Pea-Saving Partners: Bacillus and Pseudomonas Combat Downy Mildew in Pea Crops

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Pea-Saving Partners: *Bacillus* and *Pseudomonas* Combat Downy Mildew in Pea Crops

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Keywords: *Bacillus* | biological control agents | downy mildews | legumes | oomycetes | *Pseudomonas*

ABSTRACT

Downy mildew (DM) is a destructive disease that significantly reduces the yield and quality of important pulses (legumes) and horticultural crops, particularly during humid and cool seasons. This disease is caused by obligate and host-specific oomycete pathogens. Controlling the pathogen is challenging due to its long-term spore survival and rapid mutation. Although chemical pesticides have been the most effective method to control DM pathogens, their environmental hazards remain a global concern. Current research is focused on exploring the potential of microbial biological control agents (MBCA), particularly rhizobacteria strains of the genera *Bacillus* and *Pseudomonas*, which have shown suppression of plant pathogens. However, to date, no MBCA has been reported to be effective against DM pathogens in pulses. We investigated the effectiveness of *Bacillus* and *Pseudomonas* strains as potential biopesticides against the pea downy mildew pathogen *Peronospora viciae* f. sp. *pisi* (Pvp). In vitro bioassays showed 100% inhibition of Pvp spore germination compared to the control. In planta antagonism assays further demonstrated significant suppression (> 80%) of Pvp sporulation in pea plants sprayed with strains of *Bacillus velezensis* or *Pseudomonas fluorescens* or their filtrates. The drench application also showed significant effects where either a *Pseudomonas* or cold-adapted *Bacillus* strain was used. We observed a synergistic effect for the dual foliar application of the microbes compared to individual application (40%–78% suppression). Molecular biomass analysis supported these findings. Based on these results, we conclude that *Bacillus* and *Pseudomonas* MBCAs could be highly effective in combating Pvp infections in the field.

1 | Introduction

Plant pathogens have been serious and persistent threats to global crop yield and quality (Ristaino et al. 2021; Yang et al. 2022). Along with pests, they cause up to 40% crop loss globally each year, which cost the global economy billions of dollars (Jamiołkowska 2020; Pandit et al. 2022). The global concern is not only for present threats from existing plant pathogens that have persisted for centuries, but also from emerging ones as occasioned by climate change (Burdon and Zhan 2020;

Corredor-Moreno and Saunders 2020; Ristaino et al. 2021; Velasquez et al. 2018). Pathogenic attacks are one of the primary causes of global food insecurity, and their impacts could worsen by 2050 when the world's human population is projected to reach approximately 10 billion (Velasquez et al. 2018; Zhao et al. 2022). This further highlights the global urgency of reducing pathogen-induced yield loss (McDonald and Stukenbrock 2016; Savary et al. 2019).

Downy mildew (DM) is one of the world's most devastating plant diseases; it seriously reduces yield (up to 80%) and quality of globally important pulses, vegetables, fruits and ornamentals, most notably during humid-cool seasons that are usually synchronised with the cropping seasons (Salcedo et al. 2021; Siddaiah et al. 2017). The disease is caused by obligate biotrophic pathogens that exhibit host-specificity (Choi and Thines 2015; Thines 2009; van Damme et al. 2009). Some of the common downy mildew pathogens are *Peronospora viciae* f. sp. *pisi* (PvP; pea), *P. viciae* f. sp. *fabae* (faba beans), *Hyaloperonospora brassicae* (brassica), *H. parasitica* (*Capsella bursa-pastoris*), *P. belbahrii* (basil), *P. destructor* (onion), *P. manshurica* (soyabean), *P. effusa* (spinach), *Bremia lactucae* (lettuce), *Plasmopara viticola* (grapevine), *Pseudoperonospora cubensis* (cucurbits) and *Plasmopara halstedii* (sunflower) (Salcedo et al. 2021; Thines 2009; Tör et al. 2023). They attack above-ground plant parts such as the leaves, stems, flowers, pods and fruits (Koledenkova et al. 2022). The effects on plants include stunted growth, distortion and discolouration of leaves, and typical fluffy mould-like growth on the surface of the leaves (Bandamaravuri et al. 2020). The pathogens are resilient and adaptable to new environments (Delmas et al. 2016) due to their ability to survive as long-lasting spores (oospores) under harsh conditions or in the absence of host plants and to rapidly mutate to evade or overcome pesticides or host defences (Koledenkova et al. 2022).

For many years, chemical pesticides such as Wakil XL (metalaxyl-M, fludioxonil and cymoxanil) have been the most effective method to control DM pathogens such as Pvp in peas. However, indiscriminate and continuous use of these chemicals has caused a lot of short-term and long-term hazards particularly to the environment and ecosystem, and accumulation of their associated toxic residues in the food chains pose serious threats to human and animal health and wellbeing (Aktar et al. 2009; Damalas and Eleftherohorinos 2011; Lahlali et al. 2022). Strict regulations have been implemented on the timing and usage of pesticides in different countries and more restrictions will follow, with a long-term aim of achieving full-scale global sustainable crop production (Lahlali et al. 2022). Towards this aim, research is increasingly focusing on developing new alternatives for managing plant pathogens that will not only be effective, but also safe, sustainable and ecofriendly. Some of the non-chemical pesticides that hold great promise are biological/biocontrol agents or their byproducts (Jimenez-Quiros et al. 2022; Pandit et al. 2022), plant extracts (Cowan 1999), phage therapy (Erdrich et al. 2024; Villalpando-Aguilar et al. 2022) and more recently small interfering RNAs, popularly called spray-induced gene silencing (Bilir et al. 2022).

Microbial biological control agents (MBCA) have been the most broadly studied and used biopesticides (Jaiswal et al. 2022). Among them, rhizobacteria of the genera *Bacillus* and *Pseudomonas* have been shown to suppress a wide range of plant pathogens of different phyla/kingdoms (Dragana et al. 2017; Gao et al. 2012; Mnif and Ghribi 2015). We previously demonstrated that a strain of *Bacillus velezensis* (EU07), whose genome was sequenced (Baysal et al. 2024), effectively controlled *Fusarium graminearum*, the pathogen that causes Fusarium head blight disease in cereals (Jimenez-Quiros et al. 2022). Although some nonpathogenic *Fusarium* and *Trichoderma* isolates have been reported to be effective against some DM pathogens (Bakshi et al. 2001; Nandini et al. 2021; Núñez-Palenius et al. 2022), there are no reports of biocontrol of a DM pathogen that affects important legume crops such as peas. To address this critical research gap, this study aimed to investigate the effectiveness of *Bacillus* and *Pseudomonas* strains as potential biopesticides against Pvp.

2 | Materials and Methods

2.1 | Biological Agents Used and Preparation of Inoculum

We tested two *B. velezensis* strains that are commercially available as biocontrol products: Serenade (QST713) and TAEGRO370 (FZB24). Strain FZB24 is the type strain of *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011) but this taxon is now properly considered as belonging to the species *B. velezensis* (Parte 2018). We also tested a non-commercial strain, *B. velezensis* EU07 (Baysal et al. 2013; Jimenez-Quiros et al. 2022), whose genome sequence is almost identical to that of QST713 (Baysal et al. 2024). We also evaluated the cold-adapted *Bacillus* strains (K7, K9, K11, K12 and B2-6) isolated from persimmon (tree) leaf litter collected from the village of Ardiçlı, located at 1200 m a.s.l. in the Bolkar Mountains, Turkey, during the cold season after the snow melted. This region experiences significant temperature fluctuations, ranging from −27°C in winter to 20°C in summer, providing a cold-adapted microbiome in contrast to the warmer conditions of Tarsus. In addition, *Pseudomonas fluorescens* LZB 065, procured from Blades Biological Ltd., UK, was streaked on Luria Bertani (LB) agar (Bertani 1951) and incubated at 15°C or 28°C for 2 days to produce single colonies. After genetic identity verification through PCR and sequencing procedures, a colony from each strain was used to produce glycerol stocks that were flash-frozen in liquid nitrogen and stored at −80°C until needed. To make bacterial broths, bacteria were streaked on LB plates from glycerol stocks and a single colony was grown in liquid LB medium in a shaker (15°C or 28°C, 220 rpm) until OD_{600} of approximately 2 was obtained.

2.2 | Verification of *Bacillus* QST713, FZB24 and EU07 Strains

We reconfirmed the genetic identity of the *Bacillus* strains QST713, FZB24 and EU07 through a combination of colony PCR and Sanger sequencing techniques. Specifically, the PCR protocol for amplification of 16S rRNA genes involved an initial denaturation step at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min; and final extension at 72°C for 5 min. Gel electrophoresis validated the expected band

size of the PCR products. Subsequently, the purified bands underwent Sanger sequencing. To further validate our findings, we performed BLASTN analysis against known *Bacillus* 16S rRNA gene sequences in the NCBI databases (Altschul et al. 1990; Baysal et al. 2024; Jimenez-Quiros et al. 2022). Finally, these reverified bacterial colonies were maintained as glycerol stocks, and used to generate bacterial broth used for further steps of the studies. The primers used are presented in Table S1.

2.3 | Whole-Genome Sequencing of *Pseudomonas* and Cold-Adapted *Bacillus* Strains

We tested culture filtrates of five cold-adapted *Bacillus* strains (K7, K9, K11, K12 and B2-6) by spraying 4-day-old infected pea plants. The strain K11 performed the best out of these five strains to suppress the DM sporulation, and therefore we concentrated on this strain. We carried out whole-genome sequencing of the *Bacillus* K11 and *P. fluorescens* LZB 065 strains, as they had not been sequenced prior to this study. Overnight liquid cultures of the bacteria were produced from their single colonies. To harvest the pellets, 2 mL culture was centrifuged for 5 min at 8000 *g* and the supernatant was discarded. Genomic (g) DNA was extracted following the steps explained in Meridian Bioscience ISOLATE II Genomic DNA Kit (Scientific Laboratory Supplies Ltd). The quality of the gDNAs was assessed using the Agilent 4200 TapeStation to confirm that they met the required standards for genomic sequencing. The samples were sent to Novogene, UK, for whole-genome sequencing, generating 150-bp paired reads via the Illumina NovaSeq 6000 instrument.

2.4 | Genome Assembly and Annotation

Prior to genome assembly, we filtered and trimmed the raw Illumina sequence reads using TrimGalore v. 0.6.7, which incorporates Cutadapt v. 3.5 (Krueger 2019). The -q parameter was set to 30 and we used the paired option. For de novo assembly of these processed reads, we used Unicycler v. 0.5.1 (Wick et al. 2017), which incorporates SPAdes v. 4.0.0 (Bankevich et al. 2012). The command line was: 'unicycler-1 short_reads_1.fastq.gz-2 short_reads_2.fastq.gz -o output_dir'. We submitted the resulting genome sequence assemblies to GenBank (Benson 2004) via the NCBI submission portal (Sayers et al. 2019). Genome annotation was generated by the NCBI's PGAP pipeline v. 6.8 (Tatusova et al. 2016).

2.5 | Maintenance and Propagation of Pvp on Pea Plants

The purified Pvp isolate 20-1-3 (DM3), originally collected in 2020 from infected pea plants in Cambridge, UK, was obtained from the culture collection of NIAB and used throughout this study. All in planta experiments were conducted using the pea cv. Maro, a standard susceptible control cultivar for DM. It was selected to avoid confounding effects from host genetic resistance, allowing clear assessment of biocontrol efficacy. The shoots from Pvp-infected plants of pea cv. Maro were harvested, placed in a beaker with sterile water and gently agitated to shake spores off the shoots. The spore suspension was then filtered through a layer of Miracloth into clean glassware. The spore count was determined using a haemocytometer under a light microscope and adjusted to 5×10^4 spores/mL. The spore solution obtained was used to inoculate 4-day-old pregerminated pea seeds. The seedlings were immersed in the spore solution for 30 min with gentle shaking every 5 min to ensure uniform inoculation. They were then immediately sown in standard compost (Levington Advance Seed and Modular F2S Compost) and transferred to a growth cabinet (16°C, 12 h light and 12 h dark). Ten days post-inoculation (dpi), the inoculated plants were covered with transparent lids (with the edges sealed with electrical tape) for 2 days to aid the sporulation of the pathogen. The spores formed were harvested and either used for experiments or repropagated to maintain the pathogen on the host as summarised in Figure 1.

2.6 | In Vitro Antagonism Bioassays of *Bacillus* and *Pseudomonas* Strains on Pvp Spore Germination

The Pvp spores were harvested and cleaned by centrifugation at approximately 1500 *g* for 3 min and washing in ice-cold water, repeated twice, followed by resuspension in water. Full-strength cultures (OD_{600}~2) of the biocontrol *Bacillus* strains were centrifuged at 7000 *g* for 5 min to separate the cells (pellets) from the filtrates. Different concentrations of the filtrates (100%, 50%, 25% and 12.5%) and bacterial cells (OD_{600} of 1, 0.5 and 0.25) were separately mixed with the Pvp spores (final concentration of 25,000 spores/mL). 100 μL from each mix was plated on a microscope slide, two spots per slide, placed on a transparent Petri dish. The lids were covered, and the Petri dishes were placed in the growth cabinet (16°C, 12 h light and 12 h dark) for a day to allow spore germination. To quantify the antagonistic effect of *Bacillus/Pseudomonas* on Pvp, the percentage of germination from both treated and untreated spores were measured and compared using statistical analysis.

2.7 | In Planta Antagonism Assay of *Bacillus* and *Pseudomonas* Strains on Pvp Development

Two different methods were used: drenching and foliar spray applications. For the drenching method, 4-day-old pea seedlings were inoculated with Pvp spores (25,000 spores/mL) and planted in 15 multicell trays filled with standard compost (Levington Advance Seed and Modular F2S Compost). Each seedling was drenched with 25 mL of biocontrol full-strength culture (OD_{600}~2) or LB medium as a control. The plants were then moved to a growth cabinet (16°C, 12 h light and 12 h dark). Ten days after inoculation, the trays of plants were covered with transparent lids for 2 days to allow the pathogen to sporulate. The inoculated plants were then harvested, and spores were counted.

For the foliar spraying assay, culture filtrates (supernatant after centrifugation and filtering cultures through 0.22 μm filters; EMD Millipore Millex) and bacterial cells (pellets resuspended in water) were sprayed on 10-day-old Pvp-inoculated pea plants using an electric atomiser. Each plant was treated with 20 mL of either individual or combined biocontrol agents (cells or filtrates), supplemented with 0.05% Silwet L-77. Full

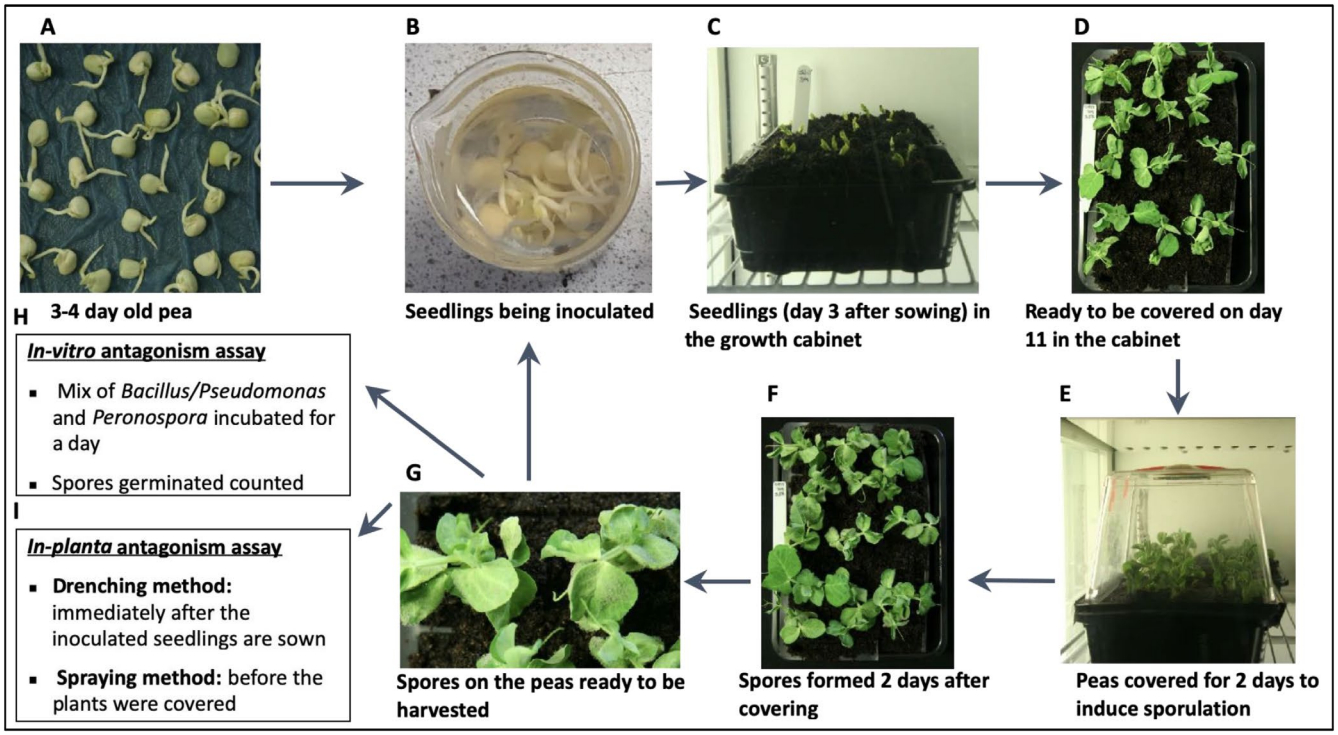

FIGURE 1 | Inoculation of pea seedlings with *Peronospora viciae* f. sp. *pisi* (Pvp). (A) Germinated pea seedlings ready for Pvp inoculation. (B) Seedlings treated with Pvp spores (25,000 spores/mL) for 30 min for inoculations. (C) Pvp-inoculated seedlings growing in the growth cabinet. (D) Inoculated plants ready to be covered. (E) Plants covered with a transparent lid to maintain humidity and induce sporulation of Pvp. (F, G) Sporulation occurred after covering the trays. (H, I) Spores were harvested and used for the biocontrol antagonism assays. [Colour figure can be viewed at wileyonlinelibrary.com]

strength filtrates and the cells with $OD_{600} = 5$ were used. LB and water were applied as controls for biocontrol filtrates and cells, respectively. The plants were allowed to air-dry for 5 min, covered with lids, and moved back to the cabinet for a further 2 days to allow the pathogen to sporulate. The sporulated plants were then harvested, and spore counts were carried out.

Wakil XL-treated seeds were obtained as precoated commercial seed stock (https://churchofbures.co.uk) and were used as a positive control in this experiment. The treatment is designed to protect against systemic infection via root infection.

2.8 | DNA Extraction

DNA was extracted from the whole Pvp-inoculated pea plants that were either drenched or sprayed with the biocontrol treatment or experimental control (LB or water). The extraction was performed using the traditional cetyltrimethylammonium bromide (CTAB) method with polyvinylpyrrolidone (PVP), as described by Koh et al. (2021).

2.9 | Downy Mildew Biomass Analysis Using Quantitative PCR

The quantitative PCR (qPCR) technique was used to measure the Pvp mycelial biomass using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystem) as the preferred master mix. For each sample, a reaction mix of 20 μL was prepared, which included 10 μL of SyGreen Mix Lo-ROX, 0.8 μL each of 10 μM forward and reverse primers, 1 μL of 100 ng DNA template and 7.4 μL of nuclease-free water. The PCR was performed in a Roche 480 II thermocycler with the following programme: 3 min at 95°C, followed by 40 cycles of 5 s at 95°C, 20 s with a touchdown step size of 0.8°C from 65°C to 60°C, and 1 min at 40°C. The Pvp-*Actin* primer pair was used to amplify a unique region of the Pvp-*Actin* gene, and the Ps-*Actin* (pea-Actin) primer pair was used for normalisation (housekeeping) of host DNA. Three biological replicates, each with two technical repeats, were used. To compare the relative abundance of Pvp-*Actin* to Ps-*Actin* for the biocontrol-treated and the mock-treated samples, the fold change was calculated relative to the control ($2^{-\Delta\Delta Ct}$) as explained by Schmittgen and Livak (2008). DNA from *Bacillus* and *Pseudomonas* was not quantified, as the focus was on pathogen suppression.

2.10 | Statistical Analysis

For experiments involving three or more treatments, a parametric one-way ANOVA was performed, followed by Tukey's B test to compare and group means based on statistically significant differences. For comparisons involving only two treatments, a two-tailed, unpaired, heteroscedastic *t* test was used. Bar plots were generated using Microsoft Excel v. 16.89.1 (24091630), while box plots were constructed using R software v. 4.4.1 ('Race for Your Life') with RStudio IDE v. 2024.04.2 + 764 and the ggplot2 package (Wickham 2016).

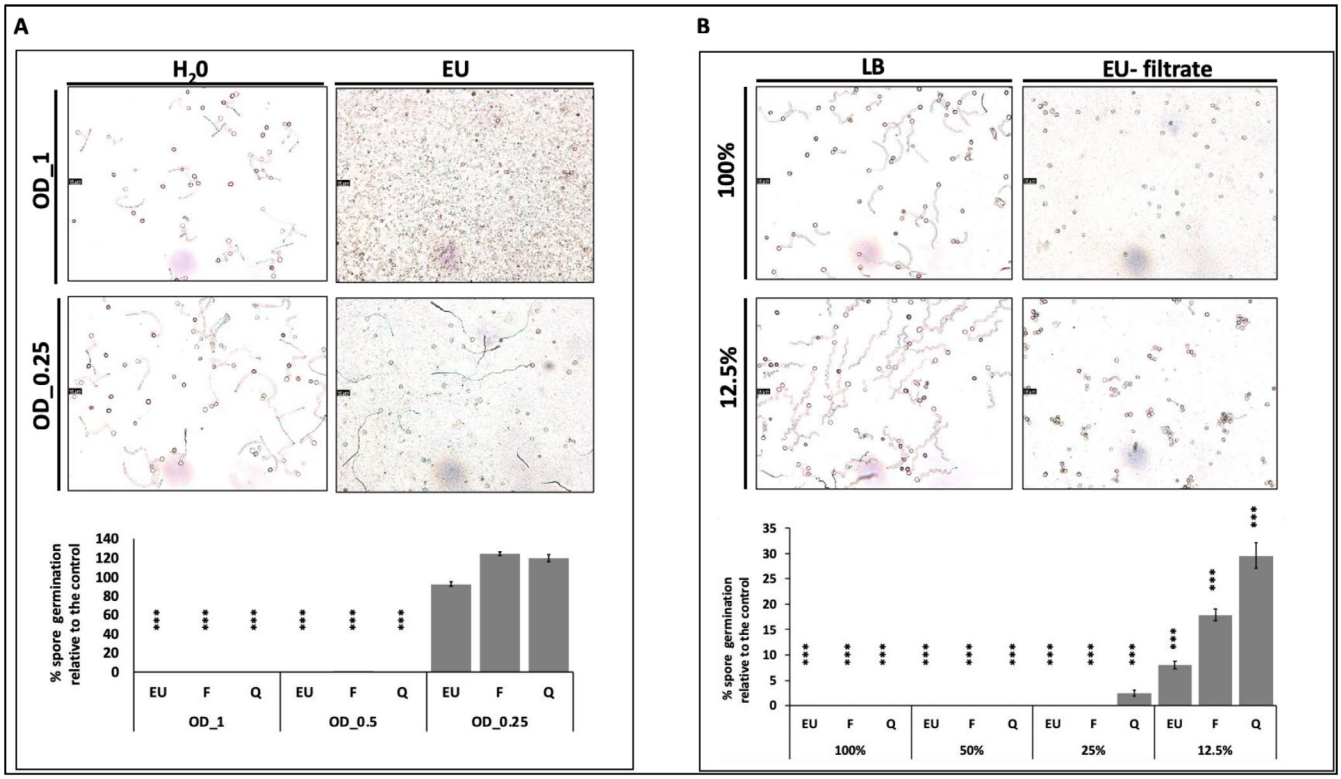

FIGURE 2 | Inhibitory effect of *Bacillus* strains on *Peronospora viciae* f. sp. *pisi* (Pvp) spore germination. *Bacillus* strains EU07 (EU), FZB24 (F) and QST713 (Q) were tested. The effects of the biocontrol are presented relative to 100% of the mock treatment. Antagonism assay of cells (A) and filtrates (B) of the three *Bacillus* strains in varying concentrations on Pvp spore germination percentage. OD_{600} of 1, 0.5 and 0.25 of bacterial cells and filtrate concentrations of 100%, 50%, 25% and 12.5% were tested, while water was used as the mock treatment for the bacterial cells and Luria Bertani medium (LB) for the filtrates. Spore germination pictures are given only for the *Bacillus* strain EU07 as a representative. The bar plots on the bottom of each panel show combined data for each of the three independent biological repeats. Error bars represent standard error from the three repeats. *t* test was used to compare the means for significant differences. ***$p < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

2.11 | Bioinformatics

For identification of bacteria to species level, we uploaded genome assemblies to the Type Strain Genome Server (TYGS; Meier-Kolthoff and Göker 2019; Meier-Kolthoff et al. 2022) at https://tygs.dsmz.de/user_requests/new.

To calculate average nucleotide identities, we used FastANI v. 1.33 (Jain et al. 2018). To generate a maximum-likelihood phylogenetic tree based on genome-wide single-nucleotide variants, we used PhaME v. 1.0.2 (Shakya et al. 2020) with FastTree v. 2.1.11 (Price et al. 2010). This generated a tree which we graphically rendered using the Interactive Tree of Life (iTOL) v. 7.0 (Letunic and Bork 2021). Essentially, we used the same protocols for ANI and phylogenomics analysis as described previously (Baysal et al. 2024) but included additional genome sequences with strain K11.

3 | Results

3.1 | Effect of *Bacillus* and *Pseudomonas* Strains on Germination of Pvp Spores In Vitro

Pvp spores were grown on pea plants, harvested and examined using in vitro antagonism bioassays. Three strains of *B. velezensis* (EU07, FZB24, QST713) and *P. fluorescens* were mixed with the Pvp spores and incubated on microscope slides overnight. The biocontrol agents completely suppressed Pvp spore germination (100%) when treated with bacterial cells at OD_{600} of 1 and 0.5. However, a lower bacterial cell concentration (OD_{600} of 0.25) did not show consistent inhibitory effects in the three biological repeats. Similarly, filtrates of the *Bacillus/Pseudomonas* strains at 100% and 50% concentrations showed 100% inhibitory effects (Figures 2 and 3, respectively). At the lowest concentration of 12.5%, there was still a significant reduction in spore germination percentage for *Bacillus/Pseudomonas*-treated spores compared the control (LB medium), although some spores (0.49%–29.5% relative to the control) were able to germinate.

3.2 | Control of DM With Wakil XL-Coated Pea Seeds

Pea seeds coated with Wakil XL were pregerminated, and the resulting seedlings were inoculated with the Pvp pathogen. As anticipated, the pea plants did not exhibit any symptoms of DM disease compared to the control plants, even when the Pvp spore concentration was doubled to 50,000 spores/mL (Figure 4A–C). In contrast, plants from untreated seeds showed full pathogen sporulation and disease symptoms (Figure 4D).

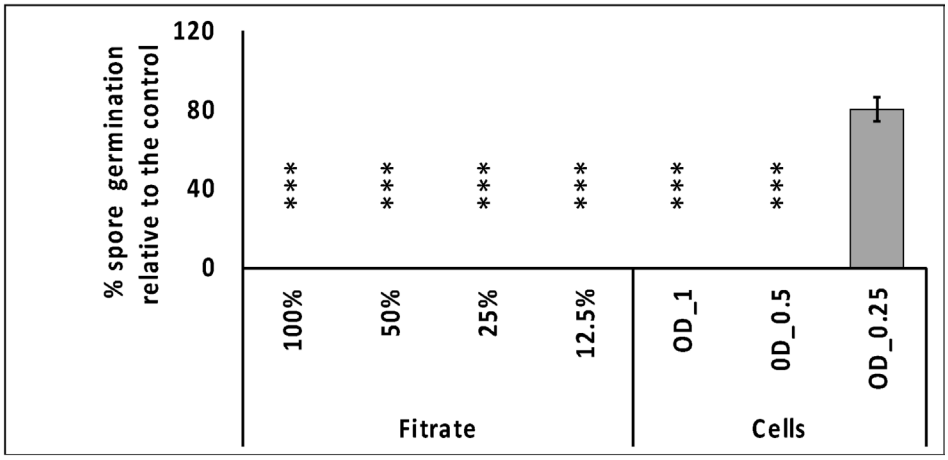

FIGURE 3 | Inhibitory effect of *Pseudomonas* strain on *Peronospora viciae* f. sp. *pisi* (Pvp) spore germination. The effect of the biocontrol is presented relative to 100% of the mock treatment. Antagonism assay of the biocontrol cells and filtrates of varying concentrations on Pvp spore germination percentage was tested. OD_{600} of 1, 0.5 and 0.25 of bacterial cells and filtrate concentrations of 100%, 50%, 25% and 12.5% were tested, while water was used as the mock treatment for the bacterial cells and Luria Bertani medium (LB) for the filtrates. Percentage of germinated spores was calculated. The bar plots show combined data for the three independent biological repeats. Error bars represent standard error from three technical replicates. *t* test was used to compare the means for significant differences. $^{***}p < 0.001$.

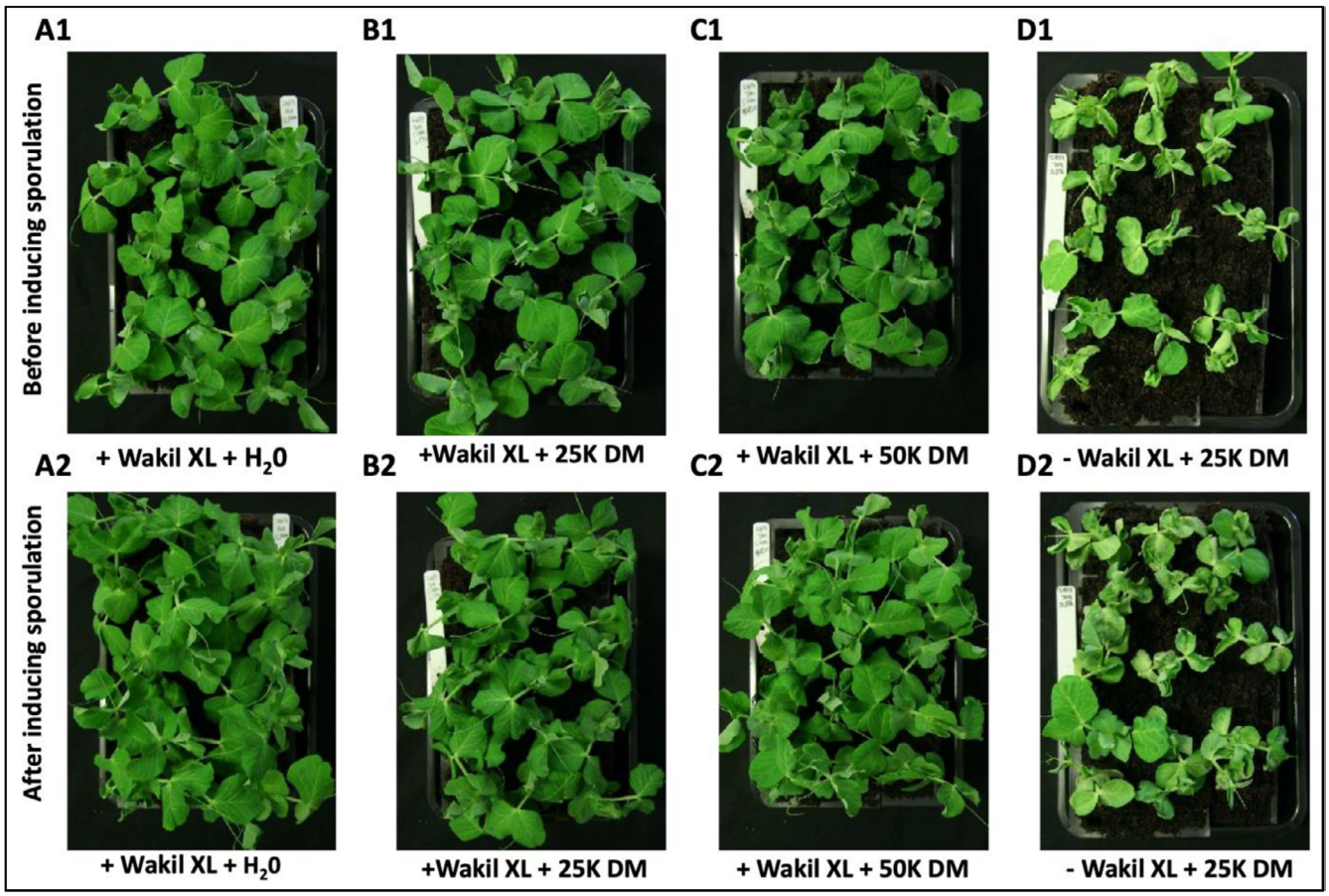

FIGURE 4 | Effectiveness of Wakil XL against *Peronospora viciae* f. sp. *pisi* (Pvp). (A1, A2) Pea plants from Wakil XL-coated seeds without Pvp inoculation, and with Pvp inoculation at (B1, B2) 25,000 spores/mL (25K DM) and (C1, C2) 50,000 spores/mL (50K DM). (D1, D2) Pea plants from control seeds (no Wakil XL) with Pvp inoculation at 25,000 spores/mL. Images in the top panel were taken 10 days post-inoculation, and those in the lower panel were taken 2 days after covering the pea plants. [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Effect of Soil Drenching With *Bacillus* and *Pseudomonas* Strains on Pvp Growth

We investigated whether soil drenching with biocontrol broths could inhibit Pvp growth. Spore count data indicated that the three tropical *Bacillus* strains (EU07, FZB24, QST713) did not consistently or significantly reduce Pvp spore counts (Figure 5). Although Repeats 1 and 2 showed higher counts than Repeat 3, this variation was not statistically significant compared to the LB control and probably reflects experimental variability. In contrast, cold-adapted *Bacillus* and *Pseudomonas* strains significantly ($p < 0.05$) reduced pathogen sporulation by up to 90% across all repeats (Figures 6 and 7 and S1).

3.4 | Effect of Soil Drenching on Pvp Biomass

We further investigated whether drenching peas with *Pseudomonas* reduced the total DNA of the pathogen. To assess this, the DNA of the Pvp-*Actin* gene, which plays a critical role in the pathogen's structure, movement and virulence, was quantified via qPCR. Consistent with the earlier spore count data, the Pvp DNA biomass analysis showed a significant ($p < 0.05$) decrease (65.8% less DNA compared to the control) in the Pvp-inoculated peas drenched with *Pseudomonas* compared to those drenched with LB medium (Figure 7D).

3.5 | Effect of Foliar Application of Biocontrol Agents or Filtrates on Pvp Growth

In addition, we tested the effectiveness of direct foliar application of selected biocontrol agents. Pea plants infected with Pvp and expected to produce spores were treated with either cells or filtrates from three *B. velezensis* (F, Q and EU) and one *P. fluorescens* strain. Only culture filtrates were tested for the cold-adapted *Bacillus* strains. The application of both cells and filtrates from all *Bacillus* strains reduced sporulation by 87.6%–96.7% for cells and 78.2%–96.5% for filtrates compared to the Pvp-infected control plants, which were not treated with *Bacillus* filtrates or cells. Similarly, *Pseudomonas* cells and filtrates reduced sporulation by up to 95.3% and 80.3%, respectively, significantly inhibiting Pvp sporulation in three separate trials (Figures 8 and 9, S2A,B and S3A,B).

To assess persistence of the biocontrol effect, plants treated with the EU strain were allowed to grow for an additional 5 days after initial Pvp sporulation. No pathogen recovery was observed on EU07-treated plants after visual inspection and examination under a stereomicroscope, while control plants continued to show Pvp sporulation (Figure 10). The extended observation was not performed for other biocontrol strains, and no quantitative spore counts were conducted during this period.

3.6 | Synergistic Effect of Dual Application of *Pseudomonas* and *Bacillus* Strains on DM Suppression

We conducted a study to determine if using both *Pseudomonas* and *Bacillus* bacterial strains together would have a greater impact on reducing pathogen growth compared to using them individually. We tested this by using the filtered byproducts of these bacteria. Considering their optimal growth temperature, we combined tropical *Bacillus* and *Pseudomonas* strains, both of which have an optimal growth temperature of 28°C. The combined application of both bacterial strains significantly decreased the pathogen Pvp spore load by 93.6%–97.1% compared to a control group using LB (Figure 11). While the combined treatment appeared to produce a greater effect (40%–78%) than the individual treatments, this difference was not statistically significant (Figure 12).

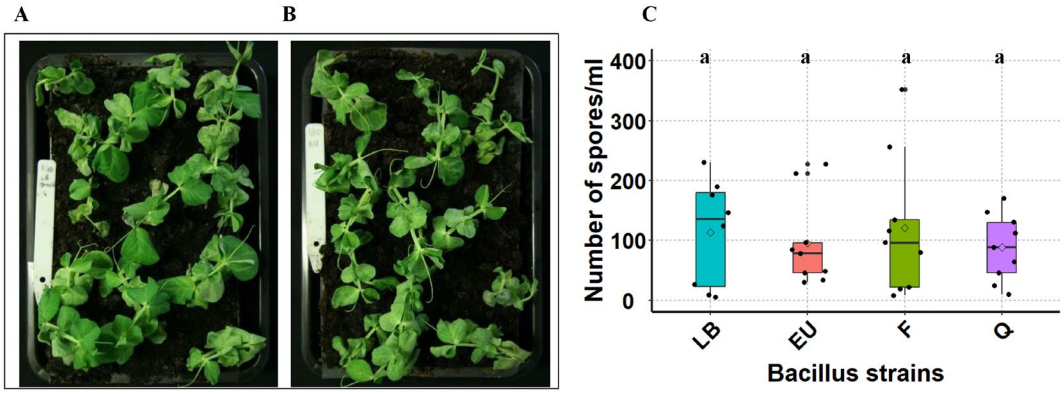

FIGURE 5 | Antagonism assay of drench application of tropical *Bacillus* broth on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old pea seedlings were inoculated with Pvp spores and sown in a standard compost. Biocontrol broths or Luria Bertani (LB) medium were applied immediately upon sowing the seedlings. After 10 days, the plants were covered for 2 days to induce Pvp spore formation. After sporulation, plants drenched with LB (A) and EU07 *Bacillus* broth (B) were photographed; mean spore counts for plants drenched with the three *Bacillus* strains EU07 (EU), FZB24 (F) and QST713 (Q) and LB (control) are shown in box plots (C). Plots show combined data from the three independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Identical lowercase letters above bars indicate no significant difference ($\alpha = 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

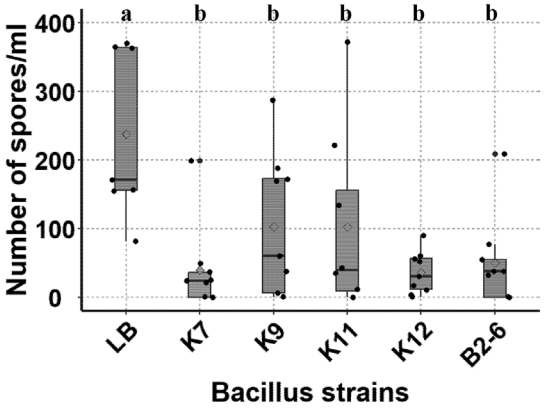

FIGURE 6 | Antagonism assay of drench application of cold-adapted *Bacillus* broth on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old pea seedlings were inoculated with Pvp spores and sown in standard compost. Biocontrol broths or Luria Bertani (LB) medium were applied immediately upon sowing the seedlings. After 10 days, the plants were covered for 2 days to induce Pvp spore formation. Mean spore counts for the plants drenched with the five *Bacillus* strains (K-7, K-9, K-11, K-12 and B2-6) and LB (control) are shown in box plots. Plots show combined data from two independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Different lowercase letters above bars indicate significant difference ($\alpha = 0.05$).

3.7 | Assessment of Side Effects on Healthy Pea After Application of Biocontrol Agent

The *Bacillus* EU07 strain (both cells and filtrates) was tested for potential visual side effects after foliar applications on healthy pea plants. As shown in Figure 13, no visual side effects were observed in the pea plants treated with either EU07 bacterial cells or corresponding filtrates compared to their respective controls. In fact, the biocontrol-treated plants, including the controls (mock), appeared as healthy and stress-free as the non-treated ones.

3.8 | Confirmation of *P. fluorescens* and Identification of the Cold-Adapted *Bacillus* K11 Using Genomic Analysis

We used genome sequences to confirm the identity of the commercially purchased *P. fluorescens* LZB 065. Our genome assembly for LZB 065 was almost identical (with average nucleotide identity [ANI] of 99.9948%) to the genome of *P. fluorescens* type strain DSM 50090 (GenBank: GCA_007858165.1). Although the vendor provides no information about the provenance of LZB 065, it is therefore probably derived from this type strain.

The genome sequence data for the *Bacillus* strain K11 provided confirmation that it belongs to the species *B. velezensis*. The

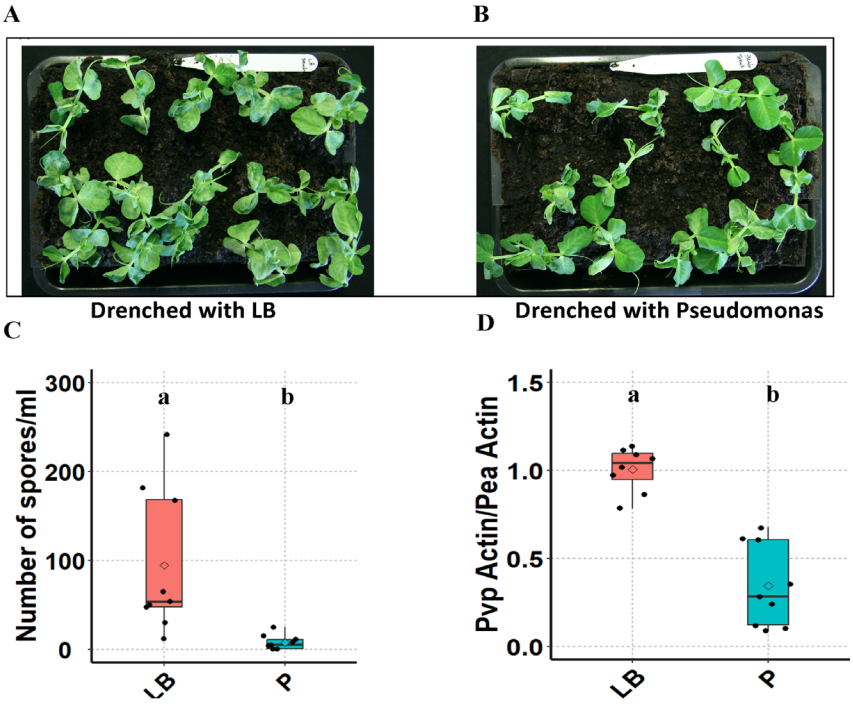

FIGURE 7 | Antagonism assay of drench application of *Pseudomonas* broth on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old pea seedlings were inoculated with Pvp spores and sown in a standard compost. *Pseudomonas* broth or Luria Bertani (LB) medium was applied immediately upon sowing the seedlings. After 10 days, the plants were covered for 2 days to induce Pvp sporulation. After the sporulation, plants drenched with LB (A) and *Pseudomonas* broth (B) were photographed; mean spore counts for plants drenched with *Pseudomonas* and LB (control) are shown in box plots (C). (D) Pvp molecular biomass quantification in *Pseudomonas* and LB-drenched pea plants. Pvp-*Actin* primer pair was used to amplify a unique region of Pvp-*Actin*, while a Pea-*Actin* primer pair was used for normalisation ('housekeeping' control). The fold change of the Pvp-*Actin*/Pea-*Actin* in the *Pseudomonas*-treated peas relative to the control (LB-treated peas) was plotted. Plots show combined data from three independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Different lowercase letters above bars indicate significant difference ($\alpha = 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

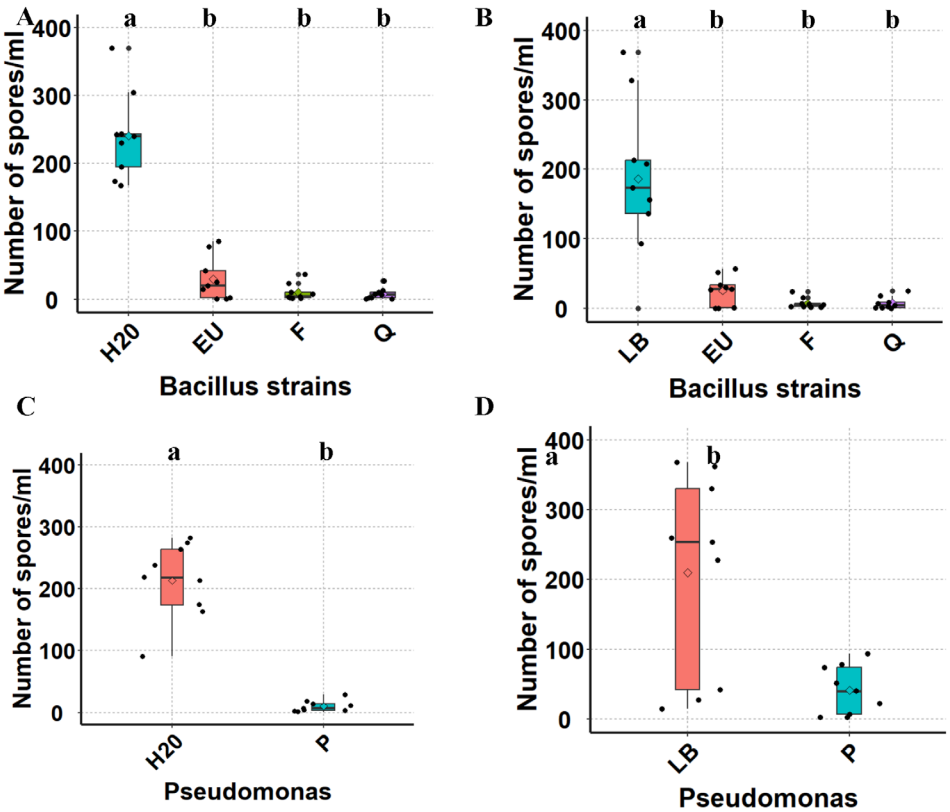

FIGURE 8 | Antagonism assay of foliar application of tropical *Bacillus* and *Pseudomonas* cells/filtrates on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old seedlings were inoculated with Pvp spores and grown in the growth cabinet. After 10 days, plants were sprayed with the biocontrol or the control and covered for 2 days to induce Pvp sporulation. After sporulation, spores were harvested and counted. Mean spore counts for plants sprayed with *Bacillus* cells and water control (A), and with *Bacillus* filtrates and Luria Bertani medium (LB) control (B). Mean spore counts for plants sprayed with *Pseudomonas* cells and water control (C) and with *Pseudomonas* filtrates and LB control (D). Plots show combined data from the three independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Different lowercase letters above bars indicate significant difference ($\alpha = 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

TYGS webserver identified K11 as belonging to this species and it shares 97.9673% ANI with the type strain. Strain K11 is phylogenetically distinct from strains FZB24 and from EU07 and QST713 (Figure 14). The most closely related genome sequence currently available is that of strain DE0372 (99.3861% ANI), isolated from an environmental sample in North Carolina, United States, in 2018 (BioSample: SAMN11792532).

4 | Discussion

The use of MBCA is a safe and sustainable alternative to chemical pesticides. It not only protects crops against pathogens but also significantly reduces pollution and negative impacts of chemical pesticides on the environment (Jaiswal et al. 2022; Lahlali et al. 2022). Additionally, MBCAs ensure the production of healthy and safe foods for human and animal consumption and well-being (Bale et al. 2008; Garvey 2022). Current research is focused on exploring the untapped potential of MBCAs (De Simone et al. 2021; El-Saadony et al. 2022; Lahlali et al. 2022). Bacteria such as *Bacillus*, *Pseudomonas*, *Streptomyces* and fungi such as *Trichoderma*, *Rhizophagus* and *Clonostachys* have been tested and commercialised as biopesticides and bioprotectants against a wide range of plant pathogens (El-Saadony et al. 2022; Jangir et al. 2021; Thambugala et al. 2020).

No specific MBCA has been reported to be effective against the DM pathogen in pulses, including pea crops. In this study, the antagonistic abilities of three *Bacillus* strains (EU07, FZB24 and QST713) and *Pseudomonas* to inhibit Pvp spore germination were demonstrated. The microbes were mixed with the Pvp spores and the mixtures were then incubated to assess the impact of the biocontrol on the spore germination percentage. This method was used because Pvp is an obligate pathogen and cannot be propagated without the host, making traditional in vitro bioassays using agar medium unsuitable. However, the system used for the bioassays in this research has been employed previously by other researchers such as Bilir et al. (2019) and Telli et al. (2020). In these bioassays, the cells (pellets suspended in water) and filtrates (supernatant after centrifugation) of the *Bacillus* and *Pseudomonas* strains were tested separately, as the filtrates could contain antimicrobial metabolites. Interestingly, the cells and filtrates of all the potential MBCAs showed complete inhibition of Pvp spore germination even at 50% concentration.

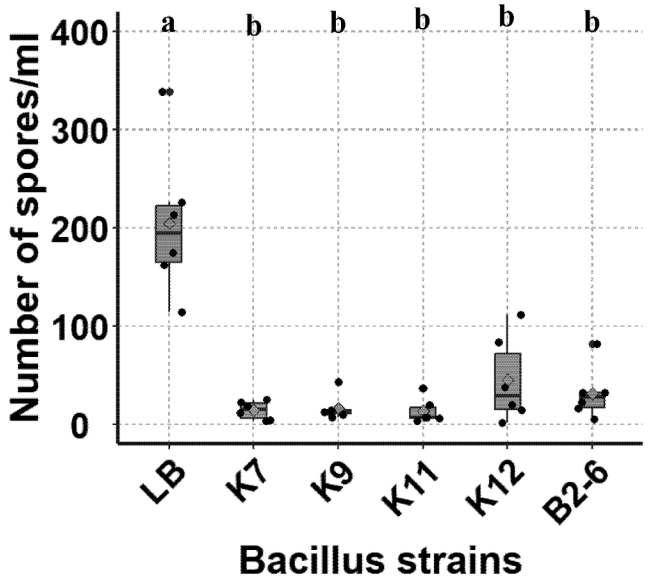

FIGURE 9 | Antagonism assay of foliar application of cold-adapted *Bacillus* filtrates on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old seedlings were inoculated with Pvp spores and grown in the growth cabinet. After 10 days, plants were sprayed with the biocontrol or the control and covered for 2 days to induce Pvp sporulation. After sporulation, spores were harvested and counted. Mean spore counts for plants sprayed with filtrates of *Bacillus* strains (K-7, K-9, K-11, K-12 and B2-6) and the control (Luria Bertani medium, LB) are displayed in box plots. Plots show combined data from three independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Different lowercase letters above bars indicate significant difference ($\alpha = 0.05$).

The positive antagonistic effects observed, especially with the filtrates, align with a significant body of literature explaining that the primary mechanism of direct antagonism of these microbial biocontrol agents is their natural ability to produce and use various antimicrobial substances such as lipopeptide, subtilin, bacilysin, mycobacillin, bacillomycin, fengycin, surfactin and iturin to inhibit the growth and proliferation of pathogenic microorganisms (Hashem et al. 2019; Ntushelo et al. 2019; Shoda 2000).

While in vitro antagonism on Pvp has not been reported in the literature, the effectiveness of *Bacillus* and *Pseudomonas* spp. and their filtrates against various pathogens has been demonstrated using agar-based in vitro systems. For example, the application of *Bacillus* species significantly inhibited *F. graminearum* by up to 79% (Jimenez-Quiros et al. 2022), *Botrytis cinerea* by up to 87% (Chen et al. 2019) and *Sclerotium rolfsii* by around 88% with cells and 100% with filtrates (Sultana and Hossain 2022).

Our in vitro assays with *Bacillus* and *Pseudomonas* strains demonstrated suppression of Pvp spore germination. However, this effect may vary in the plant–microbe interaction environment. Therefore, the antagonistic activities of the *Bacillus* and *Pseudomonas* strains against Pvp were further studied in the host crop, pea. The biocontrol applications were applied either by drenching Pvp-inoculated pea seedlings before infection developed with *Bacillus*/*Pseudomonas* broths or by foliar spray of cells/filtrates on the inoculated plants after infection developed. Drenching the soil with the MBCA was only significantly effective for cold-adapted *Bacillus* K11 and *P. fluorescens* (61.8%–91.8% reduction in spore load compared

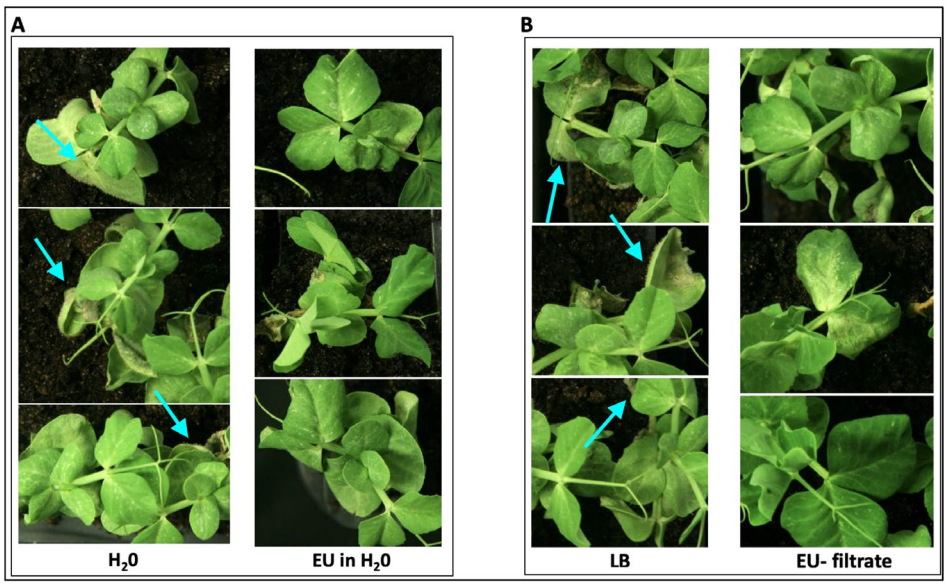

FIGURE 10 | Magnified images showing the durability of EU07 antagonism on *Peronospora viciae* f. sp. *pisi* (Pvp) sporulation in pea plants. Four-day-old seedlings were inoculated with Pvp spores and grown in the growth cabinet. After 10 days, plants were sprayed with EU07 cells or filtrates. Control plants were sprayed with water or Luria Bertani medium (LB). The plants were covered for 2 days to induce Pvp sporulation. After sporulation, the plants were uncovered and returned to the growth cabinet for 5 days. Images were taken after 5 days for those sprayed with EU07 cells or water (A) and those sprayed with EU07 filtrate or LB (B). Arrows indicate the sporulation areas in the control plants. [Colour figure can be viewed at wileyonlinelibrary.com]

to the control, respectively). However, significant suppression of Pvp sporulation occurred in pea plants sprayed with all strains of *Bacillus/Pseudomonas* (~90%) or their filtrates (more than 80%).

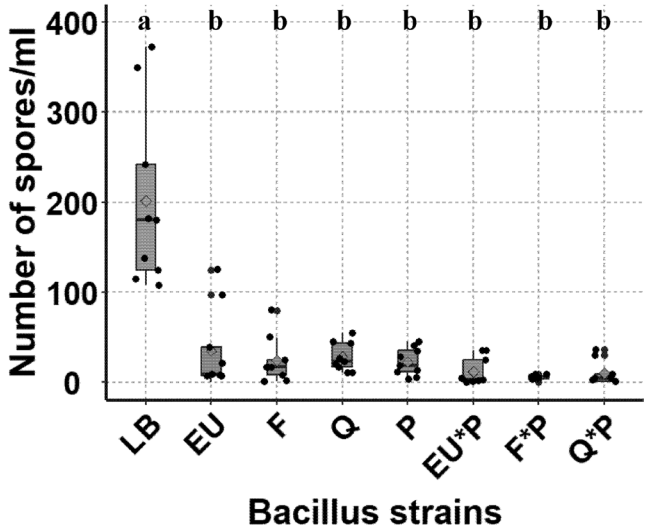

FIGURE 11 | Antagonistic effects of cocktail foliar application of *Bacillus* and *Pseudomonas* filtrates on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old seedlings were inoculated with Pvp spores and grown in the growth cabinet. After 10 days, plants were sprayed with the biocontrol agents in single or combined forms along with the control (Luria Bertani medium, LB). Plants were covered for 2 days to induce Pvp sporulation. Mean spore counts from plants sprayed with *Bacillus* strains EU07 (EU), FZB24 (F) and QST713 (Q) and/or *Pseudomonas* (P) and controls are displayed in boxplots. Plots show combined data from three independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Different lowercase letters above bars indicate significant difference ($\alpha = 0.05$).

The positive in planta antagonism supports several studies that have shown that rhizobacterial *Bacillus* and *Pseudomonas* species can suppress a wide range of plant pathogens (Dragana et al. 2017; Gao et al. 2012; Mnif and Ghribi 2015). For example, Núñez-Palenius et al. (2022) reported that foliar application of *B. subtilis* effectively controlled downy mildew disease in cucumber, caused by *Pseudoperonospora cubensis*, in a controlled environment. Kremmydas et al. (2013) also indicated that *P. fluorescens* strain X was able to suppress cucumber and sugar beet damping-off caused by the oomycete pathogen *Pythium ultimum*. The consistent results of in vitro and in planta antagonism assays in this research, in which both the cells and filtrates significantly suppressed Pvp growth and proliferation, suggest that one of the modes of action of these biocontrol agents could be their ability to produce antimicrobial substances, as observed with their filtrates (Biniarz et al. 2017; Raaijmakers et al. 2010; Shafi et al. 2017).

Deravel et al. (2014) noted that two antimicrobial compounds, mycosubtilin and surfactin, obtained from the filtrates of two *B. subtilis* strains, were highly effective in controlling lettuce DM disease caused by *Bremia lactucae*. Similar results were found by Li et al. (2019), where surfactin and fengycin purified from another *Bacillus* strain were effective against grape DM. Apart from the antibiosis mode of interaction, biocontrol agents can also use different antagonistic mechanisms, such as competing for space and nutrients, mycoparasitism or indirectly priming/activating the host resistance genes, either separately or synergistically, to inhibit the growth and activities of pathogens (Bonaterra et al. 2022; Köhl et al. 2019; Legein et al. 2020; Roca-Couso et al. 2021).

In this study, we also assessed the persistence of antagonistic actions of biocontrol agents. The findings showed that Pvp did not visually recover on plants sprayed with biocontrol agents at 5 dpi, while the control plants still had Pvp spores on them. Bardin et al. (2015) stressed the need for further research on

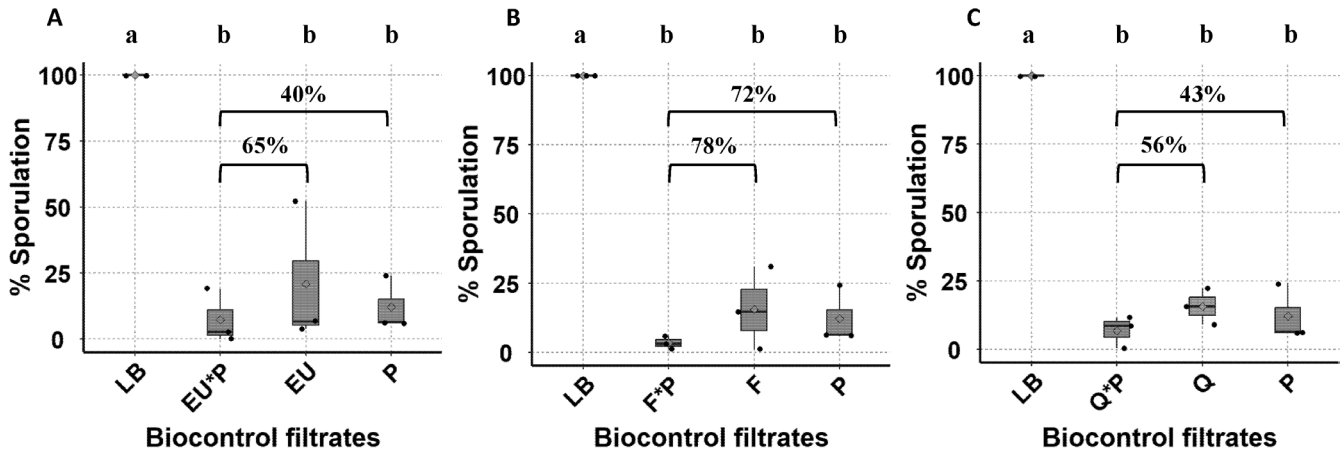

FIGURE 12 | Synergistic effects of combined foliar application of *Bacillus* and *Pseudomonas* filtrates on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Synergistic effects were calculated from the dual foliar application of *Bacillus* and *Pseudomonas*. The reduction in spore counts for each individual biocontrol treatment and their combination, relative to 100% sporulation in the Luria Bertani medium (LB) control, was determined. The synergistic values (presented as percentages) represent the reduction in spore counts due to the combined application of *Bacillus* strains EU07 (EU), FZB24 (F) and QST713 (Q) and *Pseudomonas* (P) relative to the effect from their individual applications. Plots show combined data from three independent biological repeats. ANOVA was conducted, and Tukey's B test was used to compare and group means based on statistically significant differences. Different lowercase letters above bars indicate significant difference ($\alpha = 0.05$).

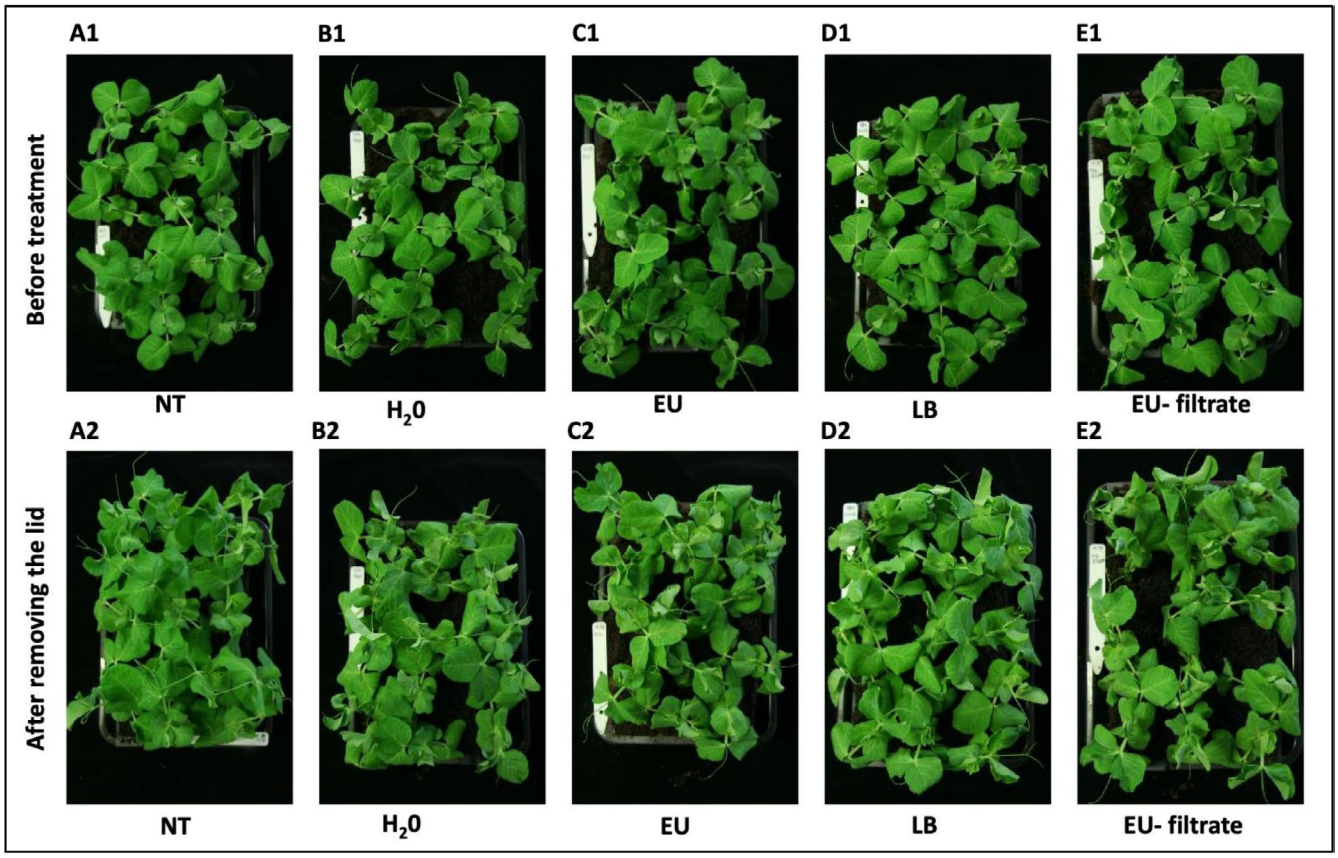

FIGURE 13 | Evaluation of negative effects of biocontrol sprays on healthy pea plants. Four-day-old seedlings were sown in pots and grown in the growth cabinet. After 10 days, pea plants were sprayed with the biocontrol. Plants were covered and moved to the growth cabinet for 2 days. Upper images show plants before being covered. Pea plants with no spray (A1), sprayed with water (B1), EU07 cells (C1), Luria Bertani (LB) medium (D1) and EU07 filtrates (E1). Lower images show pea plants after being covered for 2 days. Pea plants with no spray (A2), sprayed with water (B2), EU07 cells (C2), LB (D2) and EU07 filtrates (E2). [Colour figure can be viewed at wileyonlinelibrary.com]

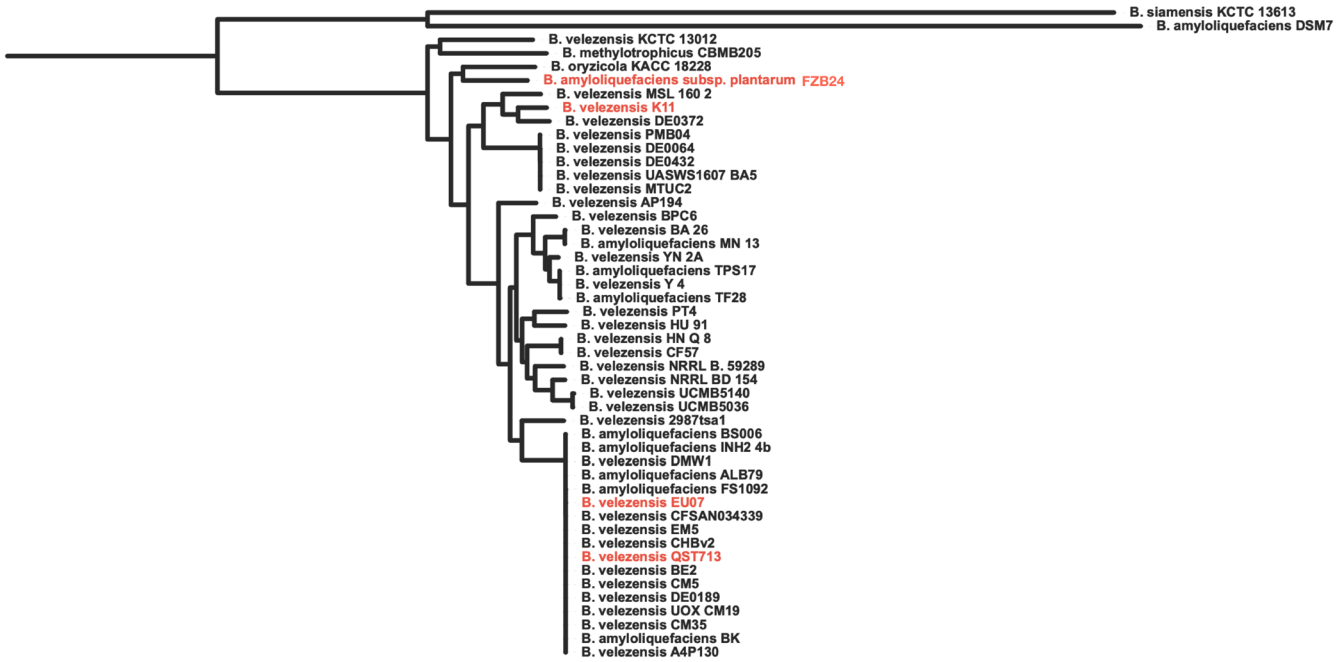

FIGURE 14 | Phylogenetic tree *of Bacillus velezensis* strains, based on genome sequence data. This maximum-likelihood tree is based on genome-wide single-nucleotide variants, using PhaME, as described in the Methods section. The strains used in this study (FZB24, K11, EU07 and QST713) are highlighted in red. The tree was rooted by including *B. siamensis* and *B. amyloliquefaciens* type strains as an outgroup. [Colour figure can be viewed at wileyonlinelibrary.com]

the durability of biocontrol agents to minimise potential failure or variations in their effectiveness, particularly in new environments. The positive results highlight the significant untapped potential that biocontrol agents offer in sustainable agriculture. In addition to investigating their durability, combining different biocontrol agents that share similar growth conditions as cocktails has been found to have synergistic effects, resulting in more effective antagonistic behaviour than when applied individually (Bardin et al. 2015; Xu et al. 2011). This is because each biocontrol agent exhibits unique features in how they demonstrate antagonistic activities; for example, some may produce distinctive types or quantities of antimicrobial substances or employ different combinations of antagonistic mechanisms. Combining them would harness all their individual attributes and positive interactions, leading to a more robust and efficient pesticidal effect on target pathogens (Köhl et al. 2019).

Synergistic effects of combining filtrates of tropical *Bacillus* strains (EU07, FZB24 and QST713) with *Pseudomonas* that have a common peak growth temperature as foliar sprays on Pvp-inoculated peas were examined. The cocktail application significantly decreased Pvp spore load compared to the control and mixed application of the two biocontrol agents showed synergistic effects (40%–78% compared to individual application). Similarly, Abeysinghe (2009) indicated that cocktail application of *B. subtilis* with *P. fluorescens* strains showed higher plant protection against *Rhizoctonia solani* and *S. rolfsii* in *Capsicum annuum* (red pepper) than in the plants treated with either of the biocontrol agents alone (up to 45%). Other researchers also reported increased antagonistic actions following application of combined biocontrol agents against different plant pathogens (Diaz-Manzano et al. 2022; El-Sharkawy et al. 2022; Palazzini et al. 2022; Panchalingam et al. 2022). Assemblage and use of diverse biocontrol agents as consortia is an effective way to increase the efficiency and durability of MBCA (Sarma et al. 2015). However, compatibility and possible interaction of the proposed biocontrol agents to be combined needs to be studied to ensure there are no negative interactions from their combination that would result in reduced efficacy relative to their individual efficacies (Niu et al. 2020; Sarma et al. 2015).

Although biocontrol agents are widely considered safe and have little to no negative effects on the environment and ecosystems (Bhat et al. 2023; El-Saadony et al. 2022; Li et al. 2022), some researchers caution that because these microbes or their byproducts are intentionally applied, often in high amounts, their biosafety, especially on non-target organisms, should be tested (Barratt et al. 2010; Delfosse 2005; Kiss 2004; Winding et al. 2004). Therefore, in the present study, a simple biosafety analysis of the biocontrol agents, using EU07 *Bacillus* strain as a representative, was conducted. Following spraying of EU07 and its filtrate on healthy pea plants, no negative effects on the plants were visually observed above ground compared to the control plants. This indicates that the type and dosage of the biocontrol agents used in this study are safe for use in crop protection, as also indicated by other researchers (Brutscher et al. 2022; Deravel et al. 2014; Lefevre et al. 2017).

The lack of information on the efficacy of potential biopesticides and the lack of credible alternatives to chemicals for controlling DM pathogens in pulses led to this research. In vitro assays showed that all *Bacillus* and *Pseudomonas* strains and their filtrates completely inhibited Pvp spore germination, while treatment of Pvp-inoculated pea seedlings with biocontrol broth via soil drenching was significantly effective only for cold-adapted *Bacillus* K11 and *P. fluorescens*, as indicated by spore assays and molecular biomass quantification. When the biocontrol agents were applied as foliar sprays on Pvp-inoculated pea plants, those treated with *Bacillus* strains, *P. fluorescens* or their filtrates showed a significant decrease in spore numbers compared to the control, and combining *Bacillus* strains and *P. fluorescens* resulted in a synergistic reduction of Pvp spore load. We also assessed the safety of using these biocontrol agents as biopesticides on healthy pea plants, and found no obvious negative effects, confirming their safety and environmental compatibility. This research, being the first on the biocontrol of pea DM, will provide a crucial foundation for further studies. Importantly, cocktails of *Bacillus* strains and *P. fluorescens* could be effective immediately in controlling pea DM disease, thus bolstering the health of a significant nitrogen-fixing crop in rotations.

Author Contributions

Emeka Chibuzor Okechukwu: data curation, formal analysis, investigation, visualization, writing – original draft, writing – review and editing. **Catherine Jimenez-Quiros:** investigation, methodology, writing – review and editing. **Ömür Baysal:** resources, writing – review and editing. **Süreyya Kocamaz:** resources, writing – review and editing. **Burhan Arıkan:** resources, writing – review and editing. **Anne Webb:** software, writing – review and editing. **Thomas Wood:** conceptualization, funding acquisition, writing – review and editing. **Sanu Arora:** writing – review and editing. **Claire Domoney:** funding acquisition, writing – review and editing. **David J. Studholme:** formal analysis, software, writing – review and editing. **Mahmut Tör:** conceptualization, funding acquisition, project administration, supervision, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author on reasonable request. All genomic data are publicly available as described in the paper. All genome sequence data have been deposited in public databases under the BioProject accession PRJNA1150624. Raw sequence reads are deposited in the Sequence Read Archive (Kodama et al. 2012) under the following accession numbers: SRX25802839 (QST713), SRX25802838 (FZB24), SRX25793480 (K-11) and SRX25793481 (LZB 065). Annotated genome assemblies are deposited in GenBank under the accession numbers GCA_045108535.1 (QST713), GCA_045108515.1 (FZB24), GCA_041520185.1 (K-11), GCA_041521055.1 (LZB 065).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Magnified pictures from antagonism assay of drench-application of *Bacillus* broth on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old pea seedlings were inoculated with Pvp spores and sown in a standard compost. *Pseudomonas* broth or Luria Bertani (LB) broth was applied immediately upon sowing the seedlings. After 10 days, plants were covered for 2 days to induce Pvp sporulation. After sporulation, images were taken for plants drenched with LB (A) or *Pseudomonas* broth (B). **Figure S2:** Magnified pictures from antagonism assay of foliar application of EU07 on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old seedlings were inoculated with Pvp spores and allowed to grow in the growth cabinet. After 10 days, plants were sprayed with EU07 or water as a control and covered for 2 days to induce Pvp sporulation. After sporulation, images were taken for plants sprayed with water (A) or EU07 cells (pellets suspended in water) (B). **Figure S3:** Magnified pictures from antagonism assay of foliar application of EU07 filtrate on *Peronospora viciae* f. sp. *pisi* (Pvp)-inoculated pea plants. Four-day-old seedlings were inoculated with Pvp spores and grown in the growth cabinet. After 10 days, plants were sprayed with EU07 filtrate or Luria Bertani (LB) medium as a control and covered for 2 days to induce Pvp sporulation. After sporulation, images were taken for plants sprayed with LB (A) or EU07 filtrate (supernatant after centrifugation) (B). **Table S1:** List of primers used in this research.