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Polyhydroxyalkanoate production by the plant beneficial rhizobacterium *Pseudomonas chlororaphis* PCL1606 influences survival and rhizospheric performance

Sandra Tienda [a,b], José Antonio Gutiérrez-Barranquero [a,b], Isabel Padilla-Roji [a,b], Eva Arrebola [a,b], Antonio de Vicente [a,b], Francisco M. Cazorla [a,b,*]

[a] *Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, Avda. Louis Pasteur 31, 29071 Málaga, Spain*
[b] *Grupo de Biología y Control de Enfermedades de Plantas, Área de Protección de Cultivos, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", IHSM-UMA-CSIC, Avda. Louis Pasteur 49, 29010 Málaga, Spain*

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ABSTRACT

Pseudomonas chlororaphis PCL1606 (PcPCL1606) is a model rhizobacterium used to study beneficial bacterial interactions with the plant rhizosphere. Many of its beneficial phenotypes depend on the production of the antifungal compound 2-hexyl, 5-propyl resorcinol (HPR). Transcriptomic analysis of PcPCL1606 and the deletional mutant in HPR production Δ*darB* strain, assigned an additional regulatory role to HPR, and allowed the detection of differentially expressed genes during the bacterial interaction with the avocado rhizosphere. Interestingly, the putative genes *phaG* (PCL1606_46820) and *phaI* (PCL1606_56560), with a predicted role in polyhydroxyalkanoate biosynthesis, were detected to be under HPR control. Both putative genes were expressed in the HPR-producing wild-type strain, but strongly repressed in the derivative mutant Δ*darB,* impaired in HPR production. Thus, a derivative mutant impaired in the *phaG* gene was constructed, characterized and compared with the wild-type strain PcPCL1606 and with the derivative mutant Δ*darB*. The *phaG* mutant had strongly reduced PHA production by PcPCL1606, and displayed altered phenotypes involved in bacterial survival on the plant roots, such as tolerance to high temperature and hydrogen peroxide, and decreased root survival, in a similar way that the Δ*darB* mutant. On the other hand, the *phaG* mutant does not have altered resistance to desiccation, motility, biofilm formation or adhesion phenotypes, as displayed by the HPR-defective Δ*darB* mutant have. Interestingly, the mutant defective in PHA production also lacked a biocontrol phenotype against the soilborne pathogenic fungus *Rosellinia necatrix*, even when the derivative mutant still produced the antifungal HPR compound, demonstrating that the final biocontrol phenotype of PcPCL1606 first requires bacterial survival and adaptation traits to the soil and rhizosphere environment.

1. Introduction

The rhizosphere, considered the soil fraction intimately associated with the plant roots, is one of the most complex, dynamic and variable habitats on earth (Prashar et al., 2014). In this environment, root exudates enable physicochemical changes in the surrounding rhizosphere, giving rise to a selection effect that shapes the root microbial community composition (Berendsen et al., 2012). This selection could favour microbe-specific traits that make them able to colonize, compete and efficiently survive in this fluctuating environment. Rhizobacterial survival in soil and plant roots mainly depends on several molecular and cellular mechanisms. Among them, motility and chemotaxis are some of the most studied, because the production of root exudates has an impact on the presence of surrounding microbes (Bais et al., 2006), and motile bacteria that can be attracted by root exudates have advantages during the initial phase of root colonization (Turnbull et al., 2001; Zboralski and Filion, 2020). After the initial bacterial contact with the plant roots, episodes of bacterial attachment and spatial organization of cells on the root surface can take place. Some bacteria have an endophytic lifestyle and penetrate inside the root structure, and others usually develop

* Corresponding author at: Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, Avda. Louis Pasteur 31, 29071 Málaga, Spain.
E-mail address: cazorla@uma.es (F.M. Cazorla).

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microcolonies or a more complex biofilm structure on the root surface (Pandin et al., 2017). During such plant-microbe interactions, the combined production of plant and bacterial secondary metabolites can influence both plant and bacterial physiology and metabolism, resulting in reshaping of the root microbiome (Pang et al., 2021; Chevrette et al., 2022). Simultaneously, microbes must evade plant immune responses, leading to the assembly and expansion of the final plant-related microbiome (Teixeira et al., 2019).

During microbiome assembly processes, a relevant strategy for the survival of rhizospheric microbes is metabolic versatility (Ortiz et al., 2021) and especially the production of energy reserves, such as polyhydroxyalkanoate (PHA) granules, that help cells overcome changes in the habitat (Kadouri et al., 2003). Bacterial PHA granules can help improve their establishment, proliferation, and survival, increasing their tolerance to stress in competitive environments such as the plant rhizosphere (Kadouri et al., 2005; Mozejko-Ciesielska et al., 2019).

Within the most prevalent rhizosphere-associated bacteria, beneficial representatives belonging to the *Pseudomonas fluorescens* complex have been widely reported to control soil-borne fungal pathogens (Raaijmakers et al., 2009). The main mechanisms associated with this plant protective activity include the production of antimicrobial compounds, the induction of plant disease resistance, and competition with the pathogen for niches and/or nutrients (Haas and Défago, 2005; Arrebola et al., 2009; Raaijmakers and Mazzola, 2012; Heredia-Ponce et al., 2021a; Heredia-Ponce et al., 2021b; Müller and Behrendt, 2021). Within the *P. fluorescens* complex, the *P. chlororaphis* species displays wide antagonistic activity against different soil-borne fungal pathogens, mainly due to the production of a great variety of metabolites and antifungal compounds, as well as by efficient plant root-colonizing features (Arrebola et al., 2019; Biessy et al., 2019).

In this work, the model biocontrol rhizobacterium *P. chlororaphis* PCL1606 (PcPCL1606) was used (Cazorla et al., 2006). PcPCL1606 produces, among others, the antifungal compound 2-hexyl, 5-propyl resorcinol (HPR), which has been directly linked to its antagonism and biocontrol-associated activities (Calderón et al., 2013). HPR belongs to the "dialkylresorcinols" (DARs) family of compounds, which have been described as novel signalling molecules in bacterial cell-to-cell communication (Brameyer et al., 2015). In PcPCL1606, HPR participates in different bacterial phenotypes involved in the multitrophic interaction between the avocado root and the pathogen *R. necatrix* but also in bacterial colonization, persistence and biofilm formation on the avocado root and *R. necatrix* hyphae (Calderón et al., 2014b; Calderón et al., 2019).

The main aim of this study was to report the role of PHA production in the *P. chlororaphis* cells, especially during the beneficial interactions of the bacterium with the avocado rhizosphere The performance of PcPCL1606 will be compared with a Δ*darB* mutant (impaired in HPR production) in order to elucidate the phenotypes influenced by HPR production and/or by PHA biosynthesis.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type strain PcPCL1606 and derivative mutant strains were grown at 25 °C in tryptone-peptone-glycerol medium (TPG; Calderón et al., 2013). M9 minimal liquid medium supplemented with 2% (w/v) glucose was used to induce the production of polyhydroxyalkanoate granules as previously described (Tal et al., 1990). The culture media were supplemented with kanamycin (50 μg/mL) and gentamicin (50 μg/mL), when necessary. Cycloheximide (100 μg/mL) was added to the media to prevent fungal growth in the bacteria cultured from roots. The *Rosellinia necatrix* CH53 strain used in this study was grown on potato dextrose agar (PDA) at 25 °C.

Table 1
Microorganisms and plasmids used for this study.

Strain	Relevant characteristics[a]	Reference
Pseudomonas chlororaphis		
PcPCL1606	Wild-type strain, isolated from avocado rhizoplane, HPR +, biocontrol strain of avocado white root rot caused by *Rosellinia necatrix*.	Cazorla et al. (2006)
PcPCL1606-GFP	Green fluorescent PCL1606 derivative strain containing the plasmid pBAH8; Gm^r	Calderon et al., 2014
PCL1606::*darB*	PCL1606 derivative insertional mutant in *darB* gene by plasmid pΔDARB379 insertion, HPR -; Km^r	Calderón et al. (2013)
Δ*darB*	PCL1606 derivative deletional mutant in *darB* gene, HPR-pGEM®-T Easy Vector; Km^r	Calderón et al. (2019)
ComB	Δ*darB* transformed with the plasmid pCOMB, HPR +, Gm^r and Km^r.	Calderón et al. (2013)
PCL1606::*phaG*	PCL1606 derivative insertional mutant in *phaG* by plasmid pΔPHAG insertion, HPR +; Km^r	This study
Escherichia coli		
DH5α	General-purpose host strain.	Boyer and Roulland-Dussoix (1969)
Rosellinia necatrix		
CH53	Wild type fungus, isolated from avocado roots; elicits symptoms of white root rot; high virulence	P é rez-Jiménez, 1997
Plasmids		
pBAH8	pBBR1MCS-5 containing $P_{Al/04/03}$-*gfp mut3*-T_0-T_1	Huber et al. (2002)
pCR2.1-TOPO® I	TA cloning vector for PCR products, Amp^r, Km^r	Invitrogen, San Diego, CA, U.S.A.
pΔDARB379	*darB* fragment (379 bp) cloned into pCR2.1-TOPO® used for construction of insertional mutant on *darB* gene of strain PcPCL1606, Amp^r, Km^r	Calderon et al., 2019
pGEM®-T Easy Vector	Linearized vector with single 3′-terminal thymidine at both ends	Promega
pΔPHAG	*phaG* fragment (342 bp) cloned into pCR2.1-TOPO® used for construction of insertional mutant on *phaG* gene of strain PcPCL1606, Amp^r, Km^r	This study

[a] HPR = 2-hexyl, 5-propyl resorcinol, + = production, - = no production. Antibiotic resistance: Amp^r = ampicillin; Gm^r = gentamycin; Km^r = kanamycin,

2.2. Experimental model of avocado root-bacteria interactions

An experimental interaction model to study the gene expression dependent on HPR production during the interaction of PcPCL1606 with avocado roots was designed (Figure S1). Briefly, PcPCL1606 and the Δ*darB* mutant (derivative strain impaired in HPR production; (Calderón et al., 2015) were grown in TPG liquid medium for 24 h under shaking conditions (180 rpm at 25 °C) to obtain a high cellular concentration. Subsequently, the cells were washed twice with 0.85% NaCl solution. The final suspension was added to M9 minimal medium with succinic acid (10 mM) as the sole carbon source (Villar-Moreno et al., 2022), and incubated for 24 h (180 rpm at 25 °C). Bacterial strains were inoculated onto avocado roots by immersion in a bacterial suspension of 10^8 cfu/mL ($OD_{600\ nm}$= 0.8). The roots were previously disinfected (20 min; 1% NaClO) and washed (sterile distilled water). The plants were placed inside square Petri dishes (Corning®, 23 ×23 cm) containing sterile quartz sand, with a hole through which the stem of the avocado plant protruded, and the system was sealed. After 48 h of incubation, three independent rhizosphere samples (sand adhered to the avocado roots and manually was removed) from the inoculated avocado plants (with the wild-type and Δ*darB* mutant) were collected and immediately processed.

2.3. Transcriptomic analysis

RNA was extracted from the rhizosphere samples using a PowerSoil® RNA extraction kit (Qiagen Iberia S.L., Madrid, Spain) following the manufacturer's instructions and its amount was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

For the RNAseq experiment, the quantity and quality of RNA were verified by the Genomics and Ultrasequencing Service Unit (University of Malaga) and subsequently sequenced using NextSeq550 equipment (Illumina). The raw reads and their subsequent processing were carried out by the Centre for Supercomputing and Bioinnovation (University of Malaga). The bacterial RNAseq data analysis was performed based on a series of software packages adapted to the experimental model. The software components of the RNAseq analysis pipeline included analysis by SeqTrimNext (v.2.0.6) to remove low-quality reads, adapters, organular DNA and contaminant sequences; BOWTIE (v.2.2.9) to align reads to the genomic reference; Samtools (v. 0.1.19), a package of programs to deal directly with the alignment files, reading, writing, editing or viewing the alignment files in SAM/BAM format (http://www.htslib.org/); and TUXEDO tools (http://cole-trapnell-lab.github.io/cufflinks/manual/), used to estimate the aligned RNAseq reads in the different transcripts and estimate their abundance. The abundance of the transcripts was measured in fragments per kilobase of fragments of exon per million reads (fpkm). Once the transcripts and their corresponding estimated fpkm have been assembled, these transcripts were annotated with the known reference set of genes obtained from the database from the annotated reference file. This pipeline is a tool developed by the Andalusian Platform for Bioinformatics (PAB; http://www.scbi.uma.es/site/omics/bioinformatics) for the study of differential expression analysis using data of RNAseq on a genomic reference. The subsequent analysis of differential expression with a method analogous to differentially expressed sequences, and the graphical representation of the expression results was done using the 'cummeRbund' R package (v. 2.42.0). The array of reads in fpkm format generated will be used to obtain a list of differentially expressed genes that showed a p-value less than 0.05.

Once the expression profile was calculated, the differentially expressed genes (P values < 0.05 and Log_2 Fold Change > 1 or < -1) between PcPCL1606 and Δ*darB* in interaction with the roots of avocado plants were obtained to identify possible genes regulated by HPR.

To validate the RNAseq expression profile results obtained, different genes (including the genes putatively involved in PHA granule biosynthesis) overexpressed and repressed by HPR were analyzed by quantitative reverse transcription PCR (qRT-PCR). Rhizosphere RNA samples obtained previously for RNAseq experiments were converted to cDNA using Invitrogen Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with random primers according to the manufacturer's instructions.

The value of relative transcript abundance was calculated using the ΔΔCT (cycle threshold) method (Livak and Schmittgen, 2001). The RT-qPCR assays were conducted in a CFX96 Touch qPCR system (Bio--Rad, USA) using SYBR Green Supermix (Bio-Rad, USA). Transcriptional data were normalized using the *gyrA* housekeeping gene as reference using the Roche LightCycler 480 Software and are presented as the change in base 2 expression (Log_2FC) compared to the expression of each gene in the wild-type strain relative to the Δ*darB* mutant. The primers used for the qRT-PCR experiments (Table S1) were designed with the online software Primer3 (http://bioinfo.ut.ee/primer3/) using the genomic sequences of each gene as a template. qRT-PCR reaction was performed at 50 °C for 40 min, followed by PCR amplification using a 40-cycle amplification program (94 °C for 30 s, 58 °C for 1 min and 68 °C for 1 min) and a final extension cycle at 68 °C for 7 min

2.4. Construction of a PCL1606::phaG insertional mutant

Among the genes overexpressed in the wild-type strain, two genes putatively involved in PHA granule biosynthesis were identified and further studied. The *phaG* gene of PcPCL1606 (locus PCL1606_46820), situated alone 1.04 Mbp upstream of the *pha* cluster where *phaI* (PCL1606_56560) is located, was disrupted by insertional mutagenesis according to the protocol described in Sambrook and Russel (2001) by single-crossover homologous recombination. Briefly, DNA fragments of 342 bp from the *phaG* ORF were amplified using specific PCR primers (Table S1) and cloned into the pCR.2.1 TOPO vector (pΔ*phaG*; Table 1). The *E. coli* DH5α strain was transformed by heat-shock (Inoue et al., 1990), and the plasmid with the DNA fragment was extracted by miniprep and used to transform the PcPCL1606 strain using a standard protocol (Choi et al., 2006). Km-resistant colonies of PcPCL1606 were selected and the correct insertion and orientation of the plasmid within the target gene was confirmed by PCR (mutant primer verification; Table S1). Characterization of the growth phenotype (growth curves measuring O.D. at 600 nm and bacterial counts) on M9 minimal medium of PCL1606::*phaG* mutants was reported in comparison with the wild-type strain (Rainey, 1999). Additionally, HPR production was confirmed for the PCL1606::*phaG* mutant using the protocol previously described by Cazorla et al. (2006).

2.5. Microscopic visualization of polyhydroxyalkanoate granules

The PHA granule visualization assay was performed using methodology previously described with minor modifications (Sharma et al., 2017). Briefly, 500 μL from suspensions adjusted to 1 ($OD_{600\ nm}$; approx. 10^9 cfu/mL) of the PcPCL1606, Δ*darB* and PCL1606::*phaG* strains were inoculated in flasks with 25 mL of M9 minimal medium supplemented with 2% glucose as the sole carbon source and incubated at 25 °C and 100 rpm. After 72 h, 200 μL of each culture was centrifuged at 6000 rpm for 5 min. For PHA granule staining, cells were resuspended in a 0.1% aqueous solution of Nile blue chloride (Ref. 222550, Sigma). After 15 min, two washes with sterile distilled water (10000 rpm for 2 min) were performed. Finally, the cells were resuspended in 200 μL of sterile distilled water. For the visualization of the PHA granules, observation in a confocal laser microscope was carried out. PHA granules stained with Nile blue were visualized with 555 nm excitation and 580 nm emission wavelengths using a green filter (636 nm). The cells that produce PHA granules are shown red.

To visualize and compare the production of PHA granules between the different strains, the cell concentration of each strain was adjusted to 10^6 cfu/mL, observed under confocal microscopy, and the fluorescence intensity of the PHA granules in each image was quantified by measuring the fluorescent pixels and normalized by the number of cells present in the observation area with the ImageJ software (Schneider et al., 2012).

2.6. Evaluation of the bacterial motility, colony morphology, cell adhesion, and biofilm formation phenotype

For bacterial motility, swimming analysis was performed. The bacteria were inoculated with a sterile toothpick into the center of a 0.3% agar plate with TPG diluted 1/20 in Milli-Q water similar to a method previously described (Friedman and Kolter, 2004). The plates were analyzed after 24 h of incubation at 25 °C. Measurements of the motility circle radius enabled the calculation of the motility area.

A colony morphology assay was performed to determine the composition of the extracellular matrix, as described by O'Toole and Kolter (1998) with modifications. Precultures of the three strains on TPG were adjusted to an O.D. of 0.8 at 600 nm (approximately 10^8 cfu/mL), and 10 μL of each strain was inoculated onto TPG plates supplemented with Congo red (40 μg/mL) and Coomassie brilliant blue (20 μg/mL). The plates were then incubated at 25 °C for 5 days, and colony images

were captured using a stereomicroscope AZ-100 (Nikon Co., Tokyo, Japan).

Biofilm formation was assayed by the ability of the cells to adhere to the wells of 96-well microtiter dishes composed of polyvinylchloride plastic (PVC, tissue culture plate 96-well, round-bottom suspension cells, Sarstedt) as previously described with modifications (O'Toole and Kolter, 1998). An exponential culture of the bacterial strains (TPG media, 10 h at 25 °C) was adjusted to an $O.D._{600\ nm}$ of 0.08 (10^7 cfu/mL) with sterile TPG medium. The different test bacterial strains were distributed (100 μL of bacterial suspension) into each well (at least six wells inoculated per strain). After inoculation, the plates were incubated at 25 °C for 3 days without movement. A total of 120 μL of a 1% crystal violet solution was added to each well to stain the cells, and the plates were incubated at room temperature for 30 min and rinsed thoroughly and repeatedly with water. Finally, 120 μL of 50% methanol was added to each well to solubilize the crystal violet at room temperature for 20 min. The amount of crystal violet present in each well was determined by absorbance at 595 nm to quantify the biofilm.

The formation of a pellicle at the air-liquid interface was visually analyzed "in vitro" for each strain. Each strain was cultured on TPG at 25 °C overnight (o/n), washed twice with fresh TPG media, and adjusted to 0.8 O.D. at 600 nm (approximately 10^8 cfu/mL). Five microliters was inoculated in 1 mL of TPG placed into each well of a plastic 24-well plate. The plates were sealed and incubated in the dark at 25 °C without movement. After 10 days of incubation, pellicle formation was visually analyzed.

2.7. Assays of desiccation, high temperature and hydrogen peroxide tolerance

The association of PHA granule production with stress tolerance was assayed following the methodology previously described (Kadouri et al., 2003). To perform the assays, the wild-type PcPCL1606 strain and the derivative mutant strains were grown in minimal medium M9 with 2% glucose for 72 h (optimal conditions for the formation of PHA granules) and compared.

The involvement of PHA granule production in the desiccation tolerance of PcPCL1606 was evaluated by the survival of the PcPCL1606, Δ*darB* and PCL1606::*phaG* strains under desiccation. One hundred microliters of each culture adjusted to an $OD_{600\ nm}$ of 0.8 (approx. 10^8 cfu/mL) was placed in the centre of a slide cover slip and allowed to dry in a laminar flow cabinet for 60 min at room temperature (approximately 25 °C). Then, the slide cover slip was washed with 5 mL of sterile saline solution to obtain a cell suspension, and the number of viable cells was counted after cultivation on TPG agar (Kadouri et al., 2003).

To determine bacterial resistance to high temperature, 10 mL of the culture as detailed above were placed in a 15 mL tube and incubated in a water bath at 50 °C for 30 min. Bacterial viability on TPG agar was determined every 10 min

To analyze sensitivity to hydrogen peroxide, 100 μL of a bacterial culture (approx. 10^8 cfu/mL) was spread using a sterile cotton swab on the surface of minimal medium M9 amended with 2% glucose agar plates. Then, 25 μL of a solution of H_2O_2 (1.5%) were placed on 13 mm Whatman discs and air-dried for 15 min. The disc was placed on each plate, and the plates were incubated at 25 °C for 24 h. The sensitivity of each strain was evaluated by measuring the growth inhibition halos around the paper discs.

2.8. Bacterial survival, root competitiveness and biocontrol activity experiments

To evaluate the involvement of PHA granules production in the survival features of PcPCL1606, the bacterial counts of wild-type strain and the mutants were obtained in both sterile quartz sand and avocado roots. Survival assays were performed as previously described (Kadouri et al., 2003) with some modifications. Bacterial strains were grown in 5 mL of TPG broth medium and incubated overnight (200 rpm and 25 °C), and the bacterial suspensions were washed and resuspended with M9 minimal medium supplemented with 2% glucose. Then, the bacterial suspensions were adjusted to an $OD_{600\ nm}$ of 1 (approx. 10^9 cfu/mL), and 500 μL of these suspensions was inoculated into flasks with 25 mL of M9 minimal medium for 72 h (100 rpm at 25 ºC; optimal conditions for the formation of PHA granules).

For survival in quartz sand assays, one milliliter of the bacterial suspensions was mixed with 5 g of sterile quartz sand and deposited in 50 mL tubes, which were left unclosed to allow gas exchange and incubated at 25 °C. One gram of quartz sand was collected at 4, 24, 48, 72 and 96 h after inoculation and mixed with 1 mL of sterile saline solution (0.85%). The number of culturable cells was counted each time.

To analyze bacterial survival in avocado roots, the previously disinfected (0.1% NaOCl at 20 min) and washed (with sterile distilled water at 20 min) roots were immersed in a bacterial suspension for 20 min. The inoculated plants were transplanted into vermiculite pots and incubated at 25 ºC (70% HR) under a regime of 16:8 h of light:dark, and the roots were sampled at 4, 24, 48, 72 and 96 h. Root samples were weighed and homogenized in a laboratory blender for 4 min with 2 mL of sterile saline solution (0.85%) and bacterial viability was determined by bacterial counts on TPG, and compared with those of PCL1606, which is considered an efficient avocado root colonizer (Calderón et al., 2014b; Tienda et al., 2020).

To investigate whether the PHA granule production participates in the root bacterial competition of PcPCL1606, avocado roots previously disinfected and washed were immersed in mixed bacterial suspensions adjusted to 0.8 ($OD_{600\ nm}$; approx. 10^8 cfu/mL) in 1:1 proportions (wt:Δ*darB* and wt:PcPCL1606::*phaG*) for 20 min. Then, the inoculated plants were transplanted into vermiculite pots. Coinoculated root samples were collected at 24 and 48 h, weighed and homogenized to obtain the bacterial counts.

Both for survival in avocado roots and for the competitiveness experiments, the PcPCL1606-GFP strain was used (resistant to gentamicin (50 μg/mL); Calderón et al., 2014b). The mutants were selected with TPG plates supplemented with kanamycin (50 μg/mL).

To determine the role of PHA granule production in the biological control response of PcPCL1606, a biocontrol assays against avocado white root rot disease were performed as previously described (Cazorla et al., 2006). Briefly, the roots of six-month-old commercial avocado plants (Brokaw España, S.L., Velez-Málaga, Spain) were disinfected by immersion in 0.1% NaOCl (for 20 min) and then washed with sterile distilled water (20 min). For bacterial inoculation, the roots were immersed in a suspension of PcPCL1606, Δ*darB* or PCL1606::*phaG* strains adjusted to 0.8 ($OD_{600\ nm}$; approx. 10^8 cfu/mL) for 20 min. Then, avocado plants were placed into plastic pots with potting soil subsequently inoculated with wheat grains infected with *Rosellinia necatrix* (8 infected wheat grains were placed per plant). Uninfected and non-inoculated plants were used as negative controls. Two independent assays were performed, each with fifteen avocado plants per treatment. To monitor *R. necatrix* disease development, the percentage disease index of foliar symptoms of avocado white root rot was calculated as previously described (Cazorla et al., 2006). From the disease index curve, the area under the disease progress curve (AUDPC) was calculated in groups of 5 plants and statistically compared with each strain (Campbell and Madden, 1990) after all values were normalized to the wild-type strain.

2.9. Data Analyses

All experiments were independently repeated at least three times. Data distributions were tested by different statistical analyses: normality test (Shapiro-Wilk), paired t test and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test with Bonferroni's correction. A significance level of $P < 0.05$ was taken for all

comparisons, otherwise it is indicated.

All data analyses were performed using Microsoft Excel 2016 (Microsoft Corporation, Alburquerque, NM, USA), IBM SPSS statistics 25 software (SPSS, Inc., Chicago, IL, USA) and SigmaPlot software (12.0, Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. The compound HPR regulates the expression of genes involved in polyhydroxyalkanoate biosynthesis in PcPCL1606

Obtained RNAseq raw data have been uploaded in www.figshare.com (https://doi.org/10.6084/m9.figshare.23660028). Differentially expressed genes (DEGs) between PcPCL1606 (wild-type strain producing HPR) and the derivative Δ*darB* mutant strain (a non-HPR producing strain) were selected with a Log_2 fold-change > 1 or < -1 with a P value < 0.05 (available at doi: 10.6084/m9.figshare.23634687). A volcano plot was used to represent genes that were differentially overexpressed (green), differentially repressed (red) and unaffected in their expression by HPR (black) (Fig. 1A). A total of 298 genes were overexpressed, while 88 genes were repressed by HPR production in the PcPCL1606 strain (Fig. 1B). The putative functions of DEGs were analyzed using "cluster of orthologous genes" (COGs) classification (Fig. 1C). The largest number of genes overexpressed by HPR were classified into the COG groups of amino acid transport and metabolism [E: 58 genes] and unknown function [S: 48 genes]. The remaining genes overexpressed by HPR were classified mainly in the categories of transcription [K: 23 genes] and in different categories of metabolism and transport [P: 10 genes, I: 13 genes, H: 6 genes, G: 21 genes, E: 58 genes and F: 12 genes]. Genes repressed by HPR were classified mainly into the categories of unknown function [S] and amino acid metabolism and transport [E].

A selection of the top 20 DEGs (overexpressed and repressed genes by

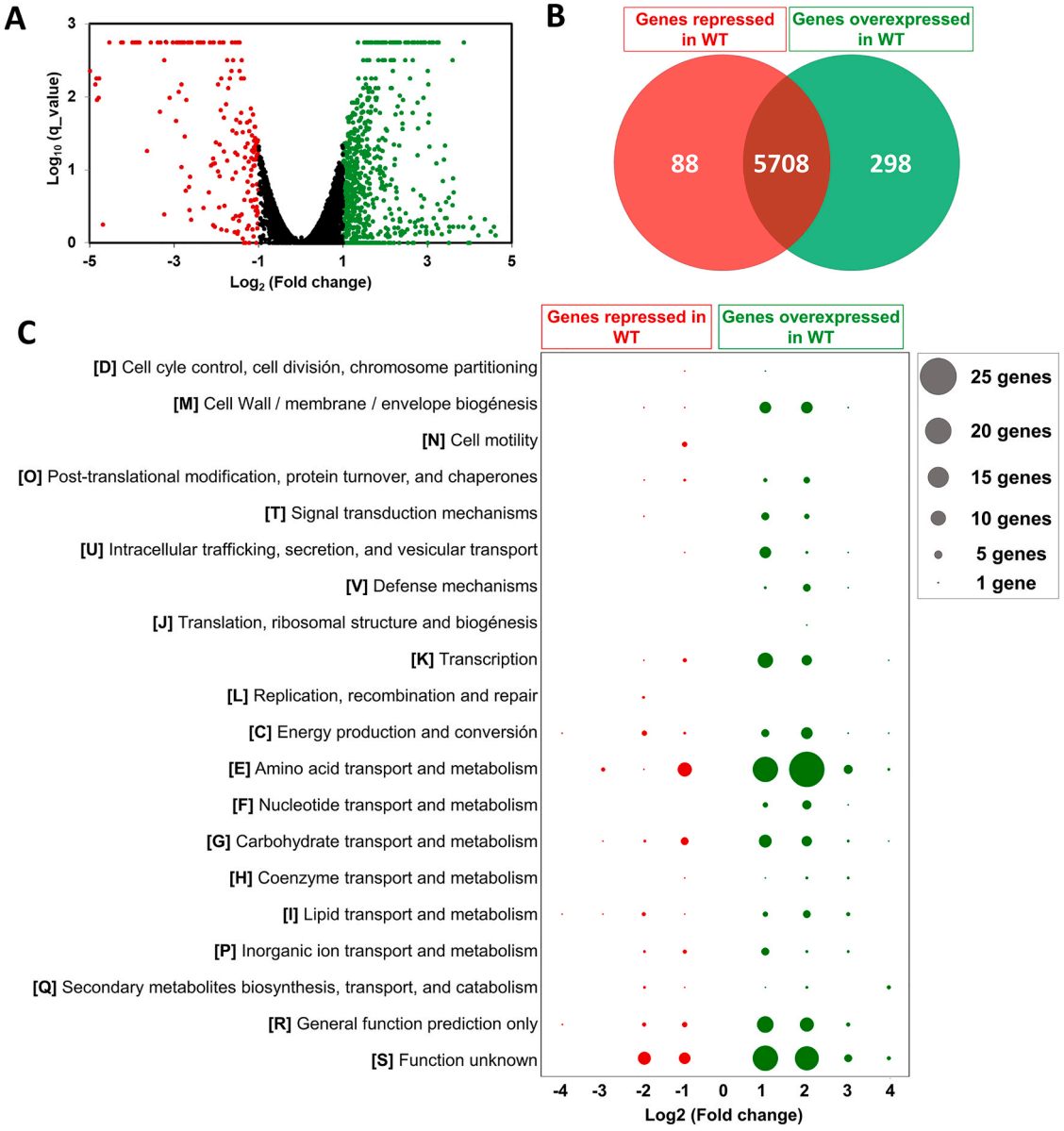

Fig. 1. Comparative analysis of differentially expressed genes (DEGs) dependent on HPR production during the interaction of PcPCL1606 with avocado roots. (A) Volcano plot: Up-and downregulated genes are reported as green and red dots, respectively. **(B)** Venn diagram showing the distribution of DEGs: genes with a Log_2 Fold change > 1 (HPR-overexpressed genes; green) and genes with a Log_2 Fold change < -1 (HPR-repressed genes; red). (C) Classification of DEGs in the cluster of orthologous genes (COG) category. Up- and downregulated genes are reported as red and green circles, respectively. A larger circle size indicates a greater number of genes within each category.

HPR production) was analyzed (Tables S2 and S3) to identify relevant genes involved in bacteria-plant interactions dependent on HPR production. Two overexpressed genes in the wild-type strain PcPCL1606 (PCL1606_56560 and PCL1606_46820), putatively involved in the polyhydroxyalkanoate granule biosynthesis (PHA granules), were selected for further analysis. These two genes were homologous to the *phaI* and *phaG* genes of *Pseudomonas aeruginosa* and showed a Log_2FC values of 3.39 and 3.01, respectively and also localized separately in the bacterial chromosome. The *phaI* gene encodes a structural protein of the PHA granule, and *phaG* encodes a transacylase that links the biosynthesis of fatty acids with the biosynthesis of PHA granules and the end of the biosynthetic pathway.

To validate the expression results obtained by RNAseq experiments, RT-qPCRs of these two genes (*phaI* and *phaG*) and of additional genes that were differentially overexpressed and repressed genes by HPR, were carried out (Figure S2). The RT-qPCR results confirmed the differential expression of these genes previously obtained in the RNAseq analysis.

3.2. *PcPCL1606 harbors a gene cluster homologous to the pha cluster of Pseudomonas aeruginosa PAO1*

A comparative analysis by amino acid sequence homology using Protein BLAST (blastp) revealed that PcPCL1606 harbors a gene cluster homologous to the *pha* cluster present in *P. aeruginosa* PAO1 (Fig. 2A). Predicted functions were deduced from the similarity results, and two putative genes encoded PHA synthases (*phaC1* and *phaC2*) separated by the *phaZ* gene with a predicted function of a PHA depolymerase, involved in granule degradation. This gene cluster also includes the *phaD* gene, which encodes a putative transcriptional regulator, and the *phaF* and *phaI* genes, encoding phasin proteins (the major proteins associated with PHA granules), which have been previously found to be associated at the surface of the PHA granules. Finally, the *phaG* gene (with a predicted transacylase function), has been described to be directly involved in PHA granule production, linking de novo fatty acid biosynthesis with PHA granule biosynthesis. This gene was positioned out of the *pha* cluster, as also occurs for *P. aeruginosa* PAO1. Therefore, among the *pha* genes differentially expressed by HPR (*phaG* and *phaI*), *phaG* was selected to further analyze its direct implication in the biosynthesis of the PHA granules.

3.3. *The phaG gene plays a key role in the production of PHA granules in PcPCL1606*

To elucidate the role of HPR and the *phaG* gene in PHA granule production, cell staining with Nile blue A and observation of red fluorescence under confocal microscopy were performed. Calculation of the ratio between the fluorescence emitted by the stained granules under confocal microscopy (Fig. 3A) and the number of cells observed under brightfield microscopy (Fig. 3B) revealed that the Δ*darB* and PcPCL1606::*phaG* mutants showed a lower ability to form PHA granules (Fig. 3C). The derivative Δ*darB* and PcPCL1606::*phaG* mutants produced significantly lower quantities of PHA granules than the wild-type strain, showing that the Δ*darB* mutant has an intermediate production phenotype between the wild-type strain and the PcPCL1606::*phaG* mutant. Remarkably, no differences in PHA granule production was observed among the wild type PCL1606 strain and the derivative Δ*darB* complemented ComB strain, with restored HPR production (Figure S3).

3.4. *The production of PHA granules by PcPCL1606 is involved in tolerance to high temperature and hydrogen peroxide abiotic stresses*

To decipher the involvement of the *darB* and *phaG* genes in different phenotypes, the wild-type strain and the mutants were tested for swimming motility, biofilm-related traits (such as colony morphology, adhesion to plastic and pellicle formation in liquid-air interphase), and tolerance to different stress conditions, such as desiccation, high temperature and hydrogen peroxide.

The derivative Δ*darB* and PcPCL1606::*phaG* mutants did not display significant differences in bacterial counts after desiccation stress when compared to the wild-type strain (Fig. 4A). No differences were observed for motility, colony morphology, pellicle formation on the liquid-air interface and adhesion to plastic (Fig. S4 and S5).

However, the bacterial counts of the Δ*darB* and PCL1606::*phaG* mutants (both affected in PHA granule production) decreased significantly faster than the wild-type strain during high temperature stress (Fig. 4B). Furthermore, the Δ*darB* and PcPCL1606::*phaG* mutants were significantly impaired in tolerance to hydrogen peroxide stress (Fig. 4C), showing a larger area of growth inhibition in comparison to the wild-type strain (Figure S4).

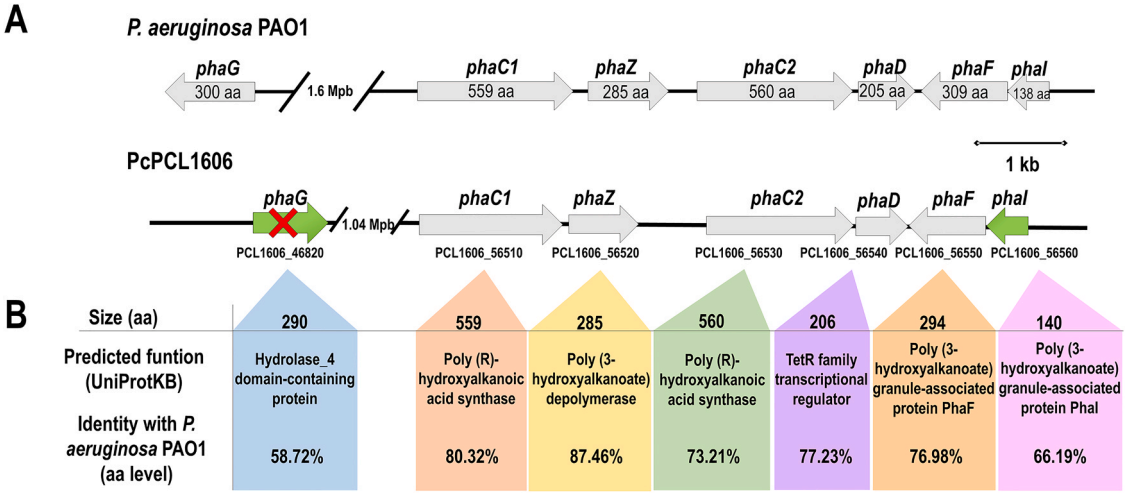

Fig. 2. Comparative analysis of the PHA cluster. (A) Structure of the *pha* gene cluster in *P. aeruginosa* PAO1 and in PcPCL1606. The *phaG* gene is outside the *phaC1C2DFIZ* cluster. Genes upregulated by HPR in this study (*phaG* and *phaI*) are shown in green and with an X in red is marked the gene interrupted in this study. **(B)** Analysis of ORFs is indicated by a color showing the size (amino acids [aa]), predicted function and identity with ORFs of *Pseudomonas aeruginosa* PAO1. Gene code are indicated in each ORF of PcPCL1606.

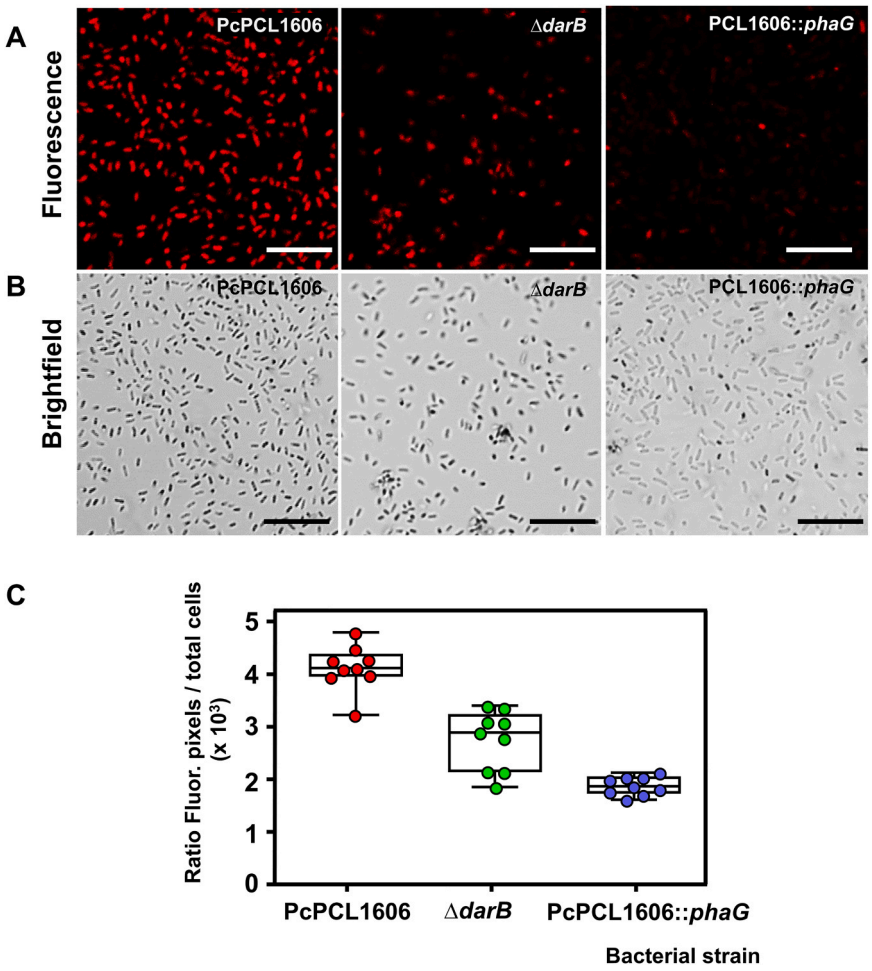

Fig. 3. Visualization of PHA granules. The presence of PHA granules was explored for the wild-type strain PcPCL1606 and its derivative mutants Δ*darB* and PCL1606::*phaG* strains. Bacteria were grown in M9 minimal medium supplemented with 2% glucose, and suspensions were analyzed. **(A)** Visualization of PHA granules (in red) under confocal laser microscopy. **(B)** Brightfield images corresponding to the same images in A. The size of the white bar represents 10 μm. **(C)** Normalization of the number of fluorescent pixels by the number of total cells on each brightfield area. The bar represents the standard error. Different letters indicate significant differences between the samples according to the analysis of variance (ANOVA; Bonferroni test; $P \leq 0.05$).

3.5. The phaG gene of PcPCL1606 is involved survival in the soil and on avocado roots and in biocontrol features

The survival and root competitiveness in sterile quartz sand at 25 ºC of constant temperature, showed that the bacterial counts of the Δ*darB* and PcPCL1606::*phaG* derivative mutants were lower in comparison to the wild-type strain during a time course experiment (6, 24, 48, 72 and 96 h; Fig. 5A). A similar result was observed in avocado roots, where the Δ*darB* and PcPCL1606::*phaG* mutants displayed a lower persistence than the wild-type strain at 6, 24, 48 and 72 h. However, at 96 h, the bacterial counts of the mutants in avocado roots reached similar levels of those of the wild-type strain (Fig. 5B).

In addition, competitiveness assays among the mutant strains compared with the wild-type strain PcPCL1606 in the avocado root revealed a reduced competitiveness of the mutants with respect to the wild-type strain, showing statistically significant differences at 24 h, but such differences were not statistically significant at 48 h (Fig. 5C).

Finally, to determine whether those phenotypes affected in the *phaG* mutant would also have an effect on plant protection against soilborne phytopathogenic fungi, biocontrol assays on the *R. necatrix*-avocado test system were performed. The area under the disease progress curve (AUDPC) results showed that avocado plants inoculated with PcPCL1606 showed 50% less disease index than the control plants without any bacterial inoculation (Fig. 5D). Interestingly, when the avocado plants were inoculated with the PcPCL1606::*phaG* mutant, the disease index was similar to that of the control plants without bacterial inoculation, even when this mutant still producing HPR similarly than the wild type strain (data not shown). These results reveal a lack of biocontrol activity by the PcPCL1606::*phaG* mutant, as also occurred for the Δ*darB* mutant, with very slightly lower AUDPC values (higher biocontrol capacity) than the control pla (nts and the plants inoculated with the PcPCL1606::*phaG* mutant, though this difference was not significant (Fig. 5D).

4. Discussion

Rhizospheric plant-beneficial *Pseudomonas* spp. have been extensively studied to decipher the molecular mechanisms of microbial interactions with plant roots (Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001). In particular, strains belonging to the *P. chlororaphis* species are some of the most adapted to grow in the rhizospheric environment, and they can show relevant features that make many of them excellent biological control agents (Arrebola et al., 2019; Zhang et al., 2020). The model rhizobacterium *P. chlororaphis* PCL1606 (PcPCL1606) is a remarkable biological control agent that produces the compound 2-hexyl, 5-propyl resorcinol (HPR), which is directly involved in the antagonism and biocontrol activity against the soil-borne fungal pathogen *R. necatrix* (Cazorla et al., 2006; Calderón et al., 2015).

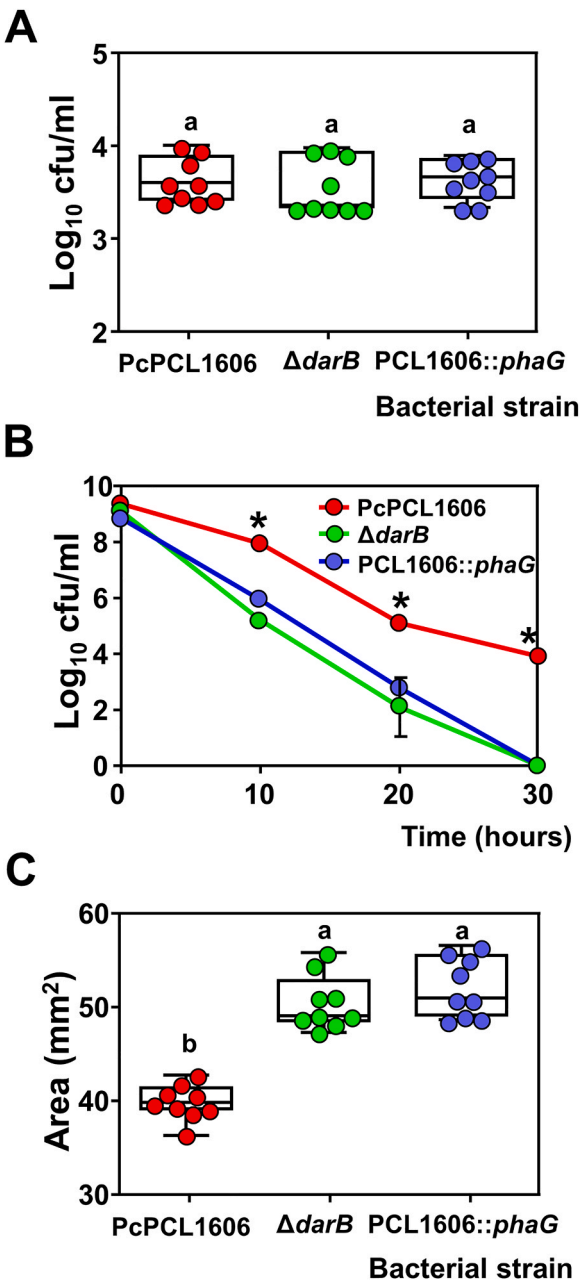

Fig. 4. Tolerance to different abiotic stresses of the PcPCL1606, Δ*darB* and PCL1606::*phaG* strains. (A) Bacterial survival under desiccation conditions, expressed in Log_{10} cfu/mL. **(B)** Bacterial survival under high-temperature conditions (50 ºC) at 0, 10, 20 and 30 min, expressed in Log_{10} cfu/mL. The asterisk indicate significant difference according to a paired Student's t test ($P < 0.05$) **(C)** Tolerance to hydrogen peroxide, expressed as the area of growth inhibition (mm^2) in the presence of hydrogen peroxide. The bar represents the standard error. Different letters (and the asterisk) indicate significant differences between the samples according to analysis of variance (ANOVA; Bonferroni test; $P \leq 0.05$).

Additionally, HPR is also involved in important biological processes in the rhizosphere, such as bacterial colonization and persistence in avocado roots, as well as biofilm formation (Calderón et al., 2014b; Calderón et al., 2019). Interestingly, the compound HPR belongs to the family of dialkyl resorcinol compounds, described as novel signaling molecules in bacterial cell-to-cell communication (Brameyer et al., 2015). In this work, we report the HPR regulon of PcPCL1606 after a transcriptomic approach on the avocado rhizosphere, and compared the gene expression in the HPR-producing wild-type strain PcPCL1606 with the Δ*darB* mutant, impaired in HPR production (Calderón et al., 2015). The results suggested that HPR may act mainly as a positive regulator of genes (many of them metabolic), pointing to HPR as an inducer involved in the fast metabolic reprogramming of the bacterial cells during the initial interaction with avocado roots.

Two genes homologous to the *phaG* and *phaI* genes of *P. aeruginosa* PAO1 (putatively involved in the production of PHA granules) were among the top 20 genes specifically overexpressed by HPR during the avocado root interaction. To study the involvement of these genes in the rhizospheric competence of PcPCL1606, an insertional mutant was constructed in the *phaG* gene. We selected *phaG* gene for mutagenesis because this gene is directly involved in linking "de novo" fatty acid biosynthesis with PHA granule biosynthesis, with a key role in the production of PHA granules in *Pseudomonas* sp. strains (Hoffmann et al., 2000; Hoffmann et al., 2002; Beckers et al., 2016). Additionally, the *phaG* gene is located outside of the *pha* cluster in *Pseudomonas* spp., and its disruption would avoid polar effects on nearby genes. The derivative mutant strain PcPCL1606::*phaG* exhibited a strong decrease in PHA granule production (approximately 50%) in comparison to the wild-type strain, confirming the involvement of this gene in the PHA granule biosynthesis by PcPCL1606. A similar result has been reported in the *P. aeruginosa* PAO1 strain, where a derivative *phaG* gene mutant showed a reduction of above 40% in PHA granule accumulation (Hoffmann et al., 2002). On the other hand, the Δ*darB* mutant (defective in HPR production) displayed an intermediate decrease in PHA production (approximately 30%) compared with the PcPCL1606::*phaG* mutant, thus explaining the regulatory effect of HPR by reducing the expression of the *phaG* gene, but not its abolishment, as occurs in the PcPCL1606::*phaG* mutant. The applied growth conditions allowed test strains to reach maximal HPR production (Calderón et al., 2014a), but interestingly, the disruption of the *phaG* gene did not have any side effect on HPR production, biofilm-related and motility phenotypes.

PHA granules are well-known energy reserve granules that bacteria use during periods of starvation that also enhance bacterial establishment, proliferation and survival in competitive environments such as the soil and rhizosphere (Okon and Itzigsohn, 1992; Hoffmann et al., 2000; Kadouri et al., 2005). Thus, bacteria that produce PHA granules also showed a substantially higher tolerance to different stress conditions (Pham et al., 2004; Mozejko-Ciesielska and Kiewisz, 2016; Obruca et al., 2020; Tribelli et al., 2020), and their derivative mutants impaired in PHA granule production, showed lower survival ability under different stresses. The role of PHA production in stress resistance in PcPCL1606, could be explained, as in other bacteria, by the degradation of PHA granules that could increase the levels of guanosine tetraphosphate (ppGpp), thus inducing the expression of the *rpoS* gene. This gene encodes a transcription factor that activates, among others, the expression of genes involved in protection against damaging stress factors (Gentry et al., 1993; Ramos-González and Molin, 1998; Ghergab et al., 2021).

In this work, the derivative PcPCL1606::*phaG* and Δ*darB* mutant strains showed lower tolerance to high temperatures, suggesting a crucial role of PHA production in PcPCL1606 performance at high temperatures, an important trait for its beneficial plant activity under a potential scenario of global warming. Similarly, lower resistance to hydrogen peroxide has been reported, indicating the participation of PHA production in the plant-bacteria interaction, and *phaG* gene would help establish bacteria with a potential endophytic lifestyle, as has been previously suggested for *Pseudomonas* sp. (Ruiz et al., 2001). This effect of PHA production on bacterial tolerance to stresses has been previously reported in other species of the genus *Pseudomonas* (Ramos-González and Molin, 1998; Mozejko-Ciesielska and Kiewisz, 2016; Obruca et al., 2020). However, the regulation of such bacterial phenotype is more complex, since the enhanced cross-tolerance to different stress agents observed, could also be due, as previously mentioned, to the increase of intracellular concentration of RpoS (Ruiz et al., 2004), pointing out to the interconnection of different regulatory networks.

In our work, the derivative mutant strains assayed were impaired in

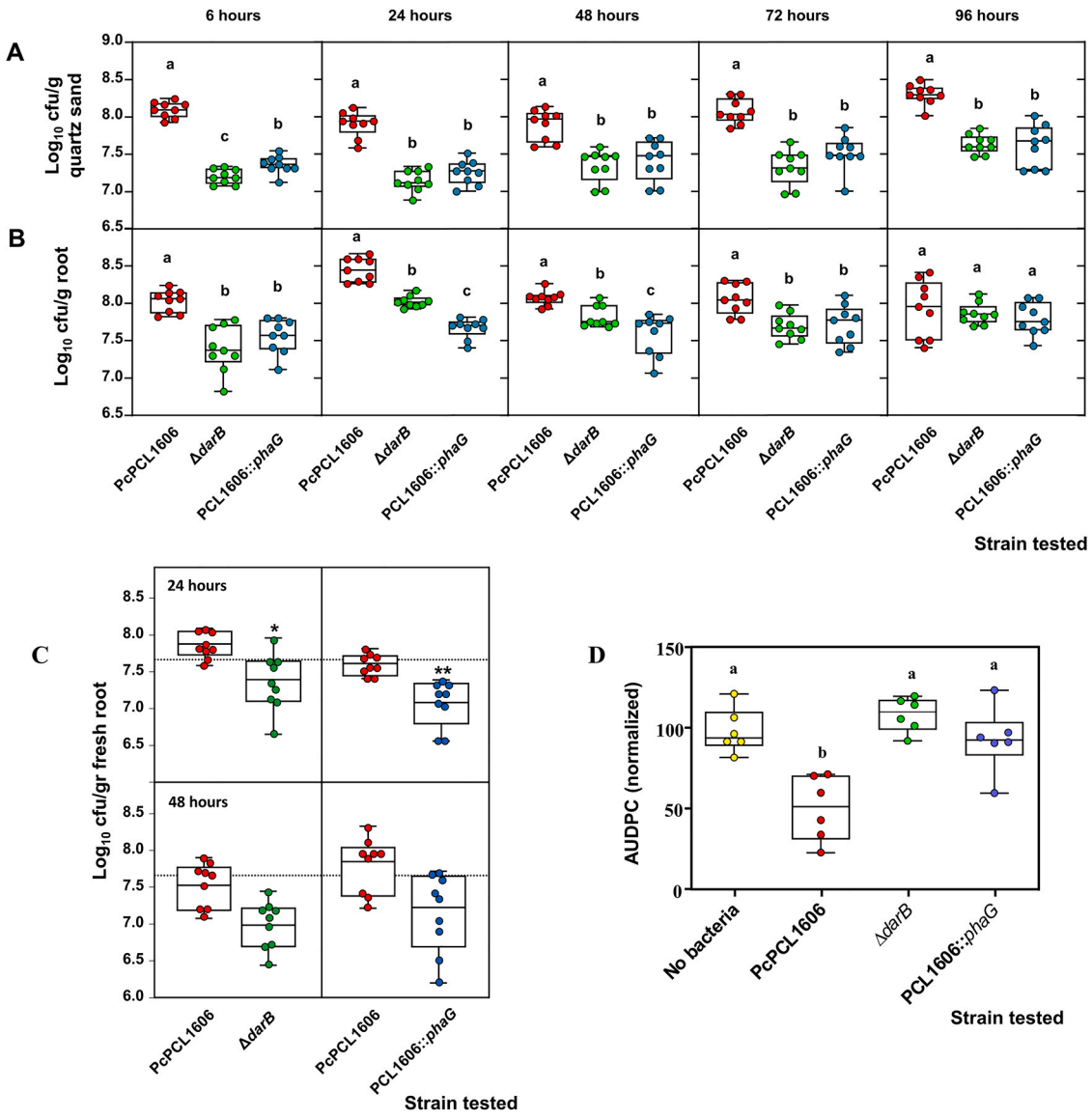

Fig. 5. Survival in competitive environments and biocontrol of PcPCL1606, Δ*darB* and PCL1606::*phaG* strains. (A) Bacterial survival in association to quartz sand and with avocado roots during a time course experiment (6, 24, 48, 72 and 96 h), expressed in $\log_{10}$ cfu/g of fresh sample. **(B)** Root competition assays. Roots were coinoculated in 1:1 proportions (wt:Δ*darB* and wt:PcPCL1606::*phaG*). **(C)** Competitiveness assay on avocado roots. Bacteria were counted after 24 and 48 h of competition. * and * * indicate significant statistical differences of the wild type strain with respect to the mutant (unpaired Student's t test) at $P \leq 0.001$ and $P = 0.002$, respectively. **(D)** Biological control against *Rosellinia necatrix* in avocado. The area under the disease progress curve (AUDPC) is represented. Every point is the average value from 5 independent avocado plants. The bar indicate the standard error. Different letters indicate significant differences between the samples according to analysis of variance (ANOVA; Bonferroni test; $P \leq 0.05$).

some survival traits, which reflects the survival and root competitiveness abilities of this bacterial strain. The derivative PcPCL1606::*phaG* and Δ*darB* mutant strains were impaired in competition for niche and nutrients in avocado roots when they were coinoculated with the wild-type PcPCL1606 strain. Similar results have been obtained for the beneficial rhizobacterium *Sinorhizobium meliloti* in alfalfa plants, where the mutant affected in PHA granule biosynthesis was also impaired in its root competition ability (Zhou et al., 2016).

Interestingly, with respect to biological control, our results confirmed that PHA production by PcPCL1606 was involved in its biological control activity against *R. necatrix*, and as far as we know, this is the first report linking biological control ability and PHA. Biological control agents must have long-term environmental persistence, and the production of PHA granules by PcPCL1606 could have a key role in increasing the root survival against stresses, thus conferring a competitive advantage in the rhizosphere (Okon and Itzigsohn, 1992; Hoffmann et al., 2000; Kadouri et al., 2005) that would be directly reflected in its biological control ability. The observed lack of competitiveness in the avocado rhizosphere displayed by the derivative mutants, could be an effect of a defective environmental adaptation to soil and rhizosphere conditions of the derivative strains impaired in PHA granule production (Willis and Walker, 1998). Indeed, the derivative strains impaired in PHA formation showed reduced survival in quartz sand, revealing the difficulties of survival under starvation conditions, but it seems to recover the wild type phenotype after four days at the end of the experiment in avocado roots, probably due to the continuous release of root exudates. These results confirmed the difficulties in adaptation and survival of the non-PHA-producing strains under starvation stress in low-nutrients environments, as it has been previously reported for *Azospirillum brasiliense*, *Ralstonia eutropha*, *Bacillus megaterium* and *P. chlororaphis* PA23 (van Elsas et al., 1992; Kadouri et al., 2003; Tribelli et al., 2020). Interestingly, as has been shown in this work for

PcPCL1606, a wild-type strain of *Azospirillum brasilense* showed higher root colonization than the non-PHA granule-producing mutant, thus indicating that PHA granule production is an important trait for root colonization by this bacterium (Tal and Okon, 1985; López et al., 1995), especially at the rhizosphere, where the C/N ratio favours PHA accumulation (Kadouri et al., 2003).

5. Conclusions

In summary, this work revealed the regulon of HPR during the interaction of PcPCL1606 with the avocado rhizosphere, confirming the HPR compound as a signalling molecule with a regulatory role. The production of HPR alters the bacterial response when interacting in the root habitat, mainly by modulation of bacterial metabolism. PHA granule biosynthesis is under the control of HPR production in the PcPCL1606 strain. PHA production improves the bacterial survival in the rhizosphere, and increases of tolerance to different stresses on plant roots. This performance has a crucial effect on bacterial survival, thus influencing bacterial competition for niches and nutrients in the avocado rhizosphere. For this competition, PHA production is directly involved in the biological control ability of PcPCL1606. Furthermore, the biosynthesis of PHA granules has gained attention in recent years from a biotechnological point of view, and this fact may suggest that PcPCL1606, a relevant biological control agent, could also be considered as a chassis vector for the production of PHA granules.

CRediT authorship contribution statement

Sandra Tienda: Data curation, Investigation, Writing – original draft. **José A. Gutiérrez-Barranquero:** Supervision, Writing – review & editing. **Isabel Padilla-Rojí :** Investigation, Data curation. **Eva Arrebola:** Methodology, Formal analysis. **Antonio de Vicente:** Conceptualization, Funding acquisition. **Francisco M. Cazorla:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

Authors declare that they have no competing interests in the present work.

Data Availability

Raw data uploaded and link cited in the text. All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127527.

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