The University of South Bohemia in České Budějovice

Faculty of Science

How endophytic actinobacteria respond to pharmaceuticals present in soil.

Bachelor's thesis

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Annotation:

Through the continuous input of antibiotics into the environment by recycling waste from wastewater treatment plants as biosolids, antibiotics start to disseminate in soil, which can lead to the spread of antibiotic resistance in resident microbes. This thesis studies the effects of different soil amendments on the frequency of antibiotic resistance in endophytes present in *Phaseolus vulgaris*.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 08.05.2024

……...

Runa Weissengruber

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Abstract

Biosolids derived from wastewater treatment plants are utilized in agriculture to enhance the content of nutrients and organic carbon in soil. Yet, they also serve as reservoirs for various micropollutants, including antibiotics. This results in the spread of antibiotic-resistance genes among soil microorganisms, impacting the bacterial community inhabiting the plant rhizosphere and subsequently the endosphere. This study investigates the response of endophytic actinobacteria, isolated from differently managed soils to commonly employed antibiotics.

Plants of *Phaseolus vulgaris* were grown in soil beds irrigated with (i) tap water, (ii) treated wastewater and amended with biosolid, and (iii) treated wastewater and amended with composted biosolid. Bacteria from the harvested plants were isolated, identified by their 16S rRNA, and assessed for antibiotic resistance by the disk diffusion technique.

Our findings revealed a distinct trend in the frequency of antibiotic resistance among the evaluated isolates, with the highest frequency observed in the control treatment using tap water and the lowest in isolates from soils amended with composted biosolid. This trend is further suspected to be influenced by the identified genus.

Notably, cephalosporins showed the highest frequency of resistance, however, the frequency of susceptibility was increased by the addition of a beta-lactamase inhibitor. Furthermore, the analysis showed a high correlation between the antibiotic responses of endophytic isolates and their clinical counterparts.

However, due to the limited number of analyzed isolates, statistical significance could not be established. To address this limitation, future research will aim to analyze a larger, more diverse pool of isolates to provide a better understanding of these relationships.

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1 Introduction

1.1 Environmental pollution

Over the last decades, environmental pollution through the emission of pharmaceuticals has become an increasing source of concern (Wilkinson et al., 2022), as these compounds continuously find their way into the environment, from their production stages to their eventual disposal. Their presence in the ecosystem stems from incomplete digestion within the gastrointestinal tract, causing their excretion via feces from both humans and animals. Upon their breakdown, their transformational products often exhibit extended half-lives compared to their parent compounds (Berkner & Thierbach, 2014). Despite standard municipal water treatment protocols, these processes are inadequately equipped to fully eliminate pharmaceutical residues. Consequently, these residues find their way into the environment through treated wastewater discharge or amalgamate with the soil through biosolid application as a method for fertilization, thereby aggravating environmental contamination (Biel-Maeso et al., 2018; Tiedje et al., 2019).

1.2 Wastewater treatment

In wastewater treatment plants, collected wastewater undergoes primary, secondary, and optionally tertiary treatment to remove contaminants and create an effluent, which meets broadly specified criteria for its reintroduction into the environment. During primary treatment, solid waste is separated through mechanical processes, firstly by gratins and secondly by sedimentation. In secondary treatment (also known as biological treatment) nutrients are removed using bacterial degradation processes under either aerobic or anaerobic conditions. Large amounts of insoluble organic material are usually degraded by anaerobic digestion driven by bacteria and archaea in the absence of oxygen. With reduced organic content, aerobic microbes are utilized through practices with activated sludge, which is created by the aeration of primarily treated wastewater. There are several processes used for the purification of wastewater including oxidation, absorption, and inclusion of organic material into flocs. In most cases, the treated wastewater can be discharged into surface waters after secondary treatment (European Legislation, 1991; Madigan et al., 2018).

The treatment of wastewater is especially important in more arid regions as the recycled water yields a constant and reliable source for irrigation without over-extracting groundwater or decreasing the available amount of freshwater. Furthermore, the disposal of larger volumes

without purification could lead to contamination of surface water, such as rivers, lakes, or streams, from the excess of nutrients, possibly leading to eutrophication (Madigan et al., 2018; Parsons et al., 2010; Toze, 2006).

1.2.1 Biosolids

The process of water purification gives rise to biosolids or sludge, which differs from sewage sludge through treatment with microbes or physical processes (Collivignarelli et al., 2019). The reclaimed organic and inorganic nutrients from wastewater treatment plants can further serve as fertilizer in agricultural settings in the form of biosolids, resulting in enhanced physical and chemical soil properties. This subsequently leads to increased metabolic activity of microorganisms present, improving soil conditions (Hudcová et al., 2019; Parsons et al., 2010). Microbial activity in soils is further influenced by seasonal changes since with warmer temperatures a reduced concentration of active compounds was observed, which is explained by an enhanced degradation during the summer months (Biel-Maeso et al., 2018; Cycoń et al., 2019).

The most commonly found traces in biosolids are polycyclic aromatic compounds, sterols, detergent metabolites, pharmaceuticals, and synthetic fragrances (Kinney et al., 2006). Particularly untreated biosolids contain various pathogens, requiring further treatment to fully achieve hygiene standards. Still, the characteristics of biosolids mostly depend on their origin and water content. Furthermore, it was shown that the amendment with biosolids significantly contributes to the total amount of nitrogen and carbon in the soil (Collivignarelli et al., 2019; Hu et al., 2019; Hudcová et al., 2019; Kumar et al., 2017; Park et al., 2013). Upon comparison to soil amended with mineral fertilizer, biosolid application might also decrease the leaching of nitrogen from the soil, leading to reduced environmental pollution in general (Hu et al., 2019). Before its introduction to agricultural soils, biosolids undergo stabilization treatments, one of which is composting, which ensures nutrient stabilization and minimizes phytotoxicity. Through the formation of aggregates and more complex structures nutrient retention is enhanced and leaching is prevented. Higher levels of aromatic aggregates are observed particularly with straw and mulch types, indicating increased retention, due to the mineralization of organic matter. (Pavlů, Balík, et al., 2023; Pavlů, Zádorová, et al., 2023; Piccolo et al., 2004; Thai et al., 2021, 2022).

1.3 Risks associated with the usage of biosolids in agriculture

As mentioned in Chapter 1.2.1, the use of biosolids as fertilizer increases the availability of nutrients in soils considerably. Still, its utilization raises serious concerns about the

environment's health, as the accumulation of harmful toxins and contaminants in our ecosystem rises as a result. Additionally, there's fear of a decrease in biodiversity, from the presence of antimicrobial components to the release of greenhouse gases and unpleasant odors (Collivignarelli et al., 2019). Furthermore, apprehension is expressed concerning the spread of pathogenic microbes as they can show persistence through endospores or through entering a viable but non-culturable (VBNC) state (Al-Gheethi et al., 2018).

1.3.1 Presence of heavy metals

Heavy metal contamination, particularly by cadmium, is a common issue associated with biosolid application. Heavy metals have been linked to antibiotic resistance, with co-selection mechanisms facilitating the spread of resistance genes (Seiler & Berendonk, 2012). This is further extended by Oyetibo et al. (2010), which correlated high frequencies of heavy metal resistance with increased occurrences of resistance against antibiotics in surface water. Furthermore, strategies such as the use of biochar have shown a reduction in the migration of heavy metals and antimicrobials, thereby reducing potential environmental risks associated with manure application (R. I. Aminov & Mackie, 2007; Bair et al., 2020; Hudcová et al., 2019; Ji et al., 2012; Li et al., 2020; Lucia Azevedo Silveira et al., 2003; Nguyen et al., 2019; Zhou et al., 2016).

1.3.2 Micropollutants

The application of biosolids on agricultural land may also lead to long-term changes in soil organic matter composition, with certain constituents aggregating over time. Studies have shown the accumulation of pharmaceutical residues, including analgesics, anti-inflammatories, and antibiotics, in soil amended with biosolids (Biel-Maeso et al., 2018; Pavlů, Balík, et al., 2023). While treatments with both composted and stabilized sewage sludge showed less leaching compared to effluent-treated soil, concerns remain regarding the migration of contaminants (Kodešová et al., 2024). The most present pharmaceuticals leached to the soil through biosolid application in Slovakia were identified as antihistamines (45.4%) followed by cardiovascular (21.2%), and psychoactive drugs (14.5%). In contrast, antibiotics had a relative abundance of 1.6%, arguably from high degradability or low sorption to sludge (Ivanová et al., 2018). The inefficient removal of active pharmaceutical compounds during wastewater treatment further poses a threat of potential antibiotic resistance issues that can negatively impact the food chain (Lucas et al., 2016). This connection is further accentuated

by the abundance of antibiotic-resistance genes in plant tissues (Araújo et al., 2017; Zhang et al., 2019).

1.3.1 Counter measurements

Long-term studies, such as that by Hu et al. (2019), have also documented shifts in microbial communities over time due to excessive biosolid application, posing a threat to soil microbes by influencing their variety, community structure, and metabolic activity. While moderate biosolid usage may enhance microbial diversity and richness, excessive application may lead to a reverse trend (Mossa et al., 2017). A promising countermeasure to increase the quality of biosolids for agricultural usage is the introduction of a composting process, as mentioned in Chapter 1.2.1. Whilst the importance of removing pharmaceuticals has been addressed only recently, both adsorption on activated charcoal and oxidative/reductive processes, which are promising, still need to be adapted for industrial scales. Active compounds further show a response to photodegradation because of aromatic and heteroaromatic constituents (Rivera-Utrilla et al., 2013).

In the European Union, Council Directive 86/278/EEC promotes the incorporation of sewage sludge in agriculture, provided that the quality of soil is not impaired. Although the heavy metal content is limited due to its known toxicity, no universal standards have been established for organic micropollutants, posing potential environmental risks (European Legislation, 1986).

1.4 Antibiotics as environmental contaminants

Antibiotics are small molecules mainly used to treat bacterial and fungal infections or occasionally to modulate immune responses. The main modes of action of antibiotics are (i) cell wall damage known as lysis, (ii) the inhibition of protein synthesis, (iii) the inhibition of RNA/DNA replication/synthesis, and (iv) the inhibition of metabolic pathways specifically concerning the folic acid synthesis (Chess, 2024). Furthermore, antibiotics can be classified after their antimicrobial action which includes bacteriostatic or bacteriocidic mechanisms. Bacteriostatic antibiotics include groups that prevent bacterial growth as opposed to bacteriocidic antibiotics, which act to kill bacteria (Nemeth et al., 2015).

Antibiotics are categorized as pseudo-persistent substances, as they pose a persistent challenge due to their continuous introduction into the environment. One such input is the application of biosolids to the soil and irrigation with wastewater, leading to widespread

contamination as previously mentioned in Chapter Biosolids1.2.1 (Chess, 2024; Cycoń et al., 2019; Pan & Chu, 2017). Even though the importance of sorption as a removal pathway for antibiotics was proven previously, in experiments with sterilized and non-sterilized biosolids some antibiotics exhibited persistence. This is associated with irreversible sorption mechanisms, which lead to the reduction of their bioavailability and hence their microbial degradation. Especially ciprofloxacin showed notable persistence in sterile and non-sterile biosolids (Wu et al., 2009). It is also already shown that the affinity of compounds to be sorbed in the soil is higher for positively charged compounds, followed by neutral compounds, and lastly negatively charged compounds. Generally, the adsorption to soil fits the principle of the Freundlich isotherm and depends largely on soil types (Biel-Maeso et al., 2019; Kodešová et al., 2024). Studies have thus demonstrated their persistence even at sub-zero temperatures over extended periods, underscoring their continual presence (Magee et al., 2018).

1.4.1 Antibiotic resistance

Intrinsic resistance represents a natural mechanism of antibiotic resistance in microbes that is unrelated to horizontal gene transfer and has evolved independently of selective antibiotic pressure in the environment (R. I. Aminov & Mackie, 2007; Rolain, 2013). In contrast, the much more common acquired resistance and its spread is driven by environmental factors, in particular the presence of antibiotics in the environment and the spread of mobile genetic elements, that carry the resistance genes (Partridge et al., 2018). This enables the exchange of these genes among bacterial communities, even under low (subinhibitory) antibiotic concentrations (Gullberg et al., 2011). Horizontal gene transfer and the subsequent selection of resistant populations are thus usually accelerated by the occurrence of antibiotics in the environment (Bengtsson-Palme et al., 2018).

Antibiotic resistance poses one of the biggest threats to human, animal, and environmental health, as the increased occurrence of diseases from resistant pathogens has become a growing problem (Finley et al., 2013). The unregulated use of wastewater in developing countries exacerbates this issue, because of its contamination with pollutants and antibiotic-resistance genes (Onalenna & Rahube, 2022). This is further elaborated by the One-Health concept, which connects the risks of antibiotic resistance with human, animal, and environmental well-being (Aslam et al., 2021). Due to the inadequate usage and disposal of antibiotics, the number of antibiotic-resistant bacteria has increased over the last decades (Lee Ventola, 2015). This resulted in the lack of effective treatment for some bacterial infections,

specifically concerning ESKAPE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species* (Rice, 2008). As a result, mortality rates have risen significantly (Finley et al., 2013; Michael et al., 2014). In the past, a major focus was laid on the evolution of resistant pathogens in clinical environments, leading to a lack of research done on their connection to the ecosystem, neglecting the traceback of these circumstances to the soil and water biomes (Allen et al., 2010; Finley et al., 2013; Tiedje et al., 2019).

1.5 Plant endophytes

Endophytes are present all over the globe and are known for colonizing the plants' inner compartments without causing damage or disease. Furthermore, they help with enhancing the availability and stability of nutrients for the plant. (Ameen et al., 2024; Santoyo et al., 2016). Endophytic bacteria, originating from the soil, typically access the inside of the plant's roots through fissures in the plant's rhizosphere. Because of this alternation between the environment (specifically the soil) and the plant, endophytic bacteria are thus described as biphasic. As endophytes, they do not alter their host's external structure, since only the internal parts of the plant get colonized. The bacteria benefit from this relationship through the utilization of the plant for nutritional purposes as well as through a more stable environment compared to soils (Hardoim et al., 2008; Madigan et al., 2018; Vurukonda et al., 2018; Yadav, 2017).

1.5.1 Actinobacterial endophytes

The main species of endophytic actinobacteria improving soil fertility and plant growth are various *Streptomyces* spp. Actinobacteria in general is a group of aerobic, high GC, gram-positive bacteria commonly found in soil that can often generate filaments. Filamentous actinobacteria can build up a mycelium consisting of vegetative hyphae from spores (Madigan et al., 2018; Wahyudi et al., 2019). Specifically, *Streptomyces* spp. possess the ability to produce immunosuppressants and antibiotic compounds thus being one of the largest antibiotic-producing classes to oppose other bacteria, fungi, and parasites. As endophytes, these mechanisms are further utilized to reduce the plant's biotic stress. (Hardoim et al., 2008; Madigan et al., 2018; Vurukonda et al., 2018). Still, some species function as pathogens, such as *Streptomyces scabies* which cause potato scab (Loria Rosemary et al., 1997).

1.5.2 Environmental influence on endophytic actinobacteria

Under the influence of effluent/biosolids, microbes showed signs of gradual stimulation, which influenced the microbe's activity, structure, and resistance gene abundance (Fér et al., 2024). Studies conducted by Yang et al. (2018) have further shown that the application of sewage sludge stimulates endophytes in lettuce and possibly triggers the development of drug resistance, with the extent of this effect depending on the quantity of sewage sludge applied. Furthermore, the abundance of antibiotic-resistance genes was found to be higher in the plant's rhizosphere compared to the phyllosphere. This confirmed the relationship between direct contact with soil amendments and the frequency of resistance (Zhang et al., 2019). In addition, the phyllosphere of organically grown crops showed a significantly higher and more diverse amount of antibiotic resistance genes than conventional ones. However, no considerable difference between organically and conventionally grown products was found when comparing their rhizosphere (Zhu et al., 2017).

Particularly, in the presence of tetracyclines, an increased production of stress proteins was observed in *Phaseolus vulgaris*, resulting in their general recline. Through the low biodegradability of tetracyclines, they further exhibit a prolonged persistence in water flows (Fiaz et al., 2021; Pan & Chu, 2017). The global issue of the spread of antibiotic-resistant genes is further aggravated by its presence in agricultural products. Concerns were raised specifically regarding leafy vegetables, such as lettuce and bok choy (Guo et al., 2021). As antibiotic-resistant endophytes have shown to persist throughout washing and disinfection processes, concerns have also been raised about the consumption of raw vegetable products. Of particular concern is their potential to transmit pathogens, since upon ingestion of contaminated products, antibiotic-resistant bacteria may colonize the gut microbiome and integrate with it (Scaccia et al., 2021).

2 Aims

This bachelor thesis aims to investigate the effect of the products from wastewater treatment plants, contaminated with various micropollutants, on endophytic actinobacteria present in *Phaseolus vulgaris*. The plants were grown in soil fertilized with biosolid and composted biosolid and watered with treated wastewater. Irrigation with tap water provided a control treatment.

We hypothesized a higher frequency of resistant endophytes isolated from the soils amended with biosolids compared to the control treatment. In addition, we expected that the composting process would decrease the frequency of resistant endophytes. Antibiograms of actinobacterial endophytes will be compared to those obtained for clinical and environmental isolates to identify specific resistance patterns caused by biosolid amendments.

This will be evaluated through:

i. The identification of actinobacteria isolated from the xylem sap of *Phaseolus vulgaris* based on 16S rRNA similarity
ii. The exclusion of identical isolates using fingerprinting methods (Box PCR).
iii. The testing of the isolates for antibiotic susceptibility testing (AST) using the disk diffusion technique.

3 Materials and Methods

The plant material was collected in an experiment held at the wastewater treatment plant (WWTP) Hrdějovice, which is treating wastewater from the regional city České Budějovice (Czech Republic). The experiment started in 2021 where raised beds filled with Cambisol were treated in three different ways:

1. Soil irrigated with tap water, which acts as a control (CMW).
2. Soil amended with anaerobically digested biosolid and irrigated with treated wastewater (CMB).
3. Soil amended with composted biosolid and irrigated with treated wastewater (CMC).

The presence of pharmaceutical residues in used biosolids, soil characteristics, and experimental settings have been published previously in Fér et al. (2024) and Kodešová et al. (2024). The irrigation continued daily during vegetation, while the biosolid or composted biosolid was amended to the soil only before plantation. The xylem sap for bacterial isolations was collected from plants of *Phaseolus vulgaris* harvested in autumn 2022 by a Scholander pressure bomb, and subsequently cultivated on different kinds of cultivation media.

3.1 Bacterial strains

The bacterial strains were isolated previously by Ing. Lucie Kotrbová and stored in a glycerol stock solution in the freezer (-76°C for long-term storage; -18°C for short-term storage). Strains were recovered by cultivation on ISP2 agar (Shirling & Gottlieb, 1966) at 28°C for 7 days. The streak plate method was used to check the strain purity.

3.2 Media preparation

3.2.1 ISP2

The solid ISP2 media was prepared as follows:

Table 1 Composition of ISP2

Yeast extract	4.0 g
Malt extract	10.0 g
Dextrose	4.0 g
Agar	20.0 g

Distilled water	1000.0 ml

(Shirling & Gottlieb, 1966)

Weighted, dehydrated components were dissolved in distilled water and the pH was adjusted to 7.2 while stirring. The medium was autoclaved at 120°C/20 min and aseptically poured into Petri dishes (10 mm in diameter) after being cooled down to approximately 55°C.

3.2.2 ISP3

For the antibiotic susceptibility testing (AST), the ISP3 medium described by Shirling & Gottlieb (1966) was used as follows:

Table 2 Composition of ISP3

Oatmeal	20.0 g
Agar	18.0 g
Trace salt mix (1g/L $FeSO_4 \cdot 7H_2O$, 1g/L $MgCl_2 \cdot 4H_2O$, 1g/L $ZnSO_4 \cdot 7H_2O$)	1.0 ml
Distilled water	1000.0 ml

(Shirling & Gottlieb, 1966)

To the ready-to-use medium (HiMedia, Maharashtra, India.) the trace salt mix was added. This was sterilized by autoclaving at 120°C/20 min and poured into Petri dishes (10 mm in diameter) aseptically after being cooled down under stirring to approximately 55°C.

3.2.3 LB (Luria-Bertani) Medium (DSMZ no. 381)

The liquid LB medium was used to obtain bacterial biomass for further DNA isolation. The medium was prepared as follows:

Table 3 Composition of L.B medium

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

(DSMZ, 2007)

3.3 Antibiotic susceptibility testing (AST)

For the evaluation of the isolate's susceptibility to antibiotics, the disk diffusion method on Müller-Hinton agar was used. The tested strains were pre-cultivated on ISP3 at 28°C for seven days. All steps of the AST were performed aseptically in a Biohazard flow box.

For the inoculation, a heavy suspension was prepared by vortexing bacterial colonies in an Eppendorf tube containing glass beads and 500 µL of a 0.9% sterile saline solution. The heavy suspension was then used to prepare a 0.5 McFarland suspension in a glass tube using the DEN-1, McFarland densitometer (Biosan, Riga, Latvia). Before inoculation, the previously prepared bacterial solution was vortexed and 200 µL was applied on a Müller-Hinton agar 90-mm plate (Dulab, Dubné, Czech Republic). The suspension was spread evenly using a sterile glass hockey stick.

For the AST, antibiotic disks (Bio-Rad, Hercules, CA, USA) were applied using a disk dispenser with a maximum of three disks per plate. The antibiotics tested, including the abbreviations used in this thesis, and their disk content are listed in **Table** *7*. Strains were cultivated at 28°C until fully grown. All strains were tested in two replicates.

3.3.1 Reading the results

The diameter of the inhibition zones was measured after 24 and 48 hours or after full growth. The values measured after the shortest cultivation period were included, except for the antibiotic Trimethoprim-sulfamethoxazole (SXT), whose values were measured after 48 hours as the shortest cultivation period.

3.4 Preparation of the cell lysates

To prepare the cell lysates, bacterial spore clusters were harvested from an ISP2 plate and suspended in 200 µL of ultra-pure water. The suspension was then frozen (-20°C) and boiled at 95°C for 5 min repeatedly (three times in total). The lysed suspension was stored in the freezer and optionally diluted before use to decrease the possible presence of PCR inhibitors.

3.5 DNA isolation

The bacterial biomass for the genomic DNA isolation was prepared by cultivating 20 ml of LB medium in 100 ml Erlenmeyer flasks at 28°C for four days at 120 rpm. The final biomass was then collected by centrifugation. An approximate amount of 40 mg of biomass was used

to isolate genomic DNA following the protocol of the NucleoSpin Microbial DNA Mini kit for DNA from microorganisms (Macherey-Nagel, Düren, Nordrhein-Westfalen Germany). The quality and quantity of the resulting DNA were tested using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at the modes A260, A280, or A260/A280. The isolated DNA was then stored at -20°C.

3.6 PCR amplification of the 16S rRNA gene

The amplification was performed using a primer pair (**Table 5**) designed by Edwards et al. (1989) and PCR conditions as previously reported by Kyselková et al. (2012). The composition per reaction is displayed in **Table 4**. The expected size of the PCR product was 1500 bp.

Table 4 Composition for PCR amplification per template

MilliQ water	10 µl
10 µM pA (forward)	0.75 µl
10 µM pH (reverse)	0.75 µl
Fast Start Master 2x (Roche, Basel, Switzerland)	12.5 µl
Template DNA	1 µl

(Kyselková et al., 2012)

Table 5 Primer Sequences

pA (forward)	5' AGA GTT TGA TCC TGG CTC AG 3'
pH (reverse)	5' AAG GAG GTG ATC CAG CCG CA 3'

(Edwards et al., 1989)

The PCR started with an initial denaturation step at 95°C for 3 minutes, followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 66°C for 30 seconds, and elongation at 72°C for 1 minute 30 seconds. As the last step the final elongation at 72°C for 5 minutes was performed.

The result of the PCR amplification was visualized by gel electrophoresis with a 1% agarose gel. Each well was loaded with 5 µL of a mix of 5 µL of the PCR-product and 1 µL DNA Gel

Loading Dye (6x) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) except one well, which was loaded with 5 µL GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The gel electrophoresis was developed at 110 V for 40 minutes. The resulting gel was then stained using ethidium bromide and visualized by the Azure 280 (Azure Biosystems, Dublin, California, United States).

3.7 Identification of the isolates

For the identification, the 16S rDNA PCR products were decoded in a sequencing facility (SEQme s.r.o., Dobříš, Czech Republic) using Sanger sequencing after enzymatic cleanup by CleanSeq. Alike isolates were further tested for similarities using Box PCR leading to the exclusion of isolates.

3.8 BOX-PCR

To evaluate only the unique endophytes, isolates identified to the same species by 16S rRNA similarity were further tested by BOX-PCR (Lanoot et al., 2004). The composition per reaction is displayed in **Table 6** Composition for Box-PCR per template.

Table 6 Composition for Box-PCR per template

MilliQ water	6.8 µL
2x LA Hot Start Master Mix (TopBio, Vestec, Czech Republic)	12.5 µL
DMSO (c=100%)	2.5 µL
BSA (5 mg/mL)	0.2 µL
BOX A1 primer (0.3µg/µl)	1 µL
DNA template	2 µL

(Lanoot et al., 2004)

The reaction included an initial denaturing step at 95°C for 7 minutes, followed by 30 cycles of denaturing at 90°C for 30 seconds, annealing at 53°C for 1 minute, and elongation at 65°C for 8 minutes. The final elongation was induced at 56 °C for 15 minutes. The results were visualized using gel electrophoresis on a 2% agar gel, at 110 V, for 1.5 hours.

3.9 Data analysis

The average zone diameter values were calculated, and the isolates were assigned as susceptible (S), intermediate (I), or resistant (R) according to breakpoint values listed in **Table** 7.

Table 7 Antibiotic breakpoints for actinobacteria

Antimicrobials	Abbreviation	Antibiotic class	Disk content	Zone diameter breakpoints (mm)		
				R≤	I	S ≥
Clarithromycin	CLR	Macrolide	15 µg	21	22-25	26
Erythromycin	ERY	Macrolide	15 µg	22	23-28	29
Tetracycline	TET	Tetracycline	30 µg	27	28-31	32
Minocycline	MNO	Tetracycline	30 µg	27	28-31	32
Ciprofloxacin	CIP	Fluoroquinolone	5 µg	24	25-29	30
Gentamycin	GMN	Aminoglycoside	10 µg	-	-	28
Vancomycin	VAN	Glycopeptide	30 µg	-	-	24
Ampicillin	AMP	Penicillin	10 µg	21	22-24	25
Amoxicillin-clavulanic acid	AMC	Penicillin	20 + 10 µg	20	21-23	23
Amoxicillin	AMX	Penicillin	25 µg	27	28-30	31
Amikacin	AKN	Aminoglycoside	30 µg	-	-	30
Chloramphenicol	CHL	Amphenicol	30 µg	23	24-26	27
Trimethoprim-sulfamethoxazole	SXT	Sulfonamide	1.25 µg + 23.75 µg	14	-	15
Rifampicin	RIF	Rifamycin	5 µg	20	21-29	30
Cefazolin	CZN	Cephalosporin	30 µg	23	24-29	30
Ceftriaxone	CRO	Cephalosporin	30 µg	23	24-29	30

(Adzitey, 2015; R. Aminov, 2017; Durand et al., 2019; Kotrbová et al., 2022; Krause et al., 2016; Van Doorslaer et al., 2014)

The obtained forward and reverse sequences were analyzed and assembled by Geneious Prime® 2023.2.1 to create a phylogenetic tree based on the 16S rRNA. The final sequences were identified by comparing them against the type strains database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The results are summarized in **Table 9**.

The phylogenetic tree was constructed using neighbor-joining with the Tamura-Nei genetic distance model and 1000 replicates (v 8.1.6, http://www.geneious.com). The closest relatives of the isolates identified by BLAST were used as reference sequences for the construction of the phylogenetic tree.

To further break down the bacterial response to the tested antibiotics, they were sorted according to their classes. Per group both the average zone diameter (in mm) as well the relative number of resistant strains according to treatment were evaluated and visualized by Microsoft Excel (Version 2403) in the form of column graphs.

4 Results

4.1 Identification of isolates

In total 32 isolates (7 from CMW, 9 from CMC, and 16 from CMB) were studied. 18 isolates were identified as *Streptomyces* spp., 9 isolates as *Micromonospora* spp., 2 isolates as *Kineococcus* spp., 1 as *Actinomadura*, and 1 as *Pseudoclavierbacter*. Additionally, 4 species were not identified as actinobacteria, of which 3 were from the genus *Sphingomonas* and 1 from the genus *Methylobacterium*. Their identification is summarized in **Table 8** further information can be found in **Table 9** in the appendix. Furthermore, treatment with composted biosolids showed the highest diversity concerning actinobacterial genera.

Table 8 Identification of the closest relatives to the isolates

CMW		CMC		CMB	
PV1	*Streptomyces argenteolus*	PV45 PV147	*Micromonospora aurantiaca*	PV65	*Micromonospora provocatoris*
PV2 PV5 PV131	*Sphingomonas cynarae*	PV46	*Streptomyces drozdowiczii*	PV81	*Micromonospora fluminis*
PV7	*Streptomyces camponoticapitis*	PV47	*Streptomyces rishiriensis*	PV88 PV90	*Streptomyces praecox*
PV9	*Streptomyces caviscabies*	PV51	*Pseudoclavibacter terrae*	PV91 PV173 PV177 PV209	*Micromonospora aurantiaca*
PV31	*Streptomyces rishiriensis*	PV148A PV148B	*Kineococcus radiotolerans*	PV168B PV169A PV169B	*Streptomyces durocortorensis*
		PV149A	*Methylobacterium goesingense*	PV170A PV170B1 PV170B2 PV170B3	*Streptomyces hainanensis*
		PV149C	*Actinomadura bangladeshensis*	PV172	*Micromonospora orduensis*

4.1.1 BOX-PCR

As there have been equal identifications by the analysis of their 16S rRNA, BOX-PCR was used to omit identical strains, for the analysis of unique strains only. The products of the BOX-PCR were visualized using gel-electrophoresis (**Figure 1**), which shows the relevant isolates' profiles as well as the control and the DNA ladder. The identity and dilution of the selected strains can be found in the appendix in **Table 9**.

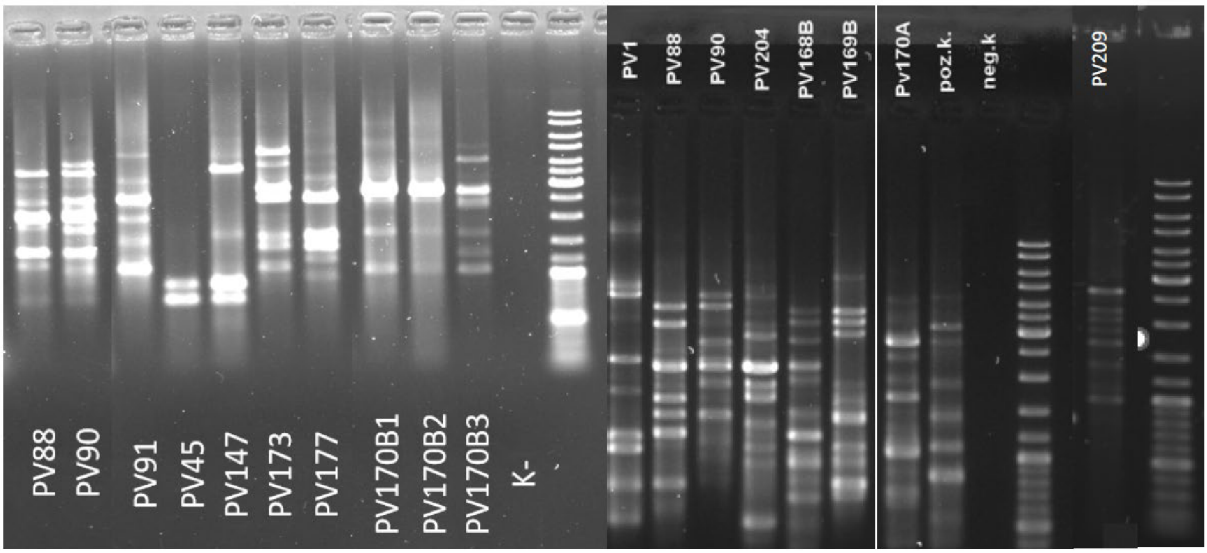

Figure 1 Gel-Electrophoresis of the Box-PCR products with positive control (poz.k.), negative control (neg.k, K-) and DNA-ladder

4.2 Antibiotic susceptibility

In **Figure 2** the results of the antibiotic susceptibility testing are summarized in the form of an antibiogram in combination with the phylogenetic tree of the tested isolates. The antibiogram shows the isolate's average diameter and the susceptibility evaluation, which can be resistant (red), susceptible (green), or intermediate (orange). Soil treatment is visualized by blue (CMW), purple (CMC), or rose (CMB) color.

In the case of isolates PV7, PV47, and PV170B3 no growth occurred after 24 hours, so the zone diameter was measured after 48 hours. For isolate PV172 the measurements were taken after 96 hours and for isolates PV173 and PV65, the diameter was determined after 120 hours of inoculation. There was no growth observed for isolate PV148A on MH agar, resulting in the usage of ISP2 agar to assess its susceptibility to antibiotics.

As visible in **Figure 2** there is a higher number of resistances per strain present in *Streptomyces* spp. compared to *Micromonospora* spp., with the maximum number being

eleven and two, respectively. The strain that displayed resistance to the highest number of antibiotics from all tested strains was PV9 isolated from the control treatment. This strain was related the most closely to *Streptomyces caviscabies* (**Table 8**). In contrast, strains PV147 and PV172 showed susceptibility to all tested antibiotics. Both were later identified as *Micromonospora* spp..

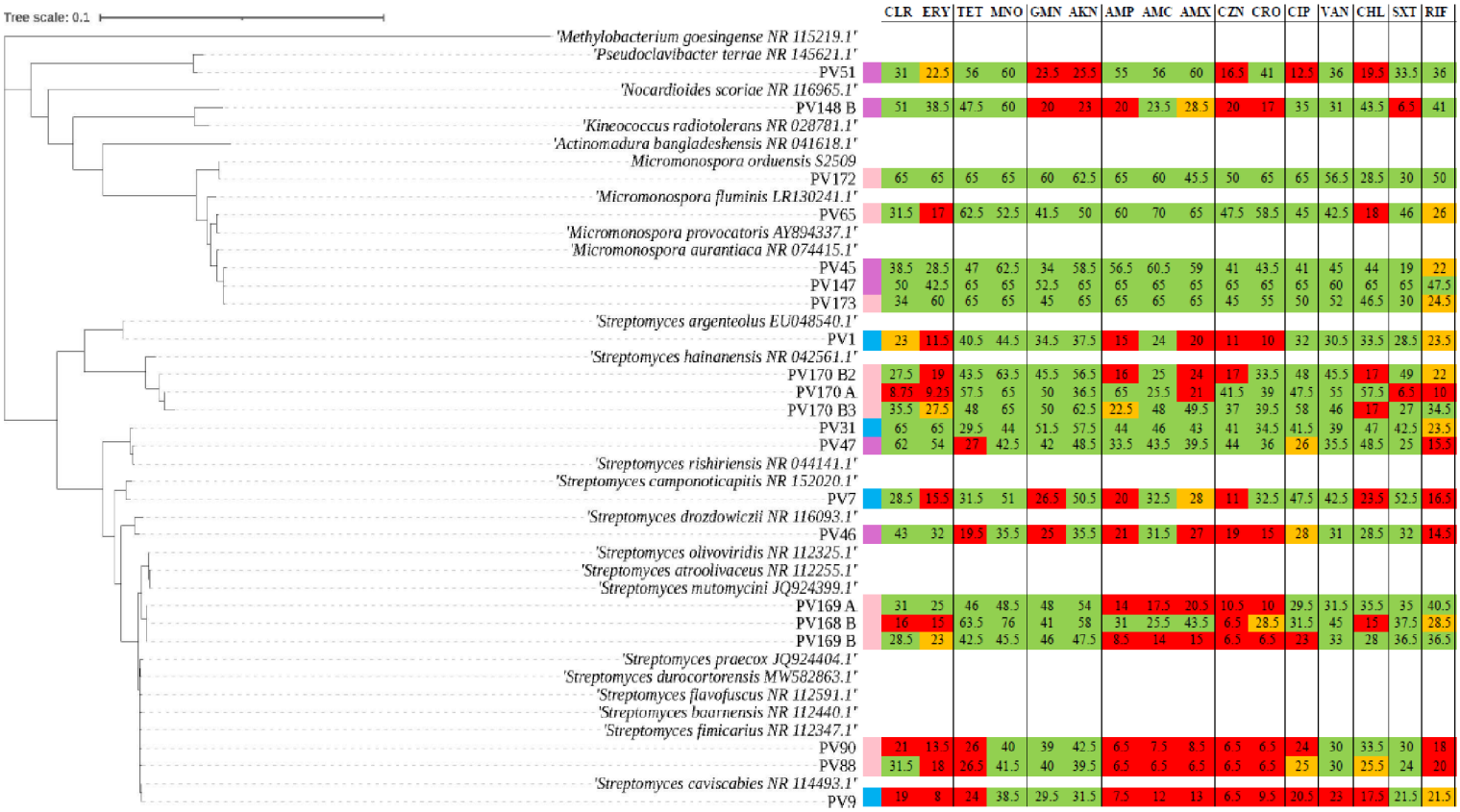

Figure 2 Phylogenetic tree with antibiogram of isolates

Notable variations in the response to antibiotics within an antibiotic group were observed with penicillin and amphenicol compounds. For both groups the frequency of resistance with composted biosolid was only half as high compared to tap water irrigation/biosolid application (**Figure 3**). Within the penicillin group, the addition of clavulanic acid to Amoxicillin (AMC) showed a decrease in the frequency of resistant strains compared to Amoxicillin (AMX) on its own (**Figure 2**).

When evaluating resistance profiles according to their antibiotic groups, the highest frequency of resistance overall was observed against cephalosporins. This further corresponded with their average zone diameter, as it was smaller compared with other antibiotic groups. Notably, there were no occurrences of resistance to certain antibiotics when using tap water irrigation, composted biosolid, or biosolid amendment. Specifically, resistance was absent against

macrolides with composted biosolid amendment, aminoglycosides with biosolid amendment, and sulfonamide with tap water irrigation. Moreover, no resistances were detected against glycopeptides with either composted biosolid or biosolid amendment. Additionally, the low frequency of resistance to tetracycline and fluoroquinolone compounds is noticeable. This is further reflected by the zone diameter, especially in the case of tetracyclines, as its average zone diameters were the highest. The results are shown in **Figure 3** and **Figure 4**, the number in the brackets displays the number of antibiotics tested per group.

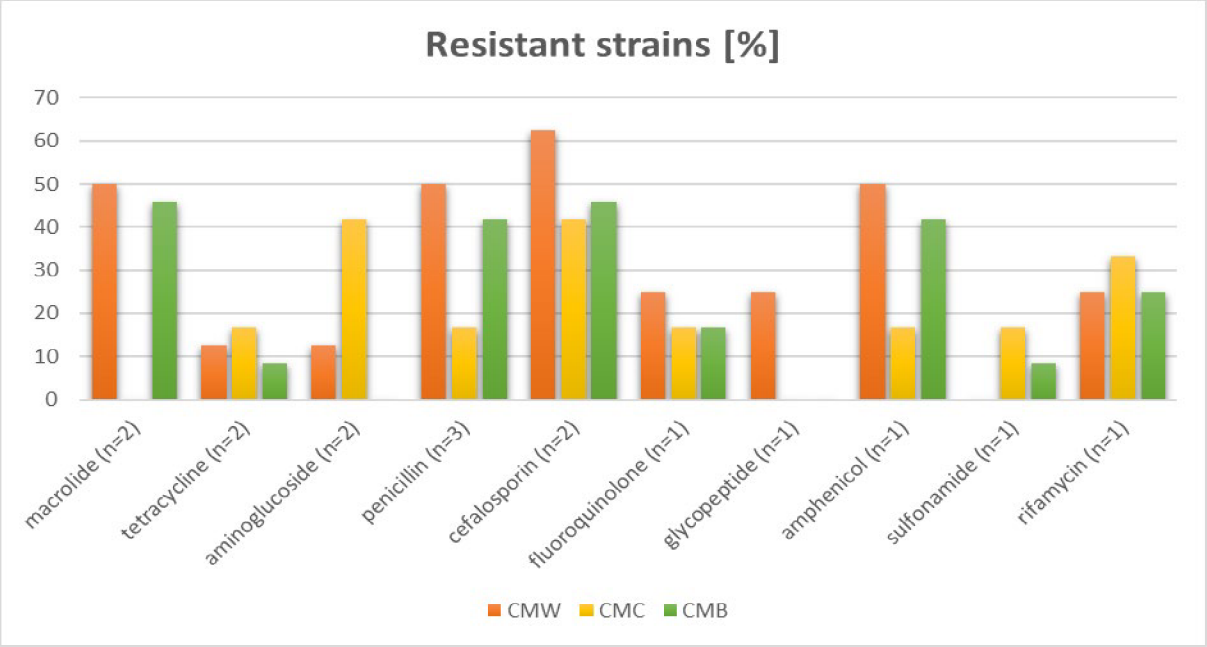

Figure 3 Relative number of resistant strains per treatment according to antibiotic classes

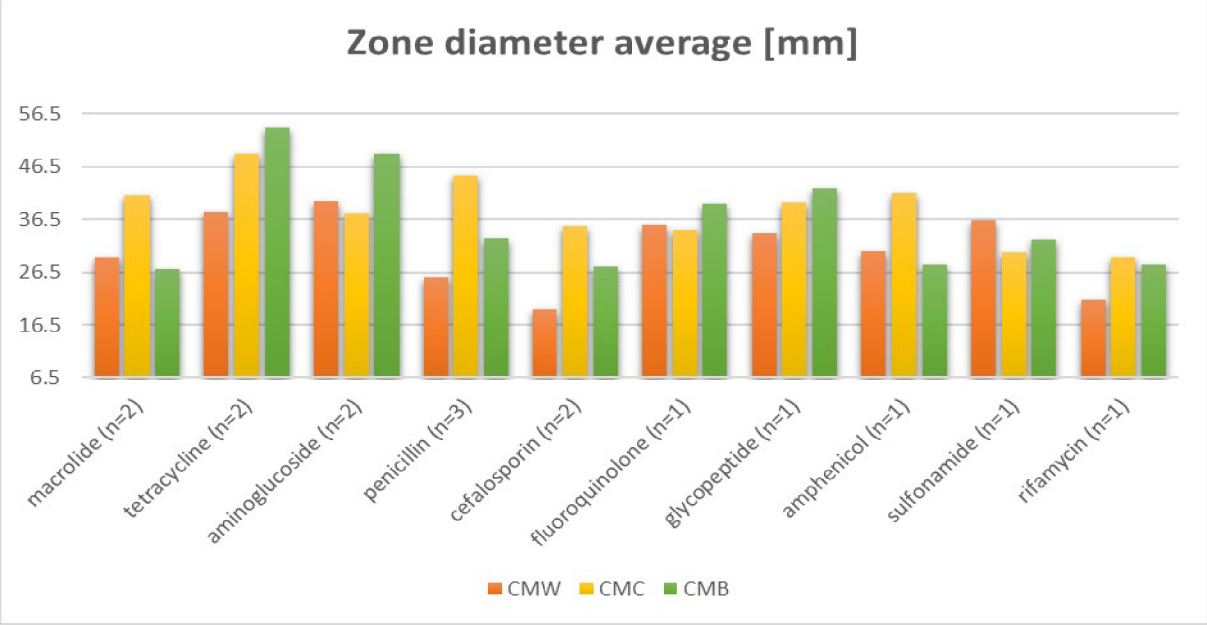

Figure 4 Average zone diameter per treatment according to antibiotic classes

5 Discussion

The increase in antibiotic-resistant bacteria in the environment and clinical settings has been broadly discussed over the last decade and represents one of the most urgent threats to humanity on a global scale. Although the transmission ways are still a subject of research, one of the possible pathways could be the contamination of soil with antibiotic-resistant bacteria and the subsequent colonization and contamination of edible plants (Scaccia et al., 2021). Therefore, the focus of this paper was on the endophytes of *Phaseolus vulgaris* growing in soil enriched with diverse types of wastewater treatment plant products, which are well-known sources of antibiotic-resistant bacteria and various micropollutants (Tiedje et al., 2019).

5.1 Assessment of diversity

Over half of the isolates were identified as *Streptomyces* spp., highlighting their prevalence in the actinobacterial endophytic community (Dinesh et al., 2017). Additionally, the high abundance of *Micromonospora* spp., which also corresponds to the previous study (Dinesh et al., 2017) stood out, as well as the rare abundance of *Kineococcus*, *Pseudoclavibacter*, and *Actinomadura*. Notable was the lowest diversity in the control treatment, as there were only *Streptomyces* spp. isolated, which can be justified by the generally frequent isolation of *Streptomyces* spp. from surface water (Ward & Bora, 2006), indicating their prevalence in this environment. This is especially prominent in contrast to the soil amendment with composted biosolid which contained *Streptomyces* spp., *Micromonospora* spp., *Kineococcus* spp., *Actinomadura,* and *Pseudoclavibacter*. The high variety of strains isolated from the composted biosolid amendment further agrees with available knowledge from the literature as microbial diversity is enhanced through composting processes (Aguilar-Paredes et al., 2023). As there was a high number of *Micromonospora* spp. isolates found from the amendment with both biosolids, it is assumed that biosolids played a role in introducing this genus to soil. This observation can be further supported by studies of Schlatter et al. (2019), which verified the high abundance of *Micromonospora* spp. in biosolids.

5.2 Pattern of resistance

Although biosolids are well known for the dispersal of antibiotic-resistance genes (Xiao et al., 2023), our results did not support these findings. Almost half of the antibiotic groups display the trend control > biosolids > composted biosolids regarding the relative abundance of

resistant strains (%), which is further supported by their average zone diameter values. This can be explained by the absence of *Micromonospora* spp. from the control treatment, which was observed to be more sensitive to selected antibiotics in comparison to *Streptomyces* spp. (Chapter 4.2). There is, however, limited research on the resistance or susceptibility of environmental strains of *Micromonospora* spp. to studied antibiotics, and the factors confirming or denying this correlation. It should also be taken into account that the same breakpoint values were used independently of the bacterial genus.

The decreased frequency of resistance concerning actinobacterial endophytes isolated after composted biosolid amendment can be explained by the enhanced microbial activity from the composting process. This depends on multiple factors such as elevated temperature, humidity, dilution of the biosolid, aeration, and the duration of the process (Francou et al., 2005; Ilani et al., 2016), which influences the decomposition of antibiotic compounds. Together with a higher content of organic matter in composted biosolid, compared to biosolid, this may result in lesser exposure of microorganisms to antibiotics. This is supported by the highly decreased frequency of resistant strains against macrolide and penicillin groups from composted biosolid amendments when compared to biosolid ones.

The resistance to the highest number of antibiotics was observed for *Streptomyces caviscabies* (PV9) from the control treatment with tap water, as this strain was resistant to 11 antibiotics. This species is a common plant pathogen present in soil causing potato scab, a disease affecting root vegetables (Liang et al., 2019). As this was the only isolate of *Streptomyces caviscabies* obtained, there is a lack of information concerning its environmental dependence.

5.3 Comparison of individual strains with the literature

In soils affected with tap water (CMW) and composted biosolids (CMC), actinobacterial species, which have been previously detected in human tissues (summarized by Kotrbová et al., 2022), were found. These species were *Streptomyces caviscabies* in CMW (PV9) and *Streptomyces drozdowiczii* in CMC (PV46). Since in many cases, the 16S rRNA sequencing does not allow the assignment of Streptomyces isolates into particular species, their similarity was assigned according to Labeda et al. (2012) as seen in **Table 10** in the appendix. The isolate *Streptomyces argenteolus* (PV1) from CMW was thus related to *Streptomyces griseus* and *Streptomyces albidoflavus* group strains *(S. hydrogenans/ S. resistomycificus/ S. griseochromogenes)* and *Streptomyces praecox* (PV 88/PV 90) from CMB was associated with *Streptomyces flavofuscus/baarnensis/fimicarius*. Upon comparison with already recorded

clinical isolates, similar patterns were observed especially concerning the high frequency of susceptibility against tetracyclines and the high frequency of resistance against penicillin and cephalosporin compounds. Additionally, isolates in this study tended to be susceptible to both CIP and SXT which was in most cases not observed in their counterparts from clinical samples (Kotrbová et al., 2022).

Since the majority of *Streptomyces* spp. can synthesize beta-lactamase, intrinsic resistance is indicated, further affecting their response to penicillins (Ogawara, 2014). This is emphasized by endophytic (PV9) and clinical (TR1318 from Kotrbová et al. (2022)) isolates of *Streptomyces caviscabies* which both expressed resistance to all penicillin compounds tested (AMP, AMC, AMX). Contrasting to this is the susceptibility to all penicillins evaluated for other strains closely related to *Streptomyces* spp. (PV31, PV47, PV168B, PV170B3), even without the addition of a beta-lactamase inhibitor (AMP, AMX).

Upon individual comparison, *Streptomyces drozdowiczii* (PV46) showed the same resistances against cephalosporin groups (CZN, CRO) and penicillin groups (AMP, AMX, AMC) as in the clinical isolate (TR978 from Kotrbová et al. (2022)). This supports the observation of increased occurrences of resistance against cephalosporins (Chapter 4.2). Opposing to the high frequency of susceptibility to tetracyclines which was observed usually, both the evaluated endophytic isolate (PV46) and the compared clinical one (TR978) exhibited resistance. Furthermore, a difference in the response to gentamycin was detected, as there were obvious differences in zone diameter, even though the breakpoint values for resistance are missing (Kotrbová et al., 2022). This might originate from the high abundance of gentamycin-resistant genes in sewage and surface water near wastewater treatment plants (Heuer et al., 2006), which would indicate horizontal gene transfer.

Streptomyces argenteolus (PV1) is phylogenetically related to *Streptomyces griseus* (NRK84) (clinical isolate from Rahdar et al. (2021), GenBank accession no. MK878410). Their comparison showed similar responses, however, differences are present in their response to fluoroquinolones (CIP) and the amoxicillin clavulanate combination. Susceptibility was observed in the endophytic isolate (PV1), but resistance in the clinical one (NRK84). This further challenges the observation of reduced frequency of resistance against amoxicillin due to the addition of a beta-lactamase inhibitor such as clavulanic acid. Through the addition of clavulanic acid, the bacteria's enzyme beta-lactamase is disturbed, which would lead to susceptibility of the induced beta-lactam, in this case, amoxicillin (Geddes et al., 2007; Kotrbová et al., 2022). When reviewing the AST of *Streptomyces argenteolus* (PV1) against

data available in the literature of *Streptomyces albidoflavus* group strains *(S. hydrogenans/ S. resistomycificus/ S. griseochromogenes)* (clinical isolates: OS17, OS18, OS20, OS21, OS32, 0S33, OS534, OS2864, OS2886A, OS3863, OS3889, OS4303, OS534, OS5590, OS5966, S6152, OS6180, OS6215, OS6618, OS6629, OS6643, OS6672, OS6764, OS6783, OS6829, OS7188, OS7560, OS8079B, OS8305, OS8560, OS8619, OS8917, TR950, TR979, TR1008, TR1011, TR1048, TR1060, TR1135, TR1206, TR1247, TR1250, TR1349, TR1353, PR198, , OS19, 0S22, 0S23, OS542, OS2243, OS3864, OS7748, OS8079A, OS10141, TR1041, TR1056, TR1059, TR1117, TR1134, TR1301; environmental isolates: BCCO 10_0258, BCCO 10_0478, BCCO 10_0550 and BCCO 10_1286, from Kotrbová et al. (2022)) similar responses were again observed. When comparing the endophytic isolate with previously studied ones, the resistance pattern of strain PV1 shares a higher similarity with the environmental isolates (BCCO strains) than with the clinical ones (Supplementary Table S2 from Kotrbová et al. (2022)), as only the clinical isolates show resistance against CIP. Still, their overall response does not differ largely. A notable deviation from both the clinical and the environmental counterparts is their resistance against SXT, which was not detected in the endophytic isolate from this study.

Upon comparison of *Streptomyces praecox* (PV88/PV90) and *Streptomyces flavofuscus/baarnensis/fimicarius* (TR1318 from Kotrbová et al. (2022)), the clinical counterpart (TR1318) showed a high level of susceptibility, which is unusual when considering the dominant resistance profile observed in the strains of this study. Differences lay especially in the susceptibility of the clinical isolate to RIF, CIP, TET, and the penicillin group. As both PV88 and PV90 were isolated from CMB, this might indicate the acquirement of these resistances from biosolids. There is however not sufficient research explaining this observation.

Furthermore, *Streptomyces rishiriensis* isolates were present in both the control treatment (PV31) and the compost amendment (PV47). When comparing their responses a higher frequency of susceptibility was detected in the control treatment opposing the observed pattern described before. *Streptomyces rishiriensis* can further be compared with an environmental isolate of *Streptomyces rishiriensis* (BCCO 10_1665) from Kotrbová et al. (2022). When contrasted the isolates were susceptible to the tested antibiotics, excluding RIF. The previously studied environmental isolate (BCCO 10_1665) also showed resistance against CIP and SXT, which was not observed in the bacterial strains of this study (PV31, PV47).

All evaluated isolates were obtained from the xylem sap of *Phaseolus vulgaris*. This was done to ensure the presence of endophytes only, as the surface sterilization procedures often fail, and lead to contamination by bacterial strains from the rhizosphere/phyllosphere. This further explains the smaller number of isolates, as the population density in the xylem is lower. It is also supposed that in the plant tissues, a bigger and more diverse quantity of actinobacteria is present (Frank et al., 2017; Petrini & Fisher, 1988).

6 Conclusion

To investigate the response of endophytic actinobacteria to different soil amendments, actinobacterial strains were isolated from the plant's xylem liquid and further identified and assessed in their response to antibiotics. Opposing to the proposed hypothesis the highest frequency of resistance was observed in the isolates from plants grown in the control soil irrigated with tap water. However, the lowest frequency of resistance was detected in plants grown in soil amended with composted biosolid. The higher frequency of resistant plant endophytes isolated from soil amended with biosolids compared to those isolated from soil amended with composted biosolids may confirm the stated hypothesis partially. It indicates that the activated microbial activity during composting affected the load and availability of antibiotics and thus the selective pressure on microbes. Moreover, the absence of *Micromonospora* spp. isolates in the control treatment and the dominance of *Streptomyces* spp. isolates in other treatments influenced the number of resistant and sensitive actinobacterial strains and at the same time opened the question of determining suitable breakpoints for different genera of actinobacteria. This observation needs to be further investigated through more extensive research in the future.

Furthermore, the correlation between endophytic and clinical/other environmental isolates was analyzed, resulting in highly similar responses when comparing equal species isolated from the environment and human tissues. The high frequency of resistance against cephalosporins was especially noteworthy, as well as the high frequency of susceptibility against tetracycline groups. Additionally, the combination of amoxicillin with a beta-lactamase inhibitor (clavulanic acid) increased the frequency of susceptibility of strains in comparison with amoxicillin only.

We are aware that the number of isolated strains for individual treatments is limited, and it is therefore difficult to confirm the observed differences by statistical means. Nevertheless, this work has yielded surprising results on the occurrence of antibiotic resistance in endophytic actinobacteria as affected by wastewater treatment plant product amendments to the soil. For more substantial assessments, future research should consider evaluating a larger number of isolates per treatment, with greater diversity to allow for robust statistical comparisons.

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8 Appendix

Table 9 Results of 16S rRNA identification – assignment to the closest relative species

DNA dilution	DNA isol.	ID	Name of the closest relative strain according to 16S rRNA similarity	% identity	%query cover	Score [max/total]	accession no.	The closest relative species
undil.		PV1	Streptomyces argenteolus strain CGMCC 4.1693 16S ribosomal RNA gene, partial sequence	99.79	99	2575/2575	EU048540.1	*Streptomyces argenteolus*
undil. extra cycles		PV2	Sphingomonas cynarae strain SPC-1 16S ribosomal RNA, partial sequence	98.59	99	2394/2394	NR_109167.1	*Sphingomonas cynarae*
undil. extra cycles		PV5	Sphingomonas cynarae strain SPC-1 16S ribosomal RNA, partial sequence	98.78	100	2475/2475	NR_109167.1	*Sphingomonas cynarae*
5x extra cycles		PV7	Streptomyces camponoticapitis strain 2H-TWYE14 16S ribosomal RNA, partial sequence	99.28	100	2521/2521	NR_152020.1	*Streptomyces camponoticapitis*
undil.		PV9	Streptomyces flavofuscus strain NBRC 100768 16S ribosomal RNA, partial sequence	99.65	100	2588/2588	NR_112591.1	*Streptomyces caviscabies*
			Streptomyces baarnensis strain NBRC 14727 16S ribosomal RNA, partial sequence	99.65	100	2588/2588	NR_112440.1	
			Streptomyces fimicarius strain NBRC 13037 16S ribosomal RNA, partial sequence	99.65	100	2588/2588	NR_112347.1	
			Streptomyces caviscabies strain ATCC 51928 16S ribosomal RNA, partial sequence	99.65	100	2588/2588	NR_114493.1	
undil.		PV31	Streptomyces rishiriensis strain NRRL B-3239 16S ribosomal RNA, partial sequence	99.44	100	2591/2591	NR_044141.1	*Streptomyces rishiriensis*
undil.		PV45	Micromonospora aurantiaca strain ATCC 27029 16S ribosomal RNA, partial sequence	99.43	100	2571/2571	NR_074415.1	*Micromonospora aurantiaca*
5x extra cycles		PV46	Streptomyces drozdowiczii strain NRRL B-24297 16S ribosomal RNA, partial sequence	99.16	100	2569/2569	NR_116093.1	*Streptomyces drozdowiczii*
undil.		PV47	Streptomyces rishiriensis strain NRRL B-3239 16S ribosomal RNA, partial sequence	99.58	100	2603/2603	NR_044141.1	*Streptomyces rishiriensis*
undil. extra cycles		PV51	Pseudoclavibacter terrae strain THG-MD12 16S ribosomal RNA, partial sequence	99.64	98	2540/2540	NR_145621.1	*Pseudoclavibacter terrae*
undil.	X	PV65	Micromonospora sp. MT25 16S ribosomal RNA gene, partial sequence	99.58	100	2579/2579	AY894337.1	*Micromonospora provocatoris*
undil.		PV81	Micromonospora sp. A38 partial 16S rRNA gene, isolate Miladis I. Camacho Pozo	99.71	100	2534/2534	LR130241.1	*Micromonospora fluminis*
undil.	X	PV88	Streptomyces praecox strain CGMCC 4.1782 clone 5 16S ribosomal RNA gene, complete sequence	99.79	99	2612/2612	JQ924406.1	*Streptomyces praecox*
undil.	X	PV90	Streptomyces praecox strain CGMCC 4.1782 clone 5 16S ribosomal RNA gene, complete sequence	99.79	99	2612/2612	JQ924406.1	*Streptomyces praecox*
undil.	X	PV91	Micromonospora aurantiaca strain ATCC 27029 16S ribosomal RNA, partial sequence	99.57	100	2560/2560	NR_074415.1	*Micromonospora aurantiaca*
5x extra		PV131	Sphingomonas cynarae strain	98.84	100	2470/2470	NR_1091	*Sphingomonas*

cycles			SPC-1 16S ribosomal RNA, partial sequence				67.1	*cynarae*
undil.	X	PV147	Micromonospora aurantiaca strain ATCC 27029 16S ribosomal RNA, partial sequence	99.57	100	2558/2558	NR_0744 15.1	*Micromonospora aurantiaca*
undil. extra cycles		PV148 A	Kineococcus radiotolerans strain SRS30216 16S ribosomal RNA, partial sequence	98.56	93	2331/2331	NR_0287 81.1	*Kineococcus radiotolerans*
undil. extra cycles		PV148 B	Kineococcus radiotolerans strain SRS30216 16S ribosomal RNA, partial sequence	98.27	94	2327/2327	NR_0287 81.1	*Kineococcus radiotolerans*
undil.	X	PV149 A	Methylobacterium goesingense strain iEII3 16S ribosomal RNA, partial sequence	99.28	100	2503/2503	NR_1152 19.1	*Methylobacterium goesingense*
undil.	X	PV149 C	Actinomadura bangladeshensis strain 3-46-b(3) 16S ribosomal RNA, partial sequence	98.73	100	2529/2529	NR_0416 18.1	*Actinomadura bangladeshensis*
10x extra cycles		PV168 B	Streptomyces durocortorensis strain RHZ10 16S ribosomal RNA gene, partial sequence	99.29	98	2543/2543	MW5828 63.1	*Streptomyces durocortorensis*
undil. extra cycles		PV169 A	Streptomyces durocortorensis strain RHZ10 16S ribosomal RNA gene, partial sequence	99.43	98	2551/2551	MW5828 63.1	*Streptomyces durocortorensis*
undil. extra cycles		PV169 B	Streptomyces durocortorensis strain RHZ10 16S ribosomal RNA gene, partial sequence	99.29	98	2543/2543	MW5828 63.1	*Streptomyces durocortorensis*
undil. extra cycles		PV170 A	Streptomyces hainanensis strain YIM 47672 16S ribosomal RNA, partial sequence	98.84	100	2471/2471	NR_0425 61.1	*Streptomyces hainanensis*
undil.	X	PV170 B1	Streptomyces hainanensis strain YIM 47672 16S ribosomal RNA, partial sequence	98.64	100	2484/2484	NR_0425 61.1	*Streptomyces hainanensis*
undil.	X	PV170 B2	Streptomyces hainanensis strain YIM 47672 16S ribosomal RNA, partial sequence	98.92	100	2494/2494	NR_0425 61.1	*Streptomyces hainanensis*
undil.	X	PV170 B3	Streptomyces hainanensis strain YIM 47672 16S ribosomal RNA, partial sequence	98.67	100	2540/2540	NR_0425 61.1	*Streptomyces hainanensis*
undil.	X	PV172	Micromonospora sp. S2509 16S ribosomal RNA gene, partial sequence	99.58	100	2582/2582	KF4948 05.1	*Micromonospora orduensis*
undil.	X	PV173	Micromonospora aurantiaca strain ATCC 27029 16S ribosomal RNA, partial sequence	99.64	100	2562/2562	NR_0744 15.1	*Micromonospora aurantiaca*
undil.	X	PV177	Micromonospora aurantiaca strain ATCC 27029 16S ribosomal RNA, partial sequence	99.72	100	2591/2591	NR_0744 15.1	*Micromonospora aurantiaca*
undil. extra cycles		PV209	Micromonospora aurantiaca strain ATCC 27029 16S ribosomal RNA, partial sequence	99.71	100	2558/2558	NR_0744 15.1	*Micromonospora aurantiaca*

Table 10 Assignment of similar isolates to the phylogenetically related clades according to (Labeda et al., 2012)

Isolate Nr.	Identification	Comparable to	Similarity assignment
PV1	*Streptomyces argenteolus*	*Streptomyces griseus*	Neighboring branch at Clade 35
PV1	*Streptomyces argenteolus*	*Streptomyces albidoflavus* group strains	Neighboring branch at Clade 35
PV88/PV90	*Streptomyces praecox*	*Streptomyces flavofuscus/baarnensis/fimicarius*	Directly neighboring branch to *Streptomyces flavofuscus*