*The role of soil properties and fertilization management in pathogen defense and plant microbial interactions in the rhizosphere of lettuce (*Lactuca sativa *L.)*

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Institute of Crop Science

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Saskia Helen Windisch

from Frankfurt am Main, Germany

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Examination Committee

Chairperson of the oral examination	Prof. Dr. Stefan Böttinger
Supervisor and Reviewer	Prof. Dr. Günter Neumann
Co-Reviewer	Prof. Dr. Gabriele Berg
Additional examiner	Prof. Dr. Ellen Kandeler

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List of Abbreviations

AMF	arbuscular mycorrhizal fungi
BIODYN2	organically managed soil (based on manure-fertilization with additional use of biodynamic preparations) of the long-term field experimental site in Therwil (FIBL)
C:N	carbon: nitrogen ratio
CONMIN	conventionally managed soil (based on mineral fertilization) of the long-term field experimental site in Therwil (FIBL)
$CuSO_4$	copper sulfate
DAMPs	damage associated molecular patterns
DOK-LTE	long-term field experimental site in Therwil (FIBL) on a silty loam for research on bio-dynamic (D), organic-biological (O) and conventional (K) cultivation of crops
ETI	effector-triggered immunity
exDNA	histone-linked extracellular DNA
HUB-LTE	long-term field experimental site in Thyrow on a loamy sand located at the Humboldt Universität zu Berlin (HUB)
HU-min	conventionally managed soil (based on mineral fertilization) of the long-term field experimental site in Thyrow
HU-org	organically managed soil (based on manure-fertilization without additional use of biodynamic preparations) of the long-term field experimental site in Thyrow
IAA	indole acetic acid
ISR	induced systemic resistance
LCOs	lipochito-oligosaccharides
LTE	long-term field experiment
MAMPs	microbe-associated molecular patterns
NLRs	Nod-like receptors
PAMPs	pathogen-associated molecular patterns
PGPMs	plant growth-promoting microorganisms
PR	pathogenesis-related proteins

PRRs	pattern recognition receptors
RET	root extracellular trap
R-genes	resistance genes
ROS	reactive oxygen species
SAR	systemic acquired resistance
VOCs	plant volatile organic compounds

Declaration

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1. The dissertation submitted on the topic

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1 Summary

Soil microorganisms are involved in nearly all relevant soil processes and considered as key players in agro-ecosystems. This is particularly relevant for the rhizosphere which is created by the activity of plant roots with dynamic impact on microbial communities, their diversity and activity. Both, beneficial but also pathogenic plant-microbial interactions in the rhizosphere are driven by root exudates and other root-induced modifications in rhizosphere chemistry, which are highly variable in space, time, composition and intensity. The physicochemical properties of the rhizosphere are influenced by numerous external factors including nutrient availability, biotic and abiotic stress, soil properties or plant genotypic variation but the related consequences for plant-microbial interactions and the consequences for plant performance and health status are still poorly understood. In this context the present study was initiated to investigate (i) the influence of the soil type on root exudation and the composition of the rhizosphere solution (ii) their impact on interactions with soil pathogens and beneficial rhizosphere microorganisms and (iii) the effect of long-term fertilization strategies (organic vs. mineral fertilization), using lettuce (*Lactuca sativa*) as a well-characterized model plant for studies on plant-microbial interactions in the rhizosphere.

Previous studies were performed at a unique field site, comprising three contrasting soils (diluvial sand - DS, alluvial loam - AL, loess loam - LL) at the Leibnitz Institute of Vegetable and Ornamental Crops, Großbeeren Germany, to study the biological control efficiency of selected beneficial microbiota as related to soil type but not influenced by local climate or different cropping history. Based on this background data set, in the present study the same soils, the same plant-beneficial bacterial strains (*Pseudomonas* sp. RU47 and *Serratia plymuthica* 3Re-4-18) and the same model pathogen (*Rhizoctonia solani* AG1-IB) were compared under controlled conditions in a minirhizotron experiment (Chapter 4.1). Lettuce plants were grown in minirhizotrons equipped with removable root observation windows to characterize antimicrobial root exudates and their possible associations with the presence of the pathogen and/ or bacterial inoculants. The observation windows allowed non-destructive micro-sampling of rhizosphere soil solution with sorption filters, placed on the surface of different root zones. Samples were subjected to GC-MS analysis. The results of plant biomass loss after pathogen infection demonstrated clear soil-type dependent expression of disease severity declining in the order DS > AL > LL soil in accordance with the results of the earlier field studies. However, the soil type effects on the expression of bottom rot disease were more strongly expressed under controlled conditions than at the field site. This underlines the importance also of the culture conditions for determining the expression of disease severity. GC-MS profiling of rhizosphere soil solutions revealed benzoic and lauric acids as antimicrobial compounds in root exudates of lettuce. Both, pathogen inoculation and pre-inoculation with the bacterial inoculants significantly increased the release of antimicrobial root exudates in a soil type-specific manner. The highest level of antimicrobial root exudates was detectable in the rhizosphere soil solutions of the loess loam with the lowest conductivity for bottom rot disease. Soil type-dependent differences were also recorded for the biocontrol effects of the two bacterial inoculants. The highest protective efficiency against lethal effects of the pathogen was recorded after double-inoculation on the AL soil. However, this was associated with a reduction of shoot growth and root hair development and a limited micronutrient status of the host plants as compared with single-strain inoculation, suggesting a competitive trade-off effect for host plant resource allocation between plant growth and defense reactions.

The defense compounds produced by the investigated lettuce plants are further characterized in chapter 5.1 based on results from experiments under controlled conditions using hydroponics, peat culture substrate and real soil culture in minirhizotrons. Based on the correlative observations obtained from the study described in chapter 4.1, suggesting a putative function of benzoic acid as defense compound released from roots of lettuce, the experiments in chapter 5.1 addressed the questions (i) whether benzoic acid is a component of root exudates in lettuce or rather a rhizosphere product of microbial origin; (ii) whether rhizosphere concentration of benzoic acid is sufficient to mediate pathogen suppressive effects; and (iii) whether the well-known sesquiterpene phytoalexin lettucenin A, accumulating in leaves of lettuce, also plays a role in the rhizosphere. Using a hydroponic culture system, avoiding soil-contact and formation of a rhizosphere effect, benzoic acid was identified as root exudate released from lettuce roots after pre-accumulation of benzoic acid esters in the root tissue. The rhizosphere concentrations determined from soil-grown plants were sufficient to inhibit hyphal growth of *R. solani in vitro* in a confrontation assay (30%) and to mitigate growth retardation (51%) and damage of fine roots (130%) in *R. solani* infected lettuce plants grown in peat culture substrate with external supplementation of benzoic acid. However, the amounts of benzoic acid were not sufficient to overcome plant growth suppression induced by root infection with the lettuce pathogen *Olpidium brassicae*. Lettucenin A is a major phytoalexin with local accumulation in affected plant tissues upon infection with pathogens and chemical elicitation ($CuSO_4$). Lettucenin A was detected in root and leaf tissues, but only in trace amounts in root exudates. The results suggest a two-stage defense mechanism with initial pathogen-induced benzoic acid exudation into the rhizosphere as first defense line upon pathogen attack followed by local accumulation of lettucenin A in affected root and leaf tissues, as a second line of defense.

To investigate the impact of organic and mineral fertilization on plant performance, soil microbiota and rhizosphere interactions, lettuce plants were grown in contrasting soils from two long-term field experiments (LTEs: HUB-LTE – loamy sand vs. DOK-LTE - silty loam) with differing fertilization history in a minirhizotron experiment (Chapter 6.1). As a site-specific effect of DOK-LTE, a high relative abundance (76-90%) of the fungal lettuce pathogen *Olpidium brassicae* was recorded in the rhizosphere, both under long-term organic and mineral fertilization. This effect was most likely due to a lower water drainage potential of the silty loam compared to the sandy HUB-LTE soils known to promote *Olpidium* infection. In the organically managed soils from both sites (HU-org; BIODYN2), the rhizospheres were characterized by increased relative abundance of plant-beneficial arbuscular mycorrhizal fungi, as expected for organic farming with reduced P availability. In addition, the organically managed soils had an increased relative abundance of fungal pathotrophs in the rhizosphere and an increased systemic expression of defense-related genes in shoot tissues of the respective plants. There was a strong plant growth depression and *Olpidium* infection in the BIODYN2 soil with organic fertilization history, which might be related to a drastic (87-97%) reduction in rhizosphere abundance of potentially plant-beneficial microbiota (*Pseudomonadaceae*, *Mortierella elongata*), which was associated with reduced concentrations of the antifungal root exudate benzoate. In contrast, high relative abundance of *Pseudomonadaceae* (61-74%) in the rhizosphere of plants grown in soils with long-term mineral fertilization without disease symptoms coincided with high rhizosphere concentrations of chemotactic dicarboxylates (succinate, malate) and defense metabolites (benzoic acid), higher concentrations of easily available monosaccharides as carbon sources for the fast growing copiotrophic beneficials and a high C (sugar)/N (amino acid) ratio.

These results suggest a complex network of belowground interactions determining the success of plant pathogen defense. Respective interactions identified in this study comprise microbial competition for rhizodeposits, the availability of suitable chemo-attractants and release of defense compounds induced by beneficial microbiota (e.g. *Pseudomonas* sp.), all influenced by fertilization history. Site-specific factors, such as soil properties and climatic conditions can interfere independently as determinants for the composition of soil microbial communities and the selective promotion of pathogen populations. A better understanding of these interactive processes is essential for the development of practical approaches in the concept of “soil biological engineering”.

2 Zusammenfassung

Bodenmikroorganismen sind an nahezu allen relevanten Bodenprozessen beteiligt und spielen daher eine Schlüsselrolle in Agrarökosystemen. Das betrifft besonders die Rhizosphäre, die durch die Aktivität von Pflanzenwurzeln mit ihrem dynamischen Einfluss auf mikrobielle Gemeinschaften im Boden, deren Diversität und deren Aktivität, gebildet wird. Sowohl nützliche als auch pathogene Interaktionen von Mikroorganismen mit ihren Wirtspflanzen werden durch Wurzelexsudate und andere wurzelinduzierte Modifikationen der Rhizosphärenchemie bestimmt, die durch eine hohe Variabilität im Hinblick auf Zusammensetzung und Intensität sowohl auf räumlicher als auch auf zeitlicher Ebene charakterisiert sind. Die physiko-chemischen Eigenschaften der Rhizosphäre werden von zahlreichen externen Faktoren beeinflusst, wie z.B. Nährstoffverfügbarkeit, biotischem und abiotischem Stress, Bodeneigenschaften oder pflanzengenotypischer Variabilität. Allerdings sind die dadurch bedingten Konsequenzen für Pflanzen-Mikroben-Interaktionen und die Auswirkungen auf die Ertragsbildung und die Pflanzengesundheit bisher nur ansatzweise verstanden. In diesem Zusammenhang wurde die vorliegende Studie durchgeführt, um (i) den Einfluss von Bodeneigenschaften auf die Wurzelexsudation und die Zusammensetzung der Rhizosphärenbodenlösung, (ii) deren Einfluss auf Interaktionen mit Bodenpathogenen und mit nützlichen Rhizosphärenmikroorganismen und (iii) den Effekt von Langzeitdüngungsstrategien (organische vs. mineralische Düngung) mit Salat (*Lactuca sativa L.*) als gut charakterisierte Modellpflanze für Pflanzen-Mikroben-Interaktionen in der Rhizosphäre zu untersuchen.

Vorgängerstudien wurden auf einer ausgewählten Feldversuchsfläche am Leibnitz-Institut für Gemüse- und Zierpflanzenbau in Großbeeren, Deutschland, durchgeführt, die durch drei kontrastierende Böden (diluvialer Sand - DS, alluvialer Lehm - AL, Loess-Lehm – LL) am selben Standort charakterisiert ist. Dieser Ansatz wurde verfolgt, um die Effizienz ausgewählter nützlicher Mikroorganismenstämme für den biologischen Pflanzenschutz in Abhängigkeit des Bodentyps, ohne den Einfluss unterschiedlicher Witterungsbedingungen oder Bewirtschaftungsmaßnahmen, zu untersuchen. Basierend auf diesen Daten wurden in der vorliegenden Studie dieselben Böden, dieselben Pflanzenwachstums-stimulierenden Bakterienstämme (*Pseudomonas* sp. RU47 und *Serratia plymuthica* 3Re-4-18) und das selbe Modell-Pathogen (*Rhizoctonia solani* AG1-IB) unter kontrollierten Bedingungen in einem Minirhizotronexperiment verglichen (Kapitel 4.1). Salatpflanzen wurden in Minirhizotronen, die mit abnehmbaren Wurzelbeobachtungsfenstern ausgestattet sind, angezogen, um antimikrobielle Wurzelexudate und deren Beziehungen zur Wirkung des Pathogens und der bakteriellen Antagonisten zu charakterisieren. Durch die Wurzelbeobachtungsfenster wurde eine nicht-destruktive Beprobung der Rhizosphären-Bodenlösung mit Sorptionsfiltern im Mikromaßstab an der Oberfläche verschiedener Wurzelzonen mit nachfolgender GC-MS-Analyse ermöglicht. Eine Verminderung der Pflanzenbiomasse nach Pathogeninfektion zeigte eine deutliche Boden-abhängige Ausprägung von Krankheitssymptomen in abnehmender Reihenfolge: DS > AL > LL, was in Einklang mit den Ergebnissen der früheren Feldstudien stand. Allerdings waren die Effekte des Bodentyps auf die Ausprägung der Krankheitsymptome unter kontrollierten Bedingungen stärker ausgeprägt als unter Feldbedingungen. Dies unterstreicht die Bedeutung der Kulturbedingungen für die Ausbildung von Pathogen-Interaktionen. Durch GC-MS-Profiling der Rhizosphärenbodenlösung wurden Benzoesäure und Laurinsäure als antimikrobielle Verbindungen in der Rhizosphärenbodenlösung von Salat nachgewiesen. Sowohl die Pathogen-Inokulation als auch die Prä-Inokulation mit antagonistischen Bakterien erhöhten die Bodentyp-abhängige Akkumulation der antimikrobiellen Verbindungen signifikant, mit den höchsten Werten in der Rhizosphärenbodenlösung des LL-Bodens, auf

dem auch die geringste Pathogenanfälligkeit für *R. solani* nachweisbar war. Bodentyp-abhängige Unterschiede wurden auch für die Pathogen-antagonistischen Wirkungen der beiden bakteriellen Inokulanzien festgestellt. Die beste Schutzwirkung gegen letale Pathogenwirkungen wurde nach Doppelinokulation beider Stämme auf dem AL-Boden festgestellt. Allerdings war dies im Vergleich zu einer Einzelstamminokulation mit einer Reduktion des Sprosswachstums und der Wurzelhaarentwicklung und mit einem verminderten Mikronährstoffstatus der Wirtspflanzen verbunden, was auf konkurrierende Effekte hinsichtlich der Verfügbarkeit von Ressourcen innerhalb der Wirtspflanze für das Pflanzenwachstum und für Abwehrreaktionen hindeutet.

Die von den untersuchten Salatpflanzen produzierten Abwehrstoffe wurden in Kapitel 5.1 auf der Grundlage von Experimenten unter kontrollierten Bedingungen in Nährlösung, Torfkultursubstrat und Bodenkultur in Minirhizotronen weiter charakterisiert. Ausgehend von den korrelativen Beobachtungen aus der in Kapitel 4.1 beschriebenen Studie, die auf eine potenzielle Funktion von Benzoesäure als einen von den Salatwurzeln abgegebenen Abwehrstoff hindeuteten, zielten die Experimente in Kapitel 5.1 auf die Fragen ab (i) ob Benzoesäure eine Komponente der Wurzelexsudate von Salat ist oder eher ein Rhizosphärenprodukt mikrobiellen Ursprungs darstellt, (ii) ob die Konzentration von Benzoesäure in der Rhizosphäre ausreicht, um pathogen-suppressive Wirkung zu entfalten und (iii) ob das bekannte Sesquiterpen-Phytoalexin Lettucenin-A, das sich in Salatblättern anreichert, auch eine Rolle in der Rhizosphäre spielt. Durch den Einsatz eines Hydrokultursystems unter Vermeidung von Bodenkontakt und der Bildung eines Rhizosphäreneffektes wurde Benzoesäure als Wurzelexsudat charakterisiert, das nach vorheriger Anreicherung von Benzoesäurekonjugaten im Wurzelgewebe abgegeben wurde. Die gemessenen Benzoesäure-Konzentrationen in der Rhizosphäre von Pflanzen in Bodenkultur waren ausreichend, um in einem Konfrontationstest das Hyphenwachstum von *R. solani in vitro* zu hemmen (30%) und durch externe Applikation bei *R. solani*-infizierten Salatpflanzen in Torfkultursubstrat Wachstumshemmungen (51%) und Schäden an den Feinwurzeln (130%) zu vermindern. Allerdings waren die Mengen an Benzoesäure nicht ausreichend, um Wachstumsdepressionen durch Wurzelbefall mit dem Salatpathogen *Olpidium brassicae* zu unterdrücken. Neben Benzoesäure ist Lettucenin A ein wichtiges Phytoalexin von Salatpflanzen, welches sich in befallenem Pflanzengewebe nach einer Infektion mit Pathogenen oder Stimulierung mit chemischem Elicitoren ($CuSO_4$) lokal anreichert. Lettucenin A wurde im Wurzel- und Blattgewebe und im Spurenbereich auch in den Wurzelexsudaten nachgewiesen. Die Ergebnisse deuten auf einen zweistufigen Abwehrmechanismus hin, mit einer Pathogen-induzierten Wurzelexsudation von Benzoesäure in die Rhizosphäre als primäre Verteidigungslinie, gefolgt von einer lokalen Anreicherung von Lettucenin A in befallenem Wurzel- und Blattgewebe.

Um den Einfluss organischer und mineralischer Düngung auf das Pflanzenwachstum, auf die Boden-Mikrobiota und auf die Rhizosphäreninteraktionen zu untersuchen, wurden Salatpflanzen in unterschiedlichen Böden zweier Langzeitfeldversuche mit unterschiedlicher Düngungshistorie (LTEs: HUB-LTE – lehmiger Sand vs. DOK-LTE – schluffiger Lehm) in einem Minirhizotronexperiment angezogen (Kapitel 6.1). Als Standort-spezifischer Effekt von DOK-LTE wurde eine hohe relative Abundanz (76-90%) des pilzlichen Salatpathogens *Olpidium brassicae* in der Rhizosphäre nachgewiesen, sowohl unter Langzeit-organischer als auch -mineralischer Düngung. Dieser Effekt war höchstwahrscheinlich auf ein geringeres Entwässerungspotential des Lehmbodens im Vergleich zu den sandigen HUB-LTE-Böden zurückzuführen, wodurch Infektionen mit *Olpidium* befördert werden können. Auf den ökologisch bewirtschafteten Flächen an beiden Standorten (HU-org; BIODYN2) war die

Rhizosphäre durch eine erhöhte relative Abundanz nützlicher arbuskulärer Mykorrhizapilze charakterisiert, was im Ökolandbau mit verminderter P-Verfügbarkeit zu erwarten ist. Darüber hinaus zeigten die ökologisch bewirtschafteten Böden eine erhöhte relative Abundanz pathotropher Pilze in der Rhizosphäre und eine erhöhte systemische Expression von Genen, die im Zusammenhang mit der Stressabwehr stehen, im Sprossgewebe der jeweiligen Pflanzen. Auf dem BIODYN2-Boden mit organischer Düngung war eine starke Depression des Pflanzenwachstums durch Befall mit *Olpidium* nachweisbar, was möglicherweise im Zusammenhang mit einer drastischen Reduktion (87-97%) der Rhizosphärenabundanz potentiell nützlicher Mikrobiota (*Pseudomonadaceae, Mortierella elongata*) steht, die auch mit verminderten Rhizosphärenkonzentrationen des antifungalen Wurzelexsudats Benzoesäure assoziiert war. Im Gegensatz dazu war eine hohe relative Abundanz (61-74%) von *Pseudomonadaceae* in der Rhizosphäre von Pflanzen, die in Böden mit Langzeit-Mineraldüngung angezogen wurden, nachweisbar, welche auch keine Krankheitssymptome zeigten. Für diese Pflanzen waren weiterhin hohe Rhizosphärenkonzentrationen von chemotaktischen Dicarboxylaten (Succinat, Malat) und von Abwehrmetaboliten (Benzoesäure), sowie erhöhte Konzentrationen von leicht-verfügbaren Einfachzuckern als Kohlenstoffquelle für schnell wachsende, copiotrophe nützliche Rhizosphärenmikrobiota nachweisbar, verbunden mit einem hohen C (Zucker) / N (Aminosäuren)-Verhältnis.

Diese Ergebnisse weisen auf ein komplexes Netzwerk von Rhizosphäreninteraktionen hin, welche den Erfolg der Pflanzenpathogenabwehr bestimmen. Die in der vorliegenden Studie charakterisierten Interaktionen umfassen die mikrobielle Konkurrenz um Wurzelausscheidungen, die Verfügbarkeit von chemotaktisch wirksamen Signalsubstanzen und die Wurzelexsudation von Abwehrstoffen, die durch nützliche Mikrobiota (z.B. *Pseudomonas* sp.) induziert, und durch Bewirtschaftungsmaßnahmen wie das Düngungsmanagement beeinflusst werden können. Standortspezifische Faktoren wie Bodeneigenschaften und klimatische Bedingungen können unabhängig davon die Zusammensetzung der mikrobiellen Gemeinschaften im Boden und die selektive Etablierung von Pathogenpopulationen beeinflussen. Ein besseres Verständnis dieser interaktiven Prozesse ist unverzichtbar für die Entwicklung praktischer Ansätze im Rahmen von Konzepten des „Bodenbiologischen Engineerings“.

3 General introduction

3.1 Plant-microbe interactions in soils

Our unique cultural landscape is largely shaped by crop cultivation and management practices. In this context, soil fertility and health are of fundamental importance for agriculture, since they provide the ground to farmers for animal and human food production (Doran and Zeiss, 2000; FAO, 2015). Successful soil fertility and health management requires adapted nutrient supply, sufficient water resources, supply and turnover of organic matter and control of pests and diseases in a stable filtering and buffering system (Doran and Zeiss, 2000; Blum, 2005). In this context, soil microorganisms are involved in nearly all relevant soil processes and are considered as key players in agro-ecosystems (Sahu, Pramod et al., 2019). Particularly the rhizosphere, created by the activity of plant roots, is an important hot-spot for soil biota with dynamic impact on microbial communities, their diversity and activity (Hartmann et al., 2008; Raaijmakers et al., 2009). Beneficial root-microbial interactions in the rhizosphere driven by rhizodeposits can support the host plant to acquire nutrients and provide other benefits during the growth phase with respect to pathogen antagonisms and induction of biotic and abiotic stress adaptations (stress priming) (Hartmann et al., 2009; Oburger and Jones, 2018). However, rhizodeposition can attract not only beneficial soil microbes but also pathogens and pests as “uninvited guests” with potential to directly attack the host plants and to counteract beneficial rhizosphere microorganisms (Snelders et al., 2020). Already in 1904, the German plant phytopathologist Lorenz Hiltner claimed that plant health and the resistance towards pathogenesis depend on the interplay of soil microbes that have settled in the rhizosphere (Hartmann et al., 2008). Consequently, various root-microbial interactions in the rhizosphere (rhizosphere effect) are key factors determining soil fertility and plant performance (Raaijmakers et al., 2009).

3.2 Rhizodeposits as key determinants for the rhizosphere effect

The “rhizosphere” defines the soil compartment, influenced by the activity of plant roots (Hiltner, 1904), which is highly variable in spatial extension and undergoes intensive modifications during all phases of plant development (Kuzyakov and Razavi, 2019). The organisms, comprising the rhizosphere biome, include bacteria, archaea, fungi, oomycetes, viruses, protozoa, algae, nematodes, and arthropods, interacting in complex food webs and

signaling systems (Buée et al., 2009; Raaijmakers et al., 2009; Mendes et al., 2013). Consequently, soil-physicochemical and biological properties in the rhizosphere (rhizosphere effect) differ from those of the bulk soil, which is not directly influenced by root activity (Vessey, 2003). Root-released organic rhizodeposits and other root-induced changes in rhizosphere chemistry (pH, redox conditions, and aggregate formation and water relationships) are regarded as major drivers for the establishment of the rhizosphere effect (Neumann and Römheld, 2002, 2007). Rhizodeposition is highly variable and the release depends on plant species, their growth stages, the root zone and abiotic and biotic influencing factors (Pausch and Kuzyakov, 2018). Rhizodeposits can originate from passive losses of organic compounds during root turnover but also from actively controlled, secretory processes with adaptive functions (Neumann and Römheld, 2007). They consist of lysates lost from damaged and decaying roots, root border cells and root exudates either lost passively via diffusion from undamaged roots or by controlled secretion processes (Hartmann et al., 2009; Narula et al., 2009; Neumann and Römheld, 2011; Vives et al., 2020). Root exudates can induce pH and redox-milieu changes, promote detoxification of potentially toxic metals and reduce the risk caused by organic pollutants (Bais et al., 2006). Furthermore, they can mobilize nutrients, stabilize soil aggregates around the root and selectively influence the water relationships in the rhizosphere soil (Narula et al., 2009; Oburger and Jones, 2018). Additionally they act as chemo-attractants in inter- and intra-plant signal exchange and contain defense molecules with antimicrobial activities (Bais et al., 2006; Badri and Vivanco, 2009; Oburger and Jones, 2018; Tian et al., 2019). Rhizodeposits can comprise any compound accumulating inside root cells but in different ratios depending on the membrane permeability, controlled release or active re-uptake of the respective compounds. This includes mono- oligo-, and polysaccharides, organic acids, amino acids, nucleotides, phenolics, alcohols, terpenoids, organic volatiles, alkaloids, peptides and proteins including secretory enzymes (Marschner, 1995; Neumann and Römheld, 2007; Tian et al., 2019). Generally, rhizodeposits act as energy source for heterotrophic organisms. Specific classes of compounds with chelating properties, such as carboxylates, phenols and phytosiderophores can be additionally involved in mobilization and acquisition of insoluble minerals. Certain carboxylates, such as malate, citrate and succinate are important chemo-attractants for beneficial rhizosphere microorganisms (i.e. *Rhizobia*, diazotrophic bacteria, strains of *Bacillus* and *Pseudomonas*) and act as precursors for bacterial siderophore production, involved in Fe-

mobilization, pathogen interactions and induction of plant defense responses (Oku et al., 2014; Sampedro et al., 2015). Due to a lack of adsorption to the soil matrix, sugars provide an easily available carbon source for microorganisms. The same holds true for most amino acids providing both, carbon skeletons and amino N to microorganisms (Neumann and Römheld, 2007). The relative proportions of sugars and amino acids significantly contribute to the available amounts of C and N for microorganisms (C:N ratio) in the rhizosphere (Juma and McGill, 1986; Jaeger et al., 1999) with important impact on the composition of rhizosphere-microbial communities and processes of carbon and nitrogen cycling in the rhizosphere. Furthermore, certain amino acids are acting as signals for root growth responses (glutamate), chemo-attraction of rhizosphere microorganisms and as precursors (tryptophane, methionine) for bacterial production of plant hormones (auxin, ethylene) (Neumann and Römheld, 2007; Canarini et al., 2019). Secondary metabolites such as phenolic acids, flavonoids and strigolactones provide signaling functions in root exudates involved in the establishment of classical symbiotic interactions for biological nitrogen fixation and arbuscular mycorrhizal associations (Narula et al., 2009). Furthermore, certain phenolics, alkaloids, terpenoids and organic volatiles have also functions as defense compounds against microbial pathogens and insect pests and can mediate interplant signaling in stress adaptations (Parales and Harwood, 2002; Martin et al., 2003; Sharifi et al., 2018; Hammerbacher et al., 2019).

3.3 Impact of agricultural management

As a result of agricultural intensification, the use of chemical fertilizers and synthetic pesticides has been established as a standard practice in conventional agriculture (Mondelaers et al., 2009). Their highly targeted and specific options for application strategies according to the plant demands have significantly increased the productivity of cropping systems and largely replaced the ecosystem services provided by beneficial plant-microbial interactions in the rhizosphere supporting nutrient acquisition and pathogen suppression. However, despite this success, the long-term high-level productivity is accompanied by consequences that can no longer maintain the natural resources of a balanced ecosystem (Hazell and Wood, 2007). Limited crop rotation favoring crops of high economic value, loss in soil quality with reduced microbial and mesofaunal biodiversity, soil degradation, and a declining availability of resistant crop cultivars and control mechanisms against pathogenic diseases over time are increasingly recognized as detrimental side effects (Oerke, 2006; Hartmann et al., 2015).

Additionally, rising concerns are related to environmental effects with respect to eutrophication of natural ecosystems due to nutrient losses and a risk of toxic pesticide residues in consumable products. This has led to demands for alternative strategies to control crop diseases (Alabouvette et al., 2006; Grosch et al., 2012; Schreiter et al., 2014b, 2018), increase nutrient use efficiency and stress resilience of crops in terms of a biological intensification of agricultural management (Bender et al., 2016).

In this context, various alternative management strategies such as permaculture, regenerative and conservation agriculture, agroforestry and organic or biodynamic farming systems are increasingly emerging. Their goal is to reduce or even avoid the use of chemical fertilizers and pesticides in closed systems of nutrient cycles with enriched organic matter, microbial biomass and soil microbial diversity (Mondelaers et al., 2009; Hartmann et al., 2015; Bender et al., 2016; Hirschfeld and Van Acker, 2021). However, due to a reduced flexibility to adapt fertilizer application and plant protection to actual crop demands, many of these strategies are confronted with lower yield, and limited options for disease control remain a major issue (Hartmann et al., 2015). This indicates that further optimization and a much better understanding of the processes and factors supporting beneficial interactions in the soil ecosystem are still required.

3.4 Microbiome management for improved crop performance

Soils with the potential to suppress diseases and to support abiotic stress resilience are important for agricultural conservation measures, integrated pest management and the goal of sustainable agriculture (Doran and Zeiss, 2000). In contrast, conductive soils, frequently characterized by a limited abundance of plant beneficial soil microbiota, are more vulnerable towards abiotic and biotic stress-conditions (Expósito et al., 2017). To ensure their survival, plants mainly recruit beneficial microbes, while at the same time they try to limit pathogen infestation as much as possible (Thomas et al., 2021). Apart from modifications of agricultural management (see 3.3, Impact of agricultural management) there are also attempts for a direct manipulation of the rhizosphere microbiome by targeted supplementation of plant beneficial rhizosphere microorganisms or strengthening plant defense adaptations by application of natural compounds with elicitor functions (Berg, 2009). The market for these so-called biostimulants and bio-pesticides is growing and currently offers a broad range of inoculum of bacterial and fungal species supporting plant nutrition and resilience to biotic and abiotic

stress factors (Narula et al., 2009). The use of a microbial inoculum depends on its mode of action. The microbial products are supposed to act as bio-fertilizers, plant strengtheners, phytostimulators and biopesticides (Lugtenberg et al., 2002), generally termed as plant growth-promoting microorganisms (PGPMs). The ability to enhance plant growth is thought to be achieved either directly by interaction between the beneficial microbes and the host plant or indirectly due to their antagonistic potential against pathogenic diseases or promotion of native beneficial soil microbiota (Berg, 2009). The main plant-microbe interactions discussed to promote plant growth and health are summarized in Figure 1. Inoculum often consists of symbiotic or associative bacteria and fungi isolated from the rhizosphere of host plants with the ability to propagate also independently in soils or on artificial growth media for technical production of inoculant strains (Vessey, 2003; Berg, 2009; Dodd and Ruiz-Lozano, 2012). They are applied as single strain formulations or more recently also as strain combinations or larger microbial consortia with the aim to exploit synergisms and to increase the flexibility under variable environmental conditions (Bradáčová et al., 2019). Application can be performed as seed or soil inoculants, which are expected to colonize the rhizosphere, the root surface or the root system internally (as endophytes). The products can participate in nutrient cycles and enhancing plant growth by atmospheric nitrogen fixation, P solubilization, enzymatic cleavage of organic N and P forms in the rhizosphere, and mobilization of iron by production of siderophores (Bhardwaj et al., 2014; Calvo et al., 2014). In this context plant benefits may arise from a direct increase of soil nutrient availability or indirectly by liberation of previously sequestered nutrients during turnover of microbial biomass involving predator-mediated grazing on rhizosphere-bacterial populations (Bonkowski, 2004). Moreover, PGPMs but also pathogens can promote plant growth, defense responses and virulence by influencing the plant hormonal balances via direct production of plant hormones such as indole acetic acid (IAA), cytokinins and gibberellins (Van Loon, 2007; Berg, 2009; Vives et al., 2020). Chemical signals play a role by interacting with the plant hormonal metabolism (quorum sensing metabolites, microbial volatiles) or by enzymatic degradation of phytohormones for instance by ACC-deaminase, reducing the stress-induced ethylene concentration in roots (Glick, 2005; Berg, 2009). They also interact via signaling pathways to regulate a wide range of physiological processes and stress adaptations, via chemical signaling molecules (inter-bacteria communication by quorum sensing), production of antibiotic compounds and production of microbe-induced plant volatiles (MIPVs). In this

manner, they inhibit pathogen growth and indirectly mediate resistance to following pathogen attacks (Narula et al., 2009; Sharifi et al., 2018; Liu et al., 2020). Especially for the widespread and difficult-to-control fungal soil pathogens *Fusarium, Phytium* and *Rhizoctonia* with a wide host plant spectrum, the literature shows that rhizo-bacteria can have a suppressive effect (Oerke, 2006; Hallmann, 2010). In this context, the bacterial genera *Streptomyces*, *Agrobacterium*, *Enterobacter*, *Erwina*, *Serratia*, *Azotobacter, Pseudomonas* and *Burkholderia* and *Bacillus* provide well-studied plant growth promoting rhizobacteria (PGPR) strains (Berg 2009; Narula et al., 2009; Raaijmakers et al., 2009; Vives et al., 2020).

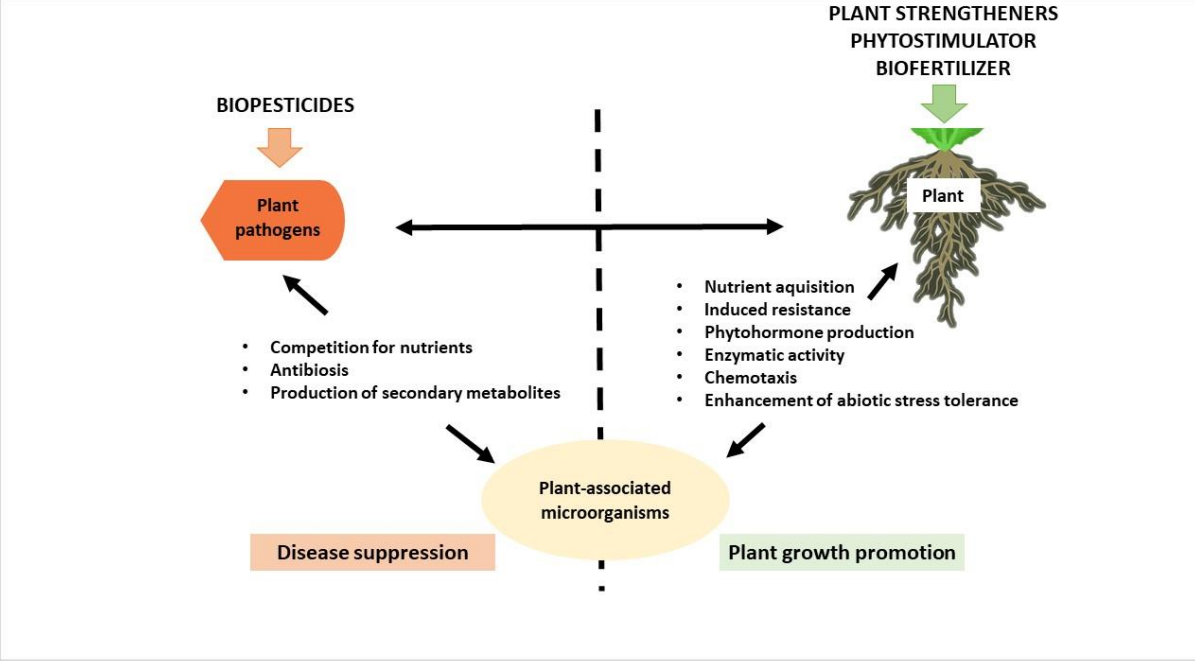

Figure 1: The application of beneficial microbial inoculum into the soil stimulates the interactions of the plants and the growth and health promoting microbial community (modified after Berg 2009).

3.5 Interactions with defense mechanisms in plants

During their development, plants are confronted with microbial interactions and abiotic stress factors influencing plant growth and health (Nobori and Tsuda, 2019), including an induction of adaptive defense responses, which already occurs before severe direct cell damage to maintain their survival (Thomas et al., 2021). Induction of resistance comprises longer lasting protective effects, still detectable even after recovery from a stress event or after perception of a stress signal (stress priming). The plant's immune status to pathogen susceptibility is not fixed, but can be fine-tuned by extrinsic and intrinsic signals during the life of the plant

(Thomas et al., 2021). Many studies on plant immunity have been primarily investigated on leaves, because root systems are more complex to study due to methodological challenges with respect to accessibility (Okubara and Paulitz, 2005; Millet et al., 2010; Chuberre et al., 2018). The two best known forms of induced resistance in plants are termed as systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is preconditioned from previous infection by virulent, avirulent and even nonpathogenic microbes, mediated via salicylic acid signaling and involving induction of pathogenesis-related (PR) proteins (e.g. glucanases, chitinases). Unlike SAR, ISR does not involve the accumulation of pathogenesis-related proteins or salicylic acid, but instead relies on pathways regulated by jasmonate and ethylene and can be mediated by PGPMs, determining the protective rhizosphere microbiome of the plants (Choudhary et al., 2007; Teixeira et al., 2019). In response to moderate abiotic stress below the threshold of cell damage, similar adaptive responses are triggered, which can increase plant resistance to abiotic and biotic stress factors.

How plants recruit beneficial microbes and at the same time limit the outbreak of diseases still raises many questions (Harris et al., 2020; Thomas et al., 2021). The outcome of any interaction between plants and microorganisms and whether the latter are pathogens, commensals or mutualists is determined by a specific exchange of molecular signals combined with the genetic potential of host and microbes (Thomas et al., 2021). This enables the plant to distinguish between friend and foe within plant-microbial interactions (Zipfel and Oldroyd, 2017). Since fungal and bacterial symbionts produce both symbiosis-inducing and immunity-inducing signals that do not allow the host to accurately distinguish pathogens from commensals, the host plant has developed distinct receptors and signaling pathways for symbiosis and immunity (Thomas et al., 2021). Receptors in the plasma membrane, called pattern recognition receptors (PRRs) and cytoplasmic Nod-like receptors (NLRs), play a central role in this context (Monteiro and Nishimura, 2018; Thomas et al., 2021). The PRRs enable a recognition of invaders and stress factors via characteristic molecular patterns, for instance for fungal chitin or bacterial flagellin, and siderophores (Zipfel, 2014; Zipfel and Oldroyd, 2017; Teixeira et al., 2019) or plant metabolites liberated during stress exposure (Blokhina et al., 2003). Accordingly, these molecular signals have been classified as general microbe-associated molecular patterns (MAMPs for bacteria, fungi and nematodes), damage associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and Lipochito-oligosaccharides (LCOs) (Barrett and Heil, 2012; Saijo et al., 2018; Thomas et al.,

2021). An interplay of these recognition receptor patterns informs the plant whether it is ultimately confronted with a mutualist or a pathogen. The plant can thus react more quickly to environmental stress factors by priming the plant's immune defense or alternatively support root and rhizosphere colonization by beneficial microbes (Thomas et al., 2021).

The plant hormones salicylic acid, jasmonic acid, and ethylene and their signaling pathways are acting upstream the PRR-mediated signal perception and play a central role in shaping physiological defense responses and thereby also functional and structural properties of the plant microbiome (Kniskern et al., 2007; Lebeis et al., 2015; Liu et al., 2020). Consequently, enhanced expression of plant genes involved in abiotic and biotic stress adaptations have been described (Chowdhury et al., 2019). For instance, after contact with PAMPs in the plant tissue, expression of the RbohD gene is upregulated which encodes an NADPH oxidase involved in a burst of production of reactive oxygen species (ROS) at the infection site for pathogen defense (Kadota et al., 2015). At the same time, the unaffected tissue is protected by activation of metabolic pathways involved in ROS detoxification, which also plays an important role in MAMP- and DAMP-induced protection against abiotic stress (Kadota et al., 2015). In addition, the production of a wide range of plant-derived chemical compounds with properties to act against diseases based on diverse secondary metabolites from different classes such as phenolics, terpenoids and alkaloids is stimulated by PRR-triggered immune reactions (Baetz and Martinoia, 2014; Thomas et al., 2021). Enzymes involved in the biosynthesis of these so-called phytoalexins (Yean et al., 2009; Talubnak et al., 2017) are formed in healthy plant tissues as a reaction to cell injuries, shortly after plant attack. The formation is induced by biotic factors (microorganisms) and abiotic factors, for instance cold stress, UV-radiation and heavy-metal toxicity (Grisebach and Ebel, 1978; Deverall, 1982; Yean et al., 2009; Talubnak et al., 2017). However, certain pathogens but also PGPMs are able to overcome the first PRR-dependent defense line by production of effector proteins with the ability to downregulate and counteract PRR-induced defense reactions (Henry et al., 2012; Prsic and Ongena, 2020). In response, plants have evolved a second line of defense against certain pathogens based on the upregulation of specific disease resistance genes (R-genes) upon perception of effector proteins, the so-called effector-triggered immunity (ETI; Henry et al., 2012). Various rhizobacteria possess skills to metabolize defense compounds released from plant roots. Phenolic compounds, for instance benzoxazinoids (tryptophan-derived secondary metabolites with allelopathic and antibiotic properties produced by cereals) act as

chemo-attractants for rhizobacteria (among them *Pseudomonas*) with plant growth-promoting properties, which settle in the rhizosphere and metabolize these compounds (Parales and Harwood, 2002; Neal et al., 2012; Kudjordjie et al., 2019).

At the cellular level, so-called root border cells are released by controlled mechanisms from the root cap to influencing root-microbe dynamics in the rhizosphere. Individual cells and small cell aggregates are released, followed by polygalcturonides (mucilage) entrapping low molecular weight exudate compounds, secondary metabolites antimicrobial proteins and histone-linked extracellular DNA (exDNA), which in the hydrated stage forms a gelatineous matrix (root extracellular trap, RET) with the ability to attract and immobilize microorganisms (Tian et al., 2019). The RET is able to modify the microbial community in the rhizosphere to ensure plant health. Both, beneficial bacteria and pathogenic microbes are attracted, whereby particularly pathogenic microbes are trapped and finally inactivated in the RET matrix. In this context, especially the exDNA in the mucilage layer plays a key role for the antibiotic RET function, since the pathogen-protective properties were completely lost after enzymatic degradation of exDNA (Hawes et al., 2012; Driouich et al., 2013).

Furthermore, also plant volatile organic compounds (VOCs) are known to play a role in plant defense towards pathogenic attack. It is assumed that they operate either in direct defense mechanisms or as signals for antimicrobial responses (Sharifi et al., 2018). The compounds are produced in the epidermal cell layer of plants. Their release takes place either by volatilization through the cell membrane or after mechanical damage of secretory structures storing VOCs caused by pathogenic microbes. Terpenes, aromatics and volatile plant hormones (methyl-jasmonate and methyl-salicylate) are considered important VOCs (Martin et al., 2003; Dudareva et al., 2004; Hammerbacher et al., 2019). A schematic representation of plant defense mechanisms, induced by rhizospheric interactions of the plant and its microbiome is summarized in Figure 2.

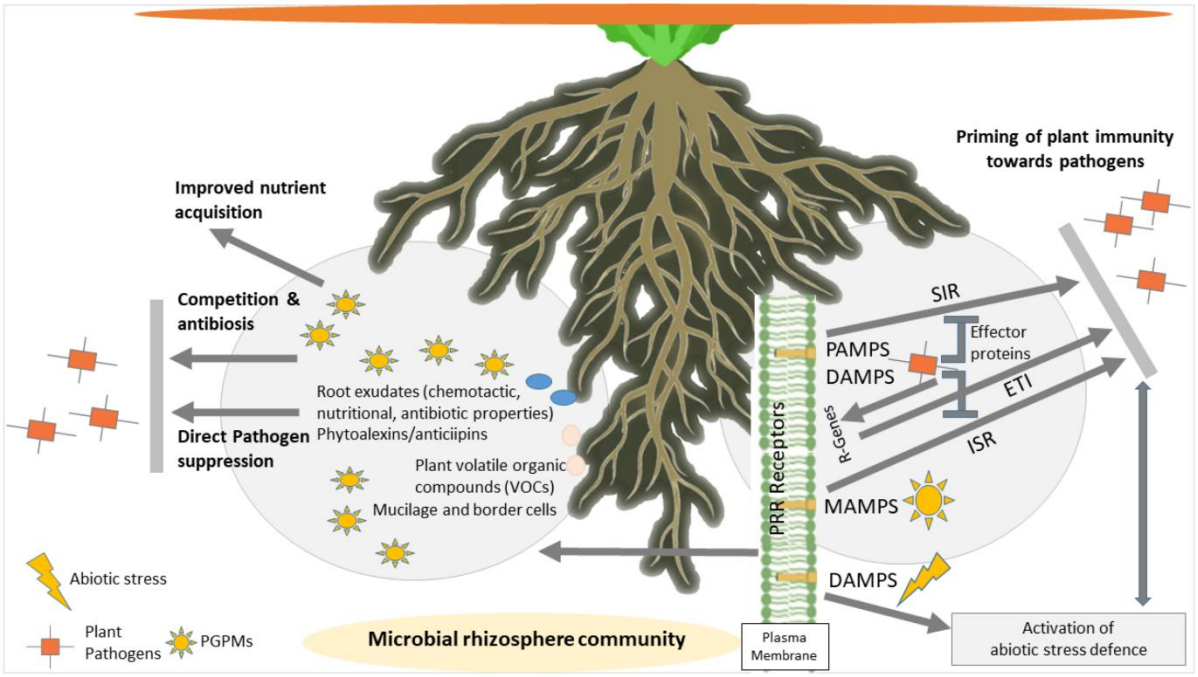

Figure 2: A schematic representation of plant defense mechanisms, induced by rhizospheric interactions of the plant and its microbiome modified after Liu et al. (2020) and Thomas et al. (2021). Plant root created by Behr, J. Final Report DiControl Project 1nd Phase FK031A560E, 2019.

3.6 Objectives and research questions

Although a wide range of microorganisms has been identified in close association with different plant species and cultivars with clear beneficial contributions in promoting plant health, nutrient acquisition and increasing stress resilience of crops, there are still significant knowledge gaps concerning the exploitation of these beneficial interactions for practical applications. Major technical and translation challenges remain, such as inconsistency in efficacy of products based on microbial inoculants under field conditions and a lack of tools and knowledge to manipulate beneficial microbiomes *in situ* by adapted management strategies (Chouhan et al., 2021). Limited yield potential and a lack of reproducibility suggest interactions with other yet unknown or poorly understood factors, which need to be considered additionally for the successful establishment of biologically intensified agricultural production systems. Root exudates and rhizodeposits are considered to play a central role in plant-microbe interactions, shaping the rhizosphere microbiota by interactions with both, beneficial soil microbes and harmful pathogens. However, due to rapid degradation processes in soil, a collection of unaltered root exudates from soil-grown plants is a difficult if not impossible task and unfortunately, there is a serious lack of studies on exudation patterns obtained from plants grown in soil environment (Oburger and Jones, 2018). Accordingly, the

majority of studies on root exudates was performed in more simple, controlled hydroponic culture systems. However, root growth and activity in hydroponics lacking the establishment of a rhizosphere can largely differ from soil-grown plants, and hydroponic culture studies are unable to capture those factors that are influenced by soil properties and plant-microbe interactions taking place simultaneously. Moreover, once released into the rhizosphere, many root exudate compounds can undergo rapid transformations, are prone to adsorption and immobilization processes to the soil matrix and are mixed with compounds originating from turnover of organic matter already present in the bulk soil (Oburger and Jones, 2018). Therefore, rhizosphere soil solutions rather contain rhizosphere products originating from these processes than pure root exudates as a basis for plant-microbial interactions. Additionally, root exudate patterns show a high spatial and temporal variability within developing root systems and are strongly influenced by external factors, such as soil properties (e.g. pH, mechanical impedance, redox status, mineral composition), abiotic stress factors and the plant nutritional status related to the fertilization regime (Neumann and Römheld, 2007). Therefore, there is still a need to understand more of the processes triggering a cascade of feedback loops between plants, rhizodeposition and associated soil microbiota (Oburger and Jones, 2018). Accordingly, the aim of the present study was to address selected factors supposed to interact with root exudation under field conditions and their impact on the composition of the rhizosphere soil solution, plant microbial interactions in the rhizosphere and finally plant performance.

Lettuce was selected as a rather “small” experimental plant, sensitive to various environmental stress factors particularly during early development, which fits well for pot or rhizotron studies. Thus, it is well suited for growth chamber experiments with limited space under controlled conditions. Similar to many other crops, lettuce cultivation faces the problem of severe plant diseases. However, due to a short cultivation period and risk of fungicide residues in consumable products, intensive fungicide application is not recommended (Talubnak et al., 2017). Therefore, the development of alternative strategies for pathogen control remains a major issue and has been intensively addressed already in earlier studies. Disease symptoms caused by soil-borne pathogens are detectable already in the early growth stages of lettuce and well characterized model patho-systems with lettuce are available (Grosch et al., 2004). Fortunately, the prospects of using biocontrol strategies in lettuce are

promising and various plant-growth promoting rhizobacteria strains have been characterized in model experiments and under field conditions, including interactions with the rhizosphere microbiome (Grosch et al., 2005; Scherwinski et al., 2008; Erlacher et al., 2014; Schreiter et al., 2014a, b, 2018). Moreover, techniques for collection and metabolic profiling of rhizosphere soil solutions from soil-grown lettuce plants have been described (Neumann et al., 2014).

Based on this solid body of already available experimental evidence, lettuce is well suited as a model plant to address the yet unknown interactions of rhizodeposits and rhizosphere chemistry with soil microbiota and microbial inoculants and their impact on plant health and stress resilience.

In this context, special emphasis was placed on (i) the role of different soil types on the expression of lettuce bottom rot diseases in a model patho-system with *Rhizoctonia solani*; (ii) the biocontrol efficiency of selected bacterial strains (*Pseudomonas* sp. RU47; *Serratia plymuthica* 3Re-4-18) with documented antagonistic potential against bottom rot disease (Schreiter et al., 2014b; 2018); (iii) the impact of long-term fertilization history (mineral vs. organic and biodynamic fertilization) on plant-microbial interactions in the rhizosphere of lettuce affecting plant performance and health status, using soils from different long-term experimental sites and (iv) the detection of metabolites involved in pathogen defense in the rhizosphere and plant tissues of lettuce.

Based on these research questions, it was postulated as working hypotheses that

(1) root exudation and consequently the composition of the rhizosphere solution is influenced by different soil types,
(2) soil-type dependent differences in the composition of the rhizosphere soil solution affect the virulence of the inoculated pathogen but also the interactions of beneficial soil microbiota with pathogen-antagonistic potential,
(3) the formation of defense compounds in lettuce reduces disease severity of soil-borne pathogens,
(4) long-term fertilization practices will result in characteristic patterns and chemical composition of the rhizosphere soil solution, which influence the recruitment of rhizosphere microbiota,

(5) the related alteration of rhizosphere microbial communities will affect the performance and health of lettuce.

4 Impact of soil type on pathogen-suppressiveness, root-released antifungal compounds and microbial antagonists in the rhizosphere of lettuce

Rhizoctonia solani-induced bottom rot disease in lettuce is difficult to control due to the limited availability of resistant varieties and fungicides (Takasugi et al., 1985; Grosch et al., 2005; Hallmann, 2010). A rising concern related to pesticide residues in consumable products has led to demands for alternative strategies to control soil-borne diseases in agriculture products (Alabouvette et al., 2006; Grosch et al., 2012; Schreiter et al., 2014a).

Previously, soil-type dependent differences in disease suppression of *Rhizoctonia solani*-induced bottom rot disease in lettuce were reported in three soils under the same cropping-history from an unique long-term experimantal system (Schreiter et al., 2014a). The identification of different bacterial community structures in the bulk soil and the correspoding lettuce rhizosphere as well as the successful application of beneficial bacterial biocontrol strains (*Pseudomonas* sp. RU47 and *Serratia plymuthica* 3Re-4-18) as soil inoculum (Schreiter et al., 2014b, 2014c) indicated a complex network of belowground plant-microbial interactions with potential disease suppression in field grown-lettuce plants. Furthermore, distinct quantitative patterns of low-molecular weight compounds in the rhizosphere soil solution of lettuce grown in minirhizotrons (Neumann et al. 2014) were detected. The cultivation of lettuce as model plant in minirhizotrons enabled a non-destructive microsampling of root exudates, followed by gas chromatography–mass spectrometry (GC-MS) analyses. Benzoic acid and lauric acid, which have been reported to have antimicrobial properties against pathogens such as *Rhizoctonia solani* and *Phytium ultimum* and other fungal diseases, are of particular interest (Walters et al., 2003; Yoon et al., 2012).

In consideration of the results of Schreiter et al. (2014a,2014b,2014c) and Neumann et al. (2014), the following study (Windisch et al. 2017; Chapter 4.1) addressed the question whether similar plant-pathogen interactions and biocontrol effects were reproducible using the same field soils in a minirhizotron study with lettuce, cultivated under controlled growth conditions in growth chamber experiments. The main objectives of this study were the characterization of (i) antifungal root exudates in the rhizosphere soil solution of lettuce and (ii) their potential relationships with the presence of the pathogen and/or the two bacterial inoculants *Pseudomonas* sp. RU47 and *Serratia plymuthica 3Re-4-18*. When this study was published, *Pseudomonas* sp. RU47 was referred to as *Pseudomonas jessenii* RU47 (Adesina et

al., 2009); though shortly after its publication whole genome sequencing revealed that this strain belongs to the *P. koreensis* cluster and likely represents a novel distinct species (Kuzmanović et al. 2018).

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4.1 *Rhizoctonia solani* and Bacterial Inoculants Stimulate Root Exudation of Antifungal Compounds in Lettuce in a Soil-Type Specific Manner

Saskia Windisch [1,*], Sebastian Bott [1], Marc-Andreas Ohler [1], Hans-Peter Mock [2], Rico Lippmann [2], Rita Grosch [3], Kornelia Smalla [4], Uwe Ludewig [1] and Günter Neumann [1]

[1] Department of Nutritional Crop Physiology, Institute of Crop Science (340h), University of Hohenheim, 70599 Stuttgart, Germany; SebastianBott@gmx.de (S.B.); Marc_Ohler@web.de (M.-A.O.); u.ludewig@uni-hohenheim.de (U.L.); guenter.neumann@uni-hohenheim.de (G.N.)

[2] Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, 06466 Gatersleben, Germany; mock@ipk-gatersleben.de (H.-P.M.); rico.lippmann@boehringer-ingelheim.com (R.L.)

[3] Department Plant Health, Leibniz Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V., 14979 Großbeeren, Germany; grosch@igzev.de

[4] Julius Kühn-Institut-Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, 38104 Braunschweig, Germany; kornelia.smalla@julius-kuehn.de

***** Correspondence: Saskia.Windisch@uni-hohenheim.de; Tel.: +49-170-459-22253

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Abstract

Previous studies conducted on a unique field site comprising three contrasting soils (diluvial sand DS, alluvial loam AL, loess loam LL) under identical cropping history, demonstrated soil type-dependent differences in biocontrol efficiency against *Rhizoctonia solani*-induced bottom rot disease in lettuce by two bacterial inoculants (*Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re-4-18). Disease severity declined in the order DS > AL > LL. These differences were confirmed under controlled conditions, using the same soils in minirhizotron experiments. Gas chromatography-mass spectrometry (GC-MS) profiling of rhizosphere soil solutions revealed benzoic and lauric acids as antifungal compounds; previously identified in root exudates of lettuce. Pathogen inoculation and pre-inoculation with bacterial inoculants significantly increased the release of antifungal root exudates in a soil type-specific manner; with the highest absolute levels detected on the least-affected LL soil. Soil type-dependent differences were also recorded for the biocontrol effects of the two bacterial inoculants; showing the highest efficiency after double-inoculation on the AL soil. However, this was associated with a reduction of shoot growth and root hair development and a limited micronutrient status of the host plants. Obviously, disease severity and the expression of biocontrol effects are influenced by soil properties with potential impact on reproducibility of practical applications.

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MDPI

Article

Rhizoctonia solani and Bacterial Inoculants Stimulate Root Exudation of Antifungal Compounds in Lettuce in a Soil-Type Specific Manner

Saskia Windisch [1,*], Sebastian Bott [1], Marc-Andreas Ohler [1], Hans-Peter Mock [2], Rico Lippmann [2], Rita Grosch [3], Kornelia Smalla [4], Uwe Ludewig [1] and Günter Neumann [1]

1 Department of Nutritional Crop Physiology, Institute of Crop Science (340h), University of Hohenheim, 70599 Stuttgart, Germany; SebastianBott@gmx.de (S.B.); Marc_Ohler@web.de (M.-A.O.); u.ludewig@uni-hohenheim.de (U.L.); guenter.neumann@uni-hohenheim.de (G.N.)
2 Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, 06466 Gatersleben, Germany; mock@ipk-gatersleben.de (H.-P.M.); rico.lippmann@boehringer-ingelheim.com (R.L.)
3 Department Plant Health, Leibniz Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V., 14979 Großbeeren, Germany; grosch@igzev.de
4 Julius Kühn-Institut-Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, 38104 Braunschweig, Germany; kornelia.smalla@julius-kuehn.de
* Correspondence: Saskia.Windisch@uni-hohenheim.de; Tel.: +49-170-459-22253

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Abstract: Previous studies conducted on a unique field site comprising three contrasting soils (diluvial sand DS, alluvial loam AL, loess loam LL) under identical cropping history, demonstrated soil type-dependent differences in biocontrol efficiency against *Rhizoctonia solani*-induced bottom rot disease in lettuce by two bacterial inoculants (*Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re-4-18). Disease severity declined in the order DS > AL > LL. These differences were confirmed under controlled conditions, using the same soils in minirhizotron experiments. Gas chromatography-mass spectrometry (GC-MS) profiling of rhizosphere soil solutions revealed benzoic and lauric acids as antifungal compounds; previously identified in root exudates of lettuce. Pathogen inoculation and pre-inoculation with bacterial inoculants significantly increased the release of antifungal root exudates in a soil type-specific manner; with the highest absolute levels detected on the least-affected LL soil. Soil type-dependent differences were also recorded for the biocontrol effects of the two bacterial inoculants; showing the highest efficiency after double-inoculation on the AL soil. However, this was associated with a reduction of shoot growth and root hair development and a limited micronutrient status of the host plants. Obviously, disease severity and the expression of biocontrol effects are influenced by soil properties with potential impact on reproducibility of practical applications.

Keywords: lettuce; soil microbiome; root exudates; plant health

1. Introduction

Pathogen-related yield losses are among the most prominent limitations in crop production [1]. The use of resistant cultivars, application of chemical pesticides, as well as crop rotation [2] represent approaches aimed to reduce the incidence and severity of pathogen attacks [1,3]. However, particularly for soil-borne pathogenic fungi with a wide host spectrum, such as *Rhizoctonia*, *Fusarium*, or *Pythium*, the development of efficient control strategies remains a highly challenging task. Long persistence of the pathogen in soils as achieved by the formation of sclerotia [4,5] and saprophytic growth stages, as well as restricted availability of resistant crop cultivars or fungicides are major limiting

factors [6]. However, distinct soil-microbial populations can induce suppression of pathogens [7], and the general suppressiveness of soils against plant pathogens [8] is a characteristic of soil quality and health [9]. This implies that biological control of pathogens via microbial antagonists is a realistic strategy, provided that it is possible to identify specific control strains, which can be transferred to pathogen-affected soils to achieve a specific soil suppressiveness [8] by harnessing the beneficial soil microbiomes against pathogens [10]. However, the exploitation of microbial inoculants as biocontrol agents in crop production systems is frequently hampered by inconsistent results at the field scale [3], likely linked to plant performance. In many cases there is still a substantial lack of knowledge about the factors determining the successful establishment of biocontrol systems.

The rhizosphere of the host plants is the major site for plant-microbial interactions, with rhizodeposition, i.e., the release of organic carbon through plant roots, as the major driving force [11,12]. During these interactions, both beneficial microorganisms and unfavorable pathogens are attracted by the plant roots [12–14] with rhizodepositions acting as signals and as carbon and nitrogen sources, but also as components of plant defense against pathogens. Constant microbiome–root interactions mediate nutrient turnover in the rhizosphere, which enables the plants to acquire nutrients and to receive benefits during growth phases [15,16], while soil-borne pathogens adapt to the rhizosphere, compete with beneficial soil microbes for nutrient availability, and can harm the host plant. Rhizodeposition is highly variable and influenced by many abiotic and biotic factors. It comprises passive losses of organic compounds, as well as highly regulated secretory processes with adaptive functions [17,18]. Due to the central role of rhizodeposits in shaping rhizosphere-microbial communities, a more detailed understanding of the factors determining rhizodeposition with impact on plant pathogens and microbial antagonists in the rhizosphere, will provide important information on the conditions required for successful establishment of biocontrol systems. As an example, biological control of *Rhizoctonia solani* (Kühn) teleomorph *Thanatephorus cucumeris* (A.B. Frank Donk) [6] by bacterial strains of *Pseudomonas jessenii* and *Serratia plymuthica* has been proposed as a promising approach against bottom rot disease in lettuce (*Lactuca sativa*) [2,19–23].

To reveal differences in biocontrol efficiency linked to the soil type and not influenced by local climate or cropping history, Schreiter et al. [23] used a unique experimental field plot system, with three different soil types stored at the same field site for 10 years under the same agricultural management. Clear soil type-dependent differences in the bacterial community structure of the bulk soils and the corresponding lettuce rhizospheres were detected. This was associated with distinct quantitative patterns of low-molecular weight compounds in the rhizosphere soil solutions, mainly representing rhizodeposits, detected by microsampling and gas chromatography-mass spectrometry (GC-MS) profiling in a parallel minirhizotron study conducted with the same soils [16]. Particularly, benzoic acid and lauric acid were of special interest besides other organic acids, various amino acids, amines, sugars, and sugar alcohols. It is documented that these two compounds exhibit antifungal activity against *Rhizoctonia solani*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, and others [24,25], and have been previously identified in root exudates of lettuce grown in soil-free systems in hydroponic culture [26]. Inoculation of the three soils with *Rhizoctonia solani* AG1-IB (*R. solani*) revealed a strong soil type-dependent effect on disease severity under field conditions, while the rhizosphere competence and the biocontrol activity of the pre-inoculated bacterial biocontrol strains *Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re-4-18 were much less affected by soil type differences [3].

Based on this background information, the present study addressed the question of whether similar plant-pathogen interactions and biocontrol effects are reproducible using the same field soils in a minirhizotron study to enable the characterization of antifungal root exudates in the rhizosphere soil solution and their potential relationships with the presence of the pathogen and/or the bacterial inoculants. Lettuce plants (*Lactuca sativa* L. cv. Tizian) were grown in minirhizotrons, equipped with removable root observation windows for non-destructive micro-sampling of rhizosphere soil solution with sorption filters applied to the surface of different root zones, followed by re-extraction and GC-MS analysis [16].

2. Results

2.1. Plant Biomass Production is Affected by Rhizoctonia solani AG1-IB

The severity of *R. solani*-induced bottom rot disease was clearly influenced by the soil type and increased in the order loess loam (LL) < alluvial loam (AL) < diluvial sand (DS) (Table 1), as indicated by declining shoot biomass production. While shoot biomass of lettuce plants grown on the LL soil was not significantly affected by *R. solani* inoculation, shoot biomass significantly declined by 38% on the AL soil. The lowest biomass of non-inoculated control plants was recorded on DS soil, where the plants died within the first week after pathogen inoculation (Table 1).

Table 1. Shoot dry mass (g plant^{-1}) of *Lactuca sativa* L. cv. Tizian grown on three different soils without (control) and with inoculation of *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). (++ Plants died within the first week after *Rhizoctonia solani* inoculation).

Treatment	Diluvial Sand	Alluvial Loam	Loess Loam
Control	0.22 ± 0.2	1.13 ± 0.1 a	1.33 ± 0.5 a
+*Rhizoctonia*	++	0.70 ± 0.3 b	1.45 ± 0.2 a

Means ± standard errors of four independent replicates. Different characters indicate significant differences for a given soil type (*t*-test, $p = 0.05$).

2.2. Biocontrol Activity of Pseudomonas jessenii RU47 and Serratia plymuthica 3Re-4-18

2.2.1. Effects on Shoot Growth

On the DS soil, no biocontrol activity of the bacterial inoculants against *R. solani* was detectable due to rapid seedling decay during the first week after pathogen inoculation (Table 1). The decline of shoot biomass on the AL soil induced by *R. solani* inoculation (Table 1) was mitigated by pre-inoculation with *P. jessenii*, reaching dry matter production not significantly different from the control treatment without *R. solani* inoculation (Figure 1b). Visual scoring of plant damage (Figure 2) revealed the most prominent biocontrol effect by double-inoculation with *P. jessenii* and *S. plymuthica* (+*P. jess./S. ply.*), indicated by survival of all plants in response to *R. solani* inoculation. Despite high biocontrol activity (Figure 2), the lowest shoot biomass production was recorded in the double-inoculated variants in both treatments with and without *R. solani* inoculation (Figure 1a,b). Single inoculation revealed that this effect could be mainly attributed to the presence of *S. plymuthica* (Figure 1b). By contrast, on the LL soil, no significant treatment effects were recorded and shoot biomass production ranged between min. 1.3 g pot^{-1} and max. 1.7 g pot^{-1} (Supplementary Figure S1).

(a)

Figure 1. *Cont.*

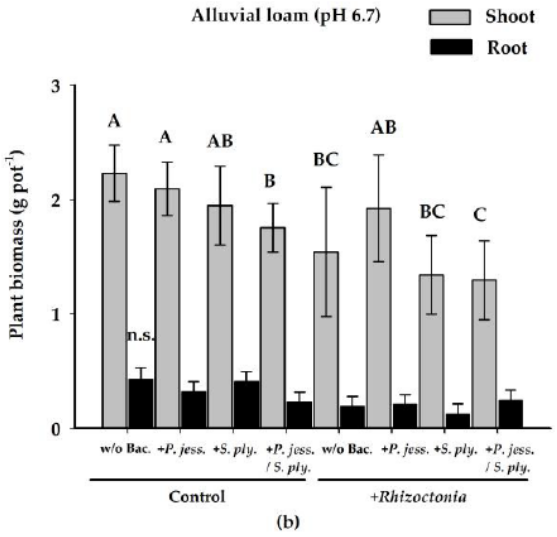

Figure 1. Habitus (**a**) and shoot and root dry mass (g pot^{-1}) (**b**) of *Lactuca sativa* L. cv. Tizian. The plants were grown on alluvial loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± standard errors of four independent replicates. Different characters indicate significant differences (one-way ANOVA, Holm-Sidak test, $p = 0.05$).

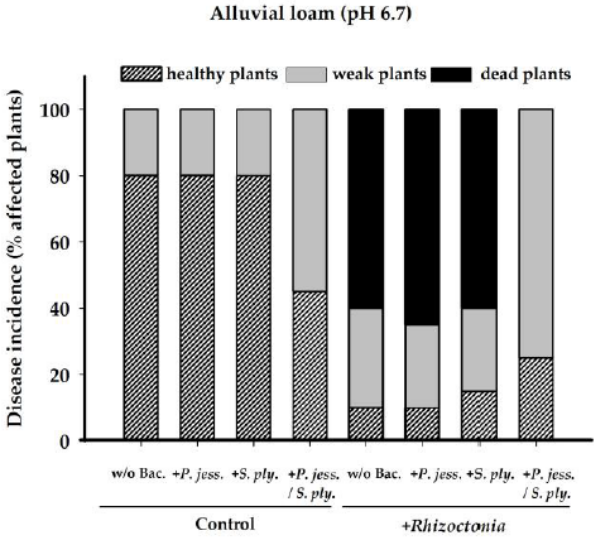

Figure 2. Visual scoring of disease incidence of *Lactuca sativa* L. cv. Tizian, expressed as percentage of healthy, weak (growth depression and leaf necrosis due to fungal infection), and dead plants. The plants were grown on alluvial loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*).

2.2.2. Effects on Root Growth and Morphology

No significant treatment effects were recorded for root biomass of lettuce plants, neither on the AL (Figure 1), nor on the LL soil. A trend for lower root biomass production was detectable for the AL soil in the treatments with *R. solani* inoculation (Figure 1). However, in all treatments with *S. plymuthica* inoculation on the AL soil, root hair length (Figure 3b) was significantly reduced by 30–50% in the presence of *R. solani*. Similarly, root hair density (Figure 3c) declined after pre-inoculation

with *S. plymuthica*, both in the control treatment and after inoculation with *R. solani*. No comparable effects were detectable on the LL soil, where root hair length ranged between 0.71 mm and 0.87 mm in all treatments (Supplementary Figure S2).

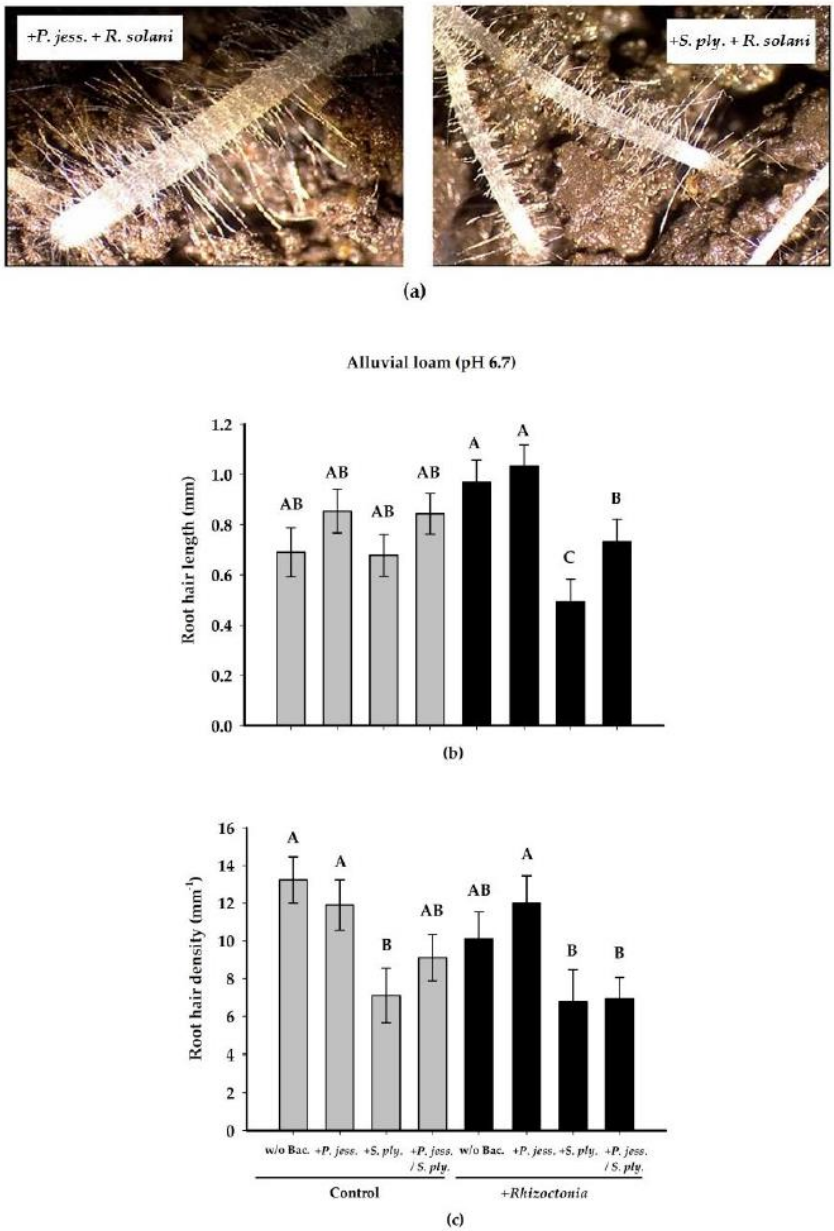

Figure 3. Root hair development (**a**), root hair length (mm) (**b**) and root hair density (mm^{-1}) (**c**) of *Lactuca sativa* L. cv. Tizian. The plants were grown on alluvial loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± standard errors of four independent replicates. Different characters indicate significant differences (one-way ANOVA, Holm-Sidak test, $p = 0.05$).

2.2.3. Plant-Nutritional Status

For the macronutrients N, P, K, and Mg supplied to the soils as fertilizers in sufficient amounts prior to plant cultivation, no significant treatment effects or characteristic deficiency symptoms were recorded for the lettuce plants grown on the AL and the LL soil (not shown). However, on AL

soil, micronutrient shoot concentrations of Zn, Mn, and Fe (Figure 4a–c) of the treatments with *R. solani* inoculation showed a trend of decline in the order w/o Bac. > *P. jessenii* > *S. plymuthica* > *P. jessenii* +*S. plymuthica*, finally reaching critical values close to the deficiency thresholds [27] for the double-inoculation. In the control treatment without *R. solani* inoculation, this effect was restricted to lettuce plants with double-inoculation with *P. jessenii* and *S. plymuthica* (+*P. jess./S. ply.*).

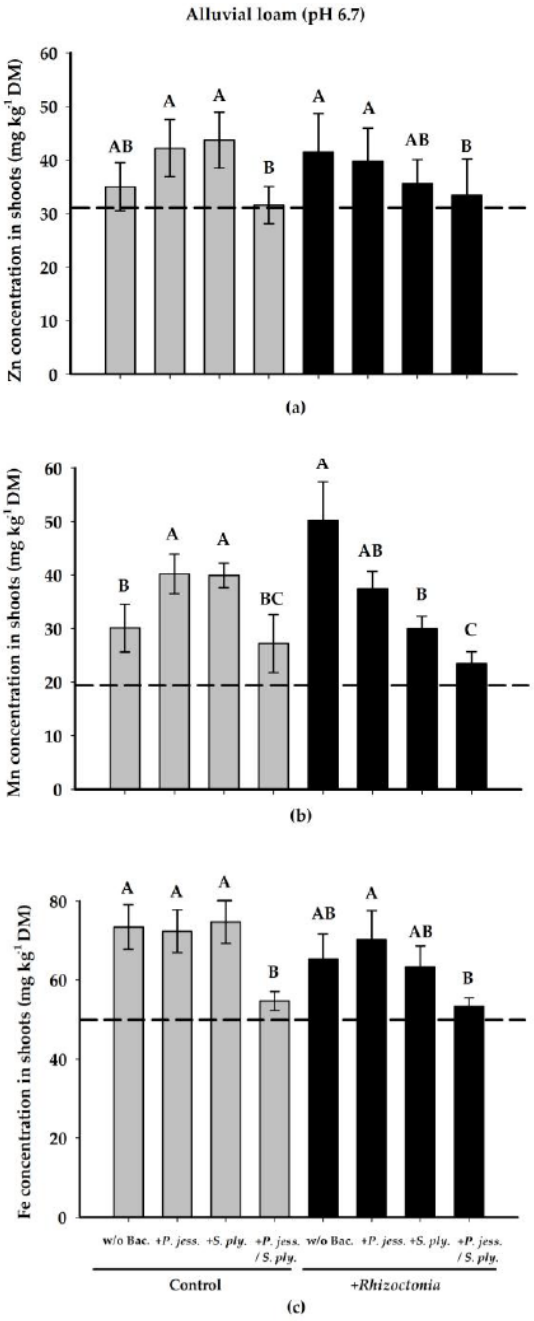

Figure 4. Micronutrient concentrations of Zn (**a**) Mn (**b**) and Fe (**c**) in shoots (mg kg^{-1} DM) of *Lactuca sativa* L. cv. Tizian. The plants were grown on alluvial loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess/S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± standard errors of four independent replicates. The dotted lines indicate the threshold levels for micronutrient deficiency. Different characters indicate significant differences (one-way ANOVA, Holm-Sidak test, $p = 0.05$).

For lettuce plants grown on LL soil, no significant treatment effects in shoot micronutrient concentrations were recorded. Shoot concentrations of Mn ranged between 29.4 mg kg^{-1} DM and 53.4 mg kg^{-1} DM, and Zn between 20.4 mg kg^{-1} DM and 29.5 mg kg^{-1} DM (Supplementary Figure S3). No nutrient analysis was conducted for the plants on DS soil due to the rapid decay already during the first week after *R. solani* inoculation.

2.2.4. Antifungal Compounds in the Rhizosphere Soil Solution

The antifungal compounds benzoic acid and lauric acid [24,25], previously identified in root exudates of lettuce [26], were detected by GC-MS analysis (Table 2) after collection by micro-sampling with sorption filters [16] from lettuce plants in 1–2 cm subapical lateral root zones and in more basal parts of the root zones (8–9 cm behind the root tip). On the AL soil, *R. solani* inoculation significantly increased the concentrations of benzoic acid (Figure 5a) collected from subapical root zones by 234% and in the basal root zones by 296%. Lauric acid concentrations (Figure 5b) tended to be increased only in the basal parts of the root zones, as compared to the control treatment without *R. solani* inoculation (Table 2). In contrast, on the LL soil, only trends for increased root exudation of benzoic acid (11% subapical, 29% basal) and lauric acids (+22% subapical) were induced by *R. solani* inoculation but no significant effects in comparison to the control treatment without *R. solani* inoculation were observed. In general, lauric acid concentrations in samples collected on the LL soil were higher than on AL soil.

Table 2. Benzoic and lauric acid concentrations in rhizosphere soil solutions collected by micro-sampling with sorption filters in 1–2 cm subapical regions of young roots and from older basal root zones (8–9 cm) of *Lactuca sativa* L. cv. Tizian. The plants were grown on alluvial loam treated without (control), with *Rhizoctonia solani* (+*R. solani*), with *Pseudomonas jessenii* (+*P. jess.*) and *Serratia plymuthica* (+*S. ply.*), with *Rhizoctonia solani* and one bacterial inoculant (+*R. solani* +*P. jess*; +*R. solani* +*S. ply.*), with a combination of both bacterial inoculants (+*P. jess*/*S. ply.*) and with *Rhizoctonia solani* and both bacterial inoculants (+*R. solani* +*P. jess./S. ply.*). Relative values based on peak areas (gas chromatography-mass spectrometry (GC-MS) analysis) after subtraction of background levels in bulk soil samples.

Treatment	Benzoic Acid				Lauric Acid			
	Subapical (Young)		Basal (Old)		Subapical (Young)		Basal (Old)	
	AL	LL	AL	LL	AL	LL	AL	LL
Control	3.92 a	5.00 a	2.77 a	6.30 a	1.76 a	4.29 a	0.14 a	14.00 a
+*R. solani*	13.07 b	5.53 a	10.97 b	8.12 a	0.85 a	5.25 a	1.36 ab	9.56 a
+*P. jess.*	12.84 b	10.54 b	12.98 b	13.58 ab	1.00 a	10.55 b	1.52 b	21.80 a
+*S. ply.*	13.08 b	10.49 b	12.75 b	9.58 ab	0.51 a	10.66 b	1.01 ab	9.40 a
+*R. solani* +*P. jess.*	10.75 b	9.62 b	13.65 b	15.53 b	0.92 a	9.29 b	1.34 ab	25.03 a
+*R. solani* +*S. ply.*	11.71 b	8.63 b	12.68 b	15.79 b	1.00 a	7.82 ab	0.94 ab	29.87 a
+*P. jess./S. ply.*	12.84 b	N.d.	12.63 b	N.d.	0.85 a	N.d.	0.93 ab	N.d.
+*R. solani* +*P. jess./S. ply.*	12.14 b	N.d.	11.31 b	N.d.	0.43 a	N.d.	1.09 ab	N.d.

Means of three independent replicates. In each column different characters indicate significant differences (one-way ANOVA, Holm-Sidak test, $p = 0.05$). N.d. = not determined. DS: Diluvial sand, AL: alluvial loam, LL: loess loam.

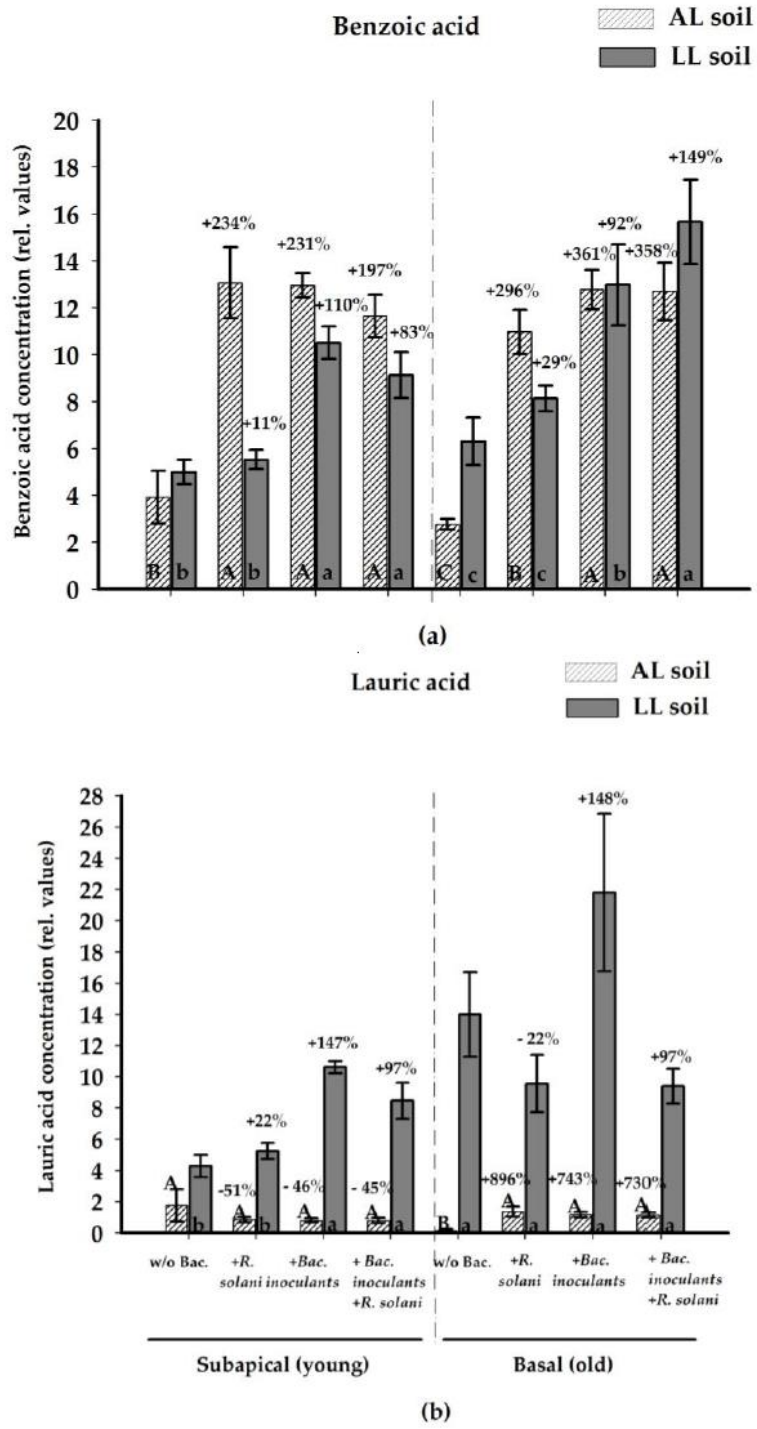

Figure 5. Concentrations and relative changes (% compared to the untreated controls) of benzoic acid (**a**) and lauric acid (**b**) in rhizosphere soil solutions collected from 1–2 cm subapical regions of young roots and from older basal root zones (8–9 cm basal roots) of *Lactuca sativa* L. cv. Tizian. The plants were grown on alluvial loam (AL) and loess loam (LL) treated without bacterial inoculation (w/o Bac.), with *Rhizoctonia solani* AG1-IB (+*R. solani*), with bacterial inoculants (+*Bac. inoculants* = *Pseudomonas jessenii* RU47 and/or *Serratia plymuthica* 3Re-4-18) and with a combination of bacterial inoculants and *Rhizoctonia solani* AG1-IB (+*Bac. inoculants* +*R. solani*). Relative values based on peak area (GC-MS analysis) after subtraction of background levels in bulk soil samples. Means ± standard errors of 3–9 independent replicates. For each soil type, different characters indicate significant differences (one-way ANOVA, Holm-Sidak test, $p = 0.05$).

Compared to the non-inoculated control treatment, pre-inoculation with *P. jessenii*, *S. plymuthica* and a combination of both (+*P. jess*/*S. ply.*) increased root exudation of benzoic acid (Figure 5a) in the subapical and basal root zones on the AL soil between 197% and 361% and on the LL soil between 83% and 149%. Since no significant differences between the bacterial inoculants were recorded (Table 2), Figure 5 summarizes the effects of all bacterial inoculants. An increased exudation of lauric acid was observed in the basal root zones on AL soil (730% and 743%) and additionally on LL soil in the subapical root zones (97% and 147%). The highest lauric acid concentrations and the highest levels of antifungal root exudates in general were recorded on LL soil (Figure 5).

3. Discussion

3.1. Plant Pathogen Interactions

Similar to previously reported field observations [3], soil type-dependent differences in severity of bottom rot disease in lettuce, induced by inoculation with *R. solani* AG1-IB (predominant in field-grown lettuce in Germany [28]), were confirmed in the present minirhizotron study. The experiment was conducted under controlled conditions on three different soils (DS: diluvial sand, pH 6.1; AL: alluvial loam, pH 6.7; LL: loess loam, pH 7.1) with the same cropping history during the last 10 years. Shoot biomass production of *R. solani*-inoculated lettuce plants declined in the order LL > AL > DS soil (Table 1), reflecting the negative impact of the pathogen on lettuce growth as previously reported on the same soils under field conditions [23]. However, in minirhizotron culture, disease symptoms on lettuce grown on DS soil appeared much faster, and in contrast to the field experiments all plants died already during the first week after pathogen inoculation. The higher conduciveness for bottom rot disease of the DS soil observed in all experiments may be caused by the bigger pore sizes and better oxygen availability in sandy soils, which enables more rapid hyphal growth of *R. solani* towards the host plant [3,29]. The more severe disease symptoms in the minirhizotron study may be attributed to higher rooting densities in the limited soil volume of the minirhizotrons and due to shorter spatial distances between the pathogen and the host plant in comparison to field conditions with larger soil volume. Moreover, the constant temperature conditions in the growth chamber (23–25 °C) in the optimum range for hyphal growth of *R. solani* AG1-IB [28] could further promote the infection process, particularly on the DS soil with the weakest plant development even in the non-inoculated control (Table 1). Accordingly, Schreiter et al. [3] reported a certain background infection potential for bottom rot disease in all investigated soils, even without artificial inoculation with *R. solani* AG1-IB, with the lowest disease severity on LL soil, in comparison to disease severity for plants grown on DS and AL soil.

On the AL soil, *R. solani* inoculation resulted in a 60% reduction in shoot biomass (Table 1) and 40% of the plants finally survived (Figure 2). In contrast, no apparent bottom rot symptoms were detected on the LL soil (Table 1) and supported the observed results of higher soil suppressiveness in the field experiment [3], reflected also by the lowest infection potential on the non-inoculated control soils [3]. Accordingly, on the AL soil, pathogen inoculation significantly increased the concentrations of benzoic and lauric acids (Table 2, Figure 5) in the rhizosphere soil solutions (234% to ca. 900%), previously identified as root exudates of lettuce with documented antifungal activity against *Rhizoctonia*, *Fusarium*, and other pathogenic fungi [24,25]. In contrast, only a non-significant trend for increased root exudation of the antifungal compounds by a maximum of 30% was detected in the rhizosphere on the LL soil with antifungal suppressive potential (Table 2; Figure 5). These findings suggest that the release of the antifungal root exudates is part of the pathogen defense response in lettuce, as previously reported also for *Fusarium*-resistant peanut cultivars [30]. *Rhizoctoniasolani*-affected plants on the AL soil obviously responded with increased exudation of antibiotic compounds, while only a weak response was triggered in the *R. solani* inoculated plants grown on the LL soil with low *R. solani* infection potential.

Besides an increased release of antifungal root exudates in response to *R. solani* infection on AL soil in basal parts of the root system close to the inoculation sites, benzoic acid was also detected in the youngest root zones (1–2 cm behind the root tip), up to 30 cm below the *R. solani* infection sites (Table 2, Figure 5). *Rhizoctonia solani* infection sites are usually located at the root-shoot junction and at the lower leaves with direct soil contact [28,29]. Thus, release of antifungal root exudates, even five weeks after inoculation with the pathogen, may indicate a systemic response of lettuce to *R. solani* infection. Accordingly, previous studies have also reported the systemic induction of biosynthetic pathways for aromatic compounds in response to *R. solani* infection and their potential role in pathogen defense in different crops, such as potato and rice [29,31].

3.2. Disease Suppression by Bacterial Inoculants

Two bacterial strains, *Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re-4-18 [32], characterized for good biocontrol effects against bottom rot disease in previous experiments [6,21], were selected as biocontrol strains used for pre-inoculation of the lettuce plants. On the DS soil with the weakest plant development even in the controls without pathogen inoculation (Table 1), and with the most severe bottom rot symptoms, no protective effects of the bacterial inoculants against *R. solani* were recorded. In contrast, on the AL soil, particularly pre-inoculation with *P. jessenii* RU47 tended to increase biomass production of *R. solani*-affected plants (Figure 1). However, the most pronounced biocontrol effect was evaluated by a double-inoculation with both bacterial strains where, in contrast to the other treatments, all plants survived (Figure 2). The increased expression of antagonistic effects after double inoculation with different biocontrol strains has been similarly reported in previous studies [33] and may be attributed to additive effects of different antibiotics, cellulolytic enzymes, and induction of plant defense responses, but also to a broader activity spectrum under different environmental conditions [34]. By contrast, no additional beneficial effects of pre-inoculation with the bacterial inoculants were observed on the least conductive LL soil (Supplementary Figure S1) where the lettuce plants did not show any visible symptoms of bottom rot disease.

Interestingly, similar to *R. solani* infection, inoculation with the bacterial inoculants also increased the exudation of benzoic and lauric acids on the AL soil. Moreover, the same response was recorded on the more suppressive LL soil where *R. solani* inoculation had only marginal effects on the release of the antifungal root exudates (Table 2, Figure 5). In accordance with the absence of disease symptoms, the highest total levels of the antifungal compounds (sum of benzoic and lauric acids) were detected in root exudates of lettuce plants grown on the LL soil (Table 2, Figure 5), which may reflect a particularly intense expression of pathogen defense mechanisms. Even in the variant without pathogen inoculation, high background levels of lauric and benzoic acids were detectable on the LL soil (Table 2, Figure 5). This raises the question of whether generally high levels of antifungal root exudates contributed to the particularly low conductivity for *Rhizoctonia* bottom rot disease on this soil. Interestingly, Schreiter et al. [3] observed a soil type-dependent rhizosphere effect of the lettuce plants on the abundance of bacteria with the capacity to degrade aromatic hydrocarbons (particularly *Sphingomonas*), declining in the order DS > AL > LL soil, which follows the soil type-dependent expression in severity of *Rhizoctonia*-induced disease symptoms (Table 1). This may reflect a declining capacity for degradation of the antifungal root exudates as secondary metabolites by members of the indigenous microflora, resulting in the highest accumulation on LL soil, which may be also responsible for the low background infection potential for bottom rot disease on this soil [3]. Apart from benzoic and lauric acids, Neumann et al. [16] also reported particularly high levels of sugars, amino acids, and organic acids in the rhizosphere soil solutions collected from lettuce plants grown on the LL soil, associated with the highest shoot biomass production (Table 1). This may indicate a generally higher capacity for root exudation due to stronger plant development on the LL soil, associated with a higher photosynthetic capacity as a major driving force for rhizodeposition [17].

The exudation of benzoic acid was more strongly induced by bacterial inoculants or by pathogen–inoculant combinations than by sole inoculation with *R. solani* (Figure 5). These findings

suggest that pre-inoculation with the bacterial inoculants can exert long-lasting priming effects on induction of plant defense reactions in the form of antifungal root exudates still detectable five to six weeks after the last inoculation, as similarly reported for the induction of pathogen defense responses by various rhizosphere bacteria [35]. This is also in line with the high rhizosphere competence of the selected bacterial inoculants reported by Schreiter et al. [23], since the observed release stimulation of the antifungal root exudates, even in the youngest roots at the end of the culture period indicates that the inoculants were still active.

Root colonization with bacterial inoculants is often preferentially located in the more basal parts of the root system [25,34] close to the initial inoculation sites. However, similar to the effects of *R. solani* inoculation, increased root exudation of benzoic and lauric acids induced by the bacterial inoculants was detectable on both soils, not only in older parts of the root system, but even in the young root tips (Table 2, Figure 5). This suggests a systemic effect for bacterial inoculants also. A systemic induction of plant defense responses in a combination of aromatic compounds has been previously proposed for the genera *Pseudomonas* and *Serratia* [36,37]. This spatial exudation pattern may contribute to a protective effect not only against *Rhizoctonia* bottom rot, but also against other more typical root pathogens [24,25].

3.3. Negative Side Effects of the Bacterial Inoculants

Although the most pronounced biocontrol effect was observed in treatments with double inoculation of *P. jessenii* and *S. plymuthica* on the AL soil, indicated by the absence of any dead plants after *R. solani* infection (Figure 2), this effect was associated with lower biomass production (Figure 1). Additionally, reduced root hair length and density (Figure 3) and a decline of the plant micronutrient status (Figure 4) close to the deficiency thresholds [27] was detectable.

The decline of plant micronutrient status was associated with the expression of typical symptoms of micronutrient limitation (Supplementary Figure S4). These effects could be attributed to the presence of *S. plymuthica*, since they were not only detectable after double-inoculations, but to a lesser extent also for single inoculation with *S. plymuthica*. The inhibitory effects were particularly expressed in *R. solani*-affected plants. Similarly, Schreiter et al. [23] reported a reduction of shoot growth under field conditions, associated with high rhizosphere abundance of *S. plymuthica*. Inhibition of plant growth and induction of micronutrient limitation may be related to the fact that both inoculant strains are effective producers of siderophores [38,39], known as efficient chelators for iron but also other divalent metal cations [40]. A comparison of the nutritional status of lettuce plants cultivated on the three investigated soils without pathogen inoculation [16] revealed the lowest micronutrient levels close to the deficiency threshold on the AL soil. Under these conditions, micronutrient availability to lettuce plants additionally stressed by *R. solani* infection may be negatively affected by competitive interactions with efficient micronutrient-chelating siderophores released by the bacterial inoculants. Moreover, bacterial siderophores are also discussed as inducers of systemic plant defense responses [38,41] and this may apply also for root exudation of benzoic acid, similarly observed in the present study.

Additionally, in *S. plymuthica*- and double-inoculated lettuce plants grown on the AL soil, both the elongation and density of the root hairs were significantly reduced. Growth and formation of root hairs are usually increased as an adaptive response to improve iron acquisition in so-called strategy I plants (dicotyledonous plants, such as lettuce). The stimulation of root hair development is triggered by Fe-deficiency-induced ethylene production [18,42]. However, a wide-spread feature of many plant growth-promoting bacteria is the suppression of excessive ethylene production with inhibitory effects on plant growth by enzymatic degradation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC desaminase [43] or by other yet unidentified mechanisms as discussed also for *S. plymuthica* [44]. This would explain the inhibitory effect on Fe deficiency-induced root hair development in *S. plymuthica*-inoculated lettuce plants (Figure 4c), which would further counteract nutrient acquisition on the AL soil with low micronutrient solubility, where root hair development is of particular importance [42]. Interestingly, these effects were not detectable on the LL soil, indicating

that the inhibitory effects of the microbial inoculants represent a soil-specific feature. As a possible explanation, plants grown on the LL soil were less affected by *R. solani* and may therefore exhibit a higher level of tolerance against nutrient limitation.

4. Materials and Methods

4.1. Lettuce Cultivation

Lettuce (*Lactuca sativa* L. cv. Tizian) cultivation, harvest, and root exudate sampling were performed as described by Neumann et al. [16]. To achieve homogenous plant development, lettuce seedlings were pre-cultivated in seed trays in a peat culture substrate–sand mixture (7:3 *w*/*w*; TKS1 Anzuchtsubstrat, Floragard, Germany) to the two-leaf stage (BBCH 12). Thereafter, minirhizotrons (36 × 11.5 × 2.5 cm) equipped with removable root observation windows were used for plant cultivation. Three contrasting soil types originating from a unique long-term field plot with a 10-year identical crop management history, located at the Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren (Germany), were used for plant cultivation. Soil characteristics were: (i) Arenic-Luvisol pH 6.1 (diluvial sand = DS) with silty sand and 5.5% clay; (ii) Gleyic-Fluvisol pH 6.7 (alluvial loam = AL) heavy sandy loam with 27.5% clay; and (iii) Luvic-Phaeozem pH 7.1 (Loess loam = LL), medium content of clayey silt with 17.2% clay. Macronutrient fertilization with 100 mg N kg^{-1} soil, 100 mg P kg^{-1} soil, 150 mg K kg^{-1} soil and 50 mg Mg kg^{-1} soil (supplied as Ca $(NO_3)_2$; Ca $(H_2PO_4)_2$; K_2SO_4; and $MgSO_4$) was applied to cover the plant demand during the culture period. The soil moisture level was adjusted to 18–20% *w*/*w* and controlled gravimetrically throughout the culture period. The transplanting of two pre-cultivated lettuce seedlings was undertaken at BBCH 12 and the minirhizotrons were fixed at an angle of 45° to stimulate root growth along the root observation window for exudate sampling. The lettuce plants were cultivated in a growth chamber with a 16 h light period at 200 µmol m^{-2} s^{-1}, 60% rel. humidity, and a day/night temperature regime of 25 °C/23 °C. Final plant harvest was conducted eight weeks after sowing (BBCH 19). For further analysis, roots were washed out from soil with sieves (0.5 to 1 mm) and their fresh weight was recorded. Root hair length (mm) and root hair density (mm^{-1}) were recorded with a video macroscope (Stemi2000-C, Zeiss, Oberkochen, Germany), and analysed with the Axion Vision 30.0 software system (Zeiss, Oberkochen, Germany). For plant analysis of mineral nutrients, the fresh shoot biomass was recorded and dried at 60 °C.

4.2. Inoculation with Bacterial Inoculants

Application of bacterial inoculants was performed according to Schreiter et al. [3]. Seed treatment was conducted with a 29 °C overnight culture of *Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re-4-18 on King's B agar (Merck KGaA, Darmstadt, Germany) supplemented with rifampicin (75 mg mL^{-1}). Bacterial cells were suspended in sterile 0.3% NaCl solution (*w*/*v*) and adjusted to a cell density of 10^8 colony forming units (CFU) mL^{-1} by spectrophotometric determination. Lettuce seeds were coated with 250 µL of the bacterial cell suspensions per 100 seeds by dripping on the seed surface under vigorous shaking. At the end of the pre-culture period of the plants on peat culture substrate (BBCH 12), a second inoculation was performed by root drenching according to Schreiter et al. [3] with 20 mL of bacterial suspension in 0.3% NaCl solution per plant.

4.3. Pathogen Inoculation

The *Rhizoctonia solani* AG1-IB isolate 7/3 from the strain collection of the Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren (Germany) was used as model pathogen. The inoculum was multiplied over three weeks at 25 °C on Petri dishes using potato extract glucose agar as the growth medium (Roth, Karlsruhe, Germany), previously inoculated with two agar blocks each (2 cm diameter) of a stock culture. The inoculation of the pathogen into the soil of the minirhizotrons was performed directly after transplanting of the lettuce seedlings (BBCH 12) from the pre-culture medium.

After opening of the root observation windows, seven *Rhizoctonia*-infected agar blocks (2 cm diameter) were inserted into the soil at a distance of 5 cm to the roots, avoiding direct root contact.

4.4. Nutrient Analysis of Plant Biomass

For analysis of mineral nutrients in the shoot biomass, 500 mg of dried plant material was ashed in a muffle furnace for 5 h at 500 °C. Thereafter, the samples were extracted twice with 2 mL of 3.4 M HNO_3 to precipitate SiO_2. The plant ash was dissolved in 2 mL of 4 M HCL and boiled for 2 min after 1:10 dilution with hot deionized water. Subsequently, 0.1 mL Cs/La buffer was added to 4.9 mL ash solution and Fe, Zn, and Mn concentrations were determined using atomic absorption spectrometry (AAS, Unicam 939, Offenbach/Main, Germany). Spectrophotometric determination of orthophosphate (Hitachi U-3300 Spectrophotometer, Hitachi Ltd. Corporation, Chiyoda, Tokio, Japan) was conducted after addition of molybdate-vanadate color reagent according to the method of Gericke and Kurmies [45]). Determination of Mg was performed by atomic absorption spectrometry, while K and Ca were measured by flame emission photometry (ELEX 6361, Eppendorf, Hamburg, Germany).

4.5. Analysis of Rhizosphere Soil Solution

Microsampling of rhizosphere soil solutions was conducted with sorption filters placed onto the surface of lateral roots growing along the root observation window in subapical root zones (1–2 cm behind the root tip) and older parts of the root system (8–9 cm behind the root tip) according to the method described by Haase et al. [46]. The exudate sampling was performed five weeks after transplanting during vegetative growth, when carbohydrate partitioning to the roots and root exudation are considered most active [47,48]. After a collection period of 4 h, for each minirhizotron, ten sorption filters of the respective root zones were pooled and stored at −20 °C for further analysis.

The sorption filters were re-extracted with 80% methanol. After centrifugation, extracts were dried at 30 °C in a Speed Vac Concentrator (Savant, Farmington, CT, USA) at 30 °C and stored at −80 °C for further analysis. For GC-MS analysis, the residues were re-dissolved in 200 µL methanol, transferred into GC-MS glass vials and evaporated to dryness at 30 °C. Derivatization was performed online directly prior to injection using a MPS Autosampler (Gerstel, Mühlheim a.d.R., Germany) by adding 25 µL methoxyhydroxymethylamine (20 mg mL^{-1} in pyridine) and incubated for 2 h at 37 °C, 350 rpm. Thereafter, 50 µL MSTFA (*N*-Trimethylsilyl-*N*-methyl trifluoroacetamide) as a silylating reagent that forms volatile derivatives for GC-MS analysis including standard alkanes from Sigma C7–C30 (0.1% *v*/*v*) were added and incubated for 30 min at 37 °C, 350 rpm. One µL aliquots were analyzed by an Agilent7890 gas chromatograph (Agilent, Santa Clara, CA, USA) in the splitless mode, coupled to a TOF mass spectrometer GCT Premier (Waters Corporation, Eschborn, Germany). Separation was performed on a Rxi®5 Sil MS Integra column (Restek, Bellefonte, PA, USA) with 0.25 mm inner diameter and 0.25 µm film thickness, including a 5 m guard column according to Lippmann et al. [49]. Injection temperature was adjusted to 240 °C. The temperature program for GC separation was: 3 min 80 °C isothermal followed by a ramp of 5 °C min^{-1} to 300 °C for 5 min. Mass spectrometry (MS) data was recorded with Mass Lynx 4.1 (Waters Corporation, Milford, Massachusetts, USA) at a rate of 10 spectra s^{-1} in a range of 50–700 *m*/*z*. The metabolites were identified automatically with the internal software ChromaLynx (Waters Corporation, Milford, Massachusetty, USA) using the NIST 5 library and interesting components were verified manually by comparison with reference spectra. Relative quantification was based on comparative analysis of peak area.

5. Conclusions

The results of the present study demonstrate that even under controlled conditions, the soil type effects on the expression of bottom rot disease severity previously described in field experiments [3,23] were still detectable and even more strongly expressed. This underlines the importance of the culture conditions for determining the expression of disease severity and for antagonistic interactions with biocontrol agents. Our study showed for the first time that the release of benzoic and lauric acids as

root exudates with antifungal activity was triggered by the presence of the pathogen *Rhizoctonia solani* and even more strongly by the bacterial inoculants *Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re4-18 in a soil type-specific manner. The highest level of antifungal root exudates was detectable in the rhizosphere soil solutions of the LL soil with the lowest conductivity for bottom rot disease. These findings strongly suggest a role of the antifungal root exudates in the defense reaction of lettuce against bottom rot disease as a biocontrol mechanism of *P. jessenii* RU47 and *S. plymuthica* 3Re4-18. This further underlines the central role of the host plant status which in turn is determined by physical and chemical soil properties, the interactions with the respective soil microbiomes, and of course climatic conditions. Obviously, high rhizosphere competence and a high antagonistic potential of bacterial inoculants are important, but not the only features determining a successful establishment of biocontrol effects in lettuce cultivation.

Supplementary Materials: The following materials are available online at www.mdpi.com/2073-4395/7/2/44/s1. Figure S1. Shoot dry mass of *Lactuca sativa* L. cv. Tizian. The plants were grown on loess loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± SE of four independent replicates. Figure S2. Root hair length (mm) of *Lactuca sativa* L. cv. Tizian. The plants were grown on loess loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± SE of four independent replicates. Figure S3. Micronutrient concentration of Zn (a) and Mn (b) in shoots (mg kg^{-1} DM) of *Lactuca sativa* L. cv. Tizian. The plants were grown on loess loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*) a combination of both (+*P. jess*/*S. ply.*) with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± SE of four independent replicates. Figure S4. Symptoms of micronutrient deficiencies (Zn, Mn, Fe) in *Lactuca sativa* L. cv. Tizian grown in a hydroponic culture system with controlled supply of mineral nutrients: 2 mM Ca $(NO_3)_2$, 0.25 mM KH_2PO_4, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 80 µM/10 µM Fe-EDTA, 10 µM H_3BO_3, 0.5 µM/0.1 µM $ZnSO_4$, 0.5 µM/0.1 µM $MnSO_4$, 0.2 µM $CuSO_4$ and 0.01 µM $(NH_4)_6Mo_7O_{24}$.

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4.2 Supplementary materials

The supplementary materials for this article can be found online at: www.mdpi.com/2073-4395/7/2/44/s1

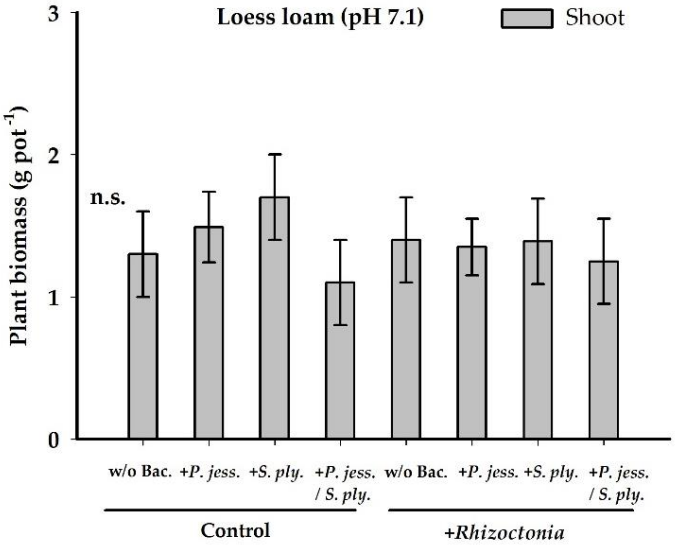

Figure S1. Shoot dry mass of *Lactuca sativa* L. cv. Tizian. The plants were grown on loess loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± SE of four independent replicates.

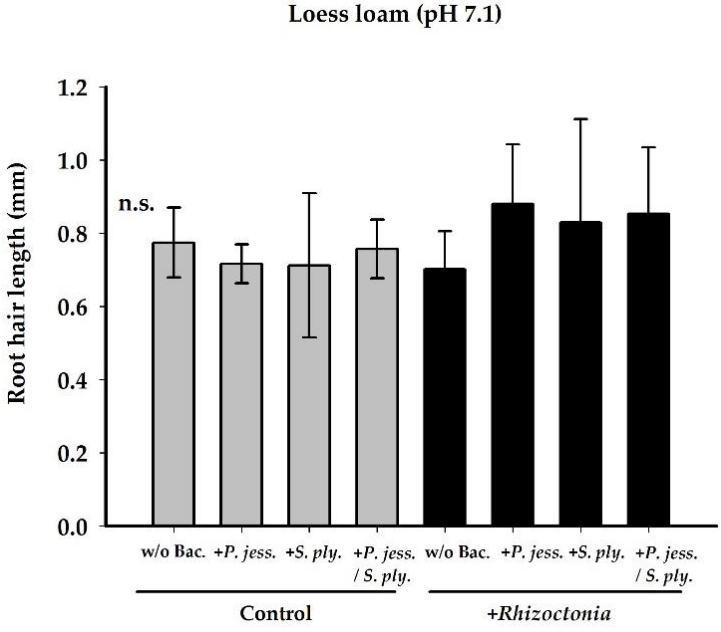

Figure S2. Root hair length (mm) of *Lactuca sativa* L. cv. Tizian. The plants were grown on loess loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*), a combination of both (+*P. jess*/*S. ply.*), with and without (Control)

subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± SE of four independent replicates.

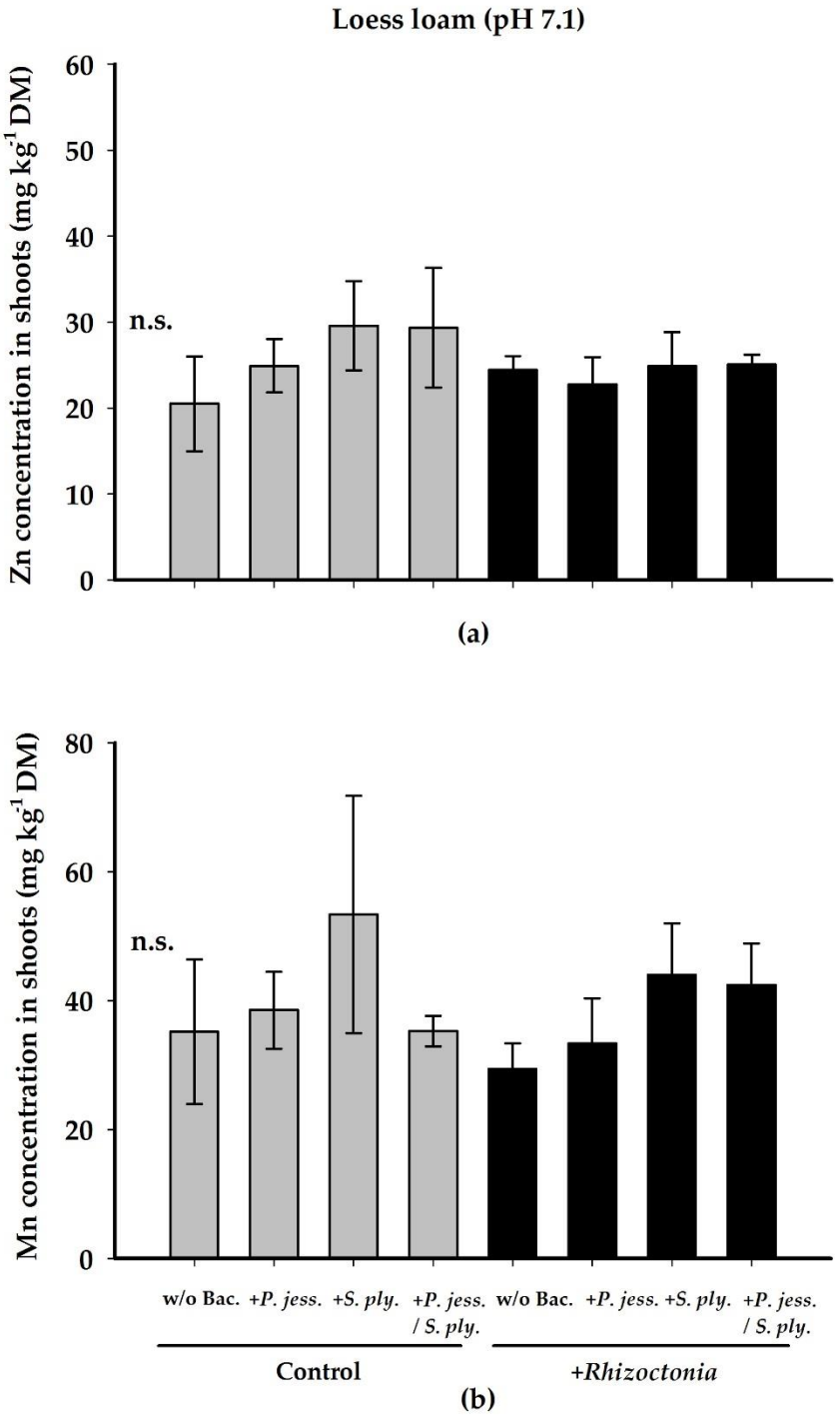

Figure S3. Micronutrient concentration of Zn (a) and Mn (b) in shoots (mg kg^{-1} DM) of *Lactuca sativa* L. cv. Tizian. The plants were grown on loess loam without bacterial inoculation (w/o Bac.), pre-inoculated with *Pseudomonas jessenii* RU47 (+*P. jess.*), *Serratia plymuthica* 3Re-4-18 (+*S. ply.*) a combination of both (+*P. jess*/*S. ply.*) with and without (Control) subsequent inoculation with *Rhizoctonia solani* AG1-IB (+*Rhizoctonia*). Means ± SE of four independent replicates.

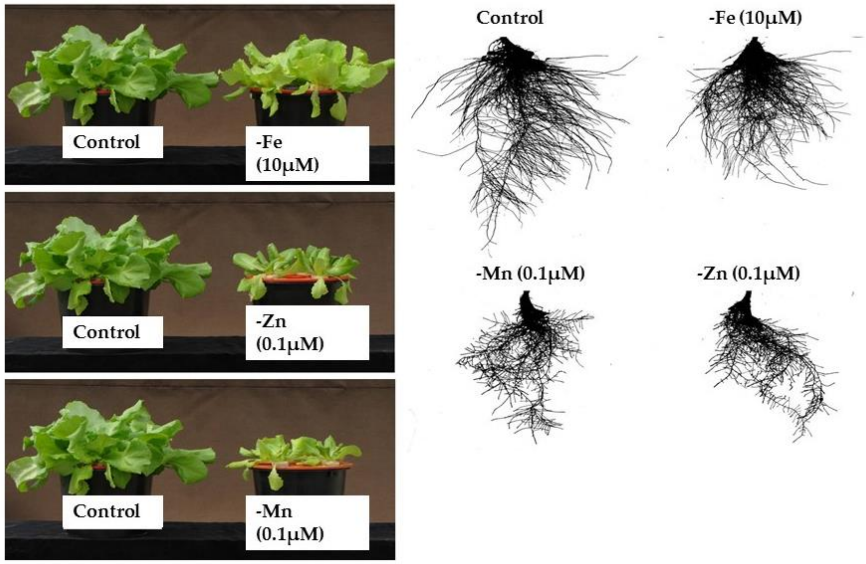

Figure S4. Symptoms of micronutrient deficiencies (Zn, Mn, Fe) in *Lactuca sativa* L. cv. Tizian grown in a hydroponic culture system with controlled supply of mineral nutrients: 2 mM Ca $(NO_3)_2$, 0.25 mM KH_2PO_4, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 80 µM/10 µM Fe-EDTA, 10 µM H_3BO_3, 0.5 µM/0.1 µM $ZnSO_4$, 0.5 µM/0.1 µM $MnSO_4$, 0.2 µM $CuSO_4$ and 0.01 µM $(NH_4)_6Mo_7O_{24}$.

5 The effect of antimicrobial compounds on soil-borne pathogens in lettuce

Plant-induced defense responses are described as plant strategies to counteract pathogen-related damage and yield loss (Grisebach and Ebel, 1978) and have been investigated in lettuce (Neumann et al., 2014; Talubnak et al., 2017; Windisch et al., 2017). In the model pathosystem of lettuce (*Lactuca sativa*) and *Rhizoctonia solani* (Windisch et al. 2017; Chapter 4.1), a relationship between the amount of benzoic acid in the rhizosphere soil solution and suppression potential of the soil against *R. solani* was found. Furthermore, a disease reducing effect of benzoic acid in the rhizosphere and two selected bacterial strains (*Pseudomonas* sp. RU47 and *Serratia plymuthica* 3Re-4-18) with antifungal properties on *R. solani* was demonstrated in model experiments in minirhizotrons as well as under field conditions (Neumann et al., 2014; Schreiter et al., 2014; Windisch et al., 2017). These results indicated that benzoic acid promotes plant defense and was enhanced by the presence of beneficial rhizosphere microorganisms in the rhizosphere of lettuce (Windisch et al., 2017). However, the question remains whether this compound is released from plant roots itself or accumulates in the rhizosphere solution as rhizosphere product of microbial origin.

In addition, sesquiterpene phytoalexins (lettucenins) (Grisebach and Ebel, 1978, 1983) have been described as antimicrobial secondary metabolites in lettuce defense mechanisms (Yean et al., 2009; Mai and Glomb, 2014; Talubnak et al., 2017). Lettucenins accumulate in the leaf tissue when exposed to microbial pathogens (i.e. *Fusarium oxysporum*, *Pythium aphanidermatum*) and in plant tissues in response to membrane damage after chemical elicitation with $CuSO_4$ or $AgNO_3$ or abiotic stress (Talubnak et al., 2017). The following study (Windisch et al. 2021b; Chapter 5.1) addressed the hypotheses that (i) benzoic acid is released from lettuce roots in response to pathogen infection by *R. solani* and accumulates in the rhizosphere in pathogen suppressive concentrations and (ii) lettucenins, which have so far only been detected in leaves play a role in pathogen defense in the rhizosphere of lettuce.

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5.1 Role of Benzoic Acid and Lettucenin A in the Defense Response of Lettuce against Soil-Borne Pathogens

Saskia Windisch [1,*], Anja Walter [1], Narges Moradtalab [1], Frank Walker [2], Birgit Höglinger [2], Abbas El-Hasan [3], Uwe Ludewig [1], Günter Neumann [1] and Rita Grosch [4]

[1] Department of Nutritional Crop Physiology, Institute of Crop Sciences, University of Hohenheim, 70599 Stuttgart, Germany; Anja.Walter2@gmx.de (A.W.); n.moradtalab@uni-hohenheim.de (N.M.); u.ludewig@uni-hohenheim.de (U.L.); guenter.neumann@uni-hohenheim.de (G.N.)

[2] Central Chemical-Analytical Laboratory, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany; frank.walker@uni-hohenheim.de (F.W.); birgit.hoeglinger@uni-hohenheim.de (B.H.)

[3] Department of Phytopathology, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany; aelhasan@uni-hohenheim.de

[4] Programme Area Plant-Microbe Systems, Leibniz Institute of Vegetable and Ornamental Crops (IGZ) e.V., 14979 Großbeeren, Germany; grosch@igzev.de

***** Correspondence: saskia.windisch@uni-hohenheim.de

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Abstract

Soil-borne pathogens can severely limit plant productivity. Induced defense responses are plant strategies to counteract pathogen-related damage and yield loss. In this study, we hypothesized that benzoic acid and lettucenin A are involved as defense compounds against *Rhizoctonia solani* and *Olpidium virulentus* in lettuce. To address this hypothesis, we conducted growth chamber experiments using hydroponics, peat culture substrate and soil culture in pots and minirhizotrons. Benzoic acid was identified as root exudate released from lettuce plants upon pathogen infection, with pre-accumulation of benzoic acid esters in the root tissue. The amounts were sufficient to inhibit hyphal growth of *R. solani* in vitro (30%), to mitigate growth retardation (51%) and damage of fine roots (130%) in lettuce plants caused by *R. solani*, but were not able to overcome plant growth suppression induced by *Olpidium* infection. Additionally, lettucenin A was identified as major phytoalexin, with local accumulation in affected plant tissues upon infection with pathogens or chemical elicitation ($CuSO_4$) and detected in trace amounts in root exudates. The results suggest a two-stage defense mechanism with pathogen-induced benzoic acid exudation initially located in the rhizosphere followed by accumulation of lettucenin A locally restricted to affected root and leaf tissues.

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[1] Department of Nutritional Crop Physiology, Institute of Crop Sciences, University of Hohenheim, 70599 Stuttgart, Germany; Anja.Walter2@gmx.de (A.W.); n.moradtalab@uni-hohenheim.de (N.M.); u.ludewig@uni-hohenheim.de (U.L.); guenter.neumann@uni-hohenheim.de (G.N.)
[2] Central Chemical-Analytical Laboratory, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany; frank.walker@uni-hohenheim.de (F.W.); birgit.hoeglinger@uni-hohenheim.de (B.H.)
[3] Department of Phytopathology, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany; aelhasan@uni-hohenheim.de
[4] Programme Area Plant-Microbe Systems, Leibniz Institute of Vegetable and Ornamental Crops (IGZ) e.V., 14979 Großbeeren, Germany; grosch@igzev.de
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Keywords: lettuce; root exudates; plant health; phytoalexin; lettucenin; benzoic acid; defense reaction

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1. Introduction

Crops are exposed to a great variety of soil microorganisms, which can act and interact as competitors, predators and pathogens [1], but also support nutrient acquisition and stress resilience of the host plant. Losses of yield and product quality, caused by soil-borne pathogens, are among the most limiting factors in plant production [2]. However, efficient control strategies, e.g., against widespread fungal soil-borne pathogens, such as *Rhizoctonia* spp., *Fusarium* spp., *Pythium* spp. or *Olpidium* ssp., are limited due to the long-term persistence of these pathogens in the soils [3–5] and poorly available resistant cultivars [6]. Adverse eco-toxicological effects of chemical fungicides urge the establishment of alternatives for disease management [7]. The development of environmentally friendly agricultural management strategies, promoted by beneficial plant–microbe interactions in the rhizosphere is a major focus of recent research activities [8]. In this context, adaptations of plants developing physical and chemical barriers against pathogen attack are of particular interest [9]. Root exudates and plant metabolites with antimicrobial,

insecticidal or allelopathic properties are described as an important component of plant defense against pathogens, pests and competitors [10–12]. Therefore, the role of root exudates and their interactions with soil microbiota for performance, stress resilience and productivity of plants is intensively studied [10,13–15]. However, characterization and quantitative determination of root exudates and their functions under real rhizosphere conditions in soil-grown plants is technically difficult, if not impossible [16]. In this regard, there are numerous interfering factors, such as rapid microbial degradation of root exudates, spatial and temporal variation in release patterns, chemical reaction with other organic compounds in the soil and adsorption to the soil matrix [16]. Therefore, concepts investigating the role of root exudates in rhizosphere interactions are frequently based on correlative observations originating from experiments conducted under controlled conditions, i.e., hydroponic culture, extraction of rhizosphere soil, analyses of soil solutions from microlysimeter studies in minirhizotrons and examination of microbial dynamics using molecular tools [16–19].

Recent studies on a model pathosystem with lettuce (*Lactuca sativa*) and *Rhizoctonia solani* have shown a positive correlation of the antimicrobial compound benzoic acid, detected in the rhizosphere soil solutions in minirhizotron experiments with the soil suppressive potential against *R. solani* [20]. A similar positive correlation between benzoic acid content in the rhizosphere and the biocontrol activities of selected bacterial inoculants (*Pseudomonas* sp. RU47 and *Serratia plymuthica 3Re-4-18*) against the fungal pathogen were also detectable in model experiments and under field conditions [17,20,21]. Accordingly, the role of root-secreted benzoic acid in plant defense modified by the presence of beneficial rhizosphere microorganisms in the lettuce rhizosphere has been discussed [20,22].

Benzoic acid is one of the oldest chemical preservatives, especially used in the cosmetic, drug and food industries [23,24]. Furthermore, this compound has been described as an antimicrobial and (auto)-allelopathic compound in root exudates of various plant species, such as barley, peanut, strawberry, tobacco and also lettuce [18,25,26]. Additionally, benzoic acid can be released by various bacteria and fungi [26–28]. Aromatic carboxylates such as benzoic acid have been shown to inhibit or kill microorganisms (i) by interfering with the permeability of the microbial cell membrane, causing uncoupling of both substrate transport and oxidative phosphorylation, (ii) by disruption of intracellular pH homeostasis and (iii) specific inhibition of various enzyme activities [23]. Moreover, defense priming with protective effects against early blight (*Alternaria solani*) in tomato has also been reported for exogenous application of benzoic acid [29].

However, investigations on root exudation of benzoic acid in lettuce have never been conducted under axenic conditions [17,18,20,22]. Therefore, it is still poorly understood whether this compound is released from plant roots or accumulates in the rhizosphere solution as rhizosphere product of microbial origin. In this study, we addressed the hypothesis that benzoic acid is released from lettuce roots in response to pathogen infection by *R. solani* and accumulates in the rhizosphere in pathogen suppressive concentrations.

Apart from benzoic acid, sesquiterpene phytoalexins (lettucenins) have also been described as antimicrobial secondary metabolites in lettuce, accumulating in the leaf tissue upon exposure to microbial pathogens (i.e., *Fusarium oxysporum*, *Pythium aphanidermatum* and others) but also as a response to membrane damage after chemical elicitation with $CuSO_4$ or $AgNO_3$ or abiotic stress [30–33]. Lettucenins and related sequiterpene lactones have been described as a major group of secondary metabolites in lettuce and the whole family of the Asteraceae. They possess multiple functions as antimicrobial compounds acting via cell wall and membrane disruption, as allelopathics, antifeedants and exhibit beneficial medical properties (reviewed by Chadwick et al. [34]). Therefore, we addressed the hypothesis that lettucenins, so far only detected in leaves, could also play a role in pathogen defense responses in lettuce rhizosphere.

2. Results

2.1. Root Exudation of Benzoic Acid

To address the question whether benzoic acid, detected in rhizosphere soil solutions of soil-grown lettuce plants in previous studies [17,20,22,25] was released from plant roots or alternatively produced by rhizosphere microorganisms, lettuce was cultivated in a soil-free hydroponic culture system. Intensive microbial root colonization was reduced by frequent replacement (2-day intervals) of the nutrient solution prepared with demineralized membrane-filtered water, since a completely axenic culture of plants in hydroponics is technically difficult and not reliable for longer time periods required for the experiment.

A successful detection of benzoic acid in root washings of hydroponically grown lettuce by UHPLC-MS (Figure 1; Table 1) was indicative of root exudation.

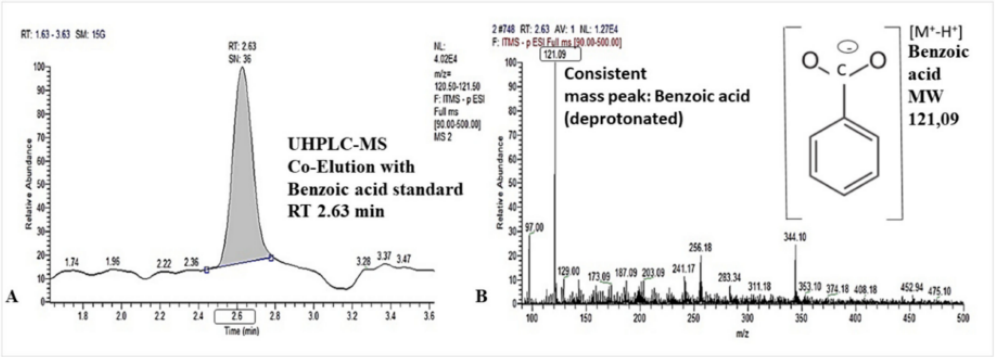

Figure 1. UHPLC-MS separation of benzoic acid in root washings of lettuce at 2.63 min retention time (**A**) and mass spectrum with [M+-H+], base peak at m/z= 121.09 (**B**).

Table 1. Detection of benzoic acid in root washings of lettuce (cv. Tizian) (**A**) and in root tissue before and after alkaline hydrolysis of methanolic plant extracts (**B**). Means ± SE of four replicates per treatment. Different lowercase letters indicate significant differences between treatments by one-way ANOVA, Tukey's test ($p \leq 0.05$).

Benzoic Acid [ng g^{-1} Root FW]		
A. Root Exudate	**B. Root Tissue**	
	Methanolic extract before alkaline hydrolysis	Methanolic extract after alkaline hydrolysis
0.04 ± 0.03 a	0.03 ± 0.01 a	0.20 ± 0.02 b

Further analyses detected benzoic acid also in the root tissue of the lettuce plants used for exudate collection. Free benzoic acid was detectable only in trace concentrations in methanolic root extracts, but significant amounts were recorded after alkaline hydrolysis of the extracts (Table 1), demonstrating that benzoic acid was present in the root tissue in conjugated form as benzoic acid esters.

2.2. Antimicrobial Activity of Benzoic Acid under Realistic Rhizosphere Concentrations

Earlier studies demonstrated a relationship between benzoic acid exudation and disease severity caused by *R. solani* [20]. However, so far, a causal relationship has not yet been demonstrated since it is still unknown whether the rhizosphere concentrations measured for benzoic acid in lettuce plants are sufficient to exert any inhibitory effects on growth and pathogenesis of *R. solani*. Based on the benzoic acid concentration determined in the rhizosphere of lettuce grown on a loamy sand with a suppressive potential against *R. solani* [22], a benzoic acid concentration of approx. 500 µg L^{-1} was calculated for the rhizosphere soil solution at a distance of 1 mm from the root surface. The addition

of benzoic acid with the respective concentration in a PDA plating assay with *R. solani* resulted in a significant reduction (30%) of mycelial growth during 72 h. Even 0.05 mg L^{-1} still mediated a reduction of 12%, demonstrating the inhibitory potential of benzoic acid in a concentration range detectable in the rhizosphere of soil-grown lettuce plants (Figure 2).

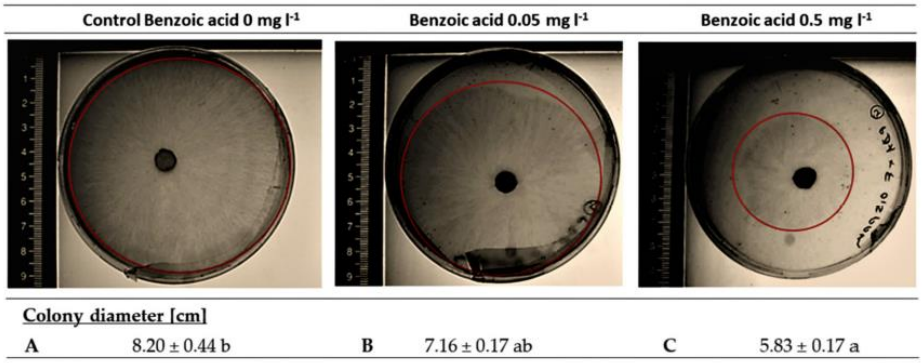

Figure 2. Mycelial growth of *R. solani* AG1-1B after 72 hours on PDA medium, spiked with 0.0 mg L^{-1} (**A**), 0.05 mg L^{-1} (**B**) and 0.5 mg L^{-1} (**C**) of benzoic acid. The red outline indicates the diameter of the mycelial growth. Means ± SE of three replicates per treatment. Different lowercase letters indicate significant differences between treatments by one-way ANOVA, Tukey's test ($p \leq 0.05$).

2.3. Protective Role of Benzoic Acid in a Lettuce-R. solani Pathosystem

Under optimum conditions for *R. solani* infection, using a peat culture substrate–sand mixture with high porosity to support hyphal spreading and an incubation temperature of 25 °C, first mycelium growth and characteristic bottom rot symptoms on stems and lower leaves were visible in lettuce plants already two days after inoculation (Figure 3B). Additionally, fine roots were preferentially affected in the topsoil by stunted growth and browning (Figure 3C). *Rhizoctonia*-typical rectangular hyphal branching was observed on infected roots (Figure 3D).

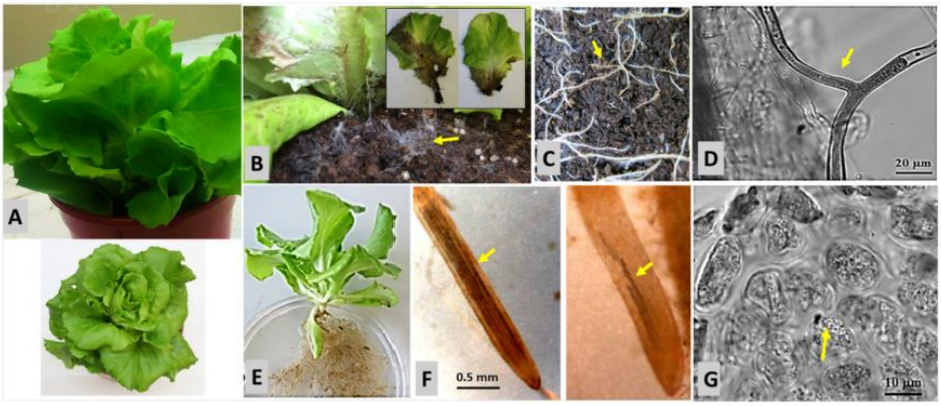

Figure 3. (**A**) Control plant of lettuce, non-infected by *R. solani* and *Olpidium* sp. (**B**) Stem base with browning of lower leaves (**C**) and stunted top-soil fine roots of lettuce (cv. Tizian) infected by *R. solani* AG1-IB (**D**) with fungal hyphae showing rectangular branching (**E**). Root system (**F**) and fine root tips of lettuce seedlings infected by naturally occurring *Olpidium* sp. with typical dark colored lines in xylem. (**G**) Sporangia formation in the root tissue by *Olpidium* sp.

Ten days after *R. solani* inoculation, the total biomass of infected plants was significantly reduced by 44.2 g, as compared with the untreated control with a biomass of

164.8 g. Benzoic acid was applied in real rhizosphere concentrations (as determined by Windisch et al. [22]). Application of benzoic acid in three doses with the irrigation water (each 110 µg kg^{-1} substrate throughout the ten days culture period), resulted in a significant suppressive effect (51%) on *R. solani*-induced reduction in plant biomass, compared with the inoculated control. However, a sole benzoic acid application in the absence of *R. solani* showed no influence on the total biomass of lettuce (Table 2).

Table 2. Decline in total fresh weight (FW) of lettuce (cv. Tizian) grown in peat culture substrate at 25 °C for 10 days with (i) application of benzoic acid (3 × 110 µg kg^{-1} substrate throughout growing period via irrigation), (ii) with *R. solani* AG1-IB inoculation and (iii) combined application of benzoic acid and *R. solani*, compared with an untreated control. * = significant difference compared with the untreated control; ** = significant difference compared with the untreated control and the *R. solani* treatment; one-way ANOVA, Tukey's test ($p \leq 0.05$).

Parameter	(i) Benzoic Acid Application [3 × 110 µg kg^{-1} Substrate]	(ii) *R. solani* Inoculation	(iii) *R. solani* + Benzoic Acid Application [3 × 110 µg kg^{-1} Substrate]
Decline in plant FW (compared with an untreated control) [g $plant^{-1}$]	0.29	44.22 (*)	21.6 (**)

Two independent growth chamber experiments at two different temperature regimes with optimal (23–25 °C) and sub-optimal (20–22 °C) temperatures for *R. solani* infection [6] confirmed similar trends. Co-application of benzoic acid to the *R. solani*-inoculated plants resulted in increased shoot and root biomass and increased fine root lengths. Biomass production was generally lower at 20–22 °C as compared with higher temperature, 23–25 °C (Table 3). However, the protective effects of benzoic acid application against bottom rot disease caused by *R. solani* were greater under the less conductive temperature regime at 20–22 °C, particularly with respect to root growth promotion and fine root formation (Table 3).

Table 3. Fresh weight (FW) of shoot and roots, root and fine root length of lettuce (cv. Tizian) grown for 10 days in peat culture substrate in two independent growth chamber experiments at different temperature regimes (23–25 °C and 20–22 °C). Plants were inoculated with *R. solani* AG1-IB with and without application of benzoic acid (3 × 110 µg kg^{-1} substrate throughout the growing period via irrigation). In each row, different lowercase letters indicate significant differences according to one-way ANOVA, Tukey's test ($p \leq 0.05$).

Incubation Temperature	23–25 °C		20–22 °C	
Treatments	*R. solani*	*R. solani* + Benzoic Acid	*R. solani*	*R. solani* + Benzoic Acid
Shoot FW [g]	113.73 a	131.62 b (+16%)	83.39 a	94.36 a (+13%)
Root FW [g]	9.82 a	11.58 b (+18%)	5.43 a	8.23 b (+52%)
Root length [cm]	1429.2 a	1510.7 a (+ 6%)	1196.7 a	2631.5 b (+120%)
Fine root length (Ø 0–0.4 mm) [cm]	1022.7 a	1182.1 a (+ 16%)	863.0 a	2040.3 b (+136%)

2.4. Lettucenin A Distribution in Plant Tissues of Lettuce

Lettucenin A was identified by thin layer chromatography, RP-HPLC, spectral characteristics and UHPLC-MS in comparison with published data [31–33] as dominant lettucenin in the leaf tissues of lettuce and in lower concentrations in the root tissues after elicitation with $CuSO_4$, $AgNO_3$ and *R. solani* (Figure 4).

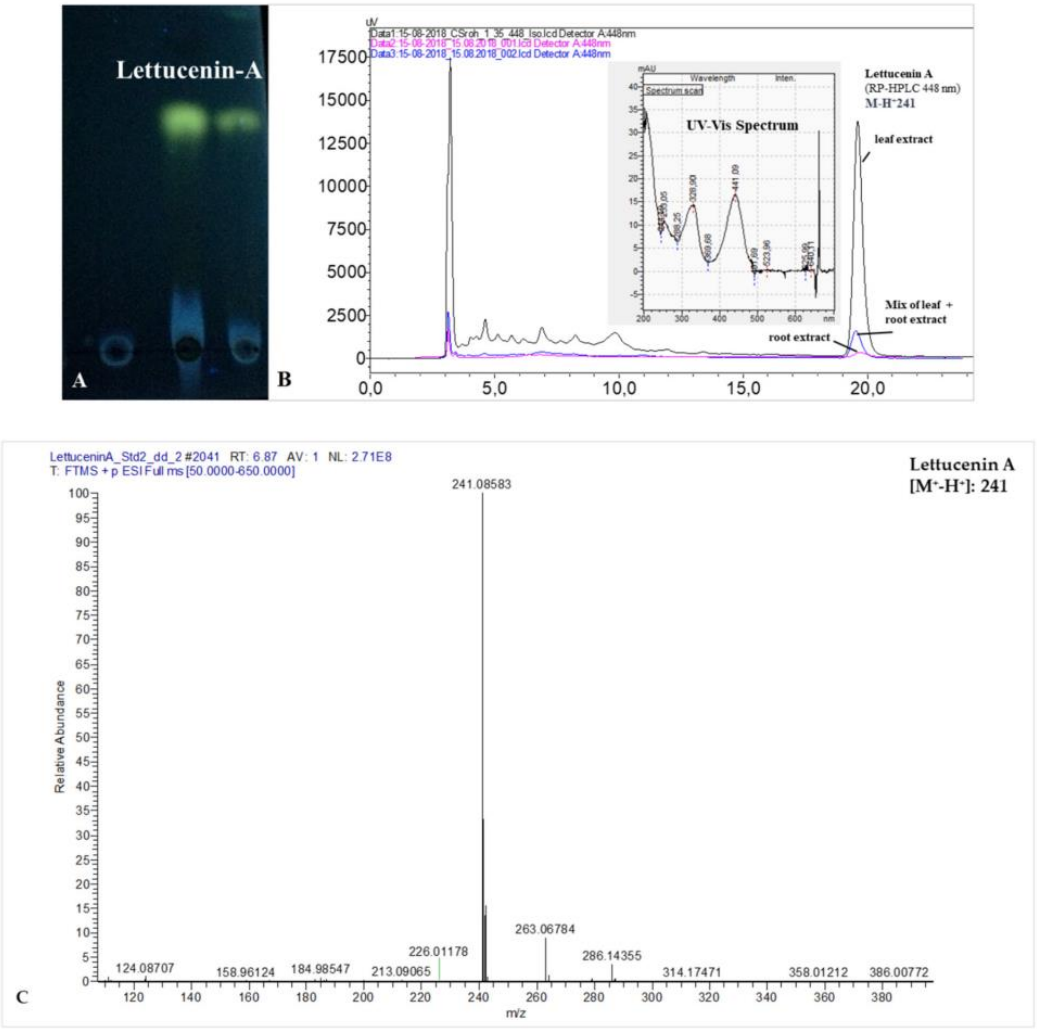

Figure 4. Detection of lettucenin A in leaf and root extracts of lettuce (cv. Tizian), using (**A**) thin-layer chromatography: yellow fluorescent lettucenin spots after elicitation of lettuce leaves with $CuSO_4$ (left) and $AgNO_3$ (right). (**B**) Identification of lettucenin A in leaf and root extracts of lettuce by comparison of retention times and spectral characteristics using RP-HPLC-UV/VIS and (**C**) by UHPLC orbitrap-MS.

In lettuce plants grown in peat culture substrate, a 46-fold increase in lettucenin A was detected in the leaf tissues treated locally with foliar sprays of $CuSO_4$ as compared with the untreated control, whereas no increase was recorded in the root tissues and in untreated leaves (Table 4). In *R. solani*-inoculated plants, lettucenin A increased in the root tissues (2-fold) and in infected leaves (7-fold), but not in non-infected leaves. Co-application of the *R. solani*-inoculant with benzoic acid in real rhizosphere concentrations resulted in a 66% reduction in lettucenin A in the *R. solani*- infected leaf tissue compared with the treatment lacking benzoic acid supply (Table 4).

Table 4. Lettucenin A content in leaf and root tissue of lettuce (cv. Tizian), grown for 10 days in peat culture substrate after treatment with biotic (*R. solani* AG1-IB inoculation) and abiotic ($CuSO_4$, 5% *w*/*v* foliar sprays) elicitors and benzoic acid application (3 × 110 µg kg^{-1} substrate throughout the growing period via irrigation) and benzoic acid application with *R. solani* inoculated plants. Means ± SE of four replicates per treatment. Significant differences (*t*-test, $p \leq 0.05$) compared with untreated control plants are indicated by *; na = not applicable.

Treatments	Analysed Plant Tissue			
	Leaves without Symptoms	Leaves with Symptoms of *R. solani*	$CuSO_4$ Treated Leaves	
		Leaf Content [µg g^{-1} FW]		Root Content [µg g^{-1} FW]
Untreated control	0.17 ± 0.01	na	na	0.18 ± 0.01
$CuSO_4$ foliar application	0.17 ± 0.06	na	7.96 ± 0.79 *	0.20 ± 0.01
R. solani inoculation	0.00	1.20 ± 0.68	na	0.42 ± 0.04 *
Benzoic acid application	0.21 ± 0.01 *	na	na	0.00
R. solani + Benzoic acid	0.12 ± 0.07	0.41 ± 0.13 *	na	0..65 ± 0.14 *

2.5. Defense Response of Soil-Grown Lettuce against Olpidium sp. Depending on Fertilization History

A high incidence of the lettuce pathogen *Olpidium* sp. was detected by visual rating, particularly in lettuce roots grown in minirhizotrons in BIODYN2 soil with biodynamic fertilization history (Figure 3; Table 5), compared with CONMIN soil with long-term mineral fertilization (see Section 4.1.3). The investigated soils were collected from two long-term field experiments [20]. A high rhizosphere abundance of *Olpidium brassicae* (syn. *virulentus*) (76–90%) was confirmed earlier by amplicon sequencing in both soils [22]. However, typical fungal structures within the tips of fine roots associated with the loss of root hairs (Figure 3F) and intracellular formation of sporangia (Figure 3G) were detected, particularly in BIODYN2 soil. The preferential *Olpidium* infection recorded in the BIODYN2 soil was associated with a significant decline of total plant biomass (47%) as compared with the plants grown in CONMIN soil (Table 5). In accordance with the higher pathogen pressure in the roots of BIODYN2 plants compared with the plants grown in CONMIN soil, the rhizosphere concentration of benzoic acid, which was released as defense compound from root tips as major infection sites of *Olpidium*, increased by 203%. A similar trend was observed for lettucenin A, although with very low concentrations close to the detection limit. Benzoic acid was detectable exclusively in the root tissue of lettuce plants, grown in BIODYN2 soil. Lettucenin A accumulated both in root and leaf tissue, with higher levels in the roots and a trend for increased concentrations in plants grown in BIODYN2 soil as compared with the CONMIN soil (Table 5).

Table 5. Plant biomass, visual rating of *Olpidium* disease severity, lettucenin A and benzoic acid concentrations in root exudates, root and leaf tissues of lettuce (cv. Tizian) grown in minirhizotrons in soils collected from fields with long-term biodynamic (BIODYN2) vs. mineral (CONMIN) fertilization history. Means of five replicates per treatment. Different lowercase letters indicate significant differences between biodynamic vs. mineral fertilization by one-way ANOVA, Tukey's HSD pairwise test, ($p \leq 0.05$). Disease severity' assessed by +++ high and + low expression of disease symptoms on roots.

Treatments	*Olpidium*-Disease Severity	Plant Biomass	Root Exudates		Root Tissue		Leaf Tissue	
	(Visual Rating)	[g $Plant^{-1}$]	Lettucenin A [ng cm^{-1} Root]	Benzoic Acid [ng cm^{-1} Root]	Lettucenin A [µg g^{-1} FW]	Benzoic Acid [µg g^{-1} FW]	Lettucenin A [µg g^{-1} FW]	Benzoic Acid [µg g^{-1} FW]
BIODYN2	+++	1.41 b	0.34 a	4.06 b	1.77 a	0.11 a	0.49 a	0.0 a
CONMIN	+	2.64 a	0.25 a	1.34 a	1.33 a	0.0 b	0.30 a	0.0 a

3. Discussion

The concept of root exudates as a component of belowground plant defense responses against pathogens has a long history. However, exploring the profiles of secreted metabolites that exhibit a defensive function in the rhizosphere is technically much more challenging compared with the analysis of antibiotic compounds in aboveground plant parts [14]. In this study, we obtained more detailed insights into the role of root exudates in plant defense responses against pathogens in a well-characterized pathosystem with lettuce and *R. solani* [20,22,35] using a combined approach of model experiments in hydroponics, peat substrates and real soil culture.

3.1. Benzoic Acid—A Root Exudate of Lettuce with Pathogen Defense Effect

Benzoic acid has been detected in the rhizosphere soil solution of soil grown lettuce plants in previous studies [17,20,22]. To address the question whether benzoic acid is a plant-released root exudate, we cultivated lettuce plants in a hydroponic culture system, avoiding soil contact and formation of a rhizosphere. Benzoic acid was detected by UHPLC-MS analyses after short-term immersion of the root systems into trap solutions (3 h to minimize microbial degradation, as a first indication that this compound was released from roots of lettuce plants (Figure 1; Table 1). Further, we detected benzoic acid also in the root tissue of the plants used for exudate collection. However, free benzoic acid was present only in trace concentrations in methanolic root extracts compared with significant amounts detected after alkaline hydrolysis of the extracts (Table 1). This finding suggests that benzoic acid in the root tissue is mainly present in form of benzoic acid esters to increase the water solubility for storage in the vacuole and reduce auto-toxic effects, as previously reported for benzoyl glucose in tobacco [36]. A release of free benzoic acid may then occur by enzymatic hydrolysis just prior or directly after release into the apoplast, similarly reported also for other secondary metabolites, such as flavonoid glycosides [37]. Since benzoic acid was detected in the esterified form in the root tissue of lettuce grown in hydroponics without any biotic or abiotic stress factor, this compound can be regarded as a phytoanticipin, which constitutively accumulates in plant tissue as a defense compound [38]. We speculate that the exudation in form of benzoic acid is triggered by a stimulation via fungal pathogens such as *R. solani* but also by inoculation with beneficial bacteria able to suppress diseases, as demonstrated by Windisch et al. [20]. This offers perspectives to manipulate root exudation of defense compounds by application of microbial inoculants for biocontrol approaches [21]. Nevertheless, under soil conditions, it cannot be ruled out that microbial benzoic acid production [26–28] could also have contributed to this effect, at least to some extent. However, this scenario still remains hypothetic, since in previous studies [20] it was not demonstrated that the concentrations measured for benzoic acid in the lettuce rhizosphere are sufficient to exert any inhibitory effects on growth and pathogenesis of the co-inoculated *R. solani* pathogen.

To address this question, in our study, benzoic acid was applied in realistic rhizosphere concentrations, determined in earlier studies [22] to a PDA growth medium in a plating assay with *R. solani*. A significant reduction (30%) in fungal growth during 72 h (Figure 2) demonstrated an inhibitory effect of benzoic acid in a concentration range detectable in the rhizosphere of soil-grown lettuce. However, during longer incubation times the fungus was able to colonize the whole plate, pointing to a potential of benzoic acid degradation by fungal activity as demonstrated in previous studies [39].

To assess the effects of benzoic acid exudation on bottom rot disease caused by *R. solani*, benzoic acid was applied with the irrigation water to lettuce co-inoculated in the rhizosphere with the pathogen and grown in a peat-sand substrate (Figure 3, Table 2). First disease symptoms at the basal leaves were detectable already at two days after inoculation and clear bottom rot leaf lesions and root infection were recorded after five days (Figure 3A), which was associated with a significant plant biomass reduction of 41 g compared with the untreated control (Table 2). By contrast, the biomass of lettuce plants with *R. solani* inoculation and simultaneous application of benzoic acid was reduced only

by 22 g, indicating a direct inhibitory effect against *R. solani*. Application of benzoic acid without *R. solani* inoculation had no effects on biomass production (Table 2) excluding any growth effects induced, e.g., by a potential bio-stimulant function of benzoic acid.

In two independent experiments, particularly root growth of *R. solani*-inoculated plants was increased after benzoic acid application, demonstrating a pathogen-suppressive effect of benzoic acid in real rhizosphere concentrations (Table 3). The protective effects were more pronounced under sub-optimal temperature conditions for root infection with *R. solani* (20–22 °C), obviously weakening the pathogenic potential of the fungus. Similar protective effects of benzoic acid in root exudates have been reported against the bacterial tobacco pathogen *Ralstonia solanacearum* already at much lower soil concentrations of 4 μg kg^{-1} substrate [25].

3.2. Lettucenin A as Phytoalexin in Lettuce

Strong local plant defense responses, which resulted in lettucenin accumulation, known as major phytoalexin in leaf tissues of lettuce, have been observed in earlier studies [31,33,40]. Accordingly, in our study we detected increased accumulation of lettucenin A in lettuce leaves as a response of plants to local chemical elicitation with $CuSO_4$ and $AgNO_3$ (Figure 4). In addition, we aimed to answer the question whether lettucenins, which have so far only been detected in leaves of lettuce, could also play a role in defense reactions in the root or even in root exudates of lettuce. Confirming the results of earlier studies [40], a local leaf elicitation with $CuSO_4$ raised the lettucenin A accumulation exclusively in the treated leaves with a 46-fold increase as compared with the untreated control. However, no increase was recorded in the root tissues. By contrast, in the presence of *R. solani*, causing the so-called bottom rot disease in lettuce, affecting both roots and the lower leaves of lettuce plants (Figure 3A), increased lettucenin A levels were detected in the infected leaves and to a lower extent also in the root tissue (Table 4). To our knowledge, this is the first report of lettucenin A accumulating as a defense compound in roots of lettuce. Interestingly, application of benzoic acid as antimicrobial compound to *R. solani*-inoculated plants resulted in a 66% reduction in lettucenin A accumulation in the leaf tissue (Table 4). This may reflect the reduced disease severity observed in the respective plants due to the antagonistic effect of benzoic acid. Accordingly, in this case, the root and shoot biomass was significantly increased (Table 3).

3.3. Chemical Defense Responses of Lettuce in Soil Culture

We conducted an additional minirhizotron experiment under real soil conditions by growing lettuce plants in a silty loam from an experimental long-term field site, which was affected by the fungal lettuce pathogen *O. brassicae* [22]. Two strategies of long-term fertilization practices were compared, namely one with organic (BIODYN2), and the other with mineral (CONMIN) fertilization history. The experiment revealed severe growth depression of lettuce (Table 5) after root infection by the biotrophic pathogen *Olpidium brassicae* grown in the organically fertilized soil of BIODYN2 in comparison with CONMIN soil with mineral fertilization history. Microscopic root examination (Figure 3) and amplicon sequencing of fungal DNA in the respective rhizosphere soils [22] confirmed *Olpidium* infection of BIODYN2 plants. The intense *Olpidium* infection in the BIODYN2 treatment was associated with an increase in benzoic acid accumulation (203%) in the rhizosphere of 1 cm apical root zones of young roots (Table 4), known as major *Olpidium* infection sites (Figure 3E). The results suggest a defense response similar to the effects observed after *R. solani* inoculation [20,22]. However, Windisch et al. [22] did not observe a comparable increase in benzoic acid accumulation in the apical root zones of the lettuce plants grown in BIODYN2 soil. The discrepancy may have resulted from the longer culture period of nine weeks [22], compared with six weeks in this study. During longer lasting plant-pathogen interactions, the fungus was obviously able to overcome and suppress the defense response of lettuce with respect to benzoic acid release. Hence, plant growth was negatively affected (Table 5). Similar suppressive effects of pathogens, counteracting

plant defense responses have been frequently reported, induced via production of effector proteins by various bacterial and fungal pathogens [41].The ability to suppress plant immunity via production of effector proteins is of particular importance for biotrophic pathogens, such as *O.brassicae*, depending on living host cells for successful colonization.

In contrast to benzoic acid, lettucenin A was detectable in trace amounts close to the detection limits in the rhizosphere soil solution of lettuce plants grown in CONMIN and BIODYN2 soils (Table 5). However, in accordance with the function as phytoalexin, lettucenin A accumulated preferentially in the root tissue of the plants, directly affected by the endophytic root pathogen *Olpidium*, whereas leaf concentrations only reached 30% of the lettucenin A levels detected in roots. The results suggest a relationship between disease severity and increased concentrations of lettucenin A in the rhizosphere soil solution (36%), root tissue (33%) and leaf tissues (61%) in the BIODYN2 soil compared with the less-affected plants grown in the CONMIN soil. However, the differences were not statistically significant (Table 5). The extremely low concentrations of lettucenin A close to the detection limits in the rhizosphere soil solution, showing similar treatment differences as compared with the root contents, suggest in contrast to benzoic acid, a rather passive (e.g., diffusion-mediated) release of lettucenin A than a controlled exudation in response to pathogen infection.

4. Materials and Methods

4.1. Plant Cultivation in Hydroponics, Peat Culture Substrate and Soil Culture

To assess defense responses in lettuce (*Lactuca sativa* cv. Tizian, Syngenta, Bad Salzuflen, Germany) against pathogens, growth chamber experiments were performed, using hydroponics-, pot experiments with peat culture substrate and soil culture in minirhizotrons.

4.1.1. Pathogen-Suppressive Effect of Benzoic Acid

To achieve homogenous plant development, lettuce seedlings were pre-cultivated in growing trays until the five-leaf stage (BBCH 15; 7 weeks) in a pre-fertilized peat culture substrate (TKS1)–sand mixture (90/10: *w*/*w*). Thereafter, the seedlings were transferred to pots with 1 kg TKS1-sand mixture (Floragard, Oldenburg, Germany). The plants were watered to 70% of substrate water-holding capacity (WHC) with demineralized water throughout the culture period. Lettuce seedlings were cultivated in a growth chamber with a 16 h light period at 420 µmol m^{-2} s^{-1}, 60% relative humidity, at 25 °C (experiment 1) and at 22 °C (experiment 2).

For pathogen inoculation, a *R. solani* AG1-IB isolate 7/3 from the collection of the Leibnitz Institute of Vegetable and Ornamental Crops (Großbeeren, Germany) was pre-cultured on potato dextrose agar (PDA, Roth, Karlsruhe, Germany), supplemented with penicillin G (Roth, 100 mg L^{-1}), streptomycin sulfate (Sigma-Aldrich, Taufkirchen, Germany; 50 mg L^{-1}) and tetracyclin (Roth, 10 mg L^{-1}) in petri dishes (9 cm diameter). The plates were incubated at 25 °C in darkness for 4 days (WTB Binder incubator, Tuttlingen, Germany). One day after transplanting (DAT), seven agar plugs (5 mm diameter) with fungal mycelium were excised from the plates, inserted into the peat culture substrate (1 cm deep) at a distance of 1–2 cm from the roots close to the rhizosphere of lettuce plants (BBCH 15) and covered with a thin layer of substrate to minimize evaporation. Non-infested agar pieces were used in the control treatments. Two independent pot experiments and a hydroponic experiment were conducted.

To investigate the pathogen-suppressive effect of benzoic acid at real rhizosphere concentrations, benzoic acid (pre-dissolved in an ethanolic stock solution at 2 mg L^{-1}) was added with the demineralized irrigation water (1 mL stock solution L^{-1}) to the lettuce plants inoculated with *R. solani* to reach a concentration of 110 µg kg^{-1} substrate. Control plants received the same amount of water with addition of pure ethanol. The respective concentration of benzoic acid was previously determined in the rhizosphere of lettuce grown on a loamy sand with suppressive potential against *R. solani* AG1-IB [22]. It corresponded to a benzoic acid concentration of approx. 500 µg L^{-1}, which has been calculated

for the rhizosphere soil solution in a distance of 1 mm from the root surface, equivalent to 110 µg kg^{-1} rhizosphere soil, assuming a volumetric soil water content of 20% and a root diameter of 1 mm [42,43]. Benzoic acid was applied three times after transplanting, to simulate the continuous release of benzoic acid from the plant roots in different stages of root development [20,22] and to account for microbial degradation during this time period [44], as suggested by the presence of bacterial rhizosphere responders of the genus *Sphingomonas* with the ability for degradation of aromatic hydrocarbons repeatedly identified in the rhzosphere of lettuce [21,22].

To induce the production of lettucenin phytoalexins, a chemical elicitation of lettuce plants by foliar sprays of three leaves with 5% (*w*/*v*) $CuSO_4$ solution was conducted at one DAT in selected treatments, whereas the remaining leaves were covered with plastic foil during the spraying process to prevent contact with $CuSO_4$. At 10 DAT, leaves were harvested and separated as "leaves with" and "without" symptoms of *R. solani* infection and $CuSO_4$ elicitation, respectively. Fresh root biomass was determined and one-half was frozen in liquid nitrogen and kept in −80 °C for metabolite determinations. The other half was kept in 60% (*v*/*v*) ethanol for root morphology analysis.

4.1.2. Benzoic Acid Released from Roots of Lettuce Cultivated in Hydroponics

After a pre-culture period in peat culture substrate and transplanting into pot culture as described in Section 4.1.1, the plants were carefully removed from the peat culture substrate at eight DAT by washing the root system with demineralized water. Thereafter, the plants (one plant per pot) were transferred to pots containing 2.8 L nutrient solution, continuously aerated with an aquarium pump (Eheim, Deizisau, Germany). Pathogen inoculation with *R. solani* was performed 6 days before transferring the plant into the hydroponic nutrient solution. The nutrient solution consisted of 1.0 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 300 µM Fe-EDTA, 0.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 1.1 mM K_2SO_4. The nutrient solution was replaced every 48 h with half-strength mineral concentrations directly after transplanting to hydroponics and proceeded with full-strength nutrient solution during an eight days-culture period and a 22 °C/20 °C day/night temperature regime. Thereafter, root washings were collected by immersion of the root system into 300 mL aerated demineralized water for 3 h [45]. Finally, root and leaf biomass of the plants was recorded, and root washings and the root tissue were frozen at −80 °C for later analysis of benzoic acid.

4.1.3. Lettuce Cultivation in Minirhizotrons with Soil Culture

For the minirhizotron experiment, lettuce seeds were sown in seedling trays filled with soils of different fertilization history and pre-cultivated until the five-leaf stage (BBCH 15). Thereafter, lettuce seedlings were transplanted to minirhizotrons filled with 0.6 kg of soil-sand mixture 70/30 (*w*/*w*) equipped with removable root observation windows to enable micro-sampling of rhizosphere soil solution by application of sorption filters onto the surface of roots growing along the observation windows as described by Windisch et al. [20,22]. The soils originated from a long-term field experiment DOK-LTE conducted by the Research Institute of Organic Agriculture (FIBL; Therwil; Switzerland) since 1978 on a Haplic Luvisol (silty loam), and compares long-term bio-dynamic compost and manure fertilization (BIODYN2) with mineral NPK (CONMIN) fertilization. Detailed soil characteristics, management practices and physicochemical parameters of the experimental soils are described in Windisch et al. [22]. Lettuce plants were cultivated under growth chamber conditions according to Windisch et al. [22] with four replicates per treatment. Regular watering of the plants was supplied to reach 60% of substrate WHC by addition of demineralized water. Microscopic evaluation of root pathogens was performed at 21 DAT with a subset of lettuce plants as described by Windisch et al. [22]. Micro-sampling of rhizosphere soil solution with sorption filters [17] was conducted at 31 DAT during 4 hours in 1 cm subapical root zones (1–2 cm behind the root tip) of young, growing roots considered as the root zones with the highest release rates of low molecular-weight root exudates [42].

For each minirhizotron, the filters of five sampling points were pooled and stored frozen at −20 °C. Thereafter, final harvest was performed with determination of shoot (total fresh shoot biomass) and root biomass. Leaves and roots were frozen in liquid nitrogen and stored at −80 °C for further analysis.

4.2. Plating Assay for the Effect of Benzoic Acid on R. Solani

To assess the pathogen suppressive potential of benzoic acid, different dosages of benzoic acid were incorporated after sterile filtration (0.2 µm) into PDA medium at 40 °C to reach final concentrations of 0 mg L^{-1}, 0.05 mg L^{-1} and 0.5 mg L^{-1}, respectively. An agar plug of an actively growing *R. solani* culture without benzoic acid was transferred onto the middle of the PDA plate using a sterile inoculation loop to assess the ability of the fungus to grow into a medium with different benzoic acid concentrations. Fungal hyphae, trying to spread into the lettuce rhizosphere with benzoic acid accumulation would face a very similar situation. The mycelial growth of the fungus on the agar plates was quantified by determining the colony diameters during a cultivation period of three days at 25 °C with three replicates per treatment. The mycelial growth inhibition percentage was calculated.

4.3. Benzoic Acid in Root Washings, Rhizosphere Soil Solution and Plant Tissues

Benzoic acid in root washings of lettuce plants grown in hydroponics (Section 4.1.2) was pre-purified via solid-phase extraction with Sep-Pac C18 cartridges (Waters Corporation, Milford, MA, USA). Conditioning of the Sep-Pac C18 cartridges was conducted with 5 mL of MeOH (100%). A 20 mL aliquot of root washing solutions was passed through the cartridge at a flow rate of 3 mL min^{-1}. Subsequent elution of hydrophobic compounds bound to the cartridge was performed with 5 mL of methanol: ethyl acetate (1:1; *v*:*v*) at a flow rate of 1 mL min^{-1}. The eluted fraction was evaporated to dryness at 30 °C with Speed-vac concentrator (Savant, Farmington, CT, USA) and the pellet was re-dissolved in 200 µL of acetonitrile: H_2O (1:4; *v*:*v*) and subjected to UHPLC-MS analysis using a Velos LTQSystem (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an YMC Triart C18 column, 3 µm particle size, 100 mm × 3 mm (YMC Europe, Dinslaken, Germany) as described by Windisch et al. [22]. Rhizosphere soil solution, collected with sorption filters (Section 4.1.3) was extracted with 0.6 mL of acetonitrile: H_2O (1:1; *v*:*v*) and aliquots of 50 µL obtained were analyzed by UHPLC-MS [22].

For determination of benzoic acid in plant tissues, 2 g of frozen leaf and root tissue were homogenized with liquid nitrogen and extracted in 4 mL methanol: H_2O (4:1; *v*:*v*) using mortar and pestle. After centrifugation for 5 min at 12,000 rpm (Microliter centrifuge Micro 24–48 R, Hettich, Tuttlingen, Germany), the supernatant was subjected to membrane filtration (PTFE 0.2 µm pore size, Roth, Karlsruhe, Germany). Aliquots of the tissue extracts (100 µL) were mixed with 300 µL demineralized of H_2O, followed by UHPLC-MS analysis [22]. To release conjugated benzoic acid, alkaline hydrolysis of the root extracts was performed with 2N NaOH at pH 8.0 for 30 min at room temperature according to Chong et al. [36]. The hydrolysate was subjected twice to liquid–liquid extraction with 2 mL ethyl acetate, and the combined upper phases were evaporated to dryness by nitrogen vaporization (Multivap 11880, Organomation, Berlin, Germany), followed by dissolving in 200 µL of acetonitrile: H_2O (1:4; *v*:*v*) for UHPLC-MS analysis [22].

4.4. Determination of Lettucenin A in Plant Tissues and Root Exudates

Frozen leaf and root material (1 g) was homogenized in liquid nitrogen and extracted with 2 mL of MeOH: H_2O (4:1; *v*:*v*) using mortar and pestle, followed by centrifugation for 5 min^{-1} and 12,000 rpm. 1.5 mL aliquots of the supernatants were membrane filtered (PTFE 0.2 µm pore size, Roth, Karlsruhe, Germany) for further analyses with RP-HPLC (Shimdazu High performance LC20 system, Kyoto, Japan). The identification and quantitative determination of lettucenin A was conducted, using a reversed phase C-18 column (GROM-SIL 120 ODS, 5 µm particle size, 290 mm × 4.6 mm equipped with a 20 mm × 4.6 mm guard column with the same stationary phase (Dr. Maisch HPLC GmbH, Ammerbuch, Germany).

The UV detection was conducted at 448 nm and isocratic elution with 50% (*v*:*v*) methanol: water at a flow rate of 0.7 mL min^{-1}, column temperature of 35 °C and an injection volume of 20 µL. The identification and quantitative analysis were performed by comparison of spectral characteristics and retention time with an external lettucinin A standard.

To obtain a pure lettucenin A standard after methanolic extraction of 2 g frozen leaf material (as described above), a solvent extraction of the methanolic extract was performed three times with 2 mL ethyl acetate each. The combined upper phases were evaporated to dryness at 30 °C using a Speed-vac concentrator (Savant, Farmington, CT, USA), followed by re-dissolving the residue in 150 µL of methanol and further purification by thin-layer chromatography (TLC) according to Yean et al. [32] and Talubnak et al. [40]. An aliquot of 30 µL methanol extract was applied onto a TLC plate (ALUGRAM SIL G/UV_{254}, 5 × 10 cm, Macherey-Nagel, Düren, Germany) and the separation was performed with a solvent system of hexane: ethyl acetate (1:1; *v*:*v*). Lettucenin A was detected as bright yellow florescent band (Rf 0.47), when examined under UV light at 365 nm wavelength. The yellow band was scratched from the TLC plate and re-extracted with 0.5 mL methanol. After 5 min centrifugation at 14,000 rpm to remove the silica particles, the supernatant was checked for purity by RP-HPLC as described above (Figure 4A). Identity was confirmed by comparison of UV-Vis absorption spectra with published data [31,33] and by mass spectrometry analysis using a Agilent 1290 U-HPLC system (Agilent Technoligies Inc., Palo Alto, USA) coupled to a QExactive Plus Orbitrap quadropoly-mass spectrometer (Thermo Fisher Scientific, Dreieich, Gemany). The separation was performed on an Acquity UPLC CSH C18 column (Waters Acquity, Milford, MA, Unites States, 1.7 µm, 2.1 × 150 mm). The elution was performed with (A) formic acid aqueous solution and (B) methanol using a gradient elution of 10% B at 0, 10−90% B at 0−10 min and 90% B at 10−15 min. Full scan mass spectra (ESI, mass range m/z 50–650) of HPLC eluates were recorded during chromatographic separation in the positive ionization mode.

The lettucenin A concentration in the purified standard solution was calculated via Lambert-Beer's law based on spectrophotometric determination (Hitachi U-3300 spectrophotometer, Hitachi Ltd., Corporation, Tokyo, Japan) of the absorption at 446 nm and the published absorption coefficient $\varepsilon\lambda$ = 32,000 [31] and the respective standard was used for quantitative determinations.

Lettucenin A in the rhizosphere soil solutions, collected in the minirhizotron experiment (Section 4.1.3) was determined together with benzoic acid by UHPLC-MS as described in Section 4.3.

4.5. Statistical Analyses

The experiments in pots, minirhizotrons and hydroponics were carried out in a completely randomized block design with one lettuce plant for each treatment in four and five replicates, respectively. Differences between treatment groups were analyzed using ANOVA followed by Tukey's test and Tukey's HSD pairwise testing ($p \leq 0.05$ significance level). Differences in lettucenin A concentration between leaf and root tissues were analyzed by *t*-test. Calculations were done in R studio (Ri386 3.4.0).

5. Conclusions

The presented data support the hypothesis, that benzoic acid is released from lettuce roots as a defense compound in response to fungal pathogen attack, which originates from esterified precursors pre-accumulating in the root tissue. At least in the investigated lettuce-*R. solani* plant–pathogensystem, the accumulated rhizosphere concentration of benzoic acid was sufficient to inhibit mycelial growth of the fungal pathogen and reduced the disease severity of infected plants. However, in the soil culture experiments, a certain contribution of microbial benzoic acid production cannot be completely excluded. In contrast to benzoic acid, lettucenin A accumulates preferentially as phytoalexin in infected tissues. However, it was not detectable in significant amounts as root exudate. These findings suggest that the release of benzoic acid represents a first defense line upon pathogen attack in the

rhizosphere, followed by local accumulation of the lettucenin A phytoalexin within the affected tissue as a second line of defense. For the first time it was demonstrated that lettucenin A is not only produced as phytoalexin upon pathogen infection in the leaf tissue of lettuce as reported in earlier studies but similarly accumulates in affected roots. However, the pathogen suppressive potential of the lettucenin A accumulation against the investigated pathogens still remains to be investigated.

The possibility to manipulate the defense responses by chemical elicitation or inoculation with beneficial microorganisms may offer perspectives for biocontrol strategies. However, the observed variability in responses with respect to the type of pathogens, soil type [7,20,21] and temperature effects (Table 3) indicates that detailed knowledge on successful application conditions is indispensable.

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6 Impact of management practices on microbial communities, rhizosphere chemistry, and plant health

The concept of plant-soil feedback describes a continuous mutual influence of the activity of plant roots and the physico-chemical and biological properties of the soil. Especially in agricultural soil, it has been postulated that the soil legacy is transferred to the next plant generation via soil microbial communities and directly influence plant performance and health (Berendsen et al., 2012; Berg et al., 2016; Lapsansky et al., 2016; Bakker et al., 2018; Babin et al., 2021). However, many of these feedback mechanisms and their role in ecosystems with respect to agricultural management practices are still poorly understood (van der Putten et al., 2016; Fitzpatrick et al., 2020). A recent study has shown that the fertilizer legacy of the soil has an impact on the composition of the bacterial communities in the rhizosphere of lettuce and provided first experimental evidence for induction of physiological adaptations under long-term organic vs. mineral fertilization that influenced how lettuce plants cope with environmental stress (Chowdhury et al., 2019).

Soils with contrasting physicochemical properties and long-term organic vs. mineral fertilization histories were investigated. The following study (Windisch et al. 2021a; Chapter 6.1) addressed the hypotheses (i) that long-term fertilization practices will result in characteristic patterns and alterations of chemical composition of the rhizosphere soil solution, which (ii) impact soil microbiota and the recruitment of rhizosphere microbiota, and (iii) these changes will affect the performance and health of the model plant lettuce.

Minirhizotron with more soil volume - A new plant cultivation system

To study the soil microbiome, plants are usually grown in pots. Minirhizotrons (rhizoboxes) with observation windows are used to investigate root exudation patterns with a non-destructive sampling methods on the root surface of plants (Neumann et al., 2006, 2014). By comparing plant growth in pots and in rhizoboxes, significant differences in plant biomass production were revealed due to the two different cultivation systems. Furthermore, Windisch et al. (2017; Chapter 4.1) showed, that infection and plant damage by pathogens in flat minirhizotrons occurred faster and more intensive than in pots with similar soil volume. The flat size of boxes and high concentration of densely root growth along the observation window induced shorter paths of pathogen infection.

In order to study the influence of rhizodeposition on the composition of rhizosphere microbiota, a new system of growing vessel was needed, in which rhizodeposits and microbial DNA could be sampled simultaneously. For this reason, minirhizotrons were developed, which combine the characteristics of growing pots and rhizoboxes. Despite a larger soil volume, the new minirhizotrons allowed sufficient root development for exudate sampling at an observation window. Windisch et al. (2021a; Chapter 6.1) showed that the new cultivation system (Figure 3) allowed good lettuce growth, non-destructive sampling of root exudates as well as DNA extraction for soil microbiome analyses.

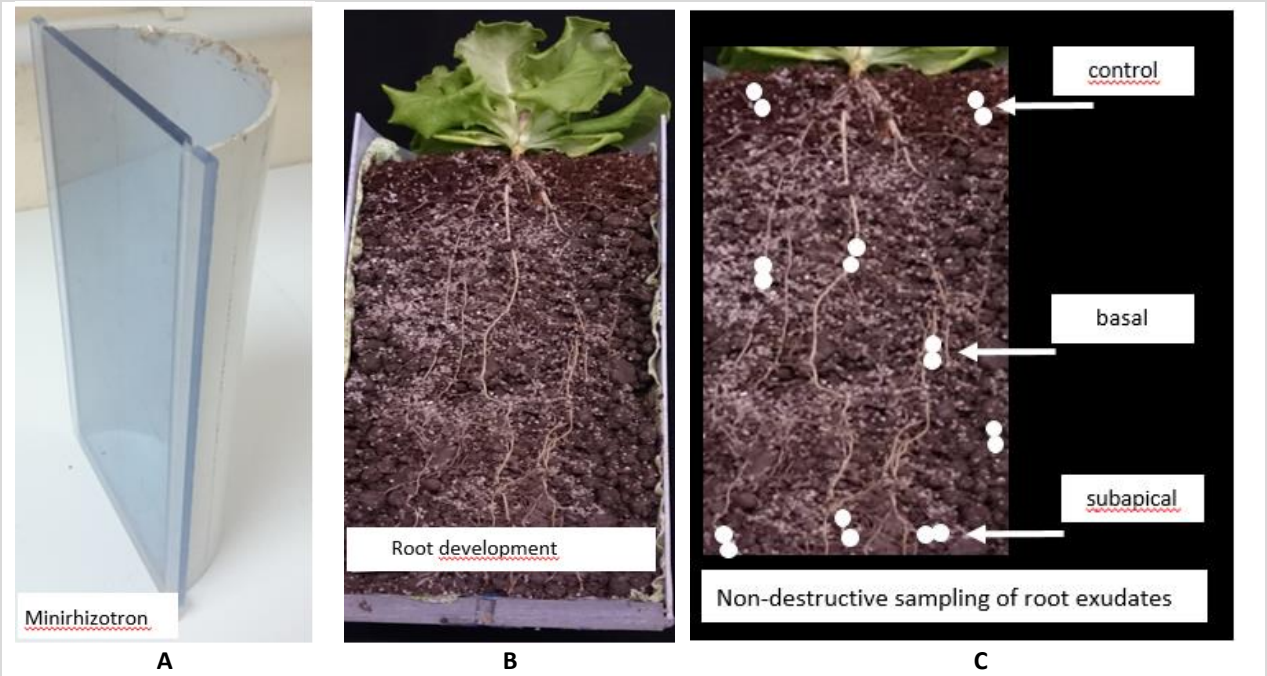
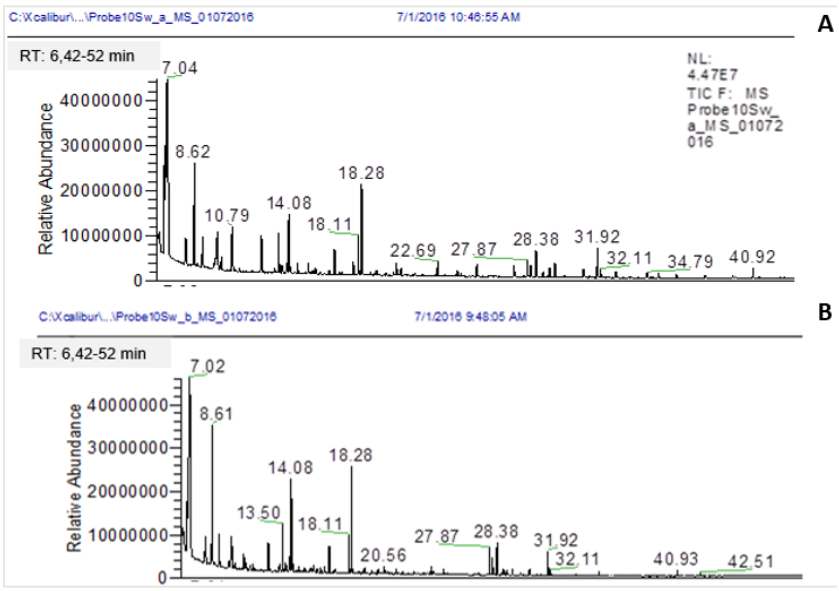


Figure 3: Minirhizotron (A). Lettuce (cv. Tizian) grown in a minirhizotron (B). Micro-sampling with sorption filters (5 mm Ø, MN815, Macherey-Nagel, Düren, Germany), placed in triplicates onto the surface of subapical root zones (1-2 cm behind the root tip), basal root zones (older, mature parts of the root system, 8-9 cm behind the root tip) and control sampling in soil zones without visible root development (soil without root contact) (C).

In order to obtain more knowledge about characteristic exudate patterns of lettuce (*Lactuca sativa*), grown in minirhizotrons in soils with different management history, a non-targeted approach of exudate sampling of lettuce roots, followed by GC-MS analyses as described in Neumann et al. (2014) was performed (Figure 4.; Windisch, unpublished). For this, lettuce plants were grown with soil of different tillage and preceding crop management history from a long-term experiment in Bernburg, Germany (Deubel et al., 2011). The detection of low-molecular weight compounds in the rhizosphere soil solution resulted in consistent exudate patterns as previously described by Neumann et al. (2014), including compounds such as

glycerol, inositol, glucose, fructose, various amino acids, carboxylate and benzoic acid. Figure 4 (C) shows detection of six new compounds, which are marked in bold black colour.



C

RT	Component
10.85	**propionic acid**
13.69	urea
13.97	benzoic acid
15.05	glycine
15.48	succinate
15.63	gylcerin
16.42	serine
17.03	threonine
18.11	**indole-3-acetic acid**
19.71	malate
24.67	**ribitol** (sugar alcohol)
27.87	D-fructose
28.38	D-glucose
29.05	**mannitol** (sugar alcohol)
31.92	**palmitic acid**
35.47	**stearic acid**

Figure 4: Chromatographic output of hydrophilic exudates in the rhizosphere soil solution of lettuce roots grown in LTE-1 soil with pre-crop rapeseed and cultivator practice (A) and intensive N-fertilization (B). List of detected exudate patterns in the rhizosphere soil solution with retention time (C).

For technical reasons, the routine analyses of the exudate samples of lettuce in the following publication (Windisch et al. 2021a; Chapter 6.1) were carried out finally with HPLC-MS instead of GC-MS but with similar precision. The analytical techniques and sampling of root exudates of lettuce based on Neumann (2006), Neumann et al. (2014) and Lippmann et al. (2009) were applied and further developed.

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6.1 Impact of Long-Term Organic and Mineral Fertilization on Rhizosphere Metabolites, Root–Microbial Interactions and Plant Health of Lettuce

Saskia Windisch [1*†], Loreen Sommermann [2†], Doreen Babin [3†], Soumitra Paul Chowdhury [4], Rita Grosch [5], Narges Moradtalab [1], Frank Walker [6], Birgit Höglinger [6], Abbas El-Hasan [7], Wolfgang Armbruster [8], Joseph Nesme [9], Søren Johannes Sørensen [9], Ingo Schellenberg [2], Jörg Geistlinger [2], Kornelia Smalla [3], Michael Rothballer [4], Uwe Ludewig [1] and Günter Neumann [1*]

[1] Department of Nutritional Crop Physiology, Institute of Crop Science, University of Hohenheim, Stuttgart, Germany,

[2] Institute of Bioanalytical Sciences (IBAS), Anhalt University of Applied Sciences, Bernburg, Germany,

[3] Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn Institute – Federal Research Centre for Cultivated Plants, Braunschweig, Germany,

[4] Institute of Network Biology, Helmholtz Zentrum München, Neuherberg, Germany,

[5] Plant-Microbe Systems, Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany,

[6] Central Chemical-Analytical Laboratory, Institute of Phytomedicine, University of Hohenheim, Stuttgart, Germany,

[7] Department of Phytopathology, Institute of Phytomedicine, University of Hohenheim, Stuttgart, Germany,

[8] Department of Food Chemistry and Analytical Chemistry, Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany,

[9] Department of Biology, Section of Microbiology, University of Copenhagen, Copenhagen, Denmark

***** Correspondence: Saskia Windisch saskia.windisch@uni-hohenheim.de;
Günter Neumann gd.neumann@t-onine.de

[†] These authors have contributed equally to this work

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Abstract

Fertilization management can affect plant performance and soil microbiota, involving still poorly understood rhizosphere interactions. We hypothesized that fertilization practice exerts specific effects on rhizodeposition with consequences for recruitment of rhizosphere microbiota and plant performance. To address this hypothesis, we conducted a minirhizotron experiment using lettuce as model plant and field soils with contrasting properties from two long-term field experiments (HUB-LTE: loamy sand, DOK-LTE: silty loam) with organic and mineral fertilization history. Increased relative abundance of plant-beneficial arbuscular mycorrhizal fungi and fungal pathotrophs were characteristic of the rhizospheres in the organically managed soils (HU-org; BIODYN2). Accordingly, defense-related genes were systemically expressed in shoot tissues of the respective plants. As a site-specific effect, high relative occurrence of the fungal lettuce pathogen *Olpidium* sp. (76–90%) was recorded in the rhizosphere, both under long-term organic and mineral fertilization at the DOK-LTE site, likely supporting *Olpidium* infection due to a lower water drainage potential compared to the sandy HUB-LTE soils. However, plant growth depressions and *Olpidium* infection were exclusively recorded in the BIODYN2 soil with organic fertilization history. This was associated with a drastic (87–97%) reduction in rhizosphere abundance of potentially plant-beneficial microbiota (*Pseudomonadaceae*, *Mortierella elongata*) and reduced concentrations of the antifungal root exudate benzoate, known to be increased in presence of Pseudomonas spp. In contrast, high relative abundance of *Pseudomonadaceae* (*Gammaproteobacteria*) in the rhizosphere of plants grown in soils with long-term mineral fertilization (61–74%) coincided with high rhizosphere concentrations of chemotactic dicarboxylates (succinate, malate) and a high C (sugar)/N (amino acid) ratio, known to support the growth of *Gammaproteobacteria*. This was related with generally lower systemic expression of plant defense genes as compared with organic fertilization history. Our results suggest a complex network of belowground interactions among root exudates, site-specific factors and rhizosphere microbiota, modulating the impact of fertilization management with consequences for plant health and performance.

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Saskia Windisch[1†], Loreen Sommermann[2†], Doreen Babin[3†], Soumitra Paul Chowdhury[4], Rita Grosch[5], Narges Moradtalab[1], Frank Walker[6], Birgit Höglinger[6], Abbas El-Hasan[7], Wolfgang Armbruster[8], Joseph Nesme[9], Søren Johannes Sørensen[9], Ingo Schellenberg[2], Jörg Geistlinger[2], Kornelia Smalla[3], Michael Rothballer[4], Uwe Ludewig[1] and Günter Neumann[1*]*

[1] Department of Nutritional Crop Physiology, Institute of Crop Science, University of Hohenheim, Stuttgart, Germany, [2] Institute of Bioanalytical Sciences (IBAS), Anhalt University of Applied Sciences, Bernburg, Germany, [3] Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn Institute – Federal Research Centre for Cultivated Plants, Braunschweig, Germany, [4] Institute of Network Biology, Helmholtz Zentrum München, Neuherberg, Germany, [5] Plant-Microbe Systems, Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany, [6] Central Chemical-Analytical Laboratory, Institute of Phytomedicine, University of Hohenheim, Stuttgart, Germany, [7] Department of Phytopathology, Institute of Phytomedicine, University of Hohenheim, Stuttgart, Germany, [8] Department of Food Chemistry and Analytical Chemistry, Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany, [9] Department of Biology, Section of Microbiology, University of Copenhagen, Copenhagen, Denmark

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Edited by:
Jacob George Malone,
John Innes Centre, United Kingdom

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Jennifer Elise Schmidt,
Mars Wrigley Davis, CA, United States
Hongmiao Wu,
Fujian Agriculture and Forestry University, China

***Correspondence:**
Saskia Windisch
Saskia.Windisch@uni-hohenheim.de
Günter Neumann
gd.neumann@t-online.de

[†]These authors have contributed equally to this work

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Fertilization management can affect plant performance and soil microbiota, involving still poorly understood rhizosphere interactions. We hypothesized that fertilization practice exerts specific effects on rhizodeposition with consequences for recruitment of rhizosphere microbiota and plant performance. To address this hypothesis, we conducted a minirhizotron experiment using lettuce as model plant and field soils with contrasting properties from two long-term field experiments (HUB-LTE: loamy sand, DOK-LTE: silty loam) with organic and mineral fertilization history. Increased relative abundance of plant-beneficial arbuscular mycorrhizal fungi and fungal pathotrophs were characteristic of the rhizospheres in the organically managed soils (HU-org; BIODYN2). Accordingly, defense-related genes were systemically expressed in shoot tissues of the respective plants. As a site-specific effect, high relative occurrence of the fungal lettuce pathogen *Olpidium* sp. (76–90%) was recorded in the rhizosphere, both under long-term organic and mineral fertilization at the DOK-LTE site, likely supporting *Olpidium* infection due to a lower water drainage potential compared to the sandy HUB-LTE soils. However, plant growth depressions and *Olpidium* infection were exclusively recorded in the BIODYN2 soil with organic fertilization history. This was associated with a drastic (87–97%) reduction in rhizosphere abundance of potentially plant-beneficial microbiota (*Pseudomonadaceae*, *Mortierella elongata*) and reduced concentrations of the antifungal root exudate benzoate, known to be increased in presence of *Pseudomonas* spp. In contrast, high relative abundance of *Pseudomonadaceae*

(Gammaproteobacteria) in the rhizosphere of plants grown in soils with long-term mineral fertilization (61–74%) coincided with high rhizosphere concentrations of chemotactic dicarboxylates (succinate, malate) and a high C (sugar)/N (amino acid) ratio, known to support the growth of Gammaproteobacteria. This was related with generally lower systemic expression of plant defense genes as compared with organic fertilization history. Our results suggest a complex network of belowground interactions among root exudates, site-specific factors and rhizosphere microbiota, modulating the impact of fertilization management with consequences for plant health and performance.

Keywords: fertilization management, root exudates, rhizosphere microbiota, high-throughout amplicon sequencing, 16S rRNA, fungal ITS2 region, stress-related gene expression

INTRODUCTION

Fertilization practices are a central component of agricultural management having a strong impact on plant performance including crop yield and quality, plant health and resistance against abiotic and biotic stresses. However, limitations in the adaptation of fertilizer inputs to actual crop demands can cause undesirable environmental effects, such as nutrient leaching, alteration of soil pH, eutrophication of surface waters, emission of greenhouse gases, and soil degradation (Loreau et al., 2001; Foley et al., 2005; Robertson and Vitousek, 2009; Geisseler and Scow, 2014). Due to the rising demand for more sustainable agricultural production systems with reduced inputs of agrochemicals and the closing of nutrient cycles to counteract detrimental ecological side effects, investigations into alternative management practices gain in importance. The availability of essential plant nutrients in farmland is controlled by interactions between fertilization management and microbial processes (Schmidt et al., 2019). Nearly all relevant soil processes are influenced by microbial activities (Mäder et al., 2002), which underlines the importance of considering plant–microbial interactions in this context. Various beneficial aspects have been linked with organic fertilization strategies, such as increased soil organic matter, stimulation of microbial activity (Lori et al., 2017), increased microbial biomass and diversity (Esperschütz et al., 2007; Hartmann et al., 2014; Francioli et al., 2016; Schmid et al., 2018), and enrichment in plant–beneficial microorganisms (Francioli et al., 2016). However, these findings cannot be generalized and are influenced by additional factors, such as soil properties, climatic conditions or specific practices of crop management. In addition, plant roots are similarly powerful drivers of the assemblage of the rhizosphere microbial community, which exhibits distinct structural and functional differences compared with the bulk soil microbiota (Turner et al., 2013). Organic rhizodeposition shapes rhizosphere microbiota (Walters et al., 2003; Narula et al., 2009) by providing nutrients, signaling compounds, and bio-active substances against pests and pathogens (Doornbos et al., 2012; Chaparro et al., 2013; Baetz and Martinoia, 2014; Windisch et al., 2017). The quantity and composition of rhizodeposits are highly variable and influenced by soil texture, plant nutritional status, abiotic and biotic stress factors, plant genotype, and rhizosphere microbiota (Neumann and Römheld, 2007). However, the impact of fertilization management on rhizodeposits, triggering the selective recruitment of rhizosphere microbiota with potential feed-back loops on plant performance and health status, still remains an open question.

A recent study demonstrated that the fertilization legacy of the soil contributed to the assemblage of rhizosphere bacterial communities in lettuce (Chowdhury et al., 2019). The crucial role of the rhizosphere microbiota for plant performance and health has been comprehensively reviewed (Berendsen et al., 2012; Berg et al., 2016). Furthermore, a relationship between plant health and agricultural management conferred via soil microorganisms has been postulated (Lapsansky et al., 2016; van der Putten et al., 2016; Bakker et al., 2018). Chowdhury et al. (2019) provided first experimental evidence for induction of physiological adaptations under long-term organic vs. mineral fertilization that helped lettuce plants to cope with environmental stresses. A better understanding of these interactions, triggering plant–beneficial interactions but also detrimental rhizosphere effects, could therefore contribute to the development of practical approaches toward improved crop productivity and agroecosystem sustainability (Schmid et al., 2018) according to the concept of "soil biological engineering" (Bender et al., 2016). However, a more detailed understanding particularly of the critical plant factors determining these interactions still represents a major knowledge gap.

In this context, our study was initiated as a complementary approach to the recently published study by Chowdhury et al. (2019). Soils with contrasting physicochemical properties and long-term organic or mineral fertilization histories were investigated. Bacterial, archaeal and fungal communities, the composition of organic compounds in the rhizosphere soil solution as well as plant performance and expression of stress-related genes were analyzed to obtain a more holistic picture of plant-microbe interactions.

We hypothesized that long-term fertilization practices will result in characteristic patterns and chemical composition of the rhizosphere soil solution, with impact on soil microbiota and the recruitment of rhizosphere microbiota. This would affect the performance and health of the model plant lettuce. The final aim was the identification of rhizosphere metabolite profiles characteristic of the investigated long-term fertilization strategies potentially related to alterations in the assemblage of rhizosphere microbiota and plant performance.

MATERIALS AND METHODS

Soil Sampling and Setup of the Minirhizotron Experiment

Field soils with contrasting properties in terms of soil type and fertilization history, originating from the two long-term fertilization experiments DOK-LTE belonging to the Research Institute of Organic Agriculture (FiBL; Therwil, Switzerland since 1978) and HUB-LTE belonging to the Humboldt-Universität zu Berlin (HUB; Thyrow, Germany since 2006) were used for plant cultivation. The field trial (i) DOK-LTE on a Haplic Luvisol (silty loam) compares bio-dynamic (compost and manure fertilizers with biodynamic preparations; BIODYN2) vs. full mineral NPK (CONMIN) fertilization. The fertilization intensity of the organic system in BIODYN2 was based on the fodder produced in the crop rotation and reflects the intensity of Swiss organic farms. The mineral fertilizer level in CONMIN was adjusted to plant-specific Swiss standard recommendations (Mäder et al., 2002). The field trial (ii) HUB-LTE on a Retisol (loamy sand) compares application of organic farmyard manure (green, and cattle manure; HU-org) vs. full mineral NPK (HU-min) fertilization. The mineral fertilizer level in HU-min was adjusted to soil fertility and yield performance in the northeast of Germany. Nitrogen was applied as calcium ammonium nitrate (KAS), phosphorus as triple-superphosphate (TSP) and potassium as Patentkali® (K + S Minerals and Agriculture GmbH, Kassel, Germany) besides an incorporation of harvested straw of winter and cover crops. The fertilization intensity of the organic system in HU-org was based on cattle and green manure from cover crops with legumes mixture. In order to obtain field soil for the minirhizotron experiment, soil sampling was performed after harvest of standing crops from the respective upper 30 cm soil layer from each field trial and combined as representative sample of 15 sampling spots. For homogenization, the soil was air-dried, sieved (4 mm mesh size) and stored in the dark at 7°C. For reactivation of the microbial communities prior to the experiment, experimental soil and soil as control without lettuce cultivation (bulk soil) was incubated for two weeks in the dark with a 20°C day/15°C night temperature regime at 100-hPA water potential (T5 tensiometer, UMS, AG, München, Germany). Detailed soil characteristics, management practices, and physicochemical parameters of the experimental soils are summarized in **Table 1**.

Lettuce (*Lactuca sativa* L. cv. Tizian, Syngenta, Bad Salzuflen, Germany) cultivation, harvest, and root exudate sampling were performed as described by Neumann et al. (2014). To achieve homogenous plant development, lettuce seedlings were pre-cultivated until the five-leaf stage (BBCH 15) in a soil-sand mixture (70/30: w/w). Thereafter, the seedlings were transferred to minirhizotrons (0.6 kg of the soil–sand mixture 70/30) made from PVC tubes (height 22 cm; diameter 9 cm) with transparent root observation windows. The minirhizotrons were fixed at an angle of 45° to stimulate root growth along the root observation window for exudate sampling.

Full mineral N fertilization (517 mg N kg^{-1} substrate as YaraLiva Calcinit [$Ca(NO_3)_2$], Yara, Oslo, Norway) was supplied to cover the plant demand during the culture period. The first half of the recommended N amount for lettuce was supplied after seedling transfer to minirhizotrons, the second half provided two weeks later. The soil moisture level was adjusted to 18–20% w/w by addition of demineralized water (25 ml kg^{-1} soil) every second day throughout the culture period. Lettuce seedlings were cultivated in a growth chamber with a 16 h light period at 420 µmol m^{-2} s^{-1}, 60% relative humidity, and a 20°C/15°C day/night temperature regime, with eight replicates per treatment in a randomized block design. Biomass of plants, root characteristics and nutritional status of lettuce shoots were analyzed. To confirm general treatment responses in plant growth and visual symptoms of pathogen infections, the experiment was performed twice with cultivation periods of six and nine weeks, respectively, with a more detailed analysis of soil microbiota, rhizosphere chemistry and gene expression after nine weeks.

Analysis of Mineral Nutrients in Soil and Shoot Tissue

The content of macro- and micronutrients in bulk soil and plant samples were analyzed according to the certified protocols of the Association of German Agricultural Analytic and Research Institutes, VDLUFA, Germany (VDLUFA, 2020). After determination of fresh shoot biomass, shoots and soil samples were oven-dried at 60°C for three days and dry biomass was recorded. Subsequently, shoots were stored in a desiccator for another two days and 200–500 mg of dry plant material and 4 g of soil were subjected to microwave digestion (Mars 6, CEM, Charlotte, NC, United States) with 5 ml of HNO_3 (65%) and 3 ml of H_2O_2 (30%) for 20–30 min.

Potasium, Na, P, Mg, Fe, Mn, Cu, and Zn concentrations in shoot tissue and soils were determined via inductively coupled plasma optical emission spectrometry (ICP-OES); total C and N were determined via elemental analysis (Elementary Vario El cube, Elementar, Langenselbold, Germany). Soil pH was determined in calcium chloride solution and salinity via electrical conductivity.

Root Morphology

For analysis of root morphological characteristics, roots of four replicates were washed from soil using sieves (mesh size 0.5–1.0 mm) and fresh and dry biomass were recorded. Morphological characteristics were determined from fresh root samples, stored in 60% (v/v) ethanol. For analysis, root samples submerged in a water film on transparent Perspex trays, were separated with forceps and subsequently digitized using a flat-bed scanner (Epson Expression 1000 XL, Tokyo, Japan). Root length, average root diameter and the proportion of fine roots of the digitized samples were measured by applying the WinRHIZO root analysis software (Regent Instruments, Quebec, QC, Canada). Root hair length was recorded non-destructively along the root observation plane of the minirhizotrons by a video microscope (Stemi 200-c, Zeiss, Oberkochen, Germany). The digitized video images were analyzed using the AxioVision, software, Version 3.1.2.1 (Zeiss, Oberkochen, Germany).

TABLE 1 | Characteristics of the field soils **(A)** and physicochemical parameters of bulk soils **(B)**.

	HUB-LTE		DOK-LTE	
	HU-org	**HU-min**	**BIODYN2**	**CONMIN**
A. Experimental soil and cultivation characteristics[1,2]				
Fertilization management	Composted farmyard manure, K as Patentkali, liming as $CaCO_3$-$MgCO_3$	Standard practice of mineral fertilizer (NPK)[5], liming as $CaCO_3$-$MgCO_3$	Composted farmyard manure (1.4 livestock unit (LU) ha^{-1} $year^{-1}$), biodynamic preparations[3]	Standard practice of mineral fertilizer (NPK)[4]
Soil management	**Tillage**	**Tillage**	**Tillage**	**Tillage**
Crop protection	No pesticides, mechanical weed control	Mechanical weed control with chemical pesticides application	Mechanical weed control, plant extracts and biodynamic preparations, biocontrol (*Bacillus thuringiensis* subsp.)	Mechanical weed control with herbicide and chemical pesticides application
Soil type	**Retisol (loamy sand)**		**Haplic Luvisol (silty loam)**	
Soil texture (0-30 cm)				
Clay (<2 μm) [%]	3		15	
Silt (2–63 μm) (%)	14		70	
Sand (63–2,000 μm) (%)	83		15	

The soils were obtained in 2016 from two long-term field experiments (LTEs) located at different field sites (DOK-LTE in Therwil, Switzerland; HUB-LTE in Thyrow, Germany) and incubated together with planted soils under the same growth chamber conditions. Data represent means ± standard errors of four independent replicates. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for the sites DOK-LTE and HUB-LTE by t-test, $p \leq 0.05$. [1]Department of Soil Sciences, Research Institute of Organic Agriculture (FiBL), Frick, Switzerland; [2]Experiment Thy_ABS "Cropping Systems" in Thyrow, Albrecht Daniel Thaer-Institute of Humboldt-Universität zu Berlin; [3]Hartmann et al., 2014; [4]Mäder et al., 2002, quantities for NPK fertilizer in CONMIN: Nitrogen soluble (kg N ha^{-1} $year^{-1}$) 125, Phosphorus (kg P ha^{-1} $year^{-1}$) 42, Potassium (kg K ha^{-1} $year^{-1}$) 253. [5]Quantities for NPK fertilizer in HU-min: Nitrogen soluble (kg N ha^{-1} $year^{-1}$) 128, Phosphorus (kg P ha^{-1} $year^{-1}$) 21, Potassium (kg K ha^{-1} $year^{-1}$) 128.

	HUB-LTE		DOK-LTE	
	HU-org	**HU-min**	**BIODYN2**	**CONMIN**
B. Physicochemical parameters – Bulk soil				
pH ($CaCl_2$)	6.50 ± 0.12 a	6.54 ± 0.04 a	6.68 ± 0.02 a	6.30 ± 0.03 b
Corg (%)	0.81 ± 0.01 a	0.77 ± 0.01 a	1.72 ± 0.01 a	1.42 ± 0.01 b
Cmic (μg g^{-1})	103.23 ± 7.83 a	95.48 ± 10.45 a	446.97 ± 22.17 a	327.86 ± 1.53 a
C/N	9.16 ± 0.56 a	9.63 ± 0.65 a	8.06 ± 0.19 a	7.64 ± 0.23 a
Electrical conductivity (EC) (μS cm^{-1})	357.60 ± 29.01 a	356.87 ± 46.94 a	442.00 ± 40.57 a	443.90 ± 51.63 a
(mg kg^{-1} soil)				
C total	8197.89 ± 18.41 a	7793.92 ± 7.59 a	17836.47 ± 4.41 a	14563.65 ± 4.34 b
N total	906.94 ± 5.44 a	821.70 ± 4.70 a	2217.17 ± 5.09 a	1912.11 ± 5.54 b
NO_3-N	362.01 ± 8.55 a	249.66 ± 6.60 a	442.55 ± 14.46 a	321.75 ± 8.91 a
DL P	92.90 ± 0.09 b	102.95 ± 0.17 a	26.32 ± 0.03 b	37.10 ± 0.03 a
DL K	75.778 ± 0.25 b	124.80 ± 0.96 a	96.30 ± 0.38 a	81.62 ± 0.29 b
Mg	71.33 ± 0.68 a	72.59 ± 0.23 a	153.34 ± 0.61 b	218.87 ± 0.54 a
Na	53.54 ± 1.27 a	32.72 ± 0.32 a	45.78 ± 0.28 b	136.00 ± 0.47 a
Cu	1.99 ± 0.00 a	1.81 ± 0.00 b	4.96 ± 0.01 b	5.81 ± 0.00 a
Fe	189.20 ± 0.25 a	190.80 ± 0.22 a	201.80 ± 0.21 b	214.00 ± 0.26 a
Mn	51.02 ± 0.12 a	49.60 ± 0.09 a	253.60 ± 0.63 a	240.40 ± 0.83 a
Zn	5.59 ± 0.01 a	5.54 ± 0.01 a	5.87 ± 0.01 a	3.45 ± 0.00 b

Corg, organic carbon; Cmic, microbial carbon; DL P and DL K, double-lactate extraction for P and K, (VDLUFA, 2020).

Plant Gene Expression

For gene expression studies, leaf samples were obtained from plants in quadruplicates. Four representative leaves per plant were pooled and snap-frozen in liquid nitrogen (Chowdhury et al., 2019). Homogenized leaf material (100 mg) was subjected to total RNA extraction using the RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). RNA was quantified by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Target genes of lettuce (Chowdhury et al., 2019) were selected based on comparisons with functional genes from *Arabidopsis thaliana* using "The Arabidopsis Information Resource" (Berardini et al., 2015)[1]. The reference gene glyceraldehyde-3-dehydrogenase was used for normalization of qPCR results. The primer pairs for qPCR were designed using the Primer3Plus software (Untergasser et al., 2007). cDNA was synthesized from 2 μg of total RNA with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, United States). The qPCR was performed with Power SYBR Green Supermix (Applied Biosystems, Foster City, CA, United States) using a peqSTAR 96Q thermal cycler (PEQLAB Biotechnologie, Erlangen, Germany). cDNA dilutions (1 μl, 1:4) were used as PCR templates. Each PCR reaction contained 12.5 μl of 2 × Power SYBR Green Supermix, 0.4 μM primers (Eurofins MVG Operon, Ebersberg, Germany), and 1 μl of template in a 25-μl reaction. PCR reactions were heated to 95°C for 3 min and then for 40 cycles with steps of 95°C for 30 s and 60°C for 30 s. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. The genes, their primer pairs and qPCR conditions used in this study are described in **Supplementary Table 1**. The 2−ΔΔCt method (Livak and Schmittgen, 2001) was employed for relative quantification. Normalization to the endogenous control for each condition was followed by logarithmic transformation to fold change differences. The standard error of the mean was calculated from the average of technical triplicates, obtained from each of four biological replicates ($n = 4$).

Sampling of Rhizosphere Soil Solutions

Samples of the rhizosphere soil solution were collected with moist sorption filters (5 mm Ø, filter paper: MN815, Macherey-Nagel, Düren, Germany) placed onto the surface of lateral roots growing along the root observation window according to Haase et al. (2007). Micro-sampling was conducted nine weeks after sowing during vegetative growth of the lettuce plants, to account for most active carbohydrate partitioning to the roots and high root exudation during this phase (Marschner, 1995). For each minirhizotron, rhizosphere sampling was conducted with two 5 mm sorption filters (equivalent to 1 cm root length), applied in triplicate in subapical root zones (1–2 cm behind the root tip) and basal root zones (older, mature parts of the root system, 8–9 cm behind the root tip). As a control, sampling was performed in soil zones without visible root development (soil without root contact). Samples for each minirhizotron were pooled after an incubation time of 4 h and kept frozen at −20°C (Neumann et al., 2014). Rhizosphere soil solution was extracted from sorption filters with 0.6 ml of acetonitrile: H_2O (1:1).

[1]www.arabidopsis.org

Analysis of Carboxylates

Aliquots of 40 μl obtained from 0.6 ml sorption filter extract were evaporated to dryness at 30°C, using a SpeedVac Concentrator (Savant, Farmington, CT, United States) and re-dissolved in 400 μl of high performance liquid chromatography (HPLC) elution buffer (18 mM KH_2PO_4, pH 2.1 adjusted with H_3PO_4; Neumann, 2006). Carboxylates were determined according to the method described by Neumann (2006), using RP-HPLC analysis in the ion suppression mode with isocratic elution (18 mM KH_2PO_4, pH 2.1). The identification and quantitative determination as organic acids (acetic, malic, lactic, citric, succinic, and fumaric acid) were conducted, using a reversed phase C-18 column (GROM-SIL 120 ODS ST, 5 μm particle size, 290 mm × 4.6 mm equipped with a 20 mm × 4.6 mm guard column with the same stationary phase, Grom, Herrenberg, Germany) with direct UV detection at 210 nm and comparison with known standards. In representative samples, the identity of detected carboxylates was additionally confirmed by commercial enzymatic tests (r-Biopharm, Darmstadt, Germany).

Analysis of Sugars

Aliquots of 400 μl from 0.6 ml sorption filter extract were evaporated to dryness at 55°C, using nitrogen evaporation and re-dissolved in 40 μl acetonitrile: H_2O (70:30). Analyses of sugars (fructose, glucose, maltose, and sucrose) in rhizosphere soil solutions of lettuce were performed by HPLC-Evaporative Light Scattering Detector (ELSD) with isocratic elution (acetonitrile: H_2O, 75:25) on a Perkin Elmer Series 200 HPLC system with a Sedex Model 80 LT ELSD system (Sedere, Orléans, France) equipped with a Shodex, Ashipak NH2P-40 3E column, 5 μm particle size, 250 mm × 3.0 mm (Shodex, München, Germany) and external standards.

Analysis of Amino Acids

A total of 20 μl aliquots from 0.6 ml sorption filter extract were mixed with 15 μl of derivatization agent (ACCQFLUOR REAG, Waters, Milford, MA, United States) and 65 μl of borate buffer, incubated 10 min at 55°C, followed by addition of 400 μl acetonitrile: H_2O (1:4) with modifications of the method of Cohen and Michaud (1993). Determination of amino acids (glutamic acid, asparagine, serine, glutamine, glycine, threonine, histidine, alanine, proline, cysteine, thyrosine, methionine, isoleucine, leucine, and phenylalanine) was performed by HPLC-MS, using a Velos LTQSystem (Thermo Fisher Scientific, Waltham, MA, United States) equipped with a ACCUTAG™ column, 4 μm particle size, 150 mm × 3.9 mm (Waters, Milford, MA, United States) and additional comparison with external standards. Gradient elution was performed with (A) ammonium formate: methanol: H_2O (40:9:60) and (B) acetonitrile.

Analysis of Benzoate

For determination of the antifungal compound benzoate 50 μl aliquots of sorption filter extract were mixed with 50 μl of H_2O followed by UHPLC-MS analysis on a Velos LTQSystem (Thermo Fisher Scientific, Waltham, MA, United States).

Identification and quantitative analysis were conducted on an ACCLAIM^{TM}C30 column, 150 mm × 3 mm (ThermoScientific, Waltham, MA, United States) with gradient elution (solvent A): H_2O: acetonitrile (95:5) and (solvent B): acetonitrile and an external standard.

Microbial Community Analyses

Total Community – DNA Extraction

The roots of the respective plants, which were studied for gene expression, were used for analysis of rhizosphere microbiota (bacteria, archaea, and fungi). At first, roots were washed with sterile tap water (Schreiter et al., 2014b). The rhizosphere fraction was obtained from 5 g of representative root samples, which were collected from complete root systems, by 1 min Stomacher treatment (Seward Ltd., Worthing, United Kingdom) followed by centrifugation (Schreiter et al., 2014a). Rhizosphere pellets were kept at −20°C for total community (TC)-DNA extraction. Root-associated soil was sampled from the soil fraction loosely adhering to the root system collected after vigorous shaking. After collection, the soil samples were immediately frozen at −20°C.

Total community-DNA was extracted from root-associated soil (0.5 g) and rhizosphere pellets using the FastPrep-24 bead-beating system and FastDNA Spin Kit for Soil. DNAs were purified with the GeneClean Spin Kit (both MP Biomedicals, Santa Ana, CA, United States).

Bacterial and Archaeal Communities

Bacterial and archaeal communities in root-associated soil and rhizosphere were characterized based on sequencing of the V3-V4 region of 16S rRNA genes amplified using the primer pair 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) originally published by Yu et al. (2005) and modified by Sundberg et al. (2013) or Caporaso et al. (2011), respectively, targeting both Bacteria and Archaea kingdoms. Detailed description of PCR amplification, purification, normalization, and amplicon sequencing on an Illumina® MiSeq® platform (2 × 250 cycles; Illumina Inc., San Diego, CA, United States) can be found in the **Supplementary Text 1**.

Unassembled raw amplicon data were submitted to NCBI Sequence Read Archive (SRA)[2] under accession number PRJNA622892. Raw sequence reads were first trimmed of primer sequences used in first PCR using cutadapt (Martin, 2011) and only read pairs for which both primers were found were retained for subsequent analysis. Primer trimmed sequences were then merged, clustered in operational taxonomic units (OTUs) using UPARSE-OTU algorithm (Edgar, 2013) and a 97% pairwise sequence similarity threshold. The taxonomic annotation of each cluster representative sequence was performed using mothur classify.seq function (Schloss et al., 2009) with default parameters and the Ribosomal Database Project trainset 14 formatted for mothur (Cole et al., 2014)[3]. Only annotations with a confidence threshold above 80% were considered. Sequences classified as chloroplasts, mitochondria, or unclassified at the domain level were removed, resulting in a total of 7,294 OTUs. The average number of quality-filtered sequences per sample was 19,969.

Fungal Communities

High-throughput sequencing based on the Internal Transcribed Spacer (ITS2) region was conducted in root-associated soil and rhizosphere. PCRs using the sample-specific barcoded NGS-primer-pair ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′; Op De Beeck et al., 2014) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990) as well as the processing of the ITS2 amplicon pool on an Illumina® MiSeq® platform (paired-end mode, 2 × 300bp) were carried out as previously described with a few modifications (Sommermann et al., 2018). Detailed information of PCR amplification and sequencing can be found in the **Supplementary Text 2**.

Unassembled raw amplicon reads were submitted to European Nucleotide Archive (ENA)[4] under BioProject accession number PRJEB39853. Barcode, primer and adapter trimming were performed based on a customized in-house perl script including the FASTX toolkit[5] followed by raw sequence merging using FLASH (Magoč and Salzberg, 2011). Subsequently, the analysis of the resulting sequences was carried out with a local version of the GALAXY Bioinformatics Platform[6] based on a database-dependent strategy (Antweiler et al., 2017) using UNITE database v7.2 (UNITE Community, 2017) by applying the closed reference approach (Carter et al., 2017). All sequences were aligned with the database (e-value 0.001) and only results with an alignment length >200 bp and a similarity >97% to the reference were kept. Furthermore, BLAST-PARSER (Antweiler et al., 2017) was used for taxonomic assignment based on the lowest e-value. The fungal OTU abundance table was generated by counting the sequences per assignment and using the SH-numbers from the database as identifier. Sequences not classified to the kingdom "fungi" (0.17%) were removed from the fungal OTU-table. Finally, a total of 1,159 OTUs was obtained with an average of 167,113 high quality sequence reads per sample.

Detection of Pathogen Infection in the Root Tissue

Colonization of *Olpidium* sp. in lettuce roots was assessed according to the method described by De Cara et al. (2008) with modifications. Soil adhering to seedling roots was removed by washing with running water for 5 min. Subsequently, the roots were assessed visually for root discoloration and small sections (2-4 mm) were randomly excised from the roots of each replicate. Root specimens were then transferred to microscopic slides, mounted with lactophenol aniline blue solution (Merck, Darmstadt, Germany) and examined for the presence of sporangia and resting spores in epidermal cells using an Axioskop microscope (Carl Zeiss Microscopy GmbH, Jena,

[2]https://www.ncbi.nlm.nih.gov/sra
[3]https://www.mothur.org/wiki/RDP_reference_files
[4]https://www.ebi.ac.uk/ena/browser/home
[5]http://hannonlab.cshl.edu/fastx_toolkit/
[6]https://galaxyproject.org

Germany) equipped with an Axiocam camera and AxioVision SE64 Rel. 4.8 software.

Statistical Analysis

For the statistical analysis of significant differences of the nutritional status of lettuce between treatment groups (separately for each LTE), a one-way ANOVA (factor fertilization) followed by a Tukey-test ($p \leq 0.05$ significance level) was performed using the SAS software 9.4 (Institute Inc., Cary, NC, United States). For the statistical evaluation of physicochemical parameters (bulk soil), the expression of stress-related genes in lettuce leaves, and the chemical composition of rhizosphere soil solution, *t*-test and Tukey's HSD pairwise testing was applied. Data are presented as means ± standard errors (SE).

Multivariate analyses of microbial communities were carried out by R using the packages *edgeR* (Robinson et al., 2010; McCarthy et al., 2012), *vegan* (Oksanen et al., 2019), *MASS* (Venables and Ripley, 2002), *ggplot2* (Wickham, 2016), *phyloseq* (McMurdie and Holmes, 2013), *pheatmap* (Kolde, 2019), *gplots* (Warnes et al., 2019), *car* (Fox and Weisberg, 2019), and *agricolae* (De Mendiburu, 2020). Alpha-diversity indices (species richness, Shannon, Pielou) were averaged per replicate over 100 randomly taken subsamples of a size corresponding to the sample with the lowest number of reads in the complete dataset (=6,744 for 16S rRNA gene, 96,750 for ITS). Indices were tested for the effect of microhabitat (root-associated soil and rhizosphere) or fertilization management, respectively, by pairwise *t*-test ($p \leq 0.05$). Non-metric multidimensional scaling (NMDS) was used to ordinate similarity between microbial communities based on relative abundances (Bray–Curtis distance). The effect of site, fertilization and habitat on the microbial community composition was tested by PERMANOVA analysis based on relative abundances (10,000 permutations, Bray–Curtis distance). The non-rarefied community data were checked for differentially abundant microbial taxa between different fertilization managements. Data were analyzed by likelihood ratio tests under negative binomial distribution and generalized linear models (edgeR). In doing so, only taxa with presence in at least three samples over the total dataset using a FDR-corrected $p \leq 0.05$ were considered. In order to study the potential relationship between rhizosphere soil solution and the bacterial and archaeal as well as fungal community composition, canonical correspondence analyses (CCA, 999 permutations) were carried out on log10 transformed relative abundances. After checking for linear dependency, environmental variables (organic compounds averaged over basal and subapical root) were fitted onto the CCA ordination by the envfit function (999 permutations). In addition, log transformed relative abundances of *Pseudomonadaceae* OTUs and fungal OTUs at least classified at genus level (both relative abundance >0.5%) that were significantly enriched (FDR < 0.05) in minerally fertilized soils (tested separately per site; edgeR) were included in envfit. The relative abundance of prevalent genera was graphically displayed in a heatmap (clustering of rows using Euclidean distance). The FUNGuild database (Nguyen et al., 2016) was used to categorize fungal communities at genus level into trophic modes (saprotroph, symbiotroph and pathotroph) and into different guilds for further classification.

Biplots of principle component analysis (PCA) were used to ordinate composition of chemical compounds of the rhizosphere soil solutions, related to their habitat and tested for the effect of fertilization management. The distribution of the data was graphically displayed in a p-dimensional cartesian coordinate system with Euclidean distance by using R Studio software 3.4.1 and prcomp and autoplot functions.

RESULTS

Plant Biomass, Root Growth Characteristics, and Nutritional Status

There were no significant differences for shoot and root dry biomasses of lettuce grown in HU-org vs. HU-min soil (**Figure 1A**). However, a significant decline in shoot and root dry biomass (by 41% and 81%) of lettuce grown in the BIODYN2 soil with organic fertilization was recorded compared to the CONMIN soil supplied with mineral fertilizers (**Figure 1B**). Growth depression was confirmed in two independent experiments in the BIODYN2 soil (**Supplementary Table 2**) and corresponded with a significant decline in total root length and fine root length (<0.4 mm diameter) (**Figure 1B** and **Supplementary Table 2**).

At the end of the culture period, shoot nutrient concentrations were recorded and deficiencies in nutrient elements such as P and K were identified in all treatments. For K, significant differences related to the fertilization management were observed, showing higher values in the HU-min vs. HU-org soil and higher levels in BIODYN2 compared to CONMIN soil. Other macro- and micronutrients in the shoot tissues, such as Ca, Mg, Mn, and Cu reached the sufficiency range in all treatments. A very moderate limitation was detected for N in all treatments. Iron concentrations were generally high, with particularly high values in DOK-LTE soils (401–842 mg kg^{-1} dry biomass, **Table 2**). Significant differences in the nutritional status were more pronounced for lettuce grown in DOK-LTE compared to HUB-LTE soils.

Expression of Stress-Related Genes in the Shoot Tissue

A qPCR-based method was used to investigate the relative expression of 14 genes, known to be involved in biotic or abiotic stress signaling pathways as previously investigated for lettuce (Chowdhury et al., 2019). Although various genes showed similar expression in plants grown in both soils with long-term organic fertilization (HU-org, BIODYN2) and long-term mineral fertilization (HU-min, CONMIN), certain stress-related genes were significantly upregulated in the treatments with long-term organic fertilization at both field sites. The results showed a significantly enhanced expression of the genes *RbohD*, *PDF1.2*, the Fe-transporter *OPT3* gene and the nitrate reductase gene *NIA1* in shoots of lettuce grown in soils with long-term organic fertilization (HU-org, BIODYN2) in

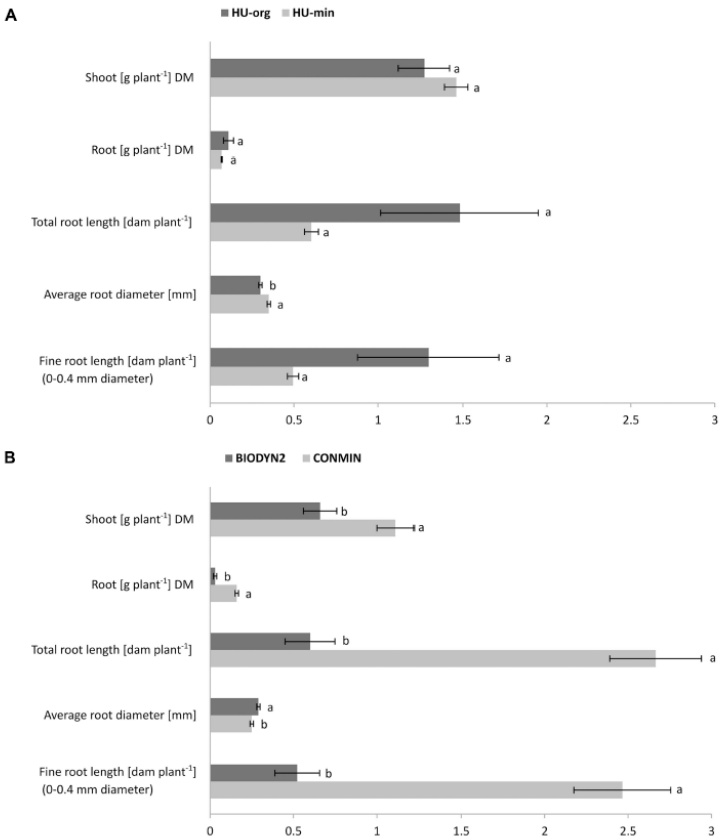

FIGURE 1 | Shoot and root dry biomass (DM) and root morphology of lettuce (cv. Tizian). The plants were grown in minirhizotron culture for nine weeks in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. Means ± standard errors of four independent replicates. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for the sites HUB-LTE **(A)** and DOK-LTE **(B)** by one-way ANOVA, Tukey's HSD pairwise test, $p \leq 0.05$.

comparison to plants grown in soils with long-term mineral fertilization (HU-min, CONMIN) (**Figure 2**). In addition, in HUB-LTE soils a significant upregulation of the genes *LOX1*, *WRKY25*, and *MYB15* in shoots of lettuce grown in organically fertilized soil (HU-org) compared to mineral fertilization (HU-min) was observed.

Chemical Composition of the Rhizosphere Soil Solution

Low molecular weight organic compounds in the rhizosphere soil solutions of lettuce grown in the various soil treatments revealed clearly different patterns depending on the fertilization history (**Figure 3**). The separation of organic compound patterns of organically and minerally fertilized soils were more distinct in DOK-LTE soils compared to HUB-LTE soils. In the different treatments, largely the same organic compounds were detectable with quantitative differences. In the rhizosphere of lettuce grown in BIODYN2 soil but also in BIODYN2 soil without root contact, exceptionally high levels of amino acids were detected (**Table 3C** and **Supplementary Table 3C**), leading to separate clustering compared to CONMIN and HUB-LTE soils as shown by PCA analysis (**Figure 3**). Moreover, higher levels particularly of low molecular weight sugars and malate in the rhizosphere soil solutions collected from lettuce plants grown in HU-min soil explained the separation from HU-org soil (**Tables 3A,B** and **Figure 3**).

The rhizosphere soil solution of lettuce was dominated by sugars, carboxylates, and amino acids (**Table 3**). Among the various mono- and di-saccharides, particularly hexoses, such as glucose and maltose, were present in significantly lower amounts in samples collected from basal (mature) root zones of lettuce plants grown in soils with long-term organic fertilization

TABLE 2 | Plant nutritional status of lettuce (cv. Tizian).

Nutrient concentration of shoot dry biomass (DM) of lettuce (cv. Tizian)					
		HUB-LTE		DOK-LTE	
		HU-org	HU-min	BIODYN2	CONMIN
Macronutrients (g kg^{-1} shoot DM)					
N	35*	31.40 ± 1.51 a	31.51 ± 1.60 a	30.68 ± 0.40 a	32.04 ± 1.21 a
P	3.0*	1.66 ± 0.25 a	2.06 ± 0.09 a	1.75 ± 0.03 a	1.91 ± 0.12 a
K	42*	20.23 ± 3.18 b	33.44 ± 0.58 a	38.11 ± 1.93 a	26.35 ± 1.47 b
Ca	12*	17.46 ± 3.11 a	18.42 ± 1.60 a	14.76 ± 0.45 b	16.59 ± 0.62 a
Mg	1.0*	3.79 ± 0.61 a	3.54 ± 0.21 a	3.53 ± 0.10 b	5.05 ± 0.29 a
S	2.5*	1.79 ± 0.27 a	2.06 ± 0.13 a	2.54 ± 0.08 a	2.36 ± 0.12 a
Na	0.6*	4.08 ± 0.93 a	2.92 ± 0.05 a	3.40 ± 0.35 b	5.59 ± 0.25 a
Micronutrients (mg kg^{-1} shoot DM)					
Cu	2.5**	2.87 ± 0.55 a	3.21 ± 0.41 a	7.10 ± 0.53 a	7.52 ± 0.64 a
Fe	50**	123.63 ± 18.00 a	289.38 ± 122.81 a	841.52 ± 203.32 a	401.24 ± 162.02 a
Mn	20**	63.37 ± 9.78 a	85.91 ± 9.48 a	80.10 ± 5.74 a	81.75 ± 12.19 a
Zn	20**	19.74 ± 2.96 a	26.47 ± 2.00 a	55.37 ± 2.06 a	33.55 ± 3.91 b

*The plants were grown in minirhizotron culture for nine weeks in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. Data represent means ± standard errors of four independent replicates per treatment. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for the sites DOK-LTE and HUB-LTE by one-way ANOVA, Tukey's HSD pairwise test, $p \leq 0.05$. *Deficiency threshold macronutrients (g kg^{-1} DM). **Deficiency threshold micronutrients (mg kg^{-1} DM), (Bergmann, 1988).*

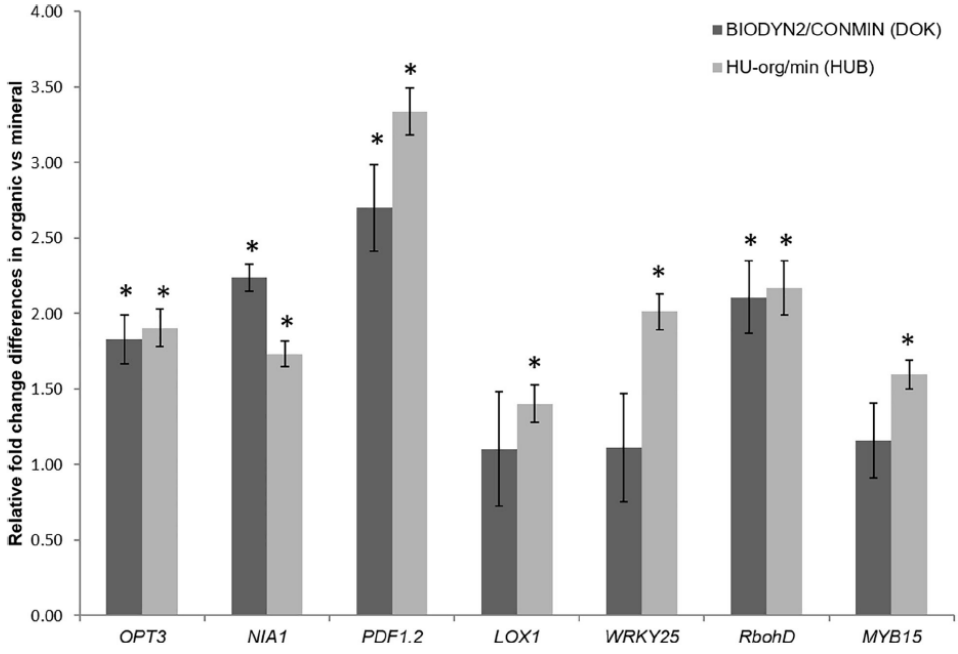

FIGURE 2 | Upregulated genes in shoots of lettuce grown over a period of nine weeks in soils with long-term organic (HU-org, BIODYN) in comparison to mineral (HU-min, CONMIN) fertilization history. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was employed for relative quantification ($n = 4$) by qPCR (see section "Materials and Methods"). Means ± standard errors of four independent replicates showing relative changes in gene expression of plants grown under long-term organic vs. mineral fertilization. Only genes showing significant ($p \leq 0.05$) differences in ΔCt values between organic vs. mineral fertilization within each site as revealed by Tukey's HSD pairwise test are shown (denoted by asterisks). Gene names, putative functions and primer sequences are described in **Supplementary Table 1**.

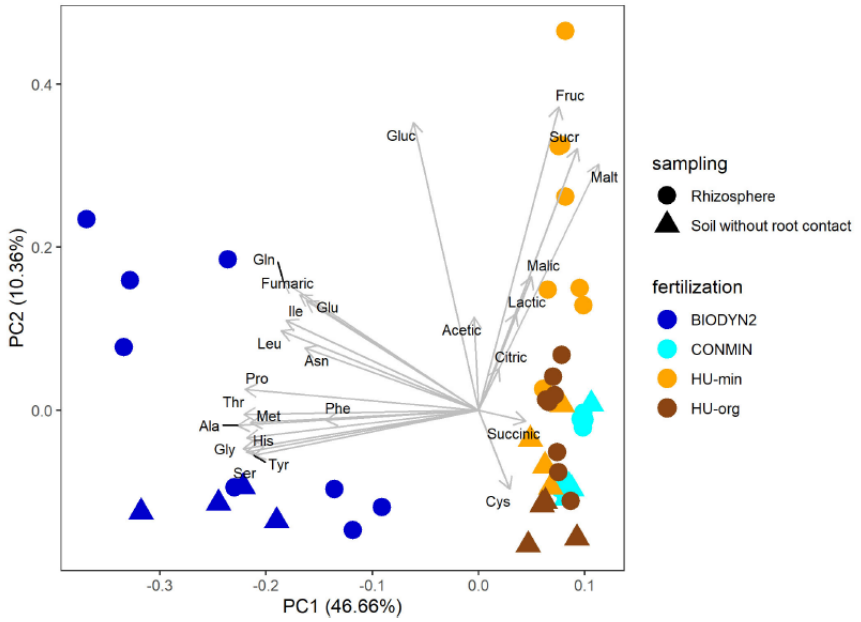

FIGURE 3 | Biplot of PCA comparing patterns of organic compounds in the rhizosphere soil solution of lettuce (cv. Tizian) and in soil without root contact under long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization. Ala, alanine; Asn, asparagine; Cys, cysteine; Gln, glutamine; Gly, glycine; Glu, glutamic acid; His, histidine; Ile, iso-leucine; Leu, leucine; Phe, phenylalanine; Pro, proline; Met, methionine; Ser, serine; Thr, threonine; Tyr, tyrosine; Fruc, fructose; Gluc, glucose; Malt, maltose, Sucr, sucrose; Acetic, acetate; Citric, citrate; Fumaric, fumarate; Malic, malate; Succinic, succinate; Lactic, lactate.

history (HU-org, BIODYN2) as compared to soils with mineral fertilization (HU-min, CONMIN). In BIODYN2 soil, all sugars ranged below the detection limit (**Table 3A**).

Lactate and acetate were the dominant monocarboxylates in the rhizosphere soil solutions (**Table 3B**) but detectable at higher levels also in samples collected from soil without root contact (**Supplementary Table 3B**). The concentrations of acetate were increased in the rhizosphere of lettuce grown in soils with organic fertilization history (HU-org, BIODYN2) (**Table 3B**). Among the di- and tri-carboxylates described also in lettuce tissues (Misaghi and Grogan, 1978), citrate was dominant in the rhizosphere soil solution of all treatments. Dicarboxylates such as malate and succinate dominated in soils with long-term mineral fertilization, with particularly high levels of succinate only in subapical (young) root zones in CONMIN soil (**Table 3B**). The antifungal compound benzoate was found in lower concentrations in the rhizosphere soil solution of older root zones of lettuce plants grown in the BIODYN2 soil compared with all other soils (**Table 3B**).

Microbial Community Analyses

Site, Habitat, and Fertilization Effects on Microbial Diversity

Alpha-diversity of bacterial and archaeal communities was lower in the rhizosphere than in root-associated soil, when assessed by Shannon, Pielou and richness indices based on 16S rRNA gene sequencing. This effect was more pronounced in HUB-LTE than in DOK-LTE soils. The long-term organic fertilization in DOK-LTE resulted in a significantly higher bacterial and archaeal diversity (Shannon, richness) in both, root-associated soil and the rhizosphere, as compared to mineral fertilization. Evenness, however, was not affected by fertilization in DOK-LTE. Organic fertilization in HUB-LTE also tended to increase the diversity (Shannon, richness) of bacterial and archaeal communities in the rhizosphere and root-associated soil but the effect was not significant (**Table 4**). Regarding fungal diversity, significantly lower alpha-diversity indices (Shannon, richness, Pielou) in the rhizosphere than in root-associated soil were observed in DOK-LTE based on ITS2 sequencing in both fertilization regimes. In HUB-LTE, this effect was detected only for fungal richness. HU-org significantly increased the fungal species richness compared to HU-min in the rhizosphere and root-associated soil (Shannon, Pielou). A similar trend was observed in the rhizosphere of DOK-LTE soils. No fertilization-dependent effect was observed on fungal evenness.

Microbial Community Composition Affected by Site, Habitat, and Fertilization History

Bacterial and archaeal community composition clearly differed depending on the habitat (R^2 = 30%, $p < 0.001$, PERMANOVA; **Figure 4A**) and the site (R^2 = 22%, $p < 0.001$). Separate

TABLE 3 | Sugars **(A)**, carboxylates **(B)**, and amino acids **(C)** in the rhizosphere soil solution of lettuce (cv. Tizian), grown in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history.

	HUB-LTE				DOK-LTE			
	HU-org	HU-min	HU-org	HU-min	BIODYN2	CONMIN	BIODYN2	CONMIN
	Basal (mature)		Subapical (young)		Basal (mature)		Subapical (young)	
A. Sugars in the rhizosphere soil solution (nmol cm^{-1} root length)								
Fructose	2.21 ± 1.04 a	2.02 ± 0.48 a	1.70 ± 0.51 a	4.34 ± 1.31 a	n.d. b	0.93 ± 0.09 a	1.47 ± 0.03 b	2.85 ± 0.03 a
Glucose	n.d. b	1.80 ± 0.49 a	1.62 ± 0.14 a	3.41 ± 0.58 a	n.d. b	1.23 ± 0.18 a	5.95 ± 1.75 a	1.22 ± 0.30 a
Sucrose	0.57 ± 0.06 a	1.53 ± 0.53 a	0.90 ± 0.20 a	1.19 ± 0.31 a	n.d.	n.d.	n.d. b	0.45 ± 0.03 a
Maltose	n.d. b	0.61 ± 0.03 a	0.47 ± 0.01 b	1.00 ± 0.10 a	n.d. b	0.36 ± 0.01 a	n.d. b	0.46 ± 0.02 a
Sum	2.79 a	5.97 a	4.70 a	9.95 a	n.d. b	2.54 a	7.43 a	4.97 a
B. Carboxylates in the rhizosphere soil solution (nmol cm^{-1} root length)								
Malate	n.d. b	2.91 ± 0.53 a	n.d. b	14.87 ± 3.11 a	n.d.	n.d.	0.72 ± 0.35 a	n.d. b
Citrate	5.42 ± 1.83 a	7.75 ± 3.10 a	2.23 ± 0.57 b	5.73 ± 1.23 a	8.83 ± 3.87 a	3.91 ± 1.46 a	3.37 ± 0.62 a	5.97 ± 1.21 a
Succinate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. b	7.78 ± 1.77 a
Fumarate	n.d. b	0.29 ± 0.05 a	0.30 ± 0.05 a	0.34 ± 0.09 a	0.39 ± 0.07 a	n.d. b	0.56 ± 0.04 a	n.d. b
Benzoate	0.15 ± 0.05 a	0.06 ± 0.01 a	0.10 ± 0.01 a	0.08 ± 0.01 a	0.02 ± 0.005 b	0.11 ± 0.004 a	0.05 ± 0.01 a	0.09 ± 0.01 a
Sum	5.57 a	11.02 a	2.63 b	21.03 a	9.25 a	4.02 a	4.71 b	13.85 a
Lactate	59.47 ± 12.41 a	61.83 ± 20.71 a	20.95 ± 4.56 a	61.15 ± 17.22 a	35.83 ± 2.87 a	12.17 ± 3.28 b	35.20 ± 7.68 a	30.96 ± 5.07 a
Acetate	66.65 ± 0.64 a	25.61 ± 11.91 b	11.27 ± 4.99 b	34.20 ± 2.70 a	30.11 ± 21.88 a	11.33 ± 3.93 a	24.52 ± 2.23 a	n.d. b
Sum	126.12 a	87.45 a	32.22 b	95.35 a	65.95 a	23.51 a	59.73 a	30.96 a
C. Amino acids in the rhizosphere soil solution (pmol cm^{-1} root length)								
Glutamic acid	8 ± 2 a	19 ± 9 a	21 ± 8 a	19 ± 5 a	90 ± 21 a	8 ± 3 b	294 ± 82 a	8 ± 2 b
Asparagine	12 ± 3 a	36 ± 21 a	15 ± 3 a	19 ± 6 a	612 ± 234 a	5 ± 1 b	1868 ± 867 a	6 ± 1 b
Serine	51 ± 6 a	40 ± 11 a	57 ± 5 a	55 ± 2 a	282 ± 18 a	42 ± 4 b	307 ± 14 a	50 ± 6 b
Glutamine	19 ± 10 a	40 ± 14 a	16 ± 6 a	77 ± 35 a	97 ± 16 a	10 ± 3 b	131 ± 9 a	12 ± 2 b
Glycine	56 ± 4 a	36 ± 10 a	48 ± 5 a	49 ± 2 a	249 ± 22 a	41 ± 1 b	290 ± 5 a	42 ± 1 b
Threonine	6 ± 1 a	6 ± 1 a	9 ± 2 a	7 ± 2 a	93 ± 11 a	11 ± 6 b	144 ± 27 a	7 ± 3 b
Histidine	n.d.	n.d.	n.d.	n.d.	15 ± 6 a	n.d. a	26 ± 3 a	n.d. b
Alanine	16 ± 4 a	9 ± 3 a	15 ± 2 a	13 ± 1 a	152 ± 17 a	6 ± 1 b	224 ± 20 a	9 ± 1 b
Proline	10 ± 2 a	6 ± 3 a	17 ± 4 a	18 ± 6 a	51 ± 5 a	6 ± 1 b	70 ± 9 a	7 ± 1 b
Cystine	2 ± 2 a	n.d. a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Thyrosine	n.d.	n.d.	n.d.	n.d.	108 ± 19 a	4 ± 1 b	94 ± 9 a	3 ± 2 b
Methionine	n.d. a	7 ± 1 a	n.d.	n.d.	119 ± 37 a	n.d. b	166 ± 23 a	n.d. b
Isoleucine	n.d. a	7 ± 2 a	n.d. b	8 ± 1 a	n.d.	n.d.	51 ± 15 a	n.d. b
Leucine	n.d. a	0.3 ± 0.3 a	0.4 ± 0.4 b	6 ± 0.4 a	16 ± 0.7 a	n.d. b	88 ± 32 a	n.d. b
Phenylalanine	19 ± 4 a	6 ± 1 b	14 ± 3 a	10 ± 0.6 a	18 ± 2 a	11 ± 2 a	54 ± 13 a	12 ± 3 b
Sum	200 a	212 a	212 a	284 a	1903 a	144 b	3808 a	156 b

The plants were cultivated in minirhizotron culture for nine weeks. Micro-sampling of soil solutions was conducted with sorption filters in 1–2 cm regions of subapical (young) roots and from basal (mature) root zones (8–9 cm). Means ± standard errors. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for the sites DOK-LTE and HUB-LTE by t-test ($p \leq 0.05$). n.d. = not detectable.

clustering of rhizosphere and root-associated soils was observed for HUB-LTE and DOK-LTE depending on mineral and organic fertilization practice. This finding was confirmed by PERMANOVA analysis which revealed an interaction effect between habitat and site ($R^2 = 7\%$, $p < 0.001$) as well as between habitat and fertilization ($R^2 = 3\%$, $p \leq 0.05$). A significant influence of the fertilization on the bacterial and archaeal communities was observed ($R^2 = 6\%$, $p < 0.001$). Fertilization-dependent clustering of bacterial and archaeal communities was more distinct in DOK-LTE, especially in the rhizosphere, compared to HUB-LTE (**Figure 4A**).

The fungal community compositions were clearly separated by site ($R^2 = 42\%$, $p < 0.001$, **Figure 4B**). Unlike the bacterial and archaeal communities, distinct clustering of rhizosphere and root-associated soils was only observed for DOK-LTE independent of fertilization regimes due to the combined effect of site and habitat ($R^2 = 19\%$, $p < 0.001$). In both LTEs, the impact of fertilization was significant but marginal ($R^2 = 5\%$, $p < 0.001$).

Site, habitat and fertilization effects were also detected in the bacterial and archaeal taxonomic community composition. Thaumarchaeota and Verrucomicrobia had higher relative abundances in DOK-LTE compared to HUB-LTE soils

TABLE 4 | Microbial alpha-diversity (Shannon diversity, species richness and Pielou's evenness) in root-associated soil (RA) and rhizosphere (RH) of lettuce (cv. Tizian).

Diversity Index	Habitat	Organism	HUB-LTE		DOK-LTE	
			HU-org	HU-min	BIODYN2	CONMIN
Microbial alpha-diversity (Shannon diversity, species richness and Pielou's evenness)						
Shannon	RA	Bacteria/Archaea	6.68 ± 0.01 Aa	6.73 ± 0.03 Aa	6.42 ± 0.03 Aa	6.33 ± 0.01 Ab
		Fungi	3.33 ± 0.04 Aa	3.39 ± 0.14 Aa	3.39 ± 0.04 Aa	3.37 ± 0.04 Aa
	RH	Bacteria/Archaea	5.29 ± 0.08 Ba	4.49 ± 0.48 Ba	6.29 ± 0.1 Aa	4.67 ± 0.65 Bb
		Fungi	3.36 ± 0.02 Aa	3.07 ± 0.06 Ab	0.68 ± 0.16 Ba	1.12 ± 0.32 Ba
Richness	RA	Bacteria/Archaea	1735.59 ± 12.45 Aa	1723.31 ± 19.86 Aa	1664.18 ± 18.29 Aa	1567.54 ± 8.59 Ab
		Fungi	376.25 ± 2.32 Aa	334.25 ± 7.41 Ab	333.50 ± 9.84 Aa	349.75 ± 5.82 Aa
	RH	Bacteria/Archaea	1007.77 ± 32.82 Ba	829.10 ± 85.15 Ba	1495.93 ± 46.57 Ba	1016.08 ± 140.83 Bb
		Fungi	285.00 ± 8.57 Ba	201.75 ± 8.31 Bb	229.33 ± 20.00 Ba	193.00 ± 14.53 Ba
Pielou	RA	Bacteria/Archaea	0.90 ± 0 Aa	0.90 ± 0 Aa	0.87 ± 0 Aa	0.86 ± 0 Aa
		Fungi	0.56 ± 0.01 Ba	0.58 ± 0.02 Aa	0.58 ± 0 Aa	0.58 ± 0.01 Aa
	RH	Bacteria/Archaea	0.77 ± 0.01 Ba	0.67 ± 0.06 Ba	0.86 ± 0.01 Aa	0.67 ± 0.08 Aa
		Fungi	0.59 ± 0 Aa	0.58 ± 0.01 Aa	0.12 ± 0.03 Ba	0.21 ± 0.06 Ba

The plants were grown in minirhizotrons for nine weeks in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. Data represent means ± standard errors of four independent replicates. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately per site, habitat and organism by t-test ($p \leq 0.05$). Different capital letters indicate significant differences between root-associated soil vs. rhizosphere tested separately per site, fertilization and organism by t-test ($p \leq 0.05$).

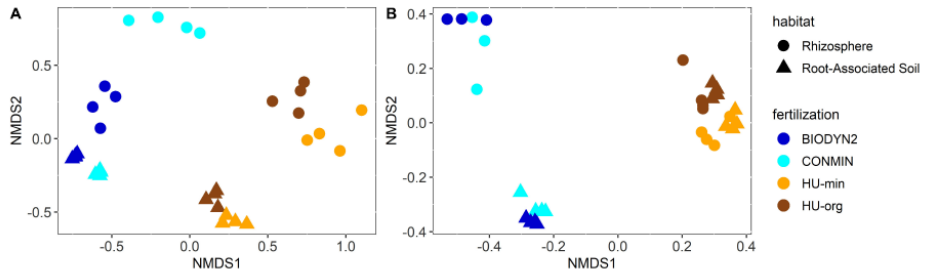

FIGURE 4 | Two-dimensional diagram of non-metric multi-dimensional scaling (NMDS) analysis of calculated Bray-Curtis distances between **(A)** bacterial and archaeal community compositions or **(B)** fungal community compositions in root-associated soil and the rhizosphere of lettuce (cv. Tizian) grown in soils from HUB-LTE (HU-org, HU-min) or DOK-LTE (BIODYN2, CONMIN). NMDS analyses are based on relative abundances. Stress = 0.11 (bacterial and archaeal communities) and 0.07 (fungal communities).

(**Supplementary Table 4A**). The rhizosphere effect was illustrated by an enrichment in Gamma-, Alpha-, and Betaproteobacteria and by a decrease in relative abundances of Acidobacteria and Firmicutes (**Supplementary Table 4A**). Heatmap analysis of the most abundant genera in root-associated soils and rhizospheres from HUB-LTE and DOK-LTE showed many taxa occurring at both sites, however most of them exhibited differential relative abundances in rhizosphere and root-associated soils (**Figure 5**). At both sites, *Bacillus* and *Nitrososphaera* were typical genera found in root-associated soils as well as sequences belonging to the family *Chitinophagaceae* or classified as acidobacterial subdivisions Gp4 and Gp6. Typical rhizosphere responders to lettuce at both sites were affiliated to e.g., *Pseudomonas*, *Rhizobium*, and *Massilia*. When lettuce was grown in HUB-LTE soils, *Variovorax*, *Devosia*, and *Asticcacaulis* were highly abundant in the rhizosphere. In contrast, *Duganella* and sequences classified as *Oxalobacteraceae* were present in the rhizosphere of lettuce grown in DOK-LTE soils. Fertilization affected the relative abundance of major rhizosphere genera. For instance, *Pseudomonas* was highly abundant in the lettuce rhizosphere of CONMIN (up to 61%) and HU-min (up to 74%) and also HU-org (up to 35%). However, a high variability among replicates was observed. In HUB-LTE soils the relative abundance of *Rhizobium* reached up to 13–17% in the rhizosphere, while the remaining major abundant taxa ranged below 10% (**Figure 5B**).

The root-associated soils of lettuce grown in HUB-LTE were enriched in the fungal phylum Ascomycota, while Mortierellomycota dominated the soils from DOK-LTE independent of fertilization regimes (**Supplementary Table 4B**). Mineral fertilization increased Basidiomycota at each site. A strong enrichment in Olpidiomycota characterized the rhizosphere in both treatments of DOK-LTE. The rhizosphere

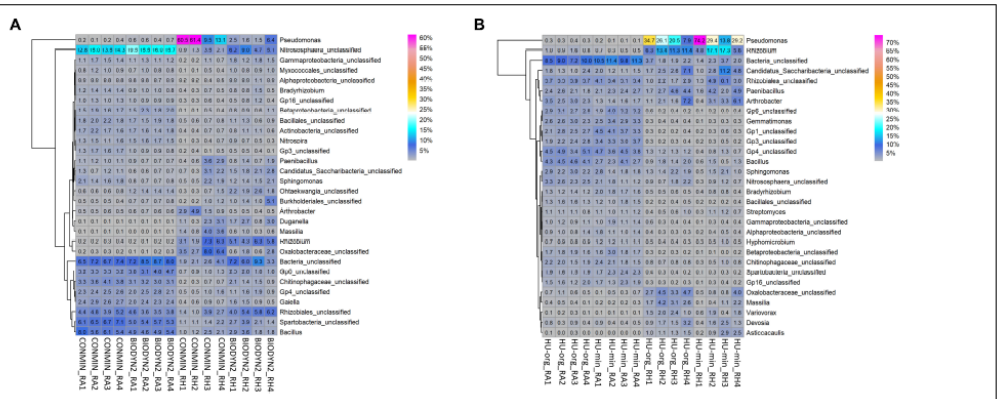

FIGURE 5 | Heatmaps displaying relative abundance distribution of top 30 most abundant bacterial and archaeal genera in root-associated soils (RA) and in the rhizosphere (RH) of lettuce (cv. Tizian) grown in **(A)** DOK-LTE soils (BIODYN2, CONMIN) and **(B)** HUB-LTE soils (HU-org, HU-min). The numbers in cells represent relative abundances (%).

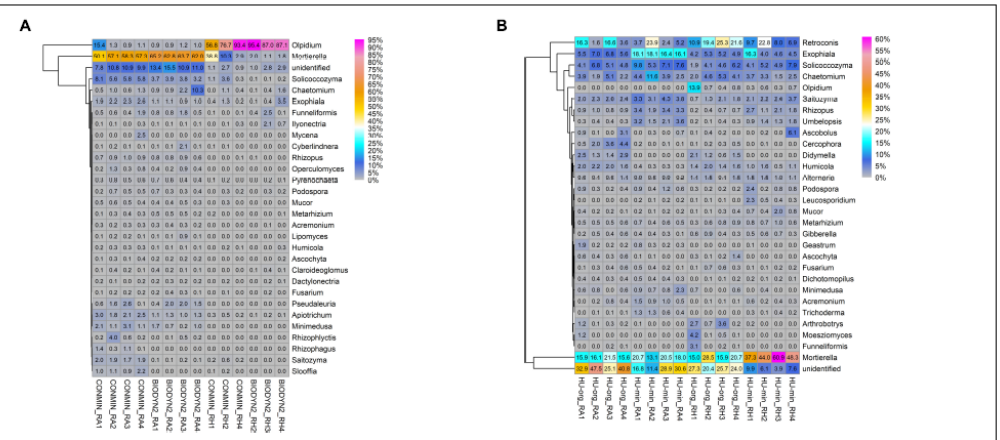

FIGURE 6 | Heatmaps displaying relative abundance distribution of top 30 most abundant fungal genera in root-associated soils (RA) and in the rhizosphere (RH) of lettuce (cv. Tizian) grown in **(A)** DOK-LTE soils (BIODYN2, CONMIN) and **(B)** HUB-LTE soils (HU-org, HU-min). The numbers in cells represent relative abundances (%).

in organic fertilization treatments of HUB-LTE also exhibited an increase of this phylum compared to mineral fertilization. Furthermore, Glomeromycota (arbuscular mycorrhizal fungi, AMF) were enriched in the rhizosphere of organic fertilization treatments of both LTEs.

Heatmap analyses of the most abundant fungal genera in root-associated soil and rhizosphere showed that almost half of the genera could be detected in both LTEs but differing in relative abundances (**Figure 6**). In general, in DOK-LTE more differences were observed between habitats than between fertilization regimes (**Figure 6A**). The root-associated soils, especially of BIODYN2, were dominated by *Mortierella* and unclassified fungi (at genus level) with a strongly reduced relative abundance in the rhizosphere. Higher relative abundances of different yeasts (*Solicoccozyma, Exophiala, Apiotrichum, Saitozyma*, and *Sloofia*) as well as AMF *Rhizophagus* were observed in minerally fertilized soil (CONMIN). *Olpidium* was the dominant rhizosphere genus in both treatments of DOK-LTE. The differences between the fertilization regimes (organic vs. mineral) were distinct in HUB-LTE (**Figure 6B**). Typical genera positively affected by organic fertilization independent of habitat were *Cercophora, Didymella*, and *Humicola*. Mineral fertilization (HU-min) enriched not only *Rhizopus* and *Umbelopsis* but also different yeasts (*Exophiala, Solicoccozyma*, and *Saitozyma*), which was in accordance with the DOK-LTE. The rhizosphere of mineral fertilization (HU-min) showed high relative abundances of the genus *Mortierella*,

while the rhizosphere of HU-org exhibited a high relative abundance of *Retroconis* and of sequences that could not be reliably classified at genus level.

Fertilization Effects on Microbial Communities

As we were interested in the effect of fertilization on plant-microbe interactions, we analyzed fertilization-dependent changes in the relative abundance of microbes in the rhizosphere of lettuce. Taxa differing significantly in relative abundance between organic vs. mineral fertilization were determined separately for each site and at several taxonomic levels [OTU (only for fungi), genus, family, order, class, phylum]. More differentially abundant bacterial and archaeal taxa were found in the rhizosphere of lettuce grown in DOK-LTE soils (>1.0% relative abundance; **Table 5**). *Pseudomonadaceae* (Gammaproteobacteria) had a significantly higher relative abundance in CONMIN rhizosphere samples compared to BIODYN2 (**Table 5B**). A similar trend was observed in the rhizosphere of HU-min vs. HU-org, however, differences were not significant. When lettuce was grown in BIODYN2 soil, a significant enrichment in taxa belonging to the Firmicutes phylum (e.g., Clostridiales) was found compared to CONMIN (**Table 5B**).

In contrast to bacteria and archaea, more differentially abundant fungal taxa were found in the rhizosphere of lettuce grown in HUB-LTE soils compared to DOK-LTE (**Table 6**). To obtain further insights into the ecological assignment of detected fungal genera, they were assessed against the FUNGuild database for classification into potential pathotrophic, saprotrophic or symbiotrophic fungi (**Supplementary Table 5**). In concordance with heatmaps (**Figure 6**), the potential pathotrophic genus *Olpidium* showed the highest relative abundance in the rhizosphere of lettuce when grown in both DOK-LTE soils and was enriched in the root-associated soil of CONMIN (**Supplementary Tables 5**, **6B**). The rhizosphere of HU-org showed the highest number of significantly enriched pathotrophs including the genera *Olpidium, Moesziomyces*, and *Ascochyta* whereas the root-associated soil of HU-min was enriched with pathotrophic-saprotrophic fungi, especially *Exophiala*. The plant pathogen *Rhizopus* was increased in the root-associated soil and in the rhizosphere of HU-min, whereas *Didymella* was increased in the root-associated soil and in the rhizosphere of HU-org. The saprotrophic genera *Cercophora* and *Humicola* as well as *Arthrobotrys* and *Plenodomus* were highly abundant in the root-associated soil and in the rhizosphere of HU-org, respectively. *Umbelopsis* was enriched in both habitats of HU-min (**Table 6A** and **Supplementary Table 6A**). Significantly more sequences classified as *Mortierella*, a saprotrophic-symbiotrophic genus, were found in the rhizosphere of lettuce grown in minerally fertilized soils (HUB-LTE and DOK-LTE) in comparison with organic fertilization. Mycorrhizal symbiotrophs (*Clariodeoglomus, Funneliformis*) were enriched in both habitats of lettuce grown in HU-org whereas the relative abundance of *Trichoderma* increased in root-associated soil of HU-min (**Supplementary Table 5**). Further results for fungal taxa in the root-associated soils are shown in **Supplementary Table 6**.

Canonical correspondence analyses revealed relationships in rhizosphere bacterial and archaeal and fungal community composition with organic compounds detected in the rhizosphere soil solution (**Figure 7**). Ordination showed that the fertilization-dependent differentiation in rhizosphere bacterial and archaeal communities of lettuce grown in DOK-LTE soils was clearly related to the concentration of succinate (CONMIN) or fumarate and amino acids (BIODYN2). HUB soils were positively associated with sugars (sucrose, maltose) which caused the separate clustering of rhizosphere bacterial and archaeal communities apart from DOK-LTE soil samples. Fertilization-dependent community differences in HUB-LTE soils were less clear as compared to DOK-LTE soils but a significant contribution of malate to the differentiation of HU-min was identified. The relative abundances of *Pseudomonadaceae* OTUs responding positively to mineral fertilization were negatively correlated with fumaric and amino acids (**Figure 7A**).

Fertilization-dependent differences of fungal rhizosphere communities were more pronounced in HUB-LTE compared to DOK-LTE (**Figure 7B**). The clear separation between organic vs. mineral fertilization (HUB-LTE) was caused by the positive correlation of several compounds in HU-min compared to only one in HU-org (acetate). Fungal communities of lettuce grown in HU-min soils showed a clear association to sugars (sucrose, maltose, and fructose) and malate, which was partly comparable with the bacterial and archaeal communities. The identified responder OTUs (*Rhizopus arrhizus, Mortierella* sp., *Umbelopsis* sp., *Mortierella hyalina*, *Mortierella humilis*, and *Nigrospora oryzae*, **Table 6**) reacted positively to mineral fertilization. Regarding all four treatments, *M. elongata* responded also positively to mineral fertilization in HUB-LTE, although this OTU was significantly enriched in CONMIN compared to BIODYN2 (**Table 6B**). Comparable with bacterial and archaeal communities, fungal rhizosphere communities of lettuce grown in DOK soils, especially BIODYN2, were clearly associated with amino acids.

DISCUSSION

Plant roots are described as powerful drivers of microbiota assemblage (Bais et al., 2006; Bakker et al., 2014). However, to which extent site-specific factors or fertilization management interact with the recruitment of rhizosphere microbiota remains largely unclear. In a holistic approach, we tried to correlate the structure of rhizosphere microbial communities with data on organic composition of the rhizosphere soil solution, root growth characteristics and aboveground plant traits (biomass, nutrient status and expression of stress-related genes). Two strategies of fertilization management were exemplarily compared by growing lettuce plants in minirhizotrons with contrasting soils from two LTEs with organic and mineral fertilization history.

Site- and Fertilization-Dependent Plant Performance

No consistent effects of long-term fertilization practice on aboveground plant biomass and root characteristics (total and

TABLE 5 | Bacterial and archaeal taxa in the rhizosphere of lettuce (cv. Tizian) differing significantly (FDR < 0.05) in relative abundance depending on long-term organic vs. mineral fertilization practice at HUB-LTE (HU-org vs. HU-min) **(A)** and DOK-LTE (BIODYN2 vs. CONMIN) **(B)**.

Kingdom	Phylum	Class	Order	Family	Genus	HU-org (%)	HU-min (%)
A. Bacterial rhizosphere taxa differing significantly (FDR < 0.05) in relative abundance in lettuce grown in long-term organically vs. minerally fertilized soils from HUB-LTE							
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonadaceae*	*Cellvibrio*	**1.1 ± 0.4**	0.0 ± 0.0
Bacteria	Proteobacteria	Betaproteobacteria	Methylophilales	*Methylophilaceae*	*Methylophilus*	**1.2 ± 0.7**	0.0 ± 0.0
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**BIODYN2 (%)**	**CONMIN (%)**
B. Bacterial and archaeal rhizosphere taxa differing significantly (FDR < 0.05) in relative abundance in lettuce grown in long-term organically vs. minerally fertilized soils from DOK-LTE							
Archaea	Thaumarchaeota					**6.3 ± 1.0**	1.9 ± 0.6
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Sphingomonadaceae*_unclassified	**1.1 ± 0.1**	0.2 ± 0.1
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis		**1.1 ± 0.3**	0.1 ± 0.0
Bacteria	Firmicutes	Clostridia				**3.5 ± 0.7**	0.8 ± 0.2
Bacteria	Firmicutes	Clostridia	Clostridiales			**3.3 ± 0.7**	0.8 ± 0.2
Bacteria	Firmicutes	Firmicutes_unclassified				**1.0 ± 0.2**	0.0 ± 0.0
Bacteria	Firmicutes	Firmicutes_unclassified	Firmicutes_unclassified			**1.0 ± 0.2**	0.0 ± 0.0
Bacteria	Firmicutes	Firmicutes_unclassified	Firmicutes_unclassified	Firmicutes_unclassified		**1.0 ± 0.2**	0.0 ± 0.0
Bacteria	Firmicutes	Firmicutes_unclassified	Firmicutes_unclassified	Firmicutes_unclassified	*Firmicutes*_unclassified	**1.0 ± 0.2**	0.0 ± 0.0
Bacteria	Cyanobacteria/Chloroplast					**1.2 ± 0.7**	0.0 ± 0.0
Bacteria	Cyanobacteria/Chloroplast	Cyanobacteria				**1.2 ± 0.7**	0.0 ± 0.0
Bacteria	Actinobacteria					7.3 ± 0.8	**8.2 ± 0.8**
Bacteria	Actinobacteria	Actinobacteria				6.9 ± 0.8	**7.9 ± 0.7**
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales			4.3 ± 0.4	**6.5 ± 0.7**
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	*Micrococcaceae*		0.5 ± 0.0	**3.2 ± 0.9**
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	*Micrococcaceae*	*Arthrobacter*	0.5 ± 0.0	**2.6 ± 0.9**
Bacteria	Proteobacteria					46.6 ± 4.7	**69.3 ± 5.3**
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*		4.2 ± 1.1	**9.7 ± 2.5**
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Massilia*	0.6 ± 0.1	**2.5 ± 0.8**
Bacteria	Proteobacteria	Gammaproteobacteria				8.6 ± 1.4	**39.8 ± 13.4**
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales			5 ± 1.2	**37.3 ± 14.1**
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonadaceae*		4.9 ± 1.2	**36.9 ± 14.1**

Only taxa with >1.0% relative abundance are displayed. Data represent means of relative abundance ± standard errors. Bold numbers indicate significant enrichment.

TABLE 6 | Relative abundance of fungal taxa in the rhizosphere of lettuce (cv. Tizian) differing significantly (FDR < 0.05) in relative abundance depending on long-term organic vs. mineral fertilization practice at HUB-LTE (HU-org vs. HU-min) **(A)** and DOK-LTE (BIODYN2 vs. CONMIN) **(B)**.

Phylum	Class	Order	Family	Genus	OTU	HU-org (%)	HU-min (%)
A. Fungal rhizosphere taxa differing significantly (FDR < 0.05) in relative abundance of lettuce grown in long-term organically vs. minerally fertilized soils from HUB-LTE							
Ascomycota	Dothideomycetes					**16.2 ± 1.6**	2.7 ± 0.4
Ascomycota	Dothideomycetes	Pleosporales				**16.1 ± 1.6**	2.7 ± 0.4
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*			**13.2 ± 1.5**	0.8 ± 0.3
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*	*Didymella*		**1.4 ± 0.3**	0 ± 0
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*	*Didymella*	*Didymella protuberans* (100%)	**1.3 ± 0.3**	0 ± 0
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*	unidentified	*Didymellaceae* sp.	**11.3 ± 1.1**	0.8 ± 0.3
Ascomycota	Orbiliomycetes					**1.8 ± 0.8**	0.1 ± 0
Ascomycota	Orbiliomycetes	Orbiliales				**1.8 ± 0.8**	0.1 ± 0
Ascomycota	Orbiliomycetes	Orbiliales	*Orbiliaceae*			**1.8 ± 0.8**	0.1 ± 0.1
Ascomycota	Orbiliomycetes	Orbiliales	*Orbiliaceae*	*Arthrobotrys*		**1.8 ± 0.8**	0.1 ± 0.1
Ascomycota	Orbiliomycetes	Orbiliales	*Orbiliaceae*	*Arthrobotrys*	*Arthrobotrys oligospora* (100%)	**1.6 ± 0.8**	0.1 ± 0.1
Ascomycota	Pezizomycetes	Pezizales	*Pezizaceae*			**2.2 ± 2.1**	0 ± 0
Ascomycota	Pezizomycetes	Pezizales	*Pezizaceae*	unidentified	*Pezizaceae* sp.	**2.2 ± 2.1**	0 ± 0
Basidiomycota	Agaricomycetes					**1.5 ± 0.4**	0.3 ± 0.1
Basidiomycota	Ustilaginomycetes					**1.2 ± 1.0**	0 ± 0
Basidiomycota	Ustilaginomycetes	Ustilaginales				**1.2 ± 1.0**	0 ± 0
Basidiomycota	Ustilaginomycetes	Ustilaginales	*Ustilaginaceae*			**1.2 ± 1.0**	0 ± 0
Basidiomycota	Ustilaginomycetes	Ustilaginales	*Ustilaginaceae*	*Moesziomyces*		**1.2 ± 1.0**	0 ± 0
Basidiomycota	Ustilaginomycetes	Ustilaginales	*Ustilaginaceae*	*Moesziomyces*	*Moesziomyces aphidis* (100%)	**1.2 ± 1.0**	0 ± 0
Glomeromycota						**1.8 ± 1.1**	0 ± 0
Glomeromycota	Glomeromycetes					**1.8 ± 1.1**	0 ± 0
Glomeromycota	Glomeromycetes	Glomerales				**1.8 ± 1.1**	0 ± 0
Glomeromycota	Glomeromycetes	Glomerales	*Glomeraceae*			**1.0 ± 0.8**	0 ± 0
Olpidiomycota						**4.0 ± 3.3**	0.5 ± 0.1
Olpidiomycota	Olpidiomycetes					**4.0 ± 3.3**	0.5 ± 0.1
Olpidiomycota	Olpidiomycetes	Olpidiales				**4.0 ± 3.3**	0.5 ± 0.1
Olpidiomycota	Olpidiomycetes	Olpidiales	*Olpidiaceae*			**4.0 ± 3.3**	0.5 ± 0.1
Olpidiomycota	Olpidiomycetes	Olpidiales	*Olpidiaceae*	*Olpidium*		**4.0 ± 3.3**	0.5 ± 0.1
Olpidiomycota	Olpidiomycetes	Olpidiales	*Olpidiaceae*	*Olpidium*	*Olpidium brassicae* (99.7%)	**4.0 ± 3.3**	0.5 ± 0.1
–	–	–	–	unidentified		**24.3 ± 1.5**	6.9 ± 1.3
Ascomycota	Pezizomycetes	Pezizales	*Ascobolaceae*			0.2 ± 0.1	**1.6 ± 1.5**
Ascomycota	Sordariomycetes	Sordariales	*Lasiosphaeriaceae*	*Podospora*		0.2 ± 0	**1.1 ± 0.5**
Ascomycota	Sordariomycetes	Sordariales	*Lasiosphaeriaceae*	*Podospora*	*Podospora* sp.	0.2 ± 0	**1.0 ± 0.5**
Basidiomycota	Tremellomycetes	Tremellales				1.5 ± 0.3	**2.6 ± 0.4**

(Continued)

TABLE 6 | Continued

Phylum	Class	Order	Family	Genus	OTU	HU-org (%)	HU-min (%)
Mortierellomycota						20.0 ± 3.1	**47.8 ± 5.0**
Mortierellomycota	Mortierellomycetes					20.0 ± 3.1	**47.8 ± 5.0**
Mortierellomycota	Mortierellomycetes	Mortierellales				20.0 ± 3.1	**47.8 ± 5.0**
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae			20.0 ± 3.1	**47.6 ± 5.0**
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*		20.0 ± 3.1	**47.6 ± 5.0**
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*	*Mortierella humilis* (100%)	0 ± 0	**1.7 ± 0.6**
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*	*Mortierella hyalina* (100%)	1.1 ± 0.3	**8.3 ± 4.0**
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*	*Mortierella* sp.	1.1 ± 0.2	**4.2 ± 1.1**
Mucoromycota						1.0 ± 0.2	**4.3 ± 0.5**
Mucoromycota	Mucoromycetes					0.8 ± 0.2	**2.9 ± 0.6**
Mucoromycota	Mucoromycetes	Mucorales				0.8 ± 0.2	**2.9 ± 0.6**
Mucoromycota	Mucoromycetes	Mucorales	Rhizopodaceae			0.5 ± 0.1	**1.9 ± 0.3**
Mucoromycota	Mucoromycetes	Mucorales	Rhizopodaceae	*Rhizopus*		0.5 ± 0.1	**1.9 ± 0.3**
Mucoromycota	Mucoromycetes	Mucorales	Rhizopodaceae	*Rhizopus*	*Rhizopus arrhizus* (100%)	0.5 ± 0.1	**1.9 ± 0.3**
Mucoromycota	Umbelopsidomycetes					0.2 ± 0	**1.4 ± 0.2**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales				0.2 ± 0	**1.4 ± 0.2**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales	Umbelopsidaceae			0.2 ± 0	**1.4 ± 0.2**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales	Umbelopsidaceae	*Umbelopsis*		0.2 ± 0	**1.4 ± 0.2**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales	Umbelopsidaceae	*Umbelopsis*	*Umbelopsis* sp.	0.2 ± 0	**1.4 ± 0.2**
Phylum	**Class**	**Order**	**Family**	**Genus**	**OTU**	**BIODYN2 (%)**	**CONMIN (%)**
B. Fungal rhizosphere taxa differing significantly (FDR < 0.05) in relative abundance of lettuce grown in long-term organically vs. minerally fertilized soils from DOK-LTE							
Glomeromycota						**1.5 ± 0.9**	0.2 ± 0
Glomeromycota	Glomeromycetes	Glomerales				**1.4 ± 0.9**	0.1 ± 0
Basidiomycota	Tremellomycetes	Filobasidiales	*Piskurozymaceae*			0.1 ± 0	**1.7 ± 1.0**
Mortierellomycota						1.6 ± 0.3	**17.3 ± 11.0**
Mortierellomycota	Mortierellomycetes	Mortierellales	*Mortierellaceae*			1.6 ± 0.3	**17.3 ± 11.0**
Mortierellomycota	Mortierellomycetes	Mortierellales	*Mortierellaceae*	*Mortierella*	*Mortierella elongata* (100%)	0.2 ± 0	**7.2 ± 5.7**

Only taxa with >1.0% relative abundance are displayed. For OTUs at species level, the percentages of similarity compared to the database are specified. Data represent means of relative abundance ± standard errors. Bold numbers indicate significant enrichment.

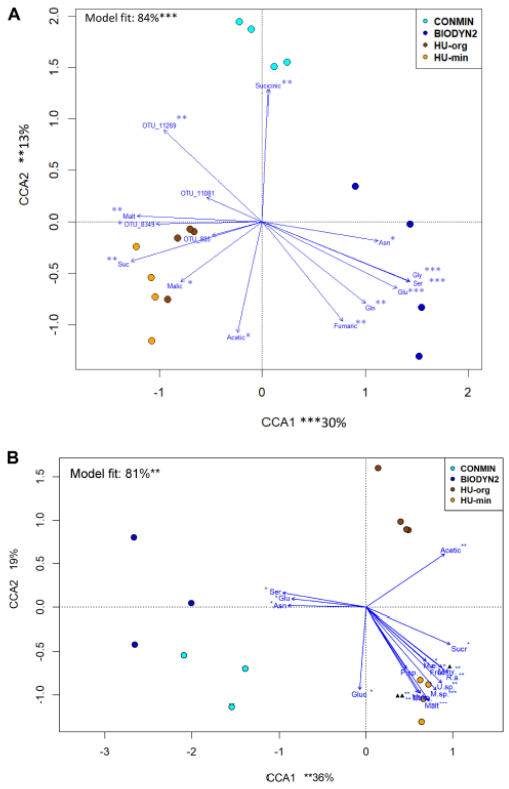

FIGURE 7 | Canonical correspondence analysis (CCA) of bacterial and archaeal **(A)** and fungal **(B)** community composition in the rhizosphere of lettuce grown in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization. CCA is based on log10 transformed relative abundances of bacterial, archaeal and fungal OTUs using organic compounds in rhizosphere soil solution averaged over basal (mature) and subapical root zones. **(A)** Bacterial and archaeal communities: Acetate (Acetic) + malate (Malic) + succinate (Succinic) + fumarate (Fumaric) + maltose (Malt) + sucrose (Suc) + asparagine (Asn) + serine (Ser) + glutamine (Gln) + glutamic acid (Glu) + glycine (Gly) as constraint variables. Significant vectors were fitted onto CCA ordination including log transformed relative abundances of *Pseudomonadaceae* OTUs that were significantly enriched (FDR < 0.05) in minerally fertilized soils and exhibited a mean relative abundance of >0.5%. Tentative taxonomic identification of OTUs based on most similar BLAST hit: OTU_11269 – *Pseudomonas corrugata* (99.77%); OTU_11081 – *P. silesiensis* (96.97%); OTU_8349 – *P. corrugata* (99.3%); OTU_885 – *P. tolaasii* (99.77%). Significant codes: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **(B)** Fungal communities: Acetate (Acetic) + malate (Malic) + fructose (Fruc) + glucose (Gluc) + sucrose (Sucr) + maltose (Malt) + glutamic acid (Glu) + asparagine (Asn) + serine (Ser) as constraint variables. Significant vectors were fitted onto CCA ordination including log transformed relative abundances of OTUs (identified at least at genus level) that were significantly enriched (FDR < 0.05) in minerally fertilized soils and exhibited a mean relative abundance of >1.0% (M.e. – *Mortierella elongata*; R.a. – *Rhizopus arrhizus*; M.sp. – *Mortierella* sp.; P.sp. – *Podospora* sp.; U.sp. – *Umbelopsis* sp.; M.hy. – *Mortierella hyalina*; M.hu. – *Mortierella humilis*). Overlapping designations: ▲ Fruc*/M.e.*/M.hy.**, ▲▲ Malic**/M.hu.**. Significant codes: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

fine root length, average diameter, and length of root hairs) were detected in this study (**Figure 1**), as similarly reported in an earlier lettuce experiment with the same soils (Chowdhury et al., 2019). The latter authors reported similar growth in DOK-LTE soils and lower plant biomass in organically fertilized soil of the HUB-LTE (Chowdhury et al., 2019). In our experiment, the results were opposite, with similar biomass in soils of the HUB-LTE and shoot and root biomass drastically reduced by 41% and 81%, respectively, in the organically fertilized soil (BIODYN2) of the DOK-LTE. This effect could not be attributed to nutrient limitations. Although at the end of the experiment, concentrations of mineral nutrients (N, P, K) in shoot tissues were below or close to the reported deficiency thresholds (Bergmann, 1988), these effects were observed similarly for all soil treatments and not only for the BIODYN2 soil (**Table 2**). Similar plant growth reductions were observed in a repeated experiment with the same soils, already detectable during early plant establishment (**Supplementary Table 2**). This suggests the presence of additional stress factors in BIODYN2 soil independent of nutrient limitations, as a site-specific effect. The discrepancy between our findings and those of Chowdhury et al. (2019) may have resulted from the collection of the soils in different years with different pre-crops. Effects of the pre-crop on soil microbial communities have been reported previously (Sommermann et al., 2018; Babin et al., 2019) and the detritusphere microbiome of pre-crop roots can even overwrite the rhizosphere effect of the current crop (Zhou et al., 2020) with potential consequences for plant performance. Furthermore, in the study of Chowdhury et al. (2019) lettuce plants were cultivated in pots, whereas our study was conducted with minirhizotrons promoting the development of high rooting densities along the root observation windows. This may lead to locally increased exudate concentrations contributing to attraction of beneficial but also of pathogenic microorganisms with potential impact on plant performance.

Soil Microbial Communities Affected by Fertilization, Site, and Habitat Effects

The LTE site and the habitats, comprising rhizosphere and root-associated soil, distinguished bacterial, archaeal, and fungal community composition as recently reported by Chowdhury et al. (2019). A significantly higher bacterial and archaeal alpha-diversity was mainly found in the rhizosphere of lettuce when grown in organically vs. minerally fertilized soils of DOK-LTE but not of HUB-LTE (**Table 4**). Conversely, the alpha-diversity of the fungal rhizosphere community was significantly increased in organically fertilized soil of HUB-LTE, but not of DOK-LTE, illustrating the impact of the soil type as a major driver determining not only the soil but also the rhizosphere microbial composition (Schreiter et al., 2014b; Chowdhury et al., 2019). The selective effect of the plant on recruitment of microbial communities was apparent by a lower alpha-diversity in the rhizosphere compared to root-associated soil (**Table 4**) as reported in various other studies (Mendes et al., 2013; Chowdhury et al., 2019).

The majority of bacterial and archaeal rhizosphere responders were classified as Gammaproteobacteria of the genus *Pseudomonas* with particularly high relative abundance (up to 60–74%) in soils with mineral fertilization history, while only marginal rhizosphere enrichment (rel. abundance 2–6%) was detectable in the BIODYN2 rhizosphere with long-term organic fertilization (**Figure 5**). Many members of this genus exert beneficial effects on plants (Roquigny et al., 2017), among them *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas brassicacearum*, although pathogens are also reported. Since we observed the lowest biomass production along with impaired fine root development in lettuce plants grown in the BIODYN2 soil (**Figure 1B**), this may indicate a relationship between the low relative abundance of potentially beneficial *Pseudomonadaceae* and plant growth, because lettuce growth was not impaired in the soils with a particularly high *Pseudomonas* relative abundance (**Figures 1**, **5**). A preliminary identification showed high similarities with members of the *P. fluorescens* complex with plant growth-promoting properties (Garrido-Sanz et al., 2016). However, strain identification based on short-read Illumina 16S rRNA gene sequences is limited. Future studies should therefore consider cultivation-based approaches for comprehensive taxonomic and functional characterization.

With regard to fungal communities, the most remarkable rhizosphere effect was the enrichment of the pathogenic genus *Olpidium* in the rhizosphere of soils with organic fertilization history (HU-org, BIODYN2), which was particularly pronounced for DOK-LTE, where its relative abundance reached 76-90% (**Figure 6** and **Supplementary Table 4B**). Lettuce is a host for *Olpidium* sp. (Olpidiomycota, formerly classified as Chytridiomycota). Zoospores of the fungus with random motility rapidly invade the tips of fine roots (Maccarone, 2013). Since these zoospores are not specifically attracted by root exudates (Westerlund et al., 1978), direct relationships with the observed site- and fertilization-dependent differences in the composition of the rhizosphere soil solution (**Figure 3** and **Table 3**) are unlikely. The pathogen can survive in soils as dormant spores for up to 20 years (Campbell, 1985). Early plant infection can result in severe growth inhibition (Navarro et al., 2004). Accordingly, in our study, growth retardation and inhibition of root hair development in the BIODYN2 soil (**Figure 1B** and **Supplementary Table 2**) was associated with typical symptoms of *Olpidium* infection. Root infection with intracellular formation of sporangia and resting spores in fine roots (**Supplementary Figure 1**) was consistent with the highest relative abundance of *Olpidium* (90%) in the BIODYN2 rhizosphere (**Figure 6** and **Supplementary Table 5**). Interestingly, in the CONMIN soil, symptoms caused by *Olpidium* were less pronounced despite similarly high relative *Olpidium* abundance in the rhizosphere of both treatments CONMIN (76%) and BIODYN2 soil (90%) (**Supplementary Table 5**). Zoospores exhibit high motility in wet clay soils to initiate infections in nearby plants (Westerlund et al., 1978). This may explain the low relative abundance of *Olpidium* in the sandy HUB-LTE soils (0.5–4%) and increased infection rates in the silty loam DOK-LTE soils with a higher water retention capacity as a site-specific effect. Nevertheless, this cannot explain the high prevalence of disease symptoms in plants from BIODYN2 soil (**Supplementary Figure 1**) with organic fertilization compared to CONMIN soil supplied with mineral fertilizers despite similar relative *Olpidium* abundance in the rhizosphere (**Supplementary Table 5** and **Figure 6**).

Along with the rhizosphere enrichment in *Olpidium*, a particularly low relative abundance of the fungal genus *Mortierella* (1.6%) was found for the BIODYN2 rhizosphere, reaching 17-48% in the other investigated treatments (**Figure 6** and **Supplementary Table 5**). Fungal endophytes of the genus *Mortierella* are classified as saprotrophic symbiotrophs (**Supplementary Table 5**). Most recently, plant growth-promoting properties and biocontrol activity against fungal pathogens via jasmonic acid-dependent plant stress signaling were reported for various *Mortierella* species (Kamzolova et al., 2014; Tamayo-Velez and Osorio, 2017; Li et al., 2018) including *Mortierella elongata* identified in our study (**Table 6B**). Therefore, we suggest that higher sensitivity toward *Olpidium* infection of lettuce plants grown in the BIODYN2 soil may be a consequence of a low relative abundance of bacterial (*Pseudomonas*) and fungal (*Mortierella*) antagonists or plant growth promoters in the rhizosphere. In contrast, a selective pathogen-suppressive effect of long-term pesticide application in the CONMIN soil (**Table 1**) seems to be unlikely in this case, since the relative *Olpidium* abundance was even higher in the root-associated CONMIN soil compared to BIODYN2 soil (**Supplementary Table 5**).

Apart from rhizosphere enrichment of potential plant pathotrophs and saprotrophs, AMF (*Funneliformis* and *Claroideoglomus*) as plant beneficial symbiothrophs, increased in organically fertilized soils (BIODYN2 and HU-org). A higher extent of AMF root colonization and increased AMF species diversity in organic farming soils has been frequently reported (Sattelmacher et al., 1991; Douds et al., 1997; Gosling et al., 2006) as a consequence of lower P availability (Nagahashi et al., 1996; Olsson et al., 2003) or reduced fertilizer and pesticide inputs. Accordingly, the lowest P-nutritional status was recorded for lettuce plants grown in BIODYN2 and HU-org soils (**Table 2**).

Components of the Rhizosphere Soil Solution Related to Rhizosphere Microbiota

In our study, significantly lower sugar concentrations (particularly glucose) were recorded in the rhizosphere soil solution of mature roots of lettuce plants grown in soils with organic compared to mineral fertilization history (**Table 3A**). Host plants provide up to 20% of photo-assimilates to mature arbuscular-mycorrhizal root systems, consumed preferentially in form of glucose by AMF. Consequently, this is frequently associated with lower sugar exudation into the rhizosphere (Jones et al., 2004). Accordingly, also in our study, lower glucose concentrations in the lettuce rhizosphere in soils with long-term organic fertilization coincided with a higher rhizosphere relative abundance of AMF (**Table 6** and **Supplementary Table 4B**). Interestingly, in lettuce plants grown in BIODYN2 soil, with the highest root infection rate by the *Olpidium* pathogen (**Figure 6** and **Supplementary Table 5**), sugar concentrations ranged close to or even below the detection limit in the rhizosphere

of mature root zones (**Table 3A**). This is most likely caused by the high carbon demand and sugar consumption of the obligate biotrophic pathogen. On the other hand, some reports show antagonistic effects of AMF colonization against *Olpidium* (Hao et al., 2019) but it remains questionable, whether this applies for competitive root colonization of *Olpidium* and AMF in the establishment phase during early growth of the host plants analyzed in our study. The extremely limited availability of glucose in the rhizosphere of the BIODYN2 plants (**Table 3A**) may also explain the particularly low relative rhizosphere abundance of *M. elongata* (**Table 6B**) with potential antagonistic properties (Li et al., 2018), since glucose supply was identified as a major carbon source for stimulation of *Mortierella* mycelium growth (Chesters and Peberdy, 1965). Noteworthy, also antagonistic effects between root colonization with AMF and *Mortierella* sp. were recently reported in avocado nurseries (Tamayo-Velez and Osorio, 2017). This might at least partially explain the higher *Mortierella* rhizosphere relative abundance in the soils with long-term mineral fertilization history (**Table 6** and **Supplementary Table 4B**).

Windisch et al. (2017) reported the ability of bacterial biocontrol inoculants (*Pseudomonas* sp. RU47; *Serratia plymuthica* 3Re-4-18) to induce root exudation of benzoate in lettuce, as a secondary metabolite with antifungal activity (Yoon et al., 2012). This was related to increased tolerance against bottom rot disease caused by *Rhizoctonia solani*. Surprisingly, in our study, the significantly reduced rhizosphere relative abundance of *Pseudomonaceae* in lettuce plants grown in the BIODYN2 soil in comparison with the CONMIN soil (**Table 5** and **Figure 5**) was associated with *Olpidium*-related growth depressions and significantly reduced benzoate concentrations in the rhizosphere soil solution (**Table 3B**). Therefore, a low rhizosphere presence of bacterial communities with antagonistic potential in the BIODYN2 soil (e.g., *Pseudomonas*) may be responsible for the limited stimulation of benzoate exudation, thereby increasing the plant sensitivity to pathogen attack. Moreover, benzoate also has chemo-attractive properties for various *Pseudomonas* species (reviewed by Sampedro et al., 2015).

In terms of di- and tri-carboxylates, characterized as intracellular compounds in lettuce (Misaghi and Grogan, 1978), high concentrations of succinate were detected exclusively in the rhizosphere soil solution of lettuce grown in the CONMIN soil (**Table 3B**) and related to a high relative abundance of *Pseudomonadaceae* (**Figure 7A**). Among other dicarboxylates, such as malate and fumarate, also detected in the rhizosphere soil solutions of the investigated lettuce plants particularly in combination with mineral fertilization (**Table 3B**), succinate has been characterized as a potent chemoattractant with importance for root colonization by various *Pseudomonas* strains with plant growth-promoting properties (Oku et al., 2014; reviewed by Sampedro et al., 2015). Moreover, succinate is the major carbon source required for production of pyoverdine siderophores by fluorescent pseudomonads (Marek-Kozaczuk and Skorupska, 1997) with biocontrol functions via iron competition with pathogens (Höfte et al., 1991) and induction of systemic plant defense responses (Audenaert et al., 2002; Adesina et al., 2007). Therefore, we speculate that a potential relationship between the enrichment of beneficial *Pseudomonadaceae* and high succinate concentrations in the CONMIN rhizosphere counteracted *Olpidium* pathogenesis.

Additionally, reduced sugar concentrations in connection with extremely high amino acid concentrations resulted in a lower C/N ratio in the rhizosphere soil solution of plants grown in the BIODYN2 soil, as compared with all other soil treatments (**Table 3**). Gammaproteobacteria are generally characterized as fast-growing, copiotrophic microorganisms, which prefer carbon-rich environments and hence a preference for high C/N substrate ratios has been described for Gammaproteobacteria (Kuramae et al., 2012; Michaud et al., 2014; Shen et al., 2016). Consequently, the relatively high N and low C availability in the rhizosphere of lettuce grown in BIODYN2 soil may have counteracted an enrichment of Gammaproteobacteria (i.e., *Pseudomonadaceae*) with potentially antagonistic properties in the rhizosphere. Accordingly, amino acid accumulation in the rhizosphere was negatively correlated with rhizosphere relative abundance of *Pseudomonadaceae* (**Figure 7A**). Similarly high amino acid concentrations were recorded also in samples collected from BIODYN2 soil without root contact (**Supplementary Table 3C**). A possible explanation for this phenomenon is the high organic N content potentially affected by long-term input of composted farmyard manure in BIODYN2 soil with the highest C_{org} and total N contents among all other investigated soils (**Table 1B**). Thus, a high mineralization potential and elevated levels of free amino acids as intermediate products may have led to the observed increase of amino acid concentrations in the soil solution largely overwriting the rhizosphere effect. Nevertheless, a wide range of amino acids has been reported as chemoattractants for *Pseudomonas* strains (reviewed by Sampedro et al., 2015) but due to the absence of a rhizosphere effect for amino acids in the BIODYN2 soil, no chemoattraction can be expected in this case. Similarly, high concentrations of acetate and lactate were detectable in the rhizosphere and in soil without root contact (**Table 3B** and **Supplementary Table 3B**), suggesting that these compounds are rather products of organic carbon turnover than directly released from plant roots.

Rhizosphere Microbiota and Stress-Related Gene Expression of the Host Plant

The rhizosphere of lettuce grown in HU-org and BIODYN2 was enriched in fungal pathotrophs and patho-saprotrophs (**Supplementary Table 5**). This could be a possible reason for a significantly higher expression of jasmonic acid signaling-dependent genes (*PDF1.2*) and lipoxigenase (*LOX*) genes in the shoot tissue of plants grown in soils with organic vs. mineral fertilization, as previously reported also by Chowdhury et al. (2019). Recent findings suggest that the rhizosphere microbiota act as an additional immune barrier for plants and can activate long distance signaling pathways, involving ethylene, salicylic and jasmonic acid, which led to systemic resistance in distal tissues (Spoel and Dong, 2012; Hacquard et al., 2017). The *RbohD* gene,

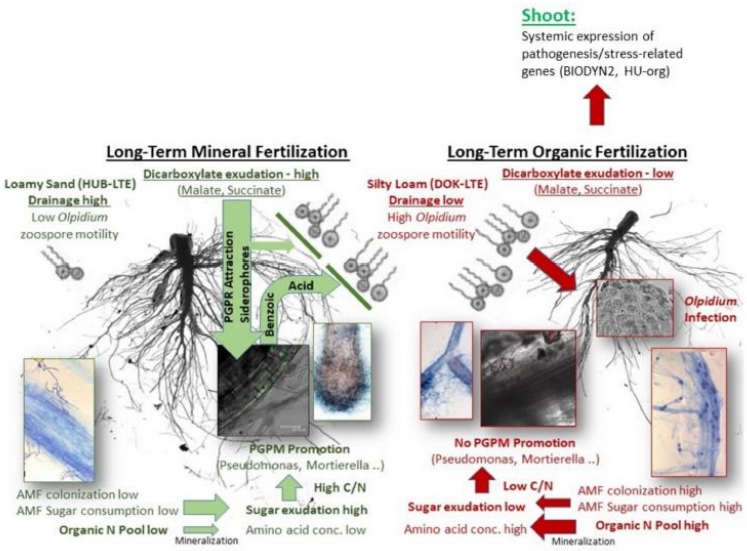

FIGURE 8 | Hypothetic model, summarizing the potential interplay between fertilization management, soil factors, root exudation and rhizosphere microbial communities in lettuce-*Olpidium* interactions. Pathogen-conducive scenarios are indicated by red labels, while green labels represent pathogen-suppressive scenarios. A high drainage potential of the loamy sand soils (HUB-LTE) counteracts the motility of *Olpidium* zoospores, which is stimulated by low moisture draining in the silty loam soils (DOK-LTE), thereby promoting *Olpidium* pathogen pressure. In the pathogen suppressive scenario, long-term mineral fertilization reduces the soil AMF inoculum (arbuscular mycorrhizal fungi). Consequently, lower AMF colonization is associated with lower sugar consumption by the fungal partner and with higher sugar exudation. The higher availability of easily accessible carbohydrates attracts rhizosphere colonization by Gammaproteobacteria (i.e., *Pseudomonadaceae*) and *Mortierella* with documented plant growth-promoting and pathogen-suppressive properties. This is further promoted by higher root exudation of dicarboxylates (i.e., succinate) with known functions as chemoattractants and substrates for siderophore production in many *Pseudomonas* species with plant growth-promoting properties. Moreover, *Pseudomonas* inoculation can stimulate root exudation of benzoate with antifungal properties in lettuce. By contrast, in the pathogen-conducive scenario, high relative abundance of AMF in the rhizosphere associated with high fungal sugar consumption drastically reduces the rhizosphere sugar concentrations of lettuce plants grown in DOK-LTE soils with long-term organic fertilization (BIODYN2), counteracting root colonization by potentially beneficial *Pseudomonadaceae* and *Mortierella* species, thereby favoring *Olpidium* infection. A low C/N ratio in the rhizosphere soil solution, promoted by high background concentrations of amino acids probably related with more intense organic N mineralization under long-term organic fertilization further reduces the relative abundance of *Pseudomonadaceae* in the rhizosphere (for details, see section "Discussion"). Photos by courtesy of Markus Weinmann, Namis Eltlbany, and Khalid Hameed.

encodes NADPH oxidase (respiratory burst oxidase), involved in free radical (ROS) production for pathogen defense after contact with pathogen-associated molecular patterns (PAMPs) and has also been reported as systemic response to elevated pathogen abundance in the root/rhizosphere (Miura and Tada, 2014; Kadota et al., 2015). Our results showed that this gene was upregulated in both soils with organic fertilization (BIODYN2, HU-org) including high relative abundances of potential pathotrophs (**Supplementary Table 5**).

Redistribution of iron upon pathogen attack is another defense response in various plant species, leading to accumulation of Fe^{3+} in the apoplast of epidermal cells, where it induces the production of H_2O_2, resulting in a defensive oxidative burst (Verbon et al., 2017). This can induce local iron deficiencies in the cytosol (Liu et al., 2007) and may explain the preferential upregulation of the *OPT3* gene in lettuce plants grown in organic HU-org and BIODYN2 soils (**Figure 2**). The gene encodes an oligopeptide transporter involved in phloem-mediated Fe transport, induced under conditions of Fe deficiency (Stacey et al., 2008). Moreover, pathogen infection can induce systemic activation of genes involved in root-induced Fe acquisition (*FRO2*, *IRT1*; Verbon et al., 2017) and may offer an explanation for the particularly high shoot Fe concentration in plants, grown in DOK-LTE soils (**Table 2**), associated with higher pathogen (*Olpidium*) enrichment in the rhizosphere compared to HUB-LTE soils (**Supplementary Table 4B**). Interestingly, the expression of the nitrate reductase gene (*NIA1*) was significantly increased in lettuce plants in soils with long-term organic fertilization history. This may be attributed to a higher N mineralization potential because of long-term manure-based fertilization in BIODYN2 and HU-org soils. However, in the experimental setup, all treatments received full N supply via nitrate fertilization, sufficient for the duration of our experiment, without any treatment differences of the N nutritional status (**Table 2**). An alternative explanation may be provided by the results of Vujinović et al. (2020), demonstrating

superior induction of nitrate reductase genes in maize by humic substances isolated from soils with long-term organic fertilization as compared with conventional mineral fertilization.

Plant defense mechanisms are complex and in soil culture where the roots are in continuous contact with diverse microbes, it is difficult to assign a direct systemic effect of particular microbial taxa to changes in the gene expression in the shoots. The generally higher expression of stress-related genes recorded in the shoots of organically grown plants are interesting indicators for a systemic defense response. However, we were not able to detect any specific response in stress gene expression to the particularly intense infection with the root pathogen *Olpidium*, affecting plants grown in BIODYN2 soil. Severe growth depressions and infection symptoms suggest that the upregulation of the respective defense genes alone was not sufficient to counteract the pathogen attack. Certain pathogens are able to overcome these primary defense lines via specific elicitors with potential to inhibit signaling pathways or the synthesis and accumulation of defense compounds by the host plant (Henry et al., 2012; Fitzpatrick et al., 2020). This scenario may be reflected by the reduced accumulation of the antifungal root exudate benzoate in the rhizosphere of the *Olpidium*-infected plants grown in BIODYN2 soil (**Table 3B**). Comparison with plants in CONMIN soil, lacking disease symptoms despite a similar relative rhizosphere abundance of the *Olpidium* pathogen and even lower systemic expression of defense genes, suggest that additional factors are required for pathogen suppression. These factors may comprise the recruitment of beneficial microorganisms (e.g., *Pseudomonas* sp., *Mortierella elongata*) via specific root exudate patterns (i.e., increased levels of succinate, malate, glucose), indicated by a reduction in stress gene expression as part of a "cry for help" strategy employed by the host plant (Fitzpatrick et al., 2020; Liu et al., 2020). Additionally, the increased production of root exudates with antifungal potential (i.e., benzoate) was stimulated by the presence of beneficial microbes (Windisch et al., 2017). A chronological investigation of the plant responses to pathogen infection and a more comprehensive characterization of metabolic stress indicators could reveal more evidence in this concern.

CONCLUSION

The present study confirmed the well-documented selective effects of the soil site and the rhizosphere as important determinants shaping the diversity and composition of soil microbial communities. However, with respect to the impact of fertilization management, frequently reported benefits of long-term organic fertilization, such as increased soil organic matter, increased microbial biomass and metabolic activity, species richness and higher abundance of plant beneficial microorganisms were only partially confirmed. A significant increase of soil organic matter was only detectable for BIODYN2 soil. The rhizosphere microbial diversity under long-term organic fertilization was not consistently increased, for both bacterial/archaeal and fungal communities. Beneficial effects on the relative abundance of plant growth-promoting microorganisms were only detectable for AMF but not generally for potential plant growth-promoting microorganisms (*Pseudomonas*, *Mortierella*), and were sometimes (BIODYN2) even associated with severe growth depressions likely caused by the lettuce pathogen *Olpidium*. A consistently increased systemic expression of stress-related genes in shoot tissue of plants growing in soils with long-term organic fertilization, previously interpreted as stress-priming effect (Chowdhury et al., 2019), was not associated with suppression of *Olpidium*. In contrast, increased rhizosphere relative abundance of *Pseudomonas* sp. and *M. elongata* and increased accumulation of the antifungal root exudate benzoic acid coincided with reduction in disease severity caused by *Olpidium* in the CONMIN soil with long-term mineral fertilization, suggesting a function in pathogen defense. Obviously, the characteristics reported for long-term organic fertilization cannot be generalized as similarly documented in previous studies (Hartman et al., 2018; Chowdhury et al., 2019).

Furthermore, the results suggest that the effects of fertilization management on the chemical composition of the rhizosphere soil solution, as a major driver for shaping rhizosphere microbial communities, are not simply controlled by root activity and rhizodeposition but also by site-specific factors e.g., turn-over of soil organic matter and substrate competitions between different groups of rhizosphere microorganisms. Additionally, soil properties such as moisture level and water-holding capacity or the availability of specific mineral nutrients may act as modulators for the assemblage of rhizosphere microbiota.

The results point to a complex network of belowground interactions between plant roots, physicochemical soil properties and different groups of rhizosphere microbiota (schematically summarized in **Figure 8**), with an important role in shaping also the aboveground characteristics of the plants.

Although the presented results, so far based on model experiments under controlled conditions are mainly based on descriptions of coinciding relationships without consideration of temporal or spatial variations and lacking detailed mechanistic explanations, the study can provide a starting point for more focused investigations into the related processes. The identification of key players in these networks and their effects on plant health and crop performance may provide important information to optimize the interactive effects of fertilization management, related to rhizosphere processes and development of novel plant growth-promoting microorganisms inoculants. Finally, our results may contribute to the development of practical approaches according to the concept of "soil biological engineering" (Bender et al., 2016).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA622892 and http://www.ebi.ac.uk/ena/data/view/PRJEB39853.

AUTHOR CONTRIBUTIONS

SW, LS, DB, GN, RG, JG, and KS conceived and designed the experiment. SW, LS, DB, and SC conducted the experiments and collected the data. SW, LS, and DB wrote the manuscript with GN, UL, RG, JG, and KS. FW, BH, NM, and SW developed and performed the UHPLC-MS analysis of amino acids and benzoic acid. WA and SW developed and performed the HPLC-ELSD analysis of sugars. SW and GN developed and performed the RP-HPLC analysis of organic acids. DB, KS, JN, and SS performed and evaluated the sequencing of bacterial and archaeal communities. LS, IS, and JG developed and evaluated the sequencing and analysis of fungal communities. SC and MR developed and performed the plant gene expression analysis. SW and AE performed the detection of pathogen infection in the root tissue. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.597745/full#supplementary-material

Supplementary Figure 1 | Habitus of lettuce plants grown for six weeks in BIODYN2 soil infected with *Olpidium* sp. (likely *Olpidium brassicae*) **(A–C)** infected fine roots with intracellular fungal structures (arrows) and inhibition of root hair development, **(D)** *Olpidium* resting spore in the root tissue **(E)** sporangium of *Olpidium* in the root tissue. Photos by courtesy of Abbas El-Hasan.

Supplementary Table 1 | List of plant genes selected for expression analysis with their corresponding loci, functions in *A. thaliana* and primer sequences.

Supplementary Table 2 | Shoot and root biomass production and root growth parameters of lettuce (cv. Tizian). The plants were grown in minirhizotron culture in two independent experiments during a culture period of nine weeks and six weeks, respectively, on soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. Means ± standard errors of four independent replicates per treatment. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for the sites DOK-LTE and HUB-LTE by one-way ANOVA, Tukey's HSD pairwise test, $p \leq 0.05$.

Supplementary Table 3 | Sugars (A), carboxylates (B) and amino acids (C) in soil solutions of samples collected without visible contact to lettuce roots grown in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. The plants were grown in minirhizotron culture for nine weeks. Exudate collection was undertaken by micro-sampling with sorption filters on bulk soil, without any root contact. Means ± standard errors. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for each long-term experimental site DOK-LTE and HUB-LTE by t-test ($p \leq 0.05$). n.d. = not detectable.

Supplementary Table 4 | Taxonomic composition of (A) bacterial/archaeal phyla and proteobacterial classes and (B) fungal phyla represented as relative abundances in root-associated soil and rhizosphere of lettuce (cv. Tizian). The plants were grown in soils under long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for each long-term experimental site and habitat by edgeR (FDR < 0.05). Means of relative abundance ± standard errors.

Supplementary Table 5 | Ecological assignment of fungal genera represented as relative abundance with FUNGuild in root-associated soils and rhizosphere of lettuce (cv. Tizian) grown in soils of DOK-LTE (BIODYN2 vs. CONMIN) and HUB-LTE (HU-org vs. HU-min). Different lowercase letters indicate significant differences (FDR < 0.05) of represented genera between organic vs. mineral fertilization tested separately for each long-term experimental site and habitat based on the results of edgeR analyses. Means of relative abundance ± standard errors. Relative abundances (> 0.5%) with significant differences are marked in bold.

Supplementary Table 6 | Fungal taxa in the root-associated soils of lettuce (cv. Tizian) differing significantly (FDR < 0.05) in relative abundance depending on long-term organic vs. mineral fertilization practice at HUB-LTE (HU-org vs. HU-min) (A) and DOK-LTE (BIODYN2 vs. CONMIN) (B). Only taxa with > 1.0% relative abundance are displayed. For OTUs on species level the similarity compared to the database are represented. Means of relative abundance ± standard errors. Bold numbers indicate significant enrichment.

Supplementary Text 1 | Materials and Methods description of microbial community analyses: Bacterial and archaeal communities.

Supplementary Text 2 | Materials and Methods description of microbial community analyses: Fungal communities.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6.2 Supplementary materials

The supplementary materials for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.597745/full#supplementary-material

Tables

Supplementary Table 1| List of plant genes selected for expression analysis with their corresponding loci, functions in *A. thaliana* and primer sequences.

Name of gene (Locus tag in *Arabidopsis thaliana*)	**Documented functions in *Arabidopsis thaliana***	**Primer sequences (5'-3')** All primers were designed in this study and have an annealing temperature of 55°C
OPT3 (AT4G16370)	Iron transporter involved in systemic iron, zinc and cadmium distribution within the plant.	OPTf - GGCTTGTCACCGGAATGATC OPTr - TGCAAGGCGAAGAACAACAA
NIA1 (AT1G77760)	Nitrate reductase, nitrate induced expression and involved in nitrate assimilation.	NIAf - ACCTTCACCATGTCCGAAGT NIAr - TGAGTATGCTGTCACTGCCA
PR1 (AT2G14610)	Pathogenesis related protein 1, Salicylic acid (SA) dependent expression, involved in resistance against broad spectrum of pathogens.	PR1f - GAGAAGGCCGATTATGATTA PR1r - ATTATTGCATTGAACCCTTG
PDF1.2 (AT5G44420)	Plant defensin factor involved in Jasmonic acid (JA)/ Ethylene (Et) dependent pathogen defense responses. Involved in Induced systemic resistance (ISR).	PDF1.2f - ACAAGATATGCGAGCGGAGA PDF1.2r - TGACAGGCTCCATGTTTTGC
LOX1 (AT1G55020)	Lipoxigenase; Upstream gene involved in the oxylipin metabolic pathway. Involved in the signaling of wounding response and JA induced defense against specific pathogens.	LOX1f - AAGAGCAGAAGCCACCCATA LOX1r - GTGGAAGGAACTGCGAGAAG
WRKY70 (AT3G56400)	Transcription factor involved in both SA- and JA-mediated signal pathways. Also involved in abiotic stress signaling.	WRKY70f - GCACACACAAAACCGACCAA WRKY70r - AGTTGTTGCAAGTATGGTGTCC
WRKY25 (AT2G30250)	Negative regulator of SA-mediated defense responses, elevated expression in response to oxidative stress, heat stress or wounding.	WRKY25f - TGTTCAATGAGGAAGAAGGTGG WRKY25r - TCGTTTGGTGGATTGTGGTTT
CAT1 (AT1G20630)	Catalase induced by hydrogen peroxide, abscisic acid (ABA), drought, and salt stress.	CAT1f - GGTCCAAGGCGATGTCTTTG CAT1r - ATGAACAGCTGGCGTTTTGT
PER50 (AT4G37520)	Peroxidase; Responses to environmental stresses such as wounding, pathogen attack and oxidative stress.	PER50f - CTGTCAACACATGGGCTTCC PER50r - TCCCACTTCGACCCGTTTTA

ERF6 (AT4G17490)	Et- Response Factor family transcription factor. Responses to oxidative stress and biotic stress induced by biotrophic and necrotrophic pathogens.	ERF6f - CAAAACGTCGCGGATCTAGG ERF6r - GACGCAACCTCAAGTGGAAA
ZAT10 (AT1G27730)	Zinc finger protein; Transcriptional repressor involved in abiotic stress responses. Positive transcriptional regulator for salinity, heat and osmotic stress.	ZATf - TCGTGACTCCTTCCACTTCC ZATr - TAGGTGGACACAAGGCTAGC
RbohD (AT5G47910)	Respiratory burst oxidase homolog D. Involved in rapid reactive oxygen species (ROS) production on perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs).	RbohDf - ACAGGGTTCTTTCGACTGGT RbohDr - AATTAGAGCAGACCTGGCGT
RbohF (AT1G64060)	Respiratory burst oxidase homolog F. Involved in hypersensitive reaction (HR)-related cell death and interaction with intercellular ROS regulating pathogen defense responses.	RbohFf - TCATCGGCTCTAAGAAGCCC RbohFr - TGCTCCAGATGACGATTACCT
MYB15 (AT3G23250)	ABA inducible abiotic stress regulator, upregulated in cold and drought stress.	MYB15nf - AGGTGGGGTTGAAGAAAGGA MYB15nr - CGTACCAGCTTTTGAAGGCA

Supplementary Table 2| Shoot and root biomass production and root growth parameters of lettuce (cv. Tizian). The plants were grown in minirhizotron culture in two independent experiments during a culture period of nine weeks and six weeks, respectively, on soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. Means ± standard errors of four independent replicates per treatment. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for the sites DOK-LTE and HUB-LTE by one-way ANOVA, Tukey´s HSD pairwise test, $p \leq 0.05$.

Fresh biomass production and root growth parameters of lettuce (cv. Tizian)								
Plant biomass	**DOK-LTE**				**HUB-LTE**			
	BIODYN2	**CONMIN**	**BIODYN2**	**CONMIN**	**HU-org**	**HU-min**	**HU-org**	**HU-min**
	9 weeks culture period		6 weeks culture period		9 weeks culture period		6 weeks culture period	
Shoot biomass [g plant^{-1}]	5.83 ± 0.89 b	9.93 ± 1.31 a	0.77 ± 0.09 b	1.58 ± 0.22 a	10.51 ± 0.82 a	12.71 ± 0.87 a	3.98 ± 0.48 b	7.55 ± 0.64 a
Root biomass [g plant^{-1}]	0.43 ± 0.15 b	1.93 ± 0.10 a	0.64 ± 0.04 b	1.06 ± 0.07 a	0.948 ± 0.20 a	0.87 ± 0.16 a	3.03 ± 0.45 b	4.94 ± 0.34 a
Total root length [cm]	600.01 ± 149.42 b	2665.42 ± 274.67 a	56.20 ± 4.49 b	85.66 ± 8.84 a	1482.12 ± 466.62 a	603.92 ± 41.1 a	142.75 ± 25.83 a	197.20 ± 26.95 a
Root hair length [mm]	Not determined	Not determined	0.16 ± 0.03 b	0.47 ± 0.02 a	Not determined	Not determined	0.37 ± 0.04 a	0.51 ± 0.08 a

Supplementary Table 3| Sugars (A), carboxylates (B) and amino acids (C) in soil solutions of samples collected without visible contact to lettuce roots grown in soils with long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization history. The plants were grown in minirhizotron culture for nine weeks. Exudate collection was undertaken by micro-sampling with sorption filters on bulk soil, without any root contact. Means ± standard errors. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for each long-term experimental site DOK-LTE and HUB-LTE by t-test ($p \leq 0.05$). n.d. = not detectable.

A. Sugars in the soil solution [nmol cm^{-1} sorption filter]				
	HUB-LTE		**DOK-LTE**	
	HU-org	**HU-min**	**BIODYN2**	**CONMIN**
	Soil solution		Soil solution	
Fructose	n.d. b	1.47 ± 0.25 a	n.d. b	0.82 ± 0.09 a
Glucose	n.d. b	1.40 ± 0.19 a	n.d. b	0.72 ± 0.02 a
Sucrose	n.d.	n.d.	n.d. b	0.46 ± 0.09 a
Maltose	n.d.	n.d.	n.d. a	0.62 ± 0.22 a
Sum	n.d. b	2.88 a	n.d. b	2.62 a

B. Carboxylates in the soil solution [nmol cm^{-1} sorption filter**]**

	HUB-LTE		DOK-LTE	
	HU-org	**HU-min**	**BIODYN2**	**CONMIN**
	Soil solution		Soil solution	
Malate	12.42 ± 3.17 a	n.d. b	n.d.	n.d.
Citrate	3.13 ± 0.94 a	5.21 ± 1.06 a	2.38 ± 0.56 a	1.64 ± 0.29 a
Succinate	n.d.	n.d.	n.d.	n.d.
Fumarate	n.d. b	0.28 ± 0.06 a	0.62 ± 0.03 a	n.d. b
Benzoate	0.06 ± 0.03 a	0.03 ± 0.005 a	0.02 ± 0.003 b	0.09 ± 0.003 a
Acetate	n.d. a	21.48 ± 9.78 a	8.44 ± 3.65 a	4.33 ± 0.78 a
Lactate	68.54 ± 12.32 a	70.99 ± 28.74 a	26.17 ± 4.93 a	10.26 ± 2.75 b
Sum	84.17 a	98.00 a	37.65 a	16.35 a

C Amino acids in the soil solution [nmol cm^{-1} sorption filter**]**

	HUB-LTE		DOK-LTE	
	HU-org	**HU-min**	**BIODYN2**	**CONMIN**
	Soil solution		Soil solution	
Glutamic acid	0.008 ± 0.005 a	n.d. a	0.006 ± 0.002 a	n.d. a
Asparagine	0.017 ± 0.010 a	0.042 ± 0.002 a	1.045 ± 0.475 a	0.004 ± 0.001 a
Serine	0.046 ± 0.004 a	0.026 ± 0.001 b	0.395 ± 0.045 a	0.046 ± 0.002 b
Glutamine	0.012 ± 0.005 a	0.038 ± 0.019 a	0.107 ± 0.014 a	n.d. b
Glycine	0.052 ± 0.003 a	0.022 ± 0.001 b	0.354 ± 0.031 a	0.047 ± 0.002 b
Threonine	0.003 ± 0.001 a	0.005 ± 0.001 a	0.109 ± 0.016 a	n.d. b
Histidine	n.d.	n.d.	0.031 ± 0.004 a	n.d. b
Alanine	0.014 ± 0.003 a	n.d. b	0.247 ± 0.023 a	0.008 ± 0.002 b
Proline	0.006 ± 0.002 a	0.005 ± 0.001 a	0.062 ± 0.004 a	0.008 ± 0.001 b
Cystine	0.003 ± 0.002 a	n.d. a	n.d.	n.d.
Thyrosine	n.d. a	0.005 ± 0.001 a	0.088 ± 0.015 a	n.d. b
Methionine	n.d. b	0.019 ± 0.004 a	0.164 ± 0.011 a	n.d. b
Isoleucine	0.006 ± 0.001 a	0.005 ± 0.001 a	0.029 ± 0.006 a	n.d. b
Leucine	0.007 ± 0.001 a	0.003 ± 0.001 b	0.039 ± 0.009 a	n.d. b
Phenylalanine	0.042 ± 0.012 a	0.006 ± 0.001 a	0.031 ± 0.002 a	0.015 ± 0.001 b
Sum	0.216 a	0.174 a	2.706 a	0.126 b

Supplementary Table 4| Taxonomic composition of (A) bacterial/archaeal phyla and proteobacterial classes and (B) fungal phyla represented as relative abundances in root-associated soil and rhizosphere of lettuce (cv. Tizian). The plants were grown in soils under long-term organic (HU-org, BIODYN2) or mineral (HU-min, CONMIN) fertilization. Different lowercase letters indicate significant differences between organic vs. mineral fertilization tested separately for each long-term experimental site and habitat by edgeR (FDR < 0.05). Means of relative abundance ± standard errors.

A. Taxonomic composition of bacterial/archaeal communities in root-associated soil and rhizosphere of lettuce (cv. Tizian)

Kingdom	Phylum/Class	Root-Associated soil				Rhizosphere			
		HUB-LTE		DOK-LTE		HUB-LTE		DOK-LTE	
		HU-org [%]	HU-min [%]	BIODYN2 [%]	CONMIN [%]	HU-org [%]	HU-min [%]	BIODYN2 [%]	CONMIN [%]
Bacteria	Acidobacteria	16.02 ± 0.82 a	19.43 ± 0.23 a	11.72 ± 0.56 b	12.42 ± 0.47 a	2.78 ± 0.14 a	2.20 ± 0.42 a	5.96 ± 0.67 a	3.55 ± 0.60 a
Bacteria	Actinobacteria	15.12 ± 0.62 a	12.72 ± 0.39 b	10.40 ± 0.29 b	12.20 ± 0.42 a	9.19 ± 2.85 a	9.03 ± 2.28 a	7.28 ± 0.82 b	8.20 ± 0.78 a
Bacteria	Bacteria_unclassified	8.68 ± 0.58 a	10.74 ± 0.38 a	8.09 ± 0.32 a	6.95 ± 0.21 a	2.43 ± 0.44 a	2.35 ± 0.49 a	6.41 ± 1.25 a	2.69 ± 0.51 a
Bacteria	Bacteroidetes	4.54 ± 0.27 a	4.26 ± 0.28 a	8.04 ± 0.04 a	7.09 ± 0.19 a	5.10 ± 0.33 a	2.47 ± 0.51 a	7.08 ± 0.19 a	3.84 ± 0.95 a
Bacteria	Candidatus_Saccharibacteria	1.63 ± 0.31 a	1.45 ± 0.21 a	0.66 ± 0.03 b	1.07 ± 0.13 a	3.49 ± 1.23 a	4.93 ± 2.22 a	2.05 ± 0.27 a	1.57 ± 0.66 a
Bacteria	Chloroflexi	1.68 ± 0.20 a	2.65 ± 0.14 a	1.53 ± 0.04 a	1.26 ± 0.05 a	0.65 ± 0.16 a	0.79 ± 0.14 a	1.82 ± 0.38 a	0.74 ± 0.14 a
Bacteria	Cyanobacteria/Chloroplast	0.08 ± 0.01 a	0.09 ± 0.04 a	0.26 ± 0.11 a	0.69 ± 0.35 a	0.03 ± 0.00 a	0.03 ± 0.02 a	1.18 ± 0.71 a	0.04 ± 0.02 b
Bacteria	Firmicutes	15.55 ± 0.68 a	13.07 ± 0.84 b	14.48 ± 0.39 a	12.42 ± 0.98 a	6.87 ± 1.08 a	5.49 ± 1.25 a	11.33 ± 1.03 a	5.60 ± 1.52 a
Bacteria	Gemmatimonadetes	2.64 ± 0.13 a	3.04 ± 0.19 a	0.43 ± 0.02 b	0.77 ± 0.06 a	0.37 ± 0.04 a	0.26 ± 0.04 a	0.22 ± 0.05 a	0.29 ± 0.06 a
Bacteria	Nitrospirae	1.02 ± 0.08 a	0.83 ± 0.07 b	1.67 ± 0.05 a	1.38 ± 0.10 a	0.16 ± 0.01 a	0.07 ± 0.01 a	0.62 ± 0.15 a	0.39 ± 0.12 a
Bacteria	Alphaproteobacteria	15.63 ± 0.89 a	15.52 ± 1.18 a	9.33 ± 0.31 b	12.15 ± 0.40 a	23.84 ± 2.36 a	25.22 ± 6.80 a	22.33 ± 1.03 a	13.95 ± 4.25 a
Bacteria	Betaproteobacteria	5.54 ± 0.33 a	4.64 ± 0.40 b	3.94 ± 0.29 a	3.43 ± 0.10 a	15.04 ± 0.80 a	5.95 ± 2.20 a	12.65 ± 3.55 a	14.34 ± 3.72 a
Bacteria	Deltaproteobacteria	2.52 ± 0.14 a	2.67 ± 0.23 a	2.62 ± 0.21 a	2.48 ± 0.12 a	0.81 ± 0.16 a	0.32 ± 0.06 a	2.34 ± 0.13 a	0.85 ± 0.26 a
Bacteria	Gammaproteobacteria	3.49 ± 0.31 a	3.57 ± 0.31 a	3.14 ± 0.08 a	3.55 ± 0.19 a	26.59 ± 5.91 a	39.31 ± 12.73 a	8.55 ± 1.36 b	39.76 ± 13.39 a
Bacteria	Proteobacteria_unclassified	0.58 ± 0.04 a	0.42 ± 0.07 b	0.36 ± 0.02 a	0.36 ± 0.01 a	0.52 ± 0.08 a	0.23 ± 0.03 a	0.67 ± 0.07 a	0.35 ± 0.12 a
Archaea	Thaumarchaeota	2.68 ± 0.20 a	1.57 ± 0.24 b	16.66 ± 0.96 a	13.91 ± 0.47 a	1.42 ± 0.36 a	0.77 ± 0.18 a	6.27 ± 0.98 a	1.94 ± 0.57 b
Bacteria	Verrucomicrobia	2.27 ± 0.11 a	2.77 ± 0.24 a	6.15 ± 0.18 b	7.57 ± 0.19 a	0.61 ± 0.11 a	0.42 ± 0.05 a	2.96 ± 0.56 a	1.75 ± 0.34 a
Bacteria	Rare (< 1%)	0.32 ± 0.01 b	0.54 ± 0.04 a	0.51 ± 0.04 a	0.31 ± 0.04 b	0.11 ± 0.01 a	0.14 ± 0.04 a	0.26 ± 0.01 a	1.15 ± 0.07 a

B. Taxonomic composition of fungal communities in root-associated soil and rhizosphere of lettuce (cv. Tizian)

Kingdom	Phylum	Root-Associated soil				Rhizosphere			
		HUB-LTE		DOK-LTE		HUB-LTE		DOK-LTE	
		HU-org [%]	HU-min [%]	BIODYN2 [%]	CONMIN [%]	HU-org [%]	HU-min [%]	BIODYN2 [%]	CONMIN [%]
Eukaryota	Ascomycota	68.23 ± 1.21 a	58.43 ± 4.65 a	21.98 ± 1.22 a	16.67 ± 2.13 a	62.72 ± 3.73 a	37.17 ± 5.27 a	5.51 ± 2.31 a	3.41 ± 1.35 a
Eukaryota	Basidiomycota	11.52 ± 0.75 b	16.98 ± 2.75 a	8.19 ± 0.65 b	16.51 ± 1.37 a	9.46 ± 0.54 a	10.12 ± 1.01 a	0.96 ± 0.41 a	3.13 ± 1.49 a
Eukaryota	Chytridiomycota	0.83 ± 0.33 a	0.22 ± 0.07 b	1.89 ± 0.27 a	2.38 ± 1.14 a	0.69 ± 0.17 a	0.08 ± 0.03 b	0.45 ± 0.27 a	0.15 ± 0.12 a
Eukaryota	Entomophthoromycota	0 ± 0 a	0 ± 0 a	0 ± 0 a	0.01 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
Eukaryota	Glomeromycota	0.18 ± 0.08 a	0.01 ± 0 b	1.74 ± 0.28 a	2.23 ± 0.23 a	1.79 ± 1.13 a	0.01 ± 0 b	1.53 ± 0.89 a	0.16 ± 0.03 b
Eukaryota	Kickxellomycota	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
Eukaryota	Mortierellomycota	17.28 ± 1.41 a	18.09 ± 1.75 a	63.45 ± 0.68 a	55.69 ± 1.89 a	20.01 ± 3.1 b	47.8 ± 4.99 a	1.64 ± 0.28 b	17.33 ± 10.96 a
Eukaryota	Mucoromycota	1.65 ± 0.06 b	5.80 ± 0.81 a	1.51 ± 0.12 a	1.74 ± 0.09 a	1.01 ± 0.24 b	4.32 ± 0.54 a	0.05 ± 0.02 a	0.17 ± 0.10 a
Eukaryota	Olpidiomycota	0.05 ± 0.01 a	0.01 ± 0 a	1.00 ± 0.07 b	4.67 ± 3.57 a	3.97 ± 3.33 a	0.48 ± 0.11 b	89.84 ± 2.77 a	75.62 ± 10.58 a
Eukaryota	unidentified	0.28 ± 0.07 a	0.47 ± 0.41 a	0.23 ± 0.02 a	0.10 ± 0.01 a	0.33 ± 0.04 a	0.03 ± 0.01 b	0.02 ± 0.01 a	0.03 ± 0.02 a

Supplementary Table 5| Ecological assignment of fungal genera represented as relative abundance with FUNGuild in root-associated soils and rhizosphere of lettuce (cv. Tizian) grown in soils of DOK-LTE (BIODYN2 vs. CONMIN) and HUB-LTE (HU-org vs. HU-min). Different lowercase letters indicate significant differences (FDR < 0.05) of represented genera between organic vs. mineral fertilization tested separately for each long-term experimental site and habitat based on the results of edgeR analyses. Means of relative abundance ± standard errors. Relative abundances (>0.5%) with significant differences are marked in bold.

Genus	Trophic Mode and Guilds	Root-Associated Soil				Rhizosphere			
		HUB-LTE		DOK-LTE		HUB-LTE		DOK-LTE	
		HU-org	HU-min	BIODYN2	CONMIN	HU-org	HU-min	BIODYN2	CONMIN
		[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
	Saprotroph	**9.3**	**6.5**	**6.5**	**7.8**	**6.2**	**7.6**	**0.7**	**1.0**
Arthrobotrys	Undefined Saprotroph	0.4 a	0 b	0.1 a	0.1 a	**1.8 a**	**0.1 b**	0 a	0 a
Cercophora	Dung Saprotroph	**2.6 a**	**0.1 b**	0 a	0 a	0.3 a	0 b	0 a	0 a
Humicola	Undefined Saprotroph-Wood Saprotroph	**1.9 a**	**0.3 b**	0.1 a	0.3 a	1.6 a	1.0 a	0.1 a	0.1 a
Leucosporidium	Soil Saprotroph-Undefined Saprotroph	0 b	0.1 a	0 a	0 a	**0.1 b**	**0.9 a**	0 a	0.1 a
Mucor	Undefined Saprotroph	0.2 a	0.2 a	0.4 a	0.5 a	**0.2 b**	**1.0 a**	0 a	0.1 a
Nigrospora	Undefined Saprotroph	0 b	0.2 a	0 a	0 a	**0 b**	**0.5 a**	0 a	0 a
Plenodomus	Undefined Saprotroph	0.1 a	0.1 a	0 b	0.1 a	**0.5 a**	**0.1 b**	0 a	0.1 a
Umbelopsis	Undefined Saprotroph	0.3 b	2.6 a	0 a	0 a	**0.2 b**	**1.4 a**	0 a	0 a
	Symbiotroph	**0.3**	**0**	**1.3**	**1.9**	**1.7**	**0**	**1.3**	**0.1**
Claroideoglomus	Arbuscular Mycorrhizal	0.1 a	0 b	0.2 a	0.2 a	**0.7 a**	**0 b**	0.2 a	0 a
Funneliformis	Arbuscular Mycorrhizal	0.1 a	0 b	1.0 a	0.8 a	**0.9 a**	**0 b**	1.0 a	0.1 a
Rhizophagus	Arbuscular Mycorrhizal	0 a	0 a	**0 b**	**0.7 a**	0 a	0 b	0 a	0 a
	Saprotroph-Symbiotroph	**17.9**	**19.1**	**63.9**	**56.2**	**20.3**	**48.7**	**1.8**	**17.5**
Mortierella	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	17.3 a	18.1 a	63.4 a	55.7 a	**20.0 b**	**47.6 a**	1.6 a	17.3 a
Podospora	Dung Saprotroph-Endophyte-Litter Saprotroph-Undefined Saprotroph	0.5 a	0.8 a	0.4 a	0.5 a	**0.2 b**	**1.1 a**	0.2 a	0.2 a
	Pathotroph	**3.2**	**1.3**	**2.3**	**5.7**	**8.2**	**2.1**	**91.3**	**75.9**

Ascochyta	Plant Pathogen	0.5 a	0.1 b	0.2 a	0.2 a	**0.5 a**	**0 b**	0.1 a	0 a
Lectera	Plant Pathogen	**0.6 a**	**0 b**	0.4 a	0.1 b	0.2 a	0 b	0 a	0 a
Moesziomyces	Plant Pathogen	0.3 a	0 b	0 a	0 a	**1.2 a**	**0 b**	0 a	0 a
Olpidium	Plant Pathogen	0 a	0 b	**1.0 b**	**4.7 a**	**4.0 a**	**0.5 b**	89.8 a	75.6 a
	Pathotroph-Saprotroph	**9.6**	**20.5**	**2.1**	**4.0**	**7.2**	**9.5**	**1.4**	**0.7**
Didymella	Animal Pathogen-Plant Pathogen-Undefined Saprotroph	**2.0 a**	**0 b**	0 a	0 a	**1.4 a**	**0 b**	0 a	0 a
Exophiala	Animal Pathogen-Undefined Saprotroph	**6.2 b**	**17.2 a**	1.0 a	2.2 a	4.9 a	7.3 a	1.3 a	0.6 a
Mycena	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	0 a	0 a	**0 b**	**0.6 a**	0 a	0 a	0 a	0 a
Rhizopus	Plant Pathogen-Undefined Saprotroph	**0.9 b**	**3.0 a**	0.8 a	0.9 a	**0.5 b**	**1.9 a**	0 a	0 a
	Pathotroph-Saprotroph-Symbiotroph	**5.0**	**8.6**	**4.4**	**1.6**	**5.8**	**4.9**	**0.9**	**0.6**
Chaetomium	Animal Pathogen-Dung Saprotroph-Endophyte-Epiphyte-Plant Saprotroph-Wood Saprotroph	3.3 a	5.6 a	**3.6 a**	**0.9 b**	4.0 a	2.8 a	0.7 a	0.5 a
Trichoderma	Endophyte-Epiphyt-Fungal Parasite-Plant Pathogen-Wood Saprotroph	**0.1 b**	**0.9 a**	0.1 a	0.2 a	0 b	0.2 a	0 a	0 a
	not classified or identified on genus level	**54.6**	**43.9**	**19.5**	**22.7**	**50.2**	**27.2**	**2.6**	**4.0**
Cyberlindnera		0 a	0 a	**0.6 a**	**0.1 b**	0 a	0 a	0 a	0.1 a
Saitozyma		2.3 a	4.2 a	**0.1 b**	**1.9 a**	1.5 a	2.6 a	0 a	0.3 a
Slooffia		0 a	0 a	**0 b**	**1.3 a**	0 a	0 a	0 b	0.1 a

Supplementary Table 6| Fungal taxa in the **root-associated soils of lettuce (cv. Tizian)** differing significantly (FDR < 0.05) in relative abundance depending on long-term organic *vs.* mineral fertilization practice at HUB-LTE (HU-org vs. HU-min) **(A)** and DOK-LTE (BIODYN2 vs. CONMIN) **(B)**. Only taxa with > 1.0% relative abundance are displayed. For OTUs on species level the similarity compared to the database are represented. Means of relative abundance ± standard errors. Bold numbers indicate significant enrichment.

A. Fungal taxa in root-associated soil differing significantly (FDR<0.05) in relative abundance in lettuce grown in long-term organically vs. minerally fertilized soils from HUB-LTE

Phylum	Class	Order	Family	Genus	OTU	HU-org [%]	HU-min [%]
Ascomycota	Dothideomycetes					**29.0 ± 4.5**	3.9 ± 0.6
Ascomycota	Dothideomycetes	Pleosporales				**28.9 ± 4.4**	3.8 ± 0.6
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*			**27.1 ± 4.3**	2.6 ± 0.4
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*	*Didymella*		**2.0 ± 0.4**	0 ± 0
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*	*Didymella*	*Didymella protuberans* (100%)	**2.0 ± 0.4**	0 ± 0
Ascomycota	Dothideomycetes	Pleosporales	*Didymellaceae*	unidentified	*Didymellaceae* sp	**24.5 ± 4.2**	2.5 ± 0.4
Ascomycota	Sordariomycetes	Sordariales	*Lasiosphaeriaceae*	*Cercophora*		**2.6 ± 0.9**	0.1 ± 0
Ascomycota	Sordariomycetes	Sordariales	*Lasiosphaeriaceae*	*Cercophora*	*Cercophora samala* (100%)	**2.3 ± 0.8**	0.1 ± 0
Ascomycota	Sordariomycetes	Sordariales	*Chaetomiaceae*	*Humicola*		**1.9 ± 0.1**	0.3 ± 0
Ascomycota	Sordariomycetes	Sordariales	*Chaetomiaceae*	*Humicola*	*Humicola grisea* (100%)	**1.9 ± 0.1**	0.3 ± 0
Ascomycota	unidentified	unidentified	unidentified	unidentified	*Ascomycota* sp	**1.9 ± 0.5**	0.2 ± 0
Mortierellomycota	Mortierellomycetes	Mortierellales	*Mortierellaceae*	*Mortierella*	*Mortierella minutissima* (100%)	**1.4 ± 0.1**	0.2 ± 0
Ascomycota	Eurotiomycetes					8.6 ± 0.5	**21.0 ± 0.6**
Ascomycota	Eurotiomycetes	Chaetothyriales				8.6 ± 0.5	**20.9 ± 0.6**
Ascomycota	Eurotiomycetes	Chaetothyriales	*Herpotrichiellaceae*	*Exophiala*		6.2 ± 0.4	**17.2 ± 0.6**
Ascomycota	Sordariomycetes	unidentified	unidentified	unidentified	*Sordariomycetes* sp	0.3 ± 0.1	**6.2 ± 5.3**
Basidiomycota						11.5 ± 0.8	**17.0 ± 2.7**
Basidiomycota	Agaricomycetes	Cantharellales				0.8 ± 0.2	**3.5 ± 2.1**
Mucoromycota						1.6 ± 0.1	**5.8 ± 0.8**
Mucoromycota	Mucoromycetes	Mucorales	*Rhizopodaceae*	*Rhizopus*		0.9 ± 0	**3.0 ± 0.4**

Mucoromycota	Umbelopsidomycetes					0.3 ± 0	**2.6 ± 0.5**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales				0.3 ± 0	**2.6 ± 0.5**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales	*Umbelopsidaceae*			0.3 ± 0	**2.6 ± 0.5**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales	*Umbelopsidaceae*	*Umbelopsis*		0.3 ± 0	**2.6 ± 0.5**
Mucoromycota	Umbelopsidomycetes	Umbelopsidales	*Umbelopsidaceae*	*Umbelopsis*	*Umbelopsis* sp	0.3 ± 0	**2.6 ± 0.5**

B. Fungal taxa in root-associated soil differing significantly (FDR<0.05) in relative abundance in lettuce grown in long-term organically vs. minerally fertilized soils from DOK-LTE

Phylum	Class	Order	Family	Genus	OTU	BIODYN2 [%]	CONMIN [%]
Ascomycota	Sordariomycetes					**11.0 ± 1.4**	6.0 ± 0.8
Ascomycota	Sordariomycetes	Sordariales				**6.4 ± 1.9**	2.2 ± 0.3
Ascomycota	Sordariomycetes	Sordariales	*Chaetomiaceae*	*Chaetomium*		**3.6 ± 2.3**	0.9 ± 0.2
Basidiomycota						8.2 ± 0.6	**16.5 ± 1.4**
Basidiomycota	Microbotryomycetes					0 ± 0	**1.3 ± 0.3**
Basidiomycota	Microbotryomycetes	Microbotryomycetes ord Incertae sedis				0 ± 0	**1.3 ± 0.3**
Basidiomycota	Microbotryomycetes	Microbotryomycetes ord Incertae sedis	*Chrysozymaceae*			0 ± 0	**1.3 ± 0.3**
Basidiomycota	Microbotryomycetes	Microbotryomycetes ord Incertae sedis	*Chrysozymaceae*	*Slooffia*		0 ± 0	**1.3 ± 0.3**
Basidiomycota	Microbotryomycetes	Microbotryomycetes ord Incertae sedis	*Chrysozymaceae*	*Slooffia*	*Slooffia cresolica* (99.6%)	0 ± 0	**1.3 ± 0.3**
Basidiomycota	Tremellomycetes					5.9 ± 0.2	**11.8 ± 1.1**
Basidiomycota	Tremellomycetes	Tremellales				0.1 ± 0	**1.9 ± 0.1**
Basidiomycota	Tremellomycetes	Tremellales	*Trimorphomycetaceae*			0.1 ± 0	**1.9 ± 0.1**
Basidiomycota	Tremellomycetes	Tremellales	*Trimorphomycetaceae*	*Saitozyma*		0.1 ± 0	**1.9 ± 0.1**

Basidiomycota	Tremellomycetes	Tremellales	*Trimorphomycetaceae*	*Saitozyma*	*Saitozyma podzolica* (100%)	0.1 ± 0	**1.9 ± 0.1**
Olpidiomycota						1.0 ± 0.1	**4.7 ± 3.6**
Olpidiomycota	Olpidiomycetes					1.0 ± 0.1	**4.7 ± 3.6**
Olpidiomycota	Olpidiomycetes	Olpidiales				1.0 ± 0.1	**4.7 ± 3.6**
Olpidiomycota	Olpidiomycetes	Olpidiales	*Olpidiaceae*	*Olpidium*		1.0 ± 0.1	**4.7 ± 3.6**

Text

Supplementary Text 1 | Materials and Methods description of microbial community analyses: Bacterial and archaeal communities

Amplification of 16S rRNA genes was performed in 25 µl volumes containing 0.625 U Hot Start Taq Polymerase (New England Biolabs GmbH, Frankfurt am Main, Germany), 1x Standard Taq Reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 1 µl of target DNA. Bovine serum albumin (final concentration 0.1 mg ml^{-1}) was added optionally. PCR conditions were previously described by Chowdhury et al. (2019). In a second PCR reaction step the primers additionally included Illumina specific sequencing adapters and a unique combination of sequence identifier tags for each sample. After both PCR reactions, amplicon products were purified using HighPrep™ PCR Clean Up System (AC-60500, MagBio Genomics Inc., Gaithersburg, MD, United States) using a 0.65:1 (beads:PCR reaction) volumetric ratio to remove DNA fragments below 100 bp in size. Samples were normalized using SequalPrep Normalization Plate (96) Kit (Invitrogen, Maryland, MD, United States) and pooled using 5 µl volumes of each sample. The final pool volume was concentrated by using the DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA, United States). The pooled library concentration was determined using the Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies, Carlsbad, CA, United States) following the specifications of the manufacturer. Before library denaturation and sequencing, the final pool concentration was adjusted to 4 nM. Amplicon sequencing was performed on an Illumina® MiSeq® platform using Reagent Kit v2 [2x250 bp] (Illumina Inc., San Diego, CA, United States). The MiSeq Controller Software Casava 1.8 (Illumina Inc., San Diego, CA, United States) was used for sequence demultiplexing and the paired-end FASTQ output files were used for the downstream sequencing analysis. Sequence analyses were performed according to acknowledged best practice guidelines (Schöler et al., 2017; Jacquiod et al., 2018).

Supplementary Text 2 | Materials and Methods description of microbial community analyses: Fungal communities

For the amplification of fungal ITS2 regions, three PCRs per sample were conducted with 10 ng TC-DNA at different annealing temperatures (56°C ± 2°C) and the number of cycles per PCR was restricted to 24 at the midpoint of exponential phase (Sommermann et al., 2018). Subsequently, samples with the same barcodes were mixed, purified by MinElute PCR Purification Kit (QIAGEN, Hilden, Germany) and eluted in 12 µl 10 mM Tris-HCl (pH 8.5). The

concentration of each sample was checked by a Qubit ® 3.0 Fluorometer (Invitrogen, Carlsbad, CA, United States) and all amplicons were pooled in equimolar amounts. The quality control and library preparation were followed by sequencing on an Illumina® MiSeq® platform (ca. 30% of an Illumina flow cell) in paired-end mode (2x 300 bp).

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7 General discussion

Soil microorganisms, soil type, agricultural management, and plant genotype are critical determinants of soil quality for maintaining soil functions and plant productivity (Chaparro et al., 2012). The majority of soil functions are influenced by microorganisms which are regarded as critical drivers not only of soil processes (Mäder et al., 2002) but also for performance, stress resilience and health status of plants (Berg, 2009; Schmidt et al., 2019). Various studies reported that agricultural land use history can impact on numerous belowground variables such as soil nutrients, pH, organic matter, and these soil properties can influence both bacterial and fungal community structures (Dignam et al., 2018; Xue et al., 2018; Turley et al., 2020). For over sixty years, it has been known that soils have the potential to suppress or support soil-borne plant pathogens (Schlatter et al., 2017). Apart from soil factors, a key role for plant microbial interactions has been attributed to the rhizosphere. This layer of soil which extends little more than a few millimetres around the roots is significantly affected by metabolic processes of both, roots and the soil biota that are attracted by organic deposits from the roots (rhizodeposits). Therefore the rhizosphere is creating a zone of intense biological activity, which differs from that in the surrounding bulk soil (Vessey, 2003; Neumann et al., 2021). However, the role of external factors, such as soil properties, management practices, microbial inoculants, their impact on plant-microbial interactions in the rhizosphere and the significance for plant health and stress resilience is still poorly understood.

In this context, the present study was initiated to investigate the role of root exudates and organic rhizodeposits shaping the composition and functions of rhizosphere-microbial communities and their impact on plant health, as well as the influence of different soil properties and agricultural management history. Lettuce (*Lactuca sativ*a cv. Tizian) was used as well-characterized model plant to investigate plant-pathogen interactions and to characterize rhizosphere microbial communities (Grosch et al., 2004; Neumann et al., 2014; Schreiter et al., 2014a,b,c).

7.1 Composition of the rhizosphere soil solution related with rhizosphere microbiota

As already postulated in the early studies of Hiltner in 1904 (Hartmann et al., 2008), organic compounds released as root exudates from intact plant roots are regarded as key components involved in the formation and subsequent shaping of the rhizosphere (Haney et al., 2015). Different exudation patterns are thought to trigger a cascade of feedback loops between plant roots and associated-soil microbiome and the exudation process has been explained as a combination of root push and microbial pull (Oburger and Jones, 2018). A large number of studies has described the relation between accumulation of certain microbial communities in the rhizosphere and the root exudation of plants (Foster and Rovira, 1976; Foster, 1986; Rudrappa et al., 2008; Shi et al., 2011; Neumann et al., 2014; Schreiter et al., 2014a).

Accordingly, also the investigations of the present study pointed to similar relationships:

(i) High suppressiveness against soil-borne fungal pathogens, such as *Rhizoctonia solani* or *Olpidium brassicae* (*syn. virulentus*) investigated in different soils, was associated with increased rhizosphere concentrations of the antimicrobial compound benzoic acid (Chapter 4.1 and 6.1; Windisch et al., 2017, 2021a) released as defense compound from roots of lettuce (Chapter 5.1; Windisch et al., 2021b).

(ii) High rhizosphere abundance of beneficial microbiota with plant growth-promoting and pathogen antagonistic properties, such as *Mortierella elongata* (Expósito et al., 2017; Li et al., 2018; Zhang et al., 2020) or members of the *Pseudomonas fluorescens* complex (Figure 5) was characteristic for a *Olpidium*-suppressive soil (CONMIN). The respective beneficials are characterized as fast growing copiotrophic microorganisms with a high demand of easily available carbon sources (Chesters and Peberdy, 1965), and were obviously promoted by high concentrations of low-molecular weight carbohydrates such as sugars (e.g. glucose) detected in the rhizosphere of lettuce plants grown in the respective soil. These effects were not detectable in *Olpidium*-affected plants grown on the pathogen conductive soil (BIODYN2) (Chapter 6.1).

(iii) The lettuce rhizosphere of the *Olpidium*-suppressive soil (CONMIN) was also characterized by increased concentration of succinic and malic acids known as

chemo-attractants and precursors for siderophore production of *Pseudomonas fluorescens* (De Weert et al., 2002), while amino acid concentrations were lower than in the conductive soil (Chapter 6.1).

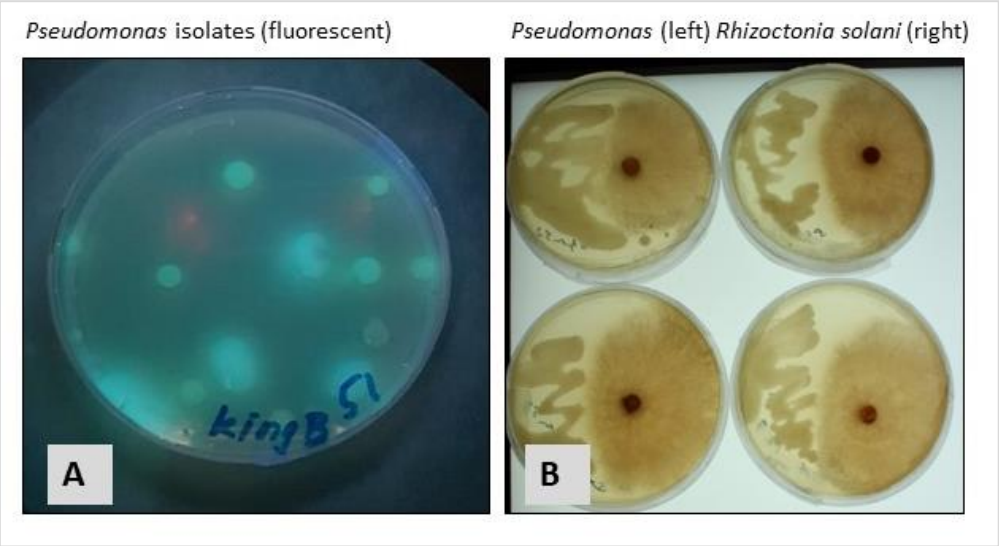

Figure 5: (A) Antagonistic potential of *Pseudomonas* isolates (fluorescent) from *Olpidium*-suppressive CONMIN soil (B) tested against the model pathogen *Rhizoctonia solani* AG1-IB in a PDA confrontation assay (Windisch, unpublished).

However, more detailed investigations revealed that root exudation of the antimicrobial defense compound benzoic acid (Chapter 5.1) was triggered by the presence of pathogen *R. solani* and similarly by pathogen antagonists, such as *Pseudomonas* sp. RU47 or *Serratia plymuthica* 3Re-4-18, as demonstrated by inoculation experiments (Chapter 4.1) pointing also to an effect of rhizosphere microorganisms on the release of root exudates. Similarly, lower sugar availability in the lettuce rhizosphere, potentially limiting the establishment of plant beneficial *Mortierella* and *Pseudomonas* sp. in the *Olpidium*-conductive BIODYN2 soil was associated with a higher rhizosphere abundance of arbuscular mycorrhizal fungi (AMF) (Chapter 6.1). Reviewed by Jones et al. (2004), reduced sugar concentrations in root exudates were frequently reported for AMF infected plants. These findings suggest that a high abundance of endophytic AMF fungi with direct access to sugar supply provided by the host plant into the root apoplast of the peri-arbuscular space of infected root cortex cells, may limit sugar availability to microorganisms colonizing the rhizosphere located in a larger distance from the root. Accordingly, higher sugar concentrations were also detected in the lettuce rhizosphere grown in a soil with a non-mycotrophic rapeseed pre-crop (Figure 6), associated with a lower diversity and abundance of AMF as compared with a mycotrophic maize or wheat

pre-crop (Sommermann et al., 2018). Moreover, in the *Olpidium*-conductive BIODYN2, the high sugar demand of the biotrophic *Olpidium* pathogen may further limit sugar exudation from infected roots down to a level, finally below the detection limit in the rhizosphere of mature root zones (Chapter 6.1). In this context, also an active limitation of sugar supply to pathogens by the host plant is possible, as recently demonstrated for a tonoplast sugar transporter of the SWEET family. This transporter was identified in roots of *Arabidopsis thaliana*, limiting sugar exudation by sequestering sugar into the vacuole upon attack by the fungal soil pathogen *Pythium* (Chen et al., 2015; Eom et al., 2015).

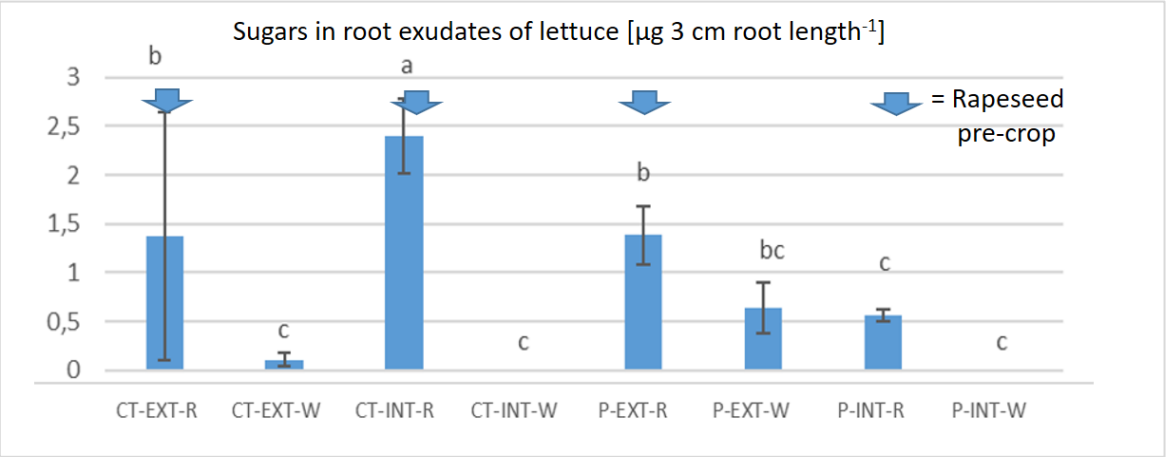

Figure 6: Sugar concentrations in root exudates of lettuce grown in different soils of a long-term crop rotation field trial, established in 1992 in Bernburg, Germany (Windisch, unpublished). Increased sugar accumulation was observed in the rhizosphere of lettuce grown in soils with the non-mycotrophic pre-crop rapeseed (R), whereby decreased sugar accumulations could be shown for the mycotrophic pre-crop wheat (W) with AMF establishment in the soil (Sommermann et al., 2018). (CT = reduced tillage; P = conventional tillage; INT, EXT = intensive and extensive nitrogen fertilization and use of fungicides.

The presented examples strongly suggest that shaping of rhizosphere microbial communities via root exudates is not simply a unidirectional process from host plants to soil microbiota. Root exudates are shaping microbiota, but at the same time microbiota are obviously shaping root exudation as previously discussed also for the stimulation of root exudation of amino acids by microbial metabolites, such as phenazine, 2,4-diacetylphloroglucinol or zearalenone (Phillips et al., 2004; Moe, 2013). The integration of both processes obviously triggers the effects on rhizosphere microbial communities and their feedback loops determining plant performance and health status.

Under certain conditions, scenarios that are even more complex are possible. In chapter 6.1, a negative correlation of low rhizosphere abundance of potentially plant beneficial *Pseudomonas fluorescens* with high rhizosphere concentrations of amino acids in *Olpidium*-affected lettuce plants grown in the pathogen conductive BIODYN2 soil was detected. The concentration of amino acids exceeded the rhizosphere concentrations of amino acids in the *Olpidium*-suppressive CONMIN soil by two orders of magnitude. However, a comparison of BIODYN2 rhizosphere samples with soil samples without root contact (bulk soil) showed similar high amino acid concentrations (Chapter 6.2). This was most probably related to mineralization processes of high organic matter inputs due to long-term manure-based fertilization in the respective soil. In this case, the rhizosphere effect with respect to amino acids was apparently overridden by the high background amino acid levels already present in the bulk soil, so that their effects on the microbial communities in the rhizosphere were determined by the mineralization process of organic fertilizers rather than by root activity. Accordingly, Zhou et al. (2020) recently demonstrated that the microbiome in the detritussphere of root canals derived from decaying roots largely overlaps with the individual rhizosphere microbiome of wheat and chickpea roots growing into root canals left by progenitor plants. These findings suggest that the process of shaping the rhizosphere microbiome involves numerous complex interactions and cannot simply be regarded as a bilateral relationship between root exudates and microbiota.

For methodological approaches, this implicates that the characterization of root exudate profiles, conducted under controlled conditions in model experiments, can provide important information on potential contributions from the plant side but is definitely not sufficient for the characterization of plant-microbial interactions in the rhizosphere. In this context, investigations of the composition of the rhizosphere soil solution influenced by additional factors as discussed above and selection of appropriate controls (e.g. bulk soil solution) should be included. The complex sampling strategy and analytical techniques applied in this study, integrating the results of model experiments in hydroponics, the use of artificial culture substrates (Chapter 5.1) and the investigation of rhizosphere samples from lettuce plants grown in field soils (Chapter 4.1 and 6.1), are attempts to consider these complex interactions between plants and microbes in the rhizosphere.

Root exudation occurs on a very small scale over time and space varying along the root axis and is described as a dynamic spatial and temporal process (Tian et al., 2019). Variations are reported in different root zones, plant developmental stages, influenced by the plant-nutritional status and even by diurnal variations (Oburger and Jones, 2018; Kuzyakov and Razavi, 2019). This indicates that both, the time point and likewise the local variability in different root zones need to be considered for sampling strategies. Highest root activities and root exudation are generally reported during exponential vegetative growth with the highest photosynthetic activity and belowground translocation of assimilates (Marschner, 1995). Therefore, the respective growth phase was selected for exudate samplings and characterization of rhizosphere microbial communities in the present study.

The most intense root exudation of low-molecular weight compounds (LMW), which are easily available for soil microorganisms has been recorded for subapical root zones of young growing roots with developing root hairs (Canarini et al., 2019). Accordingly, these root zones are known as preferential infection sites for various root pathogens (including *Olpidium;* Chapters 5.1 and 6.1) and beneficial microorganisms, as demonstrated e.g. for strains of *Bacillus, Pseudomonas, Rhizobium* or *Trichoderma* (Fan et al., 2012; Hohmann et al., 2012; Mercado-Blanco and Prieto, 2012; Mpanga et al., 2019). High molecular weight mucilage polysaccharides and root border cells are preferentially released from the root cap, that attract both, pathogens and beneficials (Hawes et al., 2016b, 2016a; Canellas and Olivares, 2017). Rhizodeposits related to sloughed-off tissues and root turnover are more characteristic for older basal located parts of the root zones (Neumann and Römheld, 2007; Kuzyakov and Razavi, 2019), resulting in different rhizosphere- microbial communities (Marschner et al., 2002; Kuzyakov and Razavi, 2019). Figure 7 represents an elegant graphical summary illustrating the spatial variation of plant-microbial interactions in the rhizosphere as recently reviewed by Kuzyakov and Razavi (2019).

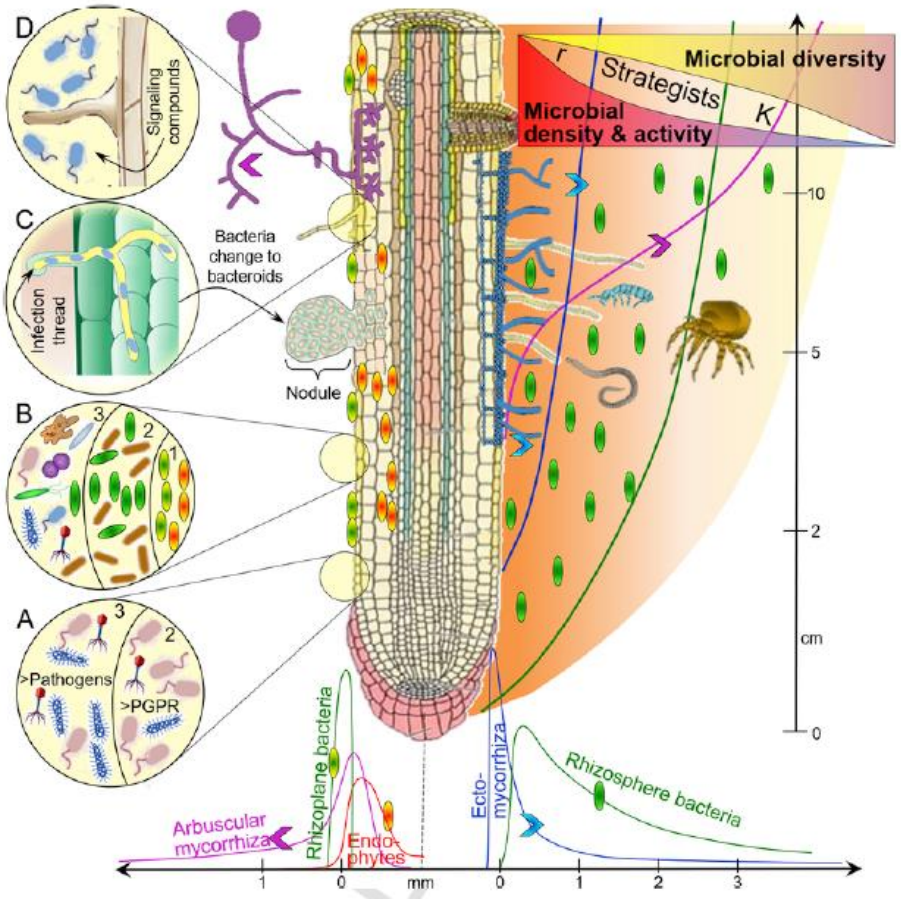

Figure 7: Habitat rhizosphere. The abundance of various microbial groups across (x-axis in mm from the root surface) and along the young root is presented by continuous color curves. Microbial groups include *Arbuscular mycorrhiza* (violet), *Ectomycorrhiza* (blue); Endophytic, Rhizoplane and Rhizosphere bacteria (green). A: higher density of plant growth promoting rhizobacteria (PGPR) compared to pathogens in 2) the rhizosphere and 3) reverse in bulk soil; B: abundance of various microbial groups 1) on rhizoplane, 2) in the rhizosphere, 3) in bulk soil; C: infection of root hairs by *rhizobia* and formation of nodules; D: release of signaling compounds and attraction of *rhizobia* and other PGPRs. The numbers in the loupes reflect: 1) rhizoplane, 2) the rhizosphere, 3) bulk soil. The schematic presentation of the abundance in individual microbial groups to the left or right of the roots made solely to avoid much overlapping of the curves (Kuzyakov and Razavi, 2019).

To account for the variability in release patterns of root exudates (Figure 7), localized samplings of rhizosphere soil solution were conducted in both, 1-2 cm subapical root zones representing the young growing part of the root and in older zones of the root 8-10 cm behind the root tip and also in soil without direct root contact as a background (control). In accordance with the results reported in the literature, significant differences between sampling sites were detectable (Figure 8). Therefore, for future investigations also samplings for rhizosphere microbial communities should consider different root zones, which was unfortunately not yet possible for technical reasons.

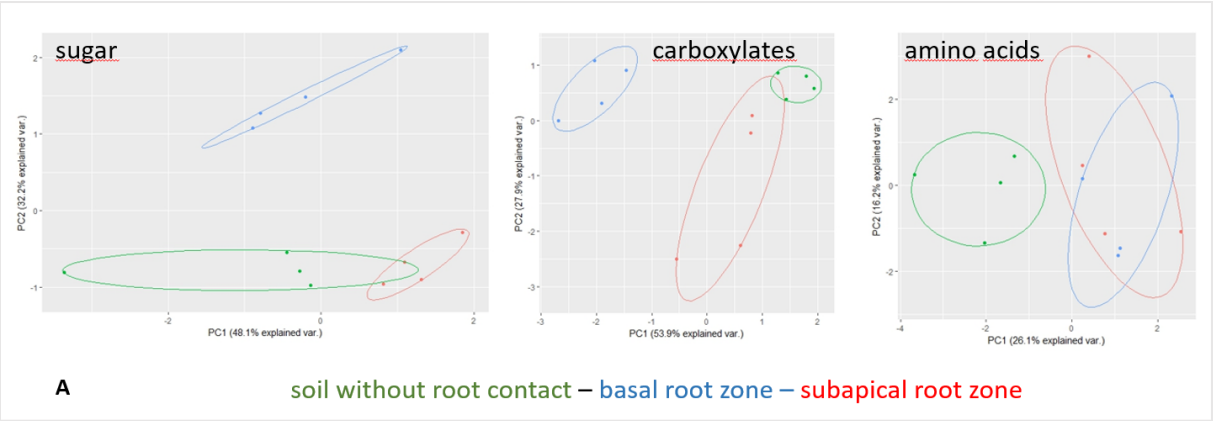

Figure 8: PCA analysis of primary metabolite classes in the soil solution depending on different root zones and distance from the root grown in CONMIN soil with long-term mineral fertilization (Windisch, unpublished).

Apart from nitrogen availability, in the present study, soil analysis revealed no nutrient limitations for lettuce growth. To minimize potential effects of N availability on the plant nutritional status and root exudation of lettuce, the experimental soils were equally fertilized with YaraLiva Calcinit ($[Ca(NO_3)_2]$, Yara, Oslo, Norway) at the recommended rate for lettuce cultivation (517 mg N kg^{-1} soil), to cover the plant requirements during the cultivation period. However, general effects of plant nutrient limitations on root exudation, the composition of the rhizosphere soil solution and related plant microbial interactions in the rhizosphere are definitely an additional aspect to be addressed more in detail in future studies. The same holds true for the impact of abiotic stress factors, which can affect plant growth and development and root exudation not only directly but also indirectly by limiting nutrient acquisition even in presence of sufficient nutrient levels in the growth substrate (Neumann and Römheld, 2007; Vives-Peris et al., 2017; Williams and de Vries, 2020).

7.2 Fertilization practice as determinant for plant interactions with rhizosphere microbiota

The nutritional status of plants, and thus also the application of fertilizers have a major influence on root-induced modifications of chemical processes in the rhizosphere, which are directly related to plant nutrient uptake. This comprises changes in rhizosphere pH, redox potential and release of organic rhizodeposits (Neumann and Römheld, 2002, 2007). However, depending on the nutritional status of the plant, these processes also act indirectly by influencing the plant-microbial interactions in the rhizosphere. For this reason, numerous studies comparing different cultivation methods have been conducted to investigate the abundance of agriculturally relevant microbes (Sommermann et al., 2018; Chowdhury et al.,

2019; Babin et al., 2021) which are influenced by fertilizer amendments (Francioli et al., 2016). In particular, organic fertilization is considered as beneficial for increasing soil-organic matter, stimulation of microbial activity (Lori et al., 2017) and increasing microbial biomass and diversity, resulting in plant beneficial properties (Esperschütz et al., 2007; Hartmann et al., 2015; Francioli et al., 2016; Schmidt et al., 2019). However, the respective benefits cannot be generalized and can be influenced by additional factors. In this context, the role of root-induced modifications (i.e. rhizodeposition) as affected by fertilization practice is still poorly understood and was therefore the major focus of this study in chapter 6.1. The model plant lettuce was grown in soils from two long-term fertilization trials with contrasting soils (loamy sand: HUB-LTE; silty loam: DOK-LTE) comparing conventional mineral fertilization (HU-min; CONMIN) vs. organic farming practices based on manure-fertilization, with (BIODYN2) and without (HU-org) additional use of biodynamic preparations. As expected, long-term organic fertilization increased the relative rhizosphere abundance of plant-beneficial AMF (Glomeromycota) in both, HUB-LTE and DOK-LTE soils. This observation is consistent with the frequently reported higher mycorrhizal dependence of plants in organic farming systems to compensate for the lower availability of readily available P forms compared to mineral fertilization (Sattelmacher et al., 1991; Douds et al., 1997; Gosling et al., 2006). On the other hand, in our study long-term organic fertilization simultaneously increased rhizosphere abundance of fungal pathotrophs, which may be suppressed by the regular use of fungicides in the treatments with mineral fertilization. Accordingly, the expression of genes involved in biotic stress adaptations was upregulated in the shoot tissue of plants grown in soils with long-term organic fertilization, which may point to a systemic response to the higher rhizosphere abundance of fungal pathotrophs (Chapter 6.1). However, the higher pathogen abundance in the rhizosphere was not necessarily associated with disease symptoms, as indicated by the absence of differences in plant performance and growth of lettuce plants grown in HUB-LTE soils independent of the fertilization history (HU-org vs. HU-min). Biocontrol effects of AMF associations against fungal pathogens have been frequently reported in the literature (Veresoglou and Rillig, 2012) and may provide an explanation for the absence of disease symptoms in lettuce plants in the HU-org soil. Moreover, all healthy plant microbiomes characterized so far, naturally also contain potential pathogens in seeds as well as in the rhizosphere (Wassermann et al., 2019; Berg et al., 2021) and may reflect the benefits of a sub-pathogenic level of potential pathotrophs by inducing stress priming effects of the host plants.

However, these findings could not be generalized, since a clear dysbiosis effect was detectable for the soil of the DOK-LTE. This was associated with a typical decline in rhizosphere alpha-diversity (Berg et al., 2021), in particular for fungal communities. They were dominated by the lettuce pathogen *Olpidium brassicae* with a relative rhizosphere abundance of 76-90%, independent of the fertilization history. However, *Olpidium* root infection and disease symptoms were preferentially recorded in the BIODYN2 soil with strong depression of plant growth and biomass production compared to the CONMIN soil (Chapter 6.1). The absence of disease symptoms despite a high rhizosphere abundance of the *Olpidium* pathogen suggests a pathogen-suppressive effect of the CONMIN soil with long-term mineral fertilization. The lower spread of the disease in the plants of the mineral soil variant CONMIN was not due to the regular application of fungicides in this treatment, since the latter even had a higher relative abundance of the fungus in the soil with a larger distance from the roots compared with the BIODYN2 soil. Moreover fungicides are frequently not effective against *Olpidium* sp. (Campbell et al., 1980).

As discussed in section 7.1 (Composition of the rhizosphere soil solution related with rhizosphere microbiota), the high *Olpidium*-disease incidence of lettuce plants grown in the BIODYN2 soil with long-term organic fertilization was associated with (i) a massive decline in the rhizosphere abundance of beneficial microorganisms such as *Mortierella elongata* and members of the *Pseudomonas fluorescens* group with pathogen suppressive properties (Figure 5); (ii) a lack of dicarboxylates with pathogen-antagonistic functions and chemotactic properties for *Pseudomonas* in the rhizosphere soil solution (succinate, malate, benzoate), and (iii) a lack of low molecular-weight compounds such as sugars as easily available carbon source for the respective beneficial microorganisms. The strong sugar limitation in the rhizosphere of lettuce grown in the BIODYN2 soil was potentially induced by the competition with endophytes, such as AMF and the biotrophic *Olpidium* pathogen that attacked the root (Lot et al., 2002). These endophytes profit from a more directly access to the sugar supply provided by the plant root compared with microorganisms preferentially colonizing the rhizosphere soil. Total amounts and relative proportions of sugars and amino acids in the rhizosphere soil solution significantly contribute to the available amounts of C and N in the rhizosphere attracting the settlement of beneficial microorganisms (Juma and McGill, 1986; Jaeger et al., 1999). Accordingly, recent studies have demonstrated an improved performance

of microbial inoculants such as *Pseudomonas, Bacillus, Trichoderma* and *Penicillium* strains in maize and tomato cultivation, when applied together with organic fertilizers (Figure 9), that provide easily available sources of carbon and nitrogen (e.g. manure-based composts, hair-, meat and feather meals (Mpanga et al., 2018; Bradáčová et al., 2019) (Thonar et al., 2017; Vinci et al., 2018a, 2018b).

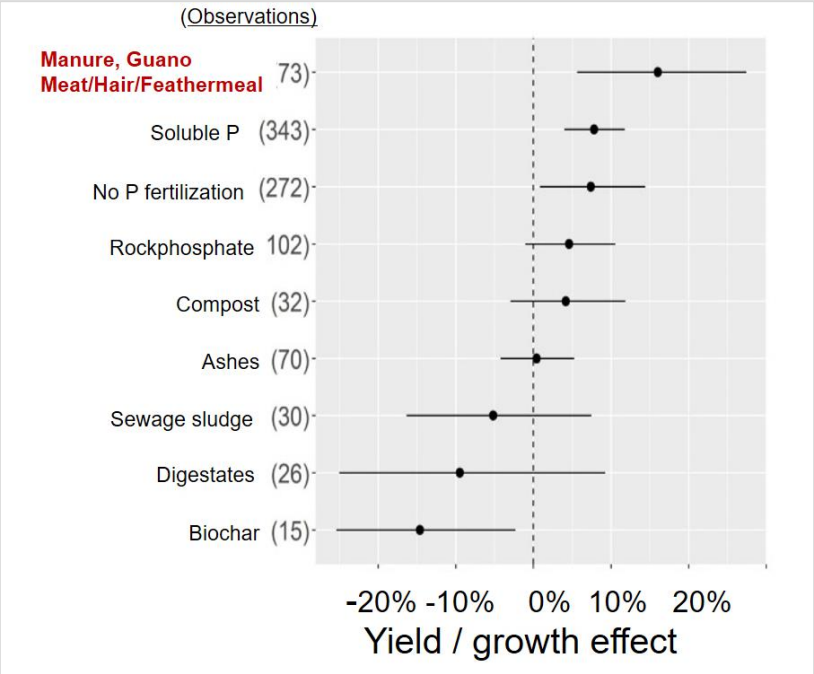

Figure 9: Efficiency of microbial and non-microbial bio-stimulants in combination with organic and inorganic fertilizers. Meta-analysis of 140 pot and field experiments (Final Report BIOFECTOR-Project, 2017).

In the context of the above-mentioned publications, the beneficial effect of carbon supply by selected organic fertilizers may reflect the well-documented carbon limitation of soil microbial life in the rhizosphere and similar benefits could be also expected for long-term manure based-fertilization in the LTE soils investigated in our study. However, in our minirhizotron experiment, all plants received an adapted mineral fertilization to achieve comparable nutrient supply, but no manure fertilizers were applied in this case. This may explain the carbohydrate limitation and competition observed for the rhizosphere microbial communities with high carbon demand established in the rhizosphere of plants grown in BIODYN2 soil (e.g. *Glomeromycota*, *Olpidium*, *Mortierella*, *Pseudomonas*). These findings suggest that not only the fertilization history but also the current fertilizer supply has an impact on the plant microbial interactions in the rhizosphere. To counteract this problem, a fertilization with adequate C-supply, expected for long-term manure based-fertilization together with external

application of a beneficial inoculum with a high rhizosphere competence (Eltlbany et al., 2019) (e.g. *Pseudomonas* sp. RU47, Chapter 4.1) could have made the difference to close the gap of missing beneficials in the lettuce rhizosphere for plant defense against fungal attack of e.g. *Olpidium* sp.

In addition, the limited soil volume in minirhizotron experiments may be a factor affecting results. By comparing lettuce growth in pots (Chowdhury et al., 2019) and in minirhizotrons, significant differences in plant biomass production and in sensitivity towards infection by *Olpidium* or *Rhizoctonia* pathogens were investigated (Chapter 6.1). Pathogen infection and plant damage by pathogens in minirhizotrons occurred faster and more intensive than in pots. This might be related to higher concentration of densely root growth along the observation window and shorter paths of hyphal growth to reach the roots of the host plant.

Taken together the results confirmed the hypothesis that the fertilization history has an impact on root exudation and the composition of the rhizosphere soil solution with potential to trigger plant microbial interactions. However, the effects can be significantly modulated by additional factors such as soil properties, current fertilizer supply, bacterial inoculum with antagonistic properties, rooting densities and available soil volume comparing different culture systems.

7.3 Soil-type effects on plant microbial interactions in the rhizosphere

Soil properties are regarded as a major driving factor for shaping the composition and function of the soil microbiome and influencing plant-released rhizosphere products and pathogen control (Hadar and Papadopoulou, 2012; Schreiter et al., 2014a). However, in this context studies under real field conditions are rare due to concomitant effects of other site-specific factors such as climatic conditions or cropping history. This problem was addressed using a unique field design with differing soils under identical management originating from the same field site (Neumann et al., 2014; Schreiter et al., 2014c, 2014a, 2014b). The respective studies postulated soil type-dependent rhizosphere competence and biocontrol of bacterial inoculant strains, as well as effects on the rhizosphere microbiome and on root exudation, triggering plant microbial interactions in the rhizosphere. In this context, the present study (Chapter 4.1,

5.1, 6.1) investigated the impact of soil types in three minirhizotron experiments with lettuce as model plant, conducted with five different soils and two lettuce pathogens (*Rhizoctonia solani, Olpidium brassicae* syn*. virulentus*) under controlled conditions. The results of all experiments indicated that soil structure has a significant effect on disease incidence, depending on the preferences of the respective pathogens. Bottom rot disease outbreak in lettuce after inoculation with *R. solani* inhibited plant growth in the order dilluvial sand > alluvial loam > loess loam (no inhibition) with similar results in minirhizotron and field experiments (Chapter 4.1; Schreiter et al., 2014b). Highest conduciveness for *Rhizoctonia*-induced bottom rot disease in the respective soils was observed for lettuce, grown in the dilluvial sandy soil. This was most likely caused by bigger pore sizes and better oxygen availability in the sand compared with the loamy soils, enabling a rapid hyphal spreading of *R. solani* towards the host plant. Similar results were reported previously by Lehtonen et al. (2008). In contrast, in chapter 6.1, lowest enrichment of the lettuce pathogen *Olpidium brassicae* was shown in the rhizosphere of lettuce grown on loamy sand of long-term field trials at the HUB-LTEs (relative abundance of 0.5-4%). In the counter trial on a silty loam at the DOK-LTEs, a remarkable rhizosphere effect with an enrichment of *Olpidium* of 76-90% in lettuce with strongest growth depression of lettuce plants grown in BIODYN2 soil was found. Zoospores of *Olpidium,* which are known to *s*urvive in soil for up to 20 years (Campbell, 1985) exhibit high motility in wet loamy soils with high water retention capacity to initiate infections in nearby plants (Westerlund et al., 1978), which may explain the higher rhizosphere abundance in the DOK-LTEs. However, unlike the *Rhizoctonia* experiments (Schreiter et al., 2014c, 2014a; Chapter 4.1), the contrasting soils used for the *Olpidium* experiments originated from different field sites (DOK and HUB-LTE) with different cropping histories which limits the comparability of the results. Furthermore, the high abundance of *Olpidium* in the rhizosphere of DOK-LTE soils resulted in increased disease incidence as affected by long-term fertilization history (Chapter 6.1) showing that the high abundance of *Olpidium* sp. was apparently not simply triggered by soil type effects.

To investigate the potential role of soil type effects on root exudation patterns with impact on rhizosphere microbial communities, benzoic acid was exemplarily selected in this study. This compound, released from lettuce roots was identified for antimicrobial defense activity against *R. solani* (Chapter 5.1*;* Walters et al., 2003; Yoon et al., 2012). As an example for a soil

type effect, highest rhizosphere concentrations of benzoic acid were detected in a loess loam soil with the highest suppressiveness against *R. solani,* even in plants without pathogen inoculation (Chapter 4.1). Inoculation experiments revealed that both, inoculation with the pathogen *R. solani* and pre-inoculation with bacterial inoculum of pathogen antagonists such as *Serratia plymuthica* 3Re4-18 and *Pseudomonas* sp. RU47 (Adesina et al., 2009; Berg and Smalla, 2009; Schreiter et al., 2014c) were able to stimulate benzoic acid exudation in the rhizosphere of lettuce (Chapter 4.1). Interestingly members of the genus *Pseudomonas* were also found to be preferentially enriched in the rhizosphere of lettuce plants grown in the *Rhizoctonia*-suppressive loess loam soil (Schreiter et al., 2014a, 2014b) with the highest benzoic acid accumulation in the rhizosphere. By contrast, in the pathogen conductive dilluvial sand, a preferential rhizosphere enrichment of the genus *Sphingomonas* was recorded, known for a pronounced potential to degrade aromatic hydrocarbons (e.g. benzoic acid), which was less expressed in the suppressive loess loam soil (Schreiter et al., 2014b). Similarly, a particularly high rhizosphere abundance of *Sphingomonadaceae* was detected in the rhizosphere of the *Olpidium*-affected lettuce plants grown in the BIODYN2 soil associated with low rhizosphere accumulation of benzoic acid (Chapter 6.1).

When taken together, these findings demonstrated potential soil type effects on the composition of the rhizosphere microbiome, which in turn affect root exudation of defense compounds, i.e. increased rhizosphere accumulation of benzoic acid in the *Rhizoctonia*-suppressive loess loam soil, which was potentially triggered by plant-beneficial members of the genus *Pseudomonas* and further promoted by limited microbial degradation of benzoic acid associated with a low rhizosphere abundance of S*phingomonas*. Besides benzoic acid, also high concentrations of lauric acid, that is also known for defense properties (Walters et al., 2003) potentially contributed to the pathogen suppressiveness against *R. solani* of the loess loam soil in the lettuce-*R. solani* pathosystem (Chapter 4.1).

This scenario illustrates an at least tripartite interaction of factors shaping the composition of the rhizosphere soil solution and related plant-microbial interactions in the rhizosphere with implications for plant health. It remains to be established to which extent also other components of the pathogen defense response in lettuce, such as phytoalexin (lettucenin A) production, shown to be induced by *R. solani* inoculation (Chapter 5.1) or expression of defense related genes (Chapter 6.1) are regulated in a similar soil-type specific manner. In

comparison to benzoic acid, lettucenin A was only detected in trace amounts close to the detection limits in the rhizosphere soil solution of lettuce plants grown in CONMIN and BIODYN2 soil. This suggests in contrast to benzoic acid, a rather passive (e.g. diffusion-mediated) release of letuccenin A, than a controlled exudation in response to pathogen infection. It was postulated that the release of benzoic acid represents a first defense line upon pathogen attack located in the rhizosphere of lettuce, followed by local accumulation of lettucenin A within the affected tissue as a second line of defense (Chapter 5.1). Distinct differences in the composition of fungal, bacterial and archaeal rhizosphere microbiota were also recorded in the soils from the two long-term field trials (HUB- LTE vs. DOK- LTE with loamy sand vs. silty loam) but additional impacts of cropping history cannot be excluded in this case (Chapter 6.1).

Soil pH is another important soil factor that affects soil microbial communities, and can be directly influenced by plant root activity. A pH-shifting can result in differences of up to three pH units in the rhizosphere compared to the bulk soil (Neumann and Römheld, 2002) with impact on promotion or suppression of soil pathogens. Soil acidity exerts suppressive effects on pathogen genera such as *Olpidium brassicae* in lettuce (Iwamoto et al., 2017), *Gaeumannomyces graminis* in wheat (Brennan, 1992), *Rhizoctonia fragariae* in strawberry (Elmer and LaMondia, 1999) and *Pseudomonas syringae* in tomato (Gonzáles-Hernández et al., 2019). On the other hand, soil acidification promotes diseases, such as club rot in cabbage, *Fusarium* wilt in cotton (Huber and Wilhelm, 1988) and bacterial wilt in tobacco, associated with a reduction in growth of antagonistic bacteria such as *Pseudomonas fluorescens* and *Bacillus cereus* (Li et al., 2017). However, for *R. solani* as lettuce pathogen investigated in chapter 4.1 and 5.1 of the present study, a broad pH spectrum between 5 and 8 has been reported in the literature (Grosch and Kofoet, 2003). Since the investigated soils in our study had a neutral to slightly acidic pH (7.3 – 6.1), strong soil type-dependent pH effects on soil microbiota are not expected. However, the influence of soil pH should be considered in future studies, also in view of possible variations of rhizosphere pH depending on plant nutritional status and fertilization (Neumann and Römheld, 2002).

7.4 Beneficial microbiomes in the lettuce rhizosphere

Soil microorganisms convey 80-90% of soil functions while they consume large amounts of nutrient-rich compounds from the rhizosphere soil solution (Mendes et al., 2013). Key soil processes e.g. carbon and nitrogen turnover influence plant-microbial community structures (Section 7.3, fertilization practice as determinant for plant interactions with rhizosphere microbiota) and attract beneficial but also harmful microorganisms, from an actively growing microbial soil population (Jones et al., 2009; Kuramae et al., 2012). It has been hypothesized that plants can even express their need for help while being under pathogen attack by a compound-release driven “cry for help” (Bakker et al., 2018), that might attract beneficial microbes into the rhizosphere (Bakker et al., 2018; Carrión et al., 2019). There is a great interest to understand more about the functional dynamics of the core microbiome supporting the host plant and their effects on plant health and growth. This aspect was exemplarily investigated in the present work and related studies using lettuce as a model plant (Chapter 6.1; Schreiter et al., 2014a, 2014c; Babin et al., 2019, 2021; Chowdhury et al., 2019). A soil type-independent enrichment of certain genera in the lettuce rhizosphere comprising the core microbiota, including *Sphingomonas*, *Rhizobium*, *Firmicutes* and *Pseudomonas* and *Mortierella* has been described (Schreiter et al., 2014a; Chowdhury et al., 2019; Babin et al., 2021). Members of these genera have been frequently associated with beneficial plant growth promoting properties (Berg, 2009). However, there are specific indications that external factors can contribute to individualization of rhizosphere community structures and assemblages (Chapter 6.1; section 7.3; Lundberg et al., 2012; Chowdhury et al., 2019). By comparing rhizosphere microbiota of lettuce grown in long-term minerally and organically fertilized soils, significant differences in the genera were revealed. Higher relative abundance of the genera U*reibacillus*, *Flavoacterium* and *Thermobacillus* as well as AMF (*Glomeromycota*) were found in organically fertilized soils (BIODYN2, HU-org), whereas higher relative abundance of *Lysobacter*, *Pseudoxanthomonas* and *Mortierella* was associated with minerally fertilized soils (CONMIN, HU-min) (Chowdhury et al., 2019; Chapter 6.1). Furthermore, also long-term management practice such as intensity of tillage, fertilization and fungicide use, as well as pre-crop effects were found to be reflected in the composition of the rhizomicrobiota of lettuce (Babin et al., 2021). Crop rotations involving rapeseed for example promoted the rapeseed pathogen *Olpidium brassicae* in the core microbiome of soils, rhizosphere and roots of rapeseed particularly in soils with extensive use of N fertilizers and fungicides (Bennett et

al., 2014; Lay et al., 2018; Sommermann et al., 2018; Babin et al., 2021). Nevertheless, lettuce plants grown in these soils had no visible *Olpidium* mediated disease symptoms, although *Olpidium* is also known as a lettuce pathogen. Increased *Olpidium* occurrence coincided with a higher relative abundance of plant growth-promoting AMF (*Glomeromycota*), which might have counteracted the pathogenic effect of *Olpidium* (Begum et al., 2019). However, *Olpidium brassicae* strains colonizing roots of rapeseed are not necessarily pathogenic to lettuce (Hartwright et al., 2010). Furthermore, Babin et al. (2021) demonstrated in the same experiment, using the LTE soils that rhizosphere bacterial and archaeal communities were primarily shaped by long-term tillage history (conservation tillage with cultivator = CT vs. conventional ploughing = P) and a major difference was seen in an enrichment of potentially plant beneficial members of the genus *Pseudomonas* (17.2 %) in the lettuce rhizosphere of soil with conservation tillage. It has been postulated that lettuce plants grown in soils, which have a history of conservation tillage might have a higher root exudation of succinic and malic acids, as chemotactic compounds attracting *Pseudomonas fluorescens* (Oku et al., 2014; Babin et al., 2021). Indeed, in a parallel minirhizotron experiment, a 70% reduction of succinate concentrations was found in the rhizosphere soil solution collected from young subapical root zones of lettuce plants grown on soil with long-term conventional tillage (rapeseed pre-crop) as compared with conservation tillage (Windisch, unpublished). Within fungal communities, *Mortierella elongata* with well-documented plant-growth promoting properties (Tamayo-Velez and Osorio, 2017; Li et al., 2018) was identified as indicator for treatments with long-term conservation tillage (Babin et al., 2021). Accordingly, comparing three experiments, a significantly higher shoot biomass in CT variants (conservation tillage) was recorded (Figure 10). Similarly, high concentration of succinate was detected, comparing soils with long-term organic vs. mineral fertilization history, exclusively in the rhizosphere soil solution of lettuce grown in the CONMIN soil with mineral fertilization. This was related to a high relative rhizosphere abundance of *Pseudomonadaceae* and also *Mortierella elongata* and a suppression of *Olpidum* disease symptoms in comparison with the BIODYN2 soil with long-term organic fertilization. (Chapter 6.1)

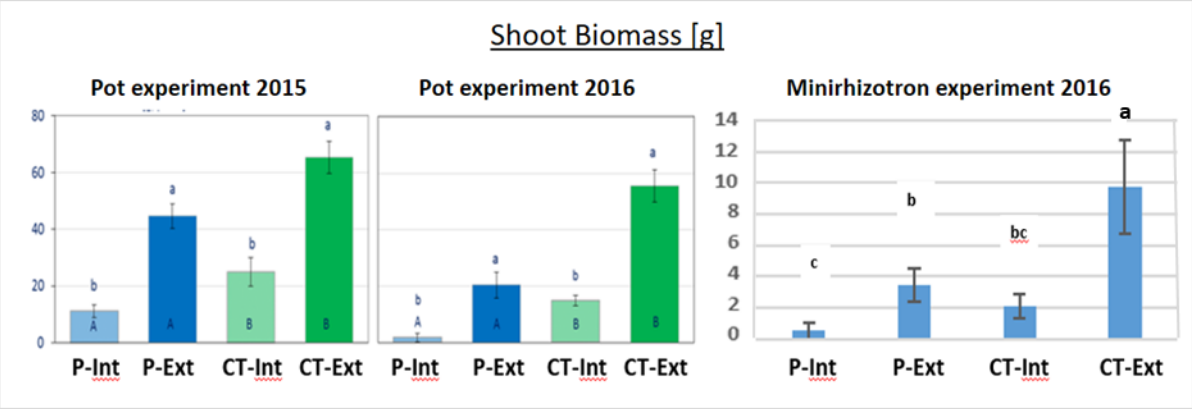

Figure 10: Shoot biomass of lettuce plants in pot and minirhizotron experiments on soils with long-term extensive (Ext) and intensive (Int) history of N fertilization and fungicide use with long-term conventional ploughing (P) vs. conservation tillage (CT) (Final Report DiControl Project 1nd Phase FK031A560E, 2019).

As an additional common feature in the range of experiments conducted on different soils in this study, disease suppression against different lettuce pathogens (*Rhizoctonia solani, Olpidium* sp.) was associated with increased rhizosphere accumulation of the antifungal compound benzoic acid. This was related with a lower rhizosphere abundance of the genus *Sphingomonas* in comparison with pathogen-affected plants reported to be involved in degradation of aromatic hydrocarbons (Schreiter et al., 2014a, 2014b, Chapter 6.1). Hence, apart from the ability to attract beneficial rhizosphere microorganisms, also the inhibited microbial degradation of pathogen-antagonistic root exudates might be a component of a pathogen-suppressive rhizosphere microbiome in lettuce.

As a practical perspective, the use of bacterial strains belonging to the beneficial lettuce core microbiome as natural biocontrol agents towards soil-borne diseases especially against *R. solani*-induced bottom rot disease in lettuce was shown to be promising (Chapter 4.1). Simultaneous application of the two bacterial strains *Pseudomonas* sp. RU47 and *Serratia plymuthica* 3Re-4-18 was most effective in pathogen suppression, strikingly demonstrated by the absence of any dead plants after inoculation with *R. solani* on the loamy soil (Chapter 4.1). However, competition with the native microbial community and mutual competition between double-inoculants in the rhizosphere (Schreiter et al., 2018; Bradáčová et al., 2019) with negative effects on plant growth can occur. The growth depression of lettuce plants in chapter 4.1 was associated with reduced plant available micronutrients, suggesting a competitive microbial-plant interaction in nutrient acquisition. The two bacterial strains are reported as

producers of siderophores, both efficient chelators for iron and other divalent metal cations in the soil (Adesina et al., 2007; Adam et al., 2016). However, a limited availability of micronutrients in lettuce plants was mainly observed on the loamy soil, where plants were already negatively stressed after *R. solani*-infection.

When taken together the results demonstrated that a beneficial core microbiome in the lettuce rhizosphere exists and is partially shared across different soil types, crop rotations and management practices in the long term. High rhizosphere abundance of plant-beneficial *Pseudomonas* and *Mortierella* species in combination with low abundance of *Sphingomonas* sp. with high potential for degradation of aromatic compounds were identified as common characteristics. These findings suggest a soil memory, which is characterized by soil heterogeneity and the ability to transfer patterns of defense to progeny via the core microbiome (Lapsansky et al., 2016). However, especially for certain bacteria there is a narrow gap between plant beneficial and pathogenic genera (Babin et al., 2021). Particularly for the pathogenic genera of *Pseudomona*s there is an overlap, as these have a similar strategy for colonizing the rhizosphere and impacting plant immune responses. Thus a harmful effect on the host plant cannot be completely circumvented by rhizosphere colonization (Brader et al., 2017; Passera et al., 2019; Yu et al., 2019; Babin et al., 2021). As shown by Babin et al. (2021) and for the data presented in chapter 6.1 as growth chamber experiments there is no chronological information about the rhizosphere colonization of lettuce, due to shifting of the individual soils prior to the establishment of the experiments. Therefore, it is difficult to clarify an actual reason for the establishment of the actual microbial community in the rhizosphere. This would be an additional aspect to be investigated in more detail in future studies. Nevertheless, given the costly production of fertilizers and pesticides in view of the social and political demands for a more sustainable agriculture, promoting the development of pathogen suppressive agricultural soils with a beneficial core microbiome may provide a promising avenue from a long-term perspective (Lapsansky et al., 2016). Additionally, the positive results on exudate patterns shown for the suppressive soils of CONMIN (Chapter 6.1) with a consistent positive influence on the structure of the microbial community in the rhizosphere may also provide a new perspective for future lettuce-breeding programs for improved plant health and productivity.

8 Concluding remarks and open questions

In the present thesis, microbial and biochemical processes in the rhizosphere of lettuce were discussed in close relation to site-specific factors, soil type, management and fertilization history as important determinants of plant health and stress resistance. Strong soil-type effects on the expression of disease severity of *R. solani* and *Olpidium* sp. in lettuce were expressed under controlled conditions in climate chamber experiments. This underlines the importance of the conditions determining the expression of disease severity and antagonistic interactions with biocontrol agents. The fungus *Olpidium* rapidly spread under favorable soil conditions in the BIODYN2 soil with suitable root exudate patterns and only few antagonistic acting microorganisms (e.g. *Mortierella* sp. and *Pseudomonas* sp.) due to carbohydrate limitations in the rhizosphere of the model plant (Chapter 6.1). These findings suggest that not only the fertilization history but also the current fertilizer supply has an impact on the plant microbial interactions in soil and plant rhizosphere. The root exudation of benzoic acid (Chapter 5.1) was triggered by the presence of pathogen *R. solani* and *Olpidium* sp. but also by pathogen antagonists (*Pseudomonas* sp. RU47, *Serratia plymuthica and Mortierella elongta*) pointing to a selective effect of rhizosphere microorganisms on the release of root exudates from lettuce with function in pathogen defense and chemo-attractants (Chapter 4.1 and 6.1). Furthermore, benzoic acid was determined to be sufficient as accumulated rhizosphere concentration to inhibit mycelial growth of *R. solani* and reduced the disease severity of infected plants (Chapter 4.1 and 5.1). Expression of lettucenin A was detected for the first time not only in the leaf tissue of lettuce but also in affected roots (Chapter 5.1). All of these findings strongly suggest that shaping of rhizosphere microbial communities via root exudates is not simply an unidirectional process from host plants to soil microbiota but vice versa, that microbiota are obviously shaping root exudation at the same time (Chapter 4.1 and 6.1).

In this thesis, crucial players in a complex network of belowground plant-microbial interactions and their effects on lettuce as a model plant for growth and health were described. The results are based on model experiments and descriptions of coinciding relationships without consideration of temporal or spatial variations. Therefore, further research should focus on (i) a variability of plant genotypes and soil type (ii) plant nutrient

limitations on root exudation influenced by soil- and rhizosphere-pH (iii) and detailed samplings for rhizosphere microbial communities in different root zones.

Finally, this thesis will contribute to a better understanding of plant-microbial interactions in agricultural soils in relation to the processes and development of the rhizosphere and will further contribute to the development of practical approaches in line with the concept of “soil biological engineering” (Bender et al., 2016).

9 References (General introduction and General discussion)

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11 Curriculum vitae

Saskia Helen Windisch
Felix-Dahn-Straße 45
70597 Stuttgart
Germany

Mobile: +49 (0) 176-82141670
Email: Saskia.Windisch@gmx.de

Personal information

Name	Saskia Helen Windisch
Date and place of birth	15 August 1987, Frankfurt am Main, Germany
Nationality	German

Academic education

Since October 2015	Doctoral candidate at the Institute of Crop Science, Nutritional Crop Physiology (340h), University of Hohenheim Supervisor: Prof. Dr. Günter Neumann Topic: *The role of soil properties and fertilization management in pathogen defense and plant microbial interactions in the rhizosphere of lettuce (*Lactuca sativa L.*)*
October 2012 - February 2015	International Master's Programme Crop Sciences, University of Hohenheim Major subject: Soil science and Plant nutrition Degree: M. Sc. Master thesis: *Effects of repeated bunch trampling by grazing cattle on the botanical composition of Argentinean grasslands*, scholarship: Dr. Herman Eiselen-Stipendium (Foundation fiat panis) Supervisor: Prof. Dr. Folkard Asch
October 2008 - April 2012	Agricultural science, University of Hohenheim Major subject: Soil science Degree: B. Sc. Bachelor thesis: *Vorkommen und Aktivität von Regenwürmern im Verlauf der fortschreitenden Veränderung der Bodeneigenschaften in einer Podsolchronosequenz in Südnorwegen*, scholarship: Soil science Institute, University of Hohenheim Supervisor: Prof. Dr. Daniela Sauer

School education

1998 - 2007	High school degree (Abitur), Ellenrieder Gymnasium, Konstanz

Internship and work experience

February - May 2015	Employee at the Institute for Agroecology and Plant production in the Tropics and Subtropics, University of Hohenheim
May 2010 - December 2013	Student assistant at the Institute of Crop Science and Institute of Soil Science, University of Hohenheim
January - May 2012	Project assistance *Eindämmungsverfahren von Feuerbrand in Obstanlagen im Bodenseekreis* at the Landwirtschaftliches Technologiezentrum Augustenberg (LTZ), Referat 33 *Biological Diagnoses and Plant Health*, Stuttgart Internship at the Ministerium für ländlichen Raum und Verbraucherschutz (MLR), Referat 23 *Plant production and production-related environmental protection*, Stuttgart Internship at the Landwirtschaftsamt Bruchsal, Karlsruhe
October 2010	Internship at Taifun (Life Food GmbH, Taifun-Tofuprodukte), Freiburg
August - September 2010	Internship at the experimental station of Kleinhohenheim, University of Hohenheim
August - September 2009	Internship at demeter farm Martin Hahn, Überlingen-Bonndorf
December 2007 - September 2008	Voluntary Service in Nicaragua with the Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ)

Saskia Windisch

Saskia Windisch
Stuttgart, 30.08.2022