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Bioeffectors and bacterial elicitors as biotechnological tools in the agrifood industry: a contribution to plant health and food safety

DOCTORAL THESIS

Presented by:

Helena Martín Rivilla

Supervised by:

Dr. Francisco Javier Gutiérrez Mañero

Dr. José Antonio Lucas García

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Supervisors:

Dr. Francisco Javier Gutiérrez Mañero Dr. José Antonio Lucas García

Helena Martín Rivilla

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“I seem to have been only like a child playing on the seashore, diverting myself in finding a smoother stone or a prettier shell than ordinary, whilst the great ocean of truth lay before my eyes with everything to discover”

Isaac Newton (1642-1727)

“Me parece haber sido solo un niño jugando en la orilla del mar, divirtiéndose y buscando una piedra más lisa o una concha más bonita de lo normal, mientras el gran océano de la verdad yacía ante mis ojos con todo por descubrir.”

Isaac Newton (1642-1727)

To my parents and grandparents

A mis padres y a mis abuelos

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Abstract

This doctoral thesis focuses on the study of the rhizosphere microbiome and the plant-beneficial microorganism relationship and their influence on plant physiology, on the defensive secondary metabolism and on the plant immune system in general. This study starts from the consideration of the rhizosphere as an especially rich source of beneficial microorganisms, which have a fundamental role in improving the adaptability of plants to changing environments, and which are adapted to the plant-microorganism system thanks to the selective pressure that plants have exerted on the microbiome during the long process of coevolution. Furthermore, the work arises from the current need to develop new, effective and sustainable biotechnology-based agricultural practices that minimize economic and material losses caused by pathogenic infections and abiotic stress conditions. Hence, the aim is to increase the yield of crops, both in quantity and quality of food, in order to be able to provide to a population in continuous growth and at the same time fulfilling the concept of food security proposed by the FAO.

For all the above, this work focuses on the use of beneficial rhizobacteria (PGPR) and molecules derived from their metabolism (metabolic elicitors) as plant inoculants capable of inducing the defensive secondary metabolism, getting plants with a stronger immune system and, therefore, plants better prepared to face to biotic and abiotic stress. Thus, the ultimate objective of the work is to develop biological plant inoculants, with an effective biostimulant capacity and therefore, capable of replacing phytosanitary products of chemical origin.

The great novelty brought by this work is the use of elicitor molecules derived from the metabolism of certain rhizobacteria. This is why the species of beneficial rhizobacterium selected to study its effect and that of its metabolic elicitors on the secondary metabolism of plants was the strain *Pseudomonas fluorescens* N 21.4, a strain

whose ability to elicit and to induce systemic resistance in different plant species has already been widely demonstrated by the research group.

The study began by testing the live strain as a positive control and different eliciting fractions of it in *Arabidopsis thaliana* seedlings under controlled laboratory conditions, studying their capacity to induce systemic resistance, the level of oxidative stress of the treated plants and the signal transduction pathways involved in the response. The live strain and its metabolic elicitors were then tested under real field conditions in commercial crops of blackberry (*Rubus* cv. Loch Ness), studying the general fitness of the plants through specific markers, as well as the accumulation of compounds of interest in the leaves and in the fruits and the specific genes involved in the ripening of the fruit.

The results showed that in the plants that had been inoculated with the treatments there was a causal relationship between the induction of systemic resistance and the reduction in oxidative stress in *A. thaliana* and a better general fitness, less oxidative stress and greater accumulation of beneficial phenolic compounds in the leaves and fruits of blackberry. Since the ability of the metabolic elicitors of the *P. fluorescens* N 21.4 strain to induce the defensive secondary metabolism was demonstrated, a chemical identification of the molecules responsible for that capacity was performed, identifying seven promising compounds for their potential antibiotic and antifungal capacity.

This study ends with the search for new PGPR strains (bioeffectors) and their metabolic elicitors and with the study of the signal transduction pathways involved in the response of plants elicited with them. From an initial group of one hundred and seventy-five rhizobacteria, two were selected for their promising abilities to be used as plant inoculants in future experiments.

Resumen

La presente tesis doctoral se centra en el estudio del microbioma de la rizosfera y en las relaciones planta-microrganismos beneficiosos y su influencia sobre la fisiología de las plantas y sobre el metabolismo secundario defensivo y el sistema inmunitario vegetal en general. Este estudio parte de la consideración de la rizosfera como una fuente especialmente rica en microorganismos beneficiosos, que tienen un papel fundamental en mejorar la capacidad de adaptación de las plantas a ambientes cambiantes y que están adaptados al sistema planta-microorganismo gracias a la presión selectiva que han ejercido las plantas sobre el microbioma durante el largo proceso de coevolución. Además, el trabajo surge ante la actual necesidad de desarrollar nuevas prácticas agrícolas de base biotecnológica, efectivas y a la vez sostenibles que minimicen las pérdidas económicas y materiales debidas a las infecciones patogénicas que atacan a los cultivos y también causadas por condiciones de estrés abiótico. De esta manera se persigue aumentar el rendimiento de los cultivos, tanto en cantidad como en calidad de alimentos, para poder proveer a una población en continuo crecimiento y a la vez cumplir con el concepto de seguridad alimentaria propuesto por la FAO.

Por todo lo anterior, el presente trabajo se centra en el uso de rizobacterias beneficiosas (PGPR) y de moléculas derivadas del metabolismo de las mismas (elicitores metabólicos) como inoculantes de plantas capaces de inducir el metabolismo secundario defensivo, dando lugar a plantas con un sistema inmunitario más fuerte y, por tanto, plantas mejor preparadas para combatir el estrés biótico y abiótico. Así, el objetivo último del trabajo es desarrollar inoculantes de plantas de origen biológico, con una capacidad bioestimulante efectiva y por ello, capaces de sustituir a los fitosanitarios químicos.

La gran novedad que aporta este trabajo es el uso de moléculas elicitoras derivadas del metabolismo de determinadas rizobacterias. Es por esto, que la especie de rizobacteria

beneficiosa seleccionada para estudiar su efecto y el de sus elicitores metabólicos sobre el metabolismo secundario de las plantas fue la cepa *Pseudomonas fluorescens* N 21.4, una cepa cuya capacidad de elicitación y de inducción de resistencia sistémica en distintas especies vegetales ya ha sido ampliamente demostrada por el grupo de investigación.

Se comenzó probando la cepa viva como control positivo y distintas fracciones elicitoras de la misma en plántulas de *Arabidopsis thaliana* en condiciones controladas de laboratorio, estudiando su capacidad de inducción de resistencia sistémica, el nivel de estrés oxidativo de las plantas tratadas y las rutas de transducción de señal implicadas en la respuesta. Después se probó la cepa viva y sus elicitores metabólicos en condiciones reales de campo en cultivos comerciales de zarzamora (*Rubus* cv. Loch Ness), estudiándose el fitness general de las plantas a través de marcadores específicos, así como la acumulación de compuestos de interés en las hojas y en los frutos y los genes específicos implicados en la maduración de los frutos.

Los resultados mostraron que en las plantas inoculadas con los tratamientos existía una relación causal entre la inducción de resistencia sistémica y una reducción del estrés oxidativo en *A. thaliana* y un mejor fitness general, menor estrés oxidativo y mayor acumulación de compuestos fenólicos beneficiosos en las hojas y frutos de zarzamora. Al demostrarse la capacidad de los elicitores metabólicos de la cepa *P. fluorescens* N 21.4 de inducir el metabolismo secundario defensivo, se procedió a la identificación química de las moléculas responsables de dicha capacidad, encontrándose siete compuestos muy prometedores dado su potencial capacidad antibiótica y antifúngica.

Este estudio termina con la búsqueda de nuevas cepas PGPR (bioefectores) y de sus elicitores metabólicos y con el estudio de las vías de transducción de señal involucradas en la respuesta de las plantas elicitadas con las mismas. De un grupo inicial

de ciento setenta y cinco rizobacterias, se seleccionaron dos por sus prometedoras capacidades para utilizarse como inoculantes de plantas en experimentos futuros.

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List of abbreviations and acronyms

- 4CL: 4-Coumarate-CoA Ligase
- ACT: Actin
- AHLs: Acyl Homoserine Lactones
- ANOVA: Analysis of Variance
- ANR: Anthocyanidin Reductase
- ANS: Anthocyanidin Synthase
- APX: Ascorbate Peroxidase
- ASC: Ascorbate
- bp: Base pairs
- BSA: Bovine Serum Albumin
- BTH: Benzothiadiazole
- C4H: Cinnamic Acid 4-Hydroxylase
- CAT: Catalase
- CEMBIO: Center of Excellence in Metabolomics and Bioanalysis
- cfu: colony forming unit
- CHI: Chalcone Isomerase
- Chl a: Chlorophyll a
- Chl b: Chlorophyll b
- CHS: Chalcone Synthase
- Ct: Cycle threshold
- cv: cultivar
- DFR: Dihydroflavonol Reductase
- DHAR: Dehydroascorbate reductase
- DMSO: Dimethyl Sulfoxide

- DPPH: 2,2-diphenyl-1-picrilhidrazil
- EIC: Extracted Ion Chromatogram
- Embrapa: Empresa Brasileira de Pesquisa Agropecuária
- ERF: Ethylene Response Factor
- ESI: Electrospray Ionization Source
- ET: Ethylene
- ETI: Effector-Triggered Immunity
- F0: Minimal fluorescence yield
- F1-F4: Fractions 1-4
- F3´H: Flavonoid-3´-Hydroxylase
- F3H: Flavanone-3-Hydroxylase
- FAO: Food and Agriculture Organization of the United Nations
- FGT: Flavonol Glucosyl Transferase
- Fig: Figure
- FLS: Flavonol Synthase
- Fm: Maximum fluorescence yield of chlorophyll
- Fp: Purest fraction
- Fv: Variable fluorescence
- Fv/Fm: Maximum quantum yield of photosystem II
- FW: Fresh Weigh
- GPX: Guaiacol Peroxidase
- GR: Glutathione Reductase
- GSH: glutathione
- hapc: hours after pathogen challenge
- HIR: Herbivore Induced Resistance

- HPLC: High Performance Liquid Chromatography
- HR: hypersensitive response
- IAA: Indole Acetic Acid
- ICS: Isochorismate Synthase
- ISR: Induction of Systemic Resistance
- JA: Jasmonic Acid
- K-3-O-G: Kaempferol-3-O-glucoside
- K-3-O-R: Kaempferol -3-O-rutinoside
- LAR: Localized Acquired Resistance
- LAR: Leucocyanidin Reductase
- LC/MS: Liquid Chromatography/Mass Spectrometry
- LOQ: Limit of Quantifying
- LOX2: Lipoxygenase 2
- MAMPs: Microbe Associated Molecular Patters
- MDA: Malondialdehyde
- MDHAR: Monodehydroascorbate Reductase
- ME: Metabolic elicitors
- MFE: Molecular Feature Extraction
- NASC: Nottingham Arabidopsis Stock Centre
- NB-LRR: Nucleotide Binding-Leucine rich repeat
- ND: Non-detected-sample
- NPR1: Nonexpressor of Pathogenesis-Related Protein 1
- PAL: Phenylalanine Ammonia-Liase
- PAMPs: Pathogen Associated Molecular Patters
- PCA: Potato Dextrose Agar

- PDF1: Plant Defensin Type 1
- PGPR: Plant Growth Promoting Rhizobacteria
- PMSF: Phenylmethylsulfonyl Fluoride
- ppm: parts per million
- PPO: Polyphenoloxidase
- PR: Pathogenesis Related
- PR1: Pathogenesis Related Protein 1
- PR2: Pathogenesis Related Protein 2
- PR3: Pathogenesis Related Protein 3
- PRRs: Pattern Recognition Receptors
- PSII: Photosystem II
- PTI: Pattern Triggered Immunity
- pv: Pathovar
- Q-3-O-G: Qurcetin-3-O-glucoside
- Q-3-O-R: Quercetin 3-O-rutinoside
- qPCR: quantitative polymerase chain reaction
- QTOF-MS: Quadrupole Time-of-Flight Mass Spectrometry Analyser
- RH: Relative Humidity
- ROS: Reactive Oxygen Species
- rpm: revolutions per minute
- RT: Reverse Transcription
- *Ru*: *Rubus*
- SA: Salicylic Acid
- SAR: Acquired Systemic Resistance
- SOD: Superoxide Dismutase

- TFA: Trifluoride Acetic Acid
- TLC: Thin Layer Chromatography
- UHPLC: Ultra-High Performance Liquid Chromatography
- VLC: Vacuum Liquid Chromatography
- XO: Xanthine Oxidase

1. Introduction

1. Introduction

The year 2020 has been declared "International Year of Plant Health" by the FAO (Food and Agriculture Organization of the United Nations (Fig. 1)). This statement of plant health is narrowly related to the current necessity of providing enough food and quality food to a continuously growing and increasingly numerous population, which is estimated to increase by 2.3 billion by 2050 (FAO, 2018), reaching 9.7 billion. This is also framed in the idea of fulfilling the concept of food security, that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life (FAO, 2011).

Figure 1 Representative scheme of the International Year of Plant Health declared by the FAO.

During the 20th century, in the relative recent Green Revolution of the 1960s, the agrifood industry was only focused on increasing crops yield and on facing to pathogen pests, but was not aware about food quality, nor soil health or environmental risks. However, during the 21st century, the awareness of food security and food quality has been growing, as well as a socio-economic and environmental awareness that pursues a sustainable development, in which the challenge is to find a perfect balance between improving people´s quality of life and the maintenance of natural resources so that our current food system could sufficiently provide for future generations (Lindgren, 2018). These challenges to sustained food security require various solutions, ranging from social

and economic change, to technological and biotechnological advance, with the improvement of crops being a priority to reach a solution (Wallace et al. 2018).

Predictions have advised that the world would need to increase crop production by 26%-68% from 2014 baseline levels (Hunter et al. 2017). This extra demand linked to the pressure that society has imposed in the agrifood industry in recent years, has favored the development of new agricultural techniques aimed at achieving quality products within the framework of sustainable production. This is why the current challenge is to find effective, ecofriendly and, at the same time, low-cost agriculture control methods that guarantee the sustainability of crop production while eliminating negative impact on the environment.

The greatest handicap that agricultural production has to face is the material and economic losses due to pests and infections, which suppose annually losses of US$ 40 billion (Miller et al. 2017; Syed Ab Raham et al. 2018), with higher loses found in food-insecure regions (Savary et al. 2019). In addition, the usual practice of monocultures is favoring the development of resistance by pathogens, and climate change and rising global temperatures have been seen as a cause of an increase in the geographic distribution and the reproductive potential of pathogens (Hirabayashi et al. 2013; Miller et al. 2017; Tietjen et al. 2017). For all these reasons, and as a mechanism to counteract the attack of pathogens, it is essential that agricultural techniques, focused on achieving food security and on the improvement and protection of crops, are based on the study of plant physiology, plant immune system, as well as on plant interactions with pathogens or with beneficial microorganisms. It is also essential to study biotic and abiotic stress suffered by the plants to know how to combat it. This is why looking for stress-resilient crops has become a priority for research (Steinward and Ronald, 2020).

For all the above, the general aim of the present work is to study the plant immune system and the plant-pathogen/beneficial microorganism relationship to improve the immune response of plants and their resistance to biotic and abiotic stress so that this knowledge can be applied to commercial crops from which to obtain a better and sustainable yield. These goals would be achieved through the inoculation of beneficial rhizobacteria and/or some of the metabolic compounds generated by them. In this way, a high-performance agricultural system, economically profitable, safe with the environment and with people's health, and, at the same time, being able to provide higher quality food and thus contributing to food security, would be settle.

To reach our objectives, laboratory experiments were carried out under controlled conditions using the model plant *Arabidopsis thaliana*, and then other experiments were performed under real field conditions in cultivars of blackberry (*Rubus* cv. Loch Ness). With our results, we would be contributing to increase the knowledge about the plant immune system and the interactions of plants with microorganisms, as well as favoring the implementation of efficient and ecofriendly agricultural techniques, all aimed at improving both production and quality of the food produced and contributing decisively to reaching the objective set by the FAO in its definition of food security.

1.1 Plant-microbe interactions in agriculture

1.1.1 The rhizosphere

The first time the term “rhizosphere” was used was in 1904 by the agronomist Lorenz Hiltner and was defined as the effect promoted by organic compound exudates from the roots of legumes, on the microbial communities that inhabit the surrounding soil (Lynch et al. 1990; Hartmann et al. 2008). Hiltner proposed that the quality of the products produced by plants could depend on the composition of the microbial communities of

their roots and that these microorganism could also influence in the resistance of plants against pathogens. However, it was not until the end of the 20th century when the rhizosphere went from being considered an effect to a complex ecosystem.

Currently, the rhizosphere is defined as the portion of soil intimately associated to the roots of growing plants, with physical, chemical and biological properties different from those of the rest of the soil and with an extraordinarily complex structure, in which a large number of variables incise, and in which a multitude of biological relationships are established. It is considered an ecosystem made up of three components: plant, soil and microorganisms (Fig. 1.1) that interact in a unique and dynamic way (Jones, 1992; Hinsinger et al. 2009; Kumar and Dubey, 2020).

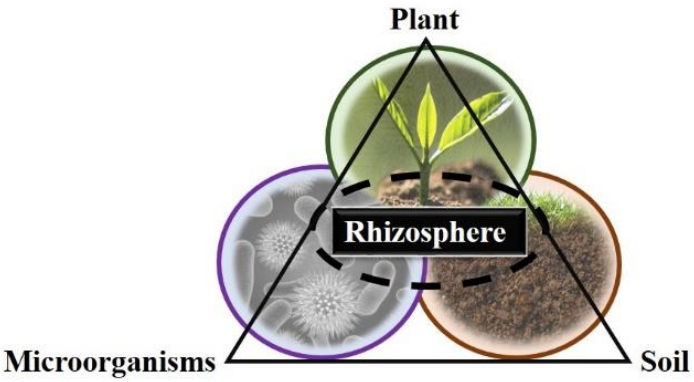

Figure 1.1 Trilogy of the soil. Representation of the interaction between the plant, the soil and the microorganisms that form the rhizosphere (Lynch, 1990).

The way plants influence in the rhizosphere is by supplying nutrients through root exudates (Fig. 1.2). It is estimated that approximately 2-21% of the photoassimilates produced by plants are released by the roots as exudates (Lugtenberg and Kamilova, 2009; Huang et al. 2014) and this is the reason why the rhizosphere has an extraordinarily microorganism density (Lugtenberg and Kamilova, 2009; Reinhold-Hurek et al. 2018). Plant roots secrete a vast range of low and high-molecular weight compounds, such as sugars, organic acids, amino acids, polysaccharides, vitamins, and other secondary

metabolites into the surrounding soil (Badri and Vivanco, 2009; Huang et al. 2014). It is remarkable that the amount and composition of root exudates varies with plant species (Lu et al. 2018; Huang et al. 2019) and with plant growth stages (Chaparro et al. 2014). The release of these exudates is an "investment" adaptive strategy (Pieterse et al. 2016), since plant can promote and select the growth of certain edaphic beneficial microorganisms that, in turn, boost plant development improving its nutrition, especially under constrained conditions (Lareen et al. 2016). Therefore, the rhizosphere is an area that locates potentially beneficial microorganisms, already selected by the plant (Lambers et al. 2009; Reinhold-Hurek et al. 2015).

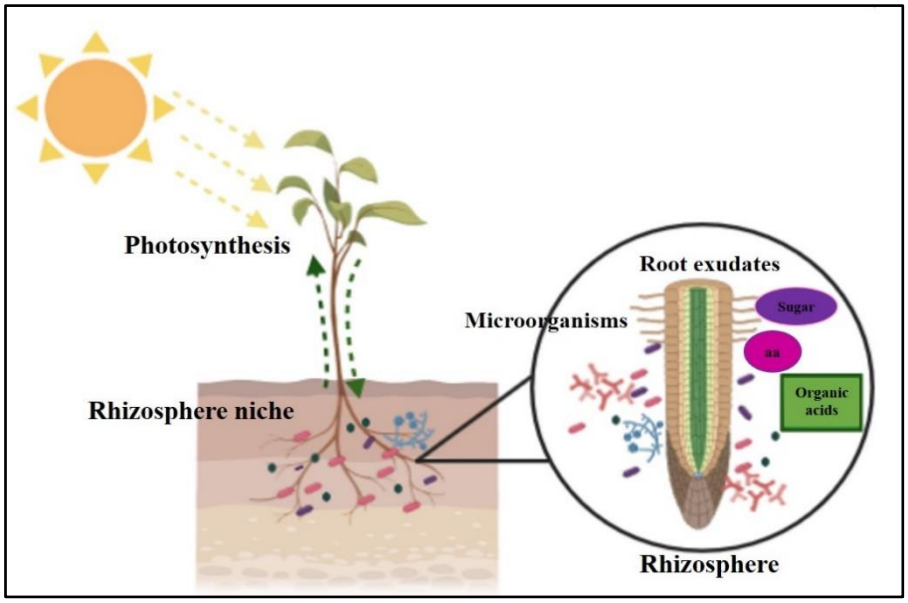

Figure 1.2 Plant-microorganism interactions in the rhizospheric system.

On the other hand, the edaphic factors that affect the plant are abiotic factors, such as the physicochemical characteristics of the soil, the pH, the concentration of O_2 and CO_2, the concentration of nutrients, pesticides residues and heavy metals, and the structure of the soil, among others (Lynch, 1990), since they are factors that influence on root development and on its capacity for exudation; and biotic factors, such as pathogen infection (Yuan et al. 2018), or the abundance or the relative proportion of the

rhizospheric microorganisms, currently and collectively named as rhizospheric microbiome (Pieterse et al. 2016).

The microbiome is known as the ‘second genome’ of an organism that strongly influences its health and general well-being (Arif et al. 2020). The plant and its microbiome form a “holobiont”, a single entity, whereby evolutionary selection between plants and microorganisms contributes to the overall stability of the system (Vandenkoornhuyse et al. 2015). Plant microbiome plays a vital role in relation to nutrient uptake, metabolic capabilities and tolerance to biotic and abiotic stress (Bulgarelli et al. 2012; Sessitsch and Mitter, 2015). It also participates in the organic matter decomposition, which boosts soil fertility and ultimately improves plant productivity (Mohanram and Kumar, 2019). The microbiome therefore eases communication between the plant and the surrounding environment and they together allow constituting a productive metagenome that leads to increase crop productivity (Zorner et al. 2018).

The current genome, metagenome and multi-omics studies have linked individual microbial taxa and genes to plant colonization, physiology and fitness (Wagner et al. 2016; Bergelson et al. 2019), and have made possible the identification and characterization of the genes that govern plant interactions with the associated microbiomes (Levy et al. 2018; Liu et al. 2018), thus increasing the understanding of how microorganisms adapt to the plant environment. However, the molecular mechanisms that govern plant–microorganism interactions at a community level are still not well understood, and to maximize the microbiome role, it is necessary to study the biochemical and molecular factors that take place in the rhizosphere, in the root and leave endosphere, and in the phyllosphere and that lead to the selective microbial enrichment (Hassani et al. 2018).

Since it has been seen that the rhizosphere and the specific rhizospheric microbiome of a plant largely determines the productivity of the whole ecosystem and therefore of the crops, its study from a biotechnological point of view has a great potential. This study of the rhizosphere should have a multidisciplinary approach due to its complexity and it should be analysed from a biophysical, biogeochemical, and edaphic point of view, but also taking into account the mechanisms of communication and metabolic adaptation between microorganisms and plants and the multitrophic interactions. Furthermore, most of the rhizospheric microorganisms are not culturable and their qualitative analysis is not possible. Hence, different culture independent techniques, such as metagenomics, transcriptomics, proteomics and metabolomics are essential to study the rhizosphere microbiome (Lin et al. 2013; Tschaplinski et al. 2014; Pascual et al. 2016; Hao et al. 2018). A multidisciplinary exploration of the rhizosphere and the plant microbiome will therefore allow for a breakthrough for the application of beneficial microorganisms in practical agricultural development (Qu et al. 2020).

For all the above explained, the present work is mainly focus on the study of the symbiotic interaction between plants and beneficial rhizobacteria known as PGPR (Plant Growth Promoting Rhizobacteria (Kloepper, 1978)). Throughout this work, the effect of some PGPR on plant physiology is studied and the characterization of one in particular is addressed.

1.1.2 PGPR/elicitors: action mechanisms and effects on plant physiology

PGPR are free-living edaphic bacteria that are beneficial to plants. PGPR have been found, belonging to a multitude of bacterial genera, being *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Pseudomonas* and *Bacillus* the most common ones (Kloepper et al. 1989; Barriuso et al. 2005; Lugtenberg and Kamilova, 2009; Babalola, 2010; Berendsen et al. 2012; Igiehon and Babalola, 2018; Hashem et al. 2019).

Since the first time the term PGPR was introduced in 1978 by Dr. Kloepper, in reference to bacteria that colonize plant roots and promote their growth, the knowledge of how these beneficial microorganisms influence on plant physiology has undergone a tremendous development. One of the main milestones of these beneficial microorganisms was the discovery of their ability to systemically protect host plants against a wide range of pathogens (Van Loon et al. 1998). Since then, studies carried out with PGPR have been focused not only to recognize the effects on plants, but also to unravel the signal transduction pathways involved and the stimuli that trigger them. The stimuli have been described as triggered by the live bacteria, which from now on we will call **bioeffectors**, or by the molecules that are directly responsible for triggering the systemic response (Pieterse et al. 2016), and which we will refer as **elicitors**. We will also differentiate between metabolic elicitors, as molecules produced by bacterial metabolism and released to the medium, and between structural elicitors, as components of the bacterial cell wall or flagellum.

The biological elicitation by PGPR and/or their metabolic or structural elicitors is proposed as a useful strategy to improve, on the one hand, plant production (biomass) if the strain used induces growth promotion of the plant to which it is applied and, on the other hand, for its potential to alter the secondary metabolism of it, the latter being of special interest to the pharmaceutical and agricultural industry (Zhao et al. 2005; Glick, 2012). All the effects that PGPR have on plant physiology are detailed below, starting with the classical direct and indirect mechanisms, to continue with the systemic induction of secondary metabolism in next sections.

Action mechanisms of PGPR

The mechanisms of action of PGPR have been divided, depending on the plant involvement, in indirect and direct (Ramos-Solano et al. 2008a).

Indirect mechanisms of action are considered when bacteria release some metabolites that, in turn, affects other rhizospheric factors that revert to an improvement or stimulation of plant growth (Kloepper and Metting Jr, 1992; Van Loon, 2007; Martínez-Viveros et al. 2010; Compant et al. 2019). In this type of stimulation, the participation of the plant is not necessary. Some examples of indirect mechanisms are:

- Production of nutrients able to mobilize substances, such as organic acids or amino acids (Jones, 1998; Vassilev et al. 2006; Rodríguez et al. 2006).
- Production of siderophores. These molecules chelate most of the Fe^{3+} present in the soil allowing PGPR to compete successfully against pathogens and letting the plant to absorb it (Kloepper et al. 1981; Weller, 1988; Radzki et al. 2013).
- Synthesis of antifungal and antibiotic molecules to control edaphic pathogens by antagonisms or competitions (Schroth et al. 1984; Chin-A-Woeng et al. 2000; Beneduzi et al. 2012; Lozano et al. 2019).
- Production of acyl homoserine lactones (AHLs) able to block or to hydrolyze "quorum-sensing" molecules produced by pathogens (Lugtenberg and Kamilova, 2009; Rutherford et al. 2012).
- Synthesis of fungal wall hydrolytic enzymes, such as ß-1, 3-glucanases or chitinases (Lim et al. 1991; Bloemberg and Lugtenberg, 2001; Ramos Solano et al. 2010b).
- Synthesis of hydrocyanic acid (HCN) with antipathogenic effect (Voisard et al. 1989; Hayat et al. 2010).

On the other hand, direct mechanisms of actions are those in which the microorganism produces a metabolite that by itself is capable of modifying the physiology of the plant and requires plant mediation (Kloepper and Metting Jr, 1992; Ramos-Solano et al. 2008a; Lugtenberg and Kamilova, 2009; Compant et al. 2019). Some direct mechanisms of action are:

- Production of growth regulators, such as auxins, abscisic acid, gibberellic acid, cytokinins, among others (Gutiérrez Mañero et al. 1996 and 2001; Spaepen et al. 2007; Brilli et al. 2019; Tahir et al. 2019).

- Symbiotic nitrogen root fixation (Baldani and Döbereiner, 1980; Dobbelaere et al. 2003; Hayat et al. 2010).

- Ethylene synthesis inhibition by the ACC deaminase enzyme (Glick et al. 1994; Glick et al. 2007; Amnaa et al. 2019).

- Symbiotic association of mycorrhizal fungus (Toro et al. 1998; Barriuso et al. 2008).

- Induction of systemic resistance (ISR) (Van Loon et al. 1998; Van Wees et al. 2008; Beneduzi et al. 2012; Martin-Rivilla et al. 2019 (data obtained during the development of the present doctoral thesis)). This is a topic of special relevance due to its close relationship with the triggering of secondary metabolism, mainly the pathways related to plant defence, and to some particular metabolites that are significant for human health and nutrition (García-Seco et al. 2015a).

For all these benefits that PGPR report to plants and that are related to better nutrition, growth and development of the plant, and protection against pathogens and abiotic stress, it is essential to study how these localized interactions occur in the rhizosphere, between the plant and the microbial communities (microbiome).

1.2 Plant immune system

1.2.1 Local and systemic resistance

Plants are sessile organisms, unable to escape from both biotic and abiotic stress, and they lack an adaptive immune system with circulating immune cells, as is the case of animals. Due to these two aspects, they have developed, as compensation, a much more complex and extensive immune system for the recognition of pathogenesis and stress than that of

animals (Dodds et al. 2010). Plants have developed a vast secondary metabolism that gives them an extraordinary ability to adapt to the environment, (Weston et al. 2012) improving their chances of survival. These environmental changes include biotic stress, like herbivores and pathogens (fungi, bacteria, nematodes, and virus), and abiotic, like UV-radiation, salt stress, drought, basic soil, temperature, etc.

Defence against herbivores triggers a metabolic response that leads to the synthesis of poorly digestive or repellent substances, or even to the synthesis of volatile compounds capable of alerting other nearby individuals to the imminent danger (Karban and Myers, 1989).

On the other hand, plants have an amazing innate ability to recognize pathogens through strategies that include the recognition of both evolutionarily conserved and variable pathogenic elicitors, while pathogens are able to manipulate plant defensive responses by secreting molecular virulence effectors (Buchanan et al. 2015). This is what is known as an "arm-race co-evolution" and takes place during the long process of coevolution between plants and pathogens (McCann et al. 2017).

Plants present two innate immunity strategies for the pathogen detection (Dodds et al. 2010):

On the one hand, plant cells have on their surface receptor proteins (Pattern Recognition Receptors (PRRs)) that recognize pathogen microbial elicitors, known as PAMPs (Pathogen Associated Molecular Patterns), or non-pathogen microbial elicitors, known as MAMPs (Microbe Associated Molecular Patterns). PAMPs are typically essential components of all kinds of pathogens, such as flagellin from bacteria or chitinase from fungi (Alabouvette et al. 1993; Yamaguchi et al. 2000; Al-Tawaha et al. 2005; Ramos-Solano et al. 2008b; Villena et al. 2018).

The stimulation of these PRRs proteins leads to an immunity triggered by PAMPs / MAMPs, known as **PTI** (Pattern Triggered Immunity) (Jones and Dangl, 2006). This defensive response focuses on the activation of the biosynthesis of defensive substances aimed at stopping the pathogenic attack (Dodds and Rathjen, 2010). Many of these PRRs interact with the BAK1 protein (Brassinosteroid Insensitive 1-Associated Kinase) to initiate the PTI signalling pathway (Fig. 1.3).

On the other hand, plants are also able to respond to endogenous molecules that pathogens released inside their cells. This type of perception involves the recognition by intracellular plant receptors of virulent pathogen molecules, called effectors, which are able to override the plant response (Miller et al. 2017) and that are highly variable, unlike PAMPs, which are conserved structures (Fig. 1.3). To counteract these attacks, plants have developed intracellular detectors, called NB-LRR (Nucleotide Binding-Leucine rich repeat). This specific recognition leads to an Effector-Triggered Immunity (Fig. 1.3 (Dodds, 2010)), (**ETI**) and to the transcription of resistance genes (PR genes).

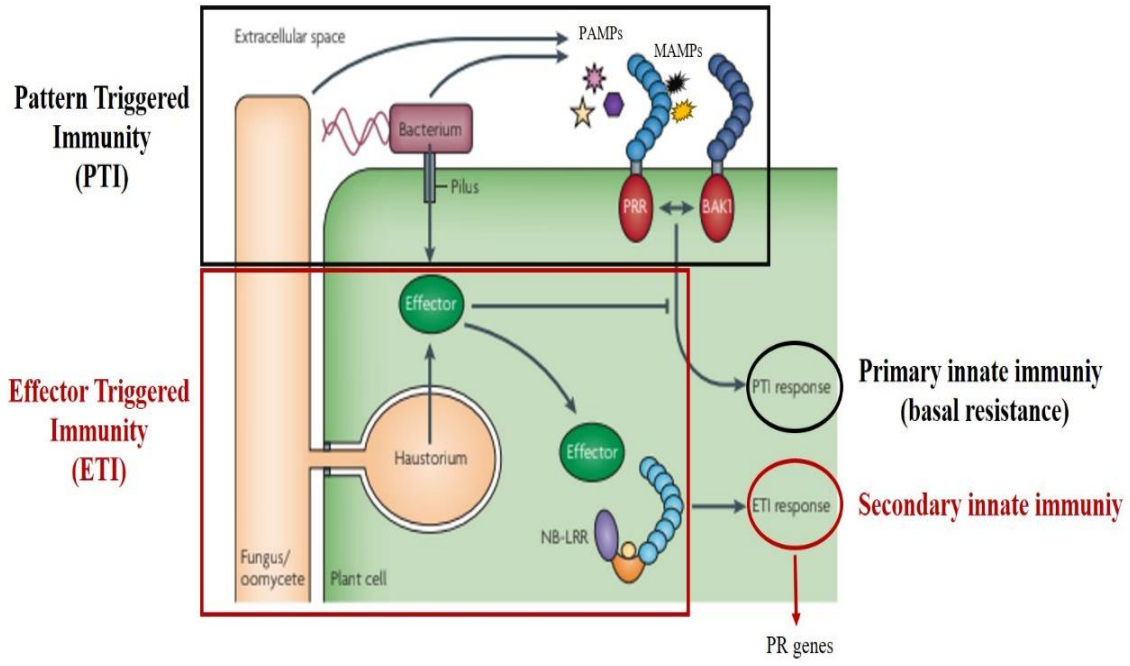

Figure 1.3 Schematic representation of Pattern Triggered Immunity (PTI), in black, and Effector Triggered Immunity (ETI), in red (Dodds et al. 2010).

Once the recognition of the pathogen by the plant has taken place, responses aimed to block the infection or to inhibit the growth of the microorganism (Boller, 1995; Nicaise et al. 2009). These responses are accompanied, by the release of reactive oxygen species (ROS), which in addition to their antibacterial effect, contribute to strengthening the cell wall and act as stress signal transduction elements, inducing other defence responses (Apel and Hirt, 2004; Jones and Dangl, 2006), such as MAPK kinase activation and changes in the phosphorylation of other proteins (Peck et al. 2001; Corwin and Kliebestein, 2017). After a few minutes, plant hormone biosynthesis and gene activation occurs (Spanu et al. 1994; Zipfel et al. 2004). Finally, after hours, callus deposition takes place (Gómez-Gómez et al. 1999) (Fig. 1.4).

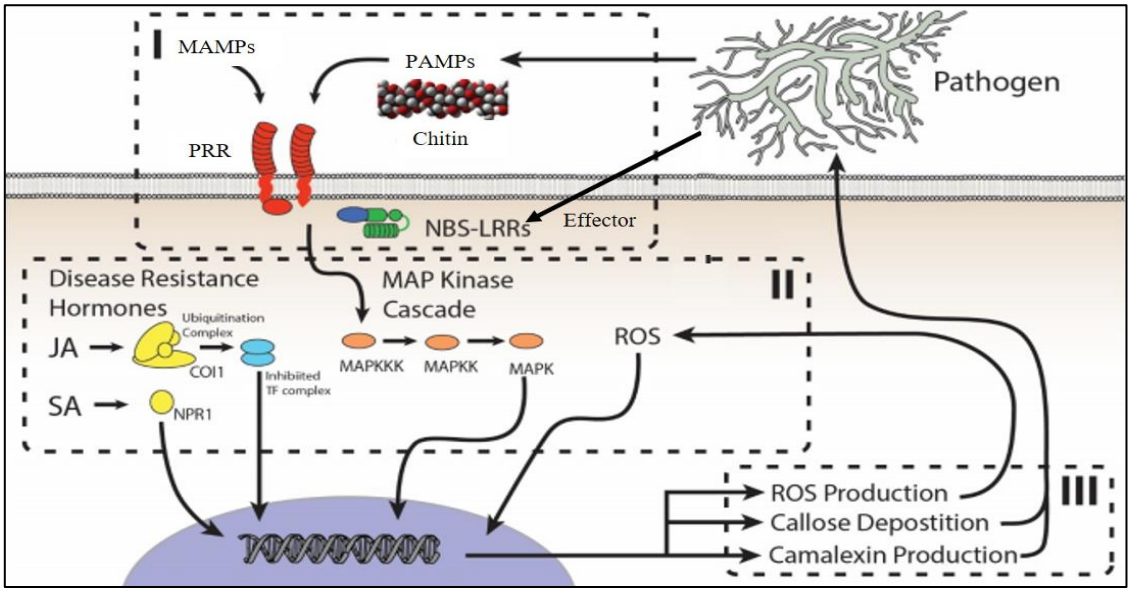

Figure 1.4 General model of the innate immune system of plants (Corwin and Kliebestein, 2017). I) The plant is able to perceive MAMPs and PAMPs via the apoplastic kinase-type receptors (PRRs) or effectors via NBS-LRRs cytoplasmic receptors; II) Signal transduction of the M / PAMPs is carried out by a cascade of MAP kinases and a number of transcription factors III) Signal transduction involves the production of specific defensive responses, such as ROS production or callus deposition. Defensive responses may go through the transduction pathway of salicylic acid (SA), against biotrophic pathogens, or of jasmonic acid (JA) against necrotrophic or herbivorous pathogens.

Generally, both PTI and ETI give rise to similar immune responses, although ETI is quantitatively more intense and faster than PTI (less specific) and frequently involves localized cell death, known as hypersensitive response (HR). This controlled cell death process prevents the pathogen from accessing nutrients, avoiding their spread through plant tissue (Boller, 1995). Such cell death is limited to a few cells at the site of infection, which are surrounded by several layers of cells in which defence-related genes are being massively expressed. However, for a specific pathogen, the basal PTI response plays a very important role in defending the plant against a large number of potential pathogens (Tao et al. 2003).

Once the hypersensitive response has taken place, plant tissues are more resistant and are prepared for future pathogenic attacks. This phenomenon is known as Acquired Systemic Resistance (**SAR**) (Ryals et al. 1996) and assumes that uninfected systemic tissues are more resistant to pathogenic attacks in response to a localized infection that has taken place anywhere in the plant (Buchanan et al. 2015. (Fig. 1.5)). In systemic tissues, SAR is characterized by increased levels of the hormone salicylic acid, which activates the transcriptional factor NPR1 (Non-expressor of PR-1) (Mukhtar et al. 2009), activating the transcription of genes related to pathogenesis (PR genes). SAR is therefore based in the ability of the plant to acquire systemic resistance after a local pathogen attack.

However, it is described another way in which plants acquire systemic resistance after being in touch with microorganisms, but in this case are non-pathogenic microorganisms. This phenomenon is called Induced Systemic Resistance (**ISR**) (Van Loon et al. 1998) and it involves inducing resistance not only locally at the site of infection, but also systemically (Fig. 1.5). The plant increases its defences acquiring resistance against a wide spectrum of pathogens after being in touch with non-pathogen

microorganisms, as could be PGPR. These PGPR (bioeffectors) and some of their elicitors (structural molecules or metabolic molecules released to the medium) also induce in plants a physiological alert state prior to stress challenge known as "**priming**" (Conrath et al. 2002). Plants in this state are able to develop a faster and/or stronger activation of defensive responses after the attack of pathogens, insects or in response to abiotic stress (Conrath et al. 2006; Beckers and Conrath, 2007; Frost et al. 2008).

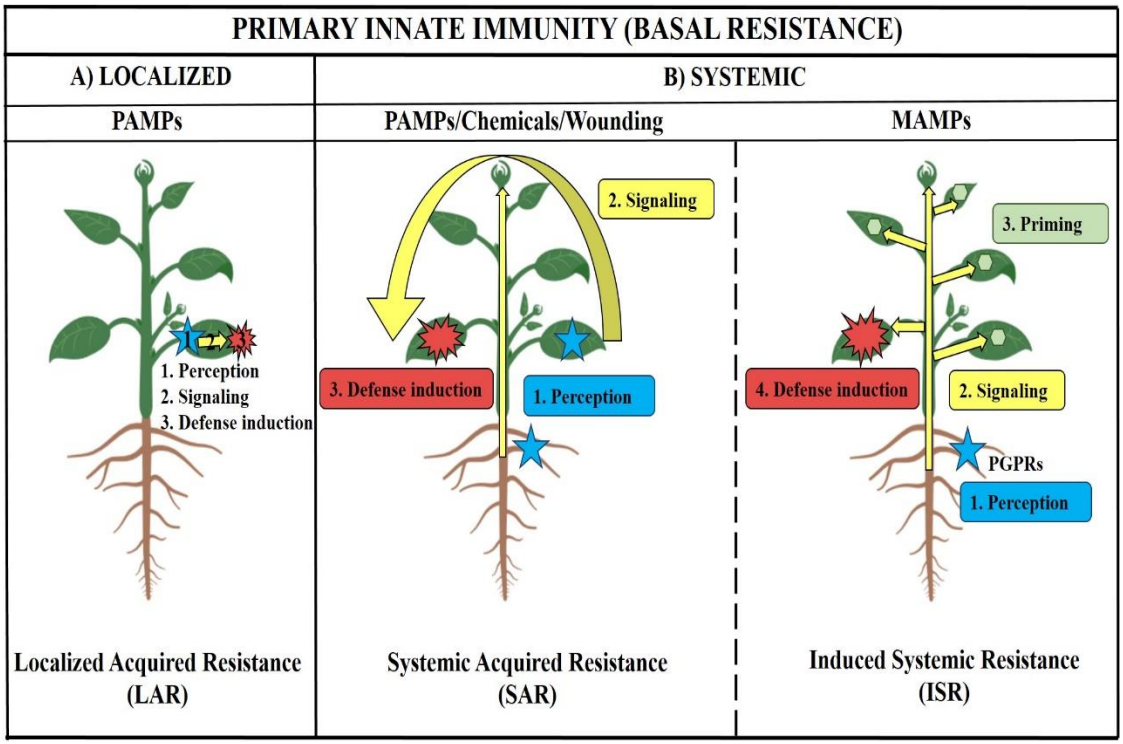

Figure 1.5 Induction of plant, local (A) and systemic (B) innate immunity. The Acquired Systemic Resistance (SAR) is activated by PAMPs, while Induced Systemic Resistance (ISR) is activated by elicitors of non-pathogenic microbial origin (MAMPs). Scheme modified from the one published by Thonart et al. 2012.

The priming state triggered by ISR, therefore, supposes that the defensive systems of the plant are on alert, elicited, and that they activate at much higher speed and more intensely when the plant is attacked by a pathogen, which significantly increases plant ability to avoid infection (Fig 1.6) (Mauch-Mani et al. 2017; Sukanya et al. 2018; Mhlongo et al. 2018). Priming can be durable and maintained throughout the plant's life

cycle and can even be transmitted to subsequent generations, therefore representing a type of plant immunological memory. In addition, priming has been seen to have a very reduced fitness cost in terms of growth and seed or fruit production (Martinez-Medina et al. 2016; Mauch-Mani et al. 2017).

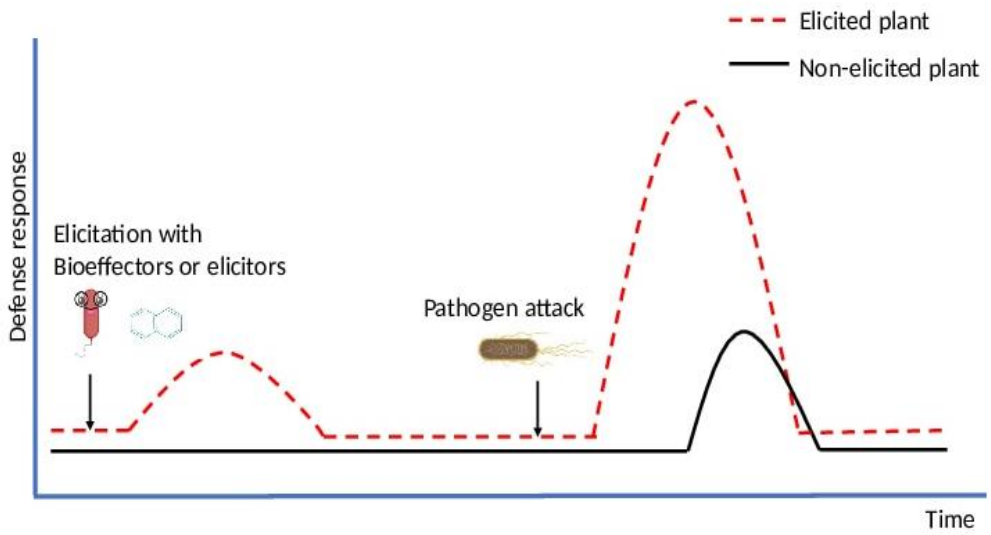

Figure 1.6 Time schematic representation of the defensive response against the attack of a pathogen of a plant elicited with a bioeffector or elicitor (in red) compared to that of a non-elicited plant (in black).

For priming to take place, the perception of the stimulus must first occur, later, changes are triggered in the plant at the physiological, transcriptional, metabolic and epigenetic levels, and the synthesis of a plethora of defensive compounds begins. This phase is called the priming phase (Fig. 1.7) and it refers to the biological process of acquiring priming, which takes place from the initial stimulation through the exposure to a challenging stress. The priming phase includes all changes that occur in the plant after the perception of a stimulus (known as the priming fingerprint) and that prepare the plant for enhanced responsiveness when a challenge occurs. These changes can occur within seconds or hours after the stimulation. Upon subsequent challenge, the plant effectively mounts a faster and/or stronger defence response (increase protein accumulation and

enzymatic activity, synthesis of defensive compounds, callose deposition, etc.) that defines the postchallenge primed state (Fig. 1.7) and results in increased resistance and/or stress tolerance.

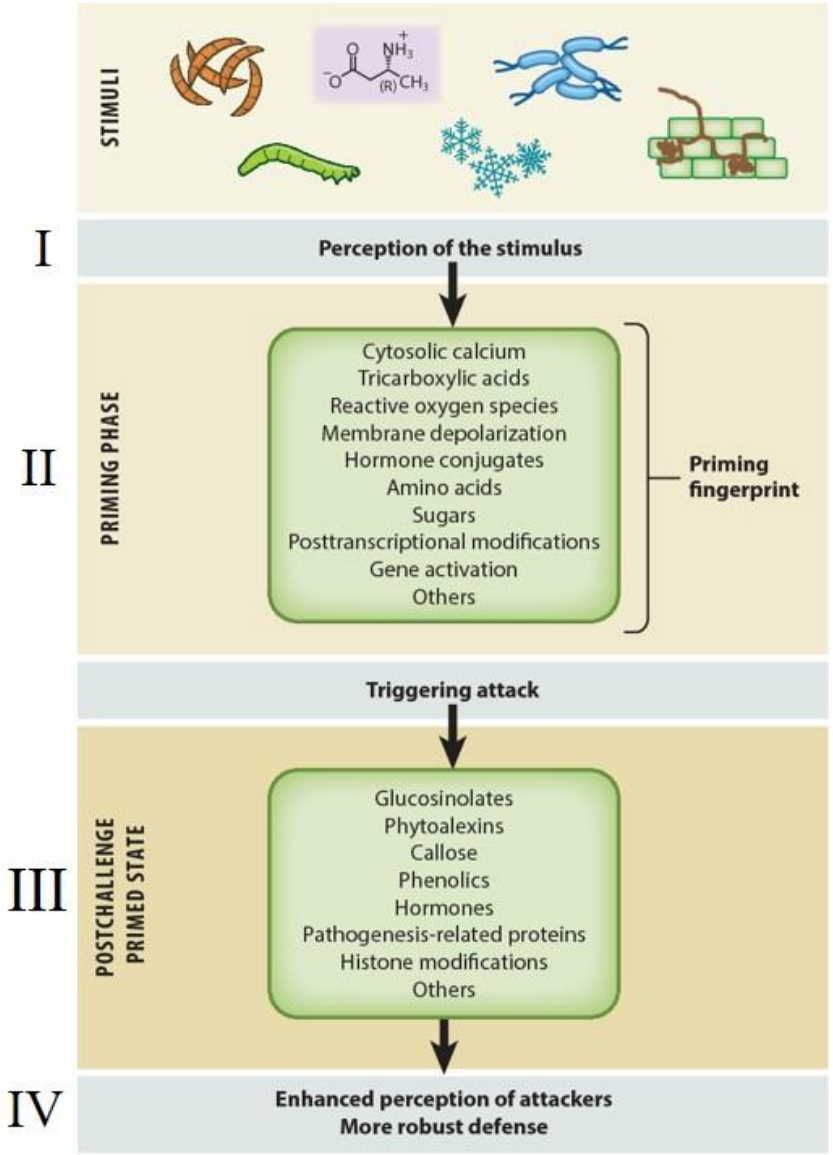

Figure 1.7 The sequential steps of defence priming: perception of the stimulus (I) from pathogenic or beneficial fungi, bacteria, arthropods, and abiotic stresses, leading to the induction of various compounds and activities (priming fingerprint) in the priming phase (II), and postchallenge primed state upon an attack (III). Finally, primed plants (IV) display an enhanced perception of the attackers and therefore are able to mount a more robust defence against them (Mauch-Mani et al. 2017).

In summary, ISR ends in molecular, structural and biochemical changes in plant cells, such as callose deposition, synthesis of phytoalexins, increase in the activity of the phenylalanine ammonia-liase (PAL) (Ramos-Solano et al. 2008a; Verhagen et al. 2010), synthesis of antioxidant metabolites, etc., with the objective of avoiding pathogen penetration into the tissues, inhibiting pathogen growth, and thus preventing the development of the disease caused by a specific plant pathogen (Edreva, 2005; Tyagi et al. 2018).

The molecular signals involved in the signal transduction pathways of SAR and ISR was first studied by Pieterse et al. 1996 and Pieterse et al. 1998. They discovered that, while SAR was dependent of SA and involved expression of PR genes, ISR response was mediated by jasmonic acid (JA), and ethylene (ET) and did not suppose expression of PR genes (Fig. 1.8). However, this model of the signal transduction pathways of SAR and ISR is nowadays much more complex, since it has been demonstrated that pathogenic and beneficial bacteria are able to trigger either one or the other signal transduction pathways or both transduction pathways at the same time (Van Hulten et al. 2006; Ramos-Solano et al. 2008b; Pieterse et al. 2014; Caarls et al. 2015; Martin-Rivilla et al. 2019; Martin-Rivilla et al. 2020c).

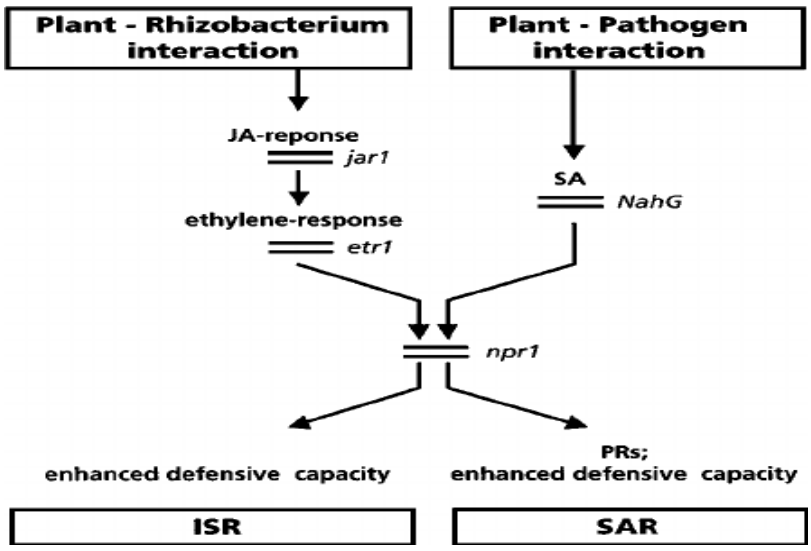

Figure 1.8 SA and JA/ET signalling pathways (Van Loon et al. 1998).

On the other hand, it has been seen that PGPR and their elicitors not only have potential to increase plant resistance against pathogens and abiotic stress, but they are also able to elicit certain metabolic pathways involved in the synthesis of secondary defensive compounds that have beneficial effects on human health and which provide added value for the pharmaceutical and agrifood industry (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015a; Martin-Rivilla et al. 2020a and 2020b).

1.2.2 Induction of the secondary defensive metabolism: elicitation

As previously explained, plants have developed a powerful secondary metabolism to adapt to environmental stress conditions (Weston et al. 2012), that is based on the synthesis of secondary metabolites with multitude defensive functions. This metabolism is associated with the primary metabolism (related to the survival, growth and reproduction of plants), since the synthesis of the secondary metabolites derives from molecules from the primary metabolism. The fundamental characteristic of this secondary metabolism is that it is only active in situations of stress (there is no constitutive production of metabolites by the plant, which limits energy losses (Pieterse et al. 2007)) and therefore, it is inducible. The process of induction or enhancement in the synthesis of secondary metabolites in plants, to guarantee their survival, persistence and competitiveness (Namdeo, 2007) is defined as **elicitation**. This phenomenon of enhancing plant immune system supposes that cells exposed to external factors activate defence mechanisms by triggering and regulating some biochemical and molecular responses, increasing the synthesis of specific molecules with a protective role (Leite et al. 1997).

Traditionally, the elicitation of the plant secondary metabolism has been focused on increasing primary production for food purposes. However, there is another current objective, with a strong biotechnological content, based on the induction of

secondary metabolism, to achieve better quality crops with higher concentration of metabolites with pharmacological activity, able to prevent human illnesses, or metabolites with defensive activity to protect plants against biotic and abiotic stress (Gutierrez Mañero et al. 2012).

Since the synthesis of secondary metabolites is highly inducible, the current challenge is the identification and management of compounds or biological agents capable of promoting the elicitation of the biosynthetic pathways of those metabolites (Moore et al. 2014). To date, multitude of natural or artificial compounds or stimuli able to elicit secondary metabolism have been identified. In general, these elicitors can be classified based on their nature, as abiotic or biotic elicitors (Fig. 1.9).

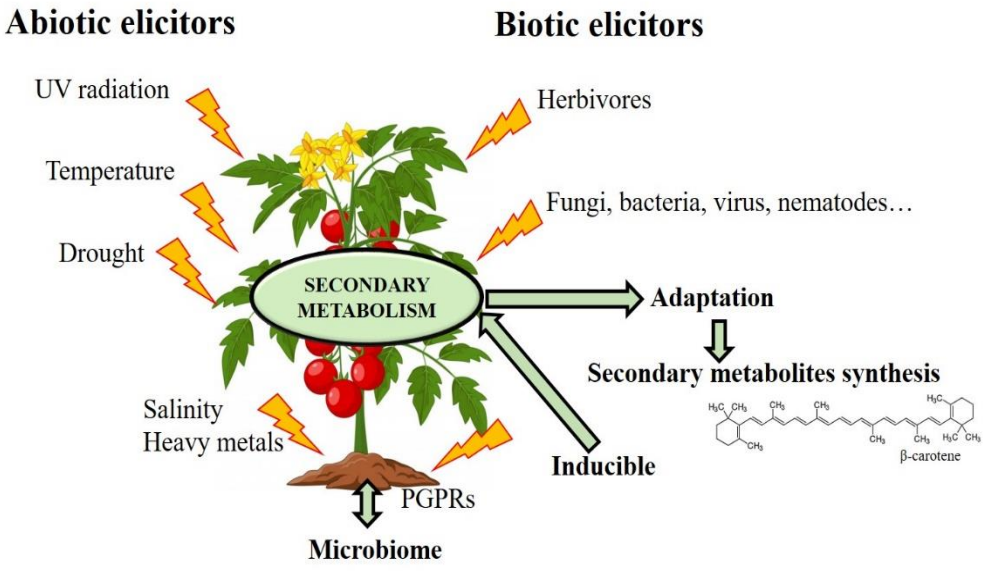

Figure 1.9 Induction of the secondary metabolism of plants, through abiotic and biotic elicitors.

Among abiotic elicitors are environmental factors like UV-radiation, salt stress, drought, basic soil, temperature, heavy metals present in the soil, etc. (Mahajan and Tuteja, 2005). However, biotic elicitors are substances of biological origin, of different chemical nature, such as lipids, proteins, polysaccharides, plant hormones such as salicylic acid or methyl jasmonate (MeJA) (Namdeo, 2007; Ramirez-Prado. 2018),

aromatic organic compounds, volatile compounds, etc. Biotic elicitors can also be live microorganisms, as PGPR, or their structural molecules, such as bacterial flagellin or metabolic derived molecules, such as antibiotics or secondary metabolites (Gozzo and Faoro, 2013; Wu et al. 2018), either from pathogenic or non-pathogenic microorganisms (Wiesel et al. 2014).

The effectiveness of elicitation as a tool to enhance the production of secondary metabolites depends on a complex interaction between the elicitor and the plant cell. The main factors that can affect the elicitation response are summarized below (Namdeo, 2007; Van Wees et al. 2008; Conrath, 2011; Erbs and Nweman, 2012):

A) Specificity of the elicitor. The same elicitor can boost the secondary metabolism of different plant species and, one species can be sensitive to different elicitors.

B) Elicitor concentration. The concentration of the elicitor affects the intensity of the response. The effective dose varies according to plant species. Furthermore, it has been shown that high doses of the same elicitor may not be able to trigger the biological effect (Ramos Solano et al. 2008a).

C) Duration of exposure to the elicitor. In general, the elicitor is in contact with the system until its collection, but the time required to obtain a maximum accumulation of secondary metabolites is characteristic of each plant species. More data is needed about this issue.

D) Development stage of the plant. The most appropriate moment to add the elicitor is highly variable, although if it were the case of live bacteria, their application during the early stages of plant development, would favor colonization of the entire rhizosphere (Bashan, 1986).

Along time, many studies have demonstrated that PGPR are biotechnological tools very effective in the processes of elicitation and obtaining of plant compounds of interest. Through their application in crops, it has been seen not only an increase in crop biomass, but also a notable enhancement in the production of high-added value bioactives, used as raw materials for the manufacture of galenic preparations or food products (Kandavel and Sekar, 2010; Gutiérrez Mañero et al. 2012; Algar et al. 2012; Bonilla et al. 2014; Martin-Rivilla et al. 2020a and 2020b). Furthermore, the application of PGPR avoids the fluctuations that the bioactives undergo during the production cycle of plants and that are associated with environmental factors (Moore et al. 2014; Garcia-Seco et al. 2015a).

In this work, certain PGPR (called bioeffectors) have been used, as well as the molecules derived from their metabolism and released into the medium (called metabolic elicitors) as plant inoculants in all the processes of elicitation of the secondary defensive metabolism of *A. thaliana* and blackberry. As some PGPR induce or elicit certain metabolic pathways, in this work it is hypothesized that their derived metabolic elicitors could mimic the capacities of the live strains. If metabolic elicitors are able to imitate bioeffectors´ abilities, it allows replacing the live strains by inert molecules, which entails many advantages in the management and maintenance of the plant inoculants made of these elicitors, since they are stable during long periods of exposure to light and/or high temperatures (Stockwell and Stack, 2007), and do not lose viability during prolonged storage. Furthermore, using molecules instead of live microorganisms avoids biosecurity problems (Ngoroyemoto et al. 2019).

Some other works have demonstrated that our hypothesis is feasible, since elicitors of different nature (structural or metabolic) have been seen capable of effectively triggering the defensive responses of the plant and lead to the production of secondary

metabolites (Zhao et al. 2005; Park et al. 2008; Algal et al. 2012; Sumayo et al. 2013; Xing et al. 2020).

Finding elicitors able to trigger plant secondary defensive metabolism will therefore allow developing effective products for agriculture aiming to minimize the consumption of chemical phytosanitaries and to contribute to a more sustainable agriculture. Purified metabolic elicitors extracted from certain PGPR would be effective, cheap to produce, easy to manage and respectful with the environment plant inoculants alternative to the chemicals and alternative or complementary to the use of live microorganisms.

For all these reasons, biological elicitation with PGPR and with their elicitors, constitute the biotechnological methodology applied in this work, to get plants with a stronger immune system and for the development of better quality food. The relationship between defensive metabolism, or metabolism that supposes an adaptive advantage of the plant, and the concentration of bioactive compounds constitute the fundamental hypothesis on which this methodology is based. All of this have the long-term aim of making ecofriendly and effective plant inoculants to fight the impact of different pests and abiotic stress in the crops.

1.3 *Pseudomonas fluorescens* N 21.4: a candidate for plant inoculants

Throughout the introduction it has been described the use of beneficial microorganisms as biotechnological tools for the elicitation of plants in order to activate their secondary defensive metabolism and to obtain more resilient plants, better prepared to face to any type of stress and at the same time, plants with a higher concentration of beneficial secondary metabolites.

The elicitor microorganism (bioeffector) most studied in the present work is the rhizobacteria *Pseudomonas fluorescens*, strain N 21.4. Elicitation experiments have been performed with this strain, first under controlled laboratory conditions, inoculating the strain in *A. thaliana* seedlings and then under field conditions in commercial blackberry cultivars (*Rubus* cv. Loch Ness). In all these experiments, both the live strain and its metabolic elicitors have been inoculated, since, the novelty of this work is to check whether metabolic elicitors are able to mimic the abilities of living microorganisms, to replace them in the manufacture of plant inoculants.

P. fluorescens N 21.4 (CECT 7620) is a non-sporulating Gram-negative bacilli, which was isolated from the rhizosphere of *Nicotiana glauca* Graham (Ramos-Solano et al. 2010b). This plant was chosen as a good source for the obtaining of effective PGPR, since it is able to colonize poor soils and because it contains the anabasin alkaloid, which indicates the existence of a complex secondary metabolism likely linked to defence (Sinclair et al. 2004; DeBoer et al. 2009).

In that sampling, the rhizosphere of *N. glauca* plants was collected in San José (Almería. Spain) in three different soils (calcareous, volcanic and quaternary) along two years (1999 and 2000) and at two sampling moments (June and November), to achieve the maximum edaphic and environmental variability. In each sampling moment, four replicates, with the roots of 15 plants, were taken. In total, 24 sampling units were collected and each one was named with the N from *Nicotiana* and the number of the sampling unit (N 1 to N 24). The nomenclature of *P. fluorescens* N 21.4 accordingly corresponds to the sampling unit 21, collected on quaternary soil in November and the strain (of the first replica) that was isolated in fourth position (Ramos-Solano et al. 2010a).

The *P. fluorescens* N 21.4 was specifically used in the present work because it has been largely demonstrated its capacity to induce systemic resistance in different plant

species. This broad-spectrum effectivity is essential to develop biological inoculants for different plant agronomic species. *P. fluorescens* N 21.4, has the ability to trigger ISR in *Solanum lycopersicum* (Ramos-Solano et al. 2010a), *A. thaliana* (Domenech et al. 2007; Martin-Rivilla et al. 2019), *Glycine max* (Ramos-Solano et al. 2010b), *Hypericum sp.* (Gutierrez Mañero et al. 2012), *Papaver sp.* (Bonilla et al. 2014) and blackberry (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015). It enhanced the isoflavone content in *Glycine max* (Algar et al. 2012), and in blackberry, it increased total fruit production and fruit quality (higher and stable bioactive content along the year (García-Seco et al. 2013; García-Seco et al. 2015a; Garcia-Seco et al. 2015b; Ramos-Solano et al. 2014; Ramos-Solano et al. 2015)). It has been also demonstrated the capacity of the metabolic elicitors of *P. fluorescens* N 21.4 to induce systemic resistance in *Glycine max* (Algar et al. 2012) and in *A. thaliana* (Martin-Rivilla et al. 2019 (data obtained during the development of the present doctoral thesis)), and to elicit flavonoid metabolism in the leaves and fruits of cultivars of blackberry (Martin-Rivilla et al. 2020a; Martin-Rivilla et al. 2020b (data obtained during the development of the present doctoral thesis)).

According to its PGPR capacities, *P. fluorescens* N 21.4 produces siderophores and chitinases (Ramos-Solano et al. 2010a) and it is also able to solubilize inorganic phosphorus.

In the agronomy field, *P. fluorescens* is well known for the beneficial role that it can play in promoting crop growth and health and for its potential as bio-fertilizer, bio-regulator and bio-control plant inoculant (Junaid et al. 2013). *P. fluorescens* strains are abundantly present within rhizosphere soil, but can also be found on both the root surface and in endophytic compartments. Having flagella is what facilitate their movement to actively look for favorable conditions in the rhizosphere (Rossez et al. 2015). In addition, various *P. fluorescens* strains have been shown to metabolize a wide variety of carbon

sources, which reinforces their ability to colonize a vast variety of plant species and environments (Simons et al. 1997; Timm et al. 2015).

Another advantage that *P. fluorescens* has to effectively colonize and survive in the rhizosphere is the ability to compete with rhizospheric pathogens by producing antimicrobial secondary metabolites, such as hydrogen cyanide, phenazine-1-carboxylic acid and 2,4- diacetylphloroglucinol (DAPG) (Siddiqui et al. 2006; Mavrodi et al. 2007; Meyer et al. 2016). Dominating the rhizosphere by controlling pathogen colonization, *P. fluorescens* can boost plant health, hence the interest for its use as plant inoculants (Frapolli et al. 2012).

It is largely known that *P. fluorescens* is one of the most important microorganisms able to produce compounds with antibiotic or elicitor activity triggering SAR (Choudhary et al. 2007) or ISR (Durrant and Dong, 2004). In addition, it has been seen a direct correlation between *P. fluorescens* colonization and ISR, with plant production of JA and ET, which leads to the activation of transcriptional co-regulator proteins, important for the triggering of genes involved in the immune defence (Wu et al. 2012).

P. fluorescens potential to suppress plant pathogen has been demonstrated in many plant species and around the world (Haas and Defágo, 2005). Literature has shown that *P. fluorescens* has extensive metabolic capabilities since it is able to produce a wide range of structurally varied bioactive molecules (Gross and Loper, 2009). Secondary metabolites isolated from *P. fluorescens* that could be used as an alternative to the use of chemical compounds in the control of plant disease include salicylic acid, phenazines, pyrrolnitrin-type antibiotics, betalactones, pyo compounds, indol derivatives, peptides, glycolipids, lipids, lipopolysaccharides, aromatic organic compounds, siderophores, aliphatic compounds, etc. (Leisinger and Margraff, 1979; Haas et al. 1991; Gross and

Loper, 2009; Neidig et al. 2011; Jankiewicz and Koltonowicz, 2012). Moreover, *P. fluorescens* can be used as efficient and not risky biocontrol agent to use in agriculture because it does not show pathogenic, allergenic or harmful risks to people or animals (Zanatta et al. 2007). For all of this, certain strains have already been developed as commercial products for the management of plant illnesses in agricultural settings (Stockwell and Stack, 2007).

The development of plant inoculants in the root environment have typically failed because they are usually studied *in vitro* rather than *in vivo*, resulting in the application of microorganisms into ecologically unsuitable environments. Therefore, and since the long-term objective of the present study is to propose *P. fluorescens* N 21.4 and/or its metabolic elicitors as plant inoculants, they were first tested in *A. thaliana* plants under controlled laboratory conditions and after that in real field conditions in cultivars of blackberry.

Furthermore, studying the genome, transcriptome, proteome and metabolome of PGPR together with their relationships with plants, and also studying the physiology of the plants challenged with these PGPR will help to further understand and develop better and more efficient microbial inoculants.

1.4 *Arabidopsis thaliana*: the model plant

As a large part of the ISR experiments that have been performed in the present doctoral thesis was carried out on *A. thaliana* seedlings under laboratory conditions, it is appropriate to add an exclusive epigraph of this model plant so widely used in research.

A. thaliana is a small, annual or winter annual, with a rapid life cycle (6-8 weeks), rosette plant (Fig. 1.10), of the *Brassicaceae* family that is native from Europe, Asia and from the northeast of Africa. Johannes Thal, in the Harz Mountains of Northern

Germany, first described it in 1577; it was featured in the Species Plantarum II (by Linnaeus) in 1753, and it received its present name, *Arabidopsis thaliana* (L.) Heynh, from Gustav Heynhold in 1842 (Kück, 2005).

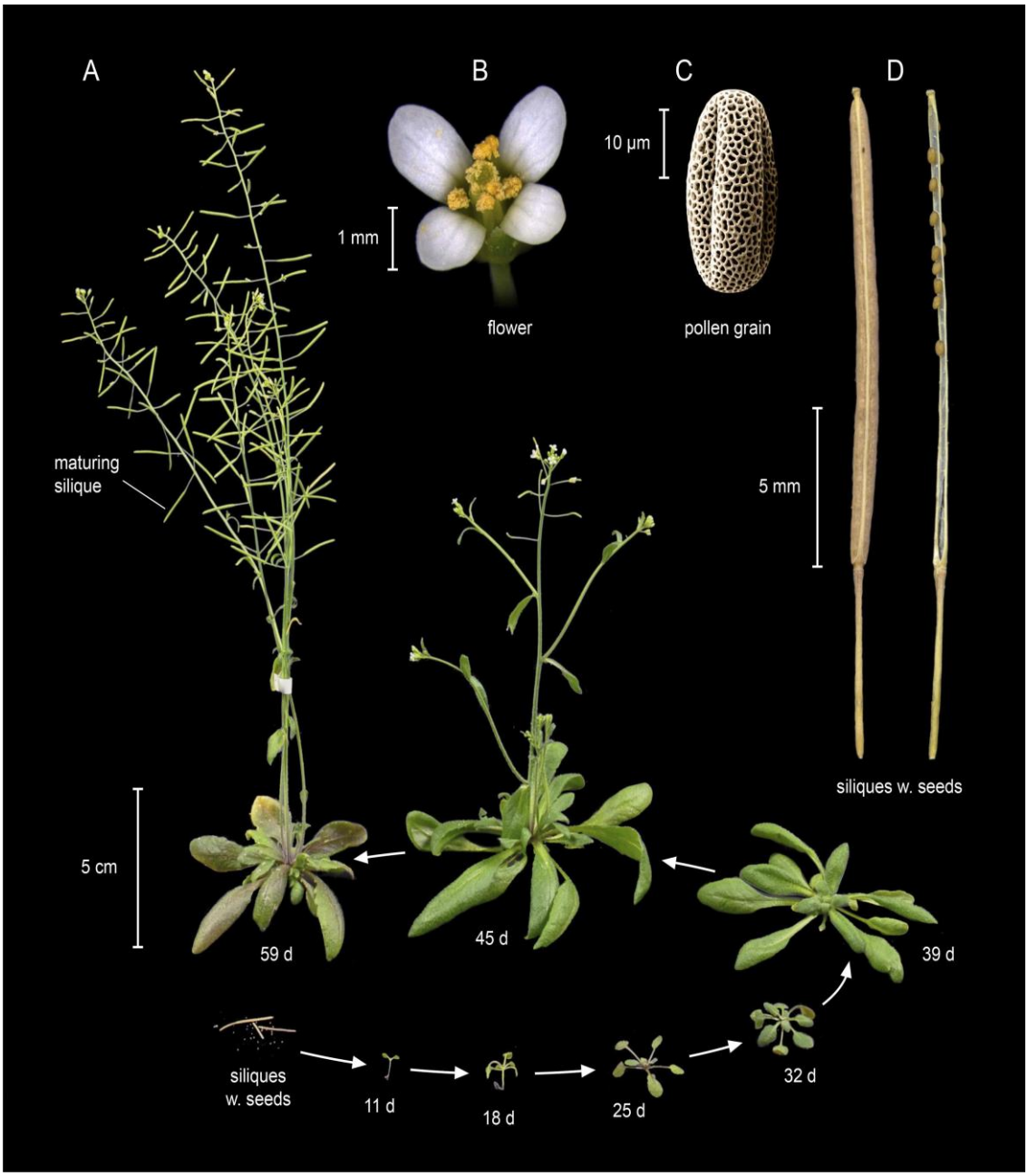

Figure 1.10 Life cycle of *A. thaliana* (Krämer, 2015): A) *A. thaliana* of the accession *Columbia* from seed to seedling (11 days), vegetative growth (39 days), and to reproductive growth (45 days); B) a flower, C) a pollen grain (scanning electron micrograph), and D) mature siliques at higher magnification. Image credits: Maria Bernal, Peter Huijser, Ines Kubigsteltig and Klaus Hagemann.

The first research of *A. thaliana* was published in 1907 by Friedrich Laibach, who presented an influential cytogenetic study that served to promote genetics in the 1940s, but the reputation of *A. thaliana* as a model plant did not start until the 1980s. After that, its use rapidly gained popularity when researchers successfully linked genetics with molecular biology methods. In 1996 scientists of a large number of different institutions created the research project, AGI (Arabidopsis Genome Initiative, 2000 (http://www.arabidopsis.org/); (Somerville and Koornneef, 2002; Koornneef and Meinke, 2010)), and in 2000, the *A. thaliana* became the first plant whose genome has been sequenced. The plant's genetic map was presented for the first time with 25,498 identified genes that encode proteins from 11,000 families. Since that milestone and thanks the successful integration of different fields of research and to the ease and speed with which experiments with *A. thaliana* can be performed, enormous progress has been made in the understanding of key concepts in biology and in the molecular principles of plant development, cell biology, metabolism, physiology, genetics and epigenetics (http://www.arabidopsisbook.org/). *A. thaliana* is now a central genetic model and the universal reference organism in plant and crop science.

A. thaliana has many advantages for research analysis, such as a short generation time, small size, large number of offspring, roots that contain only a single layer of each specialized cell type, (which is optimal for *in vivo* imaging) and that generally do not undergo mycorrhizal or rhizobial symbiosis, and a small nuclear genome (two sets of n=5 chromosomes; one of the smallest known genomes among flowering plants). These properties of *A. thaliana* have notably aided the sequencing, assembly and annotation of its genome, as well as new molecular genetics and genomics approaches. It has been also characterized at molecular level everything related to the acquisition of

inorganic nutrients (P, N, Fe…) and its efficient utilization in this plant (Abel, 2011; Hindt and Guerinot, 2012; Araya et al. 2014).

Regarding the immune system of *Arabidopsis*, it is remarkable the wide sort of general PTI (Schwessinger and Ronald, 2012) and the more-specific ETI responses that this plant is able to trigger against pathogens (Cui et al. 2014). Furthermore, *Arabidopsis* possesses effective defences against herbivory thanks to the specific synthesis of secondary metabolites. The elucidation of these immune response pathways and of the regulatory networks of *A. thaliana* has extensively contributed to the general understanding of plant defences against pathogens and to comprehend the establishment and preservation of beneficial interactions with microorganisms, such as PGPR and other beneficial microorganisms that trigger induced systemic resistance (Lugtenberg and Kamilova, 2009; Pieterse et al. 2014). This has also led to the discovery of the genetic basis of agronomically essential traits in crops, such as plant height and flowering time (Doebley et al. 2006; Olsen and Wendel, 2013), the synthesis of secondary metabolites of interest, the plant responses to biotic or abiotic stress, etc.

For all the above, in the present work, it was pursued to unravel the functioning of the defensive pathways of *A. thaliana* seedlings primed with certain PGPR and their metabolic elicitors and challenged with the pathogen *Pseudomonas syringae* pv. *tomato* DC3000. To reach this objective, some markers of the triggered ISR response were studied, such as levels of oxidative stress, activity of the pathogenesis-related proteins, activation of specific defensive marker genes, etc.

1.5 Blackberry: *Rubus* cv. Loch Ness

The blackberry (*Rubus* spp.), is a species of shrub of the *Rosaceae* family and is popularly known for its edible fruits (Fig. 1.11). The genus *Rubus* L. is widely distributed on all

continents and is present in very varied habitats. It is made up of 600-800 species (Bushakra et al. 2012) and is classified into 12 subgenres, which in turn are divided into numerous sections and series (Table 1 (Alice and Campbell, 1999)).

Table 1 Subgenres of the genus *Rubus*.

Subgenus	Number of species
Anoplobatus (Focke)	6
Chamaebatus (Focke)	5
Chamaemorus (Hill)	1
Comaropsis (Rich.)	2
Cylactis (Raf.)	14 (4 series)
Dalibarda (L.)	5
Dalibardastrum (Focke)	4
Idaeobatus (Focke)	117 (9 sections)
Lampobatus (Focke)	10
Malachobatus (Focke)	115 (7 sections)
Orobatus (Focke)	19
Rubus L. (= *Eubatus* Focke)	132 (6 sections)

Commercial species of the genus *Rubus* include the blackberry (*Rubus* cv. Loch Ness), red raspberry (*Rubus idaeus* L.) and black raspberry (*Rubus occidentalis* L.). However, our study is only focused on the blackberry (*Rubus* cv. Loch Ness), which is a lively, woody and highly invasive plant that covers extensive areas of forest, scrub and slopes. Its original distribution covers almost all of Europe, North Africa and South Asia, but it has also been introduced in America and Oceania. Morphologically (Fig 1.11), this plant is characterized by having different parts covered with hairs, glands and spines, the number and shape of which vary from one species to another.

The leaves of blackberry are composed, stipulated, and petiolate, with the margin of the lamina toothed, the upper part of the leaves glabrous, sometimes hairy, and the underside white tomentose. The number of leaflets varies between 3-5-7 and the terminal leaflet can be obovate, oval or ovate, with a rounded or slightly cordate base.

Figure 1.11 Botanical illustration of the blackberry (*Rubus* spp.) morphology.

The inflorescence is heteroclamid and hermaphrodite. The chalice comprises five imbricated, dialysepal and persistent pieces; the sepals are whole, lanceolate, and finished in a point that sometimes extends into a relatively long acumen. The external face is covered with hairs whose shape and quantity are variable. The position, erect, patent or reflex of the chalice, is characteristic for each species, and may vary, within the same species, in flowering and fruiting; most have the reflex or patent chalice and only in a few species it is erect.

The color of the petals varies between white and pink-red and is usually constant for each species, with some exceptions. The shape of the petals (transoval, oval or suborbicular) and their size are more or less constant for each species. The coloration of the stamens, in the basal part of the filament, usually coincides with that of the petals and as it ascends towards the anthers, the color is green or white. Anthers are glabrous in most

species but there are also hairy ones. The gynoecium is multicarpellar and apocarpic and from each ovary a sub terminal style emerges, which is topped with a capitulated stigma. Styles are white or green.

The fruit is a multidrupe with the drupeoles concrescent at the base, but without being totally syncarpic. The number and size of the drupeoles is variable. The color for most species in the *Rubus* section is glossy black, for those in the *Corilifolii* section is matt black or bluish black, for *R. caesius* is more or less blue and for *R. idaeus*, is red.

Blackberries are naturally distributed in temperate areas of the northern hemisphere, especially in high altitudes. They have to undergo a cold period in winter to flower in spring and to get fruits along the summer.

With the agricultural exploitation of the fruit of *Rubus* spp., it arose the necessity of the genetic improvement of wild species. This was oriented towards getting varieties with an increase in the size of the fruits, without thorns, with better organoleptic properties, resistant to diseases, etc. This improvement has been carried out by classic crosses, developing commercial varieties that have been patented. Specifically, the variety or cultivar on which this work is focus (*Rubus* cv. Loch Ness), has Scottish origin, and is a high production tetraploid hybrid complex (2n=4x=28), widely spread throughout the world, with parental SCRI 74126RA8 x SCRI 75131D2, obtained in 1998 in Invergowrie, Scotland, by DL Jennings of the Scottish Crop Research Institute (Brooks, 1997). It is a mixture of races, among which the most prominent are the ‘Comanche’ and the ‘Merton Thornless’.

The main blackberry producers are North and Central America, Europe and Asia (Kaume et al. 2012). The blackberry cultivation is usually annual, but due to the growing demand in recent years, an intensive greenhouse cultivation that allows having

production throughout the year has been stablished in temperate climates. This technique is called counter-season crop.

In southwestern Spain, commercially blackberries are produced with such counter-season production technology (Fig. 1.12), which allows the production of fruit in a way that covers market demand most of the year (Garcia-Seco et al. 2013; Ramos Solano et al. 2015).

Figure 1.12 *Rubus* cv. Loch Ness in greenhouses in Lucena del Puerto (Huelva, Spain).

Counter-season cultivation throughout the year is based on providing a cold treatment at 4 ºC of between 45 days and 5 months, (equivalent to natural exposure to low temperatures that take place during winter), to one-year-old seedlings grown in pots by hydroponic cultivation. Once this cold treatment has been carried out, the plants are placed in tunnel-type greenhouses at room temperature with drip irrigation and

hydroponic cultivation (Fig. 1.12). Then, plants begin to produce branches and leaves, and after 3-5 months (depending on the time of the year when the plants are taken to the greenhouses), the flowers appear. Thanks to the pollination assisted by bees that are part of the cultivation system, plants will gradually produce fruit approximately one month later, with a production time of about two months.

1.5.1 Blackberry secondary metabolism: flavonoids

The consumption of blackberry has grown exponentially in recent years because it has been shown the great amount of benefits that this fruits can bring to human health when they are regularly included in the diet (Tavares et al. 2013; Feresin et al. 2016; Sarkar et al. 2016; Gutierrez et al. 2017a). In fact, their pharmaceutical properties have been known in Europe at least since the 16th century, when they were used to treat some types of infections (Dai et al. 2007).

The benefits are due to the ingestion of highly antioxidant bioactive compounds of flavonoid nature that are synthesized by the secondary metabolism, and among which are flavonols, anthocyanins and catechins (Kaume et al. 2012). Furthermore, the content of these compounds is one of the most important factors affecting the organoleptic and nutritional quality of blackberries.

These compounds have demonstrated cytotoxic, anticancer, antiviral, antibacterial, anti-inflammatory, antiallergenic, antithrombotic, cardioprotective and hepatoprotective properties and they also prevent hypertension, obesity and premature aging (Alzand et al. 2012; Ferreyra et al. 2012; Rodriguez-Mateos et al. 2013; Manganaris et al. 2013; Garcia-Seco et al. 2015; Martinez-Gonzalez, 2016; Mortas and Sanlier, 2017).

However, these secondary metabolites are not synthesized by plants to have effects on the consumers of their fruits, but are synthesized because they fulfill a fundamental function to counteract stress, both biotic (fungi, bacteria, herbivores, (Kim

et al. 2011; Nile et al. 2014; Ferreyra et al. 2012) and abiotic (light, temperature, water supply, minerals, CO_2, etc. (Ramakrishna and Ravishankar, 2011; Schulz et al. 2015)), that plants have to face due to their sessile nature. These secondary metabolites also have an important role as repellents, visual attractors, phytoalexins, phytoanticipins (Ghasemzadeh and Ghasemzadeh, 2011; Jeandet et al. 2013), auxin controllers (Nakabayashi et al. 2014) and in the establishment of symbiotic bacteria relationships (Ibrahim et al. 2012).

Related to biotic stress, flavonoids are involved in the hypersensitive response and consequent programmed cell death, since they have been found in necrotic and adjacent cells to pathogen infection (Imperato et al. 2006; Nabbie et al. 2014). Furthermore, and due to their capacity to chelate metals, they can inhibit certain pathogen enzymes, mainly those responsible for digesting the cell wall, hence blocking or retarding pathogen invasion (Treutter, 2005). Flavonoids are also able to alter bacterial DNA synthesis, interacting with DNA gyrases and assembling hydrogen bonds with nucleic acid bases, or by direct interaction with the ATP binding site of the gyrases, leading to an inhibition of the synthesis of new DNA. This is probably the strategy used to fight against virus infection (Mierziak et al. 2014).

Related to abiotic stress, flavonoids are mainly associated to ROS detoxification (Ferreyra et al. 2012; Nakabayashi et al. 2014).

For all the essential functions that flavonoid compounds have in plants and for the benefits they bring to human health, the engineering of the flavonoid biosynthesis pathways for the purposeful accumulation and isolation of active molecules has been extensively used in the plant biotechnological industry (Tanaka et al. 2008).

The chemical nature of flavonoid compounds consists of a 15-carbon phenylpropanoid basic core (C6-C3-C6 system), which is arranged into two aromatic

rings (A and B) linked by a heterocyclic pyran ring (C). Ring B is always attached to C-2. The great antioxidant power of the flavonoids is due the fact that hydroxyl groups present in rings A and B are capable of reacting with different types of radicals, forming stable radicals. According to the oxidation status and saturation of the heterocyclic C ring, flavonoids are categorized into flavonols, flavones, catechins, flavanones, anthocyanins, and isoflavonoids (Jiang et al. 2016). As said above, the most abundant flavonoids present in blackberry are flavonols, anthocyanins, and catechins (Fig. 1.13).

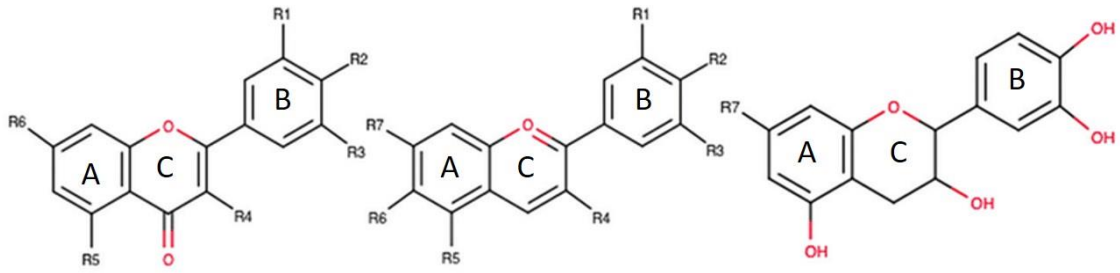

Figure 1.13 Flavonol, anthocyanin and catechin chemical structure (Gutierrez et al. 2017a).

Flavonols have a 3-hydroxyflavone (IUPAC name: 3-hydroxy-2-phenylchromen-4-one) as main structure. Their diversity derived from the different positions of the hydroxyl groups (-OH) of the phenolic ring C that are usually glycosylated and can undergo further modifications, like acylations. In this group, the three main families are derived from kaempferol (4'OH), quercetin (3', 4', 5' OH) and rutin (3', 4'OH) (Moss et al. 1995).

Anthocyanins are made up of an anthocyanidin molecule, which is the aglycone, and to which a sugar is attached by a glycosidic bond. The basic chemical structure of the aglycone is the flavylium cation (2-phenyl-benzopyrilium), which consists of a benzopyran aromatic ring, and a phenolic ring. There are six different families within the anthocyanin group, namely cyanidin, pelargonidin, delphinidin, malvinidin, peonidin and petunidin. As in the case of flavonoids, the greatest source of chemical diversity in the anthocyanin group is the number and position of sugars for glycosylation.

Catechins have two benzene rings (A and B) and a dihydropyran heterocyclic (C) with a hydroxyl group on carbon 3. Because of this structure, catechins have four diasteroisomers, two with trans configuration ((+) - catechin and (-) - catechin) and two with cis configuration ((+) - epicatechin and (-) - epicatechin). These catechins can further polymerize to form proanthocyanins, in which the diversity of structures relies on the number of monomers that polymerize and the type of bonds that stabilize them.

The biosynthesis of the basic core of all flavonoid compounds is originated from the phenylpropanoid pathway, followed by the flavonoid biosynthetic branch (Fig. 1.14). Like for many other fundamental compounds in plants, this route starts from phenylalanine. The phenylalanine ammonia-lyase (PAL) deaminates the phenylalanine, which is converted into trans-cinnamate. After that, a hydroxyl group is introduced on the phenyl ring by the cinnamic acid 4-hydroxylase (C4H), being the trans-cinnamate converted into 4-coumarate. The carboxyl group of p-4-coumarate is then activated to form 4-coumarate-CoA (by a thioester bond), catalyzed by 4-coumarate-CoA ligase (4CL). This product, 4-coumarate-CoA, is the substrate for different enzymes, so it represents a branching checkpoint of the pathway to either stilbenes of flavonoids. In the last case, 4-coumarate-CoA is then condensed with three units of malonyl-CoA by the chalcone synthase (CHS, first enzyme of the flavonoid pathway), forming the naringenin chalcone, which is transformed into naringenin by the chalcone isomerase (CHI). Flavanone-3-hydroxylase (F3H), hydroxylates naringenin until dihydrokaempferol, which is then hydroxylated by flavonoid-3-hydroxylase (F3´H) and transformed in dihydroquercetin. Flavonols are synthesized at this point by the flavonol synthase (FLS), forming kaempferol or quercetin (depending on where FLS introduces a double bond). Dihydroquercetin is then reduced by dihydroflavonol reductase (DFR) to obtain leucocyanidin. Anthocyanins are synthetized at this point by the anthocyanidin synthase

(ANS) obtaining cyanidin. (+)-Catechin is obtained when leucocyanidin reductase (LAR) reduces leucocyanidin, and (-)-epicatechin is obtained when anthocyanidin reductase (ANR) reduces cyanidin (Ferreyra et al. 2012; Mouradov et al. 2014; Jiang et al. 2016).

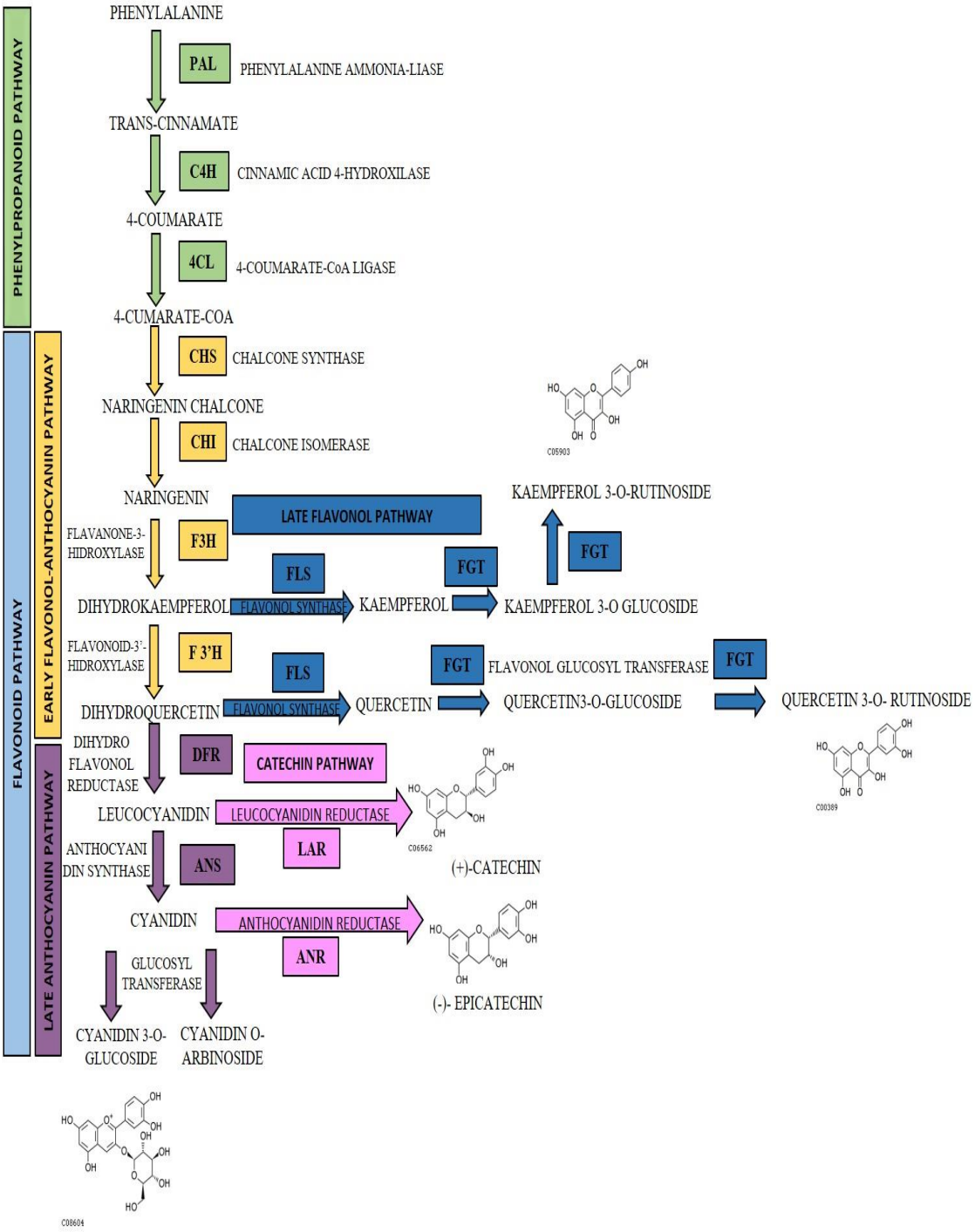

Figure 1.14 Phenylpropanoid and flavonoid pathway.

All the enzymes of the phenylpropanoid and flavonoid pathway are arranged in the different compartments within cells to improve efficiency in the synthesis of all these bioactives. The enzymes are anchored to the endoplasmic reticulum membrane, ensuring channeling of the intermediate precursors in the complex without diffusing to the cytosol, avoiding therefore metabolic interferences (Jorgensen et al. 2005; Shi et al. 2014).

All the core regulatory genes encoding these enzymes of the phenylpropanoid and the flavonoid pathways have been identified in *Rubus* cv. Loch Ness (García-Seco et al. 2015b). Furthermore, most genes corresponding to the *MYB* transcription factors (Martin and Paz-Ares, 1997; Dubos et al. 2010; Shelton et al. 2012; Gutierrez et al. 2017b; Thole et al. 2019) have also been identified. The study of the genes is crucial for modifying the accumulation of secondary metabolites of interest at the end of the route. As these genes are highly inducible, they can be elicited with external agents, such as bioeffectors and/or their metabolic elicitors. Elicitation is therefore a very interesting tool able to increase and stabilize the composition of secondary metabolites of interest (Ramos-Solano et al. 2008b; Capanoglu, 2010), allowing to achieve better crop quality in terms of higher amount of bioactives.

The blackberry (*Rubus* cv. Loch Ness) is the working material that has been chosen for this study due to the nutraceutical potential of its fruits and due to the agrifood importance, especially in recent years, of this high-added-value crop. The increased consumption of blackberries has meant that intensive production systems have the need to seek new cultivation strategies that improve the quality and sustainability of this cultivation (Carlen and Crespo, 2010).

1.6 Work plan and objectives

Elicitation, as the process of induction or enhancement in the synthesis of secondary metabolites that guarantee plant survival, persistence and competitiveness is presented as a biotechnological tool aiming to increase primary production for food purposes and to achieve crops with higher concentration of metabolites with pharmacological activity, able to prevent human illnesses, and with defensive activity to protect plants against biotic and abiotic stress. These secondary metabolites are phytonutrients that have beneficial effects on human health when are included in the diet and they can be also used as raw materials for the manufacture of nutritional supplements or functional ingredients.

Since the synthesis of secondary metabolites is highly inducible, the present work plan is based on identifying beneficial rhizobacteria (bioeffectors) and their derived metabolic elicitors capable of promoting the elicitation of the secondary defensive metabolism of *A. thaliana* and blackberry and on studying the signal transduction pathways involved in these processes. This way, the application of bioeffectors and their metabolic elicitors to crops opens a window towards the sustainable improvement of the nutritional qualities of food and the knowledge of the biosynthetic pathways of the secondary metabolites that compose them.

Our working hypothesis is established on the basis that, since some PGPR used as plant inoculants induce or elicit certain metabolic pathways, their derived metabolic elicitors could mimic the capacities of the live strains. If metabolic elicitors are able to imitate bioeffectors´ abilities, it will allow replacing the live strains by specific inert molecules, which entails many advantages in the management and maintenance of the plant inoculants made of these elicitors and also avoids biosecurity problems.

Based on the working hypothesis, the objectives designed for this thesis are established for the study of elicitation processes (by beneficial microorganisms) that

relate the secondary metabolism of plants with the improvement of agrifood products, as well as with the enhancement of the defensive capacity and general fitness of plants:

Objective 1. To obtain extracts containing metabolic elicitors of the strain *P. fluorescens* N 21.4 able to trigger protection in *A. thaliana* plants against the pathogen *P. syringae* pv. *tomato* DC3000 to determine the transduction signal pathways involved in the protection and the overall oxidative status of the elicited plants.

To reach this objective, differential expression of marker genes from the SA and JA/ET pathways was analysed, as well as enzymes involved in ROS scavenging system and proteins involved in induction systemic resistance.

With the results derived from objective 1, an article was published in the journal *AoB plants*: "Extracts from cultures of *Pseudomonas fluorescens* induce defensive patterns of gene expression and enzyme activity while depressing visible injury and reactive oxygen species in *Arabidopsis thaliana* challenged with pathogenic *Pseudomonas syringae*". https://doi.org/10.1093/aobpla/plz049

Objective 2. To evaluate the ability of the strain *P. fluorescens* N 21.4 and its metabolic elicitors to elicit the secondary defensive metabolism of blackberry plants (*Rubus* cv. Loch Ness), boosting their general fitness and achieving, at the end of the cultivation cycle, plants with leaves and fruits richer in valuable polyphenols.

To achieve this objective, photosynthetic parameters, ROS scavenging enzymes, pathogenesis-related proteins, expression of marker genes of ISR pathways mediated by SA and JA/ET and the content and variety of the phenolic compounds present in blackberry leaf were evaluated. In the fruit, it was studied the expression of structural marker genes of the phenylpropanoids pathway during blackberry ripening and the content and variety of the phenolic compounds upon beneficial rhizobacteria and metabolic elicitors challenge.

The analyses performed to reach objective 2 were conducted in two sampling moments, corresponding to two physiological plant stages: flowering (January 2018) and fruiting (April 2018).

With the results derived from objective 2, two articles were published in the following journals:

- *Journal of Agricultural and Food Chemistry*: "Improving flavonoid metabolism in blackberry leaves and plant fitness by using the bioeffector *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors: a biotechnological approach for a more sustainable crop". https://doi.org/10.1021/acs.jafc.0c01169

- *Journal of the Science of Food and Agriculture*: "Metabolic elicitors of *Pseudomonas fluorescens* N 21.4 elicit flavonoid metabolism in blackberry fruit". https://doi.org/10.1002/jsfa.10632

Objective 3. To isolate, purify and identify the compound or set of metabolic elicitor compounds of *P. fluorescens* N 21.4 responsible for plant elicitation.

Metabolic elicitors´ capacity to induce secondary metabolism was corroborated by performing experiments under controlled conditions in soybeans, to enhance isoflavone synthesis, and in *A. thaliana*, to induce systemic resistance. An analysis to obtain the profile of main compounds present in the purest fraction of the metabolic elicitors was made.

With the results derived from objective 3, an article was published in the journal *Plants*: "Identifying the compounds of the metabolic elicitors of *Pseudomonas fluorescens* N 21.4 responsible for their ability to induce plant resistance". https://doi.org/10.3390/plants9081020

Objective 4. To find beneficial bioffectors from the rhizosphere of *Nicotiana glauca*, as well as their derived metabolic elicitors, able to trigger the innate defences of *A. thaliana* plants to elucidate the mechanisms involved in the defensive response.

To achieve this objective, a screening of *N. glauca* rhizobacteria was performed to select the strains more efficient in inducing systemic resistance of *A. thaliana* plants against the pathogen *P. syringae* pv. *tomato* DC3000. The metabolic elicitors of the most effective strains were obtained and their ability to mimic bacterial response was analysed. The differential expression of marker genes from the SA and JA/ET transduction pathways of inoculated plants with selected strains and metabolic elicitors was studied.

With the results derived from objective 4, an article was sent to the journal *BMC Plant Biology* for publication: "Bioeffectors as biotechnological tools of the innate immunity: signal transduction pathways involved".

All of these objectives pursue to deepen in the study of metabolic elicitors to use them as alternative or complementary plant inoculants to live rhizospheric bacteria. This is the main novelty that brings the present work.

The blackberry (*Rubus* cv. Loch Ness) is the working material that has been chosen for this study due to the nutraceutical potential of its fruits and leaves and due to the agrifood importance of this high-added-value crop.

The present doctoral thesis is adapted to the classical standards and is divided into the following sections: abstract, general introduction, general material and methods, experimental work, general discussion, general conclusions, bibliography and annexes. Results derived from the four objectives described above are presented in the experimental work section, divided in five articles (each corresponding to one chapter), four of them already published, and another submitted to a specific journal. The memory has a final reference section including all references listed therein.

In fulfillment of the requirements applicable to obtain the International Thesis Diploma, this memory is written in two EU languages, English and Spanish, being the abstract presented in Spanish and the abstract, general introduction, general material and methods, experimental work, general discussion and general conclusions in English. Also in fulfillment of this requirement, a three-month stay in a foreign university was carried out at the State University of Londrina (Brazil) where the isolation and purification of the *P. fluorescens* N 21.4 metabolic elicitors was performed.

2. Material and methods

2. Material and methods

2.1 Materials

2.1.1 Plant material

The plant material used to perform the different experiments of the present work was the model plant *Arabidopsis thaliana*, soybeans (BS-2606 Embrapa) and blackberry plants (*Rubus* cv. Loch Ness).

The experiments with *A. thaliana* were made with *Columbia ecotype* 0 plants. Seeds were provided by the Nottingham Arabidopsis Stock Centre (NASC) and replicated in the University. Seeds were germinated in quartz sand and two-week-old or one-week-old seedlings (depending on the experiment) were then individually transplanted to 100 mL pots filled with 12:5 or 3:1 (vol/vol) peat/sand mixture (60 g per pot) or to 12-well plastic plates (5 mL) filled with 3:1 (vol/vol) peat/sand , respectively (Fig. 2). Plants grew in a culture chamber (Sanyo MLR-350H) under controlled conditions: 8 h of light (350 $\mu E\ s^{-1}\ m^{-2}$) at 24 °C and 16 h of dark at 20 °C at 70% of relative humidity.

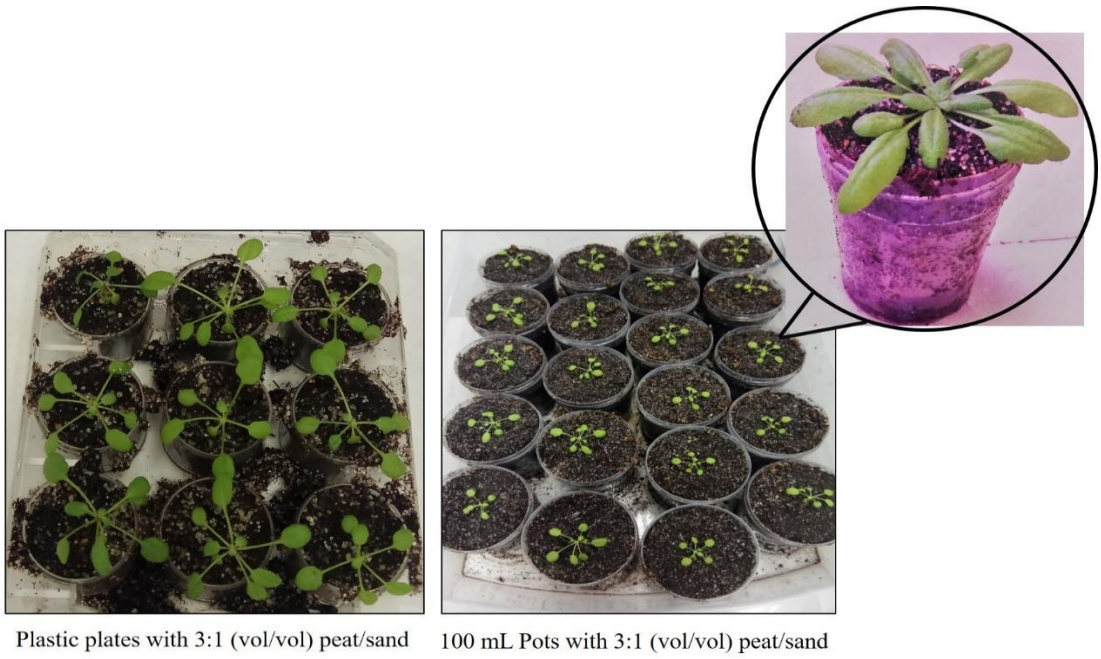

Figure 2 *A.thaliana* seedling in plastic plates (left) and in pots (right).

Soybeans (BS-2606) were kindly provided by the Embrapa ("Empresa Brasileira de Pesquisa Agropecuária" or Brazilian Agricultural Research Company). Seeds were superficially disinfected and kept imbibing in darkness at room temperature. After imbibition, a small longitudinal cut was made in the seed embryo without compromising its viability. Treatments were injected into the cut of the embryo and seeds were kept for three days in a Sanyo MLR-350H culture chamber at 27 °C in darkness and under sterile conditions (Fig. 2.1 A)).

The *Rubus* cv. Loch Ness plants used in our studies (Fig. 2.1 B)) were kindly provided by Agricola El Bosque S.L. "La Canastita" (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were handled according to regular agricultural practices (Ramos-Solano et al. 2014), under real production conditions. Plants were grown in Huelva (South Eastern Spain) from November 2017 to April 2018 under "winter cycle." Before being transplanted to greenhouses in November, plants underwent an artificial cold period in order to start their regular cycle.

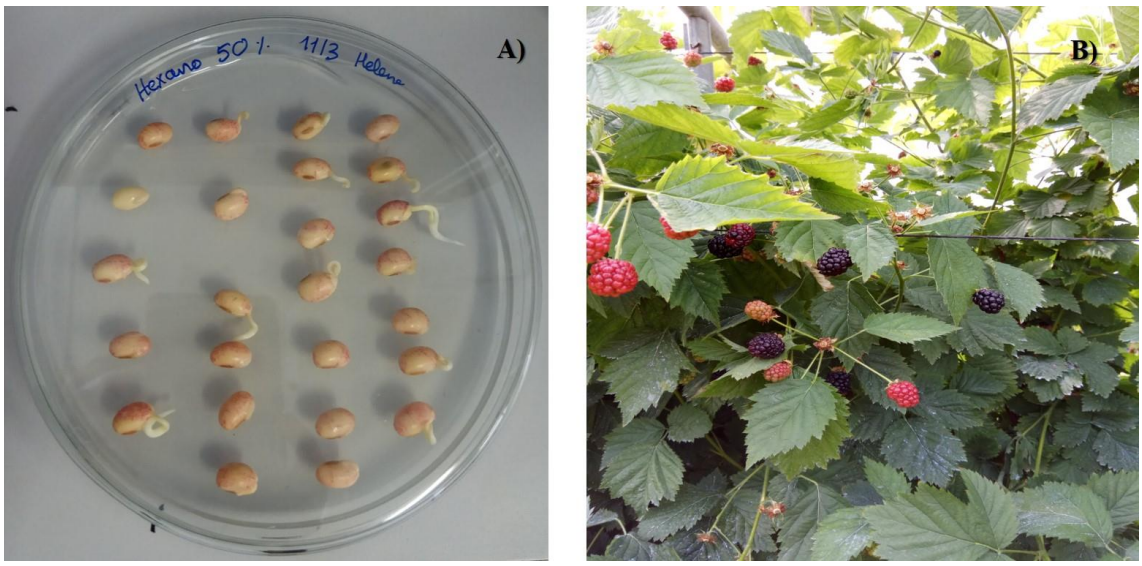

Figure 2.1 A) Soybeans germinating after treatment inoculation; B) *Rubus* cv. Loch Ness plants in the greenhouses of Lucena del Puerto (Huelva, Spain).

2.1.2 Bacterial strains

Bacterial strains analysed in the present work had been isolated from the rhizosphere of wild populations of *Nicotiana glauca* Graham in three different soils and physiological stages of the plant. The analysis of the strain *Pseudomonas fluorescens* N 21.4 (obtained in the work of Ramos-Solano et al. 2010a) was deepen. *P. fluorescens* N 21.4 is a Gram-negative bacilli. It is able to release siderophores and chitinases *in vitro* and it triggered defensive metabolism in *Solanum lycopersicum* (Ramos-Solano et al. 2010a), *A. thaliana* (Domenech et al. 2007 and Martin-Rivilla et al. 2019), *Glycine max* (Ramos-Solano et al. 2010b), *Hypericum* sp. (Gutierrez Mañero et al. 2012) and *Papaver* sp. (Bonilla et al. 2014). It also enhanced the isoflavone content in *Glycine max* (Algar et al. 2012), and in blackberry, it increased total fruit production and fruit quality (higher and stable bioactive content along the year (García-Seco et al. 2013; García-Seco et al. 2015a; Garcia-Seco et al. 2015b; Ramos-Solano et al. 2014; Ramos-Solano et al. 2015; Martin-Rivilla et al. 2020b)).

Furthermore, a subset of 175 strains from the non-assayed bacteria group of the work of Ramos-Solano et al. 2010a were evaluated in the present work, selecting the strains *Serratia rubidaea* N 12.34 and *Bacillus cereus* N 4.1 for a deeper analysis.

Pseudomonas syringae pv. *tomato* DC3000 (hereinafter abbreviated as *P. psyringae* DC 3000) was the model pathogen used in the ISR experiments. It causes visual injuries (yellow speck) on the leaves of *A. thaliana* and it is commonly used to study the model system of plant-pathogen interactions (Van Loon et al. 1998).

2.1.3 Metabolic elicitors

The metabolic elicitors (ME) inoculated in *A. thaliana* seedlings were extracted from a 24 hours-grown bacterial culture (10^9 cfu mL^{-1} (nutrient broth CONDA), grown on a rotary shaker (180 rpm) at 28 ºC), of the strains *P. fluorescens* N 21.4, *Serratia rubidaea*

N 12.34 and *Bacillus cereus* N 4.1, according to Sumayo et al. (2013), using three organic solvents (1:1 vol/vol) until obtaining n-hexane, ethyl acetate and n-butanol fractions (Fig. 2.2). Bacterial cells were eliminated by centrifugation (10 min at 2890 *x* g), and the obtained supernatant, filtrated by 0.2 μm, was used to extract the metabolic elicitors. First, an extraction with n-hexane (1:1 vol/vol) was made. The remaining aqueous phase was then extracted with ethyl acetate (1:1 vol/vol), and finally, the aqueous phase was extracted with n-butanol (1:1 vol/vol). The organic phases (n-hexane, ethyl acetate and n-butanol) were evaporated at 50 ºC and the dry residues obtained (ME) were dissolved in 10 % Dimethyl sulfoxide (DMSO).

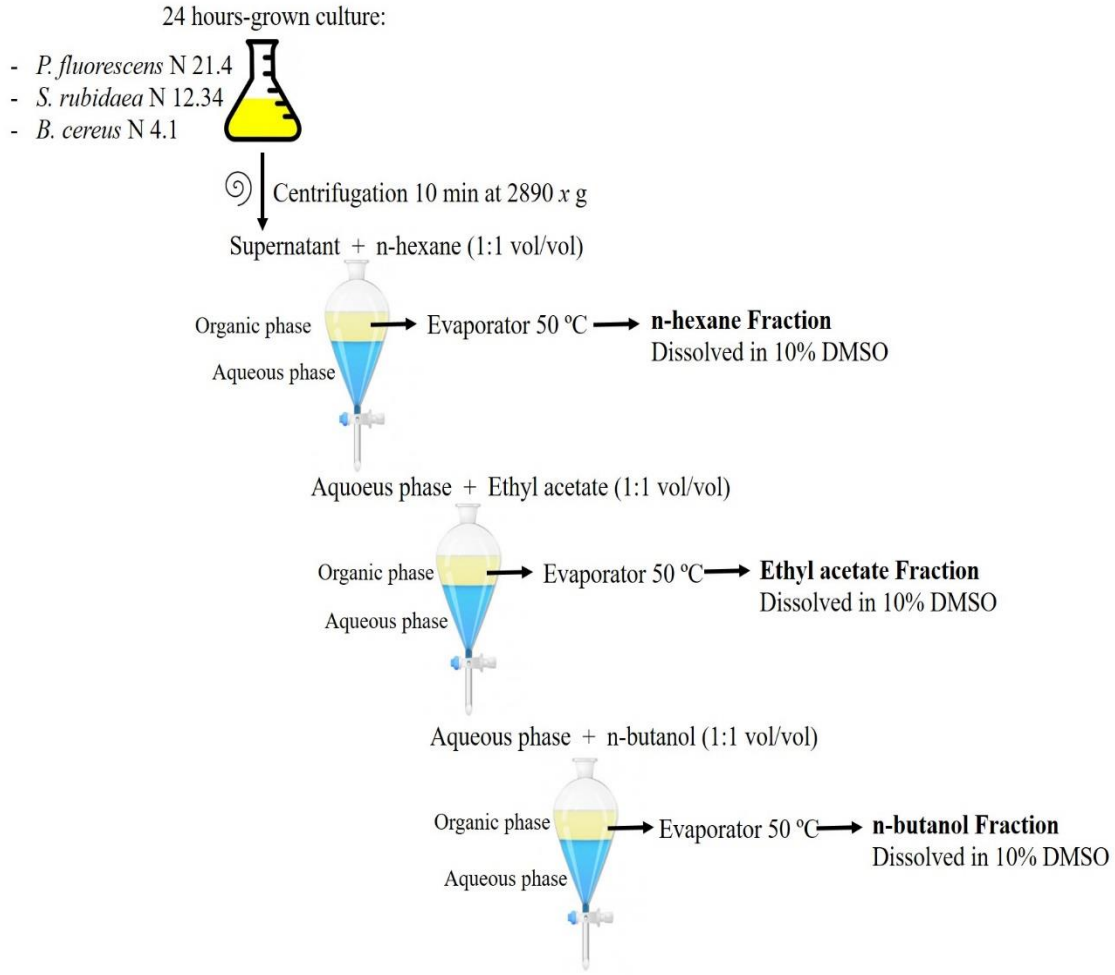

Figure 2.2 Extraction of the metabolic elicitors' fractions, according to Sumayo et al. (2013).

On the other hand, the ME inoculated in *Rubus* cv. Loch Ness were prepared by centrifuging a 24 hours-grown culture (nutrient broth (CONDA), grown at 28ºC on an orbital shaker (180 rpm) at 28 ºC), of the strain *P. fluorescens* N 21.4, discarding cells, and diluting the supernatant from 10^9 to 10^7 cfu mL^{-1}.

2.2 Methods

2.2.1 Experimental design

2.2.1.1 Experiments performed with *P. fluorescens* N 21.4 and its ME under controlled conditions: *A. thaliana* and soybean

The ability of the metabolic elicitors extracted from *P. fluorescens* N 21.4 to induce systemic resistance in *A. thaliana* against the pathogen *P. syringae* DC3000 was evaluated. From the three fractions (n-hexane, ethyl acetate and n-butanol (Fig. 2.2)), the n-hexane was the most effective one in plant protection (ISR). Hence, it was chosen for further ISR experiments to study the signal transduction pathways involved in the defensive response by evaluating the expression of marker genes of the SA signalling pathway (*NPR1*, *PR1, ICS* and *PR2*) and the JA/ET signalling pathway (*PDF1*, *MYC2*, *LOX2* and *PR3*). In addition, the oxidative stress of the n-hexane-inoculated plants was tested by determining the activity of enzymes related to the ascorbate-glutathione cycle. Activity of the pathogenesis-related proteins glucanase (PR2) and chitinase (PR3), lipoxygenase and polyphenol oxidase and malondialdehyde (MDA) levels were also measured.

The first experiment performed in *A. thaliana* seedlings to test the capacity of the three elicitor fractions to induce systemic resistance is summarized in figure 2.3. *A. thaliana* seeds were germinated in quartz sand for one week and then individually transplanted to 12-well plastic plates (5 mL) filled with 3:1 (vol/vol) peat/sand. Plants

were watered with 1 mL of tap water twice a week and with 1 mL of ½ Hoagland solution per plant once a week. Each treatment inoculated to the plants comprised three plates, each plate constituting a replicate. One week after transplanting, the three metabolic elicitor fractions were delivered to seedlings by drenching in the soil 20 µL per well. The positive control was inoculated with 1 mL of *P. fluorescens* N 21.4 culture, grown for 24 h in sterile nutrient broth (CONDA); and diluted from 10^9 to 10^8 cfu mL^{-1}, while negative controls were inoculated with 20 µL of the n-hexane, ethyl acetate and n-butanol fractions from sterile nutrient broth (CONDA) resuspended in 10% DMSO. Three days after treatment inoculation, plants were pathogen challenged with *P. syringae* DC3000. One day before pathogen challenge, plates were placed in a humidity chamber with 99% relative humidity to ensure stomata opening in order to allow disease progress. Pathogen was inoculated by placing a 5-µL drop of 10^8 cfu mL^{-1} suspension on each leaf (Sumayo et al. 2013). Seventy-two hours after pathogen inoculation, the disease index was determined as the ratio of the number of leaves with disease symptoms to the total number of leaves (Ryu et al. 2004). Results were relativized using the negative controls as 0% of protection. Plants were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 µE s^{-1} m^{-2} at 24 °C) and 16 h dark period (20 °C) at 70% relative humidity.

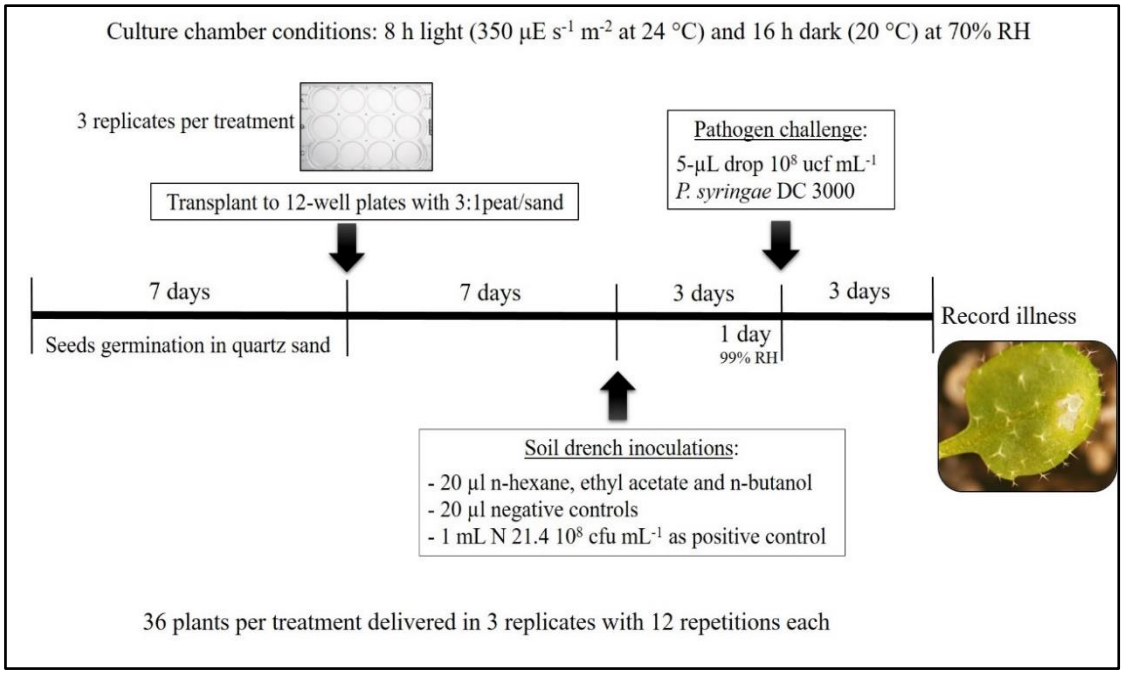

Figure 2.3 Timeline of an ISR experiment performed in 12-well plastic plates in *A. thaliana* seedlings.

Another longer ISR experiment was carried to study the signal transduction pathways involved in the response triggered by the plants that had been inoculated with the n-hexane selected fraction (Fig. 2.4). In this experiment, seeds were also germinated in quartz sand for 1 week. However, one-week-old seedlings were transplanted individually to 100 mL pots filled with 3:1 (vol/vol) peat/sand mixture (60 g per pot). Plants were watered with 5 mL of tap water twice a week, and with 5 mL of ½ Hoagland solution per plant once a week. Four-week-old seedlings were soil inoculated with 50 µL of the n-hexane fraction, and negative control seedlings with 50 µL of the n-hexane fraction from sterile nutrient broth resuspended in 10% DMSO. Four days later, plants were pathogen challenged. One day before the pathogen inoculation, plants were placed in a humidity chamber (99% relative humidity) to ensure the stomatal opening needed for the disease to establish. Pathogen inoculation was carried out by spraying the plants with 150 mL of a suspension of 10^8 cfu mL^{-1}. Seven plants per treatment were collected 6, 12 and 24 h after pathogen challenge (hapc), powdered in liquid nitrogen and stored at −80

°C until gene expression analysis by qPCR and analysis of enzymatic activity and pathogenesis-related proteins. Plants were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 µE s^{-1} m^{-2} at 24 °C) and 16 h dark period (20 °C) at 70% relative humidity.

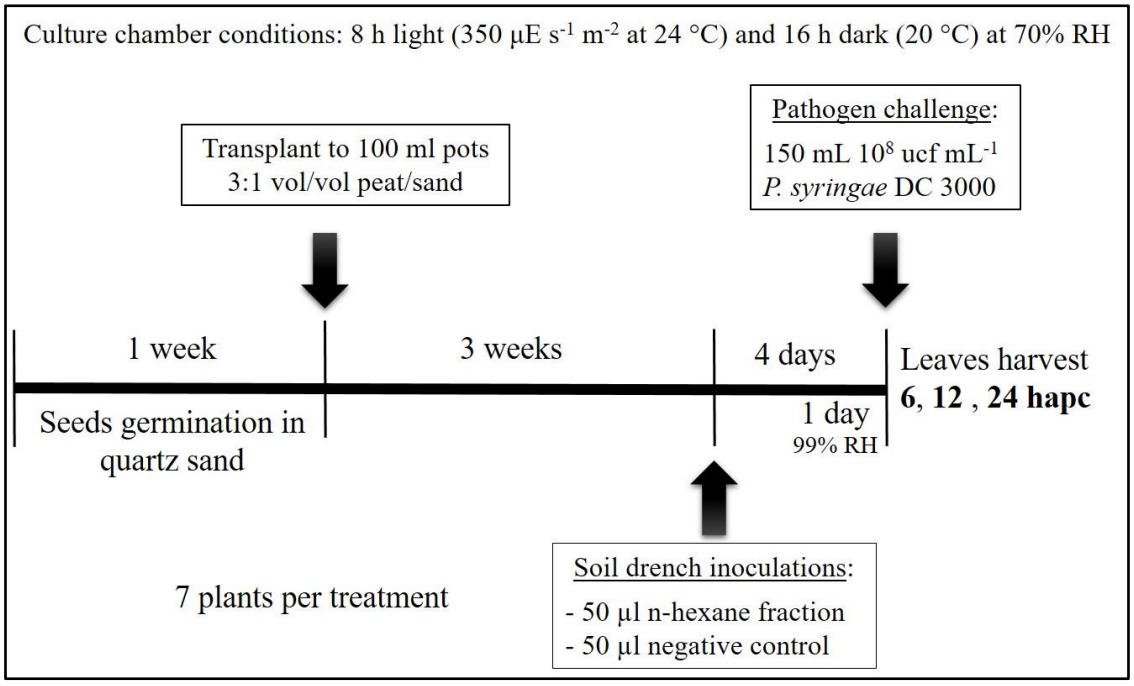

Figure 2.4 Timeline of a longer ISR experiment performed in 100 mL-pots in *A. thaliana* seedlings.

Since the efficacy of the metabolic elicitors of the *P. fluorescens* N 21.4 in the induction of systemic resistance and in the activation of the plant immune system was demonstrated, we proceeded to the fractionation and purification of them to chemically identify their composition (Fig. 2.5). Firstly, a liquid-liquid phase separation (vol/vol) with 10 L of a 24 h-grown culture of *P.fluorescens* N 21.4 (10^9 cfu mL^{-1}) and n-hexane (Sumayo et al. 2013) was carried out, followed by two sequential vacuum liquid chromatographies (VLC). All the sequentially obtained fragmented fractions were inoculated in soybean seeds to analyse their capacity to elicit isoflavone secondary metabolism. A final isolation and purification of the most fragmented fraction was

performed. Finally, the resulting fraction, the purest one, was tested in *A. thaliana* seedlings for an ISR experiment, which was performed as the longest ISR experiment from the subsection 2.2.1.1 (Fig 2.4), recording the illness as explained in the first ISR experiment (the shorter one) from subsection 2.2.1.1 (Fig. 2.3)). This fraction was finally analysed by UHPLC.

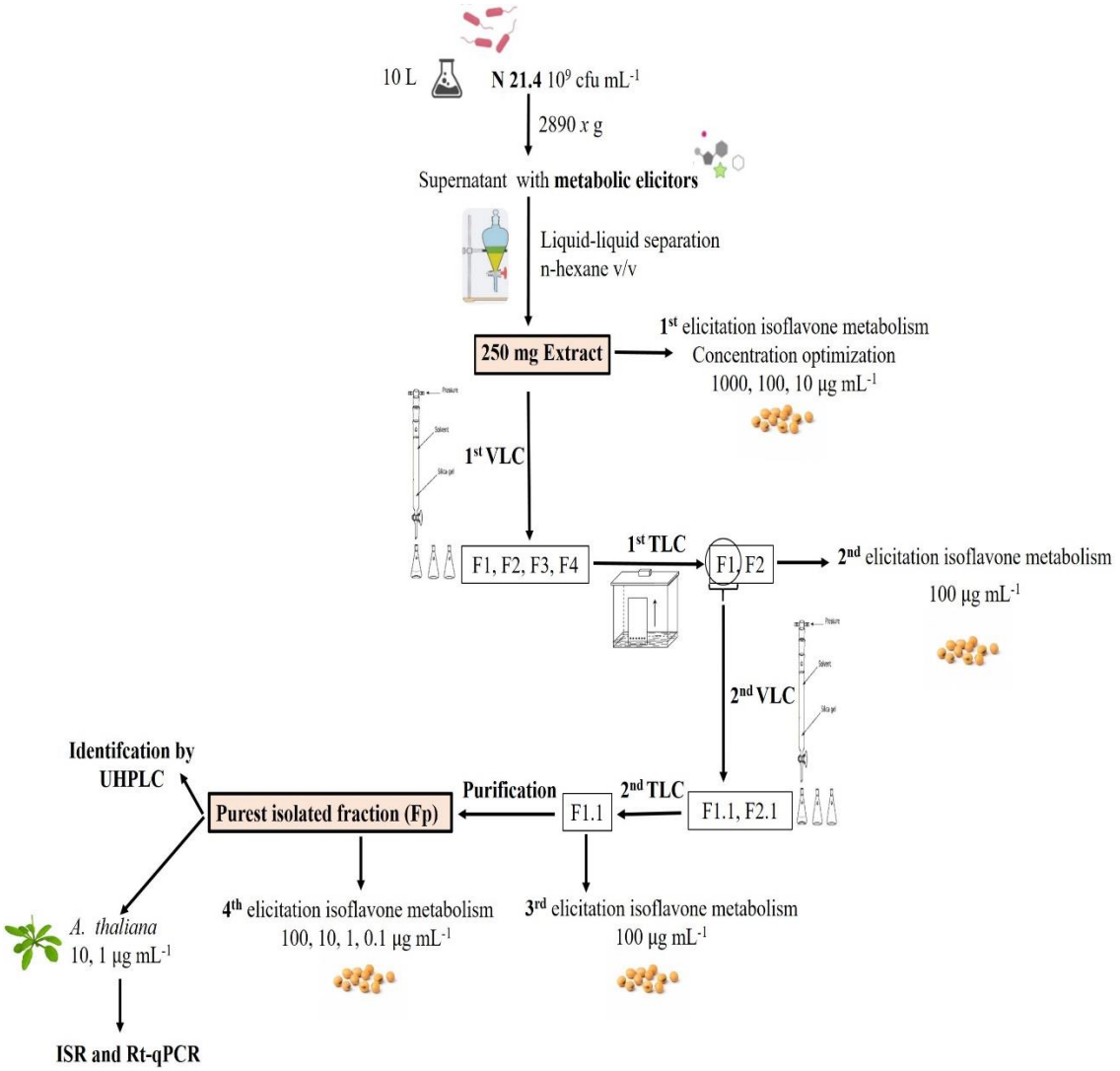

Figure 2.5 Representative scheme of the sequential extraction, fractionation and purification of the ME of *P. fluorescens* N 21.4. Shown are the growth of the bacterium in nutrient broth, the liquid–liquid phase separation with n-hexane, the process of fractionation and purification by two sequential vacuum liquid cromatographies, the four elicitation experiments of isoflavone metabolism, the ISR experiment in *A. thaliana* and the final compound identification by UHPLC.

To test the efficacy of the obtained fractions in eliciting the plant secondary metabolism, a rapid induction test was carried out in soybean seeds to measure the synthesis of isoflavones. For this, seeds were superficially disinfected with a 70% ethanol bath for 1 min, 5% sodium hypochlorite for 6 min and 5 washes with sterile distilled water. After that, seeds were kept imbibing in sterile distilled water for 4 h, in darkness and at room temperature. After imbibition, 90 seeds per treatment were distributed in 3 replicates of 30 seeds each and put to germinate in sterile Petri dishes with 1% European bacteriological agar. A small longitudinal cut was made in the seed embryo without compromising its viability. Ten µL of each treatment diluted in 10% DMSO were injected into the cut of the embryo. Ninety seeds for control treatment, inoculated with the control dissolved in 10% DMSO were also used, and another control with soybean seeds only with the cut in the embryo, but without inoculation was added. This entire procedure was carried out under sterile conditions. Once the treatments were applied, the plates were kept for 3 days in a SANYO MLR 350H camera at 27 °C in darkness.

The quantification of the isoflavones (µg mL^{-1}) was performed by interpolation of the relative area given by the HPLC detector (Fig. 2.6) on a calibration curve built for each isoflavone ($R^2 > 0.99$). The calibration curves were built with the isoflavones (LC Laboratories): daidzin, genistin and malonyl genistin.

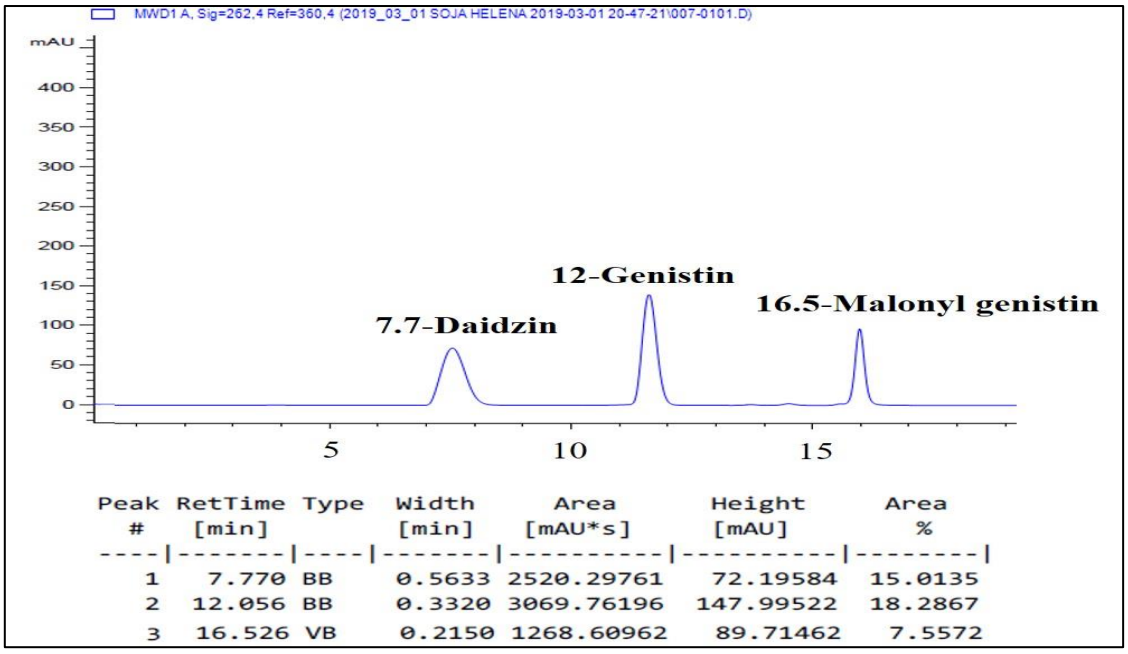

Figure 2.6 HPLC chromatogram depicting the standard isoflavones daidzin, genistin and malonyl genistin, their retention time and the width, area and height of the peaks.

2.2.1.2 Experiments performed with *P. fluorescens* N 21.4 and its ME in field conditions: *Rubus* cv. Loch Ness

The *Rubus* cv. Loch Ness plants used in our studies were kindly provided by Agricola El Bosque S.L. "La Canastita" (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were handled according to regular agricultural practices (Ramos-Solano et al. 2014), under real production conditions. Plants were grown from November 2017 to April 2018 under "winter cycle", and before being transplanted to greenhouses in November, they underwent an artificial cold period to start their regular cycle (Fig. 2.7). A total of 540 plants were in the trial, arranged in five greenhouses; each greenhouse had two lines with 120 plants in total, each line being one replicate with 60 plants. Three of the lines were inoculated with live *P. fluorescens* N 21.4 at root level, three lines were inoculated with the metabolic elicitors (ME) of N 21.4 by aerial spraying and three lines were left as non-inoculated controls. N 21.4 and ME-inoculated plants were inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum per plant.

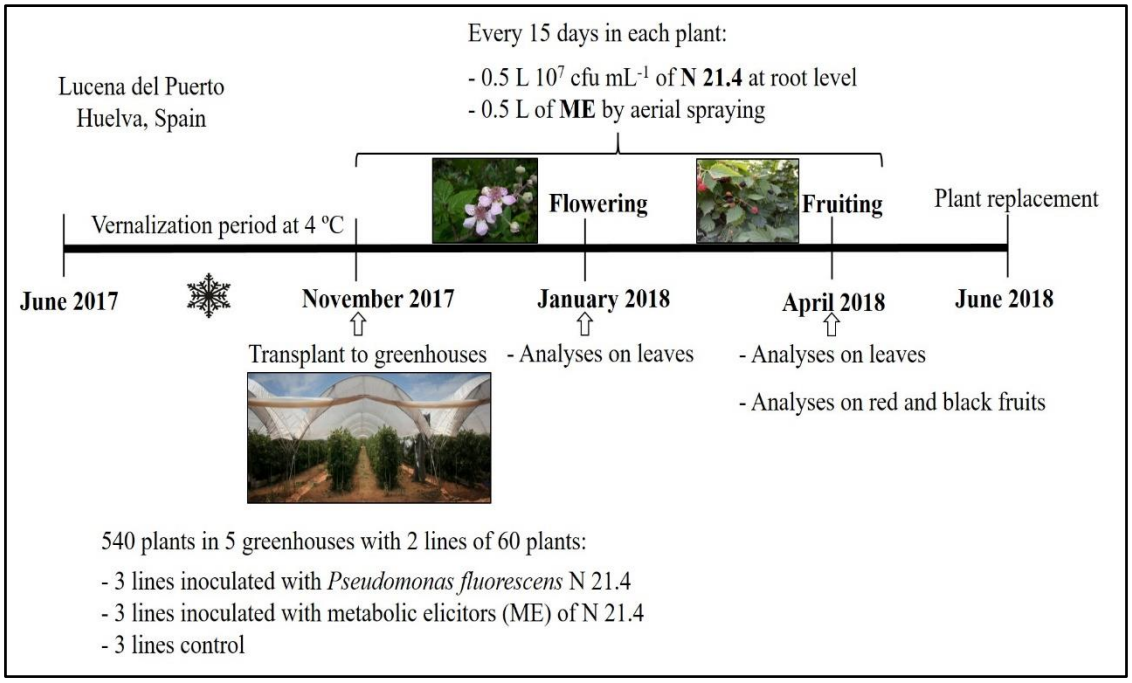

Figure 2.7 Representative timeline of the entire blackberry cultivation cycle in the greenhouses of Lucena del Puerto (Huelva, Spain).

Leaves were sampled at flowering (January 2018) and at fruiting (April 2018), and fruits, in red and black ripening stage, were harvested in April 2018 (Fig. 2.7). Leaves and fruits were rapidly frozen in liquid nitrogen and brought to the laboratory. Three replicates were taken, being each one constituted from leaves of 60 plants and 125 g of fruits that were randomly sampled of 60 plants. From all these replicates, extracts were prepared for future analysis, as described in 2.2.3 subsection.

Parameters analysed in the leaves were: photosynthesis by fluorescence measurement *in situ*, total pigments, activity of enzymes related to oxidative stress and polyphenol composition measured by UHPLC/ESI-QTOF-MS, at flowering; and photosynthesis by fluorescence measurement *in situ*, total pigments, activity of enzymes related to oxidative stress, activity of PRs, differential expression of genes encoding PRs, DPPH and polyphenol composition measured by UHPLC/ESI-QTOF-MS, at fruiting.

Parameters analysed in red and black fruits were: flavonols, phenols and anthocyanins concentration measured by colorimetry, polyphenol composition measured

by UHPLC/ESI-QTOF-MS and differential expression of marker genes of the biosynthetic flavonoid pathway.

2.2.1.3 Screening of bacterial strains isolated from the rhizosphere of *N. glauca*

To search for new bacterial strains with efficient PGPR potential, a screening of 175 isolates from the non-assayed bacteria group of the work of Ramos-Solano et al. 2010a was performed. First, biochemical tests for putative beneficial rhizobacteria traits were carried out to all isolates. The 16S rRNA partial sequencing of all isolates was analysed and a phylogenetic tree was performed with these sequences. Twenty-five strains selected based on their biochemical traits and avoiding phylogenetic redundancy were assayed to determine their ability to trigger *A. thaliana* protection (ISR). The most effective strains (5) were studied to understand the mechanisms involved in protection. Finally, metabolic elicitors were obtained from the two most effective bacteria (*S. rubidaea* N 12.34 and N *B. cereus* 4.1) to demonstrate their ability to mimic the protective response triggered by the live strains (Fig. 2.8).

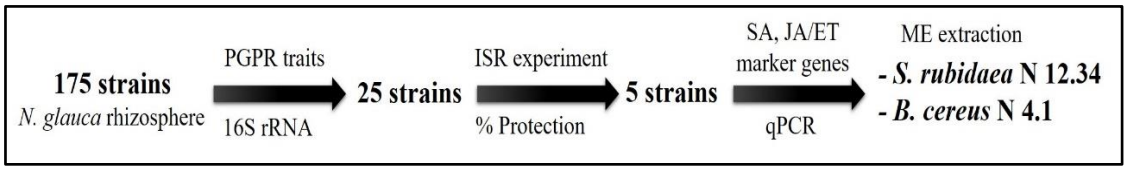

Figure 2.8 Selection of PGPR strains from the non-assayed rhizobacteria group of the work of Ramos-Solano et al. 2010a.

To perform the above procedure, four ISR experiments were carried out. They are explained below and represented summarized as a timeline in figure 2.9.

In the first ISR experiment, *A. thaliana* wild type *Columbia ecotype* 0 seeds (were germinated in quartz sand and two-week-old seedlings were then individually transplanted to 100 mL pots filled with 12:5 (vol/vol) peat/sand mixture (60 g per pot). Forty-eight plants per treatment were used; plants were arranged in three replicates, with

sixteen repetitions each. Plants were watered with 5 mL of tap water once a week and with 5 mL of half-strength Hoagland solution per plant once a week. Bacterial strains were inoculated twice by soil drench with 3 mL of a suspension of bacterial cells, grown for 24 h in nutrient broth (CONDA) at 28 ºC, and adjusted to a density of 10^8 cfu mL^{-1}, in the first and in the second week after transplant. Negative control plants were mock-inoculated by soil drench with 3 mL of sterile nutrient broth (CONDA) and positive control plants were inoculated by soil drench with 10 µL of BTH (Benzothiadiazole) 0.5 mM (Sumayo et al. 2013). Four days after the second bacterial inoculation, plants were pathogen challenged with *P. syringae* DC3000. One day before pathogen challenge, plants were maintained with 99% relative humidity to ensure stomata opening in order to allow disease progress. *P. syringae* DC3000 was centrifuged (10 min at 2890 *x* g) and cells were resuspended in 10 mM $MgSO_4$ to achieve 10^8 cfu mL^{-1}. Pathogen was inoculated by spraying the total of the plants with 250 mL. Plants were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 µE s^{-1} m^{-2} at 24 °C) and 16 h dark period (20 °C) at 70% relative humidity for 72 h, and disease severity was recorded as the number of leaves with disease symptoms relative to the total number of leaves. Results were relativized using the disease severity of negative control plants as 0% protection (Ryu et al. 2004).

In the second ISR experiment, *A. thaliana* was handled as described in the first ISR assay (Fig. 2.9). Instead of recording disease severity 72 hours after pathogen challenge (hapc), all the leaves of sixteen plants (treated with each bacteria (five selected ones)) were harvested at 6, 12 and 24 hapc, powdered in liquid nitrogen and stored at -80 ºC. These plant samples were used for gene expression analysis by qPCR to study the signal transduction pathways involved in the defensive response by evaluating expression of marker genes of the SA and the JA/ET signalling pathway.

In the third ISR experiment, *A. thaliana* was handled as described in the first ISR assay (Fig. 2.9). Three metabolic elicitors fractions (n-hexane, ethyl acetate and n-butanol) extracted from the two most effective bacteria (N 12.34 and N 4.1) were delivered to seedlings by soil drench (50 μL). Negative controls were treated with 50 μL of n-hexane, ethyl acetate and n-butanol fractions from sterile nutrient broth (CONDA) resuspended in 10% DMSO. The pathogen was also inoculated as described in the first ISR assay. Seventy-two hours after pathogen inoculation, disease severity was recorded and relativized as in the first ISR experiment.

In the fourth ISR experiment, *A. thaliana* was handled as described in the first ISR assay (Fig. 2.9). Treatments were n-hexane metabolic elicitor fraction from the strain N 12.34, ethyl acetate metabolic elicitor fraction from the strain N 12.34, n-hexane metabolic elicitor fraction from the strain N 4.1, ethyl acetate metabolic elicitor fraction from the strain N 4.1 and controls with n-hexane and ethyl acetate fractions from sterile nutrient broth (CONDA) resuspended in 10% DMSO. Plants were inoculated by soil drench (50 μL) and pathogen inoculation with *P. syringae* DC3000 was performed as explained above. All the leaves of sixteen plants were harvested at 6, 12 and 24 hapc, powdered in liquid nitrogen and stored at -80 ºC. These plant samples were used for gene expression analysis by qPCR to study the signal transduction pathways involved in the defensive response.

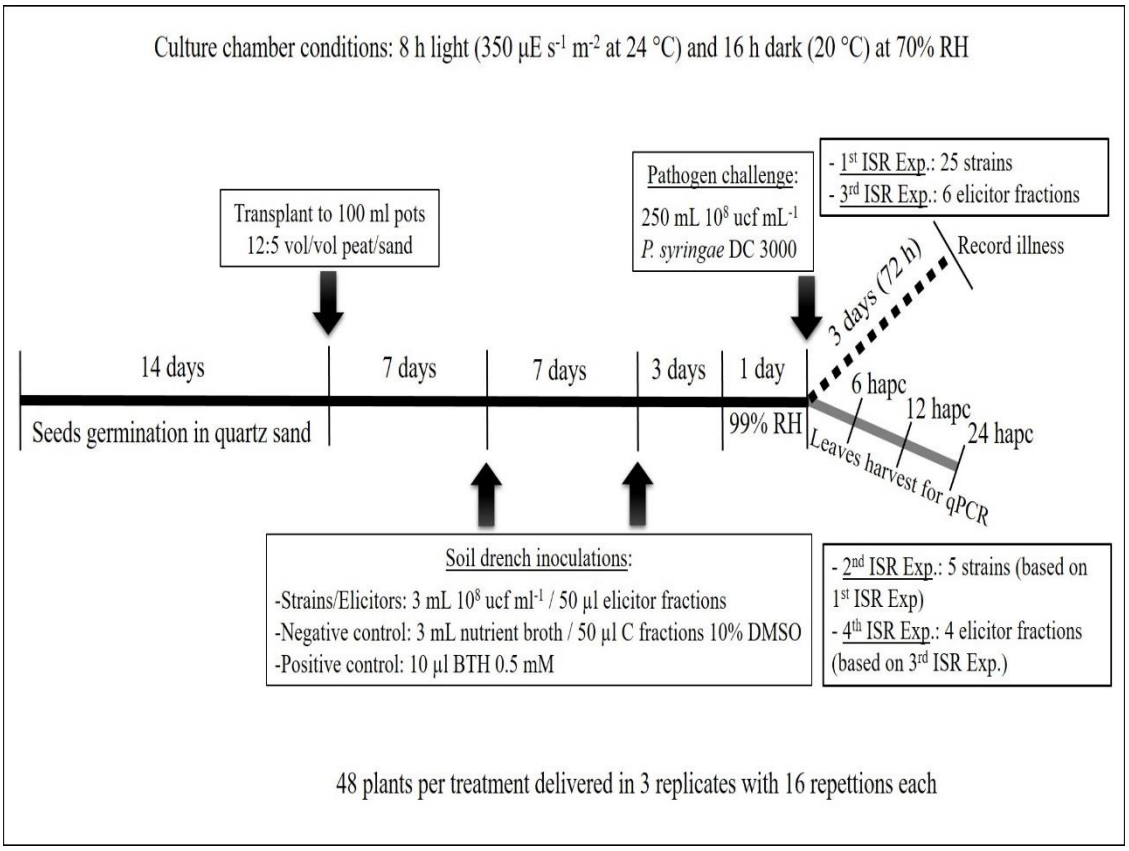

Figure 2.9 ISR experiments represented as a timeline. The black continuous line represents the part of the experimental design common to all ISR experiments; the black dashed line represents the last part of ISR experiments to assess protection against the pathogen DC 3000 (first and third experiments) and the grey continuous line represents the last part of ISR experiments to carry out differential gene expression analyses by qPCR (second and fourth experiments).

2.2.2 Inocula preparation

Bacterial strains were stored at -80 ºC in nutrient broth (CONDA) with 20% glycerol. Inocula were prepared by streaking strains from -80 ºC onto PCA (CONDA) plates, incubating them at 28°C for 24 h. After that, bacterial cells were scraped off the plates into sterile nutrient broth (CONDA) and incubated on a rotatory shaker at 28 ºC and 180 rpm for 24 h to obtain 10^9 cfu mL^{-1} inocula. Inocula were diluted from 10^9 to 10^8 cfu mL^{-1} in the case of the strains inoculated in *A. thaliana* and to10^7 cfu mL^{-1} in the case of N 21.4 inoculated in blackberry plants.

The pathogen *P. syringae* DC 3000 was stored at -80 ºC in nutrient broth with 20% glycerol. Inocula were prepared by streaking strains from -80 ºC onto PCA (CONDA) plates, incubating them at 28 °C for 24 h. Pathogen was centrifuged (10 min at 2890 *x* g) and cells were resuspended in 10 mM $MgSO_4$ to achieve 10^8 cfu mL^{-1}.

Inocula of metabolic elicitors were prepared as explained in subsection 2.1.3. Fifty µL of the different elicitor fractions were inoculated in *A. thaliana* seedlings planted in pots and twenty µL in *A. thaliana* seedlings planted in 12-well plastic plates, and ten microliters were inoculated into the soybean seeds. In the case of *Rubus* cv. Loch Ness, inocula were diluted following the same proportion as the bacterial strain (from 10^9 to 10^7 cfu mL^{-1}) and 0.5 L per plant were applied.

2.2.3 Extracts preparation

To measure the enzymatic activity of some enzymes related to oxidative stress, pathogenesis-related proteins and systemic resistance proteins, a protein extraction was firstly carried out. For this, ten mg of powdered *A. thaliana* or blackberry leaves of each sample were mixed with 1 mL of extraction buffer (cold phosphate buffer 0.1 M pH 7.0 with 2 mM of PMSF (phenylmethylsulfonyl fluoride), sonicated for 10 min and centrifuged 20 min at 10000 *x* g at 4 ºC. The remaining supernatant was frozen at -20 ºC and used as the enzymatic extract.

For the measurement of other parameters in blackberry leaves and fruits, they were powdered in liquid nitrogen and one g of the powder of each sample was mixed with 9 mL of 80% methanol for HPLC methods, sonicated for 10 min and centrifuged for 5 min at 2890 *x* g at room temperature. Supernatant of each sample was filtered (0.2 µm), and half of it was stored at 4 ºC (for bioactive measurement by colorimetry) and the other half was frozen and lyophilized.

The extraction and analysis of isoflavones from soybean seeds was performed according to Wang et al. (2002) and Lozovaya et al. (2004) with some modifications. Seeds were powdered with liquid nitrogen, mixed with 100 mL of 80% HPLC-methods methanol and maintained on an orbital shaker at 145 rpm for 15 h at 40 °C. Samples were then centrifuged at 2890 *x* g for 20 min at 20 °C. The obtained supernatant was filtered through a 0.45 µm nitrocellulose membrane, and the methanolic extract was used for analysis by HPLC.

2.2.4 Analysis of differential gene expression by RT-qPCR

In all experiments in which differential gene expression was measured by qPCR, a RNA extraction was first performed from the samples. Samples were firstly grounded to a fine powder with liquid nitrogen. Total RNA was isolated from each replicate with PureLink RNA Micro Kit (Invitrogen), DNAase treatment included, and RNA purity was confirmed using NanodropTM. Finally, a retrotranscription followed by a qPCR was performed.

The retrotranscription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were carried out using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 ºC, 30 min 42 ºC, 5 min 85 ºC, and hold at 4 ºC. Amplification was carried out with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 ºC and then 39 cycles consisting of 15 s at 95 ºC, 30 s at 55 ºC and 30 s at 72 ºC, followed by melting curve to check results. To describe the gene expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001). *Sand* gene (AT2G28390) and *Histone* H3 gene (AF304365.1) were used as reference genes. Gene primers used are shown in Table 2.

Table 2 Forward and reverse primers used in qPCR analyses.

	Forward primer	Reverse primer
AtNPR1	5´-TATTGTCAARTCTTATGTAGAT	5´-TATTGTCAARTCTRATGTAGAT
AtPR1	5´-AGTTGTTTGGAGAAAGTCAG	5´-GTTCACATAATTCCCACGA
AtICS	5´-GCAAGAATCATGTTCCTACC	5´AATTATCCTGCTGTTACGAG
AtPDF1	5´-TTGTTCTCTTTGCTGCTTTCGA	5´-TTGGCTTCTCGCACAACTTCT
AtLOX2	5´-ACTTGCTCGTCCGGTAATTGG	5´-GTACGGCCTTGCCTGTGAATG
AtMYC2	5´GATGAGGAGGTGACGGATACGGAA	5´-CGCTTTACCAGCTAATCCCGCA
AtPR2	5´-TCGTCTCGATTATGCTCTCTTC	5´-GCAGAATACACAGCATCCAAAA
AtPR3	5´-AAATCAACCTAGCAGGCCACT	5´-GAGGGAGAGGAACACCTTGACT
Sand	5′-CTGTCTTCTCATCTCTTGTC	5′-TCTTGCAATATGGTTCCTG
RuPR1	5´-TACTACACGTACGCGACAAACAC	5´-TCTCCATCATCACACACAACTCT
RuPR2	5´-TTCGTCTCGATTATGCTCTCTTC	5´-GCAGAATACACAGCATCCAAAA
RuPR3	5´-AAATCAACCTAGCAGGCCACT	5´-GAGGGAGAGGAACACCTTGACT
Histone	5´-TTCCAGAGCCATGCAGTTTTG	5´-TGGCATGAATGGCACAGAGA
RuCHS	5´ATGGTGGTTGTTGAAATTCC	5´CTGGATTGCACACCCAGGTGGCCC
RuFLS	5´CCTACAGGGAAGTCAATGAGAAA	5´CACATGGGATTTCAGTACCTTCT
RuF3´H	5´CCTATCTCCAAGCTGTCATCAAG	5´GTGGTATCCGTTGATTTCACAAC
RuDFR	5´AATCAGAAGAAGGTGAAGC	5´CATTAKSACAAGTTTGGTG
RuLAR	5´GTGGAGTCCCATACACGTACATT	5´CTGAAACTGATCTAACGGTGGAA
RuANS	5´TTGGTCTGGGATTAGAAGAAAGG	5´CTGAGGGCATTTTGGGTAGTAAT
RuANR	5´TCGCAATGTACTTCCAAGAAAC	5´CTTCATCAGCTTACGGAAATCAC
RuMYB5	5´ACTCAATCCAGACTCCTCATCTG	5´AGGAAGTGATTGGACTTTTAGGG

**At = A. thaliana*; *Ru = Rubus*

2.2.5 Determination of bioactive content in *Rubus* cv. Loch Ness

2.2.5.1 Analysis by UHPLC/ESI-QTOF-MS

Characterization and analyses of phenolic compounds and flavonoids present in the leaves and in the fruits of blackberry plants (*Rubus* cv. Loch Ness) were assessed by UHPLC/ESI-QTOF-MS. For this, ten mg of leaves/fruits powder (liophilizated) were added to 1 mL of methanol (LC-MS grade). The mixture was vortexed for 1 min, sonicated for 5 min and centrifuged at 2890 *x* g for 5 min at 4 ºC. Supernatants were

collected, filtered (0.45 μm) and stored at -20ºC until their use for LC/MS analysis. During the process, extracts were protected from light.

Samples were injected on a 1290 Infinity series UHPLC system associated to an electrospray ionization source (ESI) with Jet Stream technology to a 6550 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany).

For the molecule dissociation, a volume of two μL was injected in a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 μm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2% B (0-6 min), 2-50% B (6-10 min), 50-95% B (11-18 min), 95% B for 2 min (18-20 min), and returned to starting conditions 2% B in one min (20-21 min) to finally keep the re-equilibration with a total analysis time of 25 min.

For bioactive measurement, each sample was injected twice in six different concentrations to create calibration curves in which sample peak areas were extrapolated. UHPLC-MS data analysis was performed by MassHunter Qualitative Analysis Software (Agilent Technologies), version B.08.00, using Molecular Feature Extraction (MFE).

2.2.5.2 Analysis by colorimetry

Total flavonols, phenols and anthocyanins present in the fruit (red and black) of blackberry plants (*Rubus* cv. Loch Ness) were assessed by colorimetry.

- **Total flavonols**

Total flavonols were quantitatively determined through the test described by Jia et al. (1999), using catechin as standard (Sigma-Aldrich, St Louis, MO). One milliliter of fruit methanolic extract was added to a flask with 4 mL of distilled water and 0.3 mL of $NaNO_2$ 5% (w/v). After 5 min, 0.3 mL of $AlCl_3$ 10% were added. Five minutes later, 2 mL of

NaOH 1 M were finally added. The solution was mixed and measured at 510 nm with an UV-Visible spectrophotometer (Biomate 5). A catechin calibration curve was made (r = 0.99). Results were expressed as g of catechin equivalents per kg of FW. All samples of red and black fruits were measured in triplicate.

- **Total phenols**

Total phenols were quantitatively determined with Folin-Ciocalteu agent (Sigma. Aldrich, St Louis, MO) by a colorimetric method described by Singleton and Rossi (1965) with some modifications (Benvenuti et al. 2004). Gallic acid was used as standard (Sigma-Aldrich, St Louis, MO). Twenty µL of methanolic extract were mixed with 250 µL of Folin-Ciocalteu 2 N (Sigma-Aldrich, St Louis, MO) and 3 mL of distilled water. After 5 min at room temperature, 0.75 mL of Na_2CO_3 20% solution were added. After 8 min at room temperature, 950 µL of distilled water were added and after 2 hours in obscurity, absorbance was measured at 760 nm with an UVVisible spectrophotometer (Biomate 5). A gallic acid calibration curve was made (r = 0.99). Results were expressed as g of gallic acid equivalents per kg of FW. All samples of red and black fruits were measured in triplicate.

- **Total anthocyanins**

Total anthocyanins were quantitatively determined through the pH differential method described by Giusti and Wrolstad (2001). Methanolic extracts were diluted in pH 1 buffer (0.2 M KCl) and pH 4.5 (1M CH_3CO_2Na) in 1:15 proportion. After that, absorbance was measured at 510 and 700 nm respectively, in an UVVisible spectrophotometer (Biomate 5). A cyanidin-3-glucoside calibration curve was made (r = 0.99). Results were expressed as g of cyanidin-3-glucoside equivalents per kg of FW. All samples of red and black fruits were measured in triplicate.

2.2.6 Identification and characterization of the metabolic elicitors by UHPLC/ESI-QTOF-MS

The purest fraction obtained from the metabolic elicitors of *P. fluoresces* N 21.4 was analysed by UHPLC/ESI-QTOF-MS using the same equipment and methodology as was explained in 2.2.5.1 section.

In this case, a qualitative analysis software (MassHunter Qualitative Analysis (Agilent Technologies), version B.08.00, Santa Clara, CA, USA) was used to extract potential molecular features (MFs) and to generate the empirical formula of the compounds from their accurate mass and isotopic pattern distribution to increase the confidence of compound annotation, with a very good score (about 97-99%). The identification of compounds was carried out by comparing their retention times and the accurate masses against online databases, as FOODB (http://foodb.ca), CEU massmediator (http://ceumass.eps.uspceu.es/mediator), MetaCyc and scientific bibliography.

2.3 Statistical analyses

To check the statistical differences between the results obtained, analysis of variance (ANOVA) was used. In the experiments with only one independent variable, a one-way ANOVA was used and in experiments with two independent variables, a two-way ANOVA (factorial ANOVA) was used. In both cases, prior to ANOVA analysis, homoscedasticity and normality of the variance were checked with Statgraphics plus 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p < 0.05$), a Fisher test was used (Sokal and Rohlf, 1980).

3. Experimental work

3. Experimental work

3.1 Extracts from cultures of *Pseudomonas fluorescens* induce defensive patterns of gene expression and enzyme activity while depressing visible injury and reactive oxygen species in *Arabidopsis thaliana* challenged with pathogenic *Pseudomonas syringae*

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Extracts from cultures of *Pseudomonas fluorescens* induce defensive patterns of gene expression and enzyme activity while depressing visible injury and reactive oxygen species in *Arabidopsis thaliana* challenged with pathogenic *Pseudomonas syringae*

H. Martin-Rivilla*, A. Garcia-Villaraco, B. Ramos-Solano, F. J. Gutierrez-Mañero and J. A. Lucas

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

Extracts from cultures of *Pseudomonas fluorescens* induce defensive patterns of gene expression and enzyme activity while depressing visible injury and reactive oxygen species (ROS) in *Arabidopsis thaliana* challenged with pathogenic *Pseudomonas syringae*

Martin-Rivilla, H*., Garcia-Villaraco, A., Ramos-Solano, B., Gutierrez-Mañero, F.J., Lucas, J.A.

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

*Corresponding author: Helena Martín Rivilla, helenamartin92@gmail.com

+913 72 47 85

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Abstract

We evaluated the ability of the metabolic elicitors extracted from *Pseudomonas fluorescens* N 21.4 to induce systemic resistance (ISR) in *Arabidopsis thaliana* against the pathogen *Pseudomonas syringae* DC3000. Metabolic elicitors were obtained from bacteria-free culture medium with n-hexane, ethyl acetate and n-butanol in three consecutive extractions. Each extract showed plant protection activity. The n-hexane fraction was the most effective and was used to study the signal transduction pathways involved by evaluating expression of marker genes of the salicylic acid (SA) signalling pathway (*NPR1*, *PR1*, *ICS* and *PR2*) and the jasmonic acid/ethylene (JA/ET) signalling pathway (*PDF1*, *MYC2*, *LOX2* and *PR3*). In addition, the level of oxidative stress was tested by determining the activity of enzymes related to the ascorbate-glutathione cycle. N-hexane extracts stimulated both pathways based on overexpression of *ICS*, *PR1*, *PR2*, *PDF1* and *LOX2* genes. In addition, activity of the pathogenesis-related proteins glucanase (PR2) and chitinase (PR3), lipoxygenase and polyphenol oxidase was enhanced together with an increased capacity to remove reactive oxygen species (ROS). This was associated with less oxidative stress as indicated by a decrease in malondialdehyde (MDA), suggesting a causative link between defensive metabolism against *P. syringae* and ROS scavenging.

Keywords: *Pseudomonas fluorescens* N 21.4; metabolic elicitors; ISR; salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) signalling pathway; oxidative stress.

1. Introduction

Agricultural systems impose a range of abiotic and biotic stresses on crop plants that lower their productivity (García-Cristobal et al. 2015), thus compromising food supplies worldwide (Pechanova and Pechan, 2015; Miller et al. 2017).

Due to its significance, interactions between plants and pathogenic organisms have been studied intensively with a view to providing sustainable solutions for crop diseases, to enhance food safety by improving food quality and crop yields and to understand how plants cope with biotic stress (Silva et al. 2018).

The rapid generation of reactive oxygen species (ROS, such as $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$) represents a common plant response to pathogen attack (Noctor et al. 2014; Xia et al. 2015) and therefore represents a node from which many signalling events are generated. A rise in ROS production leads to oxidative stress (Gill and Tuteja, 2010) mainly by provoking oxidative modification of vital biomolecules including membrane lipids, cellular amino acids, proteins and DNA (Gill and Tuteja, 2010; Anjum et al. 2012). The outcomes include cell death and the arrest of plant growth and development. To maintain optimal levels of ROS, plants possess a sophisticated regulatory system consisting of enzymatic antioxidants (superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase, GPX; ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR) and non-enzymatic antioxidants (ascorbate, ASC; glutathione, GSH; carotenoids; tocopherols; phenolics compounds).

Colonization of plant roots by PGPR (Plant Growth Promoting Rhizobacteria) improves plant health by stimulating its immune system to decrease oxidative stress through improving ROS scavenging (Lucas et al. 2013; Lucas et al. 2014; García-Cristobal et al. 2015). This phenomenon is known as Induced Systemic Resistance (ISR)

and involves the induction of resistance not only locally at the site of infection, but also systemically. ISR has been primarily described as a response induced by PGPR (Pieterse et al. 2000), but it can also be induced by metabolic elicitors such as antibiotics, surfactants or other chemicals (Gozzo and Faoro, 2013). The elicitation of defensive metabolism by PGPR or elicitors leads to a physiological situation in the plant called priming (Conrath, 2011). In this situation, plants show faster and/or stronger activation of defence responses when subsequently challenged by pathogen (Conrath et al. 2006).

Despite the many studies of PGPR triggering ISR, few have focused on the molecular elicitors produced by these bacteria. However, metabolites from various bacterial genera: *Klebsiella* (Park et al. 2009), *Ochrobactrum* (Sumayo et al. 2013), *Pseudomonas* (Ongena et al. 2005) and *Bacillus* (Huang et al. 2012) have been recognized as ISR metabolic elicitors, with those from *Bacillus* being the most studied, although it is well-known that *Pseudomonas* spp. are possibly the most important producers of compounds triggering plant immune responses (Durrant and Dong, 2004; Choudhary et al. 2007). Interest in PGPR and their elicitors is heightened by their potential for developing a sustainable agriculture without pesticides or agrochemicals (Wu et al. 2018).

After a PGPR or their metabolic elicitors are sensed by a plant, salicylic acid (SA), jasmonic acid (JA) or ethylene (ET) signalling pathways are activated to trigger plant resistance (Wu et al. 2018). In the case of ISR, the response depends on JA and ET signalling and also requires NPR1 (Nonexpressor of Pathogenesis-Related Protein 1) (Pieterse and Van Loon, 2004; Pieterse and Van Loon, 2007). The JA signalling pathway has two branches controlled by the transcription factor *MYC2* and the ethylene response factor (ERF). The ERF branch of the JA pathway is associated to enhance resistance to necrotrophic pathogens and one of the marker gene of this branch is plant defensin1 (*PDF1*) (Berrocal-Lobo et al. 2002; Lorenzo et al. 2003).

The aim of the present work was (a) to obtain extracts containing bacterial metabolic elicitors able to trigger protection against pathogens in the model plant *A. thaliana* and b) to determine the transduction signal pathways involved in this protection. Three organic fractions were obtained from the culture medium of the strain *P. fluorescens* N 21.4, a gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca* (Ramos Solano et al. 2010a). This bacterium is known to trigger defensive metabolism in *Solanum lycopersicum* and *A. thaliana* (Domenech et al. 2007), to increase isoflavone content in *Glycine max* (Ramos-Solano et al. 2010b), to promote fruit production in *Rubus* sp. (Ramos-Solano et al. 2014) and to improve fruit quality of *Rubus* sp. by modifying flavonoid metabolism (Garcia-Seco et al. 2015a). We also wished to evaluate the ability of the extracts to trigger plant defence against pathovar DC 3000, a pathogenic strain of *P. syringae*. The most effective of the three fractions was then used to study the signal transduction pathway. To reach these objectives, differential gene expression of marker genes from the SA and JA/ET pathways were analysed, as well as enzymes involved in ROS scavenging system and proteins involved in induction systemic resistance, all in the context of the overall oxidative status of the plant.

2. Material and methods

2.1 Bacterial pathogen, model plant used and metabolic elicitors extraction

P. syringae (DC3000) was used as the pathogen in the experiments for challenge inoculation. This strain causes bacterial speck on the model plant *A. thaliana* and is used to study the model system for plant–pathogen interactions (Van Loon et al. 1998). The pathogen was grown for 24 h in 100 mL of nutrient broth (CONDA; gelatin peptone 5 g L^{-1} and beef extract 3 g L^{-1}) in a 250-mL Erlenmeyer flask on a shaker (180 rpm) at 28 ºC. The culture was then centrifuged (2890 *x* g for 10 min), washed with sterile water and

the pellet was suspended in sterile sufficient 10 mM MgSO4 to achieve 10^8 cfu mL^{-1}. The enumeration and calculations were carried out following the "drop method" (Hoben and Somasegaran, 1982).

A. thaliana Columbia ecotype 0 was used. Seedlings were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 μE s^{-1} m^2 at 24 °C) and 16 h dark cycle (20 °C) at 70 % relative humidity.

Metabolic elicitors from *P. fluorescens* N 21.4 were obtained according to Sumayo et al. 2013 using three separate solvents. The bacterium was first grown in nutrient broth (CONDA) on a rotary shaker (180 rpm) at 28 ºC for 24 h. Cells were separated by centrifugation at 2890 *x* g for 15 min, and 500 mL of the supernatant filtered (0.2 μm) and extracted sequentially in n-hexane, ethyl acetate and n-butanol to obtain the metabolic elicitors. The dry residues from each fraction were dissolved in 25 mL 10 % dimethylsulfoxide (DMSO).

2.2 Screening for the most effective determinant fraction to trigger systemic resistance

An induced systemic resistance (ISR) assay on *A. thaliana* plants was used to evaluate the ability of the three fractions extracted from *P. fluorescens* N 21.4 to trigger plant protection. The following five treatments were involved: i) metabolic elicitors in the n-hexane fraction, ii) metabolic elicitors in the ethyl acetate fraction, iii) metabolic elicitors in the n-butanol fraction, iv) positive control (*P. fluorescens* N 21.4, 10^8 cfu mL^{-1}) and v) negative controls (the n-hexane, ethyl acetate and n-butanol fractions from sterile nutrient broth (CONDA) resuspended in 10% DMSO, to ensure that the effects were due to metabolic elicitors and not to the DMSO). All were pathogen challenged.

A. thaliana seeds (not previously sterilized) were germinated in quartz sand for one week and then transplanted individually to 12-well plastic plates (5 mL) filled with

3:1 (vol/vol) peat/sand. Each treatment comprised three plates, each plate constituting a replicate. One week after transplanting, treatments were delivered to seedlings by drenching in the soil with 20 μL of elicitors per well. The positive control was inoculated with 1 mL of *P. fluorescens* N 21.4 culture, grown for 24 h in sterile nutrient broth (CONDA); and diluted from 10^9 to 10^8 cfu mL^{-1}, while negative controls were treated 20 μL of the n-hexane, ethyl acetate and n-butanol fractions from sterile nutrient broth (CONDA) resuspended in 10% DMSO.

Three days later, plates were placed in a humidity chamber to ensure stomata opening, and the next day challenged with pathogen *P. syringae* DC3000. The plants were inoculated by placing a 5 μL drop of a of 10^8 cfu mL^{-1} suspension on each leaf (Sumayo et al. 2013). Seventy-two hours after pathogen inoculation, the disease index was determined as the ratio of the number of leaves with disease symptoms to the total number of leaves (Ryu et al. 2004). Results were relativized using the negative controls as a 0% of protection.

2.3 Study of the signal transduction pathway of the most effective fraction

The n-hexane fraction was the most effective against pathogen infection. This was used to study signal transduction pathways based on genes overexpressed in response to this fraction during the systemic resistance assay. The genes involved are detailed below. The experimental set up included two treatments: i) metabolic elicitors in the n-hexane fraction, and ii) n-hexane negative control. Twenty-one plants per treatment were used; plants were arranged on three replicates, with seven plants each.

Seeds were germinated in quartz sand for one week. One-week-old seedlings were transplanted individually to 100 mL pots filled with 3:1 (vol/vol) peat/sand mixture (60 g per pot). Plants were watered with 5 mL of tap water twice a week, and with 5 mL of ½ Hoagland solution per plant once a week. Four-week-old seedlings were treated with 50

μL of n-hexane fraction, and negative control with 50 μL of the n-hexane fraction from sterile nutrient broth resuspended in 10% DMSO. Four days later, plants were pathogen challenged.

One day before the pathogen inoculation, plants were placed in a humidity chamber to ensure the stomatal opening needed for the disease to establish. Pathogen inoculation was carried out by spraying the plants with 150 mL of a suspension of 10^8 cfu mL^{-1}. Seven plants per treatment were collected at 6, 12 and 24 h after pathogen challenge (hapc), powdered in liquid nitrogen and stored at -80 ºC until gene expression analysis by qPCR and enzymatic activities analysis.

The genes analysed were *NPR1* (Nonexpressor of Pathogenesis Related Genes1), *PR1* (Pathogenesis-Related Gene 1), *PR2* and *ICS* (Isochorismate Synthase 1) as markers of the SA signalling pathway (Betsuyaku et al. 2017; Silva et al. 2018; Ding et al. 2018); *PDF1* (Plant Defensin 1), *LOX2* (Lipoxygenase 2), *PR3* and the transcriptional factor *MYC2* as markers of the JA/ET signalling pathway (Caarls et al. 2015).

2.4 RNA extraction and RT-qPCR Analysis

Total RNA was isolated from each replicate with PureLink RNA Micro Kit (Invitrogen), DNAase treatment included. RNA purity was confirmed using Nanodrop™. A reverse transcription was performed followed by a qPCR. Reverse transcription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad). All reverse transcription were carried out using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 ºC, 30 min 42 ºC, 5 min 85 ºC, and hold at 4 ºC. The amplification was realized with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 ºC and then 39 cycles consisting of 15 s at 95 ºC, 30 s at 55 ºC and 30 s at 72 ºC, followed by a melting curve to check the results. To describe the level of expression in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between

90 and 110 %. *Sand* gen (AT2G28390) was used as a reference gen. Primers used appear in Table 3.1.1. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

Table 3.1.1 Forward and reverse primers used in qPCR analysis.

	Forward	Reverse
AtNPR1	5´-TATTGTCAARTCTRATGTAGAT	5´-TATTGTCAARTCTRATGTAGAT
AtPR1	5´-AGTTGTTTGGAGAAAGTCAG	5´-GTTCACATAATTCCCACGA
AtICS	5´-GCAAGAATCATGTTCCTACC	5´AATTATCCTGCTGTTACGAG
AtPDF1	5´-TTGTTCTCTTTGCTGCTTTCGA	5´-TTGGCTTCTCGCACAACTTCT
AtLOX2	5´-ACTTGCTCGTCCGGTAATTGG	5´-GTACGGCCTTGCCTGTGAATG
AtMYC2	5´-GATGAGGAGGTGACGGATACGGAA	5´-CGCTTTACCAGCTAATCCCGCA
AtPR2	5´-TCGTCTCGATTATGCTCTCTTC	5´-GCAGAATACACAGCATCCAAAA
AtPR3	5´-AAATCAACCTAGCAGGCCACT	5´-GAGGGAGAGGAACACCTTGACT
Sand	*5′* -CTGTCTTCTCATCTCTTGTC	*5′* -TCTTGCAATATGGTTCCTG

**At = A. thaliana*

2.5 Pathogenesis-related proteins (PRs) and systemic resistance proteins activities

Enzymatic activities of resistance proteins glucanase (PR2), chitinase (PR3), lipoxygenase, cellulase and polyphenol oxidase were assessed. Before assessing enzymatic activities, soluble proteins were extracted from the plant powder by resuspending 100 mg in 1 mL of potassium phosphate buffer 0.1 M pH 7 containing 2 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was sonicated for 10 min and then centrifuged for 10 min at 10000 *x* g. The supernatant was divided into aliquots, frozen in liquid nitrogen and stored at -80 ºC for further analysis. All above operations were carried out at 0-4 ºC.

To measure the amount of total protein from plant extract, 250 µL of Bradford reagent, 50 µL of sample and BSA dilutions were pipetted into each well of 96-well plates, incubated for 30 min at room temperature and measured using a plate reader (MB-580 Heales) at absorbance of 595 nm. A calibration curve was constructed from commercial BSA dilutions expressed in mg mL^{-1}. The units of protein were expressed as mg mL^{-1}.

Glucanase (EC 3.2.1.6), cellulase (EC 3.2.1.4) and chitinase **(**EC 3.2.1.14**)** activities were measured as described by Lee et al. 2008. Calibration curves were made with glucose (for glucanase and cellulase) and N acetyl glucosamine (for chitinase) in acetate buffer with concentrations between 0.1 and 1 mg mL^{-1} for glucanase and cellulase, and between 0.01 and 0.1 mg mL^{-1} for chitinase. Data were expressed as µmol mg $protein^{-1}$ min^{-1}.

Lipoxygenase (E.C. 1.13.11.) activity was measured as described by Ali et al. 2005. Extinction coefficient of 25 mM^{-1} cm^{-1} was used to calculate activity. Data was expressed as µmol mg $protein^{-1}$ min^{-1}.

Polyphenol oxidase (1.14.18.1) activity was measured as described by Nawrocka et al. 2017. Extinction coefficient of 2.72 mM^{-1} cm^{-1} was used to calculate activity. Data was expressed as µmol mg $protein^{-1}$ min^{-1}.

In all assays, the blank consisted on the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer.

2.6 Enzymatic activities related to oxidative stress

Enzymatic activity of ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1), glutathione reductase (GR, EC 1.6.4.2), guaiacol peroxidase (GPX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) were measured spectrophotometrically and expressed as µmol mg $protein^{-1}$ min^{-1}.

APX was measured by the method of Garcia-Limones et al. 2002. Oxidation of ascorbate was determined by the decrease in A_{290}. An extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity.

SOD activity was determined following the specifications of the SOD activity detection kit (SOD Assay Kit-WST, Sigma-Aldrich). With this method, the rate of the reduction with O_2 is linearly related to xanthine oxidase (XO) activity and inhibited by SOD. Inhibition activity of SOD was determined colorimetrically and expressed as % inhibition mg protein^{-1}.

GR was measured by the method of Garcia-Limones et al. 2002. Oxidation of NADPH was determined by the increase in A_{340}. Extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity.

GPX was measured by the method of Garcia-Limones et al. 2002. Oxidation of guaiacol was determined by the increase in A_{470} using an extinction coefficient of 26.6 mM^{-1} cm^{-1} to calculate activity.

CAT was measured by the method of Garcia-Limones et al. 2002. The decrease in A_{240} produced by H_2O_2 breakdown was recorded and an extinction coefficient of 36 mM^{-1} cm^{-1} used to calculate activity.

MDHAR activity was measured by the method of Xu et al. 2008. Reduction of monodehydroascorbate was determined by the decrease in A_{340} using an extinction coefficient of 6.22 mM^{-1} cm^{-1} to calculate activity.

DHAR activity was measured as described by Xu et al. 2008 at 265 nm. Reduction of dehrydroascorbate was determined by the decrease in A_{265} using an extinction coefficient of 14 mM^{-1} cm^{-1} to calculate activity.

In all assays, the blank consisted of the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer. In the case of the GR assay, an additional blank without oxidized glutathione was included to account for the presence in the extracts of other enzyme activities able to oxidize NADPH.

2.7 Oxidative status of the plant: Malondialdehyde (MDA) concentration

The MDA content was determined by the method of Hu et al. (2016) with modifications. Briefly, 0.25 g of powder was mixed with 2 mL of reaction solution containing 0.5% (v/v) thiobarbituric acid (TBA) and 20 % (v/v) trichloroacetic acid (TCA). The mixture was heated at 95 °C for 30 min, then quickly cooled to room temperature, treated to eliminate air bubbles and centrifuged at 6000 *x g* for 20 min. Then, absorbance of the supernatant was determined by a spectrophotometer at 532 and 600 nm. The MDA content was calculated using the formula: MDA (nmol FW^{-1}) = [(OD532-OD600)]/(ε.FW), where FW is the fresh weight in grams and ε the extinction coefficient (155 $mM^{-1}cm^{-1}$).

2.8 Statistical analysis

One-way ANOVA was used to check the statistical differences in all data obtained in the experiments carried out. Prior to ANOVA, analysis of homoscedasticity and normality of the variance were checked with Statgraphics plus 5.1 for Windows and found to meet the requirements for analysis. When significant differences appeared ($p < 0.05$) a Fisher test was used (Sokal and Rohlf, 1980).

3. Results

3.1 Capacity of the three metabolic elicitor fractions to trigger systemic resistance

Each of the organic fractions from culture medium of *P. fluorescens* N 21.4 and the N 21.4 strain itself were able to trigger defence mechanisms in *Arabidopsis* seedlings and to improve their capacity to resist the pathogenic effects of *P. syringae* (DC 3000) (Fig. 3.1.1). The n-hexane fraction gave the highest protection percentage (91%) and was chosen to study the signal transduction pathway involved in protection by evaluating the differential gene expression (Fold Change) of selected marker genes. Negative controls

treated with the n-hexane, ethyl acetate and n-butanol fractions from sterile nutrient broth and resuspended in 10% DMSO had no protective effect.

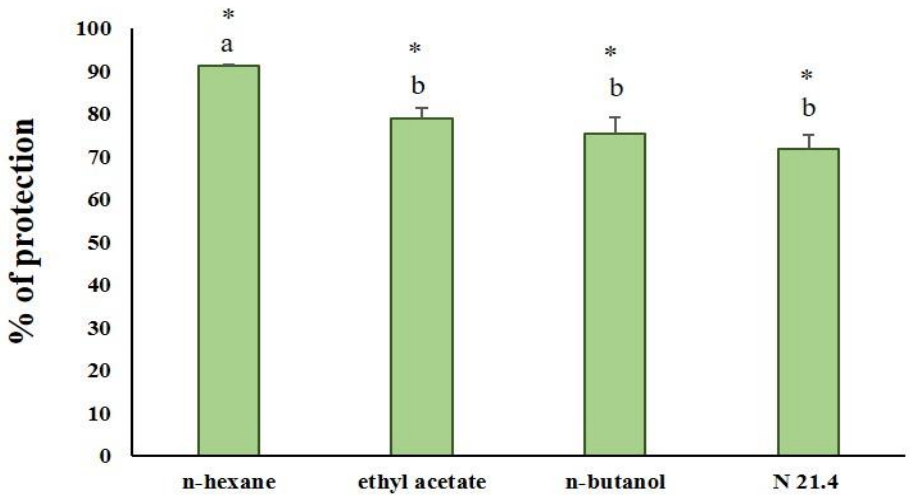

Figure 3.1.1 Percentage of protection against the pathogen *P. syringae* DC 3000 in *A. thaliana* seedlings inoculated with n-hexane, ethyl acetate and n-butanol fractions extracted from *P. fluorescens* N 21.4. Percentage of protection was based on the number of leaves with disease symptoms compared to the total of leaves (n=12 seedlings). Data were relativized to negative control (mean of the three negative controls), which was considered as 0% protection. Asterisks represent statistically significant differences ($p < 0.05$) with regard to negative control. Letters represent statistically significant differences between the four different treatments.

3.2 Study of the signal transduction pathway of the most effective fraction

Effects of the n-hexane fraction on the marker genes of the SA and JA/ET pathway are shown in figure 3.1.2. Figure 3.1.2 A) shows the SA pathway marker genes. Six hours after pathogen challenge (6 hapc) only *ICS* showed significantly higher expression (2.04), decreasing to zero values at 12 and 24 hapc. *PR1* and *PR2* showed maximum differential expression at 12 hapc (11.7), although *PR2* expression was ten times lower than that for *PR1* (1.51). None of the genes showed differential expression at 24 hapc.

Figure 3.1.2 B) shows the JA/ET pathway marker genes. Only *PDF1* (63.2) and *LOX2* (1.71) showed significant differences in gene expression. These were evident at 12 hapc, with *PDF1* values being 60 times higher than that of *LOX2*. There was no differential gene expression at 6 and 24 hours hapc. Negative control treated with 50 µL of n-hexane fraction from sterile nutrient broth) had no effect on differential expression.

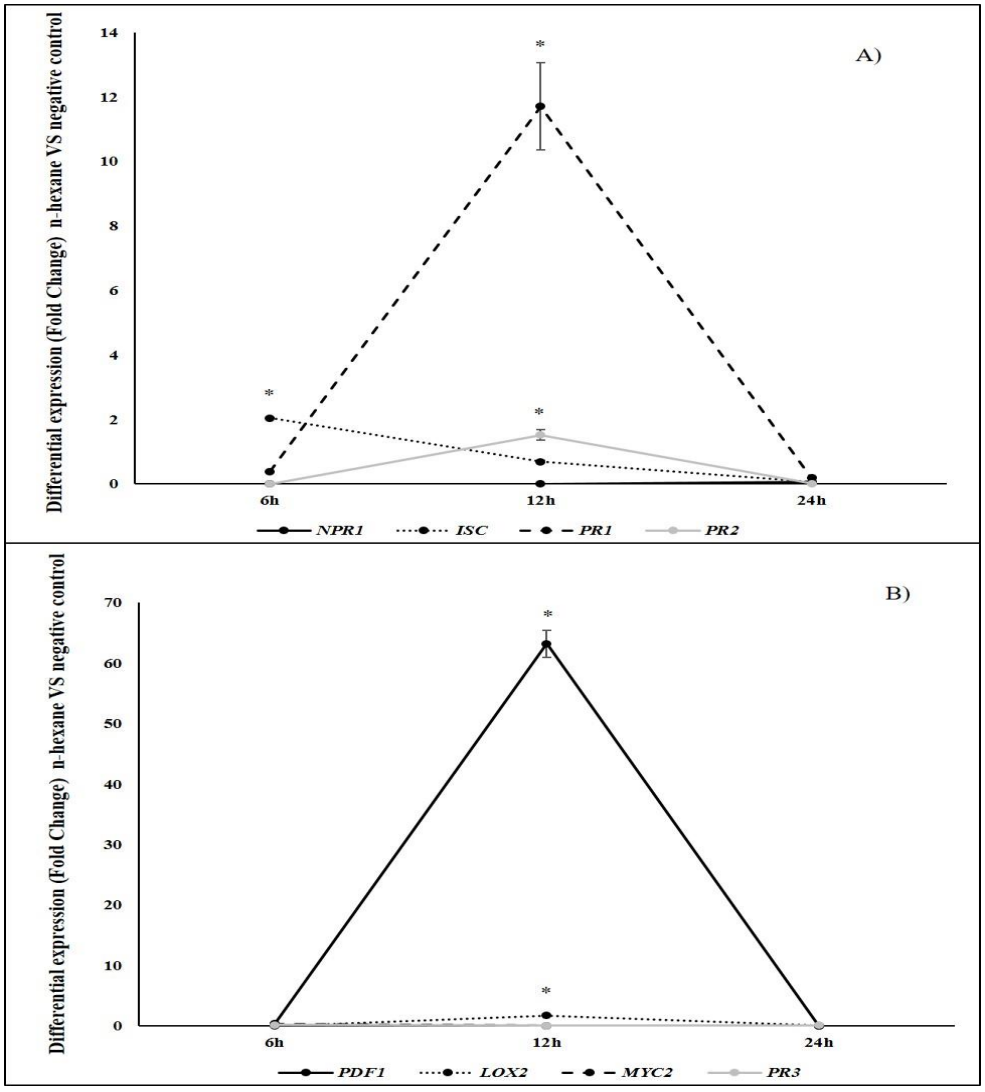

Figure 3.1.2 Differential expression (Fold Change) of SA and JA/ET pathway marker genes of *A. thaliana* seedlings treated with the n-hexane extract from *P. fluorescens* N 21.4. Results compare treatments and the negative control at 6, 12 and 24 hapc; A) *NPR1, ICS, PR1* and *PR2* genes (as SA signalling markers), B) *PDF1, LOX2, MYC2* and *PR3* (as JA/ET signalling markers). Asterisks represent statistically significant differences ($p < 0.05$) within each sampling time (6, 12 and 24 hapc; n=7).

3.3 Pathogenesis-related proteins and systemic resistance proteins activities

Activity of the pathogenesis-related proteins (PRs), glucanase (PR2) and chitinase (PR3) and the proteins related to systemic resistance against pathogens, lipoxygenase, cellulase and polyphenol oxidase were evaluated (Fig. 3.1.3). The n-hexane fraction promoted the activity of all these enzymes. The increases were statistically significant at most sampling times for chitinase (PR3; Fig. 3.1.3 A)) glucanase (PR2; Fig. 3.1.3 B)) and lipoxygenase (Fig. 3.1.3 C)). Cellulase (Fig. 3.1.3 D)) showed significant differences at 6 and 12 hapc, and polyphenol oxidase (Fig. 3.1.3 E)) at 24 hapc.

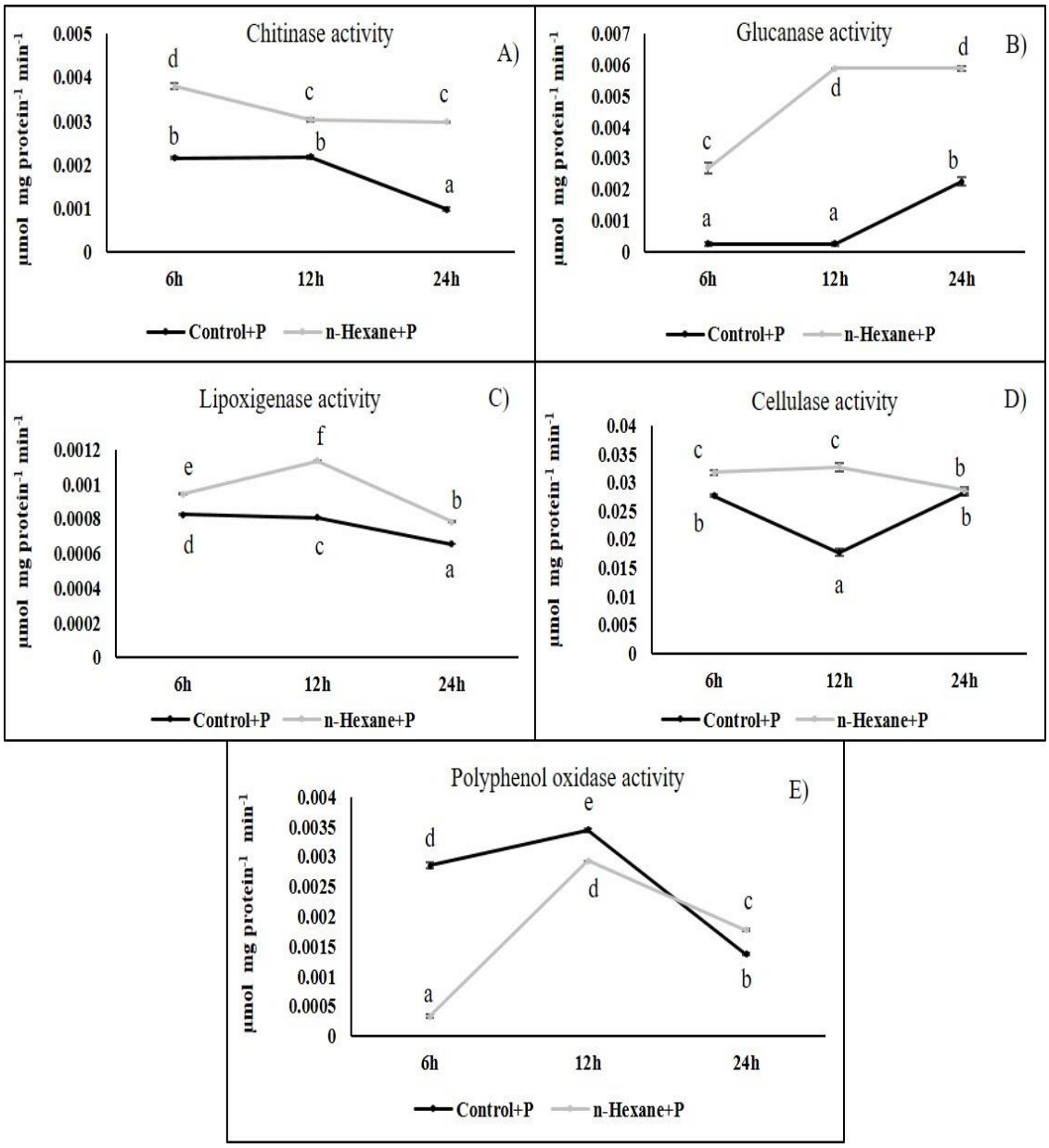

Figure 3.1.3 Pathogenesis-related proteins: A) chitinase (PR3); B) glucanase (PR2), and induced systemic resistance proteins activities: C) lipoxygenase; D) cellulase and E) polyphenol oxidase in plants of *A. thaliana* only inoculated with the pathogenic *P. syringae* DC3000 (Control+P) and in plants also treated with the elicitor fraction extracted with n-hexane (n-hexane+P) from the culture medium of *P. fluorescens* N 21.4. Activities were measured at 6, 12 and 24 hapc. Different letters indicate significant differences ($p < 0.05$) between treatments in each sampling time.

3.4 Enzymatic activities related to oxidative stress

Except for gluthathione reductase activity (GR; Fig. 3.1.4 E)), n-hexane fraction elicitors increased activity levels compared to controls. The differences were statistically significant at all three sampling times for ascorbate peroxidase (APX; Fig. 3.1.4 A)), guaiacol peroxidase (GPX; Fig. 3.1.4 D)) and monodehydroascorbate reductase (MDHAR; Fig. 3.1.4 F)). Increases in catalase (Fig. 3.1.4 E)) were significant at 12 and 24 hapc. Superoxide dismutase activity (SOD; Fig. 3.1.4 B)) was promoted at 24 hapc and dehydroascorbate reductase (DHAR; Fig. 3.1.4 G)) at 6 and 12 hapc.

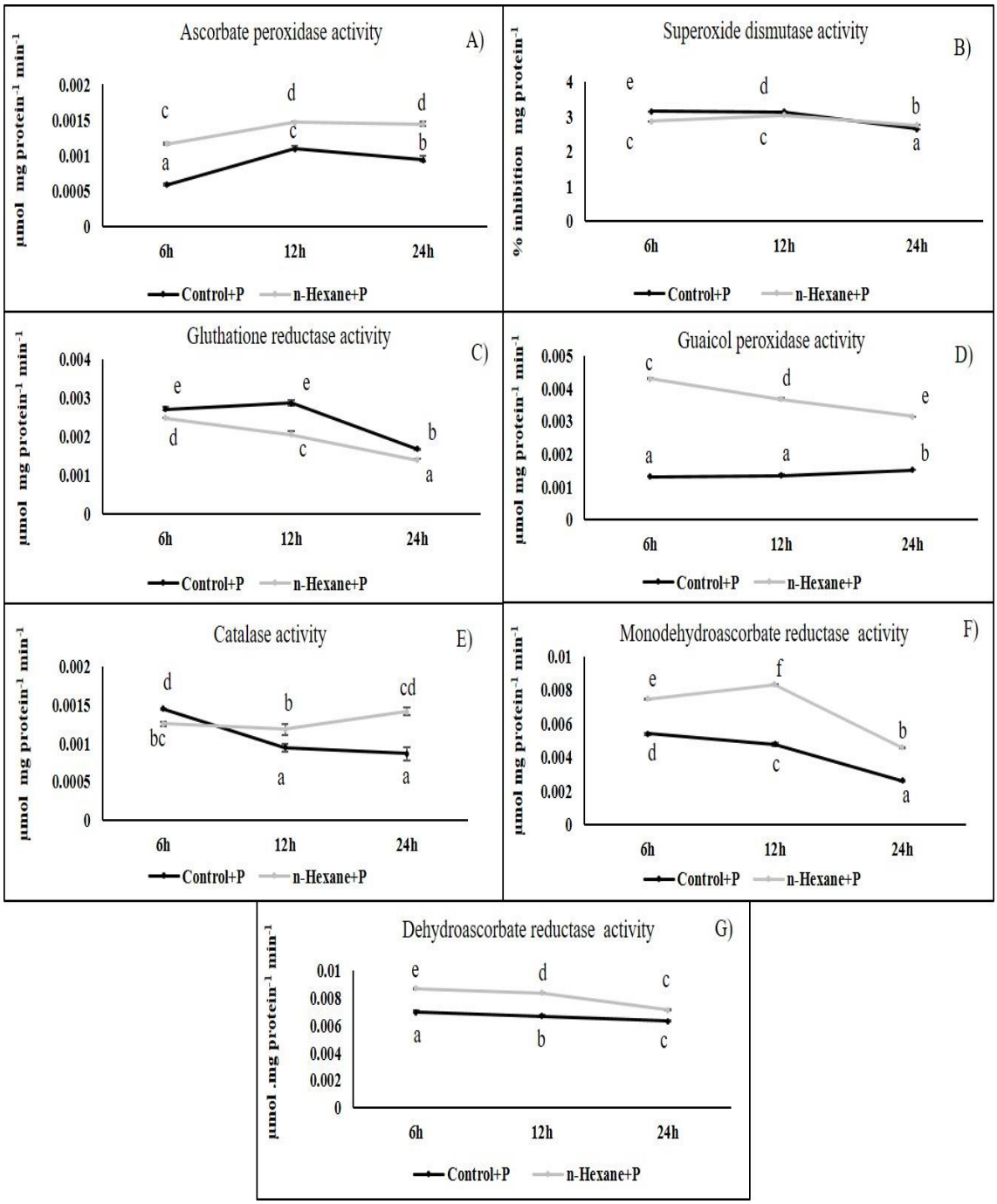

Figure 3.1.4 Enzymatic activities related to oxidative stress in *A. thaliana* seedlings inoculated with the pathogenic *P. syringae* DC3000 (Control+P) and treated with the elicitor fraction extracted with n-hexane (n-hexane+P) from the culture medium of *P. fluorescens* N 21.4. Enzymatic assays were made at 6, 12 and 24 hapc. A) ascorbate peroxidase (APX); B) superoxide dismutase (SOD); C) gluthatione reductase (GR); D) guaiacol peroxidase (GPX); E) catalase; F) monodehydroascorbate reductase (MDHAR); G) dehydroascorbate reductase (DHAR). Different letters indicate significant differences ($p < 0.05$) between treatments in each sampling time.

3.5 Oxidative status of the plant: Malondialdehyde concentration

Malondialdehyde (MDA), a marker of oxidative stress was measured at 12 hapc (Fig. 3.1.5). At this time, MDA concentrations were markedly depressed by n-hexane extract.

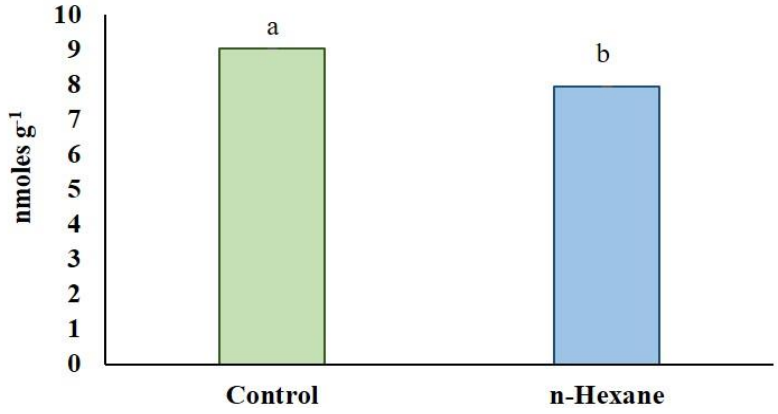

Figure 3.1.5 Malondialdehyde concentration in *A. thaliana* plants inoculated with the pathogenic *P. syringae* DC3000 and after treatment with n-hexane extracts of *P. fluorescens* N 21.4 compared to control. Different letters indicate significant differences ($p < 0.05$) between treatments.

4. Discussion

The capacity of Plant Growth Promoting Rhizobacteria (PGPR) to enhance plant defence against biotic and abiotic stress has been demonstrated many times in the past (e.g., Garcia-Cristobal et al. 2015; Beris et al. 2018; Kumar et al. 2018). However, effects of elicitors produced by these PGPR have been less studied. These substances have been reported to be either structural molecules, such as flagellin (Ramirez-Prado et al. 2018), or metabolic elicitors released to the medium (Munhoz et al. 2017; Wu et al. 2018).

The ability of the PGPR *Pseudomonas fluorescens* N 21.4 to trigger plant metabolism in different species has been described in numerous previous studies (Domenech et al. 2007; Ramos-Solano et al. 2010a; Ramos-Solano et al. 2010b; Algar et al. 2012; Ramos-Solano et al. 2015), and certain bacterial metabolic elicitors have been

classified by their molecular weight (Algar et al. 2012).The present study explores further the complex mixture of elicitors produced by *P. fluorescens* N 21.4 based on solubility in three organic solvents.

The effectiveness of all three fractions to protect plants (Fig. 3.1.1) reveals the existence of several metabolic elicitors with contrasting solvent solubilities involved in plant protection. There may also be different pattern recognition receptors (PRRs) in plants for these elicitors.

Induced systemic resistance (ISR) holds potential for activating cellular defence responses prior to pathogen attack (Akram et al. 2016). It is well known that, among others, ISR is accompanied with an augmented expression of defence related genes, increased accumulation of secondary metabolites and defence associated proteins (Conrath, 2006; Zamioudis and Pieterse, 2012). Moreover, the rapid generation of reactive oxygen species is a common protective response of plants to pathogen attack (Noctor et al. 2014; Xia et al. 2015) and therefore represents the node from which many signalling events are generated.

ISR typically relies on JA/ET signalling pathways (Pieterse et al. 2002). However, our results, at the level of gene expression and activity of proteins related to plant defence systems, (Figs. 3.1.2 and 3.1.3) indicate that elicitors from the n-hexane fraction induce the JA/ET pathway and also the SA pathway by increasing at the same time expression of marker genes *PR1* and *PDF1* (SA and JA/ET marker genes respectively; Caarls et al. 2015; Ding et al. 2018) and enzyme activities such as PR2 (marker of SA signalling pathway) and PR3, LOX, and PPO, as markers of JA/ET signalling pathway (Lucas et al. 2014; García-Cristobal et al. 2015; Silva et al. 2018; Wu et al. 2018).

This implies a versatility in the resistance mechanism, allowing attack of biotrophic and necrotrophic organisms to be opposed. These two pathways are not

necessarily antagonistic, as previously been indicated by other results (Nie et al. 2017; Betsuyaku et al. 2017).

The physiological state induced by elicitors is known as priming. It is marked by an enhanced activation of defence mechanisms readily demonstrated in pathogen challenge experiments (Maunch-Mani et al. 2017). The induction of defensive mechanisms must necessarily be mediated by elicitor detection that activates an immune response. This has been termed microbe-associated molecular pattern (MAMP) - triggered immunity (MTI). It relies on the detection of conserved microbial signature molecules (MAMPs) *via* extracellular transmembrane receptors or PRRs (Mhlongo et al. 2018). Our results add to the picture by demonstrating a stimulation of all the ascorbate-glutathione-cycle enzyme (except, gluthatione reductase) of plants treated with n-hexane extracts from *P. fluorescens* N 21.4, notably for ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and monodehydroascorbate reductase (MDHAR) (Fig. 3.1.4). These enzymes have well-established roles in stress responses (Song et al. 2009; Sultana et al. 2012; Garcia-Cristobal et al. 2015; Souza et al. 2016; Maruta and Ishikawa, 2018; Liu et al. 2018).

The results obtained with respect to the enzymatic activities related to free radical scavenging, accord with the suppressed levels of MDA (Fig. 3.1.5), a marker of oxidative damage (Lucas et al. 2017). These results are consistent with the higher protection and with the higher activity of the ROS scavenging enzymes above reported.

There are few studies that relate oxidative stress enzymes to innate immunity in plants elicited with PGPR or metabolic elicitors (Lucas et al. 2014; Garcia-Cristobal et al. 2015). However, this type of relationship helps to stablish a complete set of changes associated to plant protection. Markers related to oxidative stress metabolism will assist in improving primer fingerprinting for each bacterial strain (Maunch-Mani et al. 2017;

Gutierrez Albanchez et al. 2018). This will improve further analysis and also our understanding of the mechanisms that defend plants against pathogens. In addition, new sets of products based on metabolic elicitors or PGPR with an ability to elicit defence mechanisms against a range of stresses can be expected to be useful in practical agriculture.

5. Conclusions

The three fractions extracted from the medium in which *P. fluorescens* N 21.4 was cultured, using three different solvents, protected *A. thaliana* against the pathogen *P. syringae* DC3000, highlighting the n-hexane fraction. Elicitors in the n-hexane fraction gave higher protection than those of ethyl acetate and n-butanol fractions. The mode of action of the elicitors in the n-hexane fraction included activating the SA and JA/ET signalling pathways and the enzymatic machinery of ROS scavenging to decrease oxidative stress. Further studies are needed to chemically identify the elicitors excreted by *P. fluorescens* N 21.4. Once this is achieved, their use as biotechnological inoculants to improve the plant resistance to stress is a promising possibility.

Acknowledgements

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3.2 Improving flavonoid metabolism in blackberry leaves and plant fitness by using the bioeffector *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors: a biotechnological approach for a more sustainable crop

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Biotechnology and Biological Transformations

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Helena Martin-Rivilla, Ana García-Villaraco, Beatriz Ramos-Solano, Francisco Javier Gutierrez-Mañero, and Jose Antonio Lucas

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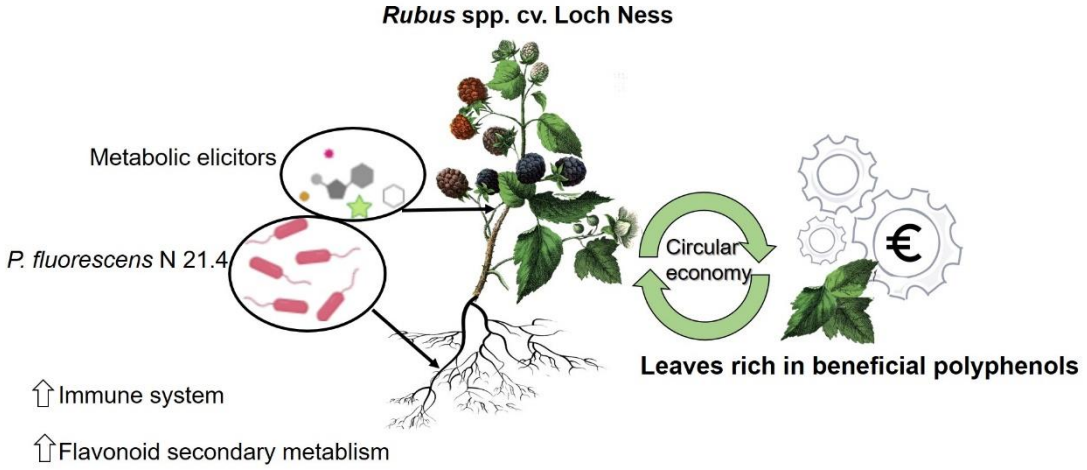

Improving flavonoid metabolism in blackberry leaves and plant fitness by using the bioeffector *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors: a biotechnological approach for a more sustainable crop

Martin-Rivilla, H*., Garcia-Villaraco, A., Ramos-Solano, B., Gutierrez-Mañero, F.J., Lucas, J.A.

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

*Corresponding author: Helena Martín Rivilla, helenamartin92@gmail.com

+913 72 47 85

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Abstract

Beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors inoculated to cultivars of blackberry (*Rubus* cv. Loch Ness) reinforced plant immune system and improved their fitness by increasing photosynthesis, decreasing oxidative stress and activating pathogenesis-related proteins. They also triggered leaves flavonoid metabolism enhancing the accumulation of beneficial phenolic compounds such as kaempferols and quercetin derivatives. The elicitation of leaves secondary metabolism allows taking advantage of the blackberry leaves (a current crop waste), following the premises of the circular economy, for the isolation and obtaining of high added value compounds. The results of this work suggest the use of *P. fluorescens* N 21.4 and/or its metabolic elicitors as plant inoculants as effective and economically and environmentally friendly agronomic alternative practices in the exploitation of blackberry crop for getting plants with a better immune system and for revalorizing the leaves pruning as a potential source of polyphenols.

Key words: *Pseudomonas fluorescens* N 21.4, metabolic elicitors, blackberry leaves, plant fitness, flavonoid metabolism, circular economy, kaempferol.

1. Introduction

Intensive blackberry cultivation is a relatively recent emerging crop for which there is a deficit of specific inoculants that improve plant resistance to pests and to abiotic stress. A widely accepted approach to create effective inoculants substituting chemical products is the use of beneficial rhizobacteria (bioeffectors) and/or structural or metabolic elicitors (Gozzo and Faoro, 2013) extracted from them able to trigger secondary defence metabolism (Ramos-Solano et al. 2008b). This process, in which defensive capacity of the entire plant increases after local exposure to bioeffectors or elicitors, is called Induced Systemic Resistance (ISR) (Pieterse et al. 2014) and can cause the *priming* (Conrath et al. 2001), a physiological state in which plants are able to activate faster and/or stronger their defences when subsequently attacked by pathogens (Conrath et al. 2006). After bioeffectors or elicitors are sensed, Salicylic acid (SA) and/or Jasmonic/Ethylene (JA/ET)) signalling pathways are activated to trigger plant resistance (Wu et al. 2028), since there is evidence that bioeffectors or elicitors can trigger either one pathway or both (Domenech et al. 2007). This improvement in plants immune system also include activation of Reactive Oxygen Species (ROS) scavenging cycle and an increase in the synthesis of antioxidant secondary metabolites, which means an enhancement of plant fitness (Garcia-Cristobal et al. 2015).

Among secondary metabolites with greater antioxidant capacity are flavonoids (Agati et al. 2012). They play an essential role in plant defence against biotic and abiotic stress (Agati and Tattini, 2010; Schulz et al. 2015) and also act as visual attractors, repellents, phytoalexins, antioxidants, and antimicrobials (Iwashina et al. 2003; Jeandet et al. 2013; Srivastava and Bezwanda, 2015). Furthermore, many flavonoid beneficial effects on human health have been reported (Mortas and Sanlier, 2017). Therefore, the engineering of the flavonoid biosynthesis pathways for the purposeful accumulation and

isolation of active molecules has been extensively used in the plant biotechnological industry (Tanaka et al. 2008).

Blackberries are one of the richest fruits in flavonoids and this is why they bring many benefits when they are included in the diet (Gutierrez et al. 2017a). Moreover, some analytical studies have shown that blackberry leaf phenolic composition is analogous to that of the fruits or even richer and higher (Ferlemi et al. 2016) and that leaves extracts also show *in vivo*/*in vitro* beneficial effects on human health, as they have antioxidant, anti-diabetic (Oszmiański et al. 2015), anti-microbial (Zia-Ul-Haq et al. 2014), analgesic, anti-inflammatory and angiogenic activity (Mukherjee et al. 1984). This suggests that leaves are a valuable source of bioactive natural compounds.

As these beneficial phenolic compounds of blackberry leaves belong to the plant secondary metabolism, which is easily inducible, their elicitation could be carried out using inoculants made of bioeffectors and/or derived elicitors. In this way, the biosynthesis and accumulation of high added value phenolic compounds could be increased in a sustainable way.

To develop biological inoculants that improve plant immune system through the elicitation of secondary defensive metabolism, effective bioeffectors used should be able to trigger a broad spectrum of plant agronomic species. The bioeffector used in the present work, *Pseudomonas fluorescens* N 21.4, has the ability to trigger ISR in *A. thaliana* (Martin-Rivilla et al. 2019), *Glycine max* (Ramos-Solano et al. 2010b), *Hypericum sp.* (Gutierrez-Mañero et al. 2012), *Papaver sp.* (Bonilla et al. 2014) and blackberry (Garcia-Seco et al. 2015a). This is why, we elicited blackberry plants with N 21.4 and with the metabolic elicitors (ME) of it, since we have previously shown that N 21.4 ME mimic bacterial effect to trigger the immune system of *A. thaliana* (Martin-Rivilla et al. 2019). Triggering secondary metabolism and improving blackberry plant immune system by

bioeffectors and/or ME would be therefore an effective and more ecofriendly approach to protect plants against pathogens and abiotic stress preventing economic loses and to improve crop yield security and human nutrition.

Hence, the aim of this study was to evaluate the ability of *P. fluorescens* N 21.4 and its ME to elicit the secondary defensive metabolism of blackberry plants (*Rubus* cv. Loch Ness), boosting their general fitness and achieving, at the end of the cultivation cycle, plants with leaves richer in valuable polyphenols. In this way, the pruning of leaves, which is a waste, could be revalorized and an added value would be given to the crop. Therefore, circular economy premises would be fulfilled by proposing blackberry leaves as the starting material to obtain usable compounds (Barreira et al. 2019).

To achieve our objectives, we evaluated different markers of plant fitness such as photosynthesis parameters, ROS scavenging enzymes, pathogenesis-related proteins and expression of marker genes of ISR pathways mediated by SA and JA/ET. Blackberry leaves polyphenol composition was also studied. All these analyses were conducted in two sampling moments, corresponding to two physiological plant stages: flowering (January) and fruiting (April).

2. Material and methods

2.1 Blackberry experimental design

The *Rubus* cv. Loch Ness plants used in this study were kindly provided by Agricola El Bosque S.L. “La Canastita” (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were handled according to regular agricultural practices (Ramos-Solano et al. 2014). Plants were grown in Huelva (South Eastern Spain) from November 2017 to April 2018 under “winter cycle.” Before being transplanted to greenhouses in November, plants underwent an artificial cold period in order to start their regular cycle. A total of 540

plants were in the trial, arranged in five greenhouses; each greenhouse had two lines with 120 plants in total, each line being one replicate with 60 plants; 3 lines were inoculated with live N 21.4 at root level, 3 lines were inoculated with the metabolic elicitors (ME) of N 21.4 by aerial spraying, and 3 lines were left as non-inoculated controls. N 21.4 and ME were inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum per plant (Fig. 3.2.1).

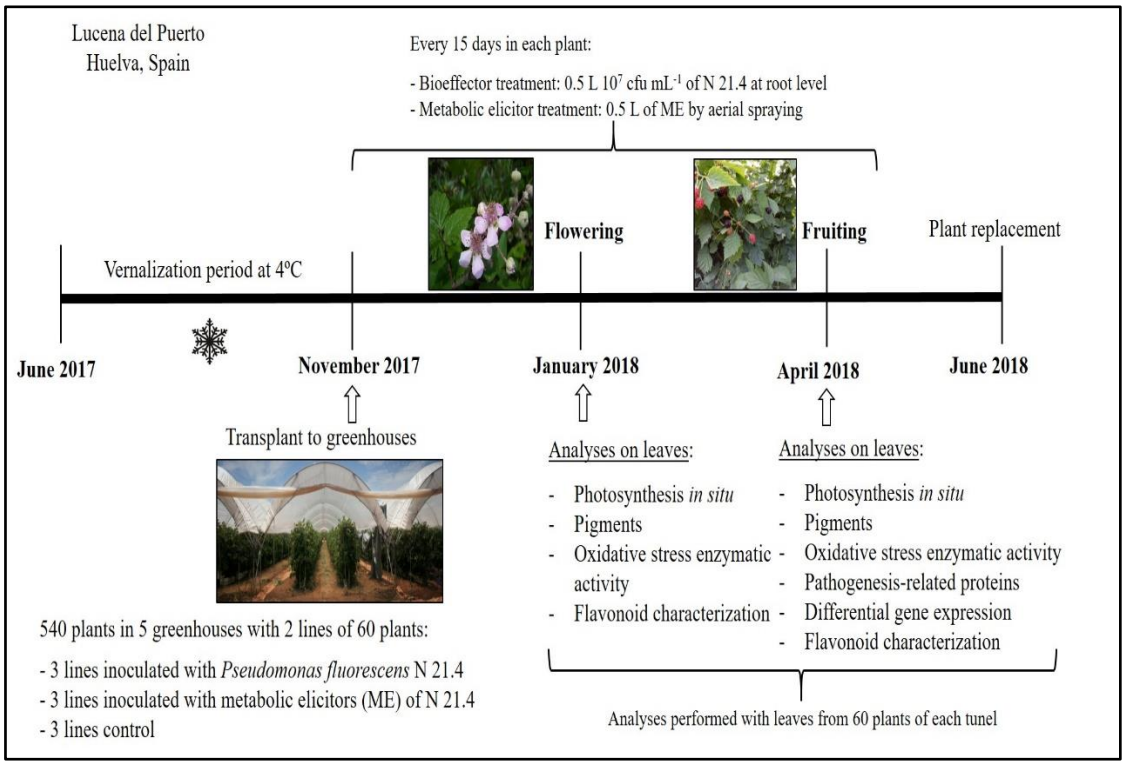

Figure 3.2.1 Representative timeline of the entire blackberry cultivation cycle in the greenhouses of Lucena del Puerto (Huelva, Spain).

2.2 Bacterial treatments

The bacterial strain used in this work was *Pseudomonas fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a Gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca* Graham in three different soils and physiological moments of the plant (Ramos-Solano et al. 2010a). It is able to release siderophores and chitinases *in vitro* and it triggers defensive metabolism in *Solanum*

lycopersicum (Ramos-Solano et al. 2010a), *A. thaliana* (Martin-Rivilla et al. 2019), *Glycine max* (Ramos-Solano et al. 2010b), *Hypericum* sp. (Gutierrez-Mañero et al. 2012) and *Papaver* sp. (Bonilla et al. 2014). It also increases fruit production in *Rubus* sp. (Ramos-Solano et al. 2014) and improves fruit quality of *Rubus* sp. by modifying flavonoid metabolism (Garcia-Seco et al. 2015b).

Bacterial strain was stored at -80 ºC in nutrient broth with 20% glycerol. Inocula were prepared by streaking strains from -80 ºC onto PCA (CONDA) plates, incubating them at 28°C for 24 h. After that, bacterial cells were scraped off the plates into sterile nutrient broth (CONDA) incubated on a rotatory shaker at 28 ºC and 180 rpm for 24 h to obtain a 10^9 cfu mL^{-1} inoculum. Inocula of N 21.4 were diluted from 10^9 to 10^7 cfu mL^{-1} and were delivered at root level to the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

ME treatment was prepared by centrifuging one liter of a 24 h culture of N 21.4, grown at 28 ºC on an orbital shaker, at 2890 *x* g during 20 min at 4 ºC. Cells were discarded and the liter of supernatant was diluted following the same proportion as the strain (from 10^9 to 10^7 cfu mL^{-1}) and it was sprayed onto the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

2.3 Photosynthesis analysis by fluorescence measurement *in situ*

As chlorophyll fluorescence analysis is a very common method to study stress effect on the photosynthetic performance, F0 and Fv/Fm, two sensitive indicators related to chlorophyll fluorescence and plant photosynthetic functioning, were measured with a pulse amplitude modulated fluorometer (Hansatech FM2, Hansatech, Inc, UK).

To determine Fv/Fm ratio (the key parameter to detect PSII photoinhibition induced by a stress (Roháček et al. 2002)), after 30 min of leaf-dark-adaptation, a weak modulated irradiation (1 μmol m^2 s^{-1}) was applied to firstly determine minimal

fluorescence yield (F0), and a 0.7 s saturating flash (10,000 μmol m^2 s^{-1}) was then superimposed to induce the maximum fluorescence yield of chlorophyll (Fm). The variable fluorescence (Fv) was calculated as the difference between the maximum fluorescence (Fm) and the minimum fluorescence (F0). The maximum photosynthetic efficiency of photosystem II was therefore calculated as Fv/Fm (Ögren and Baker, 1985).

All measurements were carried out on three plants of each treatment in each tunnel and at both sampling moments (flowering and fruiting).

2.4 Total chlorophyll and carotenoids

Total chlorophyll a, b and carotenoids (xanthophylls and carotenoids) were quantitatively determined using the formulas proposed by Lichtenthaler, 1987:

Chl a (mg g^{-1} Fresh Weigh) = [(12.24*Abs 663 nm)-(2.79*Abs 647)]*(V (mL)/FW(g))

Chl b (mg g^{-1} Fresh Weigh) = [(21.5*Abs 647 nm)-(5.1*Abs 663nm)]* (V (mL)/FW(g))

Carotenoids (mg g^{-1} Fresh Weigh) = [(1000*Abs 470nm)-(1.82*Chl a)-(85.02*Chl b)/198)]*(V (mL)/FW(g))

For this, five mg of the leaves powder were mixed with 2 mL of 80% acetone. After 24 hours at 4 ºC, the mixture was centrifuged for 10 min at 1445 *x* g at room temperature. The supernatant was collected and absorbance was measured at 663, 647 and 470 nm using a spectrophotometer Biomate 5. All measurements were carried out at flowering and fruiting.

2.5 Activity of enzymes related to oxidative stress

To measure the specific enzymatic activity of some enzymes related to oxidative stress, three steps were followed. First, a protein extraction of each sample was carried out, then the total protein of each extract was measured, and finally the specific enzymatic activity of superoxide dismutase (SOD (EC 1.15.1.1)), ascorbate peroxidase (APX (EC

1.11.1.11)) and glutathione reductase (GR (EC 1.6.4.2)) referenced to the amount of total protein was calculated.

Firstly, to prepare enzymatic extracts, 10 mg of powdered leaves of each sample were mixed with 1 mL of extraction buffer (cold phosphate buffer 0.1M pH 7.0 with 2 mM of PMSF (phenylmethylsulfonyl fluoride), sonicated for 10 min and centrifuged 20 min at 10000 *x* g at 4 ºC. The remaining supernatant was frozen at -20 ºC and used as the enzymatic extract.

After that, it was measured the amount of total protein of each enzymatic extract by mixing 250 µL of Bradford reagent, 50 µL of enzymatic extract and bovine serum albumin (BSA) dilutions from 0.05 to 2 mg mL^{-1}. This was inoculated in ELISA 96 well plates, incubated for 30 min at room temperature and measured using a plate reader at absorbance of 595 nm. A calibration curve was constructed from commercial BSA dilutions. The units of protein were expressed as mg mL^{-1}.

Finally, the specific enzymatic activity of SOD, APX, and GR enzymes related to oxidative stress were assessed spectrophotometrically in all the leaves extracts:

SOD activity was determined following the specifications of the SOD activity detection kit (SOD Assay Kit-WST, Sigma-Aldrich). With this method, the rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity and inhibited by SOD present in leaves extracts. For this, 200 µL of the Working Solution of the kit was mixed with 20 µL of enzymatic extract and with 20 µL of the enzyme working solution included in the kit. Inhibition activity of SOD was determined colorimetrically using a plate reader at absorbance of 450 nm. SOD enzymatic specific activity was calculated in relation to the amount of total protein, previously measured. The unit used for SOD activity was % inhibition mg $protein^{-1}$.

APX was measured by the method of Garcia-Limones et al. 2002. The reaction mixture consisted of 860 µL of potassium phosphate buffer 50 mM, pH 7.0, 120 µL of sodium ascorbate 2.55 mM, 120 µL of H_2O_2 50 mM and 100 µL of enzymatic extract. Adding H_2O_2 started the reaction, and after 15 s, the oxidation of ascorbate was determined by the decrease in A290. Extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity (µmol mg $protein^{-1}$ min^{-1}). APX specific activity was calculated in relation to the amount of total protein previously measured.

GR was measured by the method of Garcia-Limones et al. 2002. The assay mixture consisted of 740 µL of potassium phosphate buffer 0.2 M, pH 7.5, 120 µL of 10 mM DTNB (Ellman´s reagent), 120 µL of oxidized glutathione 10 mM, 120 µL of NADPH, 1 mM and 100 µL of enzymatic extract. Adding enzymatic extract started the reaction, and after one minute, oxidation of NADPH compound was determined by the increase in A412. Extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity (µmol mg $protein^{-1}$ min^{-1}). GR enzymatic specific activity was calculated in relation to the amount of total protein previously measured.

All measurements were carried out at both sampling moments (flowering and fruiting).

2.6 Pathogenesis-related proteins (PRs)

Glucanase (PR2) (EC 3.2.1.6) activity was measured as described by Lee et al. 2008. The reaction consists of 375 µL of sodium acetate buffer 50 mM pH 5, 25 µL of laminarina 10 mg ml^{-1} and 100 µL of leaves enzymatic extract. After an hour at 37 ºC, 1.5 mL of DNS reagent were added. The mixture was heated at 100 ºC during 5 min and finally, 550 nm absorbance was measured. A calibration curve was made with glucose in acetate buffer with concentrations between 0.1-1 mg mL^{-1}. Data was expressed as µmol mg $protein^{-1}$ min^{-1}.

This enzymatic activity was measured with the samples collected at fruiting (April 2018).

Chitinase (PR3) (EC 3.2.1.14) activity was measured as described by Lee et al. 2008. The reaction consists of 500 µL of sodium acetate 0.1 M pH 5.5, 1 % colloidal chitin in the buffer and 500 µL of enzymatic extract. After two hours of incubation at 37 ºC, 200 µL of 1 N NaOH were added and it was centrifuged at 10000 *x* g for 10 min. The supernatant was mixed with 1 mL of Schales reagent. The mixtue was heated at 100 ºC for 15 min and the absorbance at 420 nm was measured. A calibration curve was made with N acetyl glucosamine in sodium acetate buffer at concentrations between 0.01 and 0.1 mg mL^{-1}. Data was expressed as µmol mg $protein^{-1}$ min^{-1}.

This enzymatic activity was measured with the samples collected at fruiting (April 2018).

2.7 RNA Extraction and RT-qPCR analysis

Total RNA was isolated from each replicate with Plant/Fungi Total RNA Purification kit (50) (NORGENTM) (DNAase treatment included) and RNA integrity was confirmed by using NanodropTM.

The retrotranscription was carried out using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were executed using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 ºC, 30 min 42 ºC, 5 min 85 ºC, and kept at 4 ºC. The amplifications were performed with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 ºC and then 39 cycles consisting of 15 s at 95 ºC, 30 s at 50 ºC and 30 s at 72 ºC, followed by melting curve to verify the results. To explain the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Reference gene was

Histone H3 gene (AF304365.1). Primers used are in Table 3.3.1. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

RNA and RT-qPCR analysis was carried out with samples collected at fruiting (April 2018).

Table 3.2.1. Forward and reverse primers used in qPCR analysis.

	Forward primer	**Reverse primer**
RuPR1	5´-TACTACACGTACGCGACAAACAC	5´-TCTCCATCATCACACACAACTCT
RuPR2	5´-TTCGTCTCGATTATGCTCTCTTC	5´-GCAGAATACACAGCATCCAAAA
RuPR3	5´-AAATCAACCTAGCAGGCCACT	5´-GAGGGAGAGGAACACCTTGACT
Histone	5´-TTCCAGAGCCATGCAGTTTTG	5´-TGGCATGAATGGCACAGAGA

**Ru = Rubus*

2.8 Characterization of phenolics and flavonoids by UHPLC/ESI-QTOF-MS

Phenolic acids including, citric acid, gallic acid, genistic acid, salicylic acid, vanillic acid, ferulic acid and chlorogenic acid were purchased from Sigma (St. Louis, MO, USA); flavonoids including kaempferol, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, quercetin, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, luteolin, naringenin (aglycone), hesperetin, (+)-catechin, (-)-epicatechin, aesculetin, phloridzin, delphinidin, anthocyanin, delphinidin-3-O-rutinoside , and malvidin and other polyphenols like 6,7-dihydroxicoumarin were purchased from Sigma and from Extrasynthese Co.™ (Geney, France).

The standard solutions (10 ppm) were dissolved in methanol. All the solvents used, as methanol and acetonitrile (Honeywell Riedel-de Haen), were LC-MS grade. Purified water was obtained from Milli-Q Plus™ System from Millipore (Milford, MA, USA). Formic acid was purchased from Aldrich (St. Louis, MO, USA).

Sample preparation

The extraction of phenolics was conducted as follows: 10 mg of leaves powder were added to 1000 µL of methanol. The mixture was vortexed for 1 min, sonicated for 5 min and centrifuged at 2890 *x* g for 5 min at 4 ºC. Supernatants were collected and stored at -20 ºC until their use for LC/MS analysis. During the process, extracts were protected from light.

UHPLC/ESI-QTOF-MS Analysis

Samples were injected on a 1290 Infinity series UHPLC system associated to an electrospray ionization source (ESI) with Jet Stream technology to a 6550 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany).

For the dissociation, a volume of 2 µL was injected in a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2% B (0-6 min), 2-50% B (6-10 min), 50-95% B (11-18 min), 95% B for 2 min (18-20 min), and returned to starting conditions 2% B in one min (20-21 min) to finally keep the re-equilibration with a total analysis time of 25 min.

Detector was functioning in full scan mode (m/z 50 to 2000), at 1 scan s^{-1}. Accurate mass measurement was confirmed through an automated calibrator delivery system that constantly introduced a standard solution, containing masses of m/z 121.0509 (purine) and m/z 922.0098 (HP-921) in positive ESI mode; on the other hand m/z 112.9856 TFA (trifluoride acetic acid) and m/z 922.009798 (HP-921) were introduced in negative ESI mode. The capillary voltage was ± 4000 V for positive and negative ionization mode. The temperature was set at 225 °C. The nebulizer and gas flow rate were

35 psig and 11 L min^{-1} respectively, fragmentor voltage to 75V and a radiofrequency voltage in the octopole (OCT RF Vpp) of 750 V.

For the investigation, MassHunter Workstation Software LC/MS Data Acquisition (Agilent Technologies), version B.07.00, was used for control and acquisition of all data obtained with UHPLC/ESI-QTOF-MS.

For measurement, each sample was injected twice in six different concentrations to create calibration curves in which sample peak areas were extrapolated. UHPLC-MS data analysis was performed by MassHunter Qualitative Analysis (Agilent Technologies) Software version B.08.00 using Molecular Feature Extraction (MFE).

All measurements were carried out at both sampling moments.

2.9 Statistical analysis

To check the statistical differences between the results obtained, analysis of variance (ANOVA) was used. In the cases with only one independent variable (PRs and qPCR), a one-way ANOVA was used and in those data in which there were two independent variables (fluorescence, pigments and enzymatic activities related to oxidative stress), a two-way ANOVA (factorial ANOVA) was used. In both cases, prior to ANOVA analysis, homoscedasticity and normality of the variance were checked with Statgraphics 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p < 0.05$) a Fisher test was used (Sokal and Rohlf, 1980).

3. Results

3.1 Photosynthesis analysis by fluorescence measurement

Values of indicators related to chlorophyll fluorescence and representative of plant photosynthetic functioning at both sampling points appear in figure 3.2.2.

Figure 3.2.2 A) shows the minimal fluorescence yield (F0). According to two-way ANOVA, there were significant differences between sampling moments (flowering and fruiting), and differences between the control and the treatments, but there were not statistically significant differences between N 21.4 and ME. The lowest values at flowering and at fruiting were shown by the treatments.

Figure 3.2.2 B) shows the maximum photosynthetic efficiency of the photosystem II (PSII) (Fm/Fv). According to two-way ANOVA, there were significant differences between sampling moments (flowering and fruiting), but there were not differences between the control and the N 21.4 and ME treatments. Bacterial treatments showed higher Fm/Fv values at fruiting than control.

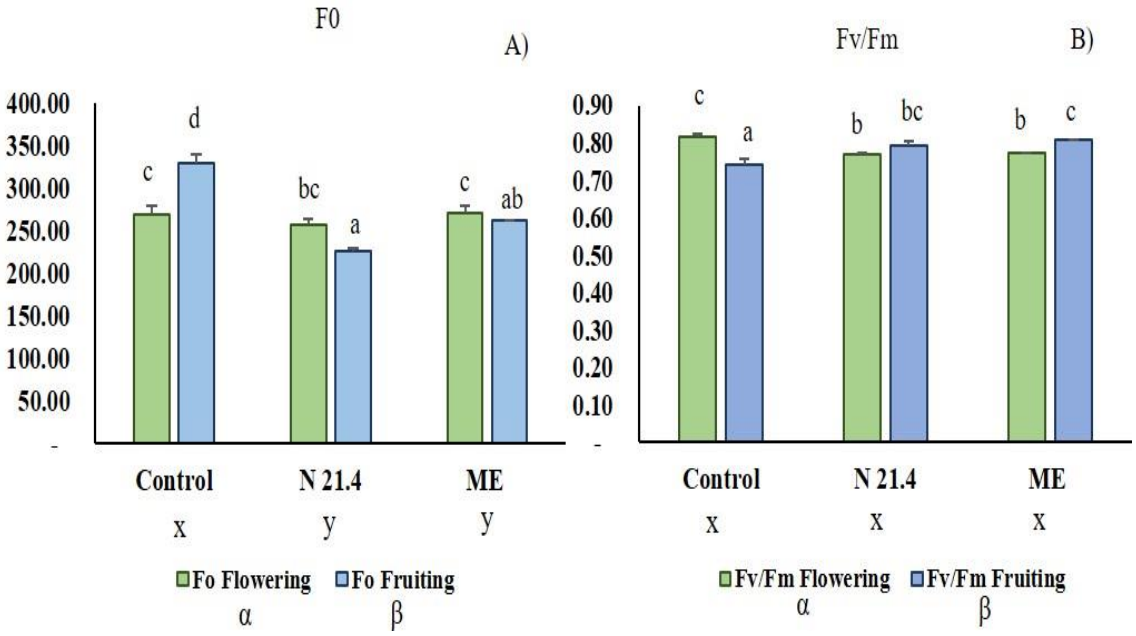

Figure 3.2.2 Measurement of chlorophyll fluorescence parameters at flowering and fruiting: A) minimal fluorescence yield (F0); B) maximum photosynthetic efficiency of the PSII (Fm/Fv) in blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls. Different letters indicate significant differences ($p < 0.05$): "α and β" indicates differences between sampling moments; "x and y" between treatments (Control, N 21.4 and ME) and "a, b, c, d" indicates differences taking into account sampling moments and treatments (interaction).

3.2 Pigments: total chlorophyll and carotenoids

Analysis of photosynthetic pigments at both sampling points appear in figure 3.2.3. Total chlorophyll a (Fig. 3.2.3 A)) and b (Fig. 3.2.3 B)) and carotenoids (Fig. 3.2.3 C)) were measured as mg per g of leaf (fresh weigh) at flowering and at fruiting.

Photosynthetic pigments were significantly different between sampling moments. Chlorophyll a (Fig. 3.2.3 A)) and carotenoids (Fig. 3.2.3 C)) were also significantly affected by both treatments, while chlorophyll b was only significantly affected by ME (Fig. 3.2.3 B)). In controls, chlorophyll b and carotenoids were significantly higher at fruiting, while chlorophyll a was not affected by the sampling moment. As regards to bacterial treatments, both induced a significant increase in chlorophyll a at flowering, being the effects of N 21.4 higher than those of ME, while at fruiting, the ME induced higher values than N 21.4 and the control; interestingly high chlorophyll a values were maintained at the two sampling moments by the ME (Fig. 3.2.3 A)). As regards to chlorophyll b, only ME induced a significant decrease compared to controls and bacterial treatment at flowering (Fig. 3.2.3 B)); carotenoids (Fig. 3.2.3 C)) were significantly lower in treated plants at fruiting.

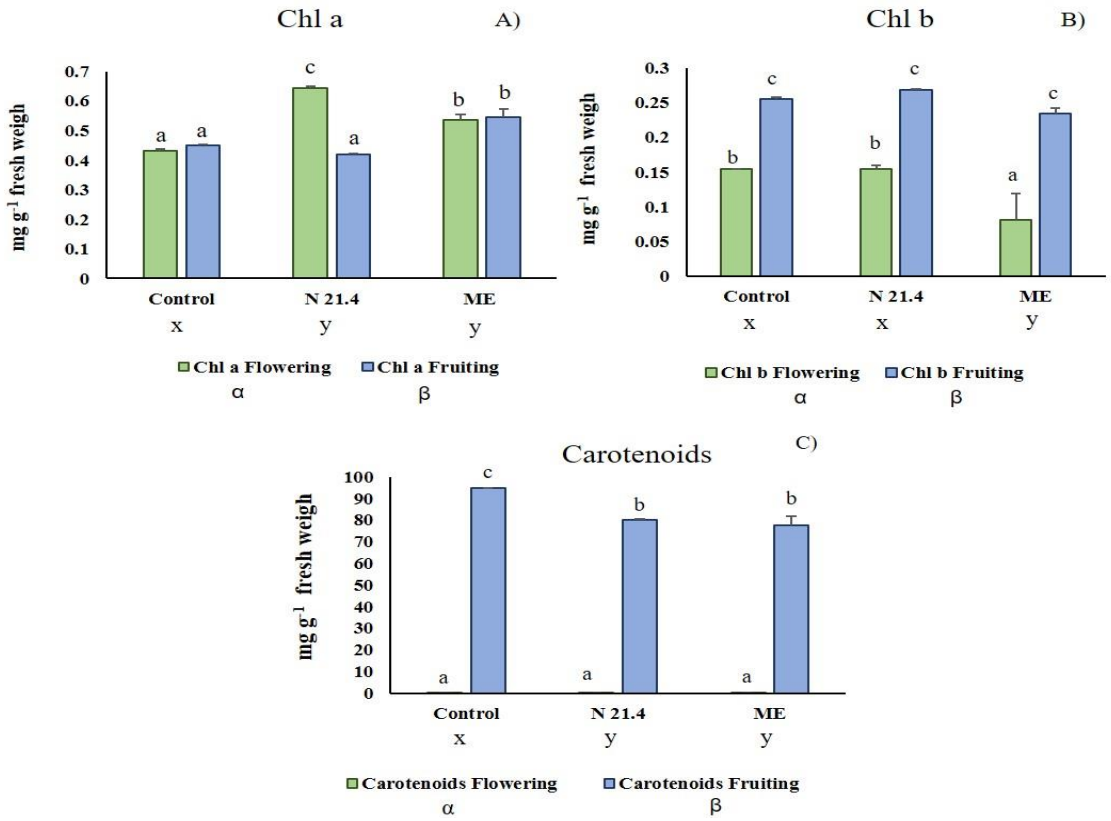

Figure 3.2.3 Pigment analysis at flowering and at fruiting: A) total chlorophyll a; B) total chlorophyll b; C) total carotenoids present in the leaves of blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls. Different letters indicate significant differences ($p < 0.05$): "α and β" indicates differences between sampling moments; "x and y" between treatments (Control, N 21.4 and ME) and "a, b and c" differences taking into account sampling campaign and treatments (interaction).

3.3 Activity of enzymes related to oxidative stress

Specific enzymatic activity of SOD, APX and GR, enzymes of the ROS scavenging cycle and related to oxidative stress, were evaluated at both sampling moments, flowering and fruiting (Fig. 3.2.4).

For all the analysed enzymes, significant differences were found between sampling moments, according to two-way ANOVA. There were also significant differences between the control and the bacterial treatments in the three cases, but there were only statistically significant differences between N 21.4 and ME in APX activity. In

general, activity of SOD (Fig. 3.2.4 A)) and APX (Fig. 3.2.4 B)) was higher at flowering, while GR (Fig. 3.2.4 C)) was higher at fruiting. Control plants showed in general the highest values at all times as compared to treated plants, being significant the differences except for GR at fruiting, which showed no differences between treatments (Fig. 3.2.4 C)). APX activity was significantly lower in treated plants at flowering, while at fruiting, only ME induced a significant increase as compared to N 21.4 treatment and controls. SOD activity (Fig. 3.2.4 A)) was significantly decreased by both treatments at flowering and fruiting; differences between bacterial strain and ME were only significant at fruiting, where ME treated plants showed the lowest values.

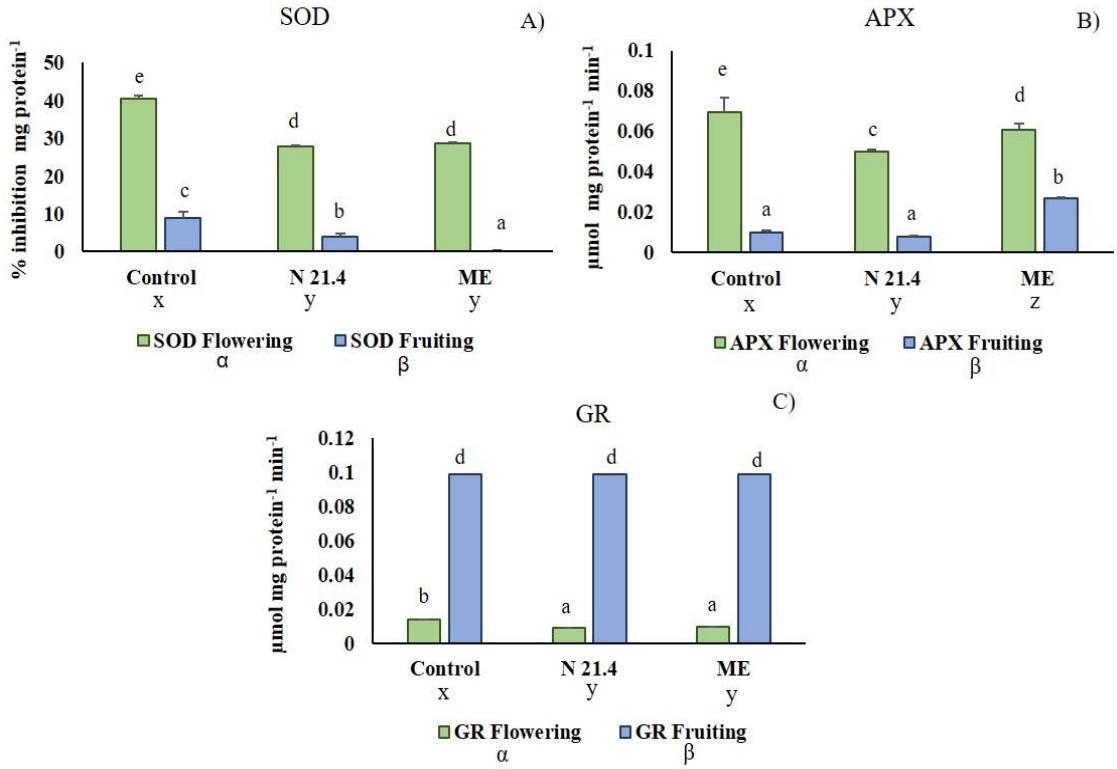

Figure 3.2.4 Enzymatic activity of: A) superoxide dismutase (SOD), B) ascorbate peroxidase (APX) and C) gluthatione reductase (GR) in the leaves of blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls at flowering and at fruiting. Different letters indicate significant differences ($p < 0.05$): "α and β" indicates differences between sampling moments; "x, y and z" between treatments (Control, N 21.4 and ME) and "a, b, c, d and e" indicates differences taking into account sampling campaign and treatments (interaction).

3.4 Pathogenesis-related proteins (PRs)

Activity of Glucanase (PR2) and Chitinase (PR3), two pathogenesis-related proteins (PRs), were also evaluated (Fig. 3.2.5). The activity values of both enzymes were higher in plants treated with the ME than those treated with the control and with N 21.4, which had even lower values than the control. These differences were significant between the three treatments for both enzymes.

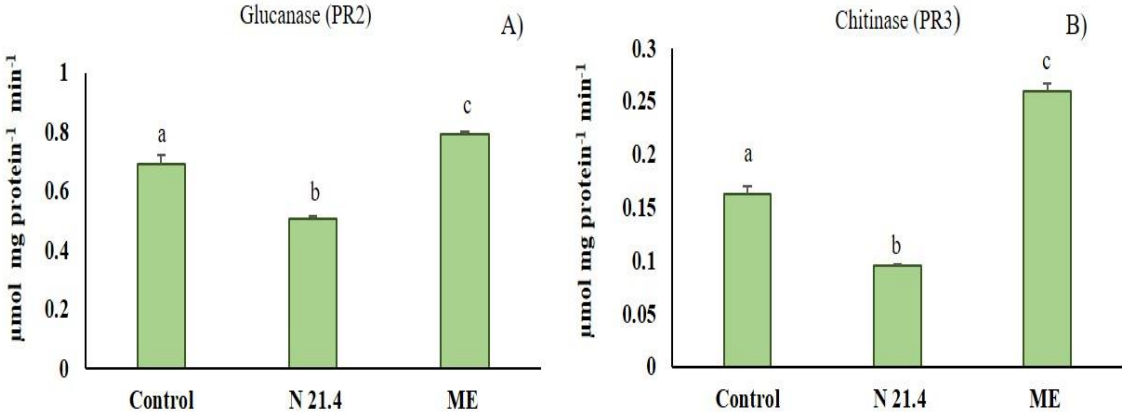

Figure 3.2.5 Activity of: A) glucanase and B) chitinase in the leaves of blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls at flowering and at fruiting. Different letters indicate significant differences ($p < 0.05$) between treatments in each sampling time.

3.5 RT-qPCR analysis

Expression of genes encoding PRs (*PR1*, *PR2* and *PR3*), indicative of ISR transduction pathways, in treated plants appears in figure 3.2.6. All genes were underexpressed in N 21.4-treated plants as compared to control plants, while ME-treated plants showed more similar expression to control. There were significant differences between N 21.4 and ME.

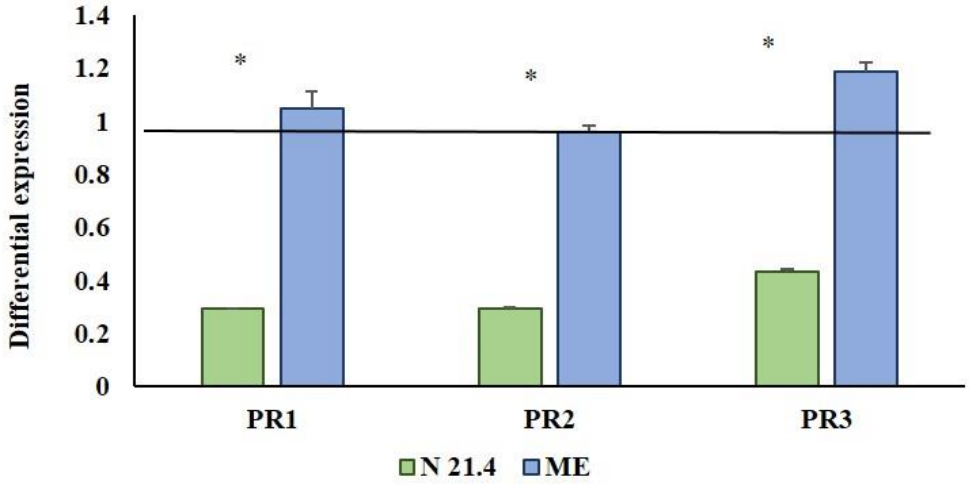

Figure 3.2.6 Differential expression of genes encoding PRs (*PR1*, *PR2* and *PR3*) in plants inoculated with N 21.4 and its metabolic elicitors (ME). Expression of 1 is that of the control (horizontal black line). Asterisks represent statistically significant differences ($p < 0.05$) between treatments.

3.6 Phenolic and flavonoid analysis by UHPLC/ESI-QTOF-MS

Methanolic extracts were analysed by UHPLC/ESI-QTOF-MS. All the compounds were identified by comparing their retention times and MS/MS spectra with reference standards. The main compounds found (13) and their concentration (µg g^{-1}) are shown in Table 3.2.2. They were grouped in flavonols (5), flavones (1), flavanones (2), flavanols (2), hydroxycoumarins (1), dihydrochalcone (1) and hydroxy benzoic acid (1). Three plant groups were compared (Control, strain N 21.4 and ME from N 21.4) at two sampling moments, flowering and fruiting (See Annex 7.1, where chromatograms of the bioactives present in blackberry leaves of both sampling moments are shown and compared).

In general, the amount of flavonols and flavanols was higher at fruiting, with outstanding presence of kaempferol derivatives (K-3-O-glucoside, K-3-O-rutinoside) and quercetin derivatives (Q-3-O-glucoside, Quercetin 3-O-rutinoside). At fruiting, strain N 21.4 and ME treated plants showed altered flavonol profiles, with marked changes in

kaemperfol derivatives: K-3-O-G registered a 2-fold increase under both treatments while K-3-O-R showed a 2.5-fold and a 2-fold increase in live strain (N 21.4) and ME-treated plants, respectively; at flowering, only ME treated plants were affected showing lower values. As regards to quercetins, Q-3-O-G was almost unaffected by treatments, while Q-3-O-R followed a similar pattern as K-3-O-R, but less intense. The amount of (-)-epicatechin extremely increased at fruiting in the three groups of plants, but with a different intensity. Salicylic acid was higher at flowering and at fruiting in treated plants than in controls.

Table 3.2.2. Main phenolic and flavonoid compounds found in blackberry leaves and their concentration in µg g^{-1}. Control, N 21.4 and ME treatments were measured at flowering and at fruiting.

		Control		N 21.4		ME	
Polyphenol class	**Compound**	**Flowering**	**Fruiting**	**Flowering**	**Fruiting**	**Flowering**	**Fruiting**
Flavonols	Kaempferol 3-O-glucoside	11.76 ± 0.975 a (x)	12.62 ± 0.77 a (α)	11.19 ± 0.175 a (x)	25.09 ± 0.185 b (β)	9.36 ± 0.17 a (x)	24.23 ± 1.09 b (β)
	Kaempferol 3-O-rutinoside	8.24 ± 0.42 a (x)	6.82 ± 0.285 a (α)	8.26 ± 0.05 a (x)	17.2 ± 0.09 b (β)	6.05 ± 0.28 a (y)	14.67 ± 0.69 b (γ)
	Quercetin (aglycone)	0.59 ± 0.035 a (x)	0 ± 0 b (α)	0.41 ± 0.01 a (y)	0 ± 0 b (α)	0.4 ± 0.01 a (y)	0 ± 0 a (α)
	Quercetin 3-O-glucoside	13.53 ± 0.13 a (x)	51.44 ± 0.4 b (α)	11.78 ± 0.255 a (y)	50.06 ± 0.05 b (α)	12.37 ± 0.235 a (y)	56.32 ± 2.955 b (α)
	Quercetin 3-O-rutinoside	7 ± 0.52 a (x)	16.5 ± 0.16 b (α)	8.19 ± 0.19 (x)	22.89 ± 0.98 b (β)	7.12 ± 0.125 a (x)	21.19 ± 0.99 b (β)
Flavone	Luteolin	1.3 ± 0.235 a (x)	0.49 ± 0.01 a (α)	1.38 ± 0.04 a (x)	1.11 ± 0.095 a (β)	0.96 ± 0.075 a (x)	1.8 ± 0.12 b (γ)
Flavanones	Naringenin (aglycone)	12.65 ± 0.05 a (x)	12.05 ± 0.05 a (α)	12.84 ± 0.025 a (y)	12.28 ± 0.035 a (α)	13.11 ± 0 a (z)	12.22 ± 0.07 b (α)
	Hespertin (S)	0.6 ± 0.065 a (x)	0.7 ± 0.03 a (α)	0.7 ± 0.1 a (x)	0.7 ± 0.03 a (α)	0.5 ± 0 a (x)	0.7 ± 0.035 b (α)
Flavanols	(+)-Catechin	0.04 ± 0 a (x)	15.63 ± 0.42 b (α)	0.0093 ± 0 a (y)	15.07 ± 0.295 b (α)	0.014 ± 0 a (y)	11.9 ± 0.3 b (β)
	(-)-Epicatechin	0.45 ± 0.015 a (x)	393.11 ± 3.425 b (α)	0.39 ± 0.005 a (x)	295.37 ± 0.425 b (β)	0.54 ± 0.02 a (y)	387 ± 12.99 b (α)
Hydroxycoumarins	Aesculetin	0.21 ± 0.015 a (x)	0.024 ± 0.001 b (α)	0.26 ± 0.005 a (y)	0.061 ± 0.005 b (β)	0.27 ± 0.005 a (y)	0.042 ± 0.007 b (α β)
Dihydrochalcone	Phloridzin	0 ± 0 a (x)	0.64 ± 0.02 b (α)	0 ± 0 a (x)	0.43 ± 0.005 b (β)	0 ± 0 a (x)	0.67 ± 0.015 b (α)
Hydroxy benzoic acid	Salicylic acid	12.07 ± 0.23 a (x)	9.09 ± 0.405 b (α)	13.99 ± 0.13 a (y)	11.84 ± 0.07 b (β)	12.31 ± 0.6 a (y)	10.44 ± 0.6 a (α β)

Letters a and b indicate significant differences between flowering and fruiting within the same treatment; x, y and z indicate significant differences between treatments at flowering; and letters α, β, γ indicate significant differences between treatments at fruiting.

4. Discussion

Blackberries and blackberry leaves are strongly rich in polyphenols that have beneficial effects on human health (Gutierrez et al. 2017a; Oszmiański et al. 2015; Martin-Rivilla et al. 2019). Blackberry leaves could be used as a profitable source to obtain high added value bioactive molecules to the pharmaceutical, nutraceutical or cosmetic industry by using what is currently a crop waste and therefore fulfilling the premises of circular economy. Hence, this study was undertaken to elicit the secondary metabolism of blackberry plants in field conditions with the beneficial *Pseudomonas fuorescens* N 21.4 (Gutierrez et al. 2017b; Garcia-Seco et al. 2015a; Ramos-Solano et al. 2014) and with its metabolic elicitors to get a sustainable crop with a stronger immune system more adapted to biotic and abiotic stress and richer in usable polyphenols. On the one hand, this study is gaining knowledge about effective biological products for agriculture and on the other hand, about the bioactive metabolism of blackberry leaves, which is a great challenge since they are considered as rich in phenolic compounds as the fruits (Ferlemi et al. 2016).

A fundamental parameter that reflects the health status of a plant is photosynthesis because the photosynthetic apparatus is the first physiological function to be affected by stress (Garcia-Cristobal et al. 2015; Garcia-Seco et al. 2015a). In our work, plants treated with N 21.4 and with its ME were less stressed, had a photosynthetic apparatus working optimally and therefore were in better health conditions. The proper photosynthesis performance in treated plants was deduced due to their lower values of F0 (minimal fluorescence yield), since high values of this parameter indicate malfunctions of the photosystem II (PSII) and general photosynthesis damage (Baker et al. 2008).

On the other hand, Fv/Fm parameter is an estimator of the maximal photochemical efficiency of PSII (PSII maximum capacity to transfer electrons to the electron transport chain (Garcia-Seco et al. 2015a)) and it is used to identify loss of function of PSII reaction

centers. A decrease in Fv/Fm ratio, especially under stress conditions means photoinhibition (Roháček et al. 2002). However, in our treated plants, higher values of Fv/Fm were observed compared to control plants (at fruiting). Therefore, the results of F0 and Fv/Fm were consistent between them and both reflected that bacterial treatments with strain N 21.4 and ME supposed a better functioning of the entire photosynthetic apparatus with greater potential to channel electrons to the electron transport chain and thus to generate more energy (Baker et al. 2008). Our treatments decreased photosynthetic damage caused by stress and improved photosynthetic performance and efficiency, increasing energy production for the plant. This extra energy could be used in the production of target secondary metabolites.

Narrowly related to photosynthesis are photosynthetic pigments (Chl a, Chl b and carotenoids). Chl a is the major pigment responsible for the absorption of light photons and the excitation of electrons that pass to the transport chain to finally generate energy (Bi Fai et al. 2007). Our results showed higher levels of Chl a in treated plants, which is associated to lower F0 values and which means that the whole photosynthetic system is working optimally. Chl b and carotenoids are accessory pigments with a protective function to light stress. That undergo a marked increase of these pigments at fruiting, when sun irradiance is getting higher (Verma et al. 2009). Carotenoids also perform an essential photoprotective role since they can act as non-enzymatic scavengers of ROS that formed within the chloroplasts (Young et al. 1991).

Plants rapidly produce ROS under stress conditions and a rise in ROS generation means oxidative stress (Gill and Tujela, 2010) and oxidative modification of vital biomolecules like membrane lipids, amino acids, proteins and DNA (Gill and Tujela, 2010), which ends in cell death and lowering the general fitness of the plant. ROS scavenging systems are classified in two groups, enzymatic and non-enzymatic

mechanisms. Within the group of enzymatic mechanisms, it stands out the activity of the ascorbate-glutathione cycle enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase, (GR), catalase and peroxidase, and within the non-enzymatic mechanisms it highlights the amount of antioxidant molecules as ascorbate, carotenoids, tocopherols, phenolic compounds, etc. (Martin-Rivilla et al. 2019). Ascorbate levels and ascorbate-glutathione cycle enzymes are tightly linked to plant tolerance to biotic and abiotic stress (Paciolla et al. 2008; Locato et al. 2012). This is why we have studied the specific enzymatic activity of key enzymes of this cycle (SOD, APX and GR). Our results suggested that treated plants were less stressed than control plants because their enzymatic activity was lower, especially SOD activity at both sampling moments and APX and GR at flowering. A decrease in the enzymatic activities suggests a more relaxed state of the plants after treatment inoculation. As treated plants were less stress, they generated less ROS and therefore, their enzymatic machinery responsible for detoxification worked in a more moderate way (Gutierrez-Albanchez et al. 2018). The results observed with carotenoids measurement also support the fact that treated plants were less stressed, since their amount of carotenoids was less than that of the control plants. Less stressed plants required less ROS-scavenging enzymatic activity and fewer antioxidant molecules, such as carotenoids.

However, ROS are necessary to start signal transduction pathways, so a lower antioxidant activity may also indicate that bacterial treatments keep ROS in levels such as to maintain a sustained systemic signalling, keeping responses activated (Gutierrez-Albanchez et al. 2018).

Activity of the pathogenesis-related proteins glucanase (PR2) and chitinase (PR3) were also evaluated because they play an essential role in the capacity of the plants to face to infection caused by pathogen attacks (Lucas et al. 2014). These PRs are plant

species-specific proteins that are produced in response to infection of pathogens, but also in response to inoculation of beneficial bioeffectors and elicitors and which maintain plants in the alert state named *priming* phase (Mauch-Mani et al. 2017).

Our study showed that the activity of both PRs was significantly higher in ME-treated-plants than in those treated with N 21.4 or control plants. This may suggests that ME prepared plants to cope with pathogen outbreaks and possibly with other stress situations. qPCR results supported this idea because analysed genes (*PR1*, *PR2*, *PR3*) of ME-treated-plants had not been downregulated as it occurred in plants inoculated with N 21.4. However, SA values were higher in plants inoculated with both treatments (N 21.4 and ME) than in control plants, which suggests that both plants inoculated with N 21.4 and with ME were more primed and prepared to face to biotic stress situations (Zhang and Li, 2019). These results were also consistent with those of enzymes related to oxidative stress, demonstrating once again that treated plants were less stressed and had therefore a better fitness than control plants to face to biotic and abiotic stress. These last discussed results were only performed at fruiting, at the end of the useful time of the crop, to assess the general physiological state of the plants after all inoculations of bacterial treatments.

Plant fitness can also be improved by the elicitation of secondary metabolism enhancing the synthesis of antioxidant metabolites. Among secondary metabolites with greater antioxidant capacity are flavonoids (Agati et al. 2012).

Flavonoid metabolism and flavonoid biosynthetic pathway has been widely studied due to its importance in plant defence against biotic and abiotic stress (Agati and Tattini, 2010; Schulz et al. 2015) and because flavonoids have many beneficial properties to human health (Mortas and Sanlier, 2015).

Although our variety of blackberry (*Rubus* cv. Loch Ness) is widely spread, and although it has been seen that leaves contain similar amount and variety of phenolic compounds (Felemi et al. 2016) as fruits, nowadays only the cultivation of the fruit is exploited. This is why in this work, the total amount of various groups of phenolic compounds of the leaves has been measured and a thorough HPLC analysis (by LC/ESI-QTOF-MS) has been carried out to specifically identify the different phenolic compounds and groups present in the leaves and their concentration. Studying in detail the phenolic compounds of the leaves of the plants inoculated with our bacterial treatments would allow to revalorize leaves pruning as a source of high-added value compounds.

LC/ESI-QTOF-MS results (Table 3.2.2), were in concordance with the results obtained by Ferlemi et al. 2016; Ozmiánki et al. 2015 and Gutierrez et al. 2017a, who found, kaempferol 3-O-glucoside, kaempferol 3-O-rutinoside, quercetin 3-O-glucoside, quercetin 3-O-rhamnoside, (+)-catechin, (-)-epicatechin and luteolin as main in blackberry leaves. Ozmiánki et al. 2015 also prove that genus *Rubus* is very rich in p-hydroxybenzoic acids.

In our study, the amount of flavonols were higher in the plants treated with N 21.4 and its ME, which means that our bacterial treatments are efficient in the elicitation of flavonoid metabolism. Highlighting results were the great increase of kaempferol derivatives (K-3-O-glucoside, K-3-O-rutinoside) and quercetin 3-O-rutinoside in treated plants. Our bacterial treatments may trigger flavonoid biosynthesis as part of an induced systemic response given the important role of this pathway in plant defence. In previous works made in blackberry fruit, it was shown that N 21.4 modified flavonoid metabolism by modifying some of the enzymes involved in the biosynthetic route (Garcia-Seco et al. 2015a), so it would be possible that it was causing similar changes in the leaves.

Kaempferol derivatives were the compounds most elicited by the treatments from flowering to fruiting, having a 2 fold increase in the case of N 21.4 inoculations and a 2.5 fold increase in the case of ME. Compared to the work of Oszmiański et al. 2015, conducted on leaves of 26 species of *Rubus*, our results showed higher kaempferol derivatives concentration.

Kaempferol and its glycosylated groups, especially glucoside and rutinoside, have demonstrated their anti-pathogenic capacity in some plants (Galleoti et al. 2008), which reinforces our idea that treated plants, with higher kaempferol values, were more protected than control plants to biotic stress. Moreover, consumption of kaempferol-rich foods is related to a decrease risk of several types of cancer due to its powerful antioxidant activity (Garcia-Closas et al. 1998). Kaempferol is also associated with a reduced mortality from cardiovascular diseases and prevention of type II diabetes (Verma et al. 2009) and it has also anti-inflammatory and anti-microbial activity (Cushnie et al. 2005). As kaempferol is poorly absorbed by gastro-intestinal tract, it has been reported that the combination of kaempferol with quercetin significantly increases the anticancer effects (Hirata et al. 2004).

Interestingly, quercetin derivatives also increased between flowering and fruiting, but in this case, differences between the treatments and the control were only observed in quercetin 3-O-rutinoside. An increase in quercetin is promising, not only for its anticancer effects, but because it has been demonstrated that a repeated quercetin supplementation can reach considerable plasma levels (Manach et al. 2005) acting as an excellent *in vitro* and *in vivo* antioxidant.

Some studies have been shown that not only quercetin aglycon is taken up in the gastro-intestinal tract, but also quercetin glycosides, such as Q-3-O-G and Q-3-O-R (present in berry leaves) can be absorbed in the intestine, and this absorption surpasses

by far that of the aglycon (Erlund et al. 2000). Hence, dietary quercetin as ingestion of fruits and vegetables or by supplements is therefore a promising agent for many diseases prevention.

In relation to flavanols, and despite the fact that there were not significant differences between the treatments and the control, it was highly remarkable the huge increase in the levels of (-)-epicatechin from flowering to fruiting present in blackberry leaves. As in the case of kaempferol, the concentration of (-)-epicatechin found in our leaves was higher than that found in other studies, such as that of Prakash et al. 2018 conducted in blackberry (114.8 µg g^{-1}), strawberry (75 µg g^{-1}) and red raspberry (50.5 µg g^{-1}) leaves.

Epicatechin high antioxidant capacity reduces oxidative stress in plants, but it has been also demonstrated its ability to induce ROS scavenging enzymes such as SOD and APX (Mahajan and Yadav, 2003). It also acts as phytoanticipins in some fruits (Guetsky et al. 2005) and that confers resistance to fungi and bacterial infection. Schoroeter et al. 2005 have demonstrated that oral administration of chemically pure (-)-epicatechin to humans (1-2 mg kg^{-1}) exert rapid cardiovascular benefits by enhancing the bioactivity of nitric oxide, a key molecule in preventing atherosclerosis. Blackberry leaves would be therefore a great source of epicatechin since 300-400 mg kg^{-1}could be extracted from them.

With our results, we have demonstrated the elicitation of flavonoid metabolism and also the improve immune system of blackberry plants by inoculating them with both the beneficial rhizobacterium *P. fluorescens* N 21.4 and with their metabolic elicitors. This way, we conclude that the treatments with N 21.4 and its ME are perfectly effective and could be economically and environmentally friendly agronomic inoculants. Furthermore, these treatments would generate a greater economic yield to the cultivation

of blackberry by allowing taking advantage of the leaves as a source for the extraction of metabolites, such as kaempferol and quercetins derivatives and epicatechin, with great value due to their enormous beneficial effects on human health.

However, results show that ME treatment had in general a more marked effect on plant physiology than N 21.4 treatment. ME-inoculated-plants were less stressed and better protected against different stress situations. ME altered both primary and secondary metabolism resulting in more resilient plants.

It cannot be forgotten the application way of each treatment (foliar (ME) vs root application (N 21.4)) because it could be partly responsible for the better effects produced by ME. Foliar application of biostimulants has been seen as a very effective technique and it is also economical and environmentally convenient (Ngoroyemoto et al. 2019). On the other hand, using molecules instead of a living bacterium reports advantages in all related to the maintenance and management of the inoculums and also prevents biosecurity problems.

For all the above explained, we conclude that the metabolic elicitors of the bioeffector *Pseudomonas fluorescens* N 21.4 would be effective, affordable, respectful with the environment and easy to manage inoculants for agriculture. Due to the promising results obtained with the ME in the present work, another parallel study is being conducted to better known its composition.

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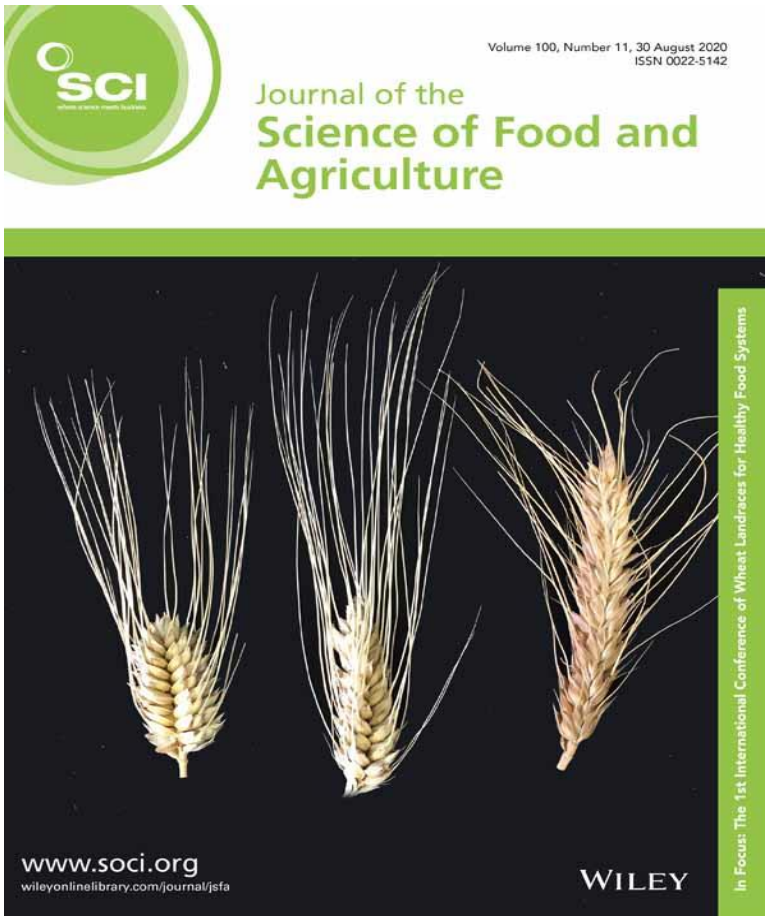
3.3 Metabolic elicitors of *Pseudomonas fluorescens* N 21.4 elicit flavonoid metabolism in blackberry fruit



Metabolic elicitors of *Pseudomonas fluorescens* N 21.4 elicit flavonoid metabolism in blackberry fruit

Martin-Rivilla, H*., Garcia-Villaraco, A., Ramos-Solano, B., Gutierrez-Mañero, F.J., Lucas, J.A.

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

*Corresponding author: Helena Martín Rivilla, helenamartin92@gmail.com

+913 72 47 85

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Abstract

BACKGROUND

Beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4, and its metabolic elicitors were inoculated in commercial cultivars of blackberry plants (*Rubus* cv. Loch Ness). Phenolic compounds present in red and black fruit and the expression of structural marker genes of the phenylpropanoid pathway during fruit ripening were studied.

RESULTS

An inverse relationship between gene expression and accumulation of metabolites was seen, except for the *RuDFR* gene, which had a direct correlation with cyanidin 3-O-glucoside synthesis, increasing its content 1.3 times when *Ru*DFR was overexpressed in red fruit of plants inoculated with the metabolic elicitors of *P. fluorescens* N 21.4, compared to red fruit of plants inoculated with N 21.4. The *RuCHS* gene also had a fundamental role in the accumulation of metabolites. Both rhizobacterium and metabolic elicitors triggered flavonoid metabolism enhancing the content of catechin, and epicatechin between 1.1 and 1.6 times in the case of red fruit and between 1.1 and 1.8 times in the case of black fruit. Both treatments also boosted anthocyanin and quercetin, and kaempferol derivatives content, highlighting the effects of metabolic elicitors in red fruit and the effects of the live rhizobacterium in black fruit.

CONCLUSION

The metabolic elicitors' capacity to modulate gene expression and to increase secondary metabolites content was demonstrated. This work therefore suggests them as effective, affordable, easily manageable, and ecofriendly plant inoculants that complement or are alternatives to beneficial rhizobacteria.

Key Words: *Pseudomonas fluorescens* N 21.4, metabolic elicitors, blackberries, flavonoid metabolism, *RuDFR* gene, *RuCHS* gene.

1. Introduction

Blackberry consumption is becoming increasingly popular due to its human health benefits (Lee et al. 2017). The increase in consumption, linked to the high economic price of blackberry in the market, makes this crop very attractive for the agrifood industry (Kaume et al. 2012).

Blackberries are among the fruits with more beneficial properties due to they are extremely rich in flavonoids, among which are flavonols, flavanols and anthocyanins (Kaume et al. 2012). These compounds have demonstrated cytotoxic, anticancer, antiviral, antibacterial, anti-inflammatory, antiallergenic, antithrombotic, cardioprotective, and hepatoprotective properties (Alzand et al. 2012: Ferreyra et al. 2012). Moreover, their content directly affects the nutritional and organoleptic quality of the fruit (Winkel-Shirley, 2001).

On the other hand, flavonoids are secondary metabolites that have a vital function in plants, acting as protectors against biotic and abiotic stress (Daayf et al. 2012). They also have an important role as repellents, visual attractors, phytoalexins, phytoanticipins (Ghasemzadeh and Ghasemzadeh, 2011; Jeandet et al. 2013), or auxin controllers (Gutierrez Mañero et al. 2012).

As flavonoids are secondary metabolites, their biosynthesis is highly inducible. An accepted and effective biotechnological practice to elicit secondary metabolism, enhancing the biosynthesis of compounds with agro-alimentary interest, is the use of beneficial rhizobacteria (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015; Martin-Rivilla et al. 2019) and their derived elicitors (structural molecules such as flagellin, (Ramirez-Prado et al. 2018) or metabolic elicitors released to the medium, such as antibiotics, surfactants, or other chemicals (Gozzo and Faoro, 2013; Wu et al. 2018).

As flavonoids have numeorus beneficial properties, the engineering of their biosynthetic pathways for the deliberate accumulation and isolation of active molecules has been extensively used in the biotechnological industry (Tanaka et al. 2008). However, the regulation of this pathway during blackberry ripening has not been deeply studied yet. Knowledge of this pathway during blackberry ripening and upon challenge with beneficial rhizobacteria and metabolic elicitors would be useful for the development of new blackberry crop techniques by stimulating the synthesis of secondary metabolites, sustainably improving fruit nutritional qualities (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015a).

For all the above, the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors (ME) were used in the present work to study their capacity to elicit the flavonoid biosynthetic pathway during blackberry fruit ripening. The *P. fluorescens* N 21.4 strain has widely demonstrated its beneficial effects on the physiology of blackberry plant, and on the flavonoid metabolism in the fruit. N 21.4 was able to trigger secondary metabolism in *Rubus fruticosus*, enhancing plant defences and fruit quality and production (Garcia-Seco et al. 2013). In the work of Ramos-Solano et al. 2014, it also demonstrated its capacity to increase fruit production and fruit quality, enhancing the quantity of phenolic compounds throughout the year, mainly when environmental conditions were tougher to the plant´s survival. Finally, it was seen that elicitation of blackberry plants by N 21.4 strain modulated gene expression in the fruit of *Rubus* cv. Loch Ness and affected the profiles of secondary metabolites during fruit ripening, boosting the expression of some flavonoid biosynthetic genes and enhancing the concentration of certain flavonoids in the fruit (Garcia-Seco et al. 2015b). This allowed the identification of regulatory genes involved in the phenylpropanoid pathway.

Since the strain N 21.4 had demonstrated an extraordinary ability to influence the physiology of blackberry plants and to elicit the flavonoid secondary metabolism, it was proposed, in the present work, to evaluate the potential of its ME to mimic the capacities of the live strain. A previous study, demonstrated the capacity of ME to elicit flavonoid metabolism in blackberry leaves, enhancing the amount of epicatechin and kaempferol, and quercetin derivatives (Martin-Rivilla et al. 2020a). It was also seen that ME were able to reinforce the blackberry plant´s immune system, activating pathogenesis-related proteins, and to improve plant fitness, reducing oxidative stress and increasing photosynthesis in the leaves (Martin-Rivilla et al. 2020a). In other plant species, the ME of N 21.4 also elicited secondary metabolism (e.g. isoflavone metabolism in *Glycine max* (Algar et al. 2012), and defensive secondary metabolism in *A. thaliana* (Martin-Rivilla et al. 2019). As a result of these data, the present study hypothesized that it was feasible that ME could modify the biosynthetic pathway of flavonoids during blackberry ripening.

The scientific contribution of the present research is therefore to advance the study of metabolic elicitors, using them as alternative plant inoculants to live rhizospheric bacteria, as they have many reported advantages related to the maintenance and management of the inoculums and because they do not cause biosecurity problems. The latter is the innovative aspect of this work, as the capacity of N 21.4 to trigger secondary metabolism in blackberry has already been demonstrated by our group (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015a and Garcia-Seco et al. 2015b).

To pursuit these goals, a thorough study of the content and variety of phenolic compounds present in blackberry fruits of plants inoculated with *P. fluorescens* N 21.4 and with its metabolic elicitors was carried out in red and in black fruit, as well as an

expression analysis of structural marker genes of the phenylpropanoids pathway in both fruit stages.

2. Material and methods

2.1 Blackberry experimental design

The *Rubus* cv. Loch Ness plants used in this work were kindly provided by Agricola El Bosque S.L. "La Canastita" (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were managed according to regular agricultural practices (Ramos-Solano et al. 2014). Plants were grown in Huelva (South Eastern Spain) from November 2017 to June 2018 under "winter cycle." Before being transplanted to greenhouses in November, plants underwent an artificial five-month cold period at 4 ºC to start their regular cycle. A total of 540 plants were in the trial, arranged in five greenhouses. Each greenhouse had two lines (200 m long each) with 120 plants in total, each line being one replicate with 60 plants. Three lines were inoculated with N 21.4 at root level; three lines were inoculated with the metabolic elicitors (ME) of N 21.4 by aerial spraying; and three lines were left as non-inoculated controls. N 21.4 and ME were inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum per plant.

Fruits for analysis were sampled in April 2018, when the two stages of the fruit (red and black) were present in the plants at the same time, and were rapidly frozen in liquid nitrogen and brought to the laboratory to be maintained at -80ºC. Three replicates were taken, each comprising 125 g of fruits, randomly sampled from 60 plants.

2.2 Bacterial experimental design

The bacterial strain used in this study was *Pseudomonas fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a Gram-negative bacilli which

was isolated from the rhizosphere of *Nicotiana glauca* Graham (Ramos-Solano et al. 2010a). The strain N 21.4 is able to trigger defence and phenolic metabolism in *Solanum lycopersicum* (Domenech et al. 2007), *A. thaliana* (Domenech et al. 2007; Martin-Rivilla et al. 2019), *Glycine max* (Ramos-Solano et al. 2010b), *Hypericum* sp. (Gutierrez Mañero et al. 2012), *Papaver* sp. (Bonilla et al. 2014) and blackberry. In blackberry, it improves fruit yield and quality by increasing flavonoid content (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015a).

Bacterial strain was stored at -80 ºC in nutrient broth (CONDA) with 20% glycerol. Inocula were prepared by streaking strains from -80 ºC onto Plate Count Agar (CONDA) plates, incubating them at 28°C for 24 h. Then, bacterial cells were scraped off the plates into sterile nutrient broth and were incubated for 24 h on an orbital shaker at 28 ºC with strong agitation, to keep the medium oxygenated and to obtain a 10^9 cfu mL^{-1} inoculum. Inocula of N 21.4 were diluted from 10^9 to 10^7 cfu mL^{-1} and they were delivered at root level to the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

Inoculum of ME was prepared by centrifuging an N 21.4-culture (grown for 24 h at 28ºC on an orbital shaker) at 2890 *x* g during 20 min at 4 ºC. Cells were discarded and the remaining supernatant was diluted following the same proportion as the live strain (from 10^9 to 10^7 cfu mL^{-1}) and it was sprayed onto the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

2.3 Fruit production

Fruit was collected throughout the campaign, from mid-March to early June. Fruits were collected every three days from all plants in each line and treatment. They were weighed and this weight was divided by the number of plants, obtaining the g per plant on each

day of harvest. Fruit production corresponds to the accumulated production of all the harvesting moments.

2.4 Extract preparation for measuring bioactives by colorimetry

The fruit extracts (red and black fruit) used to measure the total flavonols and phenols were prepared by adding 9 mL of 80% cold methanol to 1 g of fruit powder, mixing by vortex (protected from light), sonicating for 10 min and centrifuging at 2890 *x* g for 5 min at 4 ºC. The remaining supernatant was collected and stored at 4 ºC.

The fruit extracts (red and black fruit) to measure total of anthocyanins were prepared by adding 9 mL of 80% methanol and 0.1% of cold HCl to 1 g of fruit powder, mixing by vortex (protected from light), sonicating for 10 min and centrifuging at 2890 *x* g for 5 min at 4 ºC. The remaining supernatant was collected and stored at 4 ºC.

2.5 Bioactives measurement

- **Total flavonols**

Total flavonols were quantitatively determined through the test described by Jia et al. 1999, using catechin as standard (Sigma-Aldrich, St Louis, MO). One milliliter of the methanolic extract was added to a flask with 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$ (w/v). After 5 min, 0.3 mL of 10% $AlCl_3$ were added. Five min later, 2 mL of NaOH 1 M was finally added. The solution was mixed and measured at 510 nm with an UV-Visible spectrophotometer (Biomate 5). A catechin calibration curve was made (r = 0.99). Results were expressed as g of catechin equivalents per kg of fresh weigh (FW). All samples of red and black fruit were measured in triplicate.

- **Total phenols**

Total phenols were quantitatively determined with Folin-Ciocalteu agent (Sigma. Aldrich, St Louis, MO) by a colorimetric method described by Singleton and Rossi, 1965

with some modifications (Benvenuti et al. 2004). Gallic acid was used as standard (Sigma-Aldrich, St Louis, MO). Twenty µL of the methanolic extract were mixed with 250 µL of Folin-Ciocalteu 2 N (Sigma. Aldrich, St Louis, MO) and 3 mL of distilled water. After 5 min at room temperature, 0.75 mL of 20% Na_2CO_3 solution was added. After 8 min at room temperature, 950 µL of distilled water was added and after 2 h in obscurity, absorbance was measured at 760 nm with an UV Visible spectrophotometer (Biomate 5). A gallic acid calibration curve was made (r = 0.99). Results were expressed as g of gallic acid equivalents per kg of fresh weigh (FW). All samples of red and black fruit were measured in triplicate.

- **Total anthocyanins**

Total anthocyanins were quantitatively determined through the pH differential method described by Giusti and Wrolstad, 2001. Methanolic extracts were diluted in pH 1 buffer (0.2 M KCl) and pH 4.5 (1M CH_3CO_2Na) in 1:15 proportion. After that, absorbance was measured at 510 and 700 nm respectively, in an UV Visible spectrophotometer (Biomate 5). A cyanidin-3-glucoside calibration curve was made (r = 0.99). Results were expressed as g of cyanidin-3-glucoside equivalents per kg of fresh weigh (FW). All samples of red and black fruits were measured in triplicate.

2.6 Characterization of phenolics and flavonoids by UHPLC/ESI-QTOF-MS

Phenolic acids including, citric acid, gallic acid, genistic acid, salicylic acid, vanillic acid, ferulic acid, ellagic acid and chlorogenic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA); flavonoids including kaempferol, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, quercetin, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, luteolin, naringenin (aglycone), hesperetin, (+)-catechin, (-)-epicatechin, aesculetin, phloridzin, delphinidin, anthocyanin, delphinidin-3-O-rutinoside, and malvidin and other polyphenols like 6,7-

dihydroxicoumarin were purchased from Sigma-Aldrich and from Extrasynthese Co. ™ (Geney, France).

The standard solutions (10 ppm) were dissolved in methanol. All the solvents used, as methanol and acetonitrile (Honeywell Riedel-de Haen), were LC-MS grade. Purified water was obtained from Milli-Q Plus™ System from Millipore (Milford, MA, USA). Formic acid was purchased from Aldrich (St. Louis, MO, USA).

- **Sample preparation**

The extraction of phenolics was conducted as follows: 10 mg of powder of lyophilized fruit were added to 1000 µL of methanol. The mixture was vortexed for 1 min, sonicated for 5 min, and centrifuged at 2890 *x* g for 5 min at 4 ºC. Supernatants were collected and stored at -20 ºC until their use for LC/MS analysis. During the process, extracts were protected from light.

- **UHPLC/ESI-QTOF-MS Analysis**

Samples were injected on a 1290 Infinity series UHPLC system associated to an electrospray ionization source (ESI) with Jet Stream technology to a 6550 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany).

For the dissociation, a volume of 2 µL was injected in a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2% B (0-6 min), 2-50% B (6-10 min), 50-95% B (11-18 min), 95% B for 2 min (18-20 min), and returned to starting conditions 2% B in one min (20-21 min) to finally keep the re-equilibration with a total analysis time of 25 min.

The detector was functioning in full scan mode (m/z 50 to 2000), at 1 scan s^{-1}. Accurate mass measurement was confirmed through an automated calibrator delivery

system that constantly introduced a standard solution, containing masses of m/z 121.0509 (purine) and m/z 922.0098 (HP-921) in positive ESI mode; on the other hand m/z 112.9856 (TFA) and m/z 922.009798 (HP-921) were introduced in negative ESI mode. The capillary voltage was ±4000 V for positive and negative ionization mode. The temperature was set at 225 °C. The nebulizer and gas flow rate were 35 psig and 11 L min^{-1} respectively, fragmentor voltage to 75V and a radiofrequency voltage in the octopole (OCT RF Vpp) of 750 V.

For the investigation, MassHunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies) was used for control and acquisition of all data obtained with UHPLC/ESI-QTOF-MS.

For measurement, each sample was injected twice in six different concentrations to create calibration curves in which sample peak areas were extrapolated. The UHPLC-MS data analysis was performed by MassHunter Qualitative Analysis (Agilent Technologies), Software version B.08.00, using Molecular Feature Extraction (MFE).

All measurements were carried out using red and black fruit.

2.7 RNA Extraction and RT-qPCR analysis

Prior to RNA extraction, the fruits were removed from the -80 ◦C freezer and grounded to a fine powder with liquid nitrogen using an RNAase free mortar and pestle. Total RNA was isolated from each replicate with Plant/Fungi Total RNA Purification kit (50) (NORGENTM) (DNAase treatment included) and RNA integrity was confirmed by using NanodropTM.

The retrotranscription was carried out using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were executed using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 ºC, 30 min 42 ºC, 5 min 85 ºC, and hold at 4 ºC. The amplification were performed with a MiniOpticon Real Time PCR System (Bio-Rad): 3

min at 95 ºC and then 39 cycles consisting of 15 s at 95 ºC, 30 s at 50 ºC and 30 s at 72 ºC, followed by melting curve to verify the results. To explain the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Regulatory genes of the phenylpropanoids biosynthetic pathway analysed in blackberry were: *RuCHS*, *RuFLS*, *RuF3´H*, *RuDFR*, *RuLAR*, *RuANS* and *RuANR*. The transcription factor Ru*MYB5* was also analysed. Reference gene was *Actin*. The primers used are provided in Table 3.3.1. The primers were designed with the Primer3Plus program. Under design conditions, a PCR fragment size of 100-120 bp was specified. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

Table 3.3.1 Forward and reverse primers used in qPCR analysis.

Gene	Gene code	Forward primer	Tm (ºC)	Reverse primer	Tm (ºC)
RuCHS	Chalcone synthase [EC 2.3.1.74]	5´ATGGTGGTTGTTGAAATTCC	61.1	5´CTGGATTGCACACCCAGGTGGCCC	79.4
RuFLS	Flavonol synthase [EC 1.14.20.6]	5´CCTACAGGGAAGTCAATGAGAAA	63.1	5´CACATGGGATTTCAGTACCTTCT	62.9
RuF3´H	Flavonid-3´-hydroxylase [EC 1.14.14.82]	5´CCTATCTCCAAGCTGTCATCAAG	63.8	5´GTGGTATCCGTTGATTTCACAAC	64.1
RuDFR	Dihydroflavonol reductase [EC 1.1.1.219]	5´AATCAGAAGAAGGTGAAGC	55.9	5´CATTAKSACAAGTTTGGTG	50.2
RuLAR	Leucocyanidin reductase [EC 1.17.1.3]	5´GTGGAGTCCCATACACGTACATT	63.6	5´CTGAAACTGATCTAACGGTGGAA	64
RuANS	Anthocyanidin synthase [EC 1.14.20.4]	5´TTGGTCTGGGATTAGAAGAAAGG	64.2	5´CTGAGGGCATTTTGGGTAGTAAT	63.9
RuANR	Anthocyanidin reductase [EC 1.3.1.77]	5´TCGCAATGTACTTCCAAGAAAC	62.9	5´CTTCATCAGCTTACGGAAATCAC	63.6
RuMYB5	MYB family transcription factor	5´ACTCAATCCAGACTCCTCATCTG	63.5	5´AGGAAGTGATTGGACTTTTAGGG	63.2
RuACT	Actin	5´ATGTTCCCTGGTATTGCAGAC	62.7	5´CCACAACCTTGATCTTCATGC	64.4

Garcia-Seco et al. 2015b. The primers were designed with the Primer3Plus program. Under design conditions, a PCR fragment size of 100-120 bp was specified.

2.8 Statistical analysis

To check the statistical differences between the results obtained, an analysis of variance (ANOVA) was used. In the qPCR experiment and in the bioactives measurement by colorimetric methods (with only one independent variable), a one-way ANOVA was used. In the bioactive analysis by UHPLC/ESI-QTOF-MS, with two independent variables, a two-way ANOVA (factorial ANOVA) was used. In both cases, prior to ANOVA analysis, homoscedasticity and normality of the variance were checked with Statgraphics plus 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p < 0.05$) a Fisher test was used (Sokal and Rohlf, 1980).

3. Results

3.1 Fruit production

In control plants, 6167 ± 75 g per plant were recorded; in plants inoculated with N.21.4, 6277 ± 50 g per plant, and in plants inoculated with ME, 5680 ± 45 g per plant. Plants inoculated with ME showed statistically significant differences when compared with those inoculated with live strain (N 21.4) and control plants, which registered a higher fruit yield.

3.2 Bioactive content of fruit from non-inoculated plants

The differences between red and black fruit of control plants (not inoculated with any bacterial treatment) were analysed.

The amount of flavonols, measured by colorimetry, was very similar in red control fruit (0.46 ± 0 g of catechin equivalents kg^{-1} of FW) and in black control fruit (0.47 ± 0 g of catechin equivalents kg^{-1} of FW). However, in the case of phenols, measured by colorimetry, black control fruit showed significant higher content (3.02 ± 0.03 g of gallic

acid equivalents kg^{-1} of FW) than red control fruit (2.83 ± 0.02 g of gallic acid equivalents kg^{-1} of FW). In the case of anthocyanins, measured by colorimetry, black control fruit also showed significant higher content (0.63 ± 0 g of cyaniding 3-O-glucoside equivalents kg^{-1} of FW) than red control fruit (0.12 ± 0 g of cyaniding 3-O-glucoside equivalents kg^{-1} of FW).

Regarding the specific bioactives measured by UHPLC/ESI-QTOF-MS, it was seen that black control fruit had a significantly lower concentration of all the measured bioactives, except for quercetin aglycone and phloridzin (1.7 times higher in black control fruit) and cyanidin-3-O-glucoside (3.7 times higher in black control fruit), than red control fruit.

3.3 Bioactives measurement

The total amount of bioactives in red and in black fruit was measured by colorimetry. Figure 3.3.1 shows the fold-increase/decrease of bioactives in red fruit and in black fruit of each treatment compared to the bioactives present in the fruit of control plants: Figure 3.3.1 A) fold-increased in flavonols compared to control; B) fold-increased/decreased in phenols compared to control and C) fold-increased/decreased in anthocyanins compared to control.

In flavonols (Fig. 3.3.1 A)), significant differences between both treatments (N 21.4 and ME) were seen in red and in black fruit. With both treatments and in both fruit stages there was an increase in flavonol content compared with controls, the greatest increase occurring in black fruit of plants inoculated with the strain N 21.4 (1.13 fold) and in red fruit of plants inoculated with ME (1.08 fold).

In phenols (Fig. 3.3.1 B)), significant differences between both treatments (N 21.4 and ME) were seen in red and in black fruit. A slight increase was recorded in red fruit of plants inoculated with ME and in black fruit of plants inoculated with N 21.4.

However, a decrease in the amount of phenols was seen in red fruit of plants inoculated with N 21.4, and in black fruit of plants inoculated with ME.

In anthocyanins (Fig. 3.3.1 C)), significant differences between both treatments (N 21.4 and ME) were only seen in red fruit. A deep decreased in the quantity of anthocyanins was seen in the red fruit of plants inoculated with N 21.4. However, a 1.2 fold-increased was seen in the black fruit of plants inoculated with both treatments.

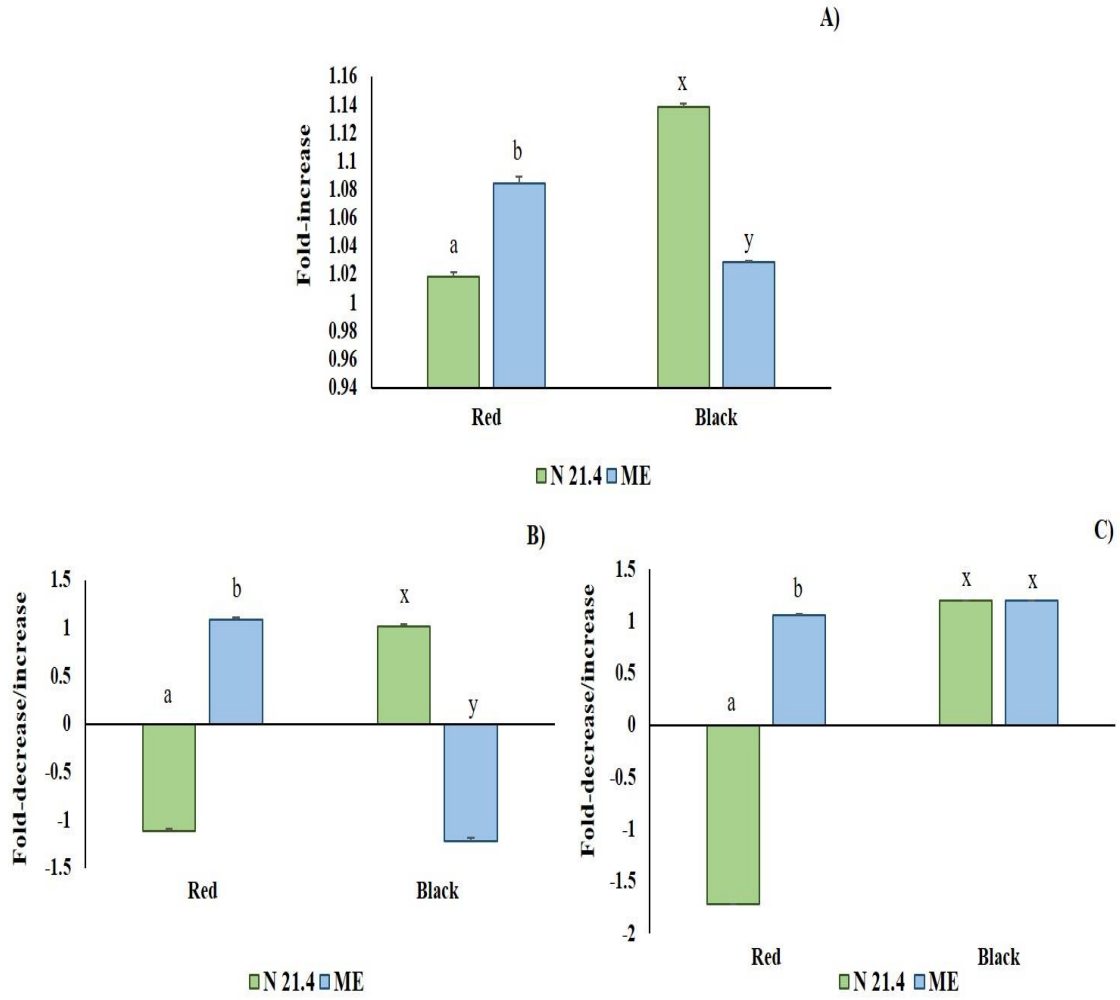

Figure 3.3.1 Fold-increase/decrease in the content of bioactives, measured by colorimetry, with respect to control: A) flavonols; B) phenols and (C) anthocyanins in red and black fruit of blackberry plants treated with *P. fluorescens* N 21.4 and with its metabolic elicitors (ME). Different letters indicate significant differences ($p < 0.05$): "a and b" indicate differences between treatments in red fruit; and "x and y" between treatments in black fruit.

3.4 Bioactives analysis by UHPLC/ESI-QTOF-MS

Methanolic extracts were analysed by LC/ESI-QTOF-MS. All the compounds were identified by comparing the retention times and spectra with reference standards. The main compounds found (11) and their concentration (μg g^{-1}) are shown in Table 3.3.2. They are grouped as flavonols (5), flavanols (2), dihydrochalcone (1), anthocyanine (1), hydroxycinnamic acid (1) and flavone (1). Red and black fruit of control and inoculated plants (N 21.4 and ME) were compared.

In red fruit, all compounds had significant higher values (between 1.2 and 1.6 times higher) in ME-treated plants than in N 21.4-treated plants. Values of N 21.4-treated-plants were similar to those of control plants, except for (-)-epicatechin and (+)-catechin values, which were 1.2 times higher in treated plants (this being significant), and for cyaniding-3-O-glucoside that were significantly lower in treated plants.

In black fruit, the opposite trend was observed: all compounds (except cyaniding 3-O-G) had significant higher values in N 21.4-treated plants (between 1.1 and 1.5 times higher) than in ME-treated plants. The values of ME-treated-plants were similar to those of control plants, except for (-)-epicatechin and (+)-catechin values that were 1.1 times significantly higher in treated plants, and for quercetin aglycone and phloridzin that were significantly lower in treated plants.

The variation of the content of (+)-catechin, (-)-epicatechin and cyaniding 3-O-G, the phenolic compounds most affected by our treatments, appear in figure 3.3.2, in a chromatogram. The rest of chromatograms, in which bioactive composition between treatments and between red and black fruit is compared, are shown in Annex 7.2.

Table 3.3.2 Main flavonoid and phenolic compounds found in blackberry fruit and their concentration in μg g^{-1}. Control, N 21.4 and ME treatments were compared in red and black fruit.

		Control		N 21.4		ME	
Polyphenol class	**Compound**	**Red**	**Black**	**Red**	**Black**	**Red**	**Black**
Flavonols	Quercetin aglycone	<LOQ	0.621±0 (α)	<LOQ	0.172±0.08 (β)	0.235±0.11 a	0.185±0.01 b (β)
	Quercetin 3-O-glucoside	13.8±0.3 a (x)	13.2±0.1 a (αβ)	13.1±0.55 a (x)	14.1±0.3a (α)	17.7±0.3 a (y)	12.9±0.4 b (β)
	Quercetin 3-O-rutinoside	7.5±0.25 a (x)	6.2±0.1 b (α)	7.4± .35 a (x)	7.6±0.15 a (β)	9.7±0.25 a (y)	6.4±0.15 b (α)
	Kaempferol 3-O-rutinoside	0.661± 0 a (x)	0.44±0.01 b (α)	0.631±0.01 a (x)	0.501±0 b (β)	0.814± .03 a (y)	0.426±0.2 b (α)
	Kaempferol 3-O-glucoside	1.1±0.05 a (x)	0.8±0.05 b (α)	0.9±0 a (y)	0.8±0.05 b (α)	1.3 ± 0 a (z)	0.7±0.05 b (α)
Flavanols	(-)-Epicatechin	302.4±8.3 a (x)	226.7±3.4 b (α)	356±0.45 a (y)	304.4±0.45 b (β)	386.8±8.05 a (z)	247.8±1.8 b (γ)
	(+)-Catechin	20.8±0 a (x)	9.5±0.35 b (α)	25.4±0.65 a (y)	17.2±0.55 b (β)	33.7±0.75 a (z)	11.1±0.25 b (γ)
Dihydrochalcone	Phloridzin	0.263±0.01 a (x)	0.442±0.02 b (α)	0.216±0 a (y)	0.397±0 b (β)	0.329±0 a (z)	0.392±0 b (β)
Anthocyanine	Cyanidin 3-O-glucoside	838.3±21.16 a (x)	3162.26±42.808 b (α)	560.74±18.18 a (y)	2968.72±46.28 b (β)	727.23±17.99 a (x)	3117.82±38.40 b (α)
Hydroxycinnamic acid	Chlorogenic acid	0.603±0.04 a (x)	0.333±0.01 a (α)	0.597±0.01 a (x)	0.256±0.21 b (α)	0.779±0.05 a (x)	0.335±0.03 b (α)
Flavone	Luteolin	0.05±0.01 a (x)	0.029±0 a (α)	0 (y)	0 (α)	0.091±0.02 a (x)	0 b (α)

Letters a and b indicate significant differences between red and black fruit within the same treatment; x, y and z indicate significant differences between treatments in red fruit; and letters α, β, γ indicate significant differences between treatments in black fruit. <LOQ means sample under the limit of quantifying.

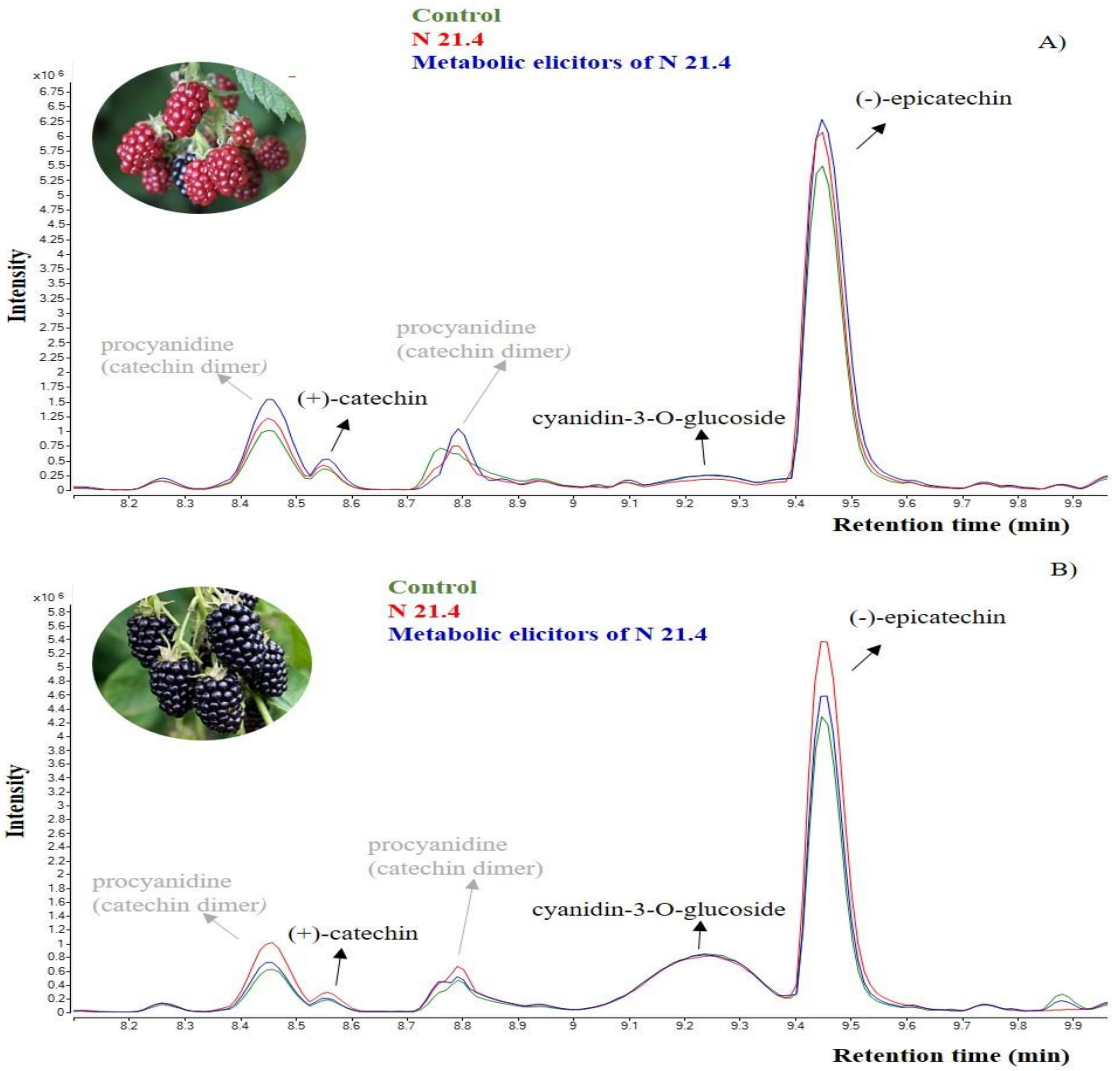

Figure 3.3.2 Enlarged chromatogram (from 8.0 to 9.9 min) visually comparing the content variation of three main phenolic compounds in red (A) and black (B) fruit and between treatments. Green line represents control, red line *P. fluorescens* N 21.4 and blue line ME treatments.

3.5 RT-qPCR analysis

The differential expression of flavonoids pathway genes in the fruit of treated plants appears in figure 3.3.3, showing red fruit (3.3.3 A)) and black fruit (3.3.3 B)).

In the red fruit, (Fig. 3.3.3 A)), of plants treated with N 21.4, *RuF3´H*, *RuFLS*, *RuDFR*, *RuANS* and *RuANR* genes appeared upregulated. *RuCHS*, *RuLAR* and *RuMYB5* were not significantly affected by this treatment. In the case of ME-treated-plants, *RuCHS* and *RuDFR* genes appeared upregulated and *RuF3´H*, *RuFLS*, *RuANS*, *RuLAR*

and *RuANR* were downregulated. Differential expression of all genes, except the one of the transcriptional factor *RuMYB5*, had significant differences (*) between treatments (N 21.4 versus ME).

In the black fruit, (Fig. 3.3.3 B)), of plants treated with N 21.4, *RuFLS*, *RuANS* and *RuANR* genes were upregulated. The rest of genes were not significantly affected by this treatment. In ME-treated plants, *RuF3´H*, *RuFLS*, *RuANS* and *RuANR* genes were upregulated and *RuLAR* was downregulated. Differential expression of all genes, except the one of the transcriptional factor *RuMYB5*, had significant differences (*) between treatments.

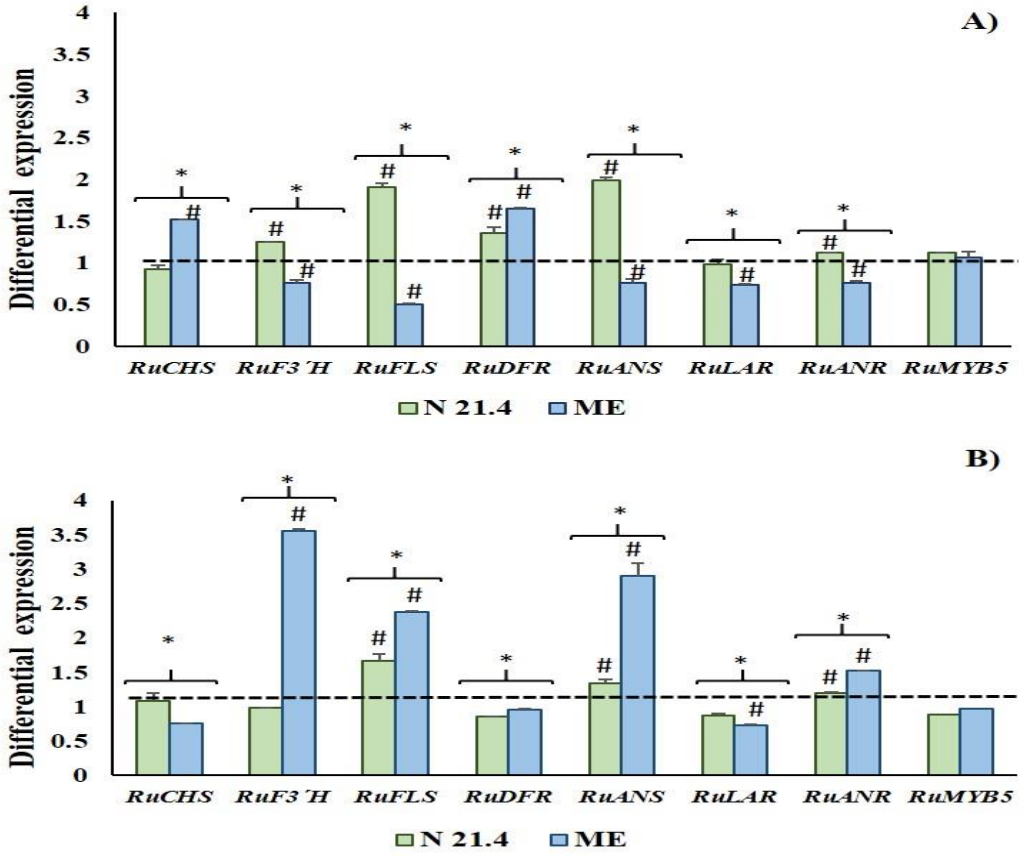

Figure 3.3.3 Differential expression of phenylpropanoids pathway genes in red fruit (A) and in black fruit (B) of blackberry plants inoculated with *P. fluorescens* N 21.4 and with its metabolic elicitors (ME). Asterisks represent statistically significant differences ($p < 0.05$) between treatments and hashtags represent statistically significant differences ($p < 0.05$) with the control. Expression of 1 is that of the control (horizontal black dashed line).

4. Discussion

Food security, as well as the improvement and protection of crops, in a more ecofriendly environment is nowadays a topic of great importance. This calls for more efficient and non-polluting agricultural methods and one of the most challenging tool to achieve this goal is the use of biological agents (Capanoglu, 2010), such as beneficial rhizobacteria or their derived elicitors. In the present work, the beneficial rhizobacterium *P. fluorescens* N 21.4 and its metabolic elicitors have been used as plant inoculants in commercial cultivars of blackberry (*Rubus* cv. Loch Ness) to elicit flavonoid metabolism in the fruit.

Blackberries are strongly rich in flavonoids, which have essential functions in plant defence against biotic (fungi, bacteria, herbivores (Kim et al. 2011; Ferreyra et al. 2012; Nile and Park, 2014)) and abiotic stress (light, temperature, water supply, minerals, CO_2, etc. (Ramakrishna and Ravishankar, 2011) and they also have many beneficial properties to human health (Rodriguez-Mateos et al. 2013; Manganaris et al. 2013) when they are included in the diet. For this reason, plant flavonoid metabolism has been studied in depth with the aim of modifying and obtaining a better performance (higher accumulation of beneficial secondary metabolites, higher antipathogenic capacity, better plant fitness, etc.).

The biosynthesis of flavonoid compounds starts from the amino acid phenylalanine and produces phenylpropanoids, which are channeled into the flavonol-anthocyanin pathway by chalcone synthase (CHS). Further reactions involve chalcone isomerase (CHI), which generates naringenin, flavanone-3-hydroxylase (F3H), which hydroxylates naringenin until dihydrokaempferol, which is then hydroxylated by flavonoid-3-hydroxylase (F3´H) and transformed in dihydroquercetin. Flavonols are synthesized at this point by the flavonol synthase (FLS), forming kaempferol or quercetin (depending on where FLS introduces a double bond). Dihydroquercetin is then reduced

by dihydroflavonol reductase (DFR) to obtain leucocyanidin. Anthocyanins are synthetized at this point by the anthocyanidin synthase (ANS) obtaining cyanidin. (+)-Catechin is obtained when leucocyanidin reductase (LAR) reduces leucocyanidin, and (-)-epicatechin is obtained when anthocyanidin reductase (ANR) reduces cyaniding (Ferreyra et al. 2012; Jian et al. 2016).

The study of regulatory genes encoding those enzymes of the phenylpropanoid and flavonol-anthocyanin pathways is crucial for modifying the accumulation of secondary metabolites of interest at the end of the route. In our work, an inverse relationship (in red and black fruit) between gene expression and accumulation of secondary metabolites has been seen, except for the *RuDFR* gene, the first gene of the anthocyanins route, which had a direct effect in the increase of cyanidin 3-O-glucoside (Fig. 3.3.3 and Table 3.3.2). However, in the study of Chen et al. 2012, some genes involved in anthocyanin and proanthocyanidin biosynthesis were investigated and the expression levels of genes agreed with the final products accumulated. Furthermore, they saw that enzymes encoded by the structural genes of the pathway had two peaks of maximum activity: at the beginning of the fruit ripening and at the end. The same pattern of enzyme activity was observed in strawberry by Halbwirth et al. 2006, except for DFR and FLS enzymes, which only had one peak of activity at red-black stage (also seen by Almeida et al. 2007). This last was consistent with our results in which *RuDFR* gene was upregulated in red stages of the fruit, but downregulated in black, suggesting that Dihydroflavonol reductase (DFR) only had one peak of activity at this stage of ripening (in red fruit).

Regarding to the effects of our treatments in red blackberry fruit, ME treatment had effects in all the studied genes, downregulating *RuF3´H*, *RuFLS*, *RuANS*, *RuLAR* and *RuANR* and upregulating *RuDFR*. This was directly linked to an increase in all the

compounds derived from the activity of the enzymes encoded by the above downregulated cited genes. The plants treated with ME had red fruit with a higher concentration of flavonoids, highlighting quercetin and kaempferol derivatives, catechin, epicatechin and anthocyanins. However, red fruits of N 21.4-treated-plants showed higher differential expression and lower concentration of flavonoid compounds, which reinforces our idea of a supposed inverse relationship between gene expression and final compounds accumulation. A hypothesis that could explain this phenomenon is that, since there were more transcripts of these genes, all the biosynthetic machinery of phenolic compounds worked faster and bioactives were accumulated in later stages of the route as derivatives of the compounds that we had measured. Probably, if compounds had been measured in subsequent steps of the route, an accumulation of them would have been found (https://www.genome.jp/kegg-bin/show_pathway?map00944; https://www.genome.jp/kegg-bin/show_pathway?map00942).

In black fruit, the same inverse relationship was observed, but in this case it was the plants treated with N 21.4 which showed less differential expression and greater accumulation of all the secondary compounds, except anthocyanins, which were more accumulated in fruits of ME-treated plants. This is also consistent with a greater expression of *RuDFR* observed with the ME treatment. *RuDFR* is the only gene in which a direct relationship between differential expression and anthocyanin accumulation was observed in red and in black fruit. *RuDFR* had the same behavior as that of *DFR* in the work of Almeida et al. 2007 and Garcia-Seco et al. 2015a and Garcia-Seco et al. 2015b.

At the same time, *RuCHS* seem to be a fundamental regulatory gene related to the accumulation of secondary metabolites. When *RuCHS* is upregulated, compound accumulation occurs along the pathway. This had been previously seen in the work of Garcia-Seco et al. 2015b.

MYB transcription factors are among the most important regulators involving the flavonoid biosynthesis (Hichri et al. 2011; Garcia-Seco et al. 2015b). In the present work, gene expression of *RuMYB5* was analysed since it was the positive regulator of *RuDFR*, *RuANR*, and *RuLAR* genes more affected by another beneficial rhizobacteria of our collection (Gutierrez et al. 2017b). In that work, the positive regulation supposed an increase in catechin synthesis, precursor of proanthocyanidins, which were accumulated in fruits. However, in our study, *RuMYB*5 did not exceed control differential expression. In the work of Thole et al. 2019, it is said that *RuMYB5* from cultivated blackberry has a peak of expression at the early intermediate ripening fruit stage, which could be related with a higher concentration of catechin and epicatechin in green-red fruit stages. In that study it is also said that Ru*MYB*5 interacts with other transcription factors (*RuTTG1* and *RubHLH1*) related to proanthocyanidins synthesis, showing decreasing transcript levels during ripening. Despite not having seeing differential gene expression of *RuMYB5* in our work, the tendency discussed in Thole et al. 2019 work has been seen, with a higher concentration of catechin and epicatechin in red fruit and a lower concentration in black fruit. However, in strawberry, *FaMYB5* transcripts have been seen to accumulate steadily during fruit development. Moreover, Chang et al. 2012, saw that not *RuMYB5*, but *RuMYB10* was the transcription factor that better controlled proanthocyanidin biosynthesis. Hence, it can be concluded that these transcription factors might be affected differently by diverse bioeffectors or by different crop conditions.

On the other hand, it has been seen that in early maturation stages (red fruit), (-)-epicatechin and (+)-catechin were the predominant phenolic compounds and their concentration decreased during ripening, when anthocyanins increased. This was consistent with the gene expression of *RuANR*, *RuLAR*, and *RuANS* genes, respectively. Chang et al. 2012 and Garcia-Seco et al. 2015b observed the same in blackberry and

Almeida et al. 2007 in strawberry. Hence, there is an obvious redirection of flavonoid biosynthesis from flavanol to anthocyanin formation during the complex developmental process of fruit ripening (Halbwirth et al. 2006; Chang et al. 2012). The different groups of polyphenols formed during fruit ripening fulfill different important functions, like herbivore deterrence with the presence of astringent flavanols in early stages (Garcia-Seco et al. 2015a) or visual attraction of ripen fruit. Anthocyanin, epicatechin and catechin have been seen as key factors affecting fruit flavor and nutrition properties in persimmon, grape, berries and many other fruits (Fernandez et al. 2007; Hümmer et al. 2008; Akagi et al. 2009). However, in grapes, catechin and epicatechin accumulation occurs immediately after fruit-set and maximum levels of accumulation are reached around véraison (Kennedy et al. 2001). Consequently, these species might be under control of different regulatory mechanisms.

In our *Rubus* cv. Loch Ness plants, treatments inoculated (rhizobacterium N 21.4 and ME) affected not only the total phenolic and flavonoids during ripening, but also the profile of different flavonols, flavanols and anthocyanins (also seen by Garcia Seco et al. 2015b with N 21.4). In general, the fruits of inoculated plants, and specially the fruits of ME-inoculated plants, accumulated secondary metabolites in greater concentration than non-inoculated control plants. A remarkable fact was a major increase of flavanols ((-)-epicatechin and (+)-catechin) at both stages of fruit ripening with both treatments. These compounds have strong antioxidant capacity and they are able to reduce oxidative stress in plants (Martinez et al. 2005; Mahajan and Yadav, 2013). They have also important cardiovascular benefits for humans (Schroeter et al. 2005; Fraga et al. 2018). Epicatechin also acts as phytoanticipins in some fruits (Guetsky et al. 2005) giving fungal and bacterial resistance to infection.

The elicitation of the secondary metabolism of *P. fluorescens* N 21.4-treated plants was again demonstrated and the elicitation of secondary metabolism of ME-treated plants was also evidenced. This secondary metabolism activation not only suppose an improvement in the nutritional quality of the fruit by increasing their metabolite content, but it also suppose that plants trigger their defences as a result of the activation of this secondary metabolism (Conrath, 2011). In general, ME had more marked effects on plant elicitation enhancing the amount of many of the metabolites accumulated in fruits. Hence, it is understood that ME-treated plants had a more active secondary defensive metabolism and therefore they were more protected against biotic and abiotic stress; they had better fitness. The fruit production in ME-inoculated plants was slightly lower, which is a clear symptom of the ME´s elicitation capacity, which improved the quality of fruit slightly compromising fruit yield. Thus, ME-treated plants were more primed, were more fit, and had slightly fewer fruits, but fruits with better nutritional quality. Previous results from our group, with the same plant and with another rhizobacteria (Gutierrez Albanchez et al. 2018), verified that elicitation supposes a redirection of resources towards defensive metabolism, slightly compromising fruit yield. They also support that metabolic changes inherent to the primed status (Martinez-Medina et al. 2016) resulted in allocation of carbon sources to defence metabolism (Mauch-Mani et al. 2017).

In summary, the effectiveness of ME as plant inoculants for the elicitation of blackberry secondary metabolism was shown, as well as their capacity to modify flavonoid biosynthetic pathway. The ME of *P. fluorescens* N 21.4 were able to modulate gene expression in fruit of *Rubus* cv. Loch Ness, and to affect the profiles of secondary metabolites, increasing the synthesis and accumulation of them mainly during red stage and potentially increasing nutritional properties of subsequent black fruit. The ME used as plant inoculants also have advantages related to the management and maintenance of

inoculums. They are effective, cheap to produce, easy to manage, environmentally friendly, and they do not cause the biosecurity problems that live rhizobacteria could cause (Ngoroyemoto et al. 2019). The application of ME of beneficial rhizobacteria as plants inoculants therefore opens a feasible new window towards the improvement of the nutritional qualities of crops using innovative and more ecofriendly agrifood techniques.

From all the above, we conclude that the efficacy of ME of *P. fluorescens* N 21.4 in the elicitation of blackberry secondary metabolism has been demonstrated. ME are efficient, profitable and ecological plant inoculants that could be alternative to agrochemicals or could be either alternatives or complementary to rhizobacteria-based products. We can also conclude that, through the study of the phenylpropanoid pathway in blackberry fruit, the regulatory role of *RuCHS* in the accumulation of secondary metabolites at the final stages of the pathway has been shown, as well as the role of *RuDFR* in the increase of synthesis and accumulation of cynidine-3-O-glucoside.

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3.4 Identifying the compounds of the metabolic elicitors of *Pseudomonas fluorescens* N 21.4 responsible for their ability to induce plant resistance

Identifying the compounds of the metabolic elicitors of *Pseudomonas fluorescens* N 21.4 responsible for their ability to induce plant resistance

Helena Martin-Rivilla [1]*, F. Javier Gutierrez-Mañero [1], Ana Gradillas [2], Miguel O.P. Navarro [3], Galdino Andrade [3], and José A. Lucas [1]

[1] Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain; jgutierrez.fcex@ceu.es; alucgar@ceu.es

[2] Centre for Metabolomics and Bioanalyses, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain; gradini@ceu.es

[3] Laboratory of Microbial Ecology, Department of Microbiology, Londrina State University, Londrina 86051-990, Brazil; micromiguel@gmail.com; andradeg@uel.br

*Corresponding author: Helena Martín Rivilla, helenamartin92@gmail.com

+913 72 47 85

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Abstract

In this work, the metabolic elicitors extracted from the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 were sequentially fragmented by vacuum liquid chromatography to isolate, purify and identify the compounds responsible for the extraordinary capacities of this strain to induce systemic resistance and to elicit secondary defensive metabolism in diverse plant species. To check if the fractions sequentially obtained were able to increase the synthesis of isoflavones and if, therefore, they still maintained the eliciting capacity of the live strain, rapid and controlled experiments were done with soybean seeds. The optimal action concentration of the fractions was established and all of them elicited isoflavone secondary metabolism, being the fractions that had been extracted with n-hexane more effective. The purest fraction was the one with the highest eliciting capacity and was also tested in *Arabidopsis thaliana* seedlings to induce systemic resistance against the pathogen *Pseudomonas syringae* pv. *tomato* DC 3000. This fraction was then analysed by UHPLC/ESI–QTOF–MS, and an alkaloid, two amino lipids, three arylalkylamines and a terpenoid were tentatively identified. These identified compounds could be part of commercial plant inoculants of biological and sustainable origin to be applied in crops, due to their potential to enhance the plant immune response and since many of them have putative antibiotic and/or antifungal potential.

Keywords: *Pseudomonas fluorescens* N 21.4; metabolic elicitors; isoflavone elicitation; induced systemic resistance; sphingolipids; terpenoids

1. Introduction

Protecting crops against diseases caused by pathogens in agricultural systems has always been a constant challenge when trying to maximize crop yields, minimize economic losses and ensure quality food worldwide (Miller et al. 2017). As 2020 has been declared International Year of Plant Health by the FAO (Food and Agriculture Organization of the United Nations), the current challenge is to find effective, ecofriendly and at the same time, low-cost agriculture control methods that guarantee the sustainability of crop production while eliminating negative impact on the environment.

It has been widely demonstrated that biological agents, such as beneficial microorganisms, are able to enhance plants' immune systems, inducing systemic resistance (ISR) and/or systemic acquired resistance (SAR) (Van Loon et al. 1998; Pieterse et al. 2014; Mauch-Mani et al. 2017). This phenomenon of enhancing a plant's immune system is called elicitation, and it supposes that cells exposed to external factors activate defence mechanisms by triggering and regulating some biochemical and molecular responses, increasing the synthesis of specific molecules with a protective role (Leite et al. 1997). After elicitor perception, signals are transported throughout the plant, triggering local and systemic responses (Pascholati et al. 2003) and leading to the generation of reactive oxygen species (ROS), phytoalexin biosynthesis, increased synthesis of antioxidant secondary metabolites, reinforcement of plant cell wall, deposition of callose, synthesis of defence enzymes, accumulation of pathogenesis-related proteins (Van Loon et al. 2006), etc.

Originally, the term elicitor referred to molecules capable of inducing the production of phytoalexins, but nowadays it is commonly used for compounds that stimulate any defensive line (Ebel and Cosio, 1994; Hahn, 1996; Nürnberger, 1999). Natural elicitor molecules derived from microorganisms can induce similar defence

responses in plants to those responses induced by the live microorganisms (Gómez-Vásquez et al. 2004). Different types of natural elicitor molecules have been characterized, including structural molecules, such as carbohydrate polymers, lipids, and bacterial flagellin (Ramirez-Prado, 2018), and metabolic elicitors, released to the medium, such as antibiotics and secondary metabolites (Gozzo and Faoro, 2013; Wu et al. 2018).

A wide range of metabolic elicitors produced by fungi and beneficial rhizobacteria have been found to induce in plants the synthesis of protective compounds, such as phytoalexins, defensins, phenolic acids, and flavonoids, that directly suppress pathogens (Baker et al. 1997). They can reduce plant diseases through elicitation of physical and chemical processes linked to systemic plant defence mechanisms (Sarma et al. 2002). This is why plant inoculants made of beneficial rhizobacteria and/or their metabolic elicitors have been seen as feasible and effective alternatives to chemical phytosanitaries to counteract the attack of pathogens and also to face to diverse biotic and abiotic stresses (Ramos-Solano et al. 2010a; Martin-Rivilla et al. 2019). However, using elicitor molecules instead of living bacteria is a way to reduce the cost and to simplify the production and subsequent management of plant inoculants. Products made of partially purified or purified compounds derived from bacterial metabolism (de Oliveira et al. 2016) are cheap to produce; easy to manage; respectful with the environment; not harmful or toxic to human health or to other organisms, as their targets are directly the plants; and do not cause biosecurity problems as bacteria could cause (Ngoroyemoto et al. 2019). Furthermore, they can be easily applied by spraying (Thakur et al. 2013) and they do not require special preservation conditions like live microorganisms because they are stable during long periods of exposure to light and/or high temperatures (Stockwell and Stack, 2007) and they do not lose viability during prolonged storage.

Among beneficial microorganisms, the genus *Pseudomonas* spp. and *Bacillus* spp. are the most studied genera concerning everything related to plant–pathogen–beneficial microorganism interactions and the improvement of plant immune system, because they are very abundant in the soil and because of their role in the suppression of pathogens (Navarro et al. 2017). Literature has shown that *Pseudomonas* spp. have extensive metabolic capabilities and adaptable biochemistry through their production of structurally varied bioactive molecules (Gross and Loper, 2009). Furthermore, it is largely known that *Pseudomonas* spp. are some of the most important microorganisms able to produce compounds with antibiotic or eliciting activity triggering SAR (Choudary et al. 2007) or ISR (Pieterse et al. 2014).

The potential of P*seudomonas* spp. to suppress plant pathogens has been demonstrated in many plant species and around the world (Haas and Defágo, 2005). They can be used as efficient and not-risky biocontrol agents to use in agriculture because they do not show pathogenic, allergenic or harmful risks to people or animals (Zanatta et al. 2007). Secondary metabolites isolated from *Pseudomonas* spp. that could be an alternative to the use of chemical compounds in the control of plant disease include phenazines, pyrrolnitrin-type antibiotics, betalactones, pyo compounds, indol derivatives, peptides, glycolipids, lipids, aromatic organic compounds and aliphatic compounds, among others (Gross and Loper, 2009; Lesinger and Margraff, 1919).

More specifically and within *Pseudomonas* spp. genus, the specie *Pseudomonas fluorescens* is a Gram-negative soil bacterium that has been widely studied in relation to its capacity to suppress other pathogenic microorganisms by producing siderophores, antibiotics and antifungal and antiparasitic compounds and to induce systemic resistance in plants through a vast variety of secondary metabolites with eliciting capacity (Lesinger and Magraff, 1919; Haas et al. 1991; Niedig et al. 2011; Jankiewicz and Koltonowicz,

2012). For all of this, certain strains have already been developed as commercial products for management of plant illnesses in agricultural settings (Stockwell and Stack, 2007).

The *P. fluorescens* strain N 21.4 was specifically used in the present work because its capacity to induce systemic resistance in different plant species, such as *Arabidopsis thaliana* (Domenech et al. 2007; Martin-Rivilla et al. 2019), *Solanum lycopersicum* (Ramos-Solano et al. 2010a), *Hypericum* sp. (Gutierrez-Manero et al. 2012), *Papaver* sp. (Bonilla et al. 2014) and blackberry (*Rubus* cv. Loch Ness) has been largely demonstrated (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015; Martin-Rivilla et al. 2020a; Martin-Rivilla 2020b). Its metabolic elicitors have also been demonstrated to induce systemic resistance in *A. thaliana* (Martin-Rivilla et al. 2019) and to elicit flavonoid metabolism in the leaves and in the fruits of cultivars of blackberry (Martin-Rivilla et al. 2020a; Martin-Rivilla 2020b).

For all the above, the objective of the present study was to isolate, purify, test and identify the compound or set of elicitor compounds of *P. fluorescens* N 21.4, obtained from sequential fractionations of its metabolic elicitors by vacuum liquid chromatography (VLC), responsible for plant elicitation. To corroborate fractions' capacities to elicit secondary metabolism, some experiments under controlled conditions were performed in soybean seeds to enhance isoflavone synthesis and in *A. thaliana* to induce systemic resistance. A final analysis to obtain the profile of main compounds present in the purest fraction of the metabolic elicitors was made by ultra-high performance liquid chromatography (UHPL), with an electrospray ionization source (ESI) and a quadrupole time-of-flight mass spectrometry analyser (QTOF-MS). Eight compounds were tentatively identified and classified into different families: alkaloids, amino lipids, terpenoids and arylalkylamines.

2. Material and methods

Sequential extraction, fragmentation and purification of the metabolic elicitors of *P. fluorescens* N 21.4 were carried out (Figure 3.4.1 and Table 3.4.1). Firstly, a liquid–liquid phase separation was made (Sumayo et al. 2013), followed by two sequential VLCs. All the sequentially fragmented fractions were inoculated in soybean seeds to analyse their capacity to elicit isoflavone secondary metabolism pathway. Final isolation and purification were performed. The resulting fraction of the entire process of fragmentation and purification was tested in an ISR experiment in *A. thaliana* seedlings. Finally, this fraction, the purest one, was analysed by UHPLC/ESI–QTOF–MS to characterize its composition.

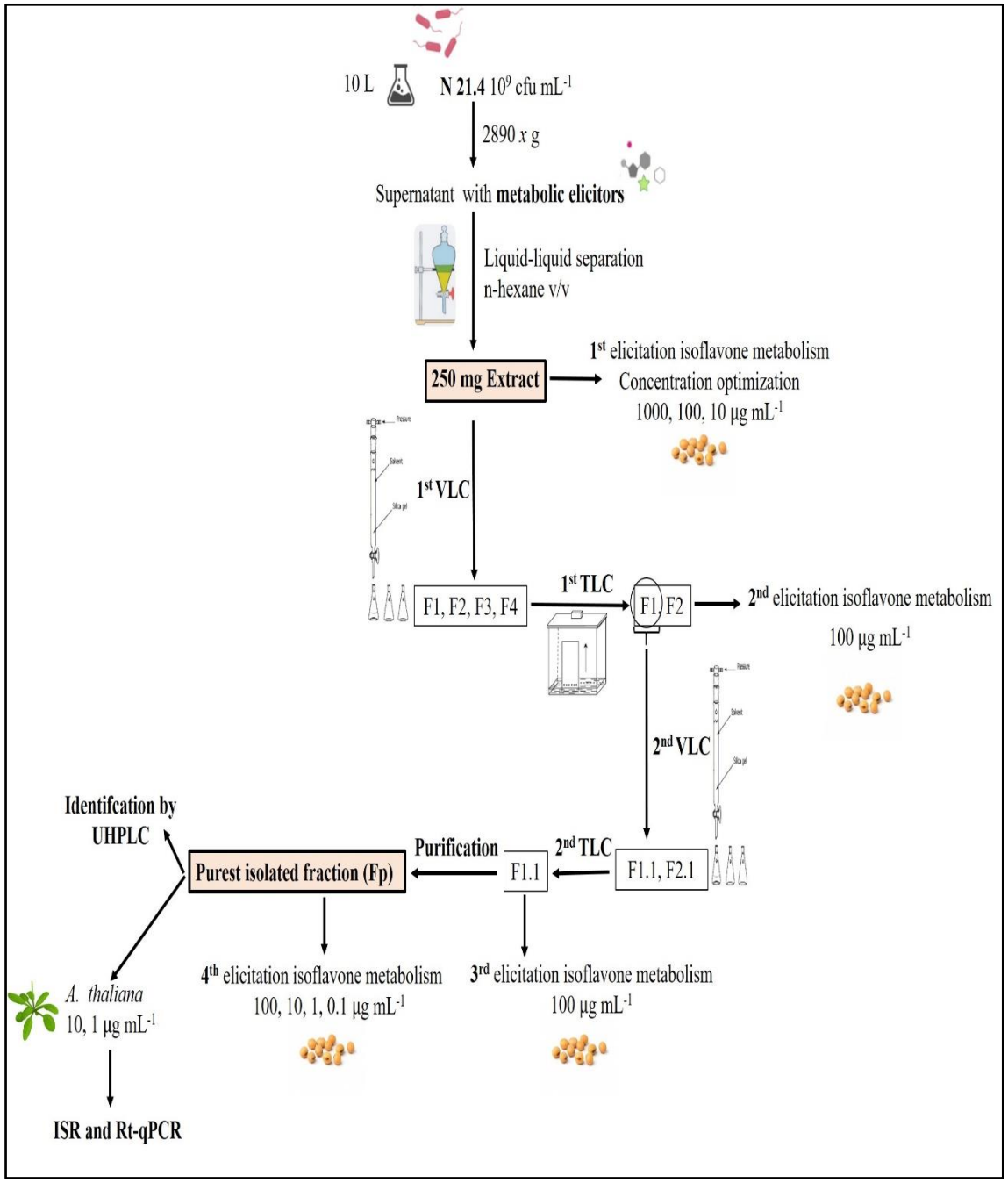

Figure 3.4.1 Representative scheme of the sequential extraction, fractionation and purification of the metabolic elicitors of *P. fluorescens* N 21.4. Shown are the growth of the bacterium in nutrient broth, the liquid–liquid phase separation with n-hexane, the process of fractionation and purification by two sequential vacuum liquid cromatographies, the four elicitation experiments of isoflavone metabolism, the ISR experiment in *A. thaliana* and the final compound identification by UHPLC.

Table 3.4.1 Experimental design and treatments used.

Method Used	Treatment	Characteristics	Use
Growth in nutrient broth	*P. fluorescens* N 21.4	10 L 10^9 cfu mL^{-1}	Liquid-Liquid separation ISR as positive control
Liquid-Liquid separation (bacterial culture)	Dry extract	250 mg Aliquots dissolved in 10% DMSO	1st Isoflavone elicitation: Concentration optimization (1000,100,10 µg mL^{-1}) 1st VLC
Liquid-Liquid separation (nutrient broth)	Control 1	Dissolved in 10% DMSO	1st,2nd,3rd and 4th isoflavone elicitation 1st and 2nd TLCs ISR as 0% of protection
Seed embryo cut	Control 2	Not inoculated soybean seeds	1st Isoflavone elicitation
1st VLC with dry extract	F1 F2 F3 F4	Hexane Dichloromethane Ethyl acetate Methanol	1st TLC 1st TLC 1st TLC 1st TLC
1st TLC with F1, F2, F3 and F4	F1 F2	100 µg mL^{-1} 100 µg mL^{-1}	2nd Isoflavone elicitation
2nd VLC with F1	F1.1 F1.2	Hexane Dichloromethane	2nd TLC 2nd TLC
2nd TLC with F1.1 and F1.2	F1.1	100 µg mL^{-1}	3rd Isoflavone elicitation
Purification of F1.1	Fp (Purest fraction)	1, 0.1 µg mL^{-1}	4th Isoflavone elicitation
ISR in *A.thaliana*	Fp	10, 1 µg mL^{-1}	Pathogen protection (%) qPCR (SA and JA/ET marker genes)
UHPLC/ESI–QTOF–MS	Fp	0.19 mg in 100 mL methanol LC-MS grade	Tentative compound identification

2.1 Bacterial strain

The bacterial strain used in this study was *P. fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a Gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca* Graham (Ramos-Solano et al. 2010a). The bacterial strain was stored at −80 °C in nutrient broth (CONDA) with 20% glycerol.

2.2 Metabolic elicitor extraction and control obtaining

The bacterial strain, stored at −80 °C in nutrient broth with 20% glycerol, was streaked onto nutrient agar (peptone 3 g $L^{-}1$, beef extract 5 g L^{-1} and agar 15 g L^{-1} pH 7) plates and cultivated for 24 h at 28 °C. After 24 h of growth, bacterial cells were scraped off the plates into 10 L of sterile nutrient broth (peptone 3 g L^{-1} and beef extract 5 g. L^{-1} pH 7) and incubated on a rotatory shaker at 28 °C and 180 rpm for 24 h.

Metabolic elicitors (released into the medium) were obtained by centrifuging the 10 L of N 21.4 culture at 2890 *x* g during 20 min at 4 °C. Cells were discarded and the remaining supernatant was evaporated in a stove at 60 °C until obtaining 1 L. This concentrated supernatant was filtrated through a 0.2 µm nitrocellulose filter and extracted with n-hexane (v/v). The extract was evaporated to dryness in a Buchi R-215 rotary evaporator at 50 °C (Sumayo et al. 2013). The dry extract was weight (250 mg) and stored at 4 °C protected from light and humidity.

To obtain the control 1, the same procedure was followed as for extracting the metabolic elicitors from the bacterium, but while carrying out the entire process exclusively with the nutrient medium (peptone 3 g $L^{-1}$and beef extract 5 g L^{-1} pH 7), in the absence of bacterium.

2.3 Elicitation of isoflavone metabolism in the soybean

To test the capacity of the sequentially obtained fractions to elicit isoflavone secondary metabolism, rapid induction tests were carried out in soybean seeds (BS-2606 Embrapa). Seeds were superficially disinfected with a 70% ethanol bath for 1 min, 5% sodium hypochlorite for 6 min and 5 washes with sterile distilled water. After that, seeds were kept imbibing in sterile distilled water for 4 h, in darkness and at room temperature. After imbibition, 90 seeds per treatment and 90 seeds for each control (control 1 and 2), were distributed in 3 replicates of 30 seeds each and put to germinate in sterile Petri dishes with

1% European bacteriological agar. A small longitudinal cut was made in the seeds embryo without compromising their viability.

For the first isoflavone elicitation experiment, three dilutions of concentration 1000, 100 and 10 µg mL^{-1} diluted in 10% DMSO were prepared from the separated aliquot of the dry extract obtained by liquid–liquid separation, and 10 µL of each dilution was inoculated into the cut of the seeds embryo. For the second isoflavone elicitation experiment, 10 µL of fractions F1 and F2 at 100 µg mL^{-1}, obtained from the first VLC, was inoculated. For the third isoflavone elicitation experiment, 10 µL of F1.1 at 100 µg mL^{-1}, obtained from the second VLC, was inoculated. For the fourth isoflavone elicitation experiment, 10 µL of Fp at concentrations 100, 10, 1 and 0.1 µg mL^{-1} was inoculated. This entire procedure was carried out under sterile conditions. Once the treatments were applied, the plates were kept in darkness for 3 days in a SANYO MLR 350H camera at 27 °C.

The extraction and analysis of isoflavones was performed according to Wang et al. 2002 and Lozovaya et al. 2004 with some modifications. Seeds were powdered with liquid nitrogen, mixed with 100 mL of 80% HPLC-methods methanol and maintained on an orbital shaker at 145 rpm for 15 h at 40 °C. Samples were then centrifuged at 2890 *x* g for 20 min at 20 °C. The obtained supernatant was filtered through a 0.2 µm nitrocellulose membrane, and the methanolic extract was used for analysis by HPLC.

The identification and quantification of isoflavone was carried out on a chromatograph Agilent Technologies 1260 Infinity HPLC system. Chromatography conditions were: UV detection: 262 nm, ZORBAZ 300SB-C18 column (4.6 µm × 250 mm x 5 µm), Gecko 2000 30, 80 °C thermostat that kept the column at 30 °C. The mobile phase consisted of water with 0.1% acetic acid (solvent A), and acetonitrile with 0.1% acetic acid (solvent B), with the following gradient: from 15% to 45% of B during 40

min, rising until 100% B during 1 min and remaining this composition for 9 min, after which it dropped to initial conditions (15% B) for 1 min and held for 9 min to equilibrate the column. The flow was 1.5 mL min^{-1} and the sample injection volume was 10 µL.

The quantification of the isoflavones (µg mL^{-1}) was performed by interpolation of the relative area given by the detector on a calibration curve built for each isoflavone ($R^2 > 0.99$). The calibration curves were built with the isoflavones (LC Laboratories): daidzin, genistin and malonyl genistin.

2.4 Vacuum liquid chromatography (VLC)

The two VLCs performed were carried out in a glass column (20 mm diameter x 350 mm H) filled with 30 g of silica gel 60 (0.063–0.200 mm, Merck) coupled to a vacuum pump with 51 kPa. The starting extract for fractionation was crushed and mixed with silica gel 60 until obtaining a fine powder, which was placed on the top of the silica column and fractionated passing through the column the following mobile phases (from lower to higher polarity): n-hexane, dichloromethane, ethyl acetate and methanol, for the first VLC, and n-hexane and dichloromethane for the second VLC. Each organic solvents (400 mL) was passed through the column and concentrated in a rotary evaporator under vacuum at 50 °C.

2.5 Thin layer chromatography (TLC)

Two thin layer chromatographies, using TLC plates of silica gel on aluminum support 60 F254 (Merk), were performed to qualitatively assess the components present in the control 1 and in the fractions obtained from the first VLC (F1, F3, F3 and F4) and in the fractions obtained from the second VLC (F1.1 and F1.2).

2.6 Purification of F1.1

F1.1 fraction, the most fractionated one, was dissolved in 1 mL of chloroform and put on a TLC plate, which was imbibed in a dichloromethane mobile phase. The band that appeared at the top of the plate (common to those bands that had appeared in the first and second TLCs) was removed by scraping the silica gel from the TLC aluminum plate. Scraped silica was mixed with 1 mL of 80% methanol for HPLC methods and centrifuged for 10 min at 6500 *x* g at room temperature. Precipitated silica was discarded and supernatant (containing the elicitor pure compound/s) was evaporated. The isolated and pure fraction (Fp) (0.39 mg) was tested in soybean seeds and stored protected from light and humidity to later perform another experiment for checking its capacity to induce systemic resistance in *A. thaliana*.

The mobile phase used was a mixture of chloroform/dichloromethane/ethyl acetate/methanol (v/v/v/v). Ultraviolet light (254 nm and 366 nm) was used for revealing the TLC plates.

2.7 ISR experiment

An aliquot of the purest fraction (Fp) was dissolved in DMSO 10% at concentrations 10 and 1 μg mL^{-1} and used for an ISR experiment in *A. thaliana*. The ISR experiment was carried out as follows:

A. thaliana wild type *Columbia ecotype* 0 seeds (provided by the Nottingham Arabidopsis Stock Centre (NASC)) were germinated in quartz sand and two-week-old seedlings were then individually transplanted to 100 mL pots filled with peat/sand mixture (12:5 vol/vol) (60 g per pot). Forty-eight plants per treatment were used; plants were arranged in three replicates, with sixteen repetitions each. Plants were watered with 5 mL of tap water once a week and with 5 mL of half-strength Hoagland solution per plant once a week. Plants were inoculated by soil drench with 50 μL of each treatment:

Fp diluted in 10% DMSO at a concentration of 10 µg mL^{-1} and at 1 µg mL^{-1}, in the first and second weeks after transplant. Control plants were-mock inoculated by soil drench with 50 µL of control 1. Another positive control was added, in which thirty-six plants (three replicates of 12 plants each) were inoculated by soil drench, in the first and second weeks after transplant, with 1 mL of a 10^9 cfu mL^{-1} *P. fluorescens* N 21.4 suspension. Four days after the second inoculation, plants were pathogen challenged with the pathogen *P. syringae* pv. *tomato* DC3000. One day before pathogen challenge, plants were maintained with 99% relative humidity to ensure stomata opening in order to allow disease progress. *P. syringae* pv. *tomato* DC3000 grown for 24 h was centrifuged (10 min at 2890 *x* g) and cells were suspended in 10 mM MgSO4 to achieve 10^8 cfu mL^{-1}. Inoculation was done by spraying all the plants with 250 mL. Plants were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 µE s^{-1} m^{-2} at 24 °C) and 16 h dark period (20 °C) at 70% relative humidity. All the leaves of twelve plants (four per replicate) of each treatment and of control 1 were harvested at 6, 12 and 24 h after pathogen challenge (hapc), powdered in liquid nitrogen and stored at −80 °C. These plant samples were used for gene expression analysis by qPCR. The 36 remaining plants (12 per replicate) of each treatment and of control 1, and the 36 plants of positive control, were used to record disease severity 72 h after pathogen inoculation as the number of leaves with disease symptoms relative to the total number of leaves. Results were relativized using the disease severity of leaves inoculated with the control 1 extract as 0% protection.

2.8 RT-qPCR experiment

Total RNA was isolated from each replicate with PureLink RNA Micro Kit (Invitrogen (Massachusetts, USA)), DNAase treatment included. RNA purity was confirmed using NanodropTM. A retrotranscription followed by RT-qPCR was performed.

The retrotranscription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad (Massachusetts, USA)). All retrotranscriptions were carried out using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 °C, 30 min 42 °C, 5 min 85 °C, and hold at 4 °C. Amplification was carried out with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 °C and then 39 cycles consisting of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, followed by melting curve to check results. To describe the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method. Sand gene (AT2G28390) was used as reference gen (Remans et al. 2008). Gene primers used are shown in Table 3.4.2.

Table 3.4.2 Forward and reverse primers used in qPCR analysis.

	Forward Primer	Reverse Primer
AtNPR1	5´-TATTGTCAARTCTRATGTAGAT	5´-TATTGTCAARTCTRATGTAGAT
AtPR1	5´-AGTTGTTTGGAGAAAGTCAG	5´-GTTCACATAATTCCCACGA
AtICS	5´-GCAAGAATCATGTTCCTACC	5´AATTATCCTGCTGTTACGAG
AtPDF1	5´-TTGTTCTCTTTGCTGCTTTCGA	5´-TTGGCTTCTCGCACAACTTCT
AtLOX2	5´-ACTTGCTCGTCCGGTAATTGG	5´-GTACGGCCTTGCCTGTGAATG
AtPR3	5´-AAATCAACCTAGCAGGCCACT	5´-GAGGGAGAGGAACACCTTGAC
Sand	5′ -CTGTCTTCTCATCTCTTGTC	5′-TCTTGCAATATGGTTCCTG

**At = A. thaliana*

2.9 Tentative identification and characterization by UHPLC/ESI–QTOF–MS

2.9.1 Sample preparation

The remaining content (0.19 mg) of the purest fraction (Fp) was dissolved in 100 µL of methanol, LC-MS grade, and vortex mixed for 3 min. The mixture was then centrifuged at 10000 *x* g for 5 min and supernatant was collected for direct analysis.

2.9.2 UHPLC-MS Analysis

Samples were analysed on a 1290 Infinity series UHPLC system coupled through an electrospray ionization source (ESI) with Jet Stream technology to a 6545 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany). For the separation, a volume of 2 µL was injected in a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 x 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2 % B (0–6 min), 2-50 % B (6–10 min), 50-95 % B (11–18 min), 95 % B for 2 min (18–20 min), and returned to starting conditions 2 % B in one minute (20–21 min) to finally keep the re-equilibration with a total analysis time of 25 min. Detector was operated in full scan mode (m/z 50 to 2000), at a scan rate of 1 scan s^{-1}. Accurate mass measurement was assured through an automated calibrator delivery system that continuously introduced a reference solution, containing masses of m/z 121.0509 (purine) and m/z 922.0098 (HP-921) in positive ESI mode; whereas m/z 112.9856 (TFA) and m/z 922.009798 (HP-921) in negative ESI mode. The capillary voltage was ± 4000 V for positive and negative ionization mode. The source temperature was 225 °C. The nebulizer and gas flow rate were 35 psig and 11 L min^{-1} respectively, fragmentor voltage to 75 V and a radiofrequency voltage in the octopole (OCT RF Vpp) of 750 V.

All the solvents used were LC-MS grade. Purified water was obtained from Milli-Q Plus™ System from Millipore (Milford, MA, USA). Formic acid was purchased from Aldrich (St. Louis, MO, USA).

For the study, MassHunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies, Santa Clara, CA, USA) was used for control and acquisition of all data obtained with UHPLC/MS-QTOF.

2.9.3 Data handling

UHPLC-MS data processing was performed by MassHunter Qualitative Analysis (Agilent Technologies, Santa Clara, CA, USA) Software version B.08.00 using "Molecular Feature Extraction (MFE)" to extract potential molecular features (MFs). The MFE algorithm creates a list of possible components that represent the full TOF mass spectral data features, which are the sum of co-eluting ions that are related by charge-state envelope, isotopic distribution and/or the presence of different adducts and dimmers. Several parameters of the algorithm were set for data extraction, applying 2000 counts as limits for the background noise. Moreover, the algorithm was applied to find co-eluting adducts for the same possible compound, selecting $^{+}$H, $^{+}$Na, $^{+}$K, and neutral water loss as possible adducts for positive ionization and $^{-}$H, $^{+}$FA, $^{+}$Cl in negative ionization. Additionally, the "Generate Formula" option in the MassHunter Qualitative Analysis software was used to generate the empirical formula from accurate mass and isotopic pattern distribution to increase the confidence of compound annotation, with a very good score (about 97–99%).

2.9.4 Compound identification

The tentative identification of compounds was carried out by comparing their retention times and the accurate masses of features (± 5-ppm error) against online databases, as FOODB, MetaCyc, CEU massmediator, and scientific bibliography.

To reach the possible annotation of peak 2, it was necessary to use the MetaCyc and to consult specific bibliography (Hillwig et al. 2013).

The identification of compounds corresponding to peaks 3, 4, 6, 7 and 8 was supported by comparison of their accurate masses in the databases for each compound, providing an accuracy error below 5 ppm. Specific bibliography was also consulted for their annotation (Siebers et al. 2016; Lenarcic et al. 2017; Ali et al. 2018). To confirm the

annotation of the peaks 4, 6 and 7, a MS/MS analysis was carried out and their final identification was supported using *in silico* prediction approaches, such as the freely available tool CFM-ID 3.0. The experimental MS/MS spectra were searched and scored against predicted spectra based on similarity.

Regarding peak 5, it was annotated after matching against different databases, based on its monoisotopic mass and molecular formula.

2.10 Statistical analyses

One-way ANOVA with replicates was used to check the statistical differences in all data obtained. Prior to ANOVA analysis, homoscedasticity and normality of the variance was checked with Statgraphics plus 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p < 0.05$) a Fisher test was used (Sokal and Rohlf, 1980).

3. Results

3.1 First elicitation of isoflavone in the soybean: concentration optimization

To test the capacity of the extract obtained from the metabolic elicitors by liquid–liquid separation to induce the isoflavone secondary metabolism, this extract was inoculated in the embryo cut of the soybean seeds at concentrations of 1000, 100 and 10 µg mL^{-1}, as is explained in section 2.3. The fractions of 1000 and 100 µg mL^{-1} significantly elicited isoflavone production over both controls (Fig. 3.4.2), and the seeds inoculated with 100 µg mL^{-1} recorded the highest induction. However, the seeds inoculated with 10 µg mL^{-1} showed an isoflavone synthesis very similar to that of control 1 and control 2 seeds or even less, in the case of malonyl genistin. Differences between the three concentrations were statistically significant.

As there were not statistical differences between both controls in the synthesis of isoflavones, it was assumed that elicitation was due to the components of the metabolic elicitors and not the components present in the nutrient broth. For that reason, the control 1 was not further purified as the metabolic elicitor extract, and for the following elicitation experiments only control 1 was used.

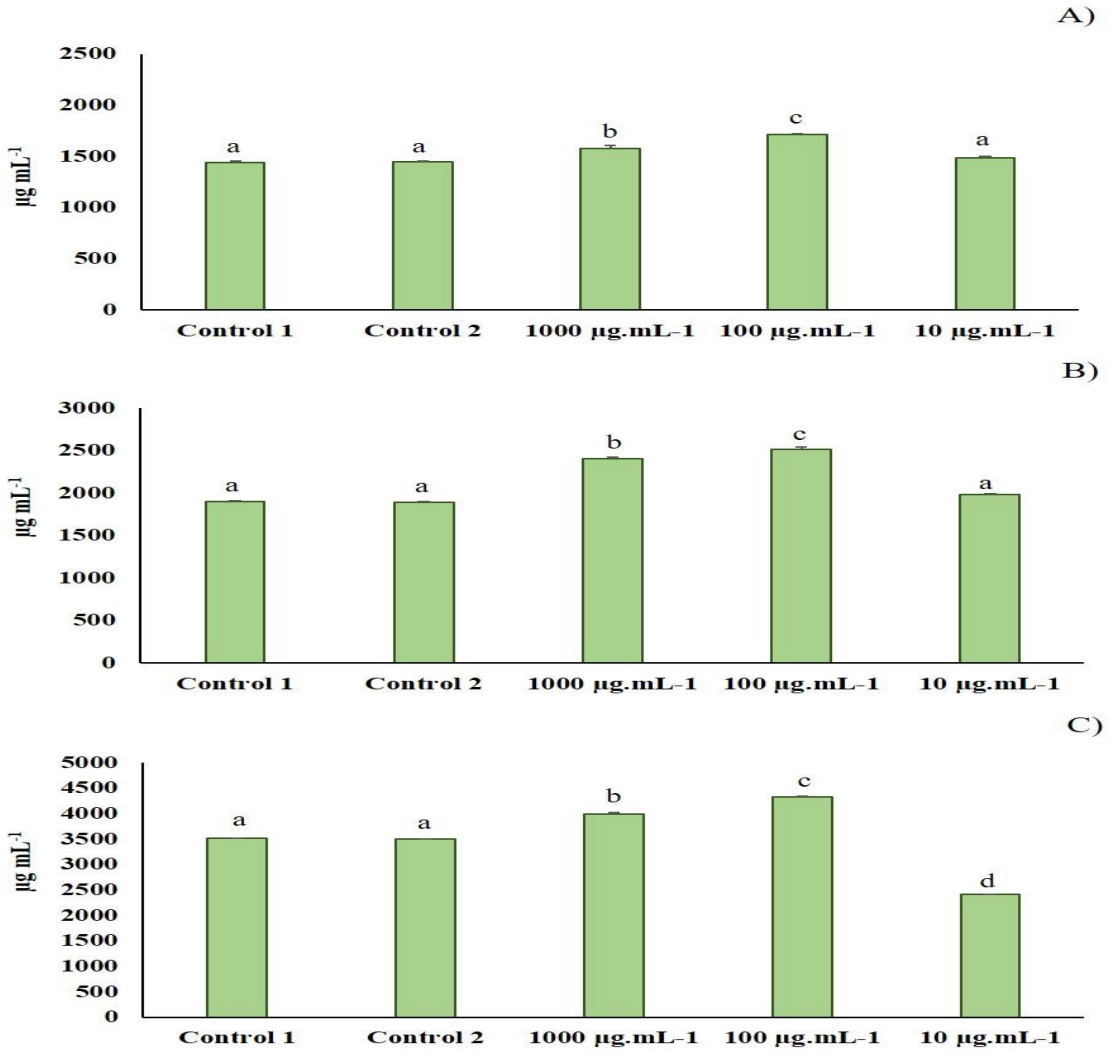

Figure 3.4.2 Daidzin A), genistin B) and malonyl genistin C) production in the seeds inoculated with the extract obtained from the metabolic elicitors at 1000, 100 and 10 µg mL^{-1} and in control 1 (extract obtained from the liquid–liquid phase separation from the culture broth without bacteria) and control 2 (non-inoculated soybean seeds). The amount of isoflavones is expressed in µg mL^{-1} (n = 30 soybean seeds x 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$).

3.2 First TLC

To visually assess the components present in the control 1 (C) and in the four fractions (F1, F2, F3 and F4) obtained from the first VLC, a TLC was performed (Fig. 3.4.3). In the TLC plate, only F1 and F2 showed separation between their components and both showed a common band at the top of the plate. Control 1 extract did not show band separation.

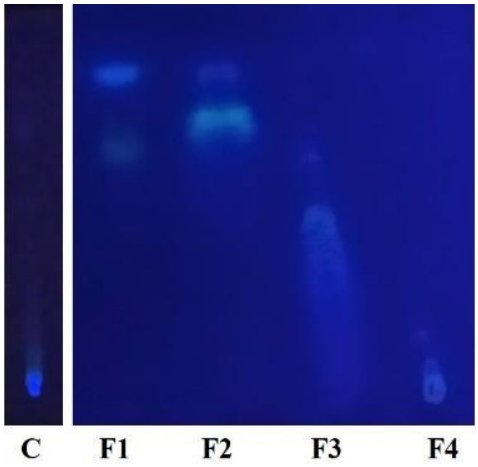

Figure 3.4.3 First TLC made with the control 1 (C) and the four fractions obtained from the first VLC. F1 is the fraction obtained with n-hexane, F2 with dichloromethane, F3 with ethyl acetate and F4 with methanol.

3.3 Second elicitation of isoflavone metabolism in the soybean

According to the results obtained from the previous TLC, only F1 and F2 (of the first VLC) were tested in soybean seeds to check their ability to elicit isoflavone secondary metabolism (Fig. 3.4.4), as F3 and F4 did not showed component fragmentation. The biosynthesis of the three isoflavones assessed was significantly higher in the seeds that were inoculated with F1 and F2 fractions compared to control 1 seeds. The highest elicitation values were obtained with F1.

When comparing this experiment with the first elicitation experiment, it was seen that the values of isoflavone elicitation obtained with the F1 and F2 fractions were similar to those obtained with the initial extract.

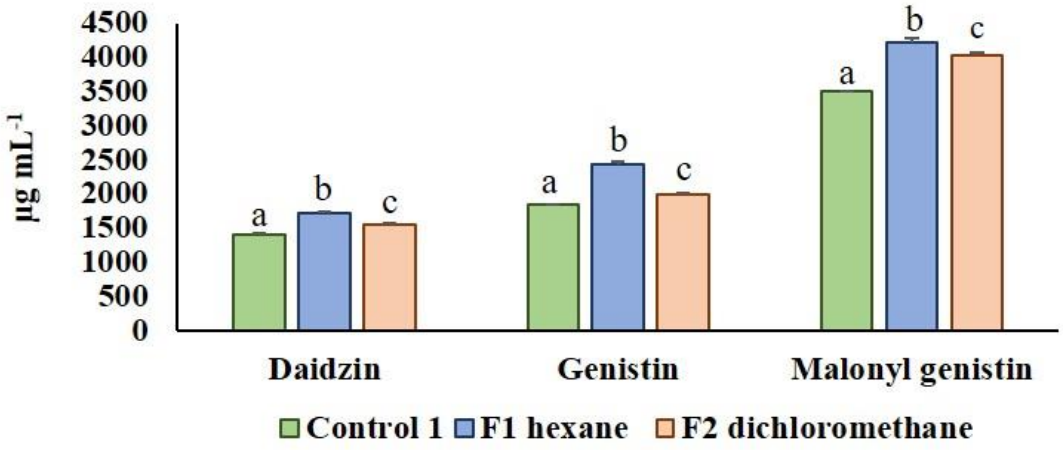

Figure 3.4.4 Daidzin, genistin and malonyl genistin production in the seeds inoculated with F1 and F2 obtained from the first VLC and in control 1 seeds. The amount of isoflavones is expressed in µg mL^{-1} (n = 30 soybean seeds x 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$).

3.4 Second TLC

Since the highest elicitation values were obtained with F1 (Fig. 3.4.4), a second VLC was performed to fragment and purify it. After the VLC, a TLC was made to visually assess the components present in the two fractions obtained (F1.1 and F2.1). F1.1 and F2.1 showed again a common band at the top of the TLC, bands of F1.1 being more intense than those of F2.1. These bands were very similar to those seen in the first TLC of F1 and F2 (Fig. 3.4.5).

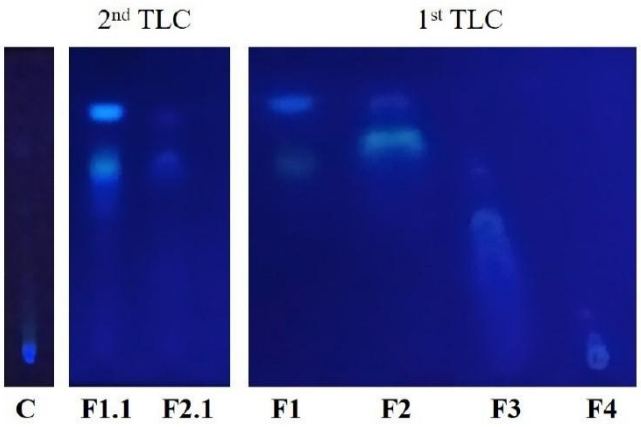

Figure 3.4.5 Comparison between the first and the second TLC. Second TLC was made with the two fractions obtained from the second VLC. F1.1 is the fraction obtained with n-hexane and F2.1 with dichloromethane.

3.5 Third elicitation of isoflavone metabolism in the soybean

As F1.1 fraction showed a similar but more intense band than F2.1 in the second TLC, it was chosen to check its capacity to elicit isoflavone secondary metabolism and it was inoculated in soybean seeds (Fig. 3.4.6). Seeds inoculated with F1.1 had a significant higher isoflavone concentration than control 1 seeds.

When comparing this experiment with the first and the second elicitation experiments, it was seen that the values of isoflavone elicitation obtained with the F1.1 fraction were 1.2 times greater than those obtained with the F1 and F2 fractions and with the initial extract.

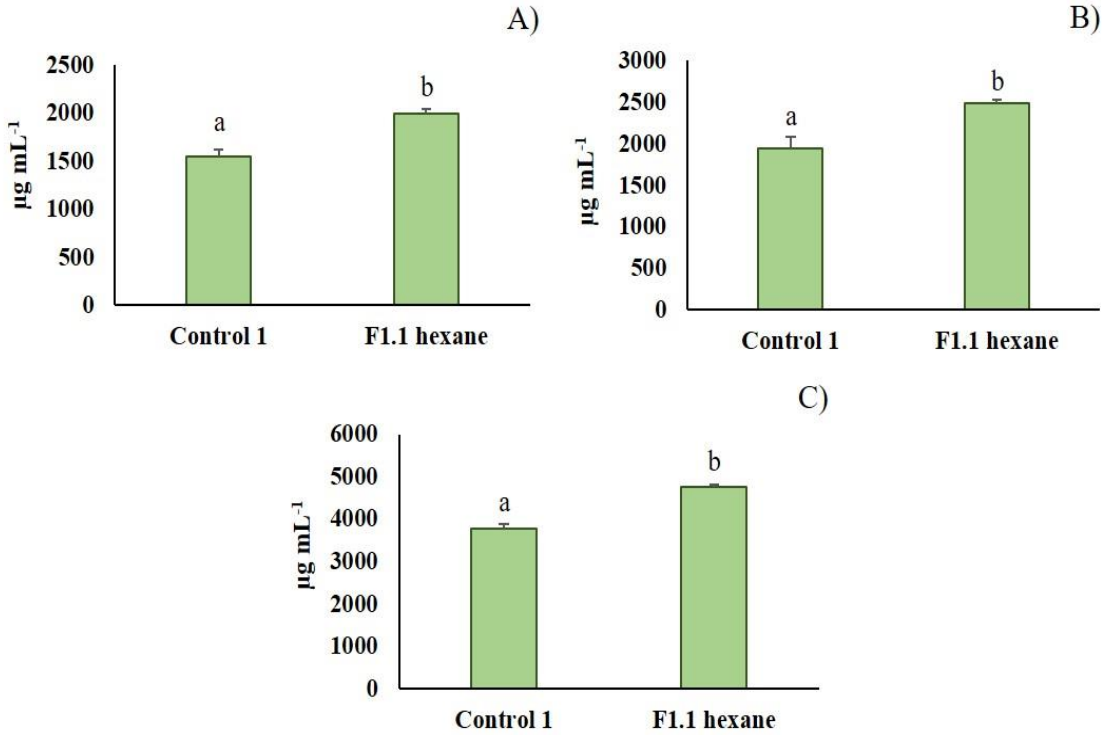

Figure 3.4.6 Daidzin A), genistin B) and malonyl genistin C) production in the seeds inoculated with F1.1 obtained from the second VLC and in control 1 seeds. The amount of isoflavones is expressed in µg mL^{-1} (n = 30 soybean seeds x 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$).

3.6 Fourth elicitation of isoflavone metabolism in the soybean

As F1.1 (the fraction obtained from the second VLC) elicited the isoflavone metabolism, it was purified and the resultant fraction (Fp) was tested at concentrations of 100, 10, 1 and 0.1 µg mL^{-1} in soybean seeds to check its capacity to elicit secondary metabolism of isoflavone (Fig. 3.4.7).

Seeds inoculated with 100 and 10 µg mL^{-1} were not able to germinate; they went black (data not shown) and they were not analysed. However, in the seeds inoculated with 1 and 0.1 µg mL^{-1}, the synthesis of daidzin and especially of genistin significantly increased with respect to control 1. The synthesis of malonyl genistin decreased compared

to control 1. No significant differences were seen between both concentrations in any of the three isoflavones.

When comparing the elicitation results obtained in this last experiment with the previous elicitation experiments (first, second and third), it was seen that the increases in the concentrations of daidzine and genistin triggered by Fp fraction (compared to the control 1) were more than double to those produced by the F1.1, F1 and F2 fractions and the initial extract.

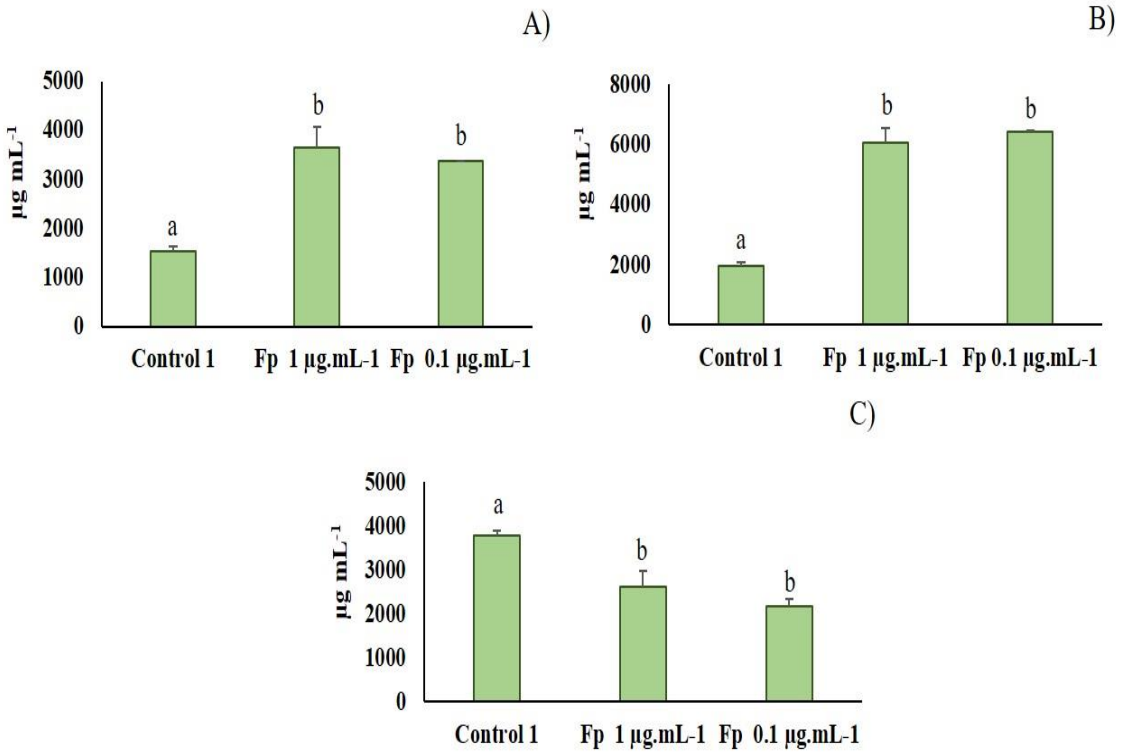

Figure 3.4.7 Daidzin A), genistin B) and malonyl genistin C) production in the seeds inoculated with the purest fraction (Fp) obtained from F1.1 and in control 1 seeds. The amount of isoflavones is expressed in µg mL^{-1} (n = 30 soybean seeds x 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$). Error bars correspond to standard deviation.

3.7 ISR experiment

As the purest fraction (Fp) elicited the isoflavone metabolism in soybean seeds, it was then inoculated in *A. thaliana* seedlings at concentrations of 10 and 1 µg mL^{-1} to carry out an ISR experiment with the objective of checking its ability to protect plants against *P. syringae* pv. *tomato* DC 3000 infection. Results of protection against infection are shown in Figure 3.4.8.

Protection rates against infection were between 40% and 50% for the Fp fraction and 70% for the live strain (used as positive control). No significant differences between the two concentrations of the Fp fraction were observed.

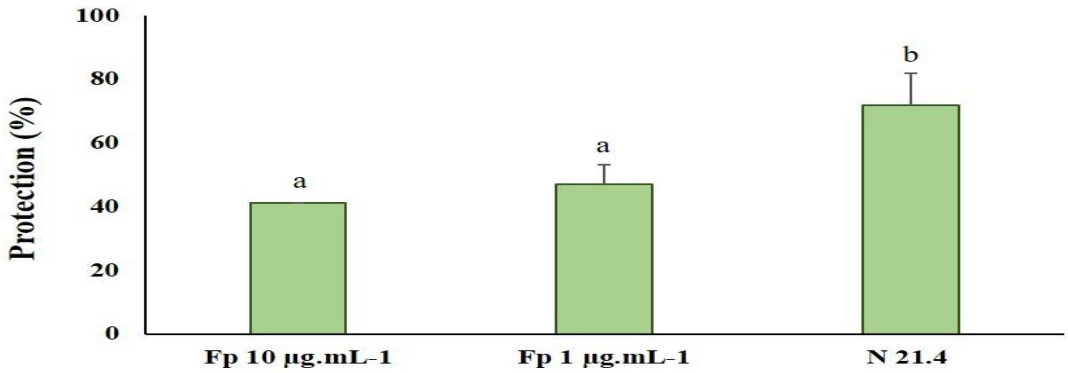

Figure 3.4.8 Protection (%) against *P. syringae* pv. *tomato* DC3000 in *A. thaliana* seedlings elicited with the purest fraction (Fp) at 10 and 1 µg mL^{-1} and with the live strain *P. fluorescens* N 21.4. The percentage was calculated based on the number of leaves with disease symptoms to the total of leaves (n = 12 seedlings per replicate). Data were relativized to control 1, which was considered as 0% protection. Different letters show significant statistical differences between treatments ($p < 0.05$).

3.8 RT-qPCR experiment

After the ISR experiment in *A. thaliana*, the differential expression of marker genes of the salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) signal transduction pathways was analysed by qPCR (Table 3.4.3). The studied marker genes of the SA signalling

pathway were *PR1*, *NPR1* and *ISC*, and those of the JA/ET pathway were *PDF1*, *PR3* and *LOX 2*. All these genes were measured at 6, 12 and 24 hapc.

The differential expression of marker genes of the SA signalling pathway at both concentrations (10 and 1 µg mL^{-1}) decreased from 6 to 12 hapc, except for *PR1*, with 10 µg mL^{-1}, which increased. With 1 µg mL^{-1}, *PR1* did not show significant differential expression, while *NPR1* and *ICS* showed slightly higher expression with 1 µg mL^{-1} than with 10 µg mL^{-1} at both sampling moments. None of the genes showed differential expression at 24 hapc.

Marker genes of the JA/ET signalling pathways, at both concentrations, only showed significant differential expression at 6 hapc, except *PR3*, which also showed significant differential expression at 12 hapc with 1 µg mL^{-1}. The expression of *PDF1* and *PR3* was higher with the concentration of 1 µg mL^{-1}. None of the genes showed differential expression at 24 hapc.

Table 3.4.3 Differential gene expression (*A. thaliana* seedlings inoculated with the purest fraction (Fp) at a concentration of 10 and 1 µg mL^{-1} vs control 1) at 6, 12 and 24 hapc (n = 12).

		10 µg mL^{-1}			**1 µg mL^{-1}**		
		6 hapc	**12 hapc**	**24 hapc**	**6 hapc**	**12 hapc**	**24 hapc**
SA	*PR1*	1.2 ± 0.02 *	5.35 ± 0.06 *	0	0.83 ± 0	0	0
	NPR1	2.44 ± 0.21 *	1.1 ± 0.05	0.73 ± 0	2.65 ± 0.13 *	1.2 ± 0.03 *	0
	ICS	1.66 ± 0.12 *	0.66 ± 0.01 *	0	2.02 ± 0.03 *	1.47 ± 0.03 *	0
JA/ET	*PDF1*	1.23 ± 0.01 *	0	0	2.36 ± 0.02 *	0	0
	LOX2	1.79 ± 0.06 *	0.73 ± 0.03	0	0.9 ± 0.01	0	0
	PR3	1.7 ± 0 *	0.72 ± 0	0	3.4 ± 0.06 *	1.31 ± 0.1 *	0

Asterisks represent statistically significant differences ($p < 0.05$) with respect to control 1 (differential expression of 1) within each sampling time.

3.9 Characterization by UHPLC/ESI-QTOF-MS

The purest fraction (Fp) obtained from the metabolic elicitors of *P. fluorescens* N 21.4 was analysed by UHPLC/ESI–QTOF–MS, as described in Material and Methods, leading to the characterization of eight peaks (corresponding to eight compounds). Figure 3.4.9 shows the extracted ion chromatograms (EICs) provided by the analysis of the extract in the positive ionization mode, which has proved to be more efficient and sensitive than in negative mode for compound characterization, and located within the chromatographic retention interval 14 – 17.5 min. The tentatively identified compounds, classified by families, and the main parameters that support their annotation, are listed in Table 3.4.4.

In the research proposed, eight compounds (Fig. 3.4.9) were detected in the extract of the purest fraction. Among them, one alkaloid (peak 2), two amino lipids (peaks 3 and 8), a terpenoid (peak 5) and three aryl alkylamines (peaks 4, 6 and 7).

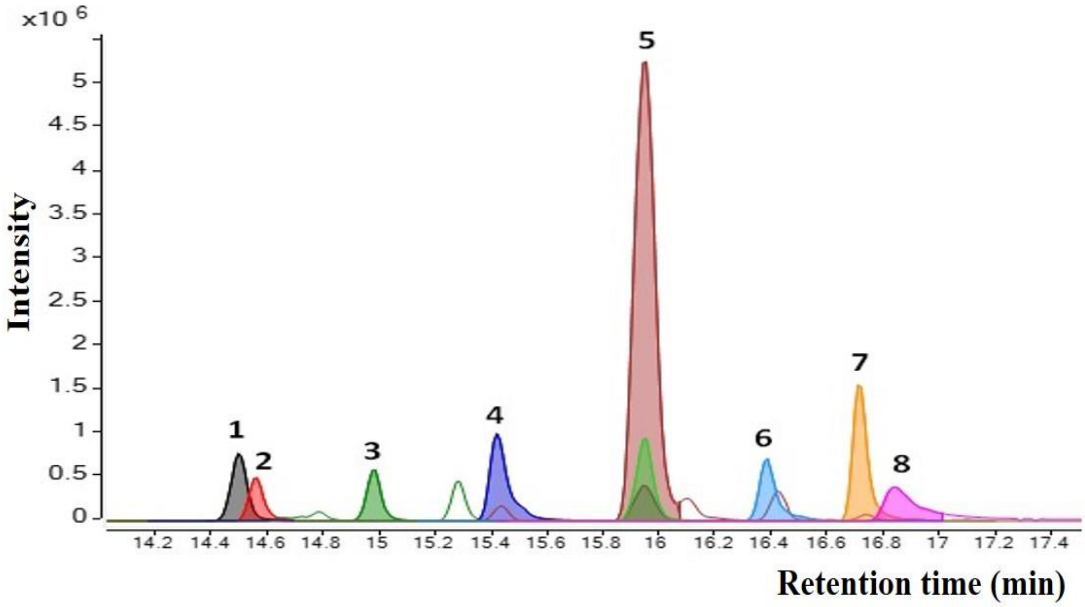

Figure 3.4.9 Overlay of the extracted ion chromatograms (EICs) for compounds present in the extract of the purest fraction (Fp). The enlargements of a part of the chromatogram are shown from 14.0 to 17.5 min.

Table 3.4.4 Retention times, mass spectral data and characterizations of the detected compounds in the UHPLC/ESI–QTOF–MS analysis in positive and negative ionization.

nº	Tentative Annotation[a]	Rt (min)	Molecular Formula	Monoisotopic Mass	m/z Experimental[b]	Fragments (MS2)
	Unknowns					
1	Unknown	14.5	$C_{17}H_{14}N_2S$	278.0878	$[M+Na]^+$ = **301.0762**	--
	Alkaloids					
2	--	14.6	$C_{25}H_{29}NO$	359.2249	$[M+H]^+$ = **360.2333**	--
	Amino lipids					
3	Sphinganine C17	14.9	$C_{17}H_{37}NO_2$	287.2824	$[M+H]^+$ = **288.2999** $[M+Na]^+$ = 310.	--
8	1-Nonadecanamine	16.8	$C_{19}H_{41}N$	283.3239	$[M+H]^+$ = **284.3320**	--
	Terpenoids					
5	--	15.9	$C_{24}H_{30}O_6$	414.2042	$[M+Na]^+$ = **437.1946** $[M+H]^+$ = 415.2121 $[M+K]^+$ = 453.1680 $[2M+Na]^+$ = 851.3989 $[M+FA-H]^-$ = **459.2029**	303.1214, 73.0661 338.4838, 325.1891, 277.1818, **141.4137**, 104.2879, 90.6591
Arylalkylamines						
4	*N*-benzyl-1-tetradecanamine	15.4	$C_{21}H_{37}N$	303.2926	$[M+H]^+$ = **304.3003**	212.2379, **91.0544**, 65.0383, 58.0652
6	*N*-benzyl-1-hexadecanamine	16.4	$C_{23}H_{41}N$	331.3239	$[M+H]^+$ = **332.3320**	240.2682, **91.0544**, 69.0694, 58.0652
7	*N*-benzyl-1-octadecanamine	17.3	$C_{25}H_{46}N$	325.3709	$[M+H]^+$ = **360.3622**	268.2993, **91.0544**, 85.0652, 58.0652

[a]Compounds identified comparing with data reported in literature and online databases.

[b]The m/z values for the base peak are given in **bold type**.

According to its monoisotopic mass molecular formula and by consulting a specific database and bibliography (Hillwig et al. 2013), the annotation Ambiguine P (a cycloheptane-containing member of the hapalindole alkaloid) could be possible for the compound corresponding to peak 2.

The family that encompasses amino derivatives was remarkable in the extract due to the number of identified compounds (compounds 3, 8, 4, 6 and 7 of the Table 3.4.4). The most important compounds in this family were those related to long chain dialkyl, monalkyl and aryl alkylamines, saturated and unsaturated. In this study, peak 3, was annotated as Sphinganine C17 and peak 8 was annotated as N-Methyl-N-stearylamine (1-nonadecanamine). The peaks 4, 6 and 7, were annotated as arylalkylamines and their identification was supported by their MS/MS spectra (Fig. 3.4.10).

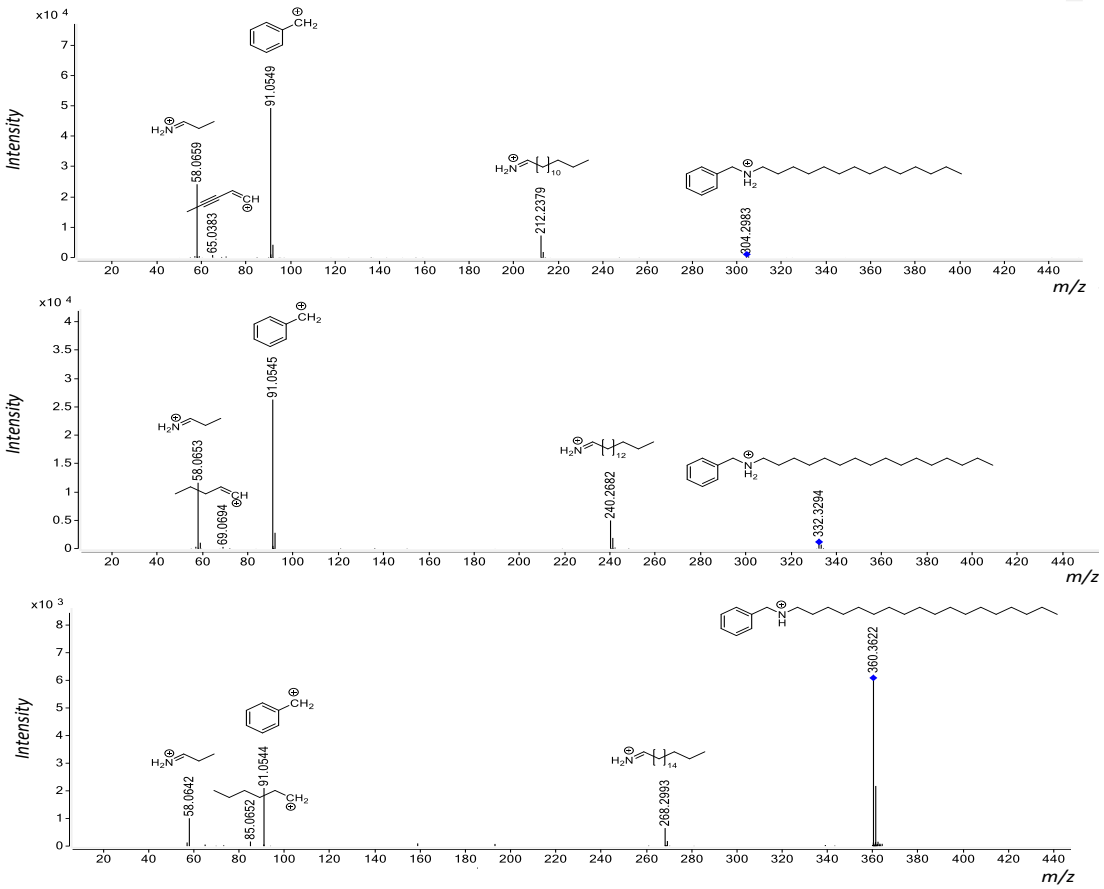

Figure 3.4.10 Experimental MS/MS spectra for the aryl alkylamines detected in the extract at the collision energy (30 eV) in positive.

Regarding peak 5, and considering different databases based on its monoisotopic mass and molecular formula (Fig. 3.4.11), two terpenoid annotations could be considered to be related: the sesquiterpenoid 4-O-methylmelleolide (alkyl resorcinol ester derivative) (Yin et al. 2012), and the diterpenoid 2-Acetoxy-3-deacetoxycaesaldekarin E (neocaesalpin AH) (Jing et al. 2019).

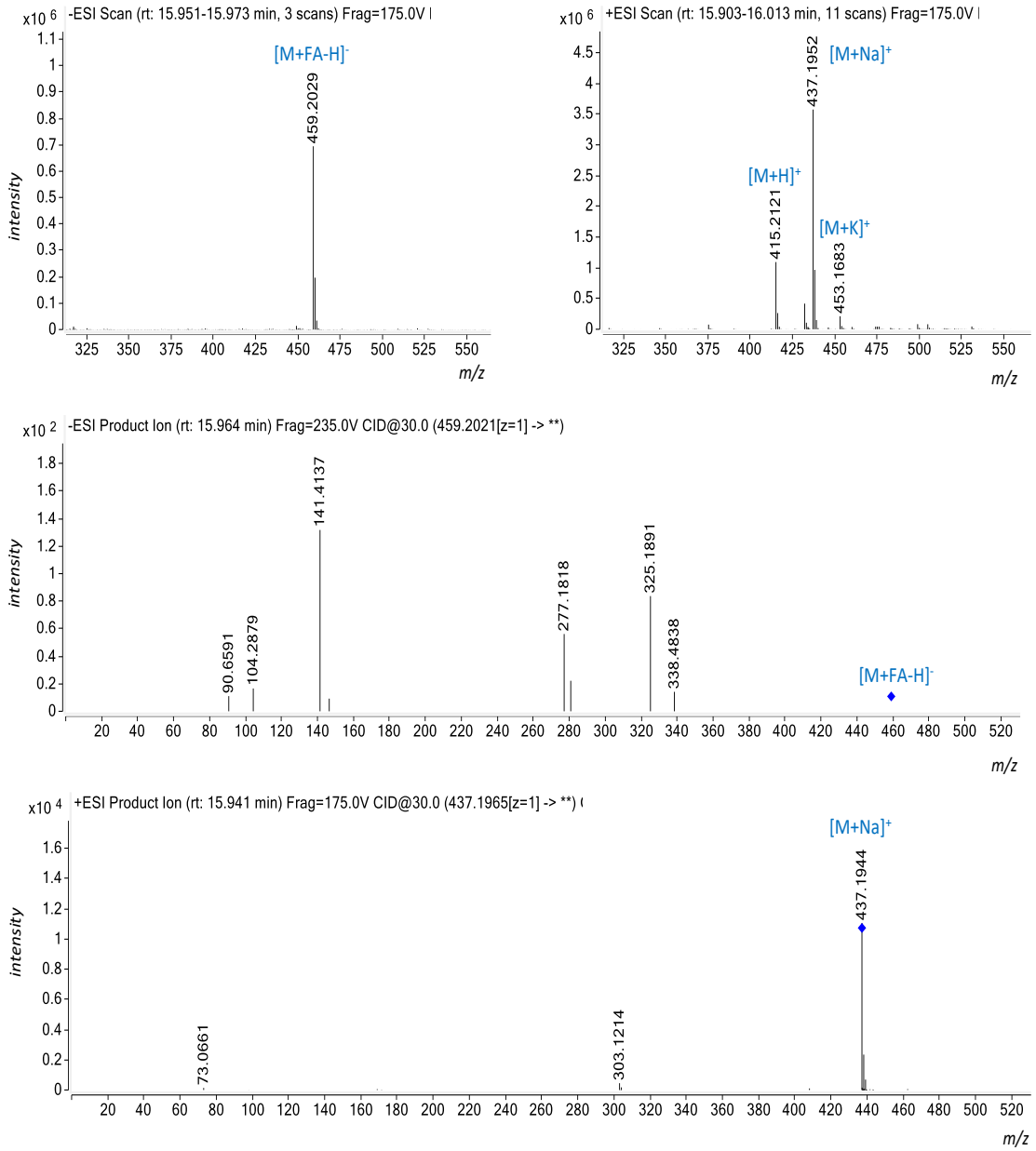

Figure 3.4.11 Experimental MS/MS spectra at the collision energy (30 eV) in positive and negative.

4. Discussion

The pressure on the demand for agriculture practices that improve yield and quality food, linked to increasing concerns about sustainability, has led to the emerge of studies about new substances that substitute environmentally harmful or health risky phytosanitaries for more ecofriendly and effective plant inoculants able to enhance plants' immune systems and plants' resistance to pests and abiotic stress. This current development of biotechnological alternatives in the agronomic field has been concentrated on the use of more efficient tools with low biological and environmental repercussions (Navarro et al. 2017). One of these reliable and non-polluting tools is the use of inductors or elicitors from beneficial microorganisms (Gozzo and Faoro, 2013). Therefore, the present work was focused on isolating, purifying and identifying the compound or set of elicitor compounds extracted from the metabolism of the beneficial rhizobacterium *P. fluorescens* N 21.4, which were able to elicit isoflavone secondary metabolism in soybean seeds and to induce systemic resistance in *A. thaliana*.

Our results have shown that metabolic elicitor fractions extracted from the strain *P. fluorescens* N 21.4 were able to enhance the synthesis of isoflavones in soybean seeds between 1.2 to 3.2 times more than controls, demonstrating their potential to elicit secondary defence metabolism (Figs. 3.4.4, 3.4.6 and 3.4.7). This capacity of elicitation was increased while the purification and concentration of the fractions (from F.1 at 100 $\mu g\ mL^{-1}$ to Fp at 1-0.1 $\mu g\ mL^{-1}$) progressed (Lopes et al. 2012).

The technique provided in the present work to verify the elicitation of secondary metabolism in the plant was an effective, simple and very fast technique, since it allowed checking the eliciting capacity of the fractions inoculated in soybean seeds in less than 90 h. This fast and effective system would allow one to carry out rapid screenings to search for new eliciting compounds in future research.

On the other hand, the results obtained in the ISR experiment in *A. thaliana* against the pathogen *P. syringae* pv. *tomato* DC 3000 supported the statement that the metabolic elicitors of the strain N 21.4 have great potential to increase plant resistance, since protection rates between 40 to 50% were seen (Fig. 3.4.8). The ISR experiment also revealed a simultaneous activation of both SA and JA/ET signalling pathways, since high levels of *PR1*, *NPR1* and *ICS* (SA marker genes), and *PDF1*, *LOX 2* and *PR3* (JA/ET marker genes) were seen. Hence, it has been again demonstrated that these two pathways are not necessarily antagonistic, as previously indicated by some authors (Liu et al. 2016; Betsuyaku et al. 2017). The importance of high concentrations of SA and JA to trigger defensive responses mediated by both hormones is nowadays widely accepted (Pieterse et al. 2014; Caarls et al. 2015). Furthermore, this experiment showed a very rapid ISR response, having high values of differential gene expression at 6 hapc. Despite not having seen big differences between both concentrations of inoculation (1 and 10 µg mL^{-1}), a trend of greater differential expression and higher protective rates against pathogen infection were seen when plants were inoculated with 1 µg mL^{-1}. The genes that showed the greatest differences of expression between the two concentrations were *ICS* and *PR3* genes, which were doubly expressed in the plants inoculated with 1 µg mL^{-1}. From a commercial point of view, having a noticeable effect at such a low concentration is very interesting when the intention is making effective, but at the same time affordable plant inoculants.

After the exhaustive analysis by UHPLC/ESI–QTOF–MS to identify and characterize the compounds responsible for the elicitation, eight peaks corresponding to eight different compounds were detected (Fig. 3.4.9). The groups of compounds found corresponded with an alkaloid, two amino lipids, a terpenoid and three arylalkylamines.

An unknown compound was also detected, but it was impossible to elucidate its identity since it was not found described in the literature nor in databases.

A tentative annotation was found for compound corresponding to peak 2, the alkaloid nature one, after consulting the MetaCyc database and specific bibliography (Siebers et al. 2016). This compound could be described as Ambiguine P, a cycloheptane-containing member of the hapalindole alkaloid. Hapalindole-type natural products are structurally diverse terpenoid indole alkaloids that, to date, have only been described as secondary metabolites produced by cyanobacteria. They have a wide range of biological activities, including insecticidal (Becher et al. 2007), antibacterial and antifungal (Smitka et al. 1992; Mo et al. 2009; Mo et al. 2010). Literature does mention other alkaloid compounds produced by *P. fluorescens*, such as pyrrolnitrin or hydropyridine-type compounds that also show antibiotic and antifungal activity (Lesinger and Margraff, 1979; Lozano et al. 2019). However, hapalindole-type natural products have never been described in *P. fluorescens*; hence, this result would require further investigation to be more conclusive.

Compounds corresponding to peaks 3 and 8 were seen to be amino lipid compounds. Sphinganine C17 (peak 3) is a type of sphingolipid that the literature describes with a signalling function in plants subjected to biotic and abiotic stress (Siebers et al. 2016; Lenarcic et al. 2017; Ali et al. 2018). In bacteria, sphingolipids are specific membrane lipids each with a monounsaturated long-chain, the sphingosine, and their biological role has not been fully understood yet. Few bacteria have been described able to synthesize sphingolipids and some of these are cytophaga-flavobacterium-bacteroidetes group bacteria, α-proteobacteria and δ-proteobacteria (*Bdellovibrio bacterivorous*, *Cystobacter fuscus*, *Myxococcus stipitatus*, *Sorangium cellulosum* and *Myxococcus xanthus* (Ali et al. 2018)). However, it has been seen that lipopolysaccharide

of Gram-negative bacteria shows structural and functional resemblance to sphingolipids of the above-cited bacteria and even to sphingolipids produced by eukaryotes. That is why in the work of Heaver et al. 2018 it was proposed that as sphingolipid produced by bacteria are very similar to those produced by their eukaryotic hosts, they could influence in their hosts immune responses.

The role that sphingolipids play in plant-microorganism interactions and as bioactive elicitors that initiate defensive responses in plants has begun to be studied (Ali et al. 2018). However, Giorni et al. 2015 and Dall'Asta et al. 2015 have already established a possible relationship between sphingolipids produced by various maize hybrids with *Fusarium verticillioides* infection.

In contrast to eukaryotes, where sphingolipids functions have been extensively studied, very little is known about sphingolipids in bacteria and their functions in the plant–pathogen relationship. Some of the sphingolipids-producing bacteria have been found to be abundant in the phyllosphere of plants (Vorholt, 2012), which hints that sphingolipid production by bacteria may be relevant for them.

The present study was the first synthesis of sphingolipids in the genus *Pseudomonas*, and its relationship with the triggering of secondary metabolism is cited. However, it is clear that more research is needed on this unknown subject.

The other amino lipid nature compound, the one corresponding to peak 8, was tentatively identified as N-methyl-N-stearylamine (1-nonadecanamine). This compound has been reported in some works for its antimicrobial potential, being more commonly found in plant species (Saïdana et al. 2006; Selim et al. 2013; Choudary and Singh, 2018). It has been also found as a secondary metabolite of *Brevibacterium casei* (Kiram et al. 2010) and *Saccharomyces cerevisiae*, with antibiotic activity against *Proteus mirabilis* (Al-Jassaci et al. 2016). Compound 8 has also been described in other bacterial and plants

extracts as a structural constituent of biological membranes, and the results were consistent with those of previous literature reports (Anderson, 1983).

The amino derivatives corresponding to arylalkylamines (peaks 4, 6 and 7) have been generally described as dialkyl and monalkyl amines, saturated and unsaturated. Further investigations will be necessary to determine whether such alkylamines may be considered functional analogs of sphingosine and which may be primarily expressed among the pathways associated with sphingolipid metabolism.

For the last identified compound, the most abundant one (peak 5), two possible annotations within the group of terpenoids were found: the sesquiterpenoid 4-O-methylmelleolide (alkyl resorcinol ester derivative) (Yin et al. 2012) and the diterpenoid 2-acetoxy-3-deacetoxycaesaldekarin E (neocaesalpin AH) (Jing et al. 2019).

Terpenoids are generally considered as secondary metabolites produced by plant or fungi, but recent sequencing of the bacterial genome and bioinformatics analyses of bacterial proteins have revealed the presence of these metabolites in bacteria (Nakano et al. 2011; Yamada et al. 2014). In bacteria, terpenoids can be found in the form of essential oils or aromatic constituents and some have antibiotic and antifungal activities (Fraga et al. 2013; Bohnert et al. 2014).

Despite terpene biosynthetic pathways in bacteria is being considered ubiquitous, few bacterial terpenes have been identified, and their biosynthesis is still poorly understood (Helfrich et al. 2019). The antiSMASH tool lists more than 4000 bacterial terpene biosynthetic gene clusters (Blin et al. 2019), but only 127 have been identified and deposited in the MIBiG database (repository of characterized biosynthetic gene clusters) (Medema et al. 2015), so far. However, literature has revealed the production of 2,5-dialkylresorcinol compounds that exhibit antifungal and antibacterial activities in specific strains of *Pseudomonas* spp. (Kanda et al. 1975; Budzikiewicz et al. 1980). These

are compounds very similar to the compound proposed by our first annotation option (alkyl resorcinol ester derivative).

The induction of systemic resistance in *A. thaliana* and the elicitation of the secondary isoflavone metabolism in soybean seeds may have been due to the effect of the sesquiterpenoid compound present in the extract of the purest fraction of the metabolic elicitors of *P. fluorescens* N 21.4 (it being the majority in the fraction) or due to a synergy between all the compounds that have been identified. Nevertheless, it is clear that deeper research will be necessary in the future to carry out more specific studies with the identified compounds in order to better characterize them and their effects in the plants.

By comparing the fractions extracted from the metabolic elicitors with the control 1, which was extracted in the same way as the fractions, it has also been possible to affirm that the inducing and protective effects were due to the compounds present in the metabolic elicitors of the bacterium and not to any compound present in the nutrient broth. Furthermore, it has been seen that the beef extract used in the nutrient broth is a concentrate of water-soluble compounds, which remained in the aqueous phase, which was discarded in the initial liquid-liquid separation.

In our work we tested the capacity to induce systemic resistance and the capacity to elicit secondary defensive metabolism in plants, but in view of our promising results and having some compounds with putative antibiotic and/or antifungal activity, further research will be performed to test their potential as antibiotics or antifungals against common pathogens present in agricultural systems. Furthermore, we will study the possibility of including all or some of these compounds, derivatives of the secondary metabolism of the *P. fluorescens* N 21.4, as commercial plant inoculants.

5. Conclusions

The results of the present study demonstrated that the fractions obtained by VLC from the metabolic elicitors of *P. fluorescens* N 21.4 induced systemic resistance in *A. thaliana* seedlings against the pathogen *P. syringae* pv. *tomato* DC3000, being able to trigger the two signalling pathways of the defensive response (SA and JA/ET) very quickly and at very low concentrations. These fractions also boosted the secondary defensive metabolism of isoflavones in soybean seeds.

Taking into account our results and those provided in previously published works, it can be concluded that the metabolic elicitors of *P. fluorescens* N 21.4 could be used to create new plant inoculants to be introduced in agricultural practices, minimizing the scope of chemical control, and thus advancing the development of ecofriendly agricultural tools. The purified and identified compounds of the metabolic elicitor fraction (mixed or individually), could result in commercial products of biological origin being applied to crops in the near future, since many of them have putative antibiotic and/or antifungal potential.

Furthermore, the elicitor screening system carried out in soybean seeds to specifically study the metabolic elicitors' effect on isoflavone metabolism, is a doubly fast and efficient system that allows one to verify the elicitation of secondary metabolism in the plant in less than 90 h, which will be very useful for future elicitor compound screenings.

In this work, in *Pseudomonas fluorescens*, we described the synthesis of certain compounds that had not been described in the literature to date, such as sphingolipids and hapalindole-type natural products.

3.5 Bioeffectors as biotechnological tools of the innate immunity: signal transduction pathways involved

Bioeffectors as biotechnological tools of the innate immunity: signal transduction pathways involved

Martin-Rivilla, H*., Garcia-Villaraco, A., Ramos-Solano, B., Gutierrez-Mañero, F.J., Lucas, J.A.

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

*Corresponding author: Helena Martín Rivilla, helenamartin92@gmail.com

+913 72 47 85

Abstract

BACKGROUND

Unraveling the complex functioning of plant immune system is essential and something in which great effort is being made since its performance is not entirely clear yet. Knowing plant immune system allows strengthening it and therefore developing a more efficient and environmentally friendly agriculture, avoiding the massive use of agrochemicals and making plants the main protagonist in the defence against pathogens.

The use of beneficial rhizobacteria (bioeffectors) and their derived metabolic elicitors are biotechnological alternatives in plant immune system elicitation. The present work aimed to check the ability of 25 bacterial strains selected from a group of 175, isolated from the rhizosphere of *Nicotiana glauca*, to trigger the innate immune system of *Arabidopsis thaliana* seedlings against the pathogen *Pseudomonas syringae* DC3000. A study of the signal transduction pathways involved in plant response was made.

RESULTS

The selected 25 strains were chosen because of their biochemical traits and avoiding phylogenetic redundancy. The 5 strains, of the previous 25, more effective in the prevention of pathogen infection were used to elucidate signal transduction pathways involved in the plant immune response, studying the differential expression of Salicylic acid and Jasmonic acid/Ethylene pathway marker genes. Some strains stimulated the two pathways with no inhibitory effects between them, while others stimulated either one or the other. Metabolic elicitors of two strains, chosen for their taxonomic affiliation and for the results obtained in the differential expression of the genes studied, were extracted using n-hexane, ethyl acetate and n-butanol, and their capacity to mimic bacterial effect to trigger the immune system of the plant was studied. N-hexane and ethyl acetate were the most effective fractions against the pathogen in both strains, achieving similar

protection rates although gene expression responses were different from that obtained by the bacteria.

CONCLUSIONS

Beneficial rhizobacteria and their metabolic elicitors have great potential as biotechnological tools since they are able to improve plant immune system through the triggering of either Salicylic acid or Jasmonic acid/Ethylene pathway or both pathways simultaneously. These results open a huge amount of biotechnological possibilities to develop biological products for agriculture in different situations and plant species.

Key words: Bioeffector, beneficial rhizobacteria, metabolic elicitor, induced systemic resistance (ISR), *Pseudomonas syringae* DC3000, SA and JA/ET transduction signal pathways.

1. Introduction

The diseases caused by different pathogen organisms in plants represent an important and persistent threat and a challenge to supply food worldwide (Pechanova et al. 2015; Miller et al. 2017). Because of that, the study of plants´ immune system as a mechanism to counteract the attack of pathogens is fundamental, especially in this year that has been declared International Year of Plant Health by the FAO (Food and Agriculture Organization of the United Nations).

Plants can activate patter-triggered-immunity (PTI) by the recognition of PAMPs/MAMPs (Pathogens Microbe-Associated Molecular Patterns), or effector-triggered immunity (Jones and Dangl, 2006) (ETI) by the recognition of pathogen effectors. PTI response activates when some specific receptors located on cells surface, called Pattern Recognition Receptors (PRRs), detect these PAMPs/MAMPs. However, plants can also respond to endogenous molecules that have been released by pathogens, which implies recognition of virulent pathogen molecules, called effectors, by intracellular receptors. This last recognition leads to a second line of defence, the Effector-Triggered Immunity (ETI) and also to the transcription of resistance genes (PR genes). These endogenous effectors recognized by plants are much more variable in structure and composition than PAMPs/MAMPs (Pel and Pieterse, 2013).

Pieterse et al. (2014) classified induced resistance triggered by pathogens with respect to the type of triggering agent in: systemic acquired resistance (SAR), herbivore induced resistance (HIR) and induced systemic resistance (ISR). SAR is a form of induced resistance that happens in plants after localized exposure to a pathogen and that depends on the accumulation of salicylic acid (SA) and the activation of the Nonexpressor of Pathogenesis-Related Protein 1 (NPR1). SA accumulates after pathogen infection, binding NPR1 and triggering induction of Pathogenesis-related genes (PR). Although

SA-mediated resistance acts against a wide plethora of pathogens, it has been reported that SAR is generally more effective facing to biotrophic and hemibiotrophic pathogens (Glazebrook, 2005; Hammerschmidt, 2009).

In contrast, Pieterse et al. 2000 described ISR as an answer triggered by non-pathogen rhizobacteria (bioeffectors). However, different elicitors such as antibiotics, surfactants or chemical inducers (Gozzo and Faoro, 2013) are also able to induce ISR. In this case, ISR response was described as dependent on jasmonic acid (JA) and ethylene (ET) signalling pathways and also needs the involvement of NPR1 (Pieterse and Van Loon, 2004; Pieterse and Van Loon, 2007). Plant defensin1 (*PDF1*) (Berrocal-Lobo et al. 2002; Lorenzo et al. 2003), and *MYC2* also play an essential role in this signalling pathway (Pozo et al. 2008; Pré et al. 2008).

These bioeffectors and some of their elicitors (structural molecules or metabolic molecules released to the medium) induce in plants a physiological alert state prior to stress challenge known as priming (Conrath et al. 2002). Plants in this state are able to develop a faster and/or stronger activation of defensive responses after the attack of pathogens, insects or in response to abiotic stress (Conrath et al. 2006). After bioeffectors or their elicitors are sensed, the SA, JA or ET signalling pathways are activated to trigger plant resistance (Wu et al. 2018). Therefore, the study of these transduction signal pathways is meaningful for understanding the plant immune system and their defences against pathogens. This can contribute to promote the use of bioeffectors and their elicitors as a useful biotechnological strategy to develop a sustainable agriculture without using agrochemicals and pesticides (Wu et al. 2018).

Taking advantage of the well-known ability of the plants to strongly select beneficial bacterial strains in the rhizosphere to survive to adverse conditions (Marilley and Aragno, 1999; Lucas Garcia et al. 2001; Berendsen et al. 2012; Stringlis et al. 2018),

bacteria isolated from the rhizosphere of *Nicotiana glauca* Graham, a *Solanaceae* native to Southern Spain with a strong secondary metabolism (Ramos-Solano et al. 2010a), were studied. The effects induced in the plants by the beneficial rhizobacteria depend on molecules (elicitors), so we considered that after the extraction of these elicitors, it would be possible to find out which ones were able to reproduce the effects of the rhizobacteria and therefore were responsible of this effect.

The general objective of this work was to find beneficial rhizobacteria (bioeffectors) from *N. glauca* rhizosphere efficient in triggering the innate defence response of *Arabidopsis thaliana* plants, as well as effective derived metabolic elicitors, trying to elucidate the mechanisms involved in the protection. To achieve this objective the following partial objectives were defined: i) to perform a screening of *N. glauca* rhizobacteria to select those strains efficient in triggering the innate response of *Arabidopsis* plants against the pathogen *Pseudomonas syringae* DC3000, ii) to study the mechanisms involved in plant defence triggered by the most effective bioeffectors against the pathogen *P. syringae* DC3000, iii) to obtain metabolic elicitors from the most effective bioeffectors and assay their ability to mimic bacterial response.

To reach our goals, ISR experiments were carried out in *A. thaliana* plants using the bioeffectors and the metabolic elicitors of the chosen strains to protect the plants against *P. syringae* DC3000; the differential expression of marker genes for the SA and JA/ET transduction pathways were studied on plants inoculated with selected strains and selected metabolic elicitors.

2. Material and methods

A screening of 175 isolates was carried out. Firstly, biochemical tests for putative beneficial rhizobacteria traits were carried out to all isolates. The 16S rRNA partial

sequencing of all isolates was analysed and a phylogenetic tree was performed with these sequences. Twenty-five strains selected based on their biochemical traits and avoiding phylogenetic redundancy were assayed to determine their ability to trigger plant protection (ISR). The most effective strains (5) were studied to understand the mechanisms involved in protection. Finally, metabolic elicitors (molecules released to the medium) were obtained from the two most effective bacteria to demonstrate their ability to mimic the protective response triggered by the live strains.

2.1 Origin of bacteria

Bacteria used in this work were isolated from the rhizosphere of wild populations of *Nicotiana glauca* Graham in three different soils and physiological stages of the plant. A total of 960 isolates were obtained and 50% were tested for their putative beneficial rhizobacteria traits, as explained in the work of Ramos-Solano et al. (2010a). In the present study, a subset of 175 strains from the non-assayed group of bacteria were used. These isolates and the pathogen *P. syringae* DC3000 were maintained in 20% glycerol, frozen at -80 ºC and plated to check viability.

2.2 16S rRNA partial sequencing phylogenetic analysis

Bacteria were identified by 16S rRNA partial sequencing phylogenetic analysis. They were grown in PCA (Plate Count Agar (CONDA)) Petri dishes for 48 h and then in nutrient broth (CONDA) under shaking for 24 h at 28 ºC in both cases. DNA was extracted from 1.8 mL of each bacterial culture by using the UltranClean Microbial DNA isolation Kit (Mo Bio, Carlsbad, CA, USA, EE.UU). DNA amount and quality were checked with a Nano Drop 2000 Thermo Scientific.

Each DNA sample was amplified with 16S rRNA universal primers: 1492R (5'TACGGYTACCTTGTTACGACTT3') and 27F (5'AGAGTTTGATCMTGGCTCAG 3'). Amplification reactions were carried out with 5µL DNA (20 ng μL^{-1}), 1 unit of DNA

polymerase (Biotools Hotsplit), 0.5 µL of Primer F (30 µM) and 0.5 µL of Primer R (30 µM), 2.5 µL of 10X standard reaction buffer with $MgCl_2$ (Biotools), 0.625 µL of dNTPs (10 mM each, Biotools), 0.375 µL of 100% DMSO (Dimehyl sulfoxide) and ultrapure water up to a volume of 25 µL.

The reaction mixtures were incubated in a thermocycler (Gene Amp PCR system 2700, Applied Biosystems, South San Francisco, CA, USA) at 94 ºC for 2 min and then subjected to 10 cycles, consisting of 94 ºC for 0.3 min, 50 ºC for 0.30 min and 72 ºC for 1 min and 20 cycles consisting of 94 ºC for 0,3 min, 50 ºC for 0,30 min and 72 ºC for 1 min. Finally, the mixtures were incubated at 72 ºC for 7 min. PCR products were purified with UltraClean PCR Clean-up DNA purification kit (MO BIO). Purified PCR products were sequenced in an ABI PRIMS" 377 DNA Sequencer (Applied Biosystems). Sequences were visualized with Sequence Scanner software v1.0. (Applied Bio- systems, Foster City, CA, USA), and editing was performed using the software Clone Manager Professional Suite v6.0. (Sci-Ed Software, Cary, NC, USA). Sequence alignment was carried out on the server MAFFT v6.0 (http://mafft.cbrc.jp/alignment/software/) and annotated by BLASTN 2.2.6. in the National Centre for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project Release 10 (RDP: http://rdp. cme.msu.edu/) databases. Finally, a phylogenetic tree was performed with the 16S rRNA sequences. The sequences reported in this work are available in the GenBank database under the accession numbers, MH571489 to MH571661.

2.3 Phylogenetic tree

An unrooted tree was performed with MEGA v4.0.2. with aligned sequences in MAFFT v6. The evolutionary distances were inferred using the neighbour-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the

associated taxa clustered together in more than 50% of the 1000 replicates of the bootstrap test are shown next to the branches. All positions containing gaps and missing data were eliminated from the data set (complete deletion option).

2.4 Biochemical tests for putative beneficial rhizobacteria traits

The following biochemical tests for putative beneficial rhizobacteria traits were performed on all bacterial isolates: phosphate solubilisation (De Freitas et al. 1997), auxin-like compounds production (Sergeeva et al. 2007), chitinases production (Frändberg and Shnurer, 1998; Rodríguez-Kábana et al. 1983) and siderophores production (Alexander and Zuberer, 1991).

2.5 First ISR experiment. Screening for isolates able to induce systemic resistance

Based on phylogenetic analysis and putative beneficial rhizobacteria traits, twenty-five strains were selected for a first induced systemic resistance (ISR) assay. These bacteria (bioeffectors) were inoculated in *A. thaliana* plants at root level and challenged with the pathogen to evaluate their ability to protect plants.

A. thaliana wild type *Columbia ecotype* 0 seeds (provided by the Nottingham Arabidopsis Stock Centre (NASC)) were germinated in quartz sand and two-week-old seedlings were then individually transplanted to 100 mL pots filled with 12:5 (vol/vol) peat/sand mixture (60 g/pot). Forty-eight plants per treatment (strains and controls) were used; plants were arranged in three replicates, with sixteen repetitions each. Plants were watered with 5 mL of tap water once a week and with 5 mL of half-strength Hoagland solution per plant once a week. Strains were inoculated twice by soil drench with 3 mL of a suspension of bacterial cells, grown for 24 h in nutrient broth (CONDA) at 28 ºC, and adjusted to a density of 10^8 cfu mL^{-1}, in the first and the second week after transplant. Negative control plants were mock-inoculated by soil drench with 3 mL of sterile nutrient broth and positive control plants were inoculated by soil drench with 10 μL of BTH

(Benzothiadiazole) 0.5 mM (Sumayo et al. 2013). Four days after the second bacterial inoculation, plants were pathogen challenged with *P. syringae* DC3000. One day before pathogen challenge, plants were maintained with 99% relative humidity to ensure stomata opening in order to allow disease progress. *P. syringae* DC3000 was centrifuged (10 min at 2890 *x* g) and cells were resuspended in 10 mM $MgSO_4$ to achieve 10^8 cfu mL^{-1}. It was inoculated by spraying the total of the plants with 250 mL. Plants were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 μE s^{-1} m^{-2} at 24 °C) and 16 h dark period (20°C) at 70% relative humidity for 72 h, and disease severity was recorded as the number of leaves with disease symptoms relative to the total number of leaves. Results were relativized using the disease severity of negative control plants as 0% protection. All the ISR experimental design is represented as a timeline in Fig. 3.5.1.

2.6 Second ISR experiment. Study of the signal transduction pathway involved in plant protection

Based on results obtained from the first ISR experiment, the most protective strains (5) were selected to perform a second experiment to analyse the signal transduction pathways involved in plant protection triggered by bacteria. The expression of some marker genes after pathogen challenge were assessed by qPCR. Genes analysed were *NPR1* (Nonexpressor of Pathogenesis Related Genes1), *PR1* (Pathogenesis-Related Gene 1) and *ICS* (Isochorismate Synthase 1) as markers of the SA signalling pathway (Wildermuth et al. 2002; Vlot et al. 2009; Niu et al. 2011; Pieterse et al. 2014; Seyfferth and Tsuda, 2014; Caarls et al. 2015; Nie et al. 2017; Ding et al. 2018; Kazan, 2018); *PDF1* (Plant Defensin 1), *LOX2* (Lipoxygenase 2) and the transcriptional factor *MYC2* as markers of the JA-ET signalling pathway (Lorenzo and Solano, 2005; Niu et al. 2011; Pangesti et al. 2014; Caarls et al. 2015; Liu et al. 2016; Nie et al. 2017; Du et al. 2017); and two pathogenesis-related proteins genes, *PR2* (encoding β-1,3-glucanase) and *PR3 (*encoding chitinase), as

SA and JA/ET markers, respectively (Van Loon and Van Strien, 1999; Spoel and Dong, 2012; Jeandet et al. 2013; Schenk and Schikora, 2013; Lemarié et al. 2015; Jiang et al. 2016; Wu et al. 2018; Silva et al. 2018).

A. thaliana was handled as described in the first ISR assay (Fig. 3.5.1). Instead of recording disease severity 72 h after pathogen challenge (hapc), all the leaves of sixteen plants (treated with each bacteria (5)) were harvested at 6, 12 and 24 hapc, powdered in liquid nitrogen and stored at -80ºC. These plant samples were used for gene expression analysis by qPCR.

2.7 RNA extraction and RT-qPCR analysis (second ISR experiment)

Prior to RNA extraction, samples were grounded to a fine powder with liquid nitrogen. Total RNA was isolated from each replicate with PureLink RNA Micro Kit (Invitrogen), DNAase treatment included. RNA purity was confirmed using NanodropTM. A retrotranscription followed by RT-qPCR was performed.

The retrotranscription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were carried out using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 ºC, 30 min 42 ºC, 5 min 85 ºC, and hold at 4 ºC. Amplification was carried out with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 ºC and then 39 cycles consisting of 15 s at 95 ºC, 30 s at 55 ºC and 30 s at 72 ºC, followed by melting curve to check results. To describe the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method. *Sand* gene (AT2G28390) was used as reference gen (Remans et al. 2008). Gene primers used are shown in Table 3.5.1.

Table 3.5.1 Forward and reverse primers used in qPCR analysis.

	Forward primer	Reverse primer
AtNPR1	5´-TATTGTCAARTCTRATGTAGAT	5´-TATTGTCAARTCTRATGTAGAT
AtPR1	5´-AGTTGTTTGGAGAAAGTCAG	5´-GTTCACATAATTCCCACGA
AtICS	5´-GCAAGAATCATGTTCCTACC	5´AATTATCCTGCTGTTACGAG
AtPdf1	5´-TTGTTCTCTTTGCTGCTTTCGA	5´-TTGGCTTCTCGCACAACTTCT
AtLOX2	5´-ACTTGCTCGTCCGGTAATTGG	5´-GTACGGCCTTGCCTGTGAATG
AtMYC2	5´GATGAGGAGGTGACGGATACGGAA	5´-CGCTTTACCAGCTAATCCCGCA
AtPR2	5´-TCGTCTCGATTATGCTCTCTTC	5´-GCAGAATACACAGCATCCAAAA
AtPR3	5´-AAATCAACCTAGCAGGCCACT	5´-GAGGGAGAGGAACACCTTGACT
Sand	*5′* -CTGTCTTCTCATCTCTTGTC	*5′* -TCTTGCAATATGGTTCCTG

**At* = A. *thaliana*

2.8 Metabolic elicitors' extraction and its capacity to induce systemic resistance. Third ISR experiment.

Based on data from qPCRs and protection from the first ISR experiment, two strains were chosen to isolate their metabolic elicitors and check their capacity to mimic bacterial protection: *Serratia rubidaea* N 12.34 because it was the one with best differential expression results and *Bacillus cereus* N 4.1 because it was the Gram-positive one with best protection against disease results.

Metabolic elicitors were extracted according to Sumayo et al. (2013) protocol until obtaining n-hexane, ethyl acetate and n-butanol fractions. Briefly, strains were grown in nutrient broth (CONDA) on a rotary shaker (180 rpm) at 28 ºC for 24 h. Cells were eliminated by centrifugation at 8000 *x* g for 15 min. Five hundred mL of the obtained supernatant was filtrated by a 0,2 μm nitrocellulose filter. This filtrate was used to extract metabolic elicitors. First, a double extraction 1:1 (v/v) with n-hexane was made. The remaining aqueous phase was extracted twice with ethyl acetate (1:1 v/v), and finally, the aqueous phase was extracted twice with n-butanol (1:1 v/v). The organic phases (n-

hexane, ethyl acetate and n-butanol) were pooled and evaporated to dryness in a rotary evaporator at 50 ºC. The dry residues obtained were dissolved in 25 mL of 10 % Dimethyl sulfoxide (DMSO).

A third ISR assay on *A. thaliana* plants to evaluate the ability of the three metabolic elicitor fractions extracted from N 12.34 and N 4.1 strains was carried out. Four treatments per strain were defined: a) metabolic elicitors in the n-hexane fraction, b) metabolic elicitors in the ethyl acetate fraction, c) metabolic elicitors in the n-butanol fraction, and e) positive control (BTH (Sumayo et al. 2013)). Additional controls (negative control) with the fractions extracted with n-hexane, ethyl acetate and n-butanol from nutrient broth (without bacteria) and dissolved in 10% DMSO were also included to ensure that elicitor effects were due to bacterial components and not to the nutrient broth or the DMSO. All were pathogen challenged.

A. thaliana was handled as described in the first ISR assay (Fig. 3.5.1). Treatments were delivered to seedlings by soil drench (50 µL of the three metabolic elicitor fractions, 10 µL of BTH (positive control), and 50 µL of each negative control fraction). The pathogen was also inoculated as described in the first ISR assay. Seventy-two hours after pathogen inoculation, disease severity was recorded and relativized as in the first ISR experiment (Fig 3.5.1).

2.9 RT-qPCR analysis of the genes triggered by metabolic elicitor fractions (fourth ISR experiment)

Based on data from the third ISR experiment, another ISR assay was carried out using the protocol explained above. The two most effective metabolic elicitor fractions against pathogen attack from each bacteria (n-hexane and ethyl acetate) were used. Differential gene expression of *NPR1*, *PR2* and *PDF1* for strain N 12.34 and *NPR1*, *PR3* and *PDF1* for strain N 4.1 were analysed. In the case of strain N 12.34, analysis was performed at 6

and 12 hapc, and in that of strain N 4.1, at 12 and 24 hapc. Genes and sampling moments were selected according to previous results of the first qPCR experiment.

A. thaliana was handled as described in the first ISR assay (Fig. 3.5.1). Treatments were n-hexane metabolic elicitor fraction from N 12.34, ethyl acetate metabolic elicitor fraction from N 12.34, n-hexane metabolic elicitor fraction from N 4.1, ethyl acetate metabolic elicitor fraction from N 4.1 and controls with n-hexane and ethyl acetate (sterile nutrient broth was used to obtain control n-hexane and control ethyl-acetate fractions). Plants were inoculated by soil drench (50 µL), and challenge inoculation with DC3000 was performed as explained above.

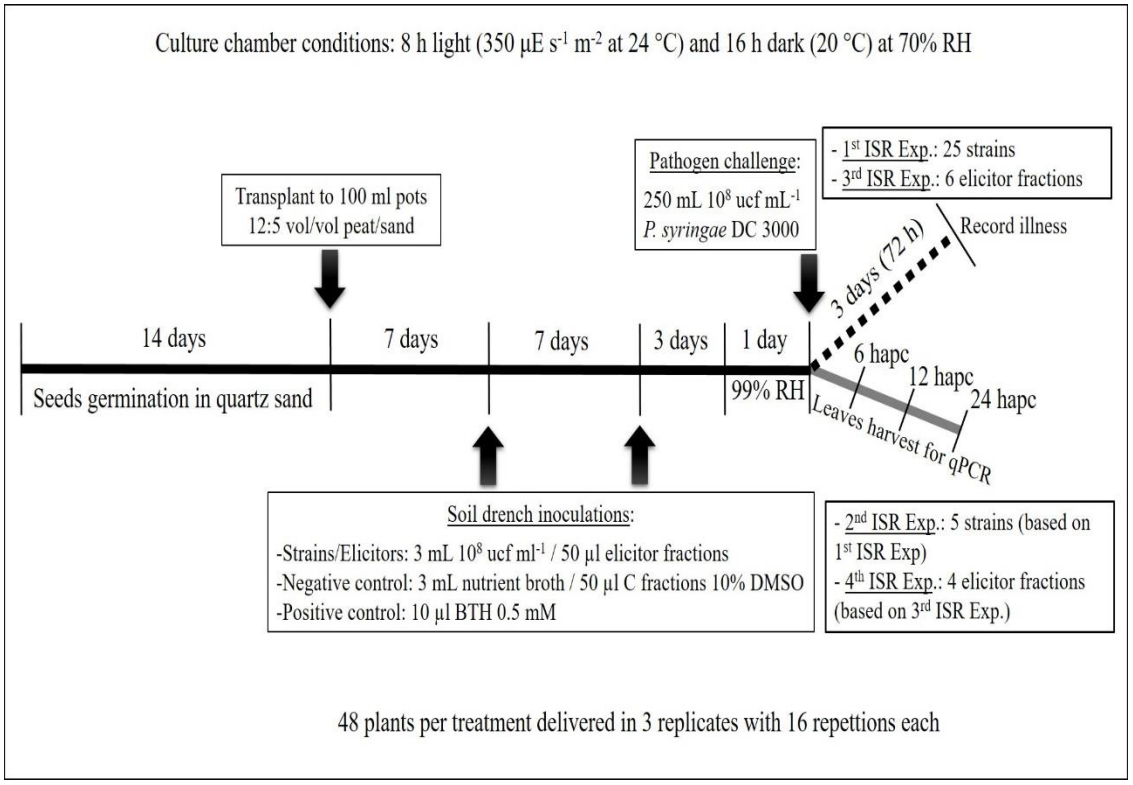

Figure 3.5.1 ISR experiments represented as a timeline. The black continuous line represents the part of the experimental design common to all ISR experiments; the black dashed line represents the last part of ISR experiments to assess protection against the pathogen DC 3000 (first and third experiments) and the grey continuous line represents the last part of ISR experiments to carry out differential gene expression analyses by qPCR (second and fourth experiments).

2.10 Statistical analysis

One-way ANOVA with replicates was used to check the statistical differences in all data obtained. Prior to ANOVA analysis, homoscedasticity and normality of the variance was checked with Statgraphics plus 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p < 0.05$) a Fisher test was used (Sokal and Rohlf, 1980).

3. Results

3.1 Beneficial rhizobacteria screening: phylogenetic tree and biochemical tests

A phylogenetic tree was performed with the 16S rRNA sequences of the 175 bacterial strains (Fig. 3.5.2). Two main groups appeared, one made up of Gram-positive (74 strains) and the other of Gram-negative bacteria (101 strains).

In the Gram-negative group, eight genera were found (*Serratia*, *Enterobacter*, *Pantoea*, *Erwinia*, *Cronobacter*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*), being *Pseudomonas* especially diverse in species (5 species identified: *P. putida*, *P. reinekei*, *P. brassicacearum*, *P. fragi* and *P. fluorescens*). In the Gram-positive group, only two genera were found, (*Bacillus* and *Brevibacterium*). Within *Bacillus*, two species were especially abundant, *Bacillus cereus* and *Bacillus megaterium* (Fig. 3.5.2).

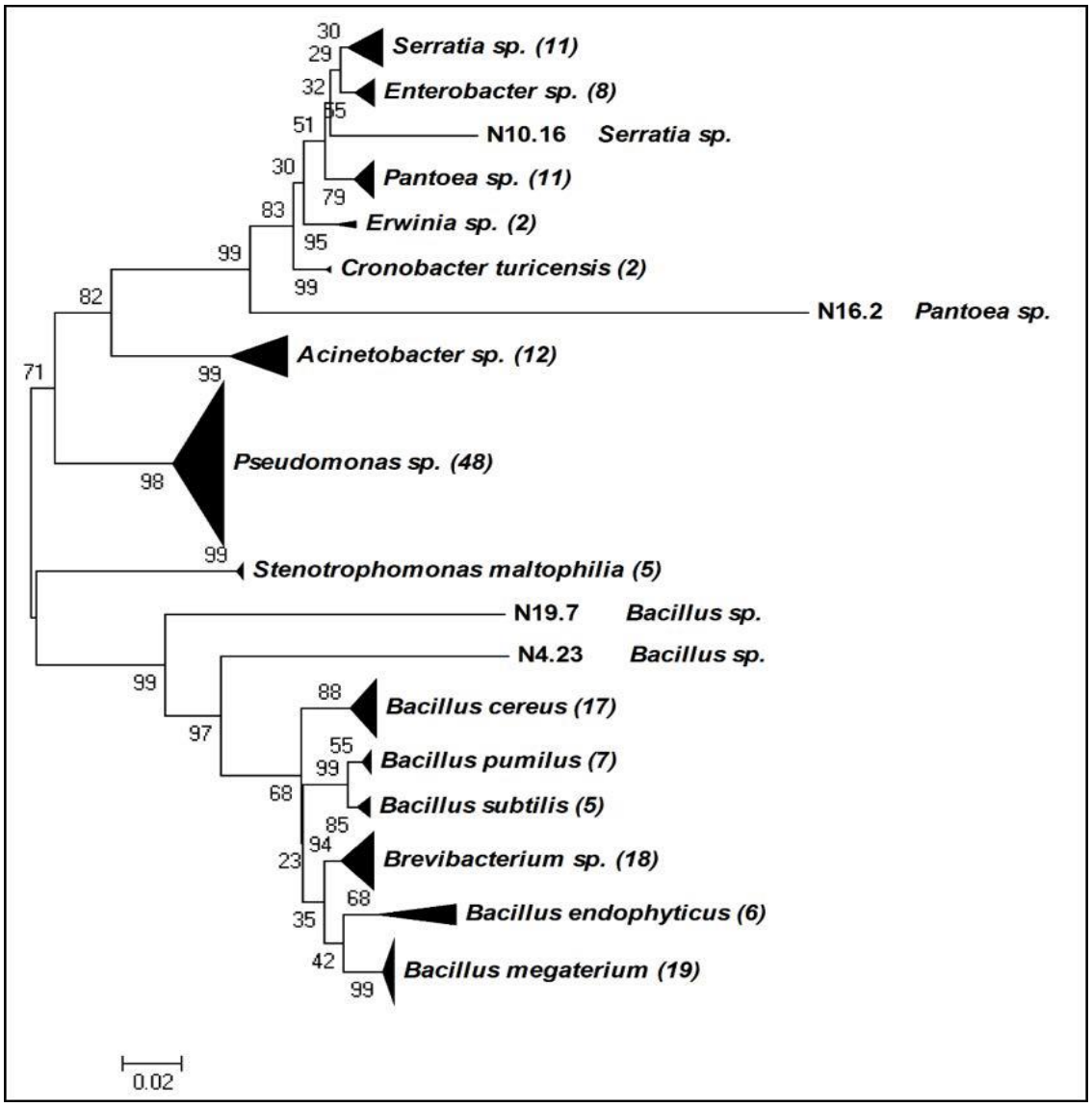

Figure 3.5.2 Phylogenetic tree performed with the 16S rRNA sequences. The evolutionary distances were inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. The tree was conducted in MEGA4. Annotation of bacteria included in the tree, were obtained from the NCBI (http://www.ncbi. nlm.nih.gov/). The number in brackets indicates number of species within each phylogenetic group.

Biochemical tests (auxin-like compounds production (Sergeeva et al. 2007), siderophores production (Alexander and Zuberer, 1991), phosphate solubilisation (De Freitas et al. 1997), and chitinases production (Rodríguez-Kábana et al. 1983; Frändberg and Shnurer, 1998)) for identifying putative beneficial rhizobacteria were carried out to

the 175 strains. The results of these tests are shown in Table 3.5.2. Within Gram-negative bacteria, *Enterobacter* was the only genus across all isolates tested that were capable of producing indole acetic acid (IAA). Siderophore producing isolates were present in all genera. *Acinetobacter* and *Pseudomonas* showed the highest percentage of phosphate solubilisers, but also isolates of *Enterobacter*, *Pantoea* and *Erwinia* were able to solubilise phosphate. Finally, all *Stenotrophomonas* isolates were able to produce chitinases (100%). Isolates able to produce siderophores and also solubilise phosphates belonged to *Enterobacter*, *Pantoea, Erwinia, Acinetobacter* and *Pseudomonas*. Those able to produce siderophores and also chitinases were present among *Stenotrophomonas* and *Pseudomonas*, although less abundant among the latter (2.08%). The unique genus that had isolates with three biochemical traits was *Enterobacter*. It was able to produce siderophores and IAA and also, to solubilise phosphate.

Within Gram-positive bacteria, none of the isolates produced IAA, however all were able to produce siderophores. Only *B. cereus, Brevibacterium* sp. and *B. megaterium* were able to solubilise phosphate. *B. cereus* and *B. subtilis* were able to produce chitinases. The isolates that were able to produce siderophores and also solubilise phosphates were *B. cereus*, *Brevibacterium* sp. and *B. megaterium*. The isolates that were able to produce siderophores and also chitinases were *B. cereus* and *B. subtilis.* The unique isolate that had three biochemical traits was *B. cereus*. It was able to produce siderophores and chitinases and also to solubilise phosphate.

Table 3.5.2. Percentage of bacteria within each genera or species (in Gram-positive group), positive for biochemical traits.

	Gram-negative group							
Biochemical traits	*Serratia*	*Enterobacter*	*Pantoea*	*Erwinia*	*Cronobacter*	*Acinetobacter*	*Pseudomonas*	*Stenotrophomonas*
IAA production	0.00	37.5	0.00	0.00	0.00	0.00	0.00	0.00
Siderophores production	100.00	87.5	100.00	100.00	100.00	83.33	100.00	100.00
Phosphate solubilisation	0.00	75.00	45.45	50.00	0.00	100.00	91.67	0.00
Chitinases production	0.00	0.00	0.00	0.00	0,00	0.00	2.08	100.00
Siderophores production and phosphate solubilisation	0.00	62.50	45.45	50.00	0.00	83.33	85.42	0.00
Siderophores and chitinases production	0.00	0.00	0.00	0.00	0.00	0.00	2.08	100.00
Siderophores and IAA production and phosphate solubilisation	0.00	25.00	0.00	0.00	0.00	0.00	0.00	0.00

	Gram-positive group					
Biochemical traits	*Bacillus cereus*	*Bacillus pumillus*	*Bacillus subtilis*	*Brevibacterium* sp.	*Bacillus endophyticus*	*Bacillus megaterium*
IAA production	0.00	0.00	0.00	0.00	0.00	0.00
Siderophores production	100.00	100.00	100.00	100.00	100.00	100.00
Phosphate solubilisation	23.08	0.00	0.00	11.76	0.00	36.84
Chitinases production	46.15	0.00	20.00	0.00	0.00	0.00
Siderophores production and phosphate solubilisation	23.08	0.00	0.00	11.76	0.00	36.84
Siderophores and chitinases production	15.38	0.00	20.00	0.00	0.00	0.00
Siderophores and chitinases production and phosphate solubilisation	7.69	0.00	0.00	0.00	0.00	0.00

Biochemical traits are Indole Acetic Acid (IAA) production, siderophores production, phosphate solubilisation, chitinases production and the combination of these traits.

3.2 ISR by beneficial rhizobacteria

According to the results obtained from the phylogenetic tree (Fig. 3.5.2) and the biochemical tests (Table 3.5.2), twenty-five strains were chosen (fifteen Gram-negative and ten Gram-positive) to develop a first protection experiment against the pathogen *P. syringae* DC3000. All selected strains had at least two or three biochemical traits, except N 10.7 *Serratia odorifera*, N 12.34 *S. rubidaea* and N 11.14 *Bacillus endophyticus* that only had one activity, but they were able to reduce growth of other strains in plate (data not shown), probably due to the production of antibiotics. The selected strains and their biochemical traits are shown in table 3.5.3.

Table 3.5.3. Twenty-five selected strains and its biochemical traits.

			Biochemical traits			
	Bacterial Strain		**IAA production**	**Siderophores production**	**Chitinases production**	**Phosphate solubilisation**
GRAM -	N 5.12	*Pseudomonas putida*		+		+
	N 8.17	*Stenotrophomonas maltophilia*		+	+	
	N 8.22	*Stenotrophomonas* sp.		+	+	
	N 9.11	*Pseudomonas reinekei*		+		+
	N 10.6	*Pseudomonas putida*		+		+
	N 10.7	*Serratia odorifera*		+		
	N 10.21	*Pseudomonas putida*		+		+
	N 12.34	*Serratia rubidaea*		+		
	N 15.23	*Pseudomonas brassicacearum*		+		+
	N 16.3	*Pantoea* sp.		+		+
	N 16.15	*Enterobacter* sp.	+			+
	N 16.23	*Pantoea agglomerans*		+		+
	N 16.24	*Enterobacter sp.*	+	+		+
	N 18.10	*Pseudomonas fragi*		+		+
	N 21.24	*Pseudomonas fluorescens*		+		+
GRAM +	N 4.1	*Bacillus cereus*		+	+	
	N 5.20	*Bacillus cereus*		+	+	+
	N 8.10	*Bacillus* sp.		+		+
	N 11.5	*Brevibacterium* sp.		+		+
	N 11.14	*Bacillus endophyticus*		+		
	N 11.20	*Bacillus atrophaeus*		+	+	
	N 11.22	*Bacillus megaterium*		+		+
	N 11.36	*Bacillus megaterium*		+		+
	N 11.40	*Bacillus megaterium*		+		+
	N 20.15	*Bacillus simplex*		+		+

Biochemical traits are Indole Acetic Acid (IAA) production, siderophores production, phosphate solubilisation and chitinases production. A positive biochemical trait of each bacteria is indicated by a + symbol.

Table 3.5.4 shows the percentage (%) of protection induced in seedlings of *A. thaliana* inoculated with the twenty-five selected strains and the percentage of protection of negative and positive control plants. All Gram-negative bacteria significantly protected against the pathogen, except N 8.22, N 10.6, N 10.21, N 15.23 and N 18.10. Protection achieved by N 16.24 was not statistically significant. N 5.12 (*P. putida*), N 8.17 (*S. maltophilia*), N 12.34 (*S. rubidaea*) and N 21.24 (*P. fluorescens*) were the Gram-negative bacteria that induced the highest protection, even above of that of the positive control. Therefore, these four strains were chosen for assessing differential gene expression of eight genes, markers of different signal transduction pathways related to plant immune system.

Within Gram-positive bacteria, all of them significantly protected against the pathogen, except N 11.14, N 11.22 and N 11.36. Strain N 4.1 (*B. cereus*) was the Gram-positive bacterium that performed best, so it was selected to assess the differential gene expression of eight genes, markers of different signal transduction pathways related to plant immune system.

Table 3.5.4 Percentage of protection (%) induced in *A. thaliana* seedlings inoculated with chosen strains against the pathogen P. syringae DC3000.

Treatment			% of protection
Controls	Negative Control	Nutrient broth	0
	Positive Control	Benzothiadiazole (BTH)	54.21 ± 4.03 *
Gram-negative strains	**N 5.12**	***Pseudomonas putida***	**57.69 ± 1.76 ***
	N 8.17	***Stenotrophomonas maltophilia***	**64.87 ± 1.79 ***
	N 8.22	*Stenotrophomonas* sp.	0
	N 9.11	*Pseudomonas reinekei*	51.44 ± 6.88 *
	N 10.6	*Pseudomonas putida*	0
	N 10.7	*Serratia odorífera*	33.76 ± 3.22 *
	N 10.21	*Pseudomonas putida*	0
	N 12.34	***Serratia rubidaea***	**56.64 ± 2.15 ***
	N 15.23	*Pseudomonas brassicacearum*	0
	N 16.3	*Pantoea* sp.	14.91 ± 2.45 *
	N 16.15	*Enterobacter* sp.	24.18 ± 1.96 *
	N 16.23	*Pantoea agglomerans*	21.21 ± 7.32 *
	N 16.24	*Enterobacter sp.*	6.93 ± 2.31
	N 18.10	*Pseudomonas fragi*	0
	N 21.24	***Pseudomonas fluorescens***	**82.08 ± 2.46 ***
Gram-positive strains	**N 4.1**	***Bacillus cereus***	**69.45 ± 0.38 ***
	N 5.20	*Bacillus cereus*	49.75 ± 0.82 *
	N 8.10	*Bacillus* sp.	22.93 ± 2.93 *
	N 11.5	*Brevibacterium* sp.	29.82 ± 1.82 *
	N 11.14	*Bacillus endophyticus*	0
	N 11.20	*Bacillus atrophaeus*	42.72 ± 3.51 *
	N 11.22	*Bacillus megaterium*	0
	N 11.36	*Bacillus megaterium*	0
	N 11.40	*Bacillus megaterium*	23.98 ± 0.18 *
	N 20.15	*Bacillus simplex*	30.83 ± 4.92 *

The percentage of protection was calculated based on the number of leaves with disease symptoms to the total of leaves (n=12 seedlings per replicate). Data were relativized to negative control (seedlings inoculated only with nutrient broth and pathogen challenged), which was considered as 0% protection. A positive control (BTH) was also used. Strains in bold are those whose percentage of protection against the pathogen *P. syringae* DC3000 exceeded that of the positive control and therefore, those that were selected for further analyses. Asterisks represent statistically significant differences ($p < 0.05$) with regard to negative control.

Differential gene expression at 6, 12 and 24 hours after pathogen challenge (hapc) of *A. thaliana* plants inoculated with selected strains (N 5.12 (*P. putida*), N 8.17 (*S. maltophilia*), N 12.34 (*S. rubidaea*), N 21.24 (*P. fluorescens*) and N 4.1 (*B. cereus*) is shown in figures 3.5.3 to 3.5.7. Three different behaviours appeared among the five strains. The first behaviour was a strong and significant increase at 6 hapc, followed by strains N 5.12 (Fig. 3.5.3) and N 21.24 (Fig. 3.5.6); N 5.12 increased the expression of *NPR1* (12.55 times), *PDF1* (376.54 times) and *PR3* (4.53 times), while N 21.24 strongly induced *ICS* (42.11 times) and *LOX2* (10.66 times) at 6 hapc. A second behaviour pattern was a significant increase in expression at 12 hapc, only followed by N 12.34 (Fig. 3.5.5) with a very high increment of the differential expression of *NPR1*(149.74 times), *PR2* (57.09 times), *PDF1* (675.98 times), *PR3* (41.37 times), and *LOX2* (32.79 times). The third pattern was a significant increase at 24 hapc, followed by strains N 8.17 (Fig. 3.5.4) and N 4.1 (Fig. 3.5.7). *ICS* (1.79 times), *PR1*(1.95 times), *PR2* (2.22 times) and *MYC2* (2.02 times) were the genes induced by N 8.17, while all genes studied were induced by N 4.1 (from 1.24 times for *MYC2* until 5.01 times for *NPR1*).

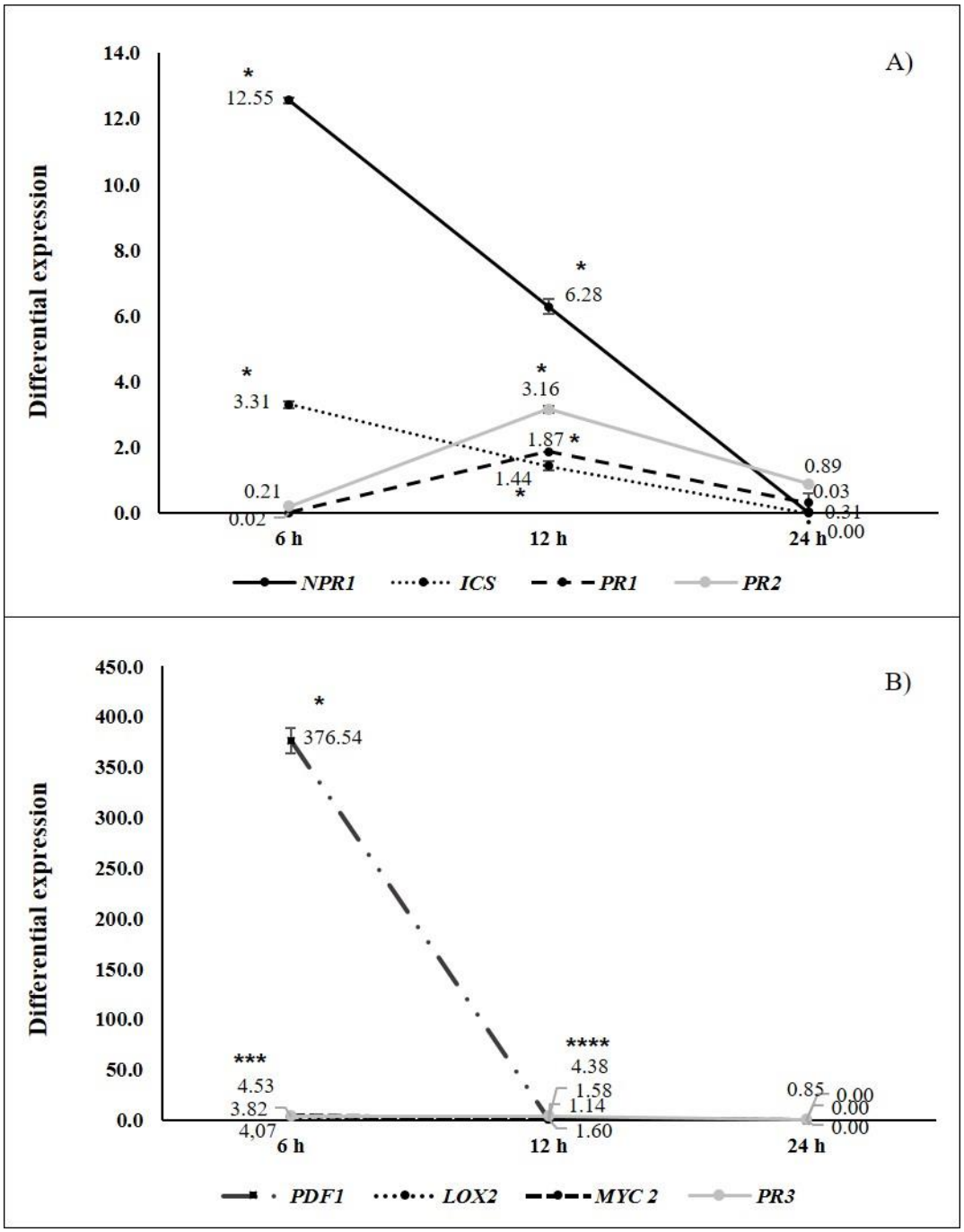

Figure 3.5.3 Differential gene expression (seedlings inoculated with N 5.12 (*Pseudomonas putida*) vs negative control) at 6 (n=16), 12 (n=16) and 24 (n=16) hapc; A) *NPR1*, *ICS*, *PR1* and *PR2* genes (as SA signalling pathway markers) and B) *PDF1*, *LOX2* , *MYC2* and *PR3* (as JA/ET signalling pathway markers). Asterisks represent statistically significant differences ($p < 0.05$) with respect to negative control (differential expression of 1).

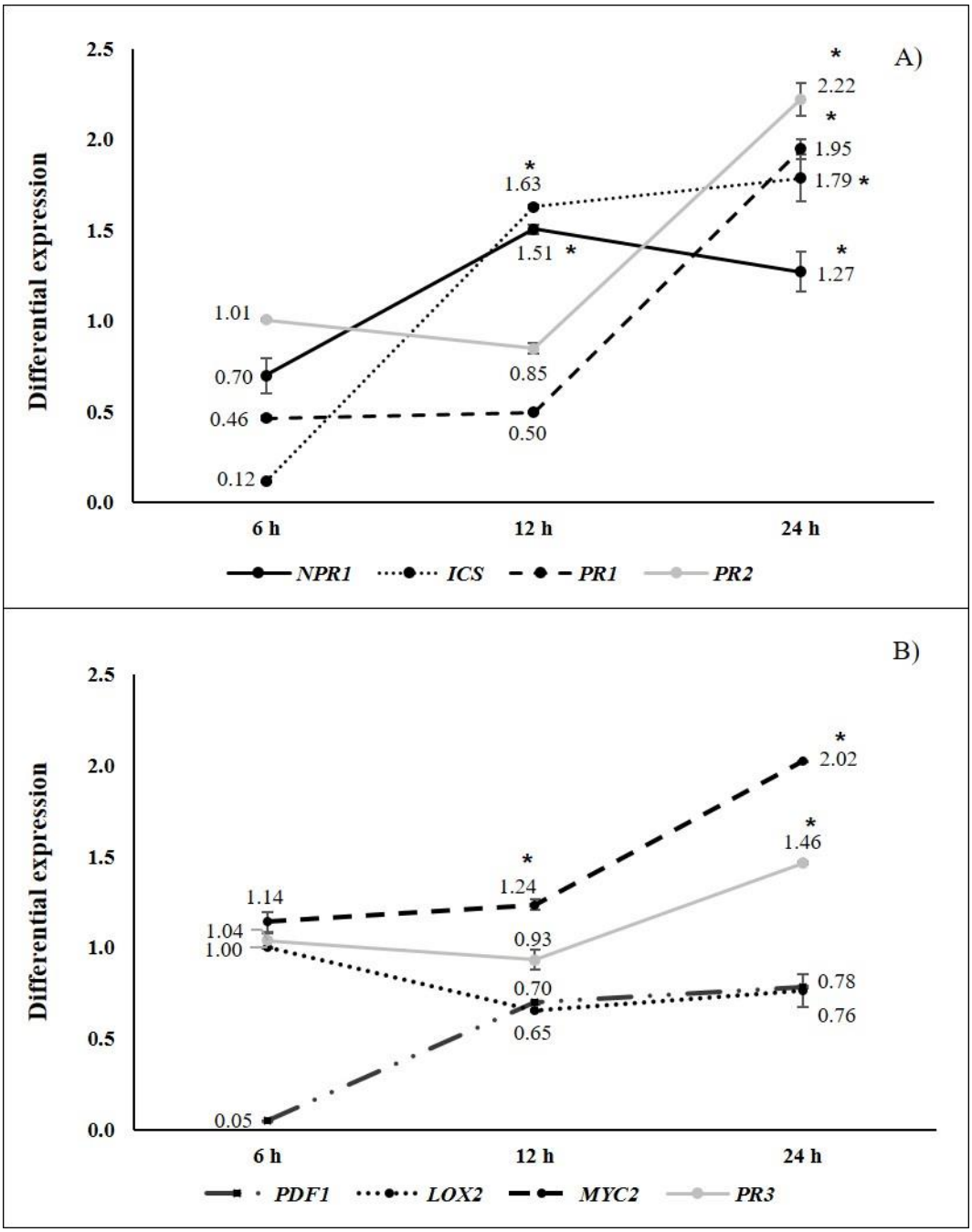

Figure 3.5.4 Differential gene expression (seedlings inoculated with N 8.17 (*Stenotrophomonas maltophilia*) vs negative control) at 6 (n=16), 12 (n=16) and 24 (n=16) hapc; A) *NPR1*, *ICS*, *PR1* and *PR2* genes (as SA signalling pathway markers) and B) *PDF1*, *LOX2* , *MYC2* and *PR3* (as JA/ET signalling pathway markers). Asterisks represent statistically significant differences ($p < 0.05$) with respect to negative control (differential expression of 1).

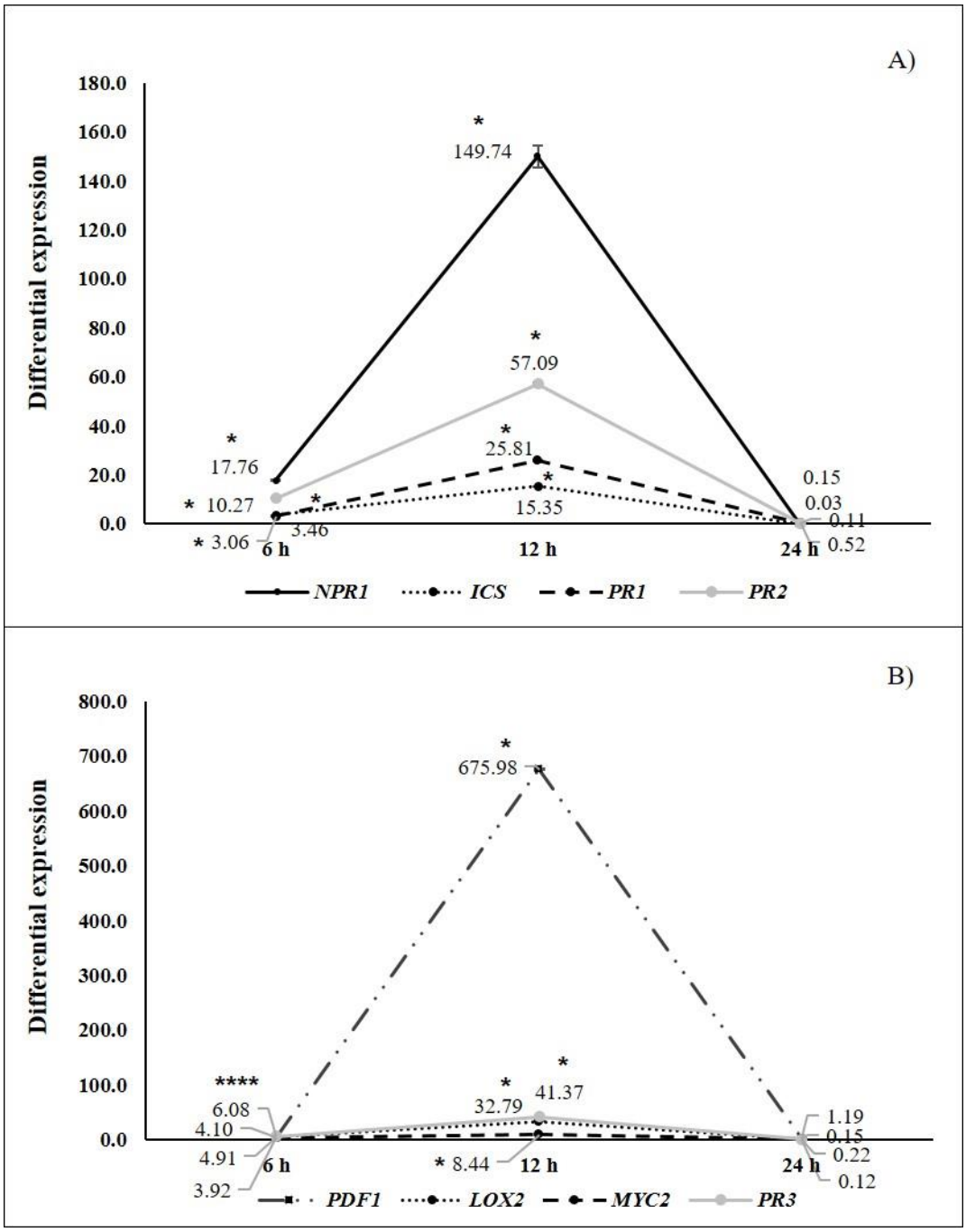

Figure 3.5.5 Differential gene expression (seedlings inoculated with N 12.34 (*Serratia rubidaea*) vs negative control) at 6 (n=16), 12 (n=16) and 24 (n=16) hapc; A) *NPR1*, *ICS*, *PR1* and *PR2* genes (as SA signalling pathway markers) and B) *PDF1*, *LOX2* , *MYC2* and *PR3* (as JA/ET signalling pathway markers). Asterisks represent statistically significant differences ($p < 0.05$) with respect to negative control (differential expression of 1).

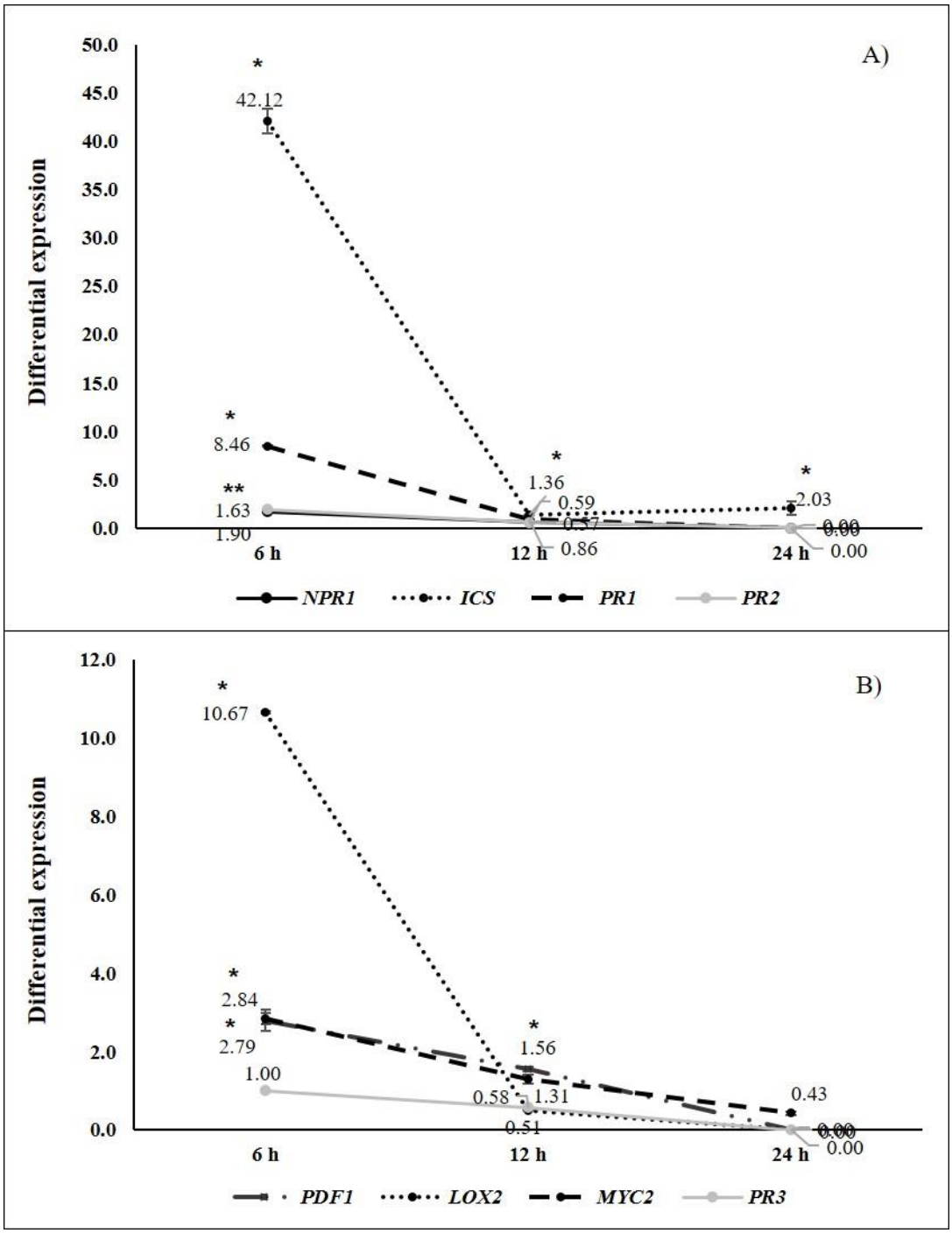

Figure 3.5.6 Differential gene expression (seedlings inoculated with N 21.24 (*Pseudomonas fluorescens*) vs negative control) at 6 (n=16), 12 (n=16) and 24 (n=16) hapc; A) *NPR1*, *ICS*, *PR1* and *PR2* genes (as SA signalling pathway markers) and B) *PDF1*, *LOX2* , *MYC2* and PR3 (as JA/ET signalling pathway markers). Asterisks represent statistically significant differences ($p < 0.05$) with respect to negative control (differential expression of 1).

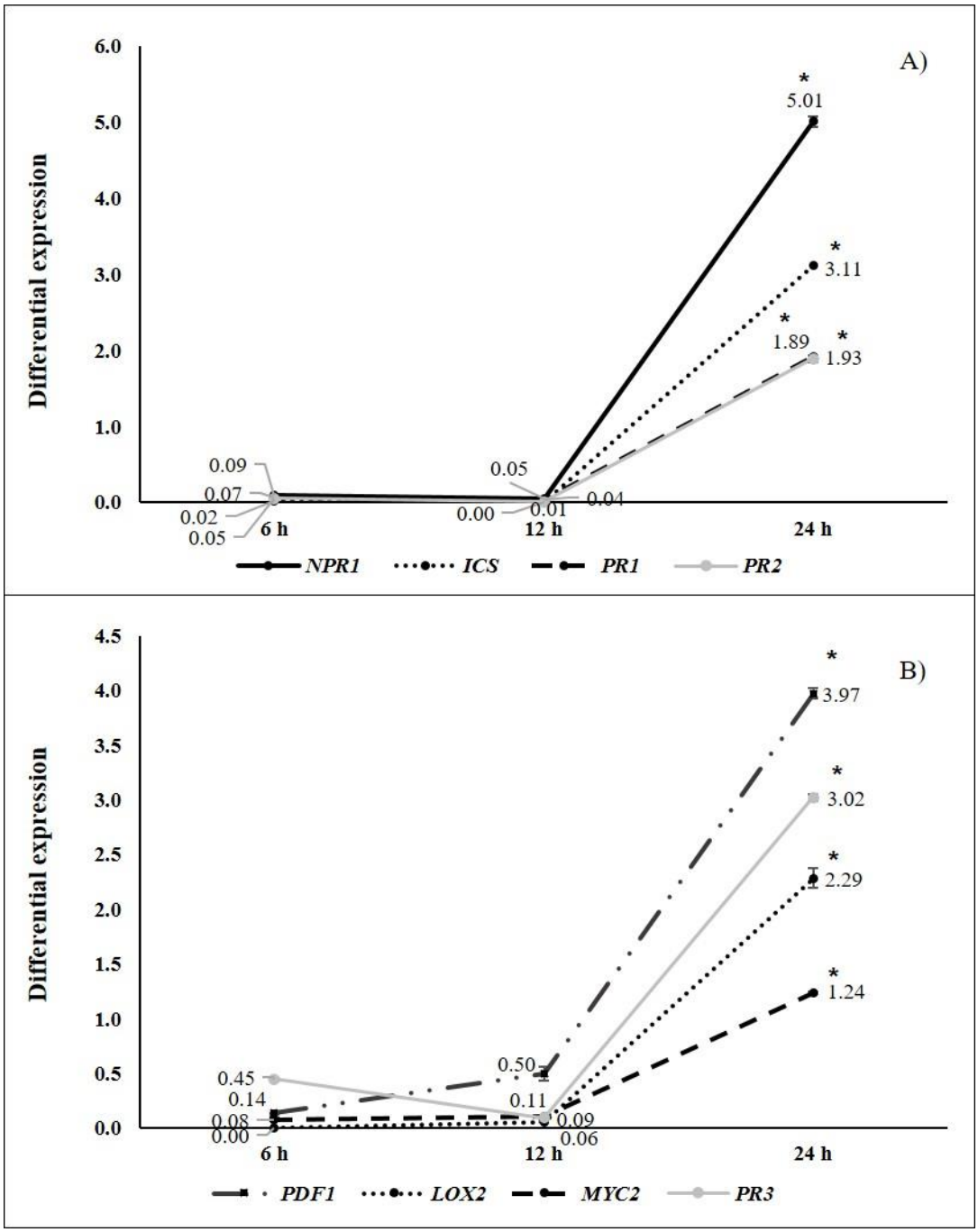

Figure 3.5.7 Differential gene expression (seedlings inoculated with N 4.1 (*Bacillus cereus*) vs negative control) at 6 (n=16), 12 (n=16) and 24 (n=16) hapc; A) *NPR1*, *ICS*, *PR1* and *PR2* genes (as SA signalling pathway markers) and B) *PDF1*, *LOX2* , *MYC2* and *PR3* (as JA/ET signalling pathway markers). Asterisks represent statistically significant differences ($p < 0.05$) with respect to negative control (differential expression of 1).

3.3 ISR by metabolic elicitors

Based on all the results, two strains were selected to extract their metabolic elicitors and to check the capacity of these metabolic elicitors to mimic protective effects of bacteria. They were selected N 12.34 (*S. rubidaea*), the Gram-negative strain that showed the highest differential expression (Fig. 3.5.5) and N 4.1 (*B. cereus*) as it was the Gram-positive strain with better protection among the Gram-positive and which ranked second among all (Table 3.5.4).

The three fractions extracted from each strain (n-hexane, ethyl acetate and n-butanol), achieved significant protection (Table 3.5.5), having an outstanding performance metabolic elicitors in the n-hexane and ethyl acetate fractions. Protection of the n-hexane (61.26%) and the ethyl acetate (54.64%) fractions of N 12.34 and protection of the n-hexane (68.11) and the ethyl acetate (67.30%) fractions of N 4.1 was similar to that obtained with the bacterial strains (56.64% for N 12.34 and 69.45% for N 4.1, respectively).

Table 3.5.5. Percentage of protection (%) induced in *A. thaliana* seedlings inoculated with elicitor fractions against DC3000.

Treatment		% of protection
Controls	Negative control	0
	Positive control (BTH)	52.09 ± 1.75 *
N 12.34	**n-Hexane**	**61.26 ± 2.23 ***
	Ethyl acetate	**54.64 ± 1.48 ***
	n-Butanol	35.42 ± 2.77 *
N 4.1	**n-Hexane**	**68.11 ± 0.76 ***
	Ethyl acetate	**67.30 ± 3.76 ***
	n-Butanol	52.31 ± 1.91 *

A. thaliana seedlings were elicited with the n-hexane, ethyl actetate and n-butanol fractions exctracted from strains N 12.34 and N 4.1. Percentages were estimated according to the number of leaves with pathogen infection symptoms with respect to the total of leaves (n=16 seedlings per replicate). Negative control was considered as 0% of protection and then data were relativized with respect to it. A positive control (BTH) was also used. Fractions in bold are those whose percentage of protection against the pathogen DC3000 exceeded that of the positive control and therefore, those that were selected for further analyses. Asterisks indicate statistically significant differences ($p < 0.05$) with respect to negative control.

Differential gene expression induced by metabolic elicitors in the n-hexane and ethyl acetate fractions (the fractions with greatest protective capacity) from N 12.34 and N 4.1 is shown in figure 3.5.8. In the case of N 12.34, analysis was performed at 6 and 12 hapc, and in that of N 4.1, at 12 and 24 hapc. Genes and sampling moments were selected according to the results obtained in the previous qPCR experiment.

The two metabolic elicitor fractions from N 12.34 induced the same behaviour in the genes studied: expression of *NPR1* and *PR2* increased from 6 to 12 hapc, while *PDF1* decreased. Both metabolic elicitor fractions from N 4.1 also had the same behaviour: expression of *NPR1* and *PDF1* decreased from 12 to 24 hapc, while *PR3* increased.

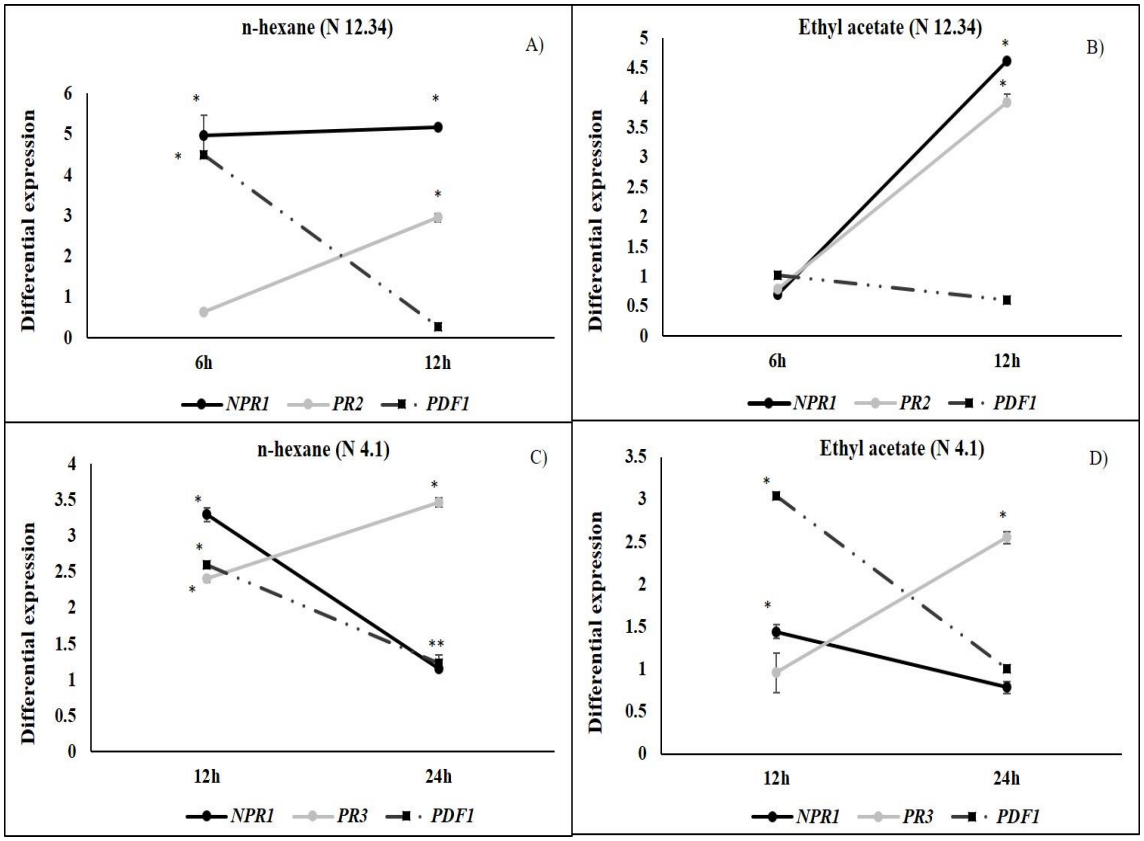

Figure 3.5.8 Differential gene expression in plants under the following treatments: A) N 12.34 elicitors from the n-hexane fraction; B) N 12.34 elicitors from the ethyl-acetate fraction; C) N 4.1 elicitors from the n-hexane fraction and D) N 4.1 elicitors from the ethyl-acetate fraction vs negative control, at 6 (n=16) and 12 (n=16) hapc in N 12.34 (A) and B)) and at 12 (n=16) and 24 (n=16) hapc in N 4.1 (C) and D)). *NPR1* and *PR2* genes as markers of the SA signalling pathway and *PDF1* as marker of the JA/ET signalling pathway in N 12.34; *NPR1* as marker of the SA signalling pathway, and *PDF1* and *PR3* as markers of the JA/ET signalling pathway in N 4.1. Asterisks represent statistically significant differences ($p < 0.05$) within each sampling time. Genes and sampling times were chosen based on results obtained by bacterial strains (Figs. 3.5.5 and 3.5.7).

4. Discussion

In the present study, the efficiency of bioeffectors and derived metabolic elicitors to trigger the immune system of *A. thaliana* conferring protection against *P. syringae* DC3000 has been shown.

The 175 strains were isolated in 2010 (Ramos-Solano et al. 2010a) from the rhizosphere of wild populations of *N. glauca*. This plant species was chosen as it was hypothesized that its very active secondary metabolism would select a good group of bacteria to ensure plant fitness.

The rationale of plant's selection capacity has been widely demonstrated, and also the use of the rhizosphere as a source of highly specialized strains (Barriuso et al. 2005; Ramos Solano et al. 2006; Lucas et al. 2013; Aarab et al. 2015; Anwar et. al 2016;), since it is one of the most complex and diverse ecosystems on earth. This suggests a definite role of plant-derived metabolites in the microbiome assemblage in the rhizosphere (Yang et al. 2017; Zhang et al. 2017). According to previous results, the common culturable bacterial genera in the rhizosphere of *N. glauca* includes *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Acinetobacter* sp., *Burkholderia* sp., *Arthrobacter* sp., and *Paenibacillus* sp. (Ramos-Solano et al. 2010a).

In the present study, almost 100% of the strains produced siderophores. Siderophore production is related to iron limiting nutrient (Raymond et al. 1984; Jin et al. 2006; Lucas et al. 2013), but also has been related to biocontrol and/or systemic induction of secondary metabolism, and therefore, siderophore-producing strains may have the ability to protect plants against pathogens through complex and inducible secondary metabolism, which is probably related to defence (Sinclair et al. 2004; Barriuso et al. 2008).

Regarding the production of auxins and the ability to solubilise insoluble phosphorus, only one genus of those of our study was capable of producing auxins (*Enterobacter* sp). However, the solubilisation of phosphates was a very abundant activity among the strains studied. Our results support that *N. glauca* selects rhizobacteria related to nutrition or biocontrol activities (phosphate solubilisation and siderophore production) rather than those able to affect plant growth regulator balance (auxins production).

The production of chitinases was well represented within the Gram-positive group, but among the Gram-negatives, only the *Stenotrophomonas* genus was able to produce them, consistent with Ramos Solano et al. (2010a). Many species of rhizosphere microorganisms produce chitinolytic enzymes to protect themselves against fungi, since chitin is a major structural component of most fungal cell walls. Therefore, these microorganisms have an excellent potential as biocontrol agents (Lorito et al. 1993; Sid et al. 2003; Adesina et al. 2007).

The strains that were selected for ISR experiment were able to produce siderophores, and they had also some other complementary capacities, mainly the production of chitinases. This selection criterion has already been used by other authors with the aim of finding bacteria capable of inducing systemic resistance in plants (Van Loon et al. 1998; Ramamoorthy et al. 2001; Ramos Solano et al. 2008b Ramos-Solano et al. 2010a). The strain N 16.15 (*Enterobacter* sp.) was the only non-siderophore producing isolate, but it was one of the two strains that produced auxins, and was chosen for this reason. Some authors have shown that auxins are related to the induction of systemic resistance (Petti et al. 2012; Akram et al. 2016). Three strains, N 10.7 (*S. odofirera*), N 12.34 (*S. rubidaea*) and N 11.14 (*B. enterophyticus*) were chosen with only one biochemical trait, because of their capacity to reduce growth of other strains in plate (data not shown), probably due to the production of antibiotics. This working scheme has

proved to be very effective, since 16 out of the 25 strains chosen induced systemic resistance against the pathogen DC3000 (Table 3.5.4).

To determine signal transduction pathways triggered by the five outstanding strains, from the 25 previously selected, the differential expression of marker genes of the SA and JA/ET signalling pathways was studied. For this experiment, the criterion followed for the bioeffector selection was the highest protection against *P. syringae* DC 3000 infection within both bacterial groups (Gram-positive and Gram-negative). To date, most bioeffectors studied for their ability to trigger ISR mechanisms belong to the group of Gram-negative bacteria, especially bacteria of the genus *Pseudomonas*. However, Gram-positive bacteria, and among them, those of the genus *Bacillus*, have gained much importance in the last decade because of the great potential to trigger resistance mechanisms against a wide range of pathogens (Kannojia et al. 2018; Gutierrez-Albanchez et al. 2018).

Three types of defensive responses were detected, according to the time needed to increase gene expression: rapid, intermediate and slow. The rapid response (at 6 hapc) was generated by strains N.5.12 (*P. putida*) (Fig. 3.5.3) and N 21.24 (*P. fluorescens*) (Fig. 3.5.6). N 5.12, induced a strong differential expression of *NPR1*, a marker of SA pathway, *PDF1* and *PR3*, markers of the JA/ET pathway. Interestingly, N 21.24 induced a strong differential expression of *ICS* and *LOX2* involved in SA and JA synthesis, respectively. The intermediate response (at 12 hapc) was produced by N 12.34 (*S. rubidaea*) (Fig. 3.5.5), which induced a strong differential expression of markers of SA pathway (*NPR1* and *PR2*), and markers of the JA/ET pathway (*PDF1* and *PR3*). The different behaviour generated by these three strains is also reflected in their defensive capacity. Although the three induced resistance above the positive control (BTH), N 5.12 and N 12.34 induced a lower protection than N 21.24, which was the most effective of all the tested. Contrary to

Caarls et al. (2015), we observed a simultaneous high expression of *NPR1* and *PDF1* at 6 hapc for N 5.12 and at 12 hapc for N 12.34, suggesting that SA is not suppressing the expression of *PDF1* as these authors indicated. This may be related to the monomerisation process of NPR1 protein, (which has not been determined in this work), as well as with the location of this protein (nucleus or cytoplasm), which plays an important role in the suppression or not of the genes involved in the synthesis of JA by SA (Leon-Reyes et al. 2009; Caarls et al. 2015). The higher protection achieved by N 21.24 (Table 3.5.4), is probably related to the high expression of the genes related to the synthesis of SA and JA (*ICS* and *LOX2*) at 6 hapc (Fig. 3.5.6), something that was specific to this strain. Nowadays, the importance of high concentrations of SA and JA to trigger defensive responses mediated by both hormones is widely accepted (Spoel and Dong, 2012; Pieterse et al. 2014; Caarls et al. 2015).

Slow response strains showed a progressive increase on expression from 0 to 24 hapc. These strains, N 8.17 (*S. maltophilia*) (Fig. 3.5.4) and N 4.1 (*B. cereus*) (Fig. 3.5.7) ranked right after N 21.24 in *Arabidopsis* protection (Table 3.5.4). N 8.17 follows the classic SA response pathway elicitation by a beneficial strain: high expression levels of *ICS* and *NPR1* and consequently, high expression levels of *PR1*, while genes related with the JA/ET pathway were not expressed. Strain N 4.1 was able to stimulate both pathways (SA and JA/ET) simultaneously, according to the high expression levels of SA markers genes (*NPR1*, *ICS* and *PR1)* and JA/ET markers (*PDF1*, *LOX 2* and *PR3)* (Fig. 3.5.7), demonstrating again that these two pathways are not necessarily antagonistic, as previously indicated by several authors (Liu et al. 2016; Betsuyaku et al. 2017).

Based on gene expression and protection results, the Gram-negative *Serratia rubidaea* N 12.34 and the Gram-positive *Bacillus cereus* N 4.1 were selected to extract and purify their metabolic elicitors. Bacterial elicitors capable of starting defensive

immune responses in plants, have been found to be structural molecules, (e.g. flagellin (Ramirez-Prado et al. 2018)), or metabolic elicitors that are released into the medium (Munhoz et al. 2017; Wu et al. 2018; Martin-Rivilla et al. 2019; Martin-Rivilla et al. 2020c). Our research delves into the study of mixtures of metabolic elicitors extracted from rhizobacteria and according to their solubility in three different organic solvents. The objective was to compare the effect of these fractions with that of the bacteria, looking for similarities or differences in the response. For this reason, the genes studied and the hapc sampling moments in each case were set according to results obtained with the bacterial strains.

For both bacteria, metabolic elicitors in the n-hexane and the ethyl acetate fractions were as efficient in triggering the defensive response in the plant as the bioeffectors (bacteria) (Tables 3.5.4 and 3.5.5). Although a lack of effect of structural elicitors cannot be ruled out, it is evidenced herein that both bacteria are capable of releasing metabolic elicitors with the ability to elicit defensive metabolism in the plant very efficiently. On the other hand, since both fractions have elicitation capacity, it seems that the diversity of elicitors is high. This has also been proven by other authors using the same fractions (Sumayo et al. 2013; Fatima and Anjum, 2017; Martin-Rivilla et al. 2019).

Although metabolic elicitors of the two fractions studied protected to the same extent as the bacteria, the expression of the analysed genes had different behaviours. The strain N 12.34 induced gene expression levels more intensely (up to 140 times. Fig. 3.5.5) than metabolic elicitors (Fig. 3.5.8 A) and B)). The different intensity could be due to either the abundance of elicitors when the bacteria is delivered alive, holding all determinants, as compared to a subset of the same elicitors delivered on fractions, or because the plant is more sensitive to elicitors not present in the n-hexane and ethyl acetate fractions. The large difference in the levels of genetic expression indicates a level

of priming also different. It is known that the priming can modify the distribution of energetic resources compromising plant growth in favour of a more production of metabolites involved in defensive response (Van Hulten et al. 2006; Lucas et al. 2014). Therefore, in this case the use of metabolic elicitors may have advantages over bioeffectors.

Interestingly, metabolic elicitors in both fractions from *S. rubidaea* N 12.34 were able to activate the SA pathway, increasing the expression of *NPR1* and *PR2* (Fig. 3.5.8 A) and B)). In both fractions, *PDF1* expression (marker of the JA/ET pathway) decreased, which indicate that the metabolic elicitors present in this fraction were only activating the SA mediated transduction pathway, while the bacterial strain activated both. These results show that the elicitors detected by the plant in both cases have to be different, and so would be the PRRs involved in that response (Tang et al. 2017).

Regarding the *B. cereus* strain N 4.1, the two metabolic elicitor fractions (Fig. 3.5.8 C) and D)) did not match the bacterium except for *PR3*, a marker of the JA/ET pathway. These results suggest a lower diversity of effective metabolic elicitors, pointing out a more relevant role of structural elicitors triggering the SA mediated pathway observed with the live strain.

All these results show the great number of possibilities offered by elicitors to trigger the immune system of plants, which opens a plethora of biotechnological solutions to different stress situations. Application of elicitors has many advantages from the agronomic point of view because it is more economical and profitable to conserve a molecule than an live bacterium, which has nutritional and environmental requirements. In addition, the use of elicitors also implies less environmental aware for possible cases of ecological niches competition between edaphic species and also avoids problems of

infectious pathogenesis and alterations of the rhizosphere (Timmusk et al. 2017; Rosier et al. 2018).

5. Conclusion

The enormous biotechnological potential of the rhizosphere as a source of bacterial strains capable of establishing a beneficial relationship with plants and of modifying their defensive metabolism, improving their ability to defend themselves from pathogen attacks, has been evidenced.

In addition, triggering SA and/or JA/ET defensive pathways by bacteria seem to be more complex than current description in the literature and the concept of simultaneous elicitation of different pathways of plant immune system has been reinforced.

Each bacterium had a different effect in the genes studied, even within the same bacterial genus. In addition, the metabolic elicitors of the two studied strains had different effects to that produced by the bacteria, confirming the presence of many different bacterial molecules able to trigger plant metabolism. This is very interesting since it opens a huge amount of biotechnological possibilities to develop biological products for agriculture in different situations and plant species

4. Discussion

4. Discussion

The current need to improve and protect crops to provide sufficient and quality food to a continuous growing population, fulfilling the concept of "food security", is nowadays linked to the development of new and more efficient and ecofriendly agricultural methods based on improving the resistance of crops against biotic and abiotic stress by enhancing the plants' immune system. One example of challenging tool to achieve this goal is the use of biological plant inoculants based on beneficial microorganisms, like PGPR or their derived molecules, which have bioestimulant capacities and therefore are progressively replacing chemical and environmentally harmful phytosanitaries.

For all the above, the present work was focused on studying the plant immune system and the plant-beneficial microorganism relationship so that this knowledge can be applied in the development of commercial plant inoculants able to boost the immune response of plants and their resistance to biotic and abiotic stress, therefore improving crops yield. This would be achieved through the inoculation of the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors, first in *A. thaliana* plants under laboratory conditions, and then under real field conditions in blackberry crops (*Rubus* cv. Loch Ness).

The capacity of this strain and its metabolic elicitors to induce systemic resistance and to elicit secondary defensive metabolism has been studied, with the aim of obtaining plants with better fitness and ability to cope with stress and, at the same time, plants with an activated secondary metabolism and therefore plants with higher content of metabolites beneficial for human health. Plants with a stronger immune system and richer in secondary metabolites of interest to the food, pharmaceutical, nutraceutical and cosmetic industry will be obtained, which would confer high added value to crops.

In this way, it is intended to demonstrate the potential of the *P. fluorescens* N 21.4 and its metabolic elicitors for the development of biostimulant products not harmful to the environment that allow to decrease chemical inputs and thus stablish more sustainable agronomic production systems. Moreover, the ability of the metabolic elicitors of the bacterium to mimic the elicitation capabilities of the live strain has been studied with the ultimate goal of developing effective biostimulants based on inert molecules and not on live bacteria, as this represents a great improvement in the management, handling and maintenance of biologic inoculants and eliminates biosecurity problems (Thakur et al. 2013; Ngoroyemoto et al. 2019). After verifying the elicitation capacity of the metabolic elicitors of *P. fluorescens* N 21.4, we proceeded to a preliminary structural elucidation of them to better known their chemical nature.

This part of the work concentrated on studying the metabolic elicitors is novel because despite the many studies of PGPR triggering ISR, few have been focused on the molecular elicitors produced by these bacteria. However, metabolites from various bacterial genera such as *Klebsiella* (Park et al. 2009), *Ochrobactrum* (Sumayo et al. 2013), *Pseudomonas* (Ongena et al. 2005) and *Bacillus* (Huang et al. 2012; Xin et al. 2020) have been recognized as ISR metabolic elicitors. Those metabolites from *Bacillus* spp. have being the most studied ones, although it is well-known that *Pseudomonas* spp. are possibly the most important producers of compounds triggering plant immune responses (Durrant and Dong 2004; Choudhary et al. 2007).

In addition to these studies with the N 21.4 strain, new rhizobacteria effective in inducing systemic resistance were sought. For this, a screening was carried out starting from 175 potential strains and selecting the best five to elucidate the signal transduction pathways involved in the defensive responses. Of these five, two strains (the ones that

showed better results) were selected for the extraction and study of the ability of their metabolic elicitors to mimic the capacities of the live strains.

All the effort made in the search of beneficial rhizobacteria to improve crops is being carried out because agricultural systems are exposed to a wide range of abiotic and biotic stresses that lower crop productivity (García-Cristobal et al. 2015), thus compromising food supplies worldwide (Pechanova and Pechan, 2015; Miller et al. 2017). This is why interactions between plants and microorganisms (pathogenic or beneficial) have been intensively studied with a view to provide sustainable solutions for crop diseases, to ensure food safety by improving food quality and crop yields and to understand how plants cope with stress (Silva et al. 2018).

The beneficial rhizobacterium *P. fluorescens* N 21.4 was chosen as the main strain for the research work because, as previously explain in the introduction, its ability to induce systemic resistance and to elicit the secondary metabolism of various plant species has already been demonstrated by our research group. This ability to boost the immune system of different plant species is essential when it comes to developing plant inoculants that can serve a large number of agronomic species. *P. fluorescens* N 21.4 has the ability to trigger ISR in *A. thaliana* (Domenech et al. 2007; Martin-Rivilla et al. 2019), *Solanum lycopersicum* (Domenech et al. 2007), *Glycine max* (Ramos-Solano et al. 2010b), *Hypericum sp.* (Gutierrez Mañero et al. 2012), *Papaver sp.* (Bonilla et al. 2014) and blackberry (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015). It enhanced the isoflavone content in *Glycine max* (Algar et al. 2012), and in blackberry, it increased total fruit production and fruit quality by modifying flavonoid metabolism (García-Seco et al. 2013; García-Seco et al. 2015a; Garcia-Seco et al. 2015b; Ramos-Solano et al. 2014; Ramos-Solano et al. 2015; Martin-Rivilla et al. 2020b (results obtained throughout the development of the present doctoral thesis)).

As the strain largely demonstrated its ISR and elicitation capacity, for the present work it was postulated the hypothesis that these defensive responses could be totally or partially triggered by secondary metabolites produced by the bacterium and released into the culture medium. This means that these metabolites, by "mimicking" the live strain's capabilities, could replace live bacteria in the creation of plant inoculants. This is why the metabolic elicitors of this strain were inoculated, first in *A. thaliana* under laboratory conditions and then in cultivars of blackberry (*Rubus* cv. Loch Ness), taking the live strain as a positive control.

The experiments in *A. thaliana* inoculated with N 21.4 and with its metabolic elicitors (three fractions in different organic solvents) and challenged with the pathogen *P. syringae* DC 3000 started with the study of the overall oxidative status of the plants, since PGPR are able to boost plant ROS scavenging (Lucas et al. 2013; Lucas et al. 2014; García-Cristobal et al. 2015).

When a plant is attacked by a pathogen, as it was the case of *P. syringae* DC 3000, a common plant response to the attack is the rapid generation of ROS (Noctor et al. 2014; Xia et al. 2015). To maintain optimal levels of ROS, plants possess a sophisticated regulatory system consisting of enzymatic antioxidants (SOD; CAT; GPX; APX; MDHAR; DHAR; GR) and non-enzymatic antioxidants (ASC; GSH; carotenoids; tocopherols; phenolics compounds).

Some of these enzymes involved in ROS scavenging (APX, SOD, GR, GPX, CAT, and DHAR) and proteins involved in ISR (glucanase (PR2), chitinase (PR3), lipoxygenase, cellulase and polyphenol oxidase), as well as the differential gene expression of marker genes from the SA and JA/ET pathways were analysed, since ISR is accompanied with an augmented expression of defence related genes, increased

accumulation of secondary metabolites and defence associated proteins (Conrath 2006; Zamioudis and Pieterse, 2012).

The analysis of all these stress and ISR markers was carried out with the *A. thaliana* seedlings that had been inoculated with the metabolic elicitors contained in the n-hexane fraction. This fraction was chosen against the ethyl acetate and n-butanol fractions because it was the one that performed the highest percentage of protection against the pathogen (Figure 3.1.1), even this protection being greater than that conferred by the live bacterium.

ISR typically relies on JA/ET signalling pathways (Pieterse et al. 2002), however our results, at the level of gene expression and activity of proteins related to plant defence systems, indicated that metabolic elicitors from the n-hexane fraction inoculated in *A. thaliana* seedlings induced the JA/ET pathway and the SA pathway (Fig. 3.1.2) by increasing at the same time expression of marker genes *PR1* and *PDF1* (SA and JA/ET marker genes respectively (Caarls et al. 2015; Ding et al. 2018)), and the enzymatic activity of PR2 (marker of SA signalling pathway) and PR3, *LOX*, and PPO, as markers of JA/ET signalling pathway (Lucas et al. 2014; García-Cristobal et al. 2015; Silva et al. 2018; Wu et al. 2018). This implies a versatility in the resistance mechanism, allowing attack of biotrophic and necrotrophic organisms to be opposed.

Furthermore, our results demonstrated a stimulation of all the enzymes of the ascorbate-glutathione-cycle of plants treated with the n-hexane extract from *P. fluorescens* N 21.4, notably of APX, GPX and MDHAR (Fig. 3.1.4) and the activity of the pathogenesis-related proteins and the proteins related to systemic resistance against pathogens (Fig. 3.1.3).

Few studies relate oxidative stress enzymes to innate immunity in plants elicited with PGPR or metabolic elicitors (Lucas et al. 2014; Garcia-Cristobal et al. 2015). This

is why the present study is valuable since markers related to oxidative stress metabolism will assist in improving primer fingerprinting for each bacterial strain (Maunch-Mani et al. 2017; Gutierrez Albanchez et al. 2018) and this will improve further analysis and the understanding of the mechanisms that defend plants against pathogens.

After demonstrating the effectiveness of the metabolic elicitors of the strain *P. fluorescens* N 21.4 under laboratory conditions, we verified this effectiveness under real field conditions in blackberry crops. In this case, the metabolic elicitors were inoculated directly from the bacterium's nutrient broth, without being extracted with any organic solvent to lower the costs of the agricultural system and to simplify and facilitate the handling of the inoculums.

The blackberry (*Rubus* cv. Loch Ness) was chosen as working material for this study since blackberries are among the fruits with more beneficial properties to human health due to they are extremely rich in flavonoids (Kaume et al. 2012). These compounds have demonstrated cytotoxic, anticancer, antiviral, antibacterial, anti-inflammatory, antiallergenic, antithrombotic, cardioprotective and hepatoprotective properties (Alzand et al. 2012; Ferreyra et al. 2012). Furthermore, some analytical studies have shown that blackberry leaf phenolic composition is analogous to that of the fruits (Ferlemi et al. 2016) and that leaves extracts also show *in vivo/in vitro* beneficial effects on human health, as they have antioxidant, anti-diabetic (Oszmiańskiet al. 2015), anti-microbial (Zia-Ul-Haq et al. 2014), analgesic, anti-inflammatory and angiogenic activity (Mukherjee et al. 1984). This suggests that leaves are also a valuable source of bioactive natural compounds.

For all these beneficial and proven properties, blackberry consumption is becoming increasingly popular (Lee et al. 2017), which has meant that intensive production systems have the need to seek new cultivation strategies that improve the quality and sustainability of this cultivation (Carlen and Crespo, 2010). Furthermore,

intensive blackberry cultivation is a relatively recent emerging crop for which there is a deficit of specific inoculants that improve plant fitness and resistance to pests and to abiotic stress. Hence, we evaluated the ability of *P. fluorescens* N 21.4 and its metabolic elicitors to trigger the secondary defensive metabolism of blackberry plants (*Rubus* cv. Loch Ness), boosting their general fitness and achieving, at the end of the cultivation cycle, plants with leaves and fruits richer in valuable polyphenols. The pruning of the leaves, which is nowadays a waste, would be revalorized by proposing them as the starting material to obtain usable polyphenolic compounds. An added value would be given to the crop while fulfilling the circular economy premises (Barreira et al. 2019).

This study with blackberry crop was designed to carry out leaf collection and analysis at two sampling moments, corresponding to two physiological plant stages: flowering (January 2018) and fruiting (April 2018). Fruits were also harvested in two stages of maturation (red and black) in the second sampling moment (April 2018).

We evaluated different markers of plant fitness such as photosynthesis parameters (fluorescence *in situ* and pigments), ROS scavenging enzymes (SOD, APX and GR), pathogenesis-related proteins (PR2 and PR3) and expression of marker genes of ISR pathways mediated by SA and JA/ET (*Ru*PR1, *Ru*PR2 and *Ru*PR3). In addition, blackberry leaves and fruits polyphenol composition was studied. Fruit production and structural marker genes of the phenylpropanoids pathway during fruit ripening were also analysed. The study of regulatory genes encoding the enzymes of the phenylpropanoid and flavonol-anthocyanin pathways would be crucial for modifying the accumulation of secondary metabolites of interest at the end of the route. Knowledge of this pathway during blackberry ripening and upon beneficial rhizobacteria and metabolic elicitors challenge, would provide useful tools to develop new blackberry crop technics by

stimulating the synthesis of secondary metabolites, improving fruit nutritional qualities in a sustainable way (Ramos-Solano et al. 2014; Garcia-Seco et al. 2015a).

The photosynthesis was analysed as the main parameter that reflects the health status of a plant since the photosynthetic apparatus is the first physiological function to be affected by a stress (Garcia-Cristobal et al. 2015). In our work, plants treated with the strain N 21.4 and with its metabolic elicitors showed lower values of F0 and higher values of Fv/Fm (Fig. 3.2.2) compared to control plants. Associated to F0 parameter, results also showed higher levels of Chl a in treated plants (Fig. 3.2.3 A)). All this supposes that the strain N 21.4 and its metabolic elicitors decreased photosynthetic damage caused by stress (Roháček et al. 2002; Baker et al. 2008) and improved photosynthetic performance and efficiency, increasing energy production for the plant. This extra energy could be used in the production of target secondary metabolites.

Furthermore, the lower enzymatic activity of treated plants, especially SOD activity at both sampling moments and APX and GR at flowering (Fig. 3.2.4), suggested that they were less stressed than control plants. As treated plants were less stress, they generated less ROS and therefore, their enzymatic machinery responsible for detoxification worked in a more moderate way (Gutierrez Albanchez et al. 2018).

Activity of the pathogenesis-related proteins glucanase (PR2) and chitinase (PR3) were also evaluated because they play an essential role in the capacity of the plants to face to infection caused by pathogen attacks (Lucas et al. 2014). Our study showed that the activity of both PRs was significantly higher in the plants treated with the metabolic elicitors (Fig. 3.2.5). This may suggests that the metabolic elicitors of N 21.4 prepared plants to cope with pathogen outbreaks and possibly with other stress situations. qPCR results supported this idea because analysed genes (*Ru*PR1, *Ru*PR2 and *Ru*PR3) of

metabolic elicitors-treated-plants were not downregulated as it occurred in plants inoculated with N 21.4 (Fig. 3.2.6).

The treatments were also efficient in the elicitation of flavonoid metabolism since the amount of flavonols were higher in the leaves (Table 3.2.2) and in the fruits (Fig. 3.3.1 A)) of plants treated with N 21.4 and with its metabolic elicitors than in the leaves and fruits of control plants. Highlighting results were the great increase of kaempferol derivatives (K-3-O-glucoside, K-3-O-rutinoside) and quercetin 3-O-rutinoside in the leaves of treated plants. Kaempferol and its glycosylated groups, especially glucoside and rutinoside, have demonstrated their anti-pathogenic capacity in some plants (Galeotti et al. 2008), which reinforces our idea that treated plants, with higher kaempferol values, were more protected than control plants to biotic stress.

The fruit of inoculated plants, and specially the fruit of ME-inoculated plants, accumulated secondary metabolites in greater concentration than non-inoculated control plants. A remarkable fact was a major increase of the flavanols (-)-epicatechin and (+)-catechin at both stages of fruit ripening (red and black) with both treatments. These compounds have strong antioxidant capacity and they are able to reduce oxidative stress in plants (Martinez et al. 2005; Mahajan et al. 2013). Epicatechin also acts as phytoanticipins in some fruits (Guetsky et al. 2005) giving fungal and bacterial resistance to infection.

The fruit production in ME-inoculated plants was slightly lower, which is a clear symptom of the metabolic elicitors´ elicitation capacity, which improved the quality of fruit slightly compromising fruit yield. Previous results from our group, in the same plant and with another rhizobacteria (Gutierrez Albanchez et al. 2018), already verified that elicitation supposes a redirection of resources towards defensive metabolism, slightly compromising fruit yield.

The study of regulatory genes of the phenylpropanoid and flavonol-anthocyanin pathways during fruit ripening revealed an inverse relationship (in red and black fruit) between gene expression and accumulation of secondary metabolites (Fig. 3.3.3 and Table 3.3.2), except for *RuDFR* gene, the first gene of the anthocyanins route, that had a direct effect in the increase of cyanidin 3-O-glucoside.

In red fruit, metabolic elicitors´ treatment had effects in all the studied genes, down-regulating *RuF3´H*, *RuFLS*, *RuANS*, *RuLAR* and *RuANR* and up-regulating *RuDFR*. This was directly linked to an increase in kaempferol and quercetin derivatives, epicatechin, catechin and cyanidin 3-O-glucoside.

In black fruit, the same inverse relationship was observed, but in this case it was the plants treated with N 21.4 which showed less differential expression and greater accumulation of all the secondary compounds, except anthocyanins, which were more accumulated in fruits of metabolic elicitors-treated plants. This is also consistent with a greater expression of *RuDFR* observed with this last treatment. *RuDFR* had the same behavior of that of *DFR* in Almeida´s et al. 2007 and Garcia-Seco et al. 2015b works.

At the same time, *RuCHS* showed a fundamental regulatory role related to the accumulation of secondary metabolites. When *RuCHS* was up-regulated (Fig. 3.3.3), compound accumulation occurred along the pathway (Table 3.3.2). This had been previously seen in the work of Garcia-Seco et al. 2015b.

In summary, the results have shown that both the live strain and its metabolic elicitors have had beneficial effects on blackberry plants, activating their secondary defensive metabolism and achieving less stressed plants, with better fitness and greater defensive capacity, and also with higher content of beneficial polyphenols accumulated in both leaves and fruits (mainly during red stage). Their capacity to modify flavonoid

biosynthetic pathway by modulating gene expression in the fruit of *Rubus* cv. Loch Ness has also been demonstrated.

However, the metabolic elicitors of the strain *P. fluorescens* N 21.4 had in general a more marked effect on plant physiology than the live strain, maybe do to the way of application of the treatments: foliar vs radical, respectively. Foliar application of biostimulants has been seen as a very effective technique (Ngoroyemoto et al. 2019) and it is economical and environmentally convenient (Thakur et al. 2013; Ngoroyemoto et al. 2019).

Once, the ability of the ME of N 21.4 to have effects *in vitro* in *A. thaliana*, and *in vivo* in *Rubus* cv. Loch Ness were demonstrated, they were sequentially fragmented by vacuum liquid chromatography and thin layer chromatography (Fig. 3.4.1) to isolate, purify and identify the compounds responsible for their capacity to induce systemic resistance and to elicit secondary defensive metabolism in diverse plant species.

Our results have shown that the metabolic elicitors´ fractions sequentially extracted from the strain *P. fluorescens* N 21.4 were able to enhance the synthesis of isoflavones in soybean seeds between 1.2 to 3.2 times more than controls, demonstrating their potential to elicit secondary defence metabolism (Figs. 3.4.4, 3.4.6 and 3.4.7) in a very low concentration (100-0.1 µg mL^{-1}). Furthermore, the results obtained in the ISR experiment in *A. thaliana*, inoculated with the purest fraction (Fp) of the ME, against the pathogen *P. syringae* DC 3000 supported the statement that ME have great potential to increase plant resistance, since protection rates between 40 to 50% were seen (Fig. 3.4.8).

After sequentially fragmenting the ME, and verifying the eliciting and inductive capacity of all of the extracted fractions, the purest one was selected, and an analysis by UHPLC/ESI-QTOF-MS to identify and characterize the compounds responsible for the elicitation was carried out. Eight compounds were tentatively identified and classified

into different families (Table 3.4.3): alkaloids (Ambiguine P (Hillwig et al. 2013); amino lipids (Sphinganine C17 and 1-Nonadecanamine (Siebers et al. 2016; Lenarcic et al. 2017; Ali et al. 2018)); terpenoids (4-O-methylmelleolide (Yin et al. 2012)) or 2-Acetoxy-3-deacetoxycaesaldekarin E (Jing et al. 2019)) and arylalkylamines (*N*-benzyl-1-tetradecanamine, *N*-benzyl-1-hexadecanamine and *N*-benzyl-1-octadecanamine).

These identified compounds could result in commercial products of biological origin to be applied in crops, since many of them have antibiotic and/or antifungal potential (Kanda et al. 1975; Becher et al. 2007; Mo et al 2010; Al-Jassaci et al. 2016; Choudary and Singh, 2018; Lozano et al. 2019).

As a result of all this work, it has been demonstrated that the metabolic elicitors of *P. fluorescens* N 21.4 are able to induce systemic resistance and to elicit secondary defensive metabolism in different plant species. Furthermore, their composition have been identified and the synthesis of certain compounds (hapalindole-type natural products and sphingolipids) have been shown for the first time in *Pseudomonas fluorescens*. Nevertheless, deeper research would be necessary in the future to carry out more specific studies, to better characterize the identified compounds and their effects in the plants and to test their antibiotic or antifungal potential against common pathogens present in agricultural systems.

To finish the experimental work of the present doctoral thesis, and after having amply demonstrated the capacities of the strain *P. fluorescens* N 21.4 and its metabolic elicitors, new effective PGPR in the induction of systemic resistance in *A. thaliana* were sought. The ultimate aim was to apply them and/or their metabolic elicitors in future experiments under real field conditions to develop commercial products based on PGPR or on their metabolic elicitors.

For this, it was tested the ability of 25 bacterial strains extracted from the rhizosphere of *N. glauca*, and selected from a subset of 175 strains from the non-assayed bacteria group of the work of Ramos-Solano et al. 2010a, to trigger the innate immune system of *A. thaliana* seedlings against the pathogen *P. syringae* DC3000. The 25 selected strains were chosen because of their PGPR activities (Table 3.5.2): auxin-like compounds production (Sergeeva et al. 2007), siderophores production (Alexander and Zuberer, 1991), phosphate solubilisation (De Freitas et al. 1997), and chitinases production (Rodríguez-Kábana et al. 1983; Frändberg and Shnurer, 1998), and avoiding phylogenetic redundancy (Fig 3.5.2).

In this study, almost the totality of the strains produced siderophores, which is related to iron limiting nutrient (Raymond et al. 1984; Jin et al. 2006; Lucas et al. 2013), but also has been related to biocontrol and/or systemic induction of secondary metabolism. Therefore, siderophore-producing strains may have the ability to protect plants against pathogens (Sinclair et al. 2004, Barriuso et al. 2008; Radzki et al. 2013).

Furthermore, the solubilisation of phosphates was a very abundant activity among the strains studied. Our results support that *N. glauca* selects rhizobacteria related to nutrition or biocontrol activities (phosphate solubilisation and siderophore production) rather than those able to affect plant growth regulator balance (e.g. auxins production).

Hence, the 25 strains selected for the ISR experiment were chosen because they were able to produce siderophores and solubilize phosphate and because they had some other complementary capacities, mainly the production of chitinases, which protects plants against pathogen fungi by decomposing fungal cell walls. Therefore, the selected microorganisms would have an excellent potential as biocontrol agents (Lorito et al. 1993; Sid et al. 2003; Adesina et al. 2007; Ramos-Solano et al. 2010a) and it was seen

that 16 out of the 25 chosen strains induced systemic resistance and protection against the pathogen DC3000 (Table 3.5.4).

The five strains of the previous twenty-five (*Pseudomonas putida* N 5.12, *Stenotrophomonas maltophilia* N 8.17, *Serratia rubidaea* N 12.34, *Pseudomonas fluorescens* N 21.24 and *Bacillus cereus* N 4.1), more effective in the prevention of pathogen infection (Table 3.5.4) were used to elucidate signal transduction pathways involved in the plant immune response by studying the differential expression of SA and JA/ET marker genes (Figs. 3.5.3-3.5.7). Some strains stimulated the two pathways with no inhibitory effects between them, while others stimulated either one or the other.

The importance of high concentrations of SA and JA to trigger defensive responses mediated by both hormones is nowadays widely accepted, as well as that both signal transduction pathways are not necessarily antagonistic (Pieterse et al. 2014; Caarls et al. 2015). This simultaneous activation of both routes has been seen throughout the entire experimental process of the present doctoral thesis, both in *A. thaliana* seedlings inoculated with the N 21.4 strain and its metabolic elicitors, and with the fractions extracted from the metabolic elicitors of N 21.4, and in the seedlings of *A. thaliana* inoculated with these five selected strains and with the metabolic elicitors of two of them.

The metabolic elicitors of two of the five selected strains (N 12.34 (*S. rubidaea*), as the Gram-negative strain that showed the highest differential expression (Fig. 3.5.5), and N 4.1 (*B. cereus*) as it was the Gram-positive strain with better protection among the Gram-positive and which ranked second among all (Table 3.5.4)), were extracted using n-hexane, ethyl acetate and n-butanol, and their capacity to mimic bacterial effect to trigger the immune system of the plant was studied. The three fractions extracted from each strain, achieved significant protection (Table 3.5.5), having an outstanding performance the metabolic elicitors in the n-hexane and ethyl acetate fractions, as it

happened in previous experiments with the ME of the strain N 21.4. N-hexane and ethyl acetate fractions of N 12.34 and N 4.1 reached protection rates similar to living bacteria, although gene expression responses were different from that obtained by the bacteria (Figure 3.5.8). This suggests that the elicitors detected by the plant have to be different (live bacteria versus metabolic elicitors in the n-hexane and ethyl acetate fractions), and so would be the PRRs involved in the response (Tang et al. 2017). Moreover, since both fractions have elicitation capacity, it seems that the diversity of elicitors is high.

In summary, it has been seen that each bacterium had a different effect in the genes studied. In addition, the metabolic elicitors of the two studied strains had different effects to that produced by the bacteria, confirming the presence of many different bacterial molecules able to trigger plant metabolism. This is very interesting since it opens a huge amount of biotechnological possibilities to develop biological products for agriculture in different situations and plant species.

In this work, it has amply demonstrated the great importance of the rhizosphere and the effects of the rhizospheric microbiome on plants. It has been also shown that plants are able to select the most convenient microorganisms to improve their fitness and that this can be directed from a biotechnological point of view to induce the defensive secondary metabolism of plants, and to obtain crops more resistant to biotic and abiotic stress and with a higher and better quality production, all within the framework of fulfilling the concept of food security.

5. General conclusions

5. General conclusions

1. The metabolic elicitors of the *Pseudomonas fluorescens* N 21.4 extracted with n-hexane induce systemic resistance in *Arabidopsis thaliana* seedlings against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000, trigger SA and JA/ET signalling pathways and the enzymatic machinery of ROS scavenging to decrease plant oxidative stress.

2. The metabolic elicitors of the *P. fluorescens* N 21.4 trigger secondary defensive metabolism in *Rubus* cv. Loch Ness and improve plant fitness by increasing photosynthesis performance and pathogenesis-related proteins activity, decreasing oxidative stress, and boosting the synthesis of kaempferol and quercetin derivatives in the leaves.

3. The metabolic elicitors of the *P. fluorescens* N 21.4 as inoculants of blackberry commercial cultivars generate a greater economic yield by allowing plant leaves to be used as a source for the extraction of metabolites with beneficial effects on human health, such as epicatechin and kaempferol and quercetins derivatives.

4. The metabolic elicitors of the *P. fluorescens* N 21.4 modulate gene expression in the fruit of *Rubus* cv. Loch Ness, and affect the profiles of secondary metabolites, increasing the synthesis and accumulation of them during fruit ripening.

5. The *RuCHS* gene has a vital role in the accumulation of secondary metabolites in blackberry fruit, as well as the *RuDFR* gene in the increase of the synthesis and accumulation of cyanidine-3-O-glucoside.

6. The metabolic elicitors of the *P. fluorescens* N 21.4 are effective, affordable, manageable and environmentally friendly agriculture inoculants that at a very low concentration (100-0.1 μg mL^{-1}) alter the primary and secondary metabolism of different plant species, resulting in more resilient plants.

7. Nine compounds comprise the metabolic elicitors of the *P. fluorescens* N 21.4: one alkaloid, two amino lipids, a terpenoid, three aryl alkylamines and one still unknown.

8. The rhizobacteria *Pseudomonas putida* N 5.12, *Stenotrophomonas maltophilia* N 8.17, *Serratia rubidaea* N 12.34, *Pseudomonas fluorescens* N 21.24 and *Bacillus cereus* N 4.1 (extracted from the rhizosphere of *Nicotiana glauca*), have diverse PGPR activities and induce systemic resistance in *A. thaliana* seedlings against the pathogen *P. syringae* pv. *tomato* DC3000, triggering both the SA and JA/ET signalling pathways, which makes them effective candidates to carry out future experiments under field conditions.

9. The metabolic elicitors in the n-hexane and ethyl acetate fractions of the *Serratia rubidaea* N 12.34 and the *Bacillus cereus* N 4.1 are as efficient in inducing systemic resistance in *A. thaliana* seedlings against the pathogen *P. syringae* pv. *tomato* DC3000 as the live bacteria, which allows them to be used as plant inoculants alternative to live bacteria.

6. Bibliography

6. Bibliography

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7. Annexes

7. Annexes

7.1 Chromatograms of the bioactives present in blackberry leaves

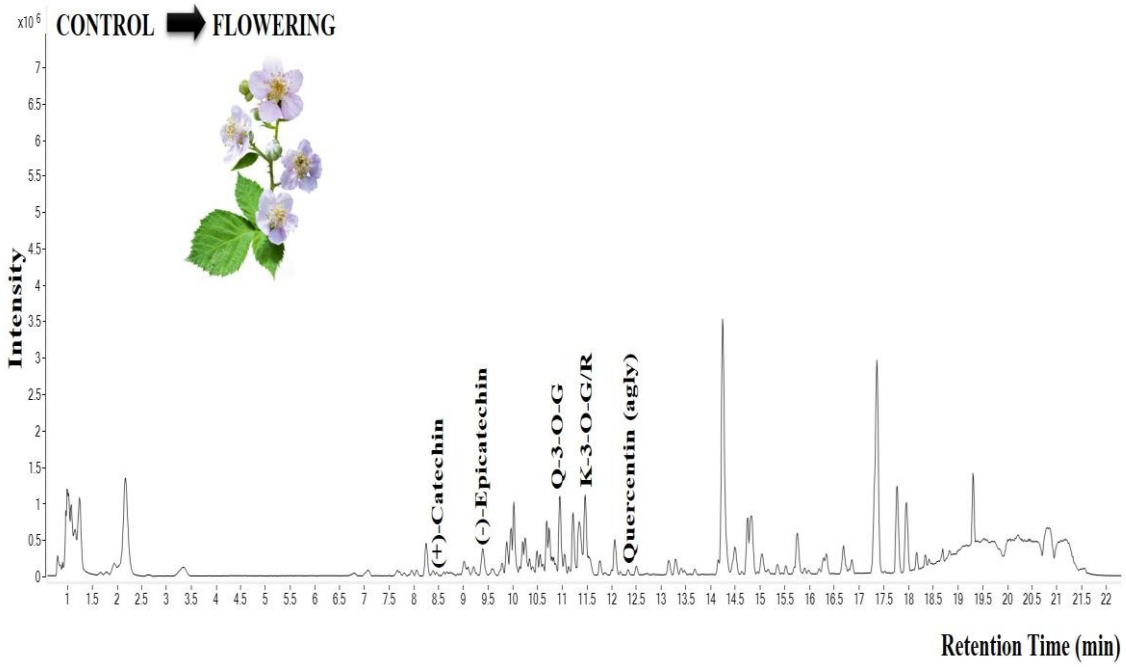


Figure 7.1.1 Representative base peak chromatogram (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of the methanolic extract of leaves samples (at flowering).

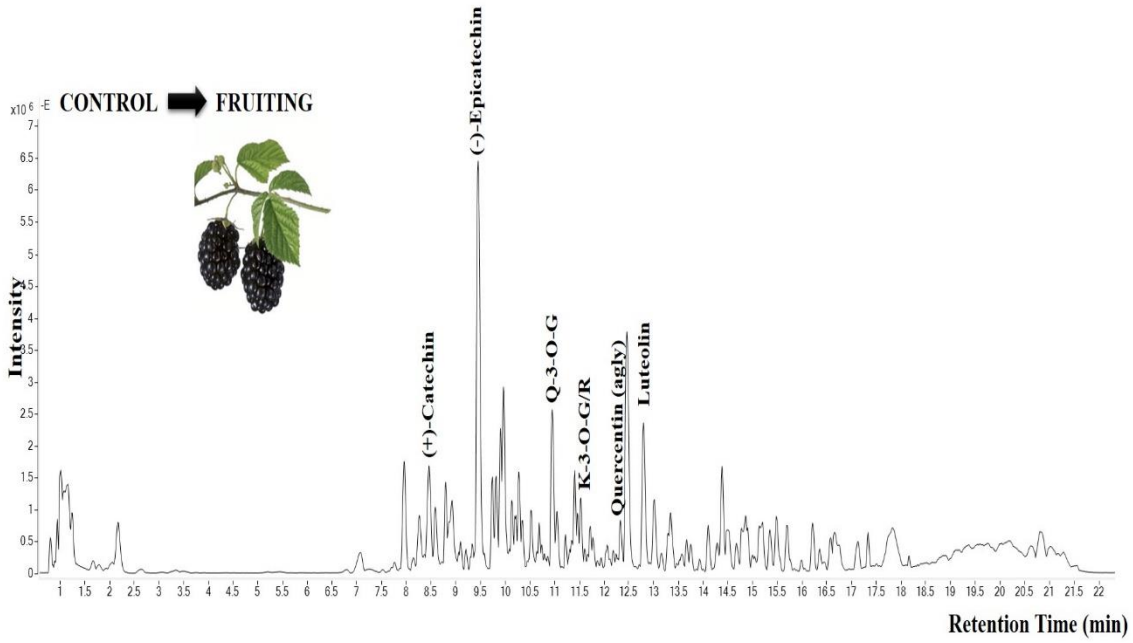


Figure 7.1.2 Representative base peak chromatogram (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of the methanolic extract of leaves samples (at fruiting).

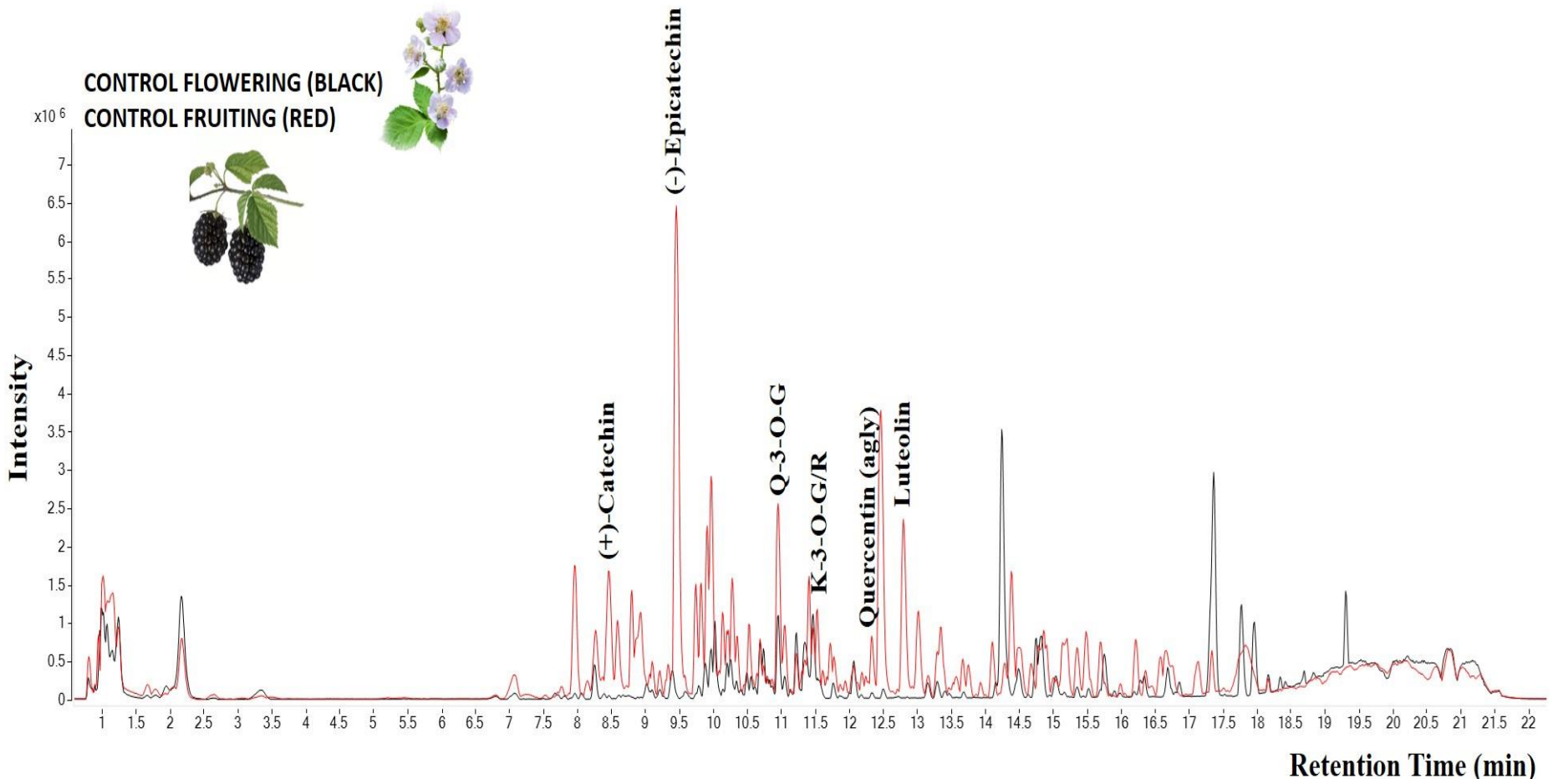


Figure 7.1.3 Overlaid base peak chromatograms (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of methanolic extract of leaves samples (at flowering (black line) and at fruiting (red line)).

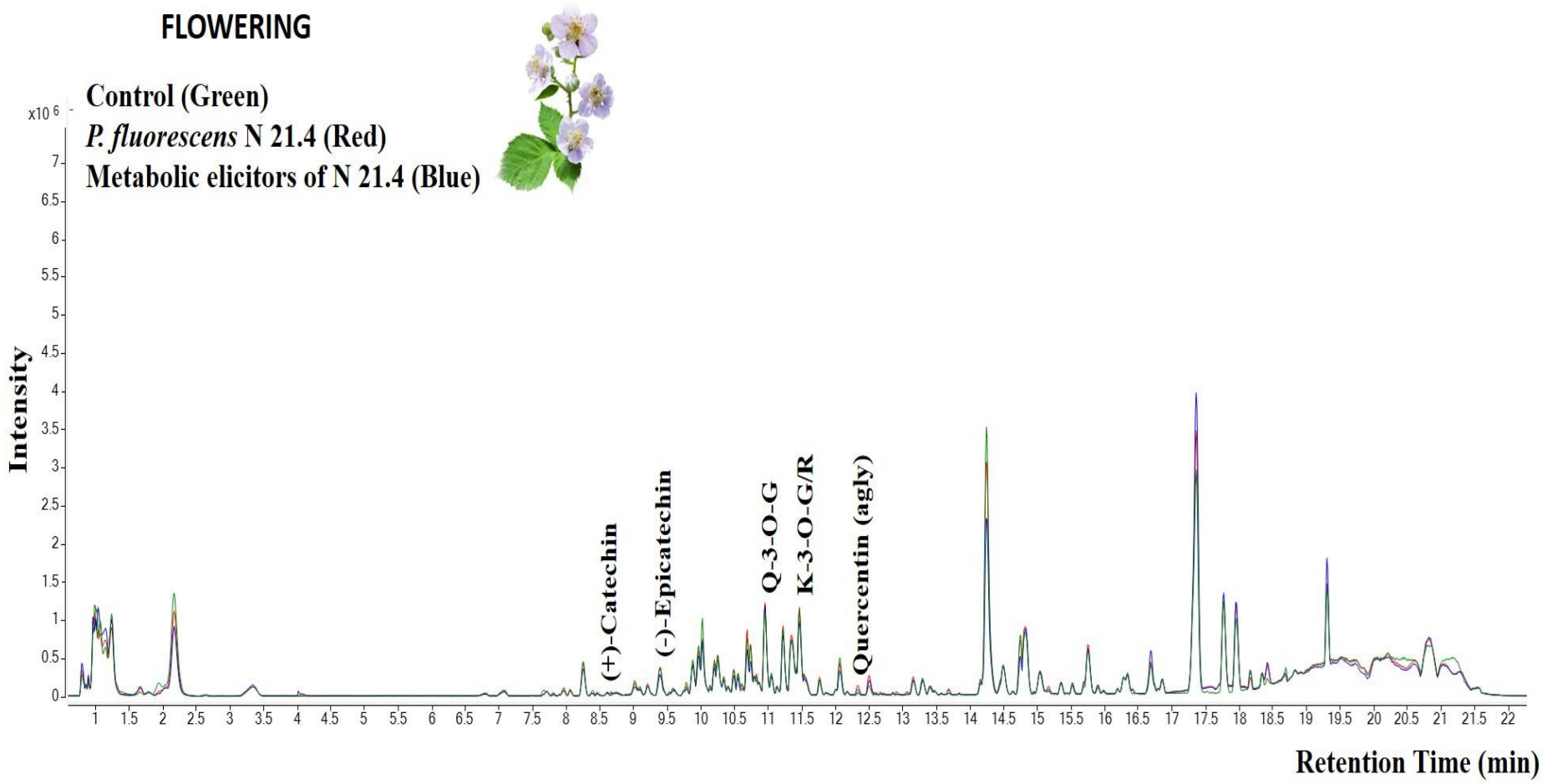


Figure 7.1.4 Overlaid base peak chromatograms (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of control, *P.fluorescens* N 21.4 and metabolic elicitors methanolic extract of leaves samples (at flowering).

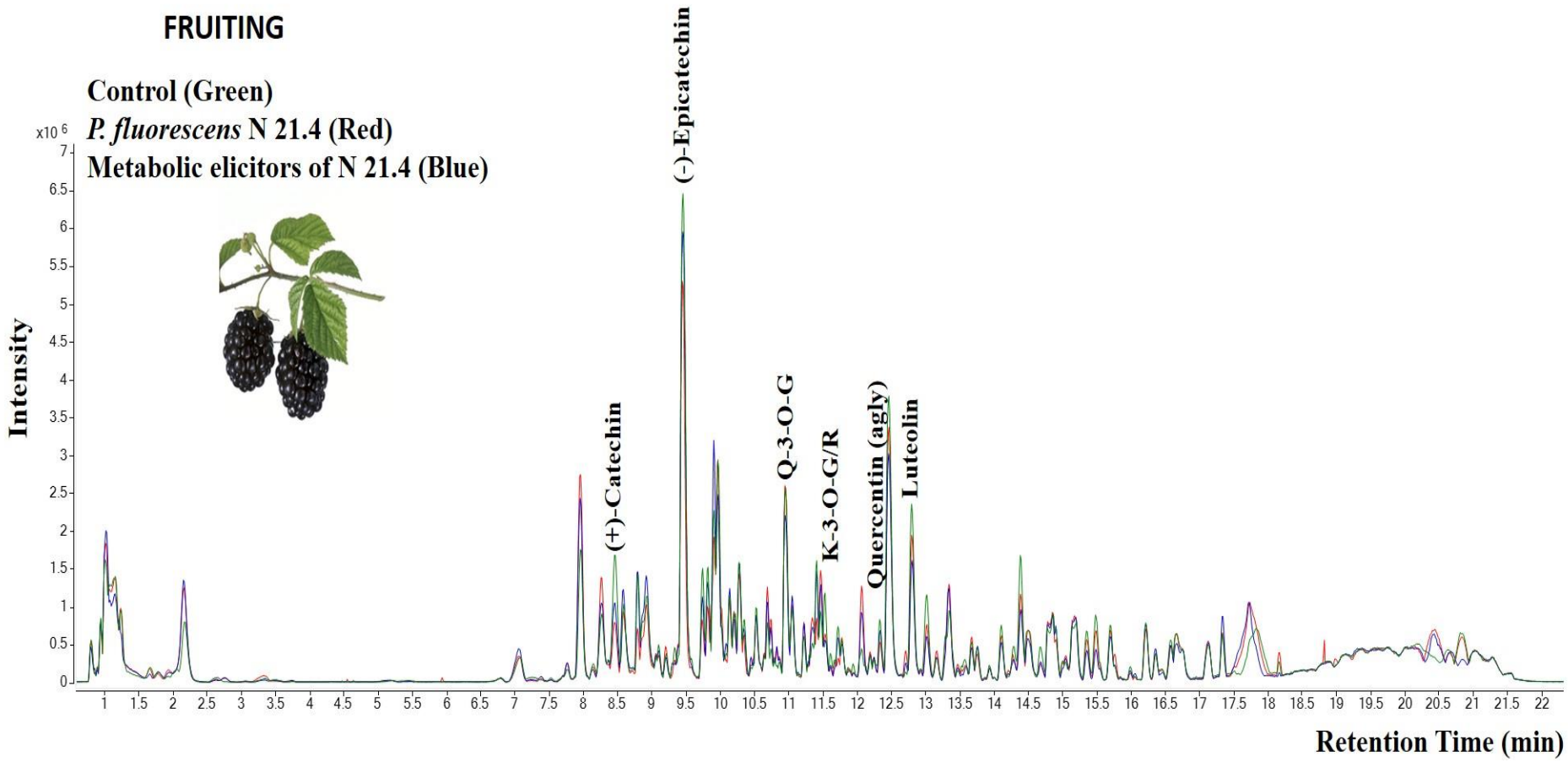


Figure 7.1.5 Overlaid base peak chromatograms (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of control, *P.fluorescens* N 21.4 and metabolic elicitors methanolic extract of leaves samples (at fruiting)

7.2 Chromatograms of the bioactives present in blackberry fruit

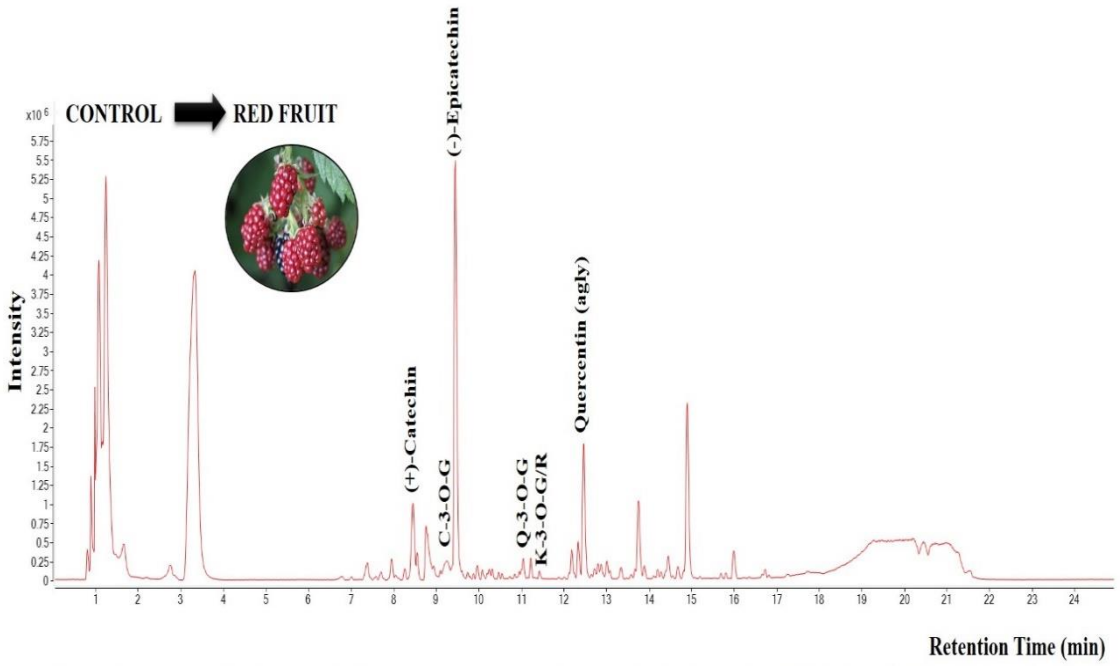


Figure 7.2.1 Representative base peak chromatogram (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of the methanolic extract of red fruit samples.

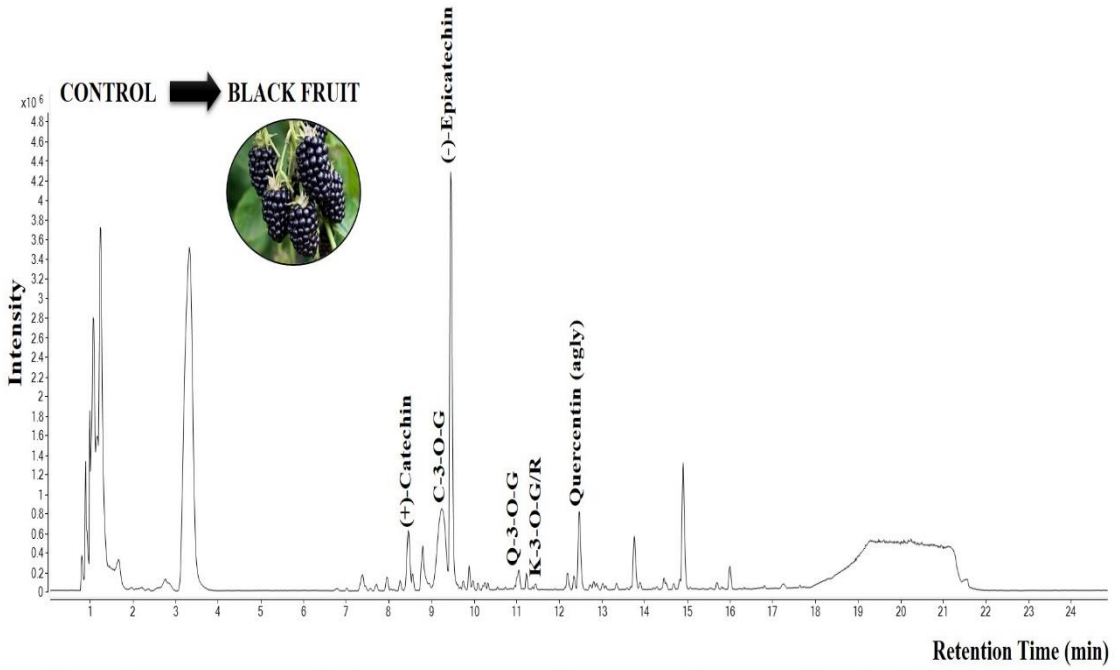


Figure 7.2.2 Representative base peak chromatogram (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of the methanolic extract of black fruit samples.

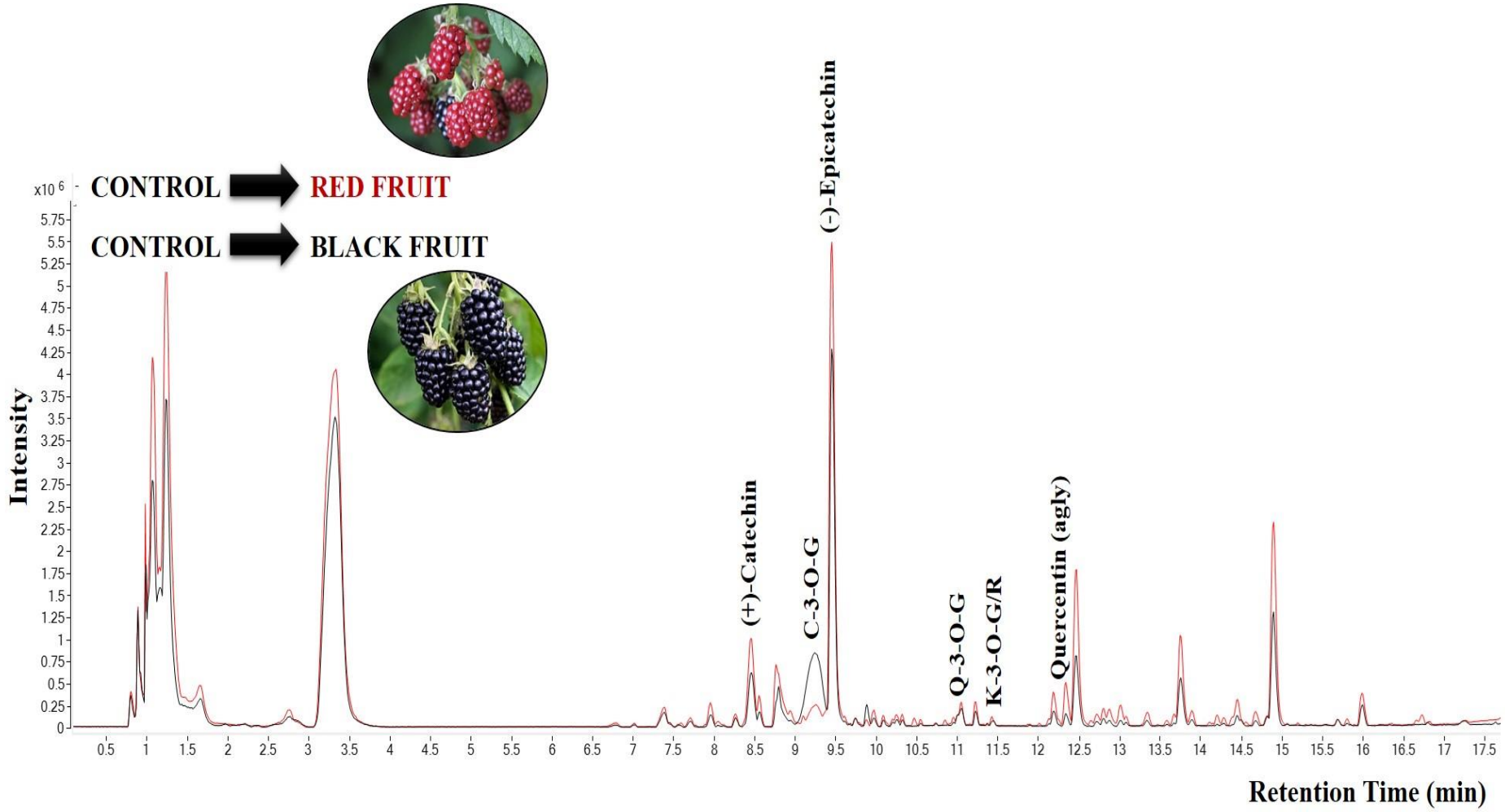


Figure 7.2.3 Overlaid base peak chromatograms (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of methanolic extract of blackberry **black** and red fruit samples. Enrarged chromatogram from 0.5 to 17.5 min.

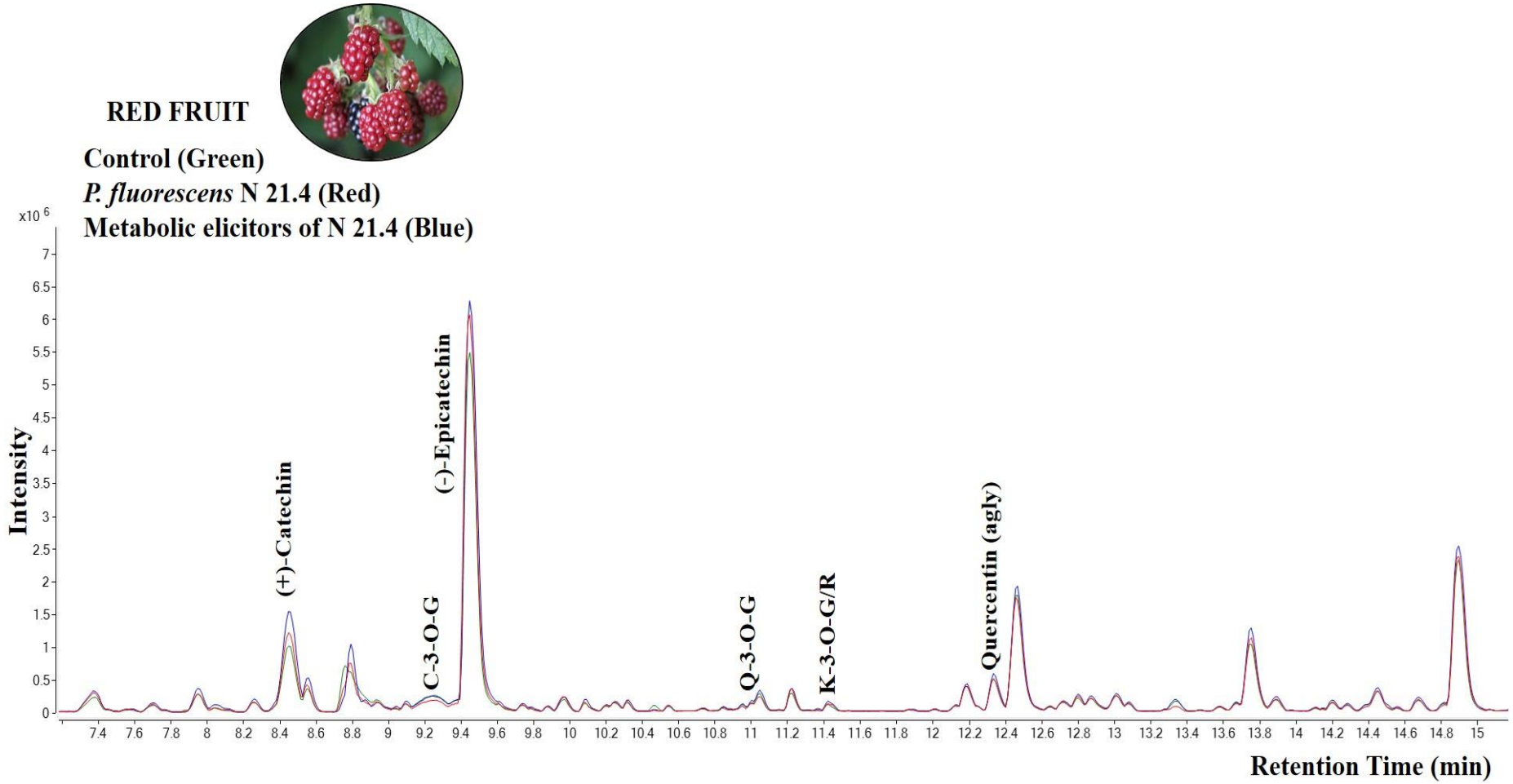


Figure 7.2.4 Overlaid base peak chromatograms (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of control, *P.fluorescens* N 21.4 and metabolic elicitors methanolic extract of blackberry red fruit samples.

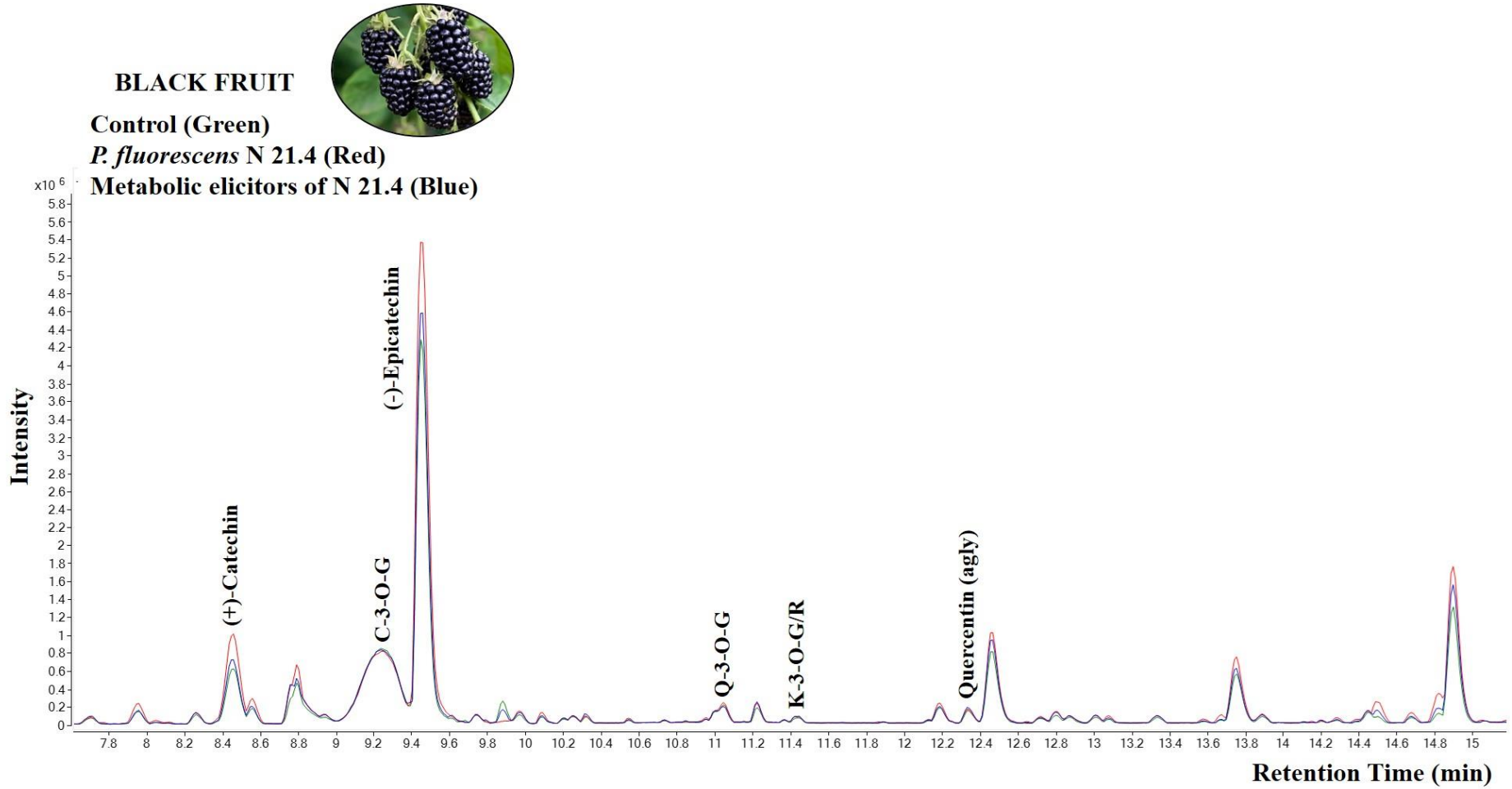
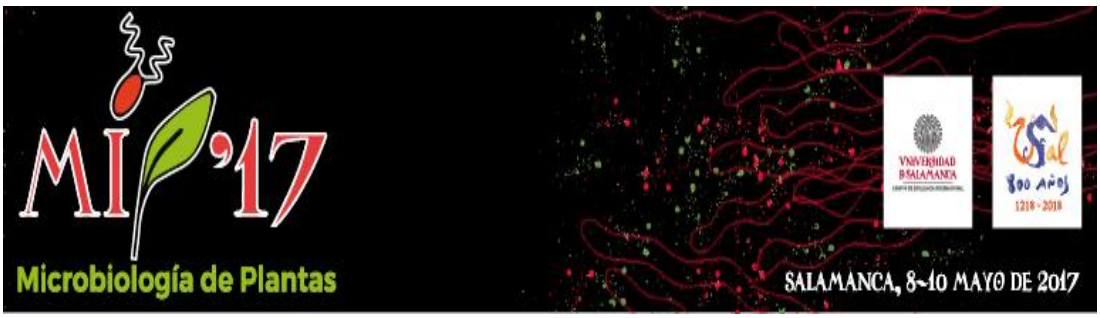


Figure 7.2.5 Overlaid base peak chromatograms (negative ion mode) obtained from UHPLC/ESI-QTOF-MS analysis of control, *P.fluorescens* N 21.4 and metabolic elicitors methanolic extract of blackberry black fruit samples.

7.3 Congress communications

Comunicación presentada en la VI Reunión del grupo especializado de Microbiología de plantas en la Universidad de Salamanca, 2017



Selección de cepas PGPR para estimular la protección frente a *Pseudomonas syringae* DC3000 en plantas de brócoli

Helena Martín-Rivilla*, Linda Lamia, Enrique Gutiérrez, José Antonio Lucas, Javier Gutierrez-Mañero, Beatriz Ramos-Solano.

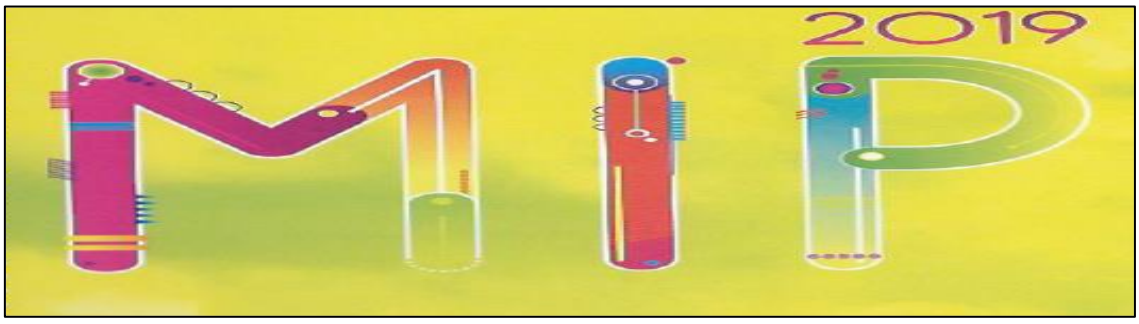
Grupo Interacción Planta-Microbioma. Facultad de Farmacia, Universidad San Pablo-CEU Universities, Boadilla del Monte, Madrid, España.

E-mail: hel.martin.ce@ceindo.ceu.es

Las bacterias promotoras del crecimiento vegetal (PGPR) están cobrando cada vez más importancia en el control de plagas, como alternativa a los productos químicos, dentro del marco de una agricultura sostenible. Algunas cepas son capaces de estimular el metabolismo defensivo de las plantas de forma que éstas se encuentran más preparadas para hacer frente al ataque repentino de un patógeno, estado metabólico denominado *priming* (Mauch-Mani et al. 2017). Dicha estimulación de las plantas está mediada por distintas moléculas bacterianas (MAMPs) y por receptores específicos, por lo que es necesario evaluar el efecto de cada cepa en cada cultivo para confirmar que existe

comunicación entre esos dos elementos concretos. Así, el presente trabajo plantea la caracterización de la potencialidad PGPR *in vitro* de 18 cepas aisladas de distintos orígenes y su identificación mediante 16S rRNA. De estas 18 cepas, se seleccionaron las 6 con el mejor potencial PGPR y de distinto genoma para evaluar su efecto sobre el crecimiento y protección de plantas de brócoli frente al patógeno *Pseudomonas syringae* DC3000. Para esto, se realizó un clásico experimento de ISR (Domenech et al. 2007) con dos aplicaciones de PGPR y una de patógeno a lo largo de 6 semanas. Una semana después de la aplicación del patógeno, se midió la fotosíntesis (fluorescencia y fijación de CO_2), se cosecharon las plantas y se determinó la incidencia de la enfermedad, el peso fresco y el peso seco. Se vió que todas las cepas fueron capaces de producir sideróforos *in vitro*, salvo la cepa E89. Sin embargo, ésta fue la única cepa capaz de producir quitinasas; el 50% de las cepas aisladas solubilizó fosfato y todas produjeron auxinas. De las 6 cepas seleccionadas, 4 protegieron a las plantas de brócoli frente al patógeno, destacando dos cepas de *Bacillus* E89 y BaA1.31. E89, disminuyó significativamente el *quenching* fotoquímico y aumentó el NPQ, manteniendo la fijación neta de CO_2 en niveles similares al control. Sorprendentemente, esto estuvo asociado a una estimulación del crecimiento (peso seco). La otra cepa que mayor protección confirió a la planta, BaC1.51, disminuyó el NPQ y también la fijación de CO_2 y, sin embargo, aumentó el peso fresco. Estos resultados ponen de manifiesto los numerosos determinantes bacterianos implicados en la comunicación planta-microorganismo, así como la especificidad genoma-genoma en la interacción. Ambas cepas tienen un enorme potencial para la formulación de productos bioestimulantes destinados a agricultura.

Comunicación presentada en la VII Reunión del grupo especializado de Microbiología de plantas en La Universidad de Osuna (Sevilla), 2019



El uso de rizobacterias para mejorar la capacidad defensiva de las plantas contra patógenos a través de la elicitación de los mecanismos de resistencia sistémica y sus interacciones con las rutas de transducción de señal mediadas por ácido salicílico y ácido jasmónico/etileno

Helena Martin-Rivilla*, Ana Garcia-Villaraco Velasco, Beatriz Ramos-Solano, Francisco Javier, Gutierrez-Mañero, José Antonio Lucas García.

Grupo Interacción Planta-Microbioma. Facultad de Farmacia, Universidad San Pablo-CEU Universities, Boadilla del Monte, Madrid, España. * hel.martin.ce@ceindo.ceu.es

INTRODUCCIÓN

Las rizobacterias promotoras del crecimiento vegetal (PGPR) se están utilizando cada vez más en el control de plagas, como alternativa biotecnológica a los agroquímicos, dentro del marco de una agricultura sostenible. Algunas cepas son capaces de inducir o estimular el metabolismo defensivo de la planta, proceso conocido como inducción de resistencia sistémica (ISR). Si el metabolismo defensivo está activado, la planta se encuentra en un estado fisiológico, llamado *priming* (Conrath et al. 2002), en el cual es capaz de

desencadenar sus defensas de manera más rápida, más fuerte o ambas cosas a la vez cuando se produce el ataque de un patógeno.

El estudio de las rutas de transducción de señal que se activan tras la detección de una PGPR o de alguno de sus elicitores es fundamental para poder entender el sistema inmunitario vegetal y su interacción con los patógenos. Estas rutas de señalización son aquellas mediadas por ácido salicílico (SA) o ácido jasmónico/etileno (JA/ET).

En este trabajo se llevó a cabo un *screening* de 175 bacterias aisladas de la rizosfera de *Nicotiana glauca* con el objetivo de seleccionar las cepas con mayor capacidad de inducción de resistencia sistémica en *Arabidopsis thaliana* frente al patógeno *Pseudomonas syringae* DC3000. Además, para conocer cuáles eran las rutas de transducción de señal elicitadas, se estudió la expresión diferencial de genes marcadores de las rutas del SA y del JA/ET.

MATERIAL Y MÉTODOS

Las bacterias empleadas en dicho trabajo fueron aisladas de la rizosfera de plantas silvestres de *N. glauca* en tres suelos diferentes (Ramos Solano et al. 2010a). Se secuenciaron e identificaron mediante técnicas de 16S rRNA y se agruparon en un árbol filogenético mediante el programa MEGA v4.0.2. A cada bacteria se le realizaron distintos test de actividad PGPR, como solubilización de fosfatos y producción de ácido indol acético, quitinasas y sideróforos.

Con los resultados obtenidos del árbol filogenético y de las pruebas PGPR se seleccionaron 25 cepas para la realización de un clásico experimento de inducción de resistencia sistémica frente al patógeno modelo *P. syringae* DC 3000. Las bacterias se inocularon a nivel radicular y el patógeno a nivel foliar en *A. thaliana*. El porcentaje de protección de las cepas se calculó en base al número de hojas con síntomas de infección

frente al total y se relativizó con respecto a un control negativo considerado como el 0% de protección.

Con las 6 bacterias con mejores índices de protección se realizó un segundo experimento de ISR para el análisis de la expresión diferencial por qPCR de genes marcadores de rutas de transducción de señal: *NPR1*, *ISC*, *PR1* y *PR2* como genes marcadores de la ruta del SA y *PDF1*, *LOX2*, *MYC2* y *PR3* como marcadores de la del JA/ET.

RESULTADOS Y DISCUSIÓN

De las 25 cepas seleccionadas para el experimento de ISR, 5 mostraron los valores más altos de protección: N 5.12, N 8.17, N 12.34, N 21.24 y N 4.1. Con estas cepas se realizaron análisis de expresión diferencial por qPCR de genes marcadores de rutas de transducción de señal a las 6, 12 y 24 horas tras la inoculación del patógeno. El efecto producido por dichas bacterias en los genes marcadores estudiados se resume en tres comportamientos: altos valores de expresión diferencial a las 6 horas (N 5.12 y N 21.24), a las12 horas (N 12.34) y a las 24 horas (N 8.17 y N 4.1) tras la inoculación del patógeno.

Los valores más altos de expresión se vieron con N 12.34 (Figura 1), donde se observan genes marcadores de ambas vías altamente expresados 12 horas tras la inoculación del patógeno. Estos resultados indican que estas rutas no siempre actúan de manera antagónica como se pensaba hasta entonces (Caarls et al. 2015).

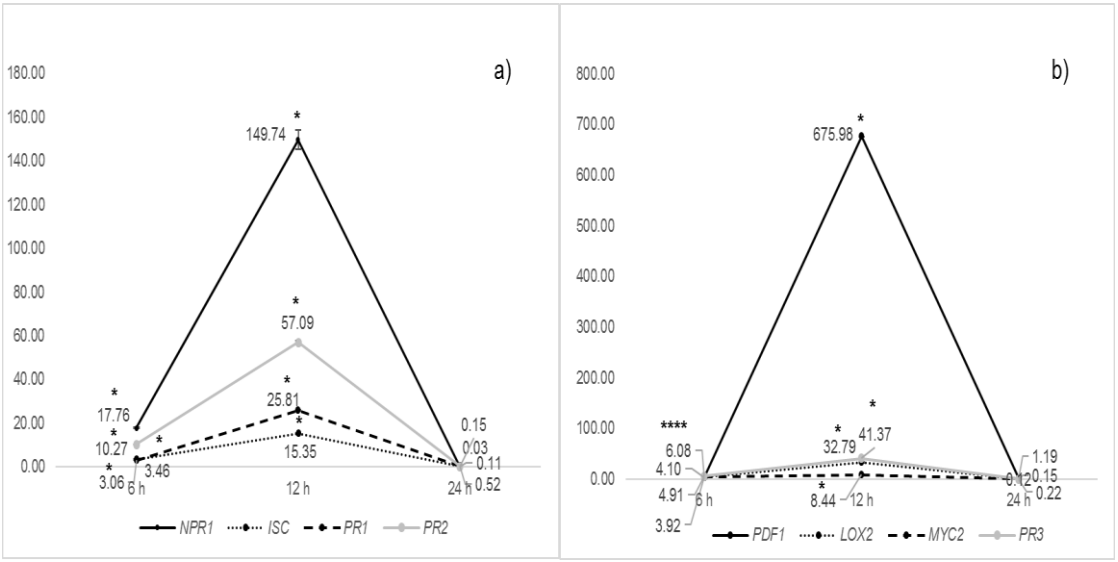


Figura 1 Expresión diferencial 6, 12 y 24 horas tras la inoculación del patógeno en plántulas de *A. thaliana* inoculadas con la cepa N 12.34 de los genes: a) *NPR1, ISC, PR1* y *PR2*, como genes marcadores de la ruta del SA y b) *PDF1*, *LOX2*, *MYC2* y PR3, como marcadores de JA-ET. Los asteriscos representan diferencias significativas ($p < 0.05$) con respecto del control.

Con este experimento, queda patente que, a pesar de tratarse de rizobacterias filogenéticamente cercanas, cada una se comporta de una manera distinta cuando se está elicitando el metabolismo defensivo de las plantas. Esto es muy interesante puesto que abre una multitud de alternativas biotecnológicas y de elicitación del metabolismo defensivo en distintas situaciones y ambientes.

7.4 Scientific publications

AoB PLANTS, 2019, 1–9

doi:10.1093/aobpla/plz049
Advance Access publication July 29, 2019
Studies

STUDIES

Extracts from cultures of *Pseudomonas fluorescens* induce defensive patterns of gene expression and enzyme activity while depressing visible injury and reactive oxygen species in *Arabidopsis thaliana* challenged with pathogenic *Pseudomonas syringae*

H. Martin-Rivilla*, A. Garcia-Villaraco, B. Ramos-Solano, F. J. Gutierrez-Mañero and J. A. Lucas

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

*Corresponding author's e-mail address: helenamartin92@gmail.com

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Abstract

We evaluated the ability of metabolic elicitors extracted from *Pseudomonas fluorescens* N21.4 to induce systemic resistance (ISR) in *Arabidopsis thaliana* against the pathogen *Pseudomonas syringae* DC3000. Metabolic elicitors were obtained from bacteria-free culture medium with *n*-hexane, ethyl acetate and *n*-butanol in three consecutive extractions. Each extract showed plant protection activity. The *n*-hexane fraction was the most effective and was used to study the signal transduction pathways involved by evaluating expression of marker genes of the salicylic acid (SA) signalling pathway (*NPR1*, *PR1*, *ICS* and *PR2*) and the jasmonic acid/ethylene (JA/ET) signalling pathway (*PDF1*, *MYC2*, *LOX2* and *PR3*). In addition, the level of oxidative stress was tested by determining the activity of enzymes related to the ascorbate-glutathione cycle. *N*-hexane extracts stimulated both pathways based on overexpression of *ICS*, *PR1*, *PR2*, *PDF1* and *LOX2* genes. In addition, activity of the pathogenesis-related proteins glucanase (PR2) and chitinase (PR3), lipoxygenase and polyphenol oxidase was enhanced together with an increased capacity to remove reactive oxygen species (ROS). This was associated with less oxidative stress as indicated by a decrease in malondialdehyde (MDA), suggesting a causative link between defensive metabolism against *P. syringae* and ROS scavenging.

Keywords: ISR; metabolic elicitors; oxidative stress; *Pseudomonas fluorescens*; salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) signalling pathway.

Introduction

Agricultural systems impose a range of abiotic and biotic stresses on crop plants that lower their productivity (García-Cristobal *et al.* 2015), thus compromising food supplies worldwide (Pechanova and Pechan 2015; Miller *et al.* 2017).

Due to its significance, interactions between plants and pathogenic organisms have been studied intensively with a view to providing sustainable solutions for crop diseases, to enhance

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food safety by improving food quality and crop yields and to understand how plants cope with biotic stress (Silva *et al.* 2018).

The rapid generation of reactive oxygen species (ROS, such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) represents a common plant response to pathogen attack (Noctor *et al.* 2014; Xia *et al.* 2015) and therefore represents a node from which many signalling events are generated. A rise in ROS production leads to oxidative stress (Gill and Tuteja 2010) mainly by provoking oxidative modification of vital biomolecules including membrane lipids, cellular amino acids, proteins and DNA (Gill and Tuteja 2010; Anjum *et al.* 2012). The outcomes include cell death and the arrest of plant growth and development. To maintain optimal levels of ROS, plants possess a sophisticated regulatory system consisting of enzymatic antioxidants (superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase, GPX; ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR) and non-enzymatic antioxidants (ascorbate, ASC; glutathione, GSH; carotenoids; tocopherols; phenolics compounds).

Colonization of plant roots by PGPR (plant growth-promoting rhizobacteria) improves plant health by stimulating its immune system to decrease oxidative stress through improving ROS scavenging (Lucas *et al.* 2013, 2014; García-Cristobal *et al.* 2015). This phenomenon is known as induced systemic resistance (ISR) and involves the induction of resistance not only locally at the site of infection, but also systemically. Induced systemic resistance has been primarily described as a response induced by PGPR (Pieterse *et al.* 2000), but it can also be induced by metabolic elicitors such as antibiotics, surfactants or other chemicals (Gozzo and Faoro 2013). The elicitation of defensive metabolism by PGPR or elicitors leads to a physiological situation in the plant called priming (Conrath 2011). In this situation, plants show faster and/or stronger activation of defence responses when subsequently challenged by pathogen (Conrath *et al.* 2006).

Despite the many studies of PGPR triggering ISR, few have focused on the molecular elicitors produced by these bacteria. However, metabolites from various bacterial genera: *Klebsiella* (Park *et al.* 2009), *Ochrobactrum* (Sumayo *et al.* 2013), *Pseudomonas* (Ongena *et al.* 2005) and *Bacillus* (Huang *et al.* 2012) have been recognized as ISR metabolic elicitors, with those from *Bacillus* being the most studied, although it is well-known that *Pseudomonas* spp. are possibly the most important producers of compounds triggering plant immune responses (Durrant and Dong 2004; Choudhary *et al.* 2007). Interest in PGPR and their elicitors is heightened by their potential for developing a sustainable agriculture without pesticides or agrochemicals (Wu *et al.* 2018).

After a PGPR or their metabolic elicitors are sensed by a plant, salicylic acid (SA), jasmonic acid (JA) or ethylene (ET) signalling pathways are activated to trigger plant resistance (Wu *et al.* 2018). In the case of ISR, the response depends on JA and ET signalling and also requires NPR1 (non-expressor of pathogenesis-related protein 1) (Pieterse and Van Loon 2004, 2007). The JA signalling pathway has two branches controlled by the transcription factor MYC2 and ethylene response factor (ERF). The ERF branch of the JA pathway is associated to enhance resistance to necrotrophic pathogens and one of the marker genes of this branch is plant defensin 1 (*PDF1*) (Berrocal-Lobo *et al.* 2002; Lorenzo *et al.* 2003).

The aim of the present work was (i) to obtain extracts containing bacterial metabolic elicitors able to trigger protection against pathogens in the model plant *Arabidopsis thaliana* and (ii) to determine the transduction signal pathways involved in this protection. Three organic fractions were obtained from the culture media of a strain of *Pseudomonas fluorescens* (N21.4), a gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca* (Ramos-Solano *et al.* 2010b). This bacterium is known to trigger defensive metabolism in *Solanum lycopersicum* and *A. thaliana* (Domenech *et al.* 2007), to increase isoflavone content in *Glycine max* (Ramos-Solano *et al.* 2010a), to promote fruit production in *Rubus* sp. (Ramos-Solano *et al.* 2014) and to improve fruit quality of *Rubus* sp. by modifying flavonoid metabolism (Garcia-Seco *et al.* 2015). We also wished to evaluate the ability of the extracts to trigger plant defence against pathovar DC3000, a pathogenic strain of *P. syringae*. The most effective of the three fractions was then used to study the signal transduction pathway. To reach these objectives differential gene expression of marker genes from the SA and JA/ET pathways was analysed as well as enzymes involved in ROS scavenging system and proteins involved in induction systemic resistance, all in the context of the overall oxidative status of the plant.

Materials and Methods

Bacterial pathogen, model plant used and metabolic elicitors extraction

Pseudomonas syringae (DC3000) was used as the pathogen in the experiments for challenge inoculation. This strain causes bacterial speck on the model plant *A. thaliana* and is used to study the model system for plant–pathogen interactions (van Loon *et al.* 1998). The pathogen was grown for 24 h in 100 mL of nutrient broth (Conda; gelatin peptone 5 g L^{-1} and beef extract 3 g L^{-1}) in a 250-mL Erlenmeyer flask on a shaker (125 rpm) at 28 °C. The culture was then centrifuged (350 × *g* for 10 min), washed with sterile water and pellet was suspended in sterile sufficient 10 mM $MgSO_4$ to achieve 10^8 cfu mL^{-1}. The enumeration and calculations were carried out following the 'drop method' (Hoben and SomesegAran 1982).

Arabidopsis thaliana Columbia ecotype was used. Seedlings were incubated in a culture chamber (Sanyo MLR-350H) with a 9 h light (350 μE s^{-1}·m^{-2} at 24 °C) and 15 h dark cycle (20 °C) at 70 % relative humidity.

Metabolic elicitors from *P. fluorescens* (N21.4) were obtained according to Sumayo *et al.* (2013) using three separate solvents. The bacterium was first grown in nutrient broth (Conda) on a rotary shaker (180 rpm) at 28 °C for 24 h. Cells were separated by centrifugation at 8000 × *g* for 15 min, and 500 mL of the supernatant filtered (0.2 μm) and extracted sequentially in *n*-hexane, ethyl acetate and *n*-butanol to obtain the metabolic elicitors. The dry residues from each fraction were dissolved in 25 mL 10 % dimethylsulfoxide (DMSO).

Screening for the most effective determinant fraction to trigger systemic resistance

An ISR assay on *A. thaliana* plants was used to evaluate the ability of three fractions from *P. fluorescens* (N21.4) to trigger plant protection. The following five treatments were involved: (i) metabolic elicitors in the *n*-hexane fraction, (ii) metabolic elicitors in the ethyl acetate fraction, (iii) metabolic elicitors in the *n*-butanol fraction, (iv) N21.4 (positive control) and (v) untreated plants (negative control). An additional control with 10 % DSMO was included to ensure that the effects were due to metabolic elicitors and not to the DMSO. All were pathogen challenged.

Arabidopsis thaliana seeds (not previously sterilized) were germinated in quartz sand for 1 week and then transplanted individually to 12-well plastic plates (5 mL) filled with peat.

Each treatment comprised three plates, each plate constituting a replicate. One week after transplanting, treatments were delivered to seedlings by drenching in the soil with 20 µL of elicitors per well. The positive control was inoculated with 1 mL of 10^8 cfu mL^{-1} of N21.4 culture, grown for 24 h in sterile nutrient broth (Conda) while negative controls were treated with 1 mL of sterile nutrient broth (Conda) or 20 µL of 10 % DMSO.

Three days later, plates were placed in a humidity chamber to ensure stomata opening, and the next day challenged with pathogen *P. syringae* DC3000. The plants were inoculated by placing a 5 µL drop of 10^8 cfu mL^{-1} suspension on each leaf (Sumayo *et al.* 2013). Seventy-two hours after pathogen inoculation, the disease index was determined as the ratio of the number of leaves with disease symptoms to the total number of leaves (Ryu *et al.* 2004). Results were relativized using the negative control as a 0 % of protection.

Study of the signal transduction pathway of the most effective fraction

The *n*-hexane fraction was the most effective against pathogen infection. This was used to study signal transduction pathways based on genes overexpressed in response to this fraction during the systemic resistance assay. The genes involved are detailed below. The experimental set-up included two treatments: (i) metabolic elicitors in the *n*-hexane fraction, and (ii) pathogen-only control (negative control). Twenty-one plants per treatment were used; plants were arranged on three replicates, with seven plants each.

Seeds were germinated in quartz sand for 1 week. One-week-old seedlings were transplanted individually to 100 mL pots filled with 3:1 (vol/vol) peat/sand mixture (60 g per pot). Plants were watered with 5 mL of tap water twice a week, and with 5 mL of ½ Hoagland solution per plant once a week. Four-week-old seedlings were treated with 50 µL of *n*-hexane fraction, and negative control with 50 µL of the *n*-hexane fraction from sterile nutrient broth. Four days later, plants were pathogen challenged.

One day before the pathogen inoculation, plants were placed in a humidity chamber to ensure the stomatal opening needed for the disease to establish. Pathogen inoculation was carried out by spraying the plants with 150 mL of a suspension of 10^8 cfu mL^{-1}; non-pathogen controls were mock inoculated with sterile nutrient broth (Conda). Seven plants per treatment were collected 6, 12 and 24 h after pathogen challenge (hapc), powdered in liquid nitrogen and stored at –80 °C until gene expression analysis by qPCR and enzymatic activities analysis.

The genes analysed were *NPR1* (non-expressor of pathogenesis-related gene 1), *PR1* (pathogenesis-related gene 1), *PR2* and *ICS* (isochorismate synthase 1) as markers of the SA signalling pathway (Betsuyaku *et al.* 2017; Ding *et al.* 2018; Silva *et al.* 2018); *PDF1*, *LOX2* (lipoxygenase 2), *PR3* and the transcriptional factor *MYC2* as markers of the JA/ET signalling pathway (Caarls *et al.* 2015).

RNA extraction and RT-qPCR analysis

Total RNA was isolated from each replicate with PureLink RNA Micro Kit (Invitrogen), DNAase treatment included. RNA purity was confirmed using Nanodrop™. A reverse transcription was performed followed by qPCR. Reverse transcription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad). All reverse transcription were carried out using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 °C, 30 min 42 °C, 5 min 85 °C, and hold at 4 °C. The amplification was realized with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 °C and then 39 cycles consisting of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a melting curve to check the results. To describe the level of expression in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110 %. *Sand* gen (AT2G28390) was used as a reference gen. Primers used appear in **Supporting Information—Table S1**. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

Pathogenesis-related proteins and systemic resistance proteins activities

Enzymatic activities of resistance proteins glucanase (PR2), chitinase (PR3), lipoxygenase, cellulase and polyphenol oxidase were assessed. Before assessing enzymatic activities, soluble proteins were extracted from the plant powder by resuspending 100 mg in 1 mL of potassium phosphate buffer 0.1 M pH 7 containing 2 mM phenylmethylsulfonyl fluoride (PMSF). These were sonicated 10 min and then centrifuged for 10 min at 14 000 rpm. The supernatant was divided into aliquots, frozen in liquid nitrogen and stored at –80 °C for further analysis. All above operations were carried out at 0–4 °C.

To measure the amount of total protein from plant extract, 250 µL of Bradford reagent, 50 µL of sample and BSA dilutions were pipetted into each well of 96-well plates, incubated for 30 min at room temperature and measured using a plate reader (MB-580 Heales) at absorbance of 595 nm. A calibration curve was constructed from commercial BSA dilutions expressed in milligrams. The units of protein were expressed as mg $µL^{-1}$.

Glucanase (EC 3.2.1.6), cellulase (EC 3.2.1.4) and chitinase (EC 3.2.1.14) activities were measured as described by Lee *et al.* (2008). Calibration curves were made with glucose (for glucanase and cellulase) and *N*-acetyl glucosamine (for chitinase) in acetate buffer with concentrations between 0.1 and 1 mg mL^{-1} for glucanase and cellulase, and between 0.01 and 0.1 mg mL^{-1} for chitinase. Data were expressed as µmol mg $protein^{-1}$ min^{-1}.

Lipoxygenase (EC 1.13.11) activity was measured as described by Ali *et al.* (2005). Extinction coefficient of 25 mM^{-1} cm^{-1} was used to calculate activity. Data were expressed as µmol mg $protein^{-1}$ min^{-1}.

Polyphenol oxidase (1.14.18.1) activity was measured as described by Nawrocka *et al.* (2018). Extinction coefficient of 2.72 mM^{-1} cm^{-1} was used to calculate activity. Data were expressed as µmol mg $protein^{-1}$ min^{-1}.

In all assays, the blank consisted on the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer.

Enzymatic activities related to oxidative stress

Enzyme activities related of APX (EC 1.11.1.11), SOD (EC 1.15.1.1), GR (EC 1.6.4.2), GPX (EC 1.11.1.7), CAT (EC 1.11.1.6), MDHAR (EC 1.6.5.4) and DHAR (EC 1.8.5.1) were measured spectrophotometrically and expressed as µmol mg $protein^{-1}$ min^{-1}.

Ascorbate peroxidase was measured by the method of Garcia-Limones *et al.* (2002). Oxidation of ASC was determined by the decrease in A_{290}. An extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity.

Superoxide dismutase activity was determined following the specifications of the SOD activity detection kit (SOD Assay Kit-WST, Sigma-Aldrich). With this method, the rate of the reduction with O_2 is linearly related to xanthine oxidase (XO) activity and inhibited by SOD. Inhibition activity of SOD was determined colourimetrically and expressed as % inhibition mg $protein^{-1}$.

Glutathione reductase was measured by the method of Garcia-Limones *et al.* (2002). Oxidation of NADPH was determined by the increase in A_{340}. Extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity.

Guaiacol peroxidase was measured by the method of Garcia-Limones *et al.* (2002). Oxidation of guaiacol was determined by the increase in A_{470} using an extinction coefficient of 26.6 mM^{-1} cm^{-1} to calculate activity.

Catalase was measured by the method of Garcia-Limones *et al.* (2002). The decrease in A_{240} produced by H_2O_2 breakdown was recorded and an extinction coefficient of 36 mM^{-1} cm^{-1} used to calculate activity.

Monodehydroascorbate reductase activity was measured by the method of Xu *et al.* (2008). Reduction of monodehydroascorbate was determined by the decrease in A_{340} using an extinction coefficient of 6.22 mM^{-1} cm^{-1} to calculate activity.

Dehydroascorbate reductase activity was measured as described by Xu *et al.* (2008) at 265 nm. Reduction of dehydroascorbate was determined by the decrease in A_{265} using an extinction coefficient of 14 mM^{-1} cm^{-1} to calculate activity.

In all assays, the blank consisted on the components of the reaction mixture except from the enzyme extract, which was replaced by an equal volume of the assay buffer. In the case of the GR assay, an additional blank without oxidized GSH was included to account for the presence in the extracts of other enzyme activities able to oxidize NADPH.

Oxidative status of the plant: malondialdehyde concentration

The malondialdehyde (MDA) content was determined by the method of Hu *et al.* (2016) with modifications. Briefly, 0.25 g of powder was mixed with 2 mL of reaction solution containing 0.5 % (vol/vol) thiobarbituric acid (TBA) and 20 % (vol/vol) trichloroacetic acid (TCA). The mixture was heated at 95 °C for 30 min, then quickly cooled to room temperature, treated to eliminate air bubbles and centrifuged at 6000 × *g* for 20 min. Then, absorbance of the supernatant was determined by a spectrophotometer at 532 and 600 nm. The MDA content was calculated using the formula: MDA (nmol/FW) = [(OD532 − OD600)]/(ε·FW), where FW is the fresh weight in grams and ε the extinction coefficient (155 mM^{-1} cm^{-1}).

Statistical analysis

One-way ANOVA was used to check the statistical differences in all data obtained in the experiments carried out. Prior to ANOVA, analysis of homoscedasticity and normality of the variance were checked with Statgraphics plus 5.1 for Windows and found to meet the requirements for analysis. When significant differences appeared ($P < 0.05$) a Fisher test was used (Sokal and Rohlf 1980).

Results

Capacity of the three metabolic elicitors fractions to trigger systemic resistance

Each of the organic fractions from culture media containing *P. fluorescens* (N21.4) and the N21.4 strain itself were able to trigger defence mechanisms in *Arabidopsis* seedlings and to improve their capacity to resist the pathogenic effects of *P. syringae* (DC3000) (Fig. 1). The *n*-hexane fraction gave the highest protection percentage (91 %) and was chosen to study the signal transduction pathway involved in protection by evaluating the differential gene expression (fold change) of selected marker genes. Negative controls treated with DMSO or sterile nutrient broth had no effect.

Study of the signal transduction pathway of the most effective fraction

Effects of the *n*-hexane fraction on SA pathway and the JA/ET pathway marker genes are shown in Fig. 2. Figure 2A shows the SA pathway marker genes. Six hours after pathogen challenge (6 hapc) only *ICS* showed significantly higher expression (2.04), decreasing to zero values 12 and 24 hapc. *PR1* and *PR2* showed maximum differential expression at 12 hapc (11.7), although *PR2* expression was 10 times lower than for *PR1* (1.51). None of the genes showed differential expression 24 hapc.

Figure 2B shows the JA/ET pathway marker genes. Only *PDF1* (63.2) and *LOX2* (1.71) showed significant differences in gene expression. These were evident 12 hapc, with *PDF1* values being 60 times higher than *LOX2*. There was no differential gene expression 6 and 24 hapc. Negative control treated with 50 µL of *n*-hexane fraction from sterile nutrient broth had no effect on differential gene expression.

Pathogenesis-related proteins and systemic resistance proteins activities

Activity of the pathogenesis-related proteins (PRs) glucanase (PR2) and chitinase (PR3) and the proteins related to systemic resistance against pathogen lipoxygenase, cellulase and polyphenol oxidase were evaluated (Fig. 3). The *n*-hexane fraction promoted the activity of all these enzymes. The increases were statistically significant at most sampling times for chitinase (PR3; Fig. 3A) glucanase (PR2; Fig. 3B) and lipoxygenase (Fig. 3C). Cellulase (Fig. 3D) showed significant differences 6 and 12 hapc, and polyphenol oxidase (Fig. 3E) 24 hapc.

Enzymatic activities related to oxidative stress

Except for glutathione reductase activity (GR; Fig. 4E), *n*-hexane fraction elicitors increased activity levels compared to controls. The differences were statistically significant at all three sampling times for APX (Fig. 4A) and GPX (Fig. 4D). Increases in CAT (Fig. 4E) and MDHAR (Fig. 4F) activity were significant 12

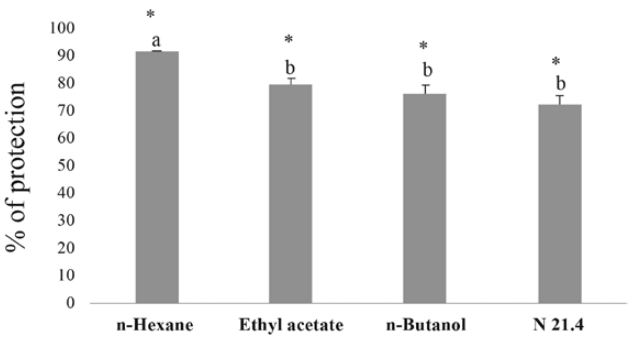

Figure 1. The extent of protection against the pathogen *Pseudomonas syringae* DC3000 to seedlings of *Arabidopsis thaliana* by extracts from culture media supporting *Pseudomonas fluorescens* (N21.4). Three solvent fractions (*n*-hexane, ethyl acetate, *n*-butanol) were tested and compared. Percentage of protection was based on the number of leaves with disease symptoms compared to the total of leaves ($n = 12$ seedlings). Data were relativized to control (i.e. seedlings inoculated only with pathogen), which was considered as 0 % protection. Asterisks represent statistically significant differences ($P < 0.05$) with regard to negative control. Letters represent statistically significant differences between the four different treatments.

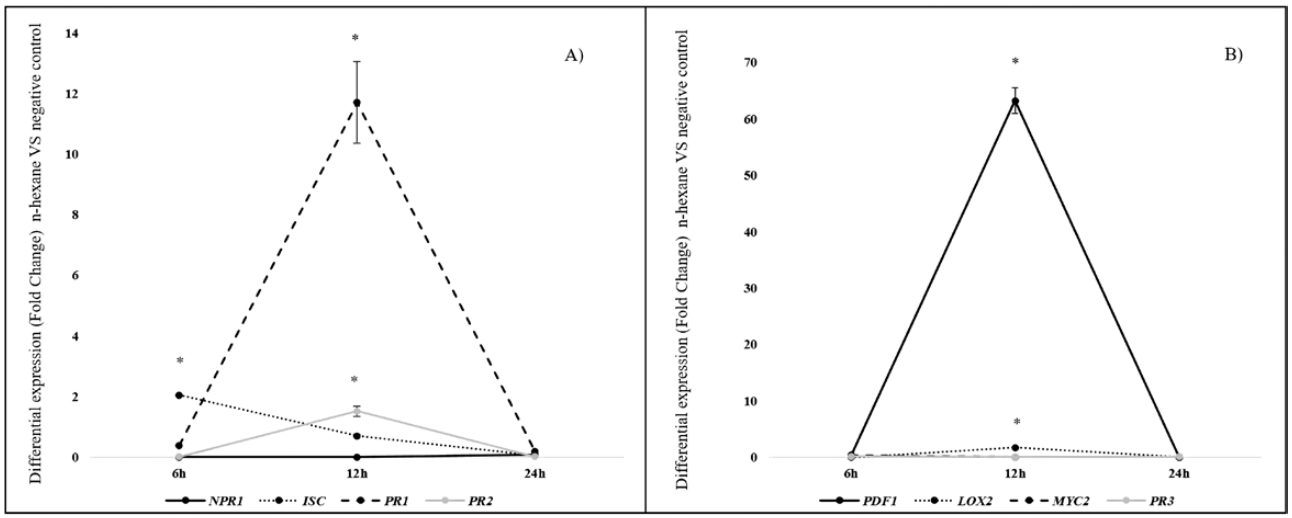

Figure 2. Differential expression (fold change) of SA pathway and JA/ET pathway marker genes by seedlings of *Arabidopsis thaliana* treated with *n*-hexane extract from culture media supporting *Pseudomonas fluorescens* N21.4. The results compare treatments against a negative control 6, 12 and 24 h after challenging with *Pseudomonas syringae* DC3000; (A) *NPR1*, *ICS*, *PR1* and *PR2* genes (as SA signalling pathway markers), (B) *PDF1*, *LOX2*, *MYC2* and *PR3* (as JA/ET signalling pathway markers). Asterisks represent statistically significant differences ($P < 0.05$) within each sampling time (6, 12 and 24 h; $n = 7$).

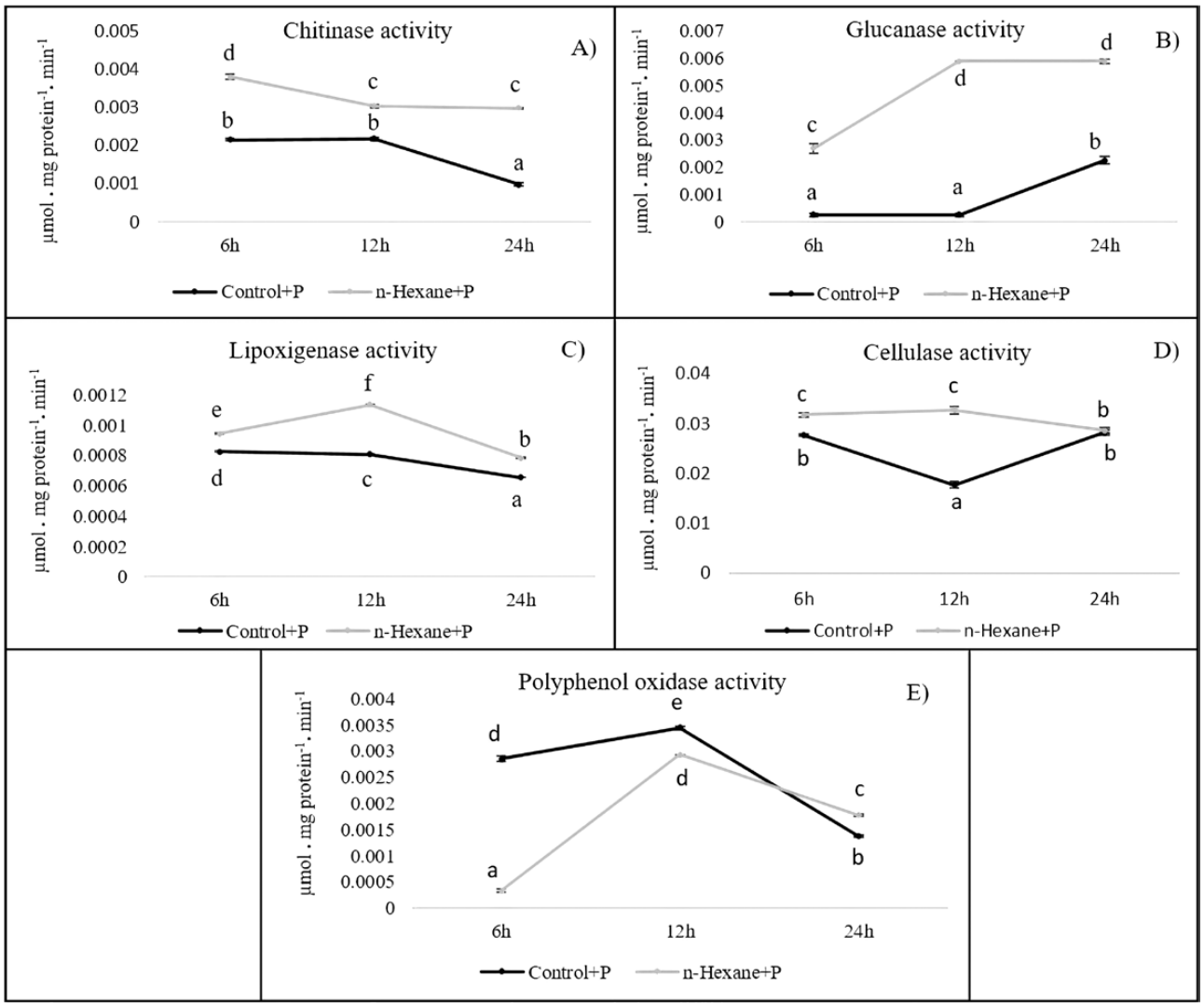

Figure 3. Pathogenesis-related proteins and ISR proteins activities in plants of *Arabidopsis thaliana* inoculated with the pathogenic *Pseudomonas syringae* DC3000 (Control + P) and treated with elicitor extract with *n*-hexane (*n*-hexane + P) taken from culture media supporting *Pseudomonas fluorescens* (N21.4). Activities were measured 6, 12 and 24 hapc. (A) Chitinase (PR3); (B) glucanase (PR2); (C) lipoxygenase; (D) cellulase and (E) polyphenol oxidase. Different letters indicate significant differences ($P < 0.05$) between treatments in each sampling time.

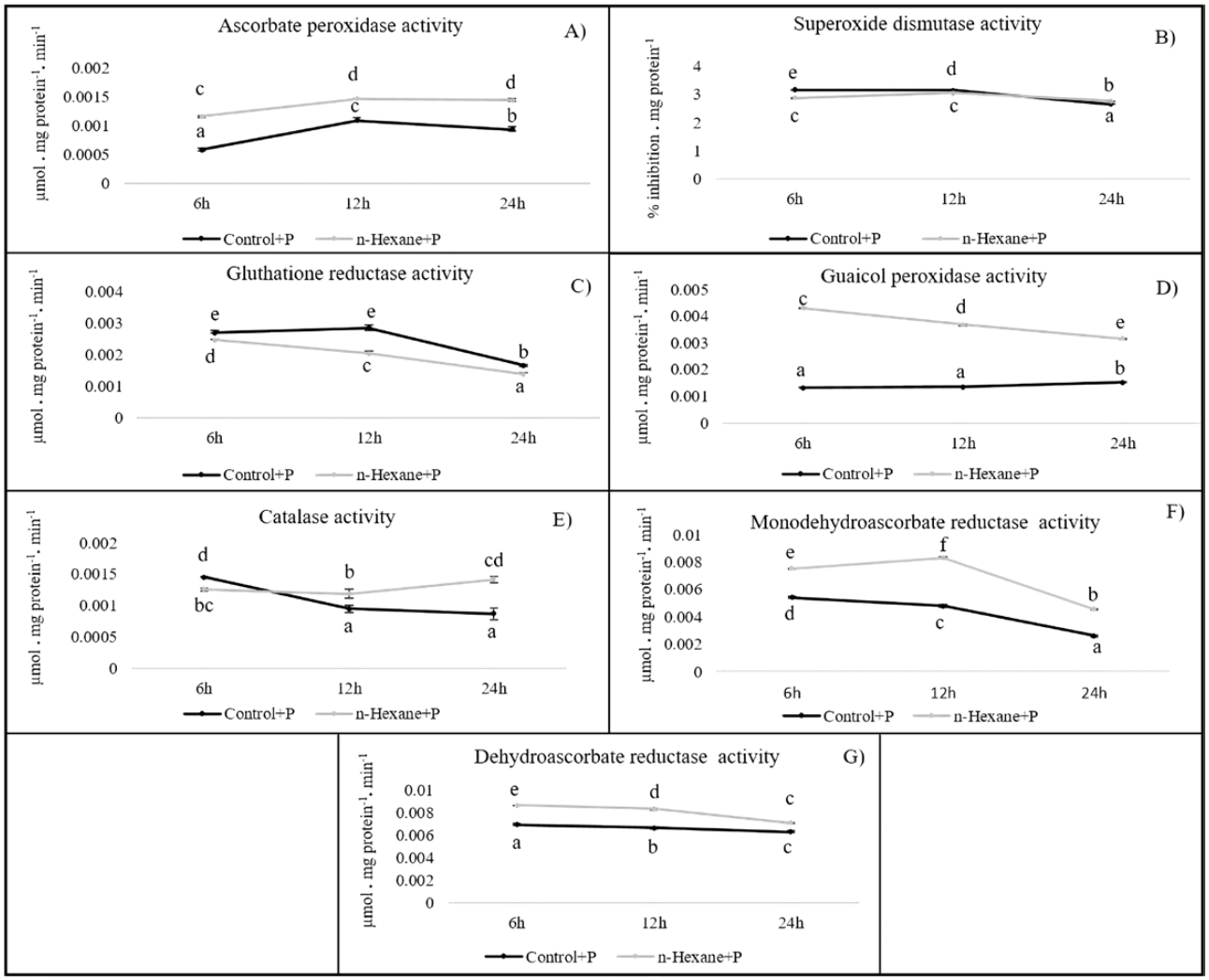

Figure 4. Enzyme activities related to oxidative stress in plants of *Arabidopsis thaliana* inoculated with the pathogenic *Pseudomonas syringae* DC3000 (Control + P) and treated with elicitor-containing *n*-hexane extract taken from culture media supporting *Pseudomonas fluorescens* (N21.4) (*n*-hexane + P). Enzyme assays were made 6, 12 and 24 hapc. (A) APX; (B) SOD; (C) GR; (D) GPX; (E) CAT; (F) MDHAR; (G) DHAR. Different letters indicate significant differences ($P < 0.05$) between treatments in each sampling time.

and 24 hapc. Superoxide dismutase activity (SOD; Fig. 4B) was promoted 6 and 24 hapc and DHAR (Fig. 4G) 12 hapc.

Oxidative status of the plant: MDA concentration

Malondialdehyde, a marker of oxidative stress was measured 12 hapc (Fig. 5). At this time, MDA concentrations were markedly depressed by *n*-hexane extract.

Discussion

The capacity of PGPR to enhance plant defence of biotic and abiotic stresses has been demonstrated many times in the past (e.g. García-Cristobal *et al.* 2015; Beris *et al.* 2018; Kumar *et al.* 2018). However, effects of elicitors produced by these PGPRs have been less studied. These substances have been reported to be either structural molecules, such as flagellin (Ramirez-Prado *et al.* 2018), or metabolic elicitors released to the medium (Munhoz *et al.* 2017; Wu *et al.* 2018).

The ability of the PGPR *P. fluorescens* N21.4 to trigger plant metabolism in different species has been described in numerous previous studies (Domenech *et al.* 2007; Ramos-Solano *et al.* 2010b; Algar *et al.* 2012; Ramos-Solano *et al.* 2015), and certain bacterial metabolic elicitors have been classified by their molecular weight (Algar *et al.* 2012). The present study explores

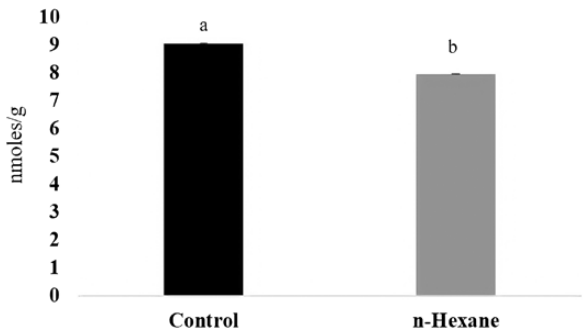

Figure 5. Malondialdehyde concentrations in plants of *Arabidopsis thaliana* inoculated with the pathogenic *Pseudomonas syringae* DC3000 and after treatment with *n*-hexane extracts of culture media supporting *Pseudomonas fluorescens* (N21.4) compared to controls. Different letters indicate significant differences ($P < 0.05$) between treatments.

further the complex mixture of elicitors produced by *P. fluorescens* based on solubility in three organic solvents.

The effectiveness of all three fractions to protect plants (Fig. 1) reveals the existence of several metabolic elicitors with contrasting solvent solubilities involved in plant protection. There may well be different pattern recognition receptors (PRRs) in plants for these elicitors.

Induced systemic resistance holds potential for activating cellular defence responses prior to pathogen attack (Akram *et al.* 2016). It is well known that, among others, ISR is accompanied with an augmented expression of defence-related genes, increased accumulation of secondary metabolites and defence-associated proteins (Conrath 2006; Zamioudis and Pieterse 2012). Moreover, the rapid generation of ROS is a common protective response of plants to pathogen attack (Noctor *et al.* 2014; Xia *et al.* 2015) and therefore represents the node from which many signalling events are generated.

Induced systemic resistance typically relies on JA/ET signalling pathways (Pieterse *et al.* 2002). Our results, at the level of gene expression and activity of proteins related to plant defence systems (Figs 2 and 3), indicate that elicitors from the *n*-hexane fraction induce the JA/ET pathway and also the SA pathway by increasing at the same time expression of marker genes *PR1* and *PDF1* (SA and JA/ET marker genes, respectively; Caarls *et al.* 2015; Ding *et al.* 2018) and enzyme activities such as PR2 (marker of SA signalling pathway) and PR3, LOX and PPO, as markers of JA/ET signalling pathway (Lucas *et al.* 2014; García-Cristobal *et al.* 2015; Silva *et al.* 2018; Wu *et al.* 2018).

This implies a versatility in the resistance mechanism, allowing attack of biotrophic and necrotrophic organisms to be opposed. These two pathways are not necessarily antagonistic, as previously been indicated by other results (Betsuyaku *et al.* 2017; Nie *et al.* 2017).

The physiological state induced by elicitors is known as priming. It is marked by an enhanced activation of defence mechanisms readily demonstrated in pathogen challenge experiments (Maunch-Mani *et al.* 2017). The induction of defensive mechanisms must necessarily be mediated by elicitor detection that activates an immune response. This has been termed microbe-associated molecular pattern (MAMP)-triggered immunity (MTI). It relies on the detection of conserved microbial signature molecules (MAMPs) via extracellular transmembrane receptors or PRRs (Mhlongo *et al.* 2018). Our results add to the picture by demonstrating a stimulation of all the ASC-GSH cycle enzyme of plants treated with *n*-hexane extracts from *P. fluorescens* (except, GR), notably for APX, GPX and MDHAR (Fig. 4). These enzymes have well-established roles in stress responses (Song *et al.* 2009; Sultana *et al.* 2012; García-Cristobal *et al.* 2015; Souza *et al.* 2016; Liu *et al.* 2018; Maruta and Ishikawa 2018).

The results obtained with respect to the enzymatic activities related to free-radical scavenging, accord with the suppressed levels of MDA (Fig. 5), a marker of oxidative damage (Lucas *et al.* 2017). These results are consistent with the higher protection and with the higher activity of the ROS scavenging enzymes reported above.

There are few studies that relate oxidative stress enzymes to innate immunity in plants elicited with PGPR or metabolic elicitors (Lucas *et al.* 2014; García-Cristobal *et al.* 2015). However, this type of relationship helps to stablish a complete set of changes associated to plant protection. Markers related to oxidative stress metabolism will assist in improving primer fingerprinting for each bacterial strain (Maunch-Mani *et al.* 2017; Gutierrez Albanchez *et al.* 2018). This will improve further analysis and also our understanding of the mechanisms that defend plants against pathogens. In addition, new sets of products based on metabolic elicitors or PGPR with an ability to elicit defence mechanisms against a range of stresses can be expected to be useful in practical agriculture.

Conclusions

Extract from media in which *P. fluorescens* N21.4 was cultured using three different solvents each protected *A. thaliana* against the pathogen *P. syringae* DC3000, highlighting the *n*-hexane fraction. Extracts in *n*-hexane gave higher protection than those of ethyl acetate and butanol. The mode of action of the elicitors in the *n*-hexane fraction included activating SA, JA or ET signalling pathways and the enzymatic machinery of ROS scavenging to decrease oxidative stress. Further studies are needed to identify chemically the elicitors excreted by *P. fluorescens*. Once this is achieved, their use as biotechnological inoculants to improve the plant resistance to stress is a promising possibility.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. Primers forward and reverse used in qPCR analysis.

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Conflict of interest

None declared.

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Biotechnology and Biological Transformations

Improving flavonoid metabolism in blackberry leaves and plant fitness by using the bioeffector Pseudomonas fluorescens N 21.4 and its metabolic elicitors: a biotechnological approach for a more sustainable crop

Helena Martin-Rivilla, Ana García-Villaraco, Beatriz Ramos-Solano, Francisco Javier Gutierrez-Mañero, and Jose Antonio Lucas

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Improving flavonoid metabolism in blackberry leaves and plant fitness by using the bioeffector *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors: a biotechnological approach for a more sustainable crop

H. Martin-Rivilla*, A. Garcia-Villaraco, B. Ramos-Solano, F. J. Gutierrez-Manero and J. A. Lucas

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain

*Corresponding author's e-mail address: helenamartin92@gmail.com

ABSTRACT

Beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors inoculated to cultivars of blackberry (*Rubus* spp. Var. Loch Ness) reinforced plant immune system and improve their fitness by increasing photosynthesis, decreasing oxidative stress and activating pathogenesis-related proteins. They also triggered leaves flavonoid metabolism enhancing the accumulation of beneficial phenolic compounds such as kaempferols and quercetin derivatives. The elicitation of leaves secondary metabolism allows taking advantage of the blackberry leaves (a current crop waste), following the premises of the circular economy, for the isolation and obtaining of high added value compounds. The results of this work suggest the use of N 21.4 and/or its metabolic elicitors as plant inoculants as effective and economically and environmentally friendly agronomic alternative practices in the exploitation of blackberry crop for getting plants with a better immune system and for revalorizing the leaves pruning as a potential source of polyphenols.

Key words: *Pseudomonas fluorescens* N 21.4, metabolic elicitors, blackberry leaves, plant fitness, flavonoid metabolism, circular economy, kaempferol.

INTRODUCTION

Intensive blackberry cultivation is a relatively recent emerging crop for which there is a deficit of specific inoculants that improve plant resistance to pests and to abiotic stress. A widely accepted approach to create effective inoculants substituting chemical products is the use of beneficial rhizobacteria (bioeffectors) and/or structural or metabolic elicitors[1] extracted from them able to trigger secondary defense metabolism[2]. This process, in which defensive capacity of the entire plant increases after local exposure to bioeffectors or elicitors, is called Induced Systemic Resistance (ISR)[3] and can cause the *priming*[4], a physiological state in which plants are able to activate faster and/or stronger their defenses when subsequently attacked by pathogens[5]. After bioeffectors or elicitors are sensed, Salicylic acid (SA) and/or Jasmonic/Etylene (JA/ET)) signaling pathways are activated to trigger plant resistance[6], since there is evidence that bioeffectors or elicitors can trigger either one pathway or both[7]. This improvement in plants immune system also include activation of Reactive Oxygen Species (ROS) scavenging cycle and an increase in the synthesis of antioxidant secondary metabolites, which means an enhancement of plant fitness[8].

Among secondary metabolites with greater antioxidant capacity are flavonoids[9]. They play an essential role in plant defense against biotic and abiotic stress[10,11] and also act as visual attractors, repellents, phytoalexins, antioxidants, and antimicrobials[12-14]. Furthermore, many flavonoid beneficial effects on human health have been reported[15]. Therefore, the engineering of the flavonoid biosynthesis pathways for the purposeful accumulation and isolation of active molecules has been extensively used in the plant biotechnological industry[16].

Blackberries are one of the richest fruits in flavonoids and this is why they bring many benefits when they are included in the diet[17]. Moreover, some analytical studies have shown that blackberry leaf phenolic composition is analogous to that of the fruits or even richer and higher[18] and that leaves extracts also show in vivo/in vitro beneficial effects on human health, as they have antioxidant, anti-diabetic[19], anti-microbial[20], analgesic, anti-inflammatory and angiogenic activity[21]. This suggests that leaves are a valuable source of bioactive natural compounds.

As these beneficial phenolic compounds of blackberry leaves belong to the plant secondary metabolism, which is easily inducible, their elicitation could be carried out using inoculants made of bioeffectors and/or derived elicitors. In this way, the biosynthesis and accumulation of high added value phenolic compounds could be increased in a sustainable way.

To develop biological inoculants that improve plant immune system through the elicitation of secondary defensive metabolism, effective bioeffectors used should be able to trigger a broad spectrum of plant agronomic species. The bioeffector used in the present work, *Pseudomonas fluorescens* N 21.4, has the ability to trigger ISR in *Arabidopsis thaliana*[22], *Glycine max*[23], *Hypericum sp.*[24], *Papaver sp.*[25] and blackberry[26]. This is why, we elicited blackberry plants with N 21.4 and with metabolic elicitors (ME) extracted from it, since we have previously shown that N 21.4 ME mimic bacterial effect to trigger the immune system of *A. thaliana*[22]. Triggering secondary metabolism and improving blackberry plant immune system by bioeffectors and/or ME would be therefore an effective and more ecofriendly approach to protect plants against pathogens and abiotic stress preventing economic loses and to improve crop yield security and human nutrition.

Hence, the aim of this study was to evaluate the ability of N 21.4 and its ME to elicit the secondary defense metabolism of blackberry plants (Rubus spp. Var. Loch Ness), boosting their general fitness and achieving, at the end of the cultivation cycle, plants with leaves richer in valuable polyphenols. In this way, the pruning of leaves, which is a waste, could be revalorized and an added value would be given to the crop. Therefore, circular economy premises would be fulfilled by proposing blackberry leaves as the starting material to obtain usable compounds[27].

To achieve our objectives, we evaluated different markers of plant fitness such as photosynthesis parameters, ROS scavenging enzymes, pathogenesis-related proteins and expression of marker genes of ISR pathways mediated by SA and JA/ET. Blackberry leaves polyphenol composition was also studied. All these analyses were conducted in two sampling moments, corresponding to two physiological plant stages: flowering (January) and fruiting (April).

MATERIAL AND METHODS

Blackberry experimental design. The *Rubus* spp. cv. Loch Ness plants used in this study were kindly provided by Agricola El Bosque S.L. “La Canastita” (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were handled according to regular agricultural practices[28]. Plants were grown in Huelva (South Eastern Spain) from November 2017 to April 2018 under “winter cycle.” Before being transplanted to greenhouses in November, plants underwent an artificial cold period in order to start their regular cycle. A total of 540 plants were in the trial, arranged in five

greenhouses; each greenhouse had two lines with 120 plants in total, each line being one replicate with 60 plants; 3 lines were inoculated with alive N 21.4 at root level, 3 lines were inoculated with metabolic elicitors (ME) from N 21.4 by aerial spraying and 3 lines were left as non-inoculated controls. N 21.4 and ME were inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum per plant (Figure 1).

Leaves were sampled at flowering (January) and at fruiting (April), and were rapidly frozen in liquid nitrogen and brought to the laboratory. Three replicates were taken, being each one constituted from plant material of 60 plants.

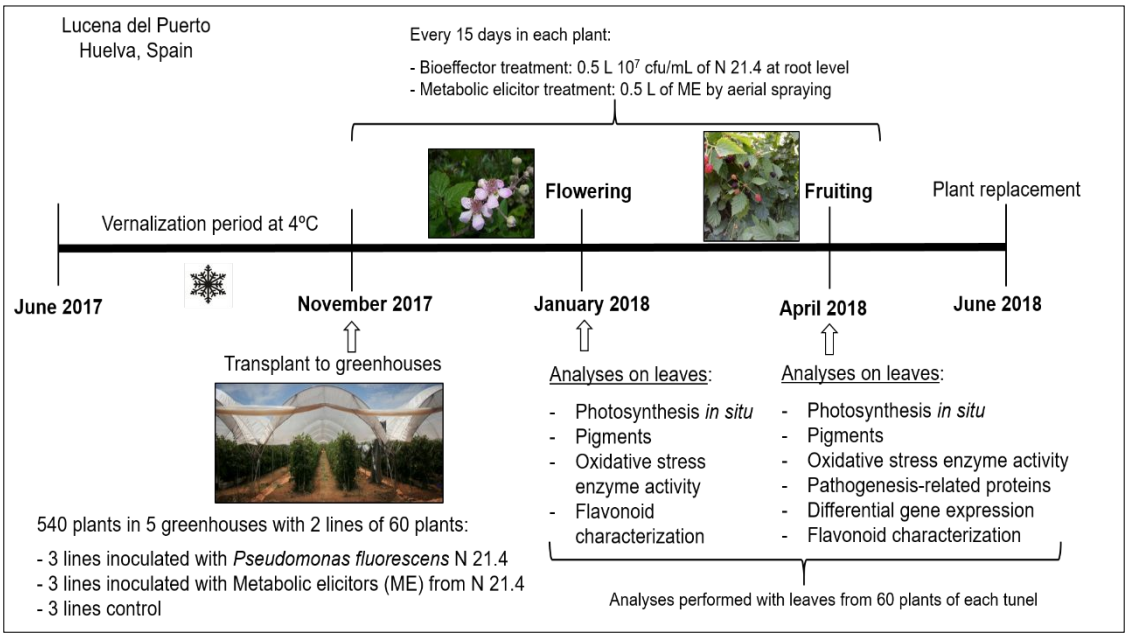


Figure 1. Representative timeline of the entire blackberry cultivation cycle in the greenhouses of Lucena del Puerto (Huelva, Spain).

Bacterial treatments. The bacterial strain used in this work was *Pseudomonas fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a Gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca* Graham in three different soils and physiological moments of the plant[29]. It is able to release siderophores and chitinases in vitro and it triggers defensive metabolism in *Solanum lycopersicum*[29], *A. thaliana*[22], *Glycine max*[23], *Hypericum sp.*[24] and *Papaver sp.*[25]. It also increases fruit production in *Rubus* sp.[28] and improves fruit quality of *Rubus* sp. by modifying flavonoid metabolism[26].

Bacterial strain was stored at -80 ºC in nutrient broth with 20% glycerol. Inoculum was prepared by streaking strains from -80 ºC onto PCA (CONDA) plates, incubating them at 28°C for 24 h. After that, bacterial cells were scraped off the plates into sterile nutrient broth (CONDA)

incubated on a rotatory shaker at 28 ºC and 180 rpm for 24 h to obtain a 10^9 cfu/mL inoculum. Inoculum of N 21.4 was diluted from 10^9 to 10^7 cfu/mL and was delivered at root level to the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

ME treatment was prepared by centrifuging one liter of a 24 h culture of N 21.4, grown at 28ºC on an orbital shaker, at 4000 rpm during 20 min at 4 ºC. Cells were discarded and the liter of supernatant was diluted following the same proportion as the strain (from 10^9 to 10^7 cfu/mL) and it was sprayed onto the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

Photosynthesis analysis by fluorescence measurement "*in situ*". As chlorophyll fluorescence analysis is a very common method to study stress effect on the photosynthetic performance, F0 and Fv/Fm, two sensitive indicators related to chlorophyll fluorescence and plant photosynthetic functioning, were measured with a pulse amplitude modulated fluorometer (Hansatech FM2, Hansatech, Inc, UK).

To determine Fv/Fm ratio (the key parameter to detect PSII photoinhibition induced by a stress[30]), after 30 min of leaf-dark-adaptation, a weak modulated irradiation (1 μmol/m^2 s^1) was applied to firstly determine minimal fluorescence yield (F0), and a 0.7 s saturating flash (10,000 μmol/m^2 s^1) was then superimposed to induce the maximum fluorescence yield of chlorophyll (Fm). The variable fluorescence (Fv) was calculated as the difference between the maximum fluorescence (Fm) and the minimum fluorescence (F0). The maximum photosynthetic efficiency of photosystem II was therefore calculated as Fv/Fm[31].

All measurements were carried out on three plants of each treatment in each tunnel and in both sampling moments (flowering and fruiting).

Total chlorophyll and carotenoids. Total chlorophyll a, b and carotenoids (xanthophylls and carotenoids) were quantitatively determined using the formulas proposed by Lichtenthaler[32]:

Chl a (mg/g Fresh Weigh) = [(12.24*Abs 663 nm)-(2.79*Abs 647)]*(V (mL)/FW(g))

Chl b (mg/g Fresh Weigh) = [(21.5*Abs 647 nm)-(5.1*Abs 663nm)]* (V (mL)/FW(g))

Carotenoids (mg/g Fresh Weigh) = [(1000*Abs 470nm)-(1.82*Chl a)-(85.02*Chl b)/198)]*(V (mL)/FW(g))

For this, five mg of the leaves powder were mixed with 2 mL of 80% acetone. After 24 hours at 4 ºC, the mixture was centrifuged for 10 min at 2000 rpm at room temperature. The supernatant was collected and absorbance was measured at 663, 647 y 470 nm using a spectrophotometer Biomate 5. All measurements were carried out at flowering and fruiting.

Activity of enzymes related to oxidative stress. To measure the specific enzymatic activity of some enzymes related to oxidative stress, three steps were followed. First, a protein extraction of each sample was carried out, then the total protein of each extract was measured, and finally the specific enzymatic activity of superoxide dismutase (SOD (EC 1.15.1.1)), ascorbate peroxidase (APX (EC 1.11.1.11)) and glutathione reductase (GR (EC 1.6.4.2)) referenced to the amount of total protein was calculated.

Firstly, to prepare enzymatic extracts, 10 mg of powdered leaves of each sample were mixed with 1 mL of extraction buffer (cold phosphate buffer 0.1M pH 7.0 with 2 mM of PMSF (phenylmethylsulfonyl fluoride), sonicated for 10 minutes and centrifuged 20 min at 14000 rpm at 4 ªC. The remaining supernatant was frozen at -20ºC and used as the enzymatic extract.

After that, it was measured the amount of total protein of each enzymatic extract by mixing 250 µL of Bradford reagent, 50 µL of enzyme extract and bovine serum albumin (BSA) dilutions from 0,05 to 2 mg/ml. This was inoculated in ELISA 96 well plates, incubated for 30 min at room temperature and measured using a plate reader at absorbance of 595 nm. A calibration curve was constructed from commercial BSA dilutions. The units of protein were expressed as mg/mL.

Finally, the specific enzymatic activity of SOD, APX, and GR enzymes related to oxidative stress were assessed spectrophotometrically in all the leaves extracts:

SOD activity was determined following the specifications of the SOD activity detection kit (SOD Assay Kit-WST, Sigma-Aldrich). With this method, the rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity and inhibited by SOD present in leaves extracts. For this, 200 µL of the Working Solution of the kit was mixed with 20 µL of enzymatic extract and with 20 µL of the enzyme working solution included in the kit. Inhibition activity of SOD was determined colorimetrically using a plate reader at absorbance of 450nm. SOD enzymatic specific activity was calculated in relation to the amount of total protein, previously measured. The unit used for SOD activity was % inhibition mg protein^{-1}.

APX was measured by the method of Garcia-Limones et al.[33]. The reaction mixture consisted of 860 µL of potassium phosphate buffer 50 mM, pH 7.0, 120 µL of sodium ascorbate 2.55 mM, 120 µL of H_2O_2 50 mM and 100 µL of enzymatic extract. Adding H_2O_2 started the reaction, and after 15 secs, the oxidation of ascorbate was determined by the decrease in A290. Extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity (µmol mg $protein^{-1}$ min^{-1}). APX specific activity was calculated in relation to the amount of total protein previously measured.

GR was measured by the method of Garcia-Limones et al.[33]. The assay mixture consisted of 740 µL of potassium phosphate buffer 0.2 M, pH 7.5, 120 µL of 10 mN DTNB (Ellman´s reagent), 120 µL of oxidized glutathione 10 mM, 120 µL of NADPH,1 mM and 100 µL of enzymatic extract. Adding enzymatic extract started the reaction, and after one minute, oxidation of NADPH compound was determined by the increase in A412. Extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity (µmol mg $protein^{-1}$ min^{-1}). GR enzymatic specific activity was calculated in relation to the amount of total protein previously measured.

All measurements were carried out at both sampling moments (flowering and fruiting).

Pathogenesis-related proteins (PRs). Glucanase (PR2) (EC 3.2.1.6) activity was measured as described by Lee et al.[34]. The reaction consists of 375 µL of sodium acetate buffer 50 mM pH 5, 25 µL of laminarina 10 mg/mL and 100 µL of leave enzymatic extract. After an hour at 37 ºC, 1.5 mL of DNS reagent were added. The mixture was heated at 100 ºC during 5 min and finally, 550 nm absorbance was measured. A calibration curve was made with glucose in acetate buffer with concentrations between 0.1-1 mg/mL. Data was expressed as µmol mg $protein^{-1}$ min^{-1}.

This enzymatic activity was measured with the samples collected at fruiting (April 2018).

Chitinase (PR3) (EC 3.2.1.14) activity was measured as described by Lee et al.[34]. The reaction consists of 500 µL of sodium acetate 0.1M pH 5.5, 1 % colloidal chitin in the buffer and 500 µL of enzymatic extract. After two hours of incubation at 37ºC, 200 µL of 1N NaOH were added and it was centrifuged at 10.000g for 10 min. The supernatant was mixed with 1mL of Schales reagent. The mix was heated at 100 ºC for 15 min and the absorbance at 420 nm was measured. A calibration curve was made with N acetyl glucosamine in sodium acetate buffer at concentrations between 0.01 and 0.1 mg/mL. Data was expressed as µmol mg $protein^{-1}$ min^{-1}.

This enzymatic activity was measured with the samples collected at fruiting (April 2018).

RNA Extraction and RT-qPCR analysis. Total RNA was isolated from each replicate with Plant/Fungi Total RNA Purification kit (50) (NORGENTM) (DNAase treatment included) and RNA integrity was confirmed by using NanodropTM.

The retrotranscription was carried out using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were executed using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25ºC, 30min 42ºC, 5 min 85ºC, and kept at 4ºC. The amplifications were performed with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95ºC and then 39 cycles consisting of 15 s at 95ºC, 30 s at 50ºC and 30 s at 72ºC, followed by melting curve to verify the results. To explain the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Reference gene was *Histone*. Primers used are in Table 1. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

RNA and RT-qPCR analysis was carried out with samples collected at fruiting (April 2018).

Table 1. Primers forward and reverse used in qPCR analysis.

	Primer Forward	**Primer Reverse**
RuPR1	5´-TACTACACGTACGCGACAAACAC-3´	5´-TCTCCATCATCACACACAACTCT-3´
RuPR2	5´-TTCGTCTCGATTATGCTCTCTTC-3´	5´-GCAGAATACACAGCATCCAAAA-3´
RuPR3	5´-AAATCAACCTAGCAGGCCACT-3´	5´-GAGGGAGAGGAACACCTTGACT-3´
Histone	5´-TTCCAGAGCCATGCAGTTTTG-3´	5´-TGGCATGAATGGCACAGAGA-3´

Characterization of phenolics and flavonoids by UHPLC/ESI-qTOF-MS. Phenolic acids including, citric acid, gallic acid, genistic acid, salicylic acid, vanillic acid, ferulic acid and chlorogenic acid were purchased from Sigma (St. Louis, MO, USA); flavonoids including kaempferol, kempherol-3-O-glucoside, kempherol-3-O-rutinoside, quercetin, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, luteolin, naringenin (aglycone), hesperetin, (+)-catechin, (-)-epicatechin, aesculetin, phloridzin,

255 delphinidin, anthocyanin, delphinidin-3-O-rutinoside , and malvidin and other polyphenols like 6,7-
256 dihydroxicoumarin were purchased from Sigma and from Extrasynthese Co.™ (Geney, France).
257 The standard solutions (10 ppm) were dissolved in methanol. All the solvents used, as
258 methanol and acetonitrile (Honeywell Riedel-de Haen), were LC-MS grade. Purified water was
259 obtained from Milli-Q Plus™ System from Millipore (Milford, MA, USA). Formic acid was
260 purchased from Aldrich (St. Louis, MO, USA).
261 **Sample preparation**
262 The extraction of phenolics was conducted as follows: 10 mg of leaves powder were added to
263 1000 µL of methanol. The mixture was vortexed for 1 min, sonicated for 5 min and centrifuged at
264 4.000 rpm for 5 min at 4 ºC. Supernatants were collected and stored at -20ºC until their use for
265 LC/MS analysis. During the process, extracts were protected from light.
266 **UHPLC/ESI-qTOF-MS Analysis**
267 Samples were injected on a 1290 Infinity series UHPLC system associated to an electrospray
268 ionization source (ESI) with Jet Stream technology to a 6550 iFunnel QTOF/MS system (Agilent
269 Technologies, Waldbronn, Germany).
270 For the dissociation, a volume of 2 µL was injected in a reversed-phase column (Zorbax
271 Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40°C. The flow rate was 0.5
272 mL/min with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol.
273 Gradient elution consisted of 2% B (0-6 min), 2-50% B (6-10 min), 50-95% B (11-18 min), 95% B
274 for 2 min (18-20 min), and returned to starting conditions 2% B in one minute (20-21 min) to finally
275 keep the re-equilibration with a total analysis time of 25 min.
276 Detector was functioning in full scan mode (m/z 50 to 2000), at 1 scan/s. Accurate mass
277 measurement was confirmed through an automated calibrator delivery system that constantly
278 introduced a standard solution, containing masses of m/z 121.0509 (purine) and m/z 922.0098
279 (HP-921) in positive ESI mode; on the other hand m/z 112.9856 TFA (trifluoride acetic acid) and
280 m/z 922.009798 (HP-921) were introduced in negative ESI mode. The capillary voltage was
281 ±4000 V for positive and negative ionization mode. The temperature was set at 225 °C. The
282 nebulizer and gas flow rate were 35 psig and 11 L/min respectively, fragmentor voltage to 75V
283 and a radiofrequency voltage in the octopole (OCT RF Vpp) of 750 V.

For the investigation, MassHunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies) was used for control and acquisition of all data obtained with UHPLC/ESI-qTOF-MS.

For measurement, each sample was injected twice in six different concentrations to create calibration curves in which sample peak areas were extrapolated. UHPLC-MS data analysis was performed by MassHunter Qualitative Analysis (Agilent Technologies) Software version B.08.00 using Molecular Feature Extraction (MFE).

All measurements were carried out at both sampling moments (flowering and fruiting).

Statistical analysis. To check the statistical differences between the results obtained, analysis of variance (ANOVA) was used. In the cases with only one independent variable (PRs and qPCR), a one-way ANOVA was used and in those data in which there were two independent variables (fluorescence, pigments and enzyme activities related to oxidative stress), a two-way ANOVA (factorial ANOVA) was used. In both cases, prior to ANOVA analysis, homoscedasticity and normality of the variance were checked with Statgraphics 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p< 0.05$) a Fisher test was used[35].

RESULTS

Photosynthesis analysis by fluorescence measurement. Values of indicators related to chlorophyll fluorescence and representative of plant photosynthetic functioning at both sampling points appear in figure 2.

Figure 2 A) shows the minimal fluorescence yield (F0). According to two-way ANOVA, there were significant differences between sampling moments (flowering and fruiting), and differences between the control and the treatments, but there were not statistically significant differences between N 21.4 and ME. The lowest values in flowering and in fruiting were shown by the treatments.

Figure 2 B) shows the maximum photosynthetic efficiency of the photosystem II (PSII) (Fm/Fv). According to two-way ANOVA, there were significant differences between sampling moments (flowering and fruiting), but there were not differences between the control and the N 21.4 and ME treatments. Bacterial treatments showed higher Fm/Fv values in fruiting than control.

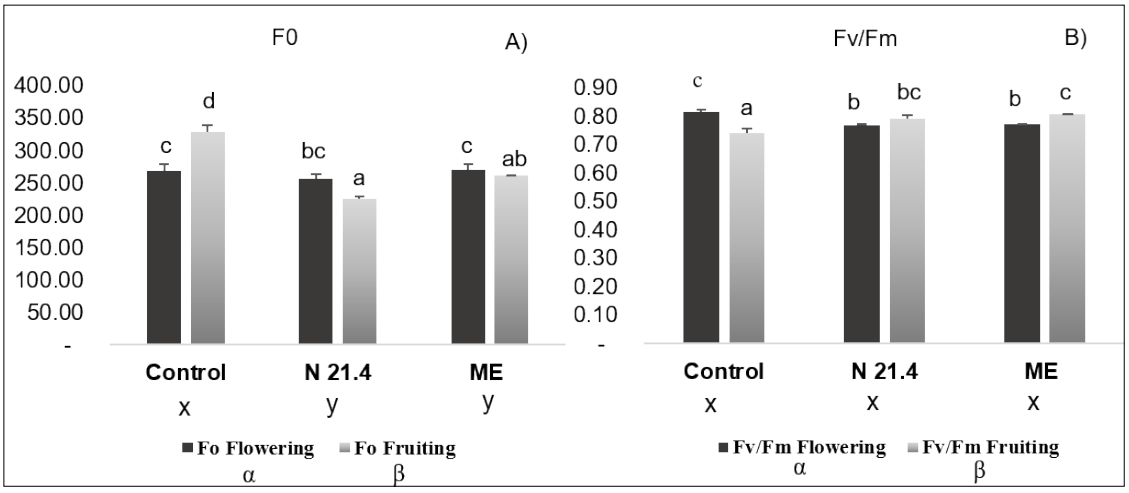


Figure 2. Measurement of chlorophyll fluorescence parameters at flowering and fruiting: A) minimal fluorescence yield (F0); B) maximum photosynthetic efficiency of the PSII (Fm/Fv) in blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls. Different letters indicate significant differences ($p<0.05$): "α and β" indicates differences between sampling moments; "x and y" between treatments (Control, N 21.4 and ME) and "a, b, c, d" indicates differences taking into account sampling moments and treatments (interaction).

Pigments: total chlorophyll and carotenoids. Analysis of photosynthetic pigments at both sampling points appear in figure 3. Total chlorophyll a (Fig. 3 A) and b (Fig. 3 B) and carotenoids (Fig. 3 C) were measured as mg per g of leaf fresh weigh at flowering and at fruiting.

Photosynthetic pigments were significantly different between sampling moments. Chlorophyll a (Fig. 3 A) and carotenoids (Fig. 3 C) were also significantly affected by both treatments, while chlorophyll b was only significantly affected by ME (Fig. 3 B). In controls, chlorophyll b and carotenoids were significantly higher at fruiting, while chlorophyll a was not affected by the sampling moment. As regards to bacterial treatments, both induced a significant increase in chlorophyll a at flowering, being the effects of N 21.4 higher than those of ME, while at fruiting, the ME induced higher values than N 21.4 and the control; interestingly high chlorophyll a values were maintained at the two sampling moments by this treatment (Fig. 3 A). As regards to chlorophyll b, only ME induced a significant decrease compared to controls and bacterial treatment at flowering (Fig. 3 B); carotenoids (Fig. 3C) were significantly lower in treated plants at fruiting.

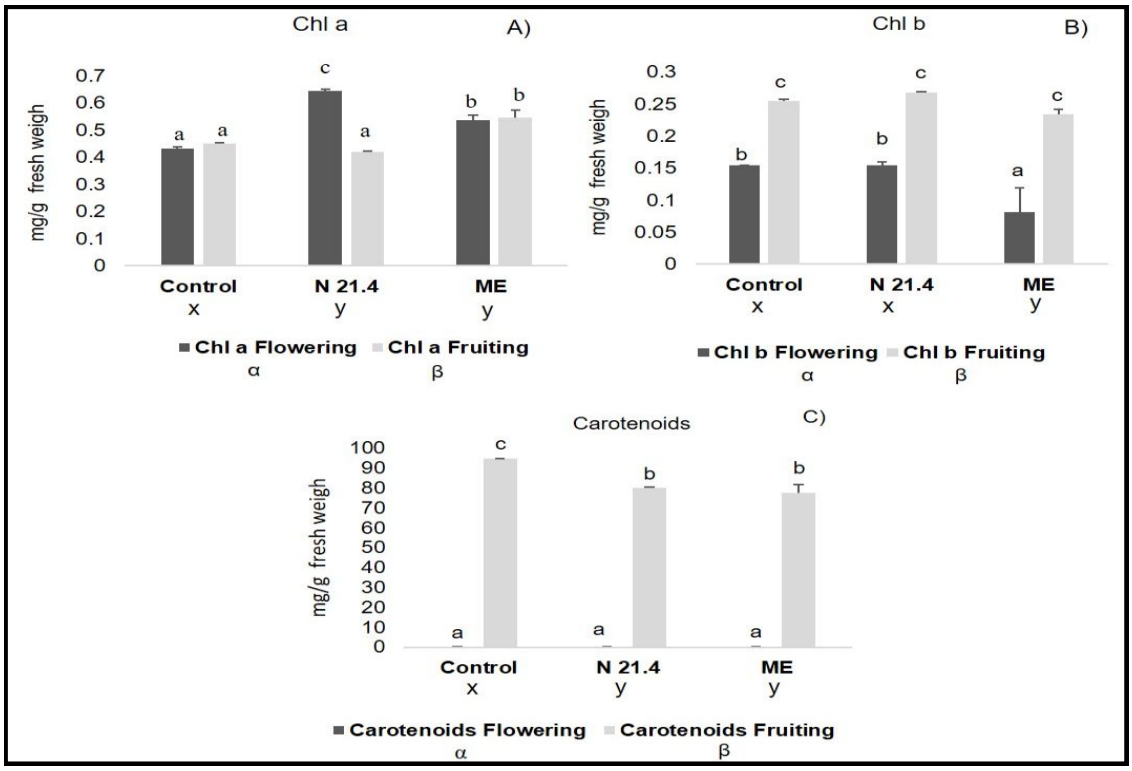
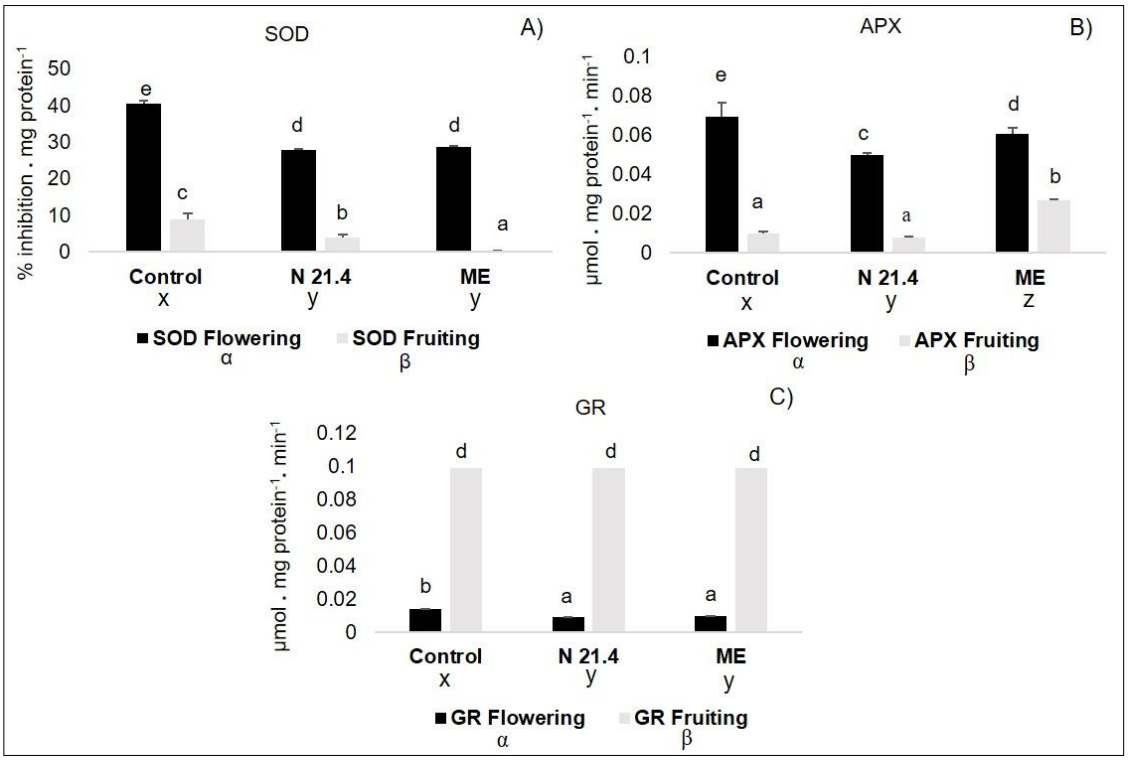


Figure 3. Pigment analysis at flowering and at fruiting: A) total chlorophyll a; B) total chlorophyll b; C) total carotenoids present in the leaves of blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls. Different letters indicate significant differences ($p<0.05$): "α and β" indicates differences between sampling moments; "x and y" between treatments (Control, N 21.4 and ME) and "a, b and c" differences taking into account sampling campaign and treatments (interaction).

Activity of enzymes related to oxidative stress. Specific enzymatic activity of SOD, APX and GR, enzymes of the ROS scavenging cycle and related to oxidative stress, were evaluated in both sampling moments, flowering and fruiting (Fig. 4).

For all the analyzed enzymes, significant differences were found between sampling moments, according to two-way ANOVA. There were also significant differences between the control and the bacterial treatments in the three cases, but there were only statistically significant differences between N 21.4 and ME in APX activity. In general, activity of SOD (Fig. 4 A) and APX (Fig. 4 B) was higher at flowering, while GR (Fig. 4 C) was higher at fruiting. Control plants showed in general the highest values at all times as compared to treated plants, being significant the differences except for GR at fruiting, which showed no differences between treatments (Fig. 4 C). APX activity was significantly lower in treated plants at flowering, while at fruiting, only ME

355 induced a significant increase as compared to N 21.4 treatment and controls. SOD activity (Fig.
356 4 A) was significantly decreased by both treatments in flowering and fruiting; differences between
357 bacterial strain and ME were only significant at fruiting, where ME treated plants showed the
358 lowest values.



359
360 **Figure 4**. Enzymatic activity of: A) superoxide dismutase (SOD), B) ascorbate peroxidase (APX)
361 and C) gluthatione reductase (GR) in the leaves of blackberry plants treated with N 21.4, its
362 metabolic elicitors (ME) and non-treated controls at flowering and at fruiting. Different letters
363 indicate significant differences ($p<0.05$): "α and β" indicates differences between sampling
364 moments; "x, y and z" between treatments (Control, N 21.4 and ME) and "a, b, c, d and e" indicates
365 differences taking into account sampling campaign and treatments (interaction).
366
367 **Pathogenesis-related proteins (PRs).** Activity of Glucanase (PR2) and Chitinase (PR3), two
368 pathogenesis-related proteins (PRs), were also evaluated (Fig. 5). The activity values of both
369 enzymes were higher in plants treated with the ME than those treated with the control and with N
370 21.4, which had even lower values than the control. These differences were significant between
371 the three treatments for both enzymes.

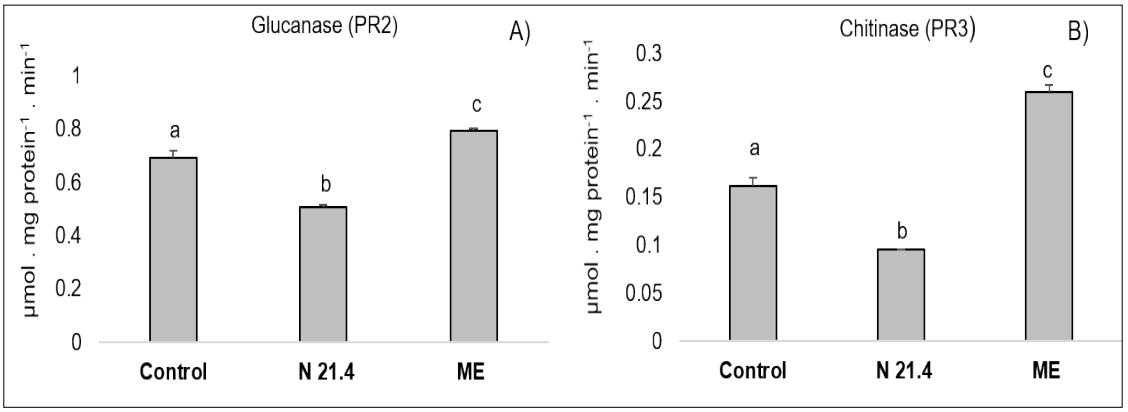


Figure 5. Activity of: A) glucanase and B) chitinase in the leaves of blackberry plants treated with N 21.4, its metabolic elicitors (ME) and non-treated controls at flowering and at fruiting. Different letters indicate significant differences ($p<0.05$) between treatments in each sampling time.

RT-qPCR analysis. Expression of genes encoding PRs (*PR1*, *PR2* and *PR3*), indicative of ISR transduction pathways, in treated plants appears in figure 6. All genes were underexpressed in N 21.4-treated plants as compared to control plants, while ME-treated plants showed more similar expression to control. There were significant differences between N 21.4 and ME.

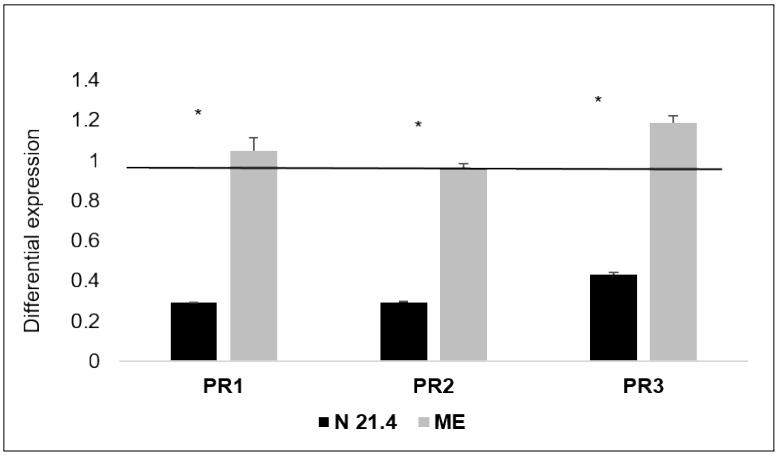


Figure 6. Differential expression of genes encoding PRs (*PR1*, *PR2* and *PR3*) in plants inoculated with N 21.4 and its metabolic elicitors (ME). Expression of 1 is that of the control (horizontal black line). Asterisks represent statistically significant differences ($p< 0.05$) between treatments.

Phenolic and Flavonoid analysis by LC/ESI-qTOF-MS. Methanolic extracts were analyzed by LC/ESI-qTOF-MS. All the compounds were identified by comparing their retention times and MS/MS spectra with reference standards. The main compounds found (13) and their

389 concentration (μg/g) are shown in Table 2. They were grouped in flavonols (5), flavones (1),
390 flavanones (2), flavanols (2), hydroxycoumarins (1), dihydrochalcone (1) and hydroxy benzoic
391 acid (1). Three plant groups were compared (Control, strain N 21.4 and ME from N 21.4) in two
392 sampling moments (flowering and fruiting).

393 In general, the amount of flavonols and flavanols was higher at fruiting, with outstanding
394 presence of kaempferol derivatives (K-3-O-glucoside, K-3-O-rutinoside) and quercetin derivatives
395 (Q-3-O-glucoside, Quercetin 3-O-rutinoside). At fruiting, strain N 21.4 and ME treated plants
396 showed altered flavonol profiles, with marked changes in kaemperfol derivatives: K-3-O-G
397 registered a 2-fold increase under both treatments while K-3-O-R showed a 2.5-fold and a 2-fold
398 increase in strain and ME-treated plants, respectively; at flowering, only ME treated plants were
399 affected showing lower values. As regards to quercetins, Q-3-O-G was almost unaffected by
400 treatments, while Q-3-O-R followed a similar pattern as K-3-O-R, but less intense. The amount
401 of (-)-epicatechin extremely increased at fruiting in the three groups of plants, but with a different
402 intensity. Salicylic acid was lower at flowering and higher at fruiting in treated plants than in
403 controls.

404 **Table 2.** Main phenolic and flavonoid compounds found in blackberry leaves and their concentration in µg/g. Control, N 21.4 and ME treatments were measured
405 at flowering and at fruiting. <LOQ means sample under the limit of quantifying and ND means a non-detected-sample.

		Control		N 21.4		ME	
		Flowering	**Fruiting**	**Flowering**	**Fruiting**	**Flowering**	**Fruiting**
Flavonols	Kaempferol 3-O-glucoside	11.76 ± 0.975	12.62 ± 0.77	11.19 ± 0.175	25.09 ± 0.185	9.36 ± 0.17	24.23 ± 1.09
	Kaempferol 3-O-rutinoside	8.24 ± 0.42	6.82 ± 0.285	8.26 ± 0.05	17.2 ± 0.09	6.05 ± 0.28	14.67 ± 0.69
	Quercetin (aglycone)	0.59 ± 0.035	0 ± 0	0.41 ± 0.01	0 ± 0	0.4 ± 0.01	0 ± 0
	Quercetin 3-O-glucoside	13.53 ± 0.13	51.44 ± 0.4	11.78 ± 0.255	50.06 ± 0.05	12.37 ± 0.235	56.32 ± 2.955
	Quercetin 3-O-rutinoside	7 ± 0.52	16.5 ± 0.16	8.19 ± 0.19	22.89 ± 0.98	7.12 ± 0.125	21.19 ± 0.99
Flavone	Luteolin	1.3 ± 0.235	0.49 ± 0.01	1.38 ± 0.04	1.11 ± 0.095	0.96 ± 0.075	1.8 ± 0.12
Flavanones	Naringenin (aglycone)	12.65 ± 0.05	12.05 ± 0.05	12.84 ± 0.025	12.28 ± 0.035	13.11 ± 0	12.22 ± 0.07
	Hespertin (S)	0.6 ± 0.065	0.7 ± 0.03	0.7 ± 0.1	0.7 ± 0.03	0.5 ± 0	0.7 ± 0.035
Flavanols	(+)-Catechin	0.04 ± 0	15.63 ± 0.42	0.0093 ± 0	15.07 ± 0.295	0.014 ± 0	11.9 ± 0.3
	(-)-Epicatechin	0.45 ± 0.015	393.11 ± 3.425	0.39 ± 0.005	295.37 ± 0.425	0.54 ± 0.02	387 ± 12.99
Hydroxycoumarins	Aesculetin	0.21 ± 0.015	0.024 ± 0.001	0.26 ± 0.005	0.061 ± 0.005	0.27 ± 0.005	0.042 ± 0.007
Dihydrochalcone	Phloridzin	0 ± 0	0.64 ± 0.02	0 ± 0	0.43 ± 0.005	0 ± 0	0.67 ± 0.015
Hydroxy benzoic acid	Salicylic acid	12.07 ± 0.23	9.09 ± 0.405	13.99 ± 0.13	11.84 ± 0.07	12.31 ± 0.6	10.44 ± 0.6

406

DISCUSSION

Blackberries and blackberry leaves are strongly rich in polyphenols that have beneficial effects on human health[17,19, 21]. Blackberry leaves could be used as a profitable source to obtain high added value bioactive molecules to the pharmaceutical, nutraceutical or cosmetic industry by using what is currently a crop waste and therefore fulfilling the premises of circular economy. Hence, this study was undertaken to elicit the secondary metabolism of blackberry plants in field conditions with the beneficial *Pseudomonas fuorescens* N 21.4[17,26,28] and with its metabolic elicitors to get a sustainable crop with a stronger immune system more adapted to biotic and abiotic stress and richer in usable polyphenols. On the one hand, this study is gaining knowledge about effective biological products for agriculture and on the other hand, about the bioactive metabolism of blackberry leaves, which is a great challenge since they are considered as rich in phenolic compounds as the fruits[18].

A fundamental parameter that reflects the health status of a plant is photosynthesis because the photosynthetic apparatus is the first physiological function to be affected by stress[8,26]. In our work, plants treated with N 21.4 and with its ME were less stressed, had a photosynthetic apparatus working optimally and therefore were in better health conditions. The proper photosynthesis performance in treated plants was deduced due to their lower values of F0 (minimal fluorescence yield), since high values of this parameter indicate malfunctions of the photosystem II (PSII) and general photosynthesis damage[36].

On the other hand, Fv/Fm parameter is an estimator of the maximal photochemical efficiency of PSII (PSII maximum capacity to transfer electrons to the electron transport chain)[26] and it is used to identify loss of function of PSII reaction centers. A decrease in Fv/Fm ratio, especially under stress conditions means photoinhibition[30]. However, in our treated plants, higher values of Fv/Fm were observed compared to control plants (at fruiting). Therefore, the results of F0 and Fv/Fm were consistent between them and both reflected that bacterial treatments with strain N 21.4 and ME supposed a better functioning of the entire photosynthetic apparatus with greater potential to channel electrons to the electron transport chain and thus to generate more energy[36]. Our treatments decreased photosynthetic damage caused by stress and improved photosynthetic performance and efficiency, increasing energy production for the plant. This extra energy could be used in the production of target secondary metabolites.

Narrowly related to photosynthesis are photosynthetic pigments (Chl a, Chl b and carotenoids). Chl a is the major pigment responsible for the absorption of light photons and the excitation of electrons that pass to the transport chain to finally generate energy[37]. Our results showed higher levels of Chl a in treated plants, which is associated to lower F0 values and which means that the whole photosynthetic system is working optimally. Chl b and carotenoids are accessory pigments with a protective function to light stress. That undergo a marked increase of these pigments at fruiting, when sun irradiance is getting higher[48]. Carotenoids also perform an essential photoprotective role since they can act as non-enzymatic scavengers of ROS that formed within the chloroplasts[38].

Plants rapidly produce ROS under stress conditions and a rise in ROS generation means oxidative stress[39] and oxidative modification of vital biomolecules like membrane lipids, amino acids, proteins and DNA[39], which ends in cell death and lowering the general fitness of the plant. ROS scavenging systems are classified in two groups, enzymatic and non-enzymatic mechanisms. Within the group of enzymatic mechanisms, it stands out the activity of the ascorbate-glutathione cycle enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase, (GR), catalase and peroxidase, and within the non-enzymatic mechanisms it highlights the amount of antioxidant molecules as ascorbate, carotenoids, tocopherols, phenolic compounds, etc.[22]. Ascorbate levels and ascorbate-glutathione cycle enzymes are tightly linked to plant tolerance to biotic and abiotic stress[40,41]. This is why we have studied the specific enzymatic activity of key enzymes of this cycle (SOD, APX and GR). Our results suggested that treated plants were less stressed than control plants because their enzymatic activity was lower, especially SOD activity at both sampling moments and APX and GR at flowering. A decrease in the enzymes activities suggests a more relaxed state of the plants after treatment inoculation. As treated plants were less stress, they generated less ROS and therefore, their enzymatic machinery responsible for detoxification worked in a more moderate way[42]. The results observed with carotenoids measurement also support the fact that treated plants were less stressed, since their amount of carotenoids was less than that of the control plants. Less stressed plants required less ROS-scavenging enzymatic activity and fewer antioxidant molecules, such as carotenoids.

However, ROS are necessary to start signal transduction pathways, so a lower antioxidant activity may also indicate that bacterial treatments keep ROS in levels such as to maintain a sustained systemic signaling, keeping responses activated[42].

Activity of the pathogenesis-related proteins glucanase (PR2) and chitinase (PR3) were also evaluated because they play an essential role in the capacity of the plants to face to infection caused by pathogen attacks[43]. These PRs are plant species-specific proteins that are produced in response to infection of pathogens, but also in response to inoculation of beneficial bioeffectors and elicitors and which maintain plants in the alert state named *priming* phase[44].

Our study showed that the activity of both PRs was significantly higher in ME-treated-plants than in those treated with N 21.4 or control plants. This may suggests that ME prepared plants to cope with pathogen outbreaks and possibly with other stress situations. qPCR results supported this idea because analyzed genes (*PR1*, *PR2*, *PR3*) of ME-treated-plants had not been downregulated as it occurred in plants inoculated with N 21.4. However, SA values were higher in plants inoculated with both treatments (N 21.4 and ME) than in control plants, which suggests that both plants inoculated with N 21.4 and with ME were more primed and prepared to face to biotic stress situations[45]. These results were also consistent with those of enzymes related to oxidative stress, demonstrating once again that treated plants were less stressed and had therefore a better fitness than control plants to face to biotic and abiotic stress. These last discussed results were only performed at fruiting, at the end of the useful time of the crop, to assess the general physiological state of the plants after all inoculations of bacterial treatments.

Plant fitness can also be improved by the elicitation of secondary metabolism enhancing the synthesis of antioxidant metabolites. Among secondary metabolites with greater antioxidant capacity are flavonoids[9].

Flavonoid metabolism and flavonoid biosynthetic pathway has been widely studied due to its importance in plant defense against biotic and abiotic stress[10,11] and because flavonoids have many beneficial properties to human health[15].

Although our variety of blackberry (*Rubus* spp. Var. Loch Ness) is widely spread, and although it has been seen that leaves contain similar amount and variety of phenolic compounds[18] as fruits, nowadays only the cultivation of the fruit is exploited. This is why in this work, the total amount of various groups of phenolic compounds of the leaves has been measured and a

thorough HPLC analysis (by LC/ESI-qTOF-MS) has been carried out to specifically identify the different phenolic compounds and groups presented in the leaves and their concentration. Studying in detail the phenolic compounds of the leaves of the plants inoculated with our bacterial treatments would allow to revalorize leaves pruning as a source of high-added value compounds.

LC/ESI-qTOF-MS results (Table 2), were in concordance with the results obtained by Ferlemi et al.[18]; Ozmiánki et al.[19] and Gutierrez et al.[17], who found, Kaempferol 3-O-glucoside, Kaempferol 3-O-rutinoside, Quercetin 3-O-glucoside, Quercetin 3-O-rhamnoside, (+)-catechin, (-)-epicatechin and luteolin as main in blackberry leaves. Ozmiánki et al.[19] also prove that genus *Rubus* is very rich in p-hydroxybenzoic acids.

In our study, the amount of flavonols were higher in the plants treated with N 21.4 and its ME, which means that our bacterial treatments are efficient in the elicitation of flavonoid metabolism. Highlighting results were the great increase of kaempferol derivatives (K-3-O-glucoside, K-3-O-rutinoside) and quercetin 3-O-rutinoside in treated plants. Our bacterial treatments may trigger flavonoid biosynthesis as part of an induced systemic response given the important role of this pathway in plant defense. In previous works made in blackberry fruit, it was shown that N 21.4 modified flavonoid metabolism by modifying some of the enzymes involved in the biosynthetic route[26], so it would be possible that it was causing similar changes in the leaves.

Kaempferol derivatives were the compounds most elicited by the treatments from flowering to fruiting, having a 2 fold increase in the case of N 21.4 inoculations and a 2.5 fold increase in the case of ME. Compared to the work of Oszmiański et al.[19] conducted on leaves of 26 species of *Rubus*, our results showed higher kaempferol derivatives concentration.

Kaempferol and its glycosylated groups, especially glucoside and rutinoside, have demonstrated their anti-pathogenic capacity in some plants[46], which reinforces our idea that treated plants, with higher kaempferol values, were more protected than control plants to biotic stress. Moreover, consumption of Kaempferol-rich foods is related to a decrease risk of several types of cancer due to its powerful antioxidant activity[47]. Kaempferol is also associated with a reduced mortality from cardiovascular diseases and prevention of type II diabetes[48] and it has also anti-inflammatory and anti-microbial activity[49]. As kaempferol is poorly absorbed by gastro-intestinal tract, it has been reported that the combination of kaempferol with quercetin significantly increases the anticancer effects[50].

Interestingly, quercetin derivatives also increased between flowering and fruiting, but in this case, differences between the treatments and the control were only observed in quercetin 3-O-rutinoside. An increase in quercetin is promising, not only for its anticancer effects, but because it has been demonstrated that a repeated quercetin supplementation can reach considerable plasma levels[51] acting as an excellent in vitro and in vivo antioxidant.

Some studies have been shown that not only quercetin aglycon is taken up in the gastro-intestinal tract, but also quercetin glycosides, such as Q-3-O-G and Q-3-O-R (present in berry leaves) can be absorbed in the intestine and this absorption surpasses by far that of the aglycon[52]. Hence, dietary quercetin as ingestion of fruits and vegetables or by supplements is therefore a promising agent for many diseases prevention.

In relation to flavanols, and despite the fact that there were not significant differences between the treatments and the control, it was highly remarkable the huge increase in the levels of (-)-epicatechin from flowering to fruiting present in blackberry leaves. As in the case of kaempferol, the concentration of (-)-epicatechin found in our leaves was higher than that found in other studies, such as that of Prakash et al.[53] conducted in blackberry (114.8 μg/g), strawberry (75 μg/g) and red raspberry (50.5 μg/g) leaves.

Epicatechin high antioxidant capacity reduces oxidative stress in plants, but it has been also demonstrated its ability to induce ROS scavenging enzymes such as SOD and APX[54]. It also acts as phytoanticipins in some fruits[55] and that confers resistance to fungus and bacterial infection. Schoroeter et al.[56] have demonstrated that oral administration of chemically pure (-)-epicatechin to humans (1-2 mg/kg) exert rapid cardiovascular benefits by enhancing the bioactivity of nitric oxide, a key molecule in preventing atherosclerosis. Blackberry leaves would be therefore a great source of epicatechin since 300-400 mg/kg could be extracted from them.

With our results, we have demonstrated the elicitation of flavonoid metabolism and also the improve immune system of blackberry plants by inoculating them with both the beneficial rhizobacterium *P. fluorescens* N 21.4 and with their metabolic elicitors. This way, we conclude that the treatments with N 21.4 and its ME are perfectly effective and could be economically and environmentally friendly agronomic inoculants. Furthermore, these treatments would generate a greater economic yield to the cultivation of blackberry by allowing taking advantage of the leaves

as a source for the extraction of metabolites, such as kaempferol and quercetins derivatives and epicatechin, with great value due to their enormous beneficial effects on human health.

However, results show that ME treatment had in general a more marked effect on plant physiology than N 21.4 treatment. ME-inoculated-plants were less stressed and better protected against different stress situations. ME altered both primary and secondary metabolism resulting in more resilient plants.

It cannot be forgotten the application way of each treatment (foliar (ME) vs root application (N 21.4)) because it could be partly responsible for the better effects produced by ME. Foliar application of biostimulants has been seen as a very effective technique[57] and it is also economical and environmentally convenient[57]. On the other hand, using molecules instead of a living bacterium reports advantages in all related to the maintenance and management of the inoculums and also prevents biosecurity problems.

For all the above explained, we conclude that metabolic elicitors extracted from the bioeffector *Pseudomonas fluorescens* N 21.4 would be effective, affordable, respectful with the environment and easy to manage inoculants for agriculture. Due to the promising results obtained with the ME in the present work, another parallel study is being conducted to better known its composition.

ABBREVIATION USED

Metabolic elicitors (ME)

Reactive Oxygen Species (ROS)

Induced Systemic Resistance (ISR)

Salicylic acid (SA)

Jasmonic/Etylene (JA/ET)

Fresh weigh (FW)

Bovine serum albumin (BSA)

Superoxide dismutase (SOD)

Ascorbate peroxidase (APX)

Glutathione reductase (GR)

Kaempferol 3-O-glucoside (K-3-O-G)

586 Kaempferol 3-O-rutinoside (K-3-O-R)

587 Quercetin 3-O-gluconoside (Q-3-O-G)

588 Quercetin 3-O-rutinoside (Q-3-O-R)

589 Photosystem II (PSII)

590

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596

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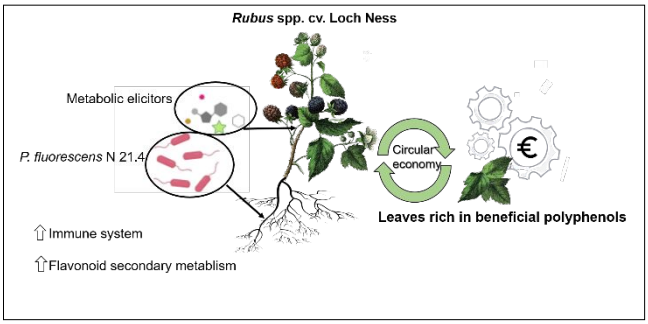

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Research Article

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Metabolic elicitors of *Pseudomonas fluorescens* N 21.4 elicit flavonoid metabolism in blackberry fruit

Helena Martin-Rivilla,* Ana Garcia-Villaraco, Beatriz Ramos-Solano, Francisco J Gutierrez-Manero and José A Lucas

Abstract

Background: The beneficial rhizobacterium, *Pseudomonas fluorescens* N 21.4, and its metabolic elicitors were inoculated in commercial cultivars of blackberry plants (*Rubus* cv. Loch Ness). Phenolic compounds present in red and black fruit and the expression of structural marker genes of the phenylpropanoid pathway during fruit ripening were studied.

Results: An inverse relationship between gene expression and accumulation of metabolites was seen, except for the *RuDFR* gene, which had a direct correlation with cyanidin 3-*O*-glucoside synthesis, increasing its content 1.3 times when *Ru*DFR was overexpressed in the red fruit of plants inoculated with the metabolic elicitors of *P. fluorescens* N 21.4, compared with red fruit of plants inoculated with N 21.4. The *RuCHS* gene also had a fundamental role in the accumulation of metabolites. Both rhizobacterium and metabolic elicitors triggered the flavonoid metabolism, enhancing the catechin and epicatechin content between 1.1 and 1.6 times in the case of red fruit and between 1.1 and 1.8 times in the case of black fruit. Both treatments also boosted the anthocyanin, quercetin, and kaempferol derivative content, highlighting the effects of metabolic elicitors in red fruit and the effects of live rhizobacterium in black fruit.

Conclusion: The metabolic elicitors' capacity to modulate gene expression and to increase secondary metabolites content was demonstrated. This work therefore suggests that they are effective, affordable, easily manageable, and ecofriendly plant inoculants that complement, or are alternatives to, beneficial rhizobacteria.

Keywords: *Pseudomonas fluorescens* N 21.4; metabolic elicitors; blackberries; flavonoid metabolism; *RuDFR* gene; *RuCHS* gene

INTRODUCTION

Blackberry consumption is becoming increasingly popular due to its human health benefits.[1] The increase in consumption, linked with the high economic price of blackberry in the market, makes this crop very attractive for the agro-food industry.[2]

Blackberries are among the fruits with more beneficial properties due to they are extremely rich in flavonoids, among which are flavonols, flavanols, and anthocyanins.[2] These compounds have demonstrated cytotoxic, anticancer, antiviral, antibacterial, anti-inflammatory, antiallergenic, antithrombotic, cardioprotective, and hepatoprotective properties.[3,4] Moreover, their content directly affects the nutritional and organoleptic quality of the fruit.[5]

On the other hand, flavonoids are secondary metabolites that have a vital function in plants, acting as protectors against biotic and abiotic stress.[6] They also have an important role as repellents, visual attractors, phytoalexins, phytoanticipins,[7,8] or auxin controlers.[9]

As flavonoids are secondary metabolites, their biosynthesis is highly inducible. An accepted and effective biotechnological practice to elicit secondary metabolism, enhancing the biosynthesis of compounds with agro-alimentary interest, is the use of beneficial rhizobacteria[10-13] and their derived elicitors (structural molecules such as flagellin,[14] or metabolic elicitors released to the medium, such as antibiotics, surfactants, or other chemicals).[15,16]

As flavonoids have numerous beneficial properties, the engineering of their biosynthetic pathways for the deliberate accumulation and isolation of active molecules has been used extensively in the biotechnological industry.[17] However, the regulation of this pathway during blackberry ripening has not been deeply studied yet. Knowledge of this pathway during blackberry ripening and upon challenge with beneficial rhizobacteria and metabolic elicitors would be useful for the development of new blackberry crop techniques by stimulating the synthesis of secondary metabolites, sustainably improving fruit nutritional qualities.[11,12]

For all the above, the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors (ME) were used in

* *Correspondence to: H Martin-Rivilla, Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668-Boadilla del Monte, Spain. E-mail: helenamartin92@gmail.com*

Plant Physiology, Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, Boadilla del Monte, Spain

the present work to study their capacity to elicit the flavonoid biosynthetic pathway during blackberry fruit ripening. The *P. fluorescens* N 21.4 strain has widely demonstrated its beneficial effects on the physiology of blackberry plant, and on the flavonoid metabolism in the fruit. N 21.4 was able to trigger secondary metabolism in *Rubus fruticosus*, enhancing plant defenses and fruit quality and production.[18] In the work of Ramos-Solano *et al.*,[11] it also demonstrated its capacity to increase fruit production and fruit quality, enhancing the quantity of phenolic compounds throughout the year, mainly when environmental conditions were tougher for the plant's survival. Finally, it was seen that elicitation of blackberry plants by the N 21.4 strain modulated gene expression in the fruit of *Rubus* cv. Loch Ness affected the profiles of secondary metabolites during fruit ripening, boosting the expression of some flavonoid biosynthetic genes and enhancing the concentration of certain flavonoids in the fruit.[12] This allowed the identification of regulatory genes involved in the phenylpropanoid pathway.

Since the N 21.4 strain had demonstrated an extraordinary ability to influence the physiology of blackberry plants and to elicit the flavonoid secondary metabolism, it was proposed, in the present work, to evaluate the potential of its ME to mimic the capacities of the live strain. A previous study demonstrated the capacity of ME to elicit flavonoid metabolism in blackberry leaves, enhancing the amount of epicatechin, kaempferol, and quercetin derivatives.[19] It was also seen that ME were able to reinforce the blackberry plant's immune system, activating pathogenesis-related proteins, and to improve plant fitness, reducing oxidative stress and increasing photosynthesis in the leaves.[19] In other plant species, the ME of N 21.4 also elicited secondary metabolism (e.g. isoflavone metabolism in *Glycine max*,[20] and defensive secondary metabolism in *Arabidopsis thaliana*).[13] As a result of these data, the present study hypothesized that it was feasible that the ME could modify the biosynthetic pathway of flavonoids during blackberry ripening.

The scientific contribution of the present research is therefore to advance the study of metabolic elicitors, using them as alternative plant inoculants to live rhizospheric bacteria, as they have many reported advantages related to the maintenance and management of the inoculums and because they do not cause biosecurity problems. The latter is the innovative aspect of this work, as the capacity of N 21.4 to trigger secondary metabolism in blackberry has already been demonstrated by our group.[11,12]

To pursue these goals, a thorough study of the content and variety of phenolic compounds present in blackberry fruits of plants inoculated with *Pseudomonas fluorescens* N 21.4 and with its metabolic elicitors was carried out in red and in black fruit, as well as an expression analysis of structural marker genes of the phenylpropanoids pathway in both fruit stages.

MATERIAL AND METHODS

Blackberry experimental design

The *Rubus* cv. Loch Ness plants used in this work were kindly provided by Agricola El Bosque S.L. 'La Canastita' (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were managed according to regular agricultural practices.[11] Plants were grown in Huelva from November 2017 to June 2018 under a 'winter cycle'. Before being transplanted to greenhouses in November, plants underwent an artificial 5-month cold period at 4 °C to start their regular cycle. A total of 540 plants were in the trial, arranged in five greenhouses. Each greenhouse had two lines (200 m long each) with 120 plants in total, each line being one replicate with 60 plants. Three lines were inoculated with N 21.4 at root level; three lines were inoculated with metabolic elicitors (ME) of N 21.4 by aerial spraying; and three lines were left as non-inoculated controls. N 21.4 and ME were inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum per plant.

Fruits for analysis were sampled in April 2018, when the two stages of the fruit (red and black) were present in the plants at the same time, and were rapidly frozen in liquid nitrogen and brought to the laboratory to be maintained at −80 °C. Three replicates were taken, each comprising 125 g of fruit, randomly sampled from 60 plants.

Bacterial experimental design

The bacterial strain used in this study was *Pseudomonas fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a gram-negative bacillus, which was isolated from the rhizosphere of *Nicotiana glauca* Graham.[21] The strain N 21.4 is able to trigger defense and phenolic metabolism in *Solanum lycopersicum*,[22] *Arabidopsis thaliana*,[13,22] *Glycine max*,[23] *Hypericum* sp.,[10] *Papaver* sp.[24] and blackberry. In blackberry, it improves fruit yield and quality by increasing flavonoid content.[11,12]

Bacterial strain was stored at −80 °C in nutrient broth (CONDA) with 20% glycerol. Inoculum was prepared by streaking strains from −80 °C onto Plate Count Agar (CONDA) plates, incubating them at 28 °C for 24 h. Then, bacterial cells were scraped off of the plates into sterile nutrient broth and were incubated for 24 h on an orbital shaker at 28 °C, with strong agitation, to keep the medium oxygenated and to obtain a 10^9 cfu mL^{-1} inoculum. Inoculum of N 21.4 was diluted from 10^9 to 10^7 cfu mL^{-1} and it was delivered at root level to the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

The inoculum of ME was prepared by centrifuging N 21.4 (grown for 24 h at 28 °C on an orbital shaker) at 2890×*g* for 20 min at 4 °C. Cells were discarded and the remaining supernatant was diluted following the same proportion as the live strain (from 10^9 to 10^7 cfu mL^{-1}) and it was sprayed onto the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

Fruit production

Fruit was collected from mid-March to early June. They were collected every 3 days from all the plants in each line and treatment. They were weighed and this weight was divided by the number of plants, obtaining the weight (in g) per plant on each day of harvest. Fruit production corresponds to the accumulated production of all the harvesting moments.

Extract preparation for measuring bioactives by colorimetry

The fruit extracts (red and black fruit) used to measure the total flavonols and phenols were prepared by adding 9 mL of 80% cold methanol to 1 g of fruit powder, mixing by vortex (protected from light), sonicating for 10 min and centrifuging at 2890×*g* for 5 min at 4 °C. The remaining supernatant was collected and stored at 4 °C.

The fruit extracts (red and black fruit) to measure total of anthocyanins were prepared by adding 9 mL of 80% methanol and 0.1% of cold HCl to 1 g of fruit powder, mixing by vortex (protected from light), sonicating for 10 min and centrifuging at 2890×*g* for 5 min at 4 °C. The remaining supernatant was collected and stored at 4 °C.

Measurement of bioactives

Total flavonols

Total flavonols were quantitatively determined through the test described by Jia *et al.*,[25] using catechin as standard (Sigma-Aldrich, St Louis, MO, USA). One milliliter of the methanolic extract was added to a flask with 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$ (w/v). After 5 min, 0.3 mL of 10% $AlCl_3$ were added. Five minutes later, 2 mL of NaOH 1 M was finally added. The solution was mixed and measured at 510 nm with an UV–visible spectrophotometer (Biomate 5). A catechin calibration curve was made ($r = 0.99$). The results were expressed as g of catechin equivalents per kg of fresh weight (FW). All samples of red and black fruit were measured in triplicate.

Total phenols

Total phenols were determined quantitatively with Folin–Ciocalteu agent (Sigma-Aldrich) by a colorimetric method described by Singleton and Rossi[26] with some modifications.[27] Gallic acid was used as standard (Sigma-Aldrich). Twenty µL of the methanolic extract were mixed with 250 µL of Folin–Ciocalteu agent 2 N (Sigma-Aldrich) and 3 mL of distilled water. After 5 min at room temperature, 0.75 mL of 20% Na_2CO_3 solution was added. After 8 min at room temperature, 950 µL distilled water was added and after 2 h in obscurity, absorbance was measured at 760 nm with an UV-visible spectrophotometer (Biomate 5). A gallic acid calibration curve was made ($r = 0.99$). Results were expressed in g of gallic acid equivalents per kg of fresh weigh (FW). All samples of red and black fruit were measured in triplicate.

Total anthocyanins

Total anthocyanins were determined quantitatively through the pH differential method described by Giusti and Wrolstad.[28] Methanolic extracts were diluted in pH 1 buffer (0.2 M KCl) and pH 4.5 (1 M CH_3CO_2Na) in 1:15 proportion. After that, absorbance was measured at 510 and 700 nm respectively, in a UV–visible spectrophotometer (Biomate 5). A cyanidin-3-glucoside calibration curve was made ($r = 0.99$). Results were expressed in g of cyanidin-3-glucoside equivalents per kg of fresh weigh (FW). All samples of red and black fruits were measured in triplicate.

Characterization of phenolics and flavonoids by ultra-high performance liquid chromatography with electrospray ionization source, coupled to quadrupole time-of-flight mass spectrometry analyzer (UHPLC/ESI-qTOF-MS)

Phenolic acids, including, citric acid, gallic acid, genistic acid, salicylic acid, vanillic acid, ferulic acid, ellagic acid, and chlorogenic acid, were purchased from Sigma-Aldrich (St Louis, MO, USA); flavonoids including kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, luteolin, naringenin (aglycone), hesperetin, (+)-catechin, (−)-epicatechin, aesculetin, phloridzin, delphinidin, anthocyanin, delphinidin-3-*O*-rutinoside, and malvidin and other polyphenols like 6,7-dihydroxicoumarin were purchased from Sigma-Aldrich and from Extrasynthese Co.™ (Geney, France).

The standard solutions (10 ppm) were dissolved in methanol. All the solvents used, as methanol and acetonitrile (Honeywell Riedel-de Haen (Seelze, Germany)) were liquid chromatography–mass spectrometry (LC/MS) grade. Purified water was obtained from the Milli-Q Plus™ system from Millipore (Milford, MA, USA). Formic acid was purchased from Sigma-Aldrich (St Louis, MO, USA).

Sample preparation

The extraction of phenolics was conducted as follows: 10 mg of powder of lyophilized fruit were added to 1000 µL of methanol. The mixture was vortexed for 1 min, sonicated for 5 min, and centrifuged at 2890×*g* for 5 min at 4 °C. Supernatants were collected and stored at −20 °C until their use for LC/MS analysis. During the process, extracts were protected from light.

UHPLC/ESI-qTOF-MS analysis

Samples were injected on a 1290 Infinity series UHPLC system associated with an electrospray ionization source (ESI) with Jet Stream technology to a 6550 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany).

For the dissociation, a volume of 2 µL was injected into a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2% B (0–6 min), 2–50% B (6–10 min), 50–95% B (11–18 min), 95% B for 2 min (18–20 min), and returned to starting conditions 2% B in 1 min (20–21 min) to finally keep the re-equilibration with a total analysis time of 25 min.

The detector was functioning in full scan mode (m/z 50 to 2000) at 1 scan/s. Accurate mass measurement was confirmed through an automated calibrator delivery system that constantly introduced a standard solution, containing m/z 121.0509 (purine) and m/z 922.0098 (HP-921) in positive ESI mode; on the other hand m/z 112.9856 (TFA) and m/z 922.009798 (HP-921) were introduced in negative ESI mode. The capillary voltage was ±4000 V for positive and negative ionization mode. The temperature was set at 225 °C. The nebulizer and gas flow rate were 35 psig and 11 L min^{-1} respectively, fragmentor voltage was 75 V, and radiofrequency voltage in the octopole (OCT RF Vpp) was 750 V.

For the investigation, MassHunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies) was used for control and acquisition of all data obtained with UHPLC/ESI-qTOF-MS.

For measurement, each sample was injected twice in six different concentrations to create calibration curves in which sample peak areas were extrapolated. The UHPLC-MS data analysis was performed by MassHunter Qualitative Analysis (Agilent Technologies) Software version B.08.00 using molecular feature extraction (MFE).

All measurements were carried out using red and black fruit.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Prior to RNA extraction, the fruits were removed from the −80 °C freezer and ground to a fine powder with liquid nitrogen using an RNAase free mortar and pestle. Total RNA was isolated from each replicate with a Plant / Fungi Total RNA Purification kit (50) (Norgen™ (Thorold, ON, Canada)) (DNAase treatment included) and RNA integrity was confirmed by using Nanodrop™ (ThermoScientific).

The retrotranscription was carried out using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were executed using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 °C, 30 min 42 °C, 5 min 85 °C, holding at 4 °C. The

Table 1 Forward and reverse primers used in qPCR analysis

Gene	Gene code	Forward primer	Tm (°C)	Reverse primer	Tm (°C)
RuCHS	Chalcone synthase [EC 2.3.1.74]	5′ATGGTGGTTGTTGAAATTCC	61.1	5′CTGGATTGCACACCCAGGTGGCCC	79.4
RuFLS	Flavonol synthase [EC 1.14.20.6]	5′CCTACAGGGAAGTCAATGAGAAA	63.1	5′CACATGGGATTTCAGTACCTTCT	62.9
RuF3′H	Flavonid-3′-hydroxylase [EC 1.14.14.82]	5′CCTATCTCCAAGCTGTCATCAAG	63.8	5′GTGGTATCCGTTGATTTCACAAC	64.1
RuDFR	Dihydroflavonol reductase [EC 1.1.1.219]	5′AATCAGAAGAAGGTGAAGC	55.9	5′CATTAKSACAAGTTTGGTG	50.2
RuLAR	Leucocyanidin reductase [EC 1.17.1.3]	5′GTGGAGTCCCATACACGTACATT	63.6	5′CTGAAACTGATCTAACGGTGGAA	64
RuANS	Anthocyanidin synthase [EC 1.14.20.4]	5′TTGGTCTGGGATTAGAAGAAAGG	64.2	5′CTGAGGGCATTTTGGGTAGTAAT	63.9
RuANR	Anthocyanidin reductase [EC 1.3.1.77]	5′ TCGCAATGTACTTCCAAGAAAC	62.9	5′CTTCATCAGCTTACGGAAATCAC	63.6
RuMYB5	MYB family transcription factor	5′ACTCAATCCAGACTCCTCATCTG	63.5	5′AGGAAGTGATTGGACTTTTAGGG	63.2
RuACT	Actin	5′ATGTTCCCTGGTATTGCAGAC	62.7	5′CCACAACCTTGATCTTCATGC	64.4

Garcia-Seco *et al.*[12] The primers were designed with the Primer3Plus program. Under the design conditions, a PCR fragment size of 100–120 bp was specified.

amplification was performed with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 °C and then 39 cycles consisting of 15 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, followed by a melting curve to verify the results. To explain the expression obtained in the analysis, a cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. The regulatory genes of the phenylpropanoids biosynthetic pathway analyzed in blackberry were: *RuCHS*, *RuFLS*, *RuF3′H*, *RuDFR*, *RuLAR*, *RuANS* and *RuANR*. The transcription factor Ru*MYB5* was also analyzed. The reference gene was *Actin*. The primers used are given in Table 1. The primers were designed with the Primer3Plus program. Under design conditions, a PCR fragment size of 100–120 bp was specified. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

To check the statistical differences in the results obtained, an analysis of variance (ANOVA) was used. For the quantitative polymerase chain reaction (qPCR) experiment and the bioactive measurements by colorimetric methods (with only one independent variable), a one-way ANOVA was used. In the bioactive analysis by UHPLC/ESI-qTOF-MS, with two independent variables, a two-way ANOVA (a factorial ANOVA) was used. In both cases, prior to ANOVA analysis, homoscedasticity and normality of the variance were checked with Statgraphics plus 5.1 for Windows, meeting the requirements for analysis. When significant differences appeared ($P < 0.05$) a Fisher test was used.[29]

RESULTS

Fruit production

In control plants, 6167 ± 75 g per plant was recorded; in plants inoculated with N.21.4, 6277 ± 50 g per plant, and in plants inoculated with ME, 5680 ± 45 g per plant was recorded. Plants inoculated with ME showed statistically significant differences when compared with those inoculated with live strain (N 21.4) and control plants, which registered a higher fruit yield.

Bioactive content of fruit from non-inoculated plants

The differences between the red and black fruit of control plants (not inoculated with any bacterial treatment) were analyzed.

The amount of flavonols, measured by colorimetry, was very similar in red control fruit (0.46 ± 0 g of catechin equivalents kg^{-1} of FW) and in black control fruit (0.47 ± 0 g of catechin equivalents kg^{-1} of FW). However, in the case of phenols, measured by colorimetry, black control fruit showed significant higher content (3.02 ± 0.03 g of gallic acid equivalents kg^{-1} of FW) than red control fruit (2.83 ± 0.02 g of gallic acid equivalents kg^{-1} of FW). In the case of anthocyanins, measured by colorimetry, black control fruit also showed significant higher content (0.63 ± 0 g of cyaniding 3-*O*-glucoside equivalents kg^{-1} of FW) than red control fruit (0.12 ± 0 g of cyaniding 3-*O*-glucoside equivalents kg^{-1} of FW).

Regarding the specific bioactives measured by UHPLC/ESI-qTOF-MS, it was seen that black control fruit had a significantly lower concentration of all the measured bioactives, except for quercetin, aglycone, and phloridzin (1.7 times higher in black control fruit) and cyanidin-3-*O*-glucoside (3.7 times higher in black control fruit than red control fruit).

Measurement of bioactives

The total amount of bioactives in red and in black fruit was measured by colorimetry. Figure 1 shows the fold increase and fold-decrease in bioactives in red fruit and in black fruit of each treatment compared to the bioactives present in the fruit of control plants: Fig. 1 (i) fold-increased in flavonols compared to control; (ii) fold-increased and fold-decreased in phenols compared to control and (iii) fold-increased and fold-decreased in anthocyanins compared to control.

In flavonols (Fig. 1(A)), significant differences between both treatments (N 21.4 and ME) were seen in red and in black fruit. With both treatments and in both fruit stages there was an increase in flavonol content compared with controls, the greatest increase occurring in black fruit of plants inoculated with the

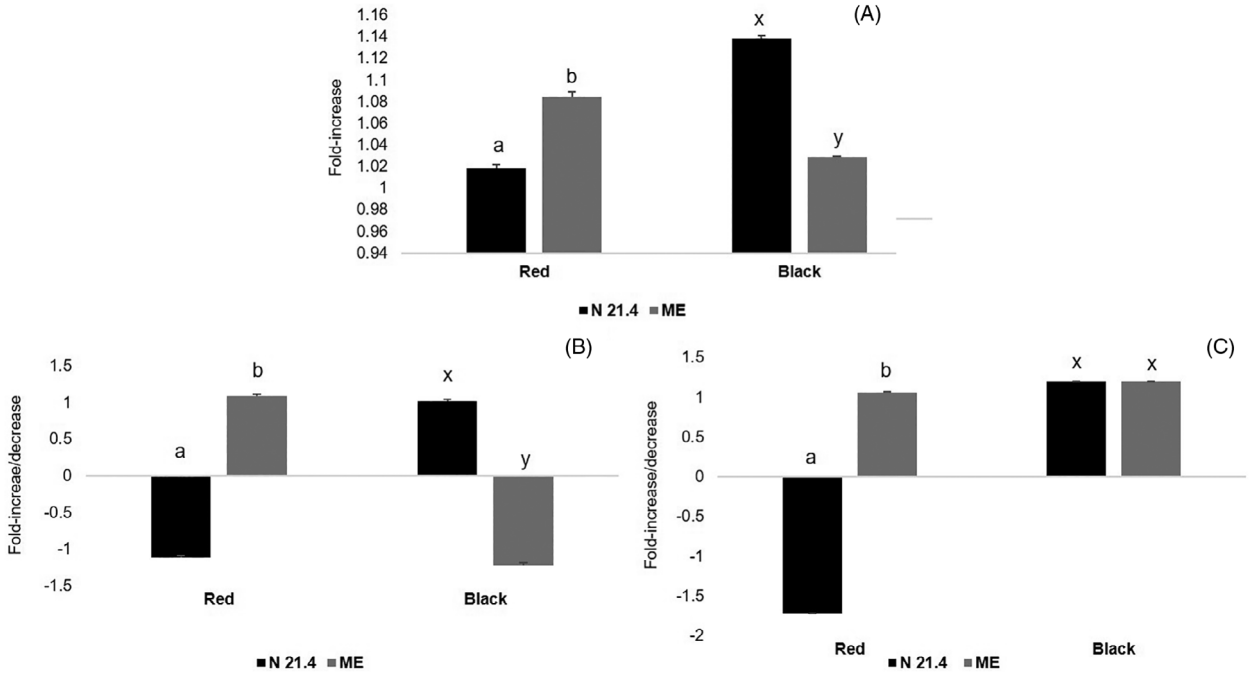

Figure 1 Fold-increase and fold decreased in the content of bioactives, measured by colorimetry, with respect to control: (A) flavonols; (B) phenols and (C) anthocyanins in red and black fruit of blackberry plants treated with *P. fluorescens* N 21.4 and with its metabolic elicitors (ME). Different letters indicate significant differences ($P < 0.05$): a and b indicate differences between treatments in red fruit; and x and y between treatments in black fruit.

strain N 21.4 (1.13 fold) and in red fruit of plants inoculated with ME (1.08 fold).

In phenols (Fig. 1(B)), significant differences between both treatments (N 21.4 and ME) were seen in red and in black fruit. A slight increase was recorded in red fruit of plants inoculated with ME and in black fruit of plants inoculated with N 21.4. However, a decrease in the amount of phenols was seen in red fruit of plants inoculated with N 21.4, and in black fruit of plants inoculated with ME.

In anthocyanins (Fig. 1(C)), significant differences between both treatments (N 21.4 and ME) were only seen in red fruit. A deep decreased in the quantity of anthocyanins was seen in the red fruit of plants inoculated with N 21.4. However, a 1.2 fold-increased was seen in the black fruit of plants inoculated with both treatments.

Bioactives analysis by UHPLC/ESI-qTOF-MS

Methanolic extracts were analyzed by liquid chromatography with electrospray ionization, coupled to quadrupole-time-of-flight mass spectrometry (LC/ESI-qTOF-MS). All the compounds were identified by comparing the retention times and spectra with reference standards. The main compounds found (11) and their concentration ($\mu g\ g^{-1}$) are shown in Table 2. They are grouped as flavonols (5), flavanols (2), dihydrochalcone (1), anthocyanine (1), hydroxycinnamic acid (1), and flavone (1). Red and black fruit of control and inoculated plants (N 21.4 and ME) were compared.

In red fruit, all compounds had significant higher values (between 1.2 and 1.6 times higher) in ME-treated plants than in N 21.4-treated plants. Values of N 21.4-treated-plants were similar to those of control plants, except for (−)-epicatechin and (+)-catechin values, which were 1.2 times higher in treated plants (this being significant), and for cyaniding-3-*O*-glucoside that were significantly lower in treated plants.

In black fruit, the opposite trend was observed: all compounds (except cyaniding 3-*O*-G) had significantly higher values in N 21.4-treated plants (between 1.1 and 1.5 times higher) than in ME-treated plants. The values for ME-treated-plants were similar to those of control plants, except for (−)-epicatechin and (+)-catechin values, which were 1.1 times significantly higher in treated plants, and for quercetin aglycone and phloridzin, which were significantly lower in treated plants.

The variation in the content of (+)-catechin, (−)-epicatechin and cyaniding 3-*O*-G, the phenolic compounds most affected by our treatments, appears in Fig. 2, in a chromatogram.

RT-qPCR analysis

The differential expression of flavonoids pathway genes in the fruit of treated plants appears in Fig. 3, showing red fruit (3 A) and black fruits (3 B).

In the red fruit, (Fig. 3(A)) of plants treated with N 21.4, *RuF3′H*, *RuFLS*, *RuDFR*, *RuANS* and *RuANR* genes appeared upregulated. *RuCHS*, *RuLAR* and *RuMYB5* were not significantly affected by this treatment. In the case of ME-treated-plants, *RuCHS* and *RuDFR* genes appeared upregulated and *RuF3′H*, *RuFLS*, *RuANS*, *RuLAR* and *RuANR* were downregulated. Differential expression of all genes, except the one for the transcriptional factor *RuMYB5*, had significant differences ($P<0.05$) between treatments (N 21.4 versus ME).

In the black fruit (Fig. 3(B)) of plants treated with N 21.4, *RuFLS*, *RuANS*, and *RuANR* genes were upregulated. The rest of genes were not significantly affected by this treatment. In ME-treated plants, *RuF3′H*, *RuFLS*, *RuANS*, and *RuANR* genes were upregulated and *RuLAR* was downregulated. Differential expression of all genes, except the one of the transcriptional factors, *RuMYB5*, gave significant differences ($P<0.05$) between treatments.

Table 2 Main flavonoid and phenolic compounds found in blackberry fruit and their concentration in µg g-1. Control, N 21.4 and ME treatments were compared in red and black fruit

Polyphenol class	Compound	Control		N 21.4		ME	
		Red	Black	Red	Black	Red	Black
Flavonols	Quercetin aglycone	<LOQ	0.621 ± 0 (α)	<LOQ	0.172 ± 0.08 (β)	0.235 ± 0.11 a	0.185 ± 0.01 b (β)
	Quercetin 3-O-glucoside	13.8 ± 0.3 a (x)	13.2 ± 0.1 a ($\alpha\beta$)	13.1 ± 0.55 a (x)	14.1 ± 0.3a (α)	17.7 ± 0.3 a (y)	12.9 ± 0.4 b (β)
	Quercetin 3-O-rutinoside	7.5 ± 0.25 a (x)	6.2 ± 0.1 b (α)	7.4 ± 0.35 a (x)	7.6 ± 0.15 a (β)	9.7 ± 0.25 a (y)	6.4 ± 0.15 b (α)
	Kaempferol 3-O-rutinoside	0.661 ± 0 a (x)	0.44 ± 0.01 b (α)	0.631 ± 0.01 a (x)	0.501 ± 0 b (β)	0.814 ± 0.03 a (y)	0.426 ± 0.2 b (α)
	Kaempferol 3-O-glucoside	1.1 ± 0.05 a (x)	0.8 ± 0.05 b (α)	0.9 ± 0 a (y)	0.8 ± 0.05 b (α)	1.3 ± 0 a (z)	0.7 ± 0.05 b (α)
Flavanols	(−)-Epicatechin	302.4 ± 8.3 a (x)	226.7 ± 3.4 b (α)	356 ± 0.45 a (y)	304.4 ± 0.45 b (β)	386.8 ± 8.05 a (z)	247.8 ± 1.8 b (γ)
	(+)-Catechin	20.8 ± 0 a (x)	9.5 ± 0.35 b (α)	25.4 ± 0.65 a (y)	17.2 ± 0.55 b (β)	33.7 ± 0.75 a (z)	11.1 ± 0.25 b (γ)
Dihydrochalcone	Phloridzin	0.263 ± 0.01 a (x)	0.442 ± 0.02 b (α)	0.216 ± 0 a (y)	0.397 ± 0 b (β)	0.329 ± 0 a (z)	0.392 ± 0 b (β)
Anthocyanine	Cyanidin 3-O-glucoside	838.3 ± 21.16 a (x)	3162.26 ± 42.808 b (α)	560.74 ± 18.18 a (y)	2968.72 ± 46.28 b (β)	727.23 ± 17.99 a (x)	3117.82 ± 38.40 b (α)
Hydroxycinnamic acid	Chlorogenic acid	0.603 ± 0.04 a (x)	0.333 ± 0.01 a (α)	0.597 ± 0.01 a (x)	0.256 ± 0.21 b (α)	0.779 ± 0.05 a (x)	0.335 ± 0.03 b (α)
Flavone	Luteolin	0.05 ± 0.01 a (x)	0.029 ± 0 a (α)	0 (y)	0 (α)	0.091 ± 0.02 a (x)	0 b (α)

The letters a and b indicate significant differences between red and black fruit within the same treatment; x, y and z indicate significant differences between treatments in red fruit; and letters α, β, and γ indicate significant differences between treatments in black fruit. < LOQ means sample under the limit of quantification.

DISCUSSION

Food security, as well as the improvement and protection of crops in a more ecofriendly environment, is nowadays a topic of great importance. This calls for more efficient and non-polluting agricultural methods and one of the most challenging tools to achieve this goal is the use of biological agents,[30] such as beneficial rhizobacteria or their derived elicitors. In the present work, the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors have been used as plant inoculants in commercial cultivars of blackberry (*Rubus* cv. Loch Ness) to elicit flavonoid metabolism in the fruit.

Blackberries are strongly rich in flavonoids, which have essential functions in plant defense against biotic (fungus, bacteria, herbivores)[4,31,32] and abiotic stress (light, temperature, water supply, minerals, CO_2, etc.)[33] and they also have many benefits for human health[34,35] when they are included in the diet. For this reason, plant flavonoid metabolism has been studied in depth with the aim of modifying and obtaining better performance (higher accumulation of beneficial secondary metabolites, higher antipathogenic capacity, better plant fitness, etc.).

The biosynthesis of flavonoid compounds starts from the amino acid phenylalanine and produces phenylpropanoids, which are channeled into the flavonol-anthocyanin pathway by chalcone synthase (CHS). Further reactions involve chalcone isomerase (CHI), which generates naringenin, flavanone-3-hydroxylase (F3H), which hydroxylates naringenin until dihydrokaempferol, which is then hydroxylated by flavonoid-3-hydroxylase (F3′H) and transformed into dihydroquercetin. Flavonols are synthesized at this point by the flavonol synthase (FLS), forming kaempferol or quercetin (depending on where FLS introduces a double bond). Dihydroquercetin is then reduced by dihydroflavonol reductase (DFR) to obtain leucocyanidin. Anthocyanins are synthetized at this point by the anthocyanidin synthase (ANS) obtaining cyanidin. (+)-Catechin is obtained when leucocyanidin reductase (LAR) reduces leucocyanidin, and (−)-epicatechin is obtained when anthocyanidin reductase (ANR) reduces cyanidin.[4,36]

The study of regulatory genes encoding those enzymes of the phenylpropanoid and flavonol-anthocyanin pathways is crucial for modifying the accumulation of secondary metabolites of interest at the end of the route. In our work, an inverse relationship (in red and black fruit) between gene expression and accumulation of secondary metabolites has been seen, except for the *RuDFR* gene, the first gene of the anthocyanins route, which had a direct effect in the increase of cyanidin 3-O-glucoside (Fig. 3 and Table 2). However, in the study by Chen *et al.*,[37] some genes involved in anthocyanin and proanthocyanidin biosynthesis were investigated and the expression levels of genes agreed with the final products accumulated. Furthermore, they saw that enzymes encoded by the structural genes of the pathway had two peaks of maximum activity: at the beginning of the fruit ripening and at the end. The same pattern of enzyme activity was observed in strawberry by Halbwirth *et al.*,[38] except for DFR and FLS enzymes, which only had one peak of activity at the red-black stage (also seen by Almeida *et al.*).[39] This last was consistent with our results in which *RuDFR* gene was upregulated in red stages of the fruit, but downregulated in black, suggesting that dihydroflavonol reductase (DFR) only had one peak of activity at this stage of ripening (in red fruits).

Regarding the effects of our treatments in red blackberry fruit, ME treatment had effects in all the studied genes, downregulating *RuF3′H*, *RuFLS*, *RuANS*, *RuLAR* and *RuANR* and upregulating *RuDFR*.

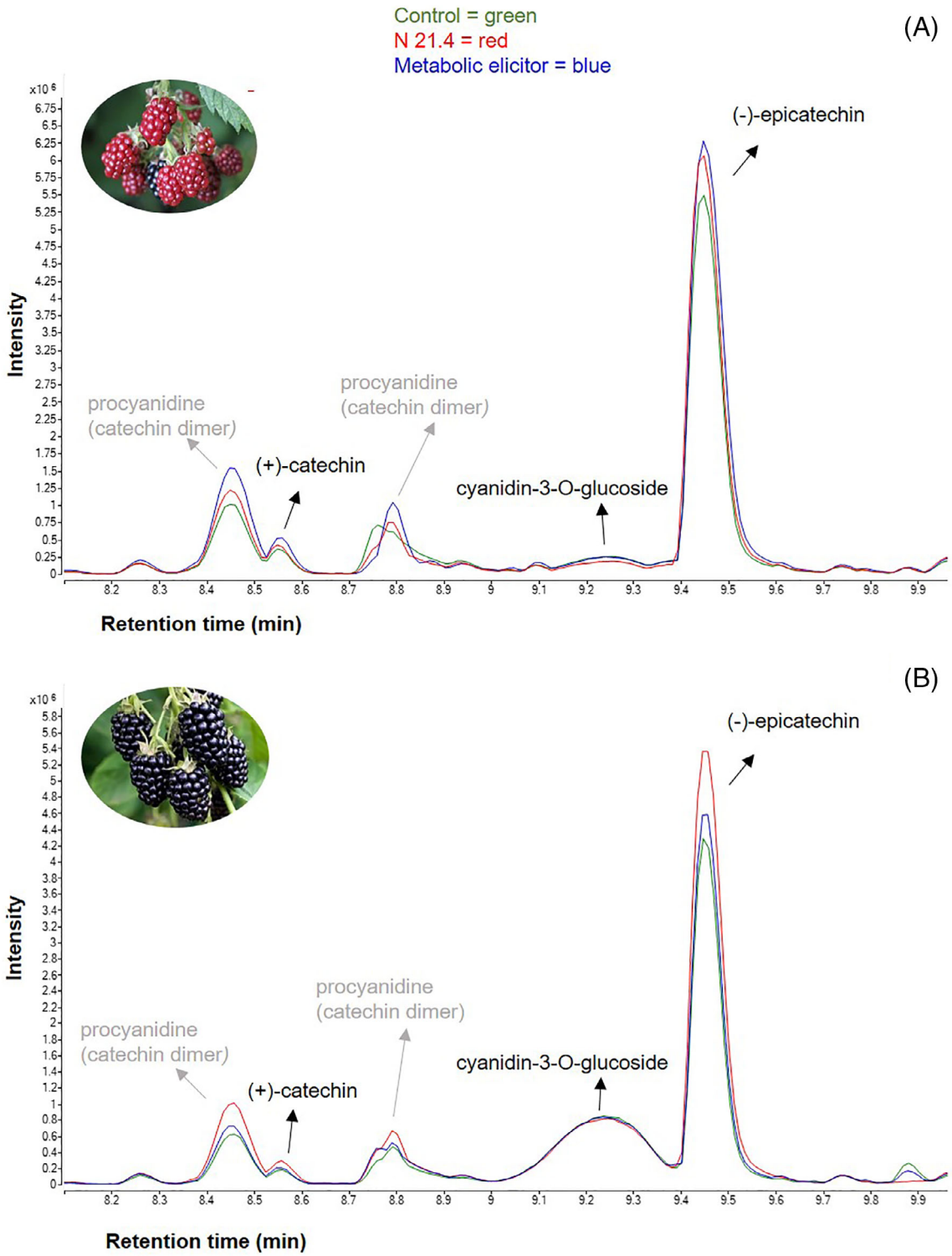

Figure 2 Enlarged chromatogram (from 8.0 to 9.9 min) visually comparing the content variation of three main phenolic compounds in red (A) and black (B) fruit and between treatments. Green line represents control, red line *P. fluorescens* N 21.4 and blue line ME treatments.

This was directly linked to an increase in all the compounds derived from the activity of the enzymes encoded by the above downregulated cited genes. The plants treated with ME had red fruit with a higher concentration of flavonoids, highlighting quercetin and kaempferol derivatives, catechin, epicatechin and anthocyanins. However, red fruits of N 21.4-treated-plants showed higher differential expression and lower concentration of flavonoid compounds, which reinforces our idea of a supposed inverse relationship between gene expression and final compound accumulation. A hypothesis that could explain this phenomenon is that, since there were more transcripts of these genes, all the biosynthetic machinery of phenolic compounds

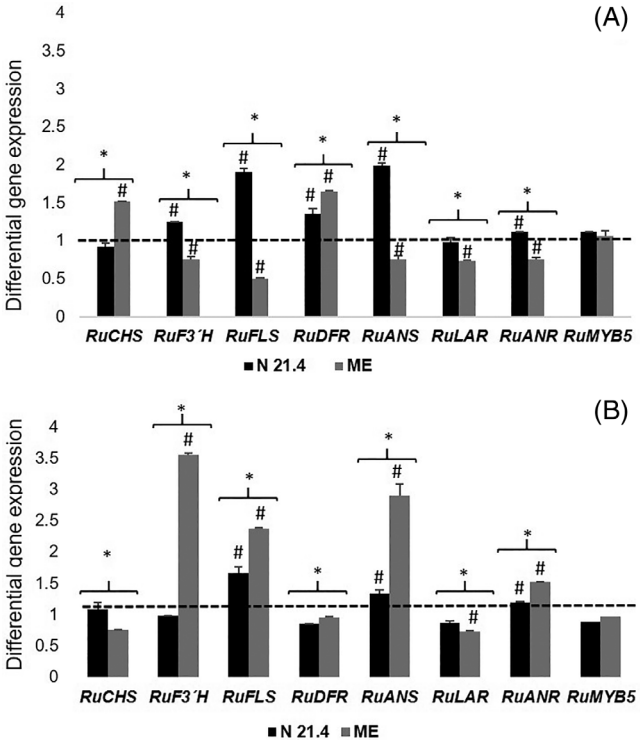

Figure 3 Differential expression of phenylpropanoids pathway genes in red fruit (A) and in black fruit (B) of blackberry plants inoculated with *P. fluorescens* N 21.4 and with its metabolic elicitors (ME). Asterisks represent statistically significant differences ($P < 0.05$) between treatments and hashtags represent statistically significant differences ($P < 0.05$) with the control. Expression of 1 is that of the control (horizontal black dashed line).

worked faster and bioactives were accumulated in later stages of the route as derivatives of the compounds that we had measured. Probably, if compounds had been measured in subsequent steps of the route, an accumulation of them would have been found (https://www.genome.jp/kegg-bin/show_pathway?map00944; https://www.genome.jp/kegg-bin/show_pathway?map00942).

In black fruit, the same inverse relationship was observed, but in this case it was the plants treated with N 21.4 that showed less differential expression and greater accumulation of all the secondary compounds, except anthocyanins, which accumulated more in fruits of ME-treated plants. This is also consistent with a greater expression of *RuDFR* observed with the ME treatment. *RuDFR* is the only gene in which a direct relationship between differential expression and anthocyanin accumulation was observed in red and in black fruit. *RuDFR* had the same behavior as that of *DFR* in the work by Almeida *et al.*[39] and Garcia-Seco *et al.*[12]

At the same time, *RuCHS* seem to be a fundamental regulatory gene related to the accumulation of secondary metabolites. When *RuCHS* is upregulated, compound accumulation occurs along the pathway. This had been previously seen in the work of Garcia-Seco *et al.*[12]

MYB transcription factors are among the most important regulators involving flavonoid biosynthesis.[12,40] In the present work, the gene expression of *Ru*MYB5 was analyzed, since in previous works and with other beneficial rhizobacteria from our collection, *Ru*MYB5 was found to be a positive regulator of the RuDFR, RuANR and RuLAR genes.[41] In that work, the positive regulation supposed an increase in catechin synthesis and their accumulation in the fruits. However, in our study, *RuMYB5* did not exceed the control differential expression. In the work of Thole *et al.*,[42] it is said that *RuMYB5* from cultivated blackberry has a peak of expression at the early intermediate ripening fruit stage, which could be related to a higher concentration of catechin and epicatechin in green-red fruit stages. In that study it is also said that Ru*MYB5* interacts with other transcription factors (*RuTTG1* and *RubHLH1*) related to proanthocyanidins synthesis, showing decreasing transcript levels during ripening. Despite not having seen differential gene expression of *RuMYB5* in our work, the tendency discussed in Thole *et al.*[42] work has been seen, with a higher concentration of catechin and epicatechin in red fruit and a lower concentration in black fruit. However, in strawberry, *FaMYB5* transcripts have been seen to accumulate steadily during fruit development. Moreover, Chang *et al.*[43] saw that not *RuMYB5*, but *RuMYB10* was the transcription factor that better controlled proanthocyanidin biosynthesis. Hence, it can be concluded that these transcription factors might be affected differently by diverse bioeffectors or by different crop conditions.

On the other hand, it has been seen that in early maturation stages (red fruit), (−)-epicatechin and (+)-catechin were the predominant phenolic compounds and their concentration decreased during ripening, when anthocyanins increased. This was consistent with the gene expression of *RuANR*, *RuLAR*, and *RuANS*, respectively. Chang *et al.*[43] and Garcia-Seco *et al.*[12] observed the same in blackberry and Almeida *et al.*[39] in strawberry. Hence, there is an obvious redirection of flavonoid biosynthesis from flavanol to anthocyanin formation during the complex developmental process of fruit ripening.[38,43] The different groups of polyphenols formed during fruit ripening fulfill different important functions, like herbivore deterrence with the presence of astringent flavanols in early stages[12] or visual attraction of ripen fruits. Anthocyanin, epicatechin, and catechin have been seen as key factors affecting fruit flavor and nutrition properties in persimmon, grape, berries, and many other fruits.[44-46] However, in grapes, catechin and epicatechin accumulation occurs immediately after fruit-set and maximum levels of accumulation are reached around véraison.[47] Consequently, these species might be under control of different regulatory mechanisms.

In our *Rubus* cv. Loch Ness plants, treatments inoculated (rhizobacterium N 21.4 and ME) affected not only the total phenolic and flavonoids during ripening but also the profile of different flavonols, flavanols, and anthocyanins (also seen by Garcia Seco *et al.*[12] with N 21.4). In general, the fruits of inoculated plants, and especially the fruits of ME-inoculated plants, accumulated secondary metabolites in greater concentrations than non-inoculated control plants. A remarkable fact was a major increase of flavanols ((−)-epicatechin and (+)-catechin) at both stages of fruit ripening with both treatments. These compounds have strong antioxidant capacity and they are able to reduce oxidative stress in plants.[48,49] They have also important cardiovascular benefits for humans.[50,51] Epicatechin also acts as phytoanticipins in some fruits[52] giving fungal and bacterial resistance to infection.

The elicitation of the secondary metabolism of *P. fluorescens* N 21.4-treated plants was again demonstrated and the elicitation of secondary metabolism of ME-treated plants was also evidenced. This secondary metabolism activation not only suggested an improvement in the nutritional quality of the fruit by increasing their metabolite content but it also suggested that plants trigger their defenses as a result of the activation of this secondary metabolism.[53] In general, ME had more marked effects on plant elicitation, enhancing the amount of many of the

metabolites accumulated in fruits. Hence, it is understood that ME-treated plants had a more active secondary defensive metabolism and therefore they were more protected against biotic and abiotic stress; they had better fitness. The fruit production in ME-inoculated plants was slightly lower, which is a clear symptom of the ME's elicitation capacity, which improved the quality of fruit slightly compromising fruit yield. Thus, ME-treated plants were more primed, were more fit, and had slightly fewer fruits, but fruits with better nutritional quality. Previous results from our group, with the same plant and with another rhizobacterium,[54] verified that elicitation supposes a redirection of resources towards defensive metabolism, slightly compromising fruit yield. They also support the view that metabolic changes inherent in the primed status[55] resulted in the allocation of carbon sources to the defense metabolism.[56]

In summary, the effectiveness of ME as plant inoculants for the elicitation of blackberry secondary metabolism was shown, as well as their capacity to modify the flavonoid biosynthetic pathway. The ME of *P. fluorescens* N 21.4 were able to modulate gene expression in the fruit of *Rubus* cv. Loch Ness, and to affect the profiles of secondary metabolites, increasing the synthesis and accumulation of them mainly during the red stage and potentially increasing nutritional properties of subsequent black fruit. The ME used as plant inoculants also have advantages related to the management and maintenance of inoculums. They are effective, cheap to produce, easy to manage, environmentally friendly, and they do not cause the biosecurity problems that live rhizobacteria could cause.[57] The application of ME of beneficial rhizobacteria as plants inoculants therefore opens a feasible new window towards the improvement of the nutritional qualities of crops using innovative and more ecofriendly agro-food techniques.

From all the above, we conclude that the efficacy of ME of *P. fluorescens* N 21.4 in the elicitation of blackberry secondary metabolism has been demonstrated. Metabolic elicitors are efficient, profitable and ecological plant inoculants that could be alternatives to agrochemicals, or could be either alternatives or complementary to rhizobacteria-based products. We can also conclude that, through the study of the phenylpropanoid pathway in blackberry fruit, the regulatory role of *RuCHS* in the accumulation of secondary metabolites at the final stages of the pathway has been shown, as well as the role of *RuDFR* in the increase of synthesis and accumulation of cynidine-3-*O*-glucoside.

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Identifying the Compounds of the Metabolic Elicitors of *Pseudomonas fluorescens* N 21.4 Responsible for Their Ability to Induce Plant Resistance

Helena Martin-Rivilla [1,*], F. Javier Gutierrez-Mañero [1], Ana Gradillas [2], Miguel O. P. Navarro [3], Galdino Andrade [3] and José A. Lucas [1]

1 Plant Physiology Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668 Madrid, Spain; jgutierrez.fcex@ceu.es (F.J.G.-M.); alucgar@ceu.es (J.A.L.)

2 Centre for Metabolomics and Bioanalyses, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, 28668 Madrid, Spain; gradini@ceu.es

3 Laboratory of Microbial Ecology, Department of Microbiology, Londrina State University, Londrina 86051-990, Brazil; micromiguel@gmail.com (M.O.P.N.); andradeg@uel.br (G.A.)

* Correspondence: hel.martin.ce@ceindo.ceu.es; Tel.: +34-913-72-47-85

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Abstract: In this work, the metabolic elicitors extracted from the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 were sequentially fragmented by vacuum liquid chromatography to isolate, purify and identify the compounds responsible for the extraordinary capacities of this strain to induce systemic resistance and to elicit secondary defensive metabolism in diverse plant species. To check if the fractions sequentially obtained were able to increase the synthesis of isoflavones and if, therefore, they still maintained the eliciting capacity of the live strain, rapid and controlled experiments were done with soybean seeds. The optimal action concentration of the fractions was established and all of them elicited isoflavone secondary metabolism—the fractions that had been extracted with n-hexane being more effective. The purest fraction was the one with the highest eliciting capacity and was also tested in *Arabidopsis thaliana* seedlings to induce systemic resistance against the pathogen *Pseudomonas syringae* pv. tomato DC 3000. This fraction was then analyzed by UHPLC/ESI–QTOF–MS, and an alkaloid, two amino lipids, three arylalkylamines and a terpenoid were tentatively identified. These identified compounds could be part of commercial plant inoculants of biological and sustainable origin to be applied in crops, due to their potential to enhance the plant immune response and since many of them have putative antibiotic and/or antifungal potential.

Keywords: *Pseudomonas fluorescens* N 21.4; metabolic elicitors; isoflavone elicitation; induced systemic resistance; sphingolipids; terpenoids

1. Introduction

Protecting crops against diseases caused by pathogens in agricultural systems has always been a constant challenge when trying to maximize crop yields, minimize economic losses and ensure quality food worldwide [1]. As 2020 has been declared International Year of Plant Health by the FAO (Food and Agriculture Organization of the United Nations), the current challenge is to find effective, ecofriendly and at the same time, low-cost agriculture control methods that guarantee the sustainability of crop production while eliminating negative impact on the environment.

It has been widely demonstrated that biological agents, such as beneficial microorganisms, are able to enhance plants' immune systems, inducing systemic resistance (ISR) and/or systemic acquired resistance (SAR) [2–4]. This phenomenon of enhancing a plant's immune system is called

elicitation, and it supposes that cells exposed to external factors activate defense mechanisms by triggering and regulating some biochemical and molecular responses, increasing the synthesis of specific molecules with a protective role [5]. After elicitor perception, signals are transported throughout the plant, triggering local and systemic responses [6] and leading to the generation of reactive oxygen species (ROS), phytoalexin biosynthesis, increased synthesis of antioxidant secondary metabolites, reinforcement of plant cell wall, deposition of callose, synthesis of defense enzymes, accumulation of pathogenesis-related proteins [7], etc.

Originally, the term elicitor referred to molecules capable of inducing the production of phytoalexins, but nowadays it is commonly used for compounds that stimulate any defensive line [8–10]. Natural elicitor molecules derived from microorganisms can induce similar defense responses in plants to those responses induced by the alive microorganisms [11]. Different types of natural elicitor molecules have been characterized, including structural molecules, such as carbohydrate polymers, lipids, and bacterial flagellin [12], and metabolic elicitors, released to the medium, such as antibiotics and secondary metabolites [13,14].

Currently, a wide range of metabolic elicitors produced by fungi and beneficial rhizobacteria have been found to induce in plants the synthesis of protective compounds—such as phytoalexins, defensins, phenolic acids, and flavonoids—that directly suppress pathogens [15]. They can reduce plant diseases through elicitation of physical and chemical processes linked to systemic plant defense mechanisms [16]. This is why plant inoculants made of beneficial rhizobacteria and/or their metabolic elicitors have been seen as feasible and effective alternatives to chemical phytosanitaries to counteract the attack of pathogens and also to face to diverse biotic and abiotic stresses [17,18]. However, using elicitor molecules instead of living bacteria is a way to reduce the cost and to simplify the production and subsequent management of plant inoculants. Products made of partially purified or purified compounds derived from bacterial metabolism [19] are cheap to produce; easy to manage; respectful with the environment; not harmful or toxic to human health or to other organisms, as their targets are directly the plants; and do not cause biosecurity problems as bacteria could cause [20]. Furthermore, they can be easily applied by spraying [21] and they do not require special preservation conditions like live microorganisms because they are stable during long periods of exposure to light and/or high temperatures [22] and they do not lose viability during prolonged storage.

Among beneficial microorganisms, the genus *Pseudomonas* spp. and *Bacillus* spp. are the most studied genera concerning everything related to plant–pathogen–beneficial microorganism interactions and the improvement of plant immune system, because they are very abundant in the soil and because of their role in the suppression of pathogens [23]. Literature has shown that *Pseudomonas* spp. have extensive metabolic capabilities and adaptable biochemistry through their production of structurally varied bioactive molecules [24]. Furthermore, it is largely known that *Pseudomonas* spp. are some of the most important microorganisms able to produce compounds with antibiotic or eliciting activity triggering SAR [25] or ISR [3].

The potential of *Pseudomonas* spp. to suppress plant pathogens has been demonstrated in many plant species and around the world [26]. They can be used as efficient and not-risky biocontrol agents to use in agriculture because they do not show pathogenic, allergenic or harmful risks to people or animals [27]. Secondary metabolites isolated from *Pseudomonas* spp. that could be an alternative to the use of chemical compounds in the control of plant disease include phenazines, pyrrolnitrin-type antibiotics, betalactones, pyo compounds, indol derivatives, peptides, glycolipids, lipids, aromatic organic compounds and aliphatic compounds, among others [24,28].

More specifically and within *Pseudomonas* spp. genus, the specie *Pseudomonas fluorescens* is a Gram-negative soil bacterium that has been widely studied in relation to its capacity to suppress other pathogenic microorganisms by producing siderophores, antibiotics and antifungal and antiparasitic compounds and to induce systemic resistance in plants through a vast variety of secondary metabolites with eliciting capacity [28–31]. For all of this, certain strains have already been developed as commercial products for management of plant illnesses in agricultural settings [22].

The *P. fluorescens* strain N 21.4 was specifically used in the present work because its capacity to induce systemic resistance in different plant species, such as *Arabidopsis thaliana* [18,32], *Solanum lycopersicum* [33], *Hypericum* sp. [34], *Papaver* sp. [35] and blackberry (*Rubus* cv. Loch Ness) has been largely demonstrated [36–39]. Its metabolic elicitors have also been demonstrated to induce systemic resistance in *A. thaliana* [18] and to elicit flavonoid metabolism in the leaves and in the fruits of cultivars of blackberry [38,39].

For all the above, the objective of the present study was to isolate, purify, test and identify the compound or set of elicitor compounds of *P. fluorescens* N 21.4, obtained from sequential fractionations of its metabolic elicitors by vacuum liquid chromatography (VLC), responsible for plant elicitation. To corroborate fractions' capacities to elicit secondary metabolism, some experiments under controlled conditions were performed in soybean seeds to enhance isoflavone synthesis and in *A. thaliana* to induce systemic resistance. A final analysis to obtain the profile of main compounds present in the purest fraction of the metabolic elicitors was made by ultra-high performance liquid chromatography (UHPL), with an electrospray ionization source (ESI) and a quadrupole time-of-flight mass spectrometry analyzer (QTOF-MS). Eight compounds were tentatively identified and classified into different families: alkaloids, amino lipids, terpenoids and arylalkylamines.

2. Material and Methods

Sequential extraction, fragmentation and purification of the metabolic elicitors of *P. fluorescens* N 21.4 were carried out (Figure 1 and Table 1). Firstly, a liquid–liquid phase separation was made [40], followed by two sequential VLCs. All the sequentially fragmented fractions were inoculated in soybean seeds to analyze their capacity to elicit isoflavone secondary metabolism pathway. Final isolation and purification were performed. The resulting fraction of the entire process of fragmentation and purification was tested in an ISR experiment in *A. thaliana* seedlings. Finally, this fraction, the purest one, was analyzed by UHPLC/ESI–QTOF–MS to characterize its composition.

2.1. Bacterial Strain

The bacterial strain used in this study was *P. fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a Gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca* Graham [33]. The bacterial strain was stored at −80 °C in nutrient broth (CONDA) with 20% glycerol.

2.2. Metabolic Elicitor Extraction and Control Obtaining

The bacterial strain, stored at −80 °C in nutrient broth with 20% glycerol, was streaked onto nutrient agar (peptone 3 g·L^{-1}, beef extract 5 g·L^{-1} and agar 15 g·L^{-1} pH 7) plates and cultivated for 24 h at 28 °C. After 24 h of growth, bacterial cells were scraped off the plates into 10 L of sterile nutrient broth (peptone 3 g·L^{-1} and beef extract 5 g·L^{-1} pH 7) and incubated on a rotatory shaker at 28 °C and 180 rpm for 24 h.

Metabolic elicitors (released into the medium) were obtained by centrifuging the 10 L of N 21.4 culture at 2890× *g* during 20 min at 4 °C. Cells were discarded and the remaining supernatant was evaporated in a stove at 60 °C until obtaining 1 L. This concentrated supernatant was filtrated through a 0.2 µm nitrocellulose filter and extracted twice with a double volume of hexane (*v*/*v*). The extract was evaporated to dryness in a Buchi R-215 rotary evaporator at 50 °C [40]. The dry extract was weight (250 mg) and stored at 4 °C protected from light and humidity.

To obtain the control 1, the same procedure was followed as for extracting the metabolic elicitors from the bacterium, but while carrying out the entire process exclusively with the nutrient medium (peptone 3 g·L^{-1} and beef extract 5 g·L^{-1} pH 7), in the absence of bacterium.

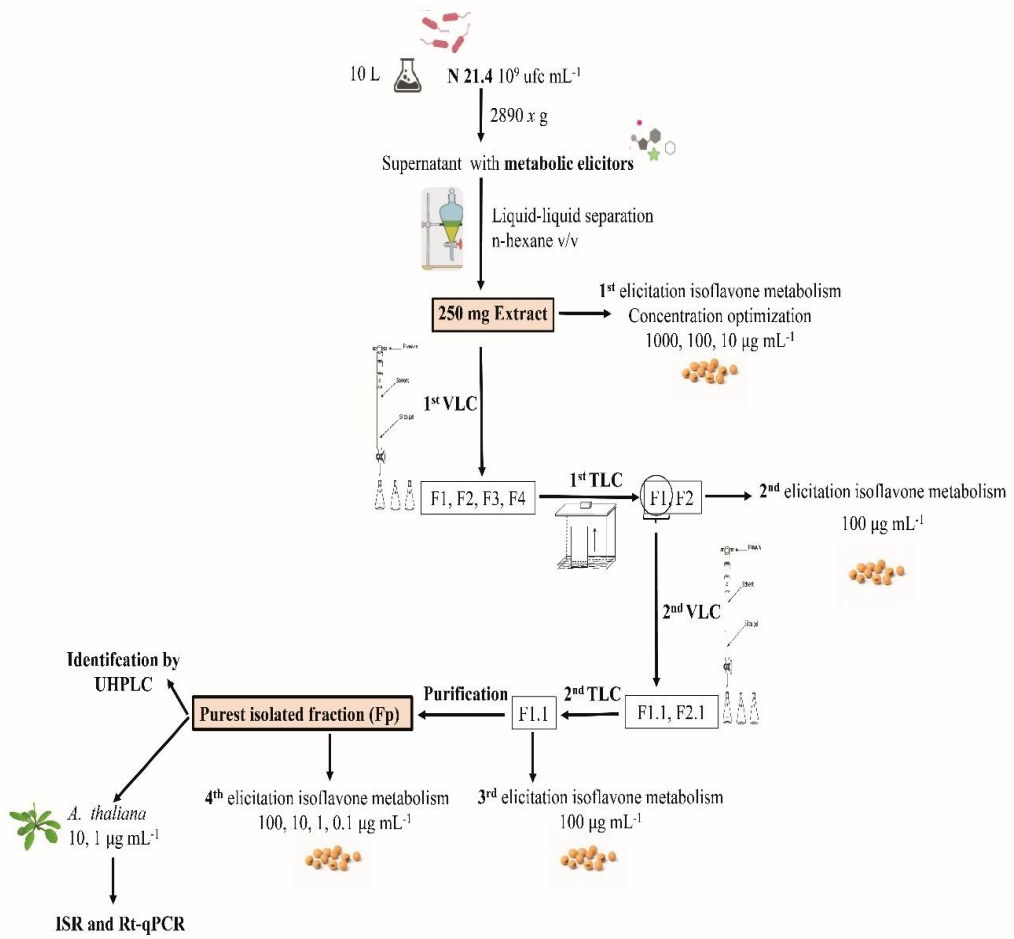

Figure 1. Representative scheme of the sequential extraction, fractionation and purification of the metabolic elicitors of *P. fluorescens* N 21.4. Shown are the growth of the bacterium in nutrient broth, the liquid–liquid phase separation with *n*-hexane, the process of fractionation and purification by two sequential vacuum liquid cromatographies, the four elicitation experiments of isoflavone metabolism, the ISR experiment in *A. thaliana* and the final compound identification by UHPLC.

Table 1. Experimental design and treatments used.

Method Used	Treatment	Characteristics	Use
Growth in nutrient broth	*P. fluorescens* N 21.4	10 L 10^9 ufc.mL^{-1}	Liquid-Liquid separation ISR as positive control
Liquid-Liquid separation (bacterial culture)	Dry extract	250 mg Aliquots dissolved in 10% DMSO	1st Isoflavone elicitation: Concentration optimization (1000,100,10 µg·mL^{-1}) 1st VLC
Liquid-Liquid separation (nutrient broth)	Control 1	Dissolved in 10% DMSO	1st,2nd,3rd and 4th isoflavone elicitation 1st and 2nd TLCs ISR as 0% of protection
Seed embryo cut	Control 2	Not inoculated soybean seeds	1st Isoflavone elicitation
1st VLC with dry extract	F1 F2 F3 F4	Hexane Dichloromethane Ethyl acetate Methanol	1st TLC 1st TLC 1st TLC 1st TLC
1st TLC with F1, F2, F3 and F4	F1 F2	100 µg·mL^{-1} 100 µg·mL^{-1}	2nd Isoflavone elicitation
2nd VLC with F1	F1.1 F1.2	Hexane Dichloromethane	2nd TLC 2nd TLC
2nd TLC with F1.1 and F1.2	F1.1	100 µg·mL^{-1}	3rd Isoflavone elicitation
Purification of F1.1	Fp (Purest fraction)	1, 0.1 µg·mL^{-1}	4th Isoflavone elicitation
ISR in *A. thaliana*	Fp	10, 1 µg·mL^{-1}	Pathogen protection (%) qPCR (SA and JA/ET marker genes)
UHPLC/ESI–QTOF–MS	Fp	0.19 mg in 100 mL methanol LC-MS grade	Tentative compound identification

2.3. Elicitation of Isoflavone Metabolism in the Soybean

To test the capacity of the sequentially obtained fractions to elicit isoflavone secondary metabolism, rapid induction tests were carried out in soybean seeds (BS-2606 Embrapa). Seeds were superficially disinfected with a 70% ethanol bath for 1 min, 5% sodium hypochlorite for 6 min and 5 washes with sterile distilled water. After that, seeds were kept imbibing in sterile distilled water for 4 h, in darkness and at room temperature. After imbibition, 90 seeds per treatment and 90 seeds for each control (control 1 and 2), were distributed in 3 replicates of 30 seeds each and put to germinate in sterile Petri dishes with 1% European bacteriological agar. A small longitudinal cut was made in the seeds embryo without compromising their viability.

For the first isoflavone elicitation experiment, three dilutions of concentration 1000, 100 and 10 $\mu g \cdot mL^{-1}$ diluted in 10% DMSO were prepared from the separated aliquot of the dry extract obtained by liquid–liquid separation, and 10 µL of each dilution was inoculated into the cut of the seeds embryo. For the second isoflavone elicitation experiment, 10 µL of fractions F1 and F2 at 100 $\mu g \cdot mL^{-1}$, obtained from the first VLC, was inoculated. For the third isoflavone elicitation experiment, 10 µL of F1.1 at 100 $\mu g \cdot mL^{-1}$, obtained from the second VLC, was inoculated. For the fourth isoflavone elicitation experiment, 10 µL of Fp at concentrations 100, 10, 1 and 0.1 $\mu g \cdot mL^{-1}$ was inoculated. This entire procedure was carried out under sterile conditions. Once the treatments were applied, the plates were kept in darkness for 3 days in a SANYO MLR 350H camera at 27 °C.

The extraction and analysis of isoflavones was performed according to Wang et al. [41] and Lozovaya et al. [42] with some modifications. Seeds were powdered with liquid nitrogen, mixed with 100 mL of 80% HPLC-methods methanol and maintained on an orbital shaker at 145 rpm for 15 h at 40 °C. Samples were then centrifuged at 2890× *g* for 20 min at 20 °C. The obtained supernatant was filtered through a 0.2 µm nitrocellulose membrane, and the methanolic extract was used for analysis by HPLC.

The identification and quantification of isoflavone was carried out on a chromatograph Agilent Technologies 1260 Infinity HPLC system. Chromatography conditions were: UV detection: 262 nm, ZORBAZ 300SB-C18 column (4.6 µm × 250 mm × 5 µm), Gecko 2000 30, 80 °C thermostat that kept the column at 30 °C. The mobile phase consisted of water with 0.1% acetic acid (solvent A), and acetonitrile with 0.1% acetic acid (solvent B), with the following gradient: from 15% to 45% of B during 40 min, rising until 100% B during 1 min and remaining this composition for 9 min, after which it dropped to initial conditions (15% B) for 1 min and held for 9 min to equilibrate the column. The flow was 1.5 $mL \cdot min^{-1}$ and the sample injection volume was 10 µL.

The quantification of the isoflavones ($\mu g \cdot mL^{-1}$) was performed by interpolation of the relative area given by the detector on a calibration curve built for each isoflavone ($R^2 > 0.99$). The calibration curves were built with the isoflavones (LC Laboratories): daidzin, genistin and malonyl genistin.

2.4. Vacuum Liquid Chromatography (VLC)

The two VLCs performed were carried out in a glass column (20 mm diameter × 350 mm H) filled with 30 g of silica gel 60 (0.063–0.200 mm, Merck) coupled to a vacuum pump with 51 kPa. The starting extract for fractionation was crushed and mixed with silica gel 60 until obtaining a fine powder, which was placed on the top of the silica column and fractionated passing through the column the following mobile phases (from lower to higher polarity): hexane, dichloromethane, ethyl acetate and methanol, for the first VLC, and hexane and dichloromethane for the second VLC. Each organic solvents (400 mL) was passed through the column and concentrated in a rotary evaporator under vacuum at 50 °C.

2.5. Thin Layer Chromatography (TLC)

Two thin layer chromatographies, using TLC plates of silica gel on aluminum support 60 F254 (Merk), were performed to qualitatively assess the components present in the control 1 and in

the fractions obtained from the first VLC (F1, F3, F3 and F4) and in the fractions obtained from the second VLC (F1.1 and F1.2).

The mobile phase used was a mixture of chloroform/dichloromethane/ethyl acetate/methanol (*v*/*v*/*v*/*v*). Ultraviolet light (254 nm and 366 nm) was used for revealing the TLC plates.

2.6. Purification of F1.1

F1.1 fraction, the most fractionated one, was dissolved in 1 mL of chloroform and put on a TLC plate, which was imbibed in a dichloromethane mobile phase. The band that appeared at the top of the plate (common to those bands that had appeared in the first and second TLCs) was removed by scraping the silica gel from the TLC aluminum plate. Scraped silica was mixed with 1 mL of 80% methanol for HPLC methods and centrifuged for 10 min at 6500× *g* at room temperature. Precipitated silica was discarded and supernatant (containing the elicitor pure compound/s) was evaporated. The isolated and pure fraction (Fp) (0.39 mg) was tested in soybean seeds and stored protected from light and humidity to later perform another experiment for checking its capacity to induce systemic resistance in *A. thaliana*.

2.7. ISR Experiment

An aliquot of the purest fraction (Fp) was dissolved in DMSO 10% at concentrations 10 and 1 $\mu g \cdot mL^{-1}$ and used for an ISR experiment in *A. thaliana*. The ISR experiment was carried out as follows:

A. thaliana wild type Columbia ecotype 0 seeds (provided by the Nottingham Arabidopsis Stock Centre (NASC)) were germinated in quartz sand and two-week-old seedlings were then individually transplanted to 100 mL pots filled with peat/sand mixture (12/5) (60 g per pot). Forty-eight plants per treatment were used; plants were arranged in three replicates, with sixteen repetitions each. Plants were watered with 5 mL of tap water once a week and with 5 mL of half-strength Hoagland solution per plant once a week. Plants were inoculated by soil drench with 50 µL of each treatment: Fp diluted in 10% DMSO at a concentration of 10 $\mu g \cdot mL^{-1}$ and at 1 $\mu g \cdot mL^{-1}$, in the first and second weeks after transplant. Control plants were-mock inoculated by soil drench with 50 µL of control 1. Another positive control was added, in which thirty-six plants (three replicates of 12 plants each) were inoculated by soil drench, in the first and second weeks after transplant, with 1 mL of a 10^9 ucf.mL^{-1} *P. fluorescens* N 21.4 suspension. Four days after the second inoculation, plants were pathogen challenged with the pathogen *Pseudomonas syringae* pv. tomato DC3000. One day before pathogen challenge, plants were maintained with 99% relative humidity to ensure stomata opening in order to allow disease progress. *P. syringae* pv. tomato DC3000 grown for 24 h was centrifuged (10 min at 2890× *g*) and cells were suspended in 10 mM $MgSO_4$ to achieve 10^8 cfu.mL^{-1}. Inoculation was done by spraying the entirety of each plant with 250 mL. Plants were incubated in a culture chamber (Sanyo MLR-350H) with an 8 h light (350 µE $s^{-1} \cdot m^{-2}$ at 24 °C) and 16 h dark period (20 °C) at 70% relative humidity. All the leaves of twelve plants (four per replicate) of each treatment and of control 1 were harvested at 6, 12 and 24 h after pathogen challenge (hapc), powdered in liquid nitrogen and stored at −80 °C. These plant samples were used for gene expression analysis by qPCR. The 36 remaining plants (12 per replicate) of each treatment and of control 1, and the 36 plants of positive control, were used to record disease severity 72 h after pathogen inoculation as the number of leaves with disease symptoms relative to the total number of leaves. Results were relativized using the disease severity of leaves inoculated with the control 1 extract as 0% protection.

2.8. RT-qPCR Experiment

Total RNA was isolated from each replicate with PureLink RNA Micro Kit (Invitrogen, Waltham, MA, USA), DNAase treatment included. RNA purity was confirmed using NanodropTM. A retrotranscription followed by RT-qPCR was performed.

The retrotranscription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). All retrotranscriptions were carried out using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 °C, 30 min 42 °C, 5 min 85 °C, and hold at 4 °C. Amplification was carried out with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 °C and then 39 cycles consisting of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, followed by melting curve to check results. To describe the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method. *Sand* gene (AT2G28390) was used as reference gen [43]. Gene primers used are shown in Table 2.

Table 2. Forward and reverse primers used in qPCR analysis.

	Forward Primer	Reverse Primer
AtNPR1	5′-TATTGTCAARTCTRATGTAGAT	5′-TATTGTCAARTCTRATGTAGAT
AtPR1	5′-AGTTGTTTGGAGAAAGTCAG	5′-GTTCACATAATTCCCACGA
AtICS	5′-GCAAGAATCATGTTCCTACC	5′AATTATCCTGCTGTTACGAG
AtPDF1	5′-TTGTTCTCTTTGCTGCTTTCGA	5′-TTGGCTTCTCGCACAACTTCT
AtLOX2	5′-ACTTGCTCGTCCGGTAATTGG	5′-GTACGGCCTTGCCTGTGAATG
AtPR3	5′-AAATCAACCTAGCAGGCCACT	5′-GAGGGAGAGGAACACCTTGACT
Sand	5′-CTGTCTTCTCATCTCTTGTC	5′-TCTTGCAATATGGTTCCTG

2.9. Tentative Identification and Characterization by UHPLC/ESI–QTOF–MS

2.9.1. Sample Preparation

The remaining content (0.19 mg) of the purest fraction (Fp) was dissolved in 100 µL of methanol, LC-MS grade, and vortex mixed for 3 min. The mixture was then centrifuged at 10,000× *g* for 5 min and supernatant was collected for direct analysis.

2.9.2. UHPLC-MS Analysis

Samples were analyzed on a 1290 Infinity series UHPLC system coupled through an electrospray ionization source (ESI) with Jet Stream technology to a 6545 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany). For the separation, a volume of 2 µL was injected in a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL·min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2 % B (0–6 min), 2–50 % B (6–10 min), 50–95 % B (11–18 min), 95 % B for 2 min (18–20 min), and returned to starting conditions 2 % B in one minute (20–21 min) to finally keep the re-equilibration with a total analysis time of 25 min. Detector was operated in full scan mode (*m*/*z* 50 to 2000), at a scan rate of 1 scan·s^{-1}. Accurate mass measurement was assured through an automated calibrator delivery system that continuously introduced a reference solution, containing masses of *m*/*z* 121.0509 (purine) and *m*/*z* 922.0098 (HP-921) in positive ESI mode; whereas *m*/*z* 112.9856 (TFA) and *m*/*z* 922.009798 (HP-921) in negative ESI mode. The capillary voltage was ± 4000 V for positive and negative ionization mode. The source temperature was 225 °C. The nebulizer and gas flow rate were 35 psig and 11 L·min^{-1} respectively, fragmentor voltage to 75 V and a radiofrequency voltage in the octopole (OCT RF Vpp) of 750 V.

All the solvents used were LC-MS grade. Purified water was obtained from Milli-Q Plus™ System from Millipore (Milford, MA, USA). Formic acid was purchased from Aldrich (St. Louis, MO, USA).

For the study, MassHunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies, Santa Clara, CA, USA) was used for control and acquisition of all data obtained with UHPLC/MS-QTOF.

2.9.3. Data Handling

UHPLC-MS data processing was performed by MassHunter Qualitative Analysis (Agilent Technologies, Santa Clara, CA, USA) Software version B.08.00 using "Molecular Feature Extraction (MFE)" to extract potential molecular features (MFs). The MFE algorithm creates a list of possible components that represent the full TOF mass spectral data features, which are the sum of co-eluting ions that are related by charge-state envelope, isotopic distribution and/or the presence of different adducts and dimmers. Several parameters of the algorithm were set for data extraction, applying 2000 counts as limits for the background noise. Moreover, the algorithm was applied to find co-eluting adducts for the same possible compound, selecting +H, +Na, +K, and neutral water loss as possible adducts for positive ionization and -H, +FA, +Cl in negative ionization. Additionally, the "Generate Formula" option in the MassHunter Qualitative Analysis software was used to generate the empirical formula from accurate mass and isotopic pattern distribution to increase the confidence of compound annotation, with a very good score (about 97–99%).

2.9.4. Compound Identification

The tentative identification of compounds was carried out by comparing their retention times and the accurate masses of features (± 5-ppm error) against online databases, as FOODB, MetaCyc, CEU massmediator, and scientific bibliography.

To reach the possible annotation of peak 2, it was necessary to use the MetaCyc and to consult specific bibliography [44].

The identification of compounds corresponding to peaks 3, 4, 6, 7 and 8 was supported by comparison of their accurate masses in the databases for each compound, providing an accuracy error below 5 ppm. Specific bibliography was also consulted for their annotation [45–47]. To confirm the annotation of the peaks 4, 6 and 7, a MS/MS analysis was carried out and their final identification was supported using in silico prediction approaches, such as the freely available tool CFM-ID 3.0. The experimental MS/MS spectra were searched and scored against predicted spectra based on similarity.

Regarding peak 5, it was annotated after matching against different databases, based on its monoisotopic mass and molecular formula.

2.10. Statistical Analysis

One-way ANOVA with replicates was used to check the statistical differences in all data obtained. Prior to ANOVA analysis, homoscedasticity and normality of the variance was checked with Statgraphics plus 5.1 for Windows, meeting requirements for analysis. When significant differences appeared ($p < 0.05$) a Fisher test was used [48].

3. Results

3.1. First Elicitation of Isoflavone in the Soybean: Concentration Optimization

To test the capacity of the extract obtained from the metabolic elicitors by liquid–liquid separation to induce the isoflavone secondary metabolism, this extract was inoculated in the embryo cut of the soybean seeds at concentrations of 1000, 100 and 10 $\mu g \cdot mL^{-1}$, as is explained in Section 2.3. The fractions of 1000 and 100 $\mu g \cdot mL^{-1}$ significantly elicited isoflavone production over both controls (Figure 2), and the seeds inoculated with 100 $\mu g \cdot mL^{-1}$ recorded the highest induction. However, the seeds inoculated with 10 $\mu g \cdot mL^{-1}$ showed an isoflavone synthesis very similar to that of control 1 and control 2 seeds or even less, in the case of malonyl genistin. Differences between the three concentrations were statistically significant.

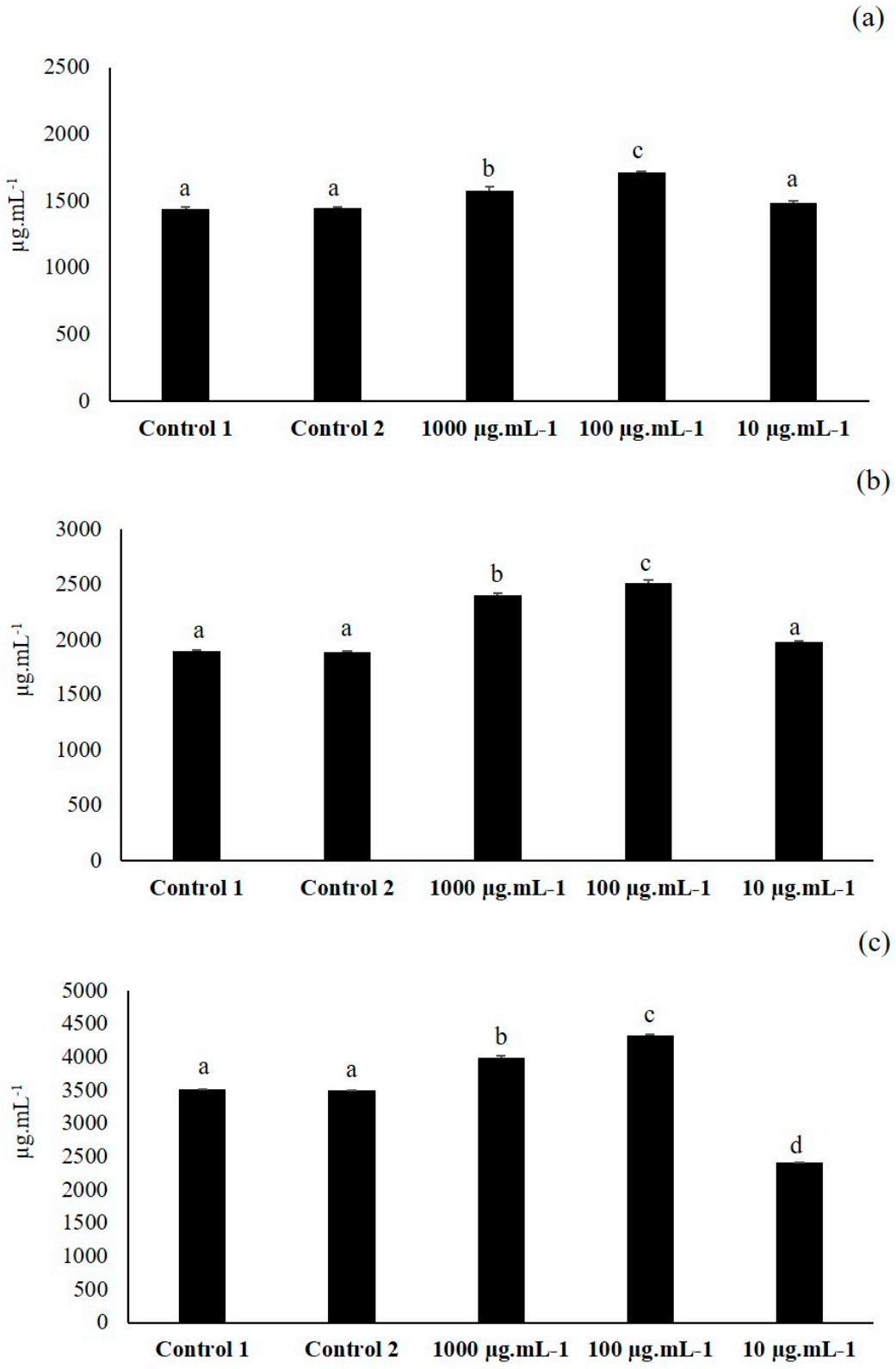

Figure 2. Daidzin (**a**), genistin (**b**) and malonyl genistin (**c**) production in the seeds inoculated with the extract obtained from the metabolic elicitors at 1000, 100 and 10 µg·mL^{-1} and in control 1 (extract obtained from the liquid–liquid phase separation from the culture broth without bacteria) and control 2 (non-inoculated soybean seeds). The amount of isoflavones is expressed in µg·mL^{-1} (n = 30 soybean seeds × 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$). Error bars correspond to standard deviation (SD).

As there were not statistical differences between both controls in the synthesis of isoflavones, it was assumed that elicitation was due to the components of the metabolic elicitors and not the components present in the nutrient broth. For that reason, the control 1 was not further purified as the metabolic elicitor extract, and for the following elicitation experiments only control 1 was used.

3.2. First TLC

To visually assess the components present in the control 1 (C) and in the four fractions (F1, F2, F3 and F4) obtained from the first VLC, a TLC was performed (Figure 3). In the TLC plate, only F1 and F2 showed separation between their components and both showed a common band at the top of the plate. Control 1 extract did not show band separation.

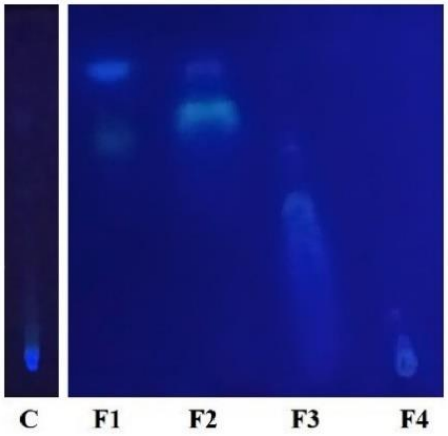

Figure 3. First TLC made with the control 1 (C) and the four fractions obtained from the fist VLC. F1 is the fraction obtained with hexane, F2 with dichloromethane, F3 with ethyl acetate and F4 with methanol.

3.3. Second Elicitation of Isoflavone Metabolism in the Soybean

According to the results obtained from the previous TLC, only F1 and F2 (of the first VLC) were tested in soybean seeds to check their ability to elicit isoflavone secondary metabolism (Figure 4), as F3 and F4 did not showed component fragmentation. The biosynthesis of the three isoflavones assessed was significantly higher in the seeds that were inoculated with F1 and F2 fractions compared to control 1 seeds. The highest elicitation values were obtained with F1.

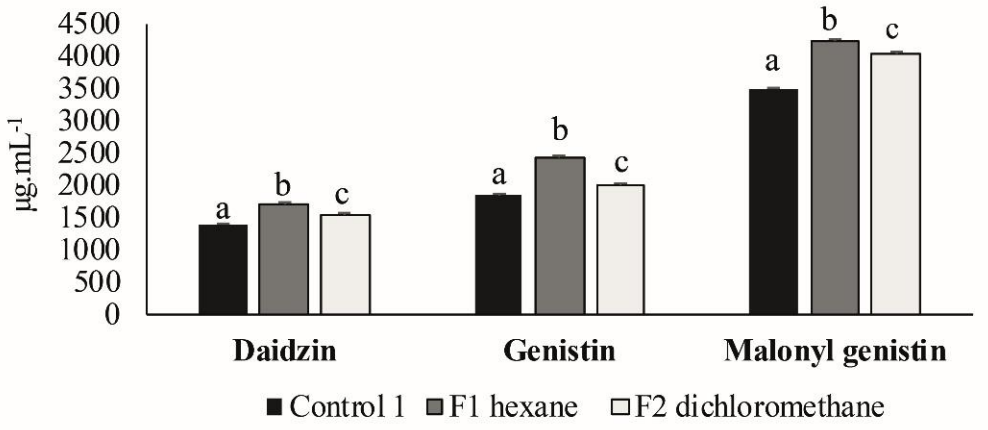

Figure 4. Daidzin, genistin and malonyl genistin production in the seeds inoculated with F1 and F2 obtained from the first VLC and in control 1 seeds. The amount of isoflavones is expressed in $\mu g \cdot mL^{-1}$ (n = 30 soybean seeds × 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$). Error bars correspond to standard deviation (SD).

When comparing this experiment with the first elicitation experiment, it was seen that the values of isoflavone elicitation obtained with the F1 and F2 fractions were similar to those obtained with the initial extract.

3.4. Second TLC

Since the highest elicitation values were obtained with F1 (Figure 4), a second VLC was performed to fragment and purify it. After the VLC, a TLC was made to visually assess the components present in the two fractions obtained (F1.1 and F1.2). F1.1 and F2.1 showed again a common band at the top of the TLC, bands of F1.1 being more intense than those of F2.1. These bands were very similar to those seen in the first TLC of F1 and F2 (Figure 5).

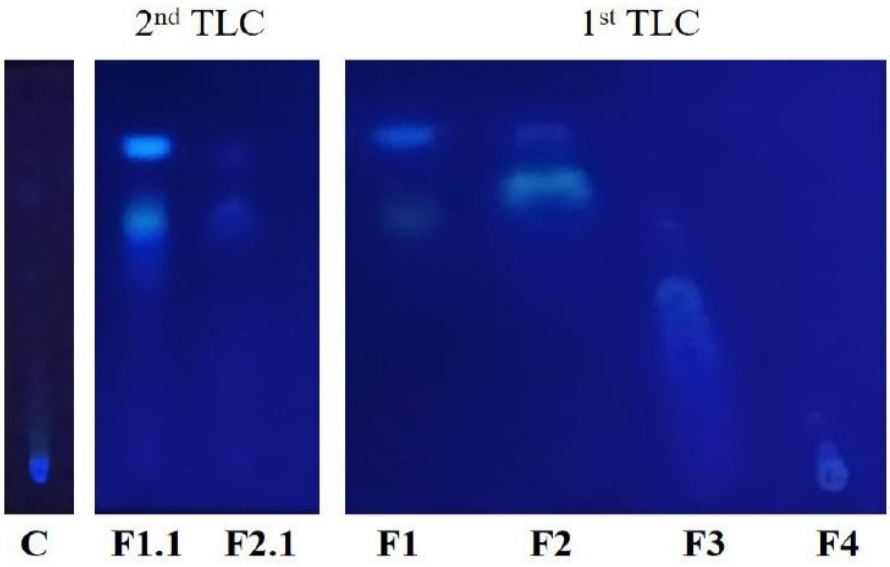

Figure 5. Comparison between the first and the second TLC. Second TLC was made with the two fractions obtained from the second VLC. F1.1 is the fraction obtained with hexane and F2.1 with dichloromethane.

3.5. Third Elicitation of Isoflavone Metabolism in the Soybean

As F1.1 fraction showed a similar but more intense band than F2.1 in the second TLC, it was chosen to check its capacity to elicit isoflavone secondary metabolism and it was inoculated in soybean seeds (Figure 6). Seeds inoculated with F1.1 had a significant higher isoflavone concentration than control 1 seeds.

When comparing this experiment with the first and the second elicitation experiments, it was seen that the values of isoflavone elicitation obtained with the F1.1 fraction were 1.2 times greater than those obtained with the F1 and F2 fractions and with the initial extract.

3.6. Fourth Elicitation of Isoflavone Metabolism in the Soybean

As F1.1—the fraction obtained from the second VLC—elicited the isoflavone metabolism, it was purified and the resultant fraction (Fp) was tested at concentrations of 100, 10, 1 and 0.1 $\mu g \cdot mL^{-1}$ in soybean seeds to check its capacity to elicit secondary metabolism of isoflavone (Figure 7).

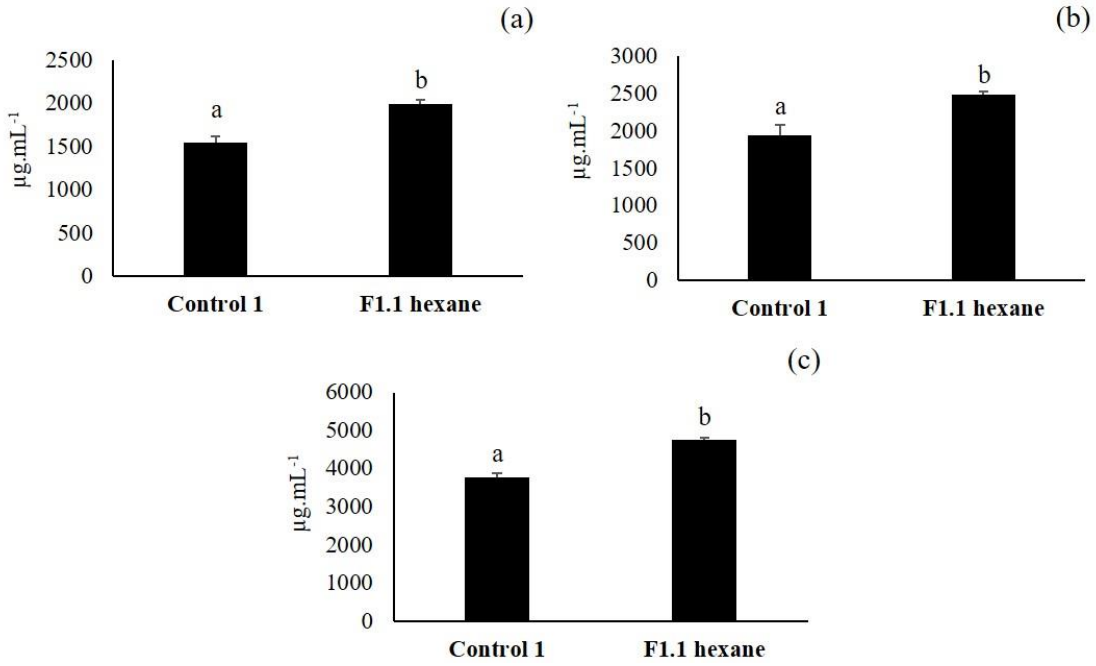

Figure 6. Daidzin (**a**), genistin (**b**) and malonyl genistin (**c**) production in the seeds inoculated with F1.1 obtained from the second VLC and in control 1 seeds. The amount of isoflavones is expressed in µg·mL^{-1} (n = 30 soybean seeds × 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$). Error bars correspond to standard deviation (SD).

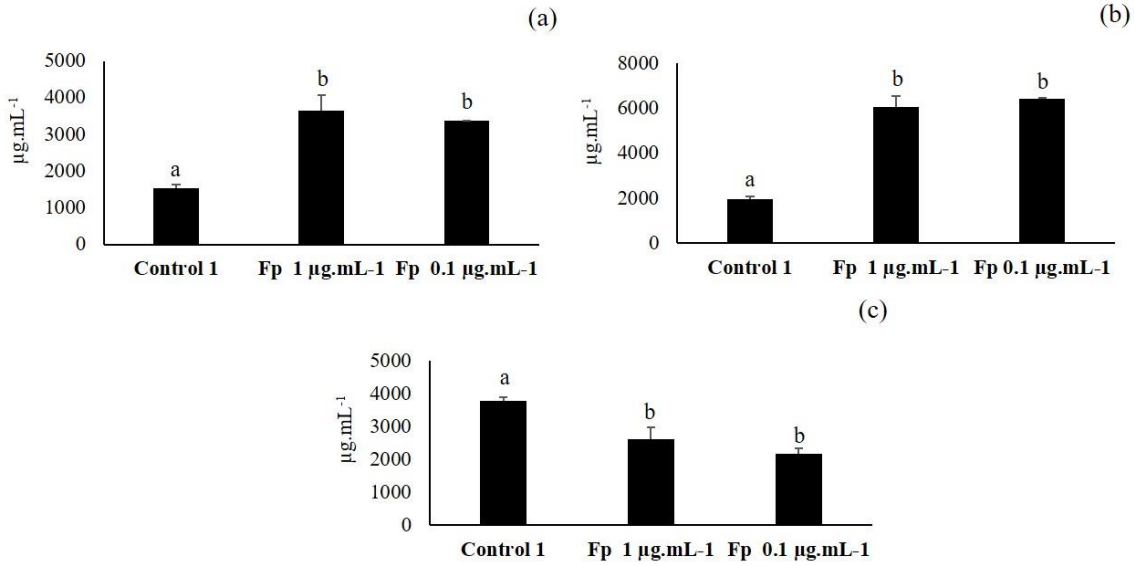

Figure 7. Daidzin (**a**), genistin (**b**) and malonyl genistin (**c**) production in the seeds inoculated with the purest fraction (Fp) obtained from F1.1 and in control 1 seeds. The amount of isoflavones is expressed in µg·mL^{-1} (n = 30 soybean seeds × 3 replicates). Different letters show significant statistical differences between treatments in each isoflavone ($p < 0.05$). Error bars correspond to standard deviation (SD).

Seeds inoculated with 100 and 10 µg·mL^{-1} were not able to germinate; they went black (data not shown) and they were not analyzed. However, in the seeds inoculated with 1 and 0.1 µg·mL^{-1}, the synthesis of daidzin and especially of genistin significantly increased with respect to control 1. The synthesis of malonyl genistin decreased compared to control 1. No significant differences were seen between both concentrations in any of the three isoflavones.

When comparing the elicitation results obtained in this last experiment with the previous elicitation experiments (first, second and third), it was seen that the increases in the concentrations of daidzine

and genistin triggered by Fp fraction (compared to the control 1) were more than double those produced by the F1.1, F1 and F2 fractions and the initial extract.

3.7. ISR Experiment

As the purest fraction (Fp) elicited the isoflavone metabolism in soybean seeds, it was then inoculated in *A. thaliana* seedlings at concentrations of 10 and 1 µg·mL^{-1} to carry out an ISR experiment with the objective of checking its ability to protect plants against *P. syringae* pv. tomato DC 3000 infection. Results of protection against infection are shown in Figure 8.

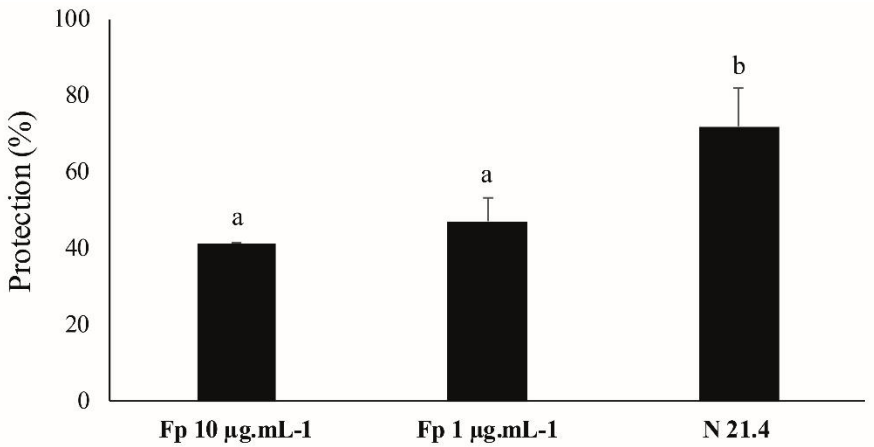

Figure 8. Protection (%) against *Pseudomonas syringae* pv. tomato DC3000 in *Arabidopsis thaliana* seedlings elicited with the purest fraction (Fp) at 10 and 1 µg·mL^{-1} and with the live strain *P. fluorescens* N 21.4. The percentage was calculated based on the number of leaves with disease symptoms to the total of leaves (n = 12 seedlings per replicate). Data were relativized to control 1, which was considered as 0% protection. Different letters show significant statistical differences between treatments ($p < 0.05$). Error bars correspond to standard deviation (SD).

Protection rates against infection were between 40% and 50% for the Fp fraction and 70% for the live strain (used as positive control). No significant differences between the two concentrations of the Fp fraction were observed.

3.8. RT-qPCR Experiment

After the ISR experiment in *A. thaliana*, the differential expressions of marker genes of the salicylic acid (SA) and jasmonic/ethylene (JA/ET) signal transduction pathways were analyzed by qPCR (Table 3). The studied marker genes of the SA signaling pathway were *PR1*, *NPR1* and *ISC*, and those of the JA/ET pathway were *PDF1*, *PR3* and *LOX 2*. All these genes were measured at 6, 12 and 24 hapc.

Table 3. Differential gene expression (*A. thaliana* seedlings inoculated with the purest fraction at a concentration of 10 and 1 µg·mL^{-1} vs. control 1) at 6 (n = 12), 12 (n = 12) and 24 (n = 12) hapc.

		10 µg·mL^{-1}			**1 µg·mL^{-1}**		
		6 hapc	**12 hapc**	**24 hapc**	**6 hapc**	**12 hapc**	**24 hapc**
SA	*PR1*	1.2 ± 0.02 *	5.35 ± 0.06 *	0	0.83 ± 0	0	0
	NPR1	2.44 ± 0.21 *	1.1 ± 0.05	0.73 ± 0	2.65 ± 0.13 *	1.2 ± 0.03 *	0
	ICS	1.66 ± 0.12 *	0.66 ± 0.01 *	0	2.02 ± 0.03 *	1.47 ± 0.03 *	0
JA/ET	*PDF1*	1.23 ± 0.01 *	0	0	2.36 ± 0.02 *	0	0
	LOX2	1.79 ± 0.06 *	0.73 ± 0.03	0	0.9 ± 0.01	0	0
	PR3	1.7 ± 0 *	0.72 ± 0	0	3.4 ± 0.06 *	1.31 ± 0.1 *	0

Asterisks represent statistically significant differences ($p < 0.05$) with respect to control 1 (differential expression of 1) within each sampling time.

The differential expression of marker genes of the SA signaling pathway at both concentrations (10 and 1 μg·mL^{-1}) decreased from 6 to 12 hapc, except for *PR1*, with 10 μg·mL^{-1}, which increased. With 1 μg·mL^{-1}, *PR1* did not show significant differential expression, while *NPR1* and *ICS* showed slightly higher expression with 1 μg·mL^{-1} than with 10 μg·mL^{-1} at both sampling moments. None of the genes showed differential expression at 24 hapc.

Marker genes of the JA/ET signaling pathways, at both concentrations, only showed significant differential expression at 6 hapc, except *PR3*, which also showed significant differential expression at 12 hapc with 1 μg·mL^{-1}. The expressions of *PDF1* and *PR3* were higher with the concentration of 1 μg·mL^{-1}. None of the genes showed differential expression at 24 hapc.

3.9. Characterization by UHPLC/ESI–QTOF–MS

The purest fraction (Fp) obtained from the metabolic elicitors of *P. fluorescens* N 21.4 was analyzed by UHPLC/ESI–QTOF–MS, as described in Material and Methods, leading to the characterization of eight peaks (corresponding to eight compounds). Figure 9 shows the extracted ion chromatograms (EICs) provided by the analysis of the extract in the positive ionization mode, which has proved to be more efficient and sensitive than in negative mode for compound characterization, and located within the chromatographic retention interval 14–17.5 min. The tentatively identified compounds, classified by families, and the main parameters that support their annotation, are listed in Table 4.

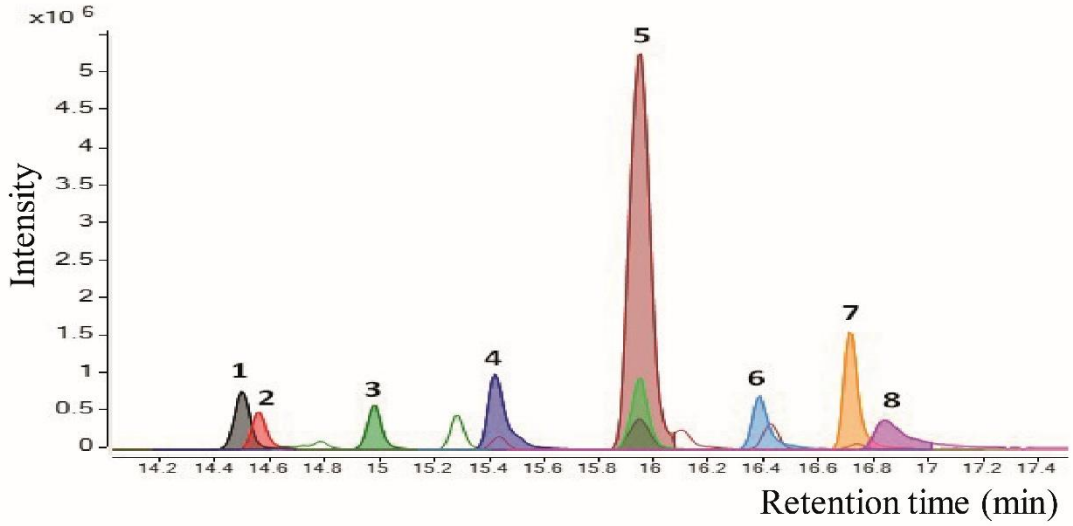

Figure 9. Overlay of the extracted ion chromatograms (EICs) for compounds present in the extract of the purest fraction. The enlargements of a part of the chromatogram are shown from 14.0 to 17.5 min.

In the research proposed, eight compounds (Figure 9) were detected in the extract of the purest fraction—among them, one alkaloid (peak 2), two amino lipids (peaks 3 and 8), a terpenoid (peak 5) and three aryl alkylamines (peaks 4, 6 and 7).

According to its monoisotopic mass molecular formula and by consulting a specific database and bibliography [44], the annotation Ambiguine P (a cycloheptane-containing member of the hapalindole alkaloid) could be possible for the compound corresponding to peak 2.

The family that encompasses amino derivatives was remarkable in the extract due to the number of identified compounds (compounds 3, 8, 4, 6 and 7 of the Table 4). The most important compounds in this family were those related to long chain dialkyl, monalkyl and aryl alkylamines, saturated and unsaturated. In this study, peak 3, was annotated as Sphinganine C17 and peak **8** was annotated as *N*-Methyl-*N*-stearylamine (1-nonadecanamine). The peaks 4, 6 and 7, were annotated as arylalkylamines and their identification was supported by their MS/MS spectra (see Figure S1 of Supplementary Material).

Regarding peak 5, (see Figure S2 of Supplementary Material for experimental MS/MS spectra), and considering different databases based on its monoisotopic mass and molecular formula, two terpenoid annotations could be considered to be related: the sesquiterpenoid 4-*O*-methylmelleolide (alkyl resorcinol ester derivative) [49], and the diterpenoid 2-Acetoxy-3-deacetoxycaesaldekarin E (neocaesalpin AH) [50].

Table 4. Retention times, mass spectral data and characterizations of the detected compounds in the UHPLC/ESI–QTOF–MS analysis in positive and negative ionization modes.

n°	Tentative Annotation [a]	Rt (min)	Molecular Formula	Monoisotopic Mass	*m/z* Experimental	Fragments (MS2)
	Unknowns					
1	Unknown	14.5	$C_{17}H_{14}N_2S$	278.0878	$[M + Na]^+$ = 301.0762	-
	Alkaloids					
2	-	14.6	$C_{25}H_{29}NO$	359.2249	$[M + H]^+$ = 360.2333	-
	Amino lipids					
3	Sphinganine C17	14.9	$C_{17}H_{37}NO_2$	287.2824	$[M + H]^+$ = 288.2999 $[M + Na]^+$ = 310.	-
8	1-Nonadecanamine	16.8	$C_{19}H_{41}N$	283.3239	$[M + H]^+$ = 284.3320	-
	Terpenoids					
5	-	15.9	$C_{24}H_{30}O_6$	414.2042	$[M + Na]^+$ = 437.1946 $[M + H]^+$ = 415.2121 $[M + K]^+$ = 453.1680 $[2M + Na]^+$ = 851.3989 $[M + FA-H]^{-}$ = 459.2029	303.1214, 73.0661 338.4838, 325.1891, 277.1818, 141.4137, 104.2879, 90.6591
	Arylalkylamines					
4	*N*-benzyl-1-tetradecanamine	15.4	$C_{21}H_{37}N$	303.2926	$[M + H]^+$ = 304.3003	212.2379, 91.0544, 65.0383, 58.0652
6	*N*-benzyl-1-hexadecanamine	16.4	$C_{23}H_{41}N$	331.3239	$[M + H]^+$ = 332.3320	240.2682, 91.0544, 69.0694, 58.0652
7	*N*-benzyl-1-octadecanamine	17.3	$C_{25}H_{46}N$	325.3709	$[M + H]^+$ = 360.3622	268.2993, 91.0544, 85.0652, 58.0652

[a] Compounds identified compared with data reported in literature and online databases.

4. Discussion

The pressure on the demand for agriculture practices that improve yield and quality food, linked to increasing concerns about sustainability, has led to the emerge of studies about new substances that substitute environmentally harmful or health risky phytosanitaries for more ecofriendly and effective plant inoculants able to enhance plants' immune systems and plants' resistance to pests and abiotic stress. This current development of biotechnological alternatives in the agronomic field has been concentrated on the use of more efficient tools with low biological and environmental repercussions [23]. One of these reliable and non-polluting tools is the use of inductors or elicitors from beneficial microorganisms [13]. Therefore, the present work was focused on isolating, purifying and identifying the compound or set of elicitor compounds extracted from the metabolism of the beneficial rhizobacterium *P. fluorescens* N 21.4, which were able to elicit isoflavone secondary metabolism in soybean seeds and to induce systemic resistance in *A. thaliana*.

Our results have shown that metabolic elicitor fractions extracted from the strain *P. fluorescens* N 21.4 were able to enhance the synthesis of isoflavones in soybean seeds between 1.2 to 3.2 times more than controls, demonstrating its potential to elicit secondary defense metabolism (Figures 4, 6 and 7). This capacity of elicitation was increased while the purification and concentration of the fractions (from F.1 at 100 $\mu g \cdot mL^{-1}$ to Fp at 1–0.1 $\mu g \cdot mL^{-1}$) progressed [51].

The technique provided in the present work to verify the elicitation of secondary metabolism in the plant was an effective, simple and very fast technique, since it allowed checking the eliciting

capacity of the fractions inoculated in soybean seeds in less than 90 h. This fast and effective system would allow one to carry out rapid screenings to search for new eliciting compounds in future research.

On the other hand, the results obtained in the ISR experiment in *A. thaliana* against the pathogen *P. syringae* pv. tomato DC 3000 supported the statement that the metabolic elicitors of the strain N 21.4 have great potential to increase plant resistance, since protection rates between 40 to 50% were seen (Figure 8). The ISR experiment also revealed a simultaneous activation of both SA and JA/ET signaling pathways, since high levels of *PR1*, *NPR1* and *ICS* (SA marker genes), and *PDF1*, *LOX 2* and *PR3* (JA/ET marker genes) were seen. Hence, it has been again demonstrated that these two pathways are not necessarily antagonistic, as previously indicated by some authors [52,53]. The importance of high concentrations of SA and JA to trigger defensive responses mediated by both hormones is nowadays widely accepted [3,54]. Furthermore, this experiment showed a very rapid ISR response, having high values of differential gene expression at 6 hapc. Despite not having seen big differences between both concentrations of inoculation (1 and 10 $\mu g \cdot mL^{-1}$), a trend of greater differential expression and higher protective rates against pathogen infection were seen when plants were inoculated with 1 $\mu g \cdot mL^{-1}$. The genes that showed the greatest differences of expression between the two concentrations were *ICS* and *PR3* genes, which were doubly expressed in the plants inoculated with 1 $\mu g \cdot mL^{-1}$. From a commercial point of view, having a noticeable effect at such a low concentration is very interesting when the intention is making effective, but at the same time affordable plant inoculants.

After the exhaustive analysis by UHPLC/ESI–QTOF–MS to identify and characterize the compounds responsible for the elicitation, eight peaks corresponding to eight different compounds were detected (Figure 9). The groups of compounds found corresponded with an alkaloid, two amino lipids, a terpenoid and three arylalkylamines. An unknown compound was also detected, but it was impossible to elucidate its identity since it was not found described in the literature nor in databases.

A tentative annotation was found for compound corresponding to peak 2, the alkaloid nature one, after consulting the MetaCyc database and specific bibliography [45]. This compound could be described as Ambiguine P, a cycloheptane-containing member of the hapalindole alkaloid. Hapalindole-type natural products are structurally diverse terpenoid indole alkaloids that, to date, have only been described as secondary metabolites produced by cyanobacteria. They have a wide range of biological activities, including insecticidal [55], antibacterial and antifungal [56–58]. Literature does mention other alkaloid compounds produced by *P. fluorescens*, such as pyrrolnitrin or hydropyridine-type compounds that also show antibiotic and antifungal activity [28,59]. However, hapalindole-type natural products have never been described in *P. fluorescens*; hence, this result would require further investigation to be more conclusive.

Compounds corresponding to peaks 3 and 8 were seen to be amino lipid compounds. Sphinganine C17 (peak 3) is a type of sphingolipid that the literature describes with a signaling function in plants subjected to biotic and abiotic stress [45–47]. In bacteria, sphingolipids are specific membrane lipids each with a monounsaturated long-chain, the sphingosine, and their biological role has not been fully understood yet. Few bacteria have been described able to synthesize sphingolipids and some of these are cytophaga-flavobacterium-bacteroidetes group bacteria, α-proteobacteria and δ-proteobacteria (*Bdellovibrio bacterivorous*, *Cystobacter fuscus*, *Myxococcus stipitatus*, *Sorangium cellulosum* and *Myxococcus xanthus* [47]. However, it has been seen that lipopolysaccharide of Gram-negative bacteria shows structural and functional resemblance to sphingolipids of the above-cited bacteria and even to sphingolipids produced by eukaryotes. That is why in the work of Heaver et al. [60] it was proposed that as sphingolipid produced by bacteria are very similar to those produced by their eukaryotic hosts, they could influence in their hosts immune responses.

The role that sphingolipids play in plant-microorganism interactions and as bioactive elicitors that initiate defensive responses in plants has begun to be studied [47]. However, Giorni et al. [61] and Dall'Asta et al. [62] have already established a possible relationship between sphingolipids produced by various maize hybrids with *Fusarium verticillioides* infection.

In contrast to eukaryotes, where sphingolipids functions have been extensively studied, very little is known about sphingolipids in bacteria and their functions in the plant–pathogen relationship. Some of the sphingolipids-producing bacteria have been found to be abundant in the phyllosphere of plants [63], which hints that sphingolipid production by bacteria may be relevant for them.

This study was the first synthesis of sphingolipids in the genus *Pseudomonas*, and its relationship with the triggering of secondary metabolism is cited. However, it is clear that more research is needed on this unknown subject.

The other amino lipid nature compound, the one corresponding to peak 8, was tentatively identified as *N*-methyl-*N*-stearylamine (1-nonadecanamine). This compound has been reported in some works for its antimicrobial potential, being more commonly found in plant species [64–66]. It has been also found as a secondary metabolite of *Brevibacterium casei* [67] and *Saccharomyces cerevisiae*, with antibiotic activity against *Proteus mirabilis* [68]. Compound 8 has also been described in other bacterial and plants extracts as a structural constituent of biological membranes, and the results were consistent with those of previous literature reports [69].

The amino derivatives corresponding to arylalkylamines (peaks 4, 6 and 7) have been generally described as dialkyl and monalkyl amines, saturated and unsaturated. Further investigations will be necessary to determine whether such alkylamines may be considered functional analogs of sphingosine and which may be primarily expressed among the pathways associated with sphingolipid metabolism.

For the last identified compound, the most abundant one (peak 5), two possible annotations within the group of terpenoids were found: the sesquiterpenoid 4-*O*-methylmelleolide (alkyl resorcinol ester derivative) [49] and the diterpenoid 2-acetoxy-3-deacetoxycaesaldekarin E (neocaesalpin AH) [50].

Terpenoids are generally considered as secondary metabolites produced by plant or fungi, but recent sequencing of the bacterial genome and bioinformatics analyses of bacterial proteins have revealed the presence of these metabolites in bacteria [70,71]. In bacteria, terpenoids can be found in the form of essential oils or aromatic constituents and some have antibiotic and antifungal activities [72,73].

Despite terpene biosynthetic pathways in bacteria being considered ubiquitous, few bacterial terpenes have been identified, and their biosynthesis is still poorly understood [74]. The antiSMASH tool lists more than 4000 bacterial terpene biosynthetic gene clusters [75], but only 127 have been identified and deposited in the MIBiG database (repository of characterized biosynthetic gene clusters) [76], so far. However, literature has revealed the production of 2,5-dialkylresorcinol compounds that exhibit antifungal and antibacterial activities in specific strains of *Pseudomonas* spp. [77,78]. These are compounds very similar to the compound proposed by our first annotation option (alkyl resorcinol ester derivative).

The induction of systemic resistance in *A. thaliana* and the elicitation of the secondary isoflavone metabolism in soybean seeds may have been due to the effect of the sesquiterpenoid compound present in the extract of the purest fraction of the metabolic elicitors of *P. fluorescens* N 21.4—it being the majority in the fraction—or due to a synergy between all the compounds that have been identified. Nevertheless, it is clear that deeper research will be necessary in the future to carry out more specific studies with the identified compounds in order to better characterize them and their effects in the plants.

By comparing the fractions extracted from the metabolic elicitors with the control 1, which was extracted in the same way as the fractions, it has also been possible to affirm that the inducing and protective effects were due to the compounds present in the metabolic elicitors of the bacterium and not to any compound present in the nutrient broth. Furthermore, it has been seen that the beef extract used in the nutrient broth is a concentrate of water-soluble compounds, which remained in the aqueous phase, which was discarded in the initial liquid-liquid separation.

In our work we tested the capacity to induce systemic resistance and the capacity to elicit secondary defensive metabolism in plants, but in view of our promising results and having some compounds with putative antibiotic and/or antifungal activity, further research will be performed to test their potential as antibiotics or antifungals against common pathogens present in agricultural systems. Furthermore,

we will study the possibility of including all or some of these compounds, derivatives of the secondary metabolism of the *P. fluorescens* N 21.4, as commercial plant inoculants.

5. Conclusions

The results of the present study demonstrated that the fractions obtained by VLC from the metabolic elicitors of *P. fluorescens* N 21.4 induced systemic resistance in *A. thaliana* seedlings against the pathogen *P. syringae* pv. tomato DC3000, being able to trigger the two signaling pathways of the defensive response (SA and JA/ET) very quickly and at very low concentrations. These fractions also boosted the secondary defensive metabolism of isoflavones in soybean seeds.

Taking into account our results and those provided in previously published works, it can be concluded that the metabolic elicitors of *P. fluorescens* N 21.4 could be used to create new plant inoculants to be introduced in agricultural practices, minimizing the scope of chemical control, and thus advancing the development of ecofriendly agricultural tools. The purified and identified compounds of the metabolic elicitor fraction (mixed or individually), could result in commercial products of biological origin being applied to crops in the near future, since many of them have putative antibiotic and/or antifungal potential.

Furthermore, the elicitor screening system carried out in soybean seeds to specifically study the metabolic elicitors' effect on isoflavone metabolism, is a doubly fast and efficient system that allows one to verify the elicitation of secondary metabolism in the plant in less than 90 h, which will be very useful for future elicitor compound screenings.

In this work, in *Pseudomonas fluorescens*, we described the synthesis of certain compounds that had not been described in the literature to date, such as sphingolipids and hapalindole-type natural products.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/8/1020/s1, Figure S1. Experimental MS/MS spectra for the aryl alkylamines detected in the extract at the collision energy (30 eV) in positive; Figure S2. Experimental MS/MS spectra at the collision energy (30 eV) in positive and negative.

Author Contributions: The results are part of the doctoral thesis of H.M.-R. whose directors and supervisors were: J.A.L. and F.J.G.-M. All authors designed the experiments described in the manuscript. H.M.-R., M.O.P.N. and G.A. carried out the metabolic elicitor fractionation and the experiments done with soybean seeds; H.M.-R. and J.A.L. performed the experiment done with *A. thaliana*, the collection of samples and the subsequent analyses; H.M.-R., J.A.L. and A.G. did the HPLC analyses; H.M.-R., J.A.L. and A.G. wrote the main manuscript; and all authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization source
Hapc	Hours after pathogen challenge
ISR	Induced systemic resistance
JA/ET	Jasmonic acid/Ethylene
QTOF-MS	Quadrupole time-of-flight mass spectrometry
SA	Salicylic acid
TLC	Thin layer chromatography
UHPLC	Ultra high performance liquid chromatography
VLC	Vacuum liquid chromatography

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