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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

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

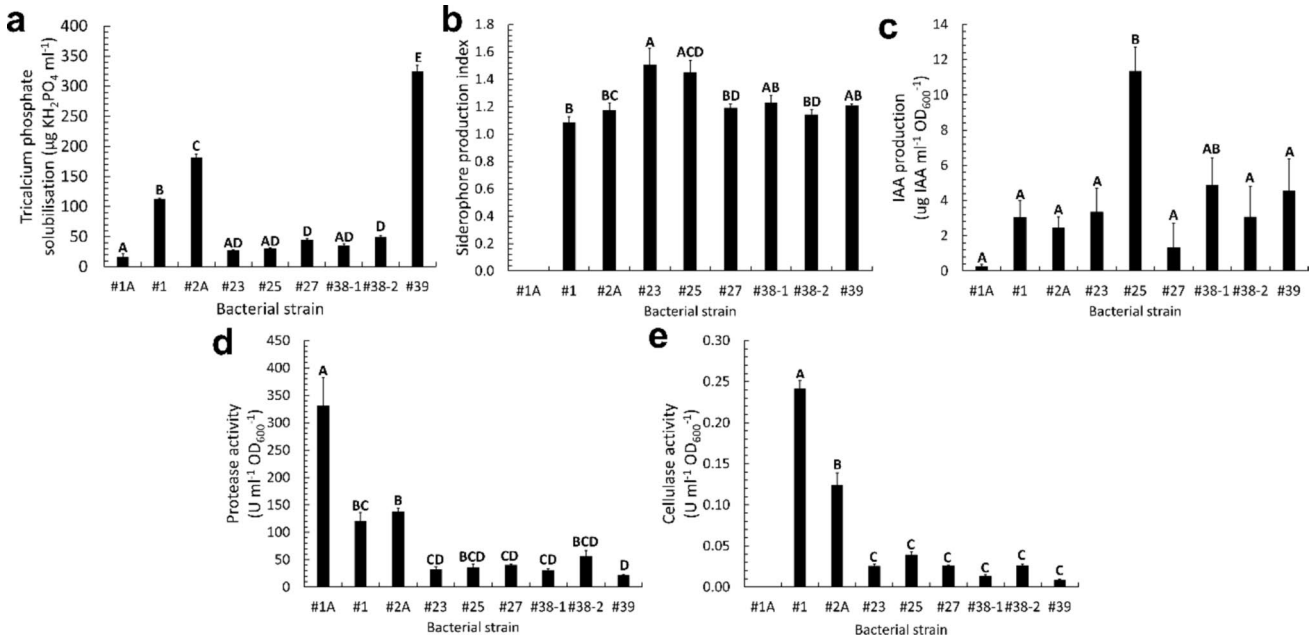

Fig. 1 Bacterial strain performance in liquid or plate plant growth-promoting assays for **a**) tricalcium phosphate solubilization, **b**) siderophore production, **c**) IAA production, **d**) protease activity and **e**) cellulase activity. The graphs show mean ± standard error (n = 3) and the different 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} $OD_{600}{}^{-1}$

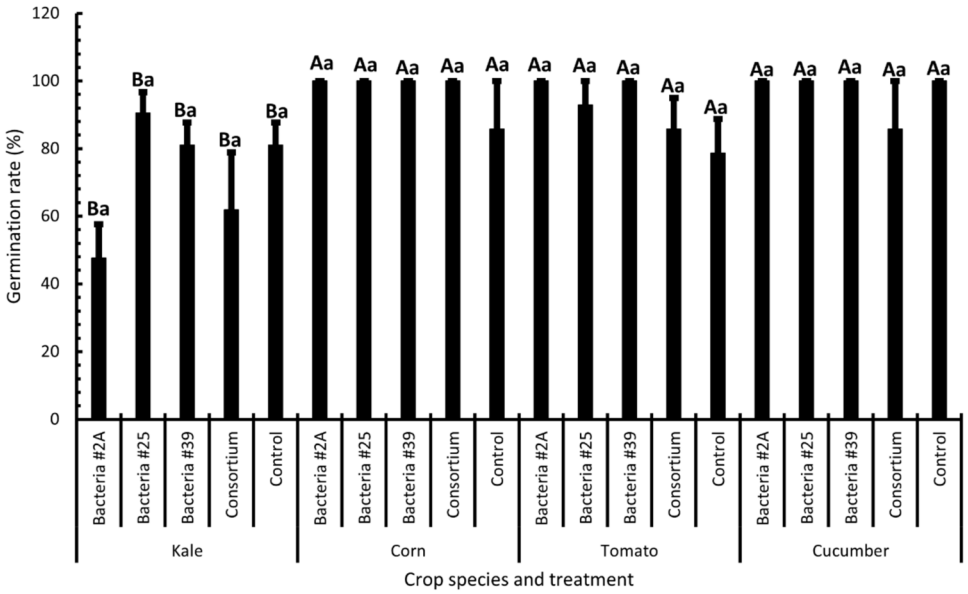

Fig. 2 Measured germination rate for kale, corn, tomato and cucumber plants and five inoculation treatments (*Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium and control). The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically 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

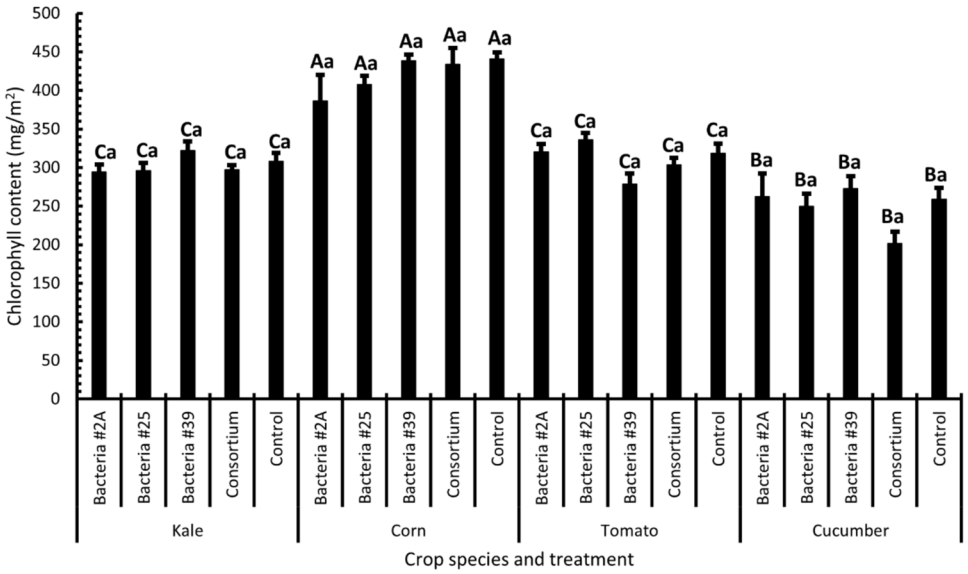

Fig. 3 Leaf chlorophyll content of four crop species (kale, corn, tomato and cucumber) inoculated with either *Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium or control. The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically 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.

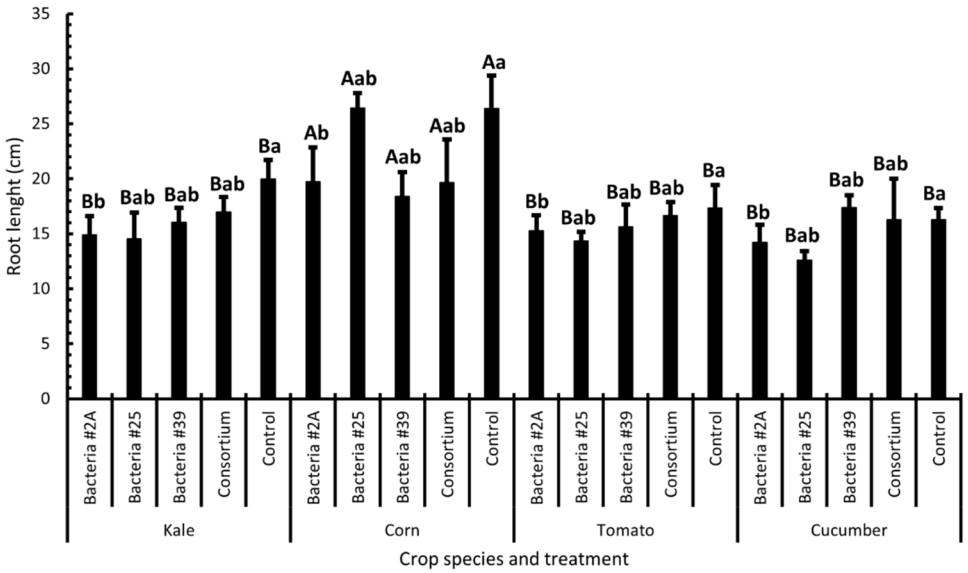

Fig. 4 Measured kale, corn, tomato and cucumber root length of plants inoculated with either *Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium or control. The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference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

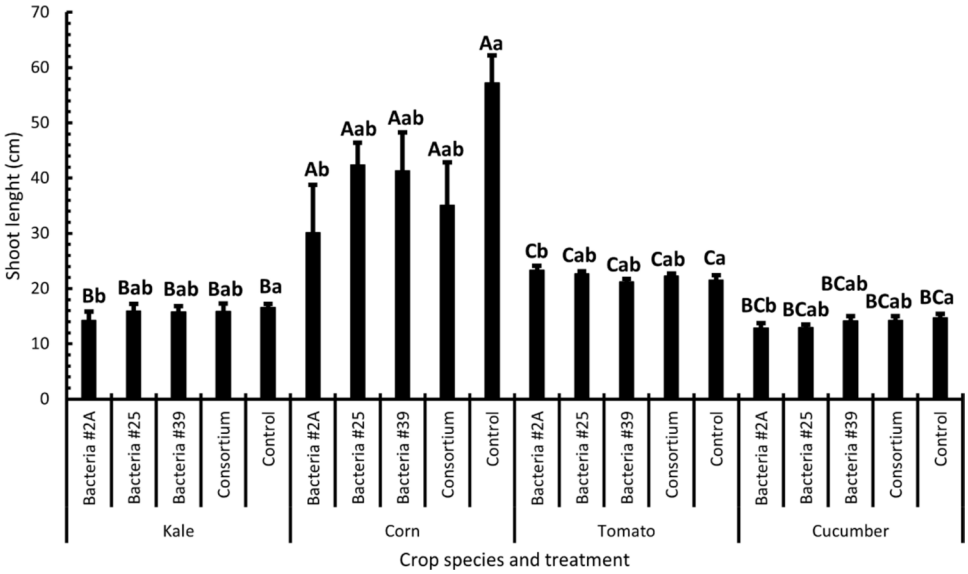

Fig. 5 Shoot length of four crop species (kale, corn, tomato and cucumber) treated with five different inoculation treatments (*Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium and control). The graph shows mean ± standard error (n = 7) and the different capital letters indicate 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

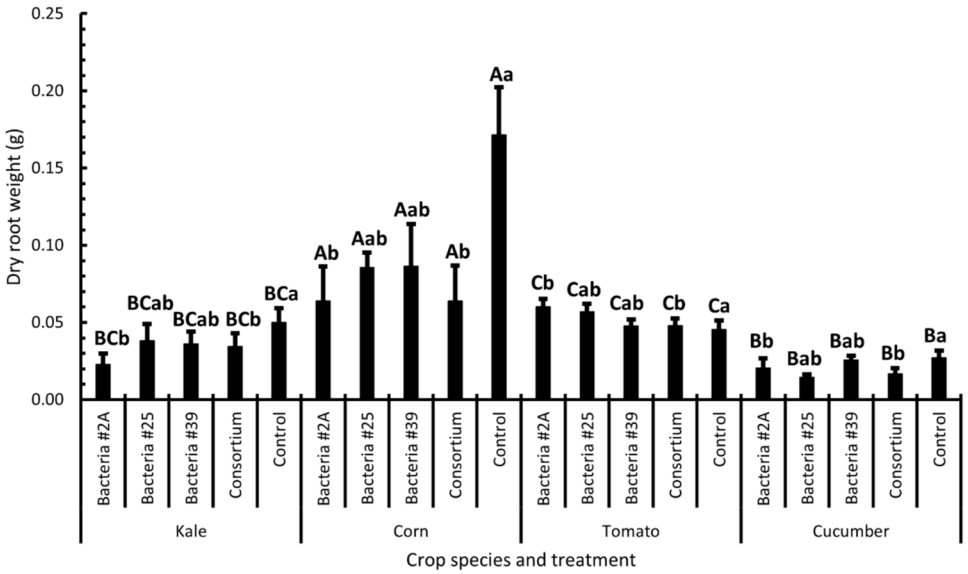

Fig. 6 Kale, corn, tomato and cucumber dry root weight for plants inoculated with five different treatments (*Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium and control). The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically 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

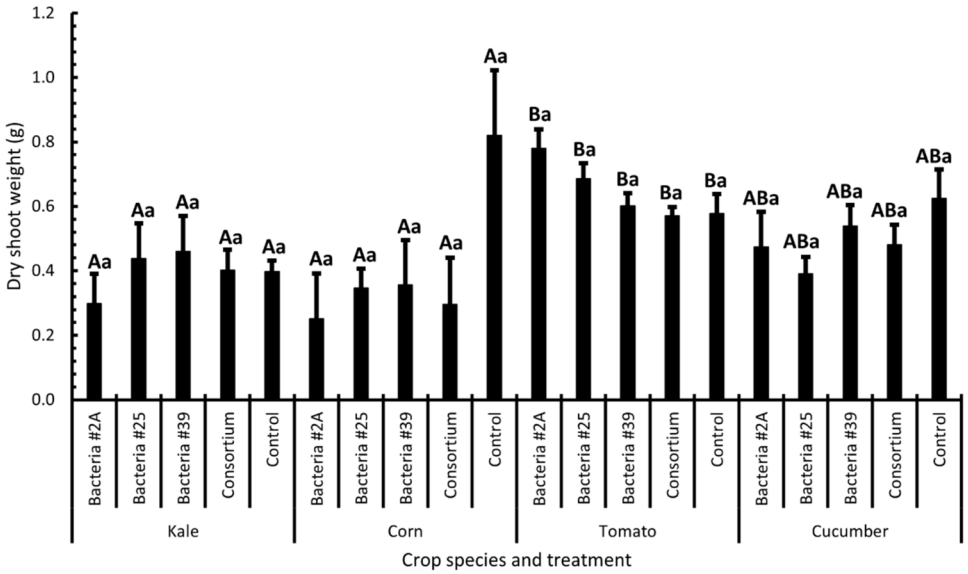

Fig. 7 Dry shoot weights of kale, corn, tomato and cucumber plants inoculated with either *Bacillus* sp. #2A, *Microbacterium* sp. #25, *P. megaterium* #39, consortium or control. The graph shows mean ± standard error (n = 7) and the different capital letters indicate a statistically significant difference 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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