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Received: 1 July 2023 | Accepted: 5 April 2024

DOI: 10.1111/1462-2920.16627

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY

Enhancement of soil aggregation and physical properties through fungal amendments under varying moisture conditions

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Funding information
Schlumberger Foundation; Plurinational State of Bolivia

Abstract

Soil structure and aggregation are crucial for soil functionality, particularly under drought conditions. Saprobic soil fungi, known for their resilience in low moisture conditions, are recognized for their influence on soil aggregate dynamics. In this study, we explored the potential of fungal amendments to enhance soil aggregation and hydrological properties across different moisture regimes. We used a selection of 29 fungal isolates, recovered from soils treated under drought conditions and varying in colony density and growth rate, for single-strain inoculation into sterilized soil microcosms under either low or high moisture (≤−0.96 and –0.03 MPa, respectively). After 8 weeks, we assessed soil aggregate formation and stability, along with soil properties such as soil water content, water hydrophobicity, sorptivity, total fungal biomass and water potential. Our findings indicate that fungal inoculation altered soil hydrological properties and improved soil aggregation, with effects varying based on the fungal strains and soil moisture levels. We found a positive correlation between fungal biomass and enhanced soil aggregate formation and stabilization, achieved by connecting soil particles via hyphae and modifying soil aggregate sorptivity. The improvement in soil water potential was observed only when the initial moisture level was not critical for fungal activity. Overall, our results highlight the potential of using fungal inoculation to improve the structure of agricultural soil under drought conditions, thereby introducing new possibilities for soil management in the context of climate change.

INTRODUCTION

Soil structure is an important aspect of soil quality and is essential to sustaining soil functioning (Mueller et al., 2013), for instance by affecting water retention and carbon sequestration (Rabot et al., 2018; Rawls et al., 2003). Soil structure is affected by physical soil degradation (Blum, 2011; Saljnikov et al., 2022), which represents a global threat to agricultural and forest soils, impacting food production and security (Bindraban et al., 2012; Costantini & Lorenzetti, 2013; Strunk, 2003). Soil degradation includes the

deformation of the inner structure due to changes in climate, human activities such as excavations, construction activities, and ongoing disruptive agricultural soil management leading to erosion (e.g., tillage, grazing, clear-cutting) (Mohr et al., 2013; Saljnikov et al., 2022). Changing land use is known to affect soil structure with adverse effects on soil hydraulic properties (e.g., soil water retention) (Chandrasekhar et al., 2018; Horel et al., 2015). This can impact agricultural productivity and environmental integrity, especially in times of climate change with predicted increased aridity and large-scale drought in the coming decades (Gelybó et al., 2018; Qi et al., 2022).

Soil aggregate stability is frequently used as an indicator of soil structure (Amézketa, 1999; Six et al., 2000). Soil aggregates are defined as the association of soil organo-mineral particles bound together with forces that are stronger than the forces between adjacent soil aggregates (Martin et al., 1955). The binding forces result from a combination of biotic and abiotic processes (Bronick & Lal, 2005), and the stability of soil aggregates reflects their ability to resist disruption as a result of mechanical forces (tillage), and hydraulic processes, for example, swelling and shrinking, and rapid wetting by raindrops (Le Bissonnais, 1996). Soil aggregates are typically grouped by size, with macroaggregates being larger than 250 μm and microaggregates being smaller than 250 μm (Tisdall & Oades, 1982). A soil structure that supports soil functioning includes a wide range of hierarchical orders of soil aggregates and is dynamically maintained through time (Dexter, 1988), with a higher turnover for soil macroaggregates as compared to microaggregates (Tisdall & Oades, 1982). For example, short events of drought significantly decrease the proportion of soil macroaggregates in the topsoil layer (Zhang et al., 2019), resulting in a degradation of soil structure. Changes in the size distribution of soil aggregates impact the pore size distribution, which influences soil hydraulic conductivity, moisture retention, and soil aeration (Gelybó et al., 2018; Lipiec et al., 2007; Witkowska-Walczak, 2000) and thereby biotic interactions (Vos et al., 2013; Wolf et al., 2013).

Microorganisms play a key role in the formation of soil structure and its dynamics over time. For instance, mycorrhizal and saprophytic fungi are involved in the formation and stabilization of soil aggregates (Lehmann & Rillig, 2015; Six et al., 2004), in the formation of the network of soil pores (Jongmans et al., 1997), and in the alteration of water distribution (Falconer et al., 2012). Soil fungi influence soil aggregate formation through a variety of different processes. Fungal hyphal networks can enmesh soil particles, which increases the formation and stability of micro and macroaggregates (Miller & Jastrow, 2000; Rashid et al., 2016; Tisdall, 1994). Lehmann et al. (2020) identified fungal biomass density as one of the main predictors of soil aggregation stability. Fungi also influence the cohesion of soil particles within soil aggregates through the secretion of proteins, notably glomalin-related soil proteins (GSRPs) associated mainly with arbuscular mycorrhiza (AMF), as well as mucilage, polysaccharides, and other extracellular compounds (Liu et al., 2020; Rillig & Mummey, 2006). In addition, fungi secrete hydrophobic proteins (hydrophobins) that reduce water infiltration, thereby preventing water from entering soil aggregates, which can disrupt them through the processes of slaking and swelling (Chenu & Cosentino, 2011; Rillig, 2005). Because of these various fungal properties, fungi enhance soil aggregate stability (Piccolo & Mbagwu, 1999). However, we have relatively little information on how effects on soil aggregation and hydrophobicity are affected by fungal strains under different levels of moisture.

Fungi have also adapted to cope with low levels of moisture, and certain groups of yeast and filamentous fungi have evolved the capacity to adapt to dry environments (Magan, 2007). Fungi can tolerate different levels of moisture (Gostinčar et al., 2009) due to their ability to cross air-filled soil pores and translocate water through their hyphal networks (Guhr et al., 2015; Miller & Fitzsimons, 2011). When soil moisture changes, fungi can also alter their internal osmolarity (Yaakoub et al., 2021), and therefore maintain their turgor (Lew & Levina, 2007) by accumulating compatible solutes such as proline and glycerol in response to lowered water potentials (Kubicek & Druzhinina, 2007; Walker & White, 2017). When soil moisture reaches very low levels and the hyphae are expected to stop growing, many fungi can survive by producing specialized cells including spores that survive long periods of dry conditions and resume growth after rewetting (Segers et al., 2016).

Fungal inoculates (e.g., *Trichoderma* spp. or *Gliocladium* spp.) have been widely used to improve soil and plant health and help control pathogens (Vinale et al., 2008). However, little is known about the impact of non-mycorrhizal fungal inoculates on soil structure under limited conditions of moisture and how such impacts affect soil hydrological properties (e.g., water retention or water repellency). Given the important role that fungi play in soil structure formation and their ability to succeed under dry conditions, inoculating soil with drought-resistant fungal strains may represent a promising strategy to improve soil aggregation, water retention, and thereby soil quality.

Our objective was to examine the effect of saprobic fungal strains on soil aggregation and soil hydrological properties under different moisture levels. To select fungal strains that are associated with dryer soils, we isolated a large collection of fungal strains from a field drought experiment in the Netherlands. From this collection, we selected 29 strains, identified them using

genomic analyses, and used them to conduct microcosm inoculation experiments with 2 contrasting moisture levels. The high moisture level was related to optimal plant growth as a reference, and the low soil moisture level was closer to drought. Microcosm experiments were used to assess the ability of fungal strains to impact soil aggregation and hydrological parameters such as soil water content, water hydrophobicity, sorptivity, and water potential. Fungal growth rate and hyphal density were also examined via plate assays as potential predictors of the ability to influence soil aggregation status. We hypothesized that (i) fungal inoculation would modify soil hydrological properties (water retention, water repellency, sorptivity, and water potential) and improve soil macroaggregate formation and stabilization, (ii) higher fungal growth rate and colony density per cm^2 in agar culture would predict improvements in soil aggregation and stabilization, and (iii) the low moisture level tested will enhance fungal biomass leading to improved soil aggregation. Taken together, the results of this study are expected to serve as a basis for the development of fungal inoculation strategies to improve soil structure and water retention under low moisture conditions.

EXPERIMENTAL PROCEDURES

Soil harvest and isolation of fungal strains

Soil samples were taken from an experimental natural grassland (*Arrhenatherum elatius* association) subjected to drought at Fort Rijnauwen in Utrecht, the Netherlands (52°04′24.8″N 5°10′32.4″ E). The experiment had 2 types of levels of drought: one level with approximately 90% of rainfall reduction in summer (pulse) (D90) whereas the 50% level (press) (D50) simulated a long-term reduction of precipitation relative to the annual average rainfall of the last 100 years in the Netherlands. The texture of the soil was classified as loamy sand and categorized as regosol (Food and Agriculture Organization of the United Nations, 2015). Samples were taken from D90, D50, and non-drought plots to a depth of 100 mm using a metal core with a diameter of 25 mm, which was flamed between samples to avoid cross-contamination. Then, soil samples were packed into plastic bags and transported in coolers with ice to the soil laboratory of the Institute of Environmental Biology at Utrecht University. Within 5 h after soil harvest in the field, the top layer of the soil samples that contained coarse organic matter (approx. 30 mm) was discarded (Janssen et al., 2002), large roots and stones were removed from the remainder, and the remaining soils were sieved through a sieve of 2 mm mesh-size and stored at 4°C until fungal isolation.

To isolate fungal strains, 1 g of field-sieved soil was suspended in 100 mL of *phosphate-buffered* saline solution (PBS) and shaken overnight at 100 rpm (orbital shaker Gerhardt, Germany). The soil suspensions were disrupted twice (1 min) using a sonicator (Sonicor Instrument Corporation, USA; Kurm et al., 2017) and filtered using a sterile medical gauze (Cutisoft). Then, the soil suspensions were diluted at 10^{-2}, 10^{-3} and 10^{-4}, and 1 mL of each dilution was inoculated onto potato dextrose agar (PDA) plates using a sterile glass spreader. To avoid bacterial growth, the medium was amended with chloramphenicol (0.05 mg/L) (Sigma-Aldrich) dissolved in absolute ethanol and sterilized using a 0.2 μm filter. Inoculated plates were incubated at 25°C for 5 days. Fungi with diverse morphotypes were selected and re-isolated on fresh PDA plates. Colonies were morphologically described and then picked for inoculation onto PDA slanted tubes incubated for 5 days and stored at 4°C until use.

Selection of fungal strains

A total of 133 fungal strains were isolated and identified (see Appendix A1). From this collection, we selected 29 fungal strains to test their impact on soil aggregate formation and stability. Strains were selected based upon at least one of the following 3 criteria: (i) fungal taxa that were abundant according to corresponding operational taxonomic units (OTUs) (Appendix A2) from the drought soil of isolation compared to the non-drought field treatment, (ii) fungal strain that belonged to taxa previously demonstrated for their presence and role in agricultural soils (e.g., *Trichoderma* spp. *Metarhizium* sp., *Purpureocillium* sp.) (Baron et al., 2020; Waghunde et al., 2016), and (iii) advised by fungal experts as interesting strains to evaluate their possible role in the formation of soil aggregates under drought, due to their frequency of isolation and their originating from drought-treated soils (Westerdijk Fungal Biodiversity Institute, personal communication) (Appendix A4, Table A).

Molecular identification of fungal species and taxonomic analysis

For each of the 29 selected fungal strains, DNA was extracted following the manufacturer's instructions using the Qiagen DNeasy Ultraclean™ kit using fungal material collected from cultures grown on malt extract agar (MEA) at 25°C for 3 days in the dark. Then, we conducted polymerase chain reaction (PCR) amplification of the internal transcribed spacer regions (ITS) and a part of the 28S rRNA gene (large subunit rDNA, LSU). The primers used for LSU amplification were LR0R (Rehner & Samuels, 1995) and LR5 (Vilgalys & Hester, 1990), and the primers used for ITS amplification were V9G (de Hoog & Gerrits van den Ende, 1998)

and LS266 (Masclaux et al., 1995). We also sequenced additional genes to better identify specific fungal species (Appendix A4, Table B). Amplification reactions were performed in a thermocycler following the protocol given by Visagie et al. (2014). The resulting PCR fragments were sequenced in both directions with the primers used for PCR amplification using the ABI Prism® Big DyeTM Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit. Samples were analysed on an ABI PRISM 3700 Genetic Analyser and contigs were assembled using the forward and reverse sequences with the program SeqMan from the LaserGene package. Sequences were compared on GenBank using BLAST and the in-house sequence database of the Westerdijk Fungal Biodiversity Institute (the Netherlands).

A maximum likelihood phylogram was created based on ITS sequences from all the fungal isolates. Bootstrap percentages were based on 1000 re-samplings; only bootstrap supported by values above 70% were presented at the nodes. Analysis was performed using the software MEGA7 (Kumar et al., 2016).

Preparation of fungal inoculants

To prepare fungal inoculants, the 29 selected fungal strains were grown on PDA plates for 7 days at 28°C. After the incubation period, several sterilized poppy seeds were added on top of the colony's edges, and plates were incubated for an additional 2–3 days to allow for fungal colonization of the seeds. Then, the seeds coated with fungi were used as carriers for fungal inoculations of soil (personal communication).

Measurement of fungal traits: Colony density and growth rate

For each fungal strain, the colony density and growth rate were determined for fungal cultures grown on soil extract agar (SEA). This nutrient-poor medium was used to mimic the nutrient conditions of the soil used in the microcosm experiment. SEA was prepared using the adapted protocol of Hamaki et al. (2005). The soil humic acids were extracted by mixing the soil with 50 mM NaOH in 1:2 w/v. Then, the mixture was incubated overnight at room temperature under gentle agitation in a sealed container using a stirring machine (Schüttelmaschine RO 20, Gerhardt). The supernatant was recovered by filtering the mixture through a 3-layer medical gauze (Cutisoft) followed by a stack of 2 sieves, with 100 μm and 50 μm mesh sizes. The resulting filtrate was collected and centrifuged at 4500 rpm for 30 min (Heraeus Megafuge 40, Thermo Fischer). Then, the supernatant was retrieved, its pH was adjusted to 6.8, and the resulting soil solution was autoclaved twice (121°C, 20 min) with 24 h in between autoclaving steps. SEA was made by mixing the soil extract obtained with demineralized water at a 4:6 v/v ratio and bacto-agar (BD Bacto™Agar, ref 214010) at 1.5%.

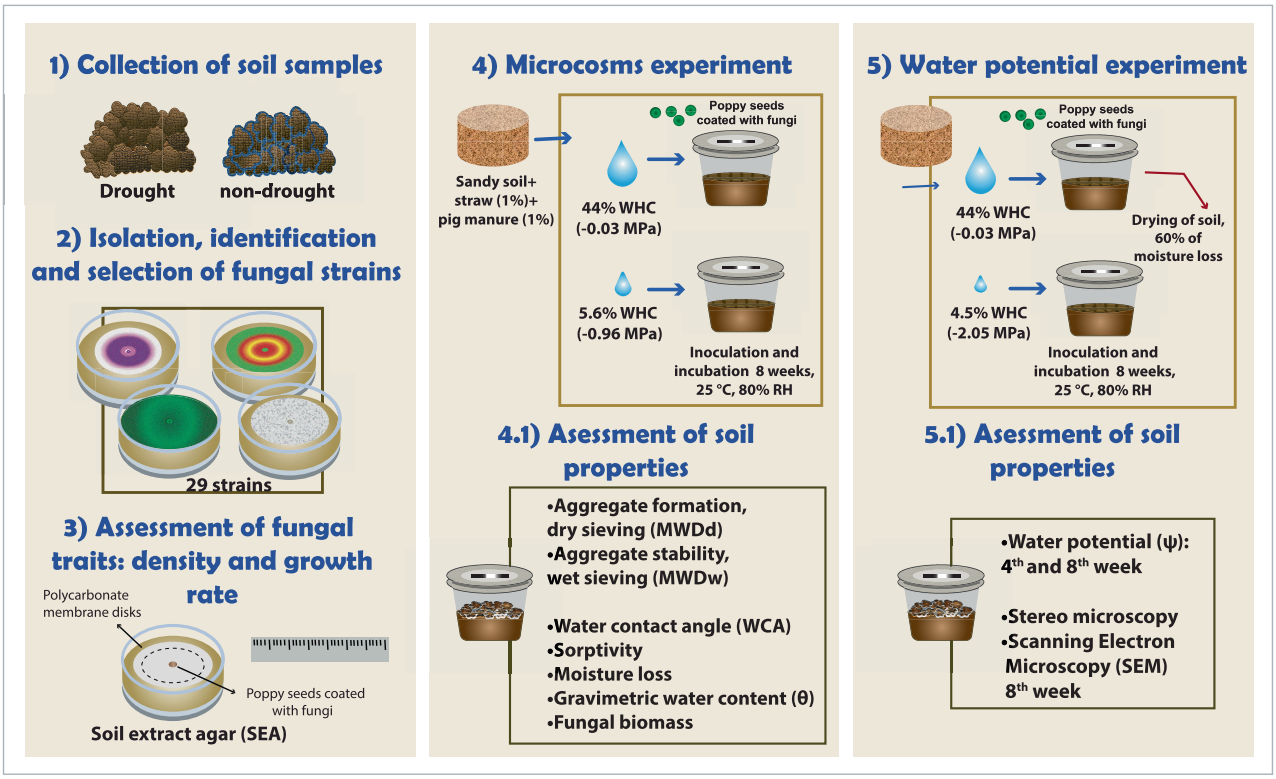
Fungal density and growth rate were measured using an adaptation of the protocol of Reeslev and Kjoller (1995) and Lehmann et al. (2020). First, polycarbonate track-etched (PCTE) membrane disks of 76 mm diameter and 0.1 μm of pore size (GVS, USA) were soaked in water, autoclaved, and placed on the top of Petri dishes filled with 30 mL of SEA. Later, the plates were wrapped in aluminium foil and heated (60°C, 30 min) to soften the agar, and 2 mL of 1.25% (w/v) agarose was added on the top of the membrane to retain moisture and allow fungal growth. The Petri dishes were kept in the flow cabinet at room temperature until the agarose dried and inoculated with one poppy seed in the middle of each Petri dish. Then, the Petri dishes were incubated at 25°C. Each fungal strain was grown on 4 replicate Petri dishes, and we maintained 4 controls without inoculation to track the sterility of the system.

For each Petri dish, we registered the fungal colony diameter every day until the colonies reached the border of the membranes or until 2 weeks of incubation. At the end of the incubation period, pictures were taken of the colonies (Nikon D3500 camera) and with the help of the software ImageJ (Schneider et al., 2012), 4 parallel lines were traced along the colony's borders pictures and the mean diameter was calculated per each plate. The biomass of the fungal colonies was also calculated as follows: the membranes were removed and heated to enable the agarose to melt and to obtain the fungal colony contained in the agarose. Each colony retrieved in this manner was dried overnight at 60°C and weighed. Fungal colony density was calculated using the biomass of the colony on the last day of incubation divided by the colony's diameter. This product was adjusted to the number of days of assessment for each strain to estimate the increase of each colony per day. For each strain, the fungal growth rate was obtained by calculating the slope of the curve for the growth during the 5 first days of incubation or until they reached the border of the membranes.

Soil aggregation assay: A microcosm experiment

Experimental design

To examine the impact of the 29 fungal strains on soil structure, we set up a fully randomized soil inoculation experiment in a microcosm. Each fungal strain was inoculated in 4 replicates at 2 levels of moisture. In addition, we set up 2 sets of control microcosms without fungal inoculation for each level of moisture, resulting in a total of 248 experimental units.



WHC = water holding capacity, MWDd=mean weight diameter after dry sieving, MWDw=mean weight diameter after wet sieving. RH=relative humidity

FIGURE 1 Scheme of fungal isolation, assessment of fungal traits, and experimental setup of the microcosm experiment and water potential experiment with 29 fungal strains and one non-inoculated control.

We selected sandy soil collected from a pig farm in the Netherlands as our model soil. The soil texture for this experiment was chosen due to the poor structure of sandy soils, thereby allowing us to potentially examine a broad range of improvements in soil structure. Although the soil used in our microcosm experiment was different from the one used for fungal strain isolation, both had a sandy texture. We prepared the soil mixture by amending the coarse sandy soil (0.5–0.25 mm, 62%) with (i) chopped (Retsch bv, Muhle, Belgium, blades 50 mm) and sieved (0.5 mm mesh) straw (1% w/w), and with (ii) gamma-irradiated (Steris company, the Netherlands) and sieved (0.5 mm mesh) pig manure (1% w/w) as a source of organic matter. The final properties of the soil used for this experiment were as follows: C/N ratio 23:1; pH 7.4, organic carbon 0.7%, clay (<2 μm) <1%; silt (2–50 μm) 8% and sand (>50 μm) 90% Then, 100 g of the soil mixture was aliquoted into plastic flasks (60 mm h × 80 mm d, Microbox filter XL, Eco2 NV) and autoclaved 3 times with time intervals of 24 h (121°C, 20 min). Then, to evaporate the remaining water, the microcosm flasks were air-dried in a flow cabinet overnight. Thereafter, soil microcosms were adjusted to 44% or 5.6% of the total soil water holding capacity (45%), corresponding to the high (ψ −0.03 MPa) and low (ψ −0.96 MPa) moisture levels, respectively. For each microcosm, 4 poppy seeds previously inoculated with fungal material (Section 2.4) were added. Three seeds were mixed with the soil material and one seed was placed at the top of the flask. The microcosm flasks were weighed to determine the initial content of moisture and then incubated for 8 weeks at 25°C and 80% of air humidity to keep the moisture in the system (Figure 1).

After the incubation period, samples were collected for the measurement of soil properties. Intact columns of soil were collected using a metal core borer (38 mm diameter) and a spatula with a flat bottom and transferred to 50 mL falcon tubes. The core samples were either kept with the original moisture or air-dried at room temperature and stored at 4°C or −20°C prior to further analyses.

Measurement of soil aggregate formation and stability

At the end of the microcosm experiment, dry sieving was used to estimate the new formation of soil aggregates acquired during the incubation period of this previously disrupted soil (as in Erktan et al., 2020). To measure soil aggregate formation, we used intact air-dried soil cores (approx. 8 g) stored at 4°C. The soil material was dry sieved through a stack of sieves with

the following meshes: 2, 1, 0.5, and <0.5 mm using an automatic siever (Retsch, Lab equipment) that stirred the soil samples during 5 s with an oscillation amplitude of 2 mm. The soil aggregates that remained on each sieve were collected, oven-dried overnight at 70°C, and later transferred to a desiccator and weighed.

At the end of the incubation period, the stability of soil aggregates was determined under the principle of breakdown by compression of air trapped (slaking) using the wet sieving technique (Kemper & Rosenau, 1986). Slaking occurs when dry aggregates are immersed in water or rapidly rewetted, thereby resulting in swelling and the release of trapped air (Le Bissonnais, 1996). An aliquot of 4 g of air-dried soil cores (stored at 4°C) was wet sieved sequentially through the following meshes: 2, 1 and 0.5 mm, resulting in 4 diameter classes: >2 mm; 2–1 mm; 1–0.5 mm, and <0.5 mm. First, the soil samples were placed on the 2 mm sieve and soaked in a thin layer of water for 30 min. Then, the 2 mm sieve was placed into an automatic sieving machine (Eijkelkamp, Germany) that raised and lowered the 2 mm sieve with an amplitude of 13 mm and a speed of 34 times/min for 3 min. At the end of the agitation period, the remaining stable soil aggregates >2 mm were collected on filter paper, and the soil fraction <2 mm was retrieved in a stainless-steel container at the bottom of the sieving machine. The soil fraction <2 mm was transferred to a sieve with a mesh of 1 mm, and the agitation process was repeated. Similarly, the soil fraction <1 mm was transferred to a sieve with a mesh of 0.5 mm and the agitation process was again repeated. After each agitation step, the remaining stable soil aggregates >1 mm, >0.5 mm and <0.5 mm were retrieved on filter paper, and together with the soil aggregates >2 mm, they were dried overnight at 105°C, placed in a desiccator and weighed.

For each soil sample and sieving method (dry and wet), the mass percentage of each soil fraction was calculated by dividing the mass of the fraction by the sum of the masses of all the soil fractions. The mean weight diameter (MWD) (Kemper & Rosenau, 1986) was calculated as follows:

$$\mathrm{MWD} = \sum_{i=1}^{n} \overline{X}_i M_i$$

where M_i is the dry mass of the soil aggregates for each size class and $\overline{X}_i$ is the mean diameter of the soil aggregate size class (mm). The MWD calculated after the wet sieving was designated $\mathrm{MWD_w}$ and this measure after dry sieving was given as $\mathrm{MWD_d}$. A stability index (SI) was adapted from Devine et al. (2014), and calculated by adding the $\mathrm{MWD_w}$ to the $\mathrm{MWD_d}$. This index helped to integrate the fungal ability to improve aggregate formation and stability into a single composite value.

Measurement of soil water contact angle and soil sorptivity

At the end of the microcosm experiment from our fungal-inoculated and control soils, we assessed the soil hydrophobicity by measuring the soil water contact angle (WCA), which assesses the wettability of solid surfaces (Marmur et al., 2017) and the soil sorptivity, which expresses the tendency of an intrinsic material to absorb and transmit a liquid by capillarity (Philip, 1957). To measure the soil WCA, we first placed a small amount of air-dried soil (stored at 4°C) between 2 glass slides, which allowed us to flatten the upper surface of the soil aliquot and achieve a standard soil thickness. Then, 10 μL of Milli-Q water was added on the top of each flattened soil sample, and the image of the droplet shape was analysed using a drop shape analysis system at room temperature (Krüss DSA 10 Mk2, Germany). For each sample, 3 measurement replicates were performed.

Soil sorptivity was measured during the 30-min soaking of the soil samples before the wet sieving and during the wet sieving (Section 2.6.2). The soil sample was settled on a 2 mm sieve on a thin layer of water that reached the bottom of the sieve. The level of water risen by capillary in the soil aggregates was used as a proxy for soil sorptivity and soil was categorized using 3 categories: (i) the soil samples were wetted during the soaking of 30 min (2), (ii) the soil samples did not get wet after 30 min of soaking but they did do after the 3 min of wet sieving (1), and (iii) the samples did not get wet after 30 min of soaking and the wet sieving of 3 min (0).

Measurement of moisture loss and soil gravimetric water content (θ)

To calculate the moisture loss, the microcosm flasks were weighed at the end of the experiment, and the difference with the initial flask weight was calculated. To calculate (θ), intact soil samples stored at −20°C were weighted ($m_{\mathrm{soil\,wet}}$). Then, the soil samples were oven-dried at 70°C until they no longer experienced weight loss and weighed again ($m_{\mathrm{soil\,dry}}$). For each soil sample, the soil gravimetric water content was then determined using the following formula:

$$\theta = \frac{m_{\mathrm{soil\,wet}} - m_{\mathrm{soil\,dry}}}{m_{\mathrm{soil\,dry}}}$$

Measurement of soil fungal biomass

At the end of the microcosm experiment, soil fungal biomass was estimated by quantifying soil ergosterol content. For each soil sample, we extracted soil ergosterol

using the protocol of Bååth (2001). Briefly, 1 g of soil (directly stored at −20°C at the end of the incubation) was mixed with 4 mL of methanol containing 10% KOH. The resulting soil suspension was then sonicated for 15 min and heated in a water bath (70°C, 90 min). After cooling, 1 mL of distilled water and 2 mL n-hexane were added, and the solution was stirred for 30 s on a vortex mixer. Then, the obtained solution was centrifuged (4500 rpm, 10 min), and 1 mL of the top phase was mixed with 1 mL of n-hexane, and centrifuged (4500 rpm, 10 min). Then, 1 mL of the supernatant was retrieved and let to evaporate overnight in a heating block at 50°C under aeration. The precipitates were dissolved in 1 mL of methanol and closed to prevent evaporation. Then, the soil precipitates were shaken for 30 s, sonicated for 4 min, and shaken again for 30 s. Finally, the mixture was filtered through a 0.2 μm filter (13 mm) and stored at −20°C before further analyses via high-performance liquid chromatography (HPLC).

The HPLC was performed at the Netherlands Institute of Ecology (NIOO-KNAW) using a UV-DAD detector, and XDB-C18 column at 25°C. Ergosterol concentrations (mg/kg soil) were calculated as follows:

$$\text{Ergosterol}_{(\text{mg/kg soil})} = \frac{\text{c} \times f}{\text{se} - (\text{se} \times \text{m})}$$

where c = initial concentration of ergosterol in mg/L, f = correction factor (1.33), se = soil wet weight used for extraction (g) and m = soil moisture fraction.

Effect of fungal inoculation on soil water potential (ψ)

One important environmental factor with major effects on fungal activity is soil water availability expressed by the soil water potential. The soil water potential represents the energy with which water is retained in the soil (Robert & Chenu, 1995; Walker & White, 2017), and water can move from areas with high to low water potential (Herman & Bleichrodt, 2022). The experiment was designed to examine the capacity of fungi to improve the water potential in soil based on the significant effects of fungal inoculation on soil aggregation and hydrophobic properties.

To examine the impact of the 29 fungal strains on soil water potential, we set up a separate fully randomized soil inoculation experiment, hereafter referred to as the 'soil water potential experiment' (Figure 1). As in the soil aggregate microcosm experiment, each fungal strain was inoculated in 4 replicate microcosms at 2 levels of moisture. In addition, we set up 2 sets of control microcosms (without fungal inoculation), resulting in a total of 248 experimental units.

We placed 60 g of the soil mixture in plastic flasks, autoclaved, and then dried it (as in Section 2.6.1). Thereafter, soil samples were adjusted to 2 matric potentials, 44% (ψ −0.03 MPa) and 4.5% (ψ −2.05 MPa) of the total soil water holding capacity (45%), representing high moisture and water stress conditions, respectively. The water stress condition is to yield conditions that are below the permanent wilting point for plants, which is approximately ψ −1.5 MPa (Palacios et al., 2014). In each microcosm, 4 poppy seeds inoculated with fungal strains were added, 3 seeds were mixed with the soil material, and one seed was placed at the bottom of the flask. The flasks were incubated for 8 weeks at 25°C at 80% relative humidity.

After 4 weeks of incubation, one intact soil sample (from one replicate) was collected with a metal ring (36 mm diameter) and dropped into disposable sample containers (Meter, Germany). At the end of the incubation period, the 3 remaining replicates were sampled similarly, and the soil water potential was measured directly for the low moisture treatment. For the treatments with the higher level of moisture, the samples were dried in blocks (one replicate in each block) in a flow cabinet until 60% of the original level of moisture was achieved, to mimic a drought event (Figure 1). For each soil sample, the soil water potential was measured using a WP4C water potential meter (Decagon Devices) using a KCl 0.5 mol/kg solution (0.984_{aw} Verification Standard) (Meter, Germany) at 25°C for calibration.

Stereomicroscopy and SEM

At the end of the water potential experiment, we examined fungal growth on a selection of intact fresh soil cores using a stereomicroscope equipped with a camera (DS-Ri2, Nikon Europe, Amstelveen, the Netherlands) and a scanning electron microscope equipped with a Cryostation (cryoSEM). Samples were selected based on the results of colony density, growth rate, and improved soil aggregation. After sampling, the soil was transferred into disposable sample containers (Meter, Germany) and kept in a polythene bag to retain moisture. To acquire images with the stereomicroscope (Nikon SMZ 25), we performed a fresh soil cut on the upper surface of the soil core with a surgical blade (no. 11, Swann-Morton, Sheffield, UK) and collected up to 30 pictures at different focal depths from the surface and the mid-part of the soil sample to reflect the development of fungal structures in contact with air and between soil particles, respectively. Image acquisition was conducted using the NIS Elements software ('Capture Z-series', version 5.11.02), and pictures were adjusted, stacked, and stored as TIFF files. To acquire images with the electron-microscope (JEOL 5600LV,

Tokyo, Japan), equipped with a cryo-station (Oxford CT1500), the soil was gently removed from the soil cores and transferred in a copper cup (6 mm depth, 12 mm diameter) for rapid freezing in liquid nitrogen using frozen tissue medium (KP-Cryoblock; Klinipath, Duiven, the Netherlands) to prevent the soil from falling out of the cup. Samples were then coated 3 times for 1 min using a gold target and electron micrographs were acquired at an acceleration voltage of 2.5–5 kV. Image acquisitions were performed at the Westerdijk Fungal Biodiversity Institute.

Statistical analyses

We tested the effect of inoculation on fungal traits: namely the density of colonies and growth rate, and on soil properties: the soil aggregate formation (MWD_d) and stabilization (MWD_w), the stability index (SI), and soil hydrological properties namely the water loss in the system, soil gravimetric water content (θ), water contact angle (WCA), water potential (ψ), and the fungal biomass in soil.

The differences between fungal strains with respect to colony density and growth rate and the effects on water loss and θ for each level of moisture were analysed using an analysis of variance (ANOVA) as a part of a linear model (LM). The effects of fungal inoculation on MWD_w, SI, WCA and soil fungal biomass were analysed using an ANOVA 'type 3' for the effect of interactions of strains and moisture level. The impact on the ψ effect was also analysed for each level of moisture using a linear mixed-effects model (LME) for the effect of blocks. The assumptions normality and homoscedasticity of the residuals were checked visually using a Q–Q plot and a plot of residuals, and the data were log or square root-transformed if necessary to meet the assumptions. When the heteroscedasticity remained, we used a generalized least squares (GLS) model and allowed the variance to be different per stratum and level of moisture using varIdent (Pinheiro & Bates, 2000), packages 'nlme' (Pinheiro et al., 2021) and 'car' (Fox & Weisberg, 2019). The non-parametric effect of fungal strains on MWD_d under the 2 levels of moisture was analysed using an ANOVA 'type 3' as part of a generalized least means (GLM) with gamma distribution through the package 'MASS' (Venables & Ripley, 2002). The pairwise comparison between the means of treatments was analysed by the test 'Tukey' through the package 'emmeans' (Lenth, 2022) and a 'Bonferroni' adjustment, and the graphics were plotted according to the fitted models. Pairwise comparisons for log or sqrt-transformed data were back-transformed using the function 'response.'

To investigate the relationships between soil physical and hydrological properties, and fungal traits, we ran a matrix of Spearman's correlations for all measured traits (package 'Hmisc') (Harrel, 2022). In addition, to test the direct effect of fungal inoculation on soil physical properties and the potential indirect effects through the modification of hydrological properties, we conducted a path analysis using the package 'piecewiseSEM' (Lefcheck, 2016). Fungal biomass, colony density, and growth rate, as well as soil hydrological properties (soil water content, soil sorptivity, soil water contact angle), were used to explain soil aggregate formation and stability under the 2 levels of moisture. The accuracy of the models was examined using χ^2 and Akaike information criteria (AIC). All analyses were conducted using the R software platform (version 4.1.2), and graphics were generated using the package 'ggplot2' (Wickham, 2016).

RESULTS

Selection and identification of fungal strains

From the 133 isolated fungal strains, we selected 29 strains based on the characteristics mentioned in Section 2.2. Two strains belonging to *Purpureocillium* sp. (strains 38 and 144) and one to *Acremonium* sp. (strain 139) were selected because these 2 genera increased in relevant abundance throughout the field drought experiment when compared to non-drought plots (data not shown). Out of the 29 strains selected, 27 belonged to the phylum Ascomycota and the other two to the Mucoromycota (Appendix A3, Figure A). Within the phylum Ascomycota, the isolated strains exhibited a broad diversity across 14 families, namely Aspergillaceae, Bionectriaceae, Chaetomiaceae, Clavicipitaceae, Coniochaetaceae, Cordycipitaceae, Hypocreaceae, Hypocreomycetidae, Nectriaceae, Onygenaceae, Ophiocordycipitaceae, Pyrenochaetopsidaceae, Stachybotryaceae and Trichocomaceae. Out of the 29 strains, 8 were isolated from the non-drought treatment, 4 from the D50 treatment, and 17 from the D90 (Appendix A3, Figure A).

Fungal colony density and growth rate

The density of colonies of 4 fungal strains (calculated per day as mentioned in Section 2.5) and growth rates of 6 fungal strains showed significantly higher values as compared to the mean of all 29 strains examined (0.06 mg/cm^2/day and 0.81 cm/day, respectively) (Figure 2). Strains 52 and 128 (*Fusarium* spp.) and strains 60 and 141 (*Trichoderma linzhiense*) exhibited the highest mycelium density (>0.08 mg/cm^2), and the last 2 were among the strains which showed the fastest growth rate (>3 cm/day). These 2 genera, and the isolates from these genera in our study, were

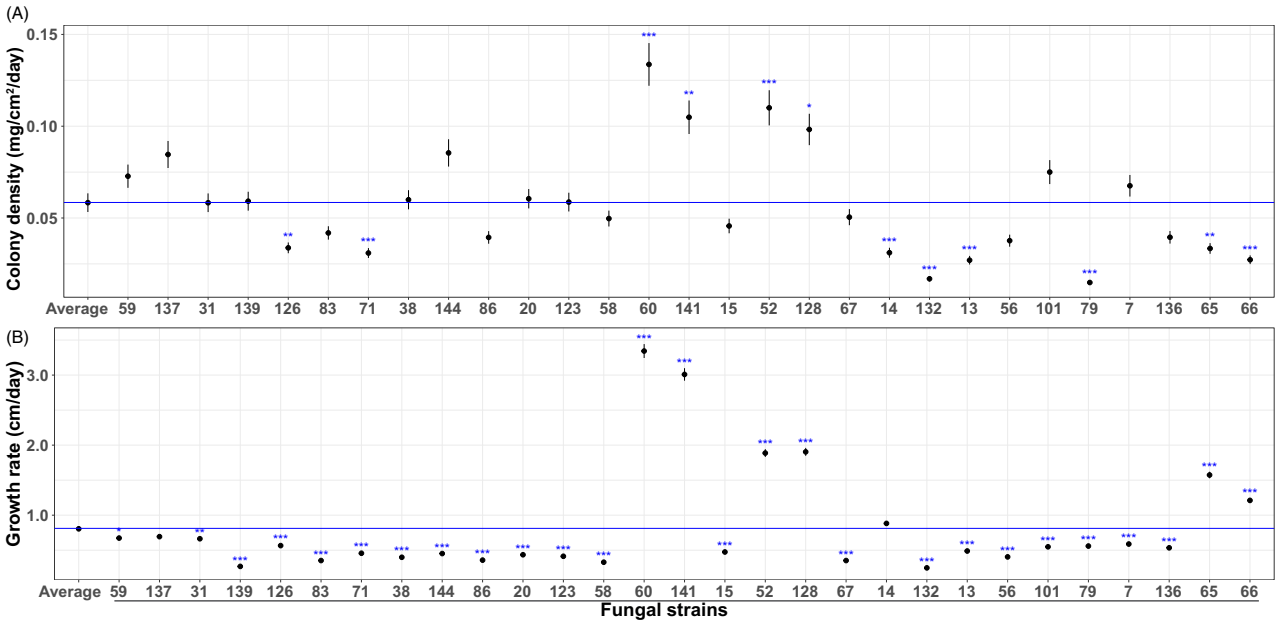


FIGURE 2 Colony density (mg/cm^2/day) (calculated using the colony's biomass divided by the colony's diameter on the last day of assessment and adjusted to the number of days of assessment for each strain) (A) and colony growth rate (cm/day) (B) of fungal colonies grown in soil extract agar (SEA). The average mean density and average mean growth rate are represented by the blue lines. Bars represent standard error. Asterisks indicate significant differences compared to the average mean: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The sequence of strains in the x-axis depicts the phylogenetic distance (Figure A, Appendix A3). The ANOVA is shown in Appendix A4, Table C. Means, standard error, p-values and significance are shown in Appendix A4, Table D. Raw data are shown in Appendix A3. Figure E.

taxonomically closely related (Appendix A3, Figure A). Some other strains, such as 65 (*Linnemannia gamsii*) and 66 (*Absidia* sp.) both belonging to Mucoromycota showed a faster growth rate than the mean but had a lower than average mycelium density, and some strains, such as strain 132 (*Auxarthron umbrinum*), showed both low density and growth rate. We also observed some other trait patterns at the genus level, such as *Penicillium* strains 7 and 136 and *Marquandomyces* strains 20 and 123, which exhibited common growth traits within each genus.

Effect of fungal inoculation on soil aggregation and hydrological properties

After 8 weeks of incubation and at both levels of soil moisture (44% and 5.6% of soil water holding capacity), the formation (MWD_d) and stability (MWD_w) of soil aggregates measured by dry and wet sieving, respectively, were affected by the interaction of individual fungal strain inoculation and soil moisture ($p < 0.05$) (Figure 3 and Appendix A4, Table C). For instance, strains 20, 52, (67, *Plenodomus chelidonii*), and 128 showed a higher impact on soil aggregate stability under high soil moisture, yet an opposite pattern was observed for strains 71 (*Paramyrothecium viridisporum*) or 126 (*Gliomastix roseogrisea*), which showed better performance at low moisture.

At the high level of moisture, 83% of the strains significantly increased soil aggregate stability (Figure 3A), with MWD_w ranging from 0.7 to 2.6 mm, and 17% of the fungal strains increased soil aggregate formation after dry sieving compared to the non-inoculated control (Figure 3B) with MWD_d ranging from 0.3 to 0.78 mm. At low soil moisture, 62% of the fungal strains improved soil aggregate stability (MWD_w ranging from 0.4 to 2.9 mm), and only a single strain, strain 56 (*Pyrenochaetopsis leptospora*), improved soil aggregate formation (MWD_d ranging from 0.3 to 0.48 mm). Strain 56 was the only strain that increased both MWD_d and MWD_w under the 2 moisture conditions. Strains 7, 14 (*Staphylotrichum acaciicola*), 15 (*Dactylonectria torresensis*), 56, (59 and 137, *Clonostachys rosea*), 83 (*Hydropisphaera* sp. nov.), 101 (*Talaromyces kabodanensis*), and strain 139, all strongly stabilized soil aggregates at both moisture conditions with a stability index (SI) ≥ 2 mm compared to 0.5 mm in the case of the non-inoculated control soil (Figure 3C). A stability index above 3 mm (6 times the control) was observed for 5 strains under low moisture conditions and 1 strain (strain 83) at high moisture.

The largest soil aggregate size fractions (>2 mm) showed the most significant contribution to soil aggregate stability under low moisture conditions (Appendix A3, Figure B). Twelve strains at low moisture and 22 strains at high moisture resulted in over 50% of the fraction size coming from >2 mm aggregates. In

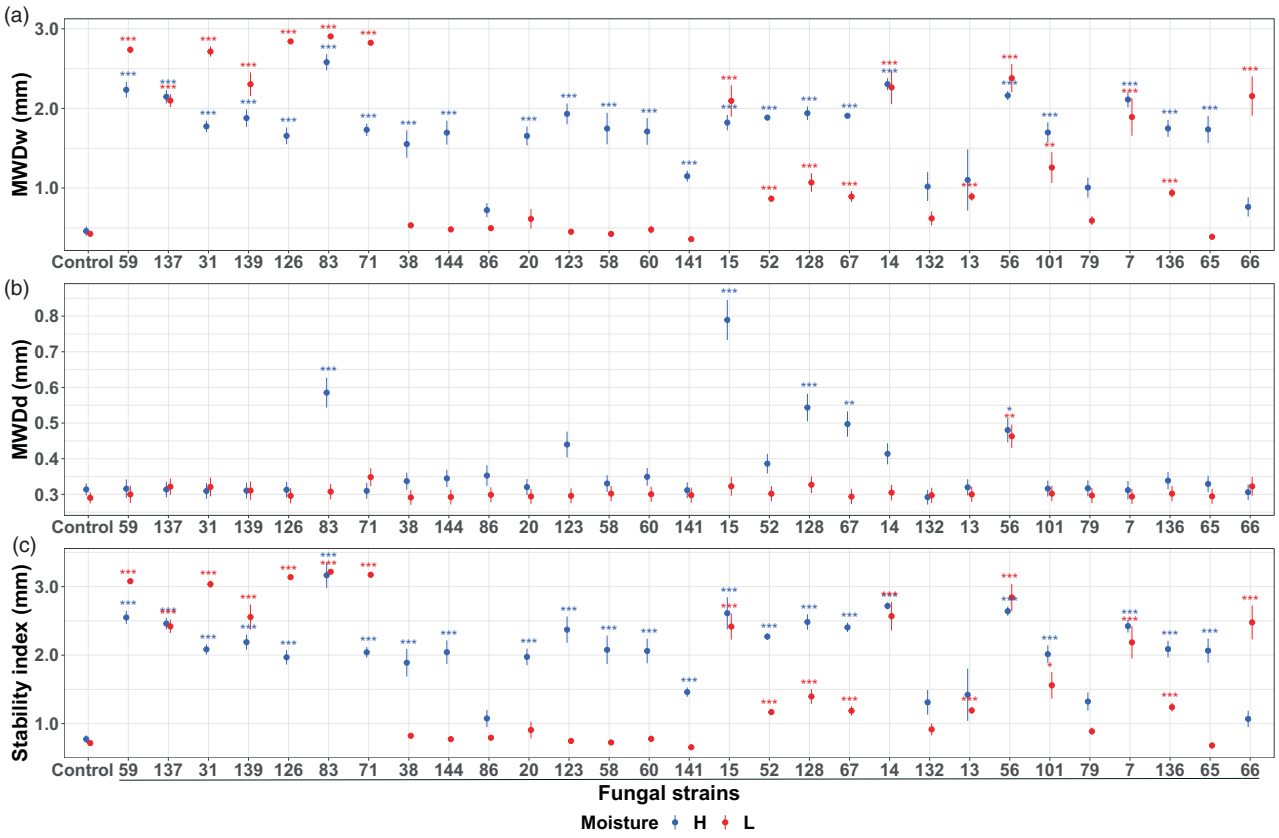


FIGURE 3 Effect of fungal strain inoculation on soil aggregate stability MWD_w (A), aggregate formation MWD_d (B), and the stability index, SI (C) which summarizes the previous 2 parameters, after 8 weeks of incubation. Bars represent standard error. Asterisks indicate significant differences compared to the non-inoculated control: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. H and L indicate high and low soil moisture, respectively. The sequence of strains in the x-axis depicts the phylogenetic distance (Figure A, Appendix A3). The ANOVA is shown in Appendix A4, Table C. Means, standard error, *p*-values, and significance are shown in Appendix A4, Table E. Raw data are shown in Appendix A3, Figure F.

comparison, soils without fungal amendment had more than 75% of the soil fractioned as <0.5 mm. A total of 83% of the strains that showed good performance under low moisture content with respect to the aggregate formation (e.g., strain 56) or aggregate stability >2.5 mm (e.g., strains 31, 59, 71, 83 and 126, which also were close taxonomically) (Appendix A3, Figure A) were isolated from one of the drought treatments (D50 and D90) plots.

In Table 1, the relation between all parameters we measured is displayed in a correlation matrix. At high soil moisture, the density of colonies measured in Petri dishes was slightly positively correlated with soil aggregate stability and formation, with strains showing higher fungal density yielding more stable aggregates. In contrast, at low soil moisture, fungal density and growth rate were not significantly correlated with soil aggregate stability.

Over the 8 weeks of incubation, the soil moisture loss was between 8% and 13% of the initial content of the higher moisture samples and between 60% and 80% in the lower moisture samples (initially set at ψ −0.96 MPa) (Appendix A3, Figure C). This loss was higher than the moisture loss in the non-inoculated controls. At high soil moisture, there was no significant effect of fungal inoculation on soil gravimetric water content (θ) at the end of incubation (Appendix A4, Table C). However, at low soil moisture, fungal inoculation reduced the θ significantly for 58.6% of the fungal strains tested (Figure 4A). We observed an interaction between the effects of soil moisture and fungal strain on soil water hydrophobicity, as indicated by the water contact angle (WCA), but with larger means under low moisture conditions (Figure 4B) (Appendix A4, Table F). In contrast, soil sorptivity (Figure 4C) decreased at low soil moisture for 17 strains out of the 29 inoculated strains. At high soil moisture, only 3 strains affected soil sorptivity, with strain 83 being the only strain to reduce sorptivity.

Soil fungal biomass

Soil fungal biomass was estimated by determining the concentration of ergosterol in soils after the 8-week incubation period. Ergosterol levels in soil samples

TABLE 1 Correlation matrix between fungal traits, soil hydrological, and physical properties. Abbreviations: MWD_w: soil aggregate stability; MWD_d: soil aggregate formation; θ: gravimetric water content, WCA: soil water contact angle.

Low moisture (*ρ*, *p*-value)	MWD_w	MWD_d	θ	WCA	Sorptivity	Soil fungal biomass	Fungal colony density	Fungal growth rate
MWD_w	—	**0.45/<0.001**	**0.41/<0.001**	**0.33/<0.001**	**−0.72/<0.001**	**0.66/<0.001**	−0.18/0.05	0.01/0.95
MWD_d		—	**0.34/ 0.002**	0.05/0.97	**−0.38 /<0.001**	**0.58/<0.001**	−0.05/0.6	0.09/0.33
θ			—	0.15/0.11	**−0.32/0.005**	**0.47/<0.001**	**−0.23/0.02**	**−0.21/0.03**
WCA				—	**−0.35/<0.001**	0.04/0.6	−0.1/0.14	−0.09/0.34
Soil sorptivity				—	—	**−0.52/<0.001**	**−0.27/0.004**	0.05/0.62
Soil fungal biomass						—	−0.12/0.66	−0.11/0.41
Fungal colony density							—	**0.35/<0.001**
Fungal growth rate								—

High moisture (*ρ*, *p*-value)	MWD_w	MWD_d	θ	WCA	Sorptivity	Soil fungal biomass	Fungal colony density	Fungal growth rate
MWD_w	—	**0.46/<0.001**	−0.03/0.75	0.05/0.57	**−0.36/<0.001**	**0.66/<0.001**	**0.27/0.004**	0.01/0.88
MWD_d		—	−0.06/0.53	−0.04/0.67	−0.03/ 0.83	**0.32/0.001**	**0.22/0.02**	0.01/0.87
θ			—	−0.11/0.22	0.03/ 0.71	0.04/0.60	0.05/0.47	0.01/0.87
WCA				—	**−0.33/0.001**	0.12/0.22	−0.11/0.24	−0.08/0.41
Soil sorptivity					—	**−0.34/<0.001**	−0.11/0.23	**0.24/0.01**
Soil fungal biomass						—	**0.21/0.02**	−0.05/0.62
Fungal colony density							—	**0.35/<0.001**
Fungal growth rate								—

Note: The Spearman's coefficients (*ρ*) and the *p*-values are in bold when $p < 0.05$.

showed a significant interaction between the effect of fungal strain and moisture level (Figure 5 and Appendix A4, Table C). Most of the low moisture samples reached higher levels of ergosterol (ranging from 0.04 to 21.7 mg ergosterol/kg soil) compared to the samples with higher moisture (0.02–9 mg ergosterol/kg soil). Eleven treated soils showed levels above 10 mg ergosterol/kg soil, all for the lower moisture regime. One of these strains (128) was also high in hyphal density and growth rate, as determined in plate assays. However, strain 65, which displayed a rapid growth rate on agar plates (Figure 2) did not appear to show ergosterol production in the soil at either level of moisture.

Relationships between fungal traits, soil hydrological, and physical properties

At high soil moisture, colony density was positively correlated with soil aggregate stability (MWD_w) and soil aggregate formation (MWD_d) (Table 1), and soil fungal biomass and colony density were also correlated. Meanwhile, at low soil moisture, water content was positively correlated to MWD_w and MWD_d as was WCA to MWD_w.

At both high and low soil moisture, MWD_d and MWD_w were positively correlated with each other. MWD_w was negatively correlated with soil sorptivity, and soil sorptivity was also negatively correlated with soil WCA. Fungal biomass was positively correlated with MWD_w and MWD_d, and negatively correlated with soil sorptivity.

By using path analyses, we were able to examine both the direct effects of fungal traits on soil physical properties, as well as indirect effects through changes in soil hydrological processes (Figure 6). These analyses showed that the fungal growth rate and WCA had no significant effect in the models tested. Therefore, we removed these parameters from the model to improve the fit. At high soil moisture, the model explained 56%

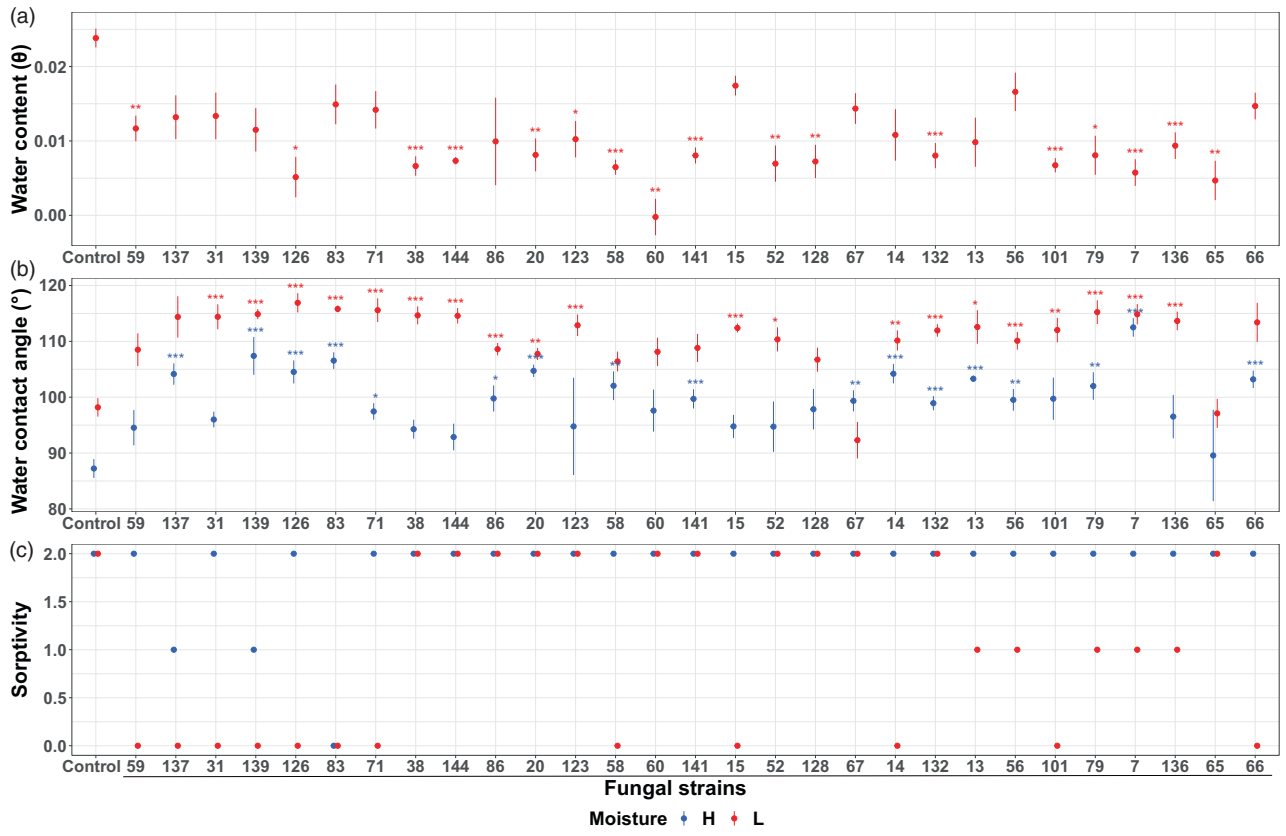


FIGURE 4 Effect of fungal inoculation on the soil hydrological properties: gravimetric water content (θ) (A), soil water hydrophobicity as indicated by the soil water contact angle (WCA) (B), and sorptivity (C) after 8 weeks of incubation. Bars represent standard error. Asterisks indicate significant differences compared to the non-inoculated control: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. H and L indicate high and low soil moisture, respectively. The sequence of strains in the x-axis depicts the phylogenetic distance (Figure A, Appendix A3). The ANOVA is shown in Appendix A4, Table C. Means, standard error, *p*-values, and significance are shown in Appendix A4, Table F. Raw data are shown in Appendix A3, Figure G.

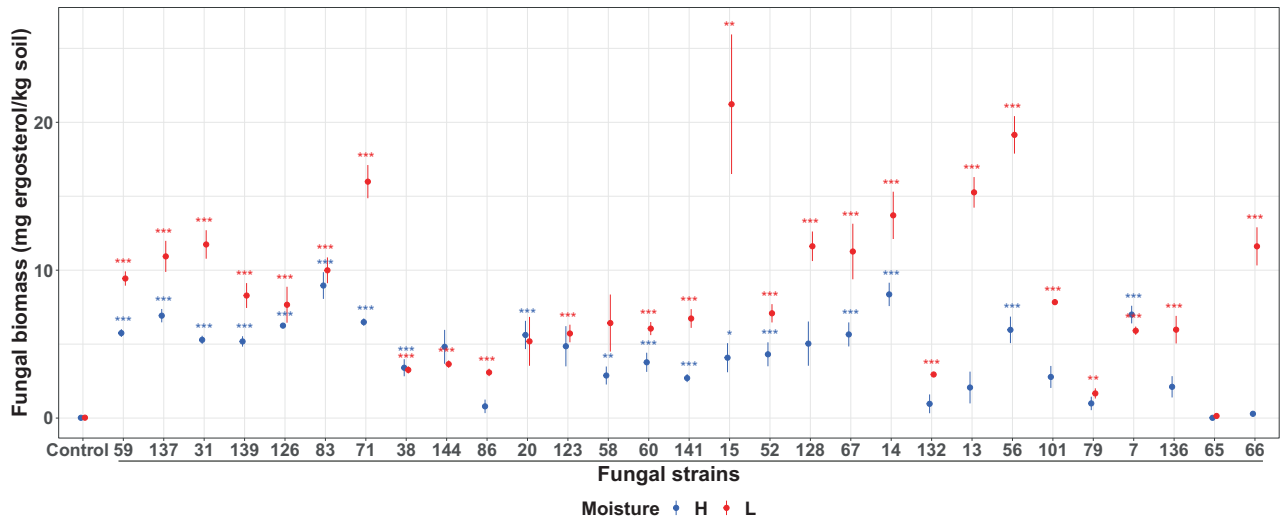


FIGURE 5 Effect of fungal strain inoculation on soil fungal biomass (ergosterol) after 8 weeks of incubation. Bars represent standard error. Asterisks indicate significant differences compared to non-inoculated control: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. H and L indicate high and low soil moisture, respectively. The sequence of strains in the x-axis depicts the phylogenetic distance (Figure A, Appendix A3). The ANOVA is shown in Appendix A4, Table C. Means, standard error, *p*-values and significance are shown in Appendix A4, Table G. Raw data are shown in Appendix A3, Figure H.

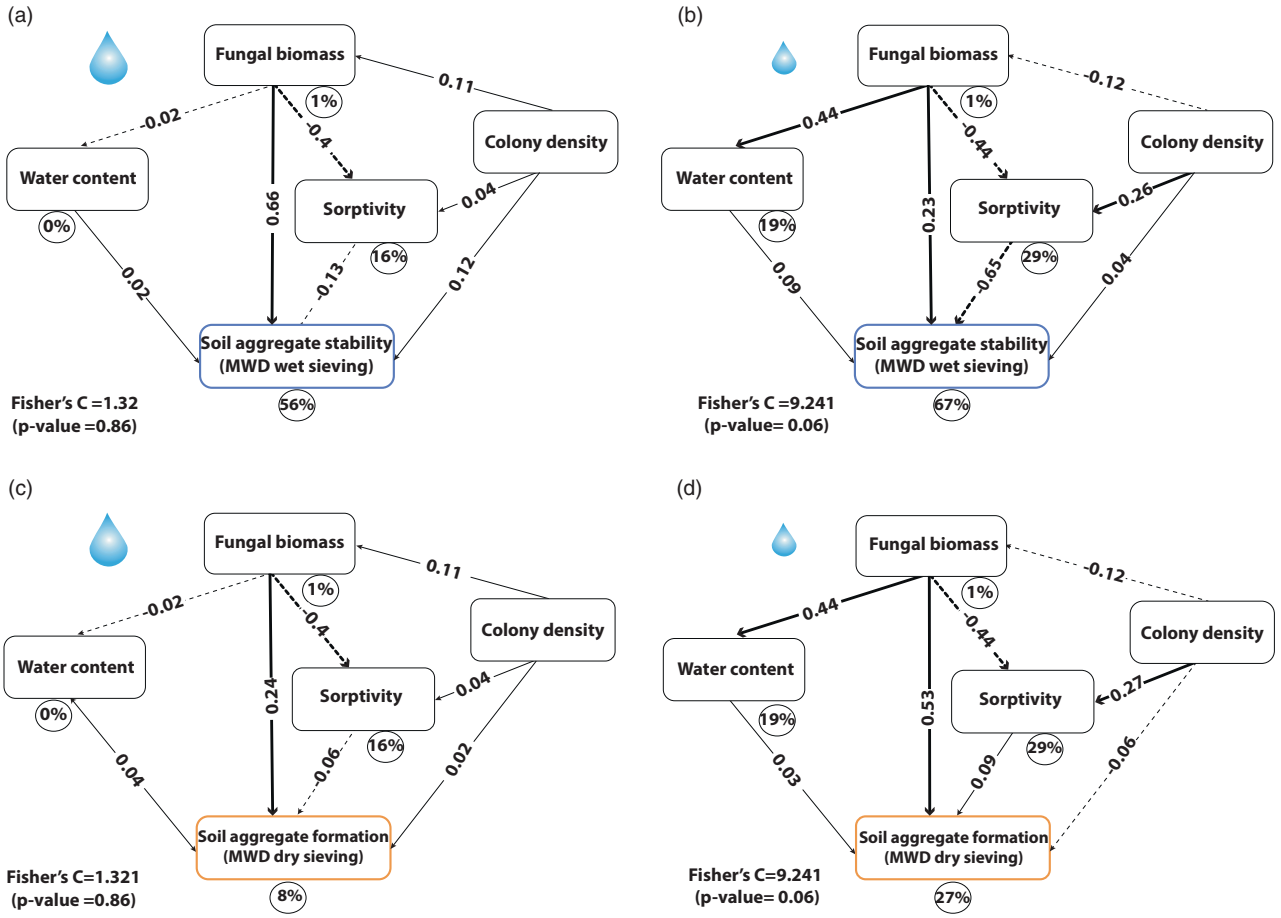


FIGURE 6 Path analyses of the direct and indirect effect of fungal traits on soil aggregate formation MWD_d and stability MWD_w. MWD_w at high soil moisture (A) and low soil moisture (B) and MWD_d (C) at high soil moisture (D) at low soil moisture. Numbers on arrows are standardized path coefficients, solid and dashed indicate positive and negative effects, respectively. Bold arrows indicate significant ($p < 0.05$) standardized path coefficients; thin arrows indicate non-significant path coefficients ($p > 0.05$). Circles indicate the % of variance explained and droplets show the level of moisture: small for low and big for high moisture content.

and 8% of the variance of MWD_w and MWD_d, respectively (Figure 6A, C), and fungal biomass, as determined by ergosterol content, was positively linked with soil MWD_w and MWD_d and negatively linked with soil sorptivity.

At low soil moisture, the model explained 67%, and 27% of the variance of MWD_w and MWD_d (Figure 6B, D), respectively, and fungal biomass directly and positively impacted soil aggregate stability and formation. Fungal biomass was also associated with soil gravimetric water content, but the latter had no significant effect on soil aggregation. This effect is correlated in Table 1, where water content at the lower level of moisture is slightly more related to fungal biomass than aggregate stability. Colony density was positively correlated to soil sorptivity but did not have a direct effect on aggregate stability on the studied path. Finally, higher biomass and lower colony density led to decreased soil sorptivity, which resulted in higher aggregate stability, but this did not affect soil aggregate formation. At the 2 levels of soil moisture, the fungal biomass measurements showed the strongest correlation with soil aggregate formation and stability.

Effect of fungal strain on soil water potential (ψ)

We examined the effects of fungal inoculation on soil water potential via an additional experiment (see Section 2.7 above). After 4 weeks of incubation, the initial levels of water potential (−0.03 and −2.05 MPa) had dropped to −0.35 and −50 MPa for the high and low levels of moisture, respectively, while the values for the non-inoculated controls at this point were around 0 and −47 MPa for the high and low moisture contents, respectively. After 8 weeks of incubation at low soil moisture, 8 of the 29 strains significantly lowered the soil water potential compared to the control (approximately −47.5 MPa), and 2 of these strains reduced soil water potential to below −60 MPa (Figure 7A). When the incubation started with a higher level of moisture

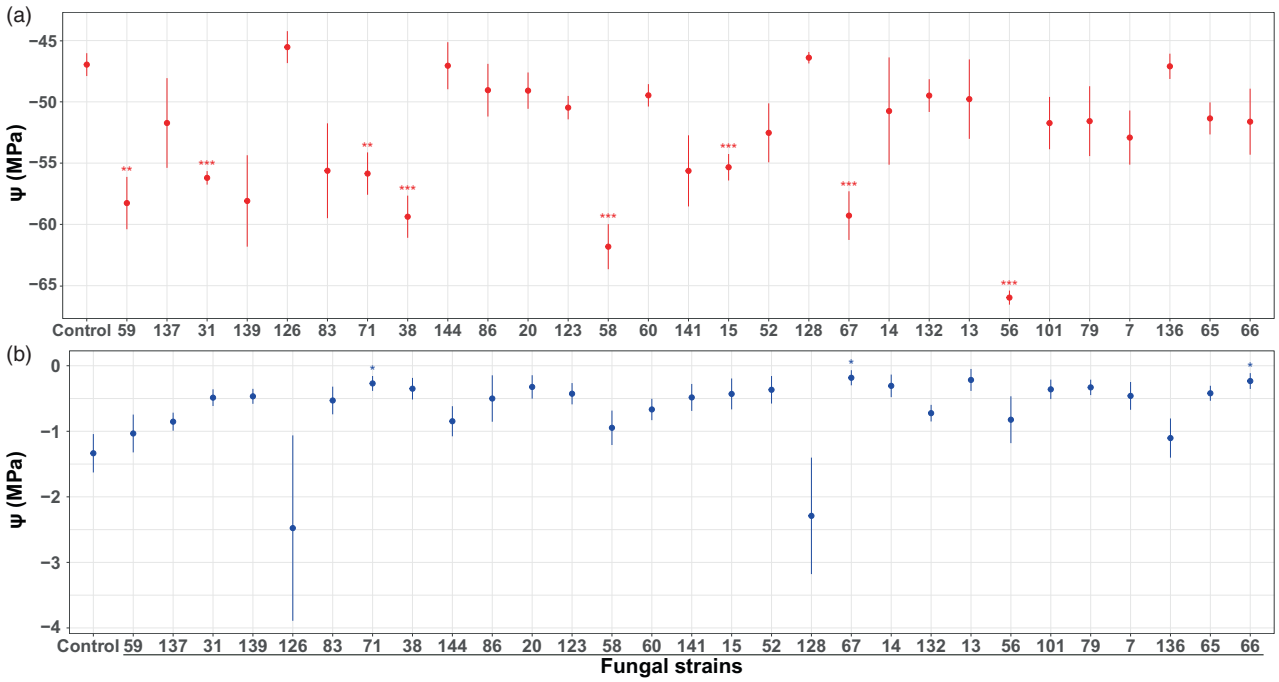


FIGURE 7 Effect of fungal strains on soil water potential (ψ) at low soil moisture (A), and a higher level of moisture followed by a decrease in the content of moisture of 60% (B). Bars represent standard error. Asterisks indicate significant differences compared to non-inoculated control: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The sequence of strains in the x-axis depicts the phylogenetic distance (Figure A, Appendix A3). The ANOVA is shown in Appendix A4, Table C. Means, standard error, *p*-values, and significance are shown in Appendix A4, Table F. Raw data are shown in Appendix A3, Figure I.

and was then diminished to 60% of the initial moisture (Figure 1), 3 of the fungal strains: 66, 67 and 71 resulted in a higher soil water potential than the non-inoculated control (Figure 7B). These 3 fungal strains also showed strong fungal biomass production under low moisture in the microcosm experiment (Figure 5), and strain 67 also showed faster than average growth in our plate assay (Figure 2).

Microscopic imaging of selected inoculated soils

Stereomicroscopic imagining was used to visualize how mycelial growth may serve to connect soil mineral particles and organic matter under our contrasted levels of soil moisture (Figure 8). As expected from the assessment of fungal biomass, we observed higher hyphal densities in inoculated soils under low soil moisture (Figure 8B, D, F), as compared to high soil moisture, where more reproductive structures were identified (Figure 8A, E). Moreover, the distribution of hyphae was not homogeneous, with specific patches of high hyphal density (e.g., Figure 8C) or a high density of reproductive structures (e.g., Figure 8E).

To get qualitative information on the fungal interactions with soil particles, we conducted scanning electron microscopy (SEM) analyses on soil samples inoculated with selected fungal strains. We selected fungal strains based on their effect on soil aggregate formation and stability from the microcosm experiment, as well as their colony density and growth rates. Specifically, we selected strain 83, which showed the highest effect on SI at low soil moisture, and strain 141, which showed a high colony density and rapid growth rate. SEM revealed that hyphae were able to bridge gaps between soil particles and capture soil particles via enmeshment at the high moisture content (Figure 9C, D). It also allowed us to observe the detailed structure of reproductive organs (Figure 9A, B). At low moisture content, we were able to observe that collapsed hyphae still could serve the function of connecting soil particles can still connect soil particles (Appendix A3, Figure D).

DISCUSSION

We found that fungal inoculation in soils enhanced soil aggregate formation and stability. Fungal inoculation also modified several soil hydrological properties, such as gravimetric water content (θ), water repellency, sorptivity and water potential (ψ), and these effects depended on soil moisture. Further, we found that the fungal capacity to colonize the soil, as determined by measurements of fungal biomass, was the strongest determinant of soil aggregation upon inoculation.

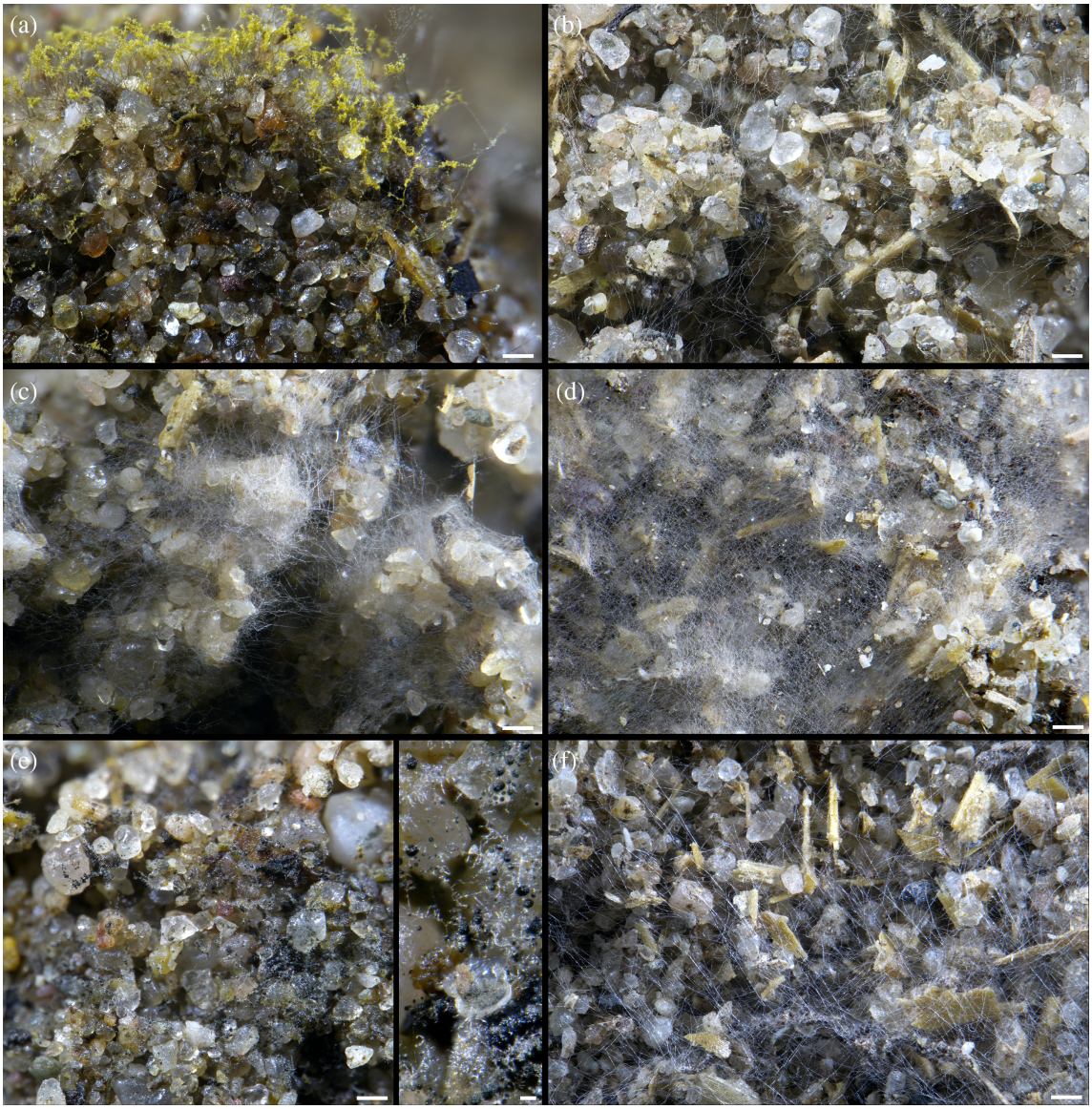


FIGURE 8 Images of fungal strains in soil (stereomicroscopy, 16×). Strain 14 (*Staphylotrichum acaciicola*) at high soil moisture (A) produced reproductive structures (yellow), which were not observed for the same strain at low soil moisture (B). This pattern was similar for strain 126 (*Gliomastix roseogrisea*) (E) which produced black reproductive structures at high soil moisture (inset shows enlargement) and more discrete for strain 56 (*Pyrenochaetopsis leptospora*) which showed patches of dense mycelia and some reproductive structures (C). At low soil moisture, strains 56 and 126 produced thick layers of mycelia (D) and (F) respectively, and no reproductive structures. Bars are 500 and 50 μm in the inset of (E).

Effect of fungal strain inoculation on soil aggregation and soil hydrological properties

The present study revealed diverse effects of fungal strain inoculation on soil structure in terms of macroaggregate formation and stabilization, which were tested using the mean weight diameter (MWD). Our results are in line with a range of studies showing that soil fungi are important determinants of soil aggregation and soil structure. For instance, Beare et al. (1997) showed that a fungicide treatment, which decreased the density of fungal mycelium in soil, led to a decrease in soil aggregation. Moreover, extracellular products like exopolysaccharides produced by basidiomycetes and *Trichomomaceae* can glue soil particles, thereby promoting soil aggregate formation and stability (Caesar-Tonthat, 2002; Daynes et al., 2012). Mycorrhizal fungi are long known to influence soil structure and soil water retention under drought through the production of glomalin-related compounds and via their mycelial network (Ji et al., 2019; Wu et al., 2008). It would have been helpful to have measures of glomalin-related soil proteins (GSRPs) and EPS in our soil microcosms. However, the appropriate measures for the quantification of GSRPs in soils have been a subject of debate

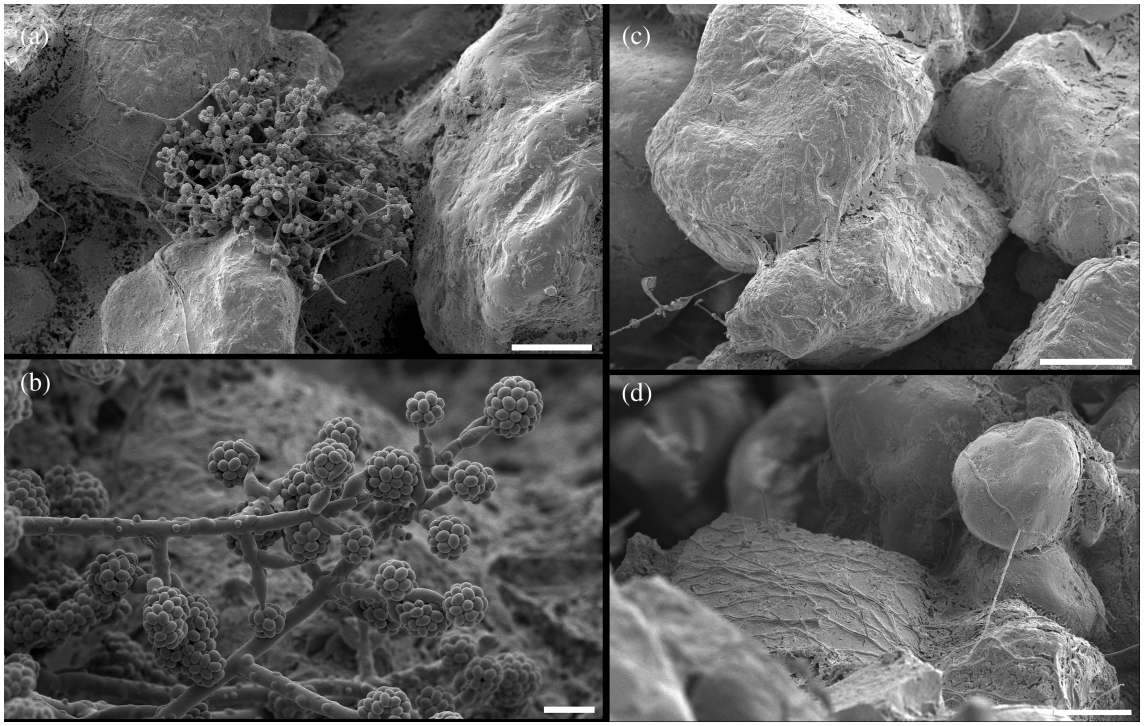


FIGURE 9 Images of fungal strains in soil (cryo-scanning-electron-microscopy) at high moisture. (A) Intermittent patches of well-developed mycelium on the soil surface (strain 141, *Trichoderma linzhiense*). Many conidiophores and conidia are also visible. Some hyphae run over the surface of the grains and one hyphal structure grows into the air space between soil grains (middle). (B) An intact conidiophore with conidia (strain 141), with warts, phialides, and slimy heads containing conidia. (C) Two soil grains bound together by several hyphae exhibit close growth and branch formation close to the surface of the particle (strain 83, *Hydropisphaera* sp. nov). (D) Crossing hyphae (strain 83) bridging 2 soil particles and illustrating the capacity of fungi to bind soil particle content. Bars are 10 μm (B) and 100 μm (A, C and D).

(Irving et al., 2021) and the quantification of EPS in soil can be extremely challenging due to inefficient methodologies (Redmile-Gordon et al., 2014). For instance, intracellular contamination by using H_2SO_4 and heating techniques (Sun et al., 2012) may co-extract large amounts of intracellular biomass and non-specific soil organic matter (SOM), leading to misestimations of soil EPS levels. More recently, the positive effect of free-living saprobic fungi on soil aggregation was demonstrated, with effects being higher for Ascomycota fungi with a higher density of mycelium (Lehmann et al., 2020).

For most of the strains we tested, soil aggregation was improved upon fungal inoculation. Isolate 83 (*Hydropisphaera* sp. nov), for instance, showed a particularly high effect on soil aggregate stability (MWD_w), which was increased by 6.7 times (2.5 mm) and 5.6 times (2.1 mm) compared to the non-inoculated control, at low and high soil moisture, respectively. We also found that 72.2% of strains with a positive impact on aggregate formation and stability under low moisture content were isolated from one of the drought-treated field plots, including strain 139, which was selected among the 3 strains determined as more abundant in the drought-treated field plots of isolation. This suggests that these strains could have used strategies to be adapted to grow under drought, but a larger number of strains would have to be examined to determine if this trend is statistically significant. Among the selected fungal strains, 97% of them belonged to the phylum Ascomycota. Interestingly, this phylum was recently suggested to have more relevance for soil aggregate formation as compared to Basidiomycota and Mucoromycota (Lehmann et al., 2020), although it remains unclear which fungal traits might contribute to these phylum-level differences. Nonetheless, some saprophytic basidiomycetes are also known to have positive impacts on soil aggregate stability (Caesar-Tonthat, 2002). We did not systematically examine whether phylogenetic affiliation was related to a strain's ability to improve soil structure, nevertheless, some closely related strains did have similar impacts. For instance, strains 7 and 136, both identified as *Penicillium* sp., and strains 20 and 123, both identified as *Marquandomyces*, showed similar behaviour for colony traits and soil properties. More systematic analyses, including a broader diversity of fungal taxa, would be necessary to identify specific fungal groups that increase soil aggregate stability.

Fungal traits, namely fungal colony density and growth rate as measured by plate assays showed a limited effect on soil aggregation. We hypothesized that

denser colonies have more hyphae per cm^2 and could theoretically entangle more soil particles causing stronger bonds between them. For most strains, the fungal colony was between 0.03 and 0.08 mg/cm^2, which corresponds to one of the most effective ranges for soil aggregate formation by the saprobic fungi tested by Lehmann et al. (2020). Also, the density of our fungal strains was slightly correlated with soil aggregate formation and stability under high soil moisture (Table 1). However, in the path analysis (Figure 6), we did not observe a direct effect of the colony density and growth rate on soil aggregate formation and/or stabilization at either level of moisture. A possible reason is that the fungal colony densities and growth rates were determined on agar plates, and thus do not necessarily translate to the conditions in the soil substrate. For instance, fungi colonizing nutrient-rich substrates typically produce dense mycelia for resource exploitation, whereas hyphae under nutrient-poor substrates branch less frequently, producing mycelia adapted for more distant resource exploration (Walker & White, 2017). In contrast, fungal biomass (ergosterol) strongly influenced soil aggregate formation and stability at both levels of soil moisture, which is in line with previous evidence (Cosentino et al., 2006; Erktan et al., 2020; Gupta & Germida, 1988; Söderström, 1979).

Soil water hydrophobicity (water contact angle) and soil sorptivity were negatively correlated, and these properties contributed to explaining soil aggregate stability under low soil moisture (Figure 6). High values of soil water repellency reduced soil sorptivity (Vogelmann et al., 2017), and a high soil hydrophobicity can promote the increased stability of soil aggregates (Chenu & Cosentino, 2011; Vogelmann et al., 2013). Soil water repellency is often linked to fungal activity (York & Canaway, 2000), and the inhibition of fungal growth decreases soil repellency (Hallett et al., 2001) Fungi secrete hydrophobins, which are proteins with potent surfactant activity and water repellency (Rillig, 2005). To restrict water loss, many mycelia produce small (15 kDa) secreted cysteine-rich hydrophobin proteins that increase the hydrophobicity of the surface, thus restricting water movement (Fricker et al., 2017). Hydrophobins allow fungi to escape from an aqueous environment and confer hyphae the ability to explore the air-filled pores to bridge soil voids (Wösten, 2001). We observed (Figures 8 and 9) hyphae bridging gaps and joining soil mineral particles. Fungal hydrophobins can also be excreted into the environment as monomers that can ensemble into insoluble complexes, thereby playing a role in the adherence of fungal hyphae to hydrophobic surfaces (Wösten et al., 1993; Wösten et al., 1994). Even though soil water repellency is also associated with reduced water infiltration, variations in water content, surface runoff, nutrient losses, and soil erosion (Doerr et al., 2000; Rillig, 2005), highly repellent coarse aggregates (2–5 mm) have been demonstrated to increase water infiltration by allowing more rapid water movement through relatively large interaggregate voids (Hillel & Berliner, 1974). This notion was corroborated by de Jonge et al. (1999) who found that the finest fractions (<0.063) of 2 water-repellent soils showed the highest degree of water repellency compared to other soil fractions. Thus, the size of aggregates was presumably an important factor in improving the stability and water infiltration capacity in our water-repellent soils.

Effects of fungal strain inoculation at contrasting soil moisture conditions

To examine whether the effect of fungal inoculation on soil structure depends on soil moisture, we examined the impacts of soil inoculations under 2 contrasting soil moisture levels. Results showed that the effect of fungal strains was affected by soil moisture. The loss of moisture during the incubation period was progressive, and the treatment under low moisture lost a higher proportion of moisture, almost 80% of the initial moisture (−0.96 MPa), than the high moisture treatment. This moisture loss had a significant effect on the gravimetric water content of the system under low moisture content. Lavee et al. (1996), showed that aggregate stability decreased with increasingly arid climatic conditions, but such effects could be at least partially mitigated by inoculation with some fungi, namely mycorrhizal fungi (Ji et al., 2019).

In our study, the effect of saprobic fungal inoculation on soil aggregate stability was positive at both levels of moisture, but higher effects were found at the lower moisture level. In contrast, the soil aggregate formation was larger at higher moisture content for a number of fungal strains. Some strains showed a higher soil aggregate formation and stability under the lower moisture content (e.g., strain 71, *Paramyrothecium viridisporum*) when compared to the control, while others, such as strain 56 (*Pyrenochaetopsis leptospora*), were better than the control at both levels of moisture. Different optimal ranges of moisture levels have been cited for different fungal taxonomic groups, which may explain why some strains perform better under specific moisture conditions. The vast majority of yeast and fungi are active within the range of 1–0.90 of water activity (a_w), an equivalent of 0 to −14.5 MPa (https://chart-studio.plotly.com/~howard.wildman/417.embed), and only a few species have been observed to grow and/or germinate at values <0.70 a_w (−49 MPa) (Stevenson et al., 2015). The high ability to overcome water stress can come at the expense of a reduced growth rate for fungi (Luard & Griffin, 1981). Some fungal traits, such as hyphal extension rates and conidial germination, decline with decreasing water availability

(Jackson et al., 1991). In the latter study, hyphal extension rates of *Trichoderma* spp. strains declined over the range of −0.7 to −14 MPa, and no growth was detected at −14 MPa. In another example, species of *Cladosporium halotolerans*, *Aspergillus niger* and *Penicillium rubens* decreased their growth rates to below 1 mm/day when the a_w reached values lower than 0.84 of a_w (∼ −25 MPa) (Segers et al., 2016). The increasing water loss in our drought experiment thus could have limited the hyphal growth and metabolism of our fungal strains.

Mycelial growth adopts different patterns of branching depending on the microenvironmental conditions (Walker & White, 2017). Fungal strains showed different growth patterns under contrasting levels of moisture (Figure 8). We observed larger production of crossing hyphal structures under low moisture, as compared to reproductive structures observed at high moisture. This indicates a shift in structure differentiation that may contribute to enhanced soil aggregate stability under low moisture. In addition, these mycelial networks can drive soil hydraulic redistribution (HR) by enabling water transport along soil water potential gradients, which has been put forth as a means of fungal resistance to low moisture levels (Guhr et al., 2016). Filamentous fungal abundance depends less on habitats with higher soil moisture than yeasts (Connell et al., 2006), as the filamentous morphology allows them to grow through air and bridge soil pores, translocating materials by an interconnected tubular network (Bielčik et al., 2019; Miller & Fitzsimons, 2011; Ritz & Young, 2004). We expect that differences in mycelial network architectures occurred in our experiment because of the contrasting levels of soil moisture and that hydraulic redistribution may have contributed to fungal biomass accumulation.

We also found that soil hydrophobicity had a negative effect on soil sorptivity, and this effect increased at low moisture content. The effect of soil moisture on soil water repellency has previously been reported by Wallis and Horne (1992). Jex et al. (1985) also showed a positive correlation between low soil water content and soil repellency, but opposite relationships have also been found (de Jonge et al., 1999). For instance, in our study, fungal strains 31, 71, and 126 resulted in higher levels of water contact angle, lower levels of sorptivity, and higher soil aggregate stability under low moisture.

Overall, our results suggest that, under low soil moisture levels, the effect of fungal strains is associated with enmeshment by high biomass of hyphae and by changes in soil water repellency. These changes in water repellency are related to hydrophobic substances that can be produced in higher quantities by fungi in dry conditions. These hydrophobic proteins also can remain in the system long after these drying periods may have negatively impacted cell activity. In addition, even in cases where the loss of moisture during the period of incubation inhibited hyphal growth, it is likely that dead hypha could still retain their capacity to connect soil particles, thereby contributing to maintaining soil structure, as dried fungal materials can possess tensile strengths of 5.0 MPa (Appels et al., 2020).

Effect of fungal strain inoculation on soil water potential

Fungi can affect soil water potential via a range of different mechanisms. Fungi, for instance, acidify the soil by secreting organic acids (Liaud et al., 2014). These organic acids will dissolve nutrients thereby releasing ions (Haynes & Swift, 1986) and lowering the water potential of the soil (Herman & Bleichrodt, 2022), but also enhancing the feeding of the plant roots. Fungal respiration will also result in water loss, but this is expected to play a relatively minor role in the total soil water budget (Flegg, 1974). Fungi can provide hydraulic redistribution and relocation of water, as discussed in Section 4.2. Fungi may also impact soil water potential by lowering water evaporation via the action of secreted hydrophobins (Fricker et al., 2017) which can self-assemble at hydrophobic-hydrophilic interfaces affecting surface tension (Rillig, 2005). A potential disadvantage of this effect is that hydrophobic soils will have a slower rewetting rate (Olorunfemi et al., 2014). Fungal EPS, biofilms (de Siqueira, 2015), and glomalin-related proteins (GSRPs) also can enhance soil hydrophobic properties (Wright et al., 1996). Moreover, fungi can also affect water retention via indirect effects through their impact on soil structure (Augé et al., 2001).

In the water potential experiment, the water potential dramatically dropped to values as low as −65 MPa for the low moisture treatment after 2 months of incubation. There is a general correlation between the number and activity of microbes and the soil water potential, with values of −10 to −40 MPa being considered limiting for many fungi (Robert & Chenu, 1995). Even highly xerophilic fungi, such as *X. bisporus* or *A. penicillioides*, which can remain active with very low moisture content, show marked decreases in activity at such soil water potential values. During part of the low moisture treatment, the soil water potential was lower than the limits of growth and metabolism of many strains. It is possible that fungal metabolism may have been altered or interrupted for different functions but the mycelium that was formed during the first stages of the treatments could still help to maintain soil structure, as can be visualized in Appendix A3, Figure D. There is limited research on the lifetime of AMF hyphae, and data are scarce from saprobic fungi. Chenu and Cosentino (2011) suggested the lifetime of aggregates depends on the lifetime of their aggregating effects.

Results also showed a higher water potential for the inoculated soil at an initially higher level of moisture

after a pulse of drying than the treatment under permanently low moisture. This shed light on the effect on water potential under conditions of lowered moisture content and how fungal activity can maintain a higher water potential in such soil systems. The results of Harris (1981) and Kieft et al. (1987) suggest that the stress experienced by soil microorganisms is not only related to the absolute values of water potential but rather to the amplitude of change caused by drying and wetting cycles. Microbial abilities to adapt to very dry soil conditions often involve the production of specialized structures or spores, which can germinate after rewetting periods. However, cycles of drying and rewetting also bring physiological consequences for fungal growth. For instance, swollen conidia, germlings and microcolonies of *A. niger* and *P. rubens* that had experienced moisture dynamics, could not reinitiate growth after being retransferred from a low relative humidity (HR) medium to another with higher one (Segers et al., 2016), and these dynamics were associated with oxidative stress (e.g., H_2O_2 and catalase activity) (Wu & Wong, 2020).

Additionally, it is important to know the amount of water stored by the fungi and the proportion that is available to the rest of the system to determine if fungal inoculation indeed enhances soil hydrological properties when facing low moisture conditions. Fungi can store water in their mycelium with strong cell walls that help prevent water loss (Guhr et al., 2015). Fungi can produce protective osmolytes (Davis et al., 2000) and biological pigments such as melanin which provide resistance to desiccation due to their hygroscopic character (Cordero & Casadevall, 2017). Fungi can also uptake water and potentially reduce the amount available for the plant. However, the total biomass of fungal mycelium in most substrates is not extremely high in absolute terms, estimated for instance as approximately 29 mg/g for compost (Vos et al., 2017). Thus, water uptake by fungi may only marginally impact water availability in soil. We did not assess fungal biomass in the water potential experiment, nonetheless, we did observe that strains 66, 67 and 71 showed higher biomass and less moisture loss than other strains under low moisture content in our microcosm experiment. Microscopic images also showed a higher density of hypha under the low level of moisture, suggesting a higher fungal biomass.

Microbial amendments to soil have been applied across a range of systems to enhance desired soil properties. While it is often assumed that changes in soil function can be attributed to the inoculated microbial strains, effects may also occur as a result of alterations in the native soil community (Xiong et al., 2017). For instance, Huang et al. (2022) found that the inoculation of 3 PGPR strains led to changes in the native bacterial communities involved in the N cycle, leading to improved N use. It is known that soil microbes impact each other through a range of beneficial and deleterious interactions, affecting both the ability of inoculated strains to establish in the community, as well as ultimate soil functionality (Brözel, 2022). In addition, the effectiveness of microorganisms on soil functioning is influenced by a diversity of ecological and evolutionary dynamics under the effect of global changes (Angulo et al., 2022).

CONCLUSIONS

Our results revealed diverse interactions between saprobic fungal strains inoculation under different moisture levels, aggregate formation and stability, and soil hydrological properties. We highlight that at low soil moisture content, fungal inoculation enhanced soil hydrophobicity and decreased soil sorptivity while improving soil aggregate stability. Fungal-induced changes in soil hydrological properties represent an indirect way to improve soil aggregates, as it prevents water from entering soil aggregates and destabilizing them, representing a potential advantage under conditions of intense drought. Fungal biomass best predicted a strain's ability to improve soil aggregate formation and stability by connecting soil particles by hyphae under both high and low moisture conditions and by modifying soil aggregate sorptivity. However, at low soil moisture content, fungal inoculation did not increase the water content in the system as we expected. Our results also suggest that initial high soil moisture may allow fungal metabolism activity which enhances the water potential under events of drought.

We propose that ecologically informed strategies of fungal inoculation could represent viable soil management options to help maintain and improve soil structure for plant productivity under conditions of drought and help mitigate the negative effects of climate change. We further advocate that future research should involve an expanded array of fungal species, as well as provide more depth of investigation into more temporal aspects, such as how fungal colonization, soil aggregation, soil water repellency, and soil water content vary under humidity dynamics.

AUTHOR CONTRIBUTIONS

Violeta Angulo: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Robert-Jan Bleichrodt:** Conceptualization (supporting); methodology (supporting); resources (supporting); writing – review and editing (supporting). **Jan Dijksterhuis:** Methodology (supporting); resources (supporting); visualization (supporting); writing – review and editing (equal). **Amandine Erktan:** Conceptualization (equal); formal analysis (equal); methodology (supporting);

supervision (supporting); writing – review and editing (supporting). **Mariet M. Hefting:** Conceptualization (supporting); project administration (lead); writing – review and editing (supporting). **Bart Kraak:** Data curation (supporting); formal analysis (supporting). **George A. Kowalchuk:** Conceptualization (equal); investigation (equal); supervision (lead); writing – review and editing (equal).

ACKNOWLEDGEMENTS

We express our gratitude to the 'One Hundred Scholarships for Technological and Scientific Sovereignty' from the Plurinational State of Bolivia and the Schlumberger Foundation, Faculty for the Future Fellowships for their financial support of this research. Our appreciation extends to Eiko Kuramae and Ciska Raaijmakers from the Netherlands Institute of Ecology (NIOO-KNAW) for their assistance with ergosterol quantification. We are also grateful to Kathryn Barry and Yann Hautier for their invaluable help with statistical analyses, and to Yann Hautier for granting access to the drought experimental field site. Finally, we would like to acknowledge Ben van der Harg and Peter Veenhuizen for their indispensable technical support.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Microcosm and water potential data are available in Zenodo: https://zenodo.org/doi/10.5281/zenodo.7648511. The sequence data are available in GenBank under accession numbers: OQ513884, OQ513885; OQ513886, OQ513887; OQ513888, OQ513889; OQ513890, OQ513891; OQ513892, OQ513893; OQ513894, OQ513895; OQ513896, OQ513897; OQ513898, OQ513899O; Q513900, OQ513901; OQ513902, OQ513903; OQ513904, OQ513905; OQ513906, OQ513907; OQ513908, OQ513909; OQ513910, OQ513911; OQ513975.

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SUPPORTING INFORMATION

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How to cite this article: Angulo, V., Bleichrodt, R.-J., Dijksterhuis, J., Erktan, A., Hefting, M.M., Kraak, B. et al. (2024) Enhancement of soil aggregation and physical properties through fungal amendments under varying moisture conditions. *Environmental Microbiology*, 26(5), e16627. Available from: https://doi.org/10.1111/1462-2920.16627