VYTAUTAS MAGNUS UNIVERSITY
LITHUANIAN RESEARCH CENTRE FOR AGRICULTURE AND FORESTRY

Dorotėja VAITIEKŪNAITĖ

TREE GROWTH STIMULATION AND PATHOGEN GROWTH INHIBITION USING ENDOPHYTIC MICROORGANISMS

Doctoral Dissertation
Agricultural Sciences, Forestry (A 004)

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Scientific Supervisor:

Dr. **Sigutė Kuusienė** (Institute of Forestry, Lithuanian Research Centre for Agriculture and Forestry, Nature Sciences, Biology N 010). 2018–2020.

Dr. **Vaida Sirgedaitė-Šėžienė** (Institute of Forestry, Lithuanian Research Centre for Agriculture and Forestry, Nature Sciences, Ecology and Environmental Sciences N 012). 2020–2023.

Dissertation will be defended at the Committee of Forestry of Vytautas Magnus University, Lithuanian Research Centre for Agriculture and Forestry:

Chairman:

Dr. **Diana Marčiulynienė** (Lithuanian Research Centre for Agriculture and Forestry, Agricultural Sciences, Forestry A 004).

Members:

Dr. **Daiva Burokienė** (Nature Research Centre, Nature Sciences, Biology N 010);

Dr. **Skaidrė Supronienė** (Lithuanian Research Centre for Agriculture and Forestry, Agricultural Sciences, Agronomy A 001);

Assoc. Prof. Dr. **Jūratė Aleinikovienė** (Vytautas Magnus University, Agricultural Sciences, Forestry A 004);

Dr. **Darta Klavina** (Latvian State Forest Research Institute "Silava", Agricultural Sciences, Forestry A 004).

The doctoral thesis will be defended in the public meeting of the Committee of Forestry at 10 o'clock on the 7th of September 2023, at the Lithuanian Research Centre for Agriculture and Forestry.

Address: Instituto al. 1, Akademija, LT-58344 Kėdainiai distr., Lithuania.

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Dorotėja VAITIEKŪNAITĖ

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Mokslinis vadovas:

Dr. **Sigutė Kuusienė** (Miškų institutas, Lietuvos agrarinių ir miškų mokslų centras, Gamtos mokslai, Biologija N 010). 2018–2020.

Dr. **Vaida Sirgedaitė-Šėžienė** (Miškų institutas, Lietuvos agrarinių ir miškų mokslų centras, Gamtos mokslai, Ekologija ir aplinkotyra N 012). 2020–2023.

Mokslo daktaro disertacija ginama Vytauto Didžiojo universiteto ir Lietuvos agrarinių ir miškų mokslų centro Miškotyros mokslo krypties Gynimo taryboje:

Pirmininkė:

Dr. **Diana Marčiulynienė** (Lietuvos agrarinių ir miškų mokslų centras, Žemės ūkio mokslai, Miškotyra A 004)

Narės:

Dr. **Daiva Burokienė** (Gamtos tyrimų centras, Gamtos mokslai, Biologija N 010);

Dr. **Skaidrė Supronienė** (Lietuvos agrarinių ir miškų mokslų centras, Žemės ūkio mokslai, Agronomija A 001);

Doc. dr. **Jūratė Aleinikovienė** (Vytauto Didžiojo universitetas, Žemės ūkio mokslai, Miškotyra A 004);

Dr. **Darta Klavina** (Latvijos miškų institutas „Silava“, Žemės ūkio mokslai, Miškotyra A 004).

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Adresas: Instituto al. 1, Akademija, 58344 Kėdainių r. sav.

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ABBREVIATIONS

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACC – 1-aminocyclopropane-1-carboxylate

BCA – biocontrol agent

CAR – carotenoids

CHA – chlorophyll *a*

CHB – chlorophyll *b*

DPPH – 2,2-diphenyl-1-picrylhydrazyl

HA – *Heterobasidion annosum*

HF – *Hymenoscyphus fraxineus*

IAA – indole-3-acetic acid (auxin)

ITS – internal prescribed spacer (DNA region for fungal identification)

LB – lysogeny broth medium

LS – *Lophodermium seditiosum*

MS – Murashige and Skoog medium

PGPM – plant growth-promoting microorganism

PGPT – plant growth-promoting trait

PGR – plant growth regulator

pH – a measure of acidity or alkalinity

PT – *Phellinus tremulae*

rRNA – ribosomal ribonucleic acid

SERS – surface-enhanced Raman spectroscopy

TFC – total flavonoid content

TPC – total phenol content

WPM – woody plant medium

INTRODUCTION

Climate change is expected to increase outbreaks of pathogens and may potentially create locally unfavorable abiotic conditions for optimal forest growth, which pose one of the greatest challenges to forest health [1,2]. Special measures must be taken to ensure forests can function normally by managing outbreaks and mitigating damages caused by abiotic stressors. Therefore, improving tree growth and health conditions is essential. However, the use of inorganic chemical substances (inorganic fertilizers and pesticides) in Europe is restricted due to adverse effects on soil, water, biodiversity, and ultimately human health [3]. Using microbial-based stimulants and fungicides could be an alternative way to deal with pathogens and enhance tree growth [3].

A relatively novel niche to find beneficial microorganisms is the plant endosphere [3], which is colonized by complex microbial communities. Endophytes, often bacteria or fungi, live within plants, both intra- and intercellularly, without causing their hosts any obvious harm [4–6]. Because they are sheltered within the tissues of their plant hosts, they can create long-lasting colonies. Endophytes can have mutualistic relationships with their host plants. In this case, they can produce various secondary metabolites that may affect plant growth and help them with stress response, increasing their resistance to harm [5,6]. They can also help their hosts by affecting their metabolic pathways [7], producing phytohormones, or enhancing nutrient bioavailability [3,8]. Endophytes can also serve as biological control agents (BCAs) in integrated pest and disease management [9,10]. Such integration of biological measures would be beneficial in commercial forestry as well as forest nurseries, as it would potentially reduce or at least limit the use of fungicides and chemical fertilizers or stimulants.

In the past, however, most studies have focused on endophytes associated with important agricultural species, and the functions of tree endophytes are in many cases as poorly understood as their diversity and community structure. Based on this limited knowledge, practical applications for forest protection based on endophytes are still rare [11–13].

Originality and novelty

Using culture-dependent methods, fungi (*Neocucurbitaria, Aspergillus, Talaromyces, Byssochlamys, Meyerozyma, Microstroma*) and bacteria (*Bacillus, Pantoea, Delftia, Pseudomonas, Paenibacillus*) species were isolated from pedunculate oak (*Quercus robur* L.) bud and leaf endosphere. Surface-enhanced Raman spectroscopy (SERS) was shown to be an effective technique to quickly evaluate within species diversity of pedunculate oak bacterial endophytes. Furthermore, *Paenibacillus tundrae* isolated from pedunculate oak was shown to

enhance model poplar (*Populus*) tree root growth and photosynthesis pigment concentration. *Q. robur* fungal and bacterial endophytes were also shown to have antagonistic potential against European forest pathogens (*Hymenoscyphus fraxineus, Phellinus tremulae, Heterobasidion annosum, Lophodermium seditiosum*).

All in all, the obtained scientific data provides knowledge about the culturable endophytes, that can be found in pedunculate oak tissues and will potentially provide the basis for the creation of eco-friendly products for silviculture.

Aim

The aim of this study was to investigate the potential of endophytes found in the leaves and buds of *Quercus robur* L. for tree growth stimulation and pathogen growth inhibition.

Hypothesis

Quercus robur (L.) bud and leaf endosphere contains bacterial and fungal strains that could be used for tree growth promotion and for forest pathogen growth inhibition in vitro.

Objectives

1. To isolate and identify *Quercus robur* (L.) leaf and bud easily culturable endophytic bacteria and fungi;
2. To evaluate if *Quercus robur* (L.) bacterial endophytes can be easily differentiated using surface-enhanced Raman spectroscopy (SERS) for efficient use in further experiments.
3. To evaluate what plant growth-promoting traits isolated endophytes qualitatively exhibit in vitro;
4. To determine if selected bacterial endophytes can enhance model poplar (*Populus*) tree growth and secondary metabolite production in vitro;
5. To evaluate the growth inhibition potential of selected isolated endophytes against forest pathogens *Heterobasidion annosum* ((Fr.) Bref.), *Hymenoscyphus fraxineus* (Baral et al.), *Lophodermium seditiosum* (Minter, Staley & Millar) and *Phellinus tremulae* (Bondartsev & P.N. Borisov) under in vitro conditions.

Defensive statements

1. *Quercus robur* (L.) endophytes have plant growth-promoting traits that may lead to tree growth promotion;
2. Surface-enhanced Raman spectroscopy (SERS) is an effective method, as compared to standard *16S rRNA* sequencing, to evaluate within species variety of *Quercus robur* (L.) bacterial endophytes;

3. Endophytic *Paenibacillus tundrae* can promote model *Populus* spp. growth in vitro and positively impact their secondary metabolism;
4. *Quercus robur* (L.) endophytes can limit the growth of *Heterobasidion annosum* ((Fr.) Bref.), *Hymenoscyphus fraxineus* (Baral et al.), *Lophodermium seditiosum* (Minter, Staley & Millar) and *Phellinus tremulae* (Bondartsev & P.N. Borisov) pathogens in vitro.

LIST OF ARTICLES

This doctoral thesis is based on the research contained in the following papers, **referred to by Arabic numerals in the text as follows**:

1. **Vaitiekūnaitė, D**., Kuusienė, S., Beniušytė, E. (2021). Oak (*Quercus robur*) Associated Endophytic *Paenibacillus* sp. Promotes Poplar (*Populus* spp.) Root Growth In Vitro. *Microorganisms, 9,* 1151. https://doi.org/10.3390/microorganisms9061151
2. **Vaitiekūnaitė, D**., Snitka, V. (2021). Differentiation of Closely Related Oak-Associated Gram-Negative Bacteria by Label-Free Surface Enhanced Raman Spectroscopy (SERS). *Microorganisms, 9,* 1969. https://doi.org/10.3390/microorganisms9091969
3. **Vaitiekūnaitė, D**., Striganavičiūtė, G., Mishcherikova, V., Sirgedaitė-Šėžienė, V. (2022). *Populus tremula* × *P. alba* Microshoot Secondary Metabolism Response after *Paenibacillus* sp. Inoculation In Vitro. *Forests*, *13*, 1702. https://doi.org/10.3390/f13101702
4. **Vaitiekūnaitė, D**., Striganavičiūtė, G., Beniušytė, E., Sirgedaitė-Šežienė, V., Augustauskaitė, M. (2023). Putative biocontrol agents for European forest pathogens found in oak (*Quercus robur*) endosphere. *Zemdirbyste-Agriculture*, *110*(1). https://doi.org/10.13080/z-a.2023.110.011

Conferences

1. International conference „Young scientists for advance of agriculture“ hosted by Lithuanian Science Academy, Vilnius, Lithuania. 2019. Oral presentation „Initiation of ancient oak tissue culture and their culture dependent endomicroflora“. Presenter – D. Vaitiekūnaitė.
2. International conference „Agrosym 2020“ hosted online in Bosnia and Hercogovina, 2020 October. Oral presentation „English oak (*Quercus robur*) bacterial endophytes and their effect on European aspen (*Populus tremula*) microshoots in vitro“. Presenter – D. Vaitiekūnaitė.
3. International conference „The Vital Nature Sign“ hosted online in Lithuania, 2020 October. Poster presentation „Diversity and plant growth-promoting traits of *Quercus robur* cultivable fungal endophytes“. Presenter – E. Beniušytė.
4. International conference „CYSENI 2021“ hosted online in Lithuania, 2021 May. Oral presentation „Growth-promoting effect of endophytic *Paenibacillus* sp. on *Populus* sp. microshoots in vitro“. Presenter – D. Vaitiekūnaitė.
5. International conference „CYSENI 2022“ hosted online in Lithuania, 2022 May. Oral presentation „Plant growth-promoting *Paenibacillus* sp. affects hybrid poplar’s (P*opulus tremula* x P. *alba*) phenolics, antioxidant activity and photosynthesis pigments“. Presenter – D. Vaitiekūnaitė.

1. LITERATURE REVIEW

1.1. Research problem

To improve growth and limit the damage from increasing pathogen attacks, silviculturists often rely on chemical assistance, i.e., pesticides and inorganic fertilizers/stimulants. Yet the current dependence on chemical substances is having a notable negative effect on ecology, biodiversity, water and soil quality, and human health, and it also negatively impacts global carbon emissions [3,14–16]. Data shows that just between 1995 and 2010 in European forests, plant emerging infectious diseases (pathogenic fungi mostly) have increased 13-fold [17]. Based on current climate change conditions, that are advantageous to fungi, these numbers are likely to rise [16]. However, while this trend is similar in both agriculture and forestry, a lot less research goes into studies relating to forest tree protection and their growth improvement.

1.2. Plant growth-promoting microorganisms

A possible eco-friendly solution to replace or limit the use of inorganic pesticides and fertilizers/stimulants could be biostimulation and disease control using microbial inoculants. Due to the current social climate, the search for microbial alternatives is expected to grow in the future [3]. Recently, plant growth-promoting microorganisms (PGPMs) have been widely studied as potential BCAs and biostimulants [3,14–16,18].

Plant growth-promoting bacteria and fungi can be used as a long-term sustainable solution for fertilization and growth stimulation or they can be used in combination with chemical fertilizers, reducing their need, resulting in reduced cost, and reduced negative impact on the environment [19,20].

PGPMs can enhance nutrient accessibility and uptake through nitrogen fixation, phosphate mineralization/solubilization, siderophore (iron transport agent) production, potassium solubilization, etc. Moreover, microorganisms can produce plant growth regulators (PGRs) [14,21,22]. Additionally, microorganisms can assist in limiting the negative effects of environmental stressors and thus affect growth via changes in plant metabolome [23,24]. Inoculants can be used for a single purpose, ex. nitrogen fixation; however, more often they can have several beneficial aspects and thus have a complex effect on their host [22,25].

As mentioned, most of the studies in this field often research agriculturally important species, which have been shown to benefit from utilizing biostimulants (maize (*Zea mays* L.) [26], tomato (*Solanum lycopersicum* L.) [27], soybean (*Glycine max* (L.) Merr.) [28], rice (*Oryza sativa* L.) [29], etc.). However, research suggests that trees and other woody plants are similarly well-suited for biostimulant use. Several inoculant species/strains or consortium of species/strains have been successfully used in both pot trials and field studies on cedar (*Thuja*

plicata Donn.) [30], avocado (*Persea americana* Mill.) [31], tea (*Camellia* sp..) [32], pine (*Pinus* spp.) [33,34] and poplar (*Populus* spp.) [35,36]. Moreover, microorganisms have been shown to work well together with chemical NPK (nitrogen, phosphorus, and potassium respectively) and organic fertilizers in several tree species: olives (*Olea europaea* L.) [37], eucalyptus (*Eucalyptus* sp.) [38], poplars (*Populus deltoides* W.Bartram ex Marshall) [20], oranges (*Citrus* spp.) [39,40]. These studies demonstrate that biostimulants can at the very least limit chemical fertilizer needs without yield losses in forestry and related enterprises.

Biocontrol agents are microorganisms that can be used for the eradication and control (growth limitation) of pathogens. BCAs work in two main ways – direct interaction via different mechanisms (antibiosis, competition, parasitism) or indirectly by inducing resistance (induced systemic resistance) [18]. This can be in the form of metabolic pathway alterations, inducing/reducing secondary metabolite production, enhancing photosynthesis system efficiency, etc. [23]. BCAs have been shown to work well in silviculture previously. The control of *Heterobasidion annosum* in the conifer forests of Northern Europe is an excellent example, though effectiveness was only achieved in certain conditions [41,42]. Other common European forest pathogens have also shown some growth inhibition by various microorganisms [43,44], however, product development and commercialization, when it comes to BCAs for silviculture use is scarce.

1.3. Endophytes

In adapting PGPMs for plant protection and growth improvement, endophytes are an especially interesting group. Endophytes are microorganisms that can be found in plant hosts for at least a part of their life cycle but don’t cause their host any harm or an evident infection. So far, every plant tested was proven to have endophytic populations [5,7,45,46], however only several percent of all plant species were tested, thus plant endosphere presents a relatively novel and expansive niche in terms of finding new beneficial species/strains [3,47].

Notably, compared to the rhizosphere and phyllosphere microorganisms, endophytes can create self-sustaining colonies within their hosts, thus limiting the impact of environmental variability that render some inoculants with restricted field applicability [15,18] and thus potentially extending their efficacy as biostimulants and BCAs [3], hence reducing the need for repeat applications.

Endophyte-plant relationships are still not fully understood. However, it is believed that microbial symbionts use their hosts for a reliable supply of nutrients and as a protective measure from environmental stress. In return, they can have influence over improved plant stress tolerance, pathogen, and pest resistance, growth, etc. [5,7,45,47,48], similar to free-ranging PGPMs.

Researchers found that endophyte group biodiversity is determined by both individual host and habitat [5,46,49,50]. For example, findings suggest that tropical areas may have a higher diversity of fungal endophytes [46]. Host age/developmental stage and environmental conditions can also be a factor [5,47,49–51]. Furthermore, endophytic microorganisms can colonize the entire body of their host: roots, stems, leaves, fruits, buds, seeds, inflorescences, etc., however, certain organs can have denser or more diverse populations than others [47,51,52]. Overall multiple factors influence endophytic biodiversity: host species, genotype, growth stage, organ type, growth site, soil, disease incidence, nutritional and cultivation status, abiotic factors, etc. [50].

Endophytes are broadly segregated into two groups based on their origin, bacterial and fungal. These two groups are considered to be the most common in plants [47]. Even though fungi are eukaryotes and bacteria are prokaryotes, how they colonize their plant hosts has many similarities [5,49]. Knowledge about how fungi colonize their hosts is limited. Some invade the plants through their root system. However, it was shown that some invade their hosts via stomata [53]. Bacteria can colonize their hosts similarly, most often through the growth sites and wounds on the roots. They often colonize vascular tissues, this likely helps with distribution within the plant [4,53].

Fungi can be recovered most frequently from their hosts and have greater biodiversity, but bacterial endophytes occur in greater numbers overall [47]. Fungal endophytes are most often of the *Glomeromycota* and *Ascomycota* phylum [47,54], while the most common bacterial endophyte taxa are *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* [50,54].

1.4. *Quercus* endosphere

Based on the previously mentioned data, the oak (*Quercus*) genus emerges as a promising source of yet-unstudied cultivable endophytes. Oaks are distributed throughout the Northern Hemisphere. Pedunculate oaks (*Quercus robur*) specifically, are the model trees for the genus. These trees are essential hardwoods, known for providing food and habitat for a variety of organisms in temperate forest environments, thus thriving in multiple habitats and ecosystems [55]. Notably, studies on pedunculate oak endophytes are relatively scarce. Commonly studied oak species in terms of their endophytic activity are cork oak (*Q. suber*) and Turkey oak (*Q. cerris*). Fungi are more thoroughly studied both as potential pathogens and as possible BCAs [56,57]. The cultivable bacterial endophytes of oaks are not thoroughly studied at all, nor is their use as biostimulants. Additionally, more fungal endophytes for oaks seem to have been identified than bacteria.

A 2004 study found that *Fusarium* sp. endophytic fungus from *Q. variabilis* produced two compounds, one of which was previously unknown. Both of these compounds had an

antibacterial effect on *Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens* [58]. Another study done with *Q. variabilis* showed that out of 67 endophytic fungi isolates, about half showed growth inhibition on harmful test subjects (various pathogenic fungi and bacteria). About 20% showed strong pathogen inhibition on a broader antimicrobial spectrum. Of this 20%, all were effective against bacteria, but only a few showed antifungal activities. The most reactive strain with antifungal properties was *Cladosporium* sp. Analysis of its secondary metabolites showed that it produces brefeldin A – an antifungal compound [59]. Cork oak endophyte *Trichoderma citrinoviride* was shown to have a negative effect on several fungal pathogens that are associated with oak decline. A group of polypeptide antibiotics that showed antifungal activity against several forest tree pathogens were found [60].

Oak endophytes have shown potential as BCAs. Brooks et al. tried to use *Q. fusiformis* endophytic bacteria as a biological control agent against oak wilt (caused by *Ceratocystis fagacearum*). They isolated 889 bacteria, 189 of which inhibited pathogen growth *in vitro*. Six isolates were tested on young trees (*Q. texana* and live oaks) in growth chambers. Of these *Pseudomonas denitrificans* reduced the diseased trees by half and reduced crown loss by 17% [9]. Fungi isolated from several *Quercus* species showed promising inhibition of *Raffaelea quercus-mongolicae* wilt-causing fungus in dual plate assays (up to 68%) [61]. Kwaśna and Szewczyk tested multiple fungi isolated from *Q. robur* on seedling growth and found that some enhanced root growth by up to 200% and leaf weight by up to 300% [62]. A 2016 article on oak endophytic bacteria describes that *Pseudomonas, Bacillus,* and *Stenotrophomonas* strains were isolated. The researchers tested them for varied plant growth-promoting traits (PGPTs). All produced PGRs, some were able to produce siderophores, while others solubilized phosphate. Several strains showed growth inhibition against *Pseudomonas syringae* pv. s*yringae* in vitro [63].

All this data combined shows that *Q. robur* endosphere has the potential as a niche for microorganisms with beneficial traits. Thus, the discovery of new pedunculate oak-associated species and strains that can enhance tree health, growth, and pathogen resistance could very likely be beneficial not only to oaks but, as research suggests, to other forest trees.

1.5. Microorganism variety

An issue researchers in this field encounter is that plant-associated samples often contain substantial amounts of diverse microbial species and potentially multiple, difficult-to-differentiate, strains within a single genus (ex. *Pseudomonas*) [64,65], which is also true for trees. Since future research in this field will rely on the screening of new microbial species and strains, it will require methods that can efficiently and effectively differentiate between them, as different species and different strains can have vastly different effects on plant growth [66].

In the field of plant-associated microbiology, many methods (DNA sequencing, analytical profile index, immuno-assays, etc.) can be utilized to differentiate between species/strains. However, the currently widely used genomic approach is not without limitations. To genetically differentiate isolates at strain level is comparatively costly and requires experience and time. An alternative could be surface-enhanced Raman spectroscopy (SERS). It relies on molecule excitation with light and a subsequent photon scattering effect on altered surfaces. Different molecules produce different scatter patterns, i.e. spectra, and thus can be differentiated. SERS is beneficial in microbiology because it's a low cost, low volume, non-destructive, broad information content, rapid, high specificity, and sensitivity diagnostic method [67], and also because the analysis can be easily performed in an aqueous environment without interface or extensive pretreatments. In bacteriology, the efficacy of SERS was demonstrated with pure cultures, mixed samples, and single-cell experiments [68].

Thus, the use of SERS could potentially improve endophyte screening at a relatively early stage and allow to speed up the research into new and beneficial strains to be used in silviculture.

To sum up, due to the negative impact inorganic fungicides and fertilizers have on the environment and human health, their use should be avoided or at the very least limited. An alternative means to stimulate growth and fight pathogens can be microorganisms. Plant growth-promoting microorganisms can positively impact plant growth in a variety of ways: through enhanced nutrient availability, phytohormone, antimicrobial or pesticidal agent production, or by inducing systemic resistance. One of the directions this field is taking is the search and research of new microorganism species or strains that could be effectively adapted to use in silviculture.

Plant endosphere is an excellent choice for this search. Only a small number of plants have been studied in relation to their endophytes (microorganisms living within plant tissues asymptomatically) and all the plants tested thus far were shown to be inhabited by them. Endophytes may provide several key benefits as biostimulants and biocontrol agents, namely, they can be internalized by plants, also they can create long-lasting colonies. This would be economically beneficial and potentially solve and issue of successful field application.

Pedunculate oak endosphere is not widely researched, especially in terms of their cultivable endomicrobiota, thus they provide a novel niche to find bacteria and fungi that could potentially be utilized for forest protection and tree growth enhancement.

An additional problem in this field is the shear amount of microorganism species to test, thus effective and sensitive means for strain differentiation are necessary.

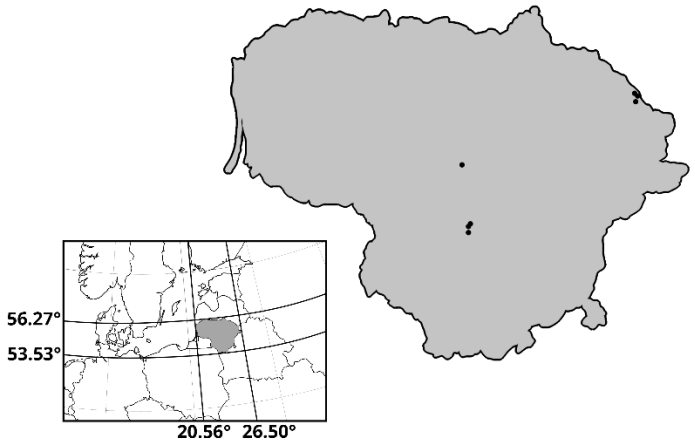
2. MATERIALS AND METHODS

All in vitro experiments were conducted in the Laboratory of Forest Plant Biotechnology, Institute of Forestry, Lithuanian Research Centre of Agriculture and Forestry in Girionys, Lithuania during 2018–2022. SERS experiments were done in collaboration with Valentinas Snitka, PhD, at Kaunas University of Technology, Research Centre for Microsystems and Nanotechnology, Kaunas, Lithuania.

The research object was pedunculate oak (*Quercus robur*) easily cultivable endophytes.

2.1. Field sampling (Article 1 and 4)

Field samples were collected in May of 2019 from pedunculate oaks at 8 random locations in Lithuania (Fig. 1). Trees were not differentiated based on bud flushing phenology, age, or growth site. Branches were transported to the lab and force flushed to limit additional infection. Due to the same reason, buds and primary leaves were chosen for endophyte isolation studies.



Research objects / Tyrimo objektai	Coordinates/Koordinatės	Forest district / Girininkija	Stand type / Medyno tipas
S	55.829832, 26.217380	Antazavės	Stand/Medynas
S2	55.802318, 26.1948466	Antazavės	Single/Pavienis
D	55.324223, 23.9769252	Kėdainių	Single/Pavienis
V	54.839992, 24.039341	Vaišvydavos	Single/Pavienis
I	54.8392751, 24.104978	Vaišvydavos	Single/Pavienis
I1	54.8577924, 24.042968	Vaišvydavos	Stand/Medynas
I2	54.8577745, 24.0429516	Vaišvydavos	Stand/Medynas
M	55.8301132, 26.2168633	Antazavės	Stand/Medynas

Fig. 1. *Growth sites and stand types of field samples collected from 8 Quercus robur trees in Lithuania*

1 pav. *Aštuonių paprastųjų ąžuolų, iš kurių buvo paimti mėginiai, augavietės ir medynų tipai Lietuvoje*

2.2. Endophyte isolation and identification (Articles 1, 2, 4 and unpublished data)

After flushing, buds and primary leaves were collected, and their surface was sterilized. Then to determine if surface sterilization was successful, explants were pressed onto a nutrient medium. The medium was observed for over a week for signs of microbial growth (Article 1, subsection 2.1). Explants were further grown on universal medium meant for woody plants, i.e., Woody plant medium (WPM, $CuSO_4 \times 5H_2O$ 0.25 mg/l, FeNaEDTA 36.7 mg/l, H_3BO_3 6.2 mg/l, $MnSO_4 \times H_2O$ 22.3 mg/l, $Na_2MoO_4 \times 2H_2O$ 0.25 mg/l, $ZnSO_4 \times 7H_2O$ 8.6 mg/l, $CaCl_2$ 72.5 mg/l, $Ca(NO_3)_2 \times 4H_2O$ 471.26 mg/l, KH_2PO_4 170.0 mg/l, K_2SO_4 990.0 mg/l, $MgSO_4$ 180.54 mg/l, NH_4NO_3 400.0 mg/l, gelrite 4.0 g/l, pH 5.6±0.1). Emerging microbial colonies were transferred as needed onto universal bacteria medium, i.e., solid Lysogeny Broth (LB, tryptone 10 g/l, NaCl 5 g/l, yeast extract 5 g/l, agar 10g/l, pH 7.2±0.1) medium (bacteria-like colonies) or solid malt medium (fungi-like colonies) (maltose 22 g/l, yeast extract 8 g/l, tryptone 6 g/l, glucose 20 g/l, gelrite 15 g/l, pH 5.5±0.1), the latter developed in Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry, Laboratory of Forest Plant Biotechnology for fungi propagation. Bacteria-like colonies were purified using the quadrant streaking method until they all had uniform colony morphology (Article 2, subsection 2.1.1). Mycelial fungi colonies were purified by multiple transfers and visually differentiated (Article 4). Bacterial and yeast samples were differentiated using a microscope by cell form and evidence of budding (Article 1 and 4).

Further on several physiological tests were carried out for bacterial samples for further strain separation. Namely cell size and form were observed under a microscope (10 000x magnification). Then biofilm formation, carbohydrate use (Phenol red test), and antibiotic susceptibility (Kirby-Bauer disk diffusion test) (as described in Article 2, subsections 2.1.2–2.1.4) tests were done. A string test (3% KOH) was used in lieu of Gram staining [69], and standard oxidase and catalase tests were done [70]. Additionally, an oxygen requirement test was performed using thioglycolate broth to determine if isolates were facultative anaerobes or obligate aerobes [71].

Moreover, endospore formation was evaluated. Bacteria were grown overnight in liquid LB. The next day they were washed three times in NaCl (0.9%) solution by centrifuge (3500x*g*). After the last wash, the bacteria were resuspended in NaCl solution. Then a microtube with this suspension was heated in a water bath at 80°C and 70°C (two groups) for 20 minutes. Afterwards, 100 µl of the bacterial suspension was pipetted and spread onto solid LB plates. If bacterial growth with the same colony morphology as before appeared within a week (22°C), the bacteria were determined to be potential endospore formers.

DNA extraction and genetic identification of the isolates were performed at *Macrogen* sequencing center in The Netherlands using selective primer pairs (*16S rRNA* for bacteria and *ITS* region for mycelial fungi and yeast samples) (Article 1, subsection 2.1 and Article 4, Materials and Methods section respectively).

2.3. Bacterial strain differentiation assay (Article 2)

To evaluate whether SERS technique can be useful in bacterial strain differentiation compared to standard *16S rRNA* gene sequencing (Article 1, subsection 2.1), bacterial endophyte isolates from two oak trees were randomly selected (Article 2, section 2).

Isolates were grown overnight in liquid LB cultures and prior to testing washed in NaCl solution as noted in the previous section. Subsequently, while the bacteria were still viable, SERS measurements were taken. Sample spectra were later processed and collected data were analyzed using multivariate cluster analyses – principal component analysis and discriminant function analysis (Article 2, subsection 2.2). In SERS, spectral band and peak positions lead to bacterial differentiation based on biochemical differences.

2.4. Plant growth-promoting traits (Articles 1, 4 and unpublished data)

Qualitative PGPT assays were performed on varied selective media in vitro. Isolates were tested for nitrogen fixation on nitrogen-free media (just bacteria), siderophore production on media enriched with insoluble from of iron, inorganic phosphate solubilization on media enriched with insoluble phosphorus source $Ca_3(PO_4)_2$ and organic phosphate mineralization on media with an organic source of phosphorus, i.e., soy lecithin, PGR – indole-3-acetic acid (IAA) production on media enriched with IAA precursor (Article 1, subsection 2.2; Article 4, Materials and Methods section) and potassium solubilization and ACC (1-aminocyclopropane-1-carboxylate) deaminase activity (just selected bacteria) tests.

Potassium solubilization assay was performed using selective media – namely preprepared Alexandrow's agar ($MgSO_4$ 0.5 g/l, $CaCO_3$ 0.1 g/l, $AlKO_6Si_2$ 2 g/l, glucose 5 g/l, $FeCl_3$ 0.005 g/l, $Ca_3(PO_4)_2$ 2 g/l, agar 20 g/l, pH 7.2±0.1) (Himedia, India), where potassium is in an insoluble form [72,73]. Briefly, solid media was prepared per the manufacturer's instructions, then microorganisms were placed on top of the medium (bacteria and yeast by stabbing the medium with an isolate swab on an inoculation needle, and by using mycelial fragments from the fungi). If clear areas appeared around the inoculation site within 2 weeks, the test was considered positive.

Additionally, selected bacterial samples were tested for ACC deaminase activity, as it is reported to have properties relating to reduced plant stress [24]. Briefly, selective minimal

Dworkin and Foster medium was prepared (0,4 % KH_2PO_4, 0,6 % Na_2HPO_4, 0,02 % $MgSO_4 \times 7H_2O$, 0,2 % glucose, 0,2 % gluconic acid, 0,2 % citric acid, 0,01 % $FeSO_4 \times 7H_2O$, 0,01 % H_3BO_3, 0,01 % $MnSO_4 \times H_2O$, 0,1 % $ZnSO_4 \times 7H_2O$, 0,8 mg $CuSO_4 \times 5H_2O$, 0,001 % MoO_3 for negative control; with added 0,2 % $(NH_4)_2SO_4$ for positive control; with added 3 mM ACC for the selective medium; (pH 7.2±0.1)), with ACC being the only nitrogen source [74]. Concurrently, plates with the same media composition, but without ACC or any other N source were used as a negative control. This test can only show if non-diazotrophic bacteria could produce ACC deaminase enzyme. This would be clear if no growth would appear on the inoculated (using inoculation needle) negative control plate, but colony growth would be observed on the plate with ACC.

2.5. *Populus* spp. growth promotion in vitro (Article 1)

Poplars – *Populus tremula* (in vitro culture establishment described by Vaičiukynė et al. [75]) and *P. tremula* × *P. alba* hybrid (IBL 91/78, established at the Forest Research Institute, Poland, from the vegetative buds of 6–7-year-old cloned trees) – were used for the in vitro experiments. Poplars were chosen due to several factors. *Populus* spp. are considered to be model organisms representative of all trees and other woody perennials [76]. They also are a common case study in plant-microbial interaction research [77].

Both in vitro clonal microshoot cultures were kept in the Laboratory of Forest Plant Biotechnology for 5 years before use in the experiments described here under continuous bimonthly propagation via microcuttings in standard composition Murashige and Skoog (MS, $CoCl_2 \times 6H_2O$ 0.025 mg/l, $CuSO_4 \times 5H_2O$ 0.025 mg/l, FeNaEDTA 36.7 mg/l, H_3BO_3 6.2 mg/l, KI 0.83 mg/l, $MnSO_4 \times H_2O$ 16.9 mg/l, $Na_2MoO_4 \times 2H_2O$ 0.25 mg/l, $ZnSO_4 \times 7H_2O$ 8.6 mg/l, $CaCl_2$ 332.02 mg/l, KH_2PO_4 170.0 mg/l, KNO_3 1900.0 mg/l, $MgSO_4$ 180.54 mg/l, NH_4NO_3 1650.0 mg/l, glycine 2.0 mg/l, myo-inositol 100.0 mg/l, nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, thiamine HCl 0.1 mg/l, pH 5.6±0.1) medium.

The experimental photoperiod was 16/8h (day/night) with growth chamber temperature at 25/20°C respectively. The light source was fluorescent white lamps (irradiance 30 μmol m^{-2} s^{-2}). All tests were done using 3 biological replicates with 10 individual samples per each replicate. The data for statistical analysis were pooled (Article 1, subsection 2.3).

Paenibacillus tundrae strain was selected for in vitro growth promotion assay based on the shear abundance in tested samples and literature review (specific strain was selected at random from the *Paenibacillus tundrae* genetic group). Uniform inoculated shoots were grown in WPM for 2 months. Afterward, to determine the advantages of using the selected strain, several morphological growth parameters were measured. Namely, vegetative growth parameters such

as root length and number, lateral root number, shoot height, leaf width, shoot number, and biomass. Lateral root density was also later calculated (Article 1, subsection 2.3). Statistical analysis was done via ANOVA (Analysis of variance) and Student's *t* test.

2.6. Plant-endophyte interaction impact on host secondary metabolism (Article 3)

As before, the same bacterial *Paenibacillus tundrae* isolate was further researched to determine if alongside potential morphological changes in poplars, it could also impact poplar metabolomics. Specifically, poplar photosynthesis pigment content (chlorophyll *a* (CHA) and *b* (CHB), carotenoids (CAR)), antioxidant activity (via ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assays) and secondary metabolite concentration (total phenol content (TPC) and total flavonoid content (TFC)). Thus, concurrently uninoculated and inoculated poplar microshoots were grown in vitro in the same manner as described before and samples were collected at 3 stages (4, 6, and 8 weeks) (Article 3, subsection 2.1).

This study was conducted using spectroscopy at various wavelengths on methanolic (phenolics and antioxidants) or acetone (photosynthesis pigments) extracts from aboveground plant biomass (leaves and stems) (Article 3, subsection 2.2 and 2.3). The sample size was the same as previously described. Statistical analysis was conducted as described (Article 3, subsection 2.4).

2.7. Antagonism assay (Article 4)

Fungal and bacterial endophytes were selected for an antagonism assay against 4 common European forest pathogens: *Heterobasidion annosum* (HA), *Hymenoscyphus fraxineus* (HF), *Lophodermium seditiosum* (LS) and *Phellinus tremulae* (PT).

For this assay, a dual in vitro culture method was selected (Article 4, Materials and Methods section). At least five biological replicates were done, each using fresh colonies. Inoculants and pathogens were tested prior to the assay to determine a medium that would be optimal for pathogen growth, but on which potential antagonists would grow as well. All 6 isolated fungi grew well on pathogen-compatible media, however, only 5 bacteria from the oak endophyte isolate group were able to sustain the necessary viability to be further used in the antagonism assay.

Growth inhibition by endophytic microorganisms was measured as radial growth inhibition as compared to the control plates.

3. RESULTS AND DISCUSSION

3.1. Isolation, identification, and PGPTs (Articles 1, 2, 4 and unpublished data)

Endophytic microorganisms were successfully isolated from 8 *Q. robur* trees (buds and leaves). In total 55 microorganisms were obtained. Per the morphological identification, 49 were rod-shaped bacteria, 2 – yeasts, and 4 – mycelial fungi. Further morphological analysis allowed fungi separation based on colony morphology. Based on colony color and visual appearance all 6 fungi were determined to be potentially different species. Bacteria were differentiated into 7 morphotypes (Supplementary table 1 and partly Article 2, Table 2 and 3, Figure 1).

All isolates were successfully sequenced. Sequencing data showed that the isolated bacteria could be assigned to *Firmicutes* and *Proteobacteria* phyla. Both of these groups were previously found in various woody plants and accounted for a large part of the bacterial diversity [6,78–81], including *Quercus robur* [82,83]. Isolates were assigned to *Bacillus*, *Delftia*, *Paenibacillus*, *Pantoea,* and *Pseudomonas* genera. *Paenibacillus* spp. and *Pseudomonas* spp. were the most common bacteria isolated in this study. All of these taxa were previously isolated from other woody plants [43,66,80,84–89].

Neocucurbitaria, *Aspergillus*, *Talaromyces,* and *Byssochlamys* molds were also isolated from oaks as were yeasts *Meyerozyma* and *Microstroma. Aspergillus* spp. have been isolated from *Q. robur* before [62,90], and so were *Microstroma* sp. [91] and *Neocucurbitaria quercina* [92]. Data on *Meyerozyma guilliermondii* (syn. *Candida guilliermondii*) endophytism in oaks were not found, but other *Candida* spp. have been previously isolated from *Quercus* [91] and other forest [93] and fruit trees [94,95]. *Talaromyces amestolkiae* has been isolated from mangrove trees [96] and *Talaromyces* sp. was isolated from pines (*Pinus massoniana* Lamb.) before [97]. *Byssochlamys spectabilis* (syn. *Paecilomyces variotii*) was previously isolated from pistachio trees (*Pistacia vera* L.) [25], while *Paecilomyces formosus* was linked with *Quercus brantii* dieback in Iran [98].

PGPTs (phosphate solubilization and mineralization, IAA and siderophore production) were evaluated for all isolates qualitatively (Article 1, Table 1 and Article 4, Table 2). All endophytes were shown to produce phytohormone IAA and degrade inorganic phosphates. All isolates apart from 2 *Bacillus* sp. samples were able to mineralize organic phosphates. Out of the bacterial samples, only *Pseudomonas* spp. were capable of siderophore production, along with yeast *Microstroma*, molds *Neocucurbitaria*, *Aspergillus,* and *Byssochlamys*. Among the bacterial samples nitrogen fixation was not ubiquitous – bacteria from the *Paenibacillus* genus were not able to grow on a nitrogen-free medium (Article 1, Table 1), while only selected bacteria were tested for potassium solubilization and ACC deaminase activity (Table 1,

unpublished data). All tested *Pantoea* spp. and *Pseudomonas* spp. were positive for both, while both *Bacillus* spp. and *Paenibacillus tundrae* were negative for both. *Delftia* sp. was positive for ACC activity and negative for potassium solubilization.

Table 1. *Results of potassium solubilization tests and ACC deaminase enzyme activity performed on selected bacterial isolates*

1 lentelė. *Kalio tirpumo ir ACC deaminazės aktyvumo testų rezultatai atlikti naudojant selektyvias terpes*

Isolate no.	**Closest genetic match / Artimiausias genetinis atitikmuo**	**Potassium solubilization / kalio tirpinimas**	**ACC deaminase activity / ACC deaminazės aktyvumas**
44.1	*Pseudomonas brenneri*	+	+
34	*Pantoea agglomerans*	+	+
24	*Pseudomonas proteolytica*	+	+
29	*Pseudomonas azotoformans*	+	+
27	*Pantoea agglomerans*	+	+
13	*Delftia lacustris*	-	+
23.1	*Bacillus aryabhattai*	-	-
55.2	*Bacillus aryabhattai*	-	-
43.1	*Paenibacillus tundrae*	-	-
32	*Pseudomonas paralactis*	+	+

+/- denotes positive or negative test result respectively/. +/- atitinkamai nurodo teigiamą ir neigiamą testo rezultatą.

Bacillus, *Delftia*, *Pantoea*, and *Pseudomonas* bacteria have been reported to exhibit multiple PGPTs previously [99–105]. Notably, *Bacillus* taxa is already utilized in commercial biostimulants [103]. Tested fungi also were capable of several PGPTs [106–108]. *Paenibacillus* spp. have been studied previously for plant growth promotion, exhibiting promising results as well [85,109–111].

Byssochlamys spectabilis was reported as an effective plant growth promoter for peppers (*Capsicum annuum* L.) and tomatoes (*S. lycopersicum*). Reported results show that it was capable of siderophore and IAA production and solubilization of phosphates [112]. *Meyerozyma guilliermondii* was shown to promote the growth of durum wheat (*Triticum turgidum* subsp. *Durum* Desf.) [113]. Improvements to the growth of maize (*Zea* sp.) and rice (*Oryza* sp.) were achieved by applying *Aspergillus awamori* [114] and *Aspergillus niger* [115] respectively. *Q. robur* seedlings exhibited increased root, stem, and leaf biomass after inoculation with *Aspergillus niger* [62]. *Aspergillus* sp. and *Talaromyces* sp. isolates were previously reported to

solubilize phosphates [116], while *Microstroma* sp. has been shown to produce high levels of IAA [117,118].

3.2. Bacterial analysis via SERS (Article 2)

In this experiment, SERS led to the separation of 3 genetically homologous (based on *16S rRNA* gene fragment sequencing) *Pantoea agglomerans* strains into 2 groups (Article 2, Figure 4 and 5) and 3 *Pseudomonas* samples were also separated into 3 main groups (Article 2, Figure 4 and 7), while both tested *Paenibacillus tundrae* strains were clustered together, denoting that they were genetically and biochemically very close, even though they were isolated from two different oak trees (Article 2, Figure 4 and 6). In comparison, all *Pantoea agglomerans* samples originated from a single tree.

Based on these data, it was demonstrated that SERS in tandem with multivariate cluster analyses can be an effective alternative in bacterial differentiation (subspecies) to standard *16S rRNA* gene sequencing in plant-associated samples. The SERS data also provide knowledge on the studied bacterial cell composition. Previous research showed that SERS works for human and food pathogens [119–122]. However, it's been noted before that this technique was not utilized for plant-associated bacteria [123]. SERS spectra are inherently complex, due to spectral peak overlap and shifts [71], therefore chemometric multivariate analyses are used to improve the interpretation of experimental data [124]. Species differentiation using the SERS platform is not uncommon [71,125], and successful differentiation of closely related bacterial samples can be best showcased by the differentiation of several genoserogroups within a single species of *Listeria monocytogenes* [126]. While the exact nature of the bacterial SERS bands is difficult to assign, some similarities with spectra from previous studies were noted: *Pantoea agglomerans* [127], *Pantoea* sp. [128]. *Pseudomonas aeruginosa* – a commonly studied pseudomonad, was shown to exhibit some similarities to the *Pseudomonas* spp. spectra observed in this work [129,130]. Also, plant-associated *Paenibacillus validus* has exhibited many of the same peaks, as those reported in our study [123].

These results showcase the innovative applicability of surface-enhanced Raman spectroscopy in tree endophyte research.

3.3. Growth promotion and *Paenibacillus* impact on tree metabolism in vitro using *Populus* as a model system (Articles 1 and 3)

In this study, *Paenibacillus tundrae* was used for poplar microshoot inoculation in vitro. *P. tundrae* was first isolated from the soil in North America [131] and later from barley [132]. The results from this study demonstrated that *Paenibacillus tundrae* had a growth-enhancing

effect mainly concentrated on poplar roots (Article 1, Table 2, Figures 2-4). While both tested genotypes were affected in a similar manner, it was evident that the hybrid poplar was impacted more. Inoculated *P. tremula* microshoots exhibited increased lateral root numbers (44.7%), lateral root density (66%), fresh and dry root biomass (101.9% and 63.6% respectively). *P. tremula* × *P. alba* microshoots exhibited increased lateral root number and density (213.7% and 125.6% respectively). Fresh and dry root biomass increased too (197.1% and 144.8% respectively). Hybrid microshoots also exhibited on average longer adventitious roots (102%, sum length), the length of the longest adventitious root increased by 79.5%. The number of adventitious roots was enhanced by 65% too. It's also noteworthy that *P. tremula* x *P. alba* hybrid's aboveground biomass was affected negatively as compared to the control group (25%).

The inoculum of *Paenibacillus tundrae* also showed IAA production. This means that the promotion of root growth of the investigated poplar genotypes could be the result of IAA, due to its reported biochemical properties [6,85,87,110,133].

Bacterial inoculation can also alter plant metabolism [134–138] and the changes in plant metabolic signature can show how a plant reacts to new conditions [139]. During this work, measurements of phenolics, antioxidant capacity, and pigment concentrations after inoculation of *P. tremula* x *P. alba* with *Paenibacillus tundrae* showed an impact on the host's metabolic profile depending on the time elapsed after inoculation. Specifically, after 4 weeks the effect was insignificant, while after 6 weeks the differences were already evident. Parameters of TPC, TFC, ABTS, and DPPH, CHA, and CAR were negatively affected, while CHB increased compared to controls. After 8 weeks, TPC, TFC, ABTS, and DPPH parameters remained negatively affected, while the concentrations of photosynthetic pigments increased (Article 3, Figure 1). Since flavonoids have an antioxidant capacity and can also regulate the interaction between plants and microorganisms [140,141], it is possible that changes in their profile caused a negative effect on DPPH and ABTS assay results. In addition, correlation and principal component analyses showed how inoculation with *Paenibacillus tundrae* influences the interactions between the studied variables (pigment concentration versus phenolics and antioxidant capacity parameters) (Article 3, Figures 2 and 3).

In several studies, enhanced plant pigment concentration is linked to the general vigor of the plant [31,109,142,143]. However, CAR, CHA, and CHB have secondary functions. CAR and chlorophylls may act as antioxidants, while CAR can also be a precursor to phytohormones [139,144,145].

In a meta-analysis performed by Wallis and Galarneau (2008 to 2017 data), it was reported that total phenolics increase after colonization of bacteria, but the type of bacteria has no difference (beneficial or pathogenic) [146]. This was confirmed by other studies [27] but

does not fit the results from this study. It may be that the inoculated poplars exhibited lower amounts of phenolics because they do not recognize *Paenibacillus tundrae* colonization. That could potentially stem from the bacterium's ability to alter signal transduction [140]. It's worth noting that *Paenibacillus* genera have been reported as inhabitants of several tree species [43,106,147–151] and were called "predominant" within the tree endosphere [85].

3.4. Antagonism assay (Article 4)

During this work, each tested pathogen was inhibited by at least one tested endophytic isolate (>30% inhibition rate, $p \leq 0.05$). Overall, yeast *Meyerozyma* sp., *Pseudomonas* spp., and *Pantoea* spp. expressed broad-spectrum inhibition. *Pseudomonas* sp. demonstrated the highest inhibition of LS (68.4%) (Figure 2a) and PT (57.5%) (Figure 2b), *Meyerozyma* sp. – of HA (45.7%) (Figure 3a), *Byssochlamys* sp. – of HF (59.8%) (Figure 2c).

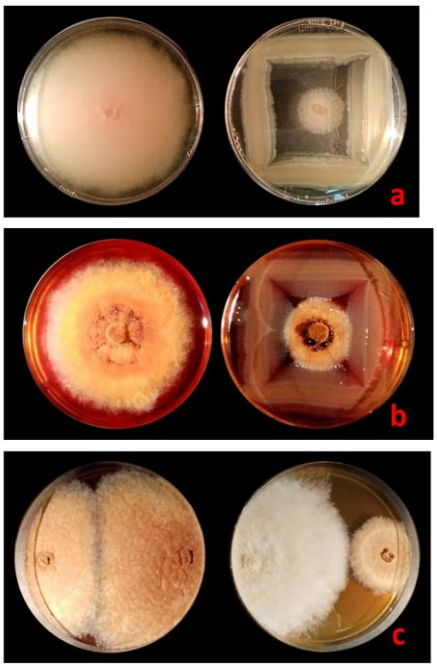


Fig. 2 *Dual culture antagonism assay exhibiting highest mean percentage of antagonism: a) Lophodermium seditiosum and Pseudomonas sp. no. 32 (68.4%***), b) Phellinus tremulae and Pseudomonas sp. no. 32 (57.5%**), c) Hymenoscyphus fraxineus (HF) and Byssochlamys sp. (59.8%**); HF negative control is on the left. HF negative control plug was transferred 6 days after the initial pathogen infection. p<0.01 – **, p<0.001 – ***, based on post hoc Dunn's test.*

2 pav. *Antagonisto ir patogeno kokultivacija in vitro. Efektyviausi radialinio augimo slopinimo pavyzdžiai: a) Lophodermium seditiosum ir Pseudomonas sp. no. 32 (68,4 %***), b) Phellinus tremulae ir Pseudomonas sp. no. 32 (57,5 %**), c) Hymenoscyphus fraxineus (HF) ir Byssochlamys sp. (59,8 %**); HF neigiama kontrolė yra kairėje. HF neigiamos kontrolės micelis į kokultivacinę terpę buvo perkeltas 6 dienos po pirminio patogeno perkėlimo. p<0,01 – **, p<,.001 – ***, pagal post hoc Dunn testą.*

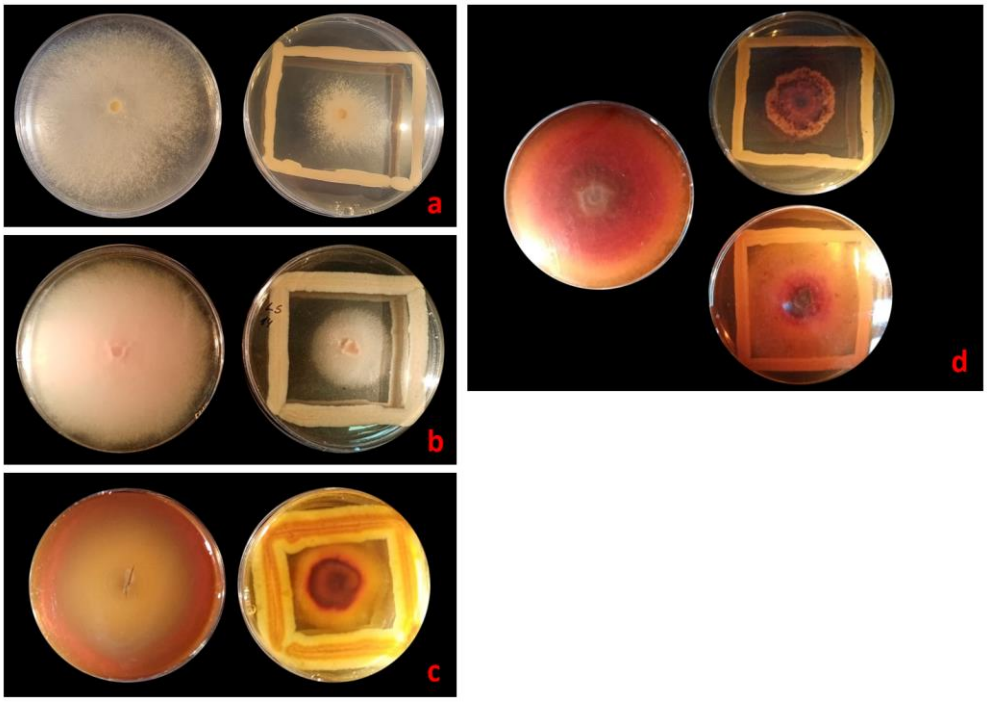


Fig. 3. *Dual culture antagonism assay with yeast Meyerozyma sp., which exhibited good antagonism (>30%) against 3 of the tested pathogenic fungi: a) Heterobasidion annosum (45.7%**), b) Lophodermium seditiosum (41.7%**), c) Phellinus tremulae (PT) (52.1%**), d) Hymenoscyphus fraxineus (HF) (showing variation in HF growth within the experimental group, but no significant antagonism). HF and PT plates were inoculated 6 and 7 days respectively after the initial pathogen infection. p<0.01 – **, based on post hoc Dunn's test.*

3 pav. *Antagonisto ir 4 tirtų patogenų kokultivacija in vitro su Meyerozyma sp. miele, efektyviai slopinusia 3 patogenų radialinį augimą (>30 %): a) Heterobasidion annosum (45,7 %**), b) Lophodermium seditiosum (41,7 %**), c) Phellinus tremulae (52,1 %**), d) Hymenoscyphus fraxineus (dėl stebėtos HF augimo variacijos kontrolinėje grupėje, neužfiksuotas statistiškai patikimas HF antagonizmas). HF ir buvo inokuliuoti atitinkamai 6 ir 7 dienos prieš potencialių antagonistų inokuliaciją. p<0,01 – **, pagal post hoc Dunn testą.*

Hymenoscyphus fraxineus causes severe ash dieback. Several microbial species previously demonstrated an antagonistic effect against HF in vitro: *Botrytis cinerea* and *Phoma macrostoma* var. *incolorata* [152], *Bacillus* sp., *Pantoea* sp., *Pseudomonas* sp. [43], *Papiliotrema flavescens*, *Vishniacozyma carnescens*, *Aureobasidium pullulans* [153], *Meyerozyma guilliermondii* [154], and *Hypoxylon rubiginosum* [155].

Lophodermium seditiosum is a conifer needle cast-causing agent. Though information on the biocontrol of LS is very rare, T. Poolak reports 19.4 to 44.1% inhibition observed using endophytic fungi [156]. *Heterobasidion annosum* also attacks conifers. Previous studies show that *Bacillus subtilis* strain was tested in a dual culture assay and exhibited notable inhibition

[157] as did *Pseudomonas protegens* [41]. Mesanza et al. tested *Pseudomonas fluorescens* and *Bacillus simplex* against HA [158].

M. guilliermondii was previously studied and expressed inhibition of pear (*Pyrus* sp.), apple (*Malus* sp.), lemon and mandarin (*Citrus* spp.) post-harvest pathogens [94,95,117,159–161]. However, efforts are mainly oriented toward orchard protection and post-harvest diseases [162–167].

Out of the 4 tested molds in this study, only *Byssochlamys* sp. and *Aspergillus* sp. showed biocontrol characteristics against tested pathogens. *Byssochlamys spectabilis* has been reported as BCA before [168–170]. Moreover, crude extracts of *B. spectabilis* are being used as BCAs in China [171].

Aspergillus fungi have been reported as BCAs too [172,173]. Mahendran et al. report that *Aspergillus terreus* exhibited inhibition of two rubber tree (*Hevea brasiliensis* Müll.Arg.) pathogens in vitro and in vivo [174], while *Aspergillus flavus* has been reported as a BCA in almond (*Prunus* sp.), fig (*Ficus* sp.) and pistachio (*Pistacia* sp.) trees [175].

4. CONCLUSIONS

1. *Quercus robur* endophytic fungi (*Neocucurbitaria, Aspergillus, Talaromyces, Byssochlamys, Meyerozyma, Microstroma*) and bacteria (*Bacillus, Pantoea, Delftia, Pseudomonas, Paenibacillus*) isolates tested positive for at least three plant growth-promoting traits. Inorganic phosphate solubilization and phytohormone indole-3-acetic acid (IAA) production was ubiquitous. This shows that tested isolates have the potential to be used for tree growth promotion.
2. Surface-enhanced Raman spectroscopy (SERS) can be effectively applied to differentiate endophytic bacteria with minimal sample pretreatment. This innovation can lead to faster endophyte differentiation and speed up biostimulant and biocontrol agent research at an early stage.
3. Model poplar (*Populus*) tree growth was improved by inoculation with endophytic *Paenibacillus tundrae* isolated from *Quercus robur*. An increase in root system parameters was observed. This isolate also positively impacted *Populus* sp. metabolome, specifically photosynthesis pigment concentrations, hence showing that it can potentially be used to promote tree growth and secondary metabolite production in vitro.
4. *Quercus robur* endophytes can limit the radial growth of forest pathogens (*Heterobasidion annosum, Hymenoscyphus fraxineus, Lophodermium seditiosum,* and *Phellinus tremulae*) in vitro. Bacteria *Pantoea agglomerans*, *Pseudomonas azotoformans,* and yeast *Meyerozyma guilliermondii* exhibited significant antagonistic properties against three out of four tested pathogens. Further investigation of these endophytic species may lead to advances in creating new management strategies for these pathogens.

SANTRAUKA

ĮVADAS

Tikėtina, kad dėl klimato kaitos padaugės patogenų protrūkių ir gali susidaryti nepalankios vietinės abiotinės sąlygos optimaliam miško augimui, o tai yra vienas didžiausių iššūkių miškų sveikatai (1,2). Reikia imtis specifinių priemonių, kad miškai galėtų normaliai funkcionuoti, valdant ligų protrūkius ir mažinant abiotinių streso veiksnių daromą žalą. Tad labai svarbu gerinti medžių augimo sąlygas ir sveikatos būklę. Tačiau neorganinių cheminių medžiagų (neorganinių trąšų ir pesticidų) naudojimas Europoje ribojamas dėl neigiamo poveikio dirvožemiui, vandeniui, biologinei įvairovei ir žmonių sveikatai (3). Mikroorganizmų pagrindu sukurtų stimuliatorių ir fungicidų naudojimas galėtų būti alternatyvus būdas kovoti su patogenais bei skatinti medžių augimą (3).

Palyginti nauja niša, kurioje galima rasti naudingų mikroorganizmų, yra augalų endosfera (3). Endofitai, dažnai bakterijos ar grybai, gyvena augaluose tiek viduląstelinėse, tiek tarpląstelinėse ertmėse, nesukeldami savo šeimininkams pastebimos žalos (4-6). Kadangi jie yra įsikūrę savo augalų šeimininkų audiniuose, jie gali sukurti ilgalaikes kolonijas. Endofitai gali palaikyti mutualistinius santykius su augalais šeimininkais. Tokiu būdu jie gali padėti savo šeimininkams, paveikdami jų medžiagų apykaitos kelius (7), gamindami fitohormonus arba didindami maisto medžiagų biologinį prieinamumą (3,8). Endofitai taip pat gali būti biologinės kontrolės agentai kompleksinėje kenkėjų ir ligų valdymo sistemoje (9,10). Toks biologinių priemonių integravimas būtų naudingas komercinėje miškininkystėje, taip pat medelynuose, nes potencialiai sumažintų arba bent jau apribotų fungicidų ir cheminių trąšų ar stimuliatorių naudojimą.

Visgi praeityje daugiausia dėmesio buvo skiriama endofitams, susijusiems su svarbiomis žemės ūkio rūšimis, o medžių endofitų funkcijos daugeliu atvejų yra taip pat menkai suprantamos, kaip ir jų įvairovė bei bendrijų struktūra. Remiantis šiomis ribotomis žiniomis, praktinis miško apsaugos pritaikymas remiantis endofitais vis dar yra retas (11-13).

Naujumas

Naudojant nuo kultivacijos priklausomus metodus, grybai (*Neocucurbitaria, Aspergillus, Talaromyces, Byssochlamys, Meyerozyma, Microstroma*) ir bakterijos (*Bacillus, Pantoea, Delftia, Pseudomonas, Paenibacillus*) buvo išskirtos iš paprastojo ąžuolo (*Quercus robur* L.) pumpurų ir lapų endosferos. Sustiprinto paviršiaus Ramano spektroskopija (SERS) pasirodė esantis veiksmingas būdas, leidžiantis greitai įvertinti paprastojo ąžuolo bakterinių endofitų vidurūšinę įvairovę. Be to, įrodyta, kad *Paenibacillus tundrae*, išskirtos iš paprastojo ąžuolo,

didina modelinių tuopų (*Populus*) šaknų augimą ir fotosintezės pigmentų koncentraciją. Paprastųjų ąžuolų grybiniai ir bakteriniai endofitai taip pat pasižymėjo antagonistiniu potencialu prieš Europos miškų patogenus (*Hymenoscyphus fraxineus, Phellinus tremulae, Heterobasidion annosum, Lophodermium seditiosum*).

Apibendrinant galima teigti, kad gauti moksliniai duomenys suteikia žinių apie kultivuojamus endofitus, aptinkamus paprastųjų ąžuolų audiniuose, ir potencialiai taps pagrindu kuriant ekologiškus miško auginimui skirtus produktus.

Tikslas

Šio tyrimo tikslas – ištirti paprastojo ąžuolo (*Quercus robur* L.) lapuose ir pumpuruose aptinkamų endofitų potencialą skatinti medžių ir slopinti patogenų augimą.

Hipotezė

Paprastojo ąžuolo (*Quercus robur* L.) pumpurų ir lapų endosferoje yra bakterijų ir grybų padermių, kurios galėtų būti naudojamos medžių augimui skatinti ir miško patogenų augimui slopinti *in vitro*.

Uždaviniai

1. Išskirti ir identifikuoti paprastojo ąžuolo lapų ir pumpurų lengvai kultivuojamas endofitines bakterijas ir grybus;
2. Įvertinti, ar paprastojo ąžuolo bakterinius endofitus galima lengvai atskirti naudojant sustiprinto paviršiaus Ramano spektroskopiją (SERS), kad juos būtų galima efektyviai panaudoti tolesniuose eksperimentuose;
3. Įvertinti, kokiomis augalų augimą skatinančiomis savybėmis kokybiškai pasižymi išskirti endofitai *in vitro*;
4. Nustatyti, ar atrinkti bakteriniai endofitai gali pagerinti modelinių tuopų (*Populus*) augimą ir antrinių metabolitų gamybą *in vitro*;
5. Įvertinti atrinktų endofitų potencialą slopinti miško patogenų *Heterobasidion annosum* ((Fr.) Bref.), *Hymenoscyphus fraxineus* (Baral et al.), *Lophodermium seditiosum* (Minter, Staley & Millar) ir *Phellinus tremulae* (Bondartsev & P.N. Borisov) augimą *in vitro* sąlygomis.

Ginamieji teiginiai

1. Paprastojo ąžuolo endofitai pasižymi augalų augimą skatinančiomis savybėmis, dėl kurių gali būti skatinamas medžių augimas;
2. Sustiprinto paviršiaus Ramano spektroskopija (SERS) yra veiksmingas metodas, palyginti su standartine *16S rRNR* sekoskaita, siekiant įvertinti paprastojo ąžuolo bakterinių endofitų vidurūšinę įvairovę;

3. Endofitinės *Paenibacillus tundrae* gali skatinti modelinių *Populus* medžių augimą *in vitro* ir teigiamai veikti jų antrinį metabolizmą;

4. Paprastojo ąžuolo endofitai gali apriboti *Heterobasidion annosum* ((Fr.) Bref.), *Hymenoscyphus fraxineus* (Baral et al.), *Lophodermium seditiosum* (Minter, Staley & Millar) ir *Phellinus tremulae* (Bondartsev & P. N. Borisov) patogenų augimą *in vitro.*

METODIKA

Visi *in vitro* eksperimentai buvo atlikti Lietuvos agrarinių ir miškų mokslų centro Miškų instituto Miško augalų biotechnologijos laboratorijoje Girionyse 2018–2022 m. Sustiprinto paviršiaus Ramano spektroskopijos eksperimentai atlikti bendradarbiaujant su daktaru Valentinu Snitka Kauno technologijų universiteto Mikrosistemų ir nanotechnologijų mokslų centre, Kaune.

Tyrimų objektas buvo paprastojo ąžuolo (*Quercus robur*) lengvai kultivuojami endofitai.

Mėginių rinkimas (Straipsnis Nr. 1 ir Nr. 4)

Lauko mėginiai buvo surinkti 2019 m. gegužės mėn. iš paprastųjų ąžuolų 8 atsitiktinai parinktose Lietuvos vietose (1 pav.). Medžiai nebuvo diferencijuojami pagal pumpurų sprogimo fenologiją, amžių ar augimo vietą. Šakos buvo gabenamos į laboratoriją, siekiant apriboti šalutinių užkratų tikimybę. Dėl tos pačios priežasties endofitų išskyrimo tyrimams buvo pasirinkti pumpurai ir pirminiai lapai.

Endofitų izoliacija ir identifikacija (Straipsnis Nr.1, Nr.2, Nr.4 ir nepublikuoti duomenys)

Prasiskleidus pumpurams, jie ir pirminiai lapai buvo surinkti ir jų paviršius sterilizuotas. Tada, siekiant nustatyti, ar paviršiaus sterilizacija buvo sėkminga, eksplantai buvo įspausti į mitybinę terpę. Daugiau kaip savaitę buvo stebima, ar terpėje nėra mikroorganizmų augimo požymių (1 straipsnio 2.1 poskyris). Toliau eksplantai buvo auginami ant universalios sumedėjusiems augalams skirtos terpės (WPM). Atsiradusios mikroorganizmų kolonijos pagal poreikį perkeltos į universalią bakterijų terpę, t. y. kietą LB terpę (į bakterijas panašios kolonijos) arba kietą maltozės terpę, sukurtą Lietuvos agrarinių ir miškų mokslų centro Miškų instituto Miško augalų biotechnologijų laboratorijoje grybams dauginti (į grybus panašios kolonijos). Bakterijų ir grybų mėginiai buvo išgryninti, o bakterijų ir mielių mėginiai vėliau diferencijuojami naudojant mikroskopą pagal ląstelių formą ir pumpuravimo požymius (1 ir 4 straipsniai).

Izoliatų identifikavimui buvo naudojami tiek morfologiniai, tiek genetiniai metodai. Buvo atlikti keli fiziologiniai bakterijų mėginių tyrimai, kad būtų galima toliau atskirti jų padermes. Ląstelių dydis ir forma buvo stebimi mikroskopu (10 000 kartų padidinimas). Tada buvo atlikti bioplėvelės formavimosi, angliavandenių naudojimo ir jautrumo antibiotikams (kaip aprašyta 2 straipsnio 2.1.2–2.1.4 poskyriuose) tyrimai. Vietoj Gram dažymo buvo naudojamas 3 % KOH testas (69), atlikti standartiniai oksidazės ir katalazės testai (70). Be to, buvo atliktas deguonies poreikio testas, naudojant tioglikolato sultinį, siekiant nustatyti, ar izoliatai yra fakultatyviniai anaerobai, ar obligatiniai aerobai (71).

Be to, buvo įvertintas bakterijų endosporų susidarymas. Bakterijos buvo auginamos per naktį skystoje LB terpėje. Kitą dieną jos buvo tris kartus nuplautos NaCl (0,9 %) tirpale centrifuguojant (3500x*g*). Po paskutinio plovimo bakterijos buvo resuspenduotos NaCl tirpale. Tada mėgintuvėlis su šia suspensija buvo kaitinamas vandens vonelėje 80 °C ir 70 °C temperatūroje (dvi grupės) 20 minučių. Po 20 minučių 100 µl šios suspensijos buvo pipete užlašinta ant LB terpės. Jei per savaitę (22 °C temperatūroje) buvo pastebėtas bakterijų su tokia pačia kolonijų morfologija kaip ir anksčiau augimas, buvo laikoma, kad bakterijos yra potencialios endosporų formuotojos.

Izoliatų DNR išskyrimas ir genetinis identifikavimas atliktas Macrogen sekoskaitos centre Nyderlanduose, naudojant selektyvias pradmenų poras (*16S rRNR* bakterijoms ir *ITS* sritis grybų ir mielių mėginių grybams) (atitinkamai 1 straipsnio 2.1 poskyris ir 4 straipsnio skirsnis "Medžiaga ir metodai").

Bakterinių padermių atskyrimas (Straipsnis Nr.2)

Siekiant įvertinti, ar sustiprinto paviršiaus Ramano spektroskopija (SERS) gali būti naudinga diferencijuojant bakterijų padermes, palyginti su standartiniu *16S rRNR* geno sekos nustatymu (1 straipsnio 2.1 poskyris), atsitiktinai buvo atrinkti bakterijų endofitų izoliatai iš dviejų ąžuolų (2 straipsnio 2 poskyris).

Izoliatai buvo auginami per naktį skystose LB terpėse ir prieš tyrimą nuplauti NaCl tirpale kaip minima ankstesniame skyrelyje. Vėliau, kol bakterijos dar buvo gyvybingos, buvo atlikti SERS matavimai. Po to buvo apdoroti mėginių spektrai ir surinkti duomenys analizuoti taikant daugiamatę klasterinę analizę – principinių komponenčių analizę ir diskriminantinių funkcijų analizę (2 straipsnis, 2.2 skirsnis). Naudojant SERS, spektrinės juostos ir pikų padėtys leidžia atskirti bakterijas pagal jų biocheminius skirtumus.

Augalų augimą skatinančios savybės (Straipsnis Nr.1, Nr.4 ir nepublikuoti duomenys)

Kokybiniai augalų augimą skatinančių savybių tyrimai buvo atliekami įvairiose selektyviose terpėse *in vitro*. Izolatai buvo tiriami terpėje be azoto (tik bakterijos) siekiant nustatyti ar jie yra azoto fiksatoriai. Taip pat terpėje, praturtintoje netirpia geležimi, siekiant nustatyti ar jie geba gaminti sideroforus. Terpėje, praturtintoje netirpiu fosforo šaltiniu, norint sužinoti ar jie geba tirpinti neorganinius fosfatus, bei terpėje, praturtintoje organiniu fosforo šaltiniu siekiant įvertinti jų gebėjimus mineralizuoti organinius fosfatus. Buvo įvertinta fitohormono indolo-3-acto rūgšties gamyba terpėje, praturtintose šio fitohormono prekursoriumi (1 straipsnio 2.2 poskirsnis; 4 straipsnio skirsnis "Medžiaga ir metodai").

Taip pat nustatyta izoliatų geba tirpinti kalį bei jų ACC (1-aminociklopropan-1-karboksilato) deaminazės aktyvumas (tik pasirinktos bakterijos). Kalio tirpinimo tyrimas buvo atliktas naudojant selektyvią terpę, t. y. iš Aleksandrovo agarą (Himedia, Indija), kuriame kalis yra netirpioje formoje (72,73). Trumpai, terpė buvo paruošta pagal gamintojo instrukcijas, tada ant terpės buvo dedami mikroorganizmai (bakterijos ir mielės, smeigiant terpę izoliacine adata ir naudojant grybienos fragmentus miceliniams grybams). Jei per 2 savaites aplink inokuliacijos vietą atsirado permatomų zonų, testas buvo laikomas teigiamu.

Be to, buvo ištirtas atrinktų bakterijų ACC deaminazės aktyvumas, nes žinoma, kad ji turi savybių, susijusių su augalų streso reguliavimu (24). Trumpai, buvo paruošta selektyvi Dworkin ir Foster terpė, kurioje ACC buvo vienintelis azoto šaltinis (74). Tuo pačiu metu kaip neigiama kontrolė buvo naudojamos lėkštelės su ta pačia terpe, bet be ACC ar bet kokio kito azoto šaltinio. Šis testas gali parodyti tik tai, ar nediazotrofinės bakterijos gali gaminti ACC deaminazės fermentą. Tai būtų aišku, jei ant užkrėstos (naudojant inokuliavimo adatą) neigiamos kontrolinės plokštelės augimas neatsirastų, bet lėkštelėje su ACC būtų stebimas kolonijų augimas.

Augalų augimo skatinimas *in vitro* (Straipsnis Nr.1)

In vitro eksperimentams naudotos modeliniu sumedėjusius augalu *in vitro* tyrimuose laikomos tuopos – *Populus tremula* ir *P. tremula* × *P. alba* hibridas. Abi *in vitro* kloninės mikroūglių kultūros buvo laikomos Miško augalų biotechnologijos laboratorijoje 5 metus prieš panaudojant šiame darbe aprašytuose eksperimentuose, kas du mėnesius dauginant mikroūgliais standartinės sudėties MS terpėje. Eksperimentinis šviesos ciklas buvo 16/8 val., kai augimo kameros temperatūra buvo atitinkamai 25/20 °C. Šviesos šaltinis buvo fluorescencinės baltos lempos (švita 30 μmol m^{-2} s^{-2}).

Tuopos eksperimentams pasirinktos dėl kelių veiksnių. *Populus* spp. yra laikomi modeliniais organizmais, reprezentuojančiais visus medžius ir kitus sumedėjusius daugiamečius augalus (76). Jie taip pat yra dažnas augalų ir mikrobų sąveikos tyrimų objektas (77).

Visi bandymai buvo atlikti naudojant 3 biologinius pakartojimus su 10 mėginių kiekviename pakartojime. Duomenys statistinei analizei buvo apjungti (1 str. 2.3 poskyris).

Paenibacillus tundrae bakterijos buvo atrinktos *in vitro* augimo skatinimo tyrimui, remiantis jų gausa tirtuose mėginiuose (konkreti padermė buvo atrinkta atsitiktinai iš *Paenibacillus tundrae* genetinės grupės) ir literatūros apžvalga. Siekiant nustatyti pasirinktos padermės naudojimo pranašumus, buvo išmatuoti keli morfologiniai tuopų mikroūglių augimo parametrai: šaknų ilgis ir skaičius, šoninių šaknų skaičius, ūglių aukštis, lapų plotis, ūglių skaičius, biomasė, šoninių šaknų tankis (1 straipsnio 2.3 poskyris) po 2 mėnesių inkubacijos. Duomenys apdoroti naudojant ANOVA ir Stjudento *t* testą.

Augalų-endofitų sąveikos įtaka augalų antriniam metabolizmui (Straipsnis Nr.3)

Paenibacillus tundrae izoliatas buvo toliau tiriamas siekiant nustatyti, ar kartu su galimais morfologiniais tuopų augimo pokyčiais jis taip pat gali turėti įtakos tuopų antriniam metabolizmui. Konkrečiai, tuopų fotosintezės pigmentų koncentracijai (chlorofilas *a* ir *b*, karotenoidai), antioksidaciniam aktyvumui (naudojant ABTS ir DPPH laisvųjų radikalų pašalinimo tyrimus) ir bendrai fenolių ir flavonoidų koncentracijai. Neinokuliuoti ir inokuliuoti tuopų mikroūgliai buvo auginami *in vitro*, o mėginiai analizėms buvo renkami 3 etapais (po 4, 6 ir 8 savaičių) (3 straipsnio 2.1 poskyris).

Šis tyrimas buvo atliktas naudojant antžeminės augalų biomasės metanolio (fenolių ir antioksidantų) arba acetono (fotosintezės pigmentų) ekstraktų spektroskopinę analizę (3 straipsnio 2.2 ir 2.3 poskyriai). Statistinė analizė atlikta naudojant ANOVA, principinių komponenčių ir koreliacinę analizes (3 str. 2.4 poskyris).

Antagonizmas *in vitro* (Straipsnis Nr.4)

Ąžuolų grybiniai endofitų izoliatai ir 5 bakterijos buvo atrinkti antagonizmo tyrimui prieš 4 labiausiai paplitusius ir didžiausius ekonominius nuostolius darančius Europos miškų patogenus: pušinę pintį (*Heterobasidion annosum*), uosių chalarą (*Hymenoscyphus fraxineus*), paprastąją spygliakritę (*Lophodermium seditiosum*) ir drebulinę pintį (*Phellinus tremulae*).

Šiam tyrimui buvo pasirinktas kokultivacijos *in vitro* metodas (4 straipsnio metodikos skyrelis). Buvo atlikti 5 biologiniai pakartojimai, kiekvienas jų iš šviežių kolonijų. Inokuliantai ir patogenai buvo išbandyti prieš tyrimą, siekiant nustatyti terpę, kuri būtų optimali patogenų

augimui, bet kurioje augtų ir galimi antagonistai. Visi 6 izoliuoti grybai gerai augo su patogenais suderinamose terpėse, tačiau tik 5 bakterijos iš bakterinių izoliatų grupės sugebėjo išlaikyti būtiną gyvybingumą. Patogenų augimo slopinimas naudojant endofitinius mikroorganizmus buvo matuojamas kaip radialinis augimo slopinimas, palyginti su kontrolinėmis lėkštelėmis.

REZULTATAI

Izoliacija, identifikacija ir augalų augimą skatinančios savybės (Straipsnis Nr.1, Nr.2, Nr.4 ir nepublikuoti duomenys)

Endofitiniai mikroorganizmai buvo sėkmingai išskirti iš 8 *Q. robur* medžių. Iš viso buvo išskirti 55 mikroorganizmai. Pagal morfologinį identifikavimą 49 buvo lazdelės formos bakterijos, 2 – mielės ir 4 – miceliniai grybai. Remiantis kolonijų spalva ir išvaizda, buvo nustatyta, kad visi 6 grybai gali būti skirtingos rūšys. Bakterijos buvo suskirstytos į 7 morfotipus (Priedų 1 lentelė ir iš dalies 2 straipsnis, 2 ir 3 lentelės, 1 pav.).

Ši morfologinė diferenciacija leido efektyviai atlikti augalų augimą skatinančių savybių testus, kol vyko genetinis identifikavimas. Sekoskaita buvo sėkmingai atlikta ir jos duomenys apdoroti. Visi izoliatai buvo identifikuoti bent rūšies lygyje (1 straipsnio 1 lentelė ir 4 straipsnio 1 lentelė). Genetiniai duomenys sutapo su morfologiniu vertinimu. Buvo identifikuotos šešios grybų gentys (mielės – *Meyerozyma* ir *Microstroma*, miceliniai grybai – *Talaromyces, Neocucurbitaria, Aspergillus* ir *Byssochlamys*) (4 straipsnio 1 lentelė) ir 5 bakterijų gentys (*Bacillus, Pseudomonas, Delftia, Paenibacillus* ir *Pantoea* (1 straipsnis, 1 lentelė). Šiame tyrime dažniausiai išskirtos *Paenibacillus* spp. ir *Pseudomonas* spp. bakterijos.

Visų izoliatų augalų augimą skatinančios savybės buvo įvertintos kokybiškai. Įrodyta, kad visi endofitai gamina fitohormoną indol-3-acto rūgšį ir skaido neorganinius fosfatus. Visi izoliatai, išskyrus 2 *Bacillus* sp. mėginius gebėjo mineralizuoti organinius fosfatus. Iš bakterijų mėginių tik *Pseudomonas* spp. galėjo gaminti sideroforus kartu su *Microstroma* mielėmis, *Neocucurbitaria, Aspergillus* ir *Byssochlamys* grybais. Tarp bakterijų mėginių azoto fiksacija nepasižymėjo tik *Paenibacillus* genties bakterijos.

Visų tirtų *Pantoea* spp. ir *Pseudomonas* spp. kalio tirpinimo ir ACC deaminazės aktyvumo rodikliai buvo teigiami, o *Bacillus* spp. ir *Paenibacillus tundrae* – neigiami. *Delftia* sp. rodikliai buvo teigiami ACC aktyvumo atžvilgiu ir neigiami kalio tirpinimo atžvilgiu (1 lentelė).

Bakterijų įvairovės analizė (Straipsnis Nr.2)

Dėl didelės endofitinių mikroorganizmų įvairovės augalų mėginiuose reikėjo būdo juos atskirti, siekiant efektyviai išbandyti atrinktus mikroorganizmus tolimesniuose eksperimentuose, neeikvojant išteklių.

SERS spektrams gauti buvo naudojama nedidelė pasirinktų bakterijų grupė. Tada surinkti duomenys buvo naudojami chemometrinei analizei. Principinių komponenčių ir diskriminantinių funkcijų analizių rezultatai buvo palyginti su genetinių ir fiziologinių tyrimų duomenimis. Metodas leido išskirti 3 genetiškai homologiškus (remiantis *16S rRNR* geno fragmentų sekos nustatymu) *Pantoea agglomerans* padermės izoliatus į 2 grupes (2 straipsnis, 4 ir 5 pav.), o 3 *Pseudomonas* mėginiai taip pat buvo atskirti į 3 grupes (straipsnis Nr.2, 4 ir 7 pav.). Abu tirti *Paenibacillus tundrae* izoliatai buvo sugrupuoti kartu, o tai reiškia, kad jie buvo genetiškai labai artimi, nors buvo išskirti iš dviejų skirtingų ąžuolų (2 straipsnis, 4 ir 6 paveikslai). Palyginimui, visi *Pantoea agglomerans* mėginiai kilę iš vieno medžio.

Tuopų augimo skatinimas ir *Paenibacillus* įtaka jų metabolizmui *in vitro* (Straipsnis Nr.1 ir Nr.3)

Iš visų izoliatų *Paenibacillus tundrae* bakterija buvo atrinkta tuopų mikroūglių inokuliacijai *in vitro*. Pirmoje bandymo dalyje buvo auginamos *Populus tremula* ir hibridinės tuopų kultūros, kurių morfologiniai augimo parametrai buvo išmatuoti po 2 mėnesių augimo periodo. Surinktų duomenų analizė parodė, kad *Paenibacillus tundrae* turėjo augimą skatinantį poveikį, daugiausia pastebimą šaknyse (1 straipsnis, 2 lentelė, 2–4 pav.). Inokuliuoti *P. tremula* mikroūgliai turėjo padidiną šoninių šaknų skaičių (44,7 %), šoninių šaknų tankį (66 %), šviežių ir sausų šaknų biomasę (atitinkamai 101,9 % ir 63,6 %). Hibridinių tuopų (*P. tremula* × *P. alba*) mikroūglių šoninių šaknų skaičius ir tankis taip pat padidėjo (atitinkamai 213,7 % ir 125,6 %). Padidėjo ir jų šviežių ir sausų šaknų biomasė (atitinkamai 197,1 ir 144,8 %). Hibridų mikroūgliai taip pat pasižymėjo vidutiniškai ilgesnėmis pridėtinėmis šaknimis (102 %, bendras ilgis), o ilgiausios pridėtinės šaknies ilgis padidėjo 79,5 %. Pridėtinių šaknų skaičius padidėjo 65 %. Taip pat pažymėtina, kad *P. tremula* x *P. alba* hibrido antžeminė biomasė buvo neigiamai paveikta, palyginti su kontroline grupe (25 %).

Kitam bandymui buvo naudojama ta pati inokuliavimo procedūra, tačiau naudota tik hibridinė tuopa. Šio tyrimo metu biomasės mėginiai buvo paimti praėjus 4, 6 ir 8 savaitėms po inokuliacijos. Išmatuotas augalų fenolių ir fotosintezės pigmentų koncentracijos ir antioksidacinis aktyvumas. Rezultatai parodė, kad *Paenibacillus tundrae* izoliatas turėjo įtakos šeimininko metaboliniam profiliui. Po 4 savaičių poveikis buvo nežymus, o po 6 savaičių

atsirado statistiškai patikimi skirtumai. Fenolių ir antioksidantų, chlorofilo *a* ir karotinoidų rodikliai buvo neigiamai paveikti, o chlorofilo *b* kiekis padidėjo, palyginti su kontrolinėmis grupėmis. Po 8 savaičių fenolių ir antioksidantų parametrai buvo neigiamai paveikti, o fotosintezės pigmentų koncentracijos padidėjo (3 straipsnis, 1 pav.).

Be to, koreliacijos ir principinių komponenčių analizės parodė, kad inokuliacija *Paenibacillus tundrae* bakterija paveikė kaip tiriami kintamieji (pigmentų kiekis, palyginti su fenoliais ir antioksidantais) sąveikauja tarpusavyje (3 straipsnis, 2 ir 3 pav.).

Antagonizmas *in vitro* (Straipsnis Nr.4)

Atrinktos bakterijos (5 individai) ir visi 6 grybų izoliatai buvo naudojami *in vitro* kokultivacijos antagonizmo tyrime prieš 4 patogeninius grybus.

Kiekvienas patogenas buvo slopinamas bent vienu iš ištirtų izoliatų (>30 % slopinimas, $p \leq 0,05$). Mielė *Meyerozyma* sp., bakterijos *Pseudomonas* spp. ir *Pantoea* spp. pasižymėjo plataus spektro slopinimu. *Pseudomonas* sp. parodė didžiausią LS (68,4 %) ir PT (57,5 %) slopinimą, *Meyerozyma* sp. – HA (45,7 %), *Byssochlamys* sp. – HF (59,8 %) (2.3 pav.). *Meyerozyma* sp. buvo vienintelis izoliatas, slopinantis HA augimą (3 pav.).

IŠVADOS

1. Visi tirti paprastojo ąžuolo endofitinių grybų (*Neocucurbitaria, Aspergillus, Talaromyces, Byssochlamys, Meyerozyma, Microstroma*) ir bakterijų (*Bacillus, Pantoea, Delftia, Pseudomonas, Paenibacillus*) izoliatai pasižymėjo bent trimis augalų augimą skatinančiomis savybėmis. Neorganinių fosfatų tirpinimas ir fitohormono indol-3-acto rūgšties gamyba buvo aptikti visuose izoliatuose. Tai rodo, kad tirti izoliatai gali būti naudojami medžių augimui skatinti.

2. Sustiprinto paviršiaus Ramano spektroskopija gali būti veiksmingai taikoma endofitinėms bakterijoms diferencijuoti, minimaliai apdorojant mėginius. Ši naujovė gali padėti greičiau diferencijuoti endofitus ir paspartinti biostimuliatorių ir biokontrolės agentų tyrimus ankstyvoje stadijoje.

3. Modelinių tuopų (*Populus*) augimas pagerėjo po inokuliacijos endofitinėmis *Paenibacillus tundrae* bakterijomis, išskirtomis iš paprastojo ąžuolo. Pastebėtas šaknų sistemos parametrų padidėjimas. Šis izoliatas taip pat teigiamai veikė tuopų antrinį metabolizmą, ypač fotosintezės pigmentų koncentraciją, taip parodant, kad jis potencialiai gali būti naudojamas medžių augimui ir antrinių metabolitų gamybai *in vitro* skatinti.

4. Paprastojo ąžuolo endofitai gali apriboti miško patogenų (*Heterobasidion annosum, Hymenoscyphus fraxineus, Lophodermium seditiosum* ir *Phellinus tremulae*) radialinį augimą *in vitro*. Bakterijos *Pantoea agglomerans, Pseudomonas azotoformans* ir mielė *Meyerozyma guilliermondii* pasižymėjo reikšmingu antagonistiniu poveikiu trims iš keturių tirtų patogenų. Tolesni šių endofitinių rūšių tyrimai gali padėti sukurti naujus šių patogenų valdymo būdus.

Supplementary table 1. *Morphological and genetic identification data on all bacterial isolates in this study*

1 lentelė. *Endofitinių bakterijų izoliuotų tyrimo metu morfologinių testų ir genetinio identifikavimo duomenys*

Isolate no. / Izoliato numeris	Closest NCBI match / Artimiausias atitikmuo NCBI duomenų bazėje	String test (Gram +/-) / Virvelės testas	Biofilm formation / Bioplevelės formavimas	Catalase test / Katalazės testas	Cell form / Ląstelės forma	Oxidase test / Oksidazės testas	Oxygen requirement / Deguonies poreikis	Lactose use / Laktozės naudojimas	Maltose use / Maltozės naudojimas	Sucrose use / Sacharozės naudojimas	Glucose use / Gliukozės naudojimas	Fructose use / Fruktozės naudojimas
24	*Pseudomonas brenneri*	-	Moderate/ Vidutinis	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
29	*Pseudomonas proteolytica*	-	Moderate / Vidutinis	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
35	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
37	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
47.1	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
47.2	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
49	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
46.1	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
46.2	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-

40	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
36	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
34	*Pantoea agglomerans*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
33	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
33.1	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
32	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
30	*Pantoea agglomerans*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/fakulta-tyvinis anaerobas	A	A	A	A	A
27	*Pantoea agglomerans*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
21	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
13	*Delftia lacustris*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-
22	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A

23.1	*Bacillus aryabhattai*	+	-	+	Rod-shaped/ Lazdelė	-	obligate aerobe/ obligatinis aerobas	A	A	A	A	A
23.2	*Bacillus aryabhattai*	+	-	+	Rod-shaped/ Lazdelė	-	obligate aerobe/ obligatinis aerobas	A	A	A	A	A
25	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-
26	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
28	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
31	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/fakulta-tyvinis anaerobas	A	A	A	A	A
38	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
39	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
39.1	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-
41.1	*Bacillus aryabhattai*	+	-	+	Rod-shaped/ Lazdelė	-	obligate aerobe/ obligatinis aerobas	A	A	A	A	A
41.2	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A

42.1	*Pseudomonas proteolytica*	-	Moderate / Vidutinis	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-
42.2	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-
43.1	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/fakulta-tyvinis anaerobas	A	A	A	A	A
43.3	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
44.1	*Pseudomonas paralactis*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
44.2	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
48.1	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
48.2	*Bacillus firmus*	+	-	+	Rod-shaped/ Lazdelė	-	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
50	*Paenibacillus oceanisediminis*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
52	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
53	*Delftia lacustris*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-

54.1	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
55.1	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
55.2	*Bacillus firmus*	+	-	+	Rod-shaped/ Lazdelė	-	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
56.1	*Pseudomonas azotoformans*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	A	-
57	*Delftia lacustris*	-	-	+	Rod-shaped/ Lazdelė	+	obligate aerobe/ obligatinis aerobas	-	-	-	-	-
58	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
201.1	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A
201.2	*Paenibacillus tundrae*	-	-	+	Rod-shaped/ Lazdelė	-	facultative anaerobe/ fakultatyvinis anaerobas	A	A	A	A	A

+/- indicate a positive and negative test result respectively; A – acid production/. +/- atitinkamai rodo teigiamą ir neigiamą testo rezultatą; A – rūgšties gamyba.

Table 1. *Continued.*
1 lentelė*. Tęsinys*

		Antibiotic susptability test/ Atsparumo antibiotikams testas					
Isolate no./ Izoliato numeris	Endospore formation; Viability after 3 days / Endosporų formavimas; Gyvybingumas po 3 dienų	AM-10	CTX-30	C-30	K-30	STP-10	TIC-75
24	-	R	R	R	S	R	R
29	-	R	R	R	S	R	R
35	+	S	R	S	S	R	R
37	-	R	R	R	S	R	R
47.1	-	R	R	R	S	R	R
47.2	-	R	R	R	S	R	R
49	-	R	R	R	S	R	R
46.1	-	R	R	R	S	R	R
46.2	-	R	R	R	S	R	R
40	-	R	R	R	S	R	R
36	-	R	R	R	S	R	R
34	-	R	S	S	S	S	R
33	-	R	R	R	S	R	R
33.1	+	S	R	S	S	R	R
32	-	R	R	R	S	R	R
30	-	S	S	S	S	S	R
27	-	S	S	S	S	S	R
21	+	S	R	S	S	R	R
13	-	R	S	I	R	R	R
22	+	S	R	S	S	R	R
23.1	+	S	S	I	S	S	S
23.2	+	S	S	S	S	S	I
25	-	R	R	R	S	S	R
26	+	S	R	S	S	R	R
28	+	S	R	S	S	R	R
31	+	S	R	S	S	R	R
38	+	S	R	S	S	R	R
39	+	S	R	S	S	R	R
39.1	-	R	R	R	S	S	R
41.1	+	R	R	S	S	S	R
41.2	+	S	R	S	S	R	R
42.1	-	R	R	R	S	R	R
42.2	-	R	R	R	S	I	R
43.1	+	R	R	R	S	R	R
43.3	+	S	R	S	S	R	R
44.1	-	R	R	R	S	R	R
44.2	+	S	R	S	S	R	R
48.1	+	S	R	S	S	R	R
48.2	-*	S	S	S	S	S	R

50	+	S	R	S	S	R	R
52	+	S	R	S	S	R	R
53	-	R	R	I	R	R	R
54.1	-	R	R	R	S	R	R
55.1	+	S	R	S	S	R	R
55.2	-*	S	S	S	S	S	R
56.1	-	R	R	R	S	R	R
57	-	R	R	I	R	R	R
58	+	S	R	S	S	R	R
201.1	+	S	R	S	S	R	R
201.2	+	S	R	S	S	R	R

+/- indicate a positive and negative test result respectively; R – resistant, I – intermediate, S – sensitive. * - test was positive at 70°C / +/- atitinkamai indikuoja teigiamą ir neigiamą testo rezutatą; R – atsparus, I – vidutiniškai atsparus, S – jautrus. * - testas buvo teigiamas esant 70°C.

Table 1. *Continued.*
1 lentelė*. Tęsinys*

	Colony morphology/ Kolonijų morfologija								
Isolate no. / Izoliato numeris	Diame-ter / Skersmuo	Shape / Forma	Elevation / Iškilumas	Margin / Pakraštys	Smoothness / Glotnumas	Pigmentation / Spalva	Opacity / Permatomumas	Appearance / Išvaizda	Consistency / Tąsumas
24	0,326± 0,033	circular/ apvali	raised/ iškilusi	entire/ ištisas	rough at center, smooth at the edge/ glotni pakraščiuose, bet ne centre	whitish/ balsva	translucent/ permatoma	glistening on the edge/ blizgi pakrašty	butyrous/ sviestinė
29	0,284± 0,014	circular/ apvali	raised/ iškilusi	entire/ ištisas	rough at center, smooth at the edge/ glotni pakraščiuose, bet ne centre	buff/ smėlio	translucent/ permatoma	glistening on the edge/ blizgi pakrašty	butyrous/ sviestinė
35	0,043± 0,042	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
37	0,34±0, 023	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
47.1	0,043± 0,073	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
47.2	0,45±0, 042	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
49	0,405± 0,042	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
46.1	0,426± 0,036	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
46.2	0,338± 0,028	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
40	0,427± 0,037	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
36	0,447± 0,047	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
34	0,438± 0,028	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
33	0,338± 0,03	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
33.1	0,361±	circular/	flat/	undulate/	smooth/ glotni	whitish, grey	translucent at the edge/	glistening/ blizgi	mucoid/

	0,039	apvali	plokščia	vingiuotas		bulls' eye/ balsva su pilku centru	permatoma pakrašty		mukoidinė
32	0,366± 0,021	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	whiteish/ balsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
30	0,352± 0,04	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
27	0,324± 0,041	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
21	0,444± 0,03	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
13	0,275± 0,037	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	cream/ kreminė	opaque in center, translucent at edges/ permatoma pakrašky, bet ne centre	glistening/ blizgi	butyrous/ sviestinė
22	0,321± 0,042	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
23.1	0,581± 0,072	circular/ apvali	craterifor m/ kraterio formos	entire/ ištisas	smooth/ glotni	cream/ kreminė	opaque/ nepermatoma	glistening/ blizgi	butyrous/ sviestinė
23.2	0,524± 0,069	circular/ apvali	craterifor m/ kraterio formos	entire/ ištisas	smooth/ glotni	cream/ kreminė	opaque/ nepermatoma	glistening/ blizgi	butyrous/ sviestinė
25	0,201± 0,028	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	buff/ smėlio	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
26	0,309± 0,032	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
28	0,305± 0,029	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
31	0,340± 0,039	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė

38	0,328± 0,045	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
39	0,286± 0,036	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
39.1	0,252± 0,02	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
41.1	0,653± 0,066	circular/ apvali	craterifor m/ kraterio formos	entire/ ištisas	smooth/ glotni	cream/ kreminė	opaque/ nepermatoma	glistening/ blizgi	butyrous/ sviestinė
41.2	0,414± 0,032	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
42.1	0,219± 0,025	circular/ apvali	raised/ iškilusi	entire/ ištisas	rough at center, smooth at the edge/ glotni pakraščiuose, bet ne centre	buff/ smėlio	translucent/ permatoma	matt at center, glistening at edge/ matinis centras, blizgūs pakraščiai	butyrous/ sviestinė
42.2	0,242± 0,022	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
43.1	0,389± 0,038	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
43.3	0,482± 0,056	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
44.1	0,338± 0,033	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth at the center, rougher edge/ glotni centre, bet ne pakraščiuose	yellowish/ gelsva	translucent/ permatoma	glistening, matt edge/ blizgi, matiniai pakraščiai	mucoid/ mukoidinė
44.2	0,431± 0,064	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
48.1	0,420± 0,043	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
48.2	0,335±	circular/	raised/	entire/	smooth/ glotni	peach/ persikinė	opaque/ nepermatoma	glistening, slightly	butyrous/

	0,028	apvali	iškilusi	ištisas				grainy/ blizgi ir grūdėta	sviestinė
50	0,640± 0,055	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
52	0,524± 0,039	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
53	0,336± 0,029	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	cream/ kreminė	opaque in center, translucent at edges/ permatoma pakrašky, bet ne centre	glistening/ blizgi	butyrous/ sviestinė
54.1	0,300± 0,022	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
55.1	0,474± 0,077	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
55.2	0,327± 0,014	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	peach/ persikinė	opaque/ nepermatoma	glistening, slightly grainy/ blizgi ir grūdėta	butyrous/ sviestinė
56.1	0,380± 0,023	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	yellowish/ gelsva	translucent/ permatoma	glistening/ blizgi	butyrous/ sviestinė
57	0,376± 0,028	circular/ apvali	raised/ iškilusi	entire/ ištisas	smooth/ glotni	cream/ kreminė	opaque in center, translucent at edges/ permatoma pakrašky, bet ne centre	glistening/ blizgi	butyrous/ sviestinė
58	0,443± 0,053	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
201.1	0,366± 0,029	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė
201.2	0,351± 0,035	circular/ apvali	flat/ plokščia	undulate/ vingiuotas	smooth/ glotni	whitish, grey bulls' eye/ balsva su pilku centru	translucent at the edge/ permatoma pakrašty	glistening/ blizgi	mucoid/ mukoidinė

ARTICLE 1 / STRAIPSNIS NR. 1

microorganisms

MDPI

Article

Oak (*Quercus robur*) Associated Endophytic *Paenibacillus* sp. Promotes Poplar (*Populus* spp.) Root Growth In Vitro

Dorotėja Vaitiekūnaitė [1,*], Sigutė Kuusienė [1] and Emilija Beniušytė [2]

[1] Lithuanian Research Centre for Agriculture and Forestry, Laboratory of Forest Plant Biotechnology, Institute of Forestry, Liepu st. 1, Girionys, LT-53101 Šlienava, Lithuania; sigutekuus@gmail.com
[2] Faculty of Natural Sciences, Vytautas Magnus University, Universiteto st. 10, Akademija, LT-53361 Ringaudai, Lithuania; emilija.beniusyte@stud.vdu.it
* Correspondence: doroteja.vaitiekunaite@lammc.lt

Abstract: Soil fertilization is necessary for high-demand crop production in agriculture and forestry. Our current dependence on chemical fertilizers has significant harmful side effects. Biofertilization using microorganisms is a sustainable way to limit the need for chemical fertilizers in various enterprises. Most plant endophytic bacteria have thus far been unstudied for their plant growth promoting potential and hence present a novel niche for new biofertilizer strains. We isolated English oak (*Quercus robur*) endophytic bacteria and tested them for plant growth promoting traits (PGPTs) such as nitrogen fixation, phosphate mineralization/solubilization, siderophore and indole-3-acetic acid (IAA) production. We also investigated the effect the selected isolate had on poplar (*Populus* spp.) microshoot vegetative growth parameters in vitro. In total 48 bacterial strains were isolated, attributed to *Bacillus*, *Delftia*, *Paenibacillus*, *Pantoea* and *Pseudomonas* genera. All the isolates displayed at least three PGPTs, with 39.6% of the isolates displaying all five (all were *Pseudomonas* spp.) and 18.75% displaying four. Based on relative abundance, *Paenibacillus* sp. isolate was selected for the poplar microshoot inoculation study. The isolate had a significant positive effect on poplar microshoot root growth and development. Two tested poplar genotypes both had increased lateral root number and density, fresh and dry root biomass. Furthermore, one genotype had increased length and number of adventitious roots as well as a decrease in fresh aboveground biomass. The root enhancement was attributed to IAA production. We propose this isolate for further studies as a potential biofertilizer.

Keywords: biofertilizer; *Paenibacillus*; *Populus*; *Quercus robur*; endophyte; bacteria; plant growth promotion; in vitro, microshoots

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1. Introduction

Fertilization is a key aspect of continuous and efficient industrial production of various crop species in agriculture and forestry. However, our current dependence on chemical fertilizers is having a significant negative effect on ecology, soil and water quality, and human health, as well as having a global impact on carbon emissions [1–7]. Microorganisms (usually bacteria or fungi) used as biofertilizers can enhance the nutritional status of their host plants through a variety of mechanisms, thus enhancing crop yields [2,5]. These beneficial microbes are an eco-friendly way to reduce and perhaps even eliminate the need for chemical fertilization [1–3,5,8–10]. Due to their benefits, the demand for microbial-based biofertilizers and biocontrol agents is expected to rise in the future [2,4].

In open systems, plant growth may be limited by chemical insufficiencies or bioavailability of certain minerals. By inhabiting plant endospheres and rhizospheres, biofertilizer bacteria can enhance plant nutrient uptake and accessibility through nitrogen fixation, phosphate solubilization/mineralization, iron transport agent-siderophore production, etc. [11–14]. Additionally, bacteria can produce plant growth regulators, such as indole-3-acetic acid (IAA), which has been shown to positively affect plant growth and devel-

opment [5]. Moreover, bacterial biofertilizers can help impede and/or limit the negative effects of both abiotic and biotic stressors [2,5]. Bacterial inoculants can be used for a single purpose, ex. N-fixation; however, more often bacteria can have several beneficial aspects and thus have a complex effect on their host [2,3,5,15,16].

Plant growth promoting bacteria (PGPB or PGPR, plant growth promoting rhizobacteria) can be used as a sustainable long-term solution for soil fertilization [3] or they can be used in tandem with chemical fertilizers, thus limiting the need for them, resulting in lower costs overall, a reduced negative impact on the ecosystem and a more sustainable end-product [3,8]. Moreover, it is likely that beneficial bacteria can create self-sustaining colonies in perennial plants, thus potentially extending their efficacy through multiple growing seasons [17–21].

Agriculturally important species have been shown to benefit from additional use of bacterial inoculants for sustainable crop production in previous studies [1,2,5,8]. In this case, the most thoroughly researched PGPB are the symbionts of legumes [10,15]. The effect of diazotroph inoculation on sugarcane has also been well documented [2,15]. Furthermore, works on other food species, such as wheat [22], apples [23], peanuts [24], tomatoes [25], etc., also show highly promising results.

Many biofertilizer trials have been focused on agricultural crops; however, studies suggest that trees and other woody plants are well suited for biofertilizer use. Several different bacterial inoculants have been successfully used both in pot trials and field studies on cedar [20], avocado [19], tea plant [16] and pine [26]. Furthermore, biofertilizers have been shown to work well concurrently with NPK (nitrogen, phosphorus and potassium) fertilizers as well as organic-matter-based fertilizer systems in several tree species: eucalyptus [7], orange trees [27], *Cordia africana*, *Croton macrostachyus*, *Erythrina brucei* and *Millettia ferruginea* [28], olive trees [29] and poplars [30]. Collectively these studies demonstrate that, at the very least, biofertilizers can limit the need for chemical fertilization without yield losses.

It is believed that in the future, one of the main goals in biofertilizer science will be the search for and thorough studies of new and/or yet unresearched microbe species [4,10]. In this aspect the endosphere of plants presents a novel niche, as so far all tested plants were shown to be inhabited by endophytic microorganisms [31]; however, just around 2% of all known plants were studied [32]. Oaks are promising as a source of yet-unstudied cultivable endophytic bacteria, as the genus is distributed widely throughout the Northern Hemisphere. English oaks (*Quercus robur*), specifically, are the model trees for the genus. They are essential hardwoods, known for providing habitat and food for a great variety of organisms in temperate forests, thus successfully thriving in multiple varied ecosystems. Oaks are also valuable for industrial forestry and other enterprises. Studies on endophytic oak bacteria are scarce. In the field of applicable endosymbionts, oak-associated fungal species are investigated as biocontrol agents for oak pathogen inhibition [33,34]; however, their cultivable bacterial endophytes are not thoroughly studied, nor is their use as biofertilizers.

Thus, the aim of this study was to investigate easily cultivable oak bacterial endophytes from oak phyllosphere and their plant growth promoting traits (PGPTs), as well as the effect selected bacteria may have on poplar (*Populus* spp.) microshoots in vitro for potential use as biofertilizers in future trials.

Five bacterial genera were isolated from the oak endosphere: *Bacillus*, *Delftia*, *Paenibacillus*, *Pantoea* and *Pseudomonas* (48 isolates in total). All the tested bacteria had at least three PGPTs. One IAA producing *Paenibacillus* sp. isolate was used for poplar inoculation study, demonstrating significant positive increases in lateral root number and density, fresh and dry root biomass, as well as a significant enhancement in adventitious root growth parameters.

2. Materials and Methods

2.1. Isolation and Identification

Seven field-grown English oaks were chosen for this study. Tree branches were gathered in late spring and force flushed in the lab to limit additional infection. Buds and leaves were used for endophyte isolation and sterilized using a modified surface sterilization method [35]. Leaves and buds were separated from the branches and first washed in a detergent solution (Tween 80) (1 drop/125 mL ddH_2O (double distilled water)) for 5 min, then in 50% commercial bleach solution in ddH_2O for 2 min. The third wash was done using 80% ethanol solution for 40 s. After each wash, samples were rinsed with ddH_2O three times, 2 min each time.

To ensure that surface sterilization was effective, a modified method described by Sherling et al. [36] was used, whereupon explants were pressed against Woody plant medium (WPM) (Duchefa Biochemie, Haarlem, The Netherlands) [37] and observed for a month for microbial growth (+22 ± 1 °C). If no growth was observed, the sterilization was concluded to be a success and the bacteria isolated from these explants were considered to be endophytic.

After visualization, emerging morphologically different putative bacterial samples were selected and transferred from WPM onto Lysogeny broth (LB) agarized medium [38] (Duchefa Biochemie). Isolates were grown in a growth chamber at +22 ± 1 °C. Pure cultures were achieved by re-streaking the samples multiple times until single colonies appeared. As bacteria and yeast colonies look similar on agar medium, yeasts had to be separated out. To separate them, samples were observed under a microscope (1000× magnification) and distinguished on the basis of cell form and evidence of budding.

Bacterial isolates were sent to *Macrogen* sequencing center for DNA extraction and *16S rRNA* gene sequencing (Amsterdam, Netherlands). Universal 27F/800R and 518F/1492R primer set was used. Reverse primer sequences were transformed to forward complement sequences using open access *Chromas 2.6.6* software. Sequences were then edited using open access *Bioedit 7.2.5* software. National Center for Biotechnology Information's (NCBI) *BLASTn* alignment tool was used to align sequences using standard parameters. Fragments were then matched with the NCBI database (BLAST Targeted Loci Nucleotide system) for *Bacteria* and *Archeae* (megablast) using ≥99% query coverage and ≥99% identity.

2.2. Plant Growth Promoting Traits

Screening for PGPTs was done in independent biological triplicates using fresh colonies each time. Bacteria were grown at +22 ± 1 °C. Isolates were tested for nitrogen fixation, phosphate solubilization and mineralization, siderophore and IAA production.

To screen for putative nitrogen fixation, as per Jasim et al. [39], nitrogen-free Jensen's medium (Himedia, Mumbai, India) was used. The bacteria were spot inoculated on the medium (one sample per plate) and incubated for up to a week. Colony growth was evaluated and colonies with well-defined growth zones were considered putative diazotrophs.

Bacterial isolates were tested for phosphate solubilization and mineralization using an agar plate method [40]. Per Chen and Liu, two different phosphate sources were utilized: tricalcium phosphate—to test for inorganic phosphate solubilization (Pi)—and soy lecithin—to test for organic phosphate mineralization (Po). Bacteria were spot inoculated on the media and incubated for up to a week. The appearance of clear zones around the colonies indicated phosphate solubilization and/or mineralization.

To test for siderophore production, a modified Chromeazurol S (CAS) assay was used [41]. Prior to testing, all glassware was washed with 1mol/L HCl and then with ddH_2O. Sterile CAS reagent was freshly prepared as described by Schwyn and Neilands before every test [42]. For the experiment, CAS reagent was mixed 1:9 with LB agarized medium. Bacterial isolates were then spot inoculated and incubated for up to a week. Orange/yellow zones around bacterial colonies were indicative of siderophore production.

To check if the bacteria were capable of tryptophan-dependent IAA production, a test with Salkowski reagent was used [43]. Bacteria were grown in LB broth (Duchefa

Biochemie) enriched with tryptophan (0.15% *w/v*) in a thermalshaker (90 rpm) at 25 °C for 24 h in the dark. After that, 1.5 mL of this suspension was transferred to a microtube and centrifuged at 16300× g for 5 min. Then, 0.5 mL of supernatant was transferred to a cuvette and an equal amount of Salkowski reagent (1 mL 0.5M FeCl3 with 49 mL of 35% HClO4 *v/v*) was added. The cuvettes were incubated in the dark for 30 min. Afterwards, optical density was measured at 530nm with T80+ UV/VIS spectrophotometer (PG Instruments LTD, Lutterworth, UK). Uninoculated tryptophan enriched LB broth and Salkowski reagent 1:1 was used as control. IAA production was estimated based on color change to red and checked using a standard curve.

2.3. Poplar Microshoot Inoculation

Based on relative abundance in all tested trees, one representative isolate was selected for this study. In vitro plant growth promoting effect of the selected isolate was tested on two model poplar tree genotypes, *Populus tremula* and *P. tremula* × *P. alba* hybrid. Both of these genotypes have been cultivated in vitro for at least 3 years. Poplars were chosen due to their status as model plants to study woody perennials as well as plant-microbiome interactions [44,45].

A modified method by Scherling et al. was used [36]. For both genotypes, the test was done under the same conditions, using WPM (additionally 4% gelrite, 2% sucrose). Two-month-old poplar microshoot culture was used, with 30 explants per group (divided into three independent replicates). For control groups microshoot leaves were removed and the stems were divided into 10 mm segments with 2–3 growing nodes each. Every segment was individually placed into ⌀20 mm glass tube with 5.25 mL of WPM. The tubes were capped and placed into a growth chamber at +22 ± 1 °C with 16/8 h day cycles.

For groups inoculated with bacteria, the bacterial inoculant was prepared first. A swab from a single bacterial colony was transferred to a fresh plate with LB medium and grown at +22 ± 1 °C overnight. The next day, prior to the transfer of microshoot segments, each tube was inoculated using an inoculation needle. Microshoot segments were then placed into the stab area. Otherwise, everything else was done in the same manner as with control groups.

After a 2-month incubation period, microshoot growth parameters were measured: shoot number and length, adventitious root number and length, width of the largest leaf, lateral root number, fresh and dry biomass (explants were dried for 2–3 h in a laboratory oven at 100 °C). Lateral root density was calculated. Data was analyzed using *Student's* t-test and ANOVA (*Microsoft Excel*).

3. Results

In this study we assessed cultivable bacterial endophytes associated with English oak phyllosphere, their plant growth promoting traits and the effect selected isolate had on poplar microshoot vegetative growth parameters in vitro.

3.1. Isolation and Identification

After surface sterilization, putative bacteria created visible colonies on the medium surface or within the medium at the base of the explants within 2–3 weeks of incubation. In total, 48 viable bacterial samples were isolated.

Partial *16S rRNA* gene sequences from all 48 isolates were successfully obtained. Sequencing data is listed in Table 1. DNA sequencing yielded fragments that were 1324–1494 bp (base pairs) in length. The isolates were shown to belong to two different phyla of bacteria, *Proteobacteria* (52%) and *Firmicutes* (48%). Isolates were separated into five genera: *Bacillus*, *Delftia*, *Paenibacillus*, *Pantoea* and *Pseudomonas*. *Pseudomonas* spp. and *Paenibacillus* spp. were the most abundant, representing 39.6% and 37.5% of the total amount respectively, with *Bacillus* spp. representing 10.4%, and *Pantoea* spp. and *Delftia* spp. representing 6.25% of the total amount each.

Table 1. Sequence analysis of the *16S rRNA* gene and plant growth promoting properties of the 48 bacterial endophytes isolated from *Q. robur* phyllosphere (highlighted—isolate chosen for further inoculation study).

Sequencing Analysis					Plant Growth Promoting Traits				
Source Tree Designation	Fragment Length, bp	Closest NCBI Match, Accession no.	% Identity	Query Coverage, %	Siderophore Production	Phosphate Solubilization/Mineralization		Nitrogen Fixation	IAA Production, µg/mL
						Pi	Po		
4	1464	*Delftia lacustris* 332, NR_116495.1	99.86	100	-	+	+	+	16.36
1	1477	*Paenibacillus tundrae* A10b, NR_044525.1	99.32	99	-	+	+	-	25
3	1486	*Paenibacillus tundrae* A10b, NR_044525.1	99.53	99	-	+	+	-	29.2
I1	1483	*Bacillus aryabhattai* B8W22, NR_115953.1	99.93	99	-	+	+	+	7.4
I1	1483	*Bacillus aryabhattai* B8W22, NR_115953.1	99.80	99	-	+	+	+	13.6
2	1469	*Pseudomonas brenneri* CFML 97-391, NR_025103.1	99.86	99	+	+	+	+	17.5
I2	1462	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.73	99	+	+	+	+	17.5
3	1483	*Paenibacillus tundrae* A10b, NR_044525.1	99.46	100	-	+	+	-	27.2
1	1395	*Pantoea agglomerans* DSM 3493, NR_041978.1	99.64	100	-	+	+	+	50
I2	1486	*Paenibacillus tundrae* A10b, NR_044525.1	99.39	99	-	+	+	-	27.22
2	1473	*Pseudomonas proteolytica* CMS 64, NR_025588.1	99.59	99	+	+	+	+	13.2
1	1411	*Pantoea agglomerans* DSM 3493, NR_041978.1	99.65	100	-	+	+	+	67.6
I2	1485	*Paenibacillus tundrae* A10b, NR_044525.1	99.53	99	-	+	+	-	28.4
1	1461	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.66	99	+	+	+	+	18.2
1	1470	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.66	99	+	+	+	+	19.2
1	1488	*Paenibacillus tundrae* A10b, NR_044525.1	99.46	99	-	+	+	-	25.6
1	1459	*Pantoea agglomerans* DSM 3493, NR_041978.1	99.97	99	-	+	+	+	41.7
2	**1484**	***Paenibacillus tundrae* A10b, NR_044525.1**	**99.39**	**99**	**-**	**+**	**+**	**-**	**27**
1	1469	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.66	99	+	+	+	+	16.4

Table 1. *Cont.*

Sequencing Analysis					Plant Growth Promoting Traits				
Source Tree Designation	Fragment Length, bp	Closest NCBI Match, Accession no.	% Identity	Query Coverage, %	Siderophore Production	Phosphate Solubilization/Mineralization		Nitrogen Fixation	IAA Production, µg/mL
						Pi	Po		
2	1472	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.73	99	+	+	+	+	18.3
I1	1486	*Paenibacillus tundrae* A10b, NR_044525.1	99.33	99	-	+	+	-	31.6
3	1490	*Paenibacillus tundrae* A10b, NR_044525.1	99.39	99	-	+	+	-	26.9
3	1467	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.73	99	+	+	+	+	19.2
1	1469	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.66	99	+	+	+	+	17
S2	1492	*Bacillus aryabhattai* B8W22, NR_115953.1	99.80	99	-	+	+	+	8.8
S2	1481	*Paenibacillus tundrae* A10b, NR_044525.1	99.53	99	-	+	+	-	25.2
3	1474	*Pseudomonas proteolytica* CMS 64, NR_025588.1	99.66	99	+	+	+	+	17
3	1472	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.73	99	+	+	+	+	17.5
3	1494	*Paenibacillus tundrae* A10b, NR_044525.1	99.53	99	-	+	+	-	22.4
3	1487	*Paenibacillus tundrae* A10b, NR_044525.1	99.53	99	-	+	+	-	26.4
3	1324	*Pseudomonas paralactis* DSM 29164, NR_156987.1	99.85	100	+	+	+	+	17.6
3	1354	*Paenibacillus tundrae* A10b, NR_044525.1	99.34	99	-	+	+	-	25.02
1	1466	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.73	99	+	+	+	+	16.4
1	1454	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.72	99	+	+	+	+	11.6
2	1467	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.73	99	+	+	+	+	17.5
2	1460	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.79	99	+	+	+	+	15
3	1487	*Paenibacillus tundrae* A10b, NR_044525.1	99.53	100	-	+	+	-	25

Table 1. *Cont.*

Sequencing Analysis					Plant Growth Promoting Traits				
Source Tree Designation	Fragment Length, bp	Closest NCBI Match, Accession no.	% Identity	Query Coverage, %	Siderophore Production	Phosphate Solubilization/Mineralization		Nitrogen Fixation	IAA Production, µg/mL
						Pi	Po		
3	1475	*Bacillus firmus* NBRC 15306, NR_112635.1	98.17	100	-	+	-	+	13.2
1	1465	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.66	99	+	+	+	+	24.4
I2	1326	*Paenibacillus oceanisediminis* L10, NR_118217.1	98.93	99	-	+	+	-	25.04
I2	1484	*Paenibacillus tundrae* A10b, NR_044525.1	99.46	99	-	+	+	-	21.08
4	1339	*Delftia lacustris* 332, NR_116495.1	99.85	99	-	+	+	+	15.2
3	1493	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	98.18	99	+	+	+	+	18.16
3	1483	*Paenibacillus tundrae* A10b, NR_044525.1	99.46	100	-	+	+	-	30
3	1483	*Bacillus firmus* NBRC 15306, NR_112635.1	98.92	99	-	+	-	+	11.2
4	1468	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1	99.79	99	+	+	+	+	27
4	1489	*Delftia lacustris* 332, NR_116495.1	98.39	100	-	+	+	+	17
I2	1490	*Paenibacillus tundrae* A10b, NR_044525.1	99.60	99	-	+	+	-	25

3.2. Plant Growth Promoting Traits

All 48 isolates were screened for five PGPTs: nitrogen fixation, phosphate solubilization and mineralization, siderophore and IAA production (Table 1, Figure 1).

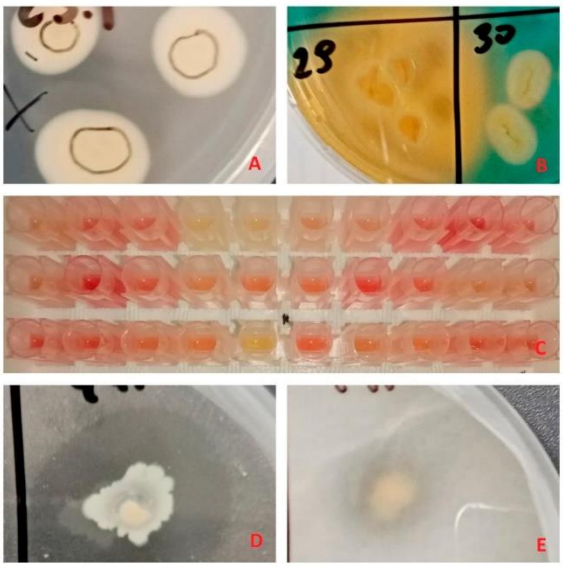

Figure 1. Examples of plant growth promoting trait tests: (**A**) bacterial growth on Jensen's medium, indicating putative nitrogen fixation capabilities, (**B**) orange zones on Chromeazul S (CAS) medium, indicating siderophore production, (**C**) supernatant color change to red after addition of Salkowski reagent, indicating indole-3-acetic acid (IAA) production, (**D**,**E**) clear zones in insoluble phosphate enriched media, indicating phosphate mineralization and solubilization respectively.

All isolates were capable of inorganic phosphate solubilization and IAA production. In the case of IAA production, amounts of produced IAA showed moderate alignment with bacterial genera, with *Pantoea* isolates producing relatively higher concentrations, *Paenibacillus* spp. and *Pseudomonas* spp. producing moderate amounts and *Bacillus* and *Delftia* isolates producing the least amount of IAA after 24 h incubation (Table 1).

Of all isolates, 95.8% showed organic phosphate mineralization. This ability was present in all the genera and absent in just two isolates, both of which showed high similarity to *Bacillus firmus* (>98% Identity), while 62.5% of the isolates were able to grow on nitrogen-free Jensen's medium, suggesting atmospheric nitrogen fixation capabilities. The only isolates that failed to display any growth were from the *Paenibacillus* genus, and 39.6% of the isolates showed positive results for siderophore production. All of these were from the *Pseudomonas* genus.

All the isolates displayed at least three PGPTs, with 39.6% of the isolates displaying all five (all were *Pseudomonas* spp.) and 18.75% displaying four.

The representative isolate used in later studies was able to produce IAA from tryptophan, on average at 27 $\pm$ 0.002 µg/mL after 24 h incubation. It tested positive for phosphate solubilization and mineralization and tested negative for nitrogen fixation and siderophore production.

3.3. Poplar microshoot inoculation

Of all isolates, 37.5% had the same colony morphology after a 2-day incubation: colonies were circular, off white, with a grey bull's eye, mucous, cells were rod-shaped, gram-negative. Partial *16S rRNA* gene sequencing results showed that all the isolates

with this morphotype belong to genus *Paenibacillus*, and that almost all isolates in this group were closely related to *Paenibacillus tundrae* (NR_044525.1). Moreover, bacteria with this morphotype were found in 6 out of 7 trees tested. Based on this information, we selected one isolate (*P. tundrae* % Identity—99.39%, Query coverage—99%) from this group at random for poplar microshoot inoculation study.

After a 2-month incubation, inoculated poplar microshoots had visible differences in regard to their respective control groups (Figure 2).

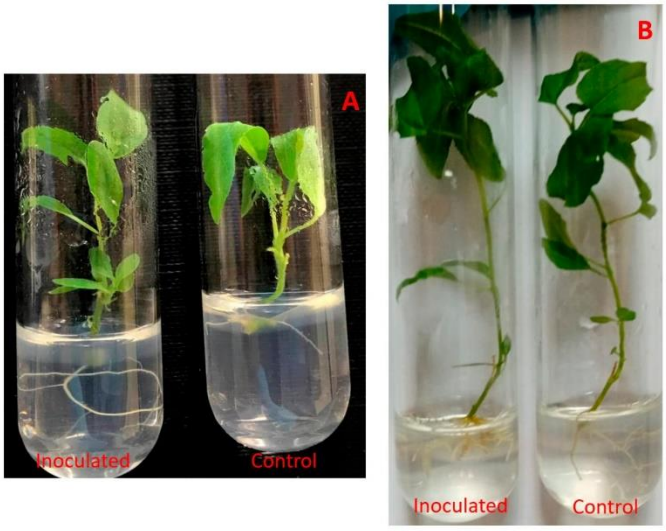

Figure 2. *Paenibacillus* sp. inoculated *P. tremula* microshoots after 2–3 weeks (**A**) and after 2 months (**B**) of incubation.

Inoculation with *Paenibacillus* sp. had a significant positive effect on the growth of both genotypes (Table 2), which was most noticeable in the root systems. Based on ANOVA, on average, in inoculated *P. tremula* microshoots lateral root number significantly increased by 44.7%, lateral root density by 66%, fresh and dry root biomass by 101.9% and 63.6% respectively (Figure 3).

Table 2. Microshoot growth parameter measurements taken after 2 months of incubation and their statistical analysis data.

Averages ± SD of:	Shoot Length, mm	Shoot Number	Largest Leaf Width, mm	Number of Adventitious Roots	Number of Lateral Roots	Sum Length of Adventitious Roots, mm	Longest Adventitious Root, mm	Fresh Aboveground Biomass, g	Fresh Root Biomass, g	Dry Aboveground Biomass, g	Dry Root Biomass, g	Lateral Root Density, Number/mm
Uninoculated *P. tremula*	43.3 ± 9.2	1.2 ± 0.5	9.6 ± 2.4	3.6 ± 1.4	26.0 ± 12.7	50.9 ± 18.1	25.1 ± 10.9	0.065 ± 0.024	0.024 ± 0.015	0.013 ± 0.005	0.003 ± 0.001	0.51 ± 0.21
Inoculated *P. tremula*	43.5 ± 6.2	1 ± 0.0	11.3 ± 1.4	4 ± 1.1	37.7 ± 9.7 (↑44.7%)	47.6 ± 16.4	18.9 ± 7.7	0.075 ± 0.012	0.049 ± 0.021 (↑101.9%)	0.015 ± 0.002	0.004 ± 0.002 (↑63.6%)	0.85 ± 0.27 (↑66%)
Student's t-test	NS	NS	**	NS	**	NS	*	NS	****	*	***	****
ANOVA	NS	NS	NS	NS	F (11.3), **	NS	NS	NS	F (19.4), ****	NS	F (15.4), ***	F (20.1), ****
Uninoculated *P. tremula* × *P. alba*	48.2 ± 14.2	1.0 ± 0.2	9.8 ± 2.3	1.6 ± 0.6	10.8 ± 13.0	34.9 ± 20.6	26 ± 14.9	0.157 ± 0.05	0.018 ± 0.021	0.028 ± 0.009	0.002 ± 0.002	0.23 ± 0.23
Inoculated *P. tremula* × *P. alba*	39.1 ± 12.1	1.0 ± 0.2	11.3 ± 2.8	2.7 ± 1.4 (↑65%)	33.7 ± 16.0 (↑213.7%)	70.5 ± 32.1 (↑102%)	46.7 ± 20.7 (↑79.5%)	0.118 ± 0.029 (↓25%)	0.053 ± 0.043 (↑197.1%)	0.022 ± 0.007	0.006 ± 0.004 (↑144.8%)	0.52 ± 0.26 (↑125.6%)
Student's t-test	NS	NS	NS	***	****	***	***	**	**	*	***	***
ANOVA	NS	NS	NS	F(11.7), **	F(27.4), ****	F(19.5), ****	F(14.7), ***	F(9.9), **	F(12.2), **	NS	F(17.1), ***	F(15.0), ***

NS-nonsignificant. $p > 0.05$, *—$p \leq 0.05$, **—$p \leq 0.01$, ***—$p \leq 0.001$, ****—$p \leq 0.0001$.

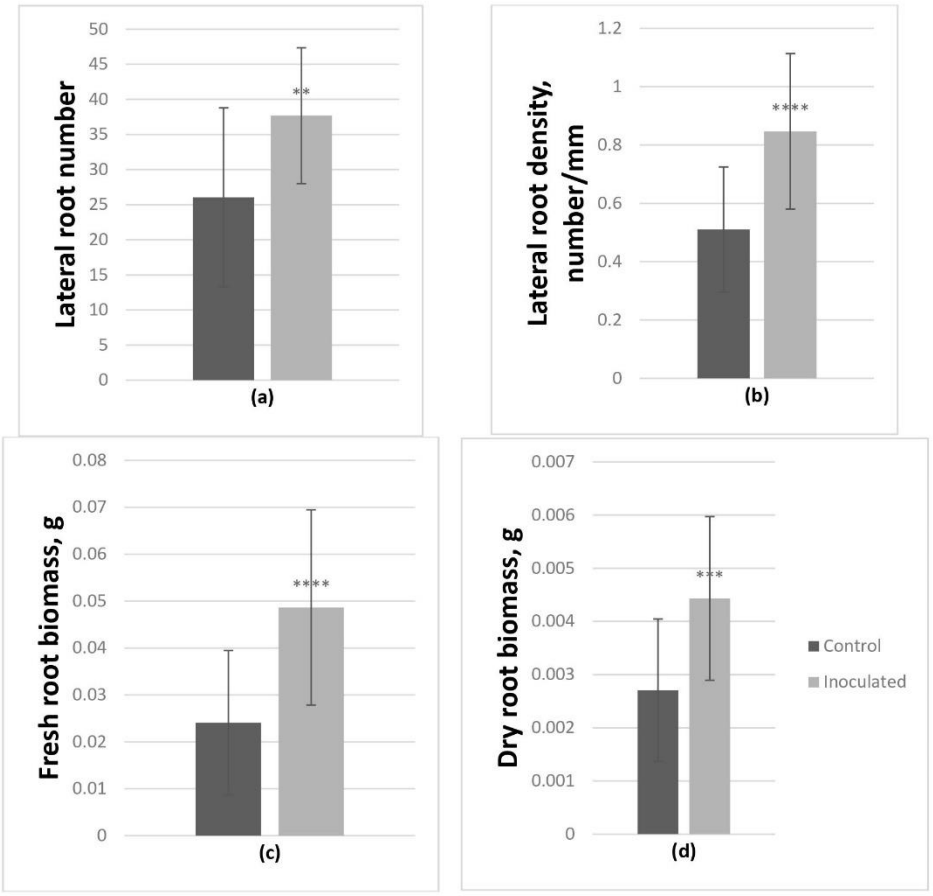

Figure 3. Effect of *Paenibacillus* sp. inoculation on *P. tremula* microshoots in vitro after 2-month incubation. On average lateral root number (**a**) and density (**b**) increased by 44.7% and 66% respectively, fresh root biomass (**c**) increased by 101.9% and dry root biomass (**d**) by 63.6% (average from 30 explants ± SD, **—$p \leq 0.01$, ***—$p \leq 0.001$, ****—$p \leq 0.0001$).

On average, *P. tremula* × *P. alba* hybrid microshoots also had significantly positively affected lateral root number and density; they increased by 213.7% and 125.6% respectively. Fresh and dry root biomass increased by 197.1% and 144.8% respectively. Inoculated microshoots also had longer adventitious roots by 102% (sum length), and the length of the longest adventitious root increased by 79.5% (Figure 4); the number of these roots was also enhanced by 65%. In this genotype, inoculation had a negative effect on fresh aboveground biomass, it decreased by 25%.

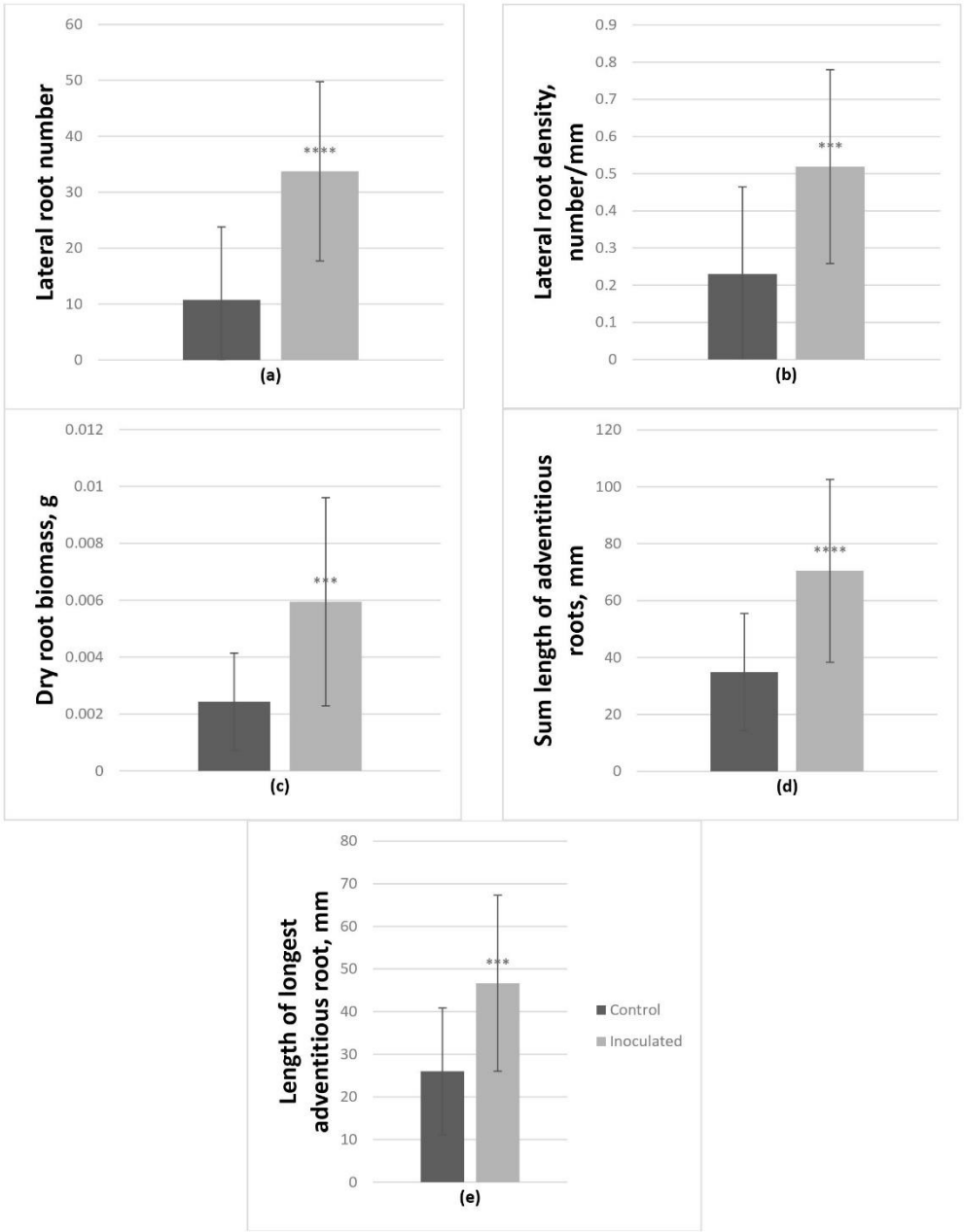

Figure 4. Effect of *Paenibacillus* sp. inoculation on *P. tremula* × *P. alba* microshoots in vitro after 2-month incubation. On average lateral root number (**a**) and density (**b**) increased by 213.7% and 125.6% respectively, dry root biomass (**c**) increased by 144.8%. Adventitious roots were also affected, on average their sum length (**d**) increased by 102% and the number of adventitious roots (**e**) increased by 65% (average from 30 explants ± SD, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4. Discussion

Obtained sequencing results showed that the isolated bacteria belong to two phyla, *Firmicutes* and *Proteobacteria*, in equal amounts. This coincides with other studies, that show that both of these phyla are often found in various woody plant hosts, accountable for much of the diversity of endophytic bacteria [5,35,46–48]. These two bacterial phyla were also found in two metabarcoding studies of English oak endophytes [49,50]. All the genera, except *Delftia* and *Pantoea*, found in this study, were also previously isolated from English oaks [51,52].

In our study bacterial isolates were categorized into 5 genera: *Bacillus*, *Delftia*, *Paenibacillus*, *Pantoea* and *Pseudomonas*, with *Paenibacillus* spp. and *Pseudomonas* spp. representing the bulk amount. Thus far, all 5 genera were isolated from several woody perennials [23,48,53–61]. Moreover, genera *Bacillus*, *Delftia*, *Pantoea* and *Pseudomonas* all have been shown to possess multiple PGPTs in earlier studies [16,39,40,62–65].

The representative isolate selected for poplar inoculation experiment was found to be closely related to *Paenibacillus tundrae*, a relatively novel species, first isolated from the soil in North America [66]. While, to our knowledge, this species was never sourced from trees, it has been shown to inhabit barley [67].

Collectively *Paenibacillus* are known as benign inhabitants of plant rhizospheres [67]. Thus far some have been isolated from trees such as poplar, larch and spruce [58], ash [54], Scots pine and rowan [55], western red cedar and lodgepole pine [56], coffee tree [59] and tea shrub [48].

Previously *Paenibacillus* spp. have been tested for plant growth promoting capabilities, mostly in agricultural species, showing promising results [24,68]. Furthermore, a nitrogen fixing *Paenibacillus* isolate (closely related to *P. humicus*) was previously tested on poplar microshoots. It had a significant effect on plant metabolic signature; additionally, it enhanced microshoot root length and number [36,58]. In our study, poplar microshoots were positively affected as well. This was especially evident in the root systems, where there was a significant increase in biomass, lateral root number and density in both tested genotypes.

Furthermore, while most inoculation effects were of similar nature in both tested genotypes, the hybrid poplar additionally had increased adventitious root length and number as well as decreased fresh aboveground biomass. Since no corresponding results were observed in *P. tremula* microshoots or dry aboveground biomass of the hybrid and hybrid poplars are known to grow faster than *P. tremula* [69,70], we attributed this decrease to nutrient and water depletion due to microshoot growth and feeding of the bacteria [71,72]. However, to test this hypothesis, further research is required.

The inoculant in our study tested positive for IAA production. Based on the results of earlier research and the biochemical properties of this plant growth regulator, it is likely that the effect on root growth in our in vitro study was instigated by IAA [5,73]. IAA is an auxin, which have been linked to promotion in root formation, due to enhanced stimulation of cell division, elongation and differentiation. Endophytic IAA production facilitates root system growth, which in due course boosts nutrient and water uptake [1,5,15,74,75]. Bal et al. reported that IAA producing *Paenibacillus* had had a positive effect on root growth as well. Inoculated rice seedlings demonstrated an increase in root length, dry and fresh root biomass, as well as shoot length and both dry and fresh shoot biomass, with a positive correlation between root elongation and IAA production [76]. Similar studies with IAA producing bacteria in other species, showed comparable results [77–79].

Moreover, trials with prototype biofertilizers of IAA producing *Paenibacillus* sp. demonstrated a positive effect not only in in vitro trials, but in pot trials as well. Inoculated mung beans showed a positive increase in dry biomass (17%) and sapling length (28%) ($p < 0.05$) [68].

5. Conclusions

Thus, in conclusion, we report that five different bacterial endophyte genera, *Bacillus*, *Delftia*, *Paenibacillus*, *Pantoea* and *Pseudomonas*, were isolated from English oak phyllosphere.

All isolates were tested for nitrogen fixation, phosphate solubilization/mineralization, IAA and siderophore production. All tested bacteria had at least three of these traits. One isolate, closely related to *Paenibacillus tundrae*, was used in a poplar microshoot inoculation study. The study revealed that root growth of the inoculated plants was significantly increased. Lateral root density, number, fresh and dry root biomass were affected in both tested genotypes. Additionally, one of the genotypes tested had increased adventitious root growth. This root growth enhancement was attributed to IAA production by the isolate. We propose that, after further research, this isolate could potentially be used as a biofertilizer in forestry enterprises.

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References

1. Kour, D.; Rana, K.L.; Yadav, A.N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H.S.; Saxena, A.K. Microbial biofertilizers: Bioresources and eco-friendly technologies for agricultural and environmental sustainability. *Biocatal. Agric. Biotechnol.* **2020**, *23*, 101487. [CrossRef]
2. García-Fraile, P.; Menendez, E.; Rivas, R. Role of bacterial biofertilizers in agriculture and forestry. *AIMS Environ. Sci.* **2015**, *2*, 183–205. [CrossRef]
3. Suhag, M. Potential of Biofertilizers to Replace Chemical Fertilizers. *Int. Adv. Res. J. Sci. Eng. Technol.* **2016**, *3*, 163–167.
4. Pirttilä, A.M.; Mohammad Parast Tabas, H.; Baruah, N.; Koskimäki, J.J. Biofertilizers and Biocontrol Agents for Agriculture: How to Identify and Develop New Potent Microbial Strains and Traits. *Microorganisms* **2021**, *9*, 817. [CrossRef] [PubMed]
5. Suman, A.; Yadav, A.N.; Verma, P. Endophytic microbes in crops: Diversity and beneficial impact for sustainable agriculture. In *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 1: Research Perspectives*; Springer: New Delhi, India, 2016; pp. 117–143. [CrossRef]
6. Grady, E.N.; MacDonald, J.; Liu, L.; Richman, A.; Yuan, Z.C. Current knowledge and perspectives of Paenibacillus: A review. *Microb. Cell Fact.* **2016**, *15*, 203. [CrossRef] [PubMed]
7. Ren, H.; Qin, X.; Huang, B.; Fernández-García, V.; Lv, C. Responses of soil enzyme activities and plant growth in a eucalyptus seedling plantation amended with bacterial fertilizers. *Arch. Microbiol.* **2020**, *202*, 1381–1396. [CrossRef] [PubMed]
8. Khosro, M.; Yousef, S. Bacterial Biofertilizers for Sustainable Crop Production: A Review. *ARPN J. Agric. Bol. Sci.* **2012**, *7*, 307–316.
9. Prasad, H.; Sajwan, P.; Kumari, M.; Solanki, S. Effect of organic manures and biofertilizer on plant growth, yield and quality of horticultural crop: A review. *Int. J. Chem. Stud.* **2017**, *5*, 217–221.
10. Fan, D.; Subramanian, S.; Smith, D.L. Plant endophytes promote growth and alleviate salt stress in Arabidopsis thaliana. *Sci. Rep.* **2020**, *10*, 12740. [CrossRef]
11. Kalayu, G. Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers. *Int. J. Agron.* **2019**, *2019*, 1–7. [CrossRef]
12. Gothwal, R.K.; Nigam, V.K.; Mohan, M.K.; Sasmal, D.; Ghosh, P. Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants. *Appl. Ecol. Environ. Res.* **2008**, *6*, 101–109. [CrossRef]
13. Ferreira, C.M.H.; Vilas-Boas, Â.; Sousa, C.A.; Soares, H.M.V.M.; Soares, E.V. Comparison of five bacterial strains producing siderophores with ability to chelate iron under alkaline conditions. *AMB Express* **2019**, *9*, 78. [CrossRef] [PubMed]
14. Mani, R.; Rajkumar, M.; Moreno, A.; Zhang, C.; Freitas, H. Serpentine endophytic bacterium Pseudomonas azotoformans ASS1 accelerates phytoremediation of soil metals under drought stress. *Chemosphere* **2017**, *185*, 75–85.
15. Vessey, J.K. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* **2003**, *255*, 571–586. [CrossRef]
16. Chakraborty, U.; Chakraborty, B.; Basnet, M. Plant growth promotion and induction of resistance in Camellia sinensis by Bacillus megaterium. *J. Basic Microbiol.* **2006**, *46*, 186–195. [CrossRef]
17. Govindasamy, V.; Senthilkumar, M.; Magheshwaran, V.; Kumar, U.; Bose, P.; Sharma, V.; Annapurna, K. Bacillus and Paenibacillus spp.: Potential PGPR for Sustainable Agriculture. *Plant Growth Health Promot. Bact.* **2010**, *18*, 333–356.
18. Puri, A.; Padda, K.P.; Chanway, C.P. Can naturally-occurring endophytic nitrogen-fixing bacteria of hybrid white spruce sustain boreal forest tree growth on extremely nutrient-poor soils? *Soil Biol. Biochem.* **2020**, *140*, 107642. [CrossRef]

19. Barra, P.J.; Inostroza, N.G.; Mora, M.L.; Crowley, D.E.; Jorquera, M.A. Bacterial consortia inoculation mitigates the water shortage and salt stress in an avocado (*Persea americana* Mill.) nursery. *Appl. Soil Ecol.* **2017**, *111*, 39–47. [CrossRef]
20. Anand, R.; Chanway, C. N2-fixation and growth promotion in cedar colonized by an endophytic strain of *Paenibacillus polymyxa*. *Biol. Fertil. Soils* **2012**, *49*, 235–239. [CrossRef]
21. Oliveira, A.L.M.; Urquiaga, S.; Döbereiner, J.; Baldani, J.I. The effect of inoculating endophytic N2-fixing bacteria on micropropagated sugarcane plants. *Plant Soil* **2002**, *242*, 205–215. [CrossRef]
22. Iniguez, A.L.; Dong, Y.; Triplett, E.W. Nitrogen Fixation in Wheat Provided by Klebsiella pneumoniae 342. *Mol. Plant-Microbe Interactions* **2004**, *17*, 1078–1085. [CrossRef]
23. Tamošiūnė, I.; Stanienė, G.; Haimi, P.; Stanys, V.; Rugienius, R.; Baniulis, D. Endophytic Bacillus and Pseudomonas spp. Modulate Apple Shoot Growth, Cellular Redox Balance, and Protein Expression Under In vitro Conditions. *Front. Plant Sci.* **2018**, *9*, 889. [CrossRef]
24. Liu, D.; Yang, Q.; Ge, K.; Hu, X.; Qi, G.; Du, B.; Liu, K.; Ding, Y. Promotion of iron nutrition and growth on peanut by Paenibacillus illinoisensis and Bacillus sp. strains in calcareous soil. *Braz. J. Microbiol.* **2017**, *48*, 656–670. [CrossRef] [PubMed]
25. Adesemoye, A.O.; Torbert, H.A.; Kloepper, J.W. Plant Growth-Promoting Rhizobacteria Allow Reduced Application Rates of Chemical Fertilizers. *Microb. Ecol.* **2009**, *58*, 921–929. [CrossRef] [PubMed]
26. Song, Z.; Lu, Y.; Liu, X.; Wei, C.; Oladipo, A.; Fan, B. Evaluation ofPantoea eucalyptiFBS135 for pine (Pinus massoniana) growth promotion and its genome analysis. *J. Appl. Microbiol.* **2020**, *129*, 958–970. [CrossRef] [PubMed]
27. Abd El-Migeed, M.M.M.; Saleh, M.M.S.; Mostafa, E.A.M. The Beneficial Effect of Minimizing Mineral Nitrogen Fertilization on Washington Navel Orange Trees by Using Organic and Biofertilizers. *World J. Agric. Sci.* **2007**, *3*, 80–85.
28. Dobo, B.; Asefa, F.; Asfaw, Z. Phosphorus Requirement for Colonization by Arbuscular Mycorrhizal Fungi (AMF) and Effect of AMF Inoculants on Growth of Perennial Crops and Agroforestry Trees. *East Afr. J. Sci.* **2016**, *10*, 77–86.
29. Abd-Alhamid, N.; Hassan, H.S.A.; Haggag, L.F.; Hassan, A.M. Effect of mineral and bio-fertilization on vegetative growth, leaf mineral contents and flowering of manzanillo olive trees. *Int. J. ChemTech Res.* **2015**, *8*, 51–61.
30. Abdou, M.A.H.; Ahmed, E.E.T.; Ahmed, A.A.; Abdel-Mola, M.A.M. Response of *Populus nigra*, L. seedlings to compost, biofertilizers and mineral npk fertilization. *Minia J. Agric. Res. Dev.* **2014**, *34*, 31–47.
31. Gouda, S.; Das, G.; Sen, S.K.; Shin, H.-S.; Patra, J.K. Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. *Front. Microbiol.* **2016**, *7*, 1538. [CrossRef]
32. Strobel, G. The Emergence of Endophytic Microbes and Their Biological Promise. *J. Fungi* **2018**, *4*, 57. [CrossRef] [PubMed]
33. Campanile, G.; Ruscelli, A.; Luisi, N. Antagonistic activity of endophytic fungi towards *Diplodia corticola* assessed by in vitro and in planta tests. *Eur. J. Plant Pathol.* **2007**, *117*, 237–246. [CrossRef]
34. Shiono, Y.; Koyama, H.; Murayama, T.; Koseki, T. New sesquiterpenes from the endophyte *Microdiplodia* sp. TT-12 and their antimicrobial activity. *Phytochem. Lett.* **2015**, *14*, 143–147. [CrossRef]
35. Ulrich, K.; Ulrich, A.; Ewald, D. Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiol. Ecol.* **2008**, *63*, 169–180. [CrossRef]
36. Scherling, C.; Ulrich, K.; Ewald, D.; Weckwerth, W. A Metabolic Signature of the Beneficial Interaction of the Endophyte *Paenibacillus* sp. Isolate and In Vitro–Grown Poplar Plants Revealed by Metabolomics. *Mol. Plant-Microbe Interact.* **2009**, *22*, 1032–1037. [CrossRef]
37. Lloyd, G.; McCown, B.H. Commercially feasible micropropagation of mountain laurel, (Kalmia latifolia) by use of shoot tip culture. *Comb. Proc. Int. Plant Propagators' Soc.* **1980**, *30*, 421–427.
38. Bertani, G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* **1951**, *62*, 293–300. [CrossRef]
39. Jasim, B.; Anish, M.C.; Shimil, V.; Jyothis, M.; Radhakrishnan, E.K. Studies on Plant Growth Promoting Properties of Fruit-Associated Bacteria from Elettaria cardamomum and Molecular Analysis of ACC Deaminase Gene. *Appl. Biochem. Biotechnol.* **2015**, *177*, 175–189. [CrossRef]
40. Chen, Q.; Liu, S. Identification and Characterization of the Phosphate-Solubilizing Bacterium Pantoea sp. S32 in Reclamation Soil in Shanxi, China. *Front. Microbiol.* **2019**, *10*, 2171. [CrossRef]
41. Arora, N.K.; Verma, M. Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech* **2017**, *7*, 381. [CrossRef]
42. Schwyn, B.; Neilands, J. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **1987**, *160*, 47–56. [CrossRef]
43. Gordon, S.A.; Weber, R.P. Colorimetric estimation of indole-3-acetic acid. *Plant Pysiol.* **1950**, *72*, 192–195.
44. Gottel, N.R.; Castro, H.; Kerley, M.; Yang, Z.; Pelletier, D.A.; Podar, M.; Karpinets, T.; Uberbacher, E.; Tuskan, G.A.; Vilgalys, R.; et al. Distinct Microbial Communities within the Endosphere and Rhizosphere of *Populus deltoides* Roots across Contrasting Soil Types. *Appl. Environ. Microbiol.* **2011**, *77*, 5934–5944. [CrossRef]
45. Cregger, M.A.; Carper, D.L.; Christel, S.; Doktycz, M.J.; Labbé, J.; Michener, J.K.; Dove, N.C.; Johnston, E.R.; Moore, J.A.M.; Vélez, J.M.; et al. Plant–Microbe Interactions: From Genes to Ecosystems Using Populus as a Model System. *Phytobiomes J.* **2021**, *5*, 29–38. [CrossRef]

46. Beckers, B.; De Beeck, M.O.; Thijs, S.; Truyens, S.; Weyens, N.; Boerjan, W.; Vangronsveld, J. Performance of *16s rDNA* Primer Pairs in the Study of Rhizosphere and Endosphere Bacterial Microbiomes in Metabarcoding Studies. *Front. Microbiol.* **2016**, *7*, 650. [CrossRef] [PubMed]
47. Carrell, A.A.; Frank, A.C. Pinus flexilis and Piceae engelmannii share a simple and consistent needle endophyte microbiota with a potential role in nitrogen fixation. *Front. Microbiol.* **2014**, *5*, 333. [CrossRef]
48. Gulati, A.; Sood, S.; Rahi, P.; Thakur, R.; Chauhan, S.; Chawla, I. Diversity analysis of diazotrophic bacteria associated with the roots of tea (*Camellia sinensis* (L.) O. Kuntze). *J. Microbiol. Biotechnol.* **2011**, *21*, 545–555. [CrossRef] [PubMed]
49. Sapp, M.; Lewis, E.; Moss, S.; Barrett, B.; Kirk, S.; Elphinstone, J.G.; Denman, S. Metabarcoding of Bacteria Associated with the Acute Oak Decline Syndrome in England. *Forests* **2016**, *7*, 95. [CrossRef]
50. Meaden, S.; Metcalf, C.J.E.; Koskella, B. The effects of host age and spatial location on bacterial community composition in the English oak tree (*Quercus robur*). *Environ. Microbiol. Rep.* **2016**, *8*, 649–658. [CrossRef] [PubMed]
51. Weyens, N.; Taghavi, S.; Barac, T.; van der Lelie, D.; Boulet, J.; Artois, T.; Carleer, R.; Vangronsveld, J. Bacteria associated with oak and ash on a TCE-contaminated site: Characterization of isolates with potential to avoid evapotranspiration of TCE. *Environ. Sci. Pollut. Res.* **2009**, *16*, 830–843. [CrossRef]
52. Bueno-Gonzalez, V.; Brady, C.; Denman, S.; Allainguillaume, J.; Arnold, D. Pseudomonas kirkiae sp. nov., a novel species isolated from oak in the United Kingdom, and phylogenetic considerations of the genera Pseudomonas, Azotobacter and Azomonas. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 2426–2434. [CrossRef]
53. Akbari Kiarood, S.L.; Rahnama, K.; Golmohammadi, M.; Nasrollanejad, S. Quorum-quenching endophytic bacteria inhibit disease caused by *Pseudomonas syringae* pv. syringae in Citrus cultivars. *J. Basic Microbiol.* **2020**. [CrossRef] [PubMed]
54. Ulrich, K.; Becker, R.; Behrendt, U.; Kube, M.; Ulrich, A. A Comparative Analysis of Ash Leaf-Colonizing Bacterial Communities Identifies Putative Antagonists of *Hymenoscyphus fraxineus*. *Front. Microbiol.* **2020**, *11*, 966. [CrossRef]
55. Izumi, H.; Anderson, I.C.; Killham, K.; Moore, E.R.B. Diversity of predominant endophytic bacteria in European deciduous and coniferous trees. *Can. J. Microbiol.* **2008**, *54*, 173–179. [CrossRef] [PubMed]
56. Bal, A.; Anand, R.; Berge, O.; Chanway, C.P. Isolation and identification of diazotrophic bacteria from internal tissues of pinus contorta and thuja plicata. *Botany* **2012**, *90*, 891–896. [CrossRef]
57. Brooks, D.; Gonzalez, C.; Appel, D.; Filer, T. Evaluation of Endophytic Bacteria as Potential Biological-Control Agents for Oak Wilt. *Biol. Control.* **1994**, *4*, 373–381. [CrossRef]
58. Ulrich, K.; Stauber, T.; Ewald, D. Paenibacillus—A predominant endophytic bacterium colonising tissue cultures of woody plants. *Plant Cell Tissue Organ Cult. (PCTOC)* **2008**, *93*, 347–351. [CrossRef]
59. Hoang, H.; Tran, L.H.; Nguyen, T.H.; Nguyen, D.A.T.; Nguyen, H.H.T.; Pham, N.B.; Trinh, P.Q.; De Boer, T.; Brouwer, A.; Chu, H.H. Occurrence of endophytic bacteria in Vietnamese Robusta coffee roots and their effects on plant parasitic nematodes. *Symbiosis* **2019**, *80*, 75–84. [CrossRef]
60. Kong, P. Evaluation of a novel endophytic Pseudomonas lactis strain for control of boxwood blight1. *J. Environ. Hortic.* **2019**, *37*, 39–43. [CrossRef]
61. Ferreira, A.; Quecine, M.C.; Lacava, P.T.; Oda, S.; Azevedo, J.L.; Araújo, W.L. Diversity of endophytic bacteria from Eucalyptus species seeds and colonization of seedlings by *Pantoea agglomerans*. *FEMS Microbiol. Lett.* **2008**, *287*, 8–14. [CrossRef]
62. Radhakrishnan, R.; Hashem, A.; Allah, E.F.A. Bacillus: A Biological Tool for Crop Improvement through Bio-Molecular Changes in Adverse Environments. *Front. Physiol.* **2017**, *8*, 667. [CrossRef]
63. Ubalde, M.C.; Braña, V.; Sueiro, F.; Morel, M.A.; Martínez-Rosales, C.; Marquez, C.; Castro-Sowinski, S. The Versatility of Delftia sp. Isolates as Tools for Bioremediation and Biofertilization Technologies. *Curr. Microbiol.* **2012**, *64*, 597–603. [CrossRef] [PubMed]
64. Feng, Y.; Shen, D.; Song, W. Rice endophyte Pantoea agglomerans YS19 promotes host plant growth and affects allocations of host photosynthates. *J. Appl. Microbiol.* **2006**, *100*, 938–945. [CrossRef] [PubMed]
65. da Silveira, A.P.D.; Iório, R.d.F.; Marcos, F.C.C.; Fernandes, A.O.; de Souza, S.A.C.D.; Kuramae, E.E.; Cipriano, M.A.P. Exploitation of new endophytic bacteria and their ability to promote sugarcane growth and nitrogen nutrition. *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **2019**, *112*, 283–295. [CrossRef] [PubMed]
66. Nelson, D.M.; Glawe, A.J.; Labeda, D.P.; Cann, I.K.O.; Mackie, R.I. *Paenibacillus tundrae* sp. nov. and *Paenibacillus xylanexedens* sp. nov., psychrotolerant, xylan-degrading bacteria from Alaskan tundra. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1708–1714.
67. Rasimus, S.; Mikkola, R.; Andersson, M.A.; Teplova, V.V.; Venediktova, N.; Ek-Kommonen, C.; Salkinoja-Salonen, M. Psychrotolerant Paenibacillus tundrae Isolates from Barley Grains Produce New Cereulide-Like Depsipeptides (Paenilide and Homopaenilide) That Are Highly Toxic to Mammalian Cells. *Appl. Environ. Microbiol.* **2012**, *78*, 3732–3743. [CrossRef]
68. Goswami, D.; Parmar, S.; Vaghela, H.; Dhandhukia, P.; Thakker, J. Describing Paenibacillus mucilaginosus strain N3 as an efficient plant growth promoting rhizobacteria (PGPR). *Cogent Food Agric.* **2015**, *1*, 1000714. [CrossRef]
69. Yu, Q.; Tigerstedt, P.M.A.; Haapanen, M. Growth and phenology hybrid aspen clones (*Populus tremula* L. x *Populus tremuloides* Michx.). *Silva Fenn.* **2001**, *35*, 15–25. [CrossRef]
70. Tullus, A.; Tullus, H.; Vares, A.; Kanal, A. Early growth of hybrid aspen (*Populus*×*wettsteinii* Hämet-Ahti) plantations on former agricultural lands in Estonia. *For. Ecol. Manag.* **2007**, *245*, 118–129.
71. Khasa, D.; Fung, M.; Logan, B. Early growth response of container-grown selected woody boreal seedlings in amended composite tailings and tailings sand. *Bioresour. Technol.* **2005**, *96*, 857–864. [CrossRef]

72. Fu, J.; Sampalo, R.; Gallardo, F.; Cánovas, F.M.; Kirby, E.G. Assembly of a cytosolic pine glutamine synthetase holoenzyme in leaves of transgenic poplar leads to enhanced vegetative growth in young plants. *Plant, Cell Environ.* **2003**, *26*, 411–418. [CrossRef]
73. Idris, E.E.; Iglesias, D.J.; Talon, M.; Borriss, R. Tryptophan-Dependent Production of Indole-3-Acetic Acid (IAA) Affects Level of Plant Growth Promotion by Bacillus amyloliquefaciens FZB42. *Mol. Plant-Microbe Interactions* **2007**, *20*, 619–626. [CrossRef]
74. Wang, S.; Ouyang, L.; Ju, X.; Zhang, L.; Zhang, Q.; Li, Y. Survey of Plant Drought-Resistance Promoting Bacteria from Populus euphratica Tree Living in Arid Area. *Indian J. Microbiol.* **2014**, *54*, 419–426. [CrossRef] [PubMed]
75. Tiwari, S.; Lata, C.; Chauhan, P.S.; Nautiyal, C.S. *Pseudomonas putida* attunes morphophysiological, biochemical and molecular responses in *Cicer arietinum* L. during drought stress and recovery. *Plant Physiol. Biochem.* **2016**, *99*, 108–117. [CrossRef] [PubMed]
76. Bal, H.B.; Das, S.; Dangar, T.K.; Adhya, T.K. ACC deaminase and IAA producing growth promoting bacteria from the rhizosphere soil of tropical rice plants. *J. Basic Microbiol.* **2013**, *53*, 972–984. [CrossRef] [PubMed]
77. Qin, S.; Miao, Q.; Feng, W.-W.; Wang, Y.; Zhu, X.; Xing, K.; Jiang, J.-H. Biodiversity and plant growth promoting traits of culturable endophytic actinobacteria associated with *Jatropha curcas* L. growing in Panxi dry-hot valley soil. *Appl. Soil Ecol.* **2015**, *93*, 47–55. [CrossRef]
78. Goryluk-Salmonowicz, A.; Orzeszko-Rywka, A.; Piórek, M.; Rekosz-Burlaga, H.; Otłowska, A.; Gozdowski, D.; Błaszczyk, M. Plant Growth Promoting Bacterial Endophytes Isolated from Polish Herbal Plants. *Acta Sci. Pol. Hortorum Cultus* **2018**, *17*, 101–110. [CrossRef]
79. Devi, K.A.; Pandey, G.; Rawat, A.K.S.; Sharma, G.D.; Pandey, P. The Endophytic Symbiont—Pseudomonas aeruginosa Stimulates the Antioxidant Activity and Growth of *Achyranthes aspera* L. *Front. Microbiol.* **2017**, *8*, 1897. [CrossRef]

ARTICLE 2/STRAIPSNIS NR. 2

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Article

Differentiation of Closely Related Oak-Associated Gram-Negative Bacteria by Label-Free Surface Enhanced Raman Spectroscopy (SERS)

Dorotėja Vaitiekūnaitė [1,*] and Valentinas Snitka [2]

[1] Laboratory of Forest Plant Biotechnology, Institute of Forestry, Lithuanian Research Centre for Agriculture and Forestry, Liepų Str. 1, Girionys, 53101 Kaunas, Lithuania
[2] Research Center for Microsystems and Nanotechnology, Kaunas University of Technology, Studentu Str. 65, 51369 Kaunas, Lithuania; vsnitka@ktu.lt
* Correspondence: doroteja.vaitiekunaite@lammc.lt

Abstract: Due to the harmful effects of chemical fertilizers and pesticides, the need for an eco-friendly solution to improve soil fertility has become a necessity, thus microbial biofertilizer research is on the rise. Plant endophytic bacteria inhabiting internal tissues represent a novel niche for research into new biofertilizer strains. However, the number of species and strains that need to be differentiated and identified to facilitate faster screening in future plant-bacteria interaction studies, is enormous. Surface enhanced Raman spectroscopy (SERS) may provide a platform for bacterial discrimination and identification, which, compared with the traditional methods, is relatively rapid, uncomplicated and ensures high specificity. In this study, we attempted to differentiate 18 bacterial isolates from two oaks via morphological, physiological, biochemical tests and SERS spectra analysis. Previous *16S rRNA* gene fragment sequencing showed that three isolates belong to *Paenibacillus*, 3—to *Pantoea* and 12—to *Pseudomonas* genera. Additional tests were not able to further sort these bacteria into strain-specific groups. However, the obtained label-free SERS bacterial spectra along with the high-accuracy principal component (PCA) and discriminant function analyses (DFA) demonstrated the possibility to differentiate these bacteria into variant strains. Furthermore, we collected information about the biochemical characteristics of selected isolates. The results of this study suggest a promising application of SERS in combination with PCA/DFA as a rapid, non-expensive and sensitive method for the detection and identification of plant-associated bacteria.

Keywords: plant-associated bacteria; surface enhanced Raman spectroscopy; SERS; label-free; *Paenibacillus*; *Pseudomonas*; *Pantoea*; principal component analysis; discriminant function analysis

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1. Introduction

One of the limitations in microbiological research stems from the inability to easily differentiate bacterial samples at the species and/or strain level [1–5]. Over the years, many methods have been developed for microbial identification: morphological assessment, analytical profile index (API), immuno-assays (ex. ELISA), DNA sequencing, etc. The current "gold" standard, DNA sequencing, allows such discrimination based on the minute differences in bacterial genetic code, however, this method can be time consuming and economically inefficient, hence situationally ineffective [2,6–14].

Vibrational spectroscopy is a technique that has been used for the analysis of various chemicals, and in recent years has been successfully adapted for microbial research, showing great promise in becoming a novel diagnostic system in this field [4–6,8,9,15–18].

This technique stems from the fact that under excitation by light, analyte molecules will experience observable photon scattering. Raman scattering happens when the excitation energy is not the same as that of the scattered energy post interaction with the analyte molecule. Due to the low emission rate of scattered photons, an integration time of minutes

is required to obtain useful spectra, however, ways to enhance this intrinsic aspect have been developed [18,19].

Raman spectroscopy, and its more sensitive variant (offering enhancements of up to 10^{15}-fold [8,16,20,21])—surface enhanced Raman spectroscopy (SERS), are effective ways to discern bacterial samples up to strain level based on qualitative differences in cell chemistry [6,8–10,15,16,22]. This is sometimes referred to as vibrational "fingerprinting" or "barcoding", as each organism presents different and unique spectra [4,23,24].

The sensitivity of the SERS technique is facilitated by substrate surface modifications. Analyte bioparticles are adsorbed on or placed in close proximity to a noble metal like silver (Ag) or gold (Au), nanoparticle (NP)-covered surfaces. In a sense this creates "hot spots", which focus and thus strengthen the signal that is emitted by the analyte molecules under excitation [14,16,20,22,25]. This allows for shorter integration times and increased sensitivity compared to standard Raman spectroscopy. SERS is advantageous due to its potential as a low cost, rapid, low volume, non-destructive, broad information content, high specificity and sensitivity diagnostic method [1,9,10,18,20,22], also in part because analysis is easily performed in aqueous environments without interface [4,25]. In bacteriology, the efficacy of SERS was demonstrated with purified cultures, mixed and even single cell samples [6–9,13,15,20].

Spectra generated by SERS, provide the ability to carry out multivariate cluster analysis (ex. principal component analysis (PCA), discriminant function analysis (DFA), hierarchical cluster analysis (HCA), etc. or a combination thereof) that help the separation of different bacterial strains by determining both unique and common aspects of given spectra [8,9,15,16,22]. Moreover, these spectra help inform about the cell's molecular structure and composition, because spectral features correspond to functional groups [6,7,20,22,26].

Despite the aforementioned advancements, the field is generally geared towards food and medical pathogen identification [1,2,6–10,12,14,18,20,22,27,28]. Furthermore, most experiments are conducted using well-studied species (ex. *E. coli, Bacillus* sp.). Wider use of this technique for bacteria sourced from environmental samples (ex. soil, plants) is, at the moment, rare [29].

In recent years, due to the limitations and negative effects of pesticides and chemical fertilizers, bacteria have been widely studied as potential biocontrol agents and biofertilizers [30]. This search for microbiological alternatives is expected to grow [31–33]. Future research in this field will rely heavily on the screening of new and/or yet thoroughly unstudied bacterial species [32–34]. Plant associated samples often contain substantial amounts of diverse bacterial species and potentially multiple, difficult to differentiate, strains within a single genus (ex. *Pseudomonas* spp.) [35–40]. In the field of plant-associated bacteriology, using SERS for the differentiation and identification of bacteria would likely prove to be highly beneficial and efficient [16,22,29,30,41], as different species and even different strains of the same species may have vastly different effects on plant growth [42].

In this study we aimed to determine whether genetically highly homologous representative endophytic bacterial species from two oak trees from the same site could be effectively distinguished using SERS as an analytical tool in addition to other morphological, physiological and biochemical tests, to help in future biofertilizer research. Also, we aimed to produce specific SERS spectra for different oak-associated *Pantoea*, *Pseudomonas* and *Paenibacillus* isolates for public library building and future use.

With the help of PCA and DFA techniques, we were able to differentiate tested isolates up to strain level based on their SERS spectra. It is noteworthy, that the spectral characteristics of plant-associated bacteria homologous with *Pseudomonas azotoformans* and *Paenibacillus tundrae* species have not yet been described using the SERS method. This SERS-based protocol can be seen as an alternative, cost-effective and fast method for differentiating, identifying and characterizing different types of plant-associated bacteria and promote research in this and associated fields.

2. Materials and Methods

Eighteen bacterial samples isolated from two English oaks (*Quercus robur*) were chosen for this study from our previously created library [35], 12 isolates from oak α and six from oak β. Both trees are from the same site, located in Lithuania (Table 1).

Table 1. Tree locations and their associated isolate identification numbers.

Tree	Site (GPS Coordinates)	Isolate Identification No.
Alfa (α)	55.829832, 26.217380	21, 27, 30, 32, 33, 33.1,34, 36, 40, 46.1, 46.2, 49
Bravo (β)	55.8301132, 26.2168633	24, 29, 35, 37, 47.1, 47.2

2.1. Morphological, Physiological and Biochemical Analysis

Morphological, physiological and biochemistry tests were done in triplicates using fresh colonies, grown on low salt lysogeny broth (LB) agarized medium (pH of 7.2 throughout the experiments) (Duchefa Biochemie, Haarlem, the Netherlands) each time. Bacteria were grown at 25 °C. All media were autoclaved prior to use at 121 °C for 15 min. Aseptic techniques were employed throughout the experiments.

2.1.1. Colony Morphology

Colony morphology was observed. Colony form, elevation, margin, color, opacity, smoothness, consistency and overall appearance on LB medium after 2 days of incubation were determined. Additionally, bacteria samples from overnight liquid LB cultures were visualized using 0.1% Gentian violet dye under 10,000× magnification. Bacteria shape, arrangement and size (average from three biological replicates and 10 technical replicates each) were observed.

2.1.2. Biofilm Formation

Bacterial ability to form biofilms was tested. A modified tissue culture plate method was used [43]. Bacteria were grown overnight in liquid LB. The next day 2 μL of this suspension was pipetted into a sterile flat-bottomed 96-well polystyrene tissue culture plate, then each cell was filled with 198 μL of LB medium supplemented with 1% glucose. 200 μL of LB medium supplemented with 1% glucose was used as control. The plate was incubated overnight. After incubation, the plate was washed three times in a new container of sterile water each time. Then the plate was left to air dry. Subsequently, the biofilm layer was dyed using 0.1% Gentian violet solution for 15 min. Afterward, the plate was washed and dried as previously described. After the fixation step, the biofilm layer was solubilized in ethanol (95%) for 30 min. Optical density (OD) was measured using Synergy HT Multi-Mode Microplate Reader (Biotek Instruments Inc., Bad Friedrichshall, Germany) at 630 nm (95% ethanol as control). Optical density cut-off (ODc) was calculated: ODc = average OD of control + 3 times the standard deviation of control. Biofilm formation capabilities were evaluated: weak biofilm ~ODc, moderate—2–4 ODc, strong biofilm—more than 4 ODc.

2.1.3. Carbohydrate Use

A modified phenol red test was used to determine how and which carbohydrates could these isolates use as a carbon source [44]. Lactose (L), fructose (F) (Merck, Darmstadt, Germany), maltose (M) (Avantor, Radnor, PA, USA), sucrose (Su) and glucose (G) (Duchefa Biochemie) were tested. LB liquid medium supplemented with 1% of one selected carbohydrate in each tube and 0.0018% of phenol red dye (Merck) was used. Carbohydrate solutions were filter sterilized and added to the medium after autoclaving. To check for gas production, an upside-down Durham tube was placed in each test tube. The tubes were then inoculated, gently mixed and incubated overnight in a stationary position. This allowed for the positive identification of isolates capable of anaerobically fermenting tested carbohydrates. To discern whether the bacteria were capable of aerobic use of carbohydrates, samples were also placed in a thermal shaker overnight. In both cases, color

changes from red to yellow were observed. Bubbles in Durham tubes were indicative of gas production and color changes (from red to yellow) indicate a pH change due to acid production, hence the capacity for carbohydrate use.

2.1.4. Antibiotic Susceptibility

Bacterial susceptibility to various antibiotics was determined by using a modified Kirby-Bauer disk diffusion test [45]. Ampicillin (AM), cefotaxime (CTX), chloramphenicol (C), streptomycin (STP), ticarcillin (TIC) (Duchefa Biochemie) and kanamycin (K) (Panpharma, La Selle-en-Luitré, France) were used. Bacteria were grown overnight in liquid LB medium. The next day the bacterial suspension was adjusted to approximately 1.5×10^8 cfu/mL. The suspension was cross-streaked on Mueller-Hinton agar (Condalab, Madrid, Spain) using sterile cotton swabs. Then sterile 0.5 mm paper disks were placed on top (6 disks per D8;9 cm plate, equally spaced). Filter sterilized antibiotic solutions were then pipetted onto the disks so that each disk contained a desired amount of antibiotics (10 µg of AM and STP, 30 µg of CTX, C, K and 75 µg of TIC per disk). The plates were incubated overnight in the dark. Inhibition zones were measured the next day and bacterial susceptibility was determined using antibiotic susceptibility charts [46–48].

2.2. SERS Analysis

We used SERS for bacterial vibrational fingerprinting. This method allowed us to sort bacterial isolates into groups using their vibrational patterns and to tentatively ascertain their molecular composition.

2.2.1. Experimental Set Up for SERS Spectra Acquisition

SERS spectra were recorded using Raman spectrometer (NTEGRA Spectra, NT-MDT Inc., Moscow, Russia) in an "upright" configuration with 532 nm laser as the excitation source. All spectra were calibrated to the first-order silicon longitudinal-optical (LO) phonon peak at 520 cm^{-1}. The instrument is equipped with 2 mW power at the sample, a 100× objective (NA: 0.7). A thermoelectrically cooled (−60 °C) charge-coupled device (CCD) was used as a detector. The spectral resolution was 1.1 cm^{-1}.

2.2.2. SERS Substrate Preparation

The preparation of SERS substrates was based on direct silver ions reduction by elemental silicon [49]. Silicon slides were cut into small pieces (1.5 × 1.5 cm). Then they were polished (2 min) with glass paper to rough up the silicon surface. Such prepared slides with etched 100 mm deep wells were washed with pure ethanol, then dried under nitrogen flow and kept in closed Petri dishes until use.

Preprepared HF (24%) and $AgNO_3$ (20 mM) solutions were mixed in a ratio of 1:1 *v/v*. Polished silicon slides were immersed in the reaction mixture for 2 s, then immediately transferred to a container with distilled water (dH_2O) and finally dried under nitrogen flow. The dried substrate slides were immediately used for SERS spectra measurements.

2.2.3. Bacteria Sample Preparation for SERS

Bacteria from our library were transferred using a plastic loop into liquid LB. Overnight cultures (~10^6 cfu/mL) were centrifuged at 3500× *g* and washed 3 times with 0.9% NaCl solution. After the last wash bacteria were placed in 200 µL of 0.9% NaCl [12,24]. Using a sterile pipette, 20 µL of the suspension was then placed on the substrate silicon slide and immediately transferred to the Raman microscope for data acquisition. It should be noted that the SERS spectra measurements were performed in the presence of bacteria in liquid suspension and by scanning the sample, thus reducing the thermal effect of the laser on the bacteria, i.e., scanning live samples [50].

2.2.4. SERS Spectra Acquisition

To ensure the reproducibility of the SERS spectra, 50 spectra from each bacterium were obtained from the suspension drop on the SERS substrate. The single spectrum was acquired as a summary spectrum by scanning 100 × 100 micron area during the SERS spectra acquisition to optimize the Raman signal strength. The bacterial spectra dataset was collected from the 50 randomly selected spots in the sample. The acquisition time of Raman scattering signal was 20 s. From the 50 acquired spectra, 16 spectra, based on their signal-to-noise ratio, were selected for processing. The resulting SERS spectra were analyzed and edited using Nova 1.1.0.1840 (NT-MDT Inc., Moscow, Russia) and SpectraGryph 1.2.14 software (Dr. Friedrich Menges, Obersdorf, Germany) with cropping to 600–1800 cm^{-1}, removal of background fluorescence, normalization to the intensity of maximum amplitude, baseline correction—5% coarseness [51].

Vibrational bands were noted (peak finding threshold—0.5%, position tolerance—0.4%) and tentative band assignments were determined based on literature sources.

2.2.5. Multivariate Cluster Analyses

Bacterial differentiation was done based on cluster map methodology using PCA and DFA with Raman processing software [52] in the MATLAB (2012) environment (MathWorks, Inc., Natick, MA, USA). PCA was employed for this study to highlight the variability existing in the spectral data set. The reference spectrum for a single isolate used for DFA was produced as an average spectrum of the 16 experimentally acquired spectra. In DFA a leave-one-out cross-validation method was used.

3. Results

3.1. Morphological, Physiological and Biochemical Analysis

Eighteen bacterial isolates were studied. Twelve from oak α and six from oak β. Previously *16S rRNA* gene fragments were successfully sequenced for all the isolates [35]. All of them were identified to genus level (Table 2). Colony morphology and DNA sequencing results allowed to presumptively divide 18 isolates into four morphotypes, identified as A–D (Table 2, Figure 1). Morphotypes A and D were isolated from both trees, while morphotypes B and C were only isolated from different trees each. NCBI Blast results showed that morphotype A was from the *Paenibacillus* genus and was closely related to *Paenibacillus tundrae*, morphotype B was closely related to *Pantoea agglomerans*, morphotype C—to *Pseudomonas brenneri/proteolytica* and morphotype D—to *Pseudomonas azotoformans*.

Table 2. Colony morphology and *16S rRNA* sequencing results of 18 bacterial isolates and their corresponding morphotypes (in part based on [35]).

Isolate Identification Code	Colony Morphology	Morphotype	Closest NCBI Match, Accession No., % Identity
21, 33.1, 35	Colonies are circular, flat with a slightly undulate margin, smooth and glistening, off-white with a grey bull's eye in the center, translucent and mucoid.	A	*Paenibacillus tundrae* A10b, NR_044525.1, 99.32–99.46%
27, 30, 34	Colonies are circular, flat, cream colored, translucent, smooth and glistening, butyrous, the margin is entire. Changes LB agar medium color to bright yellow. A small spindle formation can be observed at the center of the colony with 40× magnification.	B	*Pantoea agglomerans* DSM 3493, NR_041978.1, 99.64–99.97%
24, 29	Colonies are circular, raised, buff color, glistening and butyrous, the center of the colony is rough, and the edges are smooth, the margin is entire. The colonies change color of LB agar medium to bright yellow.	C	*Pseudomonas brenneri* CFML 97–391, NR_025103.1, 99.86%; *Pseudomonas proteolytica* CMS 64, NR_025588.1, 99.59%
32, 33, 36, 37, 40, 46.1, 46.2, 47.1, 47.2, 49	Colonies are circular, flat, cream colored, translucent, smooth and glistening, butyrous, the margin is entire. Changes LB agar medium color to bright yellow.	D	*Pseudomonas azotoformans* NBRC 12693, NR_113600.1, 99.66–99.79%

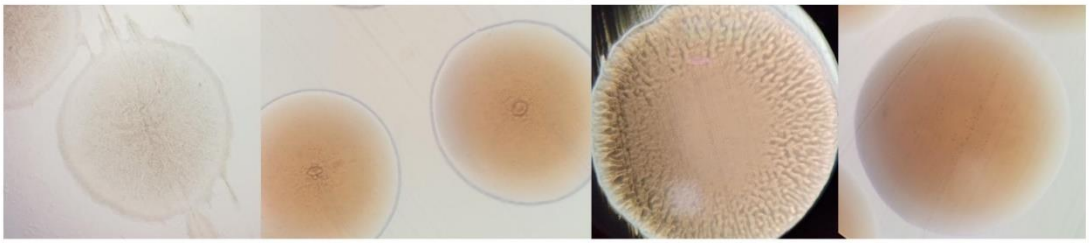

Figure 1. Colony morphology of 4 morphotypes (**A**–**D**) under 40× magnification on LB agar medium after 2 days incubation.

Bacteria were all similar in diameter—0.28–0.45 µm. Results from morphological, physiological and biochemistry tests also divided the isolates into four distinct groups that coincided with previously described morphotypes (Table 3).

Table 3. Analyzed properties (size, biofilm formation, carbohydrate use, antibiotic susceptibility) of 18 bacterial isolates used in this study (in bold—isolates selected for further SERS analysis) *.

Morphotype	Isolate Identification No.	Average Diameter ± SD, µm	Biofilm Production	Antibiotic Disk Diffusion Test						Carbohydrate Use				
				AM-10	CTX-30	C-30	K-30	STP-10	TIC-75	L	M	Su	G	F
A	21	0.444 ± 0.03	–	S	R	S	S	R	R	Ac	Ac	Ac	Ac	Ac
	33.1	0.361 ± 0.039	–	S	R	S	S	R	R	Ac	Ac	Ac	Ac	Ac
	35	0.43 ± 0.042	–	S	R	S	S	R	R	Ac	Ac	Ac	Ac	Ac
B	**27**	0.324 ± 0.041	–	S	S	S	S	S	R	Ac	Ac	Ac	Ac	Ac
	30	0.352 ± 0.04	–	S	S	S	S	S	R	Ac	Ac	Ac	Ac	Ac
	34	0.438 ± 0.028	–	S	S	S	S	S	R	Ac	Ac	Ac	Ac	Ac
C	**24**	0.326 ± 0.033	moderate	R	R	R	S	R	R	–	–	–	Ac	–
	29	0.284 ± 0.014	moderate	R	R	R	S	R	R	–	–	–	Ac	–
D	32	0.366 ± 0.021	–	R	R	R	S	R	R	–	–	–	Ac	–
	33	0.338 ± 0.03	–	R	R	R	S	R	R	–	–	–	Ac	–
	36	0.447 ± 0.047	–	R	R	R	S	R	R	–	–	–	Ac	–
	37	0.34 ± 0.023	–	R	R	R	S	R	R	–	–	–	Ac	–
	40	0.427 ± 0.037	–	R	R	R	S	R	R	–	–	–	Ac	–
	46.1	0.426 ± 0.036	–	R	R	R	S	R	R	–	–	–	Ac	–
	46.2	0.338 ± 0.028	–	R	R	R	S	R	R	–	–	–	Ac	–
	47.1	0.43 ± 0.073	–	R	R	R	S	R	R	–	–	–	Ac	–
	47.2	0.45 ± 0.042	–	R	R	R	S	R	R	–	–	–	Ac	–
	49	0.405 ± 0.042	–	R	R	R	S	R	R	–	–	–	Ac	–

*—signifies a negative result; R: resistant, S: sensitive, Ac: acid production.

Morphotype A was sensitive to AM, C and K, and capable of fermenting all the carbohydrates tested. Morphotype B was resistant to TIC and capable of fermenting all the carbohydrates tested. Morphotype C formed biofilms, was sensitive to K and capable of using G as a nutrient. Morphotype D was sensitive to K and capable of using G as a nutrient.

3.2. SERS Analysis

3.2.1. Structural Analysis Based on SERS Spectra

Eight isolates were selected for SERS analysis. As *Pseudomonas* sp. are difficult to differentiate to species level via *16S rRNA* gene sequencing and since isolates 24 and 29 are of the same origin, we treated them as equal. Thus, for further analysis, isolate 24 and isolates 37 and 49 from the pseudomonad group were chosen. Isolates 37 and 49 were highly homologous and from different sources. Isolates 33.1 and 35 were selected from the *Paenibacillus* sp. group, because based on genetic tests and additional experiments, they were identical, but of different origins. Moreover, isolates 27, 30 and 34, representing *Pantoea agglomerans*, were selected for vibrational analysis. They were all sourced from the same tree, however, they exhibited differences in plant hormone, indole-3-acetic acid (IAA), production in previous studies [35]. To determine the efficacy of the proposed bacterial differentiation methodology, the focus was put on within-group differences of isolates 33.1/35, 27/30/34 and 37/49.

Band peaks and their respective intensities are used to sort bacteria in relation to one another [7,29,50]. Representative spectra acquired during this study are shown in Figure 2. The stacked mean spectra of all 8 isolates are shown in Figure 3.

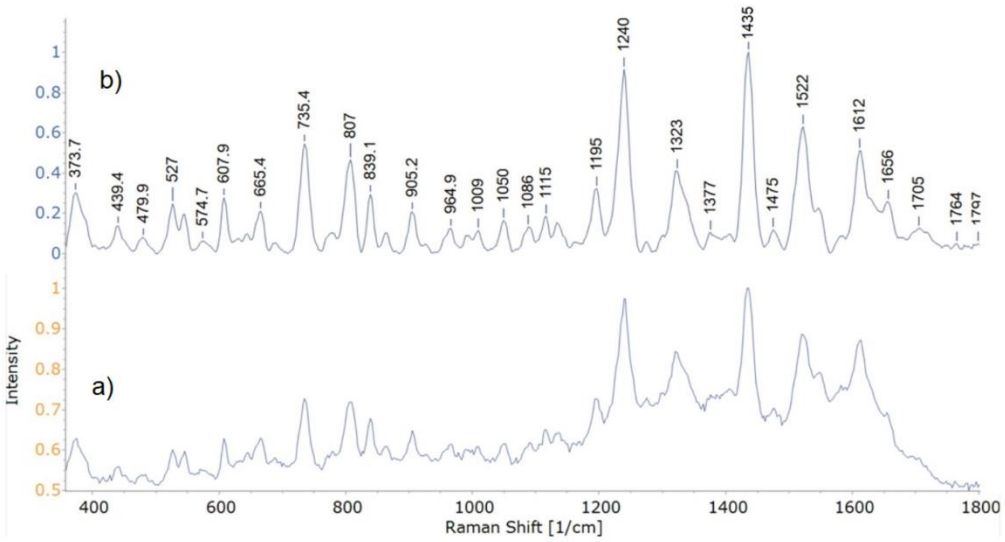

Figure 2. Representative experimental (**a**) and preprocessed (**b**) spectra of isolate 27.

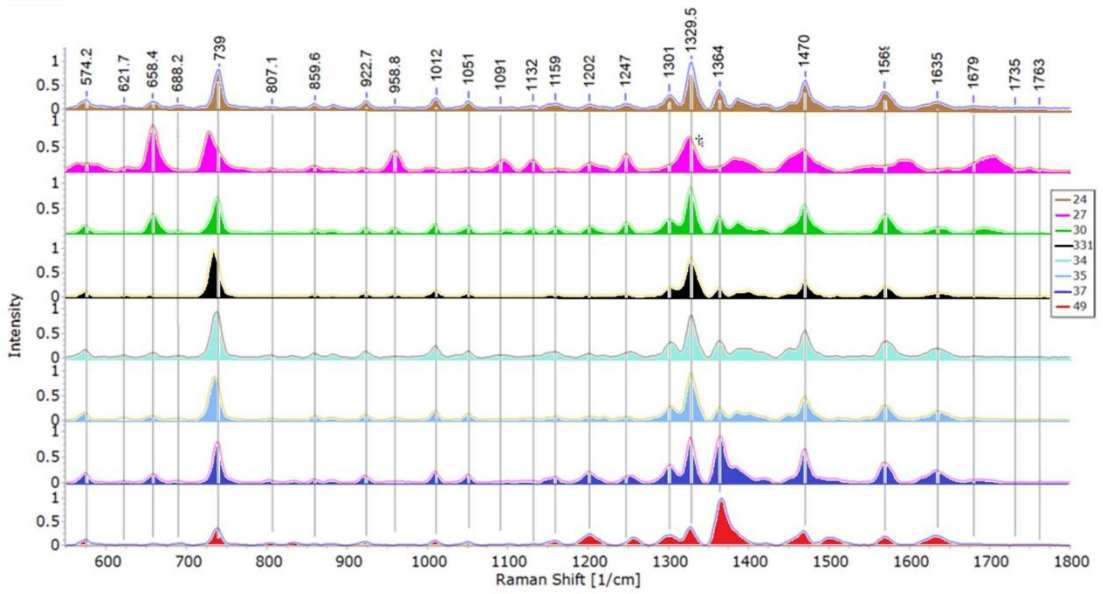

Figure 3. Stacked mean spectra of 8 bacterial isolates with peak values for the most notable bands.

As mentioned previously, peaks in the SERS spectra are linked with functional groups [6,7,20,22,26]. These groups represent components of bacterial cells [8,17], most often either extracellular polymers [17] or more likely degradation metabolites [50,53] or parts of the outer membrane in Gram negative bacteria [13]. We present tentative SERS spectra band assignments in Table 4.

Table 4. Tentative assignments of the most notable surface enhanced Raman spectral bands of mean spectra from 8 tested isolates.

Peak Wavenumber, cm^{-1}								Tentative Band Assignments *
24	**27**	**30**	**33.1**	**34**	**35**	**37**	**49**	
	563.38							T, G [10,54]
574.21		573.52	573.01	573.47	572.08	573.63	573.57	Deformation of C=O–C in lipids [55] or Trp [54,56,57] or carbohydrates [58]
621.65	625.66	622.17	623.31	620.93	622.5	622.03		C–C twisting mode of Phe ring [7,10,29,54,56]
658.43	657.8	658.53	657.42	657.71	657.09	657.93	658.28	G, ring breathing mode [9,59–61] or amino acids COO–[62]
688.19				689.83	689.18	689.93	692.23	C–S stretch [26,57] or Gly [54]
739.19	727.8	737.96	733.83	737.14	734.21	738.08	737.93	A, glycosidic ring breathing [9,10,15,26,63,64]
807.14	804.25	804.8		805.45	807.23	801.95	804.72	O–P–O [1,26] or C–N stretch [20]
836.42		832.58		831.81		830.52	832.87	O–P–O stretching in T [10,59] or Tyr [29,59]
859.65	858		858	859.14	859.65	858.69		Phosphodiester, deoxyribose related to T [4,10] or Tyr [29]
882.32		879.93		882.2		880.81		T, ring bending [10], stretching of C–N or C–O–N or deformation of C–C–H [65]
922.65	919.72	922.49	920.2	922.4	922.32	922.51	922.53	C–COO– stretch in carbohydrates [26,66]

Table 4. *Cont.*

Peak Wavenumber, cm^{-1}								Tentative Band Assignments *
24	**27**	**30**	**33.1**	**34**	**35**	**37**	**49**	
958.79	959.67	957.92	958.24		958.22			C–N stretching [7,10,29,67] or C–C/C–O stretching in membrane proteins [10]
						966.22		C–N stretch [26] or C=C deformation in G [61]
	1005.6							Phe [7,10,29]
1011.9		1009.1	1009.5	1010	1009.6	1009.5	1009.4	Phe [68,69] or Trp [54,57]
1051	1050.3	1050.6	1050.9	1050.8	1051.1	1050.4	1050	Phenylalanine (the in-plane C–H bending mode) [69] or stretching of C–O/CH_2–OH in lipids [70]
1091.2	1094.7	1099.2		1089.9				PO_2^- of nucleic acid stretching [10,20,29] or deformation in carbohydrates(C–C, C–O, –COH) [7,65,67]
					1116.4	1119.2		Trp [65]
	1131.1	1129.6						C–N and C–C stretching in carbohydrates [61,69] or =C–C= in unsaturated fatty acids in lipids [16,29]
1159.3	1161.7	1159.8	1158.5	1158.5	1159.6	1158.3	1158.7	C–C/C–N stretching in proteins [10] or carotenoids [15,29]
1202	1201.9	1203	1200.8	1201.5	1201.6	1201.1	1201.7	=C–C= in lipids [69] or aromatic amino acids in proteins [71]
1247.2	1247.4	1247.6	1246.4	1254.8	1250.9	1252.5	1256.9	Amide III [10,26,29,62,65]
1301.4		1301.7		1303.1		1301.6	1300.4	CH_2 twist in lipids [10,55]
1328.4	1325.2	1327.7	1328.2	1328.5	1328	1327.4	1327.3	A [9,62,63,70,72]
1363.7		1363.3	1363.5	1363.8	1363.8	1364.7	1366.4	Trp [10] or C–H deformation in proteins/COO– deformation [5,73]
	1383	1387.1		1388.8	1386.3			COO– stretching in proteins [66,74] or CH_3 bending [29]
			1398.8					COO– symmetric stretching [1,75] or deformation of CH_3 [76]
1421.2								CH_2 deformation in lipids [50,66,68] or A, G [66,71]
1470.4	1467.8	1469.6	1470	1470	1470.2	1469.6	1468.8	Lipids [9,10] or deformation of C–H in proteins [65,69]
							1501.2	Fatty acids in lipids [5,73] or carotenoids [29] or amino acids [74]
			1511.1		1511.5			Carotenoids [29,77] or Phe [70]
1569.4		1570.5	1570.1	1570.9	1569.9	1569.5	1569.3	Tyr/proteins [69,70] or A/G [78]
	1592.4							Proteins [10] or A/G [1,7] or Tyr [72,79]
1634.5		1634.7	1636.8	1634.5	1635.5	1633.1	1633	Amide I in lipids [10,26,72]
	1647.9							Amide I [69] or T [29,68]
1679.2		1694	1681.1	1684.3	1676.6		1682.8	Amide I [10,62,71,75]
	1703.4							C=O [5,71]
	1750.2							C=O stretching [5,21]

* T: thymine, G: guanine, Trp: tryptophan, Phe: phenylalanine, Gly: glycine, A: adenine, Tyr: tyrosine.

Minor Raman shifts in various references seen in Table 4 are due to methodological variations [7,15,17] as well as indicative of molecular differences [50], facilitating successful differentiation.

As can be seen from Figure 4a,b, isolate 27 diverged greatly from other tested isolates. It exhibits several peaks, that weren't observed in other test subjects (peaks at 563, 1005, 1592, 1647, 1703 and 1750 cm^{-1}). While peaks at 563, 1005, 1592 and 1647 cm^{-1}, are likely

indicative of a shift, bands in the 1700 cm^{-1} range, linked with C=O deformation, are wholly unique to this isolate. Isolates 30 and 34 from *Pantoea agglomerans* group didn't show such differences, however, they diverged by the absence of peaks at 688, 858 and 958 cm^{-1}. Additionally, isolate 30 exhibited a peak at 1131 cm^{-1}, related to deformations of C–C, C–N in carbohydrates or =C–C= in lipids, alongside isolate 27, while this peak was absent from the spectra of isolate 34.

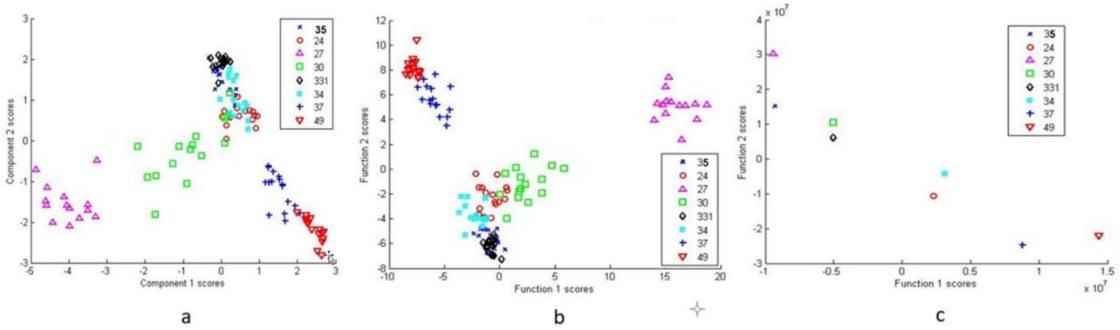

Figure 4. Principal component analysis (PCA) score plot for all 8 isolates (**a**), discriminant function analysis (DFA) for all eight isolates based on PCA scores (**b**) and DFA of all eight isolates using raw spectral data (**c**).

Isolates 33.1 and 35 from *Paenibacillus* sp. group exhibited similarities in their spectra and were grouped close in PCA and DFA score maps (Figures 4a and 6a). However, as can be seen by their raw data DFA (Figure 6b), there were differences. Isolate 33.1 has a notable peak at 1398 cm^{-1}, linked with COO– or CH_3 deformations, and lacks notable peaks at 688, 804 and 1383 cm^{-1}, while isolate 35 has a notable band at 1116 cm^{-1}, potentially related to Trp. The peak at 1398 cm^{-1} is likely related to peaks at ~1388 cm^{-1}, indicating a shift, rather than an absence. Moreover, the peak at 1116 cm^{-1} is unique for isolate 35, since isolate 33.1 doesn't exhibit a peak related to that area.

Isolate 24 exhibits a peak at 1421 cm^{-1}, linked with lipids or carbohydrates, that the other two isolates, 37 and 49, from the pseudomonads group lack, while missing a peak at 1383 cm^{-1}. Isolate 37 has peaks at 966 cm^{-1} and 1119 cm^{-1} and lacks a peak at 1679 cm^{-1} (all linked with proteins), that isolate 49 exhibits. The peak at 966 cm^{-1} can potentially be related to the peak at ~958 cm^{-1}, both, based on past studies, linked with C–N deformations. Furthermore, isolate 49 doesn't show notable peaks at 622, 858 or 882 cm^{-1}, while exhibiting a peak at 1501 cm^{-1}, which according to our findings, is linked with various organic compounds, carotenoids among them. Most of these peaks are unique to isolate 49, except for the peak near 1500 cm^{-1}, which may be a shift from the carotenoid band at ~1510 cm^{-1}.

3.2.2. Differentiation via Multivariate Cluster Analyses

Cluster analysis methods were used for bacterial differentiation. Figure 4a shows the PCA scatter plot of the eight bacterial isolates based on 14 principal components (PCs) accounting for 95.1% of the variance. Here, the scatter plot presents the ability of the SERS spectral analysis to differentiate among the different types of bacteria.

SERS data of the tested isolates were also classified using DFA. Eleven PC results were used as independent input variables in DFA (Figure 4b), which further reduced the spectral dimension, however, the groupings remained similar to those of the PCA. Furthermore, DFA from raw spectra data is presented in Figure 4c. Each isolate is sorted out as an individual, but close relationships within and between groups are noticeable. Two DF scores were calculated for each spectrum for the three bacterial cell types.

For in-depth within-group separation DFA was used further (Figures 5–7). PCA of group 27/30/34 shows a clear disassociation of isolate 27 (Figure 5a). DFA from PC scores of isolates 34, 30 and 27 was able to correctly classify 100% of each group's subjects (Figure 5b). DFA based on raw data with cross-validation between isolates 30 and 27 was able to correctly classify 100% of subjects in the groups and DFA based on raw data with cross-validation between isolate 30 and 34 was able to correctly classify 100% of subjects in the group 34 and 93.8% of subjects in the group 30 (Figure 5c). Thus, the results show that isolate 27 can be effectively differentiated from the other two isolates in the group.

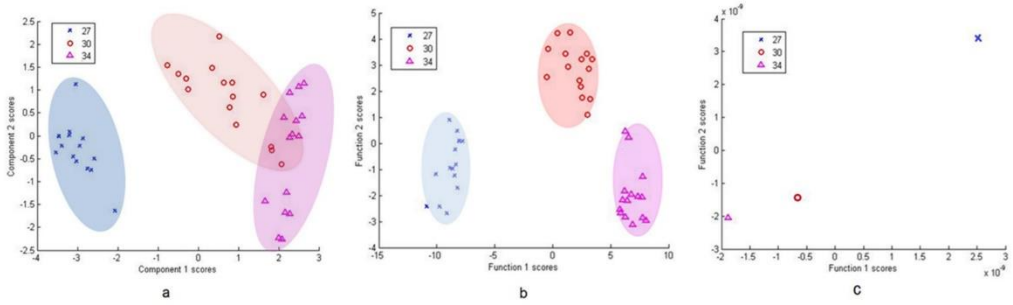

Figure 5. PCA of *Pantoea agglomerans* group (27, 30, 34) (**a**), DFA from PC scores (**b**) and DFA from raw data (**c**). Isolate 27 is individually grouped in all 3 analyses, while DFA facilitates clear separation between all the tested isolates.

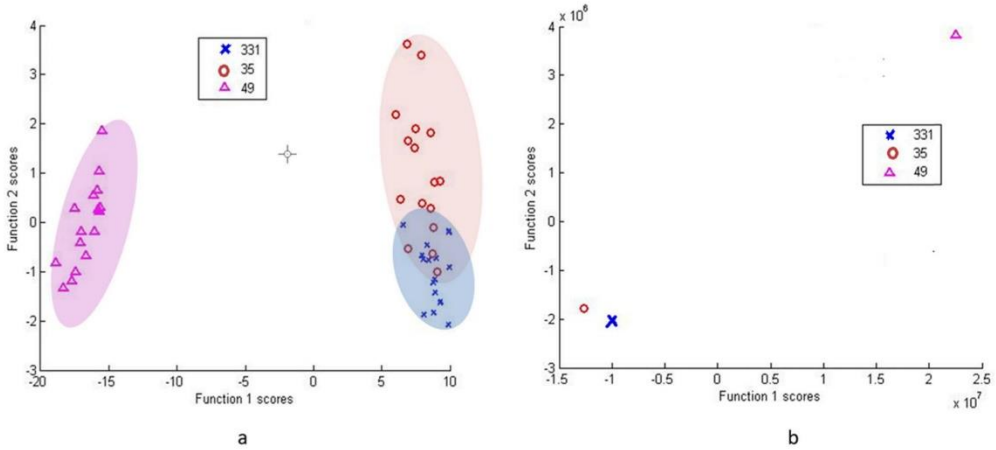

Figure 6. DFA of *Paenibacillus tundrae* group (33.1 and 35) with *Pseudomonas* isolate 49 as an outlier, as 33.1/35 are highly similar. While DFA from PCA scores shows differentiation with overlaps (**a**), DFA from raw data (**b**), while still demonstrating homology, is able to separate isolates 33.1 and 35.

DFA based on PC scores with cross-validation between isolates 33.1 and 35 was able to correctly classify 87.5% of the group 33.1 subjects and 79.1% of the group 35 subjects (Figure 6a). The DFA using raw data with cross-validation between 33.1 and 35 was able to correctly classify 100% of subjects in both groups (Figure 6b).

DFA based on PC scores and from raw data with cross-validation between isolate 37, 49 and 24 was able to correctly classify 100% of subjects in all the groups (Figure 7).

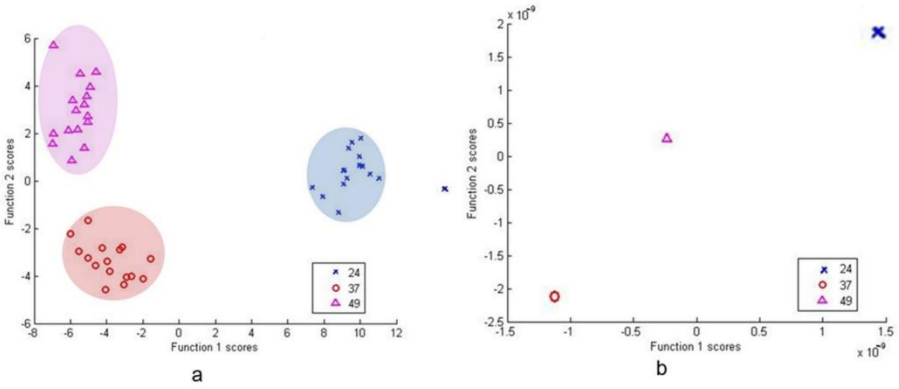

Figure 7. DFA based on PCA scores (**a**) and raw data (**b**) of *Pseudomonas* sp. group (37, 49 and 24).

4. Discussion

This study showcases that SERS coupled with multivariate cluster analyses can serve as an effective means to achieve bacterial differentiation in plant-associated species, as opposed to standard *16S rRNA* gene sequencing and additional antibiotic susceptibility, carbohydrate use, biofilm formation and phenotyping tests.

Additional tests performed during this experiment were able to account for genus level separation. Colony morphology and antibiotic susceptibility tests were more effective than biofilm formation and carbohydrate use studies. Antibiotic susceptibility is considered a strain-level response [80]. Bacterial strains also have been shown to be able to adapt to utilizing new carbohydrates through mutation [81]. *Pseudomonas* sp. are known biofilm producers [82] and indeed biofilm formation test was able to separate two pseudomonads capable of this. Biofilms are extracellular structures often containing polysaccharides as well as other compounds [3], thus it is possible that evidence of them may be noted in the SERS data, as was shown previously with several species [83–85]. However, it's worth noting that the methodology chosen in this study isn't ideal for yielding data on biofilms created by isolate 24.

While comparable studies showed that SERS works for pathogenic, medicine and food related, strains [1,8,9,20], based on available information, the usefulness of this technique was not widely studied for plant-associated bacteria [29], or even more specifically for endophytes.

The complexity of the SERS spectrum makes interpretation of the data challenging. Statistical procedures or chemometric multivariate analyses are designed to improve the use and interpretation of experimental data [22]. Chemometrics is defined as a mathematical method used to extract useful information from measured physicochemical data [86].

In this study PCA and DFA, multivariate data analysis techniques, were applied to the SERS spectral data. PCA is one of the multidimensional descriptive methods in chemometrics, particularly fitting for the study of spectral data. This technique provides a synthetic image by presenting factor maps (2D or 3D), in which each spectrum is represented by a dot. The primary variables are replaced with synthetic ones (principal components), which contain all the information [15,87], thus interpreting PCA maps makes it relatively easier to understand the structure of the spectral data [88].

DFA allows for the rapid sorting/grouping of unknown spectra based on between-group variability while minimizing within-group variability [6,52]. It also facilitates immediate validation of spectral reproducibility, as very similar spectra should have very similar discriminant function scores and should consequently be closely grouped in DFA. All in all, DFA and PCA are similar in that they both reduce the dimension of the data, but DFA provides better separation between groups of experimental data in comparison to PCA.

Additionally, while DFA may need a certain level of a priori knowledge about the spectra, PCA is used to examine raw data [87].

PCA has been successfully used for SERS spectra analysis in other bacterial differentiation studies [1,29,69,89]. DFA has been used as well, however, less often [52,63,77].

It is noteworthy, that the label-free SERS spectra acquisition protocol presented in this study is an easily replicated approach for procuring bacterial spectra, as bacteria are in an aqueous solution, requiring minimal preparation. Most often comparable procedures either use colloidal solutions or dry out the sample, hence facing difficulties with thermal damage [9,15], which may affect spectra acquisition, as often carbon associated peaks arise in the biologically relevant range [9].

Based on current knowledge, the methodology used in this study likely showcases the metabolic degradation of the tested bacteria, as they are in a no-nutrient environment (salt solution). Nevertheless, as this is linked with specific enzymes each strain may produce and unique metabolic pathways, it too ultimately relates to biochemical differences and thus strain-specific differentiation [50,53]. Another recent study demonstrates that SERS peaks may also derive from the constituents of bacterial outer membrane (Gram-) [13].

Moreover, nucleotides are rarely seen in extracellular regions, but notable bands for them have been found in various studies [9,10,15,26,29]. Bands in the same regions have been observed in this study as well. For example, an intense peak at ~730 cm^{-1} is commonly assigned to adenine-type compounds [90]. Furthermore, adenine molecules are part of adenosine triphosphate (ATP), which bacteria use for energy, hence it is possible that their degradation metabolites would end up outside of the bacterial cells, as have been shown with *E. coli* placed in starvation mode [91] and other studies [50,92].

In this study, bacteria from three different genera were examined. Although certain similarities can be observed in all the tested subjects, i.e., the aforementioned adenosine band at ~730 cm^{-1}, unique vibrational signatures for all of them were successfully obtained. Similar results have been presented previously, whereby *E. coli*, *Listeria monocytogenes* and *B. subtilis* strains were shown to have different SERS spectra [11,67,89,93]. Furthermore, Premasiri et al. state that mutations and even genealogy may be observed in their SERS data [11].

While the exact nature of the bacterial Raman/SERS bands is difficult to assign, without mutant bacteria studies, due to peak overlap and minor shifts, bacterial differentiation is still possible [7,15].

Bacterial strains genetically homologous with *Pantoea agglomerans* have been investigated in previous Raman studies [94]. *P. agglomerans* vibrational fingerprint reported by Guicheteau et al. resembles those obtained in this study. There were differences though, for example, the peak at 536 cm^{-1}, that the authors attributed to cysteine, asparagine or glutamine, was found shifted in the spectra of isolate 27, and absent from the other two tested isolates. Moreover, isolate 34 didn't have a notable peak at 958 cm^{-1}. Several peaks demonstrated shifts (ex. at ~1004, 1142, 1544 cm^{-1}), while others are absent in the spectra of the strain tested in the cited work [94]. Furthermore, other species from the *Pantoea* genus have been studied as well [30,77]. A study on IAA-producing *Pantoea* sp. has shown that some notable bands are produced by carotenoids, notably, bands at 1002, 1158 and 1520 cm^{-1}, of which analogs were found in our research as well (~1005, 1159, 1570 cm^{-1}). Additionally, authors in this study discuss the possibility that the IAA production capacity of this strain may have also been observable, through Trp peaks, as IAA and Trp have similar chemical structures (in fact Trp is a precursor to IAA) [95]. Several Trp associated peaks have been noted in our research as well. This too may potentially be linked to IAA production [35].

The most widely researched *Pseudomonas* species are *P. aeruginosa* and *P. fluorescens*. To our knowledge, pseudomonads homologous to those analyzed in this work, have not yet been characterized using Raman techniques. However, data on *P. aeruginosa*, have shown some similarities to the pseudomonad spectra examined in this work. Spectra reported by Yang et al. are similar, as they share some peaks with very minor shifts (ex. 655, 730, 920,

1328, 1470 cm^{-1}). A peak near 854 cm^{-1} (related to G) was not observed in isolate 49, while peaks at 957 and 1091 cm^{-1}, linked with hypoxanthine, A, G and guanosine, were only noted in isolate 24. Furthermore, authors report peaks at 518, 1219 and 1528 cm^{-1}, which were not obtained in this study [63]. Another study on *P. aeruginosa* also demonstrates peaks, that are comparable to those found in this study [70]. However, most of them are expressed differently in different isolates, peaks at ~690 and 832 cm^{-1} (related to G, C and C-O-C stretching according to the authors) are observed only in the data of isolates 37 and 49, while peaks near 958 and 1421 cm^{-1} (linked with phospholipids or C-O deformations) have only been noted in isolate 24. A study done on *P. fluorescens* resulted in the SERS peak at 1495 cm^{-1} [78]. During our research, this peak was found to be one of the discerning factors of isolate 49, homologous with *P. azotoformans*. This peak has been linked with lipids, proteins and carotenoids (see Table 4).

Plant-associated *Paenibacillus validus* has exhibited many of the same peaks, as those reported in our study for isolates 35 and 33.1 (958, 1009, 1158, 1363, 1470, 1511, 1570, 1680 cm^{-1}) [29]. Nonetheless, the article didn't enlighten on the origins of peaks that discerned the *Paenibacillus* sp. isolates in our experiment (1398 and 1116 cm^{-1}).

5. Conclusions

A rapid SERS strategy for bacteria differentiation was successfully established by using low-cost AgNP/Si substrates, where other methods (biochemical and genetic testing) have failed. It was found that AgNP 3D film on Si surface was interacting with the bacteria, resulting in strong and reproducible SERS spectra. Surface enhanced Raman spectroscopy coupled with advanced statistical techniques (PCA and DFA) were used to discriminate between different plant bacterial strains of *Paenibacillus*, *Pseudomonas* and *Pantoea* genera by probing the molecular components of their cells. This is the first time, SERS peaks characteristic to bacteria closely related to *Pseudomonas azotoformans* and *P. brenneri/proteolytica* have been obtained. Moreover, so far as we were able to determine, this is one of the first studies on the SERS spectra characteristics of *Paenibacillus* sp. This work progresses the current knowledge of bio-spectroscopy and may help with the introduction of SERS-based bacterial identification technique as a standard method of analysis in plant-associated bacteriology.

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References

1. Witkowska, E.; Korsak, D.; Kowalska, A.; Księżopolska-Gocalska, M.; Niedziółka-Jönsson, J.; Roźniecka, E.; Michałowicz, W.; Albrycht, P.; Podrażka, M.; Hołyst, R.; et al. Surface-enhanced Raman spectroscopy introduced into the International Standard Organization (ISO) regulations as an alternative method for detection and identification of pathogens in the food industry. *Anal. Bioanal Chem.* **2017**, *409*, 1555–1567. [CrossRef] [PubMed]
2. Ahmad, W.; Wang, J.; Li, H.; Jiao, T.; Chen, Q. Trends in the bacterial recognition patterns used in surface enhanced Raman spectroscopy. *TrAC-Trends Anal. Chem.* **2021**, *142*, 116310. [CrossRef]
3. Chisanga, M.; Muhamadali, H.; Ellis, D.I.; Goodacre, R. Surface-Enhanced Raman Scattering (SERS) in Microbiology: Illumination and Enhancement of the Microbial World. *Appl. Spectrosc.* **2018**, *72*, 987–1000. [CrossRef] [PubMed]
4. Efrima, S.; Zeiri, L. Understanding SERS of bacteria. *J. Raman Spectrosc.* **2009**, *40*, 277–288. [CrossRef]

5. Colniţă, A.; Dina, N.E.; Leopold, N.; Vodnar, D.C.; Bogdan, D.; Porav, S.A.; David, L. Characterization and discrimination of gram-positive bacteria using raman spectroscopy with the aid of principal component analysis. *Nanomaterials* **2017**, *7*, 28–31. [CrossRef] [PubMed]
6. Jarvis, R.; Clarke, S.; Goodacre, R. Rapid analysis of microbiological systems using SERS. *Top. Appl. Phys.* **2006**, *103*, 397–408. [CrossRef]
7. Kairyte, K.; Luksiene, Z.; Pucetaite, M.; Sablinskas, V. Differentiation of bacterial strains by means of surface enhanced ft-raman spectroscopy. *Lith. J. Phys.* **2012**, *52*, 276–283. [CrossRef]
8. Kotanen, C.N.; Martinez, L.; Alvarez, R.; Simecek, J.W. Surface enhanced Raman scattering spectroscopy for detection and identification of microbial pathogens isolated from human serum. *Sens. Bio-Sens. Res.* **2016**, *8*, 20–26. [CrossRef]
9. Dina, N.E.; Zhou, H.; Colniţă, A.; Leopold, N.; Szoke-Nagy, T.; Coman, C.; Haisch, C. Rapid single-cell detection and identification of pathogens by using surface-enhanced Raman spectroscopy. *Analyst* **2017**, *142*, 1782–1789. [CrossRef]
10. Lemma, T.; Saliniemi, A.; Hynninen, V.; Hytönen, V.P.; Toppari, J.J. SERS detection of cell surface and intracellular components of microorganisms using nano-aggregated Ag substrate. *Vib. Spectrosc.* **2016**, *83*, 36–45. [CrossRef]
11. Premasiri, R.; Moir, D.; Jones, G.; Ziegler, L.D. Characterization of the Surface Enhanced Raman Scattering of Bacteria on a Novel Sers Substrate. *J. Phys. Chem. B* **2005**, *109*, 312–320. [CrossRef]
12. De Plano, L.M.; Fazio, E.; Rizzo, M.G.; Franco, D.; Carnazza, S.; Trusso, S.; Neri, F.; Guglielmino, S.P.P. Phage-based assay for rapid detection of bacterial pathogens in blood by Raman spectroscopy. *J. Immunol. Methods.* **2018**, *465*, 45–52. [CrossRef]
13. Yan, Y.; Nie, Y.; An, L.; Tang, Y.Q.; Xu, Z.; Wu, X.L. Improvement of Surface-Enhanced Raman Scattering Method for Single Bacterial Cell Analysis. *Front. Bioeng. Biotechnol.* **2020**, *8*, 1–13. [CrossRef]
14. Zhou, X.; Hu, Z.; Yang, D.; Xie, S.; Jiang, Z.; Niessner, R.; Haisch, C.; Zhou, H.; Sun, P. Bacteria Detection: From Powerful SERS to Its Advanced Compatible Techniques. *Adv. Sci.* **2020**, *7*, 1–23. [CrossRef]
15. Mosier-Boss, P.A. Review on SERS of bacteria. *Biosensors* **2017**, *7*, 51. [CrossRef]
16. Jarvis, R.M.; Brooker, A.; Goodacre, R. Surface-enhanced Raman scattering for the rapid discrimination of bacteria. *Faraday Discuss.* **2005**, *132*, 281–292. [CrossRef]
17. Smith-Palmer, T.; Douglas, C.; Fredericks, P. Rationalizing the SER spectra of bacteria. *Vib. Spectrosc.* **2010**, *53*, 103–106. [CrossRef]
18. Almasoud, N.; Muhamadali, H.; Chisanga, M.; Alrabiah, H.; Lima, C.A.; Goodacre, R. Discrimination of bacteria using whole organism fingerprinting: The utility of modern physicochemical techniques for bacterial typing. *Analyst* **2021**, *146*, 770–788. [CrossRef] [PubMed]
19. Tamer, U.; Torul, H.; Doğan, Ü.; Eryılmaz, M.; Gümüştaş, A.; Boyacı, İ.H.; Özkan, S.A.; Uslu, B. SERS Sensor Applications in Environmental Analysis and Biotechnology. In *Nanotechnology Applications in Health and Environmental Sciences*; Springer: Cham, Switzerland, 2021; pp. 197–236. [CrossRef]
20. Liu, Y.; Chen, Y.R.; Nou, X.; Chao, K. Potential of surface-enhanced Raman spectroscopy for the rapid identification of Escherichia Coli and Listeria Monocytogenes cultures on silver colloidal nanoparticles. *Appl. Spectrosc.* **2007**, *61*, 824–831. [CrossRef]
21. Harz, M.; Rösch, P.; Popp, J. Vibrational spectroscopy-A powerful tool for the rapid identification of microbial cells at the single-cell level. *Cytom. Part A* **2009**, *75*, 104–113. [CrossRef] [PubMed]
22. Stöckel, S.; Kirchhoff, J.; Neugebauer, U.; Rösch, P.; Popp, J. The application of Raman spectroscopy for the detection and identification of microorganisms. *J. Raman Spectrosc.* **2016**, *47*, 89–109. [CrossRef]
23. Ashton, L.; Lau, K.; Winder, C.L.; Goodacre, R. Raman spectroscopy: Lighting up the future of microbial identification. *Future Microbiol.* **2011**, *6*, 991–997. [CrossRef] [PubMed]
24. Patel, I.S.; Premasiri, W.R.; Moir, D.T.; Ziegler, L.D. Barcoding bacterial cells: A SERS-based methodology for pathogen identification. *J. Raman Spectrosc.* **2008**, *39*, 1660–1672. [CrossRef]
25. Snitka, V.; Batiuskaite, D.; Bruzaite, I.; Lafont, U.; Butenko, Y.; Semprimoschnig, C. Surface-enhanced Raman scattering sensors for biomedical and molecular detection applications in space. *CEAS Sp. J.* **2021**, *13*, 509–520. [CrossRef]
26. Çulha, M.; Adigüzel, A.; Yazici, M.M.; Kahraman, M.; Şahin, F.; Güllüce, M. Characterization of thermophilic bacteria using surface-enhanced Raman scattering. *Appl. Spectrosc.* **2008**, *62*, 1226–1232. [CrossRef] [PubMed]
27. Franco, D.; De Plano, L.M.; Rizzo, M.G.; Scibilia, S.; Lentini, G.; Fazio, E.; Neri, F.; Guglielmino, S.P.P.; Mezzasalma, A.M. Bio-hybrid gold nanoparticles as SERS probe for rapid bacteria cell identification. *Spectrochim. Acta-Part A Mol. Biomol. Spectrosc.* **2020**, *224*, 117394. [CrossRef]
28. Ho, C.S.; Jean, N.; Hogan, C.A.; Blackmon, L.; Jeffrey, S.S.; Holodniy, M.; Banaei, N.; Saleh, A.A.E.; Ermon, S.; Dionne, J. Rapid identification of pathogenic bacteria using Raman spectroscopy and deep learning. *Nat. Commun.* **2019**, *10*, 4927. [CrossRef]
29. Paret, M.L.; Sharma, S.K.; Green, L.M.; Alvarez, A.M. Biochemical characterization of Gram-positive and Gram-negative plant-associated bacteria with micro-raman spectroscopy. *Appl. Spectrosc.* **2010**, *64*, 433–441. [CrossRef]
30. Polisetti, S.; Bible, A.N.; Morrell-Falvey, J.L.; Bohn, P.W. Raman chemical imaging of the rhizosphere bacterium Pantoea sp. YR343 and its co-culture with Arabidopsis thaliana. *Analyst* **2016**, *141*, 2175–2182. [CrossRef]
31. Kour, D.; Rana, K.L.; Yadav, A.N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H.S.; Saxena, A.K. Microbial biofertilizers: Bioresources and eco-friendly technologies for agricultural and environmental sustainability. *Biocatal. Agric. Biotechnol.* **2020**, *23*, 101487. [CrossRef]
32. Pirttilä, A.M.; Mohammad Parast Tabas, H.; Baruah, N.; Koskimäki, J.J. Biofertilizers and Biocontrol Agents for Agriculture: How to Identify and Develop New Potent Microbial Strains and Traits. *Microorganisms* **2021**, *9*, 817. [CrossRef] [PubMed]

33. Fan, D.; Subramanian, S.; Smith, D.L. Plant endophytes promote growth and alleviate salt stress in Arabidopsis thaliana. *Sci. Rep.* **2020**, *10*, 12740. [CrossRef] [PubMed]
34. Takikawa, Y. Studies on identification and taxonomy of plant pathogenic bacteria. *J. Gen. Plant. Pathol.* **2012**, *78*, 409–412. [CrossRef]
35. Vaitiekūnaitė, D.; Kuusienė, S.; Beniušytė, E. Oak (Quercus robur) Associated Endophytic Paenibacillus sp. Promotes Poplar (Populus spp.) Root Growth In Vitro. *Microorganisms* **2021**, *9*, 1151. [CrossRef]
36. Oukala, N.; Aissat, K.; Pastor, V. Bacterial endophytes: The hidden actor in plant immune responses against biotic stress. *Plants* **2021**, *10*, 1012. [CrossRef]
37. Hardoim, P.R.; van Overbeek, L.S.; Berg, G.; Pirttilä, A.M.; Compant, S.; Campisano, S.; Döring, M.; Sessitsch, A. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiol. Mol. Biol Rev.* **2015**, *79*, 293–320. [CrossRef]
38. Bueno-Gonzalez, V.; Brady, C.; Denman, S.; Allainguillaume, J.; Arnold, D. Pseudomonas kirkiae sp. Nov., a novel species isolated from oak in the united kingdom, and phylogenetic considerations of the genera pseudomonas, azotobacter and azomonas. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 2426–2434. [CrossRef]
39. Govindasamy, V.; Raina, S.K.; George, P.; Kumar, M.; Rane, J.; Minhas, P.S.; Vittal, K.P.R. Functional and phylogenetic diversity of cultivable rhizobacterial endophytes of sorghum [*Sorghum bicolor* (L.) Moench]. *Antonie Leeuwenhoek* **2017**, *110*, 925–943. [CrossRef]
40. Compant, S.; Mitter, B.; Colli-Mull, J.G.; Gangl, H.; Sessitsch, A. Endophytes of Grapevine Flowers, Berries, and Seeds: Identification of Cultivable Bacteria, Comparison with Other Plant Parts, and Visualization of Niches of Colonization. *Microb. Ecol.* **2011**, *62*, 188–197. [CrossRef]
41. Premasiri, W.R.; Moir, D.T.; Ziegler, L.D. Vibrational fingerprinting of bacterial pathogens by surface enhanced Raman scattering (SERS). *Chem. Biol. Sens. VI* **2005**, *5795*, 19. [CrossRef]
42. Tamošiūnė, I.; Stanienė, G.; Haimi, P.; Stanys, V.; Rugienius, R.; Baniulis, D. Endophytic Bacillus and Pseudomonas spp. Modulate Apple Shoot Growth, Cellular Redox Balance, and Protein Expression Under in Vitro Conditions. *Front. Plant. Sci.* **2018**, *9*, 889. [CrossRef] [PubMed]
43. Mohamed, A.; Mhand Rajaa, A.; Khalid, Z.; Fouad, M.; Naima, R. Comparison of Three Methods for the Detection of Biofilm Formation by Clinical Isolates of Staphylococcus aureus Isolated in Casablanca. *Int. J. Sci. Res.* **2013**, *5*, 2319–7064. [CrossRef]
44. Johnson, T.R.; Case, C.L. *Laboratory Experiments in Microbiology*, 11th ed.; Pearson: London, UK, 2015.
45. Bauer, A.W.; Kirby, W.M.; Sherris, J.C.; Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **1966**, *45*, 493–496. [CrossRef]
46. Sarker, M.M.R.; Islam, K.N.; Huri, H.Z.; Rahman, M.; Imam, H.; Hosen, M.B.; Mohammad, N.; Sarker, M.Z.I. Studies of the impact of occupational exposure of pharmaceutical workers on the development of antimicrobial drug resistance. *J. Occup. Health* **2014**, *56*, 260–270. [CrossRef]
47. Abdelaziz, E.; Ramadan, H.; Rasha, B.; Shadi, S.; Wael, E.-N. Antimicrobial susceptibility and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) typing of Gram negative bacteria isolated from urinary tract infections in Mansoura, Egypt. *J. Microbiol. Antimicrob.* **2014**, *6*, 43–50. [CrossRef]
48. Fouad, Z. Antimicrobial Disk Diffusion Zone Interpretation Guide. 2011. [CrossRef]
49. Ramanauskaite, L.; Snitka, V. Surface enhanced Raman spectroscopy of l-alanyl-l-tryptophan dipeptide adsorbed on Si substrate decorated with triangular silver nanoplates. *Chem. Phys. Lett.* **2015**, *623*, 46–50. [CrossRef]
50. Premasiri, W.R.; Lee, J.C.; Sauer-Budge, A.; Théberge, R.; Costello, C.E.; Ziegler, L.D. The biochemical origins of the surface-enhanced Raman spectra of bacteria: A metabolomics profiling by SERS. *Anal. Bioanal. Chem.* **2016**, *408*, 4631–4647. [CrossRef]
51. Available online: https://www.effemm2.de/spectragryph/ (accessed on 9 August 2021).
52. Reisner, L.A.; Cao, A.; Pandya, A.K. An integrated software system for processing, analyzing, and classifying Raman spectra. *Chemom. Intell. Lab. Syst.* **2011**, *105*, 83–90. [CrossRef]
53. Kurochkin, I.N.; Eremenko, A.V.; Evtushenko, E.G.; Nechaeva, N.L.; Durmanov, N.N.; Guliev, R.R.; Lagarkov, A.N. SERS for Bacteria, Viruses, and Protein Biosensing. In *Macro, Micro, and Nano-Biosensors*; Rai, M., Reshetilov, A., Plekhanova, Y., Ingle, A.P., Eds.; Springer: Cham, Switzerland, 2021. [CrossRef]
54. De Gelder, J.; De Gussem, K.; Vandenabeele, P.; Moens, L. Reference database of Raman spectra of biological molecules. *J. Raman Spectrosc.* **2007**, *38*, 1133–1147. [CrossRef]
55. Czamara, K.; Majzner, K.; Pacia, M.Z.; Kochan, K.; Kaczor, A.; Baranska, M. Raman spectroscopy of lipids: A review. *J. Raman Spectrosc.* **2015**, *46*, 4–20. [CrossRef]
56. Movasaghi, Z.; Rehman, S.; Rehman, I.U. Raman spectroscopy of biological tissues. *Appl. Spectrosc. Rev.* **2007**, *42*, 493–541. [CrossRef]
57. Podstawka, E.; Ozaki, Y.; Proniewicz, L.M. Adsorption of S-S containing proteins on a colloidal silver surface studied by surface-enhanced Raman spectroscopy. *Appl. Spectrosc.* **2004**, *58*, 1147–1156. [CrossRef] [PubMed]
58. Tian, H.; Zhuang, G.; Ma, A.; Jing, C. Arsenic interception by cell wall of bacteria observed with surface-enhanced Raman scattering. *J. Microbiol. Methods.* **2012**, *89*, 153–158. [CrossRef] [PubMed]
59. Su, L.; Zhang, P.; Zheng, D.; Wang, Y.; Zhong, R. Rapid detection of Escherichia coli and Salmonella typhimurium by surface-enhanced Raman scattering. *Optoelectron. Lett.* **2015**, *11*, 157–160. [CrossRef]

60. Otto, C.; van den Tweel, T.J.J.; de Mul, F.F.M.; Greve, J. Surface-enhanced Raman spectroscopy of DNA bases. *J. Raman Spectrosc.* **1986**, *17*, 289–298. [CrossRef]
61. Kögler, M.; Paul, A.; Anane, E.; Birkholz, M.; Bunker, A.; Viitala, T.; Maiwald, M.; Junne, S.; Neubauer, P. Comparison of time-gated surface-enhanced raman spectroscopy (TG-SERS) and classical SERS based monitoring of Escherichia coli cultivation samples. *Biotechnol. Prog.* **2018**, *34*, 1533–1542. [CrossRef] [PubMed]
62. Li, J.; Wang, C.; Kang, H.; Shao, L.; Hu, L.; Xiao, R.; Wang, S.; Gu, B. Label-free identification carbapenem-resistant Escherichia coli based on surface-enhanced resonance Raman scattering. *RSC Adv.* **2018**, *8*, 4761–4765. [CrossRef]
63. Yang, D.; Zhou, H.; Dina, N.E.; Haisch, C. Portable bacteria-capturing chip for direct surfaceenhanced Raman scattering identification of urinary tract infection pathogens. *R. Soc. Open Sci.* **2018**, *5*, 180955. [CrossRef]
64. Meng, X.; Wang, H.; Chen, N.; Ding, P.; Shi, H.; Zhai, X.; Su, Y.; He, Y. A Graphene-Silver Nanoparticle-Silicon Sandwich SERS Chip for Quantitative Detection of Molecules and Capture, Discrimination, and Inactivation of Bacteria. *Anal. Chem.* **2018**, *90*, 5646–5653. [CrossRef] [PubMed]
65. Xie, Y.; Xu, L.; Wang, Y.; Shao, J.; Wang, L.; Wang, H.; Qian, H.; Yao, W. Label-free detection of the foodborne pathogens of Enterobacteriaceae by surface-enhanced Raman spectroscopy. *Anal. Methods* **2013**, *5*, 946–952. [CrossRef]
66. Wei, C.; Li, M.; Zhao, X. Surface-enhanced raman scattering (SERS) with silver nano substrates synthesized by microwave for rapid detection of foodborne pathogens. *Front. Microbiol.* **2018**, *9*, 1–9. [CrossRef] [PubMed]
67. Walter, A.; März, A.; Schumacher, W.; Rösch, P.; Popp, J. Towards a fast, high specific and reliable discrimination of bacteria on strain level by means of SERS in a microfluidic device. *Lab. Chip.* **2011**, *11*, 1013–1021. [CrossRef]
68. Rastogi, S.K.; Jabal JM, F.; Zhang, H.; Gibson, C.M.; Haler, K.J.; Qiang, Y.; Branen, A.L. Antibody@silica coated iron oxide nanoparticles: Synthesis, capture of E. coli and SERS titration of biomolecules with antibacterial silver colloid. *J. Nanomed. Nanotechnol.* **2011**, *2*, 1000121. [CrossRef]
69. Cozar, I.B.; Colniţă, A.; Szöke-Nagy, T.; Gherman, A.M.R.; Dina, N.E. Label-Free Detection of Bacteria Using Surface-Enhanced Raman Scattering and Principal Component Analysis. *Anal. Lett.* **2019**, *52*, 177–189. [CrossRef]
70. Mungroo, N.A.; Oliveira, G.; Neethirajan, S. SERS based point-of-care detection of food-borne pathogens. *Microchim. Acta* **2016**, *183*, 697–707. [CrossRef]
71. Ahmed, M.; Almagedi, S.; Yao, W. Sers Signatures of Foodborne Pathogenic Zoonotic Bacteria Using Gold Colloid. *Int. J. Eng. Sci. Technol.* **2013**, *5*, 810–821.
72. Fan, C.; Hu, Z.; Mustapha, A.; Lin, M. Rapid detection of food- and waterborne bacteria using surface-enhanced Raman spectroscopy coupled with silver nanosubstrates. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 1053–1061. [CrossRef]
73. Jin, H.; Wang, J.; Jin, S.; Jiang, L.; Zou, Y. Raman spectroscopy of potential bio-hazards commonly found in bio-aerosols. *Spectrochim. Acta-Part A Mol. Biomol. Spectrosc.* **2020**, *243*, 118753. [CrossRef]
74. Alula, M.T.; Krishnan, S.; Hendricks, N.R.; Karamchand, L.; Blackburn, J.M. Identification and quantitation of pathogenic bacteria via in-situ formation of silver nanoparticles on cell walls, and their detection via SERS. *Microchim. Acta* **2017**, *184*, 219–227. [CrossRef]
75. Huang, H.; Liu, M.; Wang, X.; Zhang, W.; Yang, D.-P.; Cui, L.; Wang, X. Label-Free 3D Ag Nanoflower-Based Electrochemical Immunosensor for the Detection of Escherichia coli O157:H7 Pathogens. *Nanoscale Res. Lett.* **2016**, *11*, 507. [CrossRef] [PubMed]
76. Uusitalo, S.; Kögler, M.; Välimaa, A.L.; Popov, A.; Ryabchikov, Y.; Kontturi, Y.; Siitonen, S.; Petäjä, J.; Virtanen, T.; Laitinen, R.; et al. Detection of: Listeria innocua on roll-to-roll produced SERS substrates with gold nanoparticles. *RSC Adv.* **2016**, *6*, 62981–62989. [CrossRef]
77. Pérez, M.; Contreras, H.; Sosa Herrera, J.A.; Ávila, J.; Tobías, H.; Martínez, F.D.; Ramírez, R.F.; Vázquez, Á. Detection of clavibacter michiganensis subsp. Michiganensis assisted by micro-raman spectroscopy under laboratory conditions. *Plant. Pathol. J.* **2018**, *34*, 381–392. [CrossRef] [PubMed]
78. Liu, S.; Li, H.; Hassan, M.M.; Zhu, J.; Wang, A.; Ouyang, Q.; Zareef, M.; Chen, Q. Amplification of Raman spectra by gold nanorods combined with chemometrics for rapid classification of four Pseudomonas. *Int. J. Food Microbiol.* **2019**, *304*, 58–67. [CrossRef] [PubMed]
79. Kashif, M.; Irfan Majeed, M.; Nawaz, H.; Rashid, N.; Abubakar, M.; Ahmad, S.; Ali, S.; Hyat, H.; Bashir, S.; Batool, F.; et al. Surface-enhanced Raman spectroscopy for identification of food processing bacteria. *Spectrochim. Acta. Part A Mol. Biomol. Spectrosc.* **2021**, *261*, 119989. [CrossRef]
80. Lamrabet, O.; Martin, M.; Lenski, R.E.; Schneider, D. Changes in intrinsic antibiotic susceptibility during a long-term evolution experiment with escherichia coli. *MBio* **2019**, *10*, 1–12. [CrossRef]
81. Blount, Z.D.; Borland, C.Z.; Lenski, R.E. Historical contingency and the evolution of a key innovation in an experimental population of Escherichia coli. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7899–7906. [CrossRef]
82. Ramey, B.E.; Koutsoudis, M.; Bodman, S.B.V.; Fuqua, C. Biofilm formation in plant-microbe associations. *Curr. Opin. Microbiol.* **2004**, *7*, 602–609. [CrossRef]
83. Bodelón, G.; Montes-garcía, V.; López-puente, V.; Hill, E.H.; Hamon, C.; Sanz-Ortiz, M.N.; Rodal-Cedeira, S.; Costas, C.; Celiksoy, S.; Pérez-Juste, I.; et al. Detection and imaging of quorum sensing in Pseudomonas aeruginosa biofilm communities by surface-enhanced resonance Raman scattering. *Nat. Mater.* **2017**, *15*, 1203–1211. [CrossRef]
84. Schkolnik, G.; Schmidt, M.; Mazza, M.G.; Harnisch, F.; Musat, N. In situ analysis of a silver nanoparticle-precipitating Shewanella biofilm by surface enhanced confocal raman microscopy. *PLoS ONE* **2015**, *10*, 7–12. [CrossRef]

85. Baig, N.; Polisetti, S.; Morales-Soto, N.; Dunham, S.J.B.; Sweedler, J.V.; Shrout, J.D.; Bohn, P.W. Label-free molecular imaging of bacterial communities of the opportunistic pathogen Pseudomonas aeruginosa. *Biosensing Nanomed. IX* **2016**, *9930*, 993004. [CrossRef]
86. Szymańska, E.; Gerretzen, J.; Engel, J.; Geurts, B.; Blanchet, L.; Buydens, L.M.C. Chemometrics and qualitative analysis have a vibrant relationship. *TrAC-Trends Anal. Chem.* **2015**, *69*, 34–51. [CrossRef]
87. Jolliffe, I.T. Principal Component Analysis. In *Encyclopedia of Statistics in Behavioral Science*, 2nd ed.; Everitt, B.S., Howell, D.C., Eds.; Springer: NewYork, NY, USA, 2002; Volume 30, p. 487. [CrossRef]
88. Guo, S.; Rösch, P.; Popp, J.; Bocklitz, T. Modified PCA and PLS: Towards a better classification in Raman spectroscopy–based biological applications. *J. Chemom.* **2020**, *34*, 1–10. [CrossRef]
89. Witkowska, E.; Korsak, D.; Kowalska, A.; Janeczek, A.; Kamińska, A. Strain-level typing and identification of bacteria–a novel approach for SERS active plasmonic nanostructures. *Anal. Bioanal. Chem.* **2018**, *410*, 5019–5031. [CrossRef] [PubMed]
90. Kubryk, P.; Niessner, R.; Ivleva, N.P. The origin of the band at around 730 cm-1 in the SERS spectra of bacteria: A stable isotope approach. *Analyst* **2016**, *141*, 2874–2878. [CrossRef]
91. Brauer, M.J.; Yuan, J.; Bennett, B.D.; Lu, W.; Kimball, E.; Botstein, D.; Rabinowitz, J.D. Conservation of the metabolomic response to starvation across two divergent microbes. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 19302–19307. [CrossRef]
92. Link, H.; Fuhrer, T.; Gerosa, L.; Zamboni, N.; Sauer, U. Real-time metabolome profiling of the metabolic switch between starvation and growth. *Nat. Methods* **2015**, *12*, 1091–1097. [CrossRef]
93. Sharma, S.K.; Dykes, A.C.; Misra, A.K.; Kamemoto, L.E.; Bates, D.E. Raman discrimination of bacterial strains using multilayered microcavity substrates. *Smart Biomed. Physiol. Sens. Technol. VIII* **2011**, *8025*, 802508. [CrossRef]
94. Guicheteau, J.; Argue, L.; Hyre, A.; Jacobson, M.; Christesen, S.D. Raman and surface-enhanced Raman spectroscopy of amino acids and nucleotide bases for target bacterial vibrational mode identification. *Chem. Biol. Sens. VII* **2006**, *6218*, 62180O. [CrossRef]
95. Polisetti, S.; Baig, N.; Bible, A.; Morrell-Falvey, J.; Doktycz, M.; Bohn, P.W. Using Raman spectroscopy and SERS for in situ studies of rhizosphere bacteria. *Biosensing Nanomed. VIII* **2015**, *9550*, 95500D. [CrossRef]

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Populus tremula × *P. alba* Microshoot Secondary Metabolism Response after *Paenibacillus* sp. Inoculation In Vitro

Dorotėja Vaitiekūnaitė [1,*], Greta Striganavičiūtė [1], Valeriia Mishcherikova [2] and Vaida Sirgedaitė-Šėžienė [1]

[1] Lithuanian Research Centre for Agriculture and Forestry, Laboratory of Forest Plant Biotechnology, Institute of Forestry, Liepu st. 1, LT-53101 Girionys, Lithuania
[2] Lithuanian Research Centre for Agriculture and Forestry, Department of Forest Protection and Game Management, Institute of Forestry, Liepu st. 1, LT-53101 Girionys, Lithuania
* Correspondence: doroteja.vaitiekunaite@lammc.lt

Abstract: Bacterial biostimulants are an eco-friendly alternative to chemical fertilizers. However, before their introduction into open ecosystems, broad-scope studies need to be carried out. *Paenibacillus* sp. was shown to positively affect poplar root growth. It was hypothesized that alongside these improvements, the *Paenibacillus* sp. inoculant may affect its host's secondary metabolism. *Populus tremula* × *P. alba* microshoots were inoculated in vitro. Microshoots were tested for chlorophyll, carotenoid, total flavonoid (TFC), total phenol content (TPC) and free radical scavenging capacity during primary growth after 4, 6 and 8 weeks. The results showed that the inoculation decreased shoot phenolics and free radical scavenging capacity after 6 and 8 weeks. Chlorophyll *b* amounts increased after 6 and 8 weeks. Carotenoid content decreased after 6 weeks, while chlorophyll *a* and carotenoid levels increased after 8 weeks. Correlation and principal component analyses showed that the inoculant changed the way in which the photosynthesis pigment content relates to TPC, TFC and radical scavenging activity. Overall, these data suggest that the inoculant does statistically significantly affect *Populus* tree secondary metabolism in the later stages of the initial growth period. This effect may potentially be compensatory in nature.

Keywords: poplar; bacteria; total phenols; total flavonoids; ABTS; DPPH; chlorophyll; carotenoids

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1. Introduction

In recent years research on bacterium-based biostimulants has been on the rise, due to the negative effects that chemical fertilization and pesticide use have on the environment [1–4]. However, most of it is directed towards food crops, and comparatively fewer studies have been carried out on biostimulant use on trees, even though, in the face of climate change and loss of biodiversity, reforestation often plays a large role.

Multiple studies have demonstrated that plant-growth-promoting bacteria (PGPB, or plant-growth-promoting rhizobacteria (PGPR)) can serve as effective biostimulants [5–10] and biocontrol agents [11–13], both in controlled settings and in field trials. PGPB may benefit their hosts in a variety of ways: through help with nutrient availability, phytohormone production, induced systemic resistance, antagonistic effects on plant pathogens, response to stress, etc. [1–3,11,12,14].

To determine the advantages of using microbial biostimulants, various variables can be taken into consideration: visually observable vegetative growth parameters [8], antagonism to pathogens [12], photosynthesis pigment content [10,14–16], antioxidant activity [17,18] and secondary metabolites (SMs) such as phenolics [15,19].

According to Schulz et al., "Most plants are healthy, in part due to metabolic interactions between the holobiont's host and its microbiome..." [20]. Hence, it is obvious that the modification of the plant's microbiome can have multifaceted outcomes for the plant. Furthermore, it has been demonstrated that modifying the plant's environment with bacteria may have both species-specific and genotype-specific outcomes [5,8,15,21–26]. Additionally,

these outcomes may also be shaped by the plant's growth stage [25] and environmental conditions [10,14]. Thus, it is extremely important to fully comprehend the consequences that may follow the introduction of new biostimulants to the microbiome of plants and the open ecosystems, especially since microorganisms are key elements in all biogeochemical cycles and could potentially affect the condition of the entire ecosystem [27].

It is noteworthy that while the field of bacterial biostimulants is expanding, broad-ranging studies into the assorted effects that one microorganism species can have on their plant host are still necessary. To our knowledge, there are not any studies that represent bacterial-inoculant-induced time-dependent metabolite changes during the initial growth stages of woody plants.

Poplars are valuable model organisms that represent all trees and other woody plants in a variety of studies. Poplar microshoot cultures also serve as a model experimental system for woody plant–microorganism interaction research. This is due to several factors, including their relatively easy in vitro propagation and clonal tissue culture initiation, fast growth, wide habitat range and the accessible information about their whole genome [27]. Moreover this genus is grown widely for profit [28].

PGPB, *Paenibacillus* sp., was recently isolated from oaks (*Quercus robur*) and tested on two distinct model poplar (*Populus* spp.) microshoot cultures, resulting in the enhancement of their root system [8]. Additionally, this bacterial species was shown to have several plant-growth-promoting properties (phytohormone indole-3-acetic acid (IAA) production; both phosphate solubilization and mineralization in vitro) [8], making it a potentially effective biostimulant for trees.

Thus, we hypothesize, that *Paenibacillus* sp. may have an impact on poplar's photosynthesis pigment content, antioxidative response and phenolics and hence could potentially induce systemic resistance, which would help with poplar's stress responses during the timeframe of initial growth.

The results of the experiments showed that *Paenibacillus* sp. inoculation had an effect on poplar microshoot secondary metabolism that is not suggestive of induced systemic resistance, although an antioxidant compensatory mechanism was also observed.

2. Materials and Methods

The experiment was conducted using a model hybrid poplar *Populus tremula* × *P. alba* in vitro clone microshoot culture, established in the laboratory of the Forest Research Institute, Poland, from the vegetative buds of 6–7-year-old cloned trees. At the time of the experiment, the culture was kept up for 4 years via bimonthly transfers (5.25 ± 0.25 mL of solid Murashige and Skoog (MS) media, supplemented with 2% sucrose and 0.4% gelrite (gelling agent), w/v, pH 5.6 ± 0.1) to fresh media and grown under a 16/18 h 25/20 °C cycle with cool white fluorescent light (Osram, Munich, Germany, 2 × 36 W, 22 µmol m^{-2} s^{-1}) in 20 mm glass tubes. All the media and other components were purchased from Duchefa Biochemie (Haarlem, The Netherlands) and used per the manufacturer's instructions.

2.1. Inoculation and Growth Conditions

Paenibacillus sp. was used to inoculate poplar microshoots based on previously documented plant-growth-promoting abilities on this exact microshoot culture [8].

The tests were performed under previously described conditions, using Woody Plant media (WPM) (additionally 0.4% gelrite, 2% sucrose, w/v, pH 5.6 ± 0.1). In total, 30 two-month-old poplar microshoots were divided into three independent replicates per one group at one time period. For the uninoculated control groups, microshoot leaves were gently removed, and the stems were divided into 10 mm segments with 2–3 growing nodes on each stem (exempting the primary apical top). Every segment was individually placed into a ⌀20 mm glass tube with 5.25 ± 0.25 mL of WPM. The tubes were capped and placed in a growth chamber.

For groups inoculated with *Paenibacillus* sp., the inoculant was prepared prior to inoculation. A swab from a single bacterial colony was transferred to a fresh plate with

low-salt Lysogeny broth (LB) medium (pH 7.2 ± 0.1, tryptone—10 g/L, NaCl—5 g/L, yeast extract—5 g/L) and grown at +25 ± 1 °C overnight. The next day, prior to the transfer of poplar segments, tubes were inoculated with a swab using an inoculation needle [8]. Then, poplar segments were carefully placed into the stab area.

Sample measurements were taken after 4, 6 and 8 weeks (this represents three different groups, as the measurements required the destruction of the samples). Chlorophyll *a* and *b* (CHA, CHB, respectively), carotenoids (CAR), total phenol content (TPC), total flavonoid content (TFC) and antioxidant capacities using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assays were measured.

2.2. Sample Collection and Preparation for Metabolic and Physiological Assays

After each growth period, samples were collected and immediately prepared for future metabolic and physiological assays.

For the photosynthesis pigment analysis, a total of 0.5 g of fresh aboveground biomass (stems as well as leaves, as at these growth stages, they still serve a purpose in photosynthesis) was collected from both the control group and the inoculated group after 4, 6 and 8 weeks at each time point. The total biomass was divided into three biological replicates per each time point. Immediately after collection and weighing, fresh biomass was stored at –20 °C until use.

For the other metabolic tests and DPPH and ABTS assays, 75% methanol (MeOH (Chempur, Piekary Śląskie, Poland)) was used for extraction. In total, 1.1 g of fresh aboveground biomass was collected from both the control group and the inoculated group after 4, 6 and 8 weeks at each time point. The total biomass was divided into seven biological replicates per each time point. Samples were homogenized by ceramic mortar and pestle and placed in inert plastic screw cap tubes; then 10 mL of 75% MeOH was added. Samples were subsequently placed in a thermal shaker for 24 h at +25 °C, 1 × g, in the dark. Next, samples were filtered using filter paper with a retention of 5–8 µm. Extracts were then stored at –20 °C until use.

2.3. Metabolic and Physiological Assays

2.3.1. Measurements of Photosynthesis Pigments

Relative amounts of CAR, CHA and CHB were measured via their absorbance. Frozen leaves were ground with mortar and pestle using pure acetone (30 mL) (Chempur) as extracting agent. This extract was then filtered as previously described and used for absorbance measurements with a UV–VIS spectrophotometer 80+ (PG Instruments, Lutterworth, UK) at 440, 662 and 664 nm (CAR, CHA and CHB, respectively). Three technical replicates were measured for each sample at each wavelength independently. For the calculation of pigment content, the following formulas were used [29] (mg g^{-1}):

$$\mathrm{CHA} = \frac{(9.784 \times \mathrm{A}_{662} - 0.99 \times \mathrm{A}_{644}) \times \mathrm{V}}{\mathrm{P} \times 1000} \quad (1)$$

$$\mathrm{CHB} = \frac{(21.426 \times \mathrm{A}_{644} - 4.65 \times \mathrm{A}_{662}) \times \mathrm{V}}{\mathrm{P} \times 1000} \quad (2)$$

$$\mathrm{CAR} = \frac{(4.695 \times \mathrm{A}_{440} - 0.268 \times (\mathrm{CHA} + \mathrm{CHB}) \times \mathrm{V}}{\mathrm{P} \times 1000} \quad (3)$$

where A—absorbance, V—total extract volume (mL), P—fresh aboveground biomass (g).

2.3.2. Total Phenol Content

The methanol extracts were used to determine TPC. A modified method utilizing the Folin–Ciocalteu reagent (VWR Chemicals, Radnor, PA, USA) was applied [30]. Briefly, 0.1 mL of extract was mixed with 0.1 mL of the reagent (2 N) and 2.5 mL of distilled water (dH_2O) and incubated for 6 min. Then, 0.5 mL of 20% (*w*/*v*) Na_2CO_3 (Molar Chemicals

KFT, Hungary) was added. The mixture was then left to incubate at 22 °C for 30 min. Absorbance was measured using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Bad Friedrichshall, Germany) at 760 nm (75% methanol as blank). Three technical replicates were measured for each sample. TPC was expressed as chlorogenic acid (TCI, Tokyo, Japan) per gram of fresh weight (mg CAE g^{-1}). The standard calibration curve equation used was y = 5.5358x − 0.0423 (R^2 = 0.9975).

2.3.3. Total Flavonoid Content

TFC was determined using a modified method [19,31]. The methanol extract (1 mL) was mixed with 0.3 mL of 5% (*w*/*v*) $NaNO_2$ (VWR Chemicals) and incubated for 5 min. Next, 0.5 mL of 2% (*w*/*v*) $AlCl_3$ (Alfa Aesar, Haverhill, MA, USA) was added and incubated for 6 min. Subsequently, 0.5 mL of NaOH (Merck, Darmstadt, Germany) (1 M) was added. The absorbance of the mixture was measured at 470 nm (75% methanol as blank) on the Synergy HT Multi-Mode Microplate Reader. Three technical replicates were measured for each sample. TFC was expressed in milligrams of catechin (Merck) per gram of fresh weight (mg CE g^{-1}). The standard calibration curve equation used for TFC was y = 11.616x + 0.0634 (R^2 = 0.9983).

2.3.4. DPPH Assay

The total free radical scavenging capacity of the extracts was determined using a modified method [19,32]. The methanol extract (0.1 mL) was mixed with 1 mL of DPPH (TCI) solution (0.1 mM; in 75% methanol) and incubated in the dark (+22 °C) for 16 min. Absorbance was measured at 515 nm on a Synergy HT Multi-Mode Microplate Reader (0.1 mL of 75% MeOH and 1 mL of DPPH solution as a blank). Three technical replicates were measured for each sample. The standard calibration curve equation used was y = 0.2074x − 0.004 (R^2 = 0.9907). The radical scavenging capacity was expressed as antioxidant Trolox (vitamin E analogue) (Merck) equivalent per gram of fresh weight (mM TE g^{-1}):

$$TE = \frac{c * V}{m} \quad (4)$$

where c—Trolox equivalent concentration (mM mL^{-1}), V—total extract volume (mL), m—fresh aboveground biomass (g).

2.3.5. ABTS Assay

The free radical scavenging capacity was also determined using the ABTS radical cation decolorization assay [19]. One day before the measurements were taken, the ABTS solution was prepared; 0.056 g of ABTS (>99% purity, Alfa Aesar) was dissolved in 50 mL of dH_2O. All of the ABTS stock solution was mixed with 200 µL of 70 nM $K_2S_2O_8$ (0.1982 g $K_2S_2O_8$ (Molar Chemicals KFT, Halásztelek, Hungary) dissolved in 10 mL dH_2O). The mixture was held in the dark at +4 °C for 16 h before it was used. Next, if necessary, the mixture was diluted with dH_2O until it reached 0.700 ± 0.2 absorbance (734 nm) (dH_2O as a blank); otherwise, 50 µL of the sample methanol extract was mixed with 2 mL of ABTS solution and placed in the dark for 10 min. Subsequently, the absorbance was measured on a Synergy HT Multi-Mode Microplate Reader (50 µL of 75% methanol and 2 mL of ABTS solution were used as a blank). Three technical replicates were measured for each sample. Trolox was used as the standard. The radical scavenging capacity was expressed as antioxidant Trolox equivalent per gram of fresh weight (mM TE g^{-1}), Formula no. 4 was used for calculations.

2.4. Statistical Analysis of the Data

Pooled data from three independent experiments were used for statistical analysis, which was performed using the open access R software (version 4.0.5; accessed on 7 January 2022) [33]. All the data points stemmed from three technical replicates and 3–7 biological replicates, depending on the metabolite group. Thirty poplar microshoots were used for

each inoculated and control group at each time point divided into three independent replicates. Data were tested for normal distribution of residuals (Shapiro-Wilk test) and homogeneity of variance (Levene's Test) with "car" package functions [34]. ANOVA was then performed using the *aov* from the "stats" function. For data that showed a significant deviation from the normal distribution of residuals, a non-parametric analog of ANOVA, Kruskal–Wallis test, was performed. Tukey Honest Significant Differences tests (HSDs) were performed for multiple pairwise comparisons between the means of groups using the *TukeyHSD* function from the "stats" package.

To build the correlation matrix, the average for each group was calculated. For log-transformed data, the Pearson correlation coefficient was calculated using the *rcorr* function from the "Hmisc" package [35]. To visualize the correlation matrix, the *corrplot* function from the "corrplot" package was used [36]. Visualization of feature distribution (principal component analysis, PCA) was carried out using the "ggplot2" package [37].

The 7 measured bioactive compounds were simplified and classified into components through PCA with *prcomp* from the "Factoextra" package [38].

3. Results

ANOVA data on the seven tested poplar secondary metabolism and physiological parameters are displayed in Figure 1. After 6 weeks, all the data were statistically significant except the amount of CHA ($p > 0.05$), and all the data after 8 weeks were statistically significant. TPC (−8.2%), TFC (−13.7%), CAR (−15.3%), ABTS (−22.7%) (this abbreviation will henceforth be used for free radical scavenging capacity measured using the ABTS assay) and DPPH (−7.7%) (this abbreviation will henceforth be used for free radical scavenging capacity measured using the DPPH assay) metrics were lower in the inoculated group, namely, the poplar microshoots inoculated with *Paenibacillus* sp., after 6-week incubation, while CHB metrics were higher by 32.9% in regard to their respective control groups.

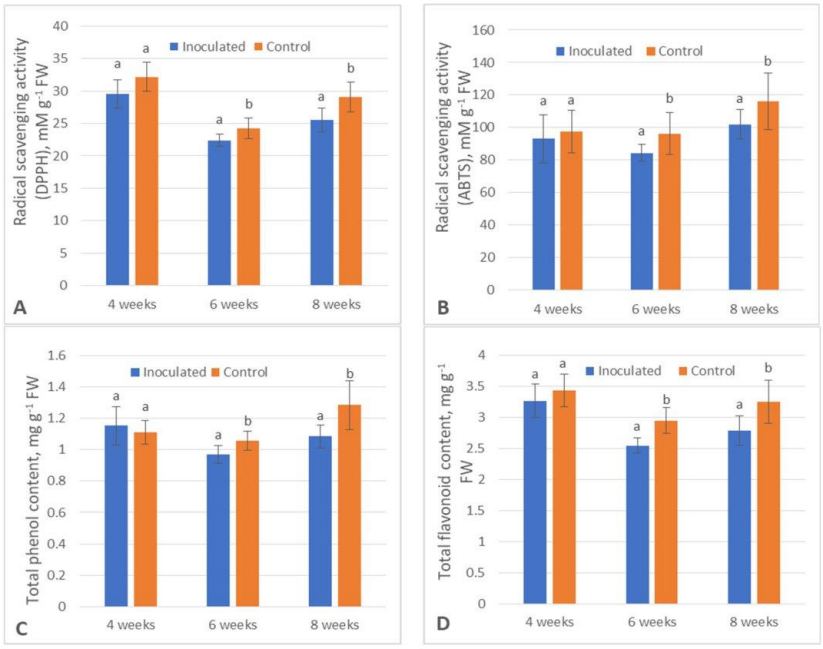

Figure 1. *Cont.*

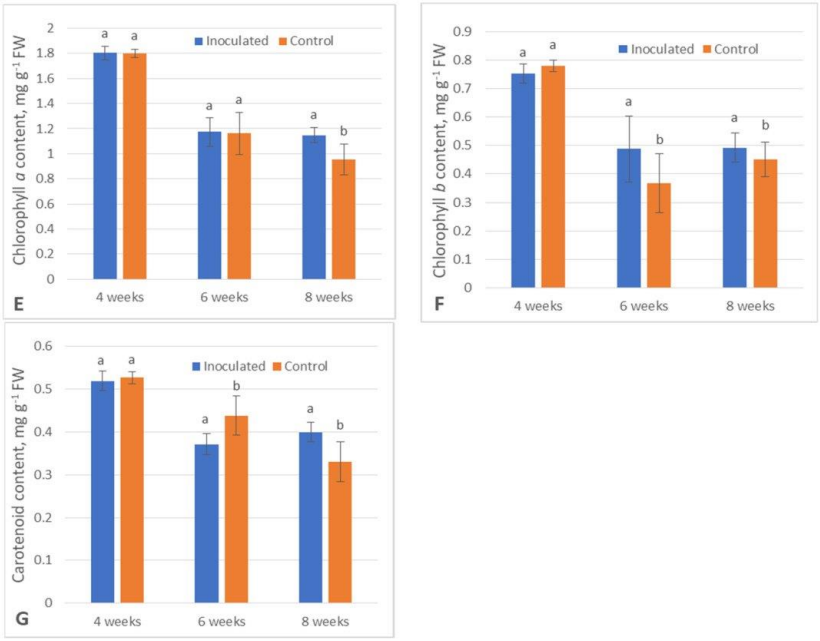

Figure 1. The concentrations of tested bioactive compounds in the control (uninoculated) and *Paenibacillus* sp. inoculated groups throughout the experiment: (**A**)—antioxidant scavenging (DPPH), (**B**)—antioxidant scavenging (ABTS), (**C**)—total phenolic content (TPC), (**D**)—total flavonoid content (TFC), (**E**)—chlorophyll a (CHA), (**F**)—chlorophyll b (CHB), (**G**)—carotenoids (CAR). Different letters above data from each week indicate significantly different results between control and inoculated groups, $p \leq 0.05$.

Comparatively, TPC, TFC, ABTS and DPPH were lower after 8 weeks by 15.6%, 14.4%, 21.4% and 12.4%, respectively, and all photosynthesis pigment contents were higher (CHA—+20.2%, CHB—+9.2%, CAR—+21.1%) in the inoculated plants in regard to their respective control groups.

There was a clear upward trend regarding the amounts of plant phenolics (TPC and TFC) as well as free radical scavenging capacity (ABTS and DPPH) over the duration of the experiment in both control and experimental groups; however, a significant divergence occurred between the two after the initial incubation period of 4 weeks.

On the other hand, the photosynthesis pigment content did not present a clear trend either way in either of the groups. After 6 weeks, the amounts of CAR decreased in the inoculated plants compared to the control, while CHB increased, and after 8 weeks, all the photosynthesis pigment contents significantly increased in the experimental group.

Correlation analysis allows for statistically significant relationships to be observed between different variables and shows whether that relationship is directly proportional or directly opposite, which, in this case, allowed for assumptions to be made regarding the varied mechanisms that regulate poplar's metabolism. Figure 2 shows the correlation between the amount of bioactive compounds in the control and experimental groups after the 8th week of incubation. In both the experimental and control groups, the amounts of TPC–TFC, TPC–ABTS, DPPH–CHB, CHA–CHB, CHA–CAR and CHB–CAR displayed moderate to strong positive correlations. Simultaneously, a moderately positive correlation between TFC and ABTS, and a moderately negative correlation between TPC and CAR and ABTS–CAR were observed in the control group, and moderately positive correlations in TFC–CHA, TFC–CHB, TFC–CAR, DPPH–CHA and DPPH–CAR were noted in the

experimental group. Specifically, inoculation lowered the correlation between TFC, TPC, ABTS and DPPH and the pigments.

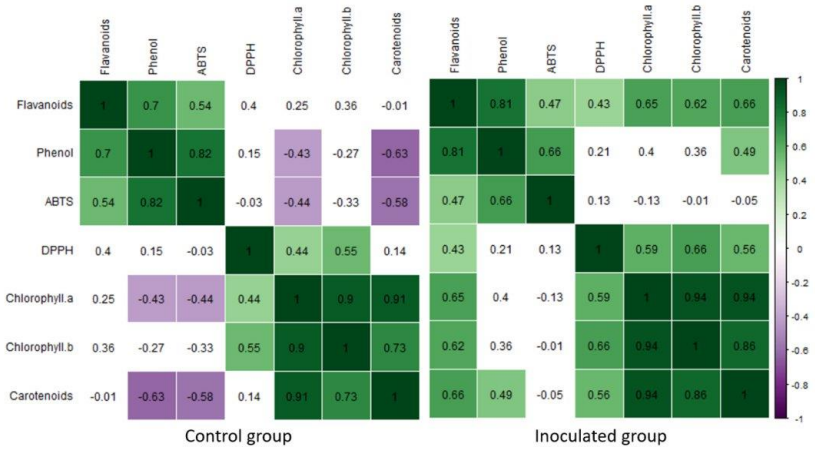

Figure 2. Correlation matrix for the data acquired after the 8th incubation week with *Paenibacillus* sp. (**right**) and their respective control groups (**left**), based on the Pearson correlation coefficient. Significant ($p \leq 0.05$) correlation coefficients are colored.

Principal component analysis is a technique used to simplify data when multiple complex data points and variables are available. This group's data, based on multivariate similarities, and similarly to correlation analysis, allows one to gauge the relationships between variables and how much they influence the grouping results and total variance. In this work, PCA analysis was performed to evaluate overall relationships between the levels of bioactive compounds after the eight experimental weeks (Figure 3). The results complimented the correlation analysis, showing some overlap between the two groups; however, there were diverging group mean values, which corresponded with the ANOVA results.

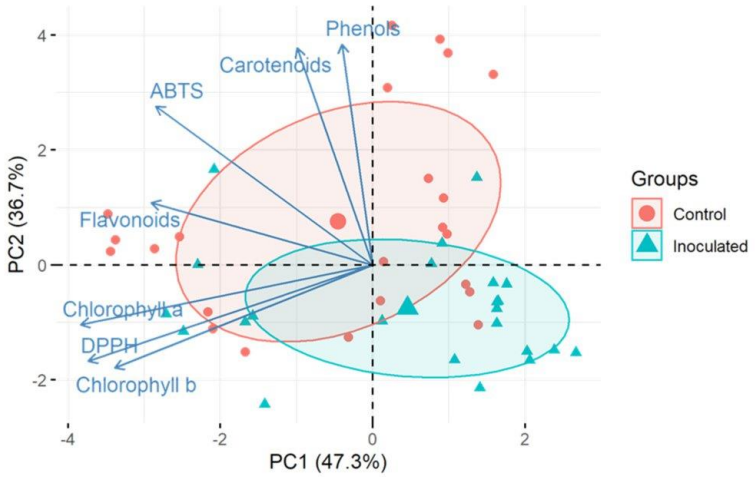

Figure 3. Concentration of tested metabolic and physiological parameters throughout the experiment via principal component analysis (PCA) displaying the main principal components (PCs), which represent 84.41% of cumulative variance. Loading vectors show the correlation between tested variables.

The seven principal components explained the total variance and three explained 92.3% of the total variance of the parameters (Table 1).

Table 1. Total variance of the cumulative data gathered throughout the experiment explained by principal components.

Importance of Components:	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Standard deviation	1.82	1.603	0.76054	0.50628	0.39011	0.31013	0.19162
Proportion of variance	0.473	0.367	0.08263	0.03662	0.02174	0.01374	0.00525
Cumulative proportion	0.473	0.84	0.92266	0.95927	0.98101	0.99475	1

Loading vector analysis showed that TPC, CAR and ABTS were all highly positively linked with principal component 2 (PC2), as was TFC, but to a lesser extent. CHA, CHB and DPPH were all negatively linked with PC1. Further relationships between the PCs and measurements can be seen in the rotated component matrix (Table 2), which suggests a possible meaning of these three principal components, as follows: PC1—pigments; PC2—ABTS and phenolics; and PC3—DPPH.

Table 2. Rotated principal component matrix and individual proportion of variance for each measured metric.

	PC1	PC2	PC3	PC4
Flavonoids	−0.37443	0.412508	−0.24301	0.143112
Phenols	−0.13049	0.563135	−0.23495	0.463554
ABTS	−0.05269	0.573278	−0.0509	−0.67733
DPPH	−0.38247	0.161549	0.869776	−0.04712
Chlorophyll *a*	−0.49104	−0.25004	−0.15893	0.064167
Chlorophyll *b*	−0.5043	−0.15491	0.022111	0.248416
Carotenoids	−0.44529	−0.26731	−0.31764	−0.48766

The results showed that the first two principal components (PCs) accounted for 84.41% of the total variation (Figure 3). PC1 (47.3%) was mainly explained by the chlorophyll and CAR content. In turn, PC2 (36.7%) was mainly explained by the ABTS activity and phenolics (although TFC to a lesser extent). PC3 could mainly be linked to DPPH data, although the total input of PC3 was only ~8.2%.

4. Discussion

Changes within a plant's metabolome illuminate how a specific plant may react to new conditions [39]. Bacterial inoculants have been shown to affect plant metabolism in previous studies [15,21,40–42]. The latest research suggests that they often act in a regulatory role [43].

Plant SMs are often linked with the plant's resistance to environmental stressors [26,44–48]. Phenols, flavonoids specifically, can act in various roles: as antioxidants [44,45], antimicrobials [45], etc. Studies pointing to the increases in SM production due to microbial inoculation posit that such a response creates induced systemic resistance (ISR) and will likely help with future responses to pathogen attacks [49,50] or abiotic stress [51]. The data of our experiment showed that the SMs and free radical scavenging activity decreased within the studied timeframe; thus, it could be assumed that the tested bacteria did not induce systemic resistance during initial growth in the tested plants.

Wallis and Galarneau conducted a meta-analysis on this subject (data from 2008 to 2017). In contrast with our own results, they confirmed that total phenolics increase when colonized by both bacteria and fungi. More importantly, their results demonstrated that the type of bacteria (beneficial or not) did not merit different reactions, but the type of fungi did (TPC increased after introduction to beneficial fungi) [52]. Other research demonstrated that

SM production decreased after fungal pathogen attack [26,53] or increased after inoculation with PGPB *Bacillus subtilis* [19], seemingly confirming these findings.

In comparison with our study, *Lolium perenne* grass inoculated with different strains of beneficial fungal endophyte *Neotyphodium lolii* expressed variations of both decreases and increases in phenolics and antioxidants that were endophyte strain dependent [54]. During our studies, both TPC and TFC were affected in a similar manner, with a significant decrease in the inoculated group after 6 and 8 weeks. Additionally, a strong positive correlation ($R > 0.7$) was observed between these effects after 8 weeks in the inoculated group as well as in the control group. This suggests that TFC is likely a large contributor to changes in TPC in our study.

In plants, flavonoids act in an antioxidative capacity, but they may also modulate plant–microorganism interactions [45]. A similar decrease in poplar phenolics, and flavonoids specifically, was noted in a study in which poplars faced a water shortage [55], showing that this type of reaction may not necessarily be unique to our studied bacteria and may potentially be indicative of stress. Consequently, the experimental design using microshoots could be the reason behind similar trends in TFC and TPC in both inoculated and control groups and provide a potential explanation as to why TFC and TPC were relatively lower in the inoculated group as a response to bacteria inoculation, indicating that they were perceived as an additional stressor.

DPPH and ABTS assays are both used to determine the total antioxidant capacity within the sample and thus are indicative of the oxidative stress level [56]. In our study, after 6 and 8 weeks of incubation, inoculated microshoot extracts exhibited a significant decrease in DPPH and ABTS metrics, suggestive of reduced free radical scavenging activity within inoculated poplars. Data on ABTS and TPC/TFC were shown to correlate irrespective of poplar inoculation status and, as phenolic compounds have been reported to act in an antioxidative capacity [44], the drop in phenolics and free radical scavenging activity could stem from the same biochemical changes.

In various studies, plant photosynthesis pigment content can be linked to the general health and vigor of the plant [10,57–59]. However, CHA, CHB and CAR have secondary functions within plants.

CAR act as secondary agents within photosynthesis; however, they also participate in antioxidative stress management and operate as precursors to phytohormones (e.g., abscisic acid, ABA) and other molecules [39,60]. Phenolics and antioxidant activity followed similar trends over the duration of our experiment, even between groups. CAR content was inconsistent throughout, with a relative decrease in the inoculated group after 6 weeks, but a complete opposite reaction after 8 weeks. Within the scope of analogue studies, the increase in CAR can be deemed to be beneficial when this increase can correlate with enhanced plant growth, as was shown in a study using an arbuscular mycorrhizal fungi–grape model [59].

The chlorophyll content within the plant is primarily linked to plant vitality [59,61]. Secondarily, chlorophylls can also work as antioxidants [62]. CHB is considered an accessory to CHA [57]. In this experiment, CHB in inoculated plants was enhanced after 6 and 8 weeks, but CHA was only significantly affected after 8 weeks, with increases post-inoculation. Just as with CAR, analogous conclusions were drawn regarding the increase in chlorophyll content for grape [59], avocado [10], cotton [7], peanut [58], eucalyptus [63] and bean [14].

A correlation analysis, performed after 8 weeks of incubation, uncovered some illuminating information. It showed that the inoculation changed the way in which photosynthesis pigments related to four other tested parameters between the control and inoculated groups. Specifically, inoculation seemed to affect TPC, TFC and free radical scavenging activity in relation to photosynthesis pigment content (see Figure 2).

Post inoculation, a moderate negative correlation emerged between ABTS and CAR, as well as between TPC and CAR. This potentially revealed a compensatory mechanism, whereby the inoculation negatively impacts TPC, which correlates with lower free radical scavenging activity (ABTS), which, in turn, raises CAR levels to, at least in part, com-

pensate for the antioxidant level drop. A similar compensation was observed in several studies [64–68]. In a 2020 study, the authors revealed a compensatory mechanism between the plastid prenyllipid antioxidants tocopherol and plastoquinol and ascorbate peroxidase (APX) enzyme [64]. In a study with tomatoes, researchers observed a negative correlation between lycopene and chlorophyll (–0.6209), also suggestive of a compensatory mechanism. This relates to results from our study, whereby after inoculation, carotenoid content was negatively linked with ABTS (-0.58) and TPC (-0.63) ($p \leq 0.05$). Thus, it can be inferred that TFC, TPC, ABTS and pigment amounts correspondingly organize in relation to each other to ensure homeostasis, as noted by Sarker and Oba [68].

PCA uncovered that the inoculated group and control group diverged based on their secondary metabolite levels, and most of this was due to changes in pigments, phenolics and ABTS, while DPPH data strongly determined a small fraction of all variation (~8.2%).

Moreover, a long-term effect on plant growth and secondary metabolism was observed after one-time inoculation, as similar results were achieved using mutant *Paeniabacillus polymyxa* on western red cedar [69] and pine [70] after a 12-month period, corn after a 3 month period [24] and *Caballeronia sordidicola* on pine and spruce after a year-long period [71]. This is potentially indicative that further changes might occur during a longer growth period, hence boosting the possibility of using *Paenibacillus* sp. as a long-acting agent. Long-term benefits would be advantageous in tree nurseries, the wood industry, or orchards, where plants are grown for years before harvest, or in cases where periodical fertilization is not feasible.

5. Conclusions

This research contains a more thorough look into *Populus* sp. metabolic and physiological changes post *Paenibacillus* sp. bacterial inoculation during the timeframe of initial growth. This is a necessary input into comprehensive studies to create biofertilizers that could be used in tree nurseries and other forestry related enterprises. These data add to the knowledge regarding the mechanisms that infer plant growth and health. It shows that bacteria may affect different aspects of secondary metabolism in different ways at the same time, i.e., different photosynthesis pigment contents as opposed to phenolic contents and antioxidant capacity. However, additional data need to be collected to further understand *Populus* tree–*Paenibacillus* interactions and pinpoint the exact compounds responsible for these changes. Overall, the data gathered here, alongside previous studies of these bacteria, provide a good example of how microbial inoculants can have complex impacts on their hosts' growth and health.

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References

1. Kour, D.; Rana, K.L.; Yadav, A.N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H.S.; Saxena, A.K. Microbial Biofertilizers: Bioresources and Eco-Friendly Technologies for Agricultural and Environmental Sustainability. *Biocatal. Agric. Biotechnol.* **2020**, *23*, 101487. [CrossRef]
2. Kalayu, G. Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers. *Int. J. Agron.* **2019**, *2019*, 4917256. [CrossRef]
3. Pirttilä, A.M.; Mohammad Parast Tabas, H.; Baruah, N.; Koskimäki, J.J. Biofertilizers and Biocontrol Agents for Agriculture: How to Identify and Develop New Potent Microbial Strains and Traits. *Microorganisms* **2021**, *9*, 817. [CrossRef]

4. Suhag, M. Potential of Biofertilizers to Replace Chemical Fertilizers. *Int. Adv. Res. J. Sci. Eng. Technol.* **2016**, *3*, 163–167. [CrossRef]
5. Puri, A.; Padda, K.P.; Chanway, C.P. In Vitro and in Vivo Analyses of Plant-Growth-Promoting Potential of Bacteria Naturally Associated with Spruce Trees Growing on Nutrient-Poor Soils. *Appl. Soil Ecol.* **2020**, *149*, 103538. [CrossRef]
6. Park, Y.G.; Mun, B.G.; Kang, S.M.; Hussain, A.; Shahzad, R.; Seo, C.W.; Kim, A.Y.; Lee, S.U.; Oh, K.Y.; Lee, D.Y.; et al. *Bacillus aryabhattai* SRB02 Tolerates Oxidative and Nitrosative Stress and Promotes the Growth of Soybean by Modulating the Production of Phytohormones. *PLoS ONE* **2017**, *12*, e0173203. [CrossRef]
7. Kumari, M.; Thakur, I.S. Biochemical and Proteomic Characterization of *Paenibacillus* sp. ISTP10 for Its Role in Plant Growth Promotion and in Rhizostabilization of Cadmium. *Bioresour. Technol. Rep.* **2018**, *3*, 59–66. [CrossRef]
8. Vaitiekūnaitė, D.; Kuusienė, S.; Beniušytė, E. Oak (*Quercus robur*) Associated Endophytic *Paenibacillus* sp. Promotes Poplar (*Populus* spp.) Root Growth In Vitro. *Microorganisms* **2021**, *9*, 1151. [CrossRef] [PubMed]
9. Ferchichi, N.; Toukabri, W.; Boularess, M.; Smaoui, A.; Mhamdi, R.; Trabelsi, D. Isolation, Identification and Plant Growth Promotion Ability of Endophytic Bacteria Associated with Lupine Root Nodule Grown in Tunisian Soil. *Arch. Microbiol.* **2019**, *201*, 1333–1349. [CrossRef] [PubMed]
10. Barra, P.J.; Inostroza, N.G.; Mora, M.L.; Crowley, D.E.; Jorquera, M.A. Bacterial Consortia Inoculation Mitigates the Water Shortage and Salt Stress in an Avocado (*Persea americana* Mill.) Nursery. *Appl. Soil Ecol.* **2017**, *111*, 39–47. [CrossRef]
11. Sahu, P.K.; Singh, S.; Gupta, A.R.; Gupta, A.; Singh, U.B.; Manzar, N.; Bhowmik, A.; Singh, H.V.; Saxena, A.K. Endophytic Bacilli from Medicinal-Aromatic Perennial Holy Basil (*Ocimum tenuiflorum* L.) Modulate Plant Growth Promotion and Induced Systemic Resistance against *Rhizoctonia solani* in Rice (*Oryza sativa* L.). *Biol. Control* **2020**, *150*, 104353. [CrossRef]
12. Ren, J.H.; Li, H.; Wang, Y.F.; Ye, J.R.; Yan, A.Q.; Wu, X.Q. Biocontrol Potential of an Endophytic *Bacillus pumilus* JK-SX001 against Poplar Canker. *Biol. Control* **2013**, *67*, 421–430. [CrossRef]
13. Castaldi, S.; Petrillo, C.; Donadio, G.; Piaz, F.D.; Cimmino, A.; Masi, M.; Evidente, A.; Isticato, R. Plant Growth Promotion Function of *Bacillus* sp. Strains Isolated from Salt-Pan Rhizosphere and Their Biocontrol Potential against *Macrophomina phaseolina*. *Int. J. Mol. Sci.* **2021**, *22*, 3324. [CrossRef]
14. Gupta, S.; Pandey, S. ACC Deaminase Producing Bacteria with Multifarious Plant Growth Promoting Traits Alleviates Salinity Stress in French Bean (*Phaseolus vulgaris*) Plants. *Front. Microbiol.* **2019**, *10*, 1506. [CrossRef]
15. Striganavičiūtė, G.; Žiauka, J.; Sirgedaitė-Šėžienė, V.; Vaitiekūnaitė, D. Priming of Resistance-Related Phenolics: A Study of Plant-Associated Bacteria and *Hymenoscyphus fraxineus*. *Microorganisms* **2021**, *9*, 2504. [CrossRef] [PubMed]
16. Jiménez-Gómez, A.; Celador-Lera, L.; Fradejas-Bayón, M.; Rivas, R. Plant Probiotic Bacteria Enhance the Quality of Fruit and Horticultural Crops. *AIMS Microbiol.* **2017**, *3*, 483–501. [CrossRef]
17. Martin, L.J.; Matar, C. Increase of Antioxidant Capacity of the Lowbush Blueberry (*Vaccinium angustifolium*) during Fermentation by a Novel Bacterium from the Fruit Microflora. *J. Sci. Food Agric.* **2005**, *85*, 1477–1484. [CrossRef]
18. Pan, F.; Su, T.J.; Cai, S.M.; Wu, W. Fungal Endophyte-Derived *Fritillaria unibracteata* Var. *Wabuensis*: Diversity, Antioxidant Capacities in Vitro and Relations to Phenolic, Flavonoid or Saponin Compounds. *Sci. Rep.* **2017**, *7*, 42008. [CrossRef]
19. Chandrasekaran, M.; Chun, S.C.; Oh, J.W.; Paramasivan, M.; Saini, R.K.; Sahayarayan, J.J. *Bacillus subtilis* CBR05 for Tomato (*Solanum lycopersicum*) Fruits in South Korea as a Novel Plant Probiotic Bacterium (PPB): Implications from Total Phenolics, Flavonoids, and Carotenoids Content for Fruit Quality. *Agronomy* **2019**, *9*, 838. [CrossRef]
20. Schulz, B.J.; Rabsch, L.; Junker, C. Chemical Warfare in the Plant Microbiome Leads to a Balance of Antagonisms and a Healthy Plant. In *Seed Endophytes: Biology and Biotechnology*; Verma, S.K., White, J.F., Jr., Eds.; Springer Nature Switzerland AG: Berlin/Heidelberg, Germany, 2019; pp. 171–189. [CrossRef]
21. Striganavičiūtė, G.; Žiauka, J.; Sirgedaitė-Šėžienė, V.; Vaitiekūnaitė, D. Impact of Plant-Associated Bacteria on the In Vitro Growth and Pathogenic Resistance against *Phellinus tremulae* of Different Aspen (*Populus*) Genotypes. *Microorganisms* **2021**, *9*, 1901. [CrossRef] [PubMed]
22. Tang, Q.; Puri, A.; Padda, K.P.; Chanway, C.P. Biological Nitrogen Fixation and Plant Growth Promotion of Lodgepole Pine by an Endophytic Diazotroph *Paenibacillus polymyxa* and Its GFP-Tagged Derivative. *Botany* **2017**, *95*, 611–619. [CrossRef]
23. Puri, A.; Padda, K.P.; Chanway, C.P. Evidence of Nitrogen Fixation and Growth Promotion in Canola (*Brassica napus* L.) by an Endophytic Diazotroph *Paenibacillus polymyxa* P2b-2R. *Biol. Fertil. Soils* **2016**, *52*, 119–125. [CrossRef]
24. Puri, A.; Padda, K.P.; Chanway, C.P. Seedling Growth Promotion and Nitrogen Fixation by a Bacterial Endophyte *Paenibacillus polymyxa* P2b-2R and Its GFP Derivative in Corn in a Long-Term Trial. *Symbiosis* **2016**, *69*, 123–129. [CrossRef]
25. Puri, A.; Padda, K.P.; Chanway, C.P. Evaluating Lodgepole Pine Endophytes for Their Ability to Fix Nitrogen and Support Tree Growth under Nitrogen-Limited Conditions. *Plant Soil* **2020**, *455*, 271–287. [CrossRef]
26. Tsers, I.; Meshcherov, A.; Gogoleva, O.; Petrova, O.; Gogoleva, N.; Ponomareva, M.; Gogolev, Y.; Korzun, V.; Gorshkov, V. Alterations in the Transcriptome of Rye Plants Following the *Microdochium nivale* Infection: Identification of Resistance/Susceptibility-Related Reactions Based on RNA-Seq Analysis. *Plants* **2021**, *10*, 2723. [CrossRef]
27. Cregger, M.A.; Carper, D.L.; Christel, S.; Doktycz, M.J.; Labbé, J.; Michener, J.K.; Dove, N.C.; Johnston, E.R.; Moore, J.A.M.; Vélez, J.M.; et al. Plant-Microbe Interactions: From Genes to Ecosystems Using *Populus* as a Model System. *Phytobiomes J.* **2021**, *5*, 29–38. [CrossRef]
28. Tullus, A.; Tullus, H.; Vares, A.; Kanal, A. Early Growth of Hybrid Aspen (*Populus* × *wettsteinii* Hämet-Ahti) Plantations on Former Agricultural Lands in Estonia. *For. Ecol. Manag.* **2007**, *245*, 118–129. [CrossRef]

29. Wettstein, D. Chlorophyll-Letale Und Der Submikroskopische Formwechsel Der Plastiden. *Exp. Cell Res.* **1957**, *12*, 427–506. [CrossRef]
30. Singleton, V.L.; Orthhofer, R.; Lamuela-Raventos, R.M. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Methods Enzymol.* **1974**, *299*, 152–178. [CrossRef]
31. Drózdz, P.; Šėžienė, V.; Wójcik, J.; Pyrzyńska, K. Evaluation of Bioactive Compounds, Minerals and Antioxidant Activity of Lingonberry (*Vaccinium vitis-idaea* L.) Fruits. *Molecules* **2018**, *23*, 53. [CrossRef]
32. Ragaee, S.; Abdel-Aal, E.S.M.; Noaman, M. Antioxidant Activity and Nutrient Composition of Selected Cereals for Food Use. *Food Chem.* **2006**, *98*, 32–38. [CrossRef]
33. R Foundation for Statistical Computing. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2013.
34. Fox, J.; Weisberg, S. *An R Companion to Applied Regression*, 3rd ed.; Sage: Thousand Oaks, CA, USA, 2019.
35. Harrell, F.E., Jr.; Dupont, C. Hmisc Package for R. 2021. Available online: https://cran.r-project.org/web/packages/Hmisc/Hmisc.pdf (accessed on 13 August 2022).
36. Wei, T.; Simko, V. R Package "Corrplot": Visualization of a Correlation Matrix. 2021. Available online: https://cran.r-project.org/web/packages/corrplot/index.html (accessed on 18 November 2021).
37. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016.
38. Kassambara, A.; Mundt, F. Extract and Visualize the Results of Multivariate Data Analyses. 2016. Available online: https://rpkgs.datanovia.com/factoextra/ (accessed on 15 October 2022).
39. Daskalchuk, T.; Ahiahonu, P.; Heath, D.; Yamazaki, Y. The Use of Non-Targeted Metabolomics in Plant Science. In *Biotechnology in Agriculture and Forestry, Plant Metabolomics*; Nagata, T., Loerz, H., Widholm, J.M., Saito, K., Dixon, R.A., Willmitzer, L., Eds.; Springer: Berlin/Heidelberg, Germany, 2006; pp. 311–313.
40. Maggini, V.; De Leo, M.; Mengoni, A.; Gallo, E.R.; Miceli, E.; Reidel, R.V.B.; Biffi, S.; Pistelli, L.; Fani, R.; Firenzuoli, F.; et al. Plant-Endophytes Interaction Influences the Secondary Metabolism in *Echinacea purpurea* (L.) Moench: An in Vitro Model. *Sci. Rep.* **2017**, *7*, 16924. [CrossRef]
41. Yu, M.; Chen, J.C.; Qu, J.Z.; Liu, F.; Zhou, M.; Ma, Y.M.; Xiang, S.Y.; Pan, X.X.; Zhang, H.B.; Yang, M.Z. Exposure to Endophytic Fungi Quantitatively and Compositionally Alters Anthocyanins in Grape Cells. *Plant Physiol. Biochem.* **2020**, *149*, 144–152. [CrossRef] [PubMed]
42. Ludwig-Müller, J. Plants and Endophytes: Equal Partners in Secondary Metabolite Production? *Biotechnol. Lett.* **2015**, *37*, 1325–1334. [CrossRef] [PubMed]
43. Pang, Z.; Chen, J.; Wang, T.; Gao, C.; Li, Z.; Guo, L. Linking Plant Secondary Metabolites and Plant Microbiomes: A Review. *Front. Plant Sci.* **2021**, *12*, 621276. [CrossRef]
44. Erb, M.; Kliebenstein, D.J. Plant Secondary Metabolites as Defenses, Regulators, and Primary Metabolites: The Blurred Functional Trichotomy. *Plant Physiol.* **2020**, *184*, 39–52. [CrossRef] [PubMed]
45. Mierziak, J.; Kostyn, K.; Kulma, A. Flavonoids as Important Molecules of Plant Interactions with the Environment. *Molecules* **2014**, *19*, 16240–16265. [CrossRef]
46. Perincherry, L.; Stepien, L.; Vasudevan, S.E. Cross-Tolerance and Autoimmunity as Missing Links in Abiotic and Biotic Stress Responses in Plants: A Perspective toward Secondary Metabolic Engineering. *Int. J. Mol. Sci.* **2021**, *22*, 11945. [CrossRef]
47. Goyal, S.; Lambert, C.; Cluzet, S.; Merillon, J.M.; Ramawat, K.G. Secondary Metabolites and Plant Defence. In *Plant Defence: Biological Control, Progress in Biological Control*; Springer: Dordrecht, The Netherlands, 2012; pp. 109–130. [CrossRef]
48. Isah, T. Stress and Defense Responses in Plant Secondary Metabolites Production. *Biol. Res.* **2019**, *52*, 39. [CrossRef]
49. Nunes da Silva, M.; Pintado, M.E.; Sarmento, B.; Stamford, N.P.; Vasconcelos, M.W. A Biofertilizer with Diazotrophic Bacteria and a Filamentous Fungus Increases *Pinus pinaster* Tolerance to the Pinewood Nematode (*Bursaphelenchus xylophilus*). *Biol. Control* **2019**, *132*, 72–80. [CrossRef]
50. Luzzatto, T.; Golan, A.; Yishay, M.; Bilkis, I.; Ben-Ari, J.; Yedidia, I. Priming of Antimicrobial Phenolics during Induced Resistance Response towards *Pectobacterium carotovorum* in the Ornamental Monocot Calla Lily. *J. Agric. Food Chem.* **2007**, *55*, 10315–10322. [CrossRef] [PubMed]
51. Ait Barka, E.; Nowak, J.; Clément, C. Enhancement of Chilling Resistance of Inoculated Grapevine Plantlets with a Plant Growth-Promoting Rhizobacterium, *Burkholderia phytofirmans* Strain PsJN. *Appl. Environ. Microbiol.* **2006**, *72*, 7246–7252. [CrossRef] [PubMed]
52. Wallis, C.M.; Galarneau, E.R.A. Phenolic Compound Induction in Plant-Microbe and Plant-Insect Interactions: A Meta-Analysis. *Front. Plant Sci.* **2020**, *11*, 580753. [CrossRef]
53. Pfabel, C.; Eckhardt, K.U.; Baum, C.; Struck, C.; Frey, P.; Weih, M. Impact of Ectomycorrhizal Colonization and Rust Infection on the Secondary Metabolism of Poplar (*Populus trichocarpa* × *deltoides*). *Tree Physiol.* **2012**, *32*, 1357–1364. [CrossRef] [PubMed]
54. Qawasmeh, A.; Obied, H.K.; Raman, A.; Wheatley, W. Influence of Fungal Endophyte Infection on Phenolic Content and Antioxidant Activity in Grasses: Interaction between *Lolium perenne* and Different Strains of *Neotyphodium lolii*. *J. Agric. Food Chem.* **2012**, *60*, 3381–3388. [CrossRef] [PubMed]
55. Popović, B.M.; Štajner, D.; Ždero-Pavlović, R.; Tumbas-Šaponjac, V.; Čanadanović-Brunet, J.; Orlović, S. Water Stress Induces Changes in Polyphenol Profile and Antioxidant Capacity in Poplar Plants (*Populus* spp.). *Plant Physiol. Biochem.* **2016**, *105*, 242–250. [CrossRef]

56. Ilyasov, I.R.; Beloborodov, V.L.; Selivanova, I.A.; Terekhov, R.P. ABTS/PP Decolorization Assay of Antioxidant Capacity Reaction Pathways. *Int. J. Mol. Sci.* **2020**, *21*, 1131. [CrossRef]
57. Nguyen, M.K.; Yang, C.M.; Shih, T.H.; Lin, S.H.; Pham, G.T.; Nguyen, H.C. Chlorophyll Biosynthesis and Transcriptome Profiles of Chlorophyll B-Deficient Type 2b Rice (*Oryza sativa* L.). *Not. Bot. Horti Agrobot. Cluj-Napoca* **2021**, *49*, 12380. [CrossRef]
58. Liu, D.; Yang, Q.; Ge, K.; Hu, X.; Qi, G.; Du, B.; Liu, K.; Ding, Y. Promotion of Iron Nutrition and Growth on Peanut by *Paenibacillus illinoisensis* and *Bacillus* sp. Strains in Calcareous Soil. *Braz. J. Microbiol.* **2017**, *48*, 656–670. [CrossRef]
59. Krishna, H.; Singh, S.K.; Sharma, R.R.; Khawale, R.N.; Grover, M.; Patel, V.B. Biochemical Changes in Micropropagated Grape (*Vitis vinifera* L.) Plantlets Due to Arbuscular-Mycorrhizal Fungi (AMF) Inoculation during Ex Vitro Acclimatization. *Sci. Hortic.* **2005**, *106*, 554–567. [CrossRef]
60. Swapnil, P.; Meena, M.; Singh, S.K.; Dhuldhaj, U.P.; Harish; Marwal, A. Vital Roles of Carotenoids in Plants and Humans to Deteriorate Stress with Its Structure, Biosynthesis, Metabolic Engineering and Functional Aspects. *Curr. Plant Biol.* **2021**, *26*, 100203. [CrossRef]
61. Li, Y.; He, N.; Hou, J.; Xu, L.; Liu, C.; Zhang, J.; Wang, Q.; Zhang, X.; Wu, X. Factors Influencing Leaf Chlorophyll Content in Natural Forests at the Biome Scale. *Front. Ecol. Evol.* **2018**, *6*, 64. [CrossRef]
62. Roca, M.; Chen, K.; Pérez-Gálvez, A. Chlorophylls. In *Handbook on Natural Pigments in Food and Beverages: Industrial Applications for Improving Food Color*; Elsevier Ltd.: Amsterdam, The Netherlands, 2016; pp. 125–158. [CrossRef]
63. Ren, H.; Qin, X.; Huang, B.; Fernández-García, V.; Lv, C. Responses of Soil Enzyme Activities and Plant Growth in a Eucalyptus Seedling Plantation Amended with Bacterial Fertilizers. *Arch. Microbiol.* **2020**, *202*, 1381–1396. [CrossRef] [PubMed]
64. Nowicka, B.; Fesenko, T.; Walczak, J.; Kruk, J. The Inhibitor-Evoked Shortage of Tocopherol and Plastoquinol Is Compensated by Other Antioxidant Mechanisms in *Chlamydomonas reinhardtii* Exposed to Toxic Concentrations of Cadmium and Chromium Ions. *Ecotoxicol. Environ. Saf.* **2020**, *191*, 110241. [CrossRef] [PubMed]
65. Vela-Hinojosa, C.; Escalona-Buendía, H.B.; Mendoza-Espinoza, J.A.; Villa-Hernández, J.M.; Lobato-Ortíz, R.; Rodríguez-Pérez, J.E.; Pérez-Flores, L.J. Antioxidant Balance and Regulation in Tomato Genotypes of Different Color. *J. Am. Soc. Hortic. Sci.* **2019**, *144*, 45–54. [CrossRef]
66. Chen, J.W.; Bai, K.D.; Cao, K.F. Inhibition of Monoterpene Biosynthesis Accelerates Oxidative Stress and Leads to Enhancement of Antioxidant Defenses in Leaves of Rubber Tree (*Hevea brasiliensis*). *Acta Physiol. Plant.* **2009**, *31*, 95–101. [CrossRef]
67. Zheng, X.T.; Yu, Z.C.; Tang, J.W.; Cai, M.L.; Chen, Y.L.; Yang, C.W.; Chow, W.S.; Peng, C.L. The Major Photoprotective Role of Anthocyanins in Leaves of *Arabidopsis thaliana* under Long-Term High Light Treatment: Antioxidant or Light Attenuator? *Photosynth. Res.* **2021**, *149*, 25–40. [CrossRef]
68. Sarker, U.; Oba, S. Drought Stress Effects on Growth, ROS Markers, Compatible Solutes, Phenolics, Flavonoids, and Antioxidant Activity in *Amaranthus tricolor*. *Appl. Biochem. Biotechnol.* **2018**, *186*, 999–1016. [CrossRef]
69. Anand, R.; Chanway, C. N2-Fixation and Growth Promotion in Cedar Colonized by an Endophytic Strain of *Paenibacillus polymyxa*. *Biol. Fertil. Soils* **2013**, *49*, 235–239. [CrossRef]
70. Anand, R.; Grayston, S.; Chanway, C. N2-Fixation and Seedling Growth Promotion of Lodgepole Pine by Endophytic *Paenibacillus polymyxa*. *Microb. Ecol.* **2013**, *66*, 369–374. [CrossRef] [PubMed]
71. Puri, A.; Padda, K.P.; Chanway, C.P. Can Naturally-Occurring Endophytic Nitrogen-Fixing Bacteria of Hybrid White Spruce Sustain Boreal Forest Tree Growth on Extremely Nutrient-Poor Soils? *Soil Biol. Biochem.* **2020**, *140*, 107642. [CrossRef]

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Putative biocontrol agents for European forest pathogens found in English oak (*Quercus robur* L.) endosphere

Dorotėja VAITIEKŪNAITĖ, Greta STRIGANAVIČIŪTĖ, Emilija BENIUŠYTĖ, Vaida SIRGEDAITĖ-ŠĖŽIENĖ, Milana AUGUSTAUSKAITĖ

Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry
Liepų St. 1, Girionys, 53101 Kaunas dist., Lithuania
E-mail: doroteja.vaitiekunaite@lammc.lt

Abstract

In Europe, pathogens such as *Heterobasidion annosum*, *Hymenoscyphus fraxineus*, *Lophodermium seditiosum*, and *Phellinus tremulae* cause extensive tree loss. Microorganisms used as biocontrol agents (BCAs) can be an eco-friendly alternative to chemical fungicides and thus help control pathogen growth and spread in a sustainable manner. Six fungi: *Byssochlamys spectabilis*, *Talaromyces amestolkiae*, *Aspergillus salwaensis*, *Neocucurbitaria quercina*, *Meyerozyma guilliermondii*, and *Microstroma bacarum*, were isolated from pedunculate English oak (*Quercus robur* L.), and together with bacteria *Bacillus aryabhattai*, *Pseudomonas azotoformans*, and *Pseudomonas paralactis*, as well as two strains of *Pantoea agglomerans* were tested in dual culture assays for their antagonism against these pathogens. Additionally, their abilities to produce indole-3-acetic acid (IAA), siderophores, solubilise potassium, organic and inorganic phosphates were determined. All the isolates produced IAA and solubilised phosphates. Only *T. amestolkiae* solubilised potassium, but this fungus and *M. guilliermondii* could not produce siderophores. *M. guilliermondii*, *Pseudomonas* spp., and *Pantoea* spp. expressed broad-spectrum antagonism. *P. azotoformans* showed the highest inhibition of *P. tremulae* (57.5%) and *L. seditiosum* (68.4%), *B. spectabilis* of *H. fraxineus* (59.8%), and *M. guilliermondii* of *H. annosum* (45.7%). Overall, these results will facilitate extensive BCA research for these forest pathogens and the potential BCA commercialisation and will promote sustainable forestry practices.

Keywords: *Heterobasidion annosum*, *Hymenoscyphus fraxineus*, *Lophodermium seditiosum*, *Phellinus tremulae*, plant growth-promoting traits.

Introduction

Pathogenic fungi cause extensive forest damage every year. Current climate conditions may benefit pathogenic fungi by extending growth periods creating favourable conditions for proliferation and simultaneously negatively affecting trees by causing abiotic stress, thus overall furthering the deleterious outcome to forests (Ghelardini et al., 2016). While it is possible to limit the spread of diseases with fungicides, the reality is that today's agriculture, horticulture, and forestry sectors largely depend on chemical assistance. Fungicides ultimately pose significant long-term disadvantages to the global ecosystem and human health. A possible alternative measure to fight fungal pathogens could be the use of microbial antagonists as biocontrol agents (BCAs). The use of antagonistic microbes could have an additional benefit in slowing down the acquired pathogen resistance to fungicides. BCAs can interact with pathogens via different mechanisms: antibiosis, competition and parasitism, they may also induce plant resistance (Ons et al., 2020).

Regarding the use of BCAs in forest pathogen management, the field remains mostly unstudied, and one of the reported limitations is the lack of commercially available BCAs. Based on recent reviews, endophytes (microorganisms inhabiting internal plant tissues asymptomatically) have been linked to tree resistance to pests and pathogens; however, significant success was limited to just several cases and scant commercialisation efforts (Witzell et al., 2014). Endophytes give a key advantage with regard to biocontrol, as they can be asymptomatically internalised by host plants, thus limiting the impact of environmental changes that render some BCAs with a limited field applicability (Ons et al., 2020; Huang et al., 2021).

Fungi *Phellinus tremulae*, *Hymenoscyphus fraxineus*, *Heterobasidion annosum*, and *Lophodermium seditiosum* can be found throughout the Northern Hemisphere costing millions in damages to the agroforestry industry and also contributing to decreased

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forest health, loss of biodiversity, and deforestation (Brazee, 2015; Jansons et al., 2020; Marčiulynas et al., 2020; Kowalski, Bilanski, 2021).

P. tremulae is a rot-causing fungus that affects aspen (*Populus* spp.) stands and plantations in Europe, Russia, and North America. As poplars are extensively grown for the agroforestry industry, the monoculture poplar forests can be widely infected (Brazee, 2015). *H. fraxineus*, on the other hand, causes significant damage in Europe's forests. It affects mainly leaves and buds causing the loss of foliage and consequently the loss of vitality in *Fraxinus excelsior* throughout the European continent (ash dieback) with no effective control means to date (Kowalski, Bilanski, 2021). Both *H. annosum* and *L. seditiosum* affect conifers. *L. seditiosum* mainly affects tree nurseries and has been reported to impact fully grown trees less. However, in tree nurseries, whereas in aspen stands the trees are grown in proximity as a monoculture, this pathogen can have a detrimental impact causing severe needle cast. *H. annosum* also infects conifers, and among these four fungi is the only success story from a biological control point of view. While this pathogen has been shown to cause major loss in pine forests, another fungus, *Phlebiopsis gigantea,* has been commercialised (biological product "Rotstop") and showed efficacy under field conditions with no reported negative impact on the surrounding microbiota, when cut stumps were inoculated thoroughly (Kenigsvalde et al., 2011; Mesanza et al., 2016; Jansons et al., 2020).

In addition, to providing plants with health benefits, microorganisms can serve as plant growth promoters. They can facilitate improved nutrient uptake via phosphate and potassium solubilisation, organic phosphate mineralisation, and iron transport agent – siderophore – production or be beneficial due to phytohormone (e.g., indole-3-acetic (IAA)) production and nitrogen fixation (bacteria). Collectively, these are called plant growth-promoting traits (PGPTs). It is not uncommon that the same microorganism species can be beneficial in several ways, both in terms of providing several PGPTs simultaneously helping with pathogen attacks (Dolatabad et al., 2017; Ons et al., 2020; Vaitiekūnaitė et al., 2021).

Thus, it was hypothesised that *Quercus robur* endophytes may possess PGPTs that could help improve plant growth and that *Q. robur* endophytes may inhibit the growth of the four (*H. annosum*, *H. fraxineus*, *L. seditiosum*, and *P. tremulae*) forest pathogens.

Material and methods

The research was conducted from the 2020s up to 2022s.

Pathogenic fungi isolation and identification. The isolation and identification of *Hymenoscyphus fraxineus* and *Phellinus tremulae* were previously described (Striganavičiūtė et al., 2021a; b). *Lophodermium seditiosum* (DSM 5029) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Putative *Heterobasidion annosum* strains were sampled by finding fruiting fungal bodies at stump level on *Pinus sylvestris* trees. The obtained fruiting bodies were surface sterilised with a gas flame placed on Petri dishes containing standard Hagem agar. Unless stated otherwise, all media and their components were purchased from Duchefa Biochemie B.V. (The Netherlands) and incubated at 20°C temperature until visible mycelium growth. Pure cultures were isolated and used for molecular identification. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) DNA extraction protocol (Doyle, Doyle, 1987). To identify/detect *H. annosum*, the polymerase chain reaction (PCR) using species-specific primers MJ-F and MJ-R was used (Hantula, Vainio, 2003). Amplification reagents were 0.25 ng μl^{-1} of template DNA, 200 µM of dNTPs, 750 µM of $MgCl_2$, 0.025 µM of DreamTaq Green polymerase (5U μl^{-1}) (Thermo Scientific, USA), and 200 nM of each primer. PCR conditions were 94°C for 10 min, 40 cycles of 95°C for 30 s, 67°C for 35 s, 72°C for 1 min, and finally 7 min at 72°C. For amplicon visualization, 1% agarose gel with gel stain Nancy-520 (Sigma-Aldrich, Sweden) and a transilluminator UVIDOC HD6 (Uvitec, UK) were used. If PCR amplicons were present, then these samples were assigned to *H. annosum*.

Oak-associated endophytic fungi isolation and identification. Five field-grown trees of English oak (*Quercus robur* L.) were used for endophytic fungi isolation. Sterilisation and isolation were done as previously described (Vaitiekūnaitė et al., 2021). Microorganisms that emerged were transferred onto a solid malt medium (MM), pH 5.6 ± 0.1 (Striganavičiūtė et al., 2021a). Colonies of morphologically different fungi were separated and purified. Yeast isolates were differentiated from bacteria using 1000× magnification with 0.1% Gentian violet dye (Carl Roth, Germany) based on cell shape and budding. Separated yeast colonies were further purified using the quadrant streaking method. Purified isolates were sent to sequencing centre for DNA extraction and *ITSrRNA* region sequencing (Macrogen Europe BV, The Netherlands). Universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (ITS1 region forward primer) and ITS4 (5'TCCTCCGCTTA TTGATATGC-3') (ITS2 region reverse primer) were used. Sequences were edited using open-access software BioEdit, version 7.2.5 (Informer Technologies, Inc.). Subsequently, fragments were matched with the National Center for Biotechnology Information's (NCBI) database (BLAST Targeted Loci Nucleotide System) for fungi.

Plant growth-promoting traits (PGPTs) screening was done in independent biological quintuplicates using fresh colonies each time: 1-week-old mycelial fungi cultures and 2-day-old yeast colonies. A stable pH of 5.6 ± 0.1 was kept throughout the experiment. Unless stated otherwise, microorganisms were kept in the dark at 22 ± 1°C temperature. Fungi were tested for phosphate solubilisation and mineralisation, potassium solubilisation, siderophore, and IAA production. Bacteria described in this study were previously tested for all PGPTs (Vaitiekūnaitė et al., 2021), except potassium solubilisation.

Indole-3-acetic acid (IAA) production. Isolates were tested for tryptophan-dependent phytohormone IAA production utilising a modified method (Vaitiekūnaitė et al., 2021). Briefly, mycelial plugs (5 mm) from the edges and scrapes from yeast colonies were transferred into individual tubes with MM with added tryptophan

(0.15% w/v) and grown in a thermal shaker (1× *g*) at 25°C temperature in the dark (3 days for yeasts and 7 days for mycelial fungi). Afterward, the methodology was the same as described by Vaitiekūnaitė et al. (2021).

Siderophore production. Isolates were tested for siderophore production using a modified chromeazurol S (CAS) assay (Vaitiekūnaitė et al., 2021). The reagent was mixed 1:9 with autoclaved solid Lysogeny broth (LB) medium prior to solidification. Yeast isolates were then spot inoculated and incubated for a week. Plugs (5 mm) from the edge of mycelial fungi cultures were placed on top of the solid LB medium (mycelium side down) and, also, incubated for a week. Orange/yellow areas around the colonies were indicative of siderophore production.

Phosphate solubilisation and mineralisation. Isolates were tested for phosphate solubilisation and mineralisation using an agar plate method (Vaitiekūnaitė et al., 2021). Inoculation of the medium was done as previously described. The phosphate solubilisation index (PSI) was calculated for both variants (Sane, Mehta, 2015).

Potassium (K) solubilisation. Isolates were also screened for K solubilisation using Alexandrov's agar (HiMedia, India), which utilises feldspar as a K source (Fatharani, Rahayu, 2018). Isolates (including the bacteria using fresh colony scrapes) were individually cultured on solid Alexandrov's medium, as previously described, for 7 days. Clear areas around the colonies were indicative of K solubilisation.

Dual culture antagonism assay. Four mycelial fungi, two yeasts, and five bacteria were tested against four tree pathogenic fungi: *H. annosum*, *H. fraxineus*, *L. seditiosum*, and *P. tremulae*. Prior to this experiment, bacteria were selected from a larger group based on growth potential on solid MM. Antagonism screening was done in independent biological quintuplicates using fresh colonies each time.

A dual co-cultivation assay for yeast and bacteria was done (Ulrich et al., 2020). Briefly, plugs (5 mm) of pathogenic fungi were transferred onto freshly prepared solid MM (filled halfway up the plate) and placed in the centre of the Petri dish (Ø9 cm for *H. annosum*, *H. fraxineus*, and *L. seditiosum* and Ø5 cm for *P. tremulae* due to very slow growth) mycelial side down. Then four smears from yeast or bacteria colonies were streaked around the plug in the shape of a square, one isolate – one pathogenic fungus per plate. A plate solely with pathogenic fungi served as a control. Due to slow growth, *H. fraxineus* was plated 6 days prior to the putative antagonists (Ulrich et al., 2020), and *P. tremulae* was plated 7 days prior (Chakravarty, Hiratsuka, 1992) as suggested. Also, in order to grow *H. fraxineus*, 50 g L^{-1} of frozen *F. excelsior* leaves were added to the MM prior to autoclaving (Becker et al., 2020; Ulrich et al., 2020). The experiment was concluded when the pathogen's mycelium reached the edges of the plate in the control group. At the end of the incubation, the radius of the mycelium was measured for each replicant in three places, then averaged and further used to calculate growth inhibition:

$$I = \left(\frac{C-T}{C}\right) \times 100 (1),$$

where I is growth inhibition, C is average radius of the fungus in the control plates, and T is average radius of the pathogenic fungus in the plates with antagonist.

Similarly, a dual co-cultivation assay was done using four mycelial fungi and four pathogens (Becker et al., 2020). Plugs (5 mm) of pathogenic fungi were transferred onto freshly prepared solid MM and placed at the side of the Petri dish (1 cm from the edge) mycelial side down. Directly on the other side of the plate, a plug of potential antagonistic fungi was placed in the same manner. *H. fraxineus* and *P. tremulae* were grown for 6 and 7 days, respectively, as previously described, prior to the addition of putative antagonists. To determine the time of experiment conclusion, a plate solely with pathogenic fungus was used, as previously stated, and a negative control (pathogen with pathogen) was used as a baseline for further calculations (equation). At the end of the incubation, the radius of the primary pathogen's mycelium was measured for each replicant in three places, and radial growth inhibition was calculated (equation). *P. tremulae* was not tested against mycelial fungi due to extensive sporulation caused by the former.

Statistical analysis. To compare mean ranks of the groups, antagonism data was checked for significant differences using the Kruskal-Wallis H test. Further pairwise comparisons were made with a *post hoc* Dunn's test with SPSS, version 28.0.1.1 (IBM Inc.). Mean values were expressed as percentages ± standard error (SE). Microorganisms exhibiting statistically significant ($p < 0.05$) >30% antagonism were considered potential candidates for future trials (Becker et al., 2020).

Results

Oak-associated endophytic fungi isolation, identification, and plant growth-promoting traits (PGPTs). All six microorganisms isolated during the experiment were successfully identified to genus level via *ITS rRNA* region sequencing. Five belong to the *Ascomycota* phylum, except for *M. bacarum*, which belongs to *Basidiomycota*. ITS1 data is shown in Table 1. Query coverage of over 98% was achieved for all isolates. Five isolates showed >97% identity match, except *M. guilliermondii*, and the closest DNA match was >93%.

All isolates were capable of IAA production with *N. quercina* and *A. salwaensis* exhibiting the highest relative values after 7-day incubation and *M. guilliermondii* exhibiting higher values than *M. bacarum* after 3-day incubation (Table 2). Similarly, all isolates were capable of both phosphate solubilisation and mineralisation. No significant siderophore production was also observed with *T. amestolkiae* and *M. guilliermondii* expressing little to no changes within the CAS culture medium. Fungi were also tested for K solubility with only *T. amestolkiae* expressing any significant solubilisation.

Additionally, five previously isolated and evaluated bacteria were also used for further antagonism assay. During the experiment, they were also tested for qualitative K solubilisation. Except for *B. aryabhattai*, all isolates were capable of K solubilisation qualitatively.

Antagonism assay. Pathogenic *H. annosum* was isolated and identified using morphological and region sequencing data. For the antagonism assay, together with purchased *L. seditiosum*, previously isolated *H. fraxineus* and *P. tremulae* were used. Isolate potential for

Table 1. Identification data of the microorganisms used in the experiment

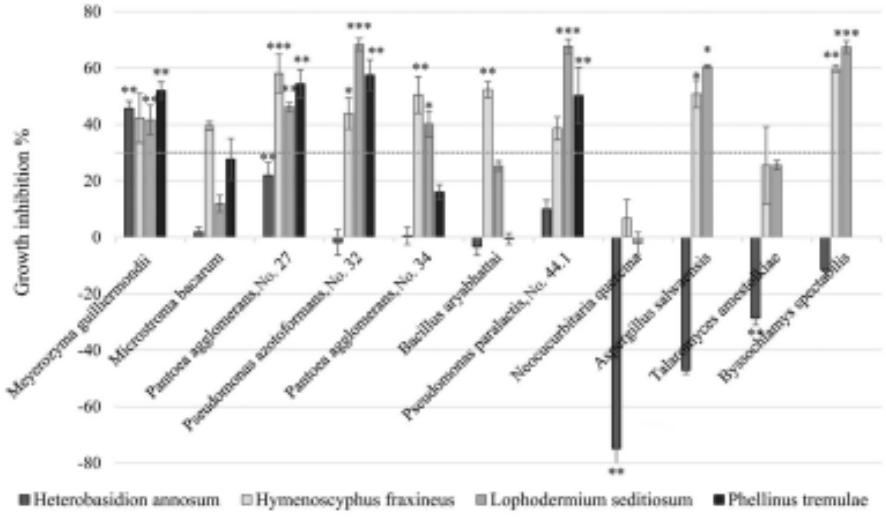
Closest NCBI match, accession No.	GenBank accession No.	Fragment length bp (ITS1)	% identity	Query coverage %
Neocucurbitaria quercina, NR_160056.1	OP896095	522	99.03	98
Aspergillus salwaensis, NR_135455.1	OP896096	539	99.3	100
Talaromyces amestolkiae, NR_120179.1	OP896097	578	99.81	100
Byssochlamys spectabilis (syn. *Paecilomyces variotii*), NR_130679.1	OP896098	567	97.19	98
Meyerozyma guilliermondii (syn.*Candida guilliermondii*), NR_111247.1	OP896099	569	93.76	98
Microstroma bacarum (syn. *Rhodotorula bacarum*, *Torulopsis bacarum*), NR_153481.1	OP896100	642	99.82	98
Pantoea agglomerans, NR_041978.1	OP895923*			
Pseudomonas azotoformans, NR_113600.1	OP895924			
Pseudomonas paralactis, NR_156987.1	OP895925	Previously reported by Vaitiekūnaitė et al. (2021)		
Pantoea agglomerans, NR_041978.1	OP895926			
Bacillus aryabhattai, NR_115953.1	OP895927			

Note. * – isolates 34 and 27 were differentiated based on biochemical differences (Vaitiekūnaitė, Snitka 2021).

Table 2. Screening results of plant growth-promoting traits (PGPTs) of all tested potential antagonists

Isolate	IAA production ± SE µl ml^{-1}	Inorganic phosphate solubilisation index (PSI)	Organic phosphate mineralisation index (PSI)	Siderophore production	Potassium solubilisation
Neocucurbitaria quercina	128.480 ± 0.012	+ (1.46)	+ (1.09)	+	–
Aspergillus salwaensis	121.700 ± 0.047	+ (0)	+ (0)	+	–
Talaromyces amestolkiae	38.367.0.001	+ (1.32)	+ (1.23)	–	+
Byssochlamys spectabilis	54.300 ± 0.002	+ (0)	+ (0)	+	–
Meyerozyma guilliermondii	54.887 ± 0.003	+ (1.47)	+ (2.44)	–	–
Microstroma bacarum	19.387 ± 0.001	+ (0)	+ (0)	+	–
Pantoea agglomerans					+
Pseudomonas azotoformans	Previously reported by Vaitiekūnaitė et al. (2021)				+
Pseudomonas paralactis					+
Pantoea agglomerans					+
Bacillus aryabhattai					–

+/– – indicates a positive or negative response, respectively



Note. Significance was calculated with Kruskal-Wallis H test on ranks followed by pairwise comparisons with Dunn's test: *, **, and *** – $p < 0.05$, $p < 0.01$, and $p < 0.001$; the dotted line denotes >30% antagonism baseline, over which an isolate is considered a potential antagonist, provided the data is significant.

Figure. Mean percentage of growth inhibition of four European forest pathogens (±SE)

antagonism in a dual culture against four tree pathogenic fungi was evaluated *in vitro*. Growth inhibition >30% was considered the minimal level for future field and *in planta* studies. Only *M. guilliermondii* was effective as antagonist to *H. annosum* (45.7%) (Figure). Interestingly, all the tested mycelial fungi increased the growth rate of *H. annosum*, but only *N. quercina* and *T. amestolkiae* did so significantly. Six isolates exhibited significant >30% antagonism against *H. fraxineus*: *B. spectabilis* 59.8%, *P. agglomerans* (isolate No. 27) 58.0%, *B. aryabhattai* 52.4%, *A. salwaensis* 50.8%, *P. agglomerans* (No. 34) 50.5%, and *P. azotoformans* 43.8%. Comparatively, seven isolates showed an antagonistic potential against *L. seditiosum* with pseudomonads being relatively the most effective: *P. azotoformans* 68.4% and *P. paralactis* 67.7%. Two mycelial fungi followed: *B. spectabilis* 67.5%, *A. salwaensis* 60.5%, then *P. agglomerans* (No. 27) 46.2%, *M. guilliermondii* 41.7%, and *P. agglomerans* (No. 34) 40.2%. *P. tremulae* had four effective antagonists among the yeasts and bacteria tested: *P. azotoformans*, *P. agglomerans* (No. 27), *M. guilliermondii*, and *P. paralactis* with antagonism values of 57.5, 54.4, 52.1, and 50.3 %, respectively.

M. guilliermondii was effective against all pathogens tested, except *H. fraxineus* exhibiting 41.7% to 52.1% effectiveness. *Pseudomonas* spp. were effective against three fungi: *P. tremulae, H. fraxineus*, and *L. seditiosum*. Similarly, *P. agglomerans* bacteria strains also exhibited a good level of antagonism against *P. tremulae, H. fraxineus*, and *L. seditiosum*. Mycelial fungi *B. spectabilis* and *A. salwaensis* visually out-grew the pathogenic fungi in their culture plates; *B. aryabhattai* only inhibited the growth of *H. fraxineus*.

Each pathogenic fungus had at least one viable antagonist among the strains studied. As previously mentioned, *M. guilliermondii* was the only antagonist to *H. annosum* (45.7%), while bacteria *P. azotoformans* expressed high effectiveness against both *P. tremulae* (57.5%) and *L. seditiosum* (68.4%). Mycelial fungus *B. spectabilis* was the most effective against ash pathogen *H. fraxineus* (59.8%).

Discussion

Endophyte isolation and plant growth promoting traits (PGPTs). In endophyte research, *Q. robur* trees are not a well-studied niche; however, species from the genus *Aspergillus* have been isolated from *Q. robur* previously (Kwaśna, Szewczyk, 2016), as were *Microstroma* sp. (Isaeva et al., 2009). While, to our knowledge, *M. guilliermondii* (syn. *Candida guilliermondii*) has never been isolated from a *Quercus* tree, other *Candida* species have been detected (Isaeva et al., 2009). Previously, *B. spectabilis* (syn. *Paecilomyces variotii*) was isolated from *Pistacia* tree (Dolatabad et al., 2017), and recently, *Talaromyces* species were isolated from *Pinus* tree (Sun et al., 2022). On the other hand, *Paecilomyces formosus* was recently linked with *Q. brantii* dieback in Iran (Sabernasab et al., 2019).

During the experiment, all tested isolates showed capacity for several PGPTs. Phosphate solubilisation and mineralisation as well as K solubilisation and siderophore production were observed. IAA production was ubiquitous. Among the mycelial fungi, the highest relative amount was produced by *N. quercina* at 128 µl ml^{-1} after a week of incubation, and *M. guilliermondii* produced more than *M. bacarum* at 55 µl ml^{-1} after 72 h of incubation. As previously mentioned, microorganisms usually serve several purposes in a plant's ecosystem – they may provide both growth-promoting benefits as well as protective capacity (Ons et al., 2020; Vaitiekūnaitė et al., 2021). Additionally, some PGPT tests may point toward a reason for potential antagonism to pathogens. For example, a siderophore-producing endophyte may lead to competition with the pathogen for available iron, i.e., protecting the host plant by outcompeting the pathogen for resources (Huang et al., 2021).

A comparative study of *B. spectabilis* reported it to be an effective plant growth promoter for tomatoes and peppers. The isolate showed high productivity of siderophores and IAA, but low solubilisation of phosphates (Moreno-Gavíra et al., 2020). Similarly, *M. guilliermondii* promoted durum wheat growth (shoot, root length, and biomass) and boosted photosynthesis and nitrogen balance rates (Kthiri et al., 2021). After inoculation with *Aspergillus niger*, *Q. robur* seedlings also exhibited enhanced root, stem, and leaf biomass (Kwaśna, Szewczyk, 2016). Both *Aspergillus* sp. and *Talaromyces* sp. were previously reported to solubilise phosphates (Doilom et al., 2020). Additionally, *Microstroma* sp. has been shown to produce a high level of IAA (Peng et al., 2018).

Dual culture antagonism assay. In recent years, the biocontrol of pathogenic *P. tremulae* has not been widely researched; however, there have been some studies related to biocontrol in the 1990s. *Peniophora polygonia* was shown to exhibit antagonistic activity against *P. tremulae* (Chakravarty, Hiratsuka, 1992). *Phoma etheridgei* is another fungus that expressed antagonistic activities both *in vitro* and on aspen wood chips (Hutchison et al., 1994). During current experiment, it was noticed that *P. tremulae* growth inhibited four of tested microorganisms: *Pseudomonas* spp., *P. agglomerans*, and *M. guilliermondii* (Figure).

In 1992, *H. fraxineus* for the first time was observed in Poland and since then has spread throughout the European continent (Kowalski, Bilanski, 2021). This invasive fungus causes severe ash dieback, which is why significant efforts are being made to study the potential of BCAs. So far as we were able to determine that widespread effective commercial treatments exist to combat this infection. However, various fungicides (e.g., Carbendazim) and urea treatments were reported and resulted in a varying level of success (Hauptman et al., 2014).

Over the past decade, many fungal and bacterial species have been reported to have an antagonistic effect on *H. fraxineus in vitro* showing promising results. Becker et al. (2020) and Ulrich et al. (2020) tested both fungi and bacteria against two genotypes of *H. fraxineus*. In their studies, *Bacillus* sp. inhibited both genotypes up to 55%, and *Pantoea* sp. and *Pseudomonas* sp. also exhibited a relatively high level of inhibition (ranging from 40% to 48%). During the experiment, *B. aryabhattai* also inhibited *H. fraxineus* (52.4%), and *Pseudomonas* spp. and two *P. agglomerans* strains inhibited *H. fraxineus*

growth from 38.6–43.8% and 50.5–58%, respectively (Figure). Furthermore, Becker et al. (2020) study with fungal antagonists revealed that some yeasts (*Papiliotrema flavescens* and *Vishniacozyma carnescens*) and yeast-like fungi (*Aureobasidium pullulans*) exhibited inhibition up to 50%, but only for one of two tested *H. fraxineus* genotypes. During current research, mycelial fungi *A. salwaensis* and *B. spectabilis* inhibited *H. fraxineus* growth by 50.8% and 59.8%, respectively (Figure).

L. seditiosum is a conifer needle cast-causing fungus that is particularly harmful to young trees. Fungicides such as Fluazinam or Azoxystrobin, among others, have been used in the past to control outbreaks (Stenström, Arvidsson, 2001), but fungicide use is typically limited to tree nurseries. There is very little information on the biocontrol of *L. seditiosum*; however, during the current experiment, seven potential antagonists of *L. seditiosum* were identified with inhibition ranging from 40.2% to 68.4% (Figure). The most successful in slowing *L. seditiosum* growth *in vitro* were *Pseudomonas* spp.; however, all the microorganisms studied expressed antagonistic activity.

H. annosum is another pathogenic fungus that attacks conifers. In terms of biocontrol, fungus *P. gigantea* under various brand names has been successfully used for some time to control the spread of *H. annosum* after tree felling, mainly by outcompeting it but also by direct antagonism. It should be noted that *P. gigantea* is a wood-decaying fungus, and its use may be limited when it comes to stopping the disease in living trees. Moreover, treatments with "Rotstop" were mostly effective for pine trees but less for spruce (Kenigsvalde et al., 2011). Other species also have been tested for biocontrol potential with varying success rates, in particular *Pseudomonas protegens* had an antagonistic effect on *H. annosum in vitro* as well as a culture filtrate *in vitro* and on wood disks. The results showed a high level of inhibition similar to that of *P. gigantea*-based products (Pellicciaro et al., 2021). Mesanza et al. (2016) tested *Pseudomonas fluorescens* and *Bacillus simplex* against *H. annosum* with promising results. In the current experiment, *B. aryabhattai* and two tested pseudomonad isolates had no observable effect on *H. annosum* growth, and only *M. guilliermondii* showed growth inhibition (45.7%) (Figure). It should be noted that *N. quercina* and *T. amestolkiae* enhanced *H. annosum* growth, possibly due to the production of bioactive compounds that stimulate *H. annosum*. Furthermore, during the experiment, *M. guilliermondii* (syn. *Candida guilliermondii*) inhibited the growth of *H. annosum*, *P. tremulae*, and *L. seditiosum* indicating broad-spectrum antagonistic properties. In recent years, *M. guilliermondii* was studied concerning antagonism to various pathogenic fungi, perhaps most notably concerning apple and lemon post-harvest pathogens (Agirman, Erten, 2020).

Similarly, during the current experiment, *P. agglomerans* strains and *Pseudomonas* spp. also exhibited antagonistic properties against three fungi: *H. fraxineus*, *P. tremulae*, and *L. seditiosum*. Study of Cañamás et al. (2008) reported the biocontrol properties of *P. agglomerans*. As with *M. guilliermondii*, the focus is mainly on orchard protection and post-harvest diseases.

Many species within the genus *Pseudomonas* have been reported to be antagonists to various pathogens. As previously mentioned, a study on antagonistic properties of *P. protegens* was reported for *H. annosum* fungus. There are also reported commercial BCAs made from *Pseudomonas* sp. (Pellicciaro et al., 2021). Overall, both pseudomonads and *Bacillus* spp. are well known for their abilities to induce plant resistance to pathogens and serve as BCAs. BCAs containing *Bacillus subtilis* are widely studied and used as well (Santoyo et al., 2012). As previously mentioned, during the current experiment, the antagonistic activity of *B. aryabhattai* against *H. fraxineus* was investigated (inhibition 52.4%).

Out of the four mycelial fungi tested, only *B. spectabilis* and *A. salwaensis* showed biocontrol potential during the experiment. During *in vitro* dual culture assay, they often overgrew their pathogenic counterparts (*H. fraxineus* and *L. seditiosum*). According to Kowalski and Bilanski (2021), such behaviour could be indicative of biocontrol potential in the field.

B. spectabilis has been reported as a BCA. Furthermore, under the brand name "ZhiNengCong" (ZNC), the crude extracts of *B. spectabilis* are used as a BCA in China. However, as with other antagonists reported in the experiment, the focus is on crop and orchard protection without any research regarding forest trees. The genus *Byssochlamys* is known to produce species with growth-promoting properties (Lu et al., 2019).

Mahendran et al. (2021) found that *Aspergillus terreus* exhibited a high level of growth inhibition (up to 100%) against both pathogens *in vitro* and *in vivo*. Interestingly, a study with *Aspergillus niger* reported trials with two *Phellinus* species, *P. badius* and *P. pachyphloeus* (mean inhibition ranging from 39% to 52%), among other wood-decaying fungi (Tiwari et al., 2011). During the current experiment, it was impossible to successfully test *A. salwaensis* and *B. spectabilis* in dual cultures with *P. tremulae* due to extreme sporulation caused by the latter.

Conclusions

1. Six fungal endophytes were isolated from *Quercus robur*. To our knowledge, *Byssochlamys spectabilis*, *Talaromyces amestolkiae*, and *Meyerozyma guilliermondii* were first isolated from the endosphere of *Q. robur*.

2. All the isolates exhibited at least several plant growth-promoting traits (PGPTs), potentially leading to plant growth enhancements *in vivo*.

3. *M. guilliermondii* yeast together with previously isolated bacteria *Pseudomonas azotoformans* and *Pantoea agglomerans* showed a broad spectrum and a high level of inhibition against significant European forest pathogens: *Heterobasidion annosum*, *Hymenoscyphus fraxineus*, *Lophodermium seditiosum*, and *Phellinus tremulae*.

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References

Agirman B., Erten H. 2020. Biocontrol ability and action mechanisms of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 against *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 causing postharvest diseases. Yeast, 37 (9–10): 437–448. https://doi.org/10.1002/yea.3501

Becker R., Ulrich K., Behrendt U., Kube M., Ulrich A. 2020. Analyzing ash leaf-colonizing fungal communities for their biological control of *Hymenoscyphus fraxineus*. Frontiers in Microbiology, 11: 590944. https://doi.org/10.3389/fmicb.2020.590944

Brazee N. J. 2015. Phylogenetic relationships among species of *Phellinus sensu stricto*, cause of white trunk rot of hardwoods, from Northern North America. Forests, 6 (11): 4191–4211. https://doi.org/10.3390/f6114191

Cañamás T. P., Viñas I., Usall J., Torres R., Anguera M., Teixidó N. 2008. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2. Part II. Effectiveness of different cell formulations. Postharvest Biology and Technology, 49 (1): 96–106. https://doi.org/10.1016/j.postharvbio.2007.12.005

Chakravarty P., Hiratsuka Y. 1992. Antagonism of two decay fungi, *Peniophora polygonia* and *Phellinus tremulae* associated with *Populus tremuloides*. European Journal of Forest Pathology, 22 (6–7): 354–361. https://doi.org/10.1111/j.1439-0329.1992.tb00307.x

Doilom M., Wei Guo J., Phookamsak R., Mortimer P. E., Karunarathna S. C., Dong W., Liao C. F., Yan K., Pem D., Suwannarach N., Promputtha I., Lumyong S., Xu J. C. 2020. Screening of phosphate-solubilizing fungi from air and soil in Yunnan, China: Four novel species in *Aspergillus*, *Gongronella*, *Penicillium*, and *Talaromyces*. Frontiers in Microbiology, 11: 585215. https://doi.org/10.3389/fmicb.2020.585215

Dolatabad H. K., Javan-Nikkhah M., Shier W. T. 2017. Evaluation of antifungal, phosphate solubilisation, and siderophore and chitinase release activities of endophytic fungi from *Pistacia vera*. Mycological Progress, 16 (8): 777–790. https://doi.org/10.1007/s11557-017-1315-z

Doyle J. J., Doyle J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, 19 (1): 11–15.

Fatharani R., Rahayu Y. S. 2018. Isolation and characterization of potassium-solubilizing bacteria from paddy rhizosphere (*Oryza sativa* L.). Journal of Physics: Conference Series, 1108 (1): 012105. https://doi.org/10.1088/1742-6596/1108/1/012105

Ghelardini L., Pepori A. L., Luchi N., Capretti P., Santini A. 2016. Drivers of emerging fungal diseases of forest trees. Forest Ecology and Management, 381: 235–246. https://doi.org/10.1016/j.foreco.2016.09.032

Hantula J., Vainio E. 2003. Specific primers for the differentiation of *Heterobasidion annosum* (s.str.) and *H.parviporum* infected stumps in Northern Europe. Silva Fennica, 37 (2): 181–187. https://doi.org/10.14214/sf.500

Hauptman T., Celar F. A., De Groot M., Jurc D. 2014. Application of fungicides and urea for control of ash dieback. IForest, 8: 165–171. https://doi.org/10.3832/ifor1272-008

Huang X., Ren J., Li P., Feng S., Dong P., Ren M. 2021. Potential of microbial endophytes to enhance the resistance to postharvest diseases of fruit and vegetables. Journal of the Science of Food and Agriculture, 101 (5): 1744–1757. https://doi.org/10.1002/jsfa.10829

Hutchison L. J., Chakravarty P., Kawchuk L. M., Hiratsuka Y. 1994. *Phoma etheridgei* sp. nov. from black galls and cankers of trembling aspen (*Populus tremuloides*) and its potential role as a bioprotectant against the aspen decay pathogen *Phellinus tremulae*. Canadian Journal of Botany, 72 (10): 1424–1431. https://doi.org/10.1139/b94-175

Isaeva O. V., Glushakova A. M., Yurkov A. M., Chernov I. Yu. 2009. The yeast *Candida railenensis* in the fruits of English oak (*Quercus robur* L.). Microbiology, 78 (3): 355–359. https://doi.org/10.1134/S002626170903014X

Jansons A., Zeltiņš P., Donis J., Neimane U. 2020. Long-term effect of *Lophodermium* needle cast on the growth of Scots pine and implications for financial outcomes. Forests, 11 (7): 718. https://doi.org/10.3390/f11070718

Kenigsvalde K., Donis J., Korhonen K., Gaitnieks T. 2011. Biological control of *Heterobasidion* root rot of coniferous stumps by *Phlebiopsis gigantea* – literature review. Mežzinātne [Forest Science], 23 (56): 25–40 (in Latvian). https://www.yumpu.com/lv/document/read/28174283/phlebiopsis-gigantea-skujkoku-celmu-bioloaiskaja-silava/13

Kowalski T., Bilanski P. 2021. Fungi detected in the previous year's leaf petioles of *Fraxinus excelsior* and their antagonistic potential against *Hymenoscyphus fraxineus*. Forests, 12: 1412. https://doi.org/10.3390/f12101412

Kthiri Z., Jabeur M., Chairi F., López-Cristoffanini C., López-Carbonell M., Serret M. D., Araus J. L., Karmous C., Hamada W. 2021. Exploring the potential of *Meyerozyma guilliermondii* on physiological performances and defense response against *Fusarium* crown rot on durum wheat. Pathogens, 10 (1): 52. https://doi.org/10.3390/pathogens10010052

Kwaśna H., Szewczyk W. 2016. Effects of fungi isolated from *Quercus robur* roots on growth of oak seedlings. Dendrobiology, 75: 99–112. https://doi.org/10.12657/denbio.075.010

Lu C., Liu H., Jiang D., Wang L., Jiang Y., Tang S., Hou X., Han X., Liu Z., Zhang M., Chu Z., Ding X. 2019. *Paecilomyces variotii* extracts (ZNC) enhance plant immunity and promote plant growth. Plant and Soil, 441 (1–2): 383–397. https://doi.org/10.1007/s11104-019-04130-w

Mahendran T. R., Thottathil G. P., Surendran A., Nagao H., Sudesh K. 2021. Biocontrol potential of *Aspergillus terreus*, endophytic fungus against *Rigidoporus microporus* and *Corynespora cassiicola*, pathogens of rubber tree. Archives of Phytopathology and Plant Protection, 54 (15–16): 1014–1032. https://doi.org/10.1080/03235408.2021.188495232

Marčiulynas A., Sirgedaitė-Šėžienė V., Žemaitis P., Jansons A., Baliuckas V. 2020. Resistance of Scots pine half-sib families to *Heterobasidion annosum* in progeny field trials. Silva Fennica, 54 (4): 10276. https://doi.org/10.14214/sf.10276

Mesanza N., Iturritxa E., Patten C. L. 2016. Native rhizobacteria as biocontrol agents of *Heterobasidion annosum* s.s. and *Armillaria mellea* infection of *Pinus radiata*. Biological Control, 101: 8–16. https://doi.org/10.1016/j.biocontrol.2016.06.003

Moreno-Gavira A., Diánez F., Sánchez-Montesinos B., Santos M. 2020. *Paecilomyces variotii* as a plant-growth promoter in horticulture. Agronomy, 10 (4): 597. https://doi.org/10.3390/agronomy10040597

Ons L., Bylemans D., Thevissen K., Cammue B. P. A. 2020. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. Microorganisms, 8 (12): 1930. https://doi.org/10.3390/microorganisms8121930

Pellicciaro M., Lione G., Giordano L., Gonthier P. 2021. Biocontrol potential of *Pseudomonas protegens* against *Heterobasidion* species attacking conifers in Europe. Biological Control, 157: 104583. https://doi.org/10.1016/j.biocontrol.2021.104583

Peng X., Wang Y., Tang L. J., Li X. X., Xiao Y. W., Zhang Z. B., Yan R. M., Yang H. L., Chang J., Zhu B., Zhu D. 2018. Yeasts from Nanfeng mandarin plants: occurrence, diversity and capability to produce indole-3-acetic acid. Biotechnology and Biotechnological Equipment, 32 (6): 1496–1506. https://doi.org/10.1080/13102818.2018.1487337

Sabernasab M., Jamali S., Marefat A., Abbasi S. 2019. Molecular and pathogenic characteristics of *Paecilomyces formosus*, a new causal agent of oak tree dieback in Iran. Forest Science, 65 (6): 743–750. https://doi.org/10.1093/forsci/fxz045

Sane S. A., Mehta S. K. 2015. Isolation and evaluation of rock phosphate solubilizing fungi as potential biofertilizer. Journal of Fertilizers and Pesticides, 06 (02): 156. https://doi.org/10.4172/2471-2728.1000156

Santoyo G., del Orozco-Mosqueda M. C., Govindappa M. 2012. Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: A review. Biocontrol Science and Technology, 22 (8): 855–872. https://doi.org/10.1080/09583157.2012.694413

Stenström E., Arvidsson B. 2001. Fungicidal control of *Lophodermium seditiosum* on *Pinus sylvestris* seedlings in Swedish forest nurseries. Scandinavian Journal of Forest Research, 16 (2): 147–154. https://doi.org/10.1080/028275801300088224

Striganavičiūtė G., Žiauka J., Sirgedaitė-Šėžienė V., Vaitiekūnaitė D. 2021 (a). Impact of plant-associated bacteria on the *in vitro* growth and pathogenic resistance against *Phellinus tremulae* of different aspen (*Populus*) genotypes. Microorganisms, 9: 1901. https://doi.org/10.3390/microorganisms9091901

Striganavičiūtė G., Žiauka J., Sirgedaitė-Šėžienė V., Vaitiekūnaitė D. 2021 (b). Priming of resistance-related phenolics: A study of plant-associated bacteria and *Hymenoscyphus fraxineus*. Microorganisms, 9: 2504. https://doi.org/10.3390/microorganisms9122504

Sun X., Xu M., Kong W., Wu F., Zhang Y., Xie X., Li D. 2022. Fine Identification and classification of a novel beneficial *Talaromyces* fungal species from masson pine rhizosphere soil. Journal of Fungi, 8 (2): 155. https://doi.org/10.3390/jof8020155

Tiwari C. K., Parihar J., Verma R. K. 2011. Potential of *Aspergillus niger* and *Trichoderma viride* as biocontrol agents of wood decay fungi. Journal of the Indian Academy of Wood Science, 8 (2): 169–172. https://doi.org/10.1007/s13196-012-0027-x

Ulrich K., Becker R., Behrendt U., Kube M., Ulrich A. 2020. A comparative analysis of ash leaf-colonizing bacterial communities identifies putative antagonists of *Hymenoscyphus fraxineus*. Frontiers in Microbiology, 11: 966. https://doi.org/10.3389/fmicb.2020.00966

Vaitiekūnaitė D., Snitka V. 2021. Differentiation of closely related oak-associated gram-negative bacteria by label-free surface enhanced raman spectroscopy (SERS). Microorganisms, 9: 1969. https://doi.org/10.3390/microorganisms9091969

Vaitiekūnaitė D., Kuusienė S., Beniušytė E. 2021. Oak (*Quercus robur*) associated endophytic *Paenibacillus* sp. promotes poplar (*Populus* spp.) root growth *in vitro*. Microorganisms, 9 (6): 1151. https://doi.org/10.3390/microorganisms9061151

Witzell J., Martin J. A., Blumenstein K. 2014. Ecological aspects of endophyte-based biocontrol of forest diseases. Verma V. C., Gange A. C. (eds.). Advances in Endophytic Research. Springer, p. 321–333. https://doi.org/10.1007/978-81-322-1575-2_17

REFERENCES

(1) Gauthier, S.; Bernier, P.; Kuuluvainen, T.; Shvidenko, A. Z.; Schepaschenko, D. G. Boreal Forest Health and Global Change. *Science (80-.).* 2015, *349* (6250), 819–822. https://doi.org/10.1126/science.aaa9092.

(2) Millar, C. I.; Stephenson, N. L. Temperate Forest Health in an Era of Emerging Megadisturbance. *Science (80-.).* 2015, *349* (6250), 823–826. https://doi.org/10.1126/science.aaa9933.

(3) Pirttilä, A. M.; Mohammad Parast Tabas, H.; Baruah, N.; Koskimäki, J. J. Biofertilizers and Biocontrol Agents for Agriculture: How to Identify and Develop New Potent Microbial Strains and Traits. *Microorganisms* 2021, *9* (4), 817. https://doi.org/10.3390/microorganisms9040817.

(4) Kandel, S.; Joubert, P.; Doty, S. Bacterial Endophyte Colonization and Distribution within Plants. *Microorganisms* 2017, *5* (4), 77. https://doi.org/10.3390/microorganisms5040077.

(5) Gouda, S.; Das, G.; Sen, S. K.; Shin, H. S.; Patra, J. K. Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. *Front. Microbiol.* 2016, *7*. https://doi.org/10.3389/fmicb.2016.01538.

(6) Suman, A.; Yadav, A. N.; Verma, P. Endophytic Microbes in Crops: Diversity and Beneficial Impact for Sustainable Agriculture. In *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 1: Research Perspectives*; 2016; pp 117–143. https://doi.org/10.1007/978-81-322-2647-5.

(7) Wani, Z. A.; Ashraf, N.; Mohiuddin, T.; Riyaz-Ul-Hassan, S. Plant-Endophyte Symbiosis, an Ecological Perspective. *Appl. Microbiol. Biotechnol.* 2015, *99* (7), 2955–2965. https://doi.org/10.1007/s00253-015-6487-3.

(8) Padda, K. P.; Puri, A.; Chanway, C. Endophytic Nitrogen Fixation - a Possible "Hidden" Source of Nitrogen for Lodgepole Pine Trees Growing at Unreclaimed Gravel Mining Sites. *FEMS Microbiol. Ecol.* 2019, *95* (11), 1–13. https://doi.org/10.1093/femsec/fiz172.

(9) Brooks, D. S.; Gonzalez, C. F.; Appel, D. N.; Filer, T. H. Evaluation of Endophytic Bacteria as Potential Biological Control Agents for Oak Wilt. *Biological Control*. 1994, pp 373–381. https://doi.org/10.1006/bcon.1994.1047.

(10) Singh, D.; Yadav, D. K.; Fatima, F. Characterization and Genetic Diversity of *Pantoea Agglomerans* Isolates Having Dual Potentiality to Suppress Growth of *Ralstonia Solanacearum* and Plant Growth Promoting Ability. *Indian Phytopathol.* 2020, *73* (4), 643–653. https://doi.org/10.1007/s42360-020-00268-1.

(11) Witzell, J.; Martin, J. A.; Blumenstein, K. Ecological Aspects of Endophyte-Based Biocontrol of Forest Diseases. In *Advances in Endophytic Research*; Verma, V. C., Gange, A. C., Eds.; Springer India, 2014; pp 321–333. https://doi.org/10.1007/978-81-322-1575-2_17.

(12) Aghai, M. M.; Khan, Z.; Joseph, M. R.; Stoda, A. M.; Sher, A. W.; Ettl, G. J.; Doty, S. L. The Effect of Microbial Endophyte Consortia on *Pseudotsuga Menziesii* and *Thuja Plicata* Survival, Growth, and Physiology across Edaphic Gradients. *Front. Microbiol.* 2019, *10* (JUN), 1–16. https://doi.org/10.3389/fmicb.2019.01353.

(13) Terhonen, E.; Blumenstein, K.; Kovalchuk, A.; Asiegbu, F. O. Forest Tree Microbiomes and Associated Fungal Endophytes: Functional Roles and Impact on Forest Health. *Forests* 2019, *10* (1). https://doi.org/10.3390/f10010042.

(14) Kour, D.; Rana, K. L.; Yadav, A. N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H. S.; Saxena, A. K. Microbial Biofertilizers: Bioresources and Eco-Friendly Technologies for Agricultural and Environmental Sustainability. *Biocatal. Agric. Biotechnol.* 2020, *23* (December 2019). https://doi.org/10.1016/j.bcab.2019.101487.

(15) Ons, L.; Bylemans, D.; Thevissen, K.; Cammue, B. P. A. Combining Biocontrol Agents with Chemical Fungicides for Integrated Plant Fungal Disease Control. *Microorganisms* 2020, *8* (12), 1–19. https://doi.org/10.3390/microorganisms8121930.

(16) De Silva, N. I.; Brooks, S.; Lumyong, S.; Hyde, K. D. Use of Endophytes as Biocontrol Agents. *Fungal Biol. Rev.* 2019, *33* (2), 133–148. https://doi.org/10.1016/j.fbr.2018.10.001.

(17) Santini, A.; Ghelardini, L.; De Pace, C.; Desprez-Loustau, M. L.; Capretti, P.; Chandelier, A.; Cech, T.; Chira, D.; Diamandis, S.; Gaitniekis, T.; Hantula, J.; Holdenrieder, O.; Jankovsky, L.; Jung, T.; Jurc, D.; Kirisits, T.; Kunca, A.; Lygis, V.; Malecka, M.; Marcais, B.; Schmitz, S.; Schumacher, J.; Solheim, H.; Solla, A.; Szabò, I.; Tsopelas, P.; Vannini, A.; Vettraino, A. M.; Webber, J.; Woodward, S.; Stenlid, J. Biogeographical Patterns and Determinants of Invasion by Forest Pathogens in Europe. *New Phytol.* 2013, *197* (1), 238–250. https://doi.org/10.1111/j.1469-8137.2012.04364.x.

(18) Latz, M. A. C.; Jensen, B.; Collinge, D. B.; Jørgensen, H. J. L. Endophytic Fungi as Biocontrol Agents: Elucidating Mechanisms in Disease Suppression. *Plant Ecol. Divers.* 2018, *11* (5–6), 555–567. https://doi.org/10.1080/17550874.2018.1534146.

(19) Khosro, M.; Yousef, S. Bacterial Biofertilizers for Sustainable Crop Production: A Review. *ARPN J. Agric. Bol. Sci.* 2012, *7* (5), 307–316.

(20) Khipla, N.; Gosal, S. K.; Gill, R. Influence of Biofertilizers and Inorganic Fertilizers on Soil Microbial Population and Enzyme Activities in Rhizosphere of Poplar. *Chem. Sci. Rev. Lett.* 2017, *6* (24), 2324–2331.

(21) Jahn, L.; Hofmann, U.; Ludwig-Müller, J. Indole-3-Acetic Acid Is Synthesized by the Endophyte

Cyanodermella Asteris via a Tryptophan-Dependent and-Independent Way and Mediates the Interaction with a Non-Host Plant. *Int. J. Mol. Sci.* 2021, *22* (5), 1–19. https://doi.org/10.3390/ijms22052651.

(22) Ma, Y.; Rajkumar, M.; Moreno, A.; Zhang, C.; Freitas, H. Serpentine Endophytic Bacterium *Pseudomonas Azotoformans* ASS1 Accelerates Phytoremediation of Soil Metals under Drought Stress. *Chemosphere* 2017, *185*, 75–85. https://doi.org/10.1016/j.chemosphere.2017.06.135.

(23) Fontana, D. C.; de Paula, S.; Torres, A. G.; de Souza, V. H. M.; Pascholati, S. F.; Schmidt, D.; Neto, D. D. Endophytic Fungi: Biological Control and Induced Resistance to Phytopathogens and Abiotic Stresses. *Pathogens* 2021, *10* (5), 1–28. https://doi.org/10.3390/pathogens10050570.

(24) Tamosiune, I.; Baniulis, D.; Stanys, V. Role of Endophytic Bacteria in Stress Tolerance of Agricultural Plants: Diversity of Microorganisms and Molecular Mechanisms. In *Probiotics in Agroecosystem*; Kumar, V., Kumar, M., Sharma, S., Prasad, R., Eds.; Springer Nature: Singapore, 2017; pp 1–29. https://doi.org/10.1007/978-981-10-4059-7_1.

(25) Dolatabad, H. K.; Javan-Nikkhah, M.; Shier, W. T. Evaluation of Antifungal, Phosphate Solubilisation, and Siderophore and Chitinase Release Activities of Endophytic Fungi from *Pistacia Vera*. *Mycol. Prog.* 2017, *16* (8), 777–790. https://doi.org/10.1007/s11557-017-1315-z.

(26) Nakayan, P.; Hameed, A.; Singh, S.; Young, L. Sen; Hung, M. H.; Young, C. C. Phosphate-Solubilizing Soil Yeast *Meyerozyma Guilliermondii* CC1 Improves Maize (*Zea Mays* L.) Productivity and Minimizes Requisite Chemical Fertilization. *Plant Soil* 2013, *373* (1–2), 301–315. https://doi.org/10.1007/s11104-013-1792-z.

(27) Chandrasekaran, M.; Chun, S. C.; Oh, J. W.; Paramasivan, M.; Saini, R. K.; Sahayarayan, J. J. *Bacillus Subtilis* CBR05 for Tomato (*Solanum Lycopersicum*) Fruits in South Korea as a Novel Plant Probiotic Bacterium (PPB): Implications from Total Phenolics, Flavonoids, and Carotenoids Content for Fruit Quality. *Agronomy* 2019, *9* (12). https://doi.org/10.3390/agronomy9120838.

(28) Park, Y. G.; Mun, B. G.; Kang, S. M.; Hussain, A.; Shahzad, R.; Seo, C. W.; Kim, A. Y.; Lee, S. U.; Oh, K. Y.; Lee, D. Y.; Lee, I. J.; Yun, B. W. *Bacillus Aryabhattai* SRB02 Tolerates Oxidative and Nitrosative Stress and Promotes the Growth of Soybean by Modulating the Production of Phytohormones. *PLoS One* 2017, *12* (3), 1–28. https://doi.org/10.1371/journal.pone.0173203.

(29) Amprayn, K. O.; Rose, M. T.; Kecskés, M.; Pereg, L.; Nguyen, H. T.; Kennedy, I. R. Plant Growth Promoting Characteristics of Soil Yeast (*Candida Tropicalis* HY) and Its Effectiveness for Promoting Rice Growth. *Appl. Soil Ecol.* 2012, *61*, 295–299. https://doi.org/10.1016/j.apsoil.2011.11.009.

(30) Anand, R.; Chanway, C. N2-Fixation and Growth Promotion in Cedar Colonized by an Endophytic Strain of *Paenibacillus Polymyxa*. *Biol. Fertil. Soils* 2013, *49*, 235–239. https://doi.org/10.1007/s00374-012-0735-9.

(31) Barra, P. J.; Inostroza, N. G.; Mora, M. L.; Crowley, D. E.; Jorquera, M. A. Bacterial Consortia Inoculation Mitigates the Water Shortage and Salt Stress in an Avocado (*Persea Americana* Mill.) Nursery. *Appl. Soil Ecol.* 2017, *111*, 39–47. https://doi.org/10.1016/j.apsoil.2016.11.012.

(32) Dutta, J.; Thakur, D. Evaluation of Multifarious Plant Growth Promoting Traits, Antagonistic Potential and Phylogenetic Affiliation of Rhizobacteria Associated with Commercial Tea Plants Grown in Darjeeling, India. *PLoS One* 2017, *12* (8), 1–24. https://doi.org/10.1371/journal.pone.0182302.

(33) Tang, Q.; Puri, A.; Padda, K. P.; Chanway, C. P. Biological Nitrogen Fixation and Plant Growth Promotion of Lodgepole Pine by an Endophytic Diazotroph *Paenibacillus Polymyxa* and Its GFP-Tagged Derivative. *Botany* 2017, *95* (6), 611–619. https://doi.org/10.1139/cjb-2016-0300.

(34) Oliveira, P.; Barriga, J.; Cavaleiro, C.; Peixe, A.; Potes, A. Z. Sustained in Vitro Root Development Obtained in *Pinus Pinea* L. Inoculated with Ectomycorrhizal Fungi. *Forestry* 2003, *76* (5), 579–587. https://doi.org/10.1093/forestry/76.5.579.

(35) Khan, Z.; Rho, H.; Firrincieli, A.; Hung, S. H.; Luna, V.; Masciarelli, O.; Kim, S. H.; Doty, S. L. Growth Enhancement and Drought Tolerance of Hybrid Poplar upon Inoculation with Endophyte Consortia. *Curr. Plant Biol.* 2016, *6*, 38–47. https://doi.org/10.1016/j.cpb.2016.08.001.

(36) Li, G. E.; Wu, X. Q.; Ye, J. R.; Hou, L.; Zhou, A. D.; Zhao, L. Isolation and Identification of Phytate-Degrading Rhizobacteria with Activity of Improving Growth of Poplar and Masson Pine. *World J. Microbiol. Biotechnol.* 2013, *29* (11), 2181–2193. https://doi.org/10.1007/s11274-013-1384-3.

(37) Haggag, L. F.; Merwad, M. A.; Shahin, M. F. M.; Fouad, A. A. Effect of NPK and Bio-Fertilizers as Soil Application on Promoting Growth of "Toffahi" Olive Seedlings under Greenhouse Condition. 2014, *10* (6), 1607–1617.

(38) Ren, H.; Qin, X.; Huang, B.; Fernández-García, V.; Lv, C. Responses of Soil Enzyme Activities and Plant Growth in a Eucalyptus Seedling Plantation Amended with Bacterial Fertilizers. *Arch. Microbiol.* 2020, *202* (6), 1381–1396. https://doi.org/10.1007/s00203-020-01849-4.

(39) Wang, P.; Wu, S. H.; Wen, M. X.; Wang, Y.; Wu, Q. S. Effects of Combined Inoculation with *Rhizophagus Intraradices* and *Paenibacillus Mucilaginosus* on Plant Growth, Root Morphology, and Physiological Status of Trifoliate Orange (*Poncirus Trifoliata* L. Raf.) Seedlings under Different Levels of Phosphorus. *Sci. Hortic. (Amsterdam).* 2016, *205*, 97–105. https://doi.org/10.1016/j.scienta.2016.04.023.

(40) Abd El-Migeed, M. M. M.; Saleh, M. M. S.; Mostafa, E. A. M. The Beneficial Effect of Minimizing

Mineral Nitrogen Fertilization on Washington Navel Orange Trees by Using Organic and Biofertilizers. *World J. Agric. Sci.* 2007, *3* (1), 80–85. https://doi.org/10.21608/jpp.2012.84988.

(41) Pellicciaro, M.; Lione, G.; Giordano, L.; Gonthier, P. Biocontrol Potential of *Pseudomonas Protegens* against *Heterobasidion* Species Attacking Conifers in Europe. *Biol. Control* 2021, *157* (March), 104583. https://doi.org/10.1016/j.biocontrol.2021.104583.

(42) Kenigsvalde, K.; Nitiša, D.; Saulite, D.; Korhonen, K.; Vulfa, L.; Nikolajeva, V.; Kļaviņa, D.; Bruna, L.; Gaitnieks, T. Impact of *Trichoderma* Sp. on the Development of *Heterobasidion Annosum* in Decayed Understory *Picea Abies* Stumps. *Proc. Latv. Acad. Sci. Sect. B Nat. Exact, Appl. Sci.* 2017, *71* (1–2), 52–56. https://doi.org/10.1515/prolas-2017-0009.

(43) Ulrich, K.; Becker, R.; Behrendt, U.; Kube, M.; Ulrich, A. A Comparative Analysis of Ash Leaf-Colonizing Bacterial Communities Identifies Putative Antagonists of *Hymenoscyphus Fraxineus*. *Front. Microbiol.* 2020, *11*. https://doi.org/10.3389/fmicb.2020.00966.

(44) Chakravarty, P.; Hiratsuka, Y. Antagonism of Two Decay Fungi, *Peniophora Polygonia* and *Phellinus Tremulae* Associated with *Populus Tremuloides*. *Eur. J. For. Pathol.* 1992, *22* (6–7), 354–361. https://doi.org/10.1111/j.1439-0329.1992.tb00307.x.

(45) Stone, J. K.; Bacon, C. W.; White, J. J. F. An Overview of Endophytic Microbes: Endophytism Defined. *Microb. endophytes* 2003, No. January, 29–33. https://doi.org/10.1163/_q3_SIM_00374.

(46) Bhardwaj, A.; Agrawal, P. K. A Review Fungal Endophytes: As a Store House of Bioactive Compound. *World J. Pharm. Pharm. Sci.* 2014, *3* (9), 228–237.

(47) Strobel, G. The Emergence of Endophytic Microbes and Their Biological Promise. *J. Fungi* 2018, *4* (57). https://doi.org/10.3390/jof4020057.

(48) Thongsandee, W.; Matsuda, Y.; Shimizu, M.; Ehara, H.; Ito, S. Isolation of Endophytic *Streptomycetes* from Above- and Belowground Organs of *Quercus Serrata*. *J. For. Res.* 2013, *18* (2), 179–189. https://doi.org/10.1007/s10310-012-0337-2.

(49) Schulz, B.; Boyle, C. What Are Endophytes? In *Microbial Root Endophytes. Soil Biology*; Springer, 2006; Vol. 9, pp 1–14. https://doi.org/10.1007/3-540-33526-9_1.

(50) Liu, H.; Carvalhais, L. C.; Crawford, M.; Singh, E.; Dennis, P. G.; Pieterse, C. M. J.; Schenk, P. M. Inner Plant Values: Diversity, Colonization and Benefits from Endophytic Bacteria. *Front. Microbiol.* 2017, *8* (DEC), 1–17. https://doi.org/10.3389/fmicb.2017.02552.

(51) Miliute, I.; Buzaite, O.; Baniulis, D.; Stanys, V. Bacterial Endophytes in Agricultural Crops and Their Role in Stress Tolerance: A Review. *Zemdirbyste* 2015, *102* (4), 465–478. https://doi.org/10.13080/z-a.2015.102.060.

(52) Rodriguez, R. J.; White Jr., J. F.; Arnold, A. E.; Redman, R. S. Fungal Endophytes Diversity and Functional Roles. *New Phytol.* 2009, *182*, 314–330. https://doi.org/10.1111/j.1469-8137.2009.02773.x.

(53) Minamiyama, H.; Shimizu, M.; Kunoh, H.; Furumai, T.; Igarashi, Y.; Onaka, H.; Yoshida, R. Multiplication of Isolate R-5 of *Streptomyces Galbus* on Rhododendron Leaves and Its Production of Cell Wall-Degrading Enzymes. *J. Gen. Plant Pathol.* 2003, *69* (1), 65–70. https://doi.org/10.1007/s10327-002-0014-y.

(54) Hardoim, P. R.; van Overbeek, L. S.; Berg, G.; Pirttilä, A. M.; Compant, S.; Campisano, A.; Döring, M.; Sessitsch, A. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiol. Mol. Biol. Rev.* 2015, *79* (3), 293–320. https://doi.org/10.1128/mmbr.00050-14.

(55) Baliuckas, V.; Pliūra, A. Genetic Variation and Phenotypic Plasticity of *Quercus Robur* Populations and Open-Pollinated Families in Lithuania. *Scand. J. For. Res.* 2003, *18* (4), 305–319. https://doi.org/10.1080/02827580310005153.

(56) Vannini, A.; Lucero, G.; Anselmi, N.; Vettraino, A. M. Response of Endophytic *Biscogniauxia Mediterranea* to Variation in Leaf Water Potential of *Quercus Cerris*. *For. Pathol.* 2009, *39* (1), 8–14. https://doi.org/10.1111/j.1439-0329.2008.00554.x.

(57) Campanile, G.; Ruscelli, A.; Luisi, N. Antagonistic Activity of Endophytic Fungi towards *Diplodia Corticola* Assessed by in Vitro and in Planta Tests. *Eur. J. Plant Pathol.* 2007, *117*, 237–246. https://doi.org/10.1007/s10658-006-9089-1.

(58) Shu, R. G.; Wang, F. W.; Yang, Y. M.; Liu, Y. X.; Tan, R. X. Antibacterial and Xanthine Oxidase Inhibitory Cerebrosides from *Fusarium* Sp. IFB-121, an Endophytic Fungus in *Quercus Variabilis*. *Lipids* 2004, *39* (7), 667–673. https://doi.org/10.1007/s11745-004-1280-9.

(59) Wang, F. W.; Jiao, R. H.; Cheng, A. B.; Tan, S. H.; Song, Y. C. Antimicrobial Potentials of Endophytic Fungi Residing in *Quercus Variabilis* and Brefeldin A Obtained from *Cladosporium* Sp. *World J. Microbiol. Biotechnol.* 2007, *23* (1), 79–83. https://doi.org/10.1007/s11274-006-9195-4.

(60) Maddau, L.; Cabras, A.; Franceschini, A.; Linaldeddu, B. T.; Crobu, S.; Roggio, T.; Pagnozzi, D. Occurrence and Characterization of Peptaibols from *Trichoderma Citrinoviride*, an Endophytic Fungus of Cork Oak, Using Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry. *Microbiology* 2009, *155* (10), 3371–3381. https://doi.org/10.1099/mic.0.030916-0.

(61) Nguyen, M. H.; Yong, J. H.; Sung, H. J.; Lee, J. K. Screening of Endophytic Fungal Isolates Against

Raffaelea Quercus-Mongolicae Causing Oak Wilt Disease in Korea. *Mycobiology* 2020, *0* (0), 1–11. https://doi.org/10.1080/12298093.2020.1830486.
(62) Kwaśna, H.; Szewczyk, W. Effects of Fungi Isolated from *Quercus Robur* Roots on Growth of Oak Seedlings. *Dendrobiology* 2016, *75*, 99–112. https://doi.org/10.12657/denbio.075.010.
(63) Tashi-Oshnoei, F.; Harighi, B.; Abdollahzadeh, J. Isolation and Identification of Endophytic Bacteria with Plant Growth Promoting. 2016. https://doi.org/10.1111/efp.12360.
(64) Bueno-Gonzalez, V.; Brady, C.; Denman, S.; Allainguillaume, J.; Arnold, D. *Pseudomonas Kirkiae* Sp. Nov., a Novel Species Isolated from Oak in the United Kingdom, and Phylogenetic Considerations of the Genera *Pseudomonas*, *Azotobacter* and *Azomonas*. *Int. J. Syst. Evol. Microbiol.* 2020, *70*, 2426–2434. https://doi.org/10.1099/ijsem.0.004055.
(65) Govindasamy, V.; Raina, S. K.; George, P.; Kumar, M.; Rane, J.; Minhas, P. S.; Vittal, K. P. R. Functional and Phylogenetic Diversity of Cultivable Rhizobacterial Endophytes of Sorghum [*Sorghum Bicolor* (L.) Moench]. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 2017, *110* (7), 925–943. https://doi.org/10.1007/s10482-017-0864-0.
(66) Tamošiūnė, I.; Stanienė, G.; Haimi, P.; Stanys, V.; Rugienius, R.; Baniulis, D. Endophytic *Bacillus* and *Pseudomonas* Spp. Modulate Apple Shoot Growth, Cellular Redox Balance, and Protein Expression Under in Vitro Conditions. *Front. Plant Sci.* 2018, *9*. https://doi.org/10.3389/fpls.2018.00889.
(67) Ahmad, W.; Wang, J.; Li, H.; Jiao, T.; Chen, Q. Trends in the Bacterial Recognition Patterns Used in Surface Enhanced Raman Spectroscopy. *TrAC - Trends Anal. Chem.* 2021, *142*, 116310. https://doi.org/10.1016/j.trac.2021.116310.
(68) Efrima, S.; Zeiri, L. Understanding SERS of Bacteria. *J. Raman Spectrosc.* 2009, *40* (3), 277–288. https://doi.org/10.1002/jrs.2121.
(69) Powers, E. M. Efficacy of the Ryu Nonstaining KOH Technique for Rapidly Determining Gram Reactions of Food-Borne and Waterborne Bacteria and Yeasts. *Appl. Environ. Microbiol.* 1995, *61* (10), 3756–3758.
(70) Bergey, D. H. *Bergey's Manual of Systematic Bacteriology - Vol 4: Bacteroidetes*; 2010. https://doi.org/10.1007/978-0-387-68572-4.
(71) Vaitiekūnaitė, D.; Bružaitė, I.; Snitka, V. Endophytes from Blueberry (*Vaccinium* Sp.) Fruit: Characterization of Yeast and Bacteria via Label-Free Surface-Enhanced Raman Spectroscopy (SERS). *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* 2022, *275* (July 5), 121158. https://doi.org/10.1016/j.saa.2022.121158.
(72) Setiawati, T. C.; Mutmainnah, L. Solubilization of Potassium Containing Mineral by Microorganisms From Sugarcane Rhizosphere. *Agric. Agric. Sci. Procedia* 2016, *9*, 108–117. https://doi.org/10.1016/j.aaspro.2016.02.134.
(73) Fatharani, R.; Rahayu, Y. S. Isolation and Characterization of Potassium-Solubilizing Bacteria from Paddy Rhizosphere (*Oryza Sativa* L.). *J. Phys. Conf. Ser.* 2018, *1108* (1). https://doi.org/10.1088/1742-6596/1108/1/012105.
(74) Tiwari, G.; Duraivadivel, P.; Sharma, S.; Hariprasad, P. 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Producing Beneficial Rhizobacteria Ameliorate the Biomass Characters of *Panicum Maximum* Jacq. by Mitigating Drought and Salt Stress. *Sci. Rep.* 2018, *8* (1), 1–12. https://doi.org/10.1038/s41598-018-35565-3.
(75) Vaičiukynė, M.; Žiauka, J.; Žūkienė, R.; Vertelkaitė, L.; Kuusienė, S. Abscisic Acid Promotes Root System Development in Birch Tissue Culture: A Comparison to Aspen Culture and Conventional Rooting-Related Growth Regulators. *Physiol. Plant.* 2019, *165* (1), 114–122. https://doi.org/10.1111/ppl.12860.
(76) Gottel, N. R.; Castro, H. F.; Kerley, M.; Yang, Z.; Pelletier, D. A.; Podar, M.; Karpinets, T.; Uberbacher, E.; Tuskan, G. A.; Vilgalys, R.; Doktycz, M. J.; Schadt, C. W. Distinct Microbial Communities within the Endosphere and Rhizosphere of *Populus Deltoides* Roots across Contrasting Soil Types. *Appl. Environ. Microbiol.* 2011, *77* (17), 5934–5944. https://doi.org/10.1128/aem.05255-11.
(77) Cregger, M. A.; Carper, D. L.; Christel, S.; Doktycz, M. J.; Labbé, J.; Michener, J. K.; Dove, N. C.; Johnston, E. R.; Moore, J. A. M.; Vélez, J. M.; Morrell-Falvey, J.; Muchero, W.; Pelletier, D. A.; Retterer, S.; Tschaplinski, T. J.; Tuskan, G. A.; Weston, D. J.; Schadt, C. W. Plant-Microbe Interactions: From Genes to Ecosystems Using Populus as a Model System. *Phytobiomes J.* 2021, *5* (1), 29–38. https://doi.org/10.1094/PBIOMES-01-20-0009-FI.
(78) Ulrich, K.; Ulrich, A.; Ewald, D. Diversity of Endophytic Bacterial Communities in Poplar Grown under Field Conditions. *FEMS Microbiol. Ecol.* 2008, *63*, 169–180. https://doi.org/10.1111/j.1574-6941.2007.00419.x.
(79) Beckers, B.; Op De Beeck, M.; Thijs, S.; Truyens, S.; Weyens, N.; Boerjan, W.; Vangronsveld, J. Performance of *16s rDNA* Primer Pairs in the Study of Rhizosphere and Endosphere Bacterial Microbiomes in Metabarcoding Studies. *Front. Microbiol.* 2016, *7*. https://doi.org/10.3389/fmicb.2016.00650.
(80) Arvind, G.; Sood, S.; Rahi, P.; Thakur, R.; Chauhan, S.; Chadha, I. C. N. Diversity Analysis of Diazotrophic Bacteria Associated with the Roots of Tea (*Camellia Sinensis* (L.) O. Kuntze). *J. Microbiol. Biotechnol.* 2011, *21* (6), 545–555. https://doi.org/10.4014/jmb.1012.12022.
(81) Carrell, A. A.; Frank, A. C. *Pinus Flexilis* and *Picea Engelmannii* Share a Simple and Consistent Needle

Endophyte Microbiota with a Potential Role in Nitrogen Fixation. *Front. Microbiol.* 2014, *5*. https://doi.org/10.3389/fmicb.2014.00333.

(82) Sapp, M.; Lewis, E.; Moss, S.; Barrett, B.; Kirk, S.; Elphinstone, J. G.; Denman, S. Metabarcoding of Bacteria Associated with the Acute Oak Decline Syndrome in England. *Forests* 2016, *7* (95). https://doi.org/10.3390/f7050095.

(83) Meaden, S.; Metcalf, C. J. E.; Koskella, B. The Effects of Host Age and Spatial Location on Bacterial Community Composition in the English Oak Tree (*Quercus Robur*). *Environ. Microbiol. Rep.* 2016, *8* (5), 649–658. https://doi.org/10.1111/1758-2229.12418.

(84) Izumi, H.; Anderson, I. C.; Killham, K.; Moore, E. R. B. Diversity of Predominant Endophytic Bacteria in European Deciduous and Coniferous Trees. *Can. J. Microbiol.* 2008, *54*, 173–179. https://doi.org/10.1139/W07-134.

(85) Ulrich, K.; Stauber, T.; Ewald, D. *Paenibacillus* - A Predominant Endophytic Bacterium Colonising Tissue Cultures of Woody Plants. *Plant Cell. Tissue Organ Cult.* 2008, *93*, 347–351. https://doi.org/10.1007/s11240-008-9367-z.

(86) Hoang, H.; Tran, L. H.; Nguyen, T. H.; Nguyen, D. A. T.; Nguyen, H. H. T.; Pham, N. B.; Trinh, P. Q.; de Boer, T.; Brouwer, A.; Chu, H. H. Occurrence of Endophytic Bacteria in Vietnamese Robusta Coffee Roots and Their Effects on Plant Parasitic Nematodes. *Symbiosis* 2019. https://doi.org/10.1007/s13199-019-00649-9.

(87) Bal, A.; Anand, R.; Berge, O.; Chanway, C. P. Isolation and Identification of Diazotrophic Bacteria from Internal Tissues of *Pinus Contorta* and *Thuja Plicata*. *Can. J. For. Res.* 2012, *42* (4), 807–813. https://doi.org/10.1139/x2012-023.

(88) Ferreira, A.; Quecine, M. C.; Lacava, P. T.; Oda, S.; Azevedo, J. L.; Araújo, W. L. Diversity of Endophytic Bacteria from Eucalyptus Species Seeds and Colonization of Seedlings by *Pantoea Agglomerans*. *FEMS Microbiol. Lett.* 2008, *287*, 8–14. https://doi.org/10.1111/j.1574-6968.2008.01258.x.

(89) Kong, P. Evaluation of a Novel Endophytic *Pseudomonas Lactis* Strain for Control of Boxwood Blight. *J. Environ. Hortic.* 2019, *37* (2), 39–43. https://doi.org/10.24266/0738-2898-37.2.39.

(90) Kwaśna, H.; Szewczyk, W.; Behnke-Borowczyk, J. Fungal Root Endophytes of *Quercus Robur* Subjected to Flooding. *For. Pathol.* 2016, *46* (1), 35–46. https://doi.org/10.1111/efp.12212.

(91) Isaeva, O. V.; Glushakova, A. M.; Yurkov, A. M.; Chernov, I. Y. The Yeast *Candida Railenensis* in the Fruits of English Oak (*Quercus Robur* L.). *Microbiology* 2009, *78* (3), 355–359. https://doi.org/10.1134/S002626170903014X.

(92) Jaklitsch, W. M.; Checa, J.; Blanco, M. N.; Olariaga, I.; Tello, S.; Voglmayr, H. A Preliminary Account of the *Cucurbitariaceae*. *Stud. Mycol.* 2018, *90*, 71–118. https://doi.org/10.1016/j.simyco.2017.11.002.

(93) Moller, L.; Lerm, B.; Botha, A. Interactions of Arboreal Yeast Endophytes: An Unexplored Discipline. *Fungal Ecol.* 2016, *22*, 73–82. https://doi.org/10.1016/j.funeco.2016.03.003.

(94) Yan, Y.; Zhang, X.; Zheng, X.; Apaliya, M. T.; Yang, Q.; Zhao, L.; Gu, X.; Zhang, H. Control of Postharvest Blue Mold Decay in Pears by *Meyerozyma Guilliermondii* and It's Effects on the Protein Expression Profile of Pears. *Postharvest Biol. Technol.* 2018, *136* (November 2017), 124–131. https://doi.org/10.1016/j.postharvbio.2017.10.016.

(95) Agirman, B.; Erten, H. Biocontrol Ability and Action Mechanisms of *Aureobasidium Pullulans* GE17 and *Meyerozyma Guilliermondii* KL3 against *Penicillium Digitatum* DSM2750 and *Penicillium Expansum* DSM62841 Causing Postharvest Diseases. *Yeast* 2020, *37* (9–10), 437–448. https://doi.org/10.1002/yea.3501.

(96) Chen, S.; Ding, M.; Liu, W.; Huang, X.; Liu, Z.; Lu, Y.; Liu, H.; She, Z. Anti-Inflammatory Meroterpenoids from the Mangrove Endophytic Fungus *Talaromyces Amestolkiae* YX1. *Phytochemistry* 2018, *146*, 8–15. https://doi.org/10.1016/j.phytochem.2017.11.011.

(97) Sun, X.; Xu, M.; Kong, W.; Wu, F.; Zhang, Y.; Xie, X.; Li, D. Fine Identification and Classification of a Novel Beneficial *Talaromyces* Fungal Species from Masson Pine Rhizosphere Soil. *J. Fungi* 2022, *8* (155). https://doi.org/10.3390/jof8020155.

(98) Sabernasab, M.; Jamali, S.; Marefat, A.; Abbasi, S. Molecular and Pathogenic Characteristics of *Paecilomyces Formosus*, a New Causal Agent of Oak Tree Dieback in Iran. *For. Sci.* 2019, *65* (6), 743–750. https://doi.org/10.1093/forsci/fxz045.

(99) Chakraborty, U.; Chakraborty, B.; Basnet, M. Plant Growth Promotion and Induction of Resistance in *Camellia Sinensis* by *Bacillus Megaterium*. *J. Basic Microbiol.* 2006, *46* (3), 186–195. https://doi.org/10.1002/jobm.200510050.

(100) Jasim, B.; Anish, M. C.; Shimil, V.; Jyothis, M.; Radhakrishnan, E. K. Studies on Plant Growth Promoting Properties of Fruit-Associated Bacteria from *Elettaria Cardamomum* and Molecular Analysis of ACC Deaminase Gene. *Appl. Biochem. Biotechnol.* 2015, *177*, 175–189. https://doi.org/10.1007/s12010-015-1736-6.

(101) Chen, Q.; Liu, S. Identification and Characterization of the Phosphate-Solubilizing Bacterium *Pantoea* Sp. S32 in Reclamation Soil in Shanxi, China. *Front. Microbiol.* 2019, *10*. https://doi.org/10.3389/fmicb.2019.02171.

(102) Ubalde, M. C.; Braña, V.; Sueiro, F.; Morel, M. A.; Martínez-Rosales, C.; Marquez, C.; Castro-Sowinski, S. The Versatility of *Delftia* Sp. Isolates as Tools for Bioremediation and Biofertilization Technologies. *Curr. Microbiol.* 2012, *64* (6), 597–603. https://doi.org/10.1007/s00284-012-0108-5.

(103) Radhakrishnan, R.; Hashem, A.; Abd Allah, E. F. *Bacillus*: A Biological Tool for Crop Improvement through Bio-Molecular Changes in Adverse Environments. *Front. Physiol.* 2017, *8*. https://doi.org/10.3389/fphys.2017.00667.

(104) da Silveira, A. P. D.; Iório, R. de P. F.; Marcos, F. C. C.; Fernandes, A. O.; de Souza, S. A. C. D.; Kuramae, E. E.; Cipriano, M. A. P. Exploitation of New Endophytic Bacteria and Their Ability to Promote Sugarcane Growth and Nitrogen Nutrition. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 2019, *112*, 283–295. https://doi.org/10.1007/s10482-018-1157-y.

(105) Feng, Y.; Shen, D.; Song, W. Rice Endophyte *Pantoea Agglomerans* YS19 Promotes Host Plant Growth and Affects Allocations of Host Photosynthates. *J. Appl. Microbiol.* 2006, *100*, 938–945. https://doi.org/10.1111/j.1365-2672.2006.02843.x.

(106) Vaitiekūnaitė, D.; Kuusienė, S.; Beniušytė, E. Oak (*Quercus Robur*) Associated Endophytic *Paenibacillus* Sp. Promotes Poplar (*Populus* Spp.) Root Growth In Vitro. *Microorganisms* 2021, *9* (6), 1151. https://doi.org/10.3390/microorganisms9061151.

(107) Kuffner, M.; De Maria, S.; Puschenreiter, M.; Fallmann, K.; Wieshammer, G.; Gorfer, M.; Strauss, J.; Rivelli, A. R.; Sessitsch, A. Culturable Bacteria from Zn- and Cd-Accumulating *Salix Caprea* with Differential Effects on Plant Growth and Heavy Metal Availability. *J. Appl. Microbiol.* 2010, *108* (4), 1471–1484. https://doi.org/10.1111/j.1365-2672.2010.04670.x.

(108) Miliūtė, I.; Buzaitė, O. IAA Production and Other Plant Growth Promoting Traits of Endophytic Bacteria from Apple Tree. *Biologija* 2011, *57* (2), 98–102. https://doi.org/10.6001/biologija.v57i2.1835.

(109) Liu, D.; Yang, Q.; Ge, K.; Hu, X.; Qi, G.; Du, B.; Liu, K.; Ding, Y. Promotion of Iron Nutrition and Growth on Peanut by *Paenibacillus Illinoisensis* and *Bacillus* Sp. Strains in Calcareous Soil. *Brazilian J. Microbiol.* 2017, *48*, 656–670. https://doi.org/10.1016/j.bjm.2017.02.006.

(110) Goswami, D.; Parmar, S.; Vaghela, H.; Dhandhukia, P.; Thakker, J. N. Describing *Paenibacillus Mucilaginosus* Strain N3 as an Efficient Plant Growth Promoting Rhizobacteria (PGPR). *Cogent Food Agric.* 2015, *1*. https://doi.org/10.1080/23311932.2014.1000714.

(111) Scherling, C.; Ulrich, K.; Ewald, D.; Weckwerth, W. A Metabolic Signature of the Beneficial Interaction of the Endophyte *Paenibacillus* Sp. Isolate and in Vitro-Grown Poplar Plants Revealed by Metabolomics. *Mol. Plant-Microbe Interact.* 2009, *22* (8), 1032–1037. https://doi.org/10.1094/MPMI-22-8-1032.

(112) Moreno-Gavíra, A.; Diánez, F.; Sánchez-Montesinos, B.; Santos, M. *Paecilomyces Variotii* as a Plant-Growth Promoter in Horticulture. *Agronomy* 2020, *10* (4). https://doi.org/10.3390/AGRONOMY10040597.

(113) Kthiri, Z.; Jabeur, M. Ben; Chairi, F.; López-Cristoffanini, C.; López-Carbonell, M.; Serret, M. D.; Araus, J. L.; Karmous, C.; Hamada, W. Exploring the Potential of *Meyerozyma Guilliermondii* on Physiological Performances and Defense Response against *Fusarium* Crown Rot on Durum Wheat. *Pathogens* 2021, *10* (1), 1–14. https://doi.org/10.3390/pathogens10010052.

(114) Mehmood, A.; Hussain, A.; Irshad, M.; Hamayun, M.; Iqbal, A.; Khan, N. In Vitro Production of IAA by Endophytic Fungus *Aspergillus Awamori* and Its Growth Promoting Activities in *Zea Mays*. *Symbiosis* 2019, *77* (3), 225–235. https://doi.org/10.1007/s13199-018-0583-y.

(115) Lubna; Asaf, S.; Hamayun, M.; Gul, H.; Lee, I. J.; Hussain, A. *Aspergillus Niger* CSR3 Regulates Plant Endogenous Hormones and Secondary Metabolites by Producing Gibberellins and Indoleacetic Acid. *J. Plant Interact.* 2018, *13* (1), 100–111. https://doi.org/10.1080/17429145.2018.1436199.

(116) Doilom, M.; Guo, J. W.; Phookamsak, R.; Mortimer, P. E.; Karunarathna, S. C.; Dong, W.; Liao, C. F.; Yan, K.; Pem, D.; Suwannarach, N.; Promputtha, I.; Lumyong, S.; Xu, J. C. Screening of Phosphate-Solubilizing Fungi From Air and Soil in Yunnan, China: Four Novel Species in *Aspergillus*, *Gongronella*, *Penicillium*, and *Talaromyces*. *Front. Microbiol.* 2020, *11* (October). https://doi.org/10.3389/fmicb.2020.585215.

(117) Peng, X.; Wang, Y.; Tang, L. J.; Li, X. X.; Xiao, Y. W.; Zhang, Z. Bin; Yan, R. M.; Yang, H. L.; Chang, J.; Zhu, B.; Zhu, D. Yeasts from Nanfeng Mandarin Plants: Occurrence, Diversity and Capability to Produce Indole-3-Acetic Acid. *Biotechnol. Biotechnol. Equip.* 2018, *32* (6), 1496–1506. https://doi.org/10.1080/13102818.2018.1487337.

(118) Ignatova, L. V.; Brazhnikova, Y. V.; Berzhanova, R. Z.; Mukasheva, T. D. Plant Growth-Promoting and Antifungal Activity of Yeasts from Dark Chestnut Soil. *Microbiol. Res.* 2015, *175*, 78–83. https://doi.org/10.1016/j.micres.2015.03.008.

(119) Kotanen, C. N.; Martinez, L.; Alvarez, R.; Simecek, J. W. Surface Enhanced Raman Scattering Spectroscopy for Detection and Identification of Microbial Pathogens Isolated from Human Serum. *Sens. Bio-Sensing Res.* 2016, *8*, 20–26. https://doi.org/10.1016/j.sbsr.2016.03.002.

(120) Dina, N. E.; Zhou, H.; Colniţă, A.; Leopold, N.; Szoke-Nagy, T.; Coman, C.; Haisch, C. Rapid Single-Cell Detection and Identification of Pathogens by Using Surface-Enhanced Raman Spectroscopy. *Analyst* 2017, *142* (10), 1782–1789. https://doi.org/10.1039/C7AN00106A.

(121) Liu, Y.; Chen, Y. R.; Nou, X.; Chao, K. Potential of Surface-Enhanced Raman Spectroscopy for the Rapid

Identification of *Escherichia Coli* and *Listeria Monocytogenes* Cultures on Silver Colloidal Nanoparticles. *Appl. Spectrosc.* 2007, *61* (8), 824–831. https://doi.org/10.1366/000370207781540060.

(122) Witkowska, E.; Korsak, D.; Kowalska, A.; Księżopolska-Gocalska, M.; Niedziółka-Jönsson, J.; Roźniecka, E.; Michałowicz, W.; Albrycht, P.; Podrażka, M.; Hołyst, R.; Waluk, J.; Kamińska, A. Surface-Enhanced Raman Spectroscopy Introduced into the International Standard Organization (ISO) Regulations as an Alternative Method for Detection and Identification of Pathogens in the Food Industry. *Anal. Bioanal. Chem.* 2017, *409* (6), 1555–1567. https://doi.org/10.1007/s00216-016-0090-z.

(123) Paret, M. L.; Sharma, S. K.; Green, L. M.; Alvarez, A. M. Biochemical Characterization of Gram-Positive and Gram-Negative Plant-Associated Bacteria with Micro-Raman Spectroscopy. *Appl. Spectrosc.* 2010, *64* (4), 433–441. https://doi.org/10.1366/000370210791114293.

(124) Stöckel, S.; Kirchhoff, J.; Neugebauer, U.; Rösch, P.; Popp, J. The Application of Raman Spectroscopy for the Detection and Identification of Microorganisms. *J. Raman Spectrosc.* 2016, *47* (1), 89–109. https://doi.org/10.1002/jrs.4844.

(125) Kairyte, K.; Luksiene, Z.; Pucetaite, M.; Sablinskas, V. Differentiation of Bacterial Strains by Means of Surface Enhanced Ft-Raman Spectroscopy. *Lith. J. Phys.* 2012, *52* (3), 276–283. https://doi.org/10.3952/physics.v52i3.2480.

(126) Witkowska, E.; Korsak, D.; Kowalska, A.; Janeczek, A.; Kamińska, A. Strain-Level Typing and Identification of Bacteria – a Novel Approach for SERS Active Plasmonic Nanostructures. *Anal. Bioanal. Chem.* 2018, *410* (20), 5019–5031. https://doi.org/10.1007/s00216-018-1153-0.

(127) Guicheteau, J.; Argue, L.; Hyre, A.; Jacobson, M.; Christesen, S. D. Raman and Surface-Enhanced Raman Spectroscopy of Amino Acids and Nucleotide Bases for Target Bacterial Vibrational Mode Identification. *Chem. Biol. Sens. VII* 2006, *6218*, 62180O. https://doi.org/10.1117/12.670294.

(128) Polisetti, S.; Bible, A. N.; Morrell-Falvey, J. L.; Bohn, P. W. Raman Chemical Imaging of the Rhizosphere Bacterium *Pantoea* Sp. YR343 and Its Co-Culture with *Arabidopsis Thaliana*. *Analyst* 2016, *141* (7), 2175–2182. https://doi.org/10.1039/c6an00080k.

(129) Yang, D.; Zhou, H.; Dina, N. E.; Haisch, C. Portable Bacteria-Capturing Chip for Direct Surfaceenhanced Raman Scattering Identification of Urinary Tract Infection Pathogens. *R. Soc. Open Sci.* 2018, *5* (9). https://doi.org/10.1098/rsos.180955.

(130) Mungroo, N. A.; Oliveira, G.; Neethirajan, S. SERS Based Point-of-Care Detection of Food-Borne Pathogens. *Microchim. Acta* 2016, *183* (2), 697–707. https://doi.org/10.1007/s00604-015-1698-y.

(131) Nelson, D. M.; Glawe, A. J.; Labeda, D. P.; Cann, I. K. O.; Mackie, R. I. *Paenibacillus Tundrae* Sp. Nov. and *Paenibacillus Xylanexedens* Sp. Nov., Psychrotolerant, Xylan-Degrading Bacteria from Alaskan Tundra. *Int. J. Syst. Evol. Microbiol.* 2009, *59*, 1708–1714. https://doi.org/10.1099/ijs.0.004572-0.

(132) Rasimus, S.; Mikkola, R.; Andersson, M. A.; Teplova, V. V.; Venediktova, N.; Ek-Kommonen, C.; Salkinoja-Salonen, M. Psychrotolerant *Paenibacillus Tundrae* Isolates from Barley Grains Produce New Cereulide-like Depsipeptides (Paenilide and Homopaenilide) That Are Highly Toxic to Mammalian Cells. *Appl. Environ. Microbiol.* 2012, *78* (10), 3732–3743. https://doi.org/10.1128/AEM.00049-12.

(133) Idris, E. S. E.; Iglesias, D. J.; Talon, M.; Borriss, R. Tryptophan-Dependent Production of Indole-3-Acetic Acid (IAA) Affects Level of Plant Growth Promotion by *Bacillus Amyloliquefaciens* FZB42. *Mol. Plant-Microbe Interact.* 2007, *20* (6), 619–626. https://doi.org/10.1094/MPMI-20-6-0619.

(134) Striganavičiūtė, G.; Žiauka, J.; Sirgedaitė-Šėžienė, V.; Vaitiekūnaitė, D. Impact of Plant-Associated Bacteria on the In Vitro Growth and Pathogenic Resistance against *Phellinus Tremulae* of Different Aspen (*Populus*) Genotypes. *Microorganisms* 2021. https://doi.org/10.3390/ microorganisms9091901.

(135) Striganavičiūtė, G.; Žiauka, J.; Sirgedaitė-Šėžienė, V.; Vaitiekūnaitė, D. Priming of Resistance-Related Phenolics: A Study of Plant-Associated Bacteria and *Hymenoscyphus Fraxineus*. *Microorganisms* 2021, *9* (12). https://doi.org/10.3390/microorganisms9122504.

(136) Maggini, V.; De Leo, M.; Mengoni, A.; Gallo, E. R.; Miceli, E.; Reidel, R. V. B.; Biffi, S.; Pistelli, L.; Fani, R.; Firenzuoli, F.; Bogani, P. Plant-Endophytes Interaction Influences the Secondary Metabolism in *Echinacea Purpurea* (L.) Moench: An in Vitro Model. *Sci. Rep.* 2017, *7* (1), 16924. https://doi.org/10.1038/s41598-017-17110-w.

(137) Yu, M.; Chen, J. C.; Qu, J. Z.; Liu, F.; Zhou, M.; Ma, Y. M.; Xiang, S. Y.; Pan, X. X.; Zhang, H. B.; Yang, M. Z. Exposure to Endophytic Fungi Quantitatively and Compositionally Alters Anthocyanins in Grape Cells. *Plant Physiol. Biochem.* 2020, *149* (February), 144–152. https://doi.org/10.1016/j.plaphy.2020.02.006.

(138) Ludwig-Müller, J. Plants and Endophytes: Equal Partners in Secondary Metabolite Production? *Biotechnol. Lett.* 2015, *37* (7), 1325–1334. https://doi.org/10.1007/s10529-015-1814-4.

(139) Daskalchuk, T.; Ahiahonu, P.; Heath, D.; Yamazaki, Y. The Use of Non-Targeted Metabolomics in Plant Science. In *Biotechnology in Agriculture and Forestry, Plant Metabolomics*; Nagata, T., Loerz, H., Widholm, J. M., Saito, K., Dixon, R. A., Willmitzer, L., Eds.; 2006; pp 311–313.

(140) Erb, M.; Kliebenstein, D. J. Plant Secondary Metabolites as Defenses, Regulators, and Primary Metabolites: The Blurred Functional Trichotomy. *Plant Physiol.* 2020, *184*, 39–52.

(141) Mierziak, J.; Kostyn, K.; Kulma, A. Flavonoids as Important Molecules of Plant Interactions with the Environment. *Molecules* 2014, *19* (10), 16240–16265. https://doi.org/10.3390/molecules191016240.

(142) Nguyen, M. K.; Yang, C. M.; Shih, T. H.; Lin, S. H.; Pham, G. T.; Nguyen, H. C. Chlorophyll Biosynthesis and Transcriptome Profiles of Chlorophyll B-Deficient Type 2b Rice (*Oryza Sativa* L.). *Not. Bot. Horti Agrobot. Cluj-Napoca* 2021, *49* (3), 1–16. https://doi.org/10.15835/nbha49312380.

(143) Krishna, H.; Singh, S. K.; Sharma, R. R.; Khawale, R. N.; Grover, M.; Patel, V. B. Biochemical Changes in Micropropagated Grape (*Vitis Vinifera* L.) Plantlets Due to Arbuscular-Mycorrhizal Fungi (AMF) Inoculation during Ex Vitro Acclimatization. *Sci. Hortic. (Amsterdam).* 2005, *106* (4), 554–567. https://doi.org/10.1016/j.scienta.2005.05.009.

(144) Swapnil, P.; Meena, M.; Singh, S. K.; Dhuldhaj, U. P.; Harish; Marwal, A. Vital Roles of Carotenoids in Plants and Humans to Deteriorate Stress with Its Structure, Biosynthesis, Metabolic Engineering and Functional Aspects. *Curr. Plant Biol.* 2021, *26*, 100203. https://doi.org/10.1016/j.cpb.2021.100203.

(145) Roca, M.; Chen, K.; Pérez-Gálvez, A. Chlorophylls. In *Handbook on Natural Pigments in Food and Beverages: Industrial Applications for Improving Food Color*; Elsevier Ltd., 2016; pp 125–158. https://doi.org/10.1016/B978-0-08-100371-8.00006-3.

(146) Wallis, C. M.; Galarneau, E. R. A. Phenolic Compound Induction in Plant-Microbe and Plant-Insect Interactions: A Meta-Analysis. *Front. Plant Sci.* 2020, *11* (December), 1–13. https://doi.org/10.3389/fpls.2020.580753.

(147) Puri, A.; Padda, K. P.; Chanway, C. P. Beneficial Effects of Bacterial Endophytes on Forest Tree Species. 2017, 111–132. https://doi.org/10.1007/978-3-319-66544-3_6.

(148) Elo, S.; Suominen, I.; Kämpfer, P.; Juhanoja, J.; Salkinoja-Salonen, M.; Haahtela, K. *Paenibacillus Borealis* Sp. Nov., a Nitrogen-Fixing Species Isolated from Spruce Forest Humus in Finland. *Int. J. Syst. Evol. Microbiol.* 2001, *51* (2), 535–545. https://doi.org/10.1099/00207713-51-2-535.

(149) Anand, R.; Grayston, S.; Chanway, C. N2-Fixation and Seedling Growth Promotion of Lodgepole Pine by Endophytic *Paenibacillus Polymyxa*. *Microb. Ecol.* 2013, *66* (2), 369–374. https://doi.org/10.1007/s00248-013-0196-1.

(150) van der Lelie, D.; Taghavi, S.; Monchy, S.; Schwender, J.; Miller, L.; Ferrieri, R.; Rogers, A.; Wu, X.; Zhu, W.; Weyens, N.; Vangronsveld, J.; Newman, L. Poplar and Its Bacterial Endophytes: Coexistence and Harmony. *CRC. Crit. Rev. Plant Sci.* 2009, *28* (5), 346–358. https://doi.org/10.1080/07352680903241204.

(151) Weyens, N.; Taghavi, S.; Barac, T.; van der Lelie, D.; Boulet, J.; Artois, T.; Carleer, R.; Vangronsveld, J. Bacteria Associated with Oak and Ash on a TCE-Contaminated Site: Characterization of Isolates with Potential to Avoid Evapotranspiration of TCE. *Environ. Sci. Pollut. Res.* 2009, *16*, 830–843. https://doi.org/10.1007/s11356-009-0154-0.

(152) Haňáčková, Z.; Havrdová, L.; Černý, K.; Zahradník, D.; Koukol, O. Fungal Endophytes in Ash Shoots – Diversity and Inhibition of *Hymenoscyphus Fraxineus*. *Balt. For.* 2017, *23* (1), 89–106.

(153) Becker, R.; Ulrich, K.; Behrendt, U.; Kube, M.; Ulrich, A. Analyzing Ash Leaf-Colonizing Fungal Communities for Their Biological Control of *Hymenoscyphus Fraxineus*. *Front. Microbiol.* 2020, *11* (October), 1–15. https://doi.org/10.3389/fmicb.2020.590944.

(154) Lahiri, A. Endophytic Diversity of *Fraxinus Excelsior* L. (European Ash) and Its Interaction with the Dieback Pathogen *Hymenoscyphus Fraxineus*, University of Dublin, Trinity College, 2020.

(155) Halecker, S.; Wennrich, J. P.; Rodrigo, S.; Andrée, N.; Rabsch, L.; Baschien, C.; Steinert, M.; Stadler, M.; Surup, F.; Schulz, B. Fungal Endophytes for Biocontrol of Ash Dieback: The Antagonistic Potential of *Hypoxylon Rubiginosum*. *Fungal Ecol.* 2020, *45*. https://doi.org/10.1016/j.funeco.2020.100918.

(156) Poolak, T. Endofüütsete Seente Antimikroobsed Omadused – Antimicrobial Properties of Endophytic Fungi, Estonian University of Life Sciences, 2015.

(157) Azeem, M.; Barba-Aliaga, M.; Borg-Karlson, A. K.; Terenius, O.; Broberg, A.; Rajarao, G. K. *Heterobasidion*-Growth Inhibiting *Bacillus Subtilis* A18 Exhibits Medium- and Age-Dependent Production of Lipopeptides. *Microbiol. Res.* 2019, *223–225* (April), 129–136. https://doi.org/10.1016/j.micres.2019.04.006.

(158) Mesanza, N.; Iturritxa, E.; Patten, C. L. Native Rhizobacteria as Biocontrol Agents of *Heterobasidion Annosum* s.s. and *Armillaria Mellea* Infection of *Pinus Radiata*. *Biol. Control* 2016, *101*, 8–16. https://doi.org/10.1016/j.biocontrol.2016.06.003.

(159) Huang, Y.; Sun, C.; Guan, X.; Lian, S.; Li, B.; Wang, C. Biocontrol Efficiency of *Meyerozyma Guilliermondii* Y-1 against Apple Postharvest Decay Caused by *Botryosphaeria Dothidea* and the Possible Mechanisms of Action. *Int. J. Food Microbiol.* 2021, *338* (November 2020), 108957. https://doi.org/10.1016/j.ijfoodmicro.2020.108957.

(160) He, F.; Zhao, L.; Zheng, X.; Abdelhai, M. H.; Boateng, N. S.; Zhang, X.; Zhang, H. Investigating the Effect of Methyl Jasmonate on the Biocontrol Activity of *Meyerozyma Guilliermondii* against Blue Mold Decay of Apples and the Possible Mechanisms Involved. *Physiol. Mol. Plant Pathol.* 2020, *109* (December 2019), 101454. https://doi.org/10.1016/j.pmpp.2019.101454.

(161) Sun, C.; Huang, Y.; Lian, S.; Saleem, M.; Li, B.; Wang, C. Improving the Biocontrol Efficacy of *Meyerozyma Guilliermondii* Y-1 with Melatonin against Postharvest Gray Mold in Apple Fruit. *Postharvest Biol. Technol.* 2021, *171* (August 2020), 111351. https://doi.org/10.1016/j.postharvbio.2020.111351.

(162) Özaktan, H.; Bora, T. Biological Control of Fire Blight in Pear Orchards with a Formulation of *Pantoea*

Agglomerans Strain Eh 24. *Brazilian J. Microbiol.* 2004, *35* (3), 224–229. https://doi.org/10.1590/S1517-83822004000200010.

(163) Xie, J.; Shu, P.; Strobel, G.; Chen, J.; Wei, J.; Xiang, Z.; Zhou, Z. *Pantoea Agglomerans* SWg2 Colonizes Mulberry Tissues, Promotes Disease Protection and Seedling Growth. *Biol. Control* 2017, *113*, 9–17. https://doi.org/10.1016/j.biocontrol.2017.06.010.

(164) Cañamás, T. P.; Viñas, I.; Usall, J.; Torres, R.; Anguera, M.; Teixidó, N. Control of Postharvest Diseases on Citrus Fruit by Preharvest Applications of Biocontrol Agent *Pantoea Agglomerans* CPA-2. Part II. Effectiveness of Different Cell Formulations. *Postharvest Biol. Technol.* 2008, *49* (1), 96–106. https://doi.org/10.1016/j.postharvbio.2007.12.005.

(165) Nunes, C.; Usall, J.; Teixidó, N.; Vias, I. Biological Control of Postharvest Pear Diseases Using a Bacterium, *Pantoea Agglomerans* CPA-2. *Int. J. Food Microbiol.* 2001, *70* (1–2), 53–61. https://doi.org/10.1016/S0168-1605(01)00523-2.

(166) Stockwell, V. O.; Johnson, K. B.; Sugar, D.; Loper, J. E. Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea Agglomerans* Strain Eh252 in Orchards. *Phytopathology* 2002, *92* (11), 1202–1209. https://doi.org/10.1094/PHYTO.2002.92.11.1202.

(167) Bonaterra, A.; Mari, M.; Casalini, L.; Montesinos, E. Biological Control of *Monilinia Laxa* and *Rhizopus Stolonifer* in Postharvest of Stone Fruit by *Pantoea Agglomerans* EPS125 and Putative Mechanisms of Antagonism. *Int. J. Food Microbiol.* 2003, *84* (1), 93–104. https://doi.org/10.1016/S0168-1605(02)00403-8.

(168) Moreno-Gavíra, A.; Diánez, F.; Sánchez-Montesinos, B.; Santos, M. Biocontrol Effects of *Paecilomyces Variotii* against Fungal Plant Diseases. *J. Fungi* 2021, *7* (6). https://doi.org/10.3390/jof7060415.

(169) Rodrigo, S.; Santamaria, O.; Halecker, S.; Lledó, S.; Stadler, M. Antagonism between *Byssochlamys Spectabilis* (Anamorph *Paecilomyces Variotii*) and Plant Pathogens: Involvement of the Bioactive Compounds Produced by the Endophyte. *Ann. Appl. Biol.* 2017, *171* (3), 464–476. https://doi.org/10.1111/aab.12388.

(170) Arora, K.; Sharma, S.; Krishna, S. B. N.; Adam, J. K.; Kumar, A. Non-Edible Oil Cakes as a Novel Substrate for DPA Production and Augmenting Biocontrol Activity of *Paecilomyces Variotii*. *Front. Microbiol.* 2017, *8* (MAY), 1–12. https://doi.org/10.3389/fmicb.2017.00753.

(171) Lu, C.; Liu, H.; Jiang, D.; Wang, L.; Jiang, Y.; Tang, S.; Hou, X.; Han, X.; Liu, Z.; Zhang, M.; Chu, Z.; Ding, X. *Paecilomyces Variotii* Extracts (ZNC) Enhance Plant Immunity and Promote Plant Growth. *Plant Soil* 2019, *441* (1–2), 383–397. https://doi.org/10.1007/s11104-019-04130-w.

(172) Zhao, J.; Liu, W.; Liu, D.; Lu, C.; Zhang, D.; Wu, H.; Dong, D.; Meng, L. Identification and Evaluation of *Aspergillus Tubingensis* as a Potential Biocontrol Agent against Grey Mould on Tomato. *J. Gen. Plant Pathol.* 2018, *84* (2), 148–159. https://doi.org/10.1007/s10327-018-0764-9.

(173) Moradi, M.; Rohani, M.; Fani, S. R.; Mosavian, M. T. H.; Probst, C.; Khodaygan, P. Biocontrol Potential of Native Yeast Strains against *Aspergillus Flavus* and Aflatoxin Production in Pistachio. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* 2020, *37* (11), 1963–1973. https://doi.org/10.1080/19440049.2020.1811901.

(174) Mahendran, T. R.; Thottathil, G. P.; Surendran, A.; Nagao, H.; Sudesh, K. Biocontrol Potential of *Aspergillus Terreus*, Endophytic Fungus against *Rigidoporus Microporus* and *Corynespora Cassiicola*, Pathogens of Rubber Tree. *Arch. Phytopathol. Plant Prot.* 2021, *54* (15–16), 1014–1032. https://doi.org/10.1080/03235408.2021.1884952.

(175) Picot, A.; Doster, M.; Islam, M. S.; Callicott, K.; Ortega-Beltran, A.; Cotty, P.; Michailides, T. Distribution and Incidence of Atoxigenic *Aspergillus Flavus* VCG in Tree Crop Orchards in California: A Strategy for Identifying Potential Antagonists, the Example of Almonds. *Int. J. Food Microbiol.* 2018, *265* (September 2017), 55–64. https://doi.org/10.1016/j.ijfoodmicro.2017.10.023.

ABOUT THE AUTHOR

Dorotėja Vaitiekūnaitė

Lithuanian Research Centre for Agriculture and Forestry,
Institute of Forestry

ORCID ID: 0000-0003-2775-9802

Tel. (8 37) 54 73 19
E-mail: doroteja.vaitiekunaite@lammc.lt
Liepų g. 1, Girionys, 53101 Kauno r.

Education

Since 2018 Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry, PhD studies in Forestry.

2016–2018 Kaunas University of Technology, Faculty of Chemical Technology, Master's in Biotechnology.

2011–2015 Vytautas Magnus University, Faculty of Natural Sciences, Bachelor's in biology.

1998–2011 Kaunas Steponas Darius and Stasys Girėnas Gimnasium.

Research fields

- Tree biotechnology
- Plant-associated microbiota
- Biofertilizers, biofungicides and biostimulants
- Plant-microorganism interaction
- Surface-enhanced Raman spectroscopy for microorganism, DNA and plant metabolome analysis
- UAV assisted forest seeding
- Deforestation due to wildfires

Professional experience

2022 10 01 – till now, Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry, junior researcher at Forest Plant Biotechnology Laboratory

2022 01 – till now, Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry, junior researcher at Forest Plant Biotechnology Laboratory (Project TREEADS – Horizon 2020)

2018 11/12 – Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry, engineer at Forest Plant Biotechnology Laboratory

2013 07/08 – Lithuanian Research Centre for Agriculture and Forestry, Institute of Agriculture, intern at Genetics and Physiology Laboratory

Involvement in programmes and projects

2022 01 – till now – Horizon 2020 project TREEADS: "Preventing and fighting extreme wildfires with the integration and demonstration of innovative means"

2022 01 – till now – national project "Evaluation of the effectiveness of different bacterial inoculants in promoting the formation of systemic antipathogenic resistance in Lithuanian confiner trees"

MAJOR RESEARCH OUTPUTS

Articles in journals indexed in *Clarivate Analytics Web of Science* database:

- Aleliūnas, A., Jonavičienė, K., Statkevičiūtė, G., **Vaitiekūnaitė, D.,** Kemešytė, V., Lübberstedt, T., Brazauskas, G. 2015. Association of single nucleotide polymorphisms in *LpIRI1* gene with freezing tolerance traits in perennial ryegrass. *Euphytica*, 204: 523.
- **Vaitiekūnaitė, D.,** Kuusienė, S., Beniušytė, E. 2021. Oak (*Quercus robur*) associated endophytic *Paenibacillus* sp. promotes poplar (*Populus* spp.) root growth in vitro. *Microorganisms,* 9(6), 1151.
- Striganavičiūtė, G., Žiauka, J., Sirgedaitė-Šėžienė, V., **Vaitiekūnaitė, D.** 2021. Priming of resistance-related phenolics: A study of plant-associated bacteria and *Hymenoscyphus fraxineus*. *Microorganisms*, 9(12), 2504.
- Striganavičiūtė, G., Žiauka, J., Sirgedaitė-Šėžienė, V., **Vaitiekūnaitė, D.** 2021. Impact of plant-associated bacteria on the in vitro growth and pathogenic resistance against *Phellinus tremulae* of different aspen (*Populus*) genotypes. *Microorganisms*, 9(9), 1901.
- **Vaitiekūnaitė, D.,** Snitka, V. 2021. Differentiation of closely related oak-associated gram-negative bacteria by label-free surface enhanced Raman spectroscopy (SERS). *Microorganisms,* 9(9), 1969.
- **Vaitiekūnaitė, D.**, Bružaitė I., Snitka, V. 2022. Endophytes from blueberry (*Vaccinium* sp.) fruit: Characterization of yeast and bacteria via label-free surface-enhanced Raman spectroscopy (SERS). Spectrochimica Acta Part A: *Molecular and Biomolecular Spectroscopy,* 275(121158).
- **Vaitiekūnaitė, D.,** Striganavičiūtė, G., Mishcherikova, V., Sirgedaitė-Šėžienė, V. 2022. *Populus tremula* × *P. alba* Microshoot Secondary Metabolism Response after *Paenibacillus* sp. Inoculation In Vitro. *Forests*, 13(10), 1702.
- **Vaitiekūnaitė, D.,** Dodoo, D., Snitka, V. 2023. Traceability of bilberries (*Vaccinium myrtillus* L.) of the Baltic-Nordic region using surface-enhanced Raman spectroscopy (SERS): DFT simulation-based DNA analysis. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, (122192).
- Kolytaitė, A., **Vaitiekūnaitė, D.,** Antanynienė, R., Baniulis, D., Frercks, B. 2022. *Monilinia fructigena* Suppressing and Plant Growth Promoting Endophytic *Pseudomonas* spp. Bacteria Isolated from Plum. *Microorganisms,* 10, no. 12: 2402.
- Beniušytė, E., Čėsnienė, I., Sirgedaitė-Šėžienė, V., **Vaitiekūnaitė, D.** 2023. Genotype-Dependent Jasmonic Acid Effect on *Pinus sylvestris* L. Growth and Induced Systemic Resistance Indicators. *Plants,* 12(2):255.

- Sirgedaitė-Šėžienė, V., Čėsnienė, I., Leleikaitė, G., Baliuckas, V., **Vaitiekūnaitė, D.** 2023. Phenolic and antioxidant compound accumulation of Quercus robur bark diverges based on tree genotype, phenology and extraction method. *Life*, 13(3), 710.
- **Vaitiekūnaitė, D.,** Striganavičiūtė, G., Beniušytė, E., Sirgedaitė-Šėžienė, V., Augustauskaitė, M. 2023. Putative biocontrol agents for European forest pathogens found in oak (*Quercus robur*) endosphere. *Zemdirbyste-Agriculture*, 110(1).

Dorotėja VAITIEKŪNAITĖ

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Edited by Author

Spausdino – Vytauto Didžiojo universitetas
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