Bringing Soil Fungi into Action

Options for forward-looking agriculture

Anna Clocchiatti

Propositions

1. Cellulose is a driving force in the stimulation of saprotrophic fungi by organic soil amendments.
   (this thesis)

2. Understanding growth responses of soil-borne pathogens to soil management is essential for development of successful disease control strategies.
   (this thesis)

3. The 'ivory tower' should be redefined as a 'knowledge incubator'.

4. Research on future ecosystems is as important for human health as research on novel medical treatments.

5. Wood properties are too good to be burned.

6. Hobbies are not there for making you a better professional (M. Gancitano and A. Colamedici, La societá della performance, Tlon, 2018).

Propositions belonging to the thesis entitled:

Bringing soil fungi into action
Options for forward-looking agriculture

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Thesis

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Chapter 1

General introduction

Soil microorganisms perform a multitude of activities that are at the core of ecosystem functioning (Delgado-Baquerizo et al., 2016; Wagg et al., 2019). The interplay between saprotrophic, mutualistic and pathogenic soil-borne microbes largely influences plant production, carbon and nutrient cycling and other soil functions in terrestrial ecosystems (van der Putten et al., 2016; Ochoa-Hueso et al., 2018; Chen et al., 2019). The importance of the microbial component of soil is increasingly recognized in the context of sustainable agriculture, while it is realized that commonly used agricultural management practices heavily interfere with microbial-mediated processes and can even degrade soils (de Vries et al., 2013; Creamer et al., 2016; FAO, 2014a). The functioning of arable soils can be enhanced by a more balanced management of biological resources, including crops and soil microbial communities (Bender et al., 2016; Arif et al., 2020). To this end, the adaptation of soil ecological concepts and the use of knowledge gathered from the study of natural ecosystems are essential for the development of integrated, sustainable soil management practices in agriculture (Lehman et al., 2015; Andreote and Pereira e Silva, 2017; Mariotte et al., 2018).

Soil microbiome engineering emerged as a prominent concept for enhancing the health and fertility of arable soils (Chaparro et al., 2012; Mazzola and Freilich, 2017; Arif et al., 2020). The goal of microbiome engineering is to manipulate soil communities so as to promote the activity of microbes that are responsible for a better soil functioning. By these means, agricultural production can become less dependent on inputs of chemical fertilizers and pesticides. For instance, mycorrhizal fungi and Rhizobia can support plant nutrition (Hart and Trevors, 2005; Chandanie et al., 2009; Korir et al., 2017), while members of *Streptomyces*, *Bacillus*, *Pseudomonas* and *Trichoderma* can improve the control of soil-borne diseases (Lugtenberg and Kamilova, 2009; Kepler et al., 2017; Araujo et al., 2019). Microbes with desired traits can be introduced into soil as single strains or synthetic communities. Introduced microbes can directly benefit soil and plant functioning, but also influence the assembly of resident microbial communities (Massart et al., 2015; Herrera Paredes et al., 2018; Araujo et al., 2019; Zhang et al., 2019; Mawarda et al., 2020). In addition to this, the activity of resident, as well as introduced, soil microbes needs to be supported and steered by appropriate management practices (Bonanomi et al., 2018; Hartman et al., 2018). This requires the re-introduction and expansion of traditional agricultural practices, such as incorporation of organic materials into the soil, a wider use of crop rotations, diversification of crops (inter- and cover cropping), reduced soil tillage and selection of crop varieties in support of specific microbial functions (Gopal and Gupta, 2016; Bonanomi et al., 2018; Arif et al., 2020).

Yet, the response of resident and exogenous soil microbes to changes in agricultural management can take a long time to develop and it has a high level of uncertainty. This is due to the high variation in environmental conditions (e.g. soil texture, moisture) and to the complexity of interactions of soil microbial communities with added soil organic materials and with plant rhizodeposits. Hence, the outcome of soil

microbiome steering approaches is highly context-dependent and benefits can become evident only in the long term. Unfortunately, this retards the large-scale use of ecology-based strategies in agriculture. The main challenge now is to find ways to target relevant microbial functional groups in an effective and reliable way (Bender et al., 2016). In this respect, saprotrophic fungi represent a promising target group, as they are involved in several essential soil functions, but are mostly present in low abundance, coinciding with low activity, in intensively managed arable soils (Djajakirana et al., 1996; Stahl et al., 1999; Bailey et al., 2002; Fierer et al., 2009; de Vries and Bardgett, 2012). Restoration of a higher fungal abundance and activity can improve the functioning of degraded soils, in particular it can contribute to the closure of nutrient cycles, a better soil structure and disease control (Frąc et al., 2018). Yet, knowledge on the ecology of saprotrophic fungi in arable soils is limited, as most research has been done in natural ecosystems, and in particular in forests. In this thesis I aim 1) to understand the possibilities to stimulate saprotrophic fungi in arable soils; 2) to examine the implications of saprotrophic fungal biomass stimulation on rhizosphere fungal and bacterial activity and community composition; 3) to study the effects on plant performance associated with stimulation of saprotrophic fungi.

Saprotrophic fungi and fungal-bacterial interactions in soil

In natural and semi-natural ecosystems, saprotrophic fungi are typically high in abundance (Bailey et al., 2002; Fierer et al., 2009; Strickland and Rousk, 2010) and play a major role in the decomposition of organic materials (Malik et al., 2016; van der Wal et al., 2013). Fungal activities have important impacts on nutrient cycling and plant nutrition, as well as on carbon fluxes and soil organic matter formation (Bailey et al., 2002; Boddy and Watkinson, 2011; Kallenbach et al., 2016; Malik et al., 2016). In turn, fungal abundance and activity are regulated by both the abiotic characteristics of soil and the amount and composition of plant-derived inputs (Eisenhauer et al., 2017; Ochoa-Hueso et al., 2018).

Although saprotrophic fungal dominance is mostly associated with the presence of recalcitrant organic matter, fungi can metabolize a wide variety of organic compounds, ranging from lignocellulose complexes to simple carbon compounds (Griffiths et al., 1998; van der Heijden et al., 2008; van der Wal et al., 2013; Eisenhauer et al., 2017). Overall, the broad range of extracellular enzymes produced by fungal communities ensures a thorough processing of organic matter entering the soil (Dighton, 2007). Fungi mediate the stabilization and transformation of plant compounds into long-term soil organic matter pools, to which also fungal necromass (i.e. dead fungi) contributes (Six et al., 2006; Cotrufo et al., 2015; Lehmann and Kleber, 2015; Kallenbach et al., 2016; Malik et al., 2016). In addition to this, fungal hyphae and fungal exudates can increment soil aggregation and water retention (Ritz and Young, 2004; Lehmann et al., 2020). Moreover, fungi regulate nutrient cycling both by

releasing minerals into the soil pore water or by capturing part of the nutrient pools into their biomass (Dighton, 2007; Boddy and Watkinson, 2011).

An important aspect of the presence of active fungi in soil is their impact on bacterial activities, and *vice versa*. Fungi and bacteria coexist in soils and, together, they constitute most of the second trophic level of the soil food web (Wardle et al., 2003; Morriën et al., 2017). Thereby, fungi and bacteria engage in a plethora of interactions, including facilitation and mutualism, as well as resource and interference competition (de Boer et al., 2005, 2007; Johnston et al., 2016). Antagonistic traits include the production of antimicrobial compounds and microbial cell-wall lytic enzymes. These are often elicited in response to competition within complex communities and cannot be easily predicted based on the behavior of microbes observed in isolation (de Boer et al., 2007; Cornforth and Foster, 2013; de Boer, 2017). In addition to this, fungal-bacterial interactions include predation and feeding off microbial-derived compounds (Ballhausen and de Boer, 2016; de Menezes et al., 2017). Fungal-bacterial interactions and, in particular, antagonism, have strong impacts on microbial community composition and - functioning (Bahram et al., 2018; Frąc et al., 2018; Wagg et al., 2019).

Saprotrophic fungi as rhizosphere inhabitants

The presence of saprotrophic fungi in the rhizosphere and inside root tissues is increasingly recognized and included in the discourse on plant performance and ecosystem functioning (Buée et al., 2009; de Vries and Caruso, 2016; van der Putten et al., 2016; Eisenhauer et al., 2017). Indeed, saprotrophic fungi are not limited to the utilization of recalcitrant plant parts, but they can compete for rhizodeposits and simple root exudates (Hannula et al., 2012). As a consequence, composition and quantity of the rhizodeposits modulate the species composition and size of the fungal communities selected by plants (Broeckling et al., 2008; Berg and Smalla, 2009). Root debris and sloughed-off root cells represent the most complex input released by living plants, whereas soluble components include simple sugars, organic acids and other metabolites (Dennis et al., 2010). Rhizosphere and root colonization by saprotrophic fungi is therefore affected by plant species, variety, plant developmental stage, and plant community diversity (Hannula et al., 2010; Eisenhauer et al., 2017; Hugoni et al., 2018). Yet, detailed information on the effect of distinct components of rhizodeposits on rhizosphere saprotrophic fungi is lacking.

Saprotrophic fungi are an important sink of plant-derived carbon. When fungi are abundant, they drive the transfer of photosythates from plants into the soil. This was evidenced, for instance, in late-successional ex-arable fields (Hannula et al., 2017; Morriën et al., 2017). The active uptake of plant-derived carbon by fungi can have broad influences on the soil, such as favoring the formation of aggregates in deep soil

layers (Baumert et al., 2018) and increasing the complexity of the soil food web (Morriën et al., 2017).

Fungal communities inhabiting the rhizosphere are distinct from those observed in bulk soil. In grasslands, about half of soil-dwelling fungi were found in the rhizosphere (Hannula et al., 2020). Rhizosphere-inhabiting and plant-C consuming saprotrophic fungi include mainly Ascomycota, Mucoromycota and basidiomycetal yeasts (Hannula et al., 2012; Hugoni et al., 2018; Hannula et al., 2020). Indeed, these taxa include many species that can efficiently utilize labile materials and thereby occupy a similar niche as bacteria. Similarly, as discussed for the soil (see previous section), also in the rhizosphere the presence of active fungi can increase the complexity of (competitive) fungal-bacterial interactions (Li et al., 2014; de Boer et al., 2015). Importantly, in the rhizosphere, this can have consequences for plant performance and health.

Effect of agricultural practices on saprotrophic and pathogenic fungi

Several studies reported that saprotrophic fungal biomass and activity in arable soils are lower as compared to natural and semi-natural soils (Djajakirana et al., 1996; Stahl et al., 1999; Ananyeva et al., 2006; Fierer et al., 2009; de Vries and Bardgett, 2012). Conversely, when arable land is converted back to nature, fungal biomass increases (van der Wal et al., 2006) and, as the ecosystem progresses towards a more complex plant community, including woody plants, also a higher fungal activity is recovered (Bailey et al., 2002; Susyan et al., 2011; Morriën et al., 2017; French et al., 2017; J. Wang et al., 2019). This can be explained by the fact that from arable soils a major part of plant biomass is removed after a cropping cycle. In this way the substrates available to fungi are limited to rhizodeposits entering the soil during the growth of crops. Long-term plant residue exclusion can be responsible for the alteration of microbial soil functioning (Paterson et al., 2011). In addition to this, tillage can sever hyphal networks or reduce the need of hyphal growth due to homogenization of resources in soil (Beare et al., 1992, 1997; Frey et al., 1999), whereas chemical fertilizers and fungicides inhibit the activity of few or many members of the fungal community (Duah-Yentumi and Johnson, 1986; Beare et al., 1997; Bittman et al., 2005; Scotti et al., 2015b).

Intensive soil management and monocropping practices not only reduce fungal abundance, but also tend to favor pathogenic over non-pathogenic fungi (Schöps et al., 2018; Gao et al., 2019; Li et al., 2019). Survival structures of soil-borne pathogens are common in natural ecosystems, however, their growth and dynamics are strongly regulated by the distribution of hosts and heterospecific plants (Ampt et al., 2019). Conversely, in the cropland, genetically uniform plants are grown at high density. In monocultures, pathogenic fungi can rapidly spread and even locally adapt to host

plants (Croll and McDonald, 2017), causing disease outbreaks (Lamichhane et al., 2017). The large-scale use of fungicides allowed to control pathogens in soils often can increase plant productivity in monocultures (Maron et al., 2011). However, fungicides can have strong off-target effects, namely inactivation or even killing of non-pathogenic fungi (Wang and Cernava, 2020). Non-pathogenic fungi, and especially saprotrophic fungi, are important regulators of the composition, diversity and activity of the plant pathogenic fungi in arable soils (Agtmaal et al., 2017). Novel soil management options should aim to boost saprotrophic fungal biomass as a way to establish more natural balance in fungal communities in arable soils.

Despite having a low fungal biomass and activity, arable soils harbor a high diversity of fungi (Moll et al., 2016; Han et al., 2017). Periods of C-starvation (bare or fallow) and other anthropogenic disturbances interfere with fungal growth several times during a year. This adds up to the dynamics in fungal biomass due to seasonal variations in moisture, temperature and pH. Nevertheless, many fungi can endure in the soil under unfavorable conditions in dormant or low-activity states, by forming propagules, spores and other resistant structures. In addition to this, fungi can enter arable soils by air-borne dispersion from other ecosystems (Cáliz et al., 2018). In this way, arable soils retain a diverse pool of fungi, which become active when conditions for growth are met (He et al., 2020). In agreement with this, during a cropping season fungal abundance can increase in the rhizosphere as a result of increased carbon inputs in the form of rhizodepositions (Hannula et al., 2012; Tavi et al., 2013; Pausch et al., 2016; Q. Wang et al., 2019). However, other studies reported high bacterial and mycorrhizal activity, but limited saprotrophic fungal activity, in the rhizosphere of crop plants (Kušlienė et al., 2014; Hünninghaus et al., 2019). Extreme soil management practices, such as maintaining fallow conditions for decades, can cause changes in the fungal community composition and the loss of fungal traits from soils, such as the ability to decompose complex organic matter (Paterson et al., 2011). However, some recent studies show that fungal communities in arable soils are highly dynamic and can respond to a variety of root-derived and litter inputs (Moll et al., 2015; Reardon and Wuest, 2016; Rahman et al., 2017). This suggests that fungal-based soil functions could be restored by the stimulation of resident fungal communities of arable soils.

Managing the soil microbiota for disease suppression

Increasing the biomass of saprotrophic fungi can improve several functions in arable soils. The most acknowledged benefits associated with higher saprotrophic fungal biomass are soil aggregation (Beare et al., 1997; Helfrich et al., 2015; Liao et al., 2018), increased carbon sequestration (Six et al., 2006) and improved nutrient cycling (de Vries et al., 2011). In addition to this, members of the fungal community were identified as keystone contributors to natural disease suppression in several arable fields (Xiong et al., 2017; Siegel-Hertz et al., 2018). In particular, the presence of soil-

borne, non-pathogenic fungi in the rhizosphere and root tissues is considered important to counteract plant diseases (Busby et al., 2016; Poli et al., 2016; Sarrocco, 2016). Active saprotrophic fungi in the soil or rhizosphere can have direct negative effects on pathogenic fungi via competitive interactions, which include the production of growth inhibiting compounds (Fravel et al., 2003; van Beneden et al., 2010; Kepler et al., 2017). Moreover, the presence of an active biomass of saprotrophic fungi can modify the activity and composition of bacterial communities (de Boer et al., 2008; de Menezes et al., 2017), in favor of fungus-suppressing and fungus-feeding bacteria (de Boer et al., 2015; Ballhausen and de Boer, 2016; Kramer et al., 2016). It would be even more relevant if competitive fungal-bacterial interactions are increased in the rhizosphere soil, in order to establish a competitive environment around the root, before the invasion of the root by pathogenic fungi (Wei et al., 2015; Chapelle et al., 2016). Overall, disease suppression is likely the result of multiple interactions between plants, beneficial microbes, pathogens and other soil inhabitants (Whipps, 2001; Chapelle et al., 2016; de Boer, 2017). Therefore, there is increasing interest in stimulating soil health by holistic approaches, such as steering the resident soil microbiome (Chaparro et al., 2012; Li et al., 2019; Mazzola and Freilich, 2017). In line with this view, I investigate the possibility to increase the activity of the resident saprotrophic fungal community in the soil and rhizosphere as a way to increase microbial competition and hence regulate soil-borne plant pathogenic fungi.

Aims of this thesis

Although active saprotrophic fungi are an essential component in soils in many natural and semi-natural ecosystems, limited knowledge is available on how a higher saprotrophic fungal biomass could be restored and maintained in intensively managed arable soils. This thesis aims to explore which organic inputs can effectively stimulate saprotrophic fungi in fungal-poor arable soils, with a focus on organic amendments and root exudates. Furthermore, this thesis investigates the effects of fungal biomass stimulation in soil on rhizosphere microbial activities and on soil-borne pathogen populations.

The first two chapters set the basis of this thesis, by examining two possible approaches for stimulation of saprotrophic fungi by the input of organic substrates into arable soils. Namely, **Chapter 2** addresses the effects of exogenous organic amendments of varying quality on soil fungi, while **Chapter 3** describes phenolic root exudates as modulators of saprotrophic fungi.

In **Chapter 2**, I explored which type of organic material can ensure a rapid and lasting stimulation of saprotrophic fungi upon incorporation in fungal-poor arable soils. With a series of pot experiments, I tested the effects of an array of materials of different quality and origin on fungal biomass and community composition. Based on the initial

results of a broad range of substrates, I focused on woody organic amendments. Wood sawdust of distinct tree species were applied in soil, alone and in combination with mineral nitrogen. The fungus-stimulating effect of wood sawdust amendment was also tested for a selection of arable soils with different soil characteristics.

In **Chapter 3**, I tested the effect of phenolic root exudates on the biomass and community composition of rhizosphere fungi. To this end, I worked with a rhizosphere model system, which allowed to directly introduce artificial exudate mixtures. Phenolic acids were tested both alone and in combination with a mixture of primary metabolites. I combined this approach with the analysis of fungal biomass in the rhizosphere of *Arabidopsis thaliana* mutants with an altered composition of phenolic root exudates.

Having established that an effective stimulation of saprotrophic fungal biomass can be obtained in soil with (ligno)cellulose-rich fresh organic amendments, I investigated whether such bulk-soil treatment can affect fungal activity and fungal-bacterial interactions in the rhizosphere. In **Chapter 4** I investigated the performance of carrot seedlings in soil amended with beech sawdust. Beech wood was chosen as a representative of deciduous tree species, as it resulted in consistent stimulation of saprotrophic fungal biomass (**Chapter 2**). In this chapter I performed ^{13}C-pulse labelling of seedlings to investigate the effects of beech sawdust on biomass (NLFA/PLFA-SIP) and community (DNA-SIP) of rhizosphere microbes using freshly photosynthesized carbon.

After individuating fungus-stimulated organic amendments (**Chapter 2**), assessing their ability to stimulate rhizosphere-competent fungi and describing their effects on bacteria (**Chapter 4**), I studied if and how woody materials and paper pulp can be used to enhance saprotrophic fungi and at the same time to control soil-borne fungal pathogens. For this, the choice was made to examine *Rhizoctonia solani*, one of the most widespread causal agents of soil-borne plant diseases. In **Chapter 5**, I exposed a *R. solani* isolate to woody materials and paper pulp in a Petri dish assay. In this way, I tested in isolation the ability of *R. solani* to grow saprotrophic and utilize these organic materials. This was followed by two bioassays, performed with an arable soil naturally infected by *Rhizoctonia*, in which I planted seeds of red beet, a host plant susceptible to the soil-borne disease. In this setting, I tested the effect of cellulose-rich amendments on seedling performance, *R. solani* growth dynamics of and total soil fungal abundance.

Finally, I conclude with a general discussion and synthesis of the main findings of this thesis (**Chapter 6**), by integrating the results of all chapters and by comparison with related research. Furthermore, I comment on practical implications with regard to the approaches explored in this thesis. Based on this, I suggest fundamental and applied research perspectives.

Chapter 2

The hidden potential of saprotrophic fungi in arable soil: Patterns of short-term stimulation by organic amendments

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Abstract

Saprotrophic fungi are abundant in soils of (semi-)natural ecosystems, where they play a major role in ecosystem functioning. On the contrary, saprotrophic fungal biomass is remarkably low in intensively managed soils and this can have a negative impact on soil functioning. Nevertheless, arable soils harbour a diverse pool of fungi, which can be stimulated by organic amendments. Management targeted towards increasing soil organic matter often coincides with an increase of fungal biomass, but it can take years before effects are seen. However, a rapid stimulation of fungal biomass at the start of the growing season could immediately benefit crop production, by improving nutrient availability, soil structure and suppression of soil-borne diseases. The objective of this study is to realize a rapid increase of saprotrophic fungal biomass with organic amendments. In controlled pot experiments, dried and milled organic materials of different quality were added to an arable sandy soil. Ergosterol based fungal biomass and ITS2-based fungal community structure were measured over a period of two months. Wood sawdust of deciduous tree species and paper pulp resulted in a high and lasting increase of fungal biomass, as opposed to transient effects given by cover crops and other non-woody plant materials. Little or no stimulation of fungi was seen for coniferous wood sawdust and agro-industrial by-products. Nitrogen immobilization induced by sawdust and paper pulp was compensated by supplementing mineral nitrogen, which enhanced the stimulation of saprotrophic fungi. The composition of the stimulated fungi was influenced by the quality of organic amendments. In particular, deciduous wood sawdust and paper pulp favoured saprotrophic ascomycete fungi (mainly Sordariomycetes), with no increment in potential plant-pathogenic fungi. Overall, our results point at a good perspective to use woody materials as sustainable soil improver via stimulation of saprotrophic fungi.

1. Introduction

Saprotrophic fungi represent an important component of soil microbial life in many terrestrial ecosystems. In particular, in (semi-)natural ecosystems with high inputs of plant litter, abundance and activity of saprotrophic fungi is typically high (Fierer et al., 2009). The decomposing activity of saprotrophic fungi contributes to important ecosystem functions in soils, such as carbon and nitrogen cycling (Fontaine et al., 2011; van der Wal et al., 2013). In addition, the hyphal networks they form are involved in soil aggregate formation, which is important for water retention and resistance against soil erosion (Beare et al., 1997). Furthermore, saprotrophic fungi have a strong influence on other soil inhabitants, for instance via competitive or mutualistic interactions with bacteria and as food for fungivorous soil biota (Ballhausen and de Boer, 2016; Kramer et al., 2016; Morriën et al., 2017; Deveau et al., 2018).

In contrast to many soils in natural ecosystems, saprotrophic fungal biomass is remarkably low in arable soils, in particular in intensively managed arable land (Djajakirana et al., 1996; van der Wal et al., 2006a; de Vries and Bardgett, 2012). This is ascribed to the application of chemical pesticides, mineral fertilizers and intensive tillage (Duah-Yentumi and Johnson, 1986; Beare et al., 1997; Frey et al., 1999; Bittman et al., 2005; Scotti et al., 2015). When arable soils are taken out of production, fungal biomass and activity increase and are followed by shifts in the soil food web structure, connectedness and soil nutrient cycling (Morriën et al., 2017).

The small size of active biomass of saprotrophic fungi in arable soils implies that these soils are bacteria-dominated and this can have negative consequence for agricultural sustainability. Increase of saprotrophic fungal biomass in arable soil has been reported to coincide with lower losses of mineral nutrients (de Vries et al., 2011), increased carbon sequestration (Six et al., 2006) and better water retention (Beare et al., 1997; Helfrich et al., 2015; Liao et al., 2018). Moreover, saprotrophic fungi can contribute to the suppression of root-infecting fungal pathogens (van Beneden et al., 2010; Xiong et al., 2017; Siegel-Hertz et al., 2018). This is ascribed to competitive interactions between pathogenic and saprotrophic fungi (Fravel et al., 2003; Kepler et al., 2017) or to fungus-suppressing bacteria that are stimulated by the presence of saprotrophic fungi soils (Leeman et al., 1996; van Beneden et al., 2010; de Boer et al., 2015).

The benefits of having high activity and biomass of saprotrophic fungi in arable soils for enhancing sustainability in agriculture are increasingly recognized (de Vries et al., 2006; Six et al., 2006; van der Wal et al., 2013; Frąc et al., 2018). Since growth of soil microbes is mostly limited by biodegradable carbon supply, one of the options is to add materials that can be particularly well degraded by fungi (Lucas et al., 2014; Arcand et al., 2016). Saprotrophic fungi are known as efficient degraders of polymers like hemicellulose, cellulose and lignin, that are present in recalcitrant fractions of plant residues. A combination of hyphal growth and production of polymer-attacking extracellular enzymes enables them to enter and decompose solid, lignocellulose

materials (van der Wal et al., 2013). Indeed, addition of lignocellulose-rich organic materials appears to be a key stimulant, as incorporation of straw and wood residues in arable or ex-arable soils were found to be associated with heightened saprotrophic fungal biomass (van der Wal et al., 2007; Moll et al., 2015; Reardon and Wuest, 2016).

Yet, a consistent increase of fungal biomass in arable soils can take several years, if not decades (van der Wal et al., 2006b; García-Orenes et al., 2013; Arcand et al., 2016; Chen et al., 2016). In addition, other management activities like tillage and use of fungicides can interfere with the stimulation of fungi by organic amendments (Williams and Hedlund, 2013; Willekens et al., 2014). Therefore, it is important to examine if there are possibilities to have a rapid increase of saprotrophic fungi at the start of the growing season. This is the period where soils are sensitive to leaching of nutrients, as more fertilizer is added than the amount taken up by seedlings. In addition, many crops suffer from pathogenic fungi at the seedling stage, such as pathogens causing damping-off diseases (Lamichhane et al., 2017). Despite the low fungal biomass, intensively managed arable soils harbour a diverse seed bank of fungal saprotrophs (de Graaff et al., 2019), which efficiently respond to new inputs of organic substrates (van der Wal et al., 2006b; Heijboer et al., 2016). In addition, in intensively managed arable soils saprotrophic fungi become active during the growth season in the root surroundings of mature crop plants (Hannula et al., 2010, 2012). However, there is little information on how a rapid and prolonged fungal biomass increase can be realized within the early growing season.

The objective of this study was to indicate organic amendments that may be used to realize a rapid and lasting stimulation of saprotrophic fungi in fungal-poor arable soils at the start of the growing season of cash crops. To this end, organic materials were selected that are commonly used in both conventional and organic farming, such as composts, agro-industrial residues, cover crop residues (Goss et al., 2013; Scotti et al., 2015) and materials rich in (ligno)cellulose, namely paper pulp and beech wood sawdust. As the latter materials resulted in prolonged fungal biomass stimulation, the study was extended with a comparison of the fungus-stimulating effect of wood sawdust obtained from other tree species. The amendment of high C:N ratio materials can have negative consequences on crop yield, as it causes immobilization of mineral nitrogen by saprotrophic microbes (Gad et al., 2015). Yield depression following the addition of (ligno)cellulose-rich materials needs to be mitigated by a supplement of mineral nitrogen (Mohanty et al., 2013; Gad et al., 2015). At the same time, nitrogen fertilization can have negative impact on fungal development (Treseder, 2008; Zhang et al., 2018). In order to test whether elevated nitrogen could interfere with fungal biomass stimulation by high-C organic amendments, paper pulp and wood sawdust were added to the soil both alone and in combination with supplemental mineral nitrogen. This study was carried out in a series of controlled pot experiments, over a period of two months, during which the response of fungal biomass and community structure were examined.

2. Materials and Methods

The fungus-stimulating effect of incorporating organic materials in arable soils was examined in three controlled pot experiments. In the first experiment (Organic Amendments, OA), fourteen organic materials were mixed with an arable sandy soil. Materials included woody and non-woody plant material, paper pulp and other organic by-products. Based on the results of the OA experiment, soil amendment with sawdust was further studied in a second experiment (Wood Amendments, WA). Here wood sawdusts of five tree species were added to the same sandy soil. Finally, the effect of wood sawdust incorporation was studied in four arable soil types (Soil Types, ST).

Table 1. Overview of the arable soils used in this study, including soil proprieties and field management.

Samplng site and batch	Exp.	Texture	Sand-Silt -Clay (%)	pH	Organic matter (%)	Management	Crop
Vredepeel, batch 1	OA	Sand	-	6.1	-	Conventional	Triticale
Vredepeel, batch 2	WA	Sand	-	6.2	-	Conventional	Oilseed radish
Vredepeel, batch 3	ST	Sand	92-6-2	5.7	6.3	Conventional	Fallow
Panningen	ST	Sandy loam	74-25-1	5.3	7.7	Conventional	Sugarbeet
Lisse	ST	Sand	95-4-1	7.5	2.3	Conventional	Hyacinth
Nagele	ST	Silty loam	36-57-1	6.7	5.6	Organic	Wheat

2.1 Soil collection and soil characteristics

Arable soil samples were collected from four sites and used for the pot experiments. The main site for this study was the experimental farm PPO-Vredepeel of Wageningen University & Research (N 51 32 19, E 5 51 05, Vredepeel, the Netherlands). Vredepeel soil has a sandy texture and has been classified as Hortic Podzol (Tab. 1; FAO, 2014). Soil was collected from a plot to which conventional management is applied (Quist et al., 2016). Three soil samples were collected from this location, namely on 1st August 2016 (Batch 1), 17th October 2016 (Batch 2) and 5th June 2017 (Batch 3) for use in experiment OA, WA and ST, respectively (Tab. 1). Batch 1 was collected in between rows of triticale, Batch 2 in between rows of the cover crop oilseed radish and Batch 3 from fallow soil on which maize had been grown during the previous summer. In June 2017 soil was collected from three additional locations, for use in the ST experiment (Tab. 2). The selected sites were arable fields nearby Panningen (N 51 32 89, E 5 97 94), Lisse (N 52 25 52, E 4 54 77) and Nagele (N 52 38 42, E 5 43 29). Panningen soil is a Plaggic Anthrosol with a sandy loam texture, Nagele soil is a Calcaric Fluvisol with as

silty loam texture and Lisse soil is a Calcaric Arenosol with a sandy texture (Tab. 1; (FAO, 2014b). All sites are located in the Netherlands, which have a temperate maritime climate. In each site soil was obtained from 0 - 10 cm depth at ca. 15 random spots within a plot of at least 20 m x 20 m. Soil collected from a site was pooled into a composite sample. This was 4-mm sieved, homogenised and stored at 4° C until use, for a maximum of two months. Soil pH, organic matter content, texture and taxonomy were determined according to standard procedures and are summarized in Tab. 1. The four soils, used in ST experiment, differed in texture, organic matter content and/or pH. These provide a small, yet representative, selection of arable soils of the Netherlands.

2.2 Experimental design and organic materials

The OA experiment was carried out as a completely randomized block design (CRBD) with three replicates per treatment. Soil amendments tested were paper pulp + N, beech wood sawdust + N, vetch, radish, Canadian pondweed, black oat, cocoa shells, soy seed meal, *Brassica* seed meal, manure/wood compost, bone meal, biophosphate and beer waste (Tab. 2). Soil without amendments was considered as control treatment. The experiment was sampled two and eight weeks after adding the materials. The selected materials comprise of by-products of farming, industry or forestry and that could gain value as soil improvers. Moreover, these materials cover a broad range in terms of biochemical quality, as shown by the variation in C:N ratios (Tab. 2). Lignocellulose-rich materials are represented by fresh sawdust of beech, whereas paper pulp consists mostly of cellulose. Non-woody plants are included as well, comprising three common winter cover crops (vetch, radish and black oat), hay and an invasive waterweed. Besides the above mentioned undecomposed materials, several residues were included, such as a composted manure/wood mixture, remainders of manure fermentation, by-products of seed and plant processing, as well as milled animal bones (Tab. 2).

The WA experiment was arranged in a CRBD with four replications per treatment. The experiment included the following materials: sawdust of different tree species (beech, willow, hazel, poplar and Douglas fir), paper pulp, green - and organic compost (Tab. 2). The experiment was sampled two and eight weeks after amendment. Each type of sawdust and paper pulp was added to the soil alone and in combination with mineral N. Both bare soil and soil amended with N only were used as controls. Beech sawdust and paper pulp were included as a repetition of the main result of the OA experiment. Sawdust of four other tree species was obtained from branches of ca. 6 cm diameter. Three of them were fast-growing deciduous tree species (willow, hazel and poplar), whereas Douglas fir was included as a representative of conifers. In addition to these recalcitrant, fresh materials, two decomposed, recalcitrant materials

Table 2. Organic materials used for experiment OA, WA and ST, with the quantification of carbon and nitrogen content (%, mean (SD), $n = 3$) C:N ratio and a brief description of the source of each material are indicated.

Material	Exp.	C %	N %	C:N	Description
Paper pulp	OA, WA	42.3 ± 0.3	0.15 ± 0.01	282	Acid-washed cellulose, (SCA Hygiene Products Suameer BV, NL)
Beech sawdust	OA, WA, ST	45.4 ± 0.9	0.14 ± 0.02	331	*Fagus sylvatica,* commercial sawdust for smoke ovens (Sänger Rollenlager GmbH & Co, Waldsolsms, D)
Vetch	OA	20.9 ± 2.8	2.44 ± 0.21	9	Cover crop *Vicia sativa*, collected in March 2016
Radish	OA	32.6 ± 1.2	2.95 ± 0.06	11	Cover crop *Raphanus sativus*, collected in March 2016
Canadian pondweed	OA	34.4 ± 1.2	3.14 ± 0.81	11	Acquatic plant, *Elodea spp.*
Black oat	OA	6.4 ± 1.9	0.28 ± 0.08	23	Cover crop *Avena strigosa*, collected in March 2016
Hay	OA	41.0 ± 0.2	1.28 ± 0.08	32	Collected from a local meadow
Cocoashells	OA	39.9 ± 1.1	2.45 ± 0.03	16	Husks of cacao beans: gardening mulch (Pokon Naturado, Venendaal, NL)
Soy seed meal	OA	41.6 ± 0.6	7.70 ± 0.45	5	Residue from protein extraction and fermentation of soy beans (Ecostyle, Oostervolde, NL)
Brassica seed meal	OA	47.2 ± 0.1	5.57 ± 0.14	8	*Brassica spp. seed meal (P.H. Petersen, Grundhof, D)*
Manure/wood composted mix	OA	33.7 ± 1.9	2.24 ± 0.17	15	Obtained from an organic farmer
Bone meal	OA	21.1 ± 0.3	5.30 ± 0.07	4	Milled residue of pig bones (Ecostyle, NL)
Biophosphate	OA	32.3 ± 9.2	2.05 ± 0.54	16	Fermented pig manure (Eurofins Agro, Wageningen, NL)
Beer waste	OA	40.7 ± 0.9	3.55 ± 0.56	11	Residue from hop fermentation (Ecostyle, NL)
Willow	WA	47.5 ± 1.0	0.33 ± 0.19	144	*Salix alba,* collected in December 2017
Hazel	WA	46.5 ± 0.4	0.30 ± 0.03	157	*Corylus avellana,* collected in December 2017
Poplar	WA	47.3 ± 0.6	0.67 ± 0.04	71	*Populus alba,* collected in December 2017
Douglas fir	WA	49.0 ± 0.2	0.18 ± 0.02	277	*Pseudotsuga menziesii,* collected in December 2017
Green compost	WA	19.4 ± 2.3	0.97 ± 0.10	20	Composted plant prunings (van Iersel Compost, Biezenmortel, NL)
Organic compost	WA	14.0 ± 2.0	1.28 ± 0.16	11	Composted organic municipal waste (Vereniging Afvalbedrijven, 's-Hertogenbosch, NL)

were tested: a commercial compost derived from plant pruning (green compost) and composted organic municipal waste (organic compost).

In the ST experiment beech sawdust together with ammonium nitrate was amended in four contrasting soils, as introduced in section 2.1. Soils with only added N acted as controls for each soil. In the ST experiment four replicates were used that were organized in a CRBD. Beech sawdust from the same batch was used in all experiments (Tab. 2). Unlike experiments OA and WA, the ST experiment was sampled at three time points, namely two, four and eight weeks after amendment.

Before addition to the soil, all materials were air-dried at 40° C for 2 to 14 days, crushed and milled (cutting mill SM 100, Retsch B.V., Haan, Germany) into a fine powder, 2-mm sieved and stored at room temperature until use. Carbon and nitrogen content of each material were determined in triplicate with Thermo flash EA 1112 (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

2.3 Soil preparation, incubation and harvesting

The three experiments were carried out the same way. Each organic material was added in the form of fine (< 2 mm), dry powder to the soils, at the concentration of 5 g kg^{-1} dry weight soil. Supplemental N was added as ammonium nitrate (170 mg N kg^{-1} soil dry weight) to a part of the treatments (as described in section 2.2). In this way, the C:N ratio of paper pulp and woody materials was adjusted to < than 15:1, which is suitable for preventing plant yield depression (Mohanty et al., 2013). Soil moisture was adjusted to 60% water holding capacity by adding and mixing the amended soils with sterile water. Pots were filled with 230 g moist soil and incubated in a dark climate chamber at 20° C. Soil moisture was kept constant by adding sterile water twice a week on basis of weight loss. Distinct replicate pots of every treatment were harvested at each time point. The upper 3 cm of soil, which experienced the largest fluctuation in moisture, was discarded from each pot. After that, the remaining soil in a pot was homogenised. For every measurement, one sub-sample per pot was taken, namely for the determination of fungal biomass (ergosterol), fungal community composition (DNA) and mineral N content. For ergosterol extraction, 1 g of fresh soil was mixed with 4 ml of 10% KOH in methanol and was stored at -20° C for up to two months before extraction. For DNA extraction, ca. 1.5 g of fresh soil were stored at -20° C for ca. four months before analysis. About 50 g of the remaining soil was collected in a paper bag and air-dried at 40° C for 7 days and stored at room temperature for ca. two months before determination of mineral nitrogen and pH.

2.4 Fungal biomass

Alkaline extraction of ergosterol was performed starting from 1 g soil samples, as described by de Ridder-Duine et al., (2006). Briefly, samples were stored in 4 ml methanol 10% KOH, processed by sonication (47 kHz, 15 min), followed by a heat

treatment (70° C for 90 min). Alkaline hydrolysis of esterified ergosterol carried out by the addition of 1 ml water and 2 ml n-hexane, combined with mechanical shaking. The hexane fraction was collected and the solvent was evaporated overnight. The pellet, containing ergosterol, was dissolved in methanol. Finally, ergosterol concentrations were quantified by LC-MSMS (UHPLC 1290 Infinity II, Agilent Technologies and 6460 Triple Quad LC-MS, Santa Clara, California, United States).

2.5 Mineral N and pH

Soil mineral nitrogen (NO^{3-}, and exchangeable NH^{4+}) and pH were determined using 10 g air-dried soil samples. Soil suspensions were obtained by mixing soil and 25 ml of water and shaking for 2 h at 250 rpm with linear movements. After measuring the pH, 25 ml 2 M KCl was added and the suspensions were shaken for 2 h. The aqueous phase was collected and centrifuged at 10 000 x g. NH^{4+} and NO^{3-} concentrations in the supernatant were determined with EAL QuAAtro SFA system (Beun-de Ronde B.V., Abcoude, the Netherlands).

2.6 Fungal community structure

For the OA experiment, the fungal community structure was analysed in soils treated with beech sawdust + N, paper pulp + N, vetch, radish, black oat and hay and the un-amended control (OA experiment). For the WA experiment, soils amended with all sawdust types and paper pulp with ammonium nitrate were analysed for fungal community structure, as well as soil amended with beech sawdust and paper pulp without supplemental N.

DNA was extracted from 0.35 g of soil using MoBio Power Soil Kit (MO BIO Laboratories, Carlsbad, California, United States) according to the indications of the manufacturer and eluted in 50 µl PCR-grade water after 15 min incubation at room temperature. The nuclear rDNA internal transcribed spacer 2 (ITS2) region was amplified with fITS9 and ITS4 primer pair (Ihrmark et al., 2012). Barcoded ITS4 primers were used for discriminating each sample. Polymerase chain reactions (PCR) were performed in 25 µl mixtures containing 200 µM each dNTP, 2.5 µl 10X PCR Buffer with $MgCl_2$, 1 µl $MgCl_2$ 25 mM, 1.25 µl BSA 4 mg ml^{-1}, 0.4 µl of each primer, 0.15 µl FastStart Expand High Fidelity polymerase (Roche Applied Sciences, Indianapolis, Indiana, USA) and 1 µl template DNA (10 ng). The PCR cycling conditions were: denaturation at 95° C for 5 min, 35 cycles of 95° C for 45 s, 54° C for 60 s, and 72° C for 90 s, followed by a final extension step at 72° C for 10 min. For each sample, two PCR reactions were performed and the products were pooled before being purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Quality of PCR-products was checked with Fragment Analyzer (Agilent Technologies, Santa Clara, California, United States). Samples were mixed in equimolar concentrations and sequenced (BGI, Shenzhen, Guangdong, China) on a MiSeq Sequencing System (Illumina, San Diego,

California, United States). The resulting sequences can be accessed at the European Nucleotide Archive (accession number PRJEB33534).

2.7 Statistical and bioinformatic analysis

The statistical analysis was carried out in R (version 3.5.1). Two-way ANOVA and a three-way ANOVA were used to compare fungal biomass and soil mineral N across organic amendments, time points (OA, WA and ST), extra N application (WA) and soil type (ST). The assumptions of normality and equality of variances were checked for each ANOVA model. Multiple comparisons were obtained by Tukey's post-hoc test (family-wise error rate 1%). Correlation between NO^{3-} and pH was tested for WA and OA experiment (Pearson).

Sequencing data were processed with the automated pipeline PIPITS (Gweon et al., 2015). Briefly, fungal sequences were prepared by joining read pairs and by quality filtering according to standard parameters. The ITS2 subregion was extracted using ITSx (Bengtsson-Palme et al., 2013). Short reads were removed (< 100 bp) and sequences were clustered based on a 97% similarity threshold using VSEARCH. Chimeric sequences were removed by comparing with UNITE uchime database. Taxonomic assignment was performed using the RDP classifier based on the UNITE fungal ITS database (Abarenkov et al., 2010; Kõljalg et al., 2013). Overall, the dataset counted 2 125 972 sequences. OTU abundances were normalized across samples by total sum scaling (TSS). All OTUs other than fungi were filtered out, as well as singletons, resulting in a dataset of 2116 fungal OTUs. Starting from the taxonomy table, fungi were classified into functional groups when possible, based on FUNGuild database v1.0 (Nguyen et al., 2016) and further manually revised based on literature. Functional groups of interest in this study were: plant pathogens, saprotrophs and fungal parasites (Tab. S7 and S8). Symbiotrophs and animal pathogens and other groups were classified as "Other". When needed, fungi were assigned to multiple guilds, in order to account for functional diversity within a taxon (e.g. *Fusarium*: plant pathogen – saprotroph).

Sequencing data were further analysed in R 3.5.1 (phyloseq and vegan). The total fungal community was analysed independently for OA and WA experiment. Permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the effects of type of organic material, time point (OA and WA) and additional mineral N (WA) on the fungal community composition. Bray-Curtis dissimilarities between fungal communities were visualized as a result of principal coordinate analysis (PCoA). Differences in relative abundance across soil treatments and time points were also analysed at phylum, class and family level by generalized linear model (GLM, glm2 and multcomp packages) based on Poisson distribution, followed by post-hoc multiple comparisons (Tukey, family wise error rate 5%). Potential plant pathogenic fungi were recorded in a conservative way, namely including all taxa classified either

as “plant pathogen” or as “plant pathogen-saptrotroph” (e.g. *Fusarium*). The relative abundance of each potential plant pathogen in this dataset was summed up in a cumulative relative abundance, which was compared across treatments and time points by two-way ANOVA ($p < 0.05$).

3. Results

3.1 Experiment OA: fungal biomass, mineral N and fungal community structure

Ergosterol concentration in Vredepeel soil changed during the two-months incubation depending on the type of added material (Tab. S1A). Addition of paper pulp and beech sawdust, in combination with ammonium nitrate, resulted in increased ergosterol concentrations as compared to the control (Fig. 1A). High ergosterol was sustained for the whole duration of the experiment and peaked at week 2 and week 8, for paper pulp + N and beech sawdust + N respectively (Fig. 1A, Tab. S2). Conversely, amendment with non-woody plant materials (vetch, radish, Canadian pondweed, black oat and hay) resulted in a transient stimulation of ergosterol. At week 2, ergosterol had increased to levels comparable to beech sawdust + N, but at week 8 it declined to levels not dissimilar to the control. These dynamics were more pronounced for N-rich plant residues (vetch, radish and Canadian pondweed) than for N-poor plant materials (black oat and hay) (Fig. 1A, Tab. S2). Finally, cocoa shells, soy seed meal, *Brassica* seed meal, manure/wood compost, bone meal, biophosphate and beer waste had little or no effect on ergosterol concentrations (Fig. 1A).

Out of the fourteen materials, seven increased mineral N concentration in the soil (Fig S1A). Of these, vetch, soy seed meal, *Brassica* seed meal, bone meal and beer waste incremented N at both time points, whereas radish and Canadian pondweed raised soil N after eight weeks. Soil N immobilization occurred after two weeks in soil amended with black oat, hay and cocoa shells, however after eight weeks N levels were again comparable to the control. At the end of the experiment, all mineral N was present as nitrate. A negative correlation was seen between the final soil pH and mineral N concentration in soil, with $R = -0.91$ (Fig. S2A).

The native fungal community of Vredepeel soil was dominated by Ascomycota, followed by Basidiomycota and Mortierellomycota. Low-abundant phyla (< 2%) were categorized as “Other” (Fig. 3). Changes in fungal community composition after soil amendment were significantly explained by both the type of added material and the time of incubation, yet most of the variation was ascribed to the type of material (PERMANOVA, $R^2 = 0.77$, $p < 0.001$, Tab. S6A). In particular, pronounced effects on fungal community composition were seen for Paper pulp + N , C-rich plant materials (beech sawdust + N and black oat) and N-rich cover crops (vetch and radish, Fig. 2A-

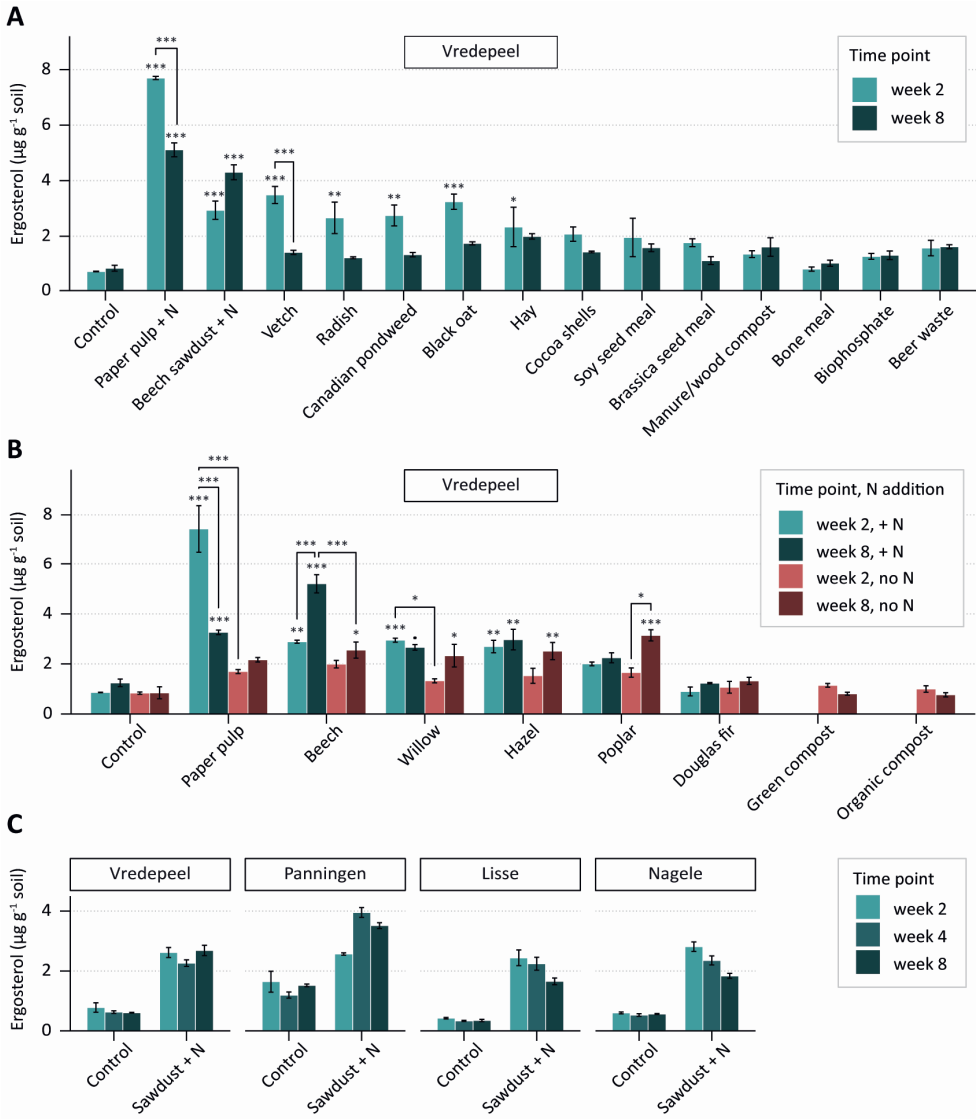

Figure 1. Effect of organic amendments on fungal biomass (ergosterol) development in arable soil. Ergosterol concentration (mean ± SE, n = 3) in Vredepeel agricultural soil amended with fourteen organic materials, sampled after two and eight weeks; Experiment OA (A). Ergosterol concentration (mean ± SE, n = 4) in Vredepeel soil amended with nine organic materials, in combination with mineral N and alone, sampled after two and eight weeks; Experiment WA (B). Significant differences between treatments and controls are indicated in A and B for both sampling time points (* on top of error bar). In addition, significant differences within organic amendments between sampling times (A and B) and between with/without nitrogen additions (B) are shown (• 0.1>p>0.05; * 0.05>p>0.01; ** 0.01>p>0.001, *** p<0.001). Experiment ST (C), ergosterol concentration (mean ± SE, n = 4) in four soils amended with beech sawdust + N, as compared to N-amended control soils. Ergosterol was measured at three time points for each soil type. The description of each soil characteristics is found in Tab. 1.

B). Soil amended with paper pulp + N was dominated by *Chaetomiaceae*, whereas beech sawdust + N favoured *Chaetomiaceae* as well as other Ascomycota (Sordariomycetes, *Lasiosphaeriaceae* and *Ramophialophora* spp). N-rich cover crops stimulated Mortierellomycota (Fig. 3C and Tab. S5A).

The cumulative relative abundance of potential plant pathogens did not increase when organic materials were added to the soil. On the contrary, the relative abundance of the sub-community of pathogens was lower, as compared to the control, in soil amended with paper pulp + N, beech sawdust + N, vetch, black oat and hay (Fig. 3A, ANOVA, $p < 0.05$). Thus, the functional analysis of the fungal community showed that organic materials shifted the indigenous soil fungal community in favor of non-pathogenic, saprotrophic groups, at the expense of potential pathogens. The only exception was radish, where the relative abundance of *Plectosphaerella* spp. was larger than in the control soil and the cumulative relative abundance of potential pathogens was comparable to the control (Fig. 3A).

3.2 Experiment WA: fungal biomass, mineral N and fungal community structure

In experiment WA the development of ergosterol concentration in response to paper pulp + N and beech sawdust + N was similar to that observed in experiment OA (Fig. 1A-B). Moreover, willow and hazel sawdust + N stimulated soil ergosterol concentrations. However, unlike beech sawdust + N, the increase in ergosterol was similar at both time points (Fig. 1B, Tab. S3). Among deciduous trees, poplar sawdust + N was the only amendment that did not significantly increase ergosterol. For sawdust of all deciduous trees, added without N supplement, ergosterol increase was seen after eight weeks. Conversely, ergosterol concentration was comparable to the control with the addition of paper pulp without N. Sawdust from Douglas fir, the only conifer species examined, had no effect on ergosterol concentrations neither with or without mineral N. Lack of stimulation of fungal biomass was also seen for green compost and organic compost addition (Fig. 1B). Addition of mineral N alone also had no effect on ergosterol.

Addition of solely paper pulp and woody materials caused a depletion of soil mineral N (Fig. S1B). When these materials were added with a dose of 170 mg N kg^{-1}, this was sufficient to maintain high N levels in the soil throughout the eight-week incubation. Only a partial N depletion was observed, which coincided with elevated ergosterol. Douglas fir was an exception to this, since it caused N immobilization without ergosterol increase. Composts had no effect on soil mineral N (Fig. S1B). Overall, soil pH had a negative correlation with mineral N concentration, with $R = -0.90$ (Fig. S2B).

In experiment WA, changes in the fungal community composition were mostly explained by the type of added material (PERMANOVA, $R^2 = 0.57$, $p < 0.001$, Tab. S6B). The fungal communities found in control soil, soil amended with paper pulp + N and beech sawdust + N were consistent with those observed in experiment OA (Fig. 3C-

D). Similar fungal communities were found in soil treated with sawdust of deciduous tree species + N (Fig. 2C-D), which was especially clear at week 8. Namely, beech, willow and hazel sawdust + N enlarged the relative abundance of Ascomycota (*Chaetomiaceae*, *Lasiosphaeriaceae* and *Ramophialophora* spp). Relative abundance of Mortierellomycota (*Mortierellaceae*) increased, albeit to a small extent, only with poplar sawdust (Tab. S5B). Douglas fir + N caused a small, yet significant increase in the relative abundance of Basidiomycota (Tremellomycetes, *Solicoccozyma* spp.). Supplemental mineral N did also affect the fungal community structure (PERMANOVA, $R^2 = 0.09$, $p < 0.001$, Tab. S6B). This was seen from the comparison of paper pulp and beech wood added to the soils with and without N (Fig. 2C-D). Paper pulp alone stimulated not only *Chaetomiaceae*, but also two additional groups,

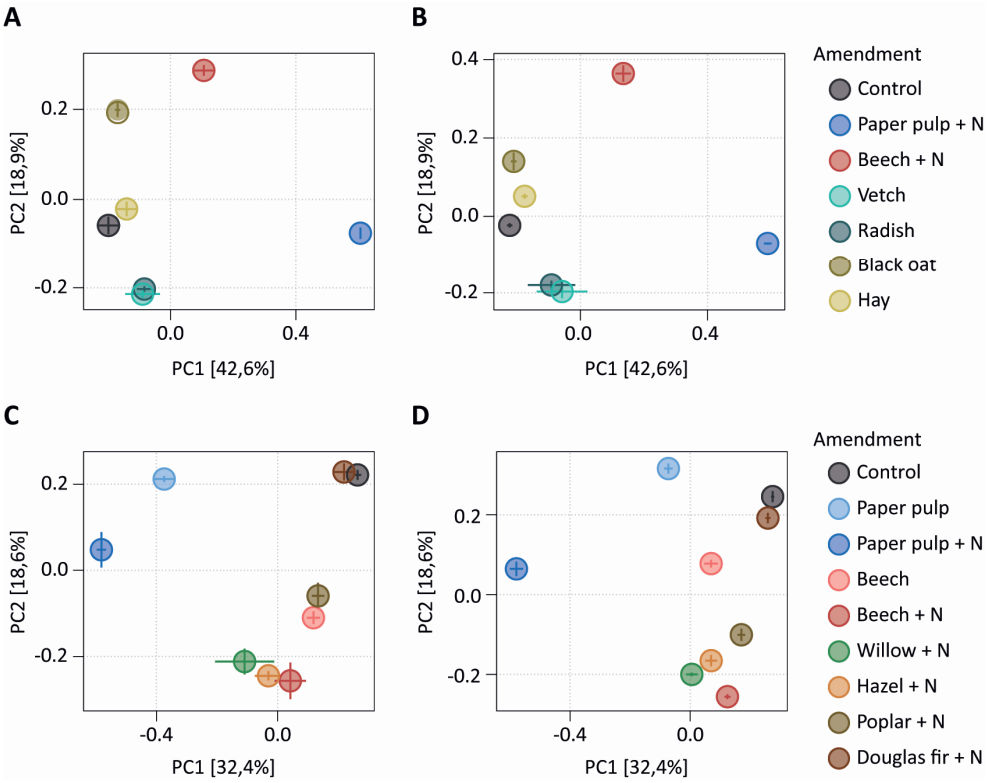

Figure 2. Effect of organic amendments on the fungal community composition in an arable soil. Ordination (PCoA based on Bray-Curtis dissimilarity matrix) was performed independently for Experiment OA and WA. A and B display the dissimilarity between fungal communities of soil amended with organic materials (Exp. OA) after two and eight weeks of incubation, respectively. Similarly, C. and D. represent the dissimilarity between fungal communities after two and eight weeks of incubation with paper pulp and five sawdust types (Exp. WA). For each amendment and time point, circles are centred on the mean and lines show SE along the first and the second Principal Coordinate.

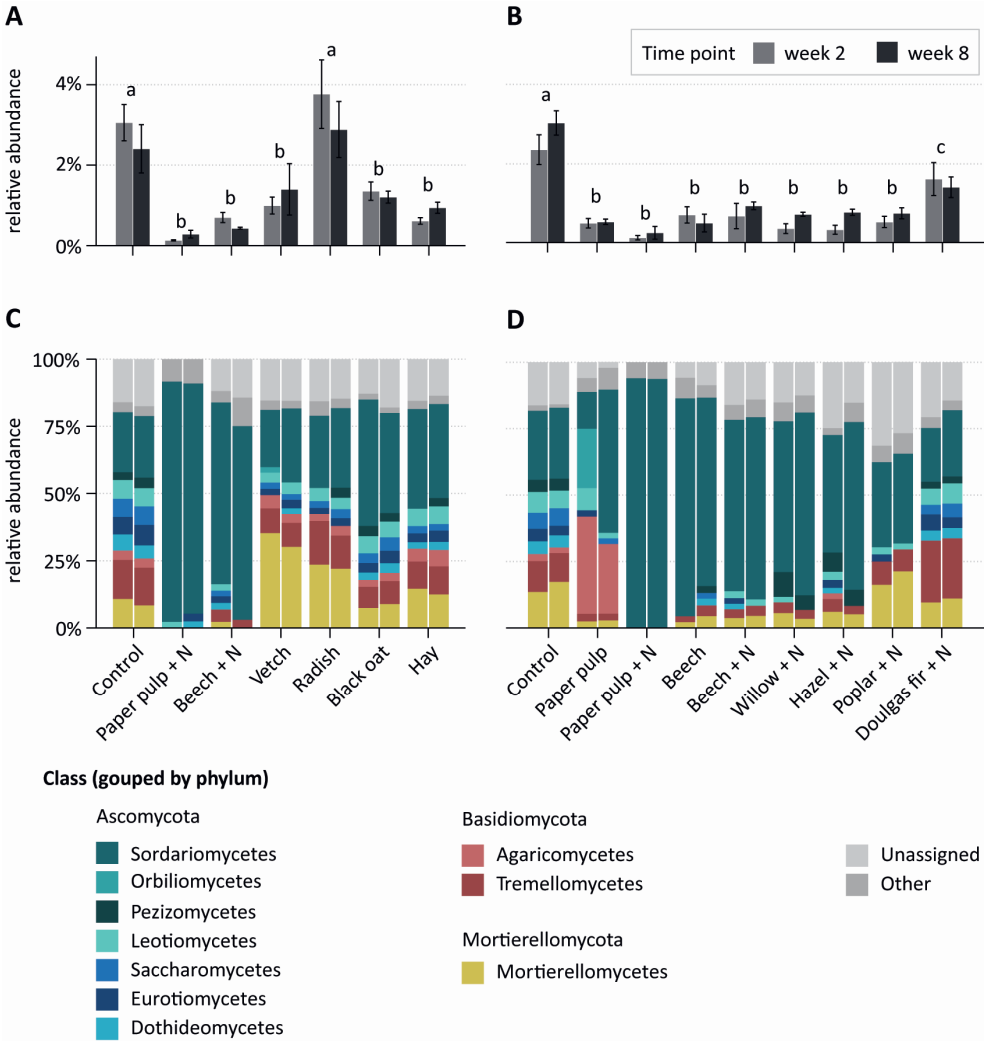

Figure 3. Effect of organic amendments on potential fungal pathogens and on fugal classes in an arable soil. Relative abundance (mean ± SE) of potential pathogens detected in soil amended with organic materials in Experiment OA (A) and WA (B) after two and eight weeks of incubation. Differences are displayed at $p < 0.05$. C and D show the composition of the total fungal community for Experiment OA and WA, respectively: relative abundances of fungal classes are represented for each soil treatment after two and eight weeks of incubation.

Orbiliomycetes (Orbiliales) and Agaricomycetes (Cantharellales) (Fig. 3D and Tab. S5B). On the contrary, the addition of beech sawdust alone led to a *Ramophialophora* spp.-dominated fungal community at week 8 (Fig. S5B).

Similarly to experiment OA, the cumulative relative abundance of the detected potential plant pathogens did not increase, rather was lower than in the control, in

soils amended with paper pulp and all sawdust types here examined (Fig. 3B, ANOVA, $p < 0.05$).

3.3 Experiment ST: fungal biomass

When added to four arable soils, beech sawdust + N significantly increased the concentrations of ergosterol, as compared to the same soils amended with ammonium nitrate only ($p < 0.01$, Fig. 1C, Tab. S4). After two weeks of incubation, ergosterol concentration was similar in all sawdust-amended soils. At later time points, ergosterol concentration further increased (week 4) in sawdust-amended Panningen soil (sandy loam, low pH, high OM), whereas it remained stable in sawdust-amended Vredepeel soil and slightly decreased in Lisse soil (sand, high pH, low OM) and Nagele soil (silty loam, high pH).

4. Discussion

4.1 Effect of organic amendments on saprotrophic fungal biomass

Ergosterol measurements indicated that fungal biomass dynamics in the soil differed strongly for the added materials. In general, three patterns of stimulation of fungi could be recognized: (I) No or slight stimulation, (II) Strong initial stimulation followed by a strong decrease, (III) Moderate or strong initial stimulation followed by gradual decrease or further increase.

Pattern (I) was seen for several industrial by-products, composts and coniferous wood sawdust. Industrial by-products and composts probably contain limited amounts of degradable organic compounds as easily available energy sources are depleted during production processes, such as fermentation, extraction, roasting or composting (Goss et al., 2013; Scotti et al., 2015). This can explain the low fungal stimulation observed here. Indeed, field studies have shown that application of compost does not increase fungal biomass, unless compost is added in large amounts (Quintern et al., 2006; Bastida et al., 2008). Fungal biomass stimulation could be expected after a longer time since the application of the by-products here tested in the short-term. On the other end, it is not clear if arable soils harbour slow-growing fungi able to further degrade energy-poor residues (Paterson et al., 2011).

Degradation of coniferous wood is a common process in forest ecosystems (van der Wal et al., 2016) and a field experiment showed that conifer wood sawdust caused a long-lasting increase in saprotrophic fungal biomass and soil C stocks, when amended yearly in a wheat monoculture (Wuest and Gollany, 2013; Reardon and Wuest, 2016). Yet, we found a lack of fungal stimulation by Douglas fir sawdust. Hence, it seems that the incubation period of two months used here is too short for Douglas fir sawdust to

cause stimulation of fungi. A slower response to conifer wood could be ascribed to higher concentrations of inhibiting secondary metabolites, resins and terpenes, as well as a structural arrangement and composition of wood polymers (Cornwell et al., 2009).

Pattern (II) was seen for non-woody plant materials. This is in line with studies showing rapid decomposition of dried material of freshly harvested plants and a transient increase in microbial biomass (Lucas et al., 2014). These materials contain a mixture of easily degradable (sugars, proteins) and slowly degradable (cellulose, lignin) compounds (Baumann et al., 2009; Lucas et al., 2014). The labile compounds promote a rapid increase of opportunistic microbes, including so-called sugar fungi. Indeed, for the non-woody plant materials we found a stimulation of the fungi belonging to phylum Mortierellomycota for which many sugar fungi have been described (see section 4.3). Yet, within the non-woody plant materials we detected differences in fungal stimulation patterns that appear to be related to the quality of the material reflected mainly in their C:N ratios. For instance, hay had the highest C:N of the non-woody plant materials and gave a lower but more consistent stimulation of fungal biomass. Our understanding of fungal stimulation could be further supported by a detailed chemical characterization of the initial composition of organic amendments (Bonanomi et al., 2018).

Pattern (III) was seen for paper pulp and deciduous woody materials. Common to these materials is that they are recalcitrant and rich in cellulose. This cellulose is crystalline, arranged in microfibrils and fibrils (Rubin, 2008). Many ascomycete and basidiomycete fungi possess a complex of hydrolytic and oxidative enzymes needed to open and degrade it (Wilson, 2011). In intact wood, the presence of lignin limits the accessibility of cellulose for fungi, but this can be partly relieved by milling (van der Wal et al., 2007; Koranda et al., 2014). During paper pulp production from wood, lignin and hemicellulose are removed (Eriksson, 1990) and as a result, paper pulp is mainly composed of cellulose fibres. This can explain the rapid increase in fungal biomass, which was observed in this study, as well as in earlier studies with paper pulp and pure cellulose (Beyer et al., 1997; van der Wal et al., 2006a). Interestingly, the fungal-stimulation effect of (beech) sawdust was consistently observed in the four arable soils tested here. This suggests that the ability to degrade woody materials is common among fungal communities of arable soils, even if they have not received woody residues since a long time. Paterson et al. (2011) showed that prolonged residue exclusion from a soil can limit the ability of resident microbes to degrade insoluble fractions of plant material. However, in that study residue exclusion lasted for fifty years, thus it was more extreme than in intensively managed soils, were periods of bare-fallow alternate with cropping. We show that fungi of such arable soils retain the ability to utilize, at least part, lignocellulose-rich materials.

4.2 Effect of nitrogen on saprotrophic fungal biomass

The initial decomposition of C-rich, fresh materials results in immobilization of mineral N because of an increase in N demands by the microbial decomposers (Mohanty et al., 2013; Gad et al., 2015). Indeed, we detected a depletion of soil mineral nitrogen with addition of wood sawdust, paper pulp and N-poor plant litter. In the field situation this could lead to N deficiency for crops (Mohanty et al., 2013; Heijboer et al., 2016). Thus, stimulation of fungi with fresh, C-rich material should be combined with extra N fertilization to compensate for N immobilization (Mohanty et al., 2013; Toenshoff et al., 2014). At the same time, elevated or prolonged nitrogen fertilization can have a negative impact on fungal biomass (Zhang et al., 2018) and especially affects lignin-degraders (Entwistle et al., 2018). Reduction in fungal biomass was observed in N-fertilized soils for a range of ecosystems (Treseder, 2008). In our study, mineral N supply did not reduce the biomass of decomposer fungi. On the contrary, mineral nitrogen stimulated fungal biomass in soil amended with paper pulp and wood sawdusts, suggesting that fungal growth was N-limited in amended soils. A similar observation has been made for birch sawdust in an ex-arable soil (van der Wal et al., 2007). In that study, the N-induced increase of fungal biomass in birch sawdust was accompanied with an increased activity of cellulose-degrading enzymes but not of lignin-degrading enzymes. As a legacy of soil management, arable soils likely harbour fungal species that are adapted to using mineral forms of N. Although we did not measure enzyme activities, the predominant stimulation of ascomycetes and not of basidiomycetes by deciduous wood sawdusts and paper pulp is in line with mainly cellulolytic activities (section 4.3).

Mineral nitrogen supply did not result in fungal biomass stimulation in soils amended with coniferous wood sawdust. This further confirms that other factors constrain the stimulation of fungi. Another exception to the positive effect of mineral nitrogen on fungal biomass stimulation is represented by poplar wood sawdust. Stimulation of fungi was higher when poplar wood was added without N. Of the studied tree species, poplar wood has the highest quality, as suggested by the relatively low C:N ratio. This was also seen for a poplar forest soil that was converted to arable land and amended with poplar post-harvest residues with and without mineral N (Toenshoff et al., 2014). These differences in fungal biomass stimulation and N dynamics after amendment with sawdust types is not fully explained by wood C:N ratio only.

In this study, measurements of mineral N pools indicated that at least 30% (paper pulp) to 50% (wood sawdust) of the added N remained available for plant nutrition. Partial immobilization of the pool of mineral nitrogen by C-rich materials can be utilized as a means for temporarily capturing fertilizer nitrogen that otherwise would be lost from the soil by leaching (Reichel et al., 2018). Our results suggest that fungi may play a major role in N capture, since the maximum nitrogen depletion corresponds with peaks in ergosterol levels, at two and eight weeks after amendment with paper pulp and beech wood, respectively. Increased retention of mineral N in

arable soil with increased fungal biomass has been reported earlier (de Vries et al., 2011).

4.3 Effect of organic amendments and mineral N on fungal community composition

The observed rapid stimulation of fungal biomass in soil amended with cover crop fragments, paper pulp and wood sawdust is attributed to an increase in fungi belonging to the phyla Ascomycota and Mortierellomycota. Indeed, such phyla comprehend generally fast-growing fungal species, that are common in arable soils and dominate organic residues in the early stages of decomposition (Poll et al., 2010; van der Wal et al., 2013; Banerjee et al., 2016; Koechli et al., 2019). Interestingly, stimulation of the phylum Mortierellomycota could be an indication of fungi growing on nitrogen-rich organic materials, as this was only seen for the N-rich non-woody plant materials and for poplar wood sawdust. On the other hand, the addition of C-rich materials (black oat, paper pulp and sawdust of deciduous tree spp.) changed the fungal community in favour of ascomycete fungi, especially Sordariomycetes. These comprise a broad diversity of saprotrophic fungi, that inhabit dung, plant litter and wood, where they participate in cellulose degradation (Koechli et al., 2019). In particular, paper pulp combined with mineral nitrogen strongly stimulated *Chaetomiaceae* spp., which are well-known for their ability to degrade cellulose (Banerjee et al., 2016; Koechli et al., 2019). Deciduous wood sawdust, and especially beech, willow and hazel sawdust, stimulated *Chaetomiaceae* and *Lasiosphaeriaceae*. The latter family has been indicated by Hartmann et al. (2015) as responsive to organic soil management. Conversely, coniferous wood caused a shift in favour of basidiomycete yeasts, even though this was not reflected in an increase of fungal biomass. Basidiomycetal yeasts are often found in agricultural soils (Hannula et al., 2012) and are stress tolerators (Treseder and Lennon, 2015), which is in line with the potential presence of antimicrobial compounds and low degradability of conifer wood, discussed above (4.1). This area of research would benefit from a detailed description of the initial C chemistry of the organic materials. This, together with profiling of feeding preferences of fungi and bacteria, is essential to accurately predict the effect of organic amendments on native microbial communities of arable soils (Bonanomi et al., 2018).

Rapidly responding fungal species can include plant-pathogenic fungi, that are commonly found as surviving propagules in resident fungal communities in arable soils (van Agtmaal et al., 2017). Pathogenic fungi are specialized in exploiting living and recently dead plant tissues. For instance, immature compost or fresh plant litter has been shown to increase the incidence of soil borne diseases (Bonanomi et al., 2010). Indeed, in this study we observed an amplification of *Plectosphaerella* spp. in soil amended with radish material. *Plectosphaerella* spp. are known for causing diseases in horticultural plants (Raimondo and Carlucci, 2018). However, other

materials did not amplify sequences of potential pathogenic fungi. Conversely, the amendment with other cover crops, and especially sawdust and paper pulp, shifted the fungal community in favour of saprotrophic fungi, with a reduction of potential pathogens. Hence, for the materials here screened for fungal community composition we did not find indications of an increased risk of pathogen proliferation.

5. Conclusions and application perspectives

This study shows that paper pulp and wood sawdust of deciduous tree species rapidly and consistently increase fungal biomass in arable fungal-poor soils, over a time-frame of two months. In particular, ascomycete saprotrophic fungi were stimulated, whereas potential pathogenic fungi did not increase. Woody materials and paper pulp required additional nitrogen to compensate for nitrogen immobilization. We showed that elevated nitrogen did not inhibit, but rather enhanced fungal biomass stimulation by fresh, high C:N materials. Hence, combining of sawdusts and paper pulp with artificial or organic nitrogen fertilizers could be done in practice. The rapid increase in saprotrophic fungal biomass of arable soils, sustained for a period of two months, can benefit the early growth of crops, for instance by increasing the suppression of soil-borne diseases and by improving the efficiency of nitrogen fertilizers. However, future research should examine these benefits, before paper pulp and woody materials can be safely and effectively implemented as soil management options. In particular, as the current study was conducted under controlled growth chamber conditions, without the presence of plants and for a limited span of time. Under field conditions, other factors could interfere with fungal stimulation, such as agricultural management practises and variable weather conditions. Thus, fungal biomass stimulation by sawdusts and paper pulp and its suggested benefits need to be evaluated in field trials, including a longer time frame. Future studies should also include the effect of such amendments on other soil inhabitants. Utilizing wood and paper wastes for sustainable agriculture would be a convenient way of disposing these materials. In particular, sawdust is an abundant waste of the wood industry, as well as of forest and urban landscape management (Harkin, 1969; Heinimö and Junginger, 2009). In recent years, an increasing amount of biomass is used for energy and heat production (Heinimö and Junginger, 2009). However, evidence is growing that large-scale use of wood for energy is a threat for the environment (Schulze et al., 2012; Griscom et al., 2017). We propose that conversion of excess woody biomass into fungal biomass for improving arable soils represents a better perspective for utilization.

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Supplementary information

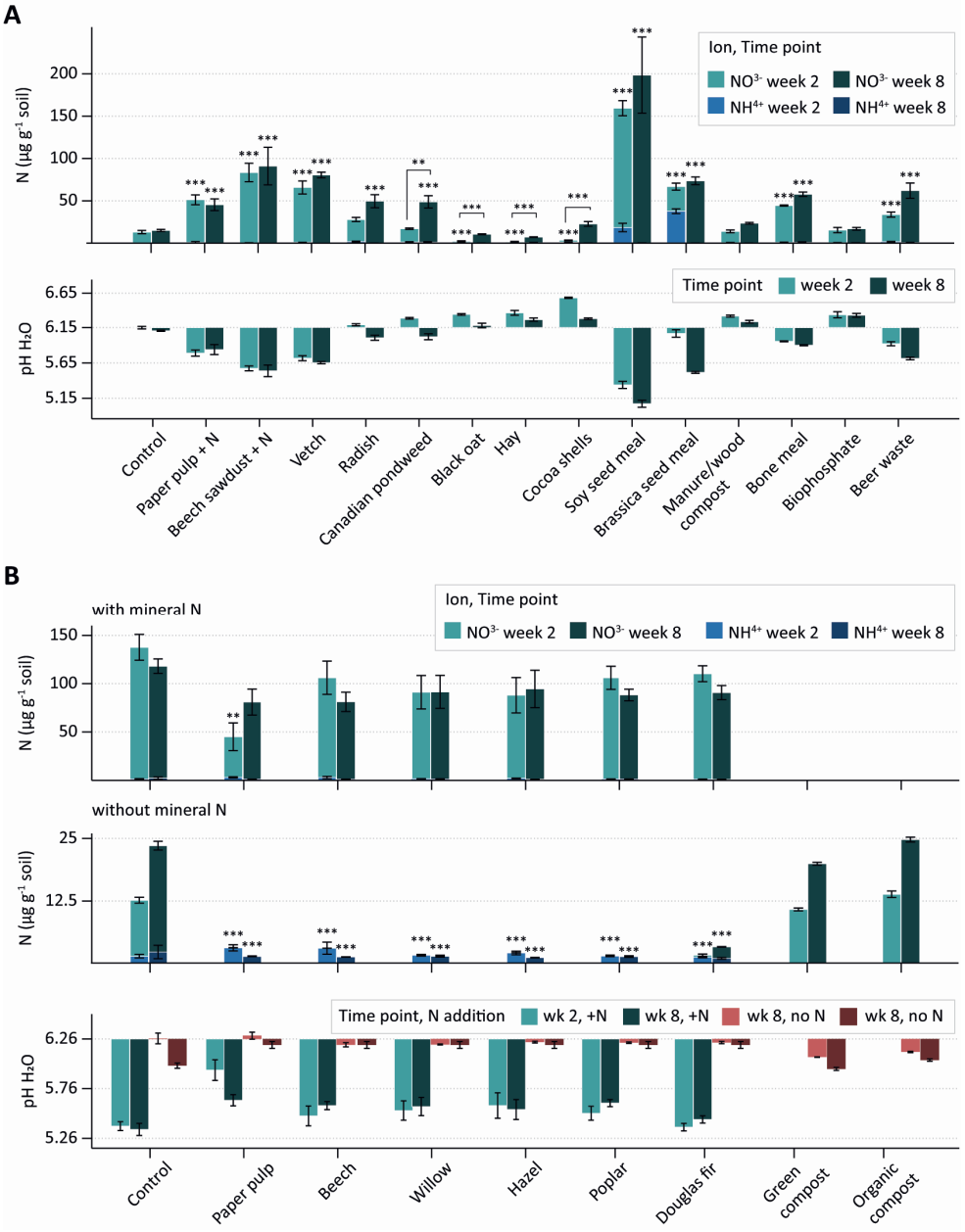

Figure S1. Effect of organic amendments on mineral N and pH in an arable soil. Concentration of available mineral N in soil (ammonium and nitrate) and soil pH, as measured in extracts from un-amended and amended soil, after two and eight weeks of incubation for experiment OA (A, mean ± SE, n = 3) and experiment WA (B, mean ± SE, n = 4). Significant differences to the control and among time points are displayed for total extractable mineral N (NH^{4+} NO^{3-}).

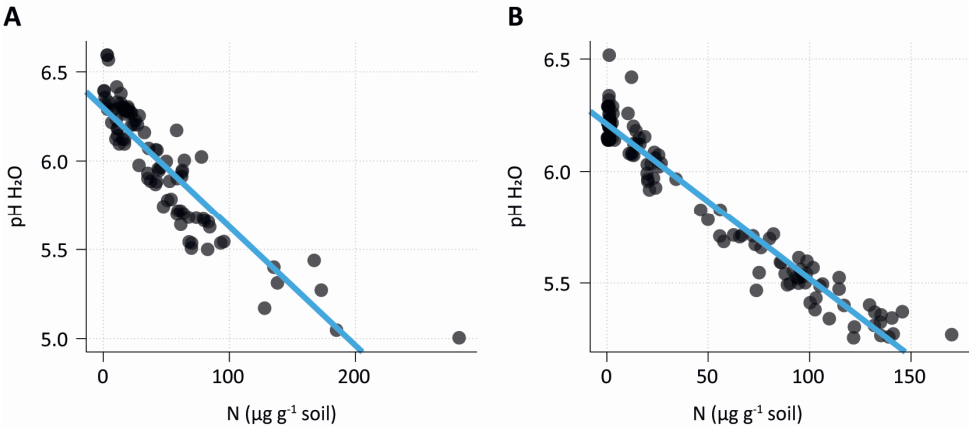

Figure S2. Correlation between pH and mineral N concentration in soil. Individual data points and linear regression line are shown for experiment OA (A) and experiment WA (B).

Table S1. Summary of ANOVA results on ergosterol as a function of organic material, time point (OA experiment, A), and supplemental mineral N (WA experiment, B). For ST experiment, ANOVA results are shown (C) for ergosterol as affected by sawdust + N amendment (Material), soil type and time point. d.f.: degrees of freedon, MS: mean sum of squares, SS: sum of squares.

A	**d.f.**	**SS**	**MS**	**F**	**p**	
Material	14	127.81	9.13	39.18	$2.2\ 10^{-6}$	***
Time point	1	7.23	7.23	31.03	$6.6\ 10^{-7}$	***
Material : Time point	14	21.30	1.52	6.52	$1.0\ 10^{-7}$	***
Residuals	59	13.748	0.233			
B	**d.f.**	**SS**	**MS**	**F**	**P**	
Material	8	115.39	14.42	52.80	$2.2\ 10^{-16}$	***
Mineral N	1	26.57	26.57	97.27	$4.0\ 10^{-16}$	***
Time point	1	1.44	1.44	5.28	0.024	*
Material : Mineral N	6	39.38	6.56	24.03	$2.2\ 10^{-16}$	***
Material : Time point	8	26.87	3.36	12.30	$7.3\ 10^{-12}$	***
Time point : Mineral N	1	5.43	5.25	19.21	$3.1\ 10^{-5}$	***
Material : Time point : Mineral N	6	22.80	3.80	13.91	$3.1\ 10^{-11}$	***
Residuals	93	25.404	0.273			
C	**d.f.**	**SS**	**MS**	**F**	**P**	
Material	1	326.82	326.82	1 369.64	$2.2\ 10^{-16}$	***
Soil type	3	62.67	20.89	87.55	$2.2\ 10^{-16}$	***
Time point	2	0.46	0.23	0.96	0.39	
Time point : Soil type	6	17.64	2.94	12.32	$2.2\ 10^{-9}$	***
Material : Time point	2	2.33	1.17	4.89	0.013	*
Material : Soil type	3	1.12	0.37	1.56	0.206	
Material : Time point : Soil type	6	12.69	2.12	8.87	$3.6\ 10^{-7}$	***
Residuals	69					

Table S2. Ergosterol concentration (µg g^{-1}, mean ± SD, n = 3), measured for fourteen materials after two and eight weeks of incubation (Experiment OA). Simple effect of Material is displayed in column A (week 2) and comlumn B (week 8). Compact letters represent differences among materials at p < 0.01.

Material	**Time point (week)**	**Ergosterol (µg g^{-1} soil)**	**A**	**B**
Control	2	0.73 ± 0.02	a	
	8	0.85 ± 0.18		a
Paper pulp + N	2	7.70 ± 0.08	e	
	8	5.11 ± 0.43		b
Beech sawd, + N	2	2.93 ± 0.57	b c d	
	8	4.30 ± 0.47		b
Vetch	2	3.48 ± 0.53	d	
	8	1.41 ± 0.13		a
Radish	2	2.09 ± 0.05	b c d	
	8	1.22 ± 0.06		a
Black oat	2	3.24 ± 0.48	c d	
	8	1.74 ± 0.09		a
Canadian Pondweed	2	2.75 ± 0.65	b c d	
	8	1.33 ± 0.13		a
Hay	2	1.62 ± 0.01	a b c d	
	8	1.99 ± 0.016		a
Cocoa shells	2	2.08 ± 0.45	a b c d	
	8	1.43 ± 0.05		a
Soy meal	2	1.96 ± 1.20	a b c d	
	8	1.58 ± 0.24		a
Seed meal	2	1.76 ± 0.24	a b c d	
	8	1.12 ± 0.25		a
Manure/wood compost	2	1.35 ± 0.21	a b	
	8	1.61 ± 0.57		a
Bone meal	2	0.81 ± 0.13	a	
	8	1.03 ± 0.19		a
Biophosphate	2	1.27 ± 0.18	a b c	
	8	1.31 ± 0.26		a
Beer waste	2	1.57 ± 0.48	a b c	
	8	1.62 ± 0.12		a

Table S3. Ergosterol concentration (µg g^{-1}, mean ± SD, n = 4), measured for eight soil amendments and control, either with and without supplemental ammonium nitrate, after two and eight weeks of incubation (Experiment WA). Simple effect of Material is displayed in column A (week 2, with mineral N), B (week 8, with mineral N), C (week 2, no mineral N) and D (week 8, no mineral N). Compact letters represent differences among materials ($p < 0.01$).

Material	Mineral N (yes,no)	Time point (week)	Ergosterol (µg g^{-1} soil)	A	B	C	D
Control	+	2	0.86 ± 0.02	a			
		8	1.24 ± 0.30		a		
	0	2	0.83 ± 0.09			a	
		8	0.84 ± 0.48				a b
Paper pulp	+	2	8.36 ± 0.23	c			
		8	3.26 ± 0.18		b		
	0	2	1.70 ± 0.16			a	
		8	2.17 ± 0.18				a b c d
Beech	+	2	2.89 ± 0.10	b			
		8	5.20 ± 0.73		c		
	0	2	1.99 ± 0.30			a	
		8	2.55 ± 0.56				a c d
Willow	+	2	2.95 ± 0.16	b			
		8	2.66 ± 0.23		a b		
	0	2	1.33 ± 0.16			a	
		8	2.33 ± 0.90				a b c d
Hazel	+	2	2.45 ± 0.06	b			
		8	2.97 ± 0.83		b		
	0	2	1.53 ± 0.60			a	
		8	2.51 ± 0.69				c d
Poplar	+	2	2.00 ± 0.15	a b			
		8	2.25 ± 0.40		a b		
	0	2	1.65 ± 0.37			a	
		8	3.14 ± 0.44				d
Douglas fir	+	2	0.90 ± 0.35	a			
		8	1.23 ± 0.05		a		
	0	2	1.07 ± 0.46			a	
		8	1.32 ± 0.28				a b c
Green compost	0	2	1.14 ± 0.15	-	-	a	
		8	0.75 ± 0.03				a b
Organic compost	0	2	1.00 ± 0.26	-	-	a	
		8	0.77 ± 0.16				b

Table S4. Ergosterol concentration (μg g^{-1}, mean ± SD, n = 4), measured in four soil types after two, four and eight weeks after amendment with either beech sawdust and ammonium nitrae or ammonium nitrate only (ST Experiment). Compact letters (column A) represent differences across sawdust-amended soils and control soils at different time points ($p < 0.01$).

Soil	Material	Time point (week)	Ergosterol (μg g^{-1} soil)	A
Vredepeel	Control N	2	0.78 ± 0.62	a b
		4	0.63 ± 0.18	a b
		8	0.61 ± 0.04	a
	Sawdust + N	2	2.62 ± 0.66	f g h
		4	2.26 ± 0.43	d e f g
		8	2.68 ± 0.67	f g h
Panningen	Control N	2	1.64 ± 0.03	c d e
		4	1.20 ± 0.40	b c
		8	1.52 ± 0.17	c d
	Sawdust + N	2	2.57 ± 0.15	f g h
		4	4.18 ± 0.98	i
		8	3.51 ± 0.38	h i
Lisse	Control N	2	0.42 ± 0.10	a
		4	0.33 ± 0.07	a
		8	0.35 ± 0.14	a
	Sawdust + N	2	2.44 ± 1.03	e f g
		4	2.24 ± 0.84	d e f g
		8	1.65 ± 0.05	c d e
Nagele	Control N	2	0.60 ± 0.12	a
		4	0.53 ± 0.17	a
		8	0.56 ± 0.06	a
	Sawdust + N	2	2.81 ± 0.61	g h
		4	2.35 ± 0.60	e f g
		8	1.84 ± 0.32	c d e f

Table S5. Effect of soil amendments on relative abundances of fungal taxa. Mean relative abundance (%) is displayed for the most abundant fungal phyla and classes (> 1.5 %). For each taxon, significant differences in relatve abundance are reported for every soil treatment, as compared to the control within the same time point. The simple effect of time is displayed for the control only (Control wk 8, as compared to Control wk2). Differences in relative abundance are also represented by colour code.

A	Control		Paper pulp + N		Beech + N		Vetch		Radish		Black oat		Hay	
	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8
Ascomycota	59	61 **	96 ***	95 ***	83 ***	88 ***	39 ***	46 ***	45 ***	50 ***	72 ***	67 ***	59	59
Sodariomycetes	22.3	23 **	89.5 ***	85.7 ***	67.6 ***	72.1 ***	21.3	27.6 ***	26.9 ***	29.7 ***	47.1 ***	37.3 ***	37.1 ***	35 ***
Chaetomiceae	4.3	1.8	86.7 ***	82.7 ***	25.3 ***	17.3 ***	11.6 ***	16.9 ***	13.3 ***	15.6 ***	2.6 ***	3.3 ***	8.9 ***	5.1 ***
Lasiosphaeriaceae	2.8	5.3 ***	1.4 ***	1.2 ***	25.3 ***	12.4 ***	2.5 ***	2.7 ***	2.8	3.4	24.6 ***	15 ***	17 ***	10.2 ***
Ramophialophora	0.1	0.1	0 ***	0 ***	2.9 ***	15.1 ***	0 *	0 ***	0 ***	0.1 ***	0.2 ***	0.3 ***	0.1	0.1
Pezizomycetes	2.9	3.8 ***	0.5 ***	0.7 ***	1.5 ***	0.7 ***	0.9 ***	1.2 ***	1.8 ***	3.7 *	3.7 ***	3.1 ***	1.9 ***	3 ***
Leotiomycetes	7	6.8	2.2 ***	1.7 ***	2.5 ***	1.6 ***	3.8 ***	4.3 ***	4.9 ***	4.3 ***	6.5 ***	5.9 ***	6.5 ***	6.7 ***
Saccharomycetes	6.7	6.9	0.5 ***	0.7 ***	2.1 ***	1.3 ***	2.3 ***	2.1 ***	2.5 ***	3.4 ***	3.5 ***	5 ***	2.7 ***	2.4 ***
Dothideomycetes	6.1	4.9 ***	0.5 ***	2.4 ***	2.4 ***	1.2 ***	1.2 ***	2.1 ***	1.8 ***	1.1 ***	2.8 ***	3.6 ***	2.4 ***	3 ***
Eurotiomycetes	6.5	7.7 ***	1.8 ***	2.9 ***	2.5 ***	2 ***	2.3 ***	3.2 ***	2.2 ***	2.8 ***	3.6 ***	4.6 ***	3.4 ***	4.3 ***
Basidiomycota	19	18	1.8 ***	2.5 ***	6.4 ***	4.2 ***	15 ***	12 ***	19	16 ***	11 ***	12 ***	15 ***	17 **
Tremellomycetes	14.5	14.1	1 ***	1.5 ***	4.6 ***	3 ***	9.2 ***	8.9 ***	16.3 ***	12.3 ***	7.9 ***	8.5 ***	10.1 ***	10.4 ***
Agaricomycetes	3.4	3.4	0.7 ***	1 ***	1.4 ***	1 ***	4.9 **	3.3 ***	2.6 ***	3.6 ***	2.5 ***	3 ***	4.8 ***	6.1 ***
Mortierellomycot	11	8.3 ***	0.6 ***	0.7 ***	2.2 ***	2 ***	35 ***	30 ***	24 ***	22 ***	7.4 ***	8.9	15 ***	13 ***

B	Control		Paper pulp + N		Paper pulp		Beech + N		Beech		Willow + N		Hazel + N		Poplar + N		Douglas fir + N	
	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8	wk 2	wk 8
Ascomycota	61	62	98 ***	97 ***	66 ***	54 ***	86 ***	87 ***	86 ***	91 ***	86 ***	82 ***	84 ***	78 ***	48 ***	60 ***	55 ***	56 ***
Sordariomycetes	26.8	26	93.7 ***	94 ***	53.9 ***	13.8 ***	68.6 ***	64.6 ***	71 ***	82.1 ***	68.8 ***	56.8 ***	63.1 ***	44.3 ***	33.8 ***	32.1 ***	25 **	20.3 ***
Chaetomiceae	2.1	1.6	84 ***	84.4 ***	45 ***	7.6 ***	10.7 ***	3.6 ***	7 ***	0.7 ***	24.6 ***	14.9 ***	18.1 ***	11 ***	6.9 ***	4.7 ***	5.9 ***	2 ***
Lasiosphaeriaceae	7.2	6.3 ***	1.3 ***	1.3 ***	4.9 ***	1.6 ***	25 ***	19.9 ***	18 ***	2.4 ***	18.1 ***	13 ***	21.2 ***	12.2 ***	15.3 ***	9.3 ***	4.9 ***	4.4 ***
Ramophialophora	0.2	0.5 ***	0 ***	0 ***	0 ***	0.6	1.1 ***	1.3 ***	8.7 ***	62 ***	0.8	0.6	1.3 ***	0.6	0.3	1 ***	0.1 ***	1.3 ***
Pezizomycetes	4.4	4.5 ***	0.3 ***	0.4 ***	1 ***	1.4 ***	1.4 ***	1.9 ***	2.6 ***	1.1 ***	5.5	9.3 ***	6.1 ***	7.2 ***	1.4 ***	1.4 ***	2.5 ***	2.5 ***
Leotiomycetes	6.7	7.9	0.6 ***	0.6 ***	2 ***	8.3 ***	2.3 ***	2.6 ***	2 ***	1.6 ***	1.6 ***	2 ***	1.5 ***	3.1 ***	2.2 ***	2.7 ***	7.7 ***	6.2 ***
Saccharomycetes	6.6	6.1	0.5 ***	0.4 ***	2.2 ***	1.2 ***	1.7 ***	2 ***	2.1 ***	0.8 ***	1.1 ***	1.5 ***	1.3 ***	1.7 ***	1.6 ***	1.5 ***	5.1 ***	3.5 ***
Orbiliomycetes	0.4	0.2	0.7 *	0.1 ***	1.9 ***	22.4 ***	0 ***	0.1 ***	0.1 ***	0 ***	0.1 ***	0.2	0.2	0.1	0.2 ***	0.1 ***	0.1 ***	0.1
Eurotiomycetes	3.5	4.6 ***	1.4 ***	0.7 ***	1.3 ***	2.4 ***	1.1 ***	2.1 ***	1.1 ***	1 ***	1.4 ***	1.9 ***	1.7 ***	3 ***	1.9 ***	2.6 ***	4 ***	6.1 ***
Dothideomycetes	4.6	4.9	0.3 ***	0.6 ***	1.6 ***	1.6 ***	1.3 ***	2 ***	2.6 ***	1.2 ***	1 ***	1.6 ***	0.9 ***	2 ***	1.4 ***	1.6 ***	3.8 ***	3.8 ***
Basidiomycota	13	15 ***	0.9 ***	1.1 ***	29 ***	40 ***	4.5 ***	4.2 ***	5 ***	3.6 ***	4.3 ***	5.5 ***	4.6 ***	7 ***	8.9 ***	9.7 ***	25 ***	26 ***
Tremellomycetes	10.8	11.6	0.7 ***	0.9 ***	2.4 ***	2.9 ***	3.8 ***	3.3 ***	4 ***	2.1 ***	3.3 ***	4 ***	3.1 ***	4.8 ***	8.2 ***	8.7	22.7 ***	23.3 ***
Agaricomycetes	2	2.6 ***	0.1 ***	0.2 ***	26.3 ***	36.6 ***	0.7 ***	0.8 ***	1 ***	1.4 ***	1 ***	1.4 ***	1.5 ***	2.1 ***	0.6 ***	0.8 ***	1.8	1.7 ***
Mortierellomycot	17	14 ***	0.5 ***	0.5 ***	2.9 ***	2.4 ***	4.5 ***	3.7 ***	4.4 ***	2.2 ***	3.5 ***	5.6 ***	5.2 ***	6.1 ***	21 ***	16 ***	11 ***	9.6 ***

Relative abundance (%) as compared to the control
higher
comparable
lower

Table S6. Permutational multivariate analysis of variance (ADONIS) using bray-curtis dissimilarity matrix partitioned by type of material, time point (OA, A) and addititional mineral N (WA, B), MS: mean sum of squares, SS: sum of squares. [a] Significance values based on 999 permutations.

A	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Material	6	5.546	0.924	27.099	0.769	0.001	***
Time point	1	0.214	0.214	6.282	0.030	0.002	**
Material : Time point	6	0.493	0.082	2.407	0.068	0.003	**
Residuals	28	0.955	0.034		0.133		
Total	41	7.207			1.000		
B	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Material	6	8.697	1.449	34.600	0.570	0.001	***
Mineral N	1	1.382	1.382	32.991	0.091	0.001	***
Time point	1	0.512	0.512	12.217	0.034	0.001	***
Material : Mineral N	1	1.099	1.099	26.235	0.072	0.001	***
Material : Time point	6	0.744	0.124	2.958	0.049	0.001	***
Time point : Mineral N	1	0.285	0.285	6.799	0.019	0.002	**
Material : Time point : Mineral N	1	0.289	0.289	6.903	0.019	0.001	***
Residuals	54	2.262	0.042		0.148		
Total	71	15.269			1.000		

Table S7. Taxonomical and functional classification of each OTU as obtained after filtering the data and as included in further analysis of the fungal community composition. Available at https://doi.org/10.1016/j.apsoil.2019.103434

Table S8. List of potential plant-pathogeninc fungi detected in this study by ITS sequencing.

kingdom	phylum	class	order	family	genus	species	Taxon
Fungi	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia		Diplodia
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium		Cladosporium
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Mycocentrospora	Mycocentrospora_acerina_SH202165.07FU	Mycocentrospora
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella		Didymella
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae			Didymellaceae
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Periconiaceae	Periconia		Periconia
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Ophiosphaerella	Ophiosphaerella_sp_SH183014.07FU	Ophiosphaerella
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria		Alternaria
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Bipolaris		Bipolaris
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Curvularia		Curvularia
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Curvularia		Curvularia
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Drechslera	Drechslera_sp_SH189947.07FU	Drechslera
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Setosphaeria	Setosphaeria_pedicellata_SH187640.07FU	Setosphaeria
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Stemphylium		Stemphylium
Fungi	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Pyrenopeziza	Pyrenopeziza_brassicae_SH640049.07FU	Pyrenopeziza
Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Hymenoscyphus	Hymenoscyphus_menthae_SH489326.07FU	Hymenoscyphus
Fungi	Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	Oidiodendron	Oidiodendron_truncatum_SH216989.07FU	Oidiodendron
Fungi	Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	Oidiodendron		Oidiodendron
Fungi	Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	Oidiodendron	Oidiodendron_echinulatum_SH217004.07FU	Oidiodendron
Fungi	Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	Oidiodendron	Oidiodendron_rhodogenum_SH217018.07FU	Oidiodendron
Fungi	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Botrytis		Botrytis
Fungi	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	Coniochaeta	Coniochaeta_cateniformis_SH191393.07FU	Coniochaeta cateniformis
Fungi	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	Coniochaeta	Coniochaeta_sp_SH191412.07FU	Coniochaeta
Fungi	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	Coniochaeta	Coniochaeta_fasciculata_SH191371.07FU	Coniochaeta
Fungi	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	Coniochaeta	Coniochaeta_fasciculata_SH191371.07FU	Coniochaeta
Fungi	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	unidentified	Coniochaetaceae_sp_SH191373.07FU	Coniochaetaceae
Fungi	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	unidentified	Coniochaetaceae_sp_SH191379.07FU	Coniochaetaceae

Table S8. Continue

kingdom	phylum	class	order	family	genus	species	Taxon
Fungi	Ascomycota	SordariomycetesGlomerellales		Glomerellaceae	Colletotrichum	Colletotrichum_coccodes_SH195651.07FU	Colletotrichum
Fungi	Ascomycota	SordariomycetesGlomerellales		Plectosphaerellaceae	Plectosphaerella		Plectosphaerella
Fungi	Ascomycota	SordariomycetesGlomerellales		Plectosphaerellaceae	Verticillium		Verticillium
Fungi	Ascomycota	SordariomycetesHypocreales		Bionectriaceae	Clonostachys	Clonostachys_divergens_SH182684.07FU	Clonostachys
Fungi	Ascomycota	SordariomycetesHypocreales		Clavicipitaceae	Metarhizium		Metarhizium
Fungi	Ascomycota	SordariomycetesHypocreales		Clavicipitaceae	Metarhizium	Metarhizium_marquandii_SH217935.07FU	Metarhizium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium		Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_rutilum_SH207064.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_curvulum_SH202931.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_furcatum_SH219559.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_persicinum_SH207979.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_sp_SH193967.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_fusidioides_SH203377.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Hypocreales fam Incertae sedis	Acremonium	Acremonium_fusidioides_SH203375.07FU	Acremonium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Cylindrocarpon	Cylindrocarpon_sp_SH174295.07FU	Cylindrocarpon
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Cylindrocarpon	Cylindrocarpon_sp_SH174295.07FU	Cylindrocarpon
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium		Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium	Fusarium_venenatum_SH215301.07FU	Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium		Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium		Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium		Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium	Fusarium_solani_SH181398.07FU	Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Nectriaceae	Fusarium		Fusarium
Fungi	Ascomycota	SordariomycetesHypocreales		Ophiocordycipitaceae	Hirsutella	Hirsutella_rhossiliensis_SH210594.07FU	Hirsutella

Table S8. Continued

kingdom	phylum	class	order	family	genus	species	Taxon
Fungi	Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	Nigrospora		Nigrospora
Fungi	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	Microdochium		Microdochium
Fungi	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae			Xylariaceae
Fungi	Basidiomycota	Agaricomycetes	Agaricales	Pluteaceae	Volvopluteus	Volvopluteus_gloiocephalus_SH221286.07FU	Volvopluteus
Fungi	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Galerina		Galerina
Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Thanatephorus		Thanatephorus
Fungi	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma	Ganoderma_adspersum_SH187221.07FU	Ganodermataceae
Fungi	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma		Ganodermataceae
Fungi	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	unidentified	Ganodermataceae_sp_SH187246.07FU	Ganodermataceae
Fungi	Basidiomycota	Agaricomycetes	Polyporales	Meripilaceae	Meripilus	Meripilus_giganteus_SH221563.07FU	Meripilus
Fungi	Basidiomycota	Agaricomycetes	Polyporales	Meripilaceae	Rigidoporus	Rigidoporus_sanguinolentus_SH189954.07FU	Rigidoporus
Fungi	Basidiomycota	Exobasidiomycetes	Tilletiales	Tilletiaceae	Tilletia	Tilletia_puccinelliae_SH191264.07FU	Tilletia
Fungi	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	Itersonilia	Itersonilia_perplexans_SH199072.07FU	Itersonilia
Fungi	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	Itersonilia	Itersonilia_pannonica_SH199073.07FU	Itersonilia
Fungi	Basidiomycota	Ustilaginomycetes	Urocystidales	Urocystidaceae	Urocystis	Urocystis_agropyri_SH185755.07FU	Urocystis
Fungi	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Sporisorium		Sporisorium
Fungi	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Ustilago	Ustilago_nunavutica_SH175968.07FU	Ustilago
Fungi	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Ustilago	Ustilago_echinata_SH005396.07FU	Ustilago
Fungi	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Ustilago		Ustilago
Fungi	Olpidiomycota	Olpidiomycetes	Olpidiales	Olpidiaceae	Olpidium	Olpidium_brassicae_SH194417.07FU	Olpidiaceae
Fungi	Olpidiomycota	Olpidiomycetes	Olpidiales	Olpidiaceae	Olpidium	Olpidium_brassicae_SH216672.07FU	

Chapter 3

Evaluation of phenolic root exudates as stimulants of saprotrophic fungi in the rhizosphere

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Submitted

Abstract

The rhizosphere microbial community of crop plants in intensively managed arable soils is strongly dominated by bacteria, especially in the initial stages of plant development. In order to establish more diverse and balanced rhizosphere microbiomes, as seen for wild plants, crop variety selection could be based on their ability to promote growth of saprotrophic fungi in the rhizosphere. We hypothesized that this can be achieved by increasing the exudation of phenolic acids, as generally higher fungal abundance is observed in environments with phenolic-rich inputs, such as exudates of older plants and litter leachates. To test this, a rhizosphere simulation microcosm was designed to establish gradual diffusion of root exudate metabolites from sterile sand into arable soil. With this system, we tested the fungus-stimulating effect of eight phenolic acids alone or in combination with primary root metabolites. Ergosterol-based fungal biomass measurements revealed that most phenolic acids did not increase fungal abundance in the arable soil layer. These results were supported by comparison of fungal biomass in the rhizosphere of wild type *Arabidopsis thaliana* plants and mutants with altered phenolic acid metabolism. Salicylic acid was the only phenolic acid that stimulated a higher fungal biomass in the arable soil layer of microcosms, but only when combined with a background of primary root metabolites. However, no such stimulation of rhizosphere fungi was seen for a salicylic acid-overproducing *A. thaliana* mutant. For three phenolic acid treatments (chlorogenic acid, salicylic acid, vanillic acid) fungal and bacterial community compositions were analyzed using amplicon sequencing. Despite having little effect on fungal biomass, phenolic acids combined with primary metabolites promoted a higher relative abundance of soil-borne fungi with the ability to invade plant roots (*Fusarium, Trichoderma* and *Fusicolla* spp.) in the simulated rhizosphere. Bacterial community composition was also affected by these phenolic acids. Although this study indicates that phenolic acids do not increase fungal biomass in the rhizosphere, we highlight a potential role of phenolic acids as attractants for root-colonizing fungi.

1. Introduction

The rhizosphere, the soil volume surrounding plant roots, is a hotspot for microbial activity (Pausch and Kuzyakov, 2018), harboring saprotrophic fungi, alongside bacteria and mycorrhizal fungi (Buée et al., 2009; van der Putten et al., 2016; Hugoni et al., 2018). Rhizosphere saprotrophic fungi can provide multiple services to the plant, such as promotion of plant growth and immunity (Kohler et al., 2007; Yadav et al., 2011; Naznin et al., 2014; Xia et al., 2019; Koike et al., 2001), as well as suppression of infection by soil-borne fungal pathogens. The presence of active saprotrophic fungi can limit root infection by soil-borne pathogens by increasing the competition for resources in the rhizosphere or by direct inhibitory activities such as antibiosis or mycoparasitism (Punja and Utkhede, 2003; Xiong et al., 2017; Latz et al., 2018). Moreover, rhizosphere saprotrophic fungi can influence beneficial rhizosphere bacteria and mycorrhizal fungi, hence indirectly influencing plant performance (Kohler et al., 2007; Saldajeno et al., 2008; de Boer et al., 2015; Qin et al., 2017; Deveau et al., 2018).

Intensively managed arable soils usually harbor low saprotrophic fungal biomass (Djajakirana et al., 1996; de Vries and Bardgett, 2012), which is also reflected in low activity of saprotrophic fungi in the rhizosphere of crop plant seedlings (Hünninghaus et al., 2019). Low fungal biomass in arable soils can be attributed to a combination of factors such as low input of organic resources (van der Wal et al., 2006a; Clocchiatti et al., 2020), use of chemical fungicides (Duah-Yentumi and Johnson, 1986; Shao and Zhang, 2017) and intensive tillage. Hence, strategies that promote high saprotrophic fungal biomass and activity in the rhizosphere of crops could be important to enhance the sustainability of agricultural cultivation.

Saprotrophic fungal abundance in the rhizosphere largely depends on the availability of appropriate energy sources. The amendment of arable soils with organic substrates, such as manure, straw, cover crop remainders or sawdust, can be used to increasie fungal biomass in the bulk soil (Lucas et al., 2014; Arcand et al., 2016; Clocchiatti et al., 2020) and also may increase fungal abundance and activity in the rhizosphere (Hannula et al., 2012). During the growth of crop plants, rhizodeposits are the main organic input into soil in conventional monocultures. However, high fungal biomass is observed in the crop rhizosphere only at late phenological stages, such as flowering and senescence, and in perennial crops (Hannula et al., 2010; Tavi et al., 2013; Pausch et al., 2016). This can be attributed to the deposition of a larger proportion of cellulose-rich root debris released by older roots (Dennis et al., 2010; Pausch and Kuzyakov, 2018). Moreover, older plants tend to exude a higher proportion of soluble secondary metabolites, including phenolic acids, as compared to younger plants (Gransee and Wittenmayer, 2000; Chaparro et al., 2013; Iannucci et al., 2013; Zhalnina et al., 2018). Hence, in order to increase the contribution of saprotrophic fungi in the rhizosphere of crop seedlings, an approach involving manipulation of the quantity and quality of

rhizodeposits may prove useful. For example, the release of fungus-stimulating root exudates could be used as selection criterion for breeding crop varieties..

Phenolic acids exert a strong selection on rhizosphere microbial communities. As phenolic acids are toxic to microbial cells at relatively low concentrations, they act as a deterrent for sensitive microbial groups, whilst they favor those groups that possess metabolic pathways for phenolic degradation (Fierer et al., 2005; Pumphrey and Madsen, 2008). The ability to degrade phenolic acids is very common among saprotrophic fungi, as it is essential to consume or tolerate free aromatic compounds released during lignocellulose depolymerization (Cain et al., 1968; Sampedro et al., 2004; Mäkelä et al., 2015). When soils receive inputs rich in simple phenolics, saprotrophic fungi are among the main microbial utilizers of such compounds (Waldrop and Firestone, 2004; Brant et al., 2006) and this can lead to a large increase in fungal abundance (Zhou et al., 2012; Wang et al., 2016; Suseela et al., 2016). This suggests that phenolic acids in root exudates could promote the colonization by saprotrophic fungi also in the rhizosphere of crop plants. However, as certain groups of bacteria can also utilize phenolic acids (Blum and Shafer, 1988), the relative stimulation of fungi by phenolic root exudates may depend on soil edaphic factors.

The objective of this study was to investigate the effect of phenolic acids on growth and composition of saprotrophic fungi in simulated and real rhizospheres in an arable soil. The first part of this study was conducted with a microcosm system that simulates root exudation via diffusion of artificial metabolite solutions. This rhizosphere simulation system was used to investigate 1) if diffusion of distinct phenolic acid compounds, alone or in combination with primary root exudate metabolites, increase fungal biomass and 2) if they modulate the community composition of fungi and bacteria. In the second part of this study, fungal development in the rhizosphere of *Arabidopsis thaliana* was compared between wild types and mutant lines *pdr2* and *sid2*, altered in the proportion of exuded phenolic acids and biosynthesis of salicylic acid, respectively. The effect of altered exudation of phenolic compounds on the rhizosphere fungal biomass was tested for two plant developmental stages. We hypothesized that the presence of phenolic acids in root exudates promotes the growth of saprotrophic fungi and increase their competitive ability to utilize other energy sources (i.e. primary metabolites) in the rhizosphere.

2. Materials and methods

The study comprised of two experiments. The first experiment (Exp. 1) was carried out in two-compartment microcosms, which were used to simulate diffusion of root exudates into arable soil (Fig. 1). Using this set-up, we investigated the effect of eight phenolic acids on abundance and community composition of fungi and bacteria. In the second experiment (Exp. 2), *A. thaliana* mutant lines with altered phenolics

exudation patterns and wild-type *A. thaliana* lines were grown in an arable soil. Fungal biomass was measured in the rhizosphere of these plants at two developmental stages.

2.1 Characteristics of soil and sand

Two batches of soil were sampled in October 2016 and July 2019 and were used for Exp. 1 and 2, respectively. The soil was sampled at the experimental farm of Wageningen University & Research located in Vredepeel (N 51 32 19, E 5 51 05, the Netherlands) from the top 10 cm soil layer, within patches of bare soil in between triticale (first batch) and maize plants (second batch), in a plot that was subject to conventional agricultural management practices. The soil was sandy and had a relatively high organic matter content (6.3%). For more details on soil management and soil characteristics see Quist et al. (2016) and **Chapter 2** (Clocchiatti et al., 2020). The soil samples were sieved through a 4-mm mesh and stored at 4° C until use. Moreover, acid-washed quartz sand (granulation 0.1–0.5 mm; Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany) was used in Exp. 1. Before use, the sand was autoclaved (121° C for 20 min.) and dried under sterile conditions.

2.2 Preparation of artificial exudates solutions

The study included eight water-soluble phenolic acid compounds that are commonly found in root exudates (Tab. 1): vanillic acid, syringic acid, gallic acid, salicylic acid, chlorogenic acid, nicotinic acid, ferulic acid and cinnamic acid. A suspension containing 0.5 mg C ml^{-1} in 50 ml sterile demi-water was prepared for each compound. The pH of all suspensions was adjusted to 6 with NaOH/KCl and the suspensions were subjected to sonication for 20 minutes at 47 kHz, in order to facilitate the dissolution of the phenolic compounds. Half of each phenolic acid solution was filter-sterilized and stored at 4° C and used in Exp. 1. The remaining solution was mixed with a stock solution of primary root exudate metabolites (PM) prepared according to Griffiths et al. (1998) (Tab. 1). The concentration of PM in the working solution was 13.4 M glucose, 13.4 mM fructose, 13.4 mM sucrose, 6.7 mM succinic acid, 6.7 mM malic acid, 3.35 mM arginine, 3.35 mM serine and 3.35 mM cysteine. Solutions containing PM and a phenolic acid had a total carbon content of 5.5 mg C ml^{-1}. These PM + P solutions were filter-sterilized and stored at 4° C until use in Exp 1.

2.3 Setup of two-compartment rhizosphere simulation microcosms

Before the start of Exp. 1, the arable soil was adjusted to 75% water holding capacity (WHC) by adding sterile demi-water and acclimatized at 21°C in a dark climate chamber for two days. Six-well plates (6-well CELLSTAR plates and ThinCert cell culture inserts, Greiner Bio-One B.V., the Netherlands) were used for setting up microcosms. Each microcosm was set up in a well containing an insert, so that it had a lower compartment and an upper compartment, separated by a permeable

Table 1. Phenolic acids and primary metabolites used in Exp. 1. For each compound are displayed: the concentration used for preparing the artificial exudates (AE) solution, the amount of added carbon per gram of sand, references to literature reporting the presence of each compound in root exudates and the product used in this study.

Compound	Concentration in exudates solution (mg ml^{-1})	Concentration in sand (mg C g^{-1})	References	Source
Phenolic acids	-	0.1		
vanillic acid	0.875	0.1	Shukla et al., 2011; Iannucci et al., 2013; Ray et al., 2018; Zhalnina et al., 2018	Sigma-Aldrich, H36001
syringic acid	0.917	0.1	Shukla et al., 2011; Iannucci et al., 2013; Zhalnina et al., 2018	Sigma-Aldrich, 86230
gallic acid	0.642	0.1	Shukla et al., 2011; Ray et al., 2018	Sigma-Aldrich 48630
salicylic acid	0.821	0.1	Shalaby et al., 2015; Badri et al., 2013; Gao et al., 2018; Zhalnina et al., 2018	Sigma-Aldrich S-7401
chlorogenic acid	0.922	0.1	Shukla et al., 2011; Shalaby et al., 2015	Merck 1.59619.0001
nicotinic acid	0.854	0.1	Shukla et al., 2011; Zhalnina et al., 2018	Sigma-Aldrich 72309
ferulic acid	0.808	0.1	Iannucci et al., 2013; Badri et al., 2013; Shalaby et al., 2015; Gao et al., 2018; Ray et al., 2018	Sigma-Aldrich 128708
cinnamic acid	0.685	0.1	Shukla et al., 2011; Shalaby et al., 2015; Gao et al., 2018	Sigma-Aldrich C80857
Primary metabolites		1	Griffiths et al., 1998; Shukla et al., 2011	
glucose	2.42	0.19		Roth 6639
fructose	2.66	0.19		Sigma-Aldrich F-0127
sucrose	4.60	0.39		Sigma-Aldrich S-9378
succinic acid	0.79	0.06		Sigma-Aldrich S3674
malic acid	0.90	0.06		Sigma-Aldrich M-9138
arginine	0.58	0.05		Sigma-Aldrich A-8094
serine	0.35	0.02		Sigma-Aldrich S-5511
cysteine	0.41	0.02		Sigma-Aldrich C7352

polyethylene terephthalate membrane (Fig. 1). The membrane had 0.4 µm pores, which enabled the diffusion of dissolved molecules between the two compartments, but hampered the migration of soil microbes. In all microcosms, the lower compartment was filled with soil equivalent to 5 g dry weight. The insert was then placed on top of the soil layer, taking care that the filter membrane adhered to the soil. The upper compartment was filled with 5 g of pure sterilized sand mixed with a solution of either phenolic acids only, PM, PM + P. The sand had a moisture of 75% WHC, which was obtained by adding the described metabolites solutions (0.2 ml g^{-1} sand) and demi-water (7.4 µl g^{-1} sand). All the materials in the upper compartment were sterile, thus the soil was the only source of microbes in the microcosm. The metabolites added in the sand compartment diffuse into the soil through the filter, simulating root exudation.

The sand in each unit received either a single phenolic acid, equivalent to 0.1 mg C g^{-1} sand, or a phenolic acid combined with PM, containing in total 1.1 mg C g^{-1} sand. In the latter mixture, the proportion of a phenolic acid was ca. 10% of the total C, which resembles the proportions observed in root exudates (Narasimhan et al., 2003). The experiment had two separate controls, namely sand receiving sterile water only and sand receiving PM only. In total, 18 types of solutions were used (eight phenolic acids × with/without PM + 2 controls). Each treatment was applied in four replicate microcosms, making up a total of 72 microcosms across 12 six-well plates. The solutions were distributed over the plates according to a complete randomized block design.

2.4 Incubation and sampling of the two-compartment rhizosphere simulation microcosms

Each plate was sealed with surgical tape (Micropore, 3M, Minnesota, United States), which allowed gas exchange, while limiting water evaporation. The two-compartment microcosms were incubated for two weeks in a dark climate chamber at 21° C. After two weeks, the sand and soil were sampled separately from all microcosms. For sand, 0.5 g samples were obtained from a 2 mm layer close to the membrane filter. These samples were used for ergosterol-based fungal biomass determination, while the rest of the sand present in the inserts was used to measure the moisture content. Similarly, 1 g samples of soil were taken from the 2 mm layer right below the filter. Half of this sample was used to determine fungal biomass with ergosterol and half was stored at -20° C to be used for DNA extraction.. Samples for ergosterol extraction were stored in methanol KOH 4% at -20° C. Soil moisture was determined by using the rest of the soil in the lower compartment. Moisture content of sand and soil were measured as weight loss following oven-drying at 105° C overnight.

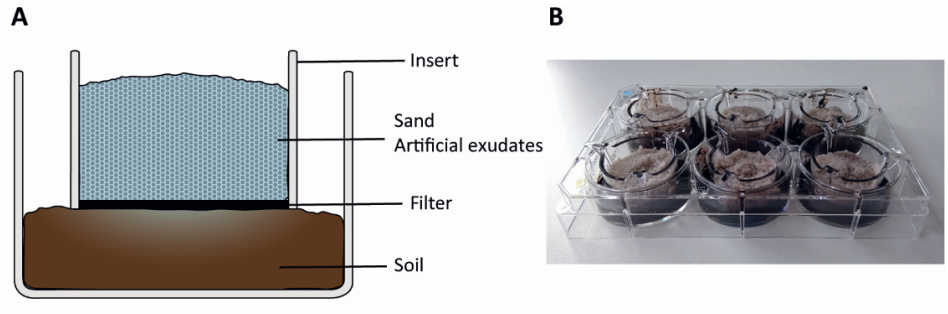

Figure 1. Setup of rhizosphere simulation microcosms in a two-compartment well with insert. A) In a microcosm, the lower compartment contained soil, the upper compartment (insert) was filled with sterile sand mixed with artificial exudates. A permeable filter separates the compartments, which allows the diffusion of artificial exudates from the sand in the soil, simulating root exudation. B) Setup of six microcosms in a six-well plate with inserts.

2.5 Fungal biomass

Ergosterol was extracted from soil and sand samples as described by de Ridder-Duine et al. (2006). Briefly, the extraction method is based on sonication, followed by heat treatment and a subsequent alkaline hydrolysis of esterified ergosterol, aided by mechanical shaking. The ergosterol concentration was quantified in the final methanol-based extract by LC-MS-MS (UHPLC 1290 Infinity II, Agilent Technologies and 6460 Triple Quad LC-MS, Santa Clara, California, United States).

2.6 Fungal and bacterial community structure and bacterial abundance

DNA was extracted from 0.25 g soil using DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was extracted for a selection of soil samples in Exp. 1, namely from microcosms of vanillic acid -, salicylic acid -, chlorogenic acid - and water treatments, both with and without PM. This selection covered phenolic acids with and without fungal stimulation effects in Exp. 1. Vanillic acid was included in the selection to allow comparisons with the numerous studies that include this compound as a representative of phenolic acids (Waldrop and Firestone, 2004; di Lonardo et al., 2017; Chen et al., 2018). The DNA was subjected to bacterial and fungal amplicon sequencing using Illumina MiSeq PE250, which was performed by McGill University and Génome Québec Innovation Centre, Montréal, Canada, with primers Eub515f/806r (Caporaso et al., 2011) and ITS4r/9f (Ihrmark et al., 2012), for bacteria and fungi, respectively. Sequencing data is accessible at the European Nucleotide Archive (primary accession PRJEB38475, secondary accession ERP121911).

The 16S rDNA gene copy number was determined by qPCR for the control soil samples and those treated with vanillic - and salicylic acid, both with and without PM. The qPCR analysis was performed on a Rotor-Gene Q Real-time PCR cycler (Qiagen). The mix comprised 0.5 µl of each primer Eub 338/518 10 µM, 7.5 µl iTaq Universal SYBR green supermix (Bio-Rad Laboratories, California, United States), 2.5 µl Nuclease-Free water (Sigma-Aldrich, Missouri, United States) and 4 µl DNA 2.5 ng μl^{-1}. The qPCR cycling program was 3 minutes at 95° C followed by 40 cycles of 30 s at 95° C, 30 s at 53° C and 30 s at 72° C. Two standard curves were established for each run (0.23 ng μl^{-1}, 0.023 ng μl^{-1}, 0.0023 ng μl^{-1}, 0.00023 ng μl^{-1}, 0.000023 ng μl^{-1}, 0.0000023 ng μl^{-1}) with a M13 plasmid containing the 16S region obtained from a *Collimonas* pure culture.

2.7 A. thaliana varieties

Four *A. thaliana* lines were used in Exp. 2, of which two were wild-type accessions (Col-0 and Col-8) and represented the controls, whereas two mutant lines (*sid2* and *pdr2*) were used to test the response of fungal biomass to altered exudate composition. *Sid2* (salicylic acid induction deficient 2) is a mutant impaired in salicylic acid production. As a consequence of a defect in the isochorismate synthase 1 (ICS1) gene, it fails to accumulate salicylic acid in its tissues (Wildermuth et al., 2001). *Pdr2* (pleiotropic drud resistance 2) is a knockdown mutant for the ATP-binding cassette transporter G30 (ABCG30), which is involved in root exudation. Badri et al. (2009) showed that this results in an altered exudation pattern with higher proportion of phenolic acids, among other secondary metabolites, and a lower proportion of sugars. Seeds of the line Col-0 were obtained from seeds available in house, whereas seeds of Col-8, *sid2* and *pdr2* were obtained from Nottingham *Arabidopsis* Stock Centre (stock IDs N60000, N16438, N66055). Seeds were propagated by growing each line in potting soil, under protective plastic covers, to prevent cross-pollination. Seeds were collected and stored at 15° C until use. Before the start of Exp. 2, seeds of each line were surface-sterilized by soaking in ethanol 70% for three minutes, followed by rinsing three times with sterile-demi water. After that, seeds were laid on wet filters in petri dishes and vernalized by incubation at 4° C for four nights.

2.8 Setup of the bioassay

Fifty pots (polyethylene, Ø 14.5 cm × 11.5 cm) were prepared for Exp. 2 as follows. Vredepeel soil was brought to 60% WHC and mixed with 0.6 g kg^{-1} of a commercial NPK fertilizer (Tuinmest 12-10-18, Pokon Naturado, the Netherlands), corresponding to the addition of 72 mg N kg^{-1}, 60 mg P kg^{-1}, 84 mg K kg^{-1}. Each pot was filled with 1.4 kg soil and sown with 80 *A. thaliana* seeds of the same line. These were distributed over 20 spots (interspace 1 cm) on the soil surface. Each spot contained four seeds. The pots were incubated in a random arrangement (CRBD) in a climate chamber at 21° C, with photoperiod 12:12. Ten days after the start of the growth period, all but one seedling per spot were removed. Hence, a maximum of 20 seedlings were grown in

each pot. The experiment comprised of ten replicate pots for each *A. thaliana* line and ten un-planted control pots. Of these, five replicates for each line were harvested 33 days after the start of the experiment, few days before the bolting stage. The other five replicates were harvested after 50 days of incubation, during the flowering stage.

2.9 Harvesting of plants and sampling

At both developmental stages, plant and soil samples were obtained as follows. Excess soil was removed from the roots by shaking and the soil adhering to the roots was collected by brushing. The rhizosphere soil of all plants in a pot was pooled to form one composite sample. Of this, 1 g was stored in methanol KOH 4% at -20° C until ergosterol extraction. In addition to this, 1 g of bulk soil was sampled from each pot and stored in the same way for ergosterol extraction. Ergosterol was extracted and quantified as described for Exp. 1. Roots and shoots were kept separate from each other but pooled per pot, frozen, freeze-dried and used as a measure of total aboveground and belowground dry biomass produced in each pot.

2.10 Statistical and bioinformatic analysis

The statistical analyses were carried out in R version 3.4.0. For Exp. 1, three-way ANOVA was performed to compare the ergosterol levels across microcosms that received different types of metabolites. The model comprised the type of phenolic acid, presence/absence of PM and compartment (soil or sand) as factors. The model included block as a factor as well. The assumptions of equality of variances and normality were verified for the model. The ergosterol concentration was compared between each metabolite type and the control by applying planned contrasts to the three-way ANOVA model (R package lsmeans), combined with Dunn–Šidák correction of p-values for multiple comparisons (Šidák, 1967). The same method was used to perform a two-way ANOVA, in order to compare 16S copy number as measured in the soil in microcosms receiving phenolic acids with and without PM.

Two-way ANOVA models were carried out for Exp. 2, after checking the assumptions of homoscedasticity and normality. ANOVA models were used for comparing four *A. thaliana* lines at two developmental stages, with block as a random factor, for the following variables: ergosterol concentration in the rhizosphere, ratio of ergosterol in the rhizosphere and bulk soil, aboveground and belowground plant biomass in a pot.

The raw sequencing data contained 2 767 341 sequences for fungi and 374 216 sequences for bacteria. The ITS2 region was extracted with ITSxpress from fungal sequences (Rivers et al., 2018) before analyzing the data with R package DADA2 , whereas 16S data was processed directly using the DADA2 pipeline without an extra filtering step. Sequences were quality filtered (maxEE = 2, truncQ = 2, only for bacteria: truncLen = 240), paired-end reads were merged, chimeric sequences were removed, sequencing errors modelled and finally sequence variants (SVs) were identified by the

DADA2 algorithm (Callahan et al., 2016). Taxonomy was assigned using the RDP classifier based on the UNITE v2019 database (Abarenkov et al., 2010) for fungi and SILVA v132 for bacteria. After removing non-fungal and non-bacterial sequences from each dataset, the fungal dataset resulted in 1 046 SVs, whereas the bacterial dataset counted 5 992 SVs. Fungal guilds were assigned to each fungal SV when possible, using the FUNGuild database v1.1 (Nguyen et al., 2016). In addition to guild, information about the growth mode and life history traits were retained in the analysis, in order to distinguish types of saprotrophic fungi (e.g. yeasts, filamentous microfungi and soft rot fungi). Permutational multivariate analysis of variance (PERMANOVA) was used to determine the effect of phenolic acids with and without a background of PM, after checking the homogeneity of multivariate variance (vegan, PERMDISP, Anderson and Walsh, 2013). Differences in relative abundance of fungal taxa between soils treated with different metabolites were analyzed at phylum, class and genus level and for fungal functional guilds. For both fungal and bacterial communities Chao1 and Shannon indexes were calculated. Differences in diversity, as well as in relative abundance were analysed with two-way ANOVA models, after verification of normality and homoscedasticity of each model. Planned contrasts between each metabolite and the control were performed for ANOVA models to extract the simple effect of a phenolic acid with or without PM. P-values were corrected with the Dunn–Šidák method. Differences in bacterial community composition between soil treated with salicylic acid + PM as compared to PM alone were highlighted by differential abundance analysis (R package DESeq2, wald test, $p < 0.01$).

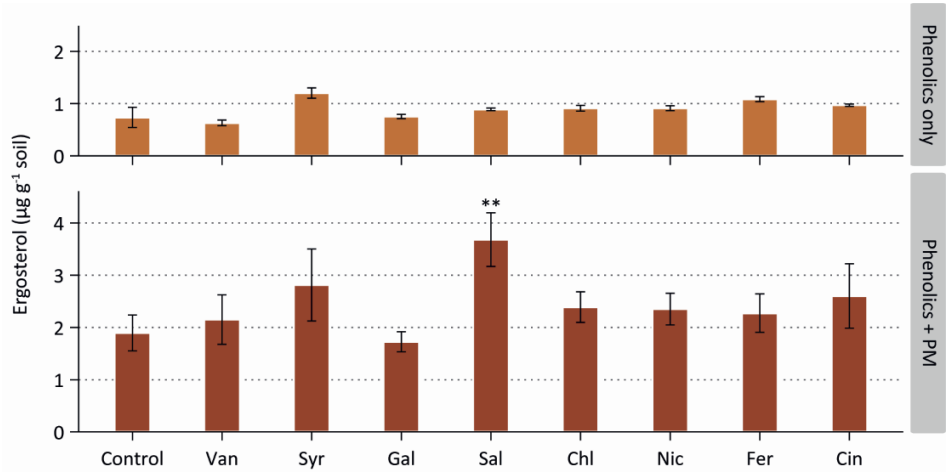

Figure 2. Effect of phenolic acids on fungal biomass, as measured in the soil compartments of rhizosphere simulation microcosms. Phenolic acids were vanillic acid (Van), syringic acid (Syr), gallic acid (Gal), salicylic acid (Sal), chlorogenic acid (Chl), nicotinic acid (Nic), ferulic acid (Fer) and cinnamic acid (Cin). Phenolic acids were added alone or with a background of soluble primary metabolites (PM). Significant differences to the control (water and PM only, respectively) are shown for each experiment and compartment (• $0.1 > p > 0.05$; * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; *** $p < 0.001$).

3. Results

3.1 Fungal biomass

The ergosterol concentration in the arable soil layer of the microcosms (Exp. 1) increased in response to the presence of primary root exudate metabolites (PM) in the upper sand layer (ANOVA, df = 1, F = 186.6, p < 0.001, Tab. S1A). Presence of phenolic acids in the upper sand layer had a smaller yet significant effect on ergosterol concentration (ANOVA, df = 8, F = 3.2, p < 0.01). However, the effect of phenolic acids was dependent on the presence of PM (seen as interaction PM x phenolic acid, Table S1A). Especially, adding salicylic acid together with PM resulted in an extra increase of ergosterol compared to the PM-only control (p < 0.01) while salicylic acid alone did not increase ergosterol concentration in the soil as compared to the water only control (Fig. 2). When compared directly with the controls, none of the other phenolic acids with or without PM had a significant effect on the ergosterol concentration in the soil layer.

Despite the small pore size (0.4 µm) of the insert membrane, fungi in the soil compartment were able to reach the sterile upper sand compartment especially in the treatments in which PM were added together with phenolic acids (Fig. S1). In the sand compartment, salicylic, nicotinic and ferulic acid added together with PM increased ergosterol concentration more than PM added alone (Fig. S1).

Bacterial numbers in the soil were not significantly affected by the addition of vanillic acid and salicylic acid alone (Fig. 3; Tab. S1). The addition of PM alone resulted in a significant increase of bacterial numbers (measured as 16S rDNA copies) in the soil, as compared to the control with addition of only water (p < 0.05; Fig. 3). Salicylic acid combined with PM had a lower bacterial abundance as compared to the addition of PM only (p < 0.05, Fig. 3, Tab. S1).

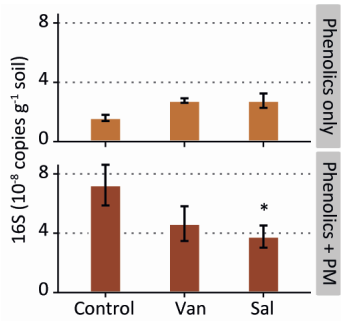

Figure 3. Effect of phenolic acids on bacterial numbers, as measured in the soil compartment of rhizosphere simulation microcosms. Phenolic acids were vanillic acid (Van) and salicylic acid (Sal), applied alone and with a background of soluble primary metabolites (PM). Significant differences to the control (water and PM only, respectively) are shown for each experiment and compartment (• 0.1 > p > 0.05; * 0.05 > p > 0.01; ** 0.01 > p > 0.001; *** p < 0.001).

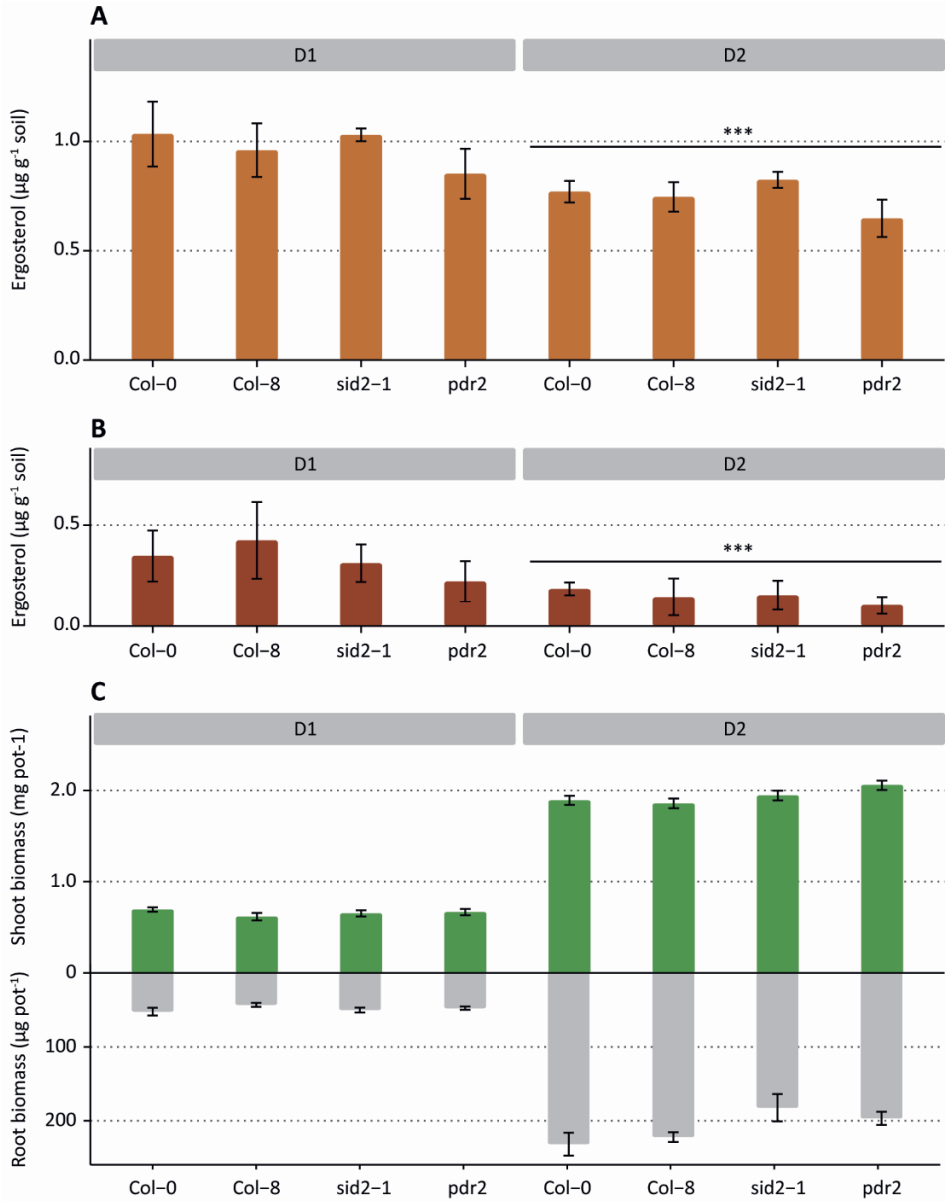

Figure 4. Fungal biomass in the rhizosphere soil of four *Arabidopsis* lines at two developmental stages (D1 and D2) measured as ergosterol concentration (A) and increase in ergosterol concentration from the level measured in the bulk soil (B). Aboveground and belowground dry plant biomass collected from each pot for four *Arabidopsis* lines at two developmental stages (C) (• 0.1 > p > 0.05; * 0.05 > p > 0.01; ** 0.01 > p > 0.001; *** p < 0.001).

In Exp 2, ergosterol concentrations were higher in the rhizosphere than the bulk soil for all *A. thaliana* accessions (Tukeys' test, $p < 0.001$ for both developmental stages; Fig. 4B). Furthermore, ergosterol content was higher in the rhizosphere of plants at the first developmental stage (D1) than at the second stage (D2) (Tukeys' test, $p < 0.001$; Fig. 4A, B). No differences were found in rhizosphere ergosterol levels among the different *A. thaliana* lines (Fig. 4; Tab. S2) and the four *A. thaliana* lines also produced comparable belowground and aboveground biomass (Fig. 4C).

3.2 Fungal and bacterial community structure

In the rhizosphere simulation microcosms, the soil fungal community was dominated by Basidiomycota, in particular Microbotryomycetes. The analysis of fungal communities in control soil and in soil receiving vanillic, salicylic and chlorogenic acid, with and without PM, revealed that the application of PM, but not of the tested phenolics, significantly affected the composition of the fungal community (PERMANOVA, df = 1, F = 0.38, $R^2 = 0.19$, $p < 0.001$; Tab. S3). In treatments with PM, increases in relative abundances were detected for Mortierellomycota (df = 1, F = 64.1, $p < 0.001$), Ascomycota (df = 1, F = 5.7, $p < 0.05$) and, among Basidiomycota, for Tremellomycetes (df = 1, F = 9.0, $p < 0.01$) (Fig. 6A). Among functional guilds, presence of PM resulted in an increase of filamentous fungi classified as saprotrophs (microfungi, pezizoid, soft rot; df = 1, F = 12.4, $p < 0.01$) and of fungi classified as endophyte-saprotroph (df = 1, F = 63.7, $p < 0.001$).

Despite the lack of overall effects of the three analyzed phenolic acids treatments on fungal community structure, specific effects were apparent. Treatments with vanillic acid + PM and salicylic acid + PM compared to PM alone had a higher proportion of Sordariomycetes ($p = 0.061$ and $p = 0.053$, respectively) (Fig. 7A). The most abundant Sordariomycetes were *Trichoderma* spp., *Fusarium* spp. and *Fusicolla* spp. (Fig. 7B-D). When combined with PM, vanillic acid and salicylic acid promoted *Trichoderma* spp. as compared to PM alone ($p < 0.05$ and $p = 0.07$, respectively). Vanillic acid + PM and salicylic acid + PM had a higher average relative abundance of *Fusarium* spp. as compared to PM only, however this effect was not significant ($p = 0.1$ and $p = 0.3$, respectively). *Fusicolla* spp. increased as compared to PM alone, with vanillic acid + PM and chlorogenic acid + PM ($p < 0.001$ and $p < 0.05$, respectively).

Bacterial community composition was affected by addition of PM and by the three analyzed phenolic acid treatments, (Fig. 5B, Tab. S3B). In particular, PM increased γ-Proteobacteria (df = 1, F = 32.0, $p < 0.001$), Bacilli (df = 1, F = 40.0, $p < 0.001$) and Clostridia (df = 1, F = 14.8, $p < 0.001$) (Fig. 6C). The phenolic acids examined altered the bacterial community composition both alone (df = 3, F = 0.04, $R^2 = 0.21$, $p < 0.01$) and with a background of PM (df = 3, F = 1.17, $R^2 = 0.23$, $p < 0.01$). In particular, salicylic acid + PM had the largest dissimilarity from the control with PM only (Fig. 5). Therefore, this treatment was analyzed in more detail. Differential abundance analysis (wald test, $p < 0.01$) showed that 210 SVs were overrepresented in the bacterial

community affected by salicylic acid + PM, as compared with PM only. Conversely, 123 SVs were overrepresented in the community affected by PM only, as compared to salicylic acid + PM (Fig. S3). Bacteria favored by salicylic acid + PM or by PM alone were distributed across all the bacterial classes, included α-, γ-, δ-Proteobacteria, Bacilli, Clostridia, Bacteroidia and Actinobacteria (Fig. S3).

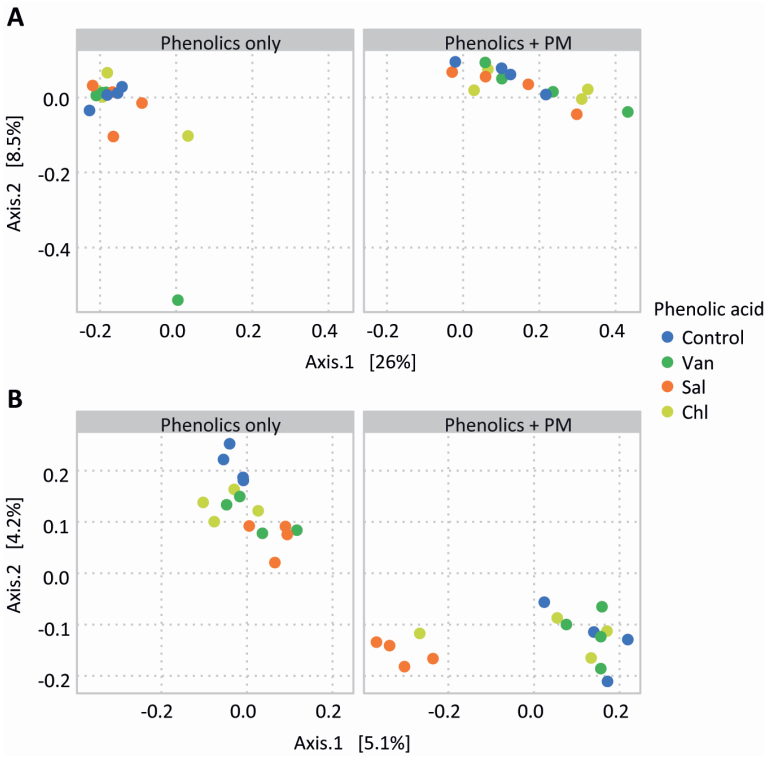

Figure 5. Effect of phenolic acids on fungal (A) and bacterial (B) communities in the arable soil layer of rhizosphere simulation microcosms. PCoA ordination plot based on Bray-Curtis distances. Four replicates are shown for the control, vanillic acid (Van), salicylic acid (Sal) and chlorogenic acid (Chl), applied alone (phenolics only) and with primary metabolites (phenolics + PM). The separate plots of samples with and without PM were obtained from the same dissimilarity matrix and ordination.

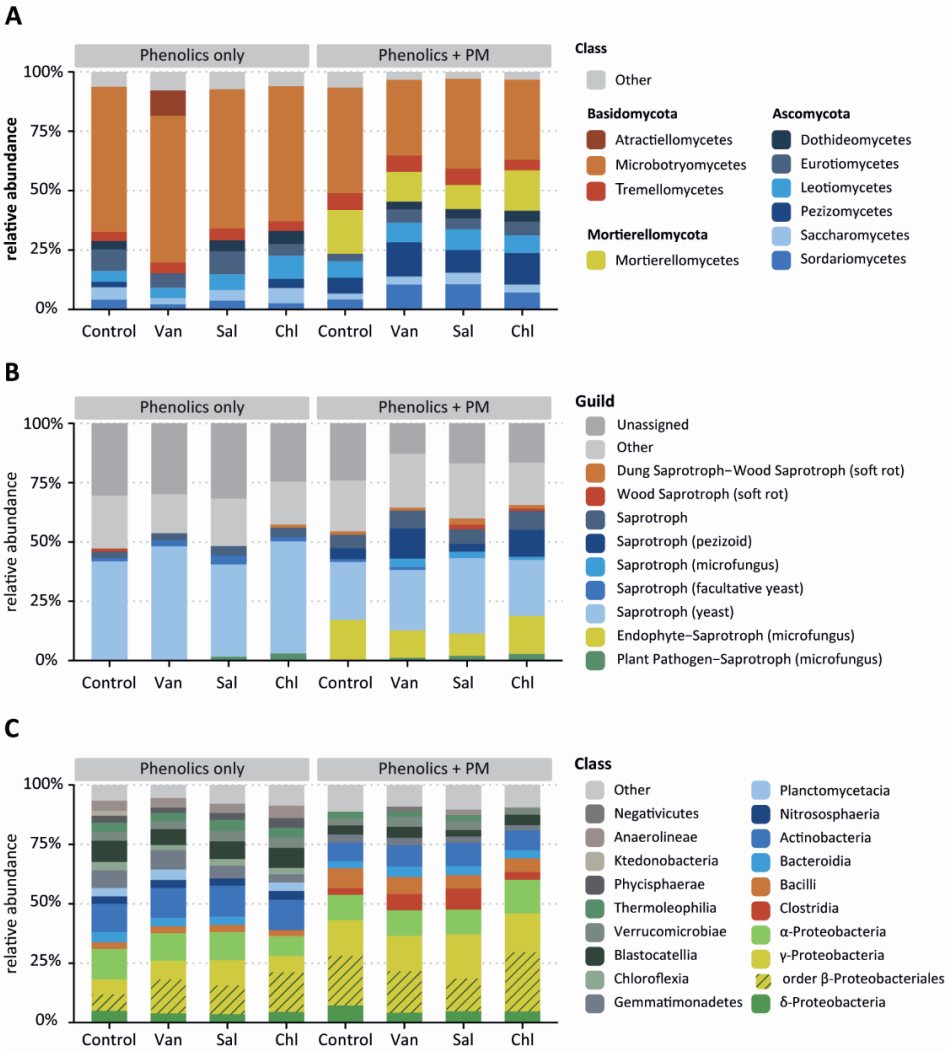

Figure 6. Effect of phenolic acids (phenolics only) and phenolic acids combined with primary metabolites (phenolics + PM) on the relative abundance of fungal classes (A), fungal functional groups (B) and bacterial classes (C), as detected in the arable soil compartment of rhizosphere simulation microcosms. Classes constituting < 2% of each community are displayed as "Other". The result is shown for the control, vanillic acid (Van), salicylic acid (Sal) and chlorogenic acid (Chl).

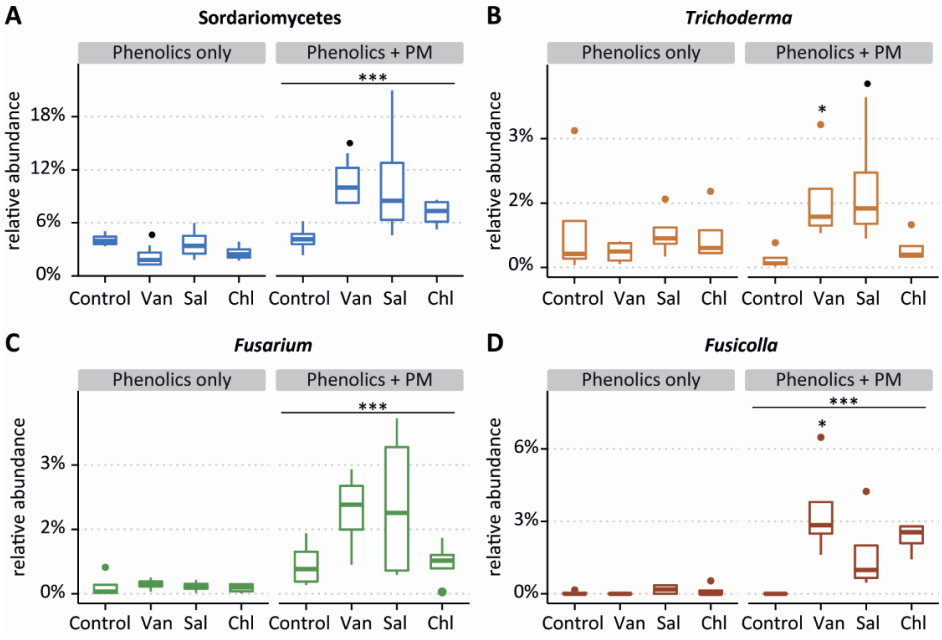

Figure 7. Relative abundance of Sordariomycetes (A) and the three most abundant sordariomycetal genera: *Trichoderma* (B), *Fusarium* (C) and *Fusicolla* (D), as found in the soil compartment of in rhizosphere simulation microcosms treated with vanillic acid (Van), salicylic acid (Sal), chlorogenic acid (Chl) or control, with and without primary metabolites (PM). The main effect of primary metabolites is shown, as well as the effect of a phenolic acid as compared to the control (• 0.1 > p > 0.05; * 0.05 > p > 0.01; ** 0.01 > p > 0.001; *** p < 0.001).

4. Discussion

4.1 Effect of phenolic acids in root exudates on abundance and community composition of fungi and bacteria

The effect of individual phenolic acids on fungal biomass was tested in rhizosphere simulation microcosms. We expected that diffusion of phenolic acids from the sterile upper sand layer into arable soil would increase saprotrophic fungal abundance. Yet, we detected no significant effect of added phenolic acids on fungal biomass in the arable soil. Diffusion of primary root exudate metabolites had a strong positive effect of fungal biomass in the arable soil layer, but combination of primary metabolites with phenolic acids did not result in an additional increase, with exception of one phenolic acid, namely salicylic acid. The analysis of fungal sequences revealed that the primary metabolites in artificial root exudates promoted the growth of Mortierellomycota, Ascomycota and basidiomycetal yeasts in the simulated rhizosphere microcosms. This is in line with what is often observed in rhizospheres and reflects the ability of many

fungi of these groups to utilize easy degradable carbon compounds (Porras-Alfaro et al., 2011; Kazerooni et al., 2017; Hugoni et al., 2018).

The absence of selective stimulation by most phenolic acids suggests that these compounds were not preferentially consumed by fungi and did also not increase their competitive ability in the simulated rhizosphere. Indeed, previous studies show that both soil fungi and bacteria can metabolize phenolic acids , although in some cases phenolic acids stimulated fungi to a larger extent than bacteria (Suseela et al., 2016; Zhou et al., 2012; Zhou and Wu, 2013). These differential responses can be ascribed to the observation that the effect of phenolic acids on fungi and bacteria appear to be concentration dependent. In particular, fungi become dominant in soils receiving high loads of simple phenolics (Blum and Shafer, 1988; Zhou and Wu, 2013). In the study of di Lonardo et al., (2017), a stimulation of fungal abundance was observed in an ex-arable soil at a high concentration of vanillic acid (0.8 mg C g^{-1}) and only in combination with supplemental nitrogen, but not at a low vanillic acid concentration (0.1 mg C g^{-1}). In monocropped soils, phenolic acids are usually found at concentrations ranging from 0.02 and 0.2 mg C g^{-1} soil (Zhou et al., 2012). In our study, phenolic acids were added at a concentration of 0.1 mg C g^{-1} soil and represented ca. 10% of the total C when mixed with primary metabolites, as based on the proportion of phenolic acids in root exudates reported by Narasimhan et al., 2003. Hence, the low, yet realistic input of phenolic acids into the arable soil may explain the lack of observable effect on fungal biomass.

Interestingly, fungi were able to invade the upper sterile sand compartment when PM were present. Probably 0.4 µm pores were enlarged as a consequence of a partial decomposition of the polyethylene membrane by soil microbes during the two weeks of incubation (Gajendiran et al., 2016). This invasion of fungi in the upper compartment could be considered as a simulation of fungi entering the root interior. It was more pronounced in combination with phenolic acids, especially with salicylic, nicotinic and ferulic acid, indicating that fungi were moving towards the source of these phenolic acids.

Fungal biomass was not stimulated in the rhizosphere of *pdr2 Arabidopsis* plants producing higher proportion of phenolic root exudates than wild type plants (Badri et al., 2009). This supports the results of the simulated rhizosphere microcosms. However, it must be noted that the mutation *pdr2* does not target specifically phenolic acids, but also impacts a number of other secondary and primary metabolites (Badri et al., 2009). In order to have better insight in the role of phenolic acids on saprotrophic fungi in the rhizosphere another possible approach would be to utilize mutants with alterations specific for phenolic acids and/or compare a broader array of plant varieties with contrasting phenolic exudation profiles (Wu et al., 2001; Zwetsloot et al., 2018; Bergelson et al., 2019). Root exudates could also be compared and/or manipulated after extraction from roots and tested in simulated rhizosphere microcosms (de Vries et al., 2019).

Although phenolic acids had little effect on the total fungal biomass, fungal community analysis for three phenolic acids revealed that they promoted specific genera, when added in combination with primary root metabolites. The analyzed phenolic acids increased the relative abundance of *Fusarium*, *Trichoderma* and *Fusicolla* spp., as compared to primary metabolites alone. Both *Fusarium* and *Trichoderma* comprise soil-borne fungi able to invade root internal tissues (Chen et al., 2018). Little information is available about *Fusicolla*, however the close relationship of this genus with *Fusarium* suggests they could share the ability to reach the plant interior (Gräfenhan et al., 2011). Catabolism of phenolic acids, in particular of salicylic acid, is required by endophytic fungi in order to cope with plant immune responses in internal plant tissues (Qi et al., 2012) which could give a competitive advantage to these fungal groups also in the rhizosphere. The ablity of *Fusarium* spp. to grow in presence of low levels of phenolic acids has been documented (Targoński et al., 1986; Chen et al., 2018). Similarly, *Trichoderma* spp. abundance in the rhizosphere was found to be promoted by vanillic acid in earlier studies (Chen et al., 2018; Zhou and Wu, 2018). Earlier research has indicated the attraction of rhizosphere- and root-inhabiting bacteria by phenolic acids (Li et al., 2012; Badri et al., 2013). Moreover, stress-induced changes in root exudation were also associated with an selective chemoattraction of *Trichoderma* (Lombardi et al., 2018). Our results indicate that phenolic root exudates could play a role in stress-induced attraction of endophytic fungi.

In this study it was not possible to determine if *Fusarium* spp. belonged to pathogenic or non-pathogenic guilds, as they were solely identified based on ITS sequences, that is not a good marker for *Fusaria*. *Trichoderma* spp. are well-known as beneficial facultative endophytes of plants (Chen et al., 2018; Kepler et al., 2017). With regard to *Fusicolla* spp., there is little information on their virulence to plants (Gräfenhan et al., 2011). This potential variety of fungal guilds promoted by phenolic acids is consistent with the evidence that phenolic acid degradation is a virulence factor found in pathogenic fungi, but that mutualistic soil-borne fungi also share this trait (Lahrmann et al., 2015). Hence, phenolic acids appear to affect both non-pathogenic and pathogenic fungal endophytes.

Besides modulating the fungal community composition, the analyzed phenolic acids also affected bacteria in the simulated rhizosphere. Bacteria belonging to all the most abundant classes in soils (i.e. α-, γ-Proteobacteria, Bacilli and Clostridia) were overrepresented in the rhizosphere receiving salicylic acid and primary metabolites, as compared to primary metabolites alone. At the same time, other members of the same classes were underrepresented in the soil receiving salicylic acid and primary metabolites. As indicated above, phenolic acids are known to exert a strong selection on rhizosphere bacterial communities (Li et al., 2012; Badri et al., 2013; Pumphrey and Madsen, 2008; Zhalnina et al., 2018). They favor those groups that possess the specialized metabolism for phenolic degradation, while acting as a deterrent for those sensitive to phenolic acids at relatively low concentrations (Blum and Shafer, 1988;

Fierer et al., 2005; Pumphrey and Madsen, 2008). Similar to this study, Lebeis et al. (2015) observed opposing effects among taxonomically closely related bacteria.

4.2 Stimulation fungal biomass and decreased bacterial numbers by salicylic acid

Salicylic acid alone did not increase fungal or bacterial abundance in the simulated rhizosphere. However, salicylic acid combined with primary metabolites increased fungal biomass and decreased bacterial numbers, hence shifting the balance between the two groups. On the other hand, *A. thaliana sid2* plants impaired in the production of salicylic acid harbored similar fungal biomass in their rhizospheres as the wild type. The fungal-stimulating effect observed in the model rhizosphere could be a consequence of a better ability of some fungi to utilize this plant hormone for growth, resulting in a competitive advantage of fungi, as compared to bacteria. Lebeis et al. (2015) showed that salicylic acid and its downstream cascade is not only an important regulator of the plant immune system, but is also a modulator of the root bacterial community composition, as it selectively promoted specific bacterial families, whilst inhibiting others. To our knowledge, saprotrophic fungi have rarely been included in studies on the effect of salicylic acid on root and rhizosphere microbiomes. Our result points at an as-yet-undefined role of salicylic acid in modulating fungal abundances in the rhizosphere. In particular, *Trichoderma* and *Fusarium* spp. are triggered by the combination of salicylic acid in a background of primary metabolites .

Yet, fungal biomass was not affected by the knockdown of salicylic acid synthesis in *sid2 A. thaliana* plants. Therefore, it is not clear whether the fungus-stimulating effect of salicylic acid observed in the rhizosphere simulation microcosm is also occurring *in planta*. Since root exudate composition was not determined, it is not possible to validate if the lack of response of fungi was indeed occurring despite different concentrations of salicylic acid. Lack of fungal biomass stimulation can be affected by the fact that a large difference in salicylic acid accumulation is seen between *sid2* and wild-type plants only after pathogen infection, whereas such a difference is less marked during constitutive salicylic acid biosynthesis (Wildermuth et al., 2001). Moreover, *sid2* plants could partially compensate for the isochorismate synthase knockdown mutation by the upregulation of alternate pathways for salicylic acid production. Considering the limitations of this approach, and apparent contrasting results in the two experiments, it will be of interest to further address whether salicylic acid operates as a modulator of size and composition of the rhizosphere fungal community.

5. Conclusions and perspectives

Our research indicates that phenolic acids, at the concentrations found in root exudates, have little effect on the biomass of saprotrophic fungi inhabiting the rhizosphere. Therefore, selecting crop varieties with a higher exudation of phenolic

acids probably does not represent an effective strategy for increasing fungal abundance in the rhizosphere of crop plants growing in fungal-poor arable soils. Nevertheless, this study shows that phenolic acids act as modulators of fungal communities by attracting soil-borne fungal endophytes. These pontentially belonged to both pathogenic and mutualistic groups. Further research should indicate if this endophyte-attraction aspect of phenolic acids in root exudates has potential for steering towards improved functioning of rhizosphere- and root microbial communities.

Acknowledgmenets

This research is supported by the Dutch Technology Foundation (STW), a branch of the Netherlands Organization for Scientific Research (NWO; grant number 14012). We thank Marc Kroonen and Harry Verstegen, who allowed us to take soil samples at the WUR Vredepeel experimental farm.

Supplementary information

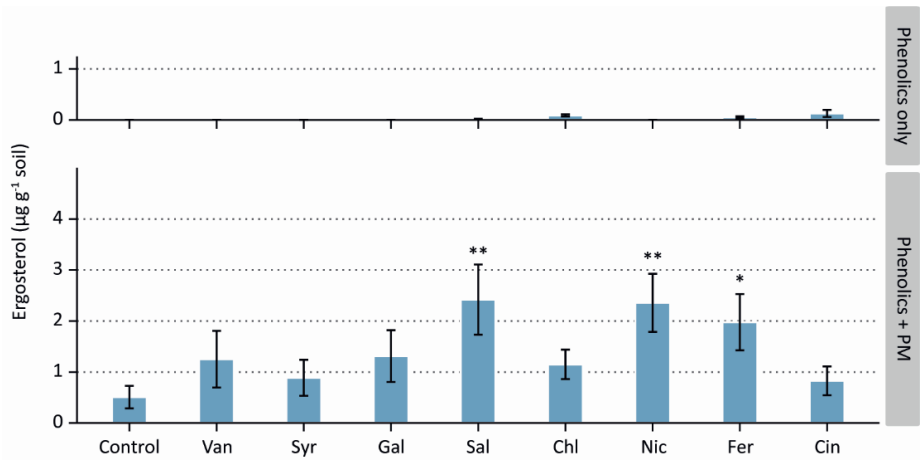

Figure S1. Effect of phenolic acids on fungal biomass, as measured in the upper sterile sand compartment of rhizosphere simulation microcosms. Phenolic acids were vanillic acid (Van), syringic acid (Syr), gallic acid (Gal), salicylic acid (Sal), chlorogenic acid (Chl), nicotinic acid (Nic), ferulic acid (Fer) and cinnamic acid (Cin). Phenolic acids were added alone or with a background of soluble primary metabolites (PM) (• 0.1 > p > 0.05; * 0.05 > p > 0.01; ** 0.01 > p > 0.001; *** p < 0.001).

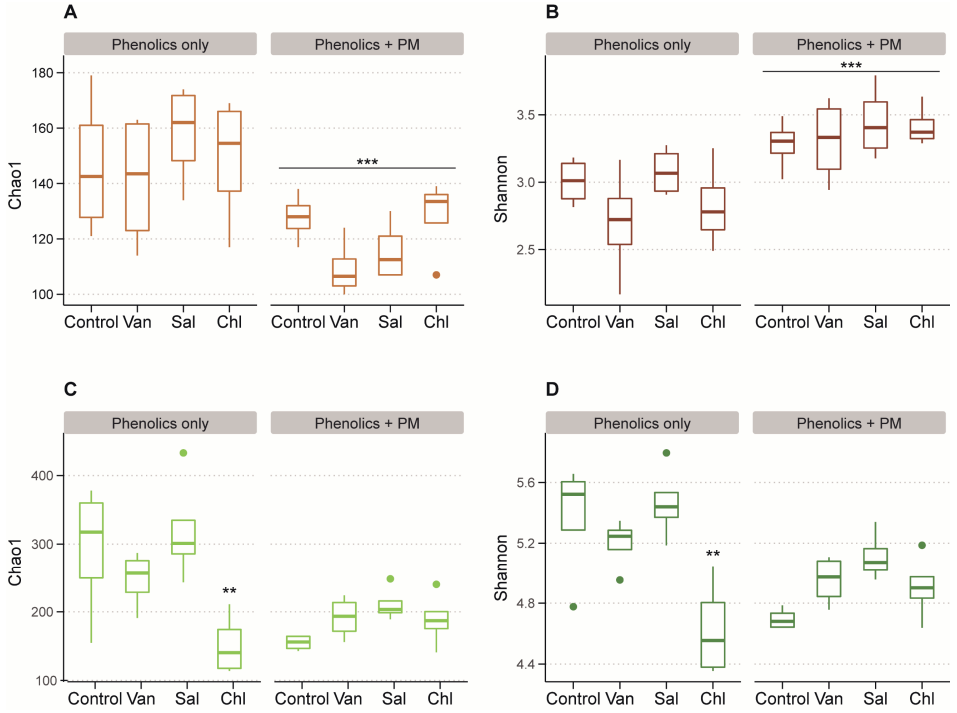

Figure S2. Effect of phenolic acids (Van = vanillic acid, Sal = salicylic acid, Chl = chlorogenic acid) alone and combined with primary metabolites (PM) on alpha diversity of the soil fungal (A, B) and bacterial community (C, D) as measured in rhizosphere simulation microcosms. Alpha diversity is shown by Chao1 (A, C) and Shannon indexes (B, D). The main effect of PM and the effect of individual phenolic acids are shown (• 0.1 > p > 0.05; * 0.05 > p > 0.01; ** 0.01 > p > 0.001; *** p < 0.001).

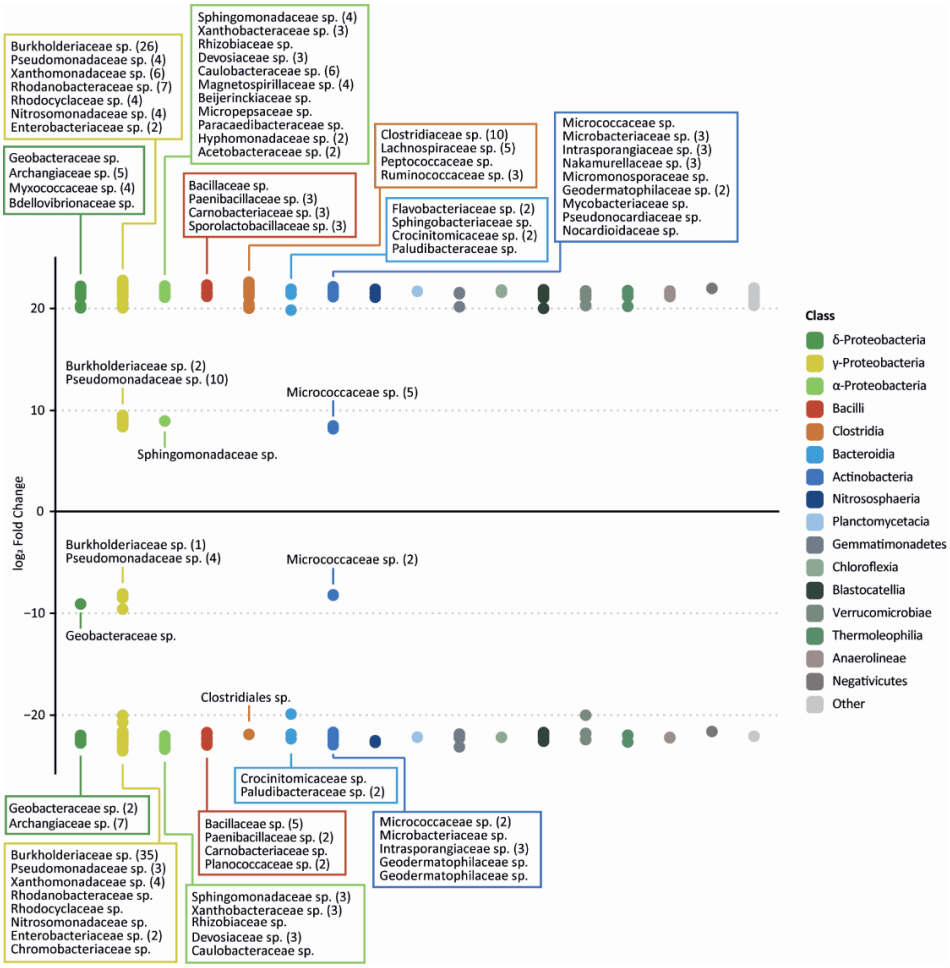

Figure S3. Effect of salicylic acid + PM as compared to PM only on bacterial taxa in the arable soil layer of rhizosphere simulation microcosms. Bacterial SVs over- (log_2 fold change > 0) and under-represented (log_2 fold change < 0) in soil treated with salicylic acid + PM were highlighted by differential abundance analysis (wald test, $p < 0{,}01$). Bacterial SVs are grouped by class and bacterial families are specified for the seven most abundant bacterial classes (δ-, γ- and α-Proteobacteria, Bacilli, Clostridia, Bacteroidia and Actinobacteria). SVs belonging to classes with < 2% relative abundance are classified as "Other".

Table S1. Summary of ANOVA results on ergosterol (A) and 16S copy number (B) as a function of the type of phenolic acid, compartment (soil/sand), and presence or absence of primary metabolites (PM) (Exp. 1). d.f..: degrees of freedon, SS: sum of squares, MS: mean sum of squares.

A	**d.f.**	**SS**	**MS**	**F**	**p**	
Phenolic acid	8	10.9	1.3	3.2	$2.9\ 10^{-3}$	**
Compartment	1	32.1	32.1	78.6	$2.2\ 10^{-11}$	***
PM	1	76.2	76.2	186.6	$2.2\ 10^{-16}$	***
block	3	3.7	1.2	3.0	0.03	*
Phenolic acid : Compartment	8	4.1	0.5	1.3	0.27	
Phenolic acid : PM	8	8.6	1.1	2.6	0.01	*
Compartment : PM	1	0.2	0.2	0.5	0.47	
Phenolic acid : Compartment : PM	8	3.5	0.4	1.1	0.39	
Residuals	105	42.9	0.4			
B	**d.f.**	**SS**	**MS**	**F**	**P**	
Phenolic acid	2	$5\ 10^{16}$	$3\ 10^{16}$	1.1	0.35	
PM	1	$5\ 10^{17}$	$5\ 10^{17}$	20.0	$4.5\ 10^{-4}$	***
Block	3	$1\ 10^{17}$	$4\ 10^{16}$	1.7	0.20	
Phenolic acid : PM	2	$2\ 10^{17}$	$1\ 10^{17}$	5.0	0.02	*
Residuals	15	$4\ 10^{17}$	$3\ 10^{17}$			

Table S2. Summary of ANOVA results on ergosterol as a function of the Arabidopsis line (A.t. line) and developmental stage (dev. stage) (Exp. 2), shown for ergosterol in the rhizosphere (A) and for the ergosterol increase in the rhizosphere from its concentration in the bulk soil (B) d.f..: degrees of freedom, SS: sum of squares, MS: mean sum of squares.

A	**d.f.**	**SS**	**MS**	**F**	**p**	
A.t. line	3	0.20	0.07	1.77	0.18	
dev. stage	1	0.63	0.63	16.48	$3.6\ 10^{-4}$	***
block	4	0.42	0.10	2.70	0.0501	.
A.t. line:dev. stage	3	0.03	0.01	0.23	0.87	
Residuals	28	1.08	0.04			
B	**d.f.**	**SS**	**MS**	**F**	**p**	
A.t. line	3	0.19	0.06	1.32	0.29	
dev. stage	1	0.36	0.36	7.60	0.01	*
block	4	0.48	0.12	2.55	0.06	.
A.t. line:dev. stage	3	0.09	0.03	0.65	0.59	
Residuals	28	1.32	0.05			

Table S3. Summary of permutational analysis of multivariate variance (PERMANOVA) applied to fungal (A) and bacterial (B) community data of the arable soil layer in rhizosphere simulation microcosms using Bray-Curtis distances. The PERMANOVA was applied for the total dataset for partitioning the variance according to two factors (type of phenolic acid x presence of additional primary metabolites, PM). The effect of type of phenolic acid was also tested alone on two subsets of data (Phenolic acid with/without PM), as these had a better homogeneity of multivariate variance. SS: sum of squares MS: mean sum of squares, p values based on 999 permutations.

A	**d.f.**	**SS**	**MS**	**F**	**R2**	**p**	
Phenolic acid x PM							
Phenolic acid	3	0.37	0.13	1.09	0.09	0.28	
PM	1	0.85	0.85	7.38	0.19	0.001	***
block	3	0.35	0.12	1.00	0.08	0.426	
Phenolic acid:PM	3	0.37	0.12	1.01	0.09	0.31	
Residuals	21	2.41	0.11		0.55		
Total	31	4.35			1.00		
Phenolic acid (without PM)							
Phenolic acid	3	0.36	0.12	1.13	0.22	0.19	
block	3	0.30	0.10	0.94	0.19	0.6	
Residuals	9	0.94	0.11		0.59		
Total	15	1.60			1.00		
Phenolic acid (with PM)							
Phenolic acid	3	0.39	0.13	0.97	0.21	0.53	
block	3	0.31	0.10	0.79	0.17	0.94	
Residuals	9	1.20	0.13		0.63		
Total	15	1.90			1.00		
B	**d.f.**	**SS**	**MS**	**F**	**R2**	**p**	
Phenolic acid x PM							
Phenolic acid	3	1.54	0.51	1.10	0.10	0.001	***
PM	1	0.61	0.61	1.30	0.04	0.001	***
block	3	1.45	0.48	1.04	0.10	0.056	.
Phenolic acid:PM	3	1.56	0.52	1.12	0.11	0.001	***
Residuals	21	9.76	0.47		0.66		
Total	31	14.92			1.00		
Phenolic acid (without PM)							
Phenolic acid	3	1.51	0.50	1.05	0.21	0.002	**
block	3	1.47	0.49	1.02	0.20	0.064	.
Residuals	9	4.31	0.47		0.59		
Total	15	7.30			1.00		
Phenolic acid (with PM)							
Phenolic acid	3	1.59	0.53	1.18	0.23	0.001	***
block	3	1.37	0.46	1.02	0.20	0.33	
Residuals	9	4.05	0.45		0.58		
Total	15	7.01			1.00		

Chapter 4

Stimulated saprotrophic fungi in arable soil extend their activities and impact to rhizosphere and root microbiomes of crop seedlings

Anna Clocchiatti, S. Emilia Hannula, Maria P. J. Hundscheid, Paulien J. A. klein Gunnewiek, Wietse de Boer

In revision

Abstract

Saprotrophic fungi play an important role in ecosystem functioning and plant performance, but their abundance in intensively managed arable soils is low. Previously we showed, that saprotrophic fungal biomass in arable soils can be enhanced with amendments of cellulose-rich materials. Here we examined if saprotrophic fungi stimulated by a cellulose-rich soil amendment (sawdust) extend their activity to the rhizosphere of crop seedlings and influence the composition and activity of other rhizosphere and root inhabitants. We grew carrot seedlings in sawdust-amended arable soil and determined fungal and bacterial biomass and community structure in roots, rhizosphere and unplanted soil. Utilization of root exudates was assessed by stable isotope probing (SIP) following $^{13}CO_2$-pulse-labeling of carrot seedlings. This was combined with the analysis of lipid fatty acids (PLFA/NLFA-SIP) and with DNA-SIP.

Sawdust amendment had a strong stimulating effect on Sordariomycetes which colonized rhizosphere and roots of carrot seedlings and actively consumed root exudates. Increased exudate utilization by fungi did not coincide with lower the abundance and activity of bacteria. Yet, shifts in bacterial community structure were seen with higher abundances of α-Proteobacteria and Bacteroidia. Biomass and activity of arbuscular mycorrhizal fungi in the carrot rhizophere increased with sawdust amendments, whereas the abundance of fungal guilds containing pathogens declined. Overall, sawdust-amendment of arable soil enhanced the exudate-consuming activity of saprotrophic fungi in the rhizosphere of crop seedlings and promoted potential beneficial microbial groups in root-associated microbiomes.

1. Introduction

In intensively managed arable soils, saprotrophic fungal biomass is low as compared to soils of natural ecosystems (de Vries and Bardgett, 2012; Djajakirana et al., 1996). This is most likely caused by a lack of decomposable plant residues, due to an efficient removal of crop parts and the predominant use of mineral fertilizers (Clocchiatti et al., 2020). Other contributing factors are the destruction of hyphal networks by tillage (Beare et al., 1997; Frey et al., 1999; Jiang et al., 2011) and inhibition of fungal growth by chemical pesticides and fungicides (Duah-Yentumi and Johnson, 1986; Nettles et al., 2016; Rahman et al., 2017; Shao and Zhang, 2017).

In natural ecosystems and less intensively managed arable ecosystems, it is known that saprotrophic fungi have major positive contributions to ecosystem functioning, e.g. by reducing nitrogen-losses (de Vries et al., 2011), forming soil aggregates (Beare et al., 1997) and suppressing root-infecting pathogenic fungi (van der Wal et al., 2013). Therefore, agricultural management practices resulting in stimulation of saprotrophic fungi in arable soils can make an important contribution to improve sustainable crop production (de Vries et al., 2012; Frąc et al., 2018). Fungal biomass stimulation can be achieved by low-intensity tillage, in the long term (Wang et al., 2017; Chen et al., 2020), whereas the use of cellulose-rich organic amendments has an immediate effect (Lucas et al., 2014; Clocchiatti et al., 2020). However, strategies that act at multiple scales, such as improving soil functioning and engineering the rhizosphere microbiome, are key for maximizing their benefits on crop performance (Chaparro et al., 2012; Bender et al., 2016). In this light, the influence of fungus-stimulating organic amendments on the performance of crops could become even more pronounced, when the stimulated fungi in bulk soil can extend their growth and activities into the rhizosphere and roots of seedlings.

Conventional arable soils are bacterial-dominated and bacteria are also the major consumers of root exudates in the rhizosphere of young plants (Hünninghaus et al., 2019). Only over time, crop plants recruit and modulate bacterial activities so that they are well suited to benefit the plant (Badri et al., 2013; Chaparro et al., 2014). Similarly, saprotrophic fungal biomass increases in the rhizosphere of crops only at later plant developmental stages (Hannula et al., 2010; Pausch et al., 2016), when they become an important microbial group consuming rhizodeposits (Hannula et al., 2012). This can be ascribed to the presence of larger amounts of cellulose-rich root debris that become available during maturation of crops (Dennis et al., 2010; Eisenhauer et al., 2017; Pausch and Kuzyakov, 2018). Moreover, older roots exude a larger share of complex soluble compounds, such as aromatic acids (Gransee and Wittenmayer, 2000; Chaparro et al., 2013; Zhalnina et al., 2018). The low fungal activity in arable soil provides limited support to the initial growth and health of plants which, conversely, are highly susceptible to the negative effects of soil-borne pathogens (Lamichhane et al., 2017) and heavily rely on exogenous chemical inputs.In a recent study, we showed that incorporation of deciduous wood sawdust in bare arable soils resulted in a rapid

and prolonged increase of saprotrophic ascomycetes in bulk soil (Clocchiatti et al., 2020). The stimulation of ascomycetes was ascribed to the accessibility of cellulose polymers in fragmented wood. Therefore, wood amendment could also represent a strategy for increasing the activity of saprotrophic ascomycetes near roots of young crop plants. Increased utilization of exudates by saprotrophic fungi is expected to have impacts on other members of rhizosphere - and root microbiomes (de Boer et al., 2008; de Menezes et al., 2017). Such interactions could be important to establish competitive suppression of root-infecting pathogens (Fravel et al., 2003; de Boer et al., 2015; Kepler et al., 2017), however they could also hamper the colonization of roots by beneficial mycorrhizal fungi (de Jaeger et al., 2010).

The aim of the current study was to examine whether fungi stimulated by sawdust in bulk soil can colonize the rhizosphere and root of seedlings and participate in competitive interactions with other microbes for root exudates. In order to test this, we determined abundance and activity of fungi and bacteria in rhizosphere and roots of carrot seedlings that were grown in arable soil amended with beech wood sawdust. For the measurement of fungal and bacterial activity in the rhizosphere, we used whole plant $^{13}CO_2$ pulse-labeling and determined ^{13}C accumulation in phospholipid fatty acids (PLFA-SIP) and microbial DNA markers (DNA-SIP).

We hypothesized that 1) stimulating fungal biomass in the bulk soil causes an increase in fungal biomass in the rhizosphere, which coincides with 2) increased activity (i.e. uptake of exudates) of saprotrophic fungi in the rhizosphere of crop seedlings and results in 3) shifts in the composition of rhizosphere - and root microbiomes. We further investigated the persistence of the effects by sowing seedlings two and six weeks after sawdust amendment.

2. Materials and Methods

2.1 Experimental setup

In August 2016, soil was sampled at the experimental farm of Wageningen University and Research located at Vredepeel (Clocchiatti et al., 2020). The soil was collected (0-10 cm) from bare patches in a plot (200 m x 18 m) that was conventionally managed and partially covered with triticale. After sieving (4-mm), soil was stored at 4° C until use. The soil was sandy (92% sand, 6% silt, 2% clay), slightly acidic (pH 6.1) and had a content of 6% organic matter.

For the experiment, the soil was divided in two batches. One batch was amended with beech sawdust (< 2 mm), 5 g kg^{-1} soil, (*Fagus sylvatica*, Sänger Rollenlager GmbH & Co, Waldsolsms, Germany) and ammonium nitrate, 170 mg kg^{-1} soil. The second batch

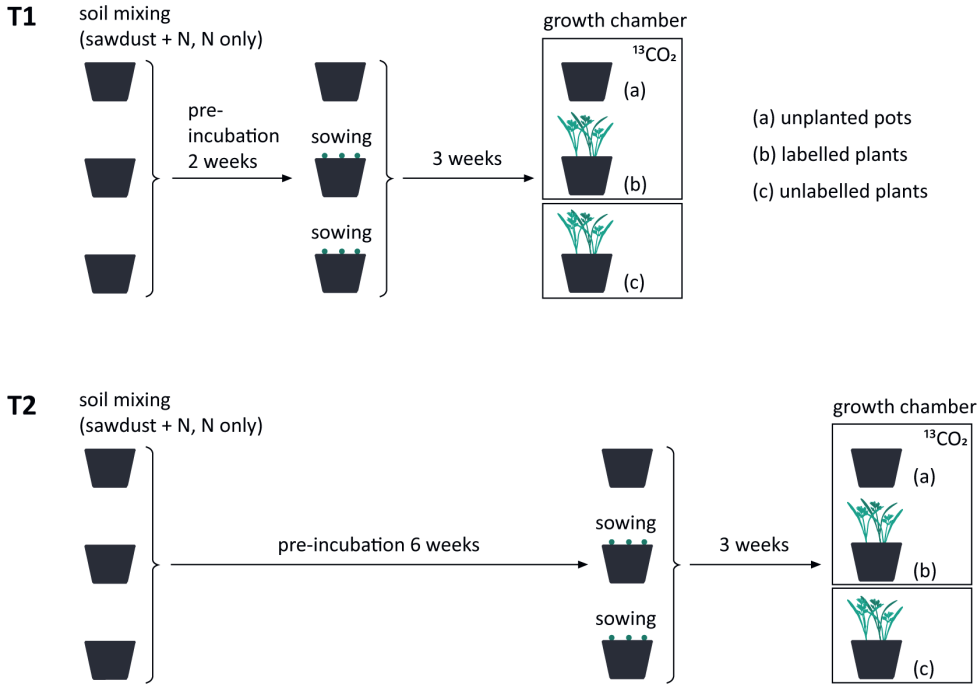

Figure 1. Schematic representation of the experimental setup. Seedlings were grown from seed for three weeks in the greenhouse before receiving, inside a growth chamber, either pulses of $^{13}CO_2$ (a, b) or atmospheric CO2 concentration (c). (T1) and (T2) only differed in the duration of the pre-incubation of sawdust-amended and control pots before sowing.

of soil was mixed with ammonium nitrate only (170 mg kg^{-1} soil), representing the control. Soil moisture was adjusted to 60% of the water holding capacity and maintained as such during the experiment. Portions of 190 g of soil were divided among pots (PET, 10 x 10 x 10 cm) and pre-incubated for two and six weeks in a greenhouse under an aluminium cover (Fig. 1). After each pre-incubation period, ten control pots and ten sawdust-amended pots were sown with carrot (40 seeds per pot, *Daucus carota* subsp. *sativus*, Bejo Zaden BV, Warmenhuizen, the Netherlands), whereas five control and five sawdust-amended pots were kept unplanted. Carrot seedlings were grown in the same greenhouse, under a combination of natural and artificial lightning (photoperiod 16:8). Two weeks after sowing, germination of carrot seedlings was scored. One week later, corresponding to the emergence of the first true leaf, $^{13}CO_2$ pulse-labelling was performed (Fig. 1).

2.2 $^{13}CO_2$ Pulse-labelling and harvesting

Planted and unplanted pots were transferred into a climate chamber and provided with pulses of 99.99 atom-% $^{13}CO_2$ (Cambridge Isotope Laboratories, Tewksbury,

Massachusetts, United States) for a total of 16 h, under artificially lit (photoperiod 16:8) and air-tight conditions. The concentration of CO_2 was monitored during the labelling period: initially CO_2 concentration was allowed to drop from ambient concentration (530 ppm) to 460 ppm, confirming photosynthetic activity of the carrot seedlings. After that, a pulse of 1.5 l of $^{13}CO_2$ was provided, restoring the initial concentration of CO_2 in the chamber. The pulse was repeated five times with 2.5 h intervals making the total amount of $^{13}CO_2$ added 12.5 liters. During the artificial night (8 h darkness), $^{13}CO_2$ was not provided and air exchange between the climate chamber and the air outside was enabled. The next day, pots were destructively harvested. As controls, the same numbers of sawdust-amended and un-amended pots were placed in an identical climate chamber, but subjected to pulses of atmospheric CO_2 (Fig. 1). We refer to them as ^{12}C controls. The labelling was performed in the same way two times, namely five weeks and nine weeks after the start of the experiment, corresponding to two and six weeks of soil pre-incubation, followed by three weeks of seedling growth. We refer to the two pre-incubtation time points as T1 and T2, respectively. Bulk soil samples were obtained from unplanted pots. A rhizosphere soil sample was obtained by pooling the rhizosphere soil from all the roots in a pot (26 ± 4 seedlings). Rhizosphere soil was obtained from a soil layer of < 1.5 mm attached to the root (Hinsinger et al., 2005; Kuzyakov and Razavi, 2019). The soil was sampled carefully, without damaging or brushing the roots, and the rhizosphere soil sample was further 1-mm sieved (Butler et al., 2003). In this way, we took care that root parts were excluded from the sample, since plants and fungi do share PLFA markers. Roots and shoots of seedlings harvested per pot were pooled in one sample. The roots were gently washed with sterile demi-water and used to extract the rhizoplane and endophytic communities together (Li et al., 2019). Root, shoot, rhizosphere and soil samples were rapidly frozen and freeze-dried. Roots and shoots were weighed and grinded to a fine powder by bead-beating (< 0.1 µm). After that, soil and plant samples were stored at -80° C until use. The determination of ^{13}C content of soil and plant parts is described in the Supporting Information.

2.3 Analysis of PLFA and NLFA, combined with stable isotope probing

PLFA and NLFA were extracted from 3 g soil using the procedure described by Frostegård et al. (1993). PLFA/NLFA concentrations were determined on a GC-FID (7890 A Agilent, Technologies, Santa Clara, California, United States). δ^{13} values were measured on a GC-c-IRMS (Trace Ultra GC equipped with a Conflo III interface and a Delta V IRMS, Thermo Scientific, Germany). Three C20:0 methyl esters (Schimmelmann, Biogeochemical Laboratories, Indiana University, United States) were used for calibrating the δ value of the in-house calibration gas. The internal standard methyl nonadecanoate fatty acid (19:0) was used for calculating concentrations. Identification of the compound was based on a BAME mix (Supelco 47080- u) and a FAME mix (Supelco 18919-1AMP). The ^{13}C enrichments for PLFA/NLFA biomarkers (excess ^{13}C pmol g^{-1} soil) were calculated as described by

(Boschker and Middelburg, 2002). Briefly, $\delta^{13}C$ values for each PLFA/NLFA were measured as ratio ($R=^{13}C/^{12}C$) by reference to the standard (Vienna Pee Dee Belemnite):

$$\delta^{13}C\ (‰) = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000$$

$\delta^{13}C$ values were used to calculate the ^{13}C fraction, $F^{13}=^{13}C/(^{13}C+^{12}C)$, for a PLFA/NLFA in a sample:

$$F^{13} = R_{sample}/\left(R_{sample} + 1\right)$$

Finally, the ^{13}C enrichment of a PLFA/NLFA in a labelled sample was obtained by adjusting the ^{13}C fraction found in a labelled sample (F_l^{13}) with the average ^{13}C fraction in unlabelled control samples (F_c^{13}). Excess ratio was converted into ^{13}C enrichment by using the total PLFA/NLFA concentration in the labelled sample ($C_{P/NLFA}$):

$$Excess\ ^{13}C\ (\text{pmol}\ ^{13}C/\text{g soil}) = (F_l^{13} - F_c^{13}) \times C_{P/NLFA}(\text{nmol C/g soil}) \times 1000$$

PLFA 18:2ω6c was used as fungal marker whereas NLFA 16:1ω5 was used as an indicator of AMF (Frostegård et al., 2011; Willers et al., 2015). The following PLFAs were used as bacterial markers: i15:0, ai15:0, i16:0, 16:1ω7c, 16:1ω6c, ai17:1ω7, i17:0, ai17:0, 17:1ω8c, cy-17:0 (Boschker and Middelburg, 2002; Mauclaire et al., 2003); Actinobacteria were indicated by 10Me-branched PLFAs (Willers et al., 2015).

2.4 DNA extraction and molecular analyses

DNA was extracted form 0.25 g freeze-dried rhizosphere, soil and root samples by DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was assessed by Qubit dsDNA HS Assay (Termo Fisher Scientific, Waltham, Massachusetts, United States). The DNA was used for quantifying ITS2 and 16S copy numbers (qPCR). ^{13}C-enriched DNA was obtained from rhizosphere samples by CsCl gradient fractionation. This, together with soil, rhizosphere and root DNA, was subjected to fungal and bacterial amplicon sequencing. For details on molecular analyses, see the Supporting Information. Sequencing data is accessible at the European Nucleotide Archive (accession ERP120799).

2.5 Statistical and bioinformatic analysis

The statistical analysis was carried out in R (version 3.6.1). After verifying the assumption of normality and equality of variances, two-way ANOVA was used for comparing microbial markers among sawdust-amended/un-amended soil and between rhizosphere and unplanted soil. Multiple comparisons were obtained by Tukey's post-hoc test at family-wise error rate of 5%. The analysis was carried out independently for each time point and performed for the following markers: the fungal PLFA 18:2ω6c (total and ^{13}C excess), the mycorrhizal NLFA 16:1ω5 (total and ^{13}C excess), total and individual bacterial PLFAs listed above (total and ^{13}C excess), ITS copy number and 16S copy number.

Sequencing data were processed in Linux and R. The ITS2 region was extracted with ITSxpress from fungal sequences (Rivers et al., 2018). After that, the R package dada2 was used for quality filtering (maxEE = 2, truncQ = 2), to join paired-end reads, to remove chimeric sequences, for modelling sequencing errors and identifying sequence variants (SVs) by the DADA2 algorithm (Callahan et al., 2016). Taxonomy was assigned by using the RDP classifier based on the UNITE v2019 database (Abarenkov et al., 2010). Bacterial sequences were processed in the same way, with exception to the quality filtering parameters (truncLen = 240, maxEE = 2, truncQ = 2) and to the database used for taxonomical assignment (SILVA v132). Overall, the fungal dataset counted 1 646 403 reads and the bacterial dataset had 4 360 774 reads. The datasets were cleaned from singletons and all SVs other than fungi and bacteria, i.e. mitochondria and chloroplasts, resulting in 4 048 fungal SVs and 16 422 bacterial SVs (R, phyloseq). Based on the taxonomy, potential functional guilds were assigned to 1 871 fungal SVs, using the FUNGuild database v1.0 (Nguyen et al., 2016), followed by manual curation of the guilds of interest in this study: saprotrophic fungi, potential plant pathogens, fungal parasites, arbuscular mycorrhizal fungi (Tab. S8).

Permutational multivariate analysis of variance (PERMANOVA) was used to determine the effect of sawdust on fungal and bacterial communities residing in the soil, rhizosphere and root and the rhizosphere communities that actively incorporated 'heavy' carbon. PERMANOVA was run independently in each compartment, after testing for homogeneity of multivariate variance (vegan, PERMDISP, Anderson and Walsh, 2013). Bray-Curtis dissimilarities between fungal communities and between bacterial communities found in two treatments (sawdust and control) and four compartments (unplanted soil, rhizosphere, ^{13}C-enriched rhizosphere and root) were visualized as a result of principal coordinate analysis (PCoA). Differences in relative abundance between sawdust and control were analyzed at phylum, class, order and family level and for fungal functional guilds. Differences between sawdust and control were analyzed for the whole dataset with three-way ANOVAs, accounting soil treatment, compartment and time point as factors. Normality and homoscedasticity were verified for each model, which also included planned contrasts for comparing sawdust vs control in each compartment, as these comparisons reflect the main

research question of this study (effect of sawdust on rhizosphere - and root microbial communities). As the variation for fungal taxa and fungal functional groups was larger in the root as compared to the other compartments, the data on the root fungal community were analyzed separately from the fungal community data of the three other compartments, by using two-way ANOVA with soil treatment and time point as factors, combined with planned contrasts for testing the effect of sawdust at each time point.

3. Results

3.1 Plant performance and ^{13}C enrichment

Germination rates and shoot biomass of carrot seedlings were not significantly different between controls and sawdust amended treatments (Tab. S1). Yet, a slight effect of pre-incubation time before sowing (T1 = 2 weeks versus T2 = 6 weeks) was apparent indicated by a small decrease in germination rates with longer pre-incubation time (Tab. S1). N content of plant parts was constant, with exception of higher N content in plants grown in T1 sawdust-amended soil.

Plant seedlings incorporated $^{13}CO_2$ from the airspace of the air-tight chamber, as indicated by a decrease in CO_2 concentration by the built-in monitoring system. This is reflected by ^{13}C incorporation in shoot and root parts of carrot seedlings, as well as in rhizosphere soil (Tab. S1). The ^{13}C values in the control rhizosphere soil and plant parts were within the normal atmospheric value range ($\delta^{13}C$ -30‰ ± 1.8). The ^{13}C enrichment of the rhizosphere soil was higher than the amount of ^{13}C in unplanted soil ($F_{1, 40} = 33.9$, $p < 0.05$), indicating that pulse-labelling resulted in ^{13}C-enriched plant-derived carbon reaching the rhizosphere (Tab. S1). The amount of ^{13}C was higher in roots than in shoots ($F_{1, 40} = 322$, $p < 0.05$), suggesting a rapid allocation of new photosynthates to the belowground parts. Pre-incubation time of soil before sowing, had an effect on the amount of ^{13}C incorporated in carrot seedlings (T1 > T2; $F_{1, 40} = 135$, $p < 0.05$).

3.2 Total and labelled biomass of saprotrophic fungi, bacteria and mycorrhizal fungi

An increase in fungal biomass (measured with fungal marker PLFA 18:2ω6,9) was found in sawdust amended treatments for both unplanted and rhizosphere soil (Fig. 2A, Tab. S2A). This was seen for both pre-sowing incubation times ($F_{1, 20} = 402.7$ for T1, $F_{1, 20} = 57.0$ for T2; for both $p < 0.001$) and the observed pattern was consistent with that of ITS2 copy numbers (Fig. S1A, $p < 0.001$ for T1, $p < 0.01$ for T2). Furthermore, the excess ^{13}C was measured in the PLFA 18:2ω6,9. This was used for quantifying the

incorporation of plant-derived carbon into the biomass of fungi. The ^{13}C signature of the fungal PLFA marker in the rhizosphere was significantly higher ($F_{1, 10} = 7.25$, $p < 0.05$, Tukey HSD $p < 0.01$, Tab. S2B) in the sawdust-amended treatment than in the control for the shortest pre-sowing incubation (T1). However, for the longer pre-sowing incubation (T2) the excess ^{13}C in the fungal PLFA was comparable for both treatments (Fig. 2B, Tab. S2B).

Based on the cumulative measure of bacterial PLFA markers, bacterial biomass was not affected by sawdust amendment for T1 (Fig. 2C, Tab. S2C, F= 4.4, p = 0.6), however for T2 the PLFA-based bacterial biomass was higher in the sawdust-amended rhizosphere as compared to the control rhizosphere (Fig. 2C, $p < 0.001$). For T1, sawdust amendment was associated with an increase in individual bacterial PLFAs. Namely, and higher abundance was seen for PLFAs i16:0, i17:0, ai17:0, 17:1ω8c, cy-17:0 in the sawdust-amended soil and rhizosphere (Tab. S3). The peak of PLFA marker 18:1ω7c/18:1ω9t, which can be present in both bacteria and fungi, was also higher in the sawdust amendmed soil as compared to the control. We did not, however, detect differences in excess ^{13}C in the rhizosphere for either individual or cumulative bacterial PLFAs between sawdust-amended and control treatments (Fig. 2D, Tab. S2D, Tab. S4).

The evaluation of the total bacterial abundance via qPCR-based quantification of 16S rRNA copy numbers, showed a more pronounced increase in bacterial abundance for T1 in both sawdust-amended soil and rhizosphere, as compared to the control. For T2, qPCR-based bacterial numbers were higher in the root tissues, but not in the bulk soil or rhizosphere, upon sawdust amendement (Fig. S1C, D).

The marker for arbuscular mycorrhizal fungi NLFA 16:1ω5 , was significantly increased in the carrot rhizosphere in the sawdust amended treatment for T1 ($F_{1, 20} = 34$, $p < 0.001$) (Fig. 2E). Moreover, a larger excess of ^{13}C ($F_{1, 10} = 10.8$, $p < 0.01$) was found in NLFA 16:1ω5 for T1, in response to sawdust amendment (Fig. 2F).

3.3 Composition of fungal and bacterial communities

Sawdust amendment strongly affected the composition of fungi present in the soil ($R^2 = 70.3\%$, $p < 0.001$, Tab. S6A), rhizosphere ($R^2 = 53.7\%$, $p < 0.001$, Tab. S6B), as well as of fungi actively incorporating plant-derived carbon ($R^2 = 17.5\%$, $p < 0.001$, Tab. S6C, Fig. 3A and 4A). While the plant had a strong selective effect on fungal community composition in the root, the effect of sawdust amendment was also seen in the root compartment ($R^2 = 15.0\%$, $p < 0.001$, Fig. 3A, Tab. S6D). The fungal community of unplanted soil without sawdust amendment was dominated by Ascomycota, followed by Basidiomycota and Mortierellomycota (relative abundance > 1.5%, Fig. 4A). Ascomycota and Basidiomycota comprised mostly saprotrophic guilds, but taxa assigned as potential plant pathogens were also detected (Fig. 4B). Sawdust amendment led to an increase in relative abundance of Sordariomycetes in the soil, rhizosphere and ^{13}C-enriched rhizosphere (Fig. 4A, $F_{1, 80} = 325.7$, $p < 0.001$). We further

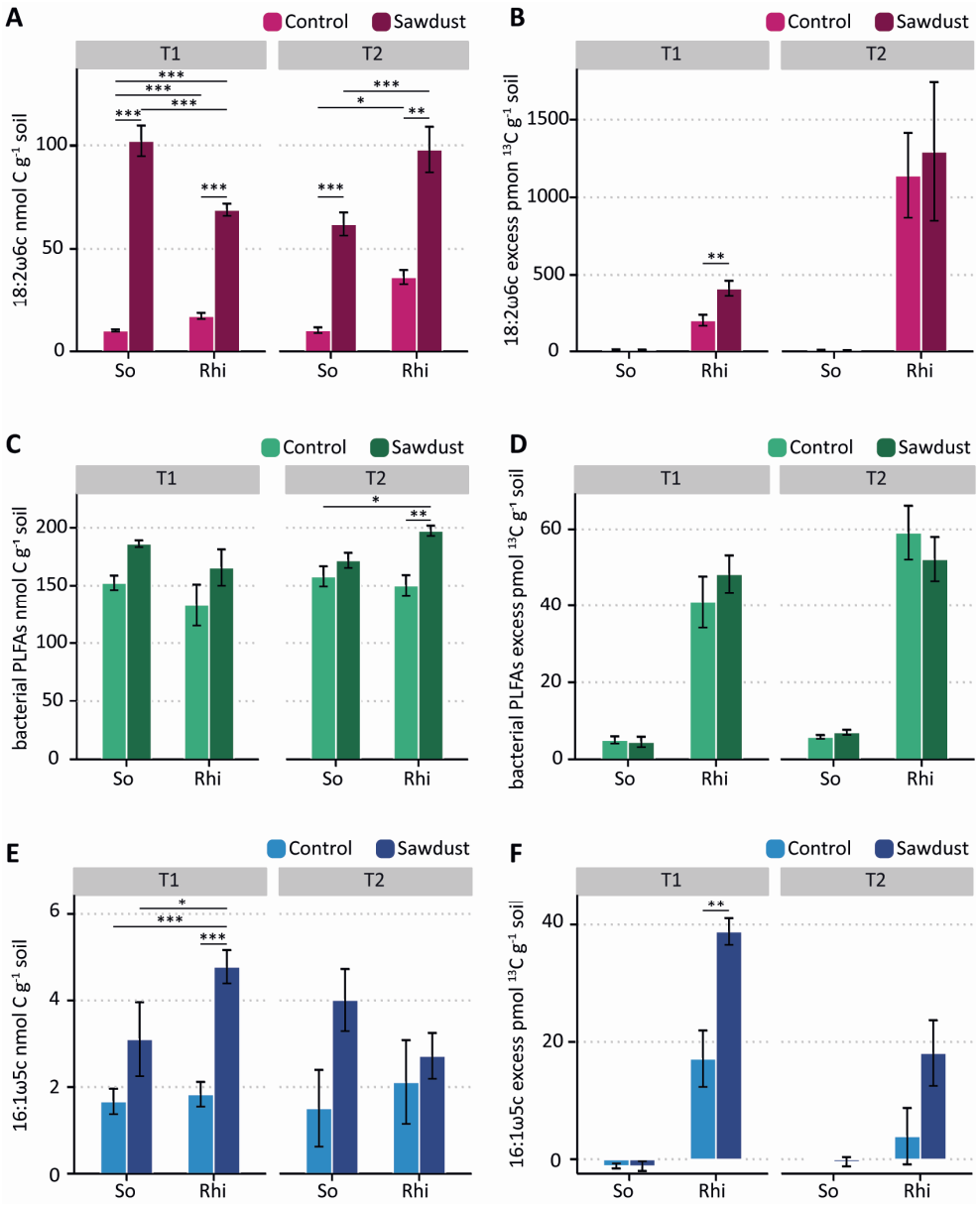

Figure 2. Effect of wood sawdust amendment on biomass and plant-C incorporation by fungi, bacteria and mycorrhizal fungi. Concentration of total C and ^{13}C excess in PLFA/NLFA markers are shown for fungi (A, B), bacteria (C, D) and arbuscular mycorrhizal fungi (E, F) in unplanted soil (So) and carrot rhizosphere soil (Rhi). T1 and T2 represent 2- and 6-weeks pre-sowing incubation times of sawdust amendments. The value for bacterial PLFAs is obtained as a sum of PLFAs i15.0, ai15.0, i16.0, 16.1w7c, 16.1w6c, ai17.1w7, i17.0, 17.1w8c, cy-17.0. ^{13}C excess expresses the net amount of labelled C incorporated in a PLFA/NLFA as a result of pulse-labelling and is obtained by subtracting the natural ^{13}C abundance found for the same PLFA/NLFA in unlabelled rhizosphere samples.

detected more Sordariomycetes (Fig. 4A, $F_{1, 40} = 13.4$, $p < 0.001$) and *Leptosphaeriaceae* (Fig. S2A, $F_{1, 40} = 20.3$, $p < 0.001$) as well as arbuscular mycorrhizal fungi (Fig. 4A, Glomeromycota, Archeosporomycetes, $F_{1, 40} = 9.0$, $p<0.01$) in the fungal community associated with roots in sawdust amended soil. Potential fungal plant pathogens were found in all compartments, but sawdust amendment decreased the relative abundance of total potential pathogens (Fig. 4D, $F_{1, 80} = 43.7$, $p < 0.001$), in favor of saprotrophic guilds (Fig. 4C, $F_{1, 80} = 137.7$, $p < 0.001$) in soil-, rhizosphere- and ^{13}C-enriched rhizosphere fungal communities. Potential plant pathogens were found in a higher proportion in roots than in soil compartments, irrespective of the sawdust treatment. Yet, sawdust reduced the relative abundance of potential pathogens in the root fungal community for T1 ($F_{1, 20} = 4.5$, $p < 0.05$), but not for T2. In the unplanted control, the most abundant potential plant pathogens were *Didymellaceae*, *Plectospherellaceae* and *Nectriaceae* (*Fusarium* spp.) (Fig. S2B). Decrease in relative abundance of *Didymellaceae* an *Plectospherellaceae* contributed to the observed decrease of the relative abundance of the total sub-community of potential pathogens in the soil ($F_{1, 20} = 24.0$, $p < 0.001$), rhizosphere ($F_{1, 40} = 14.0$, $p < 0.001$) and ^{13}C-enriched rhizosphere ($F_{1, 20} = 10.0$, $p < 0.01$) in response to sawdust amendment. On the other hand, *Nectriaceae* were not affected by sawdust in any compartment ($F_{1, 120} = 0.9$, $p = 0.76$), whereas *Olpidium* spp. increased in carrot roots grown in sawdust-amended soil for the shortest pre-sowing incubation time ($F_{1, 20} = 5.5$, $p < 0.05$).

The most abundant bacterial classes present in the soil were γ- and α-Proteobacteria, Bacilli and Actinobacteria (Fig. 5A). Overall, sawdust amendment induced a smaller shift in bacterial community composition as compared to that of fungi (Fig. 3B and 5A, Tab. S7). Yet, significant sawdust-induced shifts in the bacterial community occurred in unplanted soil ($R^2 = 7.9\%$, $p < 0.001$, Tab. S7A), rhizosphere soil ($R^2 = 3.4\%$, $p < 0.001$, Tab. S7B) and roots ($R^2 = 3.8\%$, $p < 0.01$, Tab. S7D). α-Proteobacteria increased in relative abundance in response to sawdust amendment in all compartments ($F_{1, 120} = 52.9$, $p < 0.001$) (Fig. 5B). This was also the case for Bacteroidia, although the increase was only significant for unplanted soil ($F_{1, 20} = 6.0$, $p < 0.05$) and roots ($F_{1, 40} = 13.7$, $p < 0.001$, Fig. 5C). Several members of α-Proteobacteria contributed to the effect observed at class level. *Rhizobiaceae* increased to a large extent in the root interior after sawdust amendment ($F_{1, 40} = 12.7$, $p < 0.001$), whereas in the soil the observed increase of α-Proteobacteria was not only ascribed to *Rhizobiaceae*, but also to several other members of Rhizobiales ($F_{1, 20} = 25.4$, $p < 0.001$, Fig. 5D). With regard to Bacteroidia (Fig. 5E), the relative abundance of *Chitinophagaceae* was higher after sawdust amendment than in the control ($F_{1, 120} = 5.3$, $p < 0.05$), and this was particularly observed in unplanted soil ($F_{1, 20} = 4.3$, $p < 0.05$). In addition, *Sphingobacteriaceae* contributed to the higher relative abundance of Bacteroidia ($F_{1, 120} = 9.6$, $p < 0.01$), especially in the root compartment ($F_{1, 40} = 14.1$, $p < 0.001$).

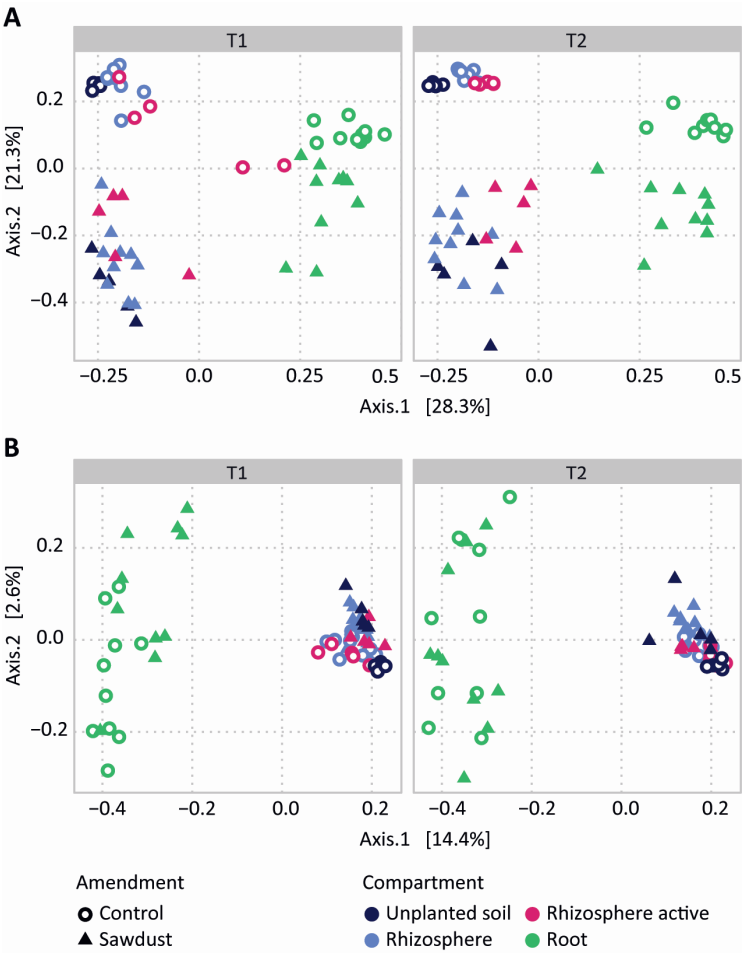

Figure 3. Effect of sawdust amendment of arable soil on the fungal and bacterial community composition in roots and rhizosphere of carrot seedlings and in bulk soil. Ordination (PCoA based on Bray-Curtis dissimilarity matrix) was performed independently for the whole fungal dataset (A) and for the whole bacterial dataset (B). For both fungi and bacteria, the dissimilarity between communities found in four compartments, with and without sawdust amendment, was displayed in separate plots for each pre-sowing incubation time (T1 = 2 weeks, T2 = 6 weeks). Communities were obtained from unplanted soil, rhizosphere, ^{13}C-enriched rhizosphere DNA and roots, after carrot seedlings had grown for three weeks.

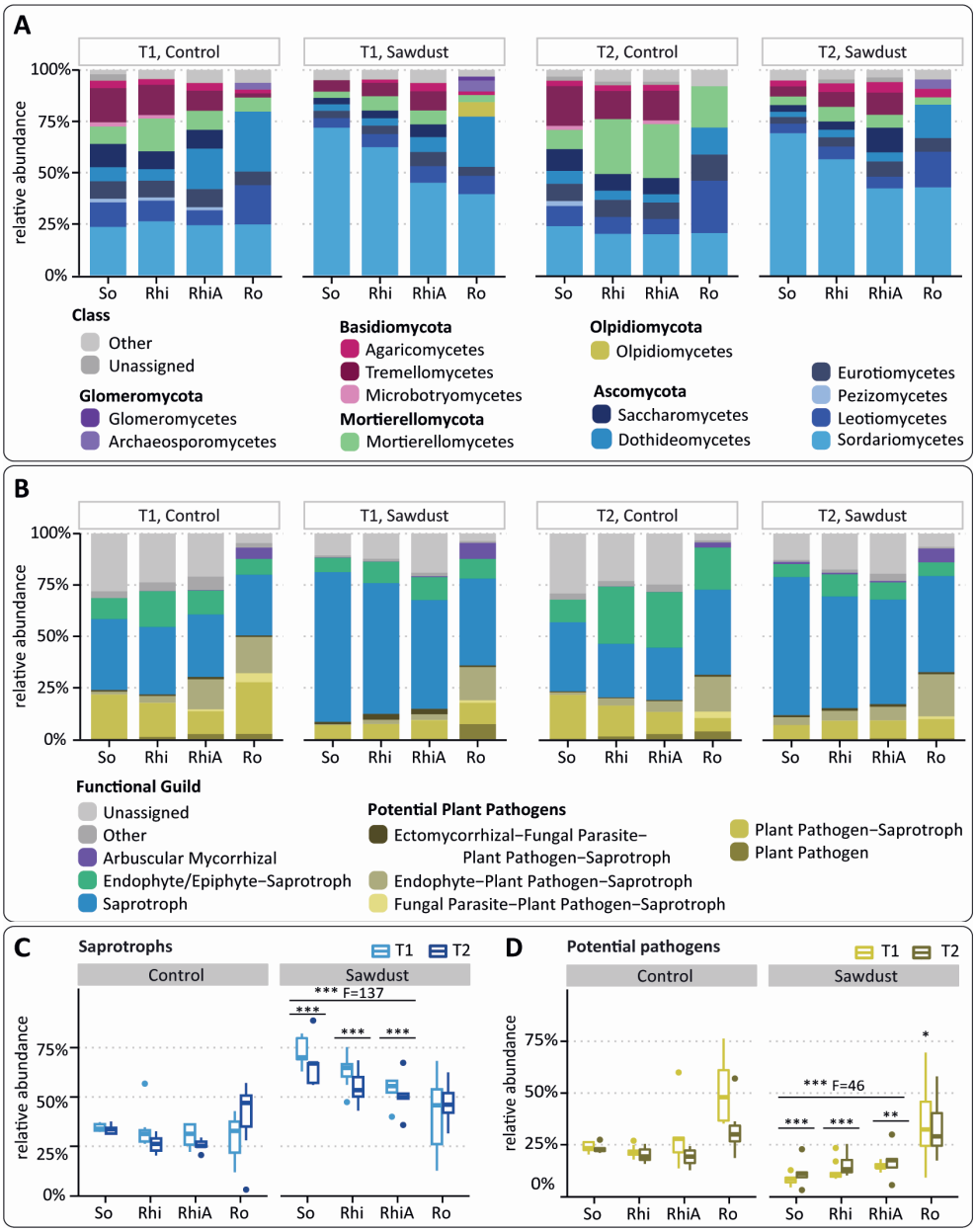

Figure 4. Effect of sawdust amendment on fungal community structure in an arable soil, as found in unplanted soil (So), rhizosphere (Rhi), ^{13}C-enriched DNA fraction in the rhizosphere (RhiA) and root (Ro). Relative abundance of fungal classes (A) and fungal functional groups (B) are displayed for soil, rhizosphere and root, for each pre-incubation time point (T1, T2) as found in sawdust-amended pots and control. Fungal taxa and guilds of relative abundance < 1.5% were classified as "Other". (C, D): relative abundance of saprotrophs and potential plant pathogens. The main effect of sawdust amendment is shown alongside the effect of sawdust in each compartment (three-way ANOVA with planned contrasts, *** p<0.001, ** p<0.01, * p<0.05).

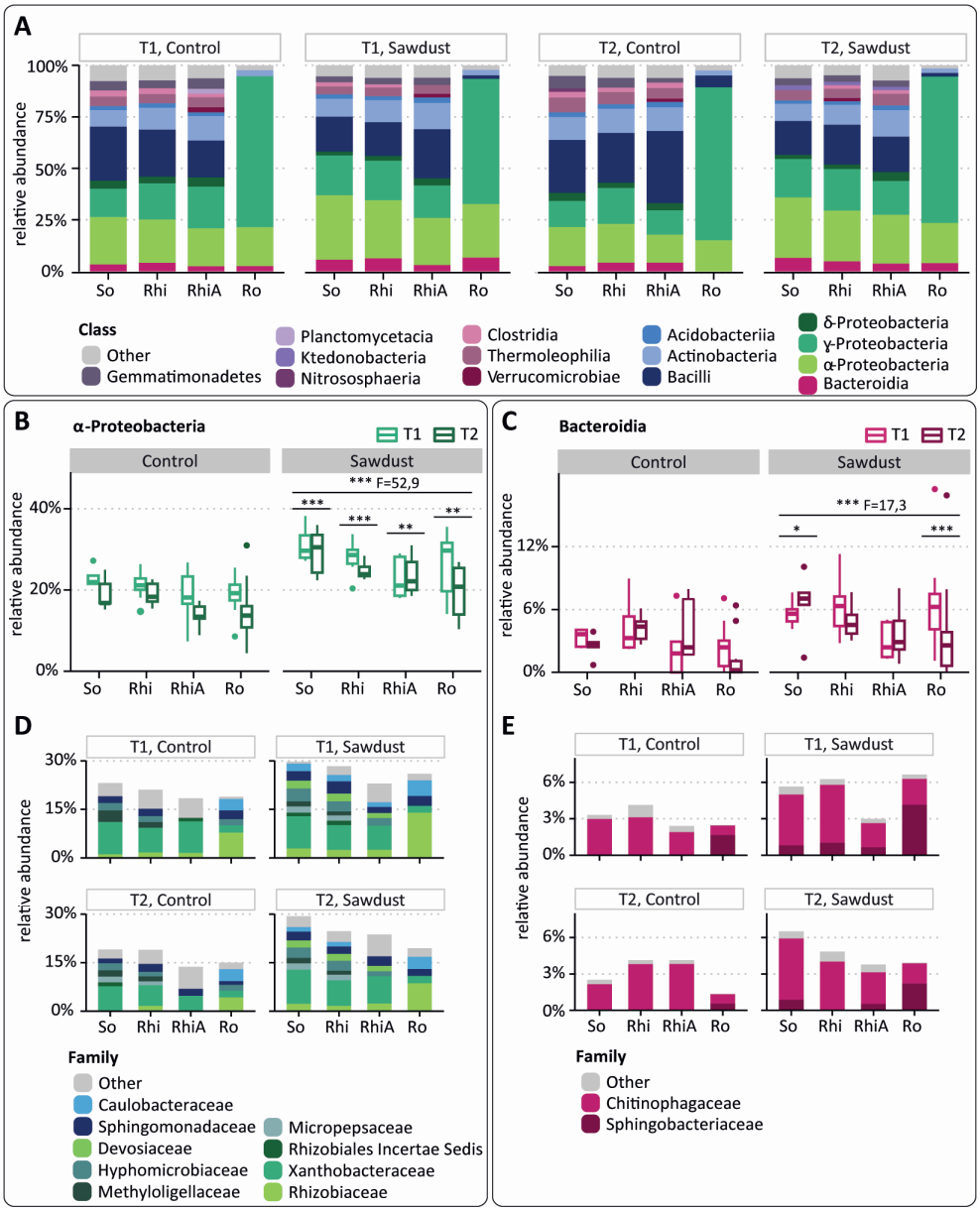

Figure 5. Effect of sawdust amendment on bacterial community composition in root and rhizosphere of carrot seedlings and soil. Results for two pre-incubation time points (T1, T2) are displayed along the x-axes for unplanted soil (So), rhizosphere soil (Rhi), ^{13}C-enriched DNA of rhizosphere (RhiA) and root (Ro). A) relative abundance of bacterial classes (classes of relative abundance<1.5% were classified as "Other"). (B, C) relative abundance of α-Proteobacteria and Bacteroidia. The main effect of sawdust is shown alongside the effect of sawdust in each compartment (three-way ANOVA with planned contrasts, *** p<0.001, ** p<0.01, * p<0.05). D, E) relative abundance of bacterial families belonging to α-Proteobacteria and Bacteroidia (respectively, families of relative abundance<1% and 0.5% were shown as "Other").

4. Discussion

4.1 Effect of sawdust addition on abundance and composition of fungi in soil, rhizosphere and roots

Free-living, saprotrophic fungi do not only occur in soil, but are also common inhabitants of the rhizosphere and are even found inside plant roots (Gkarmiri et al., 2017; Hugoni et al., 2018). However, saprotrophic fungi are negatively affected by intensive agricultural management practices, which tend to favour plant pathogenic fungi (Gao et al., 2019). Addition of organic materials, such as cover crops and straw, can increment the abundance of fungi in soil of arable fields (García-Orenes et al., 2013; Lucas et al., 2014; Rahman et al., 2017) and fragmented woody material is particularly effective (Clocchiatti et al., 2020). This study did not only confirm the stimulating effect of sawdust on fungi in unplanted arable soil but also showed that, upon subsequent sowing, fungal stimulation extended to the rhizosphere and even roots of carrot seedlings. Fungal stimulation in rhizosphere and roots was still occurring after six weeks of pre-incubation of sawdust amended soil. Saprotrophic Sordariomycetes were the most stimulated fungi in bulk soil, rhizosphere and roots. Stimulation of Sordariomycetes by sawdust is in line with their ability to efficiently decompose cellulose fractions of organic materials (Koechli et al., 2019). Under conventional agricultural practices, this group tends to associate with older plant roots (Hannula et al., 2010; Han et al., 2017).

The analysis of fungal functional guilds indicated that the relative abundance of potentially plant pathogenic populations was not increased by sawdust amendment. In the root compartment, we even noticed a slight reduction of the sub-community of potential pathogens after two weeks of sawdust incorporation, relative to the total fungal community. In particular, the relative abundance of pathogens belonging to *Didymellaceae* and *Plectospherellaceae* was reduced in roots, whereas that of *Nectriaceae* was not affected and for *Olpidiaceae* was slightly higher. This suggests that a heightened presence of rhizosphere- and root-competent saprotrophic fungi may contribute to decreased root colonization by certain plant pathogens. However, this study did not assess the absolute abundance of pathogen populations. The total abundance of fungi was higher in soil and rhizosphere after sawdust addition. Therefore, it cannot be concluded that the reduced relative abundance of potential pathogens coincides with a reduction in absolute numbers in the soil and rhizosphere. However, in roots a decrease in relative abundance of potential pathogens occurred with little change in qPCR-based fungal abundance. This suggests a decrease in size of potential pathogenic fungi in roots upon sawdust amendment. Recently, the importance of the mycobiome in reducing the severity of soil-borne diseases has been suggested (Busby et al., 2016; Poli et al., 2016; Sarrocco, 2016). We realize that our remarks on potential pathogens is only based on functional assignment of ITS sequences and that further analyses of pathogen population abundance, combined

with bioassays are required to test potential of this approach in counteracting the invasion of roots by disease-causing fungi.

An increased abundance of AMF in the rhizosphere (NLFA 16:1ω5) and in roots (ITS2 amplicon sequences) was also found following sawdust amendment. In the current study, the soil received N-fertilization only, thus the addition of sawdust could have raised the competition for available P in the soil by immobilization in saprotrophic fungal biomass (Wu et al., 2007; Zhang et al., 2018). Under low P availability, AMF increase in abundance and support plant nutrient acquisition, in particular when plant litter is added to an arable soil (Xu et al., 2018a). In natural ecosystems, AMF are often associated with plant litter (Joner and Jakobsen, 1995; Gryndler et al., 2002; Bunn et al., 2019), where they are involved in nutrient mining and have a positive feedback on saprotrophic microbes (Quist et al., 2016). To our knowledge, this is the first observation of simultaneous stimulation of AMF and saprotrophic fungi by an organic amendment in an arable soil. We suggest that this finding is worth further exploration, given the well-known beneficial effects of AMF of plant growth and health (Chandanie et al., 2009; Hu et al., 2010).

4.2 Effect of sawdust addition on root exudates consumption by fungi

Here we demonstrate that saprotrophic fungi are active in the rhizosphere of seedlings and that they consume more plant-derived C after the addition of wood sawdust to the soil, which was measured as increased ^{13}C excess accumulated in the fungal PLFA 18:2ω6,9. Contribution of saprotrophic fungi to plant-C uptake was previously observed for mature annual crop plants and perennial crops (Tavi et al., 2013; Pausch et al., 2016), but not for crop seedlings (Hünninghaus et al., 2019). For potato grown in intensively managed soils, it was shown that fungal biomass in the rhizosphere increased in advanced growth stages, probably due to presence of recalcitrant root deposits (Hannula et al., 2010). Hence, it seems that sawdust amendment creates a situation where the rhizosphere of crops can readily be colonized by root-exudate consuming saprotrophic fungi at the seedling stage, which normally requires crop maturation. Increased root C uptake after sawdust addition was ascribed mainly to sordariomycetal saprotrophic fungi, as they were prevalent within the ^{13}C-enriched fungal community. Due to the short duration of the $^{13}CO_2$ pulse (24 h), followed by immediate harvesting of rhizosphere soil and plants, mainly soluble exudates will be enriched in ^{13}C (Kaštovská et al., 2017). Thus, the observed labelling in the fungal biomarker can be explained by the uptake of soluble fraction of rhizodeposits by soil fungi. Wood sawdust and exudates greatly differ in biochemical complexity. However, saprotrophic fungi take up soluble sugars released from lignocellulose-rich organic matter by extra-cellular enzymes and are, therefore, also able to use labile monomeric exudate compounds (Buée et al., 2009; van der Wal et al., 2013; de Vries and Caruso, 2016). Rapid exudate assimilation by ascomycetal fungi was indeed found in earlier studies (Gkarmiri et al., 2017; Hannula et al., 2012; Marschner et al., 2012; Q. Wang et

al., 2019). Our study points out that sordariomycetal fungi are well-equipped for efficient decomposition of both exudates and of cellulose-rich organic materials in arable soils. The use of fine wood particles could be important to ensure bridging of fungal hyphae between wood particles and plant roots.

Earlier studies reported that AMF play a dominant role in rapid acquisition of plant-C, which is transferred only in a later stage to saprotrophic fungi and bacteria (Drigo et al., 2010; Hünninghaus et al., 2019). In the current study, the contributions of saprotrophic and arbuscular mycorrhizal fungi to plant-C acquisition were not mutually exclusive. On the contrary, a simultaneous stimulation of active incorporation of plant-C by AMF (^{13}C excess in the NLFA 16:1ω5) and saprotrophic fungi was observed in the rhizosphere of plants sown two weeks after sawdust amendment. In line with what we discussed above, this suggest the possibility that after sawdust addition, without P fertilization, no negative interactions occur between saprotrophic fungi and AMF in the root surroundings.

4.3 Effect of sawdust addition on composition and activity of bacteria

PLFA- and qPCR-based quantification of bacteria showed an increase in bacterial abundance in sawdust-amended soil, but less pronounced as compared to fungi. Similarly, sawdust caused a smaller shift in bacterial community composition than in that of fungi. Bacterial groups typically associated with cellulose degradation in arable soils, such as Acidobacteria, β-, γ-, δ-Proteobacteria and Actinobacteria (Kramer et al., 2016) did not increase in relative abundance after sawdust addition. This suggests that fungi were the main primary decomposers of sawdust, while increase of bacteria can be ascribed to the consumption of fungal-derived carbon or to the consumption of breakdown products derived from fungal sawdust decomposition (Schneider et al., 2010; Ballhausen and de Boer, 2016; de Menezes et al., 2017). Amplicon sequencing revealed that Bacteroidia and α-Proteobacteria increased after addition of sawdust in the soil. Although Bacteroidia and α-Proteobacteria could contribute in part to sawdust decomposition (Schellenberger et al., 2009; Haichar et al., 2016; Kramer et al., 2016), these groups are known to engage in fungal-bacterial interactions. Bacteroidia harbor an efficient array of chitinolytic enzymes, which confers them the ability to utilize fungal debris or predate on fungi (Wieczorek et al., 2019). The utilization of fungal-derived C by Bacteroidia is in line with the observation that they were more present in the soil- but not in the ^{13}C-enriched bacterial community, meaning that they potentially received a large share of sawdust-derived C via fungi. α-Proteobacteria are often associated with decomposing wood, where they are thought to provide an additional source of N to fungal saprotrophs in exchange of wood breakdown products (Hoppe et al., 2015; Johnston et al., 2016). Hence, the ability of α-Proteobacteria to respond to wood breakdown products may explain their increase, even though their role of N-suppliers is unlikely to be important in the current experimental setting where mineral N was supplied together with sawdust. Moreover, the presence of

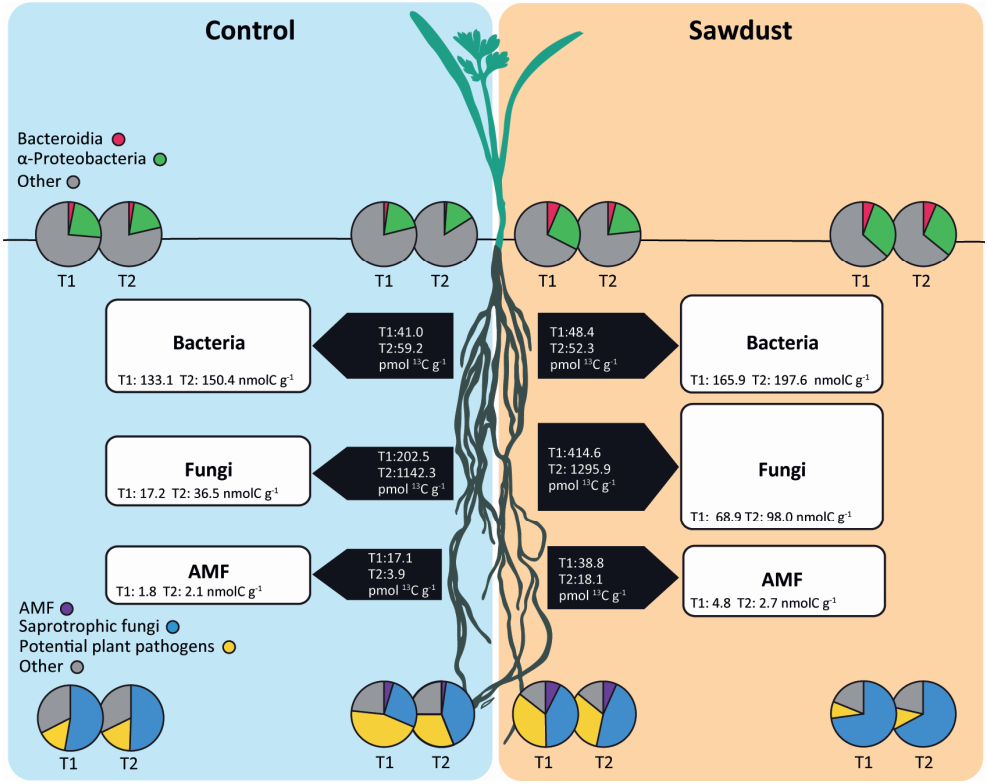

Figure 6. Effect of sawdust on fungi and bacteria in an arable soil and in the root surroundings of carrot seedlings at two pre-incubation time points (T1, T2). White boxes report the biomass of bacteria, fungi and AMF in the rhizosphere soil, based on PLFA/NLFA. The black arrows represent the incorporation of ^{13}C in fungal, bacterial and AMF PLFA/NLFA. Pie charts report the proportion of bacterial taxa (top) and fungal functional groups (bottom) that responded to sawdust amendment in the soil (external charts) and in associated with the root (central charts), based on DNA amplicon sequencing.

fungal hyphae in the bulk and rhizosphere soil could facilitate the movement of α-Proteobacteria, and Rhizobiales in particular, towards plant roots, as recently demonstrated in a legume crop (Zhang et al., 2020).

The ^{13}C incorporation in bacterial PLFAs was not affected by sawdust amendment. Thus, the increased consumption of root exudates by saprotrophic fungi did not create the hypothesized constraint for the uptake of plant-derived carbon by bacteria. This suggests that sawdust-stimulated fungi utilize an additional pool of plant C than what is used by bacteria in both the control and sawdust-amended soil. Accordingly, it seems that the total uptake of released root C is more efficient in the rhizosphere when active fungi are present. A similar pattern was reported by Morriën *et al.*, (2017), where an increased activity of fungi led to a better uptake of plant C by the food web of a restored ex-arable soil.

Even though increased fungal activity did not alter the total bacterial activity, the presence of active fungi in the rhizosphere and roots steered the composition of the plant-associated bacterial community, especially promoting Bacteroidia and α-Proteobacteria. In particular, sawdust amendment and fungal stimulation increased abundance of potentially beneficial bacteria within the roots, namely *Chitinophagaceae*, *Sphingobacteriaceae* and Rhizobiales. *Chitinophagaceae* and *Sphingobacteriaceae* could contribute to resistance to diseases by antagonistic interactions. *Chitinophaga* spp. were previously found within sugarbeet plants challenged by *Rhizoctonia solani* and were associated with a higher expression of chitinases (Carrión et al., 2019). *Spingobacteriaceae* were also found in disease suppressive soils and showed antagonistic activity against plant-pathogenic fungi, when triggered by interaction with other members of the bacterial community (de Boer et al., 2007; Gómez Expósito et al., 2017). Although Rhizobiales are mostly known as N-fixing mutualists of legumes, they can also establish mutualistic interactions with non-legume plants and contribute to plant growth stimulation and priming of the plant immune system (Garrido-Oter et al., 2018). Overall, the use of sawdust and the subsequent fungal stimulation steered the soil and rhizosphere bacterial community and favored the association of seedlings with potentially beneficial bacterial groups.

5. Conclusions and Perspectives

Stimulation of saprotrophic fungi by sawdust in arable soil was not restricted to bulk soil but extended to the rhizosphere and roots of carrot seedlings. This coincided with increased uptake of root exudates by saprotrophic fungi and higher abundance of α-Proteobacteria, Bacteroidia and arbuscular mycorrhizal fungi in rhizosphere and roots (Fig. 6), while the relative abundance of potential pathogenic fungi did not increase. This shows that an increased biomass and activity of saprotrophic fungi in the rhizosphere and root has a steering effect on other plant-associated microbes and promotes plant-beneficial microbial groups. Further research is required for assessing the benefits of such changes of on plant growth and health. We highlight the potential of saprotrophic fungi as target group for the design of sustainable agricultural practices that impact both soil and plant functioning.

Acknowledgments

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Supplementary Information

^{13}C content of soil, rhizosphere, shoot and roots

Freeze-dried, grinded plant parts, as well as freeze-dried bulk and rhizosphere soil were analysed using an elemental analyzer coupled to an isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany). $\delta^{13}C$ values were determined as an indication of the amount of photosynthates allocated to plant parts and soil compartments. Vienna Pee Dee Belamnite (V-PDB) was used as reference material. Unlabelled soil, rhizosphere, shoot and root samples obtained from unlabelled control pots were measured as well. The incorporation of ^{13}C into plant parts and soil compartments was expressed as the increase in the $\delta^{13}C$ value relative to the $\delta^{13}C$ values of un-labeled samples of the same type ($\Delta\delta^{13}C$ values). In addition, the N content of roots and shoots was measured within the same run using the elemental analyzer.

Molecular analyses: qPCR, gradient fractionation and sequencing

ITS2 and 16S rDNA copy number were quantified by qPCR, using primers ITS4r/9f (Ihrmark et al., 2012) and Eub518f/338r (Fierer et al., 2005). 1 µg of DNA of each sample was subjected to CsCl isopycnic centrifugation and subsequent density-gradient fractionation, performed as described by Neufeld et al. (2007). Fractions containing ^{13}C-enriched and ^{12}C-enriched DNA were identified by performing 16S qPCR on all fractions. In addition, the density of each fraction was measured with a AR200 refractometer (Reichert, Germany) immediately after fractionation of the density gradient. Density values were used to check that ^{13}C-enriched and ^{12}C-enriched fractions had a comparable density across all samples (Table S5). Multiple fractions were pooled into one 'heavy' and one 'light' fraction per sample, as listed in table S5. ITS2 and 16S rDNA amplicon libraries were prepared starting from DNA obtained from unplanted soil, rhizosphere soil, 'heavy' and 'light' fractions and root material. PCR was performed in 25 µl mixtures containing 200 µM each dNTP, 2.5 µl 10× PCR Buffer with MgCl2, 1µl $MgCl_2$ 25 mM, 1.25 µl BSA 4 mg ml^{-1}, 0.4 µl of each primer, 0.15 µl FastStart Expand High Fidelity polymerase (Roche Applied Sciences, Indianapolis, Indiana, United States) and 1 µl template DNA (10 ng). The PCR cycling conditions were: denaturation at 95° C for 5 min, 35 cycles of 95° C for 45 s, 54° C for 60 s, and 72° C for 90 s, followed by a final extension step at 72° C for 10 min. For each DNA sample, two PCR reactions were performed and the products were pooled before purification with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Primer pairs used were ITS4r/9f (Ihrmark et al., 2012) and Eub515f/806r (Caporaso et al., 2011). Barcoding and sequencing by Illumina MiSeq PE250 were performed at McGill University and Génome Québec Innovation Centre, Montréal, Canada.

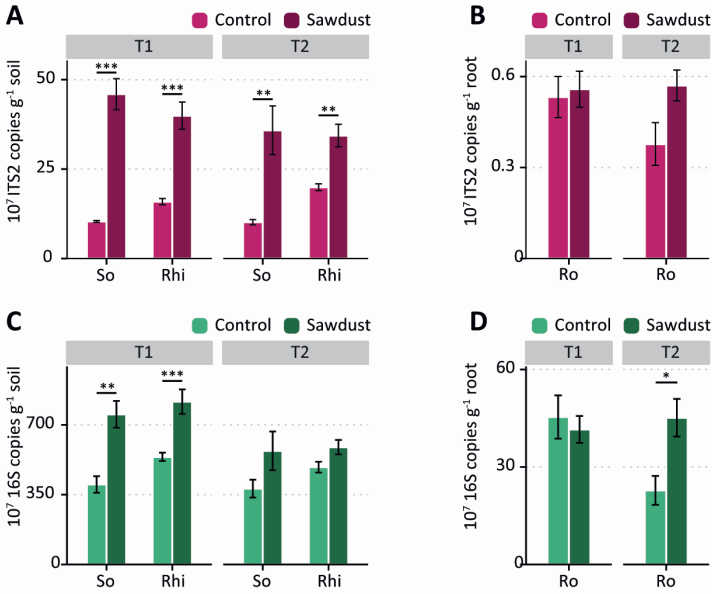

Figure S1. Total abundance of fungi, as measured by ITS copy numbers (A, B) and total abundance of bacteria, measured by 16S copy number (C, D) as measured for DNA obtained from soil (So), rhizosphere (Rhi) and root (Ro) samples at two pre-incubation time points (T1, T2). Significant differences between sawdust amendment and control are shown for each compartment and time point (*** p<0.001, ** p<0.01, * p<0.05).

Table S1. Biomass, N content and germination rate of carrot seedlings grown in control soil and sawdust-amended soil for three weeks (mean ± sd). Seedlings were sown either two weeks (T1) or six weeks (T2) from the start of the experiment. Significant differences are displayed by compact letters (ANOVA $p < 0.05$). Distribution of ^{13}C in labelled pots, as measured in unplanted control soil, rhizosphere soil, aboveground and belowground plant biomass (mean ± sd). The ^{13}C content of labelled materials is expressed as excess to the background ^{13}C content measured in the same materials obtained from unlabelled control pots of the same type (treatment and time point). ^{13}C values for both labelled and unlabelled material are relative to the Vienna PeeDee Belamnite (V-PDB) standard.

	T1		T2	
	Control	**Sawdust**	**Control**	**Sawdust**
Shoot biomass (mg)	8.74 ± 1.17 ab	8.00 ± 0.69 ab	7.36 ± 0.95 a	8.90 ± 1.38 b
Root biomass (mg)	3.25 ± 0.66 ab	3.26 ± 0.52 ab	2.63 ± 0.35 a	3.46 ± 0.70 b
Shoot N (%)	3.22 ± 0.27 a	3.68 ± 0.36 b	3.20 ± 0.24 a	3.14 ± 0.20 a
Root N (%)	3.35 ± 1.37 a	3.90 ± 0.30 b	3.43 ± 0.21 a	3.39 ± 0.18 a
Germination rate (%)	68.2 ± 9.6 a	76.0 ± 6.9 a	61.8 ± 9.4 ab	57.8 ± 10.0 b
$\Delta\delta\ ^{13}C/^{12}C$ (‰V-PDB)				
Soil	1.16 ± 1.08	1.45 ± 0.65	0.87 ± 1.04	1.19 ± 0.99
Rhizosphere	6.19 ± 1.23	5.03 ± 1.03	11.94 ± 8.35	5.06 ± 2.4
Root	3841 ± 121	3203 ± 214	2741 ± 58	2559 ± 91
Shoot	2378 ± 188	2319 ± 178	2051 ± 189	1798 ± 91

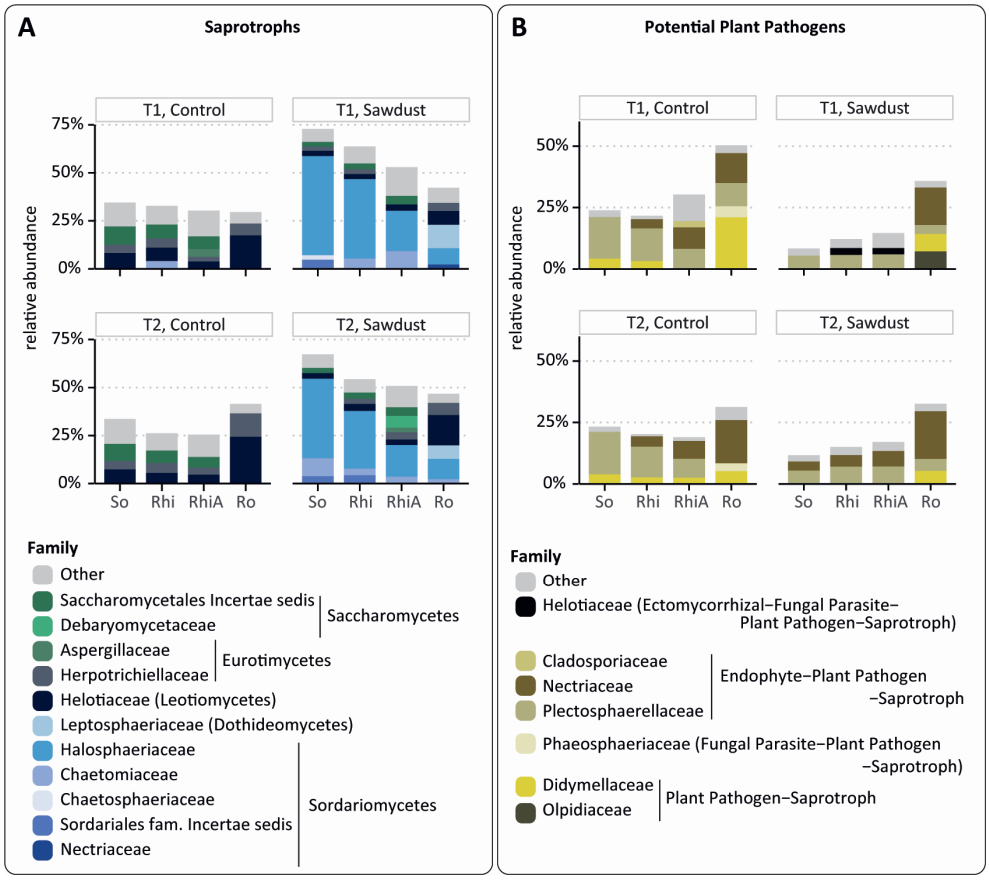

Figure S2. Effect of sawdust amendment on families assigned to saprotrophs (A) and potential plant pathogens guilds (B), as found in roots and rhizospheres of carrot seedlings and in bulk soil at pre-incubation time points (T1, T2), displayed along the x axes for unplanted soil (So), rhizosphere soil (Rhi), ^{13}C-enriched DNA fraction of rhizosphere soil (RhiA) and root (Ro). For saprotrophs and potential plant pathogens, respectively, families of relative abundance<2% and 2.5% were classified as "Other".

Table S2. Summary of ANOVA results on PLFAs and NLFA data as a function of organic soil amendment and compartment, as performed independently for each time point. (A, B) total abundance and labelled fraction of PLFA 18:2ω6c, marker for saprotrophic fungi. (C, D) total abundance and labelled fraction of bacterial PLFAs. (E, F) total abundance and labelled fraction of NLFA 16:1ω5c, marker for arbuscular mycorrhizal fungi. T1 and T2 stand for soil pre-incubation times of two and six weeks before plating, respectively. d.f.: degrees of freedom, MS: mean sum of squares, SS: sum of squares.

A		**T1**					**T2**				
	df	**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**	
Amendment	1	$1.8\,10^{4}$	$1.8\,10^{4}$	402.7	$1.3\,10^{-10}$	***	$1.3\,10^{4}$	$1.3\,10^{4}$	57.0	$6.8\,10^{-6}$	***
Compartment	1	643.5	643.5	14.5	0.002	**	$3.6\,10^{3}$	$3.6\,10^{3}$	15.4	0.002	**
Amendm.:Compartm.	1	$1.5\,10^{3}$	$1.5\,10^{3}$	34.3	$7.9\,10^{-5}$	***	89.9	89.9	0.38	0.6	
Residuals	12	532.3	44.4				$2.8\,10^{3}$	234.7			
B		**T1**					**T2**				
	df	**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**	
Amendment	1	$7.0\,10^{4}$	$7.0\,10^{4}$	12.2	0.004	**	$3.7\,10^{4}$	$3.7\,10^{4}$	0.08	0.78	
Compartment	1	$3.3\,10^{5}$	$3.3\,10^{5}$	57.8	$6.3\,10^{-6}$	***	$5.5\,10^{6}$	$5.5\,10^{6}$	12.1	0.005	***
Amendm.:Compartm.	1	$4.2\,10^{4}$	$4.2\,10^{4}$	7.2	0.01	*	$2.2\,10^{3}$	$2.2\,10^{3}$	0.049	0.82	
Residuals	12	$6.9\,10^{4}$	$5.7\,10^{3}$				$5.5\,10^{6}$	$4.5\,10^{4}$			
C		**T1**					**T2**				
	df	**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**	
Amendment	1	$1.1\,10^{4}$	$1.1\,10^{4}$	4.4	0.06	.	$1.4\,10^{4}$	$1.4\,10^{4}$	21.3	$5.9\,10^{-4}$	***
Compartment	1	$39\,10^{3}$	$3.9\,10^{3}$	1.5	0.24		932.1	932.1	1.5	0.25	
Amendm.:Compartm.	1	214.3	214.3	0.08	0.78		$3.2\,10^{3}$	$3.2\,10^{3}$	4.9	0.046	**
Residuals	12	$3.1\,10^{4}$	$2.6\,10^{3}$				$7.7\,10^{3}$	643.4			
D		**T1**					**T2**				
	df	**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**	
Amendment	1	166.4	166.4	0.94	0.35		1.8	1.8	0.02	0.87	
Compartment	1	$9.7\,10^{3}$	$9.7\,10^{3}$	54.7	$8.3\,10^{-6}$	***	$1.1\,10^{4}$	$1.1\,10^{4}$	157.2	$3.0\,10^{-8}$	
Amendm.:Compartm.	1	78.4	78.4	0.44	0.52		1.0	1.0	0.15		
Residuals	12	$2.1\,10^{3}$	177.8				811.5	67.6			
E		T1					**T2**				
	df	**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**	
Amendment	1	22.8	22.8	34.1	$1.1\,10^{-4}$	***	28.6	28.6	3.7	0.07	.
Compartment	1	3.9	3.9	5.8	0.04	*	8.5	8.5	1.1	0.31	
Amendm.:Compartm.	1	3	3	4.5	0.06	.	25.7	25.7		0.09	.
Residuals	12	7.4	0.7				91.9	7.7			
F		**T1**					**T2**				
	df	**SS**	**MS**	**F**	**p**		**SS**	**MS**	**F**	**p**	
Amendment	1	512.3	512.3	10.8	0.007	**	137.9	137.9	2.1	0.17	
Compartment	1	$2.8\,10^{3}$	$2.8\,10^{3}$	60.7	$8.4\,10^{-6}$	***	364.5	364.5	5.6	0.04	*
Amendm.:Compartm.	1	394.6	394.6	8.3	0.01	*	177.4	177.4	2.7	0.12	
Residuals	12	521.8	47.44				650.7	65.8			

Table S5. Number and density of individual fractions pooled into "heavy" and "light" DNA fractions. For each sample the initial density of the CsCl solution (before ultracetrifugation) is also indicated.

Sample	Heavy fraction nr.	Light fraction nr.	Heavy fraction density (g cm^{-3})	Light fraction density (g cm^{-3})	Initial density CsCl solution (g cm^{-3})	Heavy fraction avg density (g cm^{-3})	Light fraction avg density (g cm^{-3})
PL4	2 - 7	11 - 16	1.751 - 1.762	1.704 - 1.741	1.747	1.756	1.731
PL5	3 - 5	11 - 15	1.757 - 1.759	1.729 - 1.744	1.747	1.758	1.738
PL6	3 - 5	9 - 15	1.745 - 1.748	1.721 - 1.736	1.747	1.746	1.730
PL8	1 - 4	9 - 15	1.741 - 1.745	1.703 - 1.73	1.739	1.743	1.720
PL9	2 - 3	10 - 15	1.746 - 1.746	1.723 - 1.734	1.747	1.746	1.729
PL11	1 - 4	9 - 14	1.743 - 1.748	1.716 - 1.729	1.736	1.745	1.722
PL12	1 - 4	7 - 12	1.743 - 1.748	1.72 - 1.731	1.736	1.746	1.727
PL14	3 - 7	11 - 15	1.737 - 1.749	1.714 - 1.725	1.739	1.743	1.720
PL15	1 - 6	11 - 15	1.737 - 1.745	1.718 - 1.726	1.739	1.742	1.719
PL28	1 - 5	11 - 18	1.739 - 1.745	1.696 - 1.73	1.741	1.743	1.702
PL29	1 - 4	10 - 17	1.738 - 1.746	1.625 - 1.73	1.741	1.741	1.626
PL31	2 - 4	8 - 15	1.735 - 1.738	1.706 - 1.724	1.736	1.737	1.715
PL32	2 - 5	10 - 16	1.738 - 1.745	1.689 - 1.727	1.737	1.739	1.716
PL33	2 - 4	11 - 15	1.739 - 1.744	1.689 - 1.719	1.736	1.742	1.709
PL35	1 - 7	11 - 16	1.733 - 1.746	1.684 - 1.723	1.737	1.741	1.712
PL36	1 - 5	9 - 15	1.735 - 1.742	1.705 - 1.723	1.737	1.739	1.716
PL37	2 - 6	9 - 15	1.736 - 1.742	1.705 - 1.725	1.737	1.739	1.717
PL38	1 - 5	11 - 16	1.737 - 1.743	1.693 - 1.725	1.739	1.740	1.714
PL39	2 - 4	10 - 16	1.736 - 1.737	1.705 - 1.724	1.741	1.737	1.718
PL40	1 - 4	9 - 16	1.739 - 1.744	1.724 - 1.732	1.741	1.741	1.727
PL52	2 - 5	10 - 15	1.739 - 1.745	1.709 - 1.729	1.737	1.743	1.723
PL54	1 - 5	9 - 17	1.739 - 1.743	1.662 - 1.729	1.737	1.741	1.689
PL56	1 - 3	10 - 13	1.744 - 1.745	1.711 - 1.734	1.747	1.741	1.726
PL58	2 - 3	9 - 13	1.743 - 1.744	1.714 - 1.734	1.747	1.743	1.728
PL61	1 - 6	11 - 15	1.737 - 1.744	1.72 - 1.729	1.737	1.741	1.725
PL62	1 - 7	11 - 16	1.736 - 1.744	1.696 - 1.729	1.737	1.741	1.720
PL63	2 - 8	13 - 16	1.735 - 1.745	1.718 - 1.725	1.739	1.741	1.722
PL64	1 - 7	12 - 15	1.737 - 1.746	1.703 - 1.726	1.739	1.742	1.718
PL65	2 - 6	10 - 15	1.736 - 1.745	1.714 - 1.726	1.736	1.741	1.721
PL77	2 - 7	11 - 15	1.738 - 1.75	1.712 - 1.729	1.741	1.745	1.722
PL80	1 - 7	10 - 15	1.737 - 1.744	1.71 - 1.727	1.741	1.741	1.721
PL81	2 - 7	11 - 16	1.738 - 1.748	1.711 - 1.729	1.741	1.744	1.723
PL84	3 - 6	12 - 16	1.742 - 1.747	1.716 - 1.727	1.741	1.745	1.723
PL86	1 - 5	11 - 15	1.737 - 1.741	1.579 - 1.726	1.739	1.739	1.690
PL87	1 - 6	12 - 16	1.736 - 1.741	1.718 - 1.726	1.739	1.739	1.724
PL88	2 - 5	10 - 14	1.736 - 1.744	1.71 - 1.725	1.736	1.740	1.719
PL89	1 - 4	10 - 16	1.739 - 1.743	1.715 - 1.725	1.736	1.741	1.719
PL90	2 - 5	10 - 16	1.742 - 1.748	1.715 - 1.731	1.736	1.744	1.724

Table S6. Permutational multivariate analysis of variance (ADONIS) using Bray-Curtis dissimilarity matrix partitioned by soil treatment (sawdust, control) and pre-incubation time point (T1, T2), performed for the fungal community in each compartment separately: (A) soil, (B) rhizosphere, (C) labelled rhizosphere and (D) root. MS: mean sum of squares, SS: sum of squares. a Significance values based on 999 permutations.

A	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	2.025	2.025	44.748	0.703	0.001	***
Time point	1	0.074	0.074	1.626	0.026	0.185	
Treatment:Time point	1	0.057	0.057	1.258	0.020	0.239	
Residuals	16	0.724	0.045		0.251		
Total	19	2.880			1.000		
B	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	2.651	2.651	48.282	0.537	0.001	***
Time point	1	0.161	0.161	2.931	0.033	0.049	*
Treatment:Time point	1	0.144	0.144	2.623	0.029	0.06	
Residuals	36	1.977	0.055		0.401		
Total	39	4.932			1.000		
C	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	0.862	0.862	4.033	0.175	0.001	***
Time point	1	0.273	0.272	1.275	0.055	0.152	
Treatment:Time point	1	0.373	0.373	1.744	0.076	0.026	*
Residuals	16	3.420	0.214		0.694		
Total	19	4.927			1.000		
D	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	12.063	12.063	7.205	0.150	0.001	***
Time point	1	0.553	0.553	3.305	0.069	0.001	***
Treatment:Time point	1	0.269	0.269	1.609	0.033	0.056	
Residuals	36	6.027	0.167		0.748		
Total	39	8.056			1.000		

Table S3. Mean concentration of individual PLFAs and NLFAs (nmol C g^{-1} soil) in planted and unplanted soil, treated with either sawdust + N or N only, for two different incubation times. Compact letters show differences in concentration for each individual PLFA/NLFA marker across treatment and compartment. Differences for T1 and T2 (soil pre-incubation times of two and six weeks before plating, respectively) were analysed separetly.

		T1				**T2**			
		Control		**Sawdust**		**Control**		**Sawdust**	
		Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere
Fungi	**18:2ω6,9 PLFA**	10.2 a	17.2 a	102.2 c	68.9 b	10.4 a	36.5 ab	62.1 c	98 b
	18:2ω6 NLFA	9.3 a	14.5 a	121.2 b	108.5 b	8.5 a	36.8 a	132.4 b	156.6 b
AMF	**16:1ω5 NLFA**	1.7 a	1.8 a	3.1 b	4.8 ab	1 a	2.1 a	7 a	2.8 a
	16:1ω5 PLFA	14 a	11.4 a	15.6 a	13.8 a	13.5 a	12.4 a	14.2 ab	16.3 b
Bacteria/Fungi	18:1ω7/18:1ω9t	33.2 ab	32.2 a	57.2 c	48.9 bc	34 a	38.1 a	42.6 a	60.2 b
Bacteria	**i15:0**	54.9 a	46.8 a	65.8 a	57.3 a	57.3 ab	52.2 a	61.1 ab	68.9 b
Bacteria	**ai15:0**	35.8 a	31.8 a	42.2 a	38.5 a	36.2 a	35.4 a	38.4 ab	45 b
Bacteria	**i16:0**	19.8 a	17.7 a	25.1 b	22 b	20.8 a	20.6 a	23.2 ab	26.1 b
Bacteria	**16:1ω7**	19 a	14.7 a	41.4 a	25.2 a	28.3 ab	20.7 a	32.9 ab	46.4 b
Bacteria	**16:1ω6**	2 a	1.5 a	2.4 a	1.9 a	2.1 a	2.1 a	2.1 a	2.7 b
Bacteria	**ai17:1ω7**	4 a	3.2 a	4.4 a	3.8 a	4.1 ab	3.6 a	4.2 ab	4.9 b
Bacteria	**i17:0**	14.3 a	12.3 a	18.3 b	15.9 b	15.1 ab	14.3 a	17.7 bc	20.1 c
Bacteria	**ai17:0**	9.8 a	8.8 a	11.7 ab	10.9 b	10.2 a	10.4 a	11.1 ab	12.8 b
Bacteria	**17:1ω8**	2.1 a	2.4 a	3.3 b	3.4 b	2.4 a	2.9 ab	3.2 bc	3.6 c
Bacteria	**y-17:0**	22.3 a	20.2 ab	32.3 bc	28.1 c	22.3 a	23.3 ab	27.1 bc	35.1 c
Actinobacteria	10Me16:0	26.5 a	21.4 a	30.7 a	26.9 a	27 ab	23.9 a	30.9 bc	34.2 c
Actinobacteria	10Me17:0	2.7 a	4.3 a	4.9 a	5 a	4.6 a	4.9 a	4.8 a	5.2 a
Actinobacteria	10Me18:0	11 a	10.1 a	12.9 b	13 b	11.9 a	11.7 a	12.7 b	13.8 b
	Total	**644 ab**	**606 a**	**1045 c**	**895 bc**	**673 a**	**730 a**	**925 b**	**1147 c**

Table S4. Mean labelled fraction of individual PLFAs and NLFAs (pmol excess 13C g^{-1} soil) in planted and unplanted soil, treated with either sawdust + N or N only, for two different incubation times. Compact letters show differences in concentration for each individual PLFA/NLFA marker across treatment and compartment. Differences for T1 and T2 (soil pre-incubation times of two and six weeks before plating, respectively) were analysed separetly.

		T1				T2			
		Control		Sawdust		Control		Sawdust	
		Bulk	Rhizosphre	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere
Fungi	**18:2ω6,9 PLFA**	9.5 a	202.5 b	10 a	414.6 c	5.9 a	1296 b	6.6 a	1142 b
	18:2ω6,9 NLFA	3.4 a	44 a	-0.1 a	122.5 b	0.7 a	399.4 b	0.6 a	150 b
AMF	**16:1ω5 NLFA**	-1.1 a	17.2 b	-0.4 a	56.5 c	-0.4 a	18.1 a	0 a	3.9 a
	16:1ω5 PLFA	-1 a	6.6 b	0 a	8.8 b	-0.4 a	2.7 b	-0.7 a	-2.1 b
Bacteria/Fungi	18:1ω7/18:1ω9t	8.4 a	71.2 ab	18.9 ab	76.8 b	7.8 a	142.9 b	8 a	103.9 b
Bacteria	**i15:0**	0.7 a	9 b	1 a	10.6 b	1 a	10.3 b	1 a	11.9 b
Bacteria	**ai15:0**	0.4 a	10.2 b	0.4 a	12.5 b	0.4 a	12.4 b	0.6 a	14.7 b
Bacteria	**i16:0**	1.8 a	10.1 b	1.6 a	11.5 b	2.2 a	15.9 b	2.2 a	15.7 b
Bacteria	**16:1ω7**	0.6 a	1.1 a	0.6 a	1.5 a	0.1 a	1.7 a	0.3 a	0.5 a
Bacteria	**16:1ω6**	-1 a	6.6 b	0 a	8.8 b	-0.4 a	2.7 a	-0.7 a	-2.1 a
Bacteria	**ai17:1ω7**	-0.1 ab	0.5 b	-0.3 a	0.1 ab	0.1 a	-0.1 a	0.1 a	0.2 a
Bacteria	**i17:0**	0.3 a	3.1 b	0.6 a	3.5 b	0.6 a	3.6 b	0.4 a	3.8 b
Bacteria	**ai17:0**	0.2 a	3.4 ab	0 a	5.3 b	0.1 a	3.7 b	0 a	5.2 b
Bacteria	**17:1ω8**	1.6 a	3.2 b	1.2 a	4 b	2.4 ab	5.2 bc	1.1 a	5.6 c
Bacteria	**y-17:0**	0.3 a	4 c	0.5 ab	3.3 bc	0.2 ab	4 bc	0.3 a	3.7 c
Actinobacteria	10Me16:0	-0.1 a	1.4 a	-0.2 b	0.9 ab	0.2 a	0.9 ab	0.1 a	1.7 b
Actinobacteria	10Me17:0	0 a	1.1 b	0.1 a	1 b	0.1 a	0.9 ab	0.5 a	1.3 b
Actinobacteria	10Me18:0	0.2 a	1 b	0.2 a	1 b	0.1 a	1.1 b	0.4 a	1.3 b
	Total	**93.3 b**	**591 a**	**87 b**	**1056 c**	**55.9 a**	**2776 b**	**72.6 a**	**2217 ab**

Table S7. Permutational multivariate analysis of variance (ADONIS) using Bray-Curtis

dissimilarity matrix partitioned by soil treatment (sawdust, control) and pre-incubation time point (T_1, T_2), performed for the bacterial community in each compartment separately: A) Soil, B) Rhizosphere, C) Labelled rhizosphere and D) Root. MS: mean sum of squares, SS: sum of squares. a Significance values based on 999 permutations.

A	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	0.609	0.609	1.538	0.079	0.001	***
Time point	1	0.396	0.396	0.999	0.051	0.482	
Treatment:Time point	1	0.407	0.407	1.029	0.053	0.342	
Residuals	16	6.337	0.396		0.818		
Total	19	7.750			1.000		
B	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	0.541	0.540	1.340	0.034	0.001	***
Time point	1	0.420	0.420	1.040	0.026	0.281	
Treatment:Time point	1	0.369	0.369	0.915	0.023	0.884	
Residuals	36	14.523	0.403		0.916		
Total	39	15.853			1.000		
C	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	0.442	0.442	1.085	0.056	0.151	
Time point	1	0.417	0.417	1.023	0.053	0.33	
Treatment:Time point	1	0.520	0.520	1.275	0.066	0.021	*
Residuals	16	6.525	0.408		0.825		
Total	19	7.905			1.000		
D	**d.f.**	**SS**	**MS**	**pseudo F**	**R^2**	**p[a]**	
Treatment	1	0.434	0.434	1.537	0.038	0.01	**
Time point	1	0.389	0.389	1.378	0.034	0.051	
Treatment:Time point	1	0.430	0.430	1.524	0.038	0.019	*
Residuals	36	10.159	0.282		0.890		
Total	39	11.411			1.000		

Table S8. Taxa and functional guilds identified by ITS2 sequencing. Available upon request.

Chapter 5

Impact of cellulose-rich organic soil amendments on growth dynamics and pathogenicity of *Rhizoctonia solani*

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Abstract

Cellulose-rich amendments stimulate saprotrophic fungi in arable soils. This can increase competitive and antagonistic interactions with root-infecting pathogenic fungi, resulting in lower disease incidence. However, plant pathogenic fungi with ability to degrade cellulose may also be stimulated by cellulose-rich amendments, thereby causing an opposite effect of increased disease incidence. The current study explores these two possibilities for the notorious pathogenic fungus *Rhizoctonia solani*. First, we determined the growth of *R. solani* on fourteen pasteurized woody substrates and paper pulp in a Petri dish assay. This revealed that paper pulp is a highly suitable substrate for growth of *R. solani*, whereas its growth on woody substrates varied with tree species identity, with lowest growth on coniferous wood sawdust. In a second experiment, the effects of a subset of these materials on damping-off disease of red beet seedlings was tested with a *Rhizoctonia*-infested soil. Two weeks after application of the organic amendments, fungal biomass was determined and red beet seeds were sown. Although all substrates, with the exception of cypress wood, stimulated soil fungal biomass, only oak sawdust, elder sawdust and paper pulp significantly decreased the disease incidence of red beet seedlings. The decrease in disease suppression found for these sawdust types was not coinciding with limited *in vitro* growth of *Rhizoctonia*. In the third experiment, the same *Rhizoctonia*-infested soil was used to test the timing of organic amendments *versus* that of sowing. In this experiment, a subset of cellulose-rich amendments (paper pulp, beech and oak sawdust) was used and compared with hair meal and shrimp meal, organic amendments with reported disease suppressing effects. A strong, short-term (first week) increase of qPCR-based numbers of *Rhizoctonia* in soil was seen only for the paper pulp amendment. This coincided with increased abundance of *Rhizoctonia* in roots of beet seedlings and increase in disease symptoms. An interval of two weeks or longer between paper pulp addition and sowing resulted in disease suppression. Oak sawdust amendment, showed the strongest disease suppression independently of the time interval between amendment and sowing. Our results indicate a good perspective for the use of cellulose-rich amendments for suppression of *R. solani*. The effectiveness of *R. solani* suppression with wood sawdust depends largely on wood chemistry. Use of paper pulp as amendment requires careful timing of a pre-decomposition period in soil.

1. Introduction

Soil-borne plant pathogenic fungi represent a major threat for agricultural production (Lamichhane et al., 2017). In modern agriculture with large-scale production of monoculture crops, the negative impact of soil-borne diseases is largely controlled with the use of chemical fungicides. However, fungicides are increasingly banned by governments because of concerns on human health and negative effects on the environment. In addition, many fungicides are not effective for managing pathogen populations in the soil and can even select for resistant pathogen genotypes (Grimmer et al., 2015). As an alternative to chemical control, considerable attention is given to the development of bio-based, sustainable methods for pest and disease management. One of the options that is widely examined is the use of organic amendments to promote disease-suppressing activities in soil microbial communities (Chaparro et al., 2012; Bender et al., 2016; Bonanomi et al., 2018; Arif et al., 2020).

Incorporated organic materials serve as food for soil microbes and their addition is often followed by an increased total microbial activity and biomass (Janvier et al., 2007; Larney and Angers, 2012). Such increased microbial activity often coincides with lower incidence of soil-borne diseases (Baker and Cook, 1974; Janvier et al., 2007). This is ascribed to intensification of competition among microbes for resources, including root exudates and plant detritus (Lockwood, 1977; Garbeva et al., 2011). However also antagonistic activities, such as production of fungicidal compounds and lytic enzymes attacking the fungal cell wall can increase and are particularly important for suppressing germination and growth of pathogenic fungi (Watson and Ford, 1972; Bonanomi et al., 2018).

Stimulation of saprotrophic fungi in arable soils could be a promising approach to suppress pathogenic fungi. Saprotrophic fungi can directly compete for resources or antagonize pathogenic fungi (Kepler et al., 2017), but can also affect the suppression indirectly via stimulation of fungus-suppressing and fungus-feeding bacteria (de Boer et al., 2007; Cornforth and Foster, 2013; de Boer et al., 2015; Deveau et al., 2018). Saprotrophic fungal biomass is low in intensively managed arable soils and one of the major causal factors is the lack of decomposable organic matter input or retention (de Vries and Bardgett, 2012; Morriën et al., 2017; Clocchiatti et al., 2020). In an earlier study, we have shown that cellulose-rich materials give a rapid and lasting stimulation of saprotrophic fungal biomass (Clocchiatti et al., 2020). Therefore, amendments with cellulose-rich materials could be used to increase the suppression of soil-borne fungal diseases.

Yet, the use of organic amendments for the purpose of disease suppression should be considered carefully. It is well known that the disease control effects of organic amendments present large variability. Observed differences in suppression depend on edaphic factors, but also on the biochemical composition and decomposability of the added materials (Bonanomi et al., 2010, 2020). For instance, it has been shown that

mature composts can have disease suppressive properties, while carrying a low risk of increasing disease conductivity of soils (Hoitink and Boehm, 1999; Termorshuizen et al., 2006; Bonanomi et al., 2010).

In contrast to mature composts, fresh organic amendments can increase soil conductivity to diseases (Lockwood, 1977; Bonanomi et al., 2010). In fact, many soil-borne pathogens can grow saprotrophic and profit from an increased availability of accessible and degradable substrates (Termorshuizen and Jeger, 2008). Interestingly, an increase in disease conductivity after the addition of fresh organic materials can be followed by an increase in disease control (Lockwood, 1977; Croteau and Zibilske, 1998; Bonanomi et al., 2020). Such decline of disease incidence after an initial increase may be due to a pathogen-induced stimulation of antagonistic microorganisms (Chapelle et al., 2016). Therefore, timing of soil amendment and sowing of crops need to be considered when utilizing fresh organic materials as disease-suppressive amendments.

Rhizoctonia solani is a soil-borne pathogenic fungus, which causes worldwide diseases in several important crops (Gonzalez Garcia et al., 2006). There are indications that saprotrophic growth on certain organic amendments can increase disease severity (Henis et al., 1967; Bonanomi et al., 2020). Yet, the population dynamics and pathogenicity of *R. solani* as affected by organic substrates has received limited attention (Henis et al., 1967). *R. solani* is a basidiomycetal fungus with the ability to produce a broad array of plant cell-wall degrading enzymes (Lakshman et al., 2016; Wibberg et al., 2016), that are used for both saprotrophic and necrotrophic decomposition of cellulose in plant parts (Termorshuizen and Jeger, 2008; Horbach et al., 2011). This is considered one of the main reasons why suppression of *R. solani* by organic plant-based amendments is relatively rare, as compared to other fungal pathogens (Scheuerell et al., 2005; Termorshuizen et al., 2006; Postma and Schilder, 2015). Moreover, since *R. solani* can consume cellulose-rich substrates with a broad variety of C:N ratios (Parmeter and Parmeter, 1970), the control or population dynamics of *R. solani* is not easily predicted based on the quality of organic amendments. Hence, in order to maximize the benefits of cellulose-rich organic amendments on *R. solani* control, it is necessary to understand their impact on both pathogenic and non-pathogenic soil microbes, in relation with their effect on plant performance.

The objectives of this study were to investigate: 1) growth performance of *R. solani* on different types of sawdusts and paper pulp, 2) the relationship between the growth of *R. solani* on these cellulose-rich materials and their effect on damping-off beet seedling disease, 3) the relationship between stimulation of the total soil fungal biomass by cellulose-rich materials and *Rhizoctonia* damping-off of red beet, 4) the effect of timing of sowing of seeds after the addition of organic materials (cellulose-rich and cellulose-poor) on *Rhizoctonia* damping-off of red beet. We hypothesized that the ability of *R. solani* to grow on cellulose-rich organic materials is inversely

related to the effectiveness of the organic material to suppress *R. solani* damping-off disease. In addition, we hypothesized that substrates with little *in vitro* growth stimulating effects on *R. solani* but with high total fungus stimulating effects in soil will increase disease suppression. We further hypothesized that for organic materials increasing the *R. solani* population in soil, a time interval between the addition of the organic material and sowing would give a better control of the disease, as a result of development antagonistic interactions between the pathogenic fungus and other soil microbes. In our experiments, we combined cellulose-rich amendments with sufficient mineral fertilization, in order to study the effect of such materials on plant performance, while having no negative effects due to nitrogen (N) immobilization (Mohanty et al., 2013). The series of experiments started with testing the ability of *R. solani* to grow on pasteurized wood sawdust from different tree species and paper pulp. Next, a subset of these materials were used in a bioassay with a *R. solani* infested soil to investigate the effects on soil fungal abundance and red beet seedling performance. A second bioassay was carried out with a further subset of materials and plants were sown at three time points after amendment. In this bioassay, the growth dynamics of *Rhizoctonia* and the total fungi was monitored and related to beet seedling performance. In the latter experiment, materials rich in chitin and keratin were included to enable comparison of cellulose-rich amendment with non-cellulosic ones with reported disease suppressing properties (Postma and Schilder, 2015).

2. Materials and methods

In the first experiment, growth of *R. solani* on fourteen pasteurized woody substrates and paper pulp was assessed. The experiment consisted of an assay in Petri dishes (named hereafter: *R. solani* Performance, RsP). The second experiment consisted of a bioassay in a soil that was naturally infested with *R. solani* to test the disease suppressive effect of nine types of sawdust and paper pulp (Wood Types, WT). After sowing with seeds of red beet, germination and seedling performance was followed. In the third experiment, a second batch of the *Rhizoctonia*-infested soil was amended with five organic materials, including two wood sawdust types, paper pulp and two materials of animal origin. Red beet was sown at three time intervals after the amendment application, in order to test the effect of amendment timing on the disease suppression (Time of Sowing, ToS).

2.1 Preparation of wood sawdust, other organic materials and decomposed wood sawdust

The organic materials included in the current study were sawdust obtained from beech, oak, hazel, alder, birch, walnut, maple, elder, holly, willow, hawthorn, snowy mespilus, cypress, and Douglas fir, as well as paper pulp (SCA Hygene Products

Table 1. Wood types and organic materials used in this study in the experiments RsT, WT and ToS.

Material	Species	Exp. RsP	Exp. WT	Exp. ToS
Beech	*Fagus sylvatica*	•	•	•
10% decomposed beech	*Fagus sylvatica*		•	
20% decomposed beech	*Fagus sylvatica*		•	
Oak	*Quercus robur*	•	•	•
Hazel	*Corylus avellana*	•	•	
Black alder	*Alnus glutinosa*	•		
Birch	*Betula* sp.	•		
Walnut	*Juglans* sp.	•		
Maple	*Acer* sp.	•		
Elder	*Sambucus* sp.	•	•	
Holly	*Ilex* sp.	•	•	
Willow	*Salix alba*	•	•	
Hawthorn	*Crataegus* sp.	•		
Snowy mespilus	*Amelanchier* sp.	•		
Cypress	*Cupressus sempervirens*	•	•	
Douglas fir	*Pseudotsuga menziesii*	•		
Paper pulp	-	•	•	•
Hair meal	*Sus scrofa*			•
Shrimp meal	*Crangon crangon*			•

Suameer B.V., Sumar, the Netherlands) (Table 1). In the ToS experiment pig hair meal (Darling Ingredients, Sonac Burgum B.V., Sumar, the Netherlands), and shrimp meal (Telson B.V., Lauwersoog, the Netherlands) were included (Table 1). Beech sawdust was obtained from a local producer (Bemap Houtmeel B.V., Bemmel, the Netherlands). Wood branches of all the other tree species were obtained from trees in the forests near Wageningen in June 2018. Each branch was cut with a chainsaw and sawdust was collected and further crushed with a cutting mill (SM 100, Retsch B.V., Haan, Germany). All types of sawdust were sieved (2 mm) and stored at room temperature in a dry place until use. Paper pulp, pig hair meal and shrimp meal were dried and further sieved (2 mm) before use.

Beech sawdust and NPK fertilizer (Tuinmest 12-10-18, POKON Naturado, Veenendaal, the Netherlands) were mixed at a concentration of 0.24 g fertilizer g^{-1} wood to lower the C:N ratio of the material below 15:1 which prevents nitrogen immobilization by microbes and allows plant growth when the mixture is used in plant bioassays

(Mohanty et al., 2013). The wood-fertilizer mixture was brought to 60% WHC by adding sterile demi-water. Part of the beech sawdust was partially decomposed before use in the WT experiment. To this end, portions of the wood-fertilizer mixture corresponding to 80 g of dry sawdust were placed in eight replicate cylindric plastic jars (⌀ 10 cm x 17 cm), each closed with filter paper, and incubated at 20° C in a dark climate chamber. After that, the decomposition of the mixture was determined weekly on basis of weight loss of dry wood. Wood mass loss was 10% and 20% after 16 and 30 days, respectively. At each of these time points, four replicate jars were taken out from the climate chamber and stored at 4° C until use, in order to slow down the decomposition process until use in the WT bioassay.

2.2 Sampling and preparation of soil for the bioassays

Soil was collected from an experimental field located near the village of Burgerbrug (N 52 75 96, E 4 71 07, North-Holland, the Netherlands) in August 2018 and July 2019. The soil had a sandy loam texture and was infested with *R. solani*. The first soil batch from August 2018 was used for Exp. WT, whereas the second batch from July 2019 was used for Exp. ToS. The field (Bejo Zaden B.V., the Netherlands) was cultivated with red beet (*Beta vulgaris*). Bulk soil (0 - 10 cm) was collected in between rows from four plots (4 x 2.5 m each) where plants showed the most severe signs of disease such as low germination rate, reduced plant biomass and yellowing of leaves. Each soil sample was sieved (4 mm), homogenized with a 250 l mixer (Patriot 250, Atika, Burgau, Germany) and stored at 4° C until use, for a maximum of two months.

2.3 RsP Experiment

Assay of pathogen performance on woody substrates

A portion of 100 g sawdust of fourteen tree species (Tab. 1) and of paper pulp was pasteurized at 70° C for 24 h and dried overnight in a sterile flow cabinet. Each pasteurized dry material was then mixed under sterile conditions with NPK fertilizer (0.24 g fertilizer g^{-1} wood or paper pulp) and brought to a moisture content of 60% WHC. Portions containing ca. 4.2 g pasteurized material were equally distributed in Petri dishes (⌀ 9 cm). For each material, five replicates were prepared. The ability to grow on the woody materials and paper pulp was tested for *R. solani* AG 2-2IIIB (IRS, Bergen op Zoom, the Netherlands), a strain that causes diseases in red beet, sugar beet and other vegetable crops (Engelkes and Windels, 1996). The *Rhizoctonia* strain was pre-grown on potato dextrose agar (PDA, Oxoid, Badhoevedorp, the Netherlands) at 25° C. Plugs of mycelium covered agar (⌀ 3 mm), were taken with a cork borer and placed in the centre on top of the substrate, with the mycelium in contact with the substrate material. For each material, three additional replicate Petri dishes (⌀ 5 cm, with 2.8 g sawdust or paper pulp) were incubated without pathogen inoculation, as a

control for the absence of other decomposer microbes. The Petri dishes were incubated in a dark climate chamber at 20° C for 10 days.

Measurement of growth performance of R. solani

The growth performance of *R. solani* was evaluated by measuring the extension of hyphae (mycelial area) and ergosterol concentration in the substrate covered by the mycelium, as a proxy for mycelial density. After 10 days of growth, the area of mycelium of *R. solani* growing radially towards the edge was plotted on mica plastic sheets under a microscope (M250C, Leica Microsystems, Wetzlar, Germany) for each of the treatments. The plastic sheets were scanned and the areas of the mycelia were quantified using the WinFOLIA software (Regent Instruments Inc., Ch Ste-Foy, Canada). Next, the mycelium covered substrates were harvested and transferred in 50 ml plastic vials. After freeze-drying and homogenization, a sample of 0.25 g of each substrate sample was stored in 4 ml of 10% KOH in methanol at -20° C until ergosterol extraction.

2.4 WT Experiment

Bioassay with wood sawdust types and paper pulp

Sawdust types used as organic amendments in the WT bioassay were: paper pulp, sawdust of beech, oak, hazel, elder, holly, willow and cypress, and 10% - and 20% pre-decomposed beech sawdust. Sawdusts and paper pulp were added at the concentration of 5 g kg^{-1} dry soil and combined with NPK fertilizer. The control consisted of the addition of fertilizer only. NPK fertilizer (Tuinmest 12-10-18) was added at the concentration of 1.2 g kg^{-1} dry soil, corresponding to an input of 144 mg N, 120 mg P and 168 mg K g^{-1} soil. Decomposed beech sawdust was added to the soil (5 g kg^{-1} dry soil) without additional NPK, as the NPK was already mixed with sawdust at the beginning of the decomposition period. The moisture of the amended soil was adjusted to 60% WHC and the soil was transferred to pots (1.2 kg dry soil per pot). The experiment had five replicates for each amendment, and they were arranged in a random order within replicate blocks (CRBD). The amended pots were incubated for two weeks in the greenhouse under a dark cover, so that fungi would be stimulated before planting. After two weeks, each pot was sown with 32 seeds of a *Rhizoctonia*-sensitive red beet cultivar (*Beta vulgaris* var. conditiva 'Pablo'), at least 1 cm distance from each other. The plants were grown for three weeks before harvesting. Soil moisture was maintained constant on weight basis.

Determination of germination percentages and plant disease incidence

The number of seedlings that emerged one and two weeks after sowing were counted. Single red beet seeds of our stock can consist of clumped seeds and can give origin to either one, two or, seldom, three seedlings. The percentage of emerged seedlings (G) was calculated as:

$$G = \frac{C}{C_{max}} \times 100$$

Where C is the number of emerged seedlings in a treatment and C_{max} the number of germinated seeds under optimal conditions (disease free potting soil). The number and health status of seedlings were monitored during the three-weeks growth period. As seedlings displayed stem and root lesions to various degrees, the severity of lesions was classified in five groups at the end of the growth period for all plants (shown in Fig. 1). Classification criteria were: plants with little or no lesions in the crown area (D0), small brown lesions and/or a thinner diameter in the crown area (D1), brown or black lesions in the crown area, extending to the root and/or stem for up to 1 cm (D2), black lesions extending to most of the stem and/or roots (D3), lesions extending to the leaves and involving the whole plant (D4). Several D4 plants died during the three-weeks growth period. The disease severity index (D) was calculated for each pot as follows, where C_{DX} indicates the count of seedlings in a pot for each disease class (Chiang et al., 2017).

$$D = \frac{1 \times C_{D0} + 2 \times C_{D1} + 3 \times C_{D2} + 4 \times C_{D3} + 5 \times C_{D4}}{5 \times (C_{D0} + C_{D1} + C_{D2} + C_{D3} + C_{D4})} \times 100$$

Plants belonging to D0, D1 and D2 classes with no or minor disease symptoms were indicated as "successful" in a simplified display of the results, whereas plants classified as D3 and D4 were likely to languish and indicated as "unsuccessful". The rate of successful emerged seedlings was calculated in the same way as the germination rate.

Sampling of soil and plants

At the end of the three-week plant growth period, the remaining plants in the pots were harvested. Roots and shoots were separated from each other, after classifying the disease symptoms (see section above). Shoots obtained from the same pot were pooled together and their biomass was measured after drying at 40° C for 5 days.

One day before sowing and at the end of the plant-growth period, soil was sampled using a corer (⌀ 6 mm) in four random spots in each pot. The composite sample resulting from the four cores was homogenized, and 1 g of it was stored in 4 ml of 10% KOH in methanol at -20° C. This was used for ergosterol extraction within three months.

Figure 1. Classification of disease severity of red beet seedlings based on lesions. Arrows indicate areas with dark lesions and thinning of the crown, stem and root. Photos for D3 and D4 are shown at 2x magnification as compared to D0, D1 and D2. *R. solani* isolates were obtained from plants that had lesions and they were identified by Sanger sequencing (data not shown).

2.5 ToS Experiment

Bioassay with varying pre-incubation times of soil amendments

The ToS experiment included paper pulp, two woody materials (5 g kg^{-1} dry soil), namely beech sawdust and oak sawdust, combined with NPK fertilizer (1.2 g kg^{-1}) as well as two animal-derived materials: pig hair meal and shrimp meal added at lower concentrations (2 g kg^{-1} dry soil) and without NPK fertilizer. The lower concentration used for animal-derived materials without mineral fertilization was chosen to provide a N input comparable to the other soil treatments (Postma and Schilder, 2015). The control was amended with NPK fertilizer only. Amended soils were adjusted to 60% WHC and, for each treatment, 15 pots containing the equivalent of 1.3 kg soil (dw) were prepared, making the total 90 pots. Next, 32 red beet seeds per pot were sown 1 day after amendment in five replicate pots for each soil treatment (T1). The other pots were incubated without plants under a dark cover. Of these, five replicates for each soil treatment were sown 14 days after amendment (T2), whereas the remaining five

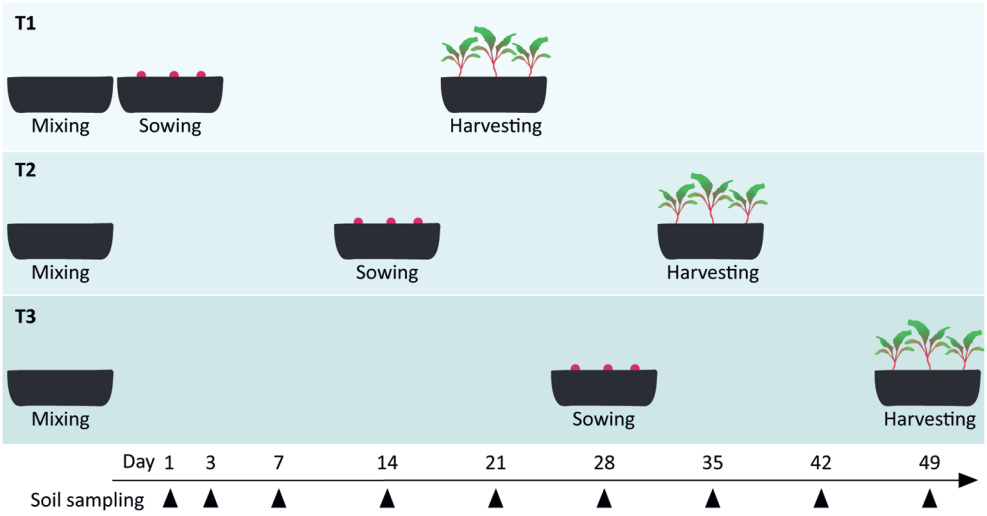

Figure 2. Experimental design of the ToS experiment. For T1, T2 and T3 the time gap in between soil organic amendments (day 0) and sowing is shown. The plant growth period was the same for T1, T2 and T3 (3 weeks). Black arrows indicate the days when soil was sampled non-destructively from all pots. Additionally, at harvesting root and shoot parts were collected. All pots were simultaneously incubated in a greenhouse.

replicates per treatment were sown 28 days after amendment (T3, Figure 2). In all cases, plants were grown for three weeks after sowing, namely until day 21, day 35 and day 49, for T1, T2 and T3, respectively. All pots were kept in the greenhouse during the experiment, randomly arranged in five blocks (CRBD). The germination rate, the rate of emergence of healthy and heavily diseased plants, and disease severity index were measured and calculated as described above for the WT experiment. Roots obtained from each pot were pooled, freeze-dried, grinded to a fine powder by beating with metal beads and stored at room temperature before DNA extraction.

Sampling of soil and plants

Soil was sampled from all the pots of the experiment non-destructively at day 1, 3, 7, 14, 21, 28, 35, 42 and 49 from the start of the experiment (Figure 2). At each sampling, a composite soil sample of about 5 g was obtained by taking four cores of soil (⌀ 6 mm) from random spots in a pot, without disturbing seeds or the plants. Part of the composite sample (1 g) was stored in 4 ml of 10% KOH in methanol at -20° C for ergosterol extraction. The rest of the sample was freeze-dried and stored at room temperature until DNA extraction.

Fungal biomass

Fungal biomass in wood (Exp. RsP) and soil from (Exp. WT and ToS) was estimated by the measurement of ergosterol concentration. For the ToS experiment, only a

selection of samples was subjected to ergosterol extraction, in order to compare ergosterol- and qPCR-based estimation of total fungal abundance. Alkaline extraction of ergosterol was performed starting from 0.25 g wood samples and 1 g soil samples, as described by de Ridder-Duine et al. (2006). Ergosterol concentration was then quantified by LC-MSMS (UHPLC 1290 Infinity II and 6460 Triple Quad LC-MS, Agilent Technologies, California, United States).

DNA extraction and qPCR of Rhizoctonia *and fungi*

DNA extraction and qPCR were performed for soil and root samples from the ToS experiment. All soil samples were included for control, paper pulp- and oak-amended soil and root samples. For beech, hair meal and shrimp meal samples of roots and soil were only taken at day 7, 21, 35, 49 as these materials had less evident effects on plant performance. DNA was extracted from 0.25 g soil or 0.25 g root with the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The abundance of *Rhizoctonia* was quantified by qPCR targeting a 174 bp fragment within the ITS region with primers ARSF5/ARSR5 (Dubey et al., 2016), whereas the total fungal abundance was quantified by targeting the ITS2 region (350-750 bp) with primers ITS9f /ITS4r (Ihrmark et al., 2012). Samples were analyzed in two technical replicates, arranged in a blocked random order. The qPCR was performed with a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, California, United States) in 20 µl mixtures containing 10 µl SYBR Green PCR Master Mix (Sigma-Aldrich, Missouri, United States), 1 µl BSA 4 mg ml^{-1}, 0.8 µl of each primer, 3.4 µl DEPC-treated water (Sigma-Aldrich) and 4µl template DNA. The qPCR cycling conditions for *R. solani* were: denaturation at 95° C for 5 min, 40 cycles of 95° C for 10 s, 54° C (*R. solani*) or 56° C (ITS2) for 20 s, and 72° C for 25 s, followed by a final extension step at 72° C for 10 min. Plasmids containing DNA amplicon from *R. solani* AG2-2-IIIB and *Trichoderma* were used as a standard for the quantification of *Rhizoctonia* and ITS2 copy number, respectively.

2.6 Statistical analysis

The statistical analysis was carried out in R (v 3.4.0). For the experiment RsP, differences in area and local ergosterol concentration of *R. solani* mycelia growing on 15 cellulose-rich substrates were analyzed using one-way ANOVA for each variable, followed by Tukey's post-hoc test for pairwise comparisons (5% family-wise error rate). Log-transformation was applied to both ergosterol and area data, in order to meet the assumptions of normality and equality of variances.

For the WT experiment, the effect of soil treatment on germination rate, number of healthy seedlings, total shoot biomass per pot and disease severity index in each pot was analyzed with one-way ANOVA models, after checking the assumptions of normality and homogeneity of variance. One-way ANOVAs had soil treatment as explanatory factor and a block as a random factor and Dunnett's post-hoc test was

used to compare the effect of each organic material to the control. For soil ergosterol concentration in the experiment WT, as measured before and after plant growth, a repeated measures ANOVA was used, with soil treatment as between subjects factor, time point as within subjects factor and a block as a random factor. Differences in ergosterol concentration between each material and the control for each time point were analyzed based on pairwise comparisons with Dunnett's post-hoc test (family-wise error rate 5%). Pearson's correlation index and its significance ($p < 0.05$) were calculated for the relation between soil ergosterol and disease severity index, as well as between soil ergosterol and the rate of successful plants. Disease severity and rate of successful plants were also correlated with the performance (mycelial area and density) of *R. solani*, measured in the RsP experiment ($p < 0.05$).

For the ToS experiment, the number of germinated plants, number of unsuccessful seedlings, as well as the disease severity were analyzed with two-way ANOVA models, with soil treatment and sowing time as fixed factors and a block as a random factor. Pairwise comparisons (Tukey post-hoc test with 5% family-wise error rate) were used to compare each variable between each soil treatment and the control, within each of three sowing times. Pairwise comparisons between treatments within each sowing time and between sowing times within control pots were checked as well. The qPCR-based abundance of fungi was analyzed using a generalized linear model with fitted gamma distribution of error. Soil treatment, sowing time (T1-3) and day of sampling were used as predictive factors, whereas block and pot identity were random factors. Moreover, the effect of each soil treatment on fungal abundance, as compared to the control, was analyzed for soil sampled one week after sowing, at harvesting and for root samples, by combining two-way ANOVA (with soil treatment and sowing time as factors) and Dunnett's post-hoc test (5% family-wise error rate), both applied on log-transformed data, in order to fit the assumption of normality and homoscendasticity. A generalized linear model was applied to qPCR-based abundance of *Rhizoctonia*, with Poisson distribution of errors, in order to account for a large amount of zeros in the dataset. Also in this case, soil treatment, sowing time (T1-3) and day of sampling were used as predictive factors, whereas block and pot identity were random factors. The effect of treatment on *Rhizoctonia* abundance was log-transformed and analysed in soil one week after sowing and in root samples by using Welch's test for two-way ANOVA combined with Dunnett's post-hoc test (5% family-wise error rate). Differences among soil treatment and sampling days were analyzed for qPCR- and ergosterol-based measurement of fungal abundance with two-way ANOVA combined with Tukey's post-hoc test.

3. Results

3.1 Experiment RsP

The pasteurization of sawdust types and paper pulp was sufficient to inhibit the growth of the natural microbial inhabitants of these materials, as assessed by the absence of development of hyphae or bacterial colonies on non-inoculated materials. *R. solani* was able to grow on all cellulose-rich substrates in absence of competition with other decomposer microorganisms. The measurement of mycelial area and local density showed that the size of the mycelium of *R. solani* varied with the type of material (ANOVA, $F_{14, 75} = 229.4$ and $F_{14, 75} = 37.4$ for area and density (ergosterol), respectively; both $p < 0.001$). The smallest mycelial areas of *R. solani* were seen for sawdust of the conifer trees (Douglas fir and cypress) and of walnut (Fig. 3, Tab. 2). Of the wood types, willow and elder had the largest mycelial area, but with low (elder) or intermediate (willow) mycelial density (Fig. 3, Tab. 2). A good performance of *R. solani* (large mycelial area, high fungal density) was seen for hazel, black alder and snowy mesiphilus. Dense growth but small area coverage was obtained with oak, holly and hawthorn (Fig. 3, Tab. 2). By far the largest biomass increase of *R. solani* was seen for paper pulp (Fig. 3, Tab. 2).

Table 2. Performance of *R. solani* on woody substrates and paper pulp. Mean and standard deviation ($n = 5$) for area and local ergosterol concentration of the substrate in the area covered by *R. solani* mycelium. Compact letters show significant differences between substrates for each measurement at $p < 0.05$.

Substrate	**area (cm^3)**		**ergosterol ($\mu g\ g^{-1}$)**	
Beech	20.7 ± 0.8	e	19.3 ± 3.6	bc
Oak	13.5 ± 0.7	d	48.8 ± 8.4	de
Hazel	26.0 ± 1.0	ef	42.9 ± 13.8	cde
Black alder	22.7 ± 1.0	ef	41.5 ± 3.5	cde
Birch	21.0 ± 2.6	e	30.3 ± 2.8	bd
Walnut	4.8 ± 1.4	b	26.5 ± 12.4	bd
Maple	12.1 ± 2.0	cd	29.1 ± 1.0	bd
Elder	44.0 ± 3.2	g	14.6 ± 3.6	b
Holly	9.3 ± 1.4	c	55.3 ± 10.0	de
Willow	54.7 ± 1.3	g	38.9 ± 4.4	cde
Hawthorn	13.2 ± 1.4	d	70.0 ± 6.5	e
Snowy mespilus	21.3 ± 2.0	e	53.7 ± 8.6	de
Cypress	0.9 ± 0.1	a	3.3 ± 3.8	a
Douglas fir	3.7 ± 0.3	b	62.1 ± 29.8	de
Paper pulp	31.7 ± 9.7	f	304.8 ± 100.0	f

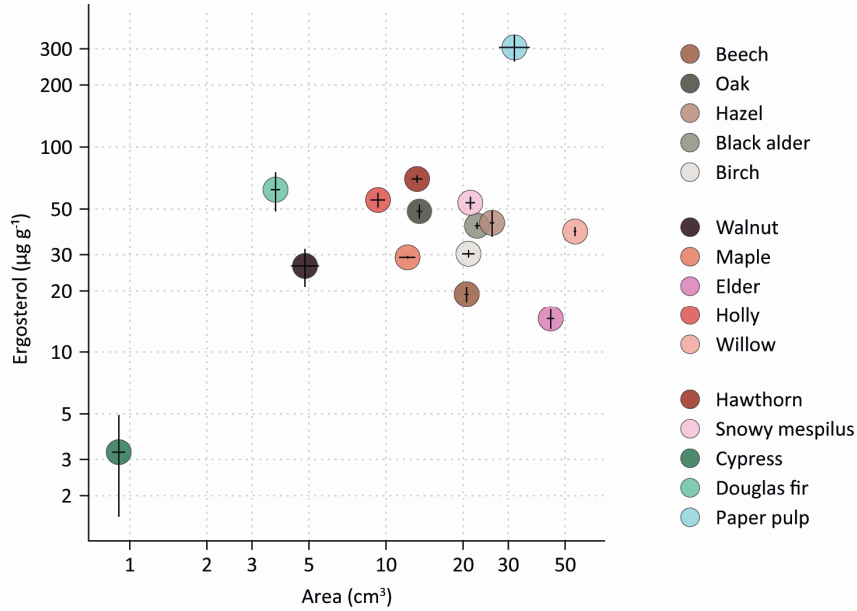

Figure 3. Performance of R. solani on 14 types of wood sawdust and paper pulp. The area of the substrates colonized by the mycelium of R. solani is shown on the x axis (mean ± se, n=5), whereas the y axis shows the ergosterol concentration of the substrate in the zone covered by Rhizoctonia hyphae, as a proxy for mycelial density (mean ± se, n=5). Both axes are log-scaled.

3.2 Experiment WT

In experiment WT, the selected cellulose-rich amendments increased fungal biomass in the soil (Fig. 4), with exception of cypress sawdust. The ergosterol-stimulating effect of the amendments was distinct at each time point (interactive term treatment x time point: $F_{10, 55} = 6.97$, $p < 0.001$). At the time of sowing red beets, two weeks after amendment, the largest increase in ergosterol was seen for paper pulp, beech, 10% and 20% pre-decomposed beech and hazel sawdust. Elder sawdust had no effect on fungal biomass at week 2, but had increased ergosterol at week 5, corresponding to the end of the plant growth period. All other deciduous wood types, as well as paper pulp, had a higher ergosterol content as compared to the control at both week 5 and week 2. Yet, ergosterol concentrations at week 5 were either similar (oak, holly) or lower as compared to week 2 (fresh and pre-decomposed beech sawdust, hazel, willow and paper pulp).

The amendment of soil with the selected organic materials had little effect on the total germination rate of red beet seeds ($F_{10, 55} = 1.62$, $p = 0.14$). Only 20% pre-decomposed beech sawdust, elder sawdust and paper pulp (Fig. 5A) increased the number of germinated seeds as compared to the control. Soil treatment significantly affected the

development of successful seedlings ($F_{10, 55}$ = 7.28, p < 0.001, Fig. 5B). In particular, paper pulp, oak and elder gave more successful seedlings than the control. Oak sawdust, elder sawdust and paper pulp also had a significantly higher aboveground biomass per pot (Fig. 5D). Disease severity index was lower in elder and paper pulp amendments as compared to the control, whereas 10% pre-decomposed beech sawdust showed a slightly increase of the disease severity index (Fig. 5C). Both disease severity and number of successful seedlings were not significantly correlated with the soil ergosterol level at the time of sowing (p = 0.3, R = 0.16 and p = 0.2, R = 0.15 respectively) or the *in vitro* performance of *R. solani* on pasteurized materials (p = 0.8, R = -0.03 and p = 0.2, R = 0.21 respectively).

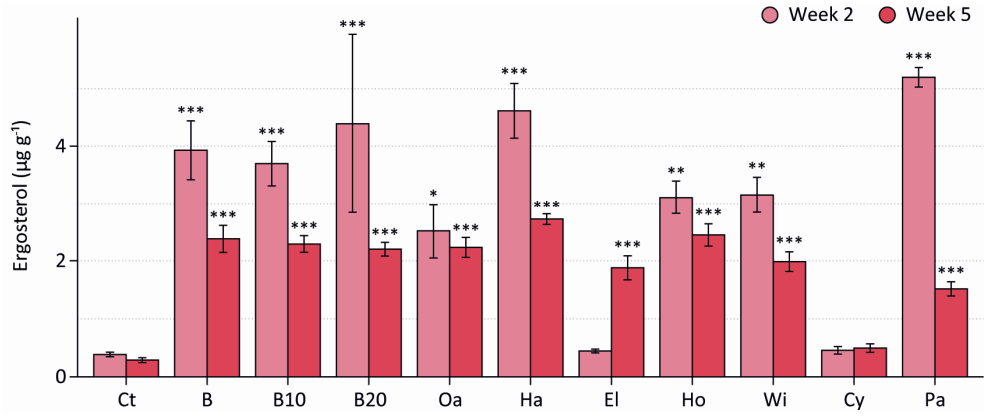

Figure 4. Fungal biomass stimulation in soil amended with seven wood types, pre-decomposed wood and paper pulp (WT experiment). Soil treatments are indicated as: Ct=control, B=beech sawdust, B10=10% pre-decomposed beech, B20=20% pre-decomposed beech, Oa=oak, Ha=hazel, El=elder, Ho=holly, Wi=willow, Cy=cypress, Pa=paper pulp. Ergosterol concentration is shown for amended and control pots at the time of red beet sowing (two weeks after amendment) and at harvesting of red beet seedlings (five weeks after amendment). Significant differences between each treatment and the control are indicated as • 0.1>p>0.05; * 0.05>p>0.01; ** 0.01>p>0.001, *** p<0.001.

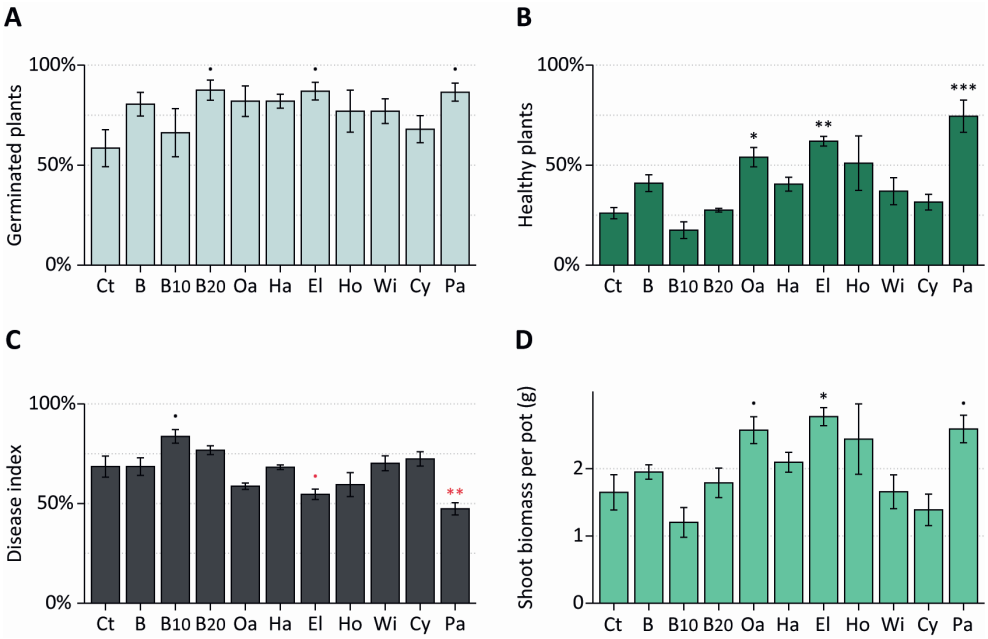

Figure 5. Performance of red beet seedlings in *Rhizoctonia*-infested soil amended with sawdust types and paper pulp (WT experiment). Soil treatments are indicated as: Ct=control, B=beech sawdust, B10=10% pre-decomposed beech, B20=20% pre-decomposed beech, Oa=oak, Ha=hazel, El=elder, Ho=holly, Wi=willow, Cy=cypress, Pa=paper pulp. A) Percentage of germinated seeds, B) percentage of seeds germinated and resulting in succesful plants, C) seedling disease severity index per pot, D) total aboveground biomass per pot. Significant differences between each treatment and the control are indicated as • 0.1>p>0.05; * 0.05>p>0.01; ** 0.01>p>0.001, *** p<0.001. Black and red simbols indicate, respectively, significant increase and decrease as compared to the control.

3.3 Experiment ToS

In the ToS bioassay, organic amendments had a significant impact on the total germination rate of red beet seeds as compared to the control ($F_{5, 90} = 10.31$, $p < 0.001$). However, the effect was dependent on the sowing time ($F_{10, 90} = 1.83$, $p = 0.08$). For T1, only hair meal resulted in increased germination rate ($p < 0.05$), whereas at T2 a higher number of germinated seedlings was seen for all soil amendments but shrimp meal (Fig. 6A). A higher germination rate as compared to the control was also seen in pots sown one month after mixing (T3) for all amendments, with exception of hair meal (Fig. 6A).

Both organic amendment and sowing time affected the amount of seeds resulting in successful plants ($F_{10, 90} = 3.5$, $p < 0.001$). Oak sawdust amendment resulted in a higher number of successful seedlings at T1, T2 and T3 as compared to the control (Fig. 6B).

An increased number of successful seedlings was seen for beech sawdust only at T3 ($p < 0.05$), whereas for hair meal only at T1 ($p < 0.1$). Paper pulp had a low number of successful plants at T1 – however the numbers were not significant different from the control. At the longer time intervals, paper pulp amendment gave a significantly higher number of successful plants (T2 $p < 0.1$ and T3 $p < 0.05$), as compared to the control. In control pots, the number of successful plants increased, albeit not significantly, with longer time gaps between amendment and sowing (T1-3, Fig. 6B).

Organic amendments had an impact on disease severity only at T1 ($F_{10, 90} = 8.6$, $p < 0.001$, Fig. 6C). Most prominent was the effect by oak ($p < 0.001$). Beech sawdust caused only a slight decrease in disease severity as compared to the control at T1 ($p < 0.1$). An opposite pattern was seen for paper pulp at T1, with an increased disease severity ($p < 0.001$). At T2 and T3 organic amendments had no significant effect on disease severity. The disease severity index in the control decreased with increasing incubation times before sowing (Fig. 6C).

Soil amendments affected the total fungal abundance in soil and roots depending on both the sampling day and, within the same day, among pots belonging to different time-interval series (GLM, interactive term Treatment x ToS x day $F_{10, 900} = 3.50$, $p < 0.001$, Table S1). A simple effect of soil amendments was seen for the abundance of *Rhizoctonia* in soil and roots (GLM, Treatment $F_{5, 900} = 19.1$, $p < 0.001$, Table S2). During plant germination (first week after sowing), the copy number of total fungi was increased in soil treated with oak, beech and paper pulp, as compared to the control, independently of the time interval between amendment and sowing. At the end of the plant growth period, fungal copy number was still higher as compared to the control for oak- and beech-amended soil, whereas paper pulp-, hair meal- and shrimp meal-amended soil had lower fungal copy number (Fig. 7, Tab. 3). Fungal colonization of roots at the end of the growth period was comparable for plants grown in control, oak-, beech- and paper pulp-amended soil, while hair meal (T1) and shrimp meal (T1 and T2) amendments resulted in lower fungal abundance in plant roots (Fig. 7, Tab. 3). An increase in fungal abundance was seen also by ergosterol measurement for control, oak- and paper pulp-amended soil (Fig. S1). Higher copy numbers of *Rhizoctonia* than in the control were detected in soil amended with paper pulp during the first week after amendment (Fig 8). Roots of seedlings sown at T1 did also contain higher *Rhizoctonia* numbers in the paper pulp amended soil (Tab. 3). For T2, plants grown in soil amended with beech, oak, hair meal and shrimp meal had a lower abundance of *Rhizoctonia* in roots as compared to the control (Fig. 8, Tab. 3).

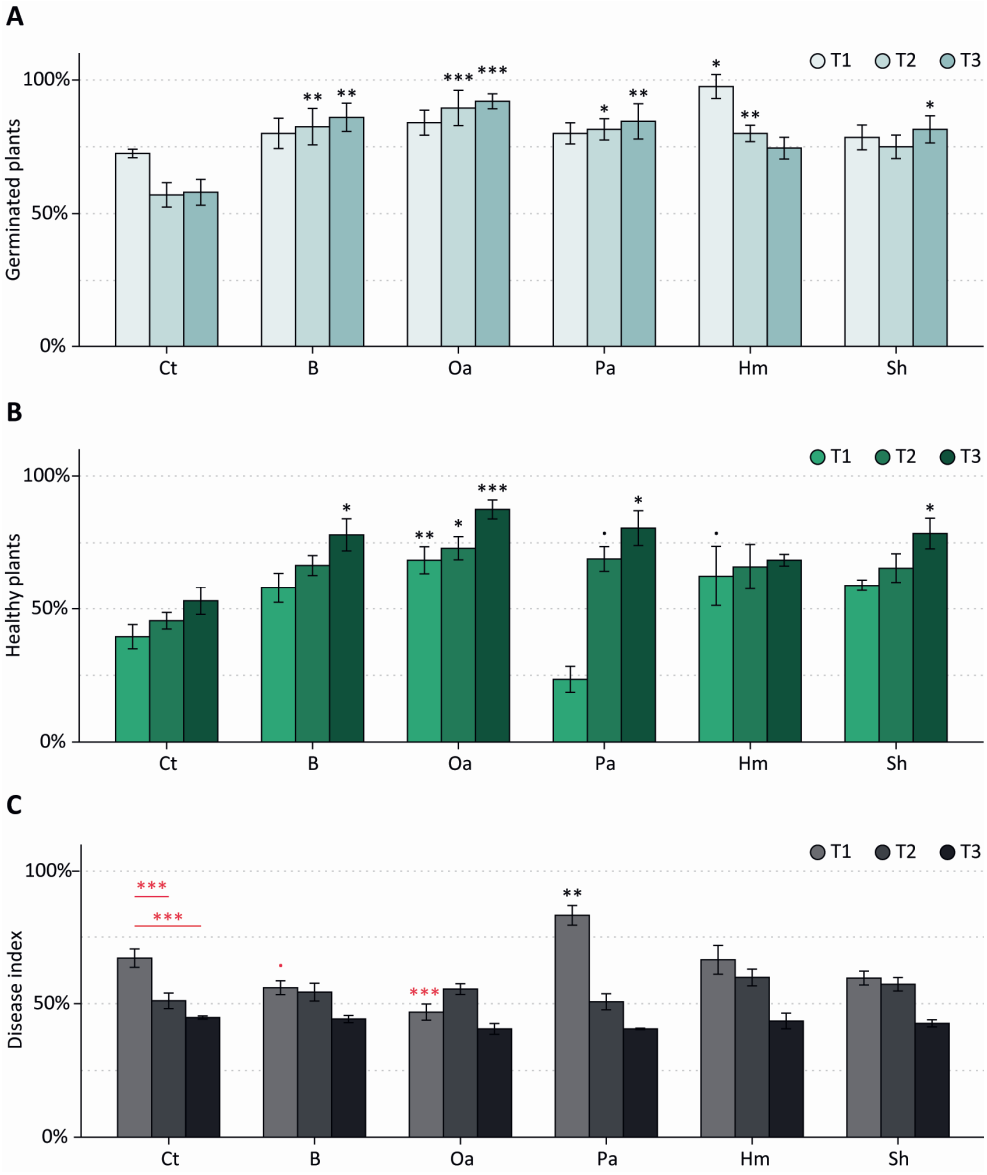

Figure 6. Performance of red beet seedlings in soil amended with five organic materials, with three time intervals (T1=1 day, T2=14 days, T3=28 days) between amendment and sowing (ToS experiment). Soil treatments are indicated as: Ct=control, B=beech sawdust, Oa=Oak sawdust, Pa=Paper pulp, Hm=hair meal, Sh=shrimp meal. A) Percentage of germinated seeds and B) percentage of seeds that resulted in succesful plants. C) Disease severity index. Significant differences between each treatment and the control within each time interval, and among time intervals for the control, are indicated as • 0.1>p>0.05; * 0.05>p>0.01; ** 0.01>p>0.001, *** p<0.001. Significant increases and decreases are shown, respectively, with black and red symbols.

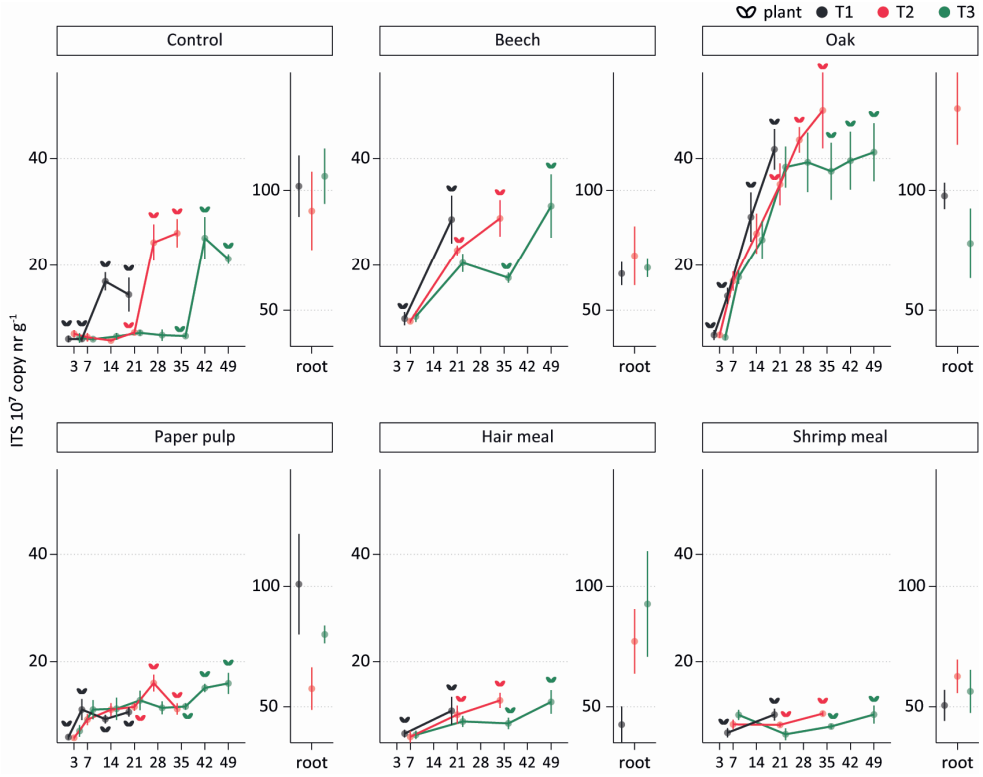

Figure 7. Quantification of fungal (ITS2) copy numbers in soil amended with five organic materials and sown at three time points after amendment. Fungal copy number was determined in soil of both planted and unplanted pots after 1, 3, 7, 14, 21, 28, 35, 42 and 49 days after amendment for control, oak and paper pulp, whereas for beech, hair meal and shrimp meal data were collected for day 7, 21, 35 and 49. For all soil treatments, fungal copy numbers in the roots are shown. Plant symbols next to each data point indicates that soil samples were obtained from planted pots.

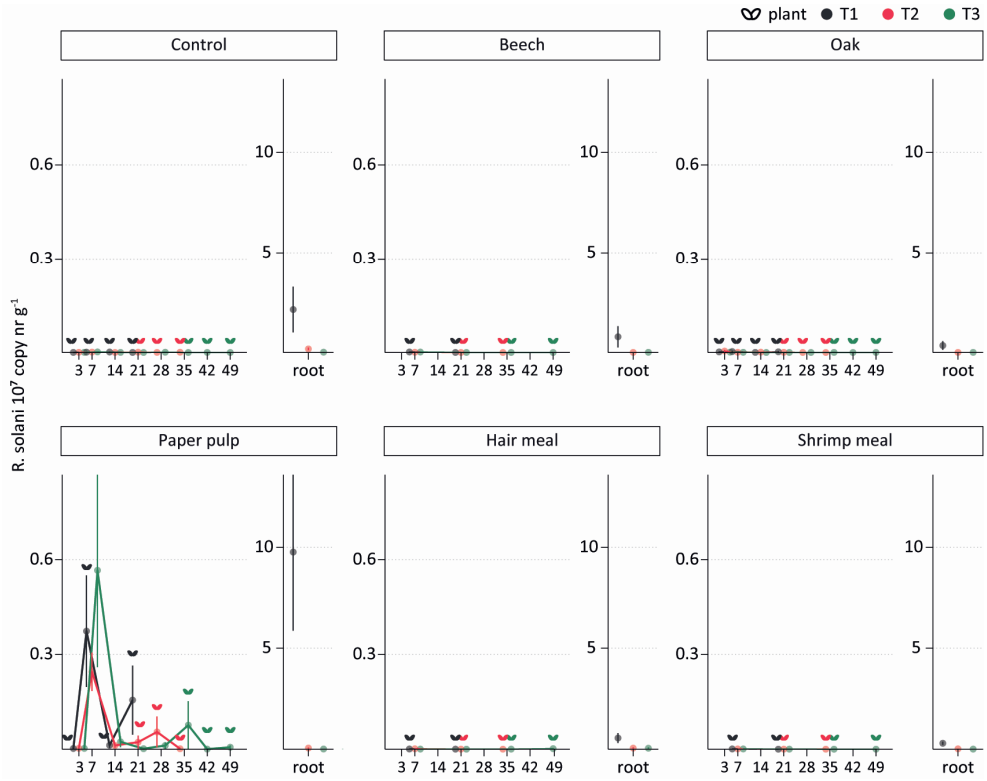

Figure 8. Quantification of *Rhizoctonia* copy numbers in soil amended with five organic materials and sown at three time points after amendment. *Rhizoctonia* copy number was determined in soil of both planted and unplanted pots after 1, 3, 7, 14, 21, 28, 35, 42 and 49 days after amendment for control, oak and paper pulp, whereas for beech, hair meal and shrimp meal data was collected for day 7, 21, 35 and 49. For all soil treatments, *Rhizoctonia* copy numbers in the roots are shown separately. Plant symbols next to each data point indicates that soil samples were obtained from planted pots.

Table 3. Fungal and *Rhizoctonia* abundance in soil and roots amended with five organic materials and sown at three time points after amendment. qPCR-based fungal (10^7 copies g^{-1} soill, mean ± sd, n = 5) and *Rhizoctonia* abundance (10^7 copies g^{-1} soill, mean ± sd, n = 5) is shown for the control and each treatment in soil sampled druring plant germination (one week after sowing), at harvesting (three weeks after sowing) and in root samples. For each column, summary statistics are given for two-way ANOVA. Significant differences are shown for amended soil as compared to the control. For soil 1 and root, significant differences are shown within the same sowing time (T1-T3), whereas for soil 2, the main effect of amendment is displayed. • 0.1>p>0.05; * 0.05>p>0.01; ** 0.01>p>0.001, *** p<0.001.

		Total fungi, soil sampled at germination		Total fungi, soil sampled at harvesting		Total fungi, root samples		*Rhizoctonia*, soil sampled at germination		*Rhizoctonia*, root samples	
Control	T1	6.3 ± 1.0		14.5 ± 7.0		101.8 ± 28.4		0 ± 0.0		2.1 ± 2.5	
	T2	7.5 ± 1.0		26.1 ± 5.9		91.5 ± 36.4		0 ± 0.0		0.2 ± 0.2	
	T3	6.8 ± 0.8		19.3 ± 4.9		105.9 ± 25.6		0 ± 0.0		0.02 ± 0.01	
Beech	T1	**10 ± 2.8**	.	**28.6 ± 10.0**	*	65.3 ± 10.7		0 ± 0.0		0.8 ± 1.2	
	T2	**22.8 ± 2.1**	***	**28.8 ± 7.6**		72.8 ± 27.5		0 ± 0.0		**0.01 ± 0.01**	*
	T3	**17.6 ± 2.1**	***	**33.3 ± 15.1**		67.7 ± 8.8		0 ± 0.0		0.01 ± 0.01	
Oak	T1	**14.3 ± 3.4**	***	**41.7 ± 8.5**	***	97.8 ± 12.2		0 ± 0.0		0.4 ± 0.5	
	T2	**35.2 ± 8.7**	***	**49 ± 15.8**		134 ± 33.5		0 ± 0.0		**0.01 ± 0.01**	*
	T3	**37.6 ± 11.9**	***	**41.2 ± 12.1**		78 ± 32.5		0 ± 0.0		0.01 ± 0.01	
Paper pulp	T1	**11 ± 4.4**	*	**10.6 ± 1.9**	***	100.9 ± 46.7		**0.4 ± 0.4**	**	**9.8 ± 8.7**	*
	T2	**11.5 ± 1.7**	*	**11.1 ± 2.4**		57.5 ± 19.7		0.02 ± 0.1		0.1 ± 0.1	
	T3	**11.6 ± 1.3**	**	**15.9 ± 4.4**		80 ± 8.2		0.1 ± 0.2		0.01 ± 0.01	
Hair meal	T1	6.6 ± 1.6		**10.8 ± 5.8**	***	**42.6 ± 16.8**	**	0 ± 0.0		0.6 ± 0.6	
	T2	10.1 ± 3.8		**12.7 ± 3.1**		77.1 ± 29.9		0 ± 0.0		**0.1 ± 0.1**	.
	T3	8.5 ± 2.4		**14.2 ± 6.0**		92.7 ± 49.0		0 ± 0.0		0.1 ± 0.1	
Shrimp meal	T1	6.7 ± 1.9		**10.1 ± 2.4**	***	**50.5 ± 14.3**	*	0 ± 0.0		0.3 ± 0.2	
	T2	8.2 ± 0.9		**10.3 ± 1.0**		62.6 ± 15.5		0 ± 0.0		**0.02 ± 0.03**	*
	T3	7.9 ± 1.0		**10.3 ± 3.8**		**56.4 ± 20.0**	*	0 ± 0.0		0 ± 0.0	
Treatment		$F_{5,90}$ = 72.4 ***		$F_{5,90}$ = 46.8 ***		$F_{5,90}$ = 7.0 ***		$F_{5,90}$ = 6.8 ***		$F_{5,90}$ = 7.6 ***	
ToS		$F_{2,90}$ = 29.1 ***		$F_{2,90}$ = 0.02 *		$F_{2,90}$ = 0.4		$F_{2,90}$ = 3.2 *		$F_{2,90}$ = 38.7 ***	
TreatmentxToS		$F_{10,90}$ = 4.0 ***		$F_{10,90}$ = 1.2		$F_{10,90}$ = 2.7 **		$F_{10,90}$ = 3.0 **		$F_{10,90}$ = 7.3 ***	

4. Discussion

4.1 Growth of Rhizoctonia *solani on woody substrates and paper pulp*

Among the tested pasteurized cellulose-rich materials, paper pulp was the most suitable substrate for the growth of *Rhizoctonia solani*. Paper pulp is constituted mainly of cellulose and is virtually devoid of lignin and hemicelluloses (Eriksson, 1990). Our result on *R. solani* performance on paper pulp is in line with previous findings, that report a good ability of *R. solani* to utilize pure cellulose for growth (Termorshuizen and Jeger, 2008; Bonanomi et al., 2020). Indeed, *R. solani* is known to produce plant cell-wall degrading enzymes, including cellulases (Lakshman et al., 2016; Wibberg et al., 2016), which enable it to grow both necrotrophic and saprotrophic (Termorshuizen and Jeger, 2008; Horbach et al., 2011). The lower performance of *Rhizoctonia solani* on wood sawdusts can be attributed to partial shielding of cellulose fibers by lignin. Although *R. solani* has been reported to produce ligninolytic enzymes (Bora et al., 2005; Wibberg et al., 2016), their actual ability to modify lignin in woody substrates is unclear. In addition, activity of ligninolytic enzymes may be inhibited by high N concentrations, provided in this study in the form of NPK fertilizer mixed with wood sawdust, both in Petri dishes and soil (Knorr et al., 2005; Treseder, 2008). Among wood types, performance of *R. solani* on coniferous wood (Douglas fir and cypress) was much lower than for most deciduous wood species. Conifer wood possesses more recalcitrant lignin as compared to deciduous tree species, given by a higher content in G-units and higher degree of crosslinks (Cornwell et al., 2009; Cesarino et al., 2012). In addition to this, biodegradability of wood is dependent on the composition and concentration of non-structural metabolites like terpenes, alkaloids and phenolics. High amounts of diterpenes and lignans, two of the major components of resins, are found in conifer wood and act as fungistatics or fungicides (Hart, 1989; Grayer and Harborne, 1994; Valette et al., 2017). In particular, wood from *Cupressaceae* species contain tropolones, which are among the strongest fungitoxic wood extractives (Hart, 1989; Valette et al., 2017).

Among deciduous tree species, low performance of *R. solani* was found on sawdust of walnut wood, which is reported as resistant to degradation due to the presence of gallic acid, 2.7-dimethylphenantheren and juglone (Hart, 1989; Hosseini Hashemi and Latibari, 2011). *R. solani* performance on the other deciduous tree species was higher but variable, both in terms of mycelial extension and density. This can be ascribed to differences in biodegradable components, cellulose, hemicellulose and non-toxic extractives, which have large variation in angiosperm wood (Weedon et al., 2009; Valette et al., 2017). Wood of birch, beech, maple, willow contain mostly decomposable extractives, such as simple phenolics, phenolic glycosides, fats and steroids (Rowe and Conner, 1979), which makes them not particularly resistant to degradation (Rowe and Conner, 1979; Hart, 1989; Bonanomi et al., 2017a). Oak wood

is characterized by the presence of hydrolyzable tannins (Rowe and Conner, 1979; Hart, 1989), whereas elder wood contains cyanogenic glycosides and lectins (Osbourn, 1996; Atkinson and Atkinson, 2002). These compounds are moderately toxic to some decay fungi (Scalbert, 1992; Tomak and Gonultas, 2018; Broda, 2020; Pospelov et al., 2020) and possibly have altered the growth or *R. solani* in this study, which developed relatively small or thin mycelia on oak and elder sawdust, respectively.

4.2 Effect of wood sawdusts and paper pulp on fungal biomass and beet seedling performance in pathogen-infested soil

In the bioassay with infested *R. solani* soil (WT experiment), the performance of red beet seedlings was assessed in soil that had received cellulose-rich amendments previous (2 weeks) to sowing. Clear positive effects on the number of healthy seedlings were seen for paper pulp, oak - and elder sawdust, whereas effects of holly and beech sawdust were smaller. Sawdusts from willow, hazelnut and cypress sawdust had no significant effect on the seedlings. Positive effects of woody materials and paper pulp on *R. solani* disease suppression were reported in previous studies, although these materials have also been associated with negative effects on plant performance due to N immobilization (Croteau and Zibilske, 1998; Bonanomi et al., 2020). In the current study, extra fertilization was applied and was sufficient for compensating the temporary N incorporation caused by the saprotrophic growth of fungi and bacteria.

Contrary to our hypothesis, the performance of *R. solani* on pasteurized cellulose-rich materials, as tested on the Petri dishes, was not predictive of the disease suppressive effects in *Rhizoctonia*-infected soil. For instance, *R. solani* had a moderately low *in vitro* growth on oak and elder sawdust, whereas it grew extensively on paper pulp. Despite such differences in supporting *in vitro R. solani* growth, all these materials had a positive effect on red beet seedling performance. The apparent contradiction between strong *in vitro* growth support of *R. solani* by paper pulp and inhibition of *Rhizoctonia* damping-off disease of beet seedlings by paper pulp amendment gave rise to examining the effect of timing between sowing and amendment in more detail (see section 4.3).

Most deciduous wood sawdust amendments stimulated fungal biomass in the two weeks before the seeds were sown. However, in contrast to our expectation, the extent of total fungal biomass stimulation in soil by sawdust types did not explain differences in their effect on the protection of seedlings against damping-off disease. This expectation was based on the possibility of increasing competitive and antagonistic interactions against pathogenic fungi, as a consequence of stimulation of saprotrophic fungal biomass and activity in the rhizosphere (**Chapter 4**). In a previous study, we showed that the composition of decomposer fungi stimulated by sawdust in soil can differ among deciduous tree species (Clocchiatti et al., 2020). Hence, wood chemistry likely influences the composition and activity of decomposer fungi, which in turn may

have determined the degree of disease suppression. In addition, presence of fungistatic compounds in certain sawdusts may have selected for tolerant decomposer species while inhibiting the growth of *R. solani* (Bhat et al., 1998; Collins et al., 2006; Mutabaruka et al., 2007; Pospelov et al., 2020). This may explain why oak and elder sawdusts gave a high suppression of *R. solani* damping off whereas the increase of total fungal biomass by the sawdust was relatively low.

The coniferous sawdust (cypress) used in this bioassay did not stimulate soil fungi and did also not affect the health of red beet seedlings. In an earlier study a low response of soil fungi was also seen after amendment with another coniferous sawdust (Douglas fir) (Clocchiatti et al., 2020). As indicated in the section 4.1, this may be due to composition and arrangement of lignin in coniferous wood. Yet, previous research showed that lignin extracted from conifer wood can reduce the viability of *R. solani* in soil due to the damage on its sclerotia by the oxidative action of ligninolytic enzymes (manganese peroxidases) of other soil fungi (van Beneden et al., 2010). It may be that longer incubation times of coniferous sawdust are needed to obtain a similar effect, although it is still unclear if ligninolytic fungi are commonly found in arable soils.

Pre-decomposed beech wood had similar effects on fungal biomass stimulation as fresh beech sawdust, although part of the stimulated fungi have probably been introduced with the pre-decomposed material. Yet, the effect of pre-decomposed sawdust on suppressing damping-off disease was lower than that of fresh sawdust. It may be that the fungi introduced with decomposed sawdust are already less active than the ones establishing on fresh sawdust and that this results in a decreased competitive interaction with pathogens for root exudates (**Chapter 4**).

4.3 Impact of timing of organic amendments and sowing on Rhizoctonia *population and disease dynamics*

In accordance to our hypothesis, paper pulp caused a transient increase in *R. solani* population in soil. This can be explained as a stimulation of the saprotrophic activity of *R. solani*, which is supported by strong *in vitro* growth of *R. solani* on pasteurized paper pulp. This coincided with an increased soil conductivity to the pathogen, as higher *Rhizoctonia* copy numbers were found in the soil one week after amendment and the disease could spread among seedlings germinating in the same time frame. Yet, the increase in *R. solani* numbers in soil enriched with paper pulp was relatively short-lived (< 2 weeks). In fact, pre-incubation of amended soil for two weeks before sowing was sufficient to observe a decline in *R. solani* abundance and to obtain a positive effect of paper pulp on seedling performance. The latter was seen in both the WT and ToS experiments. This suggests that antagonistic fungi or bacteria reduced the *R. solani* population during further stages of paper pulp decomposition. Such antagonistic microbial activities could have arisen in the soil either as a consequence of direct stimulation by paper pulp amendment, or they could have been triggered by

the *R. solani* growth (Chapelle et al., 2016). Similarly to our results, Bonanomi et al. (2020) observed that suppression of R. solani increased with time of cellulose decomposition in soil. Also Croteau and Zibilske (1998) reported about a transient increase in the concentration of *R. solani* propagules in soil after paper mill amendment. In both studies, the transient disease-conducive effect of cellulose had a total duration of at least four weeks. In the current study, the shorter decline of *R. solani* population and simultaneous increase of red beet seedling performance can be ascribed to the presence of an additional source of mineral nutrients. These do not only prevent the detrimental effects on nutrient immobilization on plant growth, but also cause a more rapid decomposition of cellulose and succession of decomposers (van der Wal et al., 2013).

Stimulation of *R. solani* in soil was not observed neither for oak or beech sawdust amendments, despite *R. solani* was able to grow *in vitro* on these substrates in absence of competitors. However, the exploitation of cellulose in woody material by *R. solani* was apparently not sufficient to compete successfully with saprotrophic soil fungi (Sarrocco et al., 2009). Consistently with this, improvement of plant performance was seen with oak sawdust at both early and delayed sowing after amendment. Although the effect of sawdust addition on pathogen stimulation or suppression could be different in other soils, reports on negative effects of sawdust on plant performance in other studies could be mainly due to the immobilization of nutrients (Bonanomi et al., 2020).

In comparison with the first bioassay (section 4.2), the second bioassay experiment had different results in terms of plant performance and fungal biomass stimulation, especially with paper pulp. Such a difference could be explained by changes in the fungal community composition or activity between the two soil batches, which where sampled one year apart. In addition to this, reduction in pathogen pressure occurred during the second bioassay. A gradual decline in *R. solani* propagule concentration in control pots was also observed in a pot experiment of Croteau and Zibilske (1998). *R. solani* abundance and activity is highly dynamic in soils and it can be influenced by changes in abiotic and biotic conditions when transferred from the field to the greenhouse (Anees et al., 2010).

Hair meal and shrimp meal restrained the abundance of total fungi, but had a positive effect on plant performance. This can be mainly ascribed to the stimulation of bacterial groups. The presence of plants stimulated saprotrophic fungi in the control, but such stimulation was not seen in presence of keratin and chitin amendments, indicating that they shifted the microbial abundance in soil, but also in the root, in favor of bacterial groups. Indeed, it has been shown that the disease suppressive effects of keratin and chitin are associated with an increased abundance bacterial groups such as Oxalobacteriaceae, Bacteroidetes and Mortierellomycota (Debode et al., 2016, Andreo Jimenez et al., *in review*). Based on the current study, *R. solani* is not

stimulated by addition of keratin or chitin in this soil and is outcompeted by mainly soil bacteria.

5. Conclusions and perspectives

This study indicates that cellulose-rich organic amendments have the potential of directly stimulating the saprotrophic growth of *R. solani* in soil. Transient *R. solani* stimulation by paper pulp coincided with transition from disease conductive to disease suppressive effects on beet seedling damping-off. In order to benefit from positive effects of paper pulp, it is essential to have a period of several weeks of decomposition in the soil before sowing. This pre-incubation period can be shortened with addition of fertilizer to compensate for nitrogen limitation. In contrast, the effect of woody materials on plant performance was affected by the quality of wood, which possibly had an impact on the activity and composition of saprotrophic fungi in the soil, but did not involve stimulation of *R. solani* in soil. Oak and elder sawdust were the most effective sawdust types in this study and their effect is associated with saprotrophic fungal stimulation. Conversely, positive effects of keratin- and chitin-rich amendments on soil health was not ascribed to the stimulation of either saprotrophic fungi or *R. solani*, but it is likely to be caused by bacteria. Our results indicate that deciduous wood sawdusts have great perspective for the stimulation of natural biocontrol of soil-borne fungal diseases. Yet, additional information is needed on the role of wood chemistry on suppression of pathogens and/or stimulation of antagonistic activities of microbes in arable soils.

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Supplementary information

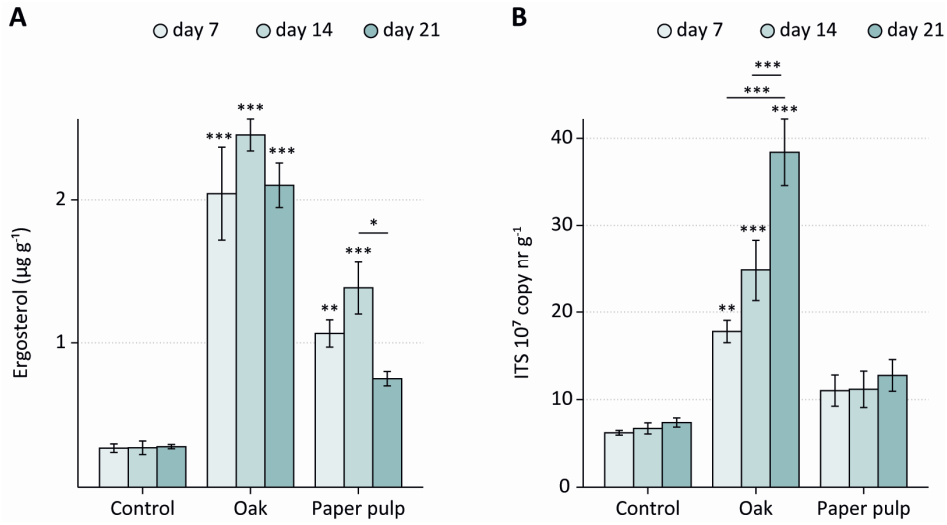

Figure S1. Fungal abundance in control soil and soil amended with oak and paper pulp, as based on qPCR and ergosterol measurement (ToS experiment). Fungal abundance is shown for pots of the T3 series, sampled on day 7, 14 and 21. Significant differences between each treatment and the control are indicated on the top of each bar as • 0.1>p>0.05; * 0.05>p>0.01; ** 0.01>p>0.001, *** p<0.001. Whereas comparisons among days within each treatment are shown above horizontal lines.

Table S1. Effect of different organic amendments on qPCR-based fungal abundance. Pots were planted at three time intervals after amendment (ToS) and fungal abundance was measured in soil at consecutive days during soil incubation and plant growth (day). Results of ANOVA after generalized linear model with gamma distribution of errors. R^2 = 0.83 (Hosmer-Lemeshow). Model χ^2 = 350.3, p < 0.001.

	df	deviance	Residual df	Residual deviance	F	Pr(>F)	
Null			509	487.6			
ToS	2	1.0	507	486.6	2.9	0.06	.
Treatment	5	30.8	502	455.8	35.2	$< 2\ 10^{-16}$	***
day	1	307.3	501	148.5	1,757.2	$< 2\ 10^{-16}$	***
ToS x Treatment	10	3.5	491	144.9	2.0	0.03	*
ToS x day	2	0.9	489	144.0	2.6	0.07	.
Treatment x day	5	54.1	484	89.9	61.9	$< 2\ 10^{-16}$	***
ToS x Treatment x day	10	6.1	474	83.9	3.50	$0{,}2\ 10^{-3}$	***

Table S2. Effect of different organic amendments on qPCR-based abundance of *Rhizoctonia*. Pots planted after three time intervals after amendment (ToS) and *Rhizoctonia* abundance was measured in soil at consecutive days during soil incubation and plant growth (day). Results of ANOVA after generalized linear model with Poisson distribution of errors. $R^2 = 0.85$ (Hosmer-Lemeshow). Model $\chi^2 = 470.3$, $p < 0.001$.

	df	**deviance**	**Residual df**	**Residual deviance**	**F**	**Pr(>F)**	
Null			506	553.0			
ToS	2	149.8	504	403.2	74.9	$< 2\ 10^{-16}$	***
Treatment	5	95.7	499	307.5	19.1	$< 2\ 10^{-16}$	***
day	1	191.3	498	116.1	191.3	$< 2\ 10^{-16}$	***
ToS x Treatment	10	1.6	488	114.5	0.2	1.00	
ToS x day	2	24.5	486	90.0	12.3	$4{,}8\ 10^{-6}$	***
Treatment x day	5	6.9	481	83.0	1.4	0.22	
ToS x Treatment x day	10	0.4	471	82.7	0.04	1.00	

Chapter 6

General Discussion

Saprotrophic soil fungi contribute to several important ecosystem functions in natural ecosystems, but their abundance in arable soils is mostly low. Therefore, management practices are needed that effectively increase growth and activity of saprotrophic fungi to also benefit from this group of organisms in arable soils. In this thesis, I examined soil management options focused on the stimulation of saprotrophic fungi in arable soils. In addition, I studied aspects of the effects of such stimulation on soil ecosystem functioning. In this chapter I summarize the results obtained during my thesis study and place them in a broader context. Finally, I give an outlook on future research directions and practical scenarios.

Characteristics of fungus-stimulating organic inputs in arable soil

Cellulose, hemicellulose and root exudates

The biochemical quality of organic materials largely determines decomposition rate and decomposer community composition (Dighton, 2007; Baumann et al., 2009; Talbot and Treseder, 2012). Cellulose and hemicelluloses are the main sources of energy for many saprotrophic fungi, and therefore cellulolytic fungi usually dominate the initial decomposition of solid plant materials (van der Wal et al., 2013; Sarrocco, 2016; Koechli et al., 2019). In line with this, a stimulation of fungal biomass was detected in arable soils after the amendment of sawdusts and paper pulp (**Chapter 2** and **Chapter 5**). Conversely, materials with a low amount of cellulose did not stimulate fungal biomass. This was the case for animal-derived materials and processed plant materials, for which processing – i.e. composting, fermentation, extraction, and roasting – probably caused depletion of accessible cellulose sources and/or enriched the materials with toxic compounds (Goss et al., 2013; Scotti et al., 2015). In contrast, wood sawdust and paper pulp harbor a large fraction of polysaccharides (Baumann et al., 2009; Cornwell et al., 2009). In a litterbag study, it was shown that fragmented woody material of birch trees can sustain high fungal biomass for long periods in abandoned arable soils (van der Wal et al., 2007). Overall, **Chapter 2** and **5** report a sustained fungal biomass stimulation in response to sawdust amendment in five distinct arable soils. Furthermore, in a study not included in this thesis, I observed that fungal biomass stimulation in arable soil upon addition of beech sawdust was still present after six months of incubation. The results obtained in this thesis study illustrate that the ability of resident fungal communities to degrade relatively complex materials is common, not only in recently abandoned arable soils, but also in soils currently used for production.

A small particle size was used for wood and all the other materials throughout this thesis. Milling wood into a small particle size increases the fungal access to cellulose and hemicellulose, which are normally locked in a lignin matrix (van der Wal et al., 2007; Koranda et al., 2014). Some ascomycetal fungi can penetrate wood blocks by

producing thin hyphae, whereas brown-rot and white-rot basidiomycete fungi can degrade or alter the structure of lignin and gain access to carbohydrates (van der Wal et al., 2013). It is not clear whether arable soils harbor such ligninolytic basidiomycetal fungi nor if these can become active in arable soils (Entwistle et al., 2018). Throughout this thesis research, the utilization of sawdust was probably important to increase the access of fungi to cellulose and ensure a homogeneous stimulation of fungi throughout the matrix of the amended soil.

In agreement with previous studies, the supply of additional N was essential for the initial growth of fungi on cellulose-rich materials (van der Wal et al., 2007) and for preventing the depletion of soil N pools by immobilization into the microbial biomass. Results also confirmed that the adjustment of the C:N ratio of wood or paper pulp amendments to ca. 15:1 is sufficient to enable both plant and fungal growth (**Chapters 4** and **5**, Mohanty et al., 2013; Toenshoff et al., 2014; Gad et al., 2015). Yet, the studies in this thesis did only deal with initial plant growth stages. Based on the work of Reichel et al., (2018), a temporal partial immobilization of nutrients, followed by a return to the soil can be expected. More experimental data are needed to determine the fluctuations of nutrient pools after soil treatment with cellulose-rich materials and their effects on plants growth in later developmental stages.

Simple carbon compounds, as those released by plant roots or present in dried, fresh plant materials (Baumann et al., 2009), favor the growth of fast-growing fungi (i.e. members of Ascomycota, Mucoromycota and basidiomycete yeasts). Indeed, vetch and radish caused a strong stimulation of saprotrophic fungi (*Mortierellaceae* and *Chaetomiaceae*), which was followed by a rapid decline in fungal biomass (**Chapter 2**). Fast-growing fungi are also found, together with bacteria, as active consumers of compounds exuded by plant roots (Hugoni et al., 2018; Hannula et al., 2012, 2020). **Chapter 4** illustrated that sawdust-stimulated fungi were able to compete for root exudates, despite the distinction in complexity between sawdust and root-derived compounds. It was indeed previously observed that saprotrophic fungi of arable soils can bridge small distances between the detritusphere and the rhizosphere (Marschner et al., 2012). The soil conditioning with sawdust was, therefore, effective in increasing the biomass of fungi in the rhizosphere of crop seedlings, which otherwise is low or takes a longer time to reach high levels, for plants growing in arable soils (Hannula et al., 2010; Hünninghaus et al., 2019). Assuming that the ascomycete fungi stimulated by sawdust were mainly cellulose and hemicellulose decomposers, they are well equipped to metabolize also sugars in root exudates. This is in line with coinciding responses of soil fungi to addition of cellulose and glucose, as reported by van der Wal et al. (2006a). Interestingly, the addition of simple phenolic compounds did not increase the fungal consumption of simple primary metabolites and root exudates (**Chapter 3,** see next section).

Lignin, phenolic acids and other secondary metabolites

Wood scraps, prunings and sawdust can be obtained from a variety of sources in industry and forestry (NL Agency Energy and Climate Change, 2013; EOS, 2019) and therefore include wood of various quality, depending on tree species, wood growth stage and geographical location (Rowe and Conner, 1979; Cornwell et al., 2009). Ultimately, wood chemistry determines its decomposability (Hart, 1989; Weedon et al., 2009; Valette et al., 2017). **Chapter 2** and **5** report the comparison of wood sawdust from different tree species and show how the extent and dynamics of fungal biomass stimulation varied based on the type of wood. Fungal biomass stimulation depends not only on the amount of available carbohydrates (see previous section), but also on the type of biochemical decomposition barriers such as presence of lignin and secondary metabolites. Lignin is markedly distinct between conifer and deciduous tree species (Jin et al., 2006; Cornwell et al., 2009). Furthermore, secondary metabolites present in wood extractives can range from highly toxic to mildly or non-toxic to fungi (Rowe and Conner, 1979; Hart, 1989; Broda, 2020). Fungitoxic compounds, together with a recalcitrant form of ligning (guaiacyl lignin) probably contribute to the low fungal stimulation in arable soil by conifer sawdust (**Chapter 2** and **5**). For the deciduous species, wood containing tannins (oak) and cyanogenic compounds (elder) may have resulted in a slower buildup of fungal biomass and had possibly a modulating effect on fungal communities (**Chapter 5**). Overall, the effect of woody inputs on soil fungi could potentially be predicted by the quantification of markers representative of carbohydrates (e.g. O-alkyl carbon in ^{13}C-NMRS spectra), lignin (e.g. ratio of guaiacyl : syringil lignin) and also by secondary metabolites present in wood extractives.

Among wood secondary metabolites, phenolic acids are considered to be of mild or low toxicity. Many fungi possess the ability to detoxify and utilize simple phenolic compounds (Shalaby and Horwitz, 2015), which they encounter not only in wood, but also in plant litter and root exudates (Narasimhan et al., 2003; Suseela et al., 2016; Wang et al., 2016). Therefore, in **Chapter 3** I tested the hypothesis that phenolic compounds would favor saprotrophic fungi in the rhizosphere and increase their competitive ability to consume primary metabolites in root exudates. Phenolic acids acted as modulators of the fungal community composition (see next section), but did not lead to an increase in fungal abundance the rhizosphere. A lack of fungal biomass stimulation was seen for both the simulated rhizosphere and in the rhizosphere of wild type *Arabidopsis thaliana* as compared to mutants. The amount of energy that fungi can obtain from phenolic acids for growth is low as compared to glucose and cellobiose (di Lonardo et al., 2017). The low concentrations of phenolic acids used in this study and found in root exudates and soils (Narasimhan et al., 2003; Zhou et al., 2012) are possibly not sufficient to increase the competitive ability of fungi in the rhizosphere. In comparison, larger inputs of phenolic acids, as found in plant litter and its leachates, can boost the growth of saprotrophic fungi (Suseela et al., 2016). In

addition to this, tolerance and utilization of phenolic acids is not a prerogative of fungi, as it is also found among bacteria (Blum and Shafer, 1988; Waldrop and Firestone, 2004; di Lonardo et al., 2017). This further contributes to the small steering effect of phenolic acids on rhizosphere fungi. Based on this, breeding crops for higher exudation rates of phenolic acids does not appear to be a suitable strategy for boosting the biomass of rhizosphere fungi. The only exception was salicylic acid, which in combination with primary root metabolites resulted in higher fungal biomass and lower bacterial abundance. This suggests that salicylic acid, a plant hormone, plays a yet-unknown role as a regulator of plant-associated fungi and bacteria. Interestingly, willow plant parts, including wood, contain a relatively high salicylic acid content (Rowe and Conner, 1979). Despite this, fungal biomass stimulation after willow sawdust amendment was not higher than other easily decomposable wood types, such as hazel and poplar (**Chapter 2** and **5**). This gives further support for predominant cellulose-driven fungal biomass stimulation by wood, while lignin and secondary metabolites play a role in inhibiting the access to cellulose by fungi and/or their activity.

Organic inputs shape fungal and bacterial communities in soil and rhizosphere

Fungal communities

Both cellulose-rich amendments and phenolic acids favored members of the fungal community belonging to Sordariomycetes (**Chapter 2, 3** and **4**). Sordariomycetes are very common ascomycete fungi in arable soils and are among the fungal groups utilizing cellulose inputs (Banerjee et al., 2016; Gkarmiri et al., 2017; Koechli et al., 2019). In line with this, the work in this thesis showed that sordariomycetal fungi are well-equipped for efficient decomposition of sawdust of deciduous tree species and paper pulp (**Chapter 2**). Moreover, they were also responsive to root exudates (**Chapter 3 and 4**), but their relative abundance in the rhizosphere was favoured by the presence of relatively complex compounds, i.e. cellulose and phenolic acids (**Chapter 3** and **4**). Indeed, Sordariomycetes are well represented in the rhizosphere and root of crops, but tend to associate with plants at later developmental stage (Hannula et al., 2012; Han et al., 2017), possibly coinciding with a more elevated deposition of root debris and complex root exudates (Chaparro et al., 2013; Pausch and Kuzyakov, 2018; Zhalnina et al., 2018). **Chapter 4** proved that these fungi have sufficient metabolic flexibility to extend from sawdust to root exudate utilization in the rhizosphere of young crop plants.

For both cellulose-rich materials and phenolic acids, a higher relative abundance of Sordariomycetes coincided with a smaller increase of Mortierellomycota in the

rhizosphere, relative to the total fungal community. Mortierellomycota were highly responsive to root exudates (**Chapter 4**), to the addition of simple primary root metabolites (**Chapter 3**) and to the amendment with non-woody, low C:N plant materials (**Chapter 2**). Indeed, Mortierellomycota include many fast-growing fungi, which consume simple carbon compounds in rhizosphere, roots and plant litter (van der Wal et al., 2013). They are also reported to participate in very early phases of cellulose degradation (e.g. few days after addition), followed by Ascomycota (Poll et al., 2010; Koechli et al., 2019).

Sordariomycetes contain both saprotrophic and pathogenic species, therefore the taxonomical analysis of community composition was combined with the analysis of potential functional guilds (Nguyen et al., 2016). Given that the assignment to functional guilds based on sequences carries a degree of uncertainty, it was performed in a conservative way, namely by assigning to the broader category of "potential pathogens" represented by taxa that contain both pathogenic and saprotrophic fungi (e.g. *Fusarium*). This confirmed that saprotrophic Sordariomycetes were favoured by the addition of paper pulp and sawdust (**Chapter 2**), while phenolic acids stimulated groups harboring both saprotrophs and potential pathogens (**Chapter 3,** Chen et al., 2018). The steering effect of phenolic acids in favor of Sordariomycetes was smaller in size, as compared to cellulose-rich materials, and it was targeted to few genera, in particular *Fusarium*, *Fusicolla* and *Trichoderma*, which include fungi that often colonize the plant endophytic compartment (**Chapter 3**). Phenolic acids are downstream elements of the plant immune system, while salicylic acid is one of the most important hormones initiating plant immune responses (Shalaby and Horwitz, 2015). Earlier studies already showed that phenolic acids regulate the concentration of microbes in internal tissues, which in turn have the ability to tolerate and establish a balance with the plant immune system (Qi et al., 2012; Lahrmann et al., 2015; Chen et al., 2018). Similarly as seen for bacteria (Lebeis et al., 2015), **Chapter 3** suggests that phenolic acids and salicylic not only regulate fungal communities inside plant tissues, but also participate in the assembly of fungal community in the rhizosphere. Perhaps, phenolic acids recruit specific fungi by chemoattraction. However, fungal receptors for phenolic compounds have not yet been examined (Shalaby and Horwitz, 2015).

Bacterial communities

The detritusphere and the rhizosphere are important hotspots of microbial activity in soil, where soil functions are determined as a result of a multitude of microbial interactions (Kuzyakov and Blagodatskaya, 2015; de Boer, 2017). In particular, the presence of active saprotrophic fungi in soil and rhizosphere is thought to have steering effects on bacterial communities and bacterial activities, as a consequence of increased complexity of interactions, such as competition, antagonism and predation (de Boer et al., 2015; Ballhausen and de Boer, 2016; de Menezes et al., 2017). The presence of active fungi in the rhizosphere, as stimulated by sawdust addition, did not

affect the uptake of plant-derived C in the bacterial biomass (**Chapter 4**). This implies that sawdust-stimulated fungi incremented the C flow into the soil and that fungi and bacteria occupied partially distinct niches in the root surroundings. This is in line with observations in late-successional ex-arable soils (Hannula et al., 2017; Morriën et al., 2017). As a consequence, the changes in bacterial community composition observed in the root, rhizosphere and soil cannot exclusively be attributed to an increased competition between fungi and bacteria for root exudates. The increase in relative abundance of Bacteroidia in the root and soil could be due to fungal-bacterial competition or feeding of bacteria on fungal cell-wall components, as many Bacteroidia are engaged in antagonistic interactions and produce chitinolytic enzymes (de Boer et al., 2007; Wieczorek et al., 2019). On the other hand, the higher proportion of Rhizobiales and arbuscular mycorrhizal fungi in roots, suggests that facilitative or even mutualistic interactions between fungi and these microbial groups took place in response to the addition of sawdust (Hoppe et al., 2015; Johnston et al., 2016; Xu et al., 2018b; Zhang et al., 2020). Overall, the addition of sawdust not only stimulated fungal activities in soil and in association with plants, but possibly enriched the complexity of microbial interactions.

Resident versus exogenous members of microbial communities

In this thesis, the changes in size and composition of microbial communities upon organic soil amendments were referred to as changes in "resident" soil microbes. However, the organic materials were mixed into the soil without prior sterilization or pasteurization. Hence, fungi and bacteria, naturally present in dry plant materials, e.g. as spores, will have been introduced (**Chapter 5**, observation not shown). This may have contributed to the observed changes in microbial community composition, as exogenous microbes can modify microbial communities composition even when their establishment in the soil is not successful (Massart et al., 2015; Mawarda et al., 2020). The choice to use non-sterilized of organic amendments was made to have a study representative for practical applications. The conclusions about which materials promote saprotrophic fungi in soil the most will not be changed by a possible contribution of introduced microbes. Yet, the use of untreated organic materials can include the risk of either introducing or stimulating potential pathogens by the amendment of plant parts (see next section).

Effect of organic inputs on pathogenic fungi and plant performance

Organic amendment inputs can bear the risk of introducing and/or stimulating the growth of soil-borne pathogenic fungi. Soil-borne pathogens are stimulated and attracted by simple compounds in root exudates, but several pathogens also possess an array of exoenzymes that enables them to utilize plant residues (Termorshuizen and Jeger, 2008; Lakshman et al., 2016). This is considered one of the main reasons

why the addition of immature compost or easily degradable plant materials can increase the conductivity of soil to soil-borne diseases (Henis et al., 1967; Lockwood, 1977; Termorshuizen and Jeger, 2008; Bonanomi et al., 2010). In **Chapter 2, 3** and **4** the relative abundance of potential plant pathogens was evaluated by the analysis of amplicon sequencing data. This method is limited by the fact that it quantifies relative abundances, which can mask changes in absolute abundances of pathogens. Moreover, sequence-based identification of fungal taxa and guilds does not allow to verify the actual pathogenicity of fungi. Despite these limitations, amplicon sequencing can rapidly provide a global picture of fungal communities in soil (Agtmaal et al., 2017; Hannula et al., 2017). **Chapter 2** and **4** show no evidence of increase in potential pathogen populations in soil after the addition of paper pulp, woody and non-woody plant materials, with the exception of radish. Only for radish, an increase in *Plectosphaerella* spp. was detected. Sawdust amendment caused no increase and even led potentially to a decrease in relative abundance of the sub-community of pathogens in soil (**Chapter 2**) and in the rhizosphere of carrot seedlings (**Chapter 4**). Furthermore, carrot seedlings appeared healthy, and their biomass and nitrogen content were not diminished by the soil treatment and fungal stimulation. Nevertheless, given that the total fungal biomass increased in soil and rhizosphere, it cannot be excluded that some pathogens populations increased in terms of absolute abundance. In order to get more insight in this matter, it was decided to determine growth dynamics and pathogenicity of *R. solani* (**Chapter 5**) in response to cellulosic and woody organic amendments.

In **Chapter 5**, it was shown that paper pulp induced a transient growth of *R. solani* seven days after amendment. This coincided with a poor performance of early-sown beet plants and is in agreement with previous reports (Croteau and Zibilske, 1998; Bonanomi et al., 2020) and with the already reported suitability of cellulose as a substrate for *R. solani* (Termorshuizen and Jeger, 2008). In **Chapter 2**, *R. solani* stimulation was not detected based on sequences. However, as the earliest sampling point was 14 days after paper pulp amendment, it is possible that the *R. solani* copy numbers were already reduced, as shown in **Chapter 5**. In addition, sequencing can fail to detect low-abundant taxa and their variations in relative abundance, especially when masked by large increases in the proportion of other taxa (e.g. *Chaetomiaceae* with paper pulp, **Chapter 2**).

Although *R. solani* was able to utilize wood sawdust for growth in isolation, its abundance in soil was not increased by the addition of sawdust (**Chapter 5**). This was accompanied by either unaltered beet plant performance (with beech sawdust) or improved plant performance (with oak sawdust). This suggests that the resident saprotrophic fungi were better competitors for woody substrates in soil. For instance, fast-growing fungi other than *R. solani* could have outcompeted the pathogen in the earliest phases of colonization of wood particles (Poll et al., 2010). Resource competition by active non-pathogenic fungi could have extended to the rhizosphere

of plant seedlings (**Chapter 4**) resulting in limited invasion of roots by *R. solani*. In addition to this, the suppression of pathogen growth may be attributed to inhibitory activities, such as antibiosis or mycoparasitism, by stimulated saprotrophic fungi (Punja and Utkhede, 2003; Xiong et al., 2017) or co-stimulated bacteria (e.g. Bacterioidia, **Chapter 4**, (de Boer et al., 2015; Deveau et al., 2018).

Research and utilization perspectives

In this thesis research, I showed that woody and paper pulp amendments can boost the biomass of saprotrophic fungi in a time scale that is relevant for practice (**Chapter 2**, **4** and **5**) and that these amendments, when applied at a proper timing, can be used for improving plant performance in soils infested with *R. solani* (**Chapter 5**). Since the experimental work was conducted in greenhouse conditions, these fungus-stimulating amendments need to be tested in field conditions. In addition, other crop-pathogen combinations and the effect on plant health at later developmental stages should be considered in follow-up experiments.

Chapter 3, together with recent works (Shalaby and Horwitz, 2015; Chen et al., 2018; Zhou and Wu, 2018), indicates that salicylic acid and phenolic acids are important for regulating plant-fungus interactions. More research is needed to understand the mechanisms of recruitment and assembly of root-infecting fungi, for instance the presence of receptors for phenolic acids in fungi has been not yet investigated (Shalaby and Horwitz, 2015). This could be important for understanding how pathogenic and beneficial fungi assemble in the rhizosphere and root interior.

Chapter 2 evidenced a capture of nitrogen pools by fungi stimulated by sawdust and paper pulp. This points at the possibility of using these amendments for retaining nitrogen in the soil, after cash crops have been harvested (Reichel et al., 2018). To this end, cover crops are grown in the winter period, but this is often not sufficient for preventing nitrogen losses. The use of cellulose-rich amendments could be used instead or in combination with winter cover crops. Changes in soil structure (Bulmer et al., 2007) and a buildup of soil carbon pools could also be expected in the long term after the addition of cellulose-rich materials, as a result of the presence of residual fractions of the added materials and recalcitrant fungal-derived compounds in the soil matrix.

Organic substrates are essential not only to fuel the growth of resident microbes, but also to enhance the establishment of microbes that are introduced into a soil (Bonanomi et al., 2018). The possibility of introducing exogenous microbes in the field as a tool for steering the resident communities and their functioning has been recently proposed (Massart et al., 2015; Mawarda et al., 2020). In this context, I highlight that cellulose-rich materials could be considered as a medium for facilitating the

establishment of exogenous microbes and their interactions with resident communities.

In practice, cellulose-rich organic amendments represent a feasible option for improving arable soils in a relatively large-scale. Paper pulp is an abundant waste product of the paper industry. After some rounds of recycling, cellulose fibers in paper pulp become too short for further utilization for paper. Mostly, exhausted pulp ends up in landfills. The redirection of this cellulose mass to arable field has already been highlighted (Curnoe et al., 2006). In this thesis, I provide further indications on the effect that paper pulp has on both saprotrophic fungi and pathogenic fungal populations. In agreement with earlier studies (Croteau and Zibilske, 1998; Bonanomi et al., 2020), in **Chapter 2** and **5** I show how paper pulp can boost soil fungi and improve the control of *R. solani* and plant performance with a proper management of nitrogen stocks and timing of paper pulp application.

Clean sawdust, wood scraps and chips are waste materials produced by wood manufacture industry, forest and landscape management (NL Agency Energy and Climate Change, 2013; EOS, 2019). In the Netherlands, the yearly production of clean wood waste is estimated as ca. 2 Mton. Most of the wood is being used for other markets, such as production of panels, floors, animal litter and pellets for "biofuel" (NL Agency Energy and Climate Change, 2013). If only 10% of the yearly wood waste would be directed for use in agriculture (i.e. 0.2 Mton), 26 000–40 000 ha of soil could be treated every year, considering an application rate of 5–7.5 ton ha^{-1}. This rate of application has been calculated based on the addition of 5 g sawdust kg^{-1} soil for a soil layer of 10 cm, with a bulk soil density range of 1–1.5 g cm^{-3}. Given a price of sawdust of ca. 150 € ton^{-1} (Visser et al., 2020), such a soil treatment would cost 750–1125 € ha^{-1}. For comparison, compost is one of the materials most widely used as a soil improver. The current demand by utilizers in the Netherlands corresponds to 1.5 Mton $year^{-1}$ (the Netherlands Enterprise Agency, 2019). Considering that the rate of application is 20–25 ton ha^{-1}, compost is currently used for treating 60 000–75 000 ha of arable soil. In addition to this, it must be considered that lower application rates of sawdust, i.e. down to 2.5 g kg^{-1} soil, also resulted in fungal biomass stimulation (data not shown) and precision application of sawdust in strips could also be tested for controlling damping-off diseases and driving the assembly of plant-associated communities during the early phases of plant growth. Overall, the use of sawdust could be considered as an additional option for improving arable soils.

According to a survey from 2013 (NL Agency Energy and Climate Change, 2013), 1 Mton $year^{-1}$ of wood waste was used for energy production. Recently, this amount has been increased by import. Unfortunately, the additional wood biomass is derived from clear cutting of native forests or tree plantations, harvested for the sole purpose of energy production (Schulze et al., 2012). This biomass cannot be considered as a by-product, but is an actual product. This type of business disrupts ecosystems and soil functioning, which could take decades, if not centuries, to fully recover (Susyan et al.,

2011). A better way for expanding the amount of sawdust available for use in arable soils, would be to derive it on a local basis, for instance by the establishment of agroforestry systems. The presence of trees brings several advantages to the landscape and carbon sequestration (Kay et al., 2019). The wood obtained from yearly pruning could be delivered to close-by arable soils. This could provide material for a possibly smaller, yet regular (Bonanomi et al., 2017b) application of wood biomass to arable soils.

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Summary

Samenvatting

Sommario

Summary

Intensively managed agricultural production of food and fiber does often coincide with negative side-effects such as environmental pollution and soil degradation. The integration of ecology-based agricultural practices has a promising perspective to bring agriculture to a more sustainable level. Many studies point at the importance of re-integrating traditional agricultural practices, such as the use of organic amendments and crop diversification (intercropping, wider crop rotations, cover crops). Other lines of research explore the possibilities of soil inoculation with microbial strains as biofertilizers and biopesticides. Arable soils harbor a diverse community of saprotrophic fungi, however their biomass and activity is mostly limited. This is due to intensive management practices, such as the use of synthetic rather than organic fertilizers, use of chemical pesticides and mechanical disturbances of soils. Having an active community of saprotrophic fungi is essential for soil functioning, as can be seen in natural and semi-natural ecosystems. In this Ph.D. thesis study, I explore options for boosting the biomass of saprotrophic fungi in arable soils. I also test the impact of stimulation of saprotrophic fungi on plants and other soil microbial inhabitants, including bacteria, pathogenic- and mycorrhizal fungi.

Saprotrophic soil fungi can respond to organic inputs both in the form of dead plant parts (litter) and rhizodeposits derived from living roots. Therefore, two possible approaches were examined for the stimulation of saprotrophic fungi in arable soil, namely the addition of exogenous organic substrates and the modification of root exudate composition.

In a series of pot experiments, organic materials of varying quality and origin were amended into arable soil, to test the response of fungi in terms of biomass and community composition (**Chapter 2**). Based on a first screening, paper pulp and beech wood sawdust amendment resulted in a rapid increase in ascomycete saprotrophic fungi (mainly Sordariomycetes), which persisted for a period of at least two months. Using the same setup, I further tested the effect of wood sawdusts from distinct tree species, alone or in combination with mineral nitrogen. The fungus-stimulating effect of wood sawdust amendment was also tested in four arable soils with different soil characteristics. The results revealed that saprotrophic fungal communities of arable soils retain a good ability to respond to wood sawdust of deciduous trees, but not to sawdust of coniferous tree species. Combination of sawdust with mineral nitrogen resulted in extra stimulation of saprotrophic fungi.

The aim of **Chapter 3** was to test the effect of phenolic root exudates on the biomass and community composition of rhizosphere fungi. To this end, an artificial rhizosphere model system was established. It allowed to introduce artificial exudate mixtures via diffusion into the soil, thereby simulating root exudation. In this way, phenolic acids were added both alone and in combination with a mixture of primary root exudate metabolites. Phenolic acids were applied at a rate consistent with the

levels found for phenolic acids deposited in soils (0.02 - 0.2 mg g^{-1} soil). Phenolic acids had little effect on the total fungal biomass in the simulated rhizosphere. The same result was obtained in a follow-up experiment, where rhizosphere fungal biomass was measured for *Arabidopsis thaliana* mutants with an altered composition of phenolic root exudates. This indicates that selecting crop varieties for a higher exudation of phenolic acids is not a promising strategy for promoting saprotrophic fungi in the rhizosphere of crop plants. This study also showed that phenolic acids promote specific root-infecting fungi (*Fusarium*, *Fusicolla* and *Trichoderma*) in the simulated rhizosphere. Consequently, this indicates phenolic root exudates as potential modulators of rhizosphere and root fungal communities.

The composition and activity of microbes in bulk soil has a strong influence on the assembly of microbial communities in the rhizosphere and roots. Having established that wood sawdust amendment causes an effective stimulation of saprotrophic fungi in arable soil, **Chapter 4** showed how this affects the biomass, activity and composition of microbial communities associated with crop seedlings. $^{13}CO_2$-pulse labelling of carrot seedlings grown in sawdust-amended and control soil revealed that sawdust-stimulated fungi in soil can extend into the rhizosphere and actively consume plant-derived carbon. The total bacterial abundance and active uptake of root exudates was not affected by sawdust addition, while AMF abundance and activity were promoted. The analysis of rhizosphere and root-associated communities using DNA(-SIP) illustrated that Sordariomycetes, Glomeromycetes, Bacteroidia and Rhizobiales increased in relative abundance. Based on sequencing data, the sub-community of potential pathogenic fungi did not increase relative to the total fungal community. Hence, the conditioning of arable soil with sawdust promotes a higher activity of saprotrophic fungi in the rhizosphere, thereby steering root-associated microbial communities.

After individuating fungus-stimulating organic amendments (**Chapter 2**), assessing their ability to stimulate rhizosphere-competent fungi and describing their effects on bacteria (**Chapter 4**), I studied if the stimulation of saprotrophic fungi by woody materials and paper pulp can be used to control *Rhizoctonia solani*, one of the most widespread causal agents of soil-borne plant diseases (**Chapter 5**). To this end, a *R. solani* isolate was exposed to woody materials and paper pulp in a Petri dish assay. This showed that paper pulp is a highly suitable substrate for the growth of *R. solani*, while its performance was lower on sawdusts, but varied according to tree species identity. This was followed by two bioassays performed with an arable soil naturally infected by *R. solani*, where I planted seeds of a red beet variety which is susceptible to this pathogen. The bioassays showed that oak and elder wood resulted in a consistent improved control of disease. In addition, paper pulp caused a short-term, transient stimulation of *R. solani*, which coincided with a poor performance of beets sown immediately after amendment. An interval of two weeks or longer between paper pulp addition and sowing resulted in disease suppression. These results point

at the importance of timing of organic amendments relative to sowing, as a factor affecting the efficacy of disease-suppressing soil treatments.

Overall, this thesis provides information on how cellulose-rich organic amendments and phenolic root exudates affect the abundance and composition of saprotrophic fungi, bacteria and soil-borne pathogenic fungi in arable soils and in the rhizosphere of crops. Based on these results, I depict a promising perspective for the use of cellulose-rich materials as a tool to promote sustainable biocontrol via managing microbial communities and microbial interactions in arable soils. In addition, a possible role of phenolic root exudates in modulating the recruitment of root-infecting fungi is indicated.

Samenvatting

Intensieve teelten in de landbouw gaan vaak gepaard met negatieve effecten, zoals milieuvervuiling en achteruitgang van bodemkwaliteit. Om de landbouw te verduurzamen, biedt de integratie van ecologisch onderbouwde landbouwmethoden een veelbelovend perspectief. Talrijke studies wijzen op het belang van herintroductie van traditionele landbouwmethoden, zoals het inwerken van organische materialen en diversificatie van gewassen (combinatieteelt, ruime gewasrotaties, groenbemesters). Tevens worden mogelijkheden onderzocht om microbiële stammen te gebruiken als biologische bestrijdingsmiddelen en – bodemverbeteraars. In landbouwgrond bevindt zich van nature een diverse gemeenschap van saprotrofe schimmels. Toch is hun biomassa en activiteit beperkt. Dit komt door intensieve beheerpraktijken, zoals het gebruik van synthetische in plaats van organische bemesting, gebruik van chemische bestrijdingsmiddelen en mechanische verstoringen van de grond. Een actieve gemeenschap van saprotrofe schimmels kan bijdragen aan het goed functioneren van bodems, zoals aangetoond is voor natuurlijke en semi-natuurlijke ecosystemen. In dit promotieonderzoek, verken ik manieren om de biomassa van saprotrofe schimmels in landbouwgrond te verhogen. Daarnaast test ik de effecten van stimulatie van saprotrofe schimmels op plantengezondheid en overig microbieel bodemleven, waaronder bacteriën, pathogene - en mycorrhiza schimmels.

Saprotrofe bodemschimmels reageren op een verhoogd aanbod van afbreekbare organische materialen. Dit kan dood plantmateriaal (strooisel) zijn, maar ook stoffen die worden afgescheiden door levende wortels (exudaten). Daarom zijn er twee mogelijke benaderingen gekozen om saprotrofe schimmels in landbouwgrond te stimuleren, namelijk het toevoegen van organische materialen en het wijzigen van de samenstelling van wortelexudaten.

In een reeks van potexperimenten werden organische materialen van variërende kwaliteit en oorsprong gemengd met landbouwgrond, om de reactie van schimmels te toetsen (**Hoofdstuk 2**). Op basis van een eerste screening, resulteerde toevoeging van papierpulp en beukenzaagsel in een snelle (2 weken) en blijvende (8 weken) toename van saprotrofe Ascomyceet schimmels (voornamelijk Sordariomyceten). Volgens dezelfde opzet, heb ik vervolgens het effect van zaagsel van verschillende boomsoorten getoetst, met of zonder toevoeging van minerale stikstof. De effecten van beukenzaagsel op de schimmels zijn ook getoetst in vier landbouwgronden met verschillende bodemeigenschappen. De resultaten laten zien dat toename van saprotrofe schimmels met zaagsel in verschillende bodems kan worden bewerkstelligd, maar dat dit alleen gebeurt met zaagsel van loofbomen. Toevoeging van mineraal N verhoogde de schimmelstimulatie door zaagsel.

Hoofdstuk 3 richt zich op het testen van effect van fenolische wortelexudaten op de biomassa en gemeenschapsstructuur van schimmels in de wortelomgeving

(rhizosfeer). Hiervoor werd een modelsysteem voor de rhizosfeer ontworpen. Dit maakte het mogelijk om mengsels van exudaten in een landbouwbodem te introduceren door middel van diffusie, vergelijkbaar met de afscheiding door wortels. Op deze manier, werden fenolische zuren, zowel met als zonder primaire wortelexudaten (suikers, organische zuren) toegevoegd. De toegevoegde hoeveelheid van fenolische zuren was vergelijkbaar met de hoeveelheden, die in de bodem gevonden zijn (0.02 - 0.2 mg g^{-1} grond). Fenolische zuren hadden weinig effect op de totale biomassa van de schimmels in deze kunstmatige rhizosfeer. Hetzelfde resultaat werd in een vervolgexperiment verkregen, waarin de biomassa van schimmels werd gemeten in de rhizosfeer van *Arabidopsis thaliana* mutanten. Deze mutanten hadden een gewijzigde samenstelling van fenolische wortelexudaten. Dit geeft aan dat het selecteren van gewasvariëteiten met een hoger gehalte aan fenolische zuren in wortelexudaten geen veelbelovende strategie is voor het bevorderen van schimmels in de rhizosfeer van gewassen. Een meer gedetailleerde analyse toonde wel een stimulerend effect aan van fenolische zuren op bepaalde schimmels die wortels kunnen infecteren (*Fusarium*, *Fusicolla* en *Trichoderma*). Mogelijk spelen fenolische wortelexudaten een rol in de herkenning van aanwezigheid van wortels door schimmels die deze vervolgens kunnen binnen groeien.

De samenstelling van microbiële gemeenschappen in de rhizosfeer en wortel wordt sterk beïnvloed door de samenstelling en activiteit van microben in de bodem. Na te hebben vastgesteld dat houtzaagsel een effectieve stimulatie van saprotrofe schimmels in landbouwgrond veroorzaakt, laat **Hoofdstuk 4** wat het effect hiervan is op de biomassa, activiteit en samenstelling van microbiële gemeenschappen in de rhizosfeer van wortelzaailingen (*Daucus carota*). "$^{13}CO_2$-pulse labelen" van zaailingen, die in bodem met en zonder zaagsel groeiden, toonde aan dat de door zaagsel gestimuleerde bodemschimmels (Sordariomyceten) zich tot in de rhizosfeer kunnen uitbreiden en daar wortelexudaten kunnen consumeren. Deze toename van actieve saprotrofe schimmels in de rhizosfeer had geen invloed op aantallen en activiteit van bacteriën, maar de samenstelling veranderde wel met toenames van Bacteroidia en Rhizobiales. Stimulatie door zaagsel van saprotrofe schimmels in de rhizosfeer had geen negatief, maar zelfs een positief effect op de hoeveelheid en activiteit van arbusculaire mycorrhiza schimmels (Glomeromyceten). Analyses van DNA-sequentie gegevens, duiden erop dat potentiële ziekteverwekkende schimmels in de onderzochte landbouwbodem niet door zaagsel toevoegingen worden gestimuleerd.

Als een eerste stap in de richting van toepassing van stimulatie van saprotrofe schimmels, onderzocht ik of toename van schimmels door houtachtige materialen en papierpulp gepaard gaat met onderdrukking van de plantpathogene bodemschimmel *Rhizoctonia solani* (**Hoofdstuk 5**). Eerst is in Petri-schalen getest of *R. solani* kan groeien op houtzaagsel en papierpulp. Hieruit bleek dat papierpulp een bijzonder geschikt substraat is voor de groei van *R. solani* was. Voor zaagsels van verschillende boomsoorten varieerde dit sterk. Hierna volgden twee bioassays met *Rhizoctonia*-

bemette grond uit proefvelden met rode biet. In deze grond werd zaagsel of papierpulp gemengd waarna, na verschillende incubatieperioden, een rode biet variëteit werd gezaaid, die vatbaar is voor *Rhizoctonia*. Verschillende zaagsels lieten een verbeterde controle van de ziekte zien, waarbij eikenzaagsel de beste resultaten gaf. Toevoeging van papierpulp resulteerde in een kortstondige toename van *Rhizoctonia*. Dit viel samen met verhoogde ziekte van bietenzaailingen, die onmiddellijk na toevoegingen van papierpulp waren gezaaid. Een interval van twee weken of meer tussen toevoegingen van papierpulp en zaaien van bieten resulteerde in ziekteonderdrukking. Dit onderzoek illustreert dat timing van toevoeging ten opzichte van het zaaien een grote invloed kan hebben op de doeltreffendheid van ziekteonderdrukking.

Samenvattend, laat dit proefschrift zien dat het goed mogelijk is om saprotrofe bodemschimmels te stimuleren in intensief bewerkte landbouwbodems en dat deze stimulatie effecten heeft op het functioneren van microbiële gemeenschappen in de rhizosfeer en op plantengezondheid. Op basis van deze resultaten, schets ik een veelbelovend perspectief voor het gebruik van schimmel-stimulerende materialen om natuurlijke ziekteonderdrukking te verhogen.

Sommario

La gestione intensiva di terreni agrari spesso comporta degli effetti collaterali negativi, quali inquinamento ambientale e degrado del suolo. L'implementazione di pratiche agrarie basate sull'ecologia rappresenta una prospettiva promettente per portare l'agricoltura ad un maggior livello di sostenibilitá. Molti studi hanno indicato l'importanza di reintegrare pratiche agrarie tradizionali, come l'uso di ammendamenti organici e la diversificazione delle colture (consociazioni colturali, maggior uso della rotazione delle colture, colture di copertura). Altre linee di ricerca hanno esplorato le possibilitá di inoculare il suolo con ceppi batterici, che funzionino come biofertilizzanti e biopesticidi. I suoli agricoli ospitano una comunitá molto diversificata di funghi saprotrofi, tuttavia la loro biomassa e attivitá sono piuttosto limitate. Questo é dovuto alle pratiche intensive di gestione del suolo agrario, come l'uso di fertilizzanti chimici piuttosto che organici, l'uso di pesticidi chimici e le lavorazioni meccaniche del terreno. La presenza di una comunitá attiva di funghi saprotrofi é essenziale per il funzionamento del suolo, come é stato ampiamente descritto per ecosistemi naturali e semi-naturali. In questa tesi di dottorato, esploro delle opzioni per aumentare la biomassa fungina nei terreni agrari. Inoltre testo qual é l'impatto della stimolazione dei funghi saprobi sulle piante e altri microbi del suolo, quali batteri, funghi patogeni e micorrize.

I funghi saprobi reagiscono al depositarsi di materiali organici nel suolo, quali parti vegetali derivate da piante senescenti e depositi radicali emessi dalle radici durante la loro crescita. Di conseguenza, in questa tesi sono stati esaminati due possibili approcci per la stimolazione dei funghi saprotrofi nei terreni agrari. Da un lato, l'aggiunta di substrati organici esogeni, dall'altro, la modificazione della composizione degli essudati radicali.

In una serie di esperimenti effettuati in condizioni controllate, materiali organici di varia qualitá ed origine sono stati ammendati in un terreno agricolo per testare il loro effetto sulla biomassa fungina e sulla composizione della comunitá fungina (**Capitolo 2**). Sulla base di tale primo screening, gli ammendamenti del suolo con polpa di carta e con segatura di legno di faggio hanno avuto come risultato un rapido incremento dei funghi ascomicoti (principalmente Sordariomycetes). Questo effetto é persistito per un periodo di almeno due mesi. Utilizzando lo stesso assetto sperimentale, ho testato l'effetto della segatura di legno proveniente da diverse specie arboree. La segatura é stata applicata sia da sola che in combinazione con un fetilizzante azotato minerale. Inoltre, l'effetto di stimolazione della biomassa fungina a seguito dell'aggiunta di segatura é stato verificato in quattro suoli agricoli aventi caratteristiche distinte. I risultati hanno rivelato che le comunitá di funghi saprotrofi presenti nei suoli agrari mantengono una buona capacitá di reagire all'applicazione di

segatura di legno di alberi latifoglie, ma non di segatura derivata da legno di conifere. La combinazione di segatura con azoto minerale ha avuto come risultato una piú elevata stimolazione dei funghi saprobi.

Lo scopo del **Capitolo 3** é stato di testare l'effetto di essudati radicali fenolici sulla biomassa e sulla composizione della comunitá fungina. A questo scopo é stato sviluppato un sistema artificiale che mima la rizosfera. Questo permette di introdurre soluzioni artificiali di essudati e, attraverso la loro diffusione nel terreno, di simulare l'essudazione radicale. In questo modo, acidi fenolici semplici sono stati introdotti ad una concentrazione simile a quella misurata solitamente nel terreno (0.02–0.2 mg g^{-1} suolo). Gli acidi fenolici hanno avuto un effetto limitato sulla biomassa fungina nella rizosfera modello. Lo stesso risultato é stato ottenuto in un esperimento successivo, in cui la biomassa fungina é stata misurata nella rizosfera di mutanti di *Arabidopsis thaliana* con una composizione alterata di essudati radicali fenolici. Questo indica che la selezone di varietá di piante coltivate sulla base di una maggiore produzione di acidi fenolici tra gli essudati radicali non é una strtegia promettente per promuovere l'attivitá dei funghi saprobi nella rizosfera delle piante coltivate. Questo studio ha anche dimostrato che gli acidi fenolici promuovono funghi capaci di infettare i tessuti radicali (*Fusarium*, *Fusicolla* e *Trichoderma*) nella rizosfera modello. Di conseguenza, gli essudati radicali fenolici potrebbero operare come modulatori delle comunitá fungine presenti nella rizosfera e nella radice.

La composizione e l'attivitá dei microorganismi presenti nel suolo ha una forte influenza sull'assemblaggio delle comunitá microbiche della rizosfera e della radice. Una volta stabilito che l'aggiunta di segatura di legno causa un'efficacie stimolazione della biomassa di funghi saprobi nel terreno agrario, il **Capitolo 4** ha dimostrato che questo influenza l'attivitá e la composizione delle comunitá microbiche associate con piantine di carota. Piantine cresciute in terreno ammendato con segatura e tereno di controllo, sono state marcate con impulsi di $^{13}CO_2$. L'analisi della quantitá di ^{13}C nei fosfolipidi di membrana ha rivelalato che i funghi stimolati dalla segatura possono estendersi nella rizosfera e consumre attivamente carbonio fissato e derivato dalla pianta. La biomassa dei batteri e il loro consumo di essudati radicali non é stato influenzato dall'aggiunta di segatura, mentre la biomassa ed attivitá dei funghi micorrizici sono aumentate. L'analisi delle comunitá associate alla rizosfera ed alla radice tramite DNA(-SIP) ha illustrato che Sordariomycetes, Glomeromycetes, Bacteroidia e Rhizobiales sono aumentati in termini di abbondanza relativa. Sulla base dei dati di sequenziamento, la comunitá di funghi potenzialmente patogeni non é aumentata relativamente alla comunitá fungina totale. Di conseguenza, il trattamento del terreno agrario con segatura di legno e promuove una maggiore attivitá fungina nella rizosfera, il che guida l'assemblamento delle comunitá microbiche associate con la radice.

Dopo aver individuato materiali organici in grado di stimolare i funghi del suolo (**Capitolo 2**), aver constatato la loro abilitá di stimolare funghi in grado di occupare la rizosfera e aver descritto i loro effetti sui batteri (**Capitolo 4**), ho studiato se e come i materiali legnosi e la polpa di carta possono essere usati per aumentare i funghi saprobi ed, allo stesso tempo, controllare la popolazione di *Rhizoctonia solani*, uno dei piú diffusi agenti causali di malattie vegetali derivate dal suolo (**Capitolo 5**). A questo scopo, un isolato di *R. solani* é stato esposto a materiali legnosi e polpa di cellulose in un saggio su piastre Petri. Questo ha mostrato che la polpa di carta é un substrato molto adatto per la crescita di *R. solani*, mentre la sua performance era minore su substrati legnosi e variabile a seconda dell'identitá della specie arborea di origine. Questo é stato seguito da due saggi effettuati in serra con un terreno agricolo naturalmente infetto da *Rhizoctonia*, dove sono stati piantati semi di bieta, una pianta ospite sensibile a *Rhizoctonia*. Tali saggi hanno mostrato che il legno di quercia e di sambuco sono risultati in un migliore contenimento della malattia delle piante. Inoltre, la polpa di carta ha causato un a stimolazione transiente di *R. solani* nel breve termine. Questa ha coinciso con una cattiva performance delle piantine di bieta seminate immediatamente dopo l'amendamento con polpa di carta. Un intervallo di due settimane o piú tra l'aggiunta della polpa di carta e la semina é risultato invece in una riduzione della malattia della bieta. Questi risultati indicano l'importanza delle giuste tempistiche dell'aggiunta di ammendamenti organici prima alla semina. Questo fattore influenza l'efficacia di trattamenti volti ad aumentare la soppressione delle malattie delle piante.

Nel complesso, questa tesi fornisce informazioni su come ammendamenti con materiali organici ricchi in cellulosa e essudati radicali fenolici influenzano l'abbondanza e la composizione dei funghi saprobi, dei batteri e dei funghi patogeni presenti nel suolo agrario e nella rizosfera di piante coltivate. Sulla base di questi risultati, tratteggio una prospettiva promettente per l'uso di materiali ricchi in cellulosa come uno strumento per il controllo biologico e sostenibile delle malattie delle colture attraverso la gestione delle comunitá microbiche e delle interazioni microbiche nei suoli agari. Inoltre, indico un possibile ruolo degli acidi fenolici semplici nel modulare l'attrazione di funghi capaci di infettare la radice.

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About the author

Anna Clocchiatti was born on March 19th, 1990 in Udine, a town in the north-east of Italy. She grew up in Udine and in the nearby village of Bagnarola. In 2009, after completing her secondary education at Liceo Scientifico Copernico, she moved to Padova to study Molecular Biology. Directly after her B.Sc. study, she continued with her education at Padova University. During her M.Sc. studies in Molecular Biology, she became interested in plant-microbe interactions and their potential application for sustainable agricultre. In 2014, she obtained a Master's degree *cum laude* at Padova University, with a thesis supervised by Prof. Barbara Baldan, on the functional and molecular traits of plant growth-promoting bacterial endophytes of *Vitis vinifera*. In 2015, she attended the Summer School in Integrative Biology and Ecology in Tolouse and a Masterclass on Enterpreneurship and Management of Startup and Innovative Projects in Udine. In 2016, she moved to Wageningen for starting her Ph.D. in soil microbial ecology. At the Netherlands Institute of Ecology, she joined the Sapro-Feed project and researched how to boost saprotrophic fungal biomass, as an option for improving the health of arable soils. She worked with the supervision of Prof. Wietse de Boer and Dr Emilia Hannula and she is continuing with her research at NIOO as a postdoc. The results of the doctoral research are presented in this thesis.

Peer-reviewed publications

Clocchiatti, A., Hannula, S.E., van den Berg, M., Korthals, G., de Boer, W., 2020. The hidden potential of saprotrophic fungi in arable soil: Patterns of short-term stimulation by organic amendments. Appl. Soil Ecol. 147, 103434. https://doi.org/10.1016/j.apsoil.2019.103434

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Submitted publications

Clocchiatti, A., Hannula, S.E., Hundscheid, M.P.J., klein Gunnewiek, P.J.A., de Boer, W. Stimulated saprotrophic fungi in arable soil extend their activities and impacts in rhizosphere and root microbiomes of crop seedlings. *In revision*

Clocchiatti, A., Hannula, S.E., van den Berg, M., Hundscheid, M.P.J., de Boer, W. Phenolic root exudates as modulators of saprotrophic fungal biomass and community structure in the rhizosphere. *Submitted*

Liu, X., Hannula, S.E., Li, X., Hundscheid, M.P.J., klein Gunnewiek, P.J.A., **Clocchiatti**, **A.**, Ding, W., de Boer, W. Decomposing cover crops modify composition and functioning of root microbiomes of cash crop seedlings. *Submitted*

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)

Review of literature (7 ECTS)

Fungal-bacterial interactions and their role in soil and plant health
Sapro-Feed: increasing crop health by managing natural microbial interactions

Post-graduate courses (6.2 ECTS)

Basic statistics; PE&RC and SENSE (2016)
Introduction to R for statistical analysis; PE&RC (2016)
R and big data; PE&RC and SENSE (2017)
New frontiers in microbial ecology; RSEE (2018)
Soil ecology, the multifunctional potential of soils; PE&RC (2019)

Invited review of (unpublished) journal manuscript (3 ECTS)

Biology and Fertility of Soils: disease suppression and soil aggregation as affected by organic fertilizers
FEMS Microbiology Ecology: fungal communities in peat moss farming fields
European Journal of Soil Science: effect of organic amendments on soil microbial communities and carbon sequestration

Competence strengthening / skills courses (3.4 ECTS)

Essentials of scientific writing and presenting; Wageningen in'to Languages (2017)
Effective behaviour in your professional surroundings; WGS (2019)
Career orientation for PhD candidates and post-docs; VU (2020)

Scientific integrity/ethics in science activities (0.6 ECTS)

Scientific integrity; WGS (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.4 ECTS)

NAEM (2016)
PE&RC Midterm weekend (2017)
PE&RC Day: the social network of nature (2018)

NAEM (2019)
Symposium: plant-soil feedback linkages between root traits and soil biota (2019)

Discussion groups / local seminars / other scientific meetings (11.3 ECTS)
PE&RC Discussion group plant-soil interactions (2016-2017)
ME Meeting with seminar (2016-2019)
STW Utilizers meeting (2016-2020)
KNPV Symposium: plant and soil microbiome relevance for crop protection (2017)
Working group soil-borne pathogens and soil microbiology (2017-2018)

International symposia, workshops and conferences (10.7 ECTS)
3rd Adam Kondrosi symposium, frontiers in beneficial plant-microbe interactions; poster presentation; Gif-sur-Yvette (2016)
MICROPE microbe-assisted crop production, opportunities, challenges and needs; poster presentation; Vienna (2017)
Ecology of soil microorganisms; poster presentation; Helsinky (2018)
4th Thünen symposium on soil metagenomics; poster presentation; Braunschweig (2019)

Lecturing/supervision of practicals/tutorials (6 ECTS)
Ecological aspects of biological Interactions (2017)
Internship students (2017-2018)

MSc Thesis supervision
Growth dynamics of soil-borne plant pathogenic fungi on fresh and decomposed woody substrates

Colophon

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