environmental microbiology reports

Environmental Microbiology Reports (2016) **8**(5), 896–904 doi:10.1111/1758-2229.12454

Role of phenazines and cyclic lipopeptides produced by *pseudomonas* sp. CMR12a in induced systemic resistance on rice and bean

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Summary

***Pseudomonas* sp. CMR12a produces two different classes of cyclic lipopeptides (CLPs) (orfamides and sessilins), which all play a role in direct antagonism against soilborne pathogens. Here we show that *Pseudomonas* sp. CMR12a is also able to induce systemic resistance to *Magnaporthe oryzae* on rice and to the web blight pathogen *Rhizoctonia solani* AG2-2 on bean. Plant assays with biosynthesis mutants of *Pseudomonas* sp. CMR12a impaired in the production of phenazines and/or CLPs and purified metabolites revealed that distinct bacterial determinants are responsible for inducing systemic resistance in these two pathosystems. In rice, mutants impaired in phenazine production completely lost their ability to induce systemic resistance, while a soil drench with pure phenazine-1-carboxamide (PCN) at a concentration of 0.1 or 1 μM was active in inducing resistance against *M. oryzae*. In bean, mutants that only produced phenazines, sessilins or orfamides were still able to induce systemic resistance against Rhizoctonia web blight, but a balanced production of these metabolites was needed. This study not only shows that *Pseudomonas* sp. CMR12a can protect rice to blast disease and bean to web blight disease, but also displays that the determinants involved in induced systemic resistance are plant, pathogen and concentration dependent.**

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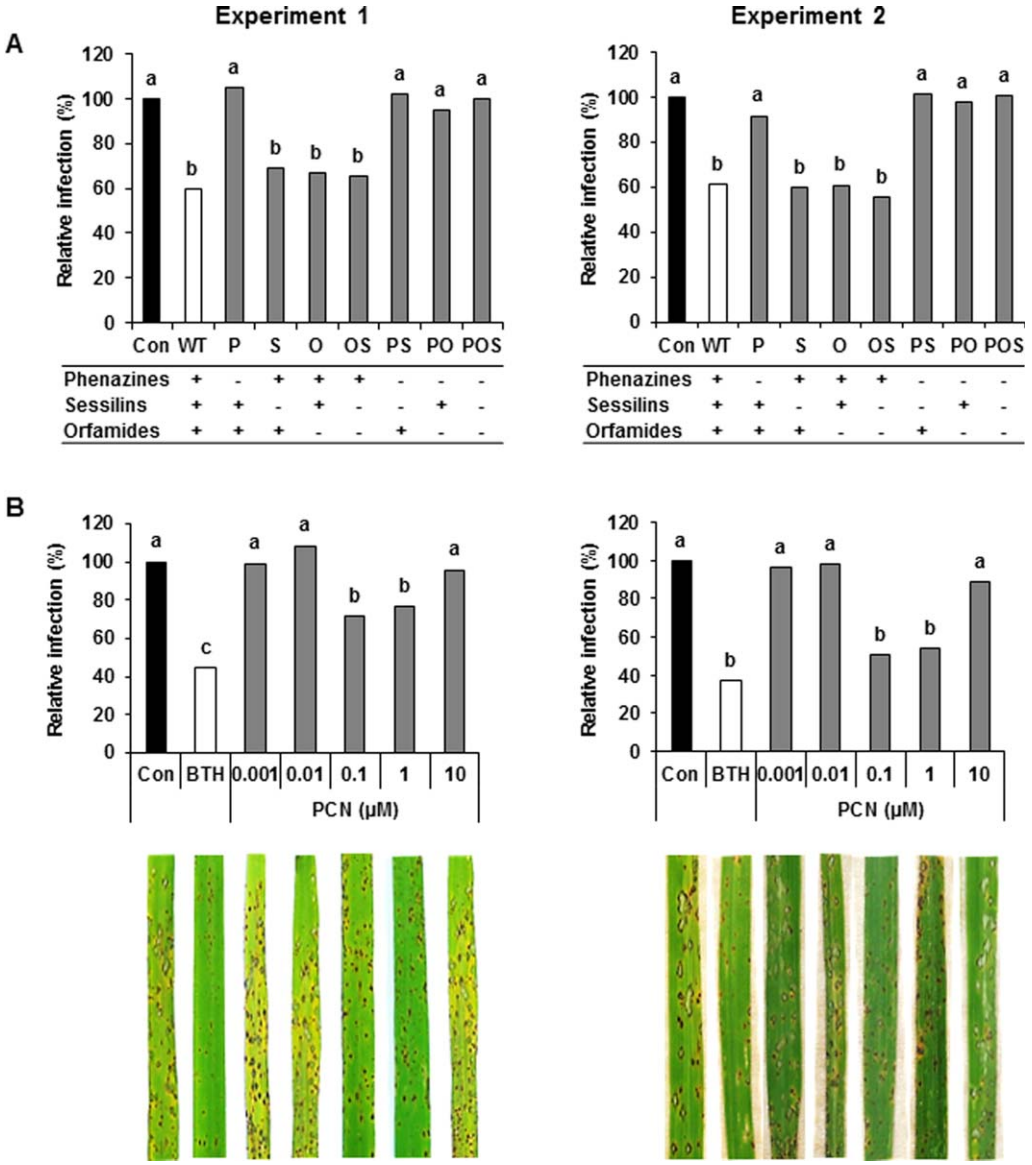
Introduction

Mechanisms involved in biocontrol of plant diseases by rhizosphere-inhabiting plant beneficial microorganisms include competition for nutrients or space, antibiosis, mycoparasitism and elicitation of the plant's innate immunity, also called induced systemic resistance (ISR) (Glick, 2012). ISR is defined as a type of systemically enhanced resistance against a broad spectrum of pathogens triggered upon root colonization by selected strains of non-pathogenic bacteria (De Vleesschauwer and Höfte, 2009) and arises as an efficient way to control plant diseases (Walters *et al.*, 2013). Numerous bacterial traits can trigger ISR, including structural compounds of the cell envelope and secreted metabolites. In this way ISR resembles PAMP-triggered immunity, an immune response in plants triggered by recognition of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) (Boller and He, 2009).

Pseudomonas spp. are among the most frequently studied model microorganisms for biocontrol and have been experimentally or commercially applied as biocontrol agents for crops (Höfte and Altier, 2010; Velivelli *et al.*, 2014; Olorunleke *et al.*, 2015a). *Pseudomonas* sp. CMR12a, a well-studied biocontrol strain, was isolated from rhizosphere of red cocoyam [*Xanthosoma sagittifolium* (L.) Schott] in Cameroon (Perneel *et al.*, 2007). *Pseudomonas* sp. CMR12a is an uncharacterized new species that is taxonomically positioned between *P. protegens* and *P. chlororaphis* in the *P. fluorescens* group (Ruffner *et al.*, 2015, Flury *et al.*, 2016). *Pseudomonas* sp. CMR12a produces phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN), and two different classes of CLPs. The strain produces orfamide-type CLPs, which are commonly produced by *P. protegens* strains (Ma *et al.*, 2016) and sessilins, which are related to tolaasins, CLPs produced by the mushroom pathogen *P. tolaasii* (D'aes *et al.*, 2014). Phenazines are nitrogen-containing heterocyclic compounds that can be produced by *Brevibacterium*, *Burkholderia*, *Pseudomonas*, *Pectobacterium* and *Streptomyces* (Budzikiewicz, 1993; Mavrodi *et al.*, 2010) Phenazines play important roles in antibiosis, antitumor, antimalaria, antiparasitic activities, biofilm formation, and can even act as virulence factors



(Laursen and Nielsen, 2004; Ramos *et al*., 2010). *Pseudomonas* derived CLPs are nonribosomal peptide synthase (NRPS) synthesized lipopeptides, harboring hydrophobic and hydrophilic properties. These NRPS-derived CLPs can interact with cell membranes, and display broad biological activities against bacteria, fungi, viruses, oomycetes and protists (Raaijmakers *et al*., 2010). Our previous studies showed that *Pseudomonas* sp. CMR12a has efficient biocontrol activity in cocoyam, bean and Chinese cabbage (Perneel *et al*., 2007; D'aes *et al*., 2011; Olorunleke *et al*., 2015b). Moreover, phenazines and CLPs play an active role in the direct antagonism of *Pseudomonas* sp. CMR12a towards *Pythium* spp. (unpublished data) and *Rhizoctonia solani* (Olorunleke *et al*., 2015b).

However, the potential role of phenazines and CLPs from *Pseudomonas* sp. CMR12a in induced systemic resistance has not yet been studied. In this study, we evaluated the ISR capacity of *Pseudomonas* sp. CMR12a and its main metabolites against rice blast caused by *M. oryzae*, and against Rhizoctonia web blight in bean. Rice (*Oryza sativa* L.) is the most important staple food in subtropical and tropical area all over the world. Rice blast disease threatens rice productivity worldwide (Dean *et al*., 2012). The causal agent, the ascomycete *M. oryzae*, is a hemibiotrophic pathogen. Web blight is a leaf disease on bean (*Phaseolus vulgaris*) caused by different anastomosis groups (AGs) of the necrotrophic fungus *R. solani* Kühn (Teleomorph: *Thanatephorus cucumeris* Frank Donk), including *R.*

solani AG2-2 (Nerey *et al*, 2010) and is a severe constraint in bean production (Gálvez *et al*. 1989).

Results and discussion

Treatment of rice roots with *Pseudomonas* sp. CMR12a resulted in a 40% reduction in the number of susceptible rice blast lesions caused by *M. oryzae* compared with the control treatment in two independent experiments (Fig. 1A). More specifically, control plants showed the typical "eye" type sporulating lesions with a grey center, which were around 3 mm in length, while lesions on plants treated with *Pseudomonas* sp. CMR12a were smaller and of the resistant type (no grey center, no sporulation). Colonization data of *Pseudomonas* sp. CMR12a on rice roots showed that the strain can maintain a stable population in rhizosphere of rice, with a concentration of approximately 10^6 to 10^7 CFU g^{-1} rice roots (Table 2). To further confirm that the disease suppression of *M. oryzae* by *Pseudomonas* sp. CMR12a was due to ISR, rice shoots and leaves were randomly selected, ground and the mixture was suspended in sterilized water, serial diluted, plated on KB plates and incubated at 28°C for 48 h for bacterial population counting. The results showed that there was no bacterial transportation from roots to shoots and leaves in rice plants (data not shown), confirming that disease reduction was due to ISR. In two independent experiments, mutants of *Pseudomonas* sp. CMR12a impaired in phenazine biosynthesis (P: CMR12a-Δ*Phz*, PS: CMR12a-Δ*Phz*-Clp1, PO: CMR12a-Δ*Phz*-ΔClp2 and POS: CMR12a-Δ*Phz*-ΔClp2-Clp1) completely lost their capacity to trigger ISR against *M. oryzae* in rice plants, while mutants impaired in CLP production that still produced phenazines (S: CMR12a-Clp1, O: CMR12a-ΔClp2 and OS: CMR12a-ΔClp2-Clp1) were able to control the disease (Fig. 1A). Root colonization data showed that all mutants maintained a population of about 10^6 to 10^7 CFU g^{-1} of fresh roots in the rhizosphere of rice plants (Table 2). These results strongly suggest that phenazines produced by *Pseudomonas* sp. CMR12a are the main bacterial determinants responsible for ISR in the *M. oryzae*-rice system.

Subsequently, we applied various concentrations of pure PCN, the major phenazine compound produced by *Pseudomonas* sp. CMR12a (Olorunleke *et al*., 2015b), as a soil drench. The resistance inducer BTH (S-methyl 1,2,3-benzothiadiazole-7-carbothioate) was used as a positive control. In two independent experiments, PCN at a concentration of 0.1 or 1 μM offered protection against *M. oryzae*, while lower or higher concentrations were not effective (Fig. 1B).

In three independent experiments we also tested the ISR capacity of *Pseudomonas* sp. CMR12a and its biosynthesis mutants against web blight, caused by leaf inoculation with *R. solani* AG2-2 on bean. As presented in Fig. 2A, *Pseudomonas* sp. CMR12a provided a significant ISR-mediated protective effect against web blight caused by *R. solani*. In all three experiments, seedlings colonized with mutants that only produced sessilins

Fig. 1. Effect of root colonization with *Pseudomonas* sp. CMR12a and mutants impaired in phenazines and/or CLPs (A), and effect of soil drench with 25 μM BTH (S-methyl 1,2,3-benzothiadiazole-7-carbothioate) or different concentrations of PCN (B) on rice blast disease in *indica* cv. CO-39 plants. Results from two representative experiments are shown. Representative disease symptoms for different concentrations of PCN or BTH treatment are shown in Fig. 1B. Con: Diseased control; WT: wild-type *Pseudomonas* sp. CMR12a; P: phenazine mutant; S: sessilin mutant; O: orfamide mutant. Strains used in this bioassay and mutant abbreviations are further explained in Table 1. The table below Fig. 1A shows the metabolites that are produced by *Pseudomonas* sp. CMR12a and its biosynthesis mutants.
Pseudomonas sp. CMR12a and its biosynthesis mutants were grown at 28°C on solid King's B (KB) medium (King *et al*., 1954) for 48 h. Bacteria were scraped from plates and suspended in sterile saline solution (0.85% sodium chloride, w/v). Bacterial density was determined by measuring the optical density (OD) at 620 nm and adjusted to a final concentration of 5×10^6 CFU g^{-1} soil. Crude phenazines were obtained from *Pseudomonas* sp. CMR12a-ΔClp2-Clp1 as described previously (Perneel *et al*., 2008). PCN was recovered from spots on preparative thin layer chromatography (Silica gel 60 F_{254}, 1000 μm, Merck; toluene/acetone (3/1, v/v) was used as solvent) with retardation factor value 0.27 and used for further bioassays. Stock solutions of PCN and BTH were prepared in DMSO, and diluted to desired concentrations for ISR assays. Rice seeds were surface sterilized with 1% (w/v) sodium hypochlorite solution for 5 min, washed three times with sterilized water, and air dried. Seeds were germinated on moistened filter paper in Petri dishes (Diameter = 9 cm) for five days, and planted into plastic trays (23 × 16 × 6 cm, 12 plants per tray). Rice plants were routinely grown in a greenhouse (12 h light, 30 ± 4°C). Pre-germinated rice seedlings were sown in sterilized potting soil (Structural; Snebbout, Kaprijke, Belgium) mixed with *Pseudomonas* sp. CMR12a and its biosynthesis mutants; Control (Con) treatment received the same amount of sterile saline. A second bacterial application was conducted as soil drench 12 days after plants were sown. Five-leaf stage (Four-week-old) rice plants were challenged with spores of *M. oryzae* VT5M1. Various concentrations of PCN and 25 μM BTH were applied as soil drench three days before pathogen challenge, as described previously (De Vleesschauwer *et al*., 2008). *M. oryzae* VT5M1 (Thuan *et al*., 2006) was routinely maintained on complete medium (Talbot *et al*., 1993) at 28°C. Spore suspensions of *M. oryzae* VT5M1 were prepared as described previously (Thuan *et al*. 2006). Conidia were collected and suspended into 0.5% (w/v) gelatin to a final concentration of 5×10^4 per milliliter. Rice plants were evenly sprayed with spores of *M. oryzae* VT5M1 using a compressor-powered airbrush gun, and trays were frequently rotated during this procedure. Infected rice plants were kept for 24 h in a dark chamber (relative humidity ≥ 90%; 25 ± 5°C), and then transferred to the greenhouse for disease development. Disease was scored 6 days post infection (dpi), by counting the number of susceptible lesions (ellipsoid to round-shaped lesions with a gray center indicative of sporulation) per 10 cm of the second youngest leaves of rice plants, as described previously (De Vleesschauwer *et al*., 2006). Bioassays were repeated independently (Experiment 1 and Experiment 2) using 12 plants per assay, Data are expressed as relative infection in comparison with the diseased control. The average number of susceptible-type blast lesions per 10 cm of the second youngest leaf of individual control plants was 55 in experiment 1A, 49 in experiment 2A, 62 in experiment 1B and 35 in experiment 2B. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney; $p = 0.05$, n = 12).

Table 1. Microorganisms used in this study and their relevant characteristics

Microorganisms	Relevant characteristics[a]	References
Pseudomonas		
CMR12a (WT)[b]	PHZ^+, $CLP1^+$, $CLP2^+$; phenazines, sessilins and orfamides	Perneel *et al*. (2007)
CMR12a-Δ*Phz* (P)	PHZ^-, $CLP1^+$, $CLP2^+$; mutant with deletion of phenazine operon, produces sessilins and orfamides	D'aes *et al*. (2011)
CMR12a-Clp1 (S)	PHZ^+, $CLP1^-$, $CLP2^+$; mutant with insertion in sessilin biosynthesis genes, Gm^R, produces phenazines and orfamides	D'aes *et al*. (2011)
CMR12a-ΔClp2 (O)	PHZ^+, $CLP1^+$, $CLP2^-$; mutant with deletion of orfamide biosynthesis genes, produces phenazines and sessilins	D'aes *et al*. (2014)
CMR12a-ΔClp2-Clp1 (OS)	PHZ^+, $CLP1^-$, $CLP2^-$; orfamide deletion and sessilin biosynthesis insertion mutant, Gm^R, produces phenazines	D'aes *et al*. (2014)
CMR12a-Δ*Phz*-Clp1 (PS)	PHZ^-, $CLP1^-$, $CLP2^+$; phenazine deletion and sessilin biosynthesis insertion mutant, Gm^R, produces orfamides	D'aes *et al*. (2011)
CMR12a-Δ*Phz*-ΔClp2 (PO)	PHZ^-, $CLP1^+$, $CLP2^-$; phenazine and orfamide deletion mutant, produces sessilins	D'aes *et al*. (2014)
CMR12a-Δ*Phz*-ΔClp2-Clp1 (POS)	PHZ^-, $CLP1^-$, $CLP2^-$; phenazine and orfamide deletlion mutant and sessilin biosynthesis insertion mutant, Gm^R	D'aes *et al*. (2014)
M. oryzae		
VT5M1	Causal agent of rice blast disease	Thuan *et al*. (2006)
R. solani		
AG 2-2 CuHav-Rs18	Web blight pathogen obtained from bean leaves	Nerey *et al*. (2010)

a. PHZ: phenazines; CLP1: sessilins; CLP2: orfamides; +: positive for trait, -: negative for trait, Gm^R: resistant to gentamycin.

b. Letters between brackets correspond to codes used to indicate bacterial strains in Fig. 1A and 2A.

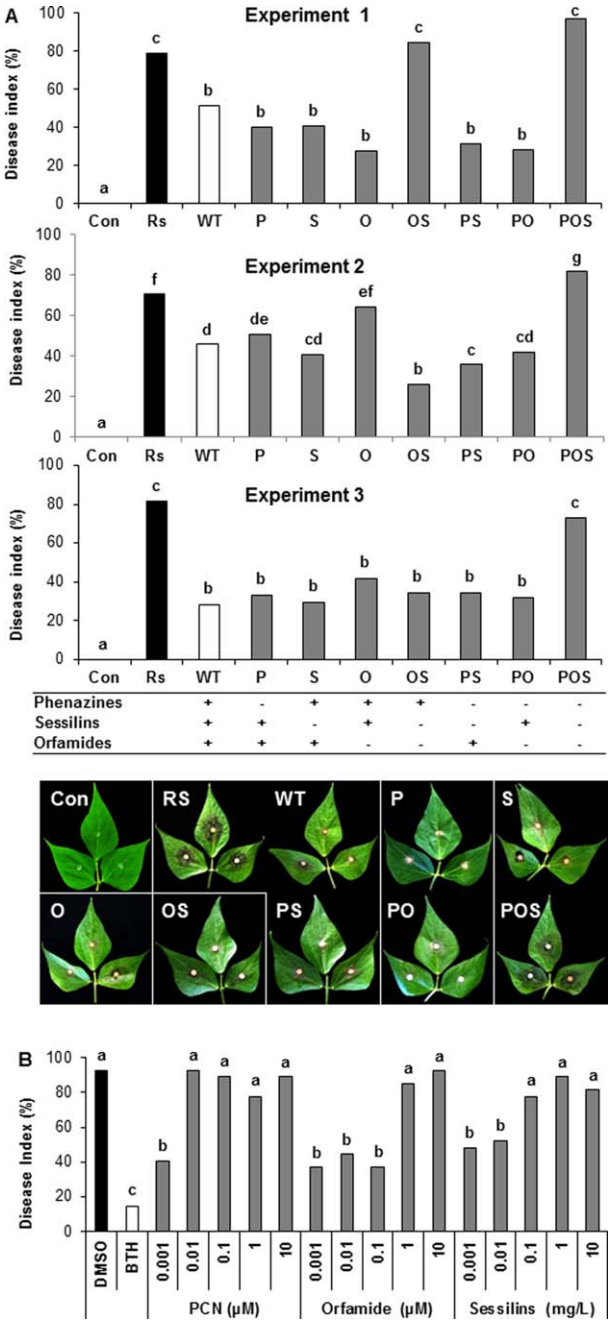
(PO) or orfamides (PS) were still very effective in triggering ISR, while a *Pseudomonas* sp. CMR12a-mutant deficient in the production of phenazines, sessilins and orfamides (POS) could no longer trigger ISR in any of the experiments. A mutant that only produced phenazines (OS) was very effective in experiment 2 and 3, but completely lost its ability to trigger ISR in experiment 1. In experiment 1, bacterial inoculum was produced on solid KB plates, while in experiments 2 and 3, the bacterial inoculum originated from liquid KB cultures. Only in experiment 2, a mutant that still produced phenazines and sessilins (O) performed worse than mutants that only produced one of the compounds (OS and PO). Taken together, results from the three experiments suggest that phenazines and the CLPs sessilin and orfamide are all determinants for ISR in bean, but that a balanced production is needed for optimal effect.

Bacterial enumeration shows that all strains could establish well on roots of bean seedlings and their population densities varied between 10^6 to 10^8 CFU g^{-1} roots (Table 3). Although low bacterial counts were found in bean stems, no bacteria were isolated from the primary leaves (Table 3). These data indicate that the observed disease suppressive effect may result from enhanced levels of plant resistance to infection by the pathogen since inducing strains and the challenging pathogen were spatially separated.

Table 2. Population of *Pseudomonas* sp. CMR12a and its biosynthesis mutants on rice roots

		Bacterial population[a] (log CFU g^{-1} fresh root)	
Pseudomonas strains	Metabolites produced	Experiment 1	Experiment 2
CMR12a (WT)	Phenazines, sessilins and orfamides	6.14 ± 0.21 a	7.73 ± 0.23 a
CMR12a-Clp1 (S)	Phenazines and orfamides	6.07 ± 0.45 a	7.23 ± 0.05 a
CMR12a-ΔClp2 (O)	Phenazines and sessilins	5.99 ± 0.11 a	7.68 ± 0.13 a
CMR12a-ΔClp2-Clp1 (OS)	Phenazines	6.16 ± 0.46 a	7.98 ± 0.21 a
CMR12a-Δ*Phz* (P)	Sessilins and orfamides	6.02 ± 0.38 a	7.74 ± 0.15 a
CMR12a-Δ*Phz*-Clp1 (PS)	Orfamides	5.96 ± 0.45 a	7.63 ± 0.18 a
CMR12a-Δ*Phz*-ΔClp2 (PO)	Sessilins	5.83 ± 0.35 a	7.81 ± 0.19 a
CMR12a-Δ*Phz*-ΔClp2-Clp1 (POS)	Null	6.02 ± 0.36 a	7.89 ± 0.38 a

a. Roots from five rice plants of each treatment were randomly chosen after disease evaluation, and rinsed carefully with sterilized water for three times to remove extra soil surrounding roots. Bacterial suspensions were obtained by grinding the roots using a mortar and pestle in sterilized saline (0.85% sodium chloride, w/v). Serial dilutions were plated on solid KB medium and incubated for 48 h at 28°C. Data were log10 transformed. Values followed by the same letter are not significantly different based on Kruskal-Wallis and Mann-Whitney tests ($P = 0.05$).



A soil drench with pure PCN, pure orfamide B and a crude extract of sessilins indicated that all metabolites could trigger ISR to web blight in bean, but at specific concentrations (Fig. 2B). PCN was only effective at a concentration of 1 nM, orfamide was effective in a range from 1 to 100 nM, while the crude extract of sessilins was active at 1 and 10 μg/L. Microscopic observation of disease responses showed a decreased penetration of mycelium of *R. solani* in bean leaves resulting from plants inoculated with *Pseudomonas* sp. CMR12a, while mycelium of *R. solani* had fully penetrated into leaves

Fig. 2. Effect of *Pseudomonas* sp. CMR12a and mutants impaired in phenazines and/or CLPs (A), and different concentrations of PCN, orfamide B and sessilins (B) on resistance to web blight caused by *R. solani* AG2-2 on bean. Representative symptoms of experiment 3 are shown in the pictures of Fig. 2A. Con: healthy control; Rs: *R. solani* AG2-2; P: phenazine mutant; S: sessilin mutant; O: orfamide mutant. Strains used in this bioassay and mutant abbreviations are further explained in Table 1. The table below Fig. 2A shows the metabolites that are produced by *Pseudomonas* sp. CMR12a and its biosynthesis mutants.
Pseudomonas sp. CMR12a and its biosynthesis mutants were grown on solid KB medium for 24 h (Fig. 2A, experiment 1) or grown in liquid KB medium on a rotary shaker (28°C, 150 rpm) for 24 h (Fig. 2A, experiments 2 and 3). The cultures were harvested and adjusted to the desired concentrations with sterile saline (0.85% sodium chloride, w/v) based on their OD_{620}. Surface-sterilized (1% sodium hypochloride, w/v) seeds of bean cv. Prelude (Het Vlaams Zaadhuis, Waarschoot, Belgium) were soaked in bacterial suspensions (10^6 CFU mL^{-1}) for 10 min. Control treated seeds received the same amount of sterile saline. Then, seeds were gently rinsed with sterile water before they were incubated in Petri dishes containing sterile moistened filter papers for three days at 28°C to allow germination. A growth substrate made of 50% (w/w) potting soil (Structural; Snebbout, Kaprijke, Belgium) and 50% (w/w) sand (Cobo garden; Belgium) was sterilized twice on two different days before it was treated with different bacterial suspensions to obtain a final density of 10^6 CFU g^{-1} substrate. Sets of four to six germinated seeds were sown in a perforated plastic tray (23 x 16 x 6 cm) containing 700 g substrate. Ten days after sowing, bacterial application was repeated as a soil drench. In healthy control and pathogen control treatments, growth substrate was treated with equal volumes of sterile saline in a similar manner. All trays were placed in a growth chamber (28°C, relative humidity = 60%, 16 h photoperiod) and seedlings were watered every two days to maintain moisture of growth substrate near field capacity. Orfamide B, the main orfamide-type CLP secreted by *Pseudomonas* sp. CMR12a (D'aes *et al.*, 2014) was purified as described previously (Ma *et al.*, 2016). Crude extracts of sessilin-type CLPs from phenazines and orfamides deficient mutant *Pseudomonas* sp. CMR12a-Δ*Phz*-Clp2 were obtained exactly as described previously (Nutkins *et al.*, 1991). Stock solutions of PCN, CLPs and BTH were made in DMSO. PCN, CLPs and BTH were applied as soil drench (300 mL/tray). DMSO (0.1%, v/v) and BTH (25 μM) treatments were used as negative and positive control, respectively. Bean leaves were detached 3 days after treatments for bioassays with PCN and CLPs. A detached leaf assay was performed following the method developed by (Takegami *et al.*, 2004), with modifications. In short, fully expanded trifoliate leaves detached from 4-week-old bean seedlings were challenged by placing a mycelial plug (diameter = 5 mm), which was collected from 7-day-old potato dextrose agar (PDA; Difco) plates of *R. solani* AG 2-2 CuHav-Rs18, in the center of each leaflet. Plugs taken from PDA plates without fungus and with fungus were used to inoculate leaflets as the healthy control (Con) and diseased control (Rs) treatment, respectively. The leaves were placed in trays containing moistened paper towels. All trays were covered to maintain a high relative humidity for disease development and they were incubated at 28°C in dark conditions. Disease severity was evaluated 6 days after fungal inoculation exactly as described previously (van Schoonhoven and Pastor-Corrales, 1987), using the scales in which 1 = no visible symptoms of disease; 3 = approximately 5% −10% of the leaf area with symptoms; 5 = approximately 20 - 30% of the leaf area with symptoms; 7 = 40 - 60% of the leaf area with symptoms; and 9 = more than 80% of the leaf area with symptoms. Five trifoliate leaves were used in each treatment and the experiment was repeated three times (Fig. 2A). Three trifoliate leaves were used for the experiment depicted in Fig. 2B and the experiment was repeated with similar results. Data are expressed as percent disease index and different letters indicate statistically significant differences among treatments (Kruskal-Wallis and Mann-Whitney tests, $p = 0.05$).

Table 3. Population of *Pseudomonas* sp. CMR12a and its biosynthesis mutants on roots of bean seedlings

		Bacterial population (log CFU g^{-1} fresh root)[a]			
		Experiment 1	Experiment 2	Experiment 3	
Pseudomonas strains	Metabolites produced	Root	Root	Root	Stem
CMR12a (WT)	Phenazines, sessilins and orfamides	6.30 ± 0.29 a	7.98 ± 0.24 bc	7.16 ± 0.30 a	4.94 ± 0.22 a
CMR12a-Δ*Phz* (P)	Sessilins and orfamides	6.21 ± 0.23 a	7.79 ± 0.20 b	7.39 ± 0.13 ab	5.20 ± 0.45 a
CMR12a-Clp1 (S)	Phenazines and orfamides	6.39 ± 0.24 a	7.44 ± 0.33 a	7.67 ± 0.39 c	4.87 ± 0.05 a
CMR12a-ΔClp2 (O)	Phenazines and sessilins	6.66 ± 0.14 c	7.98 ± 0.26 bc	7.66 ± 0.21 c	5.41 ± 0.52 b
CMR12a-ΔClp2-Clp1 (OS)	Phenazines	6.62 ± 0.35 c	7.86 ± 0.12 b	7.54 ± 0.35 bc	5.02 ± 0.33 a
CMR12a-Δ*Phz*-Clp1 (PS)	Orfamides	6.31 ± 0.22 a	8.07 ± 0.25 c	7.20 ± 0.34 a	4.96 ± 0.19 a
CMR12a-Δ*Phz*-ΔClp2 (PO)	Sessilins	6.58 ± 0.27 c	8.18 ± 0.21 c	7.51 ± 0.31 bc	5.18 ± 0.34 a
CMR12a-Δ*Phz*-ΔClp2-Clp1 (POS)	Null	6.44 ± 0.26 b	8.04 ± 0.19 c	7.42 ± 0.24 b	4.84 ± 0.16 a

a. Three seedlings per treatment were randomly chosen for determination of bacterial population on bean plants. Roots, stems (only in exp. 3) and primary leaves of the same plants were harvested after trifoliate leaves were detached for the induction assay. Bacterial population on roots as well as on surface sterilized stems and leaves were determined and counted exactly as described below Table 2. Data were log10 transformed prior to statistical analysis. Values followed by the same letter are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests ($P = 0.05$). No *Pseudomonas* sp. CMR12a-like bacteria could be detected in the leaves of bean plants (data not shown).

and caused necrosis on leaves in control treatment (data not shown).

We assessed metabolite production by *Pseudomonas* sp. CMR12a and mutants grown on solid KB plates for 48 h. There was no significant difference in phenazine production between the WT strain and mutants impaired in sessilin production (S), orfamide production (O) or impaired in the production of both CLPs (OS), while all phenazine mutants were completely unable to produce any phenazine (Fig. 3A). Mutants impaired in orfamide production (O) and/or phenazine production (PO and P) produced the same amount of sessilin as the WT (Fig.3B). No residual sessilin production was detected in the sessilin mutants. Orfamide production in the sessilin mutant (S), phenazine mutant (P) and sessilin and phenazine double mutant (PS) was clearly lower than in the WT, while no residual orfamide could be detected in any of the orfamide mutants (Fig.3C). Since an OD_{620} of 1 corresponds to about 10^9 CFU/mL it can be deduced from Fig. 3 that a density of $5x10^6$ CFU g^{-1} soil *Pseudomonas* sp. CMR12a corresponds to nM concentrations of orfamide and phenazine.

Phenazine pigments have broad spectrum activity against fungal pathogens mainly because they are involved in cellular redox cycling and induce the accumulation of toxic reactive oxygen species (ROS) (Mavrodi *et al.*, 2006). ROS generated in plants are central players in dealing with stress responses (Baxter *et al.*, 2014). The accumulation of ROS not only provides direct protective effects, but is also able to induce cellular protectant genes in surrounding cells (Lamb and Dixon, 1997). It has been shown before that the phenazine pyocyanin, produced by *P. aeruginosa*, can trigger ISR in tomato and rice (Audenaert *et al.*, 2002; De Vleesschauwer *et al.*, 2006). Pyocyanin-triggered ISR in rice was accompanied with enhanced ROS production at the site of *M. oryzae* infection (De Vleesschauwer *et al.*, 2006) and *in vitro* application of ROS in rice led to enhanced defense responses to *M. oryzae* (De Vleesschauwer *et al.*, 2009). Using an *in vitro* system, Kang *et al.* (2007) observed that a mutant of *P. chlororaphis* O6 impaired in phenazine production was less able to induce resistance to *Erwinia carotovora* (= *Pectobacterium carotovorum*) on tobacco. Intriguingly, pyocyanin is able to trigger ISR against rice blast at nanomolar or even picomolar concentrations, while high concentrations were not effective (De Vleesschauwer *et al.*, 2006). However, this study showed 0.1 to 1 μM PCN is needed to trigger ISR in rice plants, suggesting that structural differences in PCN and pyocyanin result in a different ability to trigger ISR. This may be due to the fact that pyocyanin is more reactive with oxygen compared to PCA or PCN (Wang and Newman 2008), resulting in a stronger ROS production *in situ* and a faster defense response in rice against *M. oryzae*. Intriguingly, PCN was only active at nanomolar concentrations in bean, while activity was lost at higher concentrations (Fig. 2B). We hypothesize that higher concentrations of PCN may trigger a ROS response that may favour infection by the necrotrophic pathogen *R. solani*. These results suggest that a balanced production of PCN is needed to trigger ISR and that the concentration needed is probably dependent on the plant-pathosystem used. This may probably also explain why the mutant that only produces phenazines (OS mutant) gives fluctuating results in the plant assays (Fig. 2A). We hypothesize that the phenazine concentration in experiment 1, in which the inoculum

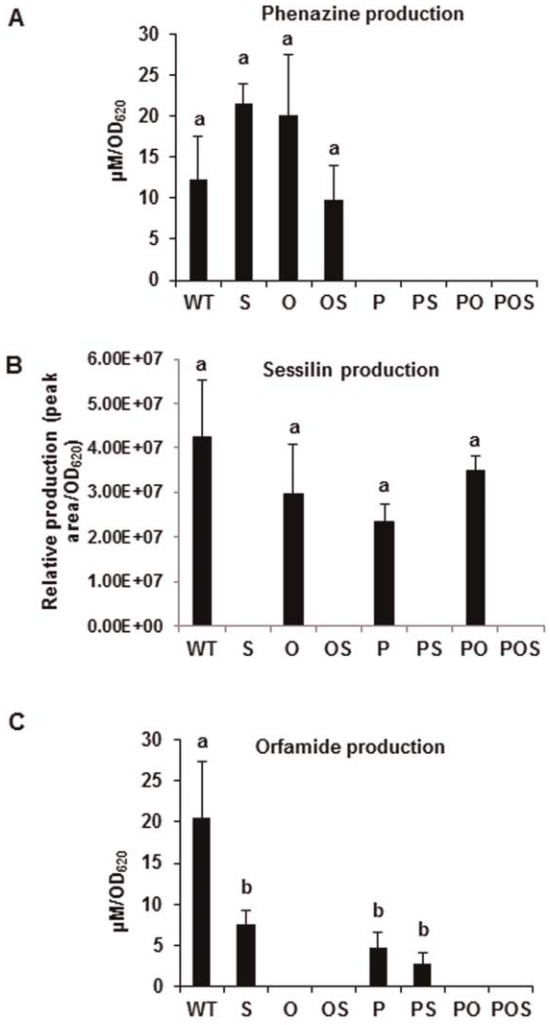


Fig. 3. Quantification of phenazine (A), sessilin (B) and orfamide (C) produced by *Pseudomonas* sp. CMR12a and its biosynthesis mutants on solid King's B (KB) plates. *Pseudomonas* strains were grown on KB plates for 48 h, and then bacteria were scraped off from KB plates and suspended in 10 mL 50% (v/v) acetonitrile solution. The optical density (OD) of bacterial solutions was recorded at 620 nm. Then, the supernatant of bacterial solution was collected after centrifugation at 12000 g for 5 min and submitted for analysis. Phenazine concentrations were determined by spectrophotometric measurements at 369 nm and using the extinction coefficient of $\varepsilon = 11{,}393\ M^{-1}\ cm^{-1}$ as described previously (D'aes *et al*., 2011). Sessilin and orfamide from bacterial supernatant were measured by ultrahigh performance liquid chromatography-mass spectrometry (UPLC-MS) by using single iron recording integration of peaks corresponding to the exact mass of sessilin and orfamide homologues as described previously (D'aes *et al*., 2014). . Orfamide was quantified based on a standard curve made using a purified orfamide sample. Different letters indicate significant differences among treatments (Tukey HSD test, $p = 0.05$). Vertical bars indicate the standard deviation (n = 3).

was obtained from solid plates, similar to the method used in the rice experiments, may have been too high.

The ISR capacity of *Pseudomonas*-derived CLPs is poorly studied, CLP massetolide A eliciting enhanced resistance of *Phytophthora infestans* in tomato is the only case (Tran *et al*., 2007). This study clearly shows that also sessilin and orfamide-type CLPs can trigger ISR, however, the mechanisms involved have not yet been deciphered. Moreover, orfamides can trigger defense-related responses in rice cell cultures and perception of orfamide by rice leads to enhanced disease resistance against the brown spot pathogen *Cochliobolus miyabeanus* (Ma *et al*., manuscript in preparation). These results show that orfamide has the capacity to elicit innate immunity in monocot and dicot plants, but the effect appears to be pathogen-dependent. Intriguingly, CLPs derived from endospore-forming *Bacillus* spp., such as surfactins, fengycins and iturins, have been intensively studied for elicitation of plant innate immunity in a broad range of plants including bean, tomato, tobacco, grapevine, rice and *Arabidopsis* (Ongena *et al*., 2007; Cawoy *et al*., 2014; Chandler *et al*., 2015; Debois *et al*., 2015; Farace *et al*., 2015; Kawagoe *et al*., 2015). An interesting study has shown surfactin elicits plant defense responses by interacting with the lipid bilayer of plant plasma membrane (Henry *et al*., 2011), and is not recognized by specific pattern recognition receptors (Trdá *et al*., 2015). However, the mechanisms by which sessilins and orfamides induce web blight resistance in bean remain to be elucidated.

Conclusions

Phenazines and CLPs produced by *Pseudomonas* sp. CMR12a are not only important in direct antagonism against soilborne pathogens as was shown before (Olorunleke *et al*., 2015b), but also play a role in enhancing plant innate immunity. Their action, however, is plant-pathosystem and concentration dependent. Phenazines appear to be the only determinant for induced systemic resistance to rice blast, while phenazines, sessilins and orfamides are able to trigger induced systemic resistance to web blight in bean, but balanced concentrations are needed for an optimal effect.

Acknowledgements

M.H. initiated, designed and supervised the study. G.K.H.H. performed experiments with bacterial strains on bean, while Z.M. conducted the experiments with pure compounds on bean and rice and with bacterial strains on rice. Z.M., G.K.H.H. and M.H. wrote the manuscript. M.O. conducted ultrahigh performance liquid chromatography-mass spectrometry (UPLC-MS) analysis, Z.M., M.O. and M.H. revised the manuscript. Z.M. sincerely acknowledges scholarships from China Scholarship Council (CSC, No. 201204910376) and a special research fund (Bijzonder Onderzoeksfonds, BOF) from Ghent University. We would like to thank N. Lemeire (Ghent University, Belgium) and I.

Delaere (Ghent University, Belgium) for technical assistance during this study.

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