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Ethylene perception by ETR3 determines plant interaction with plant growth promoting bacteria in tomato (*Solanum lycopersicum*)

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Pablo Ibort Pereda

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Memoria presentada por Pablo Ibort Pereda,

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(con la mención "Doctor Internacional")

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El doctorando Pablo Ibort Pereda y el director de la tesis Ricardo Aroca Álvarez. Garantizamos, al firmar esta tesis doctoral que el trabajo ha sido realizado por el doctorando bajo la dirección del director de tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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The doctoral candidate Pablo Ibort Pereda and the thesis supervisor Ricardo Aroca Álvarez. Guarantee, by signing this doctoral thesis, that the work has been done by the doctoral candidate under the direction of the thesis supervisor and, as far as our knowledge reaches, in the performance of the work, the rights of other authors to be cited (when their results or publication have been used) have been respected.

Granada, a 21de Mayo de 2017 / *May 21st 2017*

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"Deberíamos vivir tantas
veces como los árboles,
que pasado un año malo
echan nuevas hojas y
vuelven a empezar"

José Luis Sampedro

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Abbreviations

$^1O_2$: Singlet oxygen
50SL11: Ribosomal proteim 50SL11
AAP: Aspartyl aminopeptidase
AAT: Aspartate aminotransferase
ABA: Abscisic acid
ACC: 1-aminocyclopropane1-carboxylate
ACCd: ACC deaminase
ACO: ACC oxidase
ACS: ACC synthase
AMF: Arbuscular mycorrhizal fungi
ANOVA: Analysis of variance
AOS: Allene oxide synthase
AP2: Apetala 2
APX: Ascorbate peroxidase
ASS: Argininosuccinate synthase
ATP: Adenosine triphosphate
b-CHI: Basic chitinase
BCP: Blue copper protein
Bm: *Bacillus megaterium*
BRs: Brassinosteroids
C7: *Enterobacter* C7
Ca3: Carbonic anhydrase 3
CaPF1: *Capsicum annuum* pathogen and freezing tolerance-related protein1
CAT: Catalase
Cc: Cytochrome c
CFU: Colony-forming unities
CO_2: Carbon dioxide
COPI: Coat complex protein I
Ct: Threshold cycle
CTR1: Constitutive triple response 1
cv: Cultivar
Cyt b6/f: Cytochrome b6/f
Cytb5: Cytochrome b5
DFv/Fm': Photosynthetic efficiency
DHA: Dehydroascorbic acid
DHAR: Dehydroascorbate reductase
DRE: Dehydration responsive elements
DREB: Dehydration-responsive element-binding protein
DW: Dry weight
EBF: EIN3-binding F-box
EDS1: Enhanced disease susceptibility 1
EF: Elongation factor
EIL: EIN3-like
EIN2: Ethylene-insensitive protein 2
EIN3: Ethylene-insensitive protein 3
EIN4: Ethylene-insensitive protein 4
EIN5: Ethylene-insensitive protein 5
ePGPB: Extracellular PGPB
ER: Endoplasmic reticulum
ER21: Ethylene responsive 21
ER24: Ethylene responsive 24
ER5: Ethylene responsive 5
ERD7: Early response to dehydration
ERF: Ethylene response factor
ERF1: Ethylene responsive factor 1
ERS1: Ethylene sensor 1
ERS2: Ethylene sensor 2
ESI: Electrospray ionization
ETI: Effector-triggered immunity
ETP: EIN2-targeting protein
ETR1: Ethylene receptor 1
ETR2: Ethylene receptor 2
Fer: bHLH transcriptional regulator
Fsm1: SANT/MYB domain protein 1
FW: Fresh weight
GAs: Gibberelins
GC: Gas chromatography
GR: Glutathione reductase
GR: Green ripe protein
GRL: Green ripe-like protein
gs: Stomatal conductance
GSH: Reduced glutathione
GSL7: Glucan synthase-like7
GSSG: Oxidized gluthatione
GST: Glutathione S-transferase

H_20_2: Hydrogen peroxide
HPLC: High performance liquid chromatography
HRMS: High-resolution accurate MS
Hsp: Heat-shock protein
IAA: Indolacetic acid
iPGPB: Intracellular PGPB
ISR: Induced systemic resistance
JA: Jasmonic acid
JA-Ile: Jasmonoyl-isoleucine
JAs: Jasmonates
LC: Liquid chromatography
LDOX: Leucoanthocyanidin dioxygenase
LOX: Lipoxygenase
LPS: Lipopolisaccharides
LSD: Least significant difference
Mal d 1: Major allergen Mal d 1
MAMPs: Microbe-associated molecular patterns
MS: Mass spectrometry
MTA: 5'-methylthioadenosine
Myc: Mycorrhization factor
NADP: Nicotinamide adenine
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
Nod: Nodulation factor
NR: Never ripe
Nramp: Natural resistance-associated macrophage protein
nsLTPs: Non-specific lipid-transfer proteins
O_2^-: Superoxide anion
OD600: Optical density at 600 nm
OH-: Hydroxyl radicals
PAD4: Phytoalexin deficient 4
PAMP: Pathogen-associated molecular pattern
PC1: Principal component 1
PC2: Principal component 2
PCA: Principal component analysis
PCD: Programmed cell death
PCR: Polymerase chain reaction
PDF: Plant defensin
PGPB: Plant growth promoting bacteria
PGPR: Plant growth promoting rhizobacteria
PM: Plasma membrane
PR-10: Pathogenesis related group 10
PsbF: Cytochrome b559 beta chain
PSI: Photosystem I
PSII: Photosystem II
PTI: PAMP-triggered immunity
QRT-PCR: Quantitative Reverse transcription PCR
RAN1: Responsive to antagonist 1
RD2: Responsive to dessication 2
RD20: Responsive to dessication 20
RD29B: Responsive to dessication 29B
RGR: Relative growth rate
RLK: Receptor like kinase
RNA-seq: RNA sequencing
ROS: Reactive oxygen species
RPL4-B: Ribosomal protein L4-B
RPP3: 60S acidic ribosomal protein P3
RTE1: Reversion to ethylene sensitivity 1
Rubisco: Ribulose-1,5-bisphosphate carboxylase/oxygenase
SA: Salicylic acid
SAM: S-adenosylmethionine
SAR: Systemic acquired resistance
SlCTR: *Solanum lycopersicum* constitutive triple response
SlETR: *Solanum lycopersicum* ethylene receptor
SOD: Superoxide dismutase
SW: Substrate weight
TCTR1: Tomato CTR1
TPI: Triosephosphate isomerase
TSRF1: Tomato stress-responsive factor 1
TTSS: Type III secrtion systems
UHPLC: Ultra-HPLC
UV: Ultraviolet

V-ATPase: V-type proton ATPase
VHA: Vacuolar-type proton ATPases
VWRE: Vascular wounding responsive elements
WHC: Water-holding capacity
wpi: Weeks post-inoculation
wt: Wild type

Summary/Resumen

Summary/Resumen

La percepción de etileno a través de ETR3 determina la interacción de las plantas con bacterias promotoras del crecimiento vegetal en tomate (*Solanum lycopersicum*)

Pablo Ibort Pereda

Introducción

La intensificación sostenible de la agricultura persigue proporcionar seguridad alimentaria a una población mundial creciente y al mismo tiempo reducir los efectos negativos medioambientales de la agricultura (Tilman et al. 2011). Por ello, se deben desarrollar nuevas estrategias con el objetivo de incrementar la eficiencia de los cultivos en la utilización de recursos manteniendo los rendimientos actuales (Dodd and Ruiz-Lozano 2012). El manejo de microorganismos rizosféricos es una buena estrategia para inducir el crecimiento vegetal (Berg 2009; Singh et al. 2011), y podría disminuir la utilización de productos químicos en agricultura (Bhattacharyya and Jha 2012). Sin embargo, es necesaria una mayor investigación para comprender completamente la interacción entre plantas y microorganismos, así como los mecanismos de acción bacterianos, y utilizar dichos microorganismos de manera adecuada y efectiva a gran escala en los sistemas de agricultura integrada (Berg 2009).

Los microorganismos rizosféricos se encuentran asociados con los ciclos biogeoquímicos de los nutrientes (Barea et al. 2005), y la interacción planta-bacteria es esencial para una mejor nutrición vegetal (Ryan et al. 2009). Además, la homeostasis nutricional y hormonal de

la plantas se encuentran estrechamente relacionadas regulando finamente el crecimiento y desarrollo de la planta (Krouk et al. 2011). Las bacterias promotoras del crecimiento vegetal (PGPB) pueden actuar bien directamente o indirectamente (Ortíz-Castro et al. 2009) y existen varios mecanismos implicados en la modulación de los niveles de etileno. El etileno es una hormona vegetal inducida típicamente en respuesta a estreses ambientales como la sequía (Pierik et al. 2007). Algunas cepas de PGPB han sido definidas como reguladores del estrés (Lugtenberg and Kamilova 2009), ya que contienen la actividad 1-aminociclopropano-1-carboxílico deaminasa (ACCd) y son capaces de reducir los niveles de etileno, y en consecuencia disminuir sus efectos inhibidores del crecimiento (Abeles et al. 1992; Glick 2014).

Recientemente las técnicas -ómicas han contribuido a esclarecer la interacción entre plantas y bacterias, pero se requerire más investigación ya que los mecanismos de acción de las PGPB son a menudo específicos de cada cepa bacteriana (Long et al. 2008) y se encuentran poco caracterizados (Pühler et al. 2004). Además, las raíces son el nicho bacteriano dónde tiene lugar la interacción directa entre plantas y bacterias (Benizri et al. 2001). Las aproximaciones transcriptómicas y proteómicas, así como la información metabólica, han contribuido con información valiosa para desentrañar la interacción planta-bacteria así como para predecir cambios fisiológicos (van de Mortel et al. 2012; Feussner and Polle 2015; Su et al. 2016).

La presente Tesis Doctoral persigue principalmente arrojar luz sobre la interacción entre dos bacterias promotoras del crecimiento vegetal y plantas de tomate en relación con la sensibilidad a etileno mediante el uso de diferentes metodologías con el objetivo de elucidar

los mecanismos de acción bacteriana. En consecuencia, el mutante insensible a etileno *never ripe* (*nr*) (incapaz de percibir etileno debido a una mutación en el receptor de etileno SlETR3) (Lanahan et al. 1994; Wilkinson et al. 1995), y su parental isogénico de tipo silvestre (wild-type; wt) de tomate (*Solanum lycopersicum*) cultivar Pearson fueron seleccionados para ser inoculados con cepas PGPB aisladas de suelos áridos de la zona sur de España: *Bacillus megaterium* (Bm) (Marulanda-Aguirre et al. 2008) y *Enterobacter* sp. (en adelante *Enterobacter* C7 (C7)). Debido a que la mayoría de los estudios sobre el papel del etileno en la actividad PGPB se han centrado en bacterias que son capaces de reducir los niveles de ACC (Glick 2014), este estudio pretende utilizar bacterias sin actividad ACC deaminasa o la capacidad de producir etileno para evitar cualquier perturbación directa del metabolismo del etileno de la planta.

Capítulo 1: La sensibilidad a etileno a través de ETR3 es esencial en la interacción de tomate con *Bacillus megaterium* pero no con *Enterobacter* C7.

El primer capítulo tiene como objetivo establecer si la percepción de etileno a través de SlETR3 es crítica para la inducción del crecimiento promovida por dos cepas PGPB diferentes y evaluar los efectos bacterianos en la emisión de etileno y la expresión génica en plantas de tomate adultas. Se cultivaon plantas *never ripe* y de tipo silvestre inoculadas con *B. megaterium* o *Enterobacter* C7 hasta el estadío adulto (10 semanas de edad; 8 semanas post-inoculación; inicio de la floración) bajo condiciones de buen riego y sequía con el objetivo de analizar la promoción del crecimiento así como los efectos de la

inoculación bacteriana sobre la producción de etileno, la expresión de genes relacionados con el etileno y los perfiles transcriptómicos de la raíz.

La inoculación de *Enterobacter* C7 promovió el crecimiento de las plantas independientemente de la sensibilidad a etileno, mientras que la actividad PGPB de *B. megaterium* fue observada únicamente en plantas de tipo silvestre. Además, ambas cepas PGPB disminuyeron la expresión de genes de biosíntesis de etileno dando lugar a la mitigación de los efectos de la sequía en plantas de tipo silvestre. Sin embargo, la insensibilidad a etileno comprometió la interacción con *B. megaterium*, el cual indujo la transcripción de genes de biosíntesis y respuesta a etileno causando un ligero estrés biótico (Timmusk and Wagner 1999). La inoculación de PGPB afectó los perfiles transcriptómicos dependiendo de la cepa bacteriana, el genotipo de la planta y la sequía alterando genes implicados en respuesta frente a estrés oxidativo y la acumulación de metabolitos además del estado hormonal y nutricional de la planta. Por ello, la sensibilidad a etileno se ha propuesto como determinante para la adecuada interacción entre PGPB y las plantas de tomate. *Enterobacter* C7 podría modular el metabolismo amino acídico independientemente de la percepción de etileno. No obstante, la mutación *never ripe* causa una interacción no completamente funcional con *B. megaterium*, produciéndose un mayor estrés oxidativo y la pérdida de la actividad PGPB. De ese modo, la percepción de etileno mediante el receptor SlETR3 es crucial para la actividad promotora del crecimiento de *B. megaterium*, y afecta sólo levemente a los efectos de *Enterobacter* C7.

Capítulo 2: La inoculación de cepas PGPB modifica características fotosintéticas y el perfil metabólico de la raíz además de los estados nutricionales y hormonales de la planta con una fuerte influencia de la sensibilidad a etileno.

El segundo capítulo tiene como objetivo establecer si la percepción de etileno es determinante para la interacción planta-bacteria y la inducción del crecimiento mediado por ambas cepas PGPB en plantas juveniles, así como evaluar los efectos fisiológicos de dichas cepas en plantas de tomate juveniles y adultas. Se cultivaron plantas *never ripe* y de tipo silvestre inoculadas con *B. megaterium* o *Enterobacter* C7 hasta los estadíos juvenil (6 semanas de edad; 4 semanas post-inoculación) y adulto cuando se evaluó la biomasa, conductancia estomática y características fotosintéticas además de los estados nutricionales, hormonales y metabólicos.

No se observó promoción del crecimiento en plantas juveniles. Sin embargo, la inoculación de Bm y C7 disminuyó y aumentó la tasa de crecimiento relativo en plantas *never ripe*, respectivamente. Además, la inoculación de PGPB afectó los parámetros fisiológicos medidos y el contenido en metabolitos de la raíz en plantas juveniles, mientras que la nutrición vegetal fue fuertemente alterada dependiendo de la sensibilidad a etileno en plantas en estadío adulto. La inoculación de *B. megaterium* mejoró la asimilación de carbono en plantas de tipo silvestre. Sin embargo, la insensibilidad a etileno comprometió la actividad PGPB de *B. megaterium* afectando a la eficiencia fotosintética, la nutrición vegetal y el contenido en azúcares de la raíz.

No obstante, la inoculación de *Enterobacter* C7 modificó el contenido en amino ácidos de la raíz además de la conductancia estomática y la nutrición vegetal. Por ello, la sensibilidad a etileno determina la interacción de las plantas con las PGPB y perjudica gravemente a la interacción de *B. megaterium* con las plantas de tomate dando lugar a modificaciones fisiológicas y la pérdida de la actividad promotora del crecimiento. En cambio, la inoculación de *Enterobacter* C7 estimuló el crecimiento de la planta independientemente de la percepción de etileno y podría mejorar la asimilación de nitrógeno en plantas insensibles a etileno.

Capítulo 3: La percepción de etileno determina el resultado de la interacción planta-bacteria dando lugar a una reestructuración de la nutrición de fósforo y el estado antioxidante de la planta.

El presente capítulo tiene como objetivo arrojar luz sobre la interacción planta-bacteria y los mecanismos de acción PGPB en relación con la percepción del etileno utilizando una aproximación proteómica. Se cultivaron plantas *never ripe* y de tipo silvestre inoculadas con *B. megaterium* o *Enterobacter* C7 hasta el estadío adulto para analizar la promoción del crecimiento así como los efectos de la inoculación bacteriana sobre los perfiles proteómicos microsomales de la raíz, los cuales pueden aportar información útil sobre procesos de interacción, señalización y transporte.

Los resultados de la promoción del crecimiento vegetal fueron acordes con los obtenidos en anteriores capítulos. Además, la

inoculación de PGPB afectó al perfil proteómico de una manera dependiente de la cepa bacteriana y la sensibilidad a etileno de la planta modificando niveles de proteínas de interacción y relacionadas con el estrés. Además, la inoculación bacteriana afectó el estado antioxidante y la capacidad de adquisición de fósforo de la planta. De hecho, se evaluaron el estado redox y la nutrición de fósforo y se realizó un ensayo con condiciones de bajo fósforo en base a los resultados proteómicos obtenidos. La inoculación de *B. megaterium* incrementó y disminuyó la capacidad antioxidante en plantas de tipo silvestre y *never ripe*, respectivamente, mientras que la inoculación de C7 aumentó el estrés oxidativo en ambos genotipos de plantas. Por ello, la percepción a etileno es esencial para el adecuado reconocimiento de *B. megaterium* y su promoción del crecimiento, la cual es mediada en parte por niveles elevados de glutatión reducido mejorando la capacidad antioxidante de la planta. En cambio, *Enterobacter* C7 es capaz de mejorar la nutrición de fósforo de la planta independientemente de la sensibilidad a etileno, modulando la respuesta de estrés inducida por el bajo fósforo disponible y manteniendo de esta forma las plantas en crecimiento.

Discusión General

La inoculación con PGPB modificó los pérfiles nutricionales, transcriptómicos, metabólicos y proteómicos de una manera específica de cada cepa PGPB inoculada y dependiente de la sensibilidad a etileno de la planta en concordancia con interacciones específicas entre plantas hospedadoras y cepas PGPB (Walker et al. 2011; Weston et al. 2012), así como de mecanismos de acción también específicos de cepa y dependientes de las condiciones de crecimiento de las plantas (Ryu et

al. 2005; Long et al. 2008). La inoculación de PGPB modificó directamente los metabolitos de la raíz incluyendo amino ácidos, azúcares y ácidos orgánicos como se había descrito anteriormente (Weston et al. 2012; Su et al. 2016), los cuales podrían intervenir en la promoción del crecimiento vegetal así como en la interacción planta-bacteria. Además, la insensibilidad a etileno causó mayores diferencias que la inoculación bacteriana ya que el etileno está involucrado en varios procesos importantes en la fisiología de la planta así como en la plasticidad fenotípica (Dugardeyn and Van Der Straeten 2008).

El presente estudio describe por primera vez un mecanismo dependiente de etileno en bacterias sin actividad ACC deaminasa. Ambas PGPB (Bm y C7) fueron capaces de colonizar el sistema radicular independientemente de la sensibilidad a etileno, lo cual es determinante para la interacción con las plantas (Benizri et al. 2001). Sin embargo, aunque la sensibilidad a etileno determina la interacción de las plantas con ambas cepas PGPB, se propone la percepción del etileno a través de SlETR3 como esencial para la promoción del crecimiento mediada por Bm pero no por C7. Además, la inoculación con PGPB mejoró su eficiencia de promoción del crecimiento en plantas de tipo silvestre bajo condiciones de sequía, sugiriendo la mitigación del estrés como previamente ha sido descrito con otras cepas PGPB (Aroca and Ruiz-Lozano 2009), y la mejora de la eficiencia en la utilización de recursos (Dodd and Ruiz-Lozano 2012).

El análisis hormonal vegetal mostró que la inoculación de C7 modula el contenido de ácido abscísico (ABA) en plantas *never ripe* juveniles suprimiendo la respuesta mediada por ácido salicílico (SA) y/o ácido jasmónico (JA)/etileno (Anderson et al. 2004; Sánchez-Vallet

et al. 2012). En cambio, los niveles de ABA endógeno podrían ser esenciales para la promoción del crecimiento mediada por Bm manteniendo la producción de etileno en niveles bajos (Porcel et al. 2014). Los mayores niveles de SA, JA y jasmónico-isoleucina bajo la inoculación de Bm en plantas *never ripe* respecto a plantas de tipo silvestre sugiere que Bm activa defensas en plantas *never ripe* (Browse 2009; Vlot et al. 2009). Por ello, los efectos bacterianos sobre los niveles hormonales descritos en plantas juveniles predisponen a las plantas al crecimiento futuro ya que existe un balance entre crecimiento y defensa, el cual implica una interferencia entre las diferentes hormonas vegetales (Karasov et al. 2017).

La inoculación de *B. megaterium* en plantas *never ripe* aumentó la expresión de genes relacionados con etileno apuntando a una producción local de etileno, cómo la descrita en la interacción de las plantas con bacterias patógenas (van Loon et al. 2006), y sugiriendo que la plantas *never ripe* podrían reconocer a Bm como un microorganismo tipo patogénico. Sin embargo, se observó una regulación negativa de la señalización del etileno (Tieman et al. 2000), y la inoculación de Bm produjo una respuesta de estrés en plantas *never ripe* probablemente debido a un fallo en el reconocimiento (Zamioudis and Pieterse 2012), pero sin desencadenar completamente mecanismos de defensa que causan una reducción en el crecimiento de la planta.

Actividad promotora del crecimiento vegetal de *B. megaterium*

Los análisis transcriptómicos y proteómicos mostraron que la inoculación de Bm en plantas de tipo silvestre podría favorecer la interacción planta-bacteria y mejorar la capacidad antioxidante. Sin emabergo, la insensibilidad a etileno daña la interacción de la planta con Bm percibiéndolo como un microorganismo patogénico que aumenta el estrés oxidativo y dando lugar a una asociación que no es completamente funcional pero sin desencadenar completamente una respuesta inmune. Además, el análisis de los metabolitos de la raíz sugiere que la inoculación de Bm principalmente modifica el metabolismo de azúcares aumentando la supresión de la fotosíntesis causada por los niveles endógenos de glucosa en plantas *never ripe* (Paul and Pellny 2003). Por otro lado, los niveles reducidos de ácido fumárico (necesario para la formación del biofilm (Yuan et al. 2015)) en raíces *never ripe* inoculadas con Bm, y la competencia por el hierro propuesta en la rizosfera (Pii et al. 2015), también apoyan el fallo en la interacción funcional. La percepción de PGPB como un ligero estrés biótico ha sido descrita anteriormente (Timmusk and Wagner 1999) viéndose involucrado el etileno en este proceso (Hontzeas et al. 2004). En conformidad con lo anterior, el presente estudio apunta a la sensibilidad a etileno como regulador de la interacción de las plantas con *B. megaterium*.

El análisis de metabolitos antioxidantes confirmó los resultados transcriptómicos y proteómicos mostrando que la inoculación de Bm aumentó los niveles de glutatión reducido (GSH) en plantas de tipo silvestre. Este mecanismo había sido descrito previamente por la

inoculación de *Sphingomonas sp.* LK11 en tomate (Halo et al. 2015). Por ello, la actividad PGPB de *B. megaterium* propuesta como dependiente de la percepción de etileno a través de SlETR3 es mediada por niveles altos de glutatión reducido afectando al estado redox celular, y en consecuencia, a la capacidad antioxidante necesaria para mejorar la tolerancia frente a estreses. Sin embargo, la interacción entre *B. megaterium* y plantas *never ripe* no fue completamente establecida causando estrés oxidativo en las plantas insensibles a etileno.

Actividad promotora del crecimiento vegetal de *Enterobacter* C7

Los análisis transcriptómicos y proteómicos mostraron que la sensibilidad a etileno también determina la interacción de las plantas con *Enterobacter* C7. La presencia de *Enterobacter* C7 en raíces de tipo silvestre causó un leve estrés, pero las plantas fueron capaces de reconocer a C7 minimizando la respuesta de defensa. Además, la inoculación de C7 en plantas *never ripe* podría mejorar la nutrición vegetal, contrarrestando los efectos de estrés, ya que las plantas *never ripe* atenúan la interacción con C7 modulando proteínas implicadas en su reconocimiento. La inoculación de C7 podría mejorar la eficiencia en el uso del nitrógeno y/o modular el metabolismo amino acídico independientemente de la sensibilidad a etileno, como se observó en los resultados de metabolitos y ha sido previamente descrito para varias PGPB (Mantelin and Touraine 2004; Carvalho et al. 2014). Además, el análisis de antioxidantes mostró que la inoculación de C7 aumenta el estrés oxidativo en ambos genotipos de planta a la vez que promueve el crecimiento vegetal indicando que el mecanismo de acción de

Enterobacter C7 es independiente de la percepción del etileno y el estrés oxidativo.

El análisis proteómico también sugirió que la nutrición de fósforo está implicada en el mecanismo de acción PGPB de C7 y que la percepción de etileno a través de SlETR3 determina la interacción planta-C7. La inoculación de C7 mejoró la nutrición de plantas de tipo silvestre y *never ripe* mediado por los transportadores de fosfato *SlPT1* y *SlPT2*, respectivamente, evitando la respuesta de estrés por bajo fósforo y manteniendo el crecimiento de las plantas (Hermans et al. 2006). Por ello, *Enterobacter* C7 es capaz de promover el crecimiento vegetal mejorando la nutrición de fósforo y sorteando la insensibilidad a etileno a través de SlETR3 modulando dos transportadores de fosfato diferentes.

Trabajo futuro

Aunque los efectos de la inoculación de PGPB sobre la fisiología de las plantas representan una información útil para la aplicación en los sistemas de cultivo, es necesaria más investigación para elucidar completamente los mecanismos de acción de *B. megaterium* y *Enterobacter* C7, así como abordar otros posibles mecanismos de acción que puedan mejorar simultáneamente el crecimiento de las plantas (Martínez-Viveros et al. 2010). También los mecanismos de acción descritos deberían ser corroborados bajo condiciones de sequía. Además, la asociación beneficiosa entre plantas y PGPB requiere reconocimiento mutuo (Zamioudis and Pieterse 2012), y por ello la investigación de las características y fisiología bacterianas podría

ayudar a esclarecer la interacción planta-bacteria. Por otra parte, el gen *TCTR1* (Tomato Constitutive Triple Response 1) está implicado en la regulación negativa del etileno (Tieman et al. 2000), y podría participar en la respuesta inicial después de la inoculación de Bm. Por ello, se están obteniendo líneas transgénicas de silenciamiento y sobreexpresión de *TCTR1* para evaluar el crecimiento de las plantas así como la interacción con *B. megaterium*.

La utilización de PGPB se encuentra pobremente representada en la agricultura mundial (Banerjee et al. 2006; Timmusk 2017), pero un consorcio de microorganismos beneficiosos en combinación con la planta adecuada bajo determinadas condiciones ambientales podrá producir efectos positivos y reales dando lugar a una alternativa viable para la intensificación sostenible de la agricultura.

Conclusiones

- La percepción del etileno a través de SlETR3 es esencial para la actividad promotora del crecimiento vegetal de *Bacillus megaterium* en plantas de tomate, mientras que el mecanismo PGPB de *Enterobacter* C7 parecer ser independiente de SlETR3.

- Los efectos de la inoculación de PGPB sobre la fisiología vegetal son específicos de la cepa bacteriana y dependientes de la sensibilidad a etileno así como de las condiciones de crecimiento de la planta.

- La inoculación con PGPB afecta a la fotosíntesis, fitohormonas y metabolitos de la raíz en plantas juveniles predisponiendo a la plantas para el futuro crecimiento.

- Ambas cepas bacterianas actúan como PGPB en condiciones de buen riego y sequía en plantas de tomate sensible al etileno dando lugar a una mejora en el estado fisiológico de la planta y mitigación del estrés.

- La mutación de SlETR3 perjudica la interacción entre *Bacillus megaterium* y plantas de tomate *never ripe*, resultando en un reconocimiento no completamente funcional y causando un incremento del estrés oxidativo y la pérdida de la actividad promotora del crecimiento.

- La actividad PGPB de *Bacillus megaterium* en plantas de tomate podría estar mediada por niveles altos de glutatión reducido, y por tanto por una mejora del estado antioxidante vegetal.

- La mutación de SlETR3 determina la interacción de las plantas con *Enterobacter* C7, cuyo mecanismo PGPB implica la mejora de la nutrición de fósforo mediada por los transportadores de fosfato *SlPT1* y *SlPT2* en plantas de tipo silvestre y *never ripe*, respectivamente, evitando así la respuesta de estrés por bajo fósforo.

Introduction

Introduction

Study context

Global population is exponentially increasing and probably worldwide inhabitants will be about 9 billion by 2050 (FAO 2013). Agriculture is a key factor to provide food security (Pardey et al. 2014). Food consumption patterns are quantitatively and qualitatively varying towards diets with more food and meat. Thus, there is a increased competition for inputs, which are often overexploited, in short supply and/or used unsustainably, such as arable land and water as well as other inputs needed for food production as energy and nutrients (Foley et al. 2005; FAO 2013). From local farming communities to countries and worldwide, sustainability is a prerequisite to achieve human development (Folke et al. 2005; UN 2012).

The intensive agriculture demands for inputs which can negatively impact on environment. The intensification of agriculture, which started in the 1960s designated as "The Green revolution", was mainly based on management of crop lands with high-yielding crop varieties, chemical fertilizers and pesticides, irrigation, and mechanization (Naylor 1996). Although fertilizers provide essential nutrients to meet plant growth and development, chemical fertilization has been generally overused causing unexpected environmental impacts. For instance, nutrient washing from fertilized farms caused oxygen starvation in rivers and lakes, leading to an almost lifeless area called "the dead zone" (Malakoff 1998; Rabalais et al. 2002). Pesticides also contaminate soil and water affecting to non-target beneficial organisms including humans (Hallberg 1987; Aktar et al.

2009), and thereby diminishing biodiversity and/or soil health (Giller et al. 1997; Kibblewhite et al. 2008; Scherr et al. 2008). Moreover, performed crop practices usually resulted in soil degradation, which is a global problem especially serious in the tropics and sub-tropics (Lamb et al. 2005). In fact, the dry land surface becoming semi-arid or arid is progressively increasing during last decades (Herrmann and Hutchinson 2005), and thereby crops suffer drought periods and demands for higher water inputs. Moreover, a large amount of solid residues and plastic waste was generally produced by agriculture (Hemphill 1993), and these debris should be reduced. In addition, agriculture approximately produces a quarter of anthropogenic greenhouse gas emissions involved in climate change, which also reciprocally implies extra challenges to agriculture (Vermeulen et al. 2012). In consequence, it could be said that intensive farming practices damage the environment causing global problems.

Thus, new methodologies which can counteract negative impacts of intensive agriculture and provide food security to a growing up global population should be developed. The aim of sustainable intensification of agriculture is to simultaneously increase food production and minimize pressure of crops on the environment (Tilman et al. 2011; Garnett et al. 2013). Food production should be enhanced using existing farmlands in an environmentally-friendly way in order to do not undermine further future crop production and avoid problems caused by non-sustainable intensification (Foley et al. 2005; Bennett 2014). The new farming practices should improve efficiency use for nutrients and water without sacrificing actual yields (Ghanem et al. 2011; Dodd and Ruiz-Lozano 2012). In addition, sustainable intensification of agriculture implies a radical rethinking in systems of

food production in order to increase human and animal nutrition and welfare, as well as support rural economies and sustainable development (Garnett et al. 2013).

A plethora of soil microorganisms can establish association with plants (Gray and Smith 2005), and beneficial ones are able to stimulate plant growth (Lucy et al. 2004; Adesemoye and Kloepper 2009; Nadeem et al. 2014). These microorganisms modulate plant growth and physiology acting generally from roots (Barea et al. 2005) and some of them can even enhance plant tolerance to stresses (Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009; Glick 2014). Moreover, certain soil microorganisms can be used as biofertilizers, phytostimulators as well as biopesticides, and thereby they were pointed as an interesting way to reduce or even replace use of chemicals in agriculture (Bhattacharyya and Jha 2012). In consequence, the management of microbial populations was proposed as a cheap, versatile, and environmentally-friendly method to simultaneously enhance plant growth and reduce crop negative impacts on environment (Berg 2009; Singh et al. 2011).

Plant growth and development

Total growth of plant as biomass production results from conjunction of fixated carbon dioxide (CO_2) by photosynthesis, carbon loss by respiration processes, and mineral nutrition (Poorter 2002). Furthermore, cell expansion, which is produced by turgor pressure in response to the osmotic influx of water (Lodish et al. 2000), plays a determinant role at cellular level since cell growth determine organ

growth and morphological refinements which optimize plant growth depending on external and/or internal stimuli (Bashline et al. 2014).

Plant photosynthesis starts in chloroplast thylakoid membrane, which is enzymatically able to oxidize water dependently of light, reduce NADP and produce ATP via photosystems I and II (PSI and PSII) and ATP synthase (F-ATPase) with the cytochrome-b6 f complex transporting electrons between PSII and PSI (Nelson and Ben-Shem 2004). Chlorophylls are magnesium-tetrapyrrole molecules essential in photosynthesis. Several types of chlorophyll have been described due to substitutions on the side chains of chlorophyll a, resulting in different absorption properties to harvest sunlight at different wavelengths (Chen 2014). Chlorophylls and other pigments are contained in both photosystems and harvest light initiating the electron translocation from pigments to electron acceptors in order to provide energy for the photosynthetic process (Nelson and Ben-Shem 2004). Indeed, photosynthesis is related with chlorophyll content (Richardson et al. 2002), although the photosynthetic process is also influenced by other factors such as opening of stomata (Tanaka et al. 2005) and mesophyll conductance to CO_2 (Galmés et al. 2013; Tomás et al. 2013) as well as Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) catalytic properties (Prins et al. 2016) and other rate-limiting Calvin cycle enzymes (Zhu et al. 2007).

Photosynthesis is also affected by CO_2 concentration. CO_2 is fixed by Rubisco, which catalyzes the combination of ribulose-1,5-bisphosphate with CO_2 to yield two molecules of 3-phosphoglyceric acid. However, Rubisco also reacts with oxygen leading to photorespiration and decreasing photosynthesis efficiency (Cleland et

al. 1998). CO_2 is sensed by guard cells of stomata, which can open or close in response to CO_2 levels (Mott 1988; Assmann 1999). Abscisic acid (ABA) induces stomata closure, while ethylene can modify photosynthesis by inhibiting ABA-induced stomatal closure with a dose-dependent mechanism (Tanaka et al. 2005). Moreover, stomatal opening is induced by light (Assmann 1999). Several messengers were reported in stomatal response to CO_2 (such as apoplastic and cytosolic pH gradients, free cytosolic calcium and membrane potential), which overlap with stomatal response to ABA and light suggesting that guard cell signaling is organized as a complex network and multiple components regulate stomatal response to environmental stimuli (Hetherington and Woodward 2003; Roelfsema et al. 2006; Munemasa et al. 2015).

Additionally, plant growth and development require integration of many external and internal stimuli which in combination with the plant genetic program determine the plant phenotype. Plant hormones or phytohormones are growth regulators, which act at low concentrations, fundamental to finely orchestrate plant physiology (Gray 2004). Phytohormones include auxins, cytokinins, ethylene, ABA, gibberelins (GAs), jasmonates (JAs), brassinosteroids (BRs), salicylic acid (SA), and strigolactones. Furthermore, several cross-talk processes between phytohormones have been reported during plant development and response to environmental stimuli (Munné-Bosch and Müller 2013). As the present thesis is focused in ethylene, the functions of the other phytohormones are slightly described reporting only their main effects.

Auxins were found in plants, and indolacetic acid (IAA) is the most common and the most studied. Indeed, literature considers auxin and IAA as interchangeable terms. IAA plays crucial functions in several developmental processes such as gametogenesis, embryogenesis, seedling growth and flower development, being identified as a plant growth hormone. IAA affects plant cell division, extension and differentiation, initiates lateral and adventitious root formation and increase resistance to stress among other functions (Zhao 2010; Spaepen and Vanderleyden 2011)

Cytokinins trigger cell differentiation and thereby they are essential in several aspects of plant growth and development such as embryogenesis, vascular development and maintenance of meristems in roots and shoots as well as in response to environmental stimuli modulating root elongation, lateral root number, nodule formation, and apical dominance (Osugi and Sakakibara 2015).

Abscisic acid was firstly described as growth inhibitor. Despite of its name, ABA induces abscission zone formation but it does not control directly abscission. Abscission is controlled by auxins which control the ethylene sensitivity of abscission zone (Abeles et al. 1992; Al-Khalifah and Alderson 1999). ABA regulates several processes in plant growth and development such as cell division and elongation, embryo maturation, seed dormancy, germination, stomatal aperture, floral induction, and responses to environmental stresses such as cold, drought, salinity, UV radiation, and pathogen attack (Finkelstein 2013). Furthermore, endogenous ABA is determinant in limiting production of ethylene maintaining rather than reducing plant growth (Sharp 2002).

Gibberellins are tetracyclic diterpenoid molecules which stimulate determinant processes of plant growth and development including seed germination, stem elongation, leaf expansion, trichome development, pollen maduration and flowering (Achard and Genschik 2009).

Jasmonates are oxylipins which regulate several aspects of plant biology that range from stress responses to development being jasmonoyl-isoleucine (JA-Ile) a significant active form. Jasmonates are involved in carbon partitioning, reproductive development and senescence in healthy tissues as well as in environmental responses including defense against microbial pathogens (specially necrotrophic) and insects (herbivores), and responses to abiotic stresses such as UV radiation, drought, and ozone among others (Browse 2009). They also regulate stomatal aperture (Munemasa et al. 2011) and root water uptake (Sánchez-Romera et al. 2014).

Brassinosteroids are polyhydroxylated steroidal molecules which participate in several processes of plant growth and development including cellular expansion and proliferation, morphogenesis, differentiation of vascular tissues, development of leaves, male fertility, and time of senescence as well as improve tolerance to various stresses such as heat, salinity, drought and heavy metals (Fariduddin et al. 2014).

Salicylic acid is a phenolic compound mainly involved in plant immune response. SA is important in defense signaling pathways, which induced systemic acquired resistance (SAR) protecting plant form a wide spectrum of pathogen in a long term (Vlot et al. 2009). Furthermore, SA also plays a role in plant response to abiotic stresses

as well as in regulation of physiological and biochemical processes such as germination, flowering, photosynthesis, redox status and senescence (Rivas-San Vicente and Plasencia 2011).

Strigolactones are carotenoid derived molecules which inhibits branching in shoot tissues, stimulate symbiosis establishment with mycorrhizae and also trigger the germination of parasitic plants seeds (Gomez-Roldan et al. 2008).

Ethylene

Ethylene is a gaseous phytohormone with several roles in plant growth and development. The ethylene biosynthesis and response pathways are key players in nodulation in symbiotic nitrogen fixation (Goormachtig et al. 2004), defense against pathogens (Glazebrook 2005), regulation of flowering, fruit ripening and senescence (Abeles et al. 1992), plant architecture and regulation of the phenotypic plasticity in an environment changing continuously (Dugardeyn and Van Der Straeten 2008). Indeed, ethylene is a key player involved in response to environmental stresses such as nutritional stresses (Iqbal et al. 2013), drought (Pan et al. 2012), salinity (Tao et al. 2015), flooding response (Hattori et al. 2009), and oxidative stress (Asgher et al. 2014) among others.

Ethylene is typically reported as growth inhibitor (Abeles et al. 1992). The triple response to ethylene (inhibition of root and hypocotyls elongation, thickened hypocotyls and exaggerated apical hook formation) was firstly discovered in pea (*Pisum sativum*) seedlings growing in dark conditions, and then also described in

Arabidopsis (Fig. I1) (Guzman and Ecker 1990). Nevertheless, ethylene induction of plant growth was also reported such as strong shoot elongation of semi-aquatic plants (Vosenek and Van der Veen 1994).

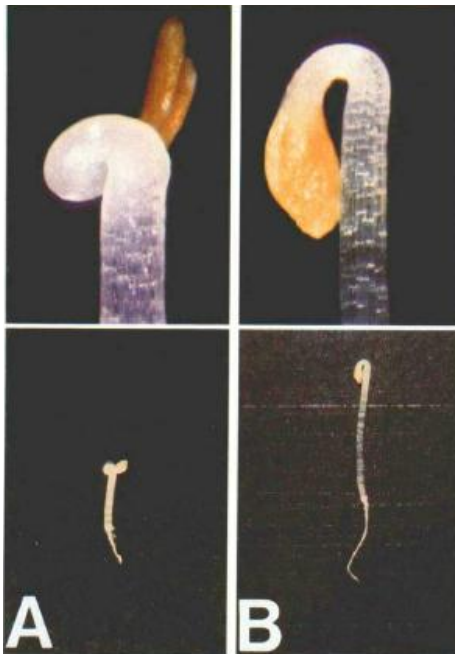

Figure I1. Morphological features of the triple response in wild type arabidopsis. Each panel is composed of two photomicrographs of an etiolated arabidopsis seedling; the upper part shows the apical region of the hypocotyl and the lower part shows the complete seedling. **(A)** Wild type displaying the triple response in the presence of 10 ml l^{-1} ethylene. **(B)** Wild type incubated without ethylene. Figure taken and adapted from Guzman and Ecker (1990).

The triple response to ethylene in dark-grown seedlings was very useful to identify ethylene insensitive mutants (Bleecker et al. 1988; Guzman and Ecker 1990; Kieber et al. 1993; Roman et al. 1995). Growth inhibitory effects mediated by ethylene were reported in several species in roots (Visser et al. 1997; Pierik et al. 1999; Swarup et al. 2007; Negi et al. 2010; Ma et al. 2014) and shoots (Smalle and Straeten 1997; Knoester et al. 1997; Fiebig and Dodd 2016). Moreover,

constitutive ethylene signaling mutants shows dwarf phenotypes with unexpanded and severely reduced cell growth (Kieber et al. 1993). Meanwhile, ethylene-insensitive mutants were considerably larger than wild type plants with larger expanded leaves resulting from enhanced cell growth (Bleecker et al. 1988; Hua et al. 1995).

On the other hand, growth stimulation of hypocotyls mediated by ethylene was reported in arabidopsis seedlings growing in light conditions. Shortage of nutrients enhanced seedling growth stimulation pointing to ethylene response is dependent on external conditions (Smalle and Straeten 1997). Moreover, light quality can also influence on ethylene responses (Pierik et al. 2004). Ethylene is able to stimulate plant growth at relatively low concentrations in several species (Smalle and Straeten 1997; Suge and Nishizawa 1997; Pierik et al. 2003). Furthermore, growth stimulation was also reported at high ethylene levels in other plant species that generally live in often flooded habitats (Voesenek et al. 1997; Kende et al. 1998; Voesenek and Sasidharan 2013).

Growth inhibition and induction produced by ethylene suggested an action mechanism with low and higher ethylene levels promoting and inhibiting plant growth, respectively, so a biphasic model was proposed (Lee and Reid 1997). The ethylene concentration required for growth stimulation or inhibition depends on integrative result of internal and external stimuli, and specific traits of plant species related in principle with their habitat (Pierik et al. 2006). One extreme in the biphasic model would be represented by aquatic and semi-aquatic plants, which showed growth promotion even at high ethylene concentrations, while the other extreme would be represented by plants

showing only inhibitory growth effects mediated by ethylene (Pierik et al. 2006). The ethylene response curves to ethylene dose usually show two phases (Fig. I2) (Lee and Reid 1997; Suge and Nishizawa 1997; Hua and Meyerowitz 1998; Fiorani et al. 2002).

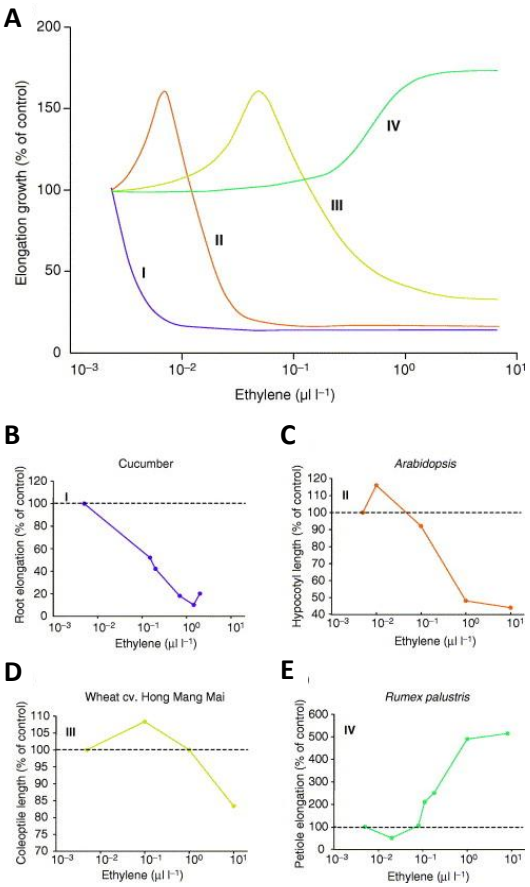

Figure I2. Ethylene biphasic response model. (A) Hypothetical dose-response curves might be shifted along the x-axis because of environmental conditions, species-specific characteristics and internal signals. Curves I–IV show variation in ethylene dose-response relationships, which are illustrated in (B-E) with examples of published data on different species and traits. Control values are set at 5×10^{-3} µl l^{-1} ethylene as the ambient ethylene concentration, but this control concentration was even lower in (C) and (D) as ethylene was experimentally removed from the air. **(B)** Root elongation in cucumber; **(C)** Hypocotyl length in dark-grown arabidopsis seedlings; **(D)** Coleoptile length in the wheat Hong Mang Mai cultivar; **(E)** Petiole elongation in Rumex palustris. Figure taken from Pierik *et al.* (2006).

Ethylene biosynthesis

Ethylene is biosynthesized from methionine via S-adenosylmethionine (SAM) and 1-aminocyclopropane1-carboxylate (ACC), which is the ethylene immediate precursor (Fig. I3). Methionine plays several physiological roles in plants including ethylene biosynthesis, sulfation, protein biosynthesis and methylation of proteins and nucleic acids. A recycling mechanism to maintain methionine pool was described in plants and it is called Yang cycle (Baur and Yang 1972).

SAM was converted to ACC releasing 5'-methylthioadenosine (MTA), which is subsequently recycled to methionine allowing continuously ethylene production without depleting the methionine pool (Miyazaki and Yang 1987). This reaction is catalyzed by ACC synthase (*ACS*) (Boller et al. 1979) and ACC oxidase (*ACO*) resulting in ethylene, carbon dioxide and cyanide (Yang and Hoffman 1984; Kende 1993), although ACC can be also conjugated in several forms such as malonyl-ACC, γ-glutamyl-ACC and jasmonyl-ACC (Fig. I3) (Van de Poel and Van Der Straeten 2014). In tomato, *ACS* and *ACO* genes were encoded by two gene families and at least nine ACS and six ACO isoforms have been described (Barry et al. 1996; Blume and Grierson 1997; Nakatsuka et al. 1998; Jiang and Fu 2000; Alexander and Grierson 2002; Sell and Hehl 2005).

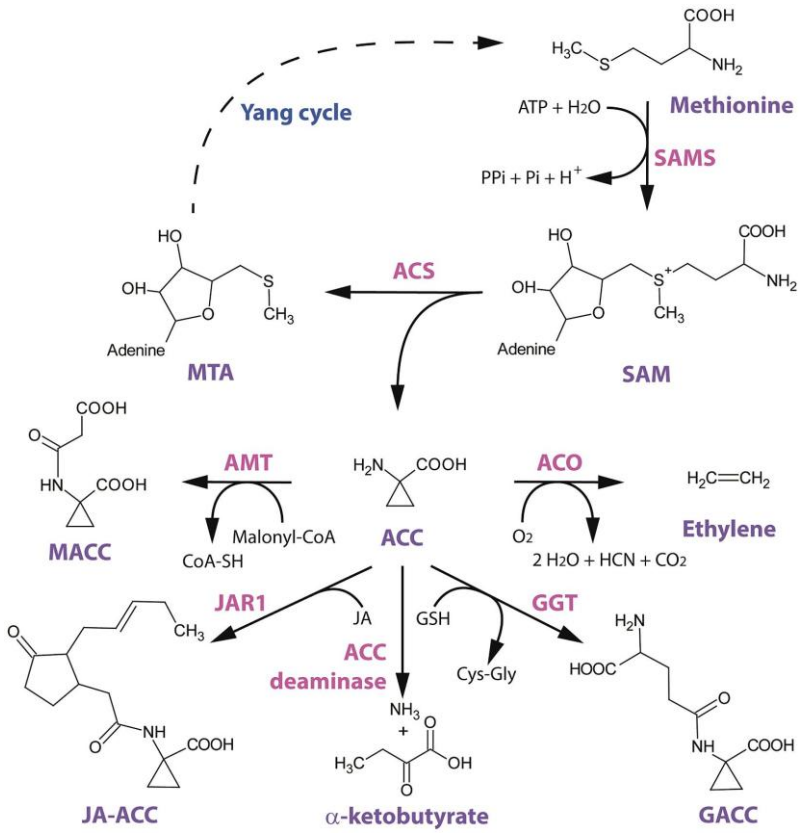

Figure I3. Structural scheme of ethylene biosynthesis and 1-aminocyclopropane-1-carboxylic acid (ACC) conjugation/metabolism. The amino acid methionine is converted to S-adenosyl-L-methionine (SAM) by SAM-synthetase (SAMS) with the requirement of ATP. The general precursor SAM is then converted to ACC by ACC-synthase (ACS). This reaction also involves the cleavage of 5′-methylthioadenosine (MTA), which is recycled back to methionine by the Yang cycle (dotted line indicates multiple enzymatic steps). ACC can be converted to ethylene by ACC-oxidase (ACO) in the presence of oxygen. ACC can also be converted to its major conjugate 1-malonyl-ACC (MACC) by the yet uncharacterized ACC-N-malonyl transferase (AMT) with the requirement of malonyl-Coenzyme-A. A second derivate of ACC is γ-glutamyl-ACC (GACC) which is formed by γ-glutamyl-transpeptidase (GGT) with the requirement of glutathione (GSH). Another novel derivate of ACC is jasmonyl-ACC (JA-ACC), which is formed by jasmonic acid resistance 1 (JAR1). ACC can also be metabolized by the bacterial (and plant) ACC deaminase into ammonium and α-ketobutyrate. Figure taken from Van de Poel and Van Der Straeten (2014).

The limiting step for ethylene biosynthesis is mainly ACS (Wang et al. 2002), but recent studies suggest that ACO could be the rate-limiting enzyme under particular conditions as low oxygen concentration (Dorling and McManus 2012). Throughout plant growth and development, *ACS* genes are expressed with cell- and tissue-specific patterns differentially regulated in function of developmental stage and in response to internal and external stimuli, in order to finely control ethylene production (Tsuchisaka et al. 2009; Dorling and McManus 2012). Furthermore, two ethylene regulatory systems were proposed in case of tomato. Basal ethylene levels were negatively feedback regulated during vegetative growth with involvement of *ACS1* and *ACS6* genes, while high ethylene levels were positively feedback regulated during fruit ripening with involvement of *ACS2* and *ACS4* genes (Barry et al. 2000; Alexander and Grierson 2002; Alba et al. 2005).

Additionally, previous studies correlated spatiotemporal *ACS* expression with ethylene production (Zarembinski and Theologis 1994; Wang et al. 2002; Sobeih et al. 2004). Nevertheless, *ACS* genes present a strong post-transcriptional regulation by phosphorylation processes (McClellan and Chang 2008; Lyzenga et al. 2012; Xu and Zhang 2014), and thereby expression patterns of *ACS* genes were not always in accordance with ethylene production.

Ethylene transduction pathway

The first step in ethylene signal transduction is the binding of ethylene to its receptors. Ethylene receptors are predominantly localized into the endoplasmic reticulum (ER), since ethylene is a

lipophilic molecule and freely diffuse up to endomembrane-located receptors (Grefen et al. 2008). Moreover, this localization might be involved in interactions with other cellular components and/or signal integration with other pathways (Ju and Chang 2012). Ethylene signal transduction is triggered when ethylene bind to its receptors via a copper cofactor (Rodríguez 1999; Woeste and Kieber 2000), diminishing the activity of a serine/threonine kinase, called CTR1 (constitutive triple response 1) which inhibits further signaling (Kieber et al. 1993; Clark et al. 1998) by ethylene-insensitive protein 2 (EIN2) and EIN3 (Chao et al. 1997; Alonso et al. 1999). Ethylene perception finally results in transcriptional changes denominated as ethylene response (Ju and Chang 2012; Vandenbussche et al. 2012; Merchante et al. 2013). The transduction pathway of ethylene is almost completely described in *Arabidopsis thaliana* since it is a model plant in basic research. Thus, ethylene transduction pathway is described in the present thesis mainly based in research performed in arabidopsis, but also the main differences found in tomato plants were detailed.

Ethylene receptors

In *Arabidopsis*, ethylene is perceived trough a family of receptors with similarity to bacterial two-component regulators called ethylene receptor 1 and 2 (ETR1, ETR2 respectively), ethylene sensor 1 and 2 (ERS1, ERS2 respectively), and ethylene insensitive 4 (EIN4) (Bleecker et al. 1988; Hua et al. 1995; Hua et al. 1998; Sakai et al. 1998). By sequence comparisons, these receptors have been classified into two subfamilies: subfamily I consisting of ETR1 and ERS1 and subfamily II consisting of ETR2, EIN4 and ESR2. All ethylene receptors present a similar N-terminal domain and a histidine kinase-

like domain, while only subfamily II presents additional amino acids which could act as a signal peptide or form an additional transmembrane helix (Fig. I4).

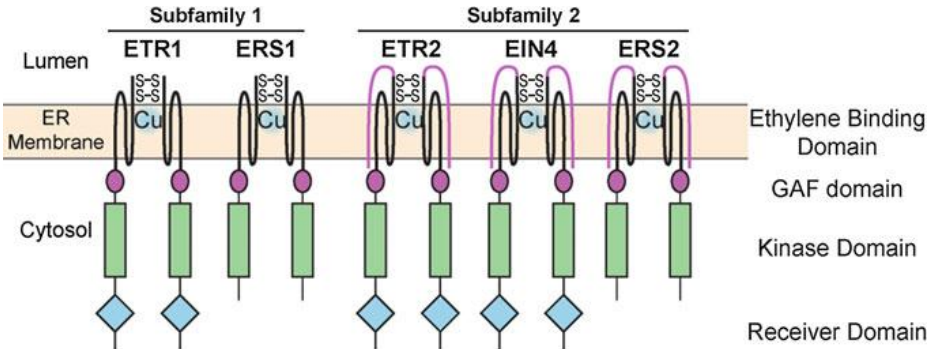

Figure I4. The domain structures of the ethylene receptors from *Arabidopsis thaliana*. Each receptor contains an ethylene binding, GAF, and kinase domains as shown. Three of the five also contain a receiver domain. ETR1 and ERS1 are in subfamily 1 and subfamily 2 includes ETR2, EIN4, and ERS2. Subfamily 2 receptors are characterized by additional amino acids at the N-terminus that may form a fourth transmembrane helix or act as a signal peptide. Figure taken from Wilson *et al.* (2015).

In tomato, a family of six ethylene receptors was found (*SlETR1-6*). They were also classified in two subfamilies according to its structure in subfamily I (*SlETR1-3*) and subfamily II (*SlETR4-6*) (Wilkinson et al. 1995; Lashbrook et al. 1998; Tieman and Klee 1999). Subfamily I contains all the essential residues for histidine kinase function, while subfamily II lack some kinase residues that are conserved in others. SlETR3 was also named Never Ripe (NR) since its mutation result in an easily recognized phenotype characterized by fruit inability to undergo ripening, delayed flower, leaf senescence and pedicel abscission, although some residual responsiveness is conserved.

Moreover, SlETR3 corresponds to ETR1 of *Arabidopsis thaliana* (Lanahan et al. 1994; Wilkinson et al. 1995).

In Arabidopsis, functional redundancy was proposed for ethylene receptors because a single receptor loss of function does not have a major effect upon ethylene signaling. However, reduction in either *SlETR4* or *SlETR6* mRNA levels produces hypersensitivity to ethylene in tomato (Tieman et al. 2000). Reduction of *SlETR3* expression by transgenic approach (antisense strategy) produced a proportionally increase in expression of *SlETR4* suggesting that tomato plants compensate for the loss of function of *SlETR3* by increasing *SlETR4* expression, while *SlETR3* overexpression in lines with decreased *SlETR4* gene expression remove the ethylene-sensitive phenotype, pointing to these ethylene receptors are functionally redundant, despite pronounced structural differences (Tieman et al. 2000; Kevany et al. 2007). Thus, functional redundancy was noticed in arabidopsis, while functional compensation and redundancy were observed for some tomato ethylene receptors.

In addition to ethylene receptors, several described genes are essential for the proper function of these receptors. The first identified gene was RAN1 (responsive to antagonist 1), a cooper transporter required for ethylene receptor biogenesis (Hirayama et al. 1999; Woeste and Kieber 2000). Another ethylene receptor regulator is RTE1 (reversion to ethylene sensitivity 1), whose mutation suppressed the weak insensitivity to ethylene in etr1 mutant in arabidopsis (Resnick et al. 2006), probably because RTE1 is involved in activation of ETR1 by conformational changes (Resnick et al. 2008), and needed for signaling of the N-terminal domain of ETR1 (Qiu et al. 2012). In tomato, the

green-ripe (gr) mutant also shows a dominant defect in fruit ripening with ethylene insensitivity or slight decrease in sensitivity to ethylene. Green ripe (GR) and green ripe-like proteins (GRL1 and GRL2) are homolog to RTE1 (Barry and Giovannoni 2006; Ma et al. 2012). Furthermore, cytochrome b5 (Cytb5) was identified as RTE1-interacting protein suggesting that Cytb5 could regulate ETR1 oxidative folding via RTE1 (Chang et al. 2014) (Fig I5).

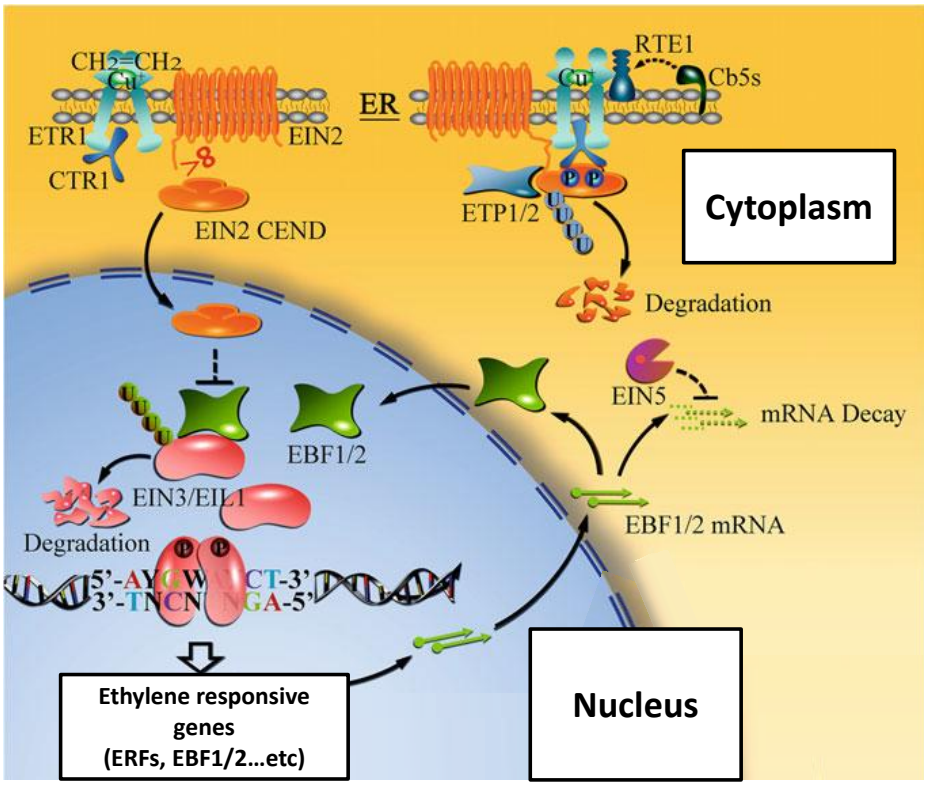

Figure I5. Ethylene signaling cascade. Figure taken and adapated from Wen *et al.* (2015).

Ethylene signaling

In arabidopsis, only one CTR1 gene constitutively expressed has been found (Kieber et al. 1993). The ethylene-insensitive phenotype of the ETR1 family members was suppressed by ctr1 mutation (Bleecker et al. 1988; Kieber et al. 1993; Hua et al. 1998; Sakai et al. 1998). In addition, direct interaction between CTR1 and ETR1 was reported pointing to CTR1 direct regulation by ethylene receptors (Clark et al. 1998).

However, four *CTR1*-like genes (*SlCTR1-4*) were reported in tomato (Leclercq et al. 2002; Adams-Phillips et al. 2004). All of them show sequence conservation of the CN motif on the N-terminal regions, which is important for interaction with ethylene receptors (Huang et al. 2003), and SlCTR1, SlCTR3 and SlCTR4 are able to restore ethylene transduction signal in arabidopsis (Adams-Phillips et al. 2004). All ethylene receptors could activate CTR1 in arabidopsis in absence of ethylene to suppress downstream responses, while the multiple ethylene receptors and CTRs are differentially regulated in response to stimuli and during development in tomato. Thus, possible specific interactions between tomato receptors and CTRs can regulate different ethylene responses (Zhong et al. 2008).

In addition, the phenotype of ctr1 mutant was suppressed by ein2 and ein3 mutations (Roman et al. 1995). EIN2 is the next step in ethylene signaling pathway (Fig I5) (Alonso et al. 1999). EIN2 N-terminal domain shows similarity to the metal ion transporters Nramp family, while C-terminal domain overexpression caused a constitutive ethylene response. Full-length expression does not produce this constitutive phenotype pointing to regulation role for N-terminal

domain and ethylene response activation function for C-terminal domain (Alonso et al. 1999). In fact, EIN2 stability is regulated via 26S-proteasome by two F-box proteins called EIN2-targeting protein 1 (ETP1) and 2 (ETP2) (Fig I5). Ethylene down-regulates ETP1/2 expression for further signaling, so their over-expression caused ethylene insensitivity (Qiao et al. 2009).

The next signaling protein involved in ethylene transduction pathway is EIN3 (Roman et al. 1995; Chao et al. 1997). After EIN2 is processed and activated, the C-terminal domain is transported to the nucleus activating EIN3, which acts a transcriptional regulator of ethylene response (Fig I5) (Chao et al. 1997). Furthermore, three EIN3-like genes (EIL1-3) were also identified in arabidopsis and EIL1 and EIL2 rescue ethylene insensitivity in ein3 mutant. EIN3 and EIL1 showed enough capacity to induce ethylene responses (Chao et al. 1997). Furthermore, the level of EIN3 protein is regulated by proteolysis via 26-proteasome pathway, and two ethylene-induced F-box proteins (EIN3-binding F-box1 (EBF1) and F-box2 (EBF2)) directly interact with EIN3 in order to mediate protein degradation (Fig I5). Ebf1 and ebf2 mutants showed hypersensitivity to ethylene, while the double mutant showed constitutive ethylene response pointing to their function as negative regulators of ethylene signaling via EIN3 and EIL1 degradation (Guo and Ecker 2003; Potuschak et al. 2003).

In addition, EIN5 gene encodes a 5'-3' exoribonuclease XRN4, which mutation affected expression of several genes including EBF1/2 (Fig I5). Thus, EIN5 function as EIN3 positive regulator by negative regulation of EBF1/2 (Olmedo et al. 2006; Potuschak et al. 2006). EIN3 can directly target the promoter of ethylene response factors

(ERFs) inducing the ethylene-response (Solano et al. 1998), which includes a transcriptional cascade which leads to differentially expression of hundreds of genes (An et al. 2010).

Ethylene response

ERFs are trans-acting factors which specifically promoters of ethylene-responsive genes (Solano et al. 1998). The ERF family belongs to the superfamily containing the apetala 2 (AP2) domain. The ERF family is composed by 122 genes in arabidopsis (Nakano et al. 2006) and 155 in *Solanum* spp. (Charfeddine et al. 2014). Additionally, the ERF type family is formed by two subfamilies: the dehydration-responsive element-binding proteins (DREB) and ethylene responsive factor (ERF) (Riechmann et al. 2000; Sakuma et al. 2002).

The ERF family is involved in multiple responses in plants such as developmental processes (Banno et al. 2001; Pirrello et al. 2006), phytohormone signaling pathways (Müller and Munné-Bosch 2015), and regulation of metabolic pathways (Broun et al. 2004; Zhang et al. 2005). ERF proteins expression results in tolerance to biotic stresses via GCC box elements, while DREB proteins were involved in tolerance to abiotic stresses such as dehydration, cold and salt stress via DRE elements (Gu et al. 2000; Guo and Ecker 2004; Huang et al. 2008). However, ERF-type bound to promoters without GCC box was also described in tomato (Chakravarthy et al. 2003), as well as ERF-type binding to vascular wounding responsive elements (VWRE) (Sasaki et al. 2007). The ERF proteins activation dependent on different stimuli or differential binding activity as well as signaling pathways crosstalk processes could simultaneously mediate regulation of multiple

responses by ERF proteins (Phukan et al. 2017). In case of tomato, ERF proteins selectively bind to GCC-box elements depending on their flanking regions displaying a specific tissue patterns enabling finely ethylene regulation of a broad range of physiological processes (Pirrello et al. 2012).

Plant nutrition

Several environmental factors are required for optimal plant growth and development including the mineral nutrients in order to meet metabolic demands. Nutrients availability is often limited in soils, and thereby several plant responses have been developed affecting the whole plant morphology and metabolism in order to cope with low availability of nutrients (López-Bucio et al. 2002). A wide range of transport proteins regulate nutrient acquisition in root cells and translocation within the plant. Moreover, nutrient bioavailability determines transporter gene expression at transcriptional and post-transcriptional level, and transporter activity is usually controlled by post-translational modifications to maintain nutrient homeostasis (Aibara and Miwa 2014). Several nutrients are acquired by transporters located in the plasma membrane and induced under limited nutritional conditions such as nitrogen (Lezhneva et al. 2014), phosphorus (Raghothama and Karthikeyan 2005), potassium (Caballero et al. 2012), magnesium (Mao et al. 2014), and manganese (Sasaki et al. 2012).

Furthermore, plant physiology is finely regulated by phytohormones, whose biosynthesis is in turn influenced by nutritional

status. In consequence, a close interrelation between nutritional and phytohormonal homeostasis as well as coordination between their signaling pathways is required for proper plant growth and development (Krouk et al. 2011). Ethylene plays a prominent role in mineral nutrition and response to low nutrient availability. Indeed, ethylene is involved in root responses to nutrient toxicities and deficiencies (Iqbal et al. 2013; García et al. 2015). Several cross-talk processes has been studied in case of nitrogen (Iqbal et al. 2011), calcium (Lau and Yang 1976), iron (Lucena et al. 2006), and potassium (Benlloch-González et al. 2010) among others. Furthermore, important processes to cope with low nutrient availability such as root elongation, lateral root proliferation and cell fate determination are regulated by ethylene (Lynch and Brown 1997). Plant nutrition includes macronutrients coming from water and photosynthesis as carbon, oxygen and hydrogen and mineral macronutrients and micronutrients, whose main functions in plants are described as follows.

Nitrogen is essential for plants because it is involved in biomass production and metabolism. Most of plant nitrogen forms part of amino acids, proteins and nucleic acids and its deficiency causes chlorosis of leaves and stunted growth. Ethylene is involved in plant response to nitrogen. Ethylene effects on plant physiology can be dependent on available nitrogen since ethylene is able to suppress root hair branching stimulated by ammonium, which in turn is enhanced by methyl jasmonate (Yang et al. 2011). Moreover, ethylene sensitivity and subsequent aerenchyma formation was increased during nitrogen deficiency in maize (He et al. 1992) showing the interrelation between ethylene and nitrogen.

Phosphorus is structural component of nucleic acids and proteins and plays important roles in a wide variety of plant metabolism processes (Cheng et al. 2011). Furthermore, plants show several physiological adaptations to cope with low phosphorus conditions (Ticconi and Abel 2004; Wasaki et al. 2009). Deficiency of phosphorus causes effects such as reduced growth, ratio between shoot and root tissues, and number of leaves. Moreover, phosphorus deficiency induced production of ethylene (Borch et al. 1999; Li et al. 2009), which in turn mediate response to low phosphorus in several plant species (Drew et al. 1989; He et al. 1992; Kim et al. 2008a; Lei et al. 2011). In addition, ethylene insensitive genotypes under low phosphorus conditions would fail to trigger some adaptive responses and show reduced growth (Feng and Barker 1992; Zhang et al. 2003). The ethylene role in growth and response to low phosphorus was studied using ethylene-insensitive mutants (*never ripe* and *etr1* in case of tomato and petunia plants, respectively) suggesting that ethylene perception regulates carbon allocation to adventitious roots and concluding that ethylene plays a key role mediating formation of adventitious roots in response to phosphorus stress (Kim et al. 2008a). Moreover, ethylene synthesis and response are also involved in root architecture response to phosphorus deficiency in common bean (Borch et al. 1999).

Potassium is involved in transpiration regulation via stomata opening and it activates several enzymes. Potassium deficiency is appreciable on leaves as yellowing from margins to the leaf inside. Ethylene signaling stimulates reactive oxygen species (ROS) production under low potassium and determines plant tolerance to low potassium availability affecting root morphology (Jung et al. 2009).

However, ethylene is involved in inhibition of stomatal closure mediated by potassium under water stress (Benlloch-González et al. 2010).

Calcium is involved in regulation of a wide range of plant processes via calmodulins as well as a key component of plant cell wall conferring rigidity (Hepler 2005; Yang et al. 2011). External addition of calcium (Ca^{2+}) was specifically able to enhance ethylene production in mung bean, although also showed synergistic effects with copper (Cu^{2+}) and kinetin (citokinin) which causes Ca^{2+} uptake on a par with ethylene production (Lau and Yang 1976). In addition, apoplastic calcium content was positively correlated with ACC oxidase induction in pea seedlings (Kwak and Lee 1997).

Magnesium is essential in photosynthesis and plays a determinant role in plant metabolism acting as cofactor of several enzymes as well as structural component in a wide variety of molecules. Magnesium deficiency results in chlorophyll degradation and thereby reduced photosynthesis and enhances enzyme inactivation (Guo et al. 2016). Under low magnesium availability or deficiency, ethylene synthesis was reported since four ACC synthase genes were strongly induced (Hermans et al. 2010).

Iron forms part of a wide range of enzymes including those in redox systems, apart from playing a key role in respiration and photosynthesis. Iron deficiency causes leaf chlorosis and stunted growth (Rout and Sahoo 2015). Response to low iron in cucumber was repressed by inhibitors of ethylene synthesis or action (Romera and Alcántara 1994). Moreover, ACC addition induced expression of genes involved in low iron response and mediated iron acquisition and

assimilation in several species including arabidopsis and tomato (Lucena et al. 2006). Indeed, ethylene biosynthesis and signaling was induced by iron deficiency in arabidopsis (García et al. 2010).

Copper plays a determinant function in protein and carbohydrate metabolism as well as acts as catalyst in photosynthesis and respiration, although it is also potentially toxic for plant cells (Yruela 2005). Copper deficiency result in plant chlorosis and finally necrosis, and high copper concentrations caused leaf toxicity and high ethylene production (Yruela 2005; Franchin et al. 2007). As abovementioned, copper is a key element of ethylene receptor functionality (Hirayama et al. 1999; Woeste and Kieber 2000), and thereby its deficiency could also result in lower ethylene responsiveness. High levels of copper are able to induce ethylene production in several species (Maksymiec 2007; Arteca and Arteca 2007). In addition, two ethylene biosynthesis and receptor genes were induced by abiotic stress caused by excessive copper in potato and broccoli (Schlagnhaufer et al. 1997; Jakubowicz et al. 2010).

Manganese plays a protective role in photosynthetic tissues and increases antioxidant capacity in root tissues (Zornoza et al. 2010). However, high levels of manganese are toxic producing necrotic lesions on par with increased ethylene levels (Fowler and Morgan 1972). Furthermore, it was reported that manganese deficiency enhanced the ozone-induced ethylene and decreased the ascorbic acid content of leaves (Mehlhorn and Wenzel 1996).

Biotic and abiotic stresses

ERFs plays a key role in tolerance to abiotic stresses such as drought, salinity, light stress, cold and heat in several species including arabidopsis (Dubois et al. 2013; Vogel et al. 2014), tomato (Hu et al. 2014; Severo et al. 2015), tobacco (Guo et al. 2004; Wu et al. 2007), and wheat (Rong et al. 2014; Djemal and Khoudi 2015). In addition, common ERF gene expression was reported under several abiotic stresses (Müller and Munné-Bosch 2015).

As abovementioned, ERF genes are able to bind to dehydration responsive elements (DRE) and GCC box. In arabidopsis, ERF1 binds to DRE elements of several genes including early response to dehydration 7 (ERD7), responsive to dessication 29B (RD29B) and RD20 (Cheng et al. 2013) conferring tolerance to various stresses including drought, heat and salinity. In addition, ERF1 is also able to bind to GCC box in promoters of jasmonic- and ethylene-responsive plant defensin (PDF1.2) and basic chitinase (b-CHI) (Solano et al. 1998), also conferring resistance to pathogens. In potato, the expression induction of pathogen and freezing tolerance-related protein1 from pepper (CaPF1; ERF pepper transcription factor) resulted in resistance to heat, freezing, heavy metal, and oxidative stress (Youm et al. 2008). Furthermore, the expression of transcriptional activator of tomato (TSRF1; ERF transcription factor) regulates osmotic stress tolerance and pathogen stress tolerance in tobacco (Zhang et al. 2007). In addition, plant response and adaptation to stress conditions need a finely coordinated phytohotmone crosstalk in order to regulate gene expression and specifically response. Unfortunately, the molecular

mechanisms underlying pathway crosstalk are still only partially unraveled (Müller and Munné-Bosch 2015).

Drought stress

Drought stress is able to induce ethylene production (Pierik et al. 2007). Drought stress causes important losses in crops since drought largely reduce plant growth, which depends on cell growth and differentiation. In addition, drought affects to a wide range of physiological processes such as stomatal conductance, respiration, transpiration, photosynthesis, and membrane functions among others (Hasanuzzaman et al. 2014). Plant mechanisms to cope water deficit are mainly mediated by ethylene and ABA (Sharp et al. 2000; Pierik et al. 2007) acting as antagonists since accumulation of ABA could modulate growth response to ethylene and vice versa (Wilkinson and Davies 2010; Wilkinson et al. 2012).

Root system is a key player in drought stress response (Steudle 2000; Chaves et al. 2003). Drought primarily diminishes aerial vegetative growth as well as produces physiological effects as gas exchange inhibition, but is mainly sensed by roots affecting root-to-shoot signaling (Deblonde and Ledent 2001; Anjum et al. 2011). Additionally, drought affected expression of several genes involved in osmotic stress showing also cross-linking with other abiotic stresses (Yamaguchi-Shinozaki and Shinozaki 2006; Shinozaki and Yamaguchi-Shinozaki 2007). ACC is transported from roots to shoots via xylem (Else and Jackson 1998), and leaf ethylene evolution was associated with ACC transport in tomato under drought stress (Sobeih

et al. 2004). In consequence, ACC was pointed as root-sourced signal and ethylene as key player in growth inhibition of leaves (Schachtman and Goodger 2008).

As observed with other abiotic stresses, drought induced the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_20_2), superoxide anion (O_2^-), singlet oxygen ($^1O_2$), and hydroxyl radicals (OH^-) in different subcellular compartments causing oxidative stress that damage cellular components and thereby cells (Mittler 2002). In addition, ROS production under stress triggers specific defense or adaptation responses with H_20_2 as secondary messenger. When plants are unable to scavenge high ROS levels, several essential processes can be affected resulting even in plant death. However, the enhanced production of ROS is kept under control through a flexible and cooperative antioxidant system, which modulates ROS concentration within the cell and adjust the redox status (Cruz de Carvalho 2008).

The main mechanism of ROS scavenging includes several enzymes and metabolites. Superoxide dismutase (SOD) quickly scavenges O_2^- radical producing oxygen and H_20_2 (Bowler et al. 1992). Catalase (CAT) and ascorbate peroxidase (APX) are the major enzymatic scavengers of H_20_2 (Willekens et al. 1997; Noctor and Foyer 1998). CAT does not require reductant and shows lower affinity (mM range) for H_20_2 scavenging, while APX needs ascorbate acting at 1000-fold lower range (Mittler 2002). Other important enzyme is glutathione reductase (GR) which participates in the ascorbate/glutathione cycle. GR maintains the glutathione pool within the cell in the reduced state (reduced glutathione; GSH) (Noctor and Foyer 1998). As ascorbic acid,

GSH can be oxidized by superoxide, singlet oxygen, and hydroxyl radicals preventing excessive oxidation of sensitive cellular components (Kataya and Reumann 2010). In addition, GSH can indirectly function as antioxidant by recycling ascorbic acid form its oxidized form (dehydroascorbic acid; DHA) via dehydroascorbate reductase (DHAR) enzyme (Morell et al. 1997; Noctor and Foyer 1998).

Biotic interactions

Ethylene emission by plants was previously reported in plant interaction with fungal and bacterial pathogens (van Loon et al. 2006). Indeed, ethylene acts as modulator of interaction between plants with several enemies, activating or repressing determined branches of the defense network in combination with SA or JA-Ile (Groen et al. 2013). Nevertheless, ethylene also plays a role in interaction with beneficial microorganisms (Zamioudis and Pieterse 2012).

Plant interaction with pathogenic organisms

Pathogen recognition by plant immune system is regulated by phytohormone signaling network. The main signaling branches are mediated by SA, JA, ethylene and phytoalexins, which interact between each others in order to provide specificity to defense response (Tsuda et al. 2009). Moreover, the plant immune network is also influenced by other phytohormones such as auxins, cytokinins, ABA, GAs, and BRs (Robert-Seilaniantz et al. 2011; Pieterse et al. 2012).

Ethylene can produce antagonistic effects on SA signaling blocking its production (Chen et al. 2009). In tomato infected with *Pseudomonas syringae*, ethylene is induced increasing its susceptibility to the pathogen (Cohn and Martin 2005). However, ethylene signaling can also act synergistically with SA in order to contribute to immunity against both necrotrophic and biotrophic pathogens (Tsuda et al. 2009). Ethylene signaling in combination with JA-Ile leads to activation of ERFs, while only JA-Ile signaling results in activation of MYC family transcription factors. Defense triggered by ERF and MYC are mutually antagonistic acting against fungi and herbivores, respectively (Lorenzo et al. 2004; Verhage 2011; Fernández-Calvo et al. 2011). Moreover, phytoalexins stimulates production of SA since mutation of PAD4 (phytoalexin deficient 4) have a defect in SA accumulation after pathogen infection, suggesting that PAD4 is involved in a positive regulatory loop to activate defense responses dependent on SA (Jirage et al. 1999). Moreover, full ethylene production after pathogen infection or other stresses relies on PAD4 activity (Heck et al. 2003; Mühlenbock et al. 2008). Furthermore, PAD4 in combination with enhanced disease susceptibility 1 (EDS1) regulates signaling during defense responses (Pathogen-associated molecular patterns (PAMP)- and effector-triggered immunities; PTI and ETI) (Rietz et al. 2011). Thus, PAD4 regulates processes determinant for antimicrobial biosynthesis during pathogen infection as previously reported with fungal infections (Glazebrook et al. 1997).

Plant interaction with beneficial organisms

Beneficial microorganisms association with plants requires mutual recognition being initially perceived as potential invaders and

thereby triggering immune responses (Zamioudis and Pieterse 2012). A common symbiosis signaling pathway (Sym pathway) is activated in plants cells after perception of mycorrhizal and rhizobial factors, called Myc and Nod factors, respectively (Oldroyd et al. 2009; Maillet et al. 2011). Furthermore, some non-symbiotic microbes as plant growth promoting bacteria (PGPB) could also activate signaling components of the Sym pathway (Sanchez et al. 2005) suggesting a partly converged signaling pathway triggered by beneficial microbes.

PGPB are able to induce immune responses (Van Wees et al. 2008), and elicitors coming from induced systemic resistance (ISR)-inducing PGPB strains are the best characterized, triggering a ROS burst, a quick increase of cytoplasmatic calcium, and expression of defense-related genes (Bakker et al. 2007; Van Loon et al. 2008). Nevertheless, PGPB are also able to suppress triggered immune response via effector secretion with involvement of ethylene since PGPB-secreted molecules could target ethylene-dependent processes (Millet et al. 2010). Indeed, several PGPB are able to reduce ethylene production by plants (Glick et al. 2007b). Additionally, several soil microbes are able to produce phytohormone-like compounds (Lugtenberg and Kamilova 2009), which can help to suppress SA signaling pathway affecting the outcome of the immune response (Pieterse et al. 2009; Verhage et al. 2010). Thus, PGPB could produce phytohormones in order to mitigate the SA signaling though hormonal cross-talk mechanisms.

In addition, bacteria can reversibly switch morphology of colonies, and thereby PGPB can use phenotypic variation or phase variation in order to avoid plant immune system (Davidson and Surette

2008). Bacterial subpopulations differ in expressed or altered molecules on the surface such as flagella or lipopolisaccharides (LPS) (Van Der Woude and Bäumler 2004). In fact, phenotypic variation processes has been reported in interaction between plants and microorganisms living in the rhizosphere (Achouak et al. 2004; Van Den Broek et al. 2005).

Furthermore, PGPB are able to secrete effectors by type III secrtion systems (TTSS) which can determinate the host-specificity suppressing innate immune system responses (Mavrodi et al. 2011). It has been reported that immune responses to PGPB are dependent on combination between host plants and bacterial strains (Van Wees et al. 1997; Van Loon et al. 1998; Ton et al. 2002), pointing to a gene-for-gene plant-bacteria interaction. However, further research is required in order to identify putative host "R proteins" which recognize effectors and modulate the interaction between plant and bacteria (Zamioudis and Pieterse 2012).

Tomato as model plant

Arabidopsis (*Arabidopsis thaliana*) was selected as model organism in plant science to integrate knowledge of classical disciplines with genetics and molecular biology (Koornneef and Meinke 2010). Nevertheless, domesticated tomato (*Solanum lycopersicum*) is the most important horticultural crop around the world, and the second vegetable consumed after potato (Schwarz et al. 2010). Tomato is broadly used as model crop for physiological, biochemical, molecular, and genetic studies as well as for fruit development (Schwarz et al. 2014). Several large seed banks provide

useful germoplasm as the Tomato Genetic Resuorce Center (TRGC) at the University of California (Davis, USA; http://tgrc.ucdavis.edu), which has been used in the present thesis. Moreover, the genomes of the tomato wild-relative (*Solanum pimpinellifollium*) and the inbred cultivar "Heinz 1706" were sequenced (Sato et al. 2012). Physical and genetic maps (http://solgenomics.net) as well as databank of gene expression (Koenig et al. 2013) and DNA polymorphisms (Causse et al. 2013) are also available providing useful information in order to analyze and compare obtained results. Moreover, simple and general methods using *Agrobacterium tumefaciens* have been developed for genetic transformation in order to obtain tomato transgenic lines with adequate efficiency in 4-6 months (Cortina and Culiáñez-Macià 2004; Qiu et al. 2007; Sun et al. 2015).

Moreover, tomato can be easily growth within growth chambers or greenhouses using nutrient solution to meet nutritional demands as well as specific nutrient surplus or deficit (Hewitt 1966), achieving seed to seed cultivation periods about 100 days at 20ºC (Schwarz et al. 2014). In consequence, tomato is a suitable model because all abovementioned traits as well as the availability of a plenty of mutants. For instance, the ethylene-insensitive mutant used in the present thesis, called *never ripe* (Lanahan et al. 1994; Wilkinson et al. 1995).

Beneficial soil microorganisms

In soil, a plethora of microorganisms are able to associate with plants. The microorganisms, which colonize plant roots, include fungi, algae, bacteria, protozoa and actinomycetes (Barea et al. 2005; Gray

and Smith 2005; Bhattacharyya and Jha 2012). These interactions can be harmful, neutral or beneficial influencing plant growth and development (Adesemoye and Kloepper 2009; Lau and Lennon 2011; Nadeem et al. 2014).

The rhizosphere can be defined as any soil volume specially influenced by plant root system or associated with the material produced by roots and plants, including the region of soil bound by plant roots and a few millimeters from the root surface (Bringhurst et al. 2001). The term "rhizosphere" was firstly described as a zone of maximum microbial activity since rhizosphere contains much more bacteria diversity than the surrounding bulk soil (Montesinos 2003) because plant exudates, that contains amino acids and sugars, provide a rich source of nutrients and energy (Burdman et al. 2000; Farrar et al. 2003). In addition, soil microorganisms can be present in the rhizosphere, rhizoplane (root surface), root tissue and/or within specialized root structures (Martínez-Viveros et al. 2010) (Fig. I6).

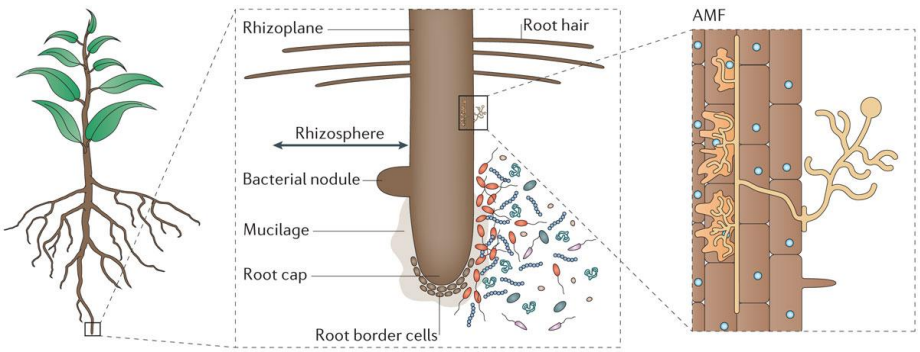

Figure I6. Schematic representation of rhizosphere. The schematic shows magnified pictures of the rhizosphere, containing saprophytic and symbiotic bacteria and fungi, including arbuscular mycorrhizal fungi (AMF). Figure taken from Philippot *et al.* (2013).

Although bacterial population is the most abundant in the rhizosphere (Schoenborn et al. 2004), fungi also inhabit the rhizosphere influencing plant growth and development. The term "mycorrhizae" designates the symbiotic association between plant roots, and fungi and have been classified in endomyccorrhizae, ectomycorrhizae, and ectendomycorrhizae regarding to hyphae penetration in root cortical cells. Mycorrhizae increases area surface of roots enhancing water and nutrient uptake, and also protects plants from abiotic stresses (Harrier 2001; Evelin et al. 2009; Martin et al. 2016).

Plant growth promoting bacteria

Among beneficial soil microorganisms, the bacteria has been studied in detail (Lugtenberg and Kamilova 2009; Pii et al. 2015; Santoyo et al. 2016), although cultivable bacterial cells in soil represents only about 1% of total number of cells present (Schoenborn et al. 2004). The term "rhizobacteria" was established to designate the bacterial community of soil that competitively colonized plant root system and promoted plant growth and/or diminish plant disease incidence (Kloepper and Schroth 1978). Later, these were named plant-growth promoting rizhobacteria or more generally plant growth promoting bacteria (PGPR and PGPB, respectively). Nowadays, PGPB concept has been limited to bacterial strains with at least two traits of the abovementioned three criteria (Weller et al. 2002; Vessey 2003).

The root colonization process is influenced by bacterial traits, root exudates and biotic and abiotic factors (Benizri et al. 2001; Haichar et al. 2014). Furthermore, a successful PGPB root colonization

is required to produce their beneficial effects (Elliott and Lynch 1984). PGPB can be classified in accordance with their association degree with the plant root cells into extracelluar (ePGPB) and intracellular (iPGPB). In addition, iPGPB can be generally found inside specialized nodular structures and ePGPB in the rhizosphere, rhizoplane or root surface and the space between cells of root cortex (Martínez-Viveros et al. 2010). Additionally, PGPB can also be classified such as biofertilizers, phytostimulators and biopesticides (Lugtenberg and Kamilova 2009), offering an attractive way to diminish or even replace the use of chemicals in agriculture (Bhattacharyya and Jha 2012).

Biofertilizer: Product which contains live microorganisms that, when is applied on the seed, plant surface or soil, is able to colonize the rhizosphere and stimulate plant growth via increasing supply of primary nutrients.

Phytostimulator: Product that contains live microorganisms, which are able to modulate phytohormone levels that finely control plant growth and development.

Biopesticide: Product that contains live microorganisms, which are able to promote plant growth by controlling phytopathogenic agents.

Plant-beneficial microbe associations are thought to be ancient and shaped during co-evolution 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.

PGPB action mechanisms

The bacteria able to stimulate plant growth include those that are free-living bacteria, bacterial endophytes able to colonize interior tissues of plants, that specifically form symbiosis with plants (such as *Rhizobia* spp. and *Frankia* spp.), and cyanobacteria (previously named blue-green algae). PGPB are able to induce plant growth either directly or indirectly (Ortíz-Castro et al. 2009), although several mechanisms may simultaneously act enhancing plant growth as a cumulative result (Martínez-Viveros et al. 2010). They all facilitate resource uptake, modulate phytohormone levels or decrease growth inhibitory effects caused by stress agents (Glick 2012) (Fig. I7).

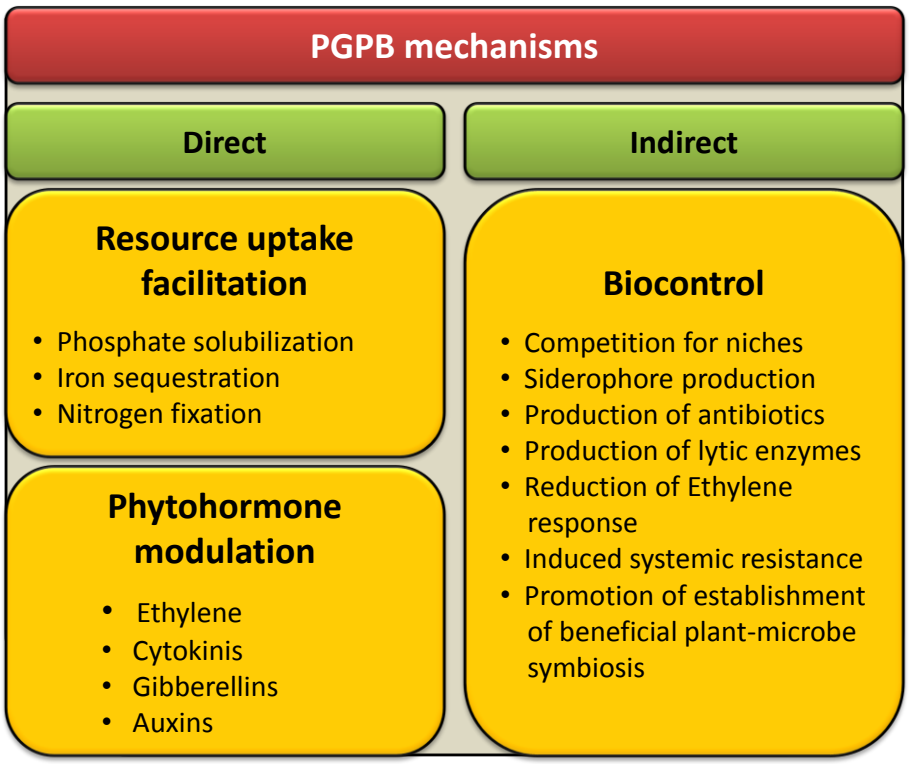

Figure I7. PGPB action mechanisms

Direct action mechanisms

Direct stimulation of plant growth by PGPB includes enhancement of plant nutrition as well as modulation of phytohormone levels (Fig. I7). Thus different action mechanisms were classified as follows.

Resource uptake facilitation

Bacterial stimulation of plant growth includes meeting plant nutritional demands with low available resources in soils such as of nitrogen, iron and phosphorous (López-Bucio et al. 2002; Colombo et al. 2014; Kiba and Krapp 2016). The 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 microbial communities of the rhizosphere are associated with nutrient biogeochemical cycles (Barea et al. 2005). To cope with nutrient limitation, several physiological and developmental responses can be triggered (López-Bucio et al. 2002) and plant association with soil microorganisms represent a suitable strategy in order to cope with low nutrient availability.

Phosphate solubilization

Phosphorus is an essential element in plant growth and development (Cheng et al. 2011). However, their bioavailability in soils is very limited since phosphate is poorly soluble (López-Bucio et al. 2002). In fact, low phosphorus availability was reported in approximately half of the agricultural lands, limiting

plant growth and resulting in crop yield losses (Lynch 2011). In consequence, phosphorus solubilization produced by phosphate solubilizing bacteria is an important feature in PGPB (Canbolat et al. 2006; Lai et al. 2008). Solubilization of phosphate is generally caused by low molecular weight acids produced by PGPB such as gluconic and citric acids, while its mineralization occurs as consequence of phosphatases excretion (Rodriguez et al. 1999). Indeed, phosphate mineralization and solubilization processes can be performed by the same bacterial strain (Tao et al. 2008).

Iron sequestration

Although iron is an abundant element on earth, it cannot be assimilated by either plants or bacteria due to its oxidation state. Ferric ion (Fe^{3+}) is poorly soluble causing a very low iron amount available for organism assimilation (Ma 2005). Plants and bacteria need a high level of iron even producing competition in the rhizosphere for iron (Loper and Buyer 1991; Pii et al. 2015). To cope with limited iron and facilitates its uptake, bacteria produce siderophores, which show high affinity for Fe^{3+} and membrane receptors able to bind complexes of Fe-siderophore for their acquisition (Hider and Kong 2010). Bacterial siderophores directly benefit plant growth since plants are also able to uptake Fe-siderophore complexes improving their fitness (Siebner-Freibach et al. 2003; Sharma et al. 2003; Jin et al. 2006; Vansuyt et al. 2007). In addition, iron nutrition enhancement by PGPB is essential under stress conditions such as heavy metal pollution (Burd et al. 2000; Belimov et al. 2005).

Nitrogen fixation

Although atmosphere contains about 78% of nitrogen gas (N_2), plants are unable to use this form and its availability in soils is very limited. Biological nitrogen fixation is performed by bacteria. In order to fixate nitrogen, an enzyme complex called nitrogenase is required as well as a large amount of energy in form of ATP. Atmospheric nitrogen is reduced to ammonia, which is a form of nitrogen that can be used by diazotrophic bacteria and plants (de Bruijn 2015). Apart from *Rhizobium* spp., several free-living bacteria such as *Azospirilumm* spp. can perform nitrogen fixation which can be taken up by plants (Bashan and Levanony 1990; Pankievicz et al. 2015). Thus, the utilization of PGPB able to fixate atmospheric nitrogen was proposed as a highest potential biotechnological tool in order to improve crop yields (Souza et al. 2014).

Phytohormone modulation

As commented above, during plant growth and development as well as in interaction and response with their environment, phytohormones play essential functions (Davies 2010). In addition, plants suffer several non-lethal stresses, which limit their growth until stress disappear or plants are able to adapt their metabolism overcoming stress effects. Under stress conditions, phytohormone levels are finely adjusted in order to diminish the negative effects on plant growth. PGPB are able to produce phytohormones or change phytohormonal levels affecting the hormonal balance of plants and thereby their stress response (De Garcia Salamone et al. 2006; Glick et al. 2007a).

Ethylene modulation

Ethylene production is typically induced in response to environmental stresses (Wang et al. 2013). The enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCd) was discovered in soil bacteria (Honma and Shimomura 1978) and reported its presence as a common trait in several PGPB (Blaha et al. 2006; Glick et al. 2007b). ACC is the immediate precursor of ethylene, and a portion is exuded by roots (Penrose and Glick 2001). PGPB are able to uptake that ACC and produce ammonia and α-ketobutyrate by ACCd activity for bacteria nutrition, reducing as a consequence ethylene produced by plant and its inhibitory growth effects (Glick et al. 1998). PGPB inoculation containing the activity ACCd, results in root elongation at short-term and shoot growth promotion at long-term (Dey et al. 2004; Contesto et al. 2008; Onofre-Lemus et al. 2009; Glick 2014).

Cytokinis and gibberellins modulation

Cytokinin and/or gibberellin production by soil bacteria was previously widely reported (Williams; and Sicardi De Mallorca 1982; Timmusk and Wagner 1999; García de Salamone et al. 2001). Additionally, plant growth stimulation by PGPB producing cytokinins or gibberellins was also reported (Joo et al. 2005; Arkhipova et al. 2007). Nevertheless, the role of microbially-produced cytokinins and gibberellins in plant physiology was proposed in function of studies with exogenous addition of hormones in plants, but little is known about their action mechanisms and regulation by plants (Glick 2012).

Auxins modulation

As abovementioned, IAA affects several processes in plant physiology such as plant cell division, extension and differentiation and increase resistance to stress among other functions (Zhao 2010; Spaepen and Vanderleyden 2011). Additionally, different IAA concentrations have differential effects regarding plant tissue and developmental stage. In consequence, the endogenous IAA concentration may be altered by soil bacteria, resulting in plant growth promotion or inhibition (Spaepen and Vanderleyden 2011). IAA production by PGPB was previously reported causing marked effects on plant growth (Patten and Glick 2002; Mohite 2013).

Indirect action mechanisms

Indirect mechanisms are basically related to biocontrol of pathogenic microorganisms (Fig. I7), but there are some differences in the molecular mechanisms and thereby they were classified as follows.

Competition for niches

The non-pathogenic microorganisms of soil quickly colonize plant surfaces in order to use available nutrients. In consequence, competition for niches on plants and nutrients between pathogenic microorganisms and PGPB has been reported limiting disease incidence and severity in some cases (Kamilova et al. 2005; Innerebner et al. 2011).

Siderophore production

In some cases, PGPB strains producing siderophores can act as biocontrol agents directly preventing that phytopathogenic

microorganisms can uptake enough iron to meet their metabolic demands and thereby limiting their growth (Kloepper et al. 1980; Dowling et al. 1996). This biocontrol mechanism is effective because PGPB siderophores show a much greater affinity for iron than pathogenic fungal ones (Miethke and Marahiel 2007). Thus, this method is proper against fungal pathogens. In addition, plant growth is generally not affected because plants are able to use the PGPB Fe-siderophore complexes (Wang et al. 1993).

Production of antibiotics and lytic enzymes

Certain PGPB strains are able to synthetize different antibiotics preventing the proliferation of plant pathogenic microorganisms (Whipps 2001; Compant et al. 2005; Mazurier et al. 2009; Beneduzi et al. 2012). Additionally, some PGPB are also able to produce lytic enzymes such as cellulases, chitinases, glucanases, proteases and lipases, which can damage structural components of pathogenic microorganisms. Generally, PGPB producing these enzymes or antibiotics are efficient against pathogenic fungi such as *Phytophtora spp.* or *Fusarium spp.* among others (Singh et al. 1999; Kim et al. 2008b).

Ethylene response reduction

Plants usually respond to phytopathogens by synthesizing ethylene which enhance stress effects on plants (van Loon et al. 2006). In consequence, reduction of ethylene levels can reduce the damage to plants caused by phytopathogens (Glick and Bashan 1997). Thus, PGPB containing ACCd activity can be very useful in biocontrol strategies (Glick 2014). For instance, inoculation with

bacteria containing ACCd activity can reduce crown gall caused by *Agrobacterium tumefaciens* infection in tomato (Toklikishvili et al. 2010).

Induced systemic resistance

Some PGPB are also able to trigger induced systemic resistance (Verhagen et al. 2004; Bakker et al. 2007). ISR involves plant ethylene and jasmonate signaling to stimulate defense response against pathogenic microorganisms. ISR is activated when plants interact with the PGPB strain, but it does not require direct interaction between pathogenic microorganism and ISR-inducing bacteria. ISR-positive plants react quicker and more strongly to attack of pathogenic microorganisms by inducing mechanisms of defense phenotypically similar to systemic acquired resistance (Pieterse et al. 2014).

Promotion of establishment of beneficial plant-microbe symbiosis

Establishment of beneficial plant-microbe symbiosis can be also modulated by PGPB. Ethylene negatively affects nodulation, in beneficial interaction between legumes and rhizobia (Guinel 2015), and mycorrhization (Azcon-Aguilar et al. 1981; Geil et al. 2001). In consequence, utilization of PGPB able to reduce ethylene levels in plants can increase mycorrhizal colonization (Gamalero et al. 2008), as well as rhizobial nodulation in several plants (Ma et al. 2004; Nascimento et al. 2012).

Ethylene and PGPB

Ethylene is involved in direct and indirect action mechanisms of PGPB, but reported studies are mainly focused in PGPB strains containing ACCd activity and thus they are able to decrease ethylene levels directly stimulating plant growth and/or indirectly favoring beneficial interactions or reducing pathogenic infections. In consequence, these PGPB containing ACCd activity (Belimov et al. 2007; Glick 2014) were defined as stress controllers (Lugtenberg and Kamilova 2009), since ethylene production is typically induced under stress conditions (Wang et al. 2013a).

In addition, it was noticed that ethylene biosynthesis or action inhibitors caused similar physiologic effects to inoculation with PGPB containing ACCd activity, suggesting that growth enhancement is consequence of ethylene level reduction (Belimov et al. 2007). Inoculation with PGPB containing ACCd activity caused lower ACC levels in seeds and roots (Mayak et al. 2004a). Moreover, effects of bacteria containing ACCd activity are not restricted to root system. Reduced ACC levels in root tissue diminishes the growth-inhibitory effects of ethylene on aerial tissues (Klee et al. 1991; Glick et al. 1998), and inoculation with PGPB containing ACCd activity caused lower ACC levels in xylem sap (Belimov et al. 2009). *Variovorax paradoxus* 5C-2 with ACCd activity increased plant vegetative growth in pea (Belimov et al. 2009) and arabidopsis (Chen et al. 2013). In addition, recovery of plant from drought was improved modulating stress-induced ethylene in tomato and pepper seedlings inoculated with PGPB containing ACCd activity (Mayak et al. 2004b).

The importance of bacterial ACCd activity for plant growth promotion was also evidenced because its disruption resulted in reduced or unnoticed plant growth stimulation (Li et al. 2000a; Madhaiyan et al. 2006; Viterbo et al. 2010). For instance, tomato inoculation with *Pseudomonas brassicacearum* Am3 deficient in ACCd showed a dose-dependent negative impact in primary root growth regarding to its wild type strain (Belimov et al. 2007).

Nevertheless, other reports suggest ethylene-independent growth promotion or that ACCd exclusively affects local regulatory mechanisms in plant roots. Inoculation of *Bacillus megaterium* in two ethylene-insensitive mutants (ein2-1 and etr1-3) and wild type *A. thaliana* produced similar promotion of shoot biomass and lateral root number (López-Bucio et al. 2007). *Arabidopsis thaliana* wild type and the ein2-1 mutant under inoculation with PGPB containing ACCd activity and ACCd-deficient mutants of four different bacterial strains showed similar effects on primary and total lateral root length, but the promotion of root hair length was significantly reduced by ACCd activity suggesting that ACCd affects local regulatory mechanisms in plant roots, but lateral root development is regulated by systemic mechanisms (Contesto et al. 2008).

Methodologies to widely study plant-PGPB interaction

Although there are plenty studies addressing interaction between plant and bacteria, most approaches were only focused on a single biochemical pathway and often miss lots of bacterial effects. The term "-omics" represents completeness. There are several kinds of -omics

technologies including genomics, transcriptomics, proteomics, metabolomics, lipidomics, secretomics or signalomics among others. All these techniques aim to depict precise pictures of the complete cellular processes (Jha et al. 2015).

Furthermore, the increasing number of sequenced plant genomes has permitted to study a wide range of biological processes regarding plant growth and development as well as response to biotic and abiotic stresses (Bolger et al. 2014; Jha et al. 2015). In consequence, various -omics approaches have been performed in order to shed light on plant-bacteria interaction (Cheng et al. 2010; Stearns et al. 2012; van de Mortel et al. 2012; Couillerot et al. 2013; Su et al. 2016), but many fundamental questions remain to be solved.

In addition, -omics studies regarding plant-bacteria interaction are mainly focused in the nitrogen-fixing rhizobia symbiosis (Mathesius 2009; Lang and Long 2015; Lardi et al. 2016) and plant-pathogen interaction (Ameline-Torregrosa et al. 2006; Mehta et al. 2008; Aliferis and Jabaji 2012; Afroz et al. 2013). However, little is known about PGPB effects on plant -omics profiles, despite of their environmental and agricultural importance. Moreover, further research is needed since PGPB action mechanisms are often strain-specific and dependent on plant growth conditions (Ryu et al. 2005; Long et al. 2008), and they are less well characterized (Pühler et al. 2004). Although PGPB inoculation produces systemic effects on aerial plant tissues, the roots are a significant bacterial niche for PGPB, where occur direct plant-bacteria interaction (Benizri et al. 2001). In consequence, PGPB effects on roots should be addressed using -omics approaches.

Transcriptomics

Transcriptomics aims to evaluate the complete set of differentially expressed genes (Jha et al. 2015). Currently, two complementary techniques are commonly used in transcriptomic studies: sequencing directly mRNA samples as RNA-seq or hybridizing them with a great number of surface-immobilized probes as in case of microarrays and BeadArrays. The two most commonly used transcriptomic methods are microarray technology and next-generation sequencing methodologies (Brady 2006).

The modern sequencing technologies such as RNA-seq allow the generation of a huge quantity of data very useful for crop improvement (Bolger et al. 2014). Both transcriptomic techniques allow the gene expression analysis in parallel of several biological samples under the same conditions (Stears et al. 2003). Sequencing methodologies require a reference genome in order to determine identity of genes and low expressed ones tend to be underrepresented. Meanwhile, microarray technology is only able to detect those genes whose probes are immobilized on the array, but low expressed genes can be easily detected if probes corresponding to them are present (Brady 2006). In addition, microarrays are commercially available for some plant species such as GeneChip® Tomato Genome Array (Affymetrix) used in the present thesis.

Several transcriptomic studies addressing plant bacteria interaction were carried out to clarify bacterial effects on plant physiology (Cartieaux et al. 2003; Verhagen et al. 2004; van de Mortel et al. 2012). Arabidopsis inoculation with *Pseudomonas thrivervalensis*

MLG45 compared to non-inoculated plants were evaluated by microarray technology showing increased transcription of defense-related genes. In fact, bacterial inoculation increased plant resistance to subsequent infections by pathogenic bacteria. Thus, these results evidenced that transcriptomic analysis could be very useful in order to predict physiological changes (Cartieaux et al. 2003). Moreover, other studies addressed the ISR by PGPB strains showing transcriptomic changes in phytohormone-related genes which are crucial to orchestrate plant responses (van de Mortel et al. 2012).

In *Arabidopsis thaliana*, root inoculation with the PGPB *Paenibacillus polymyxa* significantly affected the expression of few genes concluding that plants are responding to such PGPB presence as a mild biotic stress agent (Timmusk and Wagner 1999). In other study, the addition of an ACC deaminase-producing PGPB and its ACC deaminase negative mutant was analyzed in order to identify genes in canola roots whose expression was differentially affected. The bacteria strain with ACCd activity increase the expression of genes involved in plant growth and decrease the expression of genes involved in plant stress responses induced by ethylene suggesting that when PGPB express ACCd, they are no longer perceived as a mild biotic stress by the plant (Hontzeas et al. 2004).

In consequence, transcriptomic analysis using microarray technology is a valuable tool in order to understand plant-bacteria interaction as well as predict physiological changes that could be related with ethylene biosynthesis, signalling and/or response, but also unrelated with ethylene since the most of the genome is represented in the microarray allowing interrogate over 9200 tomato transcripts.

Proteomics

Proteomics studies the analysis of whole protein population in a subcellular compartment, cell or tissue (Jha et al. 2015). In addition, proteomic information can be interpreted as a photo on plant physiology since proteins are often final agents in plant physiology (Feussner and Polle 2015). However, post-translational processes also regulate protein activity and sub-cellular localization as well as protein turnover (Guerra et al. 2015; Nelson and Harvey Millar 2015). Some problems can be easily solved by direct analysis of certain tissues, cell or organelles. Other limitations have been addressed developing technical variations in order to analyze protein post-translational modifications such as phosphorylation using phosphoproteomics (Picotti 2015), and ubiquitination using an immunoprecipitation approach to specifically enrich ubiquitinated portion of proteins (Xu and Jaffrey 2013) among others.

Despite of limitations, proteomics can be very useful to understand bacterial effects on plant physiology and some proteomic analyses addressing plant interaction with PGPB strains have been recently performed leading to valuable information in arabidopsis (Kwon et al. 2016) and crop plants such as rice (Miché et al. 2006; Alberton et al. 2013) and maize (Cangahuala-Inocente et al. 2013; Faleiro et al. 2015).

Proteomic analysis of arabidopsis inoculation with *Paenibacillus polymyxa* E681, which is able to produce ISR, showed differential expression of proteins involved in redox and phytohormonal statuses, metabolism of amino acids and carbohydrates and photosynthesis as

well as in defense and stress responses (Kwon et al. 2016). Interaction between *Herbaspirillum seropedicae* SmR1 and rice was also proteomically assayed suggesting that bacterial inoculation promotes phytosiderophore synthesis and methionine recycling simultaneously with reduction of ethylene synthesis in roots (Alberton et al. 2013). Moreover, proteomic analysis of *Azoarcus* sp. BH72 inoculation in rice showed that stronger defense reponse was triggered in case of less compatible interaction since pathogenesis-related proteins or proteins sharing domains with receptor like kinases induced by pathogens were increased (Miché et al. 2006). Additonally, the successful association between *Azospirillum brasilense* and maize depends on plant and PGPB genotypes and a proteomic study was performed in order to clarify plant-bacteria interaction resulting in identification of several differentially expressed proteins involved in PGPB "symbiosis" (Cangahuala-Inocente et al. 2013; Faleiro et al. 2015).

Nowadays, the two most commonly used proteomic methods are two-dimensional gel electrophoresis (O'Farrell 1975) and mass spectrometry (MS) (Mann et al. 2001). However, two-dimensional gel electrophoresis presents some problems as poor resolution for basic, hydrophobic and/or low abundant proteins (Cheng et al. 2010). In contrast, shot-gun proteomics (Wolters et al. 2001; Fournier et al. 2007) can be used to perform an integral analysis of proteins extracted from plant cells, subcellular organelles and membranes (Takahashi et al. 2014), representing a valuable alternative to address PGPB effects on the whole set or a part of plant proteins.

Furthermore, plant cell membranes are key players in several cellular functions as functional separation as well as transport

(Chrispeels 1999), signalling platforms in response to abiotic (Osakabe et al. 2013) and biotic (Inada and Ueda 2014) stimuli and molecular trafficking mediated by vesicles (Chen et al. 2011; Murphy et al. 2011). Additionally, membrane protein composition mainly define the membrane functionality (Komatsu et al. 2007). The plasma membrane (PM) acts as a selectively permeable barrier which ensures the interchange of essential metabolites and ions to meet the cell requirements since several nutrients are taken up by transporters located in the plasma membrane (Chrispeels 1999). Moreover, the plasma membrane and tonoplast (vacuolar membrane) maintain the intracellular homeostasis in the cytoplasm (Sondergaard et al. 2004). In addition, plants interact with a wide variety of microorganisms and recognition and defence mechanisms have been developed to cope with them (Dodds and Rathjen 2010). Receptors, which recognize elicitors or microbe-associated molecular patterns (MAMPs), are located in cellular membranes and are able to trigger responses (Boller and Felix 2009).

Furthermore, proteins are processed along the endomembrane system. Firstly, proteins are synthesized in the endoplasmic reticulum (ER) and then transported throughout the secretory pathway to be located in the plasma membrane by exocytosis (Murphy et al. 2011). Proteins remain in the plasma membrane or are taken up by endocytosis, and stored in endocytic vesicles and recycled back to the plasma membrane when needed or targeted for degradation in lytic vacuoles (Chen et al. 2011). Plant cells can respond to microbe interaction by adapting vesicle trafficking (Ivanov et al. 2010; Dörmann et al. 2014; Inada and Ueda 2014). However, these processes have been observed with intracellular microorganisms such as

symbiotic bacteria and mycorrhizae (Nathalie Leborgne-Castel and Bouhidel 2014) and little is known about PGPB effects on secretory pathways. The microsomal fraction is enriched in membranes such as ER, Golgi, PM, tonoplast and several endosomal vesicles and compartments (Abas and Luschnig 2010). Thus, proteomic analysis of microsomal fraction is very useful for looking into plant-bacteria interaction regarding plant recognition and signalling as well as transport processes.

Metabolomics

Metabolomics pursues to define the status of a subcellular compartment, cell or tissue at a particular physiological status or developmental stage analysing the whole set of metabolites (Jha et al. 2015). Metabolites play a key role in regulatory mechanisms because they are in many cases the end-products, and thereby metabolic information is very useful to understand the plant interaction with its environment (Feussner and Polle 2015), and could be interpreted as a snapshot of bacterial effects on plant physiology. Nowadays, the metabolomics could be considered as an “emerging field” compared to transcriptomics and proteomics. The complicated nature of small molecules in combination with very little in common chemical structure makes more difficult to establish a standard methodology for metabolome analysis. Metabolomics requires methodologies for metabolite separation and detection. Separation is usually performed by chromatographic methods such as capillary chromatography (Soga et al. 2003), high performance liquid chromatography (Gika et al. 2007), or gas chromatography (Ogbaga et al. 2016). The most common used

techniques for metabolomics detection are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Barco 2013).

Furthermore, metabolomic assessment of samples rich in metabolic diversity, such as plant-microbe interaction samples, can produce a complex output in order to successfully identify all separated metabolites. However, some metabolomic studies have been performed (Weston et al. 2012; Kwon et al. 2016). In arabidopsis, metabolomic analysis showed modified levels of several metabolites such as amino acids, sugars and their derivatives as well as vitamins, phytohormones, and organic acids such as tricarboxylic acid intermediates due to bacterial inoculation that could explain the stress tolerance or promotion of plant growth conferred by the PGPB presence (Su et al. 2016).

Although performed in shoots, other studies also showed sugar modification caused by bacterial inoculation such as glucose and fructose alteration in arabidopsis (Weston et al. 2012), as well as modification of glucose, fructose, maltose, sucrose, raffinose and mannose contents in grapevine (Fernandez et al. 2012). Additionally, amino acid content modification by bacterial inoculation was also reported in arabidopsis affecting levels of tryptophan and phenylalanine (Weston et al. 2012), and proline in grapevine (Ait Barka et al. 2006). *Paenibacillus polymyxa* E681 inoculation in arabidopsis also increased levels of tryptophan, indole-3-acetic acid (IAA), and camalexin increasing resistance to *Botrytis cinerea* infection (Kwon et al. 2016). In consequence, analysis of metabolites such as phytohormones, amino acids, sugars and organic acids could shed valuable information in order to clarify interaction between plants and PGPB.

Interest of study

The present thesis mainly pursued to shed light on the role of ethylene sensitivity in interaction between plants and PGPB, focusing specifically in bacterial action mechanisms. In consequence, two tomato lines differing in ethylene sensitivity were selected in order to be inoculated with two PGPB strains isolated from arid soils in southern Spain. Indeed, non-inoculated plants were used as control treatments along all performed experiments.

The ethylene-insensitive tomato mutant *never ripe* (*nr*) and its isogenic wild-type (wt) parental Pearson cv. lines were selected because previous studies showed that despite of residual responsiveness (Lanahan et al. 1994), *never ripe* plants are largely unable to perceive ethylene due to a mutation in the sensor domain of the ethylene receptor SlETR3 (Wilkinson et al. 1995).

Two PGPB strains *Bacillus megaterium* (Bm) (Marulanda-Aguirre et al. 2008) and *Enterobacter* sp. (hereinafter *Enterobacter* C7 (C7)) were selected. Bm has shown PGPB features in previous reports (Marulanda et al. 2009, 2010; Porcel et al. 2014; Armada et al. 2014; Ortiz et al. 2015). However, *Enterobacter* C7 was evaluated here for the first time. Since most studies about the role of ethylene in the 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 ACCd activity or ethylene production capacity in order to avoid any perturbation of plant ethylene metabolism caused by the bacteria.

Aims of the Study

Aims of the Study

The present study is focused in the interaction between two plant growth-promoting bacteria and tomato plants regarding ethylene sensitivity by using different approaches in order to clarify bacterial action mechanisms and get valuable information, which could be further implemented in crop systems.

The following specific aims were defined and addressed throughout the chapters exposed in the present thesis in order to achieve this overall objective.

- Determine if ethylene sensitivity is critical for plant-bacteria interaction and growth induction by both *Bacillus megaterium* and *Enterobacter* C7 PGPB strains in juvenile and mature tomato plants.
- Evaluate *Bacillus megaterium* and *Enterobacter* C7 inoculation effects in expression of ethylene-related genes from biosynthesis to response, under both well watered and drought conditions.
- Transcriptomically assess plant interaction with *Bacillus megaterium* and *Enterobacter* C7 and PGPB mechanisms regarding to ethylene perception.
- Proteomically assess plant interaction with *Bacillus megaterium* and *Enterobacter* C7 and PGPB mechanisms regarding to ethylene perception.

- Evaluate the effects of *Bacillus megaterium* and *Enterobacter* C7 inoculation on plant nutritional and phytohormonal statuses at juvenile and mature stages.
- Determine *Bacillus megaterium* and *Enterobacter* C7 inoculation effects in root metabolite content at juvenile and mature stages.

Materials and Methods

Materials and Methods

Biological material

Seeds of *never ripe* tomato (*Solanum lycopersicum*) (LA0162) (Wilkinson et al. 1995) and its isogenic parental cv. 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* (Bm) was identified and partially characterized in a previous study (Marulanda-Aguirre *et al.*, 2008). *Enterobacter* C7 (C7) was isolated and identified as described in Armada *et al.* (2014b).

Seed sterilization and germination

Seeds were sterilized performing the following washing steps: 70% ethanol for 5 min; 5% sodium hypochlorite for 10 min; 3 washing steps with sterile water to remove any trace of chemicals. Sterilized seeds were kept in water at 4ºC overnight and placed on sterile vermiculite at 25ºC until germination. Finally seedlings were grown in a greenhouse under controlled conditions (18-24°C, 50-60% relative humidity, 16 h:8 h light (600 μmol m^{-2} s^{-1}):dark) until inoculation treatment.

Seedling inoculation with PGPB strains

Ten-days-old seedlings were inoculated during transplantation to the final substrate. Bacteria were grown in Luria broth (LB) medium with shaking (200 rpm) at 28ºC overnight. Culture optical density at 600 nm (OD_{600}) was measured, bacterial cultures were centrifuged

(2655 g, 10 min), and the pellet was resuspended with sterile distilled water until OD_{600} = 1.5, which corresponds to cell density over 10^7 colony-forming unities per milliliter (CFU ml^{-1}). One milliliter of distilled water (control plants), or a bacterial suspension: either Bm or C7 (inoculated plants) was sprinkled onto each root seedling at transplantation.

Colonization of tomato root system

Seeds were sterilized as abovementioned and germinated on filter paper soaked with sterile distilled water on Petri plates in darkness for 3 days. 10-day-old seedlings were transferred to sterilized glass bottles containing sterile peat moss:perlite (1:1, v:v, autoclaved twice at 120°C for 20 min). Seedlings were inoculated as mentioned above at transplantation. The glass bottles were closed and kept for one week in a climate-controlled growth chamber (18-24°C, 50-60% relative humidity, 16 h daylight). A one centimeter-long intermediate root segment was carefully cut and suspended in 1 ml of sterile water. Tubes were incubated for 1 hour 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 28ºC. Finally, colonies were counted and CFU cm^{-1} root values were calculated. Eight replicates of each treatment were performed (n=8). All procedures were performed under sterile conditions in a laminar flow cabinet.

Plant growth conditions

Plants were generally grown in pots of 1 l containing sterile peat moss:perlite (1:1, v:v, autoclaved twice at 120°C for 20 min) within a greenhouse under controlled climatic conditions (18-24°C, 50-60%

relative humidity, 16 h:8 h light (600 μmol 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 two days.

Watering for well watered and drought treatments (Chapter 1)

Field capacity estimation: Eight pots of 1 l containing sterile peat moss:perlite were watered until saturation and let them drain for 24 hours. Pot weights assuming to correspond to water-holding capacity of 100 % were measured resulting in weight mean of 640.10 g. These pots were dried in a forced draught oven (70ºC, 7 days) and pot dry weights corresponding to water-holding capacity of 0 % were determined again resulting in weight mean of 163.91 g. Thus, watering regime treatments were performed estimating the grams of water that one gram of substrate is able to contain by extrapolating water-holding capacity (WHC) to substrate weight (SW) in our experimental system and following the equation:

$$SW = 4.7568*WHC + 163.91$$

All plants were grown for four weeks under well watered conditions supplying water every two days in order to maintain constant soil water content close to 100 % water-holding capacity. Watering regime treatments was applied from then on watering plants up to WHC of 100 % (640.10 g) and 60 % (448.10 g) for well watered and drought conditions, respectively.

Differential phosphorus conditions bioassay (chapter 3)

Ten-day-old seedlings were transferred to 1 l plastic pots containing sterile sand:perlite (1:3, v:v, autoclaved twice at 120°C for

20 min). This inert substratum was chosen in order to control plant nutrition by using Hewitt's nutrient solution (Hewitt 1966) every two days, maintaining constant soil water content close to water-holding capacity of 100% during the whole experiment. The experiment was carried out with two phosphorus treatments: control conditions (Control P; NaH_2PO_4 1 mM) and low phosphorus conditions (Low P: NaH_2PO_4 0.2 mM).

Biomass production determination

Plant growth was determined in order to evaluate PGPB activity. Shoots were separated from root systems and their fresh weights (FW) were measured. Samples were dried in a forced draught oven (70ºC, 3 days), and their dry weights (DW) were determined.

Relative growth rate (Chapter 2)

Relative growth rate (RGR) was determined in order to know how bacterial inoculation affects plant growth between juvenile and mature stage. RGR was calculated using the classical approach (Hunt 1982) following the equation where W1 and W2 are dry weights at times t1 an t2, respectively:

$$\mathbf{RGR = (\ln W2 - \ln W1)/(t2-t1)}$$

Bacterial ACC deaminase activity bioassay

PGPB strains were tested for the ability to use ACC as a sole nitrogen source in comparison with a positive control 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 (nmoles·mg^{-1} protein·h^{-1}) according to the procedure described by Penrose and Glick (2003), thanks to the collaboration of doctors B.R. Glick and M.C. Orozco-Mosqueda from Department of Biology of Waterloo University (Canada).

Gene expression analysis (Chapters 1 and 3)

Total RNA was isolated from root tissue from 3 different plants of each treatment (n=3) using Plant RNA Isolation Mini Kit (Agilent, Cat#5185-5998, California, USA) according to instructions of manufacturer. DNase treatment of total RNA was carried on membrane of column before washing steps using RNase-free DNase (Agilent, Cat#600032-51, California, USA). RNA integrity and quality were tested by gel electrophoresis as well as measuring 260/230 and 260/280 ratios in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The expression of genes was studied by real-time PCR using Brilliant III ultra fast SYBR® Green QRT-PCR master mix (Agilent, Cat#600866, California, USA) and an iCycler 5 device (Bio-Rad, Hercules, California, USA) according to Agilent's instructions. Each 10 µl reaction contained 5 µl of 2x SYBR Green QRT-PCR Master Mix, 0.2 µl of each primer pair, 0.1 µl of 100 mM DTT, 0.5 µl of RT/RNase block, 3 µl of RNase-free H_20 and 1 µl of a dilution 1:10 of the extracted RNA (100 nmol/µl). The PCR program consisted of 10 min incubation at 50ºC to perform retro-transcription and 3 min incubation at 95°C followed by 40 cycles of 5 s at 95ºC and 10 s at 58ºC, where the fluorescence signal was measured. The specificity of

PCR amplification procedure was checked using a heat dissociation protocol ranging 60-100ºC after the last PCR cycle.

Each QRT-PCR was carried out in duplicate with three independent biological replicates (n=3). The obtained values were normalized using the threshold cycle (Ct) value for the tomato constitutive elongation factor (*EF*) gene. Different putative constitutive genes (actin, tubulin, ubiquitin and elongation factor) were tested. The elongation factor was selected as constitutive using the "Normfinder" algorithm (Andersen et al. 2004) because it was the most stable gene between all considered treatments. The relative abundance of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Negative controls without RNA were used in all PCR reactions. The primers used to amplify each analyzed gene are shown in the following tables:

Table M1 Primers of analyzed genes by QRT-PCR to validate microarray data (Chapter 1).

Gen ID	Forward	Reverse
Les.1334.1.A1_at	5'-GACTTTGGGCTTGCGAAAC-3'	5'-GTCCAACATTCCCATTAGCAG-3'
Les.1842.1.S1_at	5'-ACCCATAGGCTTGAAGAGCA-3'	5'-AGCTCCTCTGTCTCCCTTTGA-3'
Les.2063.1.A1_at	5'-GGCGTTATAAGGAACCACCA-3'	5'-GTAGGCTTGTTGAAAAGGAAAAG-3'
Les.5253.1.S1_at	5'-TACTTGAAAGGACCCGCAAC-3'	5'-CCGACATCACTGGTTGACAC-3'
Les.5416.1.S1_at	5'-GGTGGAGCCGTATACTTGGA-3'	5'-CATTACACGCACCACCTCAC-3'
LesAffx.21605.1.S1_at	5'-GGCTTATTCACCAACCCAGA-3'	5'-TTCCACGTCTCGAAACCTCT-3'
LesAffx.57363.1.S1_at	5'-AGCACAAGGGATATGGTTGC-3'	5'-ATCGATGTCTGTTCCATTGCT-3'
LesAffx.65198.1.S1_at	5'-TGTTGGAGATTCAGCTGTGG-3'	5'-TTGTCCAGCAGTGTCCCATA-3'

Table M2 Primers of ethylene-related genes analyzed by QRT-PCR (Chapter 1).

Gen ID	Forward	Reverse
Sl EF	5′-GATTGGTGGTATTGGAACTGCT-3′	5′-AGCTTCGTGGTGCATCTC-3′
Sl ACS 1	5′-CGGGCTAGTTTCAACTCAGA-3′	5′-CAACAACAACAAATCTAAGCCA-3′
Sl ACS 2	5′-GAGGTTAGGTAAAAGGCACA-3′	5′-CATACGCTAACAACTATTTCT-3′
Sl ACS 3	5′-CGGTCTCCCCGGTTTTCGCA-3′	5′-GTGGCCGCGGACACAACCAT-3′
Sl ACS 4	5′-GCTAGCTTTCATGTTGTCTGA-3′	5′-GCACGAGCCTGGGCGAATCTA-3′
Sl ACS 5	5′-CACAGTATTCGATTGGCCAAAAT-3′	5′-AAATCATGCCAACTCTGAAACCTG-3′
Sl ACS 6	5′-GGGTTTCCTGGATTTAGGGT-3′	5′-GGTACTCAGTGAAATAGTCGA-3′
Sl ACS 7	5′-TGCCTTGAGAGCAATGCTGGGT-3′	5′-ACATGCACGAAACCAACCCGGT-3′
Sl ACS 8	5′-AGAGAACGATAGTCTGTGTGAACAA-3′	5′-GGACCGAGTGCATTCTCTACA-3′
Sl ACO 1	5′-TCCGCGCTCATACAGACGCA-3′	5′--AGTGGCGCATGGGAGGAACA3′
Sl ACO 2	5′-GCATCCTTCTACAATCCAGGA-3′	5′-CATGTAGTAGGGACGCACA-3′
Sl ACO 3	5′-GAGCGTGATGCACAGAGTGA-3′	5′-CAATCACACACACATACACCA-3′
Sl ACO 4	5′-TTCGCGCTCACACGGATGCT-3′	5′-CACCTCTAGCTGATCGCCGAGG-3′
Sl ACO 5	5′-GGCCCTAGATTTGAGTCTGCC-3′	5′-ATCCTTCTTCCTCAATGCCCA-3′
Sl ACO 6	5′-GGGAATGGGAAGAAAAGATTGTT-3′	5′-CCTCTTAACATATCACACTACCAGA-3′
Sl ETR 3	5′-GATCAGGTTGCTGTCGCTCT-3′	5′-TCATCACAGCAAGGAAGTCG-3′
Sl ETR 6	5′-TACGTGGTGTGGAGGTTCTG-3′	5′-TGTAACGAGGACACAACGGG-3′
Sl TCTR 1	5′-TGAAGCTTGCTGGGCTAATG-3′	5′-TGTGTGACCTGGTGGAGGTA-3′
Sl ERF1	5′-ATTGGGGTTCTTGGGTCTCC-3′	5′-GGACCACACATTAGCCTTGC-3′
Sl ER 5	5′-TGCCAGTGAAGGTACCTCAC-3′	5′-ATGGAAGCTTGATCTCGCCG-3′
Sl ER 21	5′-TGGTGCAGGCCCTAAGATTG-3′	5′-TAGACTGGGTGAAATATCGAAGG-3′
Sl ER 24	5′-TGAAGGCCCAAGACCTGAAG-3′	5′-CGCCGCCTCATCTAGCTTTC-3′

Table M3 Phosphate transporter genes primers analyzed by QRT-PCR (Chapter 3).

Gen ID	Forward	Reverse
Sl PT1	5′-GGGAAGAGGAAACTGTAGCTG-3′	5′-TTCTAATCCCAAATACCACAA-3′
Sl PT2	5′-AGTGGGAGCGTATGGGTTCTTA-3′	5′-TTCCAAGTGCATTGATACAGCC-3′

Transcriptomic analysis (Chapter 1)

Sample preparation for microarray

Total RNA was isolated from root tissue from 3 different plants of each treatment (n=3) using RNeasy Plant Mini Kit (Qiagen, Cat#74904, California, USA). RNA quality was assessed by gel electrophoresis as well as measuring 260/230 and 260/280 ratios in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and Bioanalyzer RNA kit (Bionalyzer® 2100 Agilent Technologies).

Total RNA was processed to use on GeneChip® Tomato Genome Array (Affymetrix) according to the manufacturer's protocol by Genomic Analytical service of Cabimer (CSIC), Seville, Spain. Total RNA (10 μg) was used in a reverse transcription reaction to generate first-strand cDNA, using the SuperScript choice system (Invitrogen) with oligo(dT) 24 fused to T7 RNA polymerase promoter. After second-strand synthesis, target complementary RNA (cRNA) labelled with biotin was prepared using the BioArray high-yield RNA transcript labeling kit (Enzo Biochem, New York) in the presence of biotinylated UTP, and CTP. After purification and fragmentation, cRNA (15 μg) was used in a hybridization mixture where hybridization controls were added. Hybridization mixture (200 μl) was hybridized on arrays for 16 h at 45°C. Standard post-hybridization wash and double-stain protocols were used on an Affymetrix GeneChip fluidics station 450. Finally, arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

Microarray validation

Microarray data were validated using QRT-PCR according to Morey *et al.* (2006). A set of genes was selected for validation based on their p-value ($P < 0.05$) and assayed by QRT-PCR as before described in gene expression analysis section using the primers showed in Table M1. Relationships between microarrays and QRT-PCR date were examined by Pearson's correlation. The data input into the correlation analysis was the Log2 value of the weighted average for each gene from all replicate plants. Correlations above 0.8 between microarray and QRT-PCR results are indicative of strong agreement and consider the study validated (Morey *et al.* 2006). Correlations for selected differentially expressed genes (DEG) are showed in the following table:

Table M4 Correlation values between microarray and QRT-PCR data for microarray validation.

Gen ID	ANOVA p-value	Correlation
Les.1334.1.A1_at	5.84E-06	**0.869**
Les.1842.1.S1_at	8.89E-11	**0.985**
Les.2063.1.A1_at	4.10E-06	**0.915**
Les.5253.1.S1_at	3.30E-08	**0.957**
Les.5416.1.S1_at	2.35E-07	**0.943**
LesAffx.21605.1.S1_at	2.49E-08	**0.952**
LesAffx.57363.1.S1_at	0.00089252	**0.961**
LesAffx.65198.1.S1_at	2.89E-09	**0.974**

Microarray data analysis

In order to evaluate bacterial inoculation effects on wt and *nr* plants, the transcriptomic profiles of Bm-inoculated and C7-inoculated plants were compared with those of non-inoculated ones as well as with each others. The Plant MetGenMap Software (Joung et al. 2009) was used to identify changed pathways from genomic profile data and to visualize the profile data in a biochemical pathway context using a threshold of fold change over 2. The Venn diagram webtool software (Open Source; http://bioinformatics.psb.ugent.be/webtools/Venn/) developed by Ghent University was used to quantitatively compare transcriptomic profiles with those obtained under different experimental conditions.

Physiological Parameters (Chapter 2)

Samples were taken from the last expanded leaf for stomatal conductance, photosynthetic efficiency and chlorophyll content (n=9).

Stomatal conductance

Stomatal conductance was measured three hours 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 & Baker (1997).

Leaf chlorophyll concentration

Photosynthetic pigments were extracted from leaf samples (0.5 cm^2) in 100% methanol at 4ºC for 24 h. Pigment concentration was spectrophotometrically determined according to Lichtenthaler (1987).

Nutrient measurement (Chapters 2 and 3)

Mineral analysis was determined in shoots and roots (n=4). Carbon and nitrogen concentration (% DW) 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. Calcium, potassium, magnesium, sodium, phosphorus, sulfur, and silicon concentration (% DW) as well as copper, iron, manganese, and zinc concentration (ppm) analyses were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES Varina ICP 720-ES) and were performed by Instrumentation Service of the Estación Experimental del Zaidín (CSIC), Granada, Spain.

Phytohormone analysis

Ethylene determination

Ethylene production was analyzed using gas chromatography (GC). Samples were introduced in 20 ml vials (Supelco Analytical, Pennsylvania, USA). Vials were closed and incubated 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.

Ethylene production by tomato tissues (Chapter 1 and 2)

Completely expanded leaflets from the last developed leaf, and entire roots were chosen for shoot and root ethylene determination, respectively. Samples were introduced in vials with 200 µl of miliQ 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). Ethylene production rate (nmol ethylene h^{-1} DW g^{-1}) was evaluated using six replicates per treatment in chapter 1, while six and four replicates per treatment were used in 4 and 8 wpi harvests, respectively, in chapter 2.

Ethylene production by bacterial strains (Chapter 1)

Bacterial ethylene production was measured in order to determine possible effects of microbially-derived ethylene in plant-bacteria interaction and/or growth promotion. Bacteria were grown in Luria broth (LB) medium with shaking (200 rpm) at 28ºC overnight. Culture OD_{600} was measured and new subcultures (LB, 6 ml, OD_{600} = 0.01) were started in sterile 20 ml vials (Supelco Analytical, Pennsylvania, USA). Vials were closed and incubated at 28ºC with shaking (200 rpm). Samples of 1 ml were withdrawn from each vial and ethylene production was quantified as abovementioned at 3, 6, 9 and 24 hours after starting the culture. Six replicates per bacteria and LB without inoculum were analyzed (n=6).

Ethylene production by bacterial-inoculated seedlings (Chapter 1)

Ethylene production by tomato wt and *nr* plantlets induced by inoculation of bacterial strains was also analyzed. Seeds were sterilized as described above and directly planted within 20 ml vials (Supelco Analytical, Pennsylvania, USA) containing sterile peat moss:perlite (1:1, v:v, autoclaved twice at 120°C for 20 min). Plantlets were grown for ten days without vial lid under well watered conditions in growth chamber under controlled conditions (18-24°C, 50-60% relative humidity, 16 h:8 h light (600 μmol m^{-2} s^{-1}):dark). Bacterial strains were cultured and ten-days-old seedlings were inoculated as before mentioned. Vials were immediately closed and incubated inside the growth chamber. Moreover, vials without seedlings but containing substrate were also inoculated in order to assay ethylene production by PGPB strains and/or substrate. Ethylene production rate was evaluated on inoculation day (7 hours post-inoculation) and the day after inoculation (26 hours post-inoculation). Ethylene was determined as above mentioned using four replicates per treatment.

Other phytohormones determination

Indole-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and jasmonoyl isoleucine (JA-Ile) were analyzed using high performance liquid chromatography-electrospray ionization-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS) thanks to collaboration of doctors José María García-Mina and Ángel María Zamarreño from Department of Environmental Biology of Navarra University (Pamplona, Spain).

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 N_2) was homogenized with 2.5 ml of precooled methanol:water:HCOOH (90:9:1, v/v/v with 2.5 mM Na-diethyldithiocarbamate) and 25 µl 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 (20000 g, 10 min), shaken again for 20 min and centrifuged. 2 ml of pooled supernatants were taken and dried at 40ºC. The residue was dissolved in 500 µl of methanol:0.133% acetic acid (40:60, v/v) and centrifuged (20000 g, 10 min) before being injected in an HPLC-ESI-HRMS system. Reagents and internal standards are shown in the following table:

Table M5: Reagents and Standards used for phytohormone analysis by HPLC-ESI-HRMS.

	Compound	Source
Reagents	Indole-3-Acetic acid (IAA)	OlChemin Ltd (Olomouc, Czech Republic)
	Cis,trans-Abscisic acid (ABA	
	(-)-Jasmonic acid (JA)	
	N-(-)-Jasmonoyl Isoleucine (JA-Ile)	
	Salicylic acid (SA)	Sigma-Aldrich (St Louis USA)
Internal Standars	$^2H_5$-Indole-3-Acetic acid (D-IAA)	OlChemin Ltd (Olomouc, Czech Republic)
	$^2H_6$-(+)-cis,trans-Abscisic acid (D-ABA)	
	$^2H_2$-N-(-)-Jasmonoyl Isoleucine (D-JA-Ile)	
	$^2H_4$-Salicylic acid (D-SA)	
	2H5-Jasmonic acid (D-JA)	CDN Isotopes (Pointe-Claire, Quebec, Canada)

Hormones were quantified using a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, 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 Sci). A reverse-phase column (Synergi 4 mm Hydro-RP 80A, 150 x 2 mm; Phenomenex, Torrance, CA) 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 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 15ºC, respectively. The detection and quantification were performed using a Full MS experiment with MS/MS Confirmation in the negative-ion mode. Instrument control and data processing were performed by TraceFinder 3.3 EFS software. Instrumental parameters and compound accurate masses are reported in the following tables:

Table M6 Instrumental parameters used for HESI (II) ionization.

Instrumental parameters	Value
Sheath gas flow rate	44 au
Auxiliary gas flow rate	11 au
Sweep gas flow rate	1 au
Spray voltage	3.5 kV
Capillary temperature	340 ºC
S-lens RF level	50
Auxiliary gas heater temperature	300 ºC

Table M7 Accurate masses of the phytohormones and internal standards, and its principal fragments

Analyte	[M-H] Phytohormone	[M-H] Fragment
IAA	174.05605	130.06615
ABA	263.12888	219.13900
SA	137.02442	93.03401
JA	209.11832	59.01297
Ja-Ile	322.20238	130.08735
D-IAA	179.08744	135.09760
D-ABA	269.16654	225.17668
D-SA	141.04952	97.05915
D-JA	214.14970	61.02555
D-JA-Ile	324.21494	131.09370

Metabolite analysis (Chapter 2)

Metabolite analysis was performed by gas chromatography-mass spectrometry (GC-MS) according to Roessner *et al.* (2000). Samples were assayed by Scientific Instrumentation Service of the Estación Experimental del Zaidín (CSIC), Granada, Spain. 100 mg of frozen tissue (previously ground to a powder in a mortar with liquid N_2) was extracted with 1 ml of methanol containing internal standard (ribitol 9 µg ml^{-1} in methanol). The mixture was extracted 20 min at 4°C and 400 µl of water were added and mixed before centrifugation (18626 g, 5 min). Two aliquots of supernatant were taken for analysis of major components (10 µl) and minor components (200 µl) after dried overnight under vacuum. The residue was derivatized in two steps, metoxymation and silylation (60 µl of methoxyamine hydrochloride in pyridine, 37°C 90 min and BSTFA+TMCS, 37ºC 30 min.)

A Varian (now Bruker) 450GC 240MS system was used for GC-MS analysis. All samples were analyzed twice. 1 µl was injected at

230°C in split 1/50 mode with Pressure Pulse (30psi 0.2min). He flux at 1 ml min^{-1} and temperature ramp started at 70°C 5 min, increased at 5°C until 245°C, increased at 20°C until 310°C and kept for 1 min. Column DB-5ms (30 m x 0.25 mm, 0.25 µm). Ionization by electronic impact and mass analysis in TIC Full Scan mode acquiring masses in the range 50-600 m/z. Identification by comparison with 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.

Proteomic analysis (Chapter 3)

Proteomic analyses were performed during the short-stay in Department of Plant Physiology of Faculty of Agriculture of Iwate University (Japan) under supervision of Dr. Matsuo Uemura.

Microsomal fraction preparation

Microsomes were isolated as described in Hachez *et al.* (2006) with some modifications. Frozen root tissue (500 mg) was homogenized with 5 ml of grinding buffer (sorbitol 250 mM, Tris-HCl 50 mM (pH 8), EDTA 2 mM; and proteinase inhibitor cocktail mix: phenylmethylsulfonyl fluoride 1 mM, leupeptin 1 g l^{-1}, aprotinin 1 g l^{-1}, antipain 1 g l^{-1}, chymostatin 1 g l^{-1} and pepstatin 1 g l^{-1}. The mixture was filtered by a nylon mesh and then centrifuged (4400 g, 10 min). The supernatant was collected carefully and centrifuged (100000 g, 2 h). Finally, the supernatant was removed and the resulting pellet was

resuspended in 30 µl of suspension buffer (KH_2PO_4 5 mM, sucrose 330 mM and KCl 3 mM with a final pH of 7.8). An aliquot of the samples was used for protein quantification by Bradford assay (Bradford 1976) and the remainder was kept at -20ºC.

Sample preparation for nano-LC-MS/MS analysis

Microsomal protein samples (equivalent to 5 µg protein) were subjected to in-gel tryptic digestion for peptide analysis according to Takahashi *et al.* (2011).

Nano-LC−MS/MS analysis and data acquisition

The peptide solutions were subjected to nano-LC-MS/MS analysis. The peptide solutions were concentrated with a trap column (L-column Micro 0.3 × 5 mm; CERI, Japan) on an ADVANCE UHPLC system (MICHROM Bioresources, Auburn, CA), and eluted with formic acid 0.1 % in acetonitrile (v/v). Eluted peptides were separated by a Magic C18 AQ nano column (0.1 × 150 mm; MICHROM Bioresources) using a linear gradient of acetonitrile (from 5 to 45 % (v/v)) at a flow rate of 500 nl min^{-1}.

Then, peptides were ionized by an ADVANCE spray source (MICHROM Bioresources) with spray voltage of 1.8 kV. Mass analysis was carried out by using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Full-scan mass spectra were obtained in the range of 400 to 1800 m/z with a resolution of 30000. Collision-induced fragmentation was applied to the five most intense ions at a threshold above 500. These experiments were

performed with three three-plant pool-samples per treatment collected from 9 biologically independent plants (n=3).

Peak detection and data processing were carried out with Progenesis Ql for proteomics Software (Nonlinear Dynamics). Parameters used for file conversion were: precursor mass range (m/z 350-5000), highest and lowest charge state (0), the minimum total intensity of a spectrum (0), and the minimum number of peaks in a spectrum (1). Progenesis Ql software automatically selects a reference file (control "gel") in order to align the rest of files. A manual review process was carried out in order to get an overlapping above the threshold (70 %). Finally, Progenesis Ql for proteomics software automatically aligns all files resulting in a list of detected and quantified peptides by LTQ-Orbitrap XL.

Protein identification was performed using the Mascot search engine v. 2.5.0 (Matrix Science; London, UK) and the solanaceous protein database of international tomato annotation group (ITAG) v. 2.3. Search parameters were: monoisotopic mass accuracy, trypsin digestion, peptide mass tolerance (5 ppm), MS/MS tolerance (0.6 Da), allowance of missed cleavage, fixed modification (carbamidomethylation (Cys)), variable modification (oxidation (Met)) and peptide charges (+1, +2, +3). Positive identification was attributed with Mascot p-values under the threshold ($P< 0.05$) and false discovery rate 5 %. Progenesis Ql for proteomics software was used to associate peptide and proteins information and perform the statistical analysis of obtained data. Significant differences were assigned to proteins under the threshold ($P< 0.05$) in comparisons between treatments.

In order to evaluate bacterial inoculation effects on wt and *nr* plants, the proteomic profiles of Bm-inoculated and C7-inoculated plants were compared with those of non-inoculated ones as well as with each other. The Plant MetGenMap Software (Joung et al. 2009) was used to identify changed pathways from protein profile data and to visualize the profile data in a biochemical pathway context using a threshold of fold change over 4. The BioVenn software (Hulsen et al. 2008) was used to compare quantitatively protein profiles with those obtained under different experimental conditions.

Phosphate solubilization bioassay (Chapter 3)

Bacterial strains were tested by plate bioassay in order to evaluate phosphate solubilizing ability of PGPB strains according to Nautiyal (1999), with some variations. National Botanical Research's Institute phosphate growth medium (glucose 10 g l^{-1}, $Ca_3(PO_4)_2$ 5 g l^{-1}, $MgCl_2$.6 H_20 5 g l^{-1}, $MgSO_4$.7 H_20 0.25 g l^{-1}, KCl 0.2 g l^{-1} and $(NH4)_2S0_4$ 0.1 g g l^{-1}) supplemented with 1.5 % Bacto-agar was used in petri dishes. The medium pH was adjusted to 7.0 before autoclaving. Bacterial suspension droplets (5 µl, $D.O._{600\ nm}$=1.0) were plated and five replicates per bacterial strain were tested (n=5). A *Pseudomonas* sp. strain (C+ p12) was kindly provided by Ana. V. Lasa from the laboratory of Manuel Fernández-López from Estación Experimental del Zaidín (CSIC, Granada, Spain) and used as positive control. The colony and the halo (zone of clearance surrounding the bacterial colony) diameters were measured after 10 days of the plate incubation at 28ºC. Phosphate solubilizing index (PSI) was calculated as the ratio of halo diameter (mm) and colony diameter (mm) (Kumar and Narula 1999).

Antioxidant enzymatic activities (Chapter 3)

The extraction of enzymes were performed as described Aroca *et al.* (2003) with some variations. Frozen root sample (200 mg, previously ground to a powder in a mortar with liquid N_2) were homogenized with 1.6 ml phosphate buffer 100 mM (pH 7.0) containing KH_2PO_4 60 mM, K_2HPO_4 40 mM, DTPA 0.1 mM, and PVPP 1 % (w/v) by shaking in vortex. The homogenate was centrifuged (18000 g, 10 min, 4ºC) and the supernatant was used for enzyme activity determination. Total soluble protein was determined according to Bradford (1976), using bovine serum albumin as standard.

Glutathione reductase (EC 1.20.4.2) activity was spectrophotometrically determined by measuring the absorbance reduction at 340 nm due to the NADPH oxidation (Carlberg and Mannervik 1985). The reaction mixture (200 µl) contained buffer solution (Tris buffer 50 m*M*, $MgCl_2$ 3 m*M*, oxidized glutathione (GSSG) 1 m*M*, pH 7.5), and 10 µl of enzyme extract, and NADPH 0.3 mM was added and mixed thoroughly to begin the reaction. The results were expressed in nmol of oxidized NADPH per µg of total protein per minute, and the enzymatic activity was calculated using the initial reaction speed and the calibration curve of NADPH ranging from 0 to 0.3 mM.

Ascobate peroxidase (EC 1.11.1.11) activity was measured in a 200 µl reaction volume containing potassium phosphate buffer 80 mM (pH 7.0), hydrogen peroxide (H_20_2) 0.25 mM, and sodium ascorbate 0.5 mM and 10 µl of enzyme extract. Enzymatic activity was spectrophotometrically determined by measuring the absorbance

reduction at 290 nm during 1 min to determine the ascorbate oxidation rate when H_20_2 was added to start the reaction and the calibration curve of sodium ascorbate ranging from 0 to 0.5 mM (Amako et al. 1994).

Superoxide dismutase (EC 1.15.1.1) activity was evaluated according to Burd *et al.* (2000). SOD is able to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the enzyme amount required to inhibit the reduction rate of NBT by 50% at 25ºC.

Catalase (EC 1.11.1.6) activity was spectrophotometrically measured according to Aebi (1984). Enzymatic activity was determined by measuring consumption of H_20_2 at 240 nm for 1 min and the calibration curve of H_20_2 ranging from 0 to 8 mM. The reaction mixture consisted of potassium phosphate buffer 50 mM (pH 7.0) containing H_20_2 10 mM and 5 µl of enzyme extract in a 200 µl volume.

Antioxidant compounds determination (Chapter 3)

Root samples were used in order to measure ascorbic acid and reduced and oxidized glutathione forms (GSH and GSSG, respectively) contents. Antioxidant molecule analysis was carried out by liquid LC-MS according to Airaki *et al.* (2011) with some variations. Frozen tissue (150 mg, previously ground to a powder in a mortar with liquid N_2) was extracted with 1 ml of HCl 0.1 N. The mixture was extracted by shaking in vortex 1 min and kept at 4ºC. Homogenates were centrifuged (17000 g, 40 min, 4ºC) and the supernatants collected, filtered with a syringe through Nylon membrane filters (0.20 µm). A 10 µl aliquot of filtered fraction was immediately taken for analysis. All

procedures were done at 4ºC and protected from light to avoid potential analyte degradation.

Samples were assayed by Scientific Instrumentation Service of the Estación Experimental del Zaidín (CSIC), Granada, Spain. An alliance 2695 separation module connected to Quattro Micro triple quadrupole mass spectrometer detector (Waters) was used for LC-MS analysis. The compounds were detected by electrospray mode, using an orthogonal Z-spray electrospray interface (Quattro Micro, Waters). Instrument control, data collection, analysis and management were controlled by the MassLynx 4.1 software package. Separation was carried out using an Atlantis T3 Column (3 x 150 mm, 3 µm, Waters). Analyte concentrations were calculated from a standard curve constructed using commercial analytes.

Statistical analyses

Data were processed using R software (v3.2.2 Open Source; http://www.r-project.org/) by two- or three-way ANOVA depending on number of sources of variation in order to know if there is interaction between independent variables on the dependent variable. Significance of source of variation interaction was evaluated by P-value ($P < 0.05$).

- In case of significant interaction between factors, all treatments were compared between each other's by least significant difference (LSD) test ($P < 0.05$).

- In case of no interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA

followed by LSD's test ($P < 0.05$). Additionally, plant genotype effect was evaluated analyzing wt and *nr* plants under the same conditions by t-Student test ($P < 0.05$).

Relationships between variables were examined using the Pearson correlation coefficient ($P < 0.05$). Principal component analysis (PCA) was used to compare profiles with those obtained under different experimental conditions using Excel add-in Multibase package (v_2015 Open source; http://www.numericaldynamics.com/).

Statistical analyses in chapter 1

Plant dry weight, gene expression and shoot ethylene production data were processed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Ethylene produced by seedlings data were also processed by three-way ANOVA with plant genotype (G), time after inoculation (D) and inoculum (I) as sources of variation. Bacterial colonization and bacterial ethylene production were processed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. ACC deaminase activity was evaluated using ANOVA followed by LSD's test ($P < 0.05$).

Microarray data were analyzed with statistical tool LIMMA (Linear Models for Microarrays Analysis) by using affylmGUI (Wettenhall et al. 2006) in R software, and significant differences were assigned to genes under the threshold ($P < 0.05$) in comparisons between treatments.

Statistical analyses in chapter 2

Plant dry weight, RGR, stomatal conductance, photosynthetic efficiency, chlorophyll, nutrient, phytohormone and metabolite contents data were processed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation.

Relationships between total, shoot and root dry weights and nutrients, and metabolites were examined using the Pearson correlation coefficient. Principal component analysis (PCA) was also used to compare nutrient and metabolite profiles with those obtained under different experimental conditions.

Statistical analyses in chapter 3

Fresh weight, root phosphorus content, expression of phosphate transporter genes, antioxidant enzymatic activities, and antioxidant compounds contents obtained in main experiment of chapter 3 were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Phosphate solubilizing index was evaluated using ANOVA followed by LSD's test ($P < 0.05$).

Progenesis Ql for proteomics software was used to perform the statistical analysis of obtained proteomic data by ANOVA followed by LSD's test ($P < 0.05$). PCA was also used to compare root proteomic profiles with those obtained under different experimental conditions.

In case of differential phosphorus conditions bioassay, dry weight, root and shoot phosphorus content and expression of phosphate transporter genes were processed by three-way ANOVA with genotype (G), phosphorous regime (P), and inoculum (I) as sources of variation.

Chapter 1: Ethylene sensitivity by ETR3 is essential in tomato interaction with *Bacillus megaterium* but not with *Enterobacter* C7

Chapter 1: Ethylene sensitivity by ETR3 is essential in tomato interaction with *Bacillus megaterium* but not with *Enterobacter* C7

Objective

The present chapter aims to shed light on plant-microbe interactions as well as plant growth promotion mediated by bacterial strains regarding to ethylene sensitivity under well watered and drought conditions, with focus in ethylene transduction pathway. PGPB are able to induce plant growth either directly or indirectly (Ortíz-Castro et al. 2009). Ethylene is typically induced in response to environmental stresses as drought (Pierik et al. 2007). Some PGPB were defined as stress controllers (Lugtenberg and Kamilova 2009), since they contain ACCd activity and they are able to reduce ethylene levels (Belimov et al. 2007; Glick 2014). Furthermore, transcriptomic approaches have contributed with valuable information to clarify plant-bacteria interaction (Cartieaux et al. 2003; Verhagen et al. 2004; van de Mortel et al. 2012). However, bacteria without either ACCd activity or ethylene production capacity have been selected in the present study to avoid any direct perturbation of plant ethylene metabolism caused by the bacteria.

We pursued to determine if ethylene sensitivity is decisive for plant growth promotion in mature plants by these two PGPB and clarify plant-bacteria interaction and PGPB mechanisms regarding to ethylene perception under both well watered and drought conditions. Plant growth was determined at 8 weeks post-inoculation and expression of

ethylene-related genes as well as ethylene production was assayed. Furthermore, the expression levels of tomato genes were simultaneously analyzed by microarray technology to highlight other plant significantly changed pathways involved in PGPB activity or interaction with plants.

Experimental design

The experiment consisted of a randomized complete block design with two tomato plant lines (wt and *nr*), two watering regimes (well watered (WW) and drought (D) conditions), and three inoculation treatments: (1) non-inoculated control plants, (2) *Bacillus megaterium*-inoculated plants and (3) *Enterobacter* C7-inoculated plants. Each treatment consisted in eleven replicates (n=11). Plants were harvested at 8 weeks post inoculation (Fig. 1.1).

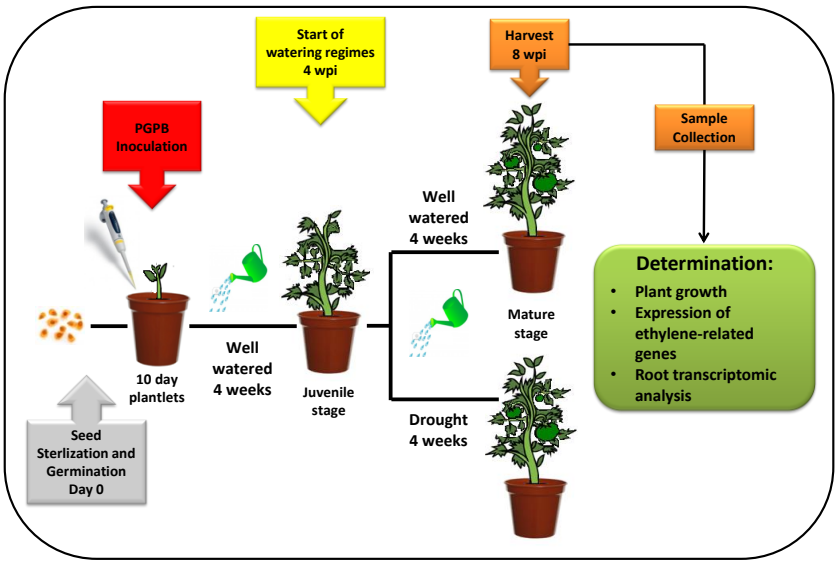

Figure 1.1 Schematic representation of experimental design of chapter 1. Weeks post-inoculation (wpi).

Results

Colonization of 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 $2x10^6$ CFU cm^{-1} root (Table 1.1). Furthermore, the PGPB strains did not show either ethylene production or the ability to cleave ACC the direct precursor of ethylene (data not shown).

Table 1.1 Bacterial root colonization. Colony-forming units (CFU) per root centimeter of *Bacillus megaterium* (Bm) and *Enterobacter C7* (C7) in wild type (wt) cv. Pearson and *never ripe* (*nr*) tomato plants. Data are means ± SE (n = 8). No significant differences were noticed according to LSD's test (P < 0.05).

Treatment		CFU root cm^{-1}	SE
Bm	wt	$2.55x10^6$	± $0.68x10^6$
	nr	$2.02x10^6$	± $0.41x10^6$
C7	wt	$1.85x10^6$	± $0.39x10^6$
	nr	$2.69x10^6$	± $0.76x10^6$

SE: standard error

Biomass production of wt and *nr* plants inoculated with *Bacillus megaterium* and *Enterobacter C7*

Dry weight of wt and *nr* plants inoculated with both PGPB (Bm or C7) showed similar growth patterns under WW and D conditions,

but interaction between main factors (GxWxI) was unnoticed (Fig. 1.2). PGPB inoculation promoted total plant growth in wt plants under WW (20.2% and 20.0% for Bm and C7, respectively) and D conditions (24.7% and 27.4% for Bm and C7, respectively). However, total DW only showed an increase in *nr* plants due to C7 inoculation (25.6% and 21.3% under WW and D conditions, respectively). Moreover, drought treatment significantly reduced total DW in both plant genotypes under all inoculation treatments (Fig. 1.2 A).

Bm inoculation only increased shoot DW in wt plants under drought conditions (26.5%). However, shoot DW was increased by C7 inoculation in both plant genotypes under both watering regimes (22.7% and 24.4% in wt plants under WW and D conditions, respectively, and 24.8% and 21.7% in *nr* plants under WW and D conditions, respectively). Furthermore, significant differences due to drought treatments were noticed in all cases excepting in wt plants under Bm inoculation (Fig. 1.2 B). Additionally, significant differences between wt and *nr* plants were only noticed in non-inoculated plants under WW conditions in total and shoot DWs, showing *nr* plants higher values than wt ones (Fig. 1.2 A, B).

In addition, root DW was increased by both PGPB inoculation in wt plants under WW (15.7% and 15.9% for Bm and C7, respectively) and D conditions (17.3% and 17.2% for Bm and C7, respectively). However, only significant differences due to C7 inoculation in *nr* plants were found under WW conditions (29.2%). Furthermore, drought treatment significantly reduced root DW in all cases, but no differences between plant genotypes were noticed (Fig. 1.2 C).

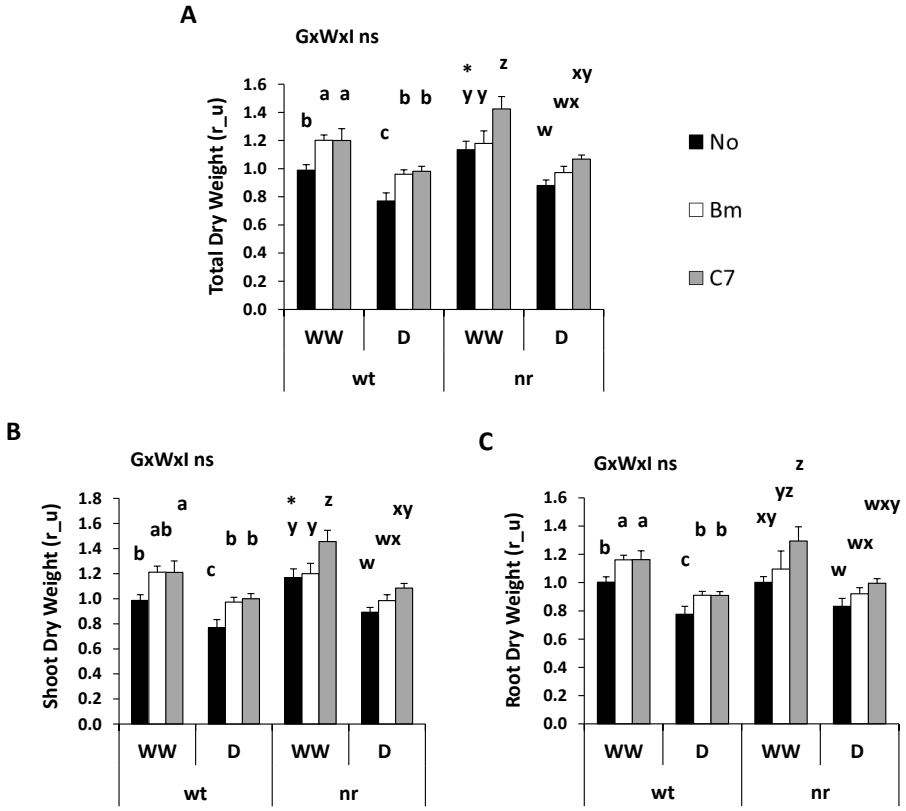

Figure 1.2 Effects of bacterial inoculation on plant dry weights under well watered and drought conditions. (A) Total, **(B)** shoot and **(C)** root dry weights of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. 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 ± SE (n = 7). Data were analyzed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxWxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. As no significant interaction between factors was noticed, inoculum effects under well watered and drought conditions were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

Modification of ethylene biosynthesis genes expression by PGPB inoculation regarding to ethylene sensitivity

PGPB inoculation, drought and ethylene insensitivity effects on expression of eight *ACS* and six *ACO* genes were evaluated (Fig. 1.3 and 1.4, respectively).

Expression of *ACS2* to *5* and *ACS8* genes in both plant tissues and *ACS7* gene in roots were unaffected by PGPB inoculation neither by plant genotype and drought treatment (data not shown). In roots, *ACS1* expression showed significant factor interaction (GxWxI). Exclusively, both bacterial strains decreased *ACS1* expression compared to non-inoculated wt plants under D conditions showing C7-inoculated plants lowest expression than Bm-inoculated ones. Moreover, drought treatment increased *ACS1* expression in roots in non- and Bm-inoculated wt plants (Fig 1.3 A).

ACS6 expression only showed significant difference between non-inoculated wt and *nr* plants under D conditions showing *nr* plants lower values than wt ones (Fig 1.3 B). Furthermore, no effects in *ACS7* expression were noticed in wt plants. Meanwhile, C7 inoculation increased *ACS7* expression in *nr* plants under WW conditions producing significant differences between C7-inoculated plants under different watering regimes (Fig 1.3 C).

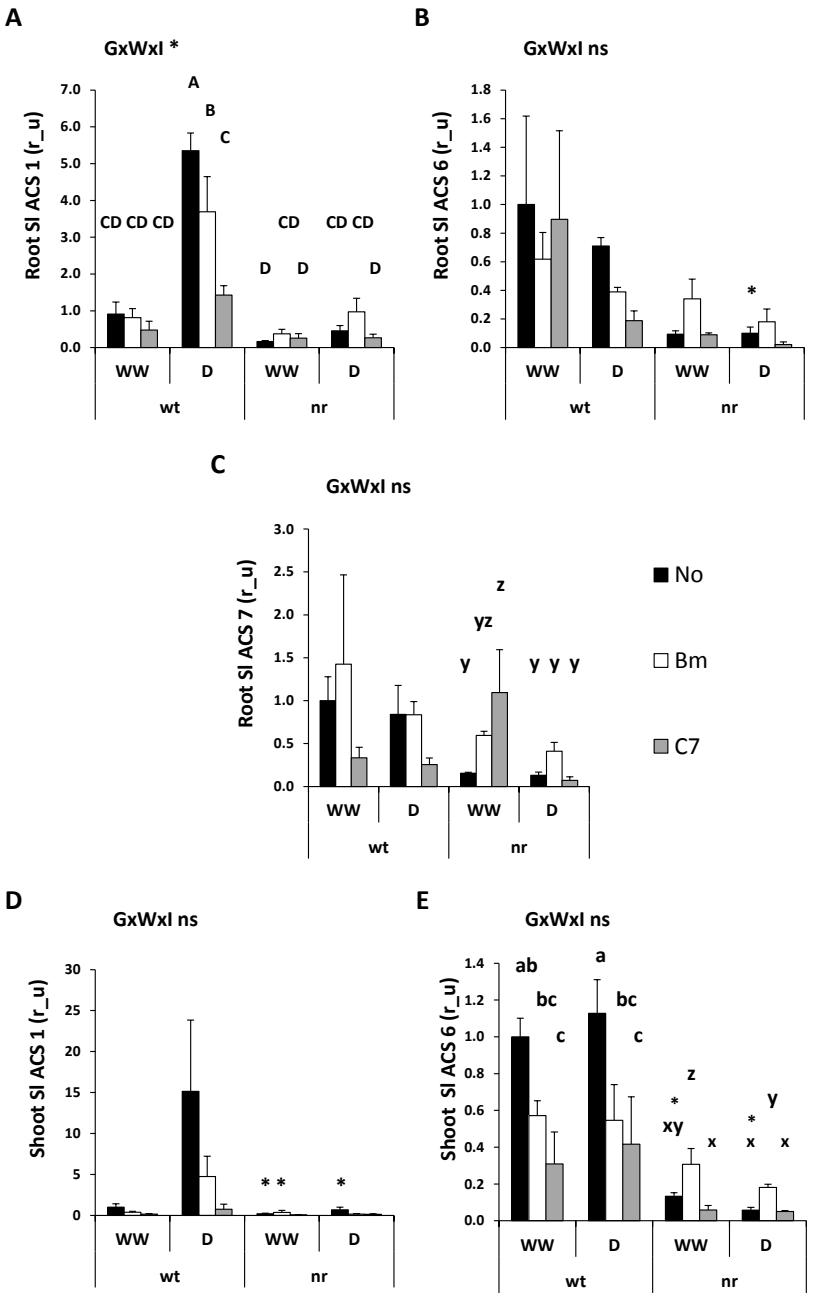

Figure 1.3 Effects of bacterial inoculation on ACC synthase (ACS) gene expression under well watered and drought conditions. Relative expression of **(A)** root ACS 1, **(B)** root ACS 6, **(C)** root ACS 7**, (D)** shoot ACS 1 and **(E)** shoot ACS 6 in wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. 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 ± SE (n = 3). Data were analyzed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxWxI) was evaluated by P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different (P < 0.05) according to LSD's test. In case of not-significant interaction between factors, inoculum effects under well watered and drought conditions were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) above *nr* means.

In shoots, *ACS1* expression was exclusively affected by plant genotype in non-inoculated plants under both watering regimes and in Bm-inoculated plants only under WW conditions always showing wt plants higher values than *nr* ones (Fig. 1.3 D). However, *ACS6* expression was affected by PGPB inoculation in both plant genotypes under both watering regimes. Bm inoculation decreased *ACS6* expression in wt plants exclusively under D conditions, while its levels were increased by Bm in *nr* plants independently of watering regime. C7 inoculation decreased *ACS6* expression in wt plants under both watering regimes, but no significant effect was noticed compared to Bm-inoculated wt plants. Furthermore, C7 inoculation did not affect *ACS6* expression in *nr* plants independently of watering regime. Significant effect of drought treatment was only observed in *nr* plants under Bm inoculation, causing a descent. In addition, significant differences between wt and *nr* plants were exclusively noticed in non-inoculated plants independently of watering regime (Fig. 1.3 E).

PGPB inoculation, drought treatment and plant genotype did not cause any effect in *ACO2* and *5* in shoots neither in ACO3 to 5 in roots (data not shown). Furthermore, no significant differences due to PGPB inoculation neither by drought treatment was noticed in root expression of *ACO1*, *2* and *6*. In these genes, only plant genotype differences were noticed, but in all cases wt plants showed higher expression values than *nr* ones (Fig. 1.4). *ACO1* and *ACO2* exclusively showed differences between plant genotypes in non-inoculated plants under WW and D conditions, respectively (Fig. 1.4 A, B). Moreover, *ACO6* showed differences between plant genotypes in non- and C7-inoculated plants only under WW conditions (Fig. 1.4 C).

In shoots, *ACO1* expression was unaffected by PGPB inoculation under D conditions in both plant genotypes. However, both bacterial strains decreased *ACO1* expression in wt plants, while Bm inoculation exclusively increased its expression in *nr* plants under WW conditions. Drought treatment decreased *ACO1* expression in non-inoculated wt plants and in Bm-inoculated plants independently of plant genotype. Moreover, significant differences between wt and *nr* plants were only noticed in non-inoculated plants under both watering regimes (Fig. 1.4 D). In *ACO3* expression, exclusively differences between plant genotypes were noticed in non- and C7-inoculated plants under WW conditions (Fig. 1.4 E). Only C7 inoculation decreased *ACO4* expression in wt plants independently of watering regime. In addition, significant differences between wt and *nr* plants were exclusively noticed in non-inoculated plants under WW conditions (Fig. 1.4 F).

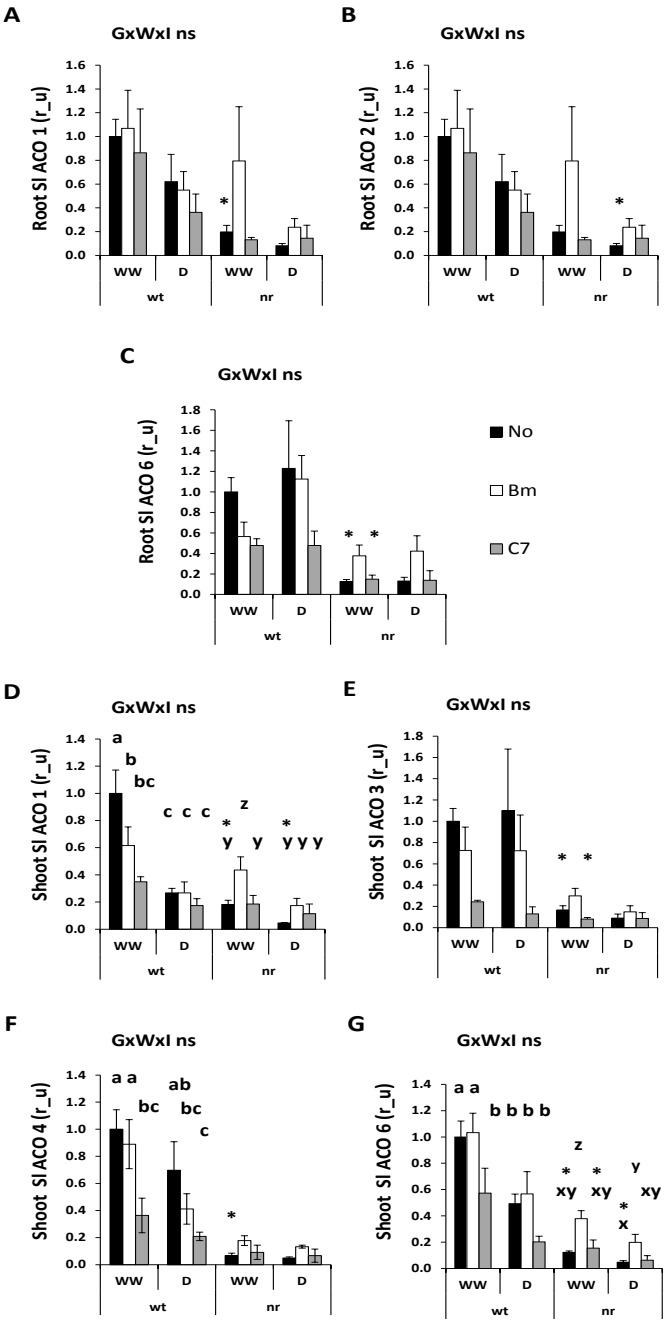

Figure 1.4 Effects of bacterial inoculation on ACC oxidase (ACO) gene expression under well watered and drought conditions. Relative expression of **(A)** root ACO 1, **(B)** root ACO 2 and **(C)** root ACO 6 **, (D)** shoot ACO 1, **(E)** shoot ACO 3, **(F)** shoot ACO 4 and **(G)** shoot ACO 6 in wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato

(*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. 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 ± SE (n = 3). Data were analyzed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxWxI)) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. As no significant interaction between factors was noticed, inoculum effects under well watered and drought conditions were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

Regarding *ACO6* expression, Bm inoculation exclusively increased its expression in *nr* plants independently of watering regime. However, C7 inoculation decreased *ACO6* expression only in wt plants under WW conditions. Significant effect of drought treatment was observed in non- and Bm- inoculated wt plants and exclusively in Bm-inoculated *nr* plants. Moreover, differences between plant genotypes were significantly noticed in non-inoculated plants independently of watering regime and in C7-inoculated plants only under WW conditions (Fig. 1.4 G).

Modification of expression of *Never Ripe* (ETR3) receptor and *TCTR1* genes by PGPB inoculation regarding to ethylene sensitivity

The effects of PGPB inoculation, drought treatment and plant genotype on expression of *ETR3* and TCTR1 (Tomato CTR1) genes were evaluated (Fig. 1.5).

In roots, Bm inoculation exclusively increased *ETR3* expression in *nr* plants under D conditions, while C7 inoculation did not show effects under both watering regimes. Moreover, differences between plant genotypes were exclusively noticed under C7 inoculation and WW conditions showing wt plants higher values than *nr* ones (Fig. 1.5 A).

In shoots, no PGPB effects were noticed in both genotypes under WW conditions. However, C7 inoculation decreased *ETR3* expression exclusively in wt plants, while Bm increased its expression in *nr* plants under D conditions. Moreover, drought treatment only increased shoot *ETR3* expression in non-inoculated wt plants and in Bm-inoculated *nr* plants. Significant differences between plant genotypes were noticed in non-inoculated plants independently of watering regime and under C7 inoculation in WW conditions always showing *nr* plants lower values than wt ones (Fig. 1.5 C).

Exclusively significant differences in root *TCTR1* expression were found between plant genotypes under Bm inoculation and D conditions, showing *nr* plants lower values than wt ones (Fig. 1.5 B). However, shoot *TCTR1* expression showed significant differences due to PGPB inoculation and between wt and *nr* plants. Bm inoculation increased *TCTR1* only under D conditions in *nr* plants. Moreover, non-inoculated *nr* plants showed lower *TCTR1* expression than wt ones under D conditions (Fig. 1.5 D). However, no significant differences were observed in wt plants (Fig. 1.5 B, D).

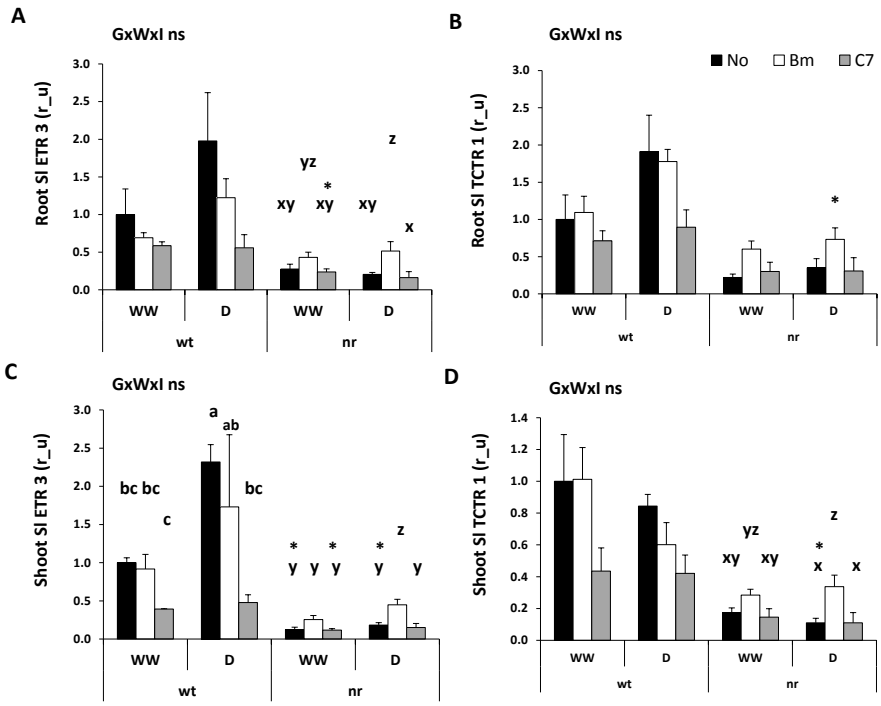

Figure 1.5 Effects of bacterial inoculation on expression of ethylene receptor (ETR3) and signaling (TCTR1) genes under well watered and drought conditions. Relative expression of **(A)** root ETR3, **(B)** root TCTR1, **(C)** shoot ETR3 and **(D)** shoot TCTR1 in wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. 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 ± SE (n = 3). Data were analyzed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxWxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ As no significant interaction between factors was noticed, inoculum effects under well watered and drought conditions were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

Modification of ethylene-responsive genes expression by PGPB inoculation regarding to ethylene sensitivity

In addition, expression of genes involved in ethylene response was also analyzed (Fig. 1.6). No significant changes were noticed in case of *ERF1* in root and shoot tissues, neither in *ER24* and *ER5* in roots (data not shown). In root, *ER21* expression interaction between main factors (GxWxI) was noticed, and exclusively Bm inoculation increased *ER21* expression in *nr* plants under WW conditions (Fig. 1.6 A).

In shoots, *ER21* expression was affected by bacterial inoculation in both plant genotypes. In wt plants, C7 inoculation reduced *ER21* expression values only under D conditions. Nevertheless, C7 induced *ER21* expression in *nr* plants exclusively under WW conditions. Moreover, *ER21* expression was increased due to Bm inoculation in *nr* plants only under D conditions. Drought treatment increased *ER21* expression in non-inoculated wt plants and in Bm-inoculated *nr* plants, while *ER21* expression was decreased by drought in *nr* plants under C7 inoculation. Differences between plant genotypes were only noticed in non-inoculated plants independently of watering regime showing *nr* plants lower values than wt ones (Fig. 1.6 B).

In addition, shoot *ER24* was exclusively affected by Bm inoculation in *nr* plants under WW conditions showing an expression induction by bacterial inoculation (Fig. 1.6 C). Regarding *ER5* expression, only non-inoculated wt plants significantly showed higher values than *nr* plants under D conditions (Fig. 1.6 D).

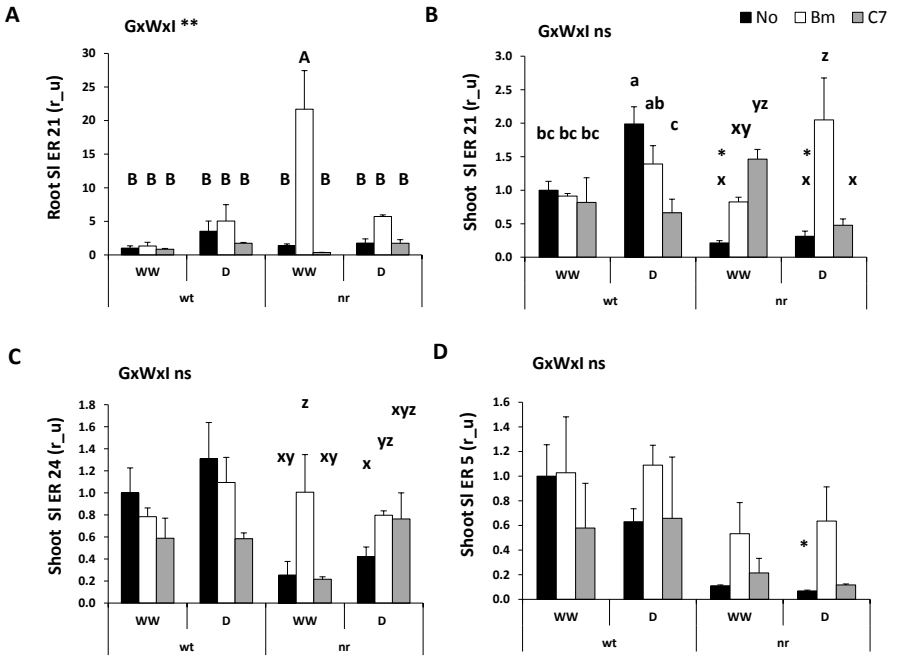

Figure 1.6 Effects of bacterial inoculation on ethylene-responsive gene expression under well watered and drought conditions. Relative expression of **(A)** root ER21, **(B)** shoot ER21, **(C)** shoot ER24, and **(D)** shoot ER5 in wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. 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 ± SE (n = 7). Data were analyzed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxWxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different ($P < 0.05$) according to LSD's test. In case of not-significant interaction between factors, inoculum effects under well watered and drought conditions were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

Modification of ethylene production by PGPB inoculation regarding to ethylene sensitivity

Ethylene emission by leaflets was determined, but no significant differences due to PGPB inoculation neither by drought treatment were observed in both plant genotypes. However, significant differences between wt and *nr* plants were noticed in C7-inoculated plants independently of watering regime, and in Bm-inoculated plants only under WW conditions and in non-inoculated plants exclusively under D conditions showing always *nr* plants higher values than wt ones (Fig. 1.7 A).

In addition, a bioassay for ethylene production was performed to test if ethylene levels emitted by seedlings are affected in response to bacterial inoculation (Fig. 1.7 B). As expected, no ethylene was detected in vials without seedlings independently of no, Bm or C7 inoculation of substrate (data not shown).

In case of wt plants, C7 inoculation exclusively induced ethylene production rate after 7 h of inoculation. In *nr* plants, only Bm increased ethylene production rate after 7 h of inoculation showing no significant effect 26 h after inoculation. In addition, differences between wt and *nr* plants in ethylene production rate were exclusively noticed under Bm inoculation 26 h post-inoculation showing *nr* plants higher ethylene production than wt plants (Fig. 1.7 B).

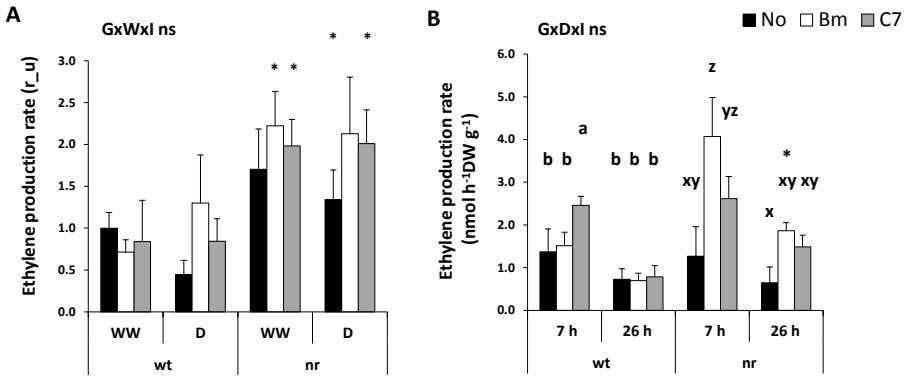

Figure 1.7 Ethylene emissions by tomato plants inoculated with PGPB under well watered and drought conditions. (A) Relative ethylene production rate in wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. Data are means ± SE (n = 6). Data were analyzed by three-way ANOVA with plant genotype (G), watering regime (W) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxWxI) was evaluated by P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001. As no significant interaction between factors neither PGPB inoculation effects were noticed, plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) above *nr* means. **(B)** Ethylene emission (nmol) per hour and gramme of dry weight (DW) in wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plantlets at 7 and 26 hours post-inoculation. Data are means ± SE (n = 4). Data were also processed by three-way ANOVA with plant genotype (G), time after inoculation (D) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxDxI) was evaluated by P-value. As no significant interaction between factors was noticed, inoculum effects under well watered and drought conditions were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test, and significant difference (P < 0.05) is showed as (*) above *nr* means. Treatments are designed as non-inoculated controls (No, black bars), *Bacillus megaterium* inoculated plantlets (Bm, white bars), and *Enterobacter C7* inoculated plantlets (C7, grey bars).

Transcriptomic analysis of wt and *nr* plants inoculated with PGPB strains

Microarray data were validated by QRT-PCR evaluating correlations between data obtained with both methodologies (Table M4). The transcriptomic analysis of root samples was performed comparing different treatments in pairs to evaluate PGPB inoculation effects on wt and *nr* plants and direct comparison between both inocula. Transcriptomic data were quantitatively evaluated using Venn diagrams to get a general overview of bacterial inoculation effects (Fig.1.8).

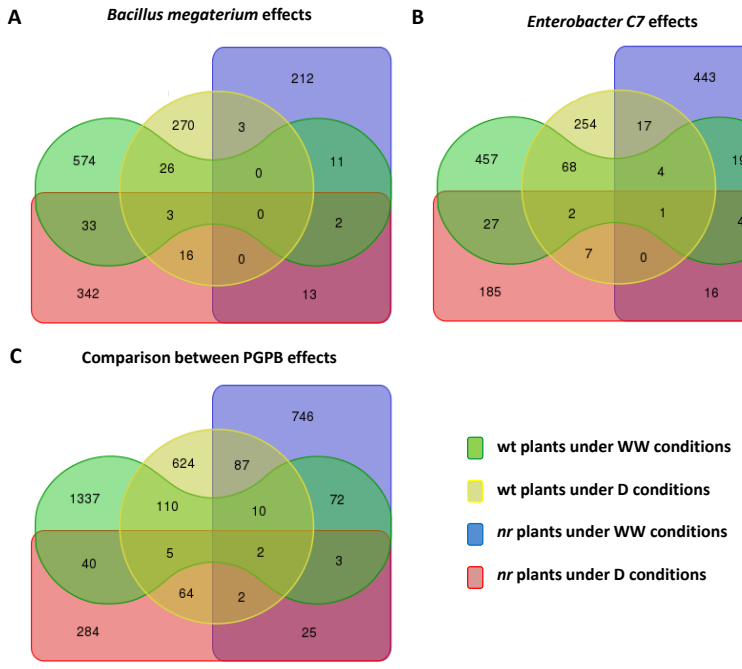

Figure 1.8 Venn diagrams of differentially expressed genes by bacterial inoculation in wild type (wt) and *never ripe* (*nr*) tomato (*Solanum lycorpesicum*) plants under under well watered (WW) and drought (D) conditions. (A) *Bacillus megaterium* effects, **(B)** *Enterobacter* C7 effects and **(C)** comparison between *Bacillus megaterium* and *Enterobacter* C7 effects. Each shape represents a comparison between two treatments. Numbers indicate genes differentially expressed (p-value <

0.05) and those within overlapping areas represents differentially expressed genes in two, three or four treatment comparisons.

Additionally, genes that were differentially expressed ($P < 0.05$) and over 2-fold change were evaluated with Plant MetGenMap to identify significantly altered pathways.

Bacillus megaterium inoculation effects on transcriptomic profiles

Most of differentially expressed genes (DEG) were specifically affected by Bm inoculation in wt and *nr* plants under WW and D conditions (Fig. 1.8 A). Bm inoculation caused 649 DEG (574 specifically; 88.4 %) in wt plants under WW conditions. 26 DEG were shared with DEG affected by Bm inoculation in wt plants under D conditions. Moreover, 11 and 33 DEG were shared with Bm-DEG in *nr* plants under WW and D conditions, respectively. In case of Bm effects in wt plants under D conditions, 318 DEG (270 specifically; 84.9 %) were noticed. 3 and 16 DEG were shared with Bm-DEG in *nr* plants under WW and D conditions, respectively. Moreover, Bm inoculation affected 241 DEG (212 specifically; 88.0 %) in *nr* plants under WW conditions. 13 DEG were shared with DEG affected by Bm in *nr* plants under D conditions. In case of Bm effects in *nr* plants under D conditions, 409 DEG (342 specifically; 83.6 %) were noticed. Furthermore, 3 Bm-DEG were noticed in common in wt plants under both watering regimes and in *nr* plants under D conditions. 2 Bm-DEG were noticed in common in *nr* plants under both watering regimes and wt plants under WW conditions. No DEG affected by Bm were in common in all cases (Fig. 1.8 A).

In addition, Bm inoculation significantly affects several DEG with a fold change over two independently of plant genotype and watering regime (Tables 1.2-5), and Plant MetGenMap identified several changed pathways. In wt plants under WW conditions, 17 DEG were noticed due to Bm inoculation which induced 3 DEG while decreased 14 DEG (Table 1.2). Anthocyanin biosynthesis pathway was induced due to increased transcription of leucoanthocyanidin dioxygenase (LDOX), while lipoxygenase and jasmonic acid biosynthesis pathways were reduced due to decreased transcription of lipoxigenase A (LOX1.1).

Table 1.2 Differentially expressed genes over 2-fold due to *Bacillus megaterium* inoculation in tomato Pearson cv. wild type under well watered conditions

Gene	ID	Fold change
Genes induced by Bm inoculation		
Proline-rich protein	LesAffx.67559.1.S1_at	2.43
Leucoanthocyanidin dioxygenase; LDOX	LesAffx.17064.1.A1_at	2.04
Peptidase C14, caspase catalytic subunit p20	LesAffx.49028.1.S1_at	2.03
Genes repressed by Bm inoculation		
Succinate dehydrogenase subunit 3 ⊥	LesAffx.15544.1.S1_at	2.71
Nitrate reductase	LesAffx.45315.4.S1_at	2.53
Lipoxygenase A; LOX1.1 *	Les.3668.1.S1_at	2.35
Extensin-like protein	Les.3560.1.S1_at	2.35
Photosystem II 44 kDa protein ⊥	LesAffx.66410.1.S1_at	2.33
No description available	Les.1542.1.A1_at	2.32
Endo-1,4-beta-glucanase; cel5	Les.3491.1.S1_at	2.21
No description available	Les.766.1.A1_at	2.17
Photosystem II 47 kDa protein ⊥	Les.4293.1.A1_at	2.12
Cytochrome c oxidase subunit 3 ⊥	LesAffx.51975.3.S1_at	2.09
No description available	Les.4279.1.A1_at	2.04
No description available	Les.1633.1.A1_at	2.03
No description available	Les.2172.1.A1_at	2.01
Cytochrome c oxidase subunit 3	LesAffx.51975.4.S1_at	2.00

*** Common genes that are differentially expressed due to *Bacillus megaterium* (Bm) inoculation in wild type and *never ripe* plants under well watered conditions**

⊥ Common genes that are differentially expressed due to both bacterial inoculations in wild type plants under well watered conditions

In wt plants under D conditions, Bm inoculation caused 7 DEG showing induction in 2 DEG and repression in 5 DEG (Table 1.3), but no significant altered pathways were noticed.

Table 1.3 Differentially expressed genes over 2-fold due to *Bacillus megaterium* inoculation in tomato Pearson cv. wild type under drought conditions

Gene	ID	Fold change
Genes induced by Bm inoculation		
AtRABA6a; GTP binding	LesAffx.65198.1.S1_at	2.41
Responsive to dessication 2; RD2	Les.2914.2.S1_at	2.07
Genes repressed by Bm inoculation		
NTGP4	Les.1842.1.S1_at	4.20
Elongation factor 1 gamma-like protein	Les.5230.1.S1_at	3.61
SANT/MYB domain protein; fsm1 ⊥	Les.3716.1.S1_at	3.11
Cytochrome b559 beta chain; psbF	LesAffx.65143.1.A1_at	2.64
Ethylene receptor-like protein; ETR6	Les.3465.1.S1_at	2.30

⊥ **Common genes that are differentially expressed due to both bacterial inoculations in wild type plants under drought conditions**

In *nr* plants under WW conditions, 34 DEG were observed due to Bm inoculation which induced 5 DEG while decreased 29 DEG (Table 1.4). Furthermore, lipoxygenase and jasmonic acid biosynthesis pathways were reduced due to decreased transcription of LOX1.1 and two allene oxide synthase genes (AOS; cytochrome P450 CYP74C3 and CYP74C4). Flavonoid biosynthesis pathway was also reduced by Bm inoculation due to a decreased transcription of chalcone synthase B and hyosciamine 6-dioxygenase. Moreover, Bm inoculation also reduced canavanine degradation pathway due to a decreased transcription of arginase 2.

Table 1.4 Differentially expressed genes over 2-fold due to *Bacillus megaterium* inoculation in tomato Pearson cv. *never ripe* under well watered conditions

Gene	ID	Fold-change
Genes induced by Bm inoculation		
Ca2+/H+ exchanger	LesAffx.23546.1.S1_at	3.10
Ribosomal protein L2	Les.4399.2.S1_at	2.32
Chalcone reductase	LesAffx.61328.1.S1_at	2.25
Ribosomal protein S7	LesAffx.33796.2.S1_at	2.22
Patatin-like protein 1	LesAffx.47467.1.S1_at	2.08
Genes repressed by Bm inoculation		
Cysteine protease inhibitor, multicystatin; TMC	Les.4820.1.S1_x_at	9.37
flavin reductase-related	Les.2173.1.A1_at	7.00
MADS-box protein 15, Wound-induced proteinase inhibitor I prepropeptide; PIIF	Les.2971.2.A1_at	6.58
Peroxidase 27; PRXR7	Les.4692.1.S1_at	6.17
MADS-box protein 15, Wound-induced proteinase inhibitor I prepropeptide; PIIF	Les.2971.1.S1_at	5.37
Polyphenol oxidase F; PPO	Les.4299.1.S1_at	4.44
Chalcone synthase B	Les.4911.1.S1_at	4.41
Cytochrome P450 CYP74C3	Les.3986.1.S1_at	3.72
Hyoscyamine 6-dioxygenase , putative	Les.4685.1.S1_at	3.63
Arginase 2; ARG2	Les.3299.2.A1_s_at	3.37
Germin-like protein	LesAffx.7583.1.S1_at	2.98
Peroxidase 1 ⊥	Les.5935.2.S1_at	2.93
Laccase	Les.5030.1.S1_at	2.88
Cationic peroxidase isozyme 40K precursor	LesAffx.53132.1.S1_at	2.77
Hydrolase, alpha/beta fold family protein	LesAffx.24204.1.S1_at	2.73
Lipoxygenase A; LOX1.1 *	Les.3668.1.S1_at	2.69
peroxidase 1	Les.5935.1.A1_at	2.66
Putative C-4 sterol methyl oxidase	LesAffx.63154.1.S1_at	2.43
Nod26-like protein	Les.5112.1.S1_at	2.35
3b-hydroxylase; 3OH-1	Les.10.1.S1_at	2.34
TPI	Les.2902.1.S1_at	2.20
No description available	Les.2922.1.S1_at	2.19
NAD-dependent epimerase/dehydratase	LesAffx.44937.1.S1_at	2.17
Basic peroxidase swpb5	LesAffx.39.1.S1_at	2.13
Cytochrome P450 CYP74C4	Les.3466.1.S1_at	2.12
No description available	Les.3210.2.A1_at	2.08
Peroxidase; TPX2	Les.203.1.S1_at	2.08
TPI	Les.2902.2.A1_at	2.02
Expansin; EXP1	Les.191.1.S1_at	2.01

*** Common genes that are differentially expressed due to *Bacillus megaterium* (Bm) inoculation in wild type and *never ripe* plants under well watered conditions**

⊥ Common genes that are diferentially expressed due to both bacterial inoculations in *never ripe* plants under well watered conditions

In *nr* plants under D conditions, Bm inoculation caused 10 DEG showing induction in 7 DEG and repression in 3 DEG (Table 1.5). As

observed in wt plants under D conditions, no significant changed pathways were noticed.

Table 1.5 Differentially expressed genes over 2-fold due to *Bacillus megaterium* inoculation in tomato Pearson cv. *never ripe* under drought conditions

Gene	ID	Fold-change
Genes induced by Bm inoculation		
Unnamed protein product	LesAffx.37715.1.S1_at	3.17
TPA: AT-hook motif nuclear localized protein 2	LesAffx.65906.1.S1_at	2.74
Phosphoglycerate/bisphosphoglycerate mutase family protein	Les.5567.1.S1_at	2.55
Intracellular ribonuclease LX; rnalx	Les.50.1.S1_at	2.38
(1-4)-beta-mannan endohydrolase, putative	LesAffx.3610.1.A1_at	2.29
Nodulin MtN3 family protein	Les.3157.1.S1_at	2.25
Thioredoxin	Les.3221.1.S1_a_at	2.21
Genes repressed by Bm inoculation		
No description available	Les.2668.1.S1_at	2.26
Cytochrome c oxidase subunit 3	LesAffx.51975.3.S1_at	2.18
Hydroxyproline-rich glycoprotein homolog	Les.3320.2.S1_at	2.10

Furthermore, LOX1.1 was the unique DEG affected in common over 2-fold change threshold by Bm inoculation independently of plant genotype under WW conditions, showing transcription repression (Tables 1.2, 1.4).

Enterobacter C7 inoculation effects on transcriptomic profiles

As observed in Bm, most of DEG were specifically affected by C7 inoculation in both plant genotypes and both watering regimes (Fig. 1.8 B). In case of C7 effects in wt plants under WW conditions, 582 DEG (457 specifically; 78.5 %) were noticed. 68 DEG were shared with DEG affected by C7 inoculation in wt plants under D conditions. Furthermore, 19 and 27 DEG were shared with C7-DEG in *nr* plants under WW and D conditions, respectively. C7 inoculation affected 353 DEG (254 specifically; 72.0 %) in wt plants under D conditions. 17 and 7 DEG were shared with DEG affected by C7 in *nr* plants under WW

and D conditions, respectively. In case of C7 inoculation effects on *nr* plants under WW conditions, 504 DEG (443 specifically; 88.0 %) were noticed. 16 DEG were shared with C7-DEG in *nr* plants under D conditions. C7 inoculation affected 242 DEG (185 specifically; 76.5 %) in *nr* plants under D conditions. Moreover, 4 and 2 C7-DEG were noticed in common in wt plants under both watering regimes and in *nr* plants under WW and D conditions, respectively. 4 C7-DEG were shared in *nr* plants under both watering regimes and wt plants under WW. Finally, only one DEG affected by C7 was shared in both plant genotypes and both watering regimes (Fig. 1.8 B).

Furthermore, several DEG were noticed due to C7 inoculation with a fold change over two in wt and *nr* plants under WW and D conditions (Tables 1.6-9) and Plant MetGenMap also identified several changed pathways. 36 DEG were observed in wt plants under WW conditions due to C7 inoculation showing induction of 3 DEG and repression of 33 DEG (Table 1.6). C7 inoculation induced cyanate degradation pathway due to increased transcription of carbonic anhydrase (Ca3).

Table 1.6 Differentially expressed genes over 2-fold due to *Enterobacter* C7 inoculation in tomato Pearson cv. wild type under well watered conditions

Gene	ID	Fold change
Genes induced by C7 inoculation		
Pathogenesis-related protein 4; P4	Les.4693.1.S1_at	6.84
Carbonic anhydrase; Ca3	Les.796.1.A1_at	4.71
Glucan endo-1,3-beta-D-glucosidase; Q`a	Les.3653.1.S1_at	4.04
Genes repressed by C7 inoculation		
Photosystem II 44 kDa protein ⊥	LesAffx.66410.1.S1_at	6.14
Cytochrome c oxidase subunit 3 ⊥	LesAffx.51975.3.S1_at	3.49
NAD(P)H-quinone oxidoreductase subunit 1; NDH subunit 1	LesAffx.44224.1.S1_at	3.15
GUN4 (Genomes uncoupled 4)	LesAffx.41330.2.S1_at	3.10
Photosystem I assembly protein Ycf4	LesAffx.482.1.S1_at	3.03
Succinate dehydrogenase subunit 3 ⊥	LesAffx.15544.1.S1_at	2.92
Cytochrome c oxidase subunit 3	LesAffx.51975.2.S1_at	2.85
Photosystem II 47 kDa protein ⊥	Les.4293.1.A1_at	2.74
Ribosomal protein S7	LesAffx.33796.2.S1_at	2.71
ATP synthase CF1 alpha chain	Les.5834.1.S1_at	2.68
Cytochrome f	LesAffx.51226.1.A1_at	2.64
ACR toxin-sensitivity inducing protein	LesAffx.3698.2.A1_at	2.52
Centromeric protein-related	Les.573.1.A1_at	2.48
Ribosomal protein L2	Les.4399.2.S1_at	2.47
Ribosomal protein S3	LesAffx.18735.1.S1_at	2.40

Cytochrome c oxidase subunit 3 ⊥	LesAffx.51975.3.S1_at	3.49
NAD(P)H-quinone oxidoreductase subunit 1; NDH subunit 1	LesAffx.44224.1.S1_at	3.15
GUN4 (Genomes uncoupled 4)	LesAffx.41330.2.S1_at	3.10
Photosystem I assembly protein Ycf4	LesAffx.482.1.S1_at	3.03
Succinate dehydrogenase subunit 3 ⊥	LesAffx.15544.1.S1_at	2.92
Cytochrome c oxidase subunit 3	LesAffx.51975.2.S1_at	2.85
Photosystem II 47 kDa protein ⊥	Les.4293.1.A1_at	2.74
Ribosomal protein S7	LesAffx.33796.2.S1_at	2.71
ATP synthase CF1 alpha chain	Les.5834.1.S1_at	2.68
Cytochrome f	LesAffx.51226.1.A1_at	2.64
ACR toxin-sensitivity inducing protein	LesAffx.3698.2.A1_at	2.52
Centromeric protein-related	Les.573.1.A1_at	2.48
Ribosomal protein L2	Les.4399.2.S1_at	2.47
Ribosomal protein S3	LesAffx.18735.1.S1_at	2.40
Unknow protein	LesAffx.71664.1.S1_at	2.39
Putative ankyrin	LesAffx.21763.2.S1_at	2.38
Ammonium transporter 1 member 3; LeAMT1;3	Les.3509.1.S1_at	2.37
Phagocytosis and cell motility protein ELMO1-related	Les.3322.1.S1_at	2.35
Photosystem II protein N	LesAffx.18338.1.S1_at	2.28
UDP-glucose:glucosyltransferase	Les.842.1.S1_at	2.26
Probable U6 snRNA-associated Sm-like protein LSm4, Glycine-rich protein 10; GRP 10	Les.2940.2.S1_at	2.26
ATP synthase CF0 A subunit	LesAffx.70834.1.S1_at	2.21
GUN4 (Genomes uncoupled 4)	LesAffx.41330.1.A1_at	2.20
Tyrosine-rich hydroxyproline-rich glycoprotein; extensin (class I)	Les.3606.1.S1_at	2.18
Probable U6 snRNA-associated Sm-like protein LSm4, Glycine-rich protein 10; GRP 10	Les.2940.2.S1_s_at	2.12
UDP-glucose:glucosyltransferase	Les.842.2.S1_a_at	2.12
NAD(P)H-quinone oxidoreductase subunit 1; NDH subunit 1	LesAffx.44224.1.A1_at	2.08
photosystem I assembly protein Ycf3	Les.4298.1.S1_s_at	2.08
ribosomal protein S12	LesAffx.3499.2.A1_at	2.05
Ribosomal protein S12	LesAffx.3499.1.S1_at	2.03
MOS4	Les.4255.2.S1_at	2.03
Sulfate transporter 2; ST2	Les.3479.1.S1_at	2.00

⊥ Common genes that are differentially expressed due to both bacterial inoculations in wild type plants under well watered conditions

In wt plants under D conditions, C7 inoculation caused 7 DEG showing induction in 3 DEG and repression in 4 DEG (Table 1.7), but no significant altered pathways were observed.

Table 1.7 Differentially expressed genes over 2-fold due to *Enterobacter* C7 inoculation in tomato Pearson cv. wild type under drought conditions

Gene	ID	Fold change
Genes induced by C7 inoculation		
Heat shock cognate 70 kDa protein 1	Les.1936.1.S1_at	3.74
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.22413.1.S1_at	2.80
Intracellular ribonuclease LX; rnlax	Les.50.1.S1_at	2.01
Genes repressed by C7 inoculation		
Non-specific lipid-transfer protein 2; LTP 2	Les.1389.1.S1_at	2.73
SANT/MYB domain protein; fms1 ⊥	Les.3716.1.S1_at	2.66
Unknown	Les.358.1.S1_at	2.08
Allantoinase/ hydrolase; ATALN	Les.3319.3.S1_at	2.05

⊥ Common genes that are differentially expressed due to both bacterial inoculations in wild type plants under drought conditions

In *nr* plants under WW conditions, 23 DEG were observed due to C7 inoculation which induced 12 DEG while decreased 11 DEG (Table 1.8). Ethylene biosynthesis from methionine and methionine salvage II pathways were altered due to induction of one *ACS* gene.

Table 1.8 Differentially expressed genes over 2-fold due to *Enterobacter* C7 inoculation in tomato Pearson cv. *never ripe* under well watered conditions

Gene	ID	Fold change
Genes induced by C7 inoculation		
IAA2	Les.3707.1.S1_at	2.60
No description available	Les.876.1.A1_at	2.53
No description available	Les.2261.1.A1_at	2.38
Putative acid phosphatase; TPSI1	Les.3614.1.S1_at	2.36
IAA2	Les.3707.1.A1_at	2.33
F-box-containing protein 1	LesAffx.41381.1.S1_at	2.33
Polygalacturonase-like protein	Les.857.1.S1_at	2.27
Peroxidase	LesAffx.53517.1.S1_at	2.17
No description available	Les.1776.1.A1_at	2.11
euFUL FRUITFULL-like MADS-box	Les.4339.1.S1_at	2.08
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.22413.1.S1_at	2.03
1-aminocyclopropane-1-carboxylate synthase	Les.3475.1.S1_at	2.01
Genes repressed by C7 inoculation		
No description available	Les.1175.2.S1_at	3.55
Peroxidase 1 ⊥	Les.5935.2.S1_at	2.84
ELI3	Les.3741.1.S1_at	2.67
UDP-glucose:glucosyltransferase	LesAffx.62420.1.S1_at	2.33
Hypothetical protein	Les.2001.1.S1_at	2.29
Shikimate kinase family protein	Les.1859.3.S1_at	2.26
protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.67592.1.S1_at	2.21
Alternative oxidase 1c	Les.4337.1.S1_at	2.15
Transporter, putative, expressed	LesAffx.55549.1.S1_at	2.10
Pathogen-related protein	LesAffx.71065.1.S1_at	2.10
bHLH transcriptional regulator; fer •	Les.3814.1.S1_at	2.07

• Common genes that are differentially expressed due to *Enterobacter* C7 (C7) inoculation in *never ripe* plants under well watered and drought conditions

⊥ Common genes that are differentially expressed due to both bacterial inoculations in *never ripe* plants under well watered conditions

In *nr* plants under D conditions, C7 inoculation only showed repression of 24 DEG (Table 1.9). Moreover, tyrosine and

phenylalanine degradation pathways were reduced due to decreased transcription of tyrosine aminotransferase.

Table 1.9 Differentially expressed genes over 2-fold due to *Enterobacter* C7 inoculation in tomato Pearson cv. *never ripe* under drought conditions

Gene	ID	Fold change
Genes induced by C7 inoculation		
-	-	-
Genes repressed by C7 inoculation		
Ribosomal protein S7	LesAffx.33796.2.S1_at	3.34
Cytochrome b6/f complex subunit IV	LesAffx.11323.1.S1_at	3.20
Cytochrome b559 beta chain; psbF	LesAffx.65143.1.A1_at	2.95
NADH dehydrogenase subunit 4L	LesAffx.35136.1.S1_at	2.95
Aquaporin 2	LesAffx.59952.2.S1_at	2.94
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	LesAffx.70764.1.S1_at	2.77
No description available	Les.1999.1.A1_at	2.71
NAD(P)H-quinone oxidoreductase subunit 1; NDH subunit 1	LesAffx.44224.1.A1_at	2.71
NADH dehydrogenase subunit 6	LesAffx.70450.1.S1_at	2.69
Tonoplast intrinsic protein 1;2	Les.4953.1.S1_at	2.67
Cytochrome b6/f complex subunit V	LesAffx.65124.1.A1_at	2.60
RNA polymerase beta subunit	LesAffx.44202.1.S1_at	2.46
Ribosomal protein S12	LesAffx.3499.2.S1_at	2.41
Putative high-affinity nitrate transporter; NRT2;1	Les.28.2.S1_a_at	2.39
Putative gag-pol polyprotein, identical	LesAffx.24116.1.S1_at	2.29
Cytochrome b6/f complex subunit V	LesAffx.65124.1.S1_at	2.19
Tyrosine aminotransferase	Les.4959.1.S1_at	2.18
Cytochrome b6	LesAffx.30946.1.S1_at	2.15
Rb7; TIP	Les.5960.1.S1_at	2.14
Probable U6 snRNA-associated Sm-like protein LSm4, Glycine-rich protein 10; GRP 10	Les.2940.2.S1_s_at	2.08
Aquaporin 2	LesAffx.59952.1.S1_at	2.08
NADH dehydrogenase subunit 4	LesAffx.44474.1.A1_at	2.07
NADH dehydrogenase subunit 4	LesAffx.44474.1.S1_at	2.04
bHLH transcriptional regulator; fer •	Les.3814.1.S1_at	2.02

• Common genes that are differentially expressed due to *Enterobacter* C7 (C7) inoculation in *never ripe* plants under well watered and drought conditions

Furthermore, bHLH transcriptional regulator (fer) was the unique DEG affected in common over 2-fold change threshold by C7 inoculation independently of watering regime, but only in *nr* plants showing transcription repression (Tables 1.8-9).

Differences between *Bacillus megaterium* and *Enterobacter C7* inoculation effects on transcriptomic profiles

Number of DEG was increased when PGPB were compared between each other regarding comparisons with non-inoculated plants, but most of DEG were also specifically affected in both plant genotypes and both watering regimes (Fig. 1.8 C). PGPB comparison showed 1579 DEG (1337 specifically; 84.7 %) in wt plants under WW conditions. 110 DEG were shared with those affected between inocula in wt plants under D conditions. Moreover, 72 and 40 DEG were shared with those in *nr* plants under WW and D conditions, respectively. Comparison between PGPB affected 904 DEG (624 specifically; 69.0 %) in wt plants under D conditions. 87 and 64 DEG were shared with those in *nr* plants under WW and D conditions, respectively. In comparison between PGPB effects on *nr* plants under WW conditions, 947 DEG (746 specifically; 78.8 %) were noticed. 25 DEG were shared with those in *nr* plants under D conditions. In PGPB comparison in *nr* plants under D conditions 425 DEG (284 specifically; 66.8 %) were noticed. Moreover, 10 and 5 DEG were shared in wt plants under both watering regimes and in *nr* plants under WW and D conditions, respectively. 3 and 2 DEG were noticed in common in *nr* plants under both watering regimes and wt plants under WW and D conditions, respectively. Finally, only 2 DEG were shared in both plant genotypes and both watering regimes (Fig. 1.8 C).

Additionally, direct comparison between both PGPB strains also showed several DEG with a fold change over two in both plant genotypes and under both watering regimes (Tables 1.10-13) and Plant MetGenMap identified several changed pathways. In wt plants under

WW conditions, 53 DEG were noticed between Bm and C7 inoculations, showing 11 DEG induction by C7 inoculation regarding Bm-inoculated plants and 42 DEG opposite pattern (Table 1.10). Five pathways were significantly reduced in C7- regarding Bm-inoculated plants. The xanthofile cycle pathway was decreased due to transcriptional reduction of violaxanthin de-epoxidase. The dTDP-L-rhamnose biosynthesis II pathway was also reduced by a transcriptional decrease of NAD-dependent epimerase/dehydratase family protein-like gene. Transcriptional reduction of glycine hydroxymethyltransferase (Cytochrome P450 76A2) decreased glycine betaine degradation, formaldehyde assimilation I and superpathway of serine and glycine biosynthesis II pathways. Moreover, four DEG were affected in common over 2-fold change in comparison between both Bm and C7 inoculations in wt plants under WW conditions. Succinate dehydrogenase subunit 3, photosystem II 44 kDa protein, photosystem II 47 kDa protein and cytochrome c oxidase subunit were repressed due to both bacterial inoculations (Tables 1.2, 1.6).

Table 1.10 Differentially expressed genes over 2-fold between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato Pearson cv. wild type under well watered conditions

Gene	ID	Fold change
Genes induced by C7 inoculation regarding Bm-inoculated plants		
Pathogenesis-related protein 4; P4	Les.4693.1.S1_at	6.82
MADS-box protein 15, Wound-induced proteinase inhibitor I prepropeptide; PIIF	Les.2971.2.A1_at	5.73
MADS-box protein 15, Wound-induced proteinase inhibitor I prepropeptide; PIIF	Les.2971.1.S1_at	4.28
26S proteasome regulatory particle non-ATPase subunit 8 •	LesAffx.31317.17.A1_at	2.69
No description available	Les.766.1.A1_at	2.35
Nodulin MtN3 family protein	Les.3157.1.S1_at	2.32
Lactuca sativa short-chain dehydrogenase/reductase 2	LesAffx.68802.1.S1_at	2.24
Thiamine biosynthesis family protein / thiC family protein	Les.4594.1.S1_at	2.17
H1 histone-like protein	Les.3317.1.S1_at	2.04
No description available	Les.1988.1.A1_at	2.03
Unnamed protein product	Les.4065.1.A1_at	2.03
Genes induced by Bm inoculation regarding C7-inoculated plants		
Glutathione S-transferase	LesAffx.57342.1.S1_at	5.85
GUN4 (Genomes uncoupled 4)	LesAffx.41330.2.S1_at	4.48
Flavonoid 3-glucosyl transferase	LesAffx.63776.1.S1_at	4.00
Putative anthocyanin permease	Les.4452.1.S1_at	3.93
Putative cell surface adhesion protein	LesAffx.46503.1.S1_at	3.65
Contains 2 PF|00400 WD40, G-beta repeats.	Les.424.2.S1_at	3.33
Ammonium transporter 1 member 3; LeAMT1;3	Les.3509.1.S1_at	3.04
GUN4 (Genomes uncoupled 4)	LesAffx.41330.1.A1_at	2.98

Gene	ID	Fold change
Genes induced by C7 inoculation regarding Bm-inoculated plants		
Pathogenesis-related protein 4; P4	Les.4693.1.S1_at	6.82
MADS-box protein 15, Wound-induced proteinase inhibitor I prepropeptide; PIIF	Les.2971.2.A1_at	5.73
MADS-box protein 15, Wound-induced proteinase inhibitor I prepropeptide; PIIF	Les.2971.1.S1_at	4.28
26S proteasome regulatory particle non-ATPase subunit 8 •	LesAffx.31317.17.A1_at	2.69
No description available	Les.766.1.A1_at	2.35
Nodulin MtN3 family protein	Les.3157.1.S1_at	2.32
Lactuca sativa short-chain dehydrogenase/reductase 2	LesAffx.68802.1.S1_at	2.24
Thiamine biosynthesis family protein / thiC family protein	Les.4594.1.S1_at	2.17
H1 histone-like protein	Les.3317.1.S1_at	2.04
No description available	Les.1988.1.A1_at	2.03
Unnamed protein product	Les.4065.1.A1_at	2.03
Genes induced by Bm inoculation regarding C7-inoculated plants		
Glutathione S-transferase	LesAffx.57342.1.S1_at	5.85
GUN4 (Genomes uncoupled 4)	LesAffx.41330.2.S1_at	4.48
Flavonoid 3-glucosyl transferase	LesAffx.63776.1.S1_at	4.00
Putative anthocyanin permease	Les.4452.1.S1_at	3.93
Putative cell surface adhesion protein	LesAffx.46503.1.S1_at	3.65
Contains 2 PF|00400 WD40, G-beta repeats.	Les.424.2.S1_at	3.33
Ammonium transporter 1 member 3; LeAMT1;3	Les.3509.1.S1_at	3.04
GUN4 (Genomes uncoupled 4)	LesAffx.41330.1.A1_at	2.98
Cysteine protease 14	LesAffx.69261.1.S1_at	2.72
Ribosomal protein S7	LesAffx.33796.2.S1_at	2.70
Cytochrome P450 76A2	Les.3127.3.S1_at	2.70
Photosystem II 44 kDa protein	LesAffx.66410.1.S1_at	2.64
Ripening-related protein-like; hydrolase-like	LesAffx.49809.1.S1_at	2.56
Contains 2 PF|00400 WD40, G-beta repeats.	Les.3981.1.S1_at	2.55
SOUL heme-binding family protein	LesAffx.59056.1.S1_at	2.51
Unknown	LesAffx.26570.1.S1_at	2.49
UDP-glucose:glucosyltransferase	Les.842.1.S1_at	2.45
Glutathione transferase	LesAffx.64054.1.S1_at	2.35
CONSTANS interacting protein 1; CIP1	Les.3376.2.S1_at	2.34
O-diphenol-O-methyl transferase, putative	Les.5506.1.S1_at	2.33
NAD-dependent epimerase/dehydratase family protein-like protein, GDP-mannose 3',5'-epimerase; GME1	Les.3214.2.S1_at	2.31
Unnamed protein product	LesAffx.71003.1.S1_at	2.27
No description available	Les.3318.2.S1_at	2.27
Membrane lipoprotein lipid attachment site-containing protein -like	LesAffx.59507.1.S1_at	2.23
Galacturonosyltransferase-like 2; GATL2	LesAffx.32379.1.S1_at	2.22
Putative aspartic proteinase nepenthesin I	LesAffx.56820.1.S1_at	2.20
Violaxanthin de-epoxidase	Les.3958.1.S1_at	2.20
No description available	Les.3006.2.S1_at	2.19
Heat shock protein binding / unfolded protein binding	LesAffx.50432.1.S1_at	2.17
No description available	Les.1816.1.S1_at	2.17
Intracellular ribonuclease LX; rnalx	Les.50.1.S1_at	2.15
UDP-glucose:glucosyltransferase	Les.842.2.S1_a_at	2.15
26S proteasome non-ATPase regulatory subunit 3	Les.3119.2.S1_at	2.12
Cytochrome P450-dependent fatty acid hydroxylase	LesAffx.295.2.S1_at	2.12
Phosphatidate cytidylyltransferase family protein	Les.3175.1.S1_at	2.11
Putative enoyl-CoA hydratase/isomerase	LesAffx.31406.1.S1_at	2.08
No description available	Les.656.1.A1_at	2.08
Os05g0404400	LesAffx.26495.1.S1_at	2.07
No description available	Les.3038.1.S1_at	2.07
Non-intrinsic ABC protein 9; ATNAP9	LesAffx.21557.1.S1_at	2.04
No description available	Les.656.2.S1_at	2.03
Elongation factor TuB; EF-TuB	Les.2942.3.S1_at	2.00

• Common genes that are differentially expressed between *Bacillus megaterium* and *Enterobacter* C7-inoculated plants in wild type plants under well watered and drought conditions

In wt plants under D conditions, 30 DEG were noticed between Bm and C7 inoculations, showing 21 DEG induction by C7 inoculation regarding Bm-inoculated plants and 9 DEG opposite pattern (Table 1.11). Moreover, several pathways were significantly induced in C7-

regarding Bm-inoculated plants. Lipoxygenase and jasmonic acid biosynthesis pathways were induced due to transcriptional induction of LOX1.1 and AOS (cytochrome P450 CYP74C3). Furthermore, C7 inoculation compared to Bm inoculation increased transcription of arginase 2 which significantly affected to canavanine degradation, arginine degradation, citrulline metabolism and urea cycle pathways. Moreover, only SANT/MYB domain protein (fsm1) was repressed in common over 2-fold change in comparison between both Bm and C7 inoculations in wt plants under D conditions (Tables 1.3, 1.7).

Table 1.11 Differentially expressed genes over 2-fold between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato Pearson cv. wild type under well watered conditions

Gene	ID	Fold change
Genes induced by C7 inoculation regarding Bm-inoculated plants		
NTGP4	Les.1842.1.S1_at	4.24
Cytochrome P450 CYP74C3	Les.3986.1.S1_at	3.86
Cytochrome b559 beta chain; psbF *	LesAffx.65143.1.A1_at	3.71
Elongation factor 1 gamma-like protein	Les.5230.1.S1_at	3.58
Cytochrome b6/f complex subunit IV *	LesAffx.11323.1.A1_at	3.57
Cytochrome b559 beta chain; psbF *	LesAffx.65143.1.S1_at	3.51
Cytochrome b6/f complex subunit V *	LesAffx.65124.1.A1_at	2.70
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.22413.1.S1_at	2.65
26S proteasome regulatory particle non-ATPase subunit 8 •	LesAffx.31317.17.A1_at	2.49
Lipoxygenase A; LOX1.1	Les.3668.1.S1_at	2.44
No description available	Les.4510.3.A1_at	2.42
NADH dehydrogenase subunit 4	LesAffx.44474.1.S1_at	2.41
Putative gag-pol polyprotein	LesAffx.24116.1.A1_at	2.40
Cytochrome b6/f complex subunit V *	LesAffx.65124.1.S1_at	2.40
Arginase 2; ARG2	Les.3299.1.S1_at	2.36
Ribosomal protein L1 family protein	Les.986.1.S1_at	2.33
Hypothetical protein Poptr_cp075	Les.4272.3.S1_at	2.26
No description available	Les.2358.1.A1_at	2.18
Zinc finger (AN1-like) family protein	LesAffx.3163.2.S1_at	2.09
Systemin	Les.266.1.S1_a_at	2.06
CYP81C6v2	LesAffx.40179.1.S1_at	2.02
Genes induced by Bm inoculation regarding C7-inoculated plants		
No description available	Les.3288.1.A1_at	2.80
TPA: AT-hook motif nuclear localized protein 2	LesAffx.65906.1.S1_at	2.44
F-box-containing protein 1	LesAffx.41381.1.S1_at	2.22
Contains 2 PF|00400 WD40, G-beta repeats.	Les.4334.2.S1_at	2.18
No description available	Les.3761.1.S1_at	2.10
Putative 3'(2'),5'-bisphosphate nucleotidase	LesAffx.69601.1.S1_at	2.07
Responsive to dessication 2; RD2 *	Les.2914.2.S1_at	2.04
Putative 3'(2'),5'-bisphosphate nucleotidase	LesAffx.69601.2.S1_at	2.00
Contains 2 PF|00400 WD40, G-beta repeats.	Les.4334.3.A1_at	2.00

*** Common genes that are differentially expressed between *Bacillus megaterium* and *Enterobacter* C7-inoculated in wild type and *never ripe* plants under drought conditions**

• Common genes that are differentially expressed between Bacillus megaterium and *Enterobacter* C7-inoculated wild type plants under well watered and drought conditions

Genes induced by Bm inoculation regarding C7-inoculated plants		
No description available	Les.3288.1.A1_at	2.80
TPA: AT-hook motif nuclear localized protein 2	LesAffx.65906.1.S1_at	2.44
F-box-containing protein 1	LesAffx.41381.1.S1_at	2.22
Contains 2 PF|00400 WD40, G-beta repeats.	Les.4334.2.S1_at	2.18
No description available	Les.3761.1.S1_at	2.10
Putative 3'(2'),5'-bisphosphate nucleotidase	LesAffx.69601.1.S1_at	2.07
Responsive to dessication 2; RD2 *	Les.2914.2.S1_at	2.04
Putative 3'(2'),5'-bisphosphate nucleotidase	LesAffx.69601.2.S1_at	2.00
Contains 2 PF|00400 WD40, G-beta repeats.	Les.4334.3.A1_at	2.00

*** Common genes that are differentially expressed between *Bacillus megaterium* and *Enterobacter* C7-inoculated in wild type and *never ripe* plants under drought conditions**

• Common genes that are differentially expressed between Bacillus megaterium and *Enterobacter* C7-inoculated wild type plants under well watered and drought conditions

In *nr* plants under WW conditions, 90 DEG were observed between Bm and C7 inoculations, showing 52 DEG induction by C7 inoculation regarding Bm-inoculated plants and 38 DEG opposite pattern (Table 1.12). Several pathways were significantly induced in C7- regarding Bm-inoculated plants. C7 regarding Bm inoculation induced transcription of two arginase 2 genes which significantly affected to canavanine degradation, arginine degradation, citrulline metabolism and urea cycle pathways. Additionally, ethylene biosynthesis from methionine and methionine salvage II pathways were also induced in C7- compared to Bm-inoculated plants due to transcriptional induction of two *ACS* genes. Moreover, only peroxidase 1 was repressed in common over 2-fold change in comparison between both Bm and C7 inoculations in *nr* plants under WW conditions (Tables 1.4, 1.8).

Table 1.12 Differentially expressed genes over 2-fold between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato Pearson cv. *never ripe* under well watered conditions

Gene	ID	Fold change
Genes induced by C7 inoculation regarding Bm-inoculated plants		
Defective in induced resistance; DIR1	LesAffx.124.1.S1_at	12.66
Defective in induced resistance; DIR1	LesAffx.66472.1.S1_at	8.18
Contains similarity to proline-rich protein	LesAffx.44139.1.S1_at	5.26
Endo-1,4-beta-glucanase precursor; cel1	Les.3667.1.S1_at	4.94
Tumor-related protein	LesAffx.823.1.S1_at	4.42
Catechol oxidase, Polyphenol oxidase F; PPO	Les.4299.1.S1_at	4.34
Germin-like protein	LesAffx.64062.1.S1_at	4.29
DNA-binding protein	LesAffx.12514.1.S1_at	4.24
Auxin-induced SAUR-like protein	LesAffx.1251.1.S1_at	4.24
Arginase 2; ARG2	Les.3299.2.A1_s_at	4.10
No description available	Les.1379.1.A1_at	4.02
Gamma-thionin	Les.3559.1.A1_at	3.89
Arginase 2; ARG2	LesAffx.1.1.S1_at	3.78
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.22413.1.S1_at	3.73
Cationic peroxidase isozyme 40K precursor	LesAffx.53132.1.S1_at	3.71
No description available	Les.2261.1.A1_at	3.67
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	Les.2696.1.S1_at	3.64
36.4 kDa proline-rich protein; TPRP-F1	Les.3273.1.S1_at	3.52

Endo-1,4-beta-glucanase precursor; cel1	Les.3667.1.S1_at	4.94
Tumor-related protein	LesAffx.823.1.S1_at	4.42
Catechol oxidase, Polyphenol oxidase F; PPO	Les.4299.1.S1_at	4.34
Germin-like protein	LesAffx.64062.1.S1_at	4.29
DNA-binding protein	LesAffx.12514.1.S1_at	4.24
Auxin-induced SAUR-like protein	LesAffx.1251.1.S1_at	4.24
Arginase 2; ARG2	Les.3299.2.A1_s_at	4.10
No description available	Les.1379.1.A1_at	4.02
Gamma-thionin	Les.3559.1.A1_at	3.89
Arginase 2; ARG2	LesAffx.1.1.S1_at	3.78
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.22413.1.S1_at	3.73
Cationic peroxidase isozyme 40K precursor	LesAffx.53132.1.S1_at	3.71
No description available	Les.2261.1.A1_at	3.67
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	Les.2696.1.S1_at	3.64
36.4 kDa proline-rich protein; TPRP-F1	Les.3273.1.S1_at	3.52
Protein GAST1	Les.827.1.S1_at	3.17
No description available	Les.647.1.A1_at	3.09
No description available	Les.2207.1.A1_at	2.93
Putative C-4 sterol methyl oxidase	LesAffx.63154.1.S1_at	2.93
Polygalacturonase-like protein	Les.857.1.S1_at	2.84
Pectin methylesterase	Les.218.3.S1_at	2.80
Proteinase inhibitor I4, serpin	LesAffx.48076.1.S1_at	2.72
1-aminocyclopropane-1-carboxylate synthase 7; ACS7B	Les.3474.1.S1_at	2.71
AMP-binding protein, putative	Les.5150.1.S1_at	2.67
Pectin methylesterase	Les.218.1.S1_at	2.65
No description available	Les.1776.1.A1_at	2.62
Phosphoethanolamine N-methyltransferase	Les.4710.1.S1_at	2.55
Drought-induced protein SDi-6, metallothionein II-like protein; MTA	Les.4077.1.S1_at	2.44
No description available	Les.1721.2.S1_at	2.42
Hydrolase, alpha/beta fold family protein	LesAffx.24204.1.S1_at	2.39
1-aminocyclopropane-1-carboxylate synthase; ACS8	Les.3475.1.S1_at	2.38
Leucine aminopeptidase; lap2	Les.84.1.S1_at	2.33
PGIP	LesAffx.56547.2.S1_at	2.27
Contains similarity to proline-rich protein	LesAffx.44139.1.A1_at	2.27
Homeobox protein knotted-1-like LET6	Les.70.1.S1_at	2.25
Phosphoesterase family protein	LesAffx.39212.1.S1_at	2.24
Sulfate transporter 2; ST2	Les.3479.1.S1_at	2.21
No description available	Les.2358.1.A1_at	2.21
No description available	Les.181.1.A1_at	2.16
Serine carboxypeptidase II-2 precursor (CP-MII.2)	Les.4810.1.S1_at	2.09
No description available	Les.477.1.S1_at	2.09
No description available	Les.2026.2.A1_at	2.06
euFUL FRUITFULL-like MADS-box	Les.4339.1.S1_at	2.05
STS14 protein precursor	Les.1394.1.A1_at	2.04
Putative ripening-related protein	LesAffx.71016.1.S1_at	2.04
Protein RSI-1	Les.3625.1.S1_at	2.03
Transferase, transferring glycosyl groups	LesAffx.30198.1.S1_at	2.02
NAD-dependent epimerase/dehydratase	LesAffx.44937.1.S1_at	2.02
Calmodulin binding protein, putative	Les.933.1.A1_at	2.01
Genes induced by Bm inoculation regarding C7-inoculated plants		
No description available	Les.195.1.S1_at	8.57
Class II small heat shock protein Le-HSP17.6	Les.3581.1.S1_at	7.32
Ca2+/H+ exchanger	LesAffx.23546.1.S1_at	5.11
Small heat shock protein; vis1	Les.3677.1.S1_at	5.03
ELI3	Les.3741.1.S1_at	4.45
TAS14 peptide	Les.3593.1.S1_at	3.79
Chalcone-flavanone isomerase	LesAffx.68320.1.S1_at	3.68
Shikimate kinase family protein	Les.1859.3.S1_at	3.45
Lipid transfer protein, putative	LesAffx.70407.1.S1_at	3.43
Flavonoid glucoyltransferase UGT73E2	Les.2403.2.S1_at	3.25
17.7 kD class I small heat shock protein	Les.4004.1.S1_a_at	3.12
ATEGY3	Les.1132.1.A1_at	3.01
4-hydroxyphenylpyruvate dioxygenase; HPPDase	Les.3415.2.S1_at	2.96
Jasmonic acid 2; JA2	Les.23.1.S1_at	2.71
Flavonoid glucoyltransferase UGT73E2	Les.2403.1.S1_at	2.63
1-aminocyclopropane-1-carboxylate oxidase 1; ACO1	Les.2560.1.S1_at	2.60
No description available	Les.3288.1.A1_at	2.54
ATP phosphoribosyltransferase	Les.4149.2.S1_a_at	2.45
Shikimate kinase family protein	Les.1859.2.S1_at	2.42
Universal stress protein (USP) family protein / early nodulin ENOD18 family protein	LesAffx.47187.1.S1_at	2.37

TAS14 peptide	Les.3593.1.S1_at	3.79
Chalcone-flavanone isomerase	LesAffx.68320.1.S1_at	3.68
Shikimate kinase family protein	Les.1859.3.S1_at	3.45
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Universal stress protein (USP) family protein / early nodulin ENOD18 family protein	LesAffx.47187.1.S1_at	2.37
Flavonoid glucoyltransferase UGT73E2	Les.5832.1.S1_at	2.32
Cytochrome P450 76A2	LesAffx.8720.1.S1_at	2.31
Gibberellin 3-beta hydroxylase	LesAffx.71330.1.S1_at	2.30
Zinc finger (C3HC4-type RING finger) family protein	Les.4360.1.S1_at	2.27
No description available	Les.1596.1.A1_at	2.25
Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LesAffx.67592.1.S1_at	2.20
Cold-induced glucosyl transferase	LesAffx.6688.1.S1_at	2.14
Oxidoreductase, 2OG-Fe(II) oxygenase family protein	LesAffx.9824.1.S1_at	2.11
Patatin-like protein 1	LesAffx.47467.1.S1_at	2.10
NAC domain protein NAC2	Les.2084.1.S1_at	2.09
MADS-box protein 15	LesAffx.4763.1.S1_at	2.09
Putative stress-responsive protein	Les.5957.1.S1_at	2.07
No description available	Les.3164.2.S1_at	2.06
AER	Les.220.1.S1_at	2.06
Pathogen-related protein	LesAffx.71065.1.S1_at	2.06
Uroporphyrinogen III synthase	LesAffx.70617.1.S1_at	2.06
Serine acetyltransferase 3;2, acetyltransferase/serine O-acetyltransferase	LesAffx.59463.1.S1_at	2.02
Cytochrome P450 76A2	LesAffx.8720.2.S1_at	2.00

In *nr* plants under D conditions, 26 DEG were noticed between Bm and C7 inoculations, showing 3 DEG induction by C7 inoculation regarding Bm-inoculated plants and 23 DEG opposite pattern (Table 1.13). Two pathways were significantly reduced in C7- regarding Bm-inoculated plants. The transcriptional reduction of glutamine synthase affected to glutamine biosynthesis pathway and ammonia assimilation cycle.

Table 1.13 Differentially expressed genes over 2-fold between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato Pearson cv. *never ripe* under drought conditions

Gene	ID	Fold change
Genes induced by C7 inoculation regarding Bm-inoculated plants		
Responsive tp dessication 2; RD2 *	Les.2914.2.S1_at	2.59
serine/threonine protein phosphatase 2A ;PP2A regulatory subunit B', putative	LesAffx.10338.1.S1_at	2.04
IAA Carboxymethyl transferase 1; S-adenosylmethionine-dependent methyltransferase	LesAffx.33082.1.S1_at	2.01
Genes induced by Bm inoculation regarding C7-inoculated plants		
Cytochrome b6/f complex subunit IV *	LesAffx.11323.1.A1_at	4.58
Cytochrome b559 beta chain; psbF *	LesAffx.65143.1.S1_at	4.41
Cytochrome b559 beta chain; psbF *	LesAffx.65143.1.A1_at	3.80
Ribosomal protein S12	LesAffx.3499.2.S1_at	3.65
NAD(P)H-quinone oxidoreductase subunit 1, NDH subunit 1	LesAffx.44224.1.A1_at	3.40
NADH dehydrogenase subunit 6	LesAffx.70450.1.S1_at	3.36
NADH dehydrogenase subunit 4L	LesAffx.35136.1.S1_at	3.28
No description available	Les.1999.1.A1_at	3.23

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Responsive tp dessication 2; RD2 *	Les.2914.2.S1_at	2.59
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Cytochrome b559 beta chain; psbF *	LesAffx.65143.1.S1_at	4.41
Cytochrome b559 beta chain; psbF *	LesAffx.65143.1.A1_at	3.80
Ribosomal protein S12	LesAffx.3499.2.S1_at	3.65
NAD(P)H-quinone oxidoreductase subunit 1, NDH subunit 1	LesAffx.44224.1.A1_at	3.40
NADH dehydrogenase subunit 6	LesAffx.70450.1.S1_at	3.36
NADH dehydrogenase subunit 4L	LesAffx.35136.1.S1_at	3.28
No description available	Les.1999.1.A1_at	3.23
Cytochrome b6/f complex subunit V *	LesAffx.65124.1.A1_at	2.95
Putative glutamine synthase; gts1	Les.224.1.S1_at	2.89
Ribosomal protein S7	LesAffx.33796.2.S1_at	2.80
Xyloglucan endotransglycosylase, putative	LesAffx.69296.1.S1_at	2.80
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	LesAffx.70764.1.S1_at	2.78
Thioredoxin	Les.3221.1.S1_a_at	2.45
Ribosomal protein S3	LesAffx.18735.1.A1_at	2.45
Cytochrome b6/f complex subunit IV	LesAffx.11323.1.S1_at	2.43
Unnamed protein product	LesAffx.37715.1.S1_at	2.31
Cytochrome b6/f complex subunit V *	LesAffx.65124.1.S1_at	2.28
Peroxidase 1	Les.5935.1.A1_at	2.24
1-aminocyclopropane-1-carboxylic acid oxidase; ACO	LesAffx.69245.1.S1_at	2.20
Retroelement pol polyprotein-like	LesAffx.71660.4.S1_at	2.15
Probable U6 snRNA-associated Sm-like protein LSm4, Glycine-rich protein 10; GRP 10	Les.2940.2.S1_s_at	2.14
No description available	Les.2033.3.S1_at	2.11

*** Common genes that are differentially expressed between *Bacillus megaterium* and *Enterobacter* C7-inoculated in wild type and *never ripe* plants under drought conditions**

In addition, several DEG were affected in common over 2-fold change in comparison between both Bm and C7 inoculations independently of plant genotype, but only under D conditions (Tables 1.11, 1.13). Responsive to dessication 2 (RD2) was the unique DEG affected showing transcription induction by Bm inoculation in wt plants and repression by C7 inoculation in *nr* plants. Nevertheless, Cytochrome b6/f complex subunit IV, Cytochrome b6/f complex subunit and Cytochrome b559 beta chain (psbF) were affected showing transcription induction by C7 inoculation in wt plants and repression by Bm inoculation in *nr* plants. Furthermore, 26S proteasome regulatory particle non-ATPase subunit 8 was the unique DEG affected in common over 2-fold change in comparison between both Bm and C7

inoculations independently of watering regime, but only in wt plants showing transcription induction by C7 inoculation (Tables 1.10, 1.11).

Discussion

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). In the present study, the colonization bioassay confirmed that Bm as well as C7 were able to colonize the roots of ethylene sensitive and insensitive plants. Moreover, the colonization rates were not significantly different (Table 1.1), suggesting that plant growth variations are not due to differences in root colonization.

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

Both Bm and C7 can be considered as PGPB due to growth promotion noticed in wt plants (Weller et al. 2002; Vessey 2003). Plant growth promotion by Bm was previously reported in several plant species (Marulanda-Aguirre et al. 2008; Marulanda et al. 2009; Porcel et al. 2014; Armada et al. 2014), but *Enterobacter* C7 was evaluated in the present study for first time. Nevertheless, noticed results in *nr* plants pointed to a PGPB mechanism dependent on ethylene sensitivity for Bm but not for C7 (Fig. 1.2). PGPB mode of action dependent on ethylene-sensitivity was previously reported in arabidopsis plants inoculated with *Variovorax paradoxus* also without differences in root colonization rate but which presenting ACC deaminase activity (Chen et al. 2013). In contrast, C7 inoculation resulted in growth promotion of

both plant genotypes (Fig. 1.2), suggesting that ethylene perception by ETR3 was not involved in its action mechanism. Other studies previously reported an ethylene-independent PGPB effect in different plant-bacteria combinations (López-Bucio et al. 2007; Meldau et al. 2012). As with many plant-bacterial interactions, discrepancies between these studies could be explained by strain-specific PGPB mechanisms, plant growth conditions (Ryu et al. 2005; Long et al. 2008), and/or differences in ethylene-transduction pathways between tomato and arabidopsis such as receptor functionality and signaling regulation (Hua and Meyerowitz 1998; Tieman et al. 2000; Kevany et al. 2007).

In addition, drought significantly reduced plant growth in all cases (Fig. 1.2 A). Yield losses caused by drought are very important in tomato (Pervez et al. 2009) and are increasing due to intensive agriculture and climate change (Misra 2014). Bacteria-inoculated wt plants under D conditions reached values of non-inoculated wt plants under WW conditions reducing potential yield losses and suggesting that PGPB can be used to diminish water inputs in agriculture or increase resource use efficiency (Dodd and Ruiz-Lozano 2012). Moreover, enhanced promotion effects were noticed in wt plants under D conditions suggesting that PGPB increased plant stress tolerance as previously reported under suboptimal environmental conditions (Glick 2004; Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009).

Nevertheless, although C7 was able to promote growth in *nr* plants independently of watering regime, the promotion was lower under D conditions. These results suggest that ethylene insensitivity affects C7 effects, despite of proposed ethylene-independent activity.

Additionally, ethylene production is typically up-regulated by drought (Wang et al. 2013), and this could result in later response and a reduction in growth promotion by C7 since *nr* plants are unable to sense it. In addition, differences between plant genotypes were only significant in non-inoculated plants pointing to less pronounced ethylene growth inhibitory effects (Pierik et al. 2006) on *nr* plants in WW conditions due to its mutation (Wilkinson et al. 1995), but not observed under D conditions probably due to ethylene induction by stress (Pierik et al. 2007).

PGPB inoculation locally affects ethylene biosynthesis and response resulting in stress alleviation by C7, and Bm miss-recognition by *nr* plants

In tomato, several isoforms of *ACS* and *ACO* genes have been identified (Blume and Grierson 1997; Jiang and Fu 2000; Alexander and Grierson 2002). The rate-limiting enzyme in ethylene biosynthesis is generally *ACS* (Wang et al. 2002), although *ACO* could be limiting under certain conditions (Dorling and McManus 2012). During plant growth and development, ethylene production is strongly regulated (Wang et al. 2002; Argueso et al. 2007). *ACS* expression shows tissue- and cell-specific patterns which are differentially regulated depending on developmental stage and in response to different stimuli, controlling finely ethylene production (Tsuchisaka et al. 2009; Dorling and McManus 2012).

Expression alteration of ethylene biosynthesis genes in tomato due to PGPB inoculation was previously reported by Porcel et al. (2014). In the present study, both PGPB modified expression of *ACS*

and *ACO* genes (Fig. 1.3 and 1.4). Either Bm as C7 decreased their expression in wt plants, although C7 inoculation affected with higher intensity in accordance with higher growth promotion. Thereby, both PGPB could diminish ethylene growth inhibitory effects independently on watering regimes (Pierik et al. 2006; Wang et al. 2013a). Nevertheless, PGPB inoculation in *nr* plants induced ethylene biosynthesis genes showing higher effects by Bm inoculation. ACC levels highly increase in response to stresses as wounding and/or pathogen infection (Kende and Boller 1981; van Loon et al. 2006) suggesting that *nr* plants could recognize Bm as a pathogen-like microorganism, which induces ethylene signaling and response.

Additionally, drought effects in ethylene biosynthesis genes pointed to higher stress alleviation by C7 inoculation, according with previously enhanced tolerance against stresses due to PGPB inoculation (Aroca and Ruiz-Lozano 2009). Ethylene emission by aerial tissues was determined to evaluate any alteration of ethylene metabolism, but neither PGPB nor drought modified ethylene production rate (Fig. 1.7 A). However, previous studies correlated spatiotemporal *ACS* expression with ethylene production (Zarembinski and Theologis 1994; Wang et al. 2002; Sobeih et al. 2004). *ACS* genes present a strong post-transcriptional regulation with high influence on ethylene production (McClellan and Chang 2008; Lyzenga et al. 2012; Xu and Zhang 2014), but ACS activity was not assayed and further research is needed to clarify these results.

Additionally, drought treatment generally increased ethylene emission (Pierik et al. 2007; Wang et al. 2013a), but the harvest was carried out one month after drought treatment started and plants could

be adapted (Pan et al. 2012). Moreover, plant homeostasis can also counteract bacterial effects since PGPB were grown in roots during two months. Another feasible explanation is that ethylene could be specifically produced by limited number of tissues or cells, leading to a strong local production, while total ethylene were maintained at low levels in the whole plant and/or organ.

Furthermore, differences between plant genotypes in ethylene biosynthesis genes were generally observed in non-inoculated plants disappearing under bacterial inoculation probably due to stress alleviation effects (Glick 2004; Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009). Moreover, ethylene production showed significant differences between plant genotypes supporting independence of ethylene for C7 activity and pointing to ethylene sensitivity as determinant in Bm-tomato interaction with interference of drought stress (Pierik et al. 2007).

In addition, a bioassay for ethylene production was performed to test if ethylene levels emitted by seedlings are affected in response to bacterial inoculation (Fig. 1.7 B). After 7 h of inoculation, Bm and C7 inoculation increased ethylene production rate in *nr* and wt plants, respectively. Although 26 h post-inoculation ethylene production rate was decreased in both cases regarding to inoculation day and no differences were noticed due to PGPB inoculation compared to non-inoculated plants, Bm-inoculated *nr* plants showed higher ethylene production rate than wt ones 26 h post-inoculation. Ethylene emission by plants after interaction with bacteria was previously reported (van Loon et al. 2006) acting as modulator of interaction between plants with several enemies (Groen et al. 2013), although also with beneficial

microorganisms (Zamioudis and Pieterse 2012). Thereby, these results also points to miss-interaction between Bm and *nr* plants.

Moreover, induction of ethylene receptors by PGPB strains was previously reported pointing to ethylene response pathway involvement in the early stages of establishment of plant-bacteria association (Ciardi et al. 2000; Vargas et al. 2012) required for proper interaction (Vacheron et al. 2013). Thus, *ETR3* expression was evaluated to determine if PGPB inoculation can modulate ethylene sensitivity by ETR3 and if ETR3 is involved in interaction between PGPB and tomato plants (Fig. 1.5 A, C). Bm inoculation induced *ETR3* expression in root and shoot tissues exclusively in *nr* plants pointing to lower ethylene sensitivity and non-completely functional association between Bm and *nr* plants. However, C7 inoculation decreased shoot *ETR3* expression only in wt shoots suggesting that aerial tissues of C7-inoculated plants could be less sensitive to ethylene. Residual responsiveness in *nr* plants (Lanahan et al. 1994), could contribute to these late response to bacterial strains.

In addition, although four *CTR1*-like genes were described in tomato, *TCTR1* expression analysis was selected because it is ethylene-inducible and associated with tissues at development stages with high ethylene levels (Zegzouti et al. 1999; Leclercq et al. 2002; Adams-Phillips et al. 2004). Induction of *TCTR1* expression in roots by Bm inoculation was noticed in *nr* plants under D conditions (although it was also partially significant induced under WW conditions) suggesting local ethylene production and in accordance with growth results (Fig. 1.5 B, D). Furthermore, *ETR3* expression on a par with *TCTR1* was reported to regulate ethylene responsiveness (Tieman et al.

2000), and Bm was able to induce both genes in *nr* plants, suggesting negatively feedback regulation in Bm-inoculated *nr* plants. Indeed, ethylene receptors and *CTRs* are differentially regulated in response to stimuli and during plant development possibly regulating different ethylene responses (Zhong et al. 2008).

Ethylene-responsive genes were also analyzed to evaluate if ethylene transduction pathway was activated in PGPB-inoculated plants despite of similar ethylene production. *ERF1* was selected because it acts as integrator between osmotic stress and ethylene pathways in tomato (Huang et al. 2004), and is induced by drought in several species (Cheng et al. 2013; Müller and Munné-Bosch 2015). Surprisingly, despite of drought treatment, no expression change was noticed pointing to plant adaptation to drought (Pan et al. 2012). Furthermore, *ERF1* is also involved in defense against pathogens (Cheng et al. 2013; Müller and Munné-Bosch 2015) suggesting that both PGPB did not induce defense mechanisms, although *nr* plants could miss-recognize Bm.

ER5, *ER21* and *ER24* expression was analyzed due to their strong ethylene-dependent alteration (Fig. 1.6). *ER5* and *ER21* are involved in stress response, while *ER24* participates in regulation of gene expression (Zegzouti et al. 1999). *ER21* encodes a tomato heat-shock protein cognate 70 (hsp70) (Sun et al. 1996). Stress can cause plant cell damage resulting in osmotic and oxidative stress and induction of heat-shock proteins (Scarpeci et al. 2008; Al-Whaibi 2011) as hsp70, which function as chaperone (Sung et al. 2001; Su and Li 2008). Bm was able to strongly induce *ER21* expression in *nr* roots suggesting that Bm

presence caused a high stress according with miss-recognition hypothesis.

ER21 expression results in shoot tissue points to Bm and C7 produced systemic stress under D and WW conditions, respectively. However, C7 inoculation could alleviate stress under D conditions in wt and *nr* plants in accordance with growth results. Furthermore, drought induction of *ER21* in Bm-inoculated *nr* shoots and in non-inoculated wt ones pointed to higher stress levels in these two treatments. Additionally, differences between plant genotypes in shoot *ER21* were only noticed in non-inoculated plants suggesting that *nr* plans were suffering less stress probably because ethylene insensitivity prevents further signaling failing to produce some adaptive responses as previously reported (Feng and Barker 1992; Zhang et al. 2003). In accordance, *ER5* is a lea-like gene (Zegzouti et al. 1999) involved in response to desiccation (Van Den Dries et al. 2011), and showed lower levels in non-inoculated *nr* than wt plants under D conditions. Furthermore, *ER24* is a transcriptional co-activator (Zegzouti et al. 1999), suggesting higher response by *nr* plants under stress triggered by Bm.

PGPB inoculation affected transcriptomic profiles showing strain-specific effects mainly dependent on ethylene insensitivity and watering conditions.

Transcriptomic analysis was carried out to evaluate PGPB inoculation effects on wt and *nr* plants and to point to other plant changed pathways. Both PGPB inoculations as well as direct comparison between inocula generally produced specific effects

dependent on bacterial strain, plant genotype and watering regime (Fig. 1.8) in accordance with PGPB effects dependent on inoculated bacteria and growth conditions (Ryu et al. 2005; Long et al. 2008). Moreover, both bacterial inoculations modified expression of a high number of genes under WW conditions suggesting than drought stress could diminish bacterial inoculation effects. Indeed, drought stress has a great impact in tomato transcriptomic profiles (Gong et al. 2010; Iovieno et al. 2016). However, Bm altered lower number of DEG in *nr* plants under WW conditions in accordance with cross-talk between drought stress and Bm interaction with *nr* plants.

***Bacillus megaterium* modulates flavonoids enhancing antioxidant status in wt plants but ethylene insensitivity impairs interaction with tomato.**

Bm inoculation showed significant effects on pathways only under WW conditions. Bm induced anthocyanin biosynthesis pathway in wt plants. LDOX produces cyanidin and is essential for biosynthesis of proanthocyanidin (Saito et al. 1999; Abrahams et al. 2003). These molecules belong to the flavonoid family, which plays a determinant role in plant interaction with beneficial microorganisms and maintain the redox status within cells by their antioxidative properties (Shirley 1996; Mierziak et al. 2014). Although flavonoids has been described in symbiotic bacteria and mychorriza (Abdel-Lateif et al. 2012), it could play also a role in interaction with bacteria living in the rhizosphere. Additionally, plant proanthocyanidins can neutralize bacterial lipopolysaccharides (Delehanty et al. 2007), which trigger plant

immune system (Newman et al. 2013), suggesting that wt plants could avoid immune response against Bm.

Nevertheless, flavonoid biosynthesis pathway was repressed by Bm inoculation in *nr* plants. Chalcone synthase is the first enzyme in the tomato flavonoid biosynthesis pathway (Schijlen et al. 2007), while hyosciamine 6-dioxygenase catalyzes hydroxylation of hyoscyamine transformation to scopolamine (Matsuda et al. 1991). Chalcone synthase expression results in accumulation of flavonoids involved in defense pathway (Dao et al. 2011), suggesting that *nr* plants did not defensively react against Bm. Induction of hyoscyamine and scopolamine accumulation was previously reported using PGPB as biotic elicitors (Ghorbanpour et al. 2010), but repression of these enzyme genes was in accordance with no completely recognition of Bm as pathogen by *nr* plants. However, among root exudates canavanine is a non-protein amino acid which is toxic to many soil bacteria (Cai et al. 2009). Canavanine degradation pathway was also reduced by Bm inoculation in *nr* plants suggesting its accumulation. Indeed, canavanine-mediated inhibition of *Alfalfa* spermosphere colonization by *Bacillus cereus* was previously reported (Emmert et al. 1998) pointing to non-completely functional colonization of *nr* roots by Bm.

Furthermore, lipoxygenase and jasmonic acid (JA) biosynthesis pathways were repressed by Bm independently of plant genotype, although AOS was exclusively reduced in *nr* plants probably because ethylene is able to induce AOS expression in tomato (Sivasankar et al. 2000). JA function as regulator of plant immune responses against pathogens (Browse 2009). Thus, these results suggest that Bm is not

completely recognized as a pathogenic microorganism despite of miss-interaction between Bm and *nr* plants.

Ethylene sensitivity determines tomato interaction with *Enterobacter C7*, which minimizes defense response in wt but improves plant fitness in *nr* plants.

C7 inoculation in wt plants under WW conditions induced cyanate degradation pathway. Carbonic anhydrase participates in transport of inorganic carbon modulating CO_2 levels in photosynthesis and respiration (Badger 1994; Price et al. 1994; Henry 1996). Carbonic anhydrase is positively correlated with photosynthesis and dry matter accumulation in shoots (Khan 2002; Khan et al. 2004) suggesting increased respiration and inorganic carbon transport in roots. Surprisingly, C7 inoculation in *nr* plants under WW conditions affected to ethylene biosynthesis and methionine salvage II pathways due to induction of an *ACS* gene. In accordance, *ACS7* expression was induced by C7 inoculation under these conditions. *ACS7* role in flooding and wound response was previously reported (Shiu et al. 1998). However, ethylene levels were unaffected by C7 inoculation and our results pointed to its involvement in plant-bacteria interaction. Indeed, several ACS genes participate in response to bacterial elicitors in arabidopsis (Denoux et al. 2008).

Under D conditions, C7 inoculation in *nr* plants decreased tyrosine and phenylalanine degradation pathways. Tyrosine and phenylalanine were used in the biosynthesis of secondary metabolites (Tzin and Galili 2010; Hyun et al. 2011) involved in cell wall structure (Bonawitz and Chapple 2010), and plant defense or response to stress

(Fraser and Chapple 2011; Lee and Facchini 2011), suggesting that higher amino acid availability in C7-inoculated *nr* plants could counteract stress.

Furthermore, a bHLH transcriptional regulator (fer) was reduced by C7 in *nr* plants independently of watering regime. Fer protein regulates iron uptake in tomato (Brumbarova and Bauer 2005), suggesting its repression that C7 inoculation could enhance Fe nutrition in *nr* plants due to noticed growth promotion. Additionally, ethylene is a signal which triggers response to Fe deficiency (Lucena et al. 2006; Lingam et al. 2011) suggesting that C7 inoculation could restore this response when plants are unable to perceive ethylene.

In addition, cyanide is a defensive metabolite in plants whose oxidation results in cyanate formation (Møller and Seigler 1999). Plants produce cyanide during ethylene biosynthesis (Peiser et al. 1984; Kende 1993) and cyanide forms part of catalytic proteins active Fe-cyanide complexes (Reissmann et al. 2003). Thus, wt plants could be able to recognize C7 decreasing defensive metabolites, while *nr* plants respond to C7 presence by enhancing ethylene biosynthesis pathway. Although growth promotion by C7 inoculation was proposed as independent of ethylene perception, these results also suggest that mutation of *ETR3* affects tomato-C7 interaction.

Bm diminishes oxidative stress only in wt plants, while C7 increase it but modulates amino acidic metabolism independently of ethylene sensitivity

Although PGPB inoculations did not significantly altered pathways compared to non-inoculated plants under D conditions, direct comparison between Bm and C7 showed multiple differences.

In wt plants under WW conditions, C7 compared to Bm inoculation repressed several pathways. The dTDP-L-rhamnose biosynthesis II pathway produces l-rhamnose, which is key component of cell wall and secondary metabolites including flavonoids and anthocyanins (Watt et al. 2004) in accordance with previous results observed when compared Bm and C7 regarding to non-inoculated plants. The xanthophyll cycle is involved in plant protection against oxidative stress (Latowski et al. 2011) pointing to higher oxidative stress in C7- than in Bm-inoculated wt plants. Although metabolites of xanthophyll cycle could present antioxidant properties in roots, oxidative protection was generally described in photosynthetic tissues (Barickman et al. 2014). However, this pathway also produced substrates for ABA production (Schwartz et al. 1997), suggesting that ABA levels could participate in PGPB activity of Bm and/or C7. In fact, normal endogenous ABA levels were proposed as essential for Bm–mediated growth promotion (keeping low ethylene production) in tomato plants (Porcel et al. 2014). Additionally, ABA was typically characterized as growth inhibitor, but it plays an important role in growth of young tissues (Finkelstein 2013). Moreover, glycine hydroxymethyltransferase plays a role in the photorespiratory pathway and contributes to minimize ROS production and avoid cellular damage

(Moreno et al. 2004) also pointing to lower oxidative stress in wt plants inoculated with Bm than with C7.

In contrast, C7 compared to Bm inoculation in wt plants under D conditions induced lipoxygenase and jasmonic acid biosynthesis pathways. This difference can be due to Bm differences with non-inoculated wt plants in those pathways not noticed with C7. Furthermore, arginase 2 induction significantly increased canavanine degradation, arginine degradation, citrulline metabolism and urea cycle pathways. C7 compared to Bm inoculation in *nr* plants under WW conditions also induced arginase 2 genes inducing those pathways suggesting a common mechanism independent on ethylene sensitivity. In tomato, jasmonic acid and wounding are able to induce arginase 2 expression (Chen et al. 2004). Arginase catalyzes the transformation of arginine pool (up to 90% of free nitrogen in vegetative tissues) into urea and ornithine, which is used in biosynthesis of glutamate, proline and to polyamines. Moreover, nitrogen mobilization has been correlated with arginase expression to meet the metabolic demands (Zonia et al. 1995; Hwang et al. 2001) suggesting that C7 could be able to induce the amino acidic metabolism and/or enhance the nitrogen use efficiency as previously reported by several PGPB (Mantelin and Touraine 2004; Tikhonovich and Provorov 2011; Carvalho et al. 2014). Furthermore, canavanine degradation is induced suggesting lower accumulation of this toxic metabolite under C7 inoculation (Cai et al. 2009).

In *nr* plants under WW conditions, ethylene biosynthesis and methionine salvage II pathways were also induced by C7 compared to Bm inoculation. As abovementioned, these processes could be affected

by *ACS7* expression induction mediated by C7 compared to non-inoculated plants. Furthermore, Peroxidase 1 was the unique repressed DEG in common by both PGPB (Tables 1.4, 1.8). Peroxidase 1 is a key enzyme in biosynthesis of lignin and suberin, which are involved in water transport and cell wall strengthen, suggesting that both PGPB strains are able to modulate root transport processes and cell wall resistance (Quiroga et al. 2000).

In *nr* plants under D conditions, C7 compared to Bm reduced glutamine synthase. This enzyme is responsible of primary ammonium assimilation and participates in ammonium detoxification released in metabolic processes as proteolysis or photorespiration (Lea and Miflin 2010), pointing to higher proteolysis under Bm inoculation in accordance with higher stress response.

In addition, several DEG showed opposite results due to Bm or C7 inoculation. Responsive to desiccation 2 (RD2) which belongs to universal stress protein A family, is involved in oxidative stress regulating negatively ROS generation in tomato plants (Gutiérrez-Beltrán et al. 2017). Thus, our results points to lower oxidative stress in Bm-inoculated wt plants and higher in C7-inoculated *nr* plants. Moreover, photosystem II (PSII), which contains psbF, and cytochrome b6/f (cyt b6/f) complex are key players in photosynthetic electron transport chain (Yamori et al. 2016). Alteration of these genes was previously reported in photosynthetic tissues, but it was suggested that PSII could function as ROS sensor in roots (Huo et al. 2015). These results suggest that C7 could increase electron transport in wt plants, while Bm reduced it in *nr* plants according with increased respiration and oxidative stress by C7 inoculation.

Conclusions

In conclusion, both bacterial strains acts as PGPB under well watered and drought conditions in ethylene-sensitive tomato plants. C7 inoculation promoted plant growth in wt and *nr* plants, and generally reduced expression of ethylene biosynthesis genes in wt plants resulting in stress alleviation. However, Bm inoculation induced expression of ethylene-biosynthesis, -signaling and -response genes in *nr* plants suggesting local ethylene emission in Bm-inoculated *nr* plants. Thus, ETR3 mutation could impair interaction between Bm and tomato plants resulting in miss-recognition and missing its PGPB activity. In consequence, *Bacillus megaterium* and *Enterobacter* C7 PGPB activity in tomato plants could be proposed as dependent and independent on ethylene perception by ETR3, respectively.

Furthermore, PGPB inoculation modified the root transcriptomic profiles in a mode dependent on strain, genotype and watering regime. Flavonoid biosynthesis was affected by Bm inoculation enhancing antioxidant status in wt plants, while *nr* plants respond avoiding immune response but inhibiting complete interaction. Moreover, *ETR3* mutation also affects to tomato-C7 interaction causing C7 inoculation in wt plants higher respiration and carbon transport, and in *nr* plants probably improved Fe nutrition leading to better growth even under stress conditions. Furthermore, C7 could also modulate amino acid metabolism and/or nitrogen use by plants. However, further research is needed to clarify if PGPB are able to modulate these processes studying PGPB effects in metabolites as well as in nutritional and antioxidant statuses.

Chapter 2: PGPB inoculation modifies photosynthetic traits and root metabolites as well as nutritional and hormonal statuses with strong influence of ethylene sensitivity

Chapter 2: PGPB inoculation modifies photosynthetic traits and root metabolites as well as nutritional and hormonal statuses with strong influence of ethylene sensitivity

Objective

The present chapter pursued to gain more insights into in plant-microbe interactions regarding to ethylene sensitivity, with focus in plant nutritional and phytohormonal statuses as well as photosynthetic traits and root metabolites at two differential developmental stages. Rhizospheric microorganisms are associated with nutrient biogeochemical cycles (Barea et al. 2005), and plant-bacterial interaction was reported as essential for better nutrition (Ryan et al. 2009). Moreover, nutritional and hormonal homeostasis are closely coordinated to finely regulate plant growth and development (Krouk et al. 2011), and metabolic information contribute with valuable information to understand plant-bacteria interaction (Feussner and Polle 2015). Additionally, PGPB colonization of plant root system causes physiological modifications as well as changes in metabolites (Su et al. 2016).

In consequence, the present study aimed to determine if ethylene sensitivity is crucial for plant-bacteria interaction and growth induction by these two PGPB strains in juvenile plants, and to evaluate the effects of bacterial inoculation on plant physiology at both juvenile and mature stages. In order to achieve exposed aims, plant growth, stomatal conductance, photosynthetic efficiency, chlorophyll contents, nutrients,

phytohormones and root metabolites were determined at 4 and 8 weeks post-inoculation (wpi).

Experimental design

The experiment consisted of a randomized complete block design with two plant lines: tomato cv. Pearson wild type and its ethylene-insensitive mutant (*never ripe*), and three inoculation treatments: (1) non-inoculated control plants, (2) *Bacillus megaterium*-inoculated plants and (3) *Enterobacter C7*-inoculated plants. Experiment consisted of 18 replicates per treatment and two different harvests were established: 4 and 8 weeks post inoculation, using nine plants at each harvest (n=9).

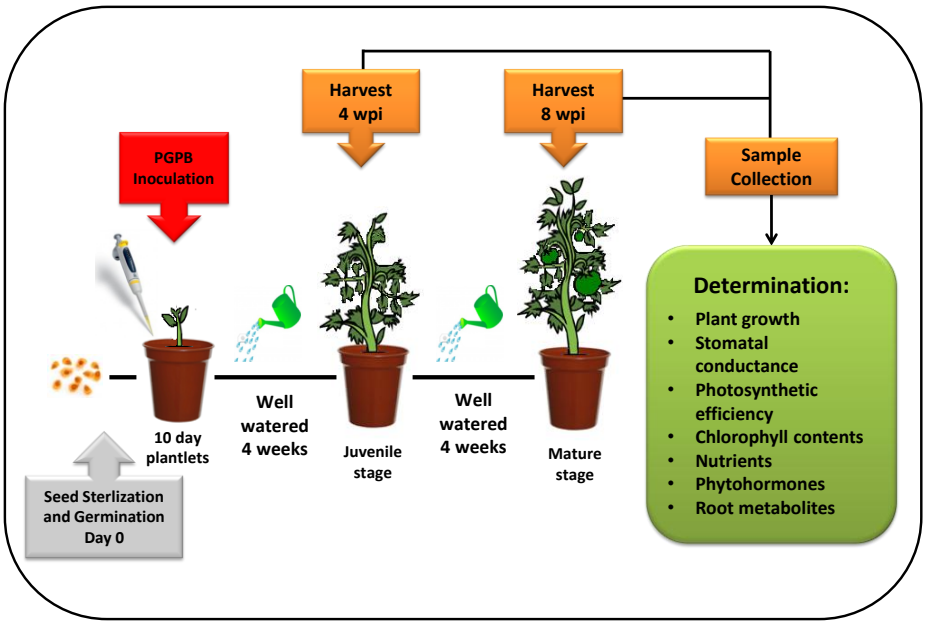

Figure 2.1 Schematic representation of experimental design of chapter 2.
Weeks post-inoculation (wpi).

Results

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

Dry weight of wild type and *never ripe* plants inoculated with PGPB strains (*B. megaterium* and *Enterobacter* C7) showed different growth patterns at 4 and 8 weeks post-inoculation (wpi), but no interaction between factors was noticed (Fig. 2.2). At 4 wpi, total and shoot DWs showed no differences due to bacteria inoculation in wt and *nr* plants neither due to plant genotype under different inoculations (Fig. 2.2 A, C). However, root DW was increased by Bm inoculation in *nr* plants, while no growth promotion in wt roots was observed. Moreover, significant difference between wt and *nr* plants was only observed in Bm-inoculated roots (Fig. 2.2 E).

At 8 wpi, total DW 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 DW was only increased by C7 inoculation (25.5%), showing no significant differences due to Bm inoculation (Fig. 2.2 B). Shoot DW 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 significant effect on shoot growth (Fig. 2.2 D). Moreover, significant difference between plant genotypes was only noticed in non-inoculated plants in total and shoot DWs (Fig. 2.2 B, D). In addition, root DW was increased by PGPB inoculation in wt plants (15.8% and 18.1% for Bm and C7, respectively), showing no significant differences in *nr* plants (Fig. 2.2 F).

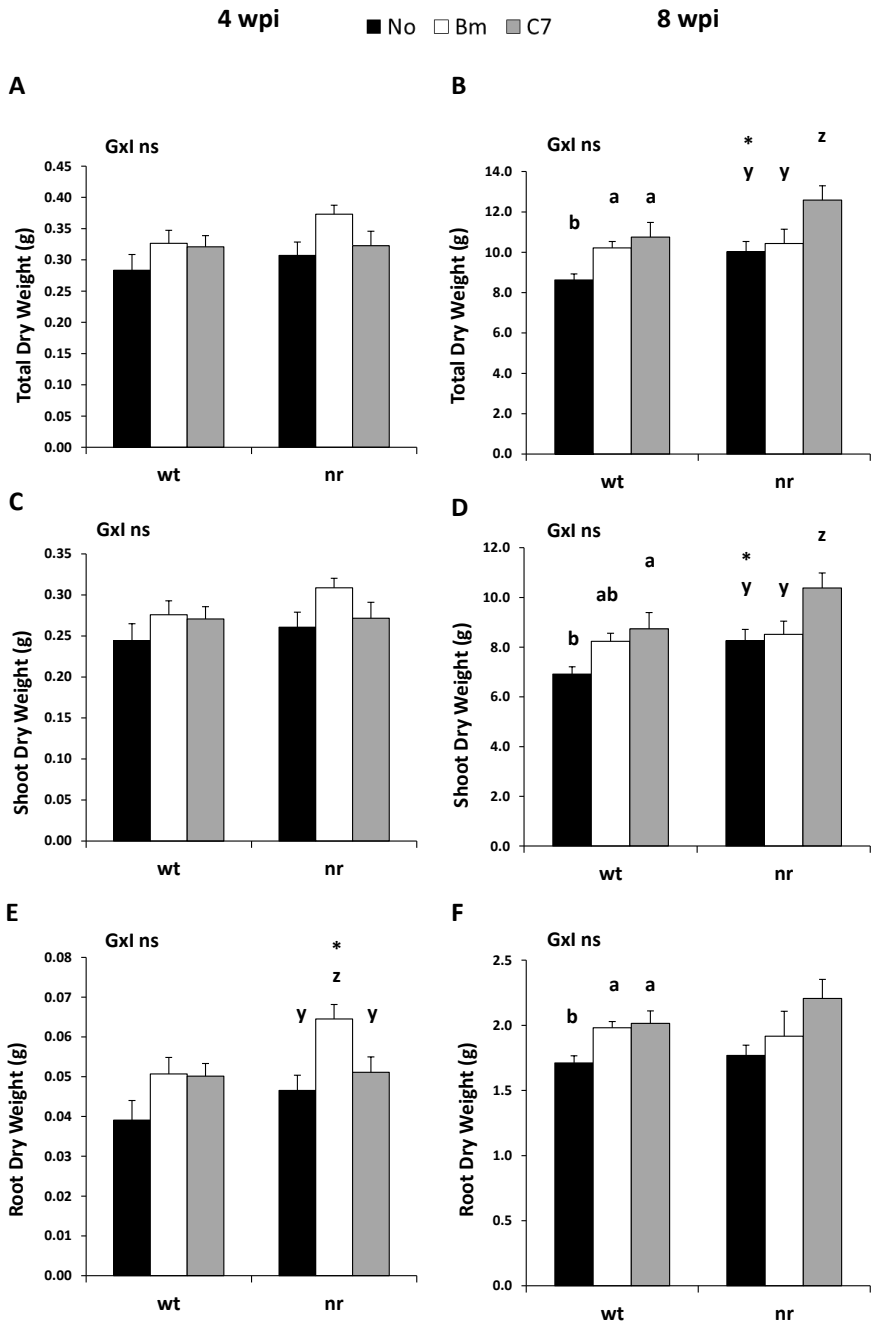

Figure 2.2 Effects of bacterial inoculation on plant dry weights at 4 and 8 weeks post-inoculation. (A) Total, **(C)** shoot and **(E)** root dry weights of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants at 4 weeks

post-inoculation (wpi). **(B)** Total, **(D)** shoot and **(F)** root dry weights of wild type cv. Pearson (wt) and *never ripe* (*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 ± SE (n = 9). Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. As no significant interaction between factors was noticed, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

In order to analyze plant growth responses between the two harvests, RGR was calculated showing interaction between factors in total and root DWs (Fig. 2.3). In wt plants, RGRs were unaffected. However, in *nr* plants RGR changed according to inoculated bacteria and plant tissue (Fig. 2.3).

Total RGR showed the highest value in C7-inoculated *nr* plants and the lowest value in Bm-inoculated ones, showing both of them significant differences with control plants. Furthermore, no differences due to plant genotype were noticed (Fig. 2.3 A). In shoots, C7-inoculated *nr* plants increased RGR, but Bm inoculation did not affect shoot RGR compared to control plants (Fig. 2.3 B). Furthermore, root RGR was only decreased by Bm inoculation in *nr* plants and significant difference between plant genotypes was exclusively noticed under Bm inoculation (Fig. 2.3 C).

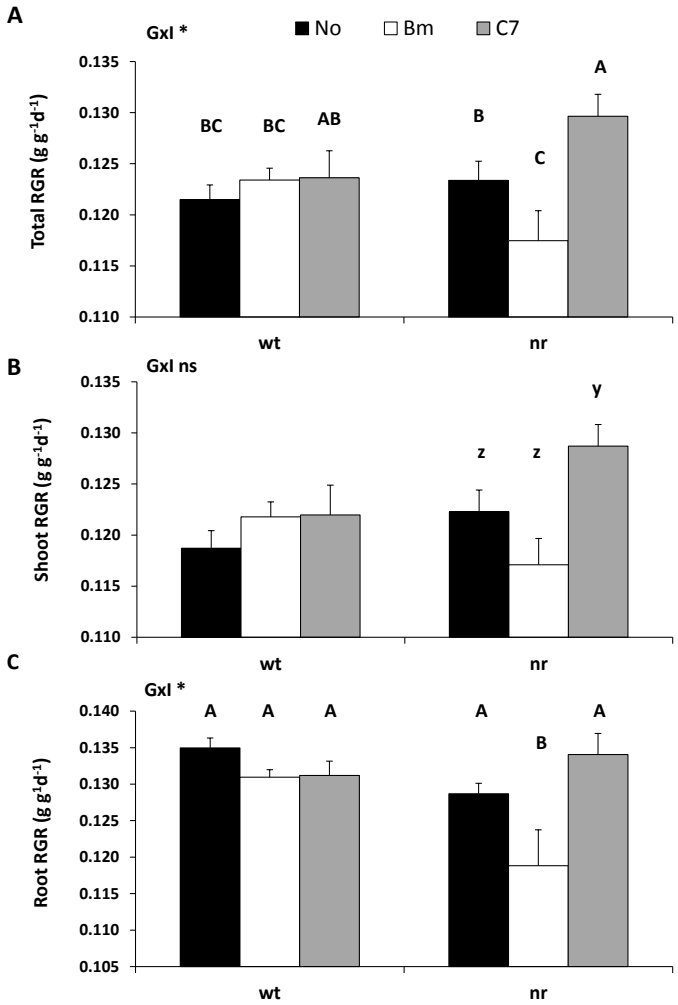

Figure 2.3 Effects of bacterial inoculation on relative growth rates. (A) Total, **(B)** shoot and **(C)** root relative growth rates (RGR) of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants at 8 weeks post-inoculation (wpi). Treatments are designed as non-inoculated controls (No, black bars), *Bacillus megaterium* inoculated plants (Bm, white bars), and *Enterobacter C7* inoculated plants (C7, grey bars). Data are means ± SE (n = 9). Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different ($P < 0.05$) according to LSD's test. In case of not-significant interaction between factors, inoculum effects were evaluated

analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) above *nr* means, but genotype effect was unnoticed in shoot RGR.

PGPB effects on stomatal conductance, photosynthetic efficiency and chlorophyll content

Factor interaction was not significant in stomatal conductance, photosynthetic efficiency and chlorophyll content (Table 2.1). Stomatal conductance was affected by bacterial inoculation in both plant genotypes only at 4 wpi, showing C7 inoculation 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, showing no effect in *nr* plants. In addition, significant differences between wt and *nr* plants in stomatal conductance were only noticed under Bm inoculation at 4 wpi (higher in *nr* plants) and under all inoculation treatments at 8 wpi (higher values in wt plants) (Table 2.1). 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 noticed 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 noticed in non- and C7-inoculated plants with higher values in wt plants (Table 2.1). Chlorophyll content was unaltered by PGPB inoculation in both plant

genotypes at both harvests. Moreover, significant plant genotype difference was only noticed under Bm inoculation at 4 wpi (Table 2.1).

Table 2.1 Effects of bacterial inoculation on stomatal conductance photosynthetic efficiency and chlorophyll content. Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 9). Significance of sources of variation interaction (GxI) was evaluated by P-value. As no significant interaction between factors was noticed, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) next to *nr* means.

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

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; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

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 (Tables 2.2 and 2.3, respectively).

In case of root nutrients, factor interaction was only significant in Ca concentration at 8 wpi (Table 2.2). Root nutrients showed no significant differences due to PGPB inoculation in wt plants at 4 wpi. However, Na level was decreased by C7 inoculation in *nr* plants at 4 wpi. Moreover, Zn concentration was higher in C7- regarding Bm-inoculated *nr* plants showing no differences compared to control ones. Furthermore, Mn concentration showed significant differences between wt and *nr* plants showing *nr* plants higher values than wt ones under all inoculation treatments (Table 2.2).

In contrast, several changes were noticed 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 to non-inoculated 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 higher level in *nr* plants, while Si showed higher levels in wt ones. Finally, Cu, Mg and S concentrations were significantly lower in *nr* than in wt plants under both bacterial inoculations (Table 2.2).

Regarding shoot nutrients, several changes were noticed in wt and *nr* plants at 4 and 8 wpi. Moreover, factor interaction was significant for Cu, Fe and P concentrations at 8 wpi (Table 2.3).

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 noticed for Cu, Mn and Zn under all inoculation treatments showing *nr* plants lower values than wt plants. Moreover, significant differences between plants genotypes were noticed in non-inoculated plants only for Mg level, and under both bacterial inoculations for Ca concentration, showing higher concentration in wt plants in all cases (Table 2.3).

Table 2.2 Effects of bacterial inoculation on root nutrient concentrations. Root nutrient concentrations in cv Pearson tomato plants at two harvest times: 4 and 8 weeks post-inoculation (wpi). Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 4). Significance of sources of variation interaction (GxI) was evaluated by P-value. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different (P < 0.05) according to LSD's test. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) above *nr* means.

	Carbon (% DW)		Nitrogen (% DW)		Calcium (% DW)		Cooper (ppm)		Iron (ppm)		Potassium (% DW)		Magnesium (% DW)		Manganese (ppm)		Sodium (% DW)		Phosphorus (% DW)		Sulphur (% DW)		Silicon (% DW)		Zinc (ppm)	
wpi	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8
wt No	41.8 +/- 0.5	44.3 +/- 0.3 **b**	2.50 +/- 0.10	1.38 +/- 0.05	0.88 +/- 0.04	0.81 +/- 0.02 **A**	5.80 +/- 0.95	15.72 +/- 2.87	306.1 2 +/- 80.85	100.1 1 +/- 20.77	0.77 +/- 0.32	0.56 +/- 0.08	0.28 +/- 0.03	0.59 +/- 0.08	113.0 2 +/- 15.74	32.13 +/- 3.00	0.26 +/- 0.05	0.63 +/- 0.06	0.27 +/- 0.06	0.18 +/- 0.02	0.18 +/- 0.06	0.202 +/- 0.016	0.10 +/- 0.06	0.052 +/- 0.006	24.14 +/- 4.84	29.86 +/- 3.55
wt Bm	41.6 +/- 0.2	45.1 +/- 0.3 **a**	2.57 +/- 0.14	1.36 +/- 0.03	0.75 +/- 0.03	0.89 +/- 0.01 **AB**	8.33 +/- 0.40	12.37 +/- 0.62	374.9 7 +/- 70.02	108.8 4 +/- 17.21	1.16 +/- 0.16	0.39 +/- 0.07	0.41 +/- 0.05	0.49 +/- 0.05	109.1 8 +/- 2.20	38.08 +/- 4.93	0.42 +/- 0.06	0.71 +/- 0.04	0.39 +/- 0.04	0.20 +/- 0.01	0.29 +/- 0.04	0.207 +/- 0.006	0.11 +/- 0.01	0.061 +/- 0.008	28.50 +/- 2.57	34.36 +/- 1.87
wt C7	41.7 +/- 0.3	45.7 +/- 0.1 **a**	2.44 +/- 0.06	1.33 +/- 0.04	0.81 +/- 0.07	0.85 +/- 0.04 **B**	6.68 +/- 0.44	12.28 +/- 0.51	460.8 1 +/- 41.25	94.27 +/- 25.84	1.17 +/- 0.19	0.42 +/- 0.05	0.37 +/- 0.01	0.51 +/- 0.04	142.6 9 +/- 9.01	30.95 +/- 2.00	0.30 +/- 0.04	0.60 +/- 0.06	0.34 +/- 0.06	0.18 +/- 0.02	0.24 +/- 0.03	0.193 +/- 0.008	0.16 +/- 0.02	0.053 +/- 0.003	30.98 +/- 4.49	34.80 +/- 4.32
P-value	na	*	na	na	na	na	na	ns	na	na	na	na	na	na	ns	na	ns	na	na	na	na	ns	na	na	na	na
nr No	42.1 +/- 0.6	45.3 +/- 0.2	2.76 +/- 0.03	1.37 +/- 0.04	0.74 +/- 0.01	0.60 +/- 0.02 **C**	6.46 +/- 0.50	10.39 +/- 0.26 **z**	173.6 9 +/- 7.89	69.79 +/- 2.21	1.20 +/- 0.11	0.34 +/- 0.04	0.28 +/- 0.01	0.34 +/- 0.01	217.8 7 +/- 14.32 *	56.92 +/- 3.86	0.37 +/- 0.03 **z**	0.51 +/- 0.03	0.37 +/- 0.03	0.18 +/- 0.01	0.28 +/- 0.01	0.191 +/- 0.009 **z**	0.06 +/- 0.01	0.047 +/- 0.002	32.55 +/- 1.59	38.24 +/- 5.47
nr Bm	42.4 +/- 0.4	45.2 +/- 0.2	2.56 +/- 0.07	1.43 +/- 0.09	0.73 +/- 0.04	0.53 +/- 0.03 **C**	6.32 +/- 0.34	8.81 +/- 0.55 **y** *	218.8 8 +/- 39.20	57.13 +/- 2.63	1.15 +/- 0.07	0.30 +/- 0.02	0.29 +/- 0.01	0.27 +/- 0.01 *	201.1 0 +/- 8.36 *	49.34 +/- 5.88 *	0.36 +/- 0.02 **z**	0.44 +/- 0.03 *	0.37 +/- 0.01	0.18 +/- 0.01	0.32 +/- 0.01	0.181 +/- 0.007 **z** *	0.07 +/- 0.01	0.040 +/- 0.002	29.62 +/- 0.38	32.11 +/- 2.32
nr C7	42.8 +/- 0.2	45.9 +/- 0.4	2.56 +/- 0.10	1.45 +/- 0.03	0.72 +/- 0.01	0.53 +/- 0.01 **C**	8.18 +/- 1.42	7.18 +/- 0.62 **x** *	303.7 9 +/- 97.43	63.58 +/- 14.19	1.31 +/- 0.02	0.25 +/- 0.04	0.29 +/- 0.02	0.25 +/- 0.01 *	246.0 9 +/- 10.55 *	42.64 +/- 3.75 *	0.26 +/- 0.01 **y**	0.42 +/- 0.02	0.40 +/- 0.01	0.16 +/- 0.01	0.29 +/- 0.02	0.150 +/- 0.002 **y** *	0.10 +/- 0.03	0.031 +/- 0.002 *	36.30 +/- 1.44	25.86 +/- 0.52
P-value	na	ns	na	na	na	na	na	**	na	na	na	na	na	na	ns	na	*	na	na	na	na	*	na	na	na	na
	Significance of source of variation																									
G x I	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

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

na: not-applicable; P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001. G x I: Genotype*Inoculum

Table 2.3 Effects of bacterial inoculation on shoot nutrient concentrations. Shoot nutrient concentrations in cv Pearson tomato plants at two harvest times: 4 and 8 weeks post-inoculation (wpi). Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 4). Significance of sources of variation interaction (GxI) was evaluated by P-value. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different (P < 0.05) according to LSD's test. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) above *nr* means.

	Carbon (% DW)		Nitrogen (% DW)		Calcium (% DW)		Cooper (ppm)		Iron (ppm)		Potassium (% DW)		Magnesium (% DW)		Manganese (ppm)		Sodium (% DW)		Phosphorus (% DW)		Sulphur (% DW)		Silicon (% DW)		Zinc (ppm)	
wpi	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8
wt No	39.0 +/- 0.2 b	39.1 +/- 0.2	5.06 +/- 0.18 a	0.84 +/- 0.02	2.29 +/- 0.10	1.85 +/- 0.07	5.63 +/- 0.22	2.65 +/- 0.05 B	88.42 +/- 3.85	23.83 +/- 0.90 B	2.06 +/- 0.06	1.48 +/- 0.08	0.26 +/- 0.01	0.31 +/- 0.01	123.0 0 +/- 2.00 a	109.8 7 +/- 3.63	0.16 +/- 0.03	0.053 +/- 0.004	0.57 +/- 0.02	0.27 +/- 0.01 A	0.59 +/- 0.03	0.32 +/- 0.02	0.057 +/- 0.05	nd	67.70 +/- 1.66	29.31 +/- 1.41
wt Bm	40.0 +/- 0.1 a	39.6 +/- 0.2	4.42 +/- 0.17 b	0.84 +/- 0.03	2.34 +/- 0.08	1.73 +/- 0.07	5.37 +/- 0.26	2.42 +/- 0.03 BC	85.10 +/- 7.52	21.71 +/- 1.69 B	1.79 +/- 0.09	1.32 +/- 0.05	0.26 +/- 0.02	0.30 +/- 0.01	114.7 7 +/- 2.10 b	105.0 0 +/- 4.32	0.12 +/- 0.01	0.048 +/- 0.004	0.49 +/- 0.03	0.27 +/- 0.01 AB	0.60 +/- 0.04	0.30 +/- 0.02	0.055 +/- 0.003	nd	60.66 +/- 3.04	28.40 +/- 1.39
wt C7	38.4 +/- 0.6 b	39.1 +/- 0.1	5.41 +/- 0.24 a	0.89 +/- 0.04	2.23 +/- 0.06	1.77 +/- 0.06	5.57 +/- 0.26	3.00 +/- 0.15 A	84.10 +/- 3.01	31.75 +/- 4.25 A	1.93 +/- 0.17	1.33 +/- 0.06	0.25 +/- 0.02	0.33 +/- 0.02	125.9 5 +/- 2.35 a	106.9 2 +/- 4.70	0.17 +/- 0.02	0.053 +/- 0.006	0.57 +/- 0.04	0.28 +/- 0.01 A	0.61 +/- 0.04	0.33 +/- 0.02	0.056 +/- 0.005	nd	69.87 +/- 2.80	30.94 +/- 1.14
P-value	*	na	*	na	na	na	na	na	na	na	na	na	na	na	**	ns	na	na	ns	na	na	na	na	na	ns	na
nr No	40.1 +/- 0.5	39.3 +/- 0.6	4.99 +/- 0.19	0.84 +/- 0.05	2.10 +/- 0.07	1.26 +/- 0.07 *	4.14 +/- 0.19 *	2.53 +/- 0.14 B	86.57 +/- 3.56	21.97 +/- 2.14 B	1.68 +/- 0.15	1.23 +/- 0.06 *	0.22 +/- 0.01 *	0.22 +/- 0.01 *	112.2 2 +/- 3.32 *	89.08 +/- 5.45 *	0.12 +/- 0.01	0.084 +/- 0.005 *	0.49 +/- 0.03	0.27 +/- 0.01 A	0.54 +/- 0.05	0.27 +/- 0.03	0.051 +/- 0.004	nd	57.39 +/- 1.67 z *	27.84 +/- 1.68
nr Bm	40.2 +/- 0.3	39.9 +/- 0.1	4.84 +/- 0.08	0.96 +/- 0.05	2.11 +/- 0.06 *	1.32 +/- 0.05 *	4.50 +/- 0.18 *	2.52 +/- 0.15 B	76.02 +/- 1.31	27.68 +/- 2.22 AB	1.76 +/- 0.09	1.31 +/- 0.07	0.22 +/- 0.01	0.23 +/- 0.01 *	105.3 7 +/- 1.74 *	92.59 +/- 6.21	0.12 +/- 0.01	0.081 +/- 0.007 *	0.46 +/- 0.02	0.29 +/- 0.02 A	0.57 +/- 0.02	0.28 +/- 0.02	0.048 +/- 0.003	nd	50.33 +/- 1.71 y *	26.46 +/- 1.69
nr C7	38.4 +/- 1.1	40.1 +/- 0.1 *	5.34 +/- 0.16	0.88 +/- 0.03	1.96 +/- 0.06 *	1.17 +/- 0.06 *	4.35 +/- 0.19 *	2.15 +/- 0.05 C	84.22 +/- 2.69	22.25 +/- 1.19 B	1.88 +/- 0.07	1.11 +/- 0.05 *	0.22 +/- 0.004	0.20 +/- 0.01 *	113.6 8 +/- 2.83 *	87.00 +/- 4.37 *	0.13 +/- 0.02	0.062 +/- 0.002	0.52 +/- 0.01	0.23 +/- 0.01 B	0.55 +/- 0.03	0.23 +/- 0.01 *	0.049 +/- 0.003	nd	56.01 +/- 1.08 z *	23.14 +/- 1.11 *
P-value	ns	na	ns	na	na	na	na	na	na	na	na	na	na	na	ns	ns	na	na	ns	na	na	na	na	na	*	na
	Significance of source of variation																									
G x I	ns	ns	ns	ns	ns	ns	ns	***	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	-	ns	ns

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).

nd: non-detected; na: not-applicable; P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

At 8 wpi, Bm only increased 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 noticed for several nutrients. Ca and Mg concentrations showed higher values in wt than in *nr* plants under all inoculation treatments. In 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 noticed for K and Mn in non- and C7-inoculated plants showing both higher values in wt plants. Finally, significant differences were exclusively noticed 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 2.3).

Nutrient concentrations and plant DWs were assessed by Pearson correlation analysis (Table 2.4). A positive correlation was observed between root S concentration and total, shoot and root DWs at 4 wpi. Meanwhile, shoot Fe concentration showed a strong negative correlation with all DWs at this time. At 8 wpi, DWs were correlated with several nutrients. Indeed, a positive correlation was found between the root C concentration and all DWs. Moreover, negative correlations were obtained between total DW and root Cu, K and S concentrations. Shoot K concentration was also negatively correlated with total DW. In addition, shoot DW showed the same correlations as total DW (Table 2.4).

Table 2.4 Pearson correlations between plant dry weights and nutrient contents.

Tissue			Root					Shoot				
Nutrient			Carbon (% DW)	Copper (ppm)	Iron (ppm)	Potassium (% DW)	Sulphur (% DW)	Carbon (% DW)	Copper (ppm)	Iron (ppm)	Potassium (% DW)	Sulphur (% DW)
4 weeks post inoculation	Total DW	r	0.376	0.177	-0.155	0.523	0.809	0.396	-0.371	-0.973	-0.540	-0.078
		P-value	0.449	0.732	0.764	0.266	0.033	0.421	0.455	0.000	0.247	0.881
	Shoot DW	r	0.359	0.179	-0.148	0.510	0.805	0.407	-0.355	-0.971	-0.535	-0.063
		P-value	0.472	0.729	0.774	0.281	0.035	0.407	0.476	0.000	0.252	0.903
	Root DW	r	0.418	0.172	0.172	-0.172	0.818	0.368	-0.411	-0.974	-0.549	-0.116
		P-value	0.393	0.745	0.740	0.739	0.030	0.459	0.402	0.000	0.237	0.823
8 weeks post inoculation	Total DW	r	0.916	-0.848	-0.502	-0.836	-0.849	0.715	-0.479	-0.016	-0.901	-0.687
		P-value	0.004	0.018	0.290	0.023	0.018	0.086	0.316	0.975	0.006	0.107
	Shoot DW	r	0.921	-0.868	-0.541	-0.854	-0.865	0.714	-0.489	-0.043	-0.920	-0.714
		P-value	0.003	0.013	0.246	0.017	0.014	0.087	0.305	0.935	0.003	0.087
	Root DW	r	0.810	-0.661	-0.225	-0.661	-0.686	0.669	-0.385	0.149	-0.714	-0.465
		P-value	0.033	0.129	0.661	0.128	0.108	0.122	0.436	0.773	0.087	0.334

DW: Dry Weight

Root nutrient concentrations and total, shoot and root DWs values were used to build a data matrix in order to perform a principal component analysis (PCA) to compare inoculation treatments in both plant genotypes (Fig. 2.4). 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, meanwhile C7-inoculated plants shared a tiny area with control ones. However, both bacterial effects were mostly overlapped. Also, both bacterial inoculations shared a little area with non-inoculated *nr* plants without overlapping each other (Fig. 2.4 A). 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. 2.4 B).

At 8 wpi, PCA (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 little area between Bm- and C7-inoculated areas (Fig. 2.4 C). Nutrients with higher contribution to variability were Ca, Fe and Mg for PC1, and P and Zn for PC2 (Fig. 2.4 D).

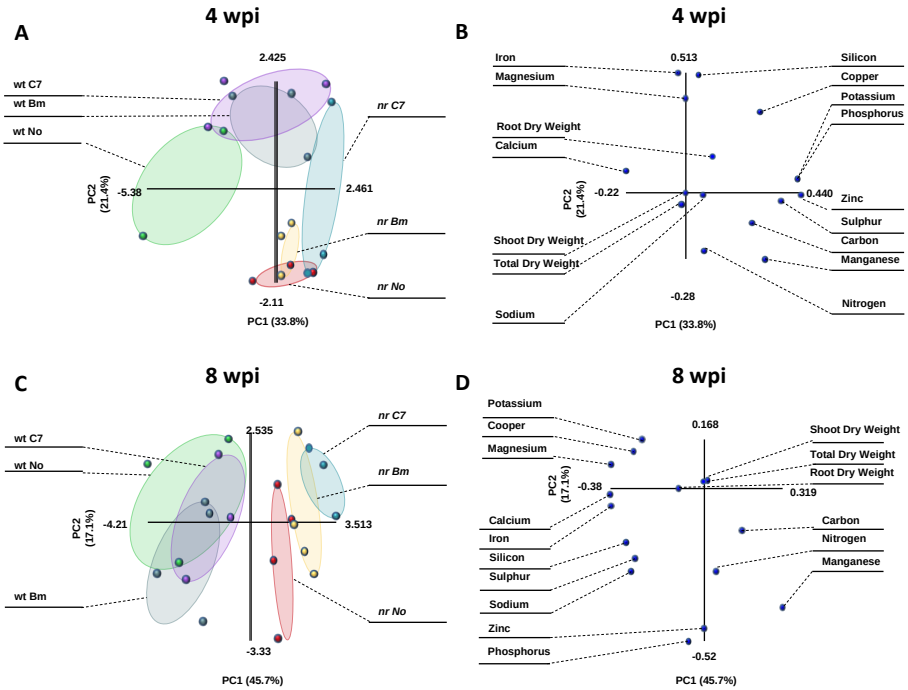

Figure 2.4 Principal component analyses of root nutrient concentrations and dry weights. Analyses were performed based on nutrient concentration and dry weight data obtained from tomato (*Solanum lycopersicum*) plants. Score plot at **(A)** 4 wpi and **(C)** 8 wpi. Treatments: non-inoculated, *Bacillus 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 were enclosed in a different colored 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 **(B)** 4 wpi and **(D)** 8 wpi. Each point represents one nutrient or dry weight.

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 2.5 and 2.6, 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 neither 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, significant difference between wt and *nr* plants was only observed in C7-inoculated roots (higher levels in *nr* plants) and unnoticed in shoots (Table 2.5). In case of ethylene, genotype differences were observed in non- and Bm-inoculated roots and in non- and C7-inoculated shoots showing *nr* plants higher ethylene levels than wt ones in all cases (Table 2.5).

Moreover, differences between wt and *nr* plants were noticed for IAA concentration in non- and C7-inoculated roots and exclusively under C7 inoculation in shoots showing *nr* plants higher levels than wt ones (Table 2.5). Finally, genotype difference in SA concentration was only noticed in Bm-inoculated shoots with higher values in *nr* plants (Table 2.5).

Table 2.5 Effects of bacterial inoculation on phytohormone concentrations at 4 weeks post-inoculation. Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 6). Significance of sources of variation interaction (GxI) was evaluated by P-value. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different (P < 0.05) according to LSD's test. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA, but inoculums effects were unnoticed. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) below *nr* means.

Tissue	Root				Shoot			
Hormone	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
P-value	na	ns	na	na	na	na	na	na
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
P-value	na	ns	na	na	na	na	na	na

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).

nd: non-detected; na: not-applicable; P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

Table 2.6 Effects of bacterial inoculation on phytohormone concentrations at 8 weeks post-inoculation. Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 4). Significance of sources of variation interaction (GxI) was evaluated by P-value. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA, but inoculum effects were unnoticed. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) below *nr* means.

Tissue	Root					Shoot				
Hormone	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
P-value	na	na	na	na	na	na	na	na	na	na
nr 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
nr 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 *
nr 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
P-value	na	na	na	na	na	na	na	na	na	na

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).

nd: non-detected; P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

At 8 wpi, PGPB inoculation did not affect any phytohormone levels in roots neither in shoots. However, significant differences between wt and *nr* plants were noticed in both tissues (Table 2.6). ABA levels were also 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 2.6). In case of IAA, genotype differences were only noticed in non-inoculated plants in root and shoot tissues, showing *nr* plants higher levels than wt ones (Table 2.6). Moreover, SA concentration showed significant differences between plant genotypes in non- and Bm-inoculated roots and exclusively in Bm-inoculated shoots, showing always higher values in *nr* plants (Table 2.6). Furthermore, although genotype differences were unnoticed for JA and JA-Ile concentration in roots, differences between wt and *nr* plants were exclusively observed under Bm inoculation in shoots showing *nr* plants higher values than wt ones (Table 2.6).

PGPB effects on root metabolite content

Root metabolites were analyzed to determine how PGPB inoculation affects their accumulation regarding to ethylene insensitivity in juvenile and mature plants (Tables 2.7 and 2.8, respectively). Several root metabolites were identified including carbohydrates (fructose, glucose, galactose, sacarose, mannose and myoinositol), amino acids (glycine, methionine, phenylalanine, threonine, valine, leucine/isoleucine, glutamine, serine, gamma-aminobutitic 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 noticed for galactose, aspartic acid, glutamic acid, fumaric acid, glutamine, isoleucine/leucine, phenylalanine, serine, threonine and valine at 4 wpi, while no factor interaction was noticed at 8 wpi.

At 4 wpi, a dozen metabolites showed different levels due to bacterial inoculation in wt and/or *nr* plants (Table 2.7 A). Carbohydrates were unaffected in wt plants. However, C7 inoculation reduced fructose, galactose and glucose in *nr* plants compared to 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 noticed in threonine and serine, showing no changes compared to control plants. 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 noticed for myoinositol, malic acid, GABA and DHA levels (Table 2.7 A).

Furthermore, differences between wt and *nr* plants were noticed in several root metabolites. GABA levels showed higher values in *nr* than in wt plants under all inoculation treatments. In case of galactose, significant difference between plant genotypes was noticed in Bm-inoculated plants (higher level in *nr* plants) and in C7-inoculated ones (higher level in wt plants). Moreover, a difference between wt and *nr* plants was only noticed in non-inoculated plants for mioinositol, fumaric acid and succinic acid showing *nr* plants higher values. In case of malic acid, genotype differences were exclusively noticed under C7 inoculation with higher values in wt plants. In addition, significant difference between plant genotypes was exclusively noticed under C7

inoculation for aspartic acid, glutamic acid, glutamine, isoleucine/leucine, phenylalanine and serine showing higher values in *nr* plants. In case of threonine and valine, significant differences between plant genotypes were noticed in non- and C7-inoculated plants showing *nr* plants higher levels than wt ones (Table 2.7 A). Correlations between plant DWs and root metabolite levels were evaluated at 4 wpi (Table 2.7 B). The galactose content was positively correlated with total, shoot and root DWs, while DHA was negatively correlated with all DWs.

PCA was also performed to compare inoculation treatment effects on root metabolite profiles at 4 wpi (Fig. 2.5). In this analysis, 57.7% of the variability was explained by the data in axes PC1 and PC2. 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 than 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. 2.5 A). The loading plot showed two clear sources of variation; amino acids (glutamine, serine, phenylalanine, valine and threonine) and carbohydrates (fructose, glucose, galactose and sacarose), which contributed to the high variability for PC1 and PC2, respectively (Fig. 2.5 B).

Table 2.7 Root metabolite contents and correlations between plant dry weights at 4 weeks post-inoculation (wpi). (A) Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 4). Significance of sources of variation interaction (GxI) was evaluated by P-value. In case of significant interaction between factors, all treatments were compared between each others. Means followed by different capital letters are significantly different (P < 0.05) according to LSD's test. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) next to *nr* means. **(B)** Significant Pearson correlations between plant dry weights and root metabolites at 4 wpi.

A		Fructose	Galactose	Glucose	Aspartic acid	Glutamic acid	Fumaric acid	Glutamine	Isoleucine/ leucine	Phenylalanine	Serine	Threonine	valine	Glicine	GABA	DHA
wt No		7.40 +/- 1.41	0.025 +/- 0.004 BC	4.47 +/- 0.90	1.08 +/- 0.07 BC	3.70 +/- 0.30 BC	0.29 +/- 0.02 B	1.08 +/- 0.08 B	0.13 +/- 0.01 B	0.086 +/- 0.009 B	0.79 +/- 0.06 BC	0.059 +/- 0.004 BC	0.053 +/- 0.010 CD	0.201 +/- 0.185	1.956 +/- 0.375	0.037 +/- 0.008
wt Bm		8.44 +/- 0.74	0.033 +/- 0.003 B	6.78 +/- 0.58	0.93 +/- 0.10 CD	3.35 +/- 0.41 C	0.33 +/- 0.02 AB	0.98 +/- 0.12 BC	0.14 +/- 0.01 AB	0.076 +/- 0.013 B	0.72 +/- 0.07 BC	0.062 +/- 0.005 B	0.066 +/- 0.018 BC	0.204 +/- 0.084	2.044 +/- 0.287	0.031 +/- 0.005
wt C7		10.71 +/- 1.84	0.032 +/- 0.005 B	5.68 +/- 1.04	0.65 +/- 0.14 D	1.87 +/- 0.42 D	0.37 +/- 0.02 A	0.67 +/- 0.16 C	0.08 +/- 0.01 C	0.041 +/- 0.005 C	0.33 +/- 0.08 D	0.040 +/- 0.007 C	0.044 +/- 0.019 D	0.258 +/- 0.042	2.154 +/- 0.389	0.031 +/- 0.004
P-value		ns	na	ns	na	*	na	na	na	na	na	na	na	na	na	na
nr No		9.22 +/- 1.01 y	0.032 +/- 0.003 B	6.37 +/- 0.63 z	1.31 +/- 0.10 B	4.61 +/- 0.27 B	0.37 +/- 0.01 A	1.27 +/- 0.07 B	0.16 +/- 0.01 AB	0.103 +/- 0.007 AB	0.95 +/- 0.06 AB	0.087 +/- 0.007 A	0.080 +/- 0.016 AB	0.338 +/- 0.117	3.305 +/- 0.740 *	0.030 +/- 0.002
nr Bm		12.11 +/- 1.17 z	0.047 +/- 0.003 A	7.88 +/- 0.75 z	0.85 +/- 0.12 CD	3.65 +/- 0.55 BC	0.29 +/- 0.03 B	1.03 +/- 0.14 B	0.16 +/- 0.02 AB	0.093 +/- 0.015 AB	0.70 +/- 0.11 C	0.062 +/- 0.007 B	0.070 +/- 0.02 ABC	0.220 +/- 0.075	2.691 +/- 0.534 *	0.026 +/- 0.004
nr C7		5.73 +/- 0.48 x	0.022 +/- 0.002 C	4.20 +/- 0.41 y	1.82 +/- 0.09 A	5.78 +/- 0.26 A	0.36 +/- 0.01 A	1.59 +/- 0.07 A	0.17 +/- 0.01 A	0.122 +/- 0.009 A	1.09 +/- 0.04 A	0.102 +/- 0.007 A	0.090 +/- 0.013 A	0.490 +/- 0.282	3.554 +/- 0.686 *	0.026 +/- 0.003
P-value		***	na	**	na	**	na	na	na	na	na	na	na	na	na	na
B		Dry Weight and Metabolite Content Correlation														
Total DW	r	0.662	0.812	0.734	-0.239	-0.071	-0.269	-0.112	0.241	0.038	-0.159	-0.050	0.216	-0.063	0.177	-0.798
	P-value	0.127	0.032	0.074	0.640	0.891	0.597	0.829	0.637	0.942	0.759	0.923	0.674	0.903	0.731	0.038
Shoot DW	r	0.675	0.818	0.742	-0.252	-0.082	-0.283	-0.124	0.236	0.029	-0.167	-0.063	0.205	-0.083	0.157	-0.786
	P-value	0.117	0.029	0.069	0.621	0.874	0.577	0.811	0.645	0.956	0.747	0.903	0.690	0.872	0.762	0.044
Root DW	r	0.628	0.793	0.710	-0.203	-0.043	-0.231	-0.082	0.254	0.062	-0.139	-0.016	0.244	-0.011	0.228	-0.827
	P-value	0.158	0.040	0.090	0.692	0.935	0.652	0.875	0.619	0.905	0.789	0.976	0.633	0.983	0.655	0.026

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). DW: Dry Weight; GABA: *gamma*-Aminobutyric acid; DHA: dehydroascorbic acid.

DW: Dry Weight; nd: non-detected; P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

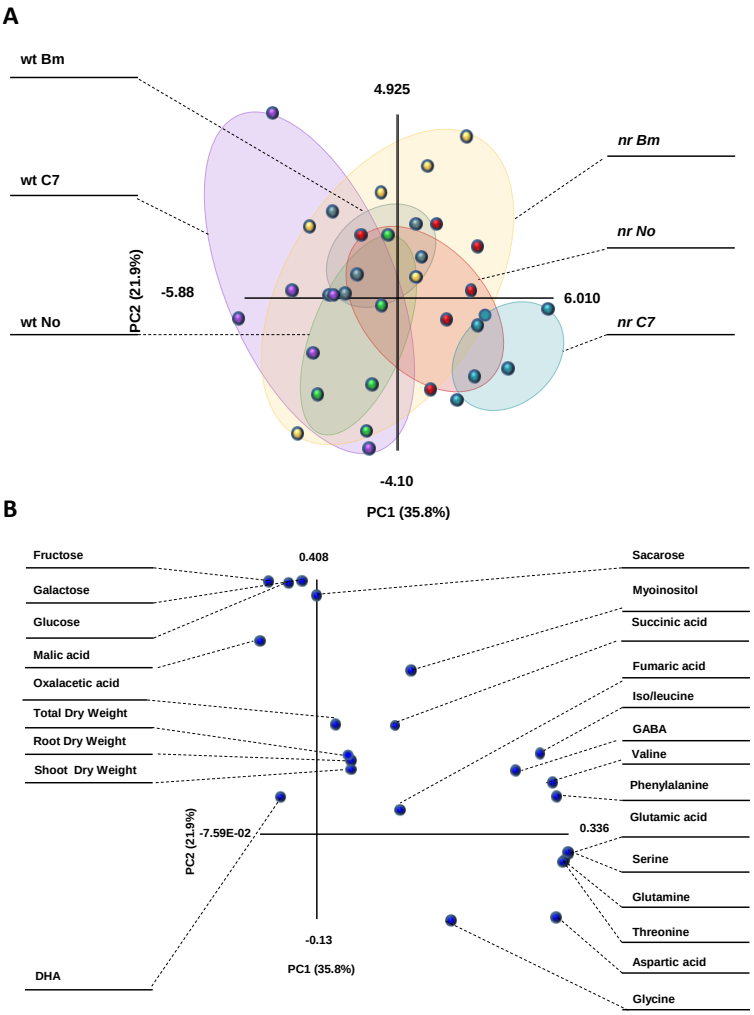

Figure 2.5 Principal component analysis of root metabolite levels and dry weights at 4 wpi. Analysis was performed based on metabolite contents and dry weight data obtained from tomato (*Solanum lycopersicum*) roots. **(A)** Score plot. Treatments: non-inoculated, *Bacillus megaoterium*-inoculated and *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 were enclosed in a different colored 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 metabolite or dry weight.

PGPB inoculation effects on root metabolite content regarding ethylene sensitivity were also assessed at 8 wpi. Nevertheless, factor interaction was unnoticed and PGPB inoculation did not affect root metabolite levels in mature plants (Table 2.8 A). Fructose levels were unaffected by plant genotype. However, differences between wt and *nr* plants were noticed for several metabolites. Mannose and serine levels showed higher values in *nr* than in wt plants under all inoculation treatments. Moreover, genotype difference was only noticed 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 showed also 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 2.8 A). Moreover, some positive correlations were observed between plant DWs and root metabolite levels at 8 wpi (Table 2.8 B). Fructose content was positively correlated with all DWs, and galactose and glucose were correlated positively with total and shoot DWs.

Treatment effects on root metabolites were also evaluated by PCA at 8 wpi (65.3% variability along axes PC1 and PC2) (Fig. 2.6). Profiles were not completely separated by plant genotype in Bm-inoculated and non-inoculated plants, but there were differences with C7-inoculated plants. In wt plants, both bacterial inoculations showed a totally different root metabolite profile compared to 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. 2.6 A).

Table 2.8 Root metabolite contents and correlations between plant dry weights at 8 weeks post-inoculation (wpi). (A) Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Data are means ± SE (n = 4). Significance of sources of variation interaction (GxI) was evaluated by P-value. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA, but inoculum effects were unnoticed. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) next to *nr* means. **(B)** Significant Pearson correlations between plant dry weights and root metabolites at 8 wpi.

A		Fructose	Galactose	Glucose	Mannose	Mioinositol	Aspartic acid	Glutamic acid	Glutamine	Isoleucine/ leucine	Phenylalanine	Serine	Threonine	valine	GABA	Metionine
wt No		68.91 +/- 1.63	0.479 +/- 0.035	41.13 +/- 3.33	0.30 +/- 0.02	6.67 +/- 1.94	0.23 +/- 0.13	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	0.35 +/- 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	0.40 +/- 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		ns	ns	na	na	na	na	na	na	na	na	na	na	na	na	na
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	0.53 +/- 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 *	0.57 +/- 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	0.59 +/- 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		ns	ns	na	na	na	na	na	na	na	na	na	na	na	na	na
B		Dry Weight and Metabolite Content Correlation														
Total DW	r	0.963	0.916	0.863	0.661	0.321	0.446	0.241	0.292	0.484	0.723	0.531	0.597	0.465	0.466	0.680
	P-value	0.000	0.004	0.014	0.128	0.523	0.358	0.638	0.563	0.312	0.082	0.257	0.187	0.334	0.333	0.113
Shoot DW	r	0.959	0.937	0.888	0.691	0.369	0.485	0.278	0.332	0.512	0.746	0.572	0.624	0.491	0.491	0.698
	P-value	0.000	0.002	0.008	0.105	0.457	0.310	0.583	0.508	0.278	0.066	0.213	0.162	0.303	0.303	0.099
Root DW	r	0.908	0.718	0.642	0.428	-0.005	0.165	-0.012	0.023	0.271	0.522	0.234	0.383	0.273	0.272	0.520
	P-value	0.005	0.085	0.145	0.381	0.993	0.750	0.981	0.965	0.594	0.267	0.647	0.438	0.591	0.592	0.270

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).

DW: Dry Weight; nd: non-detected; P-value; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

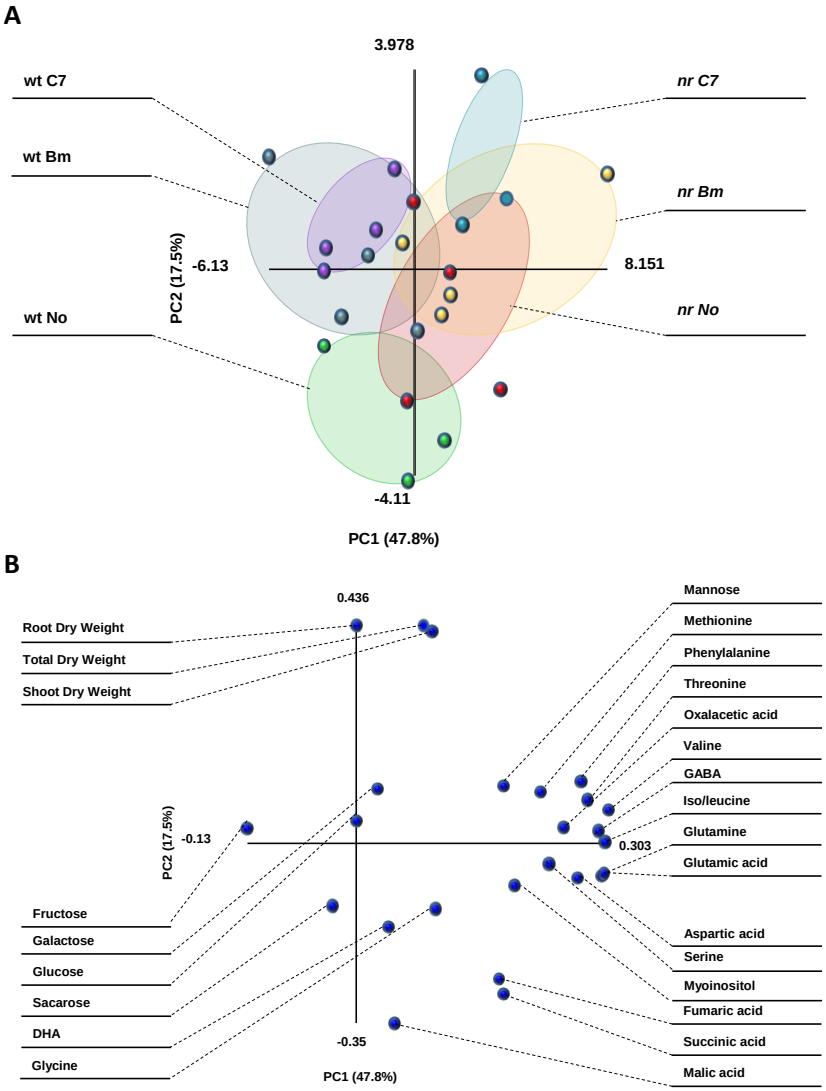

Figure 2.6 Principal component analysis of root metabolite levels and dry weights at 8 wpi. Analysis was performed based on metabolite contents and dry weight data obtained from tomato (*Solanum lycopersicum*) roots. **(A)** Score plot. Treatments: non-inoculated, *Bacillus megaoterium*-inoculated and *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 were enclosed in a different colored 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 metabolite or dry weight.

PCA also showed that PC1 variability was due to amino acids (glutamine, glutamic acid, leucine/isoleucine, valine and GABA). Unexpectedly, plant DWs were a source of variation for PC2. Moreover, malic acid also contributed to the variability of PC2 (Fig. 2.6 B).

Discussion

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

PGPB inoculation exclusively affected relative growth rate in ethylene-insensitive plants

In juvenile plants, an increase in root DW was only produced by Bm inoculation in *nr* plants resulting also in significant difference between wt and *nr* plants only under Bm inoculation (Fig. 2.2 E), suggesting that root growth promotion is related to ethylene insensitivity in 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. 2.2 B). Furthermore, phenotypic analysis only showed visible differences in plant height and number of flowers resulting in the same pattern as total DW (data not shown). These results indicate that ethylene perception is required for growth promotion in mature plants by Bm but not for C7 as discussed in the previous chapter.

Furthermore, difference between plant genotypes was only significant in non-inoculated plants pointing to less pronounced ethylene growth inhibitory effects (Pierik et al. 2006) on *nr* plants due to its mutation (Wilkinson et al. 1995). Under Bm inoculation, this genotype difference was unnoticed because growth promotion was only produced in wt plants reaching similar DW than *nr* plants, and suggesting that Bm could modulate ethylene receptor expression as previously reported by other PGPB strain (Vargas et al. 2012) and needed for the properly establishment of 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 different intensity of growth promotion. Moreover, IAA is able to induce plant growth (Zhao 2010) and higher auxin levels were exclusively noticed in non-inoculated *nr* mature plants compared to wt ones.

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 unnoticed 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. 2.3). These results points to Bm inoculation deleterious effects on mature *nr* plants, as previously reported in ABA-deficient tomato plants by Porcel *et al.* (2014). Moreover, it was also noticed 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 DWs and nutrients (Table 2.4) or metabolites (Tables 2.7 and 2.8). In juvenile plants, root sulfur concentration was positively correlated with total, shoot and root DWs. S levels were decreased by C7 inoculation in *nr* plants and a previous study reported modulation of S assimilation by PGPB inoculation (Aziz et al. 2016). Moreover, ethylene and S nutrition present links at metabolic and regulatory levels (Wawrzynska et al. 2015). In mature plants, C positively correlated with all DWs. 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 noticed between root sugar content and DWs. Shoot Fe concentration was negatively correlated with all DWs at 4 wpi. Ethylene is involved in a plant 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 microorganisms has been reported at the rhizosphere, showing that microorganisms 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 DWs positively and negatively, respectively. DHA is the oxidized form of

ascorbic acid, involved in oxidative damage prevention (Wang et al. 2013). Thus, this negative correlation could be due to reduced oxidative damage in Bm-inoculated *nr* roots which showed increased root DW. 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 signaling molecules and their availability is linked to plant growth (Rolland et al. 2006; Hanson and Smeekens 2009). In consequence, the observed correlations could be due to sugar availability.

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

PGPB inoculation effects on stomatal conductance and photosynthetic efficiency were only noticed in juvenile plants, while differences between plant genotypes showed different patterns at 4 and 8 wpi (Table 2.1). 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. 2014), lavender and salvia (Armada et al. 2014), although no effect was reported in tomato (Porcel et al. 2014). Moreover, photosynthesis is directly related with chlorophyll content (Richardson et al. 2002), but the chlorophyll content was unaffected by Bm inoculation, suggesting that the photosynthetic efficiency decrease was not linked to chlorophyll content. Ethylene is able to modify 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 the released ethylene from interaction) resulting in no change in stomatal conductance and deleterious effects as reduced photosynthetic efficiency and lower chlorophyll levels in Bm-inoculated *nr* plants compared to wt ones. Nevertheless, stomatal conductance was increased in wt and *nr* plants, while 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 in non- and C7-inoculated plants at 8 wpi, when higher ethylene values were observed in *nr* plants. Although physiological ethylene role depends on specific traits of plant species (related in principle with 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 ones, probably because ethylene is a key hormone involved in response to environmental stresses (Wang et al. 2013a; 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 altered under bacterial inoculation

PGPB effects on phytohormone levels were mainly dependent on ethylene sensitivity and only C7 inoculation directly affected root ABA (Tables 2.5 and 2.6). 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). ABA role has been historically described as growth inhibitor. However, high ABA levels were reported in young tissues and ABA-deficient mutants are severely affected in 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-inoulated *nr* plants compared to wt ones 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 neither by plant genotype in case of JA, JA-Ile and SA levels under C7 inoculation in mature plants.

However, Bm did not affect directly 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 accordance with the hypothesis pointing to plant ethylene sensitivity as a new player in Bm-tomato interaction. Indeed, although similar differences between plant genotypes were noticed in root ethylene in non- and Bm-inoculated plants, genotype difference was exclusively unnoticed in shoot ethylene under Bm inoculation. Furthermore, shoot SA levels exclusively showed higher values in *nr* plants under Bm inoculation, suggesting *nr* plants respond to Bm activating plant defenses since SA is a key factor for basal defenses establishment, effector-triggered inmmunity and both local and systemic acquired response (Vlot et al. 2009). In fact, SA, JA and JA-Ile showed also higher values in *nr* than in wt shoots only under Bm inoculation in mature plants. The best-known JA role is to regulate plant immune responses against pathogens (Browse 2009). Thus, these results also support miss-interaction resulting in Bm recognition by *nr* plants as a pathogen-like microorganism and physiologic deleterious effects. Indeed, ethylene modulates plant resistance and susceptibility to pathogens (van Loon et al. 2006) and even beneficial microorganisms can be recognized as potential invaders triggering 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 SlETR3 is not functional (Wilkinson et al. 1995). Cross-talk between ethylene and auxins is mainly produced at biosynthesis level and thus higher levels of IAA and ethylene were generally noticed in non-inoculated *nr* plants compared to wt ones. Furthermore, microbially-

derived IAA is a signaling molecule in microorganisms and interfere 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 noticed in Bm-inoculated *nr* plants compared to wt plants and IAA signaling down-regulation was reported as part of plant defense against bacteria (Spaepen and Vanderleyden 2011) in accordance with our results. Moreover, root and shoot IAA levels were increased in C7-inocualted *nr* plants compared to wt ones, predisposing plant to further growth since IAA plays crucial functions in several developmental processes being identified as a plant growth promotion hormone (Zhao 2010).

Root metabolites were directly altered by PGPB inoculation at juvenile stage showing high dependence of 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 2.7 and 2.8; Figs. 2.5 and 2.6).

Sugars and amino acids were pointed as 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, separation between Bm- and C7-inoculated juvenile plants was 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 (Fig. 2.2 and 2.6).

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 noticed 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 noticed in Bm-inoculated *nr* plants compared to wt ones at 8 wpi. Ethylene is involved in plant sensitivity to sugars and it has been reported that ethylene-insensitive plants show higher response to endogenous glucose resulting in increased photosynthesis suppression 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). Serine and derived molecules deficiencies 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 defense or response to stress (Winkel-Shirley 2001; Fraser and Chapple 2011). Thus, these results suggest that C7 inoculation could affect plant development though 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

noticed exclusively under C7 inoculation were in amino acid levels also pointing to a remodeling of amino acidic metabolism.

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

PGPB effects on nutritional status were more prominent in mature plants, showing a high dependence of ethylene sensitivity (Tables 2.2 and 2.3; Figs. 2.4). Indeed, ethylene signaling and plant nutrition interaction was reviewed by Iqbal *et al.* (2013), indicating that nutrient deficiencies are largely related with ethylene perception and biosynthesis. As observed in metabolites, separation of root nutrient profiles was clearly marked by plant genotype in mature plants, but the strain-specific effect was maintained in *nr* plants (Fig. 2.4).

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 & 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 of several nutrients were noticed 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 a biomass increase (Lawlor 2002). Moreover, Ca is a key player conferring structure and rigidity to cell wall and interacts with ethylene

signaling and plant responses to biotic attacks (Iqbal *et al.* 2013). Thus, root Ca increase mediated by Bm inoculation could enhance wt plants resistance.

Although both PGPB decreased Cu levels in *nr* roots at 8 wpi, C7 reduced it in 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 DWs. Cu is a structural component of ethylene receptors (Rodríguez 1999). Thus, lower levels of Cu could result in less 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 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, Si role in plants has been described as biotic and abiotic protector (Ma and Yamaji 2008). Thus, lower Si levels noticed in bacterial-inoculated plants could indicate that low Si could favor interaction with PGPB showing lower levels C7- 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 with 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 great impact in plant nutrition at 8 wpi. Root C concentration was increased in wt plants, while root S

levels were decreased in *nr* plants, respectively. S reduction by C7 inoculation only in *nr* plants could be due to modulation of sulfur assimilation machinery of plant (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 maintain plant on growth. However, P deficiency limits shoot and root DWs (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. 2008a) and root hair development (Zhang et al. 2003) in order to facilitate a quick recovery of stressed plants. A previous study reported that *B. 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 chapter, it has been reported that physiological parameters and root metabolites were modified by bacterial inoculation in juvenile plants rather than in mature ones, 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. 2.7).

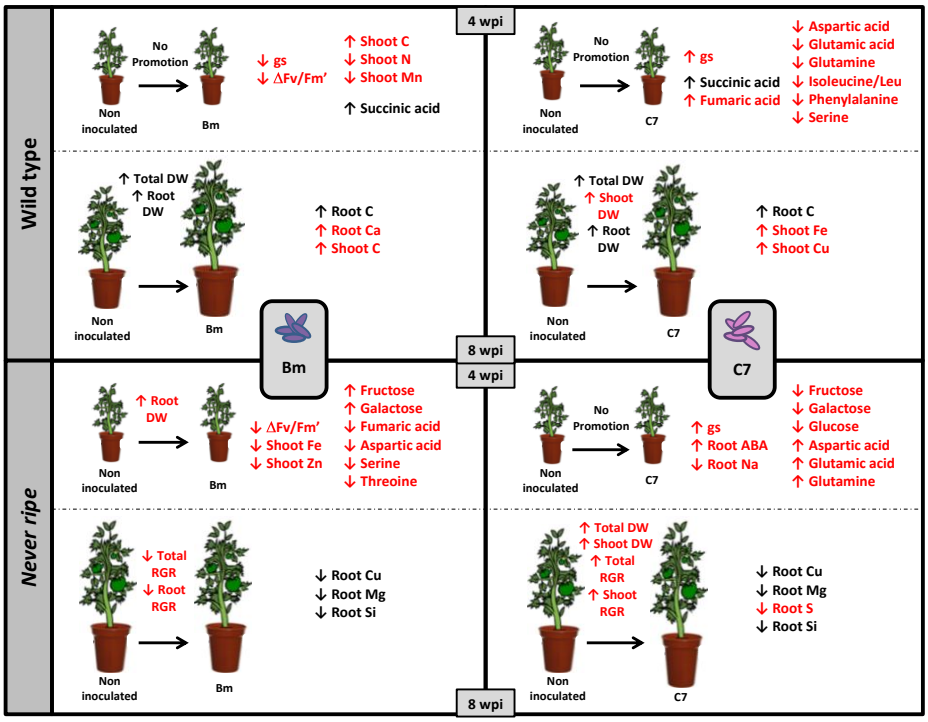

Figure 2.7 Summary of plant growth promoting bacteria (PGPB) inoculation effects on wild type and *never ripe* tomato (*Solanum lycopersicum*) plants at 4 and 8 weeks post-inoculation (wpi). Increases or decreases in measured parameters produced by PGPB inoculation are shown with ↑ and ↓ symbols, respectively. Changes induced by PGPB inoculation are shown in black and red letters for common and specific effects, respectively. Bm: *Bacillus megaterium*; C7: *Enterobacter* C7; DW: dry weight; RGR: relative growth rate; gs: stomatal conductance; DFv/Fm': 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: Sulfur.

In conclusion, the inability to perceive ethylene by the SlETR3 receptor impairs interaction between tomato plants and *Bacillus 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. 2.7). Despite of no growth promotion mediated by PGPB inoculation in juvenile plants, ethylene sensitivity could be proposed as essential for PGPB activity of *Bacillus megaterium* in tomato plants, whereas *Enterobacter* C7 PGPB mechanism seems to be SlETR3-independent.

Chapter 3: Ethylene perception determines the outcome of plant-bacteria interaction resulting in reshaping of phosphorus nutrition and antioxidant status

Chapter 3: Ethylene perception determines the outcome of plant-bacteria interaction resulting in reshaping of phosphorus nutrition and antioxidant status

Objective

The present chapter aimed to shed light on plant-bacteria interaction and PGPB mechanisms regarding to ethylene perception using a proteomic approach. Recently, -omics have clarified plant-bacteria interaction (van de Mortel et al. 2012; Su et al. 2016), but fundamental questions remain to be settled. Proteomics throws valuable information of bacterial effects on plant physiology (Feussner and Polle 2015). Nevertheless, further research is required since PGPB action mechanisms are often strain-specific (Ryu et al. 2005; Long et al. 2008).

The shot-gun proteomics (Wolters et al. 2001; Fournier et al. 2007) allows an integral analysis of proteins extracted including subcellular organelles and membranes (Takahashi et al. 2014). Plant cell membranes are determinant in transport processes (Chrispeels 1999) and molecular trafficking (Chen et al. 2011; Murphy et al. 2011) as well as in response to abiotic (Osakabe et al. 2013) and biotic stimuli (Inada and Ueda 2014). Plant cells can respond to microbe interaction by adapting vesicle trafficking (Ivanov et al. 2010; Dörmann et al. 2014; Inada and Ueda 2014), and the microsomal fraction is enriched in several membranes (Abas and Luschnig 2010). Furthermore, the roots are the bacterial niche where a direct plant-bacteria interaction occurs (Benizri et al. 2001). Thus, proteomic analysis of root microsomal

fraction can be very useful for looking into plant-bacteria interaction regarding signalling and transport processes.

The present chapter aimed to evaluate the bacterial effects on the root microsomal proteomic profiles in mature plants. Plant growth was determined at 8 weeks post-inoculation (wpi) and microsomal proteins analyzed by shot-gun proteomics. Additionally, antioxidant and phosphorus nutrition statuses were evaluated and a bioassay under low phosphorus conditions was performed based on proteomic results.

Experimental design

Main experiment The main experiment of third chapter consisted of a randomized complete block design with two tomato plant lines (wt and *nr*) and three inoculation treatments: (1) non-inoculated control plants, (2) *Bacillus megaterium*-inoculated plants and (3) *Enterobacter* C7-inoculated plants. Each treatment consisted in eleven replicates (n=11). Plants were harvested at 8 weeks post inoculation (wpi).

Differential phosphorus conditions bioassay The experiment with low phosphorus were also carried out as a randomized complete block design with the two plant lines and three inoculation treatments abovementioned, and two phosphorus treatments: control conditions (Control P; NaH_2PO_4 1 mM) and low phosphorus conditions (Low P; NaH_2PO_4 0.2 mM). Each treatment consisted of seven replicates (n=7) and plants were harvested at 4 wpi.

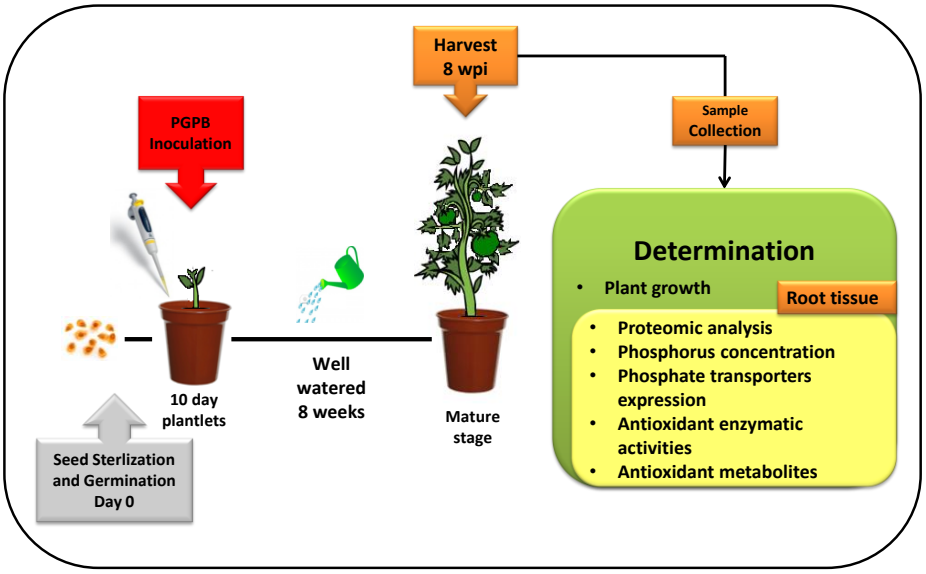

Figure 3.1 Schematic representation of experimental design of main experiment in chapter 3. Weeks post-inoculation (wpi).

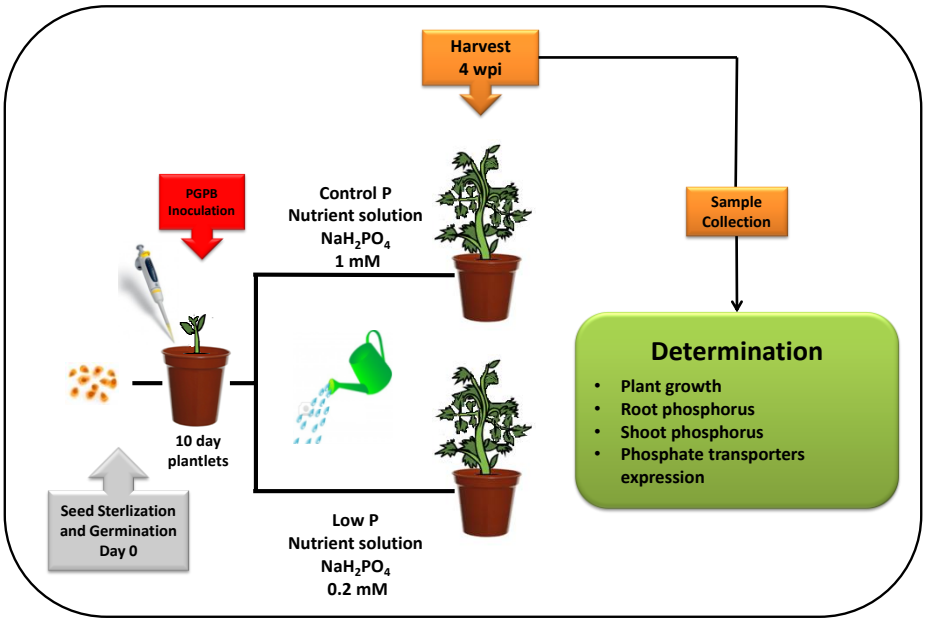

Figure 3.2 Schematic representation of experimental design of differential phosphorus conditions bioassay in chapter 3. Weeks post-inoculation (wpi).

Results

Biomass production of PGPB-inoculated wt and *nr* plants

In order to evaluate plant-growth promoting activity of both tested bacterial strains regarding to ethylene insensitivity, the biomass production was evaluated by fresh weight (FW) determination in the first experiment showing no significant interaction between main factors (GxI) (Fig. 3.3). Total FW showed an increase due to PGPB inoculation in wt plants (13.1 % and 22.6 % for Bm and C7, respectively). Nevertheless, in *nr* plants only C7 inoculation promoted plant growth (19.7 % compared to non-inoculated plants), showing Bm inoculation no significant growth promotion (Fig. 3.3 A).

Moreover, shoot FW was increased by both bacterial inoculations in both plant genotypes. Bm inoculation increased shoot FW by 17.7 % and 14.1 % in wt and *nr* plants, respectively. Meanwhile, shoot FW increases of 26.3 % and 25.9 % were noticed due to C7 inoculation in wt and *nr* plants, respectively (Fig. 3.3 B). In contrast, no significant differences were observed in root FW due to any bacterial inoculation (Fig. 3.3 C).

In addition, significant differences between plant genotypes were noticed in shoot FW independently of inoculation treatment showing higher values *nr* than wt plants. However, differences between plant genotypes were observed in non- and Bm-inoculated plants but showing *nr* plants lower root FW values than wt ones (Fig. 3.3 B, C).

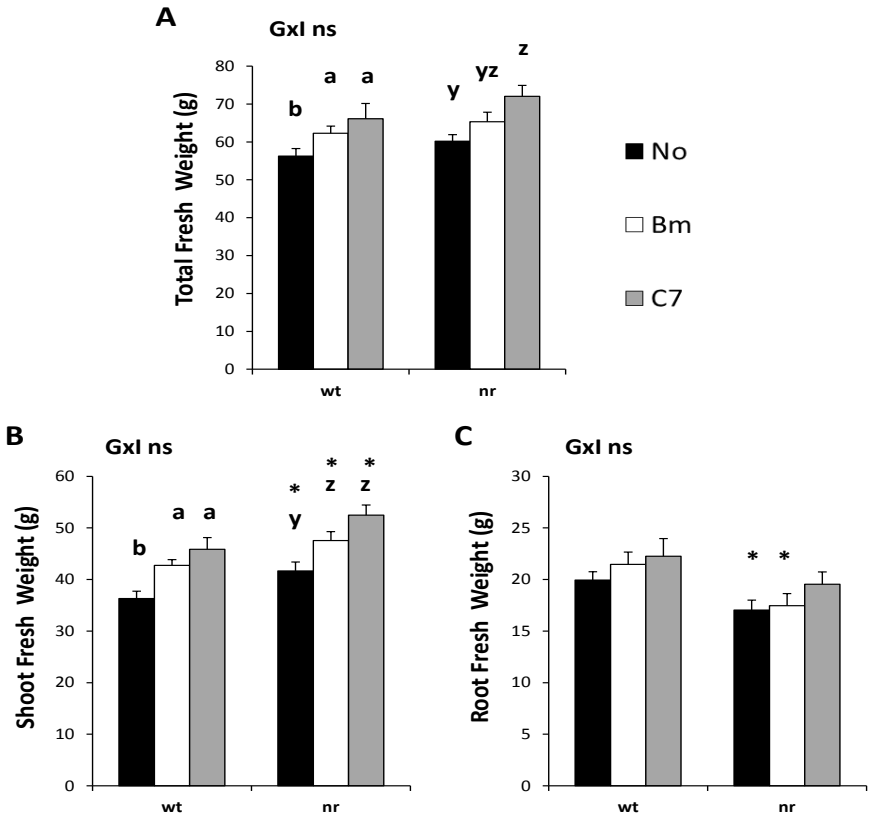

Figure 3.3 Effects of bacterial inoculation on plant fresh weights. **(A)** Total, **(B)** shoot, and **(C)** root fresh weights of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato plants at 8 weeks post-inoculation (wpi). Treatments are designed as non-inoculated controls (No, black bars), *Bacillus megaterium*-inoculated plants (Bm, white bars), and *Enterobacter* C7-inoculated plants (C7, grey bars). Data are means ± SE (n = 11). Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. As no significant interaction between factors was noticed, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

Proteomic analysis of wt and *nr* plants inoculated with PGPB strains

The proteomic analysis of microsomal root samples resulted in 1214 identified proteins. Among them, 1164 proteins were also quantified at least in all samples coming from one treatment. Different treatments were compared in pairs in order to analyze the bacterial inoculation effects on wt and *nr* plants.

Protein data obtained for each treatment were used to build a data matrix to perform a principal component analysis (PCA) in order to compare inoculation treatments in wt and *nr* plants, as well as differences between both plant genotypes (Fig. 3.4). Non-supervised PCA almost separated treatments by plant genotype. Axes PC1 and PC2 represented in the factorial plan, explained 41.9 % of data variability. In wt plants, non-inoculated plant profiles mostly overlapped with bacterial inoculated ones. Any bacterial inoculated profile approximately shared the half of their area with controls and mostly overlapped with each other. In contrast, although both bacterial inoculations partly overlapped with non-inoculated *nr* plants, the C7 inoculation produced a higher separation from non-inoculated plants than the Bm inoculation. Bm-inoculated profiles almost completely overlapped with non-inoculated ones (Fig. 3.4 A).

In addition, a supervised PCA (α=0.1) was also carried out in order to improve accuracy of analysis (Barshan et al. 2011). A clear difference between genotypes was noticed in this analysis (47.0 % variability along axes PC1 and PC2). In wt plants, both bacterial inoculations partially overlapped with non-inoculated plants without overlapping each other. Bm inoculation produced an almost completely

overlapped protein profile with non-inoculated plants, meanwhile C7-inoculated plant profile only shared a little area with non-inoculated plants. Unexpectedly in case of *nr* plants, Bm-inoculated protein profile was completely separated from non- and C7-inoculated profiles, meanwhile C7 inoculated profile only overlapped a little area with non-inoculated one (Fig. 3.4 B).

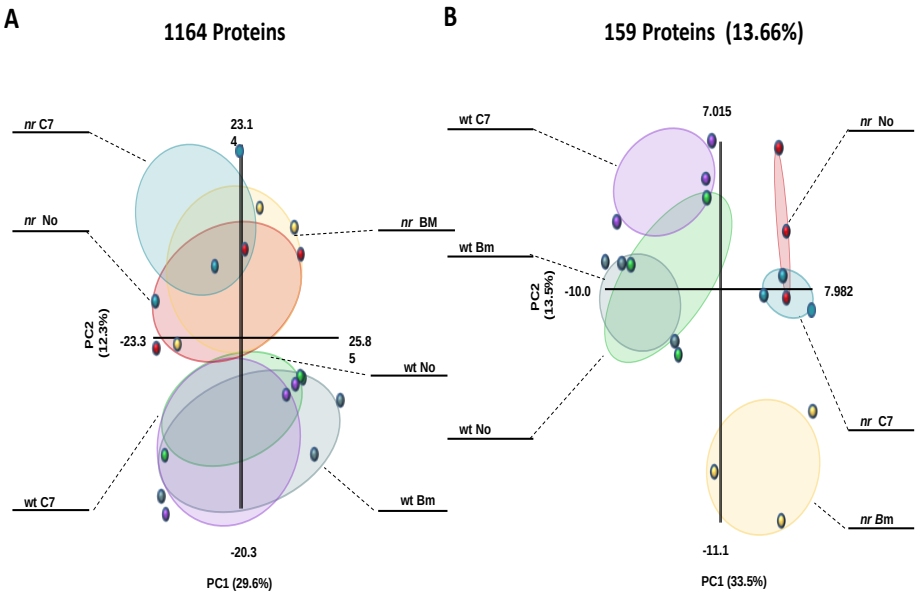

Figure 3.4 Effects of bacterial inoculation on microsomal proteomic profiles. Principal component analyses (PCA) were performed based on protein quantification data obtained from tomato roots. Treatments: non-inoculated, *Bacillus megaterium*-inoculated and *Enterobacter* C7-inoculated wild-type plants (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 a three-plant pool sample and points of the same treatment were enclosed in a different colored 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. **(A)** Non-supervised PCA; **(B)** Supervised PCA (α = 0.1%).

Furthermore, the PGPB inoculation effects on wt and *nr* plants compared with non-inoculated plants as well as between each others directly were also evaluated using a Venn diagram (Fig. 3.5).

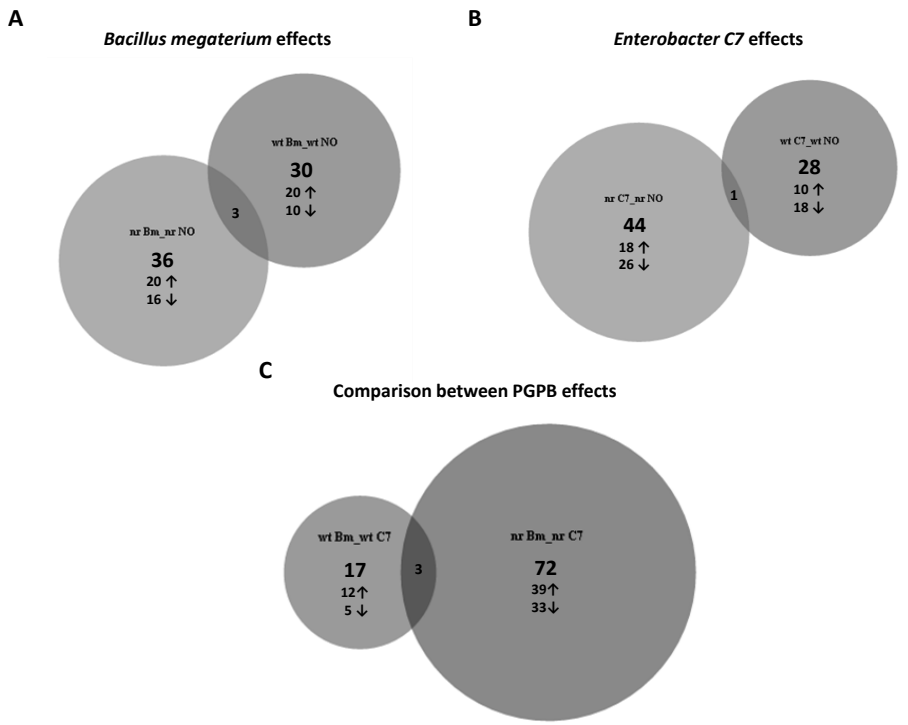

Figure 3.5 Venn diagrams for proteomic profiles comparisons. (A) *Bacillus megaterium* inoculation effects on tomato plants; **(B)** *Enterobacter* C7 inoculation effects on tomato plants; **(C)** Differences between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato plants. Each ellipse represents a comparison between two treatments. Overlapping area proportionally represents number of proteins that changes in two comparisons. Numbers indicate the number of identified proteins with significant level modification. Induced and repressed proteins by bacteria inoculation are represented by ↑ and ↓ symbols, respectively. 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).

Bacillus megaterium inoculation effects on proteomic profiles

Bacillus megaterium inoculation significantly affects several protein levels independently of plant genotype. In wt plants, Bm inoculation produced level modification of 33 proteins (Table 3.1). Among them, 22 proteins were induced and 11 proteins were repressed. Moreover, 5 proteins showed fold change over 2 between treatments. Plant MetGenMap identified several changed pathways ($P < 0.05$) due to Bm inoculation of wt plants. Dihydroxiacetone cycle (formaldehyde assimilation), Calvin-Benson-Bassham cycle, oxygenic photosynthesis, glycolysis and superpathway of glycolysis, pyruvate deshydrogenase, Krebs cycle and glyoxylate bypass were significantly affected due to modification of triosephosphate isomerase levels. Indeed, triosephosphate isomerase was undetected under Bm inoculation.

Table 3.1 Differentially expressed proteins due to *Bacillus megaterium* inoculation in tomato Pearson cv. wild type.

Protein	Solyc ID	Fold-change
Proteins induced by *Bacillus megaterium* inoculation		
60S ribosomal protein L4-B ⊥	Solyc10g084350	2.86
Inorganic phosphate transporter * ⊥	Solyc09g066410	2.82
50S ribosomal protein L11	Solyc02g082500	2.17
Solute carrier family 2, facilitated glucose transporter member 12 ⊥	Solyc04g082700	1.90
Receptor like kinase, RLK	Solyc02g071810	1.88
Harpin binding protein 1 ⊥	Solyc09g090330	1.82
40S ribosomal protein S8	Solyc06g007570	1.76
Chaperone DnaK	Solyc01g103450	1.69
Ribosomal protein L3-like *	Solyc01g103510	1.68
40S ribosomal protein S29	Solyc06g073430	1.68
60S ribosomal protein L38	Solyc03g058990	1.67
Cathepsin B-like cysteine proteinase	Solyc04g078540	1.62
Ras-related protein Rab-2-A	Solyc02g093530	1.57
Ribosomal protein L26	Solyc02g092430	1.54
Magnesium transporter MRS2-1	Solyc11g066660	1.53
40S ribosomal protein S24	Solyc06g084230	1.49
AD-dependent malic enzyme 2	Solyc08g013860	1.48
60 ribosomal protein L14	Solyc06g008260	1.43
60 ribosomal protein L14	Solyc06g083820	1.42
60S ribosomal protein L14	Solyc09g066430	1.34
Ribosomal protein	Solyc08g061850	1.32
60S ribosomal protein L23a	Solyc09g005720	1.25
Proteins repressed by *Bacillus megaterium* inoculation		

60S ribosomal protein L38	Solyc03g058990	1.67
Cathepsin B-like cysteine proteinase	Solyc04g078540	1.62
Ras-related protein Rab-2-A	Solyc02g093530	1.57
Ribosomal protein L26	Solyc02g092430	1.54
Magnesium transporter MRS2-1	Solyc11g066660	1.53
40S ribosomal protein S24	Solyc06g084230	1.49
AD-dependent malic enzyme 2	Solyc08g013860	1.48
60 ribosomal protein L14	Solyc06g008260	1.43
60 ribosomal protein L14	Solyc06g083820	1.42
60S ribosomal protein L14	Solyc09g066430	1.34
Ribosomal protein	Solyc08g061850	1.32
60S ribosomal protein L23a	Solyc09g005720	1.25
Proteins repressed by *Bacillus megaterium* inoculation		
Triosephosphate isomerase *	Solyc04g011510	Non-detected with Bm
Argininosuccinate synthase	Solyc05g012270	2.51
Reticulon family protein	Solyc08g081730	1.96
Vesicle-associated membrane protein-associated protein A	Solyc01g090910	1.83
NADH-quinone oxidoreductase subunit N	Solyc00g013130	1.79
Dynamin 2	Solyc05g050600	1.70
S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase	Solyc01g005230	1.60
tRNA pseudouridine synthase B	Solyc02g081810	1.57
LysM domain containing protein ⊥	Solyc11g012870	1.55
Aquaporin ⊥	Solyc06g075650	1.41
Vacuolar ATPase subunit H protein	Solyc07g005940	1.24

*** Common proteins that are diferentially expressed due to *Bacillus megaterium* (Bm) inoculation in wild type and *never ripe* plants**

⊥ Common proteins that are diferentially expressed due to both bacterial inoculations in wild type plants

In *nr* plants, 39 proteins were affected due to Bm inoculation (Table 3.2). 21 proteins were induced and 18 were reduced in Bm-inoculated plants compared to non-inoculated plants. 19 proteins showed fold change over 2 between treatments. As observed in Bm-inoculated wt plants, triosephosphate isomerase was undetected under Bm inoculation. Although Bm inoculation modified protein levels in higher degree in *nr* than in wt plants, pathway analysis resulted in only one significant altered pathway ($P < 0.05$) due to Bm inoculation of *nr* plants. The glutathione-mediated detoxification was affected due to increased levels of glutathione S-transferase observed in Bm-inoculated *nr* plants. Unexpectedly, although triosephosphate isomerase showed the same differential expression as in wt plants, all abovementioned altered pathways were unaffected in *nr* plants.

Table 3.2 Differentially expressed proteins due to *Bacillus megaterium* inoculation in tomato Pearson cv. *never ripe*.

Protein	Solyc ID	Fold-change
Proteins induced by *Bacillus megaterium* inoculation		
Lysosomal Pro-X carboxypeptidase	Solyc11g066590	Non-detected in controls
Glutathione S-transferase ⊥	Solyc01g081270	24.16
Band 7 stomatin family protein ⊥	Solyc06g073030	12.63
Glutathione S-transferase	Solyc08g080900	8.82
50S ribosomal protein L4 ⊥	Solyc12g088730	3.60
Ribosomal protein L3-like *	Solyc01g103510	2.66
Heat shock protein 70-3	Solyc10g086410	2.51
Non-specific lipid-transfer protein	Solyc01g105010	2.49
Neutral ceramidase	Solyc03g006140	2.43
Subtilisin-like protease	Solyc06g062950	2.04
V-type proton ATPase subunit E ⊥	Solyc08g081910	1.85
Guanine nucleotide-binding protein beta subunit-like protein	Solyc06g069010	1.80
Protein disulfide isomerase L-2	Solyc04g049450	1.79
60S ribosomal protein L34	Solyc06g069860	1.57
60S ribosomal protein L34	Solyc02g087930	1.56
Syntaxin-71 ⊥	Solyc10g084210	1.53
Phosphoglycerate kinase	Solyc07g066600	1.50
Os06g0220000 protein (Fragment) ⊥	Solyc01g105410	1.42
14-3-3 protein beta/alpha-1	Solyc02g063070	1.40
Flavoprotein wrbA	Solyc02g079750	1.39
Ribosomal protein L18	Solyc01g099900	1.31
Proteins repressed by *Bacillus megaterium* inoculation		
Triosephosphate isomerase * ⊥	Solyc04g011510	Non-detected with Bm
Reductase ⊥	Solyc08g081530	65.32
F-box family protein	Solyc04g057950	4.77
Metacaspase 7	Solyc09g098150	3.31
Erlin-2	Solyc05g012340	2.58
Mitochondrial porin (Voltage-dependent anion channel) outer membrane protein	Solyc02g092440	2.38
Receptor like kinase, RLK	Solyc12g014350	2.15
Ras-related protein Rab-8A	Solyc11g073050	2.08
Cytochrome c1	Solyc06g067920	2.07
Vesicle-associated membrane protein 7C	Solyc06g065950	1.93
Inorganic phosphate transporter * ⊥	Solyc09g066410	1.83
NADH dehydrogenase	Solyc03g007660	1.72
Acid phosphatase	Solyc04g008260	1.62
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Solyc04g082670	1.62
2-oxoglutarate/malate translocator	Solyc01g105540	1.52
Homology to unknown gene (Fragment)	Solyc11g065490	1.49
HVA22-like protein e	Solyc11g010930	1.49
REF-like stress related protein 1	Solyc05g015390	1.34

*** Common proteins that are diferentially expressed due to *Bacillus megaterium* (Bm) inoculation in wild type and *never ripe* plants**

⊥ Common proteins that are diferentially expressed due to both bacterial inoculations in *never ripe* plants

NADH dehydrogenase	Solyc03g007660	1.72
Acid phosphatase	Solyc04g008260	1.62
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Solyc04g082670	1.62
2-oxoglutarate/malate translocator	Solyc01g105540	1.52
Homology to unknown gene (Fragment)	Solyc11g065490	1.49
HVA22-like protein e	Solyc11g010930	1.49
REF-like stress related protein 1	Solyc05g015390	1.34

*** Common proteins that are diferentially expressed due to *Bacillus megaterium* (Bm) inoculation in wild type and *never ripe* plants**

⊥ Common proteins that are diferentially expressed due to both bacterial inoculations in *never ripe* plants

Furthermore, the effects on wt and *nr* plants produced by Bm inoculation was also evaluated using a Venn diagram. Most of the proteins were specifically altered in wt or *nr* roots by Bm inoculation regarding controls. Only three proteins were significantly affected in both plant genotypes by Bm inoculation, meanwhile 30 and 36 proteins showed specific level modifications in wt and *nr* plants, respectively (Fig. 3.5 A). Among these three proteins affected in both plant genotypes, an inorganic phosphate transporter was induced in wt plants due to Bm inoculation, meanwhile a reduction in its levels was noticed in Bm-inoculated *nr* plants. A triosephosphate isomerase was detected and quantified in non-inoculated wt and *nr* plants, but was undetected when plants were inoculated with Bm. Moreover, a ribosomal protein L3-like was induced in both plant genotypes due to Bm inoculation.

Enterobacter C7 inoculation effects on proteomic profiles

Enterobacter C7 inoculation significantly affects several protein levels independently of plant genotype and greater effect was observed on *nr* plants (Tables 3.3 and 3.4). In wt plants, levels of 29 proteins were significantly affected by C7 inoculation (11 induced and 18 repressed proteins). Among them, a fold change over 2 was noticed for 9 proteins (Table 3.3). However, pathway analysis showed no significant altered pathways.

Table 3.3 Differentially expressed proteins due to *Enterobacter* C7 inoculation in tomato Pearson cv. wild type.

Protein	Solyc ID	Fold-change
Proteins induced by *Enterobacter* C7 inoculation		
Aquaporin-like protein	Solyc08g066840	3.74
Non-specific lipid-transfer protein	Solyc01g005990	3.00
Inorganic phosphate transporter * ⊥	Solyc09g066410	2.53
60S ribosomal protein L4-B ⊥	Solyc10g084350	2.24
Ascorbate peroxidase	Solyc02g083630	2.09
Peroxidase 4	Solyc04g071890	1.77
Solute carrier family 2, facilitated glucose transporter member 12 ⊥	Solyc04g082700	1.74
Unknown Protein	Solyc01g020590	1.67
Dihydroxy-acid dehydratase	Solyc05g053540	1.61
Harpin binding protein 1 ⊥	Solyc09g090330	1.32
Peroxidase	Solyc03g006700	1.29
Proteins repressed by *Enterobacter* C7 inoculation		
Blue copper protein (Fragment)	Solyc07g008110	8.28
Glucan synthase like 7	Solyc01g006350	3.57
Related to ATP dependent RNA helicase	Solyc06g082100	2.42
V-type proton ATPase 16 kDa proteolipid subunit c2	Solyc02g084360	2.20
6 7-dimethyl-8-ribityllumazine synthase	Solyc08g015660	1.94
Aquaporin ⊥	Solyc06g075650	1.75
Cathepsin B-like cysteine proteinase	Solyc12g088670	1.73
LysM domain containing protein ⊥	Solyc11g012870	1.66
14-3-3 protein beta/alpha-B	Solyc04g076060	1.66
Major latex-like protein	Solyc00g323130	1.61
Protein kinase-like protein	Solyc05g009540	1.60
Remorin 2	Solyc01g094370	1.60
Aminotransferase-like protein	Solyc12g006450	1.47
Ras-related protein Rab-1A	Solyc01g103370	1.39
30S ribosomal protein S9	Solyc04g026100	1.30
26S proteasome non-ATPase regulatory subunit 4	Solyc02g083710	1.29
Flavoprotein wrbA	Solyc02g079750	1.29
Xylanase inhibitor (Fragment)	Solyc01g080010	1.09

*** Common proteins that are diferentially expressed due to *Enterobacter* C7 (C7) inoculation in wild type and *never ripe* plants**

⊥ Common proteins that are diferentially expressed due to both bacterial inoculations in wild type plants

In *nr* plants, C7 inoculation produced modification in levels of 45 proteins. Eighteen proteins were increased and 27 were reduced due to C7 inoculation with 20 proteins over a 2-fold change (Table 3.4). In fact, the dolichyl-diphosphooligosacharide-protein glycosyltransferase

subunit 2 was undetected in non-inoculated plants and triosephosphate isomerase and LRR receptor-like serine/threonine protein kinase FEI1 were undetected under C7 inoculation. Pathway analysis identified three significantly affected pathways ($P < 0.05$) due to C7 inoculation of *nr* plants. Dihydroxiacetone cycle and Calvin-Benson-Bassham cycle were altered due to triosephosphate isomerase alteration. Furthermore, ascorbate-glutathione cycle was altered by modification of reductase (carbonate anhydrase) levels observed in C7-inoculated *nr* plants.

Table 3.4 Differentially expressed proteins due to *Enterobacter* C7 inoculation in tomato Pearson cv. *never ripe.*

Protein	Solyc ID	Fold-change
Proteins induced by *Enterobacter* C7 inoculation		
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	Solyc05g046330	Non-detected in controls
Glutathione S-transferase ⊥	Solyc01g081270	33.57
Band 7 stomatin family protein ⊥	Solyc06g073030	14.90
Cytochrome P450	Solyc07g014670	5.30
Histone H2A	Solyc06g084090	3.90
60S ribosomal protein L18a	Solyc08g016180	2.94
50S ribosomal protein L4 ⊥	Solyc12g088730	2.70
Cytochrome b5	Solyc06g007930	2.51
Cytochrome P450	Solyc03g122350	2.45
V-type proton ATPase subunit E ⊥	Solyc08g081910	2.43
Aspartate aminotransferase	Solyc08g041870	2.36
Pentatricopeptide repeat-containing protein	Solyc01g096200	1.90
Syntaxin-71 ⊥	Solyc10g084210	1.87
Importin subunit beta	Solyc09g005010	1.68
B-cell receptor-associated protein 31-like containing protein	Solyc12g005910	1.64
SPFH domain / Band 7 family protein	Solyc02g067470	1.62
2 3-bisphosphoglycerate-independent phosphoglycerate mutase	Solyc07g044840	1.47
Os06g0220000 protein (Fragment) ⊥	Solyc01g105410	1.21
Proteins repressed by *Enterobacter* C7 inoculation		
Triosephosphate isomerase ⊥	Solyc04g011510	Non-detected with C7
LRR receptor-like serine/threonine-protein kinase FEI 1	Solyc03g116760	Non-detected with C7
Reductase ⊥	Solyc08g081530	115.83
Histone H3	Solyc04g074580	14.98
Coatomer protein epsilon subunit family protein	Solyc02g069590	7.27
Histone H2A	Solyc01g099410	5.55
40S ribosomal protein SA	Solyc03g019780	4.75
Glutathione S-transferase	Solyc06g009020	2.16
60S acidic ribosomal protein P3	Solyc07g009330	2.11
40S ribosomal protein S30-like	Solyc08g076340	1.97
Inorganic phosphate transporter * ⊥	Solyc09g066410	1.92
UTP-glucose 1 phosphate uridylyltransferase	Solyc11g011960	1.88
N-carbamoylputrescine amidase	Solyc11g068540	1.88

Triosephosphate isomerase ⊥	Solyc04g011510	Non-detected with C7
LRR receptor-like serine/threonine-protein kinase FEI 1	Solyc03g116760	Non-detected with C7
Reductase ⊥	Solyc08g081530	115.83
Histone H3	Solyc04g074580	14.98
Coatomer protein epsilon subunit family protein	Solyc02g069590	7.27
Histone H2A	Solyc01g099410	5.55
40S ribosomal protein SA	Solyc03g019780	4.75
Glutathione S-transferase	Solyc06g009020	2.16
60S acidic ribosomal protein P3	Solyc07g009330	2.11
40S ribosomal protein S30-like	Solyc08g076340	1.97
Inorganic phosphate transporter * ⊥	Solyc09g066410	1.92
UTP-glucose 1 phosphate uridylyltransferase	Solyc11g011960	1.88
N-carbamoylputrescine amidase	Solyc11g068540	1.88
Fasciclin-like arabinogalactan protein 13	Solyc01g091530	1.87
40S ribosomal protein S6	Solyc12g096300	1.73
40S ribosomal protein S7-like protein	Solyc06g069090	1.68
Heat shock protein 4	Solyc12g043110	1.61
26S proteasome regulatory subunit	Solyc05g053650	1.61
60S ribosomal protein L24	Solyc09g008800	1.59
Threonine synthase	Solyc03g121910	1.51
30S ribosomal protein S19	Solyc06g053820	1.49
40S ribosomal protein S24	Solyc06g084230	1.46
Proteasome subunit alpha type	Solyc10g008010	1.40
2-oxoglutarate dehydrogenase E1 component	Solyc04g011350	1.37
Phosphatidylinositol transfer protein SFH5	Solyc11g027880	1.33
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	Solyc07g006790	1.30
14-3-3 protein beta/alpha-B	Solyc04g074510	1.19

*** Common proteins that are diferentially expressed due to *Enterobacter* C7 (C7) inoculation in wild type and *never ripe* plants**

⊥ Common proteins that are diferentially expressed due to both bacterial inoculations in *never ripe* plants

In addition, Venn diagram showed that C7 inoculation effects on wt and *nr* plants were mostly genotype specific (Fig 3.5 B). While 28 and 44 proteins showed specific level modifications in wt and *nr* plants, respectively, only one protein was significantly affected in both plant genotypes due to C7 inoculation. This protein was identified as an inorganic phosphate transporter. As observed in Bm-inoculated plants, it was increased and reduced in wt and *nr* plants due to C7 inoculation, respectively.

Differences between *Bacillus megaterium* and *Enterobacter* C7 inoculation effects on proteomic profiles

Bacterial inoculation mainly produced genotype-specific effects. In addition, bacterial inoculation effects were also mostly strain-specific in both genotypes, but a common effect between bacteria was observed in both genotypes. Both bacterial inoculations affected levels of 6 and 9 proteins in wt and *nr* plants, respectively (Tables 3.1, 3.2, 3.3 and 3.4). In wt plants, all common proteins were induced or repressed independently of bacterial strain and quantitatively equivalent. In *nr* plants, although common proteins were also similarly affected by both bacteria, glutathione S-transferase, band 7 stomatin family protein and reductase were altered in a higher degree under C7 inoculation (Tables 3.2 and 3.4).

Furthermore, several differences in protein levels were noticed between Bm- and C7-inoculated plants, showing also more differences in *nr* than in wt plants (Tables 3.5 and 3.6; Fig. 3.5 C). In wt plants, the levels of 20 proteins were significantly modified between Bm and C7 inoculation treatments (Bm induced 14 and repressed 6 proteins regarding to C7-inoculated plants). Among them, only 3 protein levels were differentially affected over a 2-fold change threshold (Table 3.5). However, pathway analysis showed no significant altered pathways.

Table 3.5 Differentially expressed proteins between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato Pearson cv. wild type plants.

Protein	Solyc ID	Fold-change
Proteins induced by Bm inoculation regarding C7-inoculated plants		
Nascent polypeptide-associated complex alpha subunit-like protein	Solyc10g081030	2.83
6 7-dimethyl-8-ribityllumazine synthase	Solyc08g015660	2.35
Protein kinase-like protein *	Solyc05g009540	2.14
Calmodulin	Solyc06g068960	1.80
Eukaryotic translation initiation factor 3 subunit 2	Solyc11g017070	1.71
26S proteasome regulatory subunit	Solyc01g008370	1.67
40S ribosomal protein S12	Solyc12g042650	1.65
60S ribosomal protein L5-1	Solyc06g082870	1.62
D-xylose transporter	Solyc02g086160	1.55
Peptidyl-prolyl cis-trans isomerase	Solyc01g111170	1.52
Adenylosuccinate synthetase, chloroplastic	Solyc03g085520	1.35

Protein	Solyc ID	Fold-change
Proteins induced by Bm inoculation regarding C7-inoculated plants		
Nascent polypeptide-associated complex alpha subunit-like protein	Solyc10g081030	2.83
6 7-dimethyl-8-ribityllumazine synthase	Solyc08g015660	2.35
Protein kinase-like protein *	Solyc05g009540	2.14
Calmodulin	Solyc06g068960	1.80
Eukaryotic translation initiation factor 3 subunit 2	Solyc11g017070	1.71
26S proteasome regulatory subunit	Solyc01g008370	1.67
40S ribosomal protein S12	Solyc12g042650	1.65
60S ribosomal protein L5-1	Solyc06g082870	1.62
D-xylose transporter	Solyc02g086160	1.55
Peptidyl-prolyl cis-trans isomerase	Solyc01g111170	1.52
Adenylosuccinate synthetase, chloroplastic	Solyc02g085520	1.35
30S ribosomal protein S9	Solyc04g026100	1.35
Ectonucleoside triphosphate diphosphohydrolase 6 *	Solyc12g098540	1.31
26S proteasome non-ATPase regulatory subunit 4	Solyc02g083710	1.15
Proteins induced by C7 inoculation regarding Bm-inoculated plants		
tRNA pseudouridine synthase B	Solyc02g081810	1.74
Chitinase	Solyc10g055800	1.72
Major allergen Mal d 1 *	Solyc09g090980	1.43
Atozi1 (Fragment)	Solyc10g080190	1.39
NADH-quinone oxidoreductase subunit C	Solyc00g019950	1.29
Mitochondrial processing peptidase alpha subunit	Solyc12g008630	1.25

*** Common proteins that are diferentially expressed between *Bacillus megaterium*- and *Enterobacter* C7-inoculated wild type and *never ripe* plants**

In *nr* plants, the levels of 75 proteins were significantly modified between inoculation of Bm and C7. 42 proteins were increased and 33 were reduced due to Bm inoculation compared to C7 inoculation with 13 proteins over a 2-fold change (Table 3.6). Despite of great differences noticed between Bm- and C7-inoculated *nr* plants, no significantly affected pathways were identified.

Table 3.6 Differentially expressed proteins between *Bacillus megaterium*- and *Enterobacter* C7-inoculated tomato Pearson cv. *never ripe* plants.

Protein	Solyc ID	Fold-change
Proteins induced by Bm inoculation regarding C7-inoculated plants		
Aspartyl aminopeptidase	Solyc11g007090	23.83
40S ribosomal protein S8	Solyc06g083180	2.44
Glutathione S-transferase	Solyc06g009020	2.34
30S ribosomal protein S19	Solyc06g053820	2.29
Proteasome subunit alpha type	Solyc07g055080	1.78
Major allergen Mal d 1 *	Solyc09g090980	1.76
Unknown Protein	Solyc07g017410	1.75
Ribosomal protein L30	Solyc11g071490	1.71
4-alpha-glucanotransferase	Solyc02g020980	1.68
Ribosomal protein L7a	Solyc06g064460	1.68
Ribosomal protein L30	Solyc01g009100	1.67
Ectonucleoside triphosphate diphosphohydrolase 6 *	Solyc12g098540	1.66
N-carbamoylputrescine amidase	Solyc11g068540	1.66
60S acidic ribosomal protein P3	Solyc07g009330	1.65
Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	Solyc10g047630	1.63
Proteasome subunit beta type	Solyc11g069150	1.59

Glutathione S-transferase	Solyc06g009020	2.34
30S ribosomal protein S19	Solyc06g053820	2.29
Proteasome subunit alpha type	Solyc07g055080	1.78
Major allergen Mal d 1 *	Solyc09g090980	1.76
Unknown Protein	Solyc07g017410	1.75
Ribosomal protein L30	Solyc11g071490	1.71
4-alpha-glucanotransferase	Solyc02g020980	1.68
Ribosomal protein L7a	Solyc06g064460	1.68
Ribosomal protein L30	Solyc01g009100	1.67
Ectonucleoside triphosphate diphosphohydrolase 6 *	Solyc12g098540	1.66
N-carbamoylputrescine amidase	Solyc11g068540	1.66
60S acidic ribosomal protein P3	Solyc07g009330	1.65
Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	Solyc10g047630	1.63
Proteasome subunit beta type	Solyc11g069150	1.59
Translationally-controlled tumor protein homolog	Solyc01g099770	1.56
Histidyl-tRNA synthetase 1	Solyc03g119280	1.55
60S ribosomal protein L24	Solyc09g008800	1.51
60S ribosomal protein L34	Solyc06g069860	1.51
Phospholipase D	Solyc06g068090	1.51
Unknown Protein	Solyc01g020590	1.47
Regulator of ribonuclease activity A	Solyc04g008280	1.46
Ribosomal protein L18	Solyc01g099900	1.45
Fasciclin-like arabinogalactan protein 13	Solyc01g091530	1.45
50S ribosomal protein L25	Solyc02g090420	1.45
60S ribosomal protein L37a	Solyc08g007140	1.44
Proteasome subunit alpha type	Solyc04g080590	1.43
UTP-glucose 1 phosphate uridylyltransferase	Solyc11g011960	1.43
Proteasome subunit beta type	Solyc07g016200	1.40
Heat shock protein 4	Solyc12g043110	1.39
Protein kinase-like protein *	Solyc05g009540	1.38
Ribosomal protein L12	Solyc11g065670	1.36
60S ribosomal protein L23a	Solyc09g005720	1.35
Proteasome subunit beta type	Solyc09g082320	1.33
Ribosomal protein L3	Solyc01g104590	1.30
Proteasome subunit alpha type	Solyc12g009140	1.30
60S ribosomal protein L6	Solyc04g014720	1.26
2-oxoglutarate dehydrogenase E1 component	Solyc04g011350	1.26
Proteasome subunit alpha type	Solyc02g081700	1.25
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Solyc07g064800	1.23
26S proteasome non-ATPase regulatory subunit 13	Solyc05g053140	1.19
Proteins induced by C7 inoculation regarding Bm-inoculated plants		
F-box family protein	Solyc04g057950	5.00
Non-green plastid inner envelope membrane protein	Solyc09g015650	4.40
LETM1 and EF-hand domain-containing protein 1, mitochondrial	Solyc11g008770	2.87
Cytochrome P450	Solyc07g014670	2.84
Cytochrome c1	Solyc06g067920	2.48
Ras-related protein Rab-8A	Solyc11g073050	2.16
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Solyc04g082670	2.08
Cc-nbs-lrr, resistance protein	Solyc04g007030	2.08
Receptor like kinase, RLK	Solyc12g014350	2.06
Histone H1	Solyc06g084020	1.94
Pore protein of (OEP24)	Solyc04g080190	1.92
Receptor protein kinase	Solyc00g289230	1.91
Mitochondrial Rho GTPase 1	Solyc01g098920	1.74
Sorting and assembly machinery component 50 homolog	Solyc04g079270	1.68
Mitochondrial 2-oxoglutarate/malate carrier protein	Solyc01g005620	1.66
Cytochrome P450	Solyc03g122350	1.64
Pentatricopeptide repeat-containing protein	Solyc01g096200	1.58

Cytochrome P450	Solyc07g014670	2.84
Cytochrome c1	Solyc06g067920	2.48
Ras-related protein Rab-8A	Solyc11g073050	2.16
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Solyc04g082670	2.08
Cc-nbs-lrr, resistance protein	Solyc04g007030	2.08
Receptor like kinase, RLK	Solyc12g014350	2.06
Histone H1	Solyc06g084020	1.94
Pore protein of (OEP24)	Solyc04g080190	1.92
Receptor protein kinase	Solyc00g289230	1.91
Mitochondrial Rho GTPase 1	Solyc01g098920	1.74
Sorting and assembly machinery component 50 homolog	Solyc04g079270	1.68
Mitochondrial 2-oxoglutarate/malate carrier protein	Solyc01g005620	1.66
Cytochrome P450	Solyc03g122350	1.64
Pentatricopeptide repeat-containing protein	Solyc01g096200	1.58
Ras-related protein Rab-7a	Solyc04g072060	1.58
B-cell receptor-associated protein 31-like containing protein	Solyc12g005910	1.54
Cytochrome b5	Solyc06g007930	1.54
NADH-quinone oxidoreductase F subunit family protein	Solyc02g087240	1.49
Aquaporin	Solyc06g060760	1.48
Pre-mRNA splicing factor	Solyc04g017710	1.47
Cc-nbs-lrr, resistance protein	Solyc02g084450	1.47
NADH-quinone oxidoreductase subunit D	Solyc00g014830	1.43
Mitochondrial phosphate carrier protein	Solyc02g094470	1.38
Mitochondrial processing peptidase beta subunit	Solyc02g088700	1.36
Calcium-binding protein Calnexin	Solyc03g118040	1.35
Mitochondrial processing peptidase beta subunit	Solyc05g012480	1.33
V-type proton ATPase subunit C	Solyc03g097790	1.31
1-phosphatidylinositol-4 5-bisphosphate phosphodiesterase	Solyc05g052760	1.30
ATP synthase gamma chain	Solyc03g115110	1.25
Protein kinase	Solyc03g114210	1.18

*** Common proteins that are diferentially expressed between *Bacillus megaterium*- and *Enterobacter* C7-inoculated wild type and *never ripe* plants**

Additionally, Venn diagram showed bacterial inoculation differences in wt and *nr* plants were mostly genotype-specific (Fig. 3.5 C). 17 and 72 proteins showed specific level modifications in wt and *nr* plants, respectively, and three proteins were significantly affected in both bacterial inoculations in both plant genotypes. A protein kinase-like protein and ectonucleoside triphosphate diphosphohydrolase 6 were induced under Bm inoculation regarding C7 inoculation treatment in both plant genotypes. However, major allergen Mal d 1 showed higher protein values in C7-inoculated wt plants, meanwhile its levels were higher in Bm-inoculated *nr* plants (Tables 3.5 and 3.6).

Phosphate solubilizing bioassay

In order to check phosphorus bioavailability under different inoculation treatments, phosphate solubilization capacity of PGPB was tested and phosphate solubilization index (PSI) was calculated for each bacterial strain (Fig. 3.6). A *Pseudomonas* sp. strain named C+ p12 was used as positive control reaching a PSI-value of almost 2 and showing a clear transparent halo around bacterial colony. In contrast, Bm and C7 strains showed significant reduced values of PSI (around 1.12) and very tiny halos were noticed around colonies. Thus, no differences were noticed between Bm and C7 in ability to solubilize phosphates and both PGPB could be classified as negative phosphate solubilizers.

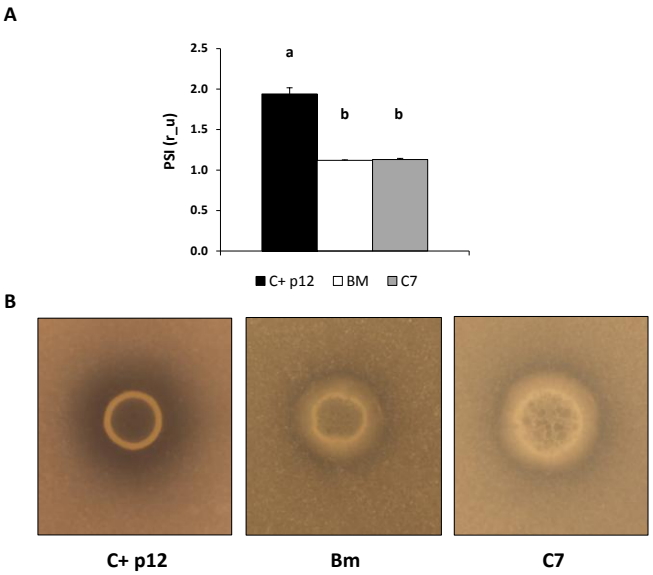

Figure 3.6 Phosphate solubilization bioassay. (A) Phosphate solubilization index values observed with NBRIP medium in plate assay**.** Positive control strain *Pseudomonas* p12 (C+ p12, black bars), *Bacillus megaterium* (Bm, white bars), and *Enterobacter C7* (C7, grey bars). Data are means ± SE (n=5). Means followed by different letters are significantly different (P < 0.05) according to LSD's test. **(B)** Pictures of bacterial colonies and phosphate solubilization halos observed with NBRIP medium in plate assay**.**

Phosphorus nutrition

In the main experiment, root phosphorus concentration and expression of phosphate transporter genes (*SlPT1* and *SlPT2*) were determined in root tissue (Fig. 3.7). Root P concentration showed interaction between factors (GxI) resulting in significant differences in wt and *nr* plants due to bacterial inoculation. In wt plants, C7 inoculation increased P concentration by 26.5 % regarding non-inoculated plants, meanwhile no effects were noticed due to Bm inoculation. In contrast, only Bm inoculation produced an increase of P concentration in *nr* roots (24.5 % regarding non-inoculated plants). In addition, significant differences between wt and *nr* plants were exclusively noticed under C7 inoculation showing *nr* plants lower values than wt ones (Fig. 3.7 A).

Furthermore, expression of phosphate transporters did not show factor interaction and was unaffected in wt plants, meanwhile different effects were observed in *nr* plants due to bacterial inoculation. Bm inoculation increased *SlPT1* expression in *nr* plants, but no expression change was noticed in *SlPT2* levels. In contrast, C7 inoculation did not affect *SlPT1* expression and induced *SlPT2* expression in *nr* plants. Moreover significant differences between wt and *nr* plants were exclusively noticed for *SlPT1* expression in non-inoculated plants (Fig. 3.7 B, C).

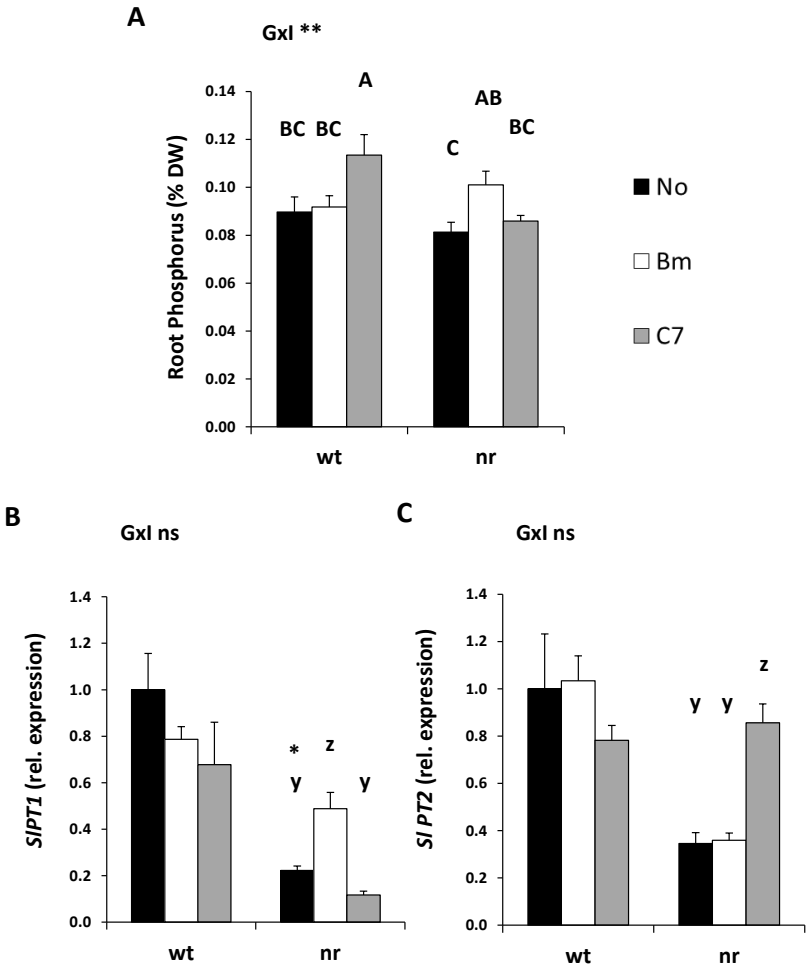

Figure 3.7 Effects of bacterial inoculation on root phosphorus concentration and expression of phosphate transporters. (A) Root phosphorus concentration (% Dry Weight (DW)) (n=7), **(B)** *Solanum lycopersicum* phosphate transporter 1 (*SlPT1*) expression (n=3), and **(C)** *Solanum lycopersicum* phosphate transporter 2 (*SlPT2*) expression (n=3) of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato plants. 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 were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. In case of significant interaction between factors, all treatments were compared between each other's. Means followed by different capital letters are significantly different ($P < 0.05$) according to LSD's test. In case of not-significant

interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different ($P < 0.05$) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference ($P < 0.05$) is showed as (*) above *nr* means.

Differential phosphorus conditions bioassay

In order to evaluate P nutrition involvement in PGPB mechanisms, a bioassay with two P nutrition regimes was performed. Biomass production was evaluated to determine importance of phosphorus in plant growth promotion by bacterial strains. Additionally, P concentration in root and shoot tissues as well as root phosphate transporters expression were evaluated to determine bacterial inoculation effects on phosphorus accumulation and acquisition, respectively. Factor interaction between main factors (GxPxI) was noticed in case of root P concentration and *SlPT2* expression (Fig. 3.8).

Several differences were noticed in biomass production due to bacterial inoculation (Fig. 3.8 A, B, C). A reduction in all DWs was observed due to low P treatment regarding control P conditions. Plant growth promotion was only noticed by C7 inoculation in *nr* plants independently of P nutrition regime. Under control P conditions, C7 inoculation in *nr* plants increased total (25.1 %) and shoot (26.2 %) DWs, but no significant difference was noticed in root DW. Under low phosphorus conditions, C7 inoculation in *nr* plants increased total (80.3 %), shoot (71.1 %) and root (116.7 %) DWs. However, no significant effects were noticed in wt plants due to C7 inoculation. In contrast, Bm inoculation only produced significant changes in wt plants under

control P conditions showing a reduction of total (21.8 %), shoot (19.2 %) and root (34.4 %) DWs, showing no significant effects on wt plants under low P conditions. Bm inoculation in *nr* plants had no growth effects under any P regime. Furthermore, significant differences between wt and *nr* plants were noticed in total, shoot and root DWs in all cases excepting under C7 inoculation and low P conditions. It was generally noticed that wt plants were more efficient than *nr* plants in biomass production under both P nutrition regimes (Fig. 3.8 A, B, C).

In addition, several effects were noticed in root P concentration (Fig. 3.8 D). No effects were noticed due to any bacterial inoculation in *nr* plants compared to non-inoculated plants. However in wt plants, Bm inoculation decreased and increased root P concentration under control and low P conditions, respectively, while C7 inoculation only increased root P concentration under control P conditions. Moreover, a reduction in root P concentration was observed due to low P treatment regarding control P conditions in non- and C7-inoculated wt plants, but not noticed under Bm inoculation treatment. In *nr* plants, this reduction due to low P conditions was observed in all cases. Furthermore, differences between inocula were noticed in wt plants under control P conditions and in *nr* plants under low P conditions. C7 inoculation increased root P concentration regarding Bm-inoculated plants in both cases. In addition, *nr* plants significantly showed higher root P concentration than wt ones in all cases excepting under C7 inoculation and control P conditions, and under Bm inoculation and low P conditions (Fig. 3.8 D). Moreover, no significant effects were observed in shoot P concentration due to PGPB inoculation neither by plant genotype, showing exclusively the low P treatment a significant decrease of P concentration (Fig. 3.8 E).

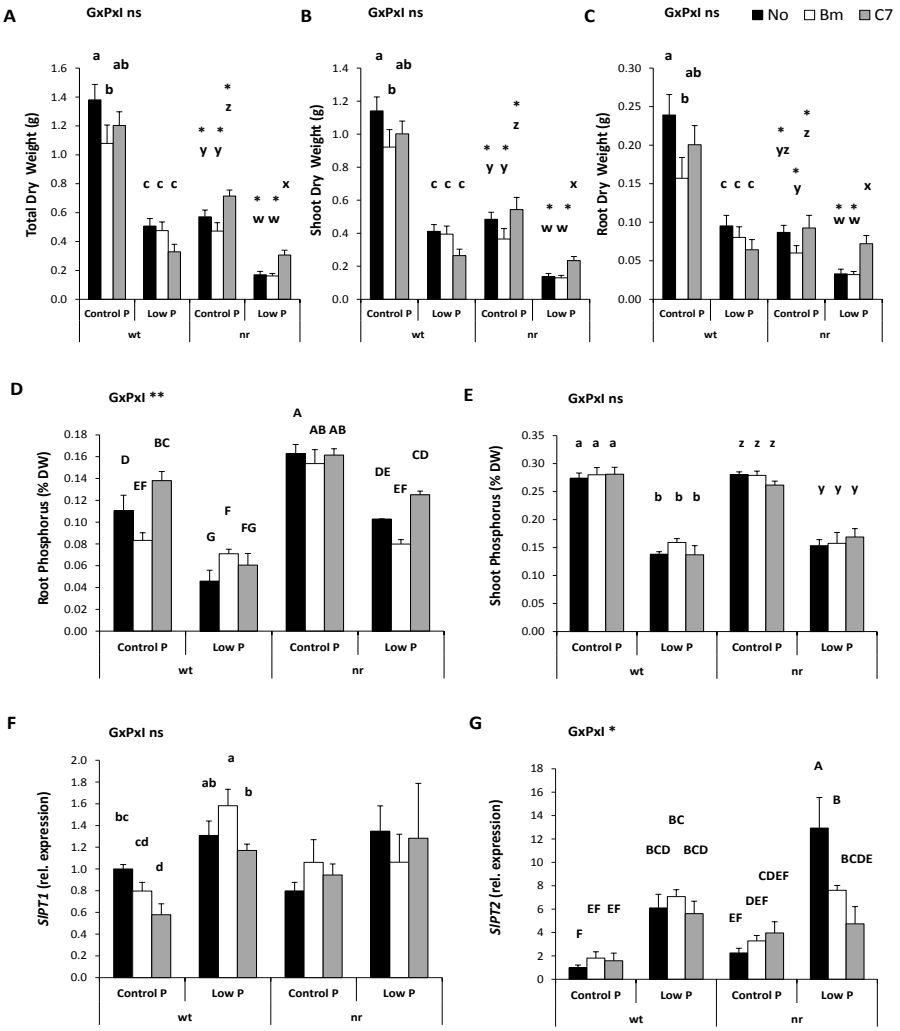

Figure 3.8 Effects of bacterial inoculation on plant growth and phosphorus nutrition under two differential phosphorus-nutrition conditions. (A) Total, **(B)** shoot and **(C)** root dry weights (n=7), **(D)** root and **(E)** shoot phosphorous content (n=4), **(F)** *Solanum lycopersicum p*hosphate transporter 1 (*SlPT1*) expression (n=3) and **(G)** *Solanum lycopersicum p*hosphate transporter 2 (*SlPT2*) expression (n=3) of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato plants irrigated with two differential phosphorus regimes: 1 and 0.2 mM. 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 ± SE. Data were analyzed by three-way ANOVA with plant genotype (G), phosphorous regime (P) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxPxI) was evaluated by P-value; ns, not significant; * P ≤ 0.05;

** P ≤ 0.01; *** P ≤ 0.001. In case of significant interaction between factors, all treatments were compared between each other's. Means followed by different capital letters are significantly different (P < 0.05) according to LSD's test. In case of not-significant interaction between factors, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) above *nr* means.

Finally, expression of phosphate transporter genes (*SlPT1* and *SlPT2*) was evaluated. On one hand, *SlPT1* expression was only affected in wt plants, not showing inoculation or low P effects on *nr* plants. Under control P conditions, C7 inoculation significantly reduced *SlPT1* expression regarding non-inoculated wt plants; meanwhile no effect was noticed due to Bm inoculation. Under low P conditions, *SlPT1* expression differences between Bm- and C7-inoculated wt plants were noticed showing Bm-inoculated wt plants higher expression than C7-inoculated ones. Furthermore, low P treatment induced *SlPT1* expression only under Bm and C7 inoculation treatments. In addition, no differences between wt and *nr* plants were noticed for *SlPT1* expression (Fig. 3.8 F). On the other hand, *SlPT2* expression showed changes in both wt and *nr* plants. In wt plants, low P treatment significantly induced *SlPT2* expression regarding control P conditions, but no PGPB inoculation effects were observed. However, *SlPT2* expression was significantly reduced by both Bm and C7 inoculations under low P conditions without differences between each inocula in *nr* plants. PGPB inoculation did not show effect in *nr* plants under control P conditions. Additionally, induction of *SlPT2* expression by low P treatment was observed under no and Bm inoculation conditions, but

not noticed under C7 inoculation. In addition, significant differences between plant genotypes were exclusively noticed in non-inoculated plants under low P conditions (Fig. 3.8 G).

Table 3.7 Effects of bacterial inoculation on antioxidant enzymatic activities. ANOVA of superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX) and catalase (CAT) activities in cv. Pearson tomato plants. SOD activity: expressed in unities of SOD μg^{-1} protein; GR activity: expressed in nmol oxidized NADPH min^{-1} μg^{-1} protein; APX activity expressed in nmol oxidized ascorbate min^{-1} μg^{-1} protein; CAT activity: expressed in nmol H_20_2 min^{-1} μg^{-1} protein. Data are means ± SE (n = 7). Significance of sources of variation interaction (GxI) was evaluated by P-value. As no significant interaction between factors was noticed, inoculum effects were evaluated analyzing separately wt and *nr* plants using ANOVA. Means followed by different letters are significantly different (P < 0.05) according to LSD's test, using as highest value from letter a onwards for wt plants, and from letter z backwards for *nr* plants. Plant genotype effect was evaluated analyzing wt and *nr* plants under the same inoculation treatment (No, Bm or C7) by t-Student test and significant difference (P < 0.05) is showed as (*) next to *nr* means.

	SOD Activity	GR activity	APX activity	CAT activity
wt No	170.50 +/- 35.53	5.08 +/- 0.59	1.98 +/- 0.09 a	914.31 +/- 142.51
wt Bm	178.51 +/- 30.87	4.47 +/- 0.53	1.54 +/- 0.13 b	631.60 +/- 54.37
wt C7	160.72 +/- 19.52	4.08 +/- 0.17	1.54 +/- 0.06 b	802.95 +/- 75.26
P-value	ns	ns	**	ns
nr No	204.78 +/- 44.97	6.62 +/- 0.90	2.58 +/- 0.64	1389.96 +/- 318.99
nr Bm	168.87 +/- 28.37	4.39 +/- 0.56	1.56 +/- 0.15	1081.88 * +/- 149.61
nr C7	185.48 +/- 28.26	5.80 +/- 0.82	1.80 +/- 0.26	1213.65 +/- 185.21
P-value	ns	ns	ns	ns

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; ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

Antioxidants enzymatic activities

Superoxide dismutase, glutathione reductase, ascorbate peroxidase and catalase activities were determined in root tissue samples of main experiment. No changes were noticed in SOD, GR, and CAT activities due to PGPB inoculation in both plant genotypes. However, APX activity showed a significant reduction due to both bacterial inoculations only in wt plants. Furthermore, differences between plant genotypes were exclusively observed for CAT activity under Bm inoculation, showing *nr* plants higher values than wt ones (Table 3.7).

Antioxidant compounds measurement

Ascorbic acid as well as reduced and oxidized glutathione forms were determined in root tissue samples of main experiment (Fig. 3.9). Ascorbic acid concentration showed a reduction due to C7 inoculation only in wt plants showing no PGPB inoculation effects in *nr* plants. Moreover, differences between plant genotypes were exclusively observed in non-inoculated plants showing *nr* plants lower values than wt ones (Fig 3.9 C). Nevertheless, glutathione forms were altered in both plant genotypes by bacterial inoculation, showing different modifications depending on sensitivity to ethylene. Bm inoculation increased reduced glutathione (GSH) in wt roots, meanwhile a decrease of GSH levels was noticed in *nr* plants. Furthermore, Bm inoculation did not alter oxidized form of glutathione (GSSG) concentration in wt roots, but increased levels were noticed in *nr* plants. On the other hand, C7 inoculation reduced GSH concentration independently of plant genotype and GSSG was increased by C7 inoculation also in both plant genotypes. Furthermore, differences between inocula were noticed in

GSH and GSSG levels in both genotypes, showing C7-inoculated plants lower GSH levels and higher GSSG levels than Bm-inoculated ones. Significant differences between wt and *nr* plants were also noticed in GSH and GSSG levels. Non-inoculated *nr* plants showed higher GSH levels and lower GSSG levels than wt ones. However, lower GSH in *nr* than in wt plants and no change in GSSG levels were noticed under Bm inoculation. In contrast, no GSH change and lower GSSG levels in *nr* than in wt plants were observed under C7 inoculation (Fig. 3.9 A, B).

In addition, total glutathione was determined to examine the glutathione pool. In wt plants, Bm inoculation increased total glutathione concentration, meanwhile it was reduced due to C7 inoculation. In *nr* plants, both Bm and C7 inoculation decreased total glutathione concentration, but lower values were noticed due to C7 inoculation. Significant differences between plant genotypes were noticed showing bacteria-inoculated *nr* plants reduced glutathione pool than wt ones and no change in non-inoculated plants (Fig. 3.9 D). Ratio between GSH and GSSG is used as a marker for oxidative stress, with higher values reflecting lower stress conditions (Mhamdi et al. 2010). Bm inoculation increased GSH:GSSG in wt roots, meanwhile GSH:GSSG was reduced in *nr* roots. Nevertheless, a decrease in GSH:GSSG was noticed due to C7 inoculation in both plant genotypes. Additionally, differences between inocula were also noticed in GSH:GSSG in both plant genotypes, showing C7-inoculated plants lower ratio values than Bm-inoculated ones. Significant differences between wt and *nr* plants were exclusively noticed in non-inoculated plants showing *nr* plants higher ratio than wt plants (Fig. 3.9 E).

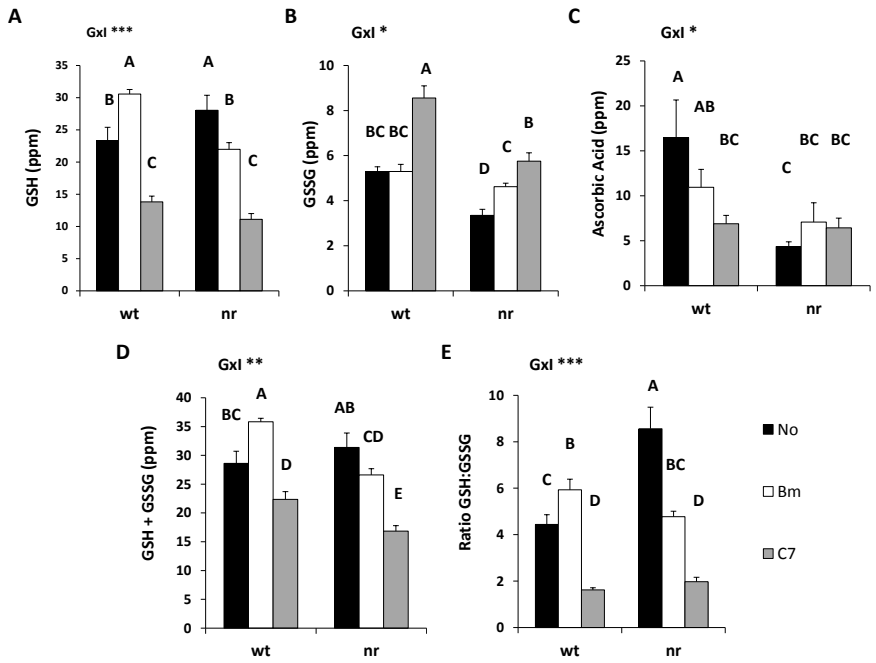

Figure 3.9 Effects of bacterial inoculation on ascorbic acid and glutathione. (A) Reduced glutathione concentration, **(B)** oxidized glutathione concentration, **(C)** ascorbic acid, **(D)** total glutathione concentration, and **(E)** ratio between reduced and oxidized glutathione forms of wild type cv. Pearson (wt) and *never ripe* (*nr*) tomato plants. 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 ± SE (n = 7). Data were analyzed by two-way ANOVA with plant genotype (G) and inoculum (I) as sources of variation. Significance of sources of variation interaction (GxI) was evaluated by P-value; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. As interaction between factors was noticed in all cases, all treatments were compared between each other's. Means followed by different capital letters are significantly different ($P < 0.05$) according to LSD's test.

Discussion

In the present chapter, Bm inoculation only promoted plant growth in wt plants. In contrast, C7 inoculation resulted in growth promotion of both plant genotypes (Fig. 3.3). These results are in accordance with previous results and PGPB mechanisms proposed as dependent on ethylene perception by SlETR3 for Bm, but independent of ethylene perception for C7 as discussed in previous chapters. The bacterial effects on roots could be responsible of noticed differences because bacterial niche is the plant root system (Benizri et al. 2001), and thereby PGPB colonization can cause physiological modifications as previously reported (Su et al. 2016).

PGPB inoculation affected proteomic profiles by different mechanisms, showing strain-specific effects dependent on ethylene insensitivity.

Root proteomic analysis of microsomal fraction was performed to examine plant-bacteria interaction. Ethylene insensitivity produced higher differences than bacterial inoculation, which additionally increased differences between plant genotypes (Fig. 3.4 A). Moreover, non-supervised PCA results suggest similar bacterial effects in wt plants, but not in *nr* plants. These results are in concordance with observed plant growth promotion suggesting that Bm inoculation could be unable to induce protein changes that conduct to a plant-growth stimulation. Specific differences between treatments were observed by supervised PCA (Fig. 3.4 B). Genotype differences were clearly noticed and a strain-specific effect was observed in wt plants

suggesting two different PGPB mechanisms. Bacterial inoculation effect was only as stronger as ethylene insensitivity effect due to Bm inoculation in *nr* plants suggesting that specific protein changes could block plant growth promotion as well. Thus, our results point to both bacterial inoculations can modify both plant proteomic profiles by different mechanisms, but the outcome is strain-specific, and dependent on ethylene sensitivity.

Bacillus megaterium could be misrecognized by _nr_ plants causing oxidative stress

Bacillus megaterium inoculation produced less and slighter protein level changes in wt than in *nr* plants and effects were mainly genotype-specific (Tables 3.1 and 3.2). Triosephosphate isomerase (TPI) was undetected in Bm-inoculated wt plants inducing significant changes in several pathways of sugar metabolism. Nevertheless, TPI was also undetected in Bm-inoculated *nr* plants, but these pathways were unaltered suggesting the involvement of ethylene perception. TPI is a sugar-metabolic enzyme and its inactivation would slow down glycolysis and tricarboxylic acid cycle, and therefore arrest mitochondrial metabolism under oxidative stress conditions resulting in prevention of deleterious reactive oxygen species (ROS) production (Cabiscol et al. 2000). Furthermore, the glutathione-mediated detoxification was affected in Bm-inoculated *nr* plants, and TPI could be target of glutathionylation for its function regulation (Ito et al. 2003). Additionally, opposed effects were noticed in inorganic phosphate transporter due to Bm inoculation pointing to a involvement of P nutrition as new player in Bm-plant interaction probably with

involvement of ethylene sensitivity. In fact, ethylene and its perception is highly related with P nutrition (Borch et al. 1999; Kim et al. 2008a; Lei et al. 2011).

On one hand, specific changes were observed in wt plants by Bm inoculation (Table 3.1). RPL4-B is involved in trafficking of vacuolar targeted proteins affecting plant growth and its role was suggested as essential for plant viability (Rosado et al. 2010). Moreover, 50SL11 plays a function in plastid ribosome activity and its mutation reduced the growth-rate affecting photosynthesis (Pesaresi et al. 2001). In contrast, argininosuccinate synthase (ASS), which is involved in arginine synthesis (Slocum 2005), was repressed. In some pathogenic fungi and bacteria, arginine is involved in growth and virulence *in planta* and its auxotrophy leads to a pathogenicity lost (Namiki et al. 2001; Ardales et al. 2009). Thus, Bm inoculation produced specific effects in wt plants that could increase protein synthesis and trafficking in vacuole and chloroplast resulting in better plant growth and reduce the possible overgrowth of Bm by decreasing arginine biosynthesis.

On the other hand, several specific changes were noticed in *nr* plants by Bm inoculation (Table 3.2). Glutathione S-transferase (GST) is a ubiquitous enzyme with antioxidant properties involved in detoxification of xenobiotics and peroxide removal (Edwards et al. 2000; Edwards and Dixon 2005). The band 7 stomatin family protein is involved in root meristem proliferation and could shape respiratory chain functioning and organization (Gehl and Sweetlove 2014). Heat shock protein 70 (Hsp70) plays role as chaperones (Sung et al. 2001; Su and Li 2008). Stress can cause damage to plant cell and lead to osmotic and oxidative stress inducing Hsps production (Scarpeci et al.

2008; Al-Whaibi 2011). In addition, the non-specific lipid-transfer proteins (nsLTPs) are fundamental in plant growth and development as well as in resistance to a/biotic stresses (Liu et al. 2015) showing differential expression in response to bacteria (Molina et al. 1993; Jung et al. 2006), and have been classified as pathogenesis-related proteins (Sels et al. 2008). NsLTPs present strong antimicrobial activity (Gizatullina et al. 2013) and their over-expression significantly increased resistance to bacterial pathogens (Molina and García-Olmedo 1997). Neutral ceramidases improve resistance to oxidative stress responses (Li et al. 2015), and subtilisin-like proteases are involved in proteolytic degradation during a/biotic programmed cell death (PCD) such as during oxidative and osmotic stresses (Vartapetian et al. 2011). Some of them are specifically induced by pathogen infection and could function as receptor activating immune signalling processes located in the plasmalemma (Figueiredo et al. 2014). Thus, induction of these proteins suggested that *nr* plants inoculated with Bm were under stress and plants responded counteracting the bacterial negative effects.

In contrast, Bm repressed some proteins in *nr* plants. Reductase or carbonic anhydrase (CA) is involved in inorganic carbon transport modulating efficient CO_2 fixation in the photosynthetic process and in respiration (Badger 1994; Price et al. 1994; Henry 1996). Furthermore, a mitochondrial porin outer membrane protein is involved as a major component of tRNA import of plant mitochondria (Salinas et al. 2006), and its mutation reduced ATP synthesis rate (Pan et al. 2014). Cytochrome c (Cc) is a redox carrier in the electron transport chain of mitochondria (Eubel et al. 2004), and is able to interact with several protein targets under homeostatic conditions affecting (among others) ascorbate biosynthesis (Leferink et al. 2008; Hervás et al. 2013).

Nevertheless, Cc in the cytoplasm and even in the nucleus is capable of interacting with other proteins under PCD (Díaz-Moreno et al. 2011; Martínez-Fábregas et al. 2013). Metacaspase 7 is also involved in induction of PCD in plant cells (Bozhkov et al. 2005). In addition, Bm inoculation reduced Ras-related protein Rab-8A protein levels (ethylene-responsive small GTP-binding protein) (Moshkov et al. 2003), suggesting involvement of ethylene. Rab GTPases play a role in intracellular membrane trafficking and would provide specificity for events of membrane fusion (Zerial and McBride 2001). Moreover, F-box proteins (involved in protein ubiquitination and degradation (Hellmann et al. 2002)) control several important processes including pathogen resistance (Lechner et al. 2006). Erlin1/2 form a complex that mediates the endoplasmic reticulum-associated degradation of inositol 1,4,5-triphosphate receptors (Pearce et al. 2009; Wang et al. 2009). These receptors are involved in Ca^{2+} signalling to control several cellular and physiological processes (Bosanac et al. 2002; Taylor and Tovey 2010) including responses to environmental stimuli in plants (Krinke et al. 2007). Finally, a Receptor like kinase (RLK) appear to play a central role in pathogen recognition, activation of plant defence and developmental control (Afzal et al. 2008; De Smet et al. 2009). Noticed reduction in these proteins pointed that Bm inoculation in *nr* plants induced a metabolism alteration but which is counteracted by plants to avoid cellular damage.

Thus, protein biosynthesis and degradation as well as trafficking were modified by Bm inoculation in *nr* plants, affecting to mitochondrial metabolism, and thus to several growth and developmental processes and responses to environmental stimuli. Bm could be perceived as a pathogenic-like bacteria by *nr* plants, since

pathogenesis-related proteins were induced. In accordance with our results, induction of a pathogenic-related protein by Bm was reported in a tomato line deficient in ABA (Porcel et al. 2014). Furthermore, several proteins involved in plant response to oxidative stress showed augmented levels pointing to an increase in oxidative stress. However, reduction in F-box, RLK, Cc and metacaspase7 suggested that plants could modulate pathogenesis related response to avoid PCD. Indeed, Bm did not reduce plant growth (Fig. 3.3) suggesting not completely recognition as pathogen. In consequence, Bm inoculation in *nr* plants may alter cell metabolism due to a fail in plant-bacteria interaction mediated by ethylene perception that ends in an increase of oxidative stress as previously observed with other beneficial microorganisms (Van Loon et al. 2008). However, plant homeostasis counteracts bacterial effects resulting in a non-pathogenic interaction and the same growth as non-inoculated *nr* plants (Fig. 3.3). Indeed, beneficial microorganisms can be recognized as potential invaders firstly triggering immune response and later detaining it to let successful beneficial association (Zamioudis and Pieterse 2012).

C7 inoculation increased oxidative stress and stimulates plant growth in wt and *nr* plants modifying different proteins

Enterobacter C7 inoculation also caused minor and less intense changes in protein levels in wt than in *nr* plants (Tables 3.3 and 3.4). Moreover, most of effects are specific of plant genotype. Only an inorganic phosphate transporter showed opposed effects suggesting that phosphorus nutrition is also involved in C7 PGPB mechanism.

Specific changes were observed in wt plants by C7 inoculation (Table 3.3). An aquaporin-like protein was induced suggesting that C7 could improve transport of water, small neutral solutes or gases in cellular membranes (Maurel et al. 2008). 60S ribosomal protein L4-B induction pointed to alteration in trafficking of vacuolar targeted proteins (Rosado et al., 2010). Additionally, APX is expressed in response to a/biotic stresses and during plant development to detoxify H_2O_2 avoiding cellular damage (Caverzan et al. 2012).

In contrast, C7 inoculation repressed some proteins in wt plants. Blue copper proteins (BCPs) transport electrons in bacteria and plants (De Rienzo et al. 2000), and are involved in lignin accumulation (Ji et al. 2015). Indeed, BCP protein seems to be responsible of cell wall-based resistance noticed in response to non-adapted bacteria (Mishina and Zeier 2007). Moreover, glucan synthase-like 7 (GSL7) protein is a callose synthase involved in phloem transport (Barratt et al. 2011). Callose plays a key role in several processes in plant growth and development as well as in response to a/biotic stresses (Chen and Kim 2009). RNA helicases are essential in RNA metabolism, regulate plant growth and development (Owttrim 2006), and stabilize the plant growth under stress-conditions (Tuteja et al. 2014). Furthermore, vacuolar-type proton ATPases (VHA) acidifies intracellular compartments to energize ion and metabolite transport.

In consequence, it could be proposed that C7 inoculation in wt plants increased oxidative stress, probably because plant-bacteria interaction induces pathogenesis related proteins such as nsLTP. However, BCP and GSL7 reductions could facilitate interaction between C7 and wt plants avoiding cell wall-based resistance.

Nevertheless, C7 inoculation also showed specific effects in *nr* plants (Table 3.4) inducing several proteins. The dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (undetected in non-inoculated plants) is fundamental in N-glycosilation of matrix polysaccharides affecting root cell growth and survival (Qin et al. 2013). The Band 7 stomatin family protein induction suggested modulation of respiratory chain and proliferation of root meristem (Gehl & Sweetlove, 2014). A GST was highly induced by C7 inoculation indicating that plants were under oxidative stress. Cytochrome P450 families are involved in chemical defence mechanisms (Mizutani 2012) catalyzing a wide variety of reactions in biosynthesis or catabolic pathways of phytohormones, antioxidants and defense compounds (Bak et al. 2011). Cytochrome b5 is involved in electron transference supplying reductant for polyunsaturated fatty acid synthesis (Shanklin and Cahoon 1998; Kumar et al. 2012), which is essential in cellular membranes (McConn and Browse 1998). The V-type proton ATPase (V-ATPase) is the dominant H+-pump localized at membranes of the secretory pathways and is essential for plant growth. Under stress conditions, cell survival depends on maintain or modulating V-ATPase activity (Dietz et al. 2001). Furthermore, it has been suggested a interaction between V-ATPase subunits and glycolytic enzymes that could allow quick ATPase access to protons and ATP generated in glycolysis (Holliday and Holliday 2014). Aspartate aminotransferase (AAT) is an enzyme with a key role in the amino acid synthesis and involved in regulation of carbon and nitrogen metabolism (Zhou et al. 2009). AAT catalyzes formation of aspartate, which is the biosynthetic precursor of several amino acids as well as

derived metabolites involved in plant growth, development, reproduction and defence (De La Torre et al. 2014).

In addition, histones are involved in cell division (Zhao et al. 2000; Nelson et al. 2002) and chromatin reorganization (Talbert et al. 2002; Mizuguchi et al. 2004). Their modifications and variants play a key role in gene expression regulation and DNA repair (Verbsky and Richards 2001; Fransz and De Jong 2002). C7 inoculation induced a histone H2A, but other H2A and H3 proteins were repressed. H3 phosphorylation was described in plants for cell cycle regulation (Houben et al. 2007). Thus, C7 inoculation could alter chromatin organization and gene expression by modulation of histone levels.

In contrast, C7 inoculation repressed some proteins in *nr* plants. TPI was undetected in C7-inoculated *nr* plants affecting significantly to dihydroxiacetone and Calvin cycles. As abovementioned, TPI inactivation would slow down glycolysis and tricarboxylic acid cycle under oxidative stress (Cabiscol et al. 2000). A LRR receptor-like serine/threonine-protein kinase FEI 1 was also undetected in C7-inoculated *nr* plants. FEI proteins are receptors that regulate cellulose biosynthesis and thereby cell wall function (Harpaz-saad et al. 2012). Its mutation disrupts anisotropic expansion suppressed by inhibition of ACC synthase, but not by blocking ethylene response (Xu et al. 2008). In consequence, although an ethylene-perception independent mechanism has been proposed for C7, the ethylene precursor ACC could be involved in C7 PGPB mechanism or C7 is able to stimulate plant growth in wt and *nr* plants by different action mechanisms. Moreover, COPI vesicles are critical in plant growth and survival taking part in retrograde protein transport in secretory pathway (Ahn et

al. 2015), and reduction of its subunits disrupted the Golgi structure and produced autolysosome-like structures accumulation. Finally, 60S acidic ribosomal protein P3 (RPP3) is an acidic phosphoprotein involved in translation. Stress is able to cause dynamic changes in the P-protein complex by protein phosphorylation (Bailey-Serres et al. 1997).

In consequence, it could be proposed that C7 inoculation in *nr* plants affected chromatin organization and gene expression regulation as well as translation. Furthermore, C7 inoculation modified mitochondrial metabolism and protein trafficking and increased oxidative stress with higher levels of GST and P450 proteins. However, noticed induction of cytochrome b5, V-ATPase subunit E proteins and processes (N-glycosilation, and amino acid biosynthesis) could counteract stress effects resulting in cell survival and growth promotion.

Interaction with *nr* plants is modulated by C7 through recognition-related proteins while Bm triggered an immune response

Furthermore, comparison between both PGPB could help to clarify action mechanisms. Our results showed mainly strain-specific bacteria in both plant genotypes and common proteins were similarly modified by both bacteria (Tables 3.1, 3.2, 3.3 and 3.4). However, quantitative changes were noticed in some proteins dependently of bacterial inoculation in *nr* plants (Tables 3.2 and 3.4) and could be related with different outputs observed. GST higher induction in C7-inoculated plants suggests higher oxidative stress than in Bm-

inoculated ones (Edwards et al. 2000). In accordance, reductase modification caused the ascorbate-glutathione cycle variation in C7-inoculated *nr* plants unnoticed in Bm-inoculated ones. Reductase was highly reduced by both bacterial inoculation, but in a higher degree by C7 suggesting a greater effect of C7 inoculation in respiration and/or inorganic carbon transport (Badger 1994; Henry 1996; Li et al. 2000b). Although, reductase is positively correlated with photosynthesis and dry matter accumulation (Khan 2002; Khan et al. 2004), it was observed in shoot tissue pointing to respiration and inorganic carbon transport as affected processes in roots. Additionally, C7 could affect mitochondrial respiratory chain more than Bm inoculation due to higher increase in band 7 stomatin family protein (Gehl and Sweetlove 2014).

Direct comparison between both PGPB also pointed to greater differences in *nr* than in wt plants (Tables 3.5 and 3.6; Fig. 3.5 C), although pathways were unaffected in both plant genotypes suggesting that bacterial effects regarding non-inoculated plants could be greater than between both bacteria despite of all noticed protein changes. Moreover, the unique common protein that showed opposed modification was the major allergen Mal d 1 induced in wt and *nr* plants by C7 and Bm, respectively. It belongs to the pathogenesis related group 10 (PR-10) which plays a role in plant defence against pathogens (Fernandes et al. 2013). Thus, Bm inoculation could trigger a higher response against bacterial colonization than C7 inoculation in *nr* plants in accordance with fail in Bm-plant interaction.

In wt plants, Bm compared to C7 inoculation could promote appropriate targeting of ribosome-nascent polypeptide complexes due

to observed changes in nascent polypeptide-associated complex alpha subunit-like protein (Beatrix et al. 2000). Furthermore, higher values of 6 7-dimethyl-8-ribityllumazine synthase or riboflavin synthase (Jordan et al. 1999) was noticed pointing to higher riboflavin levels in wt plants under Bm inoculation which could induce disease resistance due to its role as elicitor (Wang and Tzeng 1998; Dong and Beer 2000).

In *nr* plants, an aspartyl aminopeptidase (AAP) was highly induced in Bm- compared to C7-inoculated plants. AAP is a protease (Wilk et al. 1998), and function as plant susceptibility factor against bacterial pathogens (Bae et al. 2013). However, higher F-box family protein levels were observed in C7- compared to Bm-inoculated plants. As abovementioned, F-box proteins are involved in protein degradation regulating several significant processes including pathogen resistance (Hellmann et al. 2002; Lechner et al. 2006). Increased levels of LETM1 and EF-hand domain-containing protein 1 was also noticed due to C7 inoculation suggesting a higher accumulation of ATP synthase proteins (Zhang et al. 2012). Furthermore, higher cytochrome P450 levels observed in C7-inoculated suggested higher response against stress by chemical defense (Mizutani 2012). Cc-nbs-lrr is a resistance protein involved in detection of several pathogens monitoring effects of pathogen effectors in their targeted plant proteins (McHale et al. 2006; Marone et al. 2013). Higher Cc-nbs-lrr levels were observed under C7 inoculation suggesting that *nr* plants could monitor better C7 effects and thus maybe finely control their deleterious effects. In accordance, RLK levels were also higher in C7 than in Bm-inoculated *nr* plants. This receptor contribute to bacterial recognition in order to distinguish between pathogen or beneficial bacteria (Afzal et al. 2008), although the proper response critically depends on RLKs localization and

abundance at cell surface (Antolín-Llovera et al. 2014). Summarizing, Bm inoculation could be perceived as a pathogenic-like bacteria by *nr* plants, meanwhile C7 inoculation could modulate its interaction with *nr* plants affecting to recognition-related proteins as Cc-nbs-lrr and RLK.

Bm inoculation miss-regulates low phosphorus response, while C7 is able to avoid it in wt and *nr* plants.

Our results showed that phosphorus nutrition was affected by bacterial inoculation (Tables 3.1, 3.2, 3.3 and 3.4; Fig 3.7). Phosphorus is a key element in plant growth and development (Cheng et al. 2011). Additionally, their bioavailability in soils is very limited (due to poorly phosphate solubility), but fundamental for plant productivity (López-Bucio et al. 2002). Indeed, low P availability is noticed in approximately half of the agricultural lands, compromising crop yields (Lynch 2011). Several PGPB can solubilize soil phosphates increasing P availability for plant acquisition (Canbolat et al. 2006; Lai et al. 2008). PSI was evaluated resulting in poor ability to solubilize phosphates and showing no differences between Bm and C7 strains (Fig. 3.6). Thus, these results suggested similar P availability for plants, although bacterial traits could be altered under effects of plant and root exudates (Doornbos et al. 2012).

Furthermore, plants show several physiological adaptations to cope with low P conditions (Ticconi and Abel 2004; Wasaki et al. 2009). Phosphorus acquisition is performed by high-affinity phosphate transporters strongly induced under limited-P conditions in root tissue and regulated at transcriptional level mediating P acquisition across cellular membranes. In fact, P cellular concentration was proposed as

triggering signal for low P response resulting in a fine coordination between gene expression and P acquisition (Liu et al. 1998; Guo et al. 2008). Ethylene production is induced by P deficiency mediating plant response to low P availability (Borch et al. 1999; Li et al. 2009), and the ethylene signaling role in response to P starvation has been studied in several plant species (Drew et al. 1989; He et al. 1992; Kim et al. 2008a; Lei et al. 2011).

Soil microorganisms are determinant for plant nutrition and several symbiotic relationships have been developed to cope with persistent phosphorus deficiency (Raghothama and Karthikeyan 2005), such in case of mycorrhizal fungi (Harrison et al. 2002; Paszkowski et al. 2002). However, little is known about PGPB involvement in P acquisition. Only one study formerly pointed to direct modulation of P uptake by PGPB under low P conditions. *Bacillus amyloliquefaciens* promoted root growth in wheat, but reduced P uptake down-regulating phosphate transporter genes and up-regulating phosphate remobilizing ones (Talboys et al. 2014).

Thus, our results (Fig. 3.7) suggest a miss-regulation of low P response in *nr* plants due to Bm inoculation, since although higher P levels were noticed in roots, a phosphate transporter (*SlPT1*) induction was observed as in P starvation (Borch et al. 1999; Li et al. 2009). In contrast, C7 inoculation improved P concentration in wt plants and could be able to modulate P acquisition in *nr* plants via *SlPT2* suggesting an improvement of P nutrition as part of C7 PGPB mechanism.

In order to shed light on phosphorus nutrition implication in PGPB mechanisms, bacterial strains were tested under control and low

P conditions (Fig. 3.8). Low P conditions produced a DW reduction in all cases regarding corresponding control conditions pointing to an effective P shortage (Hermans et al. 2006). In addition, biomass production was generally noticed higher in wt than in *nr* plants under both P nutrition regimes. Nevertheless, higher root P concentration was generally observed in *nr* regarding wt plants. These results are in accordance with previous studies which proposed that ethylene-insensitive genotypes under low P stress would show reduced growth and would fail to produce some adaptive responses (Feng and Barker 1992; Zhang et al. 2003).

Surprisingly, no growth promotion effects due to Bm and C7 were noticed in wt plants (Fig. 3.8 A, B, C). These results could be explained because performed nutrition schedule could diminish PGPB effects since optimal nutrients concentrations were added every two days and wt plants are ethylene sensitive and thereby able to respond to low P. Furthermore, Bm inoculation did not caused effects in *nr* plants but diminished biomass production exclusively in wt plants under control P conditions suggesting that high P availability could be deleterious for Bm-interaction. In fact, Bm inoculation reduced and increased root P concentration under control and low P conditions, respectively, but no differences were noticed in root P levels between both P nutrition regimes in wt plants under Bm inoculation (Fig. 3.8 D, E). Moreover, *SlPT1* and *SlPT2* expression was induced by low P conditions under Bm inoculation suggesting a miss-regulation in P acquisition also in Bm-inoculated wt plants (Fig. 3.8 F, G).

Nevertheless, C7 inoculation stimulated plant growth in *nr* plants independently of P nutrition regime, but more efficiently under low P

conditions (Fig. 3.8 A, B, C). In fact, root DW was unaffected under control P conditions, but highly enhanced in low P conditions reaching root DW of non-inoculated *nr* plants under control P conditions. Thus, C7 inoculation could avoid low P effects on growth in *nr* plants suggesting the improvement of P nutrition as part of its action mechanism. In addition, C7 inoculation in *nr* plants under low P conditions could restore plant phenotype since plant genotype differences were noticed in all cases excepting under these conditions. However, no bacterial inoculation effects were noticed in DWs in wt plants under both P nutrition regimes, suggesting that C7 PGPB mechanism could imply P nutrition improvement even at lower levels than 0.2 mM when plants are ethylene-sensitive. Additionally, although C7 action mechanism was proposed independent on ethylene perception by SlETR3, two different C7 PGPB mechanisms in wt and *nr* plants could also explain different observed growth promotion effects.

In fact, C7 inoculation produced higher root P concentration values than wt ones under control P conditions reaching C7-inoculated *nr* plants values (Fig. 3.8 D). C7 inoculation reduced *SlPT1* expression in wt plants only under control P conditions corresponding with increased root P concentration by C7 inoculation. Moreover, *SlPT2* expression was induced by low P conditions in wt in all cases and in *nr* plants under non- and Bm-inoculation, but not under C7 inoculation suggesting that C7 inoculation could avoid low P response in wt and *nr* plants mediated by *SlPT1* and *SlPT2*, respectively (Fig. 3.8 F, G).

In tomato, *SlPT2* expression was restricted to roots, meanwhile *SlPT1* was mainly expressed in roots but faintly expressed in aerial

tissues (Liu et al. 1998). Our results showed that *SlPT1* was generally slightly induced by low P conditions in wt plants, but not in *nr* plants. In contrast *SlPT2* showed a strong induction due to low P conditions independently of plant genotypes according to previous reports that show a stronger response of *SlPT2* due to P starvation than *SlPT1* (Muneer and Jeong 2015). These results suggest that *SlPT1* response could be dependent of ethylene perception by SlETR3, while *SlPT2* regulation may be independent of this ethylene receptor.

Thus, low P response of tomato plants is dependent of ethylene and mediated by phosphate transporters. *SlPT1* expression regulation could be dependent of ethylene perception by SlETR3, but not *SlPT2*. Root P concentrations and expression of phosphate transporters observed in both experiments pointed to plants of main experiment were growing in conditions similar to control P, but *nr* plant response was similar to wt plants under low P conditions due to its inability to perceive ethylene (Fig 3.7 and 3.8). Additionally, Bm PGPB activity was proposed as dependent on ethylene perception by SlETR3 and Bm inoculation could miss-regulate low P response altering *SlPT1* expression. However C7 activity was suggested as independent of ethylene perception and could avoid low P response (keeping plant growth) mainly affecting *SlPT1* and *SlPT2* expression in wt and *nr* plants, respectively.

Bm PGPB activity could be mediated by increased GSH levels, while C7 increased oxidative stress in wt and *nr* plants

Antioxidant enzymatic activities were mainly unaffected by bacterial inoculation, excepting APX activity. However, no difference

between both PGPB strains was noticed suggesting that antioxidant metabolites could be responsible of antioxidant status alteration.

Glutathione is a key metabolite essential in plant cells (Cairns et al. 2006; Pasternak et al. 2008), and involved in several functions such as primary metabolism, redox signaling and defense/detoxification (Noctor et al. 2012). Moreover, reductive H_20_2 metabolism is linked to ascorbate in plants by the glutathione-ascorbate cycle (Noctor and Foyer 1998). GSH can undergo several redox reactions including conjugation with proteins and other electrophilic molecules (Edwards and Dixon 2010). Furthermore, GSH can be oxidized by reactive oxygen species preventing excessive oxidation of sensitive cellular components (Kataya and Reumann 2010).

Ascorbic acid was exclusively reduced by C7 inoculation in wt plants, but several changes were noticed in glutathione (Fig. 3.9). Our results showed that Bm inoculation increased GSH, total glutathione pool and GSH:GSSG ratio, but did not affect GSSG levels in wt roots. However, Bm inoculation decreased GSH and GSH:GSSG ratio, and increased GSSG concentration without altering total glutathione pool in *nr* plants. These data suggest that oxidative stress was reduced in wt plants, but it was increased in *nr* plants due to Bm inoculation agreeing with proteomic results and suggesting a PGPB mechanism dependent on ethylene sensitivity by ETR3 that affects cellular redox status. In contrast, C7 inoculation affected glutathione levels independently of ethylene sensitivity, reducing GSH, total glutathione pool and GSH:GSSG ratio, but increasing GSSG concentration. These results suggest that oxidative stress was induced by C7 inoculation, but growth

promotion was noticed in both plant genotypes pointing to an action mechanism independent of ethylene perception and oxidative stress.

Moreover, differences between wt and *nr* plants also are in accordance with these hypotheses and other studies have previously related ethylene signaling with glutathione metabolism. After ozone-induced stress in arabidopsis, ethylene insensitivity produced a decrease in GSH levels, but its external addition mitigated damage, suggesting that ethylene could induce *de novo* GSH biosynthesis protecting from injury (Yoshida et al. 2009). However, induction of an ethylene-precursor biosynthesis enzyme was induced in transgenic tobacco plants over-expressing a GSH biosynthesis enzyme, suggesting the involvement of GSH in ethylene biosynthesis (Ghanta et al. 2014). Recently, GSH-ethylene interaction was studied demonstrating that GSH stimulate ethylene production through transcriptional and post-transcriptional regulation of ACC biosynthesis enzymes. Additionally, ACC oxidase was identified as a target for S-glutathionylation. Moreover, exogenous GSH application enhanced stress tolerance in wt arabidopsis plants, but not in ethylene-insensitive ones pointing to ethylene-dependent pathway to improve stress tolerance mediated by GSH (Datta et al. 2015). GSH is involved in the synergistic multiple steps crosstalk between ethylene and salicylic acid to respond to environmental stresses (Ghanta et al. 2014). In consequence, Bm PGPB activity proposed as dependent on ethylene-perception could imply enhancement of GSH levels necessary to improve stress tolerance. Similarly, *Sphingomonas sp.* LK11 increased tomato plant growth at the same time that modulated the oxidative stress increasing GSH levels and it was suggested that could counteract toxicity mediated by

ROS via antioxidants level regulation but not by antioxidant enzymes (Halo et al. 2015).

Conclusions

In conclusion, our results showed that PGPB inoculation modified the root proteomic profiles in a mode dependent on strain and genotype. Several PGPB effects were noticed in secretory pathway proteins, but they could be spill over impacts in plant metabolism due to bacterial presence instead of direct bacterial effects. Thus, further research is needed to clarify if PGPB are able to modulate the secretory pathway.

Furthermore, SlETR3 mutation in *nr* plants impairs interaction between tomato plants and Bm, resulting in recognition as a pathogen-like microorganism and leading to increased oxidative stress and to loss of PGPB activity. Bm inoculation promoted plant growth in wt plants on a par with improvement in the redox status. Thus, the PGPB activity of *Bacillus megaterium* in tomato plants could be proposed as mediated by increased GSH and dependent on ethylene perception by SlETR3. Furthermore, low P response by *SlPT1* may be dependent on ethylene sensitivity by SlETR3, and thereby Bm inoculation in *nr* plants resulted in low P response miss-regulation.

In contrast, C7 inoculation induced plant growth and oxidative stress independently of ethylene sensitivity, but affecting different proteins depending on plant genotype. Indeed, C7 inoculation improved P nutrition mediated by *SlPT1* and *SlPT2* in wt and *nr* plants,

respectively. In consequence, although SlETR3 mutation determines plant interaction with C7 strain, *Enterobacter* C7 PGPB mechanism implies phosphorus nutrition enhancement and could be proposed as independent of ethylene perception.

General Discussion

General Discussion

The sustainable intensification of agriculture aims to provide food security to an expanding global population and simultaneously diminish harmful environmental effects of crops (Tilman et al. 2011). Thus, new strategies should be developed in order to increase efficiency in crop resource utilization maintaining present yields (Dodd and Ruiz-Lozano 2012). Management of rhizospheric microorganisms is a valuable strategy to induce plant growth (Berg 2009; Singh et al. 2011), and could diminish and even replace chemicals inputs in agriculture (Bhattacharyya and Jha 2012). Nevertheless, in-depth research is needed to completely understand interaction between plant and microorganisms as well as bacterial action mechanisms for the proper and effective large-scale use of these bacteria in integrated agricultural systems (Berg 2009). In consequence, the present study evaluate the effects of two different PGPB strains on plant growth and physiology regarding to ethylene perception in tomato in order to elucidate PGPB mechanisms and achieve determinant information for further implementation in crop systems.

Association between plants and beneficial microbes are thought to be ancient and moulded during co-evolution causing bacterial inoculation significant effects in plant physiology (Lambers et al. 2009). The present study showed that PGPB inoculation modified transcriptomic profiles in a strain-specific manner and dependently on ethylene sensitivity and watering conditions. Root metabolites, phytohormones and nutrients were also affected in function of inoculated bacteria and ethylene sensitivity. Proteomic profiles also showed strain-specific effects dependent on ethylene sensitivity. All

these results are in accordance with strain-specific interactions between host-plants and bacterial strains (Walker et al. 2011; Weston et al. 2012) as well as strain-specific PGPB mechanisms and bacterial effects depending on plant growth conditions (Ryu et al. 2005; Long et al. 2008).

Furthemore, ethylene insensitivity caused higher differences than PGPB inoculation, which additionally increased differences between plant genotypes in root transcriptomic, proteomic and nutritional profiles of mature plants. This can be explained since ethylene is involved in several key processes in plant growth and development as well as regulation of the phenotypic plasticity (Dugardeyn and Van Der Straeten 2008) to overcome environmental changes including nutritional stresses (Iqbal et al. 2013), and water stress (Hattori et al. 2009; Pan et al. 2012). Thus, ethylene-response leads to differentially expression of hundreds of genes (An et al. 2010), and ethylene-insensitive genotypes under stress would fail to adapt to stress conditions as observed in differential phosphorus bioassay (Feng and Barker 1992; Zhang et al. 2003). Moreover, plant genotype differences were significant in non-inoculated plants with higher growth in *nr* plants under well watered conditions pointing to less pronounced ethylene growth inhibitory effects (Pierik et al. 2006) due to their ethylene insensitivity (Wilkinson et al. 1995). However, this effect was unnoticed under drought conditions probably due to less adaptability to drought and higher ethylene induction by stress (Pierik et al. 2007).

Moreover, PGPB inoculation modifies plant nutrition notably in mature plants. Rhizospheric microbial communities are associated with nutrient biogeochemical cycles (Barea et al. 2005), and interaction

between plants and bacteria is fundamental for better acquisition of nutrients by plants (Ryan et al. 2009). Indeed, several PGPB action mechanisms are related with plant nutrition enhancement. PGPB inoculation directly modifies root metabolites in juvenile plants including amino acids, sugars and organic acids as observed in other studies (Weston et al. 2012; Fernandez et al. 2012; Su et al. 2016), that could explain plant growth promotion as well as interaction processes between plants and bacterial strains.

Although an ethylene-dependent mechanism was previously reported in arabidopsis (Chen et al. 2013), the present study report for first time an ethylene-dependent mechanism in bacteria without ACC deaminase activity. PGPB action mechanism related to ethylene were mainly reported for bacterial strains able to reduce ACC levels and thereby ethylene levels since they contain ACC deaminase activity (Glick et al. 2007a; Glick 2014). Nevertheless, PGPB selected in the present study did not show ACCd activity, neither ethylene production which can disturb plant ethylene metabolism. In fact, ethylene emission by plant root and shoots was unaffected directly by PGPB inoculation. Furthermore, both Bm as well as C7 were able to colonize the roots independently of plant ethylene-sensitivity, which is a determinant feature of PGPB for interaction with plants (Benizri et al. 2001). However, ethylene emission by seedlings 26 h post-inoculation was exclusively increased under Bm inoculation in *nr* plants compared to wt plants suggesting that SlETR3 plays a role in tomato plant interaction with Bm. In fact, other PGPB strains modulate ethylene receptor expression for the properly establishment of beneficial plant-bacteria association (Vargas et al. 2012; Vacheron et al. 2013). Moreover, basal ethylene levels are negatively feedback regulated

during vegetative growth (Barry et al. 2000; Alexander and Grierson 2002; Alba et al. 2005), and a similar process could be happened in Bm interaction with wt plants avoiding the induction of ethylene.

Indeed, ethylene perception by SlETR3 is proposed as essential for growth promotion mediated by Bm but no for C7, and congruent results were noticed in total growth in all chapters. However, some differences in ethylene insensitivity and PGPB effects on shoot and root growth in chapter 3 showed discordant results with chapters 1 and 2. This could be explained since fresh weights were used in chapter 3 for biomass production evaluation while dry weight were used in previous chapters suggesting that relative water content was affected by bacterial inoculation as previously reported with other PGPB (Mayak et al. 2004a; Li and Jiang 2017). However, relative water content was undetermined in these experiments, which in addition were performed in greenhouse where growth conditions can be variable depending on outside weather, affecting obtained outputs.

Moreover, the same growth promotion effects were observed under drought stress conditions and even PGPB improved their efficiency in wt plants suggesting stress alleviation due to bacterial inoculation as previously reported (Glick 2004; Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009), and enhancement of water and nutrient use efficiency (Dodd and Ruiz-Lozano 2012). Drought causes important yield losses enhanced by climate change and intensive agriculture with deep impact in tomato production (Pervez et al. 2009; Misra 2014). Thus, PGPB management can represent a suitable methodology for reduce water inputs and/or utilization of semiarid lands maintaining present yields or reducing potential yield losses.

However, drought stress has a great impact in plant physiology (Hasanuzzaman et al. 2014) as well as in transcriptomic profiles (Gong et al. 2010; Iovieno et al. 2016), which can mask PGPB inoculation effects. Actually, no significant altered pathways were reported when PGPB-inoculated plants were compared with non-inoculated ones under drought stress. Furthermore, either Bm or C7 plant growth promotion were independent of watering regime and thereby experiments in chapter 2 and 3 were carried out exclusively under well watered conditions in order to avoid that drought effects distort PGPB effects and to easily clarify plant interaction with bacterial strains as well as their action mechanisms.

In addition, PGPB inoculation affected relative growth rate exclusively in *nr* plants. RGR was decreased under Bm inoculation and increased under C7 inoculation in accordance with ethylene perception by SlETR3 essential for Bm and circumvented by C7, but suggesting that ethylene insensitivity affects plant interaction with both PGPB strains. Nevertheless, plant growth promotion effects were unnoticed in juvenile plants indicating that further research addressing bacterial effects on mature plants is necessary to completely understand bacterial effects on plant growth and achieve proper yield increases in crops, especially in tomato plants where only fruits are the profitable yield. However, PGPB inoculation at juvenile stage produced physiological effects as modification of stomatal conductance, photosynthetic efficiency and root metabolites as previously reported after PGPB colonization of plant root system (Su et al. 2016). Stomatal conductance and photosynthetic efficiency modifications were in agreement with PGPB activity dependent on ethylene perception by SlETR3 for Bm and ethylene-independent for C7.

Additionally, phytohormone levels were generally affected due to ethylene insensitivity and PGPB inoculation was able to indirectly modify plant phytohormonal status. During development and response to internal and external stimuli, several phytohormonal cross-talk processes have been reported (Munné-Bosch and Müller 2013). Transcriptomic analysis pointed to involvement of ethylene, JA and ABA in plant interaction with PGPB. ABA modulate plant growth response to ethylene and viceversa (Wilkinson and Davies 2010; Wilkinson et al. 2012), while JA acts regulating plant immune response against pathogens (Browse 2009), and SA is a key factor for basal defenses establishment (Vlot et al. 2009). Phytohormone analysis showed that C7 inoculation modulates root ABA in juvenile *nr* plants predisposing them for further growth or suppressing plant response mediated by SA and/or JA/ethylene (Anderson et al. 2004; Sánchez-Vallet et al. 2012). In contrast, endogenous ABA levels could be essential for growth promotion mediated by Bm maintaining production of ethylene at low levels (Porcel et al. 2014). Moreover, increased levels of SA, JA and JA-Ile under Bm inoculation in *nr* plants regarding wt ones suggested that Bm activate defenses in *nr* plants (Browse 2009; Vlot et al. 2009). All these bacterial effects in juvenile plants predispose plants for further growth since there is balance between plant growth and defense which implies antagonistic crosstalk among phytohormones (Karasov et al. 2017).

Furthermore, PGPB inoculation did not cause changes in ethylene evolution in root and shoot tissues, but locally affects ethylene biosynthesis, signaling and response gene expression pointing to a local production. In wt plants, Bm and C7 reduce expression of ethylene biosynthesis genes in accordance with stress alleviation (Glick 2004;

Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009). However, *nr* plantlets showed higher ethylene production rate than wt ones under Bm inoculation at 26 h post-inoculation and Bm inoculation in *nr* plants increased expression of ethylene biosynthesis, signaling and response genes pointing to a local ethylene production as reported in plant interaction with bacterial pathogens (van Loon et al. 2006), and suggesting that *nr* plants could recognize Bm as a pathogen-like microorganism and thereby non-completely functional beneficial association was established. However, ethylene negatively feedback regulation was observed in Bm-inoculated *nr* plants as previously reported by simultaneously expression of Sl*ETR3* and *TCTR1* genes (Tieman et al. 2000). Thus, Bm inoculation induced a stress response in *nr* plants probably due to a miss-recognition (Zamioudis and Pieterse 2012), but not completely trigger defense mechanisms causing plant growth reduction as noticed in biomass production (Karasov et al. 2017).

Bacillus megaterium PGPB activity

Ethylene perception by SlETR3 is proposed as essential for Bm PGPB activity. In addition, the different approaches utilized to address Bm interaction with tomato plants and clarify its action mechanism also support this hypothesis.

Transcriptomic analysis showed that Bm was able to reshape biosynthesis and degradation pathways of flavonoids that could result in improved antioxidant capacity and favored interaction in wt plants, while ethylene insensitivity impairs interaction with Bm resulting in

non-completely functional establishment of association, but without fully triggering immune response. Additionally, direct transcriptomic comparison between Bm and C7 also points to lower oxidative stress in Bm- than in C7-inoculated wt plants. Root metabolites analysis suggests that Bm inoculation mainly modify sugar metabolism and could enhance photosynthesis suppression by endogenous glucose levels in *nr* plants since ethylene perception is related to plant sensitivity to sugars (Paul and Pellny 2003). Furthermore, reduced levels in *nr* roots under Bm inoculation of fumaric acid, which is necessary for biofilm formation needed for complete colonization by *Bacillus* strains (Zhang et al. 2014; Yuan et al. 2015), suggested a failure in completely functional interaction between Bm and *nr* roots. Additionally, nutrient results also suggest an interaction failure between Bm and *nr* plants that leads to competition for iron at the rhizosphere (Pii et al. 2015).

Proteomic analysis also support that wt plants favor interaction with Bm decreasing arginine biosynthesis, and plants growth better due to Bm-mediated increase in protein synthesis and trafficking in plastids and vacuole. However, Bm could be perceived as pathogenic-like bacteria by *nr* plants increasing the oxidative stress, and affecting mitochondrial metabolism and thus to several growth and developmental processes and responses to environmental stimuli. Although *nr* plants miss-recognize Bm as a beneficial microorganism and caused stress, *nr* plants can modulate immune response in order to avoid plant cell death and further damage growing just as non-inoculated *nr* plants. Moreover, direct proteomic comparison between Bm and C7 also support that Bm could induce disease resistance in wt plants, but Bm is recognized as a pathogenic-like microorganism

inducing pathogenesis-related proteins and increasing plant susceptibility against bacterial pathogens. PGPB perception as mild biotic stress was previously reported in arabidopsis (Timmusk and Wagner 1999) and ethylene was involved in this process since PGPB with ACCd activity was not longer recognized as a stressor agent by plants (Hontzeas et al. 2004). In accordance, the present study points to ethylene sensitivity as regulator of plant interaction with PGPB.

Although phosphorus nutrition has been proposed as new player in tomato-Bm interaction with involvement of ethylene sensitivity, Bm inoculation caused miss-regulation of low phosphorus response in *nr* plants. Ethylene sensitivity by SlETR3 was proposed as essential for low phosphorus response mediated by SlPT1, and thus Bm PGPB activity does not imply phosphorus nutrition improvement. On the other hand, antioxidant metabolite results confirm transcriptomic and proteomic results showing that Bm increased GSH levels enhancing antioxidant capacity in wt plants. This action mechanism was previously reported by *Sphingomonas sp.* LK11 inoculation in tomato (Halo et al. 2015). In addition, exogenous application of GSH improves stress tolerance dependently of ethylene sensitivity (Datta et al. 2015). In consequence, Bm PGPB activity proposed as dependent on ethylene perception by SlETR3 is mediated by increased GSH levels affecting cellular redox status, and thereby antioxidant capacity necessary to improve stress tolerance. However, Bm interaction with *nr* plants was not completely successful and causes oxidative stress since ethylene sensitivity is essential in GSH biosynthesis (Fig. D1).

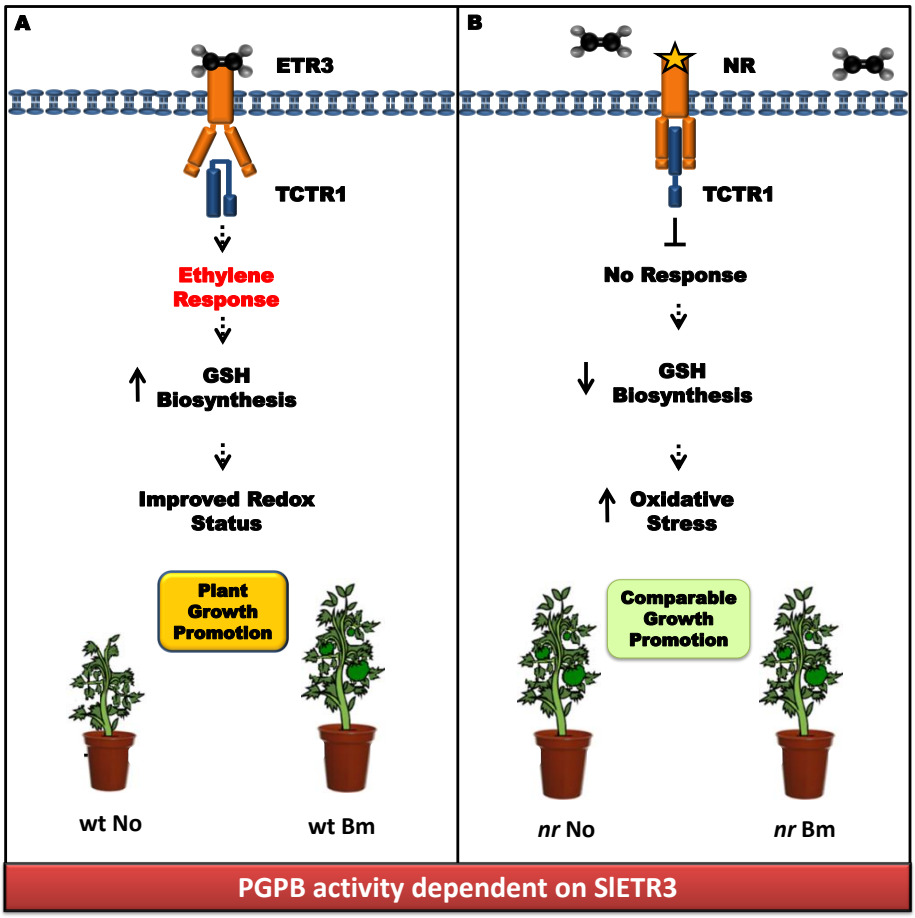

Figure D1 Summary of *Bacillus megaterium* (Bm) plant growth promotion mechanism in wild type (wt) and *never ripe (nr)* tomato (*Solanum lycopersicum*) plants. Molecular events triggered by Bm inoculation in **(A)** wt and **(B)** *nr* plants. Increases or decreases in reduced glutathione (GSH) and oxidative stress caused by Bm inoculation are shown with ↑ and ↓ symbols, respectively. Activation of the following step is represented by dotted arrows, while Inhibition is represented by ⊥ symbol. ETR3: Ethylene receptor 3; NR: Never Ripe receptor; TCTR1: Tomato Constitutive Triple Response 1.

Enterobacter C7 PGPB activity

Although C7 promoted plant growth independently on ethylene sensitivity, the different approaches indicate that SlETR3 mutation partially determines plant interaction with C7. Thus, PGPB mechanism is able to circumvent ethylene insensitivity in case of C7 strain.

Transcriptomic analysis showed that ethylene sensitivity determines plant interaction with C7 affecting different processes. C7 inoculation up-regulated transcripts of respiration in wt roots, but wt plants are able to recognize C7 minimizing defense response. However, C7 inoculation in *nr* plants could improve plant fitness enhancing plant nutrition. Additionally, C7 could modulate amino acidic metabolism and/or enhance the nitrogen use efficiency independently of ethylene sensitivity. Root metabolites results were in agreement with the above statement, since C7 inoculation could improve nitrogen assimilation in *nr* juvenile plants and points to remodeling of amino acidic metabolism by C7 independently of ethylene sensitivity affecting plant growth and development as previously reported by several PGPB (Mantelin and Touraine 2004; Tikhonovich and Provorov 2011; Carvalho et al. 2014).

Furthermore, proteomic analysis pointed to increased oxidative stress under C7 inoculation and plant growth stimulation determined by ethylene insensitivity. C7 presence in wt root caused slight stress, but plant facilitate interaction with C7 by reduction of proteins involved in cell wall resistance. In *nr* plants, C7 inoculation altered chromatin organization and gene expression together with mitochondrial metabolism and protein trafficking resulting also in oxidative stress. However, *nr* plants can counteract stress effects induced by C7.

Furthermore, direct comparison between Bm and C7 also support that C7 cause oxidative stress and affect respiratory chain, but *nr* plants tempers C7 interaction modulating recognition-related proteins. In accordance with proteomic results, antioxidant metabolite analyses showed that C7 inoculation increases oxidative stress in both wt and *nr* plant genotypes together with plant growth promotion pointing that C7 action mechanism is independent of ethylene perception and oxidative stress.

Additionally, proteomic analysis suggested that phosphorus nutrition is involved in C7 PGPB mechanism and also that ethylene perception by SlETR3 determines C7 interaction with tomato plants. Ethylene production is induced by phosphorus deficiency mediating plant response to low phosphorus availability inducing phosphate transporters expression (Borch et al. 1999; Li et al. 2009). C7 inoculation could improve phosphorus nutrition in wt and *nr* plants mediated by SlPT1 and SlPT2, respectively, avoiding low phosphorus stress response and keeping plants on growth (Hermans et al. 2006). Several PGPB can solubilise phosphates (Canbolat et al. 2006; Lai et al. 2008), but C7 did not show this trait and only one study formerly pointed to direct modulation of phosphorus uptake by PGPB under low phosphorus conditions also reducing expression of phosphate transporter genes (Talboys et al. 2014). Thus, C7 is able to promote plant growth independently on ethylene sensitivity improving phosphorus nutrition and circumventing ethylene insensivity by SlETR3 modulating two different phosphate transporters.

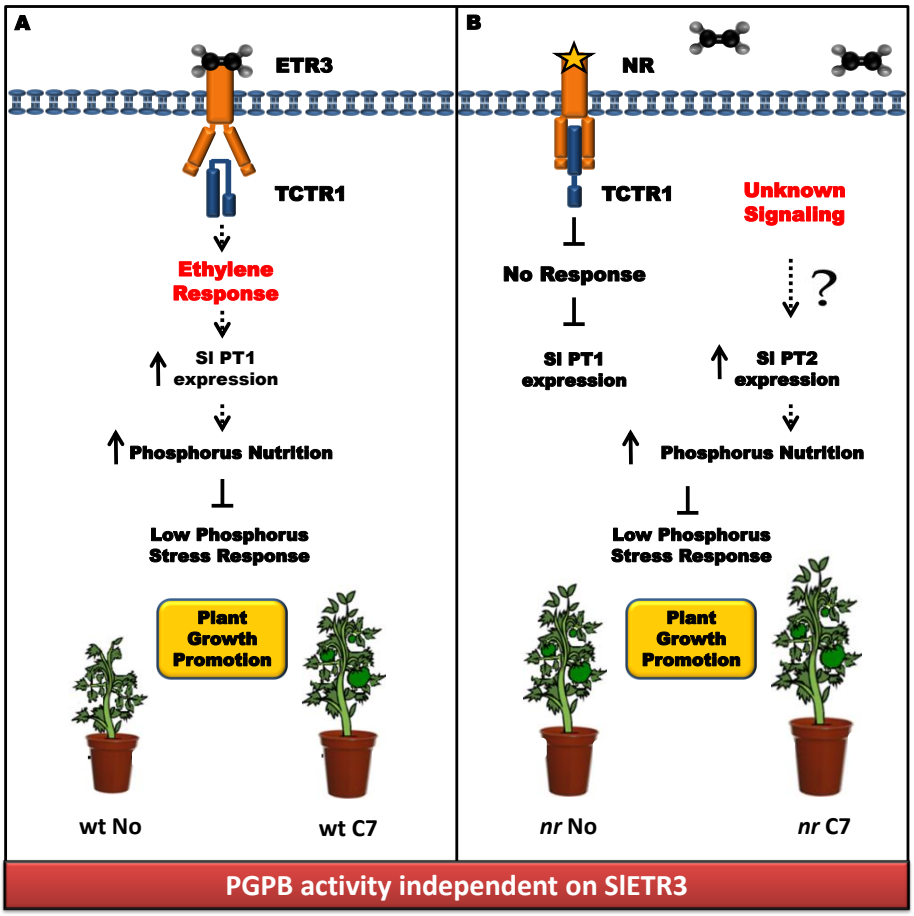

Figure D2 Summary of *Enterobacter* C7 (C7) plant growth promotion mechanism in wild type (wt) and *never ripe (nr)* tomato (*Solanum lycopersicum*) plants. Molecular events triggered by C7 inoculation in **(A)** wt and **(B)** *nr* plants. Increases in phosphate transporters expression caused by C7 inoculation are shown with ↑ symbol. Activation of the following step is represented by dotted arrows, while Inhibition is represented by ⊥ symbol. ETR3: Ethylene receptor 3; NR: Never Ripe receptor; TCTR1: Tomato Constitutive Triple Response 1; Sl PT1: *Solanum lycopersicum* Phosphate transporter 1; Sl PT2: *Solanum lycopersicum* Phosphate transporter 2

Future work

Although PGPB inoculation effects on plant physiology represent valuable information for application in crop systems, further research is needed to completely elucidate Bm and C7 action mechanisms as well as to know if several action mechanisms are simultaneously improving plant growth (Martínez-Viveros et al. 2010).

Drought stress induced the production of ROS causing oxidative stress that damage cellular components (Mittler 2002). Bm action mechanism was proposed as mediated by enhanced levels of GSH, which prevents excessive oxidation (Kataya and Reumann 2010). However, redox status was not studied under drought stress conditions neither in juvenile plants. Moreover, low phosphorus response was mediated by ethylene (Borch et al. 1999; Li et al. 2009), and ethylene is induced by drought stress (Pierik et al. 2007). C7 inoculation implies enhancement of phosphorus nutrition avoiding low phosphorus response and keeping plants on growth, but phosphorus nutrition and phosphate transporter expression was not assayed under drought conditions. In consequence, action mechanisms reported in the present study should be corroborated under drought conditions.

In addition, beneficial association between plants and PGPB requires mutual recognition (Zamioudis and Pieterse 2012), and thereby direct research addressing bacterial features and physiology can help to clarify plant-bacteria interaction as well as PGPB mechanisms. For instance, it should be addressed the PGPB phenotypic variation or phase variation to avoid plant immune system (Davidson and Surette 2008), bacterial effectors which could activate signaling components of

the Sym pathway (Sanchez et al. 2005) or determinate the host-specificity suppressing defensive responses (Mavrodi et al. 2011) as well as PGPB production of siderophores (Hider and Kong 2010). In consequence, PGPB growth and bacterial proteomic analyses under root exudates coming from wt and *nr* plants are being performed. Establishment of beneficial plant-PGPB interaction as well as immune responses against PGPB are dependent on combination between host plants and bacterial strain (Van Wees et al. 1997; Van Loon et al. 1998; Ton et al. 2002), pointing to a gene-for-gene plant-bacteria interaction. In the present study, ethylene sensitivity by SlETR3 was proposed as essential for proper Bm recognition and growth promotion suggesting that SlETR3 could function as "R-like protein". However, no clues about which protein could act as "R protein" for C7 was obtained.

In case of Bm inoculation in *nr* plants, ethylene negatively feedback regulation was observed due to induction of *SlETR3* and *TCTR1* (Tieman et al. 2000), that could be responsible of non-deleterious effects on plant growth. Moreover, *TCRT1* is ethylene-inducible protein which inhibits further ethylene signaling in tomato (Zegzouti et al. 1999; Leclercq et al. 2002; Adams-Phillips et al. 2004), and could participate in initial response after Bm inoculation in wt plants. In consequence, transgenic lines silencing and overexpressing *TCTR1* gene are being obtained in order to assay plant growth and interaction with *Bacillus megaterium*.

PGPB utilization is poorly represented in worldwide agriculture. However, several bacterial strains, including *Bacillus* spp., *Pseudomonas* spp. and *Rhizobium* spp. among others, are currently used in crop systems and commercialized despite of the limited

understanding of interaction between plants and PGPB (Lucy et al. 2004; Banerjee et al. 2006; Timmusk 2017). Total understanding of PGPB interaction with plants as well as action mechanisms would play fundamental roles in agriculture and horticulture improving productivity of crops as well as in forestry and environmental restoration. Furthermore, PGPB can be used in combination with other soil beneficial microorganisms such as other PGPB strains or mycorrhizal fungi displaying different action mechanisms showing synergistic effects on plant fitness and growth. Thus, beneficial microorganisms consortia in combination with the appropiate plant under certain environmental conditions will be able to produce real and positive effects resulting in a feasible option for sustainable intensification of agriculture.

Conclusions

Conclusions

- Ethylene perception by SlETR3 is essential for PGPB activity of *Bacillus megaterium* in tomato plants, whereas *Enterobacter* C7 PGPB mechanism seems to be SlETR3-independent.

- PGPB inoculation effects on plant physiology are strain-specific and dependent on plant ethylene sensitivity as well as on plant growth conditions.

- PGPB inoculation affects photosynthesis, phythormones and root metabolites in juvenile plants predisposing plants for further growth.

- Both bacterial strains acts as PGPB under well watered and drought conditions in ethylene-sensitive tomato plants resulting in improved plant fitness and stress alleviation.

- SlETR3 mutation impairs interaction between *Bacillus megaterium* and tomato *never ripe* plants, resulting in a non-completely functional recognition and leading to increased oxidative stress and loss of PGPB activity.

- PGPB activity of *Bacillus megaterium* in tomato plants could be proposed as mediated by increased GSH and thereby improved plant redox status.

- SlETR3 mutation determines plant interaction with *Enterobacter* C7, whose PGPB mechanism implies phosphorus nutrition enhancement mediated by phosphate transporers SlPT1 and SlPT2 in wt and *nr* plants, respectively, and avoidance of low phosphorus stress response.

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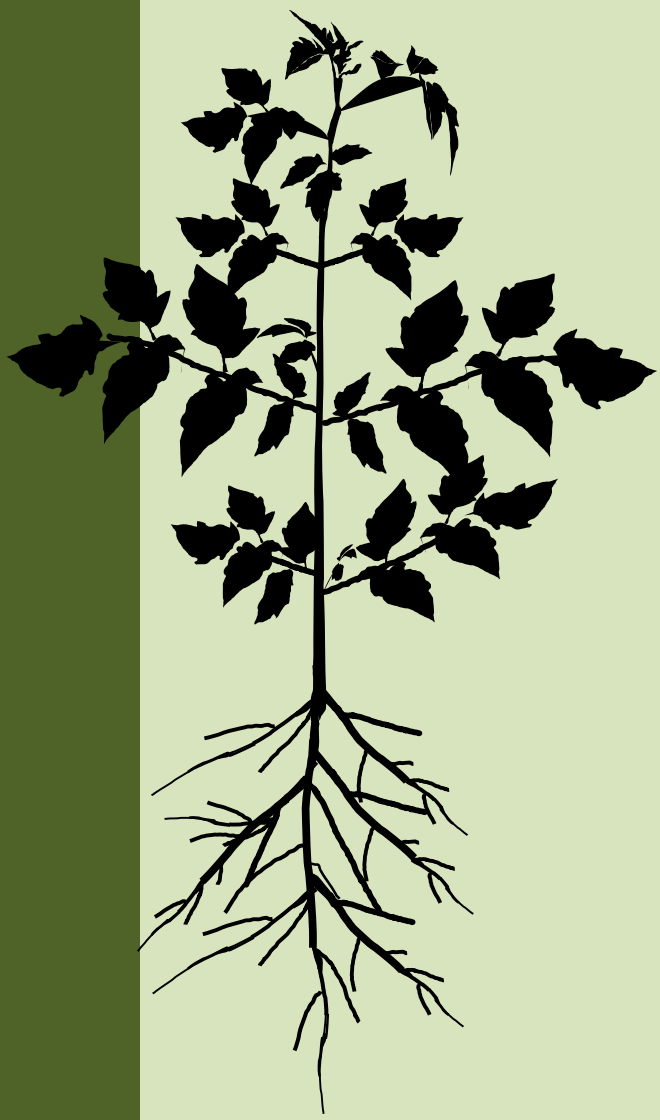

Some are leaves
some are branches
I and I are the roots ...

Robert Nesta Marley

eez
Estación Experimental del Zaidín