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RESEARCH

Soil Type and Associated Microbiome Influence Chitin's Growth Promotion Effect in Lettuce

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ABSTRACT

Chitin amendment of peat substrate has been proven effective in promoting lettuce growth and increasing phenolic compounds in lettuce seedlings. However, the effect of chitin soil amendment on lettuce growth in mineral soil remains unexplored. The effect of chitin amendment of mineral soil on lettuce growth and metabolite changes was investigated for the first time in the present study in comparison with chitin-amended peat substrate. Our findings showed that chitin addition in peat substrate increased lettuce head weight by approximately 50% at harvest, whereas this increase was 30% when chitin was added to mineral soil. Targeted metabolomics analysis indicated that chitin addition affected the phenolic compounds in lettuce seedlings, but this effect varied between soil types. Moreover, untargeted metabolomics analysis suggested that using peat substrate or mineral soil had a greater influence on produced lettuce metabolites than chitin addition. Rhizobiome analysis showed that specifically *Mortierellaceae* family members, known for chitin degradation and plant growth promotion, significantly increased in peat substrate upon chitin treatment. In mineral soil, three bacterial genera and five fungi, including known plant-growth-promoting genera, were significantly more abundant upon chitin treatment but *Mortierellaceae* family members were not. We assume that the observed effects primarily stem from soil characteristics and from chitin-induced alterations in rhizobiome composition, particularly the presence of *Mortierellaceae* members, leading to promoted lettuce growth. Despite the variability, chitin remains an environmentally friendly alternative to synthetic fertilizers in lettuce production, but its beneficial effects are dependent on rhizobiome composition, which should be considered before chitin application.

Keywords: chitin, growth promotion, lettuce, metabarcoding, metabolomics

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As a source of vitamins, antioxidants, and carotenoids, lettuce (*Lactuca sativa* L.) is a popular leafy vegetable around the world (Kim et al. 2016). From an economic standpoint, lettuce holds importance as it is cultivated in numerous countries worldwide, covering a total land area of more than 1.8 M hectares in 2021 (FAOSTAT 2021).

The utilization of chemical fertilizers plays an important role in enhancing agricultural production (Yang et al. 2019). The increase

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in global crop yields has predominantly relied on substantial investments in chemical fertilizers (Geng et al. 2019). Farmers employ high rates of fertilization to effectively manage their farmlands and sustain soil productivity. Chemical fertilizers and pesticides are also commonly employed in lettuce cultivation to achieve higher crop yields (Subbarao et al. 2017). It is known, however, that chemical fertilizers and pesticides can have a negative effect on human health and the environment (Kim et al. 2017; Mahmood et al. 2016; Sharma and Singhvi 2017). Therefore, environment-friendly fertilizers and pesticides are recommended for a sustainable agriculture (Chen et al. 2018; Kumar 2012). Chitin has drawn much attention for its use as an environment-friendly fertilizer (Shamshina et al. 2020).

After cellulose, chitin stands as the second most abundant polysaccharide present on Earth. It can be found in a wide range of organisms, such as in the exoskeletons of arthropods, the cell walls of fungi, and the spines of diatoms (Sharp 2013). Chitin is a linear polymer made up of the amino sugar *N*-acetyl-D-glucosamine (GlcNAc). The β-glycosidic bonding between GlcNAc residues leads to repetition of disaccharides concerning the position of the *N*-acetyl group. Despite the charged acetyl group, chitin remains insoluble in aqueous and nonpolar solvents (Moussian 2019).

Research has demonstrated that chitin amendment of peat substrate (PS) could significantly promote the crop production of lettuce (23.8 to 89.5%) (Debode et al. 2016; Li et al. 2023). Moreover, addition of chitin into PS potentially enriches the population of plant-growth-promoting rhizobacteria and fungi (De Tender et al. 2019, 2024). Also, chitin/chitosan addition could affect lettuce seedlings' growth on transcriptional and metabolite levels, modifying the accumulation of several phenolic acids and plant hormones, which might promote lettuce growth and disease resistance (Li et al. 2023; Pusztahelyi 2018).

The growth and defense promotion effect of chitin has been studied only in commercial PS. In agriculture practice, most lettuce plants are grown in greenhouses in mineral soil (MS), which differs from PS in texture, microbial community, and nutritional composition. The effect of chitin in MS can thus tremendously differ compared with that in PS but remains untested. Therefore, in this study, we investigated for the first time the effect of chitin as a soil amendment in MS in comparison with chitin-amended PS, focusing on lettuce growth promotion by monitoring plant physiology, metabolomic analysis (targeted and untargeted), and soil microbiome composition using metabarcoding. Targeted and untargeted metabolomic analyses were conducted in lettuce seedlings. Lettuce growth and the bacterial (16S rRNA) and fungal (internal transcribed spacer [ITS]) compositions of the lettuce rhizosphere were monitored every 2 weeks during the whole growth period of 8 weeks.

MATERIALS AND METHODS

Soil preparation. Chitin flakes obtained from crab shell were purchased from BioLog Heppe GmbH (lot 40201609; Landsberg, Germany). PS (Beroepspotgrond, NPK 12-14-24; Saniflor, Geraardsbergen, Belgium) was purchased from local gardening stores (AVEVE Lammens, Wetteren, Belgium). MS was obtained from a lettuce greenhouse (top 30 cm of soil) from PCG Kruishoutem located in East-Flanders, Belgium. Chemical characterization of MS was carried out using the method thoroughly described previously (Vandecasteele et al. 2021). For MS, 1.02 g liter^{-1} of fertilizer (PGMix fertilizer NPK 14-16-18; Haifa North West Europe BV, Mechelen, Belgium) was applied before further use. PS and MS without chitin addition were used as control. Chitin-amended soil was either PS or MS mixed with 2 g liter^{-1} of chitin (PS + CH and MS + CH, respectively). Both soils were wetted with groundwater to reach 40% water-filled pore space and incubated in a closed bag in the greenhouse for 1 week before use.

Lettuce growth and sample collection. Lettuce seedlings were germinated from pelletized butterhead lettuce seeds (*L. sativa* L. var. capitata 'Alexandria') obtained from Rijk Zwaan Distribution B.V. (De Lier, the Netherlands). PS was used to fill 77-well germination trays, and then one pelleted seed was gently pressed down with tweezers in the center of each well and covered with another thin layer of PS.

For the greenhouse experiments, seedlings were transplanted at the three-to-four-true-leaf stage into 1.3 liter pots (top Ø = 15 cm, bottom Ø = 11 cm, height = 11.5 cm) filled with 1 liter of preincubated PS, PS + CH or MS, MS + CH and grown in the greenhouse at ILVO (Flanders Research Institute for Agriculture, Fisheries and Food, Merelbeke, Belgium). Temperature, humidity, photoperiod, and light intensity were not strictly controlled and varied with the local weather (Belgium, February to April 2022). All pots were placed in a semirandom order on greenhouse tables and surrounded by border plants to avoid potential border effect (Sato and Takahashi 1983) (Supplementary Fig. S1). For 8 weeks, six plants from each treatment were sampled every 2 weeks for fresh plant weight measurement and rhizosphere collection, resulting in a total of 96 plants (6 replicates per sampling × 4 sampling points × 4 treatments). Rhizosphere microbiome samples were collected using the protocol described previously (Debode et al. 2016). Briefly, lettuce roots were pulled out of the soil and gently shaken to remove excess soil. Afterward, roots were placed in a 50-ml tube with 25 ml of sterile PBS buffer and vortexed for 2 to 3 min. Wash-off liquid was filtered through a 100-μm nylon filter and centrifuged at 3,200 × *g* for 15 min. The obtained pellet was considered as the rhizosphere pellet. Additionally, as a control, PS and MS was sampled once after the 1-week incubation period described above in Soil preparation. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and stored at –80°C before bacterial and fungal microbiome determination.

To prepare samples for metabolomics analysis, 400 young seedlings (100 plants × 4 treatments) were transplanted into 0.3-liter pots (top Ø = 9 cm, bottom Ø = 6 cm, height = 7 cm) and allowed to grow for 1 week. Subsequently, four biological replicates consisting of a collection of 25 plants per treatment were collected for both leaves and roots (25 plants per replicate × 4 replicates per treatment × 4 treatments × 2 plant parts). Roots and leaves were washed with tap water to remove any dirt attached, quickly dried on paper tissue, and frozen immediately in liquid nitrogen. All samples were manually ground to fine powder in liquid nitrogen using a mortar and pestle, freeze-dried, and sealed in vacuum bags at –20°C prior to metabolite extraction.

Fresh weight analysis. The weights of the lettuces grown in PS or MS were compared every 2 weeks between treatments. The fresh weights were analyzed using R v4.2.1 (R Core Team 2022) in RStudio Desktop v2022.07.1+554 (RStudio Team 2020). To check for significant differences in fresh weight, the weights of lettuce grown in either soil (PS or MS) were compared between treatment (PS + CH versus PS versus MS + CH versus MS). The weights were compared using the two-tailed Student's *t* test from the ggsignif v0.6.4 package (Ahlmann-Eltze and Patil 2021).

Targeted phenolic compounds and untargeted metabolomics analysis. For the targeted metabolomics analysis the same targets were used that were previously reported to be altered upon chitin soil amendments (Li et al. 2023). For both targeted and untargeted metabolomics analysis, the metabolite extraction was performed using the method described previously with slight modification (Li et al. 2023). Briefly, 50 μl of internal standard (daidzin,

100 ng μl^{-1}) and 10 ml of pure methanol (ULC/MS grade absolute methanol) were added to 500 mg of freeze-dried tissue powder, vortexed for 1 min, then sonicated using an Elma Transsonic digital S unit (Elma Schmidbauer GmbH, Singen, Germany) at 40 kHz for 15 min. Next, 10 ml of 20% (vol/vol) methanol/H_2O was added, vortexed, and sonicated as was done the first time. For samples that weighed less than 500 mg, the volumes of internal standard and extraction solvents were adapted according to the sample weight. The mixture of tissue powder and solvent was centrifuged at 3,000 × *g* for 5 min and the supernatant was filtered through a 0.22-μm polyvinyl difluoride syringe filter and analyzed with both targeted and untargeted approaches, using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and liquid chromatography high-resolution mass spectrometry (LC-HRMS), respectively.

In both analysis approaches, 5 μl of the final extract was injected onto an Acquity UPLC BEH Shield RP18 column (2.1 × 150 mm; 1.7 μm) and analyzed using an Acquity Ultra Performance liquid chromatograph (Waters, Milford, MA, U.S.A.). Details on the gradient used are given in Kips et al. (2017). For the targeted analysis the UPLC was coupled to a Xevo TQ-XS (Waters) mass spectrometer operated in MRM-mode after negative electrospray ionization. Quantitative data processing using external calibration curves was performed with TargetLynx v4.2 SCN982. For the untargeted analysis, a Synapt G2-S (Waters) high-resolution mass spectrometer was used at resolution mode (20,000 FWHM) in centroid full-scan MSe mode (data-independent acquisition [DIA]) after both positive and negative electrospray ionization. A 200 pg μl^{-1} leucine enkephalin solution was continuously infused during analysis to perform lockmass correction (*m/z* 556.2771 in positive ion mode and *m/z* 554.2615 in negative ion mode) during analysis. For quality control purposes, mixtures of equal amounts of all obtained extracts of either leaves or roots (QC) were made and analyzed throughout the untargeted LC-HRMS runs. All samples were randomized prior to the analysis. Processing of the untargeted data was done using Progenesis Qi v2.4 (Waters). Different expressed features (tested with analysis of variance and correcting for multiple testing with false discovery rate) between the treated and nontreated samples were highlighted after peak picking, sample alignment, deconvolution, and principal component analysis (PCA). For the targeted analysis, metabolite levels were compared pairwise between treatment groups using Welch's *t* test and Bonferroni correction to correct for multiple hypothesis testing.

Rhizosphere microbiome analysis. Library preparation for the 16S rRNA gene metabarcoding was carried out according to the 16S metagenomics sequencing library preparation protocol of Illumina (San Diego, CA, U.S.A.) using dual indexing (Illumina) with minor modifications. The PCR (primers in Supplementary Table S1) was run for 30 cycles using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Hoffmann-La Roche, Basel, Switzerland). Before cleanup using CleanNGS paramagnetic beads (CleanNA, Waddinxveen, Netherlands), amplicons were all checked using gel electrophoresis. A sample of the cleaned amplicons was analyzed using a Fragment Analyzer (Advanced Analytical Technologies, Orangeburg, NY, U.S.A.) with the DNF-935 Reagent Kit from Agilent Technologies (Santa Clara, CA, U.S.A.). Following the Illumina protocol, the amplicons were then dual-indexed with the Nextera XT Index Kit v2 (Illumina). Indexed amplicons were cleaned and normalized using a SequalPrep Normalization Plate (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions. The cleaned and normalized amplicons were then pooled, spiked with 7.5% 12.5 pM PhiX control v3 (Illumina) and paired-end sequenced (2 × 300 bp) on the MiSeq platform (Illumina) using the MiSeq Reagent Kit v3 (600-cycle).

For the fungal library preparation, the ITS region was targeted. The primers (Supplementary Table S1) of the Fungal Metagenomic Sequencing Demonstrated Protocol (Illumina) were used to amplify a 500-bp region of the ITS1 (between positions 195 and 695). The PCR was run for 30 cycles using HiFi HotStart ReadyMix (Kapa Biosystems). Additionally, ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, U.S.A.) was used as a positive control. The rest of the workflow was the same as for the 16S metabarcoding mentioned above. Totals of 15,483,711 and 20,214,091 paired-end reads were obtained for the 16S rRNA and the ITS1 metabarcoding, respectively.

Optimal read trimming and filter parameters were evaluated with FIGARO v1.1.2 (Sasada et al. 2020) on the demultiplexed reads. Consequently, the reads were trimmed and filtered, the error rates were learned, and the reads were merged and chimeras were removed using DADA2 v1.24.0 (Callahan et al. 2016). Taxonomic assignments for the bacterial communities were done using the SILVA v138 SSU database (Quast et al. 2013; Yilmaz et al. 2014). For species assignment using exact matching, the SILVA species assignment database v132 (Quast et al. 2013; Yilmaz et al. 2014) was queried. From the sample data and the tables produced by DADA2, a phyloseq object was created using phyloseq v1.40.0 (McMurdie and Holmes 2013). The taxonomic assignment for the ITS reads was done similarly except that the UNITE database v5.3 (Abarenkov et al. 2021) was used. All computations were run on the high-performance cluster at the Zurich University of Applied Sciences (ZHAW).

The created phyloseq object was opened with R using RStudio Desktop. The phyloseq object was transformed to a S6 microeco class using the file2meco v0.4.0 R package (Yurgel et al. 2022). Samples with fewer than 5,000 amplicon reads (Dully et al. 2021) were filtered out using the microeco v0.12.0 package (Liu et al. 2021). Only taxa assigned to the kingdom "Fungi" for the ITS samples and "Bacteria" or "Archaea" for the 16S samples were kept for downstream analysis. Assignments containing mitochondria or chloroplasts contaminants were filtered out for both data sets. For the α-diversity calculations, the samples were rarefied according to the minimum amplicon reads present in the cleaned data set present (for 16S, $n = 5{,}920$; for ITS, $n = 5{,}433$).

Shannon indices (Shannon 1948) were calculated, compared with the Kruskal-Wallis Rank Sum Test (Kruskal and Wallis 1952), and visualized using microeco. Bray-Curtis dissimilarity (Bray and Curtis 1957) were calculated on the rarefied read count. Data clustering was done with nonmetric multidimensional scaling (NMDS) and visualized with microeco. For the fungal data set, one outlier sample (R9-ITS) had to be removed. With the outlier in the data set, the stress was zero, whereas after its removal, it increased to 0.09. Relative abundance plots were created with microeco.

Differential abundance tests were done on the genus level using random forest (Beck and Foster 2014; Yatsunenko et al. 2012) combined with a nonparametric test. MeanDecreaseGini was selected as an indicator value for the analysis. The analysis and the visualization were done using microeco.

RESULTS

Soil analysis and lettuce growth. The PS used in this study is a commercial peat substrate, containing white and black peat, fertilizer, and wetting agent. Compared with PS, MS contains much less organic matter and relatively lower electrical conductivity (Table 1). The adjusted bulk density for PS and MS were 99 and 1,200 g $liter^{-1}$, respectively.

Comparing the lettuce fresh weights between PS and PS + CH, there was no significant difference in weight in the first 6 weeks. At

8 weeks posttransplanting (wpt), PS + CH showed a higher average fresh weight of 144.6 ± 6.1 g compared with the 96.2 ± 11.9 g for PS ($n = 6$, $P = 3.1 \times 10^{-5}$) (Fig. 1A). MS + CH showed a significantly higher weight at four wpt compared with MS, 7.6 ± 1.4 g compared with 4.8 ± 0.4 g, respectively ($n = 6$, $P = 0.0038$). At 6 wpt, the weight of MS + CH was still higher compared with MS but not significantly. After eight wpt, the fresh weight was also significantly higher in MS + CH compared with MS, 55.3 ± 7.7 g compared with 42.3 ± 6.0 g ($n = 6$, $P = 0.0092$) (Fig. 1B). The lettuce weight was significantly higher in PS compared with MS at each sampling point. This was the case with or without chitin treatment (Supplementary Fig. S2).

Targeted metabolomics. Of the total 47 phenolic compounds (PCs) tested, 22 were present in either leaf or root (Supplementary Table S2). Comparisons of PC contents based on chitin treatment of plants growing in each soil revealed differences in several metabolites (Table 2). Comparing the PC contents in roots between PS and PS + CH, only caffeic acid was significantly less present in PS + CH roots compared with PS roots. In leaves, quercetin-3-*O*-glucuronide, chicoric acid, apigetrin, chlorogenic acid, and luteolin were all significantly less concentrated upon chitin treatment, while ferulic acid showed significantly higher content upon chitin treatment. In lettuce roots grown in MS + CH, three metabolites (caffeic acid, quercetin-3-*O*-glucuronide, and isoquercetin) were significantly more highly concentrated when compared with the ones grown in MS. The leaves of lettuce grown in MS showed a higher concentration of rutin and astragalin compared with the ones from MS + CH.

More PCs showed significant differences in content upon comparing soil types, regardless of chitin treatment (Table 3). Analyzing the PCs in roots grown in PS and MS revealed a significantly higher level in sinapinic acid, *p*-coumaric acid, salicylic acid, chlorogenic acid, and ferulic acid in MS compared with PS. Conversely, in the leaves, PS exhibited higher concentrations of luteolin, quercetin-3-*O*-glucuronide, and chicoric acid compared with MS. In contrast, MS leaves contained significantly more salicylic acid than PS leaves.

TABLE 1
Main (nutritional) content of potting and mineral soils

Analyte	Peat substrate[a]	Mineral soil[b]
Organic matter (%)	20	2.62 ± 0.021
Dry matter (DM, %)	25	88.1 ± 0.014
pH	4.5–7	5.94 ± 0.0094
Electrical conductivity (mS m^{-1})	45	11.6
Compound fertilizer NPK 12-14-24 with trace elements	1.2 kg m^{-3}	–
NO_3-N (mg kg^{-1} DM)	–	7.4 ± 0.18
NH_4-N (mg kg^{-1} DM)	–	1.11 ± 0.094
P-AmLact (mg 100 g DM)	–	54.1 ± 1.7
K-AmLact (mg 100 g DM)	–	9.5 ± 0.33
Bulk density (g cm^{-3})	0.099	1.2
Particle density (g cm^{-3})	1.55	2.65
Porosity (%)[c]	93.61	54.72

[a] The content of the peat substrate was described by the provider.
[b] For mineral soil $n = 3$ except for the electrical conductivity, where $n = 1$.
[c] Porosity was calculated using the following formula: Soil porosity = (1 – (bulk density/particle density)) × 100.

Upon comparing samples from PS + CH with those from MS + CH, it was observed that the amounts of a total of eight metabolites were significantly higher in MS + CH (caffeic acid, chicoric acid, chlorogenic acid, ferulic acid, isoquercetin, *p*-coumaric acid, salicylic acid, and sinapinic acid). Furthermore, the leaves of MS + CH contained significantly more chlorogenic acid, quinic acid, and salicylic acid than leaves from PS + CH (Supplementary Table S3). Table 3 shows an overview of these findings.

Untargeted metabolomics. The untargeted analysis on the LC-HRMS resulted in 23,675 and 20,564 detected features in root and leaf samples, respectively (Table 4). Featured ions that showed a variation coefficient below 30% in their respective QC samples were kept for further analysis. In total, 54.2% (12,836 out of 23,675) of the features showed a clear difference upon both chitin treatment and soil types in roots, whereas 37.4% (7,688 out of 20,564) of the features were significantly different in leaf samples. A comparative analysis was conducted to assess the effects of chitin treatment and different soil types. A total of 431 marker ions were chosen for distinguishing treatment groups or soil types in root samples, and 368 marker ions were selected for leaf samples. Potential identification of selected ions was attempted using Progenesis Qi v2.4 (Supplementary Table S4). The PCA plot based on the selected marker ions showed that all samples were clearly separated from each other based on treatment or soil type. However, the soil type has a bigger effect than the chitin treatment (Supplementary Fig. S3).

Rhizosphere diversity. For PS, the α-diversity for the bacterial rhizosphere communities was decreased by the addition of chitin, although this difference was not significant ($P = 0.10$, Kruskal-Wallis test) (Supplementary Fig. S4). For MS, the α-diversity for the bacterial rhizosphere communities remained similar upon chitin amendment ($P = 0.95$, Kruskal-Wallis test) (Supplementary Fig. S4). However, both soils showed a significant decrease in the α-diversity for the fungal rhizosphere communities upon chitin treatment (Supplementary Fig. S5). The bacterial β-diversity in the rhizosphere showed no clear separation for PS, PS + CH (Fig. 2A), and MS, MS + CH (Fig. 2B). On the other hand, the fungal communities of PS, PS + CH (Fig. 2C), and MS, MS + CH (Fig. 2D) showed a clear separation upon chitin treatment.

Relative abundance of rhizosphere communities. After the 1-week incubation period the 10 most abundant bacterial genera in the bulk soil underwent a shift in PS + CH compared with PS. The relative abundance of *Rhodanobacter* species was lower in PS + CH compared with PS (6.8% compared with 11.5%). *Edaphobaculum* species also were decreased in relative abundance in PS + CH compared with PS (3.7 and 4.8%, respectively). *Cytophaga* species could not be detected in PS + CH while they had an abundance in PS of 3.4%. In PS, *Flavobacterium* species had a relative abundance of 3.2%, which decreased with the chitin addition to 0.076% abundance in PS + CH. The relative abundance of *Dyella* species increased in PS + CH to 8.1%, whereas it was 2.6% in PS. *Mucilaginibacter* and *Granulicella* organisms were also more abundant in PS + CH (6.6 and 7.6%) compared with PS (3.7 and 1.4%). An increase upon chitin amendment was also observed in *Galbitalea* species (3.1% in PS + CH compared with 0.054% in PS). The changes in MS were smaller than those in PS upon chitin amendment. The relative abundances of most of the top 10 most abundant bacterial genera remained similar. Only *Bacillus* (0.64% compared with 1.1% for MS and MS + CH) and *Streptomyces* (0.64% compared with 1.3% for MS and MS + CH) bacteria were increased slightly upon chitin amendment (Supplementary Fig. S6).

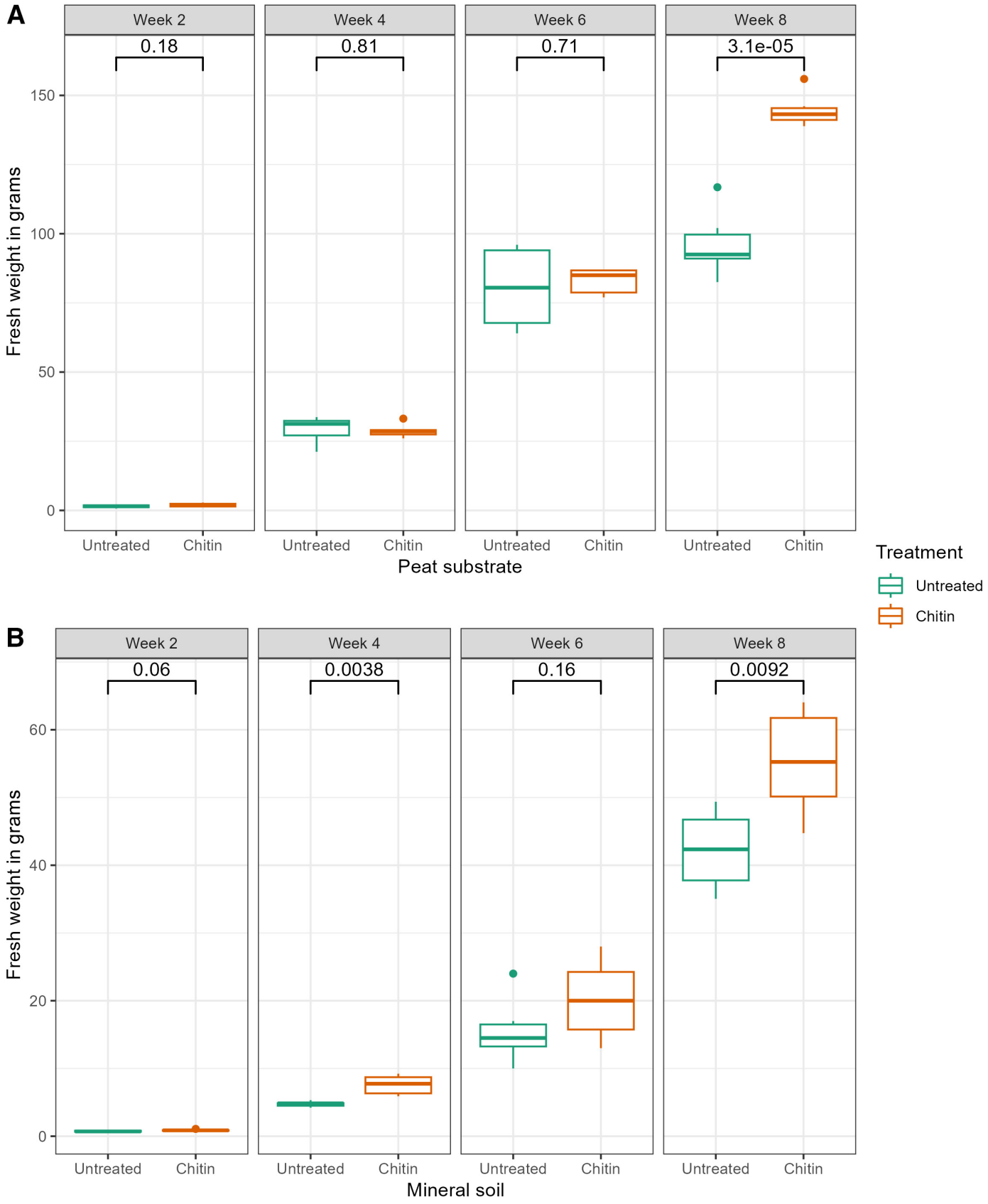

Fig. 1. A, Fresh weight of lettuce grown in peat substrate. **B,** Fresh weight of lettuce grown in mineral soil. The lettuces were grown for a period of 8 weeks, with sampling every 2 weeks. Treatments are indicated by different colors, with green for the untreated and orange for 2 g liter^{-1} of chitin addition. Fresh weights of all replicates per treatment and sampling point ($n = 6$) are indicated as boxplots. The median weight is represented as the horizontal line in the boxplots. Statistical inference is shown as a *P* value (test: two-sided Student's *t* test) plotted over the corresponding groups, focusing on the effect of chitin within a time point.

During the 8 weeks of growth, the 10 most abundant bacterial genera of the PS and PS + CH rhizosphere never made up a larger part than 40% of the total bacterial community. *Kitasatospora* species were more abundant in PS + CH from 2 to 8 wpt (2.1 ± 1.5% to 0.87 ± 0.14%) compared with PS (0.041 ± 0.071% to 0.000 ± 0.000%, 2 to 8 wpt) (Fig. 3A). *Kitasatospora* organisms were especially abundant in PS + CH, 4 wpt (8.3 ± 2.0%). *Rhodanobacter* organisms had a higher abundance in PS + CH (4.1 ± 0.48% to 1.1 ± 0.19%, 2 to 8 wpt) at any time compared with PS (2.6 ± 0.17% to 0.34 ± 0.036%, 2 to 8 wpt). The *Lacunisphaera* number increased in PS over the duration of the experiment (2.2 ± 0.28% to 3.0 ± 0.066%, 2 to 8 wpt), while its abundance decreased in PS + CH (2.1 ± 0.81% to 1.9 ± 0.55%, 2 to 8 wpt).

The 10 most abundant bacterial genera in the rhizosphere of MS or MS + CH never made up a bigger portion than 30%. *Streptomyces* bacteria were more abundant in MS + CH over the whole duration of the experiment (3.7 ± 0.27% to 2.1 ± 0.15%, 2 to 8 wpt) compared with MS (1.8 ± 0.55% to 1.2 ± 0.49%, 2 to 8 wpt). The distributions of the other genera were similar between the two treatment groups (Fig. 3B). Five of the 10 most abundant genera were the same in both soil types, including *Massilia*, *Lacunisphaera*, *Mucilaginibacter*, *Devosia*, and *Pseudomonas*. *Streptomyces* species, which were more abundant in MS + CH, were not present in the 10 most abundant genera of PS or PS + CH (0.94 ± 0.063% to 0.96 ± 0.29%, 2 to 8 wpt).

After 1 week of incubation, the compositions of the 10 most abundant fungi in the bulk soil were altered in PS compared

TABLE 2
Comparison of statistically different metabolites (confidence interval 0.95) between non-chitin-treated and chitin-treated lettuce plants grown in peat substrate (PS, PS + CH) and mineral soil (MS, MS + CH), respectively ($n = 4$)

Compound[a]	Soil[b]	Tissue[c]	Mean untreated (mg kg^{-1})[d]	Mean chitin treated (mg kg^{-1})[d]	*P* value[e]
Caffeic acid	PS	Root	10.7 ± 2.5	6.9 ± 1.5	0.05
Apigetrin		Leaf	0.026 ± 0.0032	0.019 ± 0.0026	0.02
Chicoric acid			9,500.1 ± 1,025.4	7,231.3 ± 463.3	0.01
Chlorogenic acid			1,155.0 ± 98.3	954.7 ± 34.2	0.02
Ferulic acid			0.15 ± 0.064	0.34 ± 0.12	0.05
Luteolin			0.095 ± 0.0046	0.077 ± 0.011	0.04
Quercetin-3-*O*-glucuronide			26.0 ± 0.93	20.1 ± 0.41	<0.01
Caffeic acid	MS	Root	15.3 ± 4.2	22.8 ± 2.0	0.03
Isoquercetin			0.70 ± 0.31	1.3 ± 0.34	0.04
Quercetin-3-*O*-glucuronide			13.5 ± 5.9	28.1 ± 8.6	0.04
Astragalin		Leaf	0.045 ± 0.0056	0.035 ± 0.0058	0.04
Rutin			4.97 ± 0.28	4.19 ± 0.33	0.01

[a] The measured compound.
[b] Soil type used for growing the lettuce.
[c] Tissue type.
[d] Values and the standard deviations of the corresponding compound in the untreated or treated group in mg kg^{-1}.
[e] *P* values for the comparison between the two treatment groups.

TABLE 3
Comparison of statistically different metabolites (confidence interval 0.95) between lettuce plants grown in potting soil (PS) and mineral soil (MS) with or without chitin treatment (PS + CH and MS + CH), respectively ($n = 4$)[a]

	Roots		Leaves	
Compound	Untreated	Chitin treated	Untreated	Chitin treated
Caffeic acid	–	MS	–	–
Chicoric acid	–	MS	PS	–
Chlorogenic acid	MS	MS	–	MS
Daidzin	–	–	–	MS
Ferulic acid	MS	MS	–	–
Isoquercetin	–	MS	–	–
Luteolin	–	–	PS	–
p-Coumaric acid	MS	MS	–	–
Quercetin-3-*O*-glucuronide	–	–	PS	–
Quinic acid	–	–	–	MS
Salicylic acid	MS	MS	MS	MS
Sinapinic acid	MS	MS	–	–

[a] Column 1, the analyzed metabolite. The rest of the columns show the treatment group and the tissue information, respectively, and their values indicate whether the measured compound was significantly higher in PS or in MS. No significant difference is indicated by "–".

with PS + CH. *Mortierella* and *Linnemannia* species were more abundant in PS + CH (19.8 and 6.9%) compared with PS (2.2 and 0.24%). In contrast, the *Penicillium* abundance decreased from 14.5% in PS to 8.3% in PS + CH. The relative abundances of *Phialemonium* and *Oidiodendron* organisms were also decreased in PS + CH (0.56 and 7.0%) compared with PS (3.8 and 9.5%).

After 1 week of incubation, there was also an increase in the relative abundances of *Mortierella* and *Linnemania* fungi in MS + CH (54.6 and 13.5%) compared with MS (42.7 and 8.4%). The abundances of the rest of the top 10 most relative abundant fungi remained similar (Supplementary Fig. S7).

For the fungal communities in PS and PS + CH, the 10 most abundant genera made up to 90% of the entire community. *Mortierella* was the most abundant fungal genus in PS + CH, with 64.0 ± 1.7% relative abundance at 2 wpt, decreased to 35.0 ± 5.8% at 8 wpt. The relative *Mortierella* abundance decreased from 25.6 ± 12.2% at 2 wpt to 17.2 ± 4.1% at 8 wpt in PS. *Linnemannia* fungi were more abundant in PS + CH compared with PS at all time points, starting with a relative abundance of 17.8 ± 2.3% at 2 wpt and finishing at 6.1 ± 1.5% at 8 wpt. In PS, *Linnemannia* fungi had a relative abundance of 8.0 ± 4.0% at 2 wpt and 1.4 ± 0.92% at 8 wpt. *Entomortierella* species were also more abundant in PS + CH compared with PS. In contrast to *Mortierella* fungi, the relative *Entomortierella* abundance increased in both treatments in 8 weeks (PS + CH = 4.9 ± 2.4% to 7.4 ± 1.1% and PS = 1.8 ± 0.87% to 2.2 ± 2.5%). *Penicillium* fungi were more abundant in PS compared with PS + CH over 8 weeks. In PS, its relative abundance decreased from 11.0 ± 2.1% at 2 wpt to 7.8 ± 0.96% at 8 wpt. PS + CH showed an increase of *Penicillium* fungi from 2.3 ± 0.94% at 2 wpt to 7.8 ± 0.96% at 8 wpt (Fig. 4A).

In the rhizosphere of MS and MS + CH, the 10 most abundant taxa made up to more than 90% of the fungal community. In contrast to PS + CH, the difference in relative *Mortierella* abundance between MS and MS + CH was smaller at 2 wpt (MS = 53.5 ± 12.4%, MS + CH = 48.5 ± 4.0%). From week 4 to week 8, the *Mortierella* abundance declined in MS + CH (48.0 ± 3.6% to 39.2 ± 1.2%, 4 to 8 wpt). In MS, the relative *Mortierella* abundance increased slightly (22.3 ± 8.5% to 23.2 ± 8.8%, 4 to 8 wpt). *Linnemannia* fungi were more abundant in MS + CH (6.7 ± 2.7% to 5.4 ± 0.81%, 2 to 8 wpt) compared with MS (4.1 ± 3.7% to 3.0 ± 1.6%, 2 to 8 wpt). *Entomortierella* fungi were not present in the top 10 most abundant fungi in the mineral soil. *Botryotrichum* fungi showed a relative abundance of 7.6 ± 1.7% to 13.6 ± 2.3% between 2 and 8 wpt in MS + CH. Compared with the abundance in MS + CH, the abundance was lower in MS during the 8-week growth period (2.0 ± 2.5% to 4.4 ± 4.3%, 2 to 8 wpt). *Humicola* abundance started at 19.2 ± 5.3% at 2 wpt and finished at 15.7 ± 2.6% after 8 wpt in MS + CH. In MS, it ranged between 1.5 ± 1.5% and 3.5 ± 4.8% (2 to 8 wpt). MS had higher *Dactylonectria* (6.0 ± 7.6% to 3.0 ± 3.2%, 2 to 8 wpt) and *Berkeleyomyces* (5.5 ± 1.7% to 0.81 ± 0.57%, 2 to 8 wpt) abundances compared with MS + CH (respectively, 0.23 ± 0.063% to 0.48 ± 0.11%, 2 to 8 wpt; 0.58 ± 0.35% to 0.12 ± 0.059%, 2 to 8 wpt) (Fig. 4B). Of the 10 most abundant fungal genera, three were present in both soils: *Mortierella*, *Olpidium*, and *Linnemannia*.

Differential abundance test of rhizosphere communities. The rhizosphere of lettuce grown in PS and PS + CH showed no significantly different bacterial genera. On the other hand, the rhizosphere of lettuce grown in MS contained two significantly different bacterial genera, *Kitasatospora* and *Streptomyces*, according to the random forest abundance test ($P < 0.05$) (Fig. 5A; Supplementary Table S5). For the fungal communities, *Mortierella* and *Entomortierella* genera were significantly more abundant in PS + CH compared with PS. In total, 24 fungal genera were more abundant in PS compared with PS + CH (Fig. 5B). Of those, eight had a relative abundance higher than 0.01: *Penicillium*, *Oidiodendron*, *Geomyces*, *Nematoctonus*, *Phialemonium*, *Apiotrichum*, *Candida*, and *Saitozyma* (Supplementary Table S6).

In MS + CH lettuce rhizosphere, fungi of the *Humicola*, *Syncephalis*, *Botryotrichum*, *Trichoderma*, and *Purpureocilium* genera were significantly more abundant compared with MS. In total, 14 fungal genera were significantly higher in abundance in MS compared with MS + CH (Fig. 5C). Of those, five had an abundance higher than 0.01 (*Dactylonectria*, *Berkeleyomyces*, *Plectosphaerella*, *Fusarium*, and *Gibellulopsis*) (Supplementary Table S7). The only genus that was significantly affected by the chitin treatment in both soils was *Trichoderma*. While it was less present in PS + CH compared with PS, it was more present in MS + CH compared with MS.

DISCUSSION

Chitin's effect on promoting lettuce growth in PS was reported previously (Debode et al. 2016; Li et al. 2023). However, lettuce is mainly cultivated in MS, for which the effectiveness of chitin has not been explored yet. In this study, chitin's growth promotion effect in MS was investigated for the first time in comparison with PS. Our greenhouse experiment revealed that compared with that of PS, chitin's growth promotion effect on lettuce in MS was lower. PS is a soilless cultivation medium whose physical and chemical characteristics, such as bulk density and organic content, differ greatly from real mineral soils. In MS, additional fertilizer was applied prior to use to prevent N from becoming a limiting factor during the cultivation period. Fertilizer was not applied in PS, because it tends to cause lettuce tip burn as previously observed in our experiment (*unpublished data*).

Chitin amendment clearly resulted in a higher fresh weight of the lettuce in both PS and MS. Lettuce generally grew bigger in PS than in MS with or without chitin treatment. This might be owing to the different soil types. The PS had a dry matter of 25.0% compared with 88.1% for the MS and gave less restriction on root development because of its low bulk density. Furthermore, the electric conductivity for the PS was higher, with 450 μS cm^{-1} compared

TABLE 4
Number of ions detected, significantly different (sig_diff), and selected tentative identified markers (TIM) of different treatment groups or soil types using LC-HRMS in both positive ionization (ESIpos) and negative ionization (ESIneg)

	Root			Leaf		
Method	Detected	Sig_diff	TIM markers	Detected	Sig_diff	TIM markers
ESIpos	12,931	7,695	216	11,284	4,125	137
ESIneg	10,744	5,141	215	9,280	3,563	231
Total	23,675	12,836	431	20,564	7,688	368

with 116 µS cm^{-1} for the MS. Electric conductivity of soil is an indirect measure for its nutrients' availability, which suggests that smaller amounts of nutrients were available in the MS and that a slower growth of the lettuce plants could be expected.

Chitin is known as a microbe-associated molecular pattern (MAMP), which, upon plant perception, can induce pattern-triggered immunity (PTI) (Egusa et al. 2015). Previous research showed that chitin in PS can cause transcriptional reprogramming in lettuce roots and regulate phenylpropanoid biosynthesis, which affects the accumulation of phenolic compounds in the plant (Kandel et al. 2022; Li et al. 2023). The targeted metabolomic analysis showed that chitin addition in both PS and MS affected the content of different phenolic compounds. The content of the targeted phenolic compounds, however, appeared to be more dependent on soil types. Most of the phenolic compounds had a higher amount in lettuce grown in MS compared with PS, regardless of the chitin amendment (Table 3). This is congruent with the untargeted analysis, where samples clustered more closely according to the soil type than to the chitin treatment (Supplementary Fig. S3). Consequently, we think that the disparities in phenolic

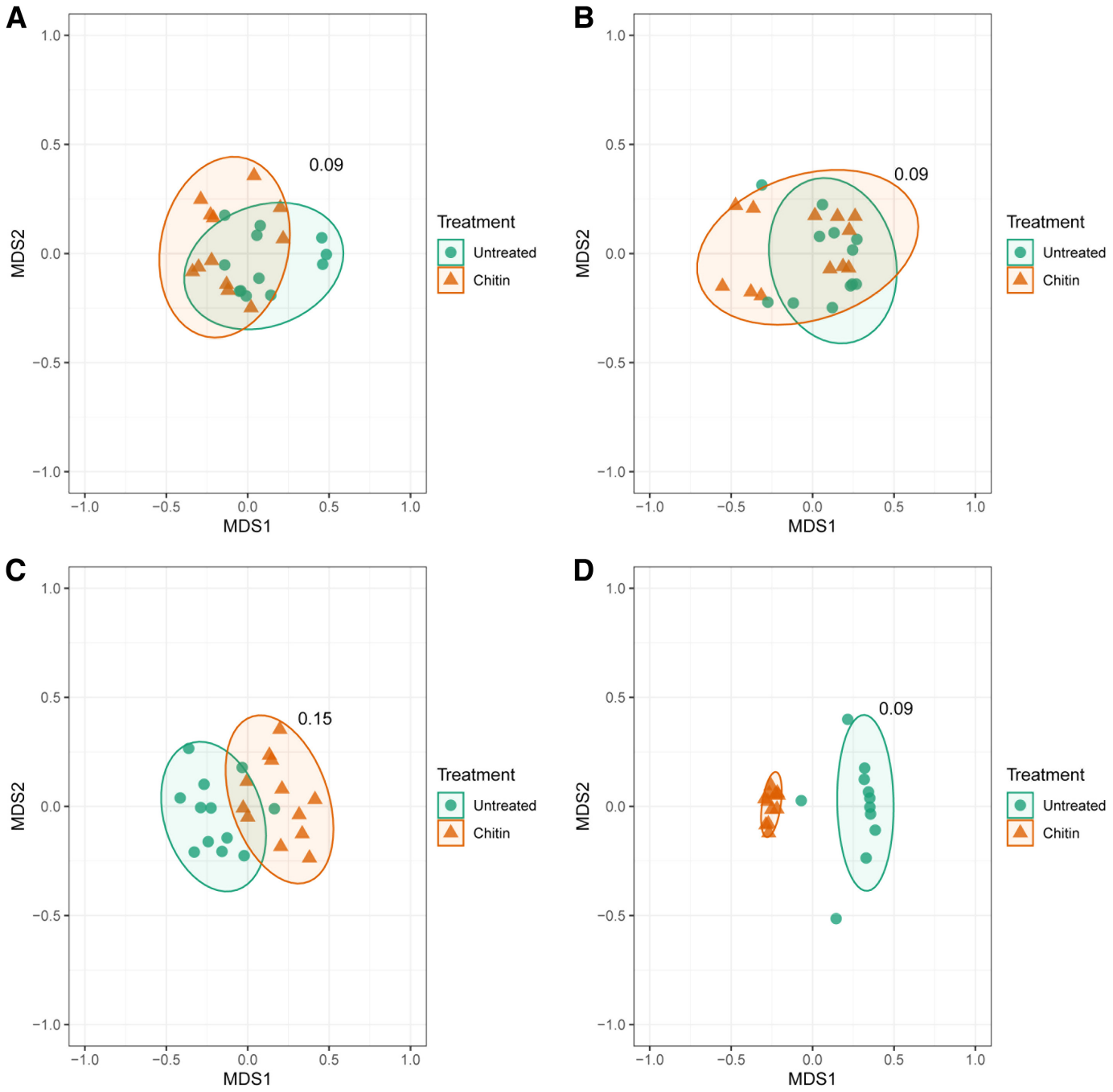

Fig. 2. Multidimensional scaling (MDS) plots illustrating the β-diversity of the **A and B,** bacterial and **C and D,** fungal communities, based on the Bray-Curtis index, between two treatments (untreated [green, circle], chitin treated [orange, triangle]) in either peat substrate (A and C) or mineral soil (B and D). Each dot represents one sample.

compounds primarily stem from the soil composition and rhizobiome composition.

The rhizobiome diversity analysis showed a significant decrease in α-diversity of the fungal communities. For the bacterial communities, the α-diversity tended to be smaller, but the difference was not significant (Supplementary Figs. S4 and S5). After the chitin treatment, the β-diversity plots showed no clear separation between the bacterial communities for the rhizospheres of the two soils. The fungal communities of the rhizosphere showed a clear separation upon chitin treatment (Fig. 2). This is in agreement with previous

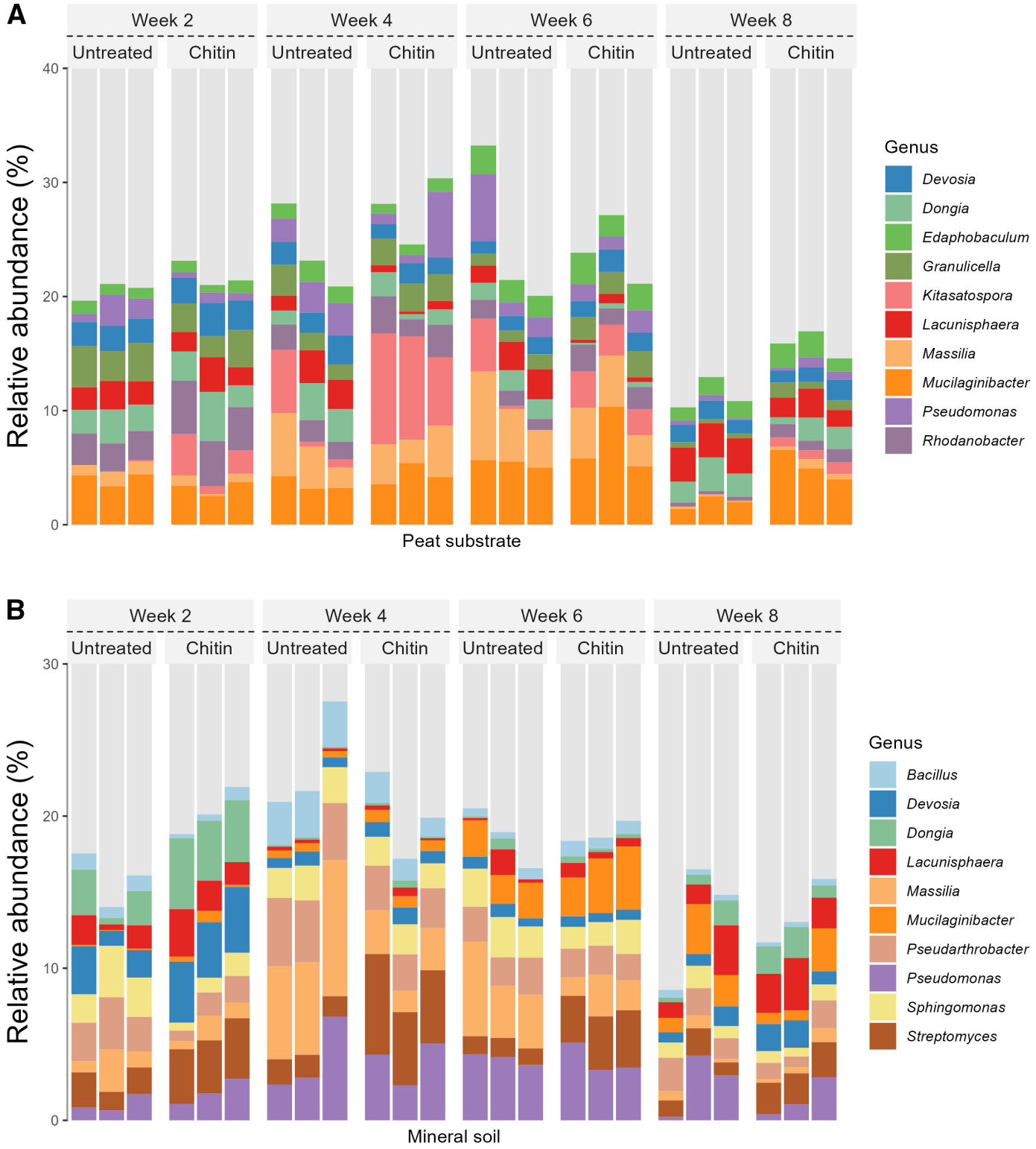

Fig. 3. Relative abundance of the 10 most abundant bacterial genera in the lettuce rhizosphere in **A,** peat substrate or **B,** mineral soil. The relative abundance is plotted over 8 weeks and split between treatment groups (untreated and chitin). The colors of the bars correspond to the different genera in the legend of each panel. All bacteria not belonging to the top 10 most relative abundant genera are shown in gray.

results that show a stronger effect on the fungal communities upon chitin soil amendment (Debode et al. 2016; De Tender et al. 2019; Randall et al. 2020). The microbial compositions of the bulk soil for PS and MS were different from the composition of the rhizosphere, indicating an active selection of the rhizosphere microbiota (Hartmann et al. 2009).

The random forest analysis showed that no bacterial genera were significantly different between PS and PS + CH. The significantly different fungal genera more abundant in PS + CH were *Mortierella* and *Entomortierella*, both belonging to the *Mortierellaceae* family. *Mortierellaceae* fungi are known chitin degraders (Telagathoti et al. 2022). *Mortierella* fungi, which showed the

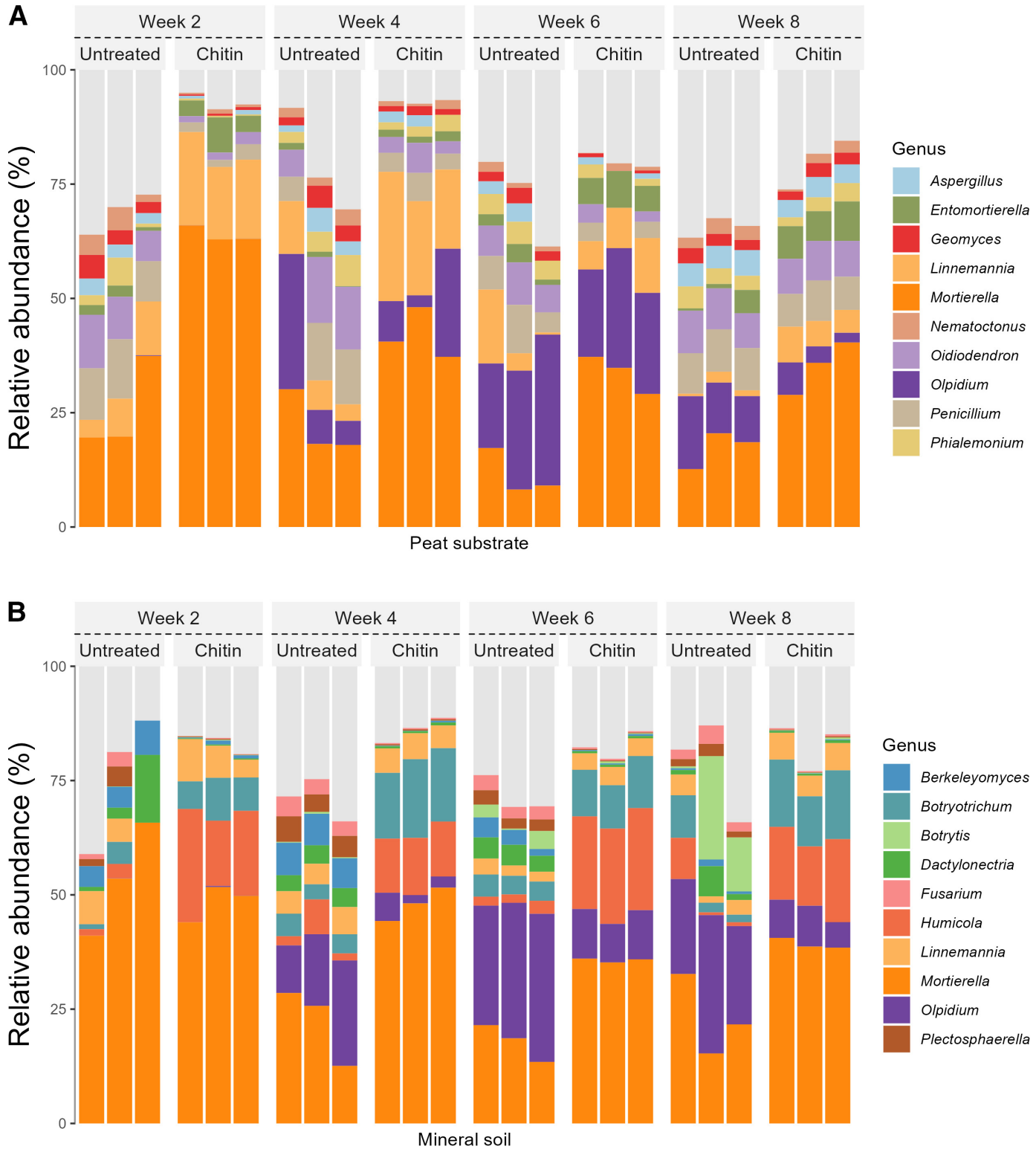

Fig. 4. Relative abundance of the 10 most abundant fungi in the lettuce rhizosphere in **A,** peat substrate or **B,** mineral soil. The abundance is plotted over 8 weeks and split between treatment groups (chitin and untreated). The colors of the bars correspond to the different genera in the legend of each panel. All fungi not belonging to the top 10 most relative abundant genera are shown in gray.

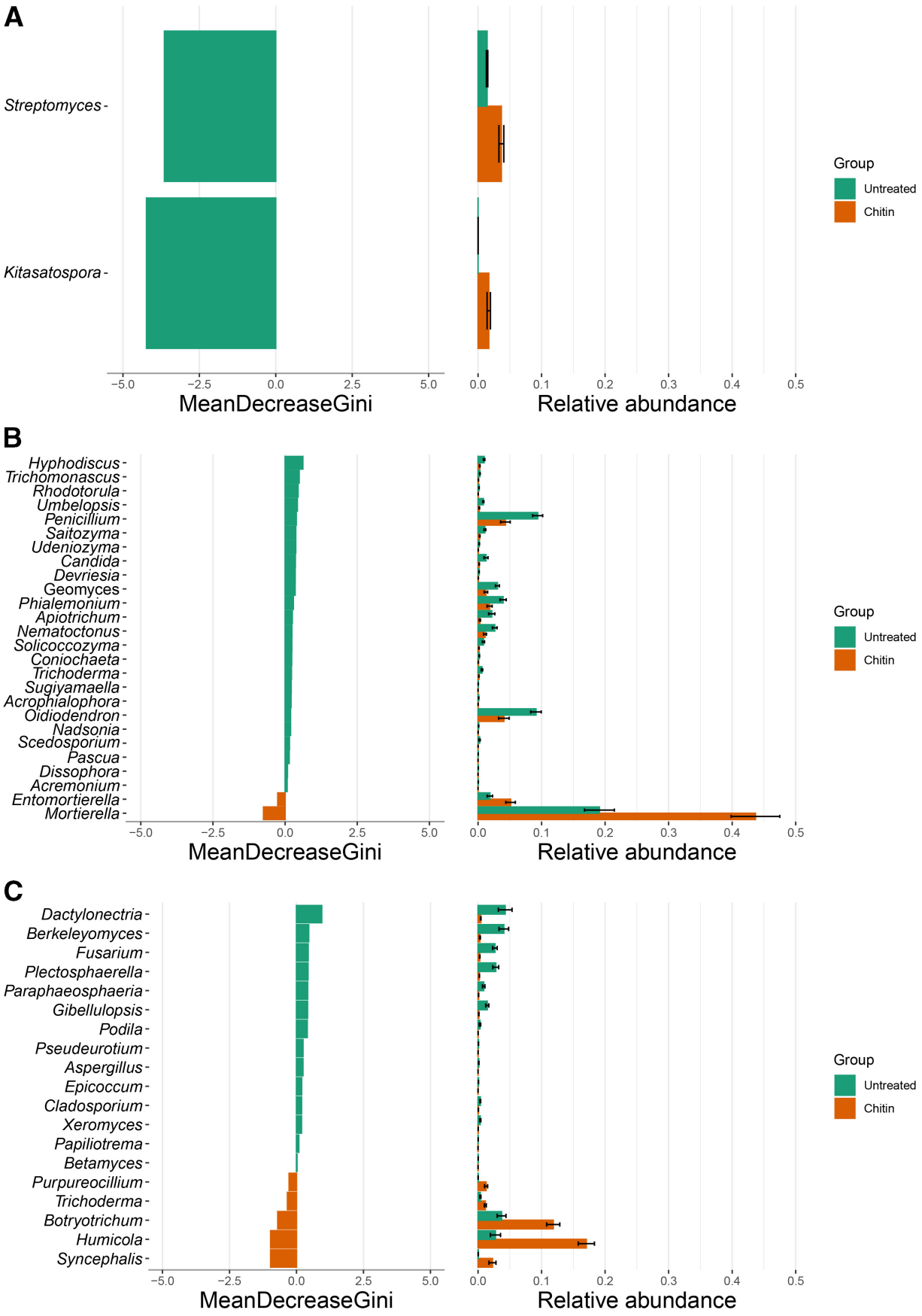

Fig. 5. **A,** Test of differential abundance between treatment groups of bacterial communities in mineral soil (MS) compared with mineral soil with chitin (MS + CH). **B,** Test of differential abundance between treatment groups of fungal communities in peat substrate (PS) compared with peat substrate with chitin (PS + CH), and **C,** MS compared with MS + CH. Bars on the left show the mean decrease in Gini of a genus, and the bars on the right show the mean corresponding abundance and the standard deviation of the treatment groups.

biggest effect in the random forest analysis, are known to promote growth in a variety of plants (Johnson et al. 2019; Li et al. 2018; Ozimek and Hanaka 2021). More specifically, it has been shown that isolated species of the *Mortierellaceae* family significantly increase the growth of *Arabidopsis* seedlings and are potential plant-growth-promoting species (De Tender et al. 2024). It is not known whether *Entomortierella* has plant-growth-promoting effects.

In the rhizosphere of MS + CH, the bacterial genera *Kitasatospora* and *Streptomyces* were found in significantly higher abundance than in MS. *Kitasatospora* and *Streptomyces* species are known to possess chitinases and chitinosanases (Mahadevan and Crawford 1997; Narayana and Vijayalakshmi 2009; Schrempf 2001; Sharma et al. 2020; Zitouni et al. 2017). *Streptomycetaceae* family strains were shown before to be associated with chitin amendment in other mineral soils (Joos et al. 2023). Furthermore, *Streptomyces* bacteria produce a broad range of secondary metabolites, including antibacterial compounds, that could repress some bacterial genera (Arn et al. 2020), and they are widely used as biocontrol agents (Enany 2018; Law et al. 2017; Sabaratnam and Traquair 2002; Trejo-Estrada et al. 1998; Vurukonda et al. 2018).

In our experiment, the fungal genera that were significantly more abundant in MS + CH included *Syncephalis*, *Humicola*, *Botryotrichum*, *Purpureocilium*, and *Trichoderma*. Some of those genera, such as *Trichoderma, Humicola,* and *Purpureocillium,* are known to include species with chitinase activity (Girardi et al. 2022; Kumar et al. 2017; Nampoothiri et al. 2004; Seidl et al. 2005). Several *Purpureocilium* and *Trichoderma* fungi are also promising biocontrol agents (Elsherbiny et al. 2021; Freeman et al. 2004; Lan et al. 2017; Sood et al. 2020). Different *Humicola* and *Purpureocillium* species were also shown to induce plant growth promotion in a variety of plants (Baron et al. 2020; Elshafie and Camele 2022; Khan and Tanaka 2023; Radhakrishnan et al. 2015).

On the other hand, some *Syncephalis* organisms are obligate mycoparasites (Benny et al. 2016).

In conclusion, our findings indicate that chitin amendments had a positive effect on lettuce growth in both types of substrates. Additionally, chitin amendments resulted in alterations of the fungal and bacterial components of the rhizobiome in both substrates. However, the rhizobiome in each soil displayed distinct changes. In the case of PS + CH, there was a significantly higher presence of plant-growth-promoting fungi (*Mortierellaceae*) compared with PS. While there was no statistically significant variance in the abundance of *Mortierellaceae* fungi between MS and MS + CH, chitin amendment led to a notable increase in other potential plant-growth-promoting fungi in MS + CH when compared with MS.

In addition to fungal genera, the random forest analysis revealed a notable increase in chitin-degrading bacteria in MS + CH compared with MS. Consequently, we hypothesize that in the presence of chitin amendments, *Mortierellacea* fungi primarily thrived in PS + CH, while in MS + CH, the principal beneficiaries were chitin-degrading bacteria.

On the basis of our findings, we believe that the introduction of chitin leads to plant growth promotion and metabolomic changes primarily resulting from the soil characteristics and the adaptation of microorganism communities. Chitin remains an eco-friendly alternative to synthetic fertilizers. However, our results show that the success of chitin soil amendment is dependent on the initial microbiome composition of the soil and for optimal results microbiome compositions should be considered before applying chitin as fertilizer.

Data availability. Sequence data are available in the ENA short read archive under the accession PRJEB70956.

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