitive sciences *even if we end up concluding that non-neural organisms are not cognitive*. This alone makes studying the cognitive ecology of non-neural organisms worthwhile. Nevertheless, as said before, for this we need reliable data backed by strong theories, and this thesis is only a small step in this direction.

I would like to conclude with a remark: I hope that the exceptional detail with which I have described our experiments, together with the transparent reporting of studies that did not succeed, or failed to support our hypotheses, provides strong evidence that I am not doing science with an agenda. Yes, I do believe at the moment that post-cognitivist approaches are the best framework to explain the extraordinary behaviours of non-neural organisms, but I also believe that the only way to test this is through meticulous experimentation and candid reporting of whatever results are found. Likewise, I will always be open to heartfelt critiques and honourable discussions.

While this thesis may have an end, the research does not. This is just the beginning of a new stage, more focused and optimised, with the potential to contribute to the growing body of evidence substantiating the claim that cognition exists beyond brains and flesh. The dominant paradigm may still be that plants and fungi are not cognitive, but slowly, evidence that challenges this view is building. Time will tell how it will be received and whether it will reshape how we think about cognition without neurons. As for me, I have adjusted the course, weighed anchor, and trimmed the canvas. I set sail, from the Island of Knowledge[2] into the unknown.

[2] A concept from Gleiser M. (2014). The Island of Knowledge: the limits of science and the search for meaning. New York: Basic Books

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