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RESEARCH ARTICLE

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Cover crop identity determines root fungal community and arbuscular mycorrhiza colonization in following main crops

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Abstract

Cover crops (CC) can promote nutrient retention and recycling for main crops yet may also promote soilborne pathogens or suppress beneficial root symbionts such as arbuscular mycorrhizal fungi (AMF). We investigated how root fungal communities of main crop are affected by preceding CC monocultures and mixtures and by main crop identity. We expected that AMF abundance and diversity in main crops are promoted by AM-host CC, and suppressed by non-AM-host CC, and that mixtures of CC species can promote beneficial and suppress pathogenic root fungi. Our full-factorial field experiment comprised crop rotation in sand soil with different CC treatments (monocultures of radish [AM non-host], ryegrass, clover, vetch [AM hosts], mixtures of radish + vetch, ryegrass + clover and fallow) and two main crops (oat and endive). At peak crop growth, we investigated the root fungal communities in the main crops using microscopy and high throughput sequencing (Illumina MiSeq). Cover crop identity was of prime importance and CC legacy overruled main crop identity in determining root fungal communities in main crops. Compared with fallow, CC with ryegrass increased AMF colonization and richness in both main crops and of non-AMF in oat. Legacies of ryegrass, ryegrass + clover and vetch resulted in distinct root fungal communities in the main crops, while the legacy of CC with radish were similar to the legacy of fallow. Root fungal community in crops after clover had highest abundance of representative fungal pathogens in contrast with the other CC treatments that resulted in fungal communities where pathogens were scarce. Oppositely to expected, CC mixtures did not enhance fungal symbionts or suppressed pathogens. Overall, fungal communities in roots of the main crops in our field experiment were determined by the preceding CC species in monoculture, rather

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than by the CC AMF preference or functional group. This research highlights that the choice of CC determines the root fungal community in main crop which may influence crop quality.

KEYWORDS

arbuscular mycorrhizal fungi, cover crops, crop diversification, legacy effects, root fungal communities

1 | INTRODUCTION

Cultivation of cover crops (CC) during fall and winter is a common agronomic practice aimed at promoting multiple ecosystem services, such as improving nutrient cycling and main crop productivity by reducing nutrient leaching and erosion, suppressing weeds and greenhouse gas emissions, and increasing soil organic carbon (Clark, 2008; Dias et al., 2015; Snapp et al., 2005). Cover crops not only affect soil physical and chemical parameters but also soil biological properties and can be used as tool to promote soil health (Vukicevich et al., 2016). Maintaining a diverse and active microbial population is of key importance to sustain and promote soil health and fertility (Bünemann et al., 2018; Chaparro et al., 2012; Lori et al., 2017). Healthy soils harbour a diversity of saprotrophic fungi and plant growth-promoting bacteria and fungi and are poor in plant pathogens. Improving soil health by making use of CC in crop rotations requires knowledge on how different CC species affect the abundance and diversity of both beneficial soil microorganisms, such as saprotrophic and root symbiotic arbuscular mycorrhizal fungi (AMF) (Benitez et al., 2016; Detheridge et al., 2016), and of plant pathogens (Abawi & Widmer, 2000). Meta-analyses of cover cropping studies show a general positive effect of CC as compared with fallow on the abundance, activity and diversity of the soil microbiome (Kim et al., 2020; Muhammad et al., 2021). However, despite proven effects of CC on soil biota in bulk soil, few field studies tested the legacy effects of different CC species and CC species mixtures on the fungal communities in the roots of the following main crops (Bainard et al., 2017; Turrini et al., 2016). From a functional perspective, the root-associated fungi are expected to be of prime importance for the health and productivity of the main crops (Porras-Alfaro et al., 2007; Rodriguez et al., 2009).

Cover crop species from different plant families or plant functional types may generate different legacy effects on the growth of subsequent plant species (Cortois et al., 2016). Brassicaceae species are popular as CC because of their fast growth and soil cover (Haramoto & Gallandt, 2004) and their promotion of soil microbial activity and nutrient mineralization after their incorporation into the soil (Barel et al., 2019; Larkin et al., 2010). However, Brassicaceae species are non-host plants for AMF and produce secondary metabolites which can suppress soilborne pathogenic and symbiotrophic fungi (Isobe et al., 2014; Karasawa & Takebe, 2012). Nevertheless, it remains unclear whether AM fungal suppression by Brassicaceae goes beyond the mere absence of an AM-host plant. Previous experiments did not find a difference of the effect of Brassicaceae on subsequent root fungal communities compared with fallow (Higo et al., 2018, 2019; White & Weil, 2010) and suggested that the suppressive mycorrhizal effect lasts and is carried over to the following main crop regardless of the subsequent crop species' identity (Higo et al., 2019).

Other commonly used CC species belong to Poaceae (grasses) or Fabaceae (legumes) because of their respective high organic matter input to soil and of the legumes' ability to fix atmospheric nitrogen (N) (Snapp et al., 2005). Plants from both families also promote soil microbial abundance and diversity (Benitez et al., 2016; Kim et al., 2020). Grasses and legumes used as CC promote different fungal (Manici et al., 2018) and AM fungal communities in their roots (Daniell et al., 2001; Higo et al., 2016). It is expected that legumes compared with grasses may enhance the abundance of AMF and

Highlights

- Legacy of cover crops determines root fungal community in succeeding main crops.
- Crop fungal community was most distinct after ryegrass, with highest arbuscular mycorrhizal (AM) fungal colonization and richness.
- Radish (non-AM cover crop) decreased AM richness but not AM colonization.
- Cover crop mixtures did not enhance AM fungi or suppressed root fungal pathogens in main crops.

saprotrophs (Benitez et al., 2016). Some studies indicate that increased AM fungal colonization and shift in AM fungal communities in roots are associated with a decrease in specific fungal root pathogens (Newsham et al., 1995; Sikes et al., 2009) and increase in plant growth promotion (Cortois et al., 2016). However, the resulting CC impact on the following main crops remains unclear as they can be overruled by main crop effects (Turrini et al., 2016). To our knowledge, no studies combined CC legacy effects of grasses, legumes and non-AMF host CC on AMF and other root-associated fungi (pathogens and saprotrophs) in several main crops within the same experiment. Further knowledge on the use of selected CC or crop rotations to enhance fungal functional diversity will contribute to improve sustainable intensification of crop production (Brito et al, 2021).

Combining different CC species in mixtures can promote the productivity of the following main crop, may reduce N leaching and may increase suppression of soilborne pathogens (Barel et al., 2018; Finney et al., 2016; Tribouillois et al., 2016; Vukicevich et al., 2016). However, effects of CC mixtures on soil microbial composition and activity can be highly dependent on the identity and abundance of the plant species in the CC mixture and their characteristics (Barel et al., 2019; Vukicevich et al., 2016). It is expected that growing plant species of the same plant family as the CC may increase plant pathogens and that the effects of species-specific plant pathogen can be diluted by mixing plant species (Garbeva et al., 2004). However, results from previous studies that show differential impacts of CC species and CC mixtures on soil fungi (Cloutier et al., 2020; Hannula et al., 2021; Thapa et al., 2021) are not always conclusive. Cloutier et al. (2020) showed that CC mixtures resulted in a distinct AM fungal community compared with monoculture, while Thapa et al. (2021) and Hannula et al. (2021) reported none or little effect of CC mixtures on the overall soil fungal community. One of the few available studies on legacy effects of CC on subsequent crop root fungal communities reported that high compared with a low diversity mixture of CC species increased AM fungal diversity in maize roots (Turrini et al., 2016). However, their study did not include the CC monocultures, and therefore, the effect of species mixtures versus the role of individual CC species in the mixture on the AM fungal communities could not be tested.

The main objective of this study was to determine the legacy effects of CC monocultures and mixtures of CC species from different plant functional groups on the root fungal community composition, richness and abundance in subsequent crops. We expected that:

i. CC composed of AM-host plants promote abundance and richness of AMF and other non-pathogenic fungi in roots of the following main crops.
ii. CC composed of non-AM-host plants suppress AM fungal abundance and richness and other fungi in main crop roots.
iii. CC mixtures increase the diversity of fungi across all fungal guilds, but lower relative abundance of fungal pathogens in main crop roots.

2 | MATERIALS AND METHODS

2.1 | Experimental design and management

The field experiment was performed at Wageningen University (Nergena, Wageningen, The Netherlands, 51°59′41.9″N 5°39′17.5″ E). The area is characterized by temperate sea climate with an annual mean temperature of 10.9°C and annual precipitation of 853.3 mm in 2015 (Royal Netherlands Meteorological Institute). Soil in this experiment is a sand soil (95% sand, 1% clay, 4% silt) with 3.9% organic matter and pH 5.7, classified as a Typic Endoaquoll (Soil Survey Staff, 2014).

The experiment comprised a full-factorial crop rotation in three phases (summer 2014, winter 2014 and summer 2015 being the test phase). Two main crops were grown during summer 2014 (MC14) and 2015 (MC15): oat (*Avena sativa* L. var. Dominik) and endive (*Cichorium endivia* L. var. Nummervijf2). The fall–winter period included seven CC treatments comprising fallow; four CC monocultures: perennial ryegrass (*Lolium perenne* L. var. Mathilde), white clover (*Trifolium repens* L. var. Alice), common vetch (*Vicia sativa* L. var. Ebena) and fodder radish (*Raphanus sativus* L. var. Terranova); and two CC mixtures: ryegrass + clover, and radish + vetch. All rotation treatments ($2 \times 7 \times 2 = 28$) were replicated five times in a randomized block design, resulting in 140 experimental plots (Table 1). The year prior to the experiment *Phacelia tanacetifolia* Benth. (var. Angelia) was grown to homogenize the cultivation history (Barel et al., 2018).

Oat was sown at the end of March and endive seedlings were transplanted early in May in both 2014 and 2015. Endive was harvested mid-July and oat on end-July 2014 while the remaining stubbles were hoed and left on the field. At the end of August, the plots were hoed by hand and CC were sown. Mid-February 2015, the CC were mown and the residues incorporated into the soil (0–15 cm depth) by a small superficially- tilling machine. All the plots were fertilized equally in March 2014 and

TABLE 1 Main crop (MC) and cover crop (CC) rotation scheme of the field experiment.

Summer 2013	Spring–summer 2014 MC14	Autumn–winter 2014 CC	Spring–summer 2015 MC15
Phacelia	Oat	Fallow	Oat
			Endive
		Ryegrass	Oat
			Endive
		Clover	Oat
			Endive
		Radish	Oat
			Endive
		Vetch	Oat
			Endive
		Ryegrass + clover	Oat
			Endive
		Radish + vetch	Oat
			Endive
	Endive	Fallow	Oat
			Endive
		Ryegrass	Oat
			Endive
		Clover	Oat
			Endive
		Radish	Oat
			Endive
		Vetch	Oat
			Endive
		Ryegrass + clover	Oat
			Endive
		Radish + vetch	Oat
			Endive

2015, according to commercial practice, with the exception that for N fertilization in 2015 half the amount given in 2014: 41 kg N ha^{-1} in March and additionally 14 kg N ha^{-1} in May 2015. Further details on the experiment, including on fertilization, irrigation and weed management, are described in Barel et al. (2018).

The properties of the CC treatments, soil and biomass responses of the main crops in this experiment are published in Barel et al. (2018) (see Table S1 for data on main crop biomass) and of soil and litter saprotroph activity in Barel et al. (2019). Barel et al. (2018) showed that the legacy effect of the different CC treatments on main crop biomass was driven by the CC biomass and N concentration of the CC. Further, in this sand soil, the residue decomposition rate and nutrient mineralization by the saprotrophic soil biota were influenced by the different CC treatments via changes in soil properties (soil microbial biomass, soil organic matter and N content), as well as by the CC residue quality (low lignin content) (Barel et al., 2019).

2.2 | Root sampling

Root samples were collected as composite samples on 17–18 June 2015 during main crop (MC15) growth using a 1-cm-diameter auger to sample 0–15 cm depth. Oat roots were collected from 12 sampling points per plot, close to plants (~2 cm from stalk). For endive, nine points per endive plot were sampled across the length of three planting rows (3 m long) and keeping 30 cm from the plot borders. Roots were washed carefully with tap water to remove adherent soil. Each composite sample was divided in two subsamples, one was stored at 4°C for root colonization measurement and the other was placed at −20°C for DNA extraction to measure fungal community diversity and composition.

2.3 | Root fungal colonization assessment (AMF and non-AMF)

Root colonization by fungi was assessed by staining fresh root subsamples of oat and endive using the ink and vinegar method (Vierheilig et al., 1998). Percentage of total AMF, arbuscles, vesicles and non-AMF (different from AMF) root colonization were determined using a light microscope according to the magnified intersections method (McGonigle et al., 1990). Non-AM fungal colonization was determined by counting intersections with non-AM fungal structures (Rillig et al., 1998). These percentages were calculated as (n intersections with the AMF/non-AMF structure)/(y total intersections) × 100, with 'y' being 100 or slightly over 100.

2.4 | Sequencing of root fungal communities

Total root fungal community composition and AM fungal community composition were determined by amplifying the ITS2 of ribosomal encoding genes using primer combination ITS4/ITS9 (Ihrmark et al., 2012) amplicon sequencing. The ITS region is the universal barcode for fungi (Schoch et al., 2012). ITS primers are known to be less specific for some of the fungal families in the

Glomeromycota phylum compared with the SSU rRNA primers (Tedersoo et al., 2018). However, previous studies have proved that ITS and SSU rRNA primers show a similar and comparable ability to detect responses of AM fungal communities to environmental shifts due to the ability to amplify the most abundant dominant taxa (Glomeraceae) in a similar manner (Berruti et al., 2017; Lekberg et al., 2018).

DNA was extracted from a 100 mg root sample per experimental plot using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) reactions were carried out in a 25 μL reaction mixture containing 1 μL template DNA, 0.5 μL of both specific fungal primers, 2.5 μL 10× Buffer with $MgCl_2$, 1 μL of 5 μM dNTPs, 1 μL of 25 mM $MgCl_2$, 1.25 μL BSA (4 mg mL^{-1}) and 0.15 μL Fast Start Taq. The PCR conditions were 94°C for 5 min, then 35 cycles of 94°C for 45 s, 54°C for 60 s and 72°C for 1.3 min, followed by a final extension of 72°C for 10 min. All PCRs were conducted in duplicates, product quality was visually verified on 1.5% agarose gel, and duplicates were pooled before PCR clean-up with Agencourt AMPure XP magnetic beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Samples were pooled in equimolar ratios after determining concentrations with a fragment analyser using a Standard Sensitivity NGS Fragment Analysis kit (1–6000 bp) and following manufacturer's instructions (Advanced Analytical Technologies GmbH, Heidelberg, Germany). Successfully amplified PCR products ($n = 135$) were sent for Illumina MiSeq sequencing to BGI (China). Since roots were washed with tap water, and there was no sterilization of root surface, the sequencing data comprise both endophytic fungi and fungi attached to the rhizoplane. This approach enabled us to evaluate root associated fungal communities including root endophytic and root adhering fungi. Information on endophytic fungal communities is also supported by the microscopy data on AM and non-AM fungal colonization of the roots of the main crops.

2.5 | Bioinformatics

Illumina MiSeq paired-end reads were analysed using a pipeline implemented in a Snakemake workflow (Köster & Rahmann, 2012). This pipeline has been successfully used to describe patterns of soil fungal communities in several studies (Koorem et al., 2020; Palomino et al., 2023; Ramirez et al., 2019). First, paired-end reads with a minimum overlap of 25 bp and at least a PHRED score of 25 were merged using the RDP extension to PANDASeq (Masella et al., 2012) named Assembler (Cole et al., 2014). ITSx 1.0.11 was used to extract ITS2 region and remove primer sequences prior to sequence clustering (Bengtsson-Palme et al., 2013). VSEARCH v. 1.0.10 (Rognes et al., 2016) was used for sequence clustering into operational taxonomic units (OTUs), using the UPARSE strategy by de-replication, sorting by abundance (with at least two sequences) and clustering using the UCLUST smallmem algorithm (Edgar, 2010). Hereafter, chimeric sequences were detected using the UCHIME algorithm (Edgar et al., 2011) implemented in VSEARCH and removed. Finally, taxonomic classification for each OTU was obtained using the UNITE database (Koljalg et al., 2013) provided by RDP. OTUs represented by singletons and samples with a low reading depth (<1000) were removed. Currently, other bioinformatic pipelines of ASV level are commonly used. A comparative study of main bioinformatic pipelines for amplicon sequences showed that OTU-level pipeline such as the USEARCH-UPARSE pipeline used in this study performs well, although they may provide lower specificity than ASV-level pipelines (Prodan et al., 2020).

The final number of root samples represented in the OTU table was 119 (Table S2). Total number of OTUs and sequences through the data cleaning process, as well as within fungal orders, is described in Table S2. There was no amplification of non-fungal sequences. The final OTU table and taxonomic classification represented the root fungal community in MC15. A separate OTU table that included only the OTUs that belonged to the Glomeromycota phylum was used to describe the AM fungal community. Both OTU tables (total fungi and AMF fungi) were used in further investigations.

2.6 | Data analysis

Lineal mixed effect (LME) models were used to test the effect of the main crop (MC15) identity and the legacy of the CC treatments and preceding main crop (MC14), as well as the interaction between CC and MC15 on the percentage of fungal colonization in the roots of the MC15 (total AMF, arbuscules and non-AMF), with block as a random factor. The treatment effects on the percentage of vesicles were not statistically tested since vesicles were almost not present. Prior to the analyses, variables that did not follow the assumptions of normality and homogeneity were arcsine square root transformed. Post hoc differences were evaluated with Fishers LSD test at $p < 0.05$. LME models were performed using 'lme4' R package (Bates et al., 2014).

Similarly, the effects of the main crop (MC15), the legacy effects of the CC and previous main crop (MC14) and the interaction between CC and MC15 on the root fungal communities in the main crops were tested

TABLE 2 Summary of the main effects and interactions of main crop identity (MC15), cover crop legacy (CC) and previous main crop legacy (MC14), on the root fungal colonization (total arbuscular mycorrhizal fungi [AMF], arbuscules and non-AMF), fungal community structure (total fungi and AMF) and fungal richness (total fungi and AMF).

		Root fungal colonization			Fungal community structure				Fungal richness	
					Total fungi		AMF			
	d.f.	Total AMF *F*	Arbuscules *F*	Non-AMF *F*	*F*	R^2	*F*	R^2	Total fungi *F*	AMF *F*
MC15	1	0.532	0.198	369.738***	7.421	0.055***	2.716	0.021**	7.871**	24.11***
CC	6	24.993***	19.063***	8.230***	2.451	0.108***	2.132	0.097***	1.429	3.125**
MC14	1	9.381**	9.575**	1.6721	2.608	0.019***	2.838	0.022**	1.669	4.121*
MC15 × CC	6	1.323	2.034	5.475***	1.145	0.051*	1.503	0.069**	0.408	1.186
CC × MC14	6	1.285	1.287	1.554	1.007	0.044	0.911	0.042	1.242	1.079
Residuals	98					0.767		0.75		
Total	118									

***$p \leq 0.001$;
**$p \leq 0.01$; *$p \leq 0.05$.

using permutational multivariate analysis of variance (PerMANOVA). The PerMANOVA model was run using the 'adonis' function from the 'vegan' R package (Oksanen et al., 2013) and tested on the log-transformed Euclidean matrix of the fungal community. Permutations were constrained within blocks (Anderson et al., 2011). Pairwise comparisons to distinguish the effect of CC on the fungal community compositions were performed using the function 'pairwise.adonis' that returns adjusted *p*-values (Arbizu, 2017). Principal coordinate analyses (PCoA) were used to graphically visualize the community composition of the different samples in the multivariate space. The legacy effects of CC was tested on OTUs richness, estimated as rarefied alpha-diversity by using 'rarefy()' function from the 'vegan' R package after rarefaction to the median read count across all samples (De Cárcer et al., 2011). Effects of the CC and main crop treatments on the overall fungal richness and the AM fungal richness were tested using the same LME model as described above. To investigate the patterns of the AM fungal communities in more detail, the same statistical analysis was done in a subset of the fungal OTUs selecting only the OTUs belonging to the Glomeromycota phylum. Information on the number of fungal OTUs and their distribution and taxonomy of the fungi that colonized the roots of the two crop species oat and endive is shown in the supplementary info (Table S2).

Indicator fungal OTUs of CC treatments were identified using the 'indicspecies' R package. Indicator-OTUs of CC treatments are fungal OTUs in which the observed distribution pattern in terms of occurrence and abundance strongly relates to the CC treatment based on the 'Indicator Value Indices' (De Cáceres et al., 2010). We used the percentage of number of sequences as a measure of relative abundance for the indicator-OTUs analysis. Indicator-OTUs with relative abundance below 0.0015% of the total number of sequences were not included; this threshold corresponds to the OTUs abundance curve inflexion point. The potential function of each indicator-OTU was subsequently evaluated by assignment of the OTU to functional guilds using FUNGuild database (Nguyen et al., 2016). The analyses classified the fungal sequences in three main categories: pathogens, saprotrophs and symbionts and three combinations thereof.

3 | RESULTS

3.1 | Legacy effects of preceding crops on fungal root colonization (AMF and non-AMF)

The AM fungal colonization in oat and endive was significantly affected by the CC legacy (Table 2; Figure 1a). Total AM fungal colonization was highest when oat and endive grew after ryegrass, or after ryegrass + clover, and AM fungal colonization was lowest in oat and endive grown after CC treatments with radish and vetch, but these levels were similar to plants grown after fallow (Figure 1a). The identity of the previous main crop (MC14) also caused a legacy effect on AM root colonization in the main crop of the following year, despite the different CC treatments that were grown in between both main crop phase (Table 2). The legacy of oat as compared with endive resulted in a higher root colonization by AMF (14.40 ± 1.15% vs. 11.44 ± 0.87% respectively). The average root colonization of oat and endive by AMF was

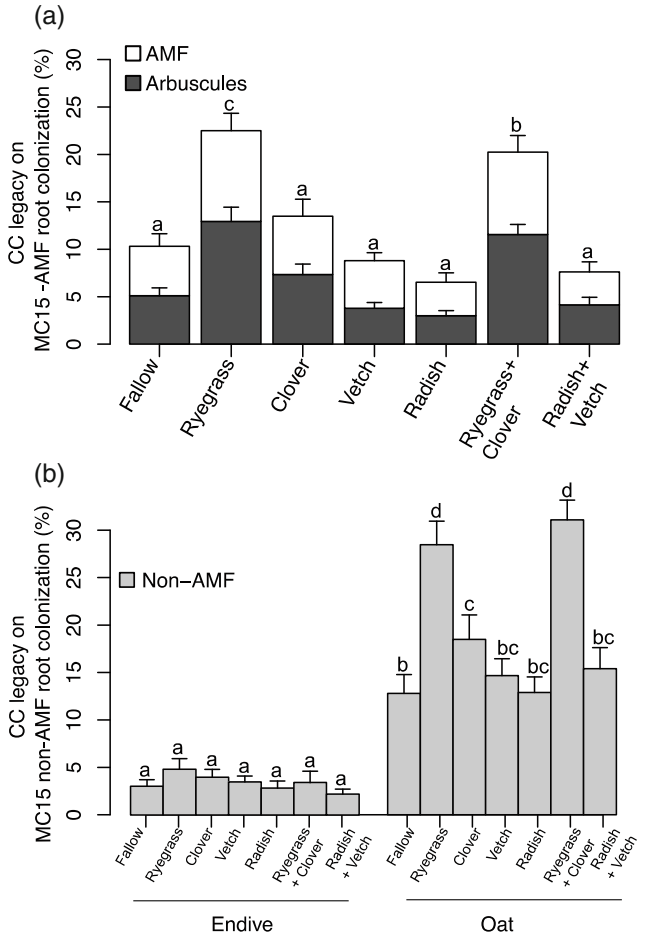

FIGURE 1 Legacy effects of the cover crops (CC) on (a) the percentage of total arbuscular mycorrhizal fungi (AMF) and arbuscules root length colonization and (b) percentage of non-AMF root colonization. Different letters above the bars indicate significant differences between treatments (p-value ≤ 0.05).

similar in MC15 (12.92 ± 0.73%), with good colonization by arbuscles (6.9 ± 0.47%) and very low abundance of vesicles (0.07 ± 0.02%). The root colonization by arbuscules followed the same pattern as total AM fungal colonization (Table 2, Figure 1a).

Non-AMF root colonization was more abundant in oat than in endive (Table 2, Figure 1b). In oat, the root colonization by non-AMF was also affected by the CC legacy with higher abundance of non-AMF in oat growing after ryegrass and ryegrass + clover, whereas the other CC legacies were not distinct from fallow (Table 2. Figure 1b).

3.2 | Legacy effects of CC monocultures and mixtures on root fungal community composition and diversity

Oat and endive roots were colonized by distinct fungal communities (Table 2; Figure 2a), yet the CC legacy effect on the fungal communities was larger than the effect of the main crop identity in terms of explained model variation (Table 2; Figure 2b). Main crop identity (MC15) explained 5.5% of the variation across the total root fungal communities, whereas the variation explained by the legacy of the CC was almost double (10.8%) (R^2 values in Table 2). The root fungal communities were also affected by the legacy of the previous main crop (MC14), but this explained only a minor part of the variation (1.9%). There was a significant interaction between the crop identity (MC15) and the CC treatments (Table 2) although post-hoc pairwise

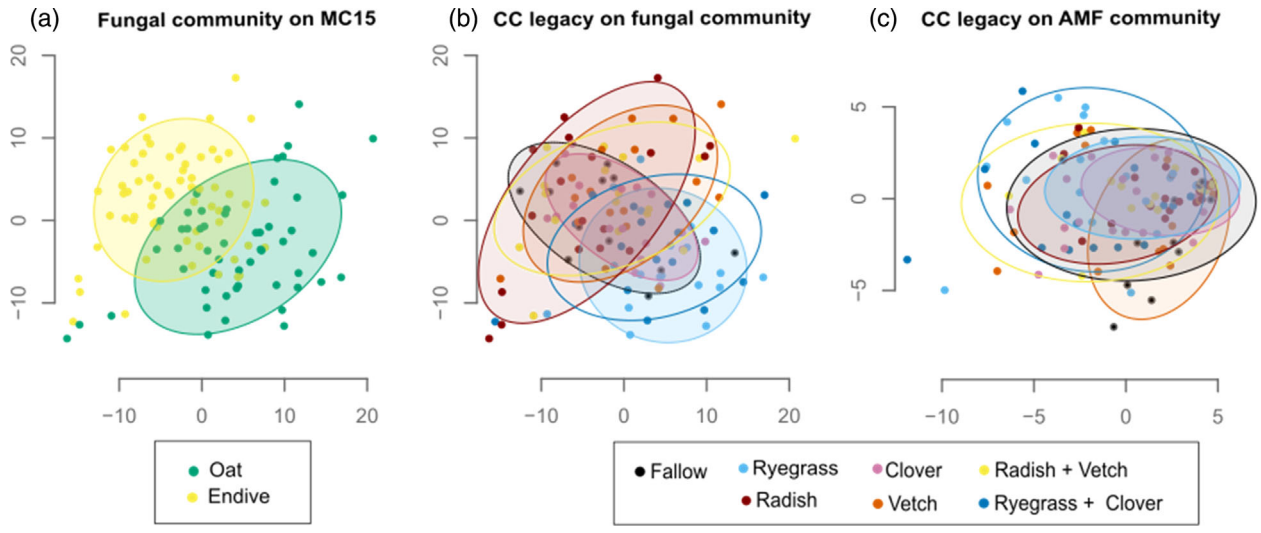

FIGURE 2 Principal coordinate analyses of (a) the fungal communities in main crop roots (MC15), (b) the legacy effects of cover crop (CC) treatments on the total fungal community and (c) the legacy effects of CC treatments on arbuscular mycorrhizal fungi (AMF) community. Ellipses delimit the 75% confidence interval around centroids. Ellipses from monocrops CC legacies and fallow legacy are shadowed while ellipses from mixture CC legacies are not shadowed.

comparisons did not show significant differences (see Table S3). Therefore, we report the overall effect of CC regardless the interaction with main crop identity. Relative abundance of fungal phyla in main crop roots and as a legacy effect of the CC treatments is represented in Figure S1.

Fungal communities colonizing the roots of the main crops were affected by CC treatments (Table 3, Figure 2b). Compared with fallow treatment, ryegrass, ryegrass + clover and vetch resulted in a distinct fungal community. Besides, the legacy of ryegrass on the root fungal communities in the main crops was different from all the other CC treatments (except from ryegrass + clover). Legume monocultures (clover and vetch) resulted in distinct main crop root fungal communities; however, their legacy effect was not different from the legacy of the non-AMF CC radish. The legacy effect of the CC mixture treatments followed similar patterns of the CC species in monoculture according to their realized abundance in the CC mixture. Thus, legacy of radish + vetch resembled legacy of radish and vetch in monoculture and differed from the legacies of the other CC species. Legacy of ryegrass + clover resembled ryegrass monoculture, since ryegrass was prevalent in the mixture (Barel et al., 2018).

The CC and main crop treatments had significant legacy effects on the AM fungal community structure in oat and endive roots (Table 2; Figure 2c). Most variation was explained by the CC legacies (9.7%) and a minor part by the current main crop (MC15, 2.1%) and the main crop of the preceding year (MC14, 2.2%) (Table 2). The

TABLE 3 R^2 values of pairwise comparison between cover crop (CC) legacy effects on the main crop (MC15) root fungal community.

	CC legacy effect on total fungal and AMF communities in roots of main crops MC15 (R^2 values)						
	Fallow	**Ryegrass**	**Clover**	**Vetch**	**Radish**	**Ryegrass + clover**	**Radish + vetch**
Fallow		**0.09***	0.05	**0.07***	0.06	**0.06***	0.07
Ryegrass	**0.13***		**0.08***	**0.1***	**0.1***	0.03	**0.09***
Clover	0.05	0.05		**0.06***	0.05	**0.06***	**0.06***
Vetch	0.04	0.05	0.02		0.05	**0.08***	0.04
Radish	0.07	**0.12***	0.07	0.04		**0.08***	0.03
Ryegrass + clover	0.09	0.02	0.04	0.05	**0.11***		**0.07***
Radish + vetch	0.08	0.07	0.05	0.03	0.03	0.07	

Note: Total fungal community (upper triangle; shaded gray) and arbuscular mycorrhizal fungal (AMF) community (lower triangle). Bold numbers indicate significantly different communities.
*Adjusted p-value ≤ 0.05.

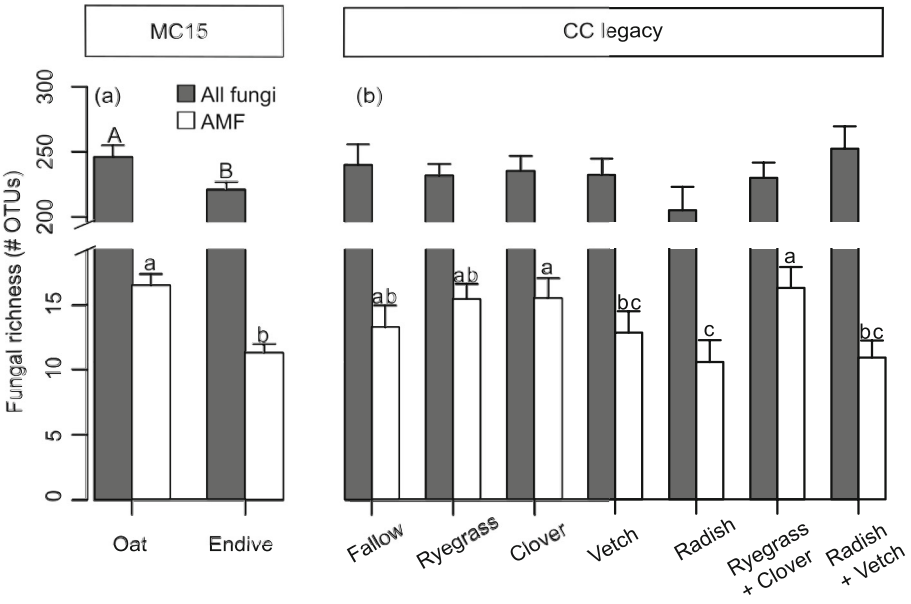

FIGURE 3 Fungal richness (total fungi and arbuscular mycorrhizal fungi [AMF]) in (a) main crop 2015 roots (MC15) and (b) as a result of the legacy effects of cover crops (CC). Vertical bars represent means ± 1 SE. Different letters above the bars indicate significant differences between treatments (p-values ≤ 0.05). OTUs, operational taxonomic units.

interaction between main crop identity and CC treatments explained 6.9% of the AM fungal community variability. Overall, compared with fallow, only the legacy of ryegrass resulted in distinct AM fungal communities in roots of oat and endive, and the legacies of ryegrass and ryegrass + clover were also distinct from that of radish (Table 3, Figure 2c). The same as for the total fungal community, there was an interactive effect of the MC15 and CC (Table 2), but post-hoc pairwise comparisons of the interaction were not significant, and therefore, we explained the overall legacy effect of the CC treatments.

Fungal communities in oat roots were more diverse than in endive roots, with higher richness of total fungal and AMF-OTUs (Table 2; Figure 3). The CC treatments and preceding main crops also influenced the AM fungal richness in the roots of the main crops but not the total fungal richness (Table 2). The CC treatments ryegrass + clover and clover stimulate AM fungal richness (although it was not different from fallow's legacy) and radish reduced AM fungal richness in oat and endive roots (Figure 3). Previous MC14 oat also stimulated AM fungal richness in the roots of the main crops grown the following year (15 ± 0.8 vs. 12 ± 0.8 number of OTUs; legacy of MC14 oat vs. endive).

The legacy of ryegrass CC resulted in the highest abundance of indicator OTUs in main crop roots (Table 4, Figure S2). Oppositely, the main crop root fungal community after both CC mixtures had the lowest abundance of indicator OTUs. Saprotroph indicator-OTUs were more abundant after legume CC and after fallow. Symbiotroph indicator OTUs were recovered after fallow, ryegrass, clover and ryegrass + clover, but not after vetch, radish, or radish + vetch (Table 4, Figure S2). Pathotrophs indicator OTUs were only detected after clover and after radish, although abundance after radish was almost null (0.004%). More details on the species assigned to each OTUs and relative abundance is described in Table S4.

All the AMF OTUs (61 AMF OTUs) were shared by oat and endive (Figure S3). AMF OTUs comprised all four Glomeromycota orders: 62.1% of AMF sequences were assigned to *Glomerales* (31 OTUs), 13.1% *Archaeosporales* (12 OTUs), 5.7% *Diversisporales* (7 OTUs) and 2% *Paraglomerales* OTUs (4 OTUs) (Table S2). Unclassified Glomeromycota represented 17% of the AMF sequences. Among the AMF orders, the more common were *Glomerales*, *Archaeosporales* and *Diversisporales*. These orders, mainly *Glomerales*, are generally found in arable fields (Daniell et al., 2001; Higo et al., 2015; Oehl et al., 2010), but their functional differences are not yet well known (Burleigh et al., 2002; Munkvold et al., 2004).

TABLE 4 Cover crop legacy effects on the number and relative abundance (% of sequences) of indicator OTUs per fungal guild in the fungal communities colonizing main crop roots (MC15, oat and endive).

	Fungal indicator OTUs													
	Fallow		Ryegrass		Clover		Vetch		Radish		Ryegrass + clover		Radish + vetch	
Fungal guild	OTUs (#)	Sequences (%)	OTUs (#)	Sequences (%)	OTUs (#)	Sequences (%)	OTUs (#)	Sequences (%)	OTUs (#)	Sequences (%)	OTUs (#)	Sequences (%)	OTUs (#)	Sequences (%)
Saprotroph	7	2.99	2	0.1	1	0.92	2	1.52	0	0	0	0	1	0.01
Symbiotroph	2	0.02	1	0.04	2	0.01	0	0	0	0	2	0.09	0	0
Pathotroph	0	0	0	0	2	0.51	0	0	1	0.004	0	0	0	0
Patho/Saprotroph	1	0.08	0	0	0	0	0	0	0	0	0	0	0	0
Patho/Sapro/Symbiotroph	1	0.05	0	0	0	0	0	0	0	0	0	0	2	0.004
Unknown	4	0.99	5	19.79	3	0.75	9	1.59	5	3.81	5	0.21	8	0.05
Total	15	4.13	8	19.93	8	2.19	11	3.11	6	3.814	7	0.3	11	0.064

4 | DISCUSSION

Our results show that the legacy of CC determines the abundance, richness and structure of fungal communities associated to the roots of subsequent main crops. The CC legacy had higher impact on the main crop root fungal community structure than the identity of the main crop itself. Similarly, AM fungal root colonization of the main crop was determined by the CC legacy and not by the main crop identity. The distinct abundance and composition of the root fungal communities in the main crops as a result of the CC legacy may be due to differences in CC root properties such as root biomass, root length and root exudates, which modulate root and soil fungal communities (Broeckling et al., 2008). Thereby, different CC species can select for distinct soil and root associated microbial populations (Finney et al., 2017; Zhou et al., 2017) which can be carried over to the next crop.

Soil texture together with cover crops have been shown to shape soil fungal communities in crop rotations (Cloutier et al., 2020; Wakelin et al., 2008). Also, soil texture may affect root growth (Schenk & Jackson, 2002) and thus plant-fungal associations. Hence, responsiveness of associated root fungal communities to cover crops may differ with soil textural properties. Here, we discuss in more detail the different legacy effects of CC species in monocultures and in mixtures as well as the effect of the main crops in an arable sand soil.

4.1 | Legacy of cover crop species in monoculture and in mixtures

We expected that CC composed of AM-host plants would promote AMF over other fungi. However, our results showed that the legacy of CC on subsequent root fungal community is not linked to the CC AM-host preference. Ryegrass had a strong effect on fungal communities (both total fungi and AMF) colonizing the roots of the main crop (MC15), while the effect of clover and vetch was not always different to fallow. The legacy of ryegrass and ryegrass + clover increased the AM fungal colonization in the roots of the main crops. These results are in line with previous field experiments and confirm grasses as good AM hosts (García-González et al., 2016; Kabir & Koide, 2000; White & Weil, 2010). The legacy of the mixture ryegrass + clover improved AM colonization compared with that of clover alone, likely because the grass provided more AM-colonized roots given the larger root length density of the grass compared with clover (Barceló et al., 2020; Barel et al., 2018).

Similarly, ryegrass and ryegrass + clover legacies also resulted in higher root colonization by non-AMF in oat roots. The high non-AMF abundance in roots of crops grown after ryegrass and ryegrass + clover may be stimulated by the high root biomass of ryegrass and its high C/N ratio compared with the legume species (Barel et al., 2018), which may serve as resources for non-AM endophytic fungi that are opportunistic decomposers until the following crop is grown (Peay et al., 2016; Reeleder et al., 2006). Accordingly, Thapa et al. (2021) recently showed that a grass CC (oat) promoted non-AMF as well as AMF to a greater extent than pea or canola in soil, which results in different soil microbial pools for the following crops.

Furthermore, the legacy of ryegrass and its mixture with clover resulted in a distinct community composition compared with the legacies of the other CC species and the fallow treatment. Thus, abundance of indicator OTUs was highest after ryegrass (5–9 times higher than fallow and the other CC monocultures). Also, ryegrass was the only CC treatment that resulted in an AM fungal community that was distinct to the one after fallow and after radish. In summary, from the seven CC treatments tested in our experiment, ryegrass and its mixture with clover stood out in promoting a distinct fungal community in the roots of the following crops.

In contrast to our second hypothesis, AM fungal root colonization after radish (non-AM-host) was not lower compared with fallow, although it resulted in the significantly lowest AM fungal richness. Compared with ryegrass, clover or the ryegrass + clover mixture, the radish legacy generally reduced AM fungal root colonization and richness. The impact of Brassicaceous CC relative to AM-host CC on AM fungal communities in roots of main crops seems context-dependent as some studies reported no effect (Higo et al., 2018), or effects being dependent on main crop identity (Higo et al., 2019), or soil management, such as tillage (Higo et al., 2020). Nevertheless, the radish CC legacy resulted in a less distinct root fungal community (for both total fungi and AMF) in the roots of the main crops. Total fungal and AM fungal communities after radish were only different to the communities after ryegrass and ryegrass + clover. Accordingly, fungal community after radish had the least number of indicator OTUs which suggests the presence of fewer plant specific root fungi of radish that also colonize oat and endive.

Lastly, we expected that CC mixtures would increase the diversity of fungi, and as a result, there would be less representation of pathogenic OTUs. However, we found similar fungal and AM fungal richness after the CC mixtures compared with the monocultures. Overall, we found that legacy of CC mixtures resembled the legacy effect of the CC species in monoculture. Within the ryegrass + clover mixture, the ryegrass legacy dominated over the clover legacy with respect to both colonization

and community structure of the fungi in the roots of the main crops. These results are in line with recent work on soil microbial communities of Ulcuango et al. (2021) who found that the legacy of a barley–vetch mixture resulted in soil microbial communities which resembled the microbial communities of the grass and differed from that of the legume. Interestingly, we found few indicator-OTUs classified as pathotrophic, yet several of these appeared to be indicator-OTUs for the clover CC treatment. Other studies also reported higher levels of potential plant fungal pathogens from legumes used as CC or in rotation compared with Brassicaceae or grasses (Bainard et al., 2017; Manici et al., 2018; Wagg et al., 2021). As there were no pathotrophic indicator-OTUs found in the ryegrass + clover mixture, it may indicate ryegrass + clover could suppress specific clover associated fungal pathotrophs which would be a beneficial effect of CC mixing.

Overall, in our study, the CC treatments that promoted crop AM fungal colonization and diversity in crops did not result in the highest crop yield (see Barel et al., 2018; Table S1) likely due to an overruling effect of nutrient mineralization in this sand soil, which was largest in radish and radish + vetch legacies compared with fallow and ryegrass legacies (Barel et al., 2019). Besides, soil organic matter and fungal soil biomass was higher after radish (Barel et al., 2018, 2019), which may indicate better soil structure and soil nutrient retention (Six et al., 2004). These soil properties are relevant for crop growth on sandy soils due to the high risk of nutrient leaching. The beneficial role of radish containing cover crops on subsequent main crop growth and nutrient retention across cropping cycles has recently also been shown in a multi-year field experiment on a similar sandy soil and climate as in our experiment (Elhakeem et al., 2023).

4.2 | Main crop effects and legacy of previous main crop

The main aim and novelty of our research is to test the CC legacy on root fungal communities in subsequent main crops. Additionally, with our data set, we also see the effect of the of main crop identity and legacy of the previous main crop, which we discuss in this section. Fungal community colonizing oat and endive were different. While oat and endive show the same levels of AM fungal root colonization, percentage of non-AMF fungi were five times more abundant in oat than in endive roots. These results may be due to the relatively larger stimulation of non-AMF by grasses as compared with forbs (Cortois et al., 2016). Fungal and AM fungal richness were also higher in oat roots than in endive roots. Interestingly, the legacy effect of the main crop grown the year before (MC14) on AM fungal root length colonization was still detectable. Growing oat instead of endive in the first year slightly stimulated AM fungal colonization in the main crops the following year, irrespective of the CC treatments that were grown in between. The AMF promotion by oat may be due to a more fibrous and dense root system of oat that is more favorable for building-up AM fungal inoculum potential for following plants compared with the taproot system of endive (Wilson & Hartnett, 1998; Zadworny & Eissenstat, 2011), as well as to a longer growing season for oat than for endive and thus longer time for roots to interact with the soil microbes for oat compared with endive.

Overall, our results support that legacy of previous crops (cover and main crops) influence fungal properties in agroecosystems. The legacy effect may decrease with time from crop growth but some fungal properties, likely linked to the existing soil fungal inoculum, may be more perdurable in time.

5 | CONCLUSIONS

The choice of CC species and species mixtures has significant impacts on the root-associated fungal community structure and abundance in the roots of the following main crops. While ryegrass and ryegrass + clover promoted AM fungal abundance and richness in the roots of the main crops, the non-AM-host radish decreased main crop AM fungal richness but did not significantly suppress AM abundance compared with fallow. CC legacy effects in mixtures resembled the legacy of the CC species in monocultures and did not increase fungal diversity or abundance (AMF and non-AMF).

Cover crop legacy effects on main crop root fungal communities may affect crop growth through beneficial root fungal symbionts and pathogens. In our study, the CC treatments that promoted crop AM fungal colonization and diversity in subsequent crops did not show highest crop yield (see Barel et al., 2018; Table S1). Further studies should aim for a holistic approach and unravel the functions of the fungal communities associated with specific CC species, species mixtures and their legacies to ecosystem services related to the crop and the agroecosystem that are perdurable in time.

AUTHOR CONTRIBUTIONS

Laura B. Martínez-García: Writing – original draft; writing – review and editing; conceptualization; formal analysis; investigation; data curation; visualization. **Irene García-González:** Writing – original draft; writing – review and editing; conceptualization; methodology;

formal analysis; investigation; data curation. **Janna M. Barel:** Conceptualization; writing – review and editing; methodology; investigation; data curation. **Henk Martens:** Methodology. **L. Basten Snoek:** Software. **Chiquinquirá Hontoria:** Writing – review and editing; conceptualization; methodology; investigation; funding acquisition. **Gerlinde B. De Deyn:** Conceptualization; writing – review and editing; funding acquisition; methodology; investigation; resources; data curation; supervision; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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