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Interaction of citrus root exudates with plant growth promoting rhizobacteria under abiotic stress conditions

Tesis Doctoral

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Interacción de los exudados radiculares de cítricos con bacterias promotoras del crecimiento vegetal en condiciones de estrés abiótico

Memoria presentada por Vicente Vives Peris para optar al grado de doctor por la Universitat Jaume I.

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Abbreviations

AACT: Acetoacetyl CoA thioliase

ABA: Abscisic Acid

ABC: ATP-Binding Cassette

Ap: Ampicillin

ACC: 1-Aminocyclopropane-1-Carboxylate

AMF: Arbuscular Mycorrhizal Fungi

ATH: Arabidopsis Twinkle Homolog

ATP: adenosine triphosphate

ALMT: Aluminium-Activated-Malate Transporters

CC: Carrizo citrange

CFU: Colony Forming Units

Cm: Chloramphenicol

CIN: Cinnamic Acid

CM: *Citrus macrophylla*

CPT: Camptothecin

DAPG: 2,4-diacetylphloroglucinol

DTX: Detoxification

DVS: Dual Vessel System

E: Transpiration

ECM: Ectomycorrhiza

FRDL: Ferric Reductase Defective Like

F_v/F_m: Maximum efficiency of photosystem II

GC-MS: Gas Chromatography coupled to Mass Spectrometry

g_s: stomatal conductance

HLB: Huanglongbing

HPLC: High Performance Liquid Chromatography

IAA: 3-Indole Acetic Acid

JA: Jasmonic Acid

LB: Lysogeny Broth

LC-MS: Liquid Chromatography coupled to Mass Spectrometry

L-Hyp: L-Hydroxyproline

L-Pro: L-Proline

MATE: Multidrug and toxic compound extrusion

MDA: Malondialdehyde

MeJA: Methyl Jasmonate

Me-SA: Methyl Salicylate

MRP: Multidrug Resistance-associated Protein

MS: Murashige and Skoog

Na-Benz: Sodium Benzoate

Na-SA: Sodium Salicylate

NBD: Nucleotide-Binding Domains

NI: Non-Inoculated

NO: Nitric Oxide

ONPG: *Ortho*-Nitrophenyl-β-Galactoside

p-coum: *p*-Coumaric Acid

PDR: Pleiotropic Drug Resistance

PEZ: Phenolics Efflux Zero

PGP: P-glycoprotein

PGPR: Plant Growth Promoting Rhizobacteria

Phe: L-Phenylalanine

PTFE: Polytetrafluoroethylene

Φ_{PSII}: Quantum efficiency of PSII photochemistry

QUAC: Quick Anion Channels

Rif: Rifampicin

RITA: Recipient à Immersion Temporaire Automatique

R-type: Rapid-type

SA: Salicylic Acid

S-type: Slow-type

SLAC: Slow Anion Channels

Tc: Tetracycline

t-cin: *t*-Cinnamic Acid

TIS: Temporary Immersion System

TMD: Transmembrane Domains

UV: Ultraviolet

VAM: Vesicular Arbuscular Mycorrhiza

Summary

In nature, plants are constantly releasing a mixture of metabolites through the roots known as root exudates. Its composition can be affected by a wide variety of stimuli, including chemical, physical and biological factors. Among these factors there are different abiotic stresses, such as salinity or high temperatures, which can negatively affect the growth and production of different crops, among which are citrus fruits. In addition, root exudates modulate the different interactions that occur between plants and other organisms in the rhizosphere, including other plants of the same or other species, fungi, bacteria, nematodes or insects. In this context, the present doctoral thesis has studied how saline stress and high temperatures affect the composition of root exudates of different citrus rootstocks, as well as their role in the rhizosphere.

In Chapter 1, it was compared how saline stress and high temperatures affect root exudation in two citrus rootstocks with different tolerance to these stresses. To this end, the rootstocks citrange Carrizo (which is sensitive to salinity and tolerant to high temperatures) and *Citrus macrophylla* (which is tolerant to salinity and sensitive to high temperatures) were used. The results obtained in this chapter show that plants subjected to stress conditions release to the rhizosphere different concentrations of certain metabolites, such as proline or phytohormones, abscisic acid, salicylic acid or indoleacetic acid. In addition, the concentration of these metabolites was also affected depending on the rootstock. Consequently, in Chapter 2 the effect of root exudates on microorganisms of the rhizosphere has been studied, showing that the citrus root exudates favour the growth of the rhizobacteria *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a. Also, when the root exudates come from plants subjected to salt stress or high temperatures, this positive effect on the growth of both bacteria is increased, which is correlated with the higher levels of certain metabolites, such as proline or salicylates, observed in these plants, as shown in Chapter 1. Additionally, the determinations of the expression of the P_{putA} and P_{pahA} promoters of *P. putida* KT2442 and *Novosphingobium* sp. HR1a respectively, have revealed that both bacteria are able to detect the proline and salicylates present in the root exudates respectively, which could be used as carbon and nitrogen source. Finally, once observed the positive effect that plants have, through their root exudates, on the growth of beneficial bacteria, in Chapter 3 the effect of these bacteria on *Citrus macrophylla* plants under saline stress conditions

has been studied. The results showed a palliative effect of both bacteria on the adverse effects that salt stress produces in physiological parameters such as chlorophyll fluorescence, transpiration and stomatal conductance, as well as in the accumulation of chlorides, proline and phytohormones.

In conclusion, this work has deepened the study of how citrus plants are able to modulate the composition of root exudates to promote the development of bacteria present in the rhizosphere and promote their development, favouring their growth to a greater extent when plants are subjected to stress, in order to improve their tolerance to these adverse conditions.

Resumen

En la naturaleza las plantas están constantemente liberando una mezcla de metabolitos a través de las raíces conocida como exudados radiculares. Su composición puede verse afectada por una gran variedad de estímulos, incluyendo factores químicos, físicos y biológicos. Entre estos factores se encuentran diferentes estreses abióticos, tales como la salinidad o las elevadas temperaturas, que pueden afectar negativamente al crecimiento y producción de diferentes cultivos, entre los que se encuentran los cítricos. Además, los exudados radiculares modulan las diferentes interacciones que se producen entre las plantas y otros organismos en la rizosfera, incluyendo otras plantas de la misma u otras especies, hongos, bacterias, nematodos o insectos. En este contexto, la presente tesis doctoral ha estudiado cómo afectan el estrés salino y por altas temperaturas a la composición de los exudados radiculares de diferentes portainjertos de cítricos, así como su papel en la rizosfera.

En el Capítulo 1, se comparó cómo afectan el estrés salino y el causado por elevadas temperaturas a la exudación radicular en dos portainjertos de cítricos con diferente tolerancia a estos estreses. Para ello, se emplearon los portainjertos citrange Carrizo (que es sensible a la salinidad y tolerante a elevadas temperaturas) y *Citrus macrophylla* (que es tolerante a la salinidad y sensible a elevadas temperaturas). Los resultados obtenidos en este capítulo demuestran que las plantas sometidas a condiciones de estrés liberan a la rizosfera diferentes concentraciones de ciertos metabolitos, tales como prolina o las fitohormonas ácido abscísico, ácido salicílico o ácido indolacético. Además, la concentración de estos metabolitos también se vio afectada en función del portainjertos. En consecuencia, en el Capítulo 2 se ha estudiado el efecto de los exudados radiculares sobre microorganismos de la rizosfera, viéndose que los exudados radiculares de cítricos favorecen el crecimiento de las rizobacterias *Pseudomonas putida* KT2440 y *Novosphingobium* sp. HR1a. Asimismo, cuando los exudados radiculares proceden de plantas sometidas a estrés salino o por elevadas temperaturas, este efecto positivo sobre el crecimiento de ambas bacterias se ve incrementado, lo que se correlaciona con los mayores niveles de ciertos metabolitos, tales como prolina o salicilatos, observados en estas plantas, tal como pone de manifiesto en el Capítulo 1. Adicionalmente, las determinaciones de la expresión de los promotores P_{putA} y P_{pahA} de *P. putida* KT2442 y *Novosphingobium* sp. HR1a respectivamente, han revelado que

ambas bacterias son capaces de detectar la prolina y los salicilatos presentes en los exudados radiculares respectivamente, los cuales podrían emplear como fuente de carbono y nitrógeno. Finalmente, una vez observado el efecto positivo que tienen las plantas, a través de sus exudados radiculares, sobre el crecimiento de bacterias beneficiosas, en el Capítulo 3 se ha estudiado el efecto que tienen estas bacterias sobre plantas de *Citrus macrophylla* en condiciones de estrés salino. Los resultados mostraron un efecto paliativo de ambas bacterias sobre los efectos adversos que produce el estrés salino en parámetros fisiológicos como la fluorescencia de clorofilas, transpiración y conductancia estomática, así como en la acumulación de cloruros, prolina y fitohormonas.

En conclusión, este trabajo ha profundizado en el estudio de cómo las plantas de cítricos son capaces de modular la composición de los exudados radiculares para promover el desarrollo de bacterias presentes en la rizosfera y que promueven su desarrollo, favoreciendo en mayor medida su crecimiento cuando las plantas están sometidas a estrés, para así poder mejorar su tolerancia a estas condiciones adversas.

Resum

En la naturalesa les plantes estan constantment alliberant una barreja de metabòlits a través de les arrels coneguda com exsudats radiculars. La seva composició es pot veure afectada per una gran varietat d'estímuls, incloent factors químics, físics i biològics. Entre aquests factors es troben diferents estressos abiòtics, com ara la salinitat o les elevades temperatures, que poden afectar negativament el creixement i producció de diferents cultius, entre els quals es troben els cítrics. A més, els exsudats radiculars modulen les diferents interaccions que es produeixen entre les plantes i altres organismes en la rizosfera, incloent altres plantes de la mateixa o altres espècies, fongs, bacteris, nematodes o insectes. En aquest context, la present tesi doctoral ha estudiat com afecten l'estrès salí i per altes temperatures a la composició dels exsudats radiculars de diferents portaempelts de cítrics, així com el seu paper en la rizosfera.

En el Capítol 1, es va comparar com afecten l'estrès salí i el causat per elevades temperatures a l'exsudació radicular en dos portaempelts de cítrics amb diferent tolerància a aquests estressos. Per a això, es van emprar els portaempelts citrange Carrizo (que és sensible a la salinitat i tolerant a elevades temperatures) i *Citrus macrophylla* (que és tolerant a la salinitat i sensible a elevades temperatures). Els resultats obtinguts en aquest capítol demostren que les plantes sotmeses a condicions d'estrès alliberen la rizosfera diferents concentracions de certs metabòlits, com ara prolina o les fitohormones àcid abscísic, àcid salicílic o àcid indolacètic. A més, la concentració d'aquests metabòlits també es va veure afectada en funció del portaempelts. En conseqüència, en el Capítol 2 s'ha estudiat l'efecte dels exsudats radiculars sobre microorganismes de la rizosfera, veient que els exsudats radiculars de cítrics afavoreixen el creixement dels rizobacteris *Pseudomonas putida* KT2440 i *Novosphingobium* sp. HR1a. Així mateix, quan els exsudats radiculars procedeixen de plantes sotmeses a estrès salí o per elevades temperatures, aquest efecte positiu sobre el creixement d'ambdues bacteris es veu incrementat, el que es correlaciona amb els nivells més alts de certs metabolits, com ara prolina o salicilats, observats en aquestes plantes, tal com posa de manifest en el Capítol 1. a més, les determinacions de l'expressió dels promotors P_{putA} i P_{pahA} de *P. putida* KT2442 i *Novosphingobium* sp. HR1a respectivament, han revelat que els dos bacteris són capaços de detectar la prolina i els salicilats presents en els exsudats radiculars respectivament, els quals podrien emprar com a font de carboni i nitrogen. Finalment, un

cop observat l'efecte positiu que tenen les plantes, a través dels seus exsudats radiculars, sobre el creixement de bacteris beneficiosos, en el Capítol 3 s'ha estudiat l'efecte que tenen aquests bacteris sobre plantes de *Citrus macrophylla* en condicions d'estrès salí. Els resultats van mostrar un efecte pal·liatiu dels dos bacteris sobre els efectes adversos que produeix l'estrès salí en paràmetres fisiològics com la fluorescència de clorofil·les, transpiració i conductància estomàtica, així com en l'acumulació de clorurs, prolina i fitohormones.

En conclusió, aquest treball ha aprofundit en l'estudi de com les plantes de cítrics són capaços de modular la composició dels exsudats radiculars per promoure el desenvolupament de bacteris presents a la rizosfera i que promouen el seu desenvolupament, afavorint en major mesura el seu creixement quan les plantes estan sotmeses a estrès, per així poder millorar la seva tolerància a aquestes condicions adverses.

Introduction

Root exudation process, what do we know?

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Abstract

Metabolites secreted to the rhizosphere by roots are involved in several processes. By the modulation of root exudates composition, plants can modify soil properties in order to get adapted and ensuring their survival under adverse conditions by through several strategies, as i) changing soil pH in order to solubilize nutrients into assimilable forms, ii) chelating toxic compounds, iii) attracting beneficial microbiota or iv) releasing toxic substances for pathogens, etc. In this work, the composition of root exudates has been reviewed, and the different mechanisms of root exudation have been described. Moreover, this work has also reviewed the different existing methodologies for root exudates obtaining, indicating their advantages and disadvantages. Several factors affecting root exudation have been exposed, including physical, chemical and biological agents which can produce qualitative and quantitative changes in exudates composition. Finally, since root exudates play an important role in the recruitment of mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR), the interaction mechanisms between plants and the beneficial microbiota has been highlighted.

Keywords: mycorrhiza, rhizosphere, rhizobacteria, root exudates

Abbreviations: AACT: Acetoacetyl CoA thioliase; ABC: ATP-Binding Cassette; ACC: 1-Aminocyclopropane-1-Carboxylate; AMF: Arbuscular Mycorrhizal Fungi; ATH: Arabidopsis Twinkle Homolog; ATP: adenosine triphosphate; ALMT: Aluminium-Activated-Malate Transporters; CPT: Camptothecin; DTX: Detoxification; DVS: Dual Vessel System; ECM: Ectomycorrhiza; FRDL: Ferric Reductase Defective Like; GC-MS: Gas Chromatography coupled to Mass Spectrometry; JA: Jasmonic Acid; LC-MS: Liquid Chromatography coupled to Mass Spectrometry; MATE: Multidrug and toxic compound extrusion; MRP: Multidrug Resistance-associated Protein; NBD: Nucleotide-Binding Domains; NO: Nitric Oxide; PDR: Pleiotropic Drug Resistance; PEZ: Phenolics Efflux Zero; PGP: P-glycoprotein; PGPR: Plant Growth Promoting Rhizobacteria; QUAC: Quick Anion Channels; RITA: Recipient à Immersion Temporaire Automatique; R-type: Rapid-type; SA: Salicylic Acid; S-type: Slow-type; SLAC: Slow Anion Channels; TIS: Temporary Immersion System; TMD: Transmembrane Domains; VAM: Vesicular Arbuscular Mycorrhiza

Root exudates composition

In nature, plants are permanently releasing different compounds to their surrounding media. This secretion process is known as exudation, and it can be carried out by different organs, including leaves, shoots or roots, which can secrete substance in solid, liquid or gaseous forms to their surrounding environment. This work is focused in root exudates, which are involved in numerous interactions within the rhizosphere, contributing to the circulation of carbon and nitrogen, which can be secreted from roots and taken up from the soil (Jones et al., 2009).

Table I.1 Compounds secreted by plant roots. Modified from Dakora and Phillips (2002) and Dennis et al. (2010)

Amino acids	α-Alanine, β-alanine, γ-aminobutyric, α-aminoadipic, arginine, asparagine, aspartic, citrulline, cystathionine, cysteine, cystine, deoxymugineic, 3-epihydroxymugineic, glutamine, glutamic, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, mugineic, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine
Sugars	Arabinose, fructose, galactose, glucose, maltose, mannose, mucilages of various compositions, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose, deoxyribose
Organic acids	Acetic, aconitic, ascorbic, aldonic, benzoic, butyric, caffeic, citric, p-coumaric, erythronic, ferulic, formic, fumaric, glutaric, glycolic, lactic, glyoxilic, malic, malonic, oxalacetic, oxalic, p-hydroxybenzoic, piscidic, propionic, pyruvic, succinic, syringic, tartaric, tetronic, valeric, vanillic
Fatty acids	Linoleic, linolenic, oleic, palmitic, stearic
Sterols	Campesterol, cholesterol, sitosterol, stigmasterol
Growth factors and vitamins	*p*-Amino benzoic acid, biotin, choline, inositol, N-methyl nicotinic acid, niacin, pathothenic, pantothenate, pyridoxine riboflavin, strigolactones, thiamine
Enzymes	Amylase, invertase, peroxidase, phenolase, acid/alkaline phosphatase, polygalacturonase, protease
Flavonoids	Chalcone, coumarine, flavones, flavonols, flavanones, flavonones, isoflavones
Nucleotides/purines	Adenine, guanine, uridine/cytidine
Others	Al-induced polypeptides, alcohols, alkyl sulphides, auxins, camalexin, dihydroquinone, ethanol, glucosides, glucosinolates, glycinebetaine, hydrocyanic acid, inorganic ions and gaseous molecules (e.g. CO_2, H_2, H^+, OH^-, HCO_3), isothiocyanates, unidentified ninhydrin positive compounds, unidentifiable soluble proteins, reducing compounds, scopoletin, sorgoleone, strigolactones

By this, it is estimated that among 5 and 25% of the carbon fixed by the photosynthesis is exuded to the rhizosphere through roots, although this percentage can vary depending on plant species, age or plant nutritional status (Bais et al., 2006; Jones et al., 2009).

Root exudates are a mix of a wide variety of compounds, including primary and secondary metabolites (Tab I.1). Primary metabolites including carbohydrates, amino acids or organic acids, are secreted in larger quantities than secondary metabolites, as flavonoids, glucosinolates, auxins… (Badri and Vivanco 2009). Several works have identified and quantified these metabolites in different plant species, including arabidopsis, soybean, rice or common bean (Strehmel et al., 2014; Suzuki et al., 2009; Tawaraya et al., 2014a; Tawaraya et al., 2014b). However, most of the works focused in root exudates metabolite identification are focused in herbaceous plants and shrubs, whereas similar studies with trees are limited to a few species as apple (*Malus pumila*), peach (*Prunus persica*) or jujube (*Ziziphus jujube*), although in these cases root exudate analyses did not identify as many metabolites as in herbaceous plants (Zhang et al., 2007).

In these cases, root exudates analyses for the identification and quantification of secreted metabolites have been generally accomplished with chromatographic tools, including GC-MS and LC-MS for analyses of primary and secondary metabolites. However, although the techniques for root exudates analysis is generally common in the majority of these studies, the methodology for the obtaining of root exudates differs substantially among the different studies, due to the complexity and inaccessibility of the root system (Oburger et al., 2014).

Mechanisms and genes involved in root exudation

Plants have developed several mechanisms to secrete these metabolites to the rhizosphere, including different types of passive and active transport. Traditionally, the secretion of root exudates has been considered a passive process, and includes the transport through the root membrane by diffusion, ionic channels and vesicles transport (Baetz and Martinoia 2014). Each secretion process is responsible of the exudation of some compounds depending on their chemical properties (Fig. I.1).

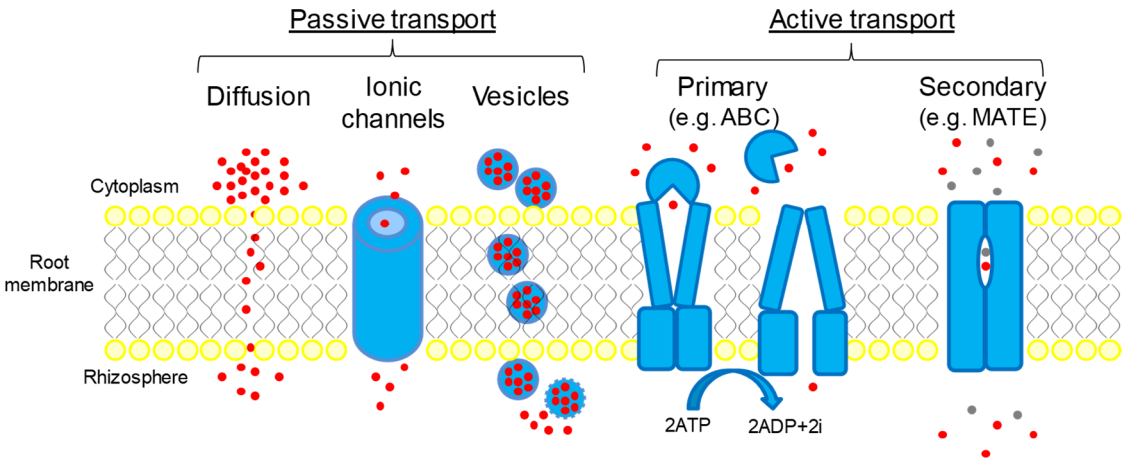

Figure I.1 Representation of the different mechanisms of root exudation. Red circles represent molecules released to the rhizosphere

Thereby, diffusion is responsible of the exudation of compounds of low molecular weight, including sugars, amino acids, carboxylic acids and phenolics. This process is due to the gradient created by the different concentrations between the cytoplasm of root membrane cells and the rhizosphere, and it can be affected by root membrane permeability, the state of root cells and the polarity of the compounds (Badri and Vivanco 2009; Bertin et al., 2003).

Ionic channels are responsible of the secretion of carbohydrates and specific carboxylates as malate or oxalate, which are also exuded in high concentrations and not always can be secreted by diffusion, being this transport mediated by proteins. In this case two different anionic channels have been described: SLow Anion Channels (SLACs), originally named S-type (Slow-type), which need several seconds to be activated; and QUick Anion Channels (QUACs), originally named R-type (Rapid-type), which can be activated in the low millisecond range (Dreyer et al., 2012). In this group, Aluminium-Activated-Malate Transporters (ALMT), has been widely studied. This group consists in several proteins involved in several physiological processes, being the exudation of organic acids, mainly malate, in presence of toxic Al^{+3} ions in the soil, the most studied. Since the secreted organic acids chelate and inactivate Al^{+3} toxic ions, these anion channels are responsible of conferring aluminium tolerance to the plants, being activated in aluminium stress conditions (Sharma et al., 2016). The ALMT family of membrane transporters has been evolutionary classified in five different clades, and consists in 13 members in *Arabidopsis thaliana*, 12 in *Vitis vinifera* (grape vine), and 8 in *Oryza sativa* (rice) (Sharma et al.,

2016). Although ALMT transporters family is highly activated in presence of aluminium and the major contributor to aluminium tolerance, is not the only protein family involved in plant responses against this stress, being MATE family of active membrane transporters independently activated, and responsible of citrate exudation in this situation (Liu et al., 2009a). Moreover, the malate secretion caused by the overexpression of *AtALMT1* in presence of aluminium, also mediates in the recruitment of beneficial rhizobacteria that induce plant immunity (Kobayashi et al., 2013).

The last group of passive transport is the vesicle transport, which is used to secrete metabolites with high molecular weight stored in vesicles (Badri and Vivanco 2009; Bertin et al., 2003). This process is also known as exocytosis, and the exuded metabolites proceed from the endoplasmic reticulum or Golgi, and contributes to the secretion of compounds involved in the protection against pathogens (Weston et al., 2012).

On the other hand, root secretion of metabolites through an active transport mechanism is mediated by proteins located in the root plasmatic membrane (Baetz and Martinoia 2014). In this context, there are two big families of membrane transporters, including ABC ("ATP-Binding Cassette") and MATE ("Multidrug and toxic compound extrusion") (Kang et al., 2011; Yazaki et al., 2008). Root exudation mediated by proteins can derive in three different situations depending on their specificity: one transporter that can secrete different metabolites; one metabolite which can be released to the rhizosphere through different membrane transporters; or specific compounds which can be exuded by only one transporter. ABC transporters family is extended in a huge variety of living organisms, including mammals, and are considered primary transporters since they utilize the energy from ATP (adenosine triphosphate) hydrolysis to translocate a wide variety of solutes (Jones and George 2002). This family of transporters is one of the most extended in living organisms, and includes 130 members in *Arabidopsis thaliana*, and have been classified in different families (Kang et al., 2011). The nomenclature of the genes that regulate these transporters have been changed over the time. However, a new unified classification has been developed depending on the organisation of the domains TMD and NBD, grouping the different members in 9 families named with letters from A to I, although family H is not present in plants (Verrier et al., 2008). Some reports have analysed the role of ABC transporters in root exudation and have determined their importance in this process. In *A. thaliana*, works analysing root exudates obtained from ABC transporters knockout mutants *Atpdr6*, *Atpdr2*, *Atmrp2*, *Atath6* and *Atpgp4-1* have

revealed that these transporters are involved in root exudation, since there were differences in root exudates composition in these mutants related to those obtained from control plants (Badri et al., 2008). Moreover, analysis of the rhizosphere microbiota of *Atabcg30* mutant have revealed that this transporter is also capable to modify soil microbiota. In this mediation, root exudates play an important role, since their analysis in this mutant have revealed that it exudes more phenolic compounds and less sugars (Badri et al., 2009).

In relation to MATE family in plants, these transporters were originally identified in *A. thaliana*, with 56 members named with the initials DTX (from detoxification) (Li et al., 2002), but over the time there have been identified a total amount of 58 members, which are reported as secondary active transporters that use an electrochemical gradient of other ions, such as sodium or protons, to allow the movement of different compounds across membranes (Weston et al., 2012). The importance of MATE transporters in root exudation has been tested in different crops, as occurs with some genes in sorghum (*SbMATE1*), rice (*OsFRDL4*) barley (*HvAACT1*), and arabidopsis (*AtMATE1*) (Magalhaes et al., 2007; Liu et al., 2009b; Yokosho et al., 2011; Zhou et al., 2013) which regulate citrate exudation in response to aluminium stress, chelating the Al^{+3} toxic ions (Delhaize et al., 2007). Moreover, other works in rice suggest that *OsPEZ1* and *OsPEZ2* are involved in the transport of phenolic compounds, which can be involved in the root exudation of these metabolites (Ishimaru et al., 2011; Takanashi et al., 2014).

Root exudates collection

There exist several methodologies to obtain root exudates from plants, although their composition can be different quantitatively and qualitatively depending on the technique used for their collection. By this, root exudates obtained from plants cultured under field conditions are more representative of those found in the nature, but due to the difficulty to obtain them in these conditions and their low reproducibility, other methodologies as hydroponic or *in vitro* cultures are more extended. In these situations, other parameters in the cultures, including the aeration in hydroponic cultures, or the *in vitro* culture in temporary immersion systems (TIS) not only affect plant development but also the composition of root exudates, affecting the representation and reproducibility of these methodologies (Vranova et al., 2013) (Fig. I.2). It has been demonstrated that *in vitro*

culture plants respond to different environmental conditions following the same trend than under field conditions, as an example citrus plants subjected to different stresses as high salinity or heat stress exhibit similar contents of phytohormones, proline or malondialdehyde (López-Climent et al., 2008; Montoliu et al., 2009; Vives-Peris et al., 2017; Zandalinas et al., 2016), and some authors recommend this system for exudates collection in order to avoid the presence of microorganisms present in the rhizosphere which can affect root exudates composition (Kujiken et al., 2015; Vranova et a. 2013).

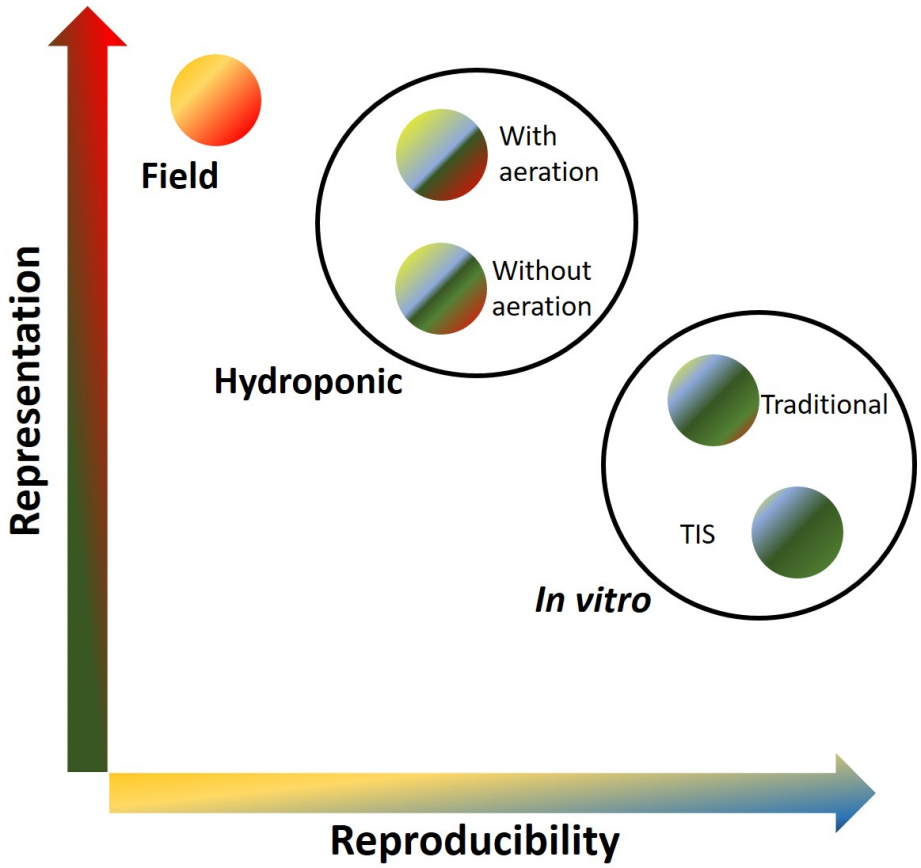

Figure I.2 Classification of the methodologies of root exudates collection according to their representation and reproducibility

Although most of the studies use root exudates obtained from hydroponic or *in vitro* cultures, where the liquid medium, with or without aeration, is collected, other studies use soil, being the root exudates obtained by different physicochemical processes, including extraction processes with different chemicals or solid phase extraction (Dundek et al., 2011), or through sorption filters buried in the ground (Neumann et al., 2014). It has been also reported the collection of exudates obtained from the culture of excised

roots instead of whole plants (Marin et al., 2010). In some cases exudation takes place in hydroponic cultures with liquid media supplemented with nutrients as Murashige and Skoog salts (Murashige and Skoog 1962) or Hoagland solution (Hoagland and Arnon 1938) which can affect root exudates analysis (Kitazawa et al., 2005). On the contrary, other studies let the plants exudate directly in distilled water in order to avoid interferences in the chromatographic analysis from the exogenously supplement salts and sucrose (Badri et al., 2013; Barbas et al., 1999).

Notwithstanding the foregoing, there are some works which compile all these techniques and commend *in vitro* systems to study root exudates, due to the facility to control environmental parameters and because that culture system offers higher reproducibility of the experiment conditions (Vranova et al., 2013). *In vitro* techniques allow the culture of entire plants or excised organs as roots or shoots. This fact can affect root exudates composition, since several studies have reported that the presence of shoots in the culture affect root exudation as they are responsible of the CO_2 assimilation by photosynthesis, and changes in the aerial tissues, as changing the scion of grafted plants affects root exudation processes in watermelon (Ling et al., 2013) and in the combination of tomato and eggplant (Liu et al., 2009a).

In addition to traditional stationary methodologies of culture for root exudates collection from *in vitro* cultured plant tissues, in the last years several automated TIS have been developed, including more than ten different systems, some of which allow the forced ventilation and CO_2 enrichment and even allow to culture hairy roots and cell suspensions. These systems generally consist in two different vessels (or one vessel with two differentiated compartments), one containing the plants and other the liquid media, which is periodically transferred to the plant material (Georgiev et al., 2014). Among all these systems RITA® (*Recipient à Immersion Temporaire Automatique*) is one of the most popular, which is one of the first temporary immersion systems (Alvard et al., 1993; Mordocco et al., 2009). Although the original purpose of RITA TIS was plant mass propagation, it has been also proved to be a good TIS for obtaining metabolites from root cultures since it reduces hyperhydricity and has lower consumable and work costs (McAlister et al., 2005). The main advantage of TIS is that generally increase biomass and metabolite production, hence their use for obtaining specific metabolites from root exudates is being more spreaded (Paek et al., 2005; Wilken et al., 2005). Moreover, some of these systems allow the filtration of the liquid medium for root exudates obtaining (Ziv

2005). However, since root exudation are produced in higher quantities in TIS, and are usually obtained from hairy roots or cell suspension cultures, the composition of the root exudates obtained from these systems can be quantitatively and/or qualitatively different than the root exudates from plants grown in field conditions. For this reason, TIS systems generally used for the obtaining of specific metabolites with commercial interest in larger quantities (Georgiev et al., 2007). Some examples of metabolites obtained from root cultures in TIS are betalains from *Beta vulgaris* (beet), which have antioxidant and colorant properties (Pavlov and Bley 2006), and other compounds with pharmacological interest, as 3,4-Dihydroxy-L-phenylalanine, obtained from root cultures of *Stizolobium hassjoo*, with therapeutic properties against Parkinson's disease (Sung and Huang 2005), or the anticancer 6-Methoxy-podophyllotoxin, obtained from transformed root cultures of *Linum album* and *Linum persicum* (Wink et al., 2005).

Factors affecting root exudates composition

Root exudation pattern can be affected quantitatively and qualitatively depending on several conditions, including physical, chemical and biological factors (Fig. I.3).

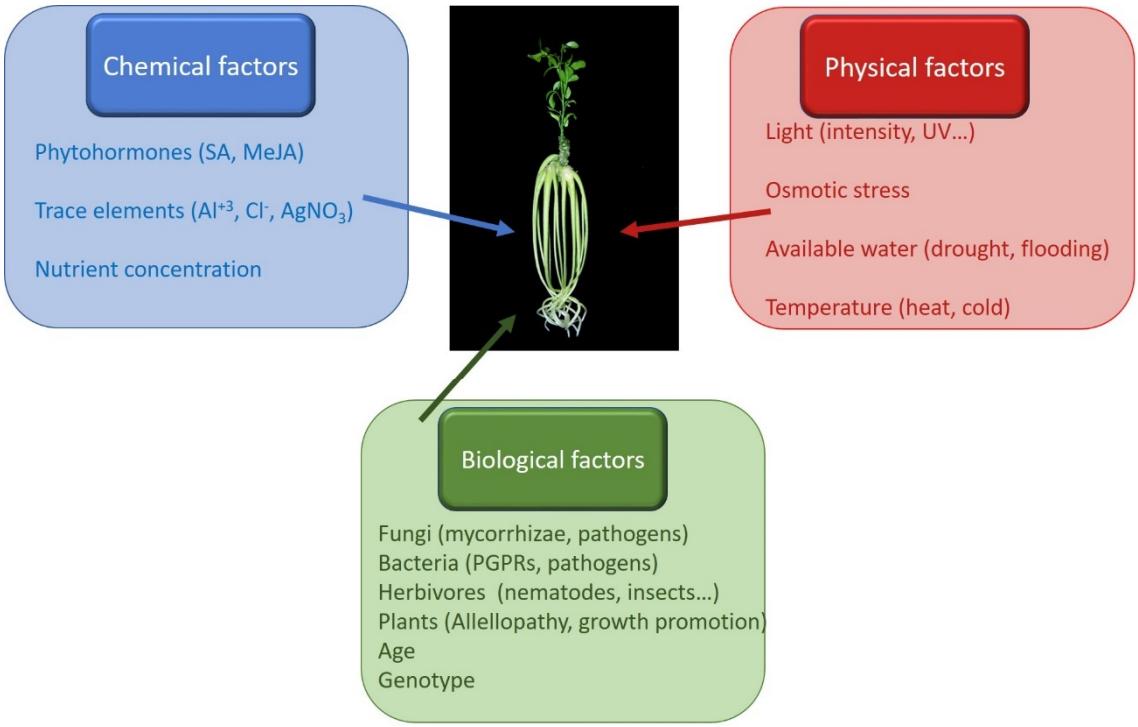

Figure I.3 Classification of the different factors affecting root exudation processes

Different factors, intrinsical from the plant, can also affect root exudation, including the genotype and the age of the plant. Thereby, genotype can produce several changes in root exudation of some compounds, as occurs with Johnsongrass and Shattercane sorghum accessions, whose exudates production is approximately 10 up-fold and 50% lower in comparison with the data obtained from other sorghum cultivars respectively (Czarnota et al., 2003). Moreover, genetic changes influence root exudates production and can strongly modulate changes in the microbial community growing in the rhizosphere in several crops, including *A. thaliana* (Micallef et al., 2009), *Solanum tuberosum* (potato) (Inceoğlu et al., 2010) and *Zea mays* (maize) plants (Aira et al., 2010).

The age of the plant also affects the metabolite secretion to the rhizosphere. It has been reported that the exudation pattern in *A. thaliana* plants vary depending on the growth stage, being sugars the most exuded compounds in the early stages of development, whereas in older plants of 28-31 days, amino acids and phenolics are secreted in higher quantities. Consequently, this fact affects to the establishment of microbial communities in the rhizosphere (Chaparro et al., 2013). Similarly, in potato plants, age also affects betaproteobacterial communities in the rhizosphere, increasing their population in the later stages of plant development (Inceoğlu et al., 2010).

Most of the chemical and physical factors which can affect root exudation can be considered abiotic stress, such as drought, high salinity, flooding, extreme temperatures or nutrient starvation. One of the most studied chemical factors which can produce changes in root exudates is nutrient availability, including not only soil nutrients but also atmospheric CO_2. In this case, plant can modify the compounds that are released to the rhizosphere depending on the quantity of nutrients present in the root environment, being affected by the lack or excess of nutrients. P deficiency, that is one of the most studied lacks affecting root exudation, induces an increase in the secretion of some organic acids as citramalic acid and salicylic acid (SA) in sugar beet plants, which contributes to solubilize soil phosphorus by the acidification of soil pH (Khorassani et al., 2011). Moreover, studies with maize plants cultured with low quantities of N, P, K and Fe have revealed different modifications over the exudation rates of amino acids, carbohydrates and organic acids depending on the low nutrient fertilization. Low N fertilization reduces amino acid exudation, but increases organic acid presence in root exudates. Whereas the lack of P promotes sugar exudation and K starvation inhibits it, and Fe deficiency

increased the exudation of glutamate, glucose, ribitol and citrate (Carvalhais et al., 2011). CO_2 concentration also affects root exudation since carbon rhizodeposition can vary in the range between 11 and 44% of the quantity fixed by the photosynthesis (Jones et al., 2009; Paterson and Sim 2000). Furthermore, studies with different grass species have concluded that plant biomass affects directly carbon rhizodeposition, being increased when the plant biomass is higher, what is also related with the total fixed CO_2 (Baptist et al., 2015).

Plant water status also induces changes in root exudates composition, affecting differentially depending on whether water is applied in excess or in absence, although both situations generally increase the organic carbon existing in root exudates. In this context, Henry et al. (2007) revealed that in plants of *Agropyron cristatum*, drought increased a 71% the total organic carbon in the rhizosphere, with higher exuded quantities of succinic, fumaric and malic acids, whereas flooding increased a 45% the total organic carbon present in root exudates, with higher secretion of oxalic and malonic acids.

Salt stress, including the osmotic and the ion toxicity components also affects root exudation pattern. Analyses of root exudates obtained from *Glycine max* (soybean) and *Phaesolus vulgaris* (common bean) have identified several flavonoids in root exudates of both species, which concentration generally increases when plants are grown in salt stress conditions (Dardanelli et al., 2010, 2012). It has been also reported that excised roots of almond tree cultured under *in vitro* conditions exuded proline in larger quantities in presence of salt stress, being the exudation pattern different depending on the genotype (Marin et al., 2010).

Changes in the temperature can also alter root exudates composition, although little information exists about how heat and cold can affect the secretion of metabolites to the rhizosphere. By this, Pramanik et al. (2000) revealed that increasing the day/night temperature from 25º/20º C to 30º/25º C in cucumber plants cultured in hydroponic conditions promotes considerably the root exudation of some organic acids as benzoic, 4-hydroxy-benzoic, phtallic and palmitic acids.

Other abiotic stress which can affect root exudation is the presence of toxic ions, including trace elements as heavy metal ions. In *A. thaliana*, the stress caused by Al toxicity induces a higher secretion of malate and citrate, due to the activation of *AtALMT1* and *AtMATE1*,

which are responsible of Al^{+3} ion chelation and the recruitment of beneficial rhizobacteria as *Bacillus subtilis* (Kobayashi et al., 2013; Liu et al., 2009a). However, the activation of ALMT transporters seems to be more effective in Al tolerance in comparison with MATE transporters, since barley plants overexpressing wheat *TaALMT1* gene showed higher tolerance than plants overexpressing *MATE* genes (Gruber et al., 2011; Zhou et al., 2014). In this interplay between ALMT and MATE transporters families, the transcription factor STOP1 has been described as necessary for the activation of both kinds of membrane transporters, which encodes a putative zinc finger protein and it is important to resist low pH conditions in arabidopsis (Iuchi et al., 2007; Liu et al., 2009a). Application of Cd stress has also revealed changes in root exudates from the hyperaccumulator plant *Sedum alfredii*, where analyses of root exudates by GC-MS have revealed differences in the concentrations of 20 compounds, demonstrating that trehalose, erythritol, naphthalene, d-pinitol and n-octacosane might be related to cadmium stabilization, phosphoric acid, tetradecanoic acid, oxalic acid, threonic acid and glycine could be involved in cadmium mobilization (Luo et al., 2014). Moreover, treatment with Pb in this plant species also affects root exudation, with 15 identified compounds which can be used as biomarkers whose concentration increases in presence of Pb, including amino acids as L-alanine and L-proline; organic acids as oxalic and glyceric acids; and some phenolic compounds as 4-methylphenol and 2-methoxyphenol (Luo et al., 2017).

Respect to the relation of light with root exudation, its role in this process is evident since CO_2 fixed in the photosynthesis is finally secreted to the rhizosphere, being this process influenced not only by light wavelength, but also by photoperiod duration. In this context, light type affects the composition of root exudates obtained from plants of *Avena fatua* grown with far-IR-enriched radiation (Pomilio et al., 2000). Moreover, longer photoperiods promotes higher exudation of some organic acids as benzoic acid, 4-hydroxy-benzoic in cucumber plants grown with a photoperiod of 14 hours of light in comparison with a photoperiod of 10 hours (Pramanik et al., 2000). In addition, the exposition of roots of *Alnus glutinosa* for 5 days to 16 hours white light photoperiod also increases root exudates of flavonols as quercetin and kaempferol (Hughes et al., 1999). The exudation of other flavonoids as catechin and catechol by roots of *Centaurea stoebe* (spotted knapweed) plants is also affected by the illumination, being the maximum exudation rate after 6 hours of light (Tharayil et al., 2010). However, although light wavelength and photoperiod clearly affect root exudation, other studies support that the

circadian rhythm does not influence as much as wavelength and photoperiod, since in *A. thaliana*, some ABC genes are not affected by diurnal rhythm, being only the secretion of some specific compounds (7 of 390 identified metabolites) affected by this process (Badri et al., 2010).

Root exudates mediation in the interaction between plants and other organisms

Different biological factors, as the presence of roots of other plants of the same or a different species, some herbivores as insects or nematodes, fungi or bacteria can affect root exudation pattern. By this, the presence of other living organisms can affect plant growth positively or negatively, being these relationships widely modulated by plant root exudates.

On the other hand, root exudates mediate the interaction among plants of the same or different species, affecting positively or negatively depending on root exudates composition and the mechanisms of the relationship. Regarding on the negative interactions, there exist different types of mechanisms to modulate these relationships, including allelopathy, consisting in the releasement of phytotoxins to the rhizosphere in order to be in advantage over other competitors. Most of phytotoxins exuded to the rhizosphere have a similar chemical structure, with aromatic components and hydroxyl or ketone groups, including metabolites as flavonoids, quinones, quinolines and hydroxamic acids (Bais et al., 2006). Allelochemicals can affect negatively other plants by inducing changes in cell structures, inhibiting cell division and elongation, destabilizing the antioxidant system, increasing membrane permeability, affecting plant growth regulators and enzymes and influencing respiration, photosynthesis, metabolism and water and nutrient uptake (Cheng and Cheng 2015). However, with the adaptation of the affected plants to the secreted allelochemicals, the adverse effect of these phytotoxins is evolutionary reduced, being more important in situations with invasive plants, where the invasive and the host plants are not adapted to the phytotoxins released by other plants. Consequently, some of these compounds could be used as herbicides against invasive plants, although their specificity and efficacy are usually limited (Bhadoria 2011). For example, root exudates of *Impatiens glandulifera*, containing the phytochemical 2-

methoxy-1,4-naphthoquinone have been reported as inhibitors of the seed germination of *Hieracium murorum* and *Scrophularia nodosa* (Ruckli et al., 2014), whereas rice plants exude different allelochemicals, including momilactone B, which also has a inhibitory effect in the neighbouring plants (Kato-Noguchi 2004). On the other hand, root exudation of other compounds can also affect positively plant growth, inducing resistance against herbivores, as occurs with *Hordeum vulgare* (barley) plants treated with root exudates from *Elytrigia repens* containing carboline, that repel aphids (Glinwood et al., 2003). Root exudates can also mediate in the attraction of predators for herbivores, as it has been described in the modification of root exudates composition from *Vicia faba* (broad bean) plants infected by the aphid *Acyrthosiphon pisum*, which induce the production of volatile compounds in unaffected plants that attract the parasitoid *Aphidius ervi* (Guerrieri et al., 2002). Plants can also directly affect the growth of other plants, as the root exudation of plant growth regulators as auxinic compounds or strigolactones which can promote plant growth, and have been found in root exudates (Haichar et al., 2014), although the positive role of strigolactones and their interaction with auxins is still not clear (Sun et al., 2016).

Plant root exudates also affect insect, microbial and nematode populations since they exudate some compounds as sugars or amino acids which can be used as sources of organic C and N, or other secondary metabolites that can affect their growth negatively, as phytoalexins or defence proteins. In addition to soil nutrient availability, plants can also vary soil pH through changes in root exudates composition, which affects pathogenic and beneficial bacteria and fungi, as well as nematodes (Lareen et al., 2016). For instance, pyrosequencing analyses of the bacterial communities are very sensitive to changes in phenolic-related compounds present in root exudates from *A. thaliana*, which can stimulate or inhibit the growth of different community members (Badri et al., 2013). Other works also reveal that some legume species are capable to release specific combinations of flavonoids in order to attract symbiont nitrogen-fixing bacteria (Hassan and Mathesius 2012). In relation to fungi, several works have reported the inhibitor effect of root exudates in some fungi species, as occurs with *Citrullus lanatus* (watermelon) plants cultivated in companion of *Triticum aestivum* (wheat) plants, whose root exudates reduces the infection of the pathogenic fungi *Fusarium oxysporum*, exhibiting the watermelon plants less damage in the presence of wheat plants (Xu et al., 2015). On the other hand, some works have also reported the positive effect of root exudates over beneficial fungi, recruiting and promoting the colonisation by mycorrhizal fungi, having

flavonoids and strigolactones an important role in this mutualism (Steinkellner et al., 2007). In consequence, the published data confirms that plants can modify the composition of root exudates to modify soil microbiome composition, recruiting beneficial microbes or inhibiting the growth of pathogenic microbes.

Plant roots can establish mutualistic relations with distinct species of fungi and bacteria by modulating root exudates composition. By this, two separate groups of beneficial microbes have been widely studied: mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR).

Mycorrhizal interactions with plants

In reference to mycorrhizal fungi, these species can be classified in different groups depending on their colonization mechanisms, including endomycorrhiza, also known as arbuscular mycorrhizal fungi (AMF) or vesicular arbuscular mycorrhiza (VAM), and ectomycorrhiza (ECM). Whereas AMFs colonize plants by penetrating until the root cortex zone and extend their hyphae outside the root, ECM live only outside the root surface, surrounding root cortex. However, ECM hyphae can sometimes penetrate plant cells, in that case these ECMs are called ectendomycorrhiza. In addition, the taxonomic classification of these fungi are also different, since most of AMFs belong to Glomeromycota phylum and ECMs belong to Basidiomycota, Ascomycota, and Zygomycota phylum (Brundrett 2004). The colonization by mycorrhizae induces several changes in the host plants, whose growth is positively affected. Among these changes, more than 500 protein-coding genes are differentially regulated, including nutrient transporters for phosphate and ammonium transporters, improving N and P nutrition, and a generalized metabolic change is produced in the host plants, having some phytohormones as ethylene, abscisic acid, SA or jasmonates, an important role in mycorrhiza colonization and plant growth promotion (Bonfante and Genre 2010). Moreover, mycorrhizae can also promote plant growth by adjusting the osmotic potential, enhancing the photosynthesis rate, alleviating the effect of allelochemicals, and increasing the resistance against different biotic and abiotic stresses. In this case, AMFs have been also reported as responsible of root architecture modification, enhancing the enzymatic and non-enzymatic antioxidant systems, or increasing water use efficiency (Nadeem et al., 2014).

There are many examples of mycorrhizal associations with plants, being estimated that in nature, about 80-90 % of plants are colonized by mycorrhizae. In the next table (Tab. I.2) some examples of works that report the beneficial effect alleviating plant abiotic stresses are listed.

Table I.2 Examples of positive effects of mycorrhizae in plants subjected to abiotic stress conditions

Stress	Mycorrhiza	Plant	Reference
Salinity	*Glomus iranicum*	*Lactuca sativa* (lettuce)	Vicente-Sánchez et al., 2014
Salinity	*Rhizophagus irregularis*	*Triticum aestivum* (wheat)	Zhu et al., 2016
Drought	*Rhizophagus intraradices*	*Punica granatum* (pomegranate)	Bompadre et al., 2014
Drought	*Rhizophagus irregularis*	*Zea mays* (maize)	Quiroga et al., 2017
Drought	*Funneliformis mosseae*	*Poncirus trifoliata* (trifoliate orange)	Huang et al., 2017
Flooding	*Rhizophagus irregularis*	*Solanum lycopersicum* (tomato)	Calvo-Polanco et al., 2014
Flooding	*Gigaspora margarita*	*Prunus persica* (peach)	Rutto et al., 2002
Cadmium	*Glomus versiforme* *Rhizophagus intraradices*	*Lonicera japonica*	Jiang et al., 2016
Cold	*Funneliformis mosseae*	*Solanum lycopersicum* (tomato)	Liu et al., 2016
Cold, heat	*Glomus mosseae*	*Cucumis sativus* (cucumber)	Haghighi et al., 2015
Heat	*Glomus fasciculatum*	*Cyclamen persicum* (cyclamen)	Maya and Matsubara 2013

Plant growth promoting rhizobacteria interactions with plants

Apart from mycorrhizae, PGPRs can also bring several benefits for increasing plant growth and enhancing their tolerance to different abiotic and biotic stresses. Most of them belong to the genera *Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Serratia* and *Streptomyces*. These microorganisms live close to plant roots due to the presence of root exudates, needing some of them dependent of the presence of root exudates to survive. Among the mechanisms that benefit plant growth, it is necessary to difference

between direct and indirect mechanisms. Direct mechanisms include fixation of atmospheric N, P solubilization, siderophore production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, which decrease ethylene concentrations in the plant, which induce salt tolerance and reduce drought stress in plants, and phytohormone production as indole acetic and gibberellic acids, which promote root growth and development. On the other hand, indirect mechanisms of PGPRs include the competition for nutrients with other pathogenic microorganisms in the rhizosphere, the production of antibiotics, the production of lytic enzymes and the detoxification and degradation of virulence factors of some pathogens (Parray et al., 2016).

In the next table (Tab. I.3) some works with PGPRs alleviating distinct kinds of abiotic stress are listed as examples.

Table I.3 Examples of positive effects of PGPRs in plants subjected to abiotic stress conditions

Stress	PGPR	Plant	Reference
Salinity	*Staphylococcus kloosii* *Kocuria erythromyxa*	*Fragaria* x *ananassa* (strawberry)	Karlidag et al., 2013
Salinity	*Enterobacter* sp.	*Triticum aestivum* (wheat)	Singh and Jha 2016
Salinity	*Dietzia natronolimnaea*	*Triticum aestivum* (wheat)	Bharti et al., 2016
Salinity, drought	*Burkholdera cepacia* *Promicromonospora* sp. *Acinetobacter calcoaceticus*	*Cucumis sativus* (cucumber)	Kang et al., 2014
Drought	*Bacillus licheniformis*	*Capiscum annuum* (pepper)	Lim and Kim 2013
Drought	*Bacillus* spp.	*Sorghum* sp. (sorghum)	Grover et al., 2014
Osmotic	*Arthrobacter* sp. *Bacillus* sp.	*Capiscum annuum* (pepper)	Sziderics et al., 2007
Flooding	*Enterobacter cloacae* *Pseudomonas putida*	*Lycopersicon esculentum* (tomato)	Grichko and Glick 2001
Heat	*Pseudomonas putida*	*Triticum* sp. (wheat)	Ali et al., 2011
Cold	*Burkholderia phytofirmans*	*Arabidopsis thaliana*	Su et al., 2015

Biotechnological tools for root exudates obtainment

Some metabolites which are exuded through plant roots have commercial interest, and their production in a large scale can contribute to the development of new and cheaper technologies for their obtainment. In this way, *in vitro* TIS are the most used systems due to the purity, the higher concentration of metabolites and easiness for obtaining root

exudates. Most of these cultures in *in vitro* TIS use hairy roots instead of whole plants since this tissue produces higher quantities of root exudates, and use genetically transformed plant material to enhance their production. In this case, the addition of some compounds, known as elicitors, to the growth media can promote metabolite root exudation, inducing the production of the metabolite of interest. In the next table, the production of some metabolites with commercial interest is listed, including the methodology and elicitors used (Tab. I.4).

Table I.4 Examples of metabolites obtained from *in vitro* root exudates. More examples can be found in Georgiev et al. 2007

Plant	Metabolite	Use	Elicitor	Method	Reference
Beta vulgaris (red beet)	Betalains	Pigments, antioxidants	None	RITA, hairy roots	Pavlov and Bley 2006
Artemisia annua (sweet wormwood)	Artemisinin	Antimalarial	*Aspergillus oryzae*	Flasks, hairy roots	Liu et al., 1997
Camptotheca acuminata (happy tree)	Camptothecin (CPT) and 10-hydroxyCPT	Anticancer	None	RITA, DVS	Sankar-Thomas and Lieberei 2011
Ocimum basilicum (basil)	Rosmarinic acid	Antimicrobial	SA, JA, chitosan, phytophthora	Flasks, hairy roots	Bais et al., 2002
Datura stramonium (jimsonweed)	Tropane alkaloids	Pharmaceutical	MeJA	Petri dishes	Amdoun et al., 2009
Harpagophytum procumbens (Devil's claw)	Iridoid glycosides	Pharmaceutical	None	Flasks, hairy roots	Georgiev et al., 2006

Elicitors can be divided into two wide groups, including abiotic elicitors, which are chemical compounds as jasmonic acid (JA), methyl jasmonate (MeJA), SA, nitric oxide (NO) or $CdCl_2$, or biotic elicitors, which are microorganism added to the growth media as *Enterobacter sakazaki* or *Phytophthora parasitica* (Badri et al., 2008 b; Georgiev et al., 2007). These signalling compounds have been described as root exudation elicitors and they can be added to the growth media in order to modify the quantity and diversity

of compounds released to the rhizosphere. For example, studies with root exudates from *Brassica rapa* ssp. in presence of SA and MeJA, revealed an overproduction of indolic glucosinolates (which can be used as bioactive additives in functional foods and nutraceuticals) to levels between two and four times higher than those obtained from control plants (Schreiner et al., 2011). By this, the addition of chemical elicitors is used for the obtaining of higher quantities of some compounds with commercial interest, as occurs with MeJA, which has been described as one of the most important chemical elicitors in root exudates production, affecting to the secretion of several secondary metabolites, including indole glucosinolates, camalexin, triterpens, rosmarinic acid, scopoletin, caffeic acid, etc... in different plant species (Badri and Vivanco 2009).

Concluding remarks

Plants are constantly secreting a wide variety of compounds to the rhizosphere through their roots by means of different passive and active transport mechanisms. The mix of these compounds is also known as root exudates, whose composition can be quantitatively and qualitatively affected by several physical, chemical and biological factors. In consequence, the alteration of their composition can contribute to facilitate the plant to face adverse conditions, as attracting beneficial microbiota, chelating toxic compounds from the soil, changing soil pH or solubilizing nutrients into assimilable forms. In consequence, promoting root exudation processes under these adverse situations could help the plants to cope with them, which shows the importance of enlarging the knowledge in this area.

Moreover, root exudates are also considered as key mediators in the interaction between plants and soil microbiota, having a clear effect in the recruitment of beneficial species of bacteria and fungi. By this, the potentiation of root exudation could enhance the colonization by these beneficial species and improve plant growth and yield without the requirement of treating with high quantities of fertilizers. In addition, this beneficial effect of PGPRs and mycorrhizal fungi manifest the possibility of their use under different stressful situations, as drought or salt stress, which are exacerbated due to the climate change. In this case, treatments of biostimulation, biofertilization or bioaugmentation can contribute to facilitate root colonization by these beneficial microorganisms, improving plant growth and yield.

The improvement of the knowledge about root exudates composition can derive in the development of new techniques for the obtainment of plant metabolites in a large scale.

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Objectives

Objectives

The main objective of this work consists in the evaluation of citrus root exudation process in plants cultured under different abiotic stress conditions and decipher their involvement in the interaction with PGPRs.

In order to achieve this aim, the following partial objectives were established:

1. Determine the different tolerance or sensitivity of two citrus genotypes (Carrizo citrange and *Citrus macrophylla*) to salt and heat stress.

2. Study the root exudation of proline and phytohormones in plants of both genotypes subjected to salt and heat stress, focusing in the different tolerance of each genotype to these abiotic stress conditions.

3. Evaluate the effect of root exudates from salt- and heat-stressed citrus plants in the growth of two rhizobacteria.

4. Evaluate the protective role of two rhizobacteria strains on citrus plants subjected to salt stress.

Partial objectives 1 and 2 will be addressed in Chapter 1 of this Doctoral thesis report, partial objective 3 in Chapter 2 and partial objective 4 in Chapter 3.

Results

Chapter 1

Citrus plants exude proline and phytohormones under abiotic stress conditions

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Chapter 1: Citrus plants exude proline and phytohormones under abiotic stress conditions

Abstract

Plants are constantly releasing several compounds to the rhizosphere through their roots, including primary and secondary metabolites. Root exudation can be affected by growth conditions, including pH, nutrient availability, soil salinity or temperature. *In vitro* cultured plants of two citrus genotypes with contrasting tolerance to salt and heat stress conditions, were used as plant material. Proline and phytohormone contents in root exudates from plants subjected to salt or high temperature conditions were evaluated. In addition, tissue damage and lipid peroxidation together with endogenous levels of chloride, proline, and phytohormones were determined in roots and shoots. Proline was released in larger quantities to the rhizosphere when plants were subjected to salt or heat stress. In each stress condition, the concentration of this amino acid was higher in the exudates obtained from plants tolerant to this particular stress condition. On the other hand, root exudation of phytohormones salicylic acid, indole acetic acid, abscisic acid and jasmonic acid generally increased under both adverse conditions. Results confirm a phytohormone exudation in citrus plants, which had not been described previously and can have an important role in the rhizosphere communication. Moreover, stress conditions and the different tolerance of each genotype to the particular stress significantly modify the exudation pattern both quantitatively and qualitatively.

Keywords*:* abiotic stress, citrus, root exudates, phytohormone, proline

Abbreviations: ABA: Abscisic acid; AMF: Arbuscular mycorrhizal fungi; CC. Carrizo citrange; CIN: *t*-Cinnamic acid; CM: *Citrus macrophylla;* IAA: 3-Indole acetic acid; JA: Jasmonic acid; MDA: Malondialdehyde; SA: Salicylic acid.

Key message: This article describes the root exudation of proline and phytohormones in citrus and their involvement in salt- and heat-stress responses.

Introduction

In nature, plants are constantly secreting a huge variety of biochemical compounds to the rhizosphere, which are known as root exudates. Several compounds are susceptible of being released, including primary metabolites such as sugars, amino acids or organic acids, and secondary metabolites as flavonoids or anthocyanins. The composition of root exudates can be altered both quantitatively and qualitatively by biotic and abiotic stress conditions (Badri and Vivanco, 2009).

Root exudates can induce positive or negative interactions between the plant and the biota present in the rhizosphere, including bacteria, fungi, nematodes, and insects, or other plants of the same or different species. Whereas some compounds released to the rhizosphere in high quantities, such as amino acids or sugars, generally promote their development, some other toxic compounds negatively affect the performance of these organisms (Bais et al., 2006).

Apart from the biotic factors, abiotic conditions including the availability of nutrients or water, soil salinity, pH or temperature can also modify the composition of root exudates. These changes can contribute to the modification of soil properties, including microorganism populations, soil oxygen pressure, nutrient availability or electrical conductivity (Henry et al., 2007).

Root exudation has been traditionally considered a passive process mediated by the transport of compounds through the root membrane by means of diffusion, ion channels and vesicular transport (Baetz and Martinoia 2014). Diffusion is produced by a different concentration gradient between the cytoplasm of root cells and the rhizosphere, and it is affected by the permeability of the membrane, the condition of root cells and the polarity of the compounds. This process is responsible for the exudation of low molecular weight compounds, such as sugars, amino acids, carboxylic acids and phenolics. Meanwhile, ionic channels secrete carbohydrates and specific carboxylates to the rhizosphere, such as citrate, malate or oxalate, which are usually exuded in high concentrations and not always can be secreted by the diffusion process. Finally, root exudation mediated by vesicles is used for secretion of high molecular weight compounds stored in vesicles (Badri and Vivanco 2009; Bertin et al., 2003).

Recent studies also describe root exudation through an active transport mediated by proteins located in the plasma membrane of the root cell (Baetz and Martinoia 2014). There are several families of protein transporters involved in root exudation processes, two of the most important being ABC ("ATP-Binding Cassette") and MATE ("Multidrug and toxic compound extrusion") (Kang et al., 2011; Yazaki et al., 2008).

Citrus is one of the most economically important fruit crops worldwide whose productivity can be negatively affected by several environmental stresses, such as drought, salinity or extreme temperatures. Therefore, it is important to study its tolerance to different stresses. In this work two different citrus rootstocks have been selected due to their different tolerance to salt and heat stress: Carrizo citrange (CC), which is salt sensitive and heat tolerant and *Citrus macrophylla* (CM), which is salt tolerant and its behavior under heat condition is not well known (García-Legaz et al., 1993; Iglesias et al., 2004; Zandalinas et al., 2016, 2017).

Although there is no information in the literature related to the process of exudation in this citrus, it has been reported that in common bean and soybean, the production of root exudates is modified by stress conditions such as high salinity, and can vary depending on the plant stress tolerance, as with the almond tree (Dardanelli et al., 2010, 2012; Marin et al., 2010). In this context, it is important to explore if citrus plants are capable of exuding stress-related metabolites, and if root exudate composition is modified in plants subjected to stress conditions.

In previous studies with citrus subjected to abiotic stress conditions, proline has been determined as a stress marker that protects the plant acting as an osmoprotectant. Generally, endogenous content of proline increased in stressed plants, but there is a controversy about the capability of this amino acid to counteract stress damage (Arbona et al., 2013). Other studies have reported that this amino acid is also released in high quantities to the rhizosphere (Vílchez et al., 2000). In addition to proline, phytohormones such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) or 3-indole acetic acid (IAA) have also been described as important mediators in several processes, including abiotic stress responses (Gómez-Cadenas et al., 2015). Scarce information on phytohormone exudation is found in the literature. ABA has been detected in soybean (Tawaraya et al., 2014) and rice (Tawaraya et al., 2013) root exudates, but their function in the rhizosphere remains unclear.

The purpose of the present work was to study root exudation of proline and phytohormones in citrus plants cultured under different abiotic stress conditions, and decipher how the exudation pattern was affected by the different tolerance of each genotype to specific adverse culture conditions.

Materials and Methods

Plant material, treatments and root exudate collection

Seeds of the citrus rootstocks Carrizo citrange (*Citrus sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.; CC) and *Citrus macrophylla* Wester (CM) were used in the different experiments. Seed coats were removed in order to facilitate seed germination. Peeled seeds were disinfected for 10 minutes with a 2% v/v sodium hypochlorite solution containing 0.1% Tween 20 as moisturizer, and rinsed three times with sterile distilled water (Pérez-Clemente et al., 2012).

Seeds were individually sown *in vitro* into 150 x 20 mm culture tubes with 25 ml MS medium containing the inorganic salts of Murashige and Skoog (1962), supplemented with 0.55 mM myo-inositol, 4.86 µM pyridoxine-HCl, 0.59 µM thiamine-HCl, 8.12 µM nicotinic acid and 87.64 mM sucrose (Duchefa Biochemie, Haarlem, Netherlands). Culture medium pH was adjusted to 5.7 and was solidified with European bacteriological agar at 0.9 % (Conda, Madrid, Spain). Plant material was cultivated at 26 °C in darkness for 2 weeks and 2 more weeks with a 16 h photoperiod and illumination of 150 mmol m^{-2} s^{-1}. After this period, plants were transferred to MS liquid medium. Roots were pruned during the transplant to induce the formation of adventitious roots.

Once plants reached between 6 and 8 centimeters length, they were transferred to tubes containing sterile deionized water, and the different abiotic stresses were applied. Salt stress was applied by adding 0 (Control), 60 and 90 mM sodium chloride, while heat stress was applied by incubating plants in different culture chambers with temperatures of 25 (Control), 30 or 40 ºC. Root exudates were collected at 1, 3 and 10 days after the stress imposition, frozen with liquid nitrogen and stored at -80 ºC. Concurrently, shoot and root tissues were also sampled and frozen in liquid nitrogen, and root exudates were freeze-dried before the analysis. The absence of contaminations in root exudates was

tested by culturing a 20 µl aliquot in petri dishes with potato dextrose agar medium (Conda, Madrid, Spain). No contamination was detected in the exudates used for the analysis.

Leaf damage

Variations in plant appearance and performance were assessed. Depending on the symptoms, three different damage levels were established: i) Asymptomatic plants; ii) Plants with some yellowish leaves (mild damage); and iii) Plants with some burned leaves (severe damage). If a plant presented different damage degrees, the most severe damaged was considered.

Chloride content

Chloride content in root and shoot tissues of both genotypes subjected to salt stress was measured by automatic titration as described in López-Climent et al. (2008). Briefly, samples were ground and incubated for 12 hours with 0.1 N HNO_3 (Panreac, Barcelona, Spain) and 10% glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA). Finally, the chloride concentration was obtained by measuring 0.5 ml of the solution with a chloride meter (Model 626, Sherwood Scientific Ltd., Cambridge, UK).

Malondialdehyde concentration

Malondialdehyde (MDA) concentration was measured by spectrophotometry following the methodology described by Hodges et al. (1999). This methodology consisted of homogenization of 0.5 g of fresh weight in 80% absolute ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes using a sonicator (Elma S30, Elma, Singen, Germany). Samples were centrifuged and two aliquots of the supernatant were mixed with 20% trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) or a solution of 20% trichloroacetic acid and 0.5 % thiobarbituric acid (Sigma-Aldrich, St. Louis, MO, USA). Both mixtures were incubated in a bath with water at 90 ºC for one hour. After that, samples were cooled and centrifuged in order to remove suspended particles. Finally,

absorbance was measured at 440, 534 and 600 nm against a blank in a spectrophotometer (Thermo Spectronic Genesys 10, Waltham, MA, USA). MDA concentrations were calculated as described in Arbona et al. (2008).

Proline concentration

Proline concentration was determined by spectrophotometry in plant tissue and root exudates as described in Bates et al. (1973) with some modifications. The extraction procedure had some differences depending on the material.

In shoot and root tissues, 0.05 g of sample was extracted with 5 ml of 3% sulfosalicylic acid (Panreac, Barcelona, Spain) by sonication for 30 minutes. Samples were centrifuged at 4000 g for 20 minutes at 4 ºC, and the supernatant was mixed with glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) and ninhydrin reagent (625 mg of ninhydrin in 15 ml of glacial acetic acid and 10 ml of orthophosphoric acid 6M) in a 1:1:1 proportion (v:v:v). Samples were incubated at 100 ºC for one hour in a water bath, cooled down and centrifuged 5 min at 2000 g at 4 ºC. Absorbance was measured at 520 nm with a spectrophotometer (Thermo Spectronic Genesys 10, Waltham, MA, USA). Proline quantification was performed with a standard curve made with a commercial standard of proline (Sigma-Aldrich, St. Louis, MO, USA,).

In root exudates, proline determination was performed as described above, but starting from 15 ml of freeze-dried root exudates. Sulfosalicylic acid at a concentration of 3% was used as extraction solvent by adding 5 ml to 15 ml of freeze-dried root exudates. Samples were sonicated for 30 minutes and centrifuged. Then, 1 ml of the supernatant was mixed with 1 ml of glacial acetic acid and 1 ml ninhydrin reagent and incubated at 100 ºC for one hour. Finally, samples were cooled down on ice and centrifuged before the measurement with a spectrophotometer at 520 nm. Proline content in root exudates was expressed relative to the root fresh weight. Blanks with water and sodium chloride at the studied concentrations were measured to check the absence of proline.

Phytohormone analysis

ABA, JA, SA and IAA concentrations were determined in tissue samples and root exudates 10 days after the onset of stress treatments. Moreover, *t*-cinnamic acid (CIN) was also determined in root exudates. Metabolite concentrations were determined by high performance liquid chromatography coupled online to a triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source (Durgbanshi et al., 2005). Two hundred milligrams of shoot and root tissue samples were homogenized to fine powder and extracted with water using a mill ball equipment (MillMix20, Domel, Železniki, Slovenija). [$^{2}H_{6}$]-ABA, dehydrojasmonic acid, [$^{13}C_{6}$]-SA, [$^{2}H_{2}$]-IAA and [$^{2}H_{6}$]-CIN were used as internal standards. pH was adjusted to 2.8-3.2 with acetic acid. Extracts were partitioned twice with diethyl ether and the supernatants were evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France) at room temperature. The solid residue was resuspended in 500 µl of water:methanol 90:10 and filtered through 0.22 µM PTFE filters. 20 µl of this solution were injected into the HPLC system (Acquity SDS, Waters Corp., Milford, MA, USA). In root exudates samples, the extraction protocol was the same as in plant tissue but starting from 15 ml of freeze-dried root exudates extracted by 15 minutes of sonication.

A reversed-phase C18 column (Gravity, 50 × 2.1mm 1.8-µm particle size, Macherey-Nagel GmbH, Germany) was used to achieve the chromatographical separation, using a methanol:water mixture, supplemented with 0.1% acetic acid, gradient at a flow rate of 300 µl min^{-1}. Results were processed using Masslynx v4.1 software, and the phytohormone contents were quantified with a standard curve prepared with commercial standards.

Statistical analyses

Statistical analyses were assessed with the Statgraphics Plus v.5.1. Software (Statistical Graphics Corp., Herndon, VA, United States). Data are means of three measurements per sample from three independent experiments and were subjected to one- or two-way analysis of variance (ANOVA) and a Tukey posthoc test ($p \leq 0.05$) when significant differences were detected.

Results

Phenotypic traits in response to stress

Differences in phenotypic traits were observed on the plants depending on the genotype and the applied stress. In salt stress experiments, no phenotypic change was observed in stressed plants compared to control. However, when cultured under heat stress conditions some plants of both genotypes showed different levels of damage, including yellowish and burned leaves (Fig. 1.1).

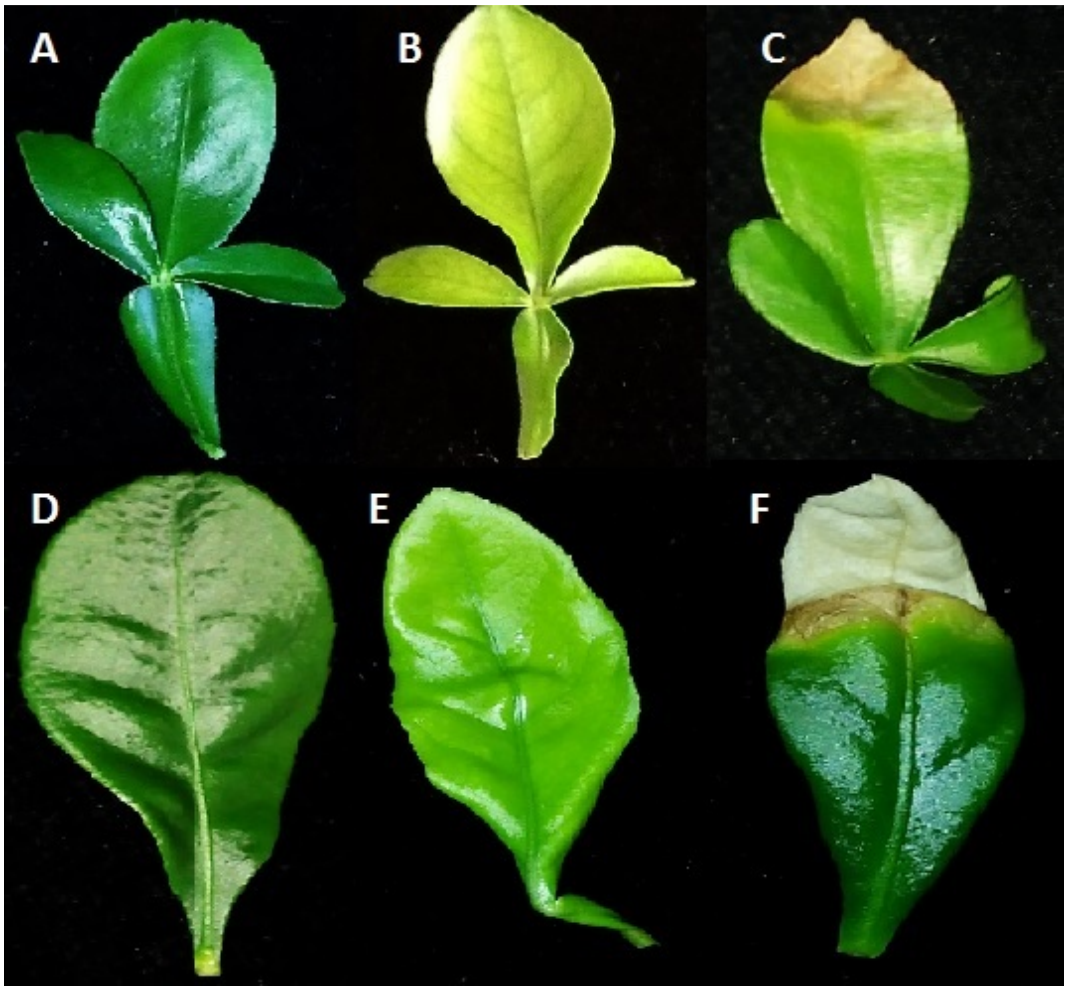

Figure 1.1 Heat-induced damage in leaves of Carrizo citrange (A-C) and *C. macrophylla* (D-F) after ten days of stress. Non-damaged leaves (A, D), mildly damaged leaves (B, E) and severely damaged leaves (C, F)

The frequency of symptom appearance depended on the rootstock. Ten days after the stress imposition, the percentage of plants exhibiting leaf damage was higher in CM (Fig.

1.2). At 30 ºC, 23.5% of CC plants showed mild damage and 11.8% showed severe damage whereas in CM, 33.3% of plants showed mild damage, and the same percentage of plants exhibited severe damage. These percentages increased in plants cultured at 40 ºC, exhibiting 56.3% and 12.5% of CC plants with mild and severe damage, respectively. In CM, 30.0% and 60.0% of plants had mild or severe damage, respectively.

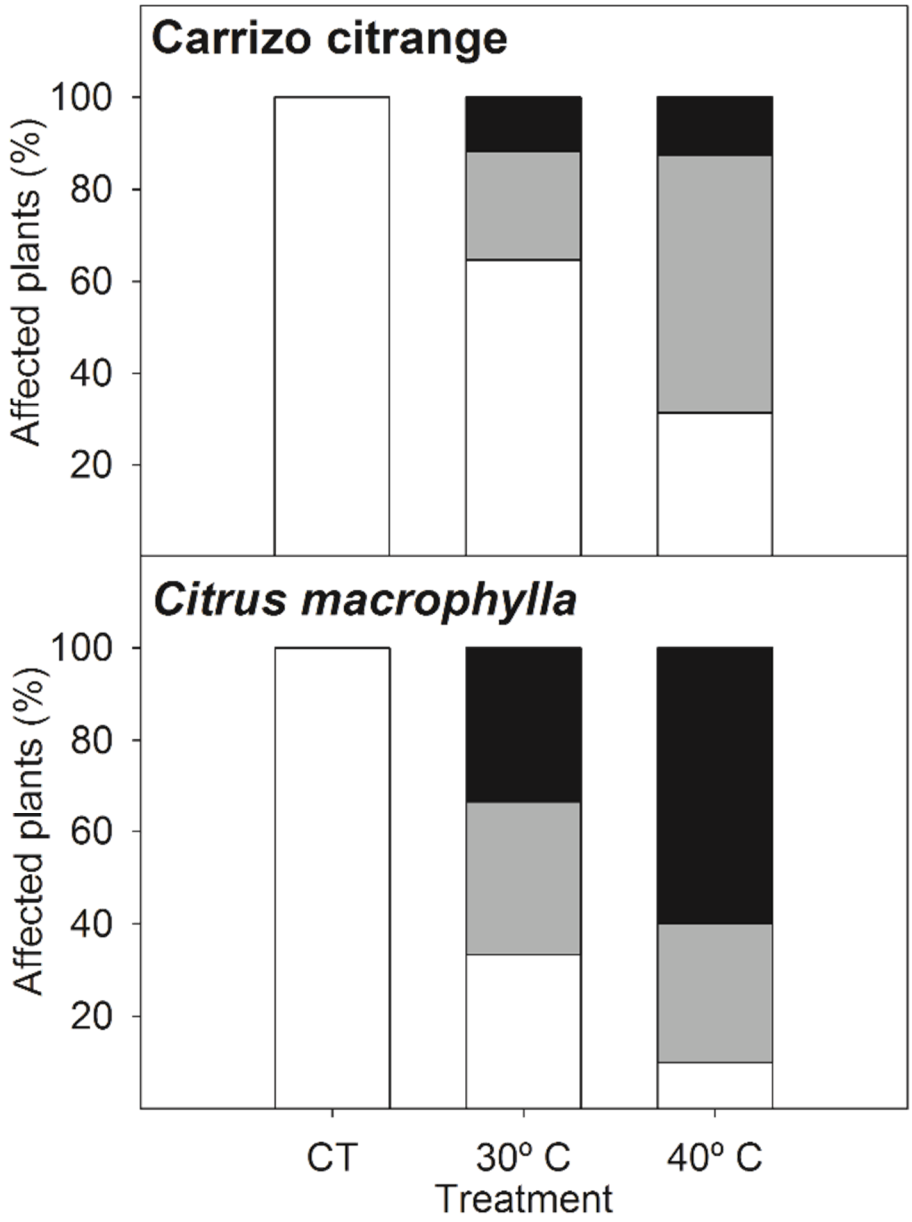

Figure 1.2 Percentage of citrus plants with heat-induced damage in leaves after ten days of stress. White: Plants without any damaged leaves. Grey: Plants with mild damaged leaves. Black: Plants with severe damaged leaves

Chloride accumulation

Endogenous chloride content increased progressively in shoots and roots of plants of both genotypes after the stress imposition although there were some differences in the accumulation pattern among tissues (Fig. 3).

In roots of plants cultured under salt stress conditions, chloride concentration increased considerably in both genotypes, being the differences in relation to control statistically significant in all the sampling points. In roots, after ten days of stress, chloride levels were similar to those determined at three days, with values 3.56 and 2.32 times higher in roots of CC and CM plants subjected to 90 mM NaCl.

Chloride accumulation pattern in shoots was sharper and ion levels were higher at the end of the experiment (Fig. 1.3). Chloride concentration in the shoots increased since the first day of stress imposition, being the accumulation pattern similar in both genotypes during the first three days, with values being among 1.04 and 1.56 times those found in control plants. After ten days of stress there was an accumulation of chloride in CM shoots, with levels 3.58 and 4.25 times higher than control in plants treated with 60 and 90 mM NaCl respectively, whereas treated CC shoots had chloride concentrations 2.06 and 3.48 times higher than those recorded in control plants, respectively.

Proline content in shoots and roots

The accumulation of this compatible osmolyte was determined in root and shoot tissues after 10 days of salt treatment or high temperature stress (Fig. 1.4).

Salt stress induced a significant decrease in proline content in roots of CM, with reductions of 12.11% and 43.79% when plants were subjected to 60 and 90 mM NaCl, respectively. No differences with respect to controls were observed in proline concentration neither in shoots of CM nor in shoots or roots of CC cultured under the different conditions assayed.

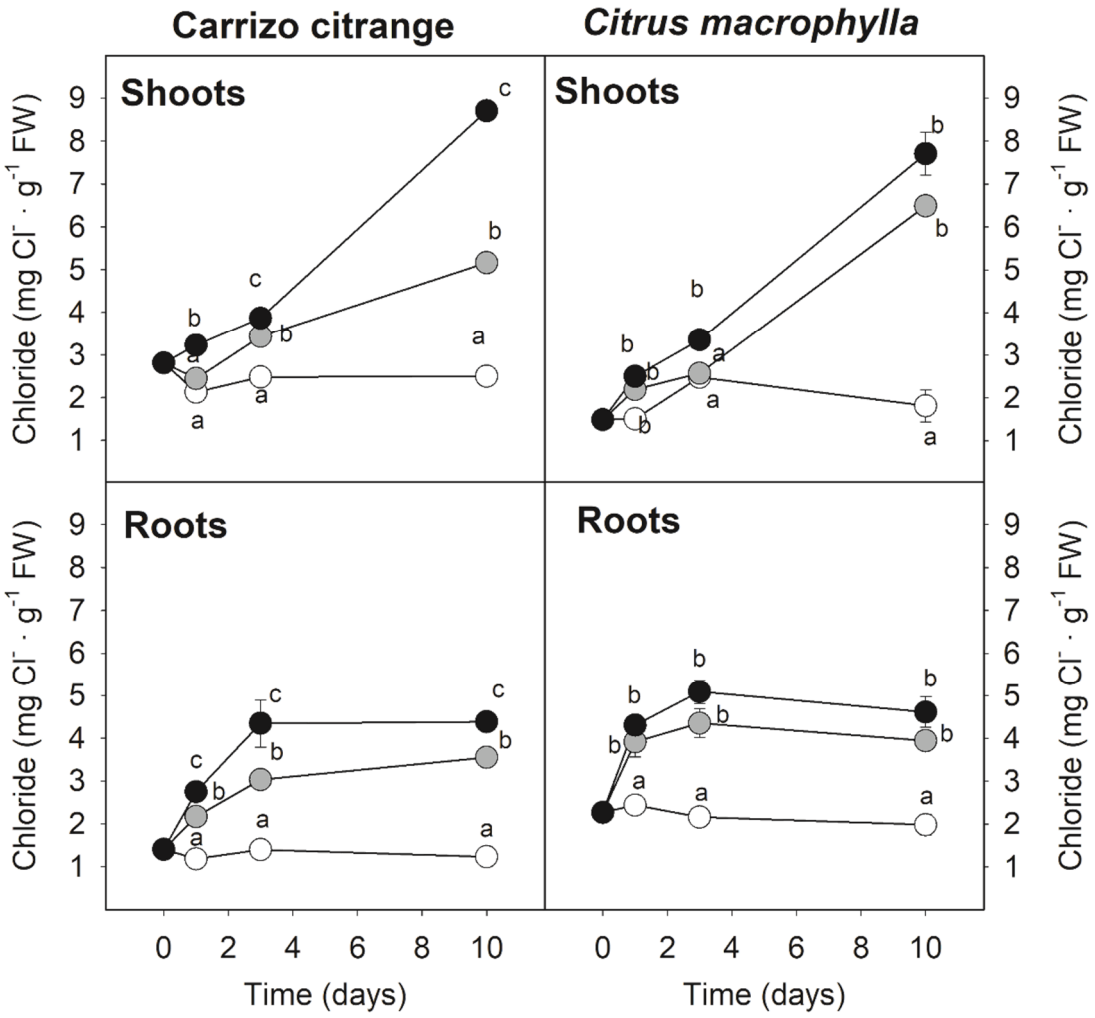

Figure 1.3 Endogenous chloride content in shoots and roots of Carrizo citrange and *C. macrophylla* subjected to salt stress. White symbols refer to control, grey to 60 mM NaCl, and black to 90 mM NaCl. Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Proline content increased in CC shoots after 10 days of heat stress, reaching values 1.29 times higher than control when cultured at 30 ºC and 40 ºC. On the contrary, the concentration of this metabolite decreased in roots exposed to 40 ºC reaching values 29.89% lower than control, while in roots of plants stressed with 30 ºC similar values to control plants were recorded.

No significant variations in proline content were detected either in root or in shoot tissue of CM plants subjected to heat stress in relation to controls (Fig. 4).

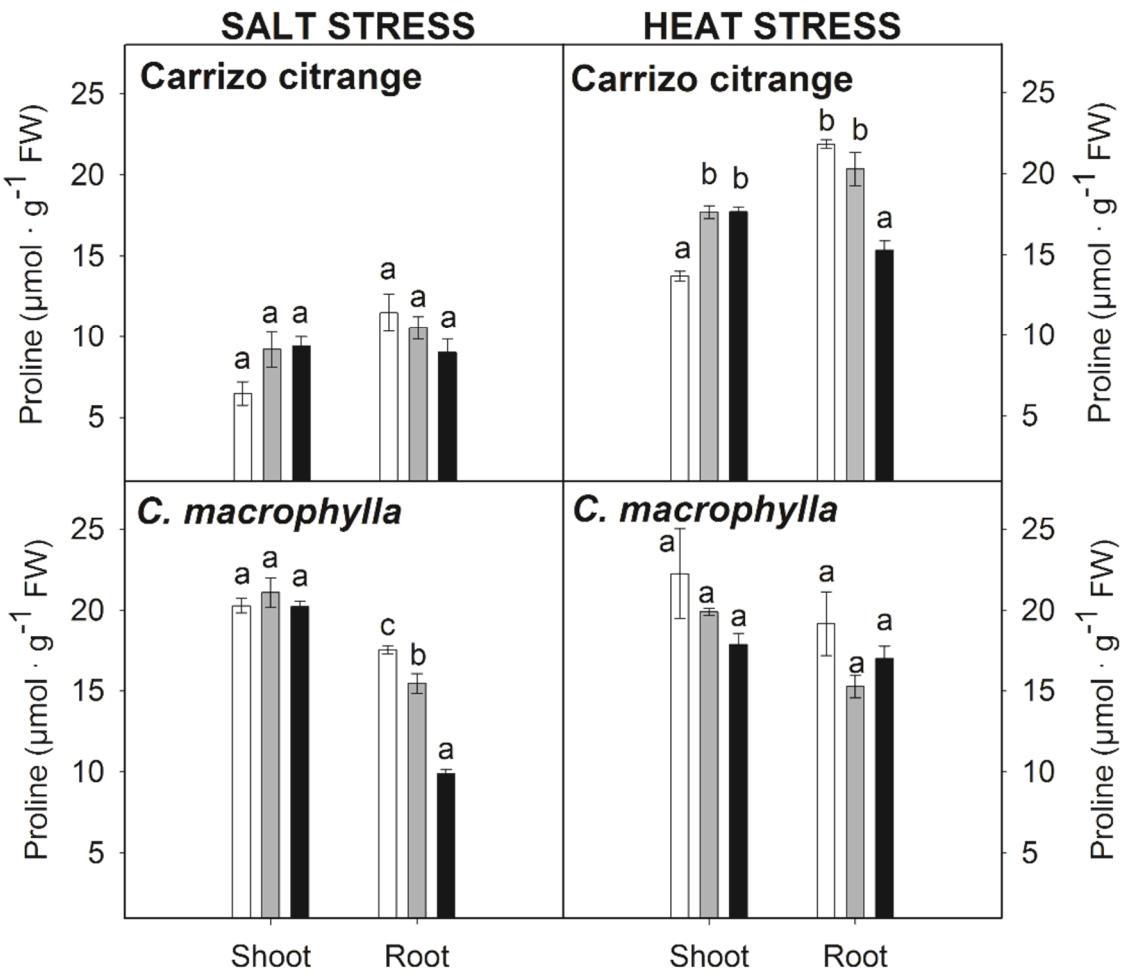

Figure 1.4 Endogenous proline content of Carrizo citrange and *C. macrophylla* shoots and roots of plants subjected to salt and heat stress for ten days. Salt stress: control (white), 60 mM (grey) and 90 mM (black). Heat stress: control (white), 30 ºC (grey) and 40 ºC (black). Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Proline exudation

The exudation rate of this amino acid was different depending on the applied stress and the genotype (Fig. 1.5).

In salt stress experiments, no differences were recorded in the root exudation of proline between stressed and control CC plants, whereas CM stressed plants released higher quantities of this amino acid to the rhizosphere from the first day of the experiment. These differences in root secretion of proline between control and stressed plants increased with the extent of the stress, independently of the NaCl concentration. Root exudates from CM

stressed plants showed proline concentrations 4.03 and 3.17 times higher than control at the third day of stress when culture medium was supplemented with 60 and 90 mM NaCl respectively. These differences increased to 8.55 and 7.67 times higher than controls at the tenth day of stress respectively.

Proline exudation also increased in plants of both genotypes subjected to 30 and 40 ºC, but in lower quantities than those detected in salt stress experiments. The differences were only statistically significant between root exudates from control plants and those obtained from plants cultured at 40 ºC. Conversely to salt stress results, when plants were cultured under high temperature, root exudates from CC showed the highest differences with respect to control, being statistically significant from the first day of stress at 40º·C, with proline content 2.52 times higher than control. This difference was maintained over time, with the amount of proline present in root exudates from plants subjected to 40 ºC 1.87 and 3.15 times higher than control at the third and the tenth day of stress respectively. On the other hand, exudates from CM plants subjected to heat stress only presented larger quantities of proline in the treatment at 40 ºC after ten days, with values 2.24 times higher than control.

Phytohormone content in shoots and roots

The endogenous content of ABA, SA, JA and IAA in shoots and roots of both genotypes was affected differently depending on the stress applied after 10 days of treatment (Tab. 1.1).

Under salt stress conditions, CC plants subjected to 90 mM NaCl exhibited increased endogenous ABA concentrations, reaching values 5.81 and 2.92 times higher than control in shoots and roots, respectively. In CM plants this increase was not as marked as in CC, being only appreciable in shoots of plants subjected to 60 mM NaCl, with values 3.83 times higher than control, whereas roots subjected to this treatment showed values 66.84% lower than control. 60 and 90 mM NaCl treatments induced an increase in SA concentration in shoots of CM, with values 4.04 and 2.45 times the control, while the content of this phytohormone did not vary in shoots of CC. Meanwhile, SA content increased in roots of CC, with values 1.65 and 3.31 times higher than control under both,

moderate and severe salt stress. In contrast, the content of SA in roots of CM subjected to 60 mM was 33.04% lower than control.

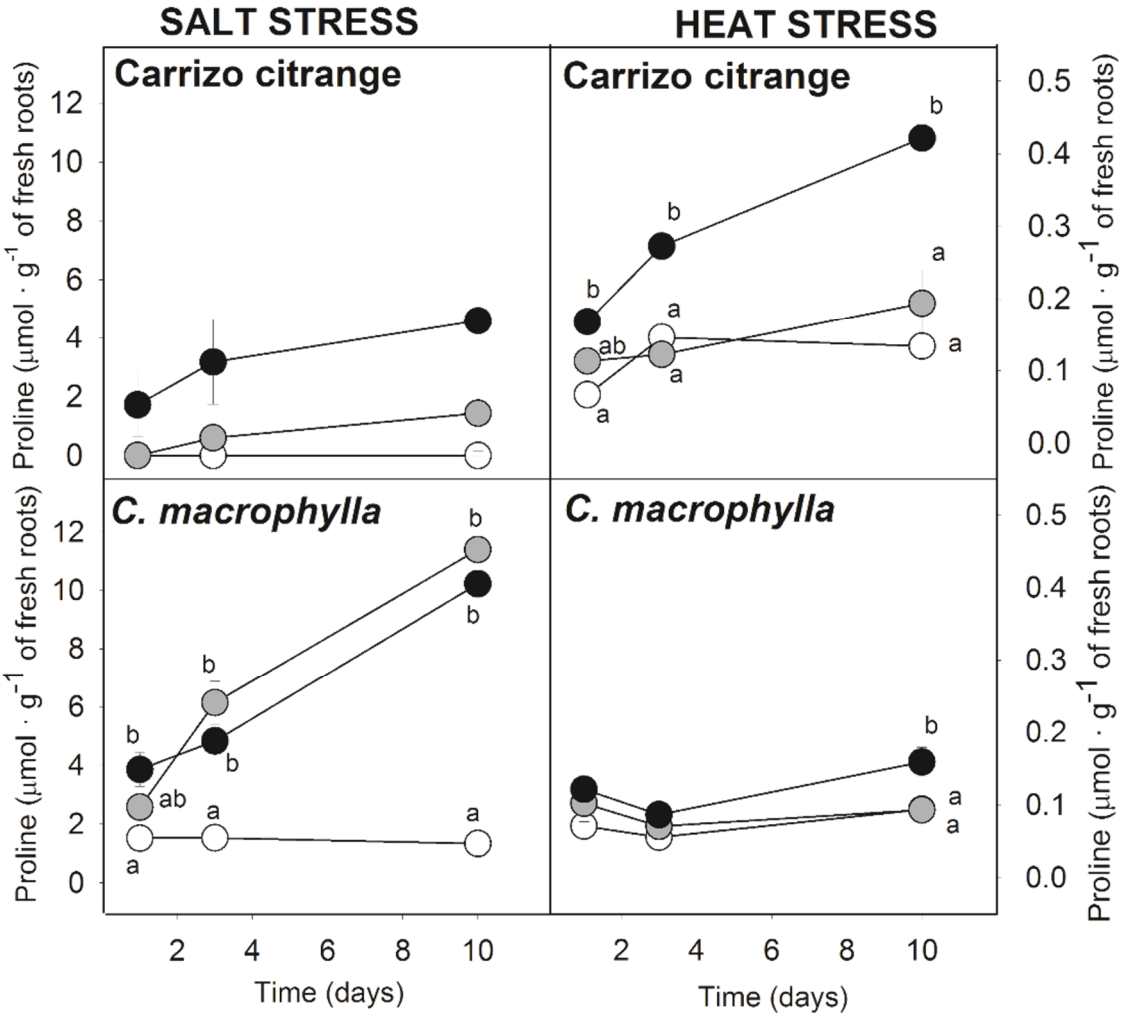

Figure 1.5 Proline levels exuded to the rhizosphere by Carrizo citrange and *C. macrophylla* plants subjected to high salinity and heat after 1, 3 and 10 days of stress. White symbols refer to root exudates obtained from control plants, grey symbols refer to root exudates obtained from mild stressed plants (60 mM or 30 ºC) and black symbols refer to root exudates obtained from severe stressed plants (90 mM or 40 ºC). Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Table 1.1 Phytohormone levels in shoot and root tissue of Carrizo citrange and *C. macrophylla* plants subjected to salt and heat stress for ten days. Values indicate the mean of three replicates ± standard error. Asterisks refer to statistically significant differences at $P \leq 0.05$

			Shoots			Roots		
			Control	Mild Stress	Severe Stress	Control	Mild Stress	Severe Stress
ABA (ng·g^{-1} FW)	Salt	Carrizo	9.38±0.41	7.06±0.46	54.51±6.48*	9.85±0.90	10.47±3.03	28.83±2.03*
		Macrophylla	3.46±0.35	13.27±0.03*	4.57±0.59	5.94±0.82	1.97±0.39*	5.43±1.53
	Heat	Carrizo	5.64±1.09	8.10±1.02	3.46±0.43	9.47±1.84	7.65±0.86	1.56±0.11*
		Macrophylla	6.47±0.31	7.23±1.65	1.32±0.53*	9.77±1.82	8.21±0.77	3.35±0.58*
SA (ng·g^{-1} FW)	Salt	Carrizo	25.05±11.05	35.93±8.57	41.37±9.25	26.43±0.43	43.60±16.03*	87.39±19.84*
		Macrophylla	27.49±6.06	111.05±11.66*	67.32±6.05*	81.90±12.16	54.84±2.67*	64.60±13.6
	Heat	Carrizo	2.70±1.06	3.48±0.46	8.81±0.59*	3.22±1.31	4.03±0.72	12.63±1.67*
		Macrophylla	9.19±0.45	10.71±1.93	52.32±0.62*	12.20±2.65	11.83±0.31	5.84±2.21*
JA (ng·g^{-1} FW)	Salt	Carrizo	<LOQ	<LOQ	13.89±0.56*	<LOQ	<LOQ	63.56±8.62*
		Macrophylla	<LOQ	<LOQ	0.55±0.55	<LOQ	7.40±1.10*	11.06±1.34*
	Heat	Carrizo	5.74±0.96	6.29±1.23	0.87±0.50*	15.53±5.05	3.96±1.53*	0.41±0.41*
		Macrophylla	7.56±0.58	8.17±1.54	6.17±2.62	11.53±2.86	10.99±3.23	1.72±0.82*
IAA (ng·g^{-1} FW)	Salt	Carrizo	0.76±0.29	0.75±0.25	0.64±0.51	0.41±0.22	0.62±0.62	1.68±0.19
		Macrophylla	7.19±0.77	14.72±3.23	8.97±1.96	4.68±1.82	1.19±0.73	0.86±0.72
	Heat	Carrizo	4.22±1.83	2.95±2.27	8.95±2.14*	3.81±2.04	7.30±2.44	2.16±0.74
		Macrophylla	7.44±2.93	7.19±5.18	10.76±0.81*	2.74±0.75	2.78±0.44	3.21±0.64

JA was only detected in shoots and roots of both genotypes subjected to stress, with values of 13.89 and 63.56 ng · g^{-1} FW in shoots and roots of plants stressed with 90 mM NaCl respectively, while in CM, statistically significant differences in relation to control were only observed in roots of plants subjected to 60 and 90 mM NaCl, with values of 7.40 and 11.06 ng · g^{-1} FW respectively.

In shoot tissue, salt stress only induced changes in endogenous IAA concentration in CM plants subjected to 60 mM NaCl. The pattern of accumulation of this phytohormone was different in roots depending on the genotype. While roots of CC plants subjected to 90 mM NaCl showed an increase of IAA levels, with values 4.10 times higher than control, roots of CM subjected to 60 and 90 mM exhibited a slight decrease of IAA contents.

On the other hand, the only heat stress condition that affected ABA endogenous levels was 40 ºC. This treatment induced decreases in ABA content of CM shoots, with values representing 79.60% lower than control, while in roots decreases were observed in both genotypes, with reductions of 83.53% and 65.71% related to controls in roots of CC and CM, respectively. Contrary to the decrease of ABA, endogenous SAcontent generally increased in plants cultured at 40 ºC. In shoots, this increase was of 3.26 and 5.69 times the control in CC and CM plants respectively. In roots, there was an increase of 3.92 times the control values in CC, while in CM SA content decreased a 52.13% related to control. Heat stress induced a reduction in endogenous JA content in stressed plants. In shoots, a decrease was only observed in CC plants subjected to 40 ºC, exhibiting values 84.84% lower than control. In roots of CC, both temperatures induced a decrease of JA content, with values 74.50 and 97.36% lower than control at 30 and 40 ºC, while roots of CM plants subjected to 40 ºC showed a decrease of 85.08% related to control, no differences in JA concentration were registered with respect to control when plants were cultured at 30ºC.

The content of IAA only varied in shoots of plants of both genotypes subjected to 40 ºC, showing an increase of its concentration of 2.12 and 1.45 times the control in shoots of CC and CM respectively.

Phytohormone content in root exudates

Phytohormone content not only varied in plant tissues but also in root exudates of both genotypes after 10 days of exposure to salt or heat stress (Tab. 1.2).

Table 1.2 Phytohormone levels in root exudates of Carrizo citrange and *C. macrophylla* plants subjected to salt and heat stress for ten days. Mild stress refers to 60 mM NaCl and 30 ºC, and severe stress refers to 90 mM NaCl and 40 ºC in salt and heat stressed plants respectively. Values indicate the mean of three replicates ± standard error. Asterisks refer to statistically significant differences at $P \leq 0.05$

			Root exudates		
			Control	Mild Stress	Severe Stress
ABA (ng·g^{-1} root)	Salt	Carrizo	4.216±0.486	8.527±1.735*	10.932±2.715*
		Macrophylla	2.959±0.446	1.456±0.211*	0.196±0.196*
	Heat	Carrizo	0.342±0.035	0.533±0.097	0.079±0.026*
		Macrophylla	0.208±0.039	0.168±0.019	<LOQ
SA (ng·g^{-1} root)	Salt	Carrizo	44.436±1.011	43.730±16.784	40.842±10.639
		Macrophylla	173.957±32.651	273.926±25.818*	176.910±11.312
	Heat	Carrizo	9.319±4.211	6.855±0.934	24.444±6.944*
		Macrophylla	18.952±1.768	16.374±4.558	70.578±9.757*
JA (ng·g^{-1} root)	Salt	Carrizo	0.944±0.545	28.104±17.421*	5.715±3.631*
		Macrophylla	1.577±0.354	72.206±17.794*	146.992±12.286*
	Heat	Carrizo	0.015±0.001	0.037±0.008*	0.024±0.002
		Macrophylla	0.018±0.004	0.019±0.002	0.059±0.009*
IAA (ng·g^{-1} root)	Salt	Carrizo	4.938±1.877	9.091±5.012	6.655±2.389
		Macrophylla	1.213±0.696	3.094±0.645*	1.502±0.638
	Heat	Carrizo	0.834±0.439	3.783±0.500*	0.659±0.316
		Macrophylla	2.873±0.958	2.768±1.206	2.389±1.280
CIN (ng·g^{-1} root)	Salt	Carrizo	0.146±0.013	0.108±0.003	0.195±0.047
		Macrophylla	3.544±1.342	15.057±1.227*	28.587±0.237*
	Heat	Carrizo	0.727±0.199	1.984±0.373	9.105±0.253*
		Macrophylla	3.428±0.834	2.094±0.359	13.710±1.887*

Under salt stress conditions, ABA concentration increased in root exudates obtained from CC plants subjected to either 60 or 90 mM NaCl, with values 2.02 and 2.59 times higher than control, while in exudates from CM plants subjected to the same stress, there was a decrease in the content of this hormone, with values 50.68 and 93.24% lower than control.

The concentration of SA and its precursor CIN in root exudates from plants subjected to salt stress was only altered in those obtained from CM plants, with an increase of SA concentration of 1.57 times the control in plants subjected to 60 mM NaCl, and an increase of CIN of 4.25 and 8.08 times the control levels in plants subjected to 60 and 90 mM NaCl respectively. Salt stress induced JA exudation in both genotypes, with values of 29.89 and 6.09 times the control in CC, and 45.70 and 93.03 times the control in root exudates obtained from CM plants subjected to 60 and 90 mM NaCl respectively. IAA

concentration in root exudates only was affected in those obtained from CM plants subjected to 60 mM NaCl, with levels of IAA 2.55 times higher than control.

After ten days of stress induced by high temperatures, plants of both genotypes subjected to 40 ºC reduced the root exudation of ABA, to a level of 23.53% the control in CC, whereas this phytohormone was not detected in CM root exudates. Contrary to the decrease of ABA, root exudates from heat stressed plants exhibited increased concentrations of SA and CIN than those from control plants. Plants of CC and CM cultured at 40 ºC exuded quantities of SA 2.62 and 3.72 times higher than control respectively, and 12.47 and 4.00 times higher in the case of CIN. Moreover, despite the decrease of endogenous levels of JA in the roots, its content increased in exudates from heat stressed plants in both genotypes, with values of 2.47 times higher than control in CC plants stressed at 30 ºC and levels 3.28 times higher than control in CM plants subjected to 40 ºC.

Heat stress induced an increase of IAA in exudates obtained from CC plants subjected to 30 ºC.

Malondialdehyde content in plants

MDA content did not vary in roots of CC plants cultured under salt stress conditions while in roots of CM a reduction of 25.66 and 37.10 % in plants treated with 60 and 90 mM NaCl respectively was recorded (Fig. 1.6). Meanwhile, in roots of plants subjected to heat stress a decrease in MDA concentration was observed. In roots of CC plants, this decrease was of 20.42 and 29.33% with respect to the control in treatments at 30 and 40 ºC. A higher decrease was observed in CM roots, exhibiting values 25.96 and 33.97% lower than the control in treatments at 30 and 40 ºC respectively.

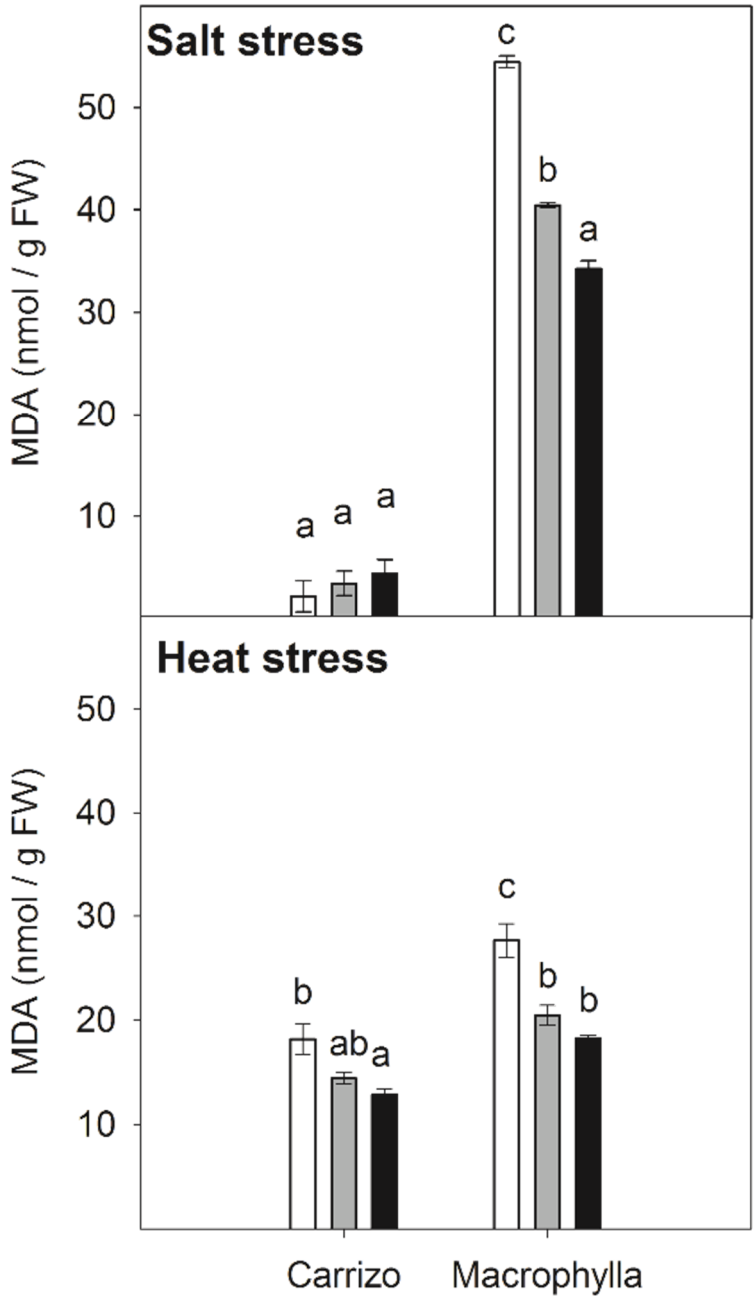

Figure 1.6 Endogenous Malondialdehyde content of Carrizo citrange and *C. macrophylla* shoots and roots of plants subjected to salt and heat stress for ten days. Salt stress: control (white), 60 mM (grey) and 90 mM (black). Heat stress: control (white), 30 ºC (grey) and 40 ºC (black). Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Discussion

The results in this work reveal that citrus plants are able to release proline and phytohormones to the rhizosphere, and their exudation is affected by salt and heat

stresses, as well as genotype tolerance to these adverse conditions. Experiments were performed with two citrus genotypes, CC and CM differing in their sensitivity to both stresses. While CC is sensitive to salt stress, CM is tolerant (García-Legaz et al., 1993; Iglesias et al. 2004), which represent an interesting plant system to study the physiological basis of citrus tolerance to high salinity. Moreover, the tolerance of these two genotypes to high temperatures seems to be opposite (Zandalinas et al., 2016; 2017), which, in our opinion made this study more comprehensive.

Seeds of both genotypes were germinated under *in vitro* conditions and salt or heat stresses were applied to the grown plants with two different intensities. Although several works have studied root exudates under field or hydroponic conditions, *in vitro* culture techniques were chosen in this work because the system allows a more rigorous control of the environmental conditions, which can affect root exudates composition both, qualitatively and quantitatively, having a higher reproducibility among the experiments (Vranova et al., 2013). To ensure that the determined amounts of proline and phytohormones in root exudates correspond to an exudation process and not to a passive output of these metabolites through damaged root cell membranes, MDA contents were determined (Fig. 1.6), since this metabolite is considered a marker of membrane degradation. Under none of the conditions assayed in this work, MDA levels increased in roots of any tested plant. This supports the validity of the system and discarding artifacts.

Our results demonstrate that under salt stress conditions, the salt tolerant genotype CM exuded higher quantities of proline related to control plants than the sensitive CC. Meanwhile, under heat stress conditions, the tolerant CC was the genotype that exuded larger quantities of this amino acid (Fig. 1.5). These results demonstrate that under both abiotic stress conditions, genotypes with lower sensitivity release higher amounts of proline to the rhizosphere. The concentration of exuded proline was proportional to the intensity of the imposed stress. Although the information on the subject is scarce, in excised roots of almond trees grown under *in vitro* conditions a release of proline to the medium was reported under salt stress conditions, and its concentration was higher in the case of tolerant rootstocks (Marin et al., 2010). Root exudation of proline has been also studied in rice and soybean plants subjected to phosphorus deprivation, detecting increases of its exudation rate in stressed plants of both genotypes (Tawaraya et al., 2013, 2014). Our results point out that citrus plants are capable of exuding proline and this

process is increased under stress conditions (Fig. 1.5). Moreover, there is a correlation between the resistance of the genotype to a specific stress situation and the amount of amino acid exuded. Differences in proline contents were evident in root exudates earlier than in shoots or roots, whose points to the detection of proline content in the rhizosphere as a non-destructive method to determine salt and heat stress in plants earlier than traditional determinations of this amino acid in shoots or roots, favoring the early use of corrective actions.

Root exudation of proline in response to abiotic stress could help plants to mitigate the adverse environmental conditions in two ways, acting as an attractant and promoting the growth of microorganisms present in the rhizosphere, and being a source of carbon and nitrogen for them. Different reports detail the benefits of proline in soil microbiota, as occurs in the case of the plant growth promoting bacteria *Pseudomonas putida* KT2442, whose growth is favored in presence of this amino acid in corn root exudates (Vílchez et al., 2000). It has been also reported that, other strains of this bacterium promote plant tolerance to some biotic and abiotic stresses, as occurs with the strain AKB28, which has been identified in citrus rhizosphere, and suggested to be involved in improving crop tolerance to huanglongbing (Trivedi et al., 2011). Another example is the strain Rs-198, which stimulates the growth of cotton plants subjected to salt stress conditions (Yao et al., 2010).

Plant hormones can modify soil properties, providing benefits for plants growing in this area; as an example, SA and CIN facilitate the solubilization of phosphorus in soil into assimilable forms (Khorassani et al., 2011; Tawaraya et al., 2014). Curiously, IAA was differently released to the medium depending on the stress applied and the rootstock tolerance (Tab. 1.2). Whereas under salt stress conditions, only the salt resistant genotype CM exuded larger quantities of this auxin, under heat stress conditions, CC, the heat-stress tolerant genotype, was the one that exuded IAA. The different exudation pattern of IAA, described as a root growth inducer, could be an adaptive mechanism to facilitate plants to explore the soil until zones with lower amounts of salts or with larger amounts of water in the case of heat stress, (Fu and Harberd 2003). IAA exudation could also facilitate the colonization by plant growth promoting rhizobacteria, which can use IAA as a source of carbon and nitrogen (Leveau and Lindow 2005).

While root exudation of JA was induced by salt and heat stress in both genotypes, ABA was exuded in lower quantities in CM under salt stress (Tab. 1.2). Root exudation of these phytohormones has been described previously in soybean, but its role in the rhizosphere remains unclear (Tawaraya et al., 2013). In contrast with the positive effect of proline in the growth of soil microbiota, the growth induction by phytohormones ABA, SA and JA is not clear and some works report that ABA, SA, MeJA and ethylene do not contribute to changes in rhizobacteria richness and evenness although phytohormones can contribute to change in community composition (Carvalhais et al., 2014). However, other authors support that SA and CIN promote their growth, using them as carbon sources (Segura, et al., 2017) and that JA has a positive effect on the growth of arbuscular mycorrhizal fungi (AMF) in garlic plants, providing benefits for the plant growth (Regvar et al., 1996).

Under salt stress conditions, no visible damage was detected in plants of the two genotypes tested, independently of their different sensitivities to high salinity conditions. The absence of visible damage may be due to the brief stress period, being the symptoms more evident in larger periods of stress as it has been previously reported (Montoliu et al., 2009). In contrast, plants of both rootstocks subjected to heat stress showed damage, with the percentage and severity of symptoms higher in CM plants, demonstrating that this genotype is more sensitive than CC to heat stress (Figs. 1.1 and 1.2). This questions its future use as rootstock in the Mediterranean citriculture, where summers will become warmer and drier in the near future, as a consequence of climate change. Previous work has demonstrated that other drought-tolerant citrus genotypes can have problems tolerating heat stress (Otero et al., 2015; Zandalinas et al., 2016).

Endogenous chloride levels rapidly increased in roots during the beginning of the experiment and these levels were maintained until the tenth day of stress (Fig. 1.3). As roots are firstly saturated of chloride ions and then, these ions are translocated to shoots, higher chloride amounts were recorded in the aerial part at the end of the experiment. Plants of both rootstocks increased the endogenous content of chloride, but the sensitive genotype CC reached higher absolute concentrations than the tolerant CM compared to the control plants. Although in comparison with control plants chloride levels of CM leaves were higher than in CC leaves, the absolute values were higher in leaves of the salt sensitive CC. These results confirm previous data indicating that salt sensitive citrus genotypes accumulate higher absolute levels of chloride than tolerant rootstocks do under

salt stress conditions (Hussain et al., 2012; López-Climent et al., 2008; Montoliu et al., 2009).

In response to environmental stresses plant leaves usually accumulate proline to regulate the osmotic potential and cope with the stress. Previous studies with citrus revealed that tolerant rootstocks accumulate higher amounts of this amino acid (Zandalinas et al., 2016). The absence of variations in the endogenous concentrations of this osmoprotectant in plants subjected to salt stress in this work may indicate that plants were not stressed enough to accumulate proline (Fig. 1.4). Moreover, since salt stress affects roots earlier than leaves, proline accumulation in leaves would occur later than under heat stress, which affects leaves directly.

However, endogenous proline content decreased in roots of the tolerant rootstock under each stress condition, which was concomitant with the higher exudation of this amino acid (Fig. 1.4). Although proline content usually increases in plant tissues under abiotic stress conditions, data in the system reported here would agree with other works which describe a reduction in proline levels in response to adverse conditions, as in wheat, with reductions in leaf proline contents in plants subjected to heat stress (Kumar et al., 2012), roots of canola plants subjected to salt stress (Saadia et al., 2012), or in roots of *Fragaria* x *ananassa* plants cultured under phosphorus starvation conditions (Valentinuzzi et al., 2015).

In general, stress-related phytohormone concentration in shoots and roots is in concordance with the different tolerance of both genotypes to salt or heat stress (Tab. 1.1). Under salt stress, ABA and JA concentrations generally increased with the stress, in a higher extent in the sensitive genotype CC than in CM (Valenzuela et al., 2016). Meanwhile, SA content followed an erratic pattern of accumulation under NaCl conditions as it has been previously reported (Montoliu et al., 2009). Results of this work also reinforce the scarce information on how citrus plants respond to heat stress conditions. Although ABA concentration generally increases under different abiotic stress conditions, there is not much information on its involvement in heat stress responses. It has been recently reported that this phytohormone content decreases in citrus plants subjected to heat stress, in parallel with the accumulation of the ABA degradation products phaseic acid and dehydrophaseic acid, revealing an induction of ABA catabolism under high temperature conditions (Zandalinas et al., 2016). Moreover, the

partial reduction in JA levels together with the lack of ABA accumulation are in consonance with other studies that demonstrated that an increase in JA levels is needed for ABA accumulation in citrus plants subjected to water stress (De Ollas et al., 2013). Also, in rice under heat stress a decrease in JA concentration has been reported (Du et al., 2013).

In conclusion, our results demonstrate that citrus plants are able to exude proline and phytohormones to the rhizosphere through their roots. Root exudation of proline increased in plants subjected to salt or heat stress, with higher increase in the tolerant genotype in both stresses. Since the increase in proline concentration was observed in root exudates earlier than in shoots or roots, its measurement in the rhizosphere could be used as an early stress marker. On the other hand, the increased quantities of phytohormones released to the rhizosphere could also help plants to cope with these stresses, either by solubilizing nutrients as occurs with SA and CIN, or inducing root growth by auxins. Meanwhile, ABA and JA exudation could improve nutrient solubilization and modify soil pH, due to their acidic character. ABA could also inhibit seed germination of competing plants.

Author contribution statement

VVP performed the experiments and wrote the manuscript. AGC and RMPC contributed in the design of the experiments and the supervision of the work.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Results

Chapter 2

Root exudates from citrus plants subjected to abiotic stress conditions have a positive effect on rhizobacteria

Chapter 2: Root exudates from citrus plants subjected to abiotic stress conditions have a positive effect on rhizobacteria

Abstract

Plants are constantly releasing root exudates to the rhizosphere. These compounds are responsible of different positive or negative interactions with other organisms, including other plants, fungi or bacteria. In this work, the effect of root exudates obtained from *in vitro* cultured plants of two citrus genotypes differing in their sensitivity to salt and heat stress on two rhizobacteria (*Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a) was evaluated. Bacteria were grown in minimal M8 medium supplemented with succinate in presence of root exudates as the sole source of N, and growth was determined measuring optical density at 660nm and colony forming units. Root exudates obtained from control plants promoted the growth of both bacteria, and this positive effect increased when exudates were obtained from stressed plants. Root exudates from salt-stressed plants of *C. macrophylla* (salt tolerant) induced an increase in bacterial growth higher than that obtained from Carrizo citrange exudates (salt sensitive). Root exudates from heat-stressed plants also had a positive effect on bacterial growth, which was more evident in the heat-sensitive *C. macrophylla*. Moreover, constructions of *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) were used to test the capability of both bacteria to detect proline and salicylates present in culture medium by measuring β-galactosidase activity. β-galactosidase activity increased in presence of root exudates obtained from stressed plants, and in a higher extent in the case of exudates obtained from the genotype resistant to each particular stress, indicating that those root exudates contain larger quantities of proline and salicylates, as it has been described previously. These results reveal that the growth of these rhizobacteria can be modulated through citrus root exudates, and it can change depending on the stress conditions and the genotype. Moreover, our data reveals that bacteria could use proline and salicylates from root exudates as sources of C and N, and they could be used as biosensors of plant stress.

Keywords: citrus, heat stress, rhizobacteria, root exudates, salt stress

Abbreviations: ACC: aminocyclopropane-1-carboxylic acid; 2: Arbuscular mycorrhizal fungi; CFU: Colony forming units; Cm: Chloramphenicol; HLB: Huanglongbing; LB: Lysogeny broth medium; MS: Murashige and Skoog salt solution; ONPG: *ortho-*

nitrophenyl-β-galactoside; PGPR: Plant growth promoting rhizobacteria; Rif: Rifampicin; SA: Salicylic acid; Tc: Tetracycline

Introduction

Plants release between 5 and 25% of net fixed carbon into the rhizosphere in the form of compounds ranging from simple organic anions to complex polymer mucilages (Bais et al., 2006). Those specialized metabolites, produced and secreted by plants, play a critical role in the interaction of plants with soil organisms at the root vicinity, generally increasing the total quantity and activity of microbes around plant roots. These microorganisms use the compounds released by the plants through their roots as major nutrient sources, mainly carbon and nitrogen, constituents of sugars and amino acids, which are the compounds released to the rhizosphere in higher quantities (Lugtenberg et al., 1999; Moe, 2013). This is the case of the plant growth promoting rhizobacteria (PGPR) *Pseudomonas putida*, which growth is positively affected by proline, one of the amino acids largely released to the rhizosphere (Vílchez et al., 2000b). In addition, flavonoids, anthocyanins or salicylates, present in root exudates in lower quantities can affect rhizosphere biotic composition (Badri and Vivanco, 2009; Cesco et al., 2012; Wu et al., 2009).

Root exudation is highly influenced by various biotic and abiotic factors in the surrounding environment, which can lead to a significant shift in the rhizosphere microbiota (Kawasaki et al., 2016). Several studies carried out with herbaceous species, reveal that PGPR and arbuscular mycorrhizal fungi (AMF) alleviate the damage induced by abiotic stresses conditions such as drought, salinity, flooding, nutrient deprivation, heavy metal or high temperatures in plants (Ali et al., 2011). The association with the bacteria *Scytonema hofmanni* alleviates the adverse effects of salt stress in rice plants (Rodríguez et al., 2006). Meanwhile, *Burkholderia phytofirmans* PsJN improves shoot and root growth and tuberization in potato plants cultured, either *in vitro* or *ex vitro*, under heat stress conditions (Bensalim et al., 1998). Wheat plants inoculated with *P. putida* AKMP7 are less damaged by high temperature stress than non-inoculated plants, showing higher levels of chlorophyll, sugars, proline, starch, protein and amino acids, and a reduction in superoxide dismutase, ascorbate peroxidase and catalase activities (Ali et al., 2011). Among the mechanisms responsible of this protection against abiotic stresses are the bacterial production of indole acetic acid or nitric oxide, which stimulate root growth and development and facilitate nutrient fixation; the decrease of plant endogenous content of ethylene by the bacterial 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase

activity or by the induction of changes in the root cell wall or membrane, such as the production of biofilms (Dimkpa et al., 2009).

Therefore, understanding the interactions among living organisms in the rhizosphere will have a great value in improving plant resistance to both biotic and abiotic stresses, which will ultimately result in an increase in crop productivity.

There is little information about the colonization of PGPRs in citrus plant rhizosphere. Isolates belonging to genera *Burkholderia*, *Pantoea*, *Pseudomonas*, *Bacilli*, *Painibacillus*, and *Serratia* were found to be associated with citrus roots, being the soil bacteria population different in plants affected by huanglongbing (HLB) (Trivedi et al., 2011) and the beneficial effect of *P. putida* in absence of any stress condition (Chiquito-Contreras et al., 2012). On the other hand, it has been reported that the AMF mitigates some biotic and abiotic stresses, including phytophthora infection (Watanarojanaporn et al., 2011), drought (Wu and Zou 2009), high salinity (Navarro et al. 2014; Satir et al. 2016; Zhang et al. 2017) or chilling (Wu and Zou 2010).

Among citrus genotypes there is a wide variability concerning to their tolerance to different abiotic or biotic stress conditions. Carrizo citrange is salt sensitive and heat stress tolerant, whereas *Citrus macrophylla* is salt tolerant and heat stress sensitive (Vives-Peris et al., 2017). In addition, our previous work demonstrates that citrus plants are capable to exude proline and salicylates as cinnamic acid or salicylic acid (SA) by their roots. These and other metabolites present in citrus root exudates could affect the growth of different bacteria present in the rhizosphere (Vílchez et al., 2000a; Vives-Peris et al., 2017; Wu et al., 2009).

In this work, the effect of root exudates of two citrus genotypes Carrizo citrange and *Citrus macrophylla* with contrasting tolerance to salt and heat stress on the bacteria *P. putida* KT2440 and *Novosphingobium* sp. HR1a has been studied. Although *P. putida* KT2440 has been previously reported as a PGPR (Planchamp et al., 2015), the role of Novosphingobium sp. HR1a as a PGPR is not clear, being limited to other strains of this genus (Zhang et al., 2016).

Materials and Methods

Root exudates obtainment

Root exudates from *in vitro* cultured plants of two citrus rootstocks, Carrizo citrange (*Citrus sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) and *Citrus macrophylla* Wester, were obtained as described in Vives-Peris et al., (2017).

Seeds of both genotypes were peeled and disinfected for 10 min in a 0.5% (vol/vol) sodium hypochlorite solution containing 0.1% (vol/vol) Tween-20 wetting agent and rinsed three times with sterile distilled water. Seeds were sown individually in 25x150 mm culture tubes with 20 mL of germination medium (MS) consisting of Murashige and Skoog salt solution (Murashige and Skoog, 1962) and 3% of sucrose as carbon source. The pH was set at 5.7 ± 0.1 with 0.1 N NaOH before autoclaving. The medium was solidified by the addition of 0.9 % agar (Conda, Madrid, Spain). The cultures were maintained at 25 ºC, first in darkness for two weeks and two more weeks with a photoperiod of 16 hours and illumination of 150 mmol m^{-2} s^{-1}. After that, plants per treatment were transferred to liquid MS medium and roots were pruned in order to favor the development of new roots.

Twenty days after the transference to MS liquid media, plants with a well-developed root system were transferred to the exudation media, composed by sterile deionized water in control and heat-stressed plants. For salt stress treatments, 60 or 90 mM NaCl was added to the exudation medium. For heat stress, plants were cultured at 30 or 40 ºC with a 16 hours photoperiod. Control and salt-stressed plants were maintained at 25 ºC with the same photoperiod. Medium was collected after ten days of exudation, frozen with liquid nitrogen and stored at -80 ºC. The absence of contaminations in root exudates was tested by culturing a 20 µL aliquot in potato dextrose agar medium (Conda, Madrid, Spain).

Bacterial strains and plasmids

To study the effect of root exudates on rhizobacterial growth, *P. putida* strain KT2440 and *Novosphingobium* sp. strain HR1a were used. For the detection of proline and salicylates, β-galactosidase assays using *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) respectively were performed. In pMIS5 (Vílchez et al., 2000a) the P_{putA} promoter was cloned before the *'lacZ* gene in plasmid pMP220

(Spaink et al., 1987) and in pPAH the P_{pahA} promoter was controlling the expression of the *'lacZ* gene (Segura et al., 2017). Chloramphenicol (Cm), rifampicin (Rif) and tetracycline were added to the bacterial cultures at final concentrations of 30, 10 and 10 µg/mL respectively when it was necessary (Table 2.1).

Table 2.1 Bacteria, plasmids and plants used. Ap^r, Cm^r, Rif^r and Tc^r refer to resistance to ampicillin, chloramphenicol, rifampicin and tetracycline respectively

Bacterial strains, plasmids and plant genotypes	Relevant characteristics	Reference or source
Bacterial strains		
P. putida KT2440	Wild type; Ap^r, Cm^r	Franklin et al. 1981
P. putida KT2442	Rif^r derivative of KT2440	Franklin et al. 1981
Novosphingobium sp. HR1a	Wild type; Tc^r	Segura et al. 2017
Plasmids		
pMIS5	Tc^r, P_{putA}*::'lacZ ori*RK2	Vílchez et al. 2000
pMP220	Tc^r, *'lacZ ori*RK2	Spaink et al. 1987
pPAH	Tc^r, P_{pahA}*::'lacZ ori*RK2	Segura et al. 2017
Plant genotypes		
Carrizo citrange	Salt sensitive, heat tolerant	Vives-Peris et al. 2017
Citrus macrophylla	Salt moderate tolerant, heat sensitive	Vives-Peris et al. 2017

Bacterial growth assays

Bacteria were cultured in glass tubes with 2 mL of liquid M8 minimal medium (Kohler et al., 2000) supplemented with 20 mM succinate as carbon source and root exudates at the original concentrations. For this, 15 mL of root exudates were freeze-dried and resuspended in 1.5 mL of sterile deionized water, being diluted 10 times when applied to the bacterial medium. Mocks with bacteria growing in M8 minimal medium containing 20 mM succinate, and supplemented with 60 or 90 mM NaCl were used to consider the salt effect on the bacteria. The concentration of the bacterial inoculum was adjusted to OD_{660nm} of 0.1. Bacterial cultures were incubated at 30 ºC in an orbital shaker at 200 rpm during 48 h.

Quantification of bacterial growth was performed both, by assessing OD_{660nm} and by counting the colony forming units (CFU). OD at a wavelength of 660 nm was recorded with a 96 well microplate spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland). Results were expressed as the variation of OD_{660nm} in comparison with the respective mock and referred to the root fresh weight (Doornbos et al., 2011; Neal et al., 2012). CFU was determined by plating appropriate bacterial culture dilutions on lysogeny broth medium (LB) supplemented with Cm and Rif for *P. putida* KT2440 and

Novosphingobium sp. HR1a cultures respectively (Bertani, 1951). CFU were counted after 24 h of incubation at 30°C (Goldman and Green, 2008).

Induction of the PputA and PpahA promoters by different metabolites

P. putida KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH), were cultured in presence of commercial standards of different metabolites related to proline and salicylates catabolism. *P. putida* KT2442 (pMIS5) was grown in M9 basal plus 20 mM succinate media supplemented with L-proline and hydroxy-L-proline, whereas *Novosphingobium* sp. HR1a (pPAH), was grown in M9 basal media plus 20 mM succinate supplemented with salicylic acid biosynthesis and conjugation pathways compounds, including methyl salicylate, sodium salicylate, L-phenylalanine, sodium benzoate, *p*-coumaric acid and *t*-cinnamic acid (Fig. Sup. 1). Tubes containing 2 mL of M9 medium plus 20 mM succinate supplemented with the standards at concentrations of 0.1 and 1 mM were inoculated to an initial OD_{660nm} of 0.1 and they were cultured at 30 °C in an orbital shaker. β-Galactosidase activity was measured after 7 and 24 hours.

β-Galactosidase activity measurement

β-Galactosidase activity was measured in *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) bacteria cultured in M8 minimal medium supplemented with 20 mM succinate and root exudates. β-Galactosidase activity was determined as described in (Miller, 1972). This methodology is based in the colorimetric reaction between *ortho*-nitrophenyl-β-galactoside (ONPG), a colorless substrate of β-galactosidase, which is hydrolyzed by this enzyme producing galactose and *o*-nitrophenol, which has a yellow color, and its concentration can be determined colorimetrically. Briefly, 1 mL of the bacterial culture was centrifuged, and the supernatant was removed. The cells were diluted in 1 mL of buffer Z with 2.7 mL L^{-1} of 2-mercaptoethanol, and the turbidity of this solution was spectrophotometrically measured at 660 nm. After this, 500 µL of the cell suspension in buffer Z were mixed with 100 µL of buffer Z in *P. putida* KT2442 (pMIS5), while 100 µL of the suspended cells in buffer Z were mixed with 500 µL of Buffer Z in *Novosphingobium* sp. HR1a (pPAH). 20 µL of chloroform and 20 µL of 0.1% SDS were added and the samples were

mixed with a vortex. Samples were incubated for 10 minutes at 30 ºC with agitation at 350 rpm, and 100 µL of ONPG were added as a substrate for the detection of β-galactosidase activity. When samples acquired a yellowish color, the reaction was stopped by adding 260 µL of Na_2CO_3 0.5M and the turbidity was measured at 420 nm. Finally, results of β-galactosidase activity were calculated in Miller units, and the results were normalized according to the root fresh weight.

Statistical analyses

Statistical analyses were assessed with the Statgraphics Plus v.5.1. Software (Statistical Graphics Corp., Herndon, VA, USA). Data are means of three independent replications and were subjected to one- or two-way analysis of variance (ANOVA) and a Tukey posthoc test ($p \leq 0.05$) when statistical significant differences were detected.

Results

Bacterial tolerance to saline conditions

In absence of root exudates, the growth of both bacterial strains was reduced during the first 8 and 2 hours in *P. putida* KT2440 and *Novosphingobium* sp. HR1a, respectively. After these periods, the cultures of both bacteria increased their turbidity, although some differences were observed depending on the salt treatment and the bacterial strain. The addition of NaCl to M8 minimal medium supplemented with 20 mM succinate had a negative effect on the turbidity of cultures of both bacterial strains, *P. putida* KT2440 and *Novosphingobium* sp. HR1a regardless the severity of the imposed stress (60 or 90 mM NaCl) in comparison with cultures growth in absence of NaCl. The diminution of the OD_{660nm} in cultures with 90 mM NaCl was sharper in the case of *P. putida* KT2440 (34.92% with respect to the control without NaCl) whereas in *Novosphingobium sp.* HR1a the decrease was of 19.07% after 24 hours. In *P. putida* KT2440 salt treatment affected cultures turbidity from 3 hours until the end of the experiment. Differently, the turbidity of *Novosphingobium sp.* HR1a cultures was only affected after 24 hours in presence of 90 mM NaCl, and after 48 hours in 60 mM NaCl treatment (Fig. 2.1).

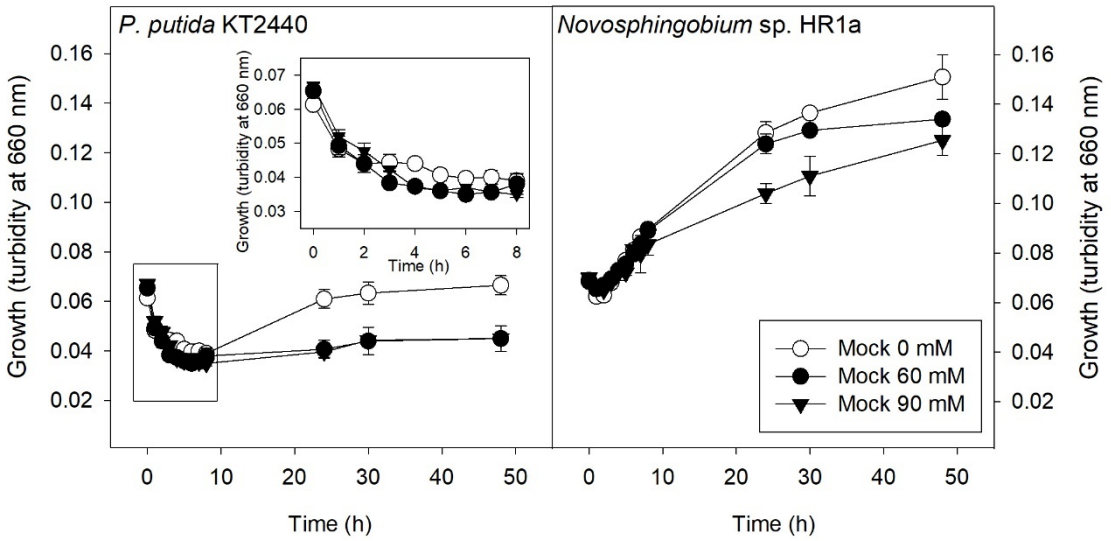

Figure 2.1 Turbidity at OD_{660nm} of cultures of *P. putida* KT2440 and *Novosphingobium* sp. HR1a in presence of 0, 60 and 90 mM NaCl in M8 medium plus 20 mM succinate. White circles refer to control; black circles refer to 60 mM NaCl and black triangles refer to 90 mM NaCl treatments. Values indicate the mean of three replicates ± standard error

Similar results were observed in the number of CFU after 6 hours of culture, when a decrease of this parameter was observed in cultures of *P. putida* KT2440, with a diminution of CFU values of 52.00 and 98.52% in bacteria grown with 60 and 90 mM, respectively, in comparison to control cultures, while no differences were observed in cultures of *Novosphingobium* sp. HR1a in presence of NaCl (Fig. 2.2).

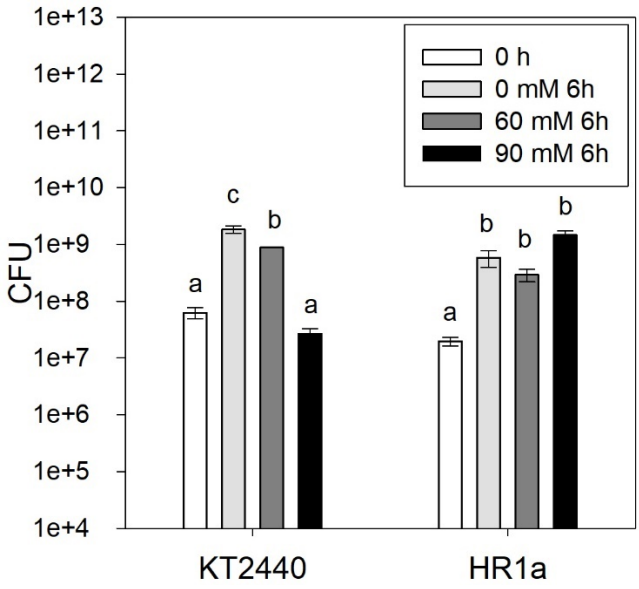

Figure 2.2 Number of CFU of cultures of *P. putida* KT2440 and *Novosphingobium* sp. HR1a in presence of 0, 60 and 90 mM NaCl in M8 medium plus 20 mM succinate, after 6 hours. White bars refer to control at 0 hours; light grey, dark grey, and black bars refer to cultures in presence of 0, 60 and 90 mM, respectively. Values indicate the mean of three replicates ± standard error

Bacterial growth in presence of citrus root exudates

Root exudates obtained from Carrizo citrange and *C. macrophylla* slightly promoted the growth of *P. putida* KT2440 and *Novosphingobium* sp. HR1a under normal growth conditions. However, exudates obtained from salt- and heat-stressed plants of both genotypes supported the growth of both strains, although this positive effect was more evident in presence of exudates from Macrophylla plants.

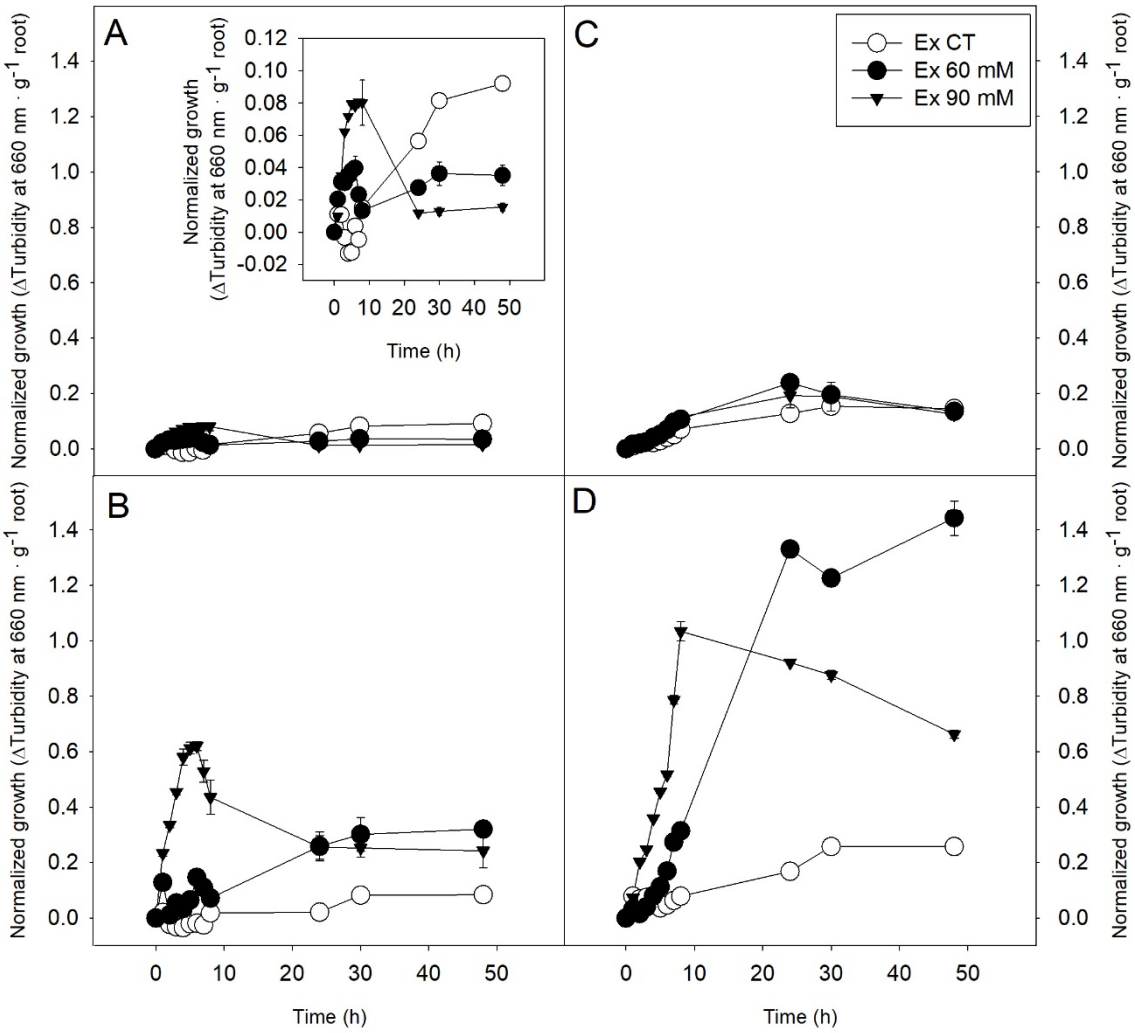

Figure 2.3 Effect of root exudates from Carrizo citrange (A and C) and *C. macrophylla* (B and D) plants subjected to salt stress in the growth of *P. putida* KT2440 (A and B) and *Novosphingobium* sp. HR1a (C and D) in M8 medium plus 20 mM succinate. White circles refer to control; black circles and black triangles refer to root exudates from plants subjected to 60 and 90 mM NaCl, respectively. Values indicate the mean of three replicates ± standard error

Root exudates from Macrophylla plants subjected to 90 mM NaCl had a clear positive effect in the growth of *P. putida* KT2440 during the first 8 hours in culture and also in *Novosphingobium* sp. HR1a, with an increase in the turbidity at 660 nm of 23.41 and

13.10 times higher than those obtained from control plants, respectively (Fig. 2.3B and 2.3D). When exudates were obtained from Carrizo plants subjected to 90 mM NaCl, it was also observed a growth increase, but it was not as marked as the observed in presence of Macrophylla exudates, with values 5.28 and 1.61 times over the control in cultures of *P. putida* KT2440 and sp. HR1a, respectively (Fig. 2.3A and 2.3C). After 24 hours, the increase of the turbidity per gram of root in cultures of *Novosphingobium* sp. HR1a in presence of root exudates from Macrophylla salt-stressed plants reached values 13.10 times higher than the growth of bacteria treated with control exudates. With exudates from Carrizo, this increase was of 1.61 times the control after 24 hours (Fig. 2.3C and 2.3D).

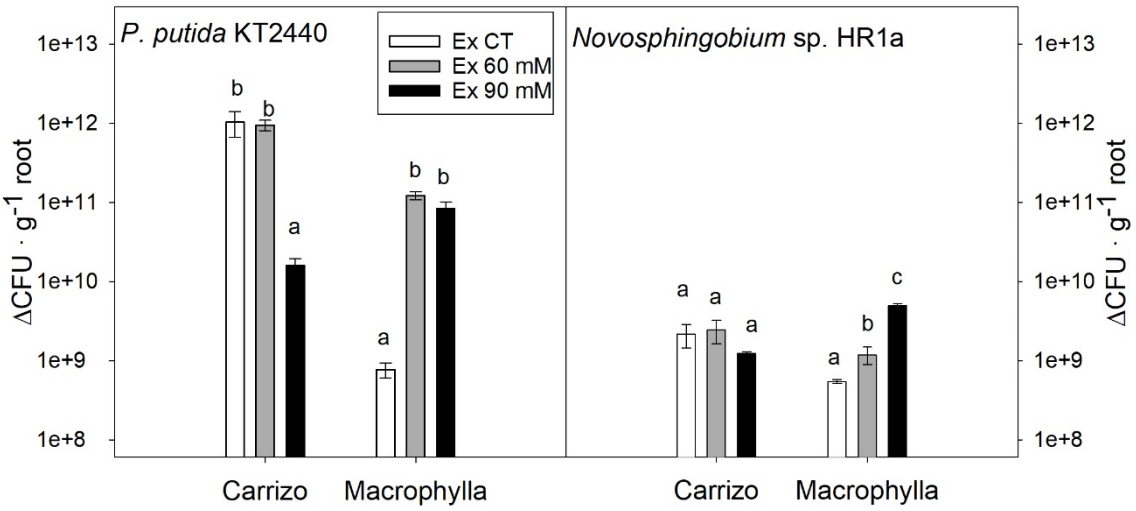

Figure 2.4 Effect of root exudates from Carrizo citrange and *C. macrophylla* salt-stressed plants in the increase of the number of CFU of *P. putida* KT2440 and *Novosphingobium* sp. HR1a in M8 medium with 20 mM succinate after 6 hours. White bars refer to control; grey bars refer to cultures with root exudates from 60 mM NaCl stressed plants, and black bars refer to cultures with root exudates from 90 mM NaCl stressed plants. Values are normalized according to root fresh weight and indicate the mean of three replicates ± standard error. Different letters to statistically significant differences at $P \leq 0.05$

Root exudates also affected differentially the number of CFU of both bacterial strains after 6 hours (Fig. 2.4). In both cases, CFU increased when root exudates from salt-stressed plants were added to the medium. This increase was different depending on the citrus genotype source of exudates. In *P. putida* KT2440, root exudates from Macrophylla salt-stressed plants increased the number of CFU reaching levels 157.82 and 108.48 times higher than control in 60 and 90 mM NaCl, respectively. Root exudates obtained from Carrizo stressed plants only affected CFU number in 90 mM NaCl, with CFU levels 98.46% lower than control. Similarly, the number of CFU also increased in cultures of

Novosphingobium sp. HR1a in presence of root exudates from Macrophylla salt-stressed plants, with values 157.82 and 108.48 times higher than control when root exudates were obtained from plants of this genotype subjected to 60 and 90 mM NaCl respectively. However, in presence of Carrizo root exudates, no differences were observed in the number of CFU in cultures of this bacterial strain.

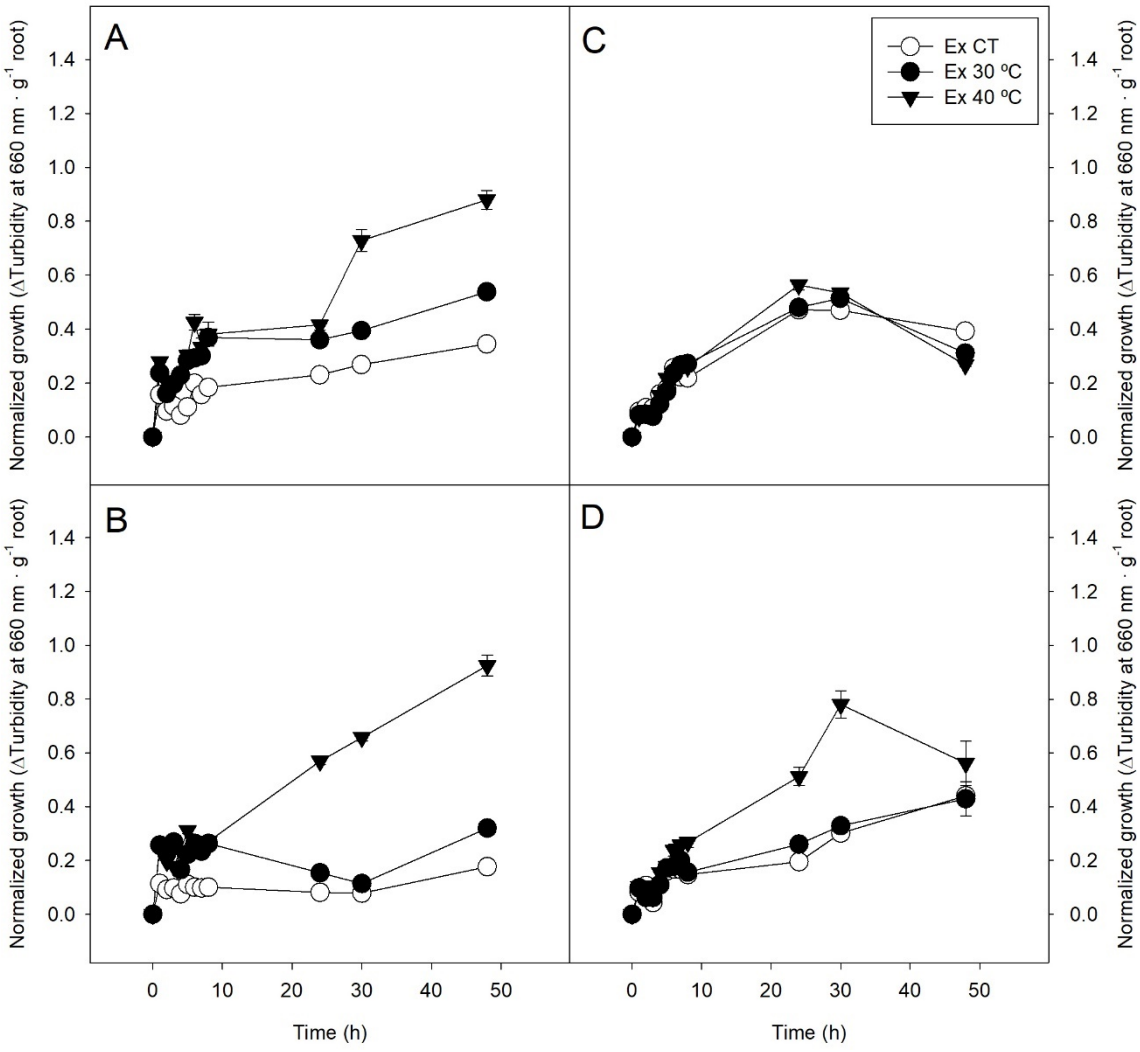

Figure 2.5 Effect of root exudates from Carrizo citrange (A and C) and *C. macrophylla* (B and D) plants subjected to heat stress in the growth of *P. putida* KT2440 (A and B) and *Novosphingobium* sp. HR1a (C and D) in M8 medium with 20 mM succinate. White circles refer to control; black circles and black triangles refer to root exudates from plants subjected to 30 ºC and 40 ºC, respectively. Values indicate the mean of three replicates ± standard error

Interestingly, exudates obtained from Carrizo and Macrophylla plants stressed at 40ºC were able to support growth of *P. putida* KT2440, while *Novosphingobium* sp. HR1a only grew when exudates from Macrophylla plants stressed at 40ºC were added, in comparison with cultures grown with exudates from control plants (Fig. 2.5). Macrophylla exudates

induced a higher growth increase in *P. putida*, reaching values 8.34 times higher than those observed in presence of control exudates after 30 hours. Root exudates from Carrizo heat-stressed plants at 40 ºC the increase of growth was 2.71 times higher than in presence of control exudates at 30 hours. Meanwhile, in the case of *Novosphingobium* sp. HR1a, this increase was not as marked as the observed in *P. putida* KT2440, reaching its maximum at 30 hours, when the root exudates from Macrophylla plants stressed at 40 ºC induced an increase of 2.58 times over the control. Root exudates from Carrizo plants cultured at 40ºC did not affect differently the *P. putida* KT2440 growth.

Results were different when exudates were obtained from heat-stressed plants. The number of CFU after 24 hours in cultures of *P. putida* KT2440 was only affected in presence of root exudates from Macrophylla plants subjected at 40ºC showing values 43.23 times higher than in presence of root exudates obtained from control plants. However, the number of *Novosphingobium* sp. HR1a CFU was not significantly affected by root exudates from any of both rootstocks after 24 hours (Fig. 2.6).

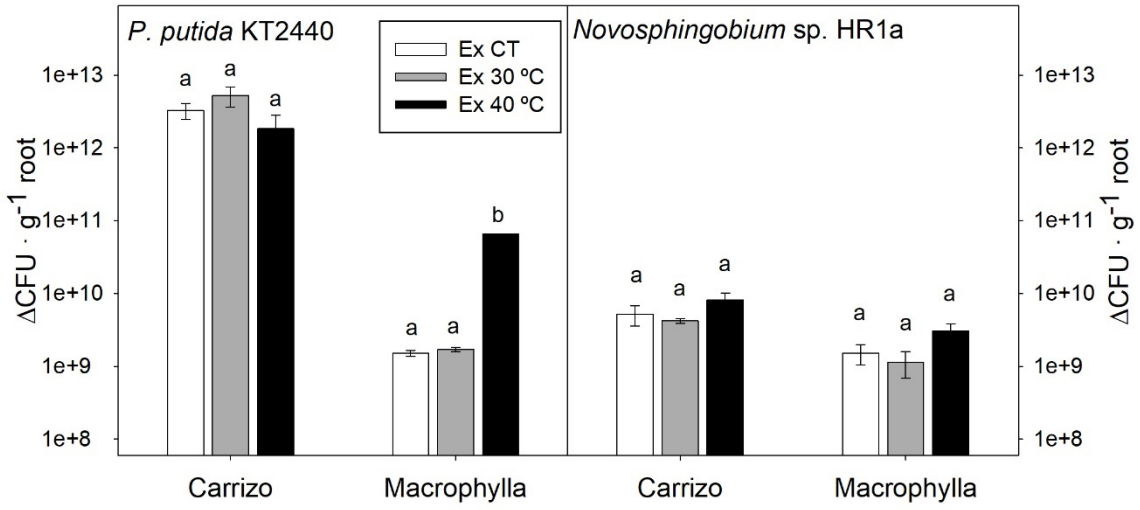

Figure 2.6 Effect of root exudates from Carrizo citrange and *C. macrophylla* heat-stressed plants in the increase of the number of CFU of *P. putida* KT2440 and *Novosphingobium* sp. HR1a in M8 medium with 20 mM succinate after 24 hours. White bars refer to control; grey bars refer to cultures with root exudates from 30 ºC stressed plants, and black bars refer to cultures with root exudates from 40 ºC stressed plants. Values are normalized according to root fresh weight and indicate the mean of three replicates ± standard error. Different letters to statistically significant differences at $P \leq 0.05$

Differential induction of the P_{putA} and P_{pahA} promoters involved in proline and salicylates detection

β-Galactosidase activity in *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) cultures was affected differently depending on the commercial standards added to the culture medium (Figs. 2.7 and 2.8).

In cultures of *P. putida* KT2442 (pMIS5), β-galactosidase activity was only affected in presence of both compounds, L-proline and hydroxy-L-proline, at 1 mM concentration. L-proline induced a higher increase of this enzymatic activity than hydroxy-L-proline did, reaching values 7.28 and 11.71 times higher than control after 7 and 24 hours respectively, while 1 mM hydroxy-L-proline only affected β-galactosidase activity after 24 hours, with values 2.73 times higher than control (Fig. 2.7).

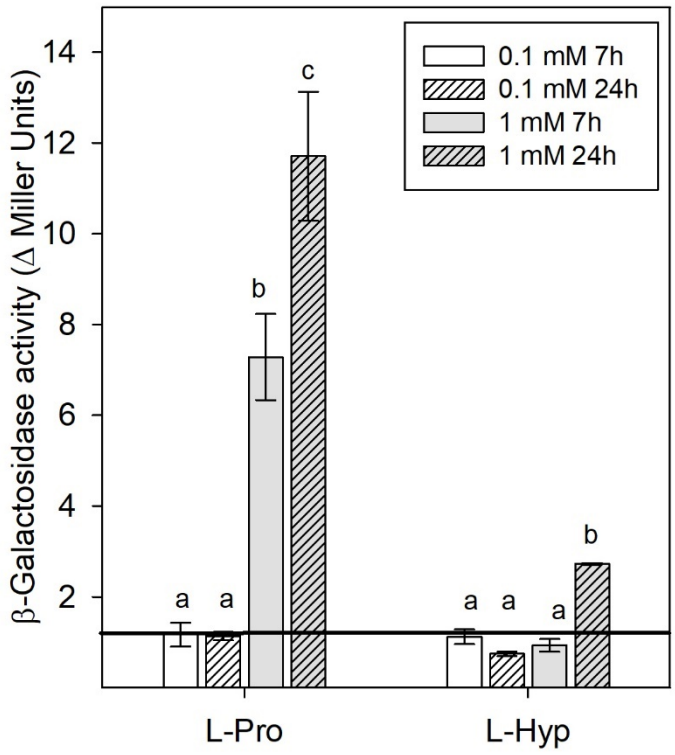

Figure 2.7 β-Galactosidase activity of cultures of *P. putida* KT2442 (pMIS5) supplemented with L-Proline (L-Pro) and L-Hydroxyproline (L-Hyp) at concentrations of 0.1 mM (white bars) and 1 mM (grey bars) after 7 (non-lined bars) and 24h (lined bars). Values are referred to mock culture and indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Regarding on β-galactosidase activity of *Novosphingobium* sp. HR1a (pPAH), it was increased by 0.1 mM methyl salicylate and sodium salicylate after 24 hours (2.74 and 5.48-fold increase compared to the control), and sodium benzoate after 7 hours, with

values 3.42 and 2.88 times higher than control at concentration 0.1 and 1 mM, respectively (Fig. 2.8).

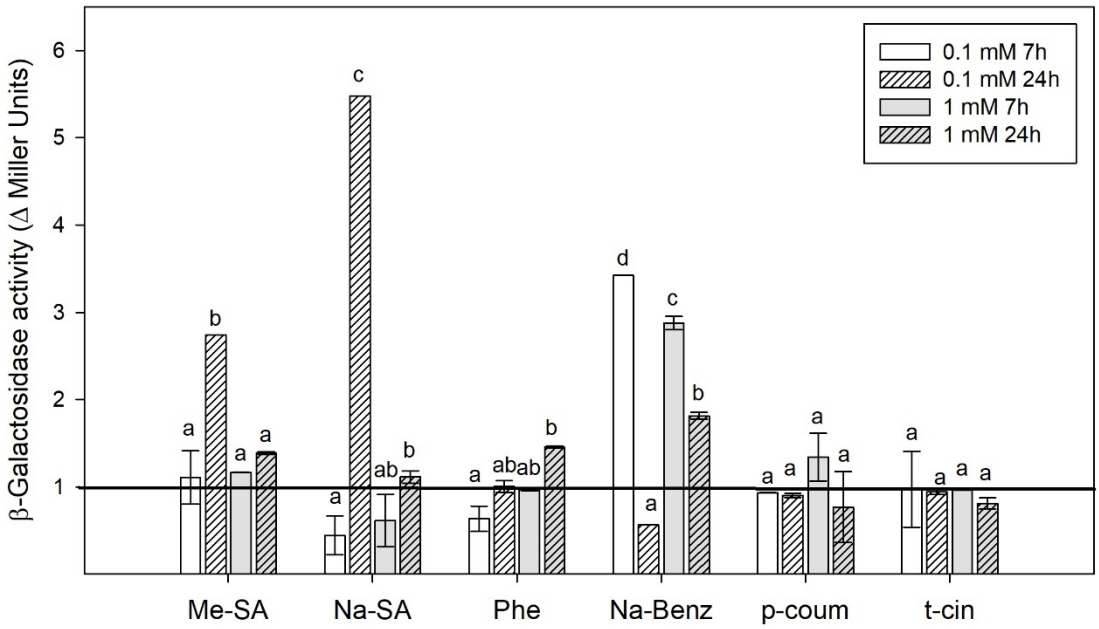

Figure 2.8 β-Galactosidase activity of cultures of *Novosphingobium* sp. HR1a (pPAH) supplemented with methyl salicylate (Me-SA), sodium salicylate (Na-SA), L-phenylalanine (Phe), sodium benzoate (Na-Benz), *p*-coumaric acid (p-coum) and *t*-cinnamic acid (t-cin) at concentrations of 0.1 mM (white bars) and 1 mM (grey bars) after 7 (non-lined bars) and 24h (lined bars). Values are referred to mock culture and indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

β-Galactosidase activity in presence of citrus root exudates

β-Galactosidase activity was measured at 7 and 24 hours after the inoculation of bacterial cultures with root exudates. Differences in β-galactosidase activity in cultures of *P. putida* KT2442 (pPAH) and *Novosphingobium* sp. HR1a (pPAH) cultures supplemented with root exudates from salt or heat-stressed citrus plants were detected depending on the applied stress and the plant genotype.

β-Galactosidase activity increased in *P. putida* KT2442 (pMIS5) cultures grown in presence of root exudates obtained from salt-stressed plants after 7 hours in culture (Fig. 2.9). Exudates from Carrizo salt-stressed plants induced an increase of this enzymatic activity (1.91- and 2.37-fold increase with respect the controls in 60 and 90 mM NaCl, respectively. The enzymatic activity increase was higher in cultures supplemented with root exudates obtained from Macrophylla, reaching values 6.17 and 7.26 times higher than control with exudates from plants treated with 60 and 90 mM NaCl, respectively. Root exudates from salt-stressed plants only those exudates obtained from Macrophylla

increased the β-galactosidase activity of *Novosphingobium* sp. HR1a (pPAH), reaching values of 6.86 and 1.40 times higher than the control for 60 and 90 mM NaCl.

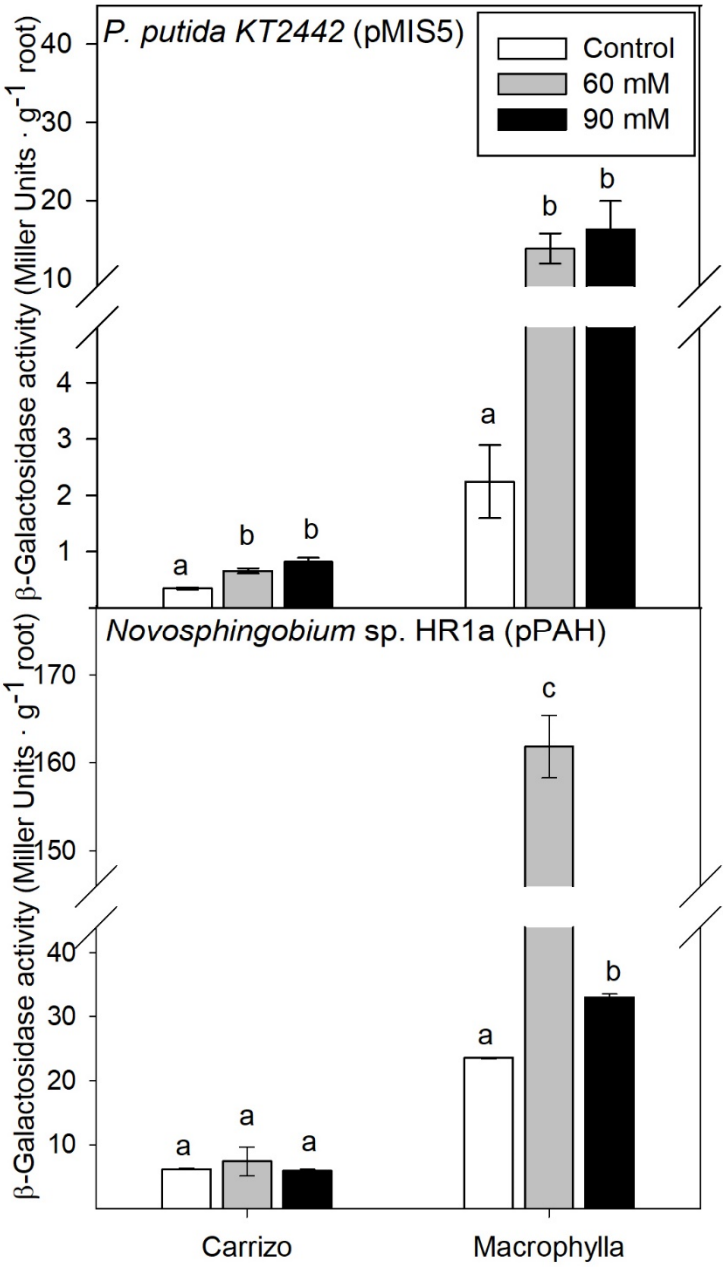

Figure 2.9 β-Galactosidase activity of *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) grown with root exudates from citrus plants subjected to salt stress for 10 days. β-Galactosidase activity was measured at 7 hours. White bars refer to control; grey bars refer to 60 mM NaCl, and black bars refer to 90 mM NaCl. Values indicate the mean of three replicates ± standard error. Different letters to statistically significant differences at $P \leq 0.05$

P. putida KT2442 (pMIS5) cultures supplemented with root exudates from Carrizo heat-stressed plants had enzymatic activity values 1.08 and 1.69 times higher than control when plants had been maintained at 30 and 40 ºC plants, respectively (Fig. 2.10).

Conversely, root exudates from heat-stressed Macrophylla plants did not induce any change in β-galactosidase activity. Only root exudates from plants subjected to 40ºC increased β-galactosidase activity in cultures of *Novosphingobium* sp. HR1a (pPAH) in comparison with control root exudates. This increase was similar in cultures with root exudates from both citrus genotypes, being 1.25 and 1.15 higher than their controls in cultures with root exudates from Carrizo and Macrophylla, respectively.

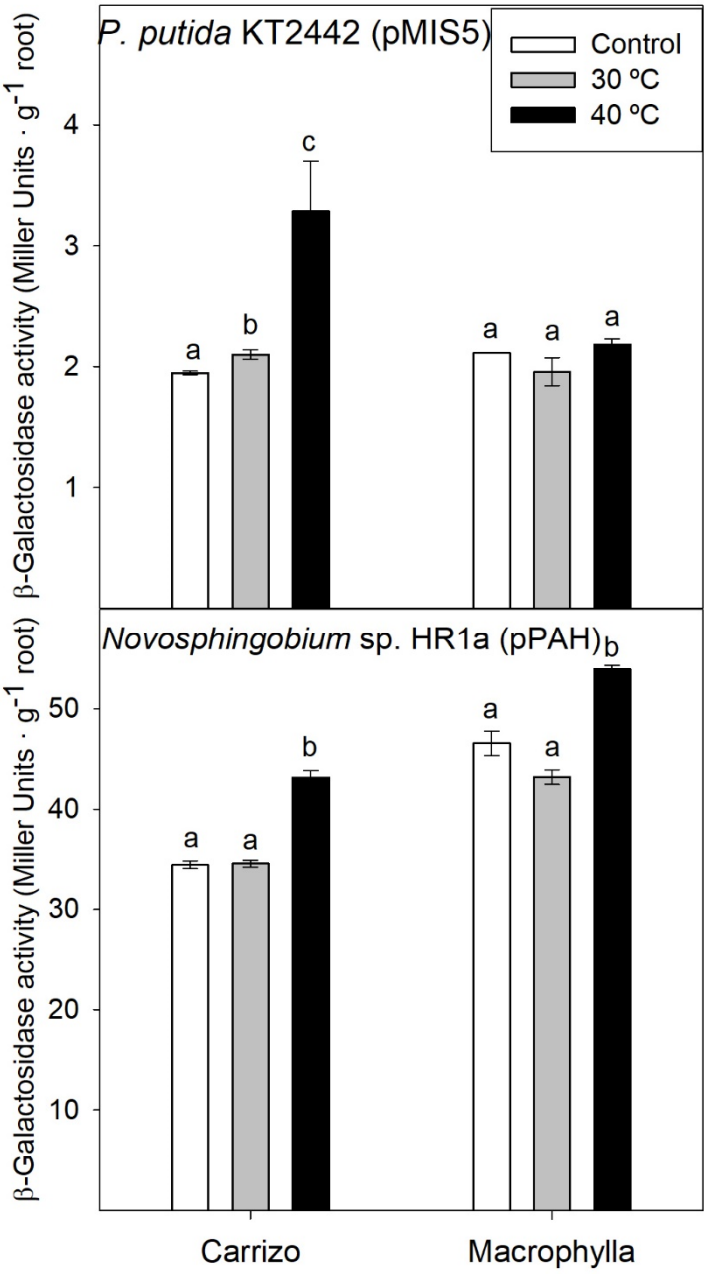

Figure 2.10 β-Galactosidase activity of *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) grown with root exudates from citrus plants subjected to heat stress for 10 days. β-Galactosidase activity was measured at 24 hours. White bars refer to control, grey bars refer to 30 ºC, and black bars refer to 40 ºC. Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Discussion

In this work, the effect of root exudates from citrus plants subjected to salt or heat stress on plant beneficial rhizobacteria *P. putida* KT2440 and *Novosphingobium sp.* HR1a has been studied. The study was performed using root exudates from two genotypes differing in their tolerance to salt and heat stress: while Carrizo is a salt-sensitive but heat-tolerant citrus rootstock, Macrophylla is salt-stress tolerant but heat-sensitive (Vives-Peris et al., 2017).

Although the role of *P. putida* as PGPR has been well established in a wide variety of plants as coffee, cocoa or coconut (Kejela et al., 2017; Khadeejath Rajeela et al., 2017), to our knowledge there is no information related the possible effect of *Novosphingobium* sp. HR1a on plants, although other strains of this genus as *Novosphingobium oryzae* or *Novosphingobium pokkalii* have been reported as PGPRs (Krishnan et al., 2017; Zhang et al., 2016).

Our results revealed that citrus root exudates promote the growth of both rhizobacterial strains when they are grown in a minimal medium without any nitrogen source apart from root exudates. This positive effect on bacterial growth can be due to the presence of extra carbon and nitrogen inputs by root exudates, generally as part of sugars and amino acids, as it has been reported by previous studies where root exudates from some plants, such as cucumber or banana, promote growth and biofilm formation of PGPRs *Bacillus amyloliquefaciens* SQR9 and *Bacillus subtilis* N11 growth (Zhang et al., 2014). However, growth promotion can be also due to the presence of other metabolites secreted to the rhizosphere in lower concentration, such as hormones or growing factors which are differentially exuded, both quantitatively and qualitatively, depending on the citrus genotype (Vives-Peris et al., 2017).

The presence of 60 and 90 mM NaCl in the culture medium inhibited bacterial growth in both strains in comparison with mocks without salt (Figs. 2.1 and 2.2). These results are in concordance with other works which confirm that size and activity of the soil microbial community is reduced under soil salinity and sodicity induced by irrigation, resulting in a reduction in the soil organic matter decomposition and the mineralization of carbon, nitrogen, sulphur and phosphorus (Rietz and Haynes, 2003). This negative effect is more evident in *P. putida* KT2440 than in *Novosphingobium* sp. HR1a, which was originally

isolated from seaside soils which commonly present high salt levels, sustaining that this strain has developed adaptive mechanisms to tolerate high salinity (Segura et al., 2017).

Therefore, it was considered essential to understand how exudates from plants subjected to salt stress could affect the growth of both rhizobacteria. Results indicate that exudates promoted this growth, even at higher levels than control exudates, during the first growth stages, being this beneficial effect higher when exudates come from the salt tolerant rootstock Macrophylla than in those obtained from Carrizo plants (Fig. 2.3). Moreover, the promotion of the development in *Novosphingobium* sp. HR1a was higher than in *P. putida* KT2440, which could be due to the higher tolerance of this strain to high salinity. The number of CFU in both bacterial strains increased in presence of root exudates from salt-stressed plants, being this beneficial effect higher in presence of root exudates from the salt tolerant genotype Macrophylla (Fig. 2.4). However, some disagreements were observed among the results observed with the turbidity measurement at 660 nm and the obtained from the measure of CFU, which could be consequence of an increase in the turbidity measurements due to the presence of lipopolysaccharides from the rhizobacteria, which can affect the turbidity determinations at this wavelength (Sakai et al., 2003). These data reveal that root exudates from the salt-tolerant genotype Macrophylla have a higher potential to promote the growth of both rhizobacteria, which could contribute to a better colonization by these bacteria and the alleviation of salt stress to the plant, as it has been reported that *P. putida* and *Novosphingobium* sp. are able to mitigate the adverse effects of this stress in herbaceous plants (He et al., 2017; Krishnan et al., 2017; Yao et al., 2010). Moreover, the higher influence of exudates from stressed plants could be due to a higher exudation of several compounds of the primary and secondary metabolisms, not only proline or phytohormones, but also other metabolites, as occurs with root exudates from *Glycine max* (soybean) and *Phaesolus vulgaris* (common bean), which have been also described as promoters of *Chryseobacterium balustinum* growth (Dardanelli et al. 2010, 2012). Previous studies with citrus plants subjected to different abiotic stresses as drought or high temperatures, have demonstrated that tolerant rootstocks have an enhanced antioxidant system (Zandalinas et al., 2017). In addition, since tolerant citrus plants subjected to salt stress maintain a higher photosynthetic rate than sensitive plants, being this parameter directly linked with the exudation rate, the exudation of metabolites obtained from the C fixed in the photosynthesis which could be used as nutrient sources by PGPRs would be increased (Bais et al., 2006; López-Climent et al., 2008). Finally, the

higher concentration of antioxidant compounds in tissue which could be related to their higher exudation, promoting the growth of PGPRs and play an important role in the mutualism among citrus and PGPRs.

When the root exudates were obtained from heat-stressed plants, those obtained from plants subjected to 30 ºC did not affect the growth of either *P. putida* KT2440 or *Novosphingobium* sp. HR1a, but root exudates from plant subjected to 40 ºC stimulate growth of both strains, principally in presence of root exudates from the heat sensitive Macrophylla (Fig. 2.5). The lack of differences in the growth of both strains in presence of root exudates from plants subjected to 30 ºC may be due to the fact that this temperature is not high enough to induce a severe stress in plants (considering that citrus is a subtropical crop) and consequently it does not affect root exudation as much as salt stress or higher temperatures such as 40 ºC (Vives-Peris et al. 2017). This difference in the composition of exudates from heat-stressed Macrophylla plants could be consequence to its higher sensitivity to this stress condition, resulting in an earlier modification of root exudate composition in this genotype in a lower threshold temperature. In addition, the higher growth of both strains in presence of exudates from salt-stressed plants in comparison with those obtained from heat-stressed ones can be due to the different character of each stress: whereas salt stress affects first to the root system, originating directly changes in this organ, heat stress mainly affects shoots, exercising the soil a buffer effect which attenuates the affection of heat stress in the root system (Zhang et al., 2005).

The differential induction of P_{putA} and P_{pahA} promoters with commercial standards revealed that the construction *P. putida* KT2442 (pMIS5) is specific for the detection of proline (Fig. 2.7), whereas the treatments with commercial standards of salicylates in *Novosphingobium* sp. HR1a (pPAH) exposed that the expression from *pahA* promoter is induced in presence of several salicylates, including methyl salicylate, sodium salicylate and sodium benzoate. Induction of the *pahA* promoter (that drives the expression of the dioxygenase involved in polycyclic aromatic hydrocarbons (PAHs) degradation in *Novosophingobium* sp. HR1a) by salicylate was previously demonstrated (Segura et al., 2017), in concordance with the capacity of *Novosphingobium* sp. HR1a to degrade PAHs *via* this aromatic compound, but our experiments demonstrate that different intermediates in the SA biosynthetic pathway in plants are able to induce its expression (Fig. 2.8), probably via salicylate.

Since previous studies reported that there is an increase in proline and salicylates content of citrus root exudates from plants subjected to salt stress (Vives-Peris et al., 2017), the expression patterns of bacterial genes related to the presence of these compounds was studied. The results of β-galactosidase activity analyses in *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) demonstrated that both bacterial strains are able to detect proline and salicylates presents in root exudates respectively, since these are promoters from the genes *putA* and *pahA* from both bacteria related to the detection of these compounds (Segura et al., 2017; Vílchez et al., 2000a). Moreover, different β-galactosidase activity was obtained depending on the rootstock and the applied stress (Figs. 2.9 and 2.10) which are in concordance with proline and SA contents measured in root exudates from citrus plants subjected to salt and heat stress (Vives-Peris et al., 2017), suggesting that the exuded proline and SA could be susceptible of being detected by these constructions and could be used as nutrient sources by soil microorganisms.

To conclude, this work reveals the positive effect of citrus root exudates in the growth of two different PGPRs, *P. putida* KT2440 and *Novosphingobium* sp. HR1a, which increases when they are obtained from salt- or heat-stressed plants. Moreover, root exudates from plants subjected to both stresses induce the expression of *putA* and *pahA* genes in both bacterial strains, revealing that they are able to detect proline and salicylates present in root exudates. The concordance between β-galactosidase activity and proline and salicylates contents quantified by other methods reveals that *P. putida* KT2442 (pMIS5) and *Novosphingobium* sp. HR1a (pPAH) can be respectively used for the detection of proline and salicylates in root exudates. On the other hand, this work reveals the importance of root exudates in the growth of PGPRs, which could be used as a fertilizer to improve PGPR colonization and therefore trigger plant benefits.

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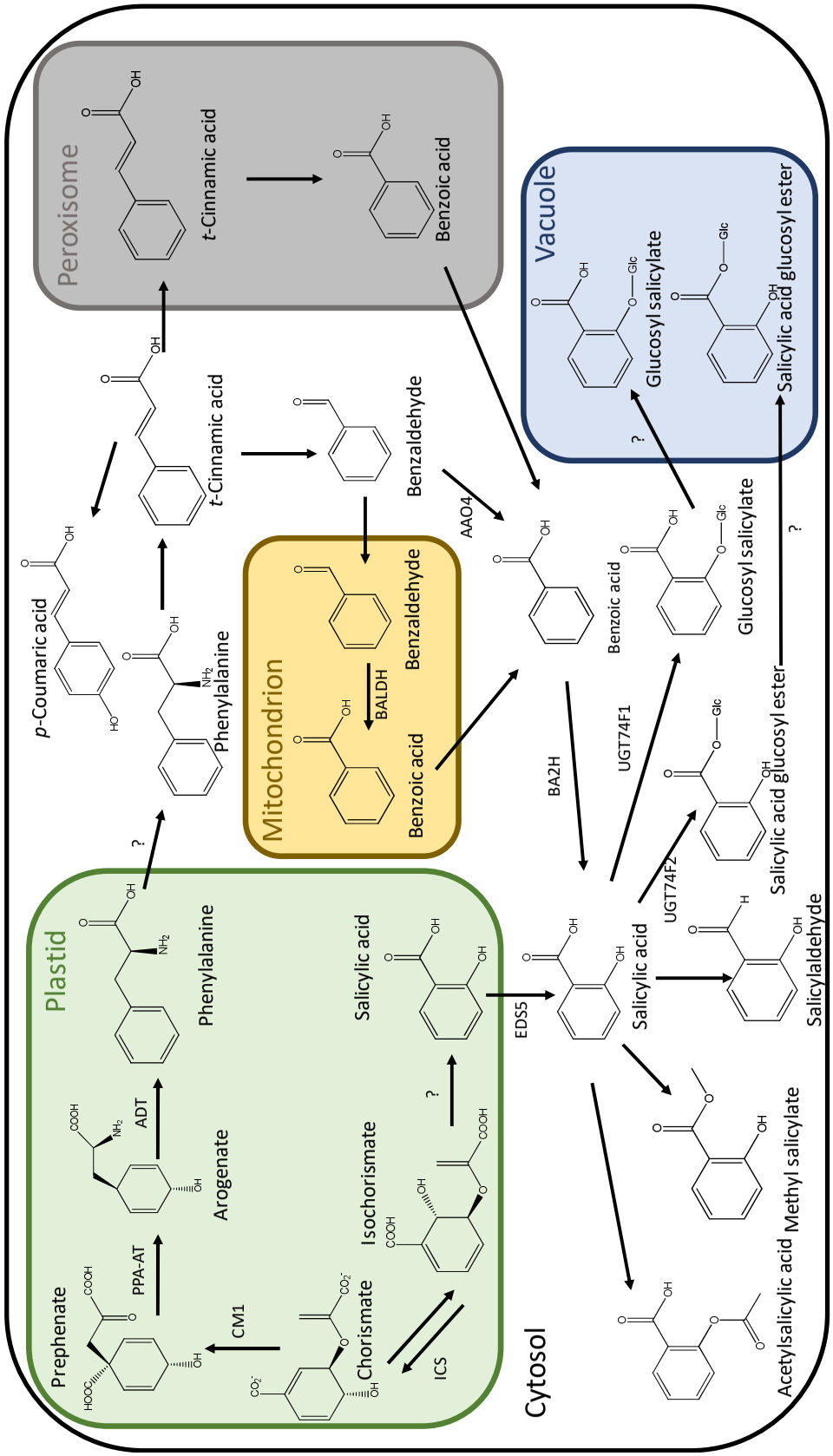
Supplementary material



Supplementary Figure S2.1 Salicylic acid biosynthesis and conjugation in plants. Adapted from Widhalm and Dudareva 2015

CHAPTER 2

Results

Chapter 3

Salt stress alleviation in citrus plants by plant growth promoting rhizobacteria *Pseudomonas putida* and *Novosphingobium* sp.

Salt stress alleviation in citrus plants by plant growth promoting rhizobacteria *Pseudomonas putida* and *Novosphingobium* sp.

Abstract

Salt stress is a kind of abiotic stress which effects can be exacerbated in the near future due to climate change. In this context, plant growth promoting rhizobacteria (PGPRs) can mitigate the negative effects of these stresses through different mechanisms. In this work, *Citrus macrophylla* (alemow) plants inoculated with the rhizobacteria *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a were subjected to salt stress for 30 days and the role of both bacterial strains in plant protection against salt stress was evaluated. Moreover, salt-stressed inoculated plants exhibited lower endogenous contents of chloride ions, proline, abscisic acid (ABA) and salicylic acid (SA) than non-inoculated stressed plants. The inoculation with both rhizobacterial strains in absence of salt stress induced a decrease of gas exchange parameters as transpiration (E) and stomatal conductance (g_s), contributing to a higher water use efficiency. Moreover, although chlorophyll fluorescence parameters as quantum yield (ΦPSII) and maximum efficiency of photosystem II photochemistry (F_v/F_m ratio) were reduced due to salt stress, the inoculation with these rhizobacteria inhibited the reduction of this parameters. All these changes leaded to a reduction of leaf abscission and the appearance of the symptoms caused by salt stress Although both bacterial species had a beneficial effect in salt-stressed citrus plants, results point out that *Novosphingobium* sp. HR1a induces more resistance to salt stress than *P. putida* KT2440. By this, our results postulate *Novosphingobium* sp. HR1a as a PGPR and demonstrate that the inoculation with PGPRs through programs of biofertilization and bioaugmentation, as well as biostimulation can improve plant tolerance under abiotic stress conditions, avoiding the utilization of chemicals for citrus plants protection against salt stress conditions.

Keywords: citrus, *Novosphingobium*, plant growth promoting rhizobacteria, *Pseudomonas*, salt stress

Abbreviations: ABA: Abscisic Acid; ACC: 1-aminocyclopropane-1-carboxylate; AMF: Arbuscular Mycorrhizal Fungi; CFU: Colony Forming Units; DAPG: 2,4-

diacetylphloroglucinol; E: Transpiration; F_v/F_m: Maximum efficiency of photosystem II; g_s: stomatal conductance; IAA: 3-Indole Acetic Acid; NI: Non-Inoculated; NO: Nitric Oxide; PGPR: Plant Growth Promoting Rhizobacteria; Φ_{PSII}: Quantum efficiency of PSII photochemistry; SA: Salicylic Acid.

Introduction

In nature, plants are constantly subjected to a wide variety of abiotic and biotic stress conditions which can reduce their growth and productivity. Moreover, climate change exacerbates the severity of these adverse conditions, affecting mainly to abiotic stress conditions as drought, heat stress or salinity (Zandalinas et al., 2017). In this context, salt concentration in groundwater of coastal regions is increasing because of saltwater intrusion due to their high exploitation, deriving in problems for the plants as a consequence of the adverse effect of salt (Klassen and Allen, 2017). High salinity levels in the substrate has two different effects which trigger plant damage: the osmotic component, that appears in the early stage of stress and restricts water absorption, producing plant dehydration and turgor loss; and the ionic component, due to the accumulation of Na^+ and Cl^- ions, that reach toxic levels in tissues (Gupta and Huang, 2014). For most species Na^+ appears to reach toxic concentrations in plant tissues before Cl^- does. On the contrary, in the case of citrus, Cl^- is considered to be the most toxic ion (Montoliu et al., 2009, Moya et al., 2002, 2003). Consequently, it is necessary to explore new strategies to maximize plant tolerance to this stress condition in order to improve plant productivity in zones affected by this adverse environmental condition, as occurs in the Mediterranean region where citrus is one of the main crops.

Several strategies to mitigate salt stress-induced damages in plants have been used, including chemical treatments, such as 24-epibrassinolide (Ekinci et al., 2012) or abscisic acid analogues (Arbona et al., 2006), improving the mineral fertilization (Rady, 2012), modifying the expression of genes related with salt stress tolerance (Vives-Peris et al., 2017b; Zhao et al., 2017), or modifying soil microbiota communities in order to potentiate plant colonization by beneficial microorganisms as arbuscular mycorrhizal fungi (AMF) or plant growth promoting rhizobacteria (PGPR) (Qin et al., 2016). PGPR can benefit the plant growth and alleviate salt stress in different ways, including an improve of nutrient levels due to phosphate solubilization or fixation of atmospheric nitrogen, the induction of root growth and development through the release of phytohormones and secondary metabolites to the rhizosphere that interfere with plant auxin biosynthesis pathway such as indole-3-acetic acid (IAA), nitric oxide (NO) and 2,4-diacetylphloroglucinol (DAPG), the production of siderophores, or the decrease of 1-aminocyclopropane-1-carboxylate (ACC) activity, due to an increase of ACC deaminase activity in the rhizosphere, which

consequently derives in a decrease in ethylene concentration in plant tissues (Nadeem et al., 2016; Vacheron et al., 2013). The reduction of ACC deaminase activity not only can mitigate the adverse effects of salt stress, but can also alleviate the damage produced by other abiotic stress conditions, such as drought, osmotic stress, flooding, extreme temperatures, nutrient starvation or heavy metal toxicity (Dimkpa et al., 2009). Several works have reported that the inoculation of plants with PGPR can derive in different benefits to the plant: i) plant growth promotion (both shoots and roots), ii) an increase in the water use efficiency by modulating transpiration and stomatal conductance, iii) an increase in the endogenous content of nutrients such as N, P, K or Ca, iv) an inhibition of the defoliation caused by ethylene, v) an increase in the production of volatile organic compounds, and vi) a decrease in the content of reactive oxygen species (Vejan et al., 2016). All these effects depend on both plant and bacteria genotypes and their interaction.

Although the alleviative effects of PGPR on plants subjected to abiotic stress conditions have been studied in a wide variety of herbaceous plants, including tomato, rice, lettuce wheat, potato, cotton, soybean, maize, chickpea, lentil or pea (Dimkpa et al., 2009; Nadeem et al., 2014), the knowledge about the effect of these beneficial microorganisms in woody plants is restricted to a few species as grapevine plants subjected to chilling (Barka et al., 2006), or *Pinus halepensis* and *Quercus coccifera* trees subjected to water stress (Rincón et al., 2008). In citrus, the knowledge about the stress mitigatory role of soil microorganisms is mainly focused on the beneficial effects of mycorrhizae in presence of different biotic and abiotic stresses as phytophthora (Watanarojanaporn et al., 2011), drought (Wu and Zou, 2009), salinity (Satir et al., 2016; Zhang et al., 2017) and low temperatures (Wu and Zou, 2010). To our knowledge, in relation to PGPRs, the only information in this crop refers to the beneficial role of the rhizobacteria *Pseudomonas putida* FCA-8 in absence of any stress condition, resulting in an increase of plant height in *Citrus* x *limonia* (Rangpur lime) plants (Chiquito-Contreras et al., 2012). Moreover, although *P. putida* KT2440 has been described as a PGPR (Planchamp et al., 2015), the positive role on plant growth of the strain *Novosphingobium* sp. HR1a has not been proved yet, being only considered as PGPRs other strains in this genus as *Novosphingobium oryzae* sp. nov. (Zhang et al., 2016).

Consequently, in this work, the putative palliative effect of two rhizobacterial strains, *P. putida* KT2440, and *Novosphingobium* sp. HR1a, over the damage caused by salt stress conditions in alemow plants has been evaluated.

Materials and methods

Plant material and treatments

Six-month-old alemow (*Citrus macrophylla* Wester) plants were acclimated in a greenhouse for two months. Culture conditions were natural photoperiod and temperatures of 25 ± 3.0°C and 18 ± 2.0°C (day/night respectively). A mixture of peat moss, perlite and vermiculite (80:10:10) was used as substrate. Plants were watered with half-strength Hoagland solution three times a week (Arbona et al., 2009).

A first experiment was carried out to optimize the inoculation and treatments methodologies. Plants about 50 cm height, grown in plastic pots containing 400 cm^3 of substrate, were selected and inoculated with *P. putida* KT2440 (Franklin et al., 1981). The inoculation was performed by watering the plants with a bacterial solution containing the necessary bacteria to inoculate the pot volume to a final optical density at 660 nm of 0.1. Previously to the inoculation, cultures of *P. putida KT2440* and *Novosphingobium sp. HR1a* were established in liquid lysogeny broth (LB) medium from glycerinated bacteria (Bertani, 1951), supplemented with chloramphenicol and tetracycline, respectively, as selective antibiotics.

Ten days after the inoculation, salt stress was applied by adding 60 and 90 mM NaCl to the watering solution twice a week. Both, non-inoculated plants, and plants watered without NaCl were added as controls. Leaf and roots samples were collected 30 days after salt stress onset (Fig. 3.1A). In this experiment, different stress related parameters were measured in order to determine the correct application of salt stress, including foliar damage, chloride content in soil, shoots and roots, as well as the colony forming units (CFU) to determine the appropriate methodology of inoculation.

In the second experiment, *C. macrophylla* plants (similar in height and age to those used in the first set of experiments) were inoculated with two different PGPRs strains, *P. putida* KT2440 and *Novosphingobium* sp. HR1a up to a final optical density at 660 nm of 0.1 (Franklin et al., 1981; Segura et al., 2017). Ten days after the inoculation with bacteria, salt stress was applied, by adding 90 mM NaCl to the watering solution twice a week (Fig. 3.1B). In this experiment, leaf and root tissues were sampled after 30 days of stress, and were used for the determination of the endogenous content of proline and phytohormones. Moreover, non-destructive analysis, including gas exchange and

chlorophyll fluorescence parameters were determined at 10, 20 and 30 days after the stress onset.

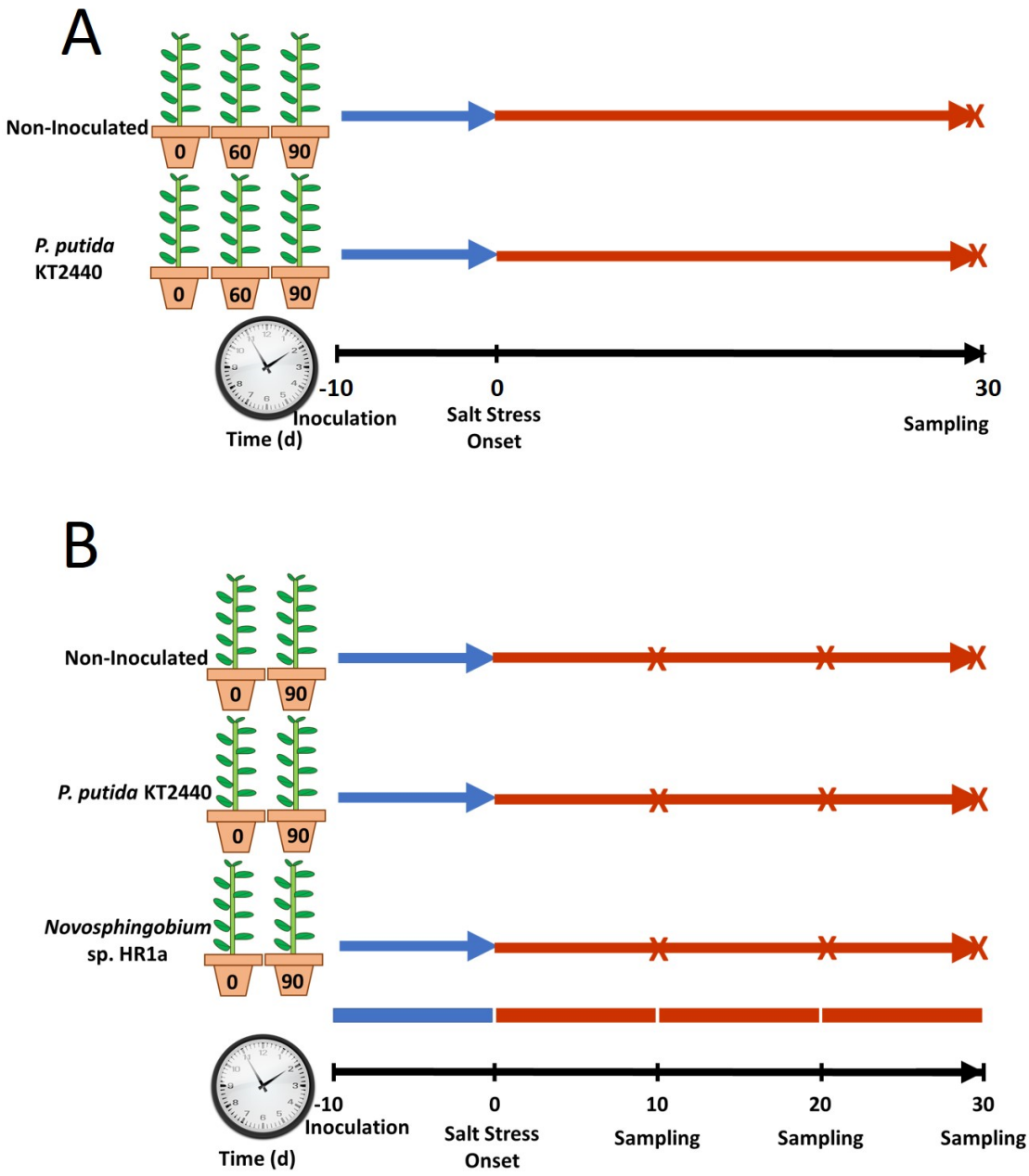


Figure 3.1 Experimental design of the first (A) and the second set of experiments (B)

Chloride analysis

Quantification of chloride ions was performed in plant tissue and soil saturated extract. Measurements in leaves and roots were performed by automatic titration with a chloride

meter (Model 626, Sherwood Scientific Ltd., Cambridge, UK) as described in López-Climent et al. (2008). Samples were extracted by adding 25 ml of the chloride extraction buffer, consisting in 0.1 N HNO_3 (Panreac, Barcelona, Spain) and 10% glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) to 0.25 g of fresh tissue, and were incubated for 12 hours at room temperature. Chloride concentration was measured by titrating 0.5 mL of the solution with the chloride meter.

Chloride measures of soil saturated extract were performed by adding water to 2 g of soil until saturation. After 24 hours at room temperature, the water was collected from the soil with a vacuum pump, and chloride concentration was measured in the chloride meter.

Determination of colonization rate

Plant colonization by the PGPRs was determined by counting the CFU. CFU were determined by washing roots with sterile deionized water and plating culture dilutions on lysogeny broth (LB) medium (Bertani, 1951). CFU were counted after 24 h of incubation at 30°C (Goldman and Green, 2008). Nine independent measurements were taken for every treatment.

Proline analysis

The concentration of proline was determined in leaf and root samples by following the methodology described in Bates et al. (1973) with some modifications. Briefly, 0.05 g of fresh material was extracted by sonication during 30 min, adding to the sample 5 mL of a solution of 3% sulfosalicylic acid (Panreac, Barcelona, Spain) in distilled water. Samples were centrifuged at 4000 rpm for 20 min and the supernatant was mixed with glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) and ninhydrin reagent (prepared with 0.625 g of ninhydrin in 15 mL of glacial acetic acid and 10 mL of orthophosphoric acid 6M), in a proportion 1:1:1 (v:v:v). Samples were incubated in a water bath at 100 ºC for one hour and centrifuged 5 min at 2000 rpm. Absorbance was measured at 520 nm with a spectrophotometer (Thermo Spectronic Genesys 10, Waltham, MA, USA), and proline was quantified by extrapolation in a standard curve made with a commercial standard of proline (Sigma-Aldrich, St. Louis, MO, USA).

Phytohormone analysis

Concentration of abscisic acid (ABA), salicylic acid (SA) and 3-indole acetic acid (IAA) were determined in the tissue samples collected along the experiment by high performance liquid chromatography coupled online to a triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source (Durgbanshi et al., 2005). Phytohormones were extracted with water from 0.2 g of fresh material reduced to fine powder by using a mill ball equipment. (MillMix20, Domel, Železniki, Slovenija). [$^{2}H_{6}$]-ABA, [$^{13}C_{6}$]-SA and [$^{2}H_{2}$]-IAA were used as internal standards. Samples were centrifuged after the extraction, and the supernatant was recovered, adjusting the pH in the range 2.8-3.2 with acetic acid. A liquid-liquid partition was performed twice with diethyl ether and the supernatant was evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The solid residue was diluted in 0.5 mL of water:methanol 90:10 and filtered through 0.22 µM PTFE filters. Finally, 20 µL of this solution were injected into the HPLC-MS system (Acquity SDS, Waters Corp., Milford, MA, USA).

Chromatographical separation was achieved by using a reversed-phase C18 column (Gravity, 50 × 2.1mm 1.8-µm particle size, Macherey-Nagel GmbH, Germany) as stationary phase, and a methanol:water gradient, both supplemented with 0.1% acetic acid, at a flow rate of 300 µL min^{-1} as mobile phase. Standard curves with the commercial standards of the different phytohormones were used for quantifying sample phytohormone concentrations. Results were processed using Masslynx v4.1 software.

Chlorophyll fluorescence parameters

Quantum yield (Φ_{PSII}) and maximum efficiency of photosystem II photochemistry, as F_v/F_m ratio, were measured between 9 and 11 h AM in nine randomly chosen undamaged leaves in every treatment using a portable fluorometer, with four measures in every leaf (FluorPen FP-MAX 100, Photon Systems Instruments, Czech Republic). Measurements of Φ_{PSII} were performed in light adapted leaves, whereas F_v/F_m measurements were performed after 30 min of dark adaptation (Zandalinas et al., 2016). F_v/F_m calculations were performed according to Murchie and Lawson (2013).

Leaf gas exchange parameters

Transpiration (E) and stomatal conductance (g_s) were measured with a LCpro+ portable infrared gas analyzer (ADC Bioscientific Ltd., Hoddesdon, UK) under ambient CO_2 and humidity. Light was provided by a photosynthetically active radiation lamp at 1000 µmol m^{-2} s^{-1} photon flux density. Air flow was set at 150 µmol mol^{-1} and all measurements were performed between 9 and 11 h AM. Three undamaged leaves of three different plants were analyzed for each treatment, and after instrument stabilization ten measures were consecutively performed in every leaf (Zandalinas et al., 2016).

Statistical analyses

Statgraphics Plus v.5.1. Software (Statistical Graphics Corp., Herndon, VA, USA) was used in order to assess the statistical analyses. Represented data are means of three independent determinations and were subjected to one- or two-way analysis of variance (ANOVA) and a Tukey posthoc test ($p \leq 0.05$) when significant differences were detected.

Results

Optimization of salt treatments and inoculation methodology

Leaf abscission

After 30 days of treatment, leaf abscission increased in non-inoculated salt stressed plants, reaching its higher level in plants subjected to 90 mM NaCl, with a percentage of leaf abscission of 17.5%, whereas in plants inoculated with *P. putida* KT2440, this percentage was of 2.6%, being similar to the observed in control plants (Fig. 3.2).

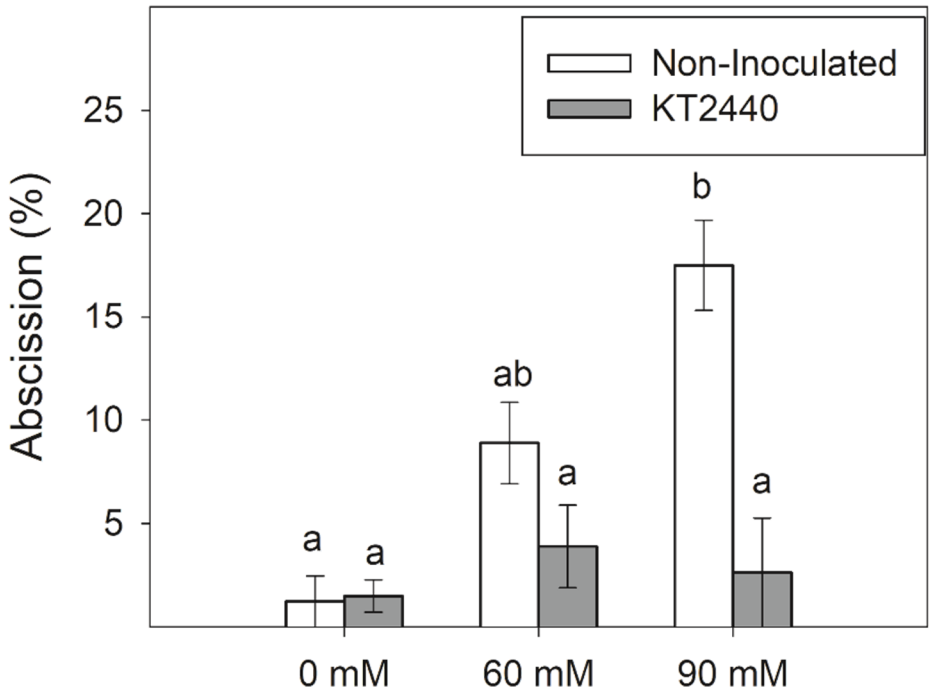


Figure 3.2 Leaf abscission in non-inoculated plants (white bars) and plants inoculated with *P. putida* KT2440 (grey bars) exposed to 0, 60 and 90 mM NaCl for 30 days. Values indicate the mean of ten replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Chloride accumulation

Chloride concentration was determined in soil, shoots and roots (Fig. 3.3). In soil, the presence of the bacteria did not influence chloride concentration, being only influenced by the salt treatments, with chloride levels about 4.5 and 9 times higher than the control in the treatments with 60 and 90 mM NaCl, respectively (Fig. 3.3A). A similar trend was observed in leaves, with an increase in the concentration of this ion depending on the stress severity exclusively, with values about 1.8 and 3 times higher than those observed in leaves of non-stressed plants in those plants subjected to 60 and 90 mM NaCl, respectively (Fig. 3.3B). In root tissue, the presence of the bacteria *P. putida* KT2440 induced a lower accumulation of chloride ions in plants subjected to 90 mM, being this reduction of 24.6% compared to non-inoculated plants subjected to the same salt concentration (Fig. 3.3C).

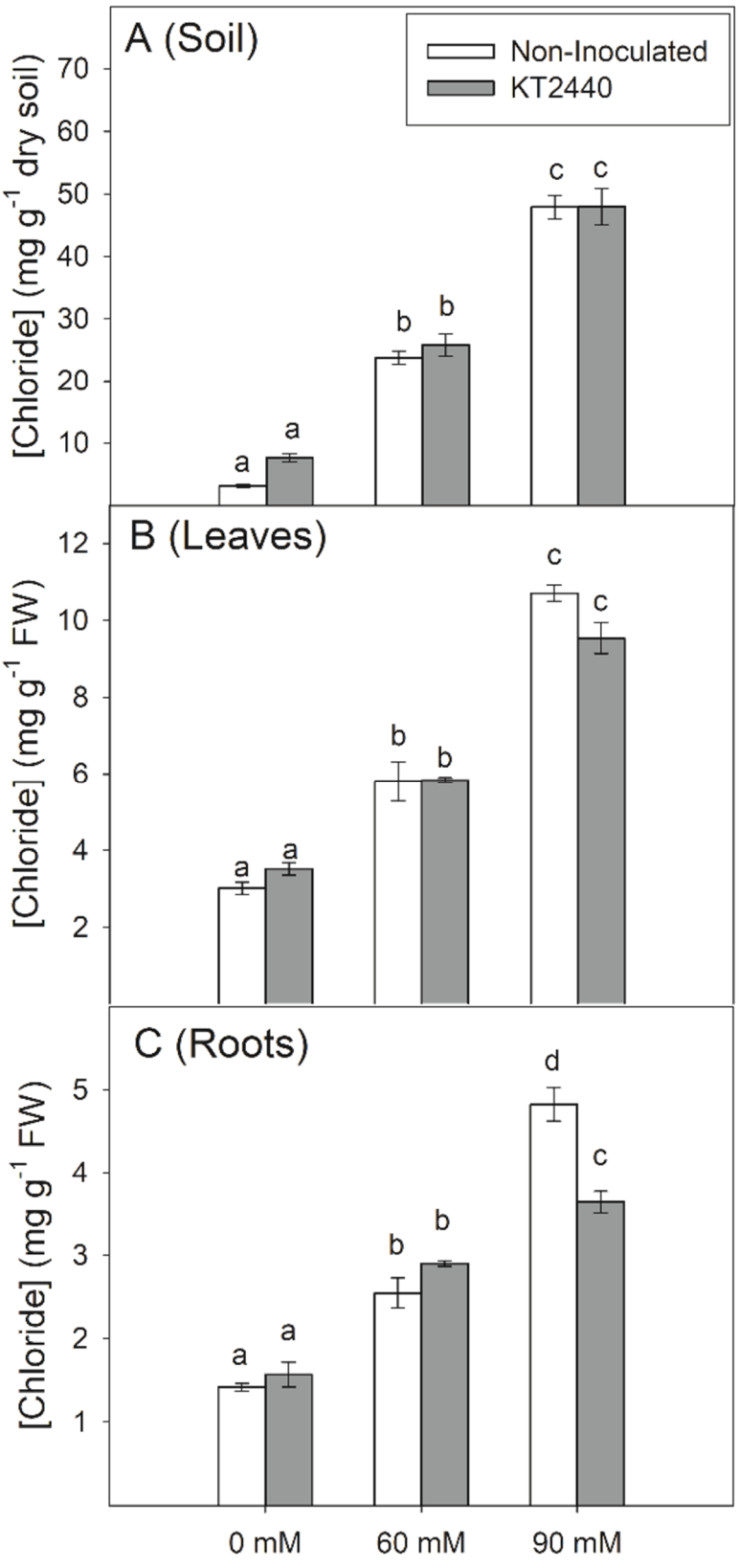


Figure 3.3 Chloride contents in soil (A), leaves (B) and roots (C) in non-inoculated plants (white bars) and plants inoculated with *P. putida* KT2440 (grey bars) exposed to 0, 60 and 90 mM NaCl. Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Colonization rate

The number of CFU was analysed at the end of the experiment. In this case, two different levels of bacterial populations were observed depending if the plants were inoculated or not (Fig. 3.4). In the case of non-inoculated plants, values of CFU were about 150,000 CFU g^{-1} root, while in plants inoculated with *P. putida* KT2440, this value was around 600,000 CFU g^{-1} root.

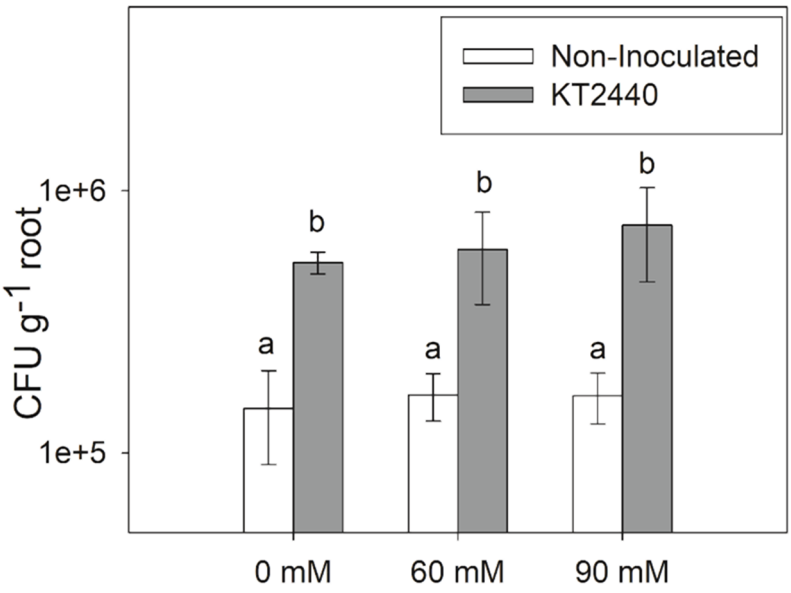


Figure 3.4 Colony forming units in roots of non-inoculated plants (white bars) and plants inoculated with *P. putida* KT2440 (grey bars) exposed to 0, 60 and 90 mM NaCl in the first set of experiments. Values indicate the mean of nine replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Evaluation of the palliative effect of both strains in plants subjected to salt stress

Appearance of symptoms induced by salt stress

Different degrees of damage were observed after 30 days of stress (Fig. 3.5). The presence of both rhizobacterial strains inhibited the frequency of the appearance of salt stress damage. While in non-inoculated plants a 68.33% of leaves were affected by salt stress, this percentage decreased in plants inoculated with *P. putida* KT2440 and *Novosphingobium* sp. HR1a, with values of 57.08 and 44.95%, respectively (Fig. 3.6).

Figure 3.5 Different levels of salt stress induced damage in leaves. 1: Non-damaged leaf; 2: Mild-damaged leaf; 3: Intermediate-damaged leaf; 4: Severe-damaged leaf; 5: Leaf abscission.

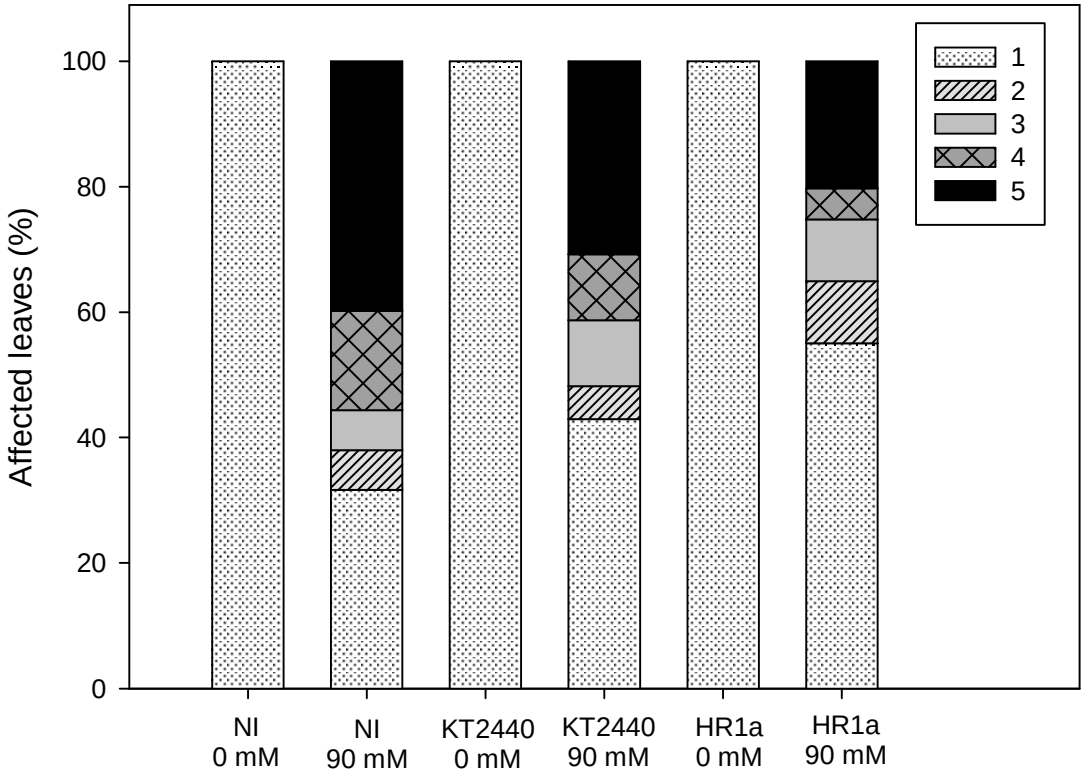


Figure 3.6 Percentage of affected leaves. Different colors and patterns refer to the different levels of leaf damage represented in the Figure 5. 1: Non-damaged leaf; 2: Mild-damaged leaf; 3: Intermediate-damaged leaf; 4: Severe-damaged leaf; 5: Leaf abscission. Represented data refers to the mean of 10 plants.

Proline concentration

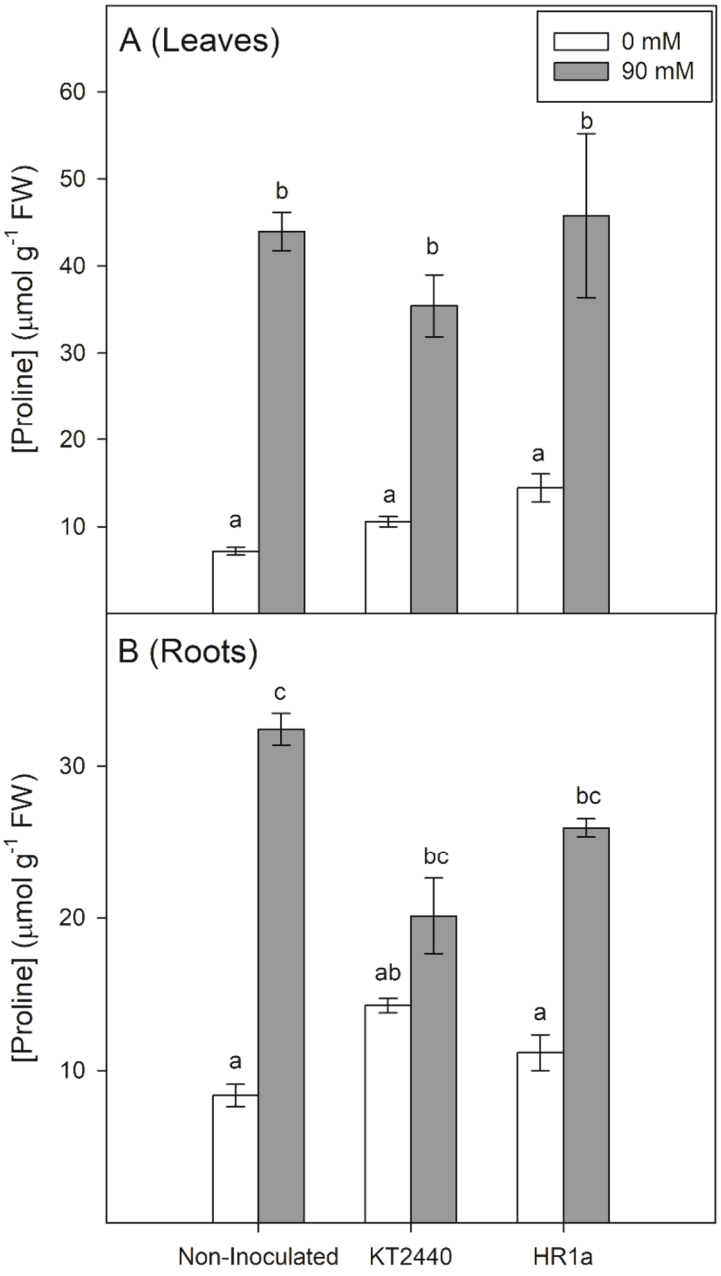


Figure 3.7 Proline concentration in leaves (A) and roots (B) of non-inoculated plants and plants inoculated with *P. putida* KT2440 and *Novosphingobium* sp. HR1a in control conditions (white bars) and 90 mM NaCl treatments (grey bars). Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

The concentration of this amino acid was determined in leaves and roots of control and non-inoculated salt-stressed plants and those inoculated with *P. putida* KT2440 or *Novosphingobium* sp. HR1a (Fig. 3.7). An increase of proline concentration was observed in leaves due to salt stress application, being this value in salt-stressed plants 6.12 times

higher than in control. The same trend was observed in inoculated plants (Fig. 3.7A). In roots of plants subjected to salt stress, there was also an increase of proline content, which was of 3.88-fold in non-inoculated plants (Fig. 3.7B). The magnitude of this increase was lower in roots of plants inoculated with both bacteria, being of 1.41 and 2.32-fold in presence of *P. putida* KT2440 and *Novosphingobium* sp. HR1a, respectively. This difference was not significative between unstressed and salt-stressed plants inoculated with *P. putida* KT2440.

Phytohormone concentration

Levels of the phytohormones ABA, SA and IAA were affected by both, the salt stress treatment and the inoculation with PGPRs (Fig. 3.8). After 30 days of salt stress, the non-inoculated plants treated with 90 mM NaCl exhibited an increase in the leaf concentration of ABA of 1.65-fold related to non-stressed plants. Contrarily, in plants inoculated with *P. putida* KT2440 the concentration of this phytohormone was reduced a 48.18%, while leaves of plants inoculated with *Novosphingobium* sp. HR1a did not show statistically significant differences with those subjected to salt stress (Fig. 3.8A). However, the inoculation of plants with *Novosphingobium* sp. HR1a induced an increase in ABA concentration in roots in presence of salt stress, with levels 2.29 times higher than non-stressed plants inoculated with this strain, being this the only case that exhibited an increment in the levels of this phytohormone in roots (Fig. 3.8B).

The endogenous content of leaf SA followed a similar pattern than that of ABA. In absence of inoculum, salt stress induced a 2.31-fold increase of SA leaf concentration in comparison with non-stressed plants. However, no differences were observed in presence of any of the bacterial strains (Fig. 3.8C). However, SA content decreased a 67.70% in roots of salt-stressed plants inoculated with *P. putida* KT2440 in comparison with non-stressed inoculated plants (Fig. 3.8D). Meanwhile, differences in the content of IAA were only recorded in leaves of plants inoculated with *Novosphingobium* sp. HR1a and treated with 90 mM NaCl, with a IAA content 2.76 times higher than the observed in leaves of non-stressed plants inoculated with this bacterial strain (Figs. 3.8E and 3.8F).

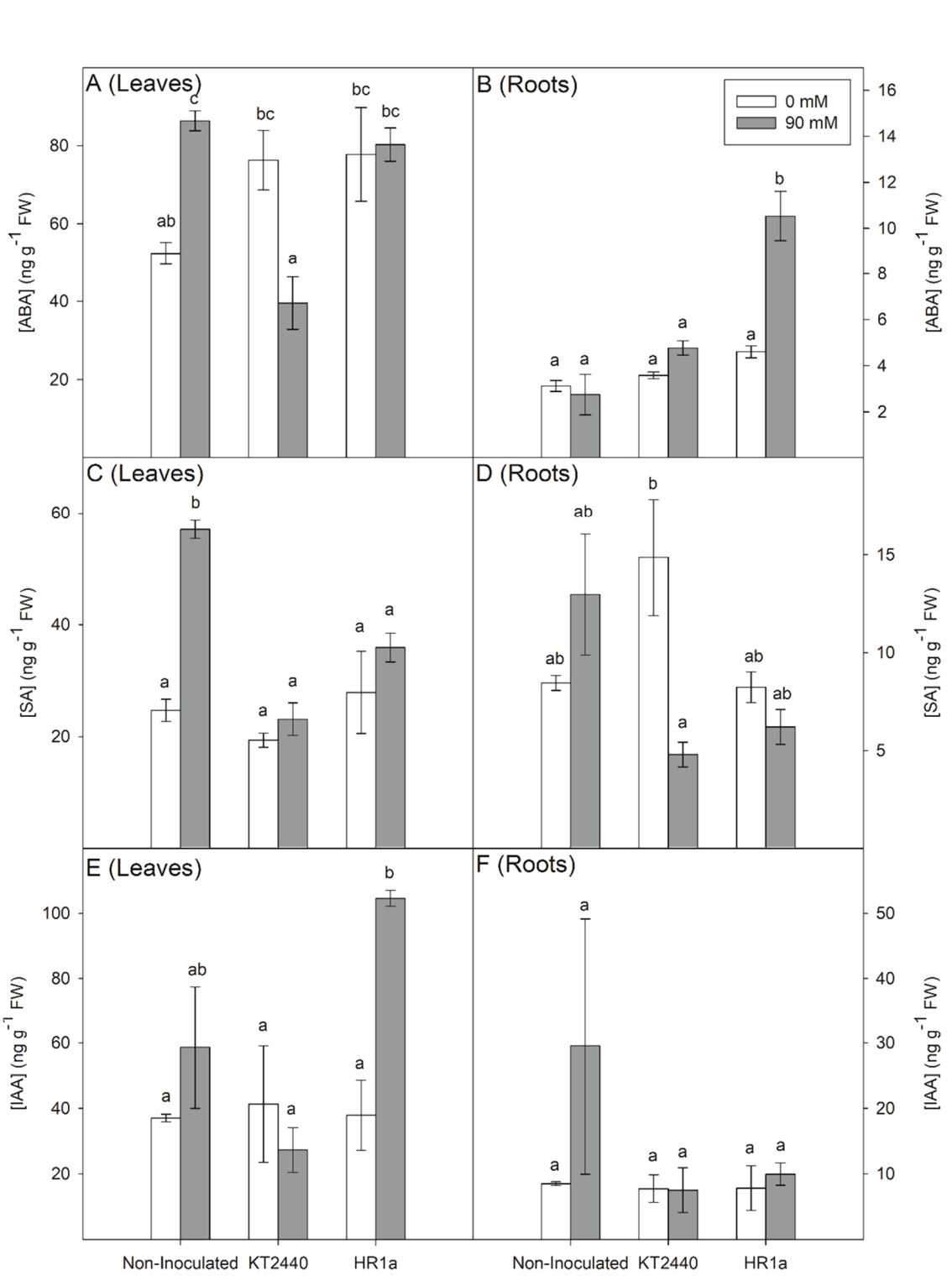


Figure 3.8 Phytohormone contents in leaves and roots of plants subjected to the different treatments. ABA (A-B), SA (C-D) and IAA (E-F) contents in leaves (A, C and E) and roots (B, D and F) of non-inoculated plants and plants inoculated with *P. putida* KT2440 and *Novosphingobium* sp. HR1a in control conditions (white bars) and 90 mM NaCl treatments (grey bars). Values indicate the mean of three replicates ± standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

Chlorophyll fluorescence parameters

Salt treatment and bacteria inoculation induced changes in the Φ_{PSII} and in the F_v/F_m ratio of treated plants (Fig. 3.9). Salt stress clearly reduced Φ_{PSII} in non-inoculated plants, with a reduction of 36.40 and 55.49% in comparison to control plants after 20 and 30 days respectively. In plants inoculated with *P. putida* KT2440, this reduction was similar to that observed in non-inoculated plants, with a diminution of Φ_{PSII} values of 23.84 and 52.10% related to control after 20 and 30 days of experiment. In plants inoculated with *Novosphingobium* sp. HR1a, Φ_{PSII} behavior was slightly different. Whereas after 20 days of salt stress the reduction of Φ_{PSII} was of 32.20% respect to control, similar to those observed in non-inoculated plants, after 30 days of salt stress this reduction was not as marked as in non-inoculated plants, being only of the 39.07% (Fig. 3.9A).

In line with the Φ_{PSII}, F_v/F_m ratio also decreased with the application of salt stress from 20 days of stress until the end of the experiment, exhibiting a reduction of 28.13 and 44.34% in non-inoculated plants at 20 and 30 days respectively. However, in plants inoculated with both bacterial strains, F_v/F_m ratio was maintained during the first 20 days of stress, and did not decrease until 30 days after the stress onset, when F_v/F_m ratio diminished until values 52.93 and 38.31% in salt stressed plants inoculated with *P. putida* KT2440 and *Novosphingobium* sp. HR1a respectively in comparison with non-stressed inoculated plants (Fig. 3.9B).

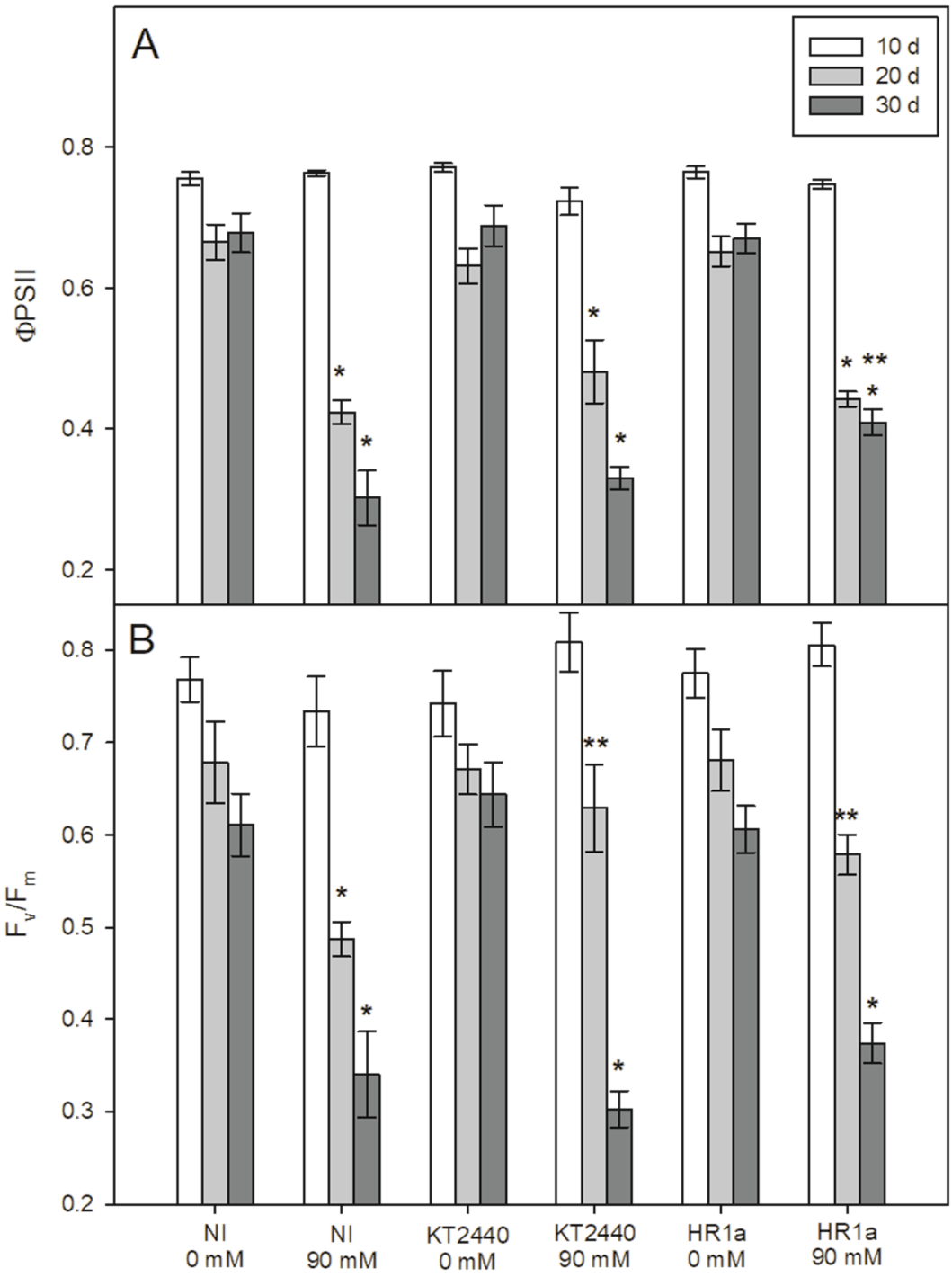


Figure 3.9 Chlorophyll fluorescence parameters in plants subjected to the different treatments. Quantum efficiency (A) and maximum efficiency of PSII photochemistry (B) of non-inoculated plants and plants inoculated with *P. putida* KT2440 and *Novosphingobium* sp. HR1a in control conditions and 90 mM treatments after 10 (white bars), 20 (light grey bars) and 30 days (dark grey bars). Values indicate the mean ± standard error. One asterisk refers to statistically significant differences among control and salt stressed plants at $P \leq 0.05$, while two asterisks refer to statistically significant differences among non-inoculated and inoculated plants at $P \leq 0.05$

Gas exchange parameters

Gas exchange parameters, including E and g_s, were measured in leaves along all the experiment after salt stress application, but no differences were observed until 20 days of stress (Fig. 3.10). Salt stress induced a decline of E between 52 and 68% after 20 days and between 82 and 84% after 30 days. Moreover, in absence of salt stress, *Novosphingobium* sp. HR1a also induced a diminution of 35.52% in this parameter after 20 days of stress in comparison with the values observed in non-inoculated plants (Fig. 3.10A).

In addition, g_s exhibited a similar tendency than the observed in E. Most of the differences observed in g_s were showed in response to salt stress, with a decrease between 58 and 74% depending on the inoculum, and after 20 days, being more evident after 30 days, with values among 85 and 88%. E was also influenced by the inoculation with *Novosphingobium* sp. HR1a in absence of salt stress, exhibiting a decrease of 47.93 and 50.94% in comparison to control plants after 20 and 30 days from the beginning of salt stress treatments respectively (Fig. 3.10B).

Discussion

The results presented in this work reveal that both bacterial strains, *P. putida* KT2440 and *Novosphingobium* sp. HR1a mitigate the negative effect of salt stress in alemow plants, confirming that *Novosphingobium* sp. HR1a has a role as a PGPR. However, although both strains have a positive effect in plant tolerance to this stress, there are common and different responses depending on the inoculated rhizobacterium.

The application of salt stress to non-inoculated plants, had a negative effect on plant growth, inducing leaf damage and abscission, Cl^-, and proline accumulation, while gas exchange and chlorophyll fluorescence parameters decreased, being in concordance with other previous reports (Hussain et al., 2012; López-Climent et al., 2008). Moreover, salt stress induced the accumulation of ABA and SA in leaves, which is widely reported in several species as *Arabidopsis thaliana* (Prerostova et al., 2017), *Cucumis sativus* (cucumber) (Chojak-Koźniewska et al., 2017) or the citrus rootstock Carrizo citrange (Gómez-Cadenas et al., 1998).

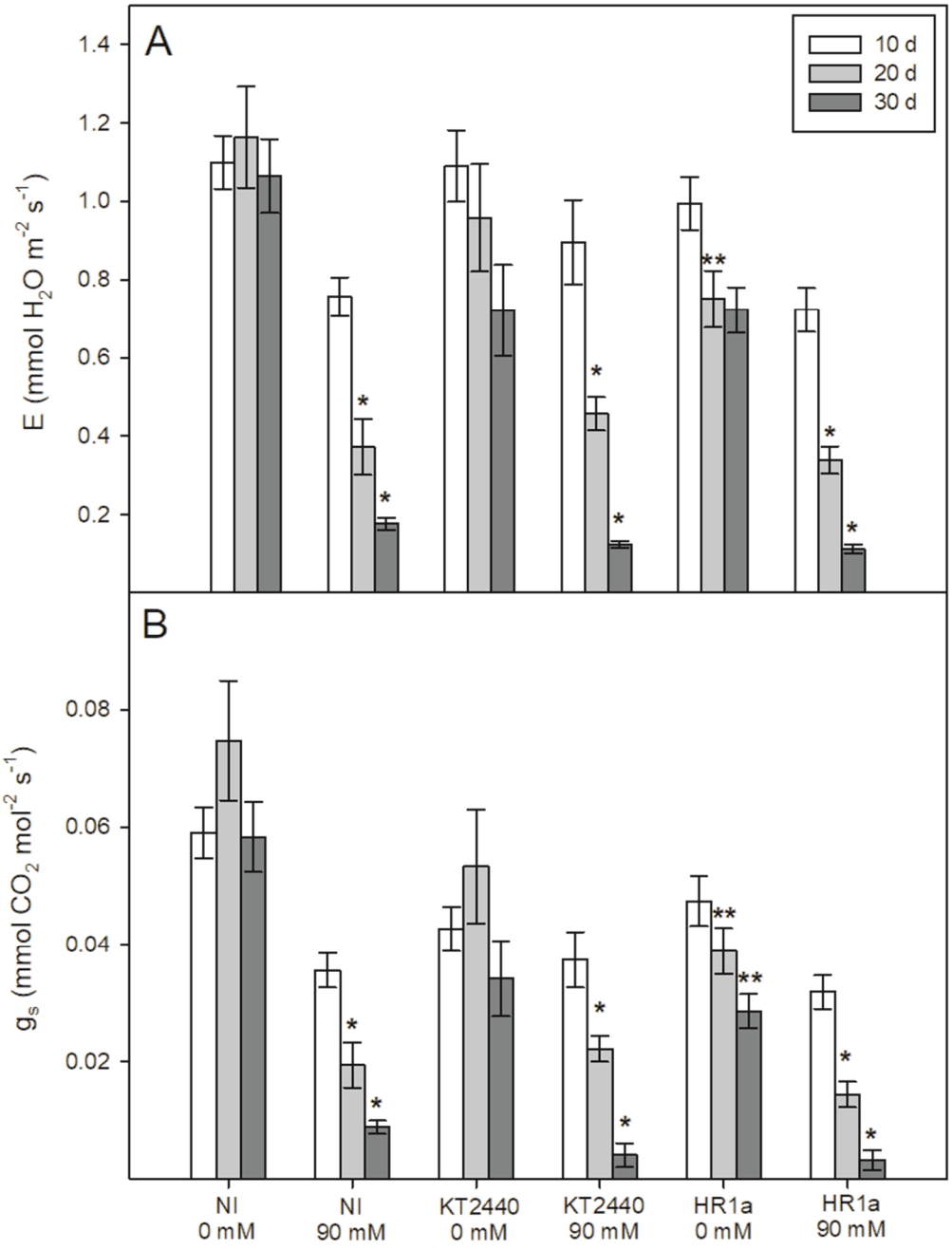


Figure 3.10 Gas exchange parameters in plants subjected to the different treatments. Transpiration (A) and stomatal conductance (B) of non-inoculated plants and plants inoculated with *P. putida* KT2440 and *Novosphingobium* sp. HR1a in control conditions and 90 mM treatments after 10 (white bars), 20 (light grey bars) and 30 days (dark grey bars). Values indicate the mean ± standard error. One asterisk refers to statistically significant differences among control and salt stressed plants at $P \leq 0.05$, while two asterisks refer to statistically significant differences among non-inoculated and inoculated plants at $P \leq 0.05$

Among the palliative effects of *P. putida* KT2440 and *Novosphingobium* sp. HR1a, both bacterial species reduced stress-induced proline accumulation in roots. This amino acid is generally accumulated in plant tissues under stress situations such as drought and salinity, being an osmoprotectant that avoids plant dehydration and turgor loss. Although the reduction of proline concentration in salt-stressed plants inoculated with both bacteria could suppose that plants are suffering salt stress in a lower measure, it has been proposed that the accumulation of proline is not a universal response associated to stress tolerance (Arbona et al., 2017). However, proline content reduction was not the only difference observed in salt stressed inoculated plants, which also exhibited maintenance of SA and ABA levels, or a decrease on ABA concentration in the case of *P. putida* KT2440, while non-inoculated plants showed an increase on the foliar amount of these phytohormones in response to salt stress. This reduction of ABA and SA concentration would further support the lower impact of salt stress in inoculated plants. It has been described previously that the ACC-deaminase activity produced by PGPRs inhibits ethylene biosynthesis in plants (Dimkpa et al., 2009). Consequently, since ethylene has a crosstalk with ABA, this chain would lead to a reduction of ABA levels (Arc et al., 2013). This lower increase in ABA has been also reported in salt and osmotic stressed cucumber plants inoculated with the PGPRs *Burkholderia* sp., *Acinetobacter* sp., and *Promicromonospora* sp. (Kang et al., 2014). In addition to the lower accumulation of ABA and SA in leaves from salt stressed inoculated plants, those plants inoculated with *Novosphingobium* sp. HR1a and subjected to salt stress exhibited an increase of IAA levels. The increase of the concentration of this phytohormone could be due to the presence of the PGPR, since some rhizobacteria, including *Novosphingobium* genus, produce IAA (Krishnan et al., 2017). In any case, IAA has been reported as a salt stress reliever in species as maize (Kaya et al., 2013). This increase of IAA could promote root development, developing lateral roots and facilitating root exploration of new soils with lower contents or toxic elements or higher quantities of water (Bao et al., 2014).

The inoculation with both rhizobacteria maintained a higher F_v/F_m ratio in salt-stressed plants, whereas *Novosphingobium* sp. HR1a also induced a maintenance of higher levels of Φ_{PSII} in salt-stressed plants. Moreover, salt stressed plants inoculated with *Novosphingobium* sp. HR1a also maintained higher levels of Φ_{PSII} after 30 days of stress in comparison with non-inoculated plants. These chlorophyll fluorescence parameters generally decrease with plant stress, as well as chlorophyll and carotenoid contents

(López-Climent et al., 2008). However, other works have revealed that PGPRs allow plants to avoid these decreases and maintaining these parameters in abiotic stress conditions. For example, in *Ocimum basilicum* L. (basil) plants inoculated with *Pseudomonades* sp. subjected to water stress exhibited higher values of the F_v/F_m ratio (Heidari and Golpayegani, 2012) than non-inoculated water-stressed plants. Moreover, PGPRs from *Bacillus megaterium* and *Enterobacter* sp. species have been also reported as inducers of chlorophylls accumulation in *Abelmoschus esculentus* L. (okra) plants subjected to salt stress (Habib et al., 2016). Although most studies regarding the effect of PGPRs on photosystem II are focused in herbaceous crops, Rincón et al. (2008) working with tree species as *Pinus halepensis* and *Quercus coccifera* inoculated with *Pseudomonas fluorescens* reported similar results under water stress conditions.

Regarding on gas exchange parameters, there is some controversy about the effect of PGPRs over E and g_s. Whereas some reports reveal a positive relation among PGPR inoculation and E and g_s rates in crops as *Vigna radiata* (mung bean) plants inoculated with *Enterobacter cloacae* and *Bacillus drentensis* and subjected to salt stress (Mahmood et al., 2016) or *Triticum durum* (wheat) plants treated with PGPRs (Zhu et al., 2014), other works indicate that E and stomatal aperture were reduced in presence of PGPRs, improving water use efficiency and consequently improving plant tolerance to stress conditions, as occurs in PGPRs inoculated *Zea mays* (maize) plants subjected to drought (Yasmin et al., 2013) or *A. thaliana* plants inoculated with *Phyllobacterium brassicacearum* and subjected to water stress (Bresson et al., 2013). The results obtained in this work showed that E and g_s were only influenced by PGPRs colonization in absence of salt stress, exhibiting a decrease in g_s in inoculated non-stressed plants independently of the used strain, and a decrease of E in plants inoculated with *Novosphingobium* sp. HR1a, suggesting that the inoculation with both PGPRs improves water use efficiency.

In addition to the physiological parameters measured, the results showed in the first experiment also exhibited a decrease in chloride ions concentration in roots of plants inoculated with *P. putida* KT2440 subjected to salt stress. The accumulation of this ion has been previously reported as the critical component of salt stress in citrus plants, being its content an important measure to quantify salt stress damage in this crop (Moya et al., 2003). By this, the avoidance of the absorption of this toxic ion, with the consequent lower endogenous concentration has been reported as an indicator of salt stress tolerance in citrus plants (Hussain et al., 2012).

Previous studies with these rhizobacterial strains have reported that Novosphingobium sp. HR1a is more tolerant to high concentrations of NaCl than P. putida KT2440, which can be due to the adaption of this strain to soils with high salinity as coastal areas (Vives-Peris et al. 2018). The higher tolerance of Novosphingobium sp. HR1a to high salinity could be crucial in the capability of this PGPR to alleviate salt stress, explaining the higher beneficial effects exerted by this strain on salt-stressed citrus plants. Moreover, the mechanisms of both PGPR strains to trigger plant benefits could be different, and consequently, affect plant growth distinctly (Vejan et al., 2016).

In conclusion, the results presented in this work reveal that both PGPRs species, *P. putida* KT2440 and *Novosphingobium* sp. HR1a have a positive role in the growth and mitigation of salt stress in citrus plants, decreasing the appearance of symptoms caused by this stress. In addition, the positive effect under salt stress conditions is more effective in plants inoculated with *Novosphingobium* sp. HR1a, what leads this strain to be a better candidate to mitigate the deleterious effect of this abiotic stress condition. Consequently, both rhizobacterial strains could be used in biofertilization and bioaugmentation programs in order to promote plant growth and prevent the damage caused by salt stress.

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General discussion

General discussion

Citrus is one of the most extended fruit crops over the world with more than 120 million of tonnes produced in the campaign 2013/2014. Spain is the sixth-largest citrus producer country in the world, and the largest producer of citrus for fresh consumption, with more than 3.5 million of tonnes exported in the campaign 2013/2014 (FAO, 2016). In nature, citrus plants must constantly cope with a variety of abiotic stress conditions that often lead to poor tree growth and reductions in fruit yield and quality. In the last years, as a consequence of the global warming, responsible of the climate change, the negative effects of stresses, such as drought or high temperature, on crop production is increasing. This situation makes necessary the development of new strategies for improving plant tolerance to these environmental adverse conditions (Zandalinas et al., 2017b). By this, the effects of a wide variety of abiotic stress conditions on plant performance has been previously studied, in citrus plants affected by salinity (López-Climent et al., 2008; Montoliu et al., 2009), flooding (Arbona and Gómez-Cadenas, 2008; Arbona et al., 2008, 2009), and drought, applied individually or simultaneously with heat stress (Zandalinas et al., 2016, 2017a, c). These abiotic stress conditions are responsible of several alterations, including modifications in the primary and secondary metabolisms, leaf turgor loss, changes in gas exchange parameters or chlorophyll degradation, which can derive in plant survival or death depending on the severity of the stress and the plant genotype sensitivity (Arbona et al., 2017). Among these changes, in other species it has been described that plants are able to alter, both in quantity and quality, the production of root exudates released to the rhizosphere through their roots. These exudates can modify soil properties such as pH or electric conductivity, as well as the growth and population of other living organisms present in the rhizosphere, including other plants of the same or different species, bacteria, fungi..., as occurs with *Arachis hypogaea* (peanut) plants, which exudates promote the growth of *Fusariym oxysporum* and *F. solani* (Li et al., 2013). This fact can contribute to a better establishment of beneficial microorganisms, including AMF and PGPR, as the metabolites released to the rhizosphere by the plant including amino acids and sugars, are metabolized by these microorganisms which can protect plant against these adverse environmental situations (Lareen et al., 2016). However, although root exudates composition and functions in the rhizosphere have been widely studied in some herbaceous plants as *Arabidopsis thaliana* (Strehmel et al., 2014), rice (Suzuki et al., 2009; Tawaraya et al., 2013), or soybean (Tawaraya et al., 2014b),

there is not much information in woody plants and the information in this topic is practically void in citrus. In this work, the importance of root exudates in plant mediation with PGPRs under abiotic stress conditions, and how these microorganisms can alleviate the adverse effects of abiotic stress conditions has been studied.

Initially, the effect of salt and heat stress on the root exudation pattern was tested in two citrus rootstocks, Carrizo citrange and *C. macrophylla* (Chapter 1). Secondly, the role of root exudates from plants of both genotypes subjected to salt or heat stresses in the rhizosphere was addressed by determining their impact on growth of two different PGPRs species, *P. putida* KT2440 and *Novosphingobium* sp. HR1a. The ability of these bacteria to detect proline and salicylates present in root exudates was investigated by evaluating the induction of P_{putA} and P_{pahA} promoters, respectively (Chapter 2). Finally, the potential contribution of both bacterial strains to the alleviation of plant damage provoked by salt stress was explored in *C. macrophylla* plants inoculated with both PGPRs species (Chapter 3).

In the Chapter 1 of this doctoral thesis project, the effect of high salinity and heat stress, on Carrizo citrange and *C. macrophylla* has been studied. In order to achieve the proposed objectives, it was developed an *in vitro* tissue culture system that allows root exudates obtaining. This methodology has been validated for the study of citrus responses to abiotic stress conditions, with similar results to those obtained in plants cultured under field conditions (Montoliu et al., 2009). Moreover, some works recommend the use of this methodology for the study of root exudates due to the sterility of the culture and the stability of root exudates composition (Kuijken et al., 2015; Vranova et al., 2013). The data obtained from the analyses of stress related parameters, including chloride accumulation and phytohormone concentrations confirmed that Carrizo citrange is salt sensitive but heat tolerant, whereas *C. macrophylla* is salt tolerant but heat sensitive. Although the different tolerance of both rootstocks to salt stress had been widely studied (López-Climent et al., 2008; Montoliu et al., 2009; Pérez-Tornero et al., 2009), there is almost no information about their behaviour under heat stress conditions. The few existing studies compare the heat tolerance of Carrizo citrange and Cleopatra mandarin (Zandalinas et al., 2016, 2017a, c). Thereby, the different tolerance of the genotypes used in this work to salinity and high temperatures makes this combination an interesting tool to decipher how plant sensitivity or tolerance affects root exudates composition under both stress conditions. The absence of MDA accumulation detected in stressed plants

reveals that there is not damage in cell membranes pointing out that the methodology used is adequate, revealing that the metabolites have been released to the rhizosphere from the roots through a root exudation process.

Analyses of root exudates composition determined that citrus roots exude proline, the phytohormones ABA, SA, JA, IAA and cinnamic acid to the rhizosphere, being this process influenced by the genotype and stress conditions. By this, root exudation of proline, SA and cinnamic acid is promoted by both stress conditions, being exuded in higher concentrations in the tolerant genotype in each adverse condition. In addition, JA and IAA also increased their concentration in root exudates from plants subjected to both stresses, while ABA increased its content in exudates from salt stressed plants but decreased its level in heat stressed plants, independently of the genotype tolerance to each stress condition, although the differences in the exudation of these phytohormones was not as marked as the observed in proline, SA and cinnamic acid. Previous works have described that some of these exuded compounds promote the growth of beneficial microorganisms, as occurs with proline (Verslues and Sharma, 2010; Vílchez et al., 2000b) or SA and cinnamic acid (Segura et al., 2017), being sources of nutrients as C and N. However, these compounds could also promote the growth of other harmful microorganisms which can colonize plants, as *Listeria monocytogenes* (Beumer et al., 1994) *Fusarium oxysporum* or *F. solani* (Li et al., 2013).

Moreover, since differences in proline concentrations were observed in root exudates before than in plant tissues, the analysis of the content in this amino acid in the rhizosphere can be postulated as an early and non-destructive stress marker, what would help to take actions before arriving to a more severe stressful situation which could irreversibly damage plants.

Although proline and phytohormones exudation by roots have been previously described in herbaceous plants as rice (Tawaraya et al., 2013), common bean (Tawaraya et al., 2014a), or soybean (Tawaraya et al., 2014b), the differential exudation depending on the plant tolerance to a particular stress condition has been practically unexplored. The only report in this topic refers to the role of SA in P solubilization in plants of sugar beet subjected to nutrient starvation conditions (Khorassani et al., 2011) or the root exudation of proline in excised root cultures of almond tree subjected to salt stress (Marin et al., 2010).

In view of the different root exudation pattern depending on genotype, and stress applied described in the first chapter of this report, the effect of citrus root exudates over the growth of two different rhizobacteria, *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a was tested in Chapter 2 (Franklin et al., 1981; Segura et al., 2017). In this work, the bacterial growth was measured by following two different approaches, including the optical density and the number of CFU. The detection of proline and salicylates present in root exudates by bacteria was evaluated through the determination of the activity of P_{putA} and P_{pahA} promoters in the strains *P. putida* KT2442 and *Novosphingobium* sp. HR1a respectively (Segura et al., 2017; Vílchez et al., 2000a). The promotor of this gene has been previously ligated to a reporter gene (in this case, *lacZ* from *Escherichia coli*), which products a detectable product (β-galactosidase in this case), that can be quantified by different techniques (Harms et al., 2006). Several constructions are available to quantify a variety of chemical compounds using this methodology, which can determine the bioavailability or the presence of some chemical compounds, including proline with *P. putida* KT2442 (pMIS5) (Vílchez et al., 2000a), polycyclic aromatic hydrocarbons with *Novosphingobium* sp. HR1a (pPAH) (Segura et al., 2017), or heavy metal ions as Pb^{2+} and Cd^{2+} with *E. coli* DH5 with plasmids pHK194 and pHK200 respectively (Kim et al., 2015).

The results obtained in this chapter showed that citrus root exudates promote the growth of both bacterial species allowing their growth in a culture media containing plant exudates as the sole source of N. Bacterial growth was higher when root exudates proceed from salt or heat stressed plants. Moreover, with root exudates obtained from salt stressed plants, the bacterial promotion of both PGPRs was higher in those cultures grown in presence of root exudates from the salt tolerant rootstock *C. macrophylla*, while under heat stress conditions similar growth induction was observed in *P. putida* KT2440 regardless plant genotype source of exudates. The growth of *Novosphingobium* sp. HR1a was also higher in presence of *C. macrophylla* root exudates. These differences in the bacterial growth in presence of root exudates could be due not only to the analysed metabolites, but also to other ones as sugars, phenolic compounds or other amino acids released to the rhizosphere in the exudation process (Dardanelli et al., 2012; Li et al., 2013). Moreover, the modulation of root exudates composition could be higher in plants subjected to salt stress than in plants stressed by high temperatures, since salt stress affects directly roots and in presence of heat stress, soil exerts as a pad.

The study of β-galactosidase activity in both constructions confirmed that *P. putida* KT2442 is capable to detect proline from citrus root exudates and *Novosphingobium* sp. HR1a is able to detect salicylates from root exudates, and could use these compounds as nutrient sources. Moreover, since the different levels of proline and salicylic acid measured in the Chapter 1 are correlated with the results obtained from β-galactosidase activity analyses, it can be concluded that both bacterial constructions can be used as biodetectors for these metabolites in root exudates, supposing a cheaper and environmentally friendly alternative to other methodologies as chromatography or colorimetry which require higher quantities of solvents and expensive equipment, although this methodology is not as sensitive as detection by chromatographical techniques (Harms et al., 2006). In concordance with the results obtained in this chapter, some works have reported the beneficial role of root exudates in the colonization and growth of beneficial microorganisms, as occurs with organic acids exuded by banana in the colonization and growth of the PGPR *Bacillus amyloliquefaciens* NJN-6 (Yuan et al., 2015) or root exudates from tomato, which stimulates the hyphal growth of the AMF *Glomus intradices* (Sun et al., 2012).

Once the beneficial effect of root exudates from citrus plants over PGPRs *P. putida* KT2440 and *Novosphingobium* sp. HR1a was proved (Chapter 2), in Chapter 3 the possible role of both bacterial strains in the mitigation of the salt stress induced damages in *C. macrophylla* was evaluated. There are a few works studying the microbiota present in citrus rhizosphere, including the characterization of bacterial populations in citrus roots (Trivedi et al., 2011), the beneficial effect of PGPRs when plants grow in optimal conditions (Chiquito-Contreras et al., 2012) or the effect of mycorrhizae in the mitigation of damages caused by phytophthora (Watanarojanaporn et al., 2011), drought (Wu and Zou, 2009) and high (Navarro et al., 2014; Satir et al., 2016; Zhang et al., 2017) or low temperatures (Wu and Zou, 2010).

Despite the fact that the palliative effect of PGPR could be more important than the alleviation produced by AMF under abiotic stress conditions (Lowe et al., 2012, Younesi and Moradi, 2014) up to date there is a lack of information in citrus.

The results obtained in the Chapter 3 showed the PGPRs *P. putida* KT2440 and *Novosphingobium* sp. HR1a trigger different mechanisms of tolerance in plants subjected to high salinity, but some differences were observed depending on the bacterial strain

inoculated to plant roots. In general terms, the inoculation with both bacterial strains reduced proline content in roots of stressed plants and ABA and SA concentration in leaves. This decrease in ABA concentration could be related to the ACC-deaminase activity produced by PGPRs, which inhibits ethylene synthesis and consequently, could lead to a reduction of ABA concentration due to the crosstalk between ABA and ethylene (Arc et al., 2013; Dimkpa et al., 2009). Both bacterial strains inhibited the reduction of chlorophyll fluorescence related parameters produced by salt stress, which usually occurs in presence of this abiotic stress condition (López-Climent et al., 2008). Moreover, apart from salt stress, the inoculation with both bacterial species produced a decrease in gas exchange related parameters, E and g_s. Although there is some controversy about the induction or inhibition of these parameters by PGPRs, with works defending both opinions (Bresson et al., 2013; Mahmood et al., 2016), it seems that their reduction can lead to a better water use efficiency and inhibit water loss due to some environmental conditions such as drought, osmotic stress or salt stress (Kudoyarova et al., 2015; Leach et al., 2017). Among the beneficial roles of PGPRs in salt stress conditions, it has also been previously described that some PGPRs species as *Pseudomonades* sp. maintain higher levels of chlorophyll fluorescence parameters Φ_{PSII} and F_v/F_m ratio, which favors plant survival to salt stress, which generally induces a decrease on the levels of these parameters (Heidari and Golpayegani, 2012; López-Climent et al., 2008), which is in concordance with the data presented in this chapter, since both strains inhibit the reduction of the F_v/F_m ratio produced by salt after 30 days stress.

Apart from the common benefits that both bacterial strains bring to alemow plant growth and tolerance, other changes in some parameters as IAA accumulation in leaves or the maintenance of Φ_{PSII} levels after 30 days of stress were observed in salt-stressed plants inoculated with *Novosphingobium* sp. HR1a. This fact would indicate that this strain is more efficient in plant stress alleviation than *P. putida* KT2440, which could be due to the higher tolerance that *Novosphingobium* sp. HR1a has to high salinity conditions, as described in Chapter 2.

All the results obtained in this doctoral thesis project, lead to elucidate that citrus root exudates play a key role in the growth and establishment of bacterial communities in the rhizosphere, modulating a mutualistic relationship which can be modified depending on the plant genotype or plant growing conditions. By this, under stress conditions, plants would release to the rhizosphere larger quantities of compounds which can be used as

nutrients by PGPRs, improving their growth and consequently increasing their own chances to survive to environmental stress conditions. However, these nutrients present in citrus root exudates could also be used as sources of C and N by other soil microorganisms, which could lead to a higher susceptibility to the attack of pathogens (Pandey et al., 2017).

This work can entail to start further investigations in the ambit of citrus root exudates and their involvement in the relation with PGPRs, including studies with different genotypes and other kinds of abiotic and biotic stresses, further analyses of primary and secondary metabolites present in root exudates, or simultaneous applications of different PGPRs with other beneficial microorganisms as AMF. Moreover, to a more realistic vision of these interaction among citrus and beneficial microbiota, similar assays with grafted plants could be researched in order to determinate the importance of the scion in root exudates production and the effect of the inoculation by PGPRs in the citrus production of commercial plants.

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Conclusions

Conclusions

Based on the results obtained in the doctoral thesis project described in this Report, the following conclusions can be drawn:

1. The studied genotypes present different sensitivities to the abiotic stresses considered: while citrange Carrizo is sensitive to salt stress and tolerant to stress due to high temperatures, *Citrus macrophylla* is tolerant to saline stress and sensitive to heat stress.

2. Citrus are able to exude, through their roots, proline, as well as the phytohormones ABA, SA, JA, IAA and cinnamic acid.

3. Root exudation pattern in citrus plants is affected by both, the genotype and the abiotic stress situations, such as salinity or the high temperatures to which the plants are exposed. In this way, proline, SA and cinnamic acid are exuded in greater quantities when the plants are subjected to stress conditions. The concentration of these metabolites in root exudates is higher in those obtained from plants tolerant to a certain stress condition.

4. Citrus root exudates promote the growth of the growth promoting bacteria *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a, being this induction of bacterial growth greater when root exudates are obtained from plants grown under abiotic stress conditions.

5. *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a are able to detect the proline and salicylates exuded through the roots. The expression of P_{putA} and P_{pahA} promoters of *Pseudomonas putida* KT2442 and *Novosphingobium* sp. HR1a is respectively correlated with the concentrations of proline, SA and cinnamic acid present in the root exudates. In addition, both strains are also capable of using citrus root exudates as the sole source of N.

6. *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a alleviate the damage caused by salt stress, promoting resistance to this stress in *Citrus macrophylla* plants. Both bacterial strains induce a lower accumulation of proline, ABA and SA in plant tissue, as well as a decrease in chlorophyll fluorescence parameters under saline stress conditions.

7. Under non-stressful culture conditions, *Pseudomonas putida* KT2440 and *Novosphingobium* sp. HR1a induce an increase in the efficiency of water use in *Citrus macrophylla* plants due to the decrease of E and g_s.

Conclusiones

A partir de los resultados obtenidos en el proyecto de tesis doctoral que se describe en esta Memoria, se pueden extraer las siguientes conclusiones:

1. Los genotipos estudiados presentan diferentes sensibilidades a los estreses abióticos considerados: mientras citrange Carrizo es sensible al estrés salino y tolerante al estrés por elevadas temperaturas, *Citrus macrophylla* es tolerante al estrés salino y sensible al estrés por calor.

2. Los cítricos son capaces de exudar, vía radicular, prolina, así como las fitohormonas ABA, SA, JA, IAA y ácido cinámico.

3. El patrón de exudación radicular en plantas de cítricos se ve afectado tanto por el genotipo como por las situaciones de estrés abiótico, como la salinidad o las altas temperaturas a las que están expuestas las plantas. De este modo, prolina, SA y ácido cinámico se exudan en mayores cantidades cuando las plantas están sometidas a condiciones de estrés. La concentración de estos metabolitos en exudados radiculares es mayor en los obtenidos de plantas tolerantes a una determinada condición de estrés.

4. Los exudados radiculares de cítricos promueven el crecimiento de las bacterias promotoras del crecimiento *Pseudomonas putida* KT2440 y *Novosphingobium* sp. HR1a, siendo esta inducción del crecimiento bacteriano mayor cuando los exudados radiculares son obtenidos a partir de plantas cultivadas en condiciones de estrés abiótico.

5. *Pseudomonas putida* KT2440 y *Novosphingobium* sp. HR1a son capaces de detectar la prolina y los salicilatos exudados a través de las raíces. La expresión de los promotores *PputA* y *PpahA* de *Pseudomonas putida* KT2442 y *Novosphingobium* sp. HR1a está respectivamente correlacionada con las concentraciones de prolina, SA y ácido cinámico presentes en los exudados radiculares. Además, ambas cepas también son capaces de usar los exudados radiculares de cítricos como única fuente de N.

6. *Pseudomonas putida* KT2440 y *Novosphingobium* sp. HR1a alivian el daño provocado por el estrés salino, promoviendo la resistencia a este estrés en plantas de *Citrus macrophylla*. Ambas cepas bacterianas inducen una menor acumulación de prolina, ABA y SA en tejido vegetal, así como un descenso de los parámetros de fluorescencia de clorofilas en condiciones de estrés salino.

7. En condiciones de cultivo no estresantes, *Pseudomonas putida* KT2440 y *Novosphingobium* sp. HR1a inducen un aumento de la eficiencia del uso del agua en plantas de *Citrus macrophylla* debido al descenso de la E y de la g_s.

Conclusions

A partir dels resultats obtinguts en el projecte de tesi doctoral que es descriu en aquesta Memòria, es poden extreure les següents conclusions:

1. Els genotips estudiats presenten diferents sensibilitats als estressos abiòtics considerats: mentre citrange Carrizo és sensible a l'estrès salí i tolerant a l'estrès per elevades temperatures, *Citrus macrophylla* és tolerant a l'estrès salí i sensible a l'estrès per calor.

2. Els cítrics són capaços d'exsudar, via radicular, prolina, així com les fitohormones ABA, SA, JA, IAA i àcid cinàmic.

3. El patró d'exsudació radicular en plantes de cítrics es veu afectat tant pel genotip com per les situacions d'estrès abiòtic, com la salinitat o les altes temperatures a les que estan exposades les plantes. D'aquesta manera, prolina, SA i àcid cinàmic s'exsuden en majors quantitats quan les plantes estan sotmeses a condicions d'estrès. La concentració d'aquests metabòlits en exsudats radiculars és major en els obtinguts de plantes tolerants a una determinada condició d'estrès.

4. Els exsudats radiculars de cítrics promouen el creixement dels bacteris promotors del creixement *Pseudomonas putida* KT2440 i *Novosphingobium* sp. HR1a, sent aquesta inducció del creixement bacterià més gran quan els exsudats radiculars són obtinguts a partir de plantes cultivades en condicions d'estrès abiòtic.

5. *Pseudomonas putida* KT2440 i *Novosphingobium* sp. HR1a són capaços de detectar la prolina i els salicilats exsudats a través de les arrels. L'expressió dels promotors P_{putA} i P_{pahA} de *Pseudomonas putida* KT2442 i *Novosphingobium* sp. HR1a està respectivament correlacionada amb les concentracions de prolina, SA i àcid cinàmic presents en els exsudats radiculars. A més, les dues soques també són capaços d'usar els exsudats radiculars de cítrics com a única font de N.

6. *Pseudomonas putida* KT2440 i *Novosphingobium* sp. HR1a alleugen el dany provocat per l'estrès salí, promovent la resistència a aquest estrès en plantes de *Citrus macrophylla*. Les dues soques bacterianes indueixen una menor acumulació de prolina, ABA i SA en teixit vegetal, així com un descens dels paràmetres de fluorescència de clorofil·les en condicions d'estrès salí.

7. En condicions de cultiu no estressants, *Pseudomonas putida* KT2440 i *Novosphingobium* sp. HR1a indueixen un augment de l'eficiència de l'ús de l'aigua en plantes de *Citrus macrophylla* a causa del descens de la E i de la g_s.

Appendix

Aurelio Gómez Cadenas, Catedrático de la Universitat Jaume I de Castellón,

HACE CONSTAR QUE:

Como coautor de las publicaciones indicadas más abajo, acepto que éstas se presenten como parte de la Tesis Doctoral de Vicente Vives Peris y renuncio expresamente a su utilización como parte de otra tesis doctoral. Además, certifico que estas publicaciones no se han incluido previamente en ninguna otra tesis doctoral.

Citrus plants exude proline and phytohormones under abiotic stress conditions. Vives-Peris, V., Gómez-Cadenas, A., Pérez-Clemente, R. M. 2017. Plant Cell Reports 36: 1971-1984. doi: 10.1007/s00299-017-2214-0.

Y para que conste a los efectos oportunos, así lo firmo

En Castellón de la Plana, diciembre de 2017

Fdo. Aurelio Gómez Cadenas

Rosa María Pérez Clemente, Profesora Titular de la Universitat Jaume I de Castellón,

HACE CONSTAR QUE:

Como coautora de las publicaciones indicadas más abajo, acepto que éstas se presenten como parte de la Tesis Doctoral de Vicente Vives Peris y renuncio expresamente a su utilización como parte de otra tesis doctoral. Además, certifico que estas publicaciones no se han incluido previamente en ninguna otra tesis doctoral.

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Fdo. Rosa María Pérez Clemente

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