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Activation of sweet pepper defense responses by novel and known biocontrol agents of the genus *Bacillus* against *Botrytis cinerea* and *Verticillium dahliae*

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Abstract The Fresno-Benavente Pepper (F-BP) Protected Geographical Indication (PGI) is a horticultural crop characterized by its great agronomic, economic and cultural importance in the region of Castilla y León (Spain). Field production is threatened by verticillium wilt caused by *Verticillium dahliae* and postharvest losses due to grey mould caused by *Botrytis cinerea*. Eight *Bacillus* spp. strains endophytically isolated from F-BP roots were used in the study. By conducting an *in vitro* antagonism study, we found that all *Bacillus* strains were effective against *B. cinerea* and five of them showed high antagonism against *V. dahliae*, with *B. siamensis* and *B. proteolyticus* strains being the most effective against both pathogens. Eight *Bacillus* strains were used for an infection test in F-BP fruits and plants to test their activity against both fungal pathogens. We report that *Bacillus thuringiensis*, *B. siamensis* and *B. pumilus* (SCFC 1–2) could control *B. cinerea* in pepper fruits through direct action and local activation of plant defences. In the case of *V. dahliae* root infection, plant roots inoculated with *B. siamensis* and *B. proteolyticus* were able to significantly decrease the occurrence of disease through direct action and local activation of jasmonic acid as a defence response. Therefore, we propose that *B. siamensis* could be used to control *B. cinerea* and *V. dahliae* in F-BP fruits and plants, respectively, through direct antagonism as well as the induction of local plant defence responses.

Keywords Pepper · *Verticillium dahliae* · *Botrytis cinerea* · Antagonism · Plant defenses · *Bacillus siamensis*

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Introduction

Sweet pepper (*Capsicum annuum* L.) is an annual herbaceous plant belonging to the Solanaceae family and originating from South America (Fratianni et al., 2020; Guevara et al., 2021). It is part of the diet especially in European countries (Fratianni et al., 2020). Sweet peppers represent an important nutritional resource of antioxidant compounds, such as carotenoids, ascorbic acid and polyphenols (Fratianni et al., 2020). The world production of this crop stands at 36 million tons per year, on 2 million hectares (FAO, 2020).

According to the European Union Council Regulation (EC) No 925/2012, the Fresno-Benavente Pepper (F-BP) ("Pimiento de Fresno-Benavente, in Spanish) is produced in three specific regions in Castilla y León

(Spain), namely Benavente and Los Valles (Zamora province) and Fresno (León province), all of them watered by the Esla and Tera rivers. F-BP was granted a Protected Geographical Indication (PGI) in 2012 (EC 925/2012). The F-BP describes a type of pepper that has been specifically adapted to its production area, including 55 municipalities in the north of the province of Zamora, 33 municipalities in the southeast of the province of León, and a municipality in the province of Valladolid. It is a "cow's nose" or "bell pepper" type, large (10 cm minimum width), red in colour, rectangular in shape and with thick and juicy flesh (EC 925/2012).

Wilt caused by *Verticillium dahliae* and grey mould caused by *Botrytis cinerea* are the two primary diseases impacting production. *V. dahliae* is a soilborne pathogen, causing serious economic losses in more than 150 different crops (Tubeileh & Stephenson, 2020). It is the most important root pathogen in pepper cultivation, causing a vascular wilt that leads to plant death, including symptoms such as a delay in plant growth, chlorosis, discoloration of the vascular bundles, and a whitish colour can be seen in the roots with serious damage that causes the loss of the outermost layers (Tyvaert et al., 2019). *B. cinerea* is a plant pathogenic fungus that causes serious diseases in more than 1400 different plant species. In peppers, *B. cinerea* can attack the leaves and fruits in the field, but the real damage occurs at post-harvest when fruits develop the characteristic grey mould that spreads rapidly and causes fruit rot (Mekawi et al., 2019; Poveda et al., 2020).

Plant endophytes are microorganisms that develop their entire life or part of it within plant tissues without causing visible damage or disease (Poveda et al., 2021). With regard to bacteria, they can be found inside different organs and plant tissues, such as seeds, tubers, roots, stems, leaves or fruits; the roots being the organ in which the greatest number and diversity of microorganisms can be found. The most commonly isolated bacterial genera are *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* (Eljounaidi et al., 2016). Endophytic bacteria can be of great importance in the agricultural system, promoting plant growth, favouring tolerance to abiotic stresses or reducing the incidence of biotic stresses. Regarding the effects against phytopathogens, endophytic bacteria reduce the damage caused to plants due to the release of chemical compounds, such as antibiotics, a mechanism known as antibiosis, through direct parasitism, competition for space and/or nutrients, or the induction of specific plant defensive responses (Eljounaidi et al., 2016; Poveda & González-Andrés, 2021).

In a previous work, different endophytic bacterial strains were isolated locally from F-BP plants (in 2010, and in the municipalities of: San Cristóbal de Entreviñas, Micereces de Tera and Fresno de la Vega) and selected for their outstanding biocontrol capacity against the root pathogenic oomycete of pepper, *Phytophthora capsici*. Out of the 122 isolated strains, two strains of the *Bacillus pumilus* species were described as the ones that most reduced the *P. capsici* infection in pepper (Barquero et al., 2016). The bacterial strains with the greatest biocontrol potential were those used in the present work.

The objective of this work was to identify effective endophytic and locally isolated bacterial biological control agents (BCAs) against *V. dahliae* and *B. cinerea* in F-BP and to assess potential modes of action. We utilized a local bacterial strain collection (described in the following section), isolated from F-BP plants grown in their geographical demarcation, because they are the bacteria that are fully adapted to the host plant and the environmental conditions of the crop. Strains were assessed for biocontrol activity through antagonistic *in vitro* assays, plant inoculations and infections *in vivo* and *in planta*, and molecular and biochemical analysis to assess potential activation of plant defences.

Materials and methods

Microbiological material

Bacterial strains used in this study (Barquero et al., 2016) included: *B. siamensis* strains SCFB 2–2 and SCFB 3–4, *B. pumilus* strains SCFC 1–2 and FVA 1–5, *B. proteolyticus* strain SCFC 2–2, *Bacillus safensis* strain MTA 1–2, *Bacillus megaterium* strain MMM 3–6 and *Bacillus thuringiensis* strain FVA 2–3, all of them identified by 16S RNA gene sequencing. Strains are preserved in glycerol at −80 °C in the collection of the Chemical, Environmental and Bioprocess Engineering Group (IQUIMAB) of the University of León (Spain), accessible to any researchers that would like to acquire them for testing. Strains are routinely grown on tryptic-soy-agar (TSA, Sigma-Aldrich, Madrid, Spain).

The pathogenic fungi used were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain): *B. cinerea* (CECT 20973) and *V. dahliae* (CECT 20246). Strains are routinely grown on potato-dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain), adding powdery tomato leaves to the medium (0.1% v/v) formulation in order to maintain the phytopathogen activity (Acosta-Morel et al., 2019).

In vitro antagonism assay

The evaluation of the antagonistic activity of *Bacillus* species against *B. cinerea* and *V. dahliae,* was assessed by the dual culture technique *in vitro*, based on the methodology described by Slama et al. (2019), with some modifications. Mycelium disks (6 mm diameter) of each pathogenic fungus, obtained from the edge of the colonies grown on PDA medium, were placed in the centre of the Petri plate with TSA medium (pathogenic fungi grow similarly on TSA medium and PDA medium: Fig. S1). From an overnight pure bacterial culture on TSA agar, a loopful of cells was suspended in 0.9% NaCl solution, applying two 5 µl drops of 10^8 CFU mL^{-1} at 30 mm distance from the fungus inoculum. Controls were performed with and without the application of 5 µl of 0.9% NaCl solution, and no difference was found between them. The inhibition rate (IR) was calculated according to the following formula (Lyousfi et al., 2021):

$$IR = (A_C - A_T)/A_C \times 100$$

where A_C means the colony area of the pathogenic fungus in the control treatment (TSA medium without bacterial treatments), and A_T means the colony area of the pathogenic fungus in bacterial treatments. Fungal areas were measured using ImageJ photographic analysis software, at 3, 5 and 7 days for *B. cinerea*, and at 7, 10 and 14 days for *V. dahliae*.

The experimental unit is represented by each of the Petri dishes. Each treatment was performed on eight plates (replicates per treatment), and the experiment was repeated twice.

In vivo biocontrol of *B. cinerea* in fruits assay

The *in vivo* study against the pathogen *B. cinerea* with the different *Bacillus* species was carried out on pepper fruits of the F-BP. For this, the methodology previously described by Shi and Sun (2017) for tomatoes was carried out, with some modifications. The fruits were superficially sterilized by washing with 70% ethanol and 2% sodium hypochlorite. A 3 × 3 mm cross-shaped wound was made in the epidermis of the fruits where each of the bacteria was inoculated with 40 µl at a concentration of 10^8 CFU ml^{-1}. Two hours after bacterial inoculation, the wounds were inoculated with 15 µl of *B. cinerea* spores at a concentration of 10^5 spores ml^{-1}. Subsequently, the fruits were placed in plastic bags to maintain constant-high humidity and kept at 23 °C and in the dark for 7 days. Different controls were used: peppers without *B. cinerea* inoculation and without bacterial treatment, peppers without *B. cinerea* inoculation and with bacterial treatment, and peppers with *B. cinerea* inoculation and without bacterial treatment.

After one week of fungal infection, measurements were taken of the areas of lesions produced by *B. cinerea* in peppers, tissue viability and reactive oxygen species (ROS) accumulation. In addition, infected tissues from the fruits were collected and immediately frozen in liquid nitrogen to analyse levels of pathogen colonization and expression of defence-related genes.

The experimental unit is represented by each fruit. Each treatment was performed on eight fruits (replicates per treatment), and the experiment was repeated three times. Treatments and *B. cinerea*-inoculations on each of the peppers were performed randomly.

In planta biocontrol of *V. dahliae* assay

F-BP seeds were surface-sterilized by vigorous sequential shaking in 70% ethanol and 5% sodium hypochlorite solutions for 10 min each and then washed thoroughly four times in sterile, distilled water. Pepper seeds were individually transferred to 1 L pots, containing a sterilized mixture of field soil/vermiculite (3:1) that was previously prepared by autoclaving a local field soil twice (24 h apart). Seeded pots were maintained in a greenhouse.

Bacillus strains were applied to three-week-old pepper seedlings as described by Mena-Violante and Olalde-Portugal (2007), with some modifications. Pepper seedlings were treated by applying a 1 ml suspension (10^7 CFU ml^{-1}) of each bacterium to individual roots. Inoculation with the pathogenic fungus *V. dahliae* was carried out as described by Mercado-Blanco et al. (2004), with some modifications. Two weeks after applying bacterial root treatments, the pepper plants were

root inoculated with 3 ml of a *V. dahliae* spores suspension (10^7 spores ml^{-1}).

Four weeks after inoculation with the fungus, the plants were collected and measurements were taken of the dry weight of the aerial part of the plant (after drying at 65 °C for 48 h), disease severity, root vitality and ROS accumulation in root tissues. In addition, roots were collected and immediately frozen in liquid nitrogen to analyse levels of pathogen colonization and defence gene expression. Disease severity was analysed by a 0–4 rating scale according to the percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant) (Mercado-Blanco et al., 2004).

The experimental unit is represented by the individual plants. Each treatment was performed on eight plants (replicates per treatment), and the experiment was repeated three times. Treatments and *V. dahliae*-inoculations on each of the plants were performed randomly. In addition, plants were randomly arranged in the greenhouse.

Vitality test and indirect quantification of ROS in tissues

To analyse and quantify the damage produced by both pathogens on F-BP fruits and roots, the vitality test and ROS quantification were assayed, as reported by Poveda (2021a).

The vitality test is based on the reduction of triphenyltetrazolium chloride (TTC) by living tissues to red-coloured insoluble triphenylformazan (TF), due to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC to TF. Pools were formed from eight fruits (*B. cinerea*-infected plants) or roots (*V. dahliae*-infected) per condition. From these pools, 100 mg was transferred to 1 mL of 1% TTC in triplicate and incubated for 48 h at 37 °C. After incubation, 200 mg of Ballotini Glass Balls (0.15–0.25 mm diameter) was added to each sample in 1.5 mL Eppendorf tubes and shaken vigorously. After centrifuging the samples for 15 min at 10,000 rpm, the supernatant was removed, and 1 mL of isopropanol was added to each tube. The samples were again agitated and centrifuged in the same way, and the supernatant was used to quantify the absorbance at 620 nm; this acted as an indirect measure of the vitality of the F-BP tissues.

The indirect quantification of reactive oxygen species (ROS) in F-BP tissues was carried out by measuring electrolyte leakage, which is a measure of cellular oxidative damage related to the production of ROS, a sign of stress in plant tissues. This methodology of indirect analysis of cell damage caused by a plant pathogen has been used in many other works, with a direct relationship between the quantified damage and the indirect ROS data obtained (Poveda, 2020, 2021a, b, 2022a, b), demonstrating its success.

From each pool formed from fruits (*B. cinerea*-infected plants) or roots (*V. dahliae*-infected), 1 cm^2 of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water at room temperature for 6 h. The conductivity of the water was measured using a Crison™ Conductimeter GLP31 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the tissues (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total concentration of ions present in the tissues (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

Tissue-colonization analysis

In order to determine the differences in F-BP tissue colonization by *B. cinerea* and *V. dahliae*, they were quantified by qPCR. For the analysis of *B. cinerea*-fruit and *V. dahliae*-root colonization, pools formed from eight fruits (*B. cinerea*-infected) or roots (*V. dahliae*-infected) per treatment were used. All material was immediately frozen with liquid nitrogen and pulverized with a mortar.

Following the methodology described by Poveda (2022a), DNA was extracted using the cetyltrimethylammonium bromide (CTAB) extraction method. A mix was prepared in a 10-μL volume using 5 μL of Brilliant SYBR Green qPCR Master Mix (Roche, Penzberg, Germany), 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. The endogenous genes of pathogens and pepper used for the quantification are represented in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), programmed for 40 cycles under the following conditions: denaturation, 95 °C for 15 s; annealing, 60 °C for 1 min; extension, 72 °C for 1 min. Each PCR was performed in triplicate by using the DNA extracted from three tissue pools of eight plants each –

Table 1 Oligonucleotides used in this work

CODE	GENE	SEQUENCE (5′-3′)	USE	REFERENCE
ACT-Ca-F ACT-Ca-R	*Actin*	CATCAGGAAGGACTTGTACGG GATGGACCTGACTCGTCATAC	Endogenous pepper gene	Kim and Hwang (2014)
PAL1-Ca-F PAL1-Ca-R	*Phenylalanine ammonia lyase 1*	GGTTTTGGTGCAACATCACATAGGAG ATTGTCAAAGTTCTCTTAGCTACTTG GC	Synthesis gene SA in pepper	Kim and Hwang (2014)
PR-1-Ca-F PR-1-Ca-R	*Pathogenesis-related protein 1*	CAGGATGCAACACTCTGGTGG ATCAAAGGCCGGTTGGTC	Response gene SA in pepper	Kim and Hwang (2014)
LOX1-Ca-F LOX1-Ca-R	*Lipoxygenase 1*	CATGTTACTGGAAAAGATTG GAATTCTATATCGACACACTGTTGGG TA	Synthesis gene JA in pepper	Hwang and Hwang (2010)
DEF1-Ca-F DEF1-Ca-R	*Defensin 1*	AAACGGCTACCACATCCAAG ACCCATCCCAAGGTTCAACT	Response gene JA in pepper	Kim and Hwang (2014)
Act-Bc-F Act-Bc-R	*Actin*	CGTCACTACCTTCAACTCCATC CGGAGATACCTGGGTACATAGT	Endogenous *B. cinerea* gene	Ren et al. (2018)
-Vd-F -Vd-R	*Actin*	CTGTACGGCAACATCG GACCATATCGCCTGTG	Endogenous *V. dahliae* gene	Klimes et al. (2008)

one for each treatment. Cycle threshold values served to calculate the amount of pathogen DNA using standard curves. The values of pathogen DNA were referred to the amount of pepper DNA in every corresponding sample.

Defence gene expression analysis

To analyse the defence responses following F-BP infection with *B. cinerea* and *V. dahliae*, an expression analysis of defence genes was performed by RT-qPCR. For gene expression studies, the pools formed from eight fruits (*B. cinerea*-infected) or roots (*V. dahliae*-infected) per treatment were used for RNA extraction with TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA). RNA was cleaned up with the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) including a DNase enzymatic treatment. cDNA was synthesized from RNA with Reverse Transcription System (Promega, Madison, WI, USA). Gene expression was analysed by RT-qPCR using an Agilent Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) with Luna ® Universal qPCR Mastermix (New England Biolabs, Ipswich, MA, USA). All PCR reactions were performed in triplicate in a total volume of 10 μL for 40 cycles under the following conditions: denaturation at 95 °C for 15 s; and annealing and extension at 60 °C for 30 s; and melt curve. Threshold cycles (CT) were calculated using the pepper *Actin* gene as an endogenous control. The primers used are shown in Table 1, as follows: phenylalanine ammonia lyase 1 (*PAL1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and defensin 1 (*DEF1*) genes. Data are expressed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Statistical analysis

Statistical analysis of the data was carried out with Statistix 8.0 software. One-way ANOVA using Tukey's multiple range test at $P < 0.05$ was used for pairwise comparisons; the different letters indicate significant differences.

Results

In vitro antagonism

The *in vitro* antagonism test between the different *Bacillus* species and the pathogens *B. cinerea* and *V. dahliae* (Fig. 1) reported positive IRs in all confrontations, with the exception of *B. megaterium* strain MMM 3–6 against *V. dahliae* (up to −22% IR) (Fig. 2b).

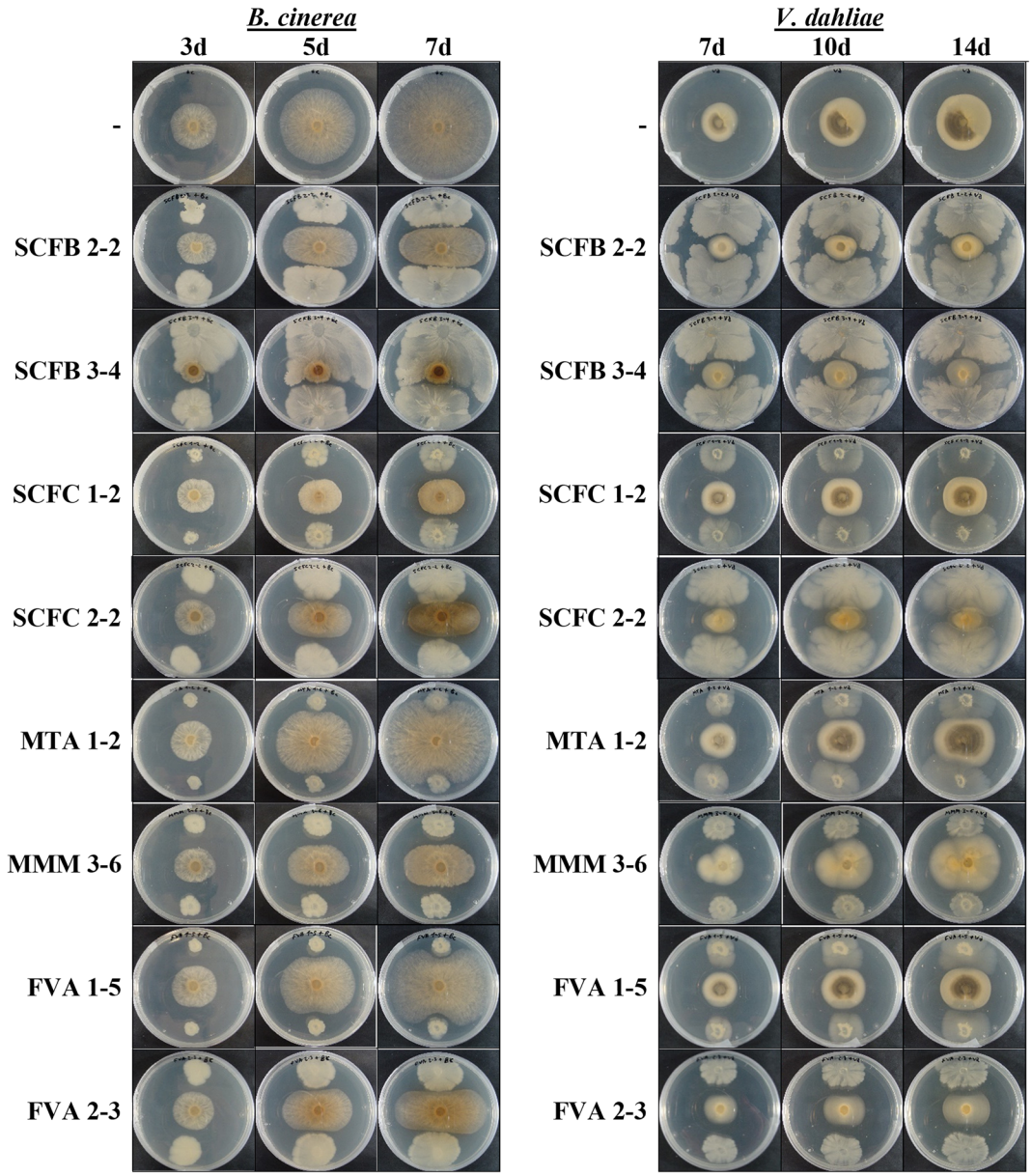

Fig. 1 Photographs of the antagonism *in vitro* study between the pathogens *B. cinerea* (3, 5 and 7 days) and *V. dahliae* (7, 10 and 14 days) and *Bacillus* endophytic bacteria. SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*

B. siamensis SCFB 3–4 and *B. pumilus* SCFC 1–2 produced a significantly higher IR against *B. cinerea* compared to other bacteria strains (between 16% and 28% higher inhibition). Furthermore, *B. proteolyticus* SCFC 2–2 and *B. megaterium* MMM 3–6 at day 7, also reduced the pathogen growth area in a significantly greater way, compared to the other bacteria strains (between 18% and 20% higher inhibition). On the other hand, *B. pumilus* FVA 1–5 produced a significantly lower IR compared to the other bacteria strains (Fig. 2a). In addition, in *B. siamensis* SCFB 2–2, *B. pumilus* SCFC 1–2, *B. proteolyticus* SCFC 2–2, *B. megaterium*

MMM 3–6, *B. pumilus* FVA 1–5 and *B. thuringiensis* FVA 2–3 confrontations, *B. cinerea* growth had zones of inhibition. A higher mycelial density was also observed in the *B. siamensis* SCFB 3–4, *B. pumilus* SCFC 1–2, *B. megaterium* MMM 3–6 and *B. thuringiensis* FVA 2–3 confrontations, and a darker coloration was observed in the *B. siamensis* SCFB 3–4, *B. proteolyticus* SCFC 2–2 and *B. thuringiensis* FVA 2–3 confrontations (Fig. 2a).

For *V. dahliae*, *B. megaterium* MMM 3–6 promoted the growth of the pathogen *in vitro*. *B. pumilus* SCFC 1–2 and FVA 1–5 and *B. safensis* MTA 1–2 inhibited the pathogen growth. Moreover, *B. siamensis* (SCFB 2–2 and SCFB 3–4) and *B. proteolyticus* (SCFC 2–2) produced significantly higher inhibition rates (between 19% and 27% higher inhibition) (Fig. 2b). Furthermore, in the confrontations with *B. siamensis* SCFB 2–2, *B. siamensis* SCFB 3–4, *B. pumilus* SCFC 1–2, *B. megaterium* MMM 3–6, *B. pumilus* FVA 1–5 and *B. thuringiensis* FVA 2–3, the growth of *V. dahliae* had zones of inhibition. A lower mycelial density was also observed in the *B. siamensis* SCFB 3–4, *B. proteolyticus* SCFC 2–2 and *B. pumilus* FVA 2–3 confrontations, and a lighter coloration was observed in the confrontations against *B. pumilus* SCFB 2–2, *B. siamensis* SCFB 3–4, *B. proteolyticus* SCFC 2–2, *B. megaterium* MMM 3–6 and *B. pumilus* FVA 2–3 (Fig. 2b).

In vivo biocontrol of *B. cinerea* in fruits

The treatment of pepper wounds with the *Bacillus* species caused a significant reduction in the lesions caused by *B. cinerea* (reductions of between 41% and 98%) (Fig. 3). The most efficient strain as BCA was *B. safensis* MTA 1–2, with a significantly smaller area of average lesions produced (reduction of 98%) by the pathogen than the following, *B. thuringiensis* FVA 2–3, *B. pumilus* SCFC 1–2 and *B. siamensis* SCFB 2–2 and SCFB 3–4 (Fig. 3b).

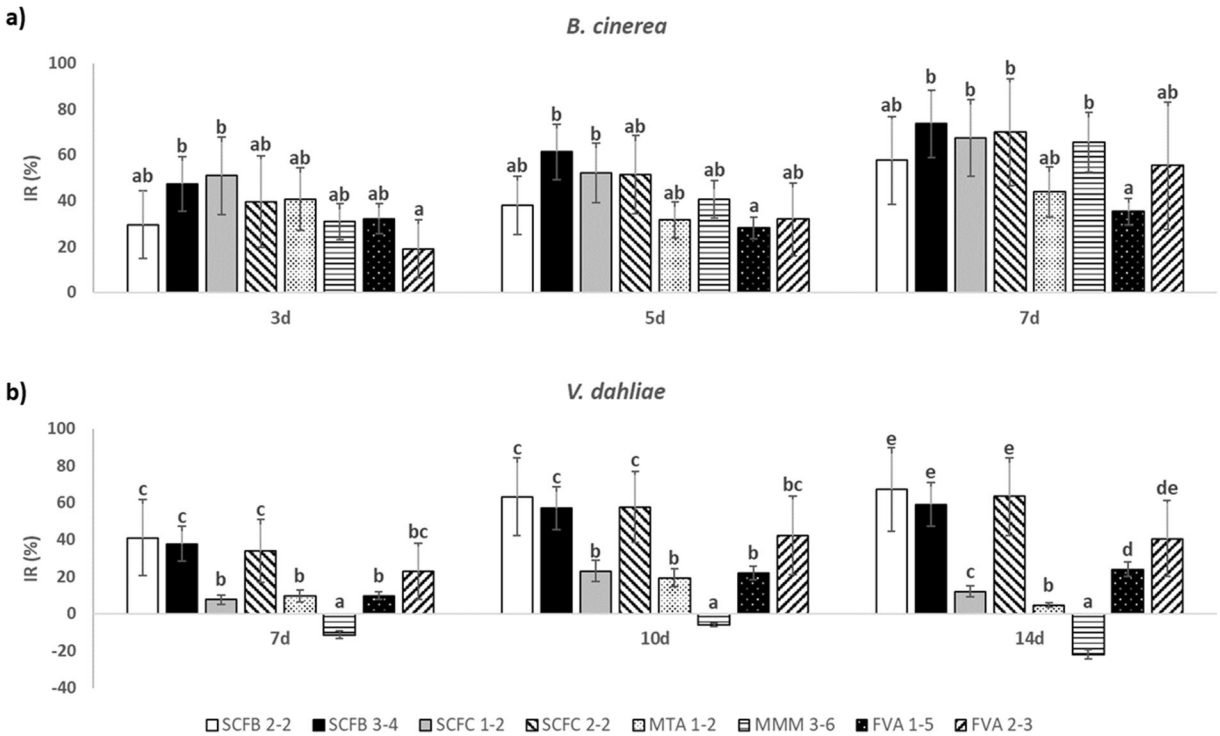

Fig. 2 Inhibition rate (IR, %) in *B. cinerea* (**a**) and *V. dahliae* (**b**) in their antagonistic confrontation *in vitro* against *B. siamensis* (SCFB 2–2), *B. siamensis* (SCFB 3–4), *B. pumilus* (SCFC 1–2), *B. proteolyticus* (SCFC 2–2), *B. safensis* (MTA 1–2), *B. megaterium* (MMM 3–6), *B. pumilus* (FVA 1–5) and *B. thuringiensis* (FVA 2–3). Data are the mean of two biological replicates for each condition with eight plates in each one. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

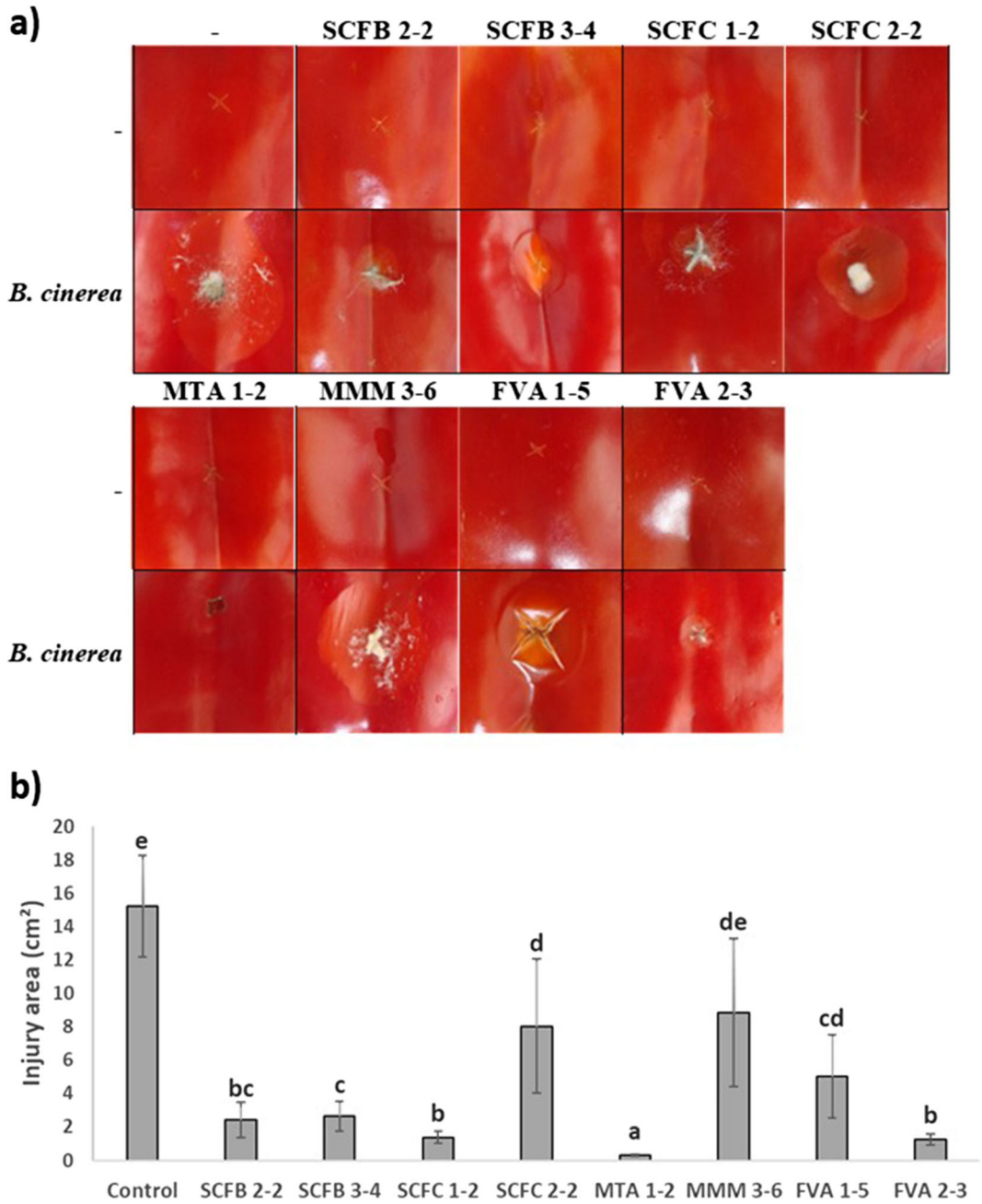

Fig. 3 Lesions produced by *B. cinerea* in peppers from the F-BP PGI. Appearance of infections caused by *B. cinerea* and wounds inoculated only with bacteria (−) (**a**) and quantification of lesions area (cm^2) (**b**). Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The columns represent the average of three biological replicates with eight fruits in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

Six out of eight *Bacillus* strains significantly improved tissue viability based on relative TTC staining, compared to the non-treated control. From the effective strains, *B. safensis* MTA 1–2 produced the highest values of viability (0.078), significantly higher than *B. siamensis* SCFB 3–4 (0.039) and *B. pumilus*

FVA1–5 (0.037); moreover *B. siamensis* SCFB 2–2 and SCFB 3–4, *B. pumilus* SCFC 1–2 and *B. thuringiensis* FVA 2–3 produced intermediate values (Fig. 4a). Concomitantly, only three strains reduced ROS levels compared with the non-treated control (4.23), namely *B. safensis* MTA 1–2 (1.22) followed by *B. thuringiensis* FVA 2–3 (2.34) and *B. pumilus* SCFC 1–2 (2.81) (Fig. 4b).

Except for strains *B. proteolyticus* SCFC 2–2 and *B. pumilus* FVA 1–5, six *Bacillus* strains reduced *B. cinerea* colonization relative to the non-treated control based on qPCR (Fig. 5). Strain *B. safensis* MTA1–2 was statistically the most effective of the *Bacillus* strains. Based on qPCR results, strain *B. safensis* MTA 1–2 reduced *B. cinerea* colonization by 92% compared to the control, whereas strains *B. siamensis* SCFB 2–2, *B. siamensis* SCFB 3–4, *B. proteolyticus* SCFC 2–2, *B. megaterium* MMM 3–6 and *B. thuringiensis* FVA 2–3 gave an intermediate reduction of 26% to 73%.

The expression analysis of several genes involved in the defensive responses of the plant was carried out in the tissues of pepper infected with *B. cinerea* (Fig. 6). Strains *B. safensis* MTA 1–2 and *B. thuringiensis* FVA 2–3 significantly reduced the expression of *PAL1* and

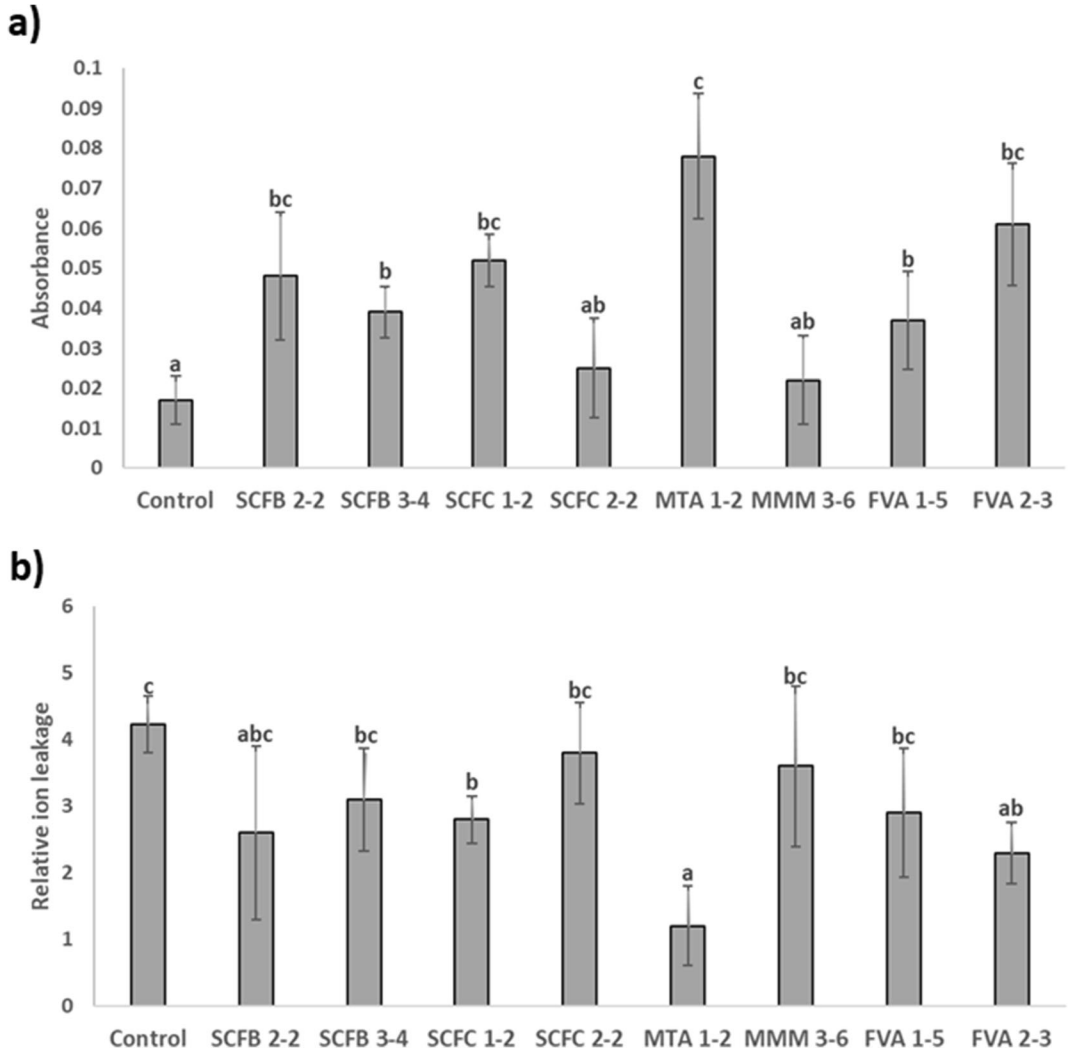

Fig. 4 Tissue vitality by TTC test (**a**) and indirect quantification of ROS (**b**) in F-BPs PGI infected with the pathogen *B. cinerea*. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B.s safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The columns represent the average of three biological replicates with eight fruits in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

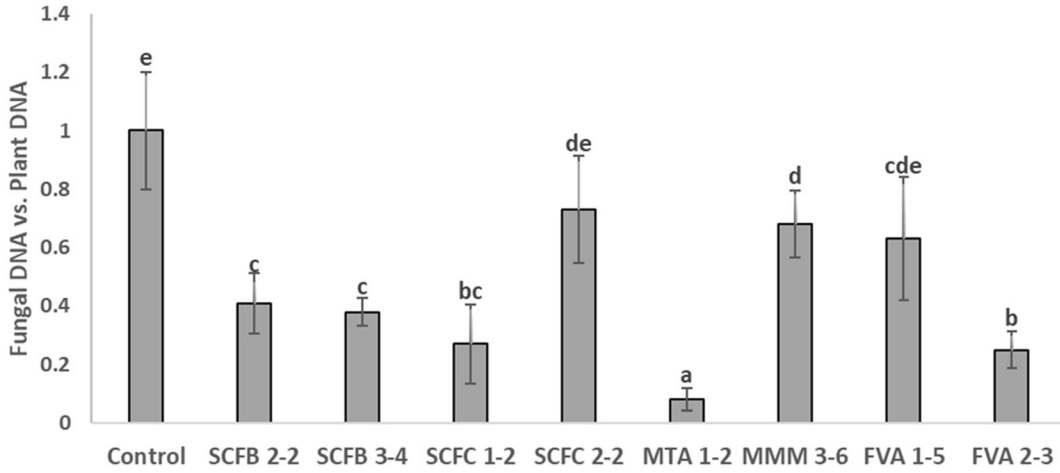

Fig. 5 Quantification of fungal-plant tissue-colonization in F-BP fruits infected with *B. cinerea*. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The columns represent the average of three biological replicates with eight fruits in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

PR-1 (88% reduction) and significantly increased the expression of *LOX1* and *DEF1* (>400% increase). Similarly, inoculation with *B. siamensis* SCFB 2–2 and SCFB 3–4 and *B. pumilus* SCFC 1–2 caused a significant increase in the expression of *LOX1* and *DEF1*, although the decrease in the expression of *PAL1* and *PR-1* was significantly less (except *PAL1* expression for *B. siamensis* SCFB2–2 and *PR-1* expression for *B. siamensis* SCFB3–4), compared to the non-treated control fruit. Fruit treated with *B. proteolyticus* SCFC 2–

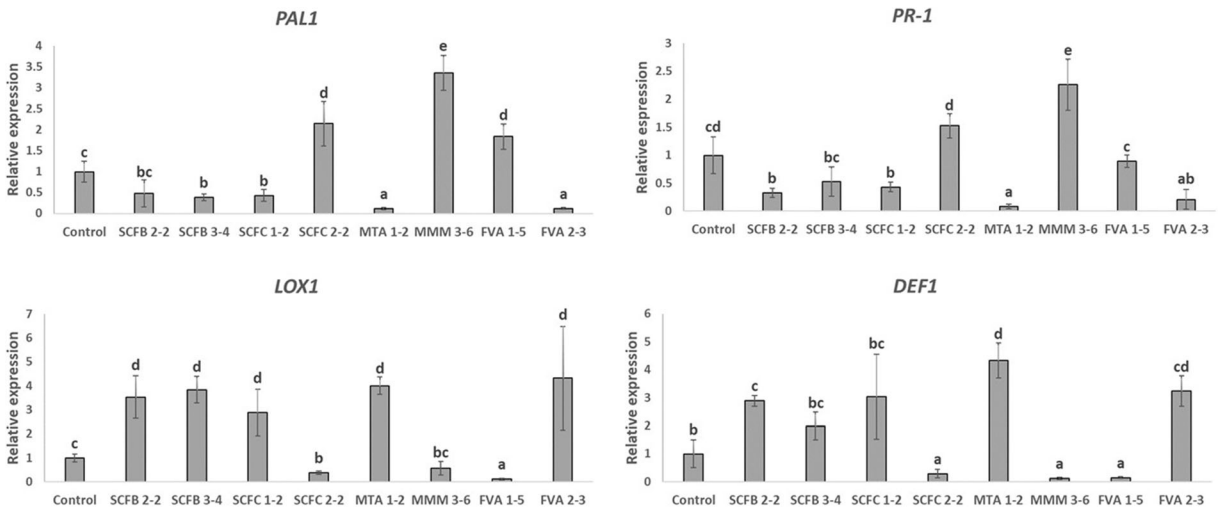

Fig. 6 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defence genes in F-BP fruits infected with *B. cinerea*. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The genes analysed were phenylalanine ammonium lyase 1 (*PAL1*), pathogenesis-related protein (*PR-1*), lipoxygenase 1 (*LOX1*) and defensin 1 (*DEF1*). The measurements were referenced to the value obtained for the plants without inoculation with Bacillus ($2^{-\Delta\Delta Ct} = 1$). The pepper *Actin* gene was used as an internal reference gene. The columns represent the average of three biological replicates with 8 fruits in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

2, *B. megaterium* MMM 3–6 and *B. pumilus* FVA 1–5 showed a significant increase in the expression of *PAL1* and *PR-1* (except expression of *PR-1* for *B. proteolyticus* SCFC2–2 and *B. pumilus* FVA1–5) and a significant decrease in *LOX1* and *DEF1* compared to the non-treated control (Fig. 6).

In planta biocontrol of *V. dahliae*

The inoculation with *V. dahliae* plus the following *Bacillus* strains produced significantly higher foliar biomass (up to 10 times greater) and lower disease severity than the non-treated control: *B. siamensis* SCFB 2–2 and SCFB 3–4, *B. proteolyticus* SCFC 2–2 and *B. megaterium* MMM 3–6 (Fig. 7). Only tissues from roots infected with *V. dahliae* and inoculated with *B. siamensis* SCFB 2–2 and SCFB 3–4 showed significantly higher viability (0.065–0.073) and lower ROS (1.22–1.28) values compared to the non-treated controls (0.021 and 3.85, respectively) (Fig. 8).

The quantification of the root colonization of pepper plants by the pathogen *V. dahliae* showed that roots

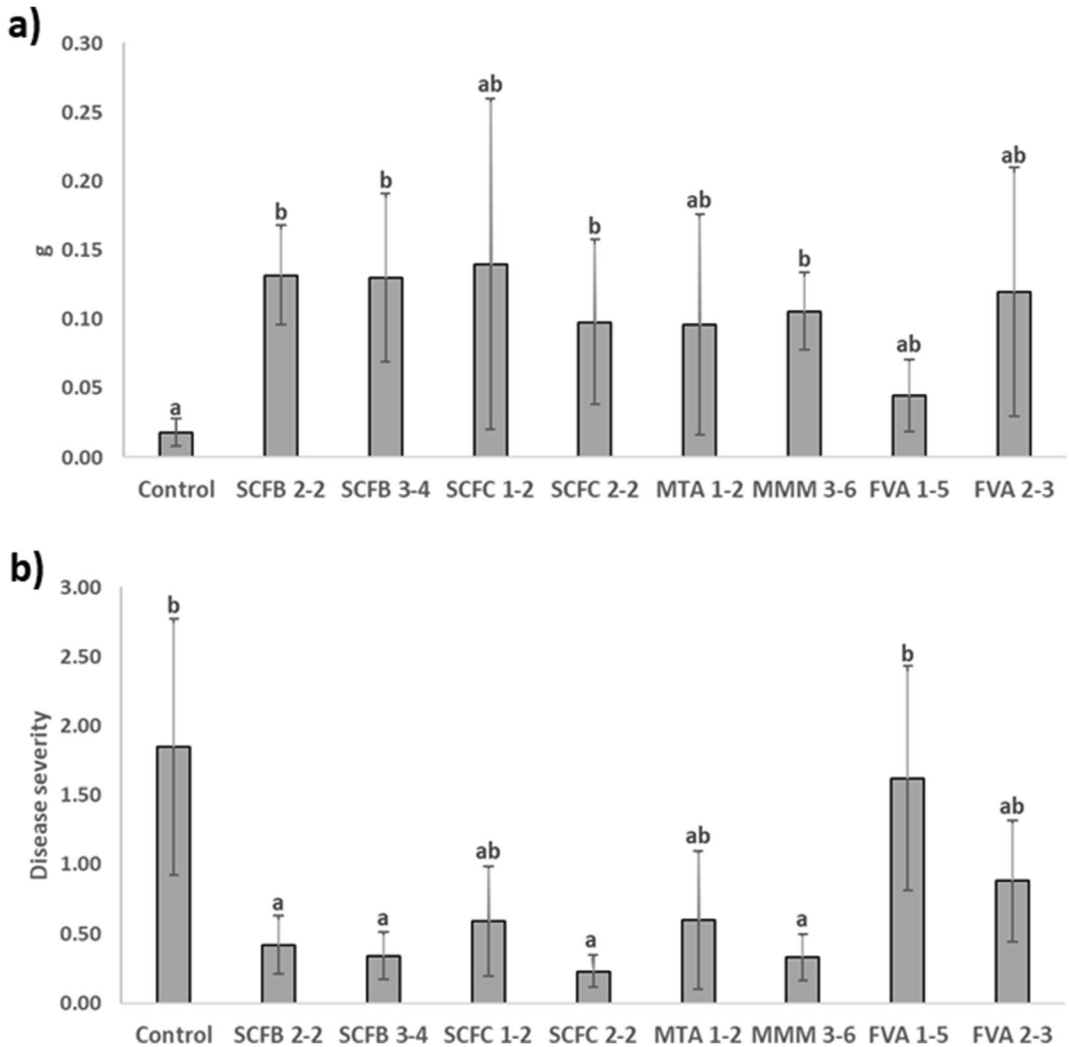

Fig. 7 Dry weight of aerial part (**a**) and disease severity (**b**) of *V. dahliae*-infected F-BP plants. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The columns represent the average of three biological replicates with eight plants in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

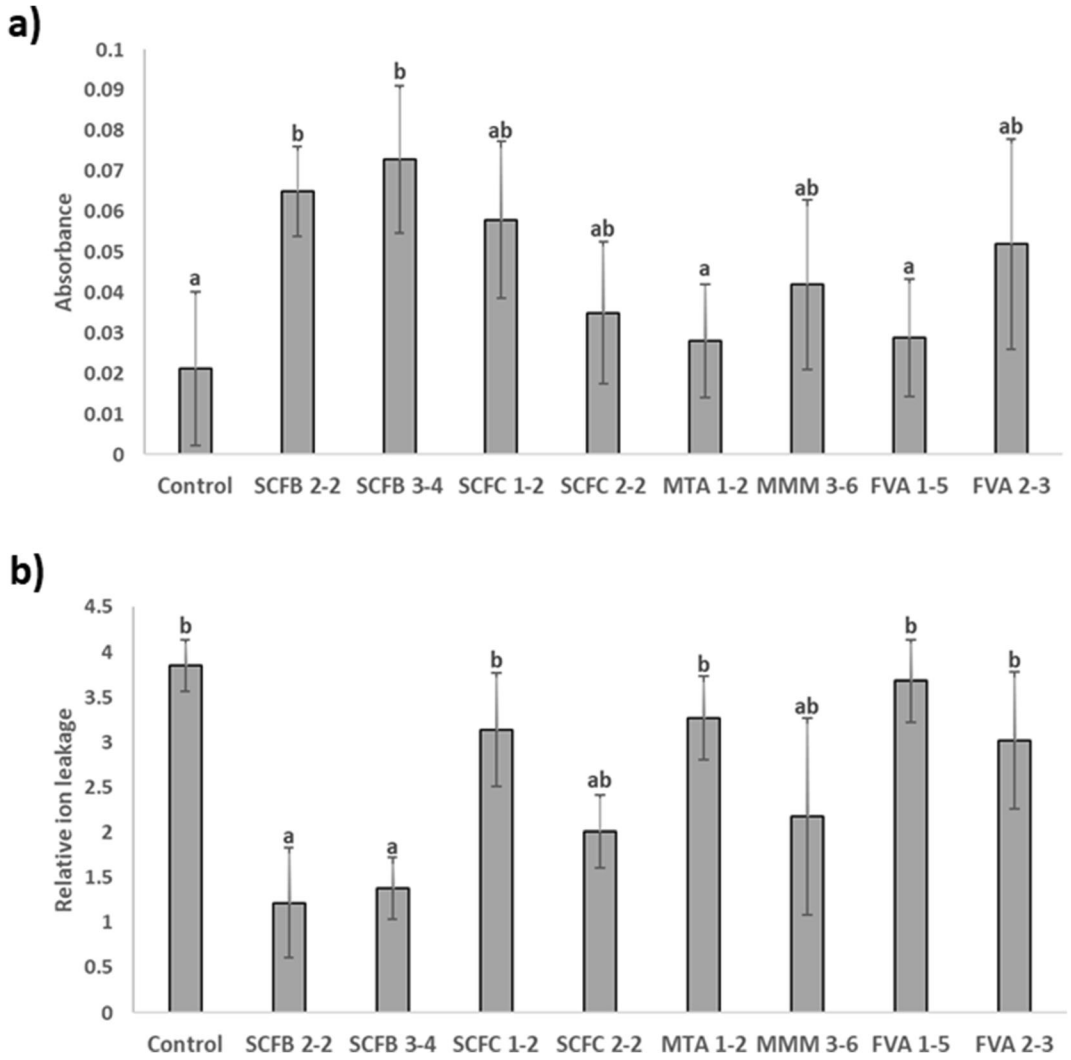

Fig. 8 Tissue vitality by TTC test (**a**) and indirect quantification of ROS (**b**) in F-BP plants infected with the pathogen *V. dahliae*. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B.s thuringiensis*. The columns represent the average of three biological replicates with eight plants in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

treated with *B. proteolyticus* SCFC 2–2, *B. siamensis* SCFB 2–2 and SCFB 3–4, and *B. megaterium* MMM 3–6, significantly reduced the root colonization by the pathogen, compared to the non-treated control (reductions of between 69% and 83%) (Fig. 9).

Regarding defence-gene expression in pepper roots infected with *V. dahliae*, the inoculation with *B. thuringiensis* FVA 2–3 increased the expression of SA-related genes (24–44% increase), compared to plants without bacterial inoculation (Fig. 10). On the contrary, the inoculations of the bacteria *B. proteolyticus* SCFC 2–2 and *B. megaterium* MMM 3–6 produced a significant reduction in the expression of *PR-1*, with an even greater reduction with the inoculations of *B. siamensis* SCFB 2–2 and SCFB 3–4 (reduction of 80–98%) (Fig. 10). Regarding JA-related genes, root inoculation with *B. siamensis* SCFB 2–2 and SCFB 3–4, *B. proteolyticus* SCFC 2–2 and *B. megaterium* MMM 3–6 bacteria significantly increased their expression (even >400% increase), compared to the non-treated control. However, inoculation with *B. pumilus* SCFC 1–2 and FVA 1–5, *B. safensis* MTA 1–2 and

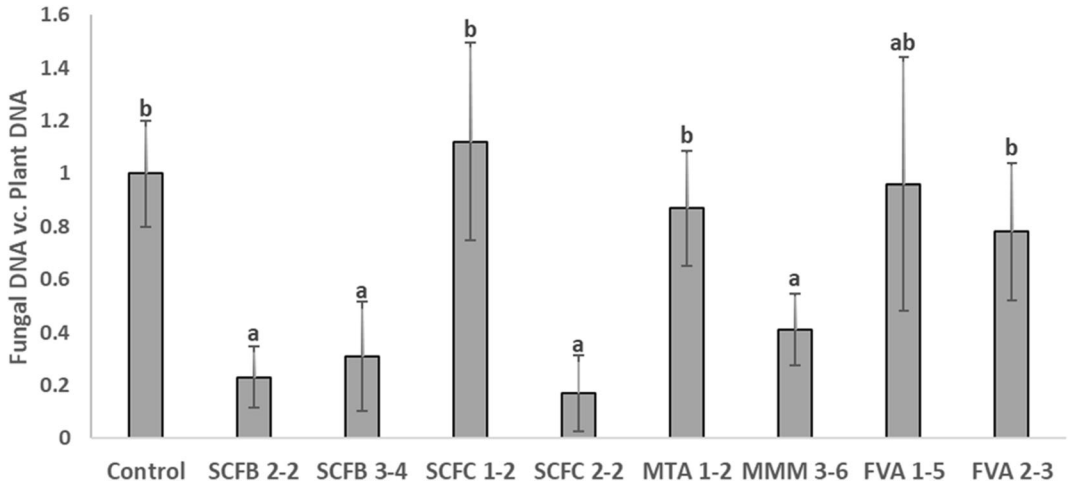

Fig. 9 Quantification of fungal-plant tissue-colonization in F-BP plants infected with *V. dahliae*. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The columns represent the average of three biological replicates with eight plants in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

B. thuringiensis FVA 2–3 bacteria significantly reduced the expression of JA-related genes (except *LOX1* in *B. pumilus* FVA 1–5), compared to plants without bacterial inoculation (Fig. 10).

Discussion

From the species included in our work, *B. proteolyticus* inhibited *in vitro* the growth of *B. cinerea* and

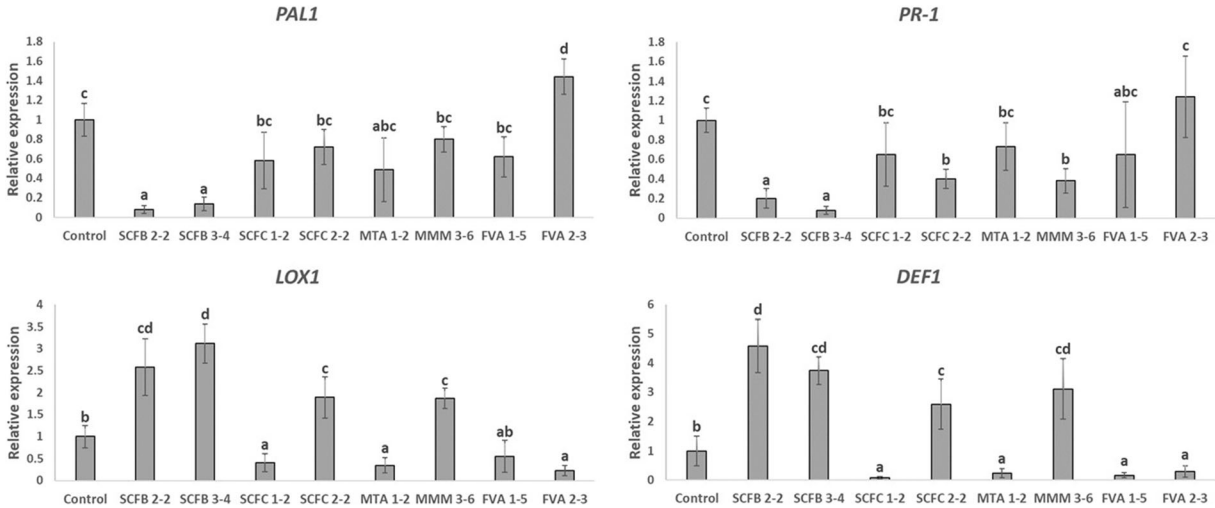

Fig. 10 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defence genes in F-BP plants infected with *V. dahliae*. Control: non-treated, inoculated control; SCFB 2–2: *B. siamensis*; SCFB 3–4: *B. siamensis*; SCFC 1–2: *B. pumilus*; SCFC 2–2: *B. proteolyticus*; MTA 1–2: *B. safensis*; MMM 3–6: *B. megaterium*; FVA 1–5: *B. pumilus*; FVA 2–3: *B. thuringiensis*. The genes analysed were phenylalanine ammonium lyase 1 (*PAL1*), pathogenesis-related protein (*PR-1*), lipoxygenase 1 (*LOX1*) and defensin 1 (*DEF1*). The measurements were referenced to the value obtained for the plants without inoculation with Bacillus ($2^{-\Delta\Delta Ct} = 1$). The pepper *Actin* gene was used as an internal reference gene. The columns represent the average of three biological replicates with eight plants in each one. One-way analysis of variance (ANOVA) was performed, followed by a Tukey test. The different letters signify significant differences ($P < 0.05$)

V. dahliae, being the first description for both pathogens, although it has recently been described as an *in vitro* antagonist of pathogens *Nigrospora sphaerica*, *Pestalotiopsis theae*, *Curvularia eragrostidis*, *Glomerella cingulata*, *Rhizoctonia solani* and *Fusarium oxysporum* (Dutta & Thakur, 2021). On the other hand, *B. siamensis* is a bacterial species widely described as a BCA against pathogens, such as *Alternaria alternata* (Xie et al., 2021), *Colletotrichum fructicola* (Park et al., 2021), or fungi producing postharvest diseases, including *B. cinerea* (You et al., 2021). The two isolates from this study belonging to *B. siamensis*, inhibited the growth of *B. cinerea*. Moreover, they also inhibited the growth of the root pathogen *V. dahliae*, previously described (Gao et al., 2021). Similarly, *B. safensis* has been widely described as a potent BCA, including pathogens such as *Aspergillus flavus* (Einloft et al., 2021), *A. alternata* (Prakash & Arora, 2021), *Magnaporthe oryzae* (Rong et al., 2020), *Plenodomus destruens* (Mateus et al., 2021) and *B. cinerea* (Berrada et al., 2012; Hassan et al., 2021); the *B. safensis* strain from this study controlled the growth of *B. cinerea in vitro* as previously reported, but it also stood out as a BCA of *V. dahliae*, never reported before, to the best of our knowledge.

On the other hand, the potential of *B. pumilus* as a BCA has been described for *R. solani* and *F. oxysporum* (Agarwal et al., 2017). In our work, we have demonstrated *in vitro* effect against *B. cinerea* and *V. dahliae*. Interestingly, the inhibitory effect was strain-dependent in the case of *B. cinerea,* with *B. siamensis* SCFC1–2 being more effective than *B. pumilus* FVA 1–5, whilst the two strains were very effective against *V. dahliae*.

Finally, *B. megaterium* has previously been identified as an efficient BCA *in vitro* against *A. flavus* (Kong et al., 2010), *Septoria tritici* (Kildea et al., 2008) or *Alternaria japonica* (Vásconez et al., 2020) and *B. cinerea*, (Donmez et al., 2011). The *B. megaterium* strain from this work inhibited *in vitro* the growth of *B. cinerea,* but unexpectedly, it promoted *V. dahliae* growth, contrary to what other authors have observed for other strains (Abada et al., 2018). However, it has been previously described that *B. megaterium* promotes the growth of other fungi such as mycorrhizal ones (Marulanda et al., 2009). In this sense, Sarathambal et al. (2022) suggested that the reason for the positive interaction could be related to the bacteria zinc solubilizing capacity.

The results obtained in the *in vivo* infection of F-BP fruits with the pathogen *B. cinerea* after the different bacterial inoculations with *Bacillus* showed that the strains that best controlled the pathogen were *B. safensis* MTA 1–2, *B. thuringiensis* FVA 2–3, *B. siamensis* SCFB 3–4 and SCFC 1–2, and *B. pumilus* SCFC 1–2. Furthermore, it has been found that the bacterial strains *B. proteolyticus* SCFC 2–2, *B megaterium* MMM 3–6 and *B. pumilus* FVA 1–5 of do not induce the activation of defence genes in colonized pepper tissues.

B. safensis MTA 1–2 was able to reduce the disease caused by *B. cinerea* in pepper fruits. However, its *in vitro* antagonism was very low. Therefore, its ability as a BCA on fruits could only be a consequence of an activation of plant defence responses. The ability of *B. safensis* to induce plant defences has been determined in rice plants root inoculated with the bacteria and infected in the aerial part with the pathogen *Bipolaris sorokiniana* (Sarkar et al., 2018). In the specific case of *B. cinerea*, the present work could be the first description of *B. safensis* as an inducer of plant defences against the pathogen, although further tests are required to confirm this, e.g., by physically separating bacterial strain and pathogen.

Based on the results obtained in the study, *B. thuringiensis* FVA 2–3 would be able to act against *B. cinerea* in pepper fruits due to a direct antagonism and the activation of plant defensive responses. The ability of *B. thuringiensis* to activate plant defence responses has been previously described with tomato root treatments, against the fungus *F. oxysporum* f. sp. *lycopersici* (Akram et al., 2013) or against the bacteria *Ralstonia solanacearum* (Takahashi et al., 2014). Against *B. cinerea*, the ability of *B. thuringiensis* to activate plant defences and reduce the disease produced by the pathogen in tomato leaves has been described due to the induction of the expression of different defence genes (Yoshida et al., 2019).

Similarly, both strains of *B. siamensis* used would be capable of reducing the ability of *B. cinerea* to infect pepper fruit tissues through direct antagonism and activation of genes related to plant defence. In this sense, it has been previously described how the root inoculation of tobacco plants with *B. siamensis* significantly reduced the foliar lesions caused by the pathogenic fungus *A. alternata*, due to the activation of plant defence genes (Xie et al., 2021). In the specific case of *B. cinerea*, our study represents the first description of activation of

plant defences against the pathogen mediated by *B. siamensis*.

Finally, regarding the two strains of *B. pumilus*, we have verified how the strain SCFC 1–2 would be able to reduce the damage caused by *B. cinerea* in pepper fruits thanks to the direct antagonism and activation of the plant defences, while the FVA 1–5 strain did not show plant defences activation. The ability of *B. pumilus* to activate plant defences has been previously described in tobacco and pepper plants against foliar infection by the bacteria *Xanthomonas axonopodis*, through the induction of SA-related genes (Li et al., 2020; Yi et al., 2013).

The results obtained in the *in planta* study showed that the bacterial species that best reduces the disease caused by *V. dahliae* was *B. siamensis*. In addition, *B. proteolyticus* and *B. megaterium* also reduced disease, to a lesser degree than *B. siamensis*.

In the control of *V. dahliae* in F-BP roots by *B. siamensis* mechanisms of both direct antagonism and activation of plant defences would be involved. In this sense, the ability of the bacterium to activate plant defences systemically when inoculated into roots has been previously described (Xie et al., 2021), its ability to activate defence genes locally in roots has not been previously described. Therefore, our study represents the first description of activation of root defences by *B. siamensis* inoculation and the first description of its effectiveness against the pathogen *V. dahliae*.

In the case of the bacteria *B. proteolyticus*, we have described its ability to control *V. dahliae* in pepper roots also through direct antagonism and activation of plant defences. These results represent a highly relevant finding since *B. proteolyticus* had never been described as a BCA against *V. dahliae* and our study places it as one of the most effective bacterial species.

Finally, *B. megaterium* would be able to reduce the disease caused by *V. dahliae* in pepper roots mainly through the activation of plant defences mediated by JA (specific response against necrotrophic pathogens, such as *V. dahliae*). The ability of *B. megaterium* to induce systemic resistance in plants has been previously described in cucumber roots against the oomycete *Pythium aphanidermatum* (Liang et al., 2011) and in *Camellia sinensis* roots against the fungus *Fomes lamaoensis* (Chakraborty et al., 2006). Against *V. dahliae*, the present work would be the first description of the ability of *B. megaterium* to control the pathogen due to the activation of plant defence responses, since in the direct *in vitro* interaction we found that the bacteria promoted the growth of the pathogenic fungus.

Our results show how the best protection effect against *B. cinerea* and *V. dahliae* occurs when SA-related genes (*PAL1* and *PR1*) are downregulated, and JA-related genes (*LOX1* and *DEF1*) are upregulated. The SA pathway activates plant defences against biotrophic pathogens, whereas the JA pathway activates plant defences against necrotrophic pathogens (Li et al., 2019; Thaler et al., 2012). Therefore, SA pathway activation is known to compromise plant defence against necrotrophes through JA pathway suppression (Li et al., 2019). Our results are consistent with those studies, by showing a clear antagonism between SA-related genes and JA-related genes activations. Those bacterial strains that downregulate SA-related genes and upregulate JA-related genes (*B. siamensis* SCFB 2–2, *B. siamensis* SCFB 3–4, *B. thuringiensis* FVA 2–3 and *B. safensis* MTA 1–2 in the case of *B. cinerea* infection; and *B. siamensis* SCFB 2–2, *B. siamensis* SCFB 3–4, *B. proteolyticus* SCFC 2–2 and *B. megaterium* MMM 3–6 in the case of *V. dahliae* infection) are the ones that show the best results in fruit injury area, aerial biomass, ion leakage and viability. JA-related genes activation explains this protective effect as *B. cinerea* and *V. dahliae* are necrotrophic pathogens. However, the mechanism used by the strains to activate the JA pathway has not been uncovered by our investigation, thus requiring further research in order to know how it is done. One possibility is the production of molecules that mimic JA by the bacteria (Thaler et al., 2012), but they have not been detected in the present study.

In conclusion, the endophytic species *B. siamensis* is the best choice as a biological control agent for the main diseases suffered by the F-BP. This choice is because *B. siamensis* can reduce the disease caused by *B. cinerea* in fruits and *V. dahliae* in roots, through direct antagonism and activation of plant defences. Although these results have been obtained under laboratory and greenhouse conditions (with local soil), the application and efficiency of the treatment in field and storage conditions can give very different results, as there are other variables, such as microbiota already present in the soils and fruits, or adverse weather conditions. Therefore, tests with *B. siamensis* should be carried out in real situations and its effectiveness in the agricultural system of the area should be determined.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-022-02575-x.

Author contribution J.P. is the Principal Researcher of the research project. J.P., M.B. and F.G.-A. thought and designed the work. J.P. and J.C. performed the assays and analyzed the results. M.B. provided the endophytic bacteria used in the study. J.P. wrote the first version of the manuscript. J.C., M.B. and F.G.-A. contributed to the manuscript correction and critical reading, as well as to the knowledge on the bacteria field. All authors have read and agreed to the published version of the manuscript.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Abada, K. A. M., Attia, A. M. F., & Zyton, M. A. L. (2018). Management of pepper *Verticillium* wilt by combinations of inducer chemicals for plant resistance, bacterial bioagents and compost. *Journal of Applied Biotechnology & Bioengineering, 5*, 117–127. https://doi.org/10.15406/jabb.2018.05.00126

Acosta-Morel, W., Marques-Costa, T. M., Santander-Gordón, D., Anta Fernández, F., Zabalgogeazcoa, I., Vázquez de Aldana, B. R., et al. (2019). Physiological and population genetic analysis of *Botrytis* field isolates from vineyards in Castilla y León, Spain. *Plant Pathology, 68*(3), 523–536. https://doi.org/10.1111/ppa.12967

Agarwal, M., Dheeman, S., Dubey, R. C., Kumar, P., Maheshwari, D. K., & Bajpai, V. K. (2017). Differential antagonistic responses of *Bacillus pumilus* MSUA3 against *Rhizoctonia solani* and *Fusarium oxysporum* causing fungal diseases in *Fagopyrum esculentum* Moench. *Microbiological Research, 205*, 40–47. https://doi.org/10.1016/j.micres.2017.08.012

Akram, W., Mahboob, A., & Javed, A. A. (2013). *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against *Fusarium* wilt. *European Journal of Microbiology and Immunology, 3*(4), 275–280. https://doi.org/10.1556/EuJMI.3.2013.4.7

Barquero, M., Terrón, A., Velázquez, E., & González-Andrés, F. (2016). Biocontrol of *fusarium oxysporum* f. sp. phaseoli and *Phytophthora capsici* with autochthonous endophytes in common bean and pepper in Castilla y León (Spain). In F. González-Andrés & E. James (Eds.), *Biological nitrogen fixation and beneficial plant-microbe interaction* (pp. 221–235). Springer. https://doi.org/10.1007/978-3-319-32528-6_19

Berrada, I., Benkhemmar, O., Swings, J., Bendaou, N., & Amar, M. (2012). Selection of halophilic bacteria for biological control of tomato gray mould caused by *Botrytis cinerea*. *Phytopathologia Mediterranea, 51*(3), 625–630. https://doi.org/10.14601/Phytopathol_Mediterr-10627

Chakraborty, U., Chakraborty, B., & Basnet, M. (2006). Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. *Journal of Basic Microbiology, 46*(3), 186–195. https://doi.org/10.1002/jobm.200510050

Donmez, M. F., Esitken, A., Yildiz, H., & Ercisli, S. (2011). Biocontrol of Botrytis cinerea on strawberry fruit by plant growth promoting bacteria. *Journal of Animal and Plant Sciences, 21*, 758–763.

Dutta, J., & Thakur, D. (2021). Diversity of culturable bacteria endowed with antifungal metabolites biosynthetic characteristics associated with tea rhizosphere soil of Assam, India. *BMC Microbiology, 21*, 1–13. https://doi.org/10.1186/s12866-021-02278-z

EC 925/2012. (2012). Council regulation (EC) no 925/2012 of 8 October 2012, entering a name in the register of protected designations of origin and protected geographical indications (Pimiento de Fresno-Benavente (PGI)). *Official Journal of the European Union, 275*, 23–24 http://data.europa.eu/eli/reg_impl/2012/925/oj

Einloft, T. C., de Oliveira, P. B., Radünz, L. L., & Dionello, R. G. (2021). Biocontrol capabilities of three *Bacillus* isolates towards aflatoxin B1 producer *a. flavus* in vitro and on maize grains. *Food Control, 125*, 107978. https://doi.org/10.1016/j.foodcont.2021.107978

Eljounaidi, K., Lee, S. K., & Bae, H. (2016). Bacterial endophytes as potential biocontrol agents of vascular wilt diseases–review and future prospects. *Biological Control, 103*, 62–68. https://doi.org/10.1016/j.biocontrol.2016.07.013

Food and Agriculture Organization of the United Nations (FAO).(2020). FAOSTAT Database. Production/Yield quantities of Chillies and peppers, green in World. Retrieved December 21, 2021, from http://www.fao.org/faostat/en/#data/QC

Fratianni, F., d'Acierno, A., Cozzolino, A., Spigno, P., Riccardi, R., Raimo, F., Pane, C., Zaccardelli, M., Tranchida Lombardo, V., Tucci, M., Grillo, S., Coppola, R., & Nazzaro, F. (2020). Biochemical characterization of traditional varieties of sweet pepper (*Capsicum annuum* L.) of the Campania region, Southern Italy. *Antioxidants, 9*(6), 556. https://doi.org/10.3390/antiox9060556

Gao, L., Ma, J., Liu, Y., Huang, Y., Mohamad, O. A. A., Jiang, H., Egamberdieva, D., Li, W., & Li, L. (2021). Diversity and biocontrol potential of cultivable endophytic bacteria associated with halophytes from the West Aral Sea basin. *Microorganisms, 9*, 1448. https://doi.org/10.3390/microorganisms9071448

Guevara, L., Domínguez-Anaya, M. Á., Ortigosa, A., González-Gordo, S., Díaz, C., Vicente, F., Corpas, F. J., Pérez del Palacio, J., & Palma, J. M. (2021). Identification of compounds with potential therapeutic uses from sweet pepper (*Capsicum annuum* L.) fruits and their modulation by nitric oxide (NO). *International Journal of Molecular Sciences, 22*(9), 4476. https://doi.org/10.3390/ijms22094476

Hassan, E. A., Mostafa, Y. S., Alamri, S., Hashem, M., & Nafady, N. A. (2021). Biosafe Management of *Botrytis* Grey Mold of strawberry fruit by novel bioagents. *Plants, 10*(12), 2737. https://doi.org/10.3390/plants10122737

Hwang, I. S., & Hwang, B. K. (2010). The pepper 9-lipoxygenase gene CaLOX1 functions in defense and cell death responses to microbial pathogens. *Plant Physiology, 152*(2), 948–967. https://doi.org/10.1104/pp.109.147827

Kildea, S., Ransbotyn, V., Khan, M. R., Fagan, B., Leonard, G., Mullins, E., & Doohan, F. M. (2008). *Bacillus megaterium* shows potential for the biocontrol of *Septoria tritici* blotch of wheat. *Biological Control, 47*(1), 37–45. https://doi.org/10.1111/jam.13171

Kim, D. S., & Hwang, B. K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *Journal of Experimental Botany, 65*(9), 2295–2306. https://doi.org/10.1093/jxb/eru109

Klimes, A., Amyotte, S. G., Grant, S., Kang, S., & Dobinson, K. F. (2008). Microsclerotia development in *Verticillium dahliae*: Regulation and differential expression of the hydrophobin gene VDH1. *Fungal Genetics and Biology, 45*(12), 1525–1532. https://doi.org/10.1016/j.fgb.2008.09.014

Kong, Q., Shan, S., Liu, Q., Wang, X., & Yu, F. (2010). Biocontrol of *Aspergillus flavus* on peanut kernels by use of a strain of marine *Bacillus megaterium*. *International Journal of Food Microbiology, 139*(1–2), 31–35. https://doi.org/10.1016/j.ijfoodmicro.2010.01.036

Li, N., Han, X., Feng, D., Yuan, D., & Huang, & L.-J. (2019). Signaling crosstalk between salicylic acid and ethylene/jasmonate in plant defense: Do we understand what they are whispering? *International Journal of Molecular Sciences, 20*(3), 671. https://doi.org/10.3390/ijms20030671

Li, W., Lee, S. Y., Cho, Y. J., Ghim, S. Y., & Jung, H. Y. (2020). Mediation of induced systemic resistance by the plant growth-promoting rhizobacteria *Bacillus pumilus* S2-3-2. *Molecular Biology Reports, 47*(11), 8429–8438. https://doi.org/10.1007/s11033-020-05883-9

Liang, J. G., Taom, R. X., Hao, Z. N., Wang, L., & Zhang, X. (2011). Induction of resistance in cucumber against seedling damping-off by plant growth-promoting rhizobacteria (PGPR) *Bacillus megaterium* strain L8. *African Journal of Biotechnology, 10*(36), 6920–6927. https://doi.org/10.5897/AJB11.260

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods, 25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Lyousfi, N., Lahlali, R., Letrib, C., Belabess, Z., Ouaabou, R., Ennahli, S., Blenzar, A., & Barka, E. A. (2021). Improving the biocontrol potential of bacterial antagonists with salicylic acid against brown rot disease and impact on nectarine fruits quality. *Agronomy, 11*(2), 209. https://doi.org/10.3390/agronomy11020209

Marulanda, A., Barea, J. M., & Azcón, R. (2009). Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments: Mechanisms related to bacterial effectiveness. *Journal of Plant Growth Regulation, 28*(2), 115–124. https://doi.org/10.1007/s00344-009-9079-6

Mateus, J. R., Dal'Rio, I., Jurelevicius, D., da Mota, F. F., Marques, J. M., Ramos, R. T. J., da Costa da Silva, A. L., Gagliardi, P. R., & Seldin, L. (2021). *Bacillus velezensis* T149-19 and *Bacillus safensis* T052-76 as potential biocontrol agents against foot rot disease in sweet potato. *Agriculture, 11*(11), 1046. https://doi.org/10.3390/agriculture11111046

Mekawi, E. M., Khafagi, E. Y., & Abdel-Rahman, F. A. (2019). Effect of pre-harvest application with some organic acids and plant oils on antioxidant properties and resistance to *Botrytis cinerea* in pepper fruits. *Scientia Horticulturae, 257*, 108736. https://doi.org/10.1016/j.scienta.2019.108736

Mena-Violante, H. G., & Olalde-Portugal, V. (2007). Alteration of tomato fruit quality by root inoculation with plant growth-promoting rhizobacteria (PGPR): *Bacillus subtilis* BEB-13bs. *Scientia Horticulturae, 113*(1), 103–106. https://doi.org/10.1016/j.scienta.2007.01.031

Mercado-Blanco, J., Rodrıguez-Jurado, D., Hervás, A., & Jiménez-Dıaz, R. M. (2004). Suppression of *Verticillium* wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biological Control, 30*(2), 474–486. https://doi.org/10.1016/j.biocontrol.2004.02.002

Park, B. R., Son, H. J., Park, J. H., Kim, E. S., Heo, S. J., Youn, H. R., Koo, Y. M., Heo, A. Y., Choi, H. W., Sang, M. K., Lee, S. W., Choi, S. H., & Hong, J. K. (2021). Chemical fungicides and *Bacillus siamensis* H30-3 against fungal and oomycete pathogens causing soil-borne strawberry diseases. *The Plant Pathology Journal, 37*(1), 79–85. https://doi.org/10.5423/PPJ.NT.12.2020.0232

Poveda, J. (2020). *Marchantia polymorpha* subsp. *ruderalis* (Bischl. & Boissel.-dub.)-arbuscular mycorrhizal fungi interaction: Beneficial or harmful? *Symbiosis, 82*, 165–174. https://doi.org/10.1007/s13199-020-00708-6

Poveda, J. (2021a). Biological control of *Fusarium oxysporum* f. sp. ciceri and *Ascochyta rabiei* infecting protected geographical indication Fuentesaúco-Chickpea by *Trichoderma* species. *European Journal of Plant Pathology, 160*, 825–840. https://doi.org/10.1007/s10658-021-02286-9

Poveda, J. (2021b). Glucosinolates profile of *Arabidopsis thaliana* modified root colonization of *Trichoderma* species. *Biological Control, 155*, 104522. https://doi.org/10.1016/j.biocontrol.2020.104522

Poveda, J. (2022a). AtCube: Performing pathogen-root infection tests on *Arabidopsis thaliana* in a completely controlled way. *Physiological and Molecular Plant Pathology, 117*, 101780. https://doi.org/10.1016/j.pmpp.2021.101780

Poveda, J. (2022b). Effect of volatile and non-volatile metabolites from *Leptosphaeria maculans* on tomato calli under abiotic stresses. *Plant Stress, 3*, 100054. https://doi.org/10.1016/j.stress.2021.100054

Poveda, J., & González-Andrés, F. (2021). *Bacillus* as a source of phytohormones for use in agriculture. *Applied Microbiology and Biotechnology, 105*(23), 8629–8645. https://doi.org/10.1007/s00253-021-11492-8

Poveda, J., Barquero, M., & González-Andrés, F. (2020). Insight into the microbiological control strategies against *Botrytis cinerea* using systemic plant resistance activation. *Agronomy, 10*(11), 1822. https://doi.org/10.3390/agronomy10111822

Poveda, J., Eugui, D., Abril-Urías, P., & Velasco, P. (2021). Endophytic fungi as direct plant growth promoters for sustainable agricultural production. *Symbiosis, 85*(1), 1–19. https://doi.org/10.1007/s13199-021-00789-x

Prakash, J., & Arora, N. K. (2021). Novel metabolites from *Bacillus safensis* and their antifungal property against *Alternaria alternata*. *Antonie Van Leeuwenhoek, 114*, 1245–1258. https://doi.org/10.1007/s10482-021-01598-4

Ren, W., Liu, N., Sang, C., Shi, D., Zhou, M., Chen, C., Qin, Q., & Chen, W. (2018). The autophagy gene BcATG8 regulates the vegetative differentiation and pathogenicity of *Botrytis cinerea*. *Applied Environmental Microbiology, 84*(11), e02455–e02417. https://doi.org/10.1128/AEM.02455-17

Rong, S., Xu, H., Li, L., Chen, R., Gao, X., & Xu, Z. (2020). Antifungal activity of endophytic *Bacillus safensis* B21 and its potential application as a biopesticide to control rice blast. *Pesticide Biochemistry and Physiology, 162*, 69–77. https://doi.org/10.1016/j.pestbp.2019.09.003

Sarathambal, C., Dinesh, R., Srinivasan, V., Sheeja, T. E., Jeeva, V., & Manzoor, M. (2022). Changes in bacterial diversity and composition in response to co-inoculation of arbuscular mycorrhizae and zinc-solubilizing bacteria in turmeric rhizosphere. *Current Microbiology, 79*(1), 1–9. https://doi.org/10.1007/s00284-021-02682-8

Sarkar, J., Chakraborty, U., & Chakraborty, B. N. (2018). Induced defense response in wheat plants against *Bipolaris sorokiniana* following application of *Bacillus safensis* and *Ochrobactrum pseudogrignonense*. *Indian Phytopathology, 71*(1), 49–58. https://doi.org/10.1007/s42360-018-0006-2

Shi, J. F., & Sun, C. Q. (2017). Isolation, identification, and biocontrol of antagonistic bacterium against *Botrytis cinerea* after tomato harvest. *Brazilian Journal of Microbiology, 48*(4), 706–714. https://doi.org/10.1016/j.bjm.2017.03.002

Slama, H. B., Cherif-Silini, H., Chenari Bouket, A., Qader, M., Silini, A., Yahiaoui, B., Alenezi, F. N., Luptakova, L., Triki, M. A., Vallat, A., Oszako, T., Rateb, M. E., & Belbahri, L. (2019). Screening for *Fusarium* antagonistic bacteria from contrasting niches designated the endophyte *Bacillus halotolerans* as plant warden against *Fusarium*. *Frontiers in Microbiology, 9*, 3236. https://doi.org/10.3389/fmicb.2018.03236

Takahashi, H., Nakaho, K., Ishihara, T., Ando, S., Wada, T., Kanayama, Y., Asano, S., Yoshida, S., Tsushima, S., & Hyakumachi, M. (2014). Transcriptional profile of tomato roots exhibiting *Bacillus thuringiensis*-induced resistance to *Ralstonia solanacearum*. *Plant Cell Reports, 33*(1), 99–110. https://doi.org/10.1007/s00299-013-1515-1

Thaler, J. S., Humphrey, P. T., & Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science, 17*(5), 260–270. https://doi.org/10.1016/j.tplants.2012.02.010

Tubeileh, A. M., & Stephenson, G. T. (2020). Soil amendment by composted plant wastes reduces the *Verticillium dahliae* abundance and changes soil chemical properties in a bell pepper cropping system. *Current Plant Biology, 22*, 100148. https://doi.org/10.1016/j.cpb.2020.100150

Tyvaert, L., Everaert, E., Lippens, L., Cuijpers, W. J. M., França, S. C., & Höfte, M. (2019). Interaction of *Colletotrichum coccodes* and *Verticillium dahliae* in pepper plants. *European Journal of Plant Pathology, 155*(4), 1303–1317. https://doi.org/10.1007/s10658-019-01857-1

Vásconez, R. D. A., Moya, E. M. T., Yépez, L. A. C., Chiluisa-Utreras, V. P., & de los Ángeles Vaca-Suquillo, I. (2020). Evaluation of *Bacillus megaterium* strain AB4 as a potential biocontrol agent of *Alternaria japonica*, a mycopathogen of *Brassica oleracea var. italica*. *Biotechnology Reports, 26*, e00454. https://doi.org/10.1016/j.btre.2020.e00454

Xie, Z., Li, M., Wang, D., Wang, F., Shen, H., Sun, G., Feng, C., Wang, X., Chen, D., & Sun, X. (2021). Biocontrol efficacy of *Bacillus siamensis* LZ88 against brown spot disease of tobacco caused by *Alternaria alternata*. *Biological Control, 154*, 104508. https://doi.org/10.1016/j.biocontrol.2020.104508

Yi, H. S., Yang, J. W., & Ryu, C. M. (2013). ISR meets SAR outside: Additive action of the endophyte *Bacillus pumilus* INR7 and the chemical inducer, benzothiadiazole, on induced resistance against bacterial spot in field-grown pepper. *Frontiers in Plant Science, 4*, 122. https://doi.org/10.3389/fpls.2013.00122

Yoshida, S., Koitabashi, M., Yaginuma, D., Anzai, M., & Fukuda, M. (2019). Potential of bioinsecticidal *Bacillus thuringiensis* inoculum to suppress gray mold in tomato based on induced systemic resistance. *Journal of Phytopathology, 167*(11–12), 679–685. https://doi.org/10.1111/jph.12864

You, W., Ge, C., Jiang, Z., Chen, M., Li, W., & Shao, Y. (2021). Screening of a broad-spectrum antagonist-*Bacillus siamensis*, and its possible mechanisms to control postharvest disease in tropical fruits. *Biological Control, 157*, 104584. https://doi.org/10.1016/j.biocontrol.2021.104584