Hinarejos, E.; Castellano Perez, M.; Rodrigo Bravo, I.; Belles Albert, JM.; Conejero Tomás, V.; López-Gresa, MP.; Lisón, P. (2016). Bacillus subtilis IAB/BS03 as a potential biological control agent. European Journal of Plant Pathology. 146(3):597-608. doi:10.1007/s10658-016-0945-3

Bacillus subtilis IAB/BS03 as a potential biological control agent

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Abstract

We describe the efficacy of *Bacillus subtilis* strain IAB/BS03 in reducing disease incidence of *B. subtilis* IAB/BS03 as a foliar treatment against *Botrytis cinerea* and *Pseudomonas syringae* on greenhouse-grown tomato (*Solanum lycopersicon*) plants. We also tested the effect of foliar treatments on lettuce (*Lactuca sativa*) against lettuce downy mildew caused by *Bremia lactucae* in multiple trials under different field conditions. All the assays indicated that *B. subtilis* IAB/BS03 reduced disease. To ascertain the mechanism of action, the induction of pathogenesis-related (PR) proteins, the accumulation of salicylic acid and the activation of peroxidase caused by foliar or root treatments with *B. subtilis* IAB/BS03 were studied in tomato. A salicylic acid-independent induction of the antifungal protein PR1 was observed after treatment with *B. subtilis* IAB/BS03, with the strongest induction due to root treatment compared with foliar application. A metabolic analysis of *B. subtilis* IAB/BS03 culture broth using Ultra Performance Liquid Chromatography coupled with ultraviolet and mass spectrometric detection determined surfactin and iturin A isomers. These compounds have been described as antifungal and antibiotic lipopeptides. The results indicated that *B. subtilis* IAB/BS03 could be effectively used as a biocontrol agent.

Keywords: *Bacillus subtilis*, biological control, Iturin, Surfactin.

Introduction

Crop disease causes major yield losses, and emerging diseases pose new threats to global food security (Wulff et al. 2011). Biological control is an environmentally sustainable alternative to using synthetic pesticides. Plant growth-promoting rhizobacteria (PGPRs), such as *Bacillus subtilis*, are widely applied to control many crop diseases (Liu et al. 2014). PGPRs are able to enhance plant growth and yield through phytohormone production (Tsavkelova et al. 2006). As application of PGPRs offers several advantages in commercial agricultural production, many products that contain *B. subtilis* are now available (Choudhary and Johri 2009). However, the implementation of these *Bacillus*-based products remains an issue (Pérez-García et al. 2011).

One of the proposed mechanisms in the PGPR-activated control of plant disease is to induce systemic resistance, known as ISR (Induced Systemic Resistance). ISR protects plants from subsequent infection by other pathogenic organisms (Van Loon 2007). Induced plant defence is regulated by pathways that involve molecules, including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Robert-Seilaniantz et al. 2007). Defence proteins, like pathogenesis-related proteins (PRs) or peroxidases, are important components of this network. PR proteins exert activity against the pathogen; for example, PR1 has been described as an antifungal protein, and is used as a marker to establish plant resistance to pathogens (Niderman et al. 1995). There is evidence to indicate that peroxidase plays an important biological role in the plant defence response against biotic or abiotic stresses (Mohammadi and Kazemi 2002). A cationic peroxidase encoded by the *Ep5C* gene has been demonstrated to be required for bacterial speck susceptibility in tomato (Coego et al. 2005).

In addition, the effect of *Bacillus* spp. on plant disease suppression has been attributed to the production of peptide antibiotics (Cho et al. 2003). Among them, two families of lipopeptides, synthesised by bacteria of the genus *Bacillus,* the iturins and surfactins are the best characterised due to their antagonistic activity against a wide range of potential pathogens (Ongena and Jacques 2008). Surfactins are powerful biosurfactants with antibacterial activity, but no marked fungitoxicity (with some exceptions). Iturins have strong antifungal action, but have only limited antibacterial activity (Pérez-García et al. 2011). Besides antibiosis, these lipopeptides can play other roles in biocontrol as they can induce plant defences (Choudhary and Johri 2009, Kawagoe et al. 2015).

The aim of this work was to test the efficacy of *B. subtilis* strain IAB/BS03 against *Botrytis cinerea* and *Pseudomonas syringae* on tomato, and against *Bremia lactucae* in lettuce. To explore the mechanisms involved, we studied the induction of the antifungal protein PR1, peroxidase activity and SA accumulation *in planta*, and we tested for the presence of antibiotics surfactin and iturin A in cultures of *B. subtilis* IAB/BS03.

Materials and methods

Efficacy of B. subtilis IAB/BS03 against *B. cinerea* and *P. syringae* in tomato plants

Tomato plants were grown in a greenhouse under natural environmental conditions and photoperiod, at an experiment station (Promo-Vert Crop Services S.L., Sevilla, Spain) in Níjar (Almería, Spain) from February to March 2012. 'Rebelion' tomato plants, used for tests against *B. cinerea*, were grown at 9-26 °C with 50-78% relative humidity (RH). 'Ramyle' tomato plants were grown at 7-23 °C with 50-90% RH and were used for tests

against *P. syringae*. Standard plant management operations (pruning to promote ventilation, removing damaged leaves, etc.) were performed to prevent excessive disease development.

Treatments included a control (untreated) and *B. subtilis* IAB/BS03 (at 10^8 cfu g^{-1}) at three doses (1, 10 and 100 g hL^{-1}). Treatments with a standard fungicide (Scala, 40% Pyrimetanil, 0.15% w/v; Bayer Cropscience, Almería, Spain) or a standard bactericide (ZZ Cuprocol, 700 g L^{-1} copper oxychloride, 0.3% w/v; Seagro SL, Sevilla, Spain) were used as positive controls (each treatment comprised three replicate plants). The experiment was performed once, and has a randomised complete block design comprising four blocks, each containing 15 plants. Five different foliar applications (A, B, C, D and E), at a rate of 1,200 L ha^{-1}, were applied at 5-8 day intervals. The first *Botrytis* assay application (A) took place at fruit maturation growth stage "BBCH77" (Zadoks et al. 1974), while the *P. syringae* assay was applied as fruit started ripening or upon colouration, "stage BBCH83". Subsequently the remaining treatments (B, C, D and E) were applied at 1-week intervals until fruit commenced ripening, or until all fruit began to soften, for the fungal and bacterial assays, respectively.

The experiments relied on natural inoculum, and plants were assessed for incidence (percentage of leaves with symptoms) and severity (percentage leaf area with symptoms). The assessments for fungal symptoms (Fig S1A, Electronic Supplementary Material 1) were recorded at 0 days post treatment (dpt) B, 0 dpt-C, 0 dpt-D, 0 dpt-E, 7 dpt-E and 16 dpt-E. In addition, symptoms were evaluated for *P. syringae* incidence and severity both on main stems and footstalks and calyxes at 0 dpt-B, 1 dpt-C, 0 dpt-D, 0 dpt-E, 8 dpt-E and 14 dpt-E (Fig S1B, Electronic Supplementary Material 1).

The tomato experiments were performed following the guidelines of Good Experimental Practice (GEP), in accordance with Guidelines PP 1/181(3), PP 1/152(3)

and PP 1/135(3) provided by the European and Mediterranean Plant Protection Organization (EPPO). Specific EPPO Guidelines PP 1/54 (3) and PP 1/121(2) were applied for *B. cinerea* and *P. syringae* assays, respectively.

Efficacy of *B. subtilis* IAB/BS03 against *Bremia lactucae* in lettuce

Different lettuce varieties were assayed in nine open field trials on growers' land at several locations: 'Modelo' (Benicarló, Spain); 'Abby', 'Carrascoy' and 'Yerga', (Vinaròs, Spain); 'Matinale' (Torres Vedras, Portugal); 'Verde Degly Ortolani' (Policoro, Italy); 'Romana' (Francolise, Italy); 'Craquante d'Avignon' (Nimes, France); and 'Kristina' (La Chapelle de Guinchay, France). The weather conditions and dates are shown in Table S1 (Electronic Supplementary Material 2).

Treatments included a control (untreated) and *B. subtilis* IAB/BS03 (10^8 cfu g^{-1}) at three different doses (50, 100 and 150 g hL^{-1}), and treatments with fungicide (Curzate M, 4% Cymoxanil and 40% Mancoceb; 0.3% w/v; Seagro SL), applied at 3,000 g ha^{-1} as the positive control (each treatment comprised three replicate plants). The experimental design was an RCB with four blocks. Five applications of each dose (A, B, C, D and E) were applied. Application A was sprayed after transplanting, and two more treatments (B and C) were successively applied, at fortnightly intervals. The last two applications (D and E) were performed thereafter at 1-week intervals.

The experiment relied on natural inoculum, and plant symptoms (Fig S1C, Electronic Supplementary Material 1) were assessed for incidence and severity of foliar symptoms at 7 dpt-C, 8 dpt-D and 11 dpt-E. Lettuce plants were checked for symptoms of phytotoxicity at each assessment.

All the lettuce trials were performed under GEP, in accordance with EPPO Guidelines PP 1/181(3), PP 1/152(3), PP 1/135(3), PP 1/65(3) and CEB 197.

Efficacy values

The efficacy of *B. subtilis* IAB/BS03 as a fungicidal or bactericidal agent was calculated according to Abbott's formula (Abbott 1925), using incidence (percentage of plant units with symptoms) and severity (percentage of the plant unit area with symptoms) data. The following formula was used:

% efficacy = (Untreated % – Treated %) × 100/ Untreated %

Induction of defence mechanisms

To study the induction of plant defence mechanisms, 'Rutgers' tomato plants were grown under standard greenhouse conditions (20-25 °C; 16/8 h light/dark photoperiod) for 3-4 weeks, and the untreated control leaves (0 h) were collected. Foliar treatments were administered by spraying plants with *B. subtilis* IAB/BS03 (10^5 cfu mL^{-1}) until run-off. Water was sprayed on the negative control plants. For a positive control, tomato leaves were inoculated with *P. syringae* DC3000 (10^8 cfu mL^{-1}) using a syringe with no needle. The equivalent control leaflets were mock-inoculated with sterile 10 mM $MgCl_2$ solution. Root treatments were performed by watering all the plants with 150 ml of a suspension of *B. subtilis* IAB/BS03 (10^5 cfu mL^{-1}) or *P. fluorescens* (10^5 cfu mL^{-1}) used as a positive control. Water was used for the negative controls. The third and

fourth leaves of the tomato plants were harvested at the times indicated in each case. All the material collected was immediately placed in liquid N_2 and stored at -80 °C until processing.

PR1 and *EP5C* analysis

Total RNA was prepared from the tomato tissues using TRIzol reagent (Invitrogen, Life Technologies, California, United States) following the manufacturer's instructions. First-strand cDNA was synthesised from 5 µg of total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Promega Biotech Ibérica, Madrid, Spain) and an oligo$(dT)_{18}$ primer. The reverse transcriptase reaction mix (5 µL) was subject to PCR using a Perkin-Elmer (Madrid, Spain) thermocycler: 94 °C denaturation for 30 s, followed by 25 (for *RPL2*) or 30 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 15 min. The primers sequences used for tomato for *PR1* were 5´-CACTAAACCTAAAGAAAAATGG-3´ (forward primer) and 5´-GTGGAATTATTCATATTAGC-3´ (reverse primer) (Tornero et al. 1997). The primer sequences used for *EP5C* gene were 5´-CTCAGCATGCCAATTCTTCA-3´ (forward primer) and 5´-GCCGAATCTCAATCCAAGAA -3´ (reverse primer) (Coego et al. 2005). The oligonucleotides used to amplify the control gene *RPL2* were 5'-GGTGACCGTGGTGTCTTTGC-3' (forward primer), and RPL2R 5'-ACCAACGTTTTGTCCAGGAGGT-3' (reverse primer) (Fleming et al. 1993). All the PCR products were detected by gel electrophoresis on 1% agarose which was stained with ethidium bromide and visualised under ultra violet.

185 Extraction and assay for peroxidase

186

187 Peroxidase activity was assayed according to previously published protocols
188 (Hammerschmidt et al. 1982). Frozen leaf samples (0.2–0.3 g) were ground in a mortar
189 in 1 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing 20 mg of
190 polyvinylpolypyrrolidone to remove phenolics that can inactivate peroxidase activity.
191 The homogenate was centrifuged for 10 min at 10,000 × g and the supernatant was used
192 to determine soluble peroxidase activity. A peroxidase assay was run in a total volume
193 of 3 mL with 50 μL of protein extract, 10 μL of guaiacol and 10 μL of 30% H_2O_2 in 0.1
194 M phosphate buffer. After incubating for 5 min at 25 °C the absorbance was measured
195 at 470 nm using a spectrophotometer (Perkin-Elmer LS 50 B). To relate peroxidase
196 activity to mg of protein, a Bradford analysis of the protein extract was conducted.
197 Peroxidase activity was expressed as increments in absorbance per minute and
198 milligram of protein for the first 5 minutes.

199

200 SA extraction and quantification

201

202 The total SA (the sum of free and glucose-conjugated SA) from tomato leaves was
203 extracted according to previously described protocols (Zacarés et al. 2007). Briefly,
204 frozen leaf material (approx. 0.25 g) was ground in 1.5 mL of methanol. Extracts were
205 vortexed, sonicated and centrifuged at 14,000 × g for 15 minutes, drying the supernatant
206 under nitrogen. The residue was hydrolysed in 1 ml of 0.1 M sodium acetate (pH 4.5),
207 which contained 10 units of almond β-glucosidase (EC 3.2.1.21) (14.3 U/mg, Fluka,
208 Buchs, Switzerland), and was incubated at 37 °C for 3 h. The reaction was stopped by

adding 77 µL of perchloric acid (70%) and the mixture was maintained for 1 h at 4 °C. After centrifuging at 14,000 × g for 15 min, the remaining supernatant was extracted with 2.5 mL of 1:1 cyclopentane/ethyl acetate using 5-mL glass tubes. The organic dried residue was resuspended in 200 µL of methanol, filtered and evaporated, before re-suspending again in 200 µL methanol. Twenty microlitres were injected using a Waters 717 (Waters Corp., Milford, MA, USA) autosampler into a reverse-phase Sun Fire 5-µm (Waters, 4.6 mm×150 mm) column equilibrated in 1% (v/v) acetic acid at room temperature. A 20-minute linear gradient of 1% (v/v) acetic acid in water to 100% methanol at a flow rate of 1 $mL \times min^{-1}$ was applied with a 600E Waters HPLC pump. SA was detected by a Waters 2475 fluorescence detector (excitation λ 313 nm, emission λ 405 nm), and was quantified with Empower (Waters) software by constructing a standard curve with an SA standard (Sigma Aldrich, Missouri, USA). Losses in the extraction procedure were determined by using anisic acid (Sigma Aldrich), as an internal standard, with losses in the range of 40 to 60% in all cases.

Isolation and analysis of iturin and surfactin lipopeptides from *B. subtilis* IAB/BS03 culture broth

A *B. subtilis* IAB/BS03 saturated culture grown overnight was used to inoculate two different flasks containing 100 mL of lysogeny broth (LB) amended with streptomycin (200 µL mL^{-1}). Flasks were incubated for 48 h at 28 °C or 37 °C to evaluate the temperature effect in the lipopeptides production (Ohno et al., 1995). The iturines and surfactines synthesised by *B. subtilis* were extracted following the protocol proposed by Chen et al. (2008). Lipopeptides were precipitated from the supernatant of the liquid cultures by adjusting to pH 2 with HCl, and maintaining overnight at 4 °C. Pellets were

collected, extracted twice with methanol and dried. Lipopeptide content was determined by reverse phase UPLC-PDA-QTof (Acquity-Micromass Q-TOF Waters) via an electrospray ionisation (ESI) interface. Separation was performed in a Waters Acquity BEH C18 column (2.1 mm × 150 mm, 1.7 µm). The solvent was 0.1% formic acid in acetonitrile (phase B) and 0.1% formic acid in ultrapure water (phase A). The gradient conditions for surfactin determination were 80% B for 5 min, and increased to 100% B for 5 more min. For iturin A identification the conditions were 40% B for 5 min, and increased to 100% B for a further 5 min. The flow rate was 0.4 ml min^{-1}, with the column and sample temperatures maintained at 40 °C and 4 °C, respectively, and the sample injection volume was 2 µL. UV spectra were acquired between 210 and 800 nm, with resolution of 1.2 nm and a sampling rate of 20 points s^{-1}. The ESI source was operated in the negative ionisation mode, with capillary and cone voltages at 3.0 kV and 35 V, respectively. The temperature of the source and desolvation was set at 120 °C and 300 °C, respectively. Cone and desolvation gas (nitrogen) flows were 500 L h^{-1} and 50 L h^{-1}, respectively. Collision energy was set at 5 eV. ESI data were collected in the Centroid mode within a full scan range from a 50 to 1500 mass-to-charge ratio [*m/z*] at 0.2 s per scan. The mass spectrometer was calibrated using a sodium formiate solution (10 ng μL^{-1}) from 200 to 1500 MW (resolution specification 5,000 FWHM, deviation < 5 ppm RMS in the presence of a known lock mass). All the data were acquired using Masslynx NT4.1 software (Waters). Iturine A and surfactin standards were obtained from Sigma Aldrich.

Statistical analysis

The disease incidence and severity data from the tomato and lettuce experiments were analysed using ANOVA (Statgraphics Centurion XVI, Warrenton VA, USA). The data were subject to Bartlett's test to ascertain variance structure. Due to heterogeneity of variance, the percentage data were arcsine transformed prior to analysis. Where significant treatment effects were determined (P = 0.05), a Student-Newman-Keuls test was applied, with different letters indicating significantly differences at $\alpha = 0.05$.

When Barlett's test (on transformed data) did not permit us to verify homogeneity of variances Kruskal-Wallis test for non-parametric data was applied. When *p*-value of Kruskal-Wallis analysis was < 0.05, a Mann-Whitney test was performed to establish the relationships between subgroups.

Results

B. subtilis IAB/BS03 confers protection to tomato plants against infection by *B. cinerea*

Disease incidence was lower on plants treated with *B. subtilis* IAB/BS03 compared with the untreated plants (Figure 1A and Table S2A Electronic Supplementary Material 2). The differences observed at 0 days after treatments B, C and D (0 dpt-B, 0 dpt-C and 0 dpt-D, respectively) were significant for all doses of *B. subtilis* IAB/BS03 applied, and for the fungicide treatment (positive control). At 0 and 7 days after applying the last treatment (0 dpt-E and 7 dpt-E, respectively), only *B. subtilis* IAB/BS03 applied at 100 g hL^{-1} resulted in a significant reduction in disease incidence. Similar results were found with disease severity (Figure 1B, Tables S2B, S2C and S2D Electronic

Supplementary Material 2). Small fluctuations in the incidence and severity at different time points were due to the removal of extremely diseased leaves.

The *B. subtilis* IAB/BS03 treatment applied to tomatoes to control *Botrytis* gave outstanding results until 0 dpt-C, achieving efficacies of between 73-88% reduction in disease incidence on leaves (Figure S2A, Electronic Supplementary Material 1). For severity, efficacy was also robust (approximately 85% reduction) at 1g hL^{-1} up to 0 dpt-C (Figure S2B, Electronic Supplementary Material 1). The efficacies of the treatments with *B. subtilis* IAB/BS03 at the maximum doses (100 g hL^{-1}) were comparable with the efficacies of the standard fungicide at all times for both incidence and severity.

B. subtilis IAB/BS03 confers protection to tomato plants against infection by *P. syringae*

B. subtilis IAB/BS03 at 100 g hL^{-1} significantly reduced incidence (Figure 2A and Table S3A Electronic Supplementary Material 2) and severity (Figure 2B and Table S3B Electronic Supplementary Material 2) on main stems as much as the standard bactericide at 0 dpt-D and 8 dpt-E, respectively. No significant effects of treatment were found at any other time point.

Reduction in the incidence of *P. syringae* on footstalks and calyxes was significant in plants treated with 100 g hL^{-1} and with 10 g hL^{-1} at 14 days after the last treatment (14 dpt-E) (Figure 3A,Tables S4A and S4B Electronic Supplementary Material 2). The effect on severity of both treatments at 14 dpt-E was also statistically different when compared not only with the untreated but also with the standard bactericide (Figure 3B, Tables S4C, S4D and S4E Electronic Supplementary Material 2).

The higher doses of *B. subtilis* IAB/BS03 (10-100 g hL^{-1}) provided similar control to the bactericide at all times for both incidence and severity. (Figures S3A and S3B, Electronic Supplementary Material 1).

B. subtilis IAB/BS03 confers protection to lettuce against *Bremia lactucae*

Results of a representative trial performed with Carrascoy lettuce plants grown at Vinaròs in Spain, shows that treatments with *B. subtilis* IAB/BS03 resulted in a marked reduction in the incidence and severity of *B. lactucae* on lettuce compared to the untreated plants (Figures 4A and 4B, Figures S4A and S4B Electronic Supplementary Material 1, and Tables S5A and S5B, Electronic Supplementary Material 2). The reduction was consistently significant compared with the untreated plants, and was similar to the effect of the fungicide. Efficacies were as high as 100% at 8 days after the fourth application (8 dpt-D) for both doses, 100 and 150 g hL^{-1}, and comparable with the commercial fungicide.

The eight other similar field trials performed in 2011 and 2012, resulted in similar effects (Tables S6 and S7, Electronic Supplementary Material 2).

Induction of defence mechanisms by *B, subtilis* IAB/BS03

PR1 mRNA was accumulating at 24 h and 120 h after foliar or root applications of *B. subtilis* IAB/BS03 (Figure 5). The root treatment provoked greater induction of *PR1* mRNA, but was not as intense as that produced by the positive controls of *P.*

fluorescens (root inoculation) or *P. syringae* (leaf infiltration). The observed induction of *PR1* mRNA in the mock-inoculated plants was due to the wounding caused by inoculation with buffer.

There was a rapid accumulation of *Ep5C* mRNA in the leaves of plants used as positive controls 4 h after inoculation with *P. syringae* (Figure 5). This enhanced gene expression remained constant until 48 h post-infection. This up-regulation parallels the peroxidase activity detected in the leaves infiltrated with *P. syringae.* Neither the root treatment with *P. fluorescens*, nor that with *B. subtilis* IAB/BS03, caused any detectable increase in *Ep5C* gene expression or enzymatic peroxidase activity.

We determined whether *PR1* up-regulation was accompanied by SA accumulation. SA accumulation occurred only in the leaves infiltrated with *P. syringae*, peaking at 24 h after inoculation (Figure 5). This prominent SA accumulation preceded the enhanced peroxidase activity expression at 48 h.

Determination of iturines and surfactines in *B. subtilis* IAB/BS03 culture

The ESI (-) mass chromatograms (Figure S5A) of methanolic extracts of *B. subtilis* IAB/BS03 cultured at 28 °C and 37 °C revealed several peaks at 4.44, 6.03, 6.51, 7.60, 8.81, and 9.10 min, which corresponded to surfactin isomers with *m/z* 1006 $[M-H]^-$, *m/z* 1020 $[M-H]^-$, *m/z* 1034 $[M-H]^-$, and *m/z* 1048 $[M-H]^-$, respectively (Tang et al. 2010).

Iturin A isomers were detected in the organic extract of *B. subtilis* IAB/BS03 cultivated exclusively at 37 °C (Figure S5B). The peaks at RT = 2.95, 4.16, 4.41, 6.72, and 6.91 min co-eluted with iturin A isomers with *m/z* 1,041 $[M-H]^-$, *m/z* 1,055 $[M-H]^-$, and *m/z* 1,069 $[M-H]^-$, respectively (Phister et al. 2004; Pryor et al. 2006). None of these peaks

were observed in the chromatogram of the methanolic extract from *B. subtilis* IAB/BS03 cultivated at 28 °C.

Discussion

Although the number of commercial biocontrol products on the market is growing, their contribution to plant health management is relatively modest. Continued research in biocontrol is needed to develop the products, ensure they are efficacious, and support their uptake for sustainable agricultural production. Alternatives should be available if recommended management tools fail (Fravel 2005). We studied the ability of *B. subtilis* IAB/BS03 to confer protection against different pathogens, and we characterised possible mechanisms involved.

Protectant effects of *B. subtilis* IAB/BS03 on greenhouse-grown tomato plants against *B. cinerea* and *P. syringae* were demonstrated and had comparable efficacies to the synthetic pesticides used as positive controls.

Progress from greenhouse trials to field trials has been described as an important step to achieve practical applications of induced resistance elicited by PGPRs. Several *Bacillus* spp. strains, which first demonstrated efficacy in reducing disease in greenhouse trials, have been tested under field conditions (Kloepper et al. 2004). We tested the potential of *B. subtilis* IAB/BS03 in open field lettuce against *B. lactucae*. Our results indicate that treatments with *B. subtilis* IAB/BS03 significantly reduced both the incidence and severity of lettuce downy mildew compared with the untreated plants. This efficacy was comparable to that of a commercially available, synthetic fungicide.

These results demonstrate that *B. subtilis* IAB/BS03 application provides protection against different diseases on different crops, and in many cases is as efficacious as synthetic pesticides. *B. subtilis* IAB/BS03 is registered and has been patented (US2014/0314718 A1 and PCT/ES2012/070310), adding it to the list of commercially available *B. subtilis* products (Choudhary and Johri 2009).

Treatments with *B. subtilis* IAB/BS03 produced an up-regulation of antifungal protein PR1 in tomato plant leaves. Similar results have been reported for *Arabidopsis* plants after root inoculation with two *B. subtilis* strains (Rudrappa et al. 2010; Niu et al. 2011). We detected neither peroxidase gene induction nor increased peroxidase enzyme activity. This is in agreement with previous results describing ISR by PGPR not requiring substantial transcriptome reprogramming since gene expression changes are usually weak or undetectable (Verhagen et al. 2004). In contrast, ISR is characterised by the establishment of a primed defence state in which further defence-related responses are induced more rapidly upon pathogen or insect attack (Conrath et al. 2002). Studying gene pattern induction in the treated plants after a pathogen challenge would provide further useful information.

Since a close relationship between SA and peroxidase induction in cucumber and *A. thaliana* infected by *P. syringae* has been described (Summermatter et al. 1995), levels of *Ep5C* mRNA and peroxidase activity were also measured in our pathosystem. We found that SA, *Ep5C* mRNA and peroxidase activity all increased in tomato leaves when inoculated with *P. syringae*. Therefore, the accumulation of *PR1* mRNA levels caused by *B. subtilis* IAB/BS03 is independent of SA accumulation, which is in agreement with previous research (Pieterse et al. 1996). Thus our results support the contention that specific signal transduction pathways activated by *Bacillus* spp. during ISR depends on the strain, the host plant and the pathogen (Kloepper et al. 2004). It

400 would be interesting to test *PR1* activation by *B. subtilis* IAB/BS03 in tomato plants that
401 have impaired SA or JA signalling.

402 Besides plant defence activation, *B. subtilis* produces secondary metabolites, which
403 could act directly against the pathogen. In fact, members of the *Bacillus* genus produce
404 a vast array of biologically active molecules that have a potential inhibitory effect
405 against phytopathogen growth (Yáñez-Mendizábal et al. 2012). Among these
406 antimicrobial compounds, cyclic lipopeptides of the surfactin and iturin families offer
407 well-recognised potential in biotechnology and biopharmaceutical applications (Ongena
408 and Jacques 2008). We detected the production of both bioactive molecules surfactin
409 and iturin by *B. subtilis* IAB/BS03 in organic culture broth grown at 37 °C. However,
410 only the surfactin isomers were identified when this strain was grown at 28 °C, which
411 indicates a temperature effect on iturin production. This contrasts with previously
412 published results obtained with *B. subtilis* RB14 during solid-state fermentation (Ohno
413 et al. 1995) , where the optimal temperature for iturin A production was 25 °C, and for
414 surfactin was 37 °C. This discrepancy might be explained by the different incubation
415 conditions or the specific strain of *Bacillus* used.

416 Iturins and surfactins have been described not only as antimicrobial compounds, but
417 also as determinants of ISR activation (Choudhary and Johri 2009, Kawagoe et al.
418 2015). Therefore, the production of these molecules by *B. subtilis* IAB/BS03 could
419 result in direct antagonism of bacterial and fungal pathogens by inducing perturbations
420 in their plasma membranes (Zeriouh et al. 2011).

421 Our results support the potential use of *B. subtilis* IAB/BS03 as a candidate for
422 integrated biocontrol programmes against fungal and bacterial diseases, particularly in
423 tomato and lettuce production.

Acknowledgements

This work was funded by IAB S. L. (*Investigaciones y Aplicaciones Biotecnológicas, S. L.*), and by grant BIO2012-33419 from the Spanish Ministry of Economy and Competitiveness. Mayte Castellano was the recipient of a research grant also funded by IAB S. L. The authors would like to thank Cristina Torres (IBMCP, UPV-CSIC) for her excellent technical assistance.

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Figure captions

Fig. 1. Evaluation of the efficacy of *B. subtilis* IAB/BS03 against *B. cinerea* in greenhouse tomato plants: foliar disease.

Tomato plants were either untreated or treated with *B. subtilis* IAB/BS03 (*B. subtilis*) at three different doses (1, 10 and 100 g hL^{-1}) compared with a standard fungicide (Scala 0.15%). Five different foliar applications (A, B, C, D and E) were applied and the percentages of leaves diseased (A) or the percentages of leaf surface diseased (B) were assessed at different timed after each application (dpt-X indicates days after the X application; 0 dpt-X indicates just prior to the X application). A total of 60 plants were tested. A Newman-Keuls means comparison (where $A \neq B \neq C$) was performed (α = 5%). * Barlett's test (on transformed data) did not permit us to verify homogeneity of variances, and Kruskal-Wallis and Mann-Whitney tests for non-parametric data were applied.

Fig. 2. Evaluation of the efficacy of *B. subtilis* IAB/BS03 against *P. syringae* in greenhouse tomato plants: disease on main stems.

Tomato plants were either untreated or treated with *B. subtilis* IAB/BS03 at three different doses (1, 10 and 100 g hL^{-1}) compared with a standard bactericide (ZZ Cuprocol 0.3%). Five different foliar applications (A, B, C, D and E) were performed, and the percentages of plants with the main stem diseased by *P. syringae* (A) and the percentages of principal stem surface diseased by *P. syringae* (B) were assessed at different timed after each application (dpt-X indicates days after the X application; 0

564 dpt-X indicates just prior to the X application). A total of 60 plants were tested. A
565 Newman-Keuls means comparison (where A ≠ B ≠ C) was performed (α = 5%).

566

567 **Fig. 3.** Evaluation of the efficacy of *B. subtilis* IAB/BS03 against *P. syringae* in
568 greenhouse tomato plants: disease on footstalks and calyxes.

569 Tomato plants were either untreated or treated with *B. subtilis* IAB/BS03 at three
570 different doses (1, 10 and 100 g hL^{-1}) compared with a standard bactericide (ZZ
571 Cuprocol 0.3%). Five different foliar applications (A, B, C, D and E) were performed,
572 and the percentages of footstalks and calyxes diseased by *P. syringae* (A) and the
573 percentages of footstalks and calyxes surface diseased by *P. syringae* (B) were assessed
574 at different timed after each application (dpt-X indicates days after the X application; 0
575 dpt-X indicates just prior to the X application). A total of 60 plants were tested. A
576 Newman-Keuls means comparison (where A ≠ B ≠ C) was performed (α = 5%). *
577 Barlett's test (on transformed data) did not permit us to verify homogeneity of
578 variances, and Kruskal-Wallis and Mann-Whitney tests for non-parametric data were
579 applied.

580

581 **Fig. 4.** Evaluation of the efficacy of *B. subtilis* IAB/BS03 against *Bremia lactucae* on
582 field-grown Carrascoy lettuce in Vinaròs (Spain): disease on leaves.

583 Lettuce plants were either untreated or treated with *B. subtilis* IAB/BS03 at three doses
584 (50, 100 and 150 g hL^{-1}). The reference product was a fungicide (Curzate M 0.3%).
585 Five different foliar applications (A, B, C, D and E) were performed. Three assessments
586 with disease symptoms were made at different timed after each application (dpt-X
587 indicates days after the X application). The percentages of diseased leaves by downy

588 mildew (A) and the percentages of leaf surface diseased (B) were recorded. A total of
589 60 plants were tested. A Newman-Keuls means comparison (where A ≠ B ≠ C) was
590 performed (α = 5%). These data correspond to the open field assay performed in
591 Vinaròs (Spain) in 2011.

592

593 **Fig. 5.** Induction of tomato defence mechanisms by *B. subtilis* IAB/BS03.

594 Tomato plants were treated with either foliar or root applied *B. subtilis* IAB/BS03. The
595 negative control was water. *P. syringae* and *P. fluorescens* were used as positive
596 controls for foliar and root treatments, respectively. *P. syringae* was infiltrated with a
597 syringe (no needle) and corresponding mock inoculated plants were also included
598 (Mock). Samples were collected at the times indicated in each case (0, 4, 24, 48 and 120
599 hours after treatment).

600 (A) HPLC-fluorescence analyses of the phenolic extracts from tomato leaves were
601 performed. FW: fresh weight. Bars indicate the total SA accumulation in the different
602 samples. Curves indicate peroxidase activity. AU: arbitrary units of absorbance. Three
603 independent replicates were performed, and the results of a representative one are
604 shown.

605 (B) *PR1* and *EP5C* expression in the different samples. RT-PCR amplification using
606 specific primers for each gene was performed (see Materials and Methods for details).
607 The *RPL2* gene was used as a control for RT (Fleming et al. 1993).

608

609 Electronic Supplementary Material 1

610

Fig. S1. (A) Symptoms of disease caused by *Botrytis cinerea* on greenhouse-grown *Solanum lycopersicum*. (B) Symptoms of disease caused by *Pseudomonas syringae* on greenhouse *Solanum lycopersicum*. (C) Symptoms of lettuce downy mildew caused by *Bremia lactucae* on field-grown *Lactuca sativa*.

Fig. S2. Evaluation of efficacy (based on Abbot's formula) of applications of *B. subtilis* IAB/BS03 against *B. cinerea* in greenhouse-grown tomato plants: foliar disease.

Tomato plants were either untreated or treated with *B. subtilis* IAB/BS03 at three different doses (1, 10 and 100 g hL^{-1}) compared with a standard fungicide (Scala 0.15%). Five different foliar applications (A, B, C, D and E) were applied, and disease incidence (A) or disease severity (B) on leaves was assessed at the different time periods after application (dpt-X indicates days after the X application; 0 dpt-X indicates just prior to the X application). Comparisons among treatments were performed by analysis of variance (ANOVA) for each time point and a Newman-Keuls means comparison (where $A \neq B \neq C$) was performed ($\alpha = 5\%$). Means with the same letter indicate that there are no significant differences. * Barlett's test (on transformed data) did not permit us to verify homogeneity of variances and Kruskal-Wallis test for non-parametric data were applied.

Fig. S3. Evaluation of efficacy (based on Abbot's formula) of applications of *B. subtilis* IAB/BS03 against *P. syringae* in greenhouse-grown tomato plants: disease on tomato footstalks and calyxes.

Tomato plants were either untreated or treated with *B. subtilis* IAB/BS03 at three different doses (1, 10 and 100 g hL^{-1}) compared with a standard bactericide (ZZ Cuprocol 0.3%). Five different foliar applications (A, B, C, D and E) were applied, and disease incidence (A) or disease severity (B) on tomato footstalks and calyxes was assessed at the different time periods after application (dpt-X indicates days after the X application; 0 dpt-X indicates just prior to the X application). Comparisons among treatments were performed by analysis of variance (ANOVA) for each time point and a Newman-Keuls means comparison (where $A \neq B \neq C$) was performed ($\alpha = 5\%$). Means with the same letter indicates that there are no significant differences. * Barlett's test (on transformed data) did not permit us to verify homogeneity of variances, and Kruskal-Wallis and Mann-Whitney tests for non-parametric data were applied.

Fig. S4. Evaluation of efficacy (based on Abbot's formula) of applications of *B. subtilis* IAB/BS03 against *Bremia lactucae* on field-grown,'Carrascoy' lettuce in Vinaròs (Spain).

Lettuce plants were either untreated or treated with *B. subtilis* IAB/BS03 at three doses (50, 100 and 150 g hL^{-1}). The reference product was a fungicide (Curzate M 0.44%). Five different foliar applications (A, B, C, D and E) were performed. Disease incidence (A) or disease severity (B) on leaves was assessed at the different time periods after application (dpt-X indicates days after the X application). Comparisons among treatments were performed by analysis of variance (ANOVA), for each time point and a Newman-Keuls means comparison (where $A \neq B \neq C$) was performed ($\alpha = 5\%$). Means with the same letter indicates that there are no significant differences (p value < 0.05).

Fig. S5. HPLC-MS (ESI-) chromatograms of methanol extracts obtained from *B. subtilis* IAB/BS03 culture broths incubated at 28 °C or 37 °C showing the (A) surfactin, (B) iturin isomers.

Electronic Supplementary Material 2

Table S1. Weather conditions for efficacy trials of *B. subtilis* IAB/BS03 against *Bremia lactucae* in lettuce in different years and locations in Europe.

Table S2. Statistical parameters of the efficacy of *B. subtilis* IAB/BS03 against *B. cinerea* in greenhouse tomato plants: foliar disease. A) Results of ANOVA test for disease incidence. B) Results of ANOVA test for disease severity. C) Kruskal-Wallis test for severity at 0 dpt-D. D) *p*-values of Mann-Whitney test for severity at 0dpt-D.

Table S3. Results of ANOVA of the efficacy of *B. subtilis* IAB/BS03 against *P. syringae* in greenhouse tomato plants: disease on main stems. A) Disease incidence. B) Disease severity.

Table S4. Statistical parameters of the evaluation of the efficacy of *B. subtilis* IAB/BS03 against *P. syringae* in greenhouse tomato plants: disease on footstalks and calyxes. A) Results of ANOVA test for disease incidence. B) Kruskal-Wallis test for incidence at 0 dpt-E. C) Results of ANOVA test for disease severity. D) Kruskal-Wallis test for severity at 0, 8 and 14 dpt-E. E) *p*-values of Mann-Whitney test for severity at 14 dpt E.

682

683 **Table S5.** Results of ANOVA of the evaluation of the efficacy of *B. subtilis* IAB/BS03
684 against *Bremia lactucae* on field-grown 'Carrascoy' lettuce in Vinaròs (Spain): disease
685 on leaves. A) Disease incidence. B) Disease severity.

686

687 **Table S6.** Results of ANOVA of the evaluation of the efficacy of *B. subtilis* IAB/BS03
688 against *Bremia lactucae* in additional experiments on lettuce. A total of 8 additional
689 experiments were performed. Several varieties were used at the different locations in
690 Europe in 2011 and 2012.

691

692 **Table S7.** Summary of the 9 efficacy trials showing the performance of *B. subtilis*
693 IAB/BS03 and reference products against *Bremia lactucae* on field-grown lettuce. A)
694 Incidence of lettuce downy mildew on leaves. B) Severity of lettuce downy mildew on
695 leaves.

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697