Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

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

Helena Martin-Rivilla (helenamartin92@gmail.com)
Universidad CEU San Pablo https://orcid.org/0000-0003-1665-5856
Ana Garcia-Villaraco
Universidad CEU San Pablo
Beatriz Ramos-Solano
Universidad CEU San Pablo
Francisco Javier Gutierrez-Mañero
Universidad CEU San Pablo
Jose Antonio Lucas
Universidad CEU San Pablo

Research article

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

Posted Date: March 27th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-19488/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Abstract

Background Unravel 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 defense against pathogens.

The use of beneficial rhizobacteria (bioeffectors) and its 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 its 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.

Background

The diseases caused by different pathogen organisms in plants represent an important and persistent threat and a challenge to supply food worldwide (Miller et al. 2017, Pechanova et al. 2015). 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 el tal. 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, a Solanaceae native to Southern Spain with a strong secondary metabolism (Ramos-Solano et al. 2010), 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 Nicotiana 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.

Results

Beneficial rhizobacteria screening: phylogenetic tree and biochemical tests

A phylogenetic tree was performed with the 16S rRNA sequences of the 175 bacterial strains. Two main groups appeared, one made up of Gram-positive (74 strains) and the other of Gram-negative bacteria (101 strains) (See Additional File 1)

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 *Bacillu*s, two species were especially abundant, *Bacillus cereus* and *Bacillus megaterium* (See Additional file 1).

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 (Frändberg and Shnurer 1998, Rodríguez-Kábana et al. 1983)) for identifying putative beneficial rhizobacteria were carried out to the 175 strains. The results of these tests are shown in Table 1. *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 *Acinetobacter*, *Pseudomonas, Enterobacter*, *Pantoea* and *Erwinia*. 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 *Bacillus cereus, B. megaterium* and *Brevibacterium* sp. 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.**ISR by beneficial rhizobacteria**According to the results obtained from the phylogenetic tree (See Additional File 1) and the biochemical tests (Table 1), twenty-five strains were chosen (fifteen Gram-negative and ten Gram-positive) to develop a first protection experiment against the pathogen 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 2. Table 3 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 8 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 8 genes, markers of different signal transduction pathways related to plant immune systems.

Table 1. Percentage of bacteria within each genera or species (in Gram-positive group), positive for biochemical traits

	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
Siderophore 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 t

Table 2. Twenty-five selected strains and its biochemical traits

			Biochemical traits		
	Bacterial Strain		IAA production	Siderophores production	Chitinases producti
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 aryabhattai*	+	+		
N 20.15	*Bacillus simplex*	+	+		

Biochemical traits are Indole Acetic Acid (IAA) production, siderophores production, phosphate solubilisation and chitinases production. A positive biochemi

Table 3. Percentage of protection (%) induced in *A. thaliana* seedlings inoculated with chosen strains against 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 odorifera*	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 aryabhattai*	23.98 ± 0.18 *
	N 20.15	*Bacillus simplex*	30.83 ± 4.92 *

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 (seedlings inoculated only with nutrient broth and pathogen challenged) was considered as 0% of protection and then data were relativized with respect to it. A positive control (BTH) was also used. Strains 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 that there were significant statistical differences ($p < 0.05$) with respect to negative control.

Differential gene expression 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 1 to 5. Three different behaviours appeared among the 5 strains. The first behaviour was a strong and significant increase 6 hapc, followed by strains N 5.12 (Fig. 1) and N 21.24 (Fig. 4); 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) 6 hapc. A second behaviour pattern was a significant increase in expression 12 hapc, only followed by N 12.34 (Fig. 3) with a very high increment of the differential expression of *NPR1*(149.74 times) , *PDF1*(675.97 times), *PR2* (57.09 times) and *PR3* (41.37 times). The third pattern was a significant increase 24 hapc, followed by strains N 8.17 (Fig. 2) and N 4.1 (Fig. 5). *ICS* (1.78 times), *PR1*(1.94 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*). **ISR by metabolic elicitors**Based on all the results, two strains were selected to extract its 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) 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).

The three fractions extracted from each strain (n-hexane, ethyl acetate and n-butanol), achieved significant protection (Table 4), 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%) 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 4. Percentage of protection (%) induced in *A. thaliana* seedlings inoculated with elicitor fractions against DC3000.

Treatment		% of protection
Controls	Negative control (DMSO)	0
	Positive control (BTH)	52.49 ± 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 (seedlings inoculated only with nutrient broth and pathogen challenged) 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 that there were significant statistical differences ($p< 0.05$) with respect to negative control.

Differential gene expression induced by metabolic elicitors in n-hexane and ethyl acetate fractions (the fractions with greatest protective capacity) from N 12.34 and N 4.1 is shown in figure 6. In the case of N 12.34, analysis was performed 6 and 12 hapc and in N 4.1, 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.

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. 2010) 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 (Anwar et. al 2016, Aarab et al. 2015, Lucas et al. 2013, Ramos Solano et al. 2006, Barriuso et al. 2005), 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 (Hacquard et al. 2017, 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. 2010).

In the present study, almost 100% of the strains produced siderophores. Siderophore production is related to iron limiting nutrient (Lucas et al. 2013, Raymond et al. 1984, Jin et al. 2006), 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. (2010). 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 (Ramos-Solano et al. 2010, Van Loon et al. 1998, Ramamoorthy et al. 2001, Ramos Solano et al. 2008). 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 (Akram et al. 2016, Petti et al. 2012). 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).

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 (6 hapc) was generated by strains N.5.12 (P. putida) (Fig. 1) and N 21.24 (P. fluorescens) (Fig. 4). 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 (12 hapc) was produced by N 12.34 (S. rubidaea) (Fig. 3), 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 6 hapc for N 5.12 and 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, present in the cytoplasm (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 (Caarls et al. 2015, Leon-Reyes et al. 2009). The higher protection achieved by N 21.24 (Table 3), is probably related to the high expression of the genes related to the synthesis of SA and JA (ICS and LOX2) 6 hapc (Fig. 4), 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 (Pieterse et al. 2014, Caarls et al. 2015, Spoel and Dong 2012).

Slow response strains showed a progressive increase on expression from 0 to 24 hapc. These strains, N 8.17 (S. maltophilia) (Fig. 2) and N 4.1 (B. cereus) (Fig. 5) ranked right after N 21.24 in Arabidopsis protection (Table 3). 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. 5), 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 (Wu et al. 2018, Munhoz et al. 2017). 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 and 4). 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 has different behaviours. The strain N 12.34 induces gene expression levels more intensely (up to 140 times. Figure 3) than metabolic elicitors (Fig. 6a 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 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 (Lucas et al. 2014, Van Hulten et al. 2006). Therefore, in this case the use of metabolic elicitors may have advantages over bioeffectors.

Interestingly, metabolic elicitors in both fractions from Serratia N 12.34 were able to activate the SA pathway, increasing the expression of NPR1 and PR2 (Fig. 6a and b). In both fractions, PDF1 expression (marker of the JA/ET pathway) decreased, which indicate that the metabolic elicitors present in these 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 Bacillus strain N 4.1, the two metabolic elicitor fractions (Fig. 6c 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 bacterium 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 alive 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).

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.

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 to demonstrate their ability to mimic the protective response triggered by the strain.

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. (2010). 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 -80ºC and plated to check viability.

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 (10mM each) Biotools, 0.375 µL of DMSO (Dimehyl sulfoxide) 100% 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 94ºC for 2 min and then subjected to 10 cycles, consisting of 94ºC for 0.3 min, 50ºC for 0.30 min and 72ºC for 1 min and 20 cycles consisting of 94ºC for 0,3 min, 50ºC for 0,30 min and 72ºC for 1 min. Finally, the mixtures were incubated at 72ºC for 7 min. PCR products were purified with UltraClean PCR Clean-up DNA purification kit (MO BIO). PCR products purified 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.

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).

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).

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.

Arabidopsis 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 mL 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 4000 rpm) 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 leaves inoculated with *P. syringae* DC3000 (negative control) as 0% protection. All the ISR experimental design is represented as a timeline in Additional File 2.

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

Based on results obtained in 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 (Pieterse et al. 2014, Ding et al. 2018, Caarls et al. 2015, Kazan 2018, Vlot et al. 2009, Seyfferth and Tsuda 2014, Niu et al. 2011, Nie et al. 2017, Wildermuth et al. 2002), *PDF1* (Plant Defensin 1), *LOX2* (Lipoxygenase 2) and the transcriptional factor *MYC2* as markers of the JA-ET signaling pathway (Caarls et al. 2015, Niu et al. 2011, Nie et al. 2017, Pangesti et al. 2014, Lorenzo and Solano 2005, Liu et al. 2016, Du et al. 2017), and two pathogenesis-related proteins genes, *PR2* (encoding b-1,3-glucanase) and *PR3 (*encoding chitinase), as SA and JA/ET markers, respectively (Wu et al.

2018, Jiang et al. 2016, Lemarié et al. 2015, Van Loon and Van Strien 1999, Spoel and Dong 2012, Jeandet et al. 2013, Schenk and Schikora 2013, Silva et al. 2018).

thaliana was handled as described in the first ISR assay (See Additional File 2). 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.

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

Prior to RNA extraction, samples were ground 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 25ºC, 30 min 42ºC, 5 min 85ºC, and hold at 4ºC. Amplification was carried out 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 55ºC and 30 s at 72ºC, 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 5.

Table 5. Primers forward and reverse used in qPCR analysis.

	Primer Forward	Primer 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

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: N 12.34 because it was the one with best differential expression results and 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 8,000 g for 15 min. Five hundred mL of the obtained supernatant was filtrated by 0,2 mm. 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 10 % Dimethyl sulfoxide (DMSO).

A third ISR assay on *A. thaliana* plants to evaluate the ability of three metabolic elicitor fractions from N 12.34 and N 4.1 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)). An additional control (negative control) with DSMO was included to ensure that elicitor effects were due to bacterial components and not to the chemical. All were pathogen challenged.

thaliana was handled as described in the first ISR assay (See Additional File 2). Treatments were delivered to seedlings by soil drench (50 mL). Negative control was treated with 50 mL of 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.

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 N 12.34 and *NPR1*, *PR3* and *PDF1* for N 4.1 were analysed. In the case of N 12.34, analysis was performed 6 and 12 hapc and in N 4.1, 12 and 24 hapc. Genes and sampling moments were selected according to previous results in the first qPCR experiment.

thaliana was handled as described in the first ISR assay (See Additional File 2). 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 mL) and challenge inoculation with DC3000 was performed as explained above.

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).

Abbreviations

PAMPs
Pathogens Associated Molecular Patterns
MAMPs
Microbe-Associated Molecular Patterns
PTI
Pattern-Triggered Immunity
ETI
Effector-Triggered Immunity
PRRs
Pattern Recognition Receptors
SAR
Systemic Acquired Resistance
HIR
Herbivore Induced Resistance
ISR
Induced Systemic Resistance
SA
Salicylic Acid
NPR1
Nonexpressor of Pathogenesis-Related Protein 1
JA
Jasmonic Acid
ET
Ethylene
PDF1
Plant defensin 1
IAA
Indole Acetic Acid
BTH
Benzothiadiazole
hapc
hours after pathogen challenge
PR1
Pathogenesis-Related Gene 1
ICS
Isochorismate Synthase 1
LOX2
Lipoxygenase 2

DMSO
Dimethyl sulfoxide

Declarations

- Ethics approval and consent to participate: “Not applicable”
- Consent for publication: “Not applicable”
- Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.
- Competing interests: The author(s) declare no competing interests.
- Funding: Ministerio de Economía y Competitividad of Spain through the project AGL-2013-45189-R. Funding came from state research funds, limited to freeing up the money without intervening in the study design, collection, analysis, and interpretation of data, or manuscript writing.
- Authors´ contributions: The results are part of the doctoral thesis of HMR whose directors are JAL and FJGM. All authors designed the experiments described in the manuscript. HMR and AGV carried out all the analyses of the strains present in the phylogenetic tree. HMR, JAL, BRS and FJGM performed the induced systemic resistance experiments in *Arabidopsis thaliana*, the collection of samples and subsequent analyses. HMR and JAL wrote the main manuscript, and all authors reviewed the manuscript.
- Acknowledgements: “Not applicable”

References

Aarab S, Ollero FJ, Megías M, Laglaoui A, Bakkali M, Arakrak. Isolation and screening of bacteria from rhizospheric soils of rice fields in Northwestern Morocco for different plant growth promotion (PGP) activities: An in vitro study. Int J Curr Microbiol App Sci. 2015; 4(1):260–269.

Adesina MF, Lembke A, Costa R, Speksnijder A, Smalla K. Screening of bacterial isolates from various European soils for in vitro antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed. Soil Biol Biochem. 2007; 39:2818–2828.

Akram W, Anjum T, Ali B. Phenylacetic Acid Is ISR Determinant Produced by Bacillus fortis IAGS162, Which Involves Extensive Re-modulation in Metabolomics of Tomato to Protect against Fusarium Wilt. Front Plant Sci. 2016; 7(1012): 897–12.

Alexander DB, Zuberer DA. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol Fertil Soils. 1991; 12:39–45.

Anwar S, Ali B, Sajid I. Screening of Rhizospheric Actinomycetes for Various In-vitro and In-vivo Plant Growth Promoting (PGP) Traits and for Agroactive Compounds. Front Microbiol. 2016; 7(198):82.

Barriuso J, Pereira MT, Lucas García JA, Megías M, Gutiérrez Mañero FJ, Ramos B. Screening for Putative PGPR to Improve Establishment of the Symbiosis Lactarius deliciosus-Pinus sp. Microb Ecol. 2005; 50(1):82–89.

Barriuso J, Ramos Solano B, Fray RG, Cámara M, Hartmann A, Gutiérrez Mañero FJ. Transgenic tomato plants alter quorum sensing in Plant Growth Promoting Rhizo- bacteria. Plant Biotechnol J. 2008; 6:442–452.

Berendsen RL, Pieterse CMJ, Bakker, PAHM. The rhizosphere microbiome and plant health. Trends in Plant Sci. 2012; 17(8):478–486.

Berrocal-Lobo M, Molina A, Solano R. Constitutive expression of Ethylene-Response-Factor1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. 2002; 29:23-32.

Betsuyaku S, Katou S, Takebayashi Y, Sakakibara H, Fukuda H. 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 Physiol. 2017 59(1):8–16.

Caarls L, Pieterse CMJ, Van Wees, SCM. How salicylic acid takes transcriptional control over jasmonic acid signaling. Front Plant Sci. 2015 6:1-11.

Conrath U, Pieterse CMJ, Mauch-Mani B. Priming in plant-pathogen interactions. Trends Plant Sci. 2002; 7(5):210–216.

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

De Freitas J, Banerjee M, Germida J. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (Brassica napus L.). Biol Fertil Soils. 1997; 24(4): 358-364.

Ding Y, Sun T, Ao K, Peng Y, Zhang Y, Li X, Zhang Y. Opposite Roles of Salicylic Acid Receptors NPR1 and NPR3/NPR4 in Transcriptional Regulation of Plant Immunity. Cell. 2018; 173(6):1454-1467.

Du M, Zhao J, Tzeng DTW, Liu Y, Deng L, Yang T, Zhai Q, Wu F, Huang Z, Zhou M, Wang Q, Chen Q, Zhong S, Li C, Li C. MYC2 orchestrates a hierarchical transcriptional cascade that regulates jasmonate-mediated plant immunity in tomato. Plant Cell. 2017; 29(8):1883–1906.

Fatima S, Anjum T. Identification of a Potential ISR Determinant from Pseudomonas aeruginosa PM12 against Fusarium Wilt in Tomato. Front Plant Sci. 2017; 8: 69.

Frändberg E, Shnurer J. Antifungal activity of chitinolytic bacteria isolated from airtight stored cereal grain. Can J Microbiol. 1998; 44:121–127.

Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol. 2005; 43:205–227.

Gozzo F, Faoro F. Systemic Acquired Resistance (50 Years after Discovery): Moving from the Lab to the Field. J Agric Food Chem. 2013; 61(51): 12473–12491.

Gutierrez Albanchez E, Garcia-Villaraco A, Lucas JA, Gutierrez FJ, Ramos-Solano B. Priming fingerprint induced by Bacillus amyloliquefaciensQV15, a common pattern in Arabidopsis thaliana and in field-grown blackberry. J. Plant Interact. 2018; 13(1):398–408.

Hacquard S, Spaepen S, Garrido-Oter R, Schulze-Lefert P. Interplay between innate immunity and the plant microbiota. Annu Rev Phytopathol. 2017; 55:565–58.

Hammerschmidt R. Systemic acquired resistance. Adv Bot Re. 2009; 51: 173–222.

Jeandet P, Clément C, Courot E, Cordelier S. Modulation of Phytoalexin Biosynthesis in Engineered Plants for Disease Resistance. Int J Mol Sci. 2013; 14(7):14136–14170.

Jiang CH, Fan ZH, Xie P, Guo JH. Bacillus cereus AR156 Extracellular Polysaccharides Served as a Novel Micro-associated Molecular Pattern to Induced Systemic Immunity to Pst DC3000 in Arabidopsis. Front. Microbiol. 2016; 7(9):977.

Jin CW, He YF, Tang CX, Wu P. Zheng, S. J. Mechanisms of microbially enhanced Fe acquisition in red clover (Trifolium pratense L.). Plant Cell Environ. 2006; 29(5):888–897.

Jones JD, Dangl JL. The plant immune system. Nature. 2006; 444:323–329.

Kannojia P, Choudhary KK, Srivastava AK, Singh AK. Chapter Four. PGPR Bioelicitors: Induced Systemic Resistance (ISR) and Proteomic Perspective on Biocontrol. PGPR Amelioration in Sustainable Agriculture 2018; 67–84. Elsevier Inc. http://doi.org/10.1016/B978-0-12-815879-1.00004-5

Kazan K. A new twist in SA signalling. Nature Plants. 2008; 4:327-328.

Lemarié S Robert-Seilaniantz A, Lariagon C, Lemoine J, Marnet N, Jubault M, Manzanares-Dauleux MJ, Gravot A. Both the jasmonic acid and the salicylic acid pathways contribute to resistance to the biotrophic clubroot agent *Plasmodiophora brassicae* in Arabidopsis. Plant Cell Physiol. 2015; 56(11):2158–2168.

Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T, Pieterse CMJ. Ethylene modulates the role of nonexpressor of pathogenesis-related genes1 in cross talk between salicylate and jasmonate signalling. Plant Physiol. 2009; 149:1797–809.

Liu L, Sonbol F.M, Huot B, Gu Y, Withers J, Mwimba M, Yao J, He SY, Dong X. Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. Nat Commun. 2016; 7:13099.

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R. Ethylene response factor integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell. 2003; 15:165-178.

Lorenzo O, Solano R. Molecular players regulating the jasmonate signalling network. Curr Opin Plant Biol. 2005; 8(5):532–540.

Lorito M, Di Pietro A, Hayes CK, Woo SL, Harman GE. Antifungal, synergistic interaction between chitinolytic enzymes from Thrichoderma harzianum and Enterobacter cloacae. Appl Environ Microbiol. 1993; 83:721–728.

Lucas Garcia JA, Probanza A, Ramos B, Gutierrez Mañero FJ. Genetic variability of rhizobacteria from wild populations of four Lupinus species based on PCR-RAPDs. J Plant Nutr Soil Sci. 2001; 164:1–7.

Lucas JA, García-Villaraco A, Ramos B, García-Cristobal J, Algar E, Gutierrez-Mañero J. Structural and functional study in the rhizosphere of *Oryza sativa* L. plants growing under biotic and abiotic stress. J Appl Microbiol. 2013; 115(1):218–235.

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

Marilley L, Aragno M. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Appl Soil Ecol. 1999; 13:127–136.

Martin-Rivilla H, Garcia-Villaraco Velasco A, Ramos-Solano B, Gutierrez-Mañero FJ, Lucas JA. 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. AoB Plants. 2019; 20:1-9.

Miller R, Ge Costa Alves GS, Van Sluys MA. Plant immunity: unravelling the complexity of plant responses to biotic stresses. Ann Bot. 2017; 119(5):681–687.

Munhoz LD, Fonteque JP, Oliveira dos Santos I, Pérez Navarro MO, Simionato AS, Goya ET, Rezende MI, Balbi-Peña MI, Gonçalves de Oliveira A, Andrade G. Control of bacterial stem rot on tomato by extracellular bioactive compounds produced by *Pseudomonas aeruginosa* LV strain. Cogent Food Agric. 2017; 35(1):1–16.

Nie P, Li X, Wang S, Guo J, Zhao H, Niu D. Induced Systemic Resistance against *Botrytis cinerea* by *Bacillus cereus* AR156 through a JA/ET- and NPR1-Dependent Signalling Pathway and Activates PAMP-Triggered Immunity in *Arabidopsis*. Front Plant Sci. 2017; 8:759–12.

Niu DD, Liu HX, Jiang CH, Wang YP, Wang QY, Jin HL, Guo JH. The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate and jasmonate/ethylene-dependent signalling pathways. Mol Plant-Microbe Interact. 2011; 24:533–542.

Pangesti N, Pineda A, Dicke M, van Loon JJA. Variation in plant-mediated interactions between rhizobacteria and caterpillars: potential role of soil composition. Plant Biology. 2014; 17(2):474–483.

Pechanova O, Pechan T. Maize-pathogen interactions: an ongoing combat from a proteomics perspective. Int J Mol Sci. 2015; 16:28429–28448.

Pel MJ, Pieterse CM. Microbial recognition and evasion of host immunity. J Exp Bot. 2013; 64(5):237–1248.

Petti C, Reiber K, Ali SS, Berney M, Doohan FM. Auxin as a player in the biocontrol of Fusarium head blight disease of barley and its potential as a disease control agent. BMC Plant Biol. 2012; 12:224.

Pieterse CMJ, Van Pelt JA, Ton J, Parchmann S, Mueller MJ, Buchala AJ, Métraux JP, Van Loon LC. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol Mol Plant Pathol. 2000; 57(3):123–134.

Pieterse CMJ, Van Loon LC. NPR1: The spider in the web of induced resistance signalling pathways. Curr Opin Plant Biol. 2004; 7:456-464.

Pieterse CMJ, Van Loon LC. Signalling cascades involved in induced resistance in: Walters D, Newton, AC, Lyon G (Eds), Induced resistance for plant defence. Blackwell, London, UK; 2007. P. 65-88

Pieterse CMJ Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA. Induced Systemic Resistance by Beneficial Microbes. Annu Rev Phytopathol. 2014; 52(1):347–375.

Pozo MJ, Van Der Ent S, Van Loon LC, Pieterse CMJ. Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in Arabidopsis thaliana. New Phytol. 2008; 180:511-523.

Pré M Atallah M, Champion A, De Vos M, Pieterse CM, Memelink J.The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. 2008; 147:13471357.

Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop. Prot. 2001; 20:1–11.

Ramirez-Prado JS, Abulfaraj AA, Rayapuram N, Benhamed M, Hirt H. Plant Immunity: From Signalling to Epigenetic Control of Defense. Trends Plant Sci. 2018; 9:833-844.

Ramos Solano B, Pereyra MT, Probanza A, Lucas García JA, Megías M, Gutierrez Mañero FJ. Screening for PGPR to improve growth of *Cistus ladanifer* seedlings for reforestation of degraded mediterranean ecosystems. Plant Soil. 2006; 287(1-2):59–68.

Ramos Solano B, Barriuso J, Pereyra MT, Domenech J, Gutierrez Mañero FJ. Systemic disease protection elicited by plant growth promoting rhizobacteria strains: relationship between metabolic responses, systemic disease protection and biotic elicitors. Phytopathol. 2008; 98:451–457.

Ramos-Solano B, Lucas Garcia J.A, Garcia-Villaraco A, Algar E, Garcia-Cristobal J Gutierrez Manero F.J.Siderophore and chitinase producing isolates from the rhizosphere of *Nicotiana glauca* Graham enhance growth and induce systemic resistance in *Solanum lycopersicum* L. Plant Soil. 2010; 334(1-2):189–197.

Raymond K, Muller G, Matzanke B. Complexation of iron by siderophores a review of their solution and structural chemistry and biological function. Top Curr Chem. 1984; 123:49–102.

Rodríguez-Kábana R, Godoy G, Morgan-Jones G, Shelby RA. The determination of soil chitinase activity: conditions for assay and ecological studies. Plant Soil. 1983; 75:95–106.

Remans T, Smeets K, Opdenakker K., Mathijsen D, Vangronsveld, J, Cuypers A. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. Planta. 2008; 227(6):1343–1349.

Rosier A, Medeiros FHV, Bais HP.Defining plant growth promoting rhizobacteria molecular and biochemical networks in beneficial plant-microbe interactions. Plant Soil. 2018; 428:35-55.

Schenk ST, Schikora A. AHL-priming functions via oxylipin and salicylic acid. Front Plant Sci. 2013; 5:784–784.

Sergeeva E, Danielle Hirkala LM, Louise NM. Production of indole-3-acetic acid, aromatic amino acid aminotransferase activities and plant growth promotion by *Pantoea agglomerans* rhizosphere isolates. Plant Soil. 2007; 297:1–13.

Seyfferth C, Tsuda K.Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. Front Plant Sc. 2014; 5:697.

Sid A, Ezziyyani M, Egea-Gilabert C, Candela ME. Selecting bacterial strains for use in the biocontrol of diseases caused by *Phytophthora capsici* and *Alternaria alteranata* in sweet pepper plants. Biol Plant. 2003; 47(4):569–574.

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

Sinclair SJ, Johnson R, Hamill JD. Analysis of would- induced gene expression in Nicotiana species with contrasting alkaloid profiles. Functional Plant Biol. 2004; 31:721–729.

Sokal RR, Rohlf FJ. Introducción a la bioestadística. Editorial Reverte SA, Barcelona, Spain; 1980. p. 362

Spoel SH, Dong X. How do plants achieve immunity? Defence without specialized immune cells. Nat Rev Immunol. 2012; 12(2): 89–100.

Stringlis IA, Kirstin Freussner KY, de Jonge R, Van Bentum S, Van Verk MC, Berendsen RL, Bakker PAHM, Feussner I, Pieterse CMJ. MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. PNAS. 2018; 115 (22) E5213-E5222.

Sumayo M, Hahm MS, Ghim SY. Determinants of Plant Growth-promoting *Ochrobactrum lupini* KUDC1013 Involved in Induction of Systemic Resistance against *Pectobacterium carotovorum* subsp. carotovorum in Tobacco Leaves Plant Pathol J. 2013; 29(2):174–181.

Tang D, Wang G, Zhou JM. Receptor Kinases in Plant-Pathogen Interactions: More Than Pattern Recognition. The Plant Cell. 2017; 29(4):618–637.

Timmusk S, Behers L, Muthoni J, Muraya A, Aronsson AC. Perspectives and challenges of microbial application for crop improvement. Front. Plant. 2017; Sci 8:49.

Van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J. Costs and benefits of priming for defense in Arabidopsis. 2006. PNAS. 2006; (14):5602-5607.

Van Loon LC, Bakker P, Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol. 1998; 36:453–483.

Van Loon LC, Van Strien EA. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol. 1999; 55(2):85–97.

Vlot AC, Dempsey DA, Klessig DF. Salicylic Acid, a Multifaceted Hormone to Combat Disease. Annu Rev of Phytopathol. 2009; 47(1):177–206.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. Corrigendum: Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature. 2002; 417(6888):571–571.

Wu G, Liu Y, Xu Y, Zhang G, Shen Q, Zhang R.Exploring elicitors of the beneficial rhizobacterium *Bacillus*. Mol Plant Microbe Interact. 2018; 31(5): 560-567.

Yang Y, Wang N, Guo X, Zhang Y, Ye B. Comparative analysis of bacterial community structure in the rhizosphere of maize by high throughput pyrosequencing. PLOS ONE. 2017; 12:178425.

Zhang X, Zhang J, Gao J, Wang X, Fan F, Ma X, Yin H, Zhang C, Feng K, Deng Y. Thirty- one years of rice-rice-green manure rotations shape the rhizosphere microbial community and enrich beneficial bacteria. Soil Biol Biochem. 2017; 104:208–217Academy of Sciences of the United States of America, 103(14):5602–5607.

Figures

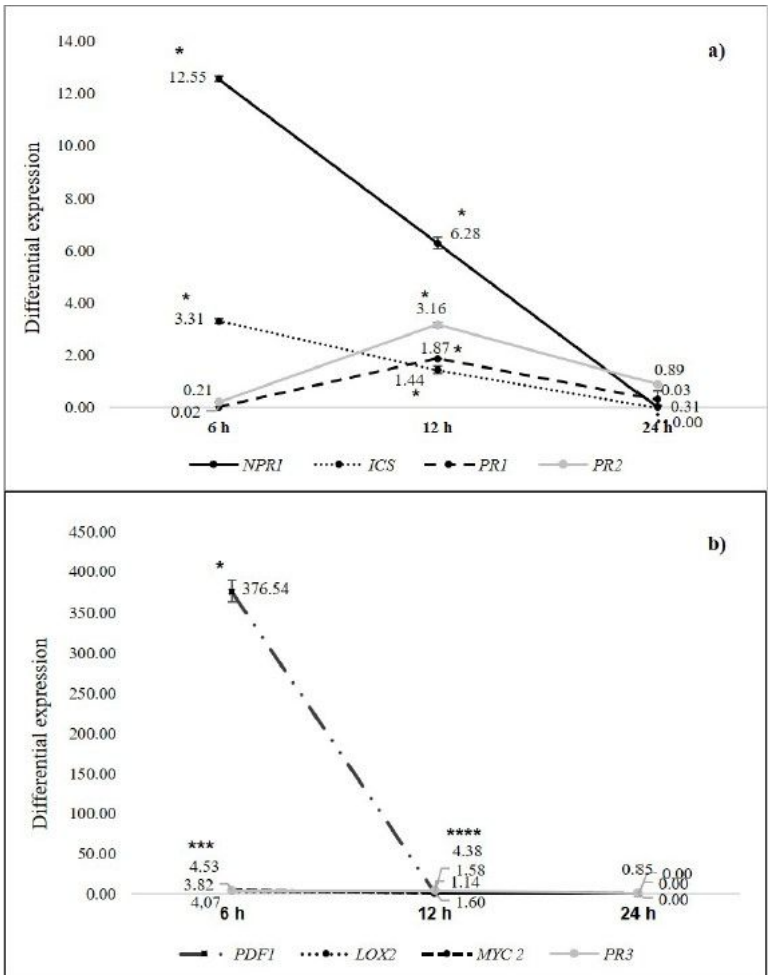

Figure 1

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) h after pathogen challenge; 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 ($p < 0.05$) between treatments within each sampling time (6, 12 and 24 h)

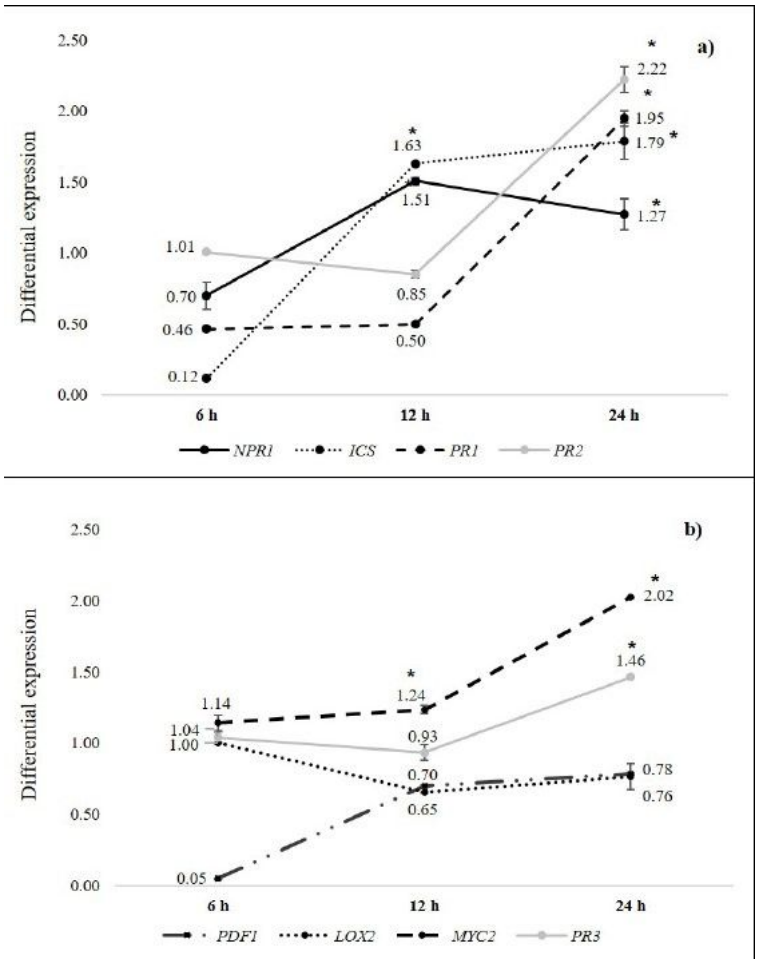

Figure 2

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) h after pathogen challenge; 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 ($p < 0.05$) between treatments within each sampling time (6, 12 and 24 h)

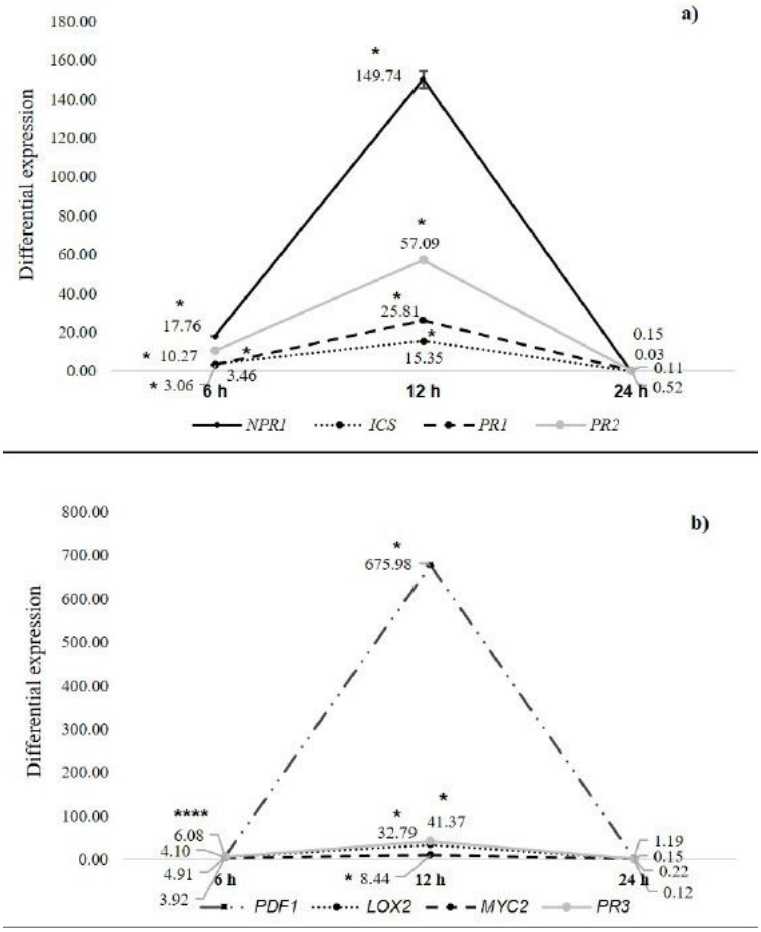

Figure 3

Differential gene expression (seedlings inoculated with N 12.34 (Serratia rubidaea) vs negative control) 6 (n=16), 12 (n=16) and 24 (n=16) h after pathogen challenge; 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 ($p < 0.05$) between treatments within each sampling time (6, 12 and 24 h)

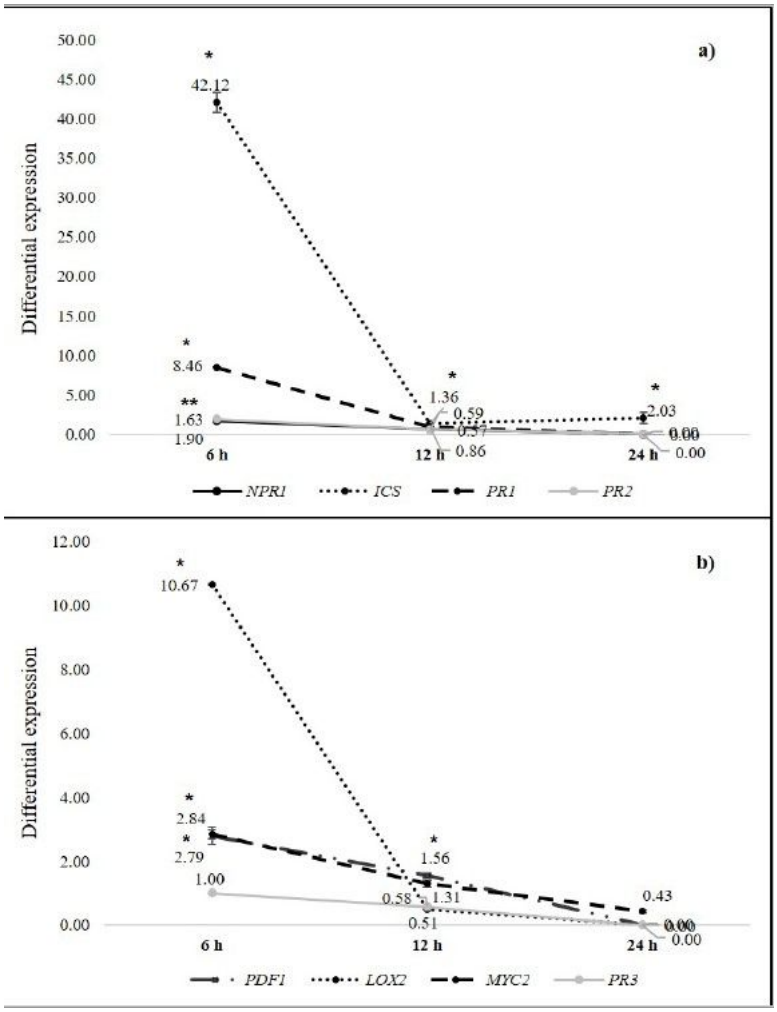

Figure 4

Differential gene expression (seedlings inoculated with N 21.24 (Pseudomonas fluorescens) vs negative control) 6 (n=16), 12 (n=16) and 24 (n=16) h after pathogen challenge; 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 ($p < 0.05$) between treatments within each sampling time (6, 12 and 24 h)

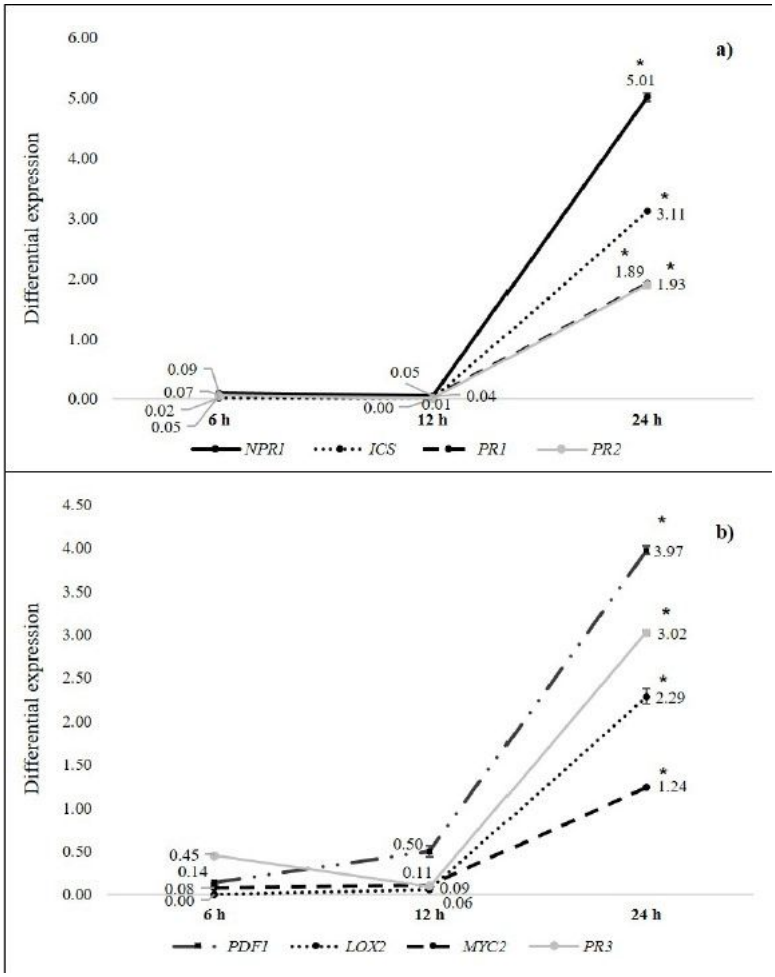

Figure 5

Differential gene expression (seedlings inoculated with N 4.1 (Bacillus cereus) vs negative control) 6 (n=16), 12 (n=16) and 24 (n=16) h after pathogen challenge; 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 ($p < 0.05$) between treatments within each sampling time (6, 12 and 24 h)

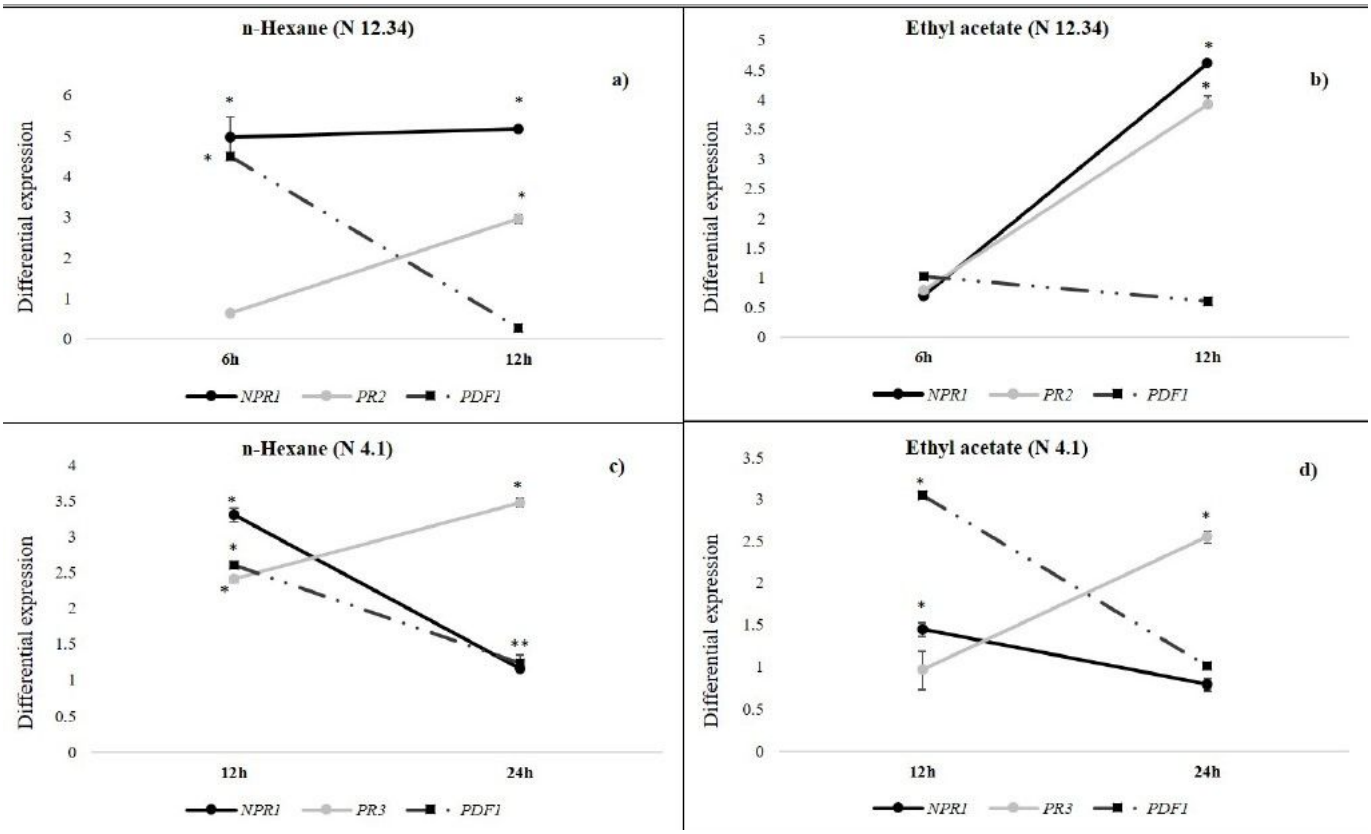

Figure 6

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) h after pathogen challenge in N 12.34 (a and b) and at 12 (n=16) and 24 (n=16) h after pathogen challenge 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 and 5).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile2.pdf
- AdditionalFile1.pdf