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Some aspects on boreal forest microbiotas and nitrogen

Tinkara Bizjak-Johansson

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Tinkara Bizjak-Johansson
Faculty of Forest Sciences
Department of Forest Genetics and Plant Physiology
Umeå

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Cover: Collecting Scots pine needles at Åheden field site for subsequent laboratory analysis (photo by Noelia Saavedra Berlanga).

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Swedish University of Agricultural Sciences, Department of Department of Forest Genetics and Plant Physiology, Umeå, Sweden

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Abstract

Forest microbiomes are integral parts of the forest ecosystem as they are involved in soil health, tree growth, plant resilience and nitrogen and carbon cycles. However, forest microbiomes are under-researched compared to microbiomes in agriculturally important environments. Therefore, the main aim of this thesis was to describe and analyse the fungal and bacterial microbiomes connected to Norway spruce and Scots pine growing in the Fennoscandian boreal forest and investigate the effect of nitrogen addition on these microbiomes. Additionally, the thesis assessed two potentially more sustainable alternatives to inorganic nitrogen fertilization. The results showed the microbiome compositions of bulk soil, humus, root and needle conifer samples, highlighted distinct microbiomes between bulk soil and needles and identified 15 culturable nitrogen-fixing bacteria isolated from Scots pine needles. The results further showed that increased nitrogen addition led to decreased fungal and bacterial abundance, changes in fungal microbiome composition and differences in the activity of several extracellular enzymes in humus. However, interestingly, nitrogen addition did not affect the activity of nitrogen-fixing bacteria inside Scots pine needles. The greenhouse study showed that controlled-release organic nitrogen-based nutrition was able to promote plant growth similar to inorganic nitrogen-based fertilizers but with lower nitrate leaching from the growth medium. Further, bacteria isolated from Scots pine needles showed plant growth-promoting properties in *in vitro* experiments but did not promote higher plant biomass of four different inoculated crop species in *in vivo* greenhouse study. In summary, the thesis offered a deeper look into diverse Fennoscandian boreal forest microbiomes and analysed the effects of inorganic nitrogen addition on both the presence and the activity of these microbiomes. Furthermore, the thesis highlighted the potential and challenges of possible sustainable alternatives to inorganic nitrogen fertilization.

Keywords: bacteria, boreal forest, fungi, inorganic and organic nitrogen, microbiome, nitrogen addition, nitrogen fixation, Norway spruce, plant growth-promoting bacteria, Scots pine

Några aspekter på skogliga svamp- och bakteriesamhällen och kväve

Sammanfattning

I denna avhandling studerades svamp- och bakteriesamhällens struktur och funktion i fält i boreal skog, samt att effekter av kvävetillförsel på dessa undersöktes. Dessutom testades två metoder för att främja tillväxt på plantor i växthus: tillsats av organiskt kvävegödsel baserat på aminosyran arginin eller tillsats av bakterier med tillväxtfrämjande egenskaper. Resultaten från fältstudierna visade distinkta svampsamhällen med avseende på artsammansättning för mark respektive barr, samt för gran- respektive tallbarr, medan många svamparter delades mellan mark och rötter. Från tallbarr identifierades och isolerades 15 odlingsbara kvävefixerande bakterier och det estimerades att kvävefixering i barr skulle kunna bidra med 15 gram växttillgängligt kväve per hektar och år till denna typ av skogsekosystem. Kvävetillförsel i form av årlig gödsling med 50 kg kväve per hektar under nästan 30 år till en grandominerad skog ledde till minskad förekomst av svampar och bakterier, samt att aktiviteten hos flera extracellulära enzymer i markens humusskikt förändrades så att de indikerade att koltillgången snarare än kvävetillgången var begränsande för deras tillväxt. Däremot påverkades inte förekomsten eller aktiviteten hos kvävefixerande bakterier inuti tallbarr av liknande nivå av kvävegödsling. Växthusstudierna visade att argininbaserad näring med kontrollerad frisättning av kvävet främjade tillväxten hos trädplantor på ett liknande sätt som ett oorganiskt kvävegödselmedel, men med lägre nitratläckage från odlingsmediet. Tillsats av bakterier isolerade från tallbarr med växttillväxtfrämjande egenskaper främjade dock inte tillväxten hos plantor av fyra grödor. Sammanfattningsvis lyfter avhandlingen fram mikrobsamhällenas komplexitet i nordliga skogsekosystem och belyser behovet av mer forskning för djupare kunskap om deras roll.

Nyckelord: bakterier, boreal skog, gran, kvävefixering, kvävetillsats, mikrobiom, oorganiskt och organiskt kväve, svampar, tall, tillväxtfrämjande bakterier

Nekateri vidiki o gozdnih mikrobiotah in dušiku

Povzetek

Mikrobiomi so ključni deli gozdnih ekosistemov, saj sodelujejo pri ohranjanju zdravja tal, rasti in odpornosti dreves ter so del dušikovega in ogljikovega cikla. Vendar pa so gozdni mikrobiomi v primerjavi s kmetijsko pomembnimi mikrobiomi premalo raziskani. Glavni cilj disertacije je bil opisati in analizirati glivne in bakterijske mikrobiome, povezane z navadno smreko in rdečim borom, ki rasteta v borealnem gozdu, ter raziskati vpliv dodatka dušika na te mikrobiome. Poleg tega sta bili v okviru disertacije raziskani dve potencialno bolj trajnostni alternativi gnojenju z anorganskim dušikom. Rezultati so pokazali sestave mikrobiomov v vzorcih prsti, humusa, korenin in iglic, izpostavili razlike med mikrobiomi v prsti in drevesnih iglicah ter identificirali 15 bakterij, izoliranih iz iglic rdečega bora, ki so sposobne vezati dušik. Rezultati so nadalje pokazali, da dodatek dušika vodi do zmanjšanja količine gliv in bakterij, do sprememb v sestavi glivnih mikrobiomov in do razlik v aktivnosti več encimov, povezanih z oksidacijo in pridobivanjem dušika in ogljika iz prsti. Poleg tega se je pokazalo, da dodatek dušika ni imel vpliva na prisotnost in aktivnost bakterij v iglicah rdečega bora, ki lahko vežejo dušik. Rezultati poskusa v rastlinjaku so pokazali, da je gnojilo, razvito na osnovi organskega dušika, imelo podoben učinek na rast sadik iglavcev, kot gnojilo na osnovi anorganskega dušika, vendar z manj izpiranja dušika. Bakterije, izolirane iz iglic rdečega bora, so pokazale lastnosti za spodbujanje rasti rastlin v laboratorijskih poskusih, vendar niso spodbudile rasti štirih različnih vrst poljščin v rastlinjaku. Raziskava v okviru disertacije je ponudila poglobljen vpogled v raznolike mikrobiome borealnega gozda ter analizirala učinke dodatka anorganskega dušika na prisotnost in aktivnost teh mikrobiomov. Poleg tega je izpostavila prednosti in izzive možnih trajnostnih alternativ za anorgansko gnojenje z dušikom.

Ključne besede: anorganski in organski dušik, bakterija, dodatek dušika, gliva, mikrobiom, navadna smreka, rast spodbujajoče bakterije, rdeči bor, tajga, vezava dušika

Preface

The idea for my PhD thesis arose already a few years before the official beginning of my PhD with the question of whether nitrogen-fixing bacteria are present in tissues such as stems and needles of conifers in the Fennoscandian boreal forest. The article published by Carrell and Frank (2014) suggested the presence of nitrogen-fixing bacteria inside needles of limber pine and Engelmann spruce growing in subalpine forest, which could represent an important source of nitrogen for trees growing in nitrogen-limited environments. Therefore, an interesting thought was if these bacteria could also be present in tissues of conifers growing in the nitrogen-limited Fennoscandian boreal forest. A few years passed, but the question was still present in my thoughts and that of my main supervisor, Annika, so my master's thesis focused on nitrogen-fixing bacteria in Scots pine needles. One of the most important thoughts from that period was definitely the sentence written in Menge et al. (2009): "The chronic nitrogen limitation that pervades mature temperate and boreal forests could easily be overcome by nitrogen fixers, who are conspicuous in these ecosystems in their absence only." After the master thesis was finished, so many questions were left unanswered and consequently, my PhD started with the same background questions, but expanded it to which bacteria and fungi are present in the needles of Scots pine and Norway spruce, are nitrogen-fixing bacteria actively fixing nitrogen when present in the needles, how is their nitrogen fixation rate affected by nitrogen addition and if fungal communities and their enzyme activities are affected by nitrogen addition. During the project, many other questions arose and some of them were just too interesting not to pursue. Therefore, my last two articles looked into using organic nitrogen-based nutrition and the use of plant growth-promoting bacteria as alternatives to inorganic nitrogen fertilization. As the papers appended at the end of this thesis describe my last

four years of research in quite a lot of detail, in this thesis, I only focused (based on my opinion) on the key results and, in some cases, tried to show the results in a different light. In the appendix, I added some results and findings that I made during the last four years that were not part of the main projects but that have taught me a lot. I think they might help future master's and PhD students researching in this area save some time or even develop into interesting new questions to be researched in the future.

I hope you will enjoy reading my thesis!

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Bizjak-Johansson, T.*,** Larsson, M., Gundale, M. J., Nordin, A. Norway spruce and Scots pine fungal and bacterial microbiomes in boreal forest common garden experiment (manuscript).

II. **Bizjak, T.**, Sellstedt, A., Gratz, R.*, Nordin, A. (2023). Presence and activity of nitrogen-fixing bacteria in Scots pine needles in a boreal forest: a nitrogen-addition experiment. Tree Physiology, 43 (8), pp 1354-1364. https://doi.org/10.1093/treephys/tpad048

III. Forsmark, B.*#, **Bizjak, T.#**, Nordin, A., Rosenstock, N. P., Wallander, H., Gundale, M. J. (2024). Shifts in microbial community composition and metabolism correspond with rapid soil carbon accumulation in response to 20 years of simulated nitrogen deposition. Science of the Total Environment, 918, 170741. https://doi.org/10.1016/j.scitotenv.2024.170741

IV. **Bizjak-Johansson, T.**, Bozaghian Bäckman, M., Nilsson, L., Holmlund, M., Skoglund, N., Näsholm, T., Gratz, R*. Novel environmentally friendly plant nutrition (manuscript).

V. **Bizjak-Johansson, T.***, Braunroth, A., Gratz, R., Nordin, A. (2025). Inoculation with *in vitro* promising plant growth-promoting bacteria isolated from nitrogen-limited boreal forest did not translate to *in vivo* growth promotion of agricultural plants. Biology and Fertility of Soils. https://doi.org/10.1007/s00374-025-01910-8

*Corresponding author
Shared first authorship

Papers II, III and V were published open access.

The contribution of Tinkara Bizjak-Johansson to the papers included in this thesis according to Contributor Roles Taxonomy (CRediT) was as follows:

I. Conceptualization, Data curation, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Project administration.

II. Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Project administration.

III. Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

IV. Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

V. Conceptualization, Funding acquisition, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Project administration.

List of figures

Abbreviations

16S	16 Svedberg
AMC	7-amido-4-methylcoumarin
ANOVA	Analysis of variance
Arginine Fe-HMP	Arginine iron-hexametaphosphate
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
Commercial CRF	Commercial controlled-release fertilizer
DNA	Deoxyribonucleic acid
GC-MS/MS	Gas chromatography - tandem mass spectrometry
HPLC	High-performance liquid chromatography
HSD	Honestly significant difference
IAA	Indole-3-acetic acid
ITS	Internal transcribed spacer
kg	Kilogram
MUB	4-methylumbelliferyl
n	Number of replicates
N_2	Dinitrogen
NH_3	Ammonia
NH_4^+	Ammonium
NO_3^-	Nitrate
OTU	Operational taxonomic unit
PGPB	Plant growth-promoting bacteria
PLFA	Phospholipid-derived fatty acids
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

1. Introduction

1.1 Nitrogen

One of the essential elements for life is nitrogen as it is a building block of nucleic acids and proteins (Canfield et al. 2010). Nitrogen has a very high abundance in our atmosphere as biologically inert dinitrogen (N_2) and is one of the least biologically available and most growth-limiting elements in the biosphere (Galloway et al. 2003; Chanway et al. 2014). To become biologically available, unreactive N_2 needs to be transformed into other nitrogen forms, for example, ammonia (NH_3), ammonium (NH_4^+) or nitrate (NO_3^-) ions (Martinez-Espinosa et al. 2011; Chanway et al. 2014). The triple bond in dinitrogen can only be broken by biological nitrogen fixation or high-temperature reactions as it requires a large amount of energy (Galloway et al. 2003; Chanway et al. 2014). Biological nitrogen fixation is a natural process of dinitrogen transformation into ammonia that can be performed by bacteria and archaea (Chanway et al. 2014). The high-temperature reactions leading to the production of biologically available nitrogen are lightning, volcanic activity, fossil fuel combustion and the Haber-Bosch process (Galloway et al. 2003; Chanway et al. 2014). As in production systems nitrogen limitation leads to decreased plant growth and yield (Chanway et al. 2014; Li & Coleman 2019), the Haber-Bosch process has been important for the production of synthetic nitrogen fertilizers, which are estimated to be responsible for feeding around half of the world's population (Fowler et al. 2013). However, the planetary boundary for anthropologically introduced nitrogen has been severely crossed (Richardson et al. 2023) and nitrogen leaching after fertilization can lead to several negative environmental effects,

such as greenhouse gas emissions, biodiversity loss and water eutrophication (Savci 2012; Andrews et al. 2013; Qiao et al. 2015).

Biologically available nitrogen can be present in inorganic and organic forms. Inorganic nitrogen refers to compounds containing nitrogen but no carbon atoms, and the most common forms include ammonium and nitrate. Organic nitrogen refers to compounds containing both nitrogen and carbon atoms, and the common forms are nucleotides, proteins, urea, amino sugars and amino acids. Both inorganic and organic nitrogen forms are present in the soil in different forms (Schulten & Schnitzer 1997). It was long thought that plants take up only inorganic nitrogen, but more recent research proves that plants can also take up different forms of organic nitrogen (Näsholm & Persson 2001; Harrison et al. 2007; Paungfoo-Lonhienne et al. 2012). Among others, it was shown that many plants can take up intact forms of amino acids such as glycine and arginine (Öhlund & Näsholm 2004; Uscola et al. 2017). Most of the commonly used fertilizers to increase plant growth are based on inorganic nitrogen, but due to their negative effects connected to nitrate leaching, the use of organic nitrogen-based nutrition is being discussed. Organic nitrogen-based nutrition can be produced from waste products, promotes plant growth similarly to inorganic fertilizers, but leads to lower nitrate leaching (Hansen et al. 2000; Paungfoo-Lonhienne et al. 2012; Gil et al. 2024).

1.2 Boreal forest and nitrogen

The boreal forest represents around ten percent of the land surface, a third of the global forest area (Shvidenko & Apps 2006; Castro et al. 2021) and is important for the global paper and timber markets (Gauthier et al. 2015). In addition to its economic value, the boreal forest has an important role in the global carbon cycle as it stores around a third of the terrestrial carbon (Pan et al. 2011; Bradshaw & Warkentin 2015), largely within the soils (Pan et al. 2024). For Fennoscandia, boreal forest is the dominant biome (Esseen et al. 1992) characterised by a harsh and cold climate, low plant species diversity and slow regeneration (Huhta et al. 1998; Castro et al. 2021; Högberg et al. 2021). Due to the cold climate leading to low rates of nitrogen fixation, slow decomposition of the soil organic matter and high nitrogen retention by mycorrhizal fungi (Högberg et al. 2017; Adamczyk et al. 2019; Fu et al. 2022), the boreal forest is severely nitrogen-limited making plant growth

highly dependent on its availability (Högberg et al. 2017). The nitrogen cycle in the boreal forest is relatively closed as the internal nitrogen turnover is much higher than any nitrogen inputs and outputs (Högberg et al. 2017). The internal nitrogen turnover is highly dependent on soil and litter decomposition by extracellular enzymes produced and exuded by microorganisms (Sinsabaugh et al. 2008; Högberg et al. 2017). The two important sources of nitrogen input are atmospheric nitrogen deposition and biological nitrogen fixation (Högberg et al. 2017), but these inputs are relatively small compared to the annual nitrogen uptake by the vegetation (Sponseller et al. 2016). Additionally, the forest can, in certain cases, be fertilized with inorganic nitrogen to increase the tree biomass production (Hedwall et al. 2014; Routa et al. 2019). The estimated nitrogen deposition, a consequence of mainly agricultural and industrial activities (Nordin et al. 2005; Zhang et al. 2021), is relatively low (below 12 kg nitrogen per hectare and year) in the Fennoscandian boreal forest (Gundale et al. 2014). The main sources for biological nitrogen fixation in boreal forests are root nodule symbiosis between actinobacteria and certain tree species, association between cyanobacteria and either mosses or lichens, and free-living nitrogen-fixing bacteria (DeLuca et al. 2002; Högberg et al. 2017). However, recently, it has been suggested that nitrogen fixation is also present in the needles of forest trees, which could be an important additional nitrogen source in nitrogen-limited environments (Carrell & Frank 2014; Carrell et al. 2016; Moyes et al. 2016; Wurzburger 2016; Puri et al. 2018).

1.3 Scots pine, Norway spruce and their production

The two main species dominating the Fennoscandian boreal forest are the conifers Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). Broadleaves that are common include birch (*Betula pubescens* and *Betula pendula*), aspen (*Populus tremula*) and willow (*Salix caprea*). As the Fennoscandian boreal forest is usually clear-cut during harvest, the common method to ensure the growth of a new forest is planting seedlings (Häggström et al. 2024). Together, Scots pine and Norway spruce represent 95% of all seedlings planted in Finland, Norway and Sweden (Solvin et al. 2021). Most of these seedlings are grown in nurseries, where they are heavily fertilized with inorganic nitrogen to ensure proper establishment and better growth upon planting (Heiskanen et al. 2009; Vaario et al. 2009). Seedling

fertilization in nurseries significantly increases plant growth, but it can lead to lower mycorrhization of conifer roots (Gruffman et al. 2012). Additionally, there is a high risk of nitrate leaching due to high nitrate mobility, low root nitrate uptake, overirrigation of growth media and application of fertilizer by mobile boom systems (Juntunen & Rikala 2001; Öhlund & Näsholm 2002). This nitrate leaching can have a detrimental effect on the surrounding groundwater (Juntunen & Rikala 2001; Öhlund & Näsholm 2002). In some nurseries, controlled-release inorganic nitrogen fertilizers are used instead as they lead to lower leaching due to their gradual nutrient release (Hunt 1989; Haase et al. 2006; Jariwala et al. 2022). However, most of the available controlled-release fertilizers are encased in non-biodegradable and fossil-fuel-based synthetic polymer coats (Vejan et al. 2021). Several Swedish nurseries have started using organic nitrogen nutrition based on the amino acid arginine (Schneider et al. 2024), as it has been shown that it can promote seedling growth and higher root biomass production (Gruffman et al. 2012), which can be important for ensuring proper water uptake after planting in the field (Grossnickle 2005). Additionally, organic nitrogen nutrition has shown no effect on seedling root mycorrhization and had lower nitrate leaching compared to inorganic nitrogen fertilizer (Öhlund & Näsholm 2002; Gruffman et al. 2012; Lim et al. 2022). However, there are no substitutes for the controlled-release inorganic nitrogen fertilizer available. Yet, it has been suggested that such plant nutrition could be produced by complexing an amino acid to polyphosphates to produce a solid-phase nutrition, where the polyphosphate length would control the nutrient release rate (Näsholm et al. 2017).

1.4 Microbiomes

In addition to the prevailing trees and other vegetation, boreal forests include a variety of microorganisms interacting with their environment (Bell-Doyon et al. 2022). Compared to agriculture, the microbiota part of forest tree species is under-researched, and our limited knowledge about forest microbiomes includes many knowledge gaps regarding their composition, function, role and importance (Terhonen et al. 2019; Padda et al. 2021).

Microbiota definition encompasses living protists, archaea, algae, bacteria and fungi present in the environment, while the microbiome definition additionally includes all genetic elements in the environment (e.g.

extracellular DNA and viruses) (Terhonen et al. 2019; Berg et al. 2020). The microbiomes are often distinguished based on their location: bulk soil microbiome, rhizosphere microbiome (microorganisms in the soil surrounding roots), phyllosphere microbiome (microorganisms living on plant surfaces) and endophytic microbiome (microorganisms living inside of plants without causing any disease symptoms). Among these, the endophytic microorganisms are the least researched. The commonly known main entrance points for endophytic microorganisms are root cracks and other tissue wounds, but recently it has become known that an alternative entry point might be through stomata (Bhattacharjee et al. 2008; Santoyo et al. 2016; Frank et al. 2017; Kandel et al. 2017). The endophytic microorganisms have been suggested to be better protected against biotic and abiotic stresses and have a closer interaction with the plant (Santoyo et al. 2016; Padda et al. 2022; Méndez-Bravo et al. 2023).

The composition of bacterial and fungal microbiomes is commonly analysed by amplicon sequencing of the 16S rRNA and ITS, respectively, as both include highly conserved and highly variable regions enabling bacterial or fungal identification. Most studies focused on either analysing the fungal or the bacterial forest microbiome (Uroz et al. 2016a; Uroz et al. 2016b), even though there is evidence of interactions between these two communities (Pohjanen et al. 2014; Marupakula et al. 2016; Rua et al. 2016). A part of the microbiome changes with time, but it is suggested that there is a more stable part called the core microbiome (Berg et al. 2020; Berg et al. 2021). The rather vague definition of the core microbiome is the community of permanent and stable microbiome species constantly associated with a specific environment or host (Berg et al. 2020). The vague core microbiome definition is therefore based on presence/absence and does not depend on the abundance of the species in the environment (Marupakula et al. 2016). Core microbiomes have been described in certain environments connected to the study-specific tree species, however, the exact definitions of the core microbiome differed between studies (Laforest-Lapointe et al. 2016; Griffin et al. 2019; Firrincieli et al. 2020). Several environmental factors, soil characteristics and plant properties are proposed to influence the composition and stability of the microbiome (Romeralo et al. 2022; Xiong et al. 2024), but their exact importance is not yet known for the boreal forest microbiome.

1.4.1 Fungi

Two important types of fungi in the boreal forest are saprotrophic and ectomycorrhizal fungi, as they play a crucial role in nutrient recycling (Kües 2015; Fernandez & Kennedy 2016). Saprotrophic fungi, mostly occupying the litter layer, obtain large parts of their carbon from non-living organic material by breaking down cell wall carbohydrates and lignin compounds (Leonowicz et al. 1999; Lindahl & Tunlid 2015; Fernandez & Kennedy 2016; Beck et al. 2018). Ectomycorrhizal fungi, mostly present in the mineral soil layers and the lower part of the humus, are known for their symbiosis with trees, where trees provide ectomycorrhizal fungi with carbon and fungi in return provide trees with nitrogen and phosphorus (Rosling et al. 2003; Näsholm et al. 2013; Chen et al. 2019; Tahovská et al. 2020). Saprotrophic fungi are present in many diverse fungal phyla (Wijayawardene et al. 2024), while ectomycorrhizal fungi mostly belong to Basidiomycota, Ascomycota and Mucuromycotina phyla (Lilleskov et al. 2019).

Sequencing studies showed that the most commonly found fungal phyla in boreal forest compartments are Ascomycota, Basidiomycota, Mucoromycota and Glomeromycota (Millberg et al. 2015; Urbanová et al. 2015; Nguyen et al. 2016a; Marciulyniene et al. 2021). A higher abundance of Basidiomycota has been observed in soils, while a higher abundance of Ascomycota has been observed in plant tissues (Terhonen et al. 2019). Distinct microbiomes have been reported for aboveground and belowground fungal communities (Haas et al. 2018; Schneider et al. 2021). The fungal microbiome composition is proposed to be strongly affected by plant species (Urbanová et al. 2015; Romeralo et al. 2022) in addition to environmental conditions, forest management practices and soil properties (Tedersoo et al. 2014; Millberg et al. 2015; Nguyen et al. 2016a).

Fungi are capable of producing extracellular enzymes whose role is to degrade complex organic compounds into simpler forms that fungi can take up (Allison et al. 2010). These enzymes mostly target compounds including nitrogen, carbon and phosphorus (Allison et al. 2010). The early stages of degradation depend on the activity of hydrolytic enzymes cleaving specific molecular bonds to decompose compounds such as cellulose and peptides (Baldrian & Štursova 2010). The latter stages of decomposition required oxidative enzymes or Fenton chemistry to decompose recalcitrant compounds such as lignin (Sinsabaugh 2010). While saprotrophic fungi are better known for their oxidative potential, it has been shown that several

ectomycorrhizal fungi retained genes for oxidative enzymes (Floudas et al. 2012; Argiroff et al. 2022). Understanding the decomposition in the boreal forest is extremely important as boreal forest soils represent a large carbon stock whose existence also depends on the fungal enzyme activities (Chodak et al. 2015; Fernandez & Kennedy 2016; Kyaschenko et al. 2017).

1.4.2 Bacteria

Bacteria are omnipresent in the boreal forest, as research even showed their presence inside Scots pine bud cells and shoot tissue cells (Pirttilä et al. 2000; Koskimaki et al. 2015). However, compared to fungal microbiomes, much less is known about bacterial microbiomes, their structure and their role in the boreal forest. Sequencing studies on boreal forest soil and plant-associated microbiomes show a high abundance of Proteobacteria, followed by Actinobacteria and Acidobacteria phyla, which all play a role in the nitrogen and carbon cycles (Tahovská et al. 2020; Choma et al. 2021; Bell-Doyon et al. 2022; Padda et al. 2022). Additionally, a high abundance of Pseudomonadota phylum has been reported for aboveground plant tissues (Terhonen et al. 2019). Similar to fungal microbiomes, several studies reported distinct microbiomes between aboveground plant tissues of boreal forest trees and belowground plant tissues or soils (Haas et al. 2018; Padda et al. 2022; Rodríguez-Rodríguez et al. 2023). It was observed that boreal forest bacterial communities can be affected by soil properties, tree species and growing season length (Priha et al. 2001; Chodak et al. 2015; Urbanová et al. 2015; Haas et al. 2018; Rodríguez-Rodríguez et al. 2023) but are more resistant to environmental changes compared to fungal microbiomes (Uroz et al. 2016a).

One special type of bacteria are nitrogen-fixing bacteria belonging to 10 different genera (Pirttilä & Frank 2018). Nitrogen-fixing bacteria can be in symbiosis with a plant, free-living or endophytic (Chanway et al. 2014; Pirttilä & Frank 2018). All nitrogen-fixing bacteria are capable of changing dinitrogen into plant-available ammonia (Bulgarelli et al. 2013). The reaction is catalyzed by nitrogenase, a multi-subunit enzyme structured from two proteins: dinitrogenase and dinitrogenase reductase (Marchal & Vanderleyden 2000; Canfield et al. 2010). Dinitrogenase is responsible for the reduction of dinitrogen into ammonia, while dinitrogenase reductase binds adenosine triphosphate (ATP) (Marchal & Vanderleyden 2000; Canfield et al. 2010). Biological nitrogen fixation is mostly limited by high

energy demand and oxygen levels as nitrogenase is oxygen sensitive (Vitousek et al. 2013). Commonly, nitrogenase activity is downregulated by higher nitrogen and upregulated by higher carbon availability, while phosphorus availability leads to various responses (Zheng et al. 2017; Avila Clasen et al. 2023). Nitrogen-fixing bacteria have been isolated from needles and stems of several, mostly North American, conifer species (e.g. lodgepole pine and hybrid white spruce) and most of these bacteria belonged to the genera *Paenibacillus*, *Bacillus*, *Pseudomonas* and *Caballeronia* (Bal et al. 2012; Padda et al. 2018; Puri et al. 2018). Furthermore, nitrogen fixation has been measured in needles of certain conifer species across diverse types of forests using acetylene-reduction assay, however, the recorded rates of nitrogen fixation differ greatly as estimates range from grams to kilograms nitrogen per hectare and year (Jones 1970; Granhall & Lindberg 1978; Jones 1982; Favilli & Messini 1990; Moyes et al. 2016). Nitrogen fixation has even been detected in Scots pine and Norway spruce fine-roots (Granhall & Lindberg 1978; Nilsson 2020) and in boreal forest logging residues (Tormanen & Smolander 2022).

In addition to nitrogen fixation, the bacteria may possess other plant growth-promoting properties contributing to higher plant growth, improved yield, better pathogen resistance and protection against certain abiotic stresses (Kong et al. 2018; Aghai et al. 2019; Iqbal et al. 2021; Ghadamgahi et al. 2022). Plant growth-promoting properties influence plant nutrition (e.g. through nitrogen fixation, and phosphorus or iron solubilization), phytohormone levels (e.g. auxins, cytokinins and gibberellins), stress response (e.g. by regulating ethylene levels) and pathogen protection (e.g. production of siderophores, hydrogen cyanide, antibiotics and cell wall degrading enzymes) (Penrose et al. 2001; Jasim et al. 2013; Olanrewaju et al. 2017; Rana et al. 2020). An idea is that the application of plant growth-promoting bacteria (PGPB) in agriculture and forestry would replace the use of synthetic fertilizers to ensure a more sustainable increase in plant growth and yield (Berg et al. 2020; Berg et al. 2021). Indeed, inoculation of conifer seedlings and certain non-conifer plants (e.g. sunflower and canola) with PGPB isolated from conifer tissues significantly improved plant biomass (Anand et al. 2013; Padda et al. 2015; Puri et al. 2015a; Puri et al. 2015b; Padda et al. 2019; Puri et al. 2020c; Song et al. 2020; Chen et al. 2021; Song et al. 2021; Younas et al. 2023). However, with conifer seedlings, the establishment of beneficial plant-bacteria interaction in some cases took up

to a year to develop (Anand et al. 2013; Tang et al. 2017). Most of the PGPB used in inoculation studies were nitrogen-fixing, and several studies showed that the inoculated plants could receive up to half of their nitrogen through biological nitrogen fixation (Anand et al. 2013; Puri et al. 2015b; Padda et al. 2019). However, most of the studies used severely nitrogen-limited growth media (e.g. sandy mixtures) without the presence of soil or seed native microbiome. Therefore, the question remains if these results can be successfully scaled up and applied to systems including natural soils (de-Bashan & Nannipieri 2024). Additionally, recent studies suggested that increased plant growth promotion can be achieved using a consortium of PGPB instead of single strains, as a consortium includes PGPB with a wider range of functions and with different roles in nutrient cycling (Ray et al. 2020; Saleem et al. 2021; Khan et al. 2022). However, similar research on PGPB in boreal forests is still lacking (Lucy et al. 2004; Jangra et al. 2024).

1.4.3 Microbiomes and nitrogen

Nitrogen addition (though deposition or fertilization) can have significant effects on the boreal forest microbiomes. Studies showed that nitrogen addition leads e.g. to decreased soil respiration and decomposition rates (Nohrstedt et al. 1989; Janssens et al. 2010; Lilleskov et al. 2019; Forsmark et al. 2021). This is suggested to be connected to a change in the fungal microbiome composition and, consequently, a change in the activity of extracellular enzymes, but the exact mechanisms are not yet known. There are contrasting results regarding an increase or decrease in microbial biomass following nitrogen addition (Janssens et al. 2010; Tahovská et al. 2020; Jörgensen et al. 2021). Many studies reported a decline of ectomycorrhizal fungi (such as *Cortinarious*, *Russula* and *Piloderma*) after nitrogen addition, while the abundance of saprotrophic fungi remained similar (Tahovská et al. 2020; Jörgensen et al. 2021; Marupakula et al. 2021). Nitrogen addition is also proposed to change the bacterial microbiome by decreasing the abundance of nitrogen-sensitive bacterial classes such as Deltaproteobacteria and Acidobacteriae and increasing the abundance of Alphaproteobacteria, Gammaproteobacteria and Actinobacteria (Tahovská et al. 2020). Furthermore, it has been shown that higher nitrogen availability can decrease biological nitrogen fixation in forests (Cusack et al. 2009; Clasen et al. 2025). For example, nitrogen addition severely negatively affected the cyanobacterial nitrogen fixation on boreal forest moss species (Gundale et

al. 2011; Gundale et al. 2013; Leppänen et al. 2013). However, little is known about the effect of nitrogen addition on conifer endophytic nitrogen-fixing bacteria.

2. Aims and research questions

2.1 Aims

The main aims were to study the composition of the Fennoscandian boreal forest fungal and bacterial microbiomes, analyse the effects of nitrogen addition on these microbiomes and their respective activity and examine if inoculation with plant growth-promoting bacteria or fertilization with organic nitrogen-based nutrition could be sustainable alternatives to inorganic nitrogen fertilization.

2.2 Research questions

1) What is the composition of the Scots pine and Norway spruce soil, endophytic root and needle fungal and bacterial communities? (Papers I, II and III)
2) What are the effects of inorganic nitrogen addition on boreal forest microbiomes, specifically Norway spruce soil and endophytic root fungi and Scots pine endophytic nitrogen-fixing bacteria? (Papers II and III)
3) Could plant growth-promoting bacteria and organic nitrogen-based nutrition be potential sustainable alternatives to inorganic nitrogen fertilizer? (Papers IV and V)

3. Material and methods

An array of molecular, microbiological and chemical methods was used in papers I-V. The exact descriptions and protocols for the methods can be found in their respective papers. Therefore, this material and methods section only includes a short overview of the methods whose results are discussed within this thesis text.

3.1 Field sites

The papers I, II, III, and V include samples collected from three field sites: Åheden, Svartberget nitrogen addition experiment, and Svartberget common garden experiment. All three sites are located close to each other outside of Vindeln, northern Sweden.

Samples for paper I were collected at the Svartberget common garden experiment (N 64° 15', E 19° 47'). The common garden was established in 1992 by planting several different conifer species in monocultures at plots sized 34 x 34 meters in a randomized block design with three replicate plots per tree species (Spitzer et al. 2025). The collected bulk soil (mixture of humus soil layer and mineral soil at their interface) and needle samples for the paper I were collected from the Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) plots.

Samples in papers II and V were collected at the Åheden field site (N 64° 14', E 19° 48'), which is a naturally regenerated boreal forest of predominately around 150-year-old Scots pine with some Norway spruce (From et al. 2016). A randomized block design was established in 2004 with five levels of nitrogen addition rates (0, 3, 6, 12 and 50 kg nitrogen per hectare and year) and six replicates for each rate. The blocks are yearly fertilized with ammonium nitrate granules. For paper II, the blocks belonging

to 0 and 50 kg N per hectare and year were used, while samples for paper V were collected in the forest adjacent to the fertilization trial. In both cases, only one-year-old needles were collected.

Paper III samples were collected at the Svartberget nitrogen addition experiment field site (N 64° 14', E 19° 46'), which is naturally regenerated around 150-year-old Norway spruce-dominated boreal forest with trees of different ages (From et al. 2016). The nitrogen addition experiment was established in 1996 with a randomized block design with three levels of nitrogen addition rates (0, 12.5 and 50 kg nitrogen per hectare and year) and with different plot sizes. The humus soil layer and fine-root samples for paper III were collected from 1000 and 2500 square meter plots, providing twelve replicates per nitrogen addition rate. The forest is fertilized yearly with ammonium nitrate granules.

3.2 Sequencing

Across all papers, bacterial identity was analysed by amplicon sequencing of the 16S rRNA region encoding a ribosomal RNA gene (Gray et al. 1984) and fungal identity by ITS region situated between large-subunit and small-subunit ribosomal RNA genes (Gardes et al. 1991). Both 16S and ITS have highly conserved and highly variable regions enabling bacterial or fungal identification (Edwards et al. 1989; Gardes et al. 1991). This identification usually provides information across all taxonomic ranks, from kingdom, phylum, class, order, family, genus to species, which reflect evolutionary relationships (Cronquist 2019). However, in certain cases, only identity at higher taxonomic orders is possible due to missing data in the databases used for taxonomic identification based on 16S and ITS sequence (Daisley & Reid 2021; Barrenechea Angeles et al. 2024).

Different sequencing methods were used in papers I, II, III and V. To identify the isolated nitrogen-fixing bacteria in papers II and V, Sanger sequencing was used. The second-generation sequencing platform Illumina was used in paper III to analyse the fungal microbiome, while the third-generation sequencing platform PacBio was used in paper I to analyse the bacterial and fungal microbiomes. Sanger sequencing is based on detecting radioactively or, more commonly, fluorescently labelled chain-terminating nucleotides (Slatko et al. 2018). Sanger sequencing is an inexpensive method used to sequence individual templates with one specific DNA primer, which

can produce accurate sequences of up to around 1000 bases, however, it cannot be used for high-throughput applications (Slatko et al. 2018). The Illumina sequencing platform is based on sequencing-by-synthesis method with fluorescently labelled reversible chain-terminating nucleotides (Hu et al. 2021). The length of the read is restricted to a maximum of 300 bases, but longer reads can be achieved with paired-end sequencing (Hu et al. 2021). The Illumina sequencing platform offers high throughput and has a very low error rate, however, short reads can lead to problems with sequence assembly and sequencing takes a relatively long time (Hu et al. 2021; Mandlik et al. 2024). The PacBio sequencing platform is based on fluorescently labelled nucleotides, whose light pulse is identified when the specific base is held by the DNA polymerase (Rhoads & Au 2015). The PacBio sequencing platform offers long reads of typically between 10 000 and 25 000 bases (Han et al. 2024). PacBio sequencing allows the detection of DNA modifications, enables an easier sequence assembly and has a faster sequencing time (Slatko et al. 2018; Hu et al. 2021). The PacBio sequencing platform used to have a quite high error rate, but it has improved significantly in recent years (Han et al. 2024).

The bioinformatic analysis of sequencing data includes several steps, but the most important parts are pre-processing of raw data, clustering or denoising and taxonomic assignment (Kim et al. 2020; Regueira-Iglesias et al. 2023). The pre-processing steps depend on the sequencing platform but usually include quality filtering of sequences and removal of chimeric sequences. The next step is based on either clustering or denoising methods. The commonly used clustering method is operational taxonomic units (OTUs) construction, where sequences sharing over 97% identity are clustered together (Kozich et al. 2013; Chiarello et al. 2022). The use of clustering decreases the number of sequencing errors and reduces the computation power needed for further analysis (Chiarello et al. 2022). More recently, the denoising method started to be used, which depends on an error model determining which sequences are a consequence of sequencing errors and which are real biological sequences defined as amplicon sequence variants (ASVs) (Chiarello et al. 2022). The use of the denoising method is proposed to capture higher biological variation, have higher accuracy and reproducibility, and in certain cases, allow easier comparison between studies (Callahan et al. 2017). The clustering method with OTUs was used in paper III, and the denoising method with ASVs was used in paper I. The

next step of the bioinformatic analysis is the sequence taxonomic assignment using a reference database such as UNITE for fungal sequences (Abarenkov et al. 2024) and SILVA 138 for bacterial sequences (Quast et al. 2013). The final step of bioinformatic analysis is data analysis and visualization using either raw or normalized data (McMurdie & Holmes 2014; Weiss et al. 2017; Cameron et al. 2021), which can focus on relative abundance, within and between sample diversity, differential abundance, core microbiome and network analysis (Peeters et al. 2021; Regueira-Iglesias et al. 2023).

Both the selection of the sequencing platform (PacBio in paper I and Illumina in paper III) and the selection of the bioinformatics pipeline, normalization method, clustering or denoising method (ASVs in paper I and OTUs in paper III), and reference database can have significant effects on the sequencing results regarding the microbiome composition (Couldrey et al. 2017; Abellan-Schneyder et al. 2021; Chiarello et al. 2022; Cook et al. 2024). More standardized state-of-the-art pipelines are needed for higher accuracy and easier comparison of sequencing results between different studies.

3.3 Isolation of nitrogen-fixing bacteria

The nitrogen-fixing bacteria were isolated from surface-sterilized Scots pine needles. The sterilization used in papers II and V was based on an unpublished pre-study, which analysed the sterilization of Scots pine needles using either ethanol, hydrogen peroxide or sodium hypochlorite with different concentrations and submersion times. The pre-study highlighted the difficulty between ensuring complete sterilization of the needle surface while preserving as much of the endophytic microbiome as possible. The two most optimal sterilization protocols proved to be submersion in 30% hydrogen peroxide for two minutes or submersion in 70% ethanol for three minutes, which were subsequently used in papers II and V.

Nitrogen-free media was used for the isolation of nitrogen-fixing bacteria in both papers II and V. An unpublished pre-study evaluated five different nitrogen-free media: combined carbon media (Baldani et al. 2014), Ashby media (Liu et al. 2012), Burk's media (Park et al. 2005), LGI-P (Baldani et al. 2014), and semi-solid NFb media (Reis et al. 2015). Based on the results, in paper II, the combined carbon media, LGI-P and semi-solid NFb media were used, while in paper V, only the combined carbon media was used. As

nitrogen-fixing bacteria have diverse nutritional preferences (Baldani et al. 2014), it is beneficial to use several different nitrogen-free media to ensure the isolation of several diverse bacterial genera. However, during the isolation of bacteria in paper II, it was noticed that bacteria growing in the semi-solid NFb media were mostly incapable of growing on solid media, which made their further characterization extremely difficult.

3.4 Microbial abundance

In paper III, phospholipid-derived fatty acids (PLFAs) were used to measure the bacterial and fungal biomass, while in papers III and IV, ergosterol measurements were used to measure fungal biomass. The PLFA method is based on the quantification of extracted phospholipid fatty acids from the cell membrane (Klamer 2004). The PLFA markers are specific for a certain group of microorganisms as the cell membranes of different microbial groups contain different phospholipid fatty acids (Klamer 2004). Ergosterol measurements are similarly based on the extraction and quantification of the ergosterol, which is a primary sterol in the cell membranes of fungi (Pasanen et al. 1999). In paper III, the ergosterol content was measured using high-performance liquid chromatography (HPLC) connected to an optical-ultraviolet detector, while in paper IV, it was measured using gas chromatography - tandem mass spectrometry (GC-MS/MS) with an internal standard to account for ergosterol degradation. Both PLFA and ergosterol measurements are considered to be reliable, relatively rapid and sensitive (Ruzicka et al. 2000; Frostegård et al. 2011), however, they both have their disadvantages. The disadvantages of ergosterol measurements are species-specific ergosterol concentrations, absence of ergosterol in cell membranes of certain primitive fungi, and longer persistence of ergosterol in dead material, which may lead to overestimation of fungal biomass (Zhao et al. 2005; Adamczyk et al. 2024). The disadvantages of PLFAs are overlapping PLFA composition between different microbial groups and the effect of environmental conditions on PLFA turnover rate (Klamer 2004; Frostegård et al. 2011).

3.5 Enzyme activities

The analysis of extracellular enzymes activities was based on exposing the humus slurry samples to labelled substrates and measuring the rate of substrate hydrolysis. The colorimetric assays are based on the detection of a colored end product, which is produced after hydrolysis by the enzyme (Jackson et al. 2013). Fluorometric assays are similar, where after the hydrolysis of the labelled substrate, the released fluorophore emits light upon light excitation (Jackson et al. 2013). In the thesis, colorimetric assays were used for peroxidase and laccase, while fluorometric assays with 4-methylumbelliferyl (MUB) labelled substrate were used for chitinase, glucosidase, xylosidase, endoglucanase and phosphatase and with 7-amido-4-methylcoumarin (AMC) labelled substrate for peptidase. Soil enzyme activity assays are usually inexpensive, rapid and accurate; however, the methods can lack the needed optimization, and the results can be misinterpreted (German et al. 2011; Nannipieri et al. 2017).

3.6 Western blot

Western blot was used to analyse the presence of nitrogenase NifH subunit protein in paper II as the NifH subunit is the building block of the dinitrogenase reductase subunit and is commonly used as a marker for nitrogen-fixing bacteria. The Western blot method includes many steps, from sample preparation, gel electrophoresis, membrane transfer, blocking, primary antibody incubation, and secondary antibody incubation to the detection of the protein (Bass et al. 2017). As the method has been extensively evolved in the last decades, there are a variety of options available for all steps (Kurien & Scofield 2006). Western blot has not been previously used to detect the NifH subunit protein in conifer needles, so a reliable protocol was developed in paper II, where SDS-PAGE was used for gel electrophoresis, the primary antibody was chicken anti-NifH, and the secondary antibody was horseradish peroxidase-conjugated goat anti-chicken. To ensure reliable and accurate data, two negative controls (protein loading buffer and *Bradyrhizobium japonicum* liquid culture protein extract) and two positive controls (soybean nodule extract and purified His-tagged NifH) were used. The advantage of using the Western blot method is high sensitivity and accessibility (Kurien & Scofield 2006; Gorr & Vogel 2015; Bass et al. 2017). Even though it can be hard to establish a reproducible

Western blot protocol due to multiple steps and various options in each step (Kurien & Scofield 2006; Gorr & Vogel 2015; Bass et al. 2017), a reproducible protocol was developed for detecting nitrogenase NifH subunit in Scots pine needle protein extract in paper II.

3.7 Acetylene-reduction assay

Acetylene-reduction assay was used to indirectly estimate the nitrogen-fixation in various sample types. It was used to estimate the nitrogen fixation inside Scots pine needles in paper II, for liquid bacterial cultures in papers II and V and for six-month-old Scots pine seedlings in paper IV. The method is based on the observation that nitrogenase, in addition to fixing nitrogen, is capable of reducing acetylene into ethylene, whose production can be measured using gas chromatography (Hardy et al. 1968; Hardy et al. 1973). The method includes exchanging 10% of the air with acetylene, incubation and subsequent measurement of the ethylene production. In papers II, IV and V, the incubation time was two hours to enable comparison between results. Usually, two controls are used: a water control and a negative control. The water control is used to account for any ethylene presence in the absence of a sample, while the negative control accounts for endogenous ethylene production of the sample in the absence of acetylene. Especially during needle acetylene-reduction assay measurements, it became evident that sample-specific negative controls are extremely important (see Appendix 1), and therefore, those have been used for all reported results in papers and this thesis. In the case of needle acetylene-reduction assay measurements, the sample-specific negative controls were needles collected from the same tree. For other measurements, sample-specific negative controls meant liquid cultures or seedlings undergoing the exact same treatment as samples. Furthermore, the effects of needle sample pre-treatment and light conditions during incubation were observed during acetylene-reduction measurements (see Appendix 2 and 3, respectively). Compared to other methods used for nitrogen fixation estimations, acetylene-reduction assay is inexpensive, rapid and sensitive (Hardy et al. 1973; Unkovich et al. 2008). However, the measurements only include ethylene production during the assay time, and the conversion ratio between ethylene production and nitrogen fixation differs across plant species and even growth stages (Unkovich et al. 2008; Soper et al. 2021).

3.8 Greenhouse experiment with arginine-iron-hexametaphosphate

A greenhouse study was used in paper IV to examine the potential of using controlled-release organic nitrogen plant nutrition in the form of arginine-iron-hexametaphosphate (referred to as Arginine Fe-HMP) to promote the growth of Scots pine seedlings. The patented technology is based on the specific, three-way interaction between arginine, polyphosphate and iron (Näsholm et al. 2017), enabling the release of the amino acid arginine and phosphate over extended periods of time, where the release rate is dependent on the length of the polyphosphate chain (Näsholm et al. 2017). The Arginine Fe-HMP is, therefore, not a fully developed product by Arevo AB, and the test is not a head-to-head comparison between products but rather a test of the potential of an alternative, organic nitrogen controlled-release formulation. In contrast to most of the inorganic nitrogen-based controlled-release fertilizers, Arginine Fe-HMP is biodegradable, as its slow nutrient release is not dependent on a synthetic polymer coat commonly used with inorganic nitrogen-based fertilizers (Vejan et al. 2021). The paper IV greenhouse study included three treatments: control, Arginine Fe-HMP and commercial controlled-release fertilizer (referred to as commercial CRF, in the case of this study, we used Osmocote, Substral). Each treatment included three cassettes with 60 planted seeds, and the nutrition was mixed into the non-fertilized limed peat just before Scots pine seeds were sown. The amount of nutrition added was calculated for each seedling to receive exactly 12 milligrams of nitrogen. Run-off water was collected the day after sowing to estimate nitrate leaching, and the germination rate was analysed daily during the first month. The survival rate was assessed monthly until the seedlings were harvested when they were approximately six months old. After harvest, the seedling length, dry shoot and root weight, chlorophyll content, carbon content, nitrogen content, and isotope ^{15}N content were measured to compare the plant growth between the three treatments. Additionally, the ethylene production from washed seedlings and root ergosterol content were measured to analyse the effects of the treatments on microbial biomass and activity.

3.9 *In vitro* plant growth-promoting property assays

In paper V, seven different *in vitro* assays were used to evaluate the plant growth-promoting properties of endophytic nitrogen-fixing bacterial strains isolated from Scots pine needles. The assays were performed on two newly isolated bacterial strains and seven bacterial strains isolated previously in paper II. The *in vitro* assays evaluated phosphorus solubilization, zinc solubilization, indole-3-acetic acid (IAA) production, hydrogen peroxide production, siderophore production and activity of protease and cellulase. For zinc solubilization, hydrogen peroxide production and siderophore production, plate assays were used, while for the other properties, colorimetric assays were performed on liquid cultures. In the phosphorus solubilization assay, three different forms of phosphorus were used (tricalcium phosphate, iron (III) phosphate and aluminium phosphate), as it has been observed that the most often used method for phosphate solubilization with tricalcium phosphate overestimates the bacterial phosphorus solubilization capability (Pérez et al. 2007; Bashan et al. 2012). Usually, plate assays are simple and inexpensive, while colorimetric assays provide higher sensitivity and better quantification but often require more expensive equipment (Hossain 2024). However, even colorimetric assays can sometimes overestimate bacterial plant growth promotion potential, for example, in the assay measuring IAA production, other indole-like molecules can be detected as well (Glickmann & Dessaux 1995; Guardado-Fierros et al. 2024).

3.10 Greenhouse inoculation experiment with plant growth-promoting bacteria

A greenhouse inoculation study in paper V was used to evaluate plant growth promotion of the three most promising bacterial strains based on *in vitro* assays. The bacterial strains selected were *Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39. The inoculation study was performed on four crop species, cucumber (*Cucumbis sativa*), corn (*Zea mays*), tomato (*Solanum lycopersicum*), and kale (*Brassica oleracea*), from different plant taxonomic orders with seven replicates each to ensure reproducibility and evaluate plant growth promotion across agriculturally important crop species. The plants were inoculated with a single strain or a consortium three times, at sowing, one week after sowing and two weeks

after sowing, to promote the bacterial establishment. However, the study did not evaluate the viability and the colonization of the inoculated bacterial strains. The plant growth promotion was assessed using chlorophyll content, root and shoot lengths and root and shoot dry weight. In the study, the soil and seeds with their native microbiome were used, and the effects of inoculation were measured using dry weight as per recent recommendations (Huang et al. 2016; de-Bashan & Nannipieri 2024). The inoculation study setups differ greatly between different studies in regards to growth media, inoculation technique (concerning both bacterial strains preparation and application), and plant growth promotion assessment methods, making it sometimes difficult to compare results between studies.

4. Key results and discussion

In this thesis, key results from papers I, II, III, IV and V will be discussed. The thesis references data from these papers and further data and details can be found in the respective papers.

4.1 Composition of Scots pine and Norway spruce microbiomes

The first research question that the thesis addressed in papers I, II, and III was about the composition of Scots pine and Norway spruce soil, roots and endophytic needle microbiomes. Very few previously published articles investigated the Fennoscandian boreal forest microbiomes and even fewer studies simultaneously analysed fungal and bacterial microbiomes. In papers I and III, the composition of the fungal and bacterial microbiomes and the presence of a core microbiome were analysed. In paper II, the endophytic nitrogen-fixing fungi were isolated and identified to provide more information about this unique group of bacteria.

4.1.1 Fungal microbiomes

Fungal microbiomes are an important part of the boreal forest ecosystem (Schneider et al. 2021), and papers I and III provided an insight into the fungal microbiome composition across several Scots pine and Norway spruce forest compartments. In paper I, the collected samples were bulk soil (a mixture of mineral soil and humus) and surface-sterilized needle samples from pine and spruce growing at separate plots in a common garden experiment. In paper III, the spruce humus soil layer and spruce surface-sterilized fine-roots were collected from plots of naturally regenerated spruce forest. The combined results showed that the two dominating fungal phyla

across all samples were Basidiomycota and Ascomycota, while the relative abundances of other fungal phyla were relatively low (Figure 1A). Basidiomycota relative abundance had a trend of decreasing from bulk soil, humus, fine-root to needle samples, while the Ascomycota relative abundance had the opposite trend of increasing from bulk soil, humus, fine-root to needle samples. The results were in accordance with previous studies showing that Basidiomycota and Ascomycota are some of the most common fungal phyla in the coniferous forest (Urbanová et al. 2015; Romeralo et al. 2022). It has been previously observed that generally, Basidiomycota have higher relative abundance in forest soils and Ascomycota in plant tissues (Terhonen et al. 2019). However, this thesis is the first to portray their relative abundance trends across two different boreal forest tree species and several compartments.

Looking deeper into taxonomic ranks, the fungal class with the highest relative abundance in bulk soil samples was Agaricomycetes (belonging to Basidiomycota phylum), whose relative abundance was dominant in both bulk soil samples but was higher in pine bulk soil compared to spruce bulk soil samples (Figure 1B). The second most abundant class in, especially, spruce soil was Leotiomycetes (belonging to Ascomycota phylum), which had a trend of increasing in relative abundance from spruce bulk soil, humus, fine-roots up to spruce needles, where it represented the dominant fungal class. Additionally, spruce bulk soil, humus and fine-roots showed a lower, but relatively similar relative abundance of Dothideomycetes (belonging to Ascomycota phylum). The most distinct samples were the needle samples, where pine had a high relative abundance of Arthoniomycetes (belonging to Ascomycota phylum), Pucciniomycetes (belonging to Basidiomycota phylum) and unknown fungal class/classes. The spruce needle samples had, in addition to the dominant fungal class Leotiomycetes (belonging to Ascomycota phylum), a high relative abundance of Lecanoromycetes (belonging to Ascomycota phylum). Several other fungal classes were identified across the samples, however, their relative abundances were below 10%.

The phyla and class taxonomic rank results were in accordance with previous studies reporting compositional differences between belowground and aboveground fungal microbiomes for Norway spruce (Haas et al. 2018; Li et al. 2018; Schneider et al. 2021). However, this thesis further highlighted

the differences in the compositions of fungal needle microbiomes between Scots pine and Norway spruce.

Already at the phyla and class taxonomic rank, there were several fungal OTUs/AVSs which had an unassigned taxonomy, and the number only increased looking further into taxonomic ranks such as family, genus and species (data not shown).

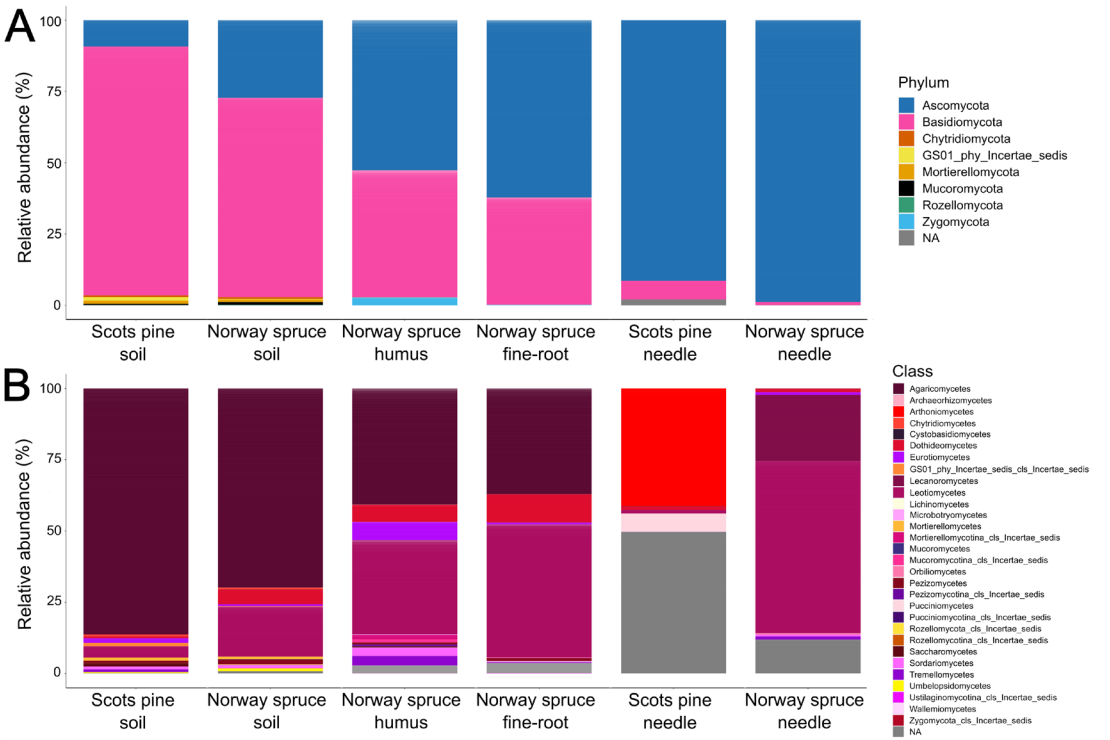

Figure 1: Fungal relative abundance based on ITS sequencing in different pine and spruce compartments looking at A) phyla and B) classes. Observe that different sequencing methods, sample sizes and field sites have been used (Scots pine bulk soil and needle: PacBio, n = 6 and Svartberget common garden, Norway spruce bulk soil and needle: PacBio, n = 8 and and Svartberget common garden, Norway spruce humus: Illumina, n = 23 and and Svartberget nitrogen addition experiment and Norway spruce fine-root: Illumina, n = 24 and Svartberget nitrogen addition experiment).

The suggested definition of the core microbiome describes it as a group of microbial species continuously associated with a specific environment (Berg et al. 2020), and Venn diagrams are one of the options for displaying it (Shade & Handelsman 2012). In this thesis, Venn diagrams were used to analyse how many OTUs/ASVs were shared across different samples by looking at their presence or absence without considering in how many samples the specific OTU/ASV occurred. In paper III, the results showed that the majority of the OTUs were shared between the Norway spruce humus layer and surface-sterilized fine-roots (Figure 2A), which indicates the presence of a core microbiome for these two spruce compartments. Humus had a high number of unique OTUs, while only eight unique OTUs were detected in the fine-root samples. A lower number of endophytic fungal species present inside plant roots compared to surrounding soil has been noticed in previous studies (Gottel et al. 2011; Cregger et al. 2018) and indicates root microbiome selection by the plant (Bulgarelli et al. 2013). Surprisingly, paper I results showed no shared ASVs at all between the bulk soil and endophytic needle microbiome in either Norway spruce (Figure 2B) or Scots pine (Figure 2C). There were some shared fungal ASVs between pine and spruce bulk soil samples (Figure 2D), while pine and spruce needle samples did not share any fungal ASVs (Figure 2E). Further composition analysis showed statistically significantly different compositions for both bulk soil and needle samples between pine and spruce (paper I). This suggests that in the paper I Svartberget common garden experiment, we could not detect the presence of a core microbiome across different compartments of the same tree species or across all compartments and both tree species. The presence of a core microbiome was expected as previous studies in diverse forests reported core microbiomes consisting of a few to several OTUs/ASVs shared across one or two compartments (Bonito et al. 2014; Castro et al. 2021; Romeralo et al. 2022), but a larger study looking at the phyllosphere microbiome of 56 different tree species did not detect a core microbiome (Redford et al. 2010).

One factor adding to the complexity concerning core microbiomes is their unclear definition, which differs between studies (see for example Griffin et al. (2019), Firrincieli et al. (2020) and Romeralo et al. (2022)), especially the requirement of occupancy (how many percentages of all samples the OTU/ASV needs to be detected in to still qualify as a core microbiome OTU/ASV). A more exact definition of a core microbiome and a more

streamlined analysis would be needed to be able to accurately compare potential core microbiomes between different studies. Additionally, the analysis of the core microbiome is commonly based on taxonomic data and not functional data (Lemanceau et al. 2017). It has been proposed that the core microbiome should instead be defined based on the functional traits of specific microbial species, as the plants probably select the species based on their function and not based on their taxonomy (Lemanceau et al. 2017). However, the functional core microbiome is hard to determine as species have more than one function (Rodriguez et al. 2009; Sikes et al. 2010; Cassán et al. 2020) and the sequencing of ITS probably does not provide enough information regarding the species functions, as taxonomically very close species can have different functions in their environment (Munkvold et al. 2004; Johnson et al. 2012). As the needle endophytic microbiome was completely distinct from the bulk soil microbiome, the results further support the hypothesis that plants can influence their microbiome compositions (Bulgarelli et al. 2013). Furthermore, no shared ASVs between the bulk soil and the needle microbiome suggest the existence of alternative entryways into the plant other than through root cracks and wounds (Santoyo et al. 2016; Kandel et al. 2017). One of the suggested alternative entryways is through leaf or needle stomata (Moore et al. 2006; Padda et al. 2022; Zhou et al. 2023), which could be the entryway for the unique needle fungal species detected in the paper I study.

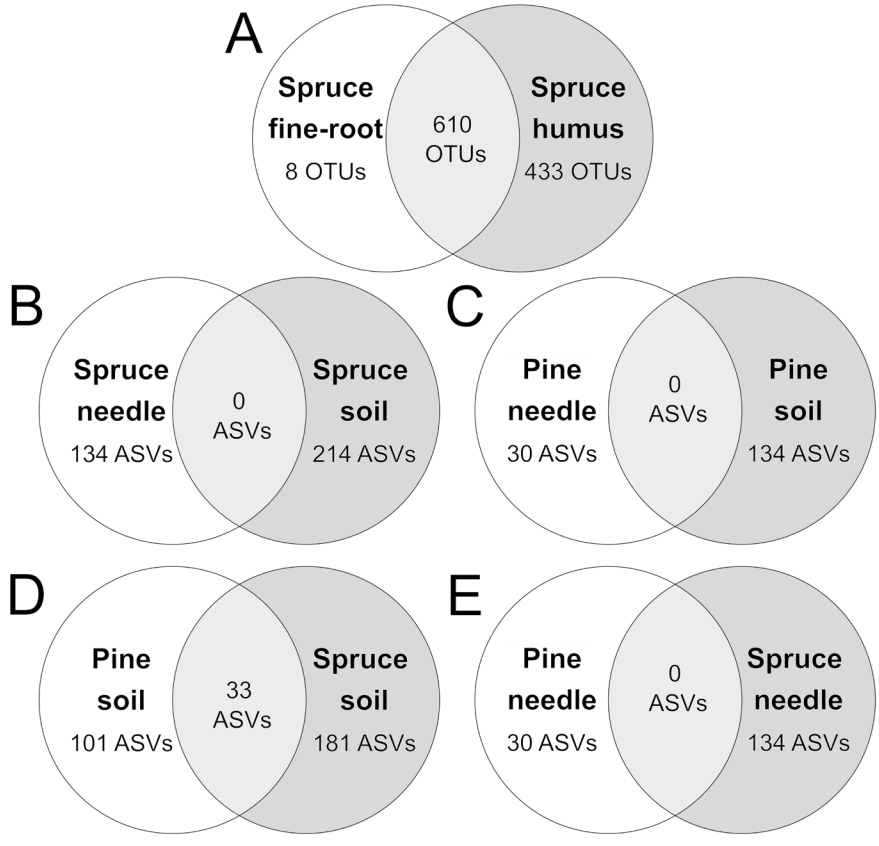

Figure 2: Shared fungal OTUs/ASVs between different compartments and tree species based on ITS sequencing: A) spruce fine-root and humus, B) spruce needle and bulk soil, C) pine needle and bulk soil, D) pine and spruce bulk soil and E) pine and spruce needle. Observe that different sequencing methods and sample sizes have been used (spruce fine-root: Illumina, n = 72 and Svartberget nitrogen addition experiment, spruce humus: Illumina, n = 71 and Svartberget nitrogen addition experiment, spruce bulk soil and needle: PacBio, n = 8 and Svartberget common garden, and pine bulk soil and needle: PacBio, n = 6 and Svartberget common garden).

4.1.2 Bacterial microbiomes

Compared to fungal microbiomes, even less is known about boreal forest bacterial microbiomes, which were investigated in paper I. The results showed the presence of eight different bacterial phyla across Scots pine and Norway spruce bulk soil samples (Figure 3A). The most abundant phylum was Actinobacteriota, with higher relative abundance in pine compared to spruce bulk soil, and Proteobacteria with similar relative abundances across

the two tree species bulk soil samples. The relative abundances of the two phyla, Acidobacteriota and Planctomycetota, were higher in spruce compared to pine samples, while the phylum Bacteroidota was mostly detected in pine bulk soil. Bacteria from the same phyla as observed in paper I study have been previously detected in association with Norway spruce and lodgepole pine (Nacke et al. 2016; Haas et al. 2018; Padda et al. 2022). Usually, higher relative abundances of Proteobacteria and Acidobacteriota were reported compared to Actinobacteriota (Carrell & Frank 2014; Nacke et al. 2016; Padda et al. 2022). In contrast to previous studies, this study found that the relative abundance of Actinobacteriota was higher than the relative abundance of Acidobacteriota, which has also been observed in a study looking at the soil microbiome of seven different forest trees (Urbanová et al. 2015).

The bacterial taxonomic classes showed a similar pattern to the bacterial phyla with a high relative abundance of Actinobacteria and a higher relative abundance of Acidobacteriariae in pine compared to spruce bulk soil (Figure 3B). Additionally, there was a high relative abundance of Alphaproteobacteria and Planctomycetes. Even though no previous studies compared the bulk soil bacterial microbiomes between the Norway spruce and Scots pine, the bacterial classes reported in this study have been observed in association with Norway spruce and diverse pine species growing in European forests (Choma et al. 2021; Romeralo et al. 2022).

For both fungi and bacteria, there were many sequences that could only be identified at the higher taxonomic ranks, but not at lower taxonomic ranks such as genus or species. Bacteria had a higher number of unassigned OTUs/ASVs at the species level than fungi (data not shown). The reason for observed unassigned OTUs/ASVs for both fungi and bacteria could be missing data in the databases used to annotate the OTU/ASV taxonomy during the bioinformatic analysis (Daisley & Reid 2021; Barrenechea Angeles et al. 2024). This suggests the need for further isolation, identification and characterization of microorganisms to be able to more accurately analyse and interpret the sequencing results, leading to better insight into the microbiome composition of a specific environment (Nguyen et al. 2016b; Barrenechea Angeles et al. 2024).

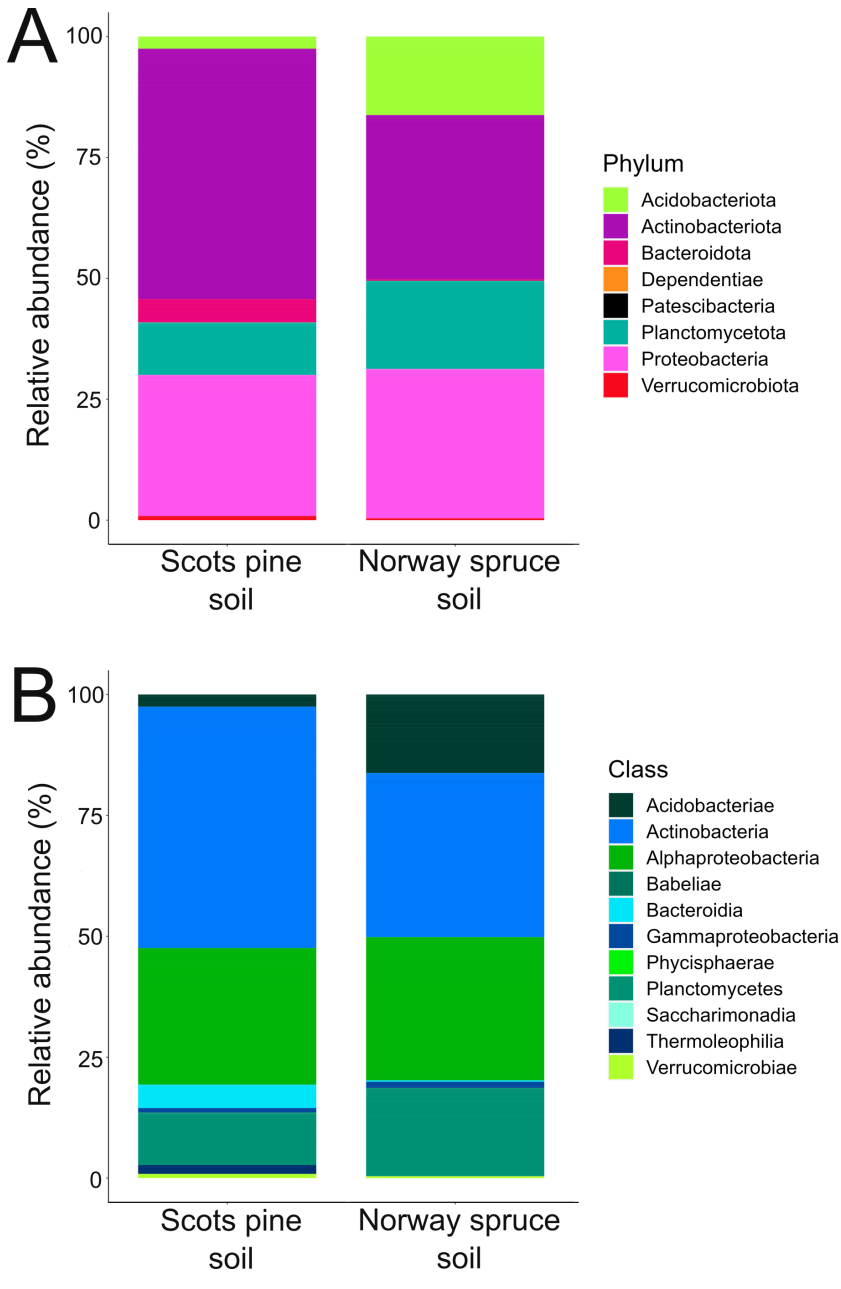

Figure 3: Bacterial relative abundance based on 16S rRNA sequencing in pine and spruce bulk soil looking at A) phylum and B) class (n = 6 for pine samples and n = 8 for spruce samples, both from the Svartberget common garden experiment and with PacBio platform sequencing).

4.1.3 Nitrogen-fixing bacteria

Bacteria with the ability to fix nitrogen from the atmosphere are widespread in nitrogen-limited ecosystems (Sepp et al. 2023), but little is known about their presence inside conifer tissues. It has been hypothesized that nitrogen-fixing bacteria might be inhabiting conifer needles in severely nitrogen-limited environments (Carrell & Frank 2014; Wurzburger 2016; Puri et al. 2018). To confirm their presence in Scots pine needles and better characterize this unique subset of bacteria, nitrogen-fixing endophytes were isolated on nitrogen-free media and identified in papers II and V. The 13 isolated bacterial strains from paper II belonged to the *Sphingomonas*, *Variovorax*, *Novosphingobium*, *Microbacterium*, *Priestia* and *Bacillus* genera (Figure 4 – bacterial strains #1, #2, #3, #14, #23, #24, #25, #26, #27, #28, #38-1, #38-2 and #39), while the two isolated bacterial strains in paper V belonged to *Bacillus* and *Robbsia* genera (Figure 4 – bacterial strains #1A and #2A). The findings of bacterial species belonging to these genera were in accordance with previous studies as bacterial strains belonging to these genera have been commonly isolated from tree species such as lodgepole pine, hybrid white spruce, poplar, birch and rowan (Moore et al. 2006; Izumi et al. 2008a; Puri et al. 2018). The only exception was *Robbsia andropogonis*, which has only been found in association with mycorrhizas of slash pine (Izumi et al. 2008b) but is otherwise a known plant pathogen with a wide host range (Duan et al. 2009). It has previously been shown that several bacterial strains belonging to *Sphingomonas*, *Variovorax*, *Novosphingobium*, *Microbacterium*, *Priestia* and *Bacillus* genera possess plant growth-promoting properties leading to increased plant growth (Çakmakçı et al. 2001; Karlidag et al. 2007; Maimaiti et al. 2007; Castanheira et al. 2014; Rangjaroen et al. 2017; Katsenios et al. 2021), therefore the bacteria isolated in papers II and V could be good candidates for plant growth promotion.

In both papers II and V, nitrogen-free media was used as that is supposed to ensure that only nitrogen-fixing bacteria are isolated, as other bacteria cannot grow without added nitrogen. However, it has been seen that certain bacterial strains without nitrogen fixation ability were capable of growing on nitrogen-free media (Doty et al. 2009; Padda et al. 2018; Puri et al. 2018). Therefore, the isolated bacterial strains were tested for their nitrogen fixation ability using the acetylene-reduction assay. The results showed that 12 out of the 15 isolated bacterial strains were able to produce ethylene, indicating

nitrogen fixation, when grown in liquid cultures (papers II and V). The other three bacterial strains (#2, #14 and #38-1) are either not nitrogen-fixing and were able to grow due to nitrogen carryover (Ozawa et al. 2003) or maybe the acetylene-reduction assay conditions were not suitable for these strains, for example due to selected media, temperature or incubation time.

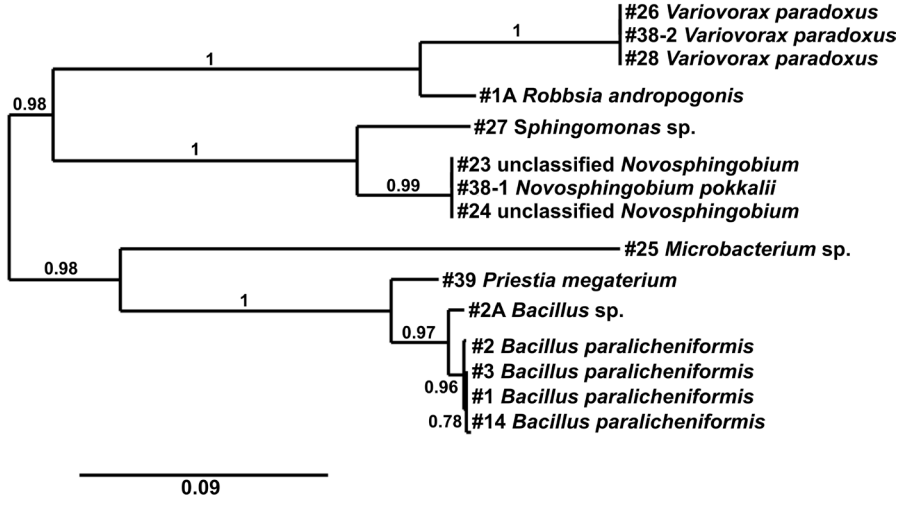

Figure 4: Phylogenetic tree of the isolated potential nitrogen-fixing bacteria from the Åheden field site surface-sterilized Scots pine needles based on 16S rRNA sequencing. The phylogenetic tree was created using Phylogeny.fr and the numbers represent the branching points´ likelihood-ratio test values.

4.2 Effects of inorganic nitrogen addition on microbiomes

The second research question investigated the effects of nitrogen addition on boreal forest microbiomes. Paper III analysed the impact of nitrogen addition on fungal and bacterial abundance, fungal microbiome composition, and the activity of several extracellular enzymes. Paper II focused on the nitrogen addition effects on the presence and activity of nitrogen-fixing bacteria inside Scots pine needles.

4.2.1 Fungal microbiomes

Addition of inorganic nitrogen, through either fertilization or atmospheric deposition, can affect the fungal communities in the boreal forest (Yan et al.

2017; Haas et al. 2018; Fu et al. 2022), however, effects may differ between sites and the exact effects on fungal community structure and function are not yet fully understood. The PLFA results from paper III showed that a high rate of nitrogen addition significantly decreased the fungal biomass in the humus soil layer (Figure 5A). Previous studies had contrasting results, as some studies reported, like paper III, a decreased fungal biomass after nitrogen addition (Janssens et al. 2010; Maaroufi et al. 2015; Maaroufi et al. 2019) and some studies reported instead an increased fungal biomass after nitrogen addition (Tahovská et al. 2020; Jörgensen et al. 2021).

The Venn diagram showed that most of the humus fungal OTUs were shared between the three nitrogen addition treatments, and there were only a few OTUs which were unique to either one or two specific nitrogen addition rates (Figure 5B). These results indicate that the observed decrease in fungal biomass might not be due to the disappearance of fungal species but rather to a changed abundance of the fungal species present in the environment. The sequencing analysis of the fungal microbiome in the humus layer showed a change in the relative abundance of the 10 most abundant OTUs (Figure 5C). The relative abundance of the species *Oidiodendron pilicola*, *Piloderma sphaerosporum* and *Cortinarious caperatus* decreased, while the relative abundance of *Tylospora fibrillose* increased with a high nitrogen addition rate. Fungi belonging to *Cortinarious* and *Piloderma* genera are known to be involved in the decomposition of lignified soil organic matter to access nitrogen, and their abundance has previously been observed to decrease with increasing nitrogen addition (Lilleskov et al. 2011; Bödeker et al. 2014). *Tylospora* genus fungi are known to be ectomycorrhizal fungi tolerant to high nitrogen levels (Prendergast-Miller et al. 2011).

Similar to the humus, the endophytic fine-root fungal microbiome also changed following the nitrogen addition treatment (Figure 5D). The relative abundance of several fungi, such as *Cenococcum geophilum*, *Piloderma sphaerosporum* and *Meliniomyces bicolor,* decreased with a higher nitrogen addition rate. But the relative abundance of *Phialocephala fortinii* and *Pezoloma ericae* increased with a higher nitrogen addition rate. Similar to *Piloderma* genus fungi, *Cenococcum* genus fungi have been seen to decrease with increasing nitrogen addition (Cox et al. 2010). In contrast to paper III results, the abundance of *Meliniomyces* has previously been seen to stay similar or even increase with nitrogen addition (Marupakula et al. 2021; Renaudin et al. 2023). *Phialocephala fortinii* and *Pezoloma ericae* are

commonly reported fungi in the boreal forest (Summerbell 2005; Orumaa et al. 2022), but not much is known about their response to nitrogen addition.

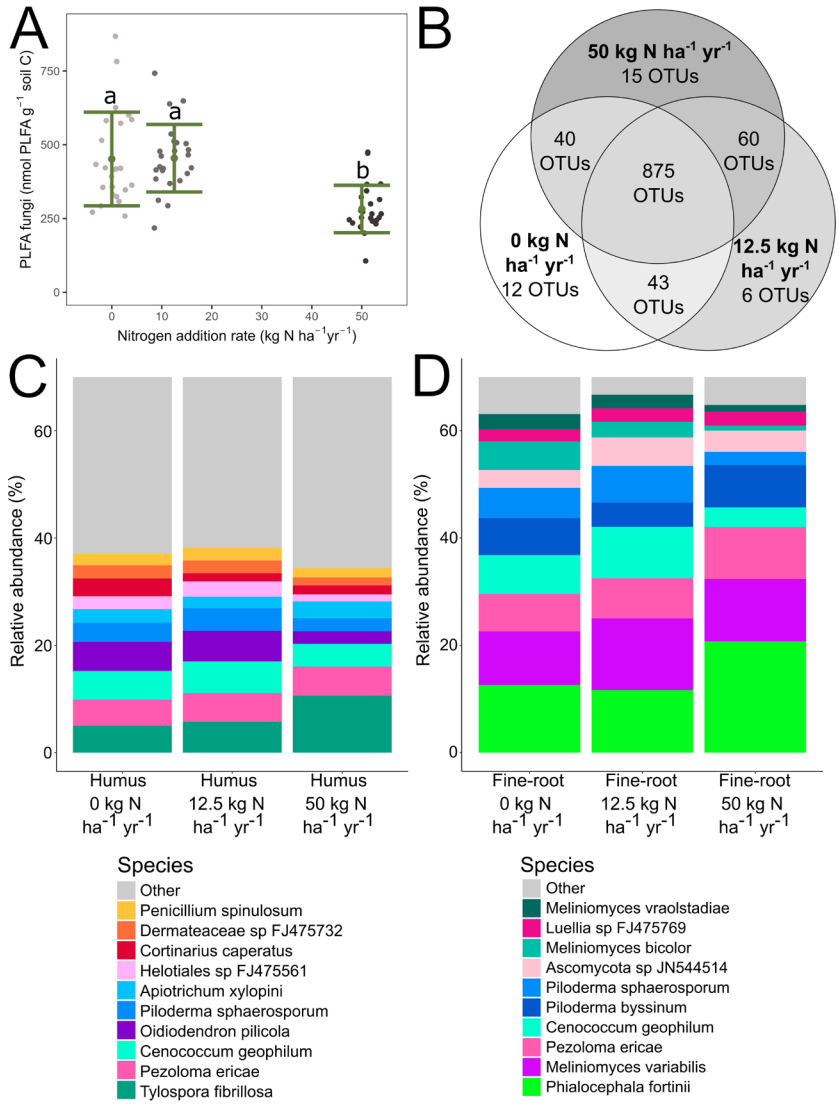

Figure 5: Effects of inorganic nitrogen addition on Norway spruce fungal microbiome at the Svartberget nitrogen addition experiment: A) fungal PLFA (different letters represent statistical differences based on linear mixed effects models with Tukey's HSD test, n = 24), B) shared OTUs between different addition treatments (n = 47), C) relative abundance of top 10 most abundant fungi in humus for different addition treatments based on ITS sequencing (n = 23) and D) relative abundance of top 10 most abundant fungi in fine-roots for different addition treatments based on ITS sequencing (n = 24).

A change in the humus fungal microbiome composition due to nitrogen addition might lead to a shift in the activity of extracellular enzymes as diverse types of fungi play different roles in the soil decomposition process (Baldrian & Štursova 2010; Sinsabaugh 2010). For example, the decreased relative abundance of fungal species known for decomposition of lignified soil organic matter observed in paper III and previous studies (Lilleskov et al. 2001; Lilleskov et al. 2002), could lead to lower activity of oxidative and nitrogen-acquiring enzymes. Therefore, paper III analysed the activity of two nitrogen-acquiring enzymes, two oxidative enzymes, three carbon-acquiring enzymes and one phosphorus-acquiring enzyme (Figure 6). Indeed, decreased enzymatic activity after high nitrogen addition was observed for the two nitrogen-acquiring enzymes (Figure 6A - peptidase and chitinase) and one of the two oxidative enzymes (Figure 6B - for peroxidase, but not laccase). It could be that there was no difference in the laccase activity as laccases are known to not only target lignin compounds but have a wider range of substrates (Thurston 1994). The activity of one carbon-acquiring enzyme (Figure 6C - glucosidase) stayed similar with nitrogen addition, but the activity of the other two carbon-acquiring enzymes increased (Figure 6C - xylosidase and endoglucanase). The observed decrease in the activity of nitrogen-acquiring enzymes and increased activity of carbon-acquiring enzymes proposes a shift from a nitrogen-limited fungal community to a carbon-limited fungal community. The increased activity of carbon-acquiring enzymes is probably not significantly affecting soil decomposition as enzymes measured in this study target labile carbon and not carbon stored in more lignified compounds. The activity of phosphorus acquiring enzyme (Figure 6D - phosphatase) did not change after nitrogen addition, which was interesting as it is predicted that the second most limiting nutrient after nitrogen in the boreal forest is phosphorus (Almeida et al. 2019; Richy et al. 2024). The unchanged activity of phosphatase indicates that at the paper III Svartberget nitrogen addition experiment field site phosphorus is probably not a growth limiting factor despite nearly 30 years of nitrogen additions of up to 50 kg nitrogen per hectare and year. Overall, the observed decrease in the activity of nitrogen-acquiring enzymes in paper III could contribute to the previously observed and reported decrease in soil respiration and organic matter decomposition after nitrogen addition (Nohrstedt et al. 1989; Janssens et al. 2010; Forsmark et al. 2021).

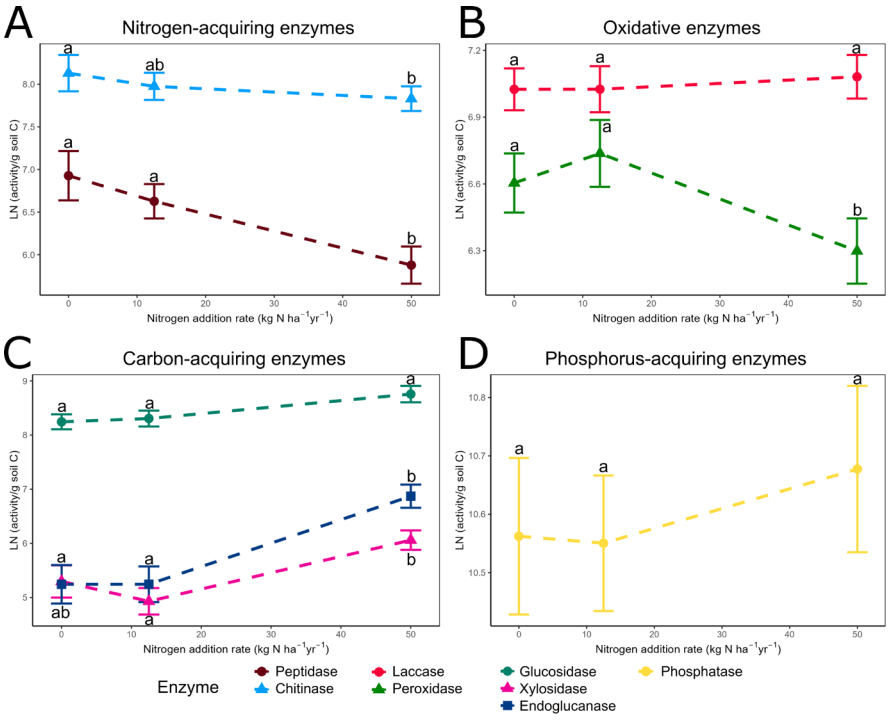

Figure 6: Combined summer and autumn potential enzyme activity across three levels of nitrogen addition at the Svartberget nitrogen addition experiment field site looking at A) nitrogen-acquiring enzymes, B) oxidative enzymes, C) carbon-acquiring enzymes and D) phosphorus-acquiring enzymes. Values are the natural logarithm of molar cleavage of enzyme-specific substrates standardized to the amount of carbon in each sample (n = 24, different letters represent statistical differences based on linear mixed effects models and Tukey's HSD test).

4.2.2 Bacterial microbiomes

Nitrogen addition has also been seen to affect the bacterial microbiomes in forests (Yan et al. 2017; Haas et al. 2018; Fu et al. 2022). In paper III, the results showed a significant decrease in the total bacterial biomass with increased nitrogen addition based on the PLFA analysis (Figure 7A). Additionally, the ratio between the gram-positive and gram-negative bacteria increased due to a lower decrease in the abundance of gram-positive bacteria compared to gram-negative bacteria after high nitrogen addition (Figure 7B). Studies in the tropical and subtropical forests showed the same trend of lower PLFA-measured bacterial biomass with a higher gram-positive to gram-negative bacteria ratio following high nitrogen addition (Wang et al. 2018;

Zhou et al. 2019). An explanation for the observed change in the gram-positive and gram-negative bacteria ratio could be a higher dependence of gram-negative bacteria on labile carbon sources, while gram-positive bacteria are capable of degrading more complex organic matter compounds containing carbon (Kramer & Gleixner 2008; Fanin et al. 2019), as increased nitrogen abundance has been associated with lower tree carbon allocation to belowground (Högberg et al. 2010; Janssens et al. 2010). Another potential explanation could be that gram-positive bacteria might be more tolerant to osmotic stress, which can temporarily incur after inorganic nitrogen fertilizer application (Eno et al. 1955; Omar & Ismail 1999; Geisseler et al. 2017). Namely, compared to gram-negative bacteria, gram-positive bacteria have a much thicker cell wall, which can sustain higher turgor pressure, better maintain cation homeostasis and is less leaky (Jordan et al. 2008; Silhavy et al. 2010; Mai-Prochnow et al. 2016; Wang et al. 2018).

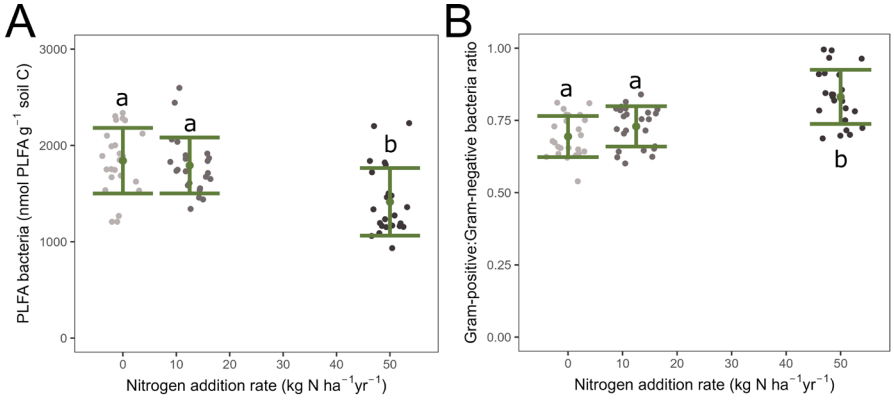

Figure 7: Effects of inorganic nitrogen addition on Norway spruce humus bacterial microbiome at the Svartberget nitrogen addition experiment field site: A) total bacteria PLFA and B) ratio between gram-positive and gram-negative bacteria. Different letters represent statistical differences based on linear mixed effects models with Tukey's HSD test (n = 24).

Additionally, higher nitrogen abundance has a documented negative effect on nitrogen fixation activity in diverse forest environments (Barron et al. 2011; Gundale et al. 2011; Zheng et al. 2017). For example, high nitrogen addition has severely decreased the nitrogen fixation of cyanobacteria associated with boreal forest mosses (Gundale et al. 2013; Leppänen et al. 2013), which has even been observed at the same Åheden field site as where samples in papers II and V were collected from (Gundale et al. 2011). What

has not yet been researched is whether nitrogen addition to the forest ecosystem can also affect the presence and activity of nitrogen-fixing bacteria inhabiting boreal forest conifer needles, which was the question addressed in paper II. The study found that the amount of nitrogenase NifH subunit protein was similar in needles from pine trees growing on the control and high nitrogen addition plots (Figure 8A), indicating no difference in the presence of nitrogen-fixing bacteria in needles between these two treatments. Furthermore, there was no significant difference in the bacterial nitrogen-fixation activity between control and high nitrogen addition plots (Figure 8B). The study expectation was decreased activity of nitrogen-fixing bacteria in needles from trees growing on high nitrogen addition plots due to previously observed decreased nitrogen fixation in connection to cyanobacteria and mosses (Gundale et al. 2013; Leppänen et al. 2013). Similar results of no correlation between nitrogen fixation and nitrogen availability have, however, been observed for limber pine needles (Moyes et al. 2016) and tropical forest tree leaves (Zheng et al. 2017). A potential explanation could be that even though nitrogen addition increases nitrogen content in needles as observed in paper II and previously (Sikström 1997; Nybakken et al. 2018), the difference is not significant enough to change the activity of nitrogen-fixing bacteria following nitrogen addition. Another potential explanation is that in addition to dependence on nitrogen availability, the nitrogen fixation activity might be dependent on carbon availability, which has been indicated previously as carbon addition has been able to promote nitrogen fixation (Welsh 2000; Smercina et al. 2019; Benavides et al. 2020). It has been shown that fertilized trees allocate more carbon to aboveground parts and have higher photosynthesis rates (Manter et al. 2005; Zhao & Liu 2012; Tarvainen et al. 2016).

The exact nitrogen contribution of needle bacterial nitrogen fixation in boreal forest is not yet known, and previously reported rates across diverse forests have a wide range from grams to kilograms of nitrogen per hectare and year (Jones 1970; Granhall & Lindberg 1978; Favilli & Messini 1990; Moyes et al. 2016). The calculated extrapolated measured rate of nitrogen fixation in Scots pine needles in paper II was in the range of between 11 and 15 grams of nitrogen per hectare and year. The rate is higher than previously reported (Granhall & Lindberg 1978), in accordance with measured rates in limber pine (Moyes et al. 2016), but notably smaller compared to rates estimated for black pine and Douglas fir (Jones 1970; Favilli & Messini

1990). The calculated rate of nitrogen fixation is most often based on many assumptions regarding seasonal, temperature, and light intensity variation, therefore, it needs to be interpreted with caution. Additionally, acetylene-reduction assay measurements only show a time-limit view of the nitrogen fixation rate. Nevertheless, the nitrogen fixed through nitrogen-fixing bacteria inhabiting Scots pine needles is very low compared to the amount needed for pine yearly growth, which is approximately 50 kg nitrogen per hectare and year (Korhonen et al. 2013), while the extrapolated rates of nitrogen fixed per hectare and year at the field site of paper II were between 11 and 15 grams. However, it might be an important long-term source of nitrogen for the Fennoscandian boreal forest, considering the forest's nitrogen cycle over centuries.

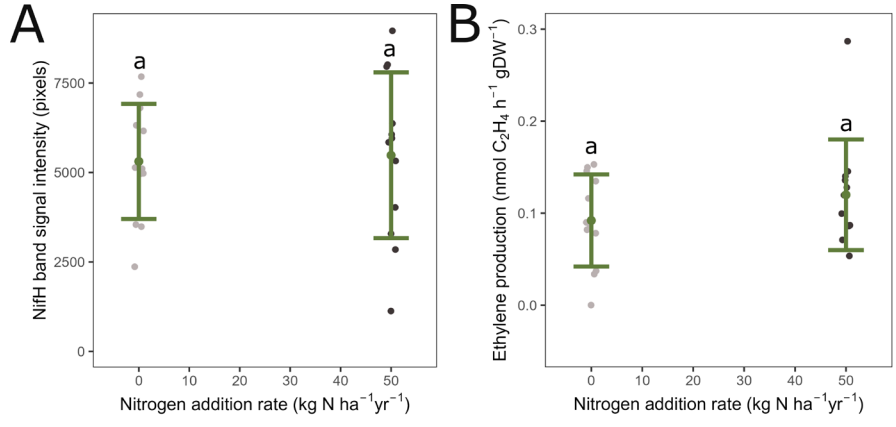

Figure 8: Inorganic nitrogen addition effects on nitrogen-fixing bacteria in Scots pine needles at Åheden field site: A) western blot detecting NifH subunit protein of nitrogenase enzyme (n = 12, different letters indicate statistical differences based on two independent samples t-test) and B) acetylene-reduction assay on needles showing ethylene production (n = 12, different letters indicate statistical differences based on two independent samples t-test).

4.3 Alternatives to inorganic nitrogen fertilization

The third research question investigated alternatives to adding inorganic nitrogen fertilizers to promote the growth of tree and crop seedlings grown under greenhouse conditions. Paper IV analysed whether a new controlled-release organic nitrogen formulation could promote the growth of Scots pine seedlings. Paper V studied the plant growth-promoting potential of bacterial

strains isolated from Scots pine needles *in vitro* and *in vivo* using four different crop species.

4.3.1 Organic nitrogen-based nutrition

One of the suggested sustainable alternatives to inorganic nitrogen fertilizers applied in conifer nurseries to promote tree seedlings growth is plant nutrition based on organic nitrogen. In comparison to inorganic nitrogen fertilizers, application of organic nitrogen may minimize nitrate leaching from the growth media, sustain associated microbiota and support a higher root-to-shoot ratio, making the seedlings more persistent to, e.g. dry soil conditions (Öhlund & Näsholm 2002; Gruffman et al. 2012). Paper IV analysed if Arginine Fe-HMP, a controlled-release organic nitrogen-based plant nutrition technology by Arevo AB, could promote plant growth similarly to a commercially available controlled-release inorganic nitrogen-based fertilizer. Arginine Fe-HMP is based on a complex between the amino acid arginine, polyphosphate and iron, and it is biodegradable as its slow nutrient release is controlled by the polyphosphate chain length (Näsholm et al. 2017). An explorative chemical analysis of Arginine Fe-HMP confirmed a successful synthesis of arginine-phosphate-iron complex with uniform elemental distribution and morphological consistency (paper IV). Results further showed the presence of expected functional groups and poor crystallinity of the Arginine Fe-HMP complex, which may improve its solubility and bioavailability to seedlings (paper IV). A greenhouse study was set up to evaluate Scots pine seedling growth for six months under three different treatments: non-fertilized control, addition of Arginine Fe-HMP or addition of commercial CRF. The results showed that Arginine Fe-HMP did promote plant growth as seedlings had higher biomass compared to the control treatment (Figure 9). The growth promotion by Arginine Fe-HMP and commercial CRF were similar as there were no significant differences in either seedling dry root or shoot weight between the two treatments (Figure 9A and 9B, respectively). Furthermore, analysis of run-off water from the growth media showed that Arginine Fe-HMP treatment had significantly lower nitrate leaching compared to commercial CRF (paper IV). No effect on the root-to-shoot ratio from the different nitrogen treatments was observed in this study (paper IV), contrasting previous studies showing a higher root-to-shoot ratio following the addition of organic nitrogen nutrition compared to inorganic nitrogen fertilization of conifer seedlings (Gruffman

et al. 2012). However, there have been a few studies showing no effect of arginine-based nutrition on the root-to-shoot ratio (Heiskanen et al. 2009; Lim et al. 2022). It could be that the root-to-shoot ratio was similar between the two nitrogen treatments in paper IV study as in contrast to previous studies using weekly additions of the arginine-based nutrition, paper IV study was the first with controlled-release nutrition formulation application only at the beginning of the study. Therefore, differences might have been seen if higher nitrogen addition rates had been used or if nutrition would have been applied more frequently. Overall, the study in paper IV showed that the controlled-release organic nitrogen-based plant nutrition Arginine Fe-HMP can promote plant growth as well as commercially available controlled-release inorganic nitrogen-based fertilizer with the benefit of being biodegradable, having slow nutrient release and having significantly lower nitrate leaching.

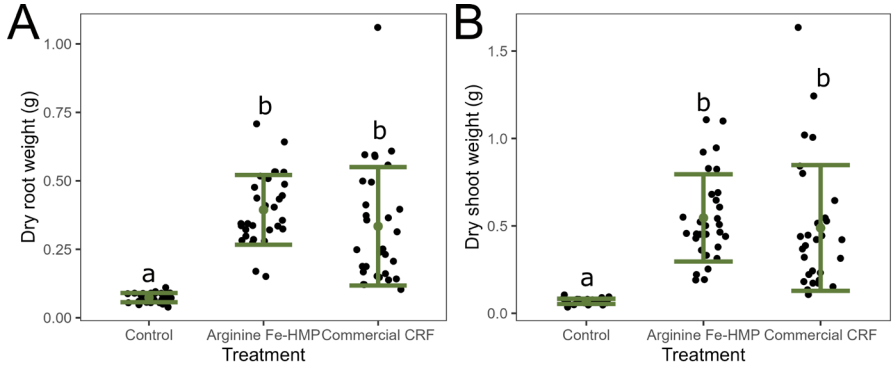

Figure 9: Scots pine seedling dry weights of A) root and B) shoot after six-months long greenhouse experiment using three different nitrogen treatments: control, the addition of inorganic nitrogen-based nutrition Arginine Fe-HMP and the addition of commercial CRF. Different letters indicate statistical significance based on one-way ANOVA followed by Tukey‘s HSD test (n = 30).

Previous studies also reported that organic nitrogen-based nutrition has a less negative effect on the mycorrhization of the conifer roots compared to inorganic nitrogen fertilization (Avolio et al. 2009; Gruffman et al. 2012). Therefore, it was hypothesized that treatment with Arginine Fe-HMP would lead to less negative effects on root fungal biomass and bacterial nitrogen fixation in Scots pine seedlings compared to commercial CRF. Ergosterol, a known fungal biomass marker (Lau et al. 2006; Gruffman et al. 2013), showed the highest ergosterol concentration for control seedlings (Figure

10A). However, as their roots were very small, the ergosterol content per whole seedling dry root was the lowest of all treatments. The ergosterol concentration was similar for both Arginine Fe-HMP and commercial CRF treatments but lower compared to control seedlings, while ergosterol contents were higher compared to control seedlings. The result was in accordance with a previous study showing similar fungal abundance for organic and inorganic nitrogen treatments (Gruffman et al. 2013). The investigation of the nitrogen-fixing capacity associated with the pine seedlings showed the highest rate for control seedlings and similarly lower for both Arginine Fe-HMP and commercial CRF (Figure 10B). As the control seedlings did not get any additional nitrogen, they were highly nitrogen-limited, and the nitrogen fixation of seedlings was expectedly the highest. It was surprising that Arginine Fe-HMP showed similar results in both ergosterol content and nitrogen-fixation capacity to commercial CRF as studies on organic nitrogen nutrition showed lesser negative treatment effects on microbiomes compared to inorganic nitrogen-based fertilization (Vaario et al. 2009; Gruffman et al. 2012). However, the previous studies used liquid arginine-based nutrition, while paper IV analysed a novel controlled-release arginine-based nutrition formulation, which could explain the difference between the results. Arginine Fe-HMP could be further developed to reach the goal of a neutral or even positive effect on the associated fungal and bacterial microbiomes and an inclusion of potassium to ensure complete nitrogen-phosphorus-potassium nutrition.

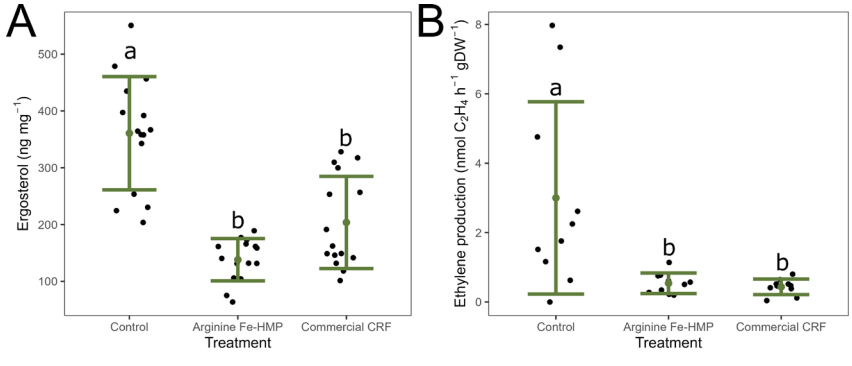

Figure 10: The effect of three different nitrogen treatments (control, Arginine Fe-HMP and commercial CRF) on A) ergosterol concentration (n = 15, different letters indicate statistical significance based on one-way ANOVA followed by Tukey‘s HSD test) and B) ethylene production in pine seedlings after six-month long greenhouse experiment (n = 10, different letters indicate statistical significance based on Kruskal-Wallis test followed by pairwise comparisons with Bonferonni‘s adjusted value).

4.3.2 Plant growth-promoting bacteria

Another sustainable alternative to inorganic nitrogen fertilizers could be the use of PGPB as inoculants to promote plant growth. In paper V, the plant growth-promoting ability of nine isolated nitrogen-fixing bacteria was assessed using *in vitro* plant growth-promoting property assays. We used bacterial strains isolated from Scots pine needles as it has been suggested that severely nitrogen-limited environments such as boreal forests harbor many excellent PGPB candidates (Ryan et al. 2008). Seven *in vitro* assays were used to assess the potential of PGPB to solubilize nutrients, and produce plant hormones, cell wall degrading enzymes and compounds involved in plant pathogen protection. All assayed bacterial strains were capable of solubilizing tricalcium phosphate (Figure 11A), however, they were unable to solubilize either iron (III) phosphate, aluminium phosphate or zinc. It has been reported previously that many bacteria are capable of solubilizing tricalcium phosphate but cannot solubilize harder-to-dissolve forms such as iron (III) phosphate and aluminium phosphate (Pérez et al. 2007; Bashan et al. 2012). The ability to solubilize nutrients, which are limited in the environment (such as zinc and phosphorus), is important as changing these nutrients into plant-available forms can have a substantial effect on plant growth (Saravanan et al. 2007; Rana et al. 2020). None of the nine bacteria were capable of hydrogen cyanide production, while all but one bacterial strain did produce siderophores (Figure 11B). Both siderophore and hydrogen cyanide production are involved in plant protection against pathogens (Jasim et al. 2013; Olanrewaju et al. 2017). It has been previously reported that the ability to produce hydrogen cyanide might be limited within the PGPB (Antoun et al. 1998). Another important plant growth-promoting ability is the production of plant hormones such as IAA, as they play a role in plant development and defence (Olanrewaju et al. 2017). The assay results showed that all bacterial strains were able to produce IAA in various amounts (Figure 11C), however, it is possible that their IAA production is overestimated as the assay might also show the presence of other indole-like molecules (Glickmann & Dessaux 1995). Further, all bacterial strains had protease activity (Figure 11D), and all but one bacterial strain showed cellulase activity (Figure 11E). The activities of both cell wall degrading enzymes are important for the endophytic bacterial lifestyle and protection against plant pathogens (Kandel et al. 2017; Puri et al. 2020b). The nine bacterial strains analysed in *in vitro* assays showed good plant growth-

promoting potential as most of them showed activity in five out of seven assays.

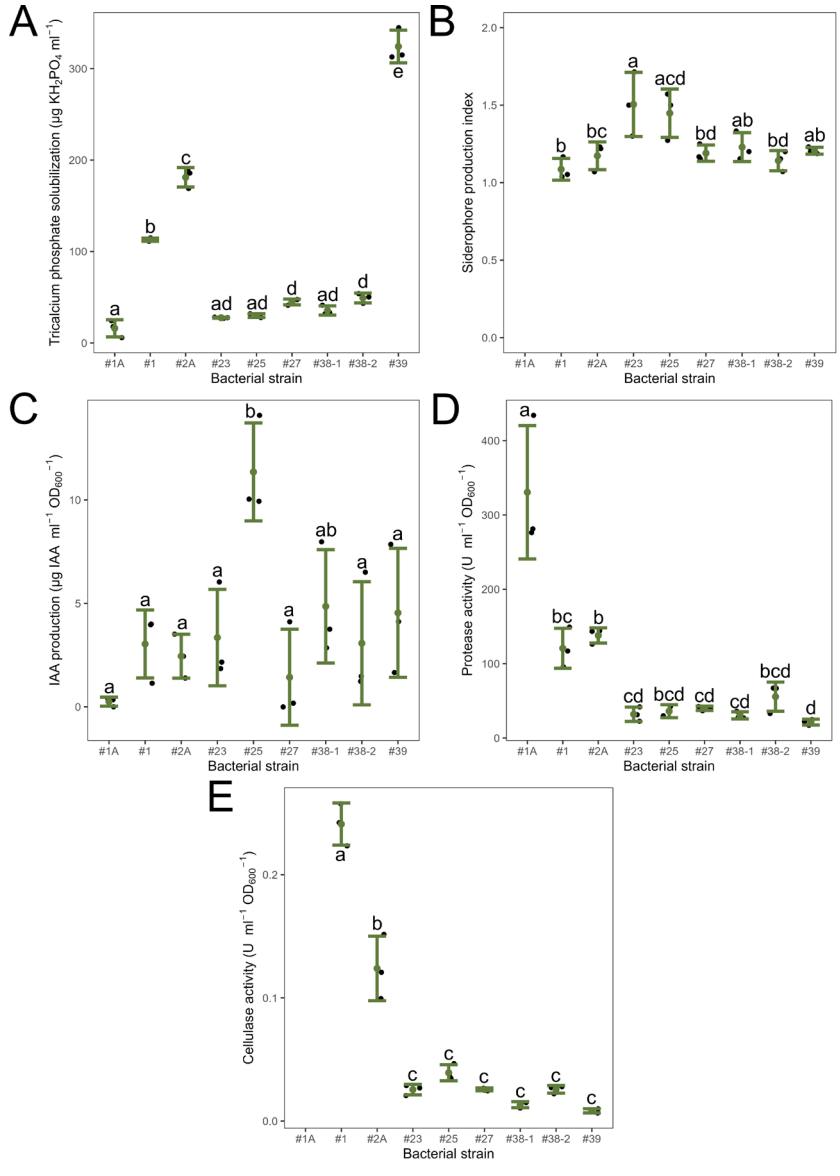

Figure 11: Plant growth-promoting property assays to evaluate A) tricalcium phosphate solubilization, B) siderophore production, C) IAA production, D) protease activity and E) cellulase activity by selected bacteria isolated from Scots pine needles at the Åheden field site (n = 3, different letters indicate statistical significance based on one-way ANOVA followed by Tukey‘s HSD test).

To further analyse the plant growth promotion of isolated bacteria, a greenhouse experiment with the three best bacterial strains based on their performance in the seven *in vitro* assays was performed. The greenhouse experiment included the use of soil as a growth medium and the presence of both soil and seed naturally occurring microbiome. This approach contrasted most of the previous studies on conifer-isolated PGPB, which were performed using a sterile sand mixture as a growth medium (Padda et al. 2015; Puri et al. 2020a; Padda et al. 2021). While studies using artificial growth media are important for a better understanding of PGPB inoculation and plant growth promotion potential, their results can rarely be translated into successful field applications (Compant et al. 2010; Gamalero & Glick 2011; Gaiero et al. 2013). Therefore, the use of soil with its native microbiome has been recommended (Etesami & Maheshwari 2018; de-Bashan & Nannipieri 2024). The greenhouse study in paper V included four crops from different taxonomic plant orders: corn, cucumber, kale and tomato, which were inoculated with either single bacterial strains *Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39 or their consortium.

The inoculation treatment with either one of the bacterial strains or the consortium did not show any promotion of plant dry weight for either corn, cucumber, kale or tomato (Figure 12). The results for chlorophyll content and root and shoot length showed a similar trend (paper V). It could be that the neutral results were observed as the inoculated bacterial strains were unable to compete with the native soil and seed microbiome (Shishido et al. 1999; Germaine et al. 2004). Another potential reason could be the use of naturally nutrient-abundant growth media, as it has been previously observed that plant growth promotion is higher in nutrient-limited growth media (Egamberdiyeva 2007; de Souza et al. 2012). Additionally, it has been suggested that PGPB might have a higher effect on plant growth promotion of their host species (Boddey & Dobereiner 1988; Lucy et al. 2004). Therefore, additional long-term experiments using Scots pine seedlings would be interesting, even though shorter unpublished pre-studies did not show promising results (Appendix 4). In conclusion, the results of paper V showed that even though bacterial strains had promising results in *in vitro* plant growth-promoting assays, they were unable to promote crop growth in the *in vivo* greenhouse experiment. It has been previously suggested that negative results in inoculation studies are under-reported in the literature

(Bacilio et al. 2017), which could lead to an overestimation of PGPB's potential as a sustainable means of promoting plant growth.

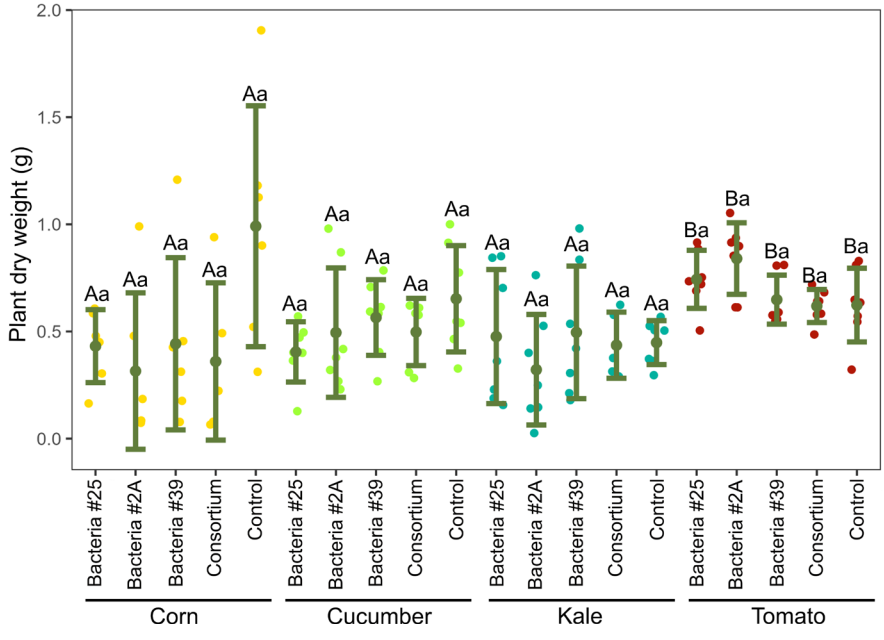

Figure 12: Total dry weight of around five-week-old corn, cucumber, kale and tomato plants after inoculation with individual bacterial strains (*Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39) or consortium compared to control non-inoculated plants. The data was analysed by two-way ANOVA followed by Tukey's HSD test (n = 7). The big letters show statistical differences between crop species, and the small letters show the statistical differences between different inoculation treatments.

5. Conclusions and future perspectives

The thesis, together with papers I-V, expanded the knowledge regarding the Scots pine and Norway spruce microbiomes in the Fennoscandian boreal forest in connection to nitrogen addition and assessed two alternatives to inorganic nitrogen fertilization to promote plant growth in greenhouse settings.

In addition to the description of pine- and spruce-associated microbiomes compositions, the microbiome analysis corroborated the suggestion that the needle endophytic microbiome is distinct from the soil and endophytic root microbiome. While humus and endophytic fine-root microbiomes shared the majority of fungal OTUs, the bulk soil and endophytic needle microbiomes did not share any fungal ASVs at all. This suggests that the needle endophytic microbiome is not a subset of the soil and root microbiomes but most probably includes species that enter the plant through alternative entry points such as stomata or above-ground wounds. Interestingly, even though pine and spruce trees were growing at the same Svartberget common garden field site, they shared only a few fungal ASVs in bulk soil samples and no fungal ASVs in the endophytic needle samples. These results corroborate the suggestion that plant species can influence the composition of their associated microbiomes.

Analysing the effects of inorganic nitrogen addition on fungal microbiomes at the Svartberget nitrogen addition experiment field site showed that even after more than 20 years of yearly fertilization, the majority of fungal OTUs were shared between the three nitrogen treatments (0, 12.5 and 50 kg nitrogen per hectare and year). These results indicate that the observed decreased fungal biomass after high nitrogen addition was probably due to changed fungal abundance and not the disappearance of fungal species. In addition to changing the fungal relative abundances, the high

nitrogen addition also resulted in statistically significantly different activities of several extracellular enzymes connected to carbon and nitrogen acquisition, which can have an important effect on the boreal forest carbon and nitrogen cycles.

The thesis results showed that the presence and activity of nitrogen-fixing bacteria in pine needles at the Åheden field site stayed similar even after years of high nitrogen addition (50 kg nitrogen per hectare and year). The results were in contrast to previously measured nitrogen fixation of cyanobacteria in association with mosses at the same field site as their nitrogen fixation decreased following high nitrogen addition (Gundale et al. 2011). One of the potential explanations for the observed difference between needles and mosses could be that nitrogen fixation rates may be connected to photosynthesis rates and carbon availability. This was additionally indicated by the observed importance of light intensity for nitrogen fixation during needle acetylene-reduction assay (Appendix 3). Previous studies showed that fertilized conifer trees have higher photosynthesis rates compared to control trees (Manter et al. 2005; Zhao & Liu 2012), which could mean a higher carbon availability for bacterial nitrogen fixation in needles. Contrary, due to bigger crowns of the fertilized pines at this field site (From et al. 2016), less light might be available to mosses, which could result in lower moss photosynthesis rates and consequently lower carbon availability for cyanobacterial and moss associated nitrogen fixation.

Two greenhouse experiments evaluated the possibility of using organic nitrogen-based controlled-release plant nutrition and inoculation with PGPB instead of the commonly used inorganic nitrogen fertilizers. Organic nitrogen-based Arginine Fe-HMP plant nutrition showed similar plant growth promotion and microbiome effects as commercial inorganic nitrogen controlled-release fertilizers with lower nitrate leaching, showing good potential as a more sustainable biodegradable alternative to commercial controlled-release fertilizers. In contrast, the inoculation of crop plants with PGPB did not result in improved plant growth. The neutral results could be due to the use of soil as growth media with naturally abundant nitrogen and the presence of the native microbiome. It could be that the native soil microbiome already includes bacteria possessing plant growth-promoting properties, as most PGPB have been isolated from natural environments. However, if PGPB are to be used in either agriculture or forestry, they must

be able to compete with the native microbiome and promote plant growth under natural settings.

Even though this thesis provided knowledge regarding boreal forest microbiomes in connection to nitrogen and analysed potential sustainable alternatives for plant growth promotion, the work is not finished, and many new questions arose during the thesis. Among others, future studies could include the microbiome composition analysis of further conifer compartments (such as stems and branches) and include more tree species to better assess the plant influence on the microbiome composition. Seasonality studies would be needed to better understand the microbiome composition changes and nitrogen-fixation rate differences throughout the year. Additional studies could investigate the potential connection between nitrogen fixation in needles and photosynthesis rates. In connection to sustainable alternatives to inorganic nitrogen fertilization, a study could test if the application of organic nitrogen-based controlled-release nutrition Arginine Fe-HPM could lead to better drought and cold acclimation as suggested for other organic nitrogen-based compounds (Sigala et al. 2020; Sigala et al. 2021). Future studies looking into PGPB would be needed to better understand the interactions between inoculated PGPB and the native microbiome to ensure a successful inoculation of plants. Ultimately, there is no lack of open questions and potential future studies due to the complexity of the boreal forest microbiomes and their multitude of interactions.

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Popular science summary

Bacteria and fungi are an important part of the boreal forest even if they are sometimes hard or impossible to notice. Both play a role in plant and soil health and growth, but compared to plants, animals and insects, not much is known about them. Therefore, this PhD thesis focused on discovering more about this intriguing part of the boreal forest by looking at Scots pine and Norway spruce associated fungal and bacterial communities.

The first question the thesis addressed was the composition of the fungal and bacterial communities in the boreal forest. The results showed that the composition of both communities was dependent on the compartment as differences were observed between soil, root and needle fungal and bacterial communities. While soil and root samples shared several fungal species, the needle samples were completely different from the soil samples for both Norway spruce and Scots pine. Part of the observed bacterial community are also nitrogen-fixing bacteria, which have the ability to change atmospheric nitrogen into a form that is available to plants for their growth. To better understand this community, several nitrogen-fixing bacteria from Scots pine needles were isolated and identified, showing the variety of these bacteria inside needles.

The second research question focused on the effect of inorganic nitrogen addition on boreal forest bacterial and fungal communities. Nitrogen is an important element in the boreal forest as tree growth is largely dependent on its availability in the environment. Therefore, inorganic nitrogen addition, through fertilization or deposition, can significantly affect the boreal forest, including bacterial and fungal communities. The results showed that the fungal and bacterial biomass declined, and the composition of fungal community changed after high nitrogen addition. The change in the fungal community consequently changed the activity of enzymes, which are exuded

by fungi to be able to take up nutrients. The thesis also analysed if inorganic nitrogen addition affected the presence or activity of nitrogen-fixing bacteria. The results showed that both their presence and activity were similar between the control and nitrogen addition boreal forest plots.

The third research question assessed two alternatives to inorganic nitrogen fertilization to promote plant growth in a greenhouse, as it is known that inorganic nitrogen can lead to negative environmental consequences. The first alternative, which was an organic nitrogen-based nutrition called Arginine Fe-HMP, showed similar plant growth promotion as currently used inorganic nitrogen fertilizers. However, as the use of this organic nitrogen fertilizer led to lower nitrate leakage and the production did not use non-biodegradable and fossil fuel-dependent substrates, Arginine Fe-HMP showed good potential as a more sustainable option than inorganic nitrogen fertilizers. The second option was the use of so-called plant growth-promoting bacteria, which are bacteria that possess certain properties that can help plants grow better. While the bacteria did possess several of these properties, they were not able to promote the growth of tomatoes, corn, cucumber or kale in a greenhouse experiment. It could be that they were unable to compete with the other bacteria and fungi already present in the soil and inside the seed.

Together, the three research questions provided more information and knowledge about the mostly hidden part of the boreal forest, which often gets overlooked due to its size.

Populärvetenskaplig sammanfattning

Bakterier och svampar är en viktig del av den boreala skogen även om de oftast är svåra eller omöjliga att se med blotta ögat. Båda organismgrupperna spelar viktiga roller för att säkerställa skogsekosystemens funktion, men jämfört med växter, djur och insekter är deras värld mindre utforskad. Denna doktorsavhandling syftade till att utforska och beskriva några tidigare inte så väl studerade frågor om svamp- och bakteriesamhällens olika roller i skogen.

Den första frågan handlade om hur artsammansättningen av svamp- och bakteriesamhällen skiljde sig mellan marken där träden växer samt trädens rötter och barr. Man fann att många svamparter var desamma i marken och inuti trädrötterna medan inuti barren fanns helt andra arter. Det fanns också betydande skillnader mellan tall och gran. I en särskild undersökning av tallbarr fann man att inuti barren fanns kvävefixerande bakterier, det vill säga bakterier som kan omvandla luftens kvävgas till kväveformer som växter kan använda för sin tillväxt. De kvävefixerande bakterierna i barren sekvenserades vilket betyder att man med hjälp av DNA analys tog reda på vilka arter av bakterier det handlade om. Kvävefixerande bakterier i barr har inte tidigare studerats i någon stor omfattning och studien bekräftade att kvävefixering i barr i skogar i norra Sverige kan bidra till skogarnas kväveförsörjning.

Den andra forskningsfrågan fokuserade på vad som händer med skogens bakterie- och svampsamhällen om man tillsätter kvävegödsel. Eftersom all tillväxt i nordliga skogar normalt sett är begränsad av kvävetillgången får kvävegödsling ofta en märkbar effekt på skogen då träden till exempel börjar växa bättre. Det är mindre väl känt vad som händer med bakterierna och svamparna när man kvävegödslar. Resultaten i denna avhandling visade att efter nästan 30 års tillsats av 50 kg kväve per hektar och år hade förekomsten av svampar och bakterier minskat. Detta gällde dock inte för de

kvävefixerande bakterierna i tallbar. Dessa påverkades inte av gödslingen utan var lika vanligt förekommande och fixerade lika mycket kväve från luften i barren på tallar som växte på ogödslade kontrollytor som på gödslade ytor.

Den tredje forskningsfrågan utvärderade två alternativ till vanlig oorganisk kvävegödsling i växthus, eftersom det är känt att till exempel läckage av kväve från växthusodling kan leda till negativa miljökonsekvenser. Det första alternativet kallas för Arginine Fe-HMP. När man gödslade trädplantor med detta eller med vanliga oorganiska kvävegödselmedel fanns det inga skillnader i planttillväxten beroende på vilket gödselmedel plantorna fick. Dock skedde mycket mindre nitratläckage från plantorna som gödslats med Arginine Fe-HMP så detta gödselmedel var mer miljövänligt än det konventionella. Det andra alternativet var tillsats av tillväxtfrämjande bakterier. I detta fall handlade det om de kvävefixerande bakterierna från tallbarren som i olika tester hade visat sig ha också många andra positiva egenskaper än kvävefixeringen. Dock kunde de inte främja tillväxten av tomater, majs, gurka eller grönkål i ett växthusexperiment. Det kan bero på att de inte kunde konkurrera med andra bakterier och svampar som redan fanns i jorden som användes för odlingen.

Avhandlingen belyser komplexiteten hos den mestadels dolda delen av den boreala skogen. Svampar och bakterier förbises ofta på grund av sin ringa storlek, men deras närvaro har avgörande betydelse för skogarnas hälsa. Ju mer vi lär om dem, desto mer förstår vi hur vi påverkar dem med olika åtgärder vi gör i skogen. Dessutom kan vi upptäcka hur vi kan ta deras egenskaper i anspråk för att tillexempel förbättra hur vi odlar olika grödor.

Acknowledgements

I think this part of the thesis might be the hardest to write…

Not because I am not thankful for all of the opportunities, support, guidance and help I received during (more than) the last four years, but because it is impossible to express all my gratefulness and to list each and every thing and person I am grateful for. But I guess I can try to do my best (with an added grain of humor):

First of all, I would like to thank my main supervisor **Annika**, who I feel has taken me under her wing when I was finishing my bachelor studies. In fact, you have been my mentor for a very long time, and I am extremely grateful for all the opportunities, feedback and support I received during that time. It has been a fun and unique experience to have a supervisor who rarely (if ever) said no to my ideas and plans, even though you did sometimes try to convince me that some of my ideas were not the best (rightfully so!). So, thank you again for your guidance and for allowing me to explore my ideas and learn from my mistakes.

I would like to thank my co-supervisor **Regina**, who stepped in as one of my co-supervisors already when I was planning my master's thesis. You were extremely helpful whenever I had a question regarding laboratory work. Through your help and guidance, I learned how the publishing system works, how to approach laboratory problems and, in general, how to be a good researcher. And it was definitely an adventure to attend my first conference with you (I mean what destination can beat Svalbard!?!).

Torgny, thank you for completing my PhD supervisor team. Even though we did not collaborate on so many projects, I always valued your ideas, comments and feedback. I always perceived you as the most critical one out

of my supervisors (maybe wrongly), but it was good as it made me re-examine and re-evaluate the ideas, methods and approaches more carefully, which helped.

All in all, I am super grateful to have had such an amazing team of supervisors, but have to admit it was incredibly hard to find a meeting time that fit everybody!

Anita, thank you for teaching me about nitrogen fixation and acetylene-reduction assay. I am grateful to have had your guidance and for your help with getting acetylene gas whenever I asked (as I was too worried to do it by myself after doing the safety assessment sheet).

Ann-Katrin, I am not sure how I would manage all the administrative tasks without your help. I feel like I had quite a few unique administrative problems during my PhD and I am so glad I received your help with all of them.

Justine, it has been such a fun experience working with you. You made every day a fun and exciting day as so many things seemed to happen when you were around. And I am still glad we managed to save the samples from the trash (it happens, right?!?).

Anne, thank you for being the best (and the only) master's student I had. It was so amazing to see you become more independent and knowledgeable during your master's thesis project and I am glad you decided to continue on with PhD studies. It must say something about my supervisor abilities, I hope?

I would also like to thank all my **co-authors** on the different papers as I could not manage without you. A special thank you to **Michael** for all the useful feedback, which made the joint papers much more cohesive and focused. And **Benjamin**, it was an honor to work with you during the last few years. You were the first PhD student I met, and you gave me so much insight and advice regarding the PhD process (even though I could also see the negative sides of being a PhD student; but good to get the real picture, right?). And I will never forget how well you handled the situation when during my cleaning of the HPLC machine, the column managed to get stuck turned the wrong way. (I was upset and distressed, but somehow you managed to save the day!)

I would also like to thank members of the **Nordin group**, who were there for part or most of my PhD journey. **Matej**, **Bodil**, **Isabella** and **Marcus**, it has been fun to discuss our PhD projects and complain about them at the same time. During all the meetings and excursions, I have also learned so much from you regarding forest management, and I appreciated your critical feedback even if I was not always happy receiving it (I mean, who is?). And Marcus, it has been an honour to share being the last two PhD students in the group, it makes us a bit special I think (just kidding!).

Thank you also to all the past and present **Silviculture group** and **C/N group** for all the interesting meetings and discussions. Even though I might not have completely understood all the details regarding your projects (sorry for that), it was always fascinating how many things are still unknown. I would also like to thank the **Soil nutrient cycling group in Skara**, as they kindly took me in during the last few months, gave me an occasional office and included me in their meetings (surprisingly, it was really nice to be able to occasionally join an in-person meeting).

Laura, I cannot imagine my PhD without you! Even though we never managed to come up with an idea for a joint article (maybe sometime in the future?), you were somehow part of most of my PhD. It was fun (and sometimes tiring) to take on the challenges such as being department PhD representatives, organising the 12th SPPS PhD conference and organising all Outside of Academia seminars together. Attending the summer school in France, a conference on Hawaii and spending some days exploring both places are some of the best memories of my PhD (even if we sometimes almost got lost). And I am glad that even though we did so many things together, we somehow still stayed friends (impressive!?!).

I am also extremely grateful to my **family**! I would not be where I am today without your guidance, help and inspiration. I have to say I have always, always felt supported by both my **grandparents** and my **parents Mateja** and **Grega**, even though (at least my grandparents) might have preferred me to stay closer to home. And I am very thankful that my parents are the type of Slovene parents who do travel to visit their child! I do not know what I would do without your help with moving (so many times during my whole studies), bringing my (amazing) car to the north and regular Skype talks.

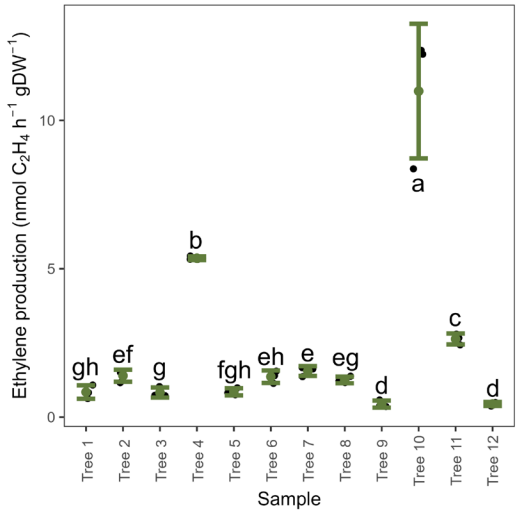
Finally, I would like to thank my husband **Anton**, who has been with me for most of my PhD journey. I know it has not always been easy to support me during this time (especially when I was in a bad mood), so I really appreciate that I could always count and lean on you during this time. Sometimes I wonder if we should have gone for the last name Bizjaksson instead of Bizjak-Johansson, but now with this thesis published with the last name Bizjak-Johansson, it is unfortunately too late to change our minds. Anyway, thank you for always being happy to see me when you come home from work! I hope that continues!

Appendices

During the PhD project, many different laboratory assays and experiments allowed me to learn more about the methods used, try a specific method on other sample types or test my ideas. Many of these experiments were not included in neither the papers nor the main thesis text, even though I learned a lot through them. Therefore, the four most important and interesting, based on my opinion, are included in this appendix. Appendices 1-3 describe the effects of specific conditions and pre-treatments on acetylene-reduction assay measurements, while Appendix 4 describes two short experiments of Scots pine seedling inoculation with PGPB.

Appendix 1

An unusually high variation in the needle negative controls was measured in the second summer of acetylene-reduction assay measurements on Scots pine needles. Negative controls are needles treated in the same way as acetylene-reduction assay samples but without injected acetylene and should account for endogenous ethylene production. A more thorough analysis of the results suggested that the endogenous ethylene production could be sample-dependent. An experiment was designed where needles from 12 different trees were sampled to look at sample-specific needle endogenous ethylene production. Three replicates for each tree were used and treated as negative controls for the acetylene-reduction assay. The measured ethylene production rates after a two-hour-long incubation revealed that endogenous ethylene production is tree-specific (Appendix Figure 1). There were several statistically significant differences in endogenous ethylene production between the twelve trees, and the variation for each tree was usually minimal. Due to the results, sample-specific negative controls were used for all acetylene-reduction assay measurements reported in either the papers or the thesis.

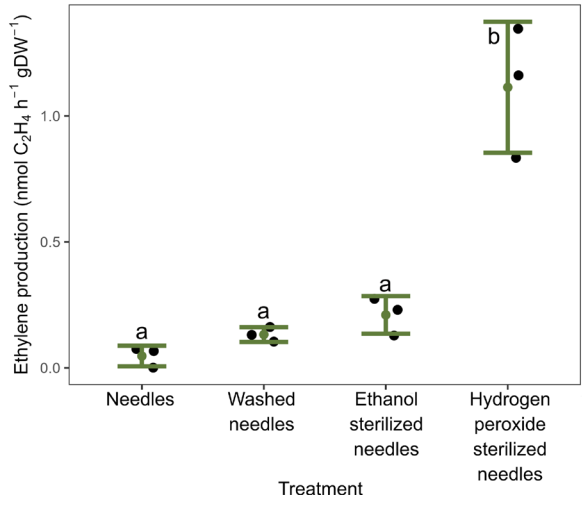


Appendix Figure 1: Ethylene production rates for acetylene-reduction assay negative controls with Scots pine needles of several trees. Different letters indicate statistical differences based on one-way ANOVA followed by Tukey's HSD (n = 3).

Appendix 2

It was additionally noticed during the acetylene-reduction assay measurements on needles that different needle pre-treatments might have an effect on the measured ethylene production. An experiment was designed with four different pre-treatments and three replicates per pre-treatment. The pre-treatments were: needles (no pre-treatment), washed needles (washed three times with sterile deionized water for 20 seconds), ethanol sterilized needles (submerged for two minutes in 70% ethanol and washed three times with sterile deionized water for 20 seconds) and hydrogen peroxide sterilized needles (submerged for two minutes in 30% hydrogen peroxide and washed three times with sterile deionized water for 20 seconds). While washed needles and ethanol sterilized needles showed a bit higher average ethylene production compared to control needles (Appendix Figure 2), the difference was not statistically significant. However, the hydrogen peroxide sterilized needles had a significantly higher ethylene production compared to other treatments. One potential explanation could be endogenous ethylene production in needles due to stress (Thiagarajan et al. 2016) caused by submersion in hydrogen peroxide. Another explanation could be a potential

correlation between ethylene production and needle hydrogen peroxide detoxifying capacity, as previous studies showed exogenous hydrogen peroxide induced an increased ethylene production in a concentration-dependent manner (Ievinsh & Tillberg 1995; Ievinsh & Ozola 1998). In any case, the experiment showed that surface sterilisation with hydrogen peroxide can lead to an overestimation of nitrogen fixation in Scots pine needles. Therefore, the needles were not surface sterilized with hydrogen peroxide or pre-treated in any other way during the acetylene reduction assay measurements for paper II.

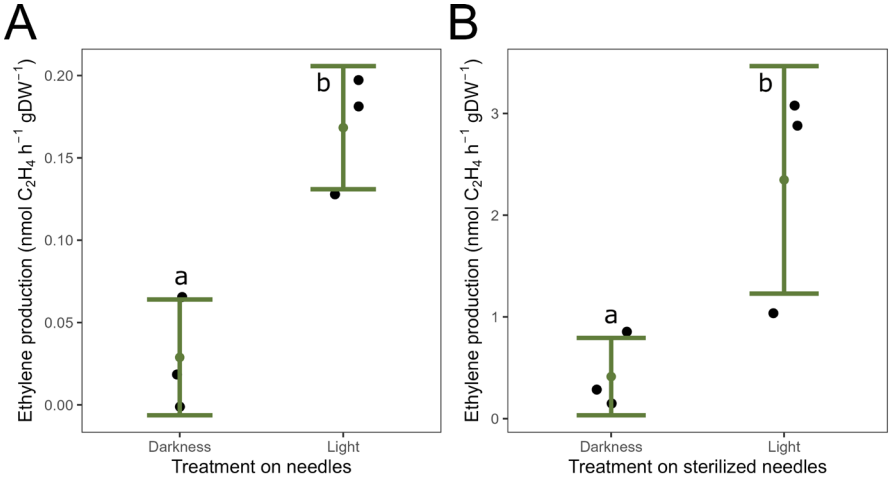


Appendix Figure 2: Ethylene production rates during acetylene-reduction assay for Scots pine needles that had undergone a washing/sterilization pre-treatment. Different letters indicate statistical differences based on one-way ANOVA followed by Tukey's HSD (n = 3).

Appendix 3

Another interesting observation during acetylene-reduction assay measurements was the effect of light on the measured ethylene production. To investigate this effect a bit better, an experiment was set up with three replicates per treatment in a way that either control non-sterilized needles or sterilized needles (submerged for two minutes in 30% hydrogen peroxide and washed three times with sterile deionized water for 20 seconds) were incubated in darkness or under light conditions overnight before the

experiment and during the acetylene-reduction assay incubation. The results showed that regardless of the needle pre-treatment, the darkness treatment showed significantly lower ethylene production compared to light treatment (Appendix Figure 3). The results indicate that the nitrogen fixation in the needles could be connected to light intensity, further suggesting a potential connection to photosynthesis. Previous studies in tropical forests did show a connection between light and nitrogen fixation rate (Bentley 1987; Taylor & Menge 2018), but more experiments would be needed to make any concrete conclusions. However, based on the results of this experiment, it was made sure that all samples were incubated under light conditions for the measurements reported in the papers.

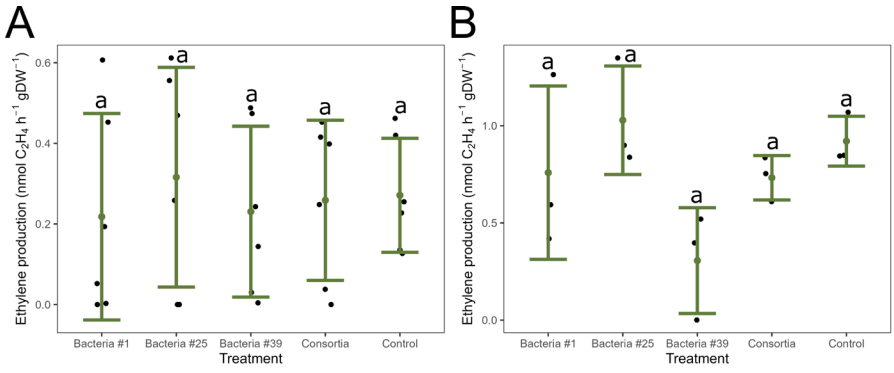


Appendix Figure 3: Ethylene production rates for: A) non-sterilized needles and B) sterilized needles, incubated under constant light or in complete darkness (overnight before the experiment and during the experiment incubation). Different letters indicate statistical differences based on two independent samples t-test (n = 3).

Appendix 4

Previous studies suggested that certain PGPB are better at promoting the growth of their original host plants compared to non-host plants (Boddey & Dobereiner 1988; Lucy et al. 2004). Two small experiments were used to test if inoculation of Scots pine seedlings with PGPB isolated from the needles of this species could result in a higher ethylene production, indirectly showing nitrogen fixation. The nitrogen fixation activity in seedlings was

used as many of the previously published articles indicated that inoculation with nitrogen-fixing bacteria caused plant growth promotion through the uptake of nitrogen introduced by bacterial nitrogen fixation (Anand et al. 2013; Padda et al. 2019). In the first experiment, one-year-old Scots pine seedlings with six replicates per treatment were used, while in the second experiment, a few-months-old seedlings with three replicates per treatment were used. In both cases, the bacteria *Bacillus paralicheniformis* #1, *Microbacterium* sp. #25 and *Priestia megaterium* #39 were used for inoculation either as individual bacterial strains or in a consortium. Seedlings were inoculated with six millilitres of bacterial liquid culture with OD_{600} = 0.4 for single bacterial strains, with two millilitres of each bacterial liquid culture with OD_{600} = 0.4 for consortium and with six millilitres of liquid Luria Broth media for control seedlings. The seedlings were inoculated again two weeks after the initial inoculation. Their nitrogen fixation rate was measured approximately two months after the initial inoculation in the first experiment and around one month after the initial inoculation in the second experiment. Both the results of the first experiment (Appendix Figure 4A) and second experiment (Appendix Figure 4B) showed no statistically significant differences between the treatments. The results indicated that seedling inoculation with nitrogen-fixing bacteria did not have any effect on the seedlings nitrogen fixation measured a few weeks after the initial inoculation. The results could be due to inefficient competition of the inoculated bacterial strains with the naturally present soil microbiome, as bacterial strain establishment was not followed (de-Bashan & Nannipieri 2024). While the two experiments did not show promising results regarding Scots pine seedling growth promotion by the selected bacteria, it would have been interesting to do a longer experiment, where the initial inoculation would be done at the time of the sowing and seedling growth would be assessed after several months of growth.



Appendix Figure 4: Ethylene production of inoculated Scots pine seedlings with individual bacterial strain, consortium or control: A) inoculated one-year-old seedlings analysed two months after initial inoculation (n = 6) and B) few months old seedlings analysed after one month after initial inoculation (n = 3). Different letters indicate statistical significance based on one-way ANOVA followed by Tukey‘s HSD test.

II

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Research paper

Presence and activity of nitrogen-fixing bacteria in Scots pine needles in a boreal forest: a nitrogen-addition experiment

Tinkara Bizjak[1], Anita Sellstedt[2], Regina Gratz[1,3,4] and Annika Nordin[1]

[1]Umeå Plant Science Centre (UPSC), Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden; [2]Umeå Plant Science Centre (UPSC), Department of Plant Physiology, Umeå University, 901 87 Umeå, Sweden; [3]Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden; [4]Corresponding author (regina.gratz@slu.se)

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Endophytic nitrogen-fixing bacteria have been detected and isolated from the needles of conifer trees growing in North American boreal forests. Because boreal forests are nutrient-limited, these bacteria could provide an important source of nitrogen for tree species. This study aimed to determine their presence and activity in a Scandinavian boreal forest, using immunodetection of nitrogenase enzyme subunits and acetylene-reduction assays of native Scots pine (*Pinus sylvestris* L.) needles. The presence and rate of nitrogen fixation by endophytic bacteria were compared between control plots and fertilized plots in a nitrogen-addition experiment. In contrast to the expectation that nitrogen-fixation rates would decline in fertilized plots, as seen, for instance, with nitrogen-fixing bacteria associated with bryophytes, there was no difference in the presence or activity of nitrogen-fixing bacteria between the two treatments. The extrapolated calculated rate of nitrogen fixation relevant for the forest stand was 20 g N ha^{-1} $year^{-1}$, which is rather low compared with Scots pine annual nitrogen use but could be important for the nitrogen-poor forest in the long term. In addition, of 13 colonies of potential nitrogen-fixing bacteria isolated from the needles on nitrogen-free media, 10 showed in vitro nitrogen fixation. In summary, 16S rRNA sequencing identified the species as belonging to the genera *Bacillus*, *Variovorax*, *Novosphingobium*, *Sphingomonas*, *Microbacterium* and *Priestia*, which was confirmed by Illumina whole-genome sequencing. Our results confirm the presence of endophytic nitrogen-fixing bacteria in Scots pine needles and suggest that they could be important for the long-term nitrogen budget of the Scandinavian boreal forest.

Keywords: diazotrophic bacteria, forest fertilization, needle endophyte, NifH, nitrogenase activity, *Pinus sylvestris*.

Introduction

To date, all plant species studied appear to be inhabited by endophytic bacteria (Santoyo et al. 2016), which are defined as microorganisms that reside in plants but do not cause any symptoms of disease (Wilson 1995). Endophytes can be symbionts, latent pathogens or harmless cohabitants (Chanway et al. 2014). Endophytic bacteria possess a diverse set of plant growth-promoting properties that can positively affect, for example, phytohormone balance (Bhattacharjee et al. 2008), nutrient acquisition (Santoyo et al. 2016), growth and yield (Ryan et al. 2008, Khan et al. 2012), protection against pathogens (Wei et al. 2014), propagation (Quambusch et al. 2014) and the response to abiotic stress. An important endophytic bacterial community that can help with nutrient acquisition comprises diazotrophic bacteria, which are capable of fixing nitrogen from the atmosphere. The multi-subunit enzyme responsible for the energy-demanding process of nitrogen fixation is nitrogenase (Doty et al. 2016), encoded by the structural genes *nifH*, *nifD* and *nifK*, and regulated by *nifA* (Marchal and Vanderleyden 2000). Of these genes, *nifH* is most commonly used as a marker for nitrogen-fixing bacteria (Zhang et al. 2007). Nitrogenase is extremely oxygen-sensitive (Marchal and Vanderleyden 2000) and its activity is upregulated by phosphorus (Reed et al. 2007, Matson et al. 2014), and probably also by carbohydrates because of the high energy demand of nitrogen fixation (Welsh 2000, Zheng et al. 2017, Benavides et al. 2020). It is less clear,

however, how a supply of externally added mineral nitrogen fertilizer affects nitrogen fixation. Many studies have shown adverse effects of external nitrogen application or higher natural nitrogen soil availability on nitrogen fixation (Barron et al. 2011, Gundale et al. 2011, Zheng et al. 2017, Nishida and Suzaki 2018), but some have shown no effects (Moyes et al. 2016, Zheng et al. 2017). Nitrogen fixation could also be downregulated by higher nitrogen concentrations in the environment, as indicated by studies where higher nitrogen-fixation rates have been measured in tissues with a higher carbon-to-nitrogen (C/N) ratio (Granhall and Lindberg 1978, Leppänen et al. 2013, Tormanen and Smolander 2022).

In the boreal forest, where plant growth is strongly nitrogen-limited, it has been suggested that in addition to the nitrogen-fixing symbiosis between cyanobacteria and mosses (DeLuca et al. 2002), endophytic nitrogen fixation provides an important additional source of nitrogen (Moyes et al. 2016). Coniferous needles could offer a good habitat for endophytic nitrogen-fixing bacteria, by protecting against adverse environmental conditions and competitors (Yang et al. 2016), and potentially a supply of carbohydrates and energy (Wurzburger 2016). The advantage for conifers would be a potential nitrogen source, either through direct supply from microbe to tree, or through degraded microbial material as a result of a relatively quick microbial turnover (Wurzburger 2016). The possible presence of nitrogen-fixing bacteria inside coniferous needles has been indicated by sequencing data (Carrell and Frank 2014, Haas et al. 2018), and complementary culturing methods have been used to isolate diverse nitrogen-fixing bacteria from a wide range of trees, including some conifers (Doty et al. 2009, Bal et al. 2012, Puri et al. 2018). Usually, their nitrogen-fixing ability is confirmed using either polymerase chain reaction (PCR) amplification to detect the presence of the *nifH* gene, or an acetylene-reduction assay (ARA) to measure their nitrogen-fixation ability (Gupta and Roper 2010, Bal et al. 2012). The most commonly occurring bacterial genera represented in nitrogen-fixing bacteria isolated from coniferous needles are *Sphingomonas*, *Bacillus*, *Rhizobium*, *Pseudomonas*, *Caballeronia* and *Paenibacillus* (Moore et al. 2006, Izumi et al. 2008, Puri et al. 2018). Using ARA, bacterial nitrogen fixation has been measured in needles from limber pine (*Pinus flexilis*) from North American subalpine forest (Moyes et al. 2016), Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) from the Scandinavian boreal forest (Granhall and Lindberg 1978), and black pine (*Pinus nigra*) and Douglas fir (*Pseudotsuga menziesii*) from Italy (Favilli and Messini 1990). Furthermore, nitrogen fixation has been measured using labelled ^{15}N in Douglas fir needles from the temperate forest (Jones 1970). However, the estimated rates of nitrogen fixation between the studies differ significantly, with the amounts of fixed nitrogen ranging from grams to kilograms per hectare and year (Jones 1970, Granhall and Lindberg 1978, Favilli and Messini 1990, Moyes et al. 2016).

In contrast to previously published studies focussing on the presence and activity of nitrogen-fixing bacteria inside needles of native conifer species in the boreal forest, our study is, as far as we know, the first to compare the activity of endophytic nitrogen-fixing bacteria using a nitrogen-addition experiment. The Scots pine was chosen as the study species because, alongside the Norway spruce, it is a dominant tree species in the nitrogen-limited Scandinavian boreal forest (Berlin et al. 2010). The study aimed to confirm the presence of endophytic nitrogen-fixing bacteria inside Scots pine needles, by measuring nitrogen fixation and assessing the effect of nitrogen fertilization on their activity, and isolating and identifying the nitrogen-fixing bacteria. The following hypotheses were addressed: (i) nitrogen-fixing bacteria are present in Scots pine needles; (ii) the bacteria are actively fixing nitrogen and their activity in needles decreases with inorganic nitrogen fertilization; (iii) isolated bacteria, identified by 16S rRNA and whole-genome sequencing, belong to similar genera as found in other conifer species; and (iv) isolated nitrogen-fixing bacteria can fix nitrogen in vitro.

Materials and methods

Field site and sample collection

The samples were collected from the Åheden research forest in northern Sweden (N 64° 14′, E 19° 48′). The area in which the field site was located has a mean annual precipitation of 600 mm, a mean annual temperature of 1 °C (Forsmark et al. 2020) and an estimated regional atmospheric nitrogen deposition of less than 2 kg N ha^{-1} $year^{-1}$ (Pihl Karlsson et al. 2012). The Åheden research forest is a naturally regenerated boreal forest dominated by approximately 150-year-old Scots pine (From et al. 2016), with some scattered Norway spruce trees in the sub-canopy. The ground vegetation is predominately lingonberry (*Vaccinium vitis-idaea*), heather (*Calluna vulgaris*), red-stemmed feathermoss (*Pleurozium schreberi*), fork mosses (*Dicranum* spp.), reindeer lichen (*Cladonia rangiferina*) and scrubby cup lichen (*Cladonia arbuscula*) (Gundale et al. 2011). In 2004, five different nitrogen treatments were established at Åheden in 0.1-ha plots; since then the plots have been fertilized yearly with 0, 3, 6, 12 and 50 kg N ha^{-1} $year^{-1}$, respectively, in the form of solid ammonium nitrate granules (NH_4NO_3). Each nitrogen treatment has six replicates in a randomized block design (Forsmark et al. 2020).

For this study, the plots fertilized with 0 kg N ha^{-1} $year^{-1}$ (referred to as control plots) and 50 kg N ha^{-1} $year^{-1}$ (referred to as fertilized plots) were selected, as they represented the most contrasting environments at the site. Samples were harvested from two trees from each of the six replicate plots per treatment (n = 12 per treatment); tree selection was based on proximity to the centre of the plot and representation of the general appearance, health and size of the trees within the plot. For immunodetection analysis and bacterial isolation, needles

from several branches of the same tree were harvested in the summer of 2021, by aseptically collecting 1-year-old (Meredieu 2002) Scots pine needles, which were stored on dry ice for transportation to the laboratory. Several branches from each tree to be used for ARA were aseptically harvested in the summer of 2022 across six sampling days, and stored on ice until analysed in the laboratory. However, at both sampling time points the samples were collected from the same trees and 1-year-old needles were selected as we wanted only healthy green needles.

Protein extraction and immunodetection of NifH protein

One-year-old Scots pine needles were surface sterilized in a vertical laminar flow hood by submersion in 30% hydrogen peroxide for 2 min with continuous shaking, followed by three washes in sterile deionized water for 20 s. Excess water was evaporated by inverting the samples onto sterile filter paper. The surface-sterilized needles were then stored at −80 °C until the start of the experiment. For total protein isolation, the needles were ground to a fine powder using a pestle and mortar, under constant cooling with liquid nitrogen. Total protein was then extracted by adding 2× protein loading buffer (124 mM Tris–HCl, pH 8.6, 5% sodium dodecyl sulfate (SDS), 4% dithiothreitol and 20% glycerol) to the sample, centrifuging at 4 °C for 5 min at maximum speed, and heating the transferred supernatant at 95 °C for 5 min. Samples were separated by SDS-PAGE (12% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad, Hercules, CA, USA), with equivalent protein amounts loaded for each sample (measured with a Qubit™ Protein Assay Kit, Invitrogen, Waltham, MA, USA). The separated proteins were transferred onto a nitrocellulose membrane (Amersham Protran, GE Healthcare, Chicago, IL, USA) and stained with Ponceau S as a control for successful transfer. Immunodetection was performed as follows: membranes were blocked for 25 min in 5% (w/v) milk solution dissolved in Tris-buffered saline, 0.1% Tween 20 (TBST) (5% milk powder in 20-mM Tris–HCl, pH 7.4, 180-mM NaCl and 0.1% Tween-20), followed by a 1.5-h incubation at room temperature in a 1:2000 dilution of the primary anti-NifH antibody (Agrisera, Vännäs, Sweden) in TBST containing 2.5% (w/v) milk. After three 15-min wash steps with TBST, the membranes were incubated for 1 h in a dilution of a secondary antibody in TBST containing 2.5% (w/v) milk. The antibody used was horseradish peroxidase-conjugated goat anti-chicken (1:20,000) (Agrisera, Vännäs, Sweden). Again, three 15-min wash steps were performed with TBST. Signal detection was carried out using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) on Azure c600 (Azure Biosystems, Dublin, CA, USA), and quantified using ImageJ (Schneider et al. 2012). To estimate the size of the bands, a PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used. To confirm antibody specificity we used positive controls (soybean nodule extract, purified NifH protein with His-tag (Agrisera)) and negative controls (2× protein loading buffer, *Bradyrhizobium japonicum* liquid culture protein extract), which did not yield in a western blot signal.

Acetylene-reduction assay

The branches harvested for ARA were stored under constant light conditions at room temperature overnight. The next day, for each sample 1-year-old needles were collected from several different branches of the same tree and divided into six subsamples; half of the samples were used to measure acetylene reduction, and half to correct for needle endogenous ethylene production. The needles from each subsample were put into 50 ml glass vials and 8 ml sterile deionized water was added. The ARA was performed as described previously (Richau et al. 2017) with the following modifications: after sealing the glass vials with a rubber septum and replacing 10% of the air with acetylene gas, the samples were incubated at room temperature for 2 h under constant light conditions before 1 ml air was removed with a syringe and analysed for ethylene production on a gas chromatograph (Shimadzu GC-8A, Kyoto, Japan). After the experiment, the needles were dried at 60 °C for 48 h and their dry mass was measured. Because of the high variation in endogenous needle ethylene production observed between individual trees, the ethylene production rates were corrected for sample-specific endogenous ethylene production. Additionally, water samples with injected acetylene were used to correct for any ethylene present due to injected acetylene gas. Specifically, both negative control values were subtracted from the measured acetylene reduction rates from samples, which affected the reported final ethylene production rate.

Carbon and nitrogen content

Dried 1-year-old Scots pine needles from the ARA were ground to a fine powder in a bead mill (Retsch, Haan, Germany), and the carbon and nitrogen content was measured using an Isotope Ratio Mass spectrometer (DeltaV, Thermo Fisher Scientific) coupled with an Elemental analyser (Flash EA 2000, Thermo Fisher Scientific) (Werner et al. 1999).

Isolation of nitrogen-fixing bacteria

Half of the 1-year-old Scots pine needles from each sample were surface sterilized by submersion in 30% hydrogen peroxide for 2 min with continuous shaking, and the other half by submersion in 70% ethanol for 3 min with continuous shaking. Two different sterilization methods were used to increase the total number of bacteria isolated. The needles were then washed three times for 20 s with sterile deionized water, and the excess water was removed by inverting the tube onto sterile filter paper. Five needle pairs were imprinted on tryptic soy agar (TSA) plates (15 g l^{-1} casein peptone, 5 g l^{-1} soy peptone, 5 g l^{-1} NaCl and 15 g l^{-1} agar), and the plates were incubated at 28 °C for 10 days to confirm the surface sterility of the samples. The

same five needle pairs were ground in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) using FastDNA Spin Kit tubes and beads and a FastPrep instrument (MP Biomedicals, Irvine, CA, USA). The crude extract was filtered using sterile Miracloth with a pore size of 22–25 μm (Merck Millipore, Burlington, MA, USA), and the filtrate was centrifuged at 5000 r.p.m. for 10 min at 8 °C. The pellet was resuspended in PBS buffer and inoculated on nitrogen-free media (semi-solid NFb (Baldani et al. 2014), combined carbon media (CCM) without yeast extract (Baldani et al. 2014) and LGI-P (Reis et al. 2015). The plates (CCM and LGI-P) and tubes (NFb) were incubated at 28 °C for 10 days. Individual colonies were observed and re-cultured on TSA plates.

16S rRNA and whole-genome bacterial sequencing

For 16S rRNA Sanger sequencing of isolated bacteria, the bacteria were grown on TSA plates overnight. Bacterial material was then transferred into an extraction buffer (0.05 M NaOH, 0.25% SDS), which was heated at 97 °C for 15 min before the sample was centrifuged for 4 min at 10,000 r.p.m. The collected supernatant was diluted with Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the extract was used in a PCR with the 16S ribosomal ribonucleic acid (rRNA) primers 27F/1492R (Heuer et al. 1997). The PCR was carried out using a DreamTaq Hot Start PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions, with 1 μl of bacterial extract, 0.5 μM forward and reverse primers, and an annealing temperature of 55 °C. Based on agarose gel separation, all the isolated bacteria had a band of around 1500 bp, so the PCR products were cleaned with ExoSAP-IT PCR Product Cleanup (Applied Biosystems, Waltham, MA, USA) before tube Sanger sequencing (Eurofins, Luxembourg City, Luxembourg). The obtained sequences were analysed using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) and compared with sequences already in the National Center for Biotechnology Information (NCBI) database. A phylogenetic tree showing the similarities between the strains was produced using Phylogeny.fr (Dereeper et al. 2008) according to Gratz et al. (2021), using MUSCLE alignment, Gblocks curation, PhyML phylogeny and TreeDyn tree rendering, but with likelihood-ratio test (minimum of SH-like and Chi2-based) instead of bootstrapping.

For whole genome sequencing, liquid Luria broth (LB) media (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl) was inoculated with isolated bacteria overnight before being centrifuged for 5 min at 10,000 r.p.m. The pellet was resuspended in a smaller amount of liquid LB media and the deoxyribonucleic acid (DNA) was isolated using a DNeasy PowerSoil Kit (Qiagen, Venlo, The Netherlands). The amount and quality of the DNA were assessed using Nanodrop before being sent for sequencing and bioinformatic analysis (CD Genomics, New York City, NY, USA through Genohub, Austin, TX, USA). For sequencing, an Illumina NovaSeq6000 (Illumina, San Diego, CA, USA) was used with pair-end 2× 150 base pair sequencing and at least 3 million reads per sample. For the bioinformatic analysis, the two pairs of reads were merged, 1000 sequences were randomly selected, and BLAST (Altschul et al. 1990) was used to compare the obtained sequences with sequences already in the NCBI database (NCBI, Bethesda, MD, USA). The identity was determined based on the sequence hit count. Additionally, Read Assembly and Annotation Pipeline Tool (RAPT) (NCBI, Bethesda, MD, USA) was used for de novo assembly of the bacterial genomes using SKESA and annotation of the genome using Prokaryotic Genome Annotation Pipeline to check for the presence of *nif* genes within the whole genome sequence.

The bacterial 16S rNRA sequences were deposited in GenBank (NCBI), and the unassembled Illumina whole genome sequences were deposited in the Sequence Read Archive (NCBI) (Sayers et al. 2022) (Table S1 available as Supplementary data at *Tree Physiology* Online). Bacterial cultures were deposited in the NCCB collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) (Table S1 available as Supplementary data at *Tree Physiology* Online).

In vitro nitrogen fixation of isolated bacteria

The nitrogen-fixation ability of isolated bacteria was measured by ARA. Bacteria were grown in either liquid CCM without yeast extract (bacteria #1, 23, 24, 25, 27, 39) or liquid LGI-P media (bacteria #2, 3, 14, 26, 28, 38-1, 38-2) for 24 h at 28 °C, and then their OD_{600} measured. The bacteria were transferred to glass vials sealed with a rubber septum; 10% of the air was replaced by acetylene and, after a 2-h incubation at 30 °C with constant shaking, the ethylene production was measured using a gas chromatograph (Shimadzu GC-8A, Kyoto, Japan). The bacterial OD_{600} was measured again after the experiment and this value was used to normalize the ethylene production. Calculated ethylene production rates were corrected for spontaneous acetylene reduction and any endogenous ethylene production by either the media or the bacteria.

Statistics

All data were analysed using SPSS Statistics 27 (IBM, Armonk, NY, USA). For data from the immunodetection of NifH protein, ARA on needles, in vitro ARA on bacterial cultures, and carbon and nitrogen content, the assumptions of normal distribution and equal variance were checked. For all datasets, both assumptions were met, as well as the assumption of independence. The data from the immunodetection of NifH protein, and carbon and nitrogen content, were then analysed using a two-sample independent *t*-test. In vitro ARA measurements on isolated bacteria were analysed using a one-way analysis of variance (ANOVA) followed by a Tukey honestly significant difference

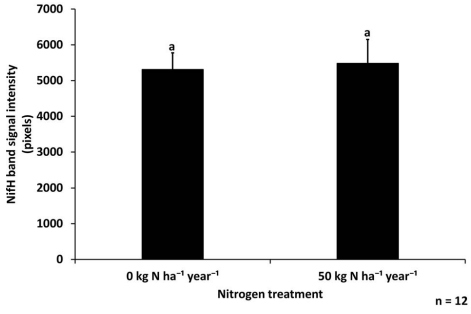

Figure 1. NifH band signal intensity for immunodetection with anti-NifH antibody (mean + SE), indicating the presence of nitrogenase enzyme in 1-year-old Scots pine needles from trees grown in control and nitrogen-fertilized plots (0 and 50 kg N ha^{-1} $year^{-1}$, respectively) (n = 12 per treatment, two-sample t-test P = 0.84, bars with different letters are significantly different).

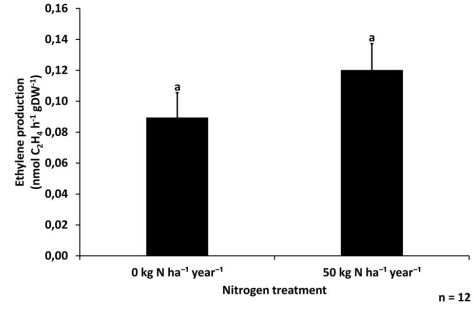

Figure 2. Ethylene production rate (mean + SE) per hour and gram dry mass of 1-year-old Scots pine needles, indicating nitrogenase enzyme activity in the needles of trees gown in control and nitrogen-fertilized plots (0 and 50 kg N ha^{-1} $year^{-1}$, respectively) (n = 12 per treatment, two-sample t-test P = 0.11, bars with different letters are significantly different).

(HSD) test. For the ARA on needles, a two-way ANOVA was used, using sampling day and nitrogen treatment as variables. A linear regression model was used to analyse the relationship between ARA in 1-year-old Scots pine needles and the C/N ratio. The regression coefficient was tested to see whether there was a statistically significant relationship between the two variables.

Results

The presence of nitrogen-fixing bacteria inside surface-sterilized needles was analysed using immunoblotting of the NifH protein, which is one of the nitrogenase enzyme's subunits. Our analyses indicated that nitrogen-fixing bacteria were present in the needles from both control and nitrogen-fertilized plots (Table S2 available as Supplementary data at *Tree Physiology* Online, Figure 1), and the signal intensity of the NifH band was similar between the two treatments (two-sample t-test, P = 0.84), with values of 5309 and 5481, respectively. This indicated a similar amount of nitrogenase protein in both treatments (Figure 1).

As the presence of nitrogenase protein does not mean active nitrogen fixation, ARA was used to measure the nitrogenase activity indirectly. As well as reducing dinitrogen to ammonia, nitrogenase enzymes can also reduce acetylene to ethylene, which can be detected with this method. We measured the nitrogenase activity in needles from both the control plots and inorganic nitrogen-fertilized plots (Table S3 available as Supplementary data at *Tree Physiology* Online). The average nitrogenase activity was slightly higher in needles from fertilized plots than in control plots (Figure 2), but the difference was not significant (two-way ANOVA, P = 0.11). The fixation rate for needles from control plots was 0.09 nmol ethylene h^{-1} and g^{-1} dry needles, and the rate for fertilized plots was 0.12 nmol ethylene h^{-1} and g^{-1} dry needles. There was, however, a significant effect of sampling day, with a difference in nitrogen fixation rates between different sampling dates (two-way ANOVA, P = 0.02), highlighting the importance of including a sufficient number of negative controls in the measurement protocol for each sampling date.

To determine the possible relevance of the nitrogen-fixation activity for the forest, the nitrogen-fixation rates were extrapolated to provide an estimate for the forest stand. We assumed equal nitrogen-fixation rates across the whole canopy regardless of needle position, an equal rate of nitrogen fixation during the whole growth period, as measured in our study, a 150-day growth period (Goude et al. 2019), a constant 12-h period of daylight (Moyes et al. 2016), and a conversion factor between ethylene production and nitrogen fixation of 3:1 (Hardy et al. 1968). Using an average leaf area index for Scots pine forest across the Swedish boreal forest (Appiah Mensah et al. 2020), the calculated nitrogen-fixation rate for control plots was approximately 11 g N ha^{-1} $year^{-1}$, and for fertilized plots, it was 15 g N ha^{-1} $year^{-1}$.

The carbon and nitrogen content of the needles was investigated to see whether they could affect the nitrogen fixation rates. The average carbon content for needles from the control plots was 51.0 g C g^{-1} dry mass and from fertilized plots 51.5 g C g^{-1} dry mass, which was not statistically significant (two-sample t-test, P = 0.07). The nitrogen content did differ between the two treatments, however, with average nitrogen content in needles from control plots of 0.93 g N g^{-1} dry mass, and from fertilized plots of 1.37 g N g^{-1} dry mass. This difference was statistically significant (two-sample t-test, $P < 0.01$). There was no significant linear regression relationship between the measured C/N ratios and ethylene production rates (ethylene production rates = $-0.0018 \times$ C/N ratio + 0.19, R^2 = 0.097, P = 0.14) of the needles.

To identify bacteria possibly responsible for the nitrogenase protein content and activity, potential nitrogen-fixing bacteria

Table 1. Potential nitrogen-fixing bacteria isolated from 1-year-old Scots pine needles. The trees were grown in control plots (0 kg N ha^{-1} $year^{-1}$) or long-term nitrogen-fertilized plots (50 kg N ha^{-1} $year^{-1}$), and the bacteria were isolated on different nitrogen-free media (CCM, LGI-P or NFb). The genus of each isolated bacterium was determined by whole-genome sequencing.

Bacteria	Nitrogen treatment	Plate	Species
1	0 kg N ha^{-1} $year^{-1}$	CCM	*Bacillus paralicheniformis*
23	0 kg N ha^{-1} $year^{-1}$	CCM	Unclassified *Novosphingobium*
24	0 kg N ha^{-1} $year^{-1}$	CCM	Unclassified *Novosphingobium*
25	0 kg N ha^{-1} $year^{-1}$	CCM	*Microbacterium* sp.
27	0 kg N ha^{-1} $year^{-1}$	CCM	*Sphingomonas* sp.
2	0 kg N ha^{-1} $year^{-1}$	LGI-P	*Bacillus paralicheniformis*
3	0 kg N ha^{-1} $year^{-1}$	LGI-P	*Bacillus paralicheniformis*
26	0 kg N ha^{-1} $year^{-1}$	LGI-P	*Variovorax paradoxus*
28	0 kg N ha^{-1} $year^{-1}$	NFb	*Variovorax paradoxus*
39	50 kg N ha^{-1} $year^{-1}$	CCM	*Priestia megaterium*
14	50 kg N ha^{-1} $year^{-1}$	LGI-P	*Bacillus paralicheniformis*
38-1	50 kg N ha^{-1} $year^{-1}$	NFb	*Novosphingobium pokkalii*
38-2	50 kg N ha^{-1} $year^{-1}$	NFb	*Variovorax paradoxus*

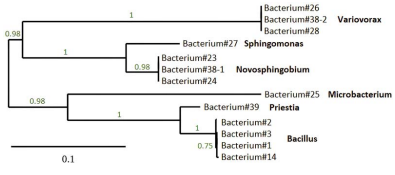

Figure 3. A phylogenetic tree (produced using Phylogeny.fr) of the 13 potentially nitrogen-fixing bacteria, based on 16S rRNA sequencing. The numbers represent the likelihood-ratio test values of the branching points.

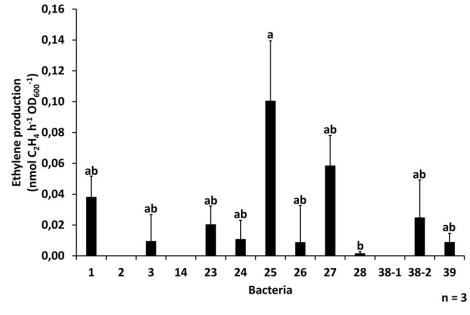

Figure 4. The nitrogenase activity, measured indirectly through ethylene production, of liquid cultures of 13 isolated bacteria (mean + SE). Bars with different letters are significantly different (one-way ANOVA followed by Tukey HSD test, $P < 0.05$).

were isolated from surface-sterilized needles from trees grown in either control or nitrogen-fertilized plots. Three different nitrogen-free media (CCM, LGI-P and NFb) were used, and 13 distinct bacterial colonies were successfully isolated. The bacteria were identified using 16S rRNA Sanger sequencing and Illumina whole-genome sequencing. The 16S rRNA sequences revealed that the isolated bacteria belonged to several different genera: *Bacillus*, *Microbacterium*, *Variovorax*, *Priestia*, *Novosphingobium* and *Sphingomonas* (Figure 3). More specifically, whole-genome sequencing identified the bacteria isolated from control plots as three *Bacillus paralicheniformis*, two unclassified *Novosphingobium*, two *Variovorax paradoxus*, one *Microbacterium* sp. and one *Sphingomonas* sp. (Table 1). Of the bacteria isolated from nitrogen-fertilized plots, one was identified as *Priestia megaterium*, one as *B. paralicheniformis*, one as *V. paradoxus* and one as *Novosphingobium pokkalii* (Table 1). The presence of *nif* genes was looked at using the assembled and annotated genome and we could detect the presence of sequence for NifU protein in bacteria 1, 2, 3, 14, 23, 24, 27, 38-1 and 39.

To confirm the ability of the 13 isolated bacteria to fix nitrogen, ethylene production was measured during ARA on liquid bacterial cultures. Ten of the 13 isolated bacterial colonies were able to fix nitrogen under specific conditions, producing ethylene to various degrees (Figure 4). Bacterium 25 (*Microbacterium* sp.) had the highest nitrogenase activity, whereas bacteria 2 (*B. paralicheniformis*), 14 (*B. paralicheniformis*) and 38-1 (*N. pokkalii*) were not capable of nitrogen fixation under the test conditions.

Discussion

It has been suggested that endophytic nitrogen fixation in conifer needles could be prevalent across temperate and boreal forests (Moyes et al. 2016), and even a small amount of nitrogen fixed by these bacteria could be ecologically important in nitrogen-limited environments (Wurzburger 2016). The main aim of our study was, therefore, to determine the presence, and measure of the activity, of endophytic nitrogen-fixing bacteria inside

1-year-old needles from Scots pine trees growing in the Scandinavian boreal forest.

By the first hypothesis, bacterial nitrogenase protein was detected in all of the samples analysed, indicating the widespread presence of nitrogen-fixing bacteria in Scots pine needles in this experimental forest area. The needles were surface sterilized before analysis, so the bacterial presence was probably endophytic. There seemed to be a similar abundance of the bacteria in the needles, as the amount of nitrogenase protein did not differ significantly between needles from control and nitrogen-fertilized plots.

The activity of nitrogen-fixing bacteria was measured using ARA. Our study corroborates results reported elsewhere that coniferous needle ethylene production can vary substantially as a result of needle age, position, season and sterilization protocol (Telewski 1992, Ievinsh and Tillberg 1995, Ievinsh and Ozola 1998, Klintborg et al. 2002). Because endogenous ethylene production was highly variable between the trees, we ensured that sample-specific production rates were measured and subtracted from the ARA ethylene production rates. The nitrogen-fixation rates were around 10 times higher than previously reported for the phyllosphere of Scots pine (Granhall and Lindberg 1978), which could be explained by different environmental or experimental factors between the two studies. It was, however, slightly lower, although in the same range, as rates reported for limber pine needles (Moyes et al. 2016), and much lower than rates reported for black pine and Douglas fir needles (Jones 1970, Favilli and Messini 1990). The nonsignificant difference in nitrogen-fixation rates between needles from trees on control and fertilized plots contradicted our second hypothesis that inorganic nitrogen fertilization would decrease the activity of nitrogen-fixing bacteria. This result is to some extent unexpected, as many studies have reported decreased nitrogen-fixation activity in biotopes enriched with inorganic nitrogen. For instance, from the same site as studied here, the nitrogen fixation of cyanobacteria associated with ground-dwelling moss surfaces was approximately eight times lower on fertilized than on control plots (Gundale et al. 2011). The same trend of decreased nitrogen fixation in response to nitrogen fertilization has also been observed in soil, forest floor and tree canopy leaves of disturbed subtropical forests in China, where the decrease between the two treatments was between 20 and 38% (Zheng et al. 2017). Additionally, it has been reported that nitrogen fixation in root nodules of individual legume trees (*Inga* sp.) from tropical forests grown in soils with a higher nitrogen content is almost nonexistent compared with those grown in soils with a lower nitrogen content (Barron et al. 2011). However, there are also studies showing no clear effect of nitrogen availability or fertilization on nitrogen fixation. For example, no correlation was found between endophytic nitrogen fixation and plant-available soil nitrogen for limber pine needles (Moyes et al. 2016). Also, in a rehabilitated subtropical forest, there was no significant effect of nitrogen fertilization on nitrogen fixation in soil and tree canopy leaves (Zheng et al. 2017). Furthermore, using a mathematical model it was suggested that in nitrogen-poor boreal forests obligate nitrogen-fixing bacteria could be prevalent due to the high cost of being facultative (Menge et al. 2009), which could explain the observed no difference in nitrogen fixation rates between control and fertilized plots.

The mechanism behind the influence of external plant nitrogen sources on nitrogen-fixation rates is not fully understood. In the case of needle endophytic nitrogen fixation for trees in a strictly nitrogen-limited environment, one speculation is that fertilization can enhance photosynthesis by increasing the supply of energy available for nitrogen fixation. Alternatively, the opposite could also be argued, as a higher C/N ratio arising from fertilization may mean that the externally supplied nitrogen renders nitrogen fixation redundant. However, in this study, whereas the C/N ratio of the needles significantly decreased with nitrogen fertilization, there was no significant relationship between the needles' C/N ratio and the nitrogen fixation rates of the needles ($P = 0.14$). A similar result has been found for logging residues of Scots pine, Norway spruce and silver birch (*Betula pendula*), where neither branches nor foliage showed a significant correlation between nitrogen fixation and C/N ratio (Tormanen and Smolander 2022).

To understand better the potential importance of needle nitrogen fixation in a nitrogen-limited forest, we extrapolated our measured nitrogen-fixation rates to a forest scale, calculating it to be less than 20 g N ha^{-1} $year^{-1}$. However, this rate needs to be interpreted with caution, as several assumptions were made, including constant nitrogen fixation during the growth season, which could be incorrect as seasonal variation in conifers has been noticed elsewhere (Favilli and Messini 1990). Our study also indicates that there is significant day-to-day variation in nitrogen-fixation rates, which was not accounted for in our extrapolation. With these caveats in mind, the estimated nitrogen contributed by nitrogen fixation in needles seems very low compared with the estimated nitrogen use of approximately 50 kg N ha^{-1} $year^{-1}$ for Scots pine growth in the boreal forest (Korhonen et al. 2013). The contribution of needle nitrogen fixation to tree growth in this forest may, therefore, be rather insignificant in the short term. However, taking into account the approximately 8500-year continuous boreal forest cover in the region (Barnekow et al. 2008), and assuming historically constant nitrogen fixation in needles, the nitrogen fixed by bacteria inside Scots pine needles could be an important nitrogen source in the longer term, contributing to the build-up of the soil nitrogen stock over the long term (Finér et al. 2003, Merilä et al. 2014).

Largely consistent with our third hypothesis, the endophytic bacteria isolated from 1-year-old Scots pine needles belonged to similar genera as found in other tree species

(Cankar et al. 2005, Moore et al. 2006, Izumi et al. 2008, Puri et al. 2018). The 13 isolated bacteria colonies were identified as belonging to the genera *Bacillus*, *Variovorax*, *Novosphingobium*, *Sphingomonas*, *Microbacterium* and *Priestia*. The genera *Penibacillus*, *Rhizobium* and *Pseudomonas* were not present, even though they are commonly found in tree species (Cankar et al. 2005, Moore et al. 2006, Izumi et al. 2008, Puri et al. 2018). Looking more closely at each genus, *Bacillus* bacteria have been detected previously in various coniferous and deciduous trees (Izumi et al. 2008, Puri et al. 2018), and have been shown to fix nitrogen and promote plant growth and yield (Çakmakçı et al. 2001, Ding et al. 2005, Yousuf et al. 2017). The Gram-positive, rod-shaped (Dunlap et al. 2015) strain of *B. paralicheniformis* has been reported as a nitrogen-fixing bacterium based on a whole-genome study (Annapurna et al. 2018). Another of the isolated bacteria belonged to the genus *Microbacterium*, which includes Gram-positive bacteria that have been shown to promote plant growth, chlorophyll content and fruit yield in a few diverse but agriculturally important plant species (Karlidag et al. 2007, Schwachtje et al. 2012, Mutai et al. 2017, Bal and Adhya 2021). This genus includes strains with the *nifH* gene and the capacity to fix nitrogen (Ruppel 1989, Zakhia et al. 2006, Lin et al. 2012), and some *Microbacterium* strains have been isolated from maple and elm trees (Shen and Fulthorpe 2015). Bacteria from *Variovorax* have been isolated from poplar trees (Moore et al. 2006), and this genus of Gram-positive bacteria includes strains capable of nitrogen fixation (Solanki et al. 2016) and promoting plant growth (Maimaiti et al. 2007). Specifically, *V. paradoxus* has been reported as a hydrogen-oxidizing plant growth-promoting bacterium (Maimaiti et al. 2007, Han et al. 2011). The Gram-positive *P. megaterium* (previously classified as *Bacillus megaterium*) has also been reported as a nitrogen-fixing bacterium (Ding et al. 2005, Yousuf et al. 2017) and shown to promote plant growth (Nascimento et al. 2020, Wang et al. 2021). *Sphingomonas* strains have been detected in willow and elm trees (Moore et al. 2006, Doty et al. 2009, Shen and Fulthorpe 2015), and the ability to fix nitrogen has been identified in a few *Sphingomonas* bacteria (Castanheira et al. 2014, Yang et al. 2014, Lowman et al. 2015). *Novosphingobium* has also been reported as a genus that includes plant growth-promoting nitrogen-fixing bacteria (Islam et al. 2009, Rangjaroen et al. 2017). *Novosphingobium pokkalii* has been described as a rhizosphere-associated bacterium with plant growth-promoting properties (Krishnan et al. 2017).

Acetylene-reduction assay (ARA) was used to check in vitro whether the isolated bacteria were endophytic diazotrophic bacteria capable of nitrogen fixation. In support of our fourth hypothesis, 10 out of the 13 bacteria colonies displayed ethylene production, with *Microbacterium* sp. being the most efficient. The three colonies that did not fix nitrogen in the ARA were identified as two species, *B. paralicheniformis* and *N. pokkalii*. It could be that these three bacterial strains did not show nitrogen fixation because of nonoptimal test conditions (Doty et al. 2009), or because their fixation rate was under the detection limit of the ARA. Not all bacteria capable of growing on nitrogen-free media demonstrate nitrogen fixation during an ARA (Doty et al. 2009, Padda et al. 2018, Puri et al. 2018). Using assembled and annotated genomes of the bacteria we could detect the sequence for the NifU protein in most of the bacteria, however, we could not detect any other *nif* genes in the sequences. This could be due to short reads limitation (as we only had 150 bp sequencing length), genome misassemblies or genome and annotation incompleteness (Chen et al. 2013, Barbitoff et al. 2020, Lobb et al. 2020).

The fact that most of the isolated endophytic bacteria were capable of nitrogen fixation makes them good candidates for plant growth-promoting bacteria. Nitrogen-fixing bacteria isolated from conifers have been used as plant growth-promoting bacteria in seedlings: the inoculated seedlings were taller and had greater biomass compared with control seedlings grown under both nitrogen-limited conditions (Puri et al. 2020) and in fertilized soil (Chen et al. 2021). However, to analyse their potential as plant growth-promoting bacteria our isolated strains would need to be tested for additional plant growth-promoting properties, such as indole-3-acetic acid production, siderophore production and 1-aminocyclopropane-1-carboxylate deaminase.

Conclusions

Our study has demonstrated that nitrogen-fixing bacteria are present and active in 1-year-old Scots pine needles; their endophytic presence was confirmed by nitrogenase protein immunodetection, and nitrogen fixation was measured using ARA. Strains of the nitrogen-fixing bacteria were isolated from sterile needles by culturing and identified using whole-genome sequencing, and their nitrogen-fixation ability was confirmed by in vitro ARA. Immunodetection of the NifH protein showed no difference between needles from control plots and fertilized plots, and the ARA showed similar fixation rates in needles from both treatments. To scale up estimates of the nitrogen-fixation rates and their impact on the boreal forest more accurately, it is important that the seasonality of nitrogen fixation is assessed, and the effect of variation in light intensity on the nitrogen-fixation capacity of endophytic bacteria inside coniferous needles determined. Even though the amount of nitrogen fixed by these bacteria might not be significant for the trees currently growing in the Scandinavian boreal forest, it could be significant in the longer term. Nitrogen fixation by bacteria within conifer needles may have provided an important source of nitrogen for the forest ecosystem's structure and function during the millennia that the boreal forest has dominated this landscape.

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Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest statement

None declared.

Authors' contributions

T.B., A.S., R.G. and A.N. conceived and designed the study, T.B. performed the experiments and carried out the statistical analysis, T.B. and A.N. wrote the manuscript, but all authors contributed equally to manuscript revision, and read and approved the submitted version.

Data availability

Sequencing data and bacterial strains are made available through GenBank, Sequence Read Archive and NCCB collection.

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Shifts in microbial community composition and metabolism correspond with rapid soil carbon accumulation in response to 20 years of simulated nitrogen deposition

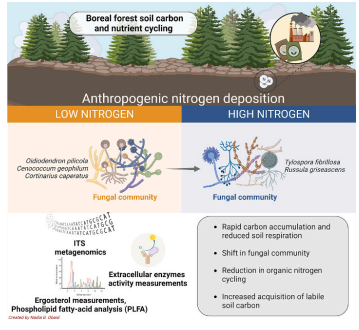
Benjamin Forsmark [a,*,1], Tinkara Bizjak [b,1], Annika Nordin [b], Nicholas P. Rosenstock [c], Håkan Wallander [d], Michael J. Gundale [a]

[a] Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden
[b] Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden
[c] Center for Environmental and Climate Research, Lund University, SE-223 62 Lund, Sweden
[d] Department of Microbial Ecology, Lund University, SE-223 62 Lund, Sweden

HIGHLIGHTS

- Nitrogen (N) addition shifted the dominance of fungal decomposers.
- Several taxa known to grow on organic N became less abundant.
- Organic N uptake and oxidative enzyme activity were suppressed.
- Higher activity of carbohydrate acquisition indicates decomposer energy limitation.
- Shifts in decomposer activity driven by N addition increase soil carbon stocks.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthropogenic nitrogen (N) deposition and fertilization in boreal forests frequently reduces decomposition and soil respiration and enhances C storage in the topsoil. This enhancement of the C sink can be as strong as the aboveground biomass response to N additions and has implications for the global C cycle, but the mechanisms remain elusive. We hypothesized that this effect would be associated with a shift in the microbial community and its activity, and particularly by fungal taxa reported to be capable of lignin degradation and organic N acquisition. We sampled the organic layer below the intact litter of a Norway spruce (*Picea abies* (L.) Karst) forest in northern Sweden after 20 years of annual N additions at low (12.5 kg N ha^{-1} yr^{-1}) and high (50 kg N ha^{-1} yr^{-1}) rates. We measured microbial biomass using phospholipid fatty-acid analysis (PLFA) and ergosterol

* Corresponding author at: Southern Swedish Forest Research Centre, SLU, P.O. Box 190, SE-234 22 Lomma, Sweden.
E-mail address: benjamin.forsmark@slu.se (B. Forsmark).
[1] Shared first authorship with equal contribution.

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measurements and used ITS metagenomics to profile the fungal community of soil and fine-roots. We probed the metabolic activity of the soil community by measuring the activity of extracellular enzymes and evaluated its relationships with the most N responsive soil fungal species. Nitrogen addition decreased the abundance of fungal PLFA markers and changed the fungal community in humus and fine-roots. Specifically, the humus community changed in part due to a shift from *Oidiodendron pilicola*, *Cenococcum geophilum*, and *Cortinarius caperatus* to *Tylospora fibrillosa* and *Russula griseascens*. These microbial community changes were associated with decreased activity of Mn-peroxidase and peptidase, and an increase in the activity of C acquiring enzymes. Our results show that the rapid accumulation of C in the humus layer frequently observed in areas with high N deposition is consistent with a shift in microbial metabolism, where decomposition associated with organic N acquisition is downregulated when inorganic N forms are readily available.

1. Introduction

Soil microbes play a central role in the global carbon (C) cycle (Liang et al., 2017). By releasing the nutrients plants need to grow, they contribute to the uptake of atmospheric CO_2; however, their respiration also contributes a significant portion of soil CO_2 emissions (Crowther et al., 2019; Paul, 2015; Smith and Read, 2008). Soils in boreal forests store a substantial fraction of global soil C (Tarnocai et al., 2009), and are affected by major changes in nutrient availability such as via atmospheric nitrogen (N) deposition (Maaroufi et al., 2015; Reay et al., 2008; Thomas et al., 2015; Tipping et al., 2017). Owing to huge C stores in boreal forests (Clemmensen et al., 2021; Deluca and Boisvenue, 2012), even small changes can have an impact on the global atmospheric C balance (Lal, 2005), yet we lack a complete mechanistic understanding of how microbial processes drive such changes in response to external N inputs (Luo et al., 2016; Schmidt et al., 2011; Stocker et al., 2016; Todd-Brown et al., 2012).

While it is clear that external N inputs enhance aboveground growth and the input of C to soils by above and below ground litter in boreal forests (Blaško et al., 2022; Forsmark et al., 2021; Leppalammi-Kujansuu et al., 2014), further understanding is needed regarding how soil microbial community composition and enzymatic activities respond. Globally, plant tissue C to N ratio is a good predictor of organic matter decomposition rates, thus lower tissue C to N ratios caused by N deposition can be expected to stimulate decomposition (Averill and Waring, 2017; Stocker et al., 2016); however, studies frequently report reduced soil respiration and decomposition rates in response to N inputs (Berg, 2014; Fog, 1988; Janssens et al., 2010; Nohrstedt et al., 1989). Recent studies of the effects of N addition on C pools and fluxes across the Swedish boreal forests have shown highly consistent responses, with as much as 1000 kg C ha^{-1} yr^{-1} accumulating in the organic soil horizon (Forsmark et al., 2020a), which is driven partly by a reduction in soil respiration by 10–50 % and increased above and belowground litter inputs (Blaško et al., 2022; Forsmark et al., 2021; Maaroufi et al., 2015). Importantly, the organic soil horizon is also a major sink for added N, frequently sequestering half of the added N or more (Gundale et al., 2014; Templer et al., 2012). Therefore, insights into how the boreal microbiome responds to changes in N availability and soil stoichiometry, and in turn influences the release of C and nutrients during decomposition are key to understand the C sinks in both plant biomass and soils in boreal forests.

Litter decomposition is largely mediated by extracellular enzymes exuded into the soil environment to catalyze the release of specific resources (Sinsabaugh et al., 2008). During early stages of decomposition abundant labile resources such as cellulose, peptides, and phosphates are released by hydrolytic enzymes (Baldrian and Stursova, 2011). During later stages of decomposition, an increasing fraction of the remaining organic matter is composed of lignin and other recalcitrant compounds that require oxidative enzymes such as laccases and peroxidases or Fenton chemistry for further degradation (Lindahl and Tunlid, 2015; Sinsabaugh, 2010). Although the fungi with the strongest oxidative potential are saprotrophic, evidence is accumulating that some ectomycorrhizal fungi (EMF) have retained these genes during evolution (Argiroff et al., 2022; Bödeker et al., 2016; Floudas et al., 2012; Kohler et al., 2015; Morgenstern et al., 2008; Nicolas et al., 2019), and that they are adapted to release N from complex organic matter (Bödeker et al., 2014; Kuyper, 2017; Talbot et al., 2008). This EMF adaptation is believed to give the fungi and the associated trees access to otherwise inaccessible organic N pools (Näsholm et al., 1998; Orwin et al., 2011; Schimel and Bennett, 2004). Indeed, EMF are more abundant during late stages of decomposition, in the lower part of the humus layer (Bending and Read, 1995; Lindahl et al., 2007; Rosling et al., 2003), which is also where the soil C stock is most responsive to N enrichment (Blaško et al., 2022; Forsmark et al., 2020a; Maaroufi et al., 2015), but the role of EMF in decomposition remains elusive. Firstly, EMF have been suggested to be major drivers of decomposition (Kyaschenko et al., 2017b; Lindahl et al., 2021; Stendahl et al., 2017), and the reduced allocation of C to EMF for N acquisition may therefore lead to decreased decomposition (Chen et al., 2014; Craine et al., 2007; Moorhead and Sinsabaugh, 2006; Talbot et al., 2008). On the contrary, organic N acquisition via EMF may inhibit decomposition by reducing soil N concentrations below the demand threshold for saprotrophic microbes, i.e. the so-called Gadgil effect (Fernandez and Kennedy, 2016; Gadgil and Gadgil, 1975; Gadgil and Gadgil, 1971; Orwin et al., 2011), and a weakening of that interaction due to N enrichment would thereby lead to increased decomposition. To shed light on these mechanisms, further data are needed on how changes in microbial community composition correspond with changes in extracellular enzyme activity.

To address this knowledge gap, we utilized a long-term experimental set up in a boreal Norway spruce forest (Table S1-S2), where N has been added annually for 20 years at a low (12.5 kg N ha^{-1} yr^{-1}) and high (50 kg N ha^{-1} yr^{-1}) rate to simulate upper level N deposition rates in the boreal region and in Europe, respectively (Gundale et al., 2011). Using this experiment, we previously reported that N addition caused soil C stocks in the humus layer to increase, while soil respiration and soil microbial biomass decreased (Maaroufi et al., 2015). Furthermore, our previous work has suggested that enhanced C accumulation in response to N has to be at least partly driven by changes in microbial activity, as litter decomposition has been shown to be reduced in N treated plots (Forsmark et al., 2020b; Maaroufi et al., 2017). Therefore, to better understand why N has this impact on the soil C cycle, we focused on describing the microbial community composition, using a combination of broad community profiling via phospholipid fatty acids (PLFA) analysis, ergosterol measurements, as well as fungal DNA sequencing for in-depth analysis of fungal taxa down to the level of species. Additionally, the activities of C, N, and P acquiring enzymes and oxidative enzymes were measured to establish the relationships between fungal community composition and soil enzyme activities. Because trees allocate more C to their root systems at the end of the growing season (Högberg et al., 2010; Kaiser et al., 2010), we sampled during summer and early autumn to capture potential seasonal variation in community composition and enzyme functioning. First, we hypothesized that the addition of N would change the microbial community through a decrease in the abundance of ectomycorrhizal decomposers previously reported to be involved in organic N uptake from lignified soil organic matter, including *Cortinarius* (Bödeker et al., 2014) and *Piloderma* (Lilleskov et al., 2011), whereas *Tylospora* and *Russula* species would increase (Kyaschenko et al., 2017a; Marupakula et al., 2021; Wallander

et al., 2010). Secondly, we hypothesized that the shifts in microbial community composition would correspond with a shift in enzyme activities, and specifically by reduced activity of N acquiring and oxidative enzymes that are known to decompose organic matter, while causing an increase in P acquiring enzymes.

2. Materials and methods

2.1. Experimental design

We used an N addition experiment in an area in northern Sweden (64°14 N, 19°46E) with low ambient N deposition (<2 kg N ha^{-1} yr^{-1}). The experiment was established as a randomized complete block design in 1996 in a Norway spruce (*Picea abies* (L.) Karst) dominated forest naturally regenerated at the beginning of the 20th century (From et al., 2016). The experiment consists of control, low and high N addition rate plots. The low N addition rate at 12.5 kg N ha^{-1} yr^{-1} (hereafter referred to as the 12.5 N treatment) corresponds to upper N deposition rates in the boreal region (Gundale et al., 2011; SMHI, 2019), whereas the high N addition rate at 50 kg N ha^{-1} yr^{-1} (hereafter referred to as the 50 N treatment) is representative of high level N deposition rates in central Europe, as well as regions of north America and China (Liu et al., 2013) and also serves as a useful comparison to other N addition experiments (Hyvönen et al., 2008). The experiment consists of plots ranging in size from 1 to 2500 m^2, arranged in blocks, with each N addition rate and plot size replicated 6 times. Here, we utilized plots at 1000 m^2 and 2500 m^2 size to reach a total replication of 12 plots per N addition rate. Nitrogen has been added annually since 1996 as solid ammonium-nitrate granules directly after snowmelt, which usually occurs by the end of May. This makes it the longest running large plot experiment with N addition treatments simulating the entire range of N deposition rates observed in the boreal zone.

2.2. Soil and fine-root sampling

The organic layer under the intact litter and down to the mineral soil was sampled in summer and autumn 2016. The two time points were chosen to represent distinct states in the seasonal development of the canopy, which influences the transfer of C and N between canopy and roots (Högberg et al., 2010; Kaiser et al., 2010). The summer sampling was done one week after the solstice (June 28–July 1), which corresponds to a time of the year with maximum light availability, shoot growth, and a phase of N depletion and accumulation of carbohydrates in photosynthesizing tissues (Linder, 1995). The autumn sampling was done around the equinox (20–27 September) at a time when both N and carbohydrates are depleted in the canopy (Linder, 1995) and when the activity in the root-zone peaks (Hasselquist et al., 2012; Högberg et al., 2001) and soil respiration is high (Maaroufi et al., 2015). While the soil temperature was similar between the summer and autumn sampling (9.7 versus 9.4 °C), the daytime air temperature was 15.4 °C in the summer sampling, and the autumn sampling was done after the first frost, with daytime temperatures of 12.8 °C (ICOS, 2023).

At both sampling occasions, the entire organic layer between the intact litter layer down to the mineral soil was collected with a sharp 22 mm soil corer at 30 locations within each plot, each spaced at least 2 m apart to ensure an appropriate coverage of the spatial variation in the fungal community (Dahlberg et al., 1997). All 30 cores from each plot were pooled to create one composite sample per plot and kept cool with ice during collection, and then were weighed and sieved (2 mm) to separate roots and humus within 1 h of collection. The sieved humus was homogenized, and then separated into two sub-samples per plot. The first subsample (approximately 10 g) was freeze-dried and used to determine gravimetric moisture content, and then was ground to a fine powder on a roller mill (Stuiver et al., 2015) for measurements of total C, N, and P, phospholipid fatty acids (PLFA), and DNA extraction as described below. The second sub-sample was kept frozen and used to measure the activity of a selection of enzymes, also described below. Roots that were isolated during sieving were further hand sorted to exclude ericaceous rhizomes, and vital living Norway spruce fine-roots (< 2 mm diameter), including associated fungal mycelium, were selected for DNA extraction.

During collection and sorting, all equipment was cleaned thoroughly with 70 % ethanol between samples and then stored on ice at 4 °C during the sampling day, and frozen for long-term storage the same evening. The fine-roots were cleaned according to a protocol developed by Gottel et al. (2011) and modified by Gundale et al. (2016). Briefly, the roots were repeatedly (5 times) vigorously shaken and rinsed in distilled water alone, then shaken and rinsed in a solution of 0.05 % Tween 20 detergent, rinsed again in distilled water, and then surface-sterilized for 2 min in a 0.27 % NaOCl solution. Roots were then washed 10 times in distilled water and then frozen, freeze-dried, and ground to a fine powder for DNA extractions. In total, 72 samples were collected in the field (3 N treatments, 2 seasons, 12 replicates) and split into soil and roots for DNA sequencing, yielding a total of 144 samples for DNA sequencing.

2.3. Phospholipid fatty acid and ergosterol analysis

We used a combination of analyses of phospholipid fatty acids (PLFA) and ergosterol on the freeze-dried samples to profile the microbial community broadly, and to represent microbial biomass. Lipids were extracted from approximately 1 g soil using the Bligh and Dyer method (Bligh and Dyer, 1959; McIntosh et al., 2012; White et al., 1979) and the abundance of individual PLFA's was measured on a gas chromatograph (Perkin-Elmer Clarus 500, Mundelein, Illinois, USA) coupled to a flame ionization detector (Waltham, MA, USA) at the Swedish University of Agriculture, SLU Umeå. A total of 28 PLFA markers were identified and described using standard nomenclature, and classified according to previous work in this study system (Maaroufi et al., 2015). The sum of all PLFA markers were used as a measure of total microbial biomass, and i-15:0, a-15:0, 15:0, i-16:0, 16:1ω9, 16:1ω7, 16:0, i-17:0, cy-17:0, a-17:0, 18:1ω7, and cy-19:0 represented the total bacterial biomass (Frostegård and Bååth, 1996). The branched PLFAs i-15:0, a-15:0, i-16:0, i-17:0, and a-17:0 represented gram-positive bacteria (Wardle et al., 2013), whereas 10me16:0, 10me17:0, and 10me18:0 were used to estimate actinobacteria, and cy-17:0, cy-19:0, and 18:1ω7 represented gram-negative bacteria. PLFA 18:2ω6 alone represented fungi (Frostegård et al., 2011; Maaroufi et al., 2019). Absolute abundances were expressed in moles per gram soil C.

Free ergosterol was measured in addition to PLFA as a general fungal biomarker intended to represent living biomass (Clemmensen et al., 2013) as a complementary measure of soil fungal biomass. Ergosterol was extracted from 35 mg of the dried soil by vigorously shaking the samples in 0.25 ml MeOH (99.8 %), and the extract cleaned by centrifugation and filtration (45 μm). The extract was injected in MeOH (isocratic) at a flow rate of 1.5 ml min^{-1} on a Shimadzu prominence HPLC and separated on a reverse-phase column (Ascentis® Express C18, 2.7 μm). After 3 min, ergosterol concentrations were detected with an optical-ultraviolet detector (SPD-20 A UV/VIS).

2.4. DNA extraction and sequencing

Nucleic acids were extracted from 0.25 g of dried powdered humus samples using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. DNA was extracted from 50 mg of freeze-dried fine-roots by first extracting and purifying in 2 % CTAB/SDS, chloroform, isopropanol and ethanol, as per Gundale et al. (2016), re-suspended in 200 ml Tris and EDTA (TE) buffer, and further purified using a Nucleospin gDNA clean up kit (Machery-Nagel, Düren, Germany).

DNA from the ITS2 region of the internal transcribed spacer (ITS) was amplified with the fungi-specific primers ITS7g (Ihrmark et al.,

2012) and ITS4 (Gardes and Bruns, 1993) which included adapter sequences for Illumina sequencing, and was subjected to a second 8-cycle amplification to attach Nextera (Illumina Inc., San Diego, CA, USA) sample barcodes. Equimolar amounts of DNA from each sample were pooled and submitted for Illumina sequencing with paired-end (325 bp forward; 275 bp reverse) sequencing on a MiSeq sequenator using the MiSeq Reagent Kit v3 chemistry (Illumina Inc., San Diego, CA, USA) at the Next-generation sequencing facility at Lund University, Lund, Sweden.

2.5. Bioinformatics

DNA sequences obtained from Illumina sequencing were trimmed and filtered using Mothur v1.34 (Schloss et al., 2009), clustered using the Gaussian mixture model clustering algorithm CROP (Hao et al., 2011) at 97 % sequence similarity, thus yielding operational taxonomic units (OTUs). All non-fungal and chimeric sequences were removed and sequences were trimmed to include only the ITS2 region using ITSx extractor v1.5.0 (Bengtsson-Palme et al., 2013). The taxonomic identity was then assigned to the set of clustered sequences by searching the Full "UNITE+INSD" (Koljalg et al., 2005; Koljalg et al., 2013) dataset (673,903 seqs, release date 2016-11-20) using the Basic Local Alignment Tool (BLASTN program 2.2.25, blast.ncbi.nlm.nih.gov). Sequences that were 96 % similar to the query sequence and top hit, with at least 80 % coverage of the query sequence length, were assigned to a taxonomic identity with genus and species. Sequences with values of 94–95 % similarity between the query sequence and top hit were assigned a taxonomic identity at the genus level only. All OTU's representing <10 total reads or occurring in only one sample were excluded. Rarefaction was performed to 31,000 reads per sample.

The ecological guild (i.e. ectomycorrhizal or non-ectomycorrhizal) of all taxa for which genera could be assigned was classified with the assistance of the database tool FunGUILD (Nguyen et al., 2016). Guild classifications were accepted, when assigned a confidence ranking of Highly Probable or Probable. EMF species identity was further verified based on the most recent knowledge of the ecology of known close relatives (genera or species) and according to Tedersoo et al. (2010). Read abundances for all OTUs for each sample were summed and the abundance of each OTU expressed as the relative abundance per sample. A matrix containing the rarefied relative abundance of each OTU per sample was then used to analyze differences in community composition. Before further analyses, 1 humus sample with very low OTU abundance and dominance of two species of *Malassizia* was removed, which gave a complete humus dataset with 71 samples and 1051 species OTUs.

2.6. Enzyme activities

The potential activity of selected enzymes originating from the soil microbiome was measured on the frozen soil according to methods in Allison (2012), that is based on methods presented by Saiya-Cork et al. (2002), and modified according to Baldrian (2009). Shortly, we used 4-methylumbelliferyl (MUB) labeled β-D-glucopyranoside, β-D-cellobioside, β-D-xylopyranoside, *N*-acetyl-β-D-glucosaminide, and phosphate to measure the activity of glucosidase (β-1.4-glucosidase, EC 3.2.1.21), endoglucanase (EC 3.2.1.4), xylosidase (β-1.4-xylosidase, EC 3.2.1.37), chitinase (β-1.4-*N*-acetylglucosaminidase, EC 3.2.1.52), and phosphatase (acid-phosphatase, EC 3.1.3.2) respectively, and 7-amido-4-methylcoumarin (AMC) labeled leucine for peptidase (leucine aminopeptidase, EC 3.4.11.1). 140 ml sodium acetate buffer (50 μM, pH 5.0) was added to 0.5 g soil. The sample was shaken for 20 min and loaded on a 96 well plate with 50 μl labeled substrate. The plate was incubated in darkness at room temperature for 10 min ($t = 0$) and fluorescence was measured with MUB after 60 min and for AMC after 20 h.

The activity of the peroxidase (Manganese(II)-peroxidase, EC 1.11.1.13) and laccase (EC 1.10.3.2) was measured as the buildup of the product of oxidized 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and 3-dimethylaminobenzoic acid (DMAB) for peroxidase and oxidation product of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) for laccase. Namely, 1 g soil was added to 12 ml Milli-Q water and shaken for 30 min. The samples were centrifuged at 9000 ×*g* for 10 min and the supernatant was frozen at −20 °C for subsequent analyses. The reaction buffer for peroxidase consisted of 1 ml Manganese (II) sulphate solution (1 mM), 0.5 ml DMAB solution in EtOH (50 mM) and 0.5 ml MBTH solution (1 mM) in 5 ml Succinate-lactate buffer (pH 4.5, 100 mM), whereas 1 ml EDTA (2 mM) substituted the Manganese (II) sulphate in the reaction buffer without Mn(II)sulphate (to account for background substrate oxidation). To each well on the 96 well plate 10 μl 1 mM H_2O_2, 140 μl reaction buffer and 50 μl of the extract were added and absorbance was measured every 5 min at 590 nm for 60 min. For laccase, 160 μl 100 mM sodium-acetate buffer (pH 5), 20 μl 50 mM ABTS and 20 μl sample were added to each well and the absorbance was measured at 420 nm every 10 min for 2 h.

All enzyme activities were estimated by regressing fluorescence or absorbance against time and were standardized to the amount of C in each sample to reduce the random variation caused by intermixing heavy mineral particles. Thus, the standardized measure is the potential enzyme activity in a completely organic sample. The activities of glucosidase, endoglucanase, and xylosidase were considered indicative of C acquisition, phosphatase of phosphorous acquisition, chitinase and peptidase of organic N acquisition, whereas peroxidase and laccase indicated oxidative decomposition potential. All measurements were made in humus samples derived after removing roots by sieving, hence all enzyme activity are related to the microbiome and not to processes occurring within plant biomass.

2.7. Statistical analyses

Linear mixed effects models were used to assess the effects of N (df = 2) and season (df = 1) and their interaction (df = 2) on the element ratios and pH, and measurements on the microbial community, including abundance of aggregated PLFA functional groups, and their ratios, free ergosterol, and the relative abundance of aggregated taxonomic or functional groups of the fungal community, and on the potential enzyme activities. Block ($n = 12$) was included as a random factor in the model and Tukey's honestly significant difference (HSD) test was used for post hoc.

Variation in the fungal community composition was explored with a combination of unconstrained and constrained ordinations (Oksanen, 2015), and multivariate analysis of variances on Bray-Curtis dissimilarity matrices. Detrended correspondence analyses (DCA) was applied to identify major gradients in the community and to visualize the relative contribution of sample type (humus vs. root), N addition rate, and season to variations in the fungal community composition. Canonical analysis of principal coordinates (CAP) was used to specifically target variations in community composition due to N and season. The direct and interactive effects of N and season on the location of fungal communities in multivariate space was tested separately for humus and roots using permutational multivariate analysis of variance (PERMANOVA), with block ($n = 12$) included as a random factor. An additional permutational test for homogeneity of multivariate dispersion was used to verify that differences were due to location, rather than differences in dispersion. Finally, for all significant effects identified with PERMANOVA, we followed up with additional 'contribution of variables to similarity' (SIMPER) analysis to identify which fungal OTUs contributed most to the similarity within groups and differences between groups. The correlation between selected responsive OTU's in humus samples from SIMPER analysis and enzyme activities as well as between responsive OTU's and microbial traits were calculated using Spearman's correlation. Only humus samples were used in this analysis as it is assumed that extracellular enzyme activities are responding to the soil environment and not root conditions. All statistics and graphs connected to sequencing were produced in R using phyloseq (McMurdie and

Holmes, 2013) and vegan packages (Dixon, 2003).

3. Results

3.1. Phospholipid fatty acid and ergosterol analysis

Based on PERMANOVA on all PLFA markers, microbial community composition in the humus differed significantly between N addition treatments (Table 1). The 50 N treatment markedly decreased the total PLFA by 25 % (Fig. 1A, Table 2), which was heavily influenced by a 37.5 % decrease in fungal (Fig. 1G) and a 27.1 % decrease in gram-negative bacterial (Fig. 1E) PLFA's. Gram-positive bacteria PLFA's (Fig. 1D) decreased less, and actinobacterial PLFA's (Fig. 1C) remained relatively unchanged, which led to an increase in the ratio between gram-positive and gram-negative bacteria (Fig. 1F). However, the total bacterial PLFA's were still lower in 50 N treatment overall (Fig. 1B) and the decrease in fungal PLFA's was bigger compared to bacterial PLFA's (Fig. 1I). The 12.5 N treatment did not differ significantly compared to the control in any of the PLFA markers measured (Fig. 1). In contrast to the fungal PLFA marker 18:2ω6, ergosterol increased by 30.2 % in the 50 N treatment (Fig. 1H) but was not significantly higher than the control in 12.5 N treatment. Season had a relatively minor effect on PLFA's and ergosterol (Table 2), although fungal PLFA tended to be higher ($p = 0.059$) in the autumn. No significant interactions between N and season were detected in the PLFA or ergosterol measurements (Table 2).

3.2. Fungal community

After all filtering steps, clustering, and rarefaction, sequencing generated 4,397,997 sequences belonging to 1051 operational taxonomic units (OTU's). Of these, 610 were present in both humus and root samples, 433 exclusively in the humus samples, and 8 OTU's exclusively in the root samples (Fig. S1A). Additionally, 1011 OTU's were shared between the seasons, 29 were unique for summer and 11 to autumn sampling (Fig. S1B). Regarding N treatment, 875 OTU's were shared between the three treatments, 12 were only found in 0 N, 6 in 12.5 N and 15 in 50 N treatment (Fig. S1C). Of the 1051 OTU's, 816 (representing 90 % of all sequence reads) were assignable to ecological guild; 295 OTU's, comprising 55 % of all sequence reads were classified as ectomycorrhizal (54 % of humus sequences, 57 % of root sequences), and 51 OTU's, comprising 33 % of all sequence reads were classified as ericoid fungi (22 % of humus sequences, 46 % of root sequences). The fungal community differed significantly between N treatments for both the humus and root community (PERMANOVA $p < 0.001$, Table 2). Differences in community composition between humus and roots contributed most of the variation in the combined fungal community, explaining 52.4 % of the variation (Fig. S2). CAP analysis constrained with N treatment and the PERMANOVA show that the fungal community composition in both humus and roots was significantly different in the 50 N treatment from the 0 N and the 12.5 N treatment (Fig. 2).

The humus community had a clear dominance of ascomycetes over basidiomycetes in the summer (57 % versus 40 %), shifting to become more evenly distributed in the autumn (46 % versus 51 %). The humus community was significantly affected by the N treatment based on PERMANOVA (Table 1). Among Agaricomycetes which alone constituted 33 % of the fungal community, and 73 % of the basidiomycetes, mixed responses to N at species level were observed. Specifically, EMF forming genera *Russula* and *Tylospora* increased with N addition (nitrophilic) based on linear mixed effects model (Table 2). Different OTU's within *Russula* responded in different directions (data not shown), notably, *Russula vinosa* was one of the most nitrophobic, whereas *Russula griseascens* was one of the most nitrophilic of all OTU's. The EMF genus *Cortinarius* sp. accounted for a large fraction of the OTUs (101 out of the total 1051 OTU's), and accounted for 12.4 % of all reads in the control treatment. At the genera level, *Cortinarius* were consistently nitrophobic as their relative abundance decreased by 45 % with N addition, a decline that was primarily driven by *Cortinarius caperatus* even though several OTU's at lower abundance were more strongly negatively correlated to the N treatments, most notably *Cortinarius alpinus*, *Cortinarius brunneifolius*, and *Cortinarius acutus*. The relative abundance of *Tylospora*, belonging to Atheliaceae, doubled in the 50 N treatment compared to the control. However, different *Piloderma* species, also belonging to Atheliaceae, usually declined with N treatment and *Piloderma bicolor* was among the most nitrophobic OTUs.

Using SIMPER analysis, the responsive fungal species were identified that contributed the most to dissimilarity between 0 N and 50 N samples (Table S3). Among the responsive species that cumulatively contributed to 30 % difference between N treatments, N increased the abundance of *Tylospora fibrillosa*, *Piloderma byssinum* and *Russula griseascens*, while *Oidiodendron pilicola*, *Cenococcum geophilum*, *Piloderma sphaerosporum* and *Cortinarius caperatus* decreased (Fig. 3A). Based on PERMANOVA analysis, the humus community also changed significantly over the season (Table 1), with the basidiomycetes *Tylospora fibrillosa*, *Cortinarius caperatus*, and *Piloderma sphaerosporum* increasing, and the ascomycetes *Oidiodendron pilicola*, *Cenococcum geophilum*, and *Pezoloma ericae* decreasing in the autumn (Table S4).

The root community was significantly different due to N treatment according to PERMANOVA (Table 2). SIMPER analysis identified taxa that contributed most to the differences between the 0 N and 50 N treatments (Table S5). For roots, the most responsive species that contributed to 40 % cumulative contribution of differences, N addition led to higher abundance of *Phialocephala fortinii*, *Piloderma byssinum*, *Piloderma ericae*, and *Meliniomyces variabilis*, and lower of *Meliniomyces bicolor*, *Piloderma sphaerosporum* and *Cenococcum geophilum* (Fig. 3B). Using PERMANOVA, season had no significant effect on the root community (Table 2), however based on SIMPER analysis the abundance of *Meliniomyces variabilis* and *Piloderma byssinum* increased in autumn, while abundance of *Leullia* sp., *Cenococcum geophilum* and *Piloderma sphaerosporum* decreased (Table S6).

Table 1
The effect of nitrogen treatment (0, 12.5, and 50 kg N ha^{-1} yr^{-1} × 20 years), season (summer vs. autumn), and their interaction on PLFA and sequenced microbial community composition assessed with permutational (9999) analysis of variances (PERMANOVA) testing the null-hypothesis that the community composition has the same centroid in multivariate space. The variation within groups did not differ significantly according to permutational test for homogeneity of multivariate dispersion (data not shown).

	Nitrogen treatment		Season		Nitrogen × season	
	Pseudo F-value	p-Value	Pseudo F-value	p-Value	Pseudo F-value	p-Value
PLFA						
Humus	21.428	**0.001**	1.642	0.188	0.138	0.976
Fungal community						
Total	7.075	**0.001**	0.956	0.364	0.462	0.923
Humus	7.373	**0.001**	1.799	**0.020**	0.612	0.879
Root	7.791	**0.001**	1.106	0.203	0.821	0.511

Significant effects (p<0.05) are highlighted in bold font.

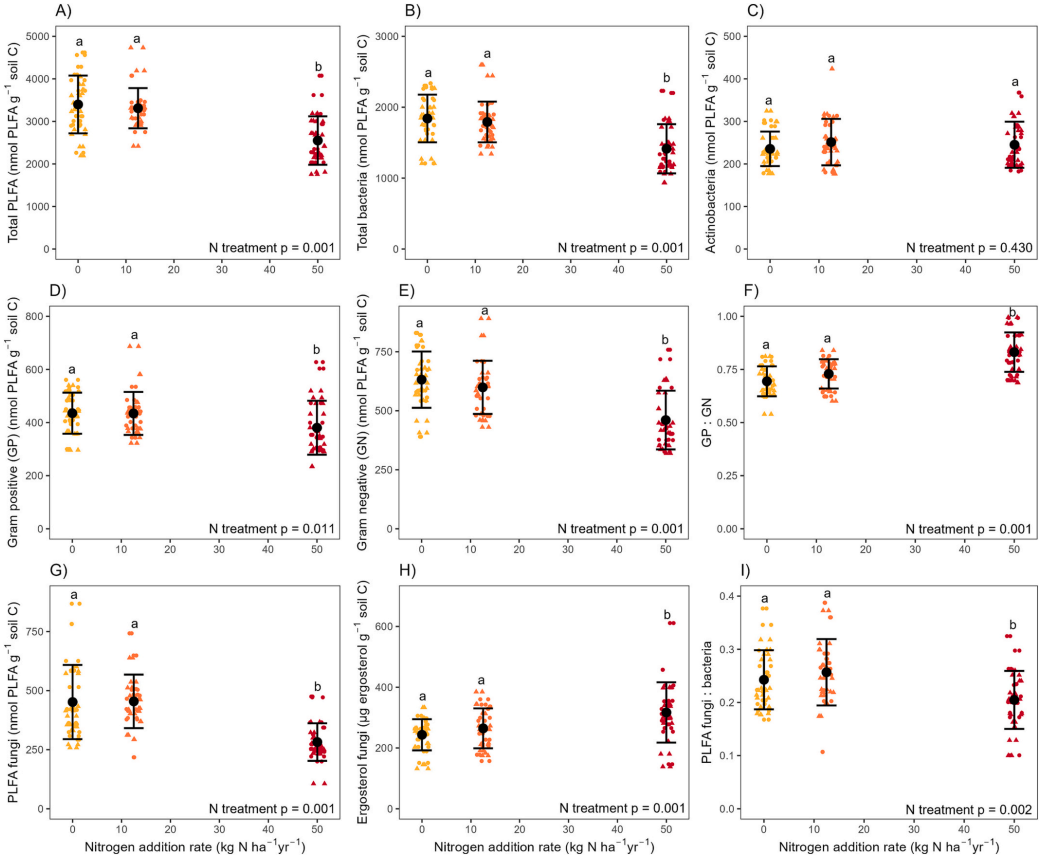

Fig. 1. Composition of the microbial community in the organic layer of a boreal forest soil sampled during summer (triangle shape) and autumn (circle shape) after 20 years of annual nitrogen addition at three levels (0, 12.5, and 50 kg N ha^{-1} yr^{-1}) based on phospholipid fatty acid (PLFA) markers and free ergosterol analysis. Black circles represent the means and the error bars represent standard deviation, the N treatment *p*-values are linear mixed effects models values (Table 1) and different letters represent a statistically significant difference based on Tukey's HSD.

3.3. Enzyme activity

The highest N addition rate (50 N) increased the activity of endoglucanase and xylosidase (β-1.4-xylosidase), decreased the activity of peptidase (Leucine aminopeptidase) and peroxidase (Manganese(II)-peroxidase), and nearly significantly ($p < 0.06$) decreased chitinase (β-1.4-*N*-acetylglucosaminidase), whereas glucosidase (β-1.4-glucosidase), phosphatase (acid-phosphatase), and laccase were unaffected (Fig. 4, Table 3). No significant differences in enzyme activities were detected between the control and the 12.5 N treatment (Fig. 4). The enzyme activity was higher in the autumn for glucosidase and peptidase, whereas the activity of phosphatase, xylosidase, chitinase, endoglucanase, peroxidase, and laccase remained constant (Fig. 4, Table 3). No interactions between N and season were detected (Table 3).

3.4. Associations between enzyme activities and humus fungal communities

Looking at association between enzyme activities and fungal species most responsive to N addition based on SIMPER analysis (Fig. 5), laccase was positively correlated with *Tylospora fibrillosa*, *Apiotrichum xylopini* and *Russula griseascens* and negatively with *Helotiales* sp. and *Piloderma sphaerosporum*. Peroxidase was positively correlated with *Oidiodendron pilicola* and *Helotiales* sp. and negatively with *Pezoloma ericae* (Fig. 5). Both chitinase and peptidase were positively correlated with *Piloderma bicolor* and peptidase was negatively correlated with *Pezoloma ericae* (Fig. 5). Phosphatase was negatively correlated with *Pezoloma ericae* (Fig. 5). *Cortinarius caperatus* was not significantly correlated with oxidative enzymes in our study (Fig. 5). In general, fungal species more abundant after N addition were positively correlated with C and P acquiring enzymes and laccase, while negatively with N acquiring enzymes (Fig. 5). Contrasting, fungal species declining with added N were positively correlated with N acquiring enzymes and peroxidase and mostly negatively with laccase, phosphatase and C acquiring enzymes (Fig. 5).

4. Discussion

The main aim of this study was to investigate a key mechanism by which N deposition enhances soil C accumulation in boreal forest, which

Table 2
The effect of nitrogen treatment (0, 12.5, and 50 kg N ha^{-1} yr^{-1} × 20 years), season (summer vs. autumn), and their interaction on aggregated microbial markers in the organic soil layer based on either Bligh & Dyer PLFA or ergosterol analysis, or the relative abundance of selected groups in the sequenced fungal community, assessed by linear mixed effects models with block (n = 12) as random factor.

	Nitrogen treatment		Season		Nitrogen × season	
	F-value	p-Value	F-value	p-Value	F-value	p-Value
Free ergosterol	8.116	**0.001**	1.703	0.197	0.533	0.590
PLFA						
Total	19.288	**0.001**	1.684	0.200	0.395	0.676
Fungi	18.884	**0.001**	3.719	0.059	0.215	0.807
Fungi:bacteria	6.731	**0.002**	2.412	0.126	0.132	0.877
Total bacteria	16.324	**0.001**	1.293	0.260	0.520	0.597
Gram positive (GP)	4.954	**0.011**	1.455	0.233	0.936	0.398
Gram negative (GN)	16.248	**0.001**	0.584	0.448	0.176	0.839
GP:GN	24.415	**0.001**	0.251	0.618	1.020	0.367
Actinobacteria	0.857	0.430	0.194	0.661	0.407	0.668
Fungal community						
Basidiomycetes: Ascomycetes	0.609	0.547	10.469	**0.002**	1.954	0.151
Ascomycetes	0.137	0.872	29.733	**0.001**	2.577	0.082
Basidiomycetes	0.154	0.858	28.895	**0.001**	2.602	0.083
Agaricomycetes	0.169	0.845	14.850	**0.001**	1.153	0.323
Russula	4.612	**0.014**	3.555	0.065	0.454	0.638
Atheliaceae	3.446	**0.039**	10.321	**0.002**	0.279	0.758
Cortinarius	9.914	**0.001**	8.414	**0.005**	1.281	0.286
Ectomycorrhiza	0.925	0.402	12.117	**0.001**	0.806	0.452

Significant effects ($p<0.05$) are highlighted in bold font.

we described in a previous study at this experimental site (Maaroufi et al., 2015). Specifically, we focused on the effect of N enrichment on microbial community composition and their associated enzyme activities using a long-term (20 years) N addition experiment. Such data are needed to explain the reduction in soil respiration and decomposition, and increase in soil C stocks that are frequently reported in boreal forests in response to external N enrichment (Blaško et al., 2022; de Vries et al., 2014; Forsmark et al., 2020a; Hyvönen et al., 2008; Janssens et al., 2010; Knorr et al., 2005).

Consistent with our first hypothesis, we found a clear shift in the microbial community composition based on PLFA and ergosterol data (Fig. 1, Tables 1 and 2). Similar to previous studies in the same experimental plots (Maaroufi et al., 2015), and a similar experiment in a Scots pine forest (Maaroufi et al., 2019), we found a clear decrease of 25 % in total PLFA between the control and 50 N treatment, but not between control and 12.5 N treatment. This effect was mainly driven by a decrease in fungal and gram-negative bacterial PLFA's, whereas the abundance of gram-positive bacterial and actinobacterial PLFA's were generally tolerant to changes in N, and in some cases even increased. Nitrogen addition significantly decreased fungal PLFA marker 18:2ω6 in the 50 N treatment but had no significant effect in the 12.5 N treatment (Fig. 1, Table 2). A reduction in fungal abundance is consistent with many studies (Fog, 1988; Janssens et al., 2010; Treseder, 2008) and it has been linked to reductions in EMF mycelial growth (Nilsson and Wallander, 2003). The abundance of this marker also showed a nearly significant ($p < 0.06$) increase by 14 %, from summer to autumn sampling which corresponds to a time of the year when the allocation of C below-ground is high (Högberg et al., 2010). Our second biomarker for fungal abundance, ergosterol, indicated a contrasting response, as this marker increased with N addition in the 50 N treatment, and was equally abundant in summer and autumn. Similar discrepancies between the two markers have been previously reported in boreal forests (Blaško et al., 2022; Kyaschenko et al., 2017b). Fungal biomass is a major pool of organic N in boreal forests, and as ergosterol is a relatively more persistent biomarker than PLFA, it may accumulate to a greater degree in soils when decomposition is disrupted, or other ecosystem perturbations occur (Clemmensen et al., 2013; Högberg, 2006; Zhao et al., 2005). For example, reductions in use of N from dead fungal necromass in response to N addition could be contributing to differences in the abundance of these two markers. Thus, the reduction in PLFA marker 18:2ω6 and accumulation of ergosterol due to N addition is likely caused by PLFA responding more strongly to a reduction in standing fungal biomass, whereas the relatively more stable ergosterol is likely responding more to the reduction in decomposition of dead fungal biomass.

DNA sequence data enabled a detailed analysis of the shifts in the fungal community composition of both humus and roots (Table 2, Fig. 2). Consistent with our hypothesis, predicting a shift from a dominance of *Cortinarius* and *Piloderma* to *Tylospora* and *Russula*, we found that the relative abundance of *Cortinarius* and *Piloderma* was 35 % and 45 % lower, respectively, in the humus samples in the 50 N treatment compared to the control. Considering that these genera together comprised one quarter of the fungal reads in the autumn, the decrease in these genera is likely to have contributed substantially to the reduction in the fungal PLFA marker 18:2ω6 we observed in response to N. Additionally, as hypothesized, the EMF genera *Russula* and *Tylospora* increased with N addition. These responses are not unique to our study system but seems to be a common response to increased availability of inorganic N (Haas et al., 2018; Jörgensen et al., 2021; Marupakula et al., 2021), and the adaptations of these taxa and the activation of specific EMF under different levels of N availability is likely to play a key role in both nutrient acquisition and C cycling. Taken together, our characterization of the fungal community revealed that the N addition not only reduced the total abundance of PLFA marker 18:2ω6 but also strongly restructured the fungal community from a dominance of EMF taxa known to be involved in organic N cycling towards taxa without the capacity to grow on organic N sources (Lilleskov et al., 2019).

For our second hypothesis, we predicted that changes in microbial community composition would correspond with shifts in soil enzyme activities. Particularly by decreasing activity of enzymes involved in organic N uptake and lignin decomposition after N addition, and increasing activity of P acquisition. Consistent with our hypothesis, the highest N addition rate markedly decreased the activity of peptidases and peroxidase, and nearly significantly decreased the activity of chitinase (Fig. 4). This decreased activity between control and 50 N for the N acquiring enzymes, peptidase and chitinase, indicates a reduction in the breakdown of microbial necromass. This decrease can indicate some combination of down-regulation of resorption of N in microbial necromass when the supply of mineral N is high, and that microbial biomass or necromass is less abundant. Based on the correlation analysis (Fig. 5), chitinase and peptidase were positively correlated with fungal species whose abundance decreased with N addition. This indicates that a reduction in abundance of species like *Cortinarius* and *Piloderma*, which are known for organic N acquisition (Lilleskov et al., 2011), is probably connected to the observed decrease in activity of N acquiring enzymes. The phosphatase activity, which we hypothesized to increase due to induced relative demand for P (Almeida et al., 2019; Vitousek et al., 2010), did not change after N addition. This observation corroborates the view that anthropogenic N deposition is unlikely to induce P limitations in boreal forests on minerogenic parent material (Forsmark et al., 2020b).

Mn-peroxidase plays a major role in mineralization of complex compounds such as lignin and other phenolics (Baldrian and Stursova, 2011; Sinsabaugh, 2010), and has been proposed as a mechanism to mineralize N when N is predominantly bound in recalcitrant organic matter (Bödeker et al., 2014). The observed decrease in its activity due to higher N availability observed in this and other studies (Moore et al., 2021), is likely to contribute to the decline in soil CO_2 efflux reported in this experiment (Maaroufi et al., 2015), and many others (Blaško et al., 2022; Forsmark et al., 2020a; Janssens et al., 2010), by decreasing extracellular oxidation of C, in addition to potential decreases in cellular

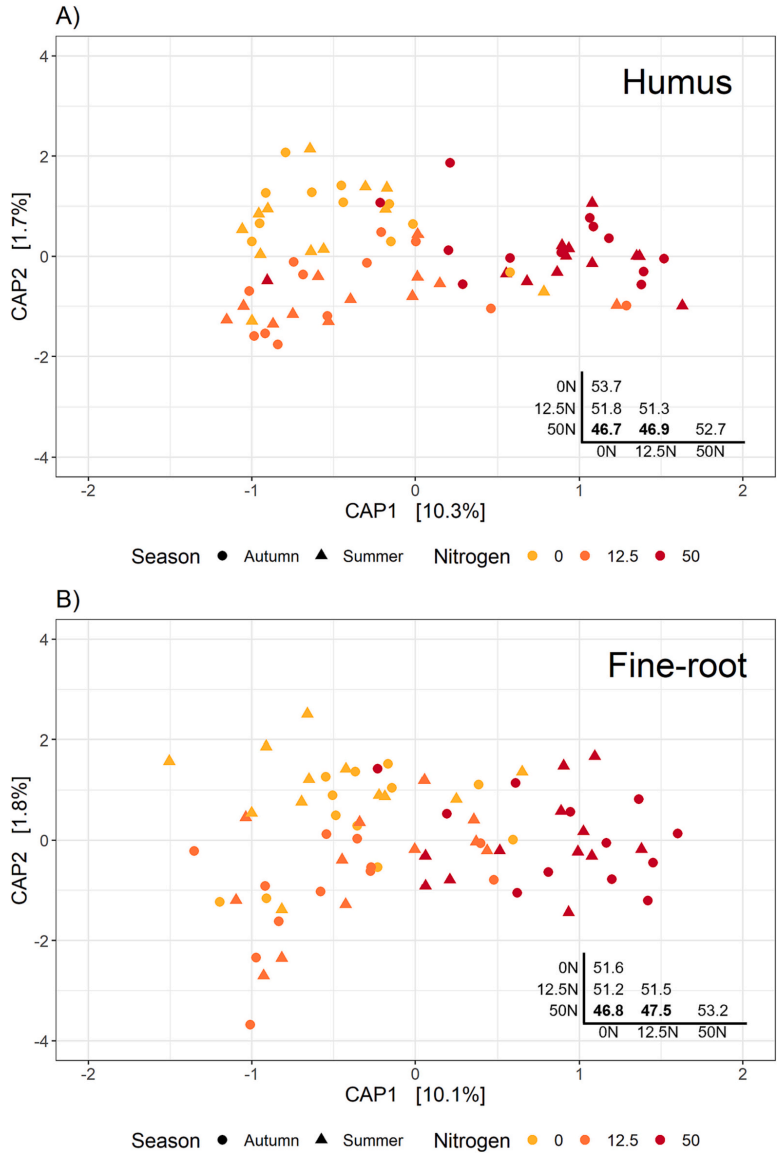

Fig. 2. Canonical analysis of principal coordinates (CAP) of fungal communities in (A) humus and (B) fine-root constrained with nitrogen addition rate (0, 12.5, and 50 kg N ha^{-1} yr^{-1} × 20 years) and season (summer (triangle shape) and autumn (circle shape)). Insert tables show percentage similarity in community composition within and between nitrogen treatments, with significant differences according to PERMANOVA in bold.

respiration due to lower microbial biomass and higher C use efficiencies. Peroxidase, like other N acquiring enzymes, was positively correlated with fungal species that are less abundant in high N environments. *Cortinarius caperatus*, which has previously been reported to be correlated with peroxidases (Bödeker et al., 2014; Lindahl et al., 2021), was not significantly correlated with peroxidase in our study, which could be due to site-specific differences. Laccase, which is an oxidative enzyme also responsible for lignin mineralization (Thurston, 1994), was unresponsive to N addition and in contrast to peroxidase was positively correlated with fungal species that increased after N addition. However, laccases are known to have a wide range of substrates in addition to lignin (Thurston, 1994), which could potentially be the reason for the difference between the observed laccase and peroxidase activity. The fungal community shift with a decrease in fungal species known for

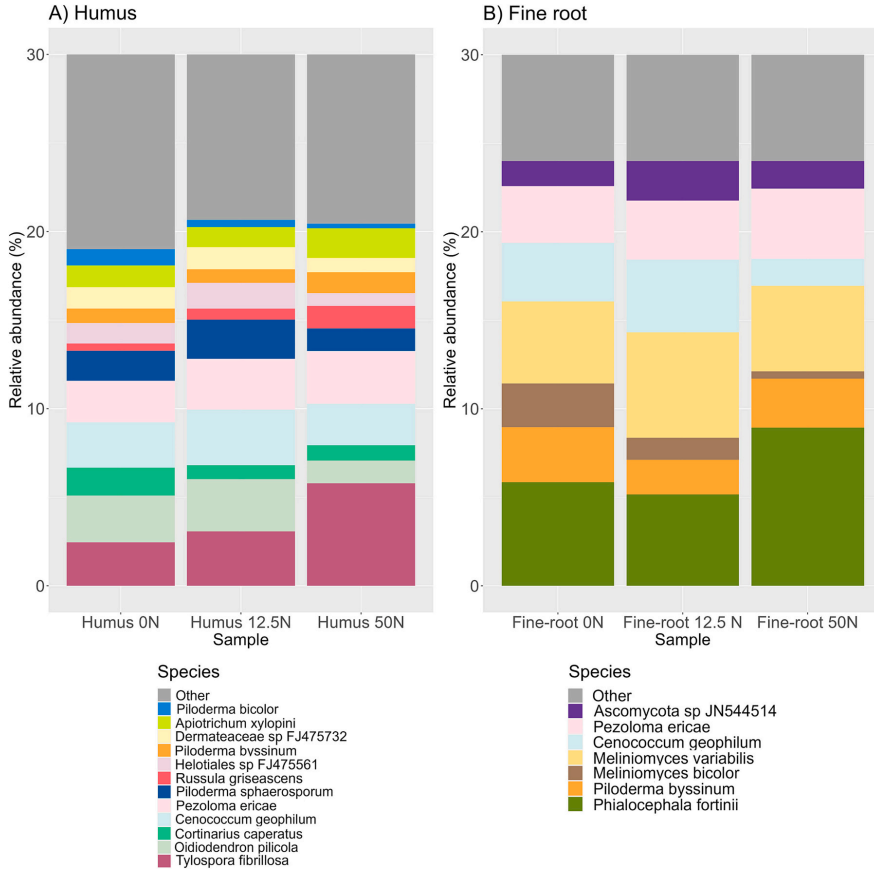

Fig. 3. The relative abundance (%) of responsive fungal species based on SIMPER analysis in (A) humus (up to 30 % cumulative contribution to differences) and (B) fine-root (up to 40 % cumulative contribution to differences) samples collected during summer and autumn on plots with annual N addition at three rates (0, 12.5, and 50 kg N ha^{-1} yr^{-1}). Species are listed by their contribution to differences based on SIMPER in increasing order, each value is the average of two sampling occasions and note that the y-axis is broken at 30 % cumulative read abundance.

organic N acquisition corresponds with a general decrease in extracellular catabolic activity associated with organic N uptake and this decrease likely reflects an increase in labile inorganic N supplied in the experimental treatments leading to reduced incentives to mine recalcitrant organic matter for organic N.

In addition to the general decrease in enzymes involved in degradation and uptake of N in recalcitrant organic matter, N also enhanced the activity of hydrolytic enzymes involved in labile C uptake (Fig. 4). The abundance of labile C in soil organic matter is minor compared to the large pool of recalcitrant fractions, and the upregulation of these enzymes is not likely to lead to any significant soil C loss, compared to the oxidative enzymes that can destabilize lignin complexes. The response may, however, shed some light on the underlying mechanism driving C accumulation in response to N enrichment (de Vries et al., 2014; Forsmark et al., 2020a; Maaroufi et al., 2015). Carbon acquiring enzymes, including glucosidase, endoglucanase and xylosidase were positively correlated with fungal species whose abundance increased after N addition, and negatively with species whose abundance decreased. Previous work in our study system has shown that N addition increases the production of plant biomass above and below ground (Forsmark et al., 2020a; From et al., 2016; Maaroufi et al., 2016), while reduces soil respiration (Forsmark et al., 2020a; Maaroufi et al., 2015) and decomposition (Forsmark et al., 2021; Maaroufi et al., 2016). In light of these previous studies, the upregulation of enzymes involved in the acquisition of labile C from soil organic matter appears to be linked to a major shift in C use in response to N enrichment, where less C is supplied to EMF and other microbes to stimulate depolymerization of soil organic matter for N acquisition (Allison et al., 2010; Janssens et al., 2010; Soong et al., 2020), and more C is used to build biomass (Campioli et al., 2015; Forsmark et al., 2021; Moorhead and Sinsabaugh, 2006; Spohn et al., 2016), eventually entering the soil organic matter pool as root and microbial litter.

5. Conclusions

Our study has several implications for understanding the impact of N deposition on soil C stocks. Firstly, our study clearly shows that N deposition can drive large shifts in the soil fungal community and their

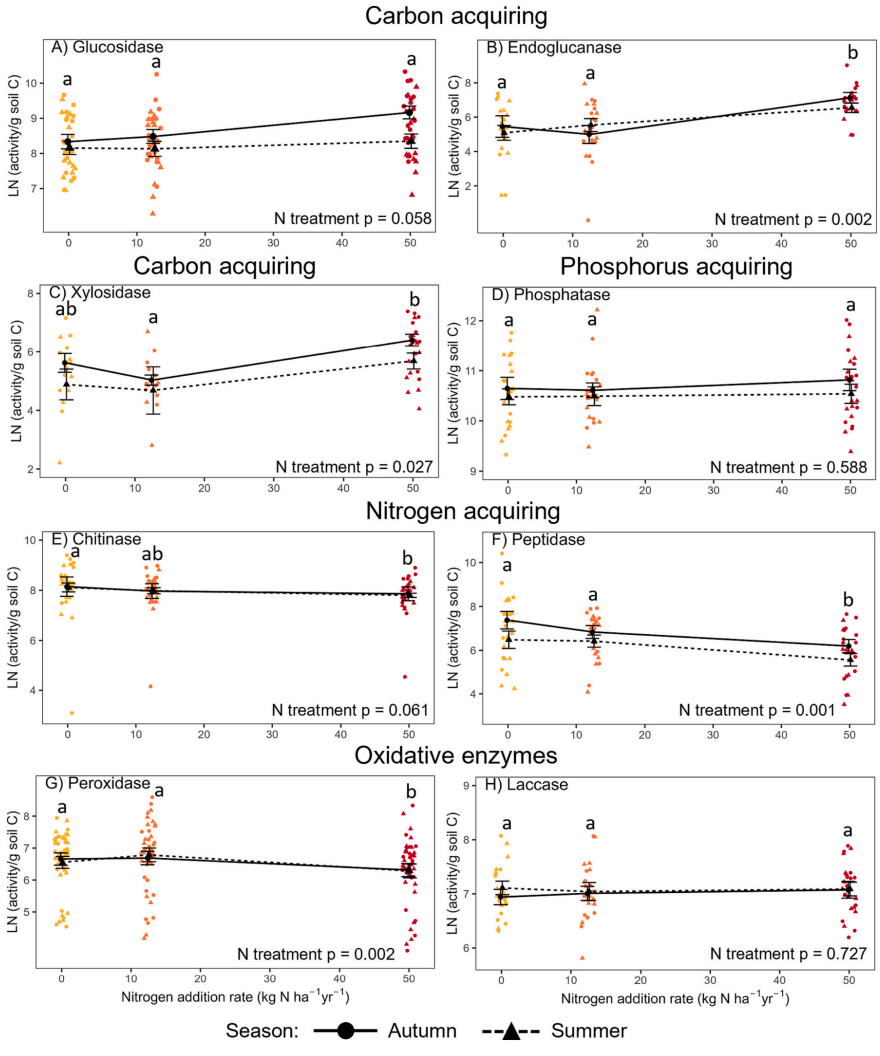

Fig. 4. Interaction plot of potential enzyme activity across three levels of nitrogen addition (0, 12.5, and 50 kg N ha^{-1} yr^{-1} × 20 years) at summer (triangle shape) and autumn (circle shape). Values are the natural logarithm of molar cleavage of enzyme specific substrates standardized to the amount of carbon in each sample (molar substrate cleavage h^{-1} g C^{-1}). Black dots represent the mean and the error bars represents standard errors of the mean (n = 12), while the N treatment p-values are values from linear mixed effects models (Table 3) and different letters represent statistical difference based on Tukey's HSD.

associated enzyme activities. In our 20 year-long experimental simulation of N deposition, these effects were most clearly pronounced for the high N addition treatment. For the low N addition treatment, corresponding with upper N deposition rates in the boreal region, no effects were detected, suggesting that very high rates of N deposition are required to drive significant changes in soil functioning. Secondly, the response of the microbial community was predictable, as the turnover of the fungal community was associated with a shift from EMF taxa capable of growing on pure organic N sources to taxa without this capability in N enriched treatments. This shift was associated with down-regulation of enzymes involved in degradation of complex organic structures and the uptake of peptides, and up-regulation of enzymes involved in uptake of simple carbohydrates. Thirdly, our results are consistent with theoretical modeling frameworks focusing on the energy demand of organic N uptake (Chen et al., 2014; Craine et al., 2007; Orwin et al., 2011), and on the role of element stoichiometry in regulating the loss of C through respiration (Chen et al., 2014; Liang et al., 2017; Luo et al., 2016). Understanding the mechanisms and functional aspects of anthropogenic N deposition effects on the changes in soil C stocks, which can have a strong effect on the global C cycle (Lal, 2005), is extremely important for a better understanding the effect of N supply on the global C cycle, especially considering a changing climate (Gruber and Galloway, 2008).

Table 3
The effect of nitrogen treatment, season, and their interaction on the natural logarithm of the potential activity (molar substrate cleavage h^{-1} g C^{-1}) of soil enzymes evaluated by linear mixed models with block (n = 12) defined as random factor.

Enzyme	Nitrogen treatment			Season			Nitrogen × season	
	Direction	F-value	p-Value	Sign	F-value	p-Value	F-value	p-Value
Glucosidase		3.000	0.058	+	11.026	**0.002**	1.632	0.205
Endoglucanase	+	7.124	**0.002**		0.251	0.619	1.405	0.256
Xylosidase	+	4.096	**0.027**		3.538	0.070	0.197	0.822
Phosphatase		0.536	0.588		2.875	0.096	0.177	0.838
Chitinase		2.951	0.061		1.965	0.167	0.003	0.997
Peptidase	−	17.966	**0.001**	+	18.859	**0.001**	0.659	0.521
Peroxidase	−	6.945	**0.002**		0.012	0.912	0.371	0.692
Laccase		0.321	0.727		1.196	0.279	0.533	0.590

Significant effects ($p<0.05$) are highlighted in bold font.

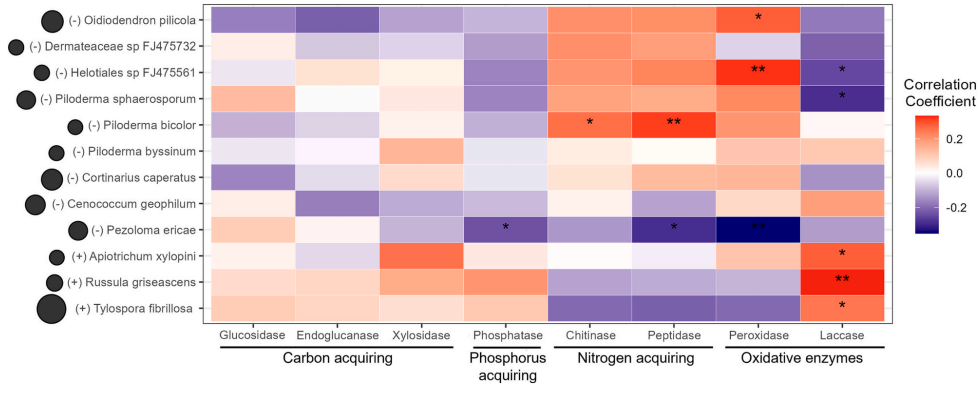

Fig. 5. Heatmap portraying Spearman's correlation coefficient between enzyme activity for carbon, phosphorus and nitrogen acquiring enzymes and oxidative enzymes and fungal species identified by SIMPER analysis to contribute to 30 % cumulative contribution. The circles by fungal species represent the individual species cumulative contribution to differences between 0 and 50 N samples according to SIMPER analysis and their sign indicates if their abundance decreased (−) or increased (+) with nitrogen addition. The statistically significant p-values are portrayed by stars (* represents $p < 0.05$, and ** $p < 0.01$).

The agreement of the data from our detailed profiling of the microbial community composition and metabolism with these modeling frameworks can therefore enable more precise predictions of the future soil C balance (Averill and Waring, 2017; Luo et al., 2016; Stocker et al., 2016; Terrer et al., 2021).

CRediT authorship contribution statement

Benjamin Forsmark: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft. **Tinkara Bizjak:** Formal analysis, Investigation, Visualization, Writing – original draft. **Annika Nordin:** Conceptualization, Supervision, Writing – review & editing. **Nicholas P. Rosenstock:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Håkan Wallander:** Conceptualization, Writing – review & editing. **Michael J. Gundale:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

AN declares employment with Stora Enso, and BF with the forestry cooperative Södra Skogsägarna. TB, NPR, HW and MJG declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are openly available on the SafeDeposit at the Swedish University of Agriculture server http://www.safedeposit.se/, reference ID: 425.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170741.

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ORIGINAL PAPER

Inoculation with in vitro promising plant growth-promoting bacteria isolated from nitrogen-limited boreal forest did not translate to in vivo growth promotion of agricultural plants

Tinkara Bizjak-Johansson[1] · **Anne Braunroth**[2] · **Regina Gratz**[2] · **Annika Nordin**[1]

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Abstract
Many yet undiscovered plant growth-promoting bacteria are proposed to be harboured in the nitrogen-limited boreal forest. These bacteria are suggested to increase plant growth not only due to their ability to fix nitrogen but also through other growth-promoting properties. Therefore, this study looked at the plant growth promotion potential of endophytic bacteria isolated from boreal forest conifer Scots pine (*Pinus sylvestris*) needles. Seven assays were used to measure the potential plant growth-promoting abilities of two newly isolated bacteria in this study and seven additionally selected bacteria isolated in our previous study. The three best-performing bacteria were used, either individually or in a consortium, to assess growth promotion on four common crop species. The greenhouse study included the presence of native soil and seed microbiota and used naturally nutrient-abundant soil. The results showed that while all bacteria were capable of multiple plant growth-promoting properties in the in vitro assays, they did not promote plant growth in the in vivo experiment as inoculated plants had similar or decreased chlorophyll content, root and shoot length and dry biomass compared to control plants. Our results show that bacterial plant growth-promoting potential does not always translate into successful plant growth increase in in vivo conditions and highlight the need for a better understanding of plant-bacteria interaction for the future establishment of successful bacterial bioinoculants.

Keywords Bioinoculants · Diazotrophic bacteria · Endophytic bacteria · Inoculation experiment · Plant growth-promoting bacteria · Scots pine

Introduction

The use of fertilizers in agriculture has been extensive in recent decades due to the growing need for food across the globe (Fowler et al. 2013). The main aim of applying fertilizers is the addition of nitrogen (in the form of nitrate and/or ammonium), which is often the main plant growth-limiting nutrient (Galloway et al. 2013). Additionally, fertilizers often include other elements that are lacking in the crop cultivation system, for example, phosphorus and potassium (Savci 2012). Fertilizer application promotes plant growth, but it has become increasingly clearer that the use of inorganic nitrogen fertilizers can have negative consequences on the environment. Namely, inorganic nitrogen leaching can lead to water pollution and nitrogen fertilization can cause increased greenhouse gas emissions, acid rain and biodiversity loss (Bhattacharjee et al. 2008; Martinez-Espinosa et al. 2011; Khan et al. 2012; Savci 2012; Galloway et al. 2013; de Souza et al. 2015). Additionally, the planetary boundary of anthropogenically introduced nitrogen in agriculture has been globally crossed, leading to serious effects on Earth and its ecosystems (Richardson et al. 2023). A more ecologically friendly alternative could be the use of plant growth-promoting bacteria (PGPB) as bioinoculants, which could help elevate nutrient limitation in addition to providing other benefits to the plant (Compant et al. 2010; Berg et al. 2021). The PGPB could be applied directly to the fields, or they could be applied together with a reduced amount of fertilizer to achieve the maximum benefits of better growth combined

✉ Tinkara Bizjak-Johansson
tinkara.bizjak-johansson@slu.se

[1] Umeå Plant Science Centre (UPSC), Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Umeå, Sweden

[2] Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, Umeå, Sweden

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with lower negative environmental effects (de Souza et al. 2012, 2015).

The PGPB are bacteria that interact beneficially with a plant and can be free-living, rhizospheric, endophytic or in a symbiotic relationship with the plant (Glick 2012). It has been suggested that endophytic PGPB, meaning PGPB living inside plant tissues without causing harm (Ryan et al. 2008), can under certain conditions be more efficient in plant growth promotion as a result of better environmental stability, more efficient communication and closer interaction with the plant (Santoyo et al. 2016; Etesami and Maheshwari 2018; Orozco-Mosqueda et al. 2022; Méndez-Bravo et al. 2023). The PGPB can be beneficial to the plant through improved nutrient acquisition, production of plant hormones, increased resistance against biotic and abiotic stresses and consequently increased plant yield (Compant et al. 2010; Olanrewaju et al. 2017; Etesami and Maheshwari 2018). The concept of the multiple mechanism hypothesis suggests that each PGPB can possess more than one plant growth-promoting property, leading to increased plant yield through various mechanisms (Cassán et al. 2020). In addition, it has been hypothesized that a consortium of bacteria might provide a more efficient plant yield increase than individual strains due to the combined array of different plant growth-promoting properties (Knoth et al. 2014; Ray et al. 2020; Chaiya et al. 2021; Saleem et al. 2021; Khan et al. 2022; Méndez-Bravo et al. 2023). However, the success of PGPB inoculation can depend on several factors, among other bacterial strain properties, diverse environmental factors and the presence of native microbiota (Foolad et al. 2000; Glick 2012; Berg et al. 2021). In fact, a decreased efficiency of PGPB has been seen in natural environments such as fields compared to more artificially set up laboratory studies (Compant et al. 2005, 2010; Gamalero and Glick 2011; Gaiero et al. 2013).

While the research on PGPB in agriculture is quite extensive, the knowledge about PGPB present in the forests and their potential application is profoundly lacking (Lucy et al. 2004; Padda et al. 2021). These environments, especially nitrogen-limited boreal forests, probably harbour many non-investigated bacteria with high plant growth-promoting potential (Ryan et al. 2008; Afzal et al. 2019), which could successfully be used as PGPB in either agriculture or forestry. As these non-investigated bacteria are growing in severely nitrogen-limited environments, it is suggested they could contribute significantly to plant growth through their ability to fix atmospheric dinitrogen into ammonia (Puri et al. 2015, 2020b). In fact, several endophytic bacteria isolated from different conifer species were shown to be nitrogen-fixing (Padda et al. 2018; Puri et al. 2018; Bizjak et al. 2023). Furthermore, some of these bacterial strains were shown to promote the growth of the host seedlings and even act non-specifically as they were able to promote the growth of non-host agricultural plants. For example, bacteria successfully increased the growth of conifer seedlings such as lodgepole pine and hybrid white spruce (Puri et al. 2018, 2020c; Song et al. 2020; Chen et al. 2021; Padda et al. 2021) and non-host agricultural plants such as sunflower, canola, corn and tomato (Padda et al. 2015; Puri et al. 2015, 2020a; Younas et al. 2023). The plant and tree seedling growth promotion was proposed to be mainly due to nitrogen fixation, which supplied a significant part of the plant or tree seedling nitrogen (Puri et al. 2015, 2020b). However, most inoculation studies with bacteria isolated from conifers focused on nitrogen fixation and only a few examined if the bacteria had any additional plant growth-promoting properties. More importantly, the majority of the experiments were performed against current recommendations under artificial conditions using surface-sterilized seeds and sand mixture growth mediums lacking necessary nutrients and the native microbiota (Bhattacharjee et al. 2008; Etesami and Maheshwari 2018; de-Bashan and Nannipieri 2024). While results from these studies are crucial for a better understanding of PGPB isolated from conifer species, significantly more knowledge is needed about the performance of these PGPB under more natural settings to evaluate their potential application as bioinoculants in agriculture or forestry.

Our study aimed to shed more light on the knowledge gap about PGPB isolated from boreal forest conifers, their plant growth-promoting properties and agricultural plant growth promotion in a greenhouse setting. Therefore, we isolated endophytic nitrogen-fixing bacteria from Scots pine trees (*Pinus sylvestris*) growing in the nitrogen-limited boreal forest in northern Europe as bacteria isolated from these environments are proposed to be excellent PGPB candidates even in agriculture due to their ability to fix nitrogen. The isolated bacteria were assessed for their plant growth-promoting potential in seven in vitro assays and tested for their application potential in in vivo inoculation experiment using four key agricultural species representing different crop families (i.e. corn (*Zea mays*), tomato (*Solanum lycopersicum*), kale (*Brassica oleracea*) and cucumber (*Cucumbis sativa*)). Uniquely, the in vivo greenhouse experiment included non-sterile seeds and naturally nutrient-abundant soil both with their native microbiota present. The addressed hypotheses were: (a) isolated and selected bacteria possess an array of plant growth-promoting properties in addition to nitrogen fixation, (b) the bacteria will be able to promote the growth of agriculturally important plants from four different crop families in a non-sterile soil pot experiment and (c) a consortium of bacteria will perform better than the individual bacterial strains.

Material and methods

Scots pine endophytic nitrogen-fixing bacteria

Scots pine endophytic bacteria were isolated from needles of trees growing at the Åheden research forest (64°13′45.3"N 19°48′00.4"E) close to Vindeln, northern Sweden. The needles were collected from five different trees under aseptic conditions, stored on ice and transported to the laboratory. To isolate endophytic bacteria, the needles were surface sterilized by submersion in 70% ethanol for 3 min, washed with sterile water three times for 20 s and the excess water was dried off by placing them on sterile Whatman filter paper. To check the sterility, the needles were imprinted on Tryptic soy agar (TSA; 15 g l^{-1} casein peptone, 5 g l^{-1} soy peptone, 5 g l^{-1} NaCl, 15 g l^{-1} agar). After sterilization, 800 µl of phosphate-buffered saline (PBS; 8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4, 0.245 g l^{-1} KH_2PO_4) was added and the needles were ground using FastPrep-24™ Instrument (MP medicals inc., USA) before being filtered through sterile Miracloth (Merck Millipore, USA). The samples were centrifuged for 10 min at 2 650 g at 8 °C and the pellet was resuspended in PBS and plated on nitrogen-free combined carbon medium (CCM) without yeast extract (Baldani et al. 2014). The plates were incubated at 28 °C for 11 days before distinct colonies were collected.

The isolated bacteria were identified by 16S rRNA gene Sanger sequencing. DNA was isolated from concentrated Luria broth (LB; 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) cultures using DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's instructions. 16S rRNA was amplified using DreamTaq Hot Start PCR Master Mix (Thermo Fisher Scientific, USA) according to the manufacturer's manual with the universally used primer pair F27: 5'-AGAGTTTGATCCTGGCTCAG-3' and R1492: 5'-ACGGCTACCTTGTTACGACTT-3' (Heuer et al. 1997). The PCR product was purified using ExoSAP-IT (Thermo Fisher Scientific, USA) and the DNA was sequenced using TubSeq Service (Eurofins, Luxemburg). The resulting forward and reverse sequences of each isolate were merged into consensus sequences using European Molecular Biology Open Software Suite (EMBOSS) cons (Rice et al. 2000) and bacterial identity was determined using Nucleotide Blast (Altschul et al. 1990).

The nitrogen fixation ability of the isolated bacteria was measured using acetylene-reduction assay. Bacteria were grown in liquid CCM media before being transferred to glass vials sealed with Suba-seal septa (Sigma Aldrich, USA). Then, acetylene replaced 10% of the air, and the samples were incubated for 2 h at 28 °C. Ethylene production (indicating nitrogen fixation) was measured on a gas chromatograph (Shimadzu GC-8A, Japan). After ethylene measurements, the OD_{600} of the cultures was measured and used to normalise the ethylene production values. Ethylene production was measured on three replicates per isolated bacterial strain.

During this study, we isolated two different bacterial strains. Therefore, to broaden the selection of bacteria and consortium formulation, we used seven additionally selected nitrogen-fixing bacteria from our endophytic Scots pine bacterial collection. The selected strains (Table 1) were

Table 1 The species, bacterial strain name and the nitrogen-fixation potential of the two nitrogen-fixing endophytic bacteria isolated in this study and of the seven previously isolated bacterial strains (Bizjak et al. 2023) selected for the study of plant growth promotion. The reported acetylene-reduction activity (mean ± standard error) was measured in triplicates per bacterial strain (n = 3) for the two bacterial strains isolated in this study, while the acetylene-reduction activity of the previously isolated bacteria has been previously reported (Bizjak et al. 2023)

	Species	Bacterial strain	Acetylene-reduction assay activity (nmol C_2H_4 h^{-1} $OD_{600}{}^{-1}$)
Isolated bacteria	*Robbsia andropogonis*	#1A	0.007 ± 0.004
	Bacillus sp.	#2A	0.008 ± 0.003
Additionally selected bacteria[a]	*Bacillus paralicheniformis*	#1	0.038 ± 0.014
	Unclassified *Novosphingobium*	#23	0.020 ± 0.012
	Microbacterium sp.	#25	0.100 ± 0.039
	Sphingomonas sp.	#27	0.058 ± 0.020
	Novosphingobium pokkalii	#38–1	/
	Variovorax paradoxus	#38–2	0.025 ± 0.025
	Priestia megaterium	#39	0.009 ± 0.006

[a]Additionally selected bacteria were isolated, identified and their acetylene-reduction activity was measured in our previous study, please see Bizjak et al. (2023)

previously isolated from Åheden research forest, Vindeln, northern Sweden. They were identified using 16S rRNA gene Sanger sequencing and their nitrogen fixation ability was confirmed using acetylene-reduction assay as previously reported by us (Bizjak et al. 2023).

In vitro plant growth-promoting properties

Phosphorus solubilization of the isolated bacteria was measured using a liquid medium assay. For the assay, a modified Pikovskaya medium (Jasim et al. 2013) without agar was used. As the only source of phosphorus, we have used either tricalcium phosphate, iron (III) phosphate or aluminium phosphate. The method was modified after Fiske and Subbarow (1925). Namely, bacterial cultures grown overnight in LB medium were concentrated by centrifugation at 20 000 g for 10 min and washed with sterile saline solution (9 g l^{-1} NaCl). Liquid Pikovskaya medium was inoculated with 10 µl of bacterial suspension and incubated for 72 h at 28 °C. Following, the supernatant was harvested by centrifugation at 8 000 g for 10 min. 250 µl of the supernatant was mixed with 125 µl of 10% trichloroacetic acid and 1 ml colour reagent (1:1:1:2 ratio of 3 M H_2SO_4, 2.5% ammonium molybdate, 10% ascorbic acid, distilled water) and incubated at room temperature for 15 min. The developed blue colour was measured using absorbance at 820 nm on a spectrophotometer (Epoch, BioTek Instruments, USA) indicating phosphorus solubilization, which was calculated for each isolate based on the standard curve of KH_2PO_4 concentrations. Three replicates were used for each bacterial strain.

Zinc solubilization was tested on TSA plates with added 0.1% zinc. Specifically, TSA with added 1.24 g l^{-1} zinc oxide was spot inoculated with 20 µl of overnight LB bacterial culture. The plates were incubated for 5 days at 28 °C and a clear halo around the bacterial colony indicated zinc solubilization by the bacteria. The assay included three replicates for each bacterial strain.

The ability of siderophore production was assessed using chrome azurol S (CAS) agar medium (Louden et al. 2011). The CAS agar medium plates were inoculated with bacterial isolates and incubated for 7 days at 30 °C. The appearance of orange colour indicated siderophore production by bacteria and the siderophore production index was calculated using the following formula: (colony diameter + halo zone diameter)/colony diameter. The siderophore production was measured on three replicates per bacterial strain.

For the HCN production assay (Lorck 1948), TSA plates with added 4.4 g l^{-1} glycine were spot inoculated with 25 µl of overnight LB bacterial culture. Sterile Whatman filter paper was dipped in picric acid solution (0.5% picric acid in 2% Na_2CO_3) and placed between the base and the lid of the plate, while parafilm was used to seal the plate. The plates were incubated in an inverted position at 28 °C for 7 days and the change in the colour of the filter paper from yellow to brown indicated HCN production. The HCN production was measured for three replicates per bacterial strain.

IAA production was measured using Salkowski solution (de Jesus Santos et al. 2014; Puri et al. 2020a). First, LB medium with added 1 mg l^{-1} L-tryptophan was inoculated with 10 µl of overnight LB bacterial culture. The plates were incubated at 28 °C for 3 days with constant shaking. OD_{600} was measured for all cultures before they were centrifuged at 10 500 g for 10 min and the supernatant was collected. 500 µl of the Salkowski solution (1:30:50 ratio of 0.5 M $FeCl_3$, 95% sulfuric acid, distilled water) was added to 250 µl of the supernatant, vortexed and incubated in the dark for 30 min. The concentration of the IAA was measured using absorbance at 530 nm on a spectrophotometer (Epoch, BioTek Instruments, USA) with an IAA calibration curve. The amount of IAA produced was normalised using the OD_{600} measurements and was measured in triplicates per bacterial strain. However, the results have to be interpreted with caution as Salkowski solution indicates the presence of all indole-like molecules and not only IAA (Glickmann and Dessaux 1995; de-Bashan and Nannipieri 2024; Guardado-Fierros et al. 2024).

Protease activity was tested using a liquid medium method (Chaiharn and Lumyong 2008), where skim-milk liquid medium (5 g l^{-1} tryptone, 2.5 g l^{-1} yeast extract, 1 g l^{-1} glucose, 7% 100 ml l^{-1} skim milk solution l^{-1}) was inoculated with 10 µl of overnight LB bacterial culture. The samples were incubated for 3 days at 28 °C with constant shaking followed by OD_{600} measurement. They were centrifuged at 8 000 g for 15 min and 100 µl of the supernatant was mixed with 100 µl 0.2 M phosphate buffer (pH 7.0) and 100 µl of 1% azocasein. The samples were incubated at 37 °C for 30 min when 400 µl of 10% trichloroacetic acid was added and everything was incubated for 5 min at room temperature. 100 µl of the sample was mixed with 200 µl of 1 M NaOH and absorbance was measured at 440 nm using a spectrophotometer (Epoch, BioTek Instruments, USA). The measurements were normalised using OD_{600} and compared against a tyrosine standard curve. One unit of enzyme catalytic activity was defined as the amount of the enzyme resulting in the release of 1 µmol of tyrosine per minute. The assay included three replicates of each bacterial strain.

For cellulase activity assay (Miller 1959; Chaiharn and Lumyong 2008; Hajiabadi et al. 2020; Puri et al. 2020a), 10 µl of overnight LB bacterial culture were inoculated in LB medium with added 1% carboxymethyl cellulose (CMC). The cultures were incubated at 28 °C for 3 days with constant shaking. The OD_{600} of the cultures was measured before centrifugation at 8 000 g at 4 °C for 15 min. 200 µl of the supernatant was mixed with 200 µl of 0.05 M citrate buffer (pH 5.0) and 200 µl 1% CMC solution. The samples were incubated for 30 min at 37 °C, then 800 µl of

3,5-dinitrosalicylic acid (DNSA) reagent (96 mM dinitro salicylic acid, 1.3 M sodium potassium tartrate in 0.5 M NaOH) was added and the samples were boiled at 100 °C for 5 min. Their absorbance at 560 nm was measured using a spectrophotometer (Epoch, BioTek Instruments, USA). The glucose content in the samples was calculated using a glucose standard curve and normalised using OD_{600} measurements. Cellulase activity was measured by the release of glucose from CMC and one unit of cellulase catalytic activity was defined as the amount of cellulase needed to release 1 µmol of glucose from CMC per minute. The cellulase activity was measured using three replicates per bacterial strain.

The bacterial compatibility was tested using a crowded plate assay (Bhatia et al. 2018) modified after Ibrahim et al. (2022) and Haque et al. (2021), where 10 µl of $OD_{600} = 1$ of the bacterial strains was spotted on LB agar plates with either spaced spots or overlapping spots. Plates were incubated at 28 °C for either one or seven days before compatibility was assessed.

In vivo inoculation experiment

To test if endophytic nitrogen-fixing Scots pine bacteria can promote the growth of agriculturally important crops, we selected cucumber (*Cucumbis sativa* Vorgebirgstrauben), corn (*Zea mays* Sweet Nugget F1), tomato (*Solanum lycopersicum* Moneymaker) and kale (*Brassica oleracea* Dwarf Green Curled) and bacterial strains *Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39. The plants were grown in soil (K-jord, NPK 14–7–15, pH 5.5–6.5, a mixture of light peat, sand, clay, lime and mineral fertilizer, Hasselfors garden) under 16-h daylight and 8-h night-time regime. Plants were inoculated with either one of the individual bacterial strains, a consortium of the three bacterial strains or with sterile LB medium as a negative control. To try to ensure reproducibility, the greenhouse experiment was based on a pre-study, each of the inoculation treatments had seven biological replicates and the inoculation effects were evaluated across the four crop species from four different plant taxonomic orders. The plants were inoculated with 2 ml of LB overnight culture with $OD_{600} = 1$ (corresponding to $2.2*10^6$ CFU/ml, $1.4*10^7$ CFU/ml and $2.4*10^5$ CFU/ml for bacteria #2A, #25 and #39, respectively) at the time of sowing, one week after sowing and two weeks after sowing by applying the liquid bacterial cultures directly to the soil near the seed or later seedling. During the ongoing experiment germination rates were recorded. The plants were harvested after approximately five weeks in the greenhouse. On the day of the harvest, the chlorophyll content of the leaves was measured using the CCM-300 chlorophyll content meter (Opti-Sciences, USA) and root and shoot lengths were measured. Plants were dried in the oven at 70 °C for at least 48 h and then their dry root and shoot weights were measured.

Statistics

SPPS Statistics 29 (IBM, USA) was used to analyse all data. The plant growth-promoting properties were analysed using a one-way analysis of variance (ANOVA) and Tukey's honestly significant difference test with bacterial strain as a variable. For the in vivo inoculation experiment, all data was analysed for each measured trait using a two-way ANOVA followed by Tukey's honestly significant difference test with inoculation treatment and crop species as variables.

Results

Scots pine endophytic nitrogen-fixing bacteria

We used a combination of two endophytic bacteria isolated in our study and seven additional endophytic bacteria (Table 1) isolated in our previous study (Bizjak et al. 2023) for the assessment of the in vitro plant growth-promoting potential of Scots pine needle endophytic nitrogen-fixing bacteria. The two isolated endophytic bacteria were identified based on 16S rRNA as gram-negative, plant-pathogenic *Robbsia andropogonis* (NCCB accession number 100967, GenBank number OR506164) and gram-positive *Bacillus* sp. (NCCB accession number 100968, GenBank number OR506163). Acetylene-reduction assay was used to confirm that the two isolated bacteria were capable of nitrogen fixation (Table 1), while nitrogen fixation ability has previously been confirmed and reported in Bizjak et al. (2023) for all the additionally selected bacteria, except for *Novosphingobium pokkalii* which did not show nitrogen fixation under the selected assay conditions.

In vitro plant growth-promoting properties

Different plate or liquid medium assays were used on the isolated and selected bacteria to test which plant growth-promoting properties the bacteria possess in addition to nitrogen fixation. One of the assays was used to test if the bacteria could solubilize various phosphorus forms and the results showed that while all bacteria were able to solubilize tricalcium phosphate (Fig. 1A, Table 2), they were not capable of solubilizing either iron (III) phosphate or aluminium phosphate (Table 2). There was a statistical difference in tricalcium phosphate solubilization between different bacterial strains (p-value < 0.001) and the three best tricalcium phosphate solubilizers were *Priestia megaterium* #39, *Bacillus* sp. #2A and *Bacillus paralicheniformis* #1. We also assessed if the bacteria were able to solubilize zinc and while all bacteria, but *Robbsia andropogonis* #1A and *Bacillus* sp. #2A were able to grow on the media with the added insoluble zinc, none of them were able to solubilize

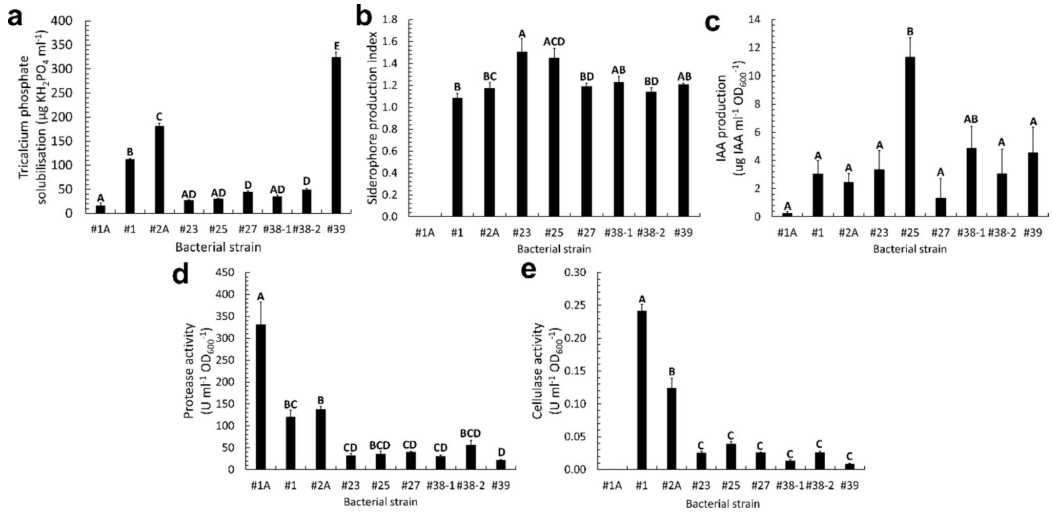

Fig. 1 Bacterial strain performance in liquid or plate plant growth-promoting assays for **a**) tricalcium phosphate solubilization, **b**) siderophore production, **c**) IAA production, **d**) protease activity and **e**) cellulase activity. The graphs show mean ± standard error (n = 3) and the different letters indicate a statistically significant difference between samples based on one-way ANOVA followed by Tukey HSD test

it (Table 2). Additionally, other than *Robbsia andropogonis* #1A, all bacteria were able to produce siderophores (Fig. 1B, Table 2). The amount of siderophores produced was significantly different between the bacterial strains (p-value = 0.002) with unclassified *Novosphingobium* #23 having the highest siderophore production. The bacteria were tested for HCN production, however, none of the bacteria had this ability (Table 2). All bacterial strains showed IAA production in varying amounts (Fig. 1C, Table 2), which was statistically significant (p-value < 0.001). The highest IAA production was measured for *Microbacterium* sp. #25, followed by *Novosphingobium pokkalii* #38–1 (Fig. 1C, Table 2). Protease activity assay showed statistically significant (p-value < 0.001) diverse protease activity for the bacteria (Fig. 1D, Table 2). The highest activity was measured for *Robbsia andropogonis* #1A and the lowest for *Priestia megaterium* #39 (Fig. 1D, Table 2). Additionally, measured cellulase activity was significantly different between the bacterial strains (p-value < 0.001). *Bacillus paralicheniformis* #1 showed the highest cellulase activity followed by *Bacillus* sp. #2A, while *Robbsia andropogonis* #1A was the only bacterial strain that did not show any cellulase activity (Fig. 1E, Table 2).

Bacterial strains were evaluated for their overall performance in the seven plant growth-promoting assays performed during this study (Table 2). Based on the evaluation, the most promising bacterial strains were *Bacillus paralicheniformis* #1, *Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39. However, due to much slower growth in liquid media for *Bacillus paralicheniformis* #1, only bacteria *Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39 were selected for the in vivo inoculation experiment. The three selected bacteria were assessed for compatibility and did not show any antagonism (data not shown).

In vivo inoculation experiment

To test if isolated endophytic nitrogen-fixing Scots pine bacterial strains could promote the growth of agricultural crop species we used kale, corn, tomato, and cucumber plants. For each of the crops, we measured germination rate, chlorophyll content, root and shoot length and dry root and shoot weight. Using two-way ANOVAs, the effects of crop species, inoculation treatment and their interaction effects were analysed for each measured plant variable. For all variables, there was a significant effect of crop species, which was expected (Tables S1-S6). The results for germination rate showed no effect of the inoculation treatment (p-value = 0.072), but they showed an effect of the interaction between the crop species and the inoculation treatment (p-value = 0.035) (Table S1, Fig. 2). Similar were the results for chlorophyll content with no effect of the bacterial inoculation treatment (p-value = 0.267) and a significant crop species and inoculation treatment interaction (p-value = 0.019) (Table S2, Fig. 3). For root length,

Table 2 Evaluation of bacterial strain performance in seven diverse plant growth-promoting assays: phosphorus solubilization (for three phosphorus forms: tricalcium phosphate, iron (III) phosphate and aluminium phosphate), zinc solubilization, siderophore production, HCN production, IAA production, protease activity and cellulase activity. The total score of the bacterial isolates is based on their overall performance and is the sum of plus signs across all seven in vitro assays. All in vitro assays were measured on three replicates per bacterial strain (n = 3)

Bacterial strain	Species	Tricalcium phosphate solubilization[a]	Iron (III) phosphate solubilization	Aluminium phosphate solubilization	Zinc solubilization	Siderophore production[b]	HCN production	IAA production[c]	Protease activity[d]	Cellulase activity[e]	Total score
#1A	*Robbsia andropogonis*	+	-	-	-	-	-	+	+ + +	-	5
#1	*Bacillus paralicheniformis*	+ +	-	-	-	+	-	+	+ +	+ + +	9
#2A	*Bacillus* sp.	+ +	-	-	-	+	-	+	+ +	+ + +	**9**
#23	Unclassified *Novosphingobium*	+	-	-	-	+ + +	-	+	+	+ +	8
#25	*Microbacterium* sp.	+	-	-	-	+ + +	-	+ + +	+	+ +	**10**
#27	*Sphingomonas* sp.	+	-	-	-	+	-	+	+	+ +	6
#38–1	*Novosphingobium pokkalii*	+	-	-	-	+ +	-	+ +	+	+	7
#38–2	*Variovorax paradoxus*	+	-	-	-	+	-	+	+ +	+ +	7
#39	*Priestia megaterium*	+ + +	-	-	-	+ +	-	+ +	+	+	**9**

[a] + if tricalcium phosphate solubilization below 100 ug ml^{-1} KH_2PO_4, + + if between 100 and 300 ug ml^{-1} KH_2PO_4, + + + if above 300 ug ml^{-1} KH_2PO_4

[b] + if siderophore production index below 1.2, + + if between 1.2 and 1.4, + + + if above 1.4

[c] + if IAA production below 4 ug ml^{-1} $OD_{600}{}^{-1}$ IAA, + + if between 4 and 10 ug ml^{-1} $OD_{600}{}^{-1}$ IAA, + + + if above 10 ug ml^{-1} $OD_{600}{}^{-1}$ IAA

[d] + if protease activity below 50 U ml^{-1} $OD_{600}{}^{-1}$, + + if between 50 and 200 U ml^{-1} $OD_{600}{}^{-1}$, + + + if above 200 U ml^{-1} $OD_{600}{}^{-1}$

[e] + if protease activity below 0.02 U ml^{-1} $OD_{600}{}^{-1}$, + + if between 0.02 and 0.1 U ml^{-1} $OD_{600}{}^{-1}$, + + + if above 0.1 U ml^{-1} $OD_{600}{}^{-1}$

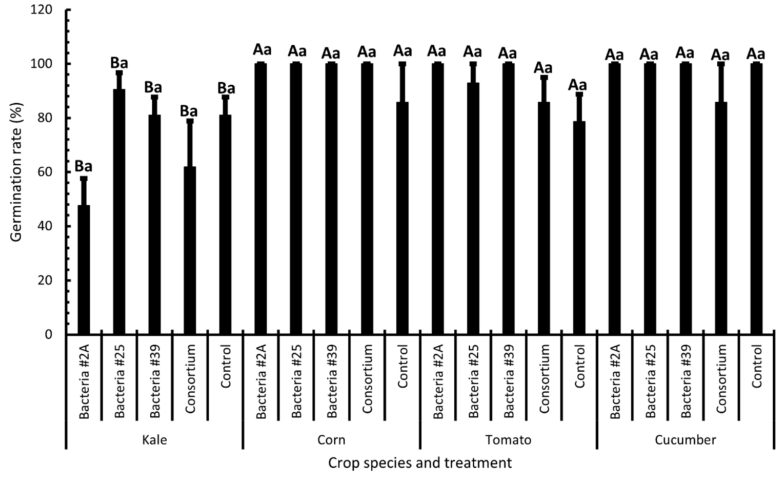

Fig. 2 Measured germination rate for kale, corn, tomato and cucumber plants and five inoculation treatments (*Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium and control). The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference between crop species and the different lowercase letters indicate a difference between inoculation treatments based on two-way ANOVA followed by Tukey HSD test

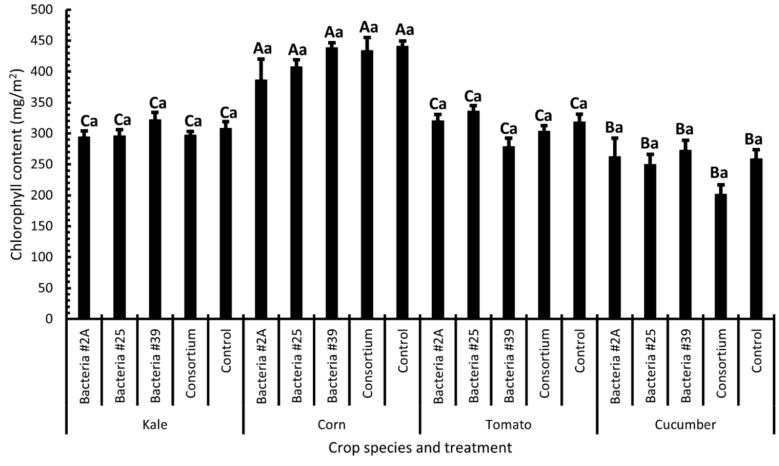

Fig. 3 Leaf chlorophyll content of four crop species (kale, corn, tomato and cucumber) inoculated with either *Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium or control. The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference between crop species and the different lowercase letters indicate a difference between inoculation treatments based on two-way ANOVA followed by Tukey HSD test

there was a nearly significant effect of the bacterial inoculation treatment (p-value = 0.056), where plants inoculated with *Bacillus* sp. #2A tended to have lower root lengths compared to control plants (Table S3, Fig. 4). However, there was no significant interaction between crop species and inoculation treatment (p-value = 0.217) (Table S3, Fig. 4). Both the inoculation treatment (p-value = 0.012) and the interaction between crop species and inoculation treatment (p-value = 0.004) were significant for plant shoot length (Table S4, Fig. 5). Comparable to root length, plants inoculated with *Bacillus* sp. #2A showed in general lower shoot length compared to control plants (Fig. 5). Furthermore, there was a significant effect of inoculation treatment (p-value < 0.001) and interaction between crop species and inoculation treatment (p-value < 0.001) on dry root weight, where consortium and *Bacillus* sp. #2A plants had lower dry root weights compared to control plants (Table S5, Fig. 6). For dry shoot weight there was no effect of the inoculation treatment (p-value = 0.078), but an effect of the inoculation treatment and crop species interaction (p-value = 0.021) (Table S6, Fig. 7).

Discussion

Nitrogen-limited boreal forests in northern Europe could harbour yet undiscovered and untested PGPB with the potential to act as bioinoculants in either forestry or agriculture.

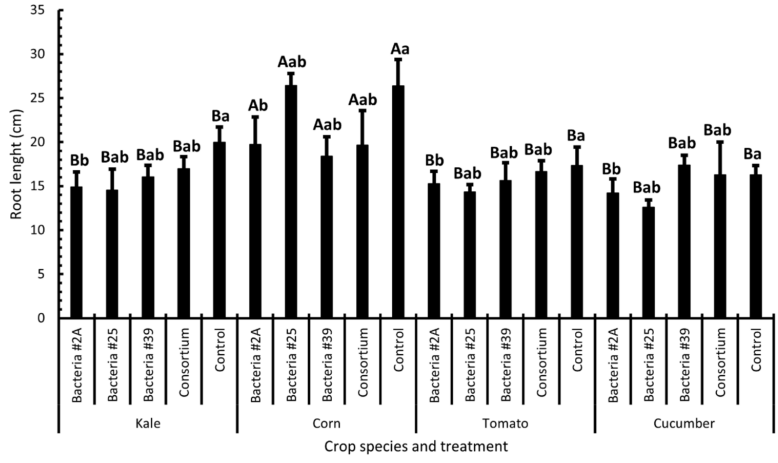

Fig. 4 Measured kale, corn, tomato and cucumber root length of plants inoculated with either *Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium or control. The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference between crop species and the different lowercase letters indicate a difference between inoculation treatments based on two-way ANOVA followed by Tukey HSD test

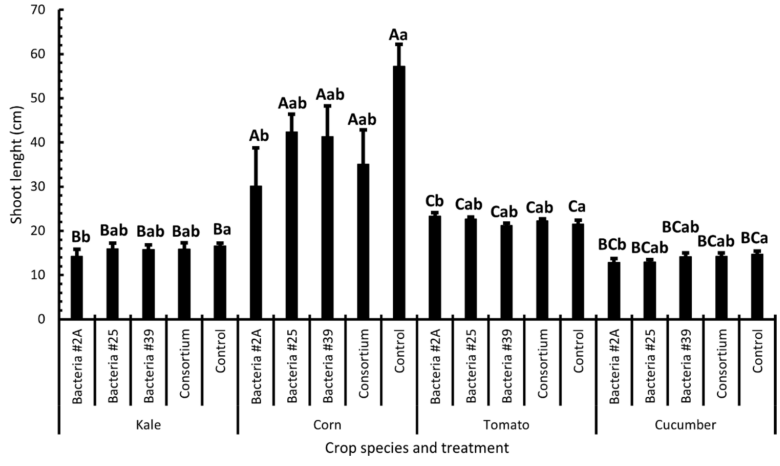

Fig. 5 Shoot length of four crop species (kale, corn, tomato and cucumber) treated with five different inoculation treatments (*Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium and control). The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference between crop species and the different lowercase letters indicate a difference between inoculation treatments based on two-way ANOVA followed by Tukey HSD test

The bacteria isolated from severely nitrogen-limited environments such as boreal forests are proposed to be great candidates for PGPB as many possess nitrogen fixation ability, which can provide significant amounts of nitrogen in inoculation treatments, hence promoting plant growth (Puri et al. 2015, 2020b). In this study, we first evaluated in vitro plant growth-promoting properties for two newly isolated and seven additionally selected nitrogen-fixing endophytic bacteria from Scots pine needles. Based on the results, we selected the three best-performing bacteria that were used in a greenhouse study, which in contrast to previous studies included seed and soil native microbiota and non-sterile conditions. The bacteria were applied either individually or in a consortium to assess their plant growth-promotion ability in vivo.

According to our first hypothesis and the concept of multiple mechanism hypothesis (Cassán et al. 2020), all bacteria used in the study did possess more than one plant growth-promoting property as most of the bacterial strains showed activity in five out of seven in vitro assays. The presence of multiple plant growth-promoting properties within one bacterial strain has previously been shown for PGPB isolated from various crop and tree species (de Souza et al. 2012; Jasim et al. 2013; Puri et al. 2020a). One of the important plant growth-promoting properties is the solubilization of nutrients that are often limited in the environment such as phosphorus, iron and zinc (Kloepper et al. 1980; Saravanan et al. 2007; Rana et al. 2020; Chen et al. 2021). All our bacterial strains were able to solubilize tricalcium phosphate, however, none of the bacteria were

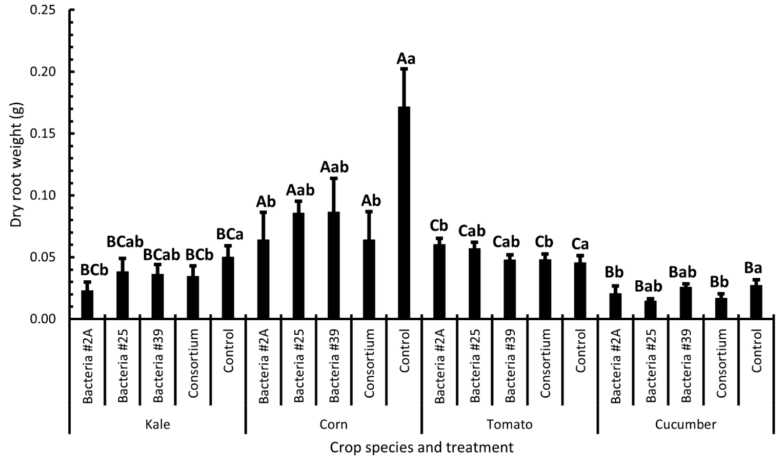

Fig. 6 Kale, corn, tomato and cucumber dry root weight for plants inoculated with five different treatments (*Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium and control). The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference between crop species and the different lowercase letters indicate a difference between inoculation treatments based on two-way ANOVA followed by Tukey HSD test

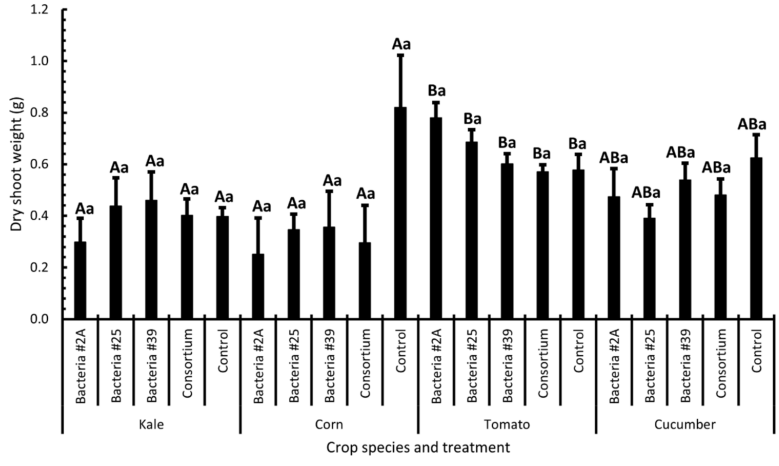

Fig. 7 Dry shoot weights of kale, corn, tomato and cucumber plants inoculated with either *Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium or control. The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference between crop species and the different lowercase letters indicate a difference between inoculation treatments based on two-way ANOVA followed by Tukey HSD test

able to solubilize either iron (III) phosphate or aluminium phosphate. It has been shown previously that the solubilization of iron (III) phosphate and aluminium phosphate is usually lower compared to tricalcium phosphate (Pradhan et al. 2022; Sen et al. 2024). Furthermore, studies showed that several tricalcium phosphate solubilizing bacteria were unable to solubilize iron (III) phosphate or aluminium phosphate and it has been recommended to use more than one phosphorus form to evaluate PGPB phosphorus solubilization (Pérez et al. 2007; Bashan et al. 2012a, b). None of the tested bacteria could solubilize zinc, even though they showed zinc tolerance. The PGPB can often synthesize plant hormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins, which can influence the plant as they play a role in the defence system and development processes (Hardoim et al. 2008; Olanrewaju et al. 2017). All bacteria in our study were capable of plant hormone IAA production, which was expected as previously reported proportions were between 75 and 97% (de Souza et al. 2012; Cueva-Yesquen et al. 2020). However, the IAA production could be overestimated as the method detects the presence of all indole-like molecules including indolepyruvic acid and indoleacetamide (Glickmann and Dessaux 1995; de-Bashan and Nannipieri 2024; Guardado-Fierros et al. 2024). Additionally, PGPB can offer pathogen protection to the plant through various mechanisms. Production of different compounds like hydrogen cyanide (HCN), antibiotics and siderophores can negatively affect the growth of pathogens competing for resources with the PGPB (Jasim et al. 2013; Olanrewaju et al. 2017). None of the tested bacteria could

produce HCN. It seems HCN producing ability is limited within the PGPB community as the previously reported proportion of HCN producing PGPB was between 1 and 3% (de Brito et al. 1995; Antoun et al. 1998). Eight out of nine bacteria used in our study were capable of siderophore production, which is per the literature reported proportions in the range of 75 to 85% (Antoun et al. 1998; Cueva-Yesquen et al. 2020). The PGPB also produce cell-wall degrading enzymes, which help with endophytic colonisation and can additionally lyse the cell walls of plant pathogens (Kandel et al. 2017; Puri et al. 2020a). This ability was seen for all bacterial strains for protease activity and for eight out of nine bacterial strains for cellulase activity.

Based on the bacterial performance in in vitro plant growth-promoting assays and their growth characteristics, three bacteria were further selected for a greenhouse experiment to test their plant growth-promoting properties in vivo. The selected bacteria were *Bacillus* sp. #2A, *Microbacterium* sp. #25 and *Priestia megaterium* #39. Different strains within the *Bacillus* genus have previously been reported as PGPB as they improved among other seed germination, shoot length, root length, plant weight and nutrient uptake (Mumtaz et al. 2017; Prakash and Arora 2019; Tang et al. 2023). Additionally, the *Bacillus* genus is described as one of the most promising plant growth-promoting genera (Song et al. 2021). Likewise, *Priestia megaterium* strains have often been shown to be able to promote the height, plant and fruit weight, mineral content and photosynthetic rates of different plant species (Katsenios et al. 2021; Ramírez-Cariño et al. 2023). Less is known about *Microbacterium* strains, but there are some studies describing their ability to promote plant growth with inoculated plants having larger diameters and increased height, leaf area and both root and shoot biomass compared to control plants (Cordovez et al. 2018; Liu et al. 2022).

The results we obtained in the greenhouse study showed contrasting results to our second hypothesis that the bacterial strains would be able to promote the growth of cucumber, tomato, corn and kale. Instead, we observed neutral effects and even some negative effects of the inoculation treatment on measured plant growth properties. For example, shoot length was lower in plants inoculated with *Bacillus* sp. #2A compared to control plants (Fig. 5). Our results are contrary to previous studies showing that bacteria isolated from conifer tissues promoted the growth of non-host plants such as agriculturally important crops canola, sunflower, tomato and corn (Padda et al. 2015; Puri et al. 2015, 2020a; Younas et al. 2023). However, unlike previous studies, the inoculated bacteria in our study had to compete with the native seed and soil microbiota as non-sterile conditions were used in the greenhouse experiment. Therefore, the neutral and negative results we observed could be due to the ineffectiveness of the inoculum in competing with the native microbiota (Kloepper et al. 1989; Shishido et al. 1999; Germaine et al. 2004). Additionally, the contrasting results could be due to using naturally nutrient-abundant soil as growth media compared to more nutrient-limited sand mixtures previously used. Studies on PGPB isolated from agricultural plants showed that the inoculation treatment has a higher effect when plants are grown in nutrient-limited media compared to more nutrient-rich growth media such as soil (Egamberdiyeva 2007; de Souza et al. 2012). Furthermore, it has been reported that the inoculation studies success can be dependent on the inoculation method, growth media, moisture, temperature and bacterial compatibility with the plant (Kloepper et al. 1989; Germaine et al. 2004; Compant et al. 2010; Kong et al. 2018). Consequently, the possible explanations for the neutral and negative effects of inoculum on plant growth in our study might be related to inefficient bacterial colonisation either because of the inoculation method, competition with the native microbiome, time of the harvest or chosen greenhouse conditions like using naturally nutrient abundant soil. Even though to our knowledge, this study is the first one reporting negative plant growth-promoting results on non-host plants of endophytic PGPB bacteria isolated from boreal forest conifers, previous studies using PGPB bacteria isolated from agricultural plants included a few bacterial strains that showed no visible positive effect on plant growth (Adjanohoun et al. 2011; da Costa et al. 2012; Ren et al. 2019). Additionally, some of those studies reported deleterious effects in the range of a 10 to 44% decrease in plant growth and yield due to bacterial inoculation (Kloepper et al. 1989; Antoun et al. 1998; Chanway et al. 2000) and in a review of *Azospirillum* inoculation studies it was calculated that only 60 to 70% of field studies resulted in a successful yield increase (Okon and Labandera-Gonzalez 1994). It has been previously reported for PGPB isolated from agricultural plants that successful laboratory studies often do not result in improved plant growth and yield in the field (Germaine et al. 2004; Mehnaz et al. 2010; Etesami and Maheshwari 2018) and that there is no correlation between in vitro assays, greenhouse and field studies (Antoun et al. 1998; Bacilio et al. 2017; Cueva-Yesquen et al. 2020). This means that sometimes PGPB showing promising results in in vitro assays do not show increased plant growth in greenhouse experiments, which was observed in this study. Field experiments may be conducted to further test if our bacteria would have a beneficial or negative effect on the chosen crops in field conditions under the presence of diverse abiotic and biotic stresses and further studies are needed to better understand the reasons behind the success or failure of bacterial inoculation in general. Especially as it has been suggested that negative results of inoculation studies are under-reported, leading to an overestimation of bacterial inoculation success and their potential application (Bacilio et al. 2017).

Interestingly, the interaction between crop species and inoculation treatment was significant for almost all measured plant traits, indicating host specificity of our bacterial strains. Similar results were observed previously for PGPB isolated from crop plants, where certain bacteria were capable of promoting the growth of various plant species, while others only promoted the growth of a few hosts (Afzal et al. 2019; Orozco-Mosqueda et al. 2022). Additionally, certain bacterial strains were better at promoting the growth of the host plants compared to non-host plants (Boddey and Dobereiner 1988; Lucy et al. 2004; Song et al. 2020). Therefore, further studies would be needed on our bacterial strains to assess their potential plant growth promotion on their host plant or other more closely related conifer species. Taken together, the results of our experiment and previously published studies indicate that at least some of the plant growth-promoting bacterial strains might have plant-specific effects and are only capable of promoting the growth of certain plant species. However, more research is needed to better understand the host specificity of PGPB and the mechanisms behind it.

The greenhouse study results also contradicted our third hypothesis stating that a consortium of bacteria will perform better compared to individual bacterial strains. The growth of consortium inoculated plants was similar to other treatments for all four crop species. The only significant difference was for dry root weight where consortium inoculated plants had lower biomass compared to control plants (Fig. 6). For PGPB isolated from agricultural plants, there have been many articles reporting better performance of consortium compared to individual strains (Rosenblueth and Martinez-Romero 2006; Knoth et al. 2014; Chaiya et al. 2021), however, there are some instances where a consortium did not perform better than individual bacterial strains (Bent and Chanway 1998; Méndez-Bravo et al. 2023). The proposed reasons for worse performance were strain incompatibility and competition for space and nutrients between different bacterial species within the consortium and within their surroundings (Méndez-Bravo et al. 2023). As our strains did not show antagonism in compatibility assay, it could be that we did not observe a positive consortium effect due to bacterial inefficiencies in competing for resources with the native microbiota (Kloepper et al. 1989; Shishido et al. 1999; Germaine et al. 2004). Our results highlight that consortium composition should be chosen carefully and be additionally tested to confirm that the selected bacteria are working synergistically to increase plant growth and yield.

Conclusions

Our study showed that even though the isolated and selected Scots pine endophytic bacteria showed excellent potential for plant growth promotion based on the seven in vitro assays, they were not able to promote the growth of four crop species (kale, corn, tomato and cucumber) in greenhouse conditions. More research on the efficiency of PGPB isolated from conifer tissues in in vivo studies using non-sterile conditions with native microbiota and naturally nutrient abundant growth media is needed to analyse if our study is an exception or if negative results are more common than reported. For example, it has been proposed that negative results in studies using PGPB isolated from agricultural plants are under-reported (Bacilio et al. 2017). Yet, to be able to develop efficient PGPB inoculates, more reported negative results are needed to be able to assess what is crucial for a successful plant growth promotion by PGPB. This is especially important in light of the big potential for the use of PGPB in agriculture and forestry (Newcombe 2011) to increase plant growth and provide protection against biotic and abiotic stresses without causing negative environmental effects.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00374-025-01910-8.

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Author's contributions All authors conceived and designed the study. T.B-J. and A.B. performed the experiments and analysed the data. T.B-J. wrote the manuscript, however, all authors contributed equally to the manuscript revision.

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Data availability The two bacterial strains isolated in the study are made available through the Netherlands Culture Collection of Bacteria (NCCB) at Westerdijk Institute and their sequencing data through GenBank at the National Center for Biotechnology Information (NCBI). *R. andropogonis* has NCCB accession number 100967 and GenBank number OR506164, while *Bacillus* sp. has NCCB accession number 100968 and GenBank number OR506163. The two datasets generated during this study are available on the SafeDeposit at Swedish University of Agriculture server accessible at https://www.safedeposit.se/projects/469 (ID = 469).

Declarations

Competing interests R.G. reports an affiliation with a commercial plant nutrition company. A.N. reports an affiliation with a commercial forestry company.

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Fungal and bacterial microbiomes are integral parts of the boreal forest ecosystem, but they remain under-researched. This thesis examined the microbiomes of several Norway spruce and Scots pine compartments and investigated the effects of nitrogen addition on their composition, soil extracellular enzyme activity and needle nitrogen fixation rate. Additionally, the thesis analysed the potential use of plant growth-promoting bacteria and organic nitrogen-based controlled-release nutrition as alternatives to inorganic nitrogen fertilization to promote plant growth.

Tinkara Bizjak-Johansson received her doctoral education in Biology at the Department of Forest Genetics and Plant Physiology at the Swedish University of Agricultural Sciences (SLU). She received her Master of Science in Molecular Biology at Umeå University.

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