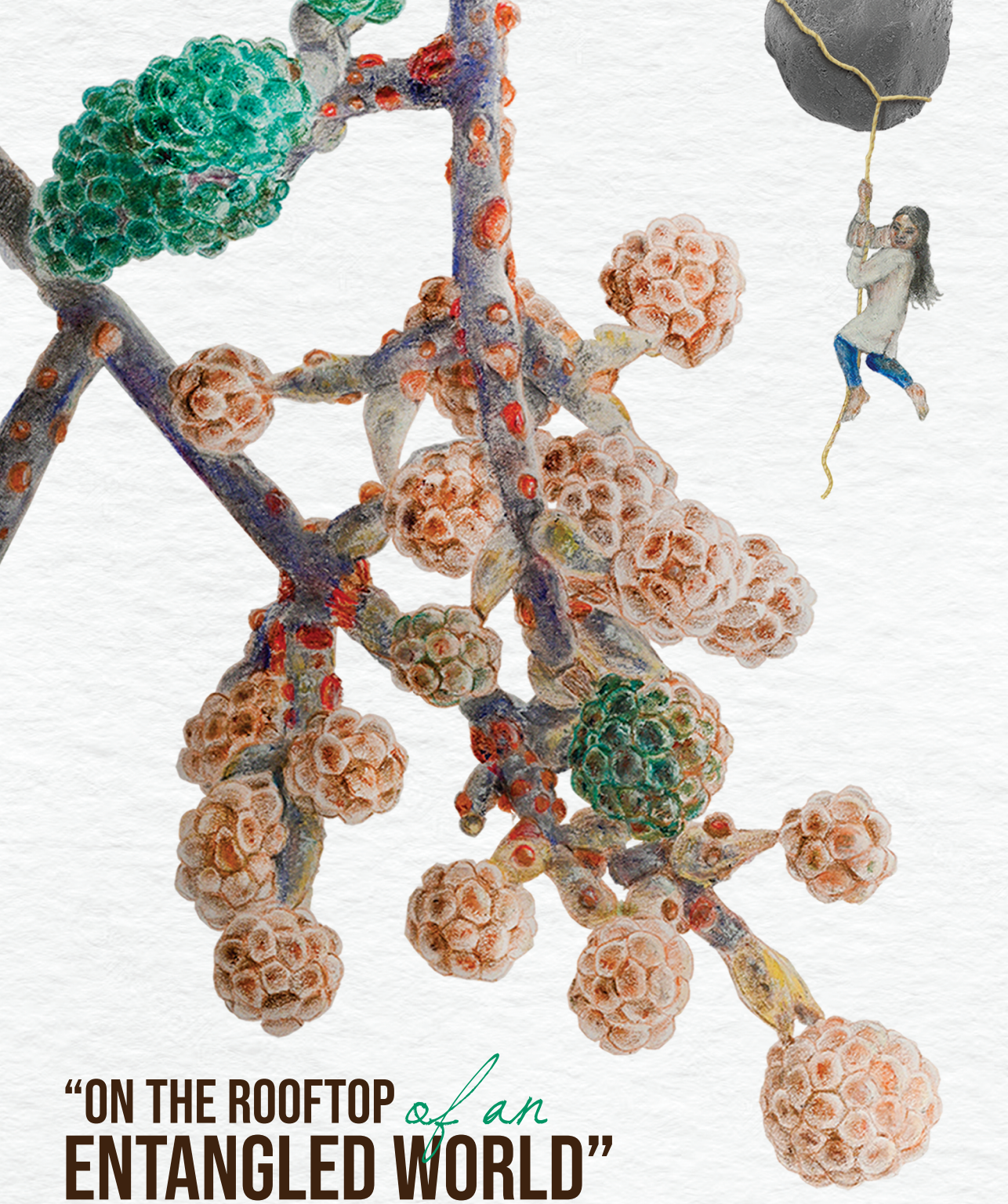

“ON THE ROOFTOP *of an* ENTANGLED WORLD”

Impacts of microbial inoculants on soil structure and plant growth under different moisture levels

Violeta Carmen Angulo Fernández

On the rooftop of an entangled world

Impacts of microbial inoculants on soil structure and plant growth under different moisture levels

Violeta Carmen Angulo Fernández

Utrecht University
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On the rooftop of an entangled world

Impacts of microbial inoculants on soil structure and plant growth under different moisture levels

Op het dak van een verstrengelde wereld
Effecten van microbiële inoculanten op de bodemstructuur en plantengroei onder verschillende vochtigheidsniveaus
(met een samenvatting in het Nederlands)

Sobre la azotea de un mundo enredado
Efectos de inoculantes microbianos en la estructura del suelo y desarrollo de plantas bajo diferentes niveles de humedad
(con un resumen en español)

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Violeta Carmen Angulo Fernández

geboren op 22 maart 1982
te La Paz, Bolivia

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Heavenly father, thank you for all the strength provided!

"But those who hope in the LORD
will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint".
Isaiah 40:31

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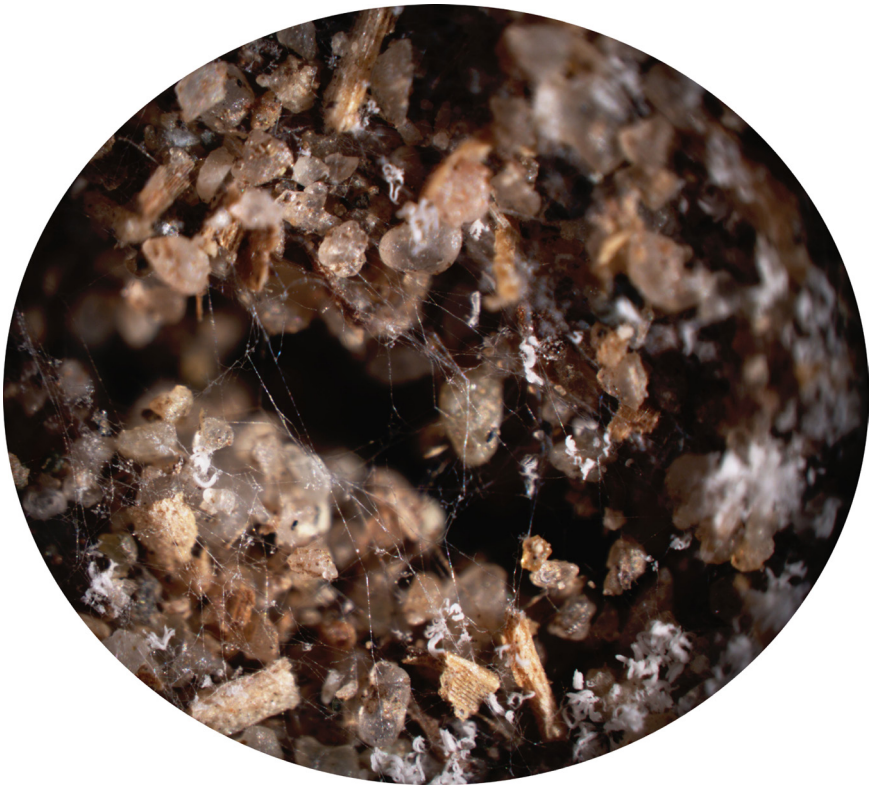

CHAPTER 1

GENERAL INTRODUCTION

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1. Soil degradation is a major global problem

The world's population has increased from an estimated 1 billion in 1800 to approximately 8 billion in 2022, and it is expected to increase further to 9,7 billion by 2050 (United Nations, 2023). The number of people affected by hunger is increasing, with 8.9% of the world's population already in severe starvation (Mumuni and Joseph Aleer, 2023), and further overpopulation (Pison, 2022) will exacerbate this problem. Intensification of agricultural systems can help increase food supplies. Nevertheless, such practices lead to critical declines in soil quality and a decrease in ecosystem services (Lal *et al.*, 1989; Feddema and Freire, 2001; Gunawardena, 2022). Furthermore, climate change is expected to bring about major changes in crop productivity and freshwater availability, with consequences for food security (Raleigh and Urdal, 2007). Droughts can occur naturally, but climate change has accelerated the hydrological events to make them set quicker and turn more intense (Mukherjee *et al*., 2018). For instance, changes in rainfall led to drought and heat waves which resulted in significant stress during critical phases of corn and soybean crops in 2012 (Al-Kaisi *et al*., 2013). Droughts and heat waves are important physical drivers of land degradation (Hermans and McLeman, 2021).

Soil degradation is defined "as a change in the soil health status resulting in a diminished capacity of the ecosystem to provide goods and services for its beneficiaries" (FAO, 2023). Soil health and soil quality are terms frequently used interchangeably to describe the "capacity of the soil to function" (Laishram *et al.*, 2012; Lehmann *et al.*, 2020). Soil degradation can be categorized into physical, chemical, biological, and ecological degradation (Fig. 1) (Lal, 2015). Soil degradation brings consequences for water and nutrient retention, availability of soil organic matter (SOM), soil erosion, and loss of biodiversity (Connolly, 1998; Jie *et al.*, 2002; Lal, 2015). The degraded soils have a diminished capacity to deliver soil functions (e.g., carbon sequestration), which act as intermediaries of the ecosystem services delivery (Gunawardena, 2022). Ecosystem services are the benefits that people derive from an ecosystem, these services are the utilitarian outcome of ecosystem functioning which in turn depends on ecosystem structure (Brussaard, 2012; Vicente-Vicente *et al.*, 2019; Gunawardena, 2022) (Fig. 1).

A range of management practices has been proposed and put into practice to help reduce soil degradation products of human activity and natural events and restore soil quality. Among these practices, reduced tillage, use of cover crops, integrated nutrient management, and sustainable irrigation use are very spread (Hillel *et al.*, 2008; Lal, 2009, 2015; Baumhardt *et al.*, 2015). Although such

measures have been shown to have considerable positive effects, additional strategies are required to help reverse the negative effects of soil degradation, especially in the face of climate change and drought events.

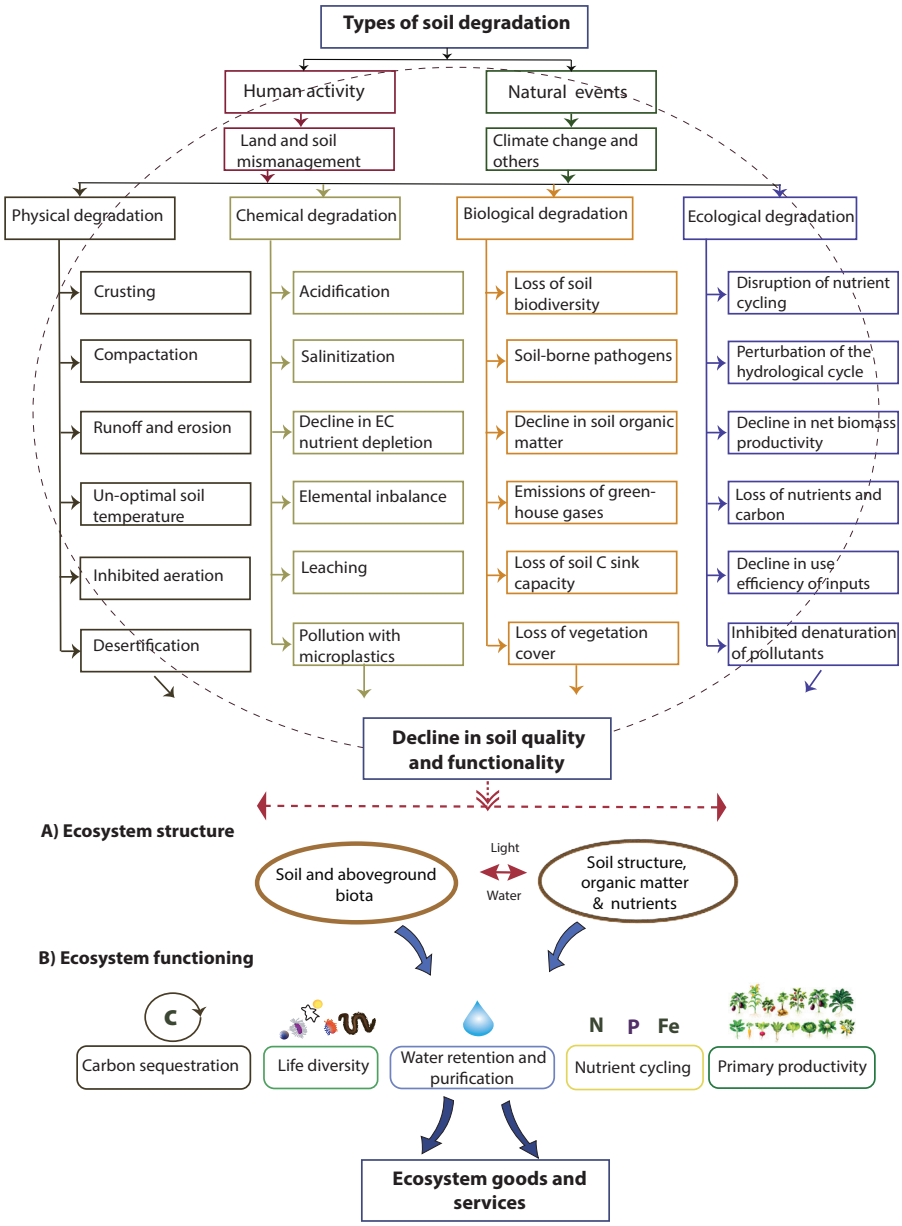


Figure 1. Types of soil degradation determined by human activity and natural events and their effect on ecosystem structure and functioning. Adapted from Brussaard, 2012; Lal, 2015 & Vicente-Vicente *et al*., 2019.

2. Soil services as driven by soil microorganisms

The proper functionality of soil is the key to ensure the continuous flow of ecological services of soil (Gunawardena, 2022). Soil functions are depicted in Fig. 1, and include the following: (a) **water purification and regulation (WR),** which has been defined as "the capacity of the soil to remove harmful compounds and the capacity of the soil to receive, store, and conduct water for subsequent use and to prevent drought, flooding and erosion" (Wall *et al.*, 2020), (b) **soil biodiversity (SB),** the SB function is defined as "the multitude of soil organisms and processes interacting in an ecosystem, providing to society with a rich diversity source and contributing to a habitat for aboveground organisms" (van Leeuwen *et al.*, 2019), (c) **nutrient cycling,** which is relevant for the mobility of plants nutrient, particularly N and P (Schröder *et al.*, 2016), (d) **climate regulation and carbon sequestration,** which refers to the transfer of atmospheric CO_2 into soil, which can help counteract the effects of anthropogenic emissions, for instance, from fossil fuel combustion, cement production, and deforestation (Lal *et al.*, 2015), and (e) **agricultural or primary productivity** (Schulte *et al.*, 2014), which is defined as the capacity of a soil to supply nutrients and water to foster the production of plant biomass for human use (Sandén *et al.*, 2019).

Microorganisms, including bacteria and fungi, represent, after plants, the second largest biomass pool on Earth, accounting for approximately 82 Gt C of the 550 Gt C in total global biomass (Bar-On *et al.*, 2018). The majority of microbial biomass and diversity is found in soil environments, where the microbiome is critical for soil and ecosystem functionality (Brussaard, 2012; Aislabie and Deslippe, 2013). A number of the most important soil functions driven by microorganisms is described in Table 1. Specifically, with respect to primary productivity, some microbial groups, such as plant growth-promoting rhizobacteria (PGPR) (Shah *et al.*, 2021), arbuscular mycorrhiza fungi (AMF) (Powell and Rillig, 2018), and plant growth-promoting fungi (PGPF) (Murali *et al.*, 2021) are known to be important determinants of plant production, health, and protection against biotic and abiotic stresses. Capitalizing on these important plant-microbe interactions has thus been suggested as an important route to the development of more sustainable agricultural practices (Agrahari *et al.*, 2020). Soil microorganisms not only interact with aboveground biota, but they can also drive belowground processes related to the build-up of soil structure (ecosystem structure, Fig. 1), organic matter, and the release of nutrients (Brussaard, 2012). As discussed below, microbes are critical agents in the soil aggregation processes (Chenu and Cosentino, 2011), and bacteria and fungi appear to have a more important role in soil aggregation than other soil organisms (Lehmann *et al.*, 2017).

Table 1. Role of microorganisms in provisioning and regulating ecosystem functioning.

Soil Function	Mechanism	Example
Water supply	Water purification: biosorption and enzymatic degradation, or a mixture of both processes (Mishra *et al.*, 2021).	A consortium of *Pseudomonas* spp. led to the decolorization of a textile effluent as well as of Reactive Orange 16 within 48 h (Jadhav *et al*., 2010).
	Water retention: physical modification of the environment by the production of extracellular polymeric substances (Guo *et al*., 2018) or glomalin proteins (Lombardo *et al*., 2019).	Soil exopolysaccharides content increased as the water content in sandy soil when it was inoculated with *Pseudomonas* sp. under desiccation (Roberson and Firestone, 1992).
Nutrient cycle	i) microbial ability to decompose organic materials and release inorganic nutrients, ii) modification of nutrient availability by solubilization, chelation, oxidation, and reduction, iii) storing nutrients in and releasing nutrients from the microbial biomass (Marschner, 2007).	Fungi can translocate nutrients and energy from one location to another through their hyphal structures (Dighton, 2007).
Microbial diversity	Soils are reservoirs for the resting phases of organisms (e.g., fungal spores, cysts) and thus are key for the rejuvenation of communities (Aislabie and Deslippe, 2013).	A higher microbial community could ameliorate better the response of legumes to counteract the effect of drought (Prudent *et al*., 2020).
Carbon sequestration	Direct: related to microbial community dynamics and the balance between the formation and degradation of microbial products. Indirect: the influence in C cycling by improving soil aggregation that physically protects soil organic matter (SOM) (Six *et al*., 2006).	Cyanobacteria strains inoculation increased levels of soil organic carbon (SOC) in natural soils (Muñoz-Rojas *et al*., 2018).
Primary productivity	Nutrient supply (nitrogen fixation, phosphorus solubilization, and iron uptake), phytohormones production, and ethylene regulation among others (Vocciante *et al*., 2022).	Inoculation of Trichoderma virens enhanced biomass production and later root development, and this was associated with auxin production (Contreras-Cornejo *et al.*, 2009).

3. Roles of bacteria and fungi in soil structure

3.1 Soil structure and soil aggregates

Soil structure may be defined as "the spatial heterogeneity of the different components or properties of soil" (Dexter, 1988) and is a key factor in soil functioning (Bronick and Lal, 2005). It regulates water retention and infiltration, soil organic matter, gaseous exchanges, and nutrient dynamics (Rabot *et al.*, 2018). Soil aggregate stability is often used as an indicator of soil structure (Six *et al.*, 2000). Aggregates are defined as "naturally occurring clusters or groups of soil particles in which the forces holding the particles together are much stronger than the forces between adjacent aggregates" (Martin *et al.*, 1955).

Soil aggregate formation occurs mainly as a result of physical forces such as events of wetting and drying and freezing and thawing (Amézketa *et al.*, 1996; Rajaram and Erbach, 1999; Oztas and Fayetorbay, 2003). Meanwhile, the stabilization of aggregates also occurs via a number of factors, in particular the quantity and quality of organic and inorganic stabilizing agents (Dalal and Bridge, 1995; Amézketa, 1999). Based on the stability of the soils against ultrasonic excitation, Edwards and Bremner, (1964, 1967) concluded that soils are built of macroaggregates (> 250 µm) and microaggregates (< 250 µm), and the association of microaggregates could lead to the formation of macroaggregates.

Further, Tisdall and Oades (1982) developed this concept of macro and microaggregates into the aggregate hierarchy model. In this model, persistent small microaggregates (2-20 µm) are formed from clay mineral particles (Cl) that are attached to organo-metallic complexes (OM) (dead bacterial, fungal debris and extracellular products) and polyvalent cations (P) (e.g., Ca, Al, Fe) to form compound particles (Cl-P-OM). These compound particles are further connected to other compounds by transient binding agents (e.g., polysaccharides) to form microaggregates (20-250 µm) indicated with $(Cl\text{-}P\text{-}OM)_x$. These microaggregates are subsequently bound together in larger macroaggregates (>250 µm) indicated with $[(Cl\text{-}P\text{-}OM)_x]_y$ by agents, such as fungal hyphae and plant roots (Edwards and Bremner, 1967; Bronick and Lal, 2005) (Fig. 2). Several factors influence aggregate stability, including physical soil properties, availability of agents of aggregation such as soil organic carbon (SOC) (Six *et al.*, 2004), and biotic interactions with plants roots, microorganisms, and soil fauna (Bronick and Lal, 2005).

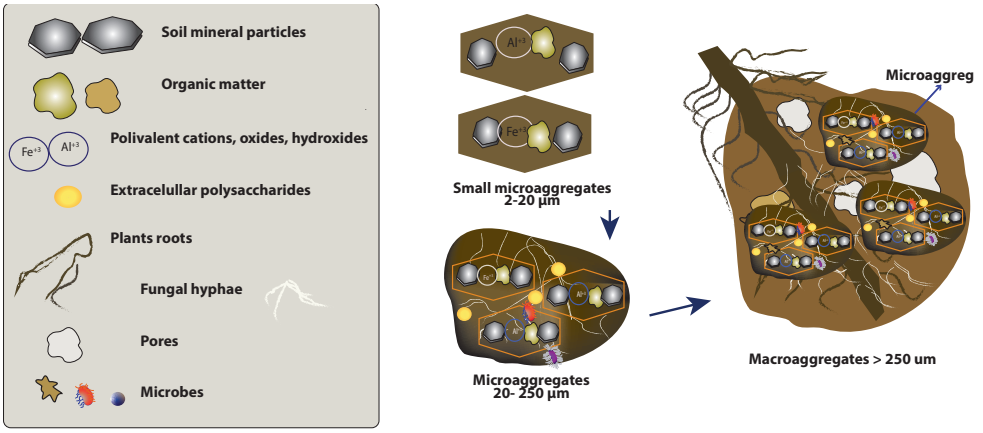


Figure 2. Conceptual diagram showing the hierarchical model of soil aggregates arrangement (Tisdall and Oades, 1982). Microaggregates are inside macroaggregates.

3.2 Microbes as agents in aggregation processes

Microorganisms act as major agents in the soil aggregation processes. They influence the organic matter content and composition, formation of organic glues, aggregate entanglement, and the production of proteins that change the water infiltration capacity of soils. The main microbial mechanisms known to be involved in aggregate processes are depicted in Fig. 3.

(1) Microbial formation, stabilization, and decay of organic matter

Microorganisms play an important role in carbon dynamics in soils (Schimel and Schaeffer, 2012), through organic matter formation, stabilization, and destruction (Chenu and Cosentino, 2011). For instance, Beare *et al.* (1997) showed that fungal biomass improved the retention of soil organic carbon (SOC) and the macroaggregation in no-tillage soil, and this effect decreased when the soil was treated with a fungicide. Microbes contribute to the organic matter with living microbial biomass and also the stabilization of recalcitrant necromass (e.g., fatty acids typical of bacterial cell membranes) (Miltner *et al.*, 2012). Living organisms can adhere to soil minerals through direct electrostatic bonds, and many of them persist after death (Totsche *et al.*, 2018) serving to preserve soil structure.

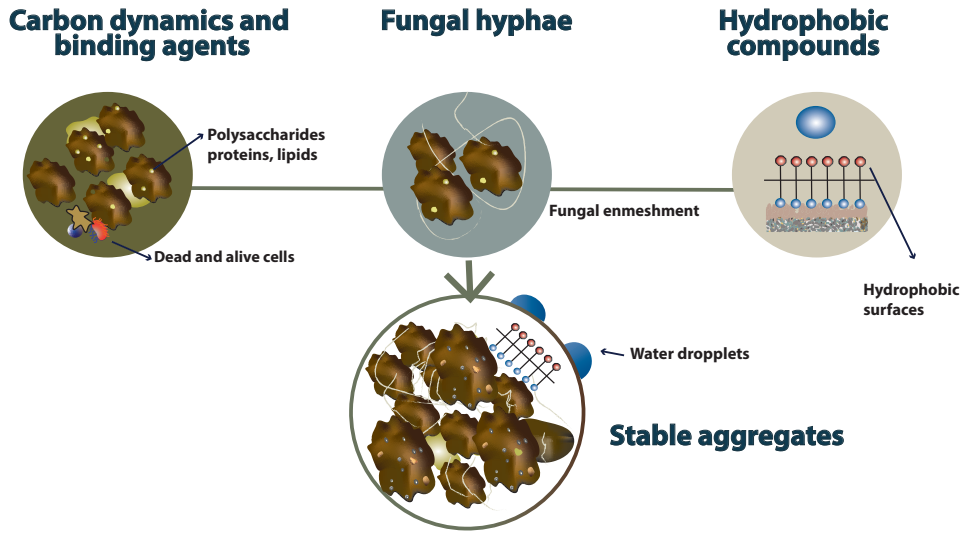


Figure 3. Scheme of main underlying mechanisms of soil aggregate stability supported by microbes.

(2) Microbial binding agents: adhesion of microbial cells to mineral particles

Microorganisms secrete exudates that can act as binding agents. Among the most important compounds comprising this exudate matrix are the biopolymers collectively referred to as extracellular polymeric substances (EPS) (Chenu, 1995; Redmile-Gordon *et al.*, 2014). EPS are composed mainly of polysaccharides, proteins, and DNA, and they are produced by bacteria (Limoli *et al.*, 2015), yeasts (Pavlova *et al.*, 2009), other fungi (Op De Beeck *et al.*, 2021), and protists (Jain *et al.*, 2005). Microbial EPS have a range of ecological functions (Costa *et al.*, 2018). The EPS matrix is involved in the adhesion of microorganisms to soil particles (Chenu, 1995) and the adsorption to mineral particles (Lin *et al.*, 2016). The EPS matrix gets adsorbed into the soil clay surfaces through cation bridges, van der Waals forces, hydrogen bondings, and anion adsorption mechanisms which help to form protective layers around soil and form soil aggregates (Tisdall and Oades, 1982). EPS can adsorb to mineral surfaces because of their surface reactivity and their high molecular weight (10^5 to > 10^6 Daltons). This allows for their adsorption to clay minerals and silicates with high affinity and low reversibility (Chenu and Cosentino, 2011). Bacterial EPS can contain carboxyl, phosphoryl, amino, and hydroxyl functional groups, and the electrostatic forces and chemical interactions play an important role in interacting with mineral surfaces (Lin *et al.*, 2016). The cross-linkage between the EPS matrix and soil particles supports small microaggregate formation and subsequent microaggregate formation (Totsche *et al.*, 2018). The inoculation of the EPS-producing *Pantoea agglomerans,* for instance, increased the fraction of water stable aggregates in the size range of > 200 µm and the mean weight diameter (MWD) in a wheat rhizosphere (Amellal *et al.*, 1998) and the mucilage produced by a basidiomycete fungus containing fucosyl residues impacted on soil aggregation (Caesar-Tonthat, 2002). Different parameters determine the production of EPS, among them the effect of C and N, pH of the culture medium, cultivation temperature, and growth phase (Saha *et al.*, 2020).

Arbuscular mycorrhizal fungi (AMF) are another group of soil organisms that can impact soil structure (Rillig and Mummey, 2006). In addition to their known role in plant nutrient acquisition, these symbiotic fungi have the ability to produce glomalin (Singh, 2012)**.** Glomalin is a fungal protein (or protein class) that is hypothesized to act as a "glue" with hydrophobic properties. Glomalin is operationally quantified from the soil as a glomalin-related protein (GRSP) (Rillig and Mummey, 2006). In soil, the concentration of glomalin can range from 2 to 15 mg g^{-1} and may contribute to 25% of total

SOM-C (SOC) (Rillig *et al.*, 2001; Singh *et al.*, 2016). Glomalin is considered ubiquitous, and it performs different ecological functions, such as improving soil aggregation, C storage, and nutrient cycling (Singh *et al.*, 2020). A model presented by (Santos *et al.*, 2020) proposed that glomalin had a direct and strong effect on organic matter accumulation within soil fractions > 2 mm and macroaggregates. Similarly, Gispert *et al*. (2013), found that glomalin and organic carbon are significantly more active in macroaggregate stability.

(3) Hyphal enmeshment

Diverse studies have described the role of fungal hyphae in entangling primary particles and thereby affecting soil aggregation (Tisdall, 1991; Ritz and Young, 2004; Chenu and Cosentino, 2011; Lehmann and Rillig, 2015). For instance, the inoculation of a saprobic fungus, *Penicillium chrysogenum,* increased the slope stability of sediments after events of avalanching (Meadows *et al.*, 1994), and this effect was supported by the network of fungal filaments observed by scanning electron microscopy (SEM). Other microscopic observations showed the physical entanglement of soil particles by fungal hyphae (Daynes *et al.*, 2012; Tisdall *et al.*, 2012; Vadakattu and Roper, 2019). This entanglement effect may depend on different factors, such as the length of hyphae (Chenu and Cosentino, 2011). For example, the increasing length of mycorrhizal hyphae contributed to the aggregate stability of sandy soil (Degens *et al.*, 1996). Another factor supporting enmeshment is the hyphal tensile strength (Lehmann and Rillig, 2015) which may remain even after the hyphae have died (Appels *et al.*, 2020). For example, the inoculation of the fungus *Stemphylium* sp. resulted in a higher tensile strength in soil (1.4 kPa) compared to 0.8 kPa in the uninoculated soil (Tisdall *et al.*, 2012). The hyphal enmeshment of aggregates can also be supported by fungal polysaccharide products (Tisdall *et al.*, 2012), and the effect of these products may be transient, depending on the nature of the extracellular adhesive materials (Daynes *et al.*, 2012).

(4) Hydrophobicity

Hydrophobicity has been identified as an important trait related to aggregate stability (Piccolo and Mbagwu, 1999; Lehmann and Rillig, 2015). Fungi play a major role in determining soil hydrophobicity and water repellency (Olorunfemi *et al.*, 2014). Fungi produce large quantities of insulating compounds in the outer wall of their hyphae, and a proportion of these materials are deposited into the soil matrix (Ritz and Young, 2004). Some of these compounds are hydrophobins, small proteins (about 100 amino acids) that play an important role in the growth and development of filamentous fungi (Wösten, 2001). Some hydrophobins have been shown to assemble into amphipathic films

at interfaces between water and hydrophobic solids, and may be involved in adherence properties (Wessels, 1996). Hydrophobins coat surfaces like fungal conidia and hyphae, as well as other surfaces in the soil such as aggregates (Rillig, 2005). By exuding hydrophobic compounds, fungi tend to increase hydrophobicity in soil organic matter, avoid the breakage of dry aggregates during rewetting, and prevent slaking in water (Coughlan *et al.*, 1973; Sullivan, 1990). The influence of fungal hydrophobicity was studied with 9 strains of ectomycorrhizal fungi. Each of these strains enhanced aggregate stability but also increased the soil water repellency in a species-specific way (Zheng *et al.*, 2014).

3.3 Aggregates as a habitat for microbes

Micro and macroaggregates within soil generate a three-dimensional system of interconnected voids, and pores ranging from nanopores to macropores and containing cracks formed between macroaggregates (Chun *et al.*, 2008). This 3D system harbors a vast range of physicochemical niches that provide the space for the growth, development, and trophic interactions of soil organisms and communities, which respond to the architecture of the soil (Young *et al.*, 2008; Vos *et al.*, 2013; Erktan, Or, *et al.*, 2020). The aggregates' interior can show different properties to the surrounding matrix. Aggregate surfaces, for instance, tend to be more dynamic environments than the micropore environment within aggregates as they are more prone to external stresses (e.g., wetting, and drying cycles) (Mummey and Stahl, 2004). Similarly, communities inhabiting aggregate surfaces may differ from those in the inner aggregate environment (Mummey and Stahl, 2004). The differences in these microhabitats can lead to sharp gradients of O_2 concentrations across a millimeter scale in particles (Sexstone *et al.*, 1985). Bacteria can be free or attached to surfaces (in individual colonies, micro-colonies, or as biofilms), located in water-filled pores, or surrounded by a film of water on the walls of air-filled pores. Fungi can also occupy these habitats but are not restricted to aqueous phases (Chenu and Stotzky, 2002). Furthermore, the aggregate structure also drives the hydrological connectivity in soil (Carminati *et al.*, 2007). During dry periods, there is an effect on the microbial community, with the isolation of the intra-aggregate communities from others and disconnecting the flow of solutes, metabolites, and microbial gene transfer (Wilpiszeski *et al.*, 2019). Microorganisms not only inhabit these aggregates but may be partly seen as architects in shaping their immediate environment (Totsche *et al.*, 2010).

4. Microbial-plant associations enhance ecosystem services under global change

Global changes, arranged from climate change to nutrient depositions, pollution, and human activities can intensify either abiotic and/or biotic stresses for both plants and organisms. Drought, for instance, impacts all living organisms, especially plants, which do not have locomotive structures that allow them to move elsewhere when resources become scarce (Silva *et al.*, 2013). Increasing drought is predicted to result in declines in microbial diversity and functions that are important for ecosystem sustainability (McHugh *et al.*, 2017). Microbes and their interaction with plants play a critical role in determining the response of terrestrial ecosystems to climate change (Bardgett, 2011). These interactions influence the soil functions from the microscale to the macroscale and landscape scale (Nannipieri *et al.*, 2020), affecting, for instance, plant nutrient acquisition, which leads to improved plant performance (Tchakounté *et al.*, 2020). Microbial communities can respond rapidly to environmental changes (Amend *et al.*, 2016). Their short generation times, large populations, and high mutation rates help support this large adaptive capacity (Bennett and Hughes, 2009). Microbes can help plant ecological responses by different mechanisms, for instance, PGPR and mycorrhizae form an intimate relationship with roots and rhizosphere and supply with nutrients acquisition under stressful environments (Nadeem *et al.*, 2014). Microbes can also interact with the plant's environment; bacterial biofilm decreased the uptake and accumulation of arsenic in plant tissues resulting in growth improvement (Mallick *et al.*, 2018). Microbes also can help plant evolutionary responses by increasing the strength of selection favoring plant traits that attract beneficial microorganisms, such as the modification of endogenous mechanisms controlling root development (Verbon and Liberman, 2016). Microbes also can improve plant responses to microbial signs (e.g. responses to phytohormones) to counteract chronic drought stress as was shown by Sayer *et al.* (2021). This collective adaptation potential may result in eco-evolutionary feedbacks between plants and their associated microorganisms (Angulo *et al.*, 2022).

5. Potential microbial strategies to improve soil functioning under global change and gaps of knowledge

As discussed in Section 2, soil microbes are known to provide a wide range of soil services that are essential to the sustainable functioning of natural and managed ecosystems. Given this high degree of functionality, it has often been suggested that microbial inoculants could be used to help boost specific

soil functions. Such approaches are considered to offer promising strategies for more sustainable agricultural systems, providing environmentally friendly ways of alleviating plant stress, improving nutrient availability, and alleviating abiotic and biotic stresses (e.g., drought) (Li *et al.*, 2022). To this end, diverse species of bacteria and fungi have been applied as biofertilizer amendments and/ or biocontrol agents, and commercial microbial inoculant products have been on the market for many years (Singh *et al.*, 2011; Stewart and Hill, 2014; Lopes *et al.*, 2021). These studies have determined that microbial inoculants' performance depends on the microbial species and crop type. Species of *Pseudomonas* and *Enterobacter,* for instance, had a greater effect on alleviating stresses than improving nutrients, and species of *Bacillus* had a similar impact across nutrients and stress categories (Li *et al.*, 2022).

Although soil amendment strategies have been examined in detail for improving soil functions related to plant disease suppression, nutrient uptake, and alleviation of abiotic stresses, relatively few studies have sought to determine the potential of microbial amendments for improving soil structure. Given the importance of soil microbes in aggregation dynamics, the exploration of such microbial amendment strategies is a necessary step in developing new methods for improving soil structure and ecosystem function in a changing world with increasing stresses like drought. Cyanobacteria application is an interesting example, where such amendments were shown to improve the status of soil nutrients and hydrology in arid and semiarid zones (Nisha *et al.*, 2007). The inoculation of EPS-producing cyanobacteria appears to be a viable strategy to improve soil aggregation and water holding capacity in different types of soil (de Caire *et al.*, 1997; Chamizo *et al.*, 2018). The application of mycorrhizal fungal amendments was also shown to lead to improvement in soil structure (Rillig and Mummey, 2006; Ortas, 2012).

Thus, although the use of microbial amendments is quite spread, the study of the microbial modification of the surrounding environment and related improvement of soil structure and subsequently plant growth under changing precipitation patterns and drought stress is still poorly studied. We propose to study the impact of bacterial and fungal inoculation in soils under drought to improve soil aggregate stability and ameliorate the stress of desiccation in soil functioning. Furthermore, we propose to study the interaction of plant-microbe in an eco-evolutive approach to counteract global change effects.

6. Aims and general research approach

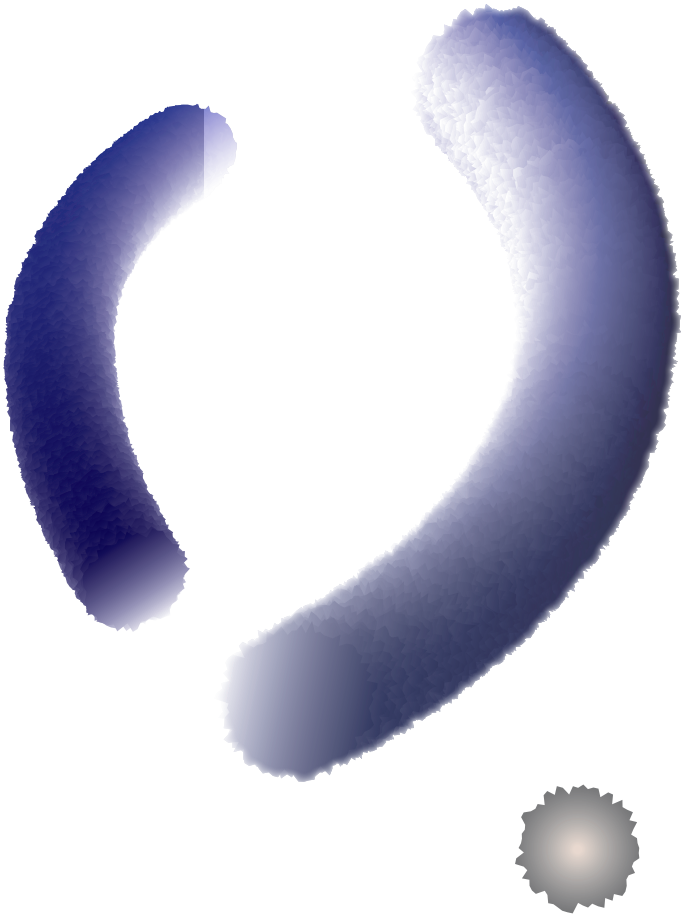
The main goal of this thesis was to examine the potential of microbial amendments as a strategy for improving soil structure and function under drought.

We defined the following specific goals: (i) determine if a trait-based approach is useful for the screening and development of bacterial inoculation strategies to improve soil aggregate stability under different moisture levels, (ii) assess the ability of fungal inoculation to change soil hydrological properties and improve soil aggregation under different moisture levels (iii) assess the effect of bacterial and fungal strain inoculations on plant growth, soil water content, and soil aggregate stability under drought and well-watered conditions, and finally (iv) explain how microbes mediate plant ecological and evolutionary responses and how environmental change drivers affect their eco-evolutionary dynamics under global change.

To examine these goals, we isolated and selected a set of bacterial and fungal strains from a drought experimental field and used a series of experiments to determine their abilities to improve soil structure. We hypothesized that bacterial and fungal strains isolated from experimental fields used for drought research will harbor specific traits and will make them efficient amendments to support physical traits of soil structure and function under conditions of limited moisture. From the bacterial collection, we selected and characterized traits with the potential to improve aggregate stability and water retention under drought and tested if the trait-based approach could be used as an effective predictor of soil aggregation. With fungi, the trait-based approach was modified, and fungal strains were selected based on a taxonomic approach and their abundance in the experimental drought fields of isolation. Then, fungal strains were tested on their ability to improve aggregate stability and soil hydrological properties. Further, a selection of bacterial and fungal strains was selected and inoculated in soils supporting the growth of tomato plants, and plant growth aboveground and soil aggregate properties and water content belowground were assessed. Finally, we studied how microbes affect plant ecological and evolutive responses facing global change and how these responses can result in eco-evolutionary feedbacks between plants and their associate microbiome.

7. Thesis outline

First, we isolated a diverse set of bacterial and fungal strains from an experimental plot with drought and non-drought (ambient control) treatments. The bacterial and fungal strains were morphologically characterized, and their taxonomy was examined using molecular methods. In **Chapter 2,** we characterized bacterial strains with traits selected for their potential to positively influence aggregate stability under moisture stress. With the help of this trait-based approach, we selected bacteria with low, medium, and high collective trait levels. Then, we inoculated these strains in a microcosm experiment to examine the impact on soil structure under two moisture levels. We found that some bacterial strains improved aggregate stability mainly under high moisture conditions, and the trait-based approach did not have a strong predictive effect as we hypothesized. In **Chapter 3,** we adjusted the trait-based approach strategy and specifically selected fungal strains based on their taxonomic characteristics and abundance in the soil of isolation. The selected fungal strains were tested under two moisture levels for their impact on aggregate stability and hydrological soil properties in a first microcosm experiment and the water potential in a second microcosm experiment. We also quantified fungal biomass and observed the ability of fungal hyphae to entangle soil particles by microscopy. We determined that fungal inoculation had a high impact on aggregation under both levels of moisture and sorptivity and fungal biomass were the most important fungal traits associated with aggregation under drought. Later, we assessed the effect of inoculation from a subgroup of bacteria and fungi on plants' growth traits, aggregates stability, and water content under well-watered and drought conditions in **Chapter 4.** Here we found that under drought, microbial inoculation had a positive effect on soil properties. Under well-watered conditions, a positive inoculation effect was also observed for plant growth. In **Chapter 5**, we studied how microbial communities can reduce or increase the selection of plants' stress tolerance traits and trigger eco-evolutionary feedbacks under global change. In **Chapter 6,** we provide a summary discussion in which we collectively examine the results of the chapters of the thesis and offer perspectives for future research directions.



CHAPTER 2

IMPACT OF BACTERIA ON SOIL AGGREGATES UNDER DIFFERENT MOISTURE LEVELS: A TRAIT-BASED APPROACH

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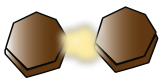


Summary

Soil physical degradation threatens soils on a global scale. It alters the functioning of terrestrial ecosystem functioning, especially under conditions of drought. The formation and stability of soil aggregates are useful indicators of the soil's physical structure and soil functioning. Bacteria contribute to soil aggregate dynamics through the production of extracellular polymeric substances (EPS) and biofilms development. In this study, we developed a bacterial trait-based approach and investigated the ability of bacterial strains to improve soil aggregation under two levels of soil moisture. We isolated 116 strains from drought-treated experimental fields and screened them for their ability to (i) resist moisture and salinity stress, (ii) produce biofilms and EPS, and (iii) display a rapid growth rate. We selected 24 strains with contrasting sets of traits to represent a range of predicted potential to influence soil aggregation. These bacterial strains were individually inoculated into sterilized soil, which were incubated under low and high soil moisture conditions (-0.96 MPa and -0.03 MPa, respectively) for 8 weeks. At the end of the incubation, soil aggregate fraction > 0.4 mm and soil water aggregate stability were measured. Soil EPS and soil bacterial density were quantified. We found that inoculated bacteria only affected soil aggregation at a higher level of soil moisture. Although some bacterial traits showed some relation to changes in soil aggregation, we did not find a clear relationship between predicted impacts, based upon measured traits, and realized levels of soil aggregation. Thus, while bacterial soil amendments hold promise for improving soil structure, more expanded, systematic analyses will be required to identify the conditions and traits that lead to the most reliable results.

1. Introduction

Soil physical structure is an important factor determining soil functionality (Bronick and Lal, 2005). The soil structure defines the flow of gases and solutes and provides the range of micro-habitats that support the unparalleled levels of biodiversity inhabiting soils (Vos *et al.*, 2013). Degradation in the soil's physical structure is recognized as an acute threat to essential ecosystem services such as food production and carbon storage (Li *et al.*, 2014; Rickson *et al.*, 2015). Intensive agriculture practices, overgrazing, soil compaction, and destruction of natural ecosystems are some of the threats that have a serious negative impact on soil's physical structure and its hydrological properties (Lal, 1993; Buytaert *et al.*, 2002; Jefferies and Rockwell, 2002; Montgomery, 2007; Dlamini *et al.*, 2014; Daliakopoulos *et al.*, 2016; Kraamwinkel *et al.*, 2021). In the last decades, it has become clear that the increased temperatures and changing precipitation patterns associated with climate change also have an increasingly negative impact on global soil physical structure (Van Lanen *et al.*, 2013). The reduction in atmospheric precipitation is expected to result in a decrease in water infiltration, water storage in the soils, and plant water supply which also impact soil forming processes, including the turnover of organic matter and structure formation (Karmakar *et al.*, 2016).

Soil aggregates are indicators of the soil's physical structure, and of soil quality (Arshad and Coen, 1992; Six *et al.*, 2000). The properties of soil aggregates mainly indicate the dynamic of the soil structure (Papadopoulos, 2011). Soil aggregates can be in the form of microaggregates (< 250 μm) that associate together to form macroaggregates (> 250 μm) (Edwards and Bremner, 1967; Tisdall and Oades, 1982). Soil aggregates are formed by the combination of primary mineral particles with inorganic and organic substances (Amézketa, 1999; Bronick and Lal, 2005), which are bound together during pedogenesis by various physical, chemical, and biological processes (Totsche *et al.*, 2018).

Bacteria play an important role in soil aggregation at the microscale (Saha *et al.*, 2020). They can, for instance, produce extracellular polymeric substances (EPS), which interact with soil minerals leading to aggregate formation and stabilization (Chenu and Stotzky, 2002). EPS are composed mainly of polysaccharides, structural proteins, and extracellular DNA (Di Martino, 2018). Bacterial EPS have a slimy texture and ionic properties, allowing it to act as a glue that can attach to clay and ions and hold soil particles together (Chenu, 1995). EPS and organic matter in interaction with cementing agents, such as oxides, hydroxides, and oxyhydroxides of iron (Fe), manganese (Mn), and aluminum (Al) (Oades and Waters, 1991) result in microaggregates formation.

Subsequently, microaggregates can be bound together into macroaggregates by temporary binding agents, such as plant roots and fungal hyphae, and transient agents, such as microbial EPS and plant-derived polysaccharides exudates (Tisdall and Oades, 1982; Rillig, 2004; Six *et al.*, 2004; Totsche *et al.*, 2018). For example, increasing the production of EPS from *Pseudomonas putida* improved the soil aggregate stability under drought (Sandhya and Ali, 2015).

EPS have other important functions in soils, being involved in microbe-plant associations, nutrient retention, and helping bacteria resist drought stress (Chenu, 1995; Wolfaardt *et al.*, 1999; Chenu and Cosentino, 2011; Costa *et al.*, 2018). Additionally, EPS improve the capacity for interactions and symbioses with other cells. When microbial communities are attached to surfaces and held together by a matrix of EPS, these microbial communities can be organized into biofilms (Chew and Yang, 2016). A biofilm is defined as "an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix" (Saha *et al.*, 2020). Such biofilm formation is also known to contribute to the formation of soil aggregates (Cai *et al.*, 2019). Since biofilms tend to have hydrophobic properties (Arnaouteli *et al.*, 2016), they can also contribute to water retention in the soil matrix and reduce desiccation stress in soil environments (Lennon and Lehmkuhl, 2016) and may improve soil aggregate stability (Vogelmann *et al.*, 2013). Given the importance of EPS and biofilm formation in processes of soil structure formation, it would be expected that bacterial populations with these properties would significantly contribute to this process.

Bacterial communities are affected by drought (de Vries *et al.*, 2018). Bacterial strains that survive soil desiccation may adopt a variety of protective mechanisms to mitigate the damage caused by the water loss. The production of EPS, and the formation of biofilms, as mentioned before are strategies used by bacteria to survive desiccation (Laskowska and Kuczyńska-Wiśnik, 2020). EPS protect bacteria against desiccation by structural modifications, trapping a reservoir of moisture and nutrients (Roberson *et al.*, 1993). Additionally, bacteria can counteract conditions of low moisture by the accumulation of compatible solutes like trehalose or stress proteins, which increase the osmotic potential inside the cell and maintain cell turgor (Yan *et al.*, 2015; Laskowska and Kuczyńska-Wiśnik, 2020). The ability to accumulate osmolytes is also often linked to bacterial resistance to high salt concentrations (Yan *et al.*, 2015). Furthermore, bacteria are also able to form drought-resistant structures such as spores (Setlow, 2016). Conditions of drought would be expected to select microbial populations well adapted to overcoming desiccation stress,

making bacterial populations with strong drought tolerance attractive targets for improving soil properties under conditions of drought.

The objective of this study was to assess the extent to which bacterial traits putatively related to soil aggregation under desiccation stress, such as EPS and biofilm production, can be used to select bacterial strains that can improve soil aggregation under two different moisture levels. First, we isolated a collection of 116 soil bacterial strains from soil under experimental drought treatments. These strains were evaluated for a range of bacterial traits, as determined by a range of *in vitro* assays. The strains were ranked based on their combined score for traits postulated to be linked with soil aggregation, namely growth properties, and desiccation resistance (drought and salinity). Based upon this ranking, we selected 24 bacterial strains that represented the full range of trait scores for inoculation into a sterilized soil substrate. After 8 weeks of incubation, under either high or low soil moisture, we examined changes in soil aggregate fraction > 0.4 mm collected by dry sieving, soil aggregate stability examined by wet sieving, and the content of EPS in the soil, as well as total bacterial density. We tested whether changes in soil aggregation could be explained by our bacterial trait-based approach. We hypothesized that (i) bacteria isolated from drought-treated soils show a higher predominance of traits related to higher survival and growth under drought conditions as tested in the laboratory, (ii) bacterial inoculation impacts soil structure properties, and bacteria with traits indicative of soil aggregation, growth properties, and adaptation to desiccation tested in the laboratory would have the most positive impacts on soil aggregation, and (iii) the effects of inoculated bacteria depend on the level of soil moisture during the incubation.

2. Materials and methods

2.1 Soil sampling and isolation of bacterial strains

Soil samples were collected from an experimental grassland (http://www.drought-net.org) located at Fort Rhijnauwen in Utrecht, Netherlands (52°04’24.8” N 5°10’32.4” E). The experiment was set up on a natural grassland (*Arrathenatum elatius* association) with rainout shelters to create two different drought treatments and a non-drought control treatment. Drought treatments consist of a pulse drought (D90) with approximately 90% of rainfall reduction in summer and re-application of rainwater in winter and a press drought (D50) with a continuous 50% reduction of rainfall, respectively. The soil texture was loamy sand and categorized as a regosol (World Reference

Base for Soil Resources, WRB) (Food and Agriculture Organization of the United Nations, 2015). Samples were taken to a soil depth of 100 mm using a metal corer (diameter 25 mm), which was flame-sterilized between sampling events. Samples were collected in triplicate for each drought condition, packed into plastic bags, and transported on ice to the soil laboratory of the Institute of Environmental Biology at Utrecht University to be processed within 5 h after collection. The top layer containing coarse organic matter such as roots and stones (approx. 30 mm) was discarded (Janssen *et al.*, 2002). Large roots and stones were removed from the remainder, and the resulting soils were sieved through a mesh of 2 mm aperture and stored at 4 °C until bacterial isolation.

For each drought condition, one g of 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). Soil suspensions were diluted at 10^{-2}, 10^{-3}, and 10^{-4}, and 1 mL of each dilution was inoculated on trypticase soy agar (TSA) at 100% and 10% plates using a sterile glass spreader and incubated at 25 ºC for 48 h. Different bacterial morphotypes were selected, and a total of 116 bacterial strains (35 isolated from D90, 48 from D50, and 33 from non-drought) were characterized and grown overnight in media Luria Bertani (LB, agitation 100 rpm, 25 ºC) and stored in glycerol at 25% at -80 ºC until use.

2.2 Molecular identification

For each of the 116 bacterial strains, we isolated the total DNA from monoclonal cultures that were grown in trypticase soy broth (TSB) (24 h at 25 ºC) according to the manufacturer's protocol for the Dneasy PowerSoil Pro kit (Qiagen, Germany). The DNA extracted was used for polymerase chain reaction (PCR) amplification of the 16S rRNA gene using universal primers 27F (5 µM) (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5 µM) (5'-GGTTACCTTGTTACGACTT-3') (Frank *et al.*, 2008) with the following thermocycling conditions, 95 °C for 3 min (1 cycle); 95 °C for 20 s, 57 °C for 30 s, and 72 °C for 1 min (33 cycles); and 72 °C for 5 min (1 cycle). PCR products were cleaned up using AMPureXP beads (Beckman Coulter, USA) and sequenced using Sanger sequencing technology, using the ABI prism sequencer 3730 (Applied Biosystems, USA), following the manufacturer's protocol. To identify the strains, the sequences were classified to the level of the genus using the package "decipher" (Wright, 2020) in the software R, version 4.1.2.

2.3 Determination of bacterial traits

The following bacterial traits were examined: drought and salinity tolerance, EPS production, and biofilm formation. The growth properties, the area under the curve (AUC), which is the cumulative biomass produced over the incubation time, and the carrying capacity (K), which is the maximum size of the population that the environment can sustain, were also measured (Fig. 1). Bacterial traits were measured in bacterial colonies grown individually. To set bacterial individual cultures, a bacterial pre-inoculum was obtained by inoculating each strain in media trypticase soy broth (TSB) at 25°C and incubating overnight (120 rpm, Gallenkamp shaker incubator). The bacterial concentration was adjusted ($OD_{600\,nm}$=0.2) and used to inoculate the respective media to examine the range of bacterial traits.

2.3.1 Drought tolerance

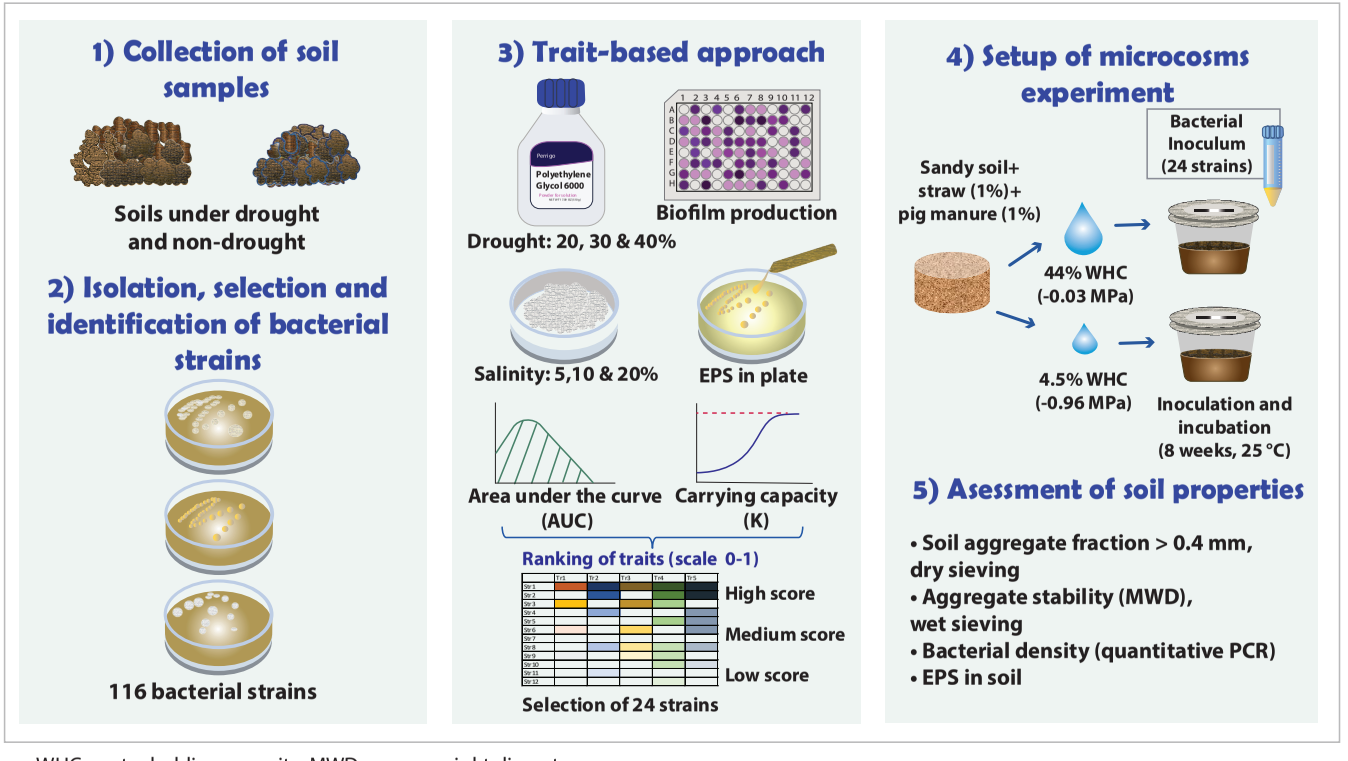
Drought tolerance was evaluated in TSB media with 20%, 30%, and 40% concentrations of polyethylene glycol (PEG6000) (Michel and Kaufmann, 1973), resulting in drought stresses equivalent to water potentials (ψ) of -1.6, -2.4, and -3.4 MPa, respectively. The concentrations were adjusted lower than the permanent wilting point of mesophilic higher plants, which is approximately ψ -1.5 MPa (Palacios *et al.*, 2014). The different concentrations of PEG were aliquoted into 96 well microplates and inoculated with 1% v/v of overnight fresh bacterial culture (Sandhya *et al.*, 2009) as obtained in step 2.3. The microplates were incubated for 24 h at 28 ºC, and growth was estimated at the end of the incubation by measuring the OD_{600} using a spectrophotometer (Spectrostar, BMG Labtech, USA). The osmotic potential was measured using a vapor pressure osmometer (Wescor vapro 5520, USA) and converted to (ψ) MPa using the formula (Money, 1989):

$$\psi_s = -RTC$$

Where: ψ_s = osmotic potential (MPa)
R = the gas constant (0.0083143 MPa L mol^{-1} K^{-1})
T = absolute temperature (°C + 273)
C = molar solute concentration (mol Kg^{-1})

2.3.2 Salinity tolerance

To examine salinity tolerance, each of the 116 bacterial strains was grown in microplates established using TSB supplemented with different levels of NaCl mimicking increased salinity stresses, namely 5%, 10%, and 20% (equivalent to 17.14, 28.1, and 41.8 mS/ cm of electrical conductivity (EC), Tresner and Hayes,



WHC=water holding capacity, MWD=mean weight diameter

Figure 1. Schematic flow of the experimental setup, including the collection of soil, isolation of bacterial strains, characterization of bacterial traits, design of the microcosm inoculation experiment, and assessment of soil properties.

1971; Sharma *et al.*, 2021). For each salinity treatment, we inoculated 1% v/v of fresh bacterial culture as obtained in step 2.3. The cultures were incubated (24 h and 28ºC), and growth at the end of the incubation was estimated using a spectrophotometer (OD_{600}) (Spectrostar, BMG Labtech, USA).

2.3.3 Bacterial growth properties

For each of the 116 bacterial strains, microplates with TSA were inoculated with 1% v/v of fresh overnight pre-inoculum as obtained in section 2.3. Bacterial growth parameters were estimated every 30 minutes over the course of 68.5 h incubation using a spectrophotometer (OD_{600}) (Spectrostar, BMG Labtech, USA). The area under the curve (AUC) was calculated using the statistic package "growthcurver" (Sprouffske, 2020) and the statistic program R version 4.1.2. The carrying capacity (K) was calculated according to the following formula (Sprouffske and Wagner, 2016):

$$N_t = \frac{K}{1+\left(\frac{K-No}{No}\right)e^{-rt}}$$

Where N_t is the number of cells (or the absorbance reading at time t), N_0 is the initial cell account (or absorbance reading), K is the carrying capacity and r is the growth rate.

2.3.4 Biofilm production

For each of the 116 bacterial strains, biofilm production was measured by spectrophotometry, using optical density as an indicator of biofilm thickness. First, 1% v/v of fresh bacterial pre-inoculum (section 2.3) was inoculated in microplates with 3% TSB for 24 h at 28°C. Then, we stained the biofilms produced using the protocol described by O'Toole (2011) with modifications. The supernatant was removed from the microplates, and microplates were washed once with sterile PBS, and 160 µL of 1% crystal violet solution was added and incubated for 15 min. Then, bacterial cultures were rinsed 3-4 times by submerging them in a tub of water, shaken, and blotted on a stack of paper towels to let the bacterial cultures dry. Biofilm formation was quantified on individual dry bacterial cultures by adding 250 µL of ethanol (70%) for 20 min. A total of 125 µL of the solution was then transferred to a new microplate, and the biofilm was quantified by absorbance in a plate reader (OD_{500}) (Spectrostar, BMG Labtech, USA) using ethanol at 70% as the control. According to their optical densities, bacterial strains were divided into the following categories: (i) OD $\leq OD_{control}$ = no biofilm producer,

(ii) $OD_{control}$ < OD ≤ (2 x $OD_{control}$) = weak biofilm producer, and (iii) (2 x $OD_{control}$) < OD = strong biofilm producer (Stepanović *et al.*, 2007).

2.3.5 Bacterial EPS production

For each of the 116 bacterial strains, we first screened for EPS exopolysaccharide-producing bacteria, according to the protocol of Mu'minah *et al*. (2015). Plates with the solid media ATCC no. 14 were inoculated with the individual bacterial strain and incubated for 7 days at 28 ºC. For each bacterial strain, the production of exopolysaccharides was assessed by their bacterial ropiness, which is the property of being cohesive and sticky, using a toothpick. Exopolysaccharide-producing bacteria showed a slime thread when the toothpick was inserted and removed from the colony. The production of exopolysaccharides was categorized into (i) high, (ii) intermediate, and (ii) non-producer. The bacterial strains with high and intermediate exopolysaccharide production (8 strains and 22 strains respectively) were subsequently tested for a quantitative assessment of exopolysaccharide production in liquid media.

The assessment of the production of exopolysaccharides in liquid media was adapted from the protocol of Pawar *et al*. (2013). For each selected bacterial strain, 50 mL of medium ATCC was adjusted to a pH of 6.5, inoculated with 1% v/v of the individual strain pre-inoculum (section 2.3), and incubated on a rotary shaker (28 ºC, 100 rpm, and 108 h). At the end of the incubation, cells were harvested by centrifugation at 4.000 rpm (Megafuge 40. Thermo Scientific, USA) for 30 min. After centrifugation, samples were precipitated in ice-cold isopropanol and collected by centrifugation (20 min at 10.000 rpm) (SL 16R Thermo Scientific Centrifuge, USA). The resulting pellets were dried at 80 ºC for 3 days and weighed. All bacterial strains were tested in triplicate.

2.3.6 Selection of strains based upon bacterial traits

We ranked bacterial strains based upon the trait values expected to impact soil aggregation under drought stress conditions. For each trait, values across all strains were normalized such that the highest value of the given trait received a value of 1, and other normalized trait values were proportional to this highest value. Categorial traits, such as biofilm formation and EPS production showed 3 categories: (i) high, (ii) intermediate, and (ii) no producer, we converted to numerical values: 1, 0.5, and 0, respectively. The subsequent values for all traits were then combined into a general cumulative metric representing the putative potential impact on soil aggregation. Based upon this cumulative metric for all 116 bacterial isolates, we selected 24 strains for soil inoculation experiments (see Supplementary Fig. S2.1) to test

their effect on soil aggregation. These strains were chosen to represent a wide range of traits to compare the impacts of strains expected to have strong, intermediate, or weak impacts of soil aggregation.

2.4 Soil aggregation assay

2.4.1 Experimental design

To assess the effect of the 24 selected bacterial strains on soil aggregation, we established a fully randomized inoculation experiment in which each bacterial strain was inoculated in five replicate microcosms at two levels of moisture (44% and 5.6% of the total field capacity (45%)) Furthermore, a set of non-inoculated microcosms was established as a control (soil without bacteria), resulting in a total of 250 experimental units. A scheme of the methodology is depicted in Fig. 1.

We selected sandy soil collected (A-horizon) from a pig farm in the Netherlands for the soil aggregation assay. The sandy soil selected for the experiment was chosen due to its relatively poor soil structure, thereby allowing us to potentially examine a broad range of changes to soil structure after inoculation. Sandy soils possess a low level of initial soil aggregation (Ciric *et al.*, 2012) and are particularly vulnerable to the impacts of drought (Mulcahy *et al.*, 2013). Although the soil used for the soil aggregation assay was different from the one used for bacterial strain isolation, both had a sandy texture. We prepared the soil mixture by amending the sandy soil (i) with 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 the soil aggregation assay 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%.

For each microcosm, eighty g of the soil mixture was aliquoted into plastic flasks (60 mm h x 80 mm d, Microbox filter XL, Eco2 NV) and autoclaved for three consecutive days (121 ºC, 20 min) with an interval of 24 h between each autoclaving step. To evaporate the remaining water, the microcosm flasks were then air-dried overnight in a flow cabinet overnight. Thereafter, soil samples were adjusted to 44% and 5.6% of the total water holding capacity (45%), corresponding to high (ψ -0.03 MPa) and low (ψ -0.96 MPa) moisture levels, respectively. For each microcosm, cracks were made with a sterilized spatula to increase connectivity and 1 mL of bacterial inoculum (OD_{600} 0.2) was added

to the soil. The microcosms flasks were weighed and then incubated for 8 weeks at 25 °C. Moisture content was assessed by weighing the flasks every two weeks, and water loss was compensated under sterile conditions. Soil moisture levels were adjusted in the 2nd, 4th, and 6th weeks of the incubation period. After 8 weeks of incubation, soil samples were dried at 30 ºC for 48 h and stored at 4 °C until further use.

2.4.2 Measurement of soil aggregate stability

The effect of bacterial inoculation on soil aggregates was measured using sieving methods. First, intact samples of approx. 40 g of dried soil were collected and sieved using a sieve of 0.4 mm which was placed into an automatic sieving machine (Retsch, Germany) for 30 s. The collected soil aggregate fraction > 0.4 mm was weighted, and the mass percentage was calculated by dividing the mass of the fraction by the total mass of the sieved soil. The soil aggregate fraction > 0.4 mm was selected with two purposes, i) to get a representative sample from our sandy soil for the later assessment of aggregate stability through wet sieving (loose texture makes it more complicated to get a homogeneous soil sample), ii) to observe the effect of bacterial inoculation without the interference of the sand size particles, which were determined to be 62% for the fraction 0.5-0.25 mm (Supplementary Fig. S2.2).

The stability of soil aggregates was determined under the principle of breakdown by compression of trapped air (slaking) using the wet sieving technique (Kemper and Rosenau, 1986). Slaking occurs when dry aggregates are immersed in water or rapidly wetted (Le Bissonnais, 2016). Briefly, 4 g of soil aggregate fraction > 0.4 mm collected in the previous step, was wet sieved through a series of meshes: 2, 1, 0.75, and 0.25 mm, resulting in 5 diameter classes for macroaggregates: > 2 mm; 2-1 mm; 1-0.75 mm, 0.75-0.25 mm and one class for microaggregates < 0.25 mm. More precisely, the soil fraction > 0.4 mm was placed on the 2 mm sieve and soaked in a thin layer of water for 10 min. Then, the 2 mm sieve was placed into a 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 during 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 0.75 mm and the agitation process was again repeated.

After each agitation step, the remaining stable soil aggregates > 1 mm, > 0.75 mm, > 0.25, and < 0.25 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 sample, the mass percentage of each soil water stable aggregate fraction (WSA) was calculated by dividing the mass of the fraction by the sum of the masses of all the soil fractions. The soil aggregate stability was determined by the mean weight diameter (MWD) (Kemper Rosenau, 1986) using the following formula:

$$MWD = \sum_{i=}^{n} \bar{X}_i M_i$$

where M_i is the dry mass of the soil aggregates for each size class and $\bar{X}_i$ is the mean diameter of the size class (mm).

2.5 Extraction of EPS from soil and quantification

EPS-polysaccharides were extracted from soil using the modified protocol described by Martens and Frankenberger (1990). For each soil microcosm, 0.5 g of soil (soil aggregate fraction > 0.4 collected in step 2.4.2) was treated with 5 mL of 0.125 M $H_2S\ O_4$ in a shaking water bath (Julabo SW22, USA) at 80 ºC and 100 rpm overnight. Then, the samples were treated with ethylenediaminetetraacetic acid (EDTA) 0.1 M (final concentration of 10 mM) to prevent co-precipitation of saccharides. The resulting samples were titrated to pH 3.5-4.0 with 5.0 M KOH and centrifuged at 4.500 rpm for 20 min (Megafuge 40. Thermo Scientific, USA). The supernatant was removed and filtered through a 0.22 µm pore microplate. Carbohydrates in the supernatant were quantified by the 2.20 Bicinchoninate-assay (BCA-assay), according to the protocol of Waffenschmidt and Jaenicke (1987). To do so, 10 mL of the supernatant was added to 1 mL of the BCA-assay working reagent and incubated (100 ºC, 15 min). Later, the samples were cooled down to room temperature for 20 min and the optical density was measured (OD_{560}) in the dark using a spectrophotometer (Spectrostar, BMG Labtech, USA). The quantification of soil exopolysaccharides was realized by comparison to a standard curve, which was calculated using: 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL of D-glucose.

2.6 Soil bacterial population density by quantitative PCR

For each microcosm, we isolated soil DNA from 0.25 g of dry soil (soil aggregate fraction > 0.4 collected in step 2.4.2) according to the manufacturer's protocol

for the Dneasy PowerSoil HTP 96 Kit (384) (QIAGEN), with resulting extracts stored at -20 ºC until use. The density of inoculated bacterial population was determined by quantitative PCR (qPCR) using specific 16S rRNA gene primers, specifically designed for these strains (Supplementary Table S2.2) based upon the 16S rRNA gene sequences used for strain identifications (see section 2.2). PCR mixtures contained 1.5 µL MQ-water, 2.5 µL of DNA template, 5 µL of 2x taq Universal SYBR Supermix, Biorad USA, and 0.5 µL of 5 µM of each forward and reverse primer. Target sequences were amplified and quantified in a 384-well thermal cycler (ViiA7, Applied Biosystems, USA) with a PCR program of 5 min at 95 °C for denaturation, followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s for annealing and elongation. The program ended with a melting curve cycle, and data were analyzed using the Quantstudio software.

All gene copy numbers were calculated from the standard curves of 16S rRNA gene copies and the specific genes by using the 1 Ct (cycle threshold) method. All qPCR amplifications were conducted in triplicate. Differences related to copy number and genome size were adjusted according to Větrovský and Baldrian, (2013). The density of target strains was quantified from the inoculated strains and non-inoculated control treatments, the difference was calculated, and log transformed. This difference was made to eliminate traces of DNA of close species present in the control soil.

2.7 Statistical analyses

We tested the effect of inoculation on bacterial traits: production of EPS in liquid media, and soil properties: soil aggregate fraction > 0.4 mm, water stable aggregates (WSA) for fractions (> 2 mm, 2-1, 0.75-0.25, < 0.25 mm), soil aggregate stability (MWD), production of EPS in soil, and soil bacterial density. The effects of the 24 experimental bacterial strains on the production of EPS in liquid media were analyzed using an analysis of variance (ANOVA). The effect of bacterial inoculation on soil aggregate fraction > 0.4 mm, WSA fractions, MWD, EPS in soil, and soil bacterial density were tested using an ANOVA "type 3" for the effect of interactions between moisture and bacterial strain. 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-transformed if necessary to meet the assumptions. When log transformation was insufficient to reduce heteroscedasticity, we used a generalized least squares (GLS) model and allowed the variance to be different per stratum and level of moisture using varIdent (Pinheiro and Bates, 2000), packages "nlme" (Pinheiro *et al.*, 2021) and "car" (Fox and Weisberg, 2019). The pairwise comparison between the means of treatments

was analyzed by the test "Tukey" through the package "emmeans" (Lenth, 2022) and a "Bonferroni" adjustment. The graphics were plotted according to the fitted models. Pairwise comparations for log data were back transformed using the function "response."

To investigate the relationships between soil aggregation and the bacterial continuous traits, we ran a matrix of Spearman's correlations (package "Hmisc") (Harrel, 2022), and for the categorical traits, we obtained the multiple R2 and the graphic representation from a linear model analysis. To determine which bacterial traits were most important for determining soil aggregation, we applied a permutation-based conditional random forest (Hapfelmeier and Ulm, 2013), using the packages "party" (Strobl *et al.*, 2007) and "permimp" (Debeer *et al.*, 2021), and calculated the adjusted R-squared (R^2). The phylogeny of the bacterial strains was added as a numeric predictor to the random forest analysis. To do so, the phylogenetic pairwise distances were calculated and incorporated into a principal component analysis (PCoA) via the cmdscale () function in the "stats" package. The cumulative sum of proportions of variance, explained by the PCoA axes on the eigenvalues was calculated and extracted for axes one and two which explained 46.9% and 44.4% of the phylogenetic variance, respectively. Then, the eigenvalues were included as 2 individual variables in the random forest analysis. All analyses were conducted using the software R (version 4.1.2) and graphics were generated using the package "ggplot2" (Wickham, 2016). The graphic of stable aggregates fractions for the wet sieving (Fig. 4) was built using the package "RColorBrewer" (Neuwirth, 2022).

We built a heatmap to connect the bacterial traits and phylogeny, Supplementary Fig. S2.1 (116 bacterial strains) and Fig. 2 (24 strains). For the construction of the heatmap for the trait-based approach, we first calculated pairwise distances from the phylogeny using the software MEGA7 (Kumar *et al.*, 2016), and these data were combined with data on the bacterial traits (growth under drought, salinity, production of biofilm, EPS in plate, K and AUC) using the TB tool (Chen *et al.*, 2020).

3. Results

3.1 Bacterial strains and characterization of bacterial traits

We isolated a total of 116 bacterial strains from the experimental grassland at Fort Rhijnauwen for subsequent trait assessments. The strains belonged to the phyla Firmicutes (50.9%), Actinobacteria (18.1%), Proteobacteria (26.7%), Bacteroidota (0.9%), and non-identified species (3.4%), see Supplementary

Fig. S2. 1. Strains belonging to phyla Firmicute and Proteobacteria were isolated from different field treatments (drought and non-drought) meanwhile for the phylum Actinobacteria, 95% were isolated from one of the drought treatments (D50 or D90). The 116 strains showed a diverse response to the tested traits. For instance, strains 15 and 92 (*Pseudomonas* spp.), were able to grow at the different levels of simulated drought (PEG 20%, 30%, and 40%). *Stenotrophomonas* sp. strain 35 had the highest growth at 10% salinity (28,1 dS/ m), although it did not show growth at 5% salinity. No strains grew at 20% salinity. Strains of the phylum Proteobacteria generally exhibited the highest growth capacity (K) and area under the curve for growth (AUC). Three strains showed strong biofilm formation, and all three belonged to the phylum Firmicutes. Eight strains were found to be strong EPS plate producers, and 22 strains showed intermediate levels of EPS production. None of the strains with strong biofilm production coincided with the ones with strong production of exopolysaccharides in the plate assay.

To confirm the production of EPS, the strong and intermediate producers of EPS using plate assays were tested in liquid media in two different experiments (Supplementary Table 2.1). The strong EPS producers on plates showed EPS productions ranging from 0.25 to 3.75 mg EPS/ml in liquid media. Within this set of producers, 7 out of 8 strains were significantly higher than the control, and strains 81 and 7 (*Bacillus* spp.) were the isolates with the highest and most significant production of EPS. The strains with intermediate production on plates yielded values from 0.46 mg to 3.44 mg of EPS/ml, which were not significantly different from their corresponding control media without inoculation. It should be noted that there was a difference between the basal level of EPS for non-inoculated media examined in both experiments, despite having used the same media protocol.

From the 116 strains analyzed and categorized, 24 strains were selected for further experiments based upon their cumulative trait scores. Our selection sought to cover a range of trait rankings so that we could compare the results of bacterial inoculations with strains predicted to have good, intermediate, and poor potential to improve soil aggregation status (see Supplementary Fig. S2.1). The 24 strains were distributed across three phyla: Actinobacteria (29%), Firmicutes (46%), and Proteobacteria (25%), Fig. 2. The three selected strains with the highest cumulative traits scores (strains 15, 92, and 99) all belonged to the genus *Pseudomonas*, and were isolated from either the non-drought or the D50 plots. Strains 141 and 118, both belonged to *Paenarthrobacter*, were amongst the strains with the lowest trait scores, and these strains were isolated from the D50 and D90 plots, respectively.

A PCoA analysis of phylogeny for the 24 selected strains resulted in one and two axes explaining 47% and 44.4 % of the taxonomy variation, respectively. Axis one depicted the distance between Actinobacteria and Firmicutes to Proteobacteria, and axis two the distance between Actinobacteria to Firmicutes and Proteobacteria.

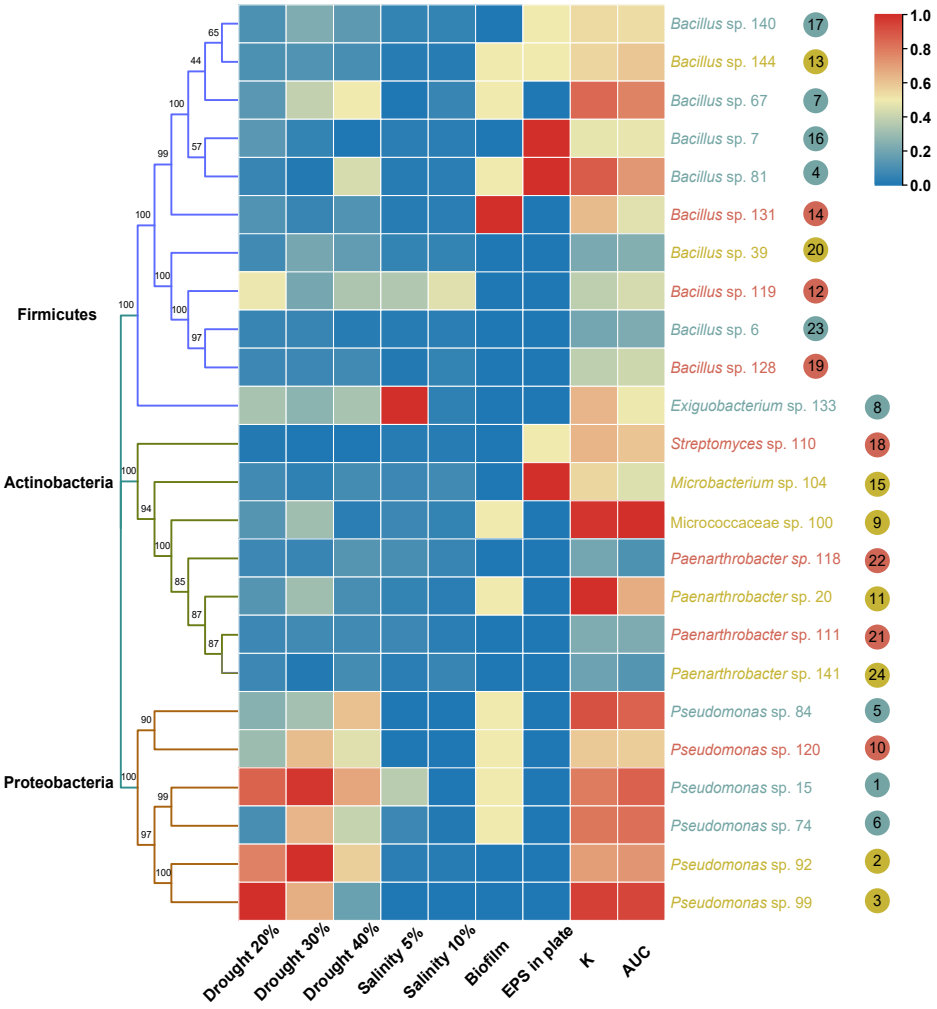


Figure 2. Heatmap showing the combined results of bacterial traits and the taxonomic relationship for the 24 selected strains. The traits drought at 20%, 30%, and 40%, salinity at 5% and 10%, growth capacity (K), and area under the curve (AUC) are shown on a scale of 0 to 1 according to the intensity of the optical density (OD), with 1 for the highest OD. Biofilm production and EPS in plates are on a scale of 1 to 3 according to the intensity of production. The numbers given in the circles on the right side of the figure depict the relative position of each strain within the 24 selected strains based on the combined trait scores. The color of the circle and type color for the strain names indicate the field drought treatment from which the strains were isolated: non-drought in blue, D50 in yellow, and D90 in red. The heatmap was built using the TB tools software.

3.2 Bacterial inoculation and aggregate stability under different moisture levels

The experimental microcosms lost approximately 8% and 66.7% of their moisture over each 2-week period before rewetting for the high and low levels of moisture, respectively. The soil aggregate fraction > 0.4 mm collected during the dry sieving under two moisture regimes after 8 weeks of incubation for each of the inoculation treatments is shown in Fig. 3. Under low moisture level, none of the strains showed better results than the non-inoculated sterile control samples. At the higher level of moisture content, 3 strains (12.5%) significantly increased the proportion of the soil fraction > 0.4 mm compared to the non-inoculated control, including strains 118 and 141 (*Paenarthrobacter* sp., Actinobacteria), and 140 (*Bacillus* sp.), which were at the bottom of the ranking based upon traits and isolated from the 3 different treatments of the drought-net field experiment.

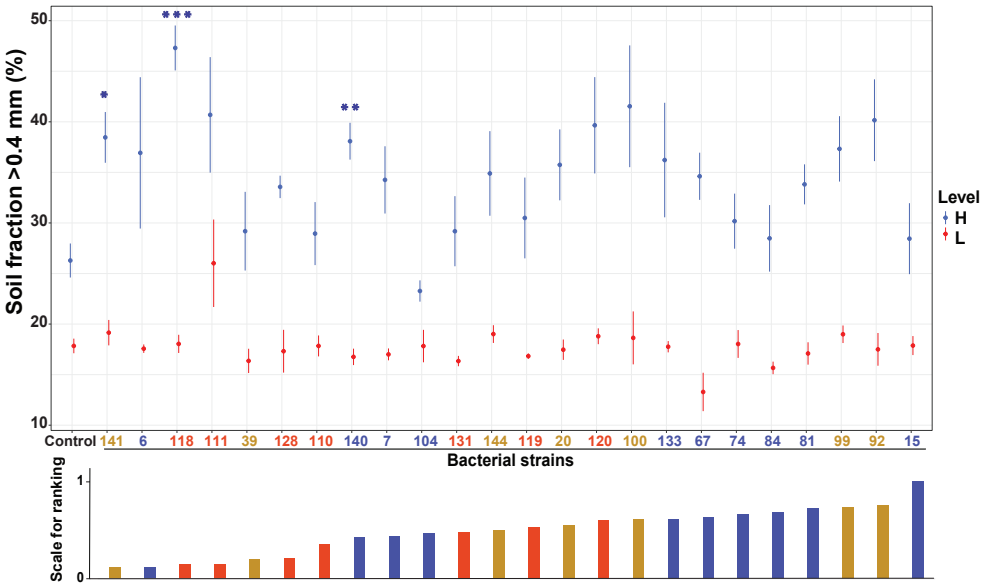


Figure 3. Soil aggregate fraction > 0.4 mm collected in dry sieving for the 24 strains after eight weeks of incubation. Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. From left to right, the strains are in increasing positions for the trait-based scale (left for the lowest grade and right for the highest). H and L indicate high and low soil moisture, respectively. The scale at the bottom shows the distance between the strains on a scale of 0-1, with 1 representing the best strain. The numbers of strains and the bars depicting relative trait scores are colored according to the field treatment from which they were isolated: non-drought in blue, D50 in yellow, and D90 in red. The analysis of variance (ANOVA) is shown in Supplementary Table S2.3 and the means and significance are shown in Supplementary Table S2.4.

The water stability of aggregates for the fractions: > 2, 2-1, 1-0.75, 0.75-0.25, and < 0.25 mm is shown in Fig. 4. At the lower moisture level, none of the strains showed a higher proportion of aggregates at any soil fraction as compared to the control (Fig. 4A). At a high level of moisture (Fig. 4B), only 2 strains significantly increased the proportion of the > 2 mm fraction: strains 110 (*Streptomyces* sp.) and strain118 (*Paenarthrobacter* sp.), and only strain 104 (*Microbacter* sp.) increased the proportion of the fraction 2-1 mm. None of the strains led to increases for the 1-0.75 and 0.75-0.25 fractions.

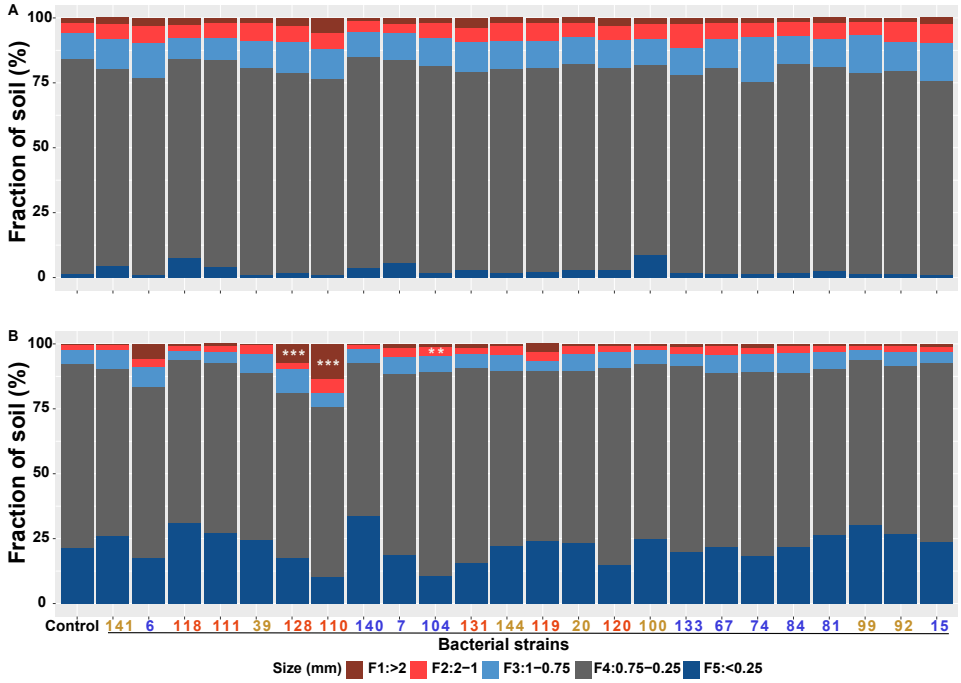


Figure 4. Mass percentage change of water stable fractions: > 2 mm, 2-1 mm, 1-0.75, 0.75-0.25 mm, and <0.25 mm of soil inoculated with 24 bacterial strains and a non-inoculated control after 8 weeks of incubation at low (A) and high moisture (B). Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. From left to right, the strains are in increasing positions for the trait-based scale (left for the lowest grade and right for the highest). The numbers of strains are colored according to the field treatment from which they were isolated: non-drought in blue, D50 in yellow, and D90 in red. H and L indicate high and low soil moisture, respectively. The analysis of variance (ANOVA) is shown in Supplementary Table S2.3 and the means and significance are shown in Supplementary Table S2.5.

Bacterial inoculation also had a modest effect on soil aggregate stability through the mean weight diameter (MWD), which summarizes aggregation across all size fractions (Fig. 5). The significant effect was only observed under the high level of moisture, and strains 128 and 110, which had the highest proportion of the > 2 mm fraction, also showed a higher MWD, which was 42% and 83% higher

than the one of control, respectively. Both strains were isolated from the drought experiment, and strain 110 showed a medium production of EPS in plate.

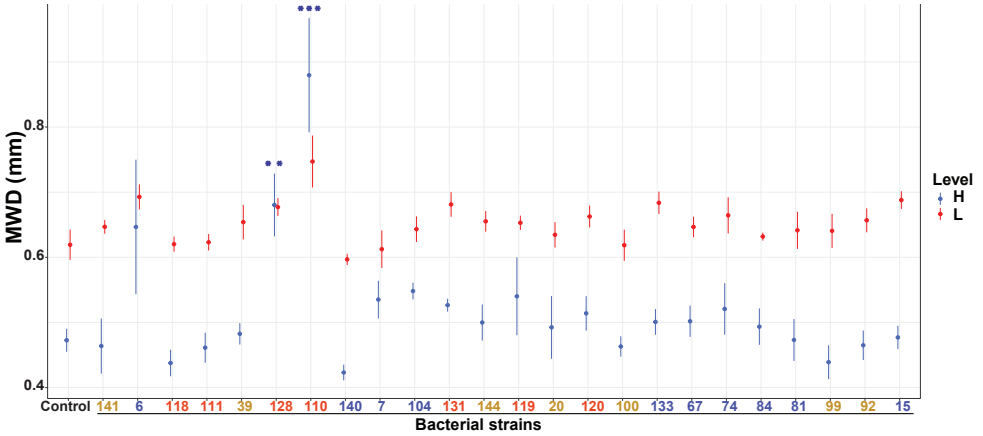


Figure 5. Mean weight diameter (MWD) for the soil fractions: > 2, 2-1, 1-0.75, 0.75-0.25, and < 0.25 mm for the 24 strains after 8 weeks of incubation. Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. From left to right, the strains are in increasing positions for the trait-based scale (left for the lowest grade and right for the highest). The numbers of strains are colored according to the field treatment from which they were isolated: non-drought in blue, D50 in yellow, and D90 in red. H and L indicate high and low soil moisture, respectively. The analysis of variance (ANOVA) is shown in Supplementary Table S2.3 and the means and significance are shown in Supplementary Table S2.5.

3.3 Production of EPS in soil

When testing the impact of bacterial inoculation on the production of EPS-exopolysaccharides in soil, we found that concentrations ranged from 26 to 46.7 µg/ml at the high moisture level and 21.5 to 43 µg/ml at low moisture content, as shown in Supplementary Fig. S2.3 and Table S2.6. Surprisingly, there were no differences in EPS production from bacterial strains when compared to the non-inoculated controls.

3.4 Bacterial population density in soil

The quantification of soil population density using the quantitative PCR (Fig. 6) showed an interaction between bacterial strains and moisture content. The concentrations at the high level of moisture ranged from 2.14 $x10^6$ to 3.5 $x10^7$ cells/ml and from 1.95 $x10^5$ to 2.38 $x10^7$ cells/ml at low moisture. Realized density often did not reflect growth behavior observed using *in vitro* assays of drought tolerance. For instance, strain 15 had the highest drought tolerance traits but did not show significant soil density than average population densities under low moisture conditions. Other strains such as 7, 84, 110, and 111 (*Paenarthrobacter* sp.) showed significantly higher

population densities than strain 15 (*Pseudomonas* sp.) but they were not higher than the average population density either. Of these strains, only strain 84 (*Pseudomonas* sp.) showed good growth performance under simulated drought conditions *in vitro*. Interestingly, strain 99 (*Pseudomonas* sp.), which scored high for drought-tolerance traits, yielded the lowest population density under low moisture conditions.

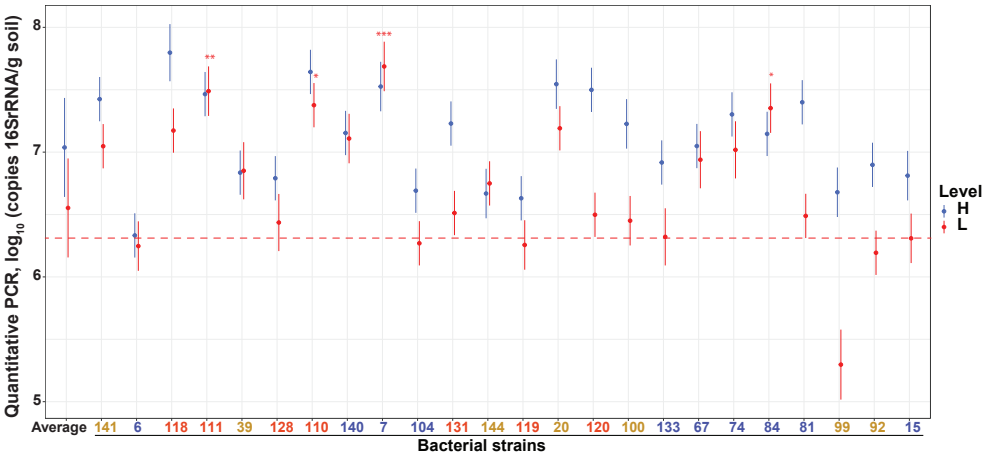


Figure 6. Bacterial population density (copies of the 16S rRNA gene) for the 24 strains and a density average. Asterisks indicate significant differences compared to strain 15 under drought: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. From left to right, the strains are in increasing positions for the trait-based scale (left for the lowest grade and right for the highest). The numbers of strains are colored according to the field treatment from which they were isolated: non-drought in blue, DL50 in yellow, and DL90 in red. H and L indicate high and low soil moisture, respectively. The analysis of variance (ANOVA) is shown in Supplementary Table S2.3 and the means and significance are shown in Supplementary Table S2.6.

3.5 Relationships between soil aggregate properties and bacterial traits

The soil aggregate fraction > 0.4 mm and aggregate stability (MWD) were correlated to measured bacterial traits for the continuous variables, as is shown in Table 1. At the low level of moisture, the soil fraction > 0.4 mm was positively correlated to realized bacterial population density and to phylogeny for axis two. In contrast, realized population density was negatively correlated to MWD, as well as response to 10% salinity. At high moisture, MWD was negatively correlated with the ability to grow under the three tested levels of drought *in vitro* and salinity at 5%. Furthermore, soil aggregate fraction > 0. 4 mm was negatively correlated to the MWD, and this relationship was driven by strains with a significant impact on soil fraction > 0.4 mm (141, 118, and 140) (Fig. 3), which also showed low values for the aggregate stabilization (Fig. 5).

Table 1. Correlation matrix for the different continuous soil properties: soil aggregate fraction > 0.4 mm and aggregate stability, MWD and the bacterial traits: drought, salinity, growth capacity (K), area under the curve (AUC), density of bacterial population, EPS in soil and phylogeny for axes one and two.

	Low moisture (ρ, p)		High moisture (ρ, p)	
Trait	**Soil fraction > 0.4 mm**	**MWD (mm)**	**Soil fraction > 0.4 mm**	**MWD (mm)**
Drought (20%)	-0.08/0.44	-0.01/0.92	-0.02/0.85	**-0.21/0.03**
Drought (30%)	-0.04/0.71	0/0.99	0.05/0.56	**-0.27/0**
Drought (40%)	-0.15/0.12	0.0/0.9	-0.08/0.38	**-0.27/0**
Salinity (5%)	0.09/0.35	-0.09/0.38	0.03/0.75	**-0.18/0.05**
Salinity (10%)	-0.11/0.26	**-0.21/0.03**	0.06/0.55	-0.01/0.96
Carrying capacity	-0.17/0.09	0.03/0.79	-0.16/0.08	-0.05/0.63
AUC	-0.15/0.13	0.04/0.72	-0.16/0.09	-0.1/0.31
Density of population	**0.2/0.04**	**-0.21/0.04**	0.12/0.22	0.05/0.6
EPS in soil	0.12/0.24	-0.15/0.14	0.06/0.5	0.08/0.4
Phylogeny axis 1	0.17/0.09	0.17/0.09	-0.08/0.41	-0.06/0.53
Phylogeny axis 2	**0.25/0.01**	-0.02/0.83	0.09/0.33	-0.09/0.36
MWD	0.17/0.09	----	**-0.51/0**	---

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

The significant interactions among soil properties and the categorial traits: biofilm production, EPS in plate, and field isolation treatment are shown in Fig. 7. The soil aggregate fraction > 0.4 mm and the field isolation treatment were positively related (Fig. 7A). We could observe this from the strains with higher means, which were isolated from one of the field drought treatments (D50 or D90), and this pattern was seen again for the aggregate stability at the high moisture content (Fig. 7C). Surprisingly, at high moisture content, the aggregates for the soil aggregate fraction > 0.4 mm were slightly negatively related to the production of EPS in plate assay (Fig. 7B).

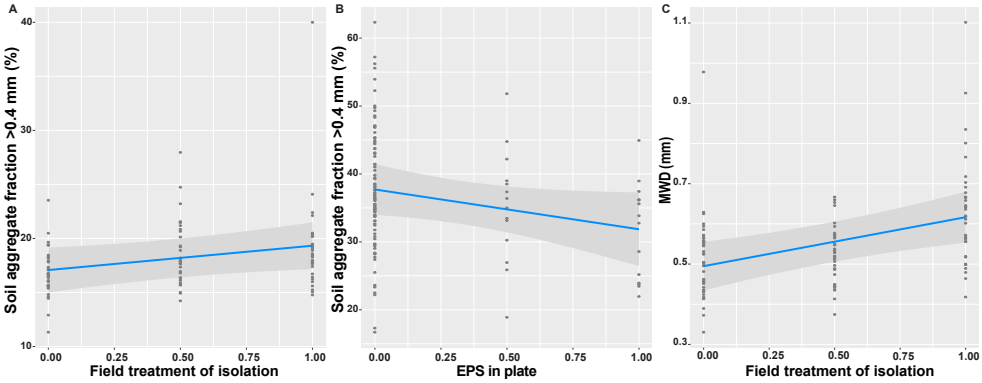


Figure. 7. Relationship between the formation of soil aggregate fraction > 0.4 mm and field treatment of isolation (R2= 0.05) at low moisture (A) and formation of EPS in plate (R2= 0.041) (B), and aggregate stability (MWD) and field treatment of isolation (R2= 0.05) at high moisture (C). Regression lines were made according to a linear model.

The random forest algorithm identified the most important traits for soil aggregate fraction > 0.4 mm and soil aggregate stability (Fig. 8). At low moisture, the most important traits for soil aggregate fraction > 0.4 mm were bacterial density and phylogeny for axis two (Fig. 8A), which is consistent with the results from the correlation Table 1. Strain 111, with one of the highest means for soil fraction > 0.4, also showed one of the highest means for realized bacterial density in soil (Fig. 3 and 6). For the aggregate stability by the MWD at low moisture, salinity 5% and phylogeny axis two were the most important explanatory traits (Fig. 8C). With respect to soil aggregate fraction > 0.4 mm at high moisture (Fig. 8B), the second axis of phylogeny, which separates Actinobacteria from Firmicutes and Proteobacteria, was the most important predictor, and the efficient strains 141 and 118 both belonged to *Paenarthrobacter* sp. For aggregate stability at high moisture, the 3 levels of drought and field isolation treatment (Fig. 8D) were the most important traits, with the 3 drought levels negatively correlated with aggregate stability (Table 1). The traits with negative relative importance were removed from the model.

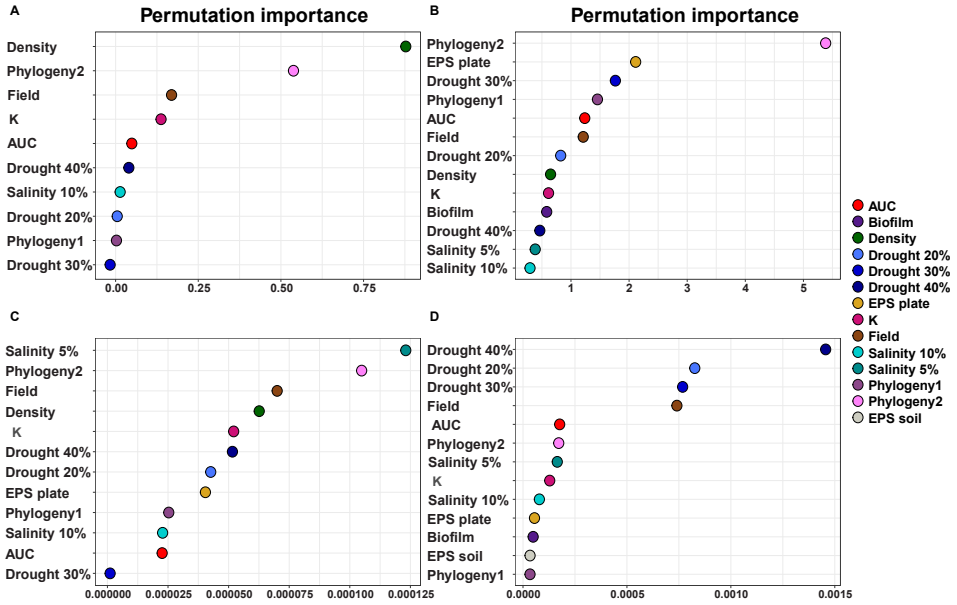


Figure 8. The importance of bacterial traits with a random forest for the soil aggregate fraction > 0.4 mm under low moisture (R^2 adj 0.93) (A), high moisture (R^2 adj 0.95) (B), and for the aggregate stability (MWD) at low (R^2 adj -0.003) (C) and high moisture (R^2 adj 0.14) (D), respectively. The traits: area under the curve (AUC), carrying capacity of growth (K), biofilm formation and drought and salinity at 3 and 2 concentrations, respectively, EPS in plate and EPS in soil, phylogeny for axes 1 and 2, the density of bacteria in soil, and field isolation treatment (field) are shown in colored circles.

4. Discussion

In our study, we focused on bacterial traits that might be presumed to be associated with the ability to colonize soils and impact soil structure under drought conditions. To this end, we subjected our collection of 116 strains to a range of *in vitro* assays to assess the following traits: growth under desiccation of drought and salinity, biofilm production, carrying capacity of growth (K), the area under the curve of growth (AUC) and production of EPS. We combined these traits to make an overall ranking of trait-predicted potential for affecting soil structure and examined the extent to which observed traits were related to the ability to improve soil structure. Although some bacterial factors could be linked with specific soil properties, only a minority of strains had significant impacts on soil structure, and our trait ranking was not sufficient to predict the outcomes of soil inoculations.

4.1 Impact of bacterial inoculation on soil aggregation

Bacterial inoculation only influenced soil aggregation at high moisture.

Three out of 24 strains had a positive effect on the formation of soil aggregate fraction > 0.4 mm (Fig. 3). This fraction was tested to observe the effect of bacterial inoculation on a substrate with 62% of sand particles between 0.5-0.25 mm (Supplementary Fig. S2.2). For water-stable aggregates, we found significant effects of bacterial inoculation on the fractions 2-1 mm and > 2 mm, which were also significant for the effect on MWD for strains 110 and 128 (Fig. 5). Soil bacteria are known to be important for the formation and stabilization of microaggregates (< 0.25 mm) (Totsche *et al.*, 2018). Although there is limited research on the impact of bacteria on the water-stable macroaggregate formation, Cheng *et al.* (2020) found that *Bacillus* sp. and *Pseudomonas* sp. with EPS production capabilities improved the ratio of water-stable macroaggregates (> 0.25 mm) by compared to non-inoculated soil. Lehmann *et al.* (2017), in a global meta-analysis also showed the strong contribution of bacteria to micro- and macroaggregates, fractions where they have been described to be well distributed (Navas *et al.*, 2021; Rui *et al.*, 2022).

Only two of our bacterial strains improved the soil aggregate stability, which means the aggregating effect of most of our bacterial strains might not have been strong enough to overcome the water disruption of wet sieving. When soil aggregates are exposed to rapid rewetting, they may break down due to the differential swelling of clay particles into the aggregates, and the air entrapped in the porosity may develop internal pressure breaking the aggregates if it overcomes the internal cohesion (Bissonnais, 1996; Chenu and Cosentino, 2011). For instance, microbial exopolysaccharides are categorized as *transient* binding agents by Tisdall and Oades (1982) and have less strength than *persistent* binding agents (organo-mineral associations). Fast wet sieving can be detrimental to extracellular polysaccharides (Tang *et al.*, 2011) and lower energy stress methods are suggested to analyze the influence of bacteria on soil aggregate stability. Strain 110 was one of the strains that impacted soil aggregate stability. This strain was identified as belonging to the actinomycetes family, a microbial group known for its ability to produce long filaments that can branch repeatedly to produce a substrate mycelium (Olanrewaju and Babalola, 2019). Thus, similar to other filamentous microorganisms (Aspiras *et al.*, 1971), this strain may have increased soil aggregation via the enmeshment of soil particles.

Bacterial impacts on soil aggregate formation and stability also depend on factors such as cell biomass and level of bacterial activity in the soil. Our results of bacterial density were modest, but some strains realized significantly higher population densities than others (Fig. 6). Lehmann *et al.* (2017) concluded

from a global meta-analysis that population density was one of the most important traits modulating effect sizes on aggregation. Other authors put more stress on the level of microbial activity, as opposed to simply biomass, when assessing impacts on soil structure (Rillig and Mummey, 2006). Cheng *et al.* (2020) found that live EPS-producing bacteria showed a significantly higher ability to promote water-stable soil macroaggregate formation than dead bacteria. Nevertheless, dead bacteria still release debris and other polymers that may also interact with soil components to help generate more stable complexes (Totsche *et al.*, 2018). Aspiras *et al.* (1971) proposed that soil-binding substances are more important than population densities and soil particle enmeshment potential for the formation and stabilization of soil aggregates (Harris *et al.*, 1964). Our results may therefore reflect the combined effects of living bacteria and residual dead biomass.

We also found a negative correlation between the aggregate stability and the soil aggregate fraction > 0.4 mm at high moisture content. These observed differences may be in part due to the different ways in which aggregates are collected for dry versus wet sieving treatments, with some stabilizing components in aggregates being vulnerable to disruption by water. Sieving techniques differ in several methodological details, such as the type of sieving, duration, loading rate, and water content (Rabot *et al.*, 2018). Sieving methods have previously been shown to not only impact the relative recovery of different fractions sizes but also the different bacterial community compositions and gene densities of specific size fractions (Blaud *et al.*, 2017). One must keep in mind that each technique has its limitations and examines different aspects of the soil's structure, highlighting the importance of including multiple parameters when examining soil structure.

4.2 Relation between bacterial traits and impacts on soil structure

We hypothesized that the bacterial strains that scored highest in traits related to aggregate formation, desiccation tolerance, and growth properties would have the most positive impacts on soil aggregation status upon inoculation. However, we did not see any relation between position in our trait ranking and observed effects on soil structure. For instance, three strains that scored within the top 5 in our trait ranking, *Pseudomonas* strains 15 and 92 and *Bacillus* strain 81, did not have any significant impact on the soil aggregate fraction > 0.4 mm or soil aggregates stability under either level of moisture. Also, with respect to aggregate stability by the MWD at high moisture content, the strains having the most positive effects were among the lowest scoring strains in our trait ranking. Thus, our trait-ranking system was ineffective

in terms of helping in the selection of the strains most effective for improving soil structure. Several reasons might explain the apparent ineffectiveness of our trait-based approach. It could be that our trait assessments, which were conducted as simplified laboratory assays and not representing the drought stress, are not indicative of actual traits as expressed in the soil. EPS, for instance, are not essential structures of bacteria in laboratory cultures, loss of EPS does not impair the growth and viability of the cells as it happens under natural conditions (Wingender *et al.*, 1999). We, therefore, suggest that the examination of bacterial traits in more realistic or *in situ* systems might provide more accurate and relevant trait data with respect to the selection of strains for field application.

We expected EPS production and associated biofilm formation potential to play a role in aggregate formation. However, although some strains displayed production of EPS using plate assays, the lack of detectable EPS production in soil, limited our ability to examine this hypothesis. Previous studies have demonstrated the importance of biofilm formation and EPS production on soil structure under stress conditions. For instance, soils inoculated with wild-type EPS-producing bacteria under acidity were able to produce larger soil aggregates and higher water-holding capacity than the EPS-minus mutant strain (Deka *et al.*, 2019). A drought-resistant *Bacillus* sp. that was able to produce EPS under different water potentials improved soil aggregation under drought (Vardharajula and Ali, 2014), and EPS and biofilm bacteria producers improved the weight of soil aggregates under concentrations of salt of 100 mM compared to non-inoculated soils (Qurashi and Sabri, 2012). The production of EPS in agricultural field conditions does not necessarily represent the optimal production in laboratory conditions (Saha *et al.*, 2020). And even when soil EPS production is optimal, there are several challenges associated with the extraction and quantification of EPS from soil biofilms, and not all the methodologies are efficient (Redmile-Gordon *et al.*, 2014). For instance, intracellular contamination by using H_2SO_4 and heating techniques (Sun *et al.*, 2012), as we used in this study, may co-extract large amounts of intracellular biomas/s and non-specific soil organic matter (SOM), leading to misestimations of EPS-polysaccharides. Even though most of the components of the extracellular polymeric substances are polysaccharides, they also comprise proteins and DNA (Costa *et al.*, 2018). Redmile-Gordon *et al.*, (2020) showed that EPS-protein was more closely related to aggregates stability than EPS-polysaccharide. We quantified soil EPS using a technique for reducing sugars (Waffenschmidt and Jaenicke, 1987) with D-glucose as the standard scale. These factors may have had an impact on our EPS estimation of inoculated soils and non-inoculated controls.

Even though our trait-ranking system proved ineffective at predicting the impacts of bacterial inoculations on soil structure, some bacterial traits showed significant explanatory power with respect to soil aggregation parameters in our predictive analysis (Fig. 8). For instance, for the formation of soil fraction > 0.4 mm at both level of moisture, the phylogeny along axis two was one of the most relevant traits. This axis was related to the difference between Actinobacteria to the other 2 phyla. Of the 3 effective strains at high moisture, 2 were affiliated with *Paenarthrobacter* spp. from the phyla Actinobacteria. *Paenarthrobacter* spp. has been related to the activity of degradation of herbicides in soils under osmotic stress (Deutch *et al.*, 2018). This taxonomic group also has been shown to improve the water productivity (WP) index, which represents the ratio between yield and water consumption, up to 30% when inoculated to plants (Riva *et al.*, 2021). For the water aggregate stability under low moisture, response to salinity at 5% was one of the most important predictors. We found that for our 24 strains selected, salinity tolerance at 5% and 10% (17.14 and 28.1 mS/ cm) was linked to the drought resistance for some of the strains (Fig. 2). It is known that tolerance to osmotic stresses from drought and salinity can use similar strategies, such as the accumulation of compatible osmolytes like K^+ or glycogen, which allows cells to maintain their turgor and metabolism (Vriezen *et al.*, 2007; Finn *et al.*, 2013). For aggregate stability at the high level of moisture, the drought resistance and field of isolation showed the highest importance. This relationship with drought resistance, which was negative (Table 1), could have stemmed from a reduced ability of drought-resistant bacteria to colonize soil under conditions of high soil moisture.

We also hypothesized that the strains isolated from drought experimental fields would be higher ranked for our aggregate trait-based ranking. However, the best strain that was at the top of the ranking was isolated from the non-drought treatment, and some of the lowest-ranking strains, such as 141, 118, and 111, were isolated from a drought treatment. Our drought field isolation treatments did not predict a good performance for bacterial traits tested facing desiccation conditions *in vitro* conditions.

4.3 Effects of soil moisture and texture on aggregation parameters

Soil moisture status affected the impacts of bacterial inoculation on soil aggregation, and we only observed a positive effect at the high level of moisture compared to the non-inoculated soil. It should be noted that moisture conditions were not constant throughout our inoculation experiments. After two weeks of incubation, the initial high and low levels of moisture (ψ -0.03

MPa and ψ -0.96 MPa) dropped by approximately 8% and 66.7%, respectively, at which point moisture was added back to restore the initial levels. Thus, our soil microcosms were exposed to a series of drying and rewetting events over the course of the incubation. Drying events may induce cell lysis in a significant proportion of microbes that are not adapted to sudden changes in water potentials (Fierer *et al.*, 2003). Bacterial groups are known to vary greatly in their ability to withstand water-limited conditions. Nitrifiers, for instance, suffer dehydration below -0.6 MPa (Stark and Firestone, 1995). Gram-positive bacteria appear to be more resistant than Gram-negative, Actinomycetes are known to be more resistant to soil drying compared to other bacteria, showing an optimal growth at -2.8 MPa but still are able to produce microcolony development at -22 MPa (Zenova *et al.*, 2007; Zvyagintsev *et al.*, 2007; Manzoni *et al.*, 2012). The lowest moisture levels experienced during our incubations in the low moisture treatment would have been at the lower range or beyond the range of bacterial activity for most groups of bacteria, which may have hampered their ability to colonize the soil and develop sufficient biomass and metabolic activity to improve aggregate stability.

We also observed differences between the non-inoculated controls under the different levels of moisture. In our study, we used sandy soil supplemented with organic matter, as the sandy texture represents a good experimental control and starting point for aggregate formation. Using such sandy soil also avoids the possibility of nonspecific aggregation caused by clay particles, a process that does not involve bacterial binding agents (Caesar-TonThat and Cochran, 2000). The stability of soil aggregates and soil water repellency has been shown to be affected by soil texture (Kiem and Kandeler, 1997), the latter also being affected by soil moisture (Wallis and Horne, 1992), with effects being either positive (Jex *et al.*, 1985) or negative (de Jonge *et al.*, 1999). Using a meta-analysis approach, Zheng *et al.* (2016) found a positive correlation between the response ratio of sandy soils and soil water repellency and aggregate stability and water repellency. Other studies have also shown that sandy soils have a higher tendency to exhibit water repellency (Cann, 2000; Nadav *et al.*, 2011). Borowik & Wyszkowska (2016) also demonstrated the importance of the texture of soil on microbial and biochemical activity in interaction with moisture content. We, therefore, suspect that the sandy soil used in our experiment could have provided interactions for water repellency under the lower level of moisture and affected the stable aggregates independently of the bacterial inoculation.

5. Conclusions

In this study, we sought to use a trait-based approach to investigate the ability of inoculated soil bacterial isolates from drought-treated fields to improve soil aggregation at two different moisture levels. We found that strains within our collection exhibited a range of traits putatively related to soil structure and drought tolerance, allowing us to make a trait-based ranking for potential soil structure enhancement capacity. However, this trait-based ranking was not related to the drought status of the field of isolation. Bacterial inoculations had a modest impact on soil structure parameters, which was effective only at high moisture content. We did not find any relationship between our ranking based upon bacterial traits and the observed impacts on soil aggregate fraction > 0.4 mm and stabilization under either level of moisture. This may be due to a discrepancy between traits measured *in vitro* and realized phenotypes in the soil environment. However, we found other traits with more explanatory power on the effect on macroaggregates, such as phylogeny related to the phylum Actinobacteria. Thus, while bacterial amendments may hold the potential for improving soil structure, greater knowledge of *in situ* traits and activities would be required to allow for more directed strategies for choosing efficient inoculants and inoculation strategies.

6. Acknowledgements

We kindly thank Kathryn Barry and Yann Hautier for their support in the statistical analyses, and the latter for providing access to the drought experimental field site. We also thank Enrique Ortega for his help with the bacterial identification and Peter Veenhuizen for his highly valuable technical support.

7. Supplementary information

Figures

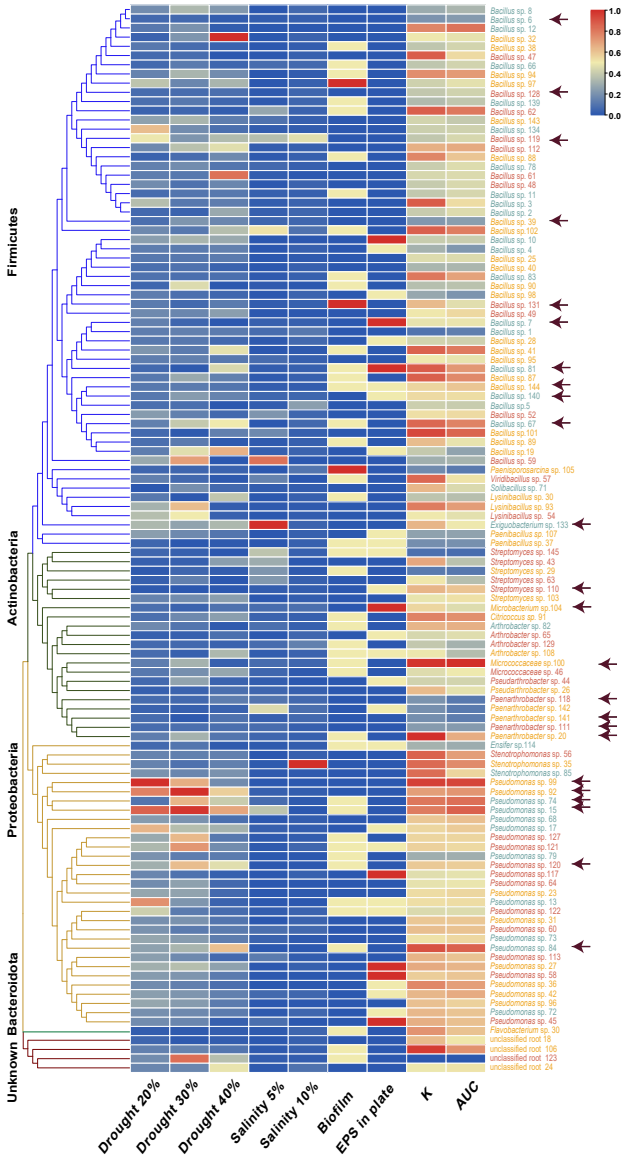


Figure S2.1. Heatmap showing the trait-based approach and the taxonomy relationship for the 116 bacterial strains isolated. The traits drought at 20%, 30%, and 40%, salinity at 5% and 10%, growth capacity (K), and area under the curve (AUC) are shown on a scale of 0 to 1 according to the intensity of the optical density (OD), with 1 for the highest OD. Biofilm production and EPS in plates are on a scale of 1 to 3 according to the intensity of production. On the right, the arrows show the 24 strains selected according to a good, intermedium, and low score. The color of the fonts indicates the field drought treatment from which the strains were isolated: non-drought in blue, D50 in yellow, and D90 in red. The heatmap was built using the TB tools software.

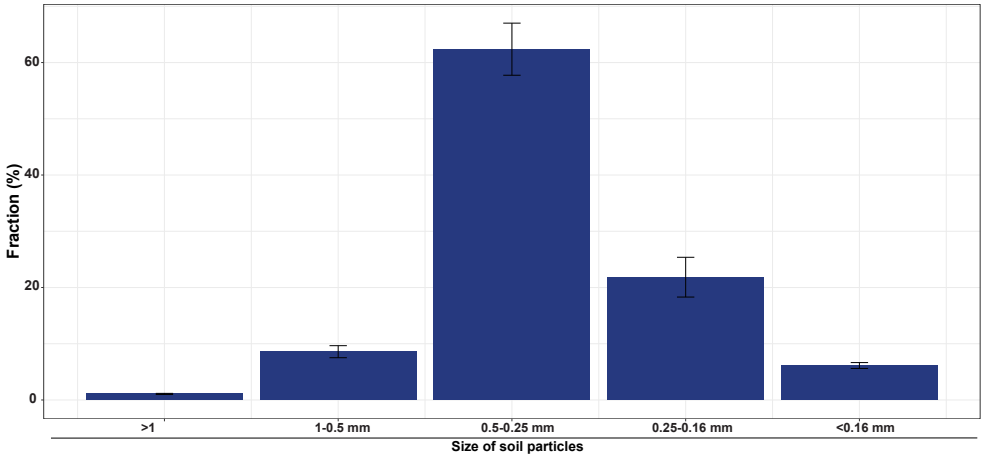


Figure S2.2. Representation of the different soil fractions for the profile of the sandy substrate used in the microcosm experiment.

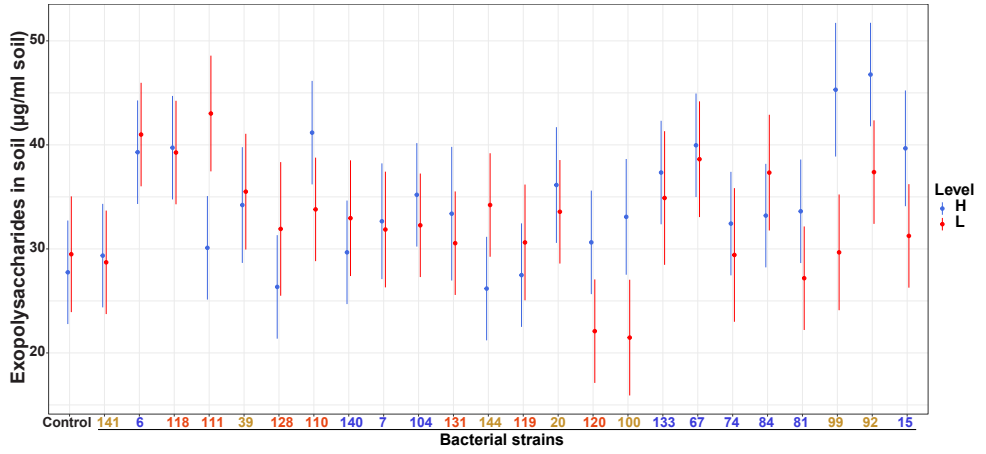


Figure S2.3. Production of EPS in soil for the 24 strains after 8 weeks of incubation. From left to right, the strains are in increasing positions for the trait-based scale (left for the lowest grade and right for the highest). The numbers of strains are colored according to the field treatment from which they were isolated: non-drought in blue, DL50 in yellow, and DL90 in red. H and L indicate high and low soil moisture, respectively. The analysis of variance (ANOVA) is shown in Supplementary Table S2.3 and means and significance are shown in Supplementary Table S2.6

Tables

Table S2.1. Effect of bacterial inoculation on the production of exopolysaccharides in liquid media with isolates showing high and intermedium production of slime on plates using media ATCC 14.

Strain	Slime in plate	EPS (mg polysaccharide /ml)	SE	Group
81	**Strong**	**3.752**	**0.1646**	**e**
7	**Strong**	**3.359**	**0.0455**	**e**
117	Strong	2.158	1.2261	abcde
10	**Strong**	**2.095**	**0.0972**	**d**
45	**Strong**	**1.86**	**0.0908**	**cd**
104	**Strong**	**1.389**	**0.0996**	**bcd**
27	**Strong**	**1.345**	**0.0653**	**bc**
58	**Strong**	**1.293**	**0.053**	**b**
Control	Strong	0.254	0.0304	a
140	Medium	3.444	0.3714	e
144	Medium	2.845	0.7058	abcde
65	Medium	1.611	0.1545	cd
4	Medium	1.585	0.303	abcde
42	Medium	1.548	0.0477	d
28	Medium	1.538	0.2382	bcd
37	Medium	1.498	0.3023	abcde
110	Medium	1.465	0.5848	abcde
72	Medium	1.388	0.1843	bcd
98	Medium	1.155	0.0973	bcd
121	Medium	1.13	0.3174	abcd
108	Medium	1.106	0.4132	abcd
107	Medium	1.073	0.2256	abcd
13	Medium	0.979	0.1939	abcd
114	Medium	0.973	0.1156	bc
44	Medium	0.964	0.0827	bc
Control	Medium	0.936	0.1101	bc
17	Medium	0.856	0.1618	abc
19	Medium	0.853	0.1631	abc
36	Medium	0.848	0.0375	b
145	Medium	0.753	0.1429	ab
122	Medium	0.545	0.265	abcd
142	Medium	0.464	0.012	a

Groups were determined by the Tukey test and Sidak adjustment for multiple comparisons. Bold means significantly different when compared to the control ($p < 0.05$).

Table S2.2 Designation and affinities of primers used for the quantitative PCR analyses to determine bacterial population density.

Positive for strains	Primer combinations	Bacterial identity on Sanger sequencing	Primer sequence	PCR fragment length (bp)
6, 119, 128	316/317	*Bacillus* sp.	GCTTCGGCTGTCACTTATG/ CAAGGTGCCAGCTTATTCAACTAG	275
7, 67, 81, 140, 144	318/319	*Bacillus* sp.	GGATCTTCTCCTTCATGGGAGATG/TCAAGGTACAAGCAGTTACTCTT	310
39	338/339	*Bacillus* sp.	GAAAGGCGGCTTCGGCTG/CAAGGCATGAGCTTATTCAACTCA	282
20, 100, 111, 118, 141	340/341	*Paenarthrobacter* sp.	GGTGGTGGAAAGCTTTTGTGG/ CCCGTACCCACTGCAGAAC	426
74	328/329	*Flavobacterium* sp.	GGGCAGTAAGCGAATACCTTG/ CTGCGCCACTAAAATCTCAAGGA	409
84, 120, 15	368/369	*Pseudomonas* sp.	CTTCGGGCCTTGCGCTATC/ CCTTCCTCCCAACTTAAAGTGC	248
92, 99	314/315	*Pseudomonas* sp.	AAGAGCTTGCTCTTCGATTC/AAAATACTCACGTATTAGGTAAG	401
104	334/335	*Microbacterium* sp.	CTAATACTGGATATGAGCTGCGATC/ CCGTGAGCCCATCCCAG	73
110	310/311	*Streptomyces* sp.	GCTCCGGCGGTGAAG /CCCGTATCGGATGCAGAC	415
131	330/331	*Brevibacterium* sp.	GATACGTTCTTTTCTCGCATGAGAG/GTCAAGGTACCAGCAGTTACTCTG	315
133	332/333	*Exiguobacterium* sp.	GCTCCGGCGTCACCTTG/ GTCAAGGTACGAGCATTACCTCTC	275

Table S2.3. Analysis of variance (ANOVA) for the effect of the bacterial inoculation on the soil properties: aggregates > 0.4 mm, water stable aggregates (> 2, 2-1, 1-0.75, 0.75-0.25 mm), aggregate stability, (MWD) and bacterial traits: production of EPS in soil, bacterial density, and production of EPS in liquid media for high and intermedium producers in plate. The analysis comprises all interactions. P-values <0.05 are considered significant and highlighted in bold.

ANOVA type 3	Strain			Moisture			Strain*moisture		
	df	**Chisq**	**p-value**	**df**	**Chisq**	**p-value**	**df**	**Chisq**	**p-value**
log (aggregates > 0.4 mm)	24	166.94	**<.0001**	1	26.24	**<.0001**	24	68.524	**<.0001**
log (water stable aggregate) (> 2 mm)	24	158.67	**<.0001**	1	7.58	**0.005**	24	58.776	**<.0001**
log (water stable aggregate) (2-1 mm)	24	85.492	**<.0001**	1	7.92	**0.005**	24	33.53	0.093
Water stable aggregate 1-0.75 mm	24	53.91	**0.0004**	1	247.61	**<.0001**	24	27.483	0.28
Water stable aggregate 0.75-0.25 mm	24	117.96	**<.0001**	1	10.10	**0.001**	24	82.65	**<.0001**
Aggregate stability (MWD)	24	141.63	**<.0001**	1	25.84	**<.0001**	24	61.260	**<.0001**
	df	**F**	**p-value**	**df**	**F**	**p-value**	**df**	**F**	**p-value**
EPS in soil	24	1.18	0.265	1	0.05	0.816	24	0.713	0.834
Bacterial density	23	4.0123	**<.0001**	1	3.205	0.075	24	1.99	**0.007**
ANOVA type 1									
log (EPS liquid media) (high producers in plate)	$F_{1,8}$=176.203		**<.0001**						
EPS liquid media (intermedium producers in plate)	$F_{1,22}$=37.487		**<.0001**						

Table S2.4. Bacterial strains information and mean ($\bar{X}$), standard error (SE) (±), p-value, and significance (sig) for bacterial soil aggregate fraction > 0.4 mm collected by dry sieving after 8 weeks of incubation under low and high levels of moisture. Asterisks indicate significant differences compared to the mean average: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Code strain	Position ranking	Molecular identity	Treatment field	Soil fraction > 0.4 mm (%) low moisture				Soil fraction > 0.4 mm (%) high moisture			
				$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
Control				17.837	0.722			26.286	1.682		
141	24	*Paenarthrobacter* sp.	D50	19.151	1.257	1.000		**38.460**	**2.509**	**0.012**	*
6	23	*Paenarthrobacter* sp.	no drought	17.566	0.418	1.000		36.930	7.483	0.996	
118	22	*Paenarthrobacter* sp.	D90	18.040	0.899	1.000		**47.305**	**2.220**	**0.000**	***
111	21	*Paenarthrobacter* sp.	D90	26.014	4.325	0.873		40.689	5.710	0.441	
39	20	*Bacillus* sp.	D50	16.360	1.201	1.000		29.185	3.890	1.000	
128	19	*Bacillus* sp.	D90	17.316	2.115	1.000		33.566	1.109	0.132	
110	18	*Streptomyces* sp.	D90	17.839	1.035	1.000		28.947	3.116	1.000	
140	17	*Bacillus* sp.	no drought	16.757	0.811	1.000		**38.084**	**1.821**	**0.002**	**
7	16	*Bacillus* sp.	no drought	17.003	0.587	1.000		34.258	3.325	0.836	
104	15	*Microbacterium* sp.	D50	17.826	1.603	1.000		23.271	1.052	0.998	
131	14	*Bacillus* sp.	D90	16.342	0.511	0.991		29.183	3.466	1.000	
144	13	*Bacillus* sp.	D50	19.012	0.877	1.000		34.895	4.183	0.923	
119	12	*Bacillus* sp.	D90	16.829	0.266	1.000		30.493	3.995	1.000	
20	11	*Paenarthrobacter* sp.	D50	17.466	1.007	1.000		35.744	3.507	0.600	
120	10	*Pseudomonas* sp.	D90	18.795	0.783	1.000		39.658	4.763	0.312	
100	9	*Micrococcaceae* sp.	D50	18.634	2.618	1.000		41.537	6.020	0.401	
133	8	*Exiguobacterium* sp.	no drought	17.760	0.557	1.000		36.220	5.663	0.970	
67	7	*Bacillus* sp.	no drought	13.288	1.899	0.953		34.615	2.333	0.351	
74	6	*Pseudomonas* sp.	no drought	18.032	1.377	1.000		30.176	2.722	1.000	
84	5	*Pseudomonas* sp.	no drought	15.667	0.618	0.826		28.478	3.289	1.000	
81	4	*Bacillus* sp.	no drought	17.094	1.106	1.000		33.816	1.979	0.389	
99	3	*Pseudomonas* sp.	D50	18.994	0.867	1.000		37.326	3.233	0.186	
92	2	*Pseudomonas* sp.	D50	17.500	1.619	1.000		40.158	4.044	0.086	
15	1	*Pseudomonas* sp.	no drought	17.875	0.939	1.000		28.446	3.509	1.000	

Mean ($\bar{X}$), standard error (SE) (±), p-value, and significance (sig) are in black bold when significantly higher than the control.

Table S2.5. Bacterial strains information and mean ($\bar{X}$), standard error (SE) (±), p-value, and significance (sig) for the bacterial soil aggregate stability (MWD) (mm) and water stability for the fraction (>2, 2-1,1-0.75, and 0.75-0.25 mm) after 8 weeks of incubation under a low and high level of moisture. Asterisks indicate significant differences compared to the mean average: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Code strain	Position ranking	Molecular identity	Treatment field	Level moist	MWD (mm)			
					$\bar{X}$	SE	p-value	sig
Control					0.619	0.023		
141	24	*Paenarthrobacter* sp.	D50	Low	0.647	0.011	1.000	
6	23	*Paenarthrobacter* sp.	no drought	Low	0.693	0.019	0.735	
118	22	*Paenarthrobacter* sp.	D90	Low	0.620	0.012	1.000	
111	21	*Paenarthrobacter* sp.	D90	Low	0.623	0.013	1.000	
39	20	*Bacillus* sp.	D50	Low	0.654	0.027	1.000	
128	19	*Bacillus* sp.	D90	Low	0.677	0.014	0.900	
110	18	*Streptomyces* sp.	D90	Low	0.747	0.040	0.426	
140	17	*Bacillus* sp.	no drought	Low	0.597	0.009	1.000	
7	16	*Bacillus* sp.	no drought	Low	0.613	0.029	1.000	
104	15	*Microbacterium* sp.	D50	Low	0.643	0.020	1.000	
131	14	*Bacillus* sp.	D90	Low	0.681	0.019	0.925	
144	13	*Bacillus* sp.	D50	Low	0.655	0.016	1.000	
119	12	*Bacillus* sp.	D90	Low	0.653	0.011	1.000	
20	11	*Paenarthrobacter* sp.	D50	Low	0.635	0.020	1.000	
120	10	*Pseudomonas* sp.	D90	Low	0.663	0.017	0.998	
100	9	*Micrococcaceae* sp.	D50	Low	0.619	0.024	1.000	
133	8	*Exiguobacterium* sp.	no drought	Low	0.684	0.017	0.861	
67	7	*Bacillus* sp.	no drought	Low	0.647	0.016	1.000	
74	6	*Pseudomonas* sp.	no drought	Low	0.664	0.028	1.000	
84	5	*Pseudomonas* sp.	no drought	Low	0.632	0.006	1.000	
81	4	*Bacillus* sp.	no drought	Low	0.642	0.028	1.000	
99	3	*Pseudomonas* sp.	D50	Low	0.641	0.026	1.000	
92	2	*Pseudomonas* sp.	D50	Low	0.657	0.018	1.000	
15	1	*Pseudomonas* sp.	no drought	Low	0.688	0.014	0.682	
Control					0.471	0.018		
141	24	*Paenarthrobacter* sp.	D50	High	0.457	0.040	1.000	
6	23	*Paenarthrobacter* sp.	no drought	High	0.625	0.090	0.970	
118	22	*Paenarthrobacter* sp.	D90	High	0.436	0.020	1.000	
111	21	*Paenarthrobacter* sp.	D90	High	0.459	0.023	1.000	
39	20	*Bacillus* sp.	D50	High	0.481	0.016	1.000	
128	19	*Bacillus* sp.	D90	High	0.673	**0.048**	**0.004**	**
110	18	*Streptomyces* sp.	D90	High	0.863	**0.084**	**0.000**	***
140	17	*Bacillus* sp.	no drought	High	0.422	0.012	0.816	
7	16	*Bacillus* sp.	no drought	High	0.533	0.029	0.977	
104	15	*Microbacterium* sp.	D50	High	0.548	0.013	0.131	
131	14	*Bacillus* sp.	D90	High	0.526	0.010	0.598	
144	13	*Bacillus* sp.	D50	High	0.497	0.027	1.000	
119	12	*Bacillus* sp.	D90	High	0.527	0.059	1.000	
20	11	*Paenarthrobacter* sp.	D50	High	0.485	0.052	1.000	
120	10	*Pseudomonas* sp.	D90	High	0.511	0.027	1.000	
100	9	*Micrococcaceae* sp.	D50	High	0.462	0.016	1.000	
133	8	*Exiguobacterium* sp.	no drought	High	0.499	0.019	1.000	
67	7	*Bacillus* sp.	no drought	High	0.499	0.025	1.000	
74	6	*Pseudomonas* sp.	no drought	High	0.515	0.040	1.000	
84	5	*Pseudomonas* sp.	no drought	High	0.490	0.027	1.000	
81	4	*Bacillus* sp.	no drought	High	0.468	0.035	1.000	
99	3	*Pseudomonas* sp.	D50	High	0.437	0.026	1.000	
92	2	*Pseudomonas* sp.	D50	High	0.463	0.021	1.000	
15	1	*Pseudomonas* sp.	no drought	High	0.476	0.018	1.000	

Table S2.5 continued. Bacterial strains information and mean ($\bar{X}$), standard error (SE) (±), p-value, and significance (sig) for the bacterial soil aggregate stability (MWD) (mm) and water stability for the fraction (>2, 2-1,1-0.75, and 0.75-0.25 mm) after 8 weeks of incubation under high and low level of moisture. Asterisks indicate significant differences compared to the mean average: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

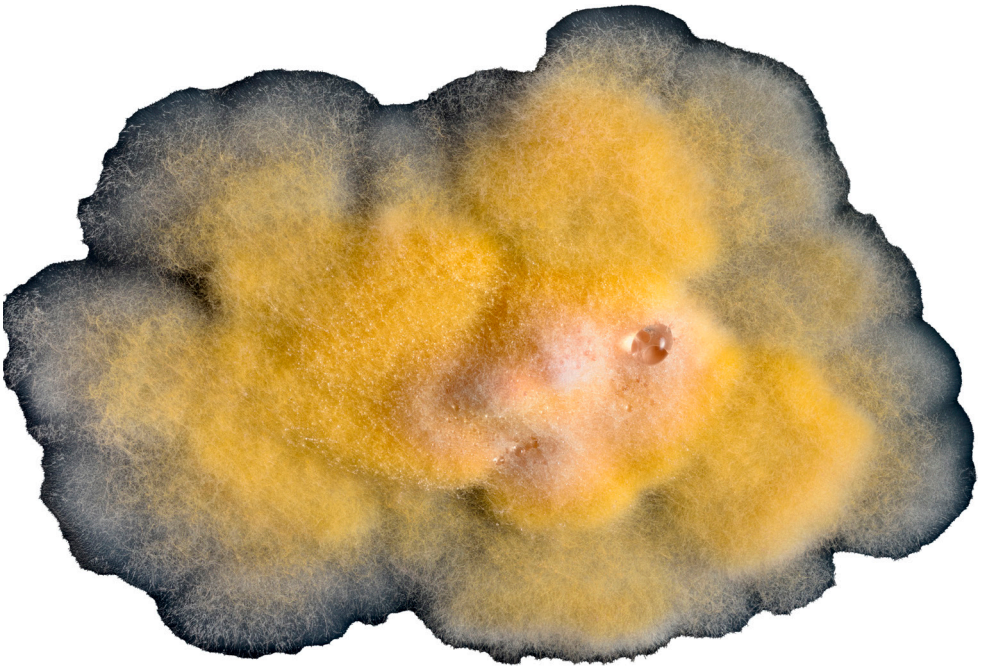
Soil fractions																
>2 mm				2-1 mm				1-0.75 mm				0.75-0.25 mm				
$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	
1.622	0.516			3.492	0.735			9.921	1.410			81.764	1.883			
2.339	0.373	1.000		5.485	0.983	1.000		11.170	1.542	1.000		75.658	1.858	0.979		
2.996	0.599	1.000		6.537	0.223	0.676		13.324	1.376	1.000		76.011	1.969	0.996		
2.588	0.273	1.000		4.967	0.315	1.000		8.155	0.580	1.000		76.623	1.090	0.970		
1.841	0.184	1.000		5.644	0.480	0.995		8.448	1.303	1.000		77.977	1.788	1.000		
1.880	0.402	1.000		6.293	1.810	1.000		9.979	0.304	1.000		78.246	2.020	1.000		
2.886	0.584	1.000		6.178	0.572	0.939		11.875	2.730	1.000		76.682	1.036	0.970		
4.706	1.371	0.946		5.645	1.382	1.000		11.424	2.180	1.000		74.780	3.522	1.000		
1.101	0.237	1.000		4.277	0.227	1.000		9.353	1.122	1.000		79.958	1.152	1.000		
2.126	0.394	1.000		2.875	1.167	1.000		10.099	1.904	1.000		77.546	1.803	1.000		
1.623	0.624	1.000		5.498	1.017	1.000		10.275	0.931	1.000		78.629	1.141	1.000		
3.475	0.684	0.998		5.404	0.222	0.997		11.398	0.678	1.000		76.321	1.041	0.926		
1.995	0.175	1.000		6.702	0.717	0.805		10.216	1.672	1.000		76.525	2.319	1.000		
2.144	0.102	1.000		6.378	1.012	0.981		10.182	0.699	1.000		77.556	1.124	0.999		
1.699	0.576	1.000		5.479	0.494	0.999		10.171	2.116	1.000		78.761	2.800	1.000		
2.928	0.508	1.000		5.401	0.239	0.998		10.821	1.513	1.000		77.103	1.845	1.000		
2.250	0.193	1.000		5.573	0.468	0.997		10.001	2.785	1.000		72.321	2.878	0.818		
2.241	0.135	1.000		8.948	1.639	0.338		10.197	1.254	1.000		76.252	1.736	0.994		
1.763	0.486	1.000		6.307	0.505	0.881		10.860	1.953	1.000		78.982	2.139	1.000		
1.948	0.268	1.000		5.107	1.232	1.000		17.008	6.197	1.000		73.804	8.941	1.000		
1.662	0.169	1.000		5.539	0.306	0.995		10.526	0.799	1.000		80.057	0.703	1.000		
1.672	0.322	1.000		5.351	1.431	1.000		10.655	1.108	1.000		76.374	2.725	1.000		
1.529	0.230	1.000		4.551	1.355	1.000		14.063	3.906	1.000		76.314	3.669	1.000		
1.561	0.182	1.000		7.458	0.921	0.539		10.442	2.147	1.000		75.778	2.228	0.997		
2.441	0.212	1.000		7.047	0.712	0.623		14.533	1.507	0.988		73.473	1.541	0.308		
0.551	0.126			1.736	0.229			5.097	0.827			69.879	3.230			
0.515	0.150	1.000		1.512	0.437	1.000		7.100	1.687	1.000		63.089	7.249	1.000		
2.333	1.991	1.000		2.975	0.560	0.972		7.323	2.083	1.000		65.173	4.417	1.000		
0.788	0.174	1.000		1.781	0.214	1.000		3.792	0.647	1.000		62.731	2.666	1.000		
0.674	0.171	1.000		2.356	0.553	1.000		4.082	0.842	1.000		64.476	2.812	1.000		
0.508	0.112	1.000		3.067	0.301	0.282		7.347	0.869	1.000		63.656	5.266	1.000		
6.512	**1.828**	**0.000**	***	1.946	0.581	1.000		9.394	2.774	1.000		63.623	1.197	1.000		
11.06	**3.447**	**0.000**	***	4.834	1.328	0.347		5.250	0.593	1.000		64.023	4.333	1.000		
0.347	0.095	1.000		1.667	0.231	1.000		5.406	0.545	1.000		58.062	2.317	0.652		
1.629	0.406	0.462		3.204	0.762	0.985		6.380	0.871	1.000		69.476	2.911	1.000		
1.069	0.159	0.957		**3.776**	**0.317**	**0.002**	**	5.794	1.297	1.000		77.926	1.708	0.991		
1.409	0.400	0.908		2.383	0.213	0.998		5.483	0.486	1.000		75.421	1.848	1.000		
0.933	0.156	1.000		3.111	0.715	0.990		5.984	1.115	1.000		65.920	3.416	1.000		
1.158	0.755	1.000		2.808	0.902	1.000		3.910	0.719	1.000		64.423	6.756	1.000		
1.022	0.098	0.940		2.577	0.882	1.000		6.281	1.822	1.000		66.308	7.031	1.000		
0.846	0.257	1.000		1.914	0.372	1.000		6.050	1.816	1.000		74.404	3.558	1.000		
0.671	0.187	1.000		1.646	0.341	1.000		5.187	0.920	1.000		66.382	2.812	1.000		
1.045	0.150	0.971		2.856	0.210	0.394		4.652	0.533	1.000		71.086	2.968	1.000		
0.842	0.211	1.000		3.180	0.496	0.659		6.894	1.140	1.000		66.042	2.465	1.000		
1.135	0.346	1.000		2.452	0.454	1.000		6.608	2.009	1.000		68.942	4.565	1.000		
0.709	0.180	1.000		2.598	0.347	0.993		7.548	2.336	1.000		66.027	2.156	1.000		
0.855	0.264	1.000		1.876	0.534	1.000		6.341	1.258	1.000		63.442	3.622	1.000		
0.773	0.175	1.000		1.463	0.346	1.000		3.658	0.397	1.000		62.674	5.569	1.000		
0.608	0.207	1.000		2.209	0.470	1.000		5.075	1.045	1.000		63.136	0.825	0.998		
1.003	0.196	0.999		2.123	0.244	1.000		4.314	0.439	1.000		68.099	3.174	1.000		

Mean ($\bar{X}$), standard error (SE) (±), p-value, and significance (sig) are in bold when significant compared to the control

Table S2.6. Bacterial strain information and bacterial population density by quantitative PCR (copies 16SrRNA/g soil) compared to the mean average population density and bacterial production of EPS in soil (µg/ml soil) compared to non-inoculated control after 8 weeks of incubation under low and high level of moisture. Asterisks indicate significant differences compared to the mean average: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Code strain	Position ranking	Molecular identity	Treatment field	Bacterial density log(copies 16SrRNA/g soil) low moisture				Bacterial density log(copies 16SrRNA/g soil) high moisture			
				$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
Mean/ control				6.553	0.397			7.038	0.397		
141	24	*Paenarthrobacter* sp.	D50	7.047	0.177	1.000		7.425	0.177	1.000	
6	23	*Paenarthrobacter* sp.	no drought	6.247	0.198	1.000		6.333	0.177	0.996	
118	22	*Paenarthrobacter* sp.	D90	7.173	0.177	0.999		7.797	0.229	0.995	
111	21	*Paenarthrobacter* sp.	D90	7.489	0.198	0.915		7.465	0.177	1.000	
39	20	*Bacillus* sp.	D50	6.851	0.229	1.000		6.836	0.177	1.000	
128	19	*Bacillus* sp.	D90	6.436	0.229	1.000		6.791	0.177	1.000	
110	18	*Streptomyces* sp.	D90	7.376	0.177	0.972		7.643	0.177	1.000	
140	17	*Bacillus* sp.	no drought	7.109	0.198	1.000		7.153	0.177	1.000	
7	16	*Bacillus* sp.	no drought	7.687	0.198	0.651		7.526	0.198	1.000	
104	15	*Microbacterium* sp.	D50	6.269	0.177	1.000		6.691	0.177	1.000	
131	14	*Bacillus* sp.	D90	6.512	0.177	1.000		7.229	0.177	1.000	
144	13	*Bacillus* sp.	D50	6.749	0.177	1.000		6.668	0.198	1.000	
119	12	*Bacillus* sp.	D90	6.256	0.198	1.000		6.630	0.177	1.000	
20	11	*Paenarthrobacter* sp.	D50	7.191	0.177	0.999		7.545	0.198	1.000	
120	10	*Pseudomonas* sp.	D90	6.498	0.177	1.000		7.499	0.177	1.000	
100	9	*Micrococcaceae* sp.	D50	6.450	0.198	1.000		7.226	0.198	1.000	
133	8	*Exiguobacterium* sp.	no drought	6.321	0.229	1.000		6.917	0.177	1.000	
67	7	*Bacillus* sp.	no drought	6.939	0.229	1.000		7.049	0.177	1.000	
74	6	*Pseudomonas* sp.	no drought	7.018	0.229	1.000		7.302	0.177	1.000	
84	5	*Pseudomonas* sp.	no drought	7.353	0.198	0.984		7.146	0.177	1.000	
81	4	*Bacillus* sp.	no drought	6.489	0.177	1.000		7.400	0.177	1.000	
99	3	*Pseudomonas* sp.	D50	5.297	0.280	0.630		6.678	0.198	1.000	
92	2	*Pseudomonas* sp.	D50	6.193	0.177	1.000		6.898	0.177	1.000	
15	1	*Pseudomonas* sp.	no drought	6.309	0.198	1.000		6.811	0.198	1.000	

Mean ($\bar{X}$), standard error (SE) (±), p-value, and significance (sig) are in bold when significant compared to the control.



Colony of strain 14, *Staphylotrichum acaciicola*

CHAPTER 3

SOIL COLONIZATION OF FUNGAL AMENDMENTS IMPROVES SOIL AGGREGATION AND SOIL PHYSICAL PROPERTIES UNDER DIFFERENT MOISTURE CONDITIONS

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Summary

Soil structure and aggregation status are fundamental for soil functioning, especially under drought conditions. Saprobic soil fungi can often withstand low moisture environments and are known to influence soil aggregate formation and stability. We examined the ability of fungal amendments to improve soil aggregation and hydrological properties across 2 moisture regimes. A selection of 29 fungal isolates recovered from drought-treated soils, varying in colony density and growth rate, was used for single-strain inoculation into sterilized soil microcosms under either low or high moisture conditions (-0.96 and -0.03 MPa, respectively). After 8 weeks, soil aggregate formation and stability were assessed, as were soil properties, including soil water content, water hydrophobicity, sorptivity, total fungal biomass, and water potential. Fungal inoculation altered soil hydrological properties and improved soil aggregation in a fungal strain and moisture level-dependent manner. Fungal biomass was correlated with enhanced soil aggregate formation and stabilization by connecting soil particles via hyphae and by modifying soil aggregate sorptivity. Water potential improved only when the initial level of moisture was not too low for fungal colonization. Our results show the potential of using fungal inoculation to improve agricultural soil's structure under drought, thereby potentially opening new possibilities in sustainable soil management.

1. 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 (Rawls *et al.*, 2003; Rabot *et al.*, 2018). 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 (Strunk, 2003; Bindraban *et al.*, 2012; Costantini and Lorenzetti, 2013). Soil degradation includes the deformation of the inner soil structure due to changes in climatic conditions, erosion, and other human activities (e.g., compaction related to the construction of roads, grazing, tillage, and land use change) (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) (Horel *et al.*, 2015; Chandrasekhar *et al.*, 2018). 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 bindings forces result from a combination of biotic and abiotic processes (Bronick and Lal, 2005), and the stability of soil aggregates reflects their ability to resist disruption as a result of mechanical forces (tillage), rapid wetting by raindrops and swelling and shrinking in clay soils caused by cycles of drought-rewetting (Papadopoulos, 2011; Fernandes *et al.*, 2015). Soil aggregates are typically grouped by size, with macro and microaggregates being larger and smaller than 250 µm, respectively (Tisdall and Oades, 1982). A soil structure that supports soil functioning includes a wide range of hierarchical orders of aggregates and is dynamically maintained through time (Dexter, 1988), with a higher turnover for soil macroaggregates as compared to microaggregates (Tisdall and 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 (Witkowska-Walczak, 2000; Lipiec *et al.*, 2007; Gelybó *et al.*, 2018) 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 (Six *et al*., 2004; Lehmann and Rillig, 2015), formation of the network of soil pores (Jongmans *et al*., 1997) and altering 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 (Tisdall, 1994; Miller and Jastrow, 2000; Rashid *et al*., 2016). Lehmann *et al*. (2020) identified fungal biomass density as one of the main predictors of soil aggregate stability. Fungi also influence the cohesion of soil particles within soil aggregates through the secretion of proteins, notably glomalin-related soil proteins associated with arbuscular mycorrhiza (AMF), as well as mucilage, polysaccharides, and other extracellular compounds (Rillig and Mummey, 2006; Liu *et al*., 2020). 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 (Rillig, 2005; Chenu and Cosentino, 2011). Together, these properties result in the improvement of soil aggregate stability (Piccolo and Mbagwu, 1999). However, the impact of specific saprobic fungal strains on soil aggregation and hydrophobicity (water repellency) under different levels of moisture is still poorly understood.

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 (i) traverse air-filled soil pores and translocate water through their hyphal networks (Miller and Fitzsimons, 2011; Guhr *et al.*, 2015), (ii) alter their internal osmolarity (Yaakoub *et al.*, 2021), and therefore maintain their turgor (Lew and Levina, 2007) by accumulating compatible solutes such as proline and glycerol in response to lowered water potentials (Kubicek and Druzhinina, 2007; Walker and White, 2017), and when the hyphae are expected to stop growing, many fungi are able to survive by means of (iii) production of specialized cells including spores that survive long periods of dry conditions and resume growth after rewetting (Dijksterhuis, 2019).

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 two 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 two contrasting moisture levels. The high moisture level was related to optimal plant growth as a reference and the low soil moisture level simulated drought. The 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, hydrophobicity, sorptivity, and water potential) and improve soil macroaggregate formation and stabilization, ii) higher fungal growth rate and colony density in agar culture would be associated with greater improvements in soil aggregation and stabilization, and iii) the effects of fungal strain inoculation would depend on the soil moisture level. 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.

2. Experimental procedures

2.1 Soil harvest and isolation of fungal strains

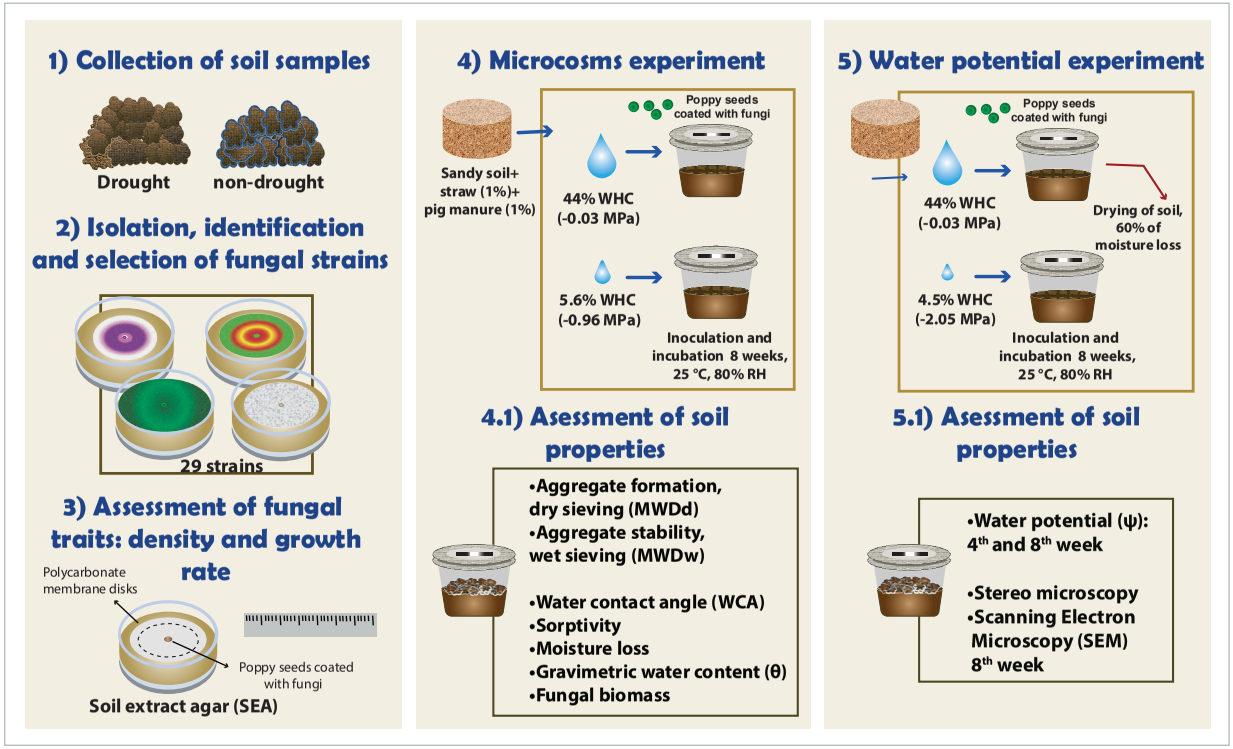
Soil samples were taken from an experimental natural grassland (*Arrhenatherum elatius* association) subjected to drought at Fort Rijnauwen in Utrecht, the Netherlands (52°04'24.8" N 5°10'32.4" E). The experiment had two types of levels of drought: one level with approximately 90% of rainfall reduction 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 ºC 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 ºC until use.

2.2 Selection of fungal strains

A total of 133 fungal strains were isolated and identified (see Supplementary Information S3.1). 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 two criteria: (i) fungal taxa that were abundant according to corresponding operational taxonomic units (OTUs) (Supplementary information S3.2) from the drought soil of isolation compared to the non-drought field treatment, and (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.) (Waghunde *et al.*, 2016; Baron, de Souza Pollo, *et al.*, 2020), and/or advised by fungal experts as interesting strains to evaluate their possible role in the formation of soil aggregates (Westerdijk Fungal Biodiversity Institute, personal communication).



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.

2.3 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 three 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 and Samuels, 1995) and LR5 (Vilgalys and Hester, 1990), and the primers used for ITS amplification were V9G (de Hoog and Gerrits van den Ende, 1998) and LS266 (Masclaux *et al.*, 1995). We also sequenced additional genes to better identify specific fungal species (Supplementary Table S3.1). 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 analyzed on an ABI PRISM 3700 Genetic Analyzer 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 1,000 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).

2.4 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).

2.5 Measurement of fungal traits: colony density and growth rate

For each fungal strain, the colony density and growth rate of fungal colonies were determined for fungal cultures grown on soil extract agar (SEA), which mimics the conditions of the soil used in the microcosm experiment (Fig. 1).

SEA was prepared using the adapted protocol of Hamaki *et al.* (2005). The first step consisted of extracting soil humic acids 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 two sieves, with 100 µm and 50 µm mesh sizes. The resulting filtrate was collected and centrifuged at 4,500 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 aluminum 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 four 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.

2.6 Soil aggregation assay: a microcosm experiment

2.6.1 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 four replicates at two levels of moisture. In addition, we set up two sets of control microcosms without fungal inoculation for each level of moisture, resulting in a total of 248 experimental units.

We selected sandy soil collected from a pig farm in the Netherlands as our model soil (coarse sand). The soil texture for this experiment was chosen due to the relatively 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 not the same as the one used for fungal strain isolation, both did have a sandy texture. We prepared the soil mixture by amending the sandy soil (i) with 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 x 80 mm d, Microbox filter XL, Eco2 NV) and autoclaved three times with time intervals of 24 h (121 ºC, 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, four 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 on 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 (Fig. 1).

After the incubation period, flasks were weighed again, and 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 overnight in a flow cabinet and stored at 4 °C or -20 °C prior to further analyses.

2.6.2 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 trapped air (slaking) using the wet sieving technique (Kemper and 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, 2016). 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:

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

where M_i is the dry mass of the soil aggregates for each size class and $\bar{X}_i$ is the mean diameter of the soil aggregate size class (mm).

The MWD calculated after the wet sieving was designated MWD_w and this measure after dry sieving was given as MWD_d. A stability index (SI) was calculated by adding the MWD_w to the MWD_d.

2.6.3 Measurement of soil water contact angle (WCA) 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 water contact angle, we first placed a small amount of air-dried soil (stored at 4 °C) between two 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 analyzed using a drop shape analysis system at room temperature (Krüss DSA 10 Mk2, Germany). Three measurement replicates were performed for each soil sample.

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 categorized using three 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).

2.6.4 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 ($m_{soil\ wet}$). Then, the soil samples were oven-dried at 70 °C until they no longer experienced weight loss and weighed again ($m_{soil\ dry}$). For each soil sample, the soil gravimetric water content was then determined using the following formula:

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

2.6.5 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 (4,500 rpm, 10 min), and 1 mL of the top phase was mixed with 1 mL of n-hexane, and centrifuged (4,500 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:

$$\text{Ergosterol}\left(\text{mg / kg soil}\right) = \frac{\text{c} * f}{\text{se} - \left(\text{se*m}\right)}$$

With 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.

2.6.6 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 and Chenu, 1995; Walker and White, 2017), and water can move from areas with high to low water potentials (Herman and Bleichrodt, 2022). 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" (Fig. 1). As in the soil aggregate microcosm experiment, each fungal strain was inoculated in four replicate microcosms at two levels of moisture. In addition, we set up two sets of control microcosms (without fungal inoculation), resulting in a total of 248 experimental units.

We placed 60 g of the soil mixture (as used in the soil aggregate microcosm experiment) in plastic flasks, autoclaved, and then dried it (as in section 2.6.1). Thereafter, soil samples were adjusted to 44% (ψ -0.03 MPa) and 4.5% (ψ -2.05 MPa) of the total water holding capacity (45%), representing high moisture and water stress conditions, respectively. The water stress condition is set to ψ -1.5 MPa to yield conditions that are below the permanent wilting point for plants. In each microcosm, four poppy seeds inoculated with fungal strains were added, three 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 three remaining replicates were sampled in a similar way, 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, as to mimic a drying event (Fig. 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.

2.6.7 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.

2.6.8 Statistical analyses

We tested the effect of inoculation on fungal traits: density of colonies and growth rate, and soil properties: soil aggregate formation (MWD_d) and stabilization (MWD_w), 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 soil fungal biomass.

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 analyzed using an analysis of variance (ANOVA). The effects of fungal inoculation on MWD_w, SI, WCA, and soil fungal biomass were analyzed using an ANOVA "type 3" for the effect of interactions of strains and moisture level. The impact on the ψ effect was also analyzed 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 and Bates, 2000), packages "nlme" (Pinheiro *et al.*, 2021) and "car" (Fox and

Weisberg, 2019). The non-parametric effect of fungal strains on MWD_d under the two levels of moisture was analyzed using an ANOVA "type 3" as part of a generalized least means (GLM) with gamma distribution through the package "MASS" (Venables and Ripley, 2002). The pairwise comparison between the means of treatments was analyzed 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 two 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).

3. Results

3.1 Selection and identification of fungal strains

From the drought experimental plots, we selected 29 strains based on the characteristics mentioned in section 2.2. Two strains belonging to *Purpureocillum* sp. (strains 38 and 144) and one to *Acremonium* sp. (strain 139) were selected based upon the fact that these two genera increased in relevant abundance over the course of 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 2 to the Mucoromycota (Supplementary Fig. S3.1). 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.

3.2 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 average of all 29 strains examined (0.06 mg/cm^2/day and 0.81 cm/day, respectively) (Fig. 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 two were among the strains which showed the fastest growth rate (> 3 cm/day). These two genera, and the isolates belonging to them examined in our study, were taxonomically closely related (Supplementary Fig. S3.1).

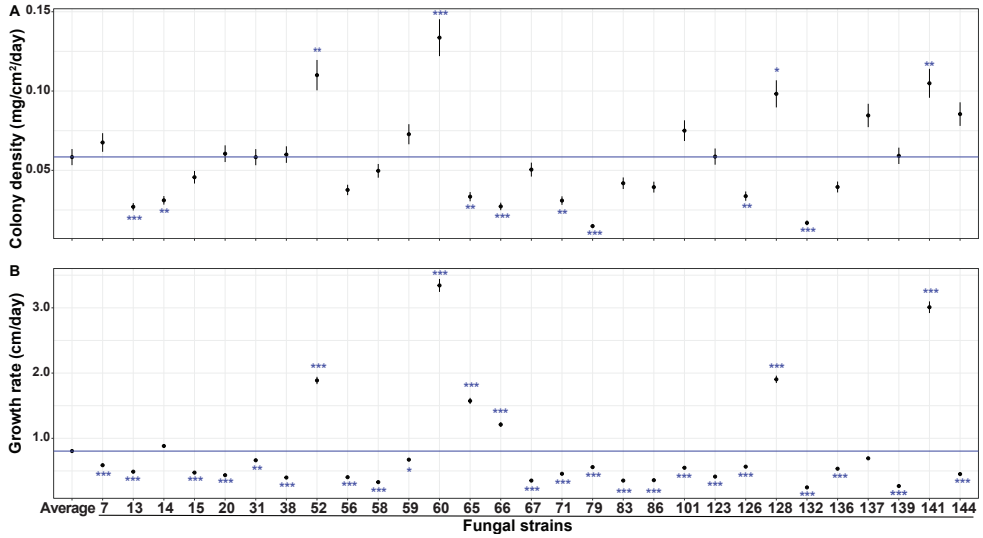


Figure 2. Colony density (mg/cm2/day) (calculated using the colony's biomass divided by the colony's diameter of the last day of assessment and adjusted to the number of days of assessment for each strain (A) and growth rate (cm/day) (B) of fungal colonies grown in soil extract agar (SEA). The average mean density and average mean growth rate are represented by the blue lines. Asterisks indicate significant differences: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ on the top or the bottom of means when they are significantly higher or lower than the respective mean average. The ANOVA is shown in Supplementary Table S3.2. Means, standard error, p-values, and significance are shown in Supplementary Table S3.3.

Some other strains, such as strains 65 (*Linnemannia gamsii*) and 66 (*Absidia* sp.; Mucoromycota) showed a faster growth rate than the mean average 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.

3.3 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 ($p < 0.05$) (Fig. 3 and Supplementary Table S3.2). For instance, strains 20, 52, 67, and 128 showed a higher impact on soil aggregate stability under high soil moisture, yet an opposite pattern was observed for strains 71 or 126, which showed better performance at low moisture.

At the high level of moisture, 83% of the strains significantly increased soil aggregate stability (Fig. 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 (Fig. 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) markedly under both moisture conditions. Strains 7, 14 (*Staphylotrichum acaciicola*), 15, 56, 59, 83 (*Hydropisphaera* sp. nov.), 101, 137, and 139, all strongly stabilized soil aggregates at both moisture conditions with a stability index (SI) at or above 2 mm compared to 0.5 mm in the case of the non-inoculated control soil. A stability index above 3 mm (6 times the control) was observed for 5 strains under low moisture conditions and for 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 (Supplementary Fig. S3.2A). 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 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 stability > 2.5 mm (e.g., strains 31, 59, 71, 83, and 126, which also were close taxonomically) (Supplementary Fig. S3.1) 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 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.

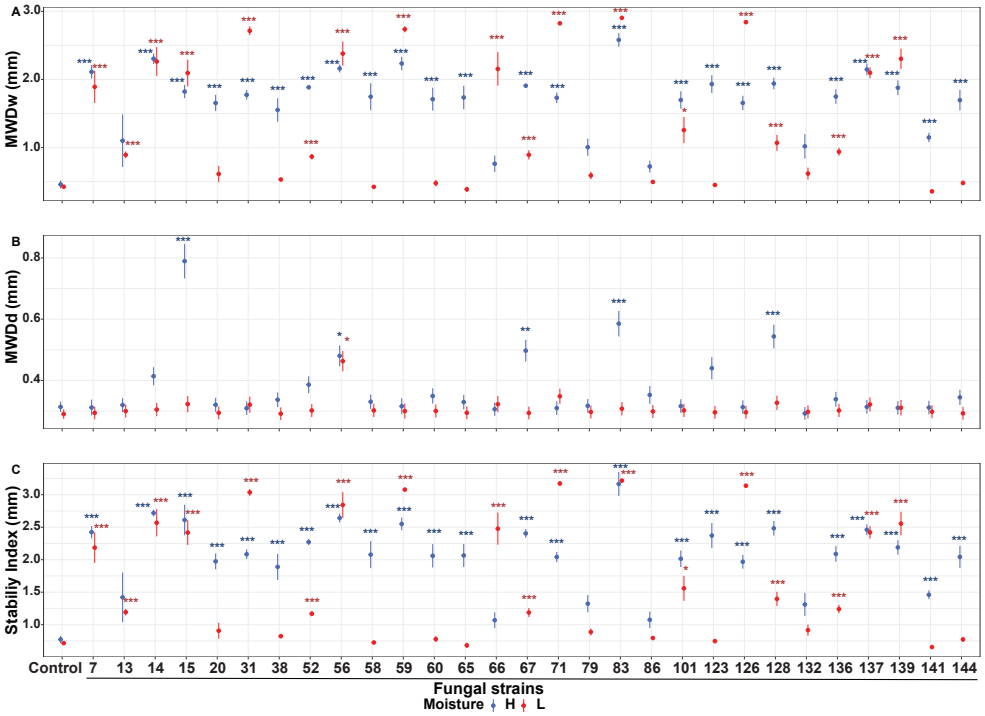


Figure 3. Effect of fungal strain inoculation on soil aggregate stability MWDw (A), aggregate formation MWDd (B), and the stability index, SI (C) which summarizes the previous two parameters, after 8 weeks of incubation. Asterisks indicate significant differences compared to the non-inoculated control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. H and L indicate high and low soil moisture, respectively. The ANOVA is shown in Supplementary Table S3.2. Means, standard error, p-values, and significance are shown in Supplementary Table S3.4.

Over the course of the 8 weeks of incubation, the soil moisture loss was between 8% to 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) (Supplementary Fig. S3.3). 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 (Supplementary, Table S3.2). However, at low soil moisture, fungal inoculation reduced the θ significantly for 58.6% of the fungal strains tested (Fig. 4A). We observed an interaction between the effects of soil moisture and fungal strain on soil water repellency, as indicated by the water contact angle (WCA), but with larger means under low moisture conditions (Fig. 4B). In contrast, soil sorptivity (Fig. 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.

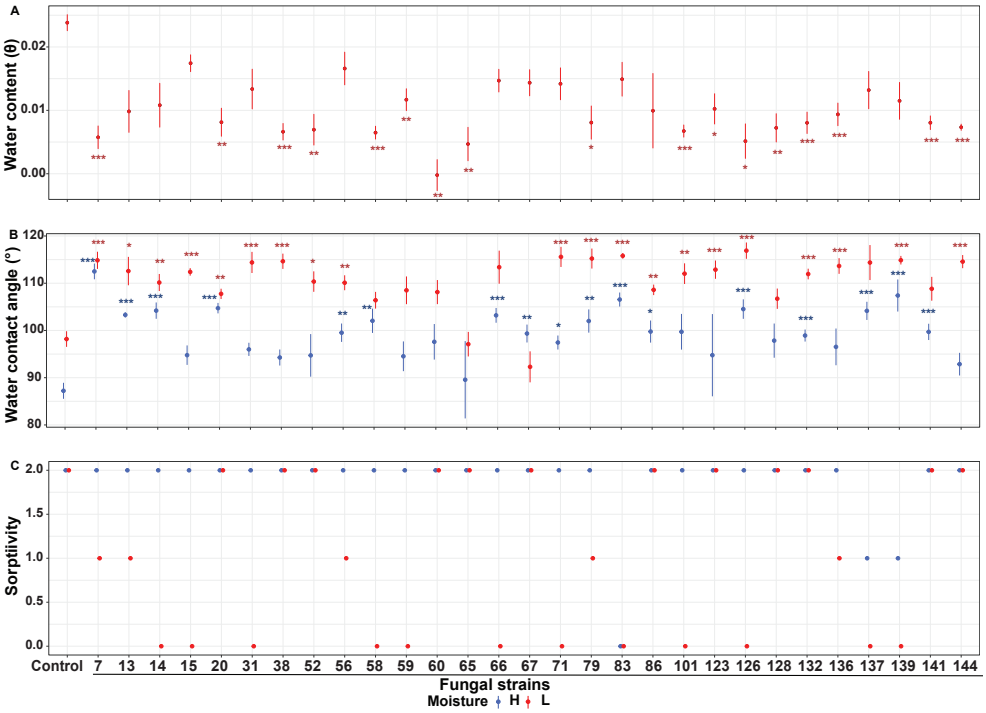


Figure 4. Effect of fungal inoculation on the soil hydrological properties: gravimetric water content (θ) (A), soil water repellency as indicated by the soil water contact angle (WCA) (B) and sorptivity (C) after 8 weeks of incubation. Asterisks indicate significant differences compared to the non-inoculated control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. H and L indicate high and low soil moisture, respectively. The ANOVA is shown in Supplementary Table S3.2. Means, standard error, p-values, and significance are shown in Supplementary Table S3.5.

3.4 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 showed a significant interaction between the effect of fungal strain and moisture level (Fig. 5 and Supplementary Table S3.2). 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 to 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 (Fig. 2) did not appear to grow well in the soil at either level of moisture.

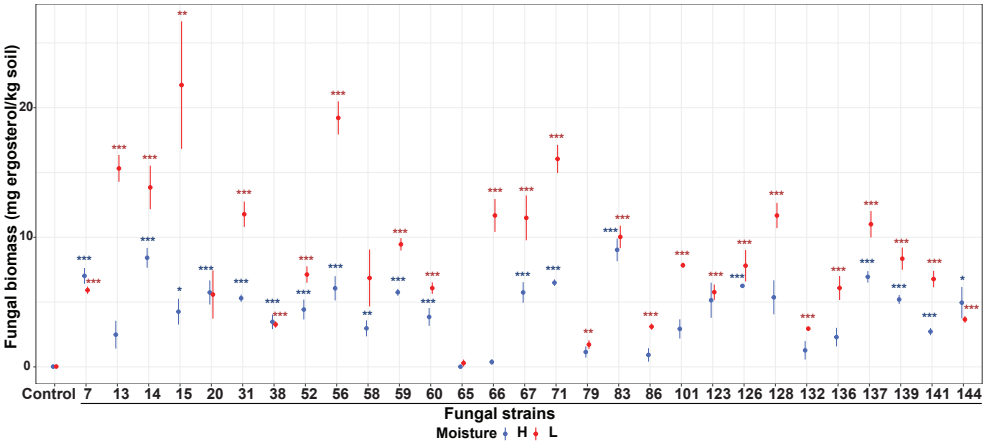


Figure 5. Effect of fungal strain inoculation on soil fungal biomass (ergosterol) after 8 weeks of incubation Asterisks indicate significant differences compared to non-inoculated control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. H and L indicate high and low soil moisture, respectively. The ANOVA is shown in Supplementary Table S3.2. Means, standard error, p-values, and significance are shown in Supplementary Table S3.6.

3.5 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, soil aggregate formation (MWD_d) and stability (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.

Table 1. Correlation matrix between fungal traits, soil hydrological, and physical properties. Abbreviations: (MWDw: soil aggregate stability; MWDd: soil aggregate formation; θ: gravimetric water content, WCA: soil water content 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								----

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

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 (Fig. 6). These analyses showed that the fungal growth rate and WCA had no significant effect in the models tested. We, therefore, removed these parameters from the model to improve the fit. At high soil moisture, the model explained 56% and 8% of the variance of MWD_w and MWD_d, respectively (Fig. 6A and 6C), 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 (Fig. 6B and 6D), 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 had no effect on soil aggregate formation. At both levels of moisture, the fungal biomass measurements showed the highest correlation between soil aggregate formation and stability.

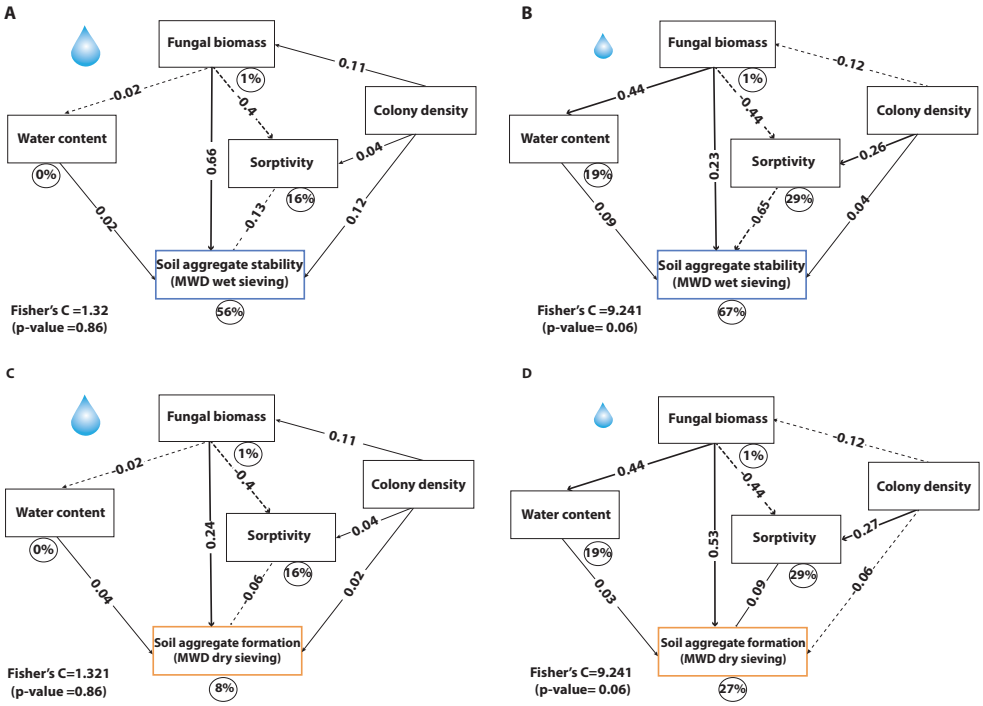


Figure 6. Path analyses of the direct and indirect effect of fungal traits on soil aggregate formation MWDd and stability MWDw. MWDw at high soil moisture (A), MWDw at low soil moisture (B), MWDd (C), and MWDd (D) at high and low moisture, respectively. Numbers on arrows are standardized path coefficients, 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.

3.6 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 MPa and -2.05 MPa) had dropped to -0.35 MPa 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 two of these strains reduced soil water potential to below -60 MPa (Fig. 7A). When the incubation started with a higher level of moisture and was then diminished to 60% of the initial moisture (Fig. 1), 3 of the fungal strains: 66, 67 (*Plenodomus chelidonii*) and 71 (*Paramyrothecium viridisporum*) resulted in a higher soil water potential than the non-inoculated control. These 3 fungal strains also showed strong fungal biomass production under low moisture in the microcosm experiment (Fig. 5), and strain 67 also showed faster than average growth in our plate assay (Fig. 2).

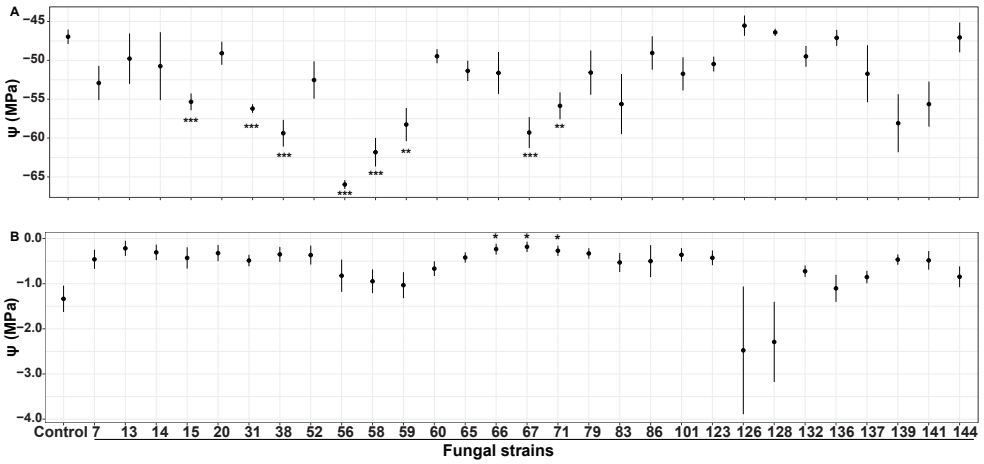


Figure 7. Effect of fungal strains on soil water potential (ψ) at low soil moisture (A), and a higher level of moisture followed by a decrease in the content of moisture of 60% (B). Asterisks indicate significant differences compared to non-inoculated control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. The ANOVA is shown in Supplementary Table S3.2. Means, standard error, p-values, and significance are shown in Supplementary Table S3.5.

3.7 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 (Fig. 8). As expected from the assessment of fungal biomass, we observed higher hyphal densities in inoculated soils under low soil moisture (Fig. 8D and 8F), as compared to high soil moisture, where more reproductive structures were identified (Fig. 8A and 8E). The distribution of

hyphae was not homogeneous, with specific patches of high hyphal density (e.g., Fig. 8C) or a high density of reproductive structures (e.g., Fig. 8E).

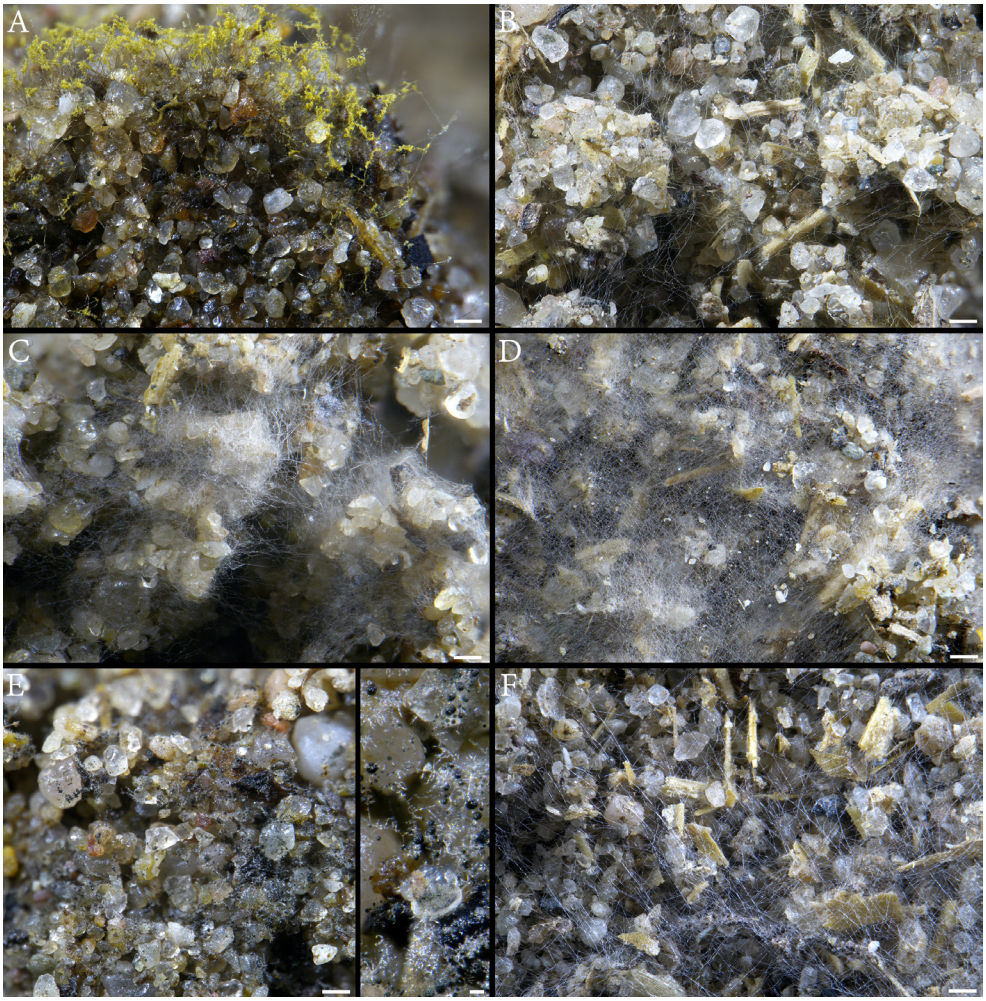


Figure 8. Images of fungal strains in soil (Stereomicroscopy, 16x). Strain 14 (*Staphylotrichum acaciicola*) at high soil moisture (A) produced reproductive structures (yellow), which were not observed for the same strain at low soil moisture (B). This pattern was similar for strain 126 (*Gliomastix 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 µm and 50 µm in the inset of (E).

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 (Fig. 9C and 9D). It also allowed us to observe the detailed structure of reproductive organs (Fig. 9A and 9B). 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 (Supplementary Fig. 3.4).

4. 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.

4.1 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 macroaggregation formation and stabilization, which were tested using the mean weight diameter (MWD). There is a range of evidence implicating soil fungi as important determinants of soil aggregation and soil structure. For instance, Beare *et al.* (1997), showed that 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 (Wu *et al.*, 2008; Ji *et al.*, 2019). 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).

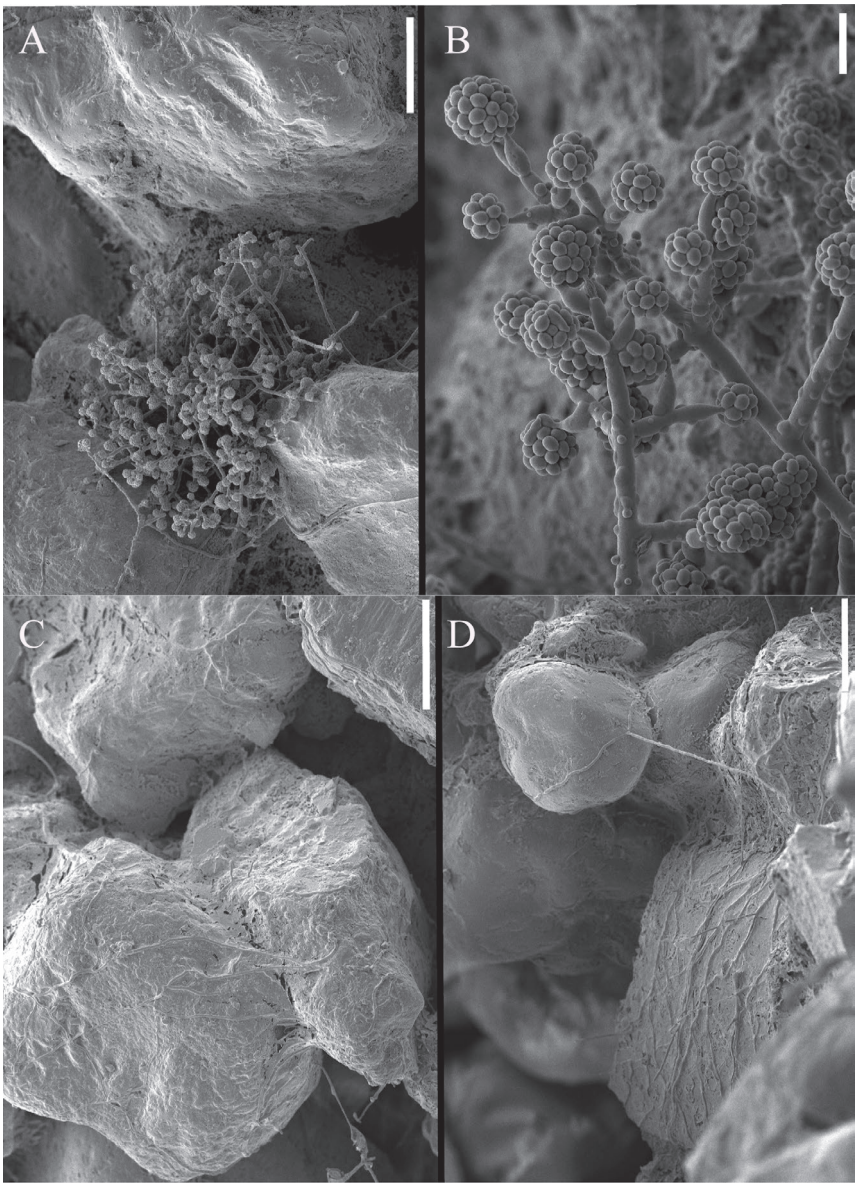


Figure 9. Images of fungal strains in soil (cryo-scanning-electron-microscopy) at high moisture. (A) Intermittent patches of well-developed mycelium on the soil surface (strain 141, *Trichoderma linzhiense*). Many conidiophores and conidia are also visible. Some hyphae run over the surface of the grains 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 two soil particles and illustrating the capacity of fungi to bind soil particle content. Bars are 10 µm (B) and 100 µm (A, C, D).

For the majority of 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% 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 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 behavior 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. The fungal colony density for most of our strains was between 0.03 to 0.08 mg/cm^2, determined as 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 (Fig. 6), we did not see 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 and 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 (Söderström, 1979; Gupta and Germida, 1988; Cosentino *et al.*, 2006; Erktan, Rillig, *et al.*, 2020).

Soil water hydrophobicity (water contact angle) and soil sorptivity were negatively correlated, and this last property partially explained soil aggregate stability under low soil moisture (Fig. 6). Vogelmann *et al.* (2017), also showed that high values of soil water repellency reduced soil sorptivity, and a high soil hydrophobicity can promote the increased stability of soil aggregates (Chenu and Cosentino, 2011; Vogelmann *et al.*, 2013). Soil water repellency is often linked to fungal activity (York and Canaway, 2000), and Hallett *et al.* (2001) showed that the inhibition of the fungal growth decreased soil repellency after 10 days of incubation in amended soils. 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 (Fig. 8 and Fig. 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 and Berliner, 1974). This notion was corroborated de Jonge *et al.* (1999) who found that the finest fractions (< 0.063 mm) of two 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.

4.2 Effects of fungal strain inoculation under different 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 two contrasting soil moisture levels, and we indeed found that the effect of fungal strains was affected by soil moisture. The loss of moisture during the period of incubation 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 means were found at the lower moisture level. In contrast, the aggregate formation was larger at higher moisture content for a number of fungal strains. Some strains showed a higher aggregate formation and stability under the lower moisture content (e.g., strain 71, *Paramyrothecium viridosporum*) 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 taxonomic groups of fungi, which may explain why some strains perform better under specific moisture conditions. The vast majority of yeasts and fungi are active within the range of 1 to 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 and Griffin, 1981). Some fungal traits, such as hyphal extension rates and conidial germination, decline with decreasing water availability as Jackson *et al.* (1991) reported. These authors showed that 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.

Fungal biomass was higher and positively correlated with soil aggregate formation and stability at the low moisture content tested. Fungi can occur across a wide range of moisture levels, but the optimal moisture level for growth may be species-specific. For instance, highly xerophilic organisms, such as *Xeromyces bisporus*, can experience optimal growth conditions at relatively low moisture levels (approx. 0.84 a_w, -22.4 MPa), yet display slow or no growth close to a high water activity of 1 (0 MPa) (Magan, 2007). Robert and Chenu (1995) suggest that the optimum moisture level for fungal growth starts at pF2 and pF 3 (ψ 0.01 MPa), and microbial activity decreases outside this range [pF = log(ψ)]. Our high soil moisture treatment was close to 0 MPa, which may explain the lower fungal biomass we observed as compared to the low moisture treatment (-0.96 MPa). In other words, our starting low-moisture treatment may have been more optimal for some of our fungal strains. Higher fungal biomass under low soil moisture levels may stem from a higher proportion of large air-filled pores, which is the preferred habitat of fungal hyphae (Soufan *et al.*, 2018).

Our fungal strains showed different growth patterns under contrasting levels of moisture (Fig. 8). Mycelial growth adopts different patterns of branching depending on the microenvironmental conditions (Walker and White, 2017). In addition, 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 fungi depend on moisture gradients less 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 (Ritz and Young, 2004; Miller and Fitzsimons, 2011; Bielčik *et al.*, 2019). 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 in our soil microcosm.

We also found that soil hydrophobicity had an inverse 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 research, 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 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).

4.3 Effect of fungal strain inoculation on soil water potential

Fungi can change the soil structure, and soil structure in turn affects water retention properties (Augé *et al.*, 2001). The water potential in our water potential experiment dropped dramatically to values as low as -65 MPa for the low moisture treatment after two months of incubation. Robert & Chenu (1995) argued that 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. 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. The soil water potential during part of our low moisture treatment was lower than the limits of growth and metabolism of many strains. It is possible the mycelium that was formed during the first stages of the treatments could still help to maintain soil structure, as can be visualized in Supplementary Fig. S3.4. 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.

The results from our experiment 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, *A. niger* and *P. rubens* that had experienced

moisture dynamics and swollen conidia, germlings, and microcolonies could not reinitiate growth after being retransferred from a low relative humidity medium to one with higher humidity (Segers *et al.*, 2016), and these dynamics were associated with oxidative stress (e.g., H_2O_2 and catalase activity) (Wu and 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 in order to determine if fungal inoculation indeed enhances soil hydrological properties when facing low moisture conditions. However, we did not find a general relationship between water potential and realized fungal biomass. 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. It is likely that a higher level of biomass could have represented a higher water content and thereby a higher water potential for the system as a whole.

5. 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 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. However, at low soil moisture content, fungal inoculation did not increase the water content in the system as we expected. 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. Our results also suggest that initial optimal soil moisture before a drying event may support sufficient fungal activity and growth to help with the mitigation of the prolonged effects of drought periods, even after fungal activity again decreases.

We propose that ecologically informed strategies of fungal inoculation could represent viable options to help maintain and improve soil structure to the service of plant productivity under conditions of drought. 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.

6. Acknowledgements

We kindly thank Kathryn Barry and Yann Hautier for their support in the statistical analyses, and the latter for providing access to the drought experimental field site. We thank Eiko Kuramae and Ciska Raaijmakers from the Netherlands Institute of Ecology (NIOO-KNAW) for their support with the quantification of ergosterol. We also thank Peter Veenhuizen for his highly valued technical support.

7. Supplementary information

S3.1. Molecular identification of fungal strains

Total DNA from monoclonal cultures that were grown for 2-4 days on malt extract agar (MEA) was isolated using the DNeasy PowerSoil Pro kit (Qiagen, Germany). The internal transcribed spacer (ITS) was sequenced, and the PCR amplification was carried out using the following set of primers: (1) ITS1F and ITS86R (Gardes and Bruns, 1993) and (2) ITS86F and ITS4 (White *et al.*, 1990). The first primer pair (ITS1) targeted the ITS1 region between the 18S rRNA gene and the 5.8S rRNA gene. The second primer pair (ITS2) targeted the ITS2 region between the 5.8S rRNA gene and the 28S. PCR thermal cycling parameters were: initial denaturation at 95 °C for 3 min, subsequent denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min repeated for 33 cycles, with a final extension at 72 °C for 5 min. The PCR reactions were carried out using the DreamTaq Green Master mix (Thermo Scientific, USA) and primers at 5 µM. Successful PCR amplification was confirmed by visualizing 5 µl of the products following electrophoresis on a 1.5% agarose TAE gel. The final PCR reactions were cleaned up using AMPureXP beads (Beckman Coulter, USA) and the DNA sequencing of the amplicon was performed by Macrogen (the Netherlands). The sequence data were assembled and nucleotide sequence alignment using the Bioedit software 7.2 and compared to the computer program Blast from the National Center for Biotechnology Information (NCBI).

S3.2. Selection of the fungal strains

From the total isolated fungi, the taxonomic units to genus level were selected and compared to the ones from a next-generation sequencing project (done in another study) from the experimental fields under drought at Fort Rhijnauwen (section 2.1). Fifty Operational Taxonomic Units (OTUs) were selected and then we made a comparison between the 6 accessions under drought, where the samples were compared against the controls from the same accessions $p < 0.05$, using a t-test

in the software R version 4.12. The OTUs for the genera *Acremonium* and *Purpureocillum* were significantly different when compared to the control and selected for the experiments on aggregation.

Supplementary Figures

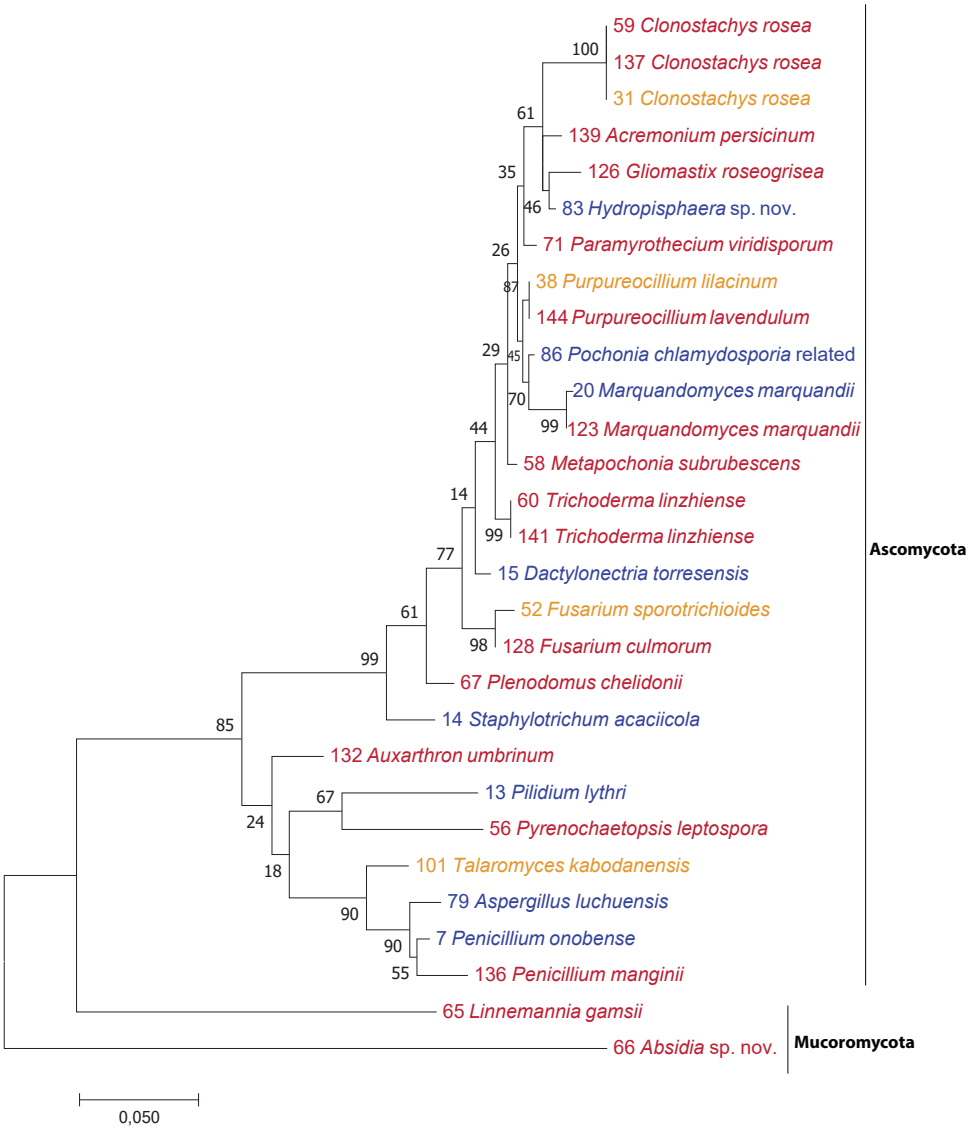


Figure S3.1. ITS phylogeny of the 29 fungal strains was inferred by the Unweighted Pair roup Method (UPGMA) with arithmetic mean analysis. Numbers at internodes refer to confidence estimates based on 1000 bootstraps. The number on the right indicates the isolate number and the species which are categorized into two subphyla: Ascomycota and Mucoromycota. The colors: red (D90), yellow (D50), and blue (non-drought) indicate the experimental field where strains were isolated.

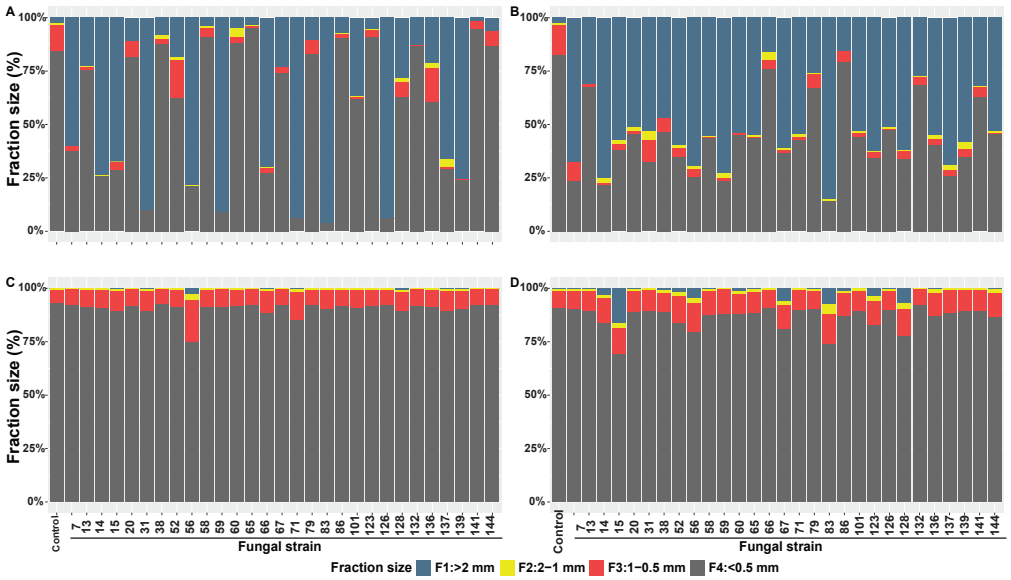


Figure S3.2. Mean percentage change in the < 0.5 mm, 0.5-1.0 mm, 1.0 - 2.0 mm, and > 2 mm fraction sizes after 8 weeks of incubation and collected by wet sieving in low (A) and high moisture (B), and under dry sieving under low (C), and (D) high moisture, respectively.

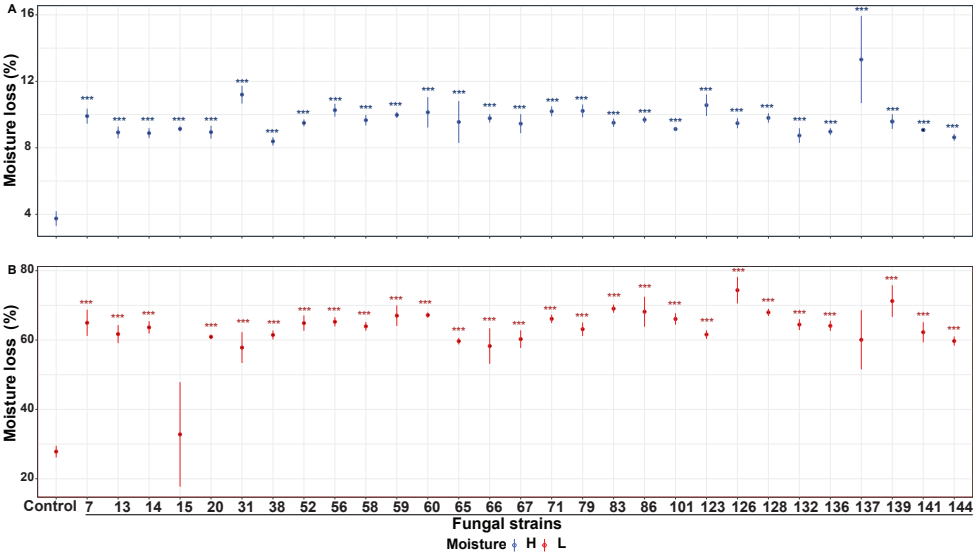


Figure S3.3. Moisture loss for soil inoculated with 29 fungal strains and one non-inoculated control after 8 weeks of incubation at high moisture (A) and low moisture content (B). Asterisks indicate significant differences when compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. The ANOVA is shown in Supplementary Table S3.2. Means, standard error, p-values, and significance are shown in Supplementary Table S3.5.

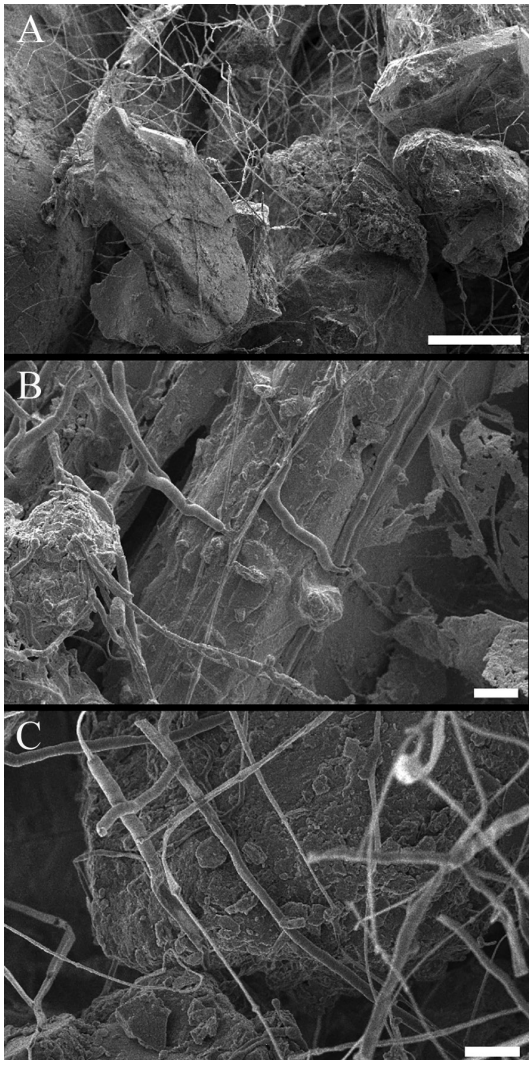


Figure S3.4. Images of soil inoculated with strain 83 (*Hydropisphaera* sp. nov.) at a lower water activity. (A) Development of numerous hyphae that connect soil particles. (B) Detail of hyphae on soil particles. Some of the cells have a turgid appearance and develop close to soil grains and other cells appear to be collapsed. This illustrates that collapsed hyphae and probably dead still can connect soil particles. (C) A second example of shriveled hyphae between soil particles. Bars are 10 µm (B and C) and A 100 µm.

Supplementary Tables

Table S3.1. Keys for identification of fungal species using different sets of primers

Genus	Name of locus	Name of gene	Forward primer	Reference	Reverse primer	Reference
Absidia	No additional loci required for species identification					
Acremonium	Partial RPB2	RPB2	5F2	(Liu *et al.*, 1999)	7cr	(Liu *et al.*, 1999)
Aspergillus	Partial beta-tubulin	BenA	BT2a	(Glass and Donaldson, 1995)	BT2b	(Glass and Donaldson, 1995)
	Partial calmodulin	CaM	CMD5	(Hong *et al.*, 2006)	CMD6	(Hong *et al.*, 2006)
Auxarthron	No additional loci required for species identification					
Clonostachys	No additional loci required for species identification					
Dactylonectria	Partial elongation factor 1 alpha	EF1a	EF1	(O'Donnell *et al.*, 1998)	EF2	(O'Donnell *et al.*, 1998)
Fusarium	Partial elongation factor 1 alpha	EF1a	EF1	(O'Donnell *et al.*, 1998)	EF2	(O'Donnell *et al.*, 1998)
	Partial RPB2	RPB2	5F2	(Liu *et al.*, 1999)	7cr	(Liu *et al.*, 1999)
Gliomastix	No additional loci required for species identification					
Hydropisphaera	No additional loci required for species identification					
Linnemannia	No additional loci required for species identification					
Marquandomyces	Partial beta-tubulin	BenA	BT2a	(Glass and Donaldson, 1995)	BT2b	(Glass and Donaldson, 1995)
Metapochonia	No additional loci required for species identification					
Paramyrothecium	Partial beta-tubulin	BenA	BT2a	(Glass and Donaldson, 1995)	BT2b	(Glass and Donaldson, 1995)
	Partial RPB2	RPB2	5F2	(Liu *et al.*, 1999)	7cr	(Liu *et al.*, 1999)
Pilidium	No additional loci required for species identification					
Penicillium	Partial beta-tubulin	BenA	BT2a	(Glass and Donaldson, 1995)	BT2b	(Glass and Donaldson, 1995)
Plenodomus	No additional loci required for species identification					
Pochonia	Partial RPB2	RPB2	5F2	(Liu *et al.*, 1999)	7cr	(Liu *et al.*, 1999)
Purpureocillium	Partial beta-tubulin	BenA	BT2a	(Glass and Donaldson, 1995)	BT2b	(Glass and Donaldson, 1995)
Pyrenochaetopsis	No additional loci required for species identification					
Staphylotrichum	Partial beta-tubulin	BenA	T1	(O'Donnell and Cigelnik, 1997)	Tub4RD	(Groenewald *et al.*, 2013)
	Partial RPB2	RPB2	5F2	(Liu *et al.*, 1999)	7cr	(Liu *et al.*, 1999)
Talaromyces	Partial beta-tubulin	BenA	BT2a	(Glass and Donaldson, 1995)	BT2b	(Glass and Donaldson, 1995)
Trichoderma	Partial elongation factor 1 alpha	EF1a	EF1-728F	(Carbone and Kohn, 1999)	EF986R	(Carbone and Kohn, 1999)

Table S3.2. Summary of the analysis of variance (ANOVA) for the inoculation of 29 strains on the fungal traits: density of colony and growth rate tested at one level of moisture and soil properties: water loss, gravimetric water content (θ), and water potential (ψ) for high and low levels of moisture independently and the two-ANOVA (error type III) for the MWD_w, MWD_d, SI, water contact angle (WCA) and fungal biomass with the effect of interaction. P-values <0.05 are considered significant and highlighted in bold.

ANOVA type 1	High moisture		Low moisture	
	F value	p-value	F value	p-value
log (density)	$F_{29,90}$ = 36.99	**<.0001**		
log (growth rate)	$F_{29,92}$ = 520.34	**<.0001**		
Water loss	$F_{1,29}$= 6.4	**<.0001**	$F_{1,29}$= 20.09	**<.0001**
θ	$F_{29,89}$= 0.88	0.6414	$F_{1,29}$= 11.73	**<.0001**
ψ	$F_{1,29}$= 19.08	**<.0001**	$F_{1,29}$= 174.01	**<.0001**

ANOVA type 3		MWD_w		MWD_d		SI		WCA		sqrt (biomass)	
	df	Chisq	p-value	F value	p-value	Chisq	p-value	Chisq	p-value	Chisq	p-value
Strain	29	1104.00	**<.0001**	12.57	**<.0001**	1194.00	**<.0001**	241.52	**<.0001**	555.72	**<.0001**
Moisture	1	0.29	0.59	1.04	**<.0001**	0.92	0.3388	21.42	**<.0001**	0.01	0.9039
Strain* Moisture	29	1467.77	**<.0001**	5.22	**<.0001**	1337.45	**<.0001**	135.53	**<.0001**	259.97	**<.0001**

Table S3.3 Mean, standard error (SE) (±), p-value, and significance for the density (mg/cm²/day), and growth rate (cm/day) for 29 fungal colonies. Asterisks indicate significant differences compared to the mean average: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test. Asterisks in red mean significantly lower than the average.

Isolate code	Molecular identity	Density (mg/cm²/day)				Growth rate (cm/day)			
		$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Mean average	0.058	0.005			0.805	0.024		
7	*Penicillium onobense*	0.068	0.006	1.000		0.588	0.017	0.000	***
13	*Pilidium lythri*	0.027	0.002	0.000	***	0.488	0.015	0.000	***
14	*Staphylotrichum acaciicola*	0.031	0.003	0.001	**	0.883	0.026	0.927	
15	*Dactylonectria torresensis*	0.046	0.004	0.973		0.474	0.014	0.000	***
20	*Marquandomyces marquandii*	0.060	0.005	1.000		0.435	0.013	0.000	***
31	*Clonostachys rosea*	0.058	0.005	1.000		0.664	0.018	0.002	**
38	*Purpureocillium lilacinum*	0.060	0.005	1.000		0.399	0.012	0.000	***
52	*Fusarium sporotrichioides*	0.110	0.010	0.001	**	1.887	0.056	0.000	***
56	*Pyrenochaetopsis leptospora*	0.038	0.003	0.119		0.404	0.012	0.000	***
58	*Metapochonia subrubescens*	0.050	0.004	1.000		0.329	0.010	0.000	***
59	*Clonostachys rosea*	0.073	0.006	0.993		0.673	0.020	0.014	*
60	*Trichoderma linzhiense*	0.134	0.012	0.000	***	3.343	0.099	0.000	***
65	*Linnemannia gamsii*	0.033	0.003	0.006	**	1.574	0.047	0.000	***
66	*Absidia* sp. nov.	0.027	0.002	0.000	***	1.211	0.036	0.000	***
67	*Plenodomus chelidonii*	0.050	0.004	1.000		0.352	0.010	0.000	***
71	*Paramyrothecium viridisporum*	0.031	0.003	0.001	**	0.457	0.012	0.000	***
79	*Aspergillus luchuensis*	0.015	0.001	0.000	***	0.559	0.017	0.000	***
83	*Hydropisphaera* sp. Nov.	0.042	0.004	0.631		0.352	0.010	0.000	***
86	*Pochonia chlamydosporia*	0.039	0.003	0.285		0.358	0.011	0.000	***
101	*Talaromyces kabodanensis*	0.075	0.007	0.964		0.548	0.016	0.000	***
123	*Marquandomyces marquandii*	0.059	0.005	1.000		0.413	0.012	0.000	***
126	*Gliomastix roseogrisea*	0.034	0.003	0.008	**	0.566	0.017	0.000	***
128	*Fusarium culmorum*	0.098	0.009	0.016	*	1.904	0.057	0.000	***
132	*Auxarthron umbrinum*	0.017	0.001	0.000	***	0.248	0.007	0.000	***
136	*Penicillium manginii*	0.039	0.003	0.294		0.534	0.016	0.000	***
137	*Clonostachys rosea*	0.085	0.007	0.392		0.693	0.021	0.120	
139	*Acremonium persicinum*, possible new sp.	0.059	0.005	1.000		0.269	0.008	0.000	***
141	*Trichoderma linzhiense*	0.105	0.009	0.002	**	3.009	0.09	0.000	***
144	*Purpureocillium lavendulum*	0.085	0.007	0.338		0.452	0.01	0.0000	***

Table S3.4. Mean, standard error (SE) (±), p-value, and significance for the MWDw (mm), MWDd (mm), and SI (mm) for the soil inoculated with 29 fungal strains after 8 weeks under low and high levels of moisture. Asterisks indicate significant differences compared to control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Isolate code	Molecular identity	Moisture	MWD_w (mm)				MWD_d (mm)				Stability index (mm)			
			$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Control	Low	0.426	0.020			0.290	0.016			0.716	0.019		
7	*Penicillium onobense*	Low	1.892	0.236	0.000	***	0.294	0.021	1.000		2.186	0.236	0.000	***
13	*Pilidium lythri*	Low	0.893	0.051	0.000	***	0.300	0.021	1.000		1.193	0.051	0.000	***
14	*Staphylotrichum acaciicola*	Low	2.264	0.210	0.000	***	0.305	0.022	1.000		2.569	0.209	0.000	***
15	*Dactylonectria torresensis*	Low	2.095	0.196	0.000	***	0.323	0.027	1.000		2.417	0.191	0.000	***
20	*Marquandomyces marquandii*	Low	0.613	0.122	1.000		0.294	0.021	1.000		0.907	0.122	1.000	
31	*Clonostachys rosea*	Low	2.715	0.065	0.000	***	0.321	0.026	1.000		3.036	0.054	0.000	***
38	*Purpureocillium lilacinum*	Low	0.531	0.025	0.441		0.291	0.021	1.000		0.823	0.026	0.456	
52	*Fusarium sporotrichioides*	Low	0.866	0.040	0.000	***	0.302	0.022	1.000		1.168	0.039	0.000	***
56	*Pyrenochaetopsis leptospora*	Low	2.380	0.177	0.000	***	0.463	0.033	0.006	**	2.843	0.198	0.000	***
58	*Metapochonia subrubescens*	Low	0.423	0.024	1.000		0.302	0.022	1.000		0.725	0.026	1.000	
59	*Clonostachys rosea*	Low	2.737	0.044	0.000	***	0.300	0.025	1.000		3.079	0.020	0.000	***
60	*Trichoderma linzhiense*	Low	0.478	0.049	1.000		0.300	0.021	1.000		0.778	0.046	1.000	
65	*Linnemannia gamsii*	Low	0.387	0.039	1.000		0.294	0.021	1.000		0.681	0.042	1.000	
66	*Absidia* sp. nov.	Low	2.155	0.247	0.000	***	0.322	0.027	1.000		2.477	0.248	0.000	***
67	*Plenodomus chelidonii*	Low	0.893	0.069	0.000	***	0.294	0.021	1.000		1.187	0.069	0.000	***
71	*Paramyrothecium viridisporum*	Low	2.825	0.026	0.000	***	0.348	0.025	1.000		3.173	0.039	0.000	***
79	*Aspergillus luchuensis*	Low	0.591	0.056	0.856		0.297	0.021	1.000		0.888	0.054	0.711	
83	*Hydropisphaera* sp. nov.	Low	2.905	0.029	0.000	***	0.308	0.022	1.000		3.217	0.026	0.000	***
86	*Pochonia chlamydosporia*	Low	0.496	0.026	0.998		0.299	0.021	1.000		0.794	0.023	0.942	
101	*Talaromyces kabodanensis*	Low	1.257	0.193	0.032	*	0.302	0.022	1.000		1.560	0.193	0.026	*
123	*Marquandomyces marquandii*	Low	0.451	0.029	1.000		0.296	0.021	1.000		0.747	0.031	1.000	
126	*Gliomastix roseogrisea*	Low	2.842	0.028	0.000	***	0.296	0.021	1.000		3.138	0.032	0.000	***
128	*Fusarium culmorum*	Low	1.069	0.117	0.000	***	0.327	0.023	1.000		1.396	0.109	0.000	***
132	*Auxarthron umbrinum*	Low	0.618	0.088	0.998		0.297	0.021	1.000		0.916	0.086	0.994	
136	*Penicillium manginii*	Low	0.938	0.058	0.000	***	0.302	0.022	1.000		1.240	0.065	0.000	***
137	*Clonostachys rosea*	Low	2.098	0.082	0.000	***	0.322	0.023	1.000		2.420	0.097	0.000	***
139	*Acremonium persicinum*, possible new sp.	Low	2.304	0.148	0.000	***	0.311	0.026	1.000		2.556	0.182	0.000	***
141	*Trichoderma linzhiense*	Low	0.357	0.010	0.639		0.298	0.021	1.000		0.655	0.008	0.777	
144	*Purpureocillium lavendulum*	Low	0.480	0.035	1.000		0.293	0.021	1.000		0.773	0.036	1.000	

Table S3.4. continued. Mean, standard error (SE) (±), p-value, and significance for the MWDw (mm), MWDd (mm), and SI (mm) for the soil inoculated with 29 fungal strains after 8 weeks under a low and high levels of moisture. Asterisks indicate significant differences compared to control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Isolate code	Molecular identity	Moisture	MWD_w (mm)				MWD_d (mm)				Stability index (mm)			
			$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Control	High	0.459	0.059			0.314	0.017			0.773	0.056		
7	*Penicillium onobense*	High	2.112	0.098	0.000	***	0.312	0.026	1.000		2.423	0.095	0.000	***
13	*Pilidium lythri*	High	1.101	0.385	1.000		0.320	0.023	1.000		1.421	0.382	1.000	
14	*Staphylotrichum acaciicola*	High	2.305	0.076	0.000	***	0.414	0.029	0.754		2.718	0.050	0.000	***
15	*Dactylonectria torresensis*	High	1.822	0.097	0.000	***	0.789	0.056	0.000	***	2.611	0.235	0.000	***
20	*Marquandomyces marquandii*	High	1.654	0.119	0.000	***	0.321	0.023	1.000		1.974	0.122	0.000	***
31	*Clonostachys rosea*	High	1.774	0.070	0.000	***	0.309	0.022	1.000		2.084	0.074	0.000	***
38	*Purpureocillium lilacinum*	High	1.552	0.173	0.000	***	0.337	0.024	1.000		1.889	0.201	0.000	***
52	*Fusarium sporotrichioides*	High	1.885	0.025	0.000	***	0.386	0.028	0.995		2.271	0.047	0.000	***
56	*Pyrenochaetopsis leptospora*	High	2.161	0.058	0.000	***	0.480	0.034	0.024	*	2.642	0.068	0.000	***
58	*Metapochonia subrubescens*	High	1.747	0.198	0.000	***	0.331	0.024	1.000		2.078	0.207	0.000	***
59	*Clonostachys rosea*	High	2.234	0.099	0.000	***	0.316	0.026	1.000		2.550	0.097	0.000	***
60	*Trichoderma linzhiense*	High	1.711	0.168	0.000	***	0.349	0.025	1.000		2.060	0.180	0.000	***
65	*Linnemannia gamsii*	High	1.736	0.172	0.000	***	0.329	0.023	1.000		2.065	0.177	0.000	***
66	*Absidia* sp. nov.	High	0.763	0.120	0.994		0.306	0.022	1.000		1.069	0.121	0.996	
67	*Plenodomus chelidonii*	High	1.908	0.029	0.000	***	0.497	0.035	0.008	**	2.405	0.067	0.000	***
71	*Paramyrothecium viridisporum*	High	1.732	0.078	0.000	***	0.310	0.022	1.000		2.041	0.079	0.000	***
79	*Aspergillus luchuensis*	High	1.006	0.126	0.105		0.317	0.023	1.000		1.323	0.132	0.135	
83	*Hydropisphaera* sp. nov.	High	2.580	0.101	0.000	***	0.585	0.042	0.000	***	3.166	0.184	0.000	***
86	*Pochonia chlamydosporia*	High	0.722	0.088	0.968		0.353	0.029	1.000		1.075	0.127	0.997	
101	*Talaromyces kabodanensis*	High	1.698	0.128	0.000	***	0.316	0.023	1.000		2.014	0.128	0.000	***
123	*Marquandomyces marquandii*	High	1.932	0.130	0.000	***	0.440	0.036	0.583		2.372	0.192	0.000	***
126	*Gliomastix roseogrisea*	High	1.655	0.105	0.000	***	0.313	0.022	1.000		1.968	0.107	0.000	***
128	*Fusarium culmorum*	High	1.940	0.085	0.000	***	0.544	0.039	0.000	***	2.484	0.113	0.000	***
132	*Auxarthron umbrinum*	High	1.019	0.181	0.761		0.292	0.021	1.000		1.311	0.179	0.803	
136	*Penicillium manginii*	High	1.750	0.107	0.000	***	0.339	0.024	1.000		2.088	0.120	0.000	***
137	*Clonostachys rosea*	High	2.147	0.086	0.000	***	0.314	0.022	1.000		2.460	0.084	0.000	***
139	*Acremonium persicinum*, possible new sp.	High	1.879	0.108	0.000	***	0.311	0.022	1.000		2.189	0.111	0.000	***
141	*Trichoderma linzhiense*	High	1.149	0.070	0.000	***	0.312	0.022	1.000		1.461	0.068	0.000	***
144	*Purpureocillium lavendulum*	High	1.696	0.151	0.000	***	0.345	0.025	1.000		2.043	0.172	0.000	***

Table S3.5. Mean, standard error (SE) (±), p-value, and significance for the moisture loss (%), gravimetric water content (θ), water contact angle (WCA), and water potential (Ψ) after 8 weeks of growth under a low and high level of moisture. Asterisks indicate significant differences compared to control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test. Asterisks in red mean significantly lower than the mean of control.

Isolate code	Molecular identity	Moisture	Moisture loss (%)				θ				WCA (°)				Ψ (Mpa)			
			$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Control	Low	27.841	1.691			1.7E-02	6.3E-04			98.186	1.655			-46.963	0.933		
7	*Penicillium onobense*	Low	64.972	3.774	0.000	***	7.9E-03	8.9E-04	0.000	***	114.850	1.818	0.000	***	-52.917	2.208	0.710	
13	*Pilidium lythri*	Low	61.713	2.600	0.000	***	9.9E-03	1.7E-03	0.331		112.575	3.005	0.044	*	-49.780	3.241	1.000	
14	*Staphylotrichum acaciicola*	Low	63.639	1.804	0.000	***	1.0E-02	1.7E-03	0.517		110.150	1.807	0.003	**	-50.753	4.377	1.000	
15	*Dactylonectria torresensis*	Low	32.790	15.075	1.000		1.4E-02	6.6E-04	0.179		112.400	0.802	0.000	***	-55.337	1.081	0.000	***
20	*Marquandomyces marquandii*	Low	60.917	0.649	0.000	***	9.1E-03	1.1E-03	0.003	**	107.750	1.090	0.004	**	-49.087	1.482	1.000	
31	*Clonostachys rosea*	Low	57.824	4.451	0.000	***	1.2E-02	1.6E-03	0.657		114.400	2.237	0.000	***	-56.203	0.551	0.000	***
38	*Purpureocillium lilacinum*	Low	61.463	1.273	0.000	***	8.3E-03	6.5E-04	0.000	***	114.650	1.615	0.000	***	-59.373	1.720	0.000	***
52	*Fusarium sporotrichioides*	Low	64.870	2.229	0.000	***	8.5E-03	1.2E-03	0.005	**	110.350	2.165	0.017	*	-52.530	2.405	0.904	
56	*Pyrenochaetopsis leptospora*	Low	65.269	1.356	0.000	***	1.3E-02	1.3E-03	0.868		110.100	1.584	0.001	**	-65.973	0.574	0.000	***
58	*Metapochonia subrubescens*	Low	63.944	1.262	0.000	***	8.2E-03	5.1E-04	0.000	***	106.400	1.770	0.392		-61.817	1.840	0.000	***
59	*Clonostachys rosea*	Low	67.028	2.962	0.000	***	1.1E-02	8.6E-04	0.001	**	108.500	2.934	0.662		-58.260	2.135	0.001	**
60	*Trichoderma linzhiense*	Low	67.185	0.761	0.000	***	4.9E-03	1.2E-03	0.002	**	108.125	2.550	0.488		-49.470	0.914	0.906	
65	*Linnemannia gamsii*	Low	59.676	0.951	0.000	***	7.3E-03	1.3E-03	0.008	**	97.100	2.599	1.000		-51.353	1.307	0.413	
66	*Absidia* sp. nov.	Low	58.269	5.117	0.000	***	1.2E-02	8.9E-04	0.062		113.400	3.496	0.101		-51.620	2.694	0.997	
67	*Plenodomus chelidonii*	Low	60.259	2.541	0.000	***	1.2E-02	1.0E-03	0.135		92.300	3.282	1.000		-59.283	1.985	0.000	***
71	*Paramyrothecium viridisporum*	Low	66.111	1.215	0.000	***	1.2E-02	1.3E-03	0.389		115.575	2.130	0.000	***	-55.853	1.730	0.003	**
79	*Aspergillus luchuensis*	Low	63.130	1.926	0.000	***	9.0E-03	1.3E-03	0.024	*	115.225	2.117	0.000	***	-51.577	2.850	0.999	
83	*Hydropisphaera* sp. nov.	Low	69.046	1.174	0.000	***	1.2E-02	1.3E-03	0.630		115.800	0.600	0.000	***	-55.623	3.870	0.914	
86	*Pochonia chlamydosporia*	Low	68.157	4.328	0.000	***	1.0E-02	2.9E-03	0.993		108.600	1.111	0.001	**	-49.050	2.149	1.000	
101	*Talaromyces kabodanensis*	Low	66.065	1.641	0.000	***	8.4E-03	4.8E-04	0.000	***	112.025	2.187	0.002	**	-51.733	2.134	0.937	
123	*Marquandomyces marquandii*	Low	61.574	1.227	0.000	***	1.0E-02	1.2E-03	0.034	*	112.875	1.935	0.000	***	-50.473	0.957	0.397	
126	*Gliomastix roseogrisea*	Low	74.370	3.823	0.000	***	7.6E-03	1.4E-03	0.014	*	116.900	1.726	0.000	***	-45.537	1.303	1.000	
128	*Fusarium culmorum*	Low	67.963	1.029	0.000	***	8.6E-03	1.1E-03	0.002	**	106.725	2.149	0.591		-46.400	0.468	1.000	
132	*Auxarthron umbrinum*	Low	64.444	1.567	0.000	***	9.0E-03	8.4E-04	0.000	***	111.950	1.127	0.000	***	-49.490	1.336	0.996	
136	*Penicillium manginii*	Low	64.093	1.483	0.000	***	9.7E-03	8.9E-04	0.000	***	113.650	1.660	0.000	***	-47.103	1.033	1.000	
137	*Clonostachys rosea*	Low	60.074	8.525	0.091		1.2E-02	1.5E-03	0.517		114.375	3.705	0.085		-51.727	3.660	1.000	
139	*Acremonium persicinum*, possible new sp.	Low	71.241	4.555	0.000	***	1.1E-02	1.5E-03	0.274		114.875	0.910	0.000	***	-58.087	3.730	0.456	
141	*Trichoderma linzhiense*	Low	62.250	2.897	0.000	***	9.0E-03	5.4E-04	0.000	***	108.825	2.522	0.294		-55.633	2.902	0.472	
144	*Purpureocillium lavendulum*	Low	59.713	1.324	0.000	***	8.7E-03	2.4E-04	0.000	***	114.575	1.392	0.000	***	-47.053	1.920	1.000	

Table S3.5. continued. Mean, standard error (SE) (±), p-value, and significance for the moisture loss (%), gravimetric water content (θ), water contact angle (WCA), and water potential (Ψ) after 8 weeks of growth under a low and high level of moisture. Asterisks indicate significant differences compared to control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Isolate code	Molecular identity	Moisture	Moisture loss (%)				θ				WCA (°)				Ψ (Mpa)			
			$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Control	High	3.575	0.462			0.161	0.008	1.000		87.229	1.693			-1.335	0.293		
7	*Penicillium onobense*	High	9.875	0.457	0.000	***	0.155	0.003	1.000		112.500	1.662	0.000	***	-0.460	0.212	0.627	
13	*Pilidium lythri*	High	8.908	0.344	0.000	***	0.150	0.004	1.000		103.300	0.567	0.000	***	-0.217	0.168	0.078	
14	*Staphylotrichum acaciicola*	High	8.867	0.305	0.000	***	0.156	0.002	1.000		104.200	1.728	0.000	***	-0.307	0.172	0.175	
15	*Dactylonectria torresensis*	High	9.136	0.156	0.000	***	0.161	0.003	1.000		94.775	2.060	0.831		-0.430	0.236	0.655	
20	*Marquandomyces marquandii*	High	8.917	0.378	0.000	***	0.162	0.005	1.000		104.725	1.106	0.000	***	-0.323	0.179	0.219	
31	*Clonostachys rosea*	High	11.165	0.523	0.000	***	0.165	0.008	1.000		96.000	1.397	0.083		-0.487	0.128	0.365	
38	*Purpureocillium lilacinum*	High	8.377	0.259	0.000	***	0.163	0.004	1.000		94.275	1.697	0.758		-0.350	0.165	0.218	
52	*Fusarium sporotrichioides*	High	9.491	0.206	0.000	***	0.166	0.008	1.000		94.725	4.510	1.000		-0.367	0.210	0.411	
56	*Pyrenochaetopsis leptospora*	High	10.242	0.395	0.000	***	0.170	0.003	1.000		99.525	1.945	0.005	**	-0.823	0.357	1.000	
58	*Metapochonia subrubescens*	High	9.647	0.313	0.000	***	0.154	0.006	1.000		102.050	2.571	0.004	**	-0.947	0.263	1.000	
59	*Clonostachys rosea*	High	9.970	0.185	0.000	***	0.159	0.005	1.000		94.533	3.148	0.999		-1.033	0.289	1.000	
60	*Trichoderma linzhiense*	High	10.025	0.838	0.000	***	0.157	0.004	1.000		97.600	3.763	0.963		-0.667	0.163	0.888	
65	*Linnemannia gamsii*	High	9.340	1.110	0.000	***	0.157	0.003	1.000		89.567	8.186	1.000		-0.420	0.115	0.197	
66	*Absidia* sp. nov.	High	9.768	0.258	0.000	***	0.157	0.011	1.000		103.200	1.562	0.000	***	-0.233	0.121	0.037	*
67	*Plenodomus chelidonii*	High	9.401	0.576	0.000	***	0.163	0.007	1.000		99.350	1.893	0.005	**	-0.183	0.115	0.020	*
71	*Paramyrothecium viridisporum*	High	10.182	0.304	0.000	***	0.164	0.004	1.000		97.450	1.482	0.013	*	-0.270	0.114	0.048	*
79	*Aspergillus luchuensis*	High	10.201	0.375	0.000	***	0.157	0.006	1.000		102.000	2.459	0.002	**	-0.330	0.117	0.093	
83	*Hydropisphaera* sp. nov.	High	9.498	0.256	0.000	***	0.162	0.004	1.000		106.550	1.486	0.000	***	-0.530	0.211	0.771	
86	*Pochonia chlamydosporia*	High	9.685	0.198	0.000	***	0.153	0.002	1.000		99.767	2.338	0.026	*	-0.500	0.354	0.978	
101	*Talaromyces kabodanensis*	High	9.128	0.069	0.000	***	0.165	0.002	1.000		99.725	3.778	0.697		-0.360	0.148	0.188	
123	*Marquandomyces marquandii*	High	10.511	0.612	0.000	***	0.160	0.003	1.000		94.767	8.706	1.000		-0.427	0.162	0.353	
126	*Gliomastix roseogrisea*	High	9.466	0.305	0.000	***	0.168	0.003	1.000		104.525	2.061	0.000	***	-2.477	1.414	1.000	
128	*Fusarium culmorum*	High	9.789	0.279	0.000	***	0.161	0.004	1.000		97.850	3.620	0.918		-2.290	0.889	1.000	
132	*Auxarthron umbrinum*	High	8.701	0.460	0.000	***	0.184	0.023	0.438		98.925	1.272	0.000	***	-0.723	0.127	0.909	
136	*Penicillium manginii*	High	8.969	0.213	0.000	***	0.163	0.004	1.000		96.525	3.888	0.997		-1.103	0.299	1.000	
137	*Clonostachys rosea*	High	12.676	2.187	0.000	***	0.158	0.008	1.000		104.150	1.923	0.000	***	-0.853	0.138	0.996	
139	*Acremonium persicinum*, possible new sp.	High	9.553	0.436	0.000	***	0.157	0.005	1.000		107.400	3.393	0.000	***	-0.467	0.114	0.281	
141	*Trichoderma linzhiense*	High	9.073	0.041	0.000	***	0.163	0.003	1.000		99.700	1.721	0.001	***	-0.483	0.206	0.654	
144	*Purpureocillium lavendulum*	High	8.621	0.196	0.000	***	0.162	0.003	1.000		92.867	2.397	1.000		-0.847	0.230	1.000	

Table S3.6. Mean, standard error (SE) (±), p-value, and significance for fungal biomass (mg ergosterol/kg soil) after 8 weeks of growth under a low and high level of moisture. Asterisks indicate significant differences compared to control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Isolate code	Molecular identity	Fungal biomass (mg ergosterol/kg soil)									
		Moisture	$\bar{X}$	SE	p-value	sig	Moisture	$\bar{X}$	SE	p-value	sig
	Control	Low	0.027	0.004			High	0.020	0.006		
7	*Penicillium onobense*	Low	5.907	0.282	0.000	***	High	6.999	0.596	0.000	***
13	*Pilidium lythri*	Low	15.263	1.034	0.000	***	High	2.066	1.074	0.987	
14	*Staphylotrichum acaciicola*	Low	13.708	1.599	0.000	***	High	8.363	0.790	0.000	***
15	*Dactylonectria torresensis*	Low	21.223	4.719	0.004	**	High	4.082	0.983	0.017	*
20	*Marquandomyces marquandii*	Low	5.188	1.651	0.309		High	5.620	0.955	0.000	***
31	*Clonostachys rosea*	Low	11.739	0.963	0.000	***	High	5.290	0.277	0.000	***
38	*Purpureocillium lilacinum*	Low	3.257	0.243	0.000	***	High	3.403	0.576	0.000	***
52	*Fusarium sporotrichioides*	Low	7.085	0.619	0.000	***	High	4.313	0.810	0.000	***
56	*Pyrenochaetopsis leptospora*	Low	19.150	1.268	0.000	***	High	5.965	0.896	0.000	***
58	*Metapochonia subrubescens*	Low	6.425	1.929	0.201		High	2.878	0.608	0.002	**
59	*Clonostachys rosea*	Low	9.438	0.484	0.000	***	High	5.749	0.266	0.000	***
60	*Trichoderma linzhiense*	Low	6.052	0.442	0.000	***	High	3.772	0.647	0.000	***
65	*Linnemannia gamsii*	Low	0.143	0.171	1.000		High	0.017	0.003	1.000	
66	*Absidia* sp. nov.	Low	11.612	1.289	0.000	***	High	0.290	0.185	1.000	
67	*Plenodomus chelidonii*	Low	11.264	1.878	0.000	***	High	5.656	0.813	0.000	***
71	*Paramyrothecium viridisporum*	Low	15.988	1.122	0.000	***	High	6.489	0.251	0.000	***
79	*Aspergillus luchuensis*	Low	1.667	0.345	0.001	**	High	0.991	0.455	0.948	
83	*Hydropisphaera* sp. nov.	Low	9.992	0.871	0.000	***	High	8.961	0.903	0.000	***
86	*Pochonia chlamydosporia*	Low	3.087	0.254	0.000	***	High	0.792	0.457	0.998	
101	*Talaromyces kabodanensis*	Low	7.839	0.194	0.000	***	High	2.782	0.744	0.068	
123	*Marquandomyces marquandii*	Low	5.719	0.595	0.000	***	High	4.857	1.359	0.106	
126	*Gliomastix roseogrisea*	Low	7.663	1.216	0.000	***	High	6.251	0.116	0.000	***
128	*Fusarium culmorum*	Low	11.617	0.999	0.000	***	High	5.034	1.496	0.185	
132	*Auxarthron umbrinum*	Low	2.947	0.140	0.000	***	High	0.963	0.636	1.000	
136	*Penicillium manginii*	Low	5.977	0.932	0.000	***	High	2.114	0.720	0.464	
137	*Clonostachys rosea*	Low	10.932	1.052	0.000	***	High	6.925	0.452	0.000	***
139	*Acremonium persicinum*, possible new sp.	Low	8.286	0.841	0.000	***	High	5.181	0.352	0.000	***
141	*Trichoderma linzhiense*	Low	6.733	0.634	0.000	***	High	2.704	0.272	0.000	***
144	*Purpureocillium lavendulum*	Low	3.648	0.251	0.000	***	High	4.818	1.145	0.014	*

CHAPTER 4

THE EFFECT OF MICROBIAL INOCULATION ON SOIL PHYSICAL PROPERTIES AND PLANT GROWTH UNDER DROUGHT AND WELL-WATERED CONDITIONS

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Summary

Climate change is affecting precipitation patterns across the globe, increasing the duration and intensity of drought events. These drought events can pose major threats to agriculture due to associated declines in yields as well as soil degradation. Soil microbes, particularly fungi, and bacteria, are known to be important determinants of plant health and soil stability during drought events. In this study, we assessed the interactions between the inoculation of fungal and bacterial species and soil moisture on soil physical properties and plant growth. Tomato plants were grown for 38 days in nonnaturalized soils that had been inoculated with individual microbial isolates, 10 fungal and three bacterial species, under well-watered and drought conditions. We assessed plant growth properties, soil aggregation (mean weight diameter, MWD), water content, and microbial density at the time of harvest. We found that inoculation generally improved soil aggregation (MWD) under both levels of moisture. Multiple inoculated strains improved plant performance under well-watered conditions, but only a single strain showed a growth effect under conditions of drought. Plant chlorophyll content was also affected by drought conditions. We observed an interactive effect of watering level and strain identity. MWD did not correlate with any plant growth parameter and water stable soil fractions 2-1 mm were positively correlated to dry and fresh shoot biomass under well-watered conditions. Improvements in both plant growth and soil structure could be related to the production of EPS, strains isolated from plots that had experienced drought, and effective microbial soil colonization. Our study suggests that microbial stimulation of improved soil status may be a useful component of future strategies for sustainable agronomic strategies that preserve soil structure while stimulating plant growth.

1. Introduction

Several global drivers threaten future food security, including population growth, reduced availability of arable lands, water scarcity, and climate change (Chakraborty and Newton, 2011; Premanandh, 2011). Climate change has severely impacted precipitation patterns throughout the globe, increasing the severity and frequency of droughts in different regions of the world (Arnell, 1999), especially in semi-arid regions (Li *et al.*, 2009; Cook *et al.*, 2018). Intensification of land use and the increased pressures of drought exacerbate soil degradation and erosion, thereby reducing soil fertility and crop production (Masroor *et al.*, 2022). For instance, the historical effects of heatwaves, and droughts between 1961 to 2018 reduced cereal yields on average by 9% and 7.3%, respectively (Brás *et al.*, 2021). Changes and limitations in water availability, therefore, pose increasing threats to agriculture, food security, the environment, and society (Wilhite *et al.*, 2007; Hazell and Hess, 2010).

Under drought conditions, plants adopt different morphological, physiological, and biochemical responses, such as delayed germination, osmotic adjustments, reduction in biomass, changes in root hydraulic conductivity, leaf transpiration, pigments content, and photosynthesis activity (Anjum *et al.*, 2011; Dubey *et al.*, 2021). For instance, shoot biomass, net photosynthesis, and starch content were shown to decrease in tomato plants when they were exposed to drought (Zhou *et al.*, 2017). Regulation of phytohormones, production of osmolytes, exogenous compounds, and proteins (Dubey *et al.*, 2021), and association with beneficial microorganisms, such as plant growth-promoting microbes (PGPM), are mechanisms by which plants can ameliorate the detrimental effects of drought (Naik *et al.*, 2019; Porter *et al.*, 2020).

Bacteria and fungi colonize the outside and inside of plants, where they play an important role in plant growth and health (Friesen *et al.*, 2011; Bulgarelli *et al.*, 2013). Beneficial microbes associated with plants can stimulate plant growth and enhance plant resistance to abiotic stresses by at least 4 different mechanisms. The first one comprises nutrient acquisition by increasing the nitrogen (N), phosphorus (P), and iron (Fe) content (Rubio *et al.*, 2002; Benidire *et al.*, 2017; Lurthy *et al.*, 2020). The second involves modification of plant gene expression, for instance by enhancing the production of reactive oxygen species (ROS) and improving PSII photochemistry and plant tolerance to water deficiency, salinity, and heavy-metal toxicity (Gururani *et al.*, 2013). The third one is related to the secretion of phytohormones, which are important growth regulators of plant defense response mechanisms against stresses (Egamberdieva *et al.*, 2017). Finally, microbes have a role in the physical

modification of the environment via the improvement of soil aggregate stability and increased moisture retention in the rhizosphere (Sandhya and Ali, 2015).

The role of soil bacteria in the stabilization of soil aggregates, improved soil structure and organic matter contents has previously been recognized (Harris *et al.*, 1964; Rashid *et al.*, 2016). Important bacterial traits associated with enhanced soil aggregation include the production of extracellular polysaccharides (EPS) and biofilms, which can bind soil particles and hold them together through a cementing or encapsulating action (Qurashi and Sabri, 2012; Costa *et al.*, 2018). Soil fungi are also known to have a large effect on soil structure and stability (Ritz and Young, 2004), which also can have impacts on plant health and growth (Passioura, 1991). Fungi affect soil structure in a number of ways. For instance, they can modulate the cracking properties of the soil through the physical extension of hyphae, which relates to the content and type of clay within the soil (Ritz and Young, 2004). Fungi can also enmesh soil particles by hyphal entanglement (Miller and Jastrow, 1992, 2000), a process that is made more efficient by the ability of hyphae to attach strongly to surfaces via the use of binding agents (Rillig and Mummey, 2006). Furthermore, fungi can increase soil hydrophobicity, which can play an important role in soil structure, aggregate stability, and water storage (Olorunfemi *et al.*, 2014; Rashid *et al.*, 2016).

Numerous studies have shown promising results regarding the use of fungal or bacterial inoculation as a means of alleviating drought symptoms in plants (Subramanian *et al.*, 2006; Marulanda *et al.*, 2009; Barnawal *et al.*, 2017; Duc *et al.*, 2018). These studies targeted a wide range of commercially important crops, including wheat (Kasim *et al.*, 2013), lettuce (Vivas *et al.*, 2003), and tomato (Eke *et al.*, 2019; Cornejo-Ríos *et al.*, 2021). Utilization of microbial associations may thus represent an alternative strategy to complement more traditional breeding efforts aimed at increasing plant traits associated with drought tolerance. Previous studies involved in microbe-assisted plant drought tolerance have focused on the direct effect of drought stress alleviation in the plant. Given the importance of bacterial and fungal activities for soil structure and function, we sought to also examine how microbial inoculants could improve plant performance under drought via their ability to modify the soil environment, thereby providing an indirect route to alleviate drought stress in plants.

In this study, we aimed to determine how the inoculation of saprobic fungi and bacteria affects plant growth parameters, soil aggregate stability, and soil water content under well-watered and drought moisture regimes. We hypothesized

that i) inoculation of selected bacterial and fungal strains would improve the aggregate stability and water content in soil and yield higher plant growth rates compared to the non-inoculated controls for both moisture regimes, and ii) this effect will be higher under conditions of drought. We selected 10 fungal and 3 bacterial strains and inoculated them in sterilized soil. The soil resident microbial community was then reintroduced into the soil for the growth of tomato plants for a period of 38 days under well-watered and drought moisture. Plant growth parameters were measured during or after the incubation period, including the following: plant height net growth, stem diameter, chlorophyll content, and fresh and dry shoot biomass. Additionally, soil parameters were monitored. These included soil water content, soil aggregate stability, and the densities of the inoculated bacterial and fungal populations. We then examined potential linkages between changes in soil parameters and observed plant growth parameters to help determine the degree to which microbial impacts on soil structure indirectly affected plant performance.

2. Materials and methods

2.1 Plants and substrate

Tomato (*Solanum lycopersicum*) was chosen as the model plant due to its importance as a worldwide crop, as well as the wealth of known physiological traits for this species (Liedl, 2013; Vats *et al.*, 2022). *Solanum lycopersicum cv.* "M82" seeds were surface sterilized using an adapted protocol from Volpe *et al*. (2018). Seeds were soaked with 70% ethanol for 1 min by gently swaying, and ethanol was filtered through a layer of Miracloth (rayon polyester, pore size of 22-25 µm, Millipore Sigma). Seeds were then rinsed with sterile water, transferred to a 2.5% bleach solution, and shaken gently (100 rpm, 10 min) (orbital shaker Gerhardt, Germany). Seeds were finally rinsed 3 times with sterile water and placed on top of sterile paper towels contained in individual plastic germination chambers. The system was wetted with 10 mL of sterile water to keep the moisture high, and the lids were closed carefully. Seeds were then incubated in a growing chamber (MCA16039, Snijders) (12 h of light, 65% humidity, and 22°C) for 4 days. To improve the germination rate, the temperature of incubation was increased to 25°C until day 7. The system was kept moist by adding a supply of water under sterile conditions.

The substrate used was a mixture of potting soil (PrimastaTM) and middle-size sand (average size 0.35-0.5 mm) in a 40:60 ratio. Potting soil and sand were previously sieved through a 1 mm mesh sieve. The final properties of the soil

mixture used were as follows: C/N ratio 28; pH 5.6, organic carbon 8.2%, clay (< 2 µm) 2%; silt (2-50 µm) 2% and sand (> 50 µm) 79%. The soil mixture was settled in special autoclaving bags and autoclaved twice, [(121°C for 20 min) followed by a new round of autoclaving (121 °C for 45 min)] 3 times to ensure sterility. Autoclaving cycles were conducted after 24 h of resting time. Bags were opened after autoclaving and dried in a flow cabinet for 48 h to allow the excess moisture to evaporate.

2.2 Microbial materials

Bacterial and fungal strains in this study were selected from a collection obtained from the studies described in Chapters 2 and 3. Microbes were isolated from the bulk soil from a drought experiment settled at Fort Rijnauwen in Utrecht, the Netherlands (52°04'24.8" N 5°10'32.4" E). The experiment was set up on a natural grassland (*Arrhenatherum elatius* association) with rainout shelters to create two different drought treatments and a (no drought) control. Drought treatments consist of a pulse drought (D90) with approximately 90% of rainfall reduction in summer and re-application of rainwater in winter and a press drought (D50) with a continuous 50% reduction of rainfall. From an identified collection of 133 fungal strains, we selected 10 fungal strains based upon at least one of the following three criteria: (i) fungal taxa that were abundant according to corresponding operational taxonomic units (OTUs) from the drought soil of isolation compared to the non-drought treatment (Supplementary information S3.2, Chapter 3), (ii) fungal strains that belonged to taxa previously demonstrated for their role in agricultural soils (e.g., *Trichoderma* spp. *Metarhizium* sp., *Purpureocillium* sp.) (Waghunde *et al.*, 2016; Baron *et al.*, 2020), and (iii) fungal strains which could produce concentrations $>10^4$ spores/ml. The three selected bacterial strains were: B84 (*Pseudomonas* sp.), associated with the production of biofilms and growth under low moisture conditions, B144 (*Bacillus* sp.), which could produce biofilms and EPS in plate experiments, and B110 (*Streptomyces* sp.), associated with intermedium production of EPS in plate and a positive impact on soil aggregation in microcosm experiments (Angulo *et al*, unpublished) (Chapter 2). The three selected bacterial strains were categorized with a strong, intermediate, or weak potential ability to impact soil aggregation according to the trait-based approach designed in Chapter 2, respectively.

2.3 Molecular identification of microbes

Bacterial strains were identified as described in Chapter 2 (Section 2.2) and fungal strains were identified as presented in Chapter 3 (Section 2.3

and Supplementary Section S3.1). The pairwise distances for fungal strains were represented in a phylogenetic tree, which was constructed by the neighbor-joining method using Software Mega 11 (Tamura *et al.*, 2021) (see supplementary Fig S4.1).

2.4 Preparation of microbial inoculants and soil inoculation

The selected fungal strains were cultured on plates of either potato dextrose agar (PDA) or handmade oatmeal agar (OA) to increase the production of spores (personal communication). The OA media was obtained by mixing a suspension of oatmeal with 1000 mL of deionized water and 15 g of agar and sterilized (120 °C, 20 min). The oatmeal suspension was obtained by cooking 30 g of oats for 15-30 min and filtering the suspension in 4 layers of gauze (Cutisoft). PDA growth colonies (7 days, 25 ºC) were used to inoculate PDA and OA plates, the inoculation was made on the full plate surface (to optimize the spores production) with the help of sterilized cotton swabs. The inoculated plates were incubated (2 weeks at 25 °C) until they produced visible sporulation. Spores were collected by pouring 10 mL of sterile Tween 80 at 0.1% into each plate and softly scraping the spores from the media with a sterilized glass spatula, according to the adapted protocol of Mwamburi *et al*. (2015). The spore suspensions were then filtered with sterile Miracloth, and 30 mL of Tween 80 at 0.1% was added. The suspension was then mixed for 10 s and centrifuged for 15 min at 4500 rpm (centrifuge Heraeus Megafuse 40). The supernatant was then removed, and the pellet was washed twice with Tween 80 at 0.1% and centrifugated for 10 min. The final pellet was resuspended in 50 mL of Tween 80 at 0.1%, diluted in serial dilutions, and the concentration of spores was determined using a Neubauer hemocytometer. The spore concentration was calculated using the following equation:

$$Spores/mL = \frac{Counted\ spores}{Counted\ surface\left(mm^2\right) * Chamber\ depth\left(mm\right) * Factor\ of\ Dilution}$$

The final fungal concentration was adjusted to reach an approx. of 10^7 spores/ml (Supplementary Table S4.1).

Bacterial inoculants were obtained by culturing the strains on nutrient agar (NA) (48 h at 28 ºC). Individual colonies were inoculated to trypticase soy broth (TSB) (overnight, 28 ºC, 100 rpm in an orbital shaker, Gallenkamp). The cultures were centrifuged for 15 min at 4500 rpm (Megafuge 40, Heraeus), washed twice with water, and finally, resuspended in Tween 80 at 0.1%. The bacterial concentration was adjusted to 1.0 (OD_{560}) for inoculation in soil.

The colony forming units (CFU) were calculated by making serial dilutions in distilled water, inoculation on NA using a sterilized glass spatula, and incubation for 24 h at 28 ºC. The CFU was calculated using the following equation:

$$CFU/mL = \frac{Number\ of\ colonies}{Volume\ transferred\ to\ the\ plate * Factor\ of\ dilution}$$

Next, each sterilized soil bag prepared in section 2.1 was assigned a random single bacterial (3 strains) or fungal treatment (10 strains), and 180 mL of the corresponding inoculum was poured into the bag under the hood using a sterile syringe. The bags were then gently hand-mixed and tied with a breathable cork and a rope that allowed air exchange. The bags were then placed in incubators in the dark at 22°C for 5 days with daily mixing by gently shuffling the bag to allow the microbial spread. The final approx. microbial concentration in the soil based on the soil volume was between 1.5 x 10^3 and 5 x 10^5 spores/g for fungi and 1.2 x 10^4 and 1.4 x 10^7 CFU/g of soil for bacteria (Supplementary Table S4.1).

2.5 Reinoculation of the original resident microbial community

In order to restore the resident soil microbial community after the specific microbial inoculation and avoid opportunistic colonization, the original microbial community was re-inoculated into each soil bag using an adapted protocol from Yan *et al*. (2015). From the original soil mixture, 44 g were put into a highspeed blender (Mix55, Proline) with 400 mL of sterile, distilled water and blended for 1 min at maximum speed. Then, the blender and its content were put on ice for 2 minutes, and these steps were repeated 4 times. Finally, the remaining suspension was filtered with Miracloth, and 180 mL of the suspension was inoculated into each previously inoculated soil bag under gentle agitation and incubated in the dark at 22°C for 2 days (Fig. 1).

2.6 Experimental setup and design

After the microbial incubation period, 320 g of inoculated mix soil was put into autoclaved plastic gas microboxes (120 mm h x 90 mm d, Microbox filter L, Eco2 NV) equipped with 6 drainage holes. The soil was gently pressed down to ensure compactness and flasks were closed in sterile conditions. The pots were then placed overnight in individual plastic containers filled with the essential nutrients half-strength sterile Hoagland's solution (Menary and Staden, 1976) (see Supplementary Table S4.2) where the soil water reached saturation. Later,

germinated seedlings, selected based on homogeny in size and development, were transferred to the pots which reached water holding capacity (WHC) (47%) after water drainage. The flasks were closed with lids from which the filter in the central part was removed to ensure protection to the system and allow space for the seedling to establish. Pots then were transferred and kept in a walk-in growth chamber (21°C, 400 ppm of CO2, 70% relative humidity, 16 h photoperiod, 250 μmol m^{-2} s^{-1} photosynthetic photon flux density). Pots were randomly then placed on five different trays in the growing chamber and seedlings were mounted vertically on autoclaved wooden holders to stabilize the plant stem development.

The total plant-pot system was separated into 2 groups: a well-watered and stimulated drought treatment (Fig. 1). The soil moisture content corresponding to the amount of water in the soil after gravitational drainage of excess water of saturated soil (Walker, 1989) was considered as the benchmark. The well-watered pots were watered 5 times to adjust the water content to the WHC (156 ml of water per 320 g of soil). Water availability was manipulated by spacing the water application of 156 ml water two times in the whole plant growth period to manipulate drought. In total, during the period of incubation, each pot with well-watered conditions received 780 ml of water and the one under drought 312 ml. Visual symptoms of drought were observed for plants subjected to the drought treatment.

After 12 days of the experiment's establishment, each pot was re-inoculated. The new inoculum was prepared (according to section 2.4), and 5 mL of inoculum was injected using a syringe into the soil of each pot. Controls were inoculated with a similar volume of Tween 80 at 0.1%. On day 27, lids were fully removed from pots to allow better growth of the tomato seedlings. Plants were harvested on day 38.

The design of the experiment included 10 fungal strains, 3 bacterial strains, and a control group without bacterial or fungal inoculation but with the native microbial community restored. Each strain was separated into 2 treatments, either "drought" or "well-watered", with 5 replicates for each treatment. The pots were placed in a completely randomized design in the growth chamber, and a total of 140 experimental units were used.

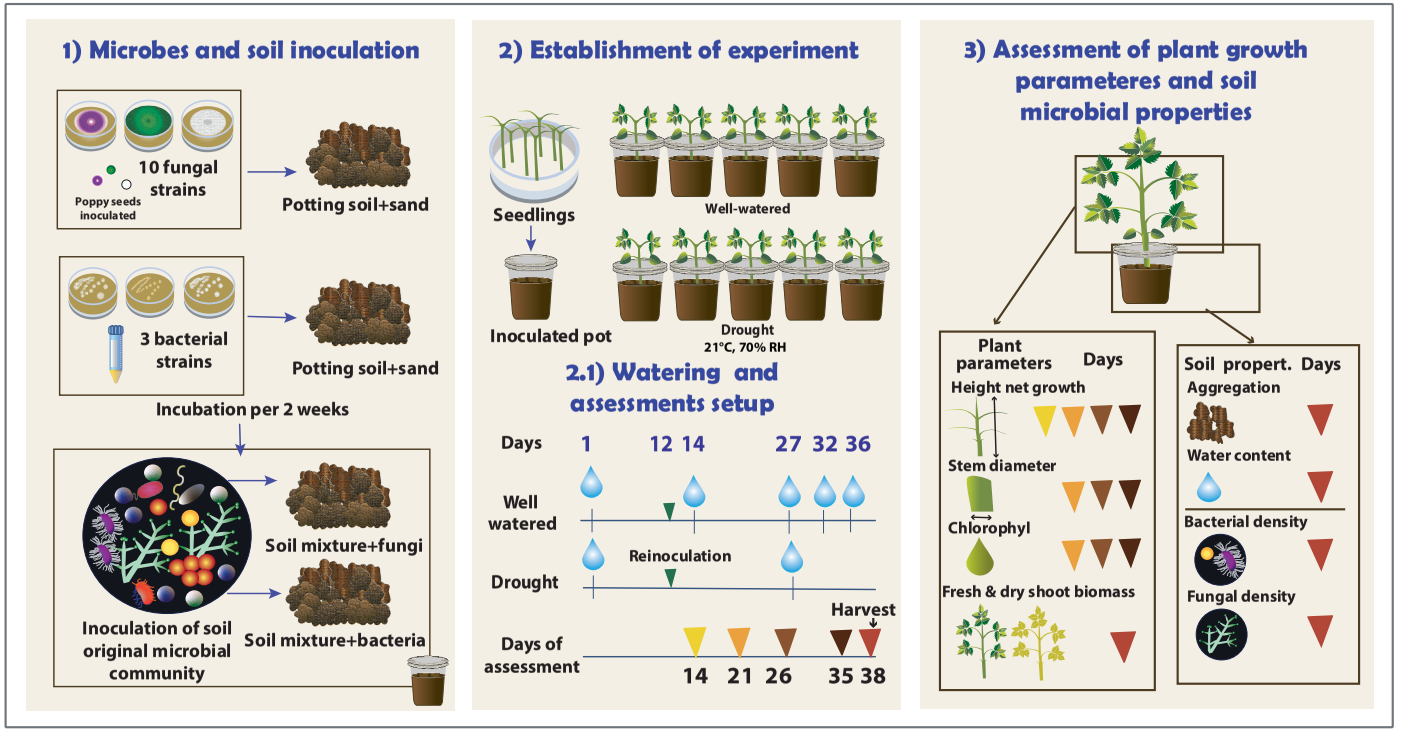

Figure 1. Scheme of the experimental inoculation of bacterial and fungal strains in soil, growth of tomato seedlings, cycles of watering and assessment periods, and assessment of plant growth parameters aboveground and soil properties belowground.

2.7 Assessment of plant growth parameters

Throughout the incubation period, different plant parameters at different times after the plant establishment were assessed (Fig. 1). These included plant height net growth (from the soil surface to the tip of the plant with the help of a ruler), stem diameter (with the help of a vernier scale) and chlorophyll content (using the SPAD-502 Plus chlorophyll meter). The SPAD 502 is a hand-held device that is used for rapid, accurate, and non-destructive measurement of chlorophyll concentrations. The measurements produce relative SPAD meter values that are proportional to the amount of chlorophyll present in the leaf (Ling *et al.*, 2011). On day 38, plants were harvested, and the fresh shoot biomass was weighed. Plants were then transferred to an oven and dried until they reached constant weight (70° C and 48 h), and dry shoot biomass was determined.

2.8 Soil aggregate stability and water content

To determine the soil aggregate stability, after harvest a core sample from the full soil profile of approximately 5 g was taken from each pot with the help of a metal core borer and stored at 4°C until use. Soil samples were then placed in an oven at 30 °C for 24 h to get a constant moisture content and then aggregate stability was determined using the ethanol technique (Bissonnais, 1996) in order to deal with soil water repellency. Approximately 5 g of each soil sample was gently immersed in a 250 mL beaker filled with 50 mL of ethanol for 10 min. Then ethanol was removed, and the remaining soil material was transferred to a 250 mL Erlenmeyer flask filled with 50 mL of deionized water. The water content was then adjusted to 200 mL, and the flasks were capped and gently agitated end over end 20 times. The mixture was allowed to settle for 30 mins and the excess water was removed. The soil mixture was transferred to a 50 µm sieve immersed in ethanol and was rotated quickly 5 times clockwise. The soil aggregates fraction > 50 µm was collected from the sieve and oven-dried at 40 °C for 24 h. After the samples were dried, they were dry sieved using sieves of sizes: 2, 1, 0.71, 0.5, and 0.25 mm, yielding a total of 6 soil fractions. Each fraction was immediately weighed. Fine roots remained in the 2 mm fraction; these roots were removed for each experimental unit. The mean weight diameter of aggregates (MWD), an index that summarized the different soil fractions (Kemper and Rosenau, 1986) was calculated using the formula:

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

where M_i is the dry mass of the soil aggregates for each size class, and $\bar{X}_i$ is the mean diameter of the soil aggregate size class (mm).

To measure the water content, we first weighed approximately 1-2 g of soil core samples collected at the time of harvest. Then samples were dried at 105°C for 48 h, and gravimetric water content (θ) was calculated according to the following formula:

$$\theta_{=} \frac{m_{soil\ wet} - m_{soil\ dry}}{m_{soil\ dry}}$$

2.9 Microbial population densities

Another soil fraction which was collected as an intact soil profile using a sterilized metal core borer was stored at -20 °C until the quantification of the microbial density by quantitative PCR (qPCR). DNA was isolated from 0.15 g of soil, according to the manufacturer's protocol for the DNeasy PowerSoil HTP 96 Kit (384) (QIAGEN), and stored at -20 ºC until use. The density of inoculated bacterial population was determined using specifically designed primers for bacterial 16S rRNA genes and ITS 1 and 2 regions for fungi (Supplementary Table S4.3). PCR mixtures contained 1.5 µL MQ-water, 2.5 µL of DNA template, 5 µL of 2x taq Universal SYBR Supermix, Biorad USA, and 0.5 µL of 5 µM of each forward and reverse primer (Supplementary Table S2.3). DNA samples were amplified and quantified in a 384-well thermal cycler (ViiA7, Applied Biosystems, USA) with a thermocycling program of 95 °C for 5 min for denaturation, followed by 40 cycles each of 95 °C for 15 s, 60 °C for 60 s for annealing and elongation. The program ended with a melting curve cycle. and data was analyzed using the Quantstudio software.

All the gene copy numbers were calculated from standard curves using the 1 Ct (cycle threshold) method. All qPCR amplifications were conducted in triplicate. Differences related to copy number and genome size were adjusted according to (Větrovský and Baldrian, 2013). The density of target strains was quantified from the inoculated strains and control treatments, both also inoculated with the resident microbial community. Data from both were log-transformed, and the difference was calculated. This procedure was necessary to eliminate the background of closely related species present in the resident microbial community from the control soil.

2.10 Statistical analyses

The effects of fungal and bacterial inoculation were assessed for the following plant growth parameters: fresh and dry shoot biomass, plant height net growth, stem diameter, chlorophyll content, as well as the following soil properties: soil gravimetric water content (θ), soil water stable aggregate fractions, MWD and inoculated bacterial and fungal population density.

The effect or the θ was analyzed for either level of moisture separately using an analysis of variance (ANOVA). The effect on soil properties: MWD and soil aggregate fractions and plant growth parameters: fresh and dry biomass, plant height net growth (14, 21, 26, and 35 days), stem diameter, and chlorophyll (21, 26, and 35 days) were analyzed using an ANOVA "type 3" for the effect of interactions between moisture and microbial strain. The assumptions of normality and homoscedasticity of the residuals were checked visually using a Q-Q plot and a plot of residuals. To counteract heteroscedasticity, we used a generalized least squares (GLS) model and allowed the variance to be different per stratum and level of moisture using varIdent (Pinheiro and Bates, 2000), packages "nlme" (Pinheiro *et al.*, 2021) and "car" (Fox and Weisberg, 2019). The pairwise comparison between the means of treatments was analyzed by the test "Tukey" through the package "emmeans" (Lenth, 2022) and a "Bonferroni" adjustment, and the graphics were plotted according to the fitted models. The effects of plant growth parameters (plant height net growth and chlorophyll) were also assessed along the periods of inoculation for each microbial strain using an ANCOVA for each level of moisture after testing the absence of interaction between strains and watering level.

Plant growth parameters at the time of harvest and soil properties were correlated using Spearman's correlation test using the "Hmisc" package (Harrel, 2022). The fungal and bacterial densities were analyzed separately, and the difference between log-transformed data for treatments and controls was calculated. Then, fungal means were compared against the fungal mean average, and the bacterial densities against each other using a Tukey test through the package "emmeans" and a "Bonferroni" adjustment. All analyses were conducted using the software R (version 4.1.2), and graphics were generated using the package "ggplot2" (Wickham, 2016). The figure depicting stable aggregate fractions for the wet sieving (Fig. 5) was built using the package "RColorBrewer" (Neuwirth, 2022).

3. Results

3.1 Effect of microbial strain inoculation and watering level on plant growth

Our results showed a range of different effects of microbial inoculations, across the three bacterial and 10 fungal strains examined concerning the impact on plant growth and soil aggregation, under the two different levels of moisture. After 38 days of incubation, the fresh and dry shoot biomass of tomato plants showed a significative interaction for strains and water regimes, where the means of well-watered conditions were higher than those under simulated drought (Fig. 2).

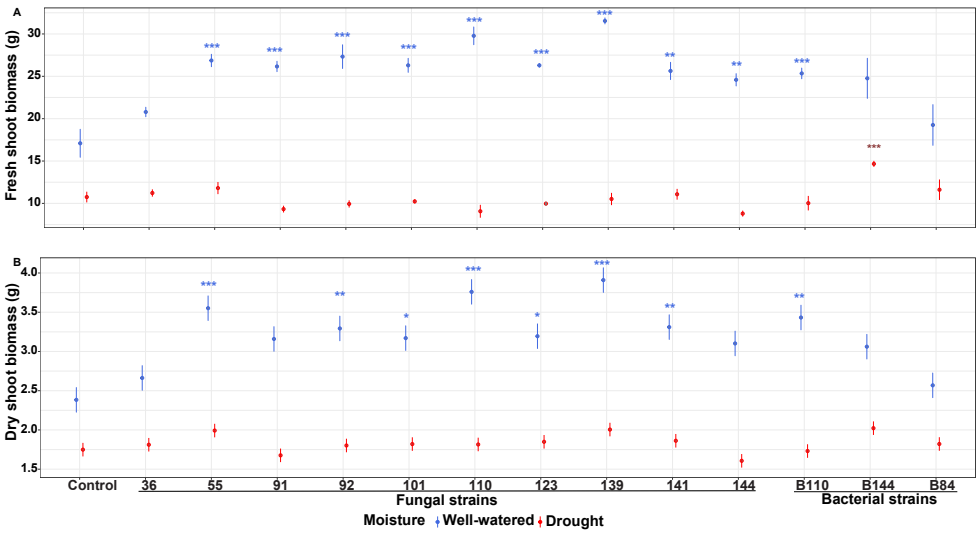

Figure 2. Fresh shoot biomass (A) and dry shoot biomass (B) of tomato plants inoculated with fungal and bacterial strains and one non-inoculated control at well-watered and drought conditions after 38 days of incubation. Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. The ANOVA is shown in Supplementary Table S4.4 and the means, standard error, p-values, and significance are shown in Supplementary Table S4.5.

When plant growth parameters of the well-watered control were compared to the inoculated treatments, 9 of the 10 fungal strains and the bacterial strain B110 (*Streptomyces* sp.) had an impact on the fresh shoot biomass. For the dry shoot biomass, similar patterns were observed except for fungal strains 91 and 144 (*Trichoderma linzhiense* and *Purpureocillium lavendulum*), which yielded values that were not significantly different from the control. For fresh shoot biomass under drought conditions, only bacterial strain B144 (*Bacillus* sp.), which had the lowest bacterial density at the inoculation time

(Supplementary Table S4.1), showed values significantly different when compared to the non-inoculated control. Under drought conditions, the effect of fungal and bacterial inoculation on dry shoot biomass was negligible.

The microbial inoculation did not result in differences in the height net growth in comparison to the non-inoculated control throughout the growth period (days 14, 21, 26, and 35). As an example, for the height net growth at day 35, which is shown in Supplementary Fig. S4.2A, some strains showed higher means than the control under well-watered conditions, and most strains led to lower means under drought conditions, but none of these trends were significant. With an absence of microbial strain and moisture interactions, the analysis of ANCOVA for each level of moisture also did not show significant differences for inoculated strains when compared to the control. Stem diameter and chlorophyll were measured on days 21, 26, and 35 when differences in watering levels began to differ most pronouncedly. Moisture level showed an effect on stem diameter on day 26, and a significative interaction was observed between moisture and strain on day 35 (Supplementary Fig. S4.2B). However, no strain resulted in better plant performance as compared to the non-inoculated control.

The effect on chlorophyll did not show interactions for moisture and strains along the different assessed times, and ANCOVA for each level of watering across measurement days showed a significative effect of microbial strain, but not the time of assessment. Under drought, fungal strain 110 (*Gliomastix* sp.) increased the chlorophyll content by a factor of 1.3 compared to the control at day 35 (Fig. 3A and supplementary Fig. S4.4), and this was significantly different. The pattern of this strain showed an increase in chlorophyll over time with an opposite effect for the control, which showed decreasing values over time. At the well-watered level (Fig. 3B), fungal strains 110 and 123 (*Marquandomyces marquandii)* showed higher levels of chlorophyll at day 26, with approximately a 20% increase compared to the control. It is important to note that on day 26, before the second watering (Fig. 1), both well-watered and drought treatments had plants with symptoms of wilting.

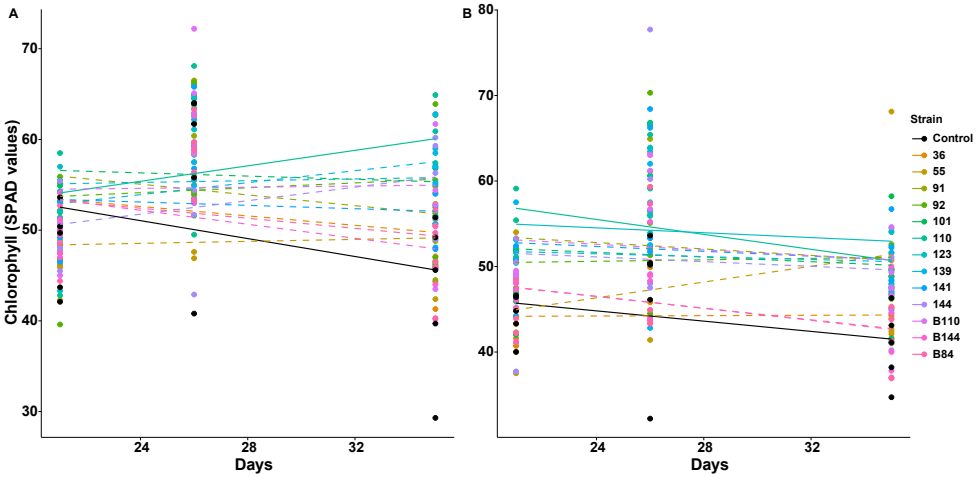

Figure 3. Chlorophyll content under drought (A) and well-watered conditions (B) showing the effect of microbial inoculation at days 21, 26, and 35. The dashed lines indicate no significant differences, and the solid lines indicate a significant effect as compared to the control ($p < 0.05$).

3.2 Effect of microbial inoculation on soil physical properties

We did not observe any significant effects of strain inoculation on the changes in gravimetric water content (θ) over time at the well-watered level (Supplementary Fig. S4.3). However, under drought conditions, fungal strain 55 (*Mortierella* sp.) and bacterial strain B144 improved the water retention of the soil when compared to the control (Fig. 4).

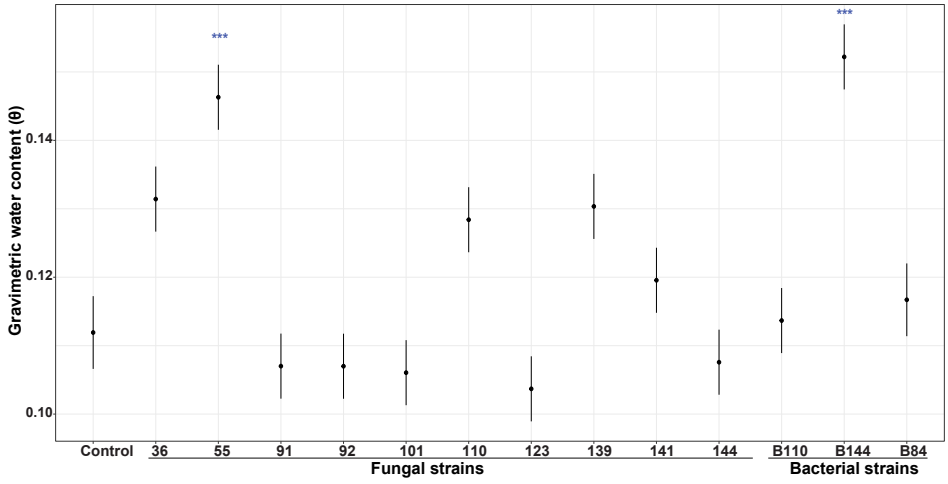

Figure 4. Gravimetric water content (θ) for fungal and bacterial inoculation and the non-inoculated control treatment under drought conditions after 38 days of incubation. Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. The ANOVA is shown in Supplementary Table S4.4 and the means, standard error, p-values, and significance are shown in Supplementary Table S4.6.

The results of microbial inoculation on the stable aggregate fractions are shown in Fig. 5. This shows that only fungal strains 123 and 144 showed an effect on the water stable aggregate fraction > 2 mm under well-watered conditions when compared to control (Fig. 5A). Under drought, strain 123 showed higher fractions of aggregates in the size class > 2 mm (Fig. 5B), and thus improved macroaggregate formation at both levels of watering.

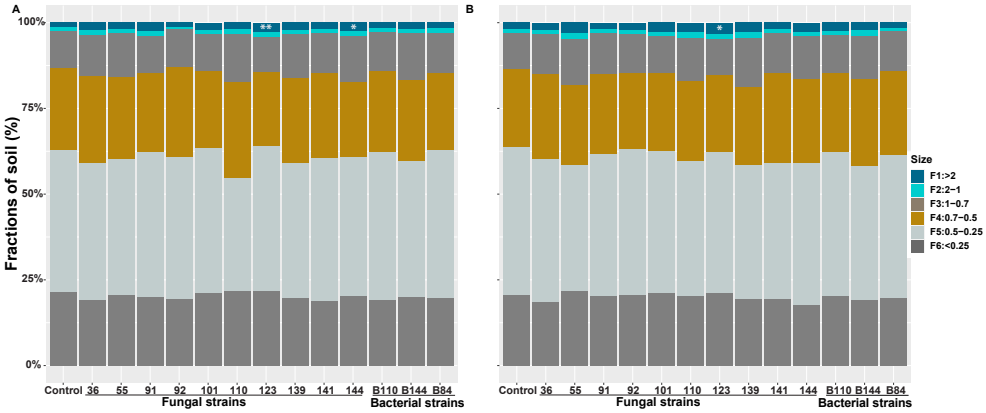

Figure 5. Mass percentage change in the < 0.25 mm, 0.25-0.5, 0.5-0.71, 0.71-1.0, 1.0-2.0, and > 2 mm fraction sizes at drought (A) and well-watered (B) respectively after 38 days of incubation and collected by wet sieving. Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. The ANOVA is shown in Supplementary Table S4.4 and the means, standard error, p-values, and significance are shown in Supplementary Table S4.6.

Nevertheless, when the summative effect of fractions was calculated by the MWD, 7 out of 10 fungal strains and 2 of the bacterial strains were significantly different from the control under well-watered conditions, with MWD values higher than 2.5 mm (Fig. 6). Also under drought, fungal strains 91, 139 (*Acremonium persicinum*) and 141 (*Trichoderma linzhiense*) were significantly different as compared to the control, with MWD values that were higher than 3 mm.

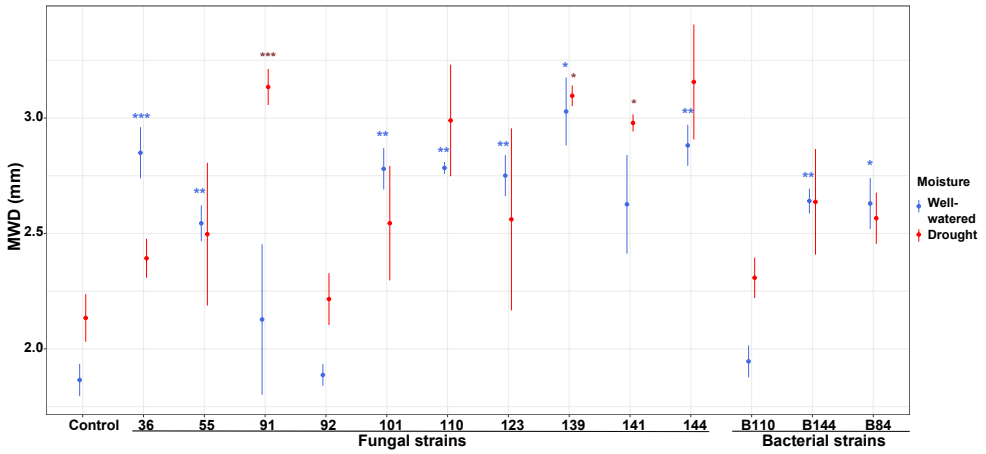

Figure 6. Mean weight diameter (MWD) for microbial strains inoculation under well-watered conditions and drought after 38 days of incubation Asterisks indicate significant differences compared to control: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. The ANOVA is shown in Supplementary Table S4.4 and the means, standard error, p-values, and significance are shown in Supplementary Table S4.6.

3.3 Interactions between plant growth and soil physical properties

Table 1 shows the correlations for the plant growth parameters and soil properties: gravimetric soil water content (θ) and aggregation properties (MWD and different aggregate fractions) from soil collected at the harvest time. Under drought, the θ had a positive correlation with the macroaggregate fraction of 1-0.71 mm and a negative correlation with the aggregate size fraction of 0.71-0.25 mm. Under well-watered conditions, dry and fresh shoot biomass were positively and negatively correlated to fractions 2-1 mm and 0.71 -0.5 mm, respectively. Surprisingly, the θ had a negative relationship only to aggregate size fractions between 0.5-0.25.

Table 1. Correlation matrix for the different continuous soil properties: soil aggregate formation (MWD), soil fractions: < 0.25 mm, 0.25-0.5, 0.5-0.71, 0.71-1.0, 1.0- 2.0, and > 2 mm and θ with plant growth parameters: dry and fresh and shoot biomass.

Drought (ρ, p-value)	MWD	> 2 mm	2-1 mm	1-0.71 mm	0.71-0.5 mm	0.5-0.25 mm	< 0.25 mm
Dry shoot biomass	-0.13/0.301	-0.04/0.757	0/0.999	0.14/0.257	-0.05/0.712	-0.1/0.424	0.11/0.384
Fresh shoot biomass	-0.16/0.192	0.03/0.831	-0.05/0.686	0.16/0.198	0.04/0.770	-0.12/0.357	-0.01/0.938
θ	-0.01/0.967	-0.07/0.556	0.22/0.078	**0.52/0.0**	**-0.42/0.001**	**-0.49/0**	-0.17/0.185
Well-watered (ρ, p-value)	**MWD**	**> 2 mm**	**2-1 mm**	**1-0.71 mm**	**0.71-0.5 mm**	**0.5-0.25 mm**	**< 0.25 mm**
Dry shoot biomass	-0.03/0.832	0.18/0.157	**0.35/0.004**	0.23/0.071	**-0.36/0.004**	-0.11/0.363	0.16/0.203
Fresh shoot biomass	0.01/0.953	0.23/0.061	**0.38/0.02**	0.1/0.415	**-0.37/0.003**	-0.15/0.236	**0.27/0.029**
θ	0.17/0.175	0.04/0.735	0.14/0.258	0.17/0.004	0.17/0.189	**-0.41/0.001**	-0.04/0.747

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

3.4 Density of inoculated microbial strains in soil

The density of the inoculated microbial populations varied considerably with some strains failing to establish significant population sizes. (Fig. 7). Fungal population densities were compared to the mean density, revealing interactions between fungal strains and the level of watering, with 5 and 3 fungal strains having above average population densities under drought and well-watered conditions, respectively. Fungal strain 101 (*Talaromyces kadodanensis*), for instance, realized a population density that was approximately 100% higher than the mean density at both levels of watering. We also observed that the fungal density for strains 36 (*Podila* sp.) and 91 (*Trichoderma* sp.) was not detected under drought. Strain 55 even showed a negative value at the well-watered level when comparing measured values in this treatment with the background signal coming from the resident community. The bacterial population density was compared between the 3 inoculated strains (Fig 7B), and interaction between moisture and the bacterial strain was observed. Under drought, B144 showed the highest abundance, while strain B110 remained undetectable.

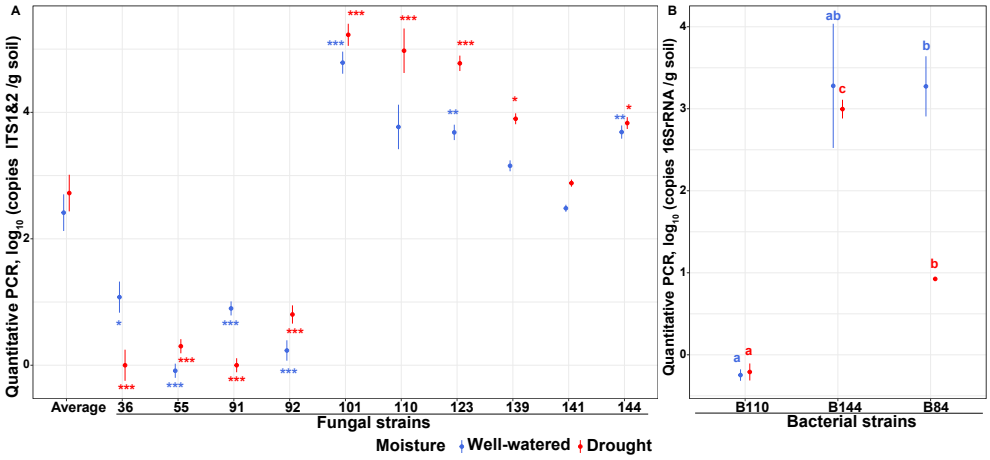

Figure 7. Fungal population density (copies of the ITS 1 and 2 regions/g soil) compared to the fungal density average (A) and bacterial population density (copies of the 16S rRNA gene /g soil) compared to each other (B) in the soil at the harvest time of tomato plants. Data of density were log-transformed, and the difference between the microbial values and the background of the strain in the resident community was calculated. Asterisks in panel A indicate significant differences compared to the average: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$, and asterisks below the average mean they are lower than the average. Letters in panel B indicate significant pairwise differences using a Tukey test. The ANOVA is shown in Supplementary Table S4.4 and the means, standard error, p-values, and significance are shown in Supplementary Table S4.6.

4. Discussion

The main objective of the present study was to examine if fungal and bacterial strain inoculation can facilitate plant growth by helping to improve soil aggregate stability and water retention under drought stress and well-watered conditions. We found that inoculation influenced water content under drought and improved aggregate stability at both water regimes. However, the effects of inoculation on plant growth were more pronounced under well-watered conditions, and there was no clear relationship between the physical soil properties and the improvement of plant growth.

4.1 Effect of fungal and bacterial inoculation on plant growth under drought and well-watered conditions

In our study, we found that strain inoculation improved plant growth in a moisture-dependent manner for most of the parameters. Plant biomass was higher under well-watered conditions, as is seen in Fig. 2. Treatments with fungal strains 110 and 139 showed values of fresh and dry shoot biomass that were approximately 80% and 60% higher than the control, respectively. The positive effect of plant growth-promoting fungi (PGPF) and plant growth-promoting rhizobacteria (PGPR) on plants is well known, and such effects have been linked to a range of consequences, including increases in germination rate (Eldridge *et al.*, 2021), enhancement of plant shoot and root growth (Gouda *et al.*, 2018; Murali *et al.*, 2021) and higher chlorophyll content (Vafadar *et al.*, 2014). Species of bacteria, such as *Pseudomonas* spp. and *Bacillus* spp. (Hashem *et al.*, 2019; Sah *et al.*, 2021), or fungi, such as *Trichoderma* spp. *Penicillium* spp. or *Aspergillus* spp. (Zin and Badaluddin, 2020; Argumedo-Delira *et al.*, 2022), have been used widely as plant growth promoters. For instance, the inoculation of PGPR and arbuscular mycorrhiza fungi (AMF) improved the above-ground biomass of tomato and corn plants, and this improvement was associated with improved phosphorus solubilization (Saia *et al.*, 2020).

Under drought, we saw an effect of microbial inoculation on levels of chlorophyll at both levels of moisture. On day 35 in the drought treatment, fungal strain 110 (*Gliomastix* sp.) increased chlorophyll, which was also the case for fungal strains 110 and 123 (*Marquandomyces marquandii*) at day 26 of the well-watered treatment (the only period when the plants were showing signs of wilting). Microbes have been extensively studied for their potential to enhance plant tolerance to abiotic stresses (Choudhary, 2012). *Trichoderma harzianum*, for instance, improved shoot and root growth, chlorophyll pigments as well as the content of total soluble proteins and proline content in tomato

plants under both well-watered and drought conditions (Mona *et al.*, 2017). Fungal strains 110 and 123 were both isolated from experimental plots under drought (D50 and D90). Thus, selection over the course of these drought treatments over time may have contributed to their ability to enhance plant chlorophyll content.

Under drought, only bacterial strain B144 (*Bacillus* sp.), increased the water content in the shoot, although it did not increase the dry biomass in comparison with the control (Fig. 2B). This bacterial strain exhibited production of EPS and biofilm formation, as determined by *in vitro* plate assays (Angulo *et al.* unpublished). Microbial EPS play multiple roles in microbial-plant-soil associations. EPS are responsible for the cohesion of microorganisms and adhesion of biofilms to surfaces, intercellular communication with other microbes and plants, carbon storage, and entrapment of nutrients and water retention and protection against desiccation and drought (Flemming, 2016; Costa *et al.*, 2018). This strain may have developed EPS products in soil, which could contribute to increased storage of water in tomato shoots.

Unlike other growth parameters, stem diameter and plant height net growth were not significantly affected by microbial inoculation at any time during our assessment regardless of watering level. We expected that the effect of the strain on plant height and steam diameter would be dependent on the level of drought, as this was the trend in other studies examining tomato growth under microbial inoculation and drought conditions (Ullah *et al.,* 2016; Ronga *et al.*, 2019). One potential factor contributing to this difference could be related to the short incubation (38 days) before tomato plants reached maturity, which may have limited our ability to observe a significant interaction between the strain and drought level. Another reason could be related to the investment of energy into other aspects of plant development, such as root length/root biomass. To assess plant biomass, we tested only shoot biomass in order to avoid disrupting the soil and allow intact soil samples to be collected for later soil aggregate stability assessment. When subjected to drought conditions, plants could use root elongation, increased rooting depth, and increased root diameter as a survival mechanism to withstand drought (Garbowski *et al.*, 2020). Plants prioritize their resource allocation and invest more in roots during drought, thereby affecting the root-shoot ratio (Eziz *et al.*, 2017). This investment allows plants to increase water uptake and maintain root water influx under drought (Bacher *et al.*, 2022). Additionally, roots are the main point for microbial interaction in soil, and plants invest additional C sources in exudates and morphological adaptations to favor particular microbial groups for drought adaptations (Shoaib *et al.*, 2022). Therefore, the plant indicators

aboveground, such as shoot biomass or plant height, might not reflect the total carbon allocation strategies being used belowground to adapt to drought conditions.

4.2 Effects of bacterial and fungal inoculation on soil properties and their relationship with plant growth

We observed an effect of microbial inoculation on the physical properties of soil, namely aggregate fractions, mean weight diameter (MWD) (which depended on the watering level), and water retention. Microbes have been studied because of their ability to impact soil structure and plant nutrition (Lehmann and Rillig, 2015; Rashid *et al.*, 2016). In our study, larger macroggregates (> 2 and 2-1 mm) were improved only by fungal inoculation at either level of watering. Fungal inoculation also had a higher impact on MWD (the index of aggregate stability) than bacterial inoculation. Fungal strain 139 was particularly effective in this respect, improving MWD by 62% and 45% when compared to the control under well-watered and drought conditions, respectively. Contrary to our expectations bacterial strain B110 that impacted MWD at high moisture in Chapter 2 did not show any effect on aggregate stability and strains B84 and B144 showed significative effects. Both bacterial strains showed a higher potential to aggregate soil according to the trait-based approach used in Chapter 2.

The formation and stability of aggregates are influenced by many factors including soil fauna, soil microorganisms, roots, inorganic binding agents, and environmental variables (Six *et al.*, 2004). For instance, the role of fungal activity in binding microaggregates < 0.25 mm into stable macroaggregates > 0.25 mm has been well documented (Tisdall, 1991; Helfrich *et al.*, 2008). The hyphae of fungi can enmesh, align, or move soil particles, thereby influencing soil aggregation status (Rillig *et al.*, 2015, Chapter 3), and, according to Bedini *et al.*, 2009, the MWD of soil aggregates was strongly correlated with hyphal length and density when inoculated with AMF. The formation of macroaggregates is also influenced by root exudates, as shown by Baumert *et al.* 2018, who used artificial exudate amendments to influence microbial community composition in favor of fungi, which in turn promoted the formation of aggregates. It is thus possible that microbial inoculations could stimulate plant activities that influence the formation of aggregates, as opposed to having direct impacts on soil aggregation.

Bacterial strain B144 also resulted in an improvement in water retention under drought, which could have led to the observed increase in the fresh weight of tomato shoots under drought. The production of EPS could have

improved soil properties and plant growth, and even though B144 did not show improvement of the MWD in soil under drought, effects were observed under well-watered conditions. Microbial EPS are involved in soil aggregation by gluing soil particles together (Totsche *et al.*, 2018). This process was studied by Vardharajula and Ali (2014), who showed that the production EPS of *Bacillus* spp. increased under drought, and this resulted in improved soil aggregation under desiccation stress. Similarly, glomalin-related soil protein (GRSP) from fungi can be important for the stabilization of soil carbon (SC) and soil aggregates (Rillig *et al.*, 2002; Singh *et al.*, 2020). EPS/biofilm formation and GRSF production can contribute to water retention in the soil matrix and reduce desiccation stress in soil environments (Lennon and Lehmkuhl, 2016).

Hydrophobicity also plays a role in aggregate stability (Piccolo and Mbagwu, 1999) and water retention in soil (Olorunfemi *et al.*, 2014). It is also important to consider that our substrate showed hydrophobicity at the time of harvest time which represented an issue to assess correctly the samples using the wet sieving technique. We used an ethanol technique instead to determine the aggregate fractions and the MWD. This technique allowed us to avoid problems related to soil hydrophobicity and also circumvent issues related to slaking (Yu Fu *et al.*, 2022). Wet sieving has been associated with slaking, which occurs during rapid wetting with water. In this process, a large amount of gas is released, and this gas participates in the destruction of soil aggregates, which can create a potential bias. Although slaking was avoided, there is still a chance that we omitted the hydrophobicity as a stabilization factor for aggregates.

Soil structure affects plant growth in several ways. For instance, macropores can provide niches for roots, water, nutrients, and microbial colonization (Passioura, 1991). In our experiment, even though we saw some correlation between some soil fractions and plant biomass, we did not see a clear relationship between MWD and plant growth for the different aboveground parameters assessed (Table 1). It is possible that the plants under stress diverted resources to different organs like roots as discussed above. Under drought, the water content showed contrasting correlations with some soil aggregation size fractions. In general, these relationships suggest that aggregate sizes of > 0.71 mm had a beneficial effect on plant shoot parameters and water content under well-watered and drought, respectively, and aggregate sizes of < 0.71, in general, had a negative effect on the same parameters.

4.3 Density of inoculated bacterial and fungal populations in soil

The quantification of microbial density for bacteria and fungi, using the 16S rRNA gene and the ITS 1 and 2 region targets, respectively, yielded a broad range of population density levels. While some strains were virtually undetectable when compared to background levels, others were able to establish themselves well in the soil environment. Soil conditions and water stress can influence microbe development. For instance, fungi disperse more readily through the soil, while bacteria have access to smaller pore spaces, and low water levels can be disadvantageous to many bacteria as compared to fungi due to their lifestyle as single-celled organisms (Lopes *et al.*, 2021).

Interestingly, of the 5 fungal strains that showed relatively high density under drought (see Fig 7A), strains 123 and 139 showed the lowest concentrations (spores/ml) at the time of inoculation (Supplementary Table S4.1). These 5 strains were isolated from experimental plots with drought treatments (D50 and D90) (section 2.2 and Supplementary Fig. S4.1), and strains 139 and 144 (designated to *Acremonium* and *Purpureocillium,* respectively) were selected based upon the fact that these two genera were higher in relevant abundance in drought plots as compared to non-drought plots (data not shown). Although these strains showed good establishment under drought, not all of them showed benefits with respect to plant and/or soil physical properties under drought. Strain 139 improved the MWD, while strain 110 (associated with *Gliomastix*) led to higher chlorophyll content. It was reported that the endophyte *Acremonium* sp. improved the performance in plants of tall fescue (*Festuca arundinacea* Schreb.) under drought (Joost, 1995). However, the effect of *Gliomastix* species' survival or enhancement of plants under drought has yet to be reported to the best of our knowledge.

For bacterial inoculation, *Bacillus* B144 (Fig. 7B) reached the highest population density under drought when compared to the other two bacterial strains. This strain, which was isolated from a drought treatment (D50), also resulted in higher water content and fresh weight of shoots under drought. Species of *Bacillus* spp. are efficient in increasing the drought tolerance of plants growing in regions with water scarcity (Minaxi *et al.*, 2012). Microbial strains isolated from drought conditions could have adapted strategies to cope with drought. For instance, drought-adaptative microbes and microbes isolated from desert plants and arid regions have been used as a strategy to alleviate the effects of drought stress in non-desertic plants (Niu *et al.*, 2018; Kour *et al.*, 2022; Naderi *et al.*, 2022).

It is noteworthy that some of our strains were not able to establish well in the soils and were undetectable above the background. It is therefore not surprising that many of these strains had negligible effects on plant growth and soil parameters. This highlights the importance of inoculum establishment for successful amendment strategies. It should also be noted that we did not work with intact soil communities, but rather microbial communities that had been reinoculated into previously sterilized soil. This may also have impacted interactions between the inoculated strains and the resident community, as well as impacts on plant and soil properties.

5. Conclusions

This study set out to improve the soil's physical properties (soil aggregation and soil water retention) and plant growth by the inoculation of bacterial and fungal strains under drought and well-watered conditions. The results showed an interactive effect of strain inoculation and level of watering. Inoculation improved mainly the plant shoot biomass under well-watered conditions, but only B144 (*Bacillus* sp.) led to an improvement in the fresh shoot biomass and soil water content under drought. Two fungal strains were also able to improve plant chlorophyll content under drought. However, we did not find a clear relationship between the improvement in soil aggregation (MWD) and plant growth as we hypothesized. Other factors could have contributed to changes in plant growth, such as the production of microbial EPS or effective microbial colonization in soil. These factors may have been selected by the drought conditions of the experimental plots from which strains were isolated. Although microbial inoculants show promise for improving plant growth and soil structure under non-stressful and stressful abiotic conditions, future research should also assess belowground plant growth parameters, as well as the impacts on the structure and functionality of the soil resident microbial community after inoculation in long-term experiments.

6. Acknowledgements

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7. Supplementary information

Figures

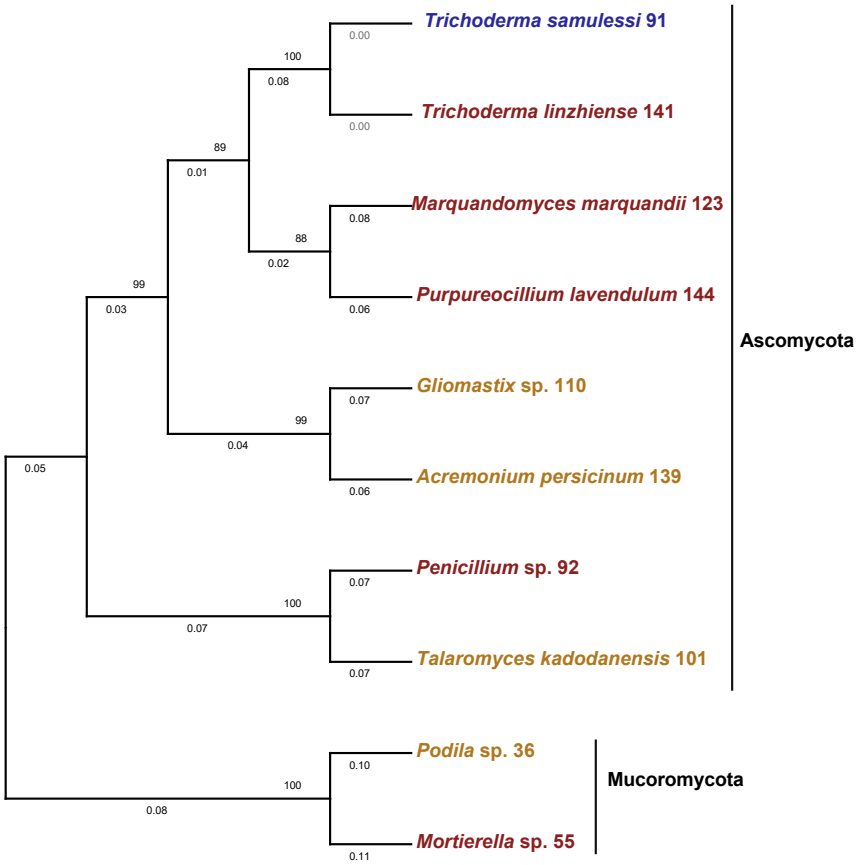


Figure S4.1. ITS phylogeny of the 10 fungal strains as inferred by neighbor-joining using the software MEGA11. Numbers at internodes refer to confidence estimates based on 1000 bootstraps. The number on the right indicates the isolate number and the species which are categorized into two subphyla: Ascomycota and Mucoromycota. The colors: red (D90), yellow (D50), and blue (control) indicate the experimental field from which strains were isolated.

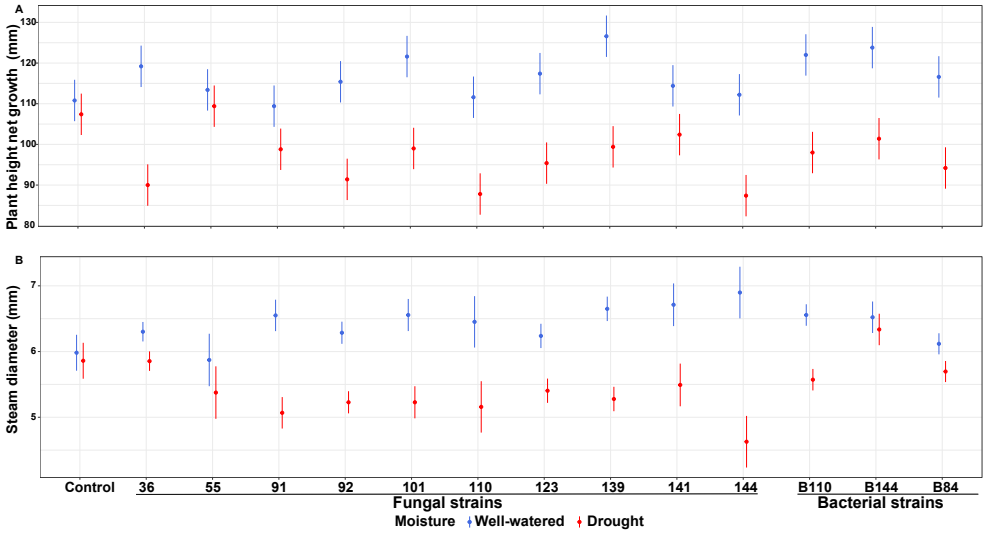


Figure S4.2. Effect of bacterial and fungal inoculation and non-inoculated control on plant height net growth (A) and diameter growth (B) after 35 days of incubation under drought and well-watered conditions. The analysis of variance (ANOVA) and the means and significance are shown in Supplementary Table S4.4 and Table S4.5, respectively.

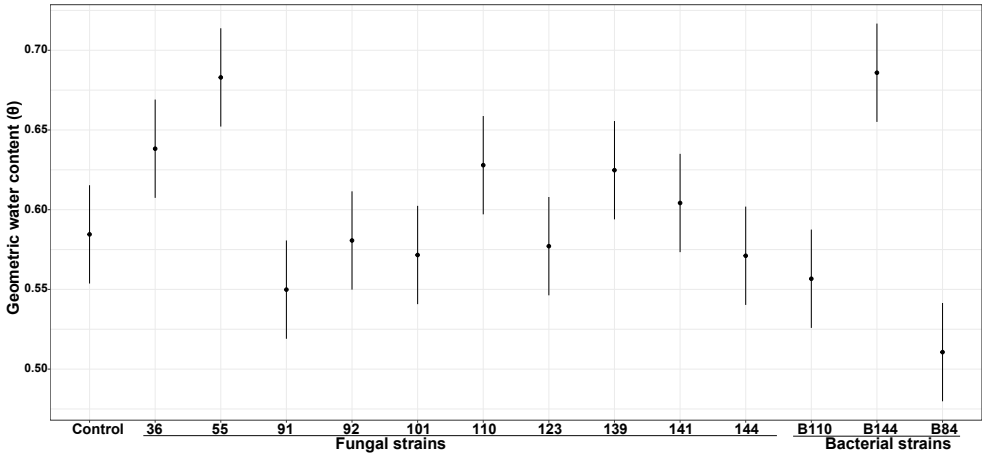


Figure S4.3. Gravimetric water content (θ) for bacterial and fungal inoculation and one non-inoculated control after 38 days under well-watered conditions. The analysis of variance (ANOVA) and the means and significance are shown in Supplementary Tables S4.4 and S4.6, respectively.

Figure S4.4. Tomato plants inoculated with fungal strain 110 (Gliomastix sp.) in soil under drought stress (bottom) and well-watered treatment (top), after 38 days of incubation.

Supplementary Tables

Table S4.1. The concentration of fungi (spores/ml) and bacterial colony-forming units (CFU) used for soil inoculation and an approx. of final concentration in soil.

Fungal strain	Identity	Initial concentration spores/mL	Final soil concentration spores/mL
36	*Podila* sp.	3×10^4	2.5×10^3
55	*Mortierella* sp.	1×10^7	5×10^5
91	*Trichoderma samuelsii*	1×10^7	5×10^5
92	*Penicillium* sp. nov.	1×10^7	5×10^5
101	*Talaromyces kadodanensis*	1×10^7	5×10^5
110	*Gliomastix* sp.	1×10^7	5×10^5
123	*Marquandomyces marquandii*	5×10^6	2.5×10^5
139	*Acremonium persicinum*	3×10^4	1.51×10^3
141	*Trichoderma linzhiense*	1×10^7	5×10^5
144	*Purpureocillium lavendulum*	1×10^7	5×10^5
Bacterial strain	**Identity**	**CFU/mL**	**CFU/mL**
B110	*Streptomyces* sp.	1.0×10^8	5×10^6
B144	*Pseudomonas* sp.	2.3×10^5	1.16×10^4
B84	*Bacillus* sp.	2.8×10^8	1.41×10^7

Table S4.2. Composition of a modified Hoagland's nutrient solution half-strength formula for growing plants.

Chemical	Stock solution (g/5 L)	Final concentration (ml/L)
KNO_3	1262	2 (5 mM KNO_3)
KH_2PO_4	680	2 (2 mM KH_2PO_4)
$MgSO_4$	1230	2 (2 mM $MgSO_4$)
Fe-EDDHA	28	2 (10 µM Fe-EDDHA)
$Ca(NO_3)_2$	2950	2 ($Ca(NO_3)_2$)
Micronutrients (2 mL/L)		
H_3BO_3	3.9	
KCl	9.3	
$MnSO_4.H_2O$	0.85	
$ZnSO_4.7H_2O$	1.45	
$CuSO_4.5H_2O$	0.32	
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.22	

Table S4.3. Bacterial and fungal designation, affinities, and sequences of primers designed using the 16S rRNA and ITS 1 and 2 respectively.

Fungal strains	Primers	Primer sequence for/rev	Region	PCR fragment length
36	342/343	TCGAGTTGAACAACACATAAAGTGTC/ TTGAGCTATCCCACGACCTTC	ITS1/ITS2	202
55	344/345	TCCTGCATCAGTCAGCACAAG/ GAGTGATCCCAACGCTTTTCCTC	ITS1/ITS2	174
91	346/347	CCGATGCGAGTGTGCAAAC/ CGCCTCTCGTAGGCGTTTC	ITS1/ITS2	315
92	370/371	CTACAGAGCGGGTGACGAAG/ AAGATTGCAGTCTGAGCGATTAGC	ITS1/ITS2	318
101	350/351	GTACTCAGACAGTCCATCTTCATC/ CCCACCCTTGTCTCTCTAC	ITS1/ITS2	135
110	352/353	CTCCCACTGAAGGGTGTG/ GTATTTACCATTTGAGTACTCTGAGTGTG	ITS1/ITS2	270
123	354/355	AGCAGGCAGGCCCCTGT/ CTAAACCCTGATTTTAATTACAGAAGTC	ITS1/ITS2	277
139	356/357	ACACCGCTCCCAGCAAAAG/ CAAACTCTTGATTGTTATAGTGGCATTC	ITS1/ITS2	247
141	358/359	GGCCCCCGTGAAGGAAG/CGCCTCTCGTAGGCGTTTC	ITS1/ITS2	248
144	360/361	GCGGCTGGTGTGCCGTC/ GACCCAAAACTACTTTTGCATTACG	ITS1/ITS2	289
Bacterial strains	**Primers**	**Primer sequence for/rev**	**Region**	**PCR fragment length**
B110	310/311	GCTCCGGCGGTGAAG/ CCCGTATCGGATGCAGAC	16S	415
B144	318/319	GGATCTTCTCCTTCATGGGAGATG/ TCAAGGTACAAGCAGTTACTCTTG	16S	275
B84	368/369	CTTCGGGCCTTGCGCTATC/ CCTTCCTCCCAACTTAAAGTGC	16S	248

Table S4.4. Analysis of variance ANOVA for the inoculation of bacteria and fungi on plant growth parameters, soil properties, and microbial density under drought and well-watered watering conditions. The analysis comprises all interactions. P values <0.05 are considered significant and highlighted in bold.

Plant growth parameter or soil property	Strain			Moisture			Strain*moisture		
ANOVA type 3	**df**	**Chisq**	**p-value**	**df**	**Chisq**	**p-value**	**df**	**F**	**p-value**
Fresh shoot biomass	13	365.297	**<0.001**	1	12.841	**<0.001**	13	184.788	**<0.001**
Dry shoot biomass	13	97.266	**<0.001**	1	12.531	**<0.001**	13	65.930	**<0.001**
Stem diameter	13	15.207	0.295	1	0.105	0.746	13	**32.215**	**0.0022**
MWD	13	504.43	**<0.001**	1	4.624	**0.031**	13	39.103	**<0.001**
Water stable aggregate > 2 mm	13	36.551	**<0.001**	1	1.191	0.2752	13	21.095	0.07
Density (fungi)	10	1428.68	**<0.001**	1	0.572	0.449	10	115.886	**<0.001**
Density (bacteria)	2	108.906	**<0.001**	1	0.093	0.761	2	37.756	**<0.001**
	df	**F**	**p-value**	**df**	**F**	**p-value**	**df**	**F**	**p-value**
Plant height net growth	13	1.068	0.3940	1	0.223	0.6375	13	1.349	0.196
ANOVA type 1	**df**	**F**	**p-value**	**df**	**F**	**p-value**	**df**	**F**	**p-value**
Chlorophyll (drought, day 35)	13	2.306	**0.0203**	1	0.201	0.6447	13	0.931	0.5220
Chlorophyll (well-watered, day 26)	13	4.25	**<0.001**	1	2.895	0.09	13	0.534	0.900
θ (drought)		$F_{13,54}$=10.52	**<0.001**						
θ (well-watered)		$F_{13,56}$=2.606	**<0.001**						

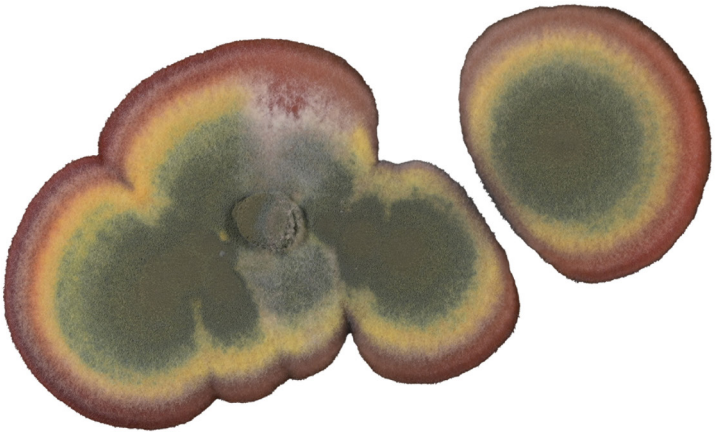
Table S4.5. Mean, standard error (SE) (±), p-value, and significance for the fresh shoot biomass (g), and dry shoot biomass (g) at 38 days. Plant height net growth (mm), and stem diameter (mm) at 35 days under drought and well-watered conditions and chlorophyll (SPAD units) at day 26 under drought and day 35 under well-watered conditions. Asterisks and bold indicate significant differences compared to the control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test.

Isolate code	Molecular identity	Watering	Fresh shoot biomass (g)				Dry shoot biomass (g)				Height growth (mm)				Stem diameter (mm)				Chlorophyll (SPAD)			
			$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Control	Drought	10.749	0.631			1.749	0.086			107.40	5.089			5.860	0.268			45.600	2.870		
36	*Podila* sp.	Drought	11.227	0.431	1.000		1.812	0.086	1.000		90.00	5.089	0.474		5.886	0.268	1.000		49.800	2.870	0.999	
55	*Mortierella* sp.	Drought	11.807	0.715	0.998		1.992	0.086	0.767		109.40	5.089	1.000		5.376	0.268	0.991		49.100	2.870	1.000	
91	*Trichoderma samuelsii*	Drought	9.326	0.399	0.819		1.676	0.086	1.000		98.80	5.089	0.995		5.068	0.268	0.707		51.800	2.870	0.960	
92	*Penicillium* sp. nov.	Drought	9.938	0.428	0.998		1.802	0.086	1.000		91.40	5.089	0.614		5.228	0.268	0.923		55.600	2.870	0.437	
101	*Talaromyces kadodanensis*	Drought	10.236	0.246	1.000		1.820	0.086	1.000		99.00	5.089	0.996		5.228	0.268	0.923		55.300	2.870	0.485	
110	*Gliomastix* sp.	Drought	9.071	0.761	0.913		1.815	0.086	1.000		87.80	5.089	0.280		5.158	0.268	0.847		**60.100**	**2.870**	**0.030**	*
123	*Marquandomyces marquandii*	Drought	9.972	0.234	0.997		1.849	0.086	1.000		95.40	5.089	0.923		5.404	0.268	0.995		55.800	2.870	0.407	
139	*Acremonium persicinum*	Drought	10.519	0.725	1.000		2.005	0.086	0.702		99.40	5.089	0.998		5.278	0.268	0.958		57.500	2.870	0.170	
141	*Trichoderma linzhiense*	Drought	11.078	0.638	1.000		1.862	0.086	1.000		102.40	5.089	1.000		5.492	0.268	0.999		52.100	2.870	0.945	
144	*Purpureocillium lavendulum*	Drought	8.795	0.352	0.290		1.607	0.086	0.996		87.40	5.089	0.250		4.628	0.268	0.082		55.900	2.870	0.396	
B110	*Streptomyces* sp.	Drought	10.027	0.857	1.000		1.731	0.086	1.000		98.00	5.089	0.989		5.572	0.268	1.000		55.000	2.870	0.556	
B144	*Bacillus* sp.	Drought	**14.666**	**0.355**	**0.000**	***	2.023	0.086	0.605		101.40	5.089	1.000		6.336	0.268	0.992		47.900	2.870	1.000	
B84	*Pseudomonas* sp.	Drought	11.613	1.209	1.000		1.821	0.086	1.000		94.20	5.089	0.856		5.854	0.268	1.000		49.400	2.870	1.000	
	Control	Well-watered	17.095	1.655			2.383	0.157			110.80	5.089			5.982	0.268			44.300	1.780		
36	*Podila* sp.	Well-watered	20.789	0.562	0.691		2.663	0.157	0.991		119.20	5.089	0.996		6.270	0.268	1.000		44.300	1.780	1.000	
55	*Mortierella* sp.	Well-watered	**26.868**	**0.735**	**0.000**	***	**3.551**	**0.157**	**0.000**	***	113.40	5.089	1.000		5.872	0.268	1.000		47.300	1.780	0.995	
91	*Trichoderma samuelsii*	Well-watered	**26.164**	**0.615**	**0.000**	***	3.159	0.157	0.051		109.40	5.089	1.000		6.550	0.268	0.965		52.500	1.780	0.074	
92	*Penicillium* sp. nov.	Well-watered	**27.324**	**1.399**	**0.001**	***	**3.293**	**0.157**	**0.009**	**	115.40	5.089	1.000		6.286	0.268	1.000		50.800	1.780	0.363	
101	*Talaromyces kadodanensis*	Well-watered	**26.295**	**0.828**	**0.000**	***	**3.170**	**0.157**	**0.045**	*	121.60	5.089	0.965		6.556	0.268	0.962		51.500	1.780	0.210	
110	*Gliomastix* sp.	Well-watered	**29.779**	**1.046**	**0.000**	***	**3.760**	**0.157**	**0.000**	***	111.60	5.089	1.000		6.452	0.268	0.993		**54.700**	**1.780**	**0.004**	**
123	*Marquandomyces marquandii*	Well-watered	**26.295**	**0.221**	**0.000**	***	**3.195**	**0.157**	**0.033**	*	117.40	5.089	1.000		6.238	0.268	1.000		**54.300**	**1.780**	**0.007**	**
139	*Acremonium persicinum*	Well-watered	**31.540**	**0.358**	**0.000**	***	**3.910**	**0.157**	**0.000**	***	126.60	5.089	0.633		6.650	0.268	0.888		51.400	1.780	0.221	
141	*Trichoderma linzhiense*	Well-watered	**25.633**	**1.020**	**0.002**	**	**3.310**	**0.157**	**0.007**	**	114.40	5.089	1.000		6.712	0.268	0.808		52.100	1.780	0.109	
144	*Purpureocillium lavendulum*	Well-watered	**24.590**	**0.726**	**0.005**	**	3.102	0.157	0.098		112.20	5.089	1.000		6.898	0.268	0.475		50.900	1.780	0.326	
B110	*Streptomyces* sp.	Well-watered	**25.342**	**0.632**	**0.001**	***	**3.433**	**0.157**	**0.001**	**	122.00	5.089	0.953		6.556	0.268	0.962		52.400	1.780	0.088	
B144	*Bacillus* sp.	Well-watered	24.763	2.367	0.319		3.061	0.157	0.151		123.80	5.089	0.869		6.522	0.268	0.977		45.900	1.780	1.000	
B84	*Pseudomonas* sp.	Well-watered	19.256	2.404	1.000		2.568	0.157	1.000		116.60	5.089	1.000		5.960	0.268	1.000		45.900	1.780	1.000	

Table S4.6. Mean, standard error (SE) (±), p-value, and significance for the soil properties: gravimetric water content (θ), water stable fraction > 2 mm, 2-1 mm, and mean weight diameter (MWD), and fungal and bacterial density in soil determined by quantitative PCR (log_{10} of copies of ITS region/g soil and 16S rRNA respectively) at 38 days under drought and well-watered conditions. Asterisks and bold indicate significant differences compared to the control: *: p < 0.05, **: p < 0.01, and ***: p < 0.001 using a Tukey test. Asterisks and bold for the fungal density indicate significant differences when compared to the mean average density and numbers in red significance lower than the average density. For the bacterial density, letters mean differences when strains are compared to each other using a Tukey test.

Isolate code	Molecular identity	Watering	θ				Fraction >2 mm			
			$\bar{X}$	SE	p-value	sig	$\bar{X}$	SE	p-value	sig
	Control	Drought	0.112	0.005			1.315	0.237		
36	*Podila* sp.	Drought	0.131	0.005	0.288		2.393	0.237	0.103	
55	*Mortierella* sp.	Drought	**0.146**	**0.005**	**0.001**	**	1.909	0.237	0.879	
91	*Trichoderma samuelsii*	Drought	0.107	0.005	1.000		2.423	0.237	0.083	
92	*Penicillium* sp. nov.	Drought	0.107	0.005	1.000		1.445	0.237	1.000	
101	*Talaromyces kadodanensis*	Drought	0.106	0.005	1.000		2.236	0.237	0.280	
110	*Gliomastix* sp.	Drought	0.128	0.005	0.554		1.836	0.237	0.950	
123	*Marquandomyces marquandii*	Drought	0.104	0.005	0.996		**2.728**	**0.237**	**0.006**	**
139	*Acremonium persicinum*	Drought	0.130	0.005	0.374		2.489	0.237	0.050	
141	*Trichoderma linzhiense*	Drought	0.120	0.005	0.998		1.788	0.237	0.977	
144	*Purpureocillium lavendulum*	Drought	0.108	0.005	1.000		**2.534**	**0.237**	**0.035**	*
B110	*Streptomyces* sp.	Drought	0.114	0.005	1.000		1.654	0.237	0.999	
B144	*Bacillus* sp.	Drought	**0.152**	**0.005**	**0.000**	***	1.760	0.237	0.986	
B84	*Pseudomonas* sp.	Drought	0.117	0.005	1.000		1.749	0.237	0.989	
	Control	Well-watered	0.585	0.031			1.745	0.315		
36	*Podila* sp.	Well-watered	0.638	0.031	0.993		2.251	0.315	0.997	
55	*Mortierella* sp.	Well-watered	0.683	0.031	0.591		3.106	0.315	0.151	
91	*Trichoderma samuelsii*	Well-watered	0.550	0.031	1.000		1.864	0.315	1.000	
92	*Penicillium* sp. nov.	Well-watered	0.581	0.031	1.000		2.262	0.315	0.996	
101	*Talaromyces kadodanensis*	Well-watered	0.572	0.031	1.000		2.819	0.315	0.487	
110	*Gliomastix* sp.	Well-watered	0.628	0.031	0.999		2.909	0.315	0.357	
123	*Marquandomyces marquandii*	Well-watered	0.577	0.031	1.000		**3.405**	**0.315**	**0.027**	*
139	*Acremonium persicinum*	Well-watered	0.625	0.031	1.000		2.570	0.315	0.842	
141	*Trichoderma linzhiense*	Well-watered	0.604	0.031	1.000		2.005	0.315	1.000	
144	*Purpureocillium lavendulum*	Well-watered	0.571	0.031	1.000		2.597	0.315	0.812	
B110	*Streptomyces* sp.	Well-watered	0.557	0.031	1.000		2.321	0.315	0.989	
B144	*Bacillus* sp.	Well-watered	0.686	0.031	0.545		2.338	0.315	0.986	
B84	*Pseudomonas* sp.	Well-watered	0.511	0.031	0.909		1.565	0.315	1.000	

Fraction 2-1 mm				MWD (mm)				Microbial soil density			
X̄	SE	p-value	sig	X̄	SE	p-value	sig	X̄	SE	p-value	sig
1.271	0.170			2.134	0.103			2.724	0.290		
1.295	0.170	1.000		2.392	0.085	0.767		**0.000**	**0.246**	**0.000**	***
1.316	0.170	1.000		2.497	0.309	0.986		**0.301**	**0.112**	**0.000**	***
1.532	0.170	0.998		**3.135**	**0.078**	**0.004**	**	**0.000**	**0.111**	**0.000**	***
0.552	0.170	0.157		2.216	0.112	1.000		**0.803**	**0.144**	**0.000**	***
1.169	0.170	1.000		2.544	0.248	0.913		**5.227**	**0.175**	**0.000**	***
1.497	0.170	1.000		2.989	0.242	0.271		**4.975**	**0.352**	**0.000**	***
1.377	0.170	1.000		2.561	0.394	0.990		**4.776**	**0.122**	**0.000**	***
1.581	0.170	0.990		**3.096**	**0.045**	**0.008**	**	**3.899**	**0.086**	**0.010**	*
1.206	0.170	1.000		**2.979**	**0.037**	**0.016**	*	2.881	0.056	1.000	
1.388	0.170	1.000		3.156	0.249	0.168		**3.831**	**0.094**	**0.022**	*
1.167	0.170	1.000		2.308	0.088	0.971		-0.209	0.104		a
1.285	0.170	1.000		2.637	0.229	0.734		**2.996**	**0.114**		**c**
1.268	0.170	1.000		2.566	0.111	0.411		0.925	0.025		b
1.277	0.170			1.865	0.070			2.414	0.290		
0.993	0.170	0.996		**2.849**	**0.111**	**0.000**	***	**1.077**	**0.246**	**0.031**	*
1.824	0.170	0.576		**2.544**	**0.078**	**0.006**	**	**-0.087**	**0.112**	**0.000**	***
1.382	0.170	1.000		2.127	0.326	0.999		**0.899**	**0.111**	**0.000**	***
0.962	0.170	0.989		1.887	0.047	1.000		**0.233**	**0.161**	**0.000**	***
1.289	0.170	1.000		**2.780**	**0.090**	**0.002**	**	**4.786**	**0.175**	**0.000**	***
1.694	0.170	0.899		**2.784**	**0.026**	**0.001**	**	3.769	0.352	0.123	
1.454	0.170	1.000		**2.751**	**0.089**	**0.007**	**	**3.683**	**0.122**	**0.006**	**
1.522	0.170	0.999		**3.028**	**0.147**	**0.011**	*	3.155	0.086	0.352	
1.139	0.170	1.000		2.626	0.214	0.257		2.483	0.056	1.000	
1.426	0.170	1.000		**2.882**	**0.089**	**0.003**	**	**3.688**	**0.105**	**0.005**	**
1.276	0.170	1.000		1.946	0.069	0.999		-0.247	0.070		a
1.614	0.170	0.980		**2.640**	**0.054**	**0.001**	**	3.279	0.757		ab
0.957	0.170	0.987		**2.630**	**0.111**	**0.043**	*	3.273	0.367		b



CHAPTER 5

PLANT-MICROBE ECO-EVOLUTIONARY DYNAMICS IN A CHANGING WORLD

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Summary

Both plants and their associated microbiomes can respond strongly to anthropogenic environmental changes. These responses can be both ecological (e.g., a global change affecting plant demography or microbial community composition) and evolutionary (e.g., a global change altering natural selection on plant or microbial populations). As a result, global changes can catalyze eco-evolutionary feedbacks. Here we take a plant-focused perspective to discuss how microbes mediate plant ecological responses to global change and how these ecological effects can influence plant evolutionary response to global change. We argue that the strong and functionally important relationships between plants and their associated microbes are particularly likely to result in eco-evolutionary feedbacks when perturbed by global changes and discuss how an improved understanding of plant-microbe eco-evolutionary dynamics could inform conservation or even agriculture.

Keywords: holobiome, microbe-mediated adaptation, rapid adaptation, species interactions, symbiosis, eco-evolutionary dynamics

Introduction

Global changes, ranging from climate change to biological invasions, nutrient deposition, pollution, and salinification, can intensify both abiotic and biotic stresses for plants and their associated microorganisms. In many cases, microorganisms can harm plants, yet beneficial microbiomes can sometimes significantly expand both the stress tolerance and the adaptive potential of plants (Kivlin *et al.*, 2013; Hawkes *et al.*, 2020; Porter *et al.*, 2020; Petipas *et al.*, 2021). When such beneficial microbes reduce the effects of global change on plant fitness, they also may reduce the strength of selection favouring the evolution of plant stress tolerance traits or increase the strength of selection favouring plant traits that attract or promote the growth of the stress-mitigating microbes. Any plant evolutionary responses might then alter plant and/or microbial ecological processes, at the population, community, or ecosystem level, potentially initiating eco-evolutionary dynamics. Such eco-evolutionary dynamics occur when ecological processes affect evolution and evolution affects ecological processes (Hendry, 2020), for example, when an evolutionary change in either the plant or microbe alters an ecological process that further changes natural selection and evolution.

Few studies have quantified the full eco-evolutionary plant-microbiome feedback resulting from a global change, but here we argue that they are likely because: (1) global changes cause strong environmental perturbations that can affect both plants and microbes (reviewed in Allison & Martiny, 2008; Blankinship *et al.*, 2011; Franklin *et al.*, 2016) and can cause strong selection on plant (e.g., Lau *et al.*, 2014; Kleynhans *et al.*, 2016) or microbial traits (Weese *et al.*, 2015), and (2) many plant-associated microbes have large population sizes, the capacity for lateral gene transfer, short generation times, and provide key ecosystem functions. We first identify the mechanisms through which microbiomes may help plants mitigate global change responses. We then outline examples by which microbiomes alter plant evolutionary responses to global change and how plant evolution might result in eco-evolutionary feedbacks between plants and their associated microbiota. We take a broad view of global changes, including both long-term, persistent changes like nutrient addition and more variable stressors like the increased frequency of drought plants in many areas will experience in the face of climate change. Both sudden and more persistent global changes, like any disturbance or shift in environmental conditions, may be particularly likely to instigate eco-evolutionary feedbacks that are mediated by microbes for the two reasons detailed above. Such plant-microbe eco-evolutionary feedbacks may also be important to population, community, and ecosystem process

given the pace of many global changes (and capacity for microbes to respond quickly), the potential for strong selection on both plants and microbes in global change contexts, and the wide range of functions driven by microbial and plant processes.

1. How do microbes affect plant ecological responses to global change?

Recent studies have illustrated the myriad ways diverse microorganisms mitigate global change effects on plants. Beneficial microbes associated with plants can stimulate plant growth and enhance plant resistance to abiotic stresses (e.g., salinity, drought, flooding) and biotic stresses (diseases) (Porter *et al.*, 2020). Beneficial microorganisms can be classic mutualists such as many plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF), and nitrogen-fixing bacteria, however, increasing evidence also suggests that diverse soil microbial communities associated with roots, leaves, and soil can also promote plant fitness under stress (Lau and Lennon, 2012; Giauque *et al.*, 2019; Hawkes *et al.*, 2020). Such microbes can influence plant responses to global changes through at least four mechanisms (Table 1).

First, the microorganism can physically alter the abiotic (often the soil) environment. Bacteria, fungi, and protists have diminutive dimensions, but they still can affect soil structure from small to large scales (Chenu and Cosentino, 2011; Erktan, Rillig, *et al.*, 2020). This structural change occurs through a variety of mechanisms. For instance, bacteria can form supracellular structures called biofilms. Biofilms are bacterial communities in which cells are embedded in a matrix of extracellular polymeric substances or exopolysaccharides (EPS). EPS can improve microbial root colonization and also can enhance the aggregation of soil particles and benefit plant growth and yield by maintaining soil moisture (Naseem and Bano, 2014; Costa *et al.*, 2018). As a result, biofilms may increase plant fitness responses to the increased drought facing many regions as a result of climate change. For instance, the EPS-producing *Pantoea* sp. had a positive effect on rhizosphere soil aggregation and microporosity and an overall positive effect on plant growth under drought (Amellal *et al.*, 1998), and a high EPS-producing *Pseudomonas fluorescens* strain stimulated seed germination and enhanced soil moisture and seedling growth under drought compared to other strains with lower production of EPS (Niu *et al.*, 2018). Similarly, AMF can produce glomalin and glomalin-related soil proteins. These compounds act as a substrate for microbes and a gluing agent for aggregates, promoting soil water holding capacity in a similar way to biofilms, potentially reducing plant drought stress

(Rillig, 2004; Singh, 2012). They can also promote the chelation of heavy metals and toxic pollutants, potentially increasing plant survival and fecundity in increasingly contaminated environments (Singh, 2012).

Table 1. Microbes can promote plant tolerance to climate change by: 1) modifying the physical environment, 2) secreting plant hormones and defence-related proteins, 3) modifying plant gene expression, and 4) promoting plant access to nutrients. Effectors (enzymes or compounds underlying the mechanism) are in italics and the details about the plant benefit provided are in bold.

Mechanism	Examples of plant stress amelioration
Physical modification of the environment	-*Glomalin, EPS, and biofilm* from fungi and bacteria **improved soil aggregate stability and increased moisture in the rhizosphere**, increasing plant survival and biomass under drought (Wu *et al*., 2008; Sandhya *et al*., 2009) and germination under salt stress (Qurashi & Sabri, 2012). -Bacterial *biofilms* **decreased uptake and accumulation of arsenic in plant tissues and improved plant growth** (Mallick *et al.*, 2018).
Secretion of phytohormones	-Rhizobial *auxins* **promoted rubisco and low molecular-weight osmolyte production**, increasing drought tolerance (Defez *et al*., 2017) and **promoted adventitious root growth** to counteract flooding (Kim *et al*., 2017). -Bacterial *cytokinins* **increased relative water content, leaf water potential, and production of root exudates** under drought (Liu *et al.,* 2013). -Endophytic fungal *gibberellins* **regulated plant hormones resulting in higher nutrient assimilation** under salt and drought stress (Waqas *et al*., 2012). -Bacterial *abscisic acid* **enhanced proline levels and photosynthetic and photoprotective pigments**, reducing plant water lost under drought (Cohen *et al.*, 2015). -*ACC-deaminase* genes in bacteria **increased root elongation and pathogen resistance** (Wang *et al*., 2000).
Modification of plant gene expression	-Bacterial *volatile organic compounds* **triggered induced systemic resistance** against a pathogen (Lee *et al*., 2012). -Bacteria enhanced mRNA expression of various **ROS-scavenging enzymes, improved PSII photochemistry and plant tolerance** to water deficit, salinity, and heavy-metal toxicity (Gururani *et al.*, 2013).
Plant nutrient acquisition	-*Nitrogenases* from *Rhizobia* **increased plant biomass and nitrogen content** under salinity (Benidire *et al*., 2017). -AMF and bacterial *phosphatases* **increased plant biomass and total phosphorus (P) content** under P deficiency in acid soils (Rubio *et al.*, 2002 and salt stress (Tchakounté *et al*., 2020). -Three distinct bacterial *ferripyoverdines* **improved iron deficiency chlorosis** (Lurthy *et al*., 2020).

Second, microorganisms can secrete chemicals that mimic plant hormones (e.g., auxins, cytokinins, abscisic acid (ABA), and gibberellins) (Friesen *et al.*, 2011). These chemicals can cause physiological changes in nearby plants that can stimulate plant growth under various stress conditions such as the increased temperature or drought plants are likely to experience under climate change (Forchetti *et al.*, 2010; Cohen *et al.*, 2015). For example, *Azospirillum sp.* produced ABA and/or increased plant produced ABA, promoting plant drought tolerance (Cohen *et al.*, 2015). The ability of microbes to synthesize

phytohormones under extreme stress where plant synthesis may be reduced can provide plants with an extra pool of these compounds, potentially helping to maintain or regain function. For example, high temperatures reduced plant production of auxin in developing anthers causing male sterility, but the exogenous application of auxin completely reversed this effect (Sakata *et al.*, 2010). In this case, the auxin was not microbially produced, but illustrates the potential for microbially produced phytohormones to maintain function. Microbes also can facilitate plant growth by decreasing hormones associated with stress like ethylene by producing enzymes that are capable of cleaving precursors in the plant ethylene pathway. For example, plant growth promoting bacterial endophytes produced one such enzyme, 1-aminocyclopropane-1-carboxylate deaminase (ACC), which reduced the build-up of salt in plants and increased plant growth and investment in reproductive structures in the face of salinity stress compared to a mutant that did not produce the enzyme (Ali *et al.*, 2014).

Third, microorganisms can alter plant gene expression, triggering physiological changes that in some cases increase tolerance to stressors imposed by the global change (e.g., Nautiyal *et al.*, 2013). For example, environmental stress can increase plant production of reactive oxygen species (ROS). Microbes can change the expression of genes involved in ROS scavenging and ethylene biosynthesis, increasing plant growth and photosynthetic performance to better tolerate global change stressors like salinity, drought and heavy metals (Gururani *et al.*, 2013; Harman and Uphoff, 2019). In other examples, volatile organic compounds emitted by some PGPR can trigger induced systemic resistance, which can prime the whole plant for enhanced defence against a broad range of pathogens and insect herbivores (Farag *et al.*, 2013; Pieterse *et al.*, 2014). Soil bacteria also can alter plant gene expression to improve plant responses to salt stress (Zhang *et al.*, 2008).

Finally, microorganisms can also mitigate the negative effects of global changes by facilitating access to limiting resources. Microbes can affect plant nutrition directly, by increasing nutrient availability (e.g., AMF or ectomycorrhizal fungi (EMF) scavenging and solubilizing phosphates, or rhizobia fixing nitrogen) or indirectly by affecting plant metabolism and growth in ways that promote plant uptake of minerals (Richardson *et al.*, 2009). Microbial promotion of nutrient access may be a major benefit to plants experiencing global changes that reduce access to nutrients (e.g., drought stress reducing access to nitrogen) or that promote increased growth that then increases nitrogen limitation (e.g., elevated CO_2 concentrations). In such cases, any negative effects of global change might be minimized (or positive effects increased in the case

of elevated CO_2) by microorganisms. For example, legumes that strongly associate with nitrogen-fixing rhizobia and plant species that associate with EMF are among those species that benefit most under elevated CO_2 (Terrer *et al.*, 2016). Ultimately, however, these benefits may require that the associated microbes are also adapted to the new environmental conditions. For instance, only salt-tolerant rhizobium strains increased *Vicia faba* biomass and nitrogen content under increasing salinity; two other tested strains did not (Benidire *et al.*, 2017).

All the mechanisms described above detail how microorganisms can benefit plants and minimize the negative consequences of global change on plant growth and fitness. However, other global changes can destabilize the plant-microbe symbiosis itself (Kiers *et al.*, 2010) and inhibit beneficial microbial functions. For example, nitrogen addition can shift plant-microbe resource mutualisms towards parasitism (Johnson *et al.*, 1997) , potentially hastening the decline or exclusion of plant taxa that benefit most from such mutualisms (e.g., legumes, Suding *et al.*, 2005). These effects are reviewed elsewhere both in the context of global changes (e.g., Toby Kiers *et al.*, 2010) and in terms of the context dependence of species interactions (e.g., Chamberlain *et al.*, 2014).

2. How do microbes affect plant evolutionary responses to global change?

Microbes affect plant ecological responses to global change (i.e., individual plant fitness) (section 1) but also can affect plant adaptive responses to global change (i. e., the strength or direction of selection acting on plant traits). Specifically, because microbes can reduce the negative consequences of global change for plant fitness, they may reduce the strength of selection favouring plant stress tolerance traits and/or increase the strength of selection favouring plant traits that attract beneficial microorganisms. Beneficial microbial communities could also strengthen selection on traits that allow plants to detect or respond more effectively to microbial signals. For example, microbes that modify the physical environment in ways that protect plants or promote nutrient acquisition (see ecological mechanisms 1 and 4 in Table 1) might both reduce selection on plant stress tolerance traits and increase selection on traits that help attract or cultivate beneficial microorganisms. Beneficial microbial communities that protect plants from global changes by secreting plant phytohormones or modifying plant gene expression could also increase selection on microbial attraction traits, but also could increase selection on traits that make plants more receptive to these microbial signals,

or even might allow for resource re-allocation away from hormone production to other plant functions. In all cases, relying on microbiomes to protect plants from global changes poses further evolutionary challenges. For example, theory suggests that such beneficial microbes will alter the evolution of immune function as plants struggle to differentiate between friend and foe, potentially making plants more susceptible to novel pathogens (Metcalf and Koskella, 2019). And theory identifying when plants should evolve to rely on microbes for stress tolerance is still limited (e.g., Hawkes *et al.*, 2020). In this section, we discuss each of the possible ways microbes might mediate plant evolutionary responses to global change. However, we note here that the ultimate evolutionary effects of global changes will also be affected by the direct selective effects of the global change on the plant and trade-offs between plant traits mediating interactions with microbes vs. plant traits directly affected by the global change. As a result, the microbiome can accelerate plant evolutionary responses to global change when the microbe-mediated selective effects act in the same direction as the direct selective effects of the global change on plant traits but can also slow plant evolutionary responses when microbe-mediate effects oppose the direct selective effects of global change.

2.1 Microbes reduce the strength of selection on plant stress tolerance traits

As described above, microbes can protect plants from the negative consequences of global changes in a number of different ways (Table 1). As a result, the direct selective effects of that global change on plant traits may be reduced. For example, if microbes increase soil water holding capacity under drought stress, there may be limited drought impacts on plant fitness and little selection favouring plant drought tolerance traits like increased investment in roots. Variation in microbial diversity or community composition certainly can alter natural selection on plant traits (Lau and Lennon, 2011; Chaney and Baucom, 2020), but few studies have assessed whether they commonly do so by reducing the negative effects of global change.

2.2 Microbes increase the strength of selection favouring plant traits that attract beneficial microorganisms

The presence of beneficial microbial communities that mitigate the effects of global change could strengthen selection favouring traits that promote interactions with these beneficial microorganisms, such as root exudation or root architecture traits (Friesen *et al.*, 2011; Verbon and Liberman, 2016). Although it can

be challenging to identify the specific traits that promote specific microbial communities, evidence from a variety of systems suggest that different genotypes recruit different microbial communities (e.g., Walters *et al.*, 2018; Kavamura *et al.*, 2020). Other studies have identified specific traits likely to contribute these interactions with microbes (e.g., Pérez-Jaramillo *et al.*, 2017). In stressful conditions, for example in flooding, plant genotypes with higher ability to form aerenchyma may promote heterotrophic, sulfur-oxidizing, methane-oxidizing, and nitrifying bacteria growth (Laanbroek, 1990; Stubner *et al.*, 1998). These bacteria in turn protect the plant from high amounts of phytotoxic compounds (e.g., reduced sulfur or excess of ammonia), which are more abundant in flooded conditions (Lamers *et al.*, 2013; Neori and Agami, 2017). Therefore, one might hypothesize that genotypes with higher aerenchyma would be highly adapted to flooding, not only because of the direct benefits of aerenchyma to plants in such anoxic waterlogged conditions (Evans, 2004), but also because aerenchyma promote the growth of certain bacterial communities. In this case, microbes may strengthen selection on this plant stress tolerance trait as the direct fitness benefits of aerenchyma combine with the benefits resulting from increased colonization from beneficial microbes.

Exudate production may be another trait under strong selection in the face of global change. For example, in the rhizophagy cycle, it is hypothesized that microbes acquire soil nutrients (especially micronutrients) in the free-living phase and enter plant roots via meristematic cells. Nutrients are then extracted oxidatively inside the plant roots. After the nutrients are exhausted, the microbes exit the plant and return to the soil through root hairs (White *et al.*, 2018). In this case, selection may favour increased exudate production to attract microbes, cell wall traits that control microbial entrance, and the production of reactive oxygen to extract nutrients from microbes (Paungfoo-Lonhienne *et al.*, 2010; White *et al.*, 2012). In contrast to rhizobial symbiosis that is limited to some plant families, the rhizophagy process may be widespread among plants. However, few studies of natural selection measure belowground traits (but see Colom & Baucom, 2020) or plant developmental traits, and as a result, we may be both misidentifying the traits commonly underlying adaptation and underestimating the role microorganisms play in plant adaptation.

2.3 Microbes strengthen selection favouring strong plant responses to microbial signals

In cases, where microbes promote plant tolerance to global change via microbial synthesis of plant phytohormones or microbial modification of plant gene expression, selection might favour plant traits promoting interactions with these microbes, but also could favour increased plant receptiveness to microbial signals. Theory suggests plants might evolve to rely on microbial signals for phenological responses, for example, because microbes might provide the most accurate environmental signal or because microbes are able to detect signals that their hosts cannot (Metcalf *et al.*, 2019). In these circumstances, plants best able to respond to those microbial cues might be favoured by selection. In other cases, microbial synthesis of plant hormones or alteration of plant gene expression might elicit stronger shifts in adaptive plant traits than simple genetic changes in the plant itself. In such scenarios, plants are predicted to evolve increased reliance on even diffuse microbiomes for stress tolerance (Hawkes *et al.*, 2020).

3. Plant-microbe eco-evolutionary feedbacks under global changes

Eco-evolutionary feedbacks describe the reciprocal effects between two pathways: how ecological change affects evolution, and how evolutionary change affects ecological processes (Hendry, 2020). The interaction between plants and microbes provides an excellent framework to study eco-evolutionary feedbacks because (1) plant-microbe interactions can strongly affect ecosystem functions that are likely to feedback to affect selection on plant and microbial traits (terHorst and Zee, 2016), and (2) microbes' short generation times, high population densities, and diverse communities make rapid ecological and evolutionary responses likely over short time-scales (Lau and Lennon, 2012; Chase *et al.*, 2021). However, even for plant-microbe interactions, often only one pathway of the eco-evolutionary feedback is empirically investigated. Here, we illustrate how plant-microbe interactions could promote eco-evolutionary feedbacks and discuss the potential prevalence of eco-evolutionary feedbacks in plant-microbe interactions under global change scenarios (Fig. 1).

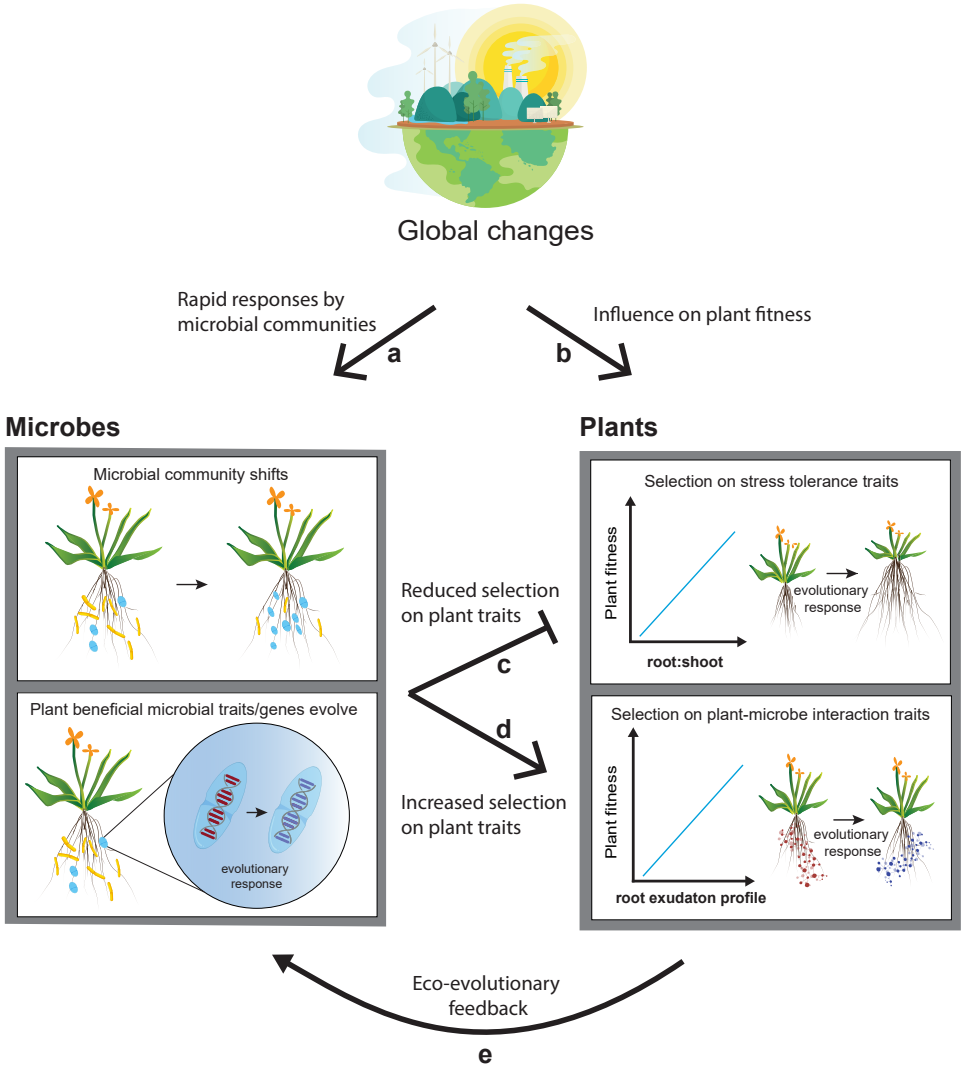
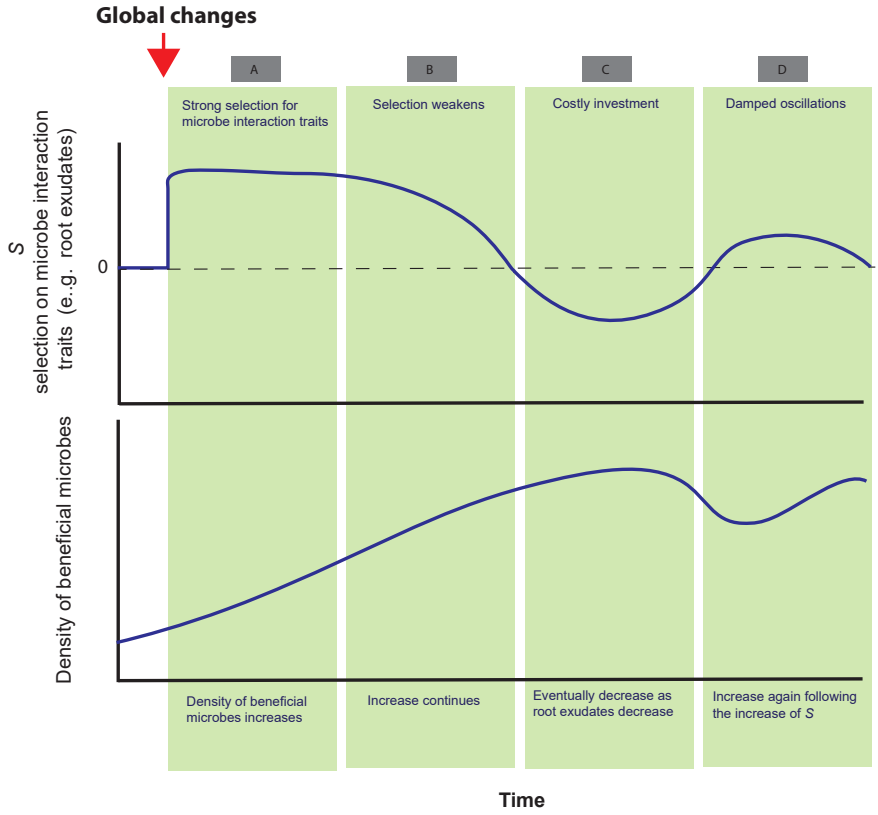


Figure 1. Global changes can cause shifts in microbial community composition or alter microbial evolution (a) and can influence plant fitness (b). These shifts in microbial community composition or microbial evolution can sometimes reduce the negative effects of the global change on plant fitness. As a result, these global change induced shifts in microbial communities or populations have the potential to reduce selection on plant stress tolerance traits (c) or increase selection on plant traits that promote interactions with beneficial microbes (d). Because many of these plant traits are likely to promote the growth of some microbes over others, evolutionary shifts in plant traits may result in further changes to microbial communities, initiating eco-evolutionary feedbacks (e).

Global changes can frequently cause rapid responses of soil microbial communities and their associated ecosystem functions (Allison and Martiny, 2008; Rillig *et al*., 2019) and can cause rapid evolution of soil microbes

(Weese *et al*., 2015) (arrow a, Fig. 1). In most cases, it is hard to distinguish the ecological changes, that is shifts in microbial community composition, from rapid evolution of microbial populations. Yet regardless of whether the microbial shift is ecological or evolutionary in nature it might influence plant fitness responses to global change (arrow b, Fig. 1) (see section 1) and ultimately selection on plant traits (see section 2). As described previously, this ecological effect caused by the shift in microbial community composition might weaken selection favouring plant stress tolerance traits (arrow c). However, if plant genotypes vary in their ability to condition the soil in ways that attract the most beneficial microbes, for example by producing certain types of exudates, then one might expect to see stronger selection favouring increased exudate production in plants (arrow d). While a number of studies have now demonstrated that microbial communities shift in ways that affect plant fitness responses to global change (Lau and Lennon, 2012; Giauque *et al*., 2019), few studies have taken the next step to show how the shifts in microbial communities affect selection on plant traits. That said, a handful of studies have demonstrated how changes in microbial diversity can influence selection on plant traits, suggesting that this latter pathway is possible (Lau and Lennon, 2011; Chaney and Baucom, 2020). Any evolutionary increase in exudate production or other traits that condition for beneficial microbes will cause further increases in the densities of those protective microbes (arrow e), amplifying the eco-evolutionary feedback. In some cases, these feedbacks can promote stronger co-evolutionary plant-microbe interactions: a recent bacterial experimental evolution study focusing on the Arabidopsis thaliana rhizosphere showed that host plants can steer the evolution of an associated Pseudomonas strain to mutualism (Li *et al.*, 2021). Despite suggestions that eco-evolutionary feedbacks mediated by plant-microbe interactions may be common and strong (terHorst and Zee, 2016), few studies demonstrate the entire feedback cycle from ecology to evolution and back to ecology. While there is potential for long-term eco-evolutionary dynamics in plant-microbe systems (Box 1), many questions remain to be answered:

Box 1: The potential for eco-evolutionary dynamics in plant-microbe systems.



Global changes have the potential to kick start eco-evolutionary feedbacks that alter plant-microbe interactions in similar ways to classic examples of eco-evolutionary feedbacks mediated by predator-prey interaction traits (e.g., Yoshida *et al*., 2003). Theory and empirical studies suggest that many potential outcomes from eco-evolutionary multispecies interactions are possible, including the cycles previously observed in the Yoshida *et al.* (2003) predator-prey system, damped oscillations (e.g., Frickel *et al*., 2016), or a complete breakdown of coexistence (Kremer & Klausmeier, 2013). In one potential scenario depicted here, some microbes benefit plants under global change. For example, perhaps certain microbes promote plant resilience to drought. Because of the increased benefit provided by these microbes in the face of global change, plants experience strong selection on traits that promote the growth or attraction of these beneficial microbes (e.g., the production of particular exudates) (A). Increases in the plant traits that attract or benefit those beneficial microbes (resulting from positive selection on those traits) will increase the abundance of those beneficial microbes. As the beneficial microbes increase in abundance in the soil microbial community, selection favouring plants that produce copious exudates weakens as there is little need to promote the growth of or attract more beneficial microbes (B) until selection may even favour reduced investment in these microbial interaction traits as there is little need to recruit more of these microbes to the rhizosphere and the costs of producing the trait outweigh any benefit (C). As a result, the frequency of plants in the population producing many exudates is reduced and beneficial microbes decline in abundance, which then begins the cycle again by causing selection to once again favour plant phenotypes with high exudate production (D).

(1) *Are eco-evolutionary feedbacks more common or stronger in tight pairwise symbioses than more diffuse interactions between plants and diverse microbial communities like those that inhabit soils or leaves*? Plant-microbe interactions can be diffuse, where plant hosts interact with the hyperdiverse microbial communities inhabiting soil or leaves, or can be tight, pairwise, coevolved symbioses, like the interactions between legumes and rhizobia. While some of the same mechanisms that stabilize and promote reliance on microbes for stress tolerance in tightly coevolved systems can apply to more diffuse interactions that are continuously reassembled from generation to generation, the evolution of plant reliance on microbes for stress tolerance may occur under a more restricted set of conditions in these diffuse systems (Hawkes *et al.*, 2020). One might predict that more tightly interacting plant-microbe partners have higher likelihood for eco-evolutionary feedbacks to occur, while more diffuse associations, like those between plants and the soil microbial community, have weaker but more stable interactions that would dampen eco-evolutionary feedbacks.

(2) *How does the type, rate or intensity of environmental change influence the likelihood or magnitude of eco-evolutionary feedback?* Across all systems, most studies documenting eco-evolutionary feedbacks occur in systems perturbed by human-caused environmental change (either natural or experimental). For example, one of the classic cases of eco-evolutionary feedbacks investigated alewives in landlocked lakes. In such lakes alewives' intensive selective grazing depleted large-body zooplankton resulting in strong selection causing a shift in alewives' foraging traits to increase predation on small-body size zooplankton (Smith *et al.*, 2020). Similarly, some of the strongest effects of microbial community responses on plant fitness arise from variables associated with climate and climate change (e.g., drought stress or aridity gradients Lau & Lennon, 2012; Giauque *et al.*, 2019), and a recent example illustrates how microbial evolution in response to nitrogen-addition affects plant communities in experimental mesocosms (Lau *et al*., unpublished manuscript). Does the prevalence of human-caused environmental change in many classic examples of eco-evolutionary feedback result from bias in choosing systems to investigate eco-evolutionary feedback or are global changes more likely to perturb systems in ways that elicit eco-evolutionary feedbacks? One might predict that large, rapid environmental changes (e.g., exceptionally warm years, extreme drought, or higher rates of nitrogen deposition) will produce strong ecological responses that alter natural selection and cause strong, persistent evolutionary responses that may feedback to affect ecological process. On the other hand, more gradual

changes might be more likely to produce stronger evolutionary responses because larger population densities can be maintained to promote adaption before extinction (Gonzalez *et al.*, 2013).

(3) How does the context dependency of plant-microbe interactions catalyse or inhibit eco-evolutionary feedbacks? Both mutualistic and antagonistic plant-microbe interactions are heavily influenced by abiotic factors ranging from resource availability to elevated temperatures, and biotic factors like the presence and diversity of other microbes, herbivores, or plant competitors (Chamberlain *et al.*, 2014). These are the same factors likely to be directly or indirectly affected by many global changes. In some cases, this context dependency could catalyse eco-evo feedbacks. For example, nitrogen addition causes shifts in the legume-rhizobium mutualism, reducing the benefits rhizobia provide to plant hosts and typically reducing plant investment in rhizobia (Streeter and Wong, 1988). Through a variety of potential mechanisms, including the reduced investment in rhizobia causing rhizobia to spend more time in non-symbiotic free-living life stages, nitrogen addition selects for less cooperative rhizobia (Weese *et al.*, 2015). Hypothetically, this evolution of reduced cooperation could then impose an additional cost on plants, accelerating legume declines in high nitrogen environments, further increasing the time rhizobia spend in free-living life stages and accelerating the evolution of reduced cooperation.

In other cases, this context dependence could dampen or inhibit eco-evolutionary feedbacks. For example, many studies, particularly those investigating evolutionary pathways in the eco-evo feedback cycle, employ single strain inoculations or otherwise simplistic growing environments (e.g., a single species host plant community, Lau & Lennon, 2012), but plant-microbe interactions are inherently diffuse, potentially involving dozens of plant species and 100s or 1000s of microbial taxa. These taxa can combine to produce novel functions. For example, when two bacterial strains interacted they produced a novel microbial volatile, not produced by any of the strains separately, with antimicrobial and quorum sensing disruption properties (Kai *et al.*, 2018). As a result, if microbial community composition shifts rapidly across space or time, selection may be so variable that strong, directional evolutionary responses are inhibited.

(4) Many global changes are occurring simultaneously - will multiple simultaneous global changes inhibit or promote plant-microbe eco-evolutionary feedbacks? Adaptation to multiple simultaneous novel selective agents is challenging. However, the diverse traits and functions of diffuse microbial communities could facilitate plant adaptation in such a scenario. If different microbial taxa

fulfil different functions or protect plants from different global changes, then multiple global changes may increase plant reliance on microbes for adaptive responses even more, potentially strengthening selection on plant traits that attract or promote the growth of diverse microbial communities. In such a scenario, then one might expect plant-microbe eco-evolutionary feedbacks to become even more likely and also more important to plant responses to global change. Alternatively, given that multiple global changes combine to reduce microbial diversity (Rillig *et al.*, 2019), the capacity for microbe-mediated adaptation may be reduced, as functional diversity is reduced and stress tolerant clades dominate.

4. Eco-evolutionary changes resulting from global changes disrupting plant-microbe symbioses

In the previous sections we considered eco-evolutionary feedbacks that result from beneficial microbes mitigating the effects of global change for their plant hosts. However, eco-evolutionary feedbacks can also result from global changes causing the breakdown of plant-microbe symbioses. For example, Evans *et al.* (2016) found that the invasive species, *Alliaria petiolata*, destroyed AMF networks that benefited native species, producing strong eco-evolutionary feedbacks. Specifically, in high interspecific competition, natural selection favoured increased production of the antimycorrhizal allelochemical sinigrin by *A. petiolata*. High sinigrin concentration inhibited the growth of competing native species that relied on AMF, facilitating *A. petiolata's* success while also shifting competition from interspecific to primarily intraspecific competition. Because high sinigrin concentrations are costly and of little benefit to intraspecific competition, selection favours reduced sinigrin production when *A. petiolata* densities become high enough. In this case, microbes mediate the effects of global change and played a large role in an eco-evolutionary feedback, not because they protect their host plants, but because they themselves are inhibited by the global change (invasion by *A. petiolata*).

Such effects may even occur in human dominated systems, although in many such cases selection on the plants is artificial rather than natural. Breeding for increased production in high resource environments has resulted in more recent agronomic cultivars benefiting less from high quality microbial partners or having less ability to impose sanctions on less-effective partners (Pérez-Jaramillo *et al.*, 2016). For example, soybeans have lost defence mechanisms against poor-quality rhizobium partners in comparison with ancestral cultivars (Kiers *et al.*, 2007). While loss of such sanctioning ability may not be costly in

high nutrient environments, it may limit soybean production in more marginal lands and increase reliance on synthetic fertilizers or other management techniques. Selection on microbes in agricultural systems also may be strong, inadvertently further favouring the development of cultivars that are less reliant on microbial symbionts. For example, conventional agriculture, tillage, and annual monocropping can reduce the diversity of potential microbial partners (Hartmann *et al.*, 2015; Bowles *et al.*, 2016; Vukicevich *et al.*, 2016) and damage AMF that help the plants take up phosphorus and nitrogen (Bowles *et al.*, 2016), perhaps even causing the evolution of less cooperative AMF or rhizobia (Kiers *et al.*, 2002). Both the selection of cultivars that have lesser interaction with the soil microbes and the reduction of potential microbial partners might restrain potentially beneficial eco-evolutionary feedbacks in these agronomic systems.

5. Conclusions

Capitalizing on a long history of research illustrating how microbes can promote plant stress tolerance, researchers are now applying these ideas to global change contexts and linking them to both plant evolution and eco-evolutionary feedbacks. Plant-microbe interactions have the potential to play important roles in plant adaptation (Petipas *et al.*, 2021), yet more empirical and theoretical work is needed to predict when microbes are likely to be most important to plant evolution and to catalyse eco-evolutionary feedbacks. Once we have a better understanding of when and how microbes promote plant adaptation to the stresses caused by rapid anthropogenic environmental changes, we can begin to identify which plants and microbes may be most affected by global change, understand how to manage for beneficial microbial communities, and manipulate the composition of microbial communities or the conditions that select for beneficial microbial communities, for applications ranging from ecological restoration to agriculture.

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7. Author Contributions

All authors jointly discussed the ideas presented in the manuscript and contributed to the editing of the manuscript. Each author wrote the initial draft of one section of the manuscript.

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CHAPTER 6

GENERAL DISCUSSION

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Soil structure plays an important role in sustaining soil functions (Rabot *et al.*, 2018). The quality of soil structure depends on the stability of aggregates (Amézketa *et al.*, 1996). The stability of aggregates depends on their ability to keep intact when exposed to different stresses (e.g., erosion) (Emerson and Greenland, 1990; Díaz-Zorita *et al.*, 2002). Soil microorganisms impact soil structure and aggregate stability via a range of mechanisms (Rashid *et al.*, 2016). This suggests that inoculating soils with bacteria and fungi with structure-enhancing properties may be a means to improve soil structure. To examine the potential of microbial inoculants as a strategy for improving soil structure and function under drought, we first isolated bacterial and fungal strains retrieved from a field experiment with precipitation manipulations to induce drought conditions. In **Chapter 2,** we screened bacterial strains for traits associated with aggregate stability and tested the predictive value of these trait combinations in a bacterial inoculation experiment. Analogous in **Chapter 3,** we tested the effect of a range of fungal strains on aggregation and hydrological properties in inoculation experiments. In **Chapter 4**, we determined the effect of both bacterial and fungal inoculations on plant growth, soil aggregation, and water retention under drought and well-watered conditions. Finally, in **Chapter 5,** we studied the plant-microbe eco-evolutionary dynamics involved with adaptation to global changes.

1. Microbial trait-based approach and soil aggregation

In **Chapter 2,** we characterized a bacterial strain collection, isolated from a drought experiment, across a range of targeted traits potentially associated with abilities to influence soil structure under conditions of drought. In our study, the targeted traits were screened in a series of plate assays. We selected "key" traits such as extracellular polymeric substances (EPS) and biofilm formation as properties that might be related to improved soil aggregation under drought. EPS and biofilm production is recognized to support the formation of soil aggregates and provide protection under desiccation (Nwodo *et al.*, 2012). Next, strains were categorized as good, intermedium, and poor with respect to potential traits to improve aggregate stability under drought and subsequently inoculated in soil under two moisture levels. We found out that only 8% of the strains improved aggregate stability compared to the uninoculated control, and this was only observed under high moisture conditions. Contrary to our expectations, strains with the top trait rankings (good aggregators) based on the screening realized on plate experiments did not significantly improve aggregation as determined by both dry and wet sieving.

Microbial EPS act as transient binding agents to join small microaggregates, according to the aggregate hierarchy theory (Tisdall and Oades, 1982). The influence that EPS have on soil aggregation depends on its molecular weight and ability to form pluri-molecular networks (Chenu and Cosentino, 2011). In **Chapter 2**, we used slaking as a disruptive method for assessing soil structure, nevertheless, this method has been considered so aggressive for extracellular polysaccharides (Tang *et al.*, 2011) and lower energy stress methods are suggested to analyze the influence of bacteria on soil aggregate stability. The extracellular bacterial EPS have a heterogeneous composition and are associated with proteins, lipids, and nucleic acids (Flemming, 2016). Redmile-Gordon *et al.* (2020) showed, for instance, that EPS-protein were more closely related to aggregate stability than EPS-polysaccharide, and soils with high EPS-proteins were correlated to a higher mean weight diameter (MWD), a standard index of aggregation. In **Chapter 2,** we measure only EPS-polysaccharides production in soil. The chemical structure and material properties of EPS also depend on the growth medium used (Kimmel and Roberts, 1998). There is a large diversity in the EPS produced by different microbial species, with a wider range of EPS biosynthesis pathways (Schmid *et al.*, 2015). Additionally, the quantification of EPS also depends on the exact methods used for its isolation and characterization (Naseem *et al.*, 2018). The quantification of EPS poses some challenges, such as intracellular or extracellular contamination from plant residues (Chenu, 1995). Redmile-Gordon *et al.* (2014) proposed a cation exchange technique to quantify EPS from soils with lower contamination from humified soil organic matter. It is important to keep these limitations when considering the results presented in **Chapter 2**, where only negligible levels of EPS-exopolysaccharide were detected in soil. Discrepancies between laboratory assays and soil measurements may indeed reflect low EPS expression in the soil microcosms examined, but it could also reflect difficulties with accurately measuring EPS in such soil systems. Nevertheless, other bacterial traits were able to explain aggregation dynamics to some extent (**Chapter 2,** Figure 8). These included the density of the bacterial population, as well as the phylogeny and the field treatment of isolation of the inoculated strains.

In **Chapter 3**, we found that the realized fungal biomass (based on ergosterol concentration) played an important role in the aggregate stability under high and low moisture content whereas the soil parameter sorptivity, was an explanatory variable in the aggregate stability under low moisture content. We also examined the effect of colony density, as this variable was selected by Lehmann *et al.* (2020) as one of the best fungal traits in their trait-based

approach. This trait was positively correlated to the MWD (Bedini *et al.*, 2009). However, we could not find a direct effect on aggregation formation and stability at either level of moisture. Conversely, in **Chapter 4,** we observed that high bacterial abundance of *Bacillus* sp. B144 may have led to significantly higher water retention and increased fresh shoot biomass under drought conditions. Even though, this EPS producer B144 did not improve the aggregate stability under drought.

In literature, numerous other soil and environmental factors have been related to aggregate dynamics and stability. For instance, internal soil factors such as electrolytes, clay mineralogy, $CaCO_3$, and organic matter, or external factors, such as climate and soil moisture, can have large effects on aggregate formation and stability dynamics (Amézketa *et al.*, 1996). Thus, it may prove difficult to extrapolate microbial traits as determined *in vitro* with activities under real soil conditions.

2. Bacteria vs. fungi on aggregate dynamics

In **Chapters 2** and **3,** we inoculated single strains of bacteria and fungi, respectively, into similarly constructed microcosms, containing a coarse sand mix substrate (0.7% organic carbon) at similar moisture levels. After 8 weeks, we found that the effects of fungi on macroaggregate stability were 2-3 fold higher than the effects of bacteria for the relative MWD (MWD normalized to their respective control) at low and high moisture levels, respectively (Fig. 1A). The larger effect on aggregation observed for fungi may be related to a range of mechanisms by which fungi can affect soil aggregation. Fungi are involved in the production of EPS, which can play a role in cementing particles leading to the formation of microaggregates (Totsche *et al.*, 2018). Fungal hyphae can enmesh soil particles, which can be a major factor in the formation and stabilization of soil macroaggregates, according to the aggregate hierarchy theory (Tisdall and Oades, 1982; Tisdall *et al.*, 1997). The fungal mycelium has been described as a "sticky string bag" (Miller and Jastrow, 2000) because of its entanglement and cementing properties (Oades and Waters, 1991). The hydrophobic properties of fungal proteins within aggregates can also restrict water infiltration and increase the water repellency of the soil which can lead to an increased fraction of water stable aggregates (Piyaruwan and Leelamanie, 2020). Fungal hydrophobicity also allows fungi to reduce water surface tension and grow through air and bridge soil pores (Ingham *et al.*, 2011), which can be advantageous under conditions of limited nutrients or other resources (Bielčik *et al.*, 2019).

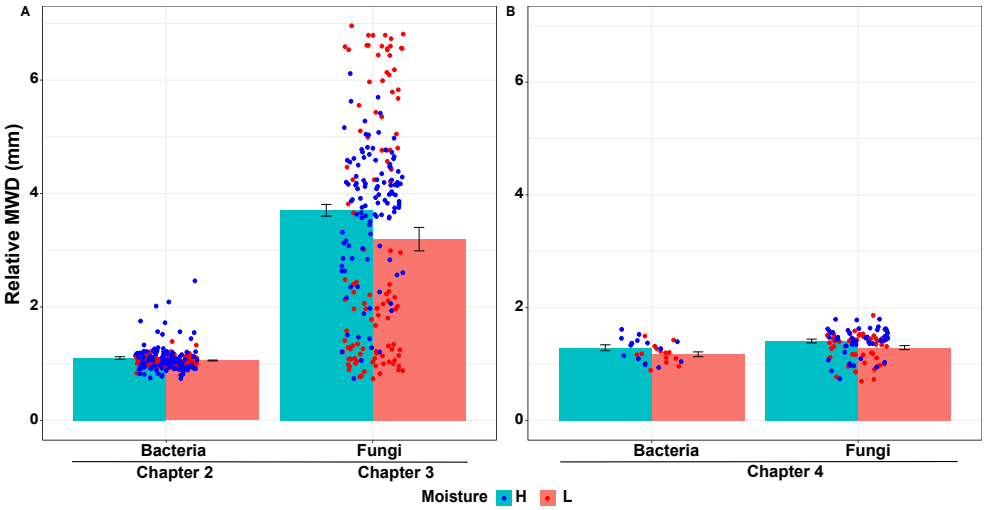

Figure 1. Relative MWD for aggregate stability (MWD normalized to the respective non-inoculated control) for bacterial and fungal inoculation in Chapters 2 and 3, respectively A), and relative MWD for aggregate stability for bacterial and fungal inoculation with plants in Chapter 4 B). Treatments were settled under high and low moisture. Bars represent standard error and dots the distribution of the data.

Furthermore, soil fungal biomass can exceed that of bacteria (in terms of milligram C per gram of soil). In a global study of carbon biomass in topsoil, He *et al.* (2020) determined a predominance of fungal biomass reaching ratios F: B as high as 8.6 in tundra ecosystems. Fungal biomass (of a single organism) can also be more spread spatially due to the nterwoven network of hyphae (the mycelium) and temporarily due to its greater longevity. An example is the network described for *Armillaria bulbosa,* which can reach a minimum of 15 ha, weigh 10,000 kg, and live more than 1,500 years (Smith *et al.*, 1992). Longer hyphae have a greater chance of connecting aggregates (Peng *et al.*, 2013; Z. Zhang *et al.*, 2019). Realized soil fungal biomass (based on ergosterol concentrations) was the most important trait explaining soil aggregation in **Chapter 3**. In total, fungi can aggregate soil at larger spatial scales than bacteria. Given all the advantages of the fungal mycelial, it is important to mention that filamentous actinomycetes resemble fungi in their morphology, branching hyphae, asexual spores, and mycelium (Olanrewaju and Babalola, 2019). It means our bacterial strain 110 belonging to *Streptomyces* could have adopted some fungal strategies to aggregate soil in **Chapter 2**.

In **Chapter 4,** the soil inoculation was carried out using a different substrate mix (potting soil and medium sand, 8.7% organic carbon) and aggregates

were collected using a different technique than the slaking approach used in **Chapters 2** and **3**. We found that fungal inoculation showed only lightly higher relative MWD as compared to bacterial inoculation at both moisture regimes (Fig. 1B). Unlike **Chapters 2** and **3,** in **Chapter 4** we established the microbial inoculants for only 2 weeks before the reinoculation of the native microbial community. This shorter period of inoculation and interactions with a microbial community could have led to lower colonization rates in soil for some strains in comparison to the earlier single-strain inoculation experiments. It is known that soil microbes impact each other through a range of beneficial and deleterious interactions, which affects the ability of inoculated strains to establish in the community as well as overall soil ecosystem functioning (Brözel, 2022). The diversity of soil microbiota also determines the efficiency of invader species' establishment (van Elsas *et al.*, 2012). Microbial interactions can also result in emerging properties. For instance, Kai *et al.*, 2018 showed that two bacterial strains could only produce a novel microbial volatile when they were interacting with each other, highlighting the need to consider microbial activities at the community level for certain properties.

In addition, the interaction with plant roots in **Chapter 4,** could either support macroaggregates by producing mucilage to bind soil particles together and harbor and provide nutrients to microorganisms (Tisdall and Oades, 1982; Materechera *et al.*, 1992). Growing plant roots can also disrupt aggregates via physical forces (Allison, 1973). These additional factors could have interfered with the bacterial and fungal microbial inoculation effects on aggregation formation and stability in **Chapter 4**, making it more difficult to compare across the different experiments presented in this thesis. Longer-term experiments (including different growth phases of plants) and higher concentrations of inoculum can help a better establishment of microbial inoculants and could help to better unravel the interaction between plants and microorganisms on soil aggregation.

3. Effects of moisture on microbial inoculation, soil, and plants

One of the main goals of this thesis was to determine the effect of microbial inoculation on aggregate stability under two different moisture regimes. To this end, in **Chapters 2** and **3**, we used the permanent wilting point for plants (-1.5 MPa) as a reference to delimit drought conditions. The moisture dynamics during the incubation period are depicted in Fig. 2A, fungal inoculation suffered from a continuous moisture loss meanwhile bacterial got the moisture re-established to the system after periodical loss. The fungal experiment did not get a reestablishment of moisture due to the high hydrophobicity shown by

some of the strains. We found that, in most of the cases, there was a significant interaction between microbial strain and moisture regimes. **In Chapter 3**, under high moisture, fungal strains on average reached higher values of aggregate stability (MWD) (represented as MWD_W in **Chapter 3**), but higher means were registered for a number of strains under drought (Fig. 1). This was also coupled with higher values of fungal biomass and lower sorptivity, which could have explained the positive effect on the aggregation. The moisture loss under drought along the incubation period showed values of -60 MPa at low moisture, which is unsuitable for fungal metabolic activity for the majority of species. However, hyphae under desiccation were still connecting soil as depicted in Fig. 3. **In Chapter 2**, the bacterial effect on aggregate stability was nevertheless low at high moisture and negligible under low moisture, even after the moisture was re-established to the system (Fig. 2A). These results can be partly explained by the difference by which fungal and bacterial metabolism is adjusted during adaptation to changes in soil moisture levels (Chotte, 2005).

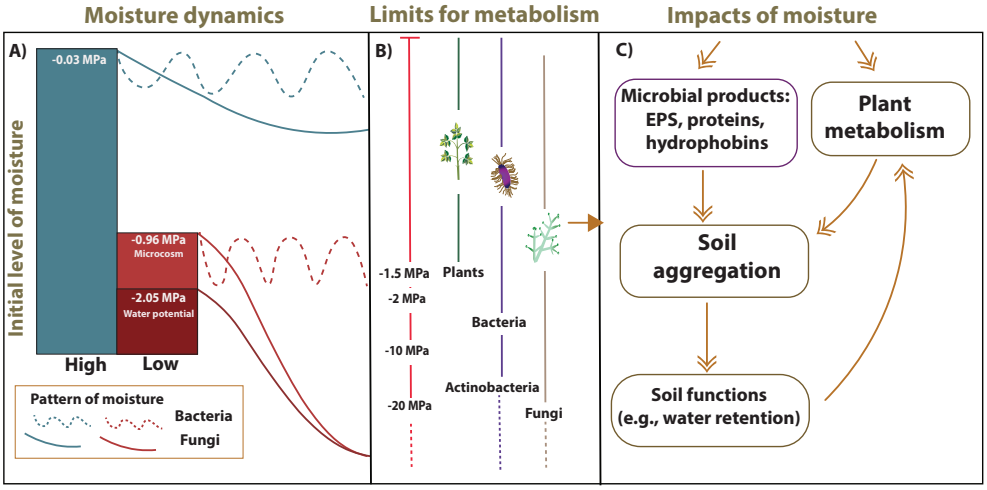

Figure 2. Schematic representation of the moisture dynamics during incubation in a microcosm experiment in Chapter 2 and microcosm and water potential experiments in Chapter 3 A). Dashed lines represent the schematic moisture dynamics for bacteria and solid for fungi. High and low levels of moisture are in blue and red, respectively. The reference threshold for microbial and plant metabolism (B), and the effects of moisture on microbial and plant functioning (C).

Microbial metabolism varies widely across soil moisture regimes. As the soil dries, conditions shift from favorable for soil fauna and bacteria to more favorable for fungi and actinomycetes (Manzoni *et al.*, 2012), this effect is depicted in Fig. 2B. Nitrifying bacteria, for instance, can suffer from

physiological damage in a water potential lower than -0.6 MPa. The lower limit for *Pseudomonas* was -5.6 MPa, -7 MPa for *Bacillus* (Potts, 1994), and -17 MPa for *Arthrobacter* spp. (Chen and Alexander, 1973). Actinomycetes tend to remain active under dryer conditions. Zenova *et al.* (2007) described -2.8 MPa as the optimal moisture for the growth of 11 actinomycetes genera, with the formation of microcolonies at -22.6 MPa and poor spore germination at -53.6 MPa. For fungi, there is a strong variability in the water stress threshold. For instance, wood decay fungi have shown optimal hyphal growth at moisture levels higher than -5 MPa (Maynard *et al.*, 2019). The Ascomycota *Gleotinia temulens* showed declination in radial growth and spore germination at -10 MPa (Alderman, 1992) and *Penicillium rubens* showed a dramatic decrease in growth rate at 0.86 a_w (approx -22.4 MPa). Interestingly, xerophilic fungi like *Xeromyces bisporus* have optimal growth around -22 MPa, but can still show activity at -60 MPa. One of the big advantages of fungi is the ability to redistribute water through their hyphae. This was shown for a saprotrophic fungus that could redistribute water from moist (-0.03 MPa) into dry (-9.5 MPa) micro-habitats. This hydraulic redistribution is likely one of the mechanisms behind the higher resistance to soil desiccation displayed by some soil fungi as compared to bacteria (Guhr *et al.*, 2015). Moisture impacts microbial activity, microbial activity on the building of aggregates, and the aggregates will support soil functions (Fig. 2C).

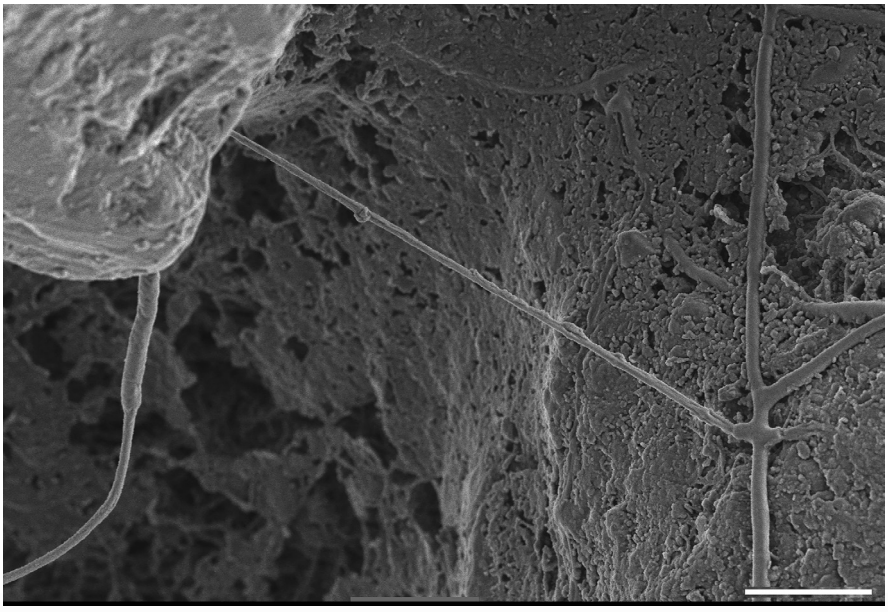

Figure 3. Hyphae of strain 141 (*Trichoderma linzhiense*) at low moisture content connecting soil particles. The bar is 100 µm.

In **Chapter 4,** microbial inoculation had a higher impact on plant growth indicators and soil properties under well-watered conditions. Under drought, we only observed a modest effect on plant growth, water retention, and soil aggregation. The interactions with the plant under contrasting levels of moisture may also have interacted with the formation and/or destabilization of soil aggregates. This was demonstrated with plants in different homogenized soil types and moisture regimes, where the interactions resulted in a diversity of effects on soil aggregates during desiccation (e.g., cracking and shrinking or changes in pore water pressure in the soil caused by water uptake by plant roots) (Materechera *et al.*, 1992). In synthesis, the changes in moisture impact plants and microbial metabolism and both may impact soil aggregate dynamics. Nevertheless, the wider range of microbial growth under desiccation may impact largely soil structure and soil functions (e.g., water retention) and thereby support plant growth to ameliorate climate change as is depicted in Fig. 2C.

In **Chapter 5,** we studied how microbes support plants to mitigate anthropogenic and environmental changes via eco-evolutionary feedbacks. First, microbes can affect plant ecological responses to global changes, thereby reducing the strength of selection favoring the evolution of plant stress-tolerance traits. Beneficial microorganisms such as many mutualistic plant growth-promoting rhizobacteria (PGPR) or symbiotic arbuscular mycorrhizal fungi (AMF) have been widely known to alleviate plant stress. These beneficial microorganisms support plants via several strategies such as the secretion of phytohormones, acquisition of primary nutrients, or modification of plant gene expression (Kumar *et al.*, 2022), as well as by indirect effects of physical alteration of the soil environment (Augé *et al.*, 2001). We presented evidence that microbes can enhance soil water holding capacity (WHC) and reduce the impact on plant fitness under desiccation by the production of gluing agents (Wu *et al.*, 2008). This acts to reduce the selection favoring plant drought tolerance traits such as the production of expensive osmoprotectant osmolytes, root modifications (Wahab *et al.*, 2022), or allocation of biomass (Eziz *et al.*, 2017). So far, very little attention has been paid to this indirect strategy of modification of the root environment, and this study, therefore, sought help to address this research gap.

Second, microbes also can increase the strength of selection by favoring: (i) plant traits that attract beneficial microorganisms and (ii) strong plant responses to microbial signals through the modification of root architecture and root exudation patterns under stress. Root exudates are characteristic of particular plant species and function as selective communication signals with

microorganisms (Badri and Vivanco, 2009). It was postulated by Williams & de Vries (2020), for instance, that fast-growing plants modify their exudates to recruit more microorganisms that facilitate a more efficient regrow after events of drought. Finally, the eco-evolutionary feedbacks are explained between two pathways which describe how ecological changes affect evolutive processes and how these evolutionary changes affect ecological processes (**Chapter 5,** Fig. 1). These eco-evolutionary feedbacks can be strong (terHorst and Zee, 2016) and may depend on the strength of plant-microbes interactions (e.g., mutualism, symbiosis) (Sachs *et al.*, 2011), the level of dependency, the level of intensity of the environmental changes and how simultaneously these events occur. This effect of eco-evolutionary dynamics can be compared schematically in **Chapter 4.** We inoculated plants with microbial populations and communities in soils that were exposed to events of drought. This stress could have triggered a microbial community shift and/or an evolution of microbial traits/genes (potential changes in microbial composition and function). These microbial changes may have increased the selection of plant traits (e.g., higher production of exudates to recruit stress-beneficial microbes) or reduced selection of plant traits through the (i) production of phytohormones or other metabolites improving plant traits under desiccation or (ii) improving soil structure and functions (e.g., water holding capacity) to counteract the effects of drought. The potential changes in plant traits are likely to promote the growth of some microbes over others, evolutionary changes in plant traits may result in further changes in microbial communities, affecting ecological processes and thereby initiating eco-evolutionary feedbacks.

Taken together, these findings highlight the importance of moisture as a factor ruling metabolism, interactions, and survival for plants and microbes. The wider range of moisture for microbial growth confers an advantage for the design of microbial inoculants that can help support ecosystem services under events of global change. Beneficial microbiomes can help plants expand their adaptative potential and stress tolerance (Hawkes *et al.*, 2020).

4. Soil water repellency in soil-plant ecosystems

Soil water repellency (hydrophobicity) is a reduction in the rate of wetting and retention of water in soil caused by the presence of hydrophobic organic soil substances coating soil particles (Hallett, 2007). These hydrophobic, aliphatic C-H compounds are produced mainly by plant-derived organic matter (Mao *et al.*, 2015) and microbes with a predominance of fungal species (Wessels, 1996; White *et al.*, 2000). Fungi have been identified as the dominant microbial group that causes water repellency in sandy soils meanwhile

bacteria may decrease water repellency (Roper, 2004). Hydrophobins are ubiquitous proteins found in filamentous fungi (Wessels, 1996), and they are characterized by low and high levels of hydrophobicity (Rillig, 2005; Bayry *et al.*, 2012). Under low moisture content in **Chapter 3**, we observed fungal growth in patches. Fungal biomass is known to grow in a patchy layout within the soil (Tibbett, 2000), and the deposition of hydrophobic compounds may have a similar patchy distribution (Salifu and El Mountassir, 2021).

Fungal hydrophobins are expressed in different stages of fungal life and they fulfill several different roles (Linder *et al.*, 2005). Hydrophobins, for instance, can lower the surface tension of interface water-air to allow hyphae growth into the air. Wösten *et al.* (1999) showed that the normal surface tension of water, 72 mJ m^{-2}, could be reduced to 24 mJ m^{-2} by adding the purified hydrophobin SC3. Fungal hydrophobins have both hydrophilic and hydrophobic ends (amphipathic) (Linder *et al.*, 2005). Hydrophobins tend to be strongly hydrophilic when wet, but when under a moisture threshold the hydrophilic surfaces bond strongly with each other and with the mineral surface leaving an exposed hydrophobic surface (Hallett, 2007). Prolonged events of wetting can reverse this effect (Clothier *et al.*, 2000), allowing water-repellent soil to regain wettability due to the nature of amphiphilic molecules (Fig. 4). Then it is expected that water-repellent soils in the field will depend on the duration of precipitation events.

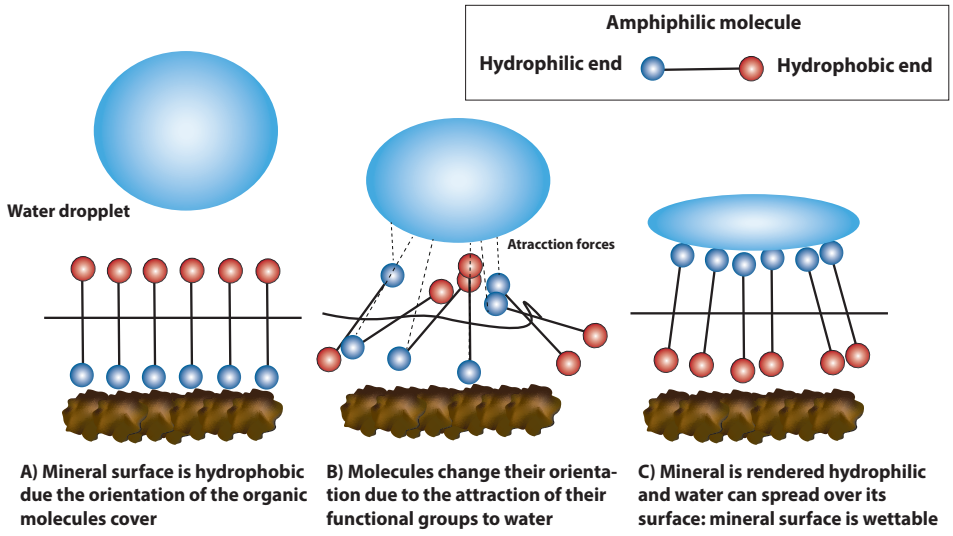

Figure 4. Schematic representation of an amphiphilic molecule and changes in its orientation on a mineral surface while in contact with water (A-C). Adapted from Doerr *et al.* (2000).

The hydrophobicity in **Chapter 3**, was measured by determining the soil water contact angle (WCA) and the sorptivity, which used the time for soil wetting prior to wet sieving as a proxy. Most of the fungal strains obtained WCA > 90° at both moisture regimes, with the highest angles at low moisture content. In contrast, fungal treatments reduced sorptivity under low moisture, and this was one of the soil properties that explained the improvement in aggregate stability. Water repellent soils show a liquid-solid contact angle > 90°, and, for extremely water repellent soils, sorptivity, and capillarity rise will be 0 (Hallett, 2007). By these measures, our soil appeared extremely water repellent after fungal inoculation.

The intensity of soil water repellency can be also explained by the amount of hydrophobic aliphatic compounds, their origin, hydration status, and intermolecular arrangement of the soil particles and soil surface area (Ruthrof *et al.*, 2019). The addition of nutrients like N, for instance, caused severe water repellency in soil aggregates (Hallett and Young, 1999). Soil texture also can impact soil hydrophobicity. Soil water repellency is not limited to climates, soil types, or land uses (Ruthrof *et al.*, 2019), but is predominantly associated with soil coarse texture, such as sand (Woche *et al.*, 2005), which has a relatively low surface area. We used a substrate with coarse sand in **Chapters 2** and **3,** and a substrate with medium size sand in **Chapter 4**. We did not measure water repellency in **Chapters 2** and **4,** but we assume the sand in the substrate mix could have affected the soil wettability, as shown by the WCA > 90° for the non-inoculated control under low moisture in **Chapter 3.**

In **Chapter 3**, we proposed fungal inoculation as an alternative to improve soil aggregation under drought. Fungal strains by increased water repellency improved aggregate stability. However, extreme soil water repellency due to the excretion of hydrophobic substances by fungal strains may affect the hydrological balance of soil bringing concerns to agricultural production (Roper, 2004). In Box 1, we discuss the benefits and drawbacks of soil water repellency for soil-plant ecosystems and some potential solutions to ameliorate these negative effects. From Box 1, we can conclude that soil water repellency is important and necessary to preserve soil aggregates, keep their structure, C storage capacity, and reduce soil evaporation, which can help preserve moisture under drought. However, soil water repellency also can become a double-edged sword for water infiltration and hydrological properties of agricultural soils when rain events are short in duration. Strategies are displayed to ameliorate the negative effects, but a holistic study of the environment and potential side effects is necessary to avoid potential negative effects.

Box 1. Water repellency: a friend or foe?

Comparison of negative and positive effects of soil water repellency in the soil-plant ecosystem and potential strategies used to ameliorate these effects.

Negative effects

- Reduced water infiltration: A group of saprobic fungi decreased water infiltration of wettable soils (Chau *et al*., 2009).
- Increased runoff and erosion: Water repellent soils showed higher runoff coefficients than soils treated with surfactants at 3 different slope levels (Lowe *et al*., 2021).
- Reduced plant growth: The basidiomycete fungus Marasmius oreades increased soil water repellency which caused considerable damage to golf greens in the UK (York and Canaway, 2000).

Positive effects

- Reduced evaporation and trap of soil moisture: A layer of hydrophobic soil was laid on top of a hydrophilic soil and evaporation rate decreased and 90% of water was retained (Gupta *et al*., 2015).
- Improved aggregate stability: Mycorrhizal fungi increased soil water repellency and aggregate stability (Rillig *et al*., 2010).
- Resistance to erosion: The hydrophobic surface of the fungus Pleurotus ostreatus causes a slipping effect at the water and soil surface reducing the shear stress at the boundaries (Zhang *et al*., 2020).

Strategies proposed to overcome soil water repellency

- The application of wetting agents alters the surface tension of irrigation water (Hallett, 2007). The application of surfactant agents on seeds helped to ameliorate the water repellency post-fire (Madsen *et al*., 2011). However, a wider environmental implication may need to be considered prior to application (Hallett, 2007).
- A group of wax-degrading bacteria dominated by actinomycetes acted as biosurfactant and emulsified hydrophobic compounds increasing the wettability of soils (Roper, 2004).
- Clay kaolinite application increased soil reactive surface areas in sandy soils (Ward and Oades, 1993).

5. Measurement of aggregation: wet sieving and slaking

There are different ways to characterize the solid phase arrangements such as the analysis of aggregate size distribution and aggregate stability (e.g., the ability of soil to retain its structure under the action of water and mechanical stresses) (Dexter, 1988). Aggregate stability is often measured by the mean weight diameter (MWD), an index that characterizes the whole soil structure by integrating the aggregates' size distribution into one number (Six *et al.*, 2000). We measured the aggregation formation and stability using the MWD by dry sieving and wet sieving, respectively. The wet sieving method is widely used to determine the size distribution and stability of aggregates caused by raindrop impact on dry soil causing slaking and surface crusting (Kemper and Rosenau, 1986). However, slaking is considered an aggressive technique, as rapid wetting produces (i) nonuniform or differential hydration and swelling of the

clay fraction, causing shear planes, and (ii) air entrapment inside capillarity pores. Only when the bonds between different structural units are strong enough to overcome these forces do the aggregates stay intact (Amézketa *et al.*, 1996). Fast sieving also can be an aggressive technique for bacterial transient binding agents (Tisdall and Oades, 1982) which determine soil aggregate stability (Tang *et al.*, 2011). Furthermore, underestimation of C and N pools in aggregates is possible due to the exclusion of water-soluble C and N and increased physical disruption of the habitat of microbial communities. Other factors, such as organic matter content, soil water content (drying of aggregates prior to sieving), and duration of wet sieving may also affect the results obtained with the wet sieving technique (Haynes and Swift, 1990). Different alternative methodologies have been used to measure aggregate stability. For instance, the use of dry sieving has been proposed to counteract these effects (Sainju, 2006). The interaction of aggregates in ethanol is also used to avoid slaking (Yu Fu *et al.*, 2022), and it also can also help counteract severe soil water repellency. Nevertheless, no general agreement or standard method exists for the determination of aggregation properties (Rivera and Bonilla, 2020), which poses a serious challenge to the use of soil structural measures as indicators for soil functionality and soil health (Rabot *et al.*, 2018).

6. Limitations and future research

The interpretation of the data in this study is specific to the set of bacterial and fungal strains (with a dominance of Ascomycota) examined and the sandy soil substrate used in our experiments. The selection of bacterial strains for **Chapter 2**, was made using a trait-based approach. However, most of these traits were collected under defined laboratory plate conditions which did not replicate the stress of desiccation applied in microcosm experiments. This could have decreased the predictive power of traits such as biofilm or EPS production. For instance, the production of EPS from *Bacillus* spp. showed an exponential increase *in vitro* and in soil when they were tested under an increased drought (Vardharajula and Ali, 2014). Additionally, in **Chapter 2** we did not evaluate the microaggregates due to the coarse nature of the sandy substrate used. Ranjard and Richaume (2001) in a qualitative and quantitative study showed that the microaggregates (2-20 µm) were the most favorable habitat for bacteria in different types of soil. Bacteria have relevance for the formation and stabilization of microaggregates (Totsche *et al.*, 2018). Furthermore, the wet sieving technique used could have been so aggressive to measure the bacterial effect on soil aggregation mediated by EPS. Another limitation of this study was our inability to collect root biomass in **Chapter 4**

due to our desire to preserve intact soil samples for a more accurate evaluation of aggregate stability. Root traits have been shown to be good indicators of plant responses to drought (Comas *et al.*, 2013), and future studies should be designed such that they could include root architecture and root properties besides the evaluation of aggregate stability.

Several questions remain to be answered in the frame of this research topic. These include the designing of a more representative strategy for determining microbial traits to facility strain selection. We think that testing strains in similar stress conditions in the laboratory as experienced *in situ* can provide more reliable results. Also, traits related to taxonomy and strain background can be considered. Further research may also seek to explore the design of multispecies communities as inoculants. Microbial co-inoculants have shown to facilitate shifts of indigenous microbial communities and improve plant yield (Wang *et al.*, 2014), and trait-based integration of different microbial species had a significative effect on soil properties and bacterial communities. Finally, further research is required to obtain a successful application of such beneficial microorganisms in order to achieve a formulation that can support microbial survival, colonization efficiency, long periods of storage, and successful regrowing under stresses such as desiccation in a changing world.

7. Concluding remarks

Our study has shown that bacterial and fungal inoculation have the potential to improve aggregate stability, and the effect of strains depended on soil moisture level. We determined that (i) the bacterial trait-based approach, as determined *in vitro*, did not provide strong predictive power for explaining effects on soil aggregation at either level of moisture, although bacterial taxonomical affiliation did show higher significant explanatory power, (ii) fungal inoculation, through realized biomass and soil hydrological changes, improved aggregation better than bacteria at both moisture contents, (ii) microbial inoculation improved better plant growth at high moisture. Yet additional studies will be required to develop effective microbial inoculation strategies to improve soil physical environments to the service of plant growth, including longer-term experiments and the design of multispecies inoculants. In addition, (iv) plant-microbe interactions are key factors in plant adaptation to global changes through ecological, evolutionary responses, and eco-evolutionary feedbacks. Taken together, these findings reaffirm the importance of microbial communities in determining soil structure and function under the events of drought, and global change and suggest new avenues by which microbial amendments can help maintain and restore soil structure and soil quality.

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Summary

Earth's human population continues to grow, and the demand for food and supplies is increasing as well. These increasing demands lead to a further intensification of agricultural activities, overgrazing, and deforestation. These, and other factors, contribute to the degradation of the physical structure and functionality of soil. In addition, climate change is intensifying extreme hydrological events, resulting in floods and more severe droughts. The proper functioning of soil is critical to sustaining the delivery of vital ecosystem services of both natural and agronomic ecosystems. Soil structure and function are highly dependent upon the stability of soil aggregates. The stability of aggregates refers to the ability to keep them intact when exposed to different stresses. The research presented in this thesis seeks to examine the potential of microbial amendments as a strategy for improving soil structure and function under drought.

We isolated and identified a collection of bacteria and fungi from a drought experimental field and utilized them in a series of experiments. In Chapter 2, a trait-based approach, relying on laboratory plate experiments, was used to select 24 bacterial strains that represented a range of predicted abilities to influence soil aggregations. These strains were inoculated individually in soil sterile microcosms under two moisture regimes (-0.03 and -0.96 MPa), considered optimal and close to permanent wilting points for plants, respectively. After 8 weeks of incubation, we found that bacteria improved aggregation better at high moisture, and bacterial traits provided a little predictive power to explain impacts on soil properties. Taxonomic affiliation had, however, a higher correlation to aggregation. In Chapter 3, we selected 29 fungal strains with higher abundance in drought field plots and taxonomy relevance to agriculture and inoculated them into soils using the same soil microcosm and moisture conditions as in the previous chapter. Fungal inoculation led to higher aggregate stability at both moisture regimes. This improvement of aggregate stability was explained by realized high soil fungal biomass and low sorptivity under drought. Fungal inoculation also showed higher soil water potential when compared to the control for the soil at high moisture after an event of drying. In Chapter 4, a selection of bacteria and fungi strains were inoculated in tomato plants, and we examined aggregate stability and plant growth under well-watered and drought regimes. The results showed that microbial inoculation improved soil aggregate stability at both moisture levels. Chlorophyll content, fresh shoot biomass, and soil water content were modified by inoculation under drought, and dry shoot biomass was affected at high moisture. However, no clear correlation was found

between soil structure improvements and plant growth. In Chapter 5, we showed how microbes mediate plant ecological and evolutionary responses to global changes and how plant responses mediate eco-evolutionary feedbacks.

We conclude that fungal inoculation showed a higher potential for improving soil aggregate stability than bacterial under both moisture levels. Bacterial and fungal inoculation improved plant growth at high moisture, and, even though a connection between soil aggregation and plant growth was not identified, we propose that additional research on the improvement of aggregates as a means of physical plant environment under drought is warranted. Long-term experiments, longer periods of microbial incubation, and the designing of multispecies communities may represent important steps forward. Although far from being completely understood, plant-microbe and microbe-soil interactions have the potential to play an important role in plant and soil adaptation in the face of global changes.

Samenvatting

De wereldbevolking op aarde blijft groeien waardoor de vraag naar voedsel toeneemt. Deze toenemende vraag leidt tot een verdere intensivering van de landbouw, overbegrazing en ontbossing. Deze, en andere factoren, dragen bij aan de degradatie van de fysieke structuur en functionaliteit van de bodem. Bovendien leidt klimaatverandering tot meer extreme hydrologische gebeurtenissen, wat resulteert in frequente overstromingen en perioden met ernstigere droogte. Een goede werking van de bodem is essentieel voor het in stand houden van vitale ecosysteemfuncties in zowel natuurlijke als agronomische ecosystemen. Een goede bodemstructuur, gevormd door samenhangende bodemdeeltjes in aggregaten, is bijvoorbeeld cruciaal voor de doorworteling, de vochthuishouding en het bodemleven. Zowel de grootte als de stabiliteit van de aanwezige bodemaggregaten zijn bepalend voor de fysische bodemkwaliteit. De aggregaatstabiliteit verwijst naar het vermogen van bodemaggregaten om intact te blijven wanneer ze worden blootgesteld aan verschillende mechanische of fysisch-chemische krachten. Het onderzoek dat in dit proefschrift wordt beschreven, onderzoekt of microbiële inoculatie kan worden gebruikt als strategie om de bodemstructuur te verbeteren in droogtegevoelige bodems.

Voor het onderzoek isoleerde en identificeerden we een verzameling bacteriën en schimmels van een aan droogte blootgesteld proefperceel en gebruikten deze in een reeks experimenten. In Hoofdstuk 2 worden de resultaten beschreven van een screening van eigenschappen die is uitgevoerd aan de verzameling geëxtraheerde bacteriën en schimmels in in-vitro experimenten. Deze eerste screening van eigenschappen is gebruikt om een selectie te maken van 24 bacteriestammen met uiteenlopende potentie om de bodemaggregatie te beïnvloeden. Vervolgens werden deze stammen individueel geïnoculeerd in gesteriliseerde bodems onder twee verschillende vochtregimes (-0,03 en -0,96 MPa), respectievelijk beschouwd als optimaal en droog (dicht bij permanente verwelkingspunten voor planten). Na 8 weken incubatie ontdekten we dat bacteriën de aggregatie beter bevorderden bij een hoog vochtgehalte en dat eerder gemeten bacteriële eigenschappen slechts een klein voorspellend vermogen hadden om de effecten op bodemstructuur te verklaren. Taxonomische verwantschap van de bacteriestammen vertoonde echter een hogere correlatie met aggregatie. In Hoofdstuk 3 hebben we vervolgens 29 schimmelstammen geselecteerd die een hogere abundantie hadden in de aan droogte blootgestelde experimentele proefpercelen en die op basis van taxonomie relevant waren voor de landbouw. We hebben deze schimmelstammen geïnoculeerd in gesteriliseerde bodems onder dezelfde

omstandigheden als in Hoofdstuk 2. Inoculatie met schimmels leidde tot een hogere stabiliteit van aggregaten bij beide vochtregimes. Deze verbetering van aggregaatstabiliteit werd verklaard door een hoge schimmelbiomassa en lage sorptie eigenschappen bij droogte. Schimmelinoculatie liet ook een hogere bodemwater potentiaal in de bodem zien in vergelijking met de controle bij de bodems met een hoog vochtgehalte na een periode van droogte. In Hoofdstuk 4 werd een selectie van bacterie- en schimmelstammen geïnoculeerd in bodems met tomatenplanten en hebben we de interactie tussen stabiliteit van aggregaten en plantengroei onder goed gehydrateerde en droge omstandigheden onderzocht. De resultaten toonden aan dat microbiële inoculatie de stabiliteit van bodemaggregaten verbeterde bij beide vochtigheidsniveaus. Chlorofylgehalte, plantbiomassa (versgewicht) en bodemvochtgehalte veranderde door inoculatie onder droge omstandigheden. Bij de bodems met een hoog vochtgehalte werden significante verschillen gevonden in het drooggewicht van de planten na inoculatie. Er werd echter geen duidelijke correlatie gevonden tussen verbeteringen in bodemstructuur en de plantengroei. In Hoofdstuk 5 hebben we laten zien hoe microben de ecologische en evolutionaire reacties van planten op wereldwijde veranderingen beïnvloeden en hoe plantenreacties eco-evolutionaire terugkoppelingen mediëren.

We concluderen dat schimmelinoculatie een groter potentieel heeft voor het verbeteren van de stabiliteit van bodemaggregaten dan bacteriële inoculatie onder verschillende vochtigheidsniveaus. Bacteriële en schimmel inoculatie verbeterden de plantengroei bij een hoog vochtgehalte, en hoewel er geen verband werd gevonden tussen bodemaggregatie en plantengroei, stellen we voor dat verder onderzoek naar de verbetering van aggregaten, in de context van het fysieke plantenmilieu, tijdens droogte gerechtvaardigd is. Langetermijn experimenten, langere microbiële incubatieperiodes en het ontwerpen van inoculatie gemeenschappen met meerdere soorten kunnen belangrijke vervolgstappen zijn in dit onderzoeksveld. Hoewel we nog niet alle mechanismen volledig doorgronden kunnen we wel stellen dat interacties tussen planten en microben en tussen microben en de bodem in potentie een belangrijke rol spelen in de weerbaarheid van het bodem-plant systeem onder invloed van mondiale veranderingen in het klimaat.

Resumen

La población humana continúa en aumento y la demanda de alimentos y suministros se encuentra incrementando también. Estas continuas demandas conducen a una continua intensificación de actividades agrícolas, sobrepastoreo y deforestación. Estos y otros factores contribuyen a la degradación de la estructura física y función del suelo. Además, el cambio climático intensifica los eventos hidrológicos extremos, los cuales resultan en inundaciones y sequías más severas. El funcionamiento adecuado del suelo es crítico para mantener la prestación de servicios que son vitales para los ecosistemas naturales y agrícolas. La estructura y función del suelo tienen una alta dependencia de la estabilidad de los agregados del suelo. La estabilidad de los agregados se refiere a la habilidad de mantenerse intactos cuándo son expuestos a diferentes estreses. La investigación presentada en esta tesis busca examinar el potencial de enmiendas microbianas como una estrategia para mejor la estructura y función del suelo bajo sequía.

Aislamos e identificamos una colección de bacterias y hongos de una parcela experimental bajo sequía y la utilizamos en una serie de experimentos. En el Capítulo 2, un enfoque basado en características bacterianas específicas evaluadas en experimentos en placas de laboratorio fue utilizado para seleccionar 24 cepas bacterianas, las cuales representaron un rango de características con capacidad predictiva para influenciar la agregación del suelo. Las cepas fueron inoculadas individualmente en un suelo microcosmos estéril bajo dos regímenes de humedad (-0.03 y -0.96 MPa), considerados cómo óptimo y cercano al punto de marchitamiento permanente en plantas, respectivamente. Después de 8 semanas de incubación, encontramos que la inoculación bacteriana incrementó la agregación en mayor grado bajo el nivel de humedad más alto, y las características bacterianas proporcionaron poco poder predictivo para explicar los efectos en las propiedades del suelo. La afiliación taxonómica tuvo, sin embargo, una mayor correlación con la agregación. En el Capítulo 3, seleccionamos 29 cepas fúngicas con mayor abundancia en parcelas bajo sequía y relevancia taxonómica en la agricultura, y las inoculamos en suelos utilizando el mismo suelo microcosmos y condiciones de humedad que en el capítulo previo. La inoculación fúngica dio lugar a una mayor estabilidad de los agregados en ambos niveles de humedad. Esta mejora en la estabilidad de los agregados fue explicada por la alta biomasa fúngica en el suelo y la baja sorción de humedad bajo sequía. La inoculación fúngica también demostró un mayor potencial hídrico para los suelos con mayor humedad después de un evento de sequía en comparación con el control. En el Capítulo 4, una selección de cepas de hongos y bacterias

fueron inoculadas en plantas de tomate y examinamos la estabilidad de los agregados y el crecimiento de las plantas bajo regímenes de buen riego y sequía. Los resultados demostraron que la inoculación microbiana incrementó la estabilidad de los agregados del suelo bajo ambos niveles de humedad. El contenido de clorofila, el peso fresco aéreo y el contenido de humedad en el suelo fueron modificados por la inoculación bajo sequía y el peso seco aéreo fue afectado con mayor riego. Sin embargo, no se encontró una clara correlación entre la mejora de la estructura del suelo y el crecimiento de las plantas. En el Capítulo 5, demostramos como los microorganismos median respuestas ecológicas y evolutivas en plantas frente a cambios globales y cómo las respuestas de las plantas median las retroalimentaciones eco-evolutivas.

Concluimos que la inoculación fúngica demostró un mayor potencial sobre el incremento de la estabilidad de los agregados del suelo bajo ambos niveles de humedad. La inoculación bacteriana y fúngica incrementaron el crecimiento de las plantas bajo mayor nivel de riego y, aún cuándo una conexión entre los agregados y el crecimiento de las plantas no fue identificado, proponemos que estudios adicionales sobre la mejora de los agregados del suelo cómo una medio para el entorno físico de las plantas es necesario. Experimentos a largo plazo, prolongados periodos de inoculación microbiana y el diseño de comunidades multi especies pueden representar importantes avances. Y lejos de ser completamente comprendidos, las interacciones de plantas-microorganismos y microorganismos-suelo tienen el potencial de jugar un importante rol en la adaptación de plantas y el suelo frente a los cambios globales.

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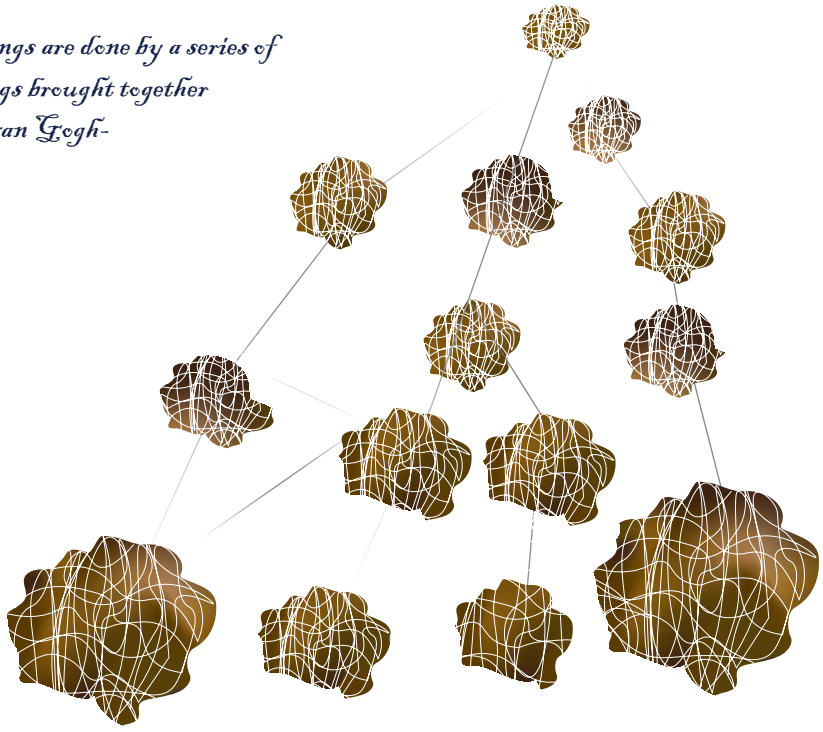
Great things are done by a series of small things brought together
- Vincet van Gogh-



¡ Gracias totales!

About the author

Violeta Carmen Angulo Fernández was born in La Paz, Bolivia. She moved with her family to Cochabamba, Bolivia when she was 4 years old and started her elementary school studies there. In this city, she spent her summer holidays exploring the countryside hills in the company of her grandmother. She developed her love for nature there. She started a biology's bachelor in 2000 at the University Mayor of San Simón (Cochabamba-Bolivia). During that period, she worked at the Center of Water and Environmental Sanitation (CASA) labs on the detection of coliform and pathogen bacteria in drinking water and wastewater. She also worked at the Center of Food and Natural Products (CAPN) detecting coliform and pathogen microorganisms in food samples. Her bachelor's thesis was focused on the effect of *Bacillus thuringiensis* on the control of the fall armyworm (*Spodoptera frugiperda*). Her thesis was part of crop-integrated management at the Foundation for the Promotion and Research of Andean Products (PROINPA). She continued working as a consultant with this foundation where she supported developing strategies in the production of microbial inoculants to improve plant growth and biocontrol of pests in crops like potato and quinoa. The research was combined with the training of farmers about the use of microbial products. During that period, she also won a scholarship from the Japan International Cooperation Agency (JICA) for a short internship at the Institute of Vegetal Physiology and Pathology (IFFIVE) in Cordoba, Argentina, where she learned molecular techniques to detect plant pathogens.

She started a master's degree with a major in microbiology in 2010 at the Concepcion University in Concepcion, Chile. She arrived there after an earthquake of 8.8 and a tsunami hit the country and the University was partially damaged. Her thesis was on the effect of plant growth-promoting rhizobacteria (PGPR) in seedlings of *Eucalyptus nitens* under the supervision of Dr. Eugenio Sanfuentes and Dr. Katherine Sossa. After, she did an internship at the Agricultural Research Center (INIA) Quilamapu in Chillán, Chile where she learned techniques about microbial biocontrol, the production of bioinoculants, and management of banks of microorganisms. After her master's, she continued working on a research project denominated

"Endolyptus" led by Dr. Sanfuentes in collaboration with the forestry company "Mininco". The project was pursuing to find bacterial and fungal inoculants to improve the rooting and growth of forestry species.

Violeta was awarded the "One Hundred Scholarships for Technological and Scientific Sovereignty" from the Plurinational State of Bolivia to start her Ph.D. project under the supervision of George Kowalchuk, Alexandre Jousset, and Mariet Hefting. Later, she was awarded the "Schlumberger Foundation, Faculty for the Future Fellowships" to finish her Ph.D. project.

With this thesis:

Angulo, V., Beriot, N., Garcia-Hernandez, E., Li, E., Masteling, R., and Lau, J. (2022) Plant-Microbe Eco-Evolutionary Dynamics in a Changing World. *New Phytologist* **234**: 1919–1928.

Angulo V., Bleichrodt R.J, Dijksterhuis J., Erktan A., Hefting MM., Kraak B., and Kowalchuk GA. Soil colonization of fungal amendments improves soil aggregation and soil physical properties under different moisture conditions. Submitted to Environmental Ecology, March 2023. In the process of correction with minor revisions.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Project literature review (4.5 ECTS)

- Degradation, transformation and stabilization of composted and vermicomposted organic matter by microorganisms
- Assessment of PGPR inoculation in compost polluted with Cadmium, plant growth promotion and microbial assembly in the rhizosphere
- Designing microbial inoculants to improve compost amendments for the restoration of degraded soilsTransformation and stabilisation of composted organic matter by microorganisms to restore degraded soils

Post-graduate courses (4.2 ECTS)

- New frontiers in Microbial ecology (2018, RSEE, PE&RC & SENSE)
- Soil Ecology, the multifunctional potential of soil (2019, PE&RC and WIMEK)
- World soils and their assessment (2021, ISRIC)

Methodological / Statistical Postgraduate Courses (2.0)

- Introduction to R (2017, PE&RC)
- Experimental designs (2017, PE&RC)
- General Linear Models (2020, PE&RC)

Deficiency, Refresh, Brush-up courses (6 ECTS)

- Basic statistics (2017, PE&RC)
- Theoretical classes of MSc course biological interactions in soil (2017, Wageningen University)
- Introductory biostatistics (2017, Julius Institute)

Laboratory training and working visits (3.4 ECTS)

- Soil Biology group, Wageningen University & Research (2021)
- Johann-Friedrich-Blumenbach Institute of Zoology and Anthropology, University of Göttingen, Germany (2018)
- Institute of Biology, Free University of Berlin, Germany (2018)
- Research for Development (IRD), Research Unit Eco&Sols, Montpellier, France (2022)

Competence strengthening / skills courses (7.8 ECTS)

- Reviewing a scientific paper (2017, PE&RC)
- Research planning and time management (2017, GSLC)
- Writing a Scientific Paper (2019, GSLC)

- Academic writing in English (2020, GSLS)
- Digital pictures: Data integrity and Display (2020, GSLS)
- Giving effective presentations (2021, GSLS)
- Scientific Artwork; Data visualization and infographics with Adobe Illustrator (2021, GSLS)
- Adobe InDesign-from dissertation layout to poster design (2022, GSLS)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.0 ECTS)

- PE&RC Day (2017)
- PhD First Years Retreat (2017)
- WGS PhD Workshop carousel (2018)
- PhD Midterm Retreat (2019)
- PE&RC Day (2021)
- PhD Last Years Retreat (2021)

Discussion groups / local seminars or scientific meetings (10.7 ECTS)

- Institute of environmental ecology PhD discussion group (2017-2021)
- Utrecht Plant Science& Industry Symposium (2018)
- Microbiology research group lab meetings (2022)
- E&B PhD discussion group (2021)

International symposia, workshops and conferences (9.4 ECTS)

- Ecology of soil microorganisms (2018, Helsinki, Finland)
- Academic Conference for Graduated students at Nanjing Agricultural University (2018, Nanjing, China)
- 8th International Symposium on Interactions of Soil Minerals with Organic Components and Microorganisms (2019, Seville, Spain)
- International Symposium on Soil Organic Matter (2022, Seoul, South Korea)

Lecturing/supervision of practicals/tutorials (5.6 ECTS)

- Biologie & Ecologie van Planten (2018, Utrecht University)
- Experiment & Statistiek (2018, Utrecht University)

Supervision of MSc students (6 ECTS)

- What are the main contaminators of compost and how can this affect plants, soil and environment?
- Saprotrophic fungi in soil aggregation under drought (Fungal colonies density).
- Effect of saprotrophic fungi on drought tolerance in plants and soil.
- Fungi and soil aggregation under drought (soil aggregation).
- The ability of saprotrophic fungi to improve soil structure (water potential).
- The role of fungal hydrophobicity in soil-plant ecosystems under climate change.

"The more I study nature, the more I stand amazed at the work of the creator. Science brings mean nearer to God"
-Louis Pasteur-

The end of this thesis.