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

Citation: Martin-Rivilla H, Garcia-Villaraco A, Ramos-Solano B, Gutierrez-Mañero FJ, Lucas JA. 2019. 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*. AoB PLANTS 11: plz049; doi: 10.1093/aobpla/plz049

**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 (Garcia-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 $O_2^{-}$, $H_2O_2$, and ‘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 (GiI and Tuteja 2010) mainly by provoking oxidative modification of vital biomolecules including membrane lipids, cellular amino acids, proteins and DNA (GiI 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; Garcia-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), Enterobacter (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 % DMSO 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 10^6 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 µL of a suspension of 10^6 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), frozen in liquid nitrogen and stored at −80 °C until gene expression analysis by qPCR and enzymatic activities analysis.

The genes analysed were NRP1 (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), DNase 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^−ΔΔCt 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-Limonnes et al. (2002). Oxidation of ASC was determined by the decrease in A290. 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$_2$O$_2$ 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 $A_{265}$ using an extinction coefficient of 14 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 at 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 lipoygenase, 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 lipoygenase (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 hpc: (A) Chitinase (PR3); (B) glucanase (PR2); (C) lipoxigenase; (D) cellulase and (E) polyphenol oxidase. Different letters indicate significant differences ($P < 0.05$) between treatments in each sampling time.
and 24 h apc. Superoxide dismutase activity (SOD; Fig. 4B) was promoted 6 and 24 h apc and DHAR (Fig. 4G) 12 h apc.

Oxidative status of the plant: MDA concentration
Malondialdehyde, a marker of oxidative stress was measured 12 h apc (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...
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 enzymes 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 establish 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.

**Sources of Funding**

The authors would like to thank the Ministerio de Economía y Competitividad of Spain for funding this work through the project AGL-2013-45189-R.

**Conflict of interest**

None declared.

**Literature Cited**

Akram W, Anjum T, Ali B. 2016. Pheny lacetic acid is ISR determinant produced by *Bacillus* fortis IAG5162, which involves extensive re-modulation in metabolomics of tomato to protect against *Fusarium* wilt. Frontiers in Plant Science 7:498.

Algar E, Gutierrez-Mañero FJ, Bonilla A, Lucas JA, Radzki W, Ramos-Solano B. 2012. *Pseudomonas fluorescens* N21.4 metabolites enhance secondary metabolism isoflavones in soybean (*Glycine max*) calli cultures. *Journal of Agricultural and Food Chemistry* 60:11080–11087.

Ali MB, Hahn EJ, Paek KY. 2005. Effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxigenase activity in *Phalaenopsis*. *Plant Physiology and Biochemistry* 43:213–223.

Anjum NA, Umar S, Ahmad A. 2012. Oxidative stress in plants: causes, consequences and tolerance. New Delhi, India: IK International Publishing House.

Beris D, Theologidis I, Skandalis N, Vassilakos N. 2018. *Bacillus amyloliquefaciens* strain MB1600 induces salicylic acid dependent resistance in tomato plants against tomato spotted wilt virus and potato virus Y. *Scientific Reports* 8:10320.

Berrocal-Lobo M, Molina A, Solano R. 2002. Constitutive expression of ethylene-response-factor1 in *Arabidopsis* confers resistance to several necrotrophic fungi. *The Plant Journal* 29:23–32.

Betsuyaku S, Katou S, Takebayashi Y, Sakakibara H, Nomura N, Fukuda H. 2017. Salicylic acid and jasmonic acid pathways are activated in spatially different domains around the infection site during effector-triggered immunity in *Arabidopsis thaliana*. *Plant & Cell Physiology* 59:8–16.

Caarls L, Pieterse CM, Van Wees SC. 2015. How salicylic acid takes transcriptional control over jasmonic acid signaling. *Frontiers in Plant Science* 6:170.
Choudhary DK, Prakash A, Johri BN. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian Journal of Microbiology 47:289–297.

Conrath U. 2011. Molecular aspects of defence priming. Trends in Plant Science 16:524–531.

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo MJ, Pugin A, Schaffrath U, Ton J, Wendehenne D, Zimmerli L, Mauch-Mani B. 2006. Priming: getting ready for battle. Molecular Plant-Microbe Interactions 19:1062–1071.

Ding Y, Sun T, Ao K, Peng Y, Zhang Y, Li X, Zhang Y. 2018. Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity. Cell 173:1454–1467.

Domenech J, Ramos SE, Probanza A, Lucas GA, Gutierrez MJ. 2007. Elicitation of systemic resistance and growth promotion of Arabidopsis thaliana by FPRs from Nicotiana glauca: a study of the putative induction pathway. Plant and Soil 290:43–50.

Durrant WE, Dong X. 2004. Systemic acquired resistance. Annual Review of Phytopathology 42:185–209.

García-Cristobal J, García-Villaraco A, Ramos B, Gutierrez-Mañero J, Lucas JA. 2015. Priming of pathogenesis-related-proteins and enzymes related to oxidative stress by plant growth promoting rhizobacteria on rice plants upon abiotic and biotic stress challenge. Journal of Plant Physiology 182:72–79.

García-Limones C, Hervás A, Navas-Cortés JA, Jiménez-Díaz RM, Tena M. 2002. Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (Cicer arietinum L.) and Fusarium oxysporum f. sp. ciceri. Physiological and Molecular Plant Pathology 61:325–337.

García-Seco D, Zhang Y, García-Villaraco A, Ramos B, Gutierrez-Mañero J, Lucas JA. 2018. Plant growth-promoting rhizobacteria (PGPR): perspective in agriculture under stressful conditions. Pest Management Science 74:1114–1117.

Gozzo F, Faoro F. 2013. Systemic acquired resistance (50 years after Mauch-Mani). Frontiers in Plant Science 4:1454–1467.

Huang CJ, Tsay JF, Chang SY, Yang HP, Wu WS, Chen CY. 2012. Dimethyl ascorbate peroxidase 6, plays an important role in the regulation of response to biotic and abiotic stresses in sugarcane. Frontiers in Plant Science 3:284–297.

Lee BR, Jing WJ, Lee BH, Avice JC, Ourry A, Kim TH. 2008. Kinetics of drought-induced pathogenesis-related proteins and its physiological significance in white clover leaves. Physiologia Plantarum 132:329–337.

Lee BR, Jung WJ, Lee BH, Avice JC, Ourry A, Kim TH. 2008. Kinetics of drought-induced pathogenesis-related proteins and its physiological significance in white clover leaves. Physiologia Plantarum 132:329–337.

Liu F, Huang N, Wang L, Ling H, Sun T, Ahmad W, Muhammad K, Guo J, Xu L, Gao S, Que Y, Su Y. 2018. A novel L-ascorbate peroxidase 6 gene, ScAPX6, plays an important role in the regulation of response to biotic and abiotic stresses in sugarcane. Frontiers in Plant Science 9:1114–1117.

Lucas JA, García-Cristobal J, Bonilla A, Ramos B, Gutierrez-Mañero J. 2014. Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. Plant Physiology and Biochemistry 82:44–53.

Lucas JA, García-Villaraco A, Ramos B, García-Cristobal J, Algar E, Gutierrez-Mañero J. 2013. Structural and functional study in the rhizosphere of Oryza sativa L. plants growing under biotic and abiotic stress. Journal of Applied Microbiology 115:218–235.

Lucas JA, García-Villaraco Velasco A, Ramos B, Gutierrez-Mañero FJ. 2017. Changes of enzyme activities related to oxidative stress in rice plants inoculated with random mutants of a Pseudomonas fluorescens strain able to improve plant fitness upon biotic and abiotic conditions. Functional Plant Biology 44:1063–1074.

Maruta T, Ishikawa T. 2018. Ascorbate peroxidases: crucial roles of antioxidant enzymes in plant stress responses. In: Munne-Bosch S, Burritt DJ, Díaz-Vivancos P, Fujita M, Lorence A, eds. Ascorbic acid in plant growth, development and stress tolerance. Switzerland: Springer International Publishing. 111–127.

Mauch-Mani B, Baccelli I, Luna E, Flors V. 2017. Defense priming: an adaptive part of induced resistance. Annual Review of Plant Biology 68:485–512.

Minhong LD, Fontepe JP, Santos IMO, Navarro MORP, Simionato AS, Goya Rezende MJ, Balbi-Peña MI, de Oliveira AG, Andrade G. 2017. Control of bacterial stem rot on tomato by extracellular bioactive compounds produced by Pseudomonas aeruginosa LV strain. Cogent Food and Agriculture 3:1–16.

Miwacka J, Malolepsza U, Szymczak K, Szczez M. 2018. Involvement of metabolic compounds, volatile compounds, PR proteins, and mechanical strengthening in multi-layer protection of cucumber plants against Rhizoctonia solani activated by Trichoderma atrovirens TR525. Protoplasma 255:359–373.

Nie P, Li X, Wang S, Guo J, Zhao H, Niu D. 2017. Induced systemic resistance against Botrytis cinerea by Bacillus cereus AR156 through a JA/ET- and NPR1-dependent signaling pathway and activates FASP-triggered immunity in Arabidopsis. Frontiers in Plant Science 8:238.

Nioro G, Mhamdi A, Foyer CH. 2014. The roles of reactive oxygen metabolism in drought: not so cut and dried. Plant Physiology 164:1636–1648.

Ongena M, Jourdan E, Schäfer M, Kech C, Budzikiewicz H, Luxen A, Noctor G, Mhamdi A, Foyer CH. 2013. Biotic elicitation of systemic resistance and growth promotion of Arabidopsis thaliana by PGPRs from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. Plant Physiology and Biochemistry 82:44–53.

Pieterse CMJ, van Wees SCM, Ton J, van Pelt JA, van Loon LC. 2002. Signalling of salicylic acid and ethylene in induced systemic resistance (ISR) in Arabidopsis thaliana. Biochimica et Biophysica Acta 1574:289–297.

Ramirez-Prado JS, Abulfaraj AA, Rayapuram N, Benhamed M, Hirt H. 2018. Plant immunity: from signaling to epigenetic control of defense. Trends in Plant Science 23:833–844.

Ramos-Solano B, Algar E, García-Villaraco A, García-Cristobal J, Lucas García JA, Gutierrez-Mañero FJ. 2010a. Induced systemic resistance (ISR) in plants upon inoculation with random mutants of a Pseudomonas fluorescens strain able to improve plant fitness upon biotic and abiotic conditions. Functional Plant Biology 44:1063–1074.

Downloaded from https://academic.oup.com/aobpla/article-abstract/11/5/plz049/5540050 by guest on 15 November 2019
isoflavone metabolism with plant growth promoting rhizobacteria in early stages of development in Glycine max var. Osumi. *Journal of Agricultural and Food Chemistry* **58**:1484–1492.

Ramos-Solano B, Algar E, Gutierrez-Mañero FJ, Bonilla A, Lucas JA, Garcia-Seco D. 2015. Bacterial bioeffectors delay postharvest fungal growth and modify total phenolics, flavonoids and anthocyanins in blackberries. *LWT-Food Science and Technology* **61**:437–443.

Ramos-Solano B, Garcia-Villaraco A, Gutierrez-Mañero FJ, Lucas JA, Bonilla A, Garcia-Seco D. 2014. Annual changes in bioactive contents and production in field-grown blackberry after inoculation with *Pseudomonas fluorescens*. *Plant Physiology and Biochemistry* **74**:1–8.

Ramos-Solano B, Lucas García JA, Garcia-Villaraco A, Algar E, García-Cristobal J, Gutierrez Mañero FJ. 2010b. Siderophore and chitinase producing isolates from the rhizosphere of *Nicotiana glauca* Graham enhance growth and induce systemic resistance in *Solanum lycopersicum* L. *Plant and Soil* **334**:189–197.

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiology* **134**:1017–1026.

Silva MS, Arraes FBM, Campos MA, Grossi-de-Sa M, Fernandez D, Cândido ES, Cardoso MH, Franco OL, Grossi-de-Sa MF. 2018. Review: potential biotechnological assets related to plant immunity modulation applicable in engineering disease-resistant crops. *Plant Science* **270**:72–84.

Sokal RR, Rohlf FJ. 1980. *Introducción a la bioestadística*. Barcelona, Spain: Reverté Ed.

Song XS, Wang YJ, Mao WH, Shi K, Zhou YH, Ngouès S, Yu JQ. 2009. Effects of cucumber mosaic virus infection on electron transport and antioxidant system in chloroplasts and mitochondria of cucumber and tomato leaves. *Physiologia Plantarum* **135**:246–257.

Souza PF, Silva FD, Carvalho FE, Silveira JA, Vasconcelos IM, Oliveira JT. 2016. Photosynthetic and biochemical mechanisms of an EMS-mutagenized cowpea associated with its resistance to cowpea severe mosaic virus. *Plant Cell Reports* **36**:219–234.

Sultana S, Khew CY, Morshed MM, Namasivayam P, Napis S, Ho CL. 2012. Overexpression of monodehydroascorbate reductase from a mangrove plant (*AeMDHAR*) confers salt tolerance on rice. *Journal of Plant Physiology* **169**:311–318.

Sumayo M, Hahn MS, Ghim SY. 2013. Determinants of plant growth-promoting *Ochrobactrum lupini* KUDCI013 involved in induction of systemic resistance against *Pectobacterium carotovorum* subsp. *carotovorum* in tobacco leaves. *The Plant Pathology Journal* **29**:174–181.

Van Loon LC, Bakker PA, Pieterse CM. 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* **36**:453–483.

Wu G, Liu Y, Xu Y, Zhang G, Shen Q, Zhang R. 2018. Exploring elicitors of the beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 to induce plant systemic resistance and their interactions with plant signaling pathways. *Molecular Plant-Microbe Interactions* **31**:560–567.

Xia XJ, Zhou YH, Shi K, Zhou J, Foyer CH, Yu JQ. 2015. Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. *The Journal of Experimental Botany* **66**:2839–2856.

Xu C, Natarajan S, Sullivan JH. 2008. Impact of solar ultraviolet-B radiation on the antioxidant defense system in soybean lines differing in flavonoid contents. *Environmental and Experimental Botany* **63**:39–48.

Zamioudis C, Pieterse CMJ. 2012. Modulation of host immunity by beneficial microbes. *Molecular Plant-Microbe Interactions* **25**:139–150.