### Title

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RESEARCH

# Exploring the Effects of *Trichoderma virens* Biofungicide on Carrot Cavity Spot and Soil Fungal Community Dynamics

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## ABSTRACT

Carrot cavity spot (CCS) has conventionally been managed with fungicides. However, fungicide resistance, their potential risks to human health and the environment, and the increasing demand for organic produce necessitate the exploration of biofungicides as alternatives. In this study, we evaluated varying concentrations of SoilGard (Certis USA, Columbia, MD), a *Trichoderma virens*-based biofungicide, for efficacy against different CCS-causing *Pythium* species in vitro. Additionally, its effects on taxonomic and functional diversities of soil fungal communities were studied in vivo in the greenhouse. To our knowledge, this is the first study reporting SoilGard's effectiveness against CCS, with emphasis on its potential as an alternative for fungicide-resistant *Pythium* isolates. Our in vitro study revealed that SoilGard efficacy was significantly dose-dependent and isolate-specific, thus highlighting the importance of selecting its application rate and the target isolate. Analysis of soil fungal communities using Illumina MiSeq sequencing revealed that SoilGard exerted a significant, albeit temporary, effect on the fungal community structure. It negatively impacted co-occurrence network complexity and alpha diversity in carrot-cultivated soil, whereas bare soil communities remained largely unaffected, thus explaining why preplant applications may yield better results. Our study showed that carrot cultivation without SoilGard enhanced fungal diversity, which was more pronounced late in the season, possibly due to carrot root-associated exudates. Our study sheds light on how complex interactions within soil fungal communities can be impacted by the application of beneficial/pathogenic microbes.

*Keywords*: co-occurrence network, Illumina MiSeq, metabolic activities, SoilGard 12G, taxonomic and functional diversity

California is the leading producer of carrots (*Daucus carota* subsp. *sativus*) in the United States, contributing about 80% of the total U.S. production (USDA-NASS 2023). However, carrot productivity faces impediments from several diseases and insect pests (Selvakumar and Kalia 2022; Suffert and Montfort 2007). Among these challenges, carrot cavity spot (CCS) emerges as one of the most economically significant diseases that causes quality losses in the United States and globally (Gossen et al. 2014; Selvakumar and Kalia 2022; Suffert and Montfort 2007). Visible carrot cavity spots render the carrots unmarketable to fresh carrot consumers and processing industries (Higgins and Hausbeck 2023). Notably, CCS prevalence has been documented to be 50% in California and Washington and 25% in Colorado (Davis 2004). CCS manifests as a sunken lesion, varying in shape from round to elliptical to irregular, spanning the taproot and significantly diminishing carrot marketability. The symptoms remain inconspicuous in the aboveground plant parts, making marketable loss particularly high, as they only become noticeable when the root approaches marketable sizes and is uprooted (Chaudhry et al. 2022). In addition, CCS ranks as the predominant postharvest disease of carrots (Heltoft and Thomsen 2023). This complex polycyclic disease is attributed to several soilborne *Pythium* species, with *P. sulcatum, P. irregulare*, *P. ultimum*, and *P. violae* being the most frequently reported causal agents (Chaudhry et al. 2022; Gossen et al. 2014; Lu et al. 2012; Suffert and Montfort 2007). Environmental factors, including temperature and rainfall, exert a significant influence on disease intensity (Saude et al. 2014).

CCS is mainly managed using fungicides. However, fungicide resistance is a major challenge in carrot production, not only in the United States but also globally (Lu et al. 2012). For example, *Pythium* species resistant to mefenoxam, the most commonly used fungicide to control CCS, have been documented (Lookabaugh et al. 2018; Lu et al. 2012). As a phenylamide fungicide (FRAC group

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4), mefenoxam inhibits rRNA biosynthesis in oomycetes, including *Pythium*. Fungicides in this group are known to have high risk of resistance development (Hermann et al. 2019; Larson et al. 2021). In addition, fungicides may readily degrade in the soil and therefore fail to control the disease even when applied at the recommended doses. Identification of fungicide-sensitive genes remains elusive (Gossen et al. 2014). Moreover, chemical pesticides not only pose dangers to human health but also carry significant environmental risks (Singh and Mazumdar 2022). In light of these challenges, biopesticides emerge as highly promising alternatives because they are considered sustainable, economical, and eco-friendly (Meher et al. 2020). In addition, they play a great role in controlling CCS (Gossen et al. 2014; Singh and Mazumdar 2022). Biofungicides are the cornerstone of organic farming, and their market is anticipated to substantially increase from $6.51 billion in 2022 to $18.15 billion by 2029 (USDA-NASS 2023). In the global biopesticide market, North America, notably the United States, leads with a substantial 46% market share, driven by the growing demand for organic food (Singh and Mazumdar 2022). *Trichoderma* is the major constituent of a globally dominant commercial biopesticides, contributing to 60% of the global biopesticide market (Meher et al. 2020). With its multipurpose plant beneficial properties, diverse mechanisms of action, and rapid colonization, *Trichoderma* stands out as an efficient biocontrol agent for soil application in environmentally friendly sustainable agriculture (Jangir et al. 2019; Woo et al. 2023).

SoilGard (Certis USA, Columbia, MD) is a *Trichoderma*-based microbial biofungicide containing the active microbe *Trichoderma virens* GL-21, formerly known as *Gliocladium virens* strain GL-21. It is EPA approved (EPA #70051-3) for *Pythium* control and is widely used in organic farming globally (Certis Biologicals 2023). SoilGard has been shown to be effective in suppressing cucumber damping-off caused by *P. ultimum* (Roberts et al. 2016). In potato cultivation, application of *T. virens*, as a spore suspension or as a commercial formulation of SoilGard, substantially reduced the incidence and severity of potato stem canker and black scurf (Brewer and Larkin 2005). However, Brewer and Larkin (2005) noted that its effectiveness diminished under field conditions, with the same soil type used in pot experiments, highlighting the influence of environmental factors. Given the reports of resistance of some *Pythium* isolates to the commercially recommended mefenoxam fungicide (Aegerter et al. 2002; Lookabaugh et al. 2015; Lu et al. 2012) and the wide application rate range of SoilGard, the aim of this study was to examine the effects of SoilGard on various *Pythium* species and across a range of application rates. Therefore, we evaluated the efficacy of SoilGard against three *Pythium* species, each represented by two different isolates.

Various *Trichoderma* species, employed as biopesticides, have demonstrated notable effects on soil fungal community structure (Hang et al. 2022). However, the effect on fungal diversity remains inconsistent. This may be attributed to differences in the species of biocontrol agents, application rates, methods of application, and physicochemical and biological properties of the soils in which they are applied. For instance, in a study by Wu et al. (2022), applying *Trichoderma harzianum* to the soil showed no negative impact on soil fungal diversity. Conversely, Sui et al. (2022) demonstrated that seed dressing with *Trichoderma atroviride* had a significant negative effect on the richness of soil fungi. Under field conditions, SoilGard (Certis USA) was found to reduce the overall fungal microbial population, but detailed compositional effects on fungal communities were not provided (Larkin 2016). In addition, previous studies have reported that SoilGard can significantly reduce microbial activity, as evaluated by the utilization of a carbon source (Brewer and Larkin 2005; Larkin 2016). Despite these findings, there is currently no documented report on the impact of SoilGard on soil fungal communities under carrot cultivation and in bare (uncultivated) soil. Given that *Trichoderma* has antifungal potential against various fungal pathogens (Woo et al. 2023) and the fact that crop cultivation is known to have a significant influence on microbial communities in the soil (de Azevedo Silva et al. 2021; Gil-Martínez et al. 2021; Shen et al. 2022), we hypothesize that fungal communities in carrot-cultivated and bare soil will exhibit varied sensitivity to SoilGard application. This study aimed to investigate the impact of SoilGard on fungal communities in both carrot-grown and bare soil across two sampling time points because understanding the impact of SoilGard on different fungal community structures under different cropping systems would be valuable for effective plant disease management.

## MATERIALS AND METHODS

The efficacy of SoilGard in controlling various *Pythium* species at different concentrations was investigated under in vitro conditions, and its effects on the taxonomic and functional diversities of soil fungal communities were studied in vivo in the greenhouse.

**In vitro assay.** The efficacy of the biofungicide SoilGard 12G (Certis USA) against three species of *Pythium* that cause CCS was compared with that of Ridomil (45% a.i. mefenoxam) (hereafter referred to as mefenoxam) in an in vitro experiment. Mefenoxam is the industry chemical standard for the management of CCS, so it was used as a positive control. SoilGard 12G (referred to as SoilGard hereafter) is a product containing the *Trichoderma virens* strain GL-21 (Certis USA) and is one of the biofungicides recommended against *Pythium* infections in carrots. The product was purchased in 2019 with the item code 180403 and the package code 586204. Twenty percent clarified V8 agar (Tuite 1969) was amended with mefenoxam/SoilGard after sterilization at the recommended maximum concentration listed by the manufacturer and poured into Petri dishes. Mefenoxam was added at a final concentration of 0.499 µl liter$^{-1}$, and SoilGard was added at 0.9 g liter$^{-1}$, which are their recommended rates. SoilGard and mefenoxam were dissolved in sterile water, then thoroughly mixed into warm clarified V8 media before pouring into Petri dishes. Clarified V8 media without SoilGard or mefenoxam served as the negative control.

Additionally, the efficacy of SoilGard at different concentrations of the greenhouse recommended rates according to the package label (Certis USA)—297 g/m$^3$ (0.3 g liter$^{-1}$), 593 g/m$^3$ (0.6 g liter$^{-1}$), and 890 g/m$^3$ (0.9 g liter$^{-1}$), and 1,187 g/m$^3$ (1.2 g liter$^{-1}$)—was evaluated using six *Pythium* isolates belonging to three different species. These isolates were chosen to represent the three species that cause CCS and were consistently observed to be pathogenic under laboratory and greenhouse conditions. Isolates CA997 and CA997A were chosen to represent *P. ultimum*, isolates C1-09 and C1-19 for *P. irregulare*, and isolates C1-67 and 3-63 for *P. sulcatum*. All isolates exhibited optimal growth at 25°C, except for *P. sulcatum,* which showed the best growth at 19°C. Different concentrations of SoilGard for each treatment were prepared by mixing it thoroughly into warm clarified V8 media. To inoculate with *Pythium*, a 5-mm plug from an actively growing 2-day-old *Pythium* culture was placed at the center of each Petri dish for the respective treatments. The Petri dishes were incubated at 25°C in the dark for 24 h. After 24 h of incubation, radial mycelial growth was determined by measuring two perpendicular diameters of the culture and averaging the values for each treatment. The experiment was conducted in a completely randomized design with five replicates per treatment.

**Greenhouse experiment: Inoculum preparation and treatment application.** One liter of vermiculite was sterilized three

times, and then 0.5 liters of autoclaved 20% V8 broth was added to it (Vivoda et al. 1991). Ten 5-mm plugs from a 2-day-old *Pythium* culture were added into the vermiculite and incubated for 21 days with regular hand shaking. The inoculum preparation method was the same for all six isolates of *Pythium* spp., except for the incubation temperatures: *P. ultimum* and *P. irregulare* were incubated at 25°C and *P. sulcatum* at 19°C. The inoculum density was determined by diluting *Pythium*-colonized vermiculite in 0.2% water agar and plating on 10% V8 agar plates amended with PARP (pimaricin 10 μg/ml, ampicillin 250 μg/ml, rifampicin 10 μg/ml, pentachloronitrobenzene 25 μg/ml) (Vivoda et al. 1991). The final total inoculum density of 4,000 CFU/g was achieved by adding 667 CFU/g of each isolate to the vermiculite and mixing with steam-sterilized UC Mix III (57% plaster sand, 43% peat moss with the addition of $KNO_3$, 0.89 kg/m$^3$ limestone flour, 0.74 kg/m$^3$ phosphate, 2.22 kg/m$^3$ dolomite, 41.5 g/m$^3$ magnesium, 18 g/m$^3$ manganese, 77.1 g/m$^3$ iron, 30 g/m$^3$ zinc, and 65 g/m$^3$ copper). To inoculate the plants, a PVC pipe with a height of 15 cm and a diameter of 3.8 cm, which had been placed in the center of each 3-liter pot (Suffert and Montfort 2007), was carefully removed, and the prepared inoculum was added in its place. Each pot was inoculated with 200 g of the inoculum. Control pots received the same amount of sterile vermiculite mixed with 20% V8 broth but without *Pythium* inoculation.

*Pythium* inoculation and the application of SoilGard/mefenoxam were performed 28 days after planting the Crispy Cut carrot variety in UC Soil Mix III (Baker 1957). Five carrots seeds were planted per pot and thinned to three carrots 3 weeks after planting. SoilGard and mefenoxam were dissolved in water and added to the soil to achieve the recommended application rates of 0.9 g liter$^{-1}$ and 0.499 μl liter$^{-1}$, respectively. Both SoilGard and mefenoxam were added to the soil on the same day as *Pythium* inoculation. The experiments were arranged in a completely randomized design, with each treatment replicated four times. Each pot served as a biological replicate. Carrots were hand-irrigated with a 1% solution of Peters Mix fertilizer (Peter's 21-5-20 Excel Multi-Purpose, Scotts, Marysville, OH) twice a week for the first month and were subsequently placed on an automatic drip irrigation system. Carrots were harvested 16 weeks after sowing.

**Assessment of carrot cavity spot.** At harvest (16 weeks after sowing), the carrots were washed, dried, and observed for lesions. The size of each lesion was measured, and treatment efficacy was assessed based on lesion density, disease incidence, and disease severity (Saude et al. 2014; Suffert and Montfort 2007). The three carrots in each pot were the sampling unit. Lesion density was determined as the mean number of lesions per root. Disease incidence was calculated as the percentage of diseased carrots in each pot. Disease severity classes were determined based on the length of horizontal lesions and calculated using a 0 to 4 scale, where 0 = no lesion; 1 = 1- to 2-mm lesion; 2 = 2.1- to 5-mm lesion; 3 = 5.1- to 10-mm lesion; and 4 = lesion greater than 10 mm. The disease severity data were converted into a disease severity index (DSI) (Saude et al. 2014).

$$\mathrm{DSI} = \frac{\sum \begin{array}{c}(\text{severity class number} \times \\ \text{number of roots in each severity class})\end{array}}{\begin{array}{c}\text{total number of roots} \times \\ (\text{maximum severity classes} - 1)\end{array}} \times 100$$

All treatments, including those uninoculated with *Pythium,* displayed small pockmarks (<1 mm) on the taproot, similar to those observed in *Pythium*-inoculated samples. However, because *Pythium* was not isolated from these small lesions, those measuring less than 1 mm were excluded from the final analysis.

**Soil sample collection, DNA extraction, and Illumina MiSeq library preparation.** Soil samples were collected at 2 and 12 weeks after SoilGard treatment and *Pythium* inoculation from both SoilGard-treated and control pots, in both carrot-grown and bare soil. The rationale behind selecting these time points (2 and 12 weeks after applying *Pythium* and SoilGard) was that (i) our preliminary studies showed that *Pythium* takes 2 weeks to establish in the soil after inoculation and (ii) CCS is a late-season disease (Vivoda et al. 1991); therefore, it was important to sample toward the end of the season to capture the microbial population associated with the disease. The samples were collected from a 5-cm depth and 3 cm away from carrot plants using a 1-cm-diameter push core. Soil samples were collected from three different spots in each pot and then combined to create a composite sample for that specific pot. DNA extraction from 0.25-g soil samples was performed using the DNeasy PowerSoil kit (Qiagen, Valencia, CA) per the manufacturer's instructions. The extracted DNA was quality checked using an Implen NanoPhotometer (Implen, Westlake Village, CA). The Illumina MiSeq library preparation was conducted following the method outlined in our previous work (McLain et al. 2023). The fungal variable region, the internal transcribed spacer 2 (ITS2) region, was PCR amplified using universal fungal primers 5.8SFun and ITS4Fun (Taylor et al. 2016). A barcode and Illumina sequencing adapters were attached in the second PCR. Purification of the first and second PCR products was carried out using the AMPure XP beads protocol (Beckman Coulter, Brea, CA). DNA concentrations of the second PCR product were determined using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and samples were pooled in equal molar concentrations of 5 nM. The quality and quantity of the final library were assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA), and the libraries were sequenced at the UC Riverside Genomics Core Facility using the Illumina MiSeq protocol (Illumina, San Diego, CA).

**Community-level physiological profiling.** We assessed the metabolic activities of soil fungal communities using a BIOLOG FF MicroPlate, comprising 95 different carbon sources (Biolog, Hayward, CA). In brief, 1 g of soil from freshly collected samples, taken 2 weeks after treatment from the same experiment used for DNA extraction, was suspended in 99 ml of sterile NaCl solution (9 g liter$^{-1}$). The mixture was vortexed at room temperature for 20 min. The bacterial growth was suppressed by adding kanamycin (100 μg ml)$^{-1}$ (Mocali et al. 2022). Aliquots (100 μl) of the suspension were added to each well of the microplate. The plates were then incubated for 6 days at 27°C (Gryta et al. 2020; Mącik et al. 2020), and absorbance was measured at 490 nm using a SpectraMax iD5 Multi-Mode microplate reader over 6 consecutive days. The soil samples used in the BIOLOG assay were collected from three randomly selected pots, with each pot considered a biological replicate. Soil samples were collected from three different spots in each pot and then combined to create a composite sample for that specific pot. Three biological replicates were used per treatment. We determined the average well color development (AWCD) and richness using the method described by Garland (1996). The AWCD and richness were also calculated for specific carbon categories: carbohydrates, carboxylic acids, polymers, amino acids, amines and amides, and miscellaneous (Preston-Mafham et al. 2002). Each biological reading was an average of three technical replicates. To visualize the change in metabolic activities of soil fungal communities across treatments during the incubation period, we employed principal component analysis.

**Bioinformatics.** Fungal raw sequences from ITS2 amplicon MiSeq sequencing were analyzed using the QIIME2 analysis workflow (version 2022.8) (Estaki et al. 2020). Briefly, raw

paired-end reads (300 bp for ITS2) were demultiplexed and barcodes trimmed using cutadapt (version 4.1) with parameters -e 1 --discard-untrimmed (Martin 2011). Demultiplexed reads were imported into QIIME2 for subsequent analysis. Imported sequences were subjected to denoising and clustering analysis using DADA2 (Callahan et al. 2016). For the ITS2 dataset, sequence data were first downloaded from the UNITE database (Nilsson et al. 2019) for QIIME, and sequences at the 99% similarity level were trained using the QIIME2 'fit-classifier-naive-bayes' module. Classification was assigned from the newly trained classifiers. Mitochondria and chloroplast reads were also filtered and removed after the initial taxonomic classification. The number of sequence reads for all samples was rarefied to an equal sampling depth of 20,114 reads per sample (Supplementary Fig. S1). The final normalized fungal microbiome dataset contained 663 amplicon sequence variants (ASVs), and taxonomy data were exported using QIIME2 and imported into R (version 4.1.3) for data processing, analysis, and visualization. We utilized a QIIME2 plugin pipeline, specifically the qiime phylogeny align-to-tree-mafft-fasttree, to align multiple sequences for the inference of a rooted phylogenetic tree. The weighted UniFrac analysis was performed using the qiime diversity-lib weighted-unifrac pipeline, with rooted tree as input. We employed fungal functional guild (FUNGuild) to predict the shift in the ecological function of fungal communities in response to the SoilGard application (Nguyen et al. 2016). Assignments of ASVs into trophic modes and guilds were made by taking into account all confidence rankings. ASVs were classified as unassigned if there was no FUNGuild database match.

**Statistical data analysis.** All downstream statistical data analysis and visualization of microbiome data were conducted using the R program software (R version 4.2.3) with various R packages, such as circlize (Gu et al. 2014), laercio, dplyr (Wickham et al. 2015), phyloseq (McMurdie and Holmes 2013), vegan (Oksanen et al. 2019), ggplot2 (Wickham 2016), and complexHeatmap (Gu et al. 2016). In vitro and in vivo data were analyzed using dplyr (Wickham et al. 2015) and agricolae (Mendiburu 2019) and visualized with ggplot2 (Wickham 2016). Homogeneity of variance and normality assumptions were tested using Leven's test, the Shapiro-Wilk test, and homogeneity of multivariate dispersion (PERMDISP) in R (Anderson et al. 2011). To increase the data normalization, fungal composition data were transformed using the centered log-ratio (Lin and Peddada 2020). The statistically significant differences between treatments based on fungal composition, alpha diversity indices, and metabolic data were compared using the agricolae R package (Mendiburu 2019). The differences in fungal community composition among treatments were determined using permutational multivariate analysis of variance (PERMANOVA) based on the weighted UniFrac distances (Anderson et al. 2011), and these distances were visualized through principal component analysis using the vegan R package (Oksanen et al. 2019). Fungal co-occurrence network analysis was conducted at the ASV level using the WGCNA package in R (version 4.2.3). Carrot-cultivated samples comprised 404 ASVs. We filtered ASVs present at less than 0.01% relative abundance. A Spearman's correlation coefficient of 0.7 or greater and $P$ value of 0.05 or less were used for analysis. The results were visualized using Gephi (Bastian et al. 2009). For this network analysis, data from *Pythium*-inoculated and noninoculated samples of carrot-cultivated soil were combined for each SoilGard-treated and nontreated control sample, resulting in two treatments. This approach was taken due to the absence of significant differences resulting from *Pythium* inoculation and because the number of samples per treatment was insufficient for separate network analysis (Kang et al. 2021).

## RESULTS

**Efficacy of SoilGard against mycelial growth and CCS in the greenhouse.** Our results from the in vitro assay showed that SoilGard had the potential to significantly ($P < 0.05$) reduce mycelial growth of some *Pythium* isolates compared with the control and mefenoxam (Fig. 1A and B). For example, mefenoxam used at the recommended rate did not suppress the mycelial growth of *P. irregulare* isolate C1-09 used in this study (Fig. 1A and B). It is worth noting that this isolate was previously reported to be resistant to mefenoxam in our previous study (data not shown). Given that SoilGard has a wide range of application rates, additional in vitro studies were conducted to investigate its efficacy at various greenhouse rates against different *Pythium* isolates. The results revealed that concentrations exceeding 0.6 g liter$^{-1}$ significantly ($P < 0.05$) inhibited the mycelial growth of *P. sulcatum* isolate C1-67, *P. irregulare* (isolate C1-09 and C1-19), and *P. ultimum* isolate CA997A but had no significant ($P > 0.05$) effect on *P. sulcatum* isolate 3-63 and *P. ultimum* isolate CA997 (Fig. 1C; Supplementary Table S1). Moreover, the effects were isolate-specific, as revealed by the highly significant ($P < 0.001$) interaction effect of *Pythium* isolates and SoilGard doses applied (Supplementary Table S2). In the SoilGard nontreated group, both isolates of *P. ultimum* exhibited the highest mycelial growth, whereas both isolates of *P. sulcatum* showed the slowest growth compared with the isolates of *P. irregulare* (Supplementary Table S3). In the greenhouse experiment, SoilGard 12G at 1.2 g liter$^{-1}$ significantly ($P < 0.05$) reduced the disease incidence, disease severity, and lesion density of CCS compared with the commercial fungicide mefenoxam (Fig. 1D to F). This shows that SoilGard has the potential to control CCS.

**Influence of SoilGard application on the dynamics of fungal diversity.** A single application of SoilGard significantly reduced fungal diversity, albeit temporarily, as indicated by Shannon diversity indices, especially when combined with *Pythium* inoculation (Fig. 2A and C; Supplementary Table S4). However, by the 12$^{th}$ week after application, no significant differences were observed among treatments (Fig. 2B and D; Supplementary Table S4), indicating that the fungal diversity likely recovered to levels found in the control by 12 weeks after SoilGard application. This observation was further supported by the twofold increase in the number of shared ASVs between control and SoilGard treatments 12 weeks after treatments compared with 2 weeks (Fig. 2E and F).

**SoilGard induces temporal shifts in fungal community structure.** Our results revealed that a one-time application of SoilGard at the recommended rate significantly (PERMANOVA $R^2 = 0.38$; $P < 0.01$) altered the soil fungal community structure (Fig. 3A to F). Two weeks after treatments, the fungal communities in SoilGard-treated soil clustered together, forming a distinct group separate from untreated control samples (Fig. 3A). However, this shift was transient. Twelve weeks after SoilGard application, there was no statistically significant difference between SoilGard-treated and control soil. This suggests that the microbial community in SoilGard-treated soil resembled the control 12 weeks after treatment (Fig. 3C and D).

There were distinct changes in fungal taxonomic composition between SoilGard-treated and nontreated samples in both *Pythium*-inoculated and noninoculated treatments. Overall, five phyla (Ascomycota, Mortierellomycota, Basidiomycota, Chytridiomycota, and Mucoromycota) constituted approximately 90% of the fungal population across all treatments. The relative abundance of Ascomycota and Mortierellomycota exhibited significant alterations within the first 2 weeks of SoilGard application. At 2 weeks, the fungal community in SoilGard-treated soil, irrespective of *Pythium* inoculation, was predominantly composed of Mortierellomycota,

whereas the untreated control soil was predominantly Ascomycota (Fig. 3E; Supplementary Table S5). This increase in the relative abundance of Mortierellomycota in SoilGard-treated soil was transient, only persisting to a lesser extent in *Pythium* noninoculated samples 10 weeks later (Fig. 3F). At 12 weeks after SoilGard application, the Ascomycota population had significantly ($P < 0.05$) increased. However, Ascomycota was dominant in the untreated control at 2 and 12 weeks irrespective of *Pythium* inoculation (Fig. 3E and F; Supplementary Table S5).

Among the top 10 taxa, *Mortierella* and *Fusarium* exhibited significant ($P < 0.05$) alterations with SoilGard application at 2 weeks after treatment (Supplementary Table S6). Interestingly, samples treated with SoilGard did not show a statistically significant ($P < 0.05$) change in the relative abundance of *Trichoderma* populations 2 weeks after treatment, suggesting the need for further investigation. Furthermore, regardless of *Pythium* inoculation, SoilGard significantly ($P < 0.05$) reduced *Fusarium* populations, whereas *Mortierella* showed substantial enrichment during the initial 2 weeks after treatment. *Fusarium* exhibited high relative abundance in *Pythium* noninoculated control samples, but both *Pythium* and SoilGard applications significantly depleted its populations (Supplementary Table S6).

**Co-occurrence network analysis of fungal communities.** The co-occurrence network analysis showed that SoilGard had a notable impact on fungal network complexity. At 2 weeks after application, SoilGard-treated samples exhibited reduced nodes, edges, and

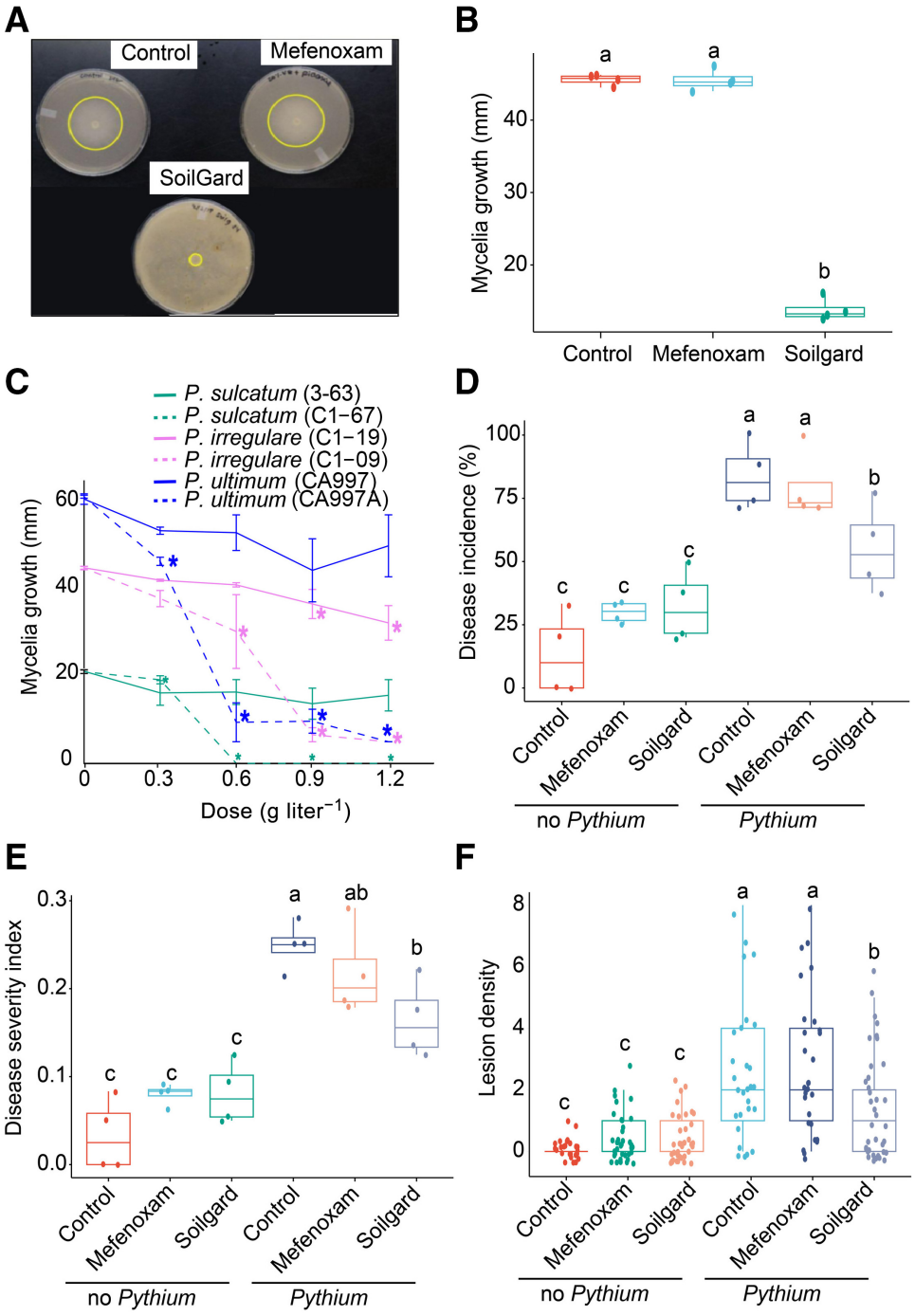


**Fig. 1.** SoilGard efficacy against *Pythium* species and carrot cavity spot. **A,** Pictorial view depicting the effectiveness of SoilGard against a mefenoxam-resistant *P. irregulare* isolate C1-09. The picture displays the bottom of the Petri dishes. **B,** In vitro assay comparing SoilGard (0.9 g liter$^{-1}$) with mefenoxam (0.499 µl liter$^{-1}$) against a mefenoxam-resistant *P. irregulare* isolate C1-09. **C,** Dose-response curve for SoilGard against different isolates of *Pythium* species. Asterisks (*) indicate statistically significant ($P < 0.05$) differences between the control and SoilGard concentrations for each isolate. Greenhouse experiment showing SoilGard efficacy in reducing **D,** disease incidence; **E,** disease severity; and **F,** lesion density compared with mefenoxam. Different letters on the bars indicate statistically significant ($P < 0.05$) differences between treatments.

degree in the fungal co-occurrence network (Fig. 4C) compared with SoilGard nontreated samples (Fig. 4A). These results suggest that SoilGard-treated soil had less compact and weakly connected fungal networks, indicating the potential for SoilGard to impact the complexity of the soil fungal community network negatively. However, fungal networks become tighter and more connected over time in both SoilGard-treated (Fig. 4D) and nontreated samples (Fig. 4B), as observed at 12 weeks after treatment. This increase in fungal network complexity at 12 weeks could be attributed to the effects of carrot cultivation and their interactions with SoilGard, but further research is needed to confirm this.

**Changes in functional diversity in response to SoilGard application.** The results from predicted ecological function at the trophic mode level indicated that both SoilGard-treated and nontreated soil fungal communities were predominantly dominated by the saprotrophs at 2 weeks (Supplementary Table S7) and 12 weeks (Supplementary Table S7). Notably, there was a significant ($P < 0.05$) reduction in the relative abundance of pathotrophs' functional

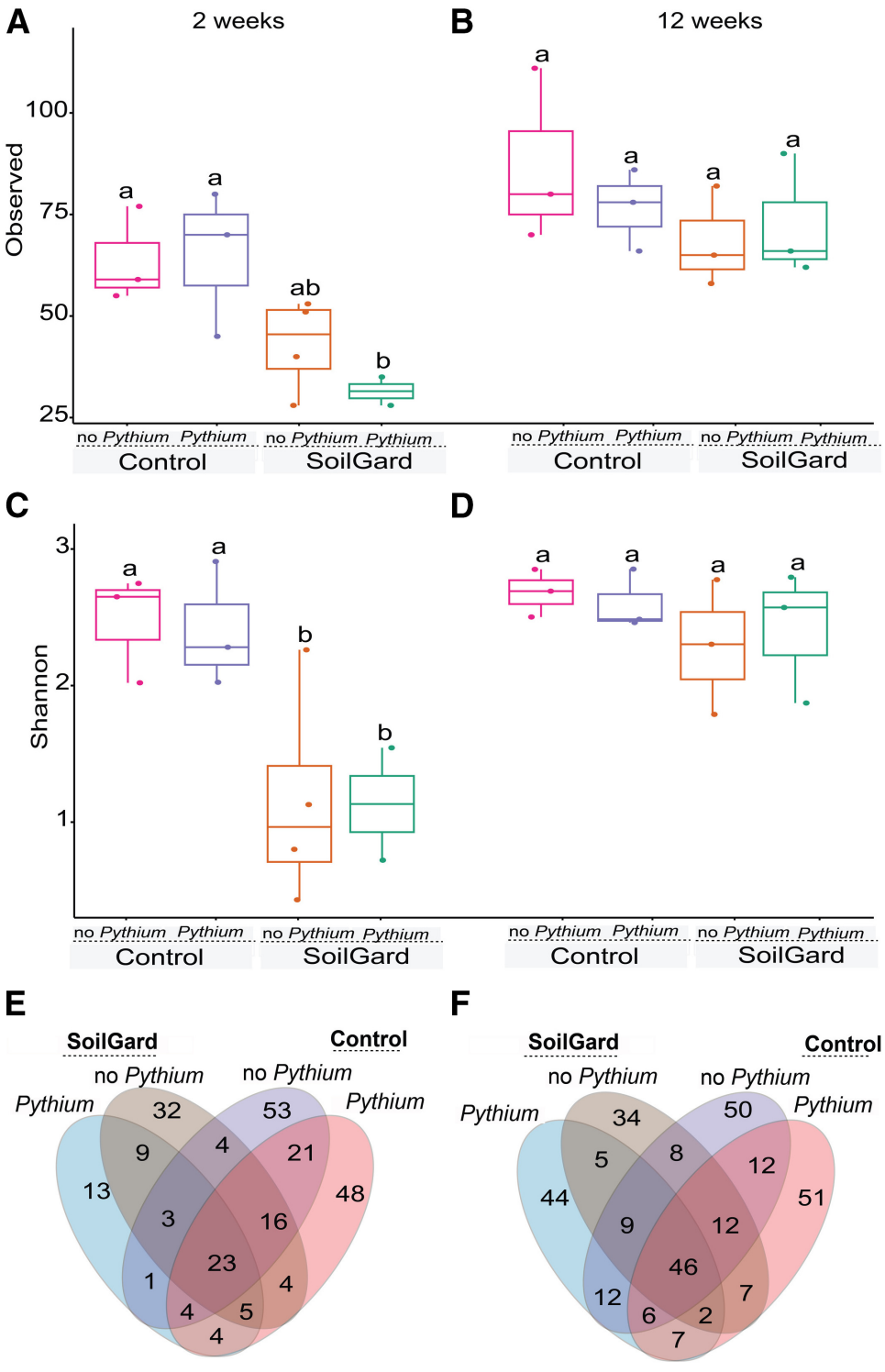


**Fig. 2.** Alpha diversity indices and Venn diagrams after treatment. Alpha diversity indices: Observed and Shannon at **A and C,** 2 weeks and **B and D,** 12 weeks after treatment. Different letters on the bars indicate statistically significant ($P < 0.05$) differences between treatments. The Venn diagram shows the unique and shared amplicon sequence variants of the treatments at **E,** 2 and **F,** 12 weeks.

guild 2 weeks after application of SoilGard (Fig. 5A; Supplementary Table S7). Application of SoilGard also reduced the relative abundance of plant pathogens and fungal parasites at 2 weeks (Fig. 5A; Supplementary Table S7). Interestingly, the symbiotrophs were significantly ($P < 0.05$) increased by the application of SoilGard, compared with the untreated control, to a greater extent at 2 weeks than at 12 weeks. This suggests that the application of SoilGard may improve some beneficial soil ecological functions.

**Community-level physiological profiling.** The change in the metabolic potential of fungal communities following SoilGard application was assessed using Biolog FF plates, which contain 95 different classes of carbon sources. The catabolic activity of carbon degradation increased with incubation time (Fig. 6A to F). There was differential utilization of the carbon sources by the fungal communities over time in the various treatments, indicating that the treatments impacted the fungal community functions uniquely. In addition, the heat map clustering (Fig. 7) revealed that treatments initially segregated according to incubation time, at 48 and 72 h. However, at later time points (96 to144 h), the clustering was more according to the soil treatment than incubation time. The highest AWCD values were observed in wells of carbohydrate carbon sources, followed by the carboxylic acid group (Fig. 6C and D). Fungal communities in soil treated with SoilGard and inoculated with *Pythium* (SGP) showed the lowest AWCD values in the utilization of most carbon sources compared with other treatments (Fig. 6B), whereas soil treated with SoilGard alone had the highest AWCD for carbohydrates, carboxylic acids, polymers, and amines-amides (Fig. 6C to F). Furthermore, SGP had the lowest richness, indicating that a low number of carbon-containing wells were utilized by SGP soil fungal communities compared with others (Fig. 6B). Among carbohydrate sources, α-D-glucose, turanose, and sucrose were more utilized by fungal communities in all

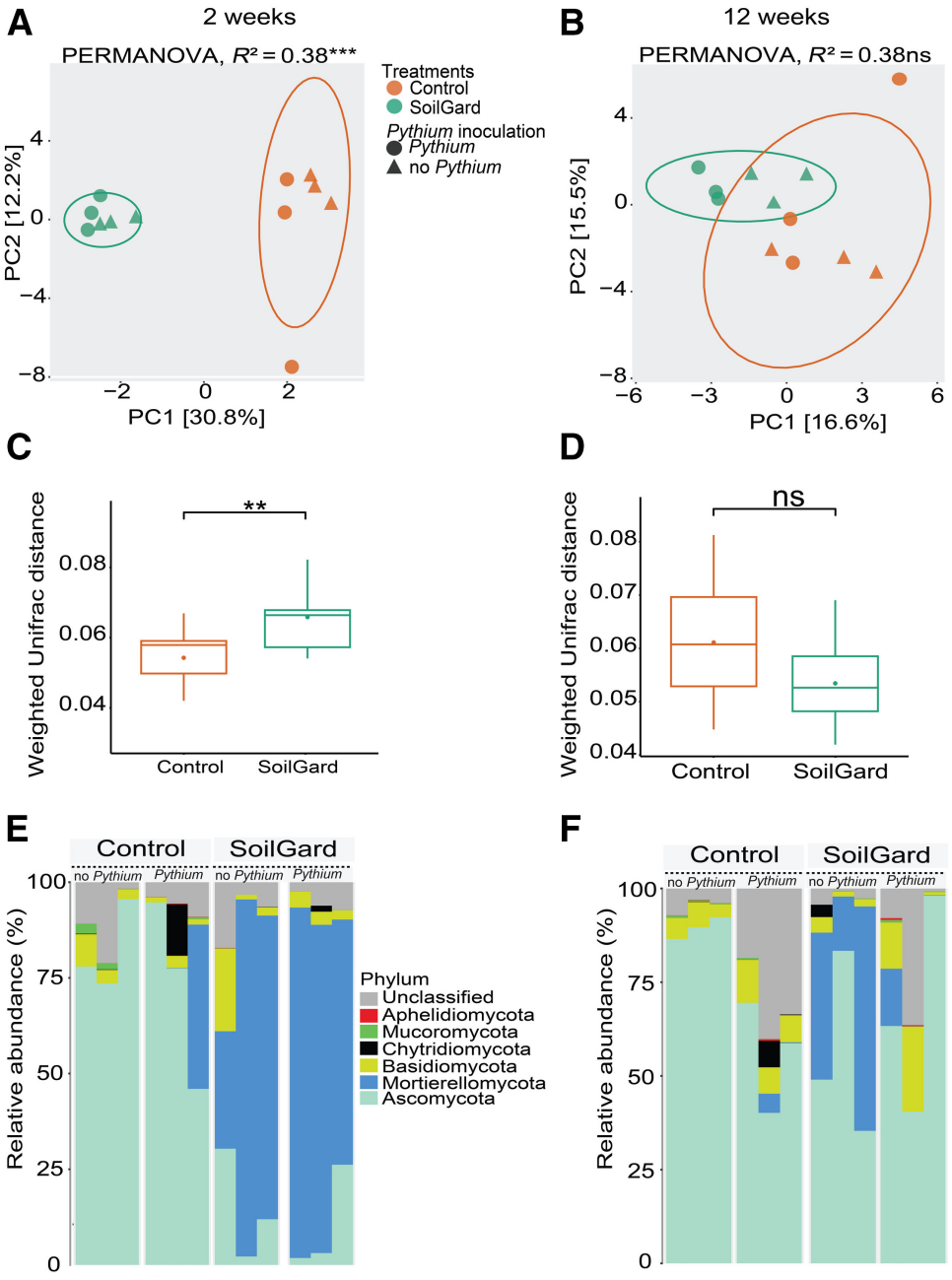


**Fig. 3.** SoilGard impacts the soil fungal community structure. Principal component (PC) analysis, based on UniFrac distances, showing fungal community structure shifts **A,** 2 and **B,** 12 weeks after SoilGard application in the presence and absence of *Pythium* inoculation. Weighted UniFrac distance at **C,** 2 and **D,** 12 weeks after treatment. Relative abundance of fungal phyla in SoilGard-treated and untreated control samples at **E,** 2 and **F,** 12 weeks after treatment. PERMANOVA, permutational multivariate analysis of variance; ns, not signficant. ** and *** denote significant differences at $P < 0.05$ and $P < 0.01$, respectively.

treatments, whereas α-methyl-D-glucoside and L-fucose exhibited the least utilization (Fig. 7). In carboxylic acid groups, sebacic acid was the least utilized, whereas succinic acid, L-malic acid, and N-acetyl-D-glucosamine, α-keto-D-gluconic-acid, fumaric acid, and D-gluconic acid were the most metabolized carbon sources (Fig. 7). The least utilized carbon sources among treatments were adenosine-5-monophosphate, followed by putrescine, which was categorized under miscellaneous and amines-amides, respectively. On the other hand, dextrin was the most degraded polymer carbon by fungal communities regardless of the treatments (Fig. 7).

**Comparison of SoilGard impact on carrot-cultivated and bare soil.** We also investigated the impact of SoilGard on soil fungal communities in bare soil that was not planted with carrots. Application of SoilGard resulted in the modification of the soil fungal community structure in bare soil (Fig. 8A and B). However, unlike in carrot-grown soil, there was no significant reduction in the fungal

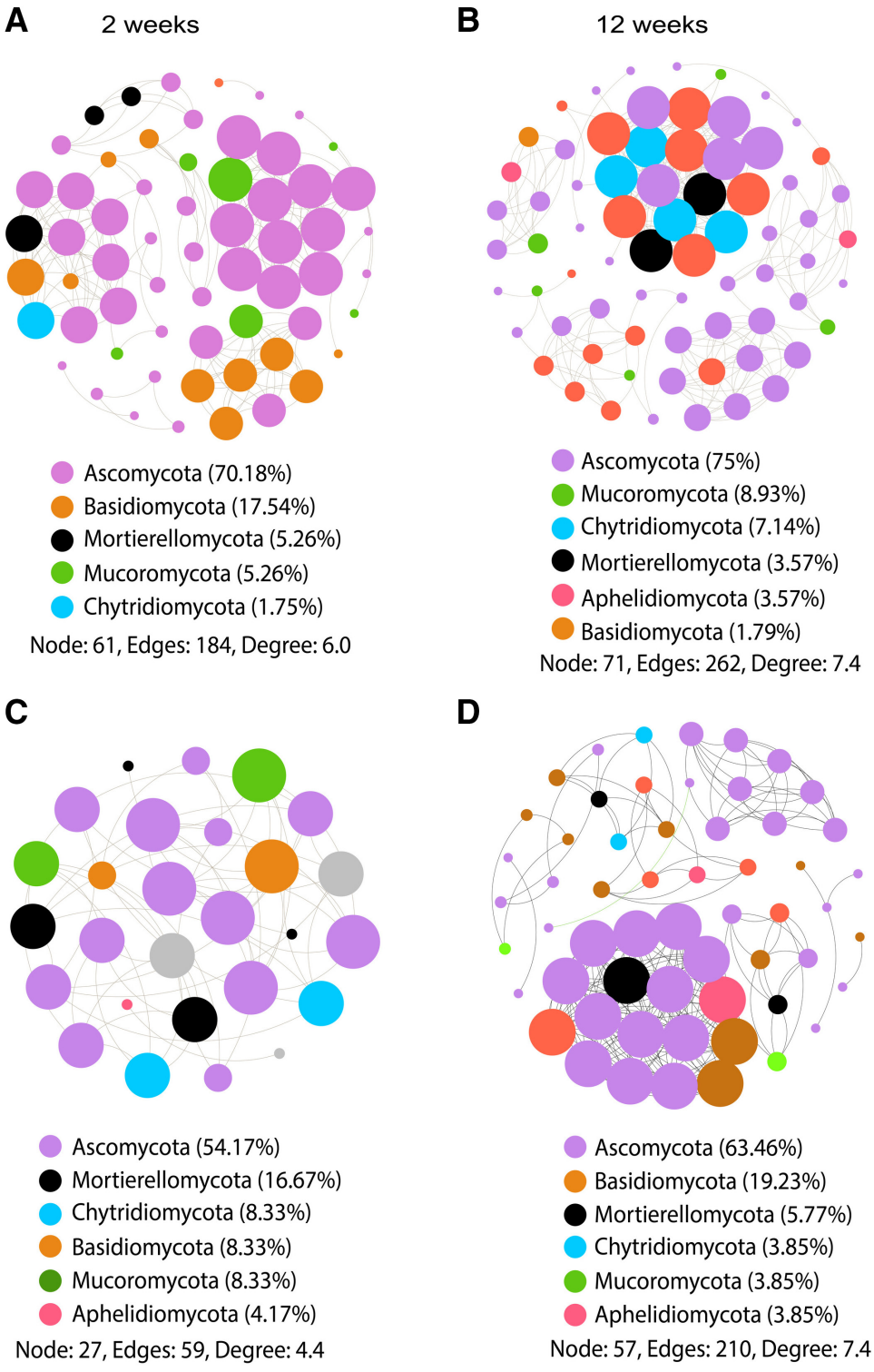


**Fig. 4.** Fungal co-occurrence network at the amplicon sequence variant (ASV) level in SoilGard-treated and nontreated samples. Co-occurrence network in **A and B,** control and **C and D,** SoilGard-treated soil samples at 2 (A and C) and 12 (B and D) weeks after treatment in carrot-cultivated soil. Node colors represent ASVs assigned to different phyla, and their size is proportional to their degrees (the number of connections).

alpha diversity (Fig. 8C). In bare soil, SoilGard application, especially without *Pythium* inoculation, increased the alpha diversity at 2 weeks and 12 weeks compared with control soil. This supported our hypothesis that fungal communities in carrot-cultivated and bare soil would exhibit varied sensitivity to SoilGard application. Our results also showed that carrot cultivation in control soil increased fungal diversity. Inoculation with *Pythium*, in combination with either SoilGard treatment or carrot cultivation, had no

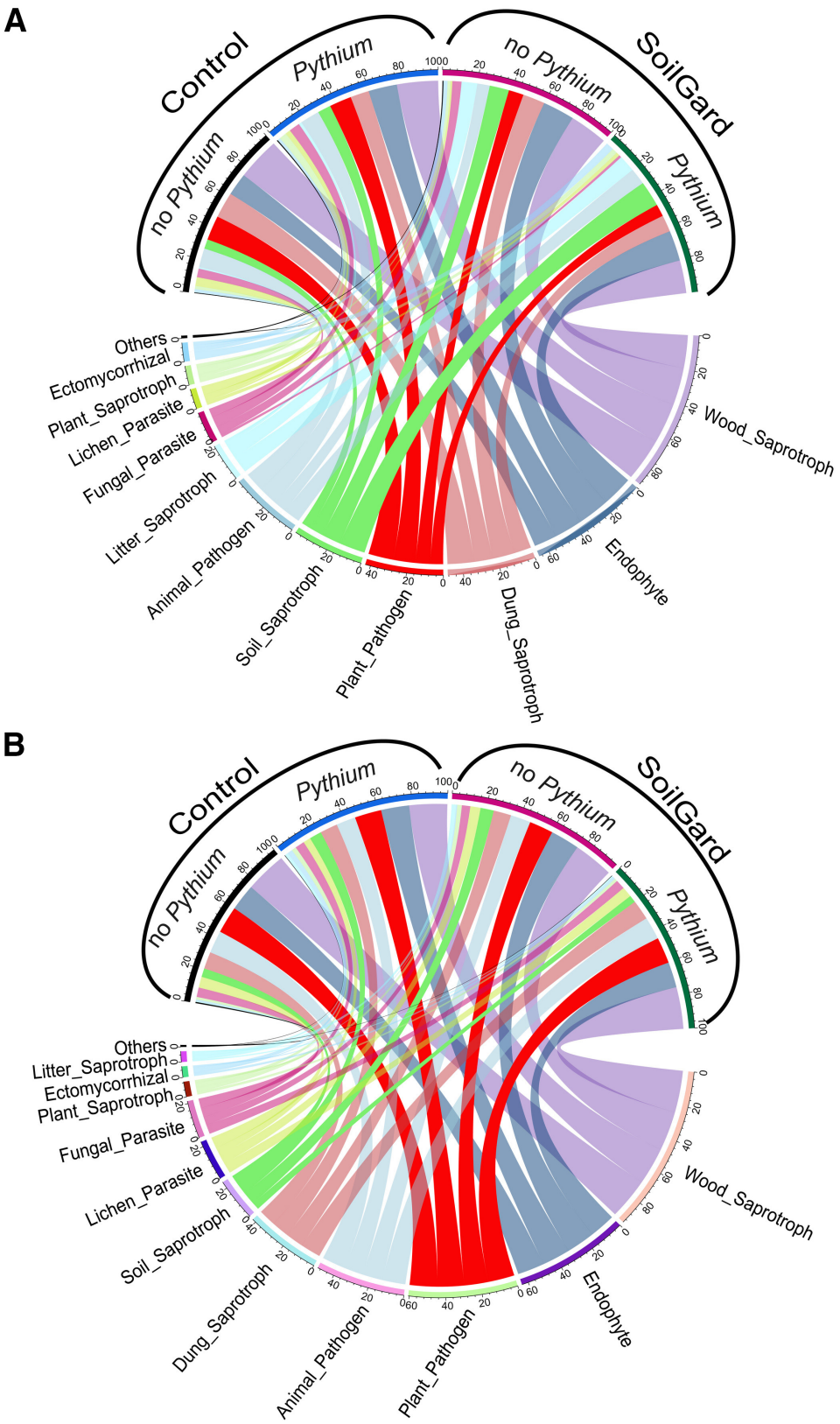


**Fig. 5.** Chord diagram showing the predicted fungal ecological functions in SoilGard-treated and untreated control samples at **A,** 2 weeks and **B,** 12 weeks after treatment.

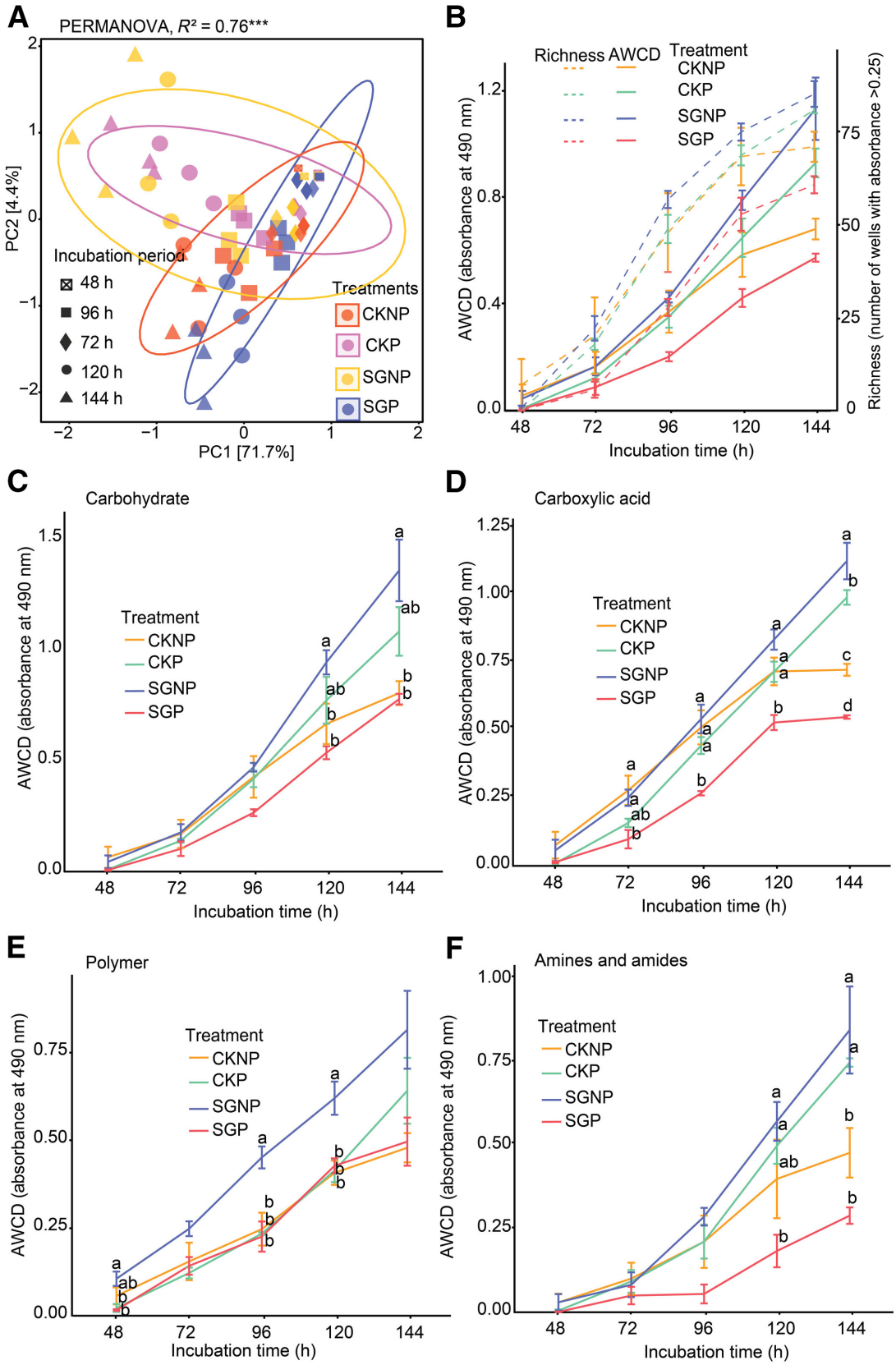


**Fig. 6.** Metabolic changes in fungal communities induced by SoilGard application. **A,** Principal component (PC) analysis illustrating shifts in the utilization of 95 distinct classes of carbon sources by soil fungal communities. Line graph depicting the utilization of carbon sources (based on average well color development [AWCD] and richness) in different categories by soil communities of each treatment between 48 and 144 h of incubation: **B,** overall 95 carbon sources; **C,** carbohydrate groups; **D,** carboxylic acid groups; **E,** polymer groups; and **F,** amines and amides groups. CKNP = control + no *Pythium*; CKP = control + *Pythium*; SGNP = SoilGard + no *Pythium*; and SGP = SoilGard + *Pythium*. PERMANOVA, permutational multivariate analysis of variance. *** denotes significant differences at $P < 0.01$. Different letter(s) on the bars indicate statistically significant differences between treatments at each incubation time ($P < 0.05$).

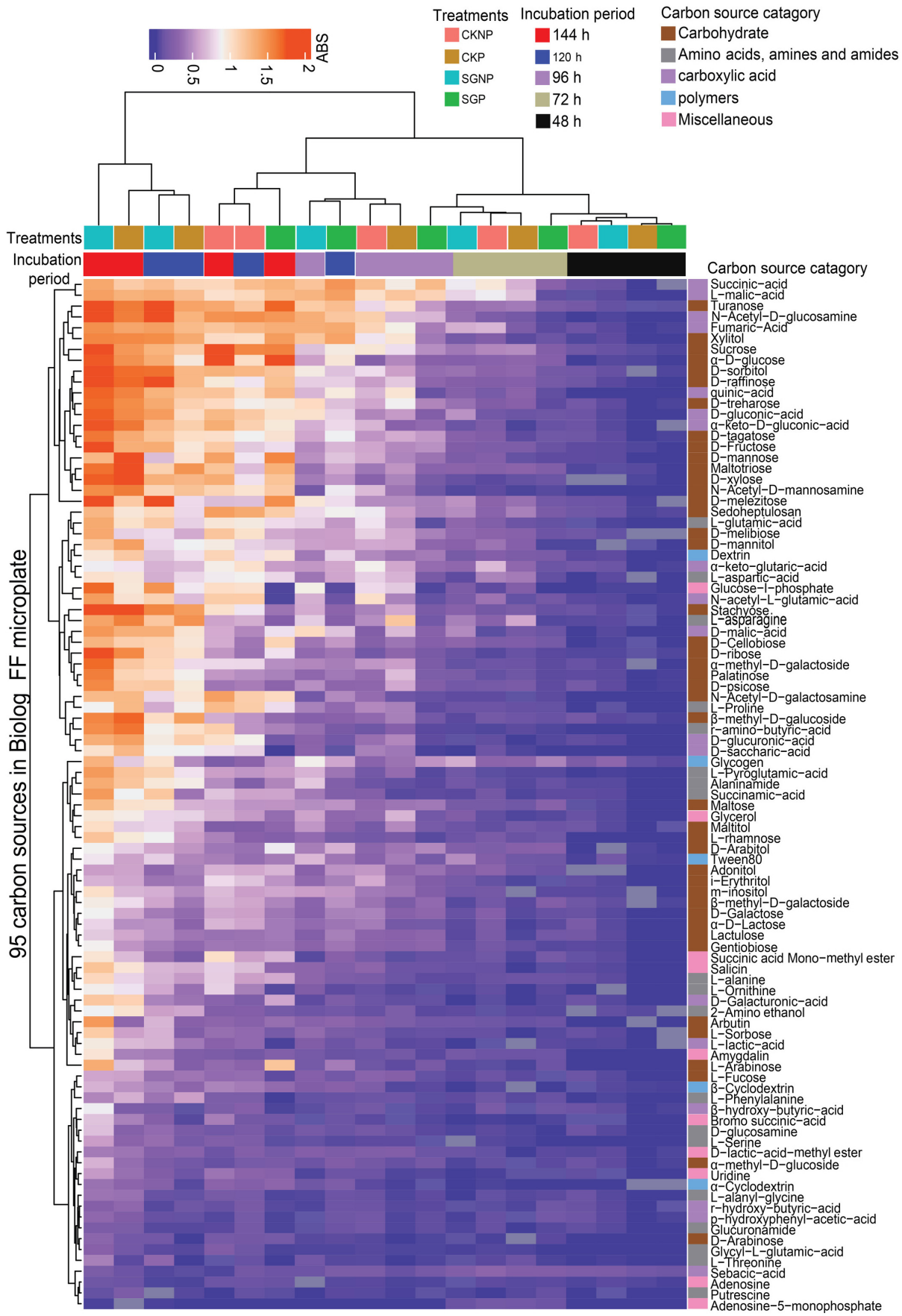


**Fig. 7.** Heatmap showing the metabolic activity shifts in carbon utilization with SoilGard application between 48 and 144 h of incubation. The data shown are absorbance values of 95 different carbon sources from the Biolog FF plates at 490 nm. The data represent the mean of three replicates. CKNP = control + no *Pythium*; CKP = control + *Pythium*; SGNP = SoilGard + no *Pythium*; and SGP = SoilGard + *Pythium.*

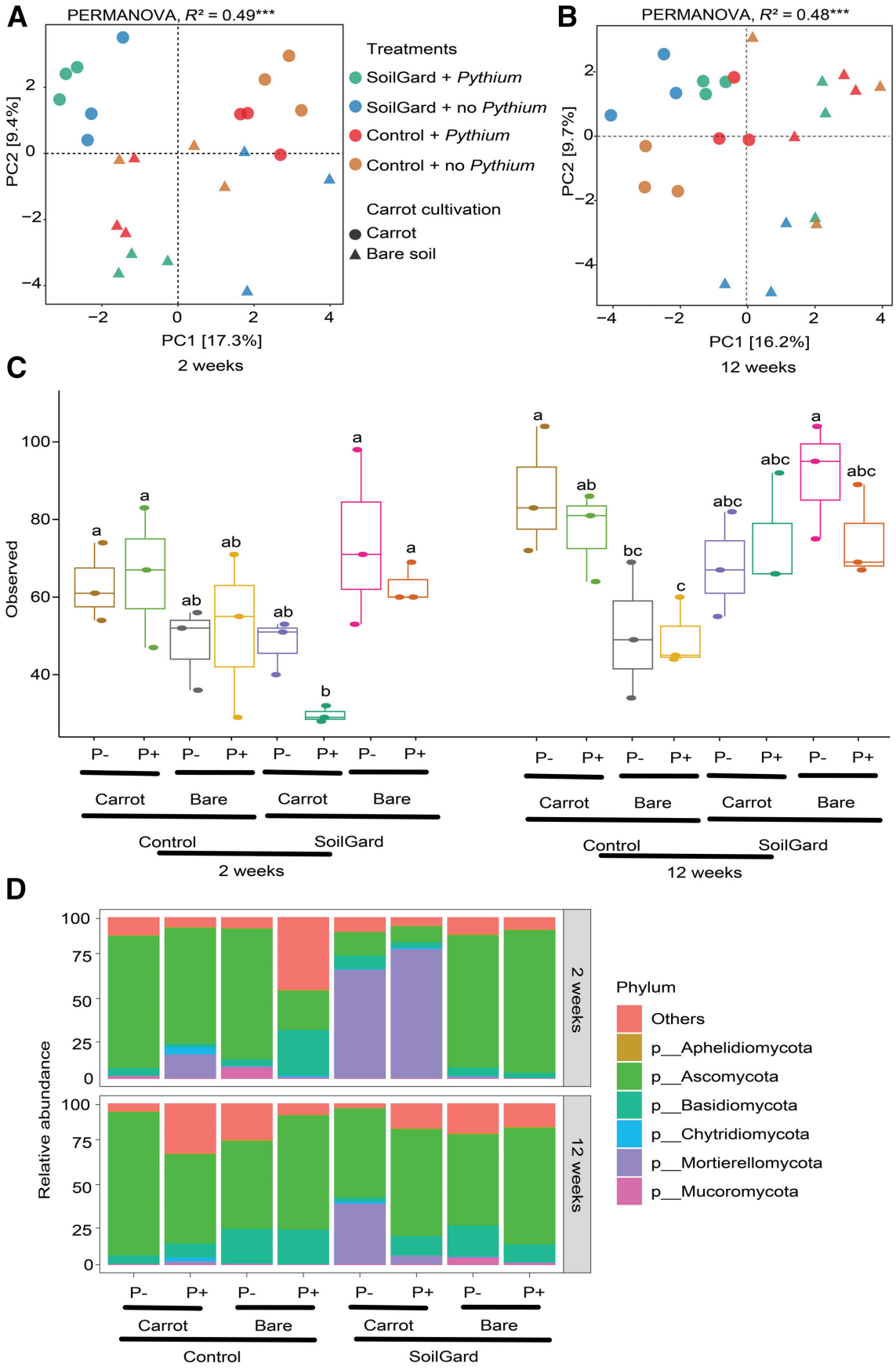


**Fig. 8.** Comparison of SoilGard impact on carrot-cultivated soil and bare soil. Beta diversity at **A,** 2 weeks and **B,** 12 weeks. **C,** Alpha diversity and **D,** community composition at the phylum level. PC, Principal component; PERMANOVA, permutational multivariate analysis of variance. *** denotes significant differences at $P < 0.01$. Different letter(s) on the bars indicate statistically significant ($P < 0.05$) differences between treatments. P– represents no *Pythium* inoculation, and P+ represents *Pythium* inoculation.

significant effect on the fungal diversity (Fig. 8C). However, inoculation with *Pythium* in bare soil in absence of SoilGard and/or carrots reduced the relative abundance of Ascomycota (Fig. 8D).

## DISCUSSION

SoilGard demonstrated variable effectiveness in inhibiting the mycelial growth of *Pythium* isolates in vitro, with its efficacy being dose-dependent and isolate-specific. This concurred with other studies that have noted variable effectiveness of *Trichoderma*-based biofungicides in controlling different isolates of fungal pathogens (Macena et al. 2020). These findings highlight the importance of carefully selecting the SoilGard application rate and considering the dominant pathogen isolate being targeted. The mode of action for SoilGard involves parasitism, antibiosis, and competition for space and nutrients (Widmer et al. 2018). Thus, our in vitro results may not perfectly reflect the scenario in soil, as the involvement of other multiple microorganisms can contribute to the outcome. Interestingly, SoilGard was effective in significantly reducing CSS disease in greenhouse conditions. Although previous studies have documented SoilGard's efficacy against *Pythium*-incited diseases in various crops (Punja and Yip 2003), this is the first study to report its effectiveness against CCS. These results underscore the potential of SoilGard as a solution to combat the development and spread of fungicide-resistant *Pythium* isolates, a common concern in agricultural production. However, it is important to note that SoilGard has been reported to lack efficiency in controlling *Pythium* diseases in some trials, with various isolates and application methods (Bailey et al. 2012; Linderman et al. 2008; Punja and Yip 2003). This suggests the necessity of performing multilocation tests in different growing soils and seasons.

Due to the aggressive and competitive nature of *Trichoderma* (Woo et al. 2023), SoilGard, which contains *Trichoderma*, was anticipated to influence the soil microbiota population. Our findings indicate that SoilGard has the potential to negatively impact soil fungal diversity. Similar reports have also suggested that the biocontrol agent *Trichoderma* can harm fungal diversity (Gao et al. 2023; Sui et al. 2022; Zhang et al. 2020). Fungal diversity is frequently associated with soil health, although results vary and depend on crop type, growth stage, and environmental factors (Banerjee and van der Heijden 2023). The association of fungal diversity with soil health is attributed to the pivotal role of soil fungi in the ecosystem, as they actively play various roles such as organic matter and nutrient recycling and establishment of symbiotic associations with plant roots (Baldrian et al. 2022). In addition, high microbial diversity is often considered more resilient and less susceptible to new pathogen intrusion resulting from a declined ability to outcompete the invading species (van Elsas et al. 2012). Furthermore, diverse microbes provide a wide range of functions (Banerjee and van der Heijden 2023). Although our study indicated that the effect of SoilGard on fungal diversity was transient, it remains uncertain whether this temporary effect is attributed to the resilience of the resident soil fungal community (Lourenço et al. 2018) or the restructuring of the soil fungal community following carrot growth (Wang et al. 2018). This indicates the need for additional research involving different crops, under field conditions, across multiple years to thoroughly assess the real impacts of *Trichoderma* or other biopesticides on soil microbial diversity.

Our results revealed that SoilGard had a significant, albeit temporary, effect on the Ascomycota population. Consistent with our findings, previous studies have documented the effect of *Trichoderma* in reducing the Ascomycota population (Sui et al. 2022). Interestingly, *Trichoderma* possesses a variety of hydrolytic enzymes that enable it to adelphoparasitize phylogenetically closely related Ascomycota species—a rare trait that *Trichoderma* acquired from plant-associated ascomycetes through lateral gene transfer (Woo et al. 2023). Other studies have also indicated that *Fusarium*, an ascomycete, is negatively impacted by SoilGard, as observed in our results (Huang et al. 2022; Sui et al. 2022; Zhang et al. 2013). *Fusarium* is a common soilborne plant pathogen that causes significant economic losses in various crops, including carrots (Leunov et al. 2021; Pascouau et al. 2023), suggesting that the application of SoilGard may help tackle *Fusarium*-incited crop diseases. Although SoilGard showed positive effects in reducing *Fusarium* disease in previous studies (Larkin and Fravel 1998), there are also contrasting reports documenting its lack of efficacy against *Fusarium* in various crops (Cummings et al. 2009; Linderman et al. 2008; Rose et al. 2004). Moreover, it is important to note that not all *Fusarium* species are pathogenic; some play a beneficial role as biocontrol agents (Bennett et al. 2023). In addition, the observed increase in the relative abundance of Mortierellomycota at 12 weeks in SoilGard-treated carrot-grown soil may be attributed to its resilience to anthropogenic disturbances (Baćmaga et al. 2022). Mortierellomycota show high relative abundance in crop-grown soil (Leng et al. 2023; Tao et al. 2023) because they are beneficial to plants and are thus frequently recruited by plant roots (Tao et al. 2023). However, further studies are needed to investigate the differential response of Mortierellomycota to SoilGard in carrot-grown soil compared with bare soil. We expected an increase in the *Trichoderma* population 2 weeks after the application of SoilGard, but our results did not show any statistically significant change in its relative abundance. Further investigation is needed to find out the reason behind these unexpected results.

Our network analysis based on random matrix theory revealed a significant impact of SoilGard on the complexity of soil fungal networks. Complex microbial interactions in soil are essential for maintaining ecological balance and serve as crucial soil health indicators (Wagg et al. 2019). Denser and more complex co-occurrence networks are critical for microbial stability (Calcagno et al. 2017) and provide benefits to plants (Fernández-González et al. 2020; Tao et al. 2018) by enhancing resistance to disturbances through greater soil ecological multifunctionality (Ding et al. 2023). Efficient resource utilization is observed in strongly connected microbial networks, contributing to a stronger community function (He et al. 2021). Results from a study by Fournier et al. (2020) concurred with our observations that biopesticides reduced the microbial co-occurrence network complexity and suggested that this may induce the loss of important soil ecological functions. This raises concern about the potential adverse effects of repeated commercial application of biopesticides (containing *Trichoderma*) on soil microbial ecology and, consequently, plant productivity. However, there are contrasting reports, with some noting that the fungal network was less complex in healthy plants compared with diseased plants (Gao et al. 2021). It is worth mentioning that some previous studies have found that the application of *Trichoderma* had no negative impact on bacterial network complexity (Gao et al. 2023), suggesting that the effect of biofungicides on microbial networks can vary. Overall, our network analysis lacked specific network statistics for each treatment because we had an insufficient number of samples to perform a separate network analysis for each treatment with replicates ($n = 3$). This limitation arises from the inherent nature of co-occurrence network analysis, which typically requires a large number of samples. However, there is no consensus on the minimum number of samples required for co-occurrence network construction, as it is influenced by various factors (Fabbrini et al. 2023; Ovens et al. 2020).

The predicted fungal ecological function based on FUNGuild showed that SoilGard application reduced pathotrophs, plant pathogens, and fungal parasites. A previous study showed that

*Trichoderma* application had a significant impact on fungal ecological functions, for example, promoting saprotroph-symbiotroph (Gao et al. 2023), which is similar to our observations. *Trichoderma* biofungicides are known for their biocontrol potential, as they inhibit plant pathogens, but they alter ecological niches to their advantage (Sui et al. 2022; Woo et al. 2023). This is attributed to the fact that *Trichoderma* secretes several compounds, such as siderophores, volatile organic compounds, reactive oxygen species, and $H_2O_2$, which either directly affect the fungal community or indirectly stimulate plant performance, thus modifying the ecological function of associated fungal communities (Woo et al. 2023).

FF microplates are widely used to assess the changes in the functional diversity of soil fungal communities (Borowik et al. 2017). Our results indicate a noticeable clustering of samples based on incubation time during the early stages, but with longer incubation, the clustering was according to soil treatments. This implies that prolonged incubation periods are needed to distinguish the utilization of various carbon sources by fungal communities across different treatments. This variability arises from the diverse capabilities of fungal communities in utilizing carbon sources, with certain fungal species degrading carbon resources faster than others (Masigol et al. 2023; Miao et al. 2022). Furthermore, the reduced utilization of carbon sources observed in SoilGard in combination with *Pythium* inoculation may be linked to the significant decrease in fungal diversity observed after the treatment. This can be attributed to the fact that less taxonomically diverse communities tend to have lower functional diversity compared with highly diverse communities, as diverse taxa may possess varying capabilities to perform different tasks (Borowik et al. 2017). However, some studies claim that taxonomic diversity is not necessarily proportional to functional diversity, citing that a small microbial community may have more genes to perform multiple functions than a large microbial community with a smaller genome (Freitas et al. 2021; Wang et al. 2023). Our observations that soil fungal communities have a preference for utilizing carbohydrates than polymers are consistent with previous studies, which show that fungi are well-adapted to the efficient use of carbohydrates compared with polymer and amide carbon source groups (Larkin 2016). This adaptation is attributed to the fact that polymer degradation is more energy consuming than carbohydrate utilization (Hage and Rosso 2021). It is also important to note that fungal communities have significant variation in their capabilities to degrade polymers (Hage and Rosso 2021).

Although SoilGard reduced fungal diversity in crop-cultivated soil, it did not have such a negative impact on the fungal diversity of bare soil. Therefore, SoilGard would not reduce fungal diversity if applied as a preplanting treatment, which may be the reason SoilGard shows better results when applied 1 day before planting (Certis USA). These differences may be attributed to variations in the sensitivity of microbial communities to the treatment due to their distinct community structures. Verdenelli et al. noted that the sensitivity of microbial communities to fungicide application depends on the nature of the microbes. They found that non-eroded soil was more sensitive to fungicide treatment compared with eroded soil microbial communities (Verdenelli et al. 2023). In contrast, some biofungicides have been shown to have no impact on fungal diversity (Fournier et al. 2020). Interestingly, our data also provide support for the positive effect of carrot cultivation on enhancing fungal diversity, which is consistent with previous studies reporting a direct proportionality between soil fungal richness and plant cover, as a result of increased soil carbon availability (de Azevedo Silva et al. 2021; Gil-Martínez et al. 2021; Shen et al. 2022). Furthermore, similar findings suggested that the enrichment of the Ascomycota population with crop cultivation may be due to the secretion of root exudates (Borowik et al. 2023).

**Conclusions.** To the best of our knowledge, this is the first study reporting SoilGard's effectiveness against CCS. Our in vitro study revealed that SoilGard efficacy was significantly dose-dependent and varied with *Pythium* isolates. Therefore, SoilGard treatment may be more effective in reducing CCS if it is optimized to target the most prevalent *Pythium* isolate. Interestingly, a single application of SoilGard demonstrated a significant, albeit temporary, impact on fungal community structure. This impact included a reduction in the Ascomycota population, particularly *Fusarium* spp., which can be either soilborne pathogens or beneficial biocontrol agents. This reduction extended to the predicted ecological functions of pathotrophs, plant pathogens, and fungal parasites without causing deleterious effects on symbiotrophs. In addition, although SoilGard had no negative impact on the fungal diversity of bare soil, it temporarily but significantly lowered the fungal diversity and complexity of soil fungal networks in carrot-cultivated soil. These differences may be attributed to variations in the sensitivity of the fungal community to SoilGard. Thus, further research involving different crops under field conditions and across different seasons may be necessary to thoroughly assess the real impacts of *Trichoderma*-based biofungicides on soil fungal community dynamics.

**Data availability.** The raw sequence reads for this study are deposited in the NCBI Sequence Read Archive (SRA) repository under the BioProject numbers PRJNA1049016, SRA27089177 to SRA27089200, and SRA27931787 to SRA27931810.

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