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Ecole doctorale SIReNa (Sciences et Ingénierie des Ressources Naturelles)

Thèse

Présentée et soutenue publiquement pour l'obtention du titre de

DOCTEUR DE l'UNIVERSITE DE LORRAINE

Mention : Biologie et écologie des forêts et des agrosystèmes

par Huayong WANG

Contribution à l'étude de la résistance à la sécheresse édaphique chez le champignon ectomycorhizien *Cenococcum geophilum* et son hôte le Hêtre (*Fagus sylvatica L.*)

Soutenue publiquement le 18 décembre 2024

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Co-directeur de thèse : Mme **Annegret KOHLER**

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UMR 1136 INRAe / Université de Lorraine, Interactions Arbres – Microorganismes
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Understanding drought stress resilience in the *Fagus sylvatica* - *Cenococcum geophilum* ectomycorrhizal symbiosis

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Table of Contents

Résumé des travaux de thèse *1*

Objectifs de la thèse *2*

Présentation simplifiée du manuscript de thèse *3*

Chapter 1 Recent advances in the biology, genetics and ecology of the ectomycorrhizal ascomycete *Cenococcum geophilum* *9*

Abstract *10*

Introduction *10*

Morphological features and Life Cycle *12*

Ecologically important ectomycorrhizal symbiont *12*

The pan-global symbiont *12*

Tolerance to N deposition, salt stress, and heavy metals *14*

Adaptation to water-stressed enviroments *14*

Heat and cold stress *14*

Host Preferences *15*

Population Structure *15*

Genomics, transcriptomics and population Genomics *16*

Applications in forestry and conservation *20*

Future directions *20*

References *21*

Chapter 2 Gene expression changes in a drought-tolerant *Cenococcum geophilum* strain during osmotic stress and recovery periods (in preparation) *25*

Abstract *26*

1. *Introduction* *27*

2. *Material and methods* *29*

2.1 Experiment 1: Fungal strains, Culturing condition and Mycelium growth of Representative Isolates under Drought Treatments 29

2.1.1 Standard curve correlating PEG-8000 concentrations and water potentials of the medium 31
2.1.2 Experimental Design and Mycelium growth 31
2.2 Experiment 2: Culture conditions, experimental design, RNA extraction, RNA-Sequencing and data analysis 32
2.2.1Culture conditions and experimental design 32
2.2.2 RNA extraction and RNA-Sequencing 33
2.2.3 RNA-Sequencing analysis 33
2.2.4 Bioinformatic analyses 34
3. *Result* 35
3.1 PEG treatment affects mycelium growth of the four strains differentially 35
3.2 Osmotic stress induced by PEG induced changes in gene expression in *C. geophilum* 1.093 mycelia after 6, 24, and 48 h 35
3.3 C. geophilum 1.093 mycelium recovered rapidly from PEG treatment after transfer to the new medium. 38
3.4 Gene co-expression network analysis 39
4. *Discussion* *43*
References *48*
Supplementary Figures *52*

Chapter 3 Effects of a controlled *Cenococcum geophilum* inoculation on the ectomycorrhizal community of beech (Fagus sylvatica L.) under drought conditions **65**

Abstract *66*
Introduction *66*
Methods and materials *66*
Plant and fungal materials 71
Experimental design 71
Sampling and measurements 72
Results *73*
Discussion *81*
Conclusions *83*
References *84*

Chapter 4 Establishment of ectomycorrhizal associations between ectomycorrhizal fungi, including *Cenococcum geophilum* and beech (Fagus sylvatica L.) seedlings using an in vitro experimental system 89

Abstract *90*

Introduction *90*

Materials and methods *92*

Plant and fungal materials 92

Experimental design 94

Results *98*

Propagating beech lines from seeds and branches 98

Tissue culture, micropropagation and rooting of beech in vitro lines 99

Establishing local beech in vitro line 99

In vitro beech ectomycorrhiza development 99

the mantle was formed, and no Hartig net was observed 100

Cross sections of the root tips among 4 ectomycorrhizal fungi and 2 beech line combinations 100

Discussion *111*

Conclusions *111*

References *112*

Chapter 5 Conclusions, Discussion and Perspectives 115

Conclusions *116*

Discussion and Perspectives *116*

Résumé 118

Abstract 119

Résumé des travaux de thèse

Ces dernières années, les événements climatiques extrêmes, en particulier les sécheresses printanières et estivales prolongées, représentent des risques importants pour les forêts, conduisant à des dépérissements qui touchent de nombreuses espèces. En particulier, ces changements climatiques entraînent une diminution des populations de hêtre (*Fagus sylvatica* L.) à travers l'Europe. Par conséquent, le développement de stratégies visant à renforcer la résilience des hêtres face à la sécheresse est devenu crucial. Notre recherche a exploré si *Cenococcum geophilum*, un champignon ascomycète ectomycorhizien cosmopolite, pouvait tolérer des conditions de sécheresse et aider ses plantes hôtes, en particulier le hêtre, à s'adapter au manque d'eau.

Par criblage phénotypique de souches fongiques, nous avons identifié une souche de *C. geophilum,* potentiellement tolérante à la sécheresse, en testant la croissance des isolats sur un milieu de croissance contenant des concentrations accrues de polyéthylène glycol (PEG) comme substitut du stress hydrique du sol ; le PEG augmentant la pression osmotique des cellules. Nous avons ensuite analysé le transcriptome de cette souche cultivé sur PEG à différents intervalles et évalué la récupération de son expression génique après un retour aux conditions normales. Six heures après le traitement au PEG, l'expression génique a changé de manière spectaculaire, activant des voies connues d'adaptation au stress osmotique et de détoxification. Le transcriptome de l'isolat est revenu à son état initial deux jours après son transfert sur un milieu de culture dépourvu de PEG.

Étant donné la tolérance de l'isolat à des changements majeurs de pression osmotique et sa récupération rapide, nous avons mené une expérience en serre pour déterminer si une inoculation mycorhizienne contrôlée avec cette souche pouvait améliorer la résistance des plantules de hêtre à la suspension de l'irrigation. Bien que la mycorhization contrôlée ait augmenté le taux de survie des semis, les isolats naturels de *C. geophilum* présents dans le sol des cultures ont surpassé notre souche sélectionnée, probablement en raison de la brève durée de l'expérience et de la formation limitée de nouvelles racines latérales.

Pour approfondir les mécanismes moléculaires de la tolérance à la sécheresse de

l'association symbiotique *Cenococcum-Fagus*, nous avons tenté de développer un système *in vitro* de culture reposant sur la micro-propagation de plantules de hêtre. Cependant, toutes les lignées de hêtre testées ont montré une formation réduite de racines latérales et de faibles taux de colonisation avec cinq champignons ectomycorhiziens, y compris *C. geophilum*. Bien qu'aucune ectomycorhize typique n'a pu être observé, nous avons mise en évidence par microscopie confocale la formation de structures intraradiculaires semblables à celles développées par les champignons endophytes.

Pour de futures études, nous recommandons de sélectionner des lignées de hêtre avec de meilleures capacités d'enracinement (Chapitre 4) et d'élargir le nombre d'isolats de *C. geophilum* testés (Chapitres 3 et 4), en isolant des souches adaptées localement.

Objectifs de la thèse

Les recherches entreprises dans ce projet visaient à étudier les mécanismes moléculaires développés par le champignon ectomycorhizien *Cenococcum geophilum* afin de tolérer la sécheresse édaphique du sol. Ces recherches ont été conduites sur le mycélium végétatif libre, ainsi que sur l'association *C. geophilum-Fagus sylvatica*. Nos objectifs principaux étaient :

1) Identifier une souche de *C. geophilum* ayant une forte tolérance à la sécheresse, c'est-à-dire capable de croître à de fortes concentrations de PEG.

2) Analyser les modifications du transcriptome suite au stress hydrique (principalement le stress osmotique, simulé par un traitement au PEG) et la récupération subséquente sous des conditions normales.

3) Utiliser la souche résistante à la sécheresse dans une étude en serre impliquant une mycorhization contrôlée de jeunes hêtres.

4) Développer un système in vitro pour établir des ectomycorhizes entre *C. geophilum* et *F. sylvatica* afin de faciliter de futures investigations moléculaires.

Présentation simplifiée du manuscript de thèse

Dans le **chapitre 1**, je présente une synthèse bibliographique de nos connaissances actuelles sur le champignon symbiotique *C. geophilum*, en me concentrant sur ses aspects biologiques, génétiques et écologiques. Les principaux sujets abordés incluent :

Aire écologique et distribution mondiale :
C. geophilum est un champignon ectomycorhizien ubiquitaire qui forme des relations symbiotiques avec plus de 200 espèces de plantes, englobant des arbres, des arbustes et des plantes herbacées. Il prospère dans des écosystèmes variés, incluant les forêts boréales, tempérées, subtropicales et les savanes. Bien qu'il soit fréquent dans les environnements alpins et arctiques, il est moins répandu dans les régions tropicales, à l'exception de certains lieux comme la Thaïlande. Réputé pour son adaptabilité, *C. geophilum* peut survivre à différentes profondeurs du sol et est particulièrement abondant dans les écosystèmes pionniers, tels que les moraines glaciaires nouvellement exposées et les déserts volcaniques. Il joue un rôle crucial dans la succession forestière précoce, s'associant aux semis et aux plantes matures, et est fréquent dans les écosystèmes alpins grâce à sa production de sclérotes. Le champignon conserve sa dominance dans les jeunes peuplements forestiers comme dans les peuplements établis, même après des incendies.

Structure et développement :
C. geophilum, un champignon mélanisé caractérisé par des hyphes septés dématiacés riches en mélanine, démontre une résistance exceptionnelle aux contraintes environnementales. Cette résilience lui permet de prospérer dans des conditions difficiles où d'autres champignons mycorhiziens échouent. Le champignon produit des sclérotes mélanisées pouvant survivre jusqu'à 40 ans, tandis que ses racines ectomycorhiziennes peuvent persister dans le sol pendant environ trois ans. *C. geophilum* synthétise la mélanine via les voies DOPA et DHN, la voie DOPA étant plus active dans les racines ectomycorhizées. Morphologiquement semblable à d'autres endophytes racinaires noirs septés, l'identification précise de *C. geophilum* nécessite des techniques morphologiques couplées à des techniques de génotypage moléculaire.

Le champignon forme également des sclérotes qui agissent comme réserves de nutriments et aident à survivre dans des conditions extrêmes, contribuant au stock de glucides dans les sols forestiers. Bien que des études moléculaires ont mis en évidence des gènes de compatibilité sexuelle (mating type) dans le génome de ce champignon ascomycète, aucune structure sexuelle n'a été observée jusqu'à présent.

Variation génétique et complexe d'espèces :

Malgré sa reproduction asexuée supposée, le champignon présente une diversité génétique significative. Les analyses phylogénétiques ont révélé que *C. geophilum* est très probablement un complexe d'espèces comprenant plusieurs lignées cryptiques. Cette variabilité génétique contribue probablement à son adaptabilité et à son succès dans des environnements diversifiés.

Tolérance au stress :

C. geophilum fait preuve d'une adaptabilité impressionnante à diverses contraintes environnementales, notamment la sécheresse, la chaleur, le froid et les métaux lourds, soulignant son importance écologique. Ce symbiote est reconnu pour sa tolérance à la sécheresse, étant fréquemment retrouvé dans des environnements soumis à des sécheresses édaphiques. Sa tolérance à la sécheresse du sol est appuyée par diverses études examinant les réponses physiologiques et l'expression des gènes liés au stress, bien que les mécanismes spécifiques demeurent en grande partie inconnus. Des facteurs tels que l'accumulation d'osmolytes compatibles (polyols), les protéines de stress thermique et les canaux membranaires de la famille des aquaporines sont supposés contribuer à sa résilience. Cependant, des recherches récentes remettent en question son efficacité en tant que symbiote résistant à la sécheresse, révélant une abondance réduite chez les plantules mycorhiziennes et dans divers écosystèmes forestiers durant les périodes de sécheresse. Ces études indiquent également que les gènes associés à la tolérance à la sécheresse ne seraient pas les plus abondants dans les sols plus secs, suggérant une interaction plus complexe – multifactorielle – entre *C. geophilum* et le stress hydrique. Les études en laboratoire montrent que si des températures de 26°C peuvent inhiber la croissance de certains isolats, l'espèce peut néanmoins former des associations mycorhiziennes après une brève exposition à un stress thermique autour de

70°C. Cependant, une combinaison de stress hydrique et de chaleur à des températures élevées peut être néfaste. L'abondance relative de *C. geophilum* et ses activités exoenzymatiques sont également influencées par la température et la sécheresse, en particulier en association avec différentes espèces de chêne. En revanche, *C. geophilum* montre une résistance remarquable face au froid, restant viable à des températures de congélation de -80°C et prospérant à des températures inférieures à 1°C, probablement grâce à sa capacité à synthétiser du mannitol, un polyol qui offre une protection contre les conditions difficiles. De plus, l'espèce présente une adaptation locale aux concentrations de métaux lourds dans les sols ultramafiques.

Relations symbiotiques et préférences pour les hôtes :

C. geophilum forme des associations ectomycorhiziennes avec un large éventail d'espèces végétales, y compris des arbres, des arbustes et des plantes herbacées. Il peut établir des ecto- et des ectendomycorrhizes, montrant une flexibilité dans sa symbiose avec divers hôtes. Nous avons résumé ici ses hôtes potentiels et les caractéristiques de leurs mycorhizes avec *C. geophilum* et fourni des éléments de preuve ainsi que des hypothèses sur ses préférences pour certains hôtes.

Génomique et biologie moléculaire :

Le génome de C. geophilum, d'environ 178 Mb, est l'un des plus grands parmi les champignons ectomycorhizien, avec une quantité significative de séquences répétées, comme les transposons, et des protéines effectrices secrétées qui sont certainement impliquées dans les relations symbiotiques avec ses plantes hôtes. Des études sur l'expression génique indiquent que 3 % de ses gènes sont induits lors de la symbiose, en particulier ceux liés aux transporteurs et aux petites protéines sécrétées (SSP), qui peuvent agir comme effecteurs pour influencer les mécanismes de défense des plantes.

Les recherches en génomique des populations révèlent une structure génétique complexe avec des lignées distinctes présentant à la fois des traits clonaux et recombinants, façonnés par des facteurs géographiques et environnementaux. Les populations de *C. geophilum* montrent des adaptations locales à des niches écologiques spécifiques, les populations associées aux angiospermes affichant une plus grande diversité génétique que celles associées aux gymnospermes. Ces découvertes

génomiques soulignent l'importance écologique de ce champignon dans le cycle des nutriments, l'amélioration de la résistance à la sécheresse et le maintien de la stabilité des écosystèmes.

Applications en foresterie et en remédiation environnementale :
C. geophilum pourrait être utilisé en gestion forestière dans le cadre des applications en foresterie durable, notamment pour améliorer la résistance à la sécheresse et l'absorption des nutriments chez les arbres. La mycorhization impliquant ce champignon pourrait également être utilisé pour la restauration des terres dégradées et dans les efforts de bioremédiation, comme le maintien des sols contaminés par les métaux lourds. Sa résilience face aux contraintes environnementales en fait un outil précieux pour s'adapter aux changements climatiques.

Pistes de recherche futures :
Nous soulignons également plusieurs pistes de recherche future, notamment une meilleure compréhension des mécanismes génétiques derrière sa tolérance au stress, sa spécificité envers les hôtes et la génomique des populations. Il est également suggéré d'explorer davantage le rôle de ce champignon ectomycorhizien dans le cycle des nutriments, ses interactions avec les communautés microbiennes du sol et son potentiel pour améliorer la résilience des forêts face aux changements environnementaux mondiaux.

Dans le **chapitre 2**, intitulé « *Modifications de l'expression génique dans une souche de Cenococcum geophilum tolérante à la sécheresse durant le stress osmotique et les périodes de récupération* », je résume nos travaux sur l'impact du stress osmotique sur l'expression génique du champignon.

En effet, dans le contexte du changement climatique, le stress hydrique pose des défis considérables aux écosystèmes forestiers. Le champignon ectomycorhizien *C. geophilum* a été fréquemment observé dans les sols forestiers soumis au stress hydrique, montrant une résilience accrue à la sécheresse (stress osmotique) par rapport à d'autres champignons ectomycorhiziens, et il peut favoriser la croissance de son hôte dans des

conditions défavorables. Cependant, les mécanismes moléculaires sous-jacents à l'adaptation de ce symbiote au stress hydrique restent à élucider.

Nous avons examiné les effets des changements de pression osmotique (utilisée comme substitut au stress hydrique) sur l'isolat *C. geophilum* 1.093, identifié comme tolérant à la sécheresse. Une analyse par séquençage d'ARN a été utilisée pour identifier les gènes liés au stress osmotique à travers l'analyse transcriptomique du mycélium cultivé sur du polyéthylène glycol (PEG), utilisé pour augmenter la pression osmotique. Le mycélium végétatif cultivé en milieu liquide, avec ou sans PEG, a été prélevé à 6, 24 et 48 h. Ensuite, le mycélium restant a été transféré dans un milieu sans PEG pendant 24 et 48 h pour évaluer sa capacité de récupération.

Le traitement au PEG a considérablement modifié l'expression des gènes dès les six premières heures d'exposition. À chaque moment, un groupe distinct de gènes a montré une régulation positive ou négative importante. Néanmoins, un ensemble commun de transcrits différemment exprimés a été identifié à tous les moments, comprenant des gènes codant pour des transporteurs membranaires et des enzymes impliquées dans le métabolisme lipidique. Ces gènes sont rapidement revenus à des niveaux d'expression constitutifs après le transfert vers un milieu sans PEG.

L'isolat *C. geophilum* 1.093, connu pour sa tolérance au stress osmotique, a montré des altérations significatives de l'expression génique en réponse aux changements de pression osmotique, un substitut au stress hydrique. De plus, ce champignon a démontré la capacité à retrouver rapidement sa viabilité après être revenu à des conditions favorables.

Dans le **chapitre 3**, intitulé « *Effet de l'inoculation contrôlée de* C. geophilum *1.093 sur la communauté ectomycorhizienne naturelle du hêtre (*Fagus sylvatica *L.) sous conditions de sécheresse.* », nous avons cherché à déterminer si la mycorhization contrôlée avec *C. geophilum* 1.093 pouvait renforcer la protection des plantules de hêtre, déjà mycorhizées par un microbiome naturel du sol, contre le stress hydrique. Une expérience pilote d'inoculation a été menée en serre en utilisant l'isolat tolérant à la sécheresse *C. geophilum* 1.093 et des semis de hêtre européen. Le stress hydrique a été induit en réduisant l'humidité du sol dans des pots contenant des plantules inoculées. Après la sécheresse expérimentale, les morphotypes des racines ectomycorhizées ont été

caractérisés, et pour certains morphotypes sélectionnés, leurs taux de colonisation ont été quantifiés. Les résultats ont révélé que l'isolat 1.093 de *C. geophilum* a pu former des associations ectomycorhiziennes avec les plantules de hêtre. Cependant, dans cette configuration expérimentale, aucun effet positif de l'inoculation contrôlée avec *C. geophilum*1.093 sur le taux de survie des plantules de hêtre n'a été observé. Ainsi, nous recommandons de tester d'autres isolats provenant de différents clades ou des souches localement adaptées de *C. geophilum* dans les recherches futures.

Dans le **chapitre 4**, intitulé : "*Établissement d'associations ectomycorhiziennes entre des champignons ectomycorhiziens, y compris Cenococcum geophilum, et des semis de hêtre (*Fagus sylvatica *L.) à l'aide d'un système expérimental in vitro.* », nous avons testé la mycorhization du hêtre. En effet, au cours des dernières décennies, plusieurs systèmes expérimentaux *in vitro* ont été développés pour explorer les mécanismes moléculaires impliqués dans le développement et le fonctionnement de la symbiose ectomycorhizienne. Cependant, aucun système *in vitro* n'a été mis en place pour étudier l'ectomycorhize du hêtre, malgré l'existence de protocoles de micro-propagation *in vitro* des boutures de hêtre. Nous avons donc testé différentes méthodes pour obtenir des cultures *in vitro* de hêtre à partir de divers explants, y compris des graines et des branches provenant de forêts locales. Des tests préliminaires ont également été réalisés afin d'établir la symbiose entre des boutures *in vitro* de lignées de hêtre et divers champignons ectomycorhiziens. Nos efforts ont permis le développement réussi d'un système d'enracinement *in vitro* pour plusieurs lignées de *F. sylvatica* (FS-ap, FS-10-4) et de *Fagus orientalis* (FO-BS, FO Clone3, FO Clone30) obtenues grâce aux Dr Castro et Martínez du CSIC (Espagne). La lignée de hêtre FS-ap peut former un manteau fongique, mais ne développe pas de réseau de Hartig en présence des champignons ectomycorhiziens sélectionnés, tels que les souches de *C. geophilum* 1.093 et 1.058, *Laccaria bicolor* S238N, ou *Lactarius subdulcis* 3. Nous avons toutefois observé par microscopie confocale la formation de structures typiques des champignons endophytes dans les extrémités racinaires de la lignée FS-ap colonisées par *C. geophilum* 1.093.

Enfin, dans le **Chapitre 5**, je discute l'ensemble de mes résultats et mes conclusions principales. Je propose également quelques perspectives pour de futures recherches, comme le développement

Chapter 1

Recent advances in the biology, genetics and ecology of the ectomycorrhizal ascomycete *Cenococcum geophilum*

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Biology, genetics, and ecology of the cosmopolitan ectomycorrhizal ascomycete *Cenococcum geophilum*

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The ascomycete *Cenococcum geophilum* is a cosmopolitan and ecologically significant ectomycorrhizal (ECM) fungus that forms symbiotic associations with diverse host plants worldwide. As the only known ECM species within the large class Dothideomycetes, *C. geophilum* exhibits several characteristics that distinguish it from other ECM fungi. This fungus significantly contributes to ecosystem stability and development as an early colonizer of primary forest succession. The capacity of this symbiont to rapidly colonize disturbed or newly formed environments promotes the development of conditions that support the growth of other plant species, thus playing a crucial role in the ecological progression and restoration of ecosystems. Several *C. geophilum* isolates are known to enhance the drought resistance of host plants, a trait that is becoming increasingly important in the context of climate change and frequent drought events. In this review, we examined genetic studies that have assessed the phylogenetic structure of *C. geophilum* populations and identified the genes associated with adaptation to environmental stress and symbiosis. The high genetic diversity of *C. geophilum* is particularly noteworthy, considering its putative asexual reproductive mode. Population genomic analyses have suggested that *C. geophilum* is not a single species but rather a species complex comprising multiple cryptic lineages. This genetic variability may contribute to its adaptability and extensive distribution across habitats from circumpolar to tropical biomes. These lineages exhibit potential host preferences, suggesting a degree of specialization within the complex. The nuclear genome of *C. geophilum* has been sequenced, providing valuable insights into the symbiont genetic traits. Notably, this genome encodes a large set of repeated sequences and effector-like small secreted proteins. Transcriptomics has been used to identify candidate genes related to symbiosis and adaptation to environmental stress. Additionally, we briefly discuss how *C. geophilum* offers potential for sustainable forestry practices by improving resilience to stress.

KEYWORDS

drought, environmental stress, forest, genomics, mycorrhizal symbiosis, population genetics

1 Introduction

A majority of land plants establish symbiotic relationships with mycorrhizal fungi, which play a critical role in terrestrial ecosystems by regulating nutrient and carbon cycles, influencing soil structure, and contributing to ecosystem multifunctionality (Martin and Van Der Heijden, 2024). Approximately 80% of plant N and P are provided by these

mutualistic fungi, and the majority of plant species depend on them for growth and survival. An estimated 20,000 fungal species, primarily belonging to the phyla Basidiomycota and Ascomycota, establish ectomycorrhizal (ECM) associations with approximately 6,000 plant species, mostly trees and shrubs (Van Der Heijden et al., 2015). ECM fungi are present in a diverse range of terrestrial ecosystems and are responsible for colonizing 60% of the trees in temperate and boreal forest ecosystems (Baldrian et al., 2023). These tree species, belonging to the Pinaceae, Fagaceae, Betulaceae, Nothofagaceae, Myrtaceae, or Dipterocarpaceae families, play crucial ecological and economic roles in both the northern and southern hemispheres.

During symbiosis development, ECM fungi differentiate the hyphal mantle, ensheathing the rootlets and an intraradical mycelial network, the so-called Hartig net, which penetrates host roots. In numerous ECM associations, an extraradical mycelium permeating the soil environment extends from ECM roots. Mycelial networks facilitate the acquisition of water and nutrients by plants and enhance their resistance to environmental stressors. ECM symbionts secrete extracellular enzymes that degrade soil organic matter (SOM) to facilitate nitrogen acquisition in their hosts (Nicolas et al., 2019). ECM fungi from different independently evolved lineages exhibit varying capacities to degrade SOM and transfer N to their hosts (Nicolas et al., 2019). In boreal and temperate forests, ECM fungi provide 70% of N flux to their hosts (Smith and Read, 2010). Consequently, ECM plays a crucial role in C and N cycles in forest soils. In exchange for soil minerals, 10–20% of photoassimilates are allocated to fungal partners by the host plant. Plant communities allocate 9.07 Gt of atmospheric CO_2 per year to their mycorrhizal symbionts (Hawkins et al., 2023).

The ascomycetous fungus *Cenococcum geophilum*, previously known as *C. graniforme*, is a cosmopolitan ECM fungus and one of the most prevalent mutualistic species found in soil fungal communities worldwide (LoBuglio, 1999; Figure 1). It forms mycorrhizal associations with over 200 trees, shrubs, and herbaceous species in boreal, temperate, and subtropical forests as well as in savannas and alpine meadows. As the only known ECM member of the class Dothideomycetes, *C. geophilum* exhibits several distinctive characteristics that distinguish it from other ECM fungi (LoBuglio, 1999; Obase et al., 2017). As an early colonizer of primary forest succession, *C. geophilum* contributes significantly to ecosystem stability and development. It is particularly important in nutrient cycling because it facilitates the transfer of nutrients, especially nitrogen (N) and phosphorus (P), from the soil to its host plants (LoBuglio, 1999). Additionally, *C. geophilum* enhances the drought resistance of its host plants (Coleman et al., 1989), a trait that is becoming increasingly important in the context of a warming world with an increased occurrence of drought events (Zheng et al., 2023). *C. geophilum* is therefore a compelling model system for research on fungal ecology, evolution, and mycorrhizal symbiosis.

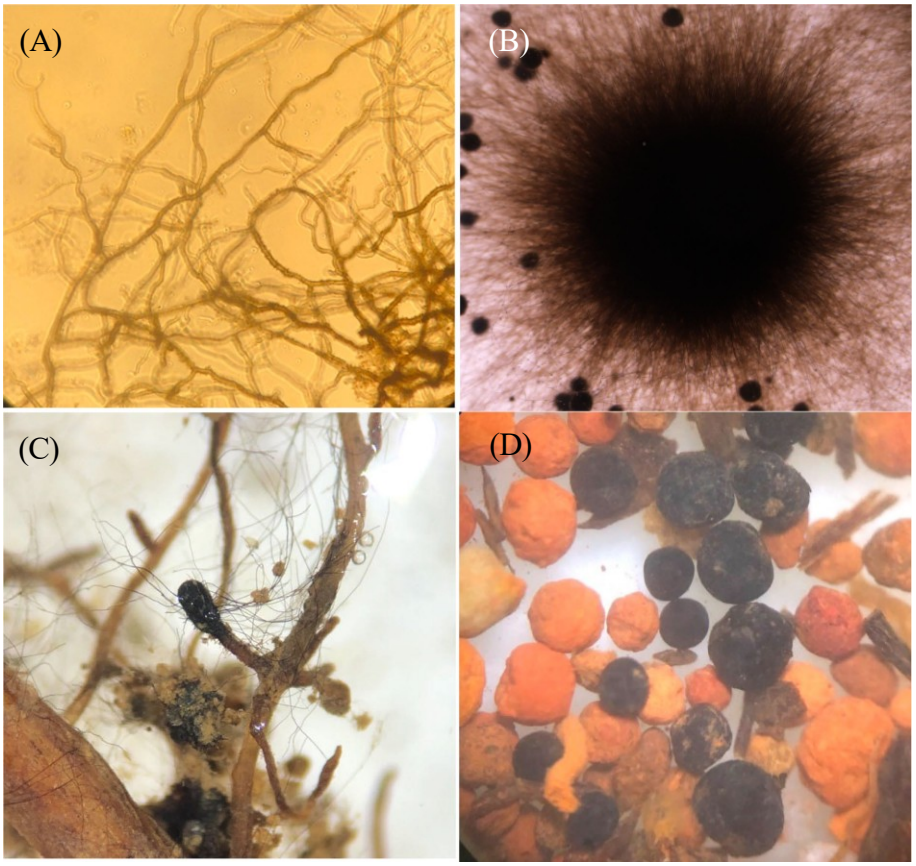


FIGURE 1
The ascomycete *Cenococcum geophilum*: **(A)** melanized mycelial hyphae, **(B)** vegetative colony of free-living mycelium, **(C)** an ectomycorrhizal root tip of the *Fagus sylvatica-Cenocccum geophilum* association with extraradical hyphae, and **(D)** melanized sclerotia.

The biological and ecological attributes of *C. geophilum* have been documented extensively (LoBuglio, 1999; Obase et al., 2017). Recently, genomics has emerged as a crucial tool for investigating the biology, evolution, and ecology of mutualistic symbionts including *C. geophilum* (Kohler et al., 2015; Peter et al., 2016; Miyauchi et al., 2020; Lebreton et al., 2021). This approach not only provides essential mechanistic insights but also identifies key genetic traits, such as adaptation to drought stress (Li et al., 2023; Zhang et al., 2024), which can be prioritized to select strains for the application of this mycorrhizal symbiont in forestry. This review provides a brief account of the biological and ecological attributes of *C. geophilum*, followed by a discussion of recent studies that have demonstrated the impact of genomics and related techniques (i.e., DNA metabarcoding, population genomics, and transcriptomics) on our understanding of this enigmatic mycorrhizal fungus. Additionally, it briefly explores the potential applications of *C. geophilum* in sustainable forestry and ecosystem restoration, highlighting the significance of understanding the functional traits and ecological roles of these ECM fungi in adapting to environmental changes. By consolidating the latest research findings, this review aims to identify knowledge gaps and suggest future research directions for this ubiquitous symbiont to address the global challenges in forestry and environmental sustainability.

2 Morphological features and life cycle

The black fungus *C. geophilum* is distinguished by its septate dematiaceous hyphae, which contain high concentrations of melanin in their cell walls (Figure 1; Fernandez and Koide, 2013). This pigmentation enables mycelia to endure various environmental challenges including UV exposure, dehydration, high temperatures, enzymatic breakdown, antimicrobial agents, and heavy metal exposure (Pal et al., 2013). The resilience of *C. geophilum* enables it to thrive in challenging environments for several years, where other mycorrhizal fungi may find it difficult to survive (McCormack et al., 2017). Its hyphae show various shapes according to the growth medium and the age of the mycelial colony (Trappe, 1962). Chlamydospore-like structures have been observed in both solid and liquid media (Massicotte et al., 1992). These chlamydospore-like structures are always intercalary and rarely terminal in the mycelia (Mikola, 1948). This structure also exists in the taxonomically related species *Glonium* spp. (Amano, 1983) and *Pseudocenococcum floridanum* (Obase et al., 2016).

Cenococcum geophilum can differentiate sclerotium (Figure 1), which is a compact mass of hardened fungal mycelium containing nutrient reserves, including carbohydrates and lipids. The sclerotia constitute an underestimated source of polysaccharides in forest soils, accounting for 3.6% of the total carbohydrates in subalpine forest soils (Murayama and Sugiura, 2021). These melanized sclerotia resist decomposition by soil microorganisms (Fernandez and Koide, 2014) and remain viable for up to 40 years under extreme environmental conditions (Nyamsanjaa et al., 2022). They host specific fungal and bacterial communities (Obase et al., 2014; Narisawa et al., 2021).

Although molecular evidence, such as recombination and diploidy (see below), suggests the presence of unknown sexual stages in the life cycle of *C. geophilum*, no sexual structures have been observed under laboratory or field conditions (Bourne et al., 2014).

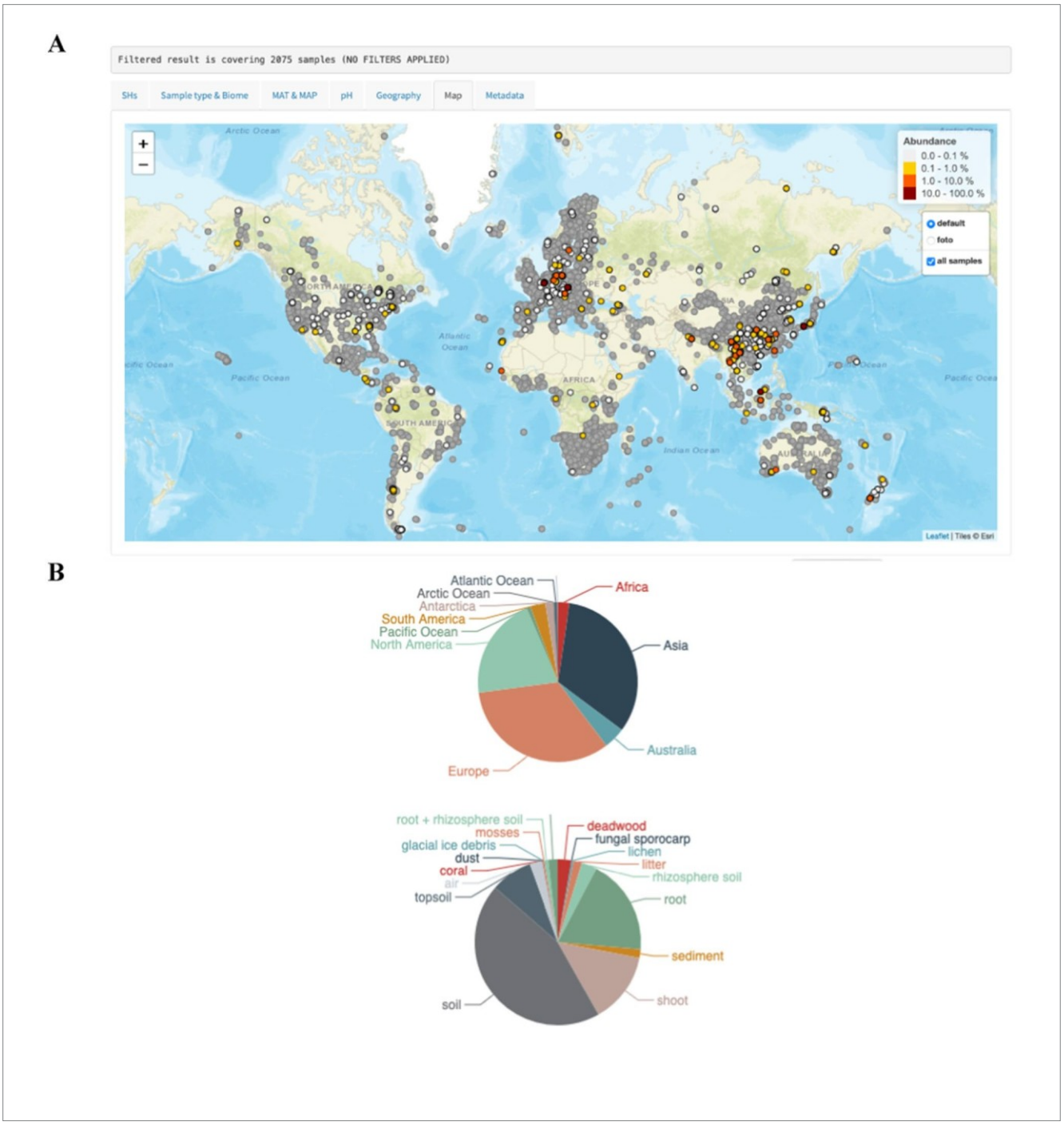
The only ECM fossil related to *C. geophilum* is *Eomelanomyces cenococcoides* gen. Spec. nov., discovered in a 52-million-year-old amber specimen from a lignite mine in Gujarat State, India (Beimforde et al., 2011). This amber was produced by representatives of Dipterocarpaceae trees in the early tropical broadleaf forests. The fossil is similar to the extant *Cenococcum*; however, it is distinguished by high variability in the branching of ECM rootlets and by the regular formation of microsclerotia and chlamydospore-like structures (Beimforde et al., 2011).

3 Ecologically important ectomycorrhizal symbiont

The identification of *C. geophilum* relies on a combination of morphological and molecular techniques, as it shares soil habitats and many physical characteristics with dark septate root endophyte (DSE) fungi such as *Piceirhiza bicolorata* and *Cadophora finlandia* (Rosling et al., 2003). DNA metabarcoding surveys have shown that this ECM fungus is a major component of the soil fungal communities in most of the forest ecosystems (Figure 2). It is considered a keystone species essential for maintaining the microbial network structure and stability (Zhu et al., 2024). As an early colonizer in primary successions, *C. geophilum* significantly contributes to ecosystem stability and development (LoBuglio, 1999). Its rapid establishment in disturbed or newly formed habitats creates favorable conditions for other plant species, thus playing a vital role in ecological succession and ecosystem recovery. Additionally, *C. geophilum* can collaborate with other bacteria to establish ECM associations under varying climatic conditions. Reis et al. (2021) examined beneficial symbiotic microorganisms, including ECM fungi and mycorrhiza helper bacteria in cork oak (*Quercus suber* L.) forests. *C. geophilum* and *Bacillus* sp. were among the most prevalent interacting microbes. Furthermore, Kataoka et al. (2009) reported that *B. subtilis* can enhance *C. geophilum* growth during symbiosis establishment. This mutual support benefits all three partners and could play a crucial role in forest resilience to future climate change.

4 The pan-global ectomycorrhizal symbiont

Cenococcum geophilum is a cosmopolitan ECM fungus and one of the most prevalent species found in soil fungal communities worldwide (Figure 2). It forms ECM or ectendomycorrhizal associations with over 200 trees, shrubs, and herbaceous species in boreal, temperate, and subtropical forests as well as in savannas. In alpine and circumpolar biomes, *C. geophilum* establishes ectendomycorrhizas or ECM with herbaceous plants such as sedges or shrubs (Obase et al., 2017). Its abundance in tropical ecosystems is relatively low (Tedersoo et al., 2010; Bakray et al., 2024), although high root colonization and genetic diversity have been reported in the dry deciduous forests of Thailand (Phosri et al., 2012). However, *C. geophilum* ECM is seldom found in African or South American



tropical forests (Becerra and Zak, 2011; Bâ et al., 2012). It has also been found at the edges of deserts (Massicotte et al., 1992) and in sandy forests of *Picea mongolica* (Bao, 2005).

Zheng et al. (2023) employed the MaxEnt model (Phillips et al., 2017) to analyze the historical shifts in geographical distribution patterns of *C. geophilum* since the Last Glacial Maximum and forecast its future spread under changing climatic conditions. They showed that this geographical distribution is closely associated with climatic factors, particularly temperature and precipitation. Temperature has greater relative importance than precipitation. This is also true for majority of the ECM fungi (Bennett and Classen, 2020). *C. geophilum* occupied a significantly smaller area during the Last Glacial Maximum and mid-Holocene, primarily concentrated in China's Qinling–Huaihe Line region and eastern Peninsular Malaysia. As global temperatures continue to rise, the model predicts a northward shift toward a suitable habitat for *C. geophilum*, resulting in an anticipated increase in suitable areas from 9 to 21%.

Cenococcum geophilum ECM rootlets are abundant in the top 0–5 cm soil layer (Rosling et al., 2003; Genney et al., 2006; Scattolin et al., 2008) but can also be found in much deeper soil layers, such as the mineral layer at a depth of 20 cm (Genney et al., 2006). *C. geophilum* is a pioneer species because of its propensity to partner with pioneer host trees such as *Salix* spp., which colonize newly exposed glacier moraines (Trappe, 1988). Moreover, the symbiont is recognized as a "multi-stage" fungus in secondary forest successions, indicating that it forms ECM associations with both seedlings and adult host plants (Visser, 1995; Danielson, 1991). In the volcanic desert of Mount Fuji, *C. geophilum* is present in both the early and later stages of vegetation

development, colonizing young and old pioneer shrubs, such as *Salix reinii*, and herbaceous species, such as *Polygonum cuspidatum* (Nara, 2006). Interestingly, the symbiont has been found in old growth forests (Peter, 2003), although it is known to primarily colonize young trees in alpine regions near treelines (Hasselquist et al., 2005). In particular, *C. geophilum* is associated with seedlings and juvenile trees of *Picea engelmannii* and *Abies lasiocarpa*, with colonization rates 20 times greater for juveniles than for seedlings (Hasselquist et al., 2005). These findings suggest that this fungus plays an important role in the early stages of forest succession. However, *C. geophilum* has not been replaced by late-stage ECM species in older forest stands. The high prevalence of *C. geophilum* in mature alpine forest ecosystems, which are known for their cold climate, slow litter breakdown, and organic matter buildup in the soil, is believed to be a consequence of the substantial presence of sclerotia (approximately 3,600 kg ha^{-1}) and synchronization of rootlet growth bursts with sclerotia germination in autumn (Vogt et al., 1982). Furthermore, *C. geophilum* is among the most frequent ECM symbionts following a short fire return interval (Buscardo et al., 2010).

Mineral weathering by *C. geophilum* can release potassium from potassium aluminosilicate minerals, such as feldspar, nepheline, biotite, muscovite, and illite (Xue et al., 2019). The symbiotic fungus can also break down the mycorrhizal necromass (Fernandez and Koide, 2014; Gray and Kernaghan, 2020), with the initial N and melanin levels strongly influencing the early decay rates and determining the remaining mass after several years.

5 Tolerance to N deposition, salt stress, and heavy metals

N addition reduced the prevalence of *C. geophilum* in the fungal communities of the humus and fine roots. Forsmark et al. (2024) analyzed the organic layer beneath undisturbed litter in a Norway spruce (*Picea abies*) forest in northern Sweden after two decades of annual N application at low (12.5 kg N ha^{-1} $yr.^{-1}$) and high (50 kg N ha^{-1} y^{r-1}) levels. N supplementation decreased *C. geophilum* abundance, suggesting that decomposition linked to organic N acquisition was suppressed when inorganic forms of N were readily accessible. These community changes were associated with a decreased activity of Mn-peroxidase and peptidase and an increase in the activity of C-acquiring enzymes.

Wen et al. (2022) studied the influence of *C. geophilum* inoculation on the growth and nutrient uptake of *Pinus thunbergii* seedlings under salt stress. Their results indicated that mycorrhizal inoculation significantly increased seedling biomass, chlorophyll, and nutrient elements (such as P, N, and K) in shoots and maintained a low Na/K ratio in roots under salt stress, suggesting that inoculation with *C. geophilum* could assist the host in overcoming salt stress. Geographical isolates of *C. geophilum* have shown patterns of local adaptation to serpentine soils, with Ni concentrations having a significant effect on fitness-related traits (Gonçalves et al., 2009; Bazzicalupo et al., 2020).

6 Adaptation to water-stressed environments

C. geophilum exhibits drought tolerance and is prevalent in water-stressed environments (Pigott, 1982; McCormack et al., 2017). Several surveys of soil fungal communities have demonstrated that the proportions of *C. geophilum* ECM and extramatrical mycelia increase under water stress conditions and are often higher during summer in natural settings (Pigott, 1982; Querejeta et al., 2009). This tolerance has been verified through *in vitro* mycelial culture experiments using osmotically adjusted media (Mexal and Reid, 1973; Coleman et al., 1989), cell damage tests following desiccation (Di Pietro et al., 2007), and respiration measurements under water stress (Jany et al., 2003). The level of tolerance varies among geographical isolates (Coleman et al., 1989; Jany et al., 2003). However, the physiological mechanisms responsible for this symbiont's success under water stress remain largely unknown. Multiple factors likely contribute to this trait, such as the accumulation of compatible osmolytes (e.g., polyols), heat shock proteins, hydrophobic proteins, and melanin in the cell walls. It has also been suggested that drought resistance in *C. geophilum* may be associated with the increased expression of aquaporin water channels (see below, Peter et al., 2016). Although *C. geophilum* is widely recognized as a drought-tolerant symbiont, this contention has recently been debated. A study utilizing *Pinus* seedlings colonized by *C. geophilum* and subjected to water shortages showed that the drought resistance of mycorrhizal plantlets was not directly correlated with that of *C. geophilum* isolates cultivated in liquid medium (Zhang et al., 2024). Xie et al. (2024) used inoculated *Quercus mongolica* and *Tilia amurensis* to investigate the responses of ECM fungal communities and their exploration types under drought conditions in a pot system. The relative abundance of *C. geophilum* in both hosts decreased. Nickel et al. (2018) examined ECM fungal community diversity changes in European beech and Norway spruce forests under drought conditions by utilizing retractable roofs to exclude rain for 3 years. The results indicated that the abundance of *C. geophilum* decreased irrespective of the depth, year, or host.

7 Heat and cold stresses

Laboratory experiments demonstrated that the growth inhibition of several *C. geophilum* isolates occurred at a temperature of 26°C (Yan et al., 2022); however, this species is capable of forming mycorrhizal associations following exposure to heat stress at approximately 70°C for a brief period or at 5°C above ambient temperature. Nevertheless, combined stress, including drought and heat stress, at a temperature of 5°C above ambient temperature and 50% precipitation can be lethal to *C. geophilum* (Kipfer et al., 2010; Gehring et al., 2020). Herzog et al. (2013) reported that increased temperature and water shortage can differentially affect the relative ECM abundance and exoenzyme activities of *C. geophilum* associated with various oak species, specifically *Q. robur*, *Q. petraea*, and *Q. pubescens*.

Furthermore, because of their prevalence as symbionts in arctic and alpine ecosystems, *C. geophilum* mycelia and ECM are likely to exhibit high tolerance to cold stress. Corbery and Le Tacon (1997) demonstrated that *C. geophilum* mycelium remained viable even when exposed to a freezing temperature of −80°C for a brief period, exhibiting greater resistance to cold than other ECM fungi. Additionally, studies have indicated that this fungus thrives at temperatures below 1°C (Vogt et al., 1982). This cold stress resistance may be attributed to its high mannitol synthesis rate (Martin et al., 1985) because mannitol is known to shield fungi from severe cryoenvironments (Weinstein et al., 1997).

8 Host preferences

C. geophilum is recognized as a mycorrhizal generalist species. This symbiont can form ecto-or ectendomycorrhizal associations with a broad host range. Based on the morphology and anatomy of mycorrhizal roots sampled in natural settings, three groups of host plants were identified (Trappe, 1962; LoBuglio, 1999): In Group 1, the hosts include members of the Salicaceae and Betulaceae families (excluding *Corylus* spp.), as well as ectotrophic genera within the Rosaceae family. The ECM root tips are typically monopodial or occasionally branched, with the mantle covering only the root tips. The Hartig net in these hosts never extends deeper than the third layer of the cortical cells, and intracellular penetration is sparse and limited to occasional cells. In Group 2, *C. geophilum* associates with *Pinus* species. The ECM root tips are monopodial, dichotomous, or occasionally irregularly branched. The mantles typically cover all short roots and have thicknesses ranging between 8 and 60 μm. The Hartig net extends inward to the innermost layer of cortical cells, and the cortex experiences strong intracellular infection. The hosts in Group 3 predominantly comprise Fagaceae, including *Corylus*, and Pinaceae, with the exception of *Pinus* spp. The root tips of these associations exhibit a range of morphologies, including monopodial, racemose, irregularly branched, long, or short structures. The mantle typically covers a significant portion or all of the short roots, and its thickness ranges from to 8 to 60 μm. The Hartig net extends to the innermost layer of the cortical cells, and intracellular infection is prevalent throughout the cortex.

Additionally, this groups includes many shrubs and herbaceous plants such as *Pedicularis capitata* (Kohn and Stasovski, 1990), *Cistus* spp. (Massicotte et al., 2010), *Bistorta vivipara* (Massicotte et al., 1998), *Carex myosuroides* (Massicotte et al., 1998), and *Rhododendron* spp. (Largent et al., 1980; Vohník et al., 2007). Unusual for an ECM symbiont, *C. geophilum* can also establish ectendomycorrhizal associations with shrubs and herbaceous plants, sharing mycelial networks with woody plants such as oak and *Helianthemum bicknellii* (Dickie and Reich, 2005), or the *Dryas octopetala–Bistorta vivipara–Salix herbacea* association (Mühlmann and Peintner, 2008). Symbiosis with herbaceous plants appears to enhance the colonization of woody plants (Dickie and Reich, 2005; Hoeksema et al., 2018). Although there is no evidence of nutrient transfer between herbaceous and woody plants sharing common mycorrhizal networks (CMNs) with *C. geophilum*, this structure could possibly act as a physical link between roots of herbaceous and woody plants, thereby enhancing *C. geophilum* colonization in sharing plants. The CMNs may also alter the bacterial communities of the hyphosphere (Vik et al., 2013).

Variations in colonization rates and/or host preferences can be attributed to genetic factors in both partners, as well as environmental factors such as soil organic matter content, total N, and available P (Wurentaoges, 2012). Zhu et al. (2024) reported that leaf photosynthesis and root morphological traits drive the topological structure of plant–fungus association networks involving *Cenococcum* species. Abundant plants may play a key role as reservoirs of symbiotic fungal diversity and thus contribute to the maintenance of ecosystem functions.

9 Population structure

As previously mentioned, *C. geophilum* is widespread and has historically posed challenges in terms of physiological and phylogenetic classification. Collections of *C. geophilum* isolates, both locally and globally, have shown remarkable genetic diversity. Genetic studies on *C. geophilum* have revealed a complex population structure, even at the soil core sample level, with evidence of both local adaptation and limited gene flow between populations (Jany et al., 2002; Douhan and Rizzo, 2005; Matsuda et al., 2015; Obase et al., 2016; Obase et al., 2017; Vélez et al., 2021). They uncovered the presence of multiple hidden clades and distinct phylogenetic groups within *C. geophilum*, supporting the widely held view that this species represents a highly diverse assemblage of ectomycorrhizal fungi at regional and global levels. The structure of symbiont populations is influenced by several factors, including geographic distance, environmental gradients, and host–plant associations. They are often structured according to environmental conditions such as soil type, moisture level, and temperature. For example, populations from dry nutrient-poor soils tend to be genetically distinct from those in more fertile environments, suggesting a local adaptation to specific ecological niches (Douhan and Rizzo, 2005; Lian et al., 2006). A subtle geographic structure with long-distance disjunction suggests complex alternation of sexual and asexual reproduction over space and time (Obase et al., 2016; Obase et al., 2017). However, gene flow between populations can occur through sclerotia dispersal, leading to a combination of local adaptation and genetic exchange.

The presence of cryptic species within *C. geophilum* has also been confirmed, with distinct genetic lineages corresponding to different ecological and geographical regions (Obase et al., 2017; Vélez et al., 2021). Obase et al. (2017) resolved seven clades with high bootstrap support among the isolates of *Cenococcum* derived from different geographical regions across the world using both single-and multi-locus and maximum likelihood (ML) analyses (Figure 3). All *Cenococcum* clades clustered together with high bootstrap support, whereas *Pseudocenococcum floridanum* isolates were resolved as a separate group. More recently, Vélez et al. (2021) examined a set of 200+ *C. geophilum* isolates obtained from the soil beneath *Populus trichocarpa* along an ~280 mile north–south corridor in the Pacific Northwest, United States. Additionally, they performed global phylogenetic analysis by incorporating 789 isolates with publicly accessible data from the United States, Japan, and Europe. This analysis identified 34 strongly supported clades using ML and Bayesian methods, with some clades exhibiting intra-and intercontinental distributions. These findings strongly indicate divergence within multiple cryptic species.

Furthermore, the genetic diversity and structure of *C. geophilum* populations were analyzed based on the rDNA ITS2 sequences of 219 ECM root samples collected from 3 plant families (Betulaceae, Fagaceae, and Pinaceae) from 10 forest sites across China (Guo et al., 2021). Analysis of molecular variance (AMOVA) confirmed that genetic differentiation was evident within each geographical population and the population in each host plant family. The Fagaceae population was distinct from the Betulaceae and Pinaceae populations, and the haplotype composition was conspicuously different among the three plant families. These cryptic species may represent locally adapted forms of *C. geophilum*, which have evolved in response to specific environmental conditions. The genetic diversity of angiosperm-associated *C. geophilum* populations is higher than that of gymnosperm-associated populations, suggesting that angiosperm and gymnosperm hosts exert different selective pressures on their

symbionts (Vélez et al., 2021). Tedersoo et al. (2024) also provided evidence for niche differentiation in tens of cryptic species of *Cenococcum*, many of which exhibit a preference toward particular partner plant genera.

Currently, it remains uncertain whether *C. geophilum* constitutes a single, highly diverse global species or whether it comprises numerous cryptic species. Subsequent studies could shed light on these local and worldwide relationships by comparing nuclear and mitochondrial genomes from a wide range of geographical isolates along with population genomics approaches.

10 Genomics, transcriptomics, and population genomics

10.1 Genomics

Within the framework of the Mycorrhizal Genome Initiative (Martin et al., 2011), the nuclear genome of *C. geophilum* (strain 1.58) has been sequenced and annotated by the U.S. Department of Energy Joint Genome Institute (Peter et al., 2016). This genome is the largest among the ECM fungi, with a mapped size of 178 Mbp and a total estimated size of 203 Mbp (Peter et al., 2016; Talhinhas et al., 2017). It is estimated to contain approximately 15,000 genes. In contrast, the genomes of the taxonomically related saprotrophic species *Glonium stellatum* and *Lepidopterella palustris* are approximately four times smaller than that of *C. geophilum*, at 41 and 46 Mbp, respectively, yet they possess similar gene counts to 14,362 and 13,870 predicted gene models, respectively. Phylogenomic analysis using single-copy conserved orthologs confirmed that *C. geophilum* belongs to the class Dothideomycetes, specifically in the order Mytilinidiales, and shares a close evolutionary relationship with the saprotrophic species *G. stellatum and L. palustris*. Despite its close taxonomic relationship with these saprotrophs, *C. geophilum* exhibits unique genomic features consistent with its ECM lifestyle. This is in agreement with the independent origin of ECM ability in *Cenococcum* within the class of otherwise saprobic Ascomycota (Dothideomycetes), with evidence that the most closely related sister group, *Glonium*, is likely saprobic and lacks mycorrhization ability. In their study, Obase et al. (2017) reported that *Pseudocenococcum floridanum* is a more closely related but distinct sister group to other *Cenococcum* lineages and that this new species likely lacks the ability to form ectomycorrhizas. Ongoing sequencing of several strains of *P. floridanum* at JGI[1] will provide new insights into the evolution of the saprotrophy-to-symbiosis transition in *Cenococcum* clades.

The *C. geophilum* gene repertoire contains 2,176 species-specific genes, including effector-like small secreted proteins (SSPs). Many of

1 https://mycocosm.jgi.doe.gov/mycocosm/home

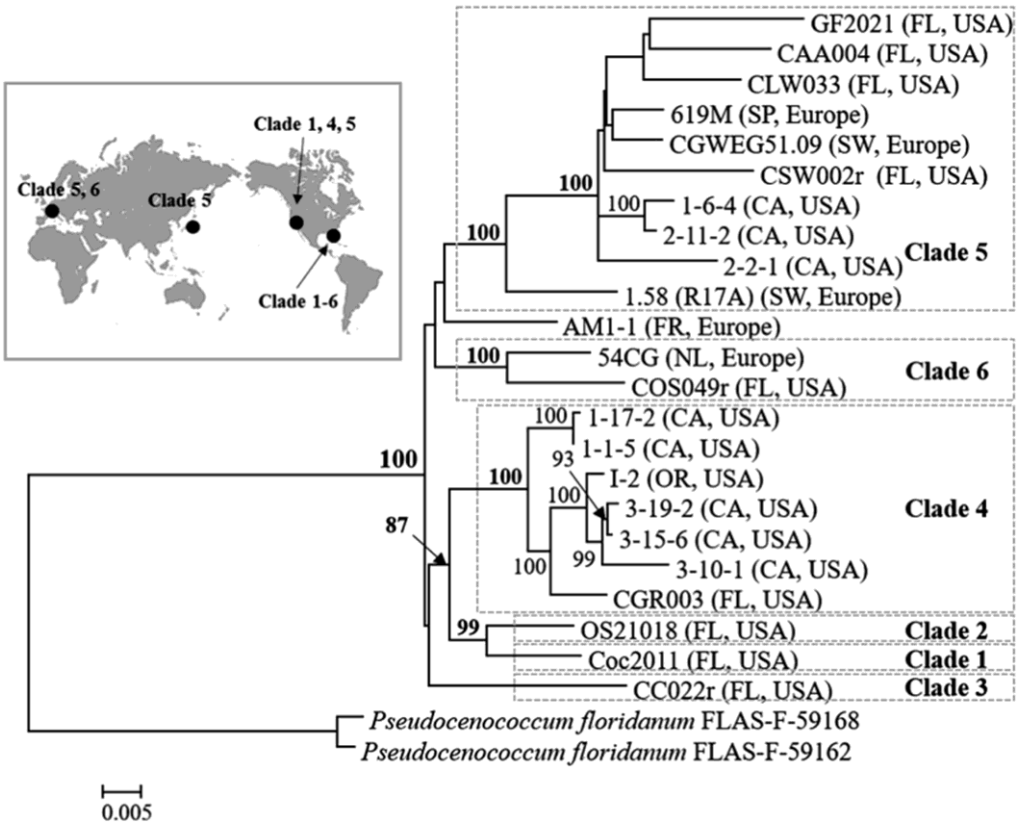


FIGURE 3
Phylogenetic tree of *Cenococcum geophilum*: major clades and their worldwide distribution (CA, California; FL, Florida; FR, France; NL, Netherlands; OR, Oregon; SP, Spain, SW, Switzerland). A maximum-likelihood phylogenetic tree was constructed using seven concatenated loci (ITS, SSU, LSU, TEF, RPB1, RPB2, and GAPDH). Isolates of *Pseudocenococcum floridanum* were used as outgroups (Obase et al., 2017).

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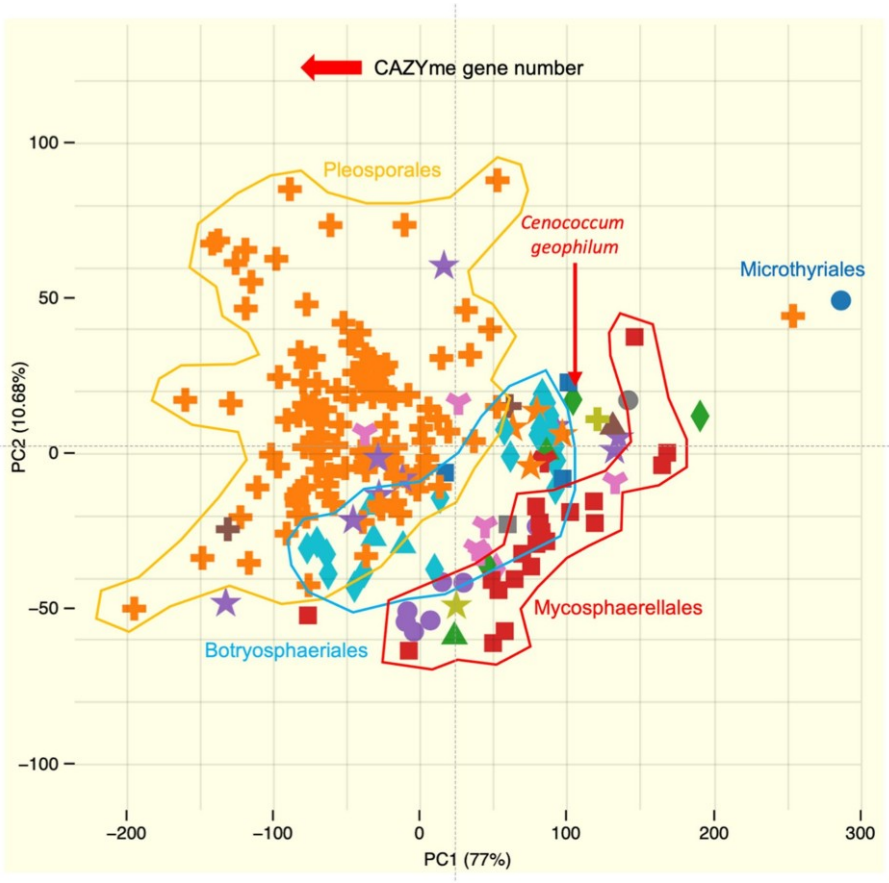


FIGURE 4
Principal component analysis (PCA) showing the distribution of the CAZyme repertoire in *Cenococcum geophilum* (red arrow) and other sequenced Dothideomycetes available in the JGI MycoCosm database (Grigoriev et al., 2014). Major orders of Dothideomycetes, such as *Botryosphaeriales*, *Mycosphaerellales*, and *Pleosporales*, are also indicated. These data were obtained after semi-manual curation of protein-filtered model sequences by the CAZy team (www.cazy.org) (Drula et al., 2022), and PCA was generated by MycoCosm.

these unique genes are involved in protein–protein interactions and signaling mechanisms, which are likely crucial for their symbiotic relationships with plants. Compared with its close relatives, the expanded genome of *C. geophilum* is attributed to its high proportion (81%) of repetitive sequences, primarily composed of transposable elements (TEs). Increased TE content is observed in numerous plant pathogenic fungi, particularly in those with (hemi-) biotrophic lifestyles. This trend is even more pronounced in symbiotic mycorrhizal fungi (Miyauchi et al., 2020; Lebreton et al., 2021). The majority of expanded gene families are associated with TEs or are involved in protein–protein interactions. These families exhibit domains typically observed in proteins related to self/non-self-recognition, which are associated with somatic incompatibility and defense mechanisms, such as HET, NACHT, and WD40 proteins. Notably, the expression of the majority of these gene families remains unregulated in functional mycorrhizas (Peter et al., 2016).

The repertoire of genes encoding Plant cell wall degrading enzymes (PCWDEs) is lower than that of the majority of saprotrophic and pathogenic Dothideomycetes but similar to that of saprotrophic and pathogenic Mycosphaerellales and Botryosphaeriales (Figure 4; Peter et al., 2016). This reduction is striking for enzymes that act on cellulose, hemicellulose, and pectin. Enzymes that act on hemicellulose, such as xylanases (GH10 and GH11), mannanases (GH26), glucuronidases (GH115), and pectin-attacking enzymes (PL1, PL3, PL4, and CE12), are also reduced from two to five members to either none or only one member. Among the sequenced ECM fungi, *C. geophilum* exhibited the most extensive PCWDE repertoire (43 enzymes). Notably, proteins that target crystalline cellulose (GH6, GH7, AA9, and CBM1) are found in the *C. geophilum* genome (Peter et al., 2016) but are frequently absent in other ECM fungi (Kohler et al., 2015; Miyauchi et al., 2020; Lebreton et al., 2021).

With the exception of polyketide synthases (PKSs), the *C. geophilum* genome did not show a reduction in the number of genes associated with the biosynthesis of secondary metabolites (Figure 5; Peter et al., 2016), many of which act as antibiotics in pathogenic interactions and microbial competition in the rhizosphere. These PKSs are typically more numerous than those found in ECM basidiomycetes (Lebreton et al., 2021). In ECM root tips, the expression of the majority of secondary metabolism-related genes is suppressed, except for two non-ribosomal peptide synthases (NRPS) and a PKS (Peter et al., 2016). Notably, one of these NRPS exhibits high protein sequence similarity (42%) to *Aspergillus fumigatus* Pes1, which is involved in the defense against oxidative

stress (Reeves et al., 2006). Oxidative stress is an unavoidable consequence of drought and is employed by plants as a defense mechanism against biotic stressors.

10.2 Transcriptomics

Peter et al. (2016) revealed that 3% of *C. geophilum* genes were upregulated during symbiosis, as determined by comparing RNA sequences from mycorrhizal roots and free-living mycelia. The most highly expressed and upregulated genes in symbiosis include aquaporins, major facilitator superfamily (MFS) membrane transporters, and small secreted proteins (SSPs), which are proteins less than 300 amino acids in length with a predicted signal peptide (Table 1). Notably, 18–23% of the upregulated genes were specific to *C. geophilum*, with SSPs being overrepresented in these taxon-specific orphan genes compared to their proportion in the overall gene repertoire. These SSPs may function as novel symbiosis-related effectors, similar to the mycorrhiza-induced small secreted proteins (MiSSPs) in *Laccaria bicolor*, which regulates defense-related pathways in host roots (Martin et al., 2016).

De Freitas Pereira et al. (2018) compared the secretome of *C. geophilum* interacting with pine and poplar trees; they reported that the levels of transcripts encoding carbohydrate-active enzymes (CAZymes) and MiSSPs were strikingly different. This may be related to the different cell wall compositions of the pine and poplar roots (Sarkar et al., 2009). Colonizing *C. geophilum* hyphae may require different cell wall-loosening enzymes to penetrate the roots and effectors to dampen the host immune system.

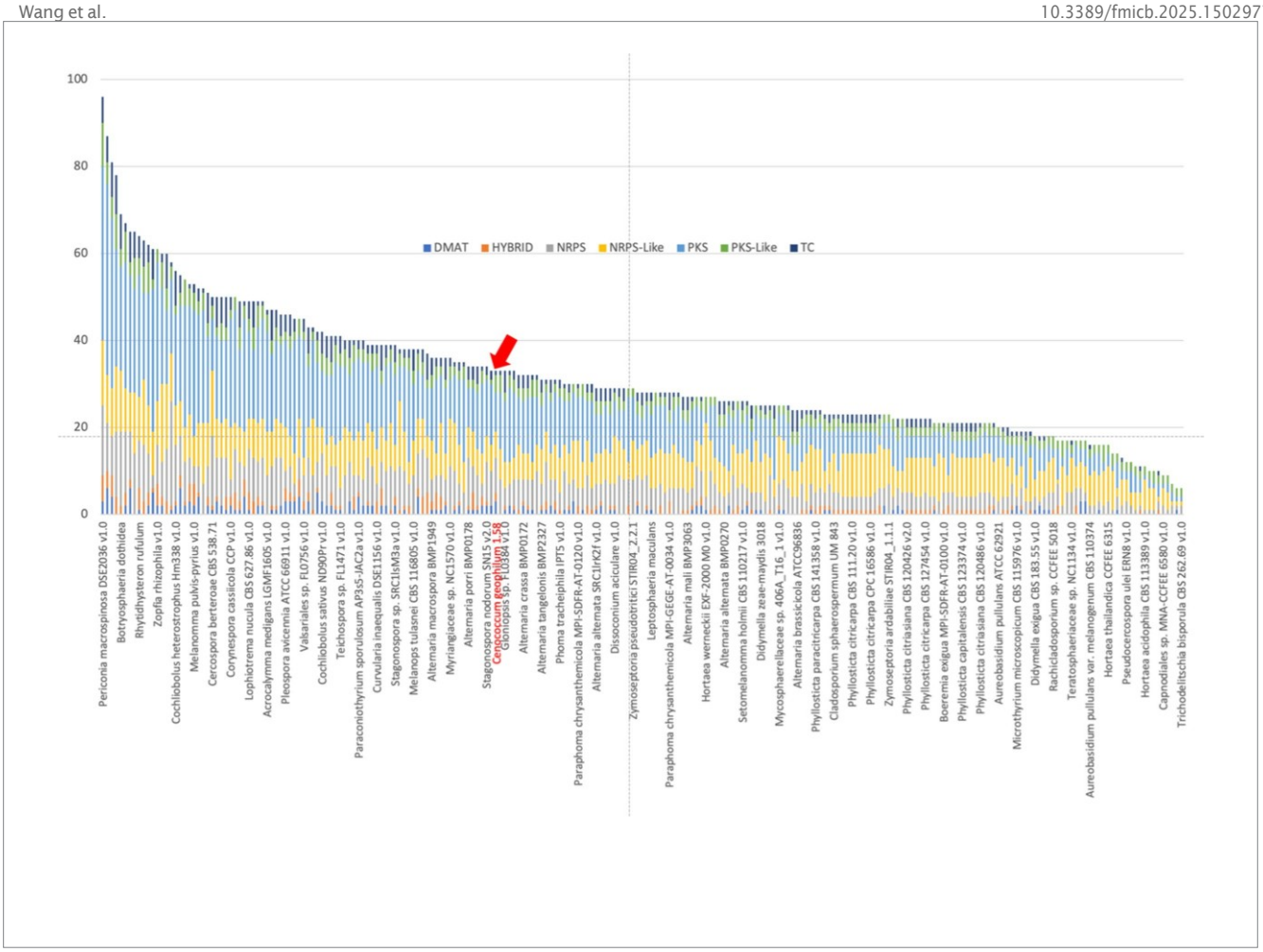
The gene expression analysis revealed significant changes in the expressions of two aquaporins (AQP) that encode water channels during symbiosis in the ECM rootlets of *Pinus sylvestris* (Table 1; Peter et al., 2016). The substantial increase in water-permeable AQPs in symbiotic rootlets may be triggered by the water and/or nutrient requirements of the plant during interactions. Studies on expression have shown downregulation and upregulation of these AQP genes under drought conditions (a shoot water potential of −3.5 MPa). Intriguingly, under well-watered control conditions, the transcript levels of the drought-induced classical AQP (Cenge3:604158) correlated best with the shoot water potential of their host plant. In the same drought/re-watering experiment, Peter et al. (2016) evaluated the conditions of mycorrhizal plants compared with non-mycorrhizal plants. They noted significantly higher needle N content, net photosynthesis, and water use efficiency in ECM pine seedlings than those in their non-mycorrhizal counterparts, confirming the mutually beneficial relationship between fungi and plants. However, mycorrhizal inoculation had no significant effect on drought treatment.

Zhang et al. (2024) investigated the effects of several *C. geophilum* ecotypes on the drought resistance of *Pinus massoniana* seedlings. They found that inoculation with various strains of *C. geophilum* improved the drought resistance of seedlings by affecting the water content, photosynthesis, osmotic adjustment substances, and antioxidant enzyme activities. The transcriptomic analysis revealed that the seedlings

TABLE 1 Most highly upregulated genes in ectomycorrhizal roots (ECM) of *Cenococcum geophilum/Pinus sylvestris* compared to free-living mycelia (FLM) (adapted from Peter et al., 2016).

RPKM FLM[1]	RPKM ECM[1]	Fold Change	Protein ID	Definition
0.6	3,849	6,133	690706	Aquaporin (major intrinsic protein)
0.4	550	1,453	600722	Sugar transporter
5.3	5,666	1,073	647346	Aquaporin (major intrinsic protein)
0.1	112	1,018	697512	Unknown function
1.0	476	484	654895	Peptidase
3.7	1,560	420	655146	Unknown function
0.5	185	410	730139	MFS transporter
0.3	91	344	612206	MFS transporter
0.2	51	341	333290	Unknown function
0.3	97	313	600293	Unknown function
2.1	636	297	698167	SSP/unknown function
0.1	36	279	616643	Unknown function
0.1	15	270	660401	SSP/unknown function
0.1	21	236	680403	SSP/unknown function
8.9	2016	227	605087	MFS transporter
0.2	39	225	610797	Amino acid permease
0.9	204	222	608762	Glucose/ribitol dehydrogenase
1.6	363	222	613185	G-protein
2.0	409	207	676136	Ricin B lectin
1.6	325	207	649427	Unknown function

Abbreviations: MFS, major facilitator superfamily; SSP, small secreted proteins.
RPKM and fold change values are represented using a gradient from white to dark red, with the intensity increasing in line with the values.
[1]Mean of three replicates; given in reads per kilobase of transcript per million reads mapped (RPKM).



primarily regulate their energy metabolism and redox reactions to cope with early drought stress. The effectiveness of inoculation did not depend on the drought tolerance of the *C. geophilum* ecotype; that is, the drought resistance of the mycorrhizal seedlings did not correlate with the inherent drought resistance of the *C. geophilum* strain itself. The beneficial effects of *C. geophilum* inoculation on the growth of pine seedlings during the early stages of drought stress suggest that this symbiont can be used in reforestation programs in drought areas. Using 1-D gel electrophoresis and LC–MS/MS, Kerner et al. (2012) identified 12 proteins that were differentially accumulated in mycelia subjected to drought conditions compared to controls. The induced responses in *C. geophilum* point toward the regulation of osmotic stress, maintenance of cell integrity, and counteracting increased levels of reactive oxygen species formed during water deprivation.

The survival of *C. geophilum* in various environments depends on its ability to regulate stress-related gene expression. Transcriptome profiling has shown that *C. geophilum* can enhance the expression of numerous genes associated with stress resistance, including those associated with osmotic/drought stress (Li et al., 2023), salt stress (Li et al., 2022), oxidative stress, heat shock responses (Yan et al., 2022), and heavy metal tolerance (Shi et al., 2022). These genes, which are involved in processes such as organic acid secretion, antioxidant activity (e.g., peroxidase, superoxide dismutase, and ubiquinone), membrane transport, and sphingolipid metabolism pathways, are regulated in a coordinated manner. This suggests that their expression is controlled by transcription factors that react to environmental changes, such as heat shock factors and elements responsive to osmotic stress. Verification of the functional roles of the numerous identified stress-related genes will necessitate genetic transformation protocols to inactivate them through RNA interference silencing or CRISPR/Cas9.

Although identifying differentially expressed genes in mycelia cultivated under laboratory conditions represents a promising approach to characterizing genes involved in drought stress adaptation, it is important to consider that gene expression in natural environments may differ significantly, as demonstrated in a recent study by Pellitier et al. (2024). They investigated fungal communities inhabiting the roots of *Populus trichocarpa* distributed across a precipitation gradient in the Pacific Northwest, United States. These communities were analyzed using taxonomic (metabarcoding) and functional (metagenomic) approaches. Their findings revealed that fungal genes associated with drought stress tolerance and plant water uptake (including genes for melanin synthesis, hydrophobins, aquaporins, trehalose synthases, and other gene families) were not predominant in drier soils.

10.3 Population genomics

Dauphin et al. (2021) conducted a study on 16 European isolates of *C. geophilum* using whole-genome resequencing. Their findings revealed divergent lineages in geographically confined

sampling locations, without strong geographic structuring. Genome-wide polymorphism analyses indicated species subdivisions and suggested two primary genetic groups: clonal and recombinant. The lineage phylogeny and groupings were largely corroborated by the numerous gene copy number variations (CNVs) discovered among the genomes. Although the clonal cluster contained nearly twice as many strains, gene diversity analyses showed a higher genetic diversity in the recombinant group. Based on Tajima's D statistics, the top candidate genes potentially under positive selection differed between the two groups. The recombinant cluster exhibited more genes from lineage-specific expanded gene families involved in self/non-self-recognition, whereas the clonal cluster showed genes related to secondary metabolism. Additionally, this study confirmed *C. geophilum* heterothallism through chromosomal synteny analysis of the mating genes *MAT1-1* and *MAT1-2* idiomorphs. It also revealed significant genetic rearrangements in the surrounding coding and non-coding regions of the strains carrying both the same and opposite *MAT1* idiomorphs. These results highlight the complex genome architecture of *C. geophilum*, possibly due to cryptic sex-and/or transposon-related mechanisms.

Lian et al. (2024) assembled five *C. geophilum* genomes representing different geographical regions and generated a pan-genome comprising 7,556 core gene families and 12,686 dispensable gene families. Genome resequencing of 304 isolates with worldwide distribution was performed to estimate the genetic diversity, structure, and demographic history of *C. geophilum* isolates. Millions of single nucleotide polymorphisms (SNPs) and 0.04–0.2% structural variations have been identified, suggesting the occurrence of several ecotypes with different drought resilience levels. Their genome-wide association and transcriptome analyses identified 161 genomic regions that were significantly associated with 9 biological and environmental adaptation traits, encompassing 2,738 potential genes, including EVM0002574, which are associated with resistance to cadmium, salt, and high-temperature stresses. These genomic resources and diversity datasets provide valuable tools and a comparative genomic framework for investigating ectomycorrhizal symbiotic relationships.

11 Applications in forestry and conservation

Cenococcum geophilum is a highly adaptable ECM fungus that demonstrates significant potential for ecological restoration and environmental remediation through microbial engineering. The symbiont forms a dense network of melanized hyphae around the roots of host plants, creating a protective sheath. This symbiotic association is particularly beneficial in water-limited environments, where *C. geophilum* helps the host tree maintain hydraulic conductivity and photosynthetic activity under drought stress. Additionally, the fungus has been shown to enhance salt tolerance of host plants, making it valuable for reclaiming saline soils. Finally, its extensive distribution, broad host range, and high stress tolerance make it particularly valuable for addressing desertification and adapting to climate change (Zhai et al., 2023). Through the utilization of genomics and other-omics techniques, we acquired a more comprehensive understanding of the molecular, physiological, and ecological mechanisms underlying the establishment and functioning of *C. geophilum* ECM under environmental stress. Candidate genes related to adaptation to these environmental stresses can be used to select appropriate strains for the mycorrhizal inoculation of tree seedlings in environments prone to drought or other abiotic stresses. Surveys of soil fungal communities using DNA metabarcoding can be used to predict the environmental conditions under which *C. geophilum* inoculation is beneficial for forest management and restoration. This enhanced knowledge should be leveraged to develop practical applications, such as mycorrhizal inoculation or microbial engineering, which would enhance ecosystem function and preservation, aid in alleviating climate change impacts, and maintain the sustainability of forest ecosystems.

Furthermore, *C. geophilum* colonizes herbaceous plants. By forming associations with both woody and non-woody plants, symbionts can contribute to the development of diverse plant communities in challenging environments. Its ability to support multiple plant species can increase soil stability, reduce erosion, and improve nutrient cycling in degraded ecosystems. In arid regions, *C. geophilum* colonizes both ECM trees and Cistaceae plants. The physical connection of *C. geophilum* mycelial networks with both tree roots and herbaceous plants could redistribute water from the deeper roots of the tree, retain a portion of the water in the upper soil layers, and facilitate enhanced nutrient acquisition by the host plants. Similarly, the mouse-tail bog sedge (*Carex (Kobresia) myosuroides*) can be incorporated into tree plantations in northern and alpine regions. In environments contaminated by industrial waste, *C. geophilum* has shown promising results in the remediation of soils affected by heavy metals and petroleum (Danielson and Visser, 1989). The fungus has also exhibited the capacity to accumulate and sequester various heavy metals, including Pb, Cd, and Zn, in its melanized cell walls (Huang et al., 2014; Azaiez et al., 2018; Shi et al., 2022; Zhang et al., 2023,). This characteristic renders *C. geophilum* a potential candidate for mycoremediation of polluted soils. Moreover, their association with host plants can enhance phytoremediation efforts by improving plant survival and growth at contaminated sites.

Urban environments often present challenging conditions for plant growth such as soil compaction, elevated temperatures, and air pollution. *C. geophilum* is frequently the most abundant ECM symbiont found in the roots of urban trees (Garbaye and Churin, 1996; Hui et al., 2017; Van Geel et al., 2018; Olchowik et al., 2021). Its ability to form symbiotic relationships can improve resilience to these stressors, potentially leading to increased tree longevity and enhanced ecosystem services in urban areas.

12 Future research

Several enduring challenges persist in utilizing genomics and other-omics approaches to enhance our understanding of the biology and ecology of *C. geophilum*, including its evolutionary history, developmental processes, functional aspects, and its resilience to environmental stress. We have identified several critical questions that require further investigation:

1 What molecular mechanisms underlie the genetic diversity of *C. geophilum*, and how does this genetic polymorphism facilitate its worldwide distribution?

2 What are the transcriptional regulators and gene networks that drive the resilience of *C. geophilum* to extreme environmental conditions including drought stress, heavy metal contamination, and high salinity?

3 What role do epigenetic modifications play in the ability of *C. geophilum* to adapt to various environments?

4 What is the significance of horizontal gene transfer (if any) in the evolutionary trajectory of *C. geophilum*?

5 How does the mutualistic association between *C. geophilum* and its plant partners fluctuate across various environmental settings?

6 What patterns have emerged in the *C. geophilum* population genomics across different geographical regions? How will climate change alter symbiont distribution worldwide?

7 How do the secondary metabolites produced by *C. geophilum*, such as melanin, influence its interactions with soil microbial communities including soil and litter decomposers?

The role of *C. geophilum* in ecosystem resilience is becoming increasingly important in the context of climate change. As extreme weather events and environmental stressors become more frequent, the capacity of this fungus and other mycorrhizal fungi to support plant growth and survival under adverse conditions may be crucial for maintaining ecosystem stability and biodiversity. Furthermore, their potential to enhance carbon sequestration through increased plant growth and soil organic matter accumulation may contribute to climate-change mitigation.

Author contributions

HW: Writing – original draft, Writing – review & editing. AK: Writing – original draft, Writing – review & editing. FM: Writing – original draft, Writing – review & editing.

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Conflict of interest

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Chapter 2

Gene expression changes in a drought-tolerant *Cenococcum geophilum* strain during osmotic stress and recovery periods (in preparation)

Gene expression changes in a drought-tolerant *Cenococcum geophilum* strain during osmotic stress and recovery periods

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Abstract

Background Drought stress presents a significant challenge to forest ecosystems in the context of climate change. The ectomycorrhizal fungus *Cenococcum geophilum* was frequently observed in forest soils experiencing drought stress, demonstrating enhanced resilience to drought (osmotic stress) relative to other ectomycorrhizal fungi, which could facilitate host growth under adverse conditions. Nevertheless, the molecular mechanisms underlying the adaptation of *C. geophilum* to drought stress remain unclear.

In the present study, we examined the effects of changes in osmotic pressure (utilized as a proxy for drought stress) on the isolate *C. geophilum* 1.093, which was previously identified as drought-tolerant. RNA sequencing was used to identify osmotic stress-related genes through transcriptome analysis of mycelia cultivated on polyethylene glycol (PEG), which was used to increase osmotic pressure. Vegetative mycelia grown in liquid growth medium with or without PEG, were sampled at 6, 24, and 48 h. Subsequently, the remaining mycelia were transferred to growth medium without PEG for 24 and 48 h to assess their recovery ability.

Results PEG treatment significantly altered gene expression as early as six hours after exposure. At each time point, a distinct group of genes exhibited substantial regulation. A common set of differentially expressed transcripts was identified across all time points, encompassing genes encoding membrane transporters and enzymes involved in lipid metabolism. These genes rapidly reverted to constitutive expression levels upon transfer to the PEG-free medium.

Conclusions *C. geophilum* isolate 1.093, selected for its tolerance to osmotic stress, exhibited significant alterations in gene expression in response to changes in osmotic pressure, a proxy for drought stress. Furthermore, this fungus demonstrated its capacity to rapidly regained viability upon transitioning back to favorable conditions.

Keywords *Cenococcum geophilum*; drought; ectomycorrhiza; transcriptome

Background

Drylands account for approximately 40% of total land area [1]. Recently, global warming has led to an increasing probability of extreme climatic events, such as drought, which threaten the stability of forest ecosystems [2,3]. It also contributes to the expansion of global drought-sensitive areas, resulting in forest decline [4]. Furthermore, drought alters the root morphological traits and increases root mortality. A global study of root traits associated with drought demonstrated that drought significantly decreased root length and root length density [5]. These alterations may result in a greater difficulty in nutrient absorption and transport into plants under higher drought intensities, leading to increased mortality. In response to drought, trees modify their underground C allocation and potentially increase their root exudation types and rates [6,7]. This likely affects the shoot growth and tree health. Drought is an increasingly critical issue in forest ecosystems.

Ectomycorrhizal fungi establish symbiotic relationships with forest plants and facilitate water acquisition through mycelial networks by expanding the soil contact area [8]. The ascomycetous fungus *Cenococcum geophilum* forms ectomycorrhizas with various woody plants and is frequently observed in extreme environmental conditions [9,10]. Herzog et al. [11] investigated the symbiotic relationships between *C. geophilum* and three oak species in response to drought and/or heat treatment. This study demonstrated that both organisms exhibited drought and heat tolerance. Coleman et al. [12] examined the drought resilience of ectomycorrhizal fungi, revealing that certain *C. geophilum* isolates can grow under -3 MPa and exhibit greater drought resilience compared to other ectomycorrhizal fungi. Although *C. geophilum* is classified as a short-distance exploring-type ectomycorrhizal fungus, long- or middle-distance exploring-type ectomycorrhizal fungi are considered to support their host in obtaining more water, the reduction of carbon from the host under drought conditions also limits

their growth [13]. The melanized mantle of ectomycorrhizal roots established by *C. geophilum* may also protect root tips of host plants from desiccation [14, 15]. Furthermore, *C. geophilum* accumulates high amounts of mannitol as a carbon source [16], a polyol that protects fungi and plants from drought stress [17, 18].

Recent research has demonstrated that several *C. geophilum* isolates not only exhibit robust growth but also enhance host growth under soil drought conditions [19,20]. These studies revealed that different *C. geophilum* isolates employ diverse drought resistance strategies. For instance, sensitive isolates absorb Na^+ or K^+ ions and upregulate peroxisome pathway genes to maintain water potential. Conversely, tolerant isolates upregulated genes encoding the terpenoid-quinone biosynthesis and sphingolipid metabolism pathways. Furthermore, studies have indicated that inoculation with *C. geophilum* isolates at an early stage can enhance the drought resistance of *Pinus massoniana* seedlings [20]. However, under laboratory conditions, the drought resilience of symbiotic associations is not tightly correlated with the drought tolerance of fungal isolates [20]. Nevertheless, in a greenhouse experiment utilizing *C. geophilum* isolate 1.58, it was demonstrated that inoculation with this fungus could facilitate host water acquisition by upregulating genes encoding aquaporins in pine seedlings [21]. Although the resilience of symbiotic plant-fungus associations and drought tolerance of fungal isolates do not always appear to be related, *C. geophilum* isolates likely adapted to osmotic stress may possess a higher probability than other *C. geophilum* isolates or ectomycorrhizal fungi that survive in extreme drought environments. Consequently, it is worth assessing whether the presence of *C. geophilum* contributes to the survival of tree seedlings the experience soil drought stress.

Although previous studies have explored *C. geophilum* drought tolerance mechanisms at the transcriptome level [20], the ability to rapidly recover from drought stress is another crucial aspect of stress management. PEG is commonly employed to simulate drought stress through changes in osmotic pressure of the growth medium. Although drought stress involves various factors, such as changes in soil physical properties that PEG cannot replicate, osmotic stress remains the primary focus when testing drought tolerance in ECM fungi. For instance, Coleman et al. [12] used PEG3350 to examine the drought tolerance of ECM fungi. Similarly, Zhang et al. [22] employed PEG6000 to assess drought tolerance in three ECM fungi and showed that

mild drought stress (-0.15MPa) enhanced their growth. Therefore, in this study, we used PEG to mimic osmotic stress in drought soils.

We identified a *C. geophilum* strain characterized by rapid growth and tolerance to high osmotic pressure (i.e., high PEG levels). Our subsequent objectives were to investigate: (1) how quickly the isolated gene expression changes when exposed to PEG-induced drought stress; (2) which specific pathways and functions are regulated in samples treated with PEG compared to untreated controls; and (3) the nature and speed of regulation of differentially expressed genes following a shift from PEG-containing to PEG-free conditions.

Methods

Experiment 1: Fungal strains, Culturing condition and Mycelium growth of Representative Isolates under Drought Treatments

Four isolates (Table 1) with different reaction patterns to drought stress were selected from previous studies (not published). *C. geophilum* strain 1.58 has its whole genome sequenced (https://mycocosm.jgi.doe.gov/Cenge3/Cenge3.home.html). The strain 1.58 and 1.278 were provided by Dr. Martina Peter (WSL, Zürich), while isolates BAARN and 1.093 were obtained from the INRAE IAM fungal collection (Champenoux, France). The internal transcribed spacer (ITS) regions of all isolates were sequenced and identified as *Cenococcum geophilum* by using the pairwise primers ITS1f and ITS4 [23].

Table 1. Information on the four isolates used in this study

Name	Sampling site	Host	Water potential levels	Strains isolated from sclerotia/root tips
1.278	Bärschwil tief, Switzerland	*Fagus sylvatica*	Wet	Sclerotia
1.58	Valais, Switzerland	*Pinus sylvestris*	Drought	Sclerotia
BAARN	Netherlands	*Picea abies*	Unknown	Unknown
1.093 (Td4-4)	Tendon, France	*Fagus sylvatica*	Drought	Sclerotia

All isolates were maintained in Cg medium [24] with 20 g/L glucose at 20 ◦C in the dark. The solid medium contained 12 g/L agarose. The four isolates were pre-cultured on solid medium on a cellophane piece (diameter = 2 cm) for one month. Sterilized pipet tips (r = 1 cm) were used to cut out sample plugs for use as inocula for preculturing. After preculturing, plugs were removed from the cellophane. Because the high concentration of PEG hindered the solidification of the medium, we transferred the mycelium with cellophane to a liquid medium bead system [25] (Figure 1) to test their growth under drought stress.

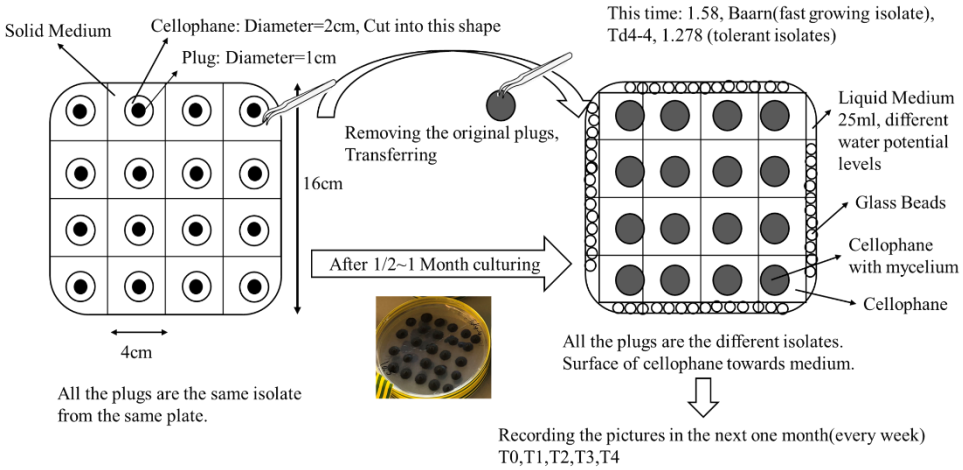


Fig. 1. Flowchart for mycelium growth test showing the pre-culturing of the isolates and the bead system.

Times points T0, T1, T2, T3, T4: week0, week1, week2, week3, week4. The isolates names: 1.58, Baarn, Td4-4, 1.278 means the *C. geophilum* isolates: 1.058, BAARN, 1.093, 1.278.

The flow chart shows the pre-culturing and testing systems used in this study. The plugs of fungi were precultured on cellophane pieces in a solid medium for half a month to one month. The plug was then removed and the remaining mycelium with a cellophane piece was transferred to the bead system. The bead system consisted of beads, liquid medium with PEG, a slide of square cellophane, and pieces of round cellophane containing *C. geophilum* strains.

Standard curve correlating PEG-8000 concentrations and water potentials of the medium

PEG 8000 (Sigma, France), a proxy for soil drought conditions, was used to change the osmotic pressure of the medium. To determine the relationship between PEG8000 concentration and medium water potential, liquid Cg medium with increasing PEG8000 concentrations (Figure 2) was measured using a Wescor vapor pressure osmometer 5500 W (WARRANTY, California, US) and Wescor (PSYPRO Water Potential System, USA) (Figure 2)

PEG8000(g/L)	Water potential (- MPa)
110	0.815
130	1.06875
150	1.305
230	1.9075
250	2.1625
304	4.41
334	4.55
362	5.03
388	7.17
410	8.06

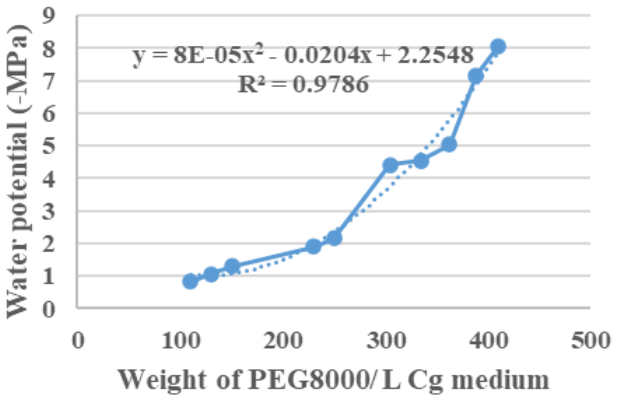


Fig. 2. Relationships and standard curve between PEG concentration in the growth medium and water potential. Cg medium: *C. geophilum* medium.

The equation linking the water potential to the medium PEG concentration is

$y = 8E\text{-}05x^2 - 0.0204x + 2.2548$

where y is the water potential (MPa) multiply by-1, x is the weight of PEG8000 in 1 L of Cg liquid medium.

Experimental Design and Mycelium growth

After the preculturing on no-PEG solid medium, the small round cellophane with mycelium were transferred to 4 different water potential levels: -0.6MPa (0 g/L), -2MPa (242 g/L), -5MPa (362 g/L), -8MPa (410 g/L). Three replicates were tested for each isolate for each condition. The growth of each isolate was recorded using a NIKON D5200, and pictures were taken from the same position once per week for four weeks.

The images were analyzed using the R package EBImage [26] as previously described [27]. Relative area was measured by counting the pixels of each colony. Data were analyzed using the R package ggplot2 [28]. Each point represents the actual number of pixels in the image.

Experiment 2: Culture conditions, experimental design, RNA extraction, RNA-Sequencing and data analysis

Culture conditions and experimental design

The drought-tolerant isolate 1.093 was selected for this experiment. The mycelium was cultivated in a 1 L Reagent Simax bottle containing 300 ml liquid Cg medium [24]. The bottle was then placed on a shaker for one month at 20 °C in the dark. Following preculture, the mycelium (300 mg/tube) was filtered using a filter (250 mL Sartorius Stedim polycarbonate filter), plastic nets, and pump (N820.3 FTP, KNF LABOPORT, Freiburg im Breisgau, Germany) and subsequently transferred to fresh Cg medium in 50 ml Falcon tubes (30 ml/tubes) for 2 days on a shaker. Fresh mycelia were used as the starting materials.

We decided to incubate the mycelium in 210 g/L PEG8000 in Cg medium (corresponding to -1.5MPa), a value close to the -1.2 MPa reported for low soil water potential in beech forests [29]. Each tube contained 300 mg mycelia and 30 ml liquid medium (Quintix Pro Precision Balance QTX120 21RO-1CFR, Göttingen, Germany). The PEG concentration was calculated from the initial experiment (Fig.2). The samples were subsequently filtered and washed thrice with liquid non-PEG medium after 6, 24, and 48 h. Subsequently, 12 tubes of mycelia were transferred back to the no PEG medium and sampled after 24 h and 48 h as recovery samples. Each treatment was performed in independent triplicate (Falcon tubes). (Figure 3). Following sampling, each replicate was divided into three aliquots, frozen in liquid nitrogen, and stored at − 80°C prior to RNA extraction.

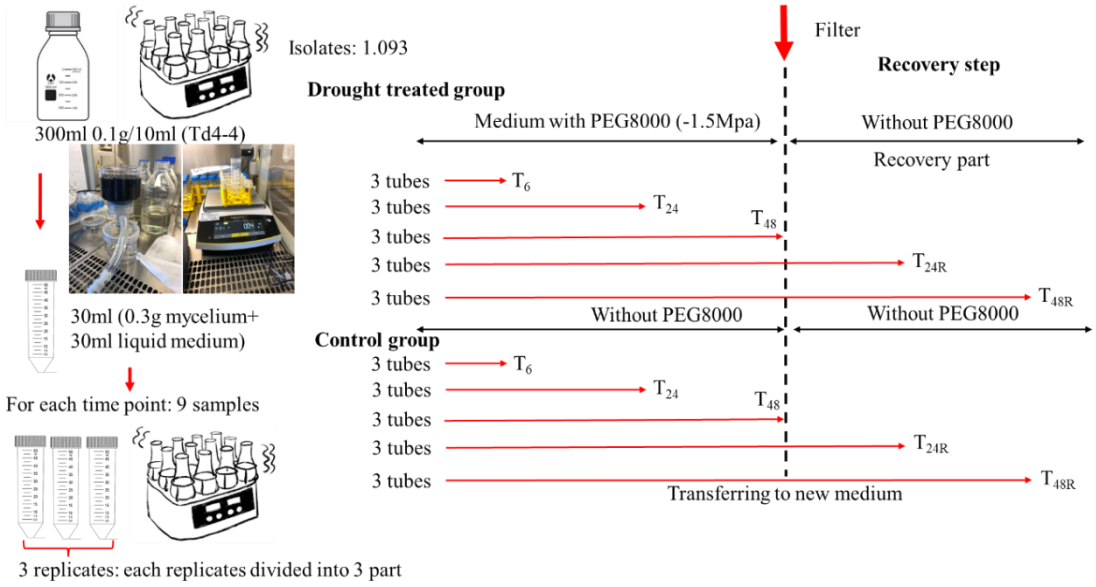


Fig. 3. Experimental design to study the effect of PEG treatment on the transcriptome of *C. geophilum*.

Workfow of PEG treatment of *C. geophilum* mycelium and subsequent transfer to no-PEG medium in order to study the capacity to recover from the treatment. 1.0. 1.093 *C.geophilum* isolate used in the experiment. R recovery; 0, 0h; 24, 24 h and 48, 48 h.

RNA extraction and RNA-Sequencing

Following sample collection, an aliquot from each of the 30 samples was homogenized into powder using steel beads (cooled on liquid N blocks) and a shaker (pre-cooled in -80 °C freezer, Retsch GmbH MM400, Haan, Germany). Total RNA was extracted using RNeasy Plant Mini Kit (100) (QIAGEN, Hilden, Germany) and RLT lysis buffer. After DNase treatment (TURBO DNA-free Kit; Thermo Fisher Scientific, France), RNA quality and quantity were assessed using a TapeStation system (Agilent, Santa Clara, California, US). RNA was sequenced using a DNBSEQ-T7 sequencer and the DNBSEQ Eukaryotic Strand-specific Transcriptome Sequencing kit (paired-end 150bp, 20 million clean reads per sample) by BGI Tech Solutions (Poland), according to the manufacturer's protocols.

RNA-Sequencing analysis

Raw reads were trimmed for low quality (quality score, 0.05) and aligned to the *C. geophilum* reference genome available in the JGI MycoCosm database (Cenge3,

https://mycocosm.jgi.doe.gov/Cenge3/Cenge3.home.html) using CLC Genomics Workbench v23.05 (Qiagen). For read mapping, the CLC genomic workbench parameters were as follows: minimum length fraction, 0.9; minimum similarity fraction, 0.8; mismatch cost = 2; insertion cost = 3; deletion cost = 3 and the maximum number of hits for a read was set to 10. The unique and total mapped read numbers for each transcript were determined and normalized to RPKM and TPM. Unique mapped reads were utilized as entry files for the identification of differentially expressed genes (DEGs) using DESeq2 [30]. DEGs were defined as $|\log2Foldchange| \geq 1$ and $p < 0.05$. The complete RNA-Seq data were submitted to NCBI GEO as GEO series xxxxxxx

Bioinformatic analyses

An upset plot graph was generated using the R package upsetplot [31] and ggplot2. Principal Component Analysis (PCA), heatmaps, volcano plot, Gene Ontology Consortium (GO) enrichment graph, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment graph, and EuKaryotic Orthologous Groups (KOG) enrichment graphs were generated using the ggfprtify package [32], Complexheatmap package [33], scRNAtoolVis package (Version 0.1.0), TopGo package [34], and Clusterprofiler package [35]. Gene function and annotation files of the reference genome *C. geophilum* 1.58 (GO, KOG, KEGG, and transporters) were downloaded from the JGI website or directly received from JGI. The polyol metabolism pathway graph was assembled from JGI Mycocosm metabolism pathway graphs. Missing proteins were identified by BlastP search against the Mycocosm Cenococcum genome using *Aspergillus fumigatus* protein.

Weighted co-expression network analysis (WGCNA) was performed using R package WGCNA [36]. After filtering for significantly regulated genes (p-value < 0.05, Fold change > $\log_2 1$) and removing of low expressed genes (mean unique reads of all conditions< 5 reads), gene expression values were imported into WGCNA to construct co-expression modules using the automatic network construction function blockwiseModules with default settings, except that the power was set to 14 (Supplementary Figure 1), TOMType was unsigned, mergeCutHeight was 0.25, and minModuleSize was 100. Pearson's correlation analysis (selecting most related timepoints and gene modules by highest Pearson's correlation coefficients comparing with others) was used to identify modules significantly associated with different time

points and drought stress treatments. Finally, the module network was mapped and visualized using Cytoscape 3.10.2.

Results

PEG treatment affects mycelium growth of the four strains differentially

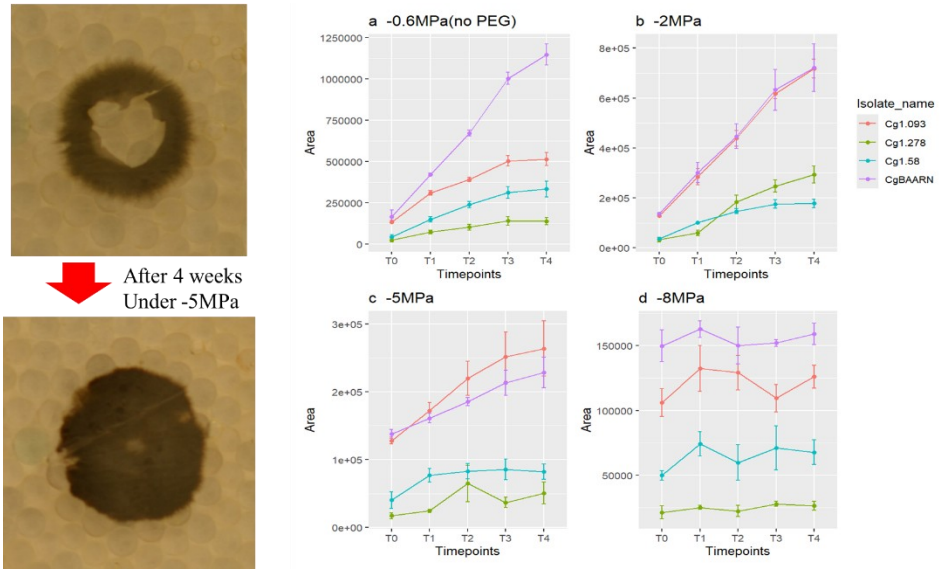


Fig. 4. Growth of *C. geophilum* isolates under different water potentials.
T0, T1, T2, T3, T4: week0, week1, week2, week3, week4. Cg: *C. geophilum*. a. showing BAARN grows faster than 1.278 under -0.6 MPa; b. showing BAARN has the same growth speed as 1.278 under -2 MPa; c. showing 1.278 grows faster than BAARN under-5 MPa.

Strains 1.278 and 1.58, respectively, exhibited a slow growth rate, whereas BAARN and 1.093 demonstrated rapid growth in the absence of PEG. Figure 4 illustrates that isolate BAARN exhibited a higher growth rate than isolate 1.093 in the absence of PEG, whereas isolate 1.093 displayed a higher growth rate than BAARN under -5 MPa conditions. Furthermore, at -5 MPa, strain 1.093 exhibited the highest growth rate among all isolates. Because of its rapid growth and high PEG tolerance, this isolate (1.093) was selected for subsequent experiments.

Osmotic stress induced by PEG induced changes in gene expression in *C. geophilum* 1.093 mycelia after 6, 24, and 48 h.

The transfer of *C. geophilum* 1.093 mycelium from Cg control medium to PEG8000 supplemented medium to simulate drought stress (primarily osmotic stress) elicited significant gene expression changes as early as 6h after PEG treatment. Principal component analysis (Figure 5) of the overall gene expression in all samples differentiated on the first axis of the PEG-treated samples at 6h, 24h and 48h (T6S, T24S, T48S) from all control samples (T0, T6C, T24C, T48C). The most substantial changes, with 643 (4.4%) upregulated and 394 (2.7%) downregulated transcripts, occurred as early as 6h after PEG treatment (Figure 5). Of the differentially expressed genes (DEGs) at 6h, only approximately one-third were also significantly regulated after 24h and 48h of PEG treatment, while the majority of the 24h and 48h DEGs were also regulated at a minimum of one of the other two time points (Figure 5).

The ten most highly upregulated DEGs at 6h include stress-related transcripts, such as glutathione S-transferase (Cenge3_673422) and heat shock protein (Cenge3_285764). At 24h and 48h, genes encoding a stress-related RTA1-like protein (Cenge3_629648), MFS transporter (Cenge3_531099), carbonic anhydrase (Cenge3_611738), and oxidoreductase activity (Cenge3_586501, Cenge3_560674) were among the most induced genes. Many of the most highly upregulated transcripts were induced at all three time points following PEG treatment (Figure 5).

At 6h after PEG treatment the DEGs included many genes known to be involved in cellular osmoregulation and homeostasis. Induced are genes coding for the *C. geophilum* homolog of the yeast HOG1 stress activated protein kinase, part of the high osmolarity glycerol (HOG) pathway (Cenge3_725216), glycerol-3-phosphate dehydrogenase (Cenge3_ 656861) involved in glycerol synthesis as well as genes linked to the sorbitol pathway (Supplementary Table 1, Supplementary Figure 2).

GO enrichment analysis (Supplementary Table 2) of the biological processes of the upregulated DEGs identified "transport" as the most significant function at all three time points. Furthermore, the GO category "response to toxic substances" is enriched at 6h, "fatty acid biosynthetic process" at 24h, and "N-acetylglucosamine metabolic process" at 48h after PEG-treatment (Supplementary Table 2).

GO enrichment analysis of the downregulated DEGs highlighted DNA-related categories (DNA replication, DNA recombination, DNA damage response, NTP biosynthesis, meiosis, and chromosome condensation) and translation (Supplementary

Table 2).

At all three time points, the upregulated transcripts enriched in KOG terms were secondary metabolite biosynthesis (KOG0023/1399), cell wall/membrane/envelope biogenesis (KOG4177), energy production (KOG1198/1575) and defense mechanisms (KOG1502, Supplementary Figure 3). In addition, the KEGG terms “oxidoreductases”, “alcoholdehydrogenases” and “with NAD+ or NADP+ as acceptor” were identified as enriched for the up-regulated transcripts in PEG-treated samples (Supplementary Figure 4).

Approximately 10% of the DEGs at all three time points coded for putative transporters, with 13% of transporter genes being induced 6h after PEG treatment (Supplementary Figure 5, Supplementary Figure 6). At 6h, 32 of the DEGs coded for MFS transporters, including certain osmotic stress response-related transporters such as glycerol-, sugar/inositol-, or sugar/polyol-transporters. Many of these genes were up-regulated after 24 and 48h. Genes encoding several Amino Acid-Polyamine-Organocation (APC) family transporters, including GABA-specific permeases, have been shown to be induced by hyperosmotic stress. Furthermore, several ABC transporters, Voltage-gated K+ Channels, auxin-related transporters, Pore-forming NADPH-dependent 1-Acyldihydroxyacetone Phosphate Reductase (Ayr1) family transporters, sulfate transporters, and aquaporins were induced 6h after PEG treatment.

Approximately 25% of the down-regulated DEGs at 6h are associated with the KOG group “Information storage and processing” and in particular the KOG classes "Chromatin structure and dynamics," "Replication, recombination and repair," "RNA processing and modification," "Transcription" as well as "Translation, ribosomal structure and biogenesis" (Supplementary Figure 6), all DNA and translation related categories previously identified by the GO enrichment analysis.

C. geophilum mycelium is highly melanized and its genome encodes multiple pathways for the synthesis of this black pigment. Supplementary Table 2 shows that all three DHN-melanin, DOPA-melanin, and L-tyrosine degradation pathways were downregulated after 6h of PEG treatment. At 48h after PEG-treatment, the gene expression of the enzymes involved in these pathways was comparable to the control and even slightly induced in the DHN-melanin pathway, potentially indicating that *C. geophilum* mycelium has adapted to PEG-induced osmotic stress and initiates growth

and secondary metabolite production again.

Up to 50% of the DEGs at the three time points were of unknown function, raising the question of whether the regulated genes after PEG treatment are conserved among fungi or whether some of them are *C. geophilum*-specific. Supplementary Figure 7 shows that the majority of DEGs at 6h and 48h share high homology with genes in the genomes of the selected fungi, indicating that they are not *C.geophilum* specific. Only appeoximately 11% of the 6h up-regulated transcripts (73/643) and 17% of the downregulated transcripts (66/394) showed no similarity with genes in the genomes of the selected fungi, with the majority having unknown functions.

C. geophilum **1.093 mycelium recovered rapidly from the PEG treatment after transfer to new medium.**

An important feature of drought tolerance is the ability to recover rapidly after cessation of stress and to resume normal metabolism and growth. At 24 h after transferring *C. geophilum* 1.093 mycelium from PEG-supplemented medium back into Cg control medium, only 52 DEGs were identified, whereas 327 transcripts were upregulated and 214 were downregulated after 48 h of recovery (Figure 5). PCA showed that the 24-hour and 48-hour recovery samples clustered with all other control samples without PEG treatment, indicating an overall similar gene expression profile (Figure 5A).

Figure 6A presents a simplified heatmap comprising 1833 DEGs regulated in at least one of the PEG treatment time points and during the recovery time points. Upregulated DEGs compared to the respective controls were assigned a value of 1, downregulated DEGs a value of -1, and DEGs without significant regulation a value of 0. The heatmap (Figure 6 A) shows that the majority of PEG-stress-regulated DEGs were no longer differentially regulated after 24 and 48 h of recovery in fresh medium without PEG when compared to the mycelium control that was not PEG-treated previously. Only a small subset of DEGs at 24 and 48 h after recovery were also PEG stress-regulated and remained up- or downregulated after transfer to the new medium (Figure 6A).

Figure 6B in Figure 6 depicts the relative gene expression values for the DEGs at 24 h and 48 h after transfer to new medium for both the samples that had been previously treated with PEG (SR) and the control samples without PEG (CR). Notably, the

transcripts with higher and lower gene expression at 48 h recovery compared to the respective controls exhibited similar gene expression at 24 h for both recovery and control samples, suggesting that the samples that had been previously PEG-treated previously maintained gene expression in an activated or repressed state for a longer duration compared to the control samples. GO enrichment analysis identified "protein phosphorylation", "regulation of DNA-templated transcription", and "signal transduction" for the upregulated transcripts as putative functions, indicating an overall resumption of cellular activities and potentially enhanced growth of the recovering mycelium compared to the control.

Gene co-expression network analysis

WGCNA was conducted using 1833 DEGs regulated in at least one of the treatments and time points. Eight modules were identified (Figure 7, upper panel). Transcripts associated with PEG treatment at 6h, 24h, and 48h clustered in the turquoise, blue, and red modules. The pink and brown modules comprised DEGs related to the 24h and 48h recovery from PEG treatment, as well as DEGs associated with the 24h control after transfer to new medium. The remaining control samples were linked to the green module (6h control), in addition to the black and yellow modules (24h control, 48h control, and 48h recovery control). WGCNA figure B illustrates the overall relationship between the modules in a network, closely associating the regulated DEGs from PEG-treated samples at 6, 24, and 48h (turquoise, blue, and red). The heatmap (WGCNA, lower right) emphasizes the co-regulation of transcripts within these modules. Regulation was primarily observed after 6h for the turquoise module, at 6h and 24h for the blue module, and at all three time points for the red module. GO enrichment analysis (Supplementary Table 3) of the biological processes identified "transport," "arginine catabolic process," and notably "carbohydrate transport" and "sulfate transport" for the turquoise module (6h), "catechol-containing compound metabolic process," DNA methylation, transport, glyoxylate cycle, and coenzyme A metabolic process for the blue module (6 and 24h), as well as fatty acid biosynthetic process, glycerol-3-phosphate metabolic/catabolic process, N-acetylglucosamine metabolic process, transport, gluconeogenesis, and polyamine biosynthetic process for the red module (all three timepoints).

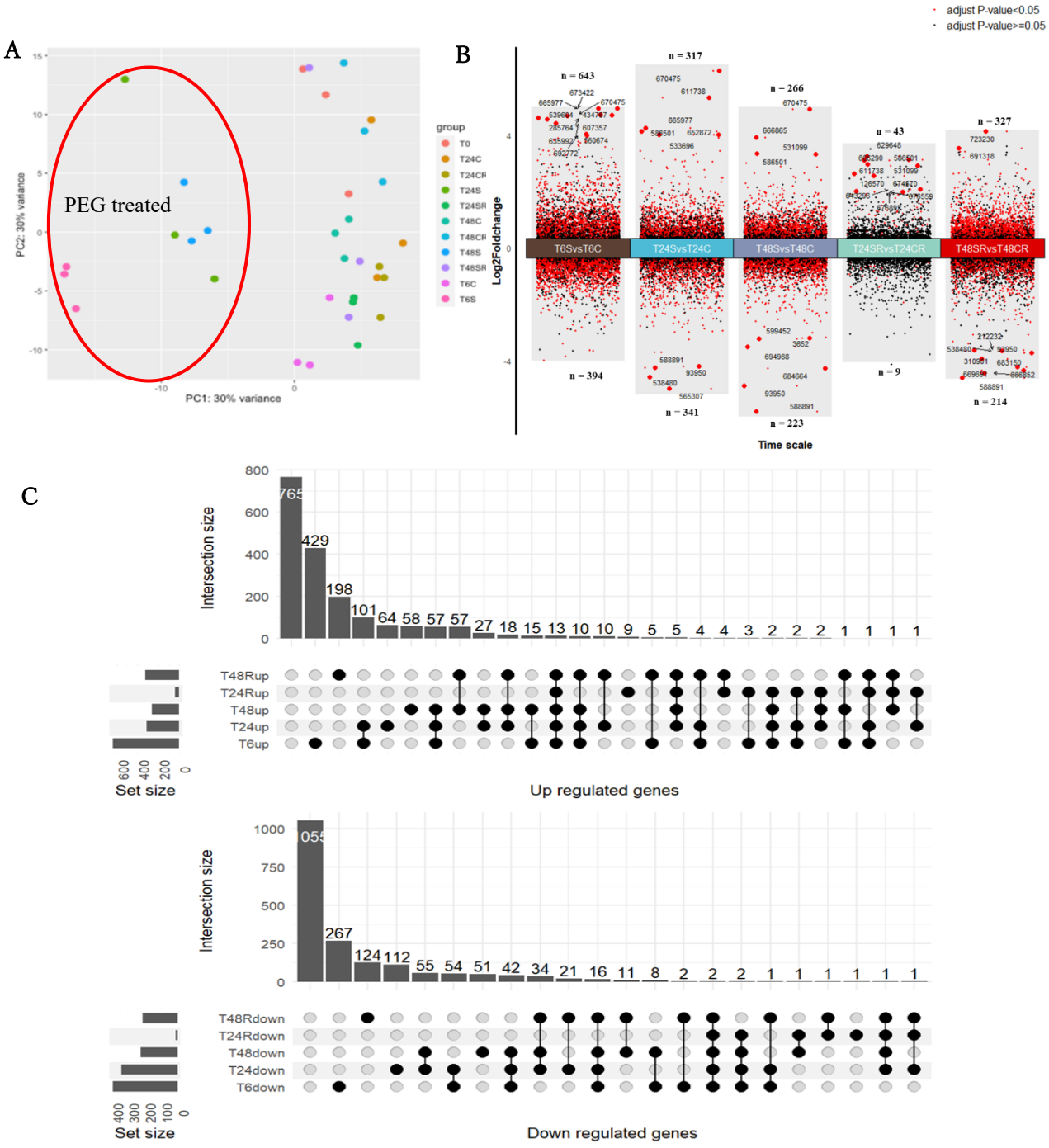


Fig. 5 The overall features of transcriptome data at different time points and drought treatments

A. The PCA plot shows the overall expression differences between PEG-treated samples and non-treated control samples. Different colors indicate different time-points and treatments. The PEG-treated samples are highlighted by a red circle. B. The volcano plot shows up- and downregulated genes, and the total number of regulated genes for each group is shown above each bar (n = x); only the top ten regulated genes are displayed by their Cenge3 protein ID. C. Upset plot showing shared DEGs between timepoints and treatments.

C, controls; S, PEG-treated; R recovery; 0, 0h; 24, 24 h and 48, 48 h. Set size, gene amount in this group; Intersection size, gene amount of different timepoint combinations, for example, there are 1055 genes not up-regulated among all timepoints.

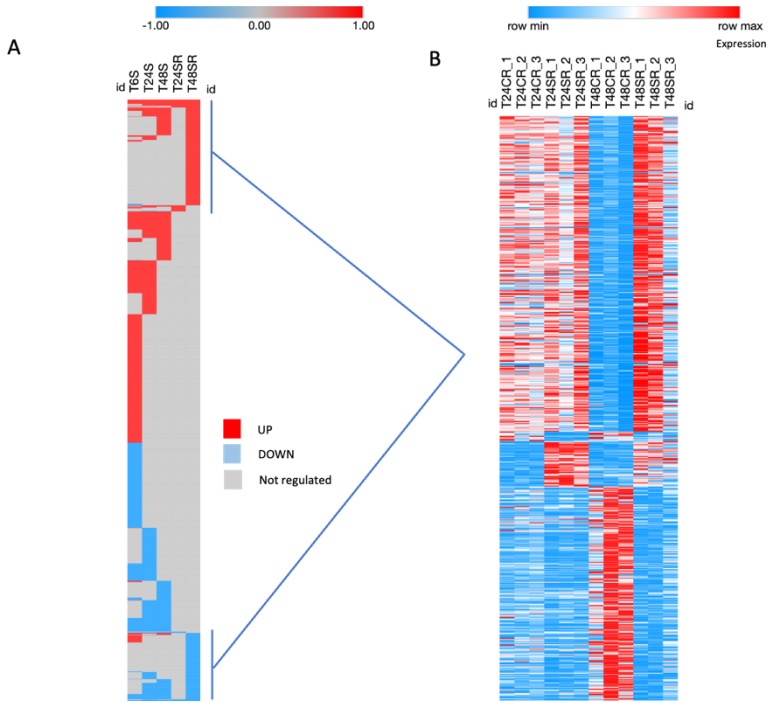


Fig. 6 Gene expression changes in the recovery period

A Simplified heatmap of transcripts regulated in minimum one of the treatments (T6S, T24S, T48S, PEG treatment 6h, 24h, 48h compared to the respective control; T24SR, T48SR, recovery period 24h, 48h compared to the respective control). UP significantly up-regulated, DOWN significantly down-regulated (Log2 Fold change>1, FDR p-value<0.05). Transcripts are ordered by their regulation during recovery.

B Heatmap showing relative gene expression in a subset of transcripts significantly regulated after 24h or 48h of recovery from PEG treatment. T24, T48: 24h or 48h after transfer to fresh medium; CR Control; SR transfer from PEG medium to control medium; 1-3, three independent biological replicates.

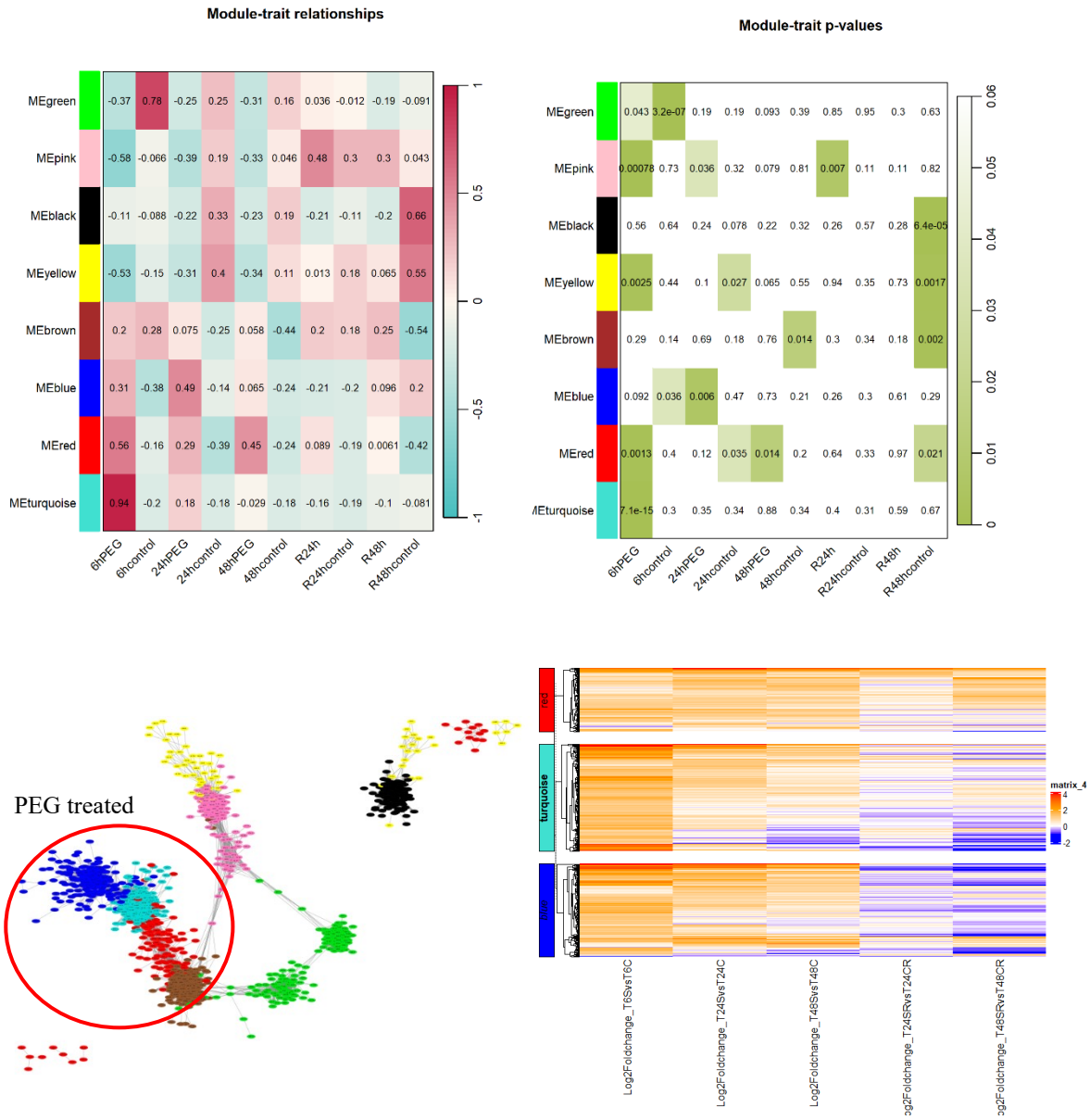


Fig. 7 Results of the Weighted Gene Co-expression Network Analysis (WGCNA).
The green blocks show the *p*-values of highly related color modules and traits (top left panel). The colored full blocks show the significance between the color modules and traits (top right panel). The PEG-treated groups (T6, T24, and T48) were highly related to the red, blue, and turquoise modules, respectively. These modules were grouped together (lower left panel). Data were obtained using WGCNA and visualized using Cytoscape). Heatmap of normalized reads of the three-color modules.

Discussion

Research has demonstrated that *C. geophilum* exhibits resilience to drought conditions and is frequently observed in soil environments characterized by limited water availability [38]. Multiple investigations examining fungal communities in soil have revealed that the abundance of *C. geophilum* ECM and its external mycelial network increases under water-stressed conditions, often attaining higher levels during summer in natural settings [37,38]. Drought tolerance has been further corroborated through laboratory experiments using media with adjusted osmotic properties [39,12], assessments of cellular damage following dehydration [40], and measurements of respiration under water-limited conditions [14]. The degree of tolerance varies among isolates from diverse geographical locations [12,14]. However, these findings, the underlying molecular mechanisms that enable this species to thrive under water stress remain largely unknown. It is hypothesized that multiple factors contribute to this characteristic, including the accumulation of compatible osmolytes (such as polyols), heat shock proteins, hydrophobic proteins, and melanin pigment in cell walls.

Although previous studies have investigated *C. geophilum* tolerance to osmotic stress at the transcriptome level [20], in this study the capacity for rapid recovery from drought stress was evaluated, by measuring growth speeds of each isolate in each water potential levels, which constitutes another critical component of stress management. Polyethylene glycol (PEG) was used in this study to alter the osmotic pressure of growth medium, which is frequently employed as a proxy to simulate drought stress. While drought stress encompasses multiple factors, including changes in soil physical properties that PEG cannot replicate, osmotic stress remains the primary focus when evaluating drought tolerance in ectomycorrhizal (ECM) fungi [12,22], demonstrating that mild drought stress (-0.15MPa) can stimulate their growth.

This study was initiated by identifying a *C. geophilum* strain known for its rapid proliferation and resilience to increased osmotic pressure, particularly in response to elevated PEG concentrations. The study's primary objectives were to examine: (1) the swiftness of gene expression modifications in the isolate when exposed to PEG-induced drought stress; (2) the particular metabolic pathways and cellular functions that are regulated in PEG-treated mycelia compared to their untreated counterparts; and (3) the

characteristics and dynamics of gene regulation for differentially expressed genes upon transitioning from PEG-containing to PEG-free environments.

Our selection of a drought-tolerant *C. geophilum* strain involved both fast- and slow-growing isolates. Isolates from both drought-prone and wet environments were selected to compare their drought tolerance, revealing that even isolates from wetter sites exhibited potential for drought tolerance. In line with Coleman et al. [12], we observed that some isolates performed best in the control medium without PEG, whereas others thrived under water potentials of -2 to -3 MPa. In this study, BAARN belonged to the first group, whereas 1.093 and 1.278 were part of the second group, with 1.58, showing slow growth regardless of water availability. We confirmed the growth differences between the fast- and slow-growing isolates.

Morel et al. [42] delineated intracellular detoxification processes in fungi, encompassing intracellular attack, conjugation, excretion, and storage, including for example, cytochrome P450 and glutathione transferase. Under PEG-induced osmotic stress, numerous genes from isolate 1.093 were upregulated as early as 6 h post-treatment. This included genes encoding cytochrome P450, glutathione transferase (among the most highly regulated transcripts at all three time points), and heavy metal transport/detoxification protein/transporters, corroborating that intracellular detoxification represents an early response to PEG-induced stress and likely constitutes one of the pathways involved in strain tolerance to osmotic stress.

Furthermore, similar to certain model organisms such as *Saccharomyces* and *Aspergillus*, 1.093 responds to osmotic shock through changes in the osmolarity glycerol (HOG) pathway, cell wall remodeling, and osmolyte production [43–46]. Genes involved in glycerol and sorbitol biosynthesis pathways, as well as those related to glycerol, inositol, and polyol transport are also involved in 1.093 response to osmotic stress (Supplementary Figure 2). This response was less pronounced than that observed with mannitol, a well-established carbon storage pond for *C. geophilum* [16], and trehalose, an osmotically active compounds previously reported for other fungi, for which we did not identify induced genes in our experiment.

While Li *et al.* [19] focused on the differences between tolerant and sensitive strains at a single time point, i.e., 24h after PEG treatment, our results revealed distinct gene expression patterns over different time points, suggesting a temporal dynamic in

gene regulation rather than differences between isolates alone. WGCNA clustered PEG-treated samples into three modules (turquoise, blue, and red) with slightly different gene expression patterns. In the turquoise module, genes showed peak of expression at 6h including many transporters and genes involved in oxidoreduction. The blue module contains more genes with high expression levels maintained at 24h in addition to 6h, like as heat shock proteins or cytochrome P450, while in the red module gene up-regulation was high at all three time points, like as for the stress-related RTA1-like protein. The highest number of DEGs compared to the control mycelium was detected as early as 6h after PEG-treatment, indicating the importance of following the response over several timepoints.

Peter et al. [21] identified two aquaporin genes upregulated in ectomycorrhizal root tips and one aquaporin upregulated in mycelium exposed to agar drying as a proxy for drought stress. In our study, one of the ECM-induced aquaporins, Cenge3_ 647346, was significantly upregulated at 6h, while the drought-stress induced aquaporin from Peter et al., Cenge3_ 604158, was not significantly upregulated during stress and downregulated after 48h recovery. This observation suggests that aquaporins are likely less effective in addressing PEG-induced osmotic stress and in regulating water shortages during drought stress.

The aforementioned finding is also applicable to another suggested component of the drought stress response in *C. geophilum*: the accumulation of melanin in cell walls. This accumulation also occurs in sclerotia, which can persist under various stress conditions for extended periods [47, 48]. In our experimental study, all three melanin synthesis pathways [49] were downregulated after 6 h of PEG treatment, suggesting that melanin synthesis is not essential for managing osmotic stress. Notably, 48 h following PEG treatment, the gene expression levels of the enzymes involved in these pathways returned to control levels, potentially indicating that *C. geophilum* mycelium has successfully adapted to PEG-induced osmotic stress and can resume growth and secondary metabolite production.

Another important feature of tolerance is the ability to recover quickly after stress has disappeared. This is what we observed for the 1.093 mycelia after the end of PEG treatment. After 24h of recovery, the overall gene expression was similar to that of the control mycelium, after 48h of recovery, genes related to energy consumption and

protein synthesis were induced compared to the control, indicating a renewed growth of 1,093.

The results of this study indicated that the tolerance of *C. geophilum* 1.093 to PEG-induced osmotic stress was achieved at the transcriptome level through a combination of rapid induction of classical detoxification and osmoregulation pathways (Figure 8), encompassing transport and rapid recovery via the swift return of gene expression to the control level. The majority of genes and pathways utilized by *C. geophilum* for this response are conserved among fungi and are not *C. geophilum*-specific; only 11% of the upregulated transcripts at 6h exhibited no homology with genes in related fungal genomes. The importance and functions of these unique and conserved genes need transformation system to study and prove.

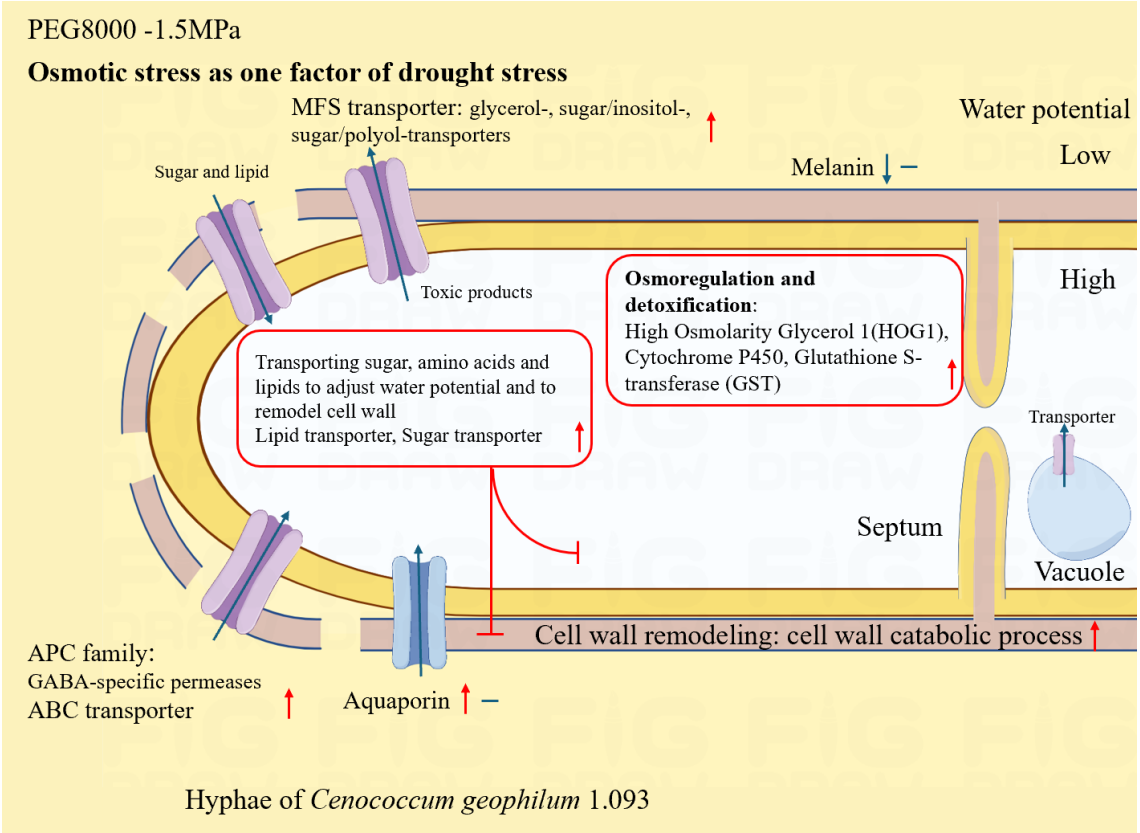


Fig. 8 Response of *C.geophilum* 1.093 to PEG-induced stress

Summary of gene expression changes in hyphae of *C.geophilum* 1.093 in response to osmotic stress. Arrows: up and down regulated gene. Blue line means not significantly regulated. Numbers is the protein ID of each gene. Yellow color phase is the membrane and flesh color phase is the cell wall. (By FigDraw tool (http://www.figdraw.com))

Supplementary Information

The online version contains supplementary material available at

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Author Contributions

HW, FM, and AK designed the experiments, analyzed the data, and wrote the manuscript. EM, BD and MB analyzed the data.

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Data availability

The complete RNA-Seq data were submitted to NCBI GEO as GEO series xxxxxxx

Declarations

Ethics approval and consent to participate

The sampling site is located outside of any protected area and ethical approval is not necessary.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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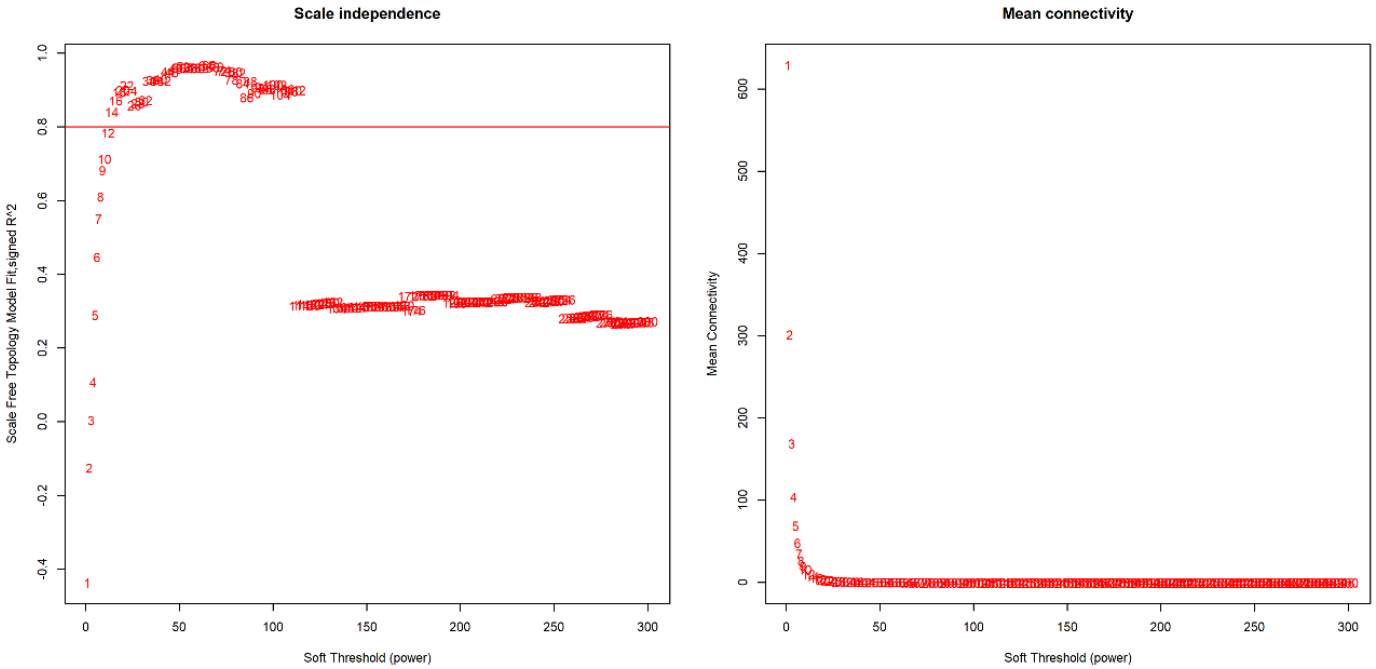
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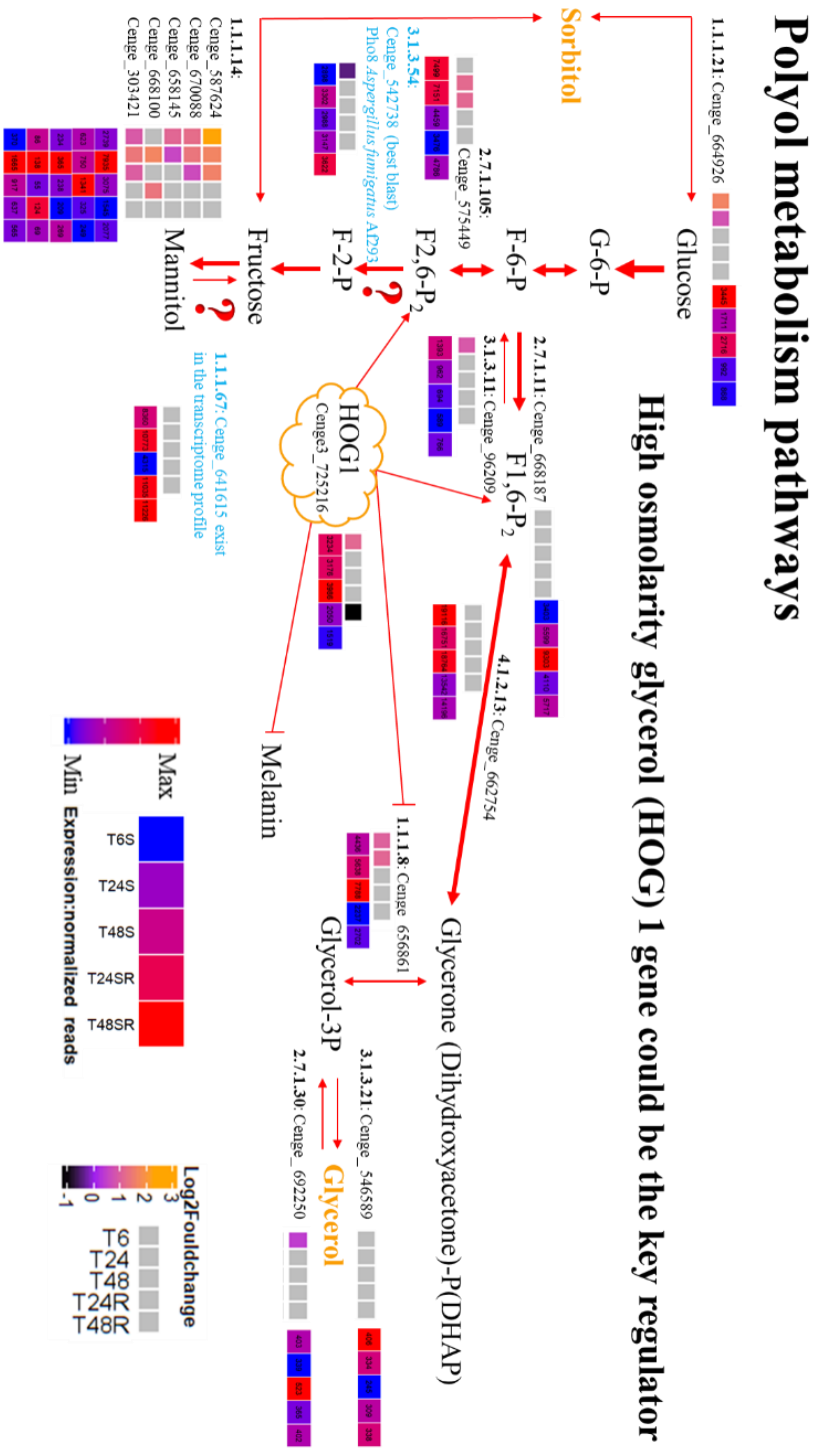
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Supplementary Figures

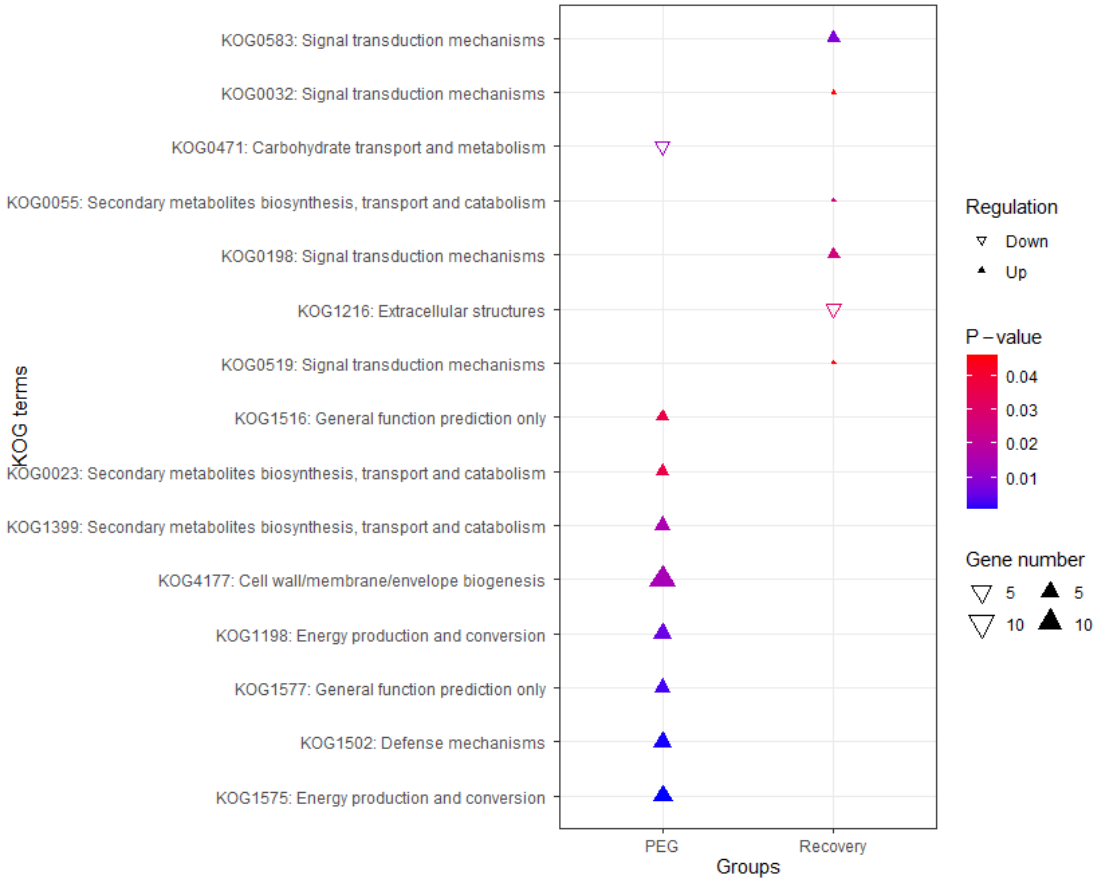


Supplementary Figure 1 Soft Threshold of Weighted co-expression network analysis
The right figure shows different soft threshold power values in a standard scale-free network, with the red line indicating the optimal power value. The left figure shows the power value and scale-free fitting index.



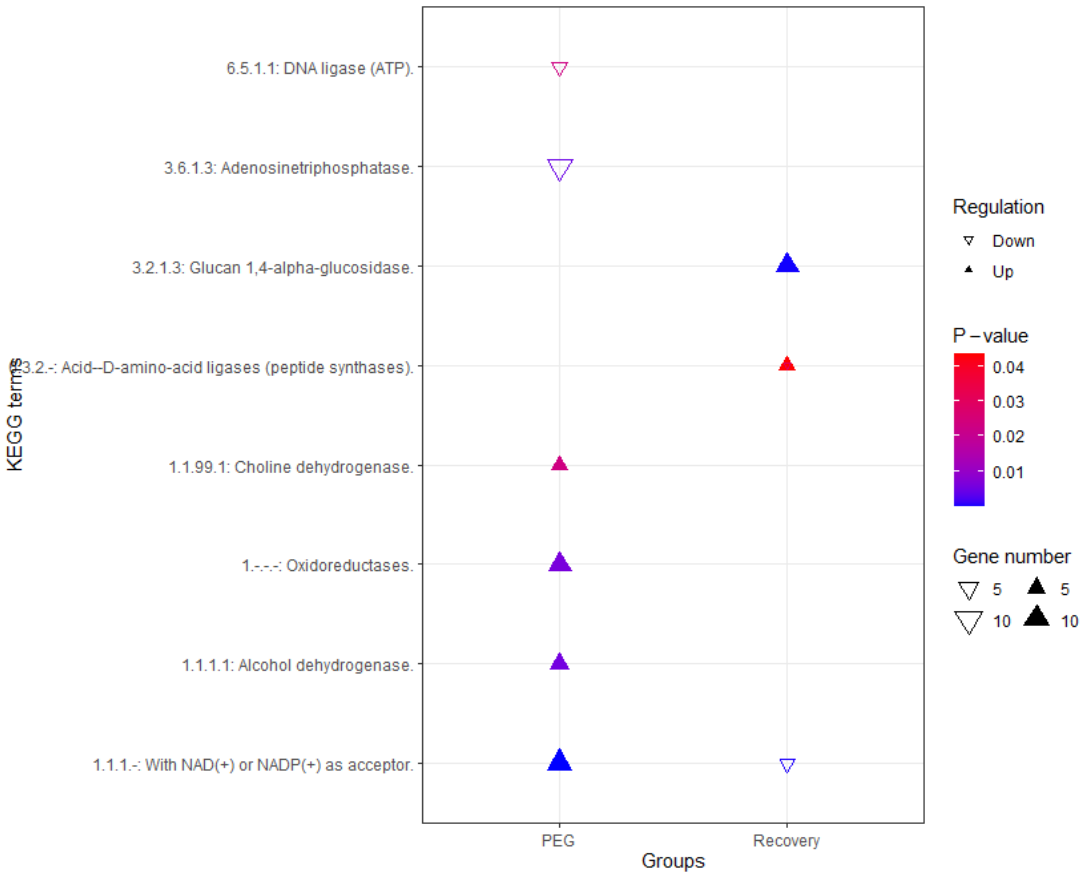
Supplementary Figure 2 KEGG Polyol pathways with *C. geophilum* 1.093 gene expression and gene regulation during PEG treatment and recovery.

Blue-red color bars show the gene expression at the different timepoints, yellow-black color bars the gene regulation as log2 fold change. "?"mean identified only by sequence homology via BlastP. The pathway numbers and gene names are given on the arrows. The thickness of the arrow corresponds to the gene expression.



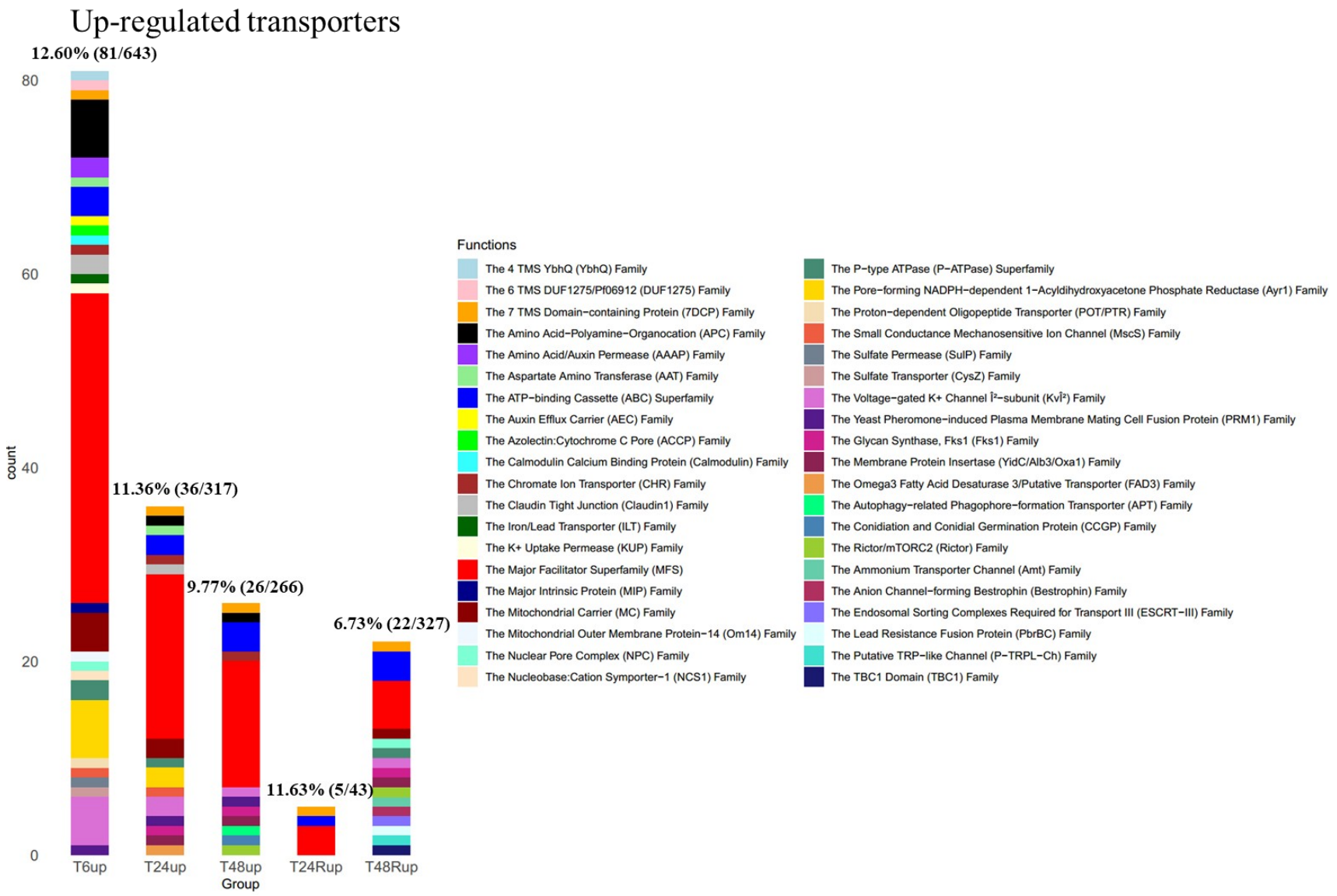
Supplementary Figure 3 KOG enrichment analyses

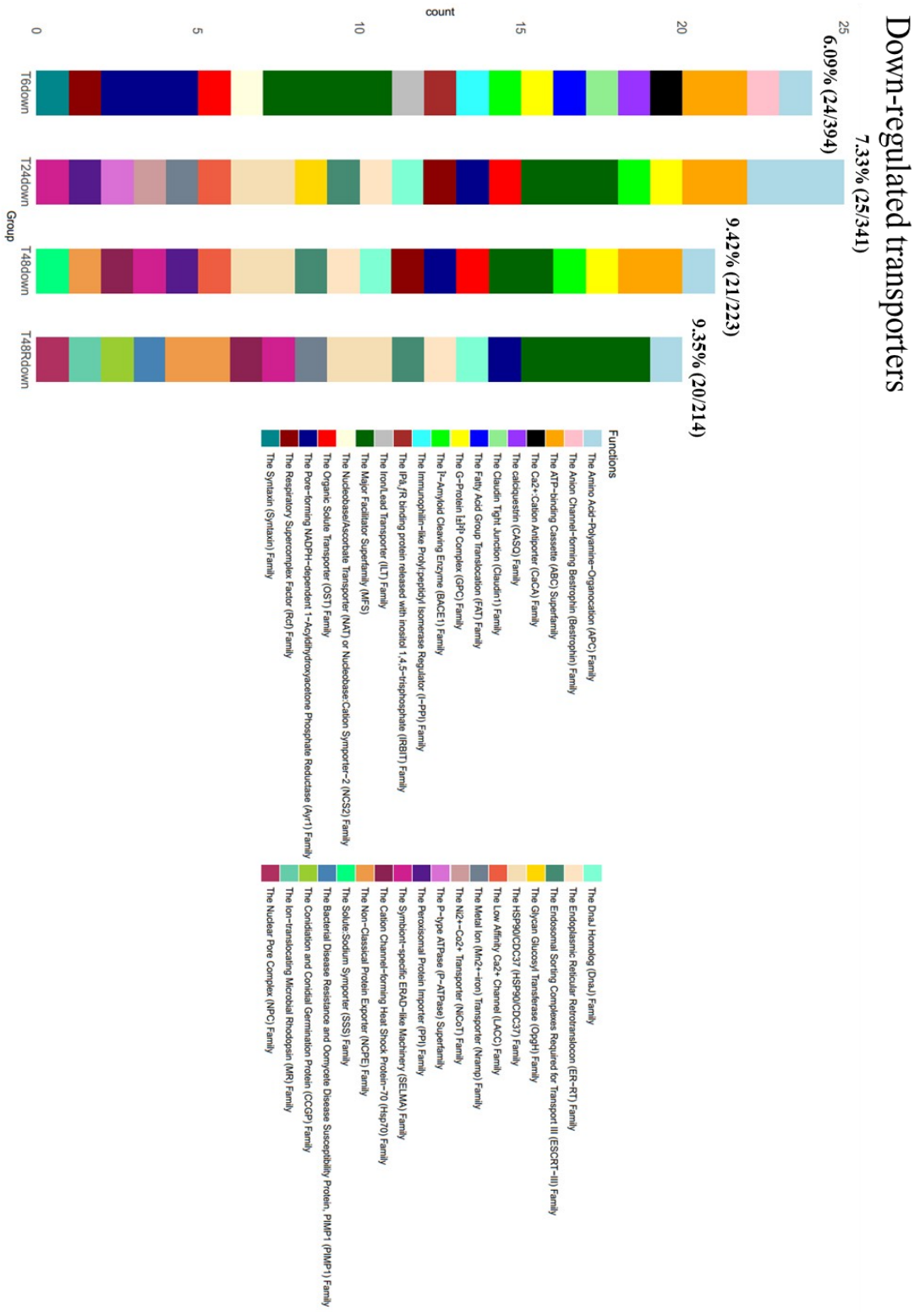
The gene number for the KOG terms are shown as well as the corresponding p-values using a color code. Up/down-triangles: up/down regulated gene functions. PEG, regulated transcripts after 6h, 24h and 48h PEG treatment; Recover



Supplementary Figure 4 KEGG enrichment analyses

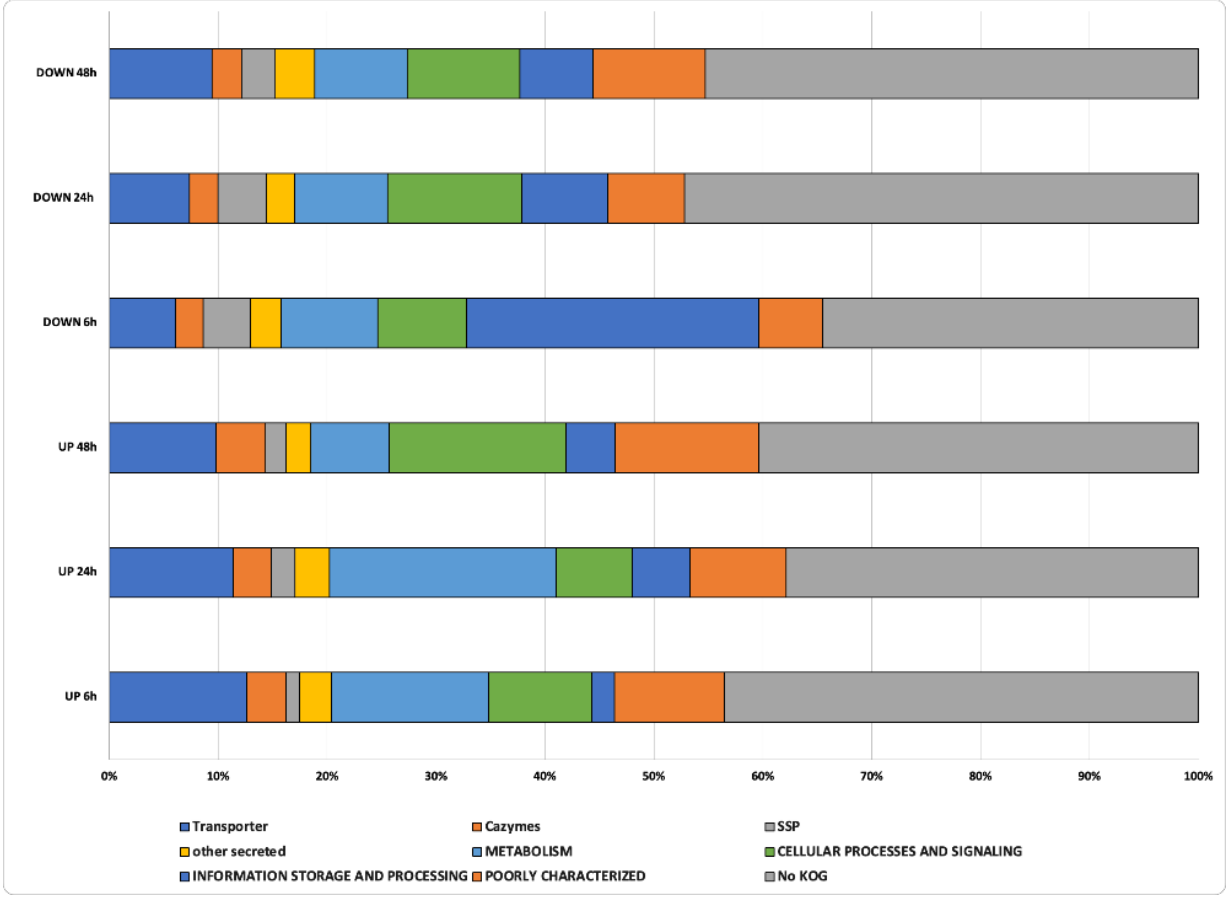
The gene number for the KEGG terms are shown as well as the corresponding p-values using a color code. Up/down-triangles: up/down regulated gene functions. PEG, regulated transcripts after 6h, 24h and 48h PEG treatment; Recover


Up-regulated transporters
12.60% (81/643)
11.36% (36/317)
9.77% (26/266)
11.63% (5/43)
6.73% (22/327)
count
0
20
40
60
80
T6up
T24up
T48up
T24Rup
T48Rup
Group
Functions
The 4 TMS YbhQ (YbhQ) Family
The 6 TMS DUF1275/Pf06912 (DUF1275) Family
The 7 TMS Domain−containing Protein (7DCP) Family
The Amino Acid−Polyamine−Organocation (APC) Family
The Amino Acid/Auxin Permease (AAAP) Family
The Aspartate Amino Transferase (AAT) Family
The ATP−binding Cassette (ABC) Superfamily
The Auxin Efflux Carrier (AEC) Family
The Azolectin:Cytochrome C Pore (ACCP) Family
The Calmodulin Calcium Binding Protein (Calmodulin) Family
The Chromate Ion Transporter (CHR) Family
The Claudin Tight Junction (Claudin1) Family
The Iron/Lead Transporter (ILT) Family
The K+ Uptake Permease (KUP) Family
The Major Facilitator Superfamily (MFS)
The Major Intrinsic Protein (MIP) Family
The Mitochondrial Carrier (MC) Family
The Mitochondrial Outer Membrane Protein−14 (Om14) Family
The Nuclear Pore Complex (NPC) Family
The Nucleobase:Cation Symporter−1 (NCS1) Family
The P−type ATPase (P−ATPase) Superfamily
The Pore−forming NADPH−dependent 1−Acyldihydroxyacetone Phosphate Reductase (Ayr1) Family
The Proton−dependent Oligopeptide Transporter (POT/PTR) Family
The Small Conductance Mechanosensitive Ion Channel (MscS) Family
The Sulfate Permease (SulP) Family
The Sulfate Transporter (CysZ) Family
The Voltage−gated K+ Channel Î²−subunit (KvÎ²) Family
The Yeast Pheromone−induced Plasma Membrane Mating Cell Fusion Protein (PRM1) Family
The Glycan Synthase, Fks1 (Fks1) Family
The Membrane Protein Insertase (YidC/Alb3/Oxa1) Family
The Omega3 Fatty Acid Desaturase 3/Putative Transporter (FAD3) Family
The Autophagy−related Phagophore−formation Transporter (APT) Family
The Conidiation and Conidial Germination Protein (CCGP) Family
The Rictor/mTORC2 (Rictor) Family
The Ammonium Transporter Channel (Amt) Family
The Anion Channel−forming Bestrophin (Bestrophin) Family
The Endosomal Sorting Complexes Required for Transport III (ESCRT−III) Family
The Lead Resistance Fusion Protein (PbrBC) Family
The Putative TRP−like Channel (P−TRPL−Ch) Family
The TBC1 Domain (TBC1) Family



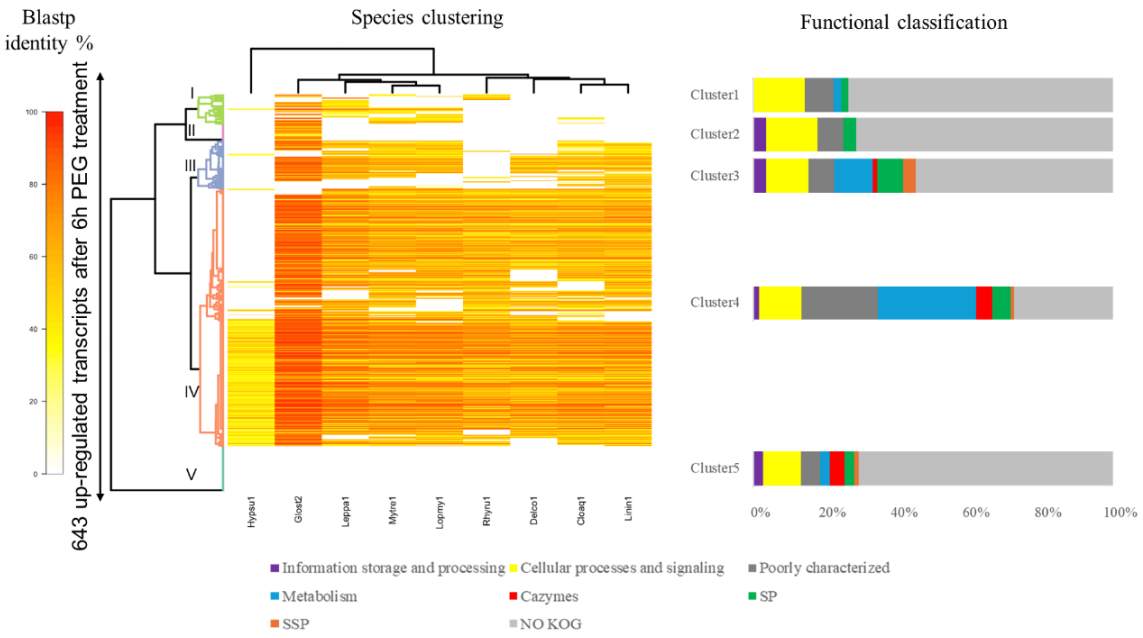
Supplementary Figure 5 DEGs coding for putative transporters

The number of up- and down-regulated transcripts coding for transporters are given for the different time points (T6 6h, T24 24h, T48 48h) and treatments (R recovery). Genes coding for transporters were classified using the transporter classification database (TCDB; https://www.tcdb.org/). The percentage of regulated transporter genes among the total number of regulated genes is given in addition.

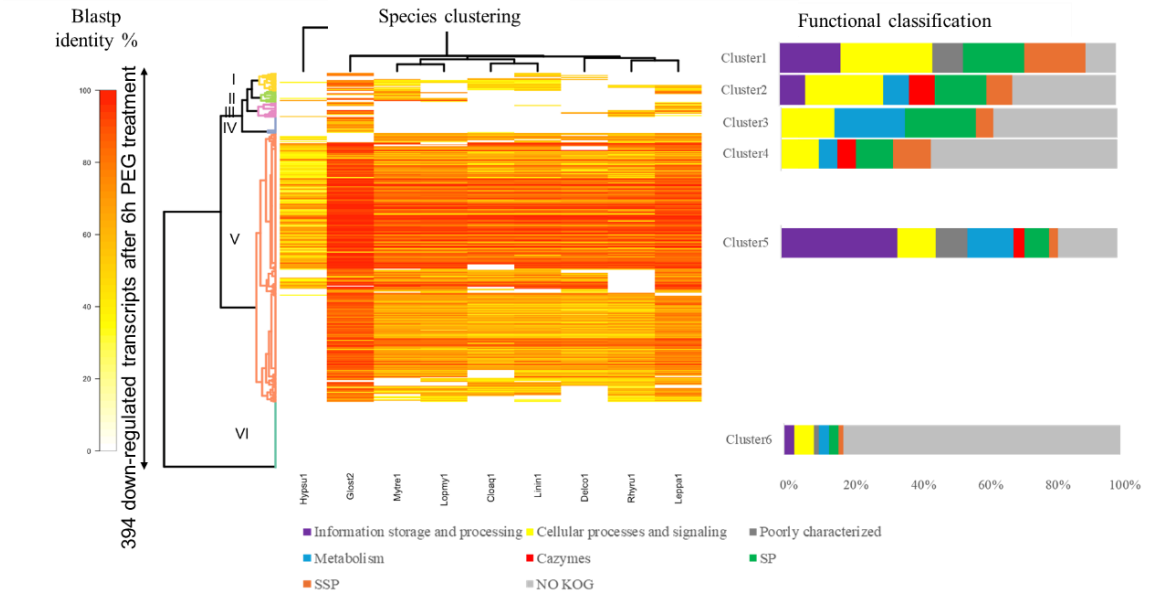


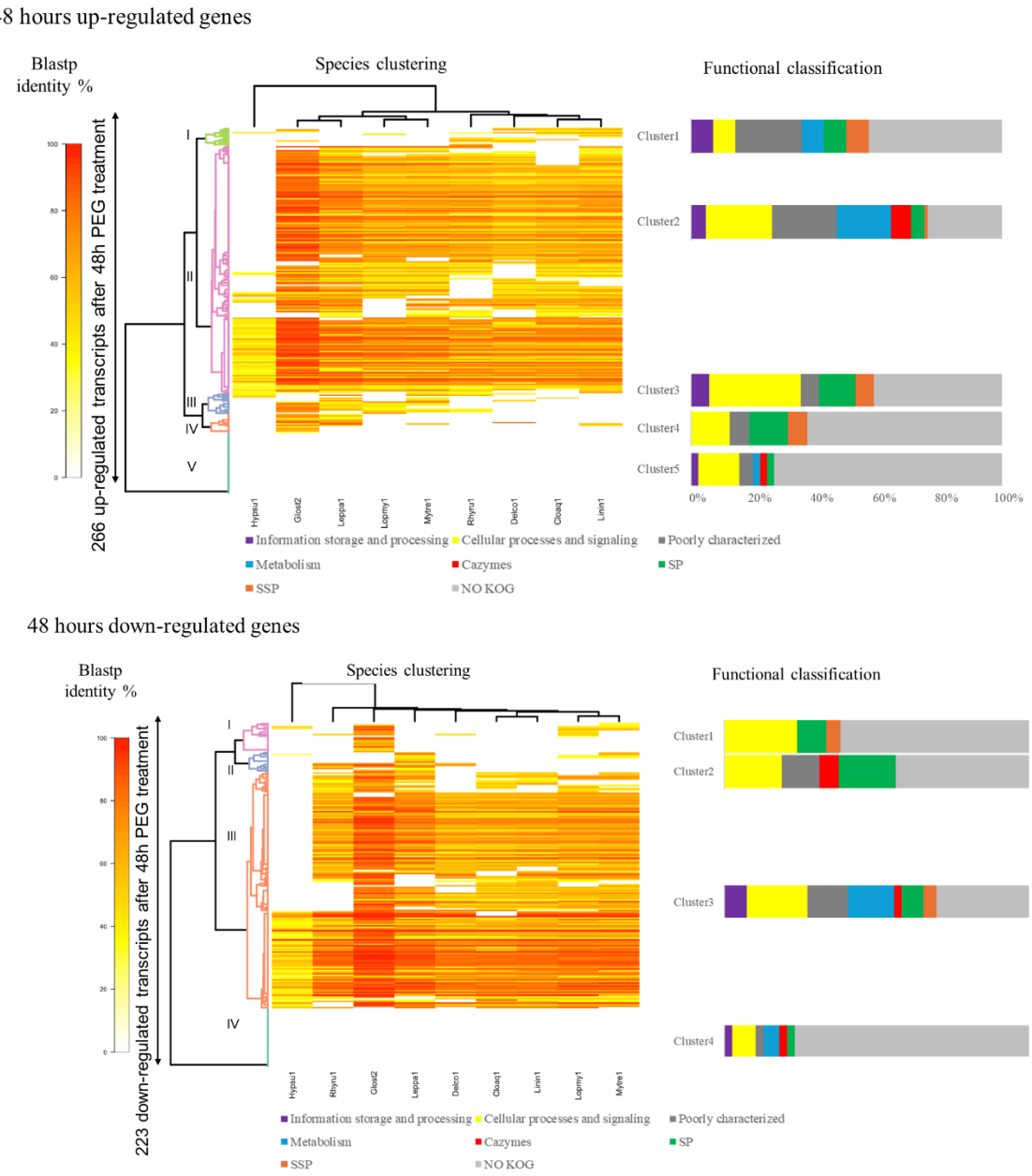
Supplementary Figure 6 Functional classification of regulated transcripts at 6, 24 and 48h. UP up-regulated, DOWN down-regulated. SSP small secreted proteins.

6 hours up-regulated genes



6 hours down-regulated genes





Supplementary Figure 7 Presence of *C. geophilum* PEG-regulated genes in other fungal genomes. The heatmap depicts a double hierarchical clustering of PEG-regulated *C. geophilum* genes at 6h and 48h (rows, fold change >5; FDR corrected P<0.05).

PEG stress-regulated genes were blasted (BLASTP) against the indicated genomes to identify homologues. Homologues are colored from yellow to red depending on the percentage of similarity. On the left of the heatmap, clusters are highlighted by branches of the same color. The percentages of putative functional categories are given for each cluster as bargrams, and the percentages of genes in each cluster are shown.

Glost2: *Glonium stellatum*; Leppa1: *Lepidopterella palustris*; Lopmy1: *Lophium mytilinum*; Mytre1: *Mytilinidion resinicola*; Rhyru1: *Rhytidhysteron rufulum*; Hyspu1: *Hysterium pulicare*; Linin1: *Lindgomyces ingoldianus*; Cloaq1: *Clohesyomyces aquaticus*; Delco1: *Delitschia confertaspora*;

Putative function	Protein ID	T6SvsT6C	24SvsT24C	T48SvsT48C	T24SRvsT24CR	T48SRvsT48CR
Detoxification						
Cytochrome P450	**542429**	**1.22**	NR	NR	NR	NR
Cytochrome P450	**653564**	**1.63**	**1.10**	NR	NR	NR
Cytochrome P450	**675498**	**1.53**	**1.29**	NR	NR	NR
Cytochrome P450	**707149**	**2.24**	**1.31**	NR	NR	NR
Cytochrome P450	**566542**	NR	**1.03**	NR	NR	NR
Cytochrome P450	**687008**	**-2.45**	**-1.93**	NR	NR	NR
Cytochrome P450	**535265**	NR	**-1.26**	NR	NR	NR
Cytochrome P450	**615589**	NR	**-1.01**	NR	NR	NR
Cytochrome P450	**645904**	NR	**-1.51**	NR	NR	NR
Cytochrome P450	**702486**	NR	**-2.07**	NR	NR	NR
Cytochrome P450	**654251**	NR	NR	**-1.28**	NR	-0.93
Glutathione S-transferase	**673422**	**4.95**	**3.10**	**1.91**	NR	NR
Glutathione S-transferase	**692323**	**1.47**	NR	NR	NR	NR
Glutathione S-transferase	**613750**	**1.22**	NR	NR	NR	NR
Glutathione S-transferase	**542381**	**1.03**	NR	NR	NR	NR
Glutathione S-transferase	**661385**	NR	**-1.01**	NR	NR	NR
Cell wall remoduling						
Carbohydrate-Binding Module Family 50 protein	**708040**	**1.25**	NR	NR	NR	NR
1,3-beta-glucan synthase/callose synthase catalytic subunit, Glycosyltransferase Family 48 protein	**359617**	**NR**	**1.35**	**1.95**	**NR**	**1.89**
Chitinase	**563333**	**-2.85**	**-2.69**	**-2.58**	**NR**	**NR**
Chitinase	**605583**	**-1.03**	**-2.14**	**-2.25**	**NR**	**NR**
Chitinase, SSP	**651565**	**-1.07**	**-1.17**	**-1.01**	**NR**	**NR**
Chitinase, SSP	**655693**	**2.32**	**2.02**	**NR**	**NR**	**NR**
Chitinase, SSP	**459921**	**NR**	**1.05**	**1.16**	**NR**	**NR**
Chitinase	**71026**	**1.74**	**1.27**	**NR**	**NR**	**NR**
Chitinase	**696694**	**NR**	**-1.65**	**-1.30**	**NR**	**NR**
Chitinase	**675249**	**NR**	**-1.13**	**NR**	**NR**	**NR**
Chitinase	**650882**	**NR**	**1.27**	**NR**	**NR**	**NR**
Chitinase, Glycoside Hydrolase Family 18 protein	**662308**	**1.69**	**NR**	**NR**	**NR**	**NR**
Glycoside Hydrolase Family 25 protein	**626952**	NR	**-1.14**	NR	**-1.37**	**-1.20**
Signaling						
Serine/threonine protein kinase	**284299**	**1.27**	**1.83**	NR	NR	0.75
MEKK and related serine/threonine protein kinases	**545846**	NR	NR	**1.31**	NR	**1.99**
Serine/threonine-protein kinase involved in autophagy	**550336**	NR	NR	**1.20**	NR	0.98
cAMP-dependent protein kinase	**566708**	NR	NR	**1.40**	NR	**2.26**
MEKK and related serine/threonine protein kinases	**578032**	NR	NR	**1.15**	NR	**1.94**
MEKK and related serine/threonine protein kinases	**652573**	**-1.26**	NR	NR	NR	NR
Mitogen-activated protein kinase	**670269**	**-1.36**	NR	NR	NR	NR
Mitotic checkpoint serine/threonine protein kinase	**632716**	NR	**-1.37**	**-1.12**	NR	NR
Osmoregulation						
Mitogen-activated protein kinase HOG1-like	**725216**	**1.12**	NR	NR	NR	**-1.07**
Sorbitol dehydrogenase	**587624**	**2.24**	**1.64**	**1.58**	NR	NR
Sorbitol dehydrogenase	**670088**	**1.21**	**1.36**	NR	NR	NR
Sorbitol dehydrogenase	**658145**	**1.09**	NR	NR	NR	NR
Sorbitol dehydrogenase	**668100**	NR	**1.66**	NR	**1.35**	NR
Sorbitol dehydrogenase	**303421**	NR	**1.39**	NR	NR	NR
Phospholipid/glycerol acyltransferase	**664212**	**2.24**	**1.39**	NR	NR	NR
glycerol-3-phosphate dehydrogenase	**653646**	NR	**1.44**	**1.00**	NR	0.78
NAD-dependent glycerol-3-phosphate dehydrogenase	**656861**	**1.00**	**1.10**	NR	NR	NR
Transport linked to osmoregulation						
γ-aminobutyric acid (GABA) permease, GabA	**627314**	**1.05**	**NR**	NR	NR	NR
γ-aminobutyric acid (GABA) permease, GabA	**632266**	**1.20**	**NR**	NR	NR	NR
γ-aminobutyric acid (GABA) permease, GabA	**636793**	NR	**-1.14**	NR	NR	NR
γ-aminobutyric acid (GABA) permease, GabA	**673432**	**1.05**	**NR**	NR	NR	NR
γ-aminobutyric acid (GABA) permease, GabA	**700015**	NR	**1.05**	1.02	NR	NR
aminobutyric acid (GABA) permease, GabA	**709552**	**1.32**	NR	NR	NR	NR
Glycerol uptake permease (Glycerol:H+ symporter) Stl1	**550496**	**2.52**	**1.95**	**1.54**	NR	NR
Aquaporin	**647346**	**1.23**	NR	NR	NR	NR

log2 fold change. Compared to the respective control. Fold changes of regulated transcripts Log2>1 are in bold, in blue down-regulated , in red up-regulated.
NR not significantly regulated, Colored blockes significantly regulated.

Supplementary Table 1 Regulated osmotic stress reacting genes in isolate 1.093 C, controls; S, PEG-treated; R recovery; 0, 0h; 24, 24 h and 48, 48 h

Gene1	A.fumigatus	Cenge3 Protein ID	remark	Description 1	Cenge3 Protein ID	T6 2	T24 2	T48 2	T24 Recovery 2	T48 Recovery 2
DHN-melanin pathway										
Pks1	Afu2g17600	605167		Polyketide synthase	605167	0.39	NR	0.47	NR	0.4
		653071			653071	**-1.02**	NR	0.81	NR	**1.0**
		580598			580598	NR 3	NR	NR	NR	NR
Ayg1	Afu2g17550	658160		Abhydrolase	658160	-0.5	NR	NR	NR	NR
Arp2	Afu2g17560	679288		1,3,6,8-Tetrahydroxynaphthalene reductase	679288	**-2.0**	NR	NR	NR	NR
		653063			653063	**-2.4**	NR	NR	NR	0.7
Arp1	Afu2g17580	658695		Scytalone dehydratase	658695	**-2.6**	-0.8	NR	NR	NR
Abr2	Afu2g17530	?		Fungal pigment MCO						
Abr1	Afu2g17540,	688486	FetC	Fungal ferroxidase	688486	0.7	NR	NR	NR	NR
Dopa-melanin pathway										
melC2	Afu3g01070	603497		Tyrosinase	603497	NR	NR	NR	NR	NR
		624786			624786	NR	NR	NR	NR	NR
melO	Afu4g14490(	677101		Laccase	677101	**-1.5**	**-1.0**	NR	NR	NR
		691085			691085	**-1.3**	-0.8	-0.8	NR	NR
		693785			693785	NR	NR	NR	NR	NR
		706698			706698	NR	NR	NR	NR	NR
		674520	contains CBM18		674520	**-1.7**	-0.8	NR	NR	0.8
		100449			100449	NR	-0.7	-0.8	NR	NR
		654471			654471	NR	NR	NR	NR	NR
		687046			687046	NR	NR	NR	NR	NR
		742834			742834	NR	NR	NR	NR	NR
		109530	truncated		109530	NR	NR	NR	NR	NR
L-tyrosine degradation pathway										
Tat	Afu2g13630	565430		Tyrosine aminotransferase	565430	NR	NR	NR	NR	NR
hppD	Afu2g04200	555620		4-Hydroxyphenylpyruvate dioxygenase	555620	NR	**-1.6**	NR	NR	NR
hmgA	Afu2g04220	630612		Homogentisate dioxygenase	630612	-0.6	NR	NR	NR	NR
fahA	Afu2g04230	547147		Fumarylacetoacetate hydrolase	547147	-0.7	NR	NR	0.8	NR
maiA	Afu2g04240	664678		Maleylacetoacetate isomerase	664678	0.9	-0.8	-0.8	NR	NR

1 Gene names and descriptions were taken from Chen et al. 2014

2 log2 fold change. Compared to the respective control. Fold changes of regulated transcripts Log2>1 are in bold, in blue down-regulated , in orange up-regulated.

3 NR not significantly regulated

Supplementary Table 2 Gene expression changes in melanin producing pathways among timepoints and drought treatments

	GO.ID	Term	Annotated	Significant	Expected	classicFisher
		GO Biological Process				
6h UP	**GO:0006810**	**transport**	**510**	**53**	**31.51**	**1.9e-05**
	GO:0009636	response to toxic substance	4	2	0.25	0.021
	GO:0006066	alcohol metabolic process	19	4	1.17	0.026
	GO:0006813	potassium ion transport	5	2	0.31	0.034
	GO:0008643	carbohydrate transport	51	7	3.15	0.035
	GO:0044237	cellular metabolic process	1147	44	70.88	0.044
	GO:0006306	DNA methylation	6	2	0.37	0.048
24 UP	**GO:0006810**	**transport**	**510**	**24**	**16.42**	**0.00077**
	GO:0008152	metabolic process	1702	51	54.81	0.0134
	GO:0006633	fatty acid biosynthetic process	6	2	0.19	0.01411
	GO:0006072	glycerol-3-phosphate metabolic process	2	2	0.06	0.03178
	GO:0018106	peptidyl-histidine phosphorylation	9	2	0.29	0.03181
	GO:0006084	acetyl-CoA metabolic process	1	1	0.03	0.0322
	GO:0006075	(1->3)-beta-D-glucan biosynthetic proces...	1	1	0.03	0.0322
	GO:0046168	glycerol-3-phosphate catabolic process	1	1	0.03	0.0322
	GO:0009058	biosynthetic process	667	12	21.48	0.04113
48h UP	**GO:0006810**	**transport**	**510**	**22**	**13.76**	**0.00072**
	GO:0006075	(1->3)-beta-D-glucan biosynthetic proces...	1	1	0.03	0.02698
	GO:0006044	N-acetylglucosamine metabolic process	1	1	0.03	0.02698
6h DOWN	**GO:0006270**	**DNA replication initiation**	**6**	**6**	**0.2**	**1.1e-09**
	GO:0006412	**translation**	**120**	**12**	**3.97**	**0.00094**
	GO:0006269	DNA replication, synthesis of RNA primer	2	2	0.07	0.00108
	GO:0006260	DNA replication	29	13	0.96	0.00151
	GO:0006310	DNA recombination	6	2	0.2	0.01485
	GO:0006974	DNA damage response	51	4	1.69	0.03247
	GO:0006275	regulation of DNA replication	1	1	0.03	0.03307
	GO:0046080	dUTP metabolic process	1	1	0.03	0.03307
	GO:0006228	UTP biosynthetic process	1	1	0.03	0.03307
	GO:0006183	GTP biosynthetic process	1	1	0.03	0.03307
24h DOWN	GO:0045132	meiotic chromosome segregation	1	1	0.02	0.022
	GO:0006183	GTP biosynthetic process	1	1	0.02	0.022
	GO:0009253	peptidoglycan catabolic process	1	1	0.02	0.022
	GO:0006228	UTP biosynthetic process	1	1	0.02	0.022
	GO:0030261	chromosome condensation	1	1	0.02	0.022
48h DOWN	GO:0006228	UTP biosynthetic process	1	1	0.01	0.013
	GO:0006183	GTP biosynthetic process	1	1	0.01	0.013
	GO:0009098	leucine biosynthetic process	1	1	0.01	0.013
	GO:0006334	nucleosome assembly	3	1	0.04	0.037
24h Recovery UP	GO:0006614	SRP-dependent cotranslational protein ta...	9	1	0.04	0.039
48h Recovery UP	**GO:0006468**	**protein phosphorylation**	**231**	**20**	**6.63**	**1.8e-05**
	GO:0006355	**regulation of DNA-templated transcriptio...**	**304**	**16**	**8.73**	**0.0099**
	GO:0007165	signal transduction	102	8	2.93	0.0214
	GO:0018106	peptidyl-histidine phosphorylation	9	2	0.26	0.0257
	GO:0006075	(1->3)-beta-D-glucan biosynthetic proces...	1	1	0.03	0.0287
24h Recovery DOWN	**GO:0009253**	**peptidoglycan catabolic process**	**1**	**1**	**0**	**0.0013**
	GO:0006308	**DNA catabolic process**	**5**	**1**	**0.01**	**0.0065**
	GO:0006644	phospholipid metabolic process	19	1	0.02	0.0246
48h Recovery DOWN	GO:0009253	peptidoglycan catabolic process	1	1	0.01	0.01
	GO:0007586	digestion	2	1	0.02	0.02
	GO:0009636	response to toxic substance	4	1	0.04	0.039
	GO:0006098	pentose-phosphate shunt	4	1	0.04	0.039
	GO:0006810	transport	510	9	5.1	0.043
	GO:0006308	DNA catabolic process	5	1	0.05	0.049

	GO.ID	Term	Annotated	Significant	Expected	classicFisher
		GO Biological Process				
Turquoise	**GO:0006810**	**transport**	**510**	**32**	**16.87**	**4.2e-05**
	GO:0006527	**arginine catabolic process**	**4**	**2**	**0.13**	**0.0062**
	GO:0008643	carbohydrate transport	51	5	1.69	0.0248
	GO:0008272	sulfate transport	1	1	0.03	0.0331
	GO:0006084	acetyl-CoA metabolic process	1	1	0.03	0.0331
blue	**GO:0009712**	**catechol-containing compound metabolic p...**	**3**	**2**	**0.07**	**0.0017**
	GO:0006306	**DNA methylation**	**6**	**2**	**0.15**	**0.0082**
	GO:0006810	transport	510	19	12.43	0.0228
	GO:0006097	glyoxylate cycle	1	1	0.02	0.0244
	GO:0015936	coenzyme A metabolic process	2	1	0.05	0.0482
red	**GO:0006633**	**fatty acid biosynthetic process**	**6**	**2**	**0.13**	**0.0068**
	GO:0006072	glycerol-3-phosphate metabolic process	2	2	0.04	0.0218
	GO:0006075	(1->3)-beta-D-glucan biosynthetic proces...	1	1	0.02	0.0222
	GO:0006044	N-acetylglucosamine metabolic process	1	1	0.02	0.0222
	GO:0046168	glycerol-3-phosphate catabolic process	1	1	0.02	0.0222
	GO:0006810	transport	510	16	11.32	0.0391
	GO:0006094	gluconeogenesis	2	1	0.04	0.0439
	GO:0006596	polyamine biosynthetic process	2	1	0.04	0.0439
brown	**GO:0006468**	**protein phosphorylation**	**231**	**18**	**5.13**	**1.7e-06**
	GO:0006355	**regulation of DNA-templated transcriptio...**	**304**	**14**	**6.75**	**0.0048**
	GO:0007165	**signal transduction**	**102**	**7**	**2.26**	**0.0089**
	GO:0051056	regulation of small GTPase mediated sign...	14	2	0.31	0.0371
	GO:0008154	actin polymerization or depolymerization	2	1	0.04	0.0439
yellow	GO:0046080	dUTP metabolic process	1	1	0.01	0.012
	GO:0006228	UTP biosynthetic process	1	1	0.01	0.012
	GO:0006183	GTP biosynthetic process	1	1	0.01	0.012
	GO:0009253	peptidoglycan catabolic process	1	1	0.01	0.012
	GO:0007586	digestion	2	1	0.02	0.024
	GO:0009636	response to toxic substance	4	1	0.05	0.048
black	GO:0009636	response to toxic substance	4	1	0.03	0.033
pink	**GO:0006260**	**DNA replication**	**29**	**8**	**0.54**	**0.00019**
	GO:0006269	**DNA replication, synthesis of RNA primer**	**2**	**2**	**0.04**	**0.00034**
	GO:0006310	**DNA recombination**	**6**	**2**	**0.11**	**0.00489**
	GO:0007018	microtubule-based movement	9	2	0.17	0.01133
	GO:0006298	mismatch repair	9	2	0.17	0.01133
	GO:0006614	SRP-dependent cotranslational protein ta...	9	2	0.17	0.01133
	GO:0006974	DNA damage response	51	5	0.95	0.01735
	GO:0006275	regulation of DNA replication	1	1	0.02	0.01871
	GO:0045132	meiotic chromosome segregation	1	1	0.02	0.01871
	GO:0009098	leucine biosynthetic process	1	1	0.02	0.01871
green	**GO:0006270**	**DNA replication initiation**	**6**	**5**	**0.1**	**6.4e-09**
	GO:0006412	**translation**	**120**	**10**	**2.04**	**1.8e-05**
	GO:0006071	glycerol metabolic process	1	1	0.02	0.017
	GO:0006979	response to oxidative stress	17	2	0.29	0.033

Supplementary Table 3 GO enrichment analysis (biological process, Fisher p-value<0.05)) for the different timepoints after PEG treatment (upper pannel) and the WGCNA modules (lower pannel).

GO terms with p-value <0.01 are highlighted in bold.

Chapter 3

Effects of a controlled *Cenococcum geophilum* inoculation on the ectomycorrhizal community of beech (*Fagus sylvatica* L.) under drought conditions

Effects of a controlled *Cenococcum geophilum* inoculation on the ectomycorrhizal community of beech (*Fagus sylvatica* L.) under drought conditions

Abstract

Ongoing climate change has led to more frequent soil droughts in forest ecosystems. Numerous beech diebacks have been reported following several years of unusually low precipitation in France and Switzerland. Consequently, the development of forest management strategies to enhance the drought resistance of forest trees has become increasingly critical. Ectomycorrhizal (ECM) fungi establish mutualistic associations with the roots of forest trees. These mutualistic fungi increase the root uptake area and facilitate the absorption of water and nutrients from the soil. The ECM fungus *Cenococcum geophilum* is cosmopolitan and prevalent in ecosystems under abiotic environmental stress, and it often shows greater resilience to drought stress than other ECM fungi. Thus, this beneficial symbiont is a suitable candidate for evaluating the capacity of ECM fungi to enhance drought adaptation in trees. The objective of this study was to assess whether controlled mycorrhiza formation by *C. geophilum* could enhance the protection of beech plantlets already mycorrhized by a natural soil microbiome from drought stress. A pilot inoculation experiment was conducted in a greenhouse using a drought-tolerant isolate of *C. geophilum* (Chapter 2) and European beech seedlings. Drought stress was induced by decreasing the soil humidity in pots containing inoculated plantlets. After a few months of experimental drought, the ECM morphotypes were characterized, and their colonization rates were quantified. The results of this experiment demonstrated that isolate 1.093 of *C. geophilum* could form ECM with beech plantlets. However, in our experimental system, no positive effect of controlled inoculation with Cg1.093 on the survival rate of European beech plantlets was observed following the application of drought stress.

Introduction

Forests play a crucial role in water and carbon cycling. They provide habitats for diverse life forms and offer ecological and economic services to humans. Recently, forests have experienced more frequent soil droughts and heat waves, resulting in widespread forest diebacks (Hammond, 2022). The frequency and duration of dry extremes have increased in most regions of the world and this trend is exacerbated

(Petrova, 2024). In Europe, forest mortality is consistently associated with drought (Senf, 2020). European beech (*Fagus sylvatica*) is the dominant timber tree species in the natural forests of Europe (Leuschner 2020). It is an economically and ecologically important species. With several unusual and combined heatwaves and droughts, severe growth reduction, early leaf senescence, leaf browning, and diebacks of beech trees have been reported across Central Europe since 2018. These observations suggest that this species is susceptible to climate change (Rukh et al. 2023). In Europe, according to validated models, beech growth has declined across large regions of its distribution in recent decades, and the reduction in growth is projected to range from −20% to more than −50% by 2090 (Martinez del Castillo et al.,2022). Consequently, developing forest management approaches to enhance the drought resistance of beech trees has become increasingly critical.

ECM fungi have been recognized as potential candidates for enhancing the drought tolerance of forest trees. Under drought conditions, they can facilitate the host plants to acquire more nutrients than non-mycorrhizal plants (Lehto & Zwiazek, 2011). Cosme (2023) employed a phylogenetic comparative method utilizing data from 1,638 extant species globally distributed to characterize the evolution of mycorrhizal strategies and drought adaptation. The detected correlated evolution revealed that gains and losses of drought tolerance occurred at accelerated rates in lineages with ecto- or ericoid mycorrhizas, which were on average approximately 15 and 300 times faster than in lineages with arbuscular mycorrhizal and naked root (non-mycorrhizal alone or with facultative arbuscular mycorrhizal) strategies, respectively, suggesting that mycorrhizas play a crucial facilitator role in the evolutionary processes of plant adaptation to critical changes in water availability across global climates. Danielsen and Polle (2014) observed that K and Mg increased exclusively in the leaves of poplar interacting with -*Paxillus involutus* under drought conditions. Li et al. (2021) investigated the Ca availability and Ca uptake of *Quercus acutissima* under drought conditions and found that compared to non-ECM (NM) treatment, the ECM fungus *Xerocomus chrysenteron* enabled *Quercus acutissima* to acquire more Ca under drought conditions. It appears that ECM fungi enhance the mechanisms that mitigate drought. A similar result was observed for *Suillus luteus-P. sylvestris* var. *mongolica* symbiosis (Yin et al., 2020). Some ECM fungi have demonstrated efficacy under moderate drought conditions.

Nilsen et al. (1998) found that moderate drought stress and nitrogen concentration result in higher colonization rates for *C. geophilum*, while other ECM fungi were not significantly influenced by drought. Pena et al. (2013) that trees colonized by *C. geophilum, Tomentella punicea or Tuber* sp. exhibited increased N uptake under drought conditions compared with NM plants.

Some compounds of ECM fungi play important roles when subjected to drought. Shi et al. (2002) found drought stress caused the accumulation of sucrose, glucose and fructose, and of fungus-derived compounds (mannitol and arabitol) in ectomycorrhizal roots. The accumulation of sugar alcohols, which are known to counteract drought stress, is species-specific. Fernandez and Koide (2013) investigated the function of melanin in Cg plants under drought stress. They used the melanin biosynthesis inhibitor tricyclazole to inhibit melanin production. Melanin inhibition only had negative effects on growth when *C. geophilum* isolates were subjected to osmotic and desiccation stress (-1.7 MPa).

ECM fungi can influence the physiology of host plants under drought stress. Sebastiana *et al.* (2019) investigated *Pisolithus tinctorius*- oak symbiosis and found improved drought tolerance of this system could be related to leaf membrane lipid metabolism, such as fatty acid content and composition. Calvo-Polanco et al. (2019) showed that the increased drought resistance of *Laccaria bicolor*-poplar symbiosis is regulated by the expression of plant aquaporins and the ABA/cytokinin status. Herzog et al. (2012) studied exoenzyme profiles of *C. geophilum* associated with *Quercus robur, Q. petraea* and *Q. pubescens* under drought stress. The results showed that the exoenzyme activities of *C. geophilum* on *Q. robur* and *Q. petraea* (but not *Q. pubescens*) significantly increased under the elevated air temperature and drought combined treatment.

ECM fungi form the mantle surrounding the root tips to cover and protect them from drying out. Some ECM fungi, such as *C. geophilum*, are drought-tolerant and abundant in water-stressed habitats (Pigott, 1982; McCormack et al., 2017). The potential respiration activity of *C. geophilum* ECM is significantly less altered than that of other ECM fungi (Jany et al. 2003). Some ECM fungi, such as many lichen-forming ascomycetes, have thick and gelatinous cell walls that maintain vitality under drought

stress. *C. geophilum*hyphae enlarged and became gelatinous when they are in contact with water, thus storing water and keeping the host root moist longer (Di Pietro 2007).

ECM inoculation is a potential method for plantation and forest restoration. Strawsine et al. (2024) studied whether pioneer dual-mycorrhizal hosts, *Leptospermum scoparium*, could facilitate the establishment of *Nothofagus* by providing an ECM inoculum. Metabarcoding analysis of the roots of monocultured and mixed pairs of cultured *Nothofagus* and *Leptospermum* seedlings indicated the potential of *Leptospermum* to promote the establishment of *Nothofagus* in restoration planting. Extreme drought events occurred during the experiment. Their results showed that the survival of *Nothofagus* seedlings was significantly improved by higher ECM root colonization (including *Cenococcum sp*., but mainly affected by *Laccaria sp.*) levels, suggesting that ECM fungi have the potential to help their hosts adapt to extreme drought stress. De Quesada et al. (2024) evaluated the transpiration rate of *Pinus sylvestris* seedlings under drought stress in greenhouse conditions by exposing seedlings to 10 ECM fungal species and three natural soil inoculums to study whether some ECM fungi increase the drought tolerance of nursery-raised seedlings. Their results showed that exposing seedlings to ECM fungi or soil inoculum had a positive effect on transpiration rate during the driest and recovery phases, suggesting that ECM fungi have the potential to enhance seedling drought tolerance in nursery plant production. A similar result was observed by Aryal et al. (2020) in a greenhouse and Mateus et al. (2024) in an *in vitro* system. In a greenhouse inoculation experiment under three months of drought treatment, *Suillus luteus* increased the colonization rates of *Pinus sylvestris* var. *mongolica* seedlings, and improved soil conditions (Yin et al., 2018). Taniguchi et al. (2021) inoculated *Pinus tabulaeformis* seedlings (a drought tolerant plant species) with three different kinds of soil: no additional adding soil (NS), soil sterilized with fungicide, and ECM-containing nontreated plantation soil (NPS) collected from a nearby *P. tabulaeformis* plantation in the Kubuqi Desert combining with “straw checkerboard barrier afforestation method” and sequenced the ECM fungi community of seedlings with different treatments. After two years, *P. tabulaeformis* survival did not differ among the three soil treatments; ECM colonization rate, total plant dry weight, leaf total nitrogen per seedling, and water use in the NPS treatment were much higher than those in the NS treatment. Structural equation modeling showed that soil

inoculation improved nitrogen uptake and water use, and increased plant growth by enhancing the ECM colonization of *P. tabulaeformis*. *Suillus, Peziza, Geopora, Tuber*, and *Tomentella* were the dominant genera in the NPS treatment group. These results indicate that soil inoculation with ECM fungi aids in afforestation with ECM plants for the restoration of drylands.

Although *C. geophilum* is widely recognized as a drought-tolerant symbiont, this assertion has recently been debated. A study utilizing *Pinus seedlings* colonized by *C. geophilum* and subjected to drought stress showed that the drought resistance of ECM plantlets was not directly correlated with that of *C. geophilum* isolates cultivated in liquid medium (Zhang et al., 2024). Xie et al. (2024a) used inoculated *Quercus mongolica* and *Tilia amurensis* to investigate the response of ECM fungal communities and their exploration types under drought conditions in a pot system. The relative abundance of *C. geophilum* in both hosts decreased. Nickel et al. (2017) examined the changes in ECM fungal community diversity of European beech and Norway spruce forests under drought conditions by utilizing retractable roofs to exclude rain for three years. The results indicated that the abundance of *C. geophilum* decreased irrespective of depth, year, or host. Pellitier et al. (2024) investigated ECM-fungal communities of *Populus trichocarpa* roots which distributed across a precipitation gradient in the Pacific Northwest USA using taxonomic (metabarcoding) and functional (metagenomics) approaches. Their results showed fungal communities in dry soils contain distinct aquaporin and hydrophobin gene sequences, and genes involved in fungal drought stress tolerance and fungal-mediated plant water uptake (including genes of melanin synthesis, hydrophobins, aquaporins, trehalose synthases, and other gene families) were not the most abundant in drier soils. Nilsen et al. (1998) investigated the effects of N addition and drought on ECM of Norway spruce trees in an outdoor pot lysimeter study. Their results showed that all mycorrhizal types were influenced by drought, but only *C. geophilum* was significant decreased. To contribute to the ongoing debate regarding the role of *C. geophilum* in drought stress mitigation, we inoculated beech seedlings with the drought-tolerant isolate Cg 1.093, as described in Chapter 2. Our hypotheses were: 1) inoculation with Cg 1.093 can facilitate beech seedling survival during extreme drought events. If not, 2) Cg1.093 inoculation cannot mitigate beech seedling death, but Cg1.093 mycelia can survive under extreme drought

conditions.

Methods and materials

Plant and fungal materials

This study was conducted in a greenhouse at room T°C at INRAE-Nancy. Fans are used to decrease T°C in summer, decreasing the greenhouse temperature by approximately 5 °C compared to the outdoor T°C. The beech seedlings were provided by Dr. Aurelie Deveau and Dr. Stéphane Uroz. In Spring 2022, *F. sylvatica* seedlings were collected from the INRAE experimental site in the Montiers-sur-Saulx forest (France). The ecosystem research site of Montiers is located in Northeastern France in the Meuse Department (48° 31' 54″ N, 5° 16' 08" E) and is managed by the BEF Research Unit (INRAE Grand Est – Nancy). Seedlings were sampled at the cotyledon stage. They were then planted in 3.5-L cylindrical plastic pots filled with natural soil, a type of clay that was sieved through a 1-cm mesh and grown under natural environmental conditions. One seedling was digged out, planted and grown in each pot. After one year of growth, they had well-developed root systems. Inoculation with C. geophilum mycelium was initiated in March 2023, using three layers of soil per pot. The top and bottom layers were composed of natural soil (3 kg/layer) from the Montiers experimental site. The middle layer was made of soil mixed homogeneously with *C. geophilum* 1.093 mycelium (5.5g/pot) from MMN liquid culture, peat, vermiculite ((V/V=1:4)) and 0.7 L MMN liquid medium (Marx, 1970) with 20 g/L glucose (for mycelium growing in the substrate, since no preculture for them) (total substrate weight: 500 g/pot) using a concrete mixer machine. A large proportion of the root system was located in the middle layer. White stones (500 g) were used to cover the surfaces of the pots (Figure 1). The drought-tolerant isolate Cg 1.093 was identified and characterized in Chapter 2. We assumed that the pre-existing soil fungal community was similar for all seedlings, as they grew in the same environment and the soil was well mixed before planting. Notable, some seedlings already have *C. geophilum* -like mycorrhizas before inoculating.

Experimental design

Three and a half months after inoculation, the mycorrhizal status of two randomly

selected beech seedlings was assessed (Figure 5). *C. geophilum* – like ECM were found on the root systems. The drought treatments were then performed from July to August 2023 and lasted for 30 d. The soil humidity of the drought-treated and untreated control groups was 25% (total weight: 3340 g/pot) and 70% (total weight: 3950 g/pot), respectively, controlled by weighing the pots (Figure 2). The relationship between the total weight and soil humidity was calculated using 100% and 0% humidity. The 100% humidity soil was weighed from the soil that was submerged in water for half an hour and after which the unabsorbed water was removed. The 0%-humidity soil was placed in an oven (> 50 °C) for > 12 h. The pots were weighed and watered every day, except on weekends. The experiment started with 34 pots as follows:

– A: no drought treatment and no inoculation (8 pots).

–B: inoculated with Cg1.093 and drought treatment (9 pots),

– C: no inoculation but without drought treatment (8 pots),

− D: no inoculation and drought treatment (9 pots), and

After three and a half months, 4 pots died naturally (didn't spread their branches). 30 seedlings were survived (7, 7, 8, and 8 seedlings as mentioned above). After the experimental treatment, all seedlings from the drought treatment, but without inoculation, died due to an unusual heat wave in the summer (Figure 2). Only12 pots of seedlings survived (4 pots for each of the remaining groups, Table 1)

Sampling and measurements

In August 2023, four pots (not transferred) from each group were selected based on their similarity, and the total fresh weight, shoot and root fresh weight, collar diameter, and height were recorded. Root systems with ECM were sampled randomly and fixed in ethanol for ECM identification and ECM rate assessment (by their phenotypes). Additional roots were collected and frozen at −80°C for future amplicon sequencing of the ECM fungal community. Owing to the high loss rate and the low remaining sample number, we opted not to perform this sequencing analysis for this pilot experiment. The ECM phenotype was identified using the DEEMY database (http://www.deemy.de/).

The Kolmogorof Smirnov test was conducted on the data of each group using R software to determine the distribution. All group data fit a normal distribution ($p > 0.05$).

Then the standard deviation for each group and overall Levine test were conducted by using R packages: “psych” (Revelle, 2024), and “car” (Fox and Weisberg, 2019) to test their homogeneity of variance. The standard deviations of each two groups were different, except for those of groups B and D (both $p = 0.01$). According to the results of the Levene test, all groups had similar variances ($p > 0.1$). Therefore, variance between different groups was analyzed by Student’s t-test (Table 2), and it was performed using Excel.

Results

C. geophilum – like and other ECM fungi, originally in the soil of Montiers experimental site, colonized on the roots of the lower soil layer and of the lower than second-class lateral roots (according to my observation). A relatively low colonization rate of *C. geophilum* was observed (approximately, < 15%, according to my observation). Colonized and non-colonized new root systems are quite different. Most of the new roots are first-class later roots, which help seedlings to have a better root system (supporting shoots fixing on the ground). They have a relatively larger diameter and are fleshier, which cannot be recognized as fine roots and are more frequently formed in spring (March to May). The colonized roots are fine roots and are more abundant in summer. Three main ECM morphotypes were identified in this study. According to our DEEMY database identification, these ECM were established by *Lactarius*, *C. geophilum,* and *Tuber* species (Figure 3). In a pre-test experiment carried out by Dr. Deveau (INRAE – Nancy) (which used same way, but no *C. geophilum* inoculation), ECM of *Tuber* spp. was found in both drought-treated and control seedlings. The ECM root tips of *C. geophilum* and *Tuber* were then counted, and their colonization rate was measured (Table 3). Since only colonized root systems were selected to count the colonization rate, the total mycorrhizal rates of the whole root system were much lower. For example, the highest colonized beech seedling, C26, had 67.3% colonized root tips, but only about less than 5% root tips were colonized in the whole root system (according to my observation). During the drought treatment, all trees in group D died. Therefore, they had more dead root tips and dead ECM. The other group (B) with drought treatment also had more dead roots than control group A (Figure1 g and h, according to my observation and obviously).

Table 1 Pot names and amounts of beech seedlings in the different experiment steps and treatments.
+/- Cg means with/without *C. geophilum 1.093* inoculation; +/- drought means with/without drought treatment

Groups by treatments	Starting pots before inoculation		Survived pots after inoculation but before drought treatment		Survived pot names afte drought treatment		Selected pots for sampling	
GroupA: +Cg -drought	18, 20, 31, 59, 65, 68, 70	7 pots	18, 20, 31, 59, 65, 68, 70	7 pots	18, 20, 31, 59, 65, 68, 70	7 pots	18, 31, 68, 70	4 pots
GroupB: +Cg +drought	2, 6, 33, 38, 52, 60,73	7 pots	2 (checked ectomycorrhizas), 33, 38, 52, 60	5 pots	2 (died during drought treatment), 33, 38, 52, 60	4 pots	33, 38, 52, 60	4 pots
GroupC: -Cg -drought	9, 10, 26, 27, 35, 45, 64, 76	8pots	9, 10, 26, 27, 35, 45, 64, 76	8pots	9, 10, 26, 27, 35, 45, 64, 76 (8pots)	8 pots	9, 10, 26, 76	4 pots
GroupD: -Cg +drought	21, 47, 49, 50, 53, 58, 61, 66	8pots	21, 47, 49, 50, 53, 58, 61, 66	8pots	21 (brown leaves), 53, 61(brown leaves) (3 pots)	3 pots	21, 53, 61	3 pots

Table 2 Statistical analysis results of *C. geophilum* colonization rates
The KS test, Levene test and test results of each treatment groups or treatment group pairs are summarized.

Group names	KS test D value	KS test p value	Standard deviation	Overall variance Levene test F value	Overall variance Levene test Pr	Group pairs	T test p-value
A	0.25475	0.8953	0.02	2.6121	0.1039	A&B	0.04356479
B	0.28803	0.8943	0.01			C&D	0.33597897
C	0.26166	0.8786	0.06			A&C	0.60847036
D	0.34598	0.7438	0.01			B&D	0.20441552

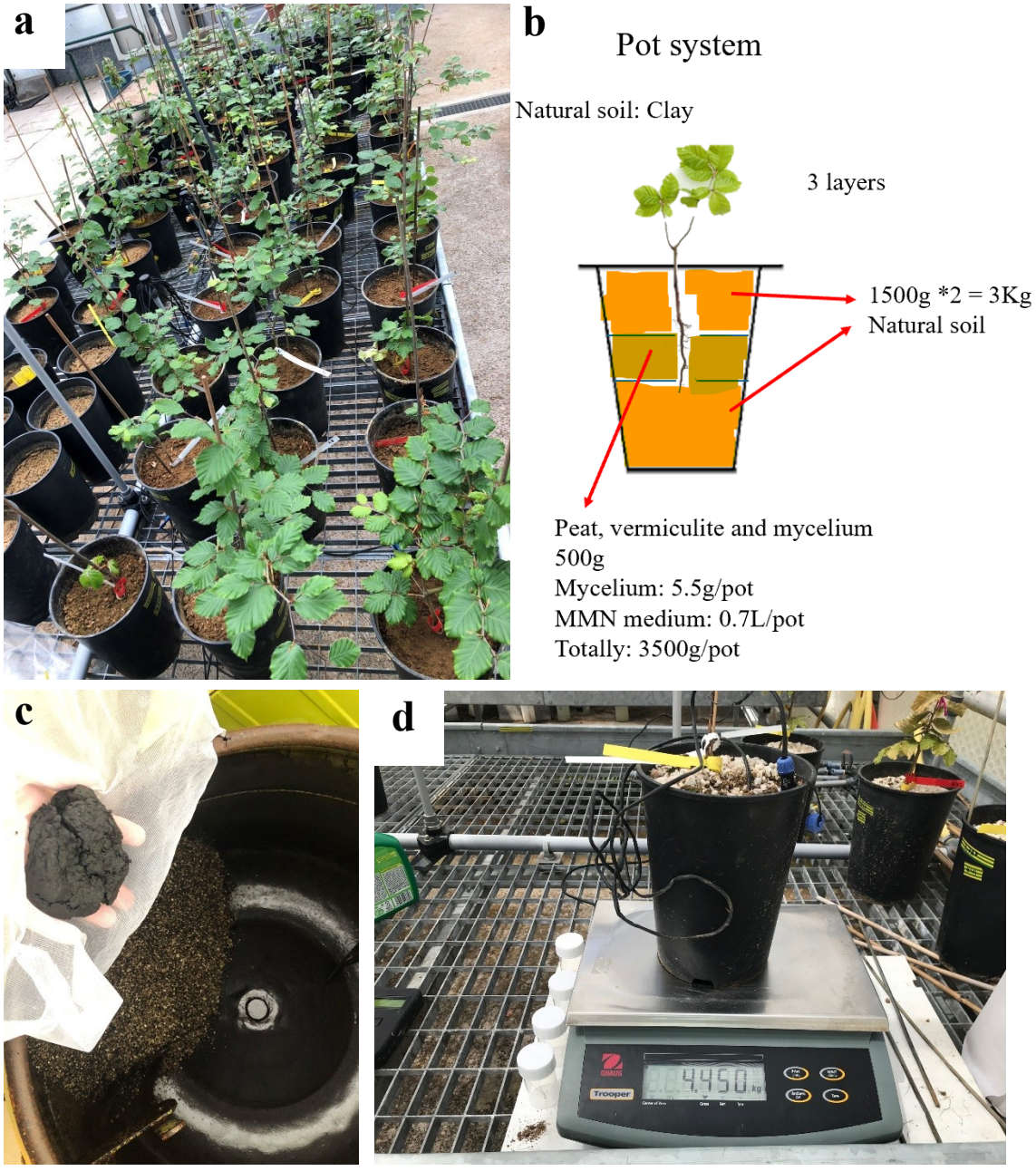
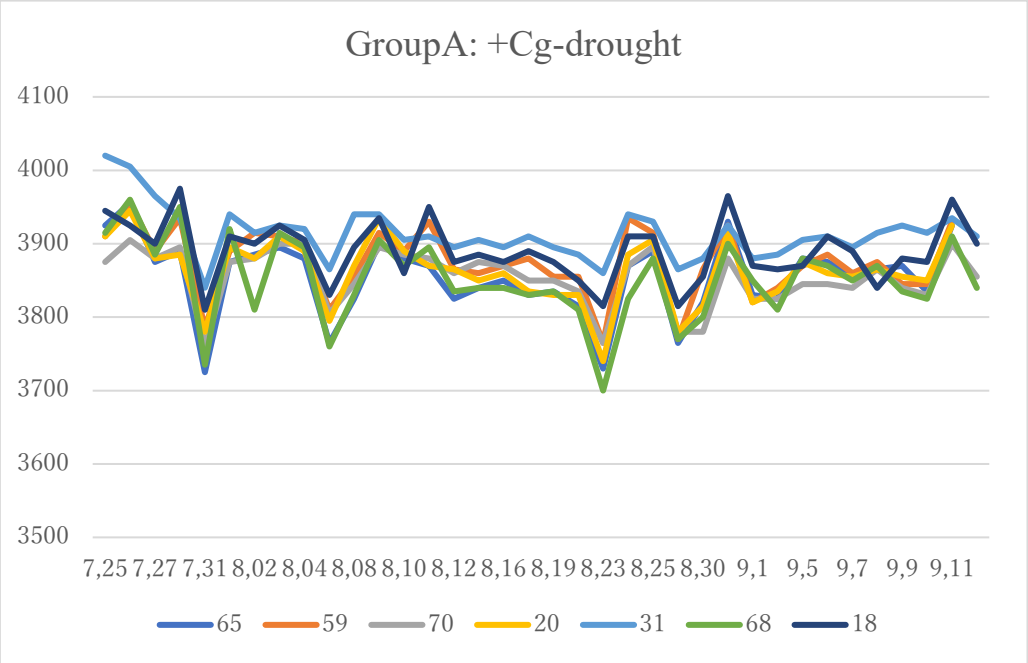
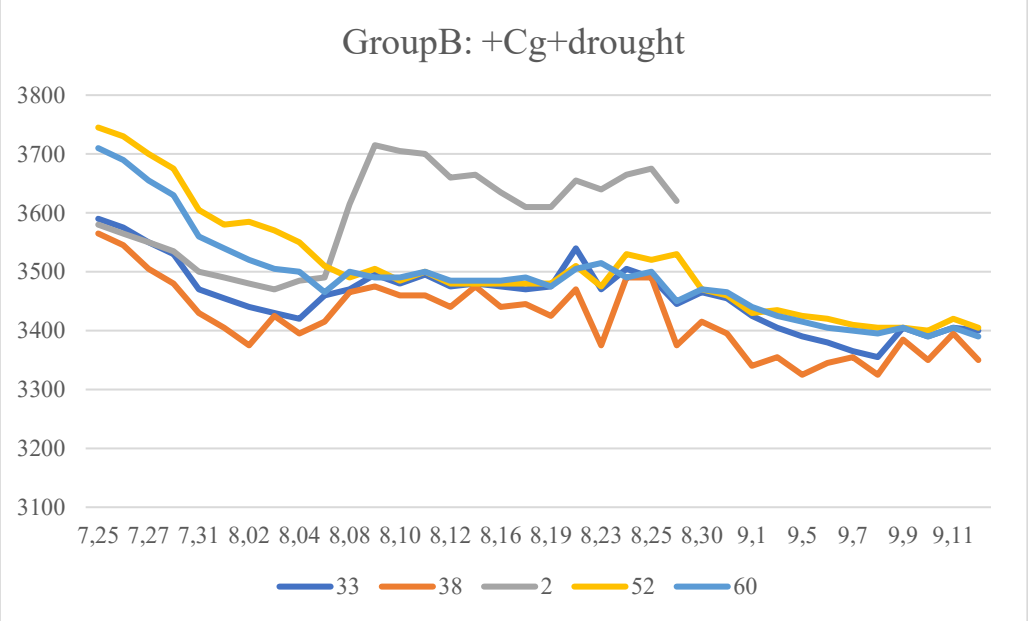
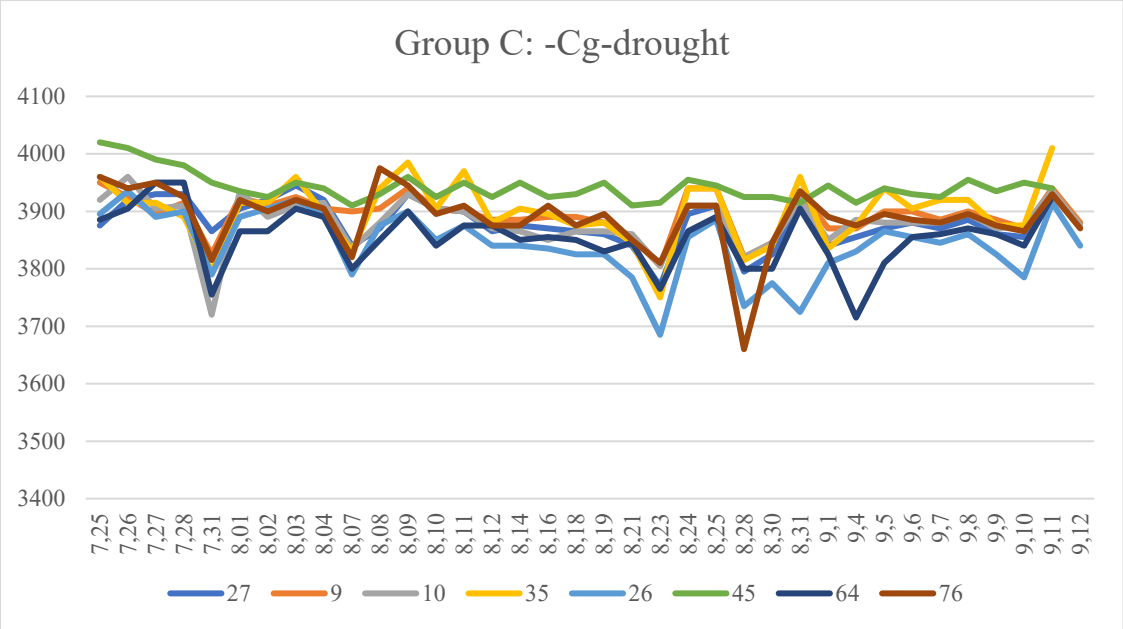


Figure 1 Experimental set-up
a. Beech seedlings in the green house b. Pot system with three layers;
c. Filtered mycelium from liquid culture mixed with peat and vermiculite;
d. Weighing of the pots for control of soil humidity.


GroupA: +Cg-drought
4100
4000
3900
3800
3700
3600
3500
7,25 7,27 7,31 8,02 8,04 8,08 8,10 8,12 8,16 8,19 8,23 8,25 8,30 9,1 9,5 9,7 9,9 9,11
65
59
70
20
31
68
18


GroupB: +Cg+drought
3800
3700
3600
3500
3400
3300
3200
3100
7,25 7,27 7,31 8,02 8,04 8,08 8,10 8,12 8,16 8,19 8,23 8,25 8,30 9,1 9,5 9,7 9,9 9,11
33
38
2
52
60



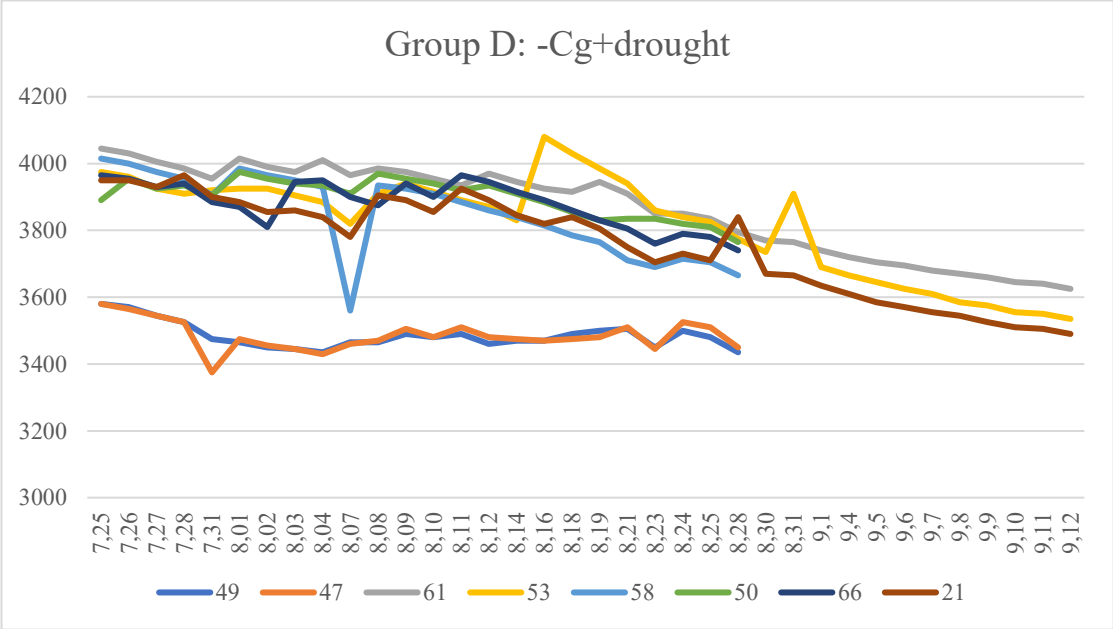


Figure 2 The record of pot weight
The pot weight reflects the soil humidity.
Y-axis showing the weight of pots; X-axis showing the dates for weighing the pots
+/- Cg means with/without *C. geophilum 1.093* inoculation;
+/- drought means with/without drought treatment
Seedlings 47,49 dead at the beginning, no watering anymore

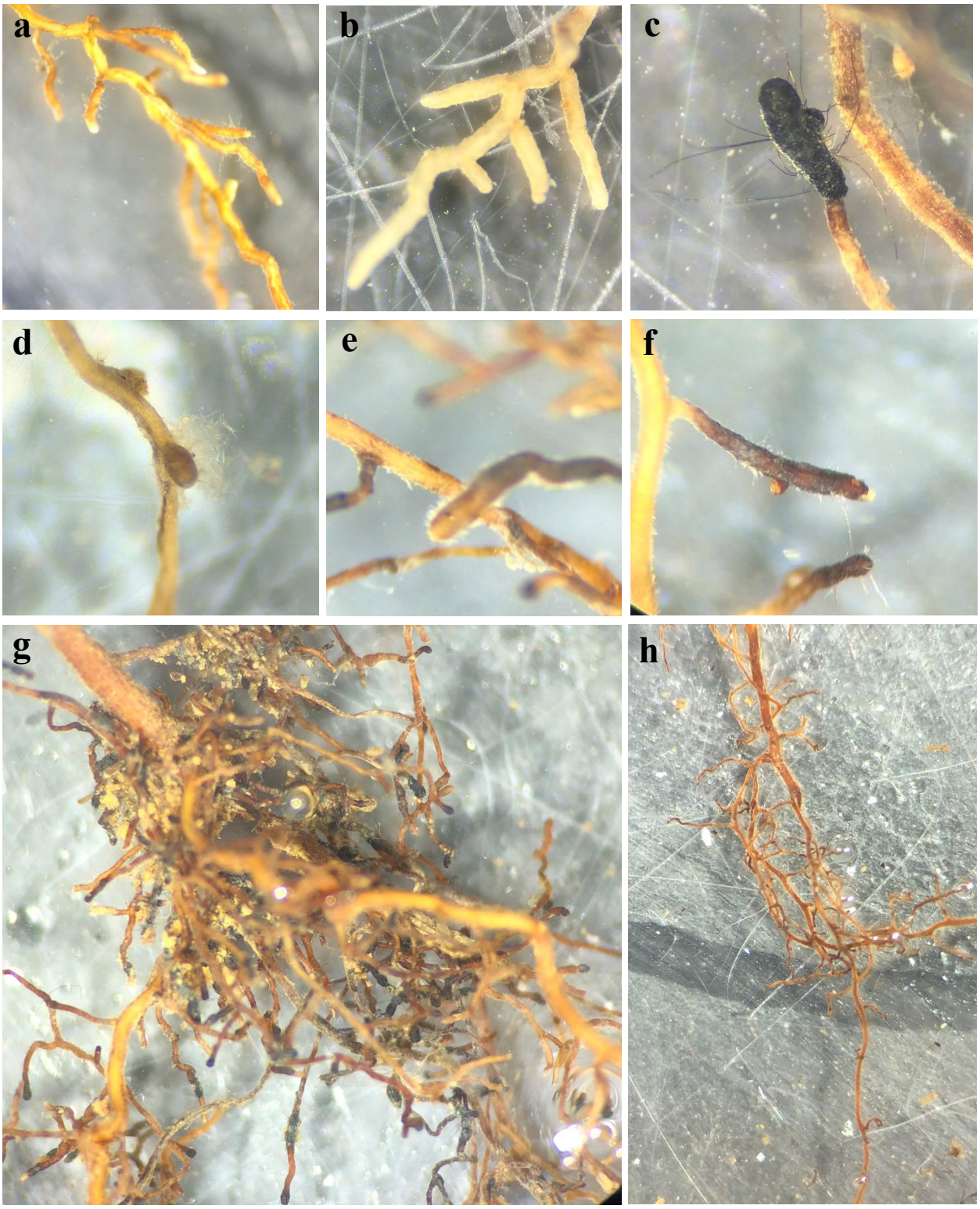


Figure 3 Selcted root tips of beech seedlings.
a. Unknown yellow color ectomycorrhizae b. *Lactarius*-like ectomycorrhizas;
c. *Cenococcum geophilum*-like ectomycorrhizas; d. *Tuber aestivum*-like ectomycorrhizas;
e. *Tuber borchii*-like ectomycorrhizas; f. Unknown black color ectomycorrhizas;
g. Dried out, dead ectomycorrhizas; e. Dried out, dead root system

Table 3 Colonization rates and identification of ectomycorrhizal fungi by morphotype in the different treatments.
+/- Cg means with/without *C. geophilum 1.093* inoculation; +/- drought means with/without drought treatment
Dead root tips were counted within the non-mycorrhiza group, not separately. Dead ectomycorrhizas were counted separately.

GroupA: +Cg-drought	Mycorhiza	Mycorrhizal rates	Non-mycorrhiza	Dead root tips (no mycorrhiza)	Dead mycorrhiza	Note for dead mycorrhiza	Cg	Cg mycorrhizal rate	*Tuber Spp.*	Tuber mycorrhizal rate	Dominant	Other notes
18	989	18.44%	4203	172	0		268	5.16%	484	9.32%	Cg	
31	1191	18.41%	4696	581	107	Only Cg	19	0.32%	37	0.62%	Cg	
68	1680	41.06%	2282	130	0		125	3.15%	207	5.22%	Yellow color mycorrhiza	
70	2531	62.17%	1311	229	0		210	5.47%	0	0.00%	Yellow and black	
GroupB: +Cg+drought												
33	1019	33.49%	1695	329	65	Dead yellow color mycorrhiza	0	0.00%	0	0.00%		
38	1526	58.92%	975	89	0		0	0.00%	0	0.00%	Yellow	
52	510	12.82%	2996	473	24		39	1.10%	0	0.00%	Light black mycorrhiza (stainned yellow by Cg mycelium?)	Old Cg
60	114	3.48%	2856	302	16		18	0.60%	0	0.00%	Dead roots	Old Cg
GroupC: -Cg-drought												
9	1392	23.94%	3419	1004	127		32	0.65%	0	0.00%	Yellow	
10	3095	39.62%	4012	704	26	Only Cg	411	5.76%	0	0.00%	Yellow	Rotted roots have Cg(dominant)
26	2609	67.38%	1247	16	0		33	0.86%	2382	61.77%	Tuber	Rotted roots have Tuber(dominant); have Cg sclerotium
76	2149	53.25%	1564	323	0		524	14.11%	515	13.87%	Yellow + Cg + Tuber	Old Cg; New Cg on no other mycorrhiza root system
GroupD: -Cg+drought												
21	590	9.26%	2943	2841	105	Cg:20	21	0.58%	15	0.41%	Dead roots	black fungi not Cg and tuber
53	3239	34.31%	3101	3101	1210		197	2.61%	21	0.28%	black fungi not Cg and tuber	
61	1113	19.01%	2451	2292	1081		38	0.82%	5	0.11%	Dead roots	black fungi not Cg and tuber

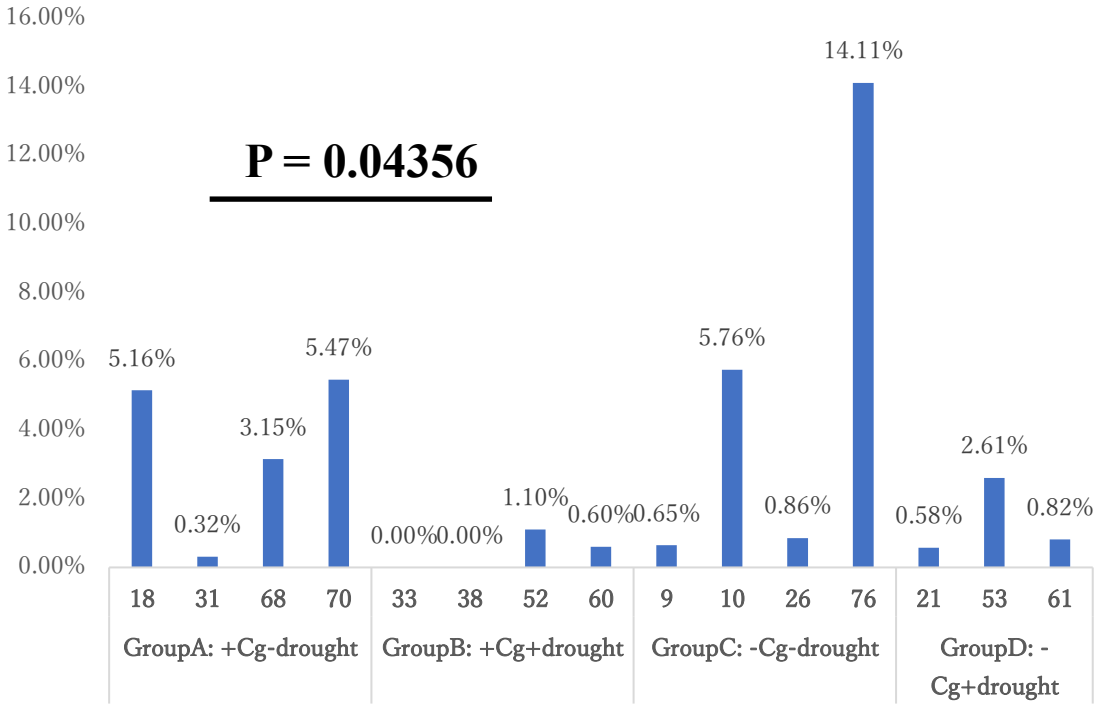


Figure 4 *C. geophilum* colonization rates in the different groups
X-axis showing the colonization rates of *C. geophilum*;
Y-axis showing the treatment groups and the names of pots;
The colonization rates of group A and B are significant different.
+/- Cg means with/without *C. geophilum 1.093* inoculation;

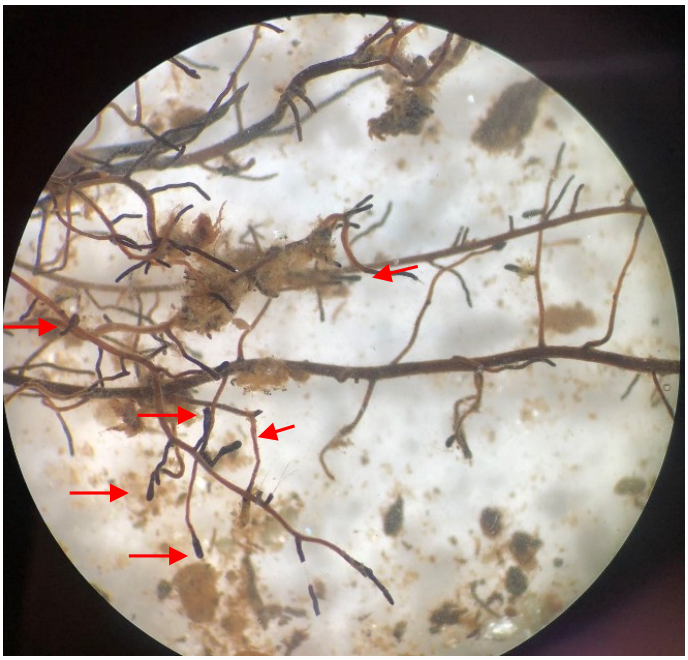


Figure 5 *C. geophilum*-like fungus is able to colonize beech seedlings
Arrows ectomycorrhizas

Drought treatment significantly reduced the colonization rate of *C. geophilum* (among the Cg-inoculation groups: p = 0.0435) and *Tuber spp.* (colonization rates variate from 0.41% to 0%), but a small number of *C. geophilum* ECM survived (Figure 4). Not all ECM root tips were affected equally by drought treatment. Two unknown ECM fungi dominated and increased their colonization rate under drought treatment: a yellow ECM fungus (Figure 3 a) in the C. *geophilum* inoculation group, and a black ECM fungus (Figure 3 f) in the control group.

Discussion

In this study, we compared beech seedlings grown in pots containing natural soil or soil mixed with *C. geophilum* 1.093 and subjected them to drought treatment or control conditions. Our results indicate that drought significantly decreased the colonization of beech seedlings by *C. geophilum*, suggesting that this fungus is unable to form additional ECM root tips on beech roots under drought conditions. Groups C and D did not receive additional inoculation with *C. geophilum* 1.093 but contained *C. geophilum* isolates from natural soil. *C. geophilum* ECM were likely formed more by these 'wild' isolates, suggesting that they are more efficient than C. geophilum 1.093 to drought stress. Consequently, it would be valuable to isolate these locally adapted strains. Furthermore*, C. geophilum* 1.093 did not play a central role in the ECM-fungal community when the host experienced drought stress. Thus, in our experimental setup, strain 1.093 was unable to improve its host survival rate. This may be related to the short duration of the experiment. The results of our experiment do not support Hypothesis 1. However, we tested only one isolate in a greenhouse. Testing other isolates from different clades (Obase et al., 2017, also see Chapter 2) and inoculating roots under field conditions would contribute to a more comprehensive understanding of *C. geophilum* under drought stress.

The leaves and fine roots of beech trees are sensitive to drought stress (Leuschner, 2020). In the present study, most of the roots died rapidly after drought stress. One reason could be that the trees were in relatively small pots, and the beech – ECM symbiosis suffered double stress, drought, and heat (over 35 °C). Furthermore, under natural conditions, beech seedlings grow under their mother trees, which provides a shaded and less stressful environment. Light conditions may also co-affect the drought

treatment, since the drought treatment in the experiment was conducted during summer. Pena et al. (2013) investigated the influence of ECM fungi (including *C. geophilum*) on the architecture of and N partitioning in young *F. sylvatica* plants in response to different light regimes and water deprivation. The results indicated that ECM fungi were able to benefit beech seedlings under mild drought, but the responses differed depending on light availability: shaded ECM plants showed a delay in the decrease of pre-dawn water potential.

Recently, several studies have shown that the exploration type of ECM fungi (Agerer, 2021) is closely linked to their colonization ability under drought stress. Long-exploring-type ECM fungi have more ECM with beech in the field under drought stress (Nickel et al., 2017). However, a potted oak seedling inoculation experiment showed that the long-exploring type of ECM fungi colonized fewer root tips under drought stress (Xie et al., 2024a) which has been shown related to carbon limitation (Xie et al., 2024b). Beech trees have higher root turnover rates (Nikolova et al., 2020). Therefore, the use of a long-exploration-type ECM fungus as a partner could be costly and detrimental. In this study, we found that the amount of contact type and short-distance type (Agerer, 2021) ECM (*Lactarius, Cenococcum*, and *Tuber*, probably linked to the inoculation source from natural soil) decreased. However, it is unknown whether these ECM fungi can recover rapidly. Unknown ECM fungi have been identified. According to their morphology, they are short – middle distance exploration types. This finding supports the energy limitation hypothesis (Xie et al., 2024b). However, both the second and the present study were conducted in pot systems and not in the forest.

It remains to be shown whether the root system, the rooting style, plays a role in the drought tolerance of beech. Danzberger et al. (2024) investigated the fungal community response of beech seedlings to drought, which were established from the same seed source and grown in three soils with different precipitation histories, but similar water retention properties. They found that seedlings grown in dry region soils with adapted fungal communities were the most affected by drought. And the result showed the drought responses of beech seedlings depend largely on their different rooting patterns and less on soil fungal communities that are adapted to long-term precipitation conditions. In our study, we used a relatively short inoculation period. However, in the inoculation period, the new roots were mainly structural roots (main root or first-class

later root) instead of absorbing roots (second- or lower-class later roots) – fine roots. Therefore, it was probably difficult for *C. geophilum* to colonize the roots. For future experiments, we would suggest waiting for beech rooting and adding the inoculum until the beech seedlings start to form new fine roots. In addition, although the results showed high ectomycorrhizal fungal colonization for sampled root system (part of whole root system) in some treatment groups of beech seedlings, they still had a low colonization rate on the whole root system level. It seems that there is an age effect in the beech root systems. Although the beech tree is known as a shallow root system-forming species, at least for 27 years, the tree maintains its deep main roots to have a better structure (Providing support for shoots. Harley, 1940). As the trees aged, the long surface branches became increasingly important as absorbing organs, and at 80 years, shallow outgoing branches of the heart root comprised the greater part of the functional root system (Harley, 1940). Therefore, the seedlings had more main roots or first-class later roots than the fine roots. In this period, although some shallow roots can be colonized, the ectomycorrhizal fungi probably have difficulties in colonizing most of the beech roots because of the physical matter – depth of the soil. Some fungi cannot use nutrients from the mineral layer – deep layers (Morel et al., 2006). Therefore, it is important to consider the living layer, exploring type of ectomycorrhizal fungi and root structure of the host tree.

In the present study, we used a three-layer pot system to inoculate beech seedlings. *C. geophilum* mycelium was in the 0–5 cm layer, and peat vermiculite has a looser soil structure than natural soil (clay). However, during drought treatment, this layer works like a filter, making the whole pot system soil inhomogeneous and making the first and second layers dry out. It stopped the water transfer from the bottom back to the upper layers through the water potential gradients and caused water to enter the lower layers. Therefore, in future experiments, it would be better to use sterile natural soil to make the inoculum or to pre-inoculate the beech in a growth chamber before being transferred to the greenhouse.

Conclusions

In our experimental setup, *the C. geophilum* isolate 1.093 failed to enhance ECM formation under drought conditions. Future studies should consider several factors: the

ECM exploration type, the status of the root system, the capacity of different-aged beech hosts to form fine roots, the inoculation technique (utilizing sterilized natural soil for a more uniform inoculum or implementing a pre-inoculation phase after beech seedlings begin developing new fine roots), and environmental factors (seasonal changes, light conditions).

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Chapter 4

Establishment of ectomycorrhizal associations between ectomycorrhizal fungi, including *Cenococcum geophilum* and beech (*Fagus sylvatica* L.) seedlings using an in vitro experimental system

Establishment of ectomycorrhizal associations between ectomycorrhizal fungi, including *Cenococcum geophilum* and beech (*Fagus sylvatica* L.) seedlings using an in vitro experimental system

Abstract

European beech (*Fagus sylvatica* L.) is an economically and ecologically important tree species in Europe. In recent decades, several *in vitro* experimental systems have been established to investigate the molecular mechanisms underlying the development and function of ectomycorrhizal symbiosis (ECM). However, no in vitro system has yet been developed to produce ECM using beech seedlings, plantlets, and ECM fungi, despite the existence of protocols for the *in vitro* micropropagation of beech cuttings. In this study, we examined various approaches to obtain *in vitro* cultures of beech from different explants, such as seeds and branches, from local forests. Additionally, we conducted preliminary tests to establish ECM symbiosis between the *in vitro* cuttings of beech lines and various ECM fungi. We successfully established an *in vitro* system for rooting *F. sylvatica* lines (FS-ap, FS-10-4), *Fagus orentalis* lines (FO-BS, FO Clone3, FO Clone30), obtained from Dr. Castro and Martínez (CSIC). We found that the beech line Fs-ap can form a mantle, but not a Hartig net, with *Cenococcum geophilum* 1.093 and 1.058, *Laccaria bicolor* S238N, and *Lactarius subdulcis* 3. *C. geophilum* 1.093 formed distinct endophytic structures in the root tips of beech Fs-ap. L. subdulcis 3 can establish a fungal mantle on roots of the beech line Fo-BS, but no Hartig net was observed.

Introduction

European beech (*Fagus sylvatica*) is an important tree species in Europe. After several unusually dry years in France and Switzerland, many beech diebacks were reported. Climate change threatens the sustainability of forest ecosystems. The development of forest management approaches to improve the drought resistance of forest trees has become increasingly important (Antonucci et al., 2021). Ectomycorrhizal (ECM) fungi form mutualistic interactions with the roots of the forest trees. They increase the root uptake area within the soil and facilitate water and nutrient uptake from the soil (Smith et al., 2021). This beneficial symbiont is a good candidate to assess the ability of ECM

fungi to enhance drought adaptation in trees.

Many ECM fungi, such as species from the genera *Laccaria*, *Lactarius*, *Cenococcum*, *Amanita*, and *Russula,* are known to form symbiotic associations with beech trees (Boeraeve, 2021). Some of these, such as *Lactarius subdulcis* and *Lactarius blennius,* are commonly found to be associated with beech trees (Eryssartier & Roux, 2017). *Cenococcum geophilum* establishes ECM associations with oak, but can form ectendomycorrhizas with beech (see Chapter 1). Both abundant and rare fungi colonize beech trees (Dietrich et al., 2022). Greenhouse experiments or field research on ECM fungi have used beech as a host material in natural soils. A series of early studies on beech ECM focused on carbohydrate metabolism. The fungus can absorb sugar from its host, trehalose and mannitol are synthesized by the fungus from glucose imported from their host. These reserve sugars are not readily available to the host (Lewis and Harley 1965 a,b,c). Anthony et al. (2022) studied both broadleaf and conifer forest (including European beech forest). Their results showed that beech tree growth was linked to mycorrhizal fungal composition and function across Europe. Audisio et al. (2024) used potted sapling pairs of European beech and North American Douglas-fir (*Pseudotsuga menziesii*) for $^{13}CO_2$ pulse-labeling. The ^{13}C transferred from beech (donor) to either beech or Douglas-fir (recipient) was compared. The results showed that beech recipients shared more ECM species and had a higher ^{13}C enrichment. However, little is known about the molecular mechanisms driving ECM development and metabolism in beech trees, mainly because of the lack of a simplified model. The development of an in vitro beech mycorrhizal system is important for closing this knowledge gap. Several model systems have been used to study ECM development at a molecular level. Among these, the *Laccaria – Populus* (Felten et al., 2009), *Suillus – Pinus* (Lofgren et al., 2024), *Pisolithus – Eucalyptus* (Burgess et al., 1996), *and Piloderma-Quercus* (Krüger, 2004) systems have been used to advance our understanding of ECM symbiosis. To the best of our knowledge, no in vitro system has yet been established to investigate ECM symbiosis in beech trees.

An *in vitro* micropropagation protocol for beech has been established previously (Chalupa, 1996). Vieitez et al. (1993) obtained *F. sylvatica* lines from the embryonic axes of seeds and cultured them on woody plant medium (WPM) supplemented with 6-benzyladenine (BA). Shoots and basal segments were transferred to WPM medium

supplemented with 0.5 mg/L BA, 2, and 0.2 mg/L 1-Naphthaleneacetic Acid (NAA). Karin and Gerhard (1994) investigated the endogenous factors necessary for the micropropagation of mature *F. sylvatica*. The results indicated that the optimal time to obtain explants was February, and the most suitable explant source was apical buds > 20 mm from 1-year-old shoots. Cuenca and Vieitez (1999) examined the effects of carbon source and concentration on shoot multiplication in *F. sylvatica* and *F. orientalis*. 3-4% glucose was determined to be the optimal carbon source and concentration. The response of shoot cultures to different carbohydrate treatments appeared to be genotype-dependent to some extent. Cuenca et al. (2000) utilized previously obtained materials to assess the bud regeneration capacity. The highest regeneration potential was observed in apical internodes, whereas those distal to the apex were the least productive. The elongated shoots of adventitious origin demonstrated the ability to proliferate via axillary branching. The optimal bud induction medium was found to be WPM containing 30 g/L sucrose, 7 g/L agar, 2.9 µM IAA and 4.5 µM thidiazuron (TDZ). The shoot proliferation medium consisted of WPM supplemented with 2.2 µM BA, 9.1 µM zeatin, 2.9 µM IAA, 20 g/L glucose and 7 g/L agar. The optimal rooting medium was determined to be 1/2 WPM with 20 g/L sucrose without any auxin.

In the present study, we established *in vitro* lines of *F. sylvatica* and *F. orientalis* under laboratory conditions and conducted preliminary experiments to test the mycorrhizal ability of a set of ECM fungi, including *Lactobacillus subdulcis* strain 3*, Cenococcum geophilum* (strains 1.58 and 1.093), and *Laccaria bicolor* strain S238N.

Materials and methods

Plant and fungal materials

We first attempted to obtain sterilized beech seedlings from seeds and explant materials from sterilized branches of beech seedlings in winter (Figure 1, test 1). Seeds were purchased from Vilmorin (FSY201 NORD-EST /France and FSY501 JURA /France, 2021) and pretreated by this company for gemination. The shells of seeds were removed, and the seeds were sterilized using 30% H_2O_2 for 8 min and transferred to MMN medium to check the contamination to have different genetic backgrounds except in

vitro beech plantlets. New shoots of *Fagus sylvatica* var. *pendula* (over 50 years), *orientalis* × *sylvatica* (over 50 years), *orientalis* (over 50 years), *sylvatica* (mixed: 30 - 40 years and less than 5 years), and var. *tortuosa* (approximately 20-30 years) were cut from trees growing in the INRAE arboretum (Champenoux, France). They were cut into 10-20 cm segments that were sterilized using copper oxychloride (purchased from Cultivers, sterilizing method described by Martínez et al., 2017), and cultured in plastic boxes filled with perlite and distilled water (Figure 2). The plastic boxes were tightly closed using tape. The culture conditions were as described by Cuenca et al. (2000) and light cycling by Felten et al. (2009).

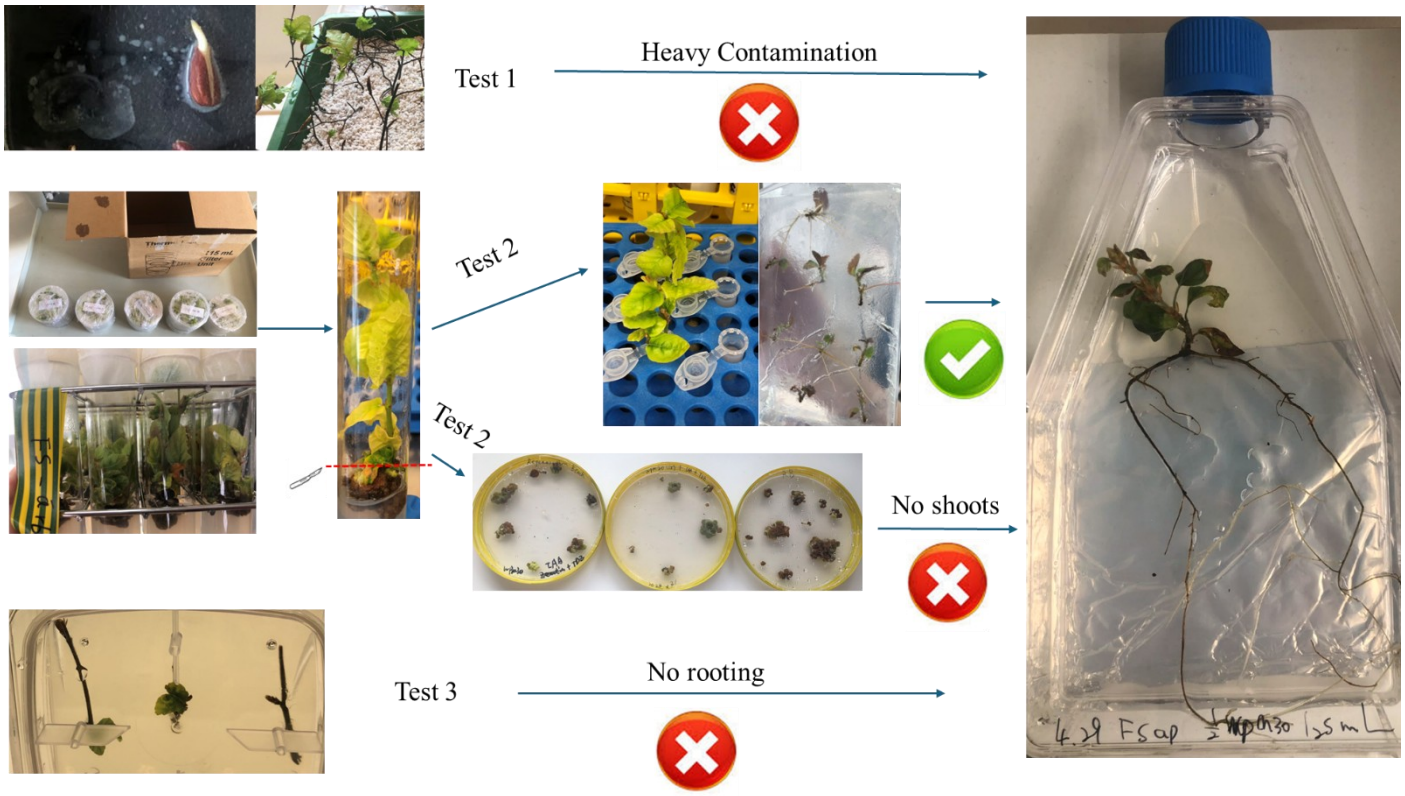
Second, we obtained *in vitro* beech cultures. *Fagus sylvatica* lines (FS-ap, FS-10-4), *Fagus orientalis* lines (FO-BS, FO Clone3, FO Clone30), and their culturing protocols were obtained from Dr. Elena Corredoira Castro and María Teresa Martínez, Consejo Superior de Investigaciones Científicas (CSIC), Spain (Vieitez et al., 1993; Cuenca and Vieitez, 1999; Cuenca et al., 2000). Briefly, cuttings were cultured in a proliferation medium (Cuenca et al., 2000). They were then rooted in a rooting medium (Figure 1, test 2). After rooting, only the FS-ap line was used for tissue culture, whereas lines FS-ap and FO-BS were used for synthesis. Petri dishes cannot provide enough space for beech growth, and tubes cannot provide enough surface area for mycelia and roots to contact each other. When using Petri dishes, in contrast to MS medium, WPM medium has more (approximately 50 ml, the volume is known after using the flasks) water at the bottom, which cannot be controlled by adding cotton rolls (up to six rolls cannot control all the water) and always causes heavy contamination. Therefore, we used Falcon® 225 cm^2 or 150 cm^2 (surface area) rectangular canted neck cell culture flasks with vented caps (Dutscher/France) for culturing and rooting the beech cuttings. Furthermore, we used TPP® Tissue Culture Flask with Vented Cap and re-closable Lid 150 cm^2 (Dutscher/France) for mycorrhiza development.

Third, we also attempted to obtain local *in vitro* cultures from young trees using the culturing protocol. One hundred branches were sampled from 20-year-old trees in the Amance State Forest, near the INRAE-Nancy Center. They were cut into 5-10 cm segments with the basal part of the leaves (Figure 1, test 3). They were then sterilized using 70% ethanol for 1-1.5 min and 5% bleach for 8 min. The culture conditions and protocol were the same as those used by our Spanish collaborators (see above).

Starting cultures of strains 1.058 and 1.093 from *C. geophilum,* S238N from *L. bicolor,* and strain 3 from *L. subdulcis* were obtained from the INRAE-IAM Fungal Collection. The mycelium was transferred by cutting 2 cm diameter plugs (5 plugs/cellophane) and cultured on MMN medium supplemented with 20 g/L glucose. Two pieces of cellophane were placed between the medium and plugs. Before contact with the roots, the mycelium was cultured on cellophane for one month (Figure 3). The plugs were removed from the cellophane. Only the outer part was used for the inoculation.

Experimental design

The beech lines and fungal mycelium (Fs-ap with *C. geophilum 1.058 and 1.093*, *L. bicolor S238N* and *L. subdulcis 3, and* Fo-BS with *L. subdulcis 3*) were placed in contact and cultured using a sandwich system (Felten et al., 2009). Since the in vitro beech, the synthesis medium for beech seedlings was beech rooting medium. Furthermore, P20 medium (Felten et al., 2009) was tested as a synthetic medium for beech lines with *C. geophilum* 1.093 and 1.058. Beech seedlings were co-cultured with fungal mycelia for 2 months. Owing to the limited number of rooted plants, only one plant was tested for each combination. Finally, 5-10 root tips for each combination were fixed in paraformaldehyde for microscopy. Cross-sections for microscopy observations were obtained as described by Basso et al. (2020).



	Explant	Step1	Step2	Step3	Result
Test 1	Purchased seeds	Sterilized with H_2O_2, 8mins	MMN medium to check contaminations		Heavy bacteria contamination
Test 1	Branches from arboretum from old tree in winter	Sterilized with copper oxychloride, 30mins	cultured in plastic boxes filled with perlite and distilled water		Spreaded new branches easily Heavy fungal contamination
Test 2	Cuttings from Spain researchers, 2 *F.sylvatica* lines and 3 *F. Orentalis* lines	The besal callus of culture from spain researchers were removed, only shoot remained	Followed the protocol from Spain researchers		Succeeded to have rooted beech plantlets
Test 2	Callus from Spain researchers, 2 *F.sylvatica* lines and 3 *F. Orentalis* lines	The besal callus of culture from spain researchers were remained	Bud inducing medium for 2 - 3 weeks	Proliferation medium	No buds (new shoots) formed
Test 3	Branches from local young beech tree	Sterilized with ethanol for 1.5-2 min and 5%bleach for 8mins	Bud inducing medium for 2 - 3 weeks	Cut off the basel callus and dipped with 1g/L IBA for 2min, then transfer the rest to rooting medium	Spreaded new branches easily No contamination but can't have root the new branches can be good materail to test rooting for the further research
Bud inducing medium: WPM + 30 g/L sucrose, 7g/L agar (for tubes, 8g/L for flasks), 2.9 µM IAA and 4.5 µM thidiazuron (TDZ)					
Proliferation medium: WPM + 20 g/L glucose, 7 g/L agar (for tubes, 8g/L for flasks), 2.2 µM BA, 9.1 µM zeatin, 2.9 µM IAA					
Rooting medium: 1/2WPM + 20g/L sucrose, 7 g/L agar (for tubes, 8g/L for flasks), without any auxin					

Figure 1 Summary of the propagation tests of beech



Figure 2 Preparing new Beech branches in winter

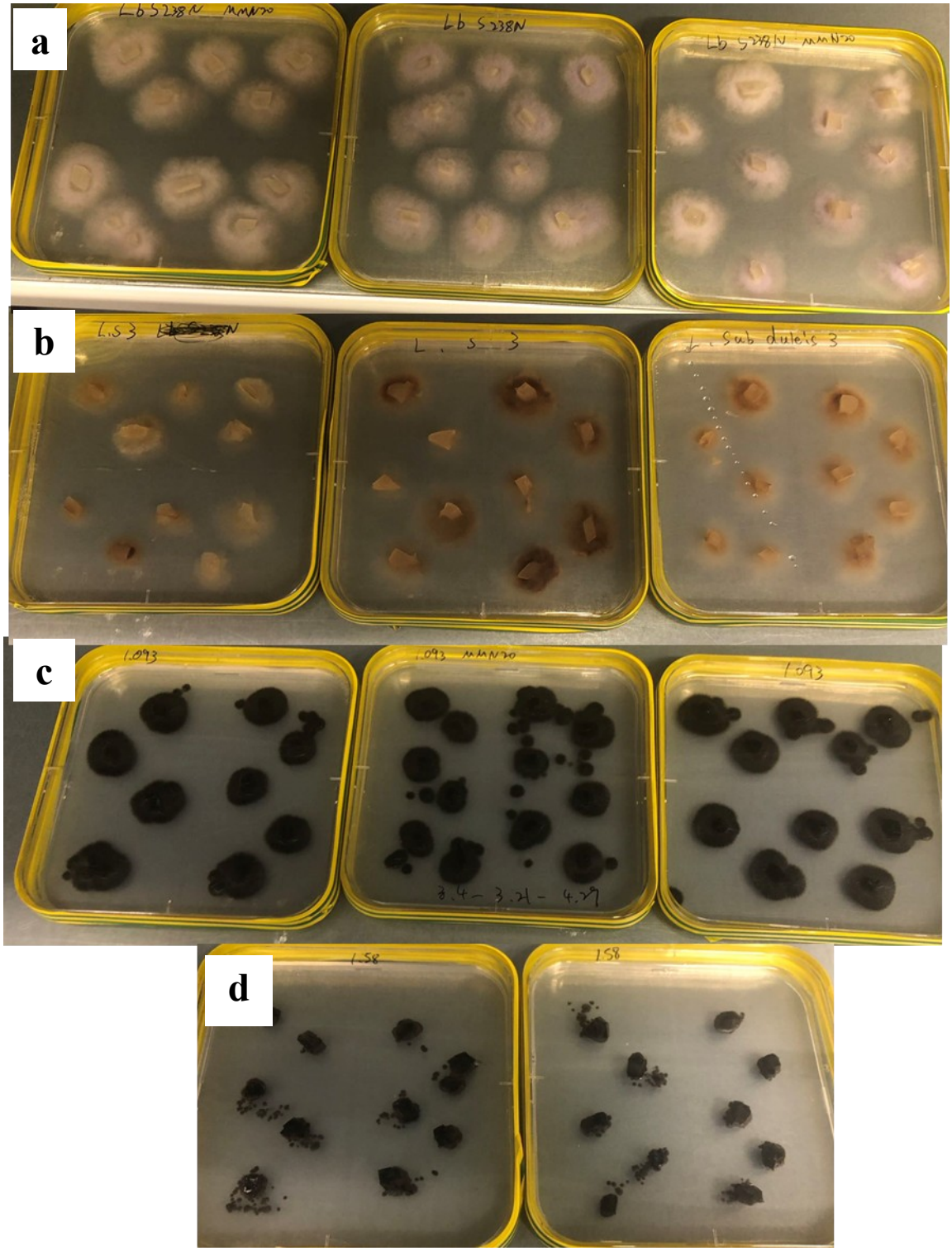


Figure 3 One-month preculturing of fungal mycelium a. *Laccaria biocolor* 238N; b. *Lactarius subdulcis 3*; c. *Cenooccum geophilum* 1.093 and d. 1.058



Figure 4 Examples of Beech seeds germination after sterilizing on MMN medium
Red rectangular showing the contaminated seed

Results

Propagating beech lines from seeds and branches

A low germination rate was observed for beech seeds following treatment with 5% bleach for 10 min and the majority of seeds remained contaminated (Figure 4). Attempts to sterilize beech seedlings from seeds were unsuccessful.

F. sylvatica exhibited the highest number of sprouted buds. In the absence of auxin and cytokinin, the beech branches developed new buds. The application of copper oxychloride proved insufficient for sterilizing beech branches. A significant proportion of beech branches was found to be contaminated. Furthermore, attempts to sterilize beech seedlings from branches of mature trees were unsuccessful.

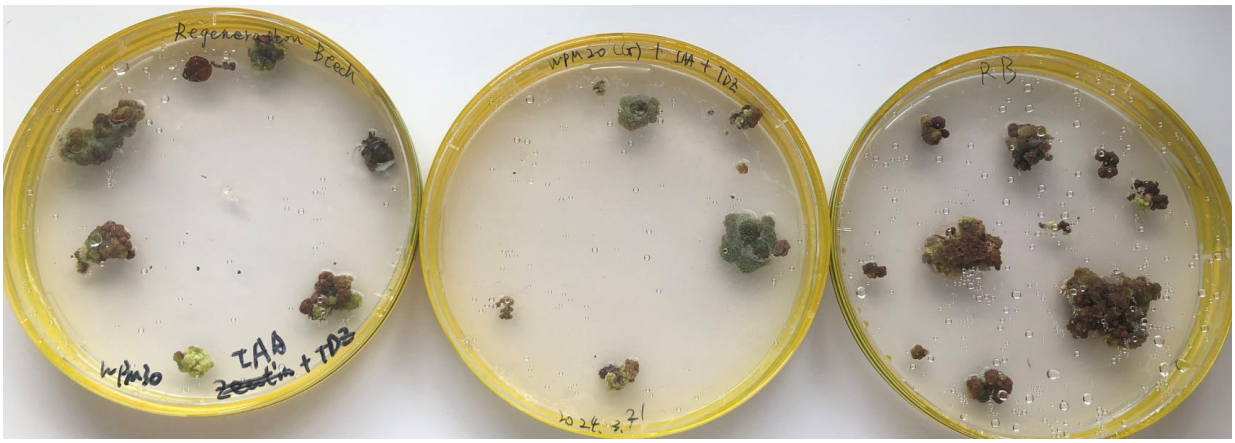


Figure 5 Tissue culture of beech

Tissue culture, micropropagation and rooting of beech in vitro lines (Figure 1, test 2)

All beech shoots grew well on the proliferation medium. Except for Fs-10-4, all lines produced roots in the rooting medium (Figure 5). The segments of in vitro cultures (cuttings) or the basal part of in vitro cultures (only tested Fs-ap) could form calluses using bud induction medium (Figure 5). With calli, cuttings grow better, but after 2-3 months, the callus became brownish and the leaves died (Figure 1, step 2). Beech cuttings had a callus at the basal part could occasionally form another bud (less than 1/50). Therefore, it was difficult to multiply the cultures using bud regeneration in our laboratory. Therefore, we tested whether calluses from the basal part of the culture (without any other shoots on the callus) could form new buds for regeneration. With all media used in published papers or protocols (Vieitez et al., 1993; Cuenca and Vieitez, 1999; Cuenca et al., 2000), the callus never formed a new bud – the beginning of new branches.

Establishing local beech in vitro line (Figure 1, test 3)

Given that callus formation did not result in new shoot development, an alternative protocol was investigated for the *in vitro* multiplication of beech shoots. Branches were collected from 20-year-old trees in the local forest. Unfortunately, these samples exhibited high contamination rates, and no callus formation was observed under our experimental growth conditions. Only two out of 100 sampled branches responded to the proliferation and rooting media by extending new branches from buds and developing without contamination. However, no root formation was observed in the rooting medium after three weeks (Figure 7).

In vitro beech ectomycorrhiza development

The P20 medium was determined to be unsuitable for the ECM formation between beech and *C. geophilum* strains 1.093 and 1.058. The roots ceased growth and exhibited browning (Fig. 8). At this stage, only a loose aggregation of hyphae – a pseudo-mantle – was observed on primary roots (Figure 8). The interaction between FS-ap and *C. geophilum* 1.058 was examined utilizing confocal microscopy on cross sections. Only

the mantle was formed, and no Hartig net was observed (Figure 9).

The tissue culture flasks are good containers for both micropropagation (Figure 6 and 7) and mycorrhiza synthesis for beech (Figure 10-13). After 2 months of coculture, although no typical ectomycorrhiza were observed, the beech line Fs-ap grow differently with the four ectomycorrhizal fungi. The *Lactarius subdulcis 3* – Fs-ap combination died (Figure 13). *Laccaria bicolor* S238N helped beech line Fs-ap grow much better than did *C. geophilum* 1.093 and 1.058 (much more green, Figure 10-12).

Cross sections of the root tips among 4 ectomycorrhizal fungi and 2 beech line combinations

No Hartig net was observed for any combination (Figure 14 and 15). However, all specimens exhibited hyphal aggregation around roots which may correspond to a pseudomantle. The structure of the root tips from beech Fs-ap with *C. geophilum* 1.093 differed from that of other combinations. The cross-section revealed that the mycelium penetrated the epidermis but did not form a Hartig net (Figure 14). It was observed that subsequent to penetration, the mycelium proliferated along the root.



Figure 6 FO-BS (right) and FS-ap (left) with roots



Figure 7 *Fagus sylvatica* cuttings from local forest in rooting medium

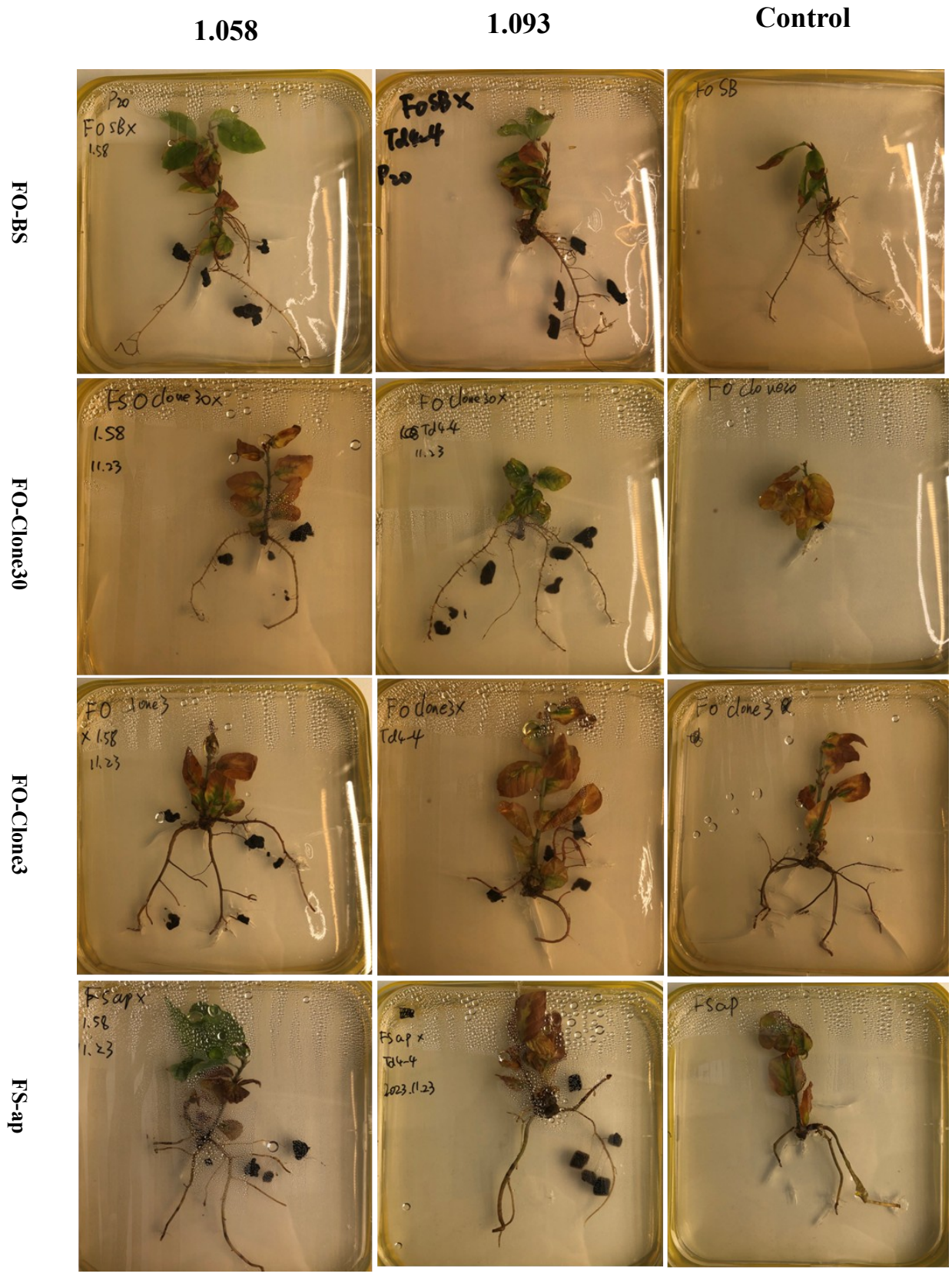


Figure 8 Beech lines with *C. geophilum* 1.058 and 1.093.

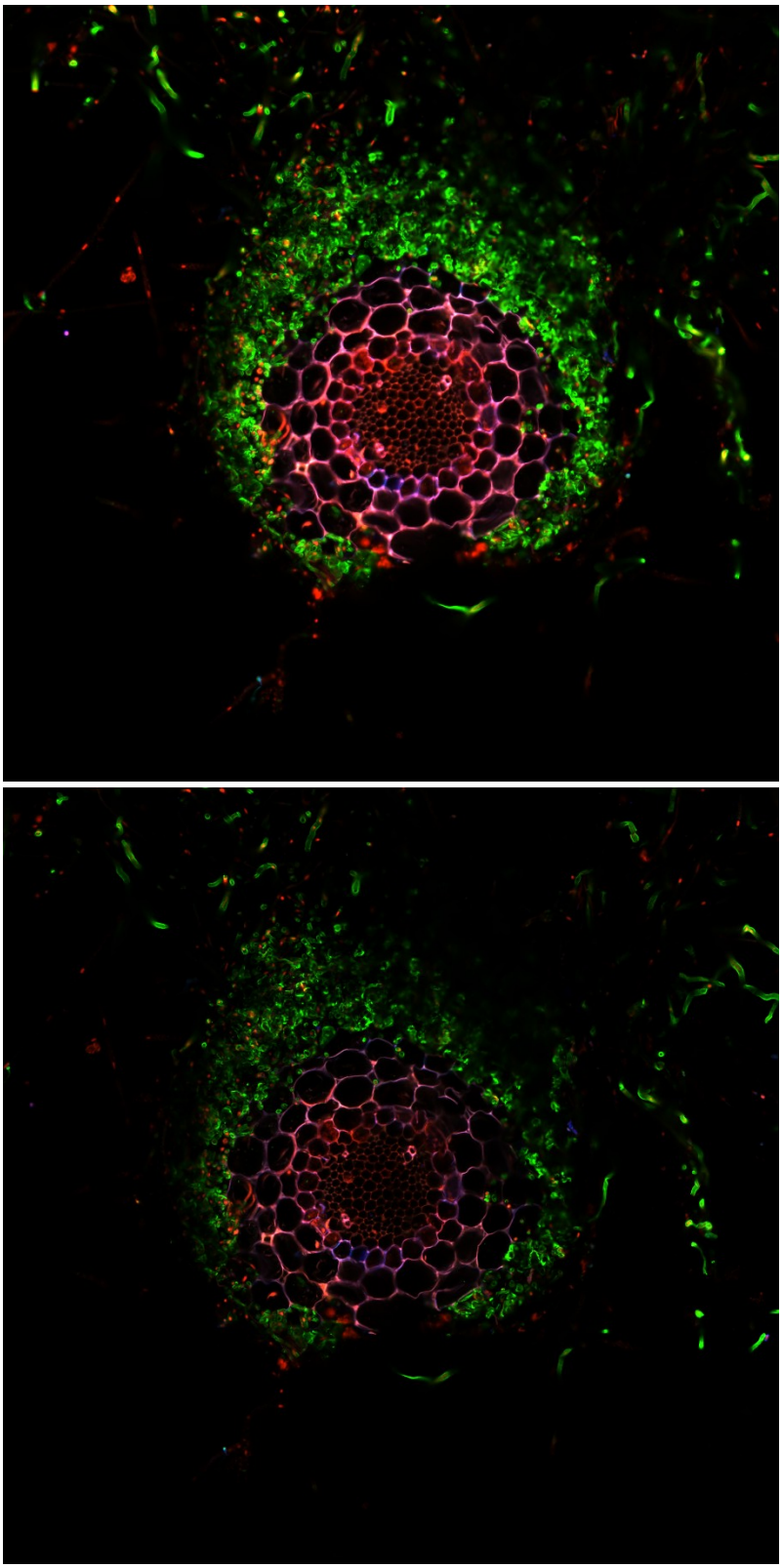


Figure 9 Examples of root tips from Fs-ap x *C. geophilum* 1.58. Sections show that no Hartig net formed, but the root tips have mantle.

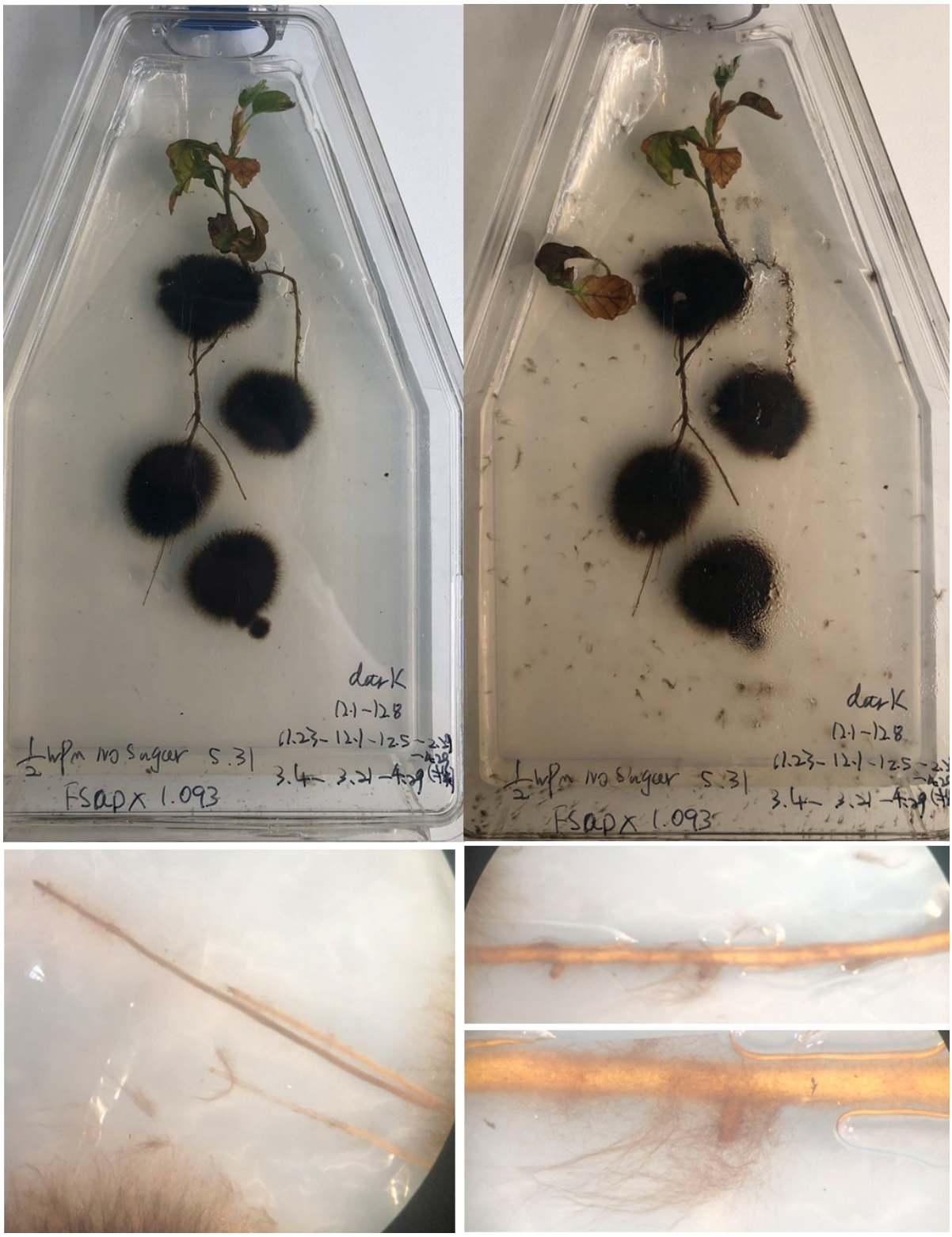


Figure 10 Fs-ap with *C. geophilum* 1.093.
Up-left: beginning of inoculation; Up- right: after 2 months
Down: root tips

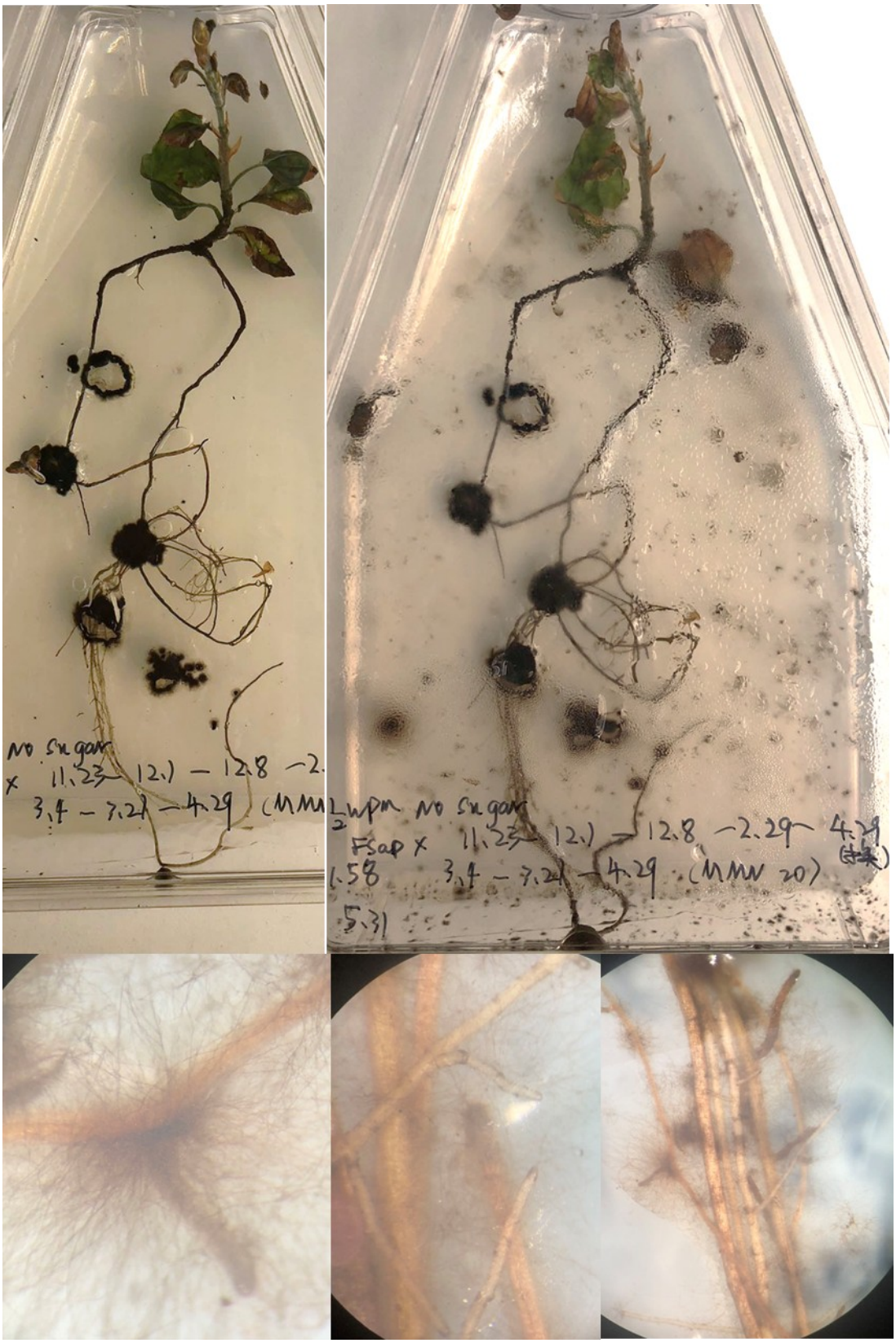


Figure 11 Fs-ap with *C. geophilum* 1.058.
Up-left: beginning of inoculation; Up- right: after 2 months
Down: root tips

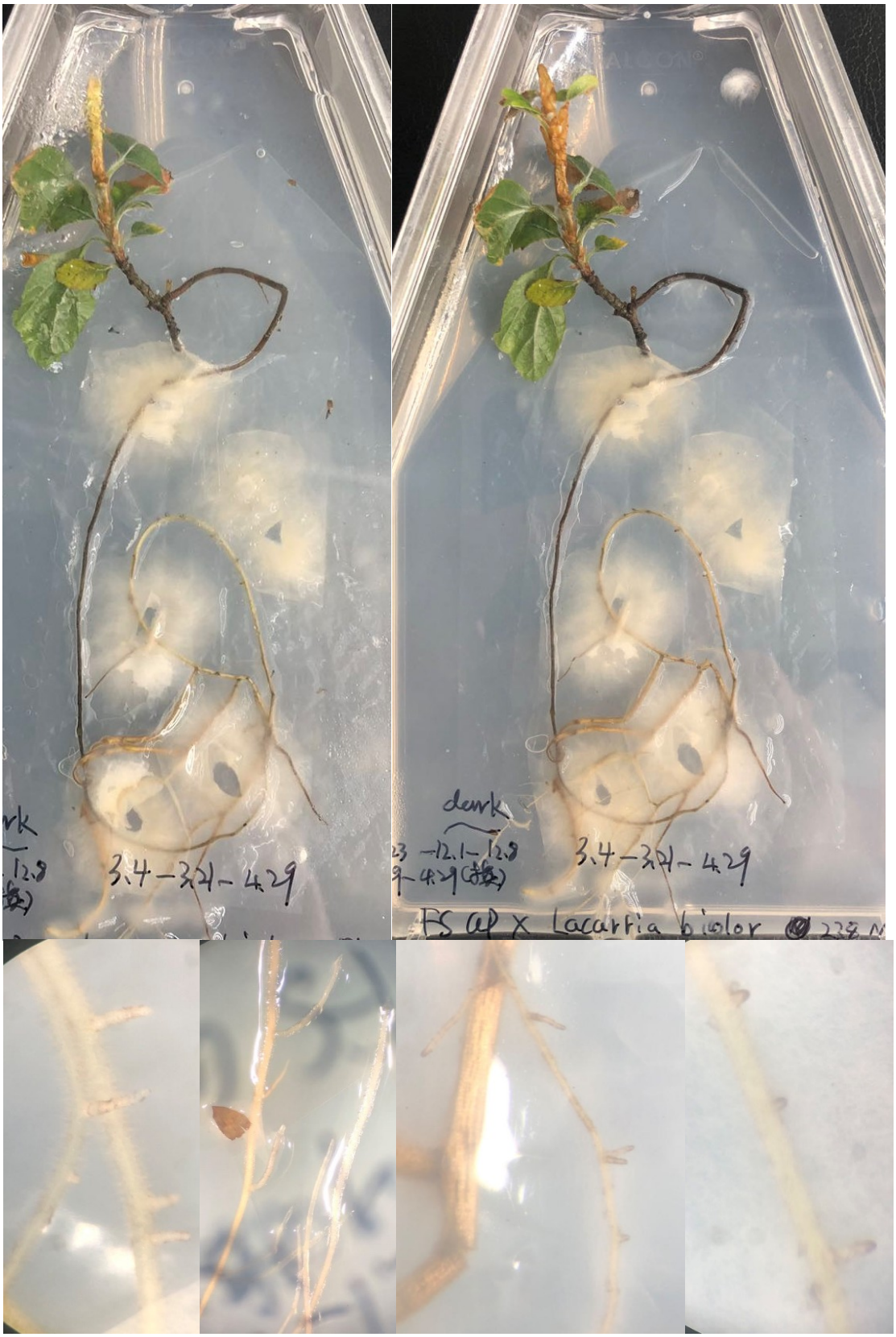


Figure 12 Fs-ap with *Laccaria biocolor* 238N.
Up-left: beginning of inoculation; Up- right: after 2 months
Down: root tips



Figure 13 Fs-ap with *Lactarius subdulcis 3* (died).
Up-left: beginning of inoculation; Up- right: after 2 months
Down: root tips

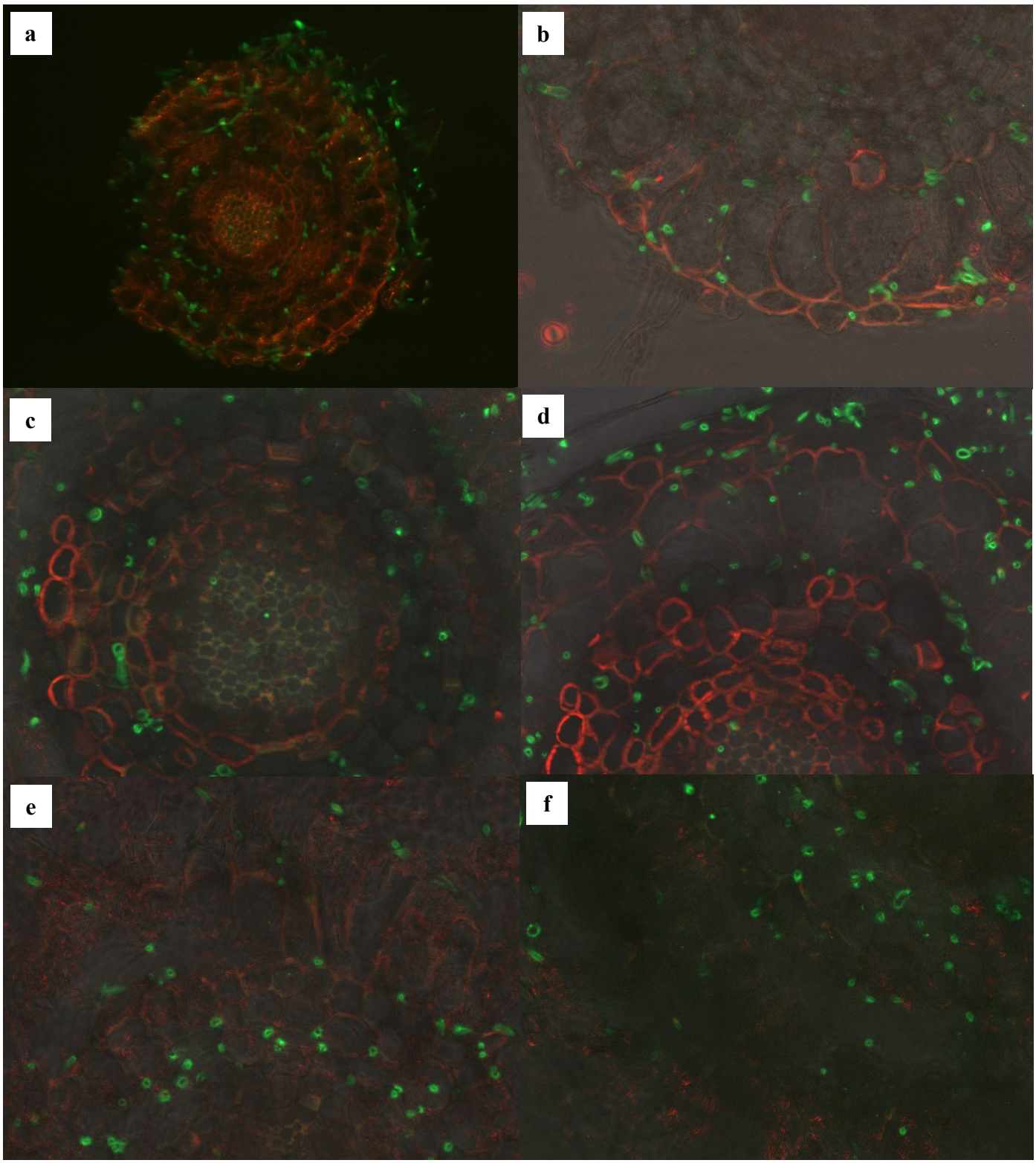


Figure 14 Cross sections of Fs-ap x *C. geophilum* 1.093.
All the figures are from the same root tip. No Hartig net formed.
The sections show the mycelium of *C. geophilum* 1.093 penetrated to epidermis and grows along roots.
0-300µm : a.; 300-600µm : b, c. = d. (upper part of c.) ; 600-900µm : e. f.

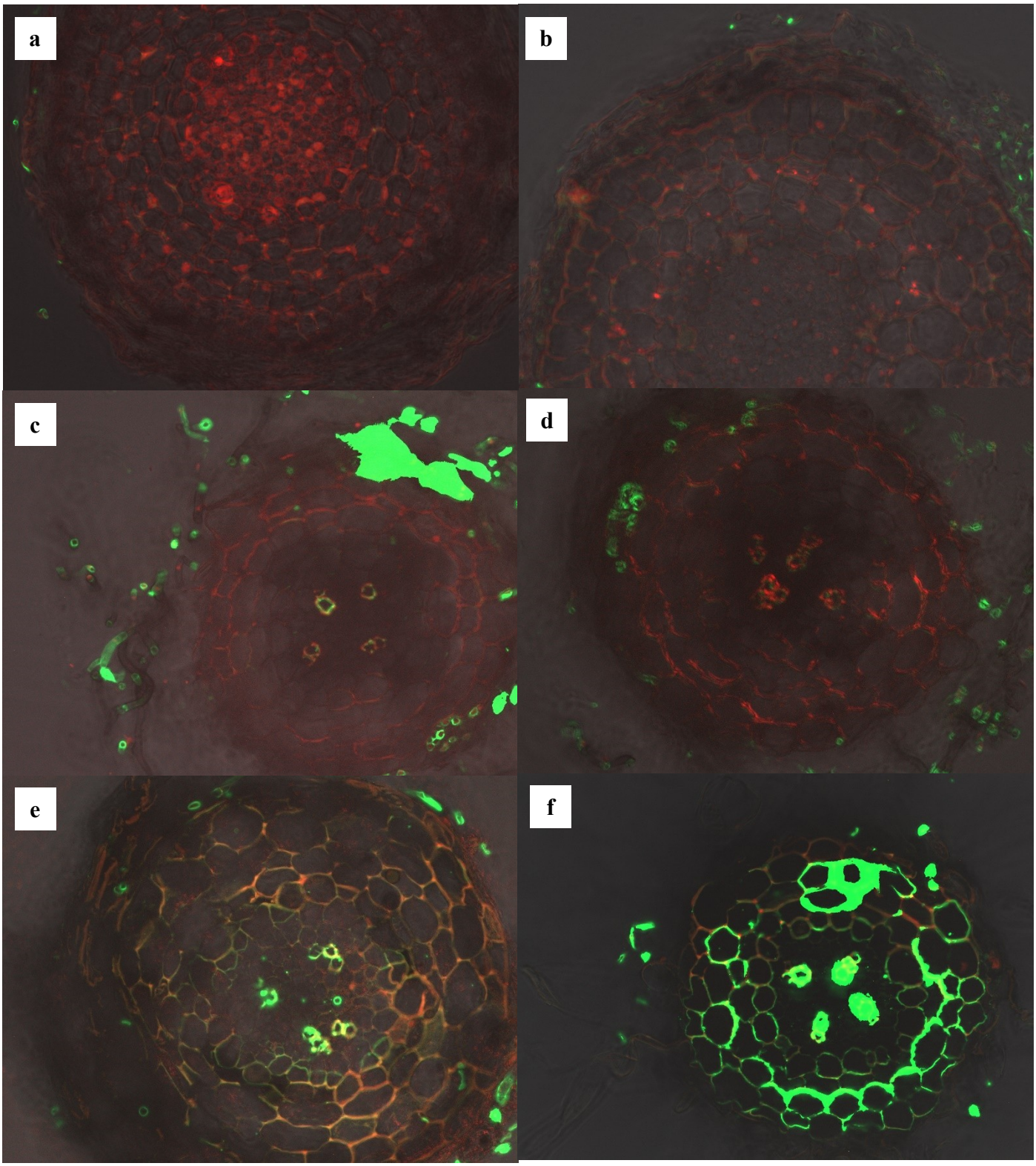


Figure 15 Cross sections of the root tips from three ectomycorrhizal fungi with two beech lines
a. and b. Fs-ap x *Laccaria biocolor* 238N;
c. and d. Fs-ap x *C.geophilum* 1.058;
e. Fo-BS x *Lactarius subdulcis 3*; f. Fs-ap x *Lactarius subdulcis 3* (died).

Discussion

All the seeds were contaminated or not germinated. It appears that beech seeds contain endophytes. Therefore, it is not possible to obtain in vitro seedlings from sterilized seeds. After 2 months, the cuttings cultured in the proliferation medium were transferred to the rooting medium to obtain roots. Without roots, the basal callus will affect the cutting and finally lose the cutting after 3 months. Compared with *Fagus orentalis* lines, the *Fagus sylvatica* line Fs-ap has a lower ability to form later roots (my observation). The main roots stopped growing until they touched the water at the bottom of the flasks and then started to have later roots. Therefore, the *Fagus sylvatica* lines had less absorption of fine roots. In natural soil, young *Fagus sylvatica* seedlings also show low ability of having more lateral roots for at least 27 years (Harley, 1940). No typical ECM structures were observed using either the two beech lines or our set of ECM fungi. *Lactarius subdulcis 3* cannot colonize FS-ap and cannot benefit the growth of beech seedlings. *Laccaria bicolor* S238N can help Fs-ap grow well (much more greenish and no falling leaf). *L. subdulcis 3* can help FO-SB grow well. More lines of *Fagus sylvatica* should be tested with these ECM fungi under in vitro conditions. Fs-ap formed a special structure with *C. geophilum* 1.093, and the root tips did not form a Hartig net but penetrated the root tips and grew along the root. According to our microscopic observations, C. *geophilum 1.093* formed endophytic structures, which did not benefit beech growth as *L. bicolor* S238N did for Fs-ap. However, more replicates are required to confirm this hypothesis.

Conclusions

New shoots can be readily obtained from *in vitro* cuttings; however, we were unable to generate new *in vitro* lines of *F. sylvatica* by using seeds or branches from 20-year-old trees. In contrast, we successfully propagated *in vitro* beech lines obtained from CSIC collaborators. These lines were rooted and used to establish symbiotic associations with a set of ECM fungi. We were unable to observe the ECM structures under our experimental conditions. Nevertheless, some of these interactions enhance the growth of beech plantlets.

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Chapter 5

Conclusions, Discussion and Perspectives

Conclusions

In my thesis project, I investigated the mechanisms underlying drought tolerance, including adaptation to osmotic stress, of the ectomycorrhizal ascomycete *Cenococcum geophilum* at various levels using different methodological approaches, which can be summarized as follows:

- Under sterile controlled culture conditions, a drought-tolerant isolate, *C. geophilum* 1.093, was identified (**Chapter 2**). Changes in gene expression during growth at high PEG concentrations were significantly and differently expressed, as early as 6h. It recovered after treatment according to its transcriptome. (**Chapter 2**).
- Under non-sterile greenhouse conditions, we found *C. geophilum* - like ectomycorrhizas, but we are not sure whether isolate 1.093 formed ectomycorrhizas with beech (*Fagus sylvatica*) roots while growing on natural forest soils in irrigated pots. And these ectomycorrhizas did not enhanced the survival rate of beech seedlings during growth under low soil water conditions (**Chapter 3**).
- An *in vitro* system using *F. sylvatica* and *F. orientalis* lines was successfully established (**Chapter 4**). Although *C. geophilum* 1.093 formed distinct endophytic structures in the beech root tips, it did not form the expected ectomycorrhizal Hartig net. In contrast, the ectomycorrhizal fungi *Laccaria bicolor* S238N and *Lactarius subdulcis* 3 were capable of forming a fungal mantle, although a Hartig net was not observed (**Chapter 4**).

Discussion and Perspectives

The identification of the drought-tolerant isolate*, C. geophilum* 1.093, described in **Chapter 2**, was pivotal for subsequent studies. Although isolates 1.058 and 1.278 demonstrated slow growth under drought stress, their potential to survive suggests that they could also be tested in greenhouse experiments. Despite the ability of strain 1.093 to form ectomycorrhizal structures, its inability to improve beech seedling survival (**Chapter 3**) requires identification and characterization of other natural isolates that may offer better performance under the same conditions. Furthermore, our transcriptomic analysis using RNA sequencing revealed several pathways and gene candidates linked to drought tolerance (i.e., high PEG concentration), although many remain functionally unknown. Unfortunately, there is currently no transformation system for *C. geophilum*, which limits further functional genomic analyses of these differentially expressed genes.

In **Chapter 3**, water potential in pots hosting mycorrhizal beech seedlings was managed via automated watering and soil weighing; however, the variable root systems of young beech trees

complicate the assessment of drought tolerance in these seedlings. The inclusion of xylem or leaf water potential measurements can provide a better understanding of the water status of these plants. The unscheduled heat stress experienced in the greenhouse due to inadequate fan cooling during summer coupled with drought stress further complicated our experimental settings and precluded the obtention of robust, replicated findings. Future experiments on ectomycorrhizal beech seedlings should be conducted under controlled temperature conditions, ideally indoors, during spring.

In **Chapter 4**, the inability of the tested beech lines to form mycorrhizas with the four selected ectomycorrhizal fungi highlighted the need for genotype-specific combinations. Future studies should involve pre-screened fungal-beech line combinations (same container but change medium to substrate) to increase the success rate of mycorrhizal formation, with an emphasis on rooting and lateral root formation (find ways to stimulate beech having more lateral roots in a short time and leave it growing until the influence removed naturally).

The differences between strains 1.093 and 1.058 for *C. geophilum* were notable. While both strains shared similar rDNA ITS sequences, only approximately 60% of sequencing reads from 1.093 could be mapped to the 1.058 reference genome, suggesting significant genetic divergence. Phylogenetically, *C. geophilum* 1.093 belonged to clade 6, which was geographically restricted, whereas 1.058 was more widely distributed. The traits and behaviors of these strains also differ: 1.093 forms endophytic structures in beech roots but does not degrade cellophane or herbaceous plant root hairs (my observation during the experiments), whereas 1.058 degrades cellophane and forms mycorrhizas with herbaceous plants.

These differences suggest that these two strains represent distinct species in the cryptic *Cenococcum* species complex. Comparative genomics has shown that genes involved in plant cell wall degradation, particularly lignin, cellulose, and hemicellulose degradation, were reduced during the evolutionary shift from saprotrophic to symbiotic lifestyles in ectomycorrhizal fungi (Kohler et al., 2015; Miyauchi et al., 2020). Strain 1.093 may have lost more saprotrophic genes, becoming more symbiotic, whereas 1.058 may retain more saprotrophic characteristics. Whole-genome sequencing of 1.093 is planned to further explore these differences and improve our understanding of *C. geophilum* physiology and symbiotic ability. Future studies should screen additional *C. geophilum* strains for their drought tolerance and explore possible correlations between phylogenetic placement, genomic features, and drought resilience.

Résumé

Ces dernières années, les événements climatiques extrêmes, en particulier les sécheresses printanières et estivales prolongées, représentent des risques importants pour les forêts, conduisant à des dépérissements qui touchent de nombreuses espèces. En particulier, ces changements climatiques entraînent une diminution des populations de hêtre (*Fagus sylvatica* L.) à travers l'Europe. Par conséquent, le développement de stratégies visant à renforcer la résilience des hêtres face à la sécheresse est devenu crucial. Notre recherche a exploré si *Cenococcum geophilum*, un champignon ascomycète ectomycorhizien cosmopolite, pouvait tolérer des conditions de sécheresse et aider ses plantes hôtes, en particulier le hêtre, à s'adapter au manque d'eau.

Par criblage phénotypique de souches fongiques, nous avons identifié une souche de *C. geophilum,* potentiellement tolérante à la sécheresse, en testant la croissance des isolats sur un milieu de croissance contenant des concentrations accrues de polyéthylène glycol (PEG) comme substitut du stress hydrique du sol ; le PEG augmentant la pression osmotique des cellules. Nous avons ensuite analysé le transcriptome de cette souche cultivé sur PEG à différents intervalles et évalué la récupération de son expression génique après un retour aux conditions normales. Six heures après le traitement au PEG, l'expression génique a changé de manière spectaculaire, activant des voies connues d'adaptation au stress osmotique et de détoxification. Le transcriptome de l'isolat est revenu à son état initial deux jours après son transfert sur un milieu de culture dépourvu de PEG.

Étant donné la tolérance de l'isolat à des changements majeurs de pression osmotique et sa récupération rapide, nous avons mené une expérience en serre pour déterminer si une inoculation mycorhizienne contrôlée avec cette souche pouvait améliorer la résistance des plantules de hêtre à la suspension de l'irrigation. Bien que la mycorhization contrôlée ait augmenté le taux de survie des semis, les isolats naturels de *C. geophilum* présents dans le sol des cultures ont surpassé notre souche sélectionnée, probablement en raison de la brève durée de l'expérience et de la formation limitée de nouvelles racines latérales.

Pour approfondir les mécanismes moléculaires de la tolérance à la sécheresse de l'association symbiotique *Cenococcum-Fagus*, nous avons tenté de développer un système *in vitro* de culture reposant sur la micro-propagation de plantules de hêtre. Cependant, toutes les lignées de hêtre testées ont montré une formation réduite de racines latérales et de faibles taux de colonisation avec cinq champignons ectomycorhiziens, y compris *C. geophilum*. Bien qu'aucune ectomycorhize typique n'a pu être observé, nous avons mise en évidence par microscopie confocale la formation de structures intraradiculaires semblables à celles développées par les champignons endophytes.

Pour de futures études, nous recommandons de sélectionner des lignées de hêtre avec de meilleures capacités d'enracinement (Chapitre 4) et d'élargir le nombre d'isolats de *C. geophilum* testés (Chapitres 3 et 4), en isolant des souches adaptées localement.

Abstract

Recently, extreme climatic events, such as extended drought periods, cause serious threats to forest ecosystems. These climate changes is leading to a decline in beech (Fagus sylvatica L.) populations across Europe. Developing forest management approaches to enhance the drought resistance of beech trees has become increasingly important.

In this study, we investigated whether the genus *Cenococcum*, host generalist, worldwide spreading ectomycorrhizal fungus and present in extreme environments, can survive and support its host (especially European beech) to adapt to drought conditions. To study it, we screened *Cenococcum* isolates on medium with increasing water potential and identified a drought tolerant isolate. Then the transcriptome of this isolate under drought condition (mimicked by polyethylene glycol (PEG) treatment) at different timepoints was investigated as well as its ability to recover after return to control condition. As early as six hours after PEG treatment, gene expression dramatically changed by inducing well-known adaptation pathways to osmotic stress and detoxification. Within two days after transferring the isolate back to the no drought treatment medium its transcriptome is similar to the non-treated. Since the isolate is drought tolerant and recovered fast after transfer to no drought stress condition, we tested whether controlled mycorrhization by this isolate in the greenhouse could improve the resistance of young beech trees to the suspension of irrigation. In our experiment controlled mycorrhization increased the survival rate of the young beech trees, but probably *Cenococcum* isolates present in the natural soil have a better performance (higher colonization rates) then the selected strain, partly probably due to the short experimental time and the lack of new lateral roots formed. To be able to study the molecular mechanisms of drought tolerance in *Cenococcum*- European beech symbiosis, we started to develop an *in vitro* system. Unfortunately, all tested European beech lines have a low ability to form lateral roots and are poorly colonized by five ectomycorrhizal fungi tested. Although no typical ectomycorrhiza was formed, we detected an endophyte-like structure formed by *Cenococcum* in the roots of in vitro European beech.

For future research, we suggest to screen for better rooting beech lines (Chapter 4) and to enlarge the number of *Cenococcum* isolates (Chapter 3 and 4) e.g. trapping locally adapted strains.