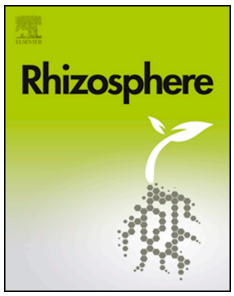

A black-pigmented pseudomonad isolate with antibacterial activity against phyllospheric pathogens

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Abstract

Bacteria of the *Pseudomonas* genus have been widely studied due to their antagonistic potential against a diverse group of fungal and bacterial phytopathogens, and their competence to colonize different plant tissues. We have isolated a rhizospheric pseudomonad that produced a black pigment, which is not a widespread trait within this genus. We confirmed that the isolate belonged to the *P. putida* complex through a MLSA analysis. We observed that the pigment synthesis was enhanced under high C:N ratios (25:1) and it was dependent of the carbon source, being maximized when we added glucose to M9. Besides, the supplementation of M9 with tryptophan inhibited the pigment production under C:N ratios of 4:1, and the addition of kojic acid reduced notably the pigment under favorable conditions. Ps. black presented several traits associated with plant-growth promoting potential with classical *in vitro* assays. Through a Tn*5* mutagenesis approach, we found 2 representative clones, PB1 and PB5, that were consistently unable to produce the pigment under several growth conditions and were not altered in their *in vitro* probiotic traits. When comparing with PB1 and PB5 performances, we observed that the pigment gives Ps. black a higher tolerance to oxidative stress and UV radiation exposure. When confronting Ps. black with different bacterial phytopathogens, we demonstrated that Ps. black could inhibit the growth of *Xanthomonas vesicatoria* Bv5-4a, *Pseudomonas syringae* pv. *tomato* DC3000, *P. syringae* pv. *syringae* B728a*, P. savastanoi* pv. *glycinea* B076 and *Clavibacter michiganensis* subsp. *michiganensis* Cm9. Except for Psg B076, this antagonism was lost for PB1 and PB5 and when performing the test for Ps. black with tryptophan supplementation. Thus, we suggest that the pigment should be involved in the bacterial antagonisms, and that Ps black contains more than one antibacterial mechanism.

Keywords

Pseudomonas - bacterial pigment – antibacterial activity – plant-growth promotion -

foliar pathogenic bacteria.

1. Introduction

Members of the *Pseudomonas* genus are broadly distributed in several ecosystems and interact with most eukaryotic taxa (Silby et al., 2011), contributing to their health or their disease (Burlinson et al., 2008; Loper et al., 2012; Mercado-Blanco and Bakker, 2007). From early studies on plant-growth promoting microorganisms, pseudomonads have shown to display multiple biochemical activities that contribute to plant fitness (Agaras et al., 2015; Hu et al., 2017; Lugtenberg and Kamilova, 2009). Besides, they have shown to colonize the rhizosphere (Lugtenberg, 2004; Mendes et al., 2011), the endosphere (Bauer et al., 2016; Ma et al., 2017; Ruiz et al., 2011) and the phyllosphere (Lindow and Brandl, 2003; Müller et al., 2016).

The phyllosphere is a harsh environment for microbial life: epiphytes are exposed to fluctuating environmental stresses, like solar radiation, low water availability and hyperosmotic stress, and nutrients are limited by the plant metabolism (Lindow and Brandl, 2003; Vorholt, 2012). Therefore, phyllospheric microbes have developed specific epiphytic-fitness traits, including their oligotrophic metabolism, several UV-protecting mechanisms (like pigmentation, high capacity of DNA repair and detoxification of reactive oxygen species), the preferential colonization of UV radiation (UVR)-protected sites of the plant, and the production of biosurfactants to move across the surfaces and of extracellular polymeric substances to protect against desiccation (Delmotte et al., 2009; Gunasekera and Sundin, 2006; Jacobs et al., 2005; Schreiber et al., 2005; Yu et al., 1999). Microbial populations in the phyllosphere can affect plant health positively or negatively. In fact, indigenous microbiota might affect the outcome of plant-pathogen interactions in the phyllosphere (Beattie and Lindow, 1995; Innerebner et al., 2011; Vorholt, 2012). Foliar bacterial pathogens cause important damages to crops, with significant economic losses and environmental impacts

(Kannan et al., 2015). Bacterial phytopathogens cause spots, blights, cankers, tissue rots, and/or hormone imbalances that lead to plant overgrowth, stunting, root branching, and leaf epinasty (Kannan et al., 2015). Foliar pathogens are difficult to control, as they can persist in seeds, plant debris, irrigation water, and weeds or non-host plants (Fayette et al., 2018; Knief et al., 2010).

The vast array of specialized compounds produced by *Pseudomonas* through their secondary metabolism (Biessy et al., 2018; Loper et al., 2012; Paterson et al., 2017) is a key aspect in the evolutionary success of this group (Silby et al., 2011). Particularly, pseudomonads have the potential to produce a wide set of antibiotics that can inhibit the growth of several plant pathogenic agents, like bacteria, fungi, insects or nematodes (Biessy et al., 2018; Garrido-Sanz et al., 2016; Haas and Défago, 2005; Haas and Keel, 2003), and they can also protect the plant indirectly by induction of systemic resistance (Bakker et al., 2007). Within the diversity of secondary metabolites produced by *Pseudomonas* species, there is a number of pigmented and chemically heterogeneous compounds, like melanins, pyoverdines and phenazines (Blankenfeldt and Parsons, 2014; Meyer, 2000; Plonka and Grabacka, 2006). Pigments are well-known because of their electron scavenger properties, offering protection to a wide range of stress conditions involving, principally, oxidative damage and UVR exposure (Sundin and Jacobs, 1999). Eventually, these pigments can act as metal chelators (Manirethan et al., 2018). In all life domains, dark black, brown and reddish pigments are associated with different forms of melanins (McGraw et al., 2005), although indigo-related compounds have also been described (Han et al., 2008). These pigments are produced by the oxidation of a hydroxylated aromatic compound from the amino acid tyrosine, leading to the accumulation of a quinone that spontaneously polymerizes under aerobic conditions. This biochemical strategy requires the presence of dedicated mono- or diphenol-oxidases, like tyrosinases or laccases (Han et al., 2008; Plonka and Grabacka, 2006). In bacteria, melanins can also be produced as a side effect in the catabolism of aromatic amino acids. Under certain conditions, like those imposed by

environmental stimuli (Pavan et al., 2020) or due to artificial constraints on the genetic background of the microorganism (Ben-David et al., 2018; Han et al., 2015; Nikodinovic-Runic et al., 2009), the accumulation of homogentisate (2,5 hydroxy-phenyl acetate) can lead to the spurious production of melanins, like pyomelanin. Representatives from different bacteria genera, like *Azospirillum, Ralstonia, Sinorhizobium, Bacillus* and *Streptomyces,* have been described to produce melanins (Pavan et al., 2020). Particularly, a few *Pseudomonas* species (*e.g.*, *P. stutzeri*, *P. aeruginosa* and *P. putida*) have been reported to produce different melanin-like compounds that fulfill several functions, in particular related to pathogenesis, motility and biofilm formation (Ganesh Kumar et al., 2013; Ketelboeter et al., 2014; Manirethan et al., 2018). Recently, some *P. fluorescens* isolates have been described to produce indigo-related pigments (Chierici et al., 2016), although the biological role of those pigments is until unknown. Therefore, dark-pigmented pseudomonads were not described yet to be involved in antibacterial activity. In this work, we describe the isolation and phenotypical characterization of a *Pseudomonas sp.* isolate from a grass rhizosphere, that produces a dark pigment under specific nutrient conditions, and we provide evidences of a functional relationship between pigment production and the inhibition of phyllospheric pathogenic bacteria.

2. Materials and Methods

2.1. Growth media conditions

Pseudomonas sp. “black” (henceforth named as Ps. black) was isolated from black-pigmented colonies developed upon plating on Gould’s S1 selective medium (Gould et al., 1985) a rhizospheric soil suspension from a natural grass patch sampled at the University campus (Agaras et al., 2012). Unless otherwise detailed for specific experiments, Ps. black was grown in minimal M9 liquid or agarized media, using 5 %

w/v glucose as the sole carbon source (M9-G5, Sambrook et al., 1989). The evaluation of the effect of different carbon sources (sucrose, citrate and glycerol; Biopack®, Argentina) and/or L-tryptophan (Trp; Biopack®, Argentina) on pigment production was performed with 1 % w/v of the corresponding carbon source and/or 2.4 mM of the amino acid.

2.2. Taxonomic assignment of Ps. black

Internal fragments of 16S rDNA, *gyrB* and *oprF* genes were amplified by PCR from thermal cell lysates following previously described procedures (Agaras and Valverde, 2018; Agaras et al., 2012) and their sequences were determined by the Sanger method at Macrogen Inc. (Seoul, Korea). In order to approach the taxonomic position within the established *Pseudomonas* complexes (Gomila et al., 2015), phylogenetic analyses were carried out by Multi Locus Sequence Analysis (MLSA), as previously described (Agaras et al., 2015), with slight modifications. Briefly, we selected 510 nt within the 5' region of the 16S rRNA gene (positions 109–618 in *Pseudomonas protegens* Pf-5, locus tag PFL_0119), 480 nt of the *oprF* gene (positions 262–741 in *P. protegens* Pf-5, locus tag PFL_1876), and 510 nt of the *gyrB* gene (positions 125–634 in *Pseudomonas protegens* Pf-5, locus tag PFL_0004). In all cases, the reference genome sequence for loci retrieval was NC_004129.6. The corresponding concatenated 16r DNA-*oprF-gyrB* sequences of 32 reference type strains were included in the analysis. Neighbor-joining trees were inferred from evolutionary distances calculated with the Kimura 2-parameter formula, using the software MEGA v7 (Kumar et al., 2016). Confidence analyses were undertaken using 1000 bootstrap replicates. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). The concatenated partial sequences of 16S rDNA, *ompA* and *gyrB* genes from *Escherichia coli* K-12 strain MG1655 (genome accession number NC_000913) were used to root the tree. 16S rDNA and *oprF* sequences have been

already published (Agaras et al., 2012). The partial *gyrB* sequence of Ps. black has been deposited into GenBank under accession number MT163171.

2.3. Tn*5* mutagenesis and screening of mutant clones with reduced pigment production

To obtain non-pigmented derivatives of Ps. black, we performed a Tn*5* mutagenesis approach by triparental conjugation within Ps. black as the acceptor strain, *Escherichia coli* CC118 λpir with the pBAMD1-2 plasmid as the Tn*5* donor strain and *E. coli* HB101 with the pRK600 plasmid as the conjugation helper strain, as previously described (Agaras et al., 2018; Martínez-García et al., 2011). Briefly, 5 ml overnight cultures were grown in nutrient yeast broth (NYB, 20 g l^{-1} nutrient broth, 5 g l^{-1} yeast extract; Biokar Diagnostics, France) that were incubated at 37 °C (for *E. coli* strains) and 28 °C for Ps. black strain, both at 200 rpm. Equal volumes (500 µl) of the three bacterial cultures were combined and centrifuged to obtain the cellular pellet; cells were resuspended in 50 µl of fresh NYB medium and transferred onto the border of an NA plate. Upon incubation at 37 °C for 5 h, cells were collected with 1 ml of fresh NYB and appropriate dilutions were plated onto M9-glucose 2 % supplemented with 100 µg ml^{-1} of kanamycin (Km) and 20 µg ml^{-1} of chloramphenicol (Cm). Clones with an altered pigmentation were conserved at -80 °C in 20% w/v glycerol. To identify the Tn*5* insertion site in each selected clone, we carried out an arbitrary nested PCR amplification with the methodology previously described (Martínez-García et al., 2011), followed by partial sequencing of the corresponding amplicons at Macrogen Inc. (Seoul, Korea).

2.4. *In vitro* characterization of plant-probiotic traits

For plate assays, we used normalized bacterial suspensions (OD_{600} = 1.0 in saline solution; SS, NaCl 0.85 %)) from overnight NYB cultures. Twenty microliters of each normalized suspension were spotted onto triplicate plates. Exoprotease and phospholipase activities were analyzed in skimmed milk agar or in egg yolk agar, respectively, as reported previously (Sacherer et al., 1994). Siderophore production was determined in CAS agar plates (Pérez-Miranda et al., 2007) and the ability of Ps. black to solubilize inorganic phosphate was studied in plates with NBRIP medium using $Ca_3(PO_4)_2$ as phosphate source (Nautiyal, 1999). The relative activity or solubilization efficiency was expressed as: (diameter of the observed halo/diameter of each bacterial spot) × 100 (Agaras et al., 2015).

HCN production was assayed qualitatively by the picrate-filter paper method (Egan et al., 1998). Lipopeptide production was evaluated qualitatively by the drop-collapse assay (Bodour and Miller-Maier, 1998). The secretion of quorum sensing signals of the N-acyl homoserine lactone (AHL) type was tested using the biosensor strains *Chromobacterium violaceum* CV026 and VIR07 (McClean et al., 1997; Morohoshi et al., 2008). For every strain, two parallel streaks of the biosensor were done along the middle of triplicate NA plates, with a separation of 1 cm from each other. At both sides of the streaks, 3 drops (10 µl) of normalized bacterial suspensions (OD_{600} = 1.0) were sown. Violacein production by the AHL reporter strains was recorded after 48 h of incubation at 28 °C (Agaras et al., 2015).

Established PCR approaches were used to evaluate the presence of four different genes related to the production of antibiotics: *phlD* for DAPG (McSpadden Gardener et al., 2001), *phzF* for phenazines (Mavrodi et al., 2010), *pltB* for pyoluteorin (Mavrodi et al., 2001) and *prnD* for pyrrolnitrin (de Souza and Raaijmakers, 2003). *Pseudomonas protegens* strain CHA0 was used as positive control for *phlD, pltB* and *prnD* PCR detection (Ramette et al., 2011), whereas *P. chlororaphis* subsp. *aurantiaca* SMMP3 served as positive control for *phzF* (Agaras et al., 2015). PCR reactions were carried out with thermal cell lysates as templates, following the cycling protocols reported for

each target gene in the aforementioned references.

2.5. Motility assays

Swimming and swarming capacity were evaluated by triplicate plate assays using M9-G5 supplemented with different agar concentrations (3 and 5 g l^{-1}, respectively) (Rashid and Kornberg, 2000). Bacterial progress was measured after 6 days of incubation at 28 °C.

2.6. Growth curve, dark pigment quantification and chemical inhibition of its production.

To evaluate the growth rate in M9-G5, Ps. black and its Tn*5* derivatives were grown in 125 ml Erlenmeyer flasks containing 20 ml of liquid culture by triplicate, and incubated at 28 °C and 200rpm. Cultures were started by appropriate dilution of saturated overnight precultures to a cell density equivalent to an OD_{600} = 0.01. After an incubation period of 12 h, 20 µl aliquots were sampled every 2 h to measure the OD_{600} of cultures, and 100 µl aliquots were centrifuged at 14000 rpm for 2 minutes, to measure the absorbance of the supernatant at λ = 400 nm (Abs_{400}), which was indicative of the pigment production (Ahmad et al., 2016; Nikodinovic-Runic et al., 2009). Under the same growth conditions, we measured the dark pigment production of Ps. black with the addition of inhibitors of the two most representative pathways for melanin biosynthesis. We used kojic acid (ARV-LAB, Argentina) at 100 and 200 µg ml^{-1} as a tyrosinase inhibitor of the L-3,4-dihydroxyphenylalanine (L-DOPA) synthesis pathway (Drewnowska et al., 2015). The operation of the 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis pathway was evaluated by the addition of tricyclazole at 125 and 250 µg ml^{-1} (BIM, DOW Agrosciences; Wheeler and Kuch, 1995).

2.7. UV sensitivity and oxidative stress assays

To evaluate the UV sensitivity of Ps. black and its Tn*5* mutants, we adapted a published protocol (Sundin and Jacobs, 1999). We grew bacterial cells up to OD_{600} ≈ 7 on liquid M9-G5, when the wild type culture is strongly pigmented. Then, 20 µl of different dilutions (from 0 to 10^{-4}) were spotted by triplicate onto M9-G5 plates and exposed to the radiation from an UV lamp (TUV30W G30T8, UV-C radiation) for 0.5, 1, 3, 5, 7, and 10 minutes, at a distance of 75 cm from the UV source. A control plate with dilutions from 10^{-5} to 10^{-8} was kept covered with its lid under the UV lamp for 10 minutes. Colonies were counted after the incubation period at 28 °C and the results were represented as the survival percentage.

Oxidative stress resistance was tested with a hydrogen peroxide disc diffusion assay. A volume of 100 µl of a cell suspension from a late exponential culture (OD_{600} ≈ 3 - 4) was spread onto M9-G5 agar plates by triplicate. Then, 8 µl of a H_2O_2 solution of 69mM, 138mM, 206mM, 275mM, 412mM y 550mM (Química Lomas, Argentina) were applied to sterile filter paper discs (5 mm diameter) gently deposited on the agar surface. After 24 h of incubation at 28 °C, the halo around the filter paper disk was measured for every tested H_2O_2 concentration. Triplicate plates were done for every strain (Nikodinovic-Runic et al., 2009).

2.8. Evaluation of the *in vitro* antagonistic potential

The bacterial phytopathogens employed in this study are listed in Table 1. We performed a confrontation of each pathogen strain with Ps. Black, by first spreading 100 µl of an overnight NYB culture of the pathogen (approximately 10^7 CFU ml^{-1}) on an M9-G5 agar plate; then, we spotted three 10 µl-drops of a suspension of Ps. black (wild type or its Tn*5* mutants; OD_{600} = 2) in every plate. After an incubation of 72 h at 28 °C, we measured the diameter of the Ps. black macrocolony and the inhibition halo of the

Table 1. Bacterial phytopathogens used in this study

Strains	Source	Reference
Xanthomonas vesicatoria Bv5-4a (LM159)	Environmental isolate from INTA Bella Vista, Corrientes, Argentina	(Richard et al., 2017)
Pseudomonas syringae pv. *tomato* DC3000	Spontaneous rifampicin-resistant strain from the wild-type isolate DC52	(Cuppels, 1986)
Pseudomonas syringae pv. *maculicola* ES4326 (Psm ES4326)	Environmental isolate from radish rhizosphere; USA	(Dong et al., 1991)
Pseudomonas syringae pv. *syringae* B728a	Environmental isolated from a snap bean leaflet in Wisconsin, USA	(Loper and Lindow, 1987)
Pseudomonas savastanoi pv. *glycinea* B076	Environmental isolate from a soybean leaflet near Champaign, Illinois, USA	(Qi et al., 2011)
Clavibacter michiganensis subsp. michiganensis Cm9	Environmental isolate from a bacterial canker of tomato, Florencio Varela, Buenos Aires, Argentina	(Romero et al., 2003)

test pathogen. The relative antagonistic activity was expressed as explained in Section 2.4.

2.1. Statistical analyses

Experiments were repeated at least twice and done by triplicate each time. Average and standard deviation values were calculated from recorded data and used to carry out ANOVA or generalized linear models (for percentage or proportion values) with the software GraphPad Prism v. 7 (GraphPad Software, La Jolla California USA, www.graphpad.com) or Infostat v. 2018 (Di Rienzo et al., 2018), respectively. When appropriate, multiple comparison tests were done with the Tukey's or DGC tests (Di Rienzo et al., 2018) to evaluate if the differences between average values were statistically significant. All the analyses were done at $p < 0.05$.

3. Results

3.1. Ps. black is a member of the *P. putida*-complex and presents plant-probiotic traits

Upon plating a suspension of a grass rhizosphere sample onto the *Pseudomonas* selective medium S1, one of the colonies draw our attention because it developed a particular diffusible black pigmentation. Thus, we decided to name it Ps. black. MLSA analysis showed that Ps. black is a member of the *P. putida* complex, with high similarity to strain *P. putida* S13.1.2 (Chong et al., 2016). To evaluate if the dark pigment influenced its fitness, we developed a genome-wide Tn*5* mutagenesis to search for colorless colonies. From nearly 600 clones screened, we obtained 6 clones with absence of pigment production by observation of the colony morphology with naked eye on M9 citrate 2% p/v plates. Among them, PB1 and PB5 clones presented non-pigmentation in all the tested growth conditions (data not shown). Thus, we decided to

Table 2. Plant-growth promoting traits evaluated *in vitro* for Ps. black

Plant-growth promoting traits		Relative activity or qualitative test result
Phospholipases *		0
Exoproteases *		0
HCN		-
pltB gene		+
prnD gene		-
phlD gene		-
phzF gene		-
Siderophores *		129.0 ± 5.3
Lipopeptides		-
QS signals	C4-C8 AHL	-
	C10-C16 AHL	+
$Ca_3(PO_4)_2$ solubilization *		147.6 ± 11.6
Motility	Simmming (mm)	51.0 ± 19.5
	Swarming (mm)	0

* Relative activity values ± SD are shown.

Plus and minus symbols mean a positive or negative result, respectively, in the qualitative assay or PCR approach.

use Tn*5* clones PB1 and PB5 as negative controls of pigment production for further analyses.

The battery of *in vitro* tests of plant-growth promoting traits revealed that Ps. black has the ability to solubilize $Ca_3(PO_4)_2$ and to produce siderophores (Table 2), with a similar performance to previously characterized environmental isolates (Agaras et al., 2015). We used a battery of PCR to reveal the presence of biosynthetic gene involved in antibiotic production (Agaras et al., 2015). Our analyses show that Ps. black contains a putative copy of the *pltB* gene, which could enable this isolate to produce pyoluteorin. Ps. black was able to synthesize long chain AHLs, but not short ones. Finally, Ps. black displayed swimming, but not swarming, motility (Table 2). The same set of assays were performed for the PB1 and PB5 Tn*5* derivatives, and in general, their performance did not differ from that of Ps. black (data not shown).

3.2. Ps. black produces the black pigment under high C:N ratios and without amino acid supplementation.

After the observation of the production of a black pigment on S1 agar plates (Figure 1a), we noticed that Ps. black did not synthesize the pigment when growing on NA plates under the same conditions (Figure 1b). As these growth media strongly differ in their carbon:nitrogen (C:N) ratio (25:1 and 4:1, respectively), we explored the effect of the C:N ratio in M9 minimal medium and we found that an increase in the C.N ratio improved the pigment synthesis (Figure 1c). However, in contrast with the performance on NA, on M9 with glucose 1 % (also C:N ratio 4:1), the pigment production was not absent, inferring an additional factor that influence the pigment synthesis other than the C:N ratio (Figures 1b and 1c). Besides, we observed an effect of the carbon source on pigment production, which was reduced in the presence of sucrose (Figure 1d). Finally, a strong inhibition, similar to that observed on NA plates, was achieved by the addition of L-tryptophan under low C:N conditions (Figure 1e).

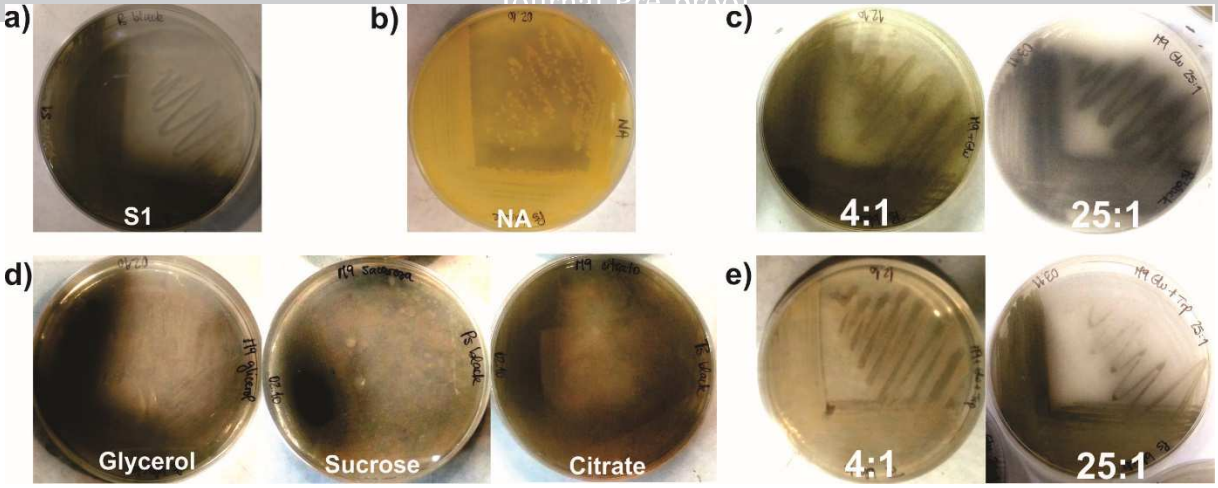

Figure 1. Growth of *Pseudomonas* sp. black on different agar media after 48 h. **a)** On Gould's S1, this isolate produces a dark blackish pigment; **b)** On NA plates, the pigmentation is lost; **c)** when comparing minimal medium M9 supplemented with 1 % (4:1) or 5 % (25:1) glucose, the pigment was brown at low C:N ratio and black at high C.N ratio; **d)** carbon sources also affected the pigment production, which was reduced when sucrose was the carbon source available; **e)** on M9-glucose, the addition of tryptophan altered the pigment production, which was inhibited under low C.N ratios (4:1).

Overall, Ps. black seems to produce two different kinds of pigments: a brownish compound under low C:N ratio or with citrate as carbon source; or a blackish compound when glucose is available at high C:N ratios. Besides, the pigment is synthesized without any amino acid supplementation (Figure 1). We cannot rule out that the brownish pigment can be chemically and spontaneously transformed into the blackish one, depending on the amount of pigment production and/or the nutrient sources in the medium

3.3. The pigment produced by Ps. black is accumulated in the culture supernatant during the stationary phase and its synthesis may be dependent of the L-DOPA biosynthesis pathways.

When we compared the growth performance of the wild type isolate and of the PB1 and PB5 mutants in M9-G5, we observed that the Tn*5* insertion did not affect their growth rate (Figure 3). Although we observed that the culture began to turn gray during the late exponential phase (OD_{600}~ 3-4), the pigment was not detected in the supernatant until Ps. black reached the stationary phase, being first observed after 27

h of growth and reaching a maximum at an OD_{600} > 5. For PB1 and PB5 mutants, the pigment was absent in the supernatant all along the growth period of 30 h (Figure 3).

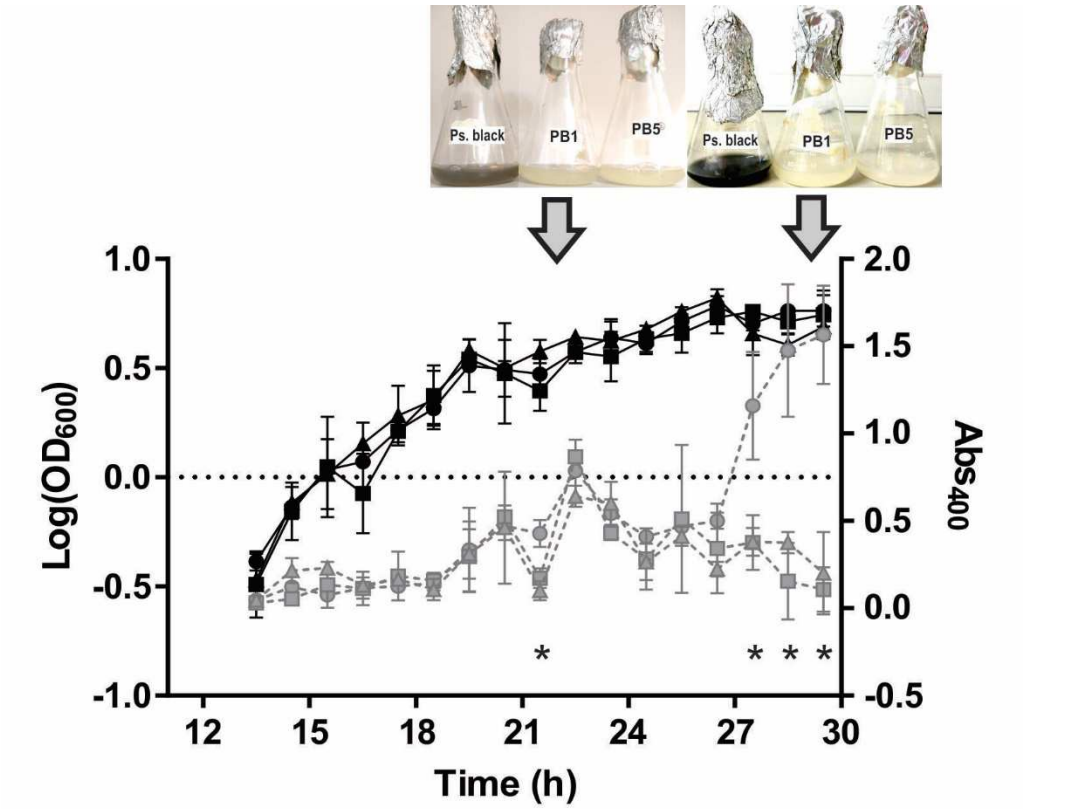

Figure 2. Growth curve (black) and pigment production (gray) of *Pseudomonas* sp. black wild type (circles), PB1 (squares) and PB5 (triangles). The pigment was traced in the supernatants by Abs_{400}. Bars indicate the SD value at each point of triplicate cultures. Asterisks denote statistically significant differences in the pigment production between the wild type and both Tn*5* mutants (Student *t* test, $p < 0.05$).

In order to get insights into the mechanism of dark pigment production in Ps. black, we employed a pharmacological approach to evaluate the changes in pigment accumulation. We used two well-known inhibitors of melanogenesis in bacteria: kojic acid, as an inhibitor of tyrosinases for the L-DOPA-dependent pathway, and the fungicide tricyclazole, which inhibits the biosynthesis of melanin using DHN as precursor (Drewnowska et al., 2015, Wheeler and Kuch, 1995). Our results showed a moderate negative effect of kojic acid on pigment accumulation, without altering the growth of Ps. black. On the other hand, tricyclazole had no effect on pigment production in M9-G (Supp. Figure 2).

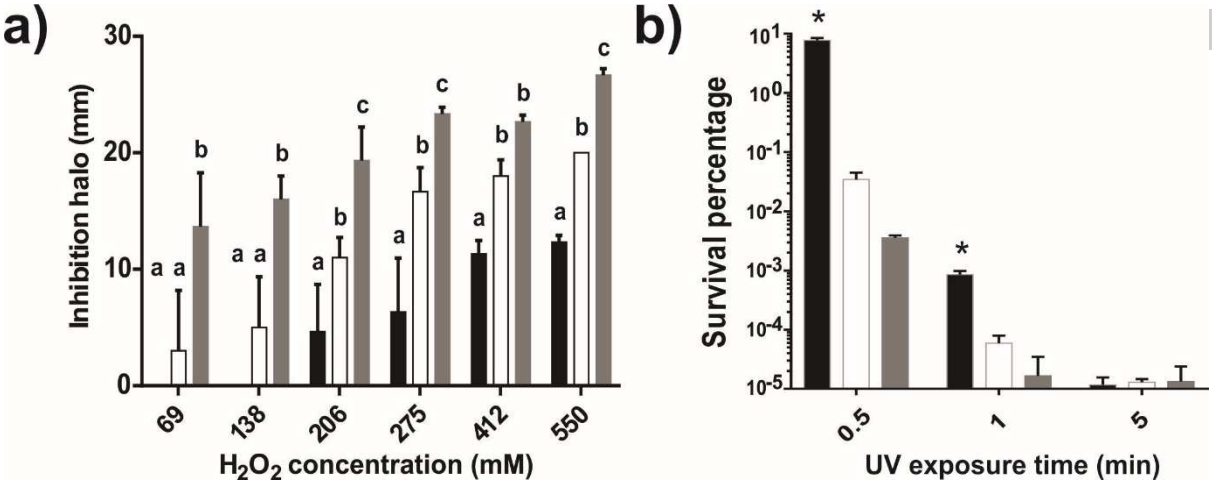

Figure 3. Sensitiveness to oxidative stress (**a**) and resistance to UV exposure (**b**) of *Pseudomonas* sp. black (black), PB1 (white) and PB5 (gray) Tn*5* mutants. a) Different letters indicate statistically significant differences in the Tukey's comparison test, after the one-way ANOVA ($p < 0.05$). b) As survival percentages do not follow a normal distribution, this experiment was analyzed with generalized linear model. Asterisks denote a statistically significant difference (LSD Fisher, $p < 0.05$).

3.4. The black pigment contributes to the tolerance of Ps. black to oxidative and UV stresses

From the disk diffusion assays, we observed that the Tn*5* derivatives of Ps. black were more sensitive to H_2O_2, being PB5 even more susceptible than PB1 (Figure 3a). Besides, Ps. black showed a higher survival after the exposure to UV-C light for up to 1 minute, when compared to PB1 and PB5 (Figure 3b).

3.5. Ps. black can inhibit the growth of bacterial phytopathogens *in vitro* and the antagonistic activity is linked to pigment production.

In confrontation assays, we found that the wild type Ps black inhibited the growth of all tested bacterial phytopathogens (Figure 4), except for Psm ES4326 (data not shown). However, the non-pigmented Tn*5* mutants PB1 and PB5 lost the antagonistic activity, except for against Psg B076 (Figure 4). Coincidentally, the same results were obtained for the wild type strain when we supplemented the growth media with tryptophan (Figure 4), which suppresses pigmentation of Ps. black in M9 under low C:N ratios (Figure 1e). These results point to the operation in Ps. black of an inhibitory

mechanism for this set of bacterial phyllospheric pathogens that would be linked to pigment production. When we analyzed the results from Psg B076 in ANOVA tests, there was not any significant treatment effect ($p < 0.05$). The latter suggests that the antagonistic activity of Ps. black against Psg B076 relies on a second and pigmentation-independent mechanism of inhibition.

4. Discussion

4.1 Regulated production of pigment in strain Ps. black

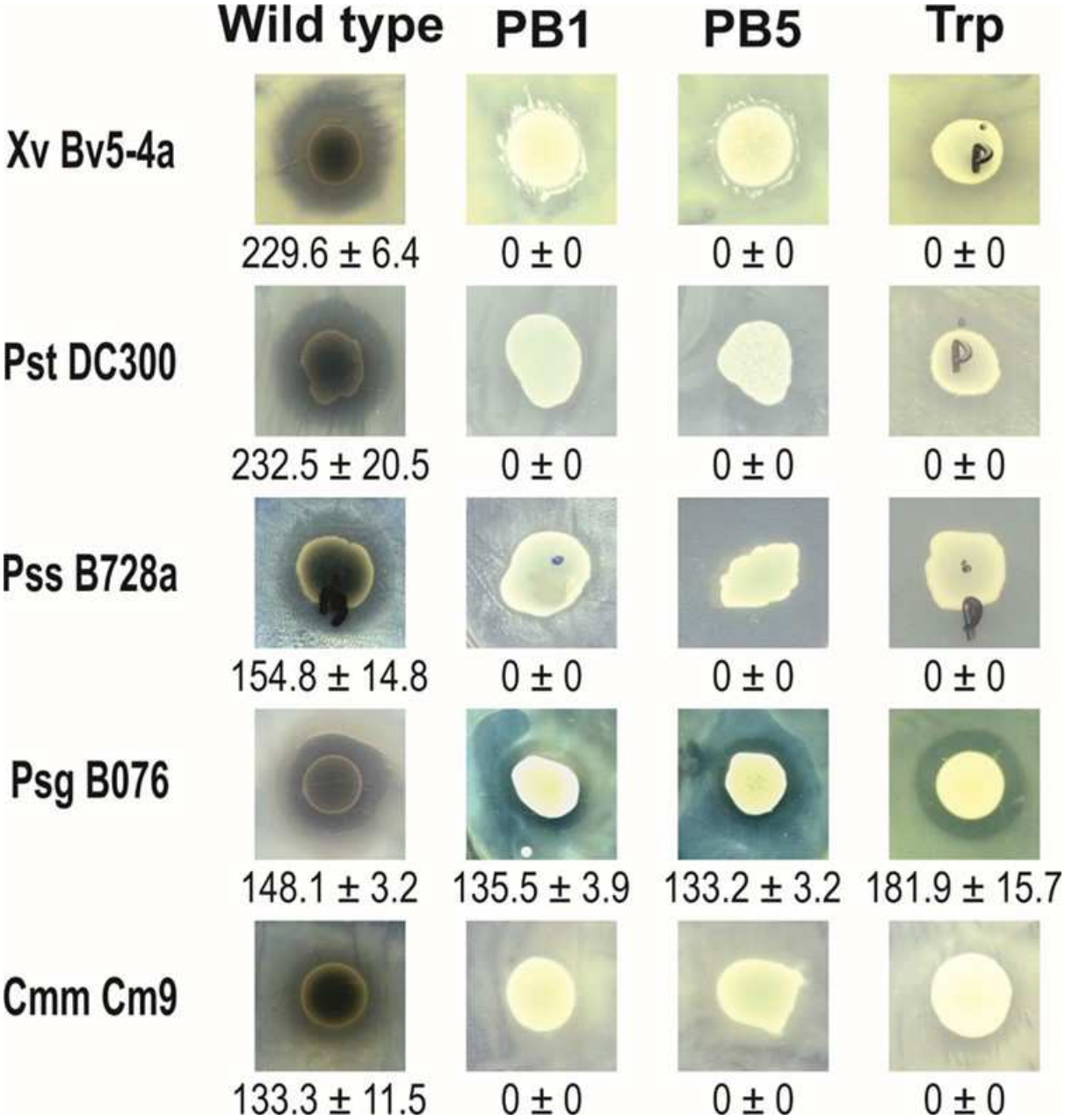

Figure 4. Relative inhibition (RI) activities of *Pseudomonas* sp. black and its Tn5 derivatives against bacterial phytopathogens. We show representative images of the inhibition halos in M9-G5 (wild type, PB1 and PB5 columns) and the images from the M9-GT (Trp column). RI values ± SD are shown below every image. Halos were measured after 96 h of incubation from triplicate plates, based on the formula explained in Section 2.4

367

368 From a grass rhizosphere sample, we obtained a blackish isolate on S1 growth
369 medium plates. Its ability to grow on this selective medium (Gould et al., 1985), and the
370 presence of the *oprF* gene allowed us to classify it as a member of the *Pseudomonas*
371 genus (Agaras et al., 2012). An MLSA allowed us to position Ps. black within the *P.*
372 *putida* complex (Supp. Figure 1). Although it showed the highest gene sequence

similarity with *P. putida* S13.1.2, the black pigmentation was not a described attribute for this strain (Chong et al., 2016).

The production of gray/black pigments has been described mainly within the *P. fluorescens* complex, for isolates involved in meal spoilage (Kröckel, 2009; Nikodinovic-Runic et al., 2009; Reichler et al., 2019; Zerrad et al., 2014; Zhao et al., 2019). Another kind of pigments, as the brownish melanin-like compounds, are produced by *P. aeruginosa* species, and they have been suggested to increase their pathogenicity (Nosanchuk and Casadevall, 2003; Solano, 2014). We found that in Ps. black, pigment production was linked to the nutritional composition of the growth medium, as this isolate was strongly pigmented in media with high C:N ratios and with glucose as the carbon source (Figure 1). A similar behavior was reported for an *Aspergillus fumigatus* strain, which also showed higher levels of melanin under a C:N ratio of 20.6, and when glucose was the main carbon source (Raman et al., 2015). In the *Vibrio cholerae* 569B strain, melanin synthesis was induced only under stressful growth conditions (*i.e.*, nutritional limitations, hyperosmotic shocks, extreme pH values, elevated temperatures) (Coyne and al-Harthi, 1992), and thus melanogenesis in *V. cholerae* strains was linked with survival in different environments and expression of virulence factors (Coyne and al-Harthi, 1992; Noorian et al., 2017; Valeru et al., 2009). Besides, for some *Pseudomonas* strains isolated from decomposed dairy meals, it has been demonstrated that the pigment production varied with the growth conditions, and it was higher under glucose-rich conditions, like on potato dextrose agar (Andreani et al., 2015b; Reichler et al., 2019). However, it does not seem to be a generalized behavior of pigment-producing microorganisms, as some bacterial species are not conditioned by amino acids or low C:N ratios and they can synthesize black pigments in rich culture media (Drewnowska et al., 2015; Ganesh Kumar et al., 2013). On the other hand, we observed that Ps. black was able to produce the pigment in M9 medium without supplementing any amino acid (Figures 1c, 1d). For several microorganisms, L-tyrosine is necessary to induce the pigment production (Almeida-Paes et al., 2012; Hoti

and Balaraman, 1993; Kotob et al., 1995; Singh et al., 2018), and sometimes L-Trp boosts it too (Mencher and Heim, 1962). Nevertheless, we found a negative effect of L-Trp on the black pigment synthesis in Ps. black (Figure 1e), like it was reported for melanin production in eukaryotic cells (Chakraborty and Chakraborty, 1993). Besides the dedicated nutritional control over pigment production, we cannot rule out the presence of another layer of regulation. As the dark-pigment accumulated during the stationary phase, and Ps. black produces long-chain AHLs (Table 1), quorum sensing could be involved in the regulation of the biosynthetic genes.

The presence of the pigment in the supernatant of batch cultures in M9-G5 medium was detected in the late exponential/stationary phases of growth (Figure 2), as reported for other bacteria (Ganesh Kumar et al., 2013; Singh et al., 2018). Although it was not completely inhibited as it have been shown for other pseudomonads (Ganesh Kumar et al., 2013), the reduction in pigment production observed with the addition of kojic acid (Supp. Figure 2), let us to infer that the synthesis of the black pigment in Ps. black would involve a typical tyrosinase (or other copper-dependent oxidase) of the L - DOPA pathway (Chang, 2009; Hoti and Balaraman, 1993). On the other hand, the absence of inhibition with tricyclazole, allow us to discard the pentaketide pathway as described for fungi and some bacteria (Lee et al., 2003, McMahon et al., 2007). Regarding the impact of L-Trp on the production of black pigment, this is the first report of an inhibitory effect of tryptophan on the synthesis of a dark pigment in bacteria. We will deepen the characterization of the collection of the colorless Tn*5* derivatives to elucidate the biosynthetic pathway of this pigment in Ps. black.

4.2 Tolerances to oxidative stress and UV exposure of strain Ps. black.

As a phyllosphere-adaptative fitness of epiphytic bacteria, pigments are linked to stress tolerance (Ahmad et al., 2016; Jacobs et al., 2005; Sundin and Jacobs, 1999). Here we showed that Ps. black is able to resist up to 138 mM of H_2O_2, and that this tolerance

was strongly reduced in the non-pigmented PB1 and PB5 mutants (Figure 3a). A comparable effect was observed with UV exposure, as Ps. black showed a higher survival percentage for up to 1 minute of exposure (Figure 3b).

4.3 Plant-growth promoting traits and antibacterial activity of strain Ps. black.

Ps. black did not show any of the typical biocontrol-related activities found in *Pseudomonas*, but the presence of the *pltB* gene for pyoluteorin synthesis (Table 2). This antibiotic was demonstrated to be involved in the growth inhibition of the oomycetes *Phytophthora* (Ohmori et al., 1978) and *Phytium* (Maurhofer et al., 1994) and of some bacterial pathogens, like *Erwinia amylovora* (Yan et al., 2017) and uropathogenic isolates (Mussa and Ziayt, 2018). However, Ps. black did not show antifungal activity against the pathogens *Macrophomina phaseolina, Fusarium oxysporum, F. solani, F. verticilloides, F. semitectum, Colletotrichum graminicola, Sclerotinia sclerotium and Phomopsis* sp. (Agaras et al., 2015) when co-cultured on potato dextrose agar plates (data not shown). Different authors have demonstrated that phenazines, tailocins and organocopper compounds were the main metabolites involved in the antagonistic activity of pseudomonads against *Xanthomonas* species (de Oliveira et al., 2016; Príncipe et al., 2018; Shanmugaiah et al., 2010; Xu et al., 2015). The Cmm antagonism by pseudomonads was attributed to the production of the antibiotics 2,4□diacetylphloroglucinol (DAPG) and HCN (Lanteigne et al., 2012; Paulin et al., 2017), whereas a bacteriocin has been described to be involved in the inhibition of *P. savastanoi* pv. *savastanoi*, although it was ineffective against a Psg strain (Lavermicocca et al., 1999). On the other hand, the mechanism of biological control of pseudomonads against *P. syringae* was primarily the activation of ISR in plants (Ji et al., 2006; Weller et al., 2012). Here, we showed with *in vitro* confrontation tests that Ps. black inhibits the growth of the tomato pathogens Xv Bv5-4a, Cmm Cm9 and Pst

DC3000, the soybean pathogen Psg B076, and the bean pathogen Pss B728a (Figure 4). Except for Psg B076, these antagonisms were lost when the M9 media was supplemented with Trp, or when we tested the PB5 clone, which does not produce the pigment but conserves all the rest of *in vitro* phenotypes evaluated for the wild type (Figure 4). Besides, the antagonistic potential was also lost when the assays were performed in NA plates (data not shown). Thus, we suggest that Ps. black possess more than one mechanism involved in its antibacterial activity, and that the inhibition of Ps. black against Xv, Pss, and Pst is functionally associated with pigment production. Besides, the black-pigmented isolate *Pseudomonas* sp. 11K1, which lacks genes for pyoluteorin synthesis, also showed antibacterial activity against *X. oryzae* RS105 and it was not linked to the cyclic lipopeptides responsible for its antifungal activity (Zhao et al., 2019). Different authors have shown the antibacterial activity of melanins against several clinical pathogens, like *Shigella* sp., *Enterobacter faecalis, Candida albicans, P. aeruginosa, E. coli, Klebsiella pneumoniae* and *Bacillus* sp. (Arun et al., 2015; Łopusiewicz, 2018; Manivasagan et al., 2013; Zerrad et al., 2014). Nevertheless, the antibacterial activity against phytopathogens was not previously reported, except for *Erwinia* (Zerrad et al., 2014), neither the inhibition mechanism was explained. We are performing analyses of the pigment nature, chemical structure and properties to specifically assess its involvement in the antagonistic potential of Ps. black and to deeply understand the mechanism.

The stress-tolerance benefits, together with the antagonistic potential against bacterial foliar pathogens, makes Ps. black a good candidate for testing its performance as a biological control agent against foliar plant diseases caused by *Xanthomonas* and *P. syringae* pathovars. In fact, the photoprotection based on melanins has been studied for *B. thuringiensis* biopesticides (Sansinenea and Ortiz, 2015). Historically, bacterial speck and spot have been controlled with copper-based bactericides (Bonn and Lesage, 1984; Jones et al., 1991). However, many copper-tolerant strains have appeared since then (Alexander et al., 1999; Marco, 1983; Martin et al., 2004),

485 including Xv BV5-4a (Richard et al., 2017). Thus, copper alternatives have been
486 investigated, combining biological control with plant activators (Ji et al., 2006; Louws et
487 al., 2001; Trueman, 2015). *In planta* and field assays should be performed to evaluate
488 if the foliar application of Ps. black can reduce the impact of the diseases caused by
489 the phytopathogens antagonized *in vitro.* Besides, as Ps. black has been isolated from
490 a grass rhizosphere, it should be possible that this isolate would be able to colonize a
491 host plant and promote its growth also by phosphate solubilization or the increase of Fe
492 availability, or even boost the ISR and enhance the plant protection (Table 2).
493 To the best of our knowledge, this is the first report showing a linkage between the
494 bacterial production of an extracellular, soluble, dark pigment and the inhibition of
495 several foliar phytopathogenic bacteria. Additional studies will be necessary to
496 determine the chemical nature of this pigment, to understand its biological role and how
497 it would help the bacterium to control foliar bacteria causing plant diseases.
498
499 4. Conclusion
500
501 A dark pigment-producer *Pseudomonas* isolate named Ps. black, which is member of
502 the *P. putida* complex, was able to antagonize several phytopathogenic bacteria. We
503 provide solid evidences that this ability is linked with the synthesis of the black pigment,
504 which in turn may be dependent of L-DOPA melanin-biosynthesis pathways. Due to its
505 ability to tolerate high UVR exposure and stressful osmotic conditions, Ps. black is a
506 good candidate for being tested for the development of an agricultural bioinput to
507 control phytopathogens through foliar spray application.
508
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510
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A black-pigmented pseudomonad isolate with antibacterial activity against phyllospheric pathogens

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: