




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

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Summary

Saprotrophic fungi play an important role in ecosystem functioning and plant performance, but their abundance in intensively managed arable soils is low. Saprotrophic fungal biomass in arable soils can be enhanced with amendments of cellulose-rich materials. Here, we examined if sawdust-stimulated saprotrophic fungi extend their activity to the rhizosphere of crop seedlings and influence the composition and activity of other rhizosphere and root inhabitants. After growing carrot seedlings in sawdust-amended arable soil, we determined fungal and bacterial biomass and community structure in roots, rhizosphere and soil. Utilization of root exudates was assessed by stable isotope probing (SIP) following ¹³CO₂-pulse-labelling of seedlings. This was combined with analysis of lipid fatty acids (PLFA/NLFA-SIP) and nucleic acids (DNA-SIP). Sawdust-stimulated Sordariomycetes colonized the seedling's rhizosphere and roots and actively consumed root exudates. This did not reduce the abundance and activity of bacteria, yet higher proportions of α -Proteobacteria and Bacteroidia were seen. Biomass and activity of mycorrhizal fungi increased with

sawdust amendments, whereas exudate consumption and root colonization by functional groups containing plant pathogens did not change. Sawdust amendment of arable soil enhanced abundance and exudate-consuming activity of saprotrophic fungi in the rhizosphere of crop seedlings and promoted potential beneficial microbial groups in root-associated microbiomes.

Introduction

In intensively managed arable soils, saprotrophic fungal biomass is low as compared with soils of natural ecosystems (Djakirana *et al.*, 1996; de Vries and Bardgett, 2012). 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, for example, 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 practises resulting in stimulation of saprotrophic fungi in arable soils can make an important contribution to improve sustainable crop production (de Vries and Bardgett, 2012; Frac *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).

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).

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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.*, 2012; 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}C pulse-labelling and determined ^{13}C accumulation in phospholipid fatty acids (PLFA-SIP) and microbial DNA markers (DNA-SIP).

We hypothesized that (i) stimulating fungal biomass in the bulk soil causes an increase in fungal biomass in the rhizosphere, which coincides with (ii) increased activity (i.e. uptake of exudates) of saprotrophic fungi in the rhizosphere of crop seedlings and results in (iii) shifts in the composition of rhizosphere – and root microbiomes. We further investigated the persistence of the effects by sowing seedlings 2 (T1) and 6 weeks (T2) after sawdust amendment.

Results

Plant performance and ^{13}C enrichment

Germination rates and shoot biomass of carrot seedlings were not significantly different between controls and sawdust amended treatments (Supporting Information Table S1). Yet, a slight effect of pre-incubation time before sowing (T1 = 2 weeks vs. T2 = 6 weeks, Fig. 1) was seen, which consisted of a small decrease in germination rates with longer pre-incubation time (Supporting Information Table 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}C from the airspace of the air-tight chamber, as indicated by a decrease in CO_2 concentration by the built-in monitoring system. This was reflected by ^{13}C incorporation in shoot and root parts of carrot seedlings, as well as in rhizosphere soil (Supporting Information Table S1). The ^{13}C values in the control rhizosphere soil and plant parts were within the normal atmospheric value range ($\delta^{13}\text{C}$ -30‰ \pm 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 (Supporting Information Table 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 plant parts. Pre-incubation time of soil with sawdust before sowing had an effect on the amount of ^{13}C incorporated in carrot seedlings (T1 > T2; $F_{1,40} = 135$, $P < 0.05$).

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

An increase in fungal biomass (measured with fungal marker PLFA 18:2 ω 6,9) was found in sawdust amended

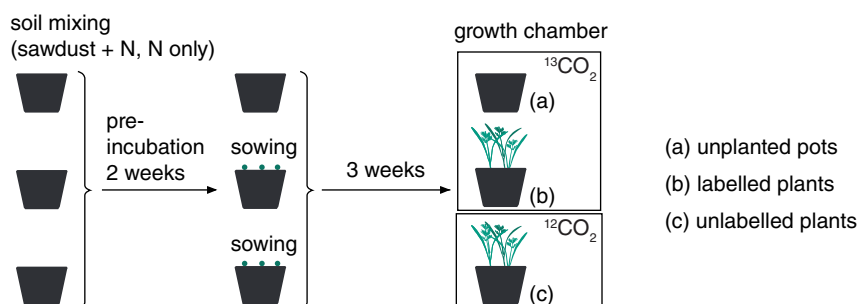
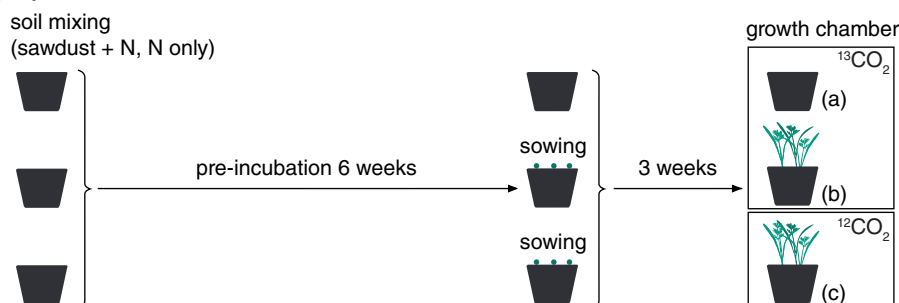
(T1)

Fig 1. Schematic representation of the experimental setup, starting with amendment of sawdust + N or N only (control), followed by sowing and growing carrot seedlings in the greenhouse and completed with ^{13}C - CO_2 labelling in a growth chamber. [Color figure can be viewed at wileyonlinelibrary.com]

(T2)

treatments for both unplanted and rhizosphere soil (Fig. 2A; Supporting Information Table 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 observed for ITS2 rRNA copy numbers (Supporting Information Fig. S1b; $P < 0.001$ for T1, $P < 0.01$ for T2) and ergosterol (Supporting Information Fig. S1e for Te, $P < 0.01$). Furthermore, the excess ^{13}C was measured in the PLFA 18:2 ω 6,9. This was used for quantifying the incorporation of plant-derived carbon into the biomass of fungi. The excess ^{13}C in the fungal PLFA marker in the rhizosphere was significantly higher ($F_{1,10} = 7.25$, $P < 0.05$, Tukey HSD $P < 0.01$; Supporting Information Table 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; Supporting Information Table S2b).

Based on the cumulative measure of bacterial PLFA markers, bacterial biomass was not affected by sawdust amendment for T1 (Fig. 2C; Supporting Information Table S2c, $F_{1,20} = 4.4$, $P = 0.6$), however for T2 the PLFA-based bacterial biomass was higher in the sawdust-amended rhizosphere as compared with the control rhizosphere (Fig. 2C; $P < 0.001$). For T1, sawdust amendment was associated with an increase in individual bacterial PLFAs, with higher abundance of PLFAs i16:0,

i17:0, ai17:0, 17:1 ω 8c, cy-17:0 in the sawdust-amended soil and rhizosphere (Supporting Information Table S3). The peak of PLFA marker 18:1 ω 7c/18:1 ω 9t, which can indicate both bacteria and fungi, was also higher in the sawdust amended 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; Supporting Information Tables S2d and 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 with the control. For T2, qPCR-based bacterial numbers were higher in the root tissues, but not in the bulk soil or rhizosphere, upon sawdust amendment (Supporting Information Fig. S1a and b).

The marker for arbuscular mycorrhizal fungi NLFA 16:1 ω 5 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 16:1 ω 5 for T1, in response to sawdust amendment (Fig. 2F).

Composition of fungal and bacterial communities

Sawdust amendment strongly affected the composition of fungi present in the soil ($R^2 = 70.3$, $P < 0.001$; Supporting

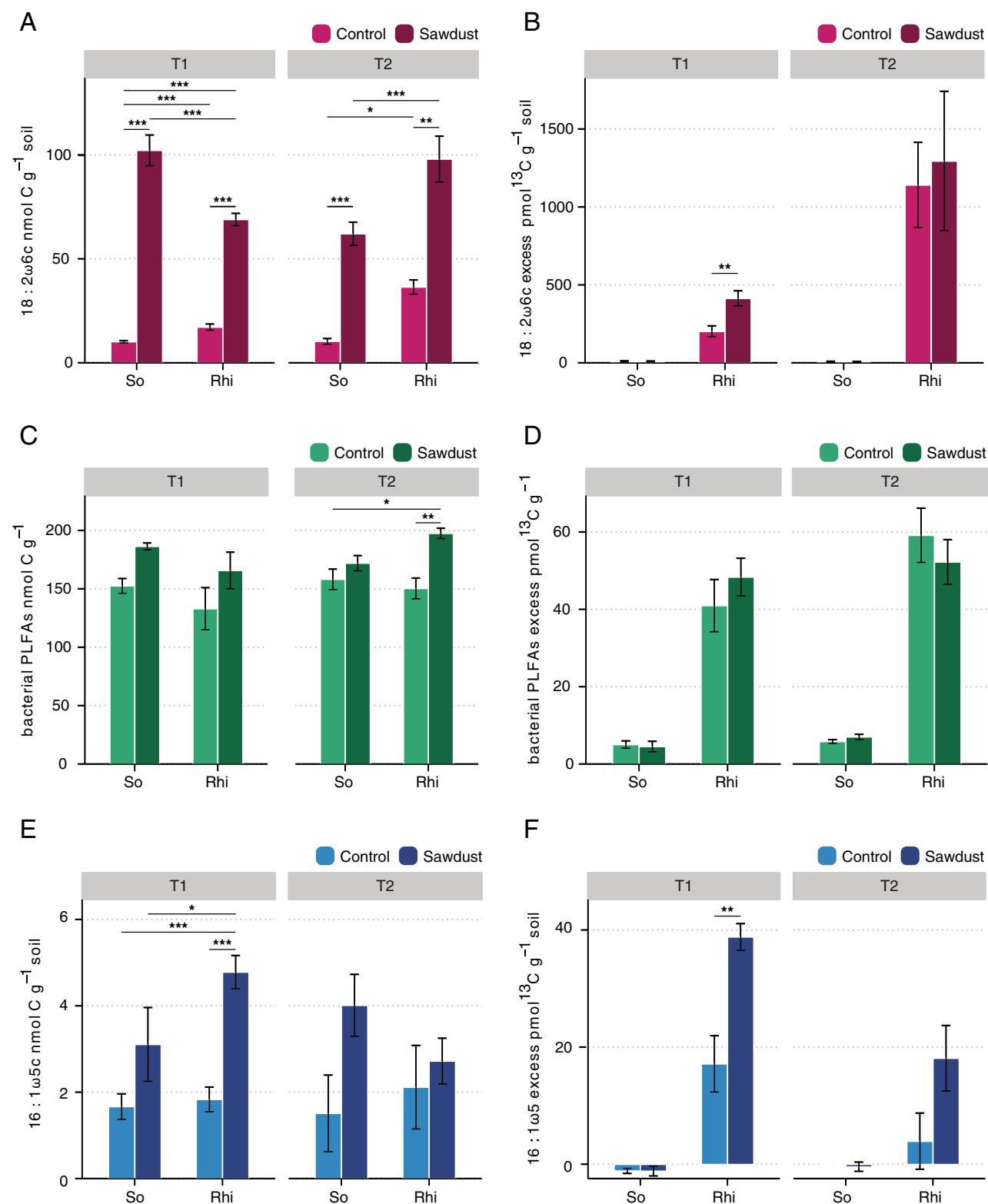


Fig 2. Effect of wood sawdust amendment on biomass and plant-C incorporation by fungi, bacteria and mycorrhizal fungi. Concentration of total C and ¹³C excess in PLFA/NLFA markers are shown for fungi (A, B), bacteria (C, D) and arbuscular mycorrhizal fungi (E, F) in unplanted soil (So) and carrot rhizosphere 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. ¹³C 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 ¹³C abundance found for the same PLFA/NLFA in unlabeled rhizosphere samples. [Color figure can be viewed at wileyonlinelibrary.com]

Information Table S6a), rhizosphere ($R^2 = 53.7$, $P < 0.001$; Supporting Information Table S6b), as well as of fungi actively incorporating plant-derived carbon ($R^2 = 17.5$, $P < 0.001$; Supporting Information Table S6c; Figs. 3A and 4A). In addition to this, the effect of sawdust amendment was seen in the root compartment ($R^2 = 15.0$, $P < 0.001$; Fig. 3A, Supporting Information Table 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 of saprotrophic guilds, but taxa assigned as plant pathogens were also detected (Fig. 4B). Sawdust amendment led to an increase in the 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 detected more Sordariomycetes (Fig. 4A; $F_{1,40} = 13.4$,

$P < 0.001$) and Leptosphaeriaceae (Supporting Information 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. Fungal SVs assigned as 'Plant pathogens' only or assigned as 'Plant pathogen or other potential function' (e.g. 'Plant pathogen-Saprotroph') were grouped in a sub-community containing potential plant pathogenic fungi. This sub-community of potential plant pathogens was found in all compartments, but sawdust amendment decreased its abundance, relative to the total fungal community (Fig. 4D, $F_{1,80} = 43.7$, $P < 0.001$), in favour of a higher relative abundance of fungal SVs assigned as 'Saprotrophs' only (Fig. 4C, $F_{1,80} = 137.7$, $P < 0.001$) in soil, rhizosphere and ^{13}C -enriched rhizosphere. Functional groups containing potential plant pathogens were found in a higher proportion

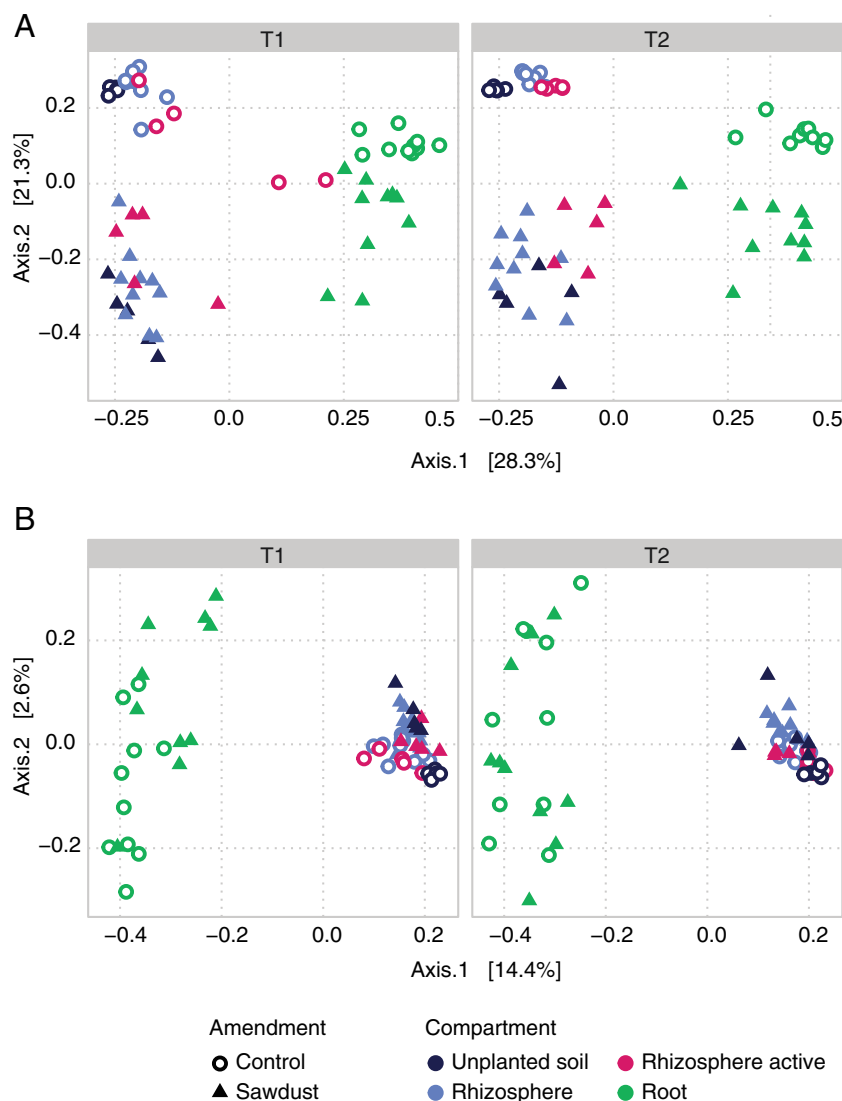


Fig 3. Effect of sawdust amendment of arable soil on the fungal and bacterial community composition in roots and rhizosphere of carrot seedlings and in bulk soil. Ordination (PCoA based on Bray–Curtis dissimilarity matrix) was performed independently for the whole fungal dataset (A) and for the whole 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 3 weeks. [Color figure can be viewed at wileyonlinelibrary.com]

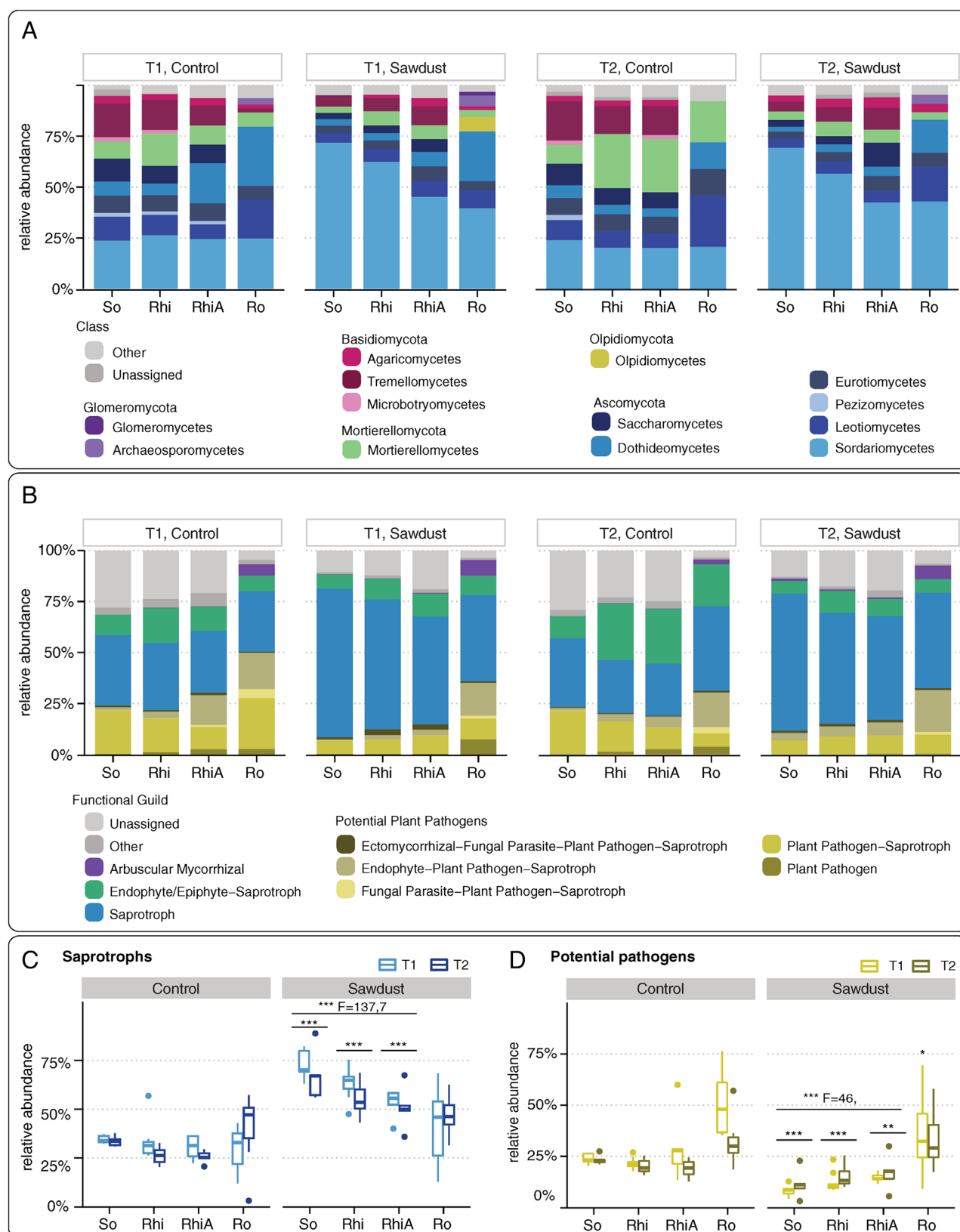


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in roots than in soil compartments, irrespective of the sawdust treatment. Yet, sawdust reduced the relative abundance of these groups in the root fungal community for T1 ($F_{1,20} = 4.5$, $P < 0.05$), but not for T2. The cumulative relative abundance of all groups potentially containing pathogenic fungi was multiplied by the total fungal biomass, thus estimating the absolute size of this sub-community. This showed a small, yet significant increase in absolute abundance of groups containing plant pathogenic fungi by sawdust amendment in the soil and rhizosphere (Supporting Information Fig. S3, simple effect of sawdust: $P < 0.001$ based on PLFA, $P < 0.001$ based on ITS). However, no change was seen in the absolute abundance of pathogen-containing groups in the ^{13}C -enriched rhizosphere and root after sawdust amendment. Conversely, the absolute abundance of the sub-community of saprotrophic fungi, estimated in the same way, was increased by sawdust in all compartments (Supporting Information Fig. S3, $P < 0.001$ based on both PLFA and ITS for soil and rhizosphere, $P < 0.001$ in ^{13}C -enriched rhizosphere, $P < 0.001$ in root).

In the unplanted control, the most abundant fungal families containing plant pathogens were Didymellaceae, Plectosphaerellaceae and Nectriaceae (*Fusarium* spp.) (Supporting Information Fig. S2b). Decrease in relative abundance of Didymellaceae and Plectosphaerellaceae contributed to the observed decrease of the relative abundance of pathogen-containing groups 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 Olpidiaceae increased in carrot roots grown in sawdust-amended soil for the shortest pre-sowing incubation time (T1: $F_{1,20} = 5.5$, $P < 0.05$).

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 with that of fungi (Figs. 3B and 5A; Supporting Information Table S7). Yet, significant sawdust-induced shifts in the bacterial community occurred in unplanted soil ($R^2 = 7.9\%$, $P < 0.001$; Supporting Information Table S7a), rhizosphere soil ($R^2 = 3.4\%$, $P < 0.001$; Supporting Information Table S7b) and roots ($R^2 = 3.8\%$, $P < 0.01$; Supporting Information Table 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 ($F_{1,40} = 14.1$, $P < 0.001$).

Discussion

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

Free-living, saprotrophic fungi not only occur in soil, they 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 practises, which tend to favour plant pathogenic fungi (Gao *et al.*, 2019). The 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 has been shown to be particularly effective (Clocchiatti *et al.*, 2020). The present study not only confirmed 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 6 weeks of pre-incubation of sawdust in soil. Saprotrophic Sordariomycetes were the most stimulated fungi in bulk soil, rhizosphere and roots. Stimulation of Sordariomycetes by sawdust is in line with their known

Fig 4. Effect of sawdust amendment on fungal community composition in an arable soil, as determined for unplanted soil (So), carrot rhizosphere (Rhi), ^{13}C -enriched DNA fraction in the carrot rhizosphere (RhiA) and carrot root (Ro). Relative abundance of fungal classes (A) and fungal functional groups (B) are displayed for soil, rhizosphere and root compartments and for each pre-sowing incubation time (T1 = 2 weeks, T2 = 6 weeks) in sawdust-amended pots and controls. Fungal taxa and functional groups of relative abundance $< 1.5\%$ were classified as 'Other'. (C, D): relative abundance of the functional fungal groups 'saprotrophs' and those potentially containing 'plant pathogens'. The main effect of sawdust amendment is shown alongside the effect of sawdust in each compartment (three-way ANOVA with planned contrasts, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

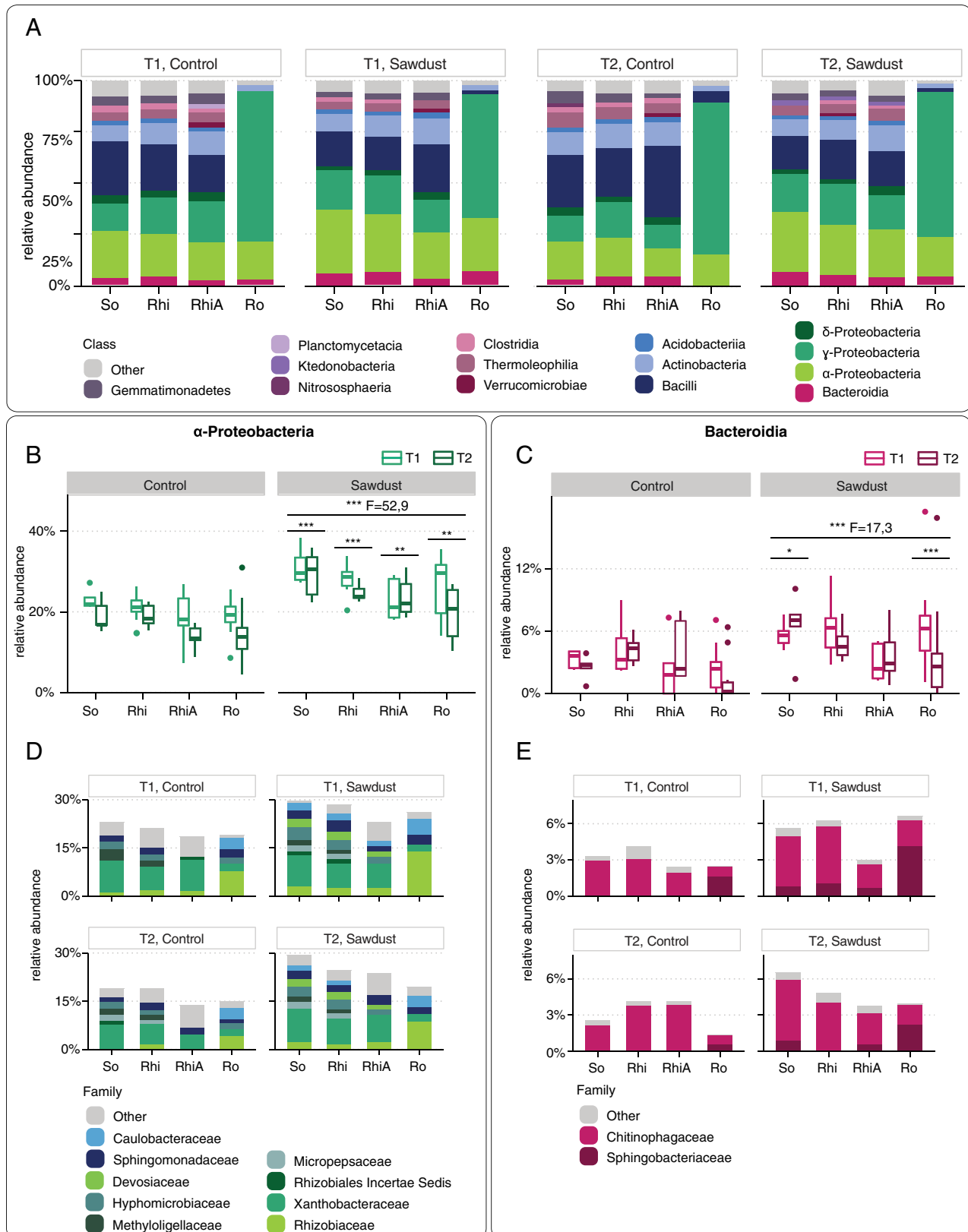


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ability to efficiently decompose cellulose fractions of organic materials (Koechli *et al.*, 2019). Under conventional agricultural practises, this group tends to associate with older plant roots (Hannula *et al.*, 2010; Han *et al.*, 2017). In order to analyse the impact of sawdust-amendment on plant-pathogenic fungi, all functional groups containing 'Pathogen' as the only function or as one of the possible functions were included. This analysis revealed that the relative abundance of the SVs assigned to these functional groups was not increased by sawdust amendment. Yet, the estimation of the absolute abundances of SVs in functional groups containing plant pathogens showed a small increase after sawdust amendment. However, this absolute increase in potential pathogens was limited to the soil and rhizosphere compartments and it was not mirrored by an increase in their uptake of root-derived ^{13}C or colonization of root tissues. Conversely, saprotrophic fungal stimulation was consistently high in sawdust-amended soil, based on both estimated absolute and relative abundance. Such promotion of sawdust-stimulated saprotrophic fungi extended also to the active root-associated fungi and to the fungi colonizing the interior of roots. Further quantification of pathogens population abundance, combined with bioassays, is required to test the potential of the present saprotroph-stimulating approach in counteracting the invasion of roots by disease-causing fungi. 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).

An increased abundance of AMF in the rhizosphere (NLFA 16:105) and in roots (ITS2 rRNA amplicon sequences) was 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 due to increased uptake by saprotrophic fungi (Wu *et al.*, 2007; Zhang *et al.*, 2018). Under conditions of 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.*, 2018). 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 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).

Effect of sawdust addition on root exudate consumption by fungi

Here, we demonstrate that saprotrophic fungi stimulated by sawdust amendment are actively consuming root-derived C in the rhizosphere of seedlings, which was measured as increased ^{13}C excess accumulated in the fungal PLFA 18:206,9. Uptake of root-derived C by saprotrophic fungi 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 the 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 fungi, as they were prevalent within the ^{13}C -enriched fungal community. Due to the short duration of the $^{13}\text{CO}_2$ pulse (24 h), followed by immediate harvesting of rhizosphere soil and plants, mainly soluble exudates were probably enriched in ^{13}C (Kaštovská *et al.*, 2017). Thus, the observed ^{13}C labelling in the fungal biomarker can be attributed to the uptake of root exudates like sugars and organic acids. The PLFA marker 18:206,9 can be found in plants; hence, care was taken when sampling the rhizosphere soil not to include plant cells. Furthermore, by using qPCR on the ITS2 region and ergosterol as additional measurements of fungal biomass, we showed that these reveal a similar pattern (Supporting Information Fig. S1) and we concluded that the marker 18:206,9 is a useful indicator of fungal biomass in the rhizosphere (Joergensen and Wichern, 2008; Kaiser *et al.*, 2010; Frostegård *et al.*, 2011), especially when combined with ^{13}C pulse-labelling.

Fig. 5. Effect of sawdust amendment on bacterial community composition in an arable soil, as determined for unplanted soil (So), carrot rhizosphere (Rhi), ^{13}C -enriched DNA fraction in the carrot rhizosphere (RhiA) and carrot root (Ro). Results are displayed for each pre-sowing incubation time (T1 = 2 weeks, T2 = 6 weeks) in sawdust-amended pots and controls.

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 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).

D,E. Relative abundance of bacterial families belonging to α -Proteobacteria and Bacteroidia (respectively, families of relative abundance < 1% and < 0.5% were classified as 'Other'). [Color figure can be viewed at wileyonlinelibrary.com]

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 (Hannula *et al.*, 2012; Marschner *et al.*, 2012; Gkarmiri *et al.*, 2017; 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 2 weeks after sawdust amendment. In line with what is discussed above, this suggests the possibility that after sawdust addition, in absence of P fertilization, no negative interactions occurred between saprotrophic fungi and AMF in the root surroundings.

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 this was 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 harbour an 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 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), in a study 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 the abundance of potentially beneficial bacteria within the roots, namely *Chitinophagaceae*, *Sphingobacteriaceae* and Rhizobiales. *Chitinophagaceae* and *Sphingobacteriaceae* could contribute to resistance against diseases by antagonistic interactions. *Chitinophaga* spp. were previously found within sugar beet plants challenged by *Rhizoctonia solani* and were associated with a higher expression of chitinases (Carrión *et al.*, 2019). *Sphingobacteriaceae* 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, 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 influenced the association of seedlings with potentially beneficial bacterial groups.

In this study, changes in size, activity and composition of the fungal and bacterial communities are mainly interpreted as a direct effect of wood particles or sawdust-stimulated fungi to the resident soil microbiome. In addition to this, it cannot be excluded that wood-inhabiting microbes were introduced into the soil and contributed to the observed effects (Massart *et al.*, 2015; Mawarda *et al.*, 2020). We argue that their role was minor, as the presence of microbes in commercial sawdust is reduced by drying at high temperatures (Ståhl *et al.*, 2004). Furthermore, we expect that the remaining sawdust-inhabiting microbes are rapidly outcompeted during wood decomposition in soil (Petrini and Fisher, 1990; van der Wal *et al.*, 2016).

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, which was associated with a higher relative abundance of α -Proteobacteria, Bacteroidia and a higher abundance of active arbuscular mycorrhizal fungi in rhizosphere and roots (Fig. 6). In soil and rhizosphere, sawdust caused a small increase in the estimated abundance of fungal groups containing potential plant pathogens; however, the root exudate consumption and root colonization by these groups did not increase after sawdust amendment. This study 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 can potentially promote beneficial microbes. Further research is required for assessing the benefits of such changes 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.

Experimental procedures

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 \times 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) at the concentration of 5 g kg⁻¹ soil, (*Fagus sylvatica*, Säger Rollenlager GmbH, Waldsölsms, Germany) and ammonium nitrate, 170 mg kg⁻¹ soil. The second batch of soil was mixed with ammonium nitrate only (170 mg kg⁻¹ soil), representing the control. Soil moisture was adjusted to 60% of its water holding capacity and it was maintained as such during the experiment. Portions of 190 g of soil were divided among pots (PET, 10 \times 10 \times 10 cm) and pre-incubated for 2 and 6 weeks in a greenhouse under an aluminium cover (Fig. 1). These pre-incubation times were chosen on basis of sawdust-induced fungal dynamics in an earlier study (Clocchiatti *et al.*, 2020). After each pre-incubation period, 10 control pots and 10 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, ¹³CO₂ pulse-labelling was performed (Fig. 1).

¹³CO₂ pulse-labelling and harvesting

Planted and unplanted pots were transferred into a climate chamber and provided with pulses of 99.99 atom-% ¹³CO₂ (Cambridge Isotope Laboratories, Tewksbury, Massachusetts) for a total of 16 h, under artificially lit (photoperiod 16:8) and air-tight conditions. The concentration of CO₂ was monitored during the labelling period: initially CO₂ 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 ¹³CO₂ was provided, restoring the initial concentration of CO₂ in the chamber. The pulse was repeated five times with 2.5 h intervals making the total amount of ¹³CO₂ added 12.5 l. During the artificial night (8 h darkness), ¹³CO₂ 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

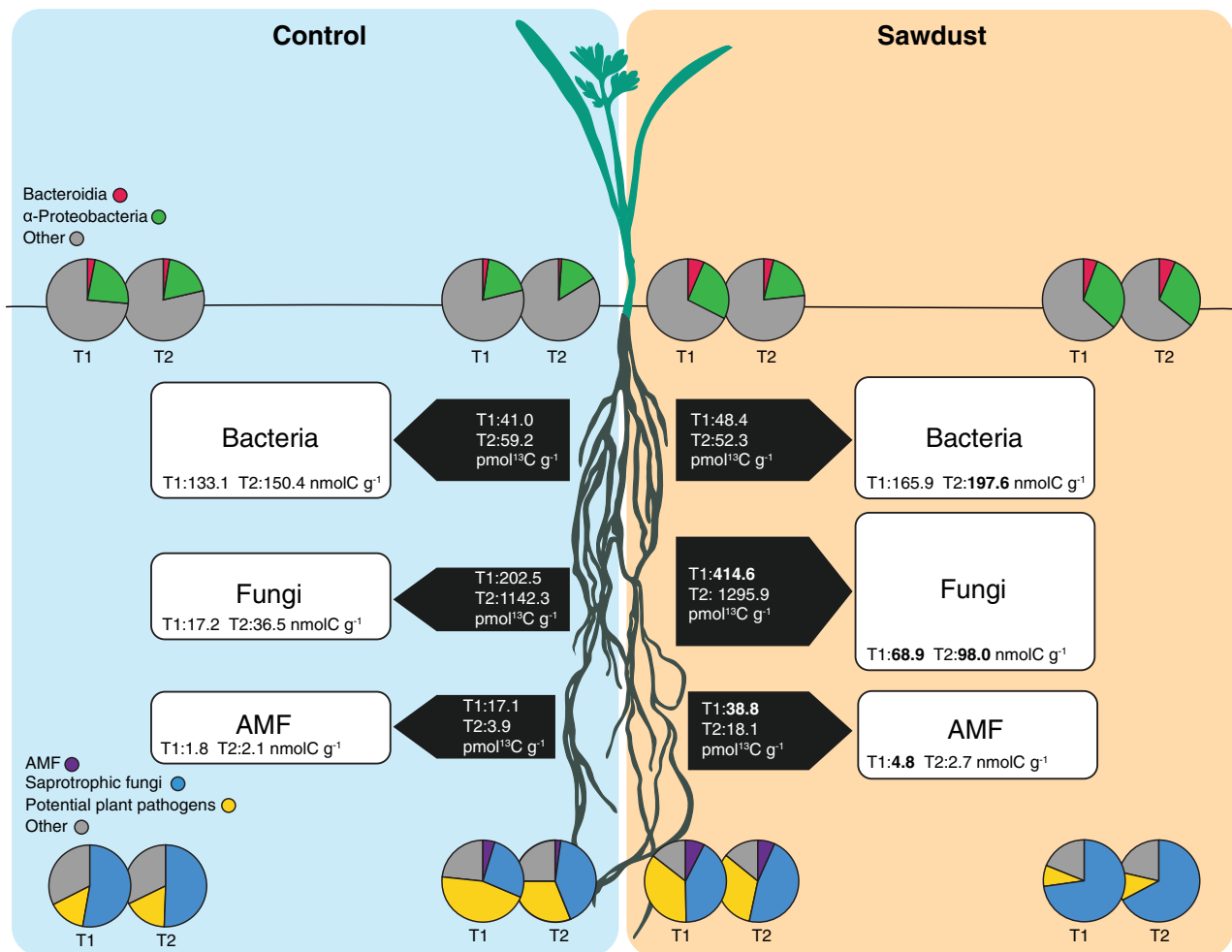


Fig 6. Effect of sawdust on fungi and bacteria in an arable soil and in the root surroundings of carrot seedlings after 2- and 6-weeks (T1, T2) pre-sowing incubation times. White boxes report the biomass of bacteria, fungi and AMF in the rhizosphere soil, based on PLFA/NLFA markers. The black arrows represent the incorporation of ¹³C in fungal, bacterial and AMF PLFA/NLFA markers. Pie charts report the proportion of bacterial taxa (top) and fungal functional groups (bottom) that responded to sawdust amendment in the soil and rhizosphere soil (external charts) and in the ¹³C-enriched rhizosphere and root (central charts), based on DNA amplicon sequencing. [Color figure can be viewed at wileyonlinelibrary.com]

atmospheric CO₂ (Fig. 1). We refer to them as ¹²C controls. The labelling was performed in the same way two times, namely 5 and 9 weeks after the start of the experiment, corresponding to 2 and 6 weeks of soil pre-incubation, followed by 3 weeks of seedling growth. We refer to the two pre-incubation 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 ¹³C content of soil and plant parts is described in the Supporting Information.

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

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

$$\delta^{13}\text{C} (0/00) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$$

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

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

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_i^{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 ($C_{P/NLFA}$):

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

PLFA 18:2 ω 6,9 was used as fungal marker, whereas NLFA 16:1 ω 5 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, 16:1 ω 7c, 16:1 ω 6c, ai17:1 ω 7, i17:0, ai17:0, 17:1 ω 8c, cy-17:0 (Boschker and Middelburg, 2002; Mauclaire *et al.*, 2003); Actinobacteria were indicated by 10Me-branched PLFAs (Willers *et al.*, 2015).

As an additional measure for fungal biomass, alkaline extraction of ergosterol was performed starting from 1 g soil, as described by de Ridder-Duine *et al.* (2006). Ergosterol was quantified by LC-MSMS (UHPLC 1290 Infinity II, Agilent Technologies and 6460 Triple Quad LC-MS, Agilent Technologies).

DNA extraction and molecular analyses

DNA was extracted from 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 (Thermo Fisher Scientific, Waltham, Massachusetts). The DNA was used for quantifying ITS2 and 16S rRNA 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.

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 18:2 ω 6,9 (total and ^{13}C excess), the mycorrhizal NLFA 16:1 ω 5 (total and ^{13}C excess), total and individual bacterial PLFAs listed above (total and ^{13}C excess), ergosterol, ITS2 and 16S rRNA copy number.

Sequencing data were processed in Linux and R. The ITS2 rRNA 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, that is, mitochondria and chloroplasts, resulting in 4048 fungal SVs and 16 422 bacterial SVs (R, phyloseq). In the latter dataset, both eubacterial and archeal SVs were retained. Based on the taxonomy, potential functions were assigned to 1871 fungal SVs, using the FUNGuild database v1.0 (Nguyen *et al.*, 2016), followed by manual curation of the functional assignment of the ecological groups of interest in this study: saprotrophic fungi, plant pathogens, fungal parasites, arbuscular mycorrhizal fungi (Supporting Information Table 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 analysed at phylum, class, order and family level and for fungal functional groups. Differences between sawdust and control were analysed 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 analysed 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.

In order to evaluate the potential effect of sawdust on the size of the sub-communities of saprotrophic and plant pathogenic fungi, the analysis of functional groups was combined with PLFA and ITS data. The cumulative relative abundance of SVs assigned as plant pathogens (alone or combined with other potential functions) was multiplied, for each sample, for the total fungal abundance, based on both PLFA 18:2 ω 6,9 (or excess ^{13}C PLFA 18:2 ω 6,9, for the ^{13}C -enriched communities) and ITS2. The same estimation was done for the group 'Saprotroph'. Differences between sawdust-amended and control soil were compared with ANOVA and Tukey's post hoc test (family-wise error rate 5%). The data required log-transformation for meeting the assumptions of homoscedasticity and normality.

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Author contributions

A.C., S.E.H. and W.d.B. designed the research, A.C., M.P.J.H. and P.J.A.k.G. carried out the experiment and the measurements, A.C. analysed the data and wrote the manuscript in consultation with S.E.H. and W.d.B.

Conflict of Interest

The authors declare that they have no competing interests.

Data Availability Statement

The data presented in this study is available at doi:10.5061/dryad.2rbnzs7nn. Sequencing data is accessible at the European Nucleotide Archive (accession PRJEB37482(ERP120799)).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Bacterial and fungal abundance in soil, rhizosphere and roots, based on qPCR and ergosterol.

Supplementary Materials and Methods: Measurement of ^{13}C content of soil, rhizosphere, shoot and roots, molecular analyses (qPCR, gradient fractionation and sequencing), bioinformatic processing of raw sequencing data.

Fig. S2. Effect of sawdust on fungal families assigned to saprotrophs and potential pathogens.

Fig. S3. Estimated effect of sawdust on the absolute abundance of fungal functional groups.

Table S1. Biomass, N content and germination rate of seedlings, ^{13}C content of labelled soil, rhizosphere and seedlings.

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.

Table S3. Mean concentration and summary of statistical differences of individual PLFAs and NLFAs (nmol C g^{-1} soil).

Table S4. Mean labelled fraction and summary of statistical differences of individual PLFAs and NLFAs ($\text{pmol excess } ^{13}\text{C g}^{-1}$ soil).

Table S5. Summary of individual fractions pooled into 'heavy' and 'light' DNA fractions.

Table S6. Summary of permutational multivariate analysis of variance for the fungal community.

Table S7. Summary of permutational multivariate analysis of variance for the bacterial community.

Table S8. Taxa and functional groups identified by ITS2 rDNA sequencing.

Appendix S1 Supporting information.