

Fungal response to drought in the maize rhizosphere after reusing cover crop root channels

Debjyoti Ghosh

`deb.jyoti.ghosh@ufz.de`

Helmholtz Centre for Environmental Research - UFZ GmbH <https://orcid.org/0009-0008-1496-1677>

Yijie Shi

Christian-Albrechts-University Kiel

Iris Zimmermann

Christian-Albrechts-University Kiel

Tobias Stürzebecher

University of Göttingen

Katja Holzhauser

Christian-Albrechts-University Kiel

Martin von Bergen

UFZ-Helmholtz-Centre for Environmental Research <https://orcid.org/0000-0003-2732-2977>

Anne-Kristin Kaster

Karlsruher Institute of Technology

Sandra Spielvogel

Christian-Albrechts University Kiel <https://orcid.org/0000-0003-2310-5975>

Michaela Dippold

University of Tuebingen <https://orcid.org/0000-0002-3657-4693>

Jochen Mueller

Karlsruhe Institute of Technology <https://orcid.org/0000-0002-0935-1523>

Harald Kellner

TU Dresden

Nico Jehmlich

Helmholtz-Centre for Environmental Research - UFZ <https://orcid.org/0000-0002-5638-6868>

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Abstract

Root channels formed by winter cover crop can enhance subsoil water and nutrient access for subsequent crops such as maize (*Zea mays* L.) yet their fungal inhabitants remain poorly understood under drought. Here, we assessed drought-induced shifts in maize rhizosphere fungal communities within reused cover crop root channels across three contrasting soil types in northern Germany (Luvisol, Podzol and Phaeozem). Using a multi-omics approach combining ITS2 amplicon sequencing, quantitative PCR and metaproteomics, we linked community composition with functional responses. Drought consistently restructured fungal communities, with increased relative abundances of *Ascomycota* and *Zoopagomycota* and declines in *Chytridiomycota* and *Mucoromycota*. Taxa within the same subkingdom occupied complementary niches, indicating functional differentiation beyond higher-level taxonomy. At the protein level, drought responses were characterised either by enhanced antioxidant defence mechanisms including catalase–glutathione peroxidase systems, superoxide dismutase, fatty acid synthesis and the methionine cycle–transsulfuration pathway or by reduced carbon and nitrogen metabolic activity, suggesting energy conservation strategies. Together, our results demonstrate substantial structural and functional plasticity of rhizosphere fungal communities in reused root channels under water limitation, highlighting their potential role in microbiome-mediated drought resilience in agroecosystems.

Introduction

Drought is a major constraint on global crop productivity and disrupts biogeochemical processes in agricultural soils. Its frequency and severity have increased in recent decades¹ with an increase of 29% since 2000² driven by rising temperatures and long-term land use change³. As topsoil dries and nutrient mobility declines, root growth is redirected to deeper, less exposed subsoil layers. Enhancing the capacity of crops to exploit subsoil resources therefore represents a promising strategy to sustain productivity under water and nutrient limitation⁴. Root channels formed by preceding crops restructure soil microhabitats by modifying physical accessibility and microbial activity^{5–7}. These stable macropores improve aeration, reduce mechanical resistance and facilitate deeper root penetration by subsequent crops.

We previously showed that maize roots reusing winter cover crop root channels harbour higher bacterial abundance and activity in the rhizosphere⁵, contributing to mitigation of drought-like conditions⁸. In particular, bacterial communities exhibited upregulation of reactive oxygen species (ROS)-detoxifying enzymes, including catalase, glutathione peroxidase and superoxide dismutase, under water limitation⁸. Whether fungal communities in these biopores display comparable adaptive responses remains unknown.

Fungi are key regulators of rhizosphere functioning, mediating soil structure, organic matter turnover, plant nutrient acquisition and stress tolerance properties^{9–11}, organic matter decomposition¹², and plant-fungi mutualistic relations. Their distribution is highly heterogenous and shaped by soil depths,

physicochemical properties and management practices^{13,14}. Soil type exerts strong control over fungal community composition. According to the World Reference Base for Soil Resources¹⁵, Luvisols and Phaeozems are characterised by high-activity clays and dark, loamy organic matter, respectively, with high base status, whereas Podzols are sandy and acidic¹⁶. Such differences in texture and chemistry influence the relative abundance of major fungal taxa and their vertical distribution along soil profiles¹⁰.

Fungi are often considered comparatively drought-tolerant due to their filamentous growth form, which enables resource redistribution under low water availability^{17,18}. However, their functional responses to drought in agricultural soils remain poorly resolved. The ecological roles of mycorrhizal symbionts, saprotrophs and pathotrophs under stress depend on their capacity to mitigate oxidative damage and maintain metabolic homeostasis^{19,20}. Although oxidative stress responses have been described in plant-associated and clinical fungi, soil fungal proteomic responses particularly within reused cover crop root channels remain largely unexplored. This knowledge gap limits our understanding of how cover cropping strategies shape fungi-mediated soil processes under climate-induced stress.

Here, we addressed this gap, through field experiments in which maize followed winter cover crop mixtures of *Brassicaceae* (Brassica), *Fabaceae* (Legume) and *Poaceae* (Grass), with drought imposed using rainout shelters. Across soils of contrasting texture, we combined ITS2 amplicon sequencing, quantitative PCR (qPCR), and metaproteomics to investigate the effects of drought (D) versus rainfall-fed (RF) maize cultivation on rhizosphere fungal communities of maize reusing pre-existing root channels. We hypothesised that fungal communities exhibit enhanced activity and distinct adaptive strategies under drought, modulated by soil type. By linking community composition with functional protein values across topsoil and subsoil, this study provides mechanistic insight to inform cover crop selection and strengthen crop resilience in water-limited agroecosystems.

Results

Fungal community structure in different soil types under drought stress

The study encompassed three soil types: Luvisol, Phaeozem, and Podzol, and two soil depths (topsoil: 0–30 cm; subsoil: 30–60, and 60–90 cm) (Fig. 1). Soil physicochemical properties differed significantly among soil types and between depths (Fig. S1, Table S1).

We obtained 37,437 unique amplicon sequencing variants (ASVs) assigned to 83 classes within 12 fungal phyla (Fig. S2). Fungal richness increased under D relative to RF when *Brassicaceae/Poaceae* was the cover crop mixture in all the soil types (Luvisol: 832_D vs 729_{RF}, Phaeozem: 742_D vs 614_{RF}, Podzol: 804_D vs 696_{RF}). Using *Fabaceae/Poaceae*, richness increased significantly in the Luvisol and Podzol but declined in the Phaeozem (Luvisol: 878_D vs 729_{RF}, Phaeozem: 511_D vs 579_{RF}, Podzol: 873_D vs 800_{RF}). The richness under fallow conditions declined in the Phaeozem compared with the Luvisol and Podzol, although no clear differences were noticeable between D and RF (Luvisol: 831_D vs 815_{RF}, Phaeozem: 599_D vs 667_{RF}, Podzol: 818_D vs 827_{RF}) (Fig. 2a, Table S2a). Evenness increased only for

Fabaceae/Poaceae ($0.774_{\text{Fabaceae/Poaceae}} > 0.766_{\text{Fallow}} > 0.764_{\text{Brassicaceae/Poaceae}}$), while no other changes were detected (Fig. 2a, Table S2b). Beta-diversity estimations using Bray-Curtis dissimilarity metrics revealed differences among Podzol fungal communities from those in the Luvisol and Phaeozem, as well as between the sampling depths (Fig. 2b, Table S2c), consistent with previously reported bacterial community behaviours in the same experimental system⁸.

Quantitative PCR analysis demonstrated a 12.8-fold reduction in ITS2 gene copy numbers from the topsoil to the subsoil ($1.60 \cdot 10^7_{\text{Topsoil}} > 1.25 \cdot 10^6_{\text{Subsoil}}$) (Fig. 2c, Table 1). Absolute abundances were highest in the Phaeozem and declined sequentially in the Podzol and the Luvisol ($1.44 \cdot 10^7_{\text{Phaeozem}} > 0.75 \cdot 10^7_{\text{Luvisol}} > 0.40 \cdot 10^7_{\text{Podzol}}$). Among cover crop treatments, *Fabaceae/Poaceae* resulted in the highest ITS2 gene copies, exceeding *Brassicaceae/Poaceae* and fallow ($9.52 \cdot 10^6_{\text{Fabaceae/Poaceae}} > 8.97 \cdot 10^6_{\text{Brassicaceae/Poaceae}} > 7.83 \cdot 10^6_{\text{Fallow}}$). Under D, fungal abundance increased within *Brassicaceae/Poaceae* root channels in the Luvisol and Podzol, but declined with *Fabaceae/Poaceae*. In the subsoil, *Fabaceae/Poaceae* exhibited significantly lower ITS2 gene abundances than *Brassicaceae/Poaceae* and fallow (subsoil: $1.69 \cdot 10^6_{\text{Brassicaceae/Poaceae}} \approx 1.52 \cdot 10^6_{\text{Fallow}} > 0.54 \cdot 10^6_{\text{Fabaceae/Poaceae}}$), whereas no significant differences were observed among treatments in the topsoil (Table 1).

Table 1

Summary of ITS2 gene copies per gram soil for different parameters in our study. We quantified the mean and SD values of ITS2 gene copies per gram soil in soils from the reused cover crop root channels for all study parameters. The significance of each category is represented using compact letter display (CLD) calculated after conducting MANOVA on the gene copies under categories of soil type, cover crop variations and soil sampling depths. The values are provided in the Supplemental Tables S4c-d. Table

Variation	Soil type	Depth	Condition	ITS2 gene copies g soil ⁻¹ (mean + SD)	CLD
Brassica/Grass	Luvisol	Topsoil	Rainfall-fed	3.09E + 06 ± 2.33E + 05	bcd
			Drought	1.25E + 07 ± 1.49E + 06	
		Subsoil	Rainfall-fed	7.46E + 06 ± 1.96E + 06	cd
			Drought	1.03E + 06 ± 1.21E + 05	
	Phaeozem	Topsoil	Rainfall-fed	3.68E + 07 ± 4.63E + 06	a
			Drought	3.17E + 07 ± 9.51E + 06	
		Subsoil	Rainfall-fed	1.39E + 05 ± 1.58E + 04	d
			Drought	6.64E + 05 ± 7.72E + 04	
	Podzol	Topsoil	Rainfall-fed	5.92E + 05 ± 6.32E + 04	bcd
			Drought	1.29E + 07 ± 3.62E + 06	
		Subsoil	Rainfall-fed	1.35E + 05 ± 1.82E + 04	d
			Drought	6.86E + 05 ± 9.41E + 04	
Fallow	Luvisol	Topsoil	Rainfall-fed	9.36E + 06 ± 1.94E + 06	abcd
			Drought	1.83E + 07 ± 1.39E + 06	
		Subsoil	Rainfall-fed	3.27E + 06 ± 6.78E + 05	cd
			Drought	8.91E + 05 ± 9.49E + 04	
	Phaeozem	Topsoil	Rainfall-fed	3.51E + 07 ± 4.06E + 06	abc
			Drought	8.54E + 06 ± 1.52E + 06	
		Subsoil	Rainfall-fed	8.87E + 04 ± 1.61E + 04	d

Variation	Soil type	Depth	Condition	ITS2 gene copies g soil ⁻¹ (mean + SD)	CLD
	Podzol	Topsoil	Drought	2.35E + 05 ± 3.37E + 04	bcd
			Rainfall-fed	1.01E + 07 ± 3.05E + 06	
		Subsoil	Drought	3.51E + 06 ± 5.36E + 05	cd
			Rainfall-fed	2.40E + 06 ± 2.50E + 05	
Legume/Grass	Luvisol	Topsoil	Drought	2.22E + 06 ± 4.68E + 05	abcd
			Rainfall-fed	2.68E + 07 ± 3.45E + 06	
		Subsoil	Drought	4.94E + 06 ± 6.83E + 05	cd
			Rainfall-fed	1.72E + 06 ± 1.87E + 05	
	Phaeozem	Topsoil	Drought	8.41E + 05 ± 1.01E + 05	ab
			Rainfall-fed	4.30E + 07 ± 1.40E + 07	
		Subsoil	Drought	1.65E + 07 ± 1.65E + 06	cd
			Rainfall-fed	6.05E + 04 ± 5.25E + 03	
	Podzol	Topsoil	Drought	2.80E + 05 ± 5.70E + 04	bcd
			Rainfall-fed	7.59E + 06 ± 2.01E + 06	
		Subsoil	Drought	6.74E + 06 ± 1.51E + 06	d
			Rainfall-fed	1.90E + 05 ± 2.01E + 04	
			Drought	1.69E + 05 ± 3.07E + 04	
			Rainfall-fed		

Although overall richness and evenness limited cover crop mixture effects, taxon-specific responses were evident at the phylum and class levels (Table S3). The *Dikarya* subkingdom, comprising *Ascomycota* and *Basidiomycota*, showed minimal change under D relative to RF (Log_2FC : 0.034 and 0.014, respectively) (Fig. 2d, Tables S4a-b). However, within these phyla, individual classes responded divergently: *Laboulbeniomycetes* (-0.218) of *Ascomycota* and *Malasseziomycetes* (-0.531) of *Basidiomycota* decreased under D. *Mucoromycota* and *Zoopagomycota* exhibited contrasting outcomes, with the former taxa declining under D against RF (-0.136) while the latter increasing (0.130). Within *Mucoromycota*, *Glomeromycetes* (arbuscular mycorrhizal fungi; 0.399) and *Mucoromycetes* (0.152), one

of the most well-studied zygomycete fungal classes, increased under D and were more abundant in the Luvisol and Phaeozem. Similarly, classes within *Zoopagomycota* were enriched in these two soils but declined upon reusing cover crop root channels (Fig. S3). *Chytridiomycota* (-0.061) and *Cryptomycota* (0.254), both typically associated with aquatic environments, displayed opposing abundance trends under D and in the drought-prone Podzol.

Across soil depths, *Dikarya* exhibited vertical niche differentiation, with *Ascomycota* predominating in the topsoil and *Basidiomycota* in the subsoil. Exceptions included *Saccharomycetes* (*Ascomycota*) and the basidiomycete classes *Atractiellomycetes* and *Cystobasidiomycetes* (Fig. S4). Similar depth stratification was observed for *Mucoromycota* (enriched in subsoil) and *Zoopagomycota* (enriched in topsoil). The flagellated *Chytridiomycota* and the chitin cell-wall-lacking *Cryptomycota* preferentially inhabit the comparatively less compact topsoil. Despite these taxonomic shifts, overall phylum-level abundances showed limited responsiveness to cover crop treatments (Fig. S5).

Correlation of fungal communities with soil physicochemical properties

Soil physicochemical characteristics were examined previously, which showed that organic carbon content varied substantially with soil type and depth⁸ (Fig. S1, Table S1). Partial Mantel tests revealed significant associations between fungal community composition and soil physicochemical properties (Fig. 3a, Tables S5a-d). In the clay-rich Phaeozem, *Ascomycota* abundance correlated positively with total nitrogen (TN) and total organic carbon (TOC) in *Fabaceae/Poaceae* under both D and RF conditions. Associations with pH depended on the soil, ranging from negative in the Phaeozem to positive in the topsoil, where *Ascomycota* also correlated positively with soil moisture. *Basidiomycota* association trends were exactly opposite to *Ascomycota* – negatively correlating with TN and TOC, and with moisture in the topsoil. *Zoopagomycota* correlated positively with moisture and pH in the topsoil, whereas negative associations were observed in the alkaline Phaeozem. The other zygosporangia-forming lineage, *Mucoromycota*, showcased the reverse pattern. Also, chitin-lacking *Cryptomycota* and the rare *Aphelidiomycota* were negatively correlated with both moisture and pH.

To link taxonomic observations with ecological attributes, fungal communities were mapped to the FUNGuild database²¹ to trophic modes and compare the changes in abundances under D versus RF (Fig. 3b). Notable shifts along the trophic modes were visible in the subsoil as compared to the topsoil (Table S6a). The most significant changes involved *Mucoromycota*, which includes arbuscular mycorrhizal fungi (*Glomeromycetes*), in the topsoil of all three soil types and in the subsoil of Luvisol (Table S6b). For *Ascomycota*, changes were limited to the Podzol. With the effect of D, topsoil pathotrophs from *Ascomycota* (plant pathotrophic *Eurotiomycetes* and *Sordariomycetes*), *Basidiomycota* (animal pathotrophic *Malasseziomycetes*) and *Mucoromycota* (animal pathotrophic *Mucoromycetes*) declined in the Podzol for *Fabaceae/Poaceae* and fallow but increased slightly for *Brassicaceae/Poaceae*. In contrast, subsoil pathotrophs increased with both cover crop treatments relative to bulk soil, particularly in the Podzol. Overall, saprotrophs and symbiotrophs generally increased following cover crop applications in the Phaeozem and Podzol. Notable exceptions were seen in the Luvisol, where

Basidiomycota (Agaricomycetes) and *Chytridiomycota* (Chytridiomycetes) declined in *Brassicaceae/Poaceae* root channels, and in the Podzol topsoil, where for *Basidiomycota* and *Mucoromycota* (Mucoromycetes) symbiotrophs decreased significantly. The information about fungal trophic modes are provided in the supplemental tables S7a and S8a.

Soil fungal metaproteome insights under drought

To assess the effects of drought on fungal biochemical pathways within reused cover crop root channels, proteins were identified by metaproteomics and assigned to taxonomic groups. Expression of fungal proteins varied significantly with soil type, depth, and cover crop mixture under drought stress (Table S9a). Overall trends in the number of protein groups were consistent with the ITS2 sequencing results, with some exceptions. Under D, protein groups increased in the topsoil of the Luvisol and Podzol for *Brassicaceae/Poaceae* compared with RF ($416_{\text{Luvisol-D}} > 356_{\text{Luvisol-RF}}$; $504_{\text{Podzol-D}} > 362_{\text{Podzol-RF}}$), but increased only in the Podzol subsoil and not in the Luvisol ($284_{\text{Luvisol-D}} < 337_{\text{Luvisol-RF}}$; $336_{\text{Podzol-D}} > 294_{\text{Podzol-RF}}$) (Table S9b). For *Fabaceae/Poaceae*, protein groups increased in the Luvisol ($429_{\text{Luvisol-D-Topsoil}} > 353_{\text{Luvisol-RF-Topsoil}}$; $341_{\text{Luvisol-D-Subsoil}} > 179_{\text{Luvisol-RF-Subsoil}}$) but showed no substantial change in Podzol topsoil, while increasing in Podzol subsoil ($470_{\text{Podzol-D-Topsoil}} < 488_{\text{Podzol-RF-Topsoil}}$; $288_{\text{Podzol-D-Subsoil}} > 226_{\text{Podzol-RF-Subsoil}}$). In contrast, no clear shifts were seen in the Phaeozem for either cover crop mixture or under fallow conditions ($364_{\text{Fabaceae/Poaceae-D-Topsoil}}$ and $361_{\text{Fabaceae/Poaceae-RF-Topsoil}}$; $213_{\text{Fabaceae/Poaceae-D-Subsoil}}$ and $229_{\text{Fabaceae/Poaceae-RF-Subsoil}}$; $365_{\text{Brassicaceae/Poaceae-D-Topsoil}}$ and $369_{\text{Brassicaceae/Poaceae-RF-Topsoil}}$; $219_{\text{Brassicaceae/Poaceae-D-Subsoil}}$ and $211_{\text{Brassicaceae/Poaceae-RF-Subsoil}}$). Relative to RF, drought altered protein expression in the moisture-limited soils, Luvisol and Podzol, following the use of cover crop mixtures. In the drought-prone sandy Podzol, overall protein expression increased only with *Brassicaceae/Poaceae* ($\text{Log2FC: } 0.532$), exceeding both fallow (0.017) and *Fabaceae/Poaceae* (-0.245) (Tables S9c-e). Similarly, protein expressions in the Luvisol increased only with *Brassicaceae/Poaceae* ($0.328_{\text{Brassicaceae/Poaceae-Log2FC}} > 0.076_{\text{Fallow-Log2FC}} > -0.470_{\text{Fabaceae/Poaceae-Log2FC}}$). By contrast, the Phaeozem had the highest water retention under D and showed reduced protein expression following cover crop introduction relative to bulk soil ($0.441_{\text{Fallow-Log2FC}} > 0.148_{\text{Brassicaceae/Poaceae-Log2FC}} > 0.044_{\text{Fabaceae/Poaceae-Log2FC}}$).

Differential expression analysis revealed a greater number of upregulated proteins in the Luvisol and the Phaeozem (Fig. 4, Table S10). Fatty-acid-synthesising 3-hydroxydecanoyl-[acyl-carrier-protein (ACP)] dehydratase, and oxidative stress regulators aldehyde dehydrogenase (NAD⁺) (ALDH) and superoxide dismutase (SOD) were upregulated under D (Table S7a). In contrast, enzymes of the pyruvate dehydrogenase complex (PDC) enzymes contributing to acetyl-CoA synthesis were downregulated, particularly in *Fabaceae/Poaceae* root channels. In the Luvisol, osmoprotectant glycerol-3-phosphate dehydrogenase (G3PDH) and glutamate racemase (Muri) of the N cycle were upregulated with *Brassicaceae/Poaceae* under D. In the Podzol, membrane translocases and catalase peroxidase (CAT-PER) were upregulated, whereas phosphoribosylformylglycinamide synthase (PFAS) was downregulated. Methionine cycle enzymes, including homocysteine methyltransferase, methionine synthase (MeSe) and

S-adenosylmethionine (SAM) synthase, were upregulated in *Fabaceae/Poaceae* root channels in the Luvisol.

Consistent with amplicon sequencing, *Ascomycota* produced the largest proportion of the identified proteins. Based on absolute label-free quantification (LFQ) intensities of highly expressed enzymes, 3-hydroxydecanoyl-[ACP]-dehydratase was expressed exclusively by *Ascomycota*, particularly in the Podzol. The same patterns were observed for MeSe and homocysteine methyltransferase, whereas aspartokinase and homoserine dehydrogenase and the oxidative stress regulator ALDH were more strongly expressed in the Luvisol (Fig. 5, S7, Table S7a). Beyond *Ascomycota*, SAM synthase expression from *Basidiomycota*, *Chytridiomycota*, *Mucoromycota* and *Zoopagomycota* was relatively higher in the Podzol, while G3PDH was expressed more in the Luvisol. ALDH expressions were similar from *Ascomycota* in both Luvisol and Podzol and from *Basidiomycota* in the Phaeozem. Additional stress responders showed soil-specific patterns: SOD was relatively upregulated in *Ascomycota* in the Phaeozem, while CAT-PER from *Basidiomycota* was overexpressed in the Podzol inside *Brassicaceae/Poaceae* root channels. The pentose phosphate pathway enzyme 6-phosphogluconolactonase (6-PGL) was more strongly expressed by *Ascomycota* in the Podzol under D. Despite a relative abundance below 1%, *Blastocladiomycota* contributed substantially to glycolytic aldolase expression. The Podzol also represented a higher abundance of N cycle enzymes, glutamate dehydrogenase and urease, primarily contributed by *Mucoromycota*, *Basidiomycota* and *Chytridiomycota*. Glutamate racemase was expressed highly in the Luvisol under D. In the topsoil, *Ascomycota*, *Basidiomycota* and *Mucoromycota* were the top taxa contributing the most to the proteome (Table S7b). On the other hand, in the subsoil, *Mucoromycota* exceeded those of *Basidiomycota*, while overall protein expressions from other phyla declined relative to topsoil. An extended heatmap of fungal protein expression across all the study parameters is provided in the Supplementary Material (Fig. S6).

Fungi-mediated lignocellulose breakdown under drought

Lignocellulose is degraded by lignocellulolytic enzymes, primarily hydrolases and some oxidoreductases, which are classified within different groups of carbohydrate-active enzymes (CAZymes). Fungal cellulases play a central role in the decomposition of lignocellulose and complex organic matter, thereby improving the availability of carbon, nutrients and water. Metaproteomics analysis revealed that glycoside hydrolases (GHs) were the most abundantly expressed CAZymes and were major contributors to lignocellulose decomposition. They were predominantly derived from the *Ascomycota* classes *Eurotiomycetes* and *Sordariomycetes*, while the other fungal taxa contributed sparsely (Fig. 6a-b).

Enzymes were further categorised according to the lignocellulosic substrates they target. Among the CAZymes, GHs were the most highly expressed, with β -glucosidases (GH1, GH3), xylanases (GH3, GH10, GH11), arabinofuranosidases (GH43, GH51), and α - and β -galactosidase (GH2, GH35) being particularly abundant. These enzymes were largely contributed by *Ascomycota* (Fig. 6a-c, S7, Tables S8a-b). Auxiliary activity (AA) enzymes were the next most expressed group, notably pyranose:acceptor oxidoreductase (AA3) derived from *Basidiomycota* and especially abundant in the Podzol. Most identified CAZymes were

extracellular, particularly those affiliated with *Basidiomycota* and *Chytridiomycota* (Fig. 6a, Table S8c). Although *Ascomycota* were the dominant producers of CAZymes, a proportion of their enzymes were predicted to be intracellular (e.g., AA4, GH2). Under D conditions, pathotrophic and symbiotrophic *Ascomycota* showed increased expression of GH12 and GH74 endoglucanases (Table S8a). In contrast, GH6 cellobiohydrolases were overexpressed by saprotrophic *Basidiomycota* but displayed reduced expression in saprotrophic *Ascomycota*. The auxiliary enzyme lytic-polysaccharide monooxygenase (LPMO; AA9), produced by *Ascomycota* across all trophic modes, was relatively upregulated under D. Hemicellulose-degrading enzymes, including acetylxytan and feruloyl esterase (CE1), declined under D in saprotrophic *Ascomycota*, but were overexpressed by saprotrophic *Basidiomycota*. Oxidative stress conditions were associated with increased expression of intracellular vanillyl-alcohol oxidase (AA4), which is involved in vanillin and H₂O₂ production. Contrastingly, enzymes involved in lignin and pectin degradation were not significantly affected by D relative to RF. Overall, contributions from *Chytridiomycota* and *Mucoromycota* were substantially lower than those from the *Dikarya* phyla.

Discussion

This study investigated the impact of drought on fungal communities and their biochemical pathways within reused winter cover crop root channels subsequently utilised by maize roots. Reuse of these root channels facilitates deeper maize root penetration by reducing mechanical resistance from the soil particles and improving access to subsoil nutrients. While previous studies demonstrated bacterial enrichment within the reused root channels^{5,8}, our findings extend this understanding to fungi, revealing coordinated structural and functional responses shaped by cover crop identity, soil type and moisture availability.

Fungal diversity and abundances varied among soil types, and were more strongly influenced by root channel reuse. In the drought-prone Luvisol and Podzol, richness and ITS2 gene copy numbers increased under drought, particularly within *Brassicaceae/Poaceae* root channels, whereas responses differed under fallow and *Fabaceae/Poaceae* treatments. Declines in *Chytridiomycota* and *Mucoromycota* under drought are consistent with previous bulk soil observations^{22,23}. In contrast, enrichment of arbuscular mycorrhizal fungi (*Glomeromycetes* and *Mucoromycetes* within *Mucoromycota*) under drought likely reflects their stress-tolerant life history traits and ability to sustain nutrient exchange with host plants under water limitation^{24,25}. *Zoopagomycota* increased under drought²⁶, despite having phylogenetic similarity to *Mucoromycota*²⁷, indicating niche differentiation among zygosporangia-forming lineages. The dominant *Dikarya* (*Ascomycota* and *Basidiomycota*) exhibit limited overall shifts, consistent with their ability to survive under resource limitations through stress-adaptive measures such as sporulation^{28,29}. Associations between fungal phyla and soil physicochemical properties further support niche partitioning across soil profiles³⁰. Fast-growing taxa such as *Ascomycota*, *Chytridiomycota* and *Zoopagomycota* preferentially exploit labile organic C and available N³¹, explaining their positive correlations with TOC and TN in the nutrient-rich Phaeozem and their predominance in the topsoil. Conversely, negative correlations for *Basidiomycota* and *Mucoromycota* with TOC and TN are consistent

with adaptation to lower nutrient conditions in subsoils. *Basidiomycota*, known to establish symbiotic relationships with maize roots³², may benefit from enhanced root penetration into deeper subsoil via reused root channels. These patterns reflect pronounced vertical stratification, whereby topsoil-dominated phyla respond to nutrient availability, while subsoil-dominating communities exhibit adaptations linked to nutrient limitations and symbiotic relationships. Rare fungal phyla in this study, *Aphelidiomycota* and *Cryptomycota*, prefer low moisture and low pH conditions³³, which explains the negative moisture and pH correlations. Drought-associated shifts in trophic modes, characterised by increased representation of saprotrophic and symbiotrophic fungi and reduced pathotrophs, likely reflect improved C sequestration besides plant defence responses and the release of plant root-derived metabolites^{34,35}.

To elucidate the functional dynamics underlying these structural changes within fungal communities in the reused root channels, we used metaproteomics. Upregulation of the fatty acid biosynthesis enzyme 3-hydroxydecanoyl-[ACP]-dehydratase advocates membrane restructuring and adjustments in energy storage under drought¹⁷, even in soils such as the Luvisol and Phaeozem that retain substantial moisture. Within *Fabaceae/Poaceae* root channels in the drought-affected Podzol, downregulation of *Ascomycota* and *Mucoromycota*-derived PDC enzymes reduces acetyl-CoA flux into the citric acid cycle, thereby conserving energy under stress³⁶. Pyruvate may instead be redirected towards proline biosynthesis, an established osmoprotectant pathway³⁷. Downregulation of the proline-degrading enzyme P5CDH in *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Mucoromycota* in *Fabaceae/Poaceae* root channels and fallow favour proline accumulation, enhancing osmotic protection and ROS scavenging³⁸. In contrast, P5CDH upregulation in *Brassicaceae/Poaceae* root channels may reflect inherent stress tolerance or regulation of preventing proline-inflicted cell toxicity³⁹. Oxidative stress regulation emerged as a prominent feature of fungal drought adaptation. Increased expression of superoxide dismutase (SOD), catalase-peroxidase (CAT-PER) and aldehyde dehydrogenase (ALDH) indicates coordinated detoxification of ROS and reactive aldehydes⁴⁰. Treatment-specific expressions of these enzymes suggest that fungi within *Brassicaceae/Poaceae* root channels may possess greater oxidative resilience and may have potentially developed 'stress memory' that invokes counter-mediating approaches under drought⁴¹. Overexpression of glycolytic aldolase in *Blastocladiomycota*, *Chytridiomycota* and *Zoopagomycota* also contributes to cellular repairs by using root exudates as primary C sources for energy⁴². Higher expression of 6-PGL in the pentose phosphate pathway, particularly in the Podzol, implies enhanced NADPH generation essential for glutathione-dependent redox buffering⁴³. Collectively, these findings point towards integrated regulation of energy metabolism and redox homeostasis under water limitation.

Increased expression of N metabolism enzymes in the Podzol underscores metabolic plasticity in nutrient-poor sandy soils. Upregulation of glutamate dehydrogenase (GDH) and urease suggests alternative N assimilation routes⁴⁴, while GDH-mediated deamination may provide C skeletons during limited carbohydrate availability^{45,46}. Concurrent overexpression of methionine cycle and

transsulfuration pathway enzymes, such as homocysteine methyltransferase, MeSe and SAM synthase across *Dikarya*, zygosporic *Mucoromycota* and *Zoopagomycota*, and zoosporic *Chytridiomycota* under drought, potentially catalyse SAM to synthesise abiotic stress-regulating polyamines⁴⁷ and glutathione, central mediators of abiotic stress tolerance⁴⁸. Notably, these metabolic adjustments were most pronounced in the coarse-textured Podzol, whereas the silt-rich Phaeozem exhibited comparatively moderate functional restructuring, underscoring the role of soil physical context in mediating drought responses.

Higher expression of CAZymes may have resulted because of the increased presence of Ascomycota (*Eurotiomycetes*, *Sordariomycetes*)⁴⁹ and *Mucoromycota* (*Mucoromycetes*)⁵⁰, all being previously reported as major fungal CAZyme producers. (Fig. 2c). The predominance of extracellular CAZymes indicates active mobilisation of complex organic matter and breaking down to simpler forms for emergency metabolic steps. Increased expression of cellulose-degrading enzymes β -glucosidase, endoglucanase, exoglucanase and LPMO by *Ascomycota* and *Mucoromycota*, enhance the breakdown of complex carbohydrates into soluble sugars⁵¹, potentially contributing to osmotic homeostasis and cellular stability during drought. These soluble sugars assist in the mobilisation of smaller molecules during water scarcity under drought. The prominence of auxiliary active oxidoreductases, particularly in the Podzol, further suggests active redox-coupled decomposition under drought. Elevated vanillyl-alcohol oxidase activity supports redox balancing through regulated H_2O_2 production. Similarly, hemicellulolytic enzymes such as acetylxyloxy/feruloyl esterase, arabinofuranosidase and xylanase from *Ascomycota* and *Basidiomycota* likely contribute to cell wall remodelling under stress and metabolic flexibility under stress, requiring simpler sugars synthesised by degrading hemicelluloses⁵².

Interpreting microbial dynamics in reused root channels is difficult because these environments are highly variable in space and time. Conditions such as soil moisture, nutrient availability, oxygen levels, and soil structure can change over short distances. These changes strongly influence how microbes grow, interact, and survive. As a result, microbial patterns in these channels are often complex and context-dependent.

The open nature of root channels means they are continuously influenced by surrounding soil properties. This makes it challenging to clearly link environmental factors to specific microbial responses. In addition, results can differ depending on the taxonomic level examined. For example, we observed an increase in arbuscular mycorrhizal fungi (class *Glomeromycetes*) under drought conditions, which agrees with the idea that plants rely more on fungal partners when water is limited. However, this pattern was not consistent at the broader phylum level (*Mucoromycota*). This shows that broad taxonomic groups may hide important ecological *differences*.

Another limitation is the small number of studies focusing on soil fungal communities, especially within rhizosphere environments and structured microsites such as reused root channels. Because of this limited evidence, it is difficult to directly compare or fully confirm our findings.

Further research is needed to better understand how fungal communities develop and respond to environmental stress belowground. Studies that combine detailed spatial sampling with functional analyses will help clarify the dynamic processes shaping fungal communities in soil.

In conclusion, drought indicates coordinated structural and functional adaptations in fungal communities inhabiting the maize rhizosphere within reused cover crop root channels. These responses involve regulation of oxidative stress pathways to counter stress and reduction of C-N metabolic pathways to conserve energy, which varied with soil type and cover crop choices. Root channels formed by mixtures of *Brassicaceae* and *Poaceae* supported fungal communities that showed strong oxidative stress regulation and improved resource management. This pattern suggests a higher tolerance to drought. Therefore, such cover crop mixtures may represent suitable choices for future cropping strategies under similar environmental conditions. Overall, our findings provide mechanistic insight into how cover crop selection can shape fungal-mediated soil processes. They support the concept of strategic cover cropping as an approach to strengthen agroecosystem resilience under climate-induced oxidative stress.

Methods

Crop cultivation and sampling regimes

Crops were grown in three agricultural fields – at experimental estates Hohenschulen of the Kiel University (Achterwehr, Germany, 54°18'44" N, 9°59'46" E), Karkendamm of the Kiel University (Bad Bramstedt, Germany, 53°55'52" N, 9°55'15" E), and Reinshof of the Georg-August-University of Göttingen (Rosdorf, Germany, 51°29'05" N, 9°53'34" E). The cash crop in this study was maize (*Zea mays* L.). Plots without cover crops during the winter (bare fallow) were established as a control and compared against two cover crop mixtures on distinct plots. The cover crops were shallow- and deep-rooting *Brassicaceae* (*Brassica napus* L., rapeseed, shallow-rooting; *Raphanus sativus* L. var. *oleiformis*, oilseed radish, deep-rooting); *Fabaceae* (*Trifolium repens* L., white clover, shallow-rooting; *Trifolium pratense* L., red clover, deep-rooting); and *Poaceae* (*Lolium perenne*, perennial ryegrass, shallow-rooting; *Festuca arundinaceae*, tall fescue, deep-rooting). The mixtures were grown as a combination of shallow- and deep-rooting cover crops of *Brassicaceae*, *Fabaceae* and *Poaceae*, complementing the niche complementarity principle, which has been reported to allow polycultures to overyield when plants compete for resources⁵³. All the cover crops were sown in October 2022 and grew until May 2023 in distinct randomised plots with four replicates of each variation. In May 2023, a herbicide formulation (Roundup, Bayer AG, Leverkusen, Germany) was applied to all experimental plots (including fallow plots) to terminate all cover crops, and subsequently maize was sown in the same plots with the cover crop variations in addition to the fallow plots. Maize was grown in the fields from May to September 2023 (Fig. 1).

To compare drought-like conditions against normal conditions, we artificially induced dry conditions using interrow rainout shelters and continued with the approach after observing differences in soil moisture trends between the sheltered and non-sheltered profiles using time-domain reflectometry

sensors (Fig. S8a-c). These specific types of rainout shelters covered half of the plot area between the maize rows, reducing 50% rainfall and restricting water infiltration to stemflow. The shelters were installed between the crop rows and below the maize canopy in June, at approximately 50 cm aboveground. This position ensured that the structures did not interfere with leaf-level photosynthesis or direct light interception by the maize plants. They were constructed with a sloped configuration to promote air flow and reduce the risk of heat accumulation or stagnant humidity. The plastic film used was standard greenhouse-grade transparent polyethene, which allows high light transmission while providing effective rain exclusion. A depiction of our rainout shelter setup used in the experiment has been provided as a supplementary figure (Fig. S9).

Before soil sampling, the soil profile was excavated to a depth of 1 m, then excavated 40 cm forward to obtain a fresh profile and fresh maize root system and to prevent any contamination by the neighbouring soil. To compare the difference inflicted by drought on microbial communities in the reused root channels of cover crops by maize, we collected soil samples from a vertical soil profile from the topsoil (0-30 cm) and the subsoil (30-60 cm and 60-90 cm) for two different conditions: 1) maize roots growing in the cover crop root biopores (MCR) from those profiles subjected to drought (D), and 2) maize roots growing in the MCR in the profiles under rainfall-fed conditions (RF). Maize rhizosphere soil collected along decayed cover crop roots was considered as originating from maize reusing cover crop root channels. The rhizosphere of maize and decayed cover crop roots was defined as extending 2 mm from the root surface. Therefore, the overlapping 2 mm rhizosphere zone of maize and decayed cover crop roots was designated as the sampling area. The sampling focused on clearly visible maize roots that were closely associated with remaining cover crop root residues, ensuring consistency in sample type across replicates and treatments to reduce subjective variation and avoid systematic error. Pictures of the reused root channels have been provided as a supplementary figure (Fig. S3). Additionally, maize rhizosphere samples from the control plot were collected, representing maize roots growing in bulk soil without reusing cover crop root channels. The sampling was done during the R1-RX growth stage of maize (bolting) grown in the Luvisol (01.08.2023), the Phaeozem (19.07.2023) and the Podzol (26.07.2023), and the profiles were maintained until sampling was done around the flowering and reproduction stage of maize. The samples were extracted from the profiles using a spatula and collected in plastic zip-lock bags. Until shipment to the laboratory, samples were stored in ice coolers containing dry ice in order to preserve microbial communities and the metabolic picture for distinct sampling time points. In the laboratory, all samples were stored at -80°C until further processing. No repetition of sampling was done from the profiles of the same plot in order to avoid bias and duplicates. To minimise potential operator bias when identifying “root overlap regions”, we used a randomised sampling scheme with the four blocks representing the four replicates and the ambient (natural rainfall) and drought (with interrow rainout shelters) plots always paired in direct proximity to each other.

Soil physicochemical properties

Soil microbial biomass carbon (C) and nitrogen (N) were determined using the chloroform fumigation extraction method⁵⁴. In brief, 7.5 g of soil was fumigated with chloroform for 24 h and then extracted with 30 mL of 0.05 M K₂SO₄ on a shaker for 1 h. C and N were measured with the N/C 2100 TOC/N analyser (Analytik Jena, Jena, Germany). MBC was calculated as the difference between extracted C from fumigated and non-fumigated soil with a conversion factor (k_C) of 0.45⁵⁵. MBN was calculated as the difference between extracted N from fumigated and non-fumigated soil with a conversion factor (k_N) of 0.54^{55,56}. The MBC and MBN were presented as $\mu\text{g g}^{-1}$ dry soil. Soil density and moisture content were also measured alongside.

Soil cylinder samples were taken to measure soil bulk density at each soil depth. Soil moisture content was also measured by water content sensors (Teros 10, Meter Group, München, Germany), which were installed in the control plots (in 0-30 cm, 30–60 cm, and 60-90 cm depth under rainout shelter and on the rainfall-fed side) at each experimental site.

To investigate the influence of soil physicochemical properties on the fungal microbiome in the maize root channels, we correlated the parameters pH, soil moisture content, TOC, TN, and soil bulk density with the ITS2 gene amplicon data using Partial Mantel's test⁵⁷ using the R package *microeco* (v1.10)⁵⁸. The soil physicochemical proteins were correlated to individual fungal phyla along the different soil types and soil moisture conditions to understand the impacts of these factors on the individual communities.

ITS2 gene amplicon sequencing

Fungal community composition in the root-vicinity samples was analysed by sequencing amplicons targeting the fungal internal transcribed spacer (ITS) regions of rRNA (2*300 bp) on an Illumina NextSeq™ 550 (Illumina, San Diego, CA, USA). DNA was extracted from 0.25 g of soil using the DNeasy® PowerSoil® Pro Kit (QIAGEN GmbH, Hilden, Germany). PCR amplicons of the ITS2 region of the fungal rRNA gene were prepared using the forward and reverse primers fITS7 and ITS4 and the NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs GmbH, Frankfurt, Germany)⁵⁹. Sequencing libraries were prepared from 100 ng of DNA according to the Illumina protocol. Dual index adapters for the sequencing were attached using the NEBNext Multiplex Oligos for Illumina. The final concentration of the libraries was 2 nM after pooling. We sequenced triplicates of samples from each soil depth and root-vicinity source per cover crop variation plot for all three sampling sites (ITS2 gene amplicons, $n=147$).

The sequencing data were analysed using QIIME2 v2023.5⁶⁰. First, the raw sequence reads were demultiplexed and quality-filtered (q-score 25) using the q2-demux plugin, followed by denoising with q2-dada2. The ITS2 regions of the fungal rRNA were trimmed to 230 bp for both the forward and reverse sequences. All amplicon sequence variants (ASVs) were aligned with q2-alignment, and then maximum-likelihood trees were constructed using q2-phylogeny. We chose ASV-based methods over OTU

approaches to limit the effect of spurious taxa on diversity indices⁶¹. Taxonomic assignment of fungal ASVs was carried out using the q2-feature-classifier and the classify-sklearn Naïve Bayes taxonomy classifier against the UNITE v10.0 database⁶² for QIIME2 (released on 04.04.2024). ASVs with a relative abundance of <0.01% were defined as rare taxa.

Quantitative PCR (qPCR)

The copy number of the fungal ITS2 gene per gram of soil was quantified by SYBR[®] Green-based qPCR using a 7500 Fast Real-Time PCR System (Applied Biosystems[™], Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of the same DNA extract utilised in amplicon sequencing were used for qPCR. Dilutions of template DNA were used to compensate for the effect of PCR inhibitors in the samples. Each sample was analysed in triplicate. A PCR amplicon of the ITS2 region derived from *Trametes versicolor* (DSM 11269) was used to generate the standard curve. Each 20 µL reaction contained 1 µL of template DNA, the forward and reverse primers fITS7 and ITS4 for the ITS2 gene⁵⁹ without adapter nucleotides and Luna[®] Universal qPCR Master Mix (NEB). Reaction conditions were an initial denaturation for 1 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 30 s. The melting curve was recorded in the temperature range of 60°C to 95°C. The gene copy numbers per gram of soil were determined in comparison against the standard essentially as before⁶³. The average efficiency value was $100.8 \pm 3.2\%$.

Metaproteomics analysis

At each timepoint, samples were collected separately from three plots for each cover crop variation at the analysed soil depths and root-vicinity sources and used for proteomic analyses following a previously described protocol⁵ ($n=119$). Approximately 4 g of soil was used for protein extraction using the SDS buffered-phenol extraction method as previously described⁵. The protein extract was purified using 1-D SDS-PAGE, and then the protein extract was further proteolytically cleaved using trypsin (Promega). A nano-HPLC system (UltiMate[™] 3000 RSLCnano system, Thermo Fisher Scientific, Waltham, MA, USA) was used to separate the peptide lysates. The system was connected to a Q Exactive HF Orbitrap LC-MS/MS system (Thermo Fisher Scientific) equipped with a nano electrospray ion source, Triversa NanoMate[®] (Advion, Ithaca, NY, USA). We searched the MS/MS data against an in-house generated proteome database containing all the defined proteomes in UniProt for the fungi identified by ITS2 gene amplicon sequencing. The database search was performed with Proteome Discoverer[™] (v2.5.0.8, Thermo Fisher Scientific) using the SEQUEST-HT algorithm, and all of the outputs are available on PRIDE (EMBL-EBI)⁶⁴. The precursor mass tolerance of the MS was set to 10 ppm, and the fragment mass tolerance of the MS/MS was 0.02 Da. Carbamidomethylation of cysteine was considered fixed, and oxidation of methionine was set as a dynamic modification. Enzyme specificity was set to trypsin with up to two missed cleavages allowed using 10 ppm peptide ions and 0.02 Da MS/MS tolerances.

Only rank-one peptides with a Percolator-estimated false discovery rate (FDR) <1% were accepted as identified. The GhostKOALA and KEGG⁶⁵ and COG⁶⁶ databases were used for protein functional annotation. Pathways with a minimum of two proteins and a minimum coverage of 5% were selected for downstream processing. The carbohydrate-active enzymes (CAZymes) were identified using Unipept Desktop (v2.0.0, Ghent University)⁶⁷. The identified CAZy enzymes and their preferred substrates provide information regarding rhizo-deposits in the soil profile along the cover crop root channels. To estimate the proportion of extracellular carbohydrate-active enzymes (CAZymes), signal peptides were predicted using Phobius⁶⁸ and proteins harbouring a signal peptide were classified as extracellular. Label-free quantification (LFQ) intensities of the identified proteins were analysed to characterise functional metabolic pathways and to assess their variation across sampling sites, soil moisture regimes, soil depths, and cover crop treatments.

A custom reference database was constructed from the UniProtKB database by selecting protein sequences corresponding to the fungal phyla identified through ITS2 gene amplicons. During database assembly, redundancy was minimised while maintaining taxonomic and functional relevance to avoid repeated assignment of already quantified proteins. Only proteins unambiguously identified by unique peptides and successfully mapped to the UniProt reference database were retained for quantification.

Taxonomic identities were retrieved using ENTREZ identifiers from the NCBI database^{69,70} employing KEGG Orthology (KO) numbers as unique protein identifiers. Mapping identified proteins to NCBI entries enabled comprehensive taxonomic annotation of their source organisms. Non-fungal proteins were excluded from further analyses. Functional pathway annotation was performed using KEGG and COG identifiers, which were linked to individual proteins. The integration of KO-based taxonomic assignments with pathway annotations enabled the generation of combined datasets capturing both community composition and functional potential.

LFQ intensities were normalised by $\log_2$ transformation using the *log* function of base R (v4.3.1)⁷¹ prior to visualisation and statistical tests. Following pathway categorisation, proteins were mapped onto biogeochemical cycles of interest to evaluate shifts associated with soil properties, moisture status, and the re-use of cover crop root biopores, thereby elucidating microbial functional responses.

To assess the impact of environmental stress following root channel re-use, fold changes in LFQ intensities under drought conditions were calculated relative to rainfall-fed controls. Proteins were considered differentially abundant when $|\log_2 \text{ fold change}| > 0.6$ and $p < 0.05$ ⁷². In addition, to quantify the effects of cover crop root channel re-use, differential abundances were determined by subtracting protein intensities measured under cover crop treatments (*Brassicaceae/Poaceae* and *Fabaceae/Poaceae* rotations) from those observed under fallow conditions.

Statistical data analysis

We used R (v4.3.1)⁷¹ to perform all statistical analyses of the sequencing and the metaproteomics data. All measures of significance were calculated using permutational multivariate analysis of variance (PERMANOVA), followed by Tukey's range post-hoc test (TukeyHSD) with package *stats* (v3.6.2)⁷³. In the ITS2 gene sequencing analysis, the ASV abundance tables were filtered with total-frequency-based filtering based on 95% sequence identity (via *q2-feature-table summarize*) and rarefied at 30,000 sequences to ensure equal sampling depth and sorting in the maximum number of samples for diversity analyses. Alpha and beta diversity metrics were calculated using the packages *phyloseq*⁷⁴ and *metacoder* from R (v4.3.1)⁷¹. Observed ASV richness measured for each cover crop variation at different sampling sites and conditions was used for estimating alpha diversity richness. Pielou's evenness is the most widely used diversity evenness index in the ecological literature⁷⁵. For beta diversity, we used Bray-Curtis dissimilarity⁷⁶ and visualised differences via Principal Coordinate Analysis (PCoA) using the *vegan* package (v2.6-4)⁷⁷. Using a four-way permutational multivariate analysis of variance (PERMANOVA), we evaluated the significantly different cover crop variations using the sampling sites, sampling conditions and sampling depths as random effects and cover crop variations as the fixed effect. This was followed by Tukey's HSD for evaluating MANOVA test outcomes with parameters of cover crop variations, depth, sampling sites, soil moisture conditions and fungal phyla. For fungal abundances under different parameters, the significance between the parameters was represented using the Compact Letter Display (CLD)⁷⁸ since we can represent multiple pairwise significances using linear models. For metaproteomics, the significantly different cover crop variations or proteins of different metabolic pathways or fungal phyla were calculated using MANOVA, using cover crop variations, sampling depths, soil moisture conditions, sampling sites and fungal phyla as fixed factors. Upon determination, they were represented by significant stars based on the adjusted *p*-values ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). All figures were generated in RStudio. Other integrated packages used for statistical analyses and figure generation were *tidyverse* (v2.0.0), and *dplyr* (v1.1.3).

Declarations

Data availability

The metaproteomics datasets generated during the current study are available in the PRIDE data repository with the sample metadata, vide PRIDE dataset identifier PXD062138 (<https://www.ebi.ac.uk/pride>). The raw sequencing data and the respective metadata generated from this study are available under the NCBI BioProject ID PRJNA1240274, which can be accessed using the link: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1240274>. The raw qPCR data, along with the sample metadata, are available on Zenodo under the DOI: <https://doi.org/10.5281/zenodo.18889777>.

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Author information

Authors and Affiliations

Department of Molecular Toxicology, Helmholtz Centre for Environmental Research – UFZ GmbH, Leipzig, Germany

Debjyoti Ghosh, Nico Jehmlich, Martin von Bergen

Institute of Biological Interfaces, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany

Jochen A. Müller, Anne-Kristin Kaster

Department of Bio- and Environmental Sciences, International Institute Zittau, Dresden Institute of Technology, Zittau, Germany

Harald Kellner

Institute of Plant Nutrition and Soil Science, Kiel University, Kiel, Germany

Yijie Shi, Iris M. Zimmermann, Sandra Spielvogel

Institute of Crop Science and Plant Breeding, Kiel University, Kiel, Germany

Katja Holzhauser

Biogeochemistry of Agroecosystems, University of Göttingen, Göttingen, Germany

Tobias Stürzebecher

Geo-Biosphere Interactions, University of Tübingen, Tübingen, Germany

Michaela A. Dippold

Institute for Biochemistry, University of Leipzig, Leipzig, Germany

Martin von Bergen

German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany

Martin von Bergen

Author contributions

N.J., J.A.M., M.A.D., and S.S. conceived and designed the study; D.G., Y.S., I.M.Z., T.S. and K.H. organised and coordinated fieldwork; D.G. performed the amplicon sequencing and metaproteomics experiments; Y.S. performed the soil physicochemical characterisations; D.G. analysed and interpreted all experimental observations; D.G., N.J., H.K and J.A.M wrote the paper with inputs from all authors; all authors read and approved the final manuscript.

Corresponding authors

Correspondence to Nico Jehmlich (nico.jehmlich@ufz.de) and Debjyoti Ghosh (debjyoti.ghosh@ufz.de).

Ethical declarations

Competing interests

The authors declare that they have no known competing interests that could have influenced the work being reported in this manuscript.

Supplementary information

Additional supplementary information and extended data can be found in the Supplemental Text, Figures S1-S10 and Tables S1-S14.

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Figures

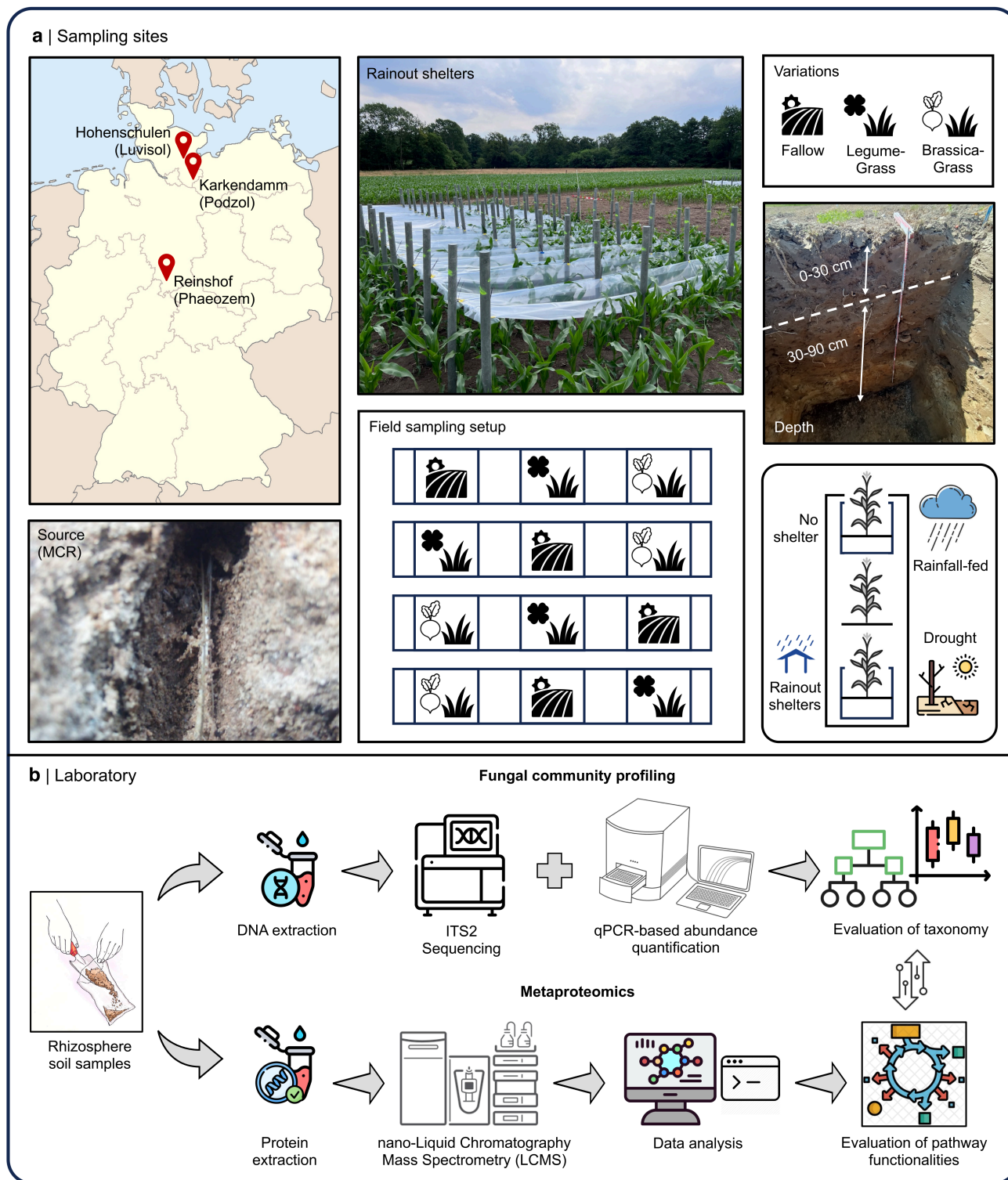


Figure 1

The experimental design for studying reuse of winter cover crop root channels to cultivate maize. a. Field sites: samples from Luvisol, Podzol, and Phaeozem in Germany with comparison of fungal microbiome associated with roots of maize (*Zea mays* L.) in root channels of cover crop [Legume/Grass (*Fabaceae/Poaceae*), Brassica/Grass (*Brassicaceae/Poaceae*)] or fallow (no cover crops) as control conditions. To replicate drought conditions, plots were covered with rainout shelters. **b.** Community

profiling using ITS2 gene-based amplicon sequencing, qPCR, and metaproteomics were used to visualise how the composition and functional role of fungal communities in the maize rhizosphere changed after being exposed to drought.

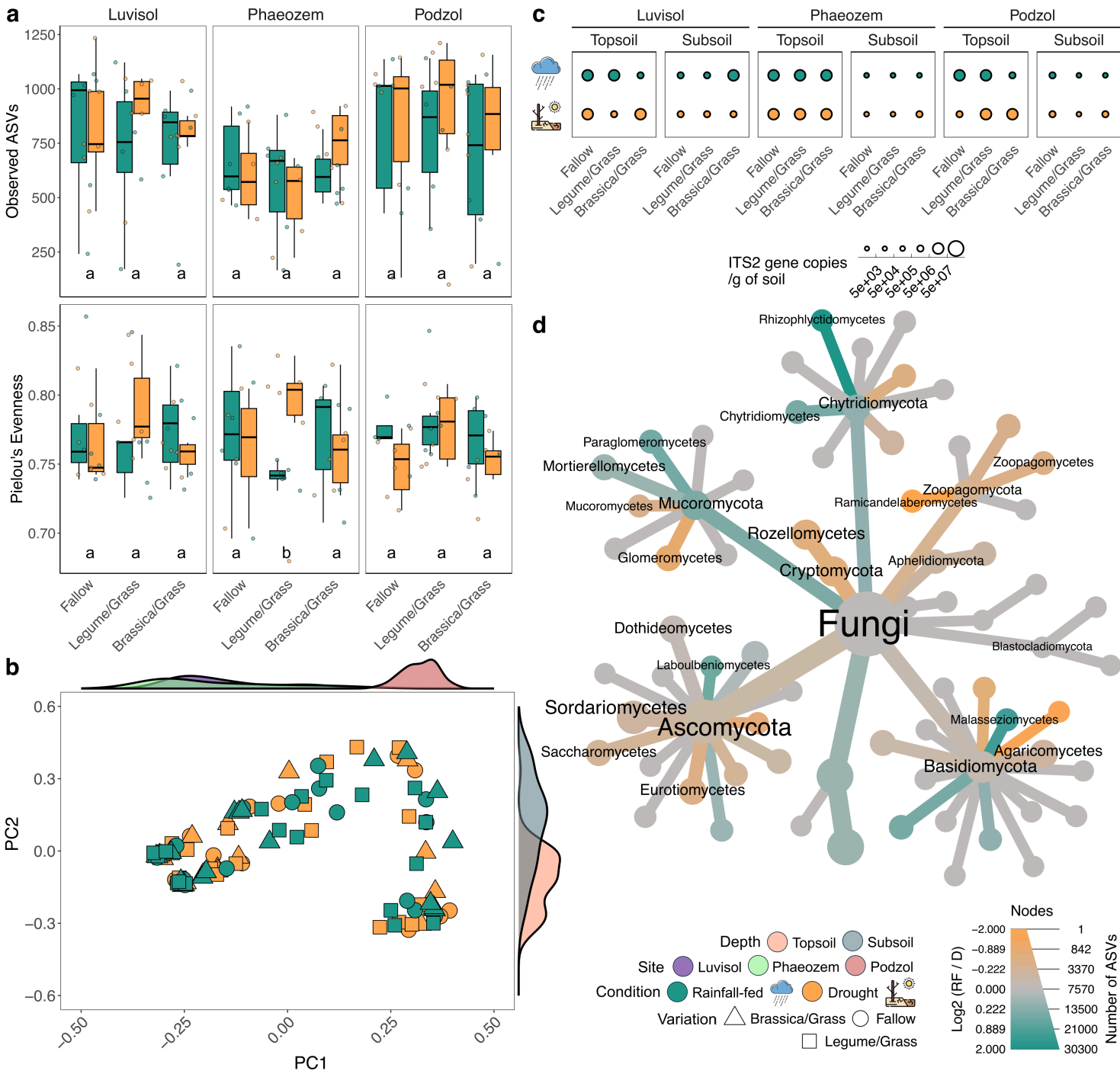


Figure 2

The taxonomic diversity and absolute abundances at the different experimental sites under soil moisture conditions. **a.** Alpha diversity richness (Observed ASVs) and evenness (Pielou's evenness index) metrics reflecting the distribution of fungal communities at the three sampling sites under drought (D) and rainfall-fed (RF) conditions. Pairwise correlation between the variations is shown using a

compact letter display representation for the significant differences between the variations, calculated using Tukey's range test (TukeyHSD); **b.** Fungal community beta-diversity visualised using PCoA ordination based on Bray-Curtis dissimilarities along the sampling sites and depths for each cover crop variation under soil moisture conditions. Distance metrics were calculated from the identified amplicon sequence variants (ASVs). The different colours of the density plots next to the axes represent the soil types and sampling depths, and peak height and shape abundance; **c.** ITS2 gene copies measured using qPCR as surrogate for absolute fungal abundances in the soil types, of each cover crop variation (Fallow, Legume/Grass and Brassica/Grass) and soil moisture conditions (drought (D) and rainfall-fed (RF)); **d.** Taxonomic heat trees represent the differential abundance of the fungal phylotypes between two parameters of soil moisture content [drought (D) vs rainfall-fed (RF)]. The yellow-ochre colour indicates increases under D conditions and green colour under RF conditions. For Luvisol, $n = 35$; for Phaeozem, $n = 36$; and for Podzol, $n = 35$; For soil moisture conditions (drought and rainfall-fed) and depth (topsoil and subsoil), $n = 106$ (statistical details in Supplemental Tables S2c-h).

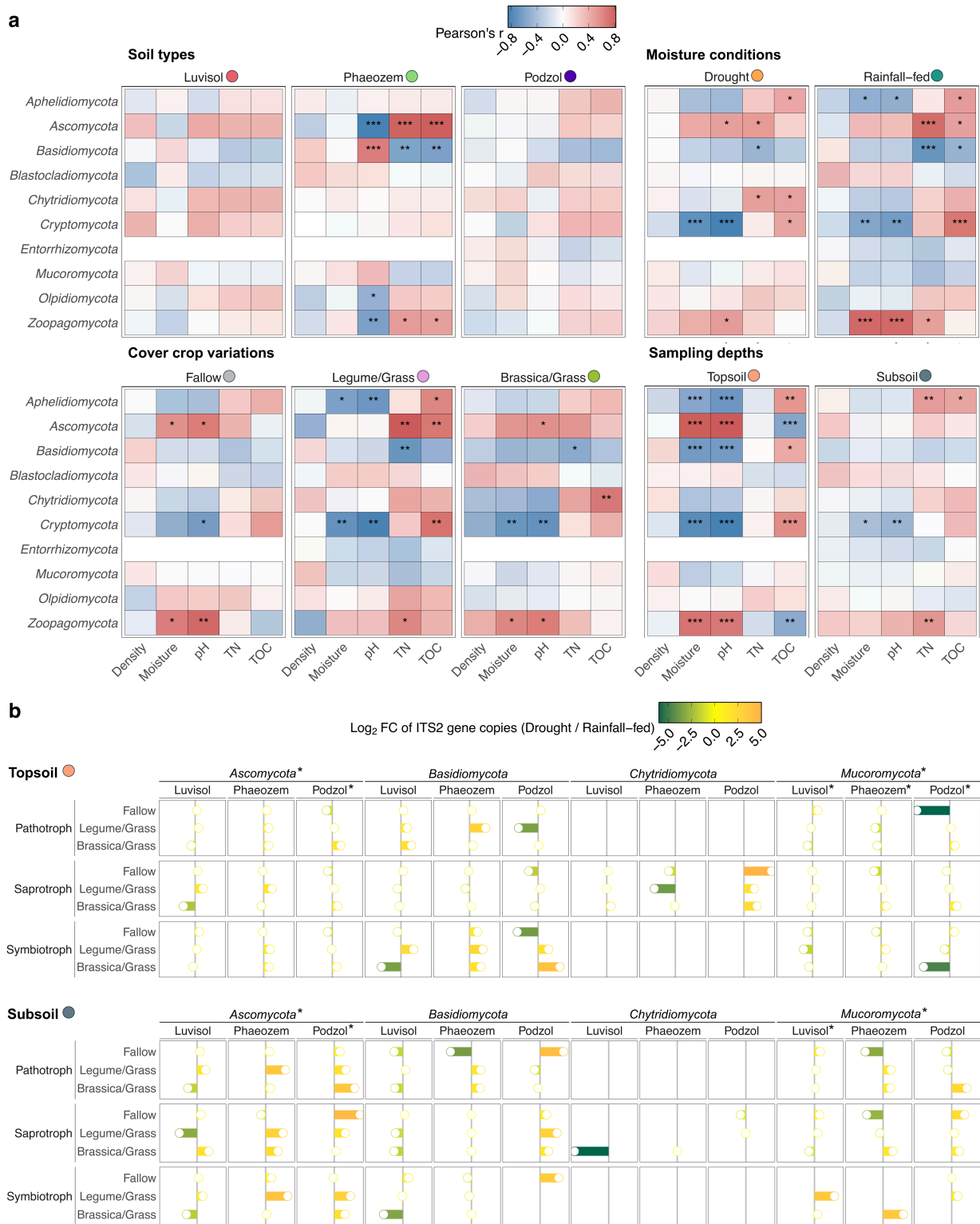


Figure 3

Correlation of individual fungal communities and the soil physicochemical properties across the parameters of study and the comparative abundance of fungal communities based on trophic modes.

a. The correlations between each fungal phylum with the physicochemical properties (TN, TOC, pH, Moisture and Density) have been estimated for each cover crop variation, soil moisture conditions, soil types and sampling depths using Partial Mantel's test. Pearson's correlation values and the significance

estimation for each physicochemical protein and each phylum are provided in the Supplemental Tables S5a-d; **b**. The comparative abundance of the fungal communities classified by trophic modes, as defined in the FUNGuild database. This was estimated by $\log_2\text{-FC}$ values of ITS2 gene copies per gram soil for drought versus rainfall-fed conditions. The yellow-ochre colour indicates increases under D conditions and green colour under RF conditions. The significant correlations are represented by asterisks inside the heatmap tiles; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

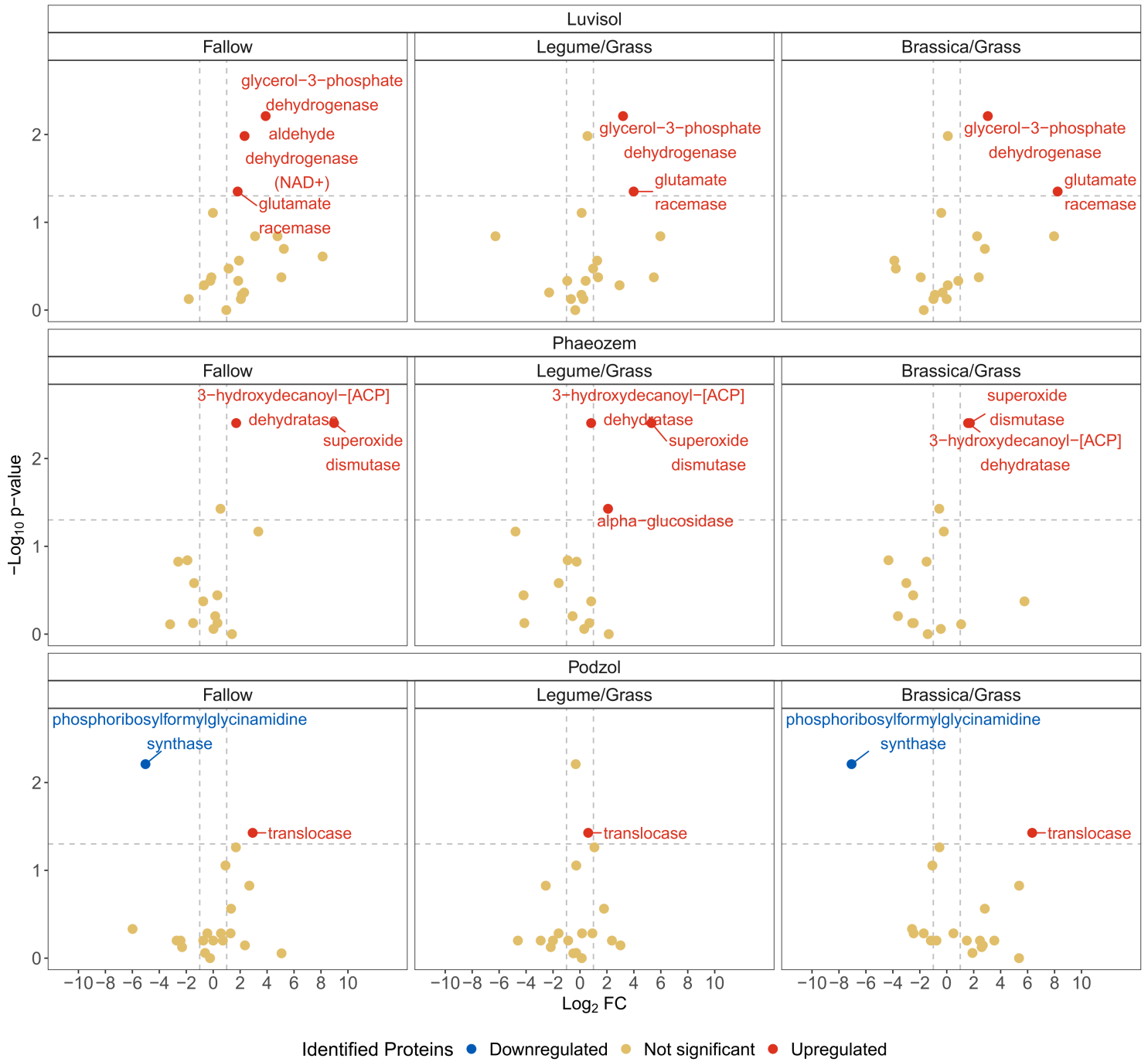


Figure 4

Upregulated and downregulated fungal proteins under drought. A volcano plot representation for the upregulation and downregulation of fungal proteins differing significantly under drought conditions at

the three sampling sites (Luvisol, Phaeozem and Podzol) for the cover crop variations (Fallow, Legume/Grass and Brassica/Grass). The x-axis represents the $\log_2$ -FC and the y-axis represents the $-\log_{10}$ p-value according to Mann-Whitney U test. The proteins having $\log_2$ fold-change (FC) values < 0.6 or > -0.6 and $p > 0.05$ were classified as 'Not significant'. $\log_2$ -FC > 0.6 and $p < 0.05$ were the 'Upregulated' proteins and $\log_2$ -FC < -0.6 and $p < 0.05$ were the proteins 'Downregulated'. Names of several identified proteins are not shown to avoid crowdedness. The information for the all the represented significantly upregulated or downregulated proteins have been provided in the Supplemental Table S10.

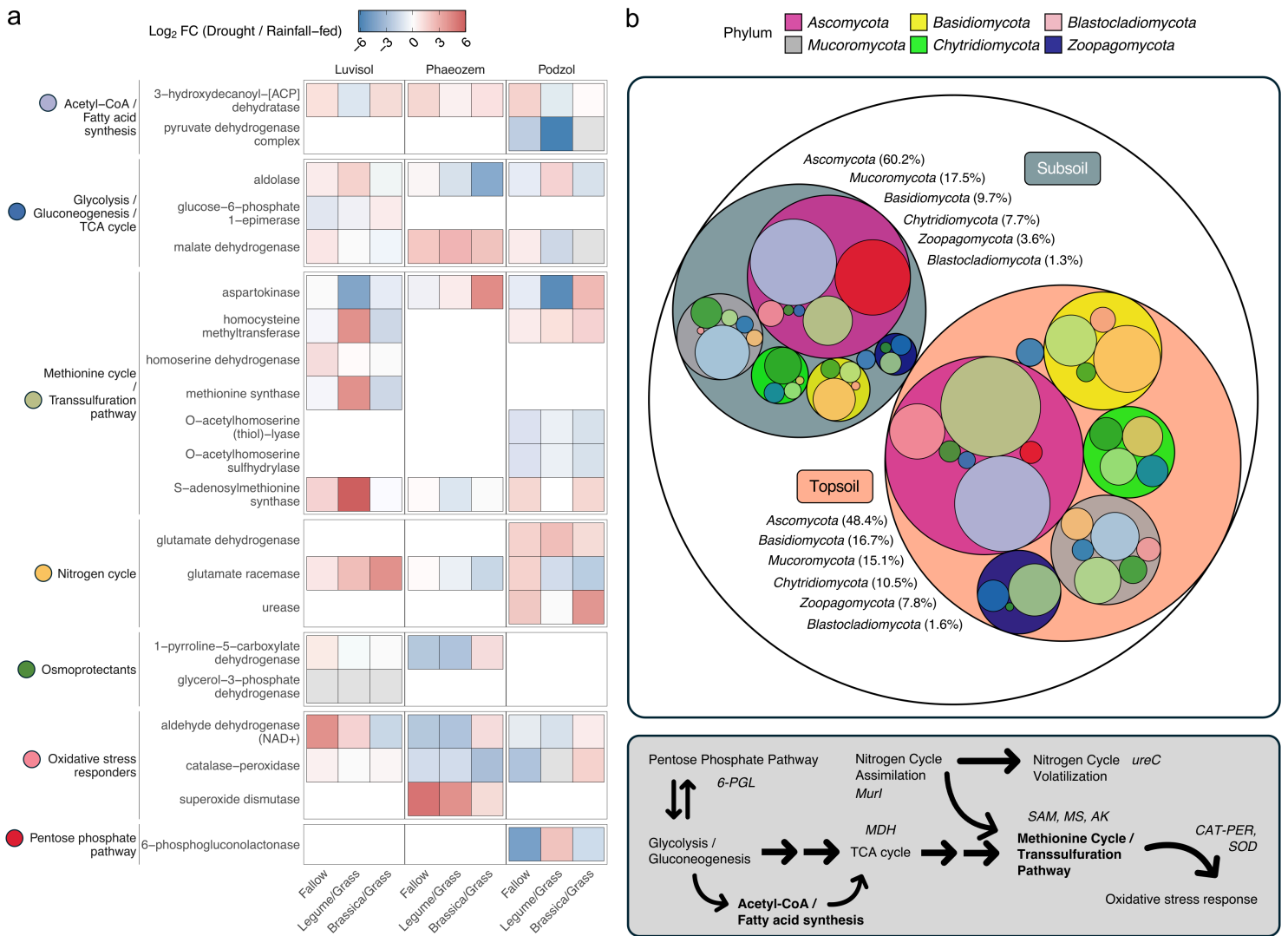


Figure 5

Overview of the functional pathways affected by drought in three soils after cover crop root-channel-reuse across the soil sampling depths. The heatmap is showing the fold change of expressions based on measured protein LFQ intensities for each protein identified under drought and rainfall-fed conditions. Positive logarithmic values of the relative change are denoted by red (higher in drought than in rainfall-fed conditions) and negative values by blue (lower in drought than in rainfall-fed conditions). The small

circles next to the names of the biochemical cycles have unique colours, which also have been used to denote the cycles in the circular packing plot, denoting the fungal protein distribution across the soil sampling depths and the contribution from the fungal communities. The significance of the changes in protein expression under drought conditions at each site was calculated using a four-way ANOVA model, significant changes in expression are marked with an asterisk ($p < 0.05$) (Supplemental Table S7a consists of a list of proteins which represented significant changes across the parameters of study); At the end, a schematic representation of the dynamics of the functional pathways observed in this study has been portrayed.

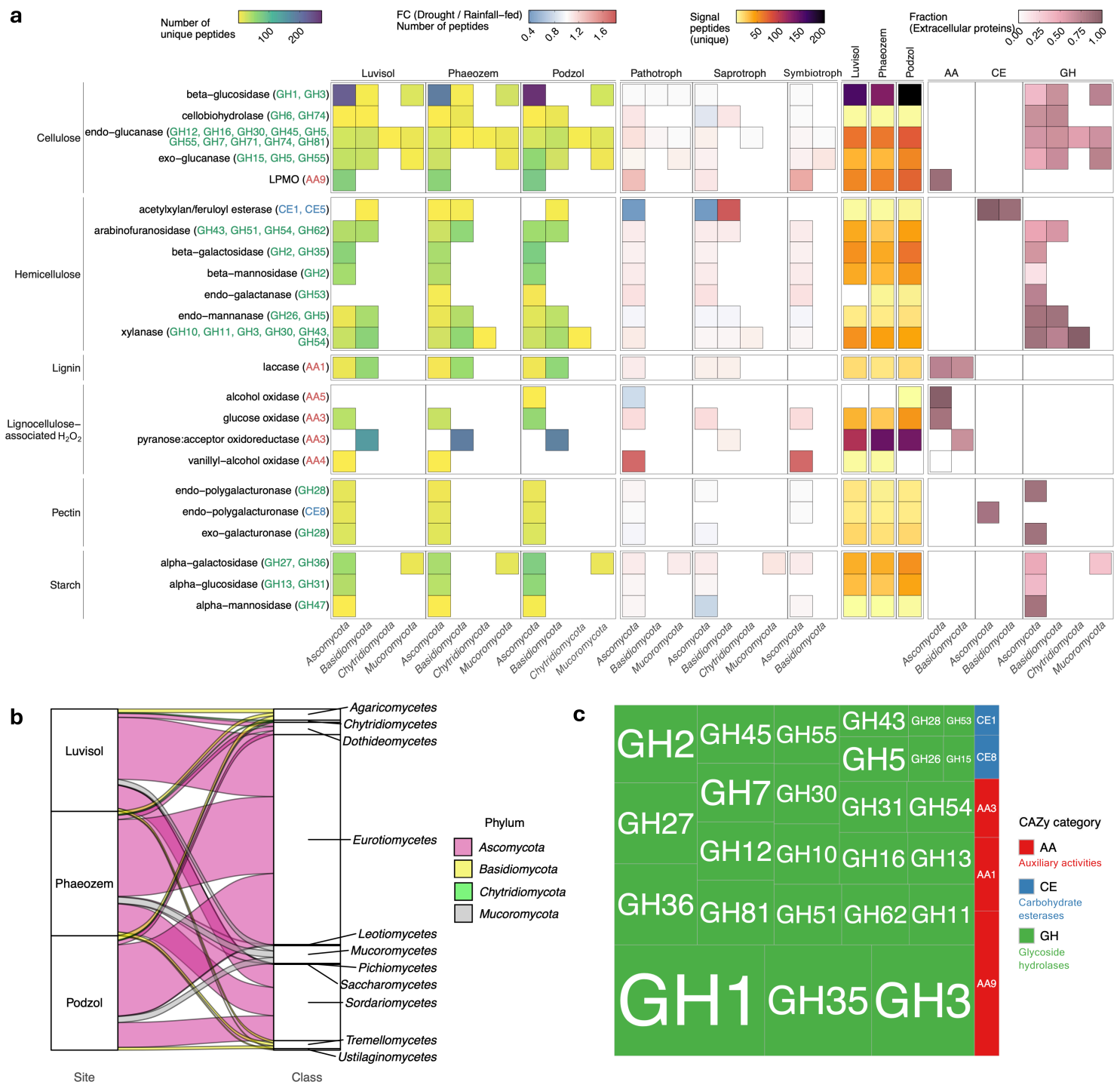


Figure 6

The behaviour of fungal CAZymes under drought stress. a. The heatmap represents a combination of several heatmaps expressing in the following order: the total number of peptides mapped to fungal CAZymes that act on lignocelluloses or are involved in supportive activities related to lignocellulose degradation; positive fold-change values of the number of peptides from CAZymes under drought against rainfall-fed are denoted by red (higher in drought than in rainfall-fed conditions) and negative values by blue (lower in drought than in rainfall-fed conditions). The CAZymes are linked to the fungal phyla, mentioned at the x-axis and at the top of the heatmap contains information about fungal trophic modes (pathotroph, saprotroph and symbiotroph); the total number of signal peptides mapped back to the CAZymes using Phobius; the quantification of the fraction of the number of extracellular proteins, detected by the presence of signal peptides. Further details about the fungal classes linked to these enzymes are represented in the Sankey diagram **(b)**, which we link to the soil types featuring the communities; **c.** A treemap representation of the CAZy database-classified categories that had the highest representation in the heatmap. The three colours are for representing the three categories of CAZymes identified – enzymes with auxiliary activity (AA) in red, carbohydrate esterase (CE) in blue and glycoside hydrolase (GH) in green. A summary of identified CAZymes is provided in Tables S8a-b and the entire list of signal peptides predicted by Phobius are provided in Tables S11a-c.

Supplementary Files

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