Fungal wars: tracing single-cell scale chemical signalling between interacting soil fungi

The INTERSPEC project, funded by a European Research Council Starting Grant, is focused on introducing a novel analytical methodology for studying single-cell scale fungal metabolism to clarify how fungal decomposition rates are affected by changing environmental (biotic and abiotic) conditions.

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Among the many microorganisms inhabiting soil, fungi play a crucial role in global biogeochemical cycles, predominantly through the decomposition of complex organic matter. Fungal community respiration resulting from decomposition is responsible for up to 25 per cent of total CO_2 flux from soil (Hawkins *et al.*, 2023). On the other hand, fungi are important for delivering carbon compounds into soil after receiving it from their symbiotic plant partners in a form of photosynthates; these can amount to 36 per cent of anthropogenic CO_2 emissions (Hawkins *et al.*, 2023). Thus, a better understanding of fungi-related processes is important for creating management practices that promote the overall increase in soil's carbon sink capacity.

Interspecific interactions and competition

Overall functioning and productivity of the fungal community are significantly affected by interspecific interactions and competition for resources. Specifically, litter-decomposing saprotrophic and plant symbiont ectomycorrhizal (ECM) fungi, although typically inhabiting different layers of soils of the world's forests, have been shown to compete for limiting nutrients (e.g. nitrogen) with great effects on the overall decomposition rates (Bödeker *et al.*, 2016; Fernandez and Kennedy, 2016). The mechanisms that determine the outcome of these interactions are not fully understood and are context-dependent. For instance, ecosystem-scale observations show that decomposition can be suppressed ('Gadgil effect'), typically in nutrient-poor forests, where the ECM fungi outcompete the saprotrophs in obtaining nitrogen (Fernandez and Kennedy, 2016). Decomposition can also be exacerbated ('priming') by the ECM fungi foraging for nitrogen in organic matter and thus producing easily accessible carbon compounds for the saprotrophs to acquire (Fernandez and Kennedy, 2016). The fragile balance of these fungal interactions is further affected by climate change and other human activity-induced changes in environmental conditions, such as shifts in vegetation (arctic greening), nutrient or water availability (fertilisation, forest clearcutting and change in precipitation), and even atmospheric CO_2 concentration and temperature. In the context of these changes, interacting fungi will either contribute to or help mitigate the carbon emissions from soils; thus, a better understanding of the fundamental mechanisms responsible for fungal metabolic responses to interactions and under changing environmental conditions is necessary to determine the direction of the overall decomposition rates.

While the field experiments yield contradicting results, laboratory studies, although excluding many important factors affecting the fungal interactions in natural environments, provide important clues about physical, chemical and metabolic responses of the mycelia at culture level (Lindahl, Stenlid and Finlay, 2001; Murphy and Mitchell, 2001; Baptista *et al.*, 2021). For example, it has been shown that interspecific fungal interactions may result in several types of mycelial responses: *intermingling* (no competition), *deadlock* (cease of growth by both counterparts) or *replacement* (one partner, the 'winner' of the competition, overtakes the other) (Leake, Donnelly and Boddy, 2003). The type of the response is determined by a range of biochemical and physiological reactions, such as up- or down-regulation of fungal primary and secondary metabolism, which determine whether the fungi are neutral, facilitative or combative towards each other. For instance, competing fungi may exhaust their immediate environment from nutrients (primary) or saturate it with toxic compounds and defend it from the ones secreted by the opponent (secondary). It is largely unknown which metabolic pathway ECM and saprotrophic fungi primarily employ to compete and how it depends on their pairing and immediate physical and nutrient environment. Furthermore, while many studies are dedicated to identifying and characterising fungal secondary metabolites, their ecological function, including ability to affect the decomposition activities of fungal individuals and/or communities, remains elusive.

Innovative techniques in soil microbiology

A major downside of fungal co-culturing on agar plates is the omission of the physical structure that fungi typically experience in natural soil environments. In addition, only the averaged response of the mycelium to competition is observed in such experiments. However, each cellular filament (hypha) in the mycelium of a fungus displays phenotypical heterogeneity in terms of growth rates and metabolic activity (Op De Beeck *et al.*, 2020). Therefore, deeper insight into the fundamental processes governing fungal interactions and competition can

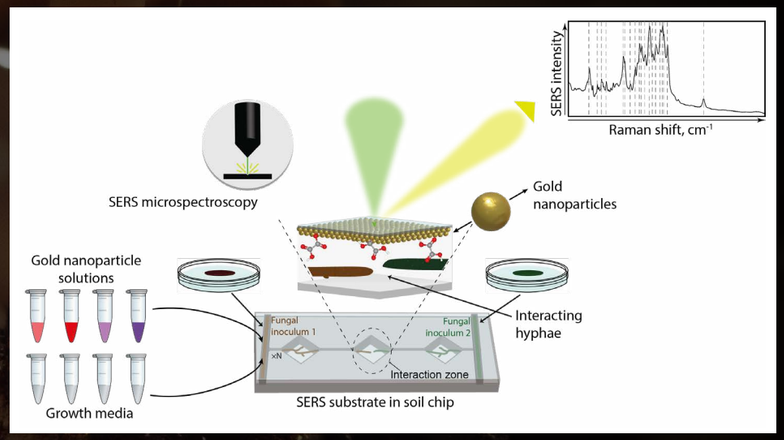

Figure 1: Schematic summary of SERS microspectroscopy in soil chips as used in INTERSPEC. Gold nanoparticle solutions of different types will be used to produce SERS substrates in the soil chip channels, which will then be filled with fungal growth media and inoculated with laboratory cultures of ECM and saprotrophic fungi, each on the opposite side of the chip. Raman microspectroscopy is used to record SERS spectra of secondary metabolome secreted by the fungi during interactions.

Photos were taken by Therese Ek. On the left, polydimethylsiloxane (PDMS) microstructured soil chip in a glass-bottom Petri dish is shown; on the right, Milda Pucetaite in the optical microscopy lab at Lund University.

be obtained by studying them directly where they occur—at the hyphal tips and in an environment similar to natural soils. Most currently used approaches for this purpose have either insufficient spatial resolution for analysis at single-cell level or are destructive, which means that observations in vivo are impossible. To solve this, an approach that makes use of two state-of-the-art techniques in soil microbiology—microstructured *soil chips* and Raman microspectroscopy—is proposed in this project (Figure 1).

Specially designed soil chips have been used and developed in the lab of Dr Edith C. Hammer at Lund University and constitute a set of interconnected microstructures engraved into an optically transparent material, which provide visual access to study otherwise obscure soil systems and serve as habitats for soil microbes that are introduced within (Mafla-Endara *et al.*, 2021). Although artificial, they can be set up at a structural and chemical complexity level which mimics real ecosystems and have already been used to reveal previously unknown fungal interaction mechanisms by confronting single hyphae of opposing fungi in a controlled and reproducible way (Gimeno *et al.*, 2021). In addition to custom optical and fluorescence microscopy, we proposed that Raman microspectroscopy can provide highly chemically specific information about the processes taking place within the chips (Pucetaite *et al.*, 2021). Raman scattering spectra are recorded by irradiating a sample with a laser beam and recording the scattering signal. Roughly one photon in a million of the incident radiation is Raman scattered, which means it gains energy from or loses energy to molecular vibrations and thus has a wavelength shifted from the original light; this shift carries information about the type of molecules in the sample. While Raman microspectroscopy measurements can be performed in vivo due to the technique's non-destructive nature, a significant challenge is the low yield of Raman scattering. The small sample amounts in the soil chip channels further compound this issue. To address this, surface-enhanced Raman scattering (SERS) microspectroscopy utilises the plasmonic properties of gold nanostructures to significantly enhance the Raman scattering signal, thereby increasing the sensitivity of the technique (Langer *et al.*, 2020). SERS holds great promise for studying microbial chemical signalling during interactions.

Goals of INTERSPEC

However, specific applications of SERS for detection and characterisation of fungal secondary metabolites during fungal-fungal interactions in soil chips require:

i. identification of experimental protocols for preparation of reproducible SERS substrates that can be built into the chips

ii. a spectral library of potential chemical substances that constitute secondary metabolome of fungi

iii. their physicochemical properties on those substrates.

Thus, the purpose of INTERSPEC is to develop such a SERS microspectroscopy-based approach for in vivo detection of hyphal secondary metabolites secreted during interactions between ECM and saprotrophic fungi in soil chips and to characterise their function and influence on organic matter decomposition. This will allow important questions in fungal ecology to be addressed: Are these fungi neutral, antagonistic or facilitative towards each other and under which conditions? What is the 'warfare strategy', or metabolic pathway, employed by the competing parties? How do physical structure and C and N availabilities affect these interactions? What determines 'the winner' of the fungal competition? How do the interactions affect organic matter decomposition? What are the ecological roles of the secondary metabolites secreted during fungal competition? The methodological approach developed during the INTERSPEC project will also ultimately offer the community of soil microbial/fungal ecologists a game-changing new tool to study ecosystem functions of secondary metabolites in more realistic settings.

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PROJECT SUMMARY

INTERSPEC is dedicated to developing a Raman spectroscopy-based approach for tracing chemical signalling between interacting soil fungal cells to answer important questions within fungal ecology: what determines whether fungi are neutral, antagonistic or facilitative towards each other? What are their 'warfare strategies'? How do the interactions affect the decomposition of organic matter in soils and, subsequently, carbon cycling?

PROJECT PARTNERS

INTERSPEC is hosted by the Department of Biology, Lund University, Sweden.

PROJECT LEAD PROFILE

Milda Pucetaite is a researcher at the Department of Biology, Lund University, Sweden. She is a chemical physicist who graduated from Vilnius University, Lithuania. Her work focuses on novel applications of microspectroscopy-based approaches to analyse soil microbial chemistry with a special interest in soil fungi and their role in carbon cycling. In 2021, she received a Swedish Research Council Starting Grant to establish her own research group aiming to use Raman microspectroscopy for monitoring hyphal metabolic processes during fungal interactions. The ERC Starting Grant received in 2023 will open a new direction of Raman spectroscopy applications for tracing chemical signalling compounds during those interactions.

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