

Tomato ethylene sensitivity determines interaction with plant growth-promoting bacteria

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- **Background and Aims** Plant growth-promoting bacteria (PGPB) are soil micro-organisms able to interact with plants and stimulate their growth, positively affecting plant physiology and development. Although ethylene plays a key role in plant growth, little is known about the involvement of ethylene sensitivity in bacterial inoculation effects on plant physiology. Thus, the present study was pursued to establish whether ethylene perception is critical for plant–bacteria interaction and growth induction by two different PGPB strains, and to assess the physiological effects of these strains in juvenile and mature tomato (*Solanum lycopersicum*) plants.
- **Methods** An experiment was performed with the ethylene-insensitive tomato *never ripe* and its isogenic wild-type line in which these two strains were inoculated with either *Bacillus megaterium* or *Enterobacter* sp. C7. Plants were grown until juvenile and mature stages, when biomass, stomatal conductance, photosynthesis as well as nutritional, hormonal and metabolic statuses were analysed.
- **Key Results** *Bacillus megaterium* promoted growth only in mature wild type plants. However, *Enterobacter* C7 PGPB activity affected both wild-type and *never ripe* plants. Furthermore, PGPB inoculation affected physiological parameters and root metabolite levels in juvenile plants; meanwhile plant nutrition was highly dependent on ethylene sensitivity and was altered at the mature stage. *Bacillus megaterium* inoculation improved carbon assimilation in wild-type plants. However, insensitivity to ethylene compromised *B. megaterium* PGPB activity, affecting photosynthetic efficiency, plant nutrition and the root sugar content. Nevertheless, *Enterobacter* C7 inoculation modified the root amino acid content in addition to stomatal conductance and plant nutrition.
- **Conclusions** Insensitivity to ethylene severely impaired *B. megaterium* interaction with tomato plants, resulting in physiological modifications and loss of PGPB activity. In contrast, *Enterobacter* C7 inoculation stimulated growth independently of ethylene perception and improved nitrogen assimilation in ethylene-insensitive plants. Thus, ethylene sensitivity is a determinant for *B. megaterium*, but is not involved in *Enterobacter* C7 PGPB activity.

Key words: *Bacillus megaterium*, *Enterobacter*, ethylene, plant growth-promoting bacteria (PGPB), plant nutrition, *Solanum lycopersicum* (tomato).

INTRODUCTION

The aim of the sustainable intensification of agriculture is to provide food security to a growing global population while minimizing any harmful environmental impacts of cropping systems (Tilman *et al.*, 2011). Consequently, the resource use efficiency of crops needs to be increased without sacrificing current yields (Dodd and Ruiz-Lozano, 2012). A wide range of soil micro-organisms are able to establish associations with plants (Gray and Smith, 2005), and dozens of reports of plant growth stimulation by beneficial soil micro-organisms can be found (Lucy *et al.*, 2004; Adesemoye and Kloepper, 2009; Nadeem *et al.*, 2014). Management of microbial populations living in the rhizosphere is a flexible, low-cost and environmentally friendly method to enhance plant growth (Berg, 2009; Singh *et al.*, 2011). The root system growth and physiology are

modulated by these micro-organisms (Barea *et al.*, 2005) and some of these can increase plant stress tolerance (Glick, 2004; Aroca and Ruiz-Lozano, 2009; Dimkpa *et al.*, 2009).

Among soil micro-organisms, plant growth-promoting bacteria (PGPB) have been studied in detail (Lugtenberg and Kamilova, 2009; Pii *et al.*, 2015; Santoyo *et al.*, 2016). The PGPB concept has been confined to bacterial strains with at least two of the three criteria such as aggressive colonization, plant growth stimulation or biocontrol (Weller *et al.*, 2002; Vessey, 2003). The root colonization process is influenced by bacterial traits, root exudates and abiotic and biotic factors (Benizri *et al.*, 2001; el Zahar Haichar *et al.*, 2014). PGPB can act either directly or indirectly (Glick, 1995; Ortíz-Castro *et al.*, 2009). Direct promotion includes increasing supply of nutrients, phytohormone modulation and induction of systemic resistance. Indirect stimulation is basically related to biocontrol

(van Loon, 2007; Lugtenberg and Kamilova, 2009). Several mechanisms may be active simultaneously, promoting plant growth as a net result (Martínez-Viveros *et al.*, 2010) and, in consequence, the use of PGPB can be described as an attractive way to replace use of chemicals in agriculture (Bhattacharyya and Jha, 2012). Plant-beneficial microbe associations are thought to be ancient and shaped during coevolution so that bacteria could have significant effects on plant physiology (Lambers *et al.*, 2009). In fact, the action mechanism of some PGPB suggests a simple interaction and responses between the two partners.

Plant–bacterial interactions and their environment are essentials for better uptake of water and nutrients by plants (Ryan *et al.*, 2009). Although nutrient availability is limited in most soils, a constant level of essential mineral nutrients needs to be maintained and rhizosphere microbial communities are associated with nutrient biogeochemical cycles (Barea *et al.*, 2005). To cope with nutrient limitation, several physiological and developmental responses can be triggered which produce modifications on whole-plant morphology and metabolism (López-Bucio *et al.*, 2002). In this regard, plant nutritional and hormonal homeostasis are closely inter-related and co-ordinated for the fine regulation of growth and development (Krouk *et al.*, 2011).

Ethylene plays a prominent role in plant physiology and, like most other phytohormones, can inhibit or promote growth depending on the cell type and plant species (Pierik *et al.*, 2006). Its production is typically upregulated in response to environmental stresses (F. Wang *et al.*, 2013). Although the interactions among ethylene, nutrients and plant responses has been reviewed, our knowledge of these interactions is incomplete (Iqbal *et al.*, 2013). Several bacterial strains can produce ethylene (Primrose and Dilworth, 1976; Saleem *et al.*, 2007) or reduce its levels. The latter have been called stress controllers (Lugtenberg and Kamilova, 2009) and induce plant growth by decreasing the content of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene in all higher plants (Glick *et al.*, 2007; Glick, 2014). However, relatively little is known about bacterial inoculation effects on plant physiology and its relationship with ethylene sensitivity of mature compared with juvenile plants.

In order to shed light on plant–bacteria interactions, various omics approaches have been used (Cheng *et al.*, 2010; Stearns *et al.*, 2012; van de Mortel *et al.*, 2012; Couillerot *et al.*, 2013; Su *et al.*, 2016). Metabolites play a key role in regulatory mechanisms because they are in many cases the end-products. Thus, metabolic information is very useful to understand a plant's interaction with its environment (Feussner and Polle, 2015) and could be interpreted as a snapshot of inoculation effects on plant physiology. Metabolomic analyses in arabidopsis showed modified amino acid and sugar levels due to bacterial inoculation (Su *et al.*, 2016). Also, bacterial root colonization can produce modifications in physiological status and metabolite content for short- and long-term periods (Su *et al.*, 2016).

The aim of this study was to gain more insights into the role of ethylene sensitivity in plant–microbe interactions, specifically PGPB activity. Tomato is the most important horticultural crop around the world, and the second most consumed vegetable after potato (Schwarz *et al.*, 2010). Moreover, mutants and previous research are available, representing a suitable model

for understanding the interaction of PGPB and other plants. The ethylene-insensitive tomato mutant *never ripe* (*nr*) and its isogenic wild-type (*wt*) parental line were used. Previous studies have shown that *nr* is largely unable to perceive ethylene, with some residual responsiveness (Lanahan *et al.*, 1994) because of a mutation in the sensor domain of the ethylene receptor LeETR3 (Wilkinson *et al.*, 1995). In addition, two PGPB strains isolated from arid soils in southern Spain have been used: *Bacillus megaterium* strain (Bm) (Marulanda-Aguirre *et al.*, 2008) and *Enterobacter* sp. [hereafter *Enterobacter* C7 (C7)]. Bm has shown PGPB features in previous reports (Marulanda *et al.*, 2009, 2010; Armada *et al.*, 2014a; Porcel *et al.*, 2014; Ortiz *et al.*, 2015). However, *Enterobacter* C7 was evaluated here for the first time. We aimed to determine if ethylene sensitivity is critical for plant–bacteria interaction and growth induction by these two PGPB strains and to evaluate the effects of bacterial inoculation on physiology in both juvenile and mature plants. Since most studies on the role of ethylene in PGPB activity have been focused on bacteria able to reduce ACC contents (Glick *et al.*, 2007; Glick, 2014), we intended to use bacteria without either ACC deaminase activity or ethylene production capacity in order to avoid any perturbation of plant ethylene metabolism caused by the bacteria. Plant growth, bacterial colonization, stomatal conductance, photosynthetic efficiency and chlorophyll contents, nutrients, phytohormones and root metabolite contents were determined at 4 and 8 weeks post-inoculation (wpi).

MATERIALS AND METHODS

Biological material

Seeds of *nr* tomato (*Solanum lycopersicum*) (LA0162) (Wilkinson *et al.*, 1995) and its isogenic parental cultivar 'Pearson' (LA0012) were obtained from the Tomato Genetics Resource Center at the University of California, Davis, CA, USA. PGPB strains were isolated from soils in southern Spain. *Bacillus megaterium* was identified and partially characterized in a previous study (Marulanda-Aguirre *et al.*, 2008). *Enterobacter* C7 was isolated and identified by E. Armada as described in Armada *et al.* (2014b).

Experimental design and growth conditions

The experiment consisted of a randomized complete block design with two plant lines: tomato 'Pearson' *wt* and its ethylene-insensitive mutant (*nr*), and three inoculation treatments: (1) non-inoculated control plants; (2) *B. megaterium*-inoculated plants; and (3) *Enterobacter* C7-inoculated plants. Experiments consisted of 18 replicates per treatment, and two different harvests were established: 4 and 8 wpi, using nine plants at each harvest ($n = 9$).

Seeds were sterilized (70 % ethanol 5 min, 5 % sodium hypochlorite 10 min and three washing steps with sterile water to remove any trace of chemicals), kept at 4 °C overnight and placed on sterile vermiculite at 25 °C until germination. Ten-day-old seedlings were transferred to 1 L plastic pots containing sterile peat moss:perlite (1:1, v/v, autoclaved twice at 120 °C for 20 min). Bacteria were grown in Luria broth (LB) medium

with shaking (200 rpm) at 28 °C overnight. The culture optical density was measured at 600 nm (OD_{600}), the cells were centrifuged (2655 g, 10 min) and the pellet was resuspended in sterile distilled water until $OD_{600} = 1.5$, corresponding to a cell density of 10^7 CFU mL⁻¹. A 1 mL aliquot of distilled water (control plants), or a bacterial suspension: Bm or C7 (inoculated plants), was added to each root seedling at transplantation. Plants were grown for 4 or 8 weeks in a greenhouse under controlled climatic conditions (18–24 °C, 50–60 % relative humidity, 16 h:8 h light (600 μ mol m⁻² s⁻¹):dark). In order to maintain constant soil water content close to water-holding capacity during the whole experiment, water was supplied every 2 d.

Biomass production

Shoots were separated from root systems at each harvest time, samples were dried in a forced draught oven (70 °C, 3 d) and dry weights were determined. The relative growth rate (RGR) was calculated using the classical approach (Hunt, 1982) following the equation: $RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$ where W_1 and W_2 are dry weights at times t_1 and t_2 , respectively.

Colonization of tomato root system

Sterilized seeds were germinated on filter paper soaked with sterile distilled water on Petri plates in darkness for 3 d. Ten-day-old seedlings were transferred to sterilized glass bottles containing the same substrate as above. At transplantation, plants were inoculated as mentioned above. Eight replicates of each were performed ($n = 8$). The glass bottles were closed and kept for 1 week in a climate-controlled growth chamber (18–24 °C, 50–60 % relative humidity, 16 h daylight). A 1 cm long intermediate root segment was carefully cut and suspended in 1 mL of sterile water. Tubes were incubated for 1 h on an orbital shaker (35 rpm) with vibration. Suspensions were serially diluted (10^{-2} – 10^{-9}). Dilutions were plated on LB agar medium and cultivated overnight at 28 °C. Finally, colonies were counted and CFU cm⁻¹ root values were calculated.

Bacterial ACC deaminase activity bioassay

The PGPB strains were tested for the ability to use ACC as a sole N source in order to evaluate their possible effect on plant ACC levels. ACC deaminase activity of cell-free extracts was determined by estimating α -ketobutyrate production (nmol mg⁻¹ protein h⁻¹) according to the procedure described by Penrose and Glick (2003).

Ethylene production by bacterial strains

Bacterial ethylene production was measured by gas chromatography (GC) in order to determine possible effects of microbially derived ethylene in plant–bacteria interaction and/or growth promotion. Bacteria were grown in LB medium with shaking (200 rpm) at 28 °C overnight. Culture OD_{600} was measured and new sub-cultures (LB, 6 mL, $OD_{600} = 0.01$)

were started in sterile 20 mL vials (Supelco Analytical, Pennsylvania, USA). Vials were closed and incubated at 28 °C with shaking (200 rpm). Samples of 1 mL were withdrawn from each vial with a syringe, and ethylene was quantified using a Hewlett Packard model 5890 gas chromatograph equipped with a Poropak-R column and a hydrogen flame ionization detector at 3, 6, 9 and 24 h after starting the culture. Six replicates per bacterium and LB without inoculum were analysed ($n = 6$).

Physiological parameters

Stomatal conductance. Stomatal conductance was measured 3 h after sunrise with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK).

Photosynthetic efficiency. A FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic) was used to measure photosystem II efficiency according to Oxborough and Baker (1997).

Leaf chlorophyll concentration. Photosynthetic pigments were extracted from leaf samples (0.5 cm²) in 100 % methanol at 4 °C for 24 h. Pigment concentration was spectrophotometrically determined according to Lichtenthaler (1987). Samples were taken from the last expanded leaf for stomatal conductance, photosynthetic efficiency and chlorophyll content ($n = 9$).

Nutrient measurement. Mineral analysis was determined in shoots and roots ($n = 4$). C and N concentration (% d. wt) were determined by mass spectrometry (ELEMENTAL LECO TruSpec CN) and were performed by the Analytical Service of the Instituto de Nutrición Animal (CSIC), Granada, Spain. Ca, K, Mg, Na, P, S and Si concentration (% d. wt) as well as Cu, Fe, Mn, and Zn concentration (ppm) analyses were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varina ICP 720-ES) and were performed by the Instrumentation Service of the Estación Experimental del Zaidín (CSIC), Granada, Spain.

Phytohormone analysis. Indole acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and jasmonoyl isoleucine (JA-Ile) were analysed using high-performance liquid chromatography-electrospray ionization-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). The extraction and purification were performed using the following method: 250 mg of frozen tissue (previously ground to a powder in a mortar with liquid N₂) was homogenized with 2.5 mL of pre-cooled methanol:water:HCOOH (90:9:1, v/v/v with 2.5 mM Na-diethyldithiocarbamate) and 25 μ L of a stock solution of 1000 ng mL⁻¹ of deuterium-labelled internal standards D-IAA, D-ABA, D-SA and D-JA, and 200 ng mL⁻¹ of D-JA-Ile in methanol. The mixture was shaken for 60 min at room temperature before being centrifuged (20 000 g, 10 min), shaken again for 20 min and centrifuged. A 2 mL aliquot of pooled supernatants was taken and dried at 40 °C. The residue was dissolved in 500 μ L of methanol:0.133 % acetic acid (40:60, v/v) and centrifuged (20 000 g, 10 min) before being injected in an HPLC-ESI-HRMS system.

Hormones were quantified using a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA),

equipped with an HESI(II) source, a quadrupole mass filter, a C-Trap, a HCD collision cell and an Orbitrap mass analyzer (Orbitrap-Focus, Thermo Scientific). A reverse-phase column (Synergi 4 mm Hydro-RP 80A, 150 × 2 mm; Phenomenex, Torrance, CA, USA) was used. A linear gradient of methanol (A), water (B) and 2 % acetic acid in water (C) was used: 38 % A for 3 min, 38 % to 96 % A in 12 min, 96 % A for 2 min and 96 % to 38 % A in 1 min, and kept for 4 min. C remains constant at 4 %. The flow rate was 0.30 mL min⁻¹, the injection volume was 40 µL and column and sample temperatures were 35 and 15 °C, respectively. The detection and quantification were performed using a Full MS experiment with tandem mass spectrometry (MS/MS) confirmation in the negative-ion mode. Instrument control and data processing were performed by TraceFinder 3.3 EFS software. Compounds as well as instrumental parameters are reported in the Supplementary Data (reagents and internal standards in Table S1, ionization source in Table S2 and compound accurate masses in Table S3).

Ethylene production was analysed using GC. Completely expanded leaflets from the last developed leaf, and entire roots were chosen for shoot and root ethylene determination, respectively. Samples were introduced in 20 mL vials (Supelco Analytical, PA, USA) with 200 µL of MilliQ water to avoid tissue drying. After 15 min to let the ethylene produced from injury escape, vials were closed and incubated for 1 h (leaflets) and 4 h (roots) at room temperature. Samples of 1 mL were withdrawn from each vial with a syringe, and ethylene was quantified using a Hewlett Packard model 5890 gas chromatograph equipped with a Poropak-R column and a hydrogen flame ionization detector. Six and four replicates per treatment were used in 4 and 8 wpi harvests, respectively.

Metabolite analysis. Metabolite analysis by GC-MS was performed according to Roessner *et al.* (2000). A 100 mg aliquot of frozen tissue (previously ground to a powder in a mortar with liquid N₂) was extracted with 1 mL of methanol containing internal standard (ribitol 9 µg mL⁻¹ in methanol). The mixture was extracted for 20 min at 4 °C, and 400 µL of water were added and mixed before centrifugation (18 626 g, 5 min). Two aliquots of supernatant were taken for analysis of major components (10 µL) and minor components (200 µL) after being dried overnight under vacuum. The residue was derivatized in two steps, methoxymation, and silylation (60 µL of methoxyamine hydrochloride in pyridine, 37 °C 90 min and BSTFA + TMCS, 37 °C 30 min). A Varian (now Bruker) 450GC 240MS system was used for GC-MS analysis. All samples were analysed twice. A 1 µL aliquot was injected at 230 °C in split 1/50 mode with Pressure Pulse (30 psi 0.2 min). Helium flux at 1 mL min⁻¹ and temperature ramp started at 70 °C for 5 min, increased at 5 °C a time until 245 °C, increased at 20 °C a time until 310 °C and kept for 1 min. The column was a DB-5ms 30 m, 0.25 mm, 0.25 µm. Ionization was by electronic impact and mass analysis in TIC Full Scan mode acquiring masses in the range 50–600 *m/z*. Identification was by comparison with the NIST08 spectra library and retention time of pure compounds. For comparative purposes, within each chromatogram, the compound peak areas were normalized by the sample fresh weight and by the internal standard peak area, resulting in relative response ratios. Six and four replicates per treatment were used for 4 and 8 wpi harvests, respectively.

TABLE 1. Bacterial root colonization

Treatment	CFU root cm ⁻¹	s.e.
Bm		
wt	2.55 × 10 ⁶	± 0.68 × 10 ⁶
nr	2.02 × 10 ⁶	± 0.41 × 10 ⁶
C7		
wt	1.85 × 10 ⁶	± 0.39 × 10 ⁶
nr	2.69 × 10 ⁶	± 0.76 × 10 ⁶

Colony-forming units (CFU) per root centimetre of *Bacillus megaterium* (Bm) and *Enterobacter* C7 (C7) in wild type 'Pearson' (wt) and *never ripe* (nr) tomato plants.

Data are means ± s.e. (*n* = 8).

No significant differences were seen (*P* < 0.05) according to two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation as well as their interaction.

Statistical analysis

Data were processed by two-way analysis of variance (ANOVA) with plant genotype (G) and inoculum (I) as sources of variation. The significance of sources of variation as well as their interaction (G × I) was evaluated by *P*-value (*P* < 0.05). In the case of significant interaction between factors, all treatments were compared against each other by least significant difference (LSD) test (*P* < 0.05). In the case of no interaction between factors, inoculum effects were evaluated analysing wt and nr plants separately using ANOVA followed by LSD test (*P* < 0.05). Plant genotype effect was evaluated by analysing wt and nr plants under the same inoculation treatment (No, Bm or C7) by Student *t*-test (*P* < 0.05). Plant dry weight, RGR, bacterial colonization, stomatal conductance, photosynthetic efficiency, photosynthetic pigments, nutrient, phytohormone and metabolite contents were analysed by this method using R software (v3.2.2 Open Source; <http://www.r-project.org/>). Relationships between total, shoot and root dry weights and nutrients, and metabolites were examined using the Pearson correlation coefficient (*P* < 0.05). Principal component analysis (PCA) was also used to compare nutrient and metabolite profiles with those obtained under different experimental conditions using Excel add-in the Multibase package (v_2015 Open source; <http://www.numericaldynamics.com/>).

RESULTS

Colonization of the tomato root system by PGPB strains

A bioassay for bacterial colonization was performed to test the ability of the PGPB strains that were used to colonize wt and nr root systems. As expected, no bacterial growth was observed in non-inoculated plants. Bm and C7 were able to colonize roots independently of plant genotype. Moreover, no significant differences were noticed in colonization rates, reaching values of around 2 × 10⁶ CFU cm⁻¹ root (Table 1). Furthermore, the PGPB strains did not show either ethylene production or the ability to cleave ACC, the direct precursor of ethylene (data not shown).

Biomass production of wt and nr plants inoculated with two PGPB strains

The dry weight of wt and nr plants inoculated with PGPB strains [*B. megaterium* (Bm) and *Enterobacter* C7] showed

different growth patterns at 4 and 8 wpi, but no interaction between factors was seen (Fig. 1). At 4 wpi, total and shoot dry weight showed no differences due to bacterial inoculation in wt and *nr* plants or due to plant genotype under different inoculations (Fig. 1A, C). However, root dry weight was increased by Bm inoculation in *nr* plants, while no growth promotion in wt roots was observed. Moreover, a significant difference between wt and *nr* plants was only observed in Bm-inoculated roots (Fig. 1E). At 8 wpi, total dry weight showed an increase due to PGPB inoculation in wt plants (18.4 and 24.6 % for Bm and C7, respectively). Nevertheless, in *nr* plants, total dry weight was only increased by C7 inoculation (25.5 %), showing no significant differences due to Bm inoculation (Fig. 1 B). Shoot dry weight was increased by C7 inoculation in both plant genotypes (26.3 % and 25.7 % in wt and *nr* plants, respectively). However, Bm inoculation did not produce a significant effect on shoot growth (Fig. 1D). Moreover, significant difference between plant genotypes was only seen in non-inoculated plants in total and shoot dry weight (Fig. 1B, D). In addition, root dry weight was increased by PGPB inoculation in wt plants (15.8 % and 18.1 % for Bm and C7, respectively), with no significant differences seen in *nr* plants (Fig. 1F).

In order to analyse plant growth responses between the two harvests, RGR was calculated, showing an interaction between factors in total and root dry weights (Fig. 2). In wt plants, RGRs were unaffected. However, in *nr* plants, RGR changed according to inoculated bacteria and plant tissue (Fig. 2A–C). Total RGR showed the highest value in C7-inoculated *nr* plants and the lowest value in Bm-inoculated *nr* plants, both of them showing significant differences from control plants. Furthermore, no differences due to plant genotype were noticed (Fig. 2A). In shoots, C7-inoculated *nr* plants increased RGR, but Bm inoculation did not affect shoot RGR compared with control plants (Fig. 2B). Furthermore, root RGR was only decreased by Bm inoculation in *nr* plants and a significant difference between plant genotypes was exclusively seen under Bm inoculation (Fig. 2 C).

PGPB effects on stomatal conductance, photosynthetic efficiency and chlorophyll content

Factor interaction was not significant in stomatal conductance, photosynthetic efficiency and chlorophyll content (Table 2). Stomatal conductance was affected by bacterial inoculation in both plant genotypes only at 4 wpi, with C7 inoculation showing increased stomatal conductance independently of plant genotype (27.1 and 36.6 % in wt and *nr* plants, respectively). Moreover, Bm decreased stomatal conductance by 17.3 % in wt plants, with no effect in *nr* plants. In addition, significant differences between wt and *nr* plants in stomatal conductance were only seen under Bm inoculation at 4 wpi (higher in *nr* plants) and under all inoculation treatments at 8 wpi (higher values in wt plants) (Table 2). C7 did not affect photosynthetic efficiency, maintaining similar values to control plants. However, Bm inoculation decreased this parameter independently of plant genotype (25.8 and 10.9 % in wt and *nr* plants,

respectively). Significant differences between wt and *nr* plants were only seen in Bm-inoculated plants, with higher values in *nr* plants. Although photosynthetic efficiency was not modified by bacterial inoculation at 8 wpi, significant plant genotype differences were seen in non- and C7-inoculated plants, with higher values in wt plants (Table 2). Chlorophyll content was unaltered by PGPB inoculation in both plant genotypes at both harvests. Moreover, a significant plant genotype difference was only seen under Bm inoculation at 4 wpi (Table 2).

PGPB effects on plant nutritional status

In order to throw some light on PGPB inoculation effects on plant nutrition regarding ethylene insensitivity, macro- and micronutrients were quantified in roots and shoots (Supplementary Data Tables S4 and S5, respectively).

In the case of root nutrients, factor interaction was only significant for Ca concentration at 8 wpi (Table S4). Root nutrients showed no significant differences due to PGPB inoculation in wt plants at 4 wpi. However, the Na level was decreased by C7 inoculation in *nr* plants at 4 wpi. Moreover, Zn concentration was higher in C7, with Bm-inoculated *nr* plants showing no differences compared with control plants. Furthermore, Mn concentration showed significant differences between wt and *nr* plants, with *nr* plants showing higher values than the wt under all inoculation treatments (Table S4). In contrast, several changes were seen due to PGPB inoculation and ethylene insensitivity at 8 wpi. In wt plants, C concentration was increased by both bacterial inoculations (1.8 and 3.2 % by Bm and C7, respectively) and Ca concentration was increased only by Bm inoculation. Most changes were found in *nr* plants, showing a general reduction by bacterial inoculation. C7 inoculation decreased Cu, Mg, S and Si concentrations in *nr* plants. In the case of Bm-inoculated *nr* plants, only Cu, Mg and Si concentrations were decreased compared with control plants. Moreover, some differences between inocula were observed in Cu and Si concentrations, decreasing to a greater extent after C7 inoculation than after Bm inoculation. Furthermore, significant differences between plant genotypes were noticed for several nutrients. Ca concentration showed higher values in wt plants under all inoculations. C concentration was higher in *nr* than wt plants only in non-inoculated plants. Exclusively under Bm inoculation, Na concentration was higher in wt plants. Under C7 inoculation, Mn showed a higher level in *nr* plants, while Si showed higher levels in wt plants. Finally, Cu, Mg and S concentrations were significantly lower in *nr* than in wt plants under both bacterial inoculations (Table S4).

Regarding shoot nutrients, several changes were seen in wt and *nr* plants at 4 and 8 wpi. Moreover, factor interaction was only significant for Cu, Fe and P concentrations at 8 wpi (Table S5). At 4 wpi, C7 inoculation did not affect shoot nutrients, while Bm inoculation modified nutrients in both plant genotypes. The C concentration was increased by Bm inoculation of wt plants. Moreover, Bm inoculation decreased N and Mn concentrations in wt plants, and Zn and Fe concentrations in *nr* plants. In addition, significant differences between wt and *nr* plants were noted for Cu, Mn and Zn under all inoculation treatments, with *nr* plants showing lower values than wt plants.

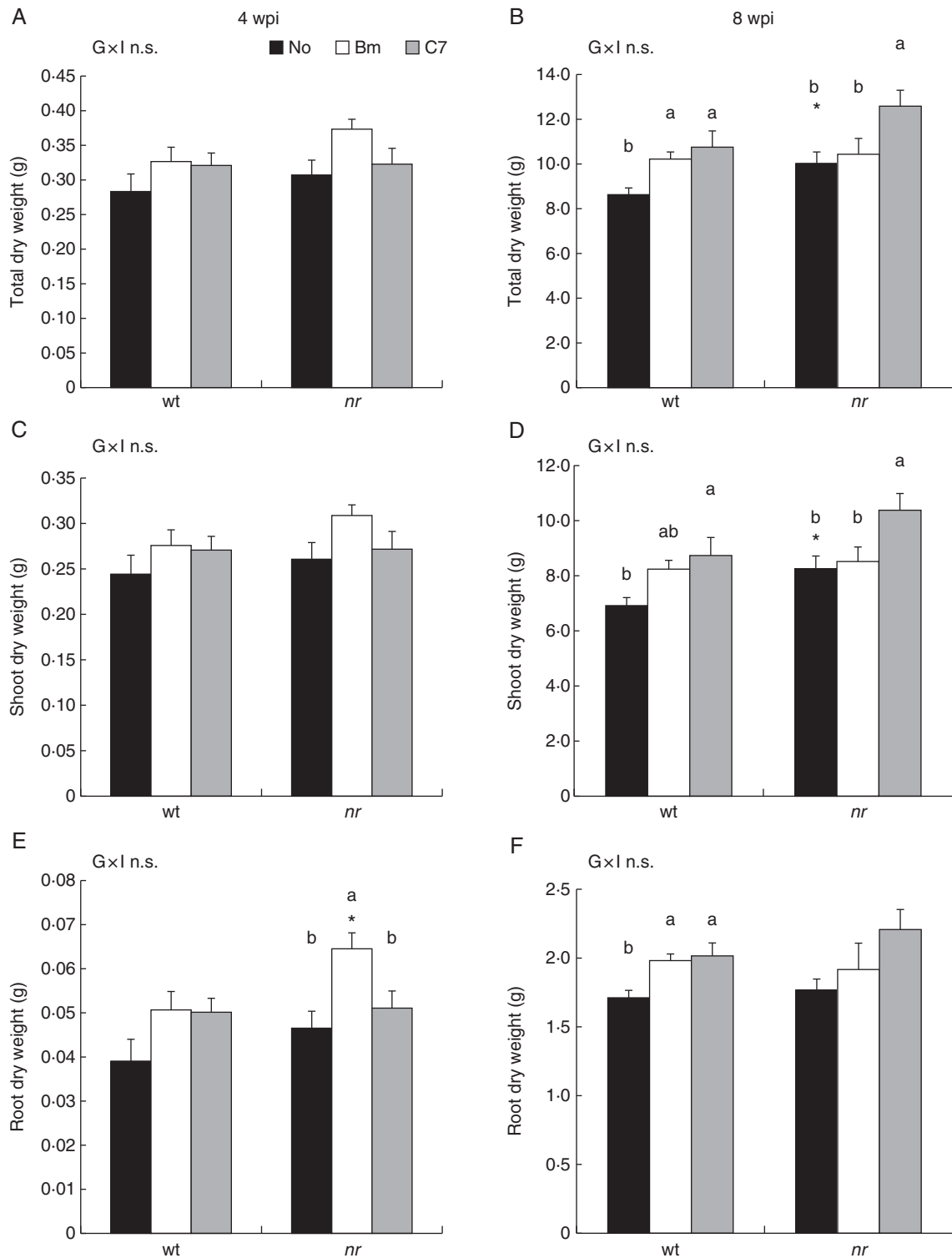


FIG. 1. Effects of bacterial inoculation on plant dry weights at 4 and 8 weeks post-inoculation (wpi). Total (A), shoot (C) and root (E) dry weights of wild-type 'Pearson' (wt) and *never ripe* (nr) tomato (*Solanum lycopersicum*) plants at 4 wpi. Total (B), shoot (D) and root (F) dry weights of wt and nr tomato plants at 8 wpi. Treatments are designed as non-inoculated controls (No, black bars), *Bacillus megaterium*-inoculated plants (Bm, white bars), and *Enterobacter C7*-inoculated plants (C7, grey bars). Data are means \pm s.e. ($n = 9$). Data were analysed by two-way ANOVA, with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation as well as their interaction (G \times I) were evaluated by P -value; n.s., not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. In the case of a significant interaction between factors, all treatments were compared with each other. In the case of a non-significant interaction between factors, inoculum effects were evaluated, analysing wt and nr plants separately using ANOVA. Means followed by different lower case letters are significantly different ($P < 0.05$) according to LSD test. Plant genotype effect was evaluated by analysing wt and nr plants under the same inoculation treatment (No, Bm or C7) by Student t -test, and a significant difference ($P < 0.05$) is shown as (*) above nr means.

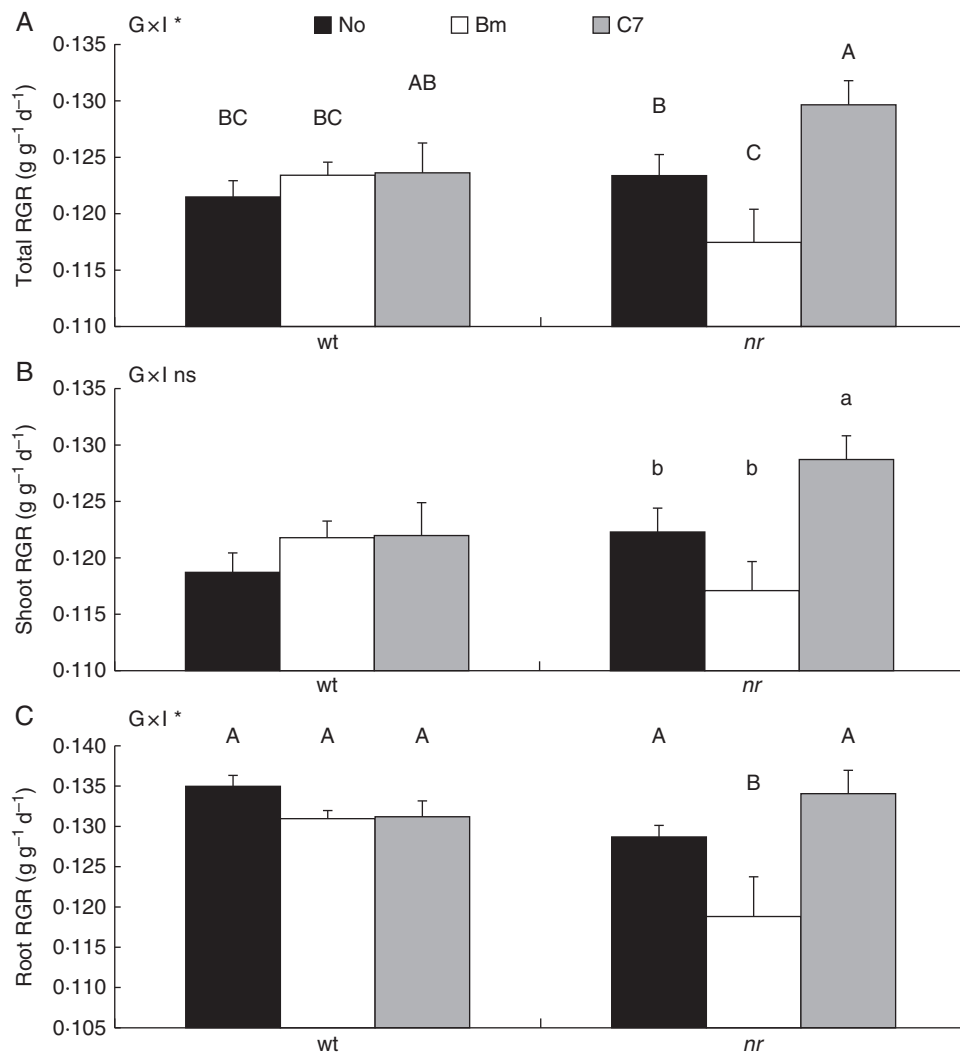


FIG. 2. Effects of bacterial inoculation on relative growth rates (RGRs). Total (A), shoot (C) and root (E) RGRs of wild-type 'Pearson'(wt) and *never ripe* (nr) tomato (*Solanum lycopersicum*) plants at 8 weeks post-inoculation (wpi). Treatments are designed as non-inoculated controls (No, black bars), *Bacillus megaterium*-inoculated plants (Bm, white bars) and *Enterobacter C7*-inoculated plants (C7, grey bars). Data are means \pm s.e. ($n = 9$). Data were analysed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation as well as their interaction (G \times I) was evaluated by P -value; n.s., not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. In the case of a significant interaction between factors, all treatments were compared with each other. Means followed by different upper case letters are significantly different ($P < 0.05$) according to LSD test. In the case of a non-significant interaction between factors, inoculum effects were evaluated by analysing wt and nr plants separately using ANOVA. Means followed by different lower case letters are significantly different ($P < 0.05$) according to LSD test. Plant genotype effect was evaluated by analysing wt and nr plants under the same inoculation treatment (No, Bm or C7) by Student t -test, and a significant difference ($P < 0.05$) is shown as (*) above nr means.

Moreover, significant differences between plants genotypes were seen in non-inoculated plants only for the Mg level and under both bacterial inoculations for Ca concentration, with a higher concentration in wt plants in all cases (Table S5). At 8 wpi, Bm only increased the C level in wt plants, showing no effect on shoot nutrition of nr plants. However, C7 inoculation increased Cu and Fe concentrations in wt plants and decreased Cu, Na and P concentrations in nr plants. Furthermore, significant differences between plant genotypes were noted for several nutrients. Ca and Mg concentrations showed higher values in wt than in nr plants under all inoculation treatments. In the case of Na, genotype differences were observed in non- and Bm-inoculated

plants, with higher values in nr plants. Moreover, significant differences between wt and nr plants were seen for K and Mn in non- and C7-inoculated plants, with both showing higher values in wt plants. Finally, significant differences were exclusively observed under C7 inoculation for C, Cu, Fe, P and Zn concentrations, showing higher values for C in nr plants and for Cu, Fe, P and Zn in wt plants (Table S5).

Nutrient concentrations and plant dry weights were assessed by Pearson correlation analysis (Table 3). A positive correlation was observed between root S concentration and total, shoot and root dry weights at 4 wpi. Shoot Fe concentration showed a strong negative correlation with all dry weights at this time. At

TABLE 2. Effects of bacterial inoculation on stomatal conductance, photosynthetic efficiency and chlorophyll content

	Stomatal conductance (mmol m ⁻² s ⁻¹)		Photosynthetic efficiency (r _u)		Total chlorophyll (mg cm ⁻²)	
	4 wpi	8 wpi	4 wpi	8 wpi	4 wpi	8 wpi
wt No	484.125 ± 30.503 ^b	131.636 ± 13.199	0.641 ± 0.017 ^a	0.727 ± 0.004	5.486 ± 0.506	2.830 ± 0.464
wt Bm	400.222 ± 27.720 ^c	136.455 ± 13.644	0.476 ± 0.026 ^b	0.720 ± 0.005	5.446 ± 0.420 ^b	2.682 ± 0.379
wt C7	615.313 ± 25.015 ^a	134.636 ± 13.304	0.590 ± 0.029 ^a	0.732 ± 0.005	5.146 ± 0.481	2.937 ± 0.389
P-value	***	n.s.	***	n.s.	n.s.	n.s.
nr No	470.833 ± 22.065 ^b	97.910 ± 4.157*	0.664 ± 0.018 ^a	0.699 ± 0.006*	5.263 ± 0.371	2.498 ± 0.389
nr Bm	499.833 ± 31.429 ^{b*}	100.273 ± 8.552*	0.592 ± 0.025 ^{b*}	0.713 ± 0.008	5.011 ± 0.233*	2.598 ± 0.580
nr C7	643.333 ± 32.067 ^a	105.500 ± 5.973*	0.660 ± 0.017 ^a	0.701 ± 0.005*	5.228 ± 0.367	2.831 ± 0.582
P-value	***	n.s.	*	n.s.	n.s.	n.s.
Significance of source of variation interaction						
G × I	n.s.	ns	ns	ns	ns	ns

Data were analysed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation.

Data are means ± s.e. ($n = 9$).

The significance of sources of variation as well as their interaction ($G \times I$) was evaluated by P -value. In the case of significant interaction between factors, all treatments were compared with each other. Means followed by different lower case letters are significantly different ($P < 0.05$) according to LSD test. In the case of a non-significant interaction between factors, inoculum effects were evaluated by analysing separately wt and nr plants using ANOVA. Plant genotype effect was evaluated by analysing wt and nr plants separately under the same inoculation treatment (No, Bm or C7) by Student t -test and significant difference ($P < 0.05$) is shown as (*) next to nr means.

Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated never ripe plants (nr No, nr Bm and nr C7, respectively).

8 wpi, dry weights were correlated with several nutrients. Indeed, a positive correlation was found between the root C concentration and all dry weights. Moreover, negative correlations were obtained between total dry weight and root Cu, K and S concentrations. Shoot K concentration was also negatively correlated with total dry weight. In addition, shoot dry weight showed the same correlations as total dry weight (Table 3).

Root nutrient concentrations and total, shoot and root dry weight values were used to build a data matrix in order to perform a PCA to compare inoculation treatments in both plant genotypes (Fig. 3). Axes PC1 and PC2, represented in the factorial plan, explained 55.2 % of data variability at 4 wpi. PCA separated treatments by plant genotype. In wt plants, Bm inoculation produced separation from control plants; however, C7-inoculated plants shared a tiny area with control plants. However, both bacterial effects mostly overlapped. Also, both bacterial inoculations shared a small area with non-inoculated nr plants without overlapping each other (Fig. 3A). The nutrients with a greater contribution to the observed variability were K, P, S and Zn for PC1, and Fe, Mg and Si for PC2 (Fig. 3B). At 8 wpi, analysis (62.8 % variability along axes PC1 and PC2) showed a clear difference between genotypes. In the case of wt plants, there was no separation due to bacterial inoculation. In contrast, both bacterial inoculations were completely separated from control nr plants, sharing a small area between Bm- and C7-inoculated areas (Fig. 3C). Nutrients with a higher contribution to variability were Ca, Fe and Mg for PC1, and P and Zn for PC2 (Fig. 3D).

PGPB effects on plant phytohormonal status

PGPB inoculation effects on phytohormones were studied regarding ethylene sensitivity in roots and shoots, evaluating

ethylene, IAA, ABA, SA, JA and JA-Ile concentrations at 4 and 8 wpi (Tables 4 and 5, respectively). Factor interaction was only noticed in root ABA concentration at 4 wpi.

At 4 wpi, JA and JA-Ile levels were unaffected by PGPB inoculation and by plant genotype (data not shown). Exclusively, C7 inoculation increased root ABA concentration in nr plants, while no changes were noticed in wt plants. Moreover, a significant difference between wt and nr plants was only observed in C7-inoculated roots (higher levels in nr plants) and were not noticeable in shoots (Table 4). In the case of ethylene, genotype differences were observed in non- and Bm-inoculated roots and in non- and C7-inoculated shoots, with nr plants showing higher ethylene levels than wt plants in all cases (Table 4). Moreover, differences between wt and nr plants were seen for IAA concentration in non- and C7-inoculated roots and exclusively under C7 inoculation in shoots, with nr plants showing higher levels than wt plants (Table 4). Finally, a genotype difference in SA concentration was only observed in Bm-inoculated shoots, with higher values in nr plants (Table 4).

At 8 wpi, PGPB inoculation did not affect any phytohormone levels in roots or in shoots. However, plant genotype had a significant effect in both plant tissues (Table 5). ABA levels were unaffected by plant genotype (data not shown). Ethylene concentration showed higher values in nr than in wt plants under all inoculation treatments in roots and shoots (Table 5). In the case of IAA, genotype differences were only seen in non-inoculated plants in root and shoot tissues, with nr plants showing higher levels than wt plants (Table 5). Moreover, SA concentration showed significant differences between plant genotypes in non- and Bm-inoculated roots, with Bm-inoculated shoots always showing higher values in nr plants (Table 5). Furthermore, although genotype differences were not seen for JA and JA-Ile concentration in

TABLE 3. Significant Pearson correlations between plant dry weights and nutrient contents

	8 weeks post-inoculation														
	4 weeks post-inoculation					Shoot									
	Root					Shoot									
	C (%)	Cu (ppm.)	Fe (ppm.)	K (%)	S (%)	C (%)	Cu (ppm)	Fe (ppm)	K (%)	S (%)	C (%)	Cu (ppm)	Fe (ppm)	K (%)	S (%)
	d. wt)	(ppm.)	(ppm.)	d. wt)	d. wt)	d. wt)	(ppm)	(ppm)	d. wt)	d. wt)	d. wt)	(ppm)	(ppm)	d. wt)	d. wt)
Total d. wt	0.376	0.177	-0.155	0.523	0.809	0.396	-0.371	-0.973	-0.540	-0.078	0.916	-0.848	-0.502	-0.836	-0.849
<i>r</i>	0.449	0.732	0.764	0.266	0.033	0.421	0.455	0.000	0.247	0.881	0.004	0.018	0.290	0.023	0.018
<i>P</i> -value	0.359	0.179	-0.148	0.510	0.805	0.407	-0.355	-0.971	-0.535	-0.063	0.921	-0.868	-0.541	-0.854	-0.865
Shoot d. wt	0.472	0.729	0.774	0.281	0.035	0.407	0.476	0.000	0.252	0.903	0.003	0.013	0.246	0.017	0.014
<i>r</i>	0.418	0.172	0.172	-0.172	0.818	0.368	-0.411	-0.974	-0.549	-0.116	0.810	-0.661	-0.225	-0.661	-0.686
Root d. wt	0.393	0.745	0.740	0.739	0.030	0.459	0.402	0.000	0.237	0.823	0.033	0.129	0.661	0.128	0.108
<i>r</i>	0.393	0.745	0.740	0.739	0.030	0.459	0.402	0.000	0.237	0.823	0.033	0.129	0.661	0.128	0.108
<i>P</i> -value	0.393	0.745	0.740	0.739	0.030	0.459	0.402	0.000	0.237	0.823	0.033	0.129	0.661	0.128	0.108

roots, differences between wt and *nr* plants were exclusively observed under Bm inoculation in shoots (Table 5).

PGPB effects on root metabolite content

Root metabolites were analysed to determine how PGPB inoculation affects their accumulation regarding ethylene insensitivity in juvenile and mature plants (Tables 6 and 7, respectively). Several root metabolites were identified by GC-MS. These include, carbohydrates (fructose, glucose, galactose, saccharose, mannose and myoinositol), amino acids [glycine, methionine, phenylalanine, threonine, valine, leucine/isoleucine, glutamine, serine, γ -aminobutyric acid (GABA), glutamic acid and aspartic acid] and organic acids [oxalacetic, fumaric, succinic and malic and dehydroascorbic (DHA)]. Factor interaction in root metabolite levels was seen for galactose, aspartic acid, glutamic acid, fumaric acid, glutamine, isoleucine/leucine, phenylalanine, serine threonine and valine at 4 wpi, while no factor interaction was observed at 8 wpi (Tables 6A and 7A).

At 4 wpi, a dozen metabolites showed different levels due to bacterial inoculation in wt and/or *nr* plants (Table 6A). Carbohydrates were unaffected in wt plants. However, C7 inoculation reduced fructose, galactose and glucose in *nr* plants compared with controls. In contrast, fructose and galactose levels were increased by Bm inoculation in *nr* plants. In the case of amino acids, aspartic acid, glutamic acid, glutamine, leucine/isoleucine, phenylalanine and serine levels were reduced in wt plants; meanwhile aspartic acid, glutamic acid and glutamine were increased in *nr* plants by C7 inoculation. Bm inoculation did not modify amino acid levels in wt plants, but aspartic acid, serine and threonine levels were decreased in *nr* plants. In addition, different levels between inocula were observed in threonine and serine, showing no changes compared with control plants. Moreover, fumaric acid was reduced by Bm inoculation in *nr* plants while C7 inoculation increased it in wt plants. Succinic acid was increased by both bacteria in wt plants. No changes due to PGPB inoculation were seen for myoinositol, malic acid, GABA and DHA levels (Table 6A). Furthermore, differences between wt and *nr* plants were observed in several root metabolites. GABA levels showed higher values in *nr* than in wt plants under all inoculation treatments. In the case of galactose, a significant difference between plant genotypes was seen in Bm-inoculated plants (a higher level in *nr* plants) and in C7-inoculated plants (a higher level in wt plants). Moreover, a difference between wt and *nr* plants was only seen in non-inoculated plants for myoinositol, fumaric acid and succinic acid, with *nr* plants showing higher values. In the case of malic acid, genotype differences were seen only under C7 inoculation, with higher values in wt plants. In addition, a significant difference between plant genotypes was seen only under C7 inoculation, with aspartic acid, glutamic acid, glutamine, isoleucine/leucine, phenylalanine and serine showing higher values in *nr* plants. In the case of threonine and valine, significant differences between plant genotypes were observed in non- and C7-inoculated plants, with higher levels in *nr* plants compared with wt plants (Table 6A).

Correlations between plant dry weights and root metabolite levels were evaluated at 4 wpi (Table 6B). The galactose content was positively correlated with total, shoot and root dry

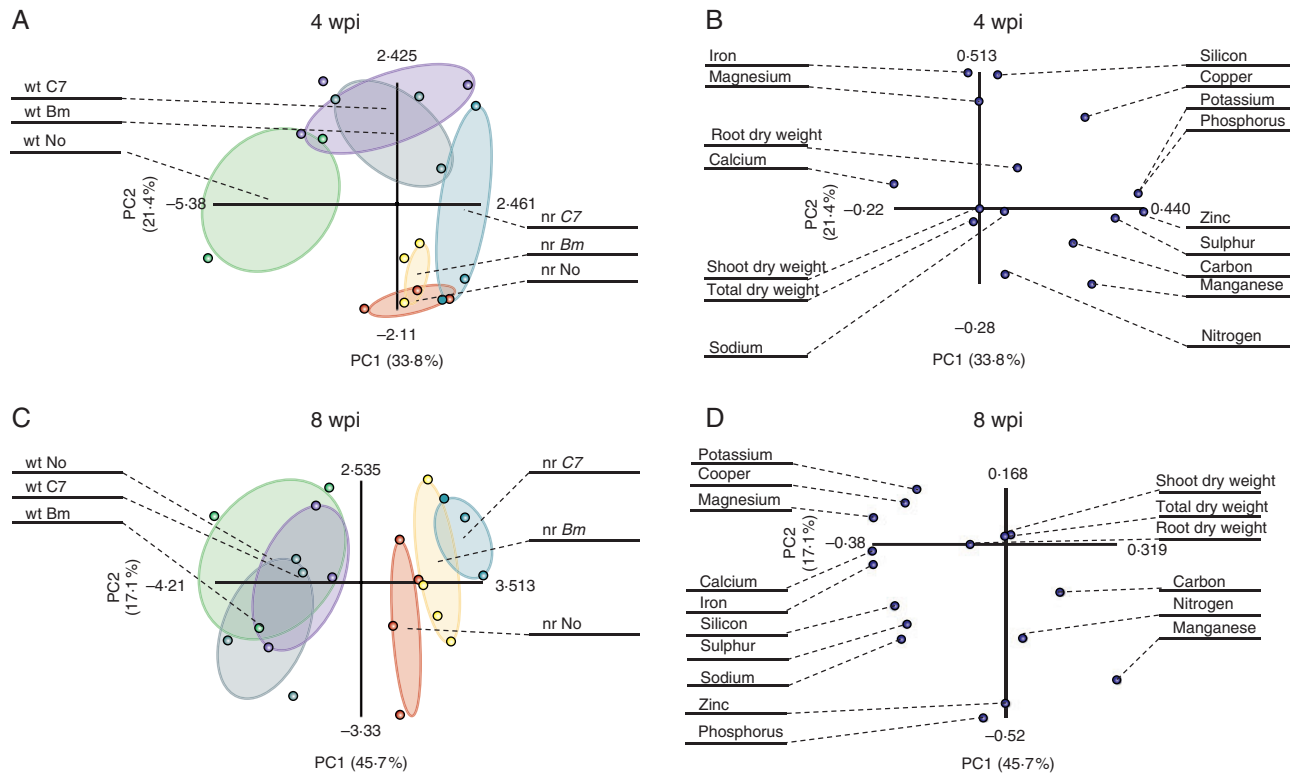


Fig. 3. Principal component analysis (PCA) of root nutrient concentrations and dry weights. Analyses were performed based on nutrient concentration and dry weight data obtained from tomato (*Solanum lycopersicum*) plants. Score plot at 4 weeks post-inoculation (wpi) (A) and 8 wpi (C). Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated never ripe plants (nr No, nr Bm and nr C7, respectively). Each point represents one plant, and points of the same treatment are enclosed in a different coloured ellipse: green for wt No, grey for wt Bm, purple for wt C7, red for nr No, yellow for nr Bm and blue for nr C7. Loading plot at 4 wpi (B) and 8 wpi (D). Each point represents one nutrient or dry weight.

TABLE 4. Effects of bacterial inoculation on phytohormone concentrations at 4 weeks post-inoculation

	Root				Shoot			
	Ethylene (nmol g ⁻¹ h ⁻¹)	IAA (pmol g ⁻¹)	ABA (pmol g ⁻¹)	SA (pmol g ⁻¹)	Ethylene (nmol g ⁻¹ h ⁻¹)	IAA (pmol g ⁻¹)	ABA (pmol g ⁻¹)	SA (pmol g ⁻¹)
wt No	3.05 ± 0.24	88.52 ± 4.38	37.65 ± 4.91 ^{BC}	80.33 ± 6.30	0.88 ± 0.05	42.07 ± 3.19	669.22 ± 36.47	224.89 ± 43.23
wt Bm	3.89 ± 0.28	79.13 ± 2.16	30.18 ± 1.96 ^C	67.65 ± 3.53	1.03 ± 0.12	38.53 ± 2.34	676.00 ± 12.40	216.23 ± 6.69
wt C7	3.55 ± 0.24	82.34 ± 3.62	32.32 ± 2.89 ^C	68.03 ± 8.60	1.21 ± 0.15	37.64 ± 1.67	655.35 ± 17.35	240.28 ± 25.36
<i>P</i> -value	n.s.	n.s.	n.a.	n.s.	n.s.	n.s.	n.s.	n.s.
nr No	6.70 ± 0.49*	108.66 ± 7.21*	42.00 ± 1.04 ^B	68.20 ± 4.60	2.02 ± 0.32*	41.95 ± 1.15	710.53 ± 28.63	226.43 ± 15.23
nr Bm	5.91 ± 0.53*	96.64 ± 6.83	37.05 ± 1.51 ^{BC}	72.13 ± 8.08	1.38 ± 0.20	44.20 ± 2.53	862.62 ± 86.44	265.35 ± 15.01*
nr C7	5.40 ± 1.07	119.92 ± 3.69*	51.42 ± 3.23 ^A	87.75 ± 11.59	2.23 ± 0.31*	46.17 ± 1.60*	693.38 ± 13.18	226.86 ± 16.30
<i>P</i> -value	n.s.	n.s.	n.a.	n.s.	n.s.	n.s.	n.s.	n.s.
Significance of source of variation interaction								
G × I	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.

Data were analysed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation.

Data are means ± s.e. ($n = 6$).

The significance of sources of variation as well as their interaction (G × I) was evaluated by *P*-value. In the case of significant interaction between factors, all treatments were compared with each other. Means followed by different upper case letters are significantly different ($P < 0.05$) according to LSD test. In the case of a non-significant interaction between factors, inoculum effects were evaluated by analysing wt and nr plants separately using ANOVA. Plant genotype effect was evaluated by analysing wt and nr plants under the same inoculation treatment (No, Bm or C7) by Student *t*-test and significant difference ($P < 0.05$) is shown as (*) next to nr means.

Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated never ripe plants (nr No, nr Bm and nr C7, respectively).

n.a., not-applicable; *P*-value; n.s., not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

TABLE 5. Effects of bacterial inoculation on phytohormone concentrations at 8 weeks post-inoculation

	Root						Shoot					
	Ethylene (nmol g ⁻¹ h ⁻¹)	IAA (pmol g ⁻¹)	SA (pmol g ⁻¹)	JA (pmol g ⁻¹)	JA-Ile (pmol g ⁻¹)	Ethylene (nmol g ⁻¹ h ⁻¹)	IAA (pmol g ⁻¹)	SA (pmol g ⁻¹)	JA (pmol g ⁻¹)	JA-Ile (pmol g ⁻¹)		
wt No	2.91 ± 0.27	22.73 ± 2.09	375.79 ± 40.53	125.56 ± 21.27	5.88 ± 0.38	0.85 ± 0.12	120.0 ± 2.98	2554.80 ± 950.65	65.88 ± 10.42	1.34 ± 0.24		
wt Bm	2.61 ± 0.55	16.20 ± 3.74	290.10 ± 35.70	91.32 ± 13.19	5.11 ± 0.99	1.30 ± 0.29	133.43 ± 14.14	1562.67 ± 375.99	50.01 ± 12.65	0.99 ± 0.02		
wt C7	3.09 ± 0.34	19.22 ± 4.25	350.86 ± 65.99	98.85 ± 15.08	4.68 ± 0.56	1.14 ± 0.13	144.52 ± 22.89	1423.38 ± 393.23	75.64 ± 6.03	1.21 ± 0.26		
<i>P</i> -value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
<i>nr</i> No	4.79 ± 0.50*	30.91 ± 1.82*	524.44 ± 35.97*	169.39 ± 14.05	6.29 ± 0.25	2.09 ± 0.37*	168.86 ± 10.06*	2581.40 ± 256.21	58.70 ± 9.32	1.54 ± 0.27		
<i>nr</i> Bm	4.69 ± 0.75*	25.66 ± 2.78	527.62 ± 78.98*	154.46 ± 37.03	6.53 ± 0.78	2.45 ± 0.41*	146.00 ± 18.12	2869.05 ± 277.71*	95.73 ± 8.34*	2.49 ± 0.10*		
<i>nr</i> C7	4.68 ± 0.53*	26.71 ± 4.69	549.53 ± 27.92	165.45 ± 36.25	7.46 ± 0.82	2.40 ± 0.51*	184.87 ± 21.29	2585.30 ± 492.29	118.31 ± 47.58	2.68 ± 0.80		
<i>P</i> -value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
<i>G</i> × <i>I</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		

Significance of source of variation interaction

Data were analysed by two-way ANOVA with plant genotype (*G*) and inoculum (*I*) as sources of variation.Data are means ± s.e. (*n* = 4).

The significance of sources of variation as well as their interaction (*G* × *I*) was evaluated by *P*-value. In the case of significant interaction between factors, all treatments were compared with each other. In the case of a non-significant interaction between factors, inoculum effects were evaluated by analysing wt and *nr* plants separately using ANOVA. Plant genotype effect was evaluated by analysing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by Student *t*-test and significant difference (*P* < 0.05) is shown as (*) next to *nr* means.

Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter* C7-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter* C7-inoculated never ripe plants (*nr* No, *nr* Bm and *nr* C7, respectively).

P-value: n.s., not significant; **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.

weights, while DHA was negatively correlated with all dry weights. PCA was also performed to compare inoculation treatment effects on root metabolite profiles at 4 wpi (Fig. 4). In these analyses, 57.7 % of the variability was explained by the data in axes PC1 and PC2. Separation by plant genotype was only noticed under C7 inoculation. In wt plants, all treatments overlapped, with C7-inoculated plants showing higher variability than Bm-inoculated and control plants. Nevertheless, Bm inoculation in *nr* plants produced a different profile from C7 inoculation, showing a tiny overlap between them. Moreover, both bacterial inoculations showed a specific overlap with non-inoculated plants. Furthermore, non-inoculated wt and *nr* profiles showed partial overlapping. The Bm-inoculated *nr* plant profile enclosed non- and Bm-inoculated wt plant profiles. However, C7 inoculation resulted in a total separation, showing less variation in *nr* plants (Fig. 4A). The loading plot showed two clear sources of variation; amino acids (glutamine, serine, phenylalanine, valine and threonine) and carbohydrates (fructose, glucose, galactose and saccharose) contributed to the high variability for PC1 and PC2, respectively (Fig. 4B).

The effects of PGPB inoculation on root metabolite content regarding ethylene sensitivity were also assessed at 8 wpi. Nevertheless, factor interaction was not seen and PGPB inoculation did not affect root metabolite levels in mature plants (Table 7A). Fructose levels were unaffected by plant genotype. However, differences between wt and *nr* plants were seen for several metabolites. Mannose and serine levels showed higher values in *nr* than in wt plants under all inoculation treatments. Moreover, a genotype difference was only seen in non-inoculated plant for galactose, with higher levels in *nr* plants. Glucose showed higher levels in *nr* plants in non- and Bm-inoculated plants. However, threonine and methionine also showed higher values in *nr* plants, but under Bm and C7 inoculations. Furthermore, myoinositol and aspartic acid showed higher levels in *nr* plants exclusively under Bm inoculation. On the other hand, differences between wt and *nr* plants were only significant under C7 inoculation for fumaric acid, glutamic acid, glutamine, isoleucine/leucine, phenylalanine, valine and GABA levels (Table 7A).

Furthermore, some positive correlations were observed between plant dry weights and root metabolite levels at 8 wpi (Table 7B). Fructose content was positively correlated with all dry weights, and galactose and glucose were correlated positively with total and shoot dry weights. Treatment effects on root metabolites were also evaluated by PCA at 8 wpi (65.3 % variability along axes PC1 and PC2) (Fig. 5). Profiles were not completely separated by plant genotype in Bm-inoculated and non-inoculated plants, but there were differences compared with C7-inoculated plants. In wt plants, both bacterial inoculations showed a totally different root metabolite profile compared with control plants, showing almost complete overlap between Bm- and C7-inoculated plants. In *nr* plants, Bm inoculation resulted in a profile that overlapped with control plants, while C7 inoculation produced a nearly complete separation from controls (Fig. 5A). PCA also showed that PC1 variability was due to amino acids (glutamine, glutamic acid, leucine/isoleucine, valine and GABA). Unexpectedly, plant dry weights were a source of variation for PC2, minimizing carbohydrate effects.

TABLE 7. Root metabolite contents and correlations between plant dry weights at 8 weeks post-inoculation (wpi)

A	Fructose	Galactose	Glucose	Mannose	Myoinositol	Aspartic acid	Fumaric acid	Glutamic acid	Glutamine	Isoleucine/ leucine	Phenylalanine	Serine	Threonine	valine	GABA	Methionine
wt No	68.91 ± 1.63	41.13 ± 3.33	41.13 ± 3.33	0.30 ± 0.02	6.67 ± 1.94	0.23 ± 0.13	0.083 ± 0.022	0.52 ± 0.19	0.16 ± 0.06	0.17 ± 0.05	0.07 ± 0.02	0.28 ± 0.05	0.023 ± 0.005	0.044 ± 0.008	2.63 ± 0.85	0.12 ± 0.02
wt Bm	75.02 ± 4.46	0.611 ± 0.068	46.37 ± 3.21	0.32 ± 0.07	5.56 ± 1.48	0.18 ± 0.07	0.059 ± 0.018	0.35 ± 0.12	0.11 ± 0.04	0.15 ± 0.07	0.06 ± 0.02	0.25 ± 0.07	0.023 ± 0.008	0.047 ± 0.024	1.90 ± 0.50	0.11 ± 0.03
wt C7	79.39 ± 3.51	0.710 ± 0.068	50.03 ± 3.25	0.33 ± 0.05	5.96 ± 1.41	0.20 ± 0.03	0.058 ± 0.003	0.40 ± 0.12	0.13 ± 0.03	0.14 ± 0.03	0.07 ± 0.02	0.26 ± 0.05	0.022 ± 0.005	0.041 ± 0.004	1.78 ± 0.18	0.11 ± 0.03
P-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
nr No	74.08 ± 4.40	0.783 ± 0.076*	56.67 ± 4.40*	0.55 ± 0.10*	9.11 ± 2.70	0.33 ± 0.09	0.070 ± 0.008	0.53 ± 0.09	0.17 ± 0.02	0.20 ± 0.06	0.08 ± 0.01	0.49 ± 0.11*	0.034 ± 0.007	0.060 ± 0.014	2.73 ± 0.67	0.13 ± 0.02
nr Bm	74.47 ± 3.03	0.781 ± 0.033	57.48 ± 3.01*	0.70 ± 0.20*	8.85 ± 1.74*	0.36 ± 0.09*	0.075 ± 0.008	0.57 ± 0.24	0.18 ± 0.07	0.24 ± 0.09	0.10 ± 0.03	0.49 ± 0.09*	0.046 ± 0.013*	0.083 ± 0.040	3.62 ± 1.44	0.18 ± 0.05*
nr C7	94.26 ± 10.54	1.013 ± 0.090	67.72 ± 5.26	0.74 ± 0.09*	8.66 ± 1.38	0.36 ± 0.12	0.075 ± 0.008*	0.59 ± 0.06*	0.18 ± 0.02*	0.24 ± 0.02*	0.11 ± 0.01*	0.53 ± 0.07*	0.044 ± 0.008*	0.071 ± 0.005*	3.91 ± 0.26*	0.20 ± 0.02*
P-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Significance of source of variation interaction																	
G × I	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Dry weight and metabolite content correlation																	
Total d. wt	0.963	0.916	0.863	0.661	0.321	0.446	-0.233	0.241	0.292	0.484	0.723	0.531	0.597	0.465	0.466	0.680	0.680
r	0.000	0.004	0.014	0.128	0.523	0.358	0.649	0.638	0.563	0.312	0.082	0.257	0.187	0.334	0.333	0.113	0.113
Shoot d. wt																	
r	0.959	0.937	0.888	0.691	0.369	0.485	-0.210	0.278	0.332	0.512	0.746	0.572	0.624	0.491	0.491	0.698	0.698
P-value	0.000	0.002	0.008	0.105	0.457	0.310	0.682	0.583	0.508	0.278	0.066	0.213	0.162	0.303	0.303	0.099	0.099
Root d. wt																	
r	0.908	0.718	0.642	0.428	-0.005	0.165	-0.357	-0.012	0.023	0.271	0.522	0.234	0.383	0.273	0.272	0.520	0.520
P-value	0.005	0.085	0.145	0.381	0.993	0.750	0.473	0.981	0.965	0.594	0.267	0.647	0.438	0.591	0.592	0.270	0.270

Data were analysed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± s.e. (n = 4). In A, the significance of sources of variation as well as their interaction (G × I) was evaluated by P-value. In the case of significant interaction between factors, all treatments were compared with each other. In the case of a non-significant interaction between factors, inoculum effects were evaluated by analysing wt and nr plants separately using ANOVA. Plant genotype effect was evaluated by analysing wt and nr plants under the same inoculation treatment (No, Bm or C7) by Student t-test and significant difference (P < 0.05) is shown as (*) next to nr means. In B, significant Pearson correlations between plant dry weights and root metabolites at 8 wpi are shown. Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated never ripe plants (nr No, nr Bm and nr C7, respectively). P-value: n.s., not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

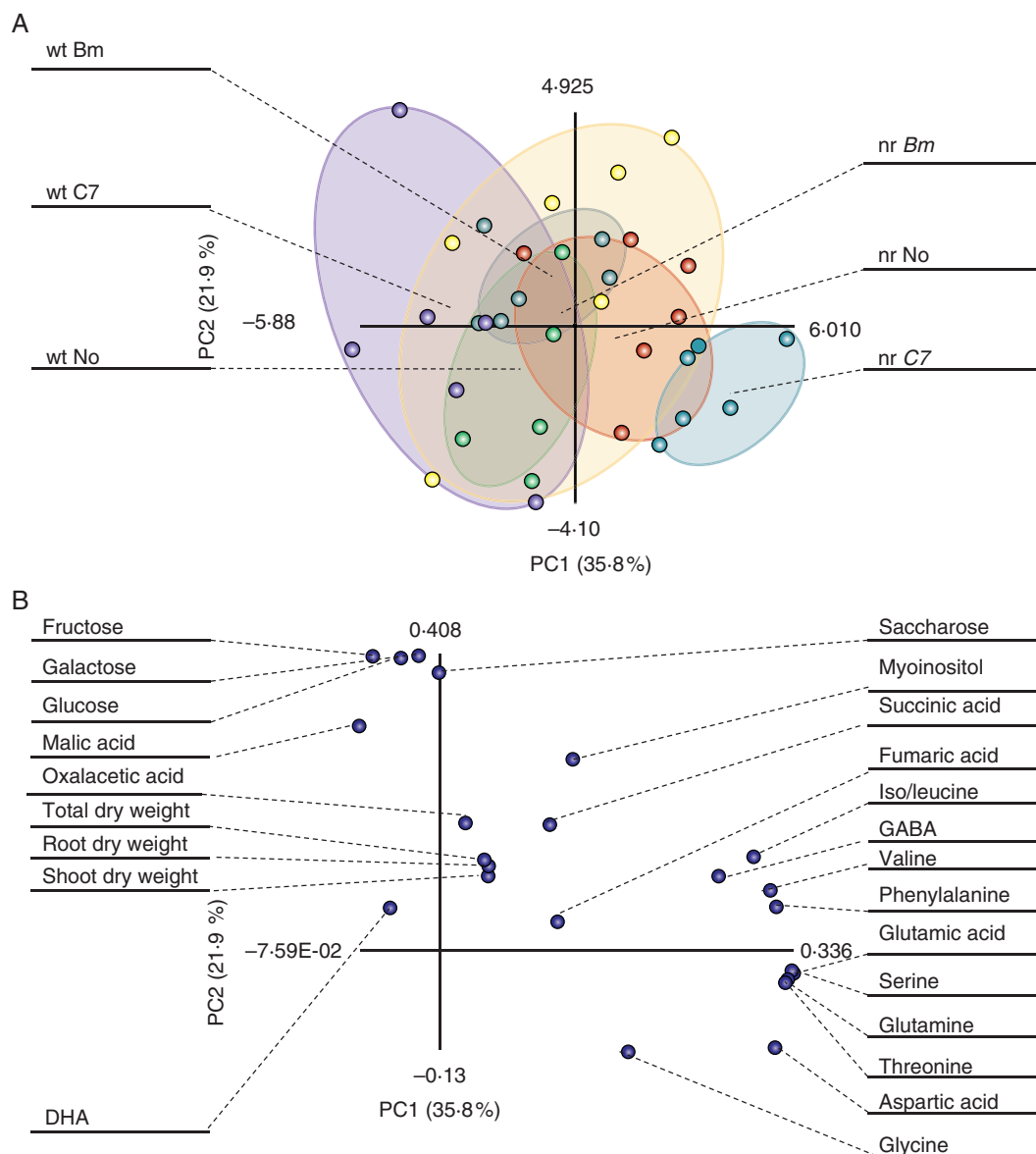


Fig. 4. Principal component analysis (PCA) of root metabolite levels and dry weights at 4 weeks post-inoculation (wpi). Analyses were performed based on metabolite contents and dry weight data obtained from tomato (*Solanum lycopersicum*) roots. (A) Score plot. Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated never ripe plants (nr No, nr Bm and nr C7, respectively). Each point represents one plant, and points of the same treatment are enclosed in a different coloured ellipse: green for wt No, grey for wt Bm, purple for wt C7, red for nr No, yellow for nr Bm and blue for nr C7. (B) Loading plot. Each point represents one nutrient or dry weight.

Moreover, malic acid also contributed to the variability of PC2 (Fig. 5B).

DISCUSSION

Management of rhizospheric micro-organisms is a valuable strategy to induce plant growth (Berg, 2009; Singh *et al.*, 2011) and could decrease chemical inputs in agriculture (Bhattacharyya and Jha, 2012). However, knowledge of PGPB effects on plants is necessary for the proper and effective large-scale use of these bacteria in crop systems. In this research, the

outcome of the inoculation of tomato plants with two different PGPB was reported regarding ethylene sensitivity at two different plant developmental stages.

Ethylene sensitivity is essential for growth promotion by Bm but not for C7

In order for a bacterium to be considered as a PGPB, colonization of the plant root system is a critical trait for plant–bacteria interaction (Benizri *et al.*, 2001). Here, a colonization bioassay confirmed that Bm as well as C7 were able to colonize

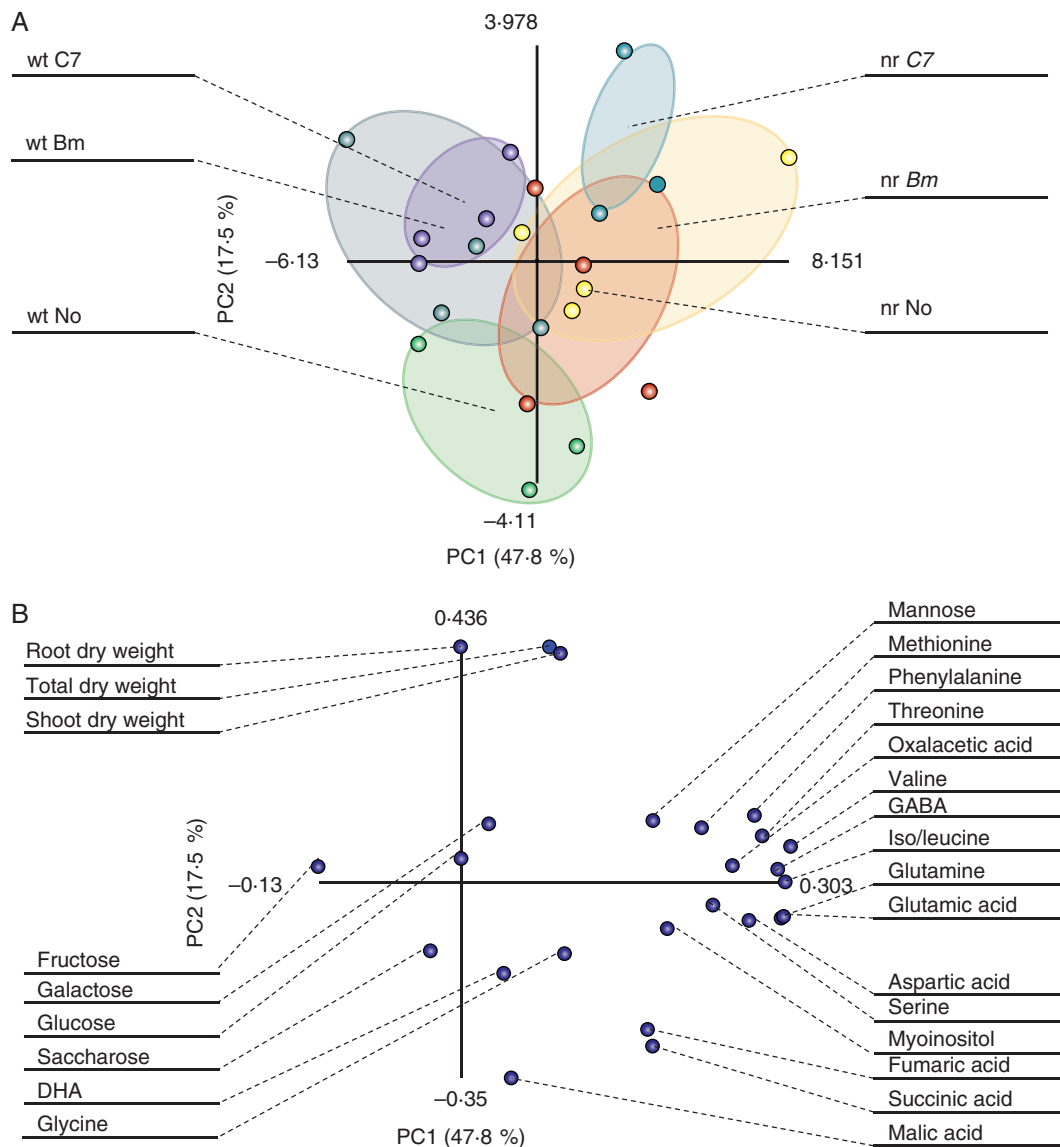


FIG. 5. Principal component analysis (PCA) of root metabolite levels and dry weights at 8 weeks post-inoculation (wpi). Analyses were performed based on metabolite contents and dry weight data obtained from tomato (*Solanum lycopersicum*) roots. (A) Score plot. Treatments: non-inoculated, *Bacillus megaoterium*-inoculated and *Enterobacter C7*-inoculated wild-type plants (wt No, wt Bm and wt C7, respectively) and non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter C7*-inoculated *never ripe* plants (nr No, nr Bm and nr C7, respectively). Each point represents one plant, and points of the same treatment are enclosed in a different coloured ellipse: green for wt No, grey for wt Bm, purple for wt C7, red for nr No, yellow for nr Bm and blue for nr C7. (B) Loading plot. Each point represents one nutrient or dry weight.

the roots of ethylene-sensitive and insensitive plants. Moreover, the colonization rates were not significantly different (Table 1), suggesting that plant growth variations are not due to differences in root colonization. In juvenile plants, an increase in root dry weight was only produced by Bm inoculation in nr plants, also resulting in a significant difference between wt and nr plants only under Bm inoculation (Fig. 1E), suggesting that root growth promotion is related to ethylene insensitivity in the case of Bm.

At 8 wpi, C7 inoculation stimulated plant growth independently of sensitivity to ethylene, while Bm inoculation

promoted plant growth only in wt plants (Fig. 1B). Furthermore, phenotypic analysis only showed visible differences in plant height and number of flowers, resulting in the same pattern as total dry weight (data not shown). These results indicate that ethylene perception is required for growth promotion by Bm but not for C7. Plant growth promotion by Bm was previously reported in several plant species (Marulanda-Aguirre *et al.*, 2008; Marulanda *et al.*, 2009; Armada *et al.*, 2014b; Porcel *et al.*, 2014). The dependence on ethylene sensitivity of PGPB action was previously reported in arabidopsis, using a *Variovorax paradoxus* strain and also showing no

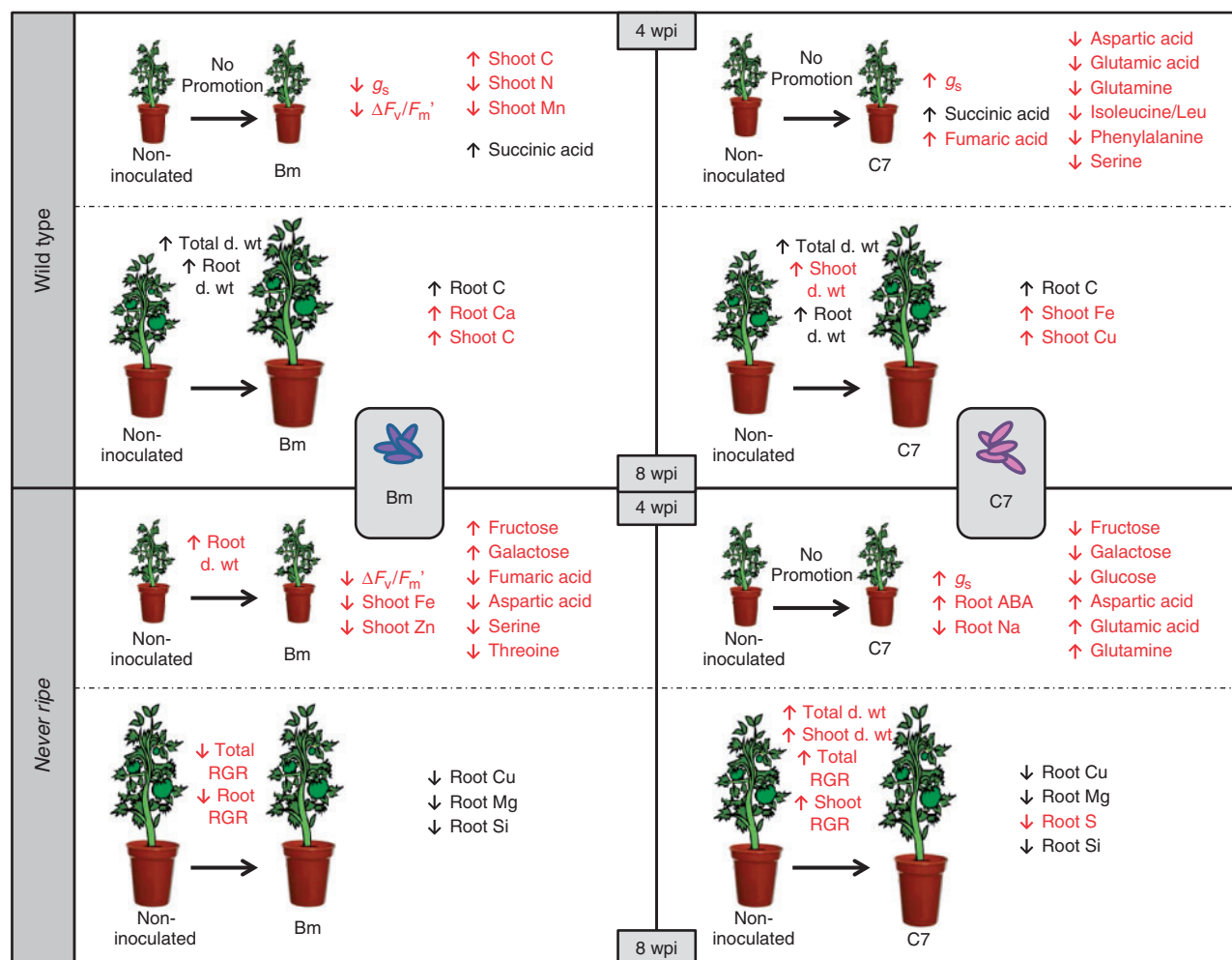


Fig. 6. Summary of plant growth-promoting bacteria (PGPB) inoculation effects on wild-type and *never ripe* tomato (*Solanum lycopersicum*) plants at 4 and 8 weeks post-inoculation (wpi). Increases or decreases in measured parameters produced by PGPB inoculation are shown with ↑ and ↓ symbols, respectively. Changes induced by PGPB inoculation are shown in black and red letters for common and specific effects, respectively. Bm, *Bacillus megaterium*; C7, *Enterobacter C7*; d. wt, dry weight; RGR, relative growth rate; g_s , stomatal conductance; $\Delta F_v/F_m'$, photosynthetic efficiency; ABA, abscisic acid; C, carbon; N, nitrogen; Mn, manganese; Ca, calcium; Fe, iron; Cu, copper; Zn, zinc; Na, sodium; Mg, magnesium; Si, silicon; S, sulphur.

differences in root colonization rate (Chen *et al.*, 2013). In contrast, other studies using inoculations of *Bacillus megaterium* UMCV1 in arabidopsis (López-Bucio *et al.*, 2007) and *Bacillus* sp B55 in *Nicotiana attenuata* (Meldau *et al.*, 2012) pointed to an ethylene-independent mechanism. As with many plant–bacterial interactions, discrepancies between these studies could be due to strain-specific PGPB mechanisms, plant growth conditions (Ryu *et al.*, 2005; Long *et al.*, 2008) and/or differences between plants in ethylene transduction pathways since functional redundancy was reported for ethylene receptors in arabidopsis (Hua and Meyerowitz, 1998).

Furthermore, the difference between plant genotypes was only significant in non-inoculated plants, indicating less pronounced ethylene growth-inhibitory effects (Pierik *et al.*, 2006) on *nr* plants due to their mutation (Wilkinson *et al.*, 1995). Under Bm inoculation, this genotype difference was not seen because growth promotion was only produced in wt plants reaching a similar dry weight to *nr* plants, and suggesting that

Bm could modulate ethylene receptor expression, as previously reported in another PGPB strain (Vargas *et al.*, 2012), and needed for the proper establishment of a beneficial plant–bacteria association (Vacheron *et al.*, 2013). Although C7 inoculation promoted growth of wt and *nr* plants, no significant difference between plant genotypes was observed, probably due to a different intensity of growth promotion. Moreover, IAA is able to induce plant growth (Zhao, 2010), and higher auxin levels were seen exclusively in non-inoculated *nr* mature plants compared with wt plants.

In addition, total RGR was not affected by ethylene insensitivity, as previously reported (Tholen *et al.*, 2004). However, our data show that bacterial inoculation produced RGR modifications in ethylene-insensitive plants not seen in wt plants. Although few PGPB studies include RGR evaluation, RGRs can be enhanced by PGPB inoculation, but these responses are strain specific (Shishido and Chanway, 2000), as observed in our results (Fig. 2). These results points to deleterious effects of

Bm inoculation on mature *nr* plants, as previously reported in ABA-deficient tomato plants by Porcel *et al.* (2014). Moreover, it was also noted that short-term growth promotion was not always linked with long-term promotion, as observed previously (Gray and Smith, 2005; Kuan *et al.*, 2016). Thus, further research addressing bacterial inoculation effects on mature plants is necessary, since several studies that propose the use of bacterial strains as PGPB have only evaluated the effects of these bacteria on juvenile plants or seedlings and/or grown *in vitro*.

Furthermore, several correlations have been found between dry weights and nutrients (Table 3) or metabolites (Tables 6 and 7). In juvenile plants, root sulphur concentration was positively correlated with total, shoot and root dry weights. Levels of S were decreased by C7 inoculation in *nr* plants, and a previous study reported modulation of S assimilation by PGPB inoculation (Aziz *et al.*, 2016), and ethylene and S nutrition have links at metabolic and regulatory levels (Wawrzynska *et al.*, 2015). In mature plants, C positively correlated with all dry weights. Although C gain is a consequence of photosynthesis, little is known about the impact of carbon availability on plant growth (Smith and Stitt, 2007). Moreover, this correlation could be linked to positive correlations seen between root sugar content and dry weights. Shoot Fe concentration was negatively correlated with all dry weights at 4 wpi. Ethylene is involved in a plant's response to Fe deficiency (Lucena *et al.*, 2006), resulting in the initiation of root hairs affected by inhibition of ethylene perception (Schmidt, 2001). Thus, lower shoot Fe levels observed in Bm-inoculated *nr* plants could be due to less Fe uptake and translocation. Competition for Fe uptake between plants and micro-organisms has been reported at the rhizosphere, showing that micro-organisms could be more competitive than plants (Pii *et al.*, 2015). Consequently, our results suggest an interaction failure between Bm and *nr* plants that leads to competition for Fe at the rhizosphere.

In juvenile plants, galactose and DHA correlated with all dry weights positively and negatively, respectively. DHA is the oxidized form of ascorbic acid, involved in prevention of oxidative damage (J. Wang *et al.*, 2013). Thus, this negative correlation could be due to reduced oxidative damage in Bm-inoculated *nr* roots which showed increased root dry weight. Moreover, the main ascorbic acid biosynthesis pathway in plants is the L-galactose pathway (Laing *et al.*, 2007), and galactose was increased by Bm inoculation in *nr* plants. In addition, sugars are immediate substrates for metabolism and signalling molecules, and their availability is linked to plant growth (Rolland *et al.*, 2006; Hanson and Smeekens, 2009). Thus, the observed correlations could be due to sugar availability.

Stomatal conductance and photosynthetic efficiency were affected by PGPB inoculation at the juvenile stage

Effects of PGPB inoculation were only seen in juvenile plants, while differences between plant genotypes showed different patterns at 4 and 8 wpi (Table 2). Stomatal conductance results also suggested a role for ethylene sensitivity in the Bm–tomato interaction. Reduction of stomatal conductance by Bm inoculation was previously reported in clover (Armada *et al.*, 2014a), lavender and salvia (Armada *et al.*, 2014b), although no effect was reported in tomato (Porcel *et al.*, 2014).

Moreover, photosynthesis is directly related to chlorophyll content (Richardson *et al.*, 2002), but the chlorophyll content was unaffected by Bm inoculation, suggesting that the decrease in photosynthetic efficiency was not linked to chlorophyll content. Ethylene also modifies photosynthesis by affecting stomatal aperture with a dose-dependent mechanism (Tanaka *et al.*, 2005), and significant differences between plant genotypes were only observed under Bm inoculation for the three parameters at 4 wpi. These results are in accordance with a Bm–tomato interaction mediated by ethylene (and thus dependent on its perception) that could decrease stomatal conductance and then photosynthetic efficiency in wt plants. However, Bm could be misrecognized by *nr* plants (unable to respond to released ethylene from the interaction), resulting in no change in stomatal conductance and deleterious effects such as reduced photosynthetic efficiency and lower chlorophyll levels in Bm-inoculated *nr* plants compared with the wt. Nevertheless, stomatal conductance was increased in wt and *nr* plants, and photosynthetic efficiency and chlorophyll content were unaffected by C7 inoculation, in accordance with a PGPB mechanism independent of ethylene sensitivity.

Furthermore, stomatal conductance was significantly lower in *nr* than in wt plants under all inoculation treatments, and photosynthetic efficiency was lower in non- and C7-inoculated plants at 8 wpi, when higher ethylene values were observed in *nr* plants. Although the physiological role of ethylene depends on specific traits of plant species (related in principle to their habitat) and the integrative result of internal and external stimuli (Pierik *et al.*, 2006), these results suggest that *nr* plants were more stressed than wt plants, probably because ethylene is a key hormone involved in response to environmental stresses (F. Wang *et al.*, 2013; Van de Poel *et al.*, 2015), and some ethylene-insensitive genotypes would fail to produce some adaptive responses (Feng and Barker, 1992; Zhang *et al.*, 2003).

Phytohormonal status was mainly affected by ethylene insensitivity and was altered under bacterial inoculation

The effects of PGPB on phytohormone levels were mainly dependent on ethylene sensitivity, and only C7 inoculation directly affected root ABA (Tables 4 and 5). Most of the previously described action mechanisms in relation to ethylene are mainly related to PGPB containing the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCd) that induces plant growth and development by decreasing the immediate ethylene precursor (ACC) and thereby the ethylene levels (Glick *et al.*, 2007; Glick, 2014). However, the PGPB used in this study did not show ACCd activity (data not shown). Furthermore, ethylene production by Bm and C7 was also assessed (data not shown), resulting in no detectable microbially derived ethylene that could affect plant growth or the root colonization process.

Root ABA concentration was exclusively increased by C7 inoculation in *nr* juvenile plants, as previously reported with others PGPB (Bresson *et al.*, 2013; Cohen *et al.*, 2015). The role of ABA has been historically described as that of a growth inhibitor. However, high ABA levels were reported in young tissues, and ABA-deficient mutants are severely affected in terms of growth (Finkelstein, 2013). Endogenous ABA is

crucial in limiting ethylene production, maintaining rather than reducing plant growth (Sharp, 2002). Furthermore, ABA is able to suppress plant resistance mechanisms mediated by JA/ethylene- and SA-dependent immune responses (Anderson *et al.*, 2004; Sánchez-Vallet *et al.*, 2012). Our results also showed higher root ABA levels in C7-inoculated *nr* plants compared with the wt, but no genotype differences in ethylene, JA and SA concentrations. Thus, although C7 did not promote plant growth at 4 wpi, these hormonal changes predispose plants to further growth. In fact, ethylene, JA, JA-Ile and SA levels were also unaffected by C7 inoculation or plant genotype in mature plants.

However, Bm did not directly affect phytohormone levels. A previous report using Bm-inoculated wt and ABA-deficient tomato lines suggested that normal endogenous ABA levels could be essential for growth promotion by maintaining production of ethylene at low levels (Porcel *et al.*, 2014). Bm only promoted growth in wt plants, and ethylene and ABA levels were unaffected, as reported in wt plants by Porcel *et al.* (2014). Thus, our results are in concordance with the hypothesis pointing to plant ethylene sensitivity as a new player in Bm–tomato interaction. Indeed, although similar differences between plant genotypes were seen in root ethylene in non- and Bm-inoculated plants, a genotype difference was exclusively not seen in shoot ethylene under Bm inoculation. Furthermore, shoot SA levels exclusively showed higher values in *nr* plants under Bm inoculation, suggesting that *nr* plants respond to Bm by activating plant defences since SA is a key factor for establishment of basal defences, effector-triggered immunity and both local and systemic acquired response (Vlot *et al.*, 2009). In fact, SA, JA and JA-Ile also showed higher values in *nr* than in wt shoots only under Bm inoculation in mature plants. The best known role of JA is its regulation of plant immune responses against pathogens (Browse, 2009). Thus, these results also support ‘misinteraction’ resulting in Bm recognition by *nr* plants as a pathogen-like micro-organism with physiologically deleterious effects. Indeed, ethylene modulates plant resistance and susceptibility to pathogens (van Loon *et al.*, 2006) and even beneficial micro-organisms can be recognized as potential invaders, triggering an immune response (Zamioudis and Pieterse, 2012).

Furthermore, ethylene and IAA affect each other’s synthesis since high IAA levels result in increased ethylene biosynthesis (Muday *et al.*, 2012), and vice versa (Stepanova *et al.*, 2005). The ethylene-insensitive tomato mutant *nr* is not able to perceive ethylene, but presents some residual responsiveness (Lanahan *et al.*, 1994) and only ETR3 is not functional (Wilkinson *et al.*, 1995). Cross-talk between ethylene and auxins is produced at the level of biosynthesis and thus higher levels of IAA and ethylene were generally seen in non-inoculated *nr* plants compared with wt plants. Furthermore, microbially derived IAA is a signalling molecule in micro-organisms and interferes with several developmental processes in *planta* (Spaepen and Vanderleyden, 2011). Although IAA production by these PGPB was not assayed, PGPB inoculation did not directly modify IAA levels and only plant genotype differences were affected under bacterial inoculations, suggesting that both Bm and C7 are unable to produce auxins. Lower IAA levels were seen in Bm-inoculated *nr* plants compared with wt plants, and IAA signaling downregulation was reported as part of plant

defence against bacteria (Spaepen and Vanderleyden, 2011) in agreement with our results. Moreover, root and shoot IAA levels were increased in C7-inoculated *nr* plants compared with wt plants, predisposing plants to further growth since IAA plays crucial roles in several developmental processes, being identified as a plant growth hormone (Zhao, 2010).

Root metabolites were directly altered by PGPB inoculation at the juvenile stage showing a high dependence on ethylene sensitivity in mature plants

Our results showed changes in root sugars, amino acids and organic acids due to bacterial inoculation. Although performed in shoots, previous studies pointed to modification of those compounds by bacterial inoculation (Weston *et al.*, 2012; Su *et al.*, 2016). Root metabolite contents were affected by bacterial inoculation in juvenile plants, but did not show changes in mature plants when genotype differences were clearly marked (Tables 6 and 7; Figs 4 and 5).

Sugars and amino acids were the main source of variation at 4 wpi, while only amino acids contributed to variability in mature plants, suggesting that modification of root sugar levels rather than amino acid content by bacterial inoculation could be relevant in growth promotion. Furthermore, ethylene perception is related to plant sensitivity to sugars (Paul and Pellny, 2003). Moreover, differences between Bm- and C7-inoculated juvenile plants were observed in *nr* plants, suggesting strain-specific interactions between bacteria and host plants, as previously reported (Walker *et al.*, 2011; Weston *et al.*, 2012). These results imply that ethylene sensitivity could affect plant–bacteria interaction because similar profiles were observed in wt plants. Root metabolic profiles were in agreement with biomass results at 8 wpi, showing high similarity in profiles of Bm- and non-inoculated *nr* plants (Figs 1 and 5). Root biomass can be increased by bacterial succinic acid (Yoshikawa *et al.*, 1993), and higher succinic acid levels coupled with higher root biomass were only observed in bacteria-inoculated wt plants. Furthermore, fumaric acid plays a crucial role in biofilm formation necessary for root colonization by *Bacillus* strains (Zhang *et al.*, 2014; Yuan *et al.*, 2015). Thus, lower fumaric acid levels only seen in Bm-inoculated *nr* plants could affect functional interaction. Moreover, differences in monosaccharide levels between both inocula were restricted to *nr* plants. Higher glucose levels were seen in Bm-inoculated *nr* plants compared with the wt plants at 8 wpi. Ethylene is involved in plant sensitivity to sugars, and it has been reported that ethylene-insensitive plants show a higher response to endogenous glucose, resulting in increased suppression of photosynthesis by carbohydrates (Zhou *et al.*, 1998; León and Sheen, 2003; Paul and Pellny, 2003). Thus, Bm inoculation could enhance this phenomenon only in *nr* plants, modifying mainly sugar metabolism.

In addition, previous studies have reported changes in plant amino acid levels by bacteria (Curzi *et al.*, 2008). Also, previous reports suggested that amino acid homeostasis could have regulatory functions in maintaining plant growth and development (Walch-Liu *et al.*, 2006; Yu *et al.*, 2013; Ros *et al.*, 2014). Several amino acids were decreased by Bm and increased by C7 in *nr* plants. These results suggest a strain-specific effect on

root amino acid levels that could lead to different growth responses. Aspartic acid is the common precursor of other amino acids in higher plants (Azevedo *et al.*, 2006). Deficiencies in serine and derived molecules have consequences such as altered mineral homeostasis and root development (Muñoz-Bertomeu *et al.*, 2009; Ros *et al.*, 2014). Thus, these specific reductions of amino acid contents in *nr* plants could affect plant growth under Bm inoculation. Moreover, aspartic acid, glutamic acid and glutamine are involved in plant nitrogen assimilation (Xu and Zhou, 2004), suggesting that C7 inoculation could improve N assimilation in *nr* juvenile plants. Furthermore, amino acid levels were reduced in wt plants by C7 inoculation. Isoleucine deficiency produces defects in cell proliferation and expansion during development of roots (Yu *et al.*, 2013). Phenylalanine is also used in the phenylpropanoid pathway leading to the biosynthesis of secondary products (Hyun *et al.*, 2011) involved in cell wall structure (Bonawitz and Chapple, 2010), and plant defence or response to stress (Winkel-Shirley, 2001; Fraser and Chapple, 2011). Thus, these results suggest that C7 inoculation could affect plant development through modification of amino acid content. As proposed by Rivero *et al.* (2015), reduction of amino acids could be due to their use for secondary metabolism. In addition, genotype differences seen exclusively under C7 inoculation were in amino acid levels, also indicating a remodelling of amino acid metabolism.

PGPB inoculation affected plant nutrition notably at the mature stage with a clear influence of ethylene sensitivity

The effects of PGPB on nutritional status were more prominent in mature plants, showing a high dependence on ethylene sensitivity (Fig. 3; Tables S4 and S5). Indeed, the interaction of ethylene signalling and plant nutrition was reviewed by Iqbal *et al.* (2013), indicating that nutrient deficiencies are greatly related to ethylene perception and biosynthesis. As observed for metabolites, separation of root nutrient profiles was separated by plant genotype in mature plants, but the strain-specific effect was maintained in *nr* plants (Fig. 3).

Bm inoculation did not alter nutrients in juvenile roots, but the leaf contents of several nutrients were affected. The C concentration was increased, while N and Mn were decreased in wt plants. Mn plays an important role in protection of photosynthetic tissues (Mehlhorn and Wenzel, 1996), and our results showed a reduction in photosynthetic efficiency of Bm-inoculated wt plants. Moreover, reduced Zn and Fe levels in *nr* shoots due to Bm inoculation could suggest that Bm affects nutrient translocation in *nr* plants (Sperotto, 2013). In mature plants, Bm inoculation increased C in roots and shoots of wt plants, but a decrease in several nutrients was seen in *nr* roots. Thus, the C:N ratio was increased by Bm inoculation in wt plants, suggesting that Bm stimulates plant growth by increasing C assimilation per N unit, leading to an increase in biomass (Lawlor, 2002). Moreover, Ca is a key player conferring structure and rigidity to the cell wall and interacts with ethylene signalling and plant responses to biotic attacks (Iqbal *et al.*, 2013). Thus, the root Ca increase mediated by Bm inoculation could enhance the resistance of wt plants.

Although both PGPB decreased Cu levels in *nr* roots at 8 wpi, C7 reduced it to a higher degree than Bm. Also, C7

inoculation decreased Cu in *nr* shoots at 8 wpi. Moreover, root Cu concentration was negatively correlated with total and shoot dry weights. Cu is a structural component of ethylene receptors (Rodríguez, 1999). Thus, lower levels of Cu could result in fewer functional ethylene receptors, and consequentially lower growth inhibition by ethylene (Pierik *et al.*, 2006). These results are in accordance with plant growth promotion observed in *nr* plants. However, C7 increased shoot Cu levels in wt plants, but the ethylene transduction pathway and regulatory mechanisms are functional, showing no growth inhibitory effects. In addition, both PGPB also affected Si levels in mature *nr* roots. Moreover, the role of Si in plants has been described as that of a biotic and abiotic protector (Ma and Yamaji, 2008). Thus, lower Si levels seen in bacteria-inoculated plants could indicate that low Si could favour interaction with PGPB, with C7-inoculated *nr* plants showing lower levels than Bm-inoculated *nr* plants.

Nevertheless, C7 inoculation only reduced root Na concentration in *nr* roots, not affecting shoot nutrients in juvenile plants. Although no saline stress was applied in this study, reduction of Na by PGPB was previously reported (Zhang *et al.*, 2008) and could be related to growth promotion because Na is toxic to plant cells and its accumulation within cells is undesirable (Pardo and Quintero, 2002). In contrast, C7 inoculation showed a large impact in plant nutrition at 8 wpi. Root C concentration was increased in wt plants, while root S levels were decreased in *nr* plants. S reduction by C7 inoculation only in *nr* plants could be due to modulation of the S assimilation machinery of plants (Aziz *et al.*, 2016) whose regulation is dependent on ethylene perception (Wawrzynska *et al.*, 2015). In addition, C7 inoculation affected shoot nutrients, increasing Fe in wt plants and decreasing P levels in *nr* plants. Fe is a key factor in photosynthesis and respiration, and its deficiency produced stunted plant growth (Iqbal *et al.*, 2013), suggesting that C7 inoculation of wt plants improves nutrient translocation (Sperotto, 2013) and maintains plant growth. However, P deficiency limits shoot and root dry weights (Borch *et al.*, 1999). Ethylene is induced in P-deficient plants and ethylene sensitivity is involved in regulating carbon allocation to adventitious roots (Kim *et al.*, 2008) and root hair development (Zhang *et al.*, 2003) in order to facilitate a rapid recovery of stressed plants. A previous study reported that *Bacillus amyloliquefaciens* reduced P uptake (Talboys *et al.*, 2014). These results are in agreement with our study but further research is necessary to know how the C7 inoculation affects P nutrition.

CONCLUSIONS

In this study, it has been reported that physiological parameters and root metabolites were modified by bacterial inoculation in juvenile plants rather than in mature plants, when plant homeostasis can counteract inoculation effects. However, PGPB effects on nutritional status were more prominent in mature plants, with a high dependence of ethylene sensitivity (Fig. 6).

In conclusion, the inability to perceive ethylene by the ETR3 receptor impairs interaction between tomato plants and *B. megaterium*, affecting photosynthetic efficiency, plant nutrition and root sugars, that leads to a loss of PGPB activity. Nevertheless, Bm could stimulate plant growth in wt plants by improving

carbon assimilation. In contrast, *Enterobacter C7* could stimulate plant growth by affecting stomatal conductance, plant nutrition and root amino acids. Moreover, *C7* inoculation in ethylene-insensitive plants could improve nitrogen assimilation (Fig. 6). Thus, ethylene sensitivity could be proposed as essential for PGPB activity of *B. megaterium* in tomato plants, whereas the *Enterobacter C7* PGPB mechanism seems to be ETR3 independent.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Table S1: reagents and standards used for phytohormone analysis by HPLC-ESI-HRMS. Table S2: instrumental parameters used for HESI (II) ionization. Table S3: accurate masses of the phytohormones and internal standards, and its principal fragments. Table S4: effects of bacterial inoculation on root nutrient concentrations. Table S5: effects of bacterial inoculation on shoot nutrient concentrations.

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