Above- and belowground interactions in Jacobaea vulgaris: zooming in and zooming out from a plant-soil feedback perspective
Liu, X.

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Chapter 4

Dissecting negative effects of two root-associated bacteria on growth of the invasive species *Jacobaea vulgaris*

Xiangyu Liu[1], Klaas Vrieling[1], Adam Ossowicki[1, 2], Suzanne T. E. Lommen[1], T. Martijn Bezemer[1, 3]

Affiliations

[1]Above-belowground interactions group, Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, The Netherlands

[2]Departamento de Microbiología, Instituto de Hortofruticultura Subtropical y Mediterránea 'La Mayora', Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Universidad de Málaga, Málaga, Spain

[3]Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 50, 6700 AB Wageningen, The Netherlands

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Abstract

Aims Understanding the effects of root-associated bacteria on plants and unraveling the underlying mechanisms are important aims in the field of host-microbe interactions. Two Gammaproteobacteria, *Serratia plymuthica* and *Pseudomonas brassicacearum*, that were isolated from the roots of the plant *Jacobaea vulgaris*, an outbreak species in Europe where it is native and an invasive species in other continents, negatively affect root growth of *J. vulgaris*. Microorganisms that suppress plant growth are interesting in the context of biological control, but little is known about the interactions between these bacteria and this host plant and the causes of these negative plant-microbe interactions.

Methods In this study, we examined whether the effects of *S. plymuthica* and *P. brassicacearum* on *J. vulgaris* through root inoculation are concentration-dependent. We further investigated whether these effects can be attributed to metabolites present in the cell-free supernatants of bacterial suspensions. We also examined whether the two bacteria negatively affect seed germination and seedling growth through volatiles. Lastly, we assessed the specificity of these two bacteria using nine other plant species.

Results Both bacteria significantly reduced the root growth of *J. vulgaris* through root inoculation, but only the effects of *S. plymuthica* exhibited a concentration-dependent pattern in-vitro. The cell-free supernatants of both bacteria had no impact on the root growth of *J. vulgaris* seedlings. Both bacteria inhibited seed germination and seedling growth of *J. vulgaris* via volatiles. The negative effects of the two bacteria were not specific to *J. vulgaris* but also affected seedling growth of several other plant species.

Conclusions Our study demonstrates that the negative effects of *S. plymuthica* and *P. brassicacearum* on *J. vulgaris* through root inoculation are caused by bacterial cells and not by the metabolites these bacteria produce. Both bacteria also produce volatiles that negatively affect germination and seedling growth of *J. vulgaris*. While the two isolated bacteria negatively affect the early growth of this unwanted outbreak plant species, the negative effects were also found on other plant species which limits the potential use of these bacteria for biological control purposes.

Keywords

Biological control, bacterial volatiles, concentration-dependent effects, common ragwort, invasive plant, *Pseudomonas brassicacearum*, plant-microbe interactions, seed germination, seedling growth, *Serratia plymuthica*

Introduction

Soil microbes play a crucial role in influencing plants (Philippot et al. 2013; Carrión et al. 2019). They can significantly influence plant health by performing numerus functions, such as nitrogen fixation, phosphorus solubilization, and suppression of antagonists (i.e., pests and pathogens) of plants (Hurek et al. 2002; Sessitsch et al. 2002; Carrión et al. 2019). Soil microbes can also be harmful to plants, causing diseases or competing with the plant for nutrients (Kuzyakov and Xu 2013). The critical interface where interactions between plants and soil microbes occur is referred to as the rhizosphere. In the vicinity of roots, many bacteria with various relationships to plants reside in the rhizoplane (root surface), the rhizosphere (soil surrounding the roots) and the endo-sphere (inside of root tissues) (Saeed et al. 2021). These root-associated bacteria exhibit relationships with plants, ranging from mutualistic to pathogenic (Kuzyakov and Xu 2013). The central focus of this research field lies in understanding the effects of root-associated bacteria on plants and unraveling the underlying mechanisms that govern their interactions (Jacoby et al. 2017; Pascale et al. 2020; Pantigoso et al. 2022; Enagbonma et al. 2023).

Root-associated bacteria can directly influence plant performance through the production of metabolites, and the effects are often dose- or concentration-dependent (Shantharaj et al. 2021). For example, the cell-free supernatants of *Burkholderia gladioli* C101, a rhizobacterial strain, showed a dose-dependent reduction in spot disease severity caused by *Xanthomonas perforans* on tomato plants (Shantharaj et al. 2021). At high concentrations, root-associated bacteria can cause overstimulation of plant hormone production, nutrient imbalances, or produce toxins that harm plant cells or tissues (Kudoyarova et al. 2019). This can result in reduced growth, stunted development, and compromised plant health. In previous experiments (Liu et al. chapter 3), we observed that two bacteria that were isolated from the roots of the plant *Jacobaea vulgaris*, *Serratia plymuthica* and *Pseudomonas brassicacearum*, had a negative impact on root growth of *J. vulgaris* in-vitro. However, these effects disappeared when the plants were grown in soil for a longer period. It is currently unclear how these bacteria influence root growth and whether these negative effects on root growth are dependent on the concentration of bacteria.

Except for secreting metabolites into the surrounding environment such as the vicinity of roots, many studies have found that root-associated bacteria can affect plants at a distance through volatiles (Ryu et al. 2003; Kai et al. 2010; Blom et al. 2011a; Garbeva and Weisskopf 2020; Martín-Sánchez et al. 2020). Bacterial volatiles contain both organic and inorganic compounds, and they can either

promote or inhibit plant growth and seed germination (Wenke et al. 2012; Yasmin et al. 2021; He et al. 2023). For example, the volatiles produced by *S. plymuthica* have been shown to significantly inhibit growth and induce Hydrogen peroxide (H_2O_2) production in *Arabidopsis* plants (Kai et al. 2010; Wenke et al. 2012). The gases hydrogen cyanide (HCN), ammonia and dimethyl disulfide (DMDS) that can be produced by bacteria can also have inhibitory effects on plants (Kai et al. 2010; Cordovez et al. 2018). Besides influencing plant growth, volatiles of *Pseudomonas pseudoalcaligenes* have been reported to increase the percentage of germinated seeds and the germination index compared to the control treatment under a drought condition (Yasmin et al. 2021). Similar to plant-bacteria interactions through direct contact, these interactions via volatiles may also be concentration-dependent. For example, plant responses to a bacterial volatile compound, DMDS, have been found to follow a dose-dependent pattern. Cordovez and colleagues (2018) showed that at concentrations of 1 μM this volatile has a significant positive effect on shoot biomass, while at concentrations higher than 1 mM it had negative effects on plant biomass. It is currently unknown whether the aforementioned two root-associated bacteria inhibit seed germination and seedling growth of *J. vulgaris* through the emitted volatiles and if so, whether these effects are concentration-dependent.

Both root-associated bacteria that are used in this study cause shortening and thickening roots of *J. vulgaris* (Liu et al. Chapter 3). This change in root morphology resembles the response of roots to the plant hormone ethylene, as documented in previous studies (Goodlass and Smith 1979; Feldman 1984; Zaat et al. 1989). Ethylene production in plants can be induced by various abiotic and biotic factors, such as flooding and infection by Rhizobium bacteria (Zaat et al. 1989; Sasidharan and Voesenek 2015). For example, Zaat et al. (1989) reported that the ethylene inhibitor aminoethoxyvinylglycine (AVG) can restore the normal nodulation by Rhizobium bacteria in *Vicia sativa* plants without exhibiting the thick and short root phenotype. Based on this, we test this assumption by assessing the effects of the two bacteria in the presence of the ethylene-inhibitor AVG and predict that the shorter and thicker root phenotype of *J. vulgaris* after exposure to these two root-associated bacteria will not appear in the presence of the ethylene-inhibitor AVG.

In this study, our first objective was to investigate whether the effects of the two bacteria, *S. plymuthica* and *P. brassicacearum*, on *J. vulgaris* through root inoculation are concentration-dependent. Next, we examined whether cell-free supernatants of these two bacteria can induce inhibitory effects on roots and whether the shorter and thicker root phenotype is absent in the presence of the

ethylene-inhibitor AVG. Subsequently, we conducted both in vitro and soil bioassays to investigate whether the two bacteria negatively affect seed germination and growth of *J. vulgaris* via emission of volatiles, and whether these effects also followed a concentration-dependent pattern. Finally, to further examine their potential to be used in biological control of *J. vulgaris*, we tested the specificity of the two bacteria and tested whether the two bacteria influence the growth of other co-occurring plant species in-vitro. The research questions that we address are as follows: (i) Do the effects of the two root-associated bacteria on *J. vulgaris* depend on the concentrations of bacteria used at inoculation? (ii) Are the effects due to bacterial metabolites produced in bacterial suspensions? (iii) Does the AVG inhibitor reduce the effects of bacteria on seedling growth of *J. vulgaris*? (iv) What are the effects of bacterial volatiles on seed germination and seedling growth of *J. vulgaris*? (v) Are the effects of bacterial volatiles on seedling growth concentration-dependent? And (vi) do the two bacteria influence seedling growth of other plant species.

Material and methods

Plants

Jacobaea vulgaris Geartn. subs. *vulgaris* (syn. *Senecio jacobaea* L.; Asteracaea) is a monocarpic perennial that is native in Europe (Harper and Wood, 1957). Plants die after the production of seeds. Seeds were collected from plants growing in Meijendel near Wassenaar in The Netherlands in a coastal dune grassland. Nine other plant species that all co-occur with *J. vulgaris* in natural grasslands in The Netherlands were used in this study to examine the specificity of the two bacteria: three grasses (*Anthoxanthum odoratum* L., *Agrostis capillaris* L., *Holcus lanatus* L.), three forbs (*Plantago lanceolata* L., *Tripleurospermum maritimum* (L.) Koch, *Achillea millefolium* L.), and three legumes (*Lotus corniculatus* L., *Trifolium dubium* Sibth., *Trifolium repens* L.). Seeds of these nine species were purchased from Cruydt-Hoeck (Nijeberkoop, The Netherlands), a supplier of seeds obtained from wild plants. Seeds were surface-sterilized by submerging in a 5% sodium hypochlorite solution for 20 minutes, followed by rinsing three times with sterilized Mill-Q water. Subsequently, the seeds were placed in containers (10 × 10 × 4 cm), filled with sterile glass beads submerged in sterilized Mill-Q water. Each container was closed with a transparent lid and put in growth cabinet (70% RH, 24 °C at day time; 16 hours and 20°C at night; 8 hours). Seedlings were grown on glass beads for one week after germination and then stored at 4°C until further use.

Concentration-dependent inhibitory effects on J. vulgaris growth through root inoculation in-vitro

The two bacterial strains that were isolated from roots of *J. vulgaris* plants, *S. plymuthica* and *P. brassicacearum*, were cultured from the glycerol stocks stored in -80°C freezer. The cultures were incubated in Erlenmeyer flasks containing liquid TSB medium on a shaker at 180 rpm and 28 °C overnight. The optical density (OD) of the bacterial cultures was measured, and the bacteria suspensions were subsequently spun down in a centrifuge and immediately resuspended in sterilized saline solution (0.9% NaCl) to achieve four OD values of 0.5, 0.8, 1.0 and 1.34 (OD_{600} was 1.34 after overnight culture). Seven days after germination of *J. vulgaris* seeds, the roots of seedlings of similar size were immersed in different concentrations of the bacterial solution or in saline solution (control) for 1 minute. Following inoculation via dipping, each seedling was immediately transferred to a square plate (10 cm × 10 cm × 2 cm) containing 25 ml of 0.5 MS medium (containing 8 g/L agar). In total, there were 38 plates and each plate had one seedling (2 bacterial strains × 4 concentrations × 4 replicate plates + 1 control × 6 replicate plates = 38 plates). The seedlings were allowed to grow for 18 days, and photographs of each seedling were taken at two-day intervals. Root morphology traits including total root length, primary root length, total lateral root length, and number of lateral roots were determined using the plugin "ObjectJ" (Vischer and Nastase 2021) from ImageJ (the software can be downloaded at https://fiji.sc/). The average total lateral root length was calculated by dividing lateral root length by number of lateral roots. The measurements were obtained from photographs taken on day 18.

After 18 days of growth, the seedlings were carefully removed from the plates, and the fresh shoot and root biomass were recorded. To determine the effects of the bacteria on plant health, chlorophyll and carotenoid content of the leaf were measured following a modified protocol of Kshetrimayum et al. (2018). Approximately 0.05 gram of fresh leaf material from each seedling was cut and placed, along with a metal ball, into a 2 ml Eppendorf tube. The weight of fresh leaf material used was recorded as well. Then, 1 ml of 95% Ethanol was added to each Eppendorf tube. The tubes were shaken for 1 minute at 1800 rpm using the TissueLyser II. After shaking, the Eppendorf tubes were centrifuged for 2 minutes at 13,200 rpm (maximum speed) at room temperature and 0.5 ml of the supernatant was mixed with 4.5ml of 95% Ethanol in a clean Falcon tube (15 ml). Then 200 µL of each mixture was pipetted into a 96-well plate. One well included 95% ethanol as the control treatment. The optical density (OD) was then measured at 649 nm, 664 nm and 470 nm using a Tecan Spark 10M Microplate Reader (Männedorf,

Switzerland). Chlorophyll and Carotenoid contents were calculated using the following standard formulas: Chlorophyll a = 13.36*A_{664} – 5.19*A_{649}; Chlorophyll b = 27.43*A_{649} – 8.12*A_{664}; Carotenoids = (1000*A_{470} –2.13*Chlorophyll a – 97.63* Chlorophyll b)/209 (Kshetrimayum et al. 2018). Chlorophyll and Carotenoid contents were then calculated per gram of fresh leaf material.

Concentration-dependent inhibitory effects on J. vulgaris growth through root inoculation in soil

Bacteria may develop differently in soil compared to on agar plates, therefore we also repeated the previous experiment and grew plants in soil. Bacterial solutions of different concentrations of *S. plymuthica* and *P. brassicacearum* (OD_{600} values of 0.5, 0.8, 1.0 and 1.34) and *J. vulgaris* seedlings were prepared as described above. Sterile transparent plastic containers (130 mm Height, 30 mm diameter) were used to test the growth of *J. vulgaris* seedlings inoculated with the bacteria but this time the seedlings were planted in soil. Each container was filled with 25 g of sterilized soil, and 2 ml of sterilized MillQ water was added. The soil was collected from Lange Dreef in Driebergen, The Netherlands and was sterilized with gamma irradiation (> 25Kgray, Isotron, Ede, The Netherlands). The soil is characterized as a sandy loam holtpodzol with a particle size distribution: 2% < 0.002 mm, 11% 0.002-0.063 mm, 84% > 0.063 mm, with ~3 % organic matter, 1,150 mg kg-1 N, 61 mg P_2O_5 100 g-1, 2.4 mmol K kg-1 and pH 5.9, and was sieved (0.5 cm mesh size) and homogenized prior to gamma irradiation. The roots of the seedlings were immersed in different concentrations (OD_{600} values of 0.5, 0.8, 1 and 1.34) of bacterial solutions for 30 minutes and then planted in the soil. Following planting, each plastic container was covered with a transparent lid. In total, there were 46 plants (2 bacterial strains × 4 concentrations × 5 replicates + 1 control × 6 replicates). After 21 days of growth, the plants were harvested and roots were hand-washed to remove the soil. To determine and the total root length for each plant, the roots were scanned with an Epson scanner. Total root length was determined using the plugin "ObjectJ" from ImageJ. The shoot and root of each seedling were then dried at 60 C for 72 hours, and the shoot and root dry mass were measured. The specific root length was calculated as total root length divided by root dry mass.

Effects of the cell-free supernatants of the two bacterial strains on J. vulgaris seedlings

To investigate whether metabolites secreted by the bacteria into the surrounding environment can attribute to the observed negative effects on roots, the effects of cell-free supernatants of the two bacterial strains on *J. vulgaris* seedlings were tested. One week before starting this experiment, *J. vulgaris* seedlings were prepared as described above. Square plates (10 cm × 10 cm × 2 cm) containing 25 ml 0.5 MS medium (8 g/L agar) were prepared. The two bacterial strains were cultured overnight at 28 °C in Erlenmeyer flasks containing liquid TSB medium. For inoculation, each of the bacterial cultures was spun down and immediately resuspended in sterilized TSB medium to an optical density (OD) of 1 at 600 nm (OD_{600}). Subsequently, the bacterial solutions were spun down again in a centrifuge, and the supernatant was separated. The supernatant of the bacteria (treatment) and fresh TSB medium (control) were filter-sterilized. Meanwhile, the bacterial pellet was immediately resuspended in sterilized saline solution. Seedlings were inoculated by dipping them for 1 minute in the supernatant of one of the bacterial solutions, or in one of the resuspended bacterial solutions, or in sterilized TSB medium (control). In total, there were 25 plates and each contained one seedling (2 bacterial strains × 2 treatments [with or without bacterial cells] × 5 replicates + 1 control [TSB medium] × 5 replicates). Plates were closed with parafilm and placed vertically in a growth cabinet (70% RH, 24°C at day time for16 hours and 20°C at night for 8 hours). The seedlings were allowed to grow for 12 days, and photographs of each seedling were taken at two-day intervals. Root morphology traits including total root length, primary root length, total lateral root length and number of lateral roots were determined using the plugin "ObjectJ" from ImageJ.

Bacterial effects on J. vulgaris plants through root inoculation with or without the ethylene-inhibitor

To examine whether the plant hormone ethylene is involved in the negative effects of the two bacteria on *J. vulgaris*, we used the ethylene inhibitor aminoethoxyvinylglycine (AVG, Sigma) to inhibit the production of ethylene in plants. Using this method, we hypothesized this would restore the root growth of *J. vulgaris* in the presence of the bacteria. AVG was dissolved in water, filter-sterilized using a GS filter with a 0.22 µm pore diameter, and stored at -20 °C prior to use and the concentration of the stock AVG solution was 0.5 mg/L. Half MS medium (8 g/L agar) was prepared and autoclaved to sterilize. When the 0.5 MS medium had cooled down to approximately 60 °C, the stock AVG solution was

added to achieve a final concentration of 5 μg/L. We also prepared 0.5 MS medium without AVG addition. Square plates (10 cm × 10 cm × 2 cm) filled with 25ml 0.5 MS medium with and without AVG were prepared. Seedlings of *J. vulgaris* and the two bacterial inoculations were prepared as described above. Bacterial cultures were diluted to an optical density (OD) of 1 at 600 nm (OD_{600}). Subsequently, the bacterial solutions were spun down in a centrifuge, and the supernatant was discarded. The bacterial pellet was immediately resuspended in sterilized saline solution. Seedlings were either inoculated by dipping their roots in one of the bacterial inoculations or in sterilized saline solution for 1 min, and then transferred to plates with 0.5 MS medium with or without AVG (each plate contained one seedling). In total, 30 seedlings were used in this experiment: 3 inoculation treatments (2 bacteria + 1 control) × AVG inhibitor (with or without) × 5 replicates. Plates were closed with parafilm and placed vertically in a growth cabinet (70% RH, 24 °C at day time for16 hours and 20°C at night for 8 hours). The seedlings were allowed to grow for 12 days, and photographs of each seedling were taken at two-day intervals. Root morphology traits including total root length, primary root length, lateral root length and number of lateral roots were determined using the plugin "ObjectJ" from ImageJ. The average total lateral root length was calculated as lateral root length divided by number of lateral roots. The measurements were obtained from photographs taken on day 12.

Effects of volatiles emitted by bacteria on J. vulgaris seed germination

To examine the effects of volatiles emitted by bacteria on seed germination, Petri dishes that were divided into two separate compartments were used (10 cm diameter). Sterilized TSA and 0.5 MS medium were prepared. Ten ml of TSA (for bacterial growth) or 10 ml of 0.5 MS medium (for seed germination) were poured in both sides of a split Petri dish. The two bacterial strains were cultured, and bacterial solutions (OD_{600} = 1) were prepared as described above. Immediately afterward, we dipped a sterile inoculation loop (1 μl) either in a bacteria solution or in a sterilized saline solution and this was smeared over the TSA side. Each plate was closed with a lid and incubated at 28 °C overnight. One day later, *J. vulgaris* seeds were surface sterilized as described above. We then used a sterilized toothpick to transfer 25 seeds on the 0.5 MS medium side of each plate. In total, there were 375 seeds in 15 plates (2 bacterial strains × 5 replicated plates × 25 seeds + 1 control × 5 replicated plates × 25 seeds). We recorded the number of freshly germinated seeds on each plate daily for 10 days and calculated the proportion of germination on each plate at day 10.

Effects of volatiles emitted by bacteria on J. vulgaris seedling growth in-vitro

To study the effects of volatiles produced by bacteria on seedling growth of *J. vulgaris* and examine whether these effects are concentration-dependent, Petri dishes that were divided into two separate compartments (10 cm diameter) were used. One week before initiating the experiment, *J. vulgaris* seeds were surface sterilized and germinated on 0.5 MS medium as described above. Sterilized TSA and 0.5 MS medium were prepared and poured in one of the sides of each split Petri dish, following the method described above. Bacteria were cultured and bacterial solutions (OD_{600} = 1) were prepared as described above. Immediately afterward, we inoculated one of the bacteria or sterilized saline solution (control) on the TSA medium by pipetting (i) 20 µl (1 spot), (ii) 3 times 20 µl (3 spots), or (iii) 5 times 20 µl (5 spots). The plates were closed with parafilm and put in an incubator at 28 °C overnight. One day after inoculation, we transferred four *J. vulgaris* seedlings onto the 0.5 MS medium side of each plate. The plates were sealed with parafilm, and photographs at fixed height were taken of each seedling. In total, there were 54 plates and each plate had 4 seedlings (2 bacterial strains × 3 concentration treatments × 6 replicated plates + 1 control × 3 concentration treatments × 6 replicated plates). Then plates were then placed vertically in a growth cabinet (70% RH, 24°C with 16 hours of light and 20 °C with 8 hours of darkness). We traced root growth of each seedling by taking photographs of each seedling at fixed height. Ten days after placing the seedlings in the plates, the experiment was ended. Total root length of the four seedlings per plate at day 10 was determined using the plugin "ObjectJ" from ImageJ. The total shoot and roots of seedlings per plate were dried at 60 C for 72 hours, and the shoot and root dry mass of seedlings per plate were measured.

Effects of volatiles emitted by bacteria on seedling growth of J. vulgaris in soil

To examine whether bacterial volatiles have an effect on *J. vulgaris* grown in soil, we utilized a pot-in-jar system with some modifications based on Martín-Sánchez et al. (2020). One week before the experiment, *J. vulgaris* seeds were surface sterilized and germinated on plates with 0.5 MS solid medium as described above. Six days after germination, we prepared sterilized TSA and TSB medium. Bacteria were cultured and bacterial solutions (OD_{600} = 1) were prepared as described above. In each sterilized plant tissue culture container (53 mm diameter, 100 mm height), we added 20 ml of sterilized TSA medium. Once the TSA medium was solid, we pipetted either 50 µl of one of the bacteria solutions or 50 µl of sterilized TSB medium onto the TSA medium in each container. We closed and sealed all

containers with a lid, then incubated them at 28 °C for 24 hours. Seven days after germination, we made twelve 2 mm diameter holes in the bottom of each 60 ml measuring cup and placed a filter paper (4 cm diameter) at the bottom. Each measuring cup was filled with 50 grams of gamma radiated sterilized soil, and the soil was the same soil as used above. One seedling was transferred into each cup. Immediately afterwards, in a sterile flow cabinet, we randomly selected a measuring cup with a grown seedling and placed it above the bacteria culture container. The connection between the measuring cup and the bacteria culture container was sealed with parafilm. In total, there were 60 plants (3 treatments [2 bacteria treatments + 1 control] × 20 replicates). Subsequently, they were placed in a growth cabinet (70% RH, 24°C with 16 hours of light and 20 °C with 8 hours of darkness). The plants were watered when needed and were harvested 28 days after planting the seedlings. The shoot and roots of each plant were dried at 60 C, and the shoot and root dry mass were recorded.

Effects of S. plymuthica and P. brassicacearum on seedling growth of J. vulgaris and nine co-occurring plant species in-vitro

One week before this experiment, sterilized seeds of *J. vulgaris* and nine other co-occurring plant species were germinated on sterilized glass beads as described above. Square plates with 0.5 MS growth medium were prepared. The two bacterial strains were cultured overnight at 28 °C in Erlenmeyer flasks containing liquid TSB medium. For inoculation, the cultures were diluted to an optical density (OD) of 1 at 600 nm (OD_{600}). Subsequently, the bacterial solutions were spun down in a centrifuge, and the supernatant was separated. The bacterial pellet was immediately resuspended in sterilized saline solution. Seedlings were inoculated by dipping them for 1 min either in one of the bacterial solutions or in sterilized saline solution. Immediately afterwards, each seedling was transferred on one square plate with 0.5 MS growth medium. In total, there were 150 plates with one seedling (10 plant species × 2 bacterial strains × 5 replicates + 1 control × 10 plant species × 5 replicates). Plates were closed with parafilm and placed vertically in a growth cabinet (70% RH, 24°C at day time for16 hours and 20°C at night for 8 hours). Photographs of each seedling were taken at two-day intervals. Root morphology traits including primary root length and total root length were determined using the plugin "ObjectJ" from ImageJ. The seedlings were allowed to grow for 12 days after which fresh shoot and root biomass of each seedling were measured.

Data analysis

Concentration-dependent inhibitory effects on J. vulgaris growth through root inoculation

In vitro and in soil, the effects of concentration of the bacterial inocula (treated as numeric variables) on primary root length, the number of lateral roots, average lateral root length, total root length, fresh shoot and root biomass, leaf chlorophyll and carotenoid contents, shoot dry mass, root dry mass, specific root length, and total root length of *J. vulgaris* were examined using linear regression models for each bacterial strain, respectively. The control treatment was not included in the linear regression models. This was followed by one-way ANOVA on all inoculation treatments including the control (treated as factor variables, 9 levels) and a Tukey post-hoc test was then used to compare the difference between bacterial inoculation and the control treatment if the one-way ANOVA was significant.

Effects of the cell-free supernatants of the two bacterial strains on J. vulgaris root growth

The effects of inoculation treatments (5 levels: control, two bacterial solutions and two supernatants of the bacterial solutions) on primary root length, the number of lateral roots, average lateral root length and total root length were examined with one-way ANOVA. A Tukey post-hoc test was then used to compare the difference among treatments.

Bacterial effects on J. vulgaris plants through root inoculation with or without the ethylene-inhibitor

The effects of inoculation treatments, the presence of the ethylene-inhibitor and its interaction on primary root length, number of lateral roots, average lateral root length and total root length were examined using two-way ANOVA. A Dunnett post-hoc test was then used to compare the difference between bacterial inoculation at a certain concentration and the control treatment.

Volatile effects of bacteria on seed germination and seedling growth of J. vulgaris

The effects of bacterial volatiles on seed germination, shoot and root dry mass and total root length of *J. vulgaris* plants when plants were grown in soil were examined using one-way ANOVA. A Tukey post-hoc test was then used to compare the difference between bacterial inoculation and the control treatment if the inoculation

treatment was significant. The effects of bacterial inoculation, concentration of bacterial inocula and its interaction on shoot and root dry mass and total root length of seedlings per plate (four seedlings together) were examined using two-way ANOVA. A Tukey post-hoc test was then used to compare the differences among treatments.

Effects of the two bacteria on the growth of J. vulgaris and other co-occurring plant species

The effects of bacterial inoculation and identity of plant species on primary root length, total root length, fresh shoot and root biomass of *J. vulgaris* and nine other co-occurring plant species were examined using two way-ANOVA. An one way-ANOVA was then used for each plant species separately. A Dunnett post-hoc test was then used to compare the difference between bacterial inoculation and the control treatment.

All linear regressions were performed with the "lm" function. All ANOVAs were carried out with the "aov" function and post-hoc tests were performed using the "glht" function with the "multcomp" package (Hothorn et al. 2008). In all analyses, residuals were checked for homogeneity of variance using a Levene's test and for normality by a Shapiro Wilk test. Primary root length, total root length, and fresh root biomass were log-transformed to fulfil requirements of normality. The Levene's test and Shapiro Wilk test were performed using the "levene_test" and "shapiro_test" function with the "rstatix" package (Kassambara, 2023).

All analyses were performed using the R statistical language, version 4.2.2 (R Core Team 2022).

Results

Concentration-dependent inhibitory effects on J. vulgaris growth through root inoculation

Bacterial inoculation significantly reduced total root length of *J. vulgaris* seedlings, both through reducing the length of the primary root and the average lateral root length (Fig S4.1). The inhibitory effects were also observed for fresh root biomass (Fig S4.1, Table S4.1). However, bacterial inoculation had no effect on fresh shoot biomass and the concentration of leaf chlorophyll and carotenoids (Fig S4.1, Table S4.1). The concentration-dependent effect was observed only for *S. plymuthica*,

where an increasing bacterial concentration led to a reduction in the negative effects on root growth in-vitro (Fig 4.1, Table S4.1). When *J. vulgaris* plants were grown in soil, *S. plymuthica* through root inoculation only significantly reduced shoot dry mass and total root length of *J. vulgaris* plants when the inoculation concentration was 0.8 at OD_{600}, compared to the control (Fig 4.2). Inoculation with *P. brassicacearum* only led to significant reduction in shoot dry mass when inoculation concentration was 1 at OD_{600} compared to the control treatment (Fig 4.2). There was no concentration-dependent pattern for both bacteria when plants were grown in soil (Table S4.2). The supernatant of the bacterial solution had no effect on root morphological traits (Fig 4.3, Table S4.3). There was no significant interaction effect between bacterial inoculation and the presence of the ethylene inhibitor AVG on root traits indicating the ethylene inhibitor AVG did not restore the negative effects of bacteria on root growth of *J. vulgaris* (Table S4.4). Instead, the ethylene inhibitor itself also exhibited negative effects on root growth (Fig 4.4, Table S4.4).

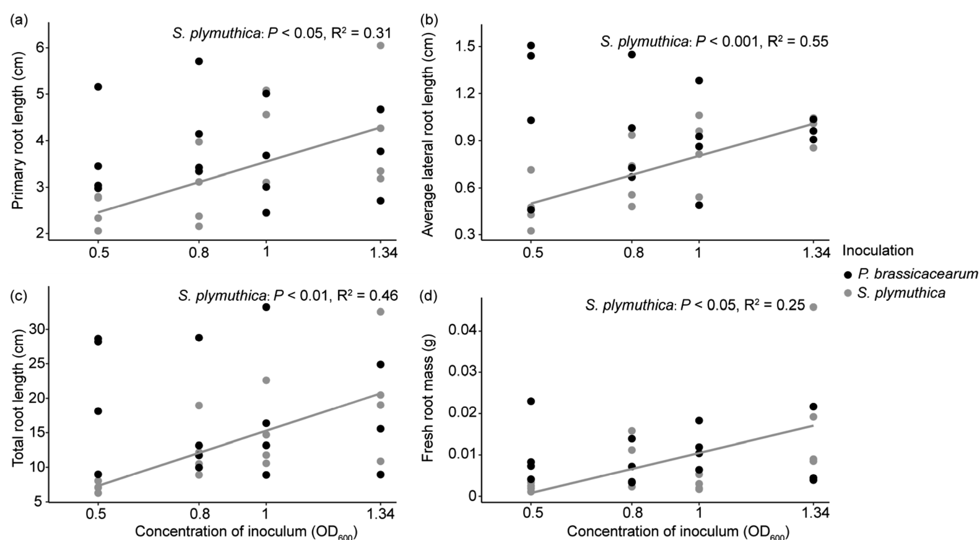

Fig 4.1. Relationship between concentration of bacterial inoculum and primary root length (a), average lateral root length (b), total root length (c) and fresh root mass (d) of *J. vulgaris* in-vitro. In (a, b, c, d), R^2 and P-values from a linear regression analysis for *S. plymuthica* are also presented. There were no significant relations for *P. brassicacearum*.

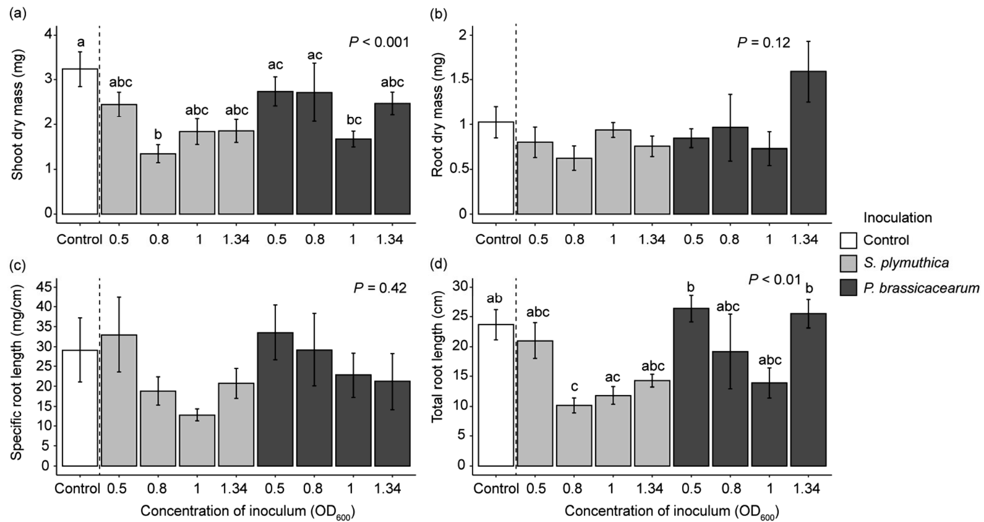

Fig 4.2. Mean (± SE) shoot dry mass (a), root dry mass (b), specific root length (c), and total root length (d) of *J. vulgaris* in the presence of one of the two bacterial inocula at varying concentrations in soil. In (a, d), letters indicate significant differences between the inoculation treatments (P < 0.05) based on a Tukey post-hoc test and bars with identical letters are not significantly different. In (b, c) there were no significant differences between the inoculation treatment**.** P values of one-way ANOVA are also presented.

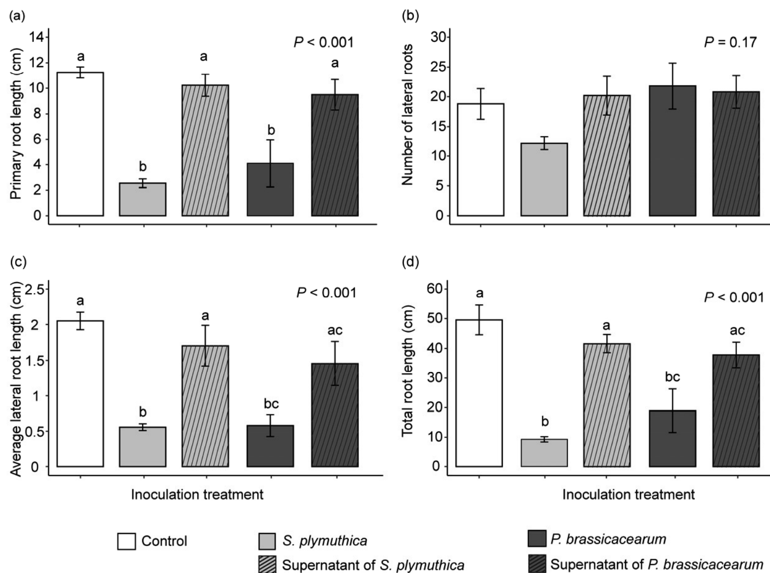

Fig 4.3. Mean (± SE) primary root length (a), number of lateral roots (b), average lateral root length (c), and total root length (d) of *J. vulgaris* seedlings with bacterial cells or supernatant of one of the two bacteria or no inoculation (control) in-vitro. In (a, c, d), letters

indicate significant differences between the inoculation treatments ($P < 0.05$) based on a Tukey post-hoc test and bars with identical letters are not significantly different. In (b) there was no significant differences between inoculation treatment. *P* values of one-way ANOVA are also presented.

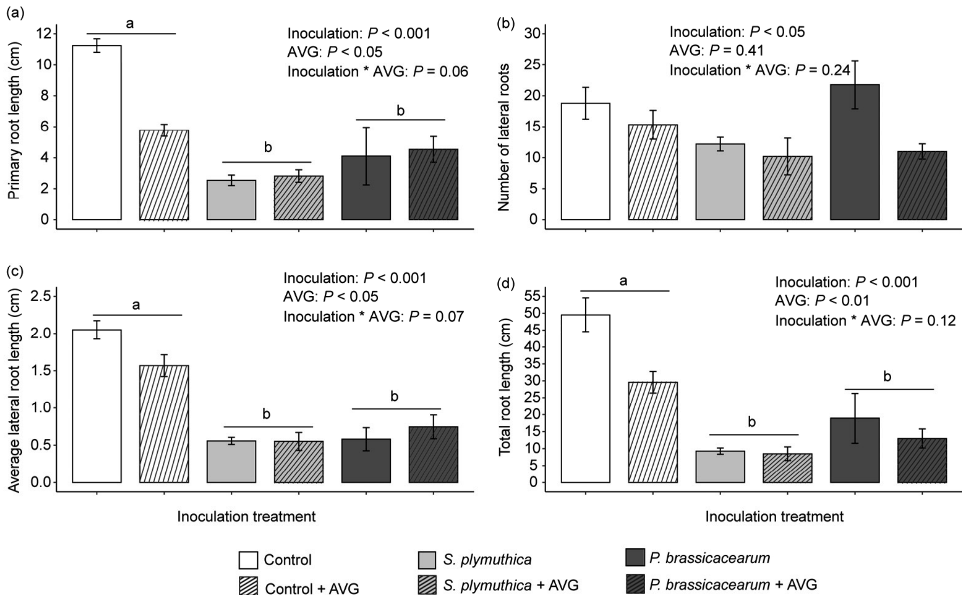

Fig 4.4. Mean (± SE) primary root length (a), number of lateral roots (b), average lateral root length (c), and total root length (d) of *J. vulgaris* seedlings after 10 days inoculated with one of bacterial inoculum or no inoculation (control) the presence of one of the two bacteria on 0.5 MS medium with or without the ethylene inhibitor (AVG). In (a, c, d), letters above the horizontal line indicate significant differences between inoculation treatments ($P < 0.05$) based on a Tukey post-hoc test and bars with identical letters are not significantly different. In (b), there were no significant differences between inocula. P values of two-way ANOVA are also presented.

Volatile effects of bacteria on seed germination and seedling performance of J. vulgaris

Bacterial volatiles significantly inhibited seed germination of *J. vulgaris* (Fig 4.5 a, b). The average proportion of germinated seeds was 0.78 (*SE* = 0.02) in the control treatment without bacterial volatiles. In presence of volatiles from *S. plymuthica* and *P. brassicacearum*, the proportion of germinated seeds was reduced to 0.16 (*SE* = 0.13) and 0, respectively (Fig 4.5 a). Shoot and root dry mass, as well as total root length of *J. vulgaris* seedlings per plate, were significantly influenced by bacterial volatiles (Fig 4.5 c, d, e and f, Table S4.5). Volatiles from *S. plymuthica* significantly reduced total root length per plate but had no influence on shoot and root dry mass of *J. vulgaris* seedlings per plate in-vitro (Fig 4.5 c, d, e). Volatiles from *P. brassicacearum* significantly reduced shoot and root dry mass, and total root length of *J. vulgaris* seedlings per plate in-vitro (Fig 4.5 c, d, e). The concentration effect was not the same for all inoculation treatments resulting in a significant interaction between inoculation treatment and concentration (Table S4.5). In particular, volatiles from *S. plymuthica* only led to a reduction in total root length at high concentrations (3 and 5 spots of bacterial inoculum) (Fig 4.5 e). In soil, exposure to volatiles from one of the two bacterial strains significantly reduced root dry mass of *J. vulgaris* ($F_{2, 55} = 6.58$, $P < 0.01$), while it had no effect on shoot dry mass (Fig 4.5 g, h and i, $F_{2, 56} = 1.01$, $P = 0.37$).

Effects of the two bacteria on growth of J. vulgaris and other co-occurring plant species

The effects of bacterial inoculation on the length of the primary root and total root length, varied significantly depending on the identity of the plant species (Fig 4.6 a and b, Table S4.6). Inoculation with *S. plymuthica* significantly reduced root length in *J. vulgaris*, as well as in two other forbs (*T. maritimum* and *P. lanceolata*), and two legumes (*T. dubium* and *T. repens*) (Fig 4.6 a and b). Similarly, inoculation with *P. brassicacearum* significantly reduced root length in *T. maritimum*, *A. capillaris*, *T. dubium* and *T. repens* (Fig 4.6 a and b). Biomass of *P. lanceolata* and *T. dubium* were significantly reduced after inoculation with *S. plymuthica*. Inoculation with *P. brassicacearum* significantly reduced biomass of *A. capillaris* and *T. dubium*, but increased root biomass of *H. lanatus* (Fig 4.6 c and d).

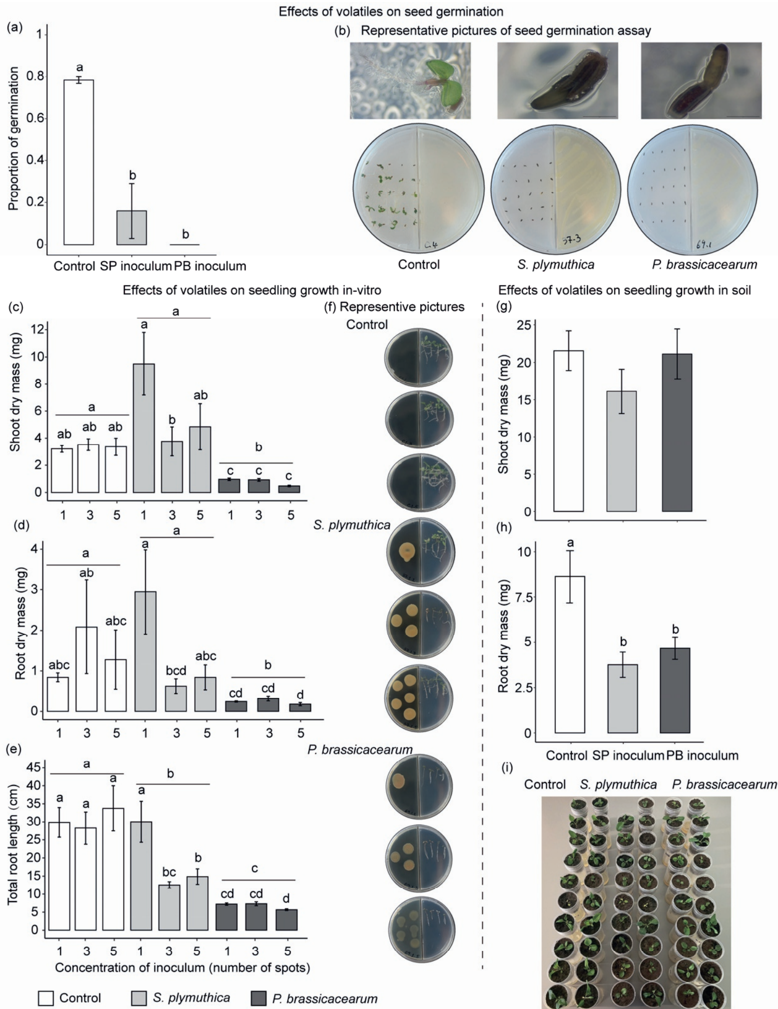

Fig 4.5. Mean (± SE) proportion of germination of *J. vulgaris* seeds 10 days after start of the experiment in presence and absence of volatiles emitted by one of the bacterial strains (a), representative pictures of the seed germination assay (b), mean (± SE) shoot dry mass (c), root dry mass (d), total root length (e), and representative pictures (f) of *J. vulgaris* seedlings per plate ten days after placing them in plates in the presence of bacterial volatiles with 20 (1 spot), 60 (3 spots) and 100 µl (5 spots) of bacterial inoculum, and shoot (g) and root dry mass (h) and a picture (i) of *J. vulgaris* plants 28 days after planting in soil after exposure to volatiles of one of the two root-associated bacteria. In (a, c, d, e and h), letters above bars indicate significant differences between treatments ($P < 0.05$) ($P < 0.05$) based on a Tukey post-hoc test and bars with identical letters are not significantly different. In (g) there were no significant differences between treatments. In (c, d, e), letters above the horizontal line indicate significant differences between bacterial inocula ($P < 0.05$) based on a Tukey post-hoc test.

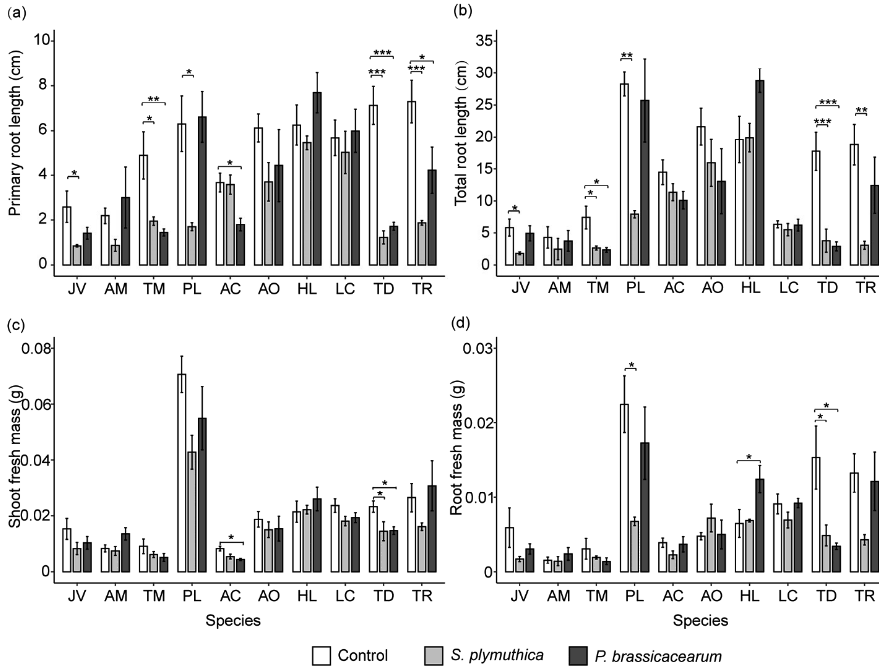

Fig 4.6. Mean (± SE) primary root length (a), total root length (b), shoot (c) and root fresh mass (d) of *J. vulgaris* and nine other plant species in the presence and absence of one of the two bacteria in-vitro. In (a, b, c and d) asterisks indicate significant differences between bacterial inoculum and control treatment based on a Dunnett post hoc test of a one-way ANOVA for each plant species. *, **, *** indicates significant differences at $P < 0.05$, 0.01 or 0.001, respectively. Abbreviations of species: JV = *Jacobaea vulgaris*, AM = *Achillea millefolium*, TM = *Tripleurospermum maritimum*, PL = *Plantago lanceolata*, AC = *Agrostis capillaris*, AO = *Anthoxanthum odoratum*, HL = *Holcus lanatus*, LC = *Lotus corniculatus*, TD = *Trifolium dubium*, TR = *Trifolium repens*.

Discussion

The aim of this study was to examine some of the mechanisms underlying the negative effects of two bacteria, *S. plymuthica* and *P. brassicacearum* isolated from roots of *J. vulgaris* on plant performance. We investigated the effects of bacterial strains on plant performance of *J. vulgaris* via root inoculation and via volatiles at various concentrations of the bacterial solution. Additionally, we explored whether the two root-associated bacteria, isolated from *J. vulgaris*, exhibit host specificity. This study reveals four main findings. First, both bacterial strains negatively affected *J. vulgaris* plants through root inoculation, while only the inoculum of *S.*

plymuthica exhibited a concentration-dependent pattern in-vitro. Second, the negative influence of the bacteria on performance of *J. vulgaris* through root inoculation was attributed to bacterial cells rather than the cell-free supernatants. Third, volatiles produced by the two bacteria inhibited seed germination and plant growth. Lastly, the effects of the two root-associated bacteria on plant growth were not specific to *J. vulgaris* and these effects varied among a range different plant species belonging to three different functional groups. Based on this study, we can conclude that the negative effects of two root-associated bacteria on *J. vulgaris* growth are mediated through root inoculation with bacterial cells rather than through the metabolites produced by these bacteria in the supernatant alone, and through volatiles produced by the bacteria.

Previous studies have reported that when root-associated bacteria impact plants through the production of metabolites, these effects are often concentration-dependent (Shantharaj et al. 2021; He et al. 2023). In our study, only *S. plymuthica* exhibited a concentration-dependent effects on *J. vulgaris* in-vitro. Furthermore, the cell-free supernatant of both bacteria did not influence *J. vulgaris* performance indicating the negative effects were likely due to direct interaction between bacterial cells and plants rather than through the production of non-volatile metabolites. Regarding to the concentration-dependent effect of *S. plymuthica*, an opposite pattern was observed in-vitro compared to soil. When bacterial inoculum was at the lowest concentration (OD_{600} = 0.5), plant performance was lowest on agar plates, but this was opposite to plant performance in soil. On agar plates, although the initial bacterial concentrations in the inoculum varied, we expected that bacteria grew rapidly, utilizing the available carbon (C) released by roots. Subsequently, this may have led to competition between bacterial cells and the plant at all concentrations and this may explain the overall decline in root growth (Kuzyakov and Xu, 2013). However, to our surprise, the negative effects of *S. plymuthica* decreased with an increasing bacterial concentration. One possible explanation is that at higher concentrations, the bacteria may exhibit a shift in their behavior or metabolism, leading to a reduction in the negative effects on root growth. This shift could result in a less deleterious interaction between the bacteria and the plant. Further investigations could focus on understanding the specific mechanisms underlying this concentration-dependent effect, such as examining the changes in gene expression in both the bacteria and the plant upon interaction at varying concentrations. When plants were grown in soil, negative effects of the two bacteria on plant growth disappeared at the lowest concentration of the inoculum. This may be because bacteria multiply more slowly in soil, or because plants were

able to uptake sufficient nutrients from the sterilized soil independent of the concentration of bacteria in the soil. This could also explain why the negative effects were more pronounced in medium than in soil. Future studies should determine how rapidly the two bacterial populations increase in soil after inoculation.

In contrast to our expectation, there were no significant interaction effects of bacterial inoculation and the presence of the ethylene inhibitor (AVG). Notably, the presence of the ethylene inhibitor itself significantly reduced root growth of *J. vulgaris*. AVG is known to inhibit the synthesis of ethylene by plants (Adams and Yang 1981; Yang and Hoffman 1984). A previous study has found that the presence of AVG suppressed the development of short and thick roots in *Vicia sativa* that are induced by Rhizobium bacteria under normal conditions (Zaat et al. 1989). In our study, the presence of AVG did not restore the root phenotype induced by bacterial cells, but instead, reduced root growth. One possible explanation is that the concentration of AVG used in the experiment was too high. Based on these results we cannot conclude that the inhibitory effects on root growth by the two bacteria are caused by the production of ethylene in plants. To confirm whether bacterial inoculation reduces root growth through the induction of the ethylene pathway, gene expression in plants needs to be examined.

Volatiles from *S. plymuthica* reduced seed germination by 62%, and volatiles from *P. brassicacearum* completely inhibited seed germination. Furthermore, volatiles from *P. brassicacearum* consistently suppressed seedling growth, whereas volatiles from *S. plymuthica* had no effects on seedling growth at the lowest concentration but inhibitory effects appeared at higher concentrations. Many *Pseudomonas* strains have been found to release hydrogen cyanide (HCN), which is extremely toxic for living organisms (Blom et al. 2011b; Bailly et al. 2014; Ossowicki et al. 2017). In our study, *P. brassicacearum* has been found to produce HCN as well (see preliminary results in Fig S4.2). Therefore, the consistent inhibitory effects of *P. brassicacearum* volatiles on seed germination and seedling growth may be attributed to the release of HCN. Previous studies have reported that the effects of bacterial volatiles on seedling growth were highly dependent on the amount of inoculum used (Blom et al. 2011). Some studies have found that *S. plymuthica* can promote plant growth, while other evidence suggests that volatiles emitted by *S. plymuthica* have negative effects on plant growth (Velázquez-Becerra et al. 2011; del Carmen Orozco-Mosqueda et al. 2013; Zamioudis et al. 2015). The reported

volatiles emitted by *S. plymuthica* include carbon dioxide, ammonia, dimethyl trisulfide (DMDS) and sodorifen (Mai 2018). Notably, we observed when the amount of inoculum of *S. plymuthica* was the lowest (1 spot), plants exhibited an enhanced growth particularly in shoot dry mass, although this did not differ from the control. One possible explanation is that plants utilized the carbon dioxide emitted by *S. plymuthica*. We speculate that at higher concentrations, the accumulation of dimethyl disulfide (DMDS) and ammonia may have led to negative effects on *J. vulgaris*. Further studies are needed to identify and examine the specific compounds present in the volatiles released by *S. plymuthica* and their effects on plant growth.

The bacteria that were used in this study were isolated from *J. vulgaris* roots, which is a species with high alkaloid concentrations in root tissues. These alkaloids are known to exhibit negatively effects on many bacterial species (Joosten and van Veen 2011). Hence we expected that these two bacteria, that apparently can survive in an environment with high alkaloid concentrations, would be specialized to *J. vulgaris*. However, contrary to our expectations, the negative effects of the two bacteria were not specific to *J. vulgaris*, but also affected some of the other plant species tested. In our screening, the grasses, overall, appeared to be less affected by bacterial inoculation. This may be because grasses often have fibrous roots, which made them less susceptible to the impact of bacterial cells (Fry et al. 2018). Interestingly, we observed that *P. brassicacearum* caused an increase in root biomass of the grass *H. lanatus*, and other studies have shown that *H. lanatus* grows well in the soil conditioned by *J. vulgaris* (Bezemer et al. 2018; Hannula et al. 2021; Steinauer et al. 2023). This suggests that *H. lanatus* may be able to utilize extracellular enzymes or other compounds released by *P. brassicacearum.*

In conclusion, our study demonstrates that both bacterial cells and volatiles from two root-associated bacteria negatively affect root growth of *J. vulgaris*. However, these effects are not specific to *J. vulgaris* and both bacteria also have negative effects on the growth of other plant species. *J. vulgaris* is an unwanted outbreak species in Europe and an invasive species in other continents. Even though the bacteria have negative effects on the growth of *J. vulgaris*, their negative effects on other plant species hampers their potential use as biocontrol agents.

Acknowledgements

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Declaration

X.Y.L., T.M.B., K.V. and S.T.E.L. designed and developed the study. X.Y.L. carried out experiments. X.Y.L. analyzed all data. X.Y.L. wrote the first version of the manuscript with revisions from T.M.B., K.V. and S.T.E.L.. All authors contributed to subsequent versions of the manuscript.

Supporting information

Table S4.1 Results of linear regression models testing the relationship between concentration of bacterial inoculation and root morphology traits, fresh shoot and root biomass, and leaf chlorophyll and carotenoid content of *J. vulgaris* seedlings through root inoculation in-vitro. Presented are estimates, *P* values and adjusted R^2 from linear regression models. *, **, or *** indicate significant difference at $P < 0.05$, 0.01 or 0.001.

	S. plymuthica inoculum			*P. brassicacearum* inoculum		
	Estimate	P value	R^2	Estimate	P value	R^2
Primary root length	2.18	0.01*	0.31	-0.10	0.91	-0.08
No. of lateral roots	6.75	0.10	0.12	-1.71	0.73	-0.07
Average lateral root length	0.61	< 0.001***	0.55	-0.19	0.53	-0.04
Total root length	15.96	< 0.01**	0.46	-4.66	0.55	-0.05
Fresh shoot biomass	0.02	0.27	0.02	-0.01	0.51	-0.04
Fresh root biomass	0.02	0.03*	0.25	0.00	0.92	-0.08
Leaf chlorophyll content	-7.33	0.50	-0.04	-13.38	0.40	-0.02
Leaf carotenoid content	-2.93	0.18	0.07	-1.57	0.59	-0.06

Table S4.2 Results of linear regression models testing the relationship between concentration of bacterial inoculation and shoot and root dry mass, specific root length, and total root length of *J. vulgaris* seedlings through root inoculation when plants were grown in soil. Presented are estimates, *P* values and R^2 from linear regression models.

	S. plymuthica inoculum			*P. brassicacearum* inoculum		
	Estimate	P value	R^2	Estimate	P value	R^2
Shoot dry mass	-0.51	0.30	0.01	-0.53	0.47	-0.03
Root dry mass	0.03	0.88	-0.05	0.83	0.11	0.09
Specific root length	-14.80	0.14	0.07	-15.34	0.21	0.04
Total root length	-6.77	0.11	0.09	-0.79	0.92	-0.06

Table S4.3 Results of one-way ANOVA testing effects of inoculum (bacterial cells or supernatant of one of two bacterial strains or control) on primary root length, number of lateral roots, average lateral root length and total root length of *J. vulgaris* in-vitro. Presented are degrees of freedom (df) and F-values. *** indicates significant difference at $P < 0.001$.

Root traits	Inoculum	
	df	F-value
Primary root length	4, 20	12.93***
Number of lateral roots	4, 20	1.78
Average lateral root length	4, 20	13.02***
Total root length	2, 36	10.00***

Table S4.4 Results of two-way ANOVA testing inoculum (3 levels: two bacterial inoculum and control) and the presence/absence of ethylene inhibitor (AVG) and their interaction on primary root length, number of lateral roots, average lateral root length and total root length of *J. vulgaris* in-vitro. Presented are degrees of freedom (df) and F-values. *, *** indicates significant difference at $P < 0.05$ or $P < 0.001$.

	Inoculum		AVG		Inoculum × AVG	
	df	F-value	df	F-value	df	F-value
Primary root length	2, 21	19.39***	1, 21	4.83*	2, 21	3.13
Number of lateral roots	2, 21	3.70*	1, 21	0.69	2, 21	1.55
Average lateral root length	2, 21	49.76***	1, 21	5.92*	2, 21	3.06
Total root length	2, 21	25.37***	1, 21	8.59**	2, 21	2.34

Table S4.5 Results of two-way ANOVA testing in the presence and absence of volatile emitted by one of two bacterial strains (3 levels) and three different amounts of inoculum and their interaction on shoot and root dry mass, and total root length of *J. vulgaris* seedlings per plate in-vitro (four seedlings together in a Petri dish). Presented are degrees of freedom (df) and F-values. *, *** indicates significant difference at $P < 0.05$ or $P < 0.001$.

	Inoculum		Amount of inoculum		Inoculum ×Amount of inoculum	
	df	F-value	df	F-value	df	F-value
Shoot dry mass	2, 39	21.65***	2, 39	0.04	4, 39	2.68*
Root dry mass	2, 39	13.79***	2, 39	0.93	4, 39	3.43*
Total root length	2, 40	34.72***	2, 40	0.38	4, 40	3.83**

Table S4.6 Results of two-way ANOVA testing the inoculum (three levels: two bacterial strains and the control), identity of plant species (10 species) and their interaction on primary root length, total root length, and fresh shoot and root biomass in-vitro. Presented are degrees of freedom (df), F-values and *P* values. *** indicates significant difference at $P < 0.001$.

	Inoculum		Plant species		Inoculum × Plant species	
	df	F-value	df	F-value	df	F-value
Primary root length	2, 120	2.88	9, 120	5.56***	18, 120	3.82***
Total root length	2, 120	2.09	9, 120	9.35***	18, 120	3.12***
Fresh shoot biomass	2, 110	2.38	9, 110	11.03***	18, 110	1.03
Fresh root biomass	2, 110	1.97	9, 110	5.57***	18, 110	1.42

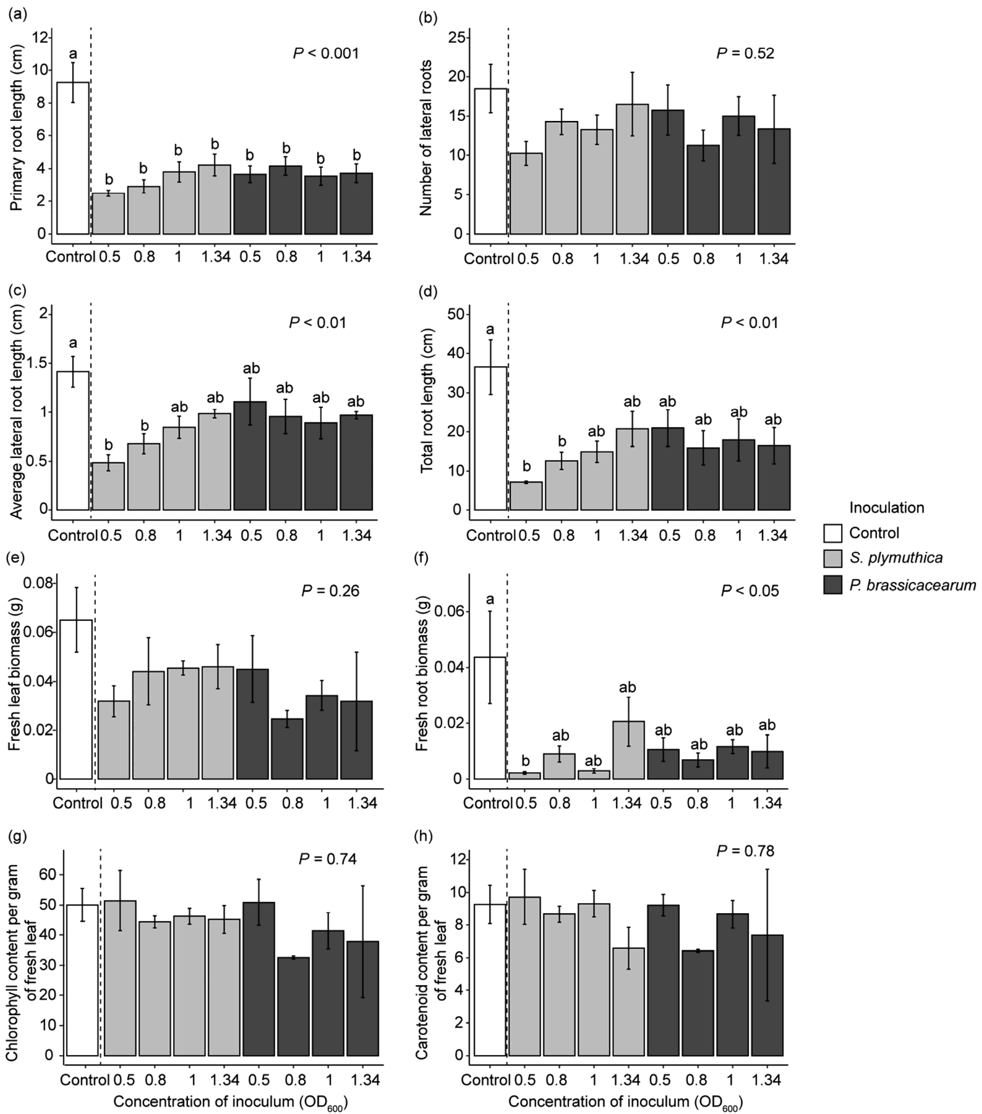

Fig S4.1. Mean (± SE) primary root length (a), number of lateral roots (b), average lateral root length (c), total root length (d), fresh shoot (e) and root biomass (f), leaf chlorophyll (g) and carotenoid content (h) in the presence and absence of one of the two bacterial inoculum at varying concentrations in-vitro. In (a, c, d, f), letters indicate significant differences between the inoculation treatments ($P < 0.05$) based on a Tukey post-hoc test and bars with identical letters are not significantly different. In (b, e, g, h) there was no significant differences ($P > 0.05$) between inoculum in a one-way ANOVA. P values of one-way ANOVA are also presented.

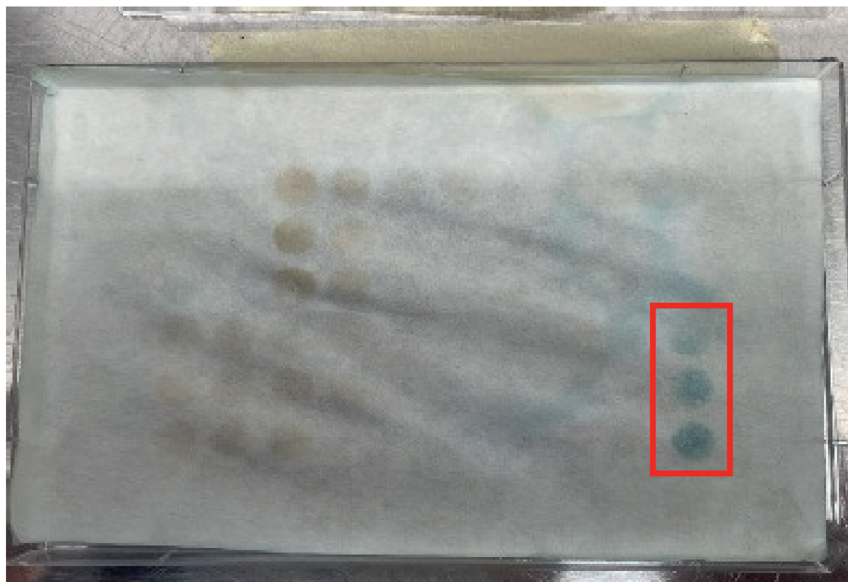

Fig S4.2. The preliminary test for hydrogen cyanide (HCN) production. The method was adapted from Ossowicki et al. (2017) and briefly introduced here: Sterile Whatmann paper (3 mm thick and size 128 × 86 mm) was soaked with suspension containing 5 mg of both 4,4'-methylenebis (N, N-dimethylaniline) and methyl acetoacetate, copper (II) (Sigma-Aldrich, USA) in chloroform and dried in sterile conditions. One hundred µl of TSB medium was poured into the wells of the 96-well plate. Wells were inoculated with or without 1 µl overnight culture of different bacteria. All bacterial cultures were diluted to OD_{600} = 1. Plate was covered with freshly prepared and dried Whatmann paper and the plastic cover. The plate was incubated 24 h at 25 C. Change of color from white to blue indicates the production of the hydrogen cyanide (indicated by the red rectangle).