Soil type and associated microbiomes influences chitin's growth-promotion effect in lettuce

Moritz Kaufmann,[1,2†] Leilei Li,[3†] Christof Van Poucke,[4] Nicola Rhyner,[1] Caroline De Tender,[5] Mieke Uyttendaele,[6] Marc Heyndrickx,[4,8] Cyril Zipfel,[2,7] Joël F. Pothier,[1] and Bart Cottyn[3]

[1] Environmental Genomics and Systems Biology Research Group, Institute of Natural Resource Sciences, Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland

[2] Department of Plant and Microbial Biology, Zurich-Basel Plant Science Center, University of Zurich, Zurich, Switzerland

[3] Plant Sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium

[4] Technology and Food Science Unit, ILVO, Melle, Belgium

[5] Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

[6] Department of Food Technology, Safety and Health, Ghent University, Ghent, Belgium

[7] The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, Norwich, United Kingdom

[8] Department of Pathobiology, Pharmacology and Zoological Medicine, Ghent University, Merelbeke, Belgium

* Corresponding author: J. F. Pothier: joel.pothier@zhaw.ch

Keywords: *Chitin, Lettuce, Metabolomics, Metabarcoding, Growth promotion*

Funding: This work was supported in Switzerland by grant no. 189340 from the Swiss National Science Foundation (SNSF) and in Belgium by grant no. G063820N from the Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO). CZ was supported by the University of Zurich. JP was supported by the Department of Life Sciences and Facility Management of the Zurich University of Applied Sciences (ZHAW) in Wädenswil. The open access article processing charges of this publication were funded by SNSF.

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 to 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 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 not *Mortierellaceae*. We assume that the observed effects primarily stem from soil physiochemical 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.

1. Introduction

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 to enhance agricultural production (Yang et al. 2019). Over the past few decades, the increase 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 2017). It is known however, that chemical fertilizers and pesticides can have a negative

effect on human health and the environment (Mahmood et al. 2016; Kim et al. 2017; Sharma and Singhvi 2017). Therefore, environmental-friendly fertilizers and pesticides are recommended for a sustainable agriculture (Kumar 2012; Chen et al. 2018). Chitin has drawn much attention in the last decades 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 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 non-polar solvents (Moussian 2019).

Previous 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; De Tender et al. 2023). Also, chitin / chitosan addition could affect lettuce seedling's growth on transcriptional and metabolite level, modifying the accumulation of several phenolic acids and plant hormones, which might promote lettuce growth and disease resistance (Pusztahelyi 2018; Li et al. 2023).

So far, the growth and defense promotion effect of chitin has only been studied in commercial PS. In agriculture practice, most lettuce plants are grown in greenhouses

in mineral soil (MS), which differs in texture, microbial community and nutritional composition compared to PS. The effect of chitin in MS can thus tremendously differ compared to PS but remains untested so far. Therefore, in this study, we investigated for the first time the effect of chitin as a soil amendment in MS in comparison to 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 analysis were conducted in lettuce seedlings. Lettuce growth and the bacterial (16S rRNA) and fungal (ITS) composition of the lettuce rhizosphere was monitored every two weeks during the whole growth period of eight weeks.

2. Materials and Methods

2.1 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 soil) from PCG Kruishoutem located in East-Flanders. Chemical characterization of MS was carried out using the method thoroughly described previously (Vandecasteele et al. 2021). For MS, 1.02 g L^{-1} fertilizer (PGMix fertilizer NPK 14-16-18, Haifa Northwest Europe) was applied before further usage. PS and MS without chitin addition were used as control. Chitin amended soil was either PS or MS mixed with 2 g L^{-1} chitin (PS+CH and MS+CH, respectively). Both soils were wetted with ground

water to reach 40% water filled pore space and incubated in a closed bag in the greenhouse for one week before using.

2.2 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). First PS was used to fill 77-well germination trays, 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 L-pots (top ⌀ = 15 cm, bottom ⌀ = 11 cm, height = 11.5 cm) filled with 1 L 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 along the local weather (Belgium, February – April 2022). All pots were placed in a semi-randomly order, on greenhouse tables and surrounded by border plants to avoid potential border effect (Sato and Takahashi 1983) (Supplementary Fig S1). For eight weeks, six plants from each treatment were sampled every two weeks for fresh plant weight measurement and rhizosphere collection, resulting in a total number 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, gently shaken to remove the excess soil attached. Afterwards, roots were placed in a 50 mL-

tube with 25 mL 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 rhizosphere pellet. Additionally, as a control, PS and MS was sampled once after the one-week incubation period described in section 2.1. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instruction 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 L- pots (top ⌀ = 9 cm, bottom ⌀ = 6 cm, height = 7 cm) and allowed to grow for one 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 grinded 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.

2.3 Fresh weight analysis

The weights of the lettuces grown in PS or MS were compared every two weeks between treatments. The fresh weights were analysed using R v4.2.1 (R Core Team 2022) in RStudio Desktop v2022.07.1+554 (Rstudio Team 2020). sTo check for significant difference in fresh weight the weights of lettuce grown in either soil (PS or MS) were compared between treatment (PS+CH vs PS vs, MS+CH vs 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).

2.4 Targeted phenolic compounds and untargeted metabolomics analysis

For the targeted metabolomics analysis the same targets were used which were previously reported to be altered upon chitin soil amendments (Li et al. 2023). For both targeted and untargeted metabolomics analysis, the metabolites extraction was performed using the method described previously with slight modification (Li et al. 2023). Briefly, 50 μL internal standard (daidzin, 100ng μL^{-1}) and 10 mL pure methanol (ULC/MS grade absolute methanol) was 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 20% (*v*/*v*) methanol/H_2O was added, vortexed and sonicated as the first time. For samples that weighed less than 500 mg, the volume 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, 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, USA). Details on the

used gradient are described by 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, a mixture 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. Data processing of the untargeted data was done using Progenesis Qi v2.4 (Waters). Different expressed features (tested with ANOVA and correcting for multiple testing with false discovery rate) between the treated and non-treated 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 using Bonferroni correction to correct for multiple hypothesis testing.

2.5 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

(Illumina, San Diego, USA, California) using dual indexing (Illumina) with minor modifications. The PCR reaction (primers in Supplementary Table S1) was run for 30 cycles using HiFi HotStart ReadyMix (Kapa Biosystems, Hofmann-La Roche, Basel, Switzerland). Before clean-up 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, USA, New York) with the DNF-935 Reagent Kit from Agilent (Agilent Technologies, Santa Clara, USA, California). 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, MS) 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 internal transcribed spacer (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 from the ITS1 (between positions 195 and 695). The PCR reaction was run for 30 cycles using HiFi HotStart ReadyMix (Kapa Biosystems). Additionally, ZymoBIOMICS Microbial Community DNA Standard (ZYMO RESEARCH, Irvine, USA, California) was used as a positive control. The rest of the workflow was the same as for the 16S metagenomics sequencing mentioned above. A total of 15'483'711 and 20'214'091

paired-end reads were obtained for the 16S rRNA and the ITS1 metabarcoding, respectively.

Optimal reads 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). Sample with less 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 datasets. For the α- diversity calculations, the samples were rarefied according to the minimum

amplicon reads present in the cleaned dataset 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 non-metric multidimensional scaling (NMDS) and visualized with microeco. For the fungal dataset, one outlier sample (R9-ITS) had to be removed. With the outlier in the dataset, 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 genus level using random forest (Beck and Foster 2014; Yatsunenko et al. 2012) combined with a non-parametric test. MeanDecreaseGini was selected as an indicator value for the analysis. The analysis and the visualization were done using microeco.

3. Results

3.1 Soil analysis and lettuce growth

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

Comparing the lettuce fresh weights between PS and PS+CH, there was no significant difference in weight within the first six weeks. At eight weeks post transplanting (wpt),

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

3.2 Targeted metabolomics

Of the total 47 phenolic compounds (PCs) tested, 22 were present in either leaf or root (Supplementary Table S2). Comparisons of PCs content based on chitin treatment of plants growing in each soil revealed differences in several metabolites (Table 2). Comparing the PCs content in roots between PS and PS+CH, only caffeic acid was significantly less present in PS+CH roots compared to 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 higher concentrated when compared to the ones grown in MS. The leaves of lettuce grown in MS showed a higher concentration of rutin and astragalin compared to 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 significant higher level in sinapinic acid, *p*-coumaric acid, salicylic acid, chlorogenic acid and ferulic acid in MS compared to PS. Conversely, in the leaves, PS exhibited higher concentrations of luteolin, quercetin-3-O-glucuronide, and chicoric acid compared to MS. In contrast, MS leaves contained significantly more salicylic acid than PS leaves.

Upon comparing samples from PS+CH with those from MS+CH, it was observed that 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.

3.3 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, while 368 marker ions were selected for leaf samples. Potential identification of selected ions was attempted using Progenesis Qi v2.4 (Supplementary Table S4**)**. 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).

3.4 Rhizosphere diversity

For PS, the α-diversity for the bacterial rhizosphere 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 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 of the fungal rhizosphere 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.

3.5 Relative abundance of rhizosphere communities

After the one-week incubation period the ten most abundant bacterial genera in the bulk soil underwent a shift in PS+CH compared to PS. The relative abundance of *Rhodonobacter*, was lower in PS+CH compared to PS (6.8% compared to 11.5%). *Edaphobaculum* also decreased in relative abundance in PS+CH compared to PS (3.7% and 4.8% respectively). *Cytophaga* could not be detected in PS+CH while it had an abundance in PS of 3.4%. In PS, *Flavobacterium* had a relative abundance of 3.2% and

decreased with the chitin addition to 0.076% abundance in PS+CH. The relative abundance of *Dyella* increased in PS+CH to 8.1% whereas it was 2.6% in PS. *Mucilaginibacter* and *Granulicella* were also more abundant in PS+CH (6.6% and 7.6%) compared to PS (3.7% and 1.4%). An increase upon chitin amendment was also observed in *Galbitalea* (3.1% in PS+CH compared to 0.054% in PS). Compared to PS, the changes in MS were smaller upon chitin amendment. The relative abundance of the majority of the top ten most abundant bacterial genera remained similar. Only *Bacillus* (0.64% compared to 1.1% for MS and MS+CH) and *Streptomyces* (0.64% compared to 1.3% for MS and MS+CH) increased slightly upon chitin amendment (Supplementary Fig S6).

During the eight weeks of growth, the ten 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* was more abundant in PS+CH from 2 wpt to 8 wpt (2.1 ± 1.5%, to 0.87 ± 0.14%) compared to PS (0.041 ± 0.071% to 0.000 ± 0.000%, 2 wpt to 8 wpt) (Fig 3A). *Kitasatospora* was especially abundant in PS+CH, 4 wpt (8.3 ± 2.0%). *Rhodanobacter* had a higher abundance in PS+CH (4.1 ± 0.48% to 1.1 ± 0.19%, 2 wpt to 8 wpt) at any time compared to PS (2.6 ± 0.17% to 0.34 ± 0.036%, 2 wpt to 8 wpt). *Lacunisphaera* increased in PS over the duration of the experiment (2.2 ± 0.28% to 3.0 ± 0.066%, 2 wpt to 8 wpt), while its abundance decreased in PS+CH (2.1 ± 0.81% to 1.9 ± 0.55%, 2 wpt to 8 wpt).

The ten most abundant bacterial genera in the rhizosphere of MS or MS+CH never made up a bigger portion than 30%. *Streptomyces* was more abundant in MS+CH over

the whole duration of the experiment (3.7 ± 0.27% to 2.1 ± 0.15%, 2 wpt to 8 wpt) compared to MS (1.8 ± 0.55% to 1.2 ± 0.49%, 2 wpt to 8 wpt). The distribution of the other genera was similar between both treatment groups (Fig 3B). Five of the ten most abundant genera were the same in both soil types, including *Massilia*, *Lacunisphaera*, *Mucilaginibacter*, *Devosia*, and *Pseudomonas*. *Streptomyces*, which was more abundant in MS+CH, was not present in the ten most abundant genera of PS or PS+CH (0.94 ± 0.063% to 0.96 ± 0.29%, 2 wpt to 8 wpt).

After one week of incubation, the composition of the ten most abundant fungi in the bulk soil was altered in PS compared to PS+CH. *Mortierella* and *Linnemannia* were more abundant in PS+CH (19.8% and 6.9%) compared to PS (2.2% and 0.24%). In contrast, the abundance of *Penicillium* decreased from 14.5% in PS to 8.3% in PS+CH. The relative abundance of *Phialemonium* and *Oidiodendron* was also decreased in PS+CH (0.56% and 7.0%) compared to PS (3.8% and 9.5%).

After one week incubation, there was also an increase in the relative abundance of *Mortierella* and *Linnemania* in GS+CH (54.6% and 13.5%) compared to GS (42.7% and 8.4%). The abundance of the rest of the top ten most relative abundant fungi remained similar (Supplementary Fig S7).

For the fungal communities in PS and PS+CH, the ten 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 abundance of *Mortierella* decreased from 25.6 ± 12.2% at 2 wpt to 17.2 ± 4.1% at 8 wpt in PS. *Linnemannia* was more abundant in PS+CH compared to 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* had a relative abundance of 8.0 ± 4.0% at 2 wpt and 1.4 ± 0.92% at 8 wpt. *Entomortierella* was also more abundant in PS+CH compared to PS. In contrast to *Mortierella*, the relative abundance of *Entomortierella* increased in both treatments during eight weeks (PS+CH 4.9 ± 2.4% to 7.4 ± 1.1% and PS 1.8 ± 0.87% to 2.2 ± 2.5%). *Penicillium* was more abundant in PS compared to PS+CH during eight 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* 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 ten most abundant taxa made up to over 90% of the fungal community. In contrast to PS+CH, the difference in relative abundance of *Mortierella* between MS and MS+CH was smaller at 2 wpt (MS = 53.5 ± 12.4%, MS+CH = 48.5 ± 4.0%). From week four to week eight, the abundance of *Mortierella* declined in MS + CH (48.0 ± 3.6% to 39.2 ± 1.2%, 4 wpt to 8 wpt). In MS, the relative abundance of *Mortierella* increased slightly (22.3 ± 8.5% to 23.2 ± 8.8%, 4 wpt to 8 wpt). *Linnemannia* was more abundant in MS+CH (6.7 ± 2.7% to 5.4 ± 0.81%, 2 wpt to 8 wpt) compared to MS (4.1 ± 3.7% to 3.0 ± 1.6%, 2 wpt to 8 wpt). *Entomortierella* was not present in the top ten most abundant fungi in the mineral soil. *Botryotrichum* showed a relative abundance of 7.6 ± 1.7% to 13.6 ± 2.3% between 2 wpt to 8 wpt in MS+CH. Compared to MS+CH, the abundance was lower in MS during the eight-week growth period (2.0 ± 2.5% to 4.4 ± 4.3%, 2 wpt to 8 wpt). *Humicola*'s 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 wpt to 8 wpt). MS had higher abundances of *Dactylonectria* (6.0 ± 7.6% to 3.0 ± 3.2%, 2 wpt to 8 wpt) and *Berkeleyomyces* (5.5 ± 1.7% to 0.81 ± 0.57%, 2 wpt to 8 wpt) compared to MS+CH (respectively, 0.23 ± 0.063% to 0.48 ± 0.11%, 2 wpt to 8 wpt; 0.58 ± 0.35% to 0.12 ± 0.059%, 2 wpt to 8 wpt) (Fig 4B). Of the ten most abundant fungal genera, three were present in both soils: *Mortierella*, *Olpidium* and *Linnemannia*.

3.6 Differential abundance test of rhizosphere communities

The rhizosphere of lettuce grown in PS and PS+CH, showed no significantly different bacteria 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 and Supplementary Table S5). For the fungal communities, *Mortierella* and *Entomortierella* were genera that were significantly more abundant in PS+CH compared to PS. In total, 24 fungal genera were more abundant in PS compared to 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 rhizosphere, *Humicola*, *Syncephalis*, *Botryotrichum*, *Trichoderma* and *Purpureocilium* were significantly more abundant in the rhizosphere compared to MS. In total, 14 fungal genera were significantly in higher abundance in MS compared to 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 to PS, it was more present in MS+CH compared to MS.

4. Discussion

Chitin's effect on promoting lettuce growth in PS has been reported previously (Debode et al. 2016; Li et al. 2023). However, lettuce is mainly cultivated in MS, for which the effectiveness of chitin was not 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 to 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 (data not shown).

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 due to the different soil types. The PS had a dry matter of 25.0% compared to 88.1% for the MS and gave less restriction on root development due to its low bulk density. Furthermore, the electric conductivity of the PS was higher with 450 $\mu S\ cm^{-1}$ compared to 116 $\mu S\ cm^{-1}$ for the MS. Electric conductivity of soil is an indirect measure for its nutrients' availability, which suggests that less 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 to 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 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 Fig S4 and Supplementary Fig S5). After the chitin treatment, the β-diversity plots showed no clear separation between the bacterial community for the rhizospheres of both soils. The fungal community of the rhizosphere showed a clear separation upon chitin treatment (Fig 2). This is in agreement with previous results that show a stronger effect on the fungal community upon chitin soil amendment (Debode et al. 2016; De Tender

et al. 2019; Randall et al. 2020). The composition of the bulk soil for PS and GS was different than the composition of the rhizosphere indicating an active selection of the bacterial rhizosphere (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* are known chitin degraders (Telagathoti et al. 2022). *Mortierella*, which showed the biggest effect in the random forest analysis, is known to promote plant 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. 2023). Up to now, 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* are known to possess chitinases and chitinosanases (Mahadevan and Crawford 1997; Schrempf 2001; Narayana and Vijayalakshmi 2009; Zitouni et al. 2017; Sharma et al. 2020). Members of *Streptomycetaceae* strains were shown before to be associated with chitin amendment in other mineral soils (Joos et al. 2023). Furthermore, *Streptomyces* 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 (Law et al. 2017; Sabaratnam and Traquair 2002; Vurukonda, Sai Shiva Krishna Prasad et al. 2018; Trejo-Estrada et al. 1998; Enany 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 like *Trichoderma, Humicola* and *Purpureocillium* are known to include species with chitinase activity (Nampoothiri et al. 2004; Seidl et al. 2005; Kumar et al. 2017; Girardi et al. 2022). Several members of *Purpureocilium* and *Trichoderma* are also promising biocontrol agents (Lan et al. 2017; Elsherbiny et al. 2021; Freeman et al. 2004; Sood et al. 2020). Different *Humicola* and *Purpureocillium* species, were also shown to induce plant growth promotion in a variety of plants (Radhakrishnan et al. 2015; Elshafie and Camele 2022; Baron et al. 2020; Khan and Tanaka 2023).

Some *Syncephalis* on the other hand 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 to PS. While there was no statistically significant variance in the abundance of *Mortierellaceae* between MS and MS+CH, chitin amendment led to a notable increase in other potential plant growth-promoting fungi in MS+CH when compared to MS.

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

Based on 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.

Supplementary Material:

Supplementary Material (PDF file)

Supplementary Table S4 (Microsoft Excel .xlsx file)

Acknowledgments: The authors would like to thank the greenhouse team of ILVO, Stijn Degroote (ILVO), Chris Van Vaes (ILVO) and Cécilia Lai (ZHAW) for their assistance in greenhouse and lab work, as well as the HPC team of the ZHAW for providing computational services.

Literature Cited

Abarenkov, K., Zirk, A., Piirmann, T., Pöhönen, R., Ivanov, F., Nilsson, R. H., and Kõljalg, U. 2021. UNITE general FASTA release for Fungi. UNITE Community.

Ahlmann-Eltze, C., and Patil, I. 2021. ggsignif: R package for displaying significance brackets for 'ggplot2'. PsyArxiv. https://doi.org/10.31234/osf.io/7awm6.

Arn, F., Frasson, D., Kroslakova, I., Rezzonico, F., Pothier, J. F., Riedl, R., and Sievers, M. 2020. Isolation and identification of *Actinomycetes* strains from Switzerland and their biotechnological potential. Chimia:382-390. https://doi.org/10.2533/chimia.2020.382.

Baron, N. C., Souza Pollo, A. de, and Rigobelo, E. C. 2020. *Purpureocillium lilacinum* and *Metarhizium marquandii* as plant growth-promoting fungi. PeerJ:e9005. https://doi.org/10.7717/peerj.9005.

Beck, D., and Foster, J. A. 2014. Machine learning techniques accurately classify microbial communities by bacterial vaginosis characteristics. PLOS ONE:e87830. https://doi.org/10.1371/journal.pone.0087830.

Benny, G. L., Ho, H.-M., Lazarus, K. L., and Smith, M. E. 2016. Five new species of the obligate mycoparasite *Syncephalis* (*Zoopagales*, *Zoopagomycotina*) from soil. Mycologia:1114-1129. https://doi.org/10.3852/15-073.

Bray, J. R., and Curtis, J. T. 1957. An ordination of the upland forest communities of Southern Wisconsin. Ecol. Monogr.:325-349. https://doi.org/10.2307/1942268.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods.:581-583. https://doi.org/10.1038/nmeth.3869.

Chen, J., Lü, S., Zhang, Z., Zhao, X., Li, X., Ning, P., and Liu, M. 2018. Environmentally friendly fertilizers: A review of materials used and their effects on the environment. Sci. Total Environ.:829-839. https://doi.org/10.1016/j.scitotenv.2017.09.186.

De Tender, C., Mesuere, B., van der Jeugt, F., Haegeman, A., Ruttink, T., Vandecasteele, B., Dawyndt, P., Debode, J., and Kuramae, E. E. 2019. Peat substrate amended with chitin modulates the N-cycle, siderophore and chitinase responses in the lettuce rhizobiome. Sci. Rep.:9890. https://doi.org/10.1038/s41598-019-46106-x.

De Tender, C., Vandecasteele, M., Ommeslag, S., Zutter, N. de, Vandenbussche, E., Haegeman, A., Audenaert, K., Li, L., Vandecasteele, B., Voorthuijzen, F., Maenhout, K., Beirinckx, S., Barneveldt, R., Goormachtig, S., and Debode, J. 2023. *Linnemannia elongata* a key species in chitin-based plant growth promotion. Phytobiomes J. https://doi.org/10.1094/PBIOMES-05-23-0031-R.

Debode, J., De Tender, C., Soltaninejad, S., van Malderghem, C., Haegeman, A., van der Linden, I., Cottyn, B., Heyndrickx, M., and Maes, M. 2016. Chitin mixed in potting soil alters lettuce growth, the survival of zoonotic bacteria on the leaves and associated rhizosphere microbiology. Front. Microbiol.:565. https://doi.org/10.3389/fmicb.2016.00565.

Dully, V., Wilding, T. A., Mühlhaus, T., and Stoeck, T. 2021. Identifying the minimum amplicon sequence depth to adequately predict classes in eDNA-based marine biomonitoring using supervised machine learning. Comput. Struct. Biotechnol. J.:2256-2268. https://doi.org/10.1016/j.csbj.2021.04.005.

Egusa, M., Matsui, H., Urakami, T., Okuda, S., Ifuku, S., Nakagami, H., and Kaminaka, H. 2015. Chitin nanofiber elucidates the elicitor activity of polymeric chitin in plants. Front. Plant Sci.:1098. https://doi.org/10.3389/fpls.2015.01098.

Elshafie, H. S., and Camele, I. 2022. Rhizospheric *Actinomycetes* revealed antifungal and plant-growth-promoting activities under controlled environment. Plants:1872. https://doi.org/10.3390/plants11141872.

Elsherbiny, E. A., Taher, M. A., Abd El-Aziz, M. H., and Mohamed, S. Y. 2021. Action mechanisms and biocontrol of *Purpureocillium lilacinum* against green mould caused by *Penicillium digitatum* in orange fruit. J. Appl. Microbiol.:1378-1390. https://doi.org/10.1111/jam.15016.

Enany, S. 2018. Basic Biology and Applications of Actinobacteria. IntechOpen, London.

FAOSTAT. 2021. Food and Agriculture Organization of the United Nations (FAO): Crops and livestock products. Accessed Oct 25, 2023. https://www.fao.org/faostat/en/#data/QCL.

Freeman, S., Minz, D., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., Nitzani, Y., Kirshner, B., Rav-David, D., Bilu, A., Dag, A., Shafir, S., and Elad, Y. 2004. *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. Eur. J. Plant Pathol.:361-370. https://doi.org/10.1023/B:EJPP.0000021057.93305.d9.

Geng, Y., Cao, G., Wang, L., and Wang, S. 2019. Effects of equal chemical fertilizer substitutions with organic manure on yield, dry matter, and nitrogen uptake of spring maize and soil nitrogen distribution. PLOS ONE:e0219512. https://doi.org/10.1371/journal.pone.0219512.

Girardi, N. S., Sosa, A. L., Etcheverry, M. G., and Passone, M. A. 2022. In vitro characterization bioassays of the nematophagous fungus *Purpureocillium lilacinum*: Evaluation on growth, extracellular enzymes, mycotoxins and survival in the surrounding agroecosystem of tomato. Fungal Biol.:300-307. https://doi.org/10.1016/j.funbio.2022.02.001.

Hartmann, A., Schmid, M., van Tuinen, D., and Berg, G. 2009. Plant-driven selection of microbes. Plant Soil:235-257. https://doi.org/10.1007/s11104-008-9814-y.

Illumina. 16S Sample Preparation Guide: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System.

Illumina. Fungal sequencing and classification with the ITS Metagenomics Protocol App Note.

Johnson, J. M., Ludwig, A., Furch, A. C. U., Mithöfer, A., Scholz, S., Reichelt, M., and Oelmüller, R. 2019. The beneficial root-colonizing fungus *Mortierella hyalina* promotes the aerial growth of

Arabidopsis and activates calcium-dependent responses that restrict *Alternaria brassicae*-induced disease development in roots. Mol. Plant Microbe Interact.:351-363. https://doi.org/10.1094/MPMI-05-18-0115-R.

Joos, L., De Tender, C., Holderbeke, A., Clement, L., Vandecasteele, B., and Debode, J. 2023. Exploring the microbial response as a potential bio-indicator for soil health: Insights from a controlled incubator experiment. Agric. Ecosyst. Environ.:108634. https://doi.org/10.1016/j.agee.2023.108634.

Kandel, S. L., Anchieta, A. G., Shi, A., Mou, B., and Klosterman, S. J. 2022. Crustacean meal elicits expression of growth and defense-related genes in roots of lettuce and tomato. PhytoFrontiers:10-20. https://doi.org/10.1094/PHYTOFR-03-21-0017-R.

Khan, M., and Tanaka, K. 2023. *Purpureocillium lilacinum* for plant growth promotion and biocontrol against root-knot nematodes infecting eggplant. PLOS ONE:e0283550. https://doi.org/10.1371/journal.pone.0283550.

Kim, K.-H., Kabir, E., and Jahan, S. A. 2017. Exposure to pesticides and the associated human health effects. Sci. Total Environ.:525-535. https://doi.org/10.1016/j.scitotenv.2016.09.009.

Kim, M. J., Moon, Y., Tou, J. C., Mou, B., and Waterland, N. L. 2016. Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). J. Food Compos. Anal.:19-34. https://doi.org/10.1016/j.jfca.2016.03.004.

Kips, L., Paepe, D. de, van Meulebroek, L., van Poucke, C., Larbat, R., Bernaert, N., van Pamel, E., Loose, M. de, Raes, K., and van Droogenbroeck, B. 2017. A novel spiral-filter press for tomato processing: process impact on phenolic compounds, carotenoids and ascorbic acid content. J. Food Eng.:27-37. https://doi.org/10.1016/j.jfoodeng.2017.06.010.

Kruskal, W. H., and Wallis, W. A. 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc.:583-621. https://doi.org/10.1080/01621459.1952.10483441.

Kumar, M., Brar, A., Vivekanand, V., and Pareek, N. 2017. Production of chitinase from thermophilic *Humicola grisea* and its application in production of bioactive chitooligosaccharides. Int. J. Biol. Macromol.:1641-1647. https://doi.org/10.1016/j.ijbiomac.2017.04.100.

Kumar, S. 2012. Biopesticides: A need for food and environmental safety. J. Fertil. Pestic. https://doi.org/10.4172/2155-6202.1000e107.

Lan, X., Zhang, J., Zong, Z., Ma, Q., and Wang, Y. 2017. Evaluation of the Biocontrol Potential of *Purpureocillium lilacinum* QLP12 against *Verticillium dahliae* in Eggplant. Biomed. Res. Int.:4101357. https://doi.org/10.1155/2017/4101357.

Law, J. W.-F., Ser, H.-L., Khan, T. M., Chuah, L.-H., Pusparajah, P., Chan, K.-G., Goh, B.-H., and Lee, L.-H. 2017. The potential of *Streptomyces* as biocontrol agents against the rice blast fungus, *Magnaporthe oryzae* (*Pyricularia oryzae*). Front. Microbiol.:3. https://doi.org/10.3389/fmicb.2017.00003.

Li, F., Chen, L., Redmile-Gordon, M., Zhang, J., Zhang, C., Ning, Q., and Li, W. 2018. *Mortierella elongata* 's roles in organic agriculture and crop growth promotion in a mineral soil. Land Degrad. Dev.:1642-1651. https://doi.org/10.1002/ldr.2965.

Li, L., Kaufmann, M., Makechemu, M., van Poucke, C., Keyser, E. de, Uyttendaele, M., Zipfel, C., Cottyn, B., and Pothier, J. F. 2023. Assessment of transcriptional reprogramming of lettuce roots in response to chitin soil amendment. Front. Plant Sci.:1158068. https://doi.org/10.3389/fpls.2023.1158068.

Liu, C., Cui, Y., Li, X., and Yao, M. 2021. microeco: an R package for data mining in microbial community ecology. FEMS Microbiol. Ecol. https://doi.org/10.1093/femsec/fiaa255.

Mahadevan, B., and Crawford, D. L. 1997. Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108. Enzyme Microb. Technol.:489-493. https://doi.org/10.1016/S0141-0229(96)00175-5.

Mahmood, I., Imadi, S. R., Shazadi, K., Gul, A., and Hakeem, K. R. 2016. Effects of pesticides on environment. Pages 253–269 in: Plant, Soil and Microbes: Volume 1: Implications in Crop Science. K. R. Hakeem, ed. Springer International Publishing AG, Cham.

McMurdie, P. J., and Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE:e61217. https://doi.org/10.1371/journal.pone.0061217.

Moussian, B. 2019. Chitin: Structure, chemistry and biology. Pages 5–18 in: Targeting Chitin-containing Organisms. Springer, Singapore.

Nampoothiri, K.M., Baiju, T.V., Sandhya, C., Sabu, A., Szakacs, G., and Pandey, A. 2004. Process optimization for antifungal chitinase production by *Trichoderma harzianum*. Process Biochem.:1583-1590. https://doi.org/10.1016/S0032-9592(03)00282-6.

Narayana, K. J. P., and Vijayalakshmi, M. 2009. Chitinase production by *Streptomyces sp*. ANU 6277. Braz. J. Microbiol.:725-733. https://doi.org/10.1590/s1517-83822009000400002.

Ozimek, E., and Hanaka, A. 2021. *Mortierella s*pecies as the plant growth-promoting fungi present in the agricultural soils. Agriculture:7. https://doi.org/10.3390/agriculture11010007.

Pusztahelyi, T. 2018. Chitin and chitin-related compounds in plant-fungal interactions. Mycology:189-201. https://doi.org/10.1080/21501203.2018.1473299.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res.:D590-6. https://doi.org/10.1093/nar/gks1219.

R Core Team. 2022. R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria.

Radhakrishnan, R., Khan, A. L., Kang, S. M., and Lee, I.-J. 2015. A comparative study of phosphate solubilization and the host plant growth promotion ability of *Fusarium verticillioides* RK01 and

Humicola sp. KNU01 under salt stress. Ann. Microbiol.:585-593. https://doi.org/10.1007/s13213-014-0894-z.

Randall, T. E., Fernandez-Bayo, J. D., Harrold, D. R., Achmon, Y., Hestmark, K. V., Gordon, T. R., Stapleton, J. J., Simmons, C. W., and VanderGheynst, J. S. 2020. Changes of *Fusarium oxysporum f.sp. lactucae* levels and soil microbial community during soil biosolarization using chitin as soil amendment. PLOS ONE:e0232662. https://doi.org/10.1371/journal.pone.0232662.

Rstudio Team. 2020. Rstudio: Integrated Development Environment for R. Rstudio, PBC.

Sabaratnam, S., and Traquair, J. A. 2002. Formulation of a *Streptomyces* biocontrol agent for the suppression of *Rhizoctonia* damping-off in tomato transplants. Biol. Control:245-253. https://doi.org/10.1006/bcon.2001.1014.

Sasada, R., Weinstein, M., Prem, A., Jin, M., and Bhasin, J. 2020. FIGARO: An efficient and objective tool for optimizing microbiome rRNA gene trimming parameters. J. Biomol. Tech.:S2.

Sato, K., and Takahashi, K. 1983. An Analysis of the Border Effect in the Rice Paddy Fields. Jpn. J. Crop Sci.:168-176. https://doi.org/10.1626/jcs.52.168.

Schrempf, H. 2001. Recognition and degradation of chitin by *Streptomycetes*. Antonie Van Leeuwenhoek:285-289. https://doi.org/10.1023/A:1012058205158.

Seidl, V., Huemer, B., Seiboth, B., and Kubicek, C. P. 2005. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. FEBS J.:5923-5939. https://doi.org/10.1111/j.1742-4658.2005.04994.x.

Shamshina, J. L., Kelly, A., Oldham, T., and Rogers, R. D. 2020. Agricultural uses of chitin polymers. Environ. Chem. Lett.:53-60. https://doi.org/10.1007/s10311-019-00934-5.

Shannon, C. E. 1948. A mathematical theory of communication. Bell Syst. tech. j.:379-423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x.

Sharma, N., and Singhvi, R. 2017. Effects of chemical fertilizers and pesticides on human health and environment: A review. Int. J. Agric. Biotechnol.:675. https://doi.org/10.5958/2230-732X.2017.00083.3.

Sharma, S., Kumar, S., Khajuria, A., Ohri, P., Kaur, R., and Kaur, R. 2020. Biocontrol potential of chitinases produced by newly isolated *Chitinophaga* sp. S167. World J. Microbiol. Biotechnol.:90. https://doi.org/10.1007/s11274-020-02864-9.

Sharp, R. 2013. A review of the applications of chitin and its derivatives in agriculture to modify plant-microbial interactions and improve crop yields. Agronomy:757-793. https://doi.org/10.3390/agronomy3040757.

Sood, M., Kapoor, D., Kumar, V., Sheteiwy, M. S., Ramakrishnan, M., Landi, M., Araniti, F., and Sharma, A. 2020. *Trichoderma*: The "secrets" of a multitalented biocontrol agent. Plants. https://doi.org/10.3390/plants9060762.

Subbarao, K. V. 2017. Compendium of lettuce diseases and pests. Second edition. The disease compendium series of the American Phytopathological Society. APS Press, St. Paul, Minnesota.

Telagathoti, A., Probst, M., Mandolini, E., and Peintner, U. 2022. *Mortierellaceae* from subalpine and alpine habitats: new species of *Entomortierella*, *Linnemannia*, *Mortierella*, *Podila* and *Tyroliellagen*. nov. Fungal Divers:25-58. https://doi.org/10.3114/sim.2022.103.02.

Trejo-Estrada, S. R., Paszczynski, A., and Crawford, D. L. 1998. Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. J. Ind. Microbiol. Biotechnol.:81-90. https://doi.org/10.1038/sj.jim.2900549.

Vandecasteele, B., Amery, F., Ommeslag, S., Vanhoutte, K., Visser, R., Robbens, J., De Tender, C., and Debode, J. 2021. Chemically versus thermally processed brown shrimp shells or Chinese mitten crab as a source of chitin, nutrients or salts and as microbial stimulant in soilless strawberry cultivation. Sci. Total Environ.:145263. https://doi.org/10.1016/j.scitotenv.2021.145263.

Vurukonda, Sai Shiva Krishna Prasad, Giovanardi, D., and Stefani, E. 2018. Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. Int. J. Mol. Sci.:952. https://doi.org/10.3390/ijms19040952.

Yang, F., Tian, J., Fang, H., Gao, Y., Xu, M., Lou, Y., Zhou, B., and Kuzyakov, Y. 2019. Functional soil organic matter fractions, microbial community, and enzyme activities in a mollisol under 35 years manure and mineral fertilization. J. Soil Sci. Plant Nutr.:430-439. https://doi.org/10.1007/s42729-019-00047-6.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., Knight, R., and Gordon, J. I. 2012. Human gut microbiome viewed across age and geography. Nature:222-227. https://doi.org/10.1038/nature11053.

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., and Glöckner, F. O. 2014. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Res.:D643-8. https://doi.org/10.1093/nar/gkt1209.

Yurgel, S. N., Nadeem, M., and Cheema, M. 2022. Microbial consortium associated with crustacean shells composting. Microorganisms. https://doi.org/10.3390/microorganisms10051033.

Zitouni, M., Viens, P., Ghinet, M. G., and Brzezinski, R. 2017. Diversity of family GH46 chitosanases in *Kitasatospora setae* KM-6054. Appl. Microbiol. Biotechnol.:7877-7888. https://doi.org/10.1007/s00253-017-8517-9.

Analyte	Peat substrate*	Mineral soil
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 100g DM)	-	54.1 ± 1.7
K-AmLact (mg 100g DM)	-	9.5 ± 0.33
Bulk density (g cm^{-3})	0.099	1.2
Particle density (g cm^{-3})	1.55	2.65
Porosity (%)#	93.61	54.72

*The content of the peat substrate was described by the provider, # porosity was calculated using the formula Soil Porosity = (1 - (Bulk Density ÷ Particle Density)) × 100

Compound	Soil	Tissue	Mean untreated in mg kg^{-1}	Mean chitin treated in mg kg^{-1}	*p*-value
Caffeic acid	PS	Root	10.7 ± 2.5	6.9 ± 1.5	0.05
Apigetrin	PS	Leaf	0.026 ± 0.0032	0.019 ± 0.0026	0.02
Chicoric acid	PS	Leaf	9,500.1 ± 1025.4	7,231.3 ± 463.3	0.01
Chlorogenic acid	PS	Leaf	1155.0 ± 98.3	954.7 ± 34.2	0.02
Ferulic acid	PS	Leaf	0.15 ± 0.064	0.34 ± 0.12	0.05
Luteolin	PS	Leaf	0.095 ± 0.0046	0.077 ± 0.011	0.04
Quercetin-3-O-glucuronide	PS	Leaf	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	MS	Root	0.70 ± 0.31	1.3 ± 0.34	0.04
Quercetin-3-O-glucuronide	MS	Root	13.5 ± 5.9	28.1 ± 8.6	0.04
Astragalin	MS	Leaf	0.045 ± 0.0056	0.035 ± 0.0058	0.04
Rutin	MS	Leaf	4.97 ± 0.28	4.19 ± 0.33	0.01

Compound	Roots		Leaves	
	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	-	-

	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

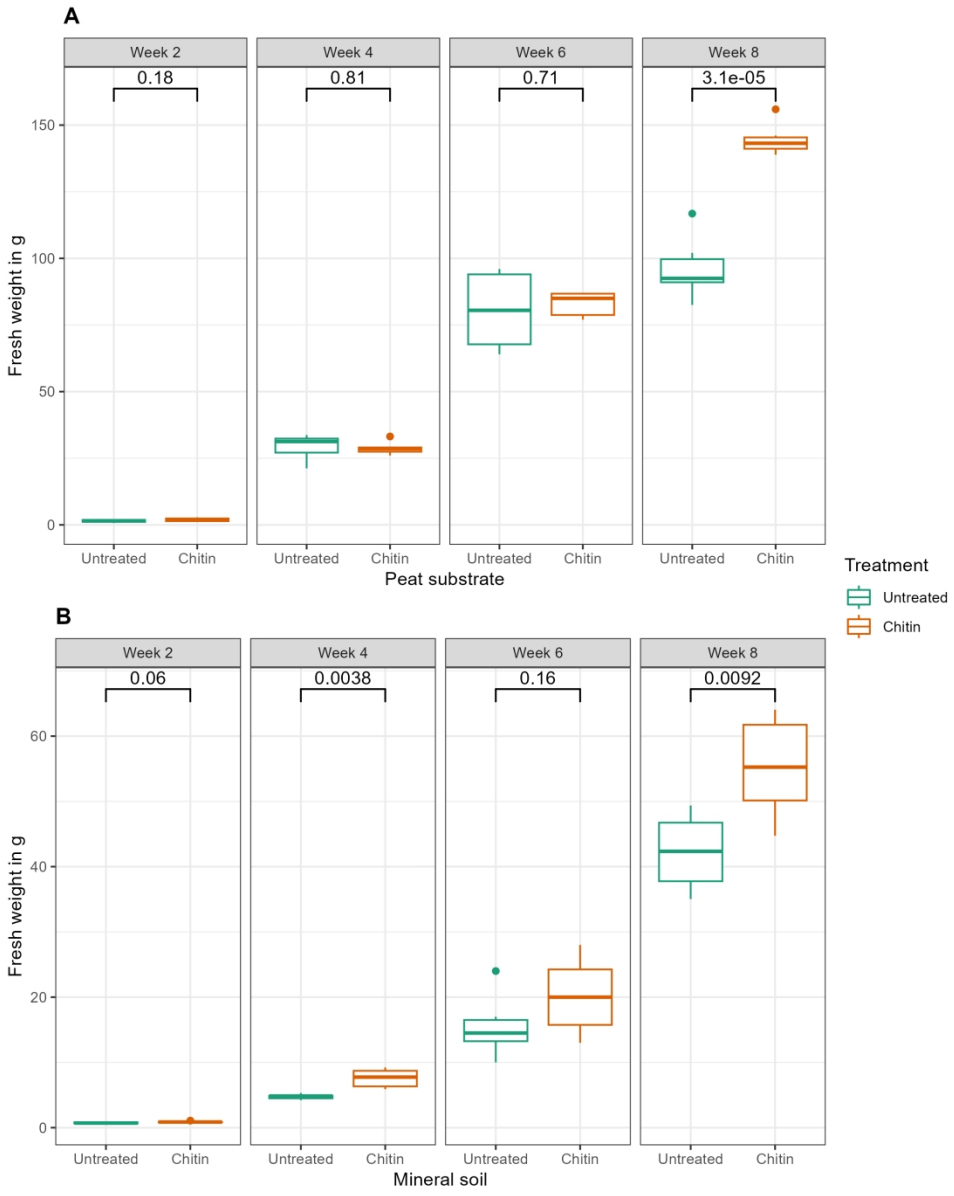


Fig. 1. A Lettuce fresh weight grown in peat substrate. **B** Lettuce fresh weight grown in mineral soil. The lettuces were grown for a period of eight weeks, with sampling each two weeks. Treatments are indicated by different colors with green for the untreated and orange for 2 g L^{-1} chitin addition. Fresh weight of all replicates per treatment and sampling point (n = 6) is indicated as a boxplot. The median weight is represented as the horizontal line in the boxplot. 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 timepoint.

199x249mm (300 x 300 DPI)

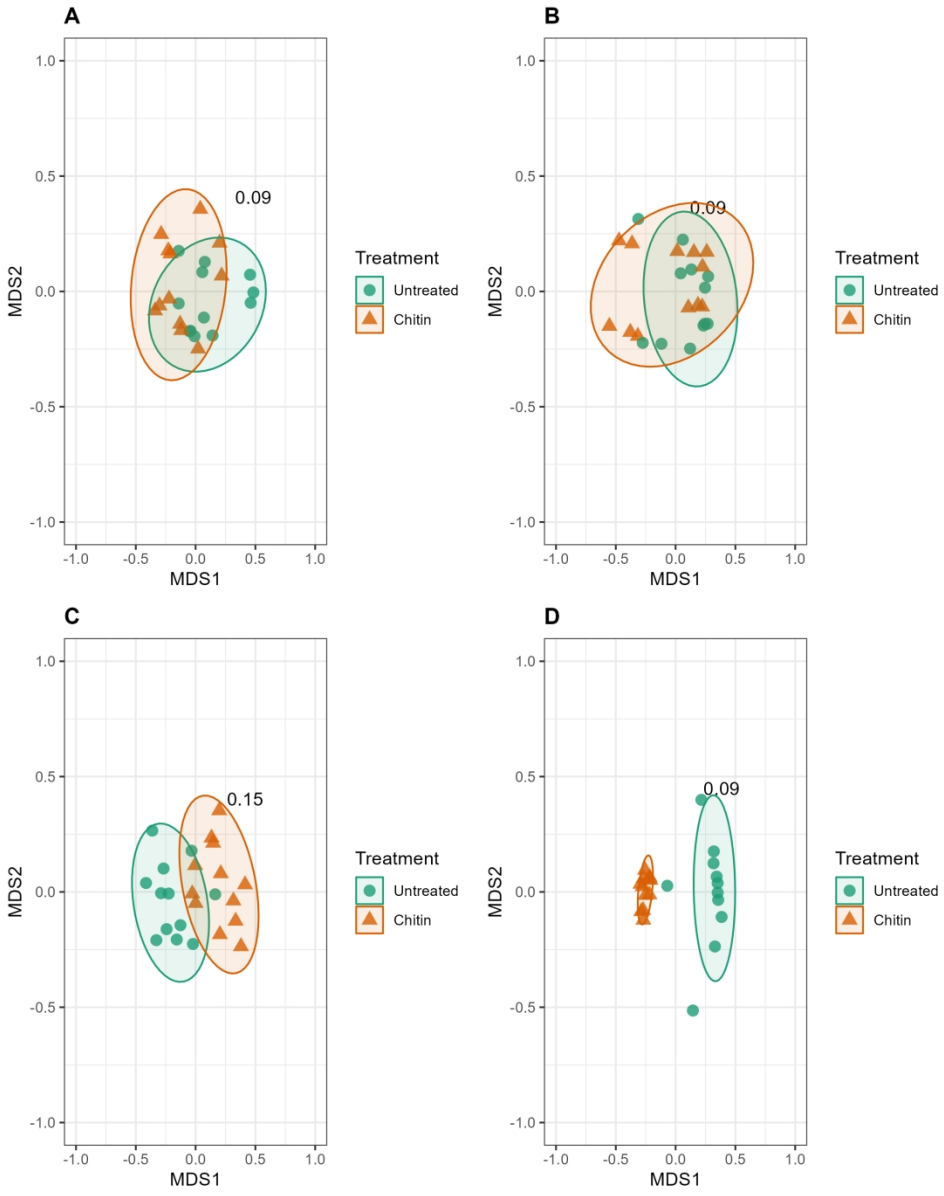


Fig. 2. MDS plot illustrating the β-diversity of the bacterial (**A**, **B**) and fungal (**C**, **D**) communities, based on the Bray-Curtis index, between two treatments (untreated (green, circle), chitin treated (orange, triangle)) in either peat substrate (**A**, **C**) or mineral soil (**B**, **D**).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

199x249mm (300 x 300 DPI)

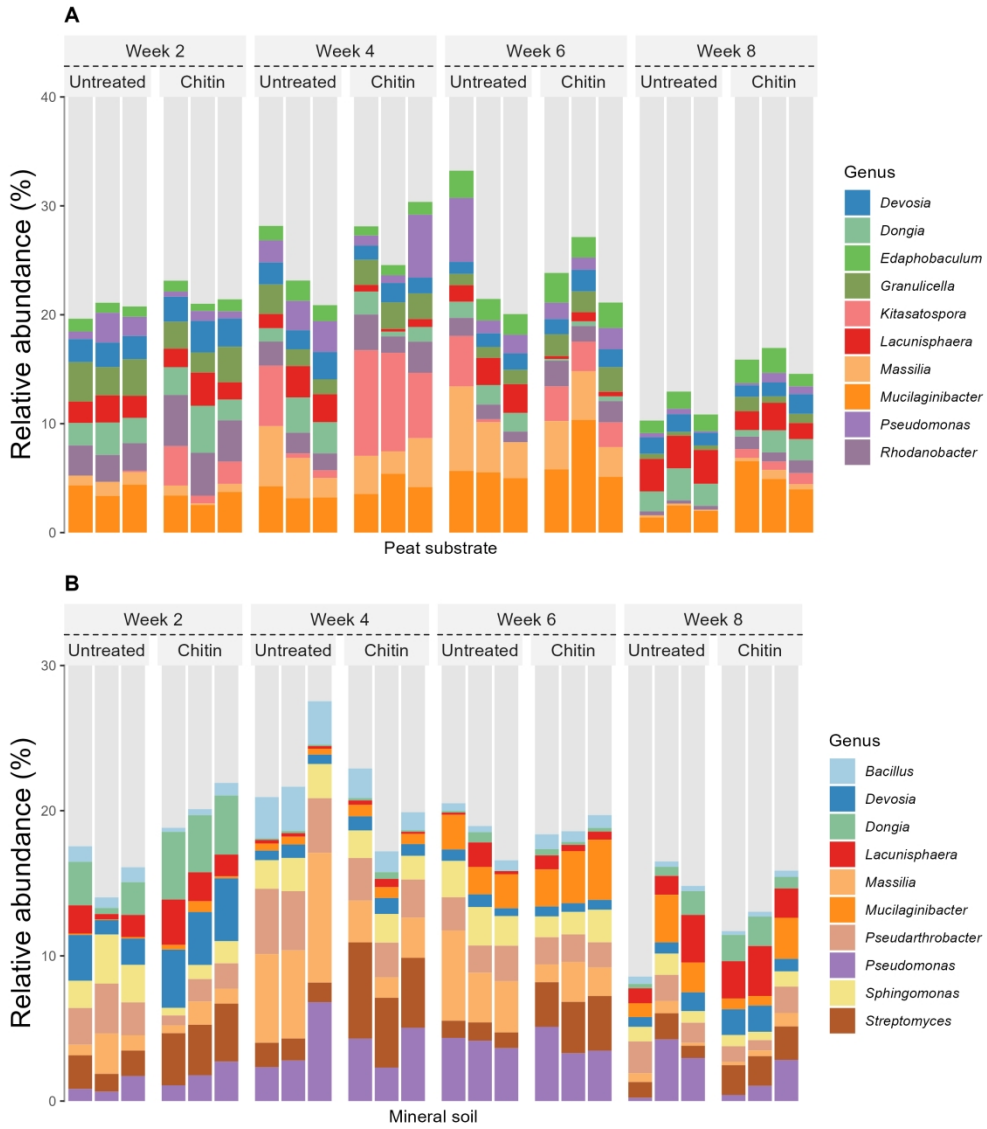


Fig. 3. A Relative abundance of the ten most abundant bacterial genera in the lettuce rhizosphere in peat substrate (PS) or **B** mineral soil (MS). The relative abundance is plotted over eight weeks and split between treatment groups (Untreated and Chitin). The colors of the bars correspond to the different genera in the legend of each panel. All bacteria not belonging to the top ten most relative abundant genera are shown in grey.

219x249mm (300 x 300 DPI)

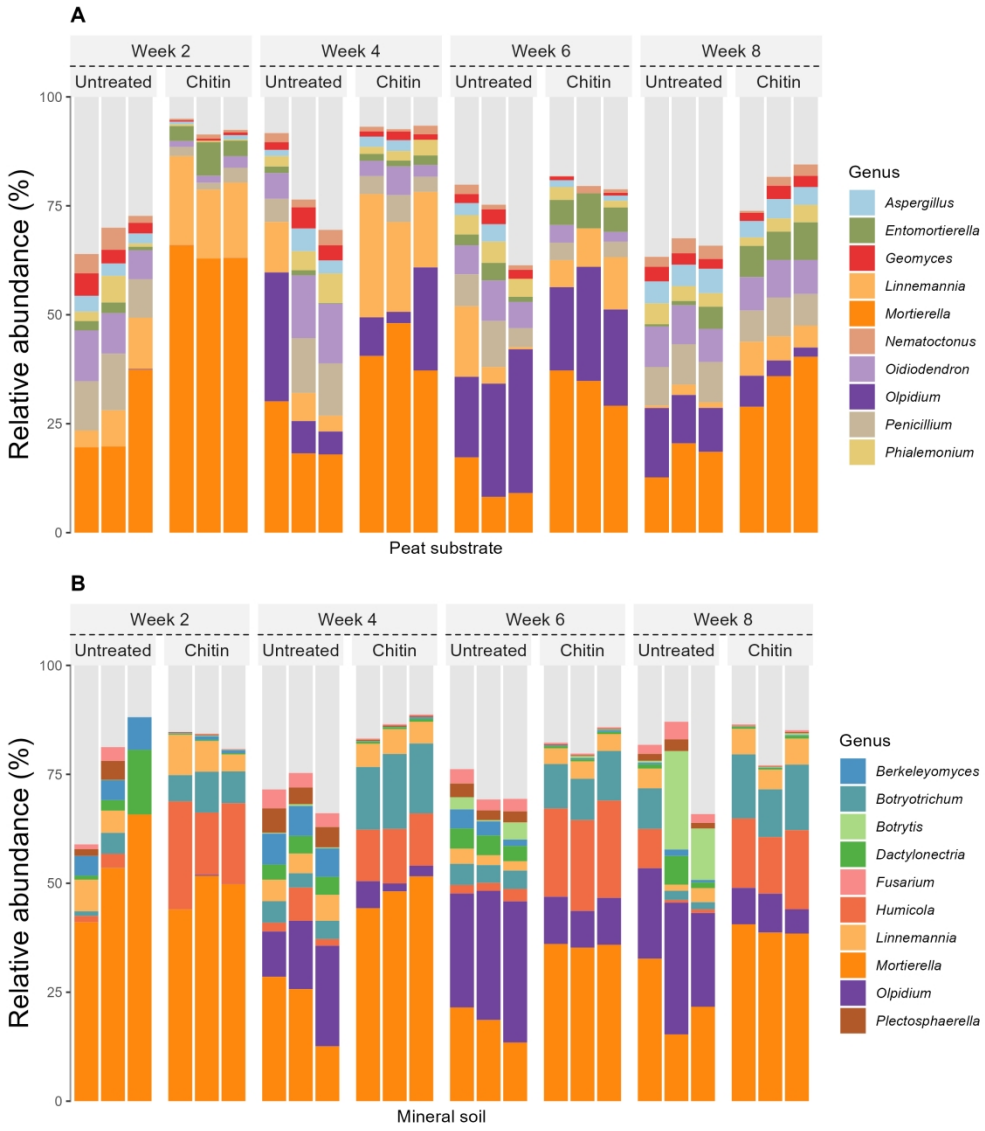


Fig. 4. A Relative abundance of the ten most abundant fungi in the lettuce rhizosphere in peat substrate or **B** mineral soil. The abundance is plotted over eight weeks and split between treatment groups (Chitin and Untreated). The colors of the bars correspond to the different genera in the legend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

219x249mm (300 x 300 DPI)

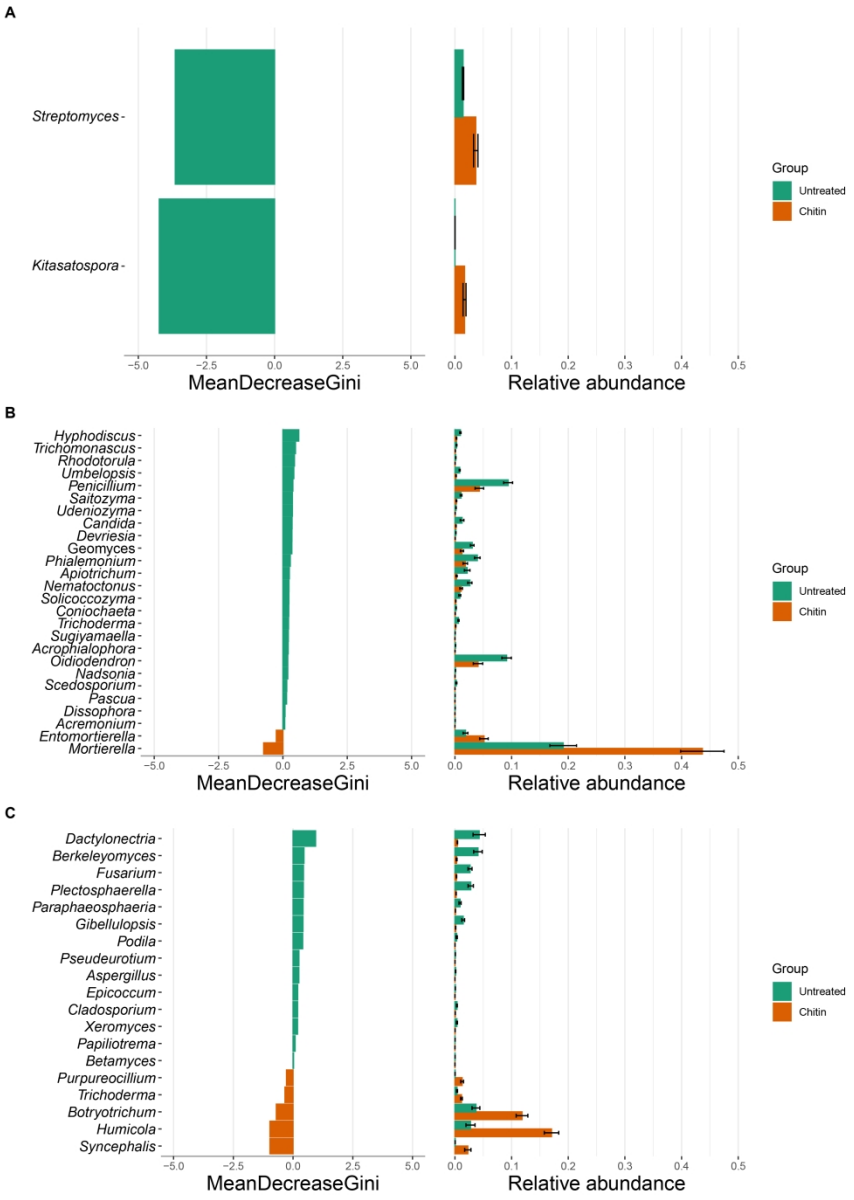


Fig. 5. A Differential abundance test between treatment groups of bacterial communities in mineral soil (MS) compared to mineral soil with chitin (MS+CH). **B** Differential abundance test between treatment groups of fungal communities in peat substrate (PS) compared to peat substrate with chitin (PS+CH) and **C** MS compared to MS+CH. Left bars show the Mean Decrease in Gini of a genus while the right bars show the mean corresponding abundance and the standard deviation of the treatment groups.

254x355mm (300 x 300 DPI)

Supplementary Material

belonging to

Soil type and associated microbiomes influences chitin's growth-promotion effect in lettuce

Moritz Kaufmann,[1,2†] Leilei Li,[3†] Christof Van Poucke,[4] Nicola Rhyner,[1] Caroline De Tender,[5] Mieke Uyttendaele,[6] Marc Heyndrickx,[4,8] Cyril Zipfel,[2,7] Joël F. Pothier,[1] and Bart Cottyn[3]

1 Supplementary Figures

Supplementary Figure S1. Schematic view of greenhouse experiment's pots layout.

Supplementary Figure S2. A Lettuce fresh weight grown in peat substrate (PS) or mineral soil (MS). **B** Lettuce fresh weight grown in peat substrate with chitin addition (PS+CH) or mineral soil with chitin addition (MS+CH).

Supplementary Figure S3. Principal component analysis plot of untargeted method on the LC-HRMS in both positive ionization (ESI pos) and negative ionization (ESI neg).

Supplementary Figure S4. A Boxplots comparing the α-diversity of the bacterial rhizosphere between peat substrate (PS) and chitin amended peat substrate (PS+CH) or **B** mineral soil (MS) and chitin amended mineral soil (MS+CH).

Supplementary Figure S5. A Boxplots comparing the α-diversity of the fungal rhizosphere between peat substrate (PS) and chitin amended peat substrate (PS+CH) or **B** mineral soil (MS) and chitin amended mineral soil (MS+CH).

Supplementary Figure S6. A Relative abundance of the ten most abundant bacteria genera in bulk soil of peat substrate (PS) and peat substrate with 2 g L^{-1} chitin addition (PS+CH) or **B** mineral soil (MS) or mineral soil with 2 g L^{-1} chitin addition (MS+CH).

Supplementary Figure S7. A Relative abundance of the ten most abundant fungal genera in bulk soil of peat substrate (PS) and peat substrate with 2 g L^{-1} chitin addition (PS+CH) or **B** mineral soil (MS) or mineral soil with 2 g L^{-1} chitin addition (MS+CH).

2 Supplementary Tables

Supplementary Table S1. Overview of the primers used for metabarcoding amplicon amplification.

Supplementary Table S2. Phenolic compounds (mg kg^{-1} ± sd%, n = 4) determined using LC-MS for non-chitin- and chitin-treated lettuce plants grown in peat substrate (PS, PS+CH) and mineral soil (GS, GS+CH), respectively.

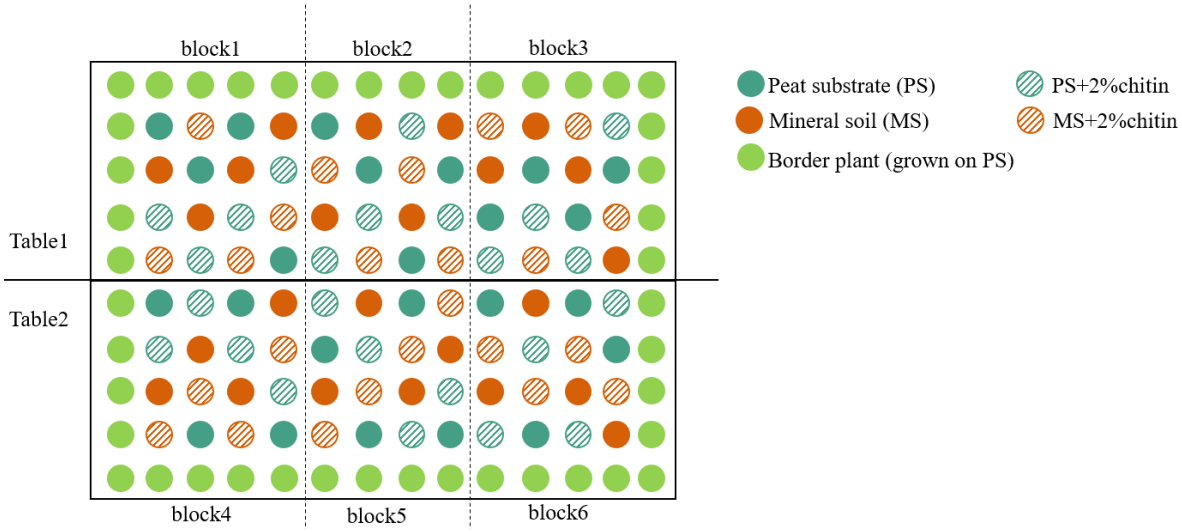
Supplementary Table S3. Comparison of statistical different metabolites (confidence interval 0.95) between lettuce plants grown in potting- and mineral soil with or without chitin treatment (PS, PS+CH) and (MS, MS+CH),

Supplementary Table S4. Separate Microsoft Excel sheet: potential identification of markers picked from comparison of differently treated samples.

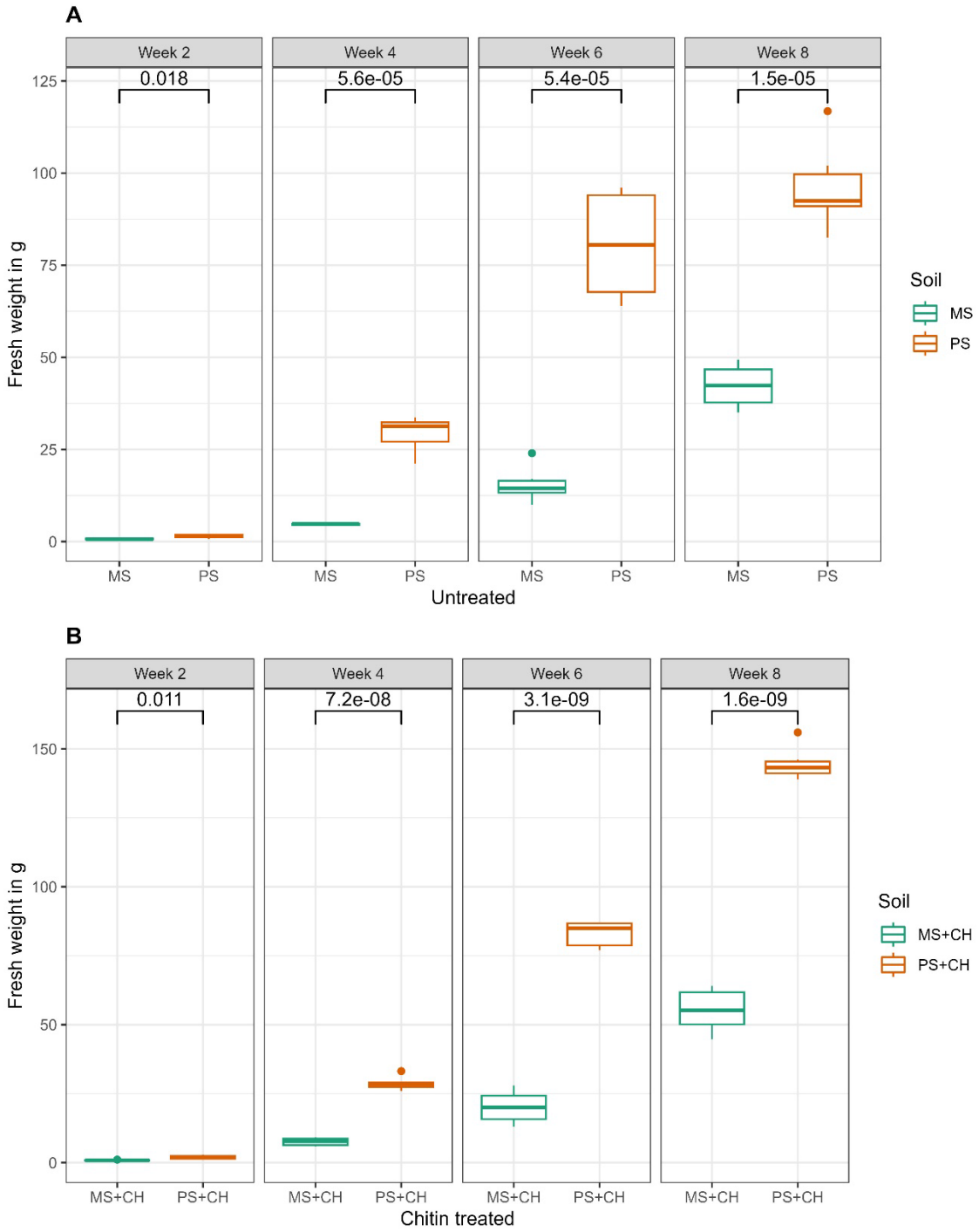
Supplementary Table S5. Bacteria with a significant difference in relative abundance ($p > 0.05$) from the random forest analysis in mineral soil (MS) compared to 2 g L^{-1} chitin amended mineral soil (MS+CH).

Supplementary Table S6. Fungi with a significant difference in relative abundance ($p > 0.05$) from the random forest analysis in mineral soil (PS) compared to 2 g L^{-1} chitin amended mineral soil (PS+CH).

Supplementary Table S7. Fungi with a significant difference in relative abundance ($p > 0.05$) from the random forest analysis in mineral soil (MS) compared to 2 g L^{-1} chitin amended mineral soil (MS+CH).

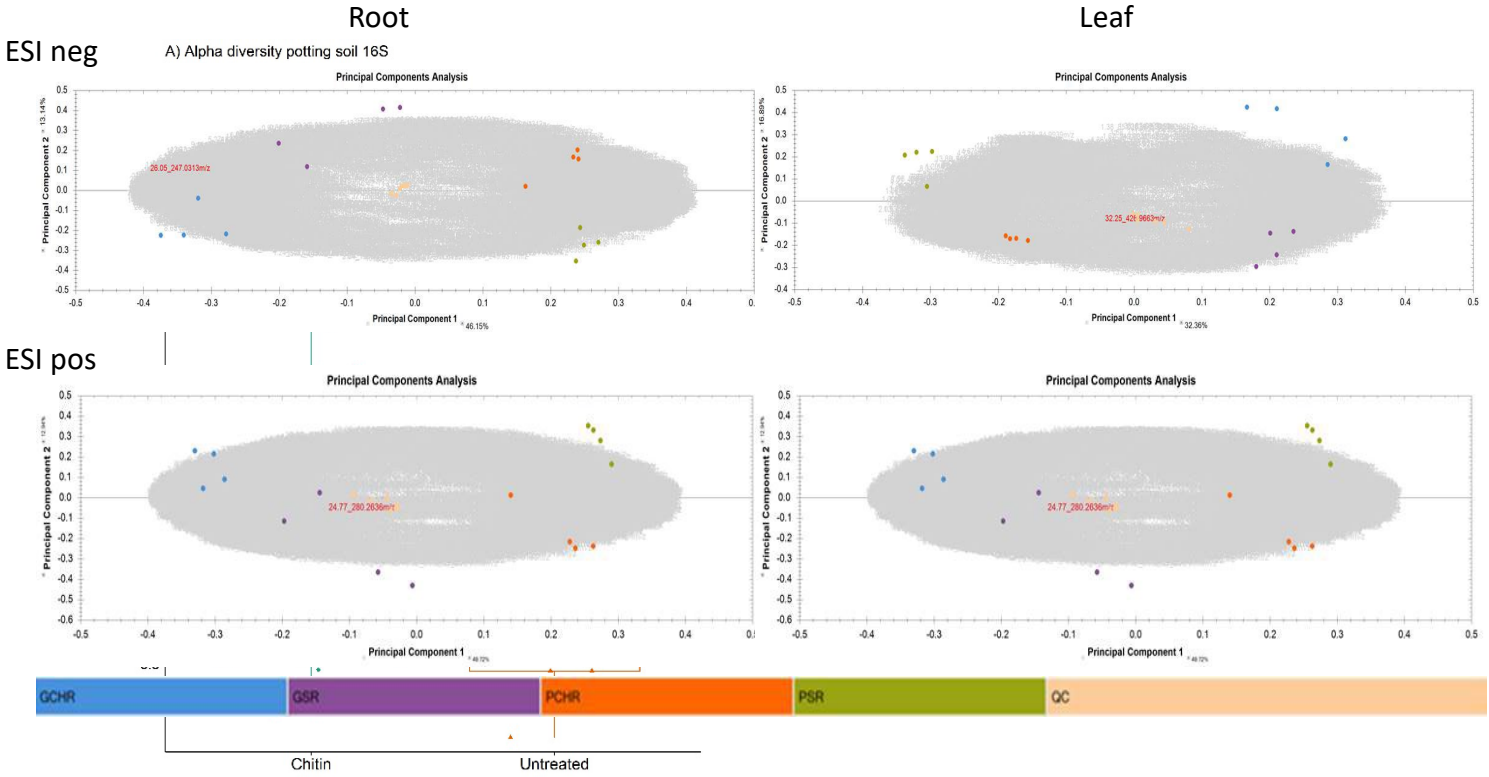


Supplementary Figure S1. Schematic view of greenhouse experiment's pots layout. Lettuce plants grown in four differently treated soils were placed semi-randomly on two greenhouse tables attached together and were surrounded with border plants. At each sampling point, one plant per treatment was randomly sampled from each of the six blocks.

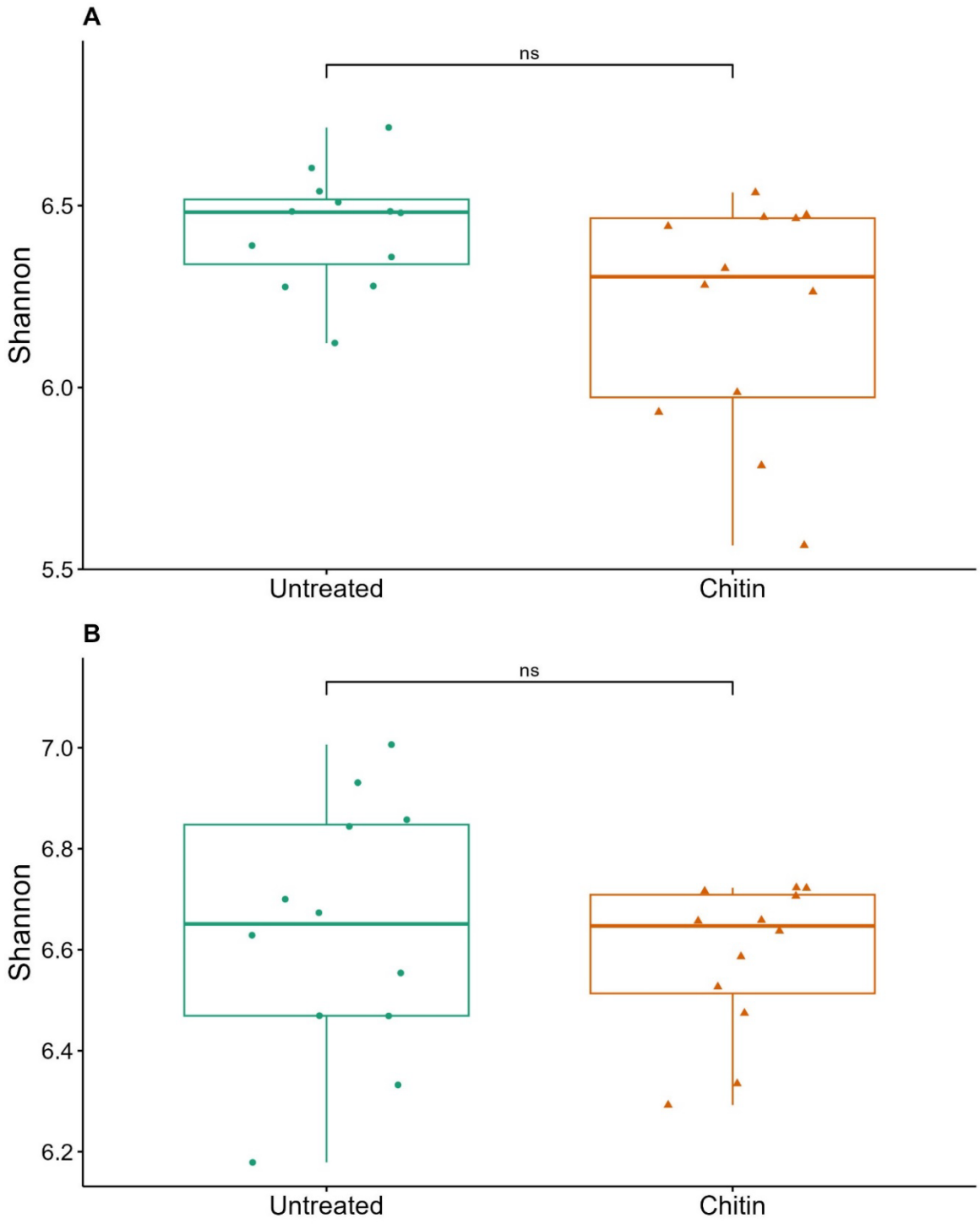


Supplementary Figure S2. **A** Lettuce fresh weight grown in peat substrate (PS) or mineral soil (MS). **B** Lettuce fresh weight grown in peat substrate with chitin addition (PS+CH) or mineral soil with chitin addition (MS+CH). The lettuces were grown for a period of eight weeks, with sampling each two weeks. Soil types are indicated by different colors with green for MS and MS+CH or orange for PS or PS+CH. Fresh weight of all replicates per treatment and sampling point ($n = 6$) is indicated as a boxplot. The median weight is represented as the horizontal line in the boxplot. Statistical inference is shown as a *p*-value (test: two-sided Student's *t*-test)

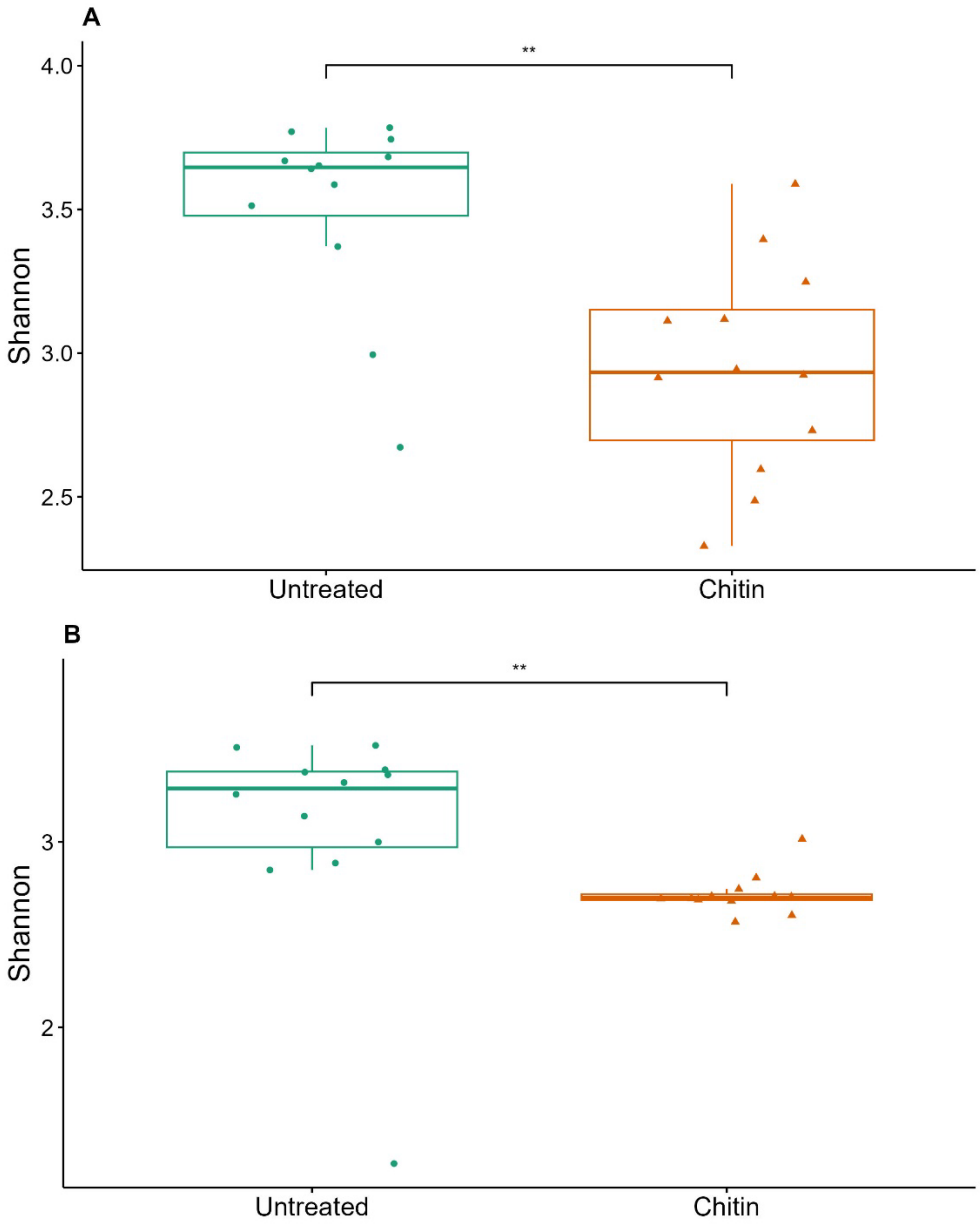
plotted over the corresponding groups, focusing on the effect of the soil type within a timepoint.



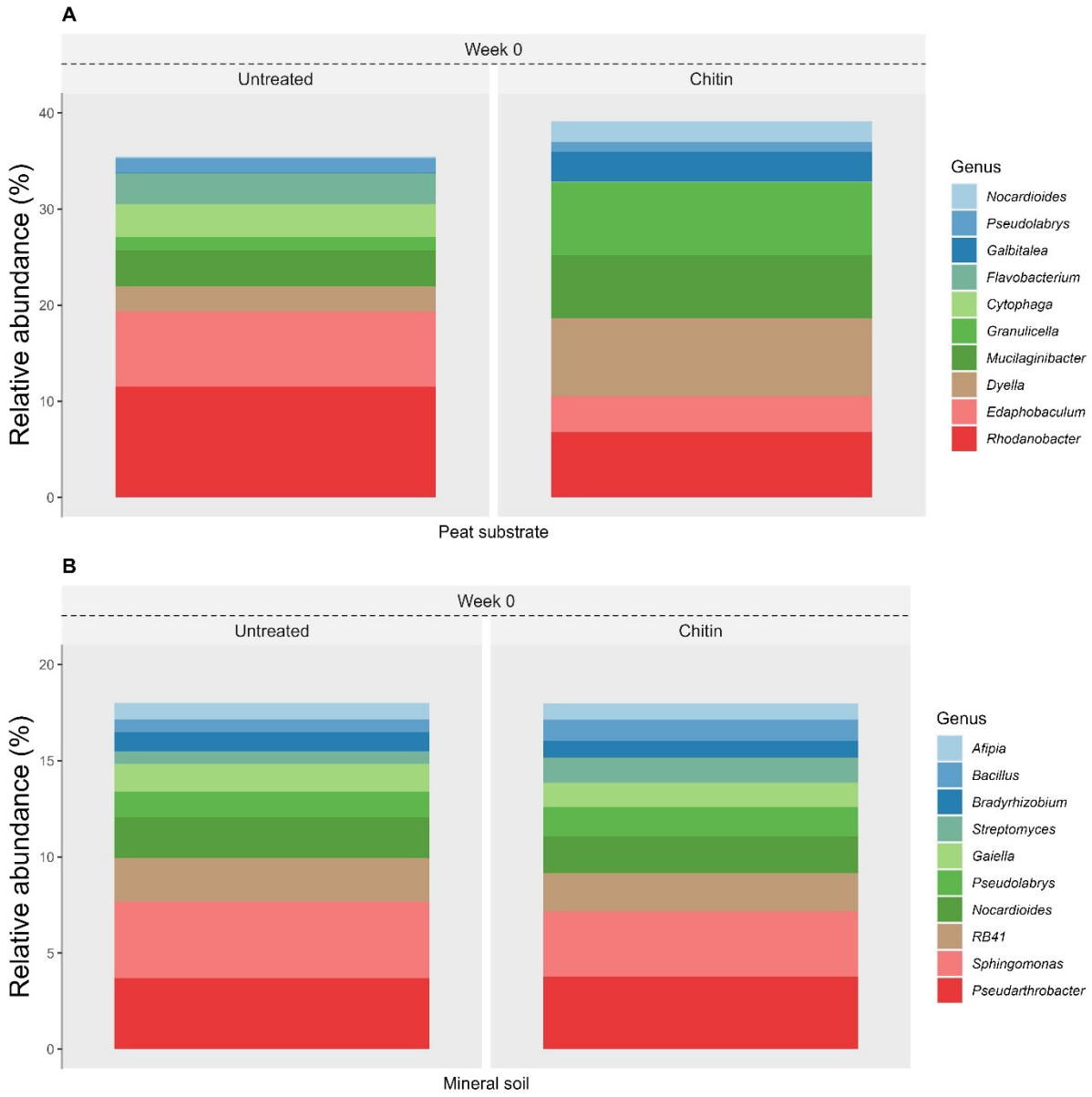
Supplementary Figure S3. Principal component analysis plot of untargeted method on the LC-HRMS in both positive ionization (ESI pos) and negative ionization (ESI neg). The grey cloud represents detected ions; the colored dots represent analyzed samples.



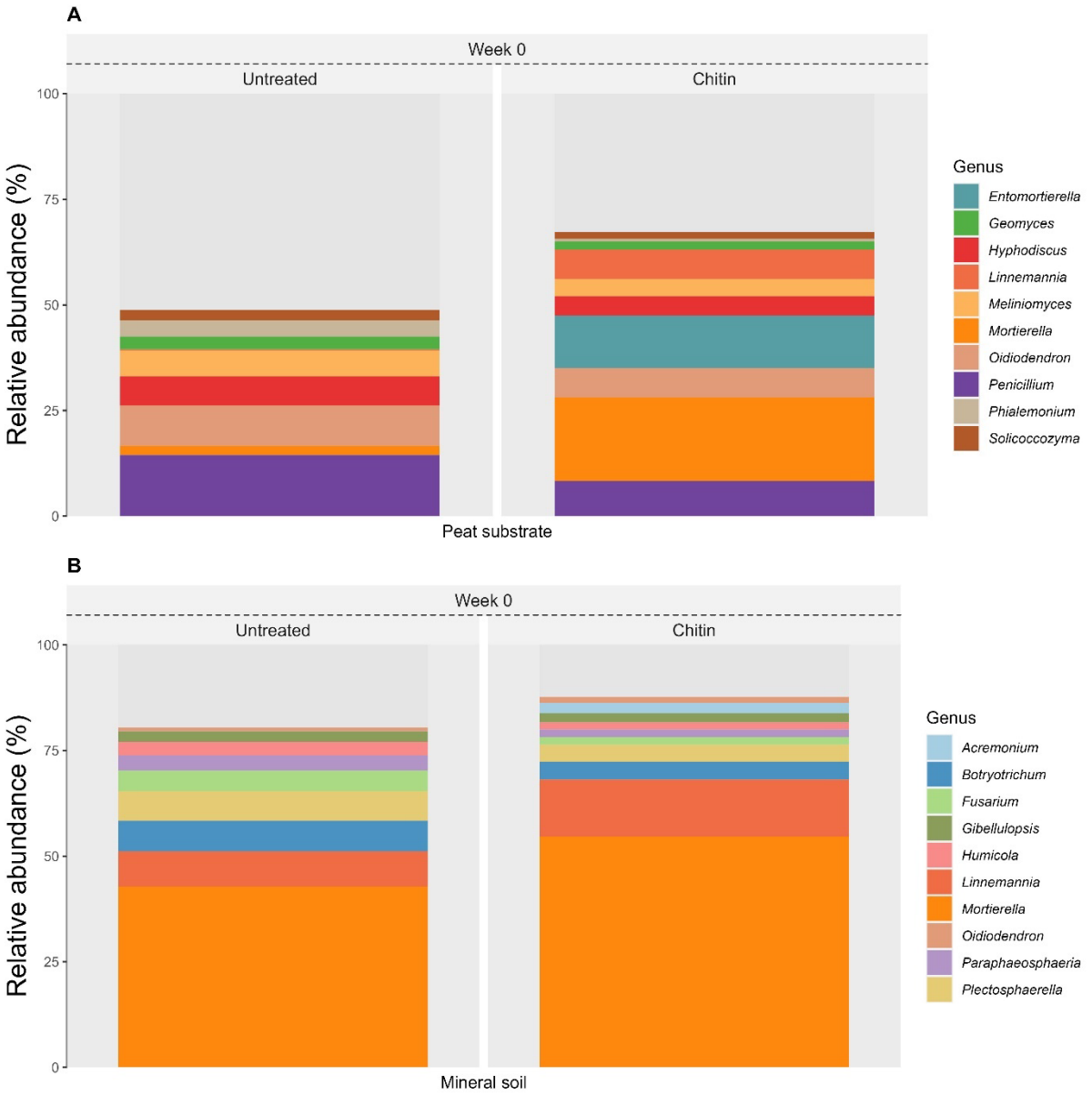
Supplementary Figure S4. **A** Boxplots comparing the α-diversity of the bacterial rhizosphere between peat substrate (PS) and chitin amended peat substrate (PS+CH) or **B** mineral soil (MS) and chitin amended mineral soil (MS+CH). Treatments are indicated by different colors with green for the untreated and orange for 2 g L^{-1} chitin addition. Shannon indices of all replicates per treatment (n = 12) is indicated as a boxplot. The median Shannon index is represented as the horizontal line in the boxplot. Statistical inference is shown as a p-value (test: Kruskal-Wallis test) plotted over the corresponding groups, focusing on the effect of chitin.



Supplementary Figure S5. A Boxplots comparing the α-diversity of the fungal rhizosphere between peat substrate (PS) and chitin amended peat substrate (PS+CH) or **B** mineral soil (MS) and chitin amended mineral soil (MS+CH). Treatments are indicated by different colors with green for the untreated and orange for 2 g L^{-1} chitin addition. Fresh weight of all replicates per treatment and sampling point (n = 12) is indicated as a boxplot. The median Shannon index is represented as the horizontal line in the boxplot. Statistical inference is shown as a p-value (test: Kruskal-Wallis test) plotted over the corresponding groups, focusing on the effect of chitin.



Supplementary Figure S6. A Relative abundance of the ten most abundant bacteria genera in bulk soil of peat substrate (PS) and peat substrate with 2 g L^{-1} chitin addition (PS+CH) or **B** mineral soil (MS) or mineral soil with 2 g L^{-1} chitin addition (MS+CH). The relative abundance is 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 ten most relative abundant genera are shown in grey.



Supplementary Figure S7. A Relative abundance of the ten most abundant fungal genera in bulk soil of peat substrate (PS) and peat substrate with 2 g L^{-1} chitin addition (PS+CH) or **B** mineral soil (MS) or mineral soil with 2 g L^{-1} chitin addition (MS+CH). The relative abundance is split between treatment groups (Untreated and Chitin). The colors of the bars correspond to the different genera in the legend of each panel. All fungi not belonging to the top ten most relative abundant genera are shown in grey.

Supplementary Table S1 Overview of the primers used for metabarcoding amplicon amplification.

Primer name	Sequence (5′-3′)
16S Fwd	CCTACGGGNGGCWGCAG
16S Rev	GACTACHVGGGTATCTAATCC
ITS Fwd 1	CTTGGTCATTTAGAGGAAGTAA
ITS Fwd 2	CTCGGTCATTTAGAGGAAGTAA
ITS Fwd 3	CTTGGTCATTTAGAGGAACTAA
ITS Fwd 4	CCCGGTCATTTAGAGGAAGTAA
ITS Fwd 5	CTAGGCTATTTAGAGGAAGTAA
ITS Fwd 6	CTTAGTTATTTAGAGGAAGTAA
ITS Fwd 7	CTACGTCATTTAGAGGAAGTAA
ITS Fwd 8	CTTGGTCATTTAGAGGTCGTAA
ITS Rev 1	GCTGCGTTCTTCATCGATGC
ITS Rev 2	GCTGCGTTCTTCATCGATGG
ITS Rev 3	GCTACGTTCTTCATCGATGC
ITS Rev 4	GCTGCGTTCTTCATCGATGT
ITS Rev 5	ACTGTGTTCTTCATCGATGT
ITS Rev 6	GCTGCGTTCTTCATCGTTGC
ITS Rev 7	GCGTTCTTCATCGATGC

Supplementary Table S2. Phenolic compounds (mg kg^{-1} ± sd%, n = 4) determined using LC-MS for non-chitin- and chitin-treated lettuce plants grown in peat substrate (PS, PS+CH) and mineral soil (GS, GS+CH), respectively. "-", not detected.

	Phenolic compounds	Leaf		Root		Leaf		Root	
		PS	PS+CH	PS	PS+CH	GS	GS+CH	GS	GS+CH
Phenolic acid	Salicylic acid	0.22±15.83%	0.25±10.59%	1.13±19.04%	1.31±13.90%	0.49±28.48%	0.49±15.54%	3.74±22.23%	5.58±19.55%
	4-OH-phenylacetic acid	6.86±9.65%	7.43±19.59%	21.71±21.10%	32.12±33.84%	6.41±14.58%	6.46±23.78%	16.53±12.18%	22.75±18.59%
	p-Coumaric acid	0.06±17.27%	0.08±17.20%	0.18±25.35%	0.14±8.29%	0.12±37.12%	0.12±27.65%	0.37±18.94%	0.31±8.30%
	Vanillic acid	-	1.16±19.66%	-	-	-	-	-	-
	Caffeic acid	33.79±24.66%	51.63±20.80%	10.74±20.03%	6.88±18.58%	55.58±32.31%	61.44±29.67%	15.34±23.78%	22.84±7.58%
	Quinic acid	102.65±6.65%	95.49±3.05%	810.31±17.13%	813.23±16.03%	109.76±6.40%	122.21±4.00%	806.13±22.91%	676.51±14.57%
	Ferulic acid	0.15±35.93%	0.34±31.13%	0.26±19.09%	0.24±12.31%	0.34±41.17%	0.39±31.00%	0.39±17.97%	0.43±4.78%
	Sinapinic acid	0.07±10.69%	0.09±14.63%	0.10±15.85%	0.10±8.97%	0.10±19.46%	0.08±19.50%	0.17±14.83%	0.13±9.11%
	Clorogenic acid	1154.96±7.37%	954.68±3.10%	2006.78±10.09%	1615.44±22.63%	1279.74±7.85%	1305.93±3.48%	4565.80±19.71%	5691.02±2.01%
	Chicoric acid	9500.06±9.35%	7231.26±5.55%	11274.32±12.81%	8947.16±11.16%	7064.06±10.53%	7887.38±8.78%	11448.65±19.10%	15034.81±2.37%
Flavonoids	Apigenin	-	-	0.01±55.58%	0.01±56.70%	-	-	0.01±44.02%	0.01±53.03%
	Daidzin	3.82±3.93%	3.78±0.88%	1.55±5.26%	1.53±4.87%	3.84±2.51%	3.94±1.22%	1.37±8.73%	1.45±3.06%
	Luteolin	0.10±4.16%	0.08±12.33%	-	-	0.07±4.88%	0.07±25.22%	-	-
	Quercetin	0.04±17.31%	0.06±23.64%	-	-	0.04±34.92%	0.04±30.76%	-	-
	Apigetrin	0.03±10.82%	0.02±11.97%	-	-	0.03±18.23%	0.03±19.57%	-	-
	Phloridzin	0.00±22.37%	0.002±40.39%	-	-	0.002±68.07%	0.002±89.36%	-	-
	Cynaroside	2.53±10.01%	2.18±7.53%	-	-	2.52±3.30%	2.32±5.17%	-	-
	Astragalin	0.04±10.59%	0.04±15.77%	-	-	0.05±10.75%	0.04±14.38%	-	-
	Isoquercetin	1.51±4.63%	1.30±14.09%	0.60±22.73%	0.66±22.10%	1.29±12.80%	1.42±16.27%	0.70±38.23%	1.30±22.40%
	Quercetin-3-O-glucuronide	26.02±3.08%	20.11±1.75%	18.18±21.16%	14.67±5.52%	17.65±11.32%	21.29±11.97%	13.47±38.09%	28.15±26.60%
	Hesperidin	-	0.02±66.44%	0.32±68.44%	0.04±54.88%	-	-	0.19±36.14%	-
	Rutin	5.29±12.14%	4.60±11.93%	2.60±16.93%	3.34±3.99%	4.97±4.89%	4.19±6.82%	4.36±29.98%	3.33±18.58%

Supplementary Table S1. Comparison of statistical different metabolites (confidence interval 0.95) between lettuce plants grown in potting- and mineral soil with or without chitin treatment (PS, PS+CH) and (MS, MS+CH), respectively (n = 4). The first column shows the analyzed metabolite. Columns two and three show the treatment group and the tissue information respectively. The fourth and fifth column show the relative values of the corresponding compound in either soil in mg kg^{-1}. The p-value for the comparison between the two treatment groups is listed in the last column.

Compound	Treatment	Tissue	Mean MS in mg kg^{-1}	Mean PS in mg kg^{-1}	p-value
Chicoric acid	Untreated	Leaf	7,064.1	9,500.1	0.01
Luteolin			0.068	0.095	< 0.01
Quercetin-3-O-glucuronide			17.6	26	< 0.01
Salicylic acid			0.49	0.23	0.04
Chlorogenic acid		Root	4,565.8	2,006.8	0.01
Ferulic acid			0.39	0.26	0.05
p-Coumaric acid			0.37	0.18	< 0.01
Salicylic acid			3.7	1.1	< 0.01
Sinapinic acid			0.17	0.10	< 0.01
Chlorogenic acid	Chitin	Leaf	1,305.9	954.7	< 0.01
Daidzin			3.9	3.8	< 0.01
Quinic acid			122.2	95.5	< 0.01
Salicylic acid			0.49	0.25	< 0.01
Caffeic acid		Root	22.8	6.9	< 0.01
Chicoric acid			1,5034.8	8,947.2	< 0.01
Chlorogenic acid			5,691	1,615.4	< 0.01
Ferulic acid			0.43	0.24	< 0.01
Isoquercetin			1.3	0.66	0.02
p-Coumaric acid			0.31	0.14	< 0.01
Salicylic acid			5.6	1.3	< 0.01
Sinapinic acid			0.13	0.10	0.02

Supplementary Table S4. Separate Microsoft Excel sheet: potential identification of markers picked from comparison of differently treated samples.

Supplementary Table S5. Bacteria with a significant difference in relative abundance ($p < 0.05$) from the random forest analysis in mineral soil (MS) compared to 2 g L^{-1} chitin amended mineral soil (MS+CH). The first two columns show the genus and the corresponding treatment. The third column shows mean relative abundance of the samples ($n = 12$). The last two columns show the standard deviation (SD) and the standard error (SE) of the relative abundance.

Genus	Treatment	Mean	SD	SE
Kitasatospora	Chitin	0.017	0.009	0.0026
	Untreated	0.000097	0.00030	0.000097
Streptomyces	Chitin	0.037	0.013	0.0038
	Untreated	0.014	0.0041	0.0012

Supplementary Table S6. Fungi with a significant difference in relative abundance ($p < 0.05$) from the random forest analysis in mineral soil (PS) compared to 2 g L^{-1} chitin amended mineral soil (PS+CH). The first two columns show the genus and the corresponding treatment. The third column shows mean relative abundance of the samples ($n = 12$). The last two columns show the standard deviation (SD) and the standard error (SE) of the relative abundance.

Genus	Treatment	Mean	SD	SE
Acremonium	Chitin	0.0000	0.0001	0.0000
	Untreated	0.0004	0.0006	0.0002
Acrophialophora	Chitin	0.0001	0.0004	0.0001
	Untreated	0.0010	0.0008	0.0002
Apiotrichum	Chitin	0.0029	0.0036	0.0010
	Untreated	0.0216	0.0170	0.0049
Candida	Chitin	0.0018	0.0013	0.0004
	Untreated	0.0126	0.0110	0.0032
Coniochaeta	Chitin	0.0005	0.0007	0.0002
	Untreated	0.0021	0.0014	0.0004
Devriesia	Chitin	0.0005	0.0003	0.0001
	Untreated	0.0017	0.0007	0.0002
Dissophora	Chitin	0.0000	0.0001	0.0000
	Untreated	0.0005	0.0005	0.0001

Entomortierella	Chitin	0.0512	0.0261	0.0075
	Untreated	0.0187	0.0147	0.0042
Geomyces	Chitin	0.0124	0.0094	0.0027
	Untreated	0.0305	0.0109	0.0031
Hyphodiscus	Chitin	0.0028	0.0015	0.0004
	Untreated	0.0096	0.0041	0.0012
Mortierella	Chitin	0.4367	0.1327	0.0383
	Untreated	0.1911	0.0813	0.0235
Nadsonia	Chitin	0.0002	0.0002	0.0001
	Untreated	0.0009	0.0006	0.0002
Nematoctonus	Chitin	0.0110	0.0079	0.0023
	Untreated	0.0263	0.0129	0.0037
Oidiodendron	Chitin	0.0407	0.0289	0.0083
	Untreated	0.0912	0.0287	0.0083
Pascua	Chitin	0.0000	0.0001	0.0000
	Untreated	0.0003	0.0003	0.0001
Penicillium	Chitin	0.0431	0.0260	0.0075
	Untreated	0.0939	0.0272	0.0079
Phialemonium	Chitin	0.0183	0.0138	0.0040
	Untreated	0.0395	0.0169	0.0049
Rhodotorula	Chitin	0.0004	0.0002	0.0001
	Untreated	0.0016	0.0009	0.0002
Saitozyma	Chitin	0.0026	0.0020	0.0006
	Untreated	0.0108	0.0051	0.0015
Scedosporium	Chitin	0.0001	0.0002	0.0001
	Untreated	0.0024	0.0036	0.0010
Solicoccozyma	Chitin	0.0015	0.0009	0.0003
	Untreated	0.0086	0.0058	0.0017
Sugiyamaella	Chitin	0.0000	0.0001	0.0000
	Untreated	0.0005	0.0005	0.0001
Trichoderma	Chitin	0.0014	0.0009	0.0003
	Untreated	0.0062	0.0037	0.0011
Trichomonascus	Chitin	0.0007	0.0005	0.0001
	Untreated	0.0030	0.0017	0.0005
Udeniozyma	Chitin	0.0004	0.0003	0.0001
	Untreated	0.0019	0.0012	0.0003
Umbelopsis	Chitin	0.0019	0.0017	0.0005
	Untreated	0.0084	0.0032	0.0009

Supplementary Table S7. Fungi with a significant difference in relative abundance ($p < 0.05$) from the random forest analysis in mineral soil (MS) compared to 2 g L^{-1} chitin amended mineral soil (MS+CH). The first two columns show the genus and the corresponding treatment. The third column shows mean relative abundance of the samples ($n = 12$). The last two columns show the standard deviation (SD) and the standard error (SE) of the relative abundance.

Genus	Treatment	Mean	SD	SE
Aspergillus	Chitin	0.0000028	0.0000096	0.0000028
	Untreated	0.0011	0.0013	0.00037
Berkeleyomyces	Chitin	0.0030	0.0025	0.00073
	Untreated	0.041	0.026	0.0076
Betamyces	Chitin	0.00000	0.00000	0.00000
	Untreated	0.00025	0.00031	0.000088
Botryotrichum	Chitin	0.12	0.036	0.010
	Untreated	0.037	0.024	0.0069
Cladosporium	Chitin	0.00053	0.00038	0.00011
	Untreated	0.0035	0.0023	0.00066
Dactylonectria	Chitin	0.0043	0.0015	0.00042
	Untreated	0.043	0.037	0.011
Epicoccum	Chitin	0.00000	0.00000	0.00000
	Untreated	0.00071	0.00045	0.00013
Fusarium	Chitin	0.0027	0.0011	0.00033
	Untreated	0.027	0.012	0.0035
Gibellulopsis	Chitin	0.0013	0.00070	0.00020
	Untreated	0.015	0.0086	0.0025
Humicola	Chitin	0.17	0.044	0.013
	Untreated	0.027	0.028	0.0080
Papiliotrema	Chitin	0.000010	0.000019	0.0000055
	Untreated	0.00020	0.00018	0.000051
Paraphaeosphaeria	Chitin	0.00087	0.00044	0.00013
	Untreated	0.0089	0.0065	0.0019
Plectosphaerella	Chitin	0.0021	0.00064	0.00018
	Untreated	0.028	0.016	0.0046
Podila	Chitin	0.000090	0.00012	0.000035
	Untreated	0.0031	0.0039	0.0011
Pseudeurotium	Chitin	0.000074	0.00014	0.000041
	Untreated	0.00063	0.00047	0.00014
Purpureocillium	Chitin	0.013	0.0085	0.0025
	Untreated	0.00025	0.00060	0.00017

Syncephalis	Chitin	0.023	0.019	0.0056
	Untreated	0.00038	0.00073	0.00021
Trichoderma	Chitin	0.011	0.0051	0.0015
	Untreated	0.0036	0.0032	0.00092
Xeromyces	Chitin	0.00032	0.00033	0.000096
	Untreated	0.0035	0.0033	0.00094