A Short-Term Response of Soil Microbial Communities to Cadmium and Organic Substrate Amendment in Long-Term Contaminated Soil by Toxic Elements

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Two long-term contaminated soils differing in contents of Pb, Zn, As, Cd were compared in a microcosm experiment for changes in microbial community structure and respiration after various treatments. We observed that the extent of long-term contamination (over 200 years) by toxic elements did not change the total numbers and diversity of bacteria but influenced their community composition. Namely, numbers of *Actinobacteria* determined by phylum specific qPCR increased and also the proportion of *Actinobacteria* and *Chloroflexi* increased in Illumina sequence libraries in the more contaminated soil. In the experiment, secondary disturbance by supplemented cadmium (doses from double to 100-fold the concentration in the original soil) and organic substrates (cellobiose or straw) increased bacterial diversity in the less contaminated soil and decreased it in the more contaminated soil. Respiration in the experiment was higher in the more contaminated soil in all treatments and correlated with bacterial numbers. Considering the most significant changes in bacterial community, it seemed that particularly *Actinobacteria* withstand contamination by toxic elements. The results proved higher resistance to secondary disturbance in terms of both, respiration and bacterial community structure in the less contaminated soil.

Keywords: respiration, diversity, actinobacteria, straw, cellobiose

HIGHLIGHTS

- Bacterial communities differed between highly (H) and low (L) contaminated soils in proportion and quantity of *Actinobacteria* but the number of total bacteria was similar.
- *Actinobacteria* increased after addition of cadmium and organic substrates in both soils.
- Respiration increased more in the H soil after addition of cellobiose and straw.
- The H soil was more sensitive to additional disturbance than the L soil.

INTRODUCTION

Ecosystem health has been defined in terms of stability and resilience in response to disturbance (Griffiths and Philippot, 2013). The amplitude of this response and time to return to the state before disturbance can serve as measures of soil health (Van Bruggen and Semenov, 2000). Many relationships in soil microbial communities are accompanied by functional redundancy, which allows conservation of important ecosystem functions in spite of changes in microbial community (Singh et al., 2014). Therefore, it seems that composition of soil bacterial community represents a sensitive measure of soil health compared to general soil characteristics such as respiration or other measures of organic matter decomposition (Hartmann et al., 2014; Singh et al., 2014).

Pollution by toxic elements represents a chemical disturbance of worldwide concern. It causes major changes to soil functioning including decomposition of organic matter or element cycling (Azarbad et al., 2013; Li et al., 2014). However, this type of disturbance can be counterweighted by adaptation of microbial communities to the contamination. Mostly because microorganisms were exposed to toxic elements since early history, which resulted in the evolution of metal resistance mechanisms, such as extracellular and intracellular sequestration of metals, exclusion by permeability barriers, enzymatic detoxification, reduction in sensitivity of cellular targets or efflux pumps (Epelde et al., 2015). Although metal toxicity to soil microorganisms leading to changes of their activities had been confirmed, it remained unclear whether they resulted from an overall decrease in microbial diversity or rather from inhibition of specific taxa. Also it was shown that different activities are unevenly affected (Niemeyer et al., 2012). With respect to that it was hypothesized that organic matter breakdown, as one of the general microbial functions, may be relatively unchanged because most microorganisms utilize different forms of organic carbon. However, respiration rates as a measure of decomposition were found both positively or negatively related to toxic elements contamination (Niemeyer et al., 2012; Epelde et al., 2015).

Actinobacteria represent a specifically interesting group for adaptation strategies in decomposition processes (Sagova-Mareckova et al., 2011). They are not only one of dominant groups of soil bacteria but they belong to the most efficient decomposers breaking down even hemicellulose and lignin.

Above that it was found that actinobacteria are more sensitive to toxic elements contamination than fungi and bacteria in general, so it seems that they have a potential to become bioindicators of this type of disturbance (Jin et al., 2014).

One specific situation of toxic elements pollution is represented by metal smelters, around which the soil was unpolluted before smelting started and then, over decades, have become highly contaminated. Therefore, soil microbial communities in such areas became adapted to metal contamination. New metal-tolerant microbial communities developed due to selection pressure and horizontal gene transfer of respective resistance genes so the soil functions were maintained (Azarbad et al., 2016). However, due to constant use of the contaminated land and possibly also the climate change, new changes of environmental conditions occur at contaminated areas. Those may be characterized as secondary disturbance and may include changing the land use for other type of crops, forest or meadow, fertilization, fire or flooding or invasion by a foreign organism. Not much is known about the outcome of such secondary disturbance in terms of microbial community structure or functions, in particular, when the effect of several disturbing factors is combined (Azarbad et al., 2016).

We tested the effect of newly added cadmium and organic substrates to see, which parameter out of respiration, quantity of total bacteria and actinobacteria and bacterial community structure will correspond to induced disturbance and how that will compare between two soils differing in the extent of long term contamination with toxic elements. Cadmium as a contaminating metal was selected because of its high toxicity and also because previous results obtained at the same site showed that Cd concentration was significantly correlated to respiration rate and metabolic quotient, parameters closely related to activities of microorganisms (Muhlbachova et al., 2015). The first organic substrate, cellobiose was selected because it is a common disaccharide, which is produced from cellulose by cellobiohydrolase. It is an easily utilized substrate, preferably consumed and may be connected to decomposition processes occurring in the root zone, which is considered a decomposition hotspot (Steinauer et al., 2016). Variability of cellobiohydrolase gene was compared in the two soils. This enzyme was selected as an example of a specific microbial function related to decomposition process. The second organic substrate, straw was chosen for its high content of lignin, which is the least degradable part of organic matter, therefore its decomposition is slower. The detailed analysis of changes in microbial community structure in the treatments using real-time PCR and Illumina amplicon sequencing was performed to show how changes in respiration are related to bacterial community. A specific attention was given to actinobacteria because of their known large pool of resistance genes. We hypothesized that the more contaminated soil will have a stronger response to additional disturbance than the less contaminated soil and that different taxonomic groups will, respectively, change their relative abundance to applied amendments in each soil. Those taxa can then serve as microbial indicators for responding to long-term and short term toxic elements contamination.

TABLE 1 | Characteristics of sites with high (H) and low (L) soil contamination.

	H	SD	L	SD
pH	5.40	0.10	5.40	0.10
Organic matter	4.72%	0.23	5.3%	0.32
Pb ***	451.3 mg.kg⁻¹	48.30	65.6 mg.kg⁻¹	6.25
Zn ***	180.6 mg.kg⁻¹	7.00	78.9 mg.kg⁻¹	4.93
As ***	30.41 mg.kg⁻¹	9.86	13.517 mg.kg⁻¹	1.02
Cd ***	15.055 mg.kg⁻¹	0.66	8.307 mg.kg⁻¹	0.37

*** $p < 0.001$, $n = 5$.

MATERIALS AND METHODS

Sites

The sampling sites were located in an area contaminated with toxic elements near a lead smelter in Pˇríbram, Czechia. Two grasslands (grass planted in old fields), H (site with high in concentration, N49˚42.327 E13˚58.516) and L (site with low concentration, N49˚42.243 E13˚56.371) were selected for this study (**Table 1**). The soil at both sites is slightly acidic modal Cambisol. The more contaminated site was about 300 m, while the less contaminated site about 3,000 m apart from the smelter chimney, which represents the major source of deposits. They differed in contamination not only due to differing distance but also due to prevailing northwest winds. The smelter processed lead ores from 1786 to 1972, when processing of secondary lead sources started. Since 1982 emissions of contaminating elements decreased 300–500 times. Contamination of the area and explanations were previously described in Muhlbachova et al. (2015). Soil was sampled November 2013. At each site, five soil cores (40 mm diameter) were taken from the upper soil horizon to depth of approximately 150 mm. Cores were collected randomly from the area of 4 m² using plastic tubes. Soil of each core after removal of grass rhizosphere was homogenized through a 2-mm sieve to produce subsamples for subsequent experiment and analyses.

Short-Term Experiment

Firstly, soils collected at the two sites were compared in terms of microbial communities using a subsample (0.5 g) from each core for DNA extraction and downstream applications. Those samples are named "original soils" and represent the long term change of the two differently contaminated sites. Secondly in a short term experiment, subsamples of 15 g of the respective cores were used in OxiTop respiration measurements. Those subsamples were treated with cadmium, cellobiose and straw according to **Table 2**. The treatments also included a "treatment" without supplements, which was subjected to respiration measurements. Dosages of cadmium were derived from its contents in the original soils (**Table 1**). The lowest dosage doubled the average original content, the following dosages were 10- and 100-fold the original content. Soil was collected after exposure in OxiTop flasks and stored at -80˚C until further processing. we chose to determine the first phase of the microbial community response to toxic elements and organic supplements because we had observed previously that in microcosms respiration rate is usually

TABLE 2 | Sample treatments in the short-term experiment applied for both the low contamination soil L and the high contamination soil H.

Sample number	Cadmium (mg.kg⁻¹)	Substrate
0	0	–
10	10	–
100	100	–
1000	1000	–
C0	0	Cellobiose (C), 3 g
C10	10	Cellobiose (C), 3 g
C100	100	Cellobiose (C), 3 g
C1000	1000	Cellobiose (C), 3 g
S0	0	Straw (S), 3 g
S10	10	Straw (S), 3 g
S100	100	Straw (S), 3 g
S1000	1000	Straw (S), 3 g

consistent only during the first 5–7 days (Li et al., 2014). Finely grated wheat straw and D-cellobiose (Sigma-Aldrich) 3 g each were added to respective treatments.

Respiration

Soil microbial respiration was measured by a pressure sensor method using OxiTop system with pressure sensor-data logger OxiTop-C heads including a capsule for NaOH solution (WTW, Weiheim, Germany). The respiration rate was calculated from linear pressure decrease equivalent to O_2 consumption between 50 and 250 h of incubation.

Soil Analysis

Soil samples were analyzed for pH, organic matter content, lead, zinc, arsenic, and cadmium contents. Soil pH was measured by a Multi 350 glass electrode WTW, in a soil-water extract, 20 g of soil being extracted with 50 ml distilled water, and set at room temperature for 12 h. Organic matter was assessed by combustion at 550˚C to constant weight. the leachable toxic elements (Pb, Zn, As, Cd), were determined in 5 g dry soil samples extracted with 50 ml 2 M nitric acid after 6 h shaking (Suprapur; Merck, Darmstadt, Germany). The filtered extract was analyzed using atomic absorption spectrometry (AAS). Determination of Cd and Pb was performed by atomizing in a graphite tube with a 240Z AA device with Zeeman background correction (Agilent Technologies, Santa Clara, CA, United States). Zn and As were determined by flame AAS on the 55 AA device, and arsenic determination was performed using Vapor Generation Accessory VGA 77 (Agilent Technologies).

Soil DNA Extraction

DNA was extracted from 0.5 g of soil samples by the method described in Sagova-Mareckova et al. (2008), where it is denoted "SK." The method is based on bead-beating and phenol/chloroform extraction. The samples are purified by incubation with cetyltrimethylammonium bromide followed by chloroform extraction and incubation with $CaCl_2$, and finally cleaned with GeneClean Turbo kit (MP Biomedicals, Santa Ana, CA, United States). DNA quality and quantity was estimated

using an agarose gel electrophoresis and the concentration was measured by NanoPhotometer (Implan, Germany).

PCR Amplification and Cloning of Bacterial Cellobiohydrolase Gene

Subsamples of two randomly selected biological replicates from controls and treatments with 100 mg/kg Cd and straw were selected for cellobiohydrolase gene sequencing. Primers Cbh1F (5[r]- CGTCRTCTACRACCTGCC -3[r]) and Cbh2R (5[r]- CCAGCCGAKCCAGCCGTG -3[r]) were designed using Primrose software included in Bioinformatic toolkit (Ashelford et al., 2002) based on the known cellobiohydrolase encoding genes retrieved from the GenBank database. The primers targeting a 346 bp part of bacterial cellobiohydrolase gene were validated by amplification and cloning from *Nocardiopsis dassonvillei* subsp. *dassonvillei* chromosomal DNA and from environmental DNA samples isolated at the study sites. The primers were designed to amplify cellobiohydrolase genes from *Actinobacteria*. According to the Carbohydrate-active enzymes database[1] (Lombard et al., 2014) the primers cover cellobiohydrolases cleaving from non-reducing end (EC3.2.1.91) classified in glycosyl hydrolases class 6 and do not amplify the genes coding for endoglucanases (EC 3.2.1.4) of the same class. PCR mixture (50 µl total volume) contained: 1×AccuPrime PCR Buffer II (containing MgCl$_2$, nucleotides), 200 nM primer (each), 5% DMSO, 1 U AccuPrime Taq DNA polymerase (Invitrogen, Carlsbad, CA, United States), and 100 ng template DNA. The amplification was performed in Bio-Rad C-1000 cycler (Bio-Rad, Hercules, CA, United States) using a touch down protocol consisting of a hold at 94 ˚C for 5 min followed by 10 cycles of denaturing at 94 ˚C for 60 s, annealing at 66–56 ˚C (each cycle the temperature decreased by 1 ˚C) for 50 s, and extension at 72 ˚C for 30 s, then 30 cycles of 94 ˚C for 60 s, 56 ˚C for 50 s, and 72 ˚C for 30 s, and final extension at 72 ˚C for 5 min.

The PCR products were purified by QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany), cloned with pGEM®-T Easy Vestor System (Promega, Madison, WI, United States) according to the manufacturer's protocol, and transformed into *Escherichia coli* JM109. The transformant colonies were transferred to 20 µl of sterile water and lysed for 5 min at 95 ˚C. A 5 µl aliquot was transferred to a PCR mixture containing in 25 µl total volume: l×DreamTaq Buffer, 400 nM pUC-M13f and pUC-M13r primers (Promega), 200 µM dNTP, 1 U DreamTaq DNA Polymerase (Fermentas, Waltham, MA, United States). The cloned inserts were amplified using the PCR protocol: initiation at 94 ˚C for 5 min, 35 cycles of denaturing at 94 ˚C for 60 s, annealing at 54 ˚C for 50 s, extension at 72 ˚C for 30 s, and final extension at 72 ˚C for 5 min. The PCR products were purified using QIAquick® PCR Purification Kit (Qiagen) and sequenced by Macrogen Europe Laboratory (Amsterdam, Netherlands). The sequences were edited in Chromas Lite Software (Technelysium, Brisbane, QLD, Australia), converted to the amino acid sequences in BioEdit Sequence Alignment Editor v. 7.0.5.3[2], aligned with Muscle v3.6 (Edgar, 2004) and analyzed

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[1] http://www.cazy.org/

[2] http://www.mbio.ncsu.edu/BioEdit/bioedit.html

in Mothur 1.39.5 (Schloss et al., 2009). Fifty sequences of each treatment were selected for the libraries, which were compared between the sites and treatments by Libshuff method (Singleton et al., 2001) implemented in Mothur.

Quantitative PCR

Primers were eub338f (5[r]-ACTCCTACGGGAGGCAGCAG-3[r]) (Lane, 1991) and eub518r (5[r]-ATTACCGCGGCTGCTGG-3[r]) (Muyzer et al., 1993) amplifying a 197 bp fragment of the 16S rRNA gene from *Bacteria*, act235f (5[r]-CGCGGCCTATCAGCTTGTTG-3[r]) (Stach et al., 2003) and eub518r, yielding a 280 bp product, for *Actinobacteria*. The reactions were done on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using 96-well plates with GoTaq qPCR Master Mix (Promega) containing SYBR Green as a double-stranded DNA binding dye. The reaction mixture contained in a total volume of 15 µl: ⋇ GoTaq qPCR Master Mix, 0.2 µM primers, and 1 ng diluted DNA sample. For both targets the PCR cycling protocol consisted of initial denaturation at 95 ˚C for 10 min, followed by 45 cycles of 95 ˚C for 15 s, 60 ˚C for 30 s and 72 ˚C for 30 s. Melting curves were recorded to ensure qPCR specificity. Baseline and threshold calculations were performed with the StepOne v. 2.2.2 software. The inhibition was tested by serial DNA dilutions from each site. All qPCR measurements were done in duplicates. The qPCR standard was prepared by cloning the fragments of the target gene from *Streptomyces europaeiscabiei* DMS 41802 as described previously (Sagova-Mareckova et al., 2015).

Illumina Amplicon Sequencing

Two randomly selected replicates of each treatment were chosen for sequencing (the number of sequenced replicates was limited to two for economic reasons after observation of low variability of replicates in qPCR). The environmental DNA samples were diluted to approximately 30 ng µl^{-1} in a volume of 50 µl and transferred to transport tubes GenTegra DNA (GenTegra, Pleasanton, CA, United States). The samples were thoroughly mixed with the protective medium, and dried in a vacuum evaporator at 37 ˚C for 240 min. Amplification of bacterial 16S rRNA gene fragment including the variable region V4 using universal primers with overhang adapters CS1-515F (5[r]-ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGG TAA-3[r]) and CS2-806R 5[r]-TACGGTAGCAGAGACTTGGTCT GGACTACHVGGGTWTCTAAT-3[r]) (Caporaso et al., 2011), construction of libraries, and sequencing by Illumina MiSeq (Illumina, San Diego, CA, United States) were done at the DNA Services Facility, Research Resources Centre University of Illinois (Chicago, IL, United States). The resulting paired sequence reads were merged, filtered, aligned using reference alignment from the Silva database (Quast et al., 2013), and chimera checked using integrated Vsearch tool (Rognes et al., 2016) according to the MiSeq standard operation procedure (Miseq SOP, February 2018; Kozich et al., 2013) in Mothur v1.39.5 (Schloss et al., 2009). A taxonomic assignment of sequence libraries was performed in Mothur using the Silva Small Subunit rRNA Database, release 132 (Yilmaz et al., 2014)

adapted for use in Mothur[3] as the reference database. Sequences of plastids, mitochondria, and those not classified in the domain *Bacteria* were discarded. The sequence library was clustered into OTUs using the Uparse pipeline in Usearch v10.0.240 software (Edgar, 2013), and the OTU table was further processed using tools implemented in the Mothur. Distance matrices describing the differences in community composition between individual samples were calculated using the Yue-Clayton theta calculator (Yue and Clayton, 2005). Analysis of molecular variance (AMOVA; Martin, 2002) was based on a matrix of Yue-Clayton theta distances. Metastats (White et al., 2009) and Lefse (Segata et al., 2011) analyses were used to detect differentially represented OTUs. A Maximum-likelihood phylogram of the OTU representative sequences was constructed using FastTree 2 (Price et al., 2010). Rarefaction curves were used as a measure of diversity because Illumina sequencing shows only relative rather than absolute quantity of individual taxa measured and the total number of OTUs in the community cannot be determined (Edgar, 2013). Figures were created using the Interactive Tree of Life online tool[4] (Letunic and Bork, 2016), and Inkscape (v0.92[5]).

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[3]https://www.mothur.org/w/images/3/32/Silva.nr_v132.tgz
[4]http://itol.embl.de
[5]http://www.inkscape.org

Statistical Analyses

Analysis of variance (ANOVA) tests were performed for respiration, copy numbers of bacterial and *Actinobacteria* 16S rRNA genes to determine differences between the treatments. *P*-values for the pairwise comparison were adjusted for multiple comparison problems with the help of the Max-abs-t-distribution method (Bretz et al., 2015). Yue-Clayton theta distances of bacterial communities were used in the testing. Differences within and between groups were determined by Adonis test, which is a permutational multivariate analysis of variance using distance matrices (McArdle and Anderson, 2001). Dispersion within groups was determined by test of multivariate dispersions using dissimilarity measures (Gijbels and Omelka, 2013). All statistical calculations were done in the R computing environment (R Core Team, 2017).

Accession Numbers

The partial cellobiohydrolase gene sequences reported in this paper have been deposited in GenBank under accession no. MF621345 – MF621548. The Illumina MiSeq 16S rRNA gene amplicon sequences have been deposited in the NCBI Sequence Read Archive[6] as BioProject PRJNA397131.
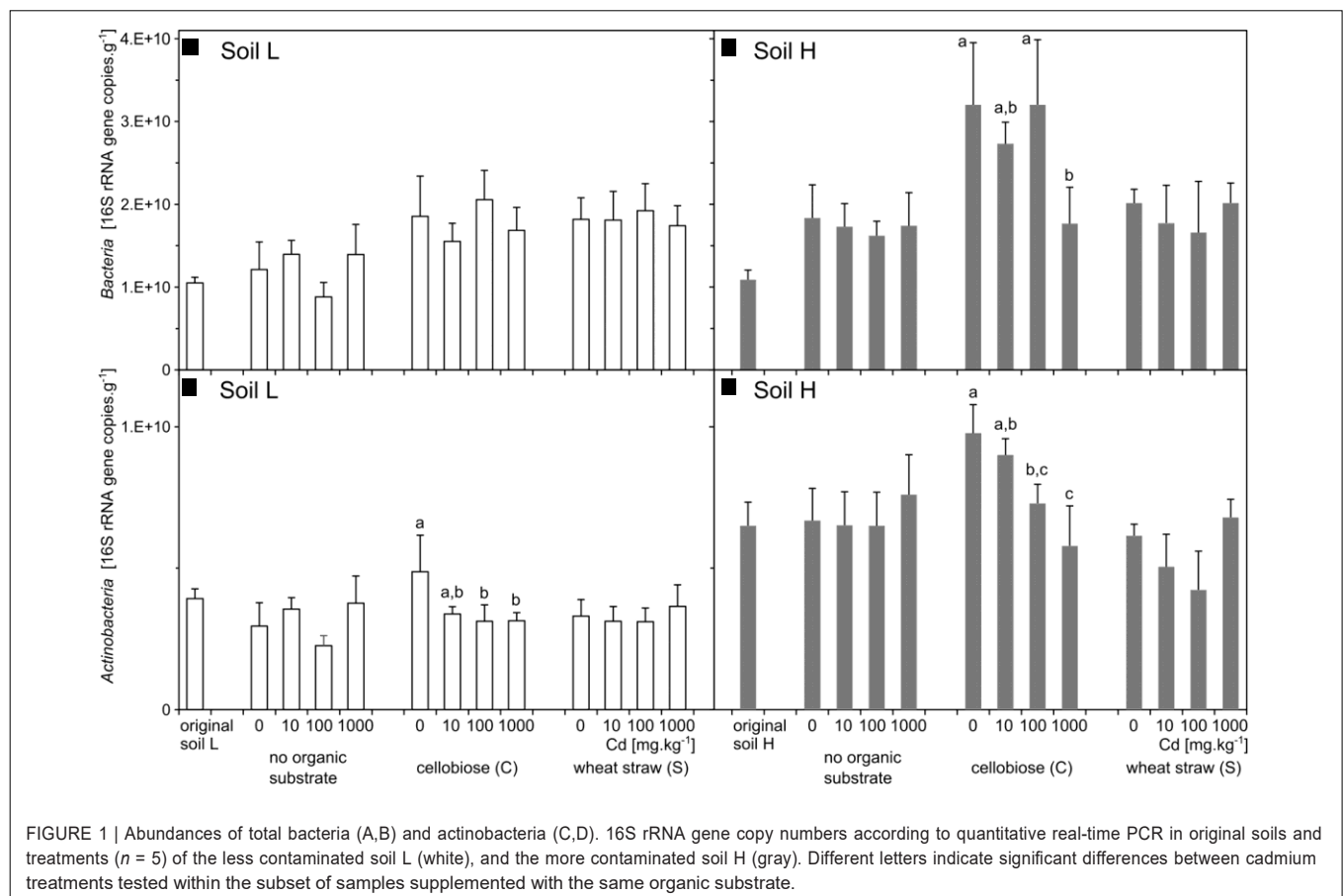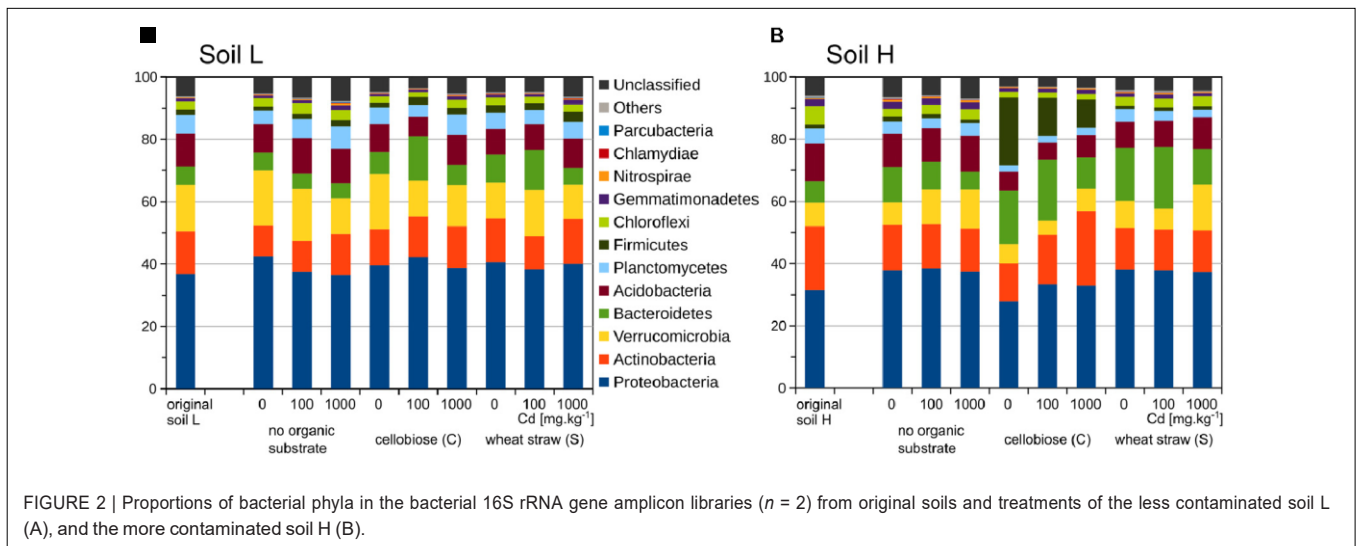
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[6]www.ncbi.nlm.nih.gov/sra

FIGURE 1 | Abundances of total bacteria (A,B) and actinobacteria (C,D). 16S rRNA gene copy numbers according to quantitative real-time PCR in original soils and treatments (*n* = 5) of the less contaminated soil L (white), and the more contaminated soil H (gray). Different letters indicate significant differences between cadmium treatments tested within the subset of samples supplemented with the same organic substrate.

FIGURE 2 | Proportions of bacterial phyla in the bacterial 16S rRNA gene amplicon libraries (*n* = 2) from original soils and treatments of the less contaminated soil L (A), and the more contaminated soil H (B).

RESULTS

Original Soils

The two original soils differed significantly by the content of toxic elements after the long term exposure to contamination. The soil with low contamination (L) had approximately half of the contaminating Pb, Zn, As, and Cd than the soil with high contamination (H) (**Table 2**). The quantity of total bacteria did not differ between the two original soils (**Figures 1A,B**), but quantity of actinobacteria was about 40% higher in H soil than L soil ($p < 0.001$, **Figures 1C,D**). The proportion of phyla was relatively similar in the two soils, only *Actinobacteria* and *Chloroflexi* were slightly higher in H soil (**Figure 2**).

Respiration

Respiration of the soil H was significantly higher than of the soil L ($p < 0.001$; **Figure 3**). The respiration increased 5–10 times in treatments supplemented with organic carbon substrates in both soils, but it increased several times more in the H soil. In both soils, respiration was significantly affected by interaction between effects of cadmium and substrate ($p < 0.001$). Differences between cadmium doses within organic substrate treatments were similar in the two soils. However, in L soil, both cadmium and substrate additions had a total significant effect on respiration ($p < 0.001$), while in H soil, only the effect of added organic carbon substrate ($p < 0.001$) was significant (**Figure 3** and **Supplementary Table S1**).

Quantity of Bacteria and Actinobacteria

The effect of treatments on bacteria and actinobacteria quantities differed in the two soils. In the treatments of H soil, the numbers of bacteria were influenced by interaction between effects of cadmium and substrate ($p = 0.022$) and the interaction was due to the treatment by cadmium 1,000 mg.kg^{-1}, which significantly decreased bacteria number in cellobiose treatment. The substrate alone also increased numbers of bacteria ($p < 0.001$), particularly after addition of cellobiose (**Figures 1A,B** and **Supplementary**

Table S1). The numbers of actinobacteria were also influenced by interaction between effect of cadmium and substrate ($p < 0.001$). That significant effect was mostly due to treatment with cadmium 1,000 mg.kg^{-1}, which increased numbers of bacteria in the straw treatment. However, both cadmium and substrate significantly influenced numbers of actinobacteria also separately ($p = 0.004$; $p < 0.001$, respectively). In the treatments of L soil, the numbers of bacteria were not influenced by interaction between the effects of cadmium and substrate and only the effect of substrate was significant ($p < 0.001$). The numbers of actinobacteria were only marginally influenced by the interaction between the effects of cadmium and substrate ($p = 0.021$) and only the individual effect of cadmium was significant ($p = 0.027$) (**Figures 1C,D** and **Supplementary Table S1**).

Cellobiohydrolase

Sequences of the gene coding for cellobiohydrolase differed significantly (Libshuff, $p < 0.05$) between the soil samples H0 and L0, which did not obtain any cadmium or organic substrate supplement and also between the samples with supplements of straw and cadmium in concentration 100 mg.kg^{-1} (**Table 3**).

Bacterial Community

Bacterial communities differed significantly between the two soils, when all treatments were included (Adonis, $p < 0.001$). Also, bacterial communities from the less contaminated soil L were more homogeneous compared to the more contaminated soil H (p disp < 0.001, **Figure 4**).

After quality filtering, a total of 3,229,710 sequences were mapped to 16,311 OTUs defined at a 97% similarity level. The long-term effect of toxic elements contamination separated the two original soils by proportions of 1,594 OTUs (Metastats, $p < 0.05$) (**Figure 5**). After incubation in the short-term experiment, samples without cadmium or organic substrate supplement (L0, H0) differed significantly from the respective original soil in proportions of 1,019 OTUs in soil L, and in proportions of 1,273 OTUs in soil H. Those samples differed
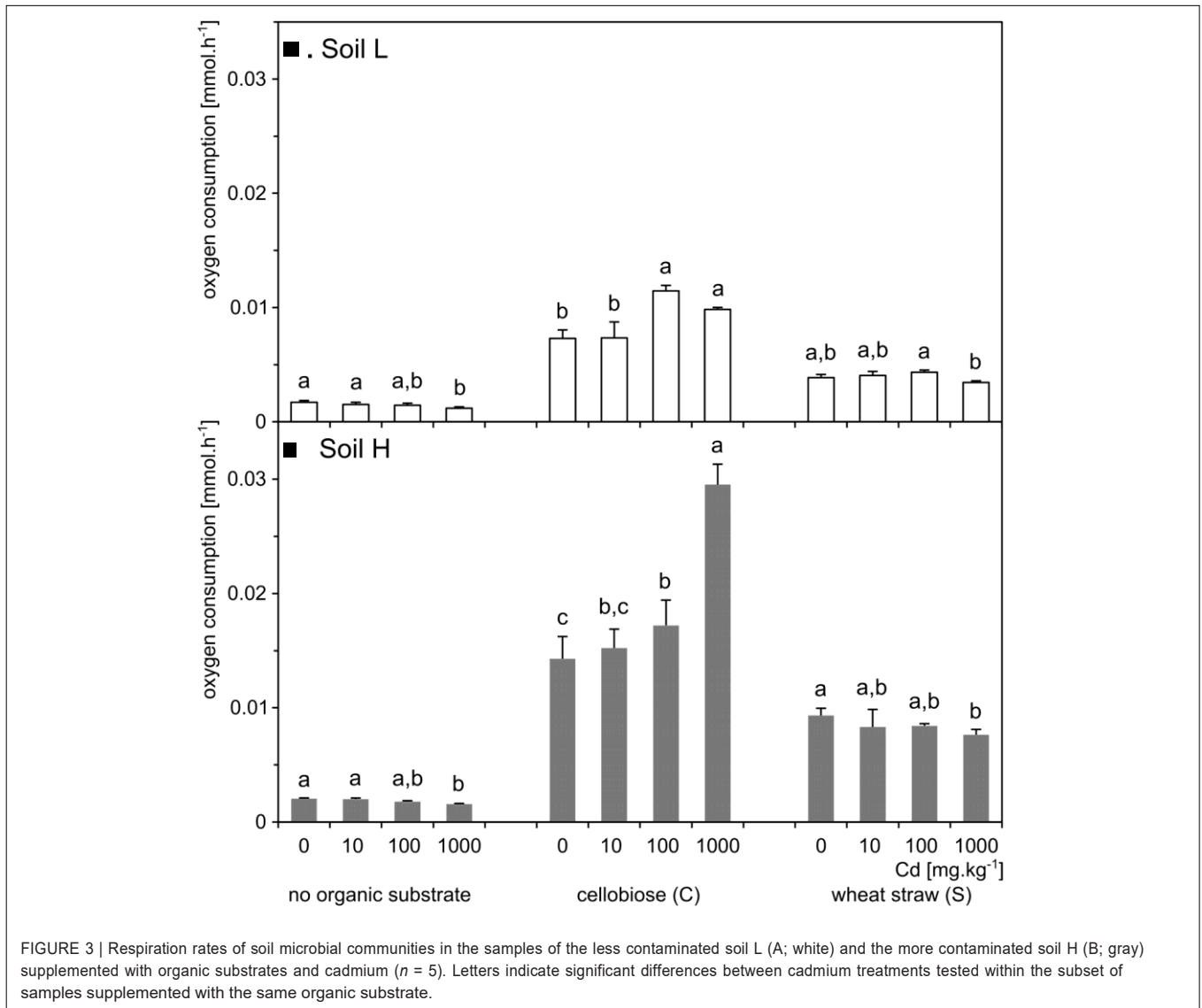
FIGURE 3 | Respiration rates of soil microbial communities in the samples of the less contaminated soil L (A; white) and the more contaminated soil H (B; gray) supplemented with organic substrates and cadmium (n = 5). Letters indicate significant differences between cadmium treatments tested within the subset of samples supplemented with the same organic substrate.

TABLE 3 | Comparison of cellobiohydrolase sequences between samples by Libshuff test.

	dCXYScore	Significance		dCXYScore	Significance
H0-L0	0.01233187	0.0002	HS100-LS100	0.00171228	0.2729
L0-H0	0.02299702	<0.0001	LS100-HS100	0.01491378	0.0002
L0-LS100	0.00139254	0.3119	H0-HS100	0.00121525	0.3241
LS100-L0	0.00141729	0.3753	HS100-H0	0.00248535	0.0885

Site H: H0 – (no Cd, no substrate), HS100 – 100 mg/kg Cd, straw; site L: L0 – (no Cd, no substrate) LS100 – 100 mg/kg Cd, straw.

between the two soils by 815 OTUs (**Figure 6**). The numbers of OTUs reacting significantly to carbon substrates were 1114 (41 reacting to both of them) in L soil, and 1043 (250 reacting to both) in soil H (Metastats, $p < 0.05$; **Figure 6**). More specifically in treatments with cellobiose, relative proportion of OTUs did not change between treatments without cadmium and with cadmium 100 mg.kg^{-1} and 1,000 mg.kg^{-1} in L soil, while in H soil the relative proportions of respective OTUs decreased (**Supplementary Figure S1**).

The rarefaction curves showed that bacterial diversity was similar in H and L soils for both the original soils exposed to long-term contamination and samples from the experiment without cadmium or organic substrate. Generally in L soil, the diversity did not reflect the treatments, while in H soil the cellobiose treated samples had the lowest diversity. Yet, diversity was mostly higher in L soil than in H soil after all treatments (**Supplementary Figure S2**). The effect of cadmium on diversity was not significant but both carbon substrates affected diversity in
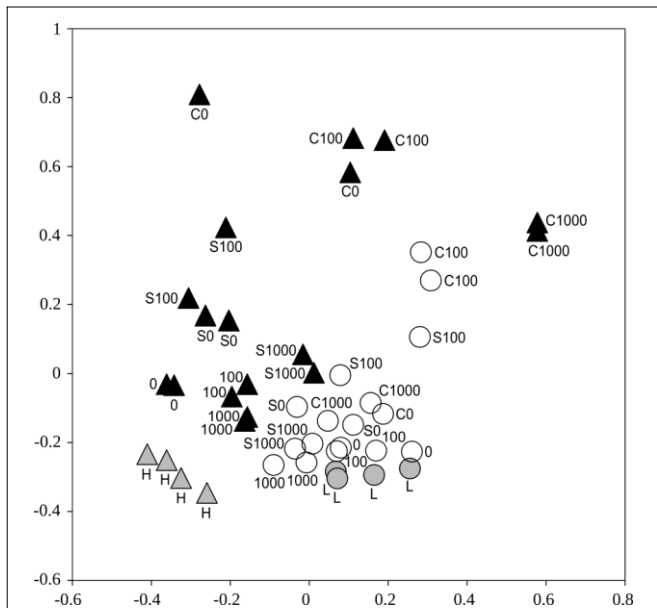
FIGURE 4 | An ordination showing distances between the bacterial communities represented by 16S rRNA gene amplicon sequence libraries from samples of the less contaminated soil L (circles) and the more contaminated soil H (triangles) after treatments with cellobiose (C), wheat straw (S), and cadmium 0–1,000 mg kg^{-1}. White and black symbols show soils after incubation in the respiration experiment (n = 2), gray symbols show the original soils (n = 4). Non-metric multidimensional scaling based on a matrix of Yue-Clayton theta distances.
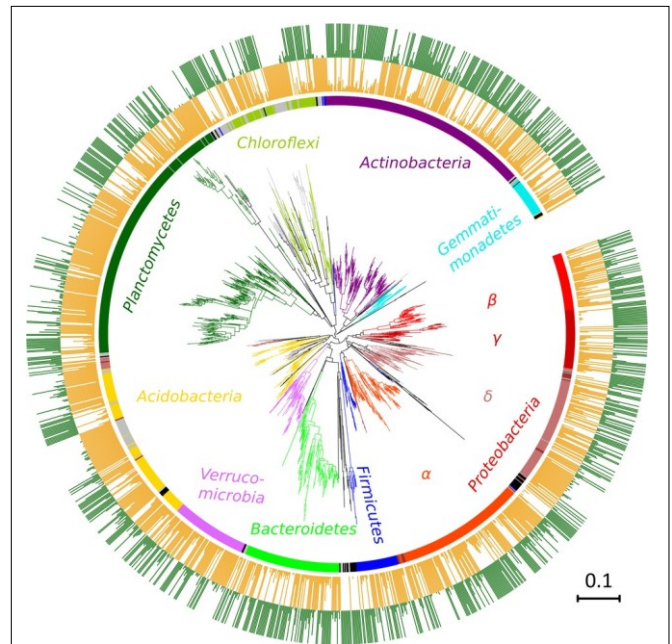

FIGURE 5 | Phylogenetic classification of OTUs differing between the two original soils, the less contaminated soil L (orange) and the more contaminated soil H (green). Colors of the branches and inner circle indicate assignment to phyla, gray depicts unclassified OTUs, and black OTUs belonging to other less frequent phyla. Lengths of bars in the outer two rings show proportions of each OTU relative to its higher value in one of the two compared soils. A maximum-likelihood method from representative sequences of a subset of 1,594 OTUs.

both soils (AMOVA, L: p = 0.009, H: p < 0.001). However, in H soil the pairwise difference occurred between all treatments, while in L soil it was significant only between the control and addition of cellobiose.

The changes in bacterial communities after treatments occurred even on the high phylogenetic level of phyla (**Figure 2**). In particular, the relative proportion of *Firmicutes* increased in H soil after addition of cellobiose but not in L soil. On the level of *Firmicutes* families, in H soil *Clostridiaceae* dominated after addition of cellobiose in treatments without cadmium and with 100 mg.kg^{-1} of cadmium but *Planococcaceae* dominated in treatments with 1,000 mg.kg^{-1} of cadmium. in *Actinobacteria*, relative proportion of *Pseudonocardiaceae* is higher in the original soil H, while proportion of Trebon clade members (defined in Kopecky et al., 2011) is higher in the original soil L. Family of *Micrococcaceae* increased after addition of carbon substrate in both soils but in L soil decreased with additions of cadmium, while in H soil further increased even in highest cadmium dosage. In *Proteobacteria*, a similar situation occurred with *Enterobacteriaceae* and *Aeromonadaceae*, which increased by addition of cellobiose in H soil and *Pseudomonadaceae*, which also increased after addition of both carbon substrates but mostly in H soil. In H soil changes were observed also in decrease of relative abundance of *Verrucomicrobia* and increase of relative abundance of *Bacteroidetes*, of which particularly *Flavobacteria* increased in H soil after cellobiose additions (**Supplementary Figures S3–S8**).
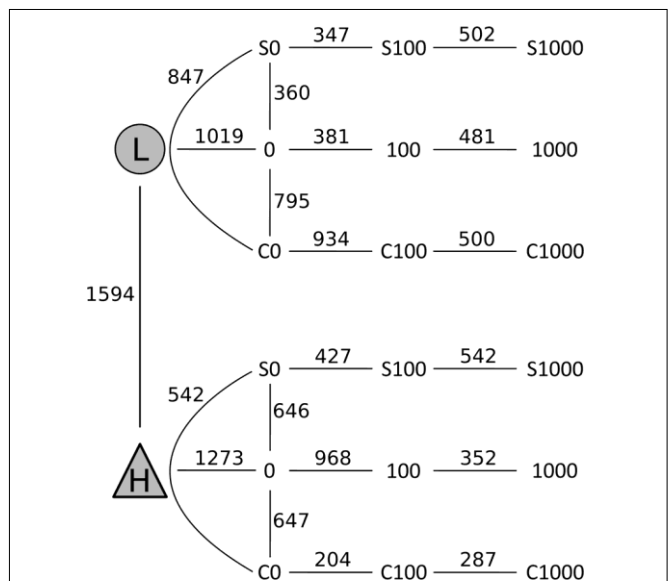

FIGURE 6 | Changes in composition of bacterial communities from samples of the less contaminated soil L (circles) and the more contaminated soil H (triangles) after treatments with cellobiose (C), wheat straw (S), and cadmium 0–1,000 mg kg^{-1}. Numbers on the lines connecting two treatments indicate the number of OTUs that significantly differed (p < 0.05) in pairwise comparisons using Metastats analysis.

DISCUSSION

The differences in bacterial diversity between the two soils occurred after addition of organic substrate and cadmium because the number of OTUs decreased in H soil and increased in L soil after treatments. In a similar experiment, microbial communities of soils differing in contamination remained similar after exposure to Cu and manure amendments (Brandt et al., 2010), while in another experiment changes in microbial communities after additions of different pollutants corresponded to the contamination history of the studied soils (Azarbad et al., 2015, 2016). Increased diversity in contaminated soils had been explained either by effects of toxic elements on diversification of the microbial populations due to resistance spreading or development of new chemical gradients, to which the microorganisms adapt (Ciarkowska et al., 2014; Yin et al., 2015). However, no clear explanation on why particular taxa respond to disturbance differently can be made as it is still only poorly understood how soil structure creates habitats and niches and how it regulates interactions (Schimel and Schaeffer, 2012).

More specifically in our study, significant changes in bacterial community structure occurred in cellobiose treatment. There, the phylum *Firmicutes* increased its proportion and became visibly sensitive to cadmium by changing the representation of *Clostridiaceae* family and a group of unclassified *Bacillales*. This may be related to both high growth rate with this carbon source and metal resistance specific to only some families within the phylum. Similar results showing higher resistance to toxic elements in *Firmicutes* were found in sites contaminated by Pb, Cu, and Zn (Ellis et al., 2003). *Actinobacteria* seemed relatively sensitive to cadmium in treatments with cellobiose because their numbers gradually decreased. High sensitivity of actinobacteria to toxic elements namely to Pb, Zn, Cr or Mn was observed previously (Yin et al., 2015) but opposite results were obtained by Li et al. (2015). A more detailed analysis showed that *Thermoleophilia*, *Propionibacteriales,* and *Pseudonocardiales* were rather tolerant to Cd, Pb, and Zn, while *Acidimicrobiales*, *Solirubrobacterales,* and *Frankiales* were not (Epelde et al., 2015). In our study, there was a strong increase of *Micrococcaceae* after organic substrate treatments with moderate tolerance to cadmium in H soil but not in L soil. *Proteobacteria* families *Pseudomonadaceae*, *Enterobacteriaceae* and *Aeromonadaceae* increased with organic substrate and seemed resistant to medium cadmium dosage. Yet, *Proteobacteria* seemed relatively sensitive to toxic elements stress in other studies (Li et al., 2015; Yin et al., 2015). *Bacteroidetes* increased after additions of both organic substrates and namely *Flavobacteria* increased after addition of cellobiose, which corresponds to their known decomposing abilities (Grondin et al., 2017). Three phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria* have been showed to possess both antibiotic and metal resistances (Li et al., 2017) so, we concluded that some taxa within those phyla might have benefited from this trait also at our site. It was shown previously that differences in resistance to metal contamination between various taxa may be related to changes of community permissiveness to plasmids carrying respective resistance genes. That occurs because although the bacterial communities possess required plasmids, there are differences in taxa, which carry particular resistances and above that type of disturbance and dose of contaminant strongly affected plasmid movement (Klümper et al., 2017). Finally, the variability in sequences of genes coding for cellobiohydrolase was specific for each of the two original soils and in the experiment it remained unchanged. Consequently it seems that cellobiohydrolase gene sequences are locally selected and decomposition of cellulose is carried out by the same taxa under treatments as in the original soils.

We demonstrated that differences between the two soils were observed in relative proportions of phyla but also in various lower taxonomic groups after both the long-term soil contamination and the short term treatments. That is supported by findings of Stefanowicz et al. (2012), who showed that community structure is a more sensitive determinant of disturbance than measurements of common respiration, biomass or catabolic abilities. It also agrees with several studies, which suggested that microbial communities respond to soil carbon with respect to their life history patterns that are deeply rooted in microbial phylogeny, i.e., that the functional groups appear at the level of families or phyla rather than species or genera (Allison and Martiny, 2008; Philippot et al., 2010; Schimel and Schaeffer, 2012; Shade et al., 2012). Consequently, we suggest that bacterial community composition reflects changes in ecosystem processes and may be used for bioindication.

The two soils had more than two centuries to gradually adapt to toxic elements pollution, so it was expected that their reaction to the additions of cadmium, cellobiose, and straw may be similar because of functional redundancy occurring in bacterial communities. However, higher respiration of the more contaminated soil H than less contaminated soil L after all treatments was in agreement with our alternative hypothesis of a stronger response of less healthy soils to secondary disturbance. Yet, that result disagreed with observations by other authors, who found decrease of respiration with increased primary contamination after secondary additions of pollutants and/or organic substrate (Brandt et al., 2010; Singh et al., 2014; Azarbad et al., 2015). In those studies, decrease in respiration was usually explained by decrease in microbial biomass and/or metabolic activities (Azarbad et al., 2013). That partially complies with our results, in which increase of respiration was paralleled with increase of bacteria numbers but mostly only after the treatment with cellobiose. However, some studies similarly as ours showed increased respiration after disturbance and reasoned that microorganisms in less polluted soils used a higher percentage of consumed carbon for assimilation and thus a smaller percentage was released as CO_2 in dissimilation processes (Zhang et al., 2016). We similarly as Niemeyer et al. (2012) suggest that the difference in reactions of respiration to disturbance may be explained by changes in microbial community structure in combination with growth rate and resistance mechanisms specific for particular taxa after substrate additions. In our study, this explanation can be applied particularly to the response of actinobacteria, whose numbers were higher in the more contaminated soil H and also increased after all treatments. The observed adaptation of actinobacteria to toxic elements can be attributed to a relationship between toxic elements and

antibiotics resistance. Actinobacteria are prominent producers of antibiotics, so they carry a wide range of resistances and it was previously demonstrated that those resistances are coupled or transferred on the same mobile genetic elements with heavy metal resistances (Seiler and Berendonk, 2012; Di Cesare et al., 2016). Besides the higher overall respiration observed in H soil, numbers of total bacteria and actinobacteria increased there after additions of both cadmium and substrate, while in the L numbers of bacteria increased slightly but numbers of actinobacteria remained the same. That again points to actinobacteria as a group with specific relationship to contamination in our soils.

CONCLUSION

We found that the more contaminated soil was more sensitive to additional disturbance than the less contaminated soil. This applied for both response amplitude of respiration and changes in bacterial community, which further indicated differences in resilience and resistance in the two soils. More specifically, respiration in low contaminated soil was more resistant to disturbance. This was attributed to growth rates and resistance to toxic elements of particular bacterial taxa. That means, respiration can be used in determining resistance or resilience in soil health assessment but only after secondary disturbance because its amplitude changed only after treatments with organic substrates. That conclusion agrees with the previous suggestions by other authors. In the contrary, bacterial community and specifically *Actinobacteria* responded to toxic elements contamination by both their quantity and community composition in both long term contamination and short term experiment. So, we propose that this phylum has a potential for bioindication of soil health. Finally, the observed changes in the contaminated soil after over century long exposure to toxic elements contamination showed that even after a very long time soil contamination significantly affects both composition of bacterial community and respiration. Therefore, some of the replaced bacterial groups were not functionally redundant in the soil because changes in bacterial community resulted in changes of functioning. That has further consequences not only on local decomposition of organic matter but more importantly on carbon cycling.

AUTHOR CONTRIBUTIONS

MS-M designed the DNA based experiments and suggesting the methodological approach. JK conducted the bioinformatic analyses. PB designed the respiration experiments. MV and YS did the toxic element analyses. TV did the field sampling. PM did the qPCR and prepared Illumina sequencing. DR did the DNA extraction. MG designed the primers for cellobiohydrolase. MO conducted statistical analyses.

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Trebonia kvetii gen. nov., sp. nov., an acidophilic actinobacterium, and proposal of the new actinobacterial family *Treboniaceae* fam. nov.

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Abstract

A novel actinobacterial strain, designated 15TR583[T], was isolated from a waterlogged acidic soil collected near the town of Trebon, Czech Republic, and was subjected to a polyphasic taxonomic characterization. Phylogenetic analysis based on 16S rRNA gene and whole-genome sequences revealed that the organism forms an individual line of descent related to the order *Streptosporangiales*, class *Actinomycetia*. The strain shared highest 16S rRNA gene sequence similarity, yet of only 92.8%, with *Actinocorallia aurea* IFO 14752[T]. The strain grew in white colonies of aerobic, Gram-stain-positive, unbranching substrate mycelium bearing single spores at hyphae tips. The major fatty acids (>10%) were iso-$C_{16:0}$, $C_{16:0}$, iso-$C_{17:1}\omega 9$ and 10-methyl-$C_{17:0}$. The fatty acid pattern differed from all patterns currently described for actinobacterial genera. The organism contained as major menaquinones MK9(H_6) and MK9(H_8), which differentiated it from other actinobacterial families. Polar lipids were composed of six unidentified glycolipids, an unidentified phosphoglycolipid, two unidentified phospholipids and two unidentified aminolipids. Whole-cell sugars contained galactose, xylose and arabinose as major components. The peptidoglycan type was A1γ *meso*-diaminopimelic acid. The genomic DNA G+C content was 69.7 mol%. The distinct phylogenetic position and unusual combination of chemotaxonomic characteristics justify the proposal of *Trebonia* gen. nov., with the type species *Trebonia kvetii* sp. nov. (type strain 15TR583[T]=CCM 8942[T]=DSM 109105[T]), within *Treboniaceae* fam. nov.

INTRODUCTION

The phylum *Actinobacteria* includes six classes: *Acidimicrobiia, Actinomycetia, Coriobacteriia, Nitriliruptoria, Rubrobacteria* and *Thermoleophilia*. The largest class *Actinomycetia,* formerly class *Actinobacteria* [1], comprises 34 orders with 60 families, while the other five classes contain only 20 families [2].

A novel actinobacterial taxon, named the Trebon Clade (TC), dominated a clone library of a waterlogged acidic soil [3]. According to Kopecky *et al*. [3], the TC forms a separate group at the level of a putative new order within the class *Actinomycetia*, sharing a common ancestral node with

Streptosporangiales, formerly suborder *Streptosporangineae* [4, 5]. Several sequences of the TC were previously found at different environmental sites characterized by acidic conditions [6–11], suggesting a special adaptation of this group to life at low soil pH. Also, four related strains were previously isolated and classified as belonging to a new putative family 'Ellin5129 group' within the class *Actinomycetia* without detailed characterization [12]. One isolate of the TC was suggested to belong to a new order or family within the order *Streptosporangiales* [13], which at present contains three families, namely *Nocardiopsaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* [14]. Another member of the TC was isolated from acidic lignite mine lake sediment and described

as *Thermomonosporaceae*-related without further description [15].

Novel taxa within the class *Actinomycetia* from unusual environments, such as highly acidic soil, may represent promising sources of novel metabolites and may have important ecological functions [16]. In the present study, a strain designated 15TR583[T] was isolated from a waterlogged acidic soil and described to belong to the TC. It appears to represent a novel family, genus and species, sharing the ancestral node with the order *Streptosporangiales*.

ISOLATION AND ECOLOGY

As described previously, the incidence of TC members is high in environments with low pH, such as waterlogged acid soils [3], pine forest soils [7], acidic mine lake sediments [15], paddy fields [10] and extremely acidic rivers [9]. The TC bacteria may thus possess enzymatic activities adapted to acidic conditions and therefore may be valuable for various biotechnological processes.

For bacterial isolation, a lower Go soil horizon was collected in January 2015. The sampling site was situated in the south of the Czech Republic near the town of Trebon (48° 58′ 27.2″ N 14° 46′ 11.5″ E) in a deciduous forest dominated by *Populus tremula*. The soil is a gleysol [17] characterized by iron reduction due to waterlogging. The soil profile consists of two horizons: (1) Ah, a 10 cm thick upper horizon of loamy-sand with a moderate humus content; and (2) Go, between 15 and 50 cm, a sandy loam lower horizon with mottling. The water table in the subsoil is regulated by a nearby fishpond (area 1.6 km^2, average depth 2 m), fluctuating over a year and reaching up to about 10 cm below the soil surface, covering the lower Go horizon. Soil pH in the lower horizon varied between 3.1 and 3.5. The TC clones were dominant among *Actinobateria* in both soil horizons in winter and summer. Further description of the site may be found in Kopecky *et al.* [3].

Strain 15TR583[T] was isolated on VL55 medium [13] using a standard dilution plating method after 30 days of incubation at 25 °C. The isolate was cultured routinely at 28 °C on/in solid and liquid R2A medium [18] with pH adjusted to 5.5 (further referred to as R2A55).

16S rRNA PHYLOGENY

The genomic DNA of strain 15TR583[T] was extracted according to the protocol described by Hopwood [19], modified by the addition of achromopeptidase (250 U ml^{-1}) to the lysis buffer. The complete 16S rRNA gene sequence (1510 nt) was recovered from the whole genome sequence after assembly with Spades 3.13.0 [20] and annotation with RAST [21] as described in the following section. The sequence identity was confirmed based on the partial 16S rRNA gene sequence (1380 nt) determined from the respective gene amplified with primers 16Seu27f [22] and pH' [23]. The 16S rRNA gene sequence was deposited at GenBank under accession number MN209171.

The top EzTaxon (www.eztaxon.org) [24] and GenBank hits were represented by 50 environmental clones or phylotypes coming from uncultured specimens and five isolated strains without taxonomic affiliation. The 16S rRNA gene sequence of strain 15TR583[T] shared 97.7–97.9% similarity with those of actinobacterial strains Ellin5129 and Ellin334 [13] isolated during the study of previously unculturable bacteria [12], and 95.6–96.8% similarity with those of '*Actinomycetales* bacterium Os2-5' (GenBank accession no. KY908302), '*Thermomonosporaceae* bacterium YE4-D4-16-CH2' (GenBank accession no. FN870346) and '*Actinobacteria* bacterium SK-25' (GenBank accession no. LC338062), isolated from acidic environments. To our knowledge, no detailed characterization of those strains has appeared in accessible resources.

Among species with validly published names, the highest similarity values were found with some members of the family *Thermomonosporaceae* of the order *Streptosporangiales*, and these values were below 93% (the nearest is *Actinocorallia aurea* IFO 14752[T] at 92.8% similarity). Other members of *Thermomonosporaceae* showed lower similarities with strain 15TR583[T] than members of other orders. For example, *Frankia alni* ACN14A[T] of the order *Frankiales* shared 91.4% 16S rRNA gene sequence similarity with strain 15TR583[T], *Micromonospora viridifaciens* DSM43909[T] of the order *Micromonosporales* shared 90.8%, while *Actinomadura livida* IMSNU22191[T] of the family *Thermomonosporaceae* and *Nocardiopsis dassonvillei* DSM 43111[T] of the family *Nocardiopsaceae* of the order *Streptosporangiales* shared only 90 and 88.6% sequence similarity, respectively.

The 16S rRNA gene sequence of strain 15TR583[T] was aligned with those of type strains from neighboring actinobacterial lineages using sILVA Incremental Aligner v.1.6.0 [25]. The best-fit model of nucleotide substitution was selected using jModelTest v.2.1.7 [26, 27]. Based on the selection, a GTR model was used to infer phylogeny through maximum-likelihood analysis in FastTree 2.1.11 [28] with built-in branch support values. The phylograms were finalized using Figtree v.1.4.2 (http://tree.bio.ed.ac.uk/).

Strain 15TR583[T] showed a distinct phylogenetic position within the class *Actinomycetia*, sharing the ancestral node with the order *Streptosporangiales* (Fig. 1). Additional phylogenetic analyses performed with strain 15TR583[T] and with sequences of the five closest related isolates and 50 environmental clones found in the GenBank database confirmed the high divergence of the TC from the representatives of described actinobacterial taxa and revealed its separation to a distinct cluster (Fig. S1, available in the online version of this article).

Thus, the phylogenetic data indicate that strain 15TR583[T] is sufficiently divergent from known bacterial species to be described as representing a novel genus. In addition, those data support the hypothesis that strain 15TR583[T] along with closely related GenBank strains represent a member of a novel family within the class *Actinomycetia*.
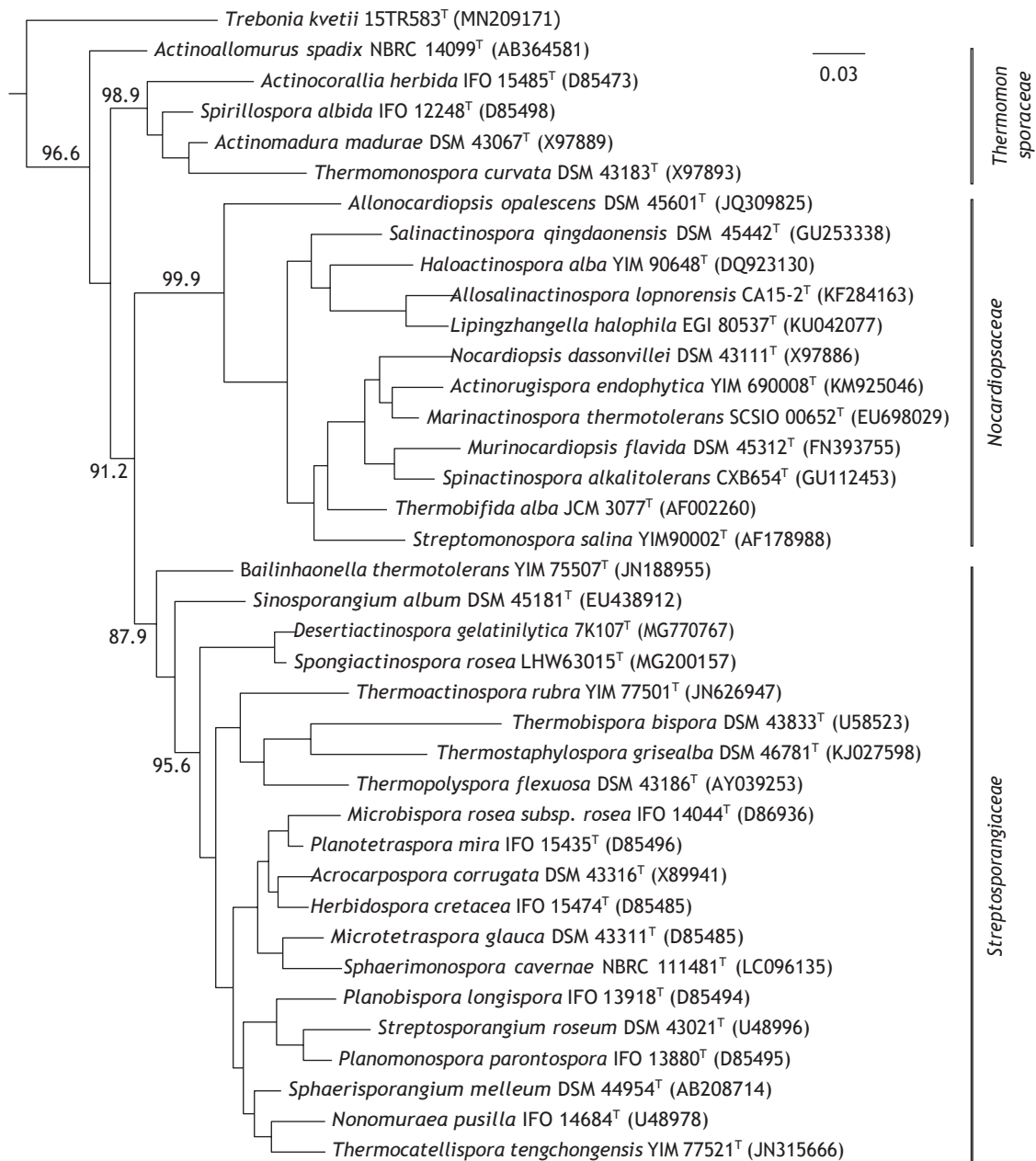
Fig. 1. Phylogenetic analysis of 16S rRNA gene sequences of strain 15TR583ᵀ with the type strains of type species of all genera within the order *Streptosporangiales*. The phylogram was inferred by maximum-likelihood analysis in FastTree [28] with the sequence of *Georgenia wutianyii* Z294ᵀ (MN020372) (not shown) as an outgroup, and the branch labels indicate the Shimodaira-Hasegawa-based 'local support' values from 1000 resamples [53]. Bar, 0.03 changes per position.

GENOME FEATURES

Next-generation sequencing data were generated via an MiSeq sequencer (Illumina) (paired-end library of 300 and 200 bp) and GridION X5 (Oxford Nanopore Technologies). The quality of Illumina reads was assessed using FastQC v0.11.7 [29], and the reads were trimmed in Cutadapt v2.4 [30]. The total number of paired-end reads was 4527308, providing 245-fold genome coverage. GridION sequences were processed by Dogfish (version 0.7.8, ONT) and base called using Guppy

(version 0.5.1, ONT). A total of 1396498 GridION reads amounted to a yield of 1.1 Gb with a mean read length of 830 bases. The whole genome was *de novo* assembled from both Illumina and GridION reads in SPAdes v3.11.0 [20]. The quality of assembly was assessed using QUAST 4.6.0 [31]. The assembly consisted of 93 contigs with a total of 9234338 bp, G+C content of 69.66%, largest contig of 1889528 bp, N50 value of 1103890 bp and L50 of 4. The obtained genome sequence of strain 15TR583ᵀ was deposited in GenBank with

the genome accession number RPFW00000000 (BioProject PRJNA497400). The genome was annotated using RAST [32] and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 4.7 [33], leading to a total of 8619 genes assigned as follows: 8545 protein coding sequences, 59 tRNAs, four rRNAs and three non-coding RNAs. Several genes for polysaccharide degradation were detected: two cellulases, one exocellobiohydrolase, eight xylanases, one chitinase and other genes for chitin utilization, and genes for lignin degradation. Among genes conferring resistance to antibiotics and toxic compounds were genes for Co, Zn, Cd, Cu, Hg, As and tetracycline resistance. Ten biosynthetic gene clusters (BGCs) for secondary metabolite production were identified with antiSMASH 5.1.2 [34]: two clusters for bacteriocin, two β-lactones, indole, NRPS-like, resorcinol, terpene, type I and type III polyketides.

The genome sequence data of strain 15TR583[T] and actinobacteria of the closest lineages according to 16S rRNA gene sequences were uploaded to the Type (Strain) Genome Server (TYGS), a free bioinformatics platform available under https://tygs.dsmz.de for a whole genome-based taxonomic analysis [35]. Briefly, the server used The Genome Blast Distance Phylogeny approach (GBDP) for the inference of phylogenetic trees from a given set of wholly (or even incompletely) sequenced genomes. First, the user genome was compared

against all type strain genomes available in the TYGS database via the MASH algorithm [36], a fast approximation of intergenomic relatedness, to determine the closest type genomes. Then, the genomes were locally pairwise aligned using the bLAST tool, which produced a set of high-scoring segment pairs (HSPs) (intergenomic matches). The total number of identical base pairs in HSPs was transformed into a single genome-to-genome distance value by use of a specific distance formula. A balanced minimum-evolution phylogenetic tree was then inferred from the distance matrices using FASTME 2.1.4 [37]. Branch support was inferred from 100 pseudo-bootstrap [38] replicates. The tree was rooted at the midpoint.

The resulting genome-based tree confirmed the unique position of strain 15TR583[T] by its clear differentiation from the order *Streptosporangiales* (Fig. 2).

Additional genome-based phylogenetic analysis was performed in Kbase using the Insert Genome Into Species Tree v2.2.0 tool [39]. First, the assembled genome was annotated with RAST [32]. The procedure continued by selecting a subset of 50 public KBase genomes closely related to the strain genome. Relatedness was determined by alignment similarity to a subset of 49 COG (Clusters of Orthologous Groups) domains of core universal genes. Next, the strain genome was

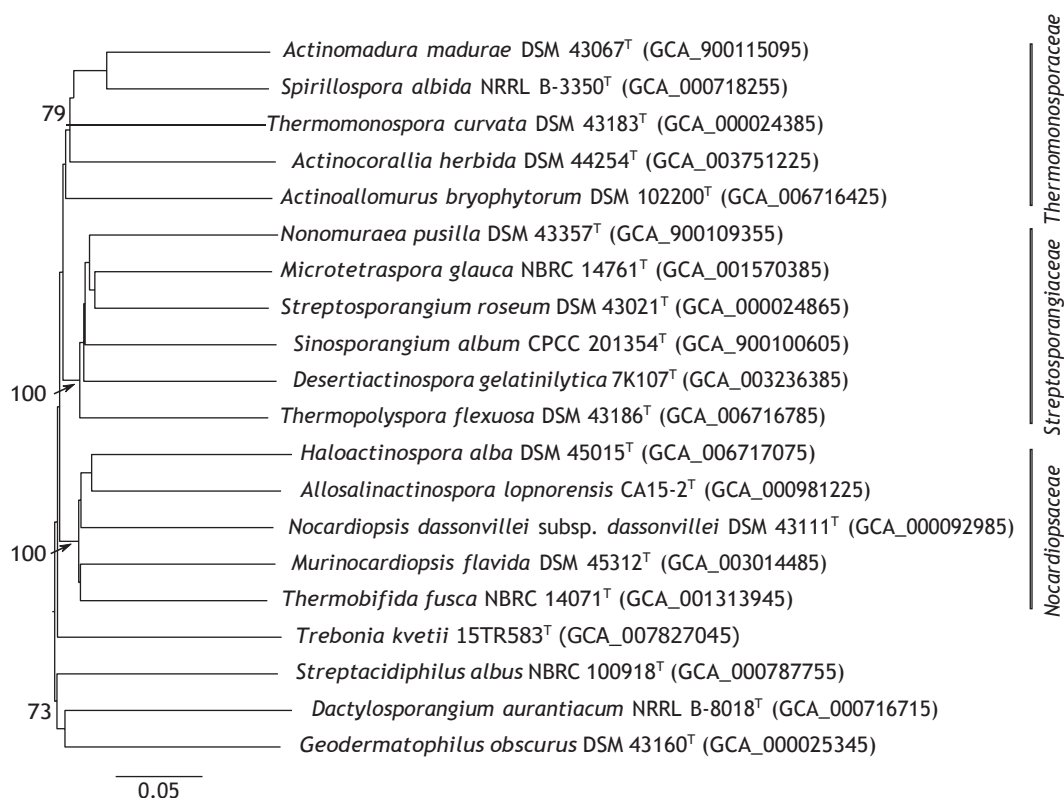Fig. 2. Genome-based phylogenetic analysis of strain 15TR583[T] and selected strains of three families of the order *Streptosporangiales* and three representatives of other actinobacterial orders. The phylogram was inferred using the Type (Strain) Genome Server (TYGS) [35] with branch labels indicating GBDP pseudo-bootstrap support values from 100 replications [38]. Bar, 0.05 changes per position.
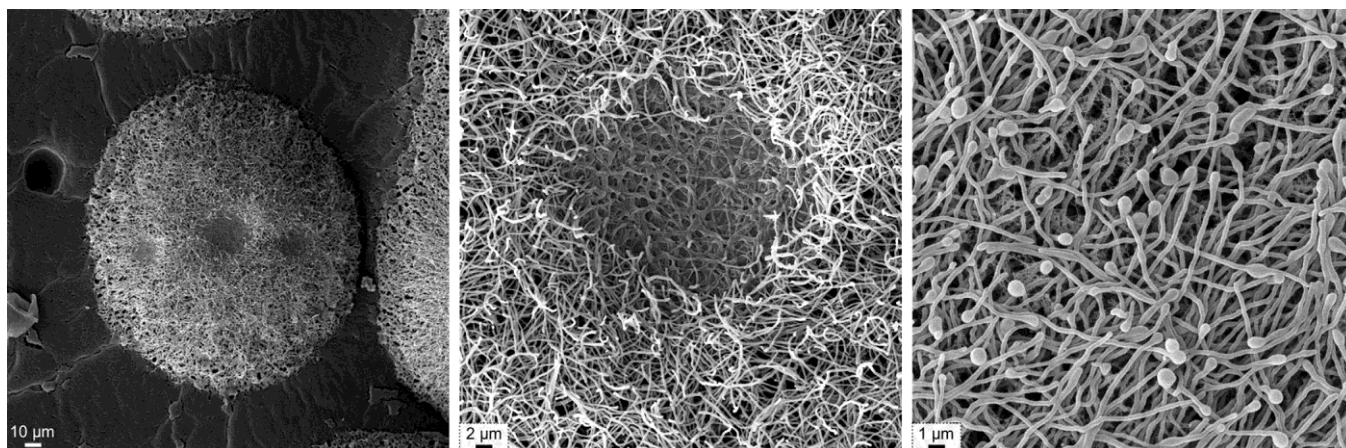
Fig. 3. Scanning electron micrographs of a strain 15TR583ᵀ colony grown on solid R2A55 medium. Bars, 10, 2, and 1 µm (left to right). The colony was cut and fixed with 3% glutaraldehyde. Subsequently, samples were dehydrated (ethanol, 30, 50, 70, 80, 90, 100%) and critical point dried (Baltec CPD-030). The samples were coated with gold using an SCD 040 Balzers gold sputter coater, and directly imaged using a TESCAN VEGA TS 5136 XM.

inserted into a curated multiple sequence alignment (MSA) for each COG family, concatenated, and a phylogenetic tree was reconstructed using maximum-likelihood by FastTree2 [40]. The resulting tree (Fig. S2) supported the unique position of the isolate compared to the other families of the order *Streptosporangiales*.

PHYSIOLOGY AND CHEMOTAXONOMY

Strain 15TR583ᵀ was an aerobic, Gram-stain-positive filamentous organism with non-branching substrate hyphae without aerial mycelium. The hyphae bore single spores at the tips (Fig. 3). The Gram-staining result was confirmed by the KOH lysis test [41]. Strain 15TR583ᵀ produced rough and highly adherent white colonies on R2A55 and VL55 agar media. Growth on several media such as inorganic salt-starch agar (Gauze 1) [42], plate count agar (PCA; Oxoid), tryptone soya agar (TSA; Oxoid), nutrient agar CM03 (Oxoid) and brain heart infusion agar (BHI; Oxoid) at 25 °C and pH 5 was evaluated. Growth at different temperatures (1, 5, 10, 15, 20, 25, 30 and 35 °C) and tolerance to various NaCl concentrations (0.5, 1, 2, and 3%, w/v) was determined on R2A55 agar plates for up to 3 weeks. The pH range for growth was tested on R2A agar plates adjusted to pH 2.0–8.0 using citrate buffer for pH 2–6, MES buffer for pH 5–7 and HEPES buffer for pH 7–8 at intervals of 1 pH unit (0.5 pH units between 5 and 6) for 3 weeks at 28 °C. Basic phenotyping was performed using conventional tube and plate tests relevant for Gram-positive rods as described previously [43, 44]. The activities of amylase and protease were tested on R2A55 agar plates supplemented with corresponding substrates [45]. Those key tests were inoculated with cells grown at 25 °C for 3 weeks on R2A55 agar medium. The utilization of different carbohydrate and nitrogen sources was studied using the GEN III MicroPlate test panel (Biolog) according to the manufacturer's instructions with minor modification of cultivation temperature

(25 °C) as well as reading time (20 days) due to the growth requirements of strain 15Tr583ᵀ. Additional biotyping using the API ZYM enzymatic test kit (bioMérieux) according to the manufacturer's instructions enabled a comprehensive characterization of isolate 15TR583ᵀ. The inoculated kit was incubated at 25 °C and the results were read after 48 h.

The complete morphological and biochemical/physiological characterization of 15TR583ᵀ strain is summarized in the species description below.

Analysis of respiratory quinones, polar lipids, whole-cell sugars and peptidoglycan structure was carried out by the Identification Service of the DSMZ. Cell biomass was obtained from a 4 week culture grown in liquid R2A55 medium.

Peptidoglycan analyses were performed by the DSMZ Identification Service according to Schumann [46]. Whole-cell sugars were assessed in 15 mg lyophilized cells after hydrolysis (0.5 M H_2SO_4, 100 °C, 2 h) and determined by TLC on cellulose plates [47]. A two-stage method described by Tindall [48, 49] was used to analyse respiratory lipoquinones and polar lipids from freeze-dried cells. Polar lipids were separated by two-dimensional silica gel TLC. The first direction was developed in chloroform/methanol/water, and the second in chloroform/methanol/acetic acid/water. Total lipid material was detected using molybdatophosphoric acid and specific functional groups were detected using spray reagents specific for defined functional groups according to Tindall [50]: anisaldehyde-sulfuric acid (for sugars), ninhydrin (for lipids containing free amino groups), periodate-Schiff (for glycerol, inositol and sugar-containing lipids), Dragendorff's reagent (for quaternary nitrogen compounds such as phosphatidylcholine) and potassium iodide with soluble starch (for secondary amines). Fatty acid methyl ester (FAME) analysis was performed using the Sherlock MIS (MIDI) system. Cells of strain 15TR583ᵀ were cultivated on R2A55 agar medium

for 4 weeks at 28 °C and subsequently incubated for 1 week at 25 °C. The complete results of the chemotaxonomic analysis are summarized in the genus description below.

The cellular fatty acid profile of strain 15TR583^T was composed of $C_{15:1}\omega6c$ (1.4%), iso-$C_{16:0}$ (33.4%), $C_{16:1}\omega7c$/$C_{16:1}\omega6c$ (4.9%), $C_{16:0}$ (15.0%), 10-methyl-$C_{16:0}$/iso-$C_{17:1}\omega9$ (20%), $C_{17:1}\omega8c$ (2.8%), $C_{17:0}$ (1.5%), 10-methyl-$C_{17:0}$ (15.6%) and 10-methyl-$C_{18:0}$ (TSBA) (1.4%) (fatty acids representing <1.0% of the total are not reported). According to Kroppenstedt [51], the fatty acid pattern of strain 15TR583^T was of complex type 3 combining both methyl-branched (type 1) and iso-/anteiso-branched (type 2) fatty acids. However, it cannot be assigned to the subtypes of the type 3 fatty acid patterns found in *Actinobacteria* [52]. The strain was distinguished by the presence of a high percentage (>15%) of methyl-branched fatty acids (10-methyl-$C_{16:0}$ and 10-methyl-$C_{17:0}$). It should be noted that as the strain is strictly acidophilic and slowly growing, it is not possible to provide standard conditions to compare fatty acid profiles with closely related actinobacterial type strains.

Strain 15TR583^T and members of the order *Streptosporangiales* shared a cell wall peptidoglycan of A1γ type, but strain 15TR583^T had a unique menaquinone, phospholipids (Fig. S3), whole-cell sugars and fatty acids pattern that has not previously been found for other actinobacterial genera and families [52]. The majority of members of the order *Streptosporangiales* are characterized by a lack of diagnostic sugars other than madurose, but strain 15TR583^T exhibited a broad range of the whole-organism sugars but not madurose. Similarly, as with *Thermomonosporaceae* and *Streptosporangiaceae*, strain 15TR583^T had major menaquinones with nine isoprene units, but they were saturated at different positions. The many environmental clones and isolates belonging to the TC originating from acidic environments may also serve as indirect proof of the uniqueness of the lineage. The comparison of chemotaxonomic and morphological features distinguishing strain 15TR583^T from the phylogenetically closest families of the order *Streptosporangiales* is shown in Table 1.

Table 1. Chemotaxonomic and morphological features distinguishing strain 15TR583^T from families of the order *Streptosporangiales* (data from the literature [14])

A. Chemotaxonomy				
Characteristic	***Streptosporangiales***			**15TR583^T**
	Thermomonosporaceae	***Streptosporangiaceae***	***Nocardiopsaceae***	
Peptidoglycan type*	A1γ *meso*-Dpm			
Major menaquinone(s)	MK-9(H$_6$)	MK-9(H$_2$), MK-9(H$_4$)	MK9, MK10, MK11	MK-9(H$_6$), MK-9(H$_8$)
Fatty acid pattern†	3a	3c or 3d	Diverse: 3d or 3e	iso-$C_{16:0}$ (33%), $C_{16:0}$ (15%), iso-$C_{17:1}\omega9$ (20%), and 10-methyl-$C_{17:0}$ (15%)‖
Phospholipid type‡	PI and PII	PIV	Diverse: PIII, PII	2AL, 6GL, 2PL, 1PGL¶
Whole-organism sugar type§	C/B	C/B	C/A	Galactose, arabinose, xylose‖
B. Cell morphology				
Substrate mycelium	Branched	Branched, with spores when aerial mycelium is absent	Absent/extensively branched, with single spores/clusters/ spore chains terminating into pseudosporangia	Non-branched with single terminal spores
Aerial mycelium	With single spores/short chains/spore vesicles with motile spores	Absent /spore /spore vesicles	Single spores on dichotomously branched sporangia/long spore chains	Absent
Spores	Motile/non-motile	Motile/non-motile	Non-motile	Non-motile
Additional characteristics	Some strains are thermophilic		Some strains are moderately or obligately halophilic	Obligately acidophilic

*According to Schumann [46].

†According to Kroppenstedt [51].

‡Phospholipid types according to Lechevalier [54, 55]. Characteristic phospholipids: I, nitrogenous phospholipids absent (phosphatidylglycerol variable); II, only phosphatidylethanolamine; III, phosphatidylcholine and characteristic phospholipid; IV, phospholipids containing glucosamine (with phosphatidylethanolamine variable).

§Whole-organism sugar patterns of actinomycetes containing *meso*-diaminopimelic acid: A, arabinose and galactose; B, madurose (3-*O*-methyl-d-galactose); C, no diagnostic sugars; D, arabinose and xylose [54-56].

‖The major components are presented; cannot be assigned to Kroppenstedt's classification.

¶Unidentified aminolipids (AL), glycolipids (GL), phospholipids (PL), phosphoglycolipids (PGL); cannot be assigned to Lechevalier's classification.

Based on its distinct phylogenetic and chemotaxonomic position, we propose 15TR583^T as the type strain of *Trebonia kvetii* gen. nov., sp. nov. Following the guidelines for the affiliation to higher hierarchical taxa in the class *Actinomycetia* based solely on phylogenetic relationships [51] together with specific chemotaxonomic traits, we propose the description of *Treboniaceae* fam. nov. to accommodate the proposed genus *Trebonia*.

DESCRIPTION OF *TREBONIA* GEN. NOV.

Trebonia (Tre.bo'ni.a. N.L. fem. n. *Trebonia* referring to the isolation site, a fishpond littoral area near Trebon in southern Bohemia, Czech Republic).

A Gram-stain-positive, acidophilic, aerobic organism forming non-branching hyphae. Aerial mycelium is absent. Single, smooth spores of 1 μm are formed on unbranched sporophores at tips of substrate hyphae. Motile elements are not produced. Colonies on R2A55 agar medium are cream-white, circular, with whole margin, convex, smooth, and 1–2 mm in diameter after 3 weeks of cultivation at 28 °C. The peptidoglycan of type A1γ *meso*-Dpm contains the amino acids alanine, glutamic acid, and *meso*-2,6 diaminopimelic acid (*meso*-Dpm). The whole-cell sugars contain galactose, xylose, mannose, glucose, arabinose, and traces of ribose and rhamnose. The major fatty acids (>10%) are iso-$C_{16:0}$, $C_{16:0}$, summed feature 9 ($C_{16:0}$ 10-methyl/iso-$C_{17:1}\omega9$) and 10-methyl-$C_{17:0}$. Contains major menaquinones MK9(H_6) and MK9(H_8), with minor menaquinones MK9(H_4), MK9(H_2) and MK10. Polar lipids are composed of six unidentified glycolipids, an unidentified phosphoglycolipid, two unidentified phospholipids and two unidentified aminolipids. The genome of the type strain is characterized by having a size of 9.25 Mbp and a G+C content of 69.7 mol%. The type species is *Trebonia kvetii*.

DESCRIPTION OF *TREBONIA KVETII* SP. NOV.

Trebonia kvetii (kve'ti.i. N.L. gen. n. *kvetii* of Kvet, referring to Dr Jan Kvet, a Czech botanist who has significantly contributed to the ecology of fishpond ecosystems by studies conducted in the area and recommended the site of collection for our study).

Chemotaxonomic and general characteristics are the same as given above for the genus. Acidophilic: grows well over a pH range from 4 to 6, optimally at pH 5.5; weak growth is observed also at pH 3, but no growth at pH 7. Aerobic, with growth occurring on R2A, VL and PCA agar media at pH 5; no growth was observed on Gauze 1, TSA, BHI or nutrient agar media with pH adjusted to 5 at 25 °C. Growth is observed between 20 and 35 °C, with optimum growth at 28 °C. Good growth on R2A medium is observed in the presence of up to 0.5 % (w/v) NaCl; weak growth occurs at 1%, but no growth with 2% NaCl. Soluble pigments are absent. An aerial mycelium is not observed. Able to utilize glucuronamide and inosine, but not acetic acid, formic acid, acetoacetic acid, *N*-acetyl neuraminic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine,

l-a lanine, γ-aminobutyric acid, D-arabitol, D-aspartic acid, L-aspartic acid, bromosuccinic acid, cellobiose, dextrin, α-D-lactose, D-fructose, D-fucose, L-fucose, D-galactonic acid lactone, D-galacturonic acid, gelatin, D-gluconic acid, D-glucose 6-phosphate, D-glucuronic acid, L-glutamic acid, glycerol, glycyl L-proline, L-histidine, *p*-hydroxy phenylacetic acid, α-hydroxybutyric acid, β-hydroxy-DL-butyric acid, α-ketobutyric acid, α-ketoglutaric acid, L-lactic acid, D-lactic acid methyl ester, D-malic acid, maltose, D-mannitol, melibiose, 3-methyl glucose, methyl pyruvate, methyl β-D-glucoside, mucic acid, *myo*-inositol, pectin, propionic acid, l-pyroglutamic acid, quinic acid, L-rhamnose, D-saccharic acid, D-salicin, D-serine, L-serine, D-sorbitol, stachyose, sucrose, trehalose or turanose. Borderline utilization of l-arginine, citric acid, D-fructose 6-phosphate, D-galactose, gentiobiose, α-D-glucose, L-malic acid, D-mannose and raffinose. Negative for hydrolysis of gelatine, starch, tyrosine and tween 80. Positive for hydrolysis of aesculin and ONPG. Positive for acid phosphatase, alkaline phosphatase (weak), catalase, esterase (C4), esterase lipase (C8), α-glucosidase (weak), β-glucosidase, leucine arylamidase, naphthol- AS-Bl-phosphohydrolase (weak) and valine arylamidase. Negative for arginine dihydrolase, cystine arylamidase, chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase, *N*-acetyl-β-glucosaminidase and trypsin.

The type strain is 15TR583^T (=CCM 8942^T=DSM 109105^T), which was isolated from waterlogged acidic soil near Trebon, Czech Republic. The genome of the type strain is characterized by having a size of 9.25 Mbp and a G+C content of 69.7 mol%. The GenBank accession number for the 16S rRNA gene sequence of 15TR583^T is MN209171. The GenBank accession number of the genome of strain 15TR583^T is RPFW00000000, BioProject PRJNA497400, BioSample SAMN10256292.

DESCRIPTION OF *TREBONIACEAE* FAM. NOV.

(Tre.bo.ni.a.ce'ae. N.L. fem. n. *Trebonia* type genus of the family; -*aceae* ending to denote a family; N.L. fem. pl. n. *Treboniaceae* the *Trebonia* family).

The description is the same as for the genus *Trebonia*. The family contains the type genus *Trebonia* gen. nov. A phylogenetic analysis is presented in the study.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Micronutrients and Soil Microorganisms in the Suppression of Potato Common Scab

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Abstract: Nature-friendly approaches for crop protection are sought after in the effort to reduce the use of agrochemicals. However, the transfer of scientific findings to agriculture practice is relatively slow because research results are sometimes contradictory or do not clearly lead to applicable approaches. Common scab of potatoes is a disease affecting potatoes worldwide, for which no definite treatment is available. That is due to many complex interactions affecting its incidence and severity. The review aims to determine options for the control of the disease using additions of micronutrients and modification of microbial communities. We propose three approaches for the improvement by (1) supplying soils with limiting nutrients, (2) supporting microbial communities with high mineral solubilization capabilities or (3) applying communities antagonistic to the pathogen. The procedures for the disease control may include fertilization with micronutrients and appropriate organic matter or inoculation with beneficial strains selected according to local environmental conditions. Further research is proposed to use metagenomics/metabolomics to identify key soil–plant–microbe interactions in comparisons of disease-suppressive and -conducive soils.

Keywords: micronutrients; mineral solubilization; inoculation; suppressive soils; antagonistic strains

1. Introduction

At present, both agriculture science and management seek to understand ecological processes which are relevant to the control of plant diseases [1,2]. New findings are expected to diminish the use of agrochemicals, which decrease the biodiversity of non-target organisms, including microbial communities in agroecosystems [3]. Proposed approaches focus on biological protection, mostly using microorganisms supporting plant growth and health [4]. However, more traditional approaches can also be used. One of the well-studied mechanisms of plant protection concerns providing plants with sufficient resources/nutrients such as nitrogen, phosphorus or potassium but also trace elements [5]. Many agricultural soils are deficient in one or more nutrients, and the production of crops depletes, particularly, micronutrients, because macronutrients are typically supplied in high amounts [6]. That leads not only to suboptimal plant growth but also to decreased plant immunity, which is influenced by their metabolic status [7,8]. Thus, composed plant nutrition is critical for disease control. That is because a delicate balance between the specific nutrient requirements of various potato cultivars and the soil chemical conditions need to be established [9–14].

Common scab (CS) is a disease potentially affected by plant mineral nutrients [15]. Previous studies associated nitrogen, phosphorus, potassium, calcium, magnesium, iron, zinc, manganese, copper and aluminum with CS severity or incidence by their content

either in soil or plant tissue [15–17]. The use of nutrients to control CS was investigated but the effects differed by location, cultivar and year [16–21].

The second studied mechanism of disease control involves plant–microbe–soil interactions, which include (1) antibiosis; (2) competition with pathogens, (3) induction of systemic defense response, (4) plant growth promotion and also (5) increased availability of nutrients, which all affect plant defense [22]. It was suggested that, particularly, the soil [23] and potato plant microbiomes [24,25] are important in controlling plant diseases.

Common scab of potatoes (CS) is a disease caused by pathogenic *Streptomyces* spp., which are distributed worldwide and, thus, adapted to various soil conditions [26]. In the past, the disease was treated by several pesticides, e.g., formaldehyde, urea formaldehyde, manganese sulphate, pentachloronitrobenzene and chloropicrin, whose use is now limited. In spite of increased research efforts, no treatments provide a reliable control of the disease across locations [26].

Most of the generally known microbial activities connected to plant disease control were also observed in CS-related interactions, particularly in suppressive soils [27–30]. Extensive research was conducted with supplementation of antagonistic strains to soil or improvement of the whole microbial communities using organic substrates reviewed in [31]. Above that, many cases of pathogen suppression by the microbial community were also related to the soil nutrient status [32,33]. Yet, no conclusive results useful for disease management were determined.

Consequently, in this review, we combine the chemical, physiological and microbiological research concerning CS to uncover the underlying processes which could be used for disease control. We focus on the effects of micronutrients because their sufficient amount is required for many physiological processes which support the defense against plant diseases. Additionally, micronutrient availability is relatively easy to manage [6]. We also recognize that microorganisms are integral components of soils, and therefore, their participation in potato plant nutrition and protection against CS needs to be considered in disease control. Finally, we recommend approaches for the most appropriate management strategies.

2. Individual Nutrients Affect CS Severity

Potatoes require optimal levels of essential nutrients throughout the growing season. Therefore, disease severity may be influenced by nutrient limitation, particularly at periods of fast growth [34]. Potato plants respond to the presence of the CS pathogen and nutrient conditions by accumulating various macro- and microelements. A significant correlation was shown between the degree of infestation by CS and the contents of Ca, Mn, K, P, Fe and Mg [15–17,35] in potato periderm or other parts of plants. The ability to accumulate different macro- and microelements also differs between cultivars and is further associated with resistance to CS [36]. However, it seems that the accumulation of different nutrients in potato periderm results from various processes, and thus, only some interactions are indicative of CS effects or can be used for its suppression.

2.1. Micronutrients

2.1.1. Calcium (Ca)

Ca relationships to CS are some of the most studied, in combination with soil pH. High total calcium levels in soil and also composed exchangeable Ca are often positively correlated with CS severity [17,27,28,37]. However, addition of Ca and K to acidic and neutral soils increased CS severity only in the neutral soil, showing that pH and Ca:K ratio are more important than the content of Ca in soil alone [38]. Similarly, the irregular relationship between soil pH, exchangeable Ca and CS development (e.g., [35]) was explained by interactions with other nutrients, including phosphorus, nitrogen and manganese [17,39] (Table 1).

Limitation of plant growth is relatively unlikely with Ca because it is a component of several primary and secondary minerals and is commonly present in ionic form (positively charged Ca^{2+}), which is considered biologically available. Limitations may occur when

it is adsorbed on soil colloidal complexes or due to human-induced acidification of soils, when Ca deficiency is caused by antagonistic plant uptake of metals such as aluminum, potassium and manganese [5,40].

However, limited Ca uptake influences the disease development because in plants, Ca affects the stability and function of membranes and cell wall structures [5,40,41]. Furthermore, Ca serves as a second messenger triggered by different environmental stimuli, including pathogens, so it is needed for plant defense [40]. In CS lesions, the Ca level is elevated [42] and it is even higher in dry soil conditions [43]. Thus, the high Ca level in the tuber periderm of diseased plants [36] shows an effect of infection rather than a cause [35]. However in healthy potatoes, a positive correlation between the Ca content and CS severity suggested that susceptible cultivars accumulate higher amounts of Ca [44].

Consequently, the total Ca content in the soil does not seem to be a good predictor of CS disease because it interacts with many other soil nutrients, pH and soil moisture and is affected by potato plant genetics. However, occasional liming of low-pH soils is recommended for prevention of Ca limitation, which also leads to increased disease severity. Above that, manipulation of soil Ca leads to fluctuations in soil pH, which may further improve CS prevention because different pathogenic streptomycetes are adapted to specific soil pH levels, and thus, its decrease or increase may suppress their populations [15,45]. In contrast, the selection of cultivars which accumulate lower amounts of Ca in the periderm may be a good strategy for CS control (Table 1).

2.1.2. Sulphur (S)

The application of elemental sulfur, calcium sulfate and ammonium sulfate reduced CS infection and severity [46,47], and above that, sulfate fertilizers enhanced biodiversity and antibiosis [46,48]. CS severity was also negatively correlated with soil S content [32]. Yet the effect of elemental sulfur and ammonium sulfate on the reduction in CS was not consistent [47].

Sulfur availability in soil depends highly on soil bacteria because more than 95% of total sulfur is bound to organic molecules in the form of sulfate esters or carbon-bonded sulfur (sulfonates or amino acid sulfur). Bacteria participate in both the formation of those compounds (sulfur immobilization) and sulfur release in the form of sulfate, which is available to plants [49]. Additionally, different microbial communities are involved in the consumption of various sulfur sources and supplying of sulfur to potato plants [50].

In the plant, organically bound sulfur in the form of various sulfur-containing metabolites is involved in cellular self-defense processes including detoxification of reactive oxygen species and other redox reactions, collectively termed sulfur-induced resistance or sulfur-enhanced defense [51]. Additionally, sulfur deficiency also has an indirect effect on plants as it reduces uptake of other elements such as P and K [40,52]. To improve CS control, it seems that the increase in decomposition processes by priming with additions of new organic matter may lead to the release of S from older soil organic matter and support microorganisms increasing S availability.

2.1.3. Magnesium (Mg)

In several studies, CS decreased and no pathogen (*Streptomyces scabiei*) was detected on potatoes grown in soils with high composed exchangeable cations including Mg [37]. Similarly, CS-suppressive soils were enriched in total Mg compared to CS-conducive soils [27]. A connection of Mg and CS suppression was also found in a resistant potato cultivar that also had a higher Mg content in the periderm compared to a susceptible cultivar [28]. However, in another study, the number of thaxtomin gene copies (*txtB*) representing the quantity of the pathogens in potato periderm was found positively correlated to periderm Mg content [32].

Mg availability in soil depends on soil weathering, moisture, pH and root–microbial activity, which are key factors determining the plant-available Mg pool [53]. Yet Mg is usually not limiting because it is present in various types of silicates and is relatively mobile

compared to other cations such as K, Ca and NH_4^+ [54]. However, similar to other cations, deficiency of Mg^{2+} in the plant may be induced not only by its low soil content but also by other cations that compete with Mg^{2+} for binding to negatively charged clay particles or root apoplast [55].

Magnesium is an important co-factor of more than 300 enzymes [53], including Ru-BisCO, a central part of a chlorophyll molecule [40], and also functions as a carrier of phosphorus in plants. Nevertheless, excess Mg^{2+} may also inhibit photosynthesis, particularly during dehydration [56] (Table 1).

Thus, for improvement of Mg-related limitation of plant growth and health, mostly a selection of cultivars with better Mg utilization may be recommended [44] or, in case of larger limitation, soil can be supplemented with dolomite, the most common Mg fertilizer.

2.1.4. Manganese (Mn)

High Mn content (Mehlich 3-extractable) was strongly correlated with low CS disease severity of soils in Canada [16]. Soil amendments which reduced Mn availability such as liming and nitrate fertilizers also increased the severity of CS [57]. Furthermore, direct Mn soil applications reduced the common scab of potato, especially when manganese sulfate was applied to Mn-deficient soils [58]. However, Barnes [59] found no effect on CS incidence when up to 125 kg/ha of $MnSO_4$ was applied on tubers or sprayed at tuber initiation (Table 1).

The availability of manganese depends on soil acidity. However, in soil, not only may limitation occur, but sometimes, a level toxic to plants was observed at some locations [57,60,61]. Yet more often, Mn is deficient, and that is connected with the increasing severity of various plant diseases.

In the plant, Mn serves as a co-factor of various enzymatic activities at low concentrations, while at high concentrations, it acts as their inhibitor [57]. Above that, Mn deficiency leads to an inhibition of cell elongation and decrease in tuber yield [61]. Thus, Mn limitation not only affects the overall growth of plants but also the thickness of the cell wall, which serves as protection for pathogen invasion [6]. Finally, Mn may affect the disease resistance of plants by controlling lignin and suberin biosynthesis, phenol biosynthesis and photosynthesis [5,41,62].

Limitation of Mn may not be related only to its availability in soil. Since Mn is required at much higher concentrations by higher plants compared to fungi or bacteria, some pathogens are known to exploit this difference in requirement [63]. For example, some plant pathogens, including *S. scabiei* oxidize Mn, making it unavailable for the plant host and, thus, increasing the plant stress [64].

Consequently, Mn limitation may strongly affect disease control and should be assessed when macronutrients are in balance. The form of supplementation might be evaluated based on soil pH and organic matter content because both inorganic and organic forms can be applied for the improvement of Mn availability. Some organic amendments such as dried grass meal may also increase the number of manganese-reducing microorganisms [65] which make manganese available to plants.

Table 1. Mineral nutrients availability in relation to common scab (CS) development.

Element	Factors of Availability to Plants	Function in Plant	Relation to Plant Defense/Pathogenesis in General	Possible Relation to CS	Main References
Cation Exchange Capacity (CEC)	Higher CEC in clay than sandy soils; low CEC and Ca especially correlated with low pH	A measure of soil capacity to hold nutrients (Ca, Mg, K, Na, Al and H)	Different effects of Ca, Mg, K and their ratio; imbalanced nutrients promote impaired biosynthesis and accumulation of low-molecular weight substances readily available for parasites	CS disease severity related to exchangeable Ca, Mg and K cations; CEC is lower in suppressive soil; the higher CEC, the greater uptake of Ca^{2+}; uptake of monovalent cations (K^+) increases at lower CEC	[5,37,60,66]
K	Leached out in acid soils; Al dominates the CEC, limits the soil's ability to absorb and hold K	Control of cation–anion homeostasis, membrane polarization, more than 60 enzymes in photosynthesis and transport of photosynthetic products to storage organs; starch synthesis; increased plant resistance to pests, diseases and abiotic stresses	Correlated with K:Mg, K:Ca and K:N ratios; decrease in some plant diseases if N and P are sufficient; high content of N increases plant susceptibility to diseases—this adverse effect can be neutralized by balanced N:K ratios of fertilizers	Special Ca:K ratio reduces scab severity and incidence, while the imbalance or excess of K or Ca promotes the disease	[38,40,64,66]
Ca	pH	Stability and function of membranes and cell walls; second messenger triggered by different stimuli including pathogens	At low Ca level, cells leak compounds used as food by parasites; supports some pathogens by stimulating the action of pectolytic enzymes dissolving plant cell wall; inhibits the activity of other pectolytic enzymes	Ca may simulate the aerial mycelium formation and spore germination of *S. scabiei*; high calcium levels in the absence of changes in pH induce scab	[35,40,41,66]
Mg	Mg deficiency can be induced by higher K supply	Allosteric activator of more than 300 enzymes; a central part of chlorophyll molecules; in the structural integrity of cell components	Decreases the susceptibility to pathogen-produced macerating enzymes as long as Ca level remains sufficient	Soils suppressive to CS disease had a higher content of Mg; the CS-resistant potato cultivar has a higher Mg content in periderm	[27,28,40,53]

Table 1. *Cont.*

Element	Factors of Availability to Plants	Function in Plant	Relation to Plant Defense/Pathogenesis in General	Possible Relation to CS	Main References
Mn	More available with lower pH; Mn uptake increased by seed inoculation with pseudomonads, organic amendments increased Mn-reducing microorganisms	Co-factor of enzymatic activity/an inhibitor at high concentrations; control of lignin and suberin biosynthesis, phenol biosynthesis, photosynthesis	Mn is required at much higher concentrations by higher plants than by fungi and bacteria	High Mn correlates with low CS; *S. scabiei* oxidize Mn making it unavailable for the plant; soil amendments reducing Mn availability (liming, nitrate fertilizers) increase CS severity; herbicide glyphosate, toxic to Mn reducing organisms	[55,57]
Fe	Predominant ferric ion (Fe^{+3}) is sparingly soluble; more available at lower pH as reduced form Fe^{+2}; bacteria supply iron to plants and backward	DNA synthesis, respiration, photosynthesis; in prosthetic groups of many enzymes (cytochromes); synthesis of chlorophyll; essential for maintenance of chloroplast structure and function	Plants, bacteria and fungi compete for Fe in the rhizosphere; microorganisms have lower Fe requirements than plants; promotes antifungals' production by soil bacteria for plant benefit; activates both enzymes involved in the infection and in plant defense	*S. scabiei* produces siderophores desferrioxamine, scabichelin and pyochelin to compete for iron; lower CS severity in soils with more available iron; higher Fe in the periderm of tubers grown in suppressive soil; enrichment with available iron and peat suppressed CS	[5,27,33,67–70]

2.1.5. Iron (Fe)

In CS-suppressive soils, potatoes were less affected due to the available iron [33]. Furthermore, the concentration of Fe in the periderm of potato tubers grown in CS-suppressive soils significantly increased during the maturation period, whereas in conductive soils, it slightly decreased [36]. Overall, although cultivars differ in Fe requirements [71], the Fe content in various cultivars was usually not related to the CS disease [44]. Finally, the enrichment of soil with soluble iron (directly or through a decrease in soil pH by peat amendments) suppressed the CS severity, although the abundance of thaxtomin biosynthetic gene copies (*txtB* genes) remained the same in the soil. This indicates that iron supports plant defense and reduces pathogen virulence rather than eliminating the pathogen population [27,33] (Table 1).

Although iron is the fourth most abundant element on Earth, it is not readily assimilated by either bacteria or plants in aerobic soils because its oxidized form, Fe^{3+}, is only sparingly soluble. Plant availability of Fe is also greatly reduced in calcareous soils (pH > 7) [7]. That is in contrast with requirements of high iron levels by both microorganisms and plants. It is particularly problematic in the rhizosphere, where plants, bacteria and fungi compete for it [69]. Plants have developed two strategies for iron uptake: (1) mostly dicots acidify the rhizosphere by the production of organic acids and phenolic compounds to activate ferric chelate reductase and Fe^{2+} transporters; while (2) monocots produce phytosiderophores and respective transporters [69]. Bacteria have developed similar strategies of producing organic acids and synthesizing low-molecular-mass siderophores (⁓400–1500 Da), molecules with a high affinity for Fe^{3+} as well as membrane receptors able to bind the Fe–siderophore complexes [72]. Many plant pathogens, including *S. scabiei*, use chelating compounds during iron uptake, and those can act as essential virulence determinants by limiting the plant's access to iron. *S. scabiei* produces three types of siderophores: desferrioxamine, scabichelin and pyochelin [73,74] (Table 1).

The effect of iron deficiency is critical for plants in metabolic processes such as DNA synthesis, respiration and photosynthesis because it serves as a co-factor of many enzymes, such as cytochromes of the electron transport chain. It is involved in the synthesis of chlorophyll and is essential for the maintenance of chloroplast structure and function [67]. Additionally, iron may participate directly in the activation of enzymes involved in infection as well as those involved in plant defense [75]. In addition, the availability of iron is important for the protective microbial community because it is required for the production of biocontrol metabolites, which may suppress various diseases [22].

The availability of iron seems to be the most overlooked factor in the protection of potato plants from different diseases, but particularly CS, because the pathogenic streptomycetes are well equipped for competition with the plants for iron supplies. The deficiency is likely in all alkaline soils but also in soils with low microbial activities. Thus, the availability of iron can be improved by supplementation with organic matter which increases microbial activities but also by fertilization by organically bound iron.

2.2. Macronutrients

Even though macronutrients are not in the main focus of CS disease control, nitrogen, phosphorus and potassium are often limiting factors and have also been demonstrated to affect CS severity, despite their regular amendments to arable soils. Similarly to micronutrients, particularly their balanced soil contents and availability are important because of many common interactions [5].

2.2.1. Nitrogen (N)

Most soils are poor in nitrogen, so it is generally supplied; yet its local recycling is often overlooked. The soil nitrogen pool is mostly replenished by ammonium released from dead biomass by microbial decomposers in natural conditions [76,77] and from the atmosphere by nitrogen fixation by symbiotic bacteria [78] or free-living bacteria and archaea [79]. The processes are attenuated by fertilizers containing large amounts of nitrogen. To

understand local nitrogen status, the proportion of ammonia-oxidizing bacteria to archaea in the rhizosphere represents a potential bioindicator because it correlates with soil health status [80]. In addition, nitrogen-rich rocks occurring at some locations should not be overlooked because they offer a potentially large pool of nitrogen, and thus, no fertilization is required [81].

In CS, some studies showed that adding N-rich soy meal, meat and bone meal to soil led to an increase in ammonia, nitrite, nitrate, pH and bacterial quantity and suppressed CS [82]. Furthermore, the temporary initial increase in soil pH to eight or higher by the addition of organic materials resulted in an increase in free ammonium levels that might be toxic to populations of *S. scabies* [83]. It was also shown that oligotrophic conditions of low soil C and N are associated with CS control, possibly because the low N content constrains the pathogens, favoring copiotrophic soil conditions [27].

The observed differences in nitrogen's impact on CS may be the result of a partially different utilization by potato cultivars [84]. A high content of N is known to increase the plant susceptibility to several diseases, but the adverse effect can be neutralized by balanced N:K ratios of fertilizers [66].

Thus, soil N content should be checked for balance with other nutrients, and fertilization by nitrogen should be evaluated carefully for CS control so as not to eliminate microbial activities. Furthermore, ammonium levels may be important indicators of microbial processes relevant to CS control.

2.2.2. Phosphorus (P)

The relationship of soil P to CS has been studied for a long time. Recently, a high soil phosphorus content was observed in CS-suppressive fields [27], and CS severity was negatively correlated with the total phosphorus content in both soil and potato periderm [32,44]. Similar to other nutrients, there is a combination of the nutrient's effects on the plant and the pathogen, which need to be disentangled to suggest an appropriate strategy for CS control.

Ecosystems begin their existence with a fixed amount of P, which cannot be readily replenished. Consequently, ecosystems with very old soils can become P-limited [2]. P in soil is subject to an extensive set of physico-chemical and biological reactions, while only a small part of total soil P is in a biologically available form [85]. In particular, in calcareous soils, precipitation of calcium phosphate is presumed to be a major factor in the loss of P availability over time [86]. As one of six macroelements, phosphorus is directly involved in all processes in the plant. Additionally, potatoes have a relatively high P requirement but are rather inefficient in soil P uptake [34], so deficiency is likely to occur.

In the plant, phosphorus deficiency results in a broad range of stress and adaptation responses [87], including changes in the root system morphology and increased expression of phosphoenolpyruvate carboxylase, resulting in root exudation of organic acids, which changes P equilibrium in the plant rhizosphere and may influence the development of specific microbial groups, including pathogens [88]. Similarly, phosphate-solubilizing microorganisms, e.g., the genera *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Acetobacter*, *Streptomyces* and *Nocardia*, release P from parent rocks and other sparingly soluble forms of soil P by secreting organic acids, and during the process, they decrease the particle size [66]. Yet, microbial communities differ by P release, possibly also due to a pressure by predators, which speeds up its recycling. This was suggested as a partial mechanism in some disease-suppressive soils [14].

However, it was observed that the content of available soil P is related to the amounts of carbon, lignin, cellulose, polyphenol and nitrogen. For this reason, their quantities may be used as predictors of the P release in various soils [86].

To assist the optimal P levels for CS disease control, solubilization activities by microorganisms can be supported by an increase in organic matter, particularly if it is slightly acidic. Selection of potato cultivars with higher capacity of P uptake is also recommended because potato cultivars vary in the efficiency of P utilization [44].

2.2.3. Potassium

Potassium has the highest concentrations in potato tubers out of all macronutrients [40, 62] but the impact of its content on CS varies greatly. Potassium concentration measured in the tuber periderm was not related to CS occurrence or severity in various potato cultivars [36]. Yet in other studies, CS severity positively correlated with the available soil K content [16], and CS was less severe in soils with a relatively high concentration of exchangeable Ca, Mg and K [37].

The sources of potassium are minerals—feldspars and micas—which release this element in the course of their weathering. Weathered potassium appears in solution as an exchangeable ion, K^+, which is adsorbed to or released from surfaces of clay particles or organic matter [89]. Therefore, the presence of K-rich minerals and soil clay content needs to be considered before its supplementation. Similar to other nutrients, K-solubilizing microorganisms assist its availability, so their enrichment may benefit plants in soils where K is present but not in forms available to plants [90].

Similar to other macronutrients, potassium in plants controls major pathways such as cation–anion homeostasis, membrane polarization and enzymatic activity [40]. K is also involved in more than 60 enzymes participating in photosynthesis, moving photosynthetic products to storage organs such as seeds and tubers [66] and starch synthesis [40]. K plays an important role in increasing plant resistance to pests, diseases and abiotic stresses [66]. Its deficiency leads to impaired synthesis of high-molecular-weight compounds (proteins, starch and cellulose), while low-molecular-weight organic compounds accumulate and are easily available to the invading plant pathogens [5].

Potassium's equilibrium with other macronutrients is particularly important for the prevention of CS. However, cultivars with a higher K uptake may be also selected to prevent K limitation because various cultivars differ strongly in K accumulation [44].

3. Organic Matter Modifies Microbial Communities and Increases Antibiosis

The application of organic amendments to soil was proposed as a strategy for the management of diseases caused by soil-borne pathogens [91]. Soil organic matter (SOM) supplementation can produce suppressive soils on which pathogens do not establish or persist [92,93]. Though many attempts have been made to suppress various plant diseases, they have generally been met with varied success [91,94].

Many studies showed also CS reductions with the addition of various organic materials, including compost [95], peat [33], green manure [92,96], several types of animal manure [83,97,98] and fish emulsion [21] (Table 2). Although the effect of organic amendments was found to be mostly beneficial, rarely were the causes for the observed effects on CS (intensification or suppression) resolved. It seems that the results depend on the organic matter quality but also on the period and frequency of application [98,99]. Therefore, a more precise understanding of organic matter supplementation is needed for successful CS control.

Currently known mechanisms of SOM impact on the development of plant diseases include (1) decrease in soil pH and increase in nutrient availability [100], (2) improvement of soil nutrient-holding capacity and stability [101] and (3) disease-suppressive activities of soil microbial communities [22]. Regarding CS specifically, direct suppression of the pathogen *S. scabies* by harmful organic compounds was achieved by fertilization with chestnut waste [102]. Finally, the connection between CS severity and SOM content may be explained by the preferential food hypothesis, according to which carbon substrate-deprived *Streptomyces* develop a pathogenic tendency, while their usual lifestyle is benign and saprophytic [103].

Table 2. Effects of organic matter amendments on incidence and severity of potato common scab.

Material	Quantity	Method	Effect	Reference
Compost	0.4 mt ha^{-1}		CS suppression 42%	[95]
Aerobic compost tea	140 L ha^{-1}	Back-pack sprayer	CS suppression 81%	
Indian mustard green manure		In the year before potatoes	CS suppression 25%	[96]
Barley/ryegrass rotations		Rotated prior to potatoes	CS suppression 13–34%	[104]
Lopsided oat (*Avena strigosa*)	150 kg ha^{1}	Rotovator prior to potatoes	CS suppression	[105]
Rice bran	3 t ha^{-1}	In the furrow with the seed tubers	CS suppression (DS)	[106]
Soybean green manure, with wheat straw		Incorporated before planting	CS suppression	[107]
Green manure (*Brassica napus*)		Grown for approx. 2 months in the fall	Decrease in CS incidence and severity	[92]
Compost	2.5 kg m^{-2}	5 days before planting	CS suppression: 61% to 28% DI, 30% to 16% DS [a]	[108]
Compost tea	1 kg m^{-2} in 5 L water	Soil drenching	CS suppression: 61% to 32% DI, 30% to 15% DS	
Fish emulsion	1%	Soil amendment	No effect	[21]
Processed swine manure	2 g per seed	Seed covering	Increased DI	[109]
Processed swine manure with chitosan	2 g per seed; 1% chitosan	Seed covering	no effect	
Chicken manure	66 t ha^{-1}	Incorporated to 15-cm depth	CS suppression	[83]
Swine manure	5.5 m^3 ha^{-1}		CS suppression	
Dairy cattle manure	100 t ha^{-1}		No effect	
Poultry manure	1.54–12.32 Mg C ha^{-1}	Applied dehydrated, pelletized prior to planting potatoes	CS suppression	[98]
Poultry manure, forestry residues and organic waste compost	45 Mg ha^{-1}	Incorporated to a depth of 15 cm in October, a year before planting	CS suppression	[97]
Meat and bone meal	37 t ha^{-1}	Incorporated to 15-cm depth	CS suppression	[16]
Soymeal	37 t ha^{-1}		CS suppression	
Poultry manure	66 t ha^{-1}		CS suppression	
Poultry manure	20 t ha^{-1}		No effect	
Nature Safe 10-2-8 (poultry feathers)	10 t ha^{-1}		CS suppression by 50–100%	
Ammonium lignosulfonate	1000 L ha^{-1}		Marketable yield 60–80% compared to 10% in the control	
Peat	2.5 L peat /10 L soil	In submerged pots	CS suppression	[33]

[a] DS—disease severity (percentage of surface covered with lesions); DI—disease incidence (proportion of infected tubers).

Green manures seem to particularly affect the availability of nutrients such as P, Mn and Zn, which are related to plant defense [91,110]. The application of peat or other low-N, low-pH SOM may also result in solubilization of nutrients, particularly Fe, but also other metals. The additions of low-pH SOM can be also followed by shifts of microbial community composition towards the increase in suppressive activities [33,91]. Green manures combined with appropriate crop rotation may also increase the protective streptomycetes soil sub-community [5,104,111].

Soil suppressiveness reinforced by activities of microbial communities was observed after addition of older compost, aerobic compost tea [95], decomposed wheat straw or soybeans [107], lopsided oat [105] or rice bran [106]. Those manipulations were later connected to increased lignin content, which can induce the production of secondary metabolites controlling the pathogen [112].

As efficient soil saprophytes, streptomycetes are especially likely to respond to the incorporation of organic material into the soil and are often implicated as microbial agents responsible for amendment-induced suppression [106,113]. This was demonstrated by organic amendments, which enriched, especially, some *Streptomyces* populations [113,114], but also with actinobacteria isolates derived from the rice bran-amended soil, which showed antagonistic activity against pathogenic *S. scabiei* and *S. turgidiscabies* [106] (Table 2). However, the use of antibiotic-resistant *S. scabies* mutants indicated that some streptomycetes strains can reduce pathogen populations even when they are not sensitive to antibiotic inhibition. It demonstrated that competition by non-pathogenic streptomycetes for space and resources rather than antibiotic activities efficiently eliminates *Streptomyces* pathogens by competitive exclusion. Thus, both antibiotic inhibition and competition between pathogenic and non-pathogenic *Streptomyces* are likely to be important mechanisms of natural suppression of potato scab [29].

In contrast, increased CS severity and/or incidence was observed after fertilization with fresh animal manure but also with compost [91,99]. In another study, CS-suppressive and -conducive soils were discriminated by specific low-molecular-weight organic compounds [27,115]. Finally, in the same study, the quantity of CS pathogens (thaxtomin biosynthetic gene *txtB* copies) was positively correlated with the soil total C and N contents, whose proportion approximates the type of SOM and its degradability by soil microorganisms [27].

It was suggested that CS pathogens can persist in soil for many years, surviving on decaying plant debris, especially where heavy loads of animal manure were applied [116], and that they possibly survive there by feeding on oligosaccharides released by ligninolytic and cellulolytic microorganisms [117,118]. This "cheating" behavior of CS pathogens suggests that competitive relationships may occur between them and ligninolytic or cellulolytic streptomycetes. The intensity of the competition might be dependent on the quality of available SOM [29,112,114]. Some of these oligosaccharides also act as environmental signals to plant pathogenic *Streptomyces* inducing the production of thaxtomin [117] so that they may further support their pathogenicity, but the regulation of those pathways is not completely resolved [119].

The inconsistent effects of various SOM additions on CS severity and incidence together with the knowledge on the importance of different carbohydrates in the induction of specific metabolic pathways in both pathogenic and beneficial streptomycetes suggest that a more precise SOM characterization is needed in future research, because only gross measurements have been provided for field testing so far [115]. Thus, we propose that organic matter supplements containing oligo- and monosaccharides should be avoided, while fresh low-pH SOM will help in nutrient release, and older, more decomposed SOM will benefit activities of antagonistic streptomycetes.

4. Rhizosphere Microorganisms Influence Nutrient Recycling and Produce Beneficial Metabolites

Microbial communities of the crop rhizosphere are essential not only for plant nutrition and health but also for nutrient cycling in agroecosystems. Therefore, there is a need to link

soil properties, especially the content and character of organic matter, with the distribution and activity of microorganisms and their use in plant protection.

Soil type and pH exert the most profound influence on the structure and function of bacterial communities [120]. In the rhizosphere, microbial communities are additionally shaped by the plant through its root exudates [121–124]. In return, the rhizosphere and also soil-derived endophytic microorganisms support the plant growth, health and metabolism [125]. The main activities of these plant-associated microorganisms are related to recycling of nutrients through mineralization of root exudates, nitrogen fixation or solubilization of minerals. Some microbes may also produce plant hormones and increase root growth [55]. This interaction is particularly important in plant management strategies because the population density of microorganisms in the rhizosphere is several times greater than that in bulk soil [126].

The composition of microbial communities in the rhizosphere is also influenced by the invasion of pathogens, which poses consequences not only for the particular disease but also for the functioning of the whole community [127]. Then, suppressive soils may act differently than conducive soils with respect to microbial community changes [22,28]. Furthermore, the suppressive effect is differentiated between induced suppressive soils of long-term monoculture and natural suppressive soils that undergo regular crop rotation [27,31,128,129].

Induced suppressive soils for CS contained a higher proportion of *Acetobacteraceae*, *Bacillaceae* and *Lysobacter* (*Xanthomonadaceae*) but also non-pathogenic *Streptomyces* (*Actinobacteria*) and lower proportions of some *Acidobacteria*, *Nocardioidaceae* and *Pseudomonadaceae* [130]. The naturally suppressive soils were enriched in *Pseudomonadaceae*, *Bradyrhizobiaceae*, *Acetobacteraceae* and *Paenibacillaceae*, which are all families of known plant growth-promoting microbes [28]. Some of the taxa occurring in suppressive soils were also enriched in soils supplemented with organic matter, which also led to CS suppression. These include *Bacillus*, *Streptomyces* [106] as well as *Solirubrobacteraceae*, *Xanthomonadaceae* and *Sphingomonadaceae* [33] (Table 3).

The suppressivity of soils is not only connected to the bottom-up control of the microbial communities, which is represented by soil physical characteristics [131], contents of nutrients (e.g., [27,33]), production of plant hormones [132] and organic matter quality [97], because the second half of the story is the top-down control by a range of bacterial and eukaryotic consumers and predators. Furthermore, predation by protists is also considered the missing link to understand soil suppressiveness because they are increasingly recognized as an essential component in nutrient recycling, shaping plant physiology, nutrition and health [14]. Those findings come from experiments conducted in controlled environments, where protists could influence the disease-suppressive ability of microbial communities directly by decreasing their numbers or via changes in the community composition [133]. Regarding CS, the micro-eukaryotic community was enriched with *Chlorophyta* together with *Myxogastria*, *Apicomplexa* and *Ciliophora* in CS-conducive soil, which consequently displayed increased micro-eukaryotic diversity and a higher number of putative interactions. Furthermore, micro-eukaryotic community was correlated with soil pH and contents of C, N, P, Ca and Fe in conducive soil but with S content in suppressive soil [28]. Thus, although the results are only preliminary, it seems that not only nutrient cycling and plant nutrition but also disease protection may be affected by trophic relationships [134].

Modification of plant-associated microbial communities can be achieved by planting various cultivars, because their tuberospheres are inhabited by cultivar-specific bacterial communities, which also comply with the cultivars' resistance to CS [28,135]. For example, *Nitrospirae* and *Acidobacteria* were enriched in a resistant cultivar together with a decreased ratio of *Thaumarchaeota*/*Euryarchaeota*. Out of these, *Nitrospirae* and *Thaumarchaeota* belong among important nitrifiers and ammonia-oxidizers, respectively, which points to their connection with nitrogen cycling. The increase in *Acidobacteria* suggests that a decrease in pH and an increase in *Euryarchaeota* points to the modification of pH, moisture or the carbon cycle [28,136]. In another study, microbial communities associated with resistant

cultivars exhibited lower abundance of bacteria, higher diversity, higher co-occurrence network complexity and increased community functioning.

Table 3. Microbial community changes associated with lowered CS severity due to soil suppressivity, potato cultivar or soil amendment.

Manipulation	Affected Bacterial Groups	Reference
Suppressive soil	Higher frequency of antagonistic pseudomonads and streptomycetes and higher proportion of pathogenic streptomycetes by cultivation; different microbial community by terminal restriction fragment analysis	[137]
Suppressive soil	Higher proportion of *Acetobacteraceae, Bacillaceae* and *Lysobacter* (*Xanthomonadaceae*), lower *Acidobacteria* group 6, *Nocardioidaceae, Pseudomonadaceae,* unclassified *Acidobacteria* group 11 and unclassified *Bacilli*	[130]
Suppressive soil	Enriched in *Pseudomonadaceae, Bradyrhizobiaceae, Acetobacteraceae* and *Paenibacillaceae*; decreased ratio of *Thaumarchaeota/Euryarchaeota* in tuberosphere of susceptible cultivar; higher proportions of Ascomycota and Basidiomycota and lower proportions of Chlorophyta, Ciliophora, Myxogastria and Apicomplexa	[28]
Resistant cultivar	Same as in suppressive soil and enriched in *Nitrospirae and Acidobacteria*; decreased tuberosphere ratio of *Thaumarchaeota/Euryarchaeota* in conducive soil; Chlorophyta and Cercozoa in lower proportions	
Resistant cultivar	More non-pathogenic streptomycetes than in susceptible cultivar	[138]
Resistant cultivar	Cloning/Sanger sequencing analysis of root, tuber and rhizosphere bacterial communities of 8 cultivars differing in resistance to CS; higher abundance of *S. turgidiscabies* in susceptible cultivars by qPCR	[30]
Susceptible cultivar	*Variovorax, Stenotrophomonas* and *Agrobacterium* were positively, and *Geobacillus, Curtobacterium* and unclassified *Geodermatophilaceae* negatively, correlated with the scab severity level, estimated absolute abundance of pathogenic *Streptomyces* and *txtAB* genes	[23]
Biocontrol strain *Pseudomonas fluorescens* LBUM223	Inoculation does not significantly alter the autochthonous rhizosphere nor geocaulosphere microbiomes in the field	[139]
Rice bran amendment	*Bacillus, Streptomyces, Chitinophaga* and *Actinomadura* significantly increased; unclassified *Koribacteraceae and* unclassified *Gaiellaceae* were significantly reduced	[106]
Iron amendment	*Bacillales* and *Gaiellales*	[33]
Peat amendment	*Rhizobiales, Burkholderiales, Xanthomonadales* and *Bacillales*	
Peat and iron amendment	*Proteobacteria* and *Bacteroidetes* increased at the proportional expense of *Actinobacteria*; proteobacterial *Burkholderiales, Xanthomonadales* and *Sphingomonadales* and actinobacterial order of *Gaiellales* were the most responsive groups; actinobacterial families *Micromonosporaceae* and *Thermomonosporaceae* were elevated	

Furthermore, a metagenomic approach showed that particularly the enrichment of antibiotic biosynthesis pathways within members of bacterial communities was typical for the tuberosphere soil of healthy tubers. In contrast, the tuberosphere soil of diseased tubers was enriched in *Variovorax, Stenotrophomonas* and *Agrobacterium* together with several ABC transporter genes, genes of bacterial secretion system, quorum sensing, cytochrome P450 and also genes for nitrogen metabolism [23]. Comparison between the metagenomes differently affected by the pathogen populations supports the previously mentioned observations that, in the vicinity of tubers, antibiosis and possibly also nitrogen-related process are important in CS disease control (Figure 1, Table 3).
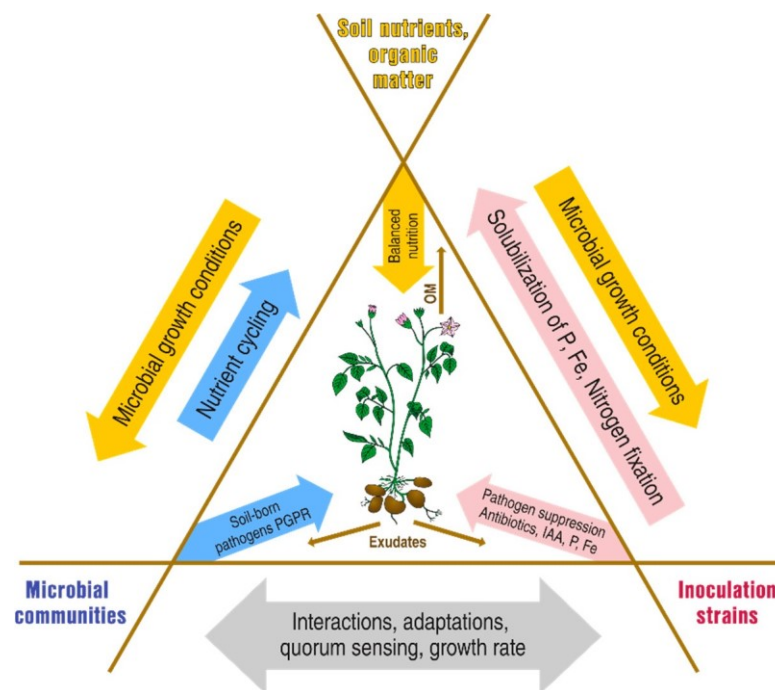
Figure 1. Interactions of soil components, microbial communities, introduced biocontrol strains and potato plants in a system affected by common scab.

The research also demonstrated that the plant–microbe interactions connected to CS severity are detectable only in the nearest vicinity to the potato tuber. In particular, out of the potato tuberosphere, rhizosphere, root zone or bulk soil, the effects of manipulations were detectable only in the tuberosphere soil [23,28]. That closely connects the soil and plant microbiomes, which seem to share not only members but also functions such as activation of both basal and inducible plant defense systems [25,140].

The changes in the microbial community associated with increased CS control might be achieved by addition of specific organic compounds, but also by addition of nutrients (see above).

Consequently, the current knowledge on microbial interactions in the rhizosphere is still limited in terms of being clearly resolved in terms of CS control. However, many examples of successful manipulation of microbial communities by supplementation or cultivars are already available to consider for the improvement of soil health.

5. Inoculation with Antagonistic Strains

Disease occurrence is often accompanied by changes in microbial communities such as altered microbial abundance, composition and function, which are studied as markers of soil health but also for their potential to set conditions favorable for plant protection. Various taxa participate in those activities, or their combinations [22,141].

CS-suppressive soils revealed that non-pathogenic *Streptomyces* act as the agents responsible for suppression [129,130]. However, different studies showed that a broad range of bacterial and fungal taxa contribute to CS suppression, with antagonistic activities found in strains of the genera *Streptomyces* [142–145], *Bacillus* [146,147], *Brevibacillus* [148] and *Pseudomonas* [149], and they were successfully used to control the disease in pot and field trials (Table 4).

Application of the individual strains or strains with organic or inorganic fertilization mostly resulted in significant decreases in CS severity (e.g., [143,144,147,150–155]). In a few studies, the suppression of pathogenic strains or the decrease in genes from the pathogenic island were also determined [149,156]. Surprisingly, in some studies, no effect on the autochthonous microbial community was observed after inoculation [111,139], while in others, proportions of several taxa were altered [146].

Table 4. Soil inoculation affecting CS severity, tuber yield and associated bacterial community.

A. Complex Inocula		
Inoculum	**Effect**	**Reference**
Swine feces with coprophilous actinobacteria	CS suppression; viable counts of *Streptomyces scabies* decreased, antagonistic fluorescent pseudomonads increased	[156]
Broth treatment and inoculation with antagonistic *Bacillus* sp. in pot assay with sterile soil	CS suppression by 40%	[154]
Pseudomonas mosselii with vermicompost	CS suppression	[150]
Bacillus subtilis GB03 and *Rhizoctonia solani* hypovirulent isolate Rhs1A1, compost	CS suppression by 10–34%, increased yield, reduction in stem and stolon canker by 20–38%, black scurf by 30–58%	[151]
Trichoderma virens, Bacillus subtilis and *Rhizoctonia solani*, compost, rapeseed rotation	Reduced disease and increased yield	[92]
Vermiculite cultures of non-pathogenic *Streptomyces* isolates mixed with soil	CS suppression	[157]
Conducive soil inoculated with *Streptomyces* isolates	No effect of inoculation; variability of streptomycete community increasing from planting to mid-season (by pyrosequencing); pathogen suppressive capacity of antagonistic streptomycetes negatively correlating with CS severity (by cultivation)	[111]
B. Individual strains		
Inoculum	**Effect**	**Reference**
Streptomyces melanosporofaciens EF-76 in chitosan beads	Increased numbers of geldanamycin-resistant actinobacteria on harvested potato tubers	[142]
Pesticide and antibiotic-resistant *Streptomyces* spp.	CS suppression by 55–>60%	[144]
S. violaceusniger AC12AB	CS reduction up to 90% in greenhouse and field, increased yield up to 26.8% in field trial	[145]
Streptomyces sp. WoRs-501	CS severity decreased by 78–94% in field pot trial	[143]
Streptomyces strain 272	CS severity reduced by 43% on susceptible potato cultivar Bintje, both disease incidence and severity reduced by 43 and 59% on the scab-tolerant cultivar Nicola	[158]
Pseudomonas fluorescens LBUM223	CS reduced by approximately 30% in plots after biweekly applications, increased yield by 46%; did not reduce pathogen soil populations, down-regulated *txtA* expression in the geocaulosphere	[149,159]
Bacillus megaterium KBA-10, *P. putida* K-19B, *B. megaterium* TV- 91C, *Pantoea agglomerans* RK-92	Biocontrol efficacy 18.7–60.3%, tuber yield increase by 20.4–40%	[160]
B. subtilis and *Trichoderma harzianum* in diatomaceous earth (225–300 kg ha^{-1})	CS index decreased by 30.6–46.1%; 19-23-fold higher *Pseudomonadales*; CS severity negatively correlated with relative abundances of *Agrobacterium*, *Achromobacter* and *Pseudomonas* and positively with *Acidobacteria*, *Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes*	[146]
Brevibacillus laterosporus AMCC100017	CS severity decreased from 2.60 to 0.77, i.e., biocontrol efficacy 70.51%; reduced pathogen, transient impact on the native bacteria community	[148]
Bacillus subtilis	Decrease in common scab severity up to 70%, and 67% in field trials	[147]
B. amyloliquefaciens Ba01	CS reduced from 14.4 ± 2.9% (naturally occurring) to 5.6 ± 1.1% in the field	[161]
B. velezensis 8-4	CS control efficiency reached 51.83 ± 8.53%, the yield increased by 19.91 ± 3.56%	[155]
Trichoderma virens	Decrease in CS incidence and severity	[92]
Phages Stsc1 and Stsc3	Prevented CS symptoms on radish seedlings	[162]

Table 4. *Cont.*

C. Mechanisms of CS suppression		
Biocontrol Strain	**Observed Property**	**Reference**
Streptomyces violaceusniger AC12AB	Production of azalomycin, indole-3-acetic acid and siderophores, nitrogen fixation and phosphate solubilization => CS reduction up to 90% in greenhouse and field trials, increased yield up to 26.8% in field trial	[145]
Pseudomonas fluorescens LBUM223	Phenazine-1-carboxylic acid (PCA) production => growth inhibition of *S. scabiei*, repression of thaxtomin biosynthesis genes (*txtA* and *txtC*); activities were lost in *phzC⁻* mutant deficient in PCA production	[163]
	Phenazine-1-carboxylic acid production => 12%–14% of all *S. scabiei* genes were differentially expressed, including key genes involved in pathogenicity/virulence, mycelium differentiation and increased oxidative stress	[164]
Fragments of *γ*-glutamyl transpeptidase from *Bacillus subtilis* BU108	In vitro growth inhibition of *S. scabiei*	[165]
S. melanosporofaciens EF-76	Geldanamycin production => disease index was reduced from 6.30 to 4.81 and from 2.83 to 2.49 in growth chamber and field experiments, respectively	[166]
Streptomyces A1RT	Production of isatropolone C and indole-3-acetic acid => reduction in average disease severity index by 82.4–95.7%	[4]
Streptomyces isolates (strains 93 and 63) and their spontaneous mutants	The mutants lost in vitro inhibitory activity against *S. scabiei* in antibiotic and co-plate assay, while retaining the biocontrol ability in soil	[167]
B. amyloliquefaciens subsp. *plantarum* FZB42	Production of cyclic lipopeptides and volatiles => pathways of induced systemic resistance	[168]
Agrobacterium tumefaciens C58 *attM* gene introduced to *S. scabiei*	No disease symptoms in planta, altered morphological differentiation—quorum quenching paralyzing *γ*-butyrolactone signalingpathway	[161]

The variation in the microbial community response might be connected to the mechanisms of suppression provided by the inoculated strains. Mostly, production of antibiotics against the pathogens is involved, while the identified compounds include geldanamycin [166], phenazine-1-carboxylic acid [163,164], isatropolone C [4], azalomycin [145] or antimicrobial peptides [154,165]. However, the strains also produce compounds such as indole-3-acetic acid or siderophores and enable nitrogen fixation and phosphate solubilization [145] or remove signaling through *γ*-butyrolactone pathway [161].

In conclusion, the inoculated strains control CS severity not only directly by antibiosis against pathogens but also through nutrition, metabolism and signaling in the plant–soil–microbe interactions. Thus, it seems that inoculation with locally isolated biocontrol strains may be a safer and more effective approach compared to application of globally distributed products, because the local strains might be more adapted to local soil and climate conditions and also to local microbial communities. That might be useful not only for lower disturbance but also for easier adaptation of inoculated strains [31,169].

6. Conclusions

Soil mineral nutrients are often in an unbalanced state due to fertilization and intensive soil exploitation by agriculture practices [64]. That negatively affects plant growth, productivity and health as well as soil quality and biodiversity. This review suggests that balanced nutrition together with promotion of suppressive microbial communities represent key components in the management of potato common scab. Two approaches can be used to improve the current state of soil, which leads to improved CS suppression. Firstly, supplying soils with the limiting nutrient and/or microbial strain(s) that solubilize the missing nutrients, and secondly, supporting microbial communities that are competitive or antagonistic to the pathogen by addition of peat or a long-decomposed and largely recalcitrant organic matter. In contrast, additions of organic matter or fertilizers containing high amounts of nitrogen should be carefully evaluated, because nitrogen recycling seems to influence CS severity in both directions. To further support CS suppression, it is necessary to carefully select potato cultivars according to their different micronutrient requirements

and accumulation [44,71]. Furthermore, it is important to consider that individual cultivars have various interactions with rhizosphere microorganisms, and those may enhance but also diminish the plant–microbe interactions [28,33,135]. Inoculated biocontrol strains affect not only the pathogen but also the potato plants, the autochthonous microbial community and the soil chemistry, so their impact on the local microbial community also needs to be evaluated prior to their application. Soil chemical status and organic matter should be regularly assessed in more detail to determine the missing elements and the quality of humus.

Focusing on future research, more attention should be paid to plant–microbe–soil interactions occurring in the nearest compartments surrounding potato tubers. These may include the transfer of microorganisms between soil and plant microbiomes, decomposition pathways which lead to compounds influencing the pathogenicity of streptomycetes or metagenomic and metabolomic studies aiming to determine the processes involved in the development of soil suppressiveness. Although long-term monoculture was suggested as the best strategy to achieve long-lasting suppression by streptomycetes [31], new knowledge about the general suppression of CS may bring novel inspiration for management of the disease.

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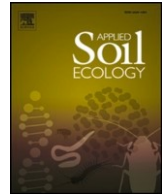
Inoculations of soil by antagonistic strains modify tuberosphere bacterial communities and suppress common scab of potatoes

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ABSTRACT

Common scab of potatoes is a soil-borne disease causing considerable economic losses worldwide. Although many management practices to control this disease have been introduced, the lack of efficiency increases an interest in effective and environmentally friendly alternatives. One of the attractive approaches to manage the disease is an application of antagonistic strains into soil. The aim of this study was to develop a selection approach for suitable biocontrol agents based on their *in vitro* properties and demonstrate their effect on soil microbial communities and host plant nutrition. Out of 56 isolates of *Actinobacteria*, three isolates and a mixture of six isolates with strong inhibitory activities against common scab pathogens and high growth rates were selected for the pot experiment to assess their biocontrol activity. The microbial community composition of the tuberosphere and the nutrient content in potato leaves was determined. One isolate significantly reduced the severity of the common scab. This isolate showed strong inhibitory activity against pathogens and reached the highest number of CFU/g of vermiculite. In the pot experiment, all the isolates affected the microbial community composition, yet the most substantial changes occurred in the treatment of the most efficient isolate. Moreover, it was determined that inoculation with isolates led to alterations in host plant nutrition suggesting plant-microbe interactions. We provided insights into relationships between antagonistic isolates, common scab pathogens, host plant and microbial communities, and their role in the disease control.

1. Introduction

Common scab of potatoes (CS), caused by *Streptomyces* spp., is a soil-borne disease occurring worldwide. The disease negatively affects potato quality by causing superficial, raised or pitted lesions on the tuber surface, which has a significant impact on the crop value. A survey of Canadian potato growers estimated the economic loss caused by CS in 2002 to be between 15.3 and 17.3 million Canadian dollars (Hill and Lazarovits, 2005). Therefore, effective and sustainable management of CS is due to its worldwide occurrence essential for maintaining the high profitability of potato cultivation.

Numerous management practices to control CS have been described to this day. Adjusting soil pH (Lambert, 1991; Waterer, 2002), irrigation early in the season (Lapwood et al., 1973), green manure, crop rotation (Wiggins and Kinkel, 2005), soil fumigation and seed treatment (Al-Mughrabi et al., 2016) are some of the traditional methods used to reduce severity or incidence of CS. However, these strategies often lead to inconsistent and/or insufficient results and can reduce potato yield or have a negative impact on the environment (Dees and Wanner, 2012). Thus, searching for efficient disease suppression has increased interest in biological control. Breeding programs focusing on the development of scab-resistant potato cultivars have shown great potential in the disease control. Several studies identified cultivars resistant to CS (Bizimungu et al., 2011; Douches et al., 2009; Jansky et al., 2018; Stark et al., 2016) but diverse environmental conditions and differences in pathogen populations are sources of noticeable variability and instability of scab resistance (Clarke et al., 2019; Haynes et al., 2010).

Inoculation of microbial strains with antagonistic activities is an alternative approach of biological control. In particular, *Streptomyces spp.* are known for the production of a wide range of antimicrobial

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compounds, some of them (*e.g.* azalomycin and Isatropolone C) were shown to participate in disease suppression by inhibiting CS pathogens. Biocontrol *Streptomyces* also exhibit plant growth promotion by the production of the plant hormone indole-3-acetic acid, siderophore production, nitrogen fixation and phosphates solubilization potential (Sarwar et al., 2018; Sarwar et al., 2019). These beneficial properties explain the capability of *Streptomyces* to significantly reduce CS symptoms, which makes them suitable candidates for biocontrol as well as growth-promoting agents.

Production of antimicrobial compounds, resource competition (Neeno-Eckwall et al., 2001), plant growth promotion and activation of plant defense response (Lin et al., 2012; Sarwar et al., 2019) have all been hypothesized to play a role in the disease control by biocontrol agents. The abundance of mechanisms controlling the disease explains considerable differences in biocontrol effectiveness observed under laboratory and field conditions. Some studies showed that *in vitro* antibiotic inhibition of pathogens by antagonistic *Streptomyces* isolates did not predict *in vivo* biocontrol efficacy (Neeno-Eckwall et al., 2001; Ryan et al., 2004). However, another study concluded that *in vitro* antibiotic inhibition as well as competitive interactions between biocontrol isolates and pathogens might improve the prediction of *in vivo* control of CS (Liu et al., 1996). Hence, the poor predictability of disease control under field conditions may be improved by a thorough comprehension of different types of interactions between antagonistic strains and pathogens.

The composition of indigenous microbial communities in the soil environment is one of the crucial factors determining the success of CS suppression by biocontrol strains (Kinkel et al., 2011; Kinkel et al., 2012). Soil community affects the ability of biocontrol agents to directly suppress CS by inhibitory activities against pathogens, and indirectly by imposing a selection for antagonistic phenotypes of indigenous microbial communities (Kinkel et al., 2012). Therefore, inoculation with biocontrol strains can lead to an increase in the relative abundance of several beneficial bacterial taxa in the rhizosphere (Li et al., 2021; Wang et al., 2019), yet in other cases, inoculants do not significantly change autochthonous microbial communities (Roquigny et al., 2018).

CS control is also strongly connected to the soil nutrient status because the deficiency of essential nutrients as well as nutrient imbalance negatively affects plant growth and resistance to soil-borne diseases (Huber and Haneklaus, 2007). Soil microorganisms alter nutrient availability and enhance or reduce nutrient uptake by plants. Microbial mineralization provides an extensive quantity of nutrients in forms available for plants; however, other processes such as nitrogen fixation and solubilization of precipitated phosphate also participate in plant nutrient acquisition (Van Der Heijden et al., 2008). Various microbial taxa are responsible for these processes and thus, changes in the composition of soil communities might have a substantial effect on plant nutrition and subsequently on a potato plant growth and protection from diseases.

The aim of this study was to find bacterial strains capable of significantly reducing CS severity. After several selection experiments in the laboratory, three strains and a mixture of six strains of *Actinobacteria* were selected based on their strong antibiotic activities and high growth rates to test their abilities to suppress the disease in a pot experiment. We further investigated the effect of biocontrol inoculants on microbial communities in the tuberosphere and nutrient content in the host plant. The results from the study will help to elucidate the relationship of the *in vitro* properties of biocontrol isolates (inhibitory activity against pathogens and growth rate) and the ability to suppress CS *in vivo*. Obtained data also provide an insight into the impact of biocontrol isolates and subsequent changes in indigenous microbial communities on CS severity and plant nutrition, which might contribute to defining an effective biological control strategy.

2. Materials and methods

2.1. Bacterial strains

A total of 56 actinobacterial strains were collected from soil and surface of potatoes including the lesions of common scab at nine field sites differing in CS incidence, soil chemistry, altitude, and climatic conditions. All field sites are located in the Czech Republic. For the inhibition assay, two CS pathogens were chosen, *Streptomyces scabiei* DSM 41658 and *Streptomyces acidiscabies* DSM 41668.

2.2. In vitro inhibition assay

Selected actinobacterial strains were assessed on their ability to inhibit the growth of two CS pathogens, *S. scabiei* and *S. acidiscabies*, by co-cultivation on agar plates. Pathogens were grown on GYM *Streptomyces* agar (Atlas, 2010) pre-inoculated with the bacterial strains. Plates were incubated for three days at 28 °C. The antagonistic properties of strains against the pathogens were evaluated by a four-degree scale based on the size of zone of inhibition (0–3, 0 no inhibition, 3 strong inhibition of the pathogen).

2.3. In vitro growth in vermiculite

Growth rate of fourteen strains with strong antagonistic activity against one or both CS pathogens was determined by cultivation in vermiculite. To prepare the inoculum, the strains were initially cultivated in 50 mL of YME medium (4 g yeast extract, 10 g malt extract and 4 g glucose per 1 L of distilled water, pH 7.2) in 250 mL flasks, shaking for 2 days at 28 °C and 180 rpm, then they were centrifuged in 50 mL sterile falcon tubes at 2,000 g, 4 °C for 15 min. The supernatant was discarded, and the culture was suspended in 10 mL of sterile distilled water in the same falcon tubes. Vermiculite was prepared in 250 mL flasks, where 50 mL of vermiculite with 8 mL of water was sterilized for 90 min and subsequently left 1 day at room temperature. This sterilization process was repeated twice. Inoculation to sterile vermiculite was done from 1 mL of suspended pellet with addition of 8 mL of the medium Ac (20 g starch, 8 g yeast extract and 4 g peptone per 1 L of distilled water, pH 6.5) to Erlenmeyer flasks with sterile vermiculite, and incubated at 28 °C in a stable incubator, mixed once a day by hand. Inoculated vermiculite was cultivated for 21, 43, 70 and 134 h at 28 °C in a stationary incubator. Aliquots of inoculated vermiculite were crushed by sterile glass rod and vortexed twice for 1 min in sterile distilled water. The growth rate was evaluated by CFU counts after plating serial dilutions of homogenized samples on GYM agar.

2.4. Pot experiment

The experiment was set up at the edge of a potato field near Pacov, Czech Republic on May 13, 2015. Thirty pots of 12 L volume were filled with soil from the field with high common scab incidence occurring there for four decades (Sagova-Mareckova et al., 2015). In the soil, 3×10^5 copies of the *txtB* gene (a gene in the biosynthetic gene cluster of thaxtomin, the main pathogenicity factor of common scab of potatoes) per g of soil have been previously determined (Sarikhani, 2020). No extra pathogenic bacteria were added into the pots. Inoculated strains were selected based on results from *in vitro* assays, all inhibited the growth of one or both pathogens and showed high growth rates. The soil was enriched with inocula of selected inoculants prepared in vermiculite particles to a final concentration of 10^8 CFU/L of soil. Three strains (14HB3D, 14HB8C and 09ZI22) were inoculated individually into pots and the treatments were labeled S1, S2 and S3, respectively. Mixed inoculum of these three strains together with the strains 09ZI42, 14SL3 and 09ZI13 was used for an additional treatment, labeled M treatment. Five replicate pots of each treatment were buried to about 30 cm depth to field soil and planted with Agria, a potato cultivar susceptible to

common scab.

2.5. Sampling

The potatoes were harvested 110 days after planting. The fourth leaves from the apex of each shoot of the potato plant were collected and dried at room temperature. Tuberosphere soil samples of approximately 30 mL were collected from a thin layer (3 mm) of soil surrounding the potato tubers using a sterile spoon, filled to 50 mL falcon tubes, transferred to laboratory at −18 °C, and stored at −80 °C before the analysis (for details see Sagova-Mareckova et al., 2015). Potato tubers were collected, washed in distilled water and CS severity was evaluated on each potato tuber using a 9-degree scale: 1, no scab; 2, 0.1%–0.8%; 3, 0.9%–2.8%; 4, 2.9%–7.9%; 5, 8.0%–18.0%; 6, 18.1%–34.0%; 7, 34.1%–55.0%; 8, 55.1%–77.0%; 9, 77.1%–100% (Wenzl and Demel, 1967).

2.6. Chemical analysis of potato leaves

Analyses of potato leaves were performed by service laboratory of the Institute of Botany (Trebon, Czech Republic). For total nitrogen analysis, 1–3 mg of dried homogenized leaves was mineralized by modified Kjeldahl method in H_2SO_4 with catalyzer at 360 °C. For total phosphorus, 20 mg of dried potato leaves was sequentially decomposed by HNO_3 and $HClO_4$. In mineralized samples, both N and P were determined by flow injection analysis with spectrophotometric detection using FIA Lachat QC 8500 analyzer (Lachat Instruments, Hach Company, Loveland, CO). Iron contents in potato leaves were determined by atomic absorption spectrometry using AAS spectrometer ContrAA 700 (Analytik Jena, Jena, Germany) after mineralization with nitro-hydrochloric acid.

2.7. DNA extraction

DNA from soil samples was extracted using the method SK developed by Sagova-Mareckova et al. (2008). This method is based on homogenization of soil samples in a bead beater and phenol/chloroform extraction followed by treatment of DNA with $CaCl_2$ and purification with GeneClean Turbo DNA kit (MP Biomedicals, Irvine, CA).

The three bacterial strains inoculated individually into pots (14HB3D, 14HB8C and 09ZI22) were grown on GYM agar plates at 28 °C. After three days, genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the protocol provided by the manufacturer.

2.8. Amplicon sequencing and analysis

DNA samples extracted from the three inoculants used individually in the treatments served as templates for PCR amplifications, in which primers 16Seu27f (5′-AGAGTTTGATCMTGGCKCAG-3′) (Čermák et al., 2008) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) (Bruce et al., 1992) were used to amplify nearly full-length 16S rRNA gene. PCRs were performed in 25 μL reaction volumes containing GoTaq G2 Hot Start Taq Polymerase (Promega, Madison, WI) using C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). PCR conditions consisted of the following steps: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s denaturation at 95 °C, 45 s annealing at 57 °C and 90 s extension at 72 °C, terminated by final extension at 72 °C for 5 min. Amplicons were sequenced by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

From soil DNA samples, universal primers CS1_515F (5′-ACACT-GACGACATGGTTCTACAGAGTGYCAGCMGCCGCGGTAA-3′) (Parada et al., 2016) and CS2_806R (5′-TACGGTAGCAGA-GACTTGGTCTACGGACTACNVGGGTWTCTAAT-3′) (Apprill et al., 2015) with linkers at the 5′-ends were used to amplify the fragments of 16S rRNA gene including the variable region V4 (Walters et al., 2016). PCRs were carried out in 25 μL reaction volumes using the Ex Taq HS

DNA Polymerase (Takara, Kusatsu, Japan). All the amplifications were performed on C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) under following conditions: 5 min initial denaturation at 95 °C, followed by 28 cycles of 30 s denaturation at 95 °C, 45 s annealing at 55 °C, 30 s extension at 72 °C, and 7 min final extension at 72 °C. Construction of amplicon libraries and sequencing on a MiSeq sequencer (Illumina, San Diego, CA) were done at the DNA Services Facility, Research Resources Center, University of Illinois (Chicago, IL). Resulting paired sequence reads were then merged, filtered, and aligned using a reference alignment from the Silva database (Quast et al., 2013), and chimera checked using the integrated Vsearch tool (Rognes et al., 2016) according to the MiSeq standard operation procedure (MiSeq SOP, February 2021) (Kozich et al., 2013) in Mothur v. 1.44.1 software (Schloss et al., 2009). Taxonomic assignment of sequence libraries was performed in Mothur using the SILVA Small Subunit rRNA Database, release 132 (Yilmaz et al., 2014) adapted for use in Mothur (https://mothur.org/w/images/3/32/Silva.nr_v132.tgz) as the reference database. Sequences of plastids and mitochondria, and those not classified in the domain *Bacteria* and *Archaea* were discarded. Using the UNOISE algorithm in Usearch v. 11.0.667 software (Edgar, 2016), the sequence library was clustered into zero-radius operational taxonomic units (ZOTUs), also called sequence variants (Callahan et al., 2016). ZOTU table was processed using tools implemented in Mothur. The 16S rRNA gene sequences of the three bacterial strains inoculated individually into pots were aligned with ZOTUs and based on the pairwise distances between aligned sequences, they were assigned into corresponding ZOTUs. Distance matrices describing the differences in community composition between treatments were calculated using Yue-Clayton theta calculator (Yue and Clayton, 2005). Matrices of the Yue-Clayton theta distances were used for calculation of analysis of molecular variance (AMOVA) (Martin, 2002). Metastats analysis (White et al., 2009) was performed to detect differentially represented ZOTUs in samples. Rarefaction curve data were calculated using the rarefaction.single command in Mothur. In R v. 4.0.3 software (R Core Team, 2020), the trapezoidal rule was applied to compute areas under the rarefaction curves. These areas were used to determine microbial diversity in samples.

2.9. Statistical analyses

Inhibition of pathogens by antagonistic strains was tested by Mann-Whitney-Wilcoxon test. Kruskal–Wallis test followed by Dunn's *post hoc* test with Bonferroni correction was used to evaluate scab severity, rarefaction areas, numbers of ZOTUs of inoculants, and nutrient content in the leaves of host plants. Diversity indices were also assessed by Kruskal-Wallis test. AMOVA was used to compare distance matrices (Yue-Clayton theta) of samples, and the distance matrices were plotted by non-metric multidimensional scaling (NMDS). Linear discriminant analysis effect size (LEfSe) algorithm was used to detect microbial taxa explaining differences between individual treatments (Segata et al., 2011). Threshold on the logarithmic LDA score was set to 2.0 and multiclass analysis was performed using all-against-all strategy. Spearman's correlation coefficients were calculated using Hmisc package. All statistical calculations were done in the R computing environment (R Core Team, 2020).

3. Results

3.1. Inhibitory activities and growth rates of selected isolates

Antagonistic isolates inhibited the growth of *S. scabiei* significantly more than the growth of *S. acidiscabies* (MWW, $p \leq 0.01$). The mean of relative inhibition level of *S. scabiei* was 1.7 compared to the mean of 1.4 for *S. acidiscabies*. Out of a total of 56 strains, twenty antagonistic strains strongly inhibited (inhibition level 3) the growth of *S. scabiei*, whereas the growth of *S. acidiscabies* was strongly inhibited by ten strains. Ten strains did not inhibit the growth of *S. scabiei* at all (inhibition level 0) in

contrast to nineteen strains that did not inhibit the growth of *S. acidiscabies*. Inhibitory activities of selected isolates with examples of inhibition levels are shown in Fig. S1.

Fourteen strains (Table S1) with strong inhibitory activity against one or both CS pathogens (eight strains strongly inhibiting the growth of both pathogens, four strains strongly inhibiting the growth of *S. scabiei*, and two strains strongly inhibiting the growth of *S. acidiscabies*) were examined for the growth rate. The highest number of CFU was reached by the strain 09ZI22 in 70 h (1.5×10^9 CFU/g of vermiculite) with the growth acceleration (number of CFU in 70 h/number of CFU in 21 h) of 150-fold, while the lowest population of CFU during the same period was reached by the strain 14HB9D (1.0×10^5 CFU/g of vermiculite) (Fig. S2).

3.2. Selection of strains for the pot assay and common scab severity

For the pot experiment, a total of six isolates with high growth rates and strong inhibitory activities were selected (Table S2). Antagonistic strains 14HB3D (S1 treatment), 14HB8C (S2 treatment) and 09ZI22 (S3 treatment) strongly inhibited one or both pathogens and showed high growth acceleration *in vitro*. In addition, these strains were amongst the top five isolates that reached the highest number of CFU in 70 h, ranging from 1.0×10^8 CFU/g of vermiculite (14HB8C) to 1.5×10^9 CFU/g of vermiculite (09ZI22). Isolates 09ZI42, 14SL3 and 09ZI13 (M treatment - together with 14HB3D, 14HB8C and 09ZI22) had also the capability to inhibit the growth of *S. scabiei* and/or *S. acidiscabies in vitro* (09ZI42 and 09ZI13 suppressed the growth of both pathogens completely). Moreover, strains 09ZI42 and 14SL3 rapidly increased the populations of CFU in 70 h (they were amongst the top five strains that reached the highest CFU counts in 70 h). Isolate 09ZI13 did not reach a high number of CFU in 70 h; however, it considerably increased the population from 70 to 134 h.

In the pot assay, CS severity differed significantly between treatments (KW, $p < 0.001$). The disease was significantly suppressed only by the S3 treatment (Dunn's test, $p < 0.05$). In this treatment, the mean of the scab scale level decreased from 4.7 (the mean of the control and the control with vermiculite) to 3.3. The S1 and S2 treatment also slightly reduced CS severity (the mean of the scab scale level was 4.2 and 4.4, respectively), whereas the mean of the M treatment (4.7) did not differ from the controls (Fig. 1). Detailed results of statistical analyses are in Table S3. Photographs of potatoes harvested from individual treatments are shown in Fig. S3.
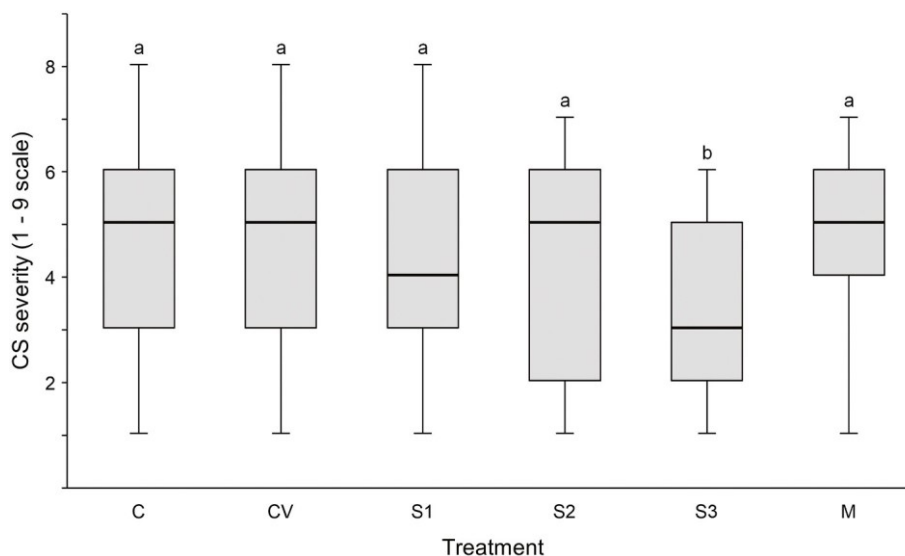
3.3. Nutrient content in potato leaves

The content of nitrogen in potato plant leaves was significantly higher in the S1 treatment (Dunn's test, $p < 0.05$) and the S2 treatment (Dunn's test, $p < 0.05$) compared to the M treatment. Between the control and the control with vermiculite, nitrogen content did not differ. Total phosphorus content was significantly lower in the leaves of the M treatment compared to the control with vermiculite (Dunn's test, $p < 0.01$) but not compared to the control. Phosphorus content of leaves in the other treatments was not different compared to both controls. No statistically significant differences between the treatments and the controls were observed in iron contents (Table 1).

3.4. Microbial community composition

Out of a total of 1,628,967 16S rRNA gene sequences, 1,451,112 sequences (*i.e.* 89.1%) were mapped into 13,239 ZOTUs. The number of sequences per sample varied from 32,017 to 71,424 and the dataset was normalized to 32,017 sequences. Antagonistic strains used for the S1, S2 and S3 treatments were assigned into corresponding ZOTUs (with a 100% sequence similarity) and the number of sequences of these ZOTUs in each treatment was examined (Fig. S4). Strain 14HB3D (S1 treatment) was assigned into ZOTU 299 and the number of respective sequences in the S1 treatment was 48.4, which was highest compared to the other treatments but significantly higher only compared to the number of ZOTU 299 sequences in the S2 treatment (Dunn's test, $p < 0.05$). In other treatments and controls, the number of ZOTU 299 sequences was not significantly different from the S1 treatment. Strain 14HB8C (S2

Table 1
Chemical content in the fourth leaves of potato plants. Data are represented as means ± standard deviations ($n = 5$). Lowercase letters indicate statistical significance ($p < 0.05$) between treatments (Dunn's test).

Sample	N	P	Fe
	[%]	[%]	[mg/kg]
control	$4.52 \pm 0.07^{a,b}$	$0.26 \pm 0.01^{a,b}$	330.38 ± 44.52^{a}
control with vermiculite	$4.64 \pm 0.19^{a,b}$	0.29 ± 0.03^{a}	362.85 ± 39.94^{a}
S1 treatment	4.85 ± 0.27^{a}	$0.27 \pm 0.02^{a,b}$	470.85 ± 343.78^{a}
S2 treatment	4.75 ± 0.15^{a}	$0.27 \pm 0.02^{a,b}$	395.37 ± 69.55^{a}
S3 treatment	$4.46 \pm 0.20^{a,b}$	$0.25 \pm 0.01^{a,b}$	450.31 ± 138.91^{a}
M treatment	4.42 ± 0.10^{b}	0.23 ± 0.02^{b}	347.86 ± 31.09^{a}

Fig. 1. CS severity in the pot experiment. Treatments S1, S2 and S3 were inoculated with three antagonistic strains individually, M treatment was inoculated with mixture of six strains. C – control, CV - control with vermiculite. Lowercase letters indicate statistical significance (p < 0.05) between treatments (Dunn's test, n = 5).

treatment) was assigned into ZOTU 907. Sequences of this ZOTU were the most abundant in the S2 treatment (average of 42.4) and differed from the S1 (Dunn's test, $p < 0.005$) and S3 treatment (Dunn's test, $p < 0.01$) but not from the M treatment and the controls. An average number of sequences of ZOTU 566 (strain 09ZI22, S3 treatment) was highest in the respective treatment (25.4) and was not significantly different from the control with non-inoculated vermiculite or other treatments. The number of ZOTU 566 sequences was significantly higher only compared to the control (Dunn's test, $p < 0.05$). All three ZOTUs of inoculated strains were assigned into *Streptomyces* spp.

Bacterial and archaeal communities of the treatments were compared using non-metric multidimensional scaling. On a NMDS plot, samples from the S3 treatment and, to a lesser extent from the S1 treatment, tended to separate from other treatments and the control/control with vermiculite, whereas other samples were closer to each other (Fig. 2). Overall, communities differed between the treatments (AMOVA, $p < 0.001$). Pairwise comparisons of communities showed significant differences between the control/control with vermiculite and the S1 treatment (AMOVA, $p < 0.05$) and also between the control/control with vermiculite and the S3 treatment (AMOVA, $p < 0.005$), but not between the control/control with vermiculite and the S2 treatment or the control/control with vermiculite and the M treatment. Besides, the control was not different from the control with vermiculite (Table 2).

Rarefaction analysis showed that enrichment with vermiculite or antagonistic strains slightly reduced diversity of *Bacteria* and *Archaea*, particularly in the S3 treatment; however, the effect of vermiculite or antagonistic strains on diversity was not significant (Fig. S5). Correspondingly, diversity indices (Simpson's and inverse Simpson's index) showed no overall differences in diversity between treatments (Fig. S6).

The relative proportion of *Actinobacteria* phylum in the sequence libraries ranged between 23.2 and 26.4%, the lowest being in the control with vermiculite and highest in the S3 treatment. Proportions of individual families did not substantially change between the treatments. The

Table 2

Analysis of molecular variance (AMOVA) between soil communities of the control/control with vermiculite and strain inoculation treatments based on a matrix of Yue-Clayton theta distances. Significance is indicated by asterisks ($p < 0.05$ *, $p < 0.01$ **, and $p < 0.001$ ***).

Pair		F-statistics	
Control *vs.*	S1 treatment	2.052	*
	S2 treatment	1.129	
	S3 treatment	3.905	**
	M treatment	1.415	
Control with vermiculite *vs.*	S1 treatment	2.574	*
	S2 treatment	0.921	
	S3 treatment	10.177	**
	M treatment	1.491	
control *vs.*	control with vermiculite	1.029	

dominant families were *Nocardioidaceae*, *Gaiellaceae*, *Micrococcaceae*, 67–14 (*Solirubrobacterales* order), *Streptomycetaceae* and uncultured family from *Gaiellales* order (Fig. S7).

Significant differences in the relative abundance of several taxa in the controls and the S1, S2 and S3 treatments were determined by LEfSe analysis ($p < 0.05$, logarithmic LDA score > 2.0). In the S3 treatment, the relative abundance of the phylum *Thaumarchaeota*, its class *Nitrososphaeria*, order *Nitrososphaerales* and family *Nitrososphaeraceae* together with the phylum *Verrucomicrobia*, its class *Verrucomicrobiae*, order *Chthoniobacterales*, family *Chthoniobacteraceae* and genus *Candidatus* Udaeobacter was significantly higher compared to the other investigated treatments. Moreover, the class of *Thermoleophilia* and two of its orders, *Gaiellales* and *Solirubrobacterales*, were enriched in this treatment. In comparison, the S2 treatment demonstrated an elevated relative abundance of the *Acidimicrobiia* class, while the S1 treatment did not show any significant differences compared to other treatments. The control was characterized by a dominance of *Firmicutes* and *Actinobacteria* phyla and the *Rhizobiaceae* family, and the control with
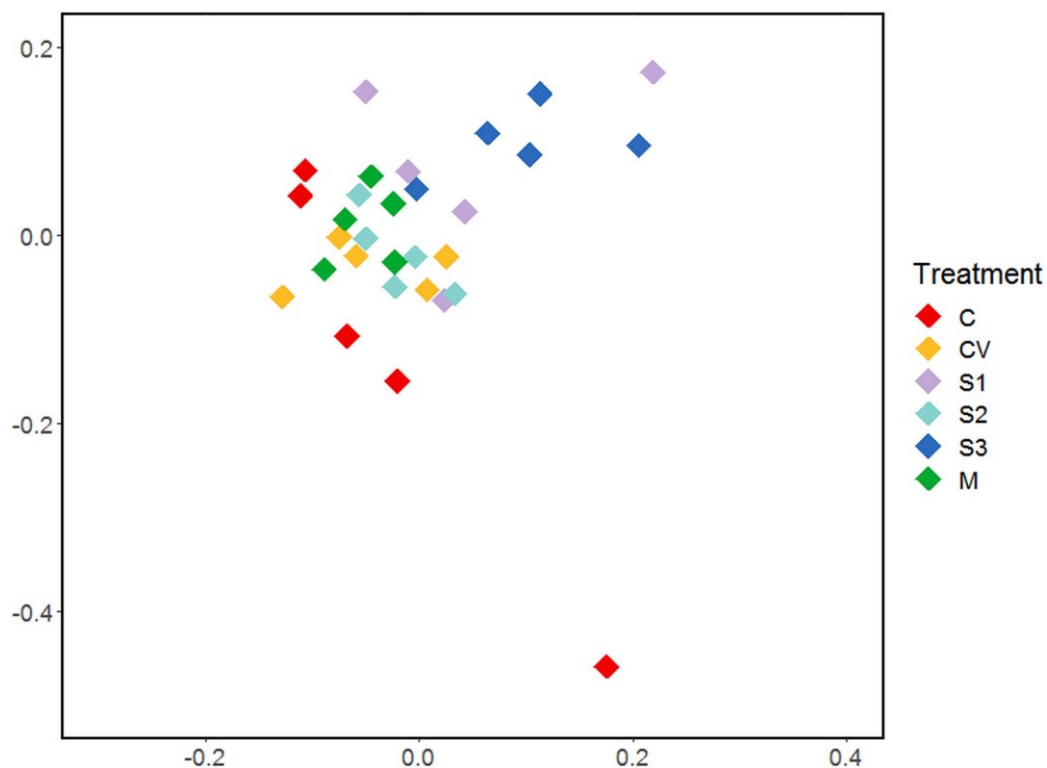

Fig. 2. Differences between soil communities of *Bacteria* and *Archaea* in the S1, S2, S3 and M treatment, the control (C) and the control with vermiculite (CV) assayed by Illumina MiSeq 16S rRNA gene amplicon sequencing. Non-metric multidimensional scaling was based on matrix of Yue-Clayton theta distances (stress value = 0.13).

vermiculite by enrichment of the order *Cytophagales* (Fig. S8).

Correlation networks based on analyses of co-occurrence of ZOTUs were determined for the S1, S2 and S3 treatment. The highest number of correlative interactions was found in the S3 treatment, followed by the S1 and S2 treatment. Furthermore, ZOTU 566 (corresponding to the 09ZI22 strain inoculated in the S3 treatment) demonstrated a higher number of correlations compared to ZOTU 299 and ZOTU 907, out of which 276 were positive and 381 negative (Table S4). Graphical representation of correlation networks of the ZOTUs corresponding to the inoculated strains in the three treatments compared to the control supported the dominance of interactions with ZOTU 566 and showed numerous positive and negative correlations, particularly with ZOTUs of *Acidobacteria*, *Actinobacteria* and *Proteobacteria* (Fig. S9). Further taxonomic assignment of ZOTUs which correlated with ZOTU 566 in the control and the S3 treatment revealed differences in the proportions of various families of positively and negatively correlated ZOTUs (Fig. S10).

3.5. Discriminant ZOTUs between controls and treatments

Treatments were evaluated based on the significantly different ZOTUs (Metastats, $p < 0.05$) compared to the control/control with vermiculite. The numbers of ZOTUs separating the controls and the treatments revealed considerable dissimilarities between the treatments. Mostly, the numbers of ZOTUs decreased in the treatments compared to the controls with the exception of the M treatment. The lowest numbers of the discriminating ZOTUs were between the control/control with vermiculite and the S2 treatment. The control and the S2 treatment differed by 549 ZOTUs (out of which 252 increased and 297 decreased in the S2 treatment) and the control with vermiculite differed from the S2 treatment by 455 ZOTUs (out of which 217 increased and 238 decreased in the S2 treatment). By contrast, the highest numbers of significantly different ZOTUs were between the control/control with vermiculite and the S3 treatment. The number of the discriminating ZOTUs was 1777 between the control and the S3 treatment (out of which 856 increased and 921 decreased in the S3 treatment) and 1824 between the control with vermiculite and the S3 treatment (out of which 864 increased and 960 decreased in the S3 treatment). The control and the control with vermiculite differed by 543 ZOTUs, out of which 287 increased and 256 decreased in the control with vermiculite (Fig. 3).

ZOTUs significantly contributing to the differences between samples were classified into taxa and proportions of the corresponding phyla were compared. Proportionally the most abundant ZOTUs separating the treatments and the control/control with vermiculite corresponded to bacterial phyla *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*, and archaeal phylum *Thaumarchaeota*. Generally, proportions of ZOTUs belonging to *Bacteroidetes* and *Firmicutes* slightly decreased, whereas the proportion of ZOTUs belonging to *Thaumarchaeota* increased in all treatments. Significantly different ZOTUs of other phyla responded variably to the individual treatments (Fig. S11A, B). Comparison between the treatments and the control with vermiculite also showed a largely increased proportion of the significantly different ZOTUs belonging to *Actinobacteria* in all the treatments (Fig. S11B).

A major decrease in the proportion of the discriminant ZOTUs of *Actinobacteria* as a result of vermiculite enrichment was also observed when the control and the control with vermiculite were compared individually. The control with vermiculite had also slightly lower proportions of ZOTUs belonging to *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia* and *Gemmatimonadetes* but had a higher proportion of the significantly different ZOTUs of *Proteobacteria*, *Acidobacteria*, *Thaumarchaeota* and *Chloroflexi* compared to the control (Fig. S12C).

The most successful treatment of the pot assay (S3 treatment) had a proportionally higher number of the discriminating ZOTUs belonging to *Actinobacteria*, *Verrucomicrobia* and *Thaumarchaeota* compared to the control and the control with vermiculite. However, an increase in the
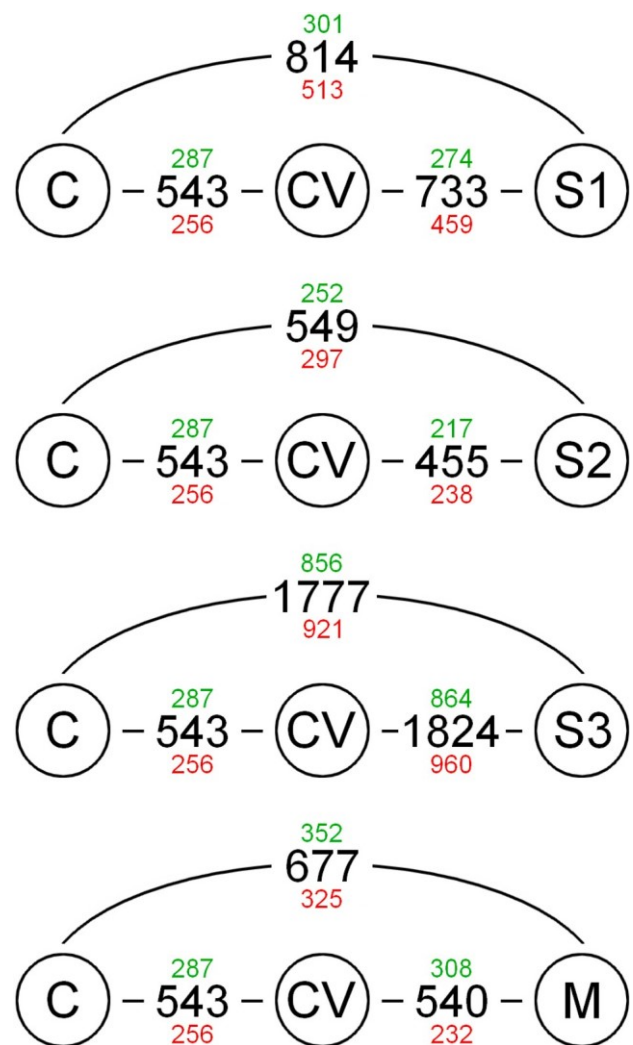

Fig. 3. Pairwise comparisons of soil communities in the control/control with vermiculite (C/CV) and the S1, S2, S3 and M treatments. Numbers in black indicate ZOTUs significantly contributing to the difference between samples in pairwise comparisons (Metastats, $p < 0.05$), out of which numbers in green represent ZOTUs increased in the treatment and numbers in red represent ZOTUs reduced in the treatment (or in the control with vermiculite in case of pairwise comparison of C and CV). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proportion of ZOTUs belonging to *Actinobacteria* was more noticeable when compared to the control with vermiculite than to the control. In contrast, proportions of ZOTUs separating the S3 treatment and the control/control with vermiculite, corresponding to *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, were decreased in the S3 treatment (Fig. S12A, B).

The most abundant ZOTUs significantly contributing to the separation between the treatments and the control/control with vermiculite, and between the two controls were further investigated. Considering the most abundant ZOTUs separating the S1 treatment and the controls, ZOTUs of *Thaumarchaeota* (ZOTUs 3, 10 compared to the control and ZOTU 3 compared to the control with vermiculite) were increased in the S1 treatment. ZOTUs of *Verrucomicrobia* (specifically *Candidatus* Udaeobacter; ZOTUs 26, 315 compared to the control and ZOTUs 26, 798, 900 compared to the control with vermiculite) were also mainly prevalent in the S1 treatment. Various ZOTUs of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes* and *Firmicutes* differed between the S1 treatment and the controls

(Table S5A, B).

When comparing the most abundant ZOTUs contributing to the differences between the S2 treatment and the controls, ZOTUs of *Thaumarchaeota* (ZOTUs 3, 10, 42 compared to the control and ZOTUs 3, 42, 919 compared to the control with vermiculite) were increased in the S2 treatment. Considering the differences at the lower taxonomic level, the class of *Acidimicrobiia* from *Actinobacteria* (ZOTUs 76, 30, 181, 403, 148, 159 compared to the control and ZOTUs 76, 181, 259, 148, 1504 compared to the control with vermiculite) and *Deltaproteobacteria* from *Proteobacteria* (ZOTUs 281, 185, 324 compared to the control and ZOTU 493 compared to the control with vermiculite) was dominant in the S2 treatment. Other phyla separated the S2 treatment and the control/control with vermiculite by diverse ZOTUs (Table S5C, D).

The S3 treatment was particularly abundant in ZOTUs of *Verrucomicrobia* (specifically *Candidatus* Udaeobacter; ZOTU 26 compared to the control and ZOTUs 26, 352 compared to the control with vermiculite) and *Thaumarchaeota* (ZOTUs 3, 2, 10 compared to both controls), and reduced in ZOTUs of *Gemmatimonadetes* (ZOTU 133 in both controls). Differences were also observed in ZOTUs of the same phyla. In the case of *Actinobacteria*, S3 treatment differed from the control by the dominance of ZOTUs belonging to the *Gaiellales* order (ZOTUs 13, 101, 395 compared to the control and ZOTUs 101, 368, 395 compared to the control with vermiculite) of the *Thermoleophilia* class, whereas controls were slightly increased in the *Pseudonocardiales* order (ZOTUs 93, 59, 356, 224 in the control and ZOTUs 59, 93 in the control with vermiculite) of the *Actinobacteria* class. Compared to the control/control with vermiculite, S3 treatment was also enriched in ZOTUs of the *Propionibacteriales* order (ZOTUs 177, 197 compared to both controls) from the class of *Actinobacteria*. From this order, ZOTUs of the *Nocardioidaceae* family were the most abundant. Regarding the difference in ZOTUs of *Proteobacteria*, the separation of the S3 treatment and the controls was particularly demonstrated by a representation of different bacterial orders from *Alphaproteobacteria* and *Gammaproteobacteria*. At the genus level, there was a prevalence of ZOTUs corresponding to the *Pseudolabrys* genus from the *Rhizobiales* order (*Alphaproteobacteria*; ZOTUs 186, 422 compared to both controls) in the S3 treatment, and several ZOTUs of the *Sphingomonas* genus from the *Sphingomonadales* order (*Alphaproteobacteria*) were also slightly increased in the S3 treatment compared to the controls (ZOTUs 58, 249 compared to the control and ZOTUs 58, 249, 536 compared to the control with vermiculite) (Table S5E, F).

ZOTUs that contributed to the difference between the M treatment and the controls corresponded to various phyla. Amongst the most abundant ZOTUs of the M treatment, ZOTUs belonging to *Thaumarchaeota* (ZOTUs 3, 10, 42 compared to both controls) predominated when compared to the controls. By contrast, both controls showed an increase in ZOTUs of *Firmicutes* (ZOTU 160 in the control and ZOTUs 164, 160, 209 in the control with vermiculite). At the class level, M treatment was enriched in ZOTUs of *Acidimicrobiia* (ZOTUs 76, 158, 181, 394, 190 compared to the control and ZOTUs 76, 158, 259, 661, 688, 2249 compared to the control with vermiculite). Other ZOTUs separating the M treatment and the controls corresponded to several phyla (Table S5G, H).

ZOTUs of various phyla participated in the separation of the control and the control with vermiculite. Considering discriminant ZOTUs of the most abundant phyla, ZOTUs from *Proteobacteria* were the most prevailing in the control with vermiculite, whereas *Actinobacteria* was the predominant phylum in the control. At the genus level, the control with vermiculite was particularly reduced in *Amycolatopsis* genus of *Actinobacteria* (ZOTUs 93, 356, 1826) (Table S5I).

Based on the heat maps, the effect of isolate addition only (comparison to the control with vermiculite) and isolate vermiculite enrichment (comparison to the control) in different treatments was observed. Regarding the S2 and M treatment, the most abundant ZOTUs in these treatments compared to the control were usually abundant in the control with vermiculite indicating the importance of vermiculite in those treatments (Table S5C, G). In contrast, in the S1 and S3 treatment,

the discriminant ZOTUs with the highest abundance in the corresponding treatments compared to the control were also strikingly increased compared to the control with vermiculite (Table S5A, E). Moreover, 16 out of 30 (*i.e.* 53%) and 22 out of 30 (*i.e.* 73%) most enriched ZOTUs in the S1 and S3 treatment, respectively, were identical when separation from the control and the control with vermiculite was performed. In the S2 and M treatment, only 6 out of 30 (*i.e.* 20%) and 8 out of 30 (*i.e.* 27%) most abundant ZOTUs, respectively, were identical when ZOTUs separating the treatments from the control and the control with vermiculite were compared.

4. Discussion

The need to effectively manage common scab and other soil-borne diseases has increased interest in biocontrol agents. In this study, three selected strains (S1, S2 and S3 treatments) were able to inhibit the growth of one or both CS pathogens (*S. scabiei* and *S. acidiscabies*) and had high growth rates *in vitro*. All three strains reduced CS severity; however, only reduction in the S3 treatment was statistically significant. The corresponding strain (09ZI22) showed a strong inhibitory effect (level 3 of inhibition) towards both CS pathogens *in vitro* (in contrast to 14HB3D and 14HB8C inhibiting strongly only *S. acidiscabies*) and reached the highest number of CFU/g in vermiculite cultivation out of all the isolates. Therefore, testing isolates on these two qualities may be a promising approach for a selection of an efficient biocontrol agent. It is consistent with a study of Liu et al. (1996), which concluded that isolates of *Streptomyces* with the ability to inhibit diverse CS pathogens and aggressive growth (forcefully growing in the presence of other strains) *in vitro* were more effective in CS control in field trials than those with poor growth and/or weak inhibition of pathogens. Our findings additionally suggest that the growth rate assessment, in particular, might be a suitable method of potential biocontrol strain evaluation as competition may be one of key mechanisms for CS suppression (Neeno-Eckwall et al., 2001).

Previously, the application of rhizosphere-derived microbial consortium (composed of *Bacillus subtilis* and *Trichoderma harzianum)* has shown a great potential in CS suppression (Wang et al., 2019). However, the predictability of biocontrol consortium effectiveness might be more challenging. In our work, all six strains of the mixture had a variety of favorable traits under laboratory conditions. Yet, the soil inoculation with the mixture (M treatment) did not suppress CS in the pot experiment, which may be explained by competitive interactions of the strains in the mixture. Besides, inhibition of indigenous antagonists by some strains of the mixture might also explain an insufficient reduction of CS severity in the M treatment (Kinkel et al., 2012).

In our study, vermiculite supplemented with cultivation medium served as a carrier that facilitated the colonization of soil by inoculated strains. The use of an inoculation carrier for antagonistic strains is an important aspect of CS suppression efficiency (Liu et al., 1995). Vermiculite is a clay mineral supporting proliferation and stability of bacterial populations (Graham-Weiss et al., 1987), and had already been used in biocontrol assays (Neeno-Eckwall et al., 2001; Sarwar et al., 2018; Wanner et al., 2014). However, compared individually to the control and all the treatments, the control soil with vermiculite was reduced in ZOTUs belonging to the phylum of *Actinobacteria*. Although a decrease of *Actinobacteria* proportion resulting from vermiculite enrichment has already been described (Barraza et al., 2020), the exact mechanism is unknown. A possible explanation is the involvement of complex interactions between vermiculite and soil microorganisms. Firstly, the filamentous morphology of some *Actinobacteria* may be a reason for poor release from vermiculite particles leading to lower DNA yield (Sagova-Mareckova et al., 2008), secondly, the presence of clay particles might affect the relative abundance of some genera due to specific surface interactions (Ding et al., 2013). Finally, the added clay might influence the disease suppressive character of soil as it might be dependent on the clay type (Almario et al., 2013). Thus, although the

stability of inoculated strains using vermiculite carrier was demonstrated by an increased numbers of sequences of S1, S2 and S3 strains compared to the control/control with vermiculite, the impact of vermiculite on indigenous communities should not be ignored, when applied to the fields. Moreover, a greater focus on the impact of vermiculite on several specific taxa is needed to fully understand the role of this otherwise promising carrier.

Biocontrol agents act directly as inhibitors of CS pathogens, but also indirectly through changes of the indigenous communities. The impact of antagonistic inoculants on soil communities has been suggested in CS suppression (Li et al., 2021; Wang et al., 2019) and agrees with the demonstrated connection between microbial community structure and CS severity in natural suppressive soils (Rosenzweig et al., 2012; Sagova-Mareckova et al., 2015). Here, all the treatments changed the tuberosphere community of *Bacteria* and *Archaea*. The alterations in soil communities were in particular demonstrated by differences in the relative proportion of certain taxa between treatments. To some extent, the inoculation might have an inhibitory effect on diverse taxonomic groups, which is indicated by an increased relative abundance of several taxa, such as *Bacillales* or *Rhizobiaceae*, in the control. In contrast, inoculation with antagonistic strains might modify microbial community by increasing the relative abundance of other taxa. For example, S2 treatment significantly differed from other treatments due to the dominance of *Acidimicrobiia*. Yet, the community of the S3 treatment was the most distinct as the majority of differentially abundant taxa were associated with this treatment, some of them possibly playing a role in the disease suppression. For example, representatives of *Gaiellales* and *Solirubrobacterales* enriched in the S3 treatment were previously shown to be prevalent in tuberosphere and bulk soils of medium CS severity compared to soils of high CS severity in susceptible cultivar Agria (Sagova-Mareckova et al., 2021). Moreover, tuberosphere of resistant cultivars was characterized by dominance of these orders suggesting they are a non-negligible part of tuberosphere community in the process of CS suppression. In addition to the most distinct changes in abundance of various taxa, interaction network of microbial community exhibited a higher complexity in this treatment, which corresponds to more complex co-occurrence networks in CS-suppressive soils (Kopecky et al., 2019; Shi et al., 2019). Thus, the most successful S3 treatment is not only a potential biocontrol strain but also an agent capable to significantly alter soil microbial community, which potentially contributes to the suppressive character of the treated soil.

Although the composition of indigenous communities differed between the treatments and the control/control with vermiculite, the application of antagonistic isolates or vermiculite did not have a significant effect on the diversity. Thus, microbial diversity was not a predictive factor of CS suppression in this work consistently with the study of Sarikhani et al. (2017) but contrary to other studies, where higher bacterial diversity was observed in suppressive compared to conducive soils (Rosenzweig et al., 2012; Shi et al., 2019). However, in all those and several other studies, the relative abundance of some bacterial taxa was connected to the disease suppression (Mendes et al., 2011; Wang et al., 2019).

In this study, the most successful S3 treatment was characterized by the enrichment of ZOTUs of *Thaumarchaeota* and *Verrucomicrobia* compared to the controls. Regarding *Actinobacteria*, the relative proportion of this phylum was also slightly raised, which corresponds with a higher relative proportion of *Actinobacteria* in suppressive soils (Kopecky et al., 2019; Tomihama et al., 2016) and increased numbers of suppressive streptomycetes connected to CS (Meng et al., 2012). However, the previous studies demonstrated only minor changes in the proportion of *Verrucomicrobia* in CS-suppressive soils, which might be related to specific soil conditions (Sagova-Mareckova et al., 2015).

At the order level, several ZOTUs of *Sphingomonadales* (*Alphaproteobacteria*) were dominant in the S3 treatment compared to the controls and *Sphingomonas* was the most enriched genus. This genus has been associated with the suppression of several soil-borne diseases (Ou et al.,

2019; Wei et al., 2019) including CS (Shi et al., 2019). The role of *Sphingomonas* in disease suppression may be relevant as several strains of *Sphingomonas* possess plant growth-promoting activities, which may result in induced systemic resistance against plant pathogens (Khan et al., 2014; Yang et al., 2014). In *Actinobacteria*, ZOTUs from *Gaiellales* and *Propionibacterales* increased in the S3 treatment, the latter constituted particularly by the *Nocardioidaceae* family, which was previously related to CS suppression (Rosenzweig et al., 2012). In addition, the *Nocardioides* genus was more abundant in the tuberosphere of potatoes with reduced CS severity (Li et al., 2021). Similarly, *Nocardioides* representatives might indicate participation in the disease suppression due to their plant growth-promoting properties (Yadav, 2017). Finally, a substantial dominance of *Candidatus* Udaeobacter from *Verrucomicrobia* in the S3 treatment may be related to its multidrug resistance and an increase in the abundance after antibiotic exposure in the soil (Willms et al., 2020), which may represent a competitive advantage for this genus.

The dominance of several taxonomic groups compared to the controls was similar in the S3 and S1 treatments. However, since the effect of the S1 treatment on CS severity was minor, the results could be attributed to the less distinct changes of the whole soil community compared to the S3 treatment. In contrast, an apparent dissimilarity of the S3 treatment from the S2 and M treatments was particularly due to an increase of *Deltaproteobacteria* (*Proteobacteria*) and/or *Acidimicrobiia* (*Actinobacteria*) and as these treatments showed the least biocontrol activity in the pot assay, we might conclude that those taxa interfered with CS suppression.

Finally, in *Proteobacteria*, the representatives of *Alphaproteobacteria* and *Gammaproteobacteria* occurred in treatments reducing CS severity although both classes were also dominant in the control/control with vermiculite. *Betaproteobacteria* were important component of suppressive soils and some representatives of this class (in this study reclassified as the order of *Betaproteobacteriales* within *Gammaproteobacteria*; Parks et al., 2018) were dominating in the S3 treatment. However, betaproteobacterial taxa *Pseudomonadaceae* and *Burkholderiaceae* previously associated with the disease suppression soils (Mendes et al., 2011; Rosenzweig et al., 2012; Sarikhani et al., 2017) were not prevalent in the S3 treatment. Overall, the observations suggest that antimicrobial activities of the inoculated strain and/or its cooperation with some indigenous antagonists made a major contribution in the CS suppression. All these findings confirm that disease suppressiveness is attributed to microbial consortia rather than a single bacterial taxon or group (Kyselková and Moënne-Loccoz, 2012; Mendes et al., 2011).

Composed plant nutrition is an important factor in plant growth and disease resistance, and the control of numerous diseases may be maintained by proper nutrient management (Huber and Haneklaus, 2007). Soil nitrogen available to plants is primarily produced by the decomposition of dead organic matter or nitrogen fixation by symbiotic and free-living bacteria or archaea, while denitrification, enhanced leaching of nitrogen (nitrates) as a result of nitrification and competition for nutrients may reduce N availability (Van Der Heijden et al., 2008). Thus, changes in the composition of the soil community may considerably alter nitrogen status in the soil environment, which could be a reason for dissimilarities in nitrogen content of potato leaves in different treatments of this study. Nitrogen content in potato leaves significantly decreased in the M treatment compared to the S1 and S2 treatments; however, N content in the most successful S3 treatment did not differ from the other treatments. Hence, it seems that nutrient-balanced systems required by specific potato cultivars are responsible for protection against CS rather than an increase or decrease of a single nutrient (Krištůfek et al., 2015). That assumption may be also relevant for phosphorus content since a significantly lower content of phosphorus occurred only in the M treatment, which may be explained by the reduction of microorganisms that enhance P availability for plants. Compared to the control with vermiculite, M treatment was reduced in ZOTUs that include strains with the P solubilization capability, such as

Bacillus (Raj et al., 2014) or *Paenibacillus* (Wang et al., 2012; Mercl et al., 2018). Overall, we determined that inoculation with different antagonistic strains influenced plant nutrition, which could be potentially relevant in CS suppression (Kopecky et al., 2021). Furthermore, we found that inoculation had an effect on the ammonia-oxidizing archaea, specifically *Thaumarchaeota*. All the treatments demonstrated an increase in the proportion of ZOTUs belonging to this phylum; however, comparison of all the relevant treatments revealed a significant enrichment of this archaeal group only in the S3 treatment. Although we cannot conclude that the dominance of *Thaumarchaeota* is directly related to CS suppression, alterations in ammonia-oxidizing archaea might change the proportion of bacteria, which is crucial for nitrogen cycling under various conditions (Clark et al., 2020; Huang et al., 2014; Mukhtar et al., 2019).

5. Conclusions

The study identified the *in vitro* characteristics of actinobacterial isolates that might help to predict biocontrol activities against CS *in vivo*. Out of the selected strains, strain 09ZI22 corresponding to the S3 treatment was the most effective biocontrol agent. The strain significantly reduced CS severity and substantially changed the soil prokaryotic community, presumably towards CS suppression. The present study provides a deeper insight into the role of certain microbial taxa in disease suppression and also highlights the importance of biocontrol agent inoculation on plant nutrition. Overall, the comprehensive assessment of the complex relationships of biocontrol agents with the host plant, pathogens and indigenous soil communities might be beneficial in selecting a suitable biocontrol candidate in various agricultural systems. In the future, a field assay should be applied to evaluate the impact of biocontrol agent on a larger scale.

Declaration of competing interest

All authors declare no conflict of interest.

Availability of data and material

Illumina MiSeq amplicon sequences of 16S rRNA genes are available in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) as BioProject PRJNA746603.

CRediT authorship contribution statement

T.P. analyzed the sequences and wrote the manuscript. I.K. conducted the experiment and extracted soil DNA, D.R. analyzed the data, S. H. extracted DNA, J.K. and M.S.-M. organized the experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104491.

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