

# Biological Control of Rhizoctonia Root Rot on Bean by Phenazine- and Cyclic Lipopeptide-Producing *Pseudomonas* CMR12a

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## ABSTRACT

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*Pseudomonas* CMR12a was previously selected as an efficient biocontrol strain producing phenazines and cyclic lipopeptides (CLPs). In this study, biocontrol capacity of *Pseudomonas* CMR12a against Rhizoctonia root rot of bean and the involvement of phenazines and CLPs in this ability were tested. Two different anastomosis groups (AGs) of *Rhizoctonia solani*, the intermediately aggressive AG 2-2 and the highly aggressive AG 4 HGI, were included in growth-chamber experiments with bean plants. The wild-type strain CMR12a dramatically reduced

disease severity caused by both *R. solani* AGs. A CLP-deficient and a phenazine-deficient mutant of CMR12a still protected bean plants, albeit to a lesser extent compared with the wild type. Two mutants deficient in both phenazine and CLP production completely lost their biocontrol activity. Disease-suppressive capacity of CMR12a decreased after washing bacteria before application to soil and thereby removing metabolites produced during growth on plate. In addition, microscopic observations revealed pronounced branching of hyphal tips of both *R. solani* AGs in the presence of CMR12a. More branched and denser mycelium was also observed for the phenazine-deficient mutant; however, neither the CLP-deficient mutant nor the mutants deficient in both CLPs and phenazines influenced hyphal growth. Together, results demonstrate the involvement of phenazines and CLPs during *Pseudomonas* CMR12a-mediated biocontrol of Rhizoctonia root rot of bean.

*Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) is a soilborne pathogen causing severe damage to a wide range of crops. To date, this fungal species is subdivided into 13 different anastomosis groups (AGs) which may possess similar characteristics, such as host preference, pathogenicity, and type of disease symptom caused (7). In the production of common bean (*Phaseolus vulgaris*), root rot caused by *R. solani* is a major constraint leading to severe crop losses, especially under tropical and subtropical climate conditions. Several AGs of *R. solani* have been identified causing root rot on bean, the most frequently reported being AG 2-2 and AG 4 (2,32,34). Knowledge about the prevalence and distribution of different AGs is important, because sensitivity to chemical control treatments and, probably, also to other control strategies varies among AGs (6,27).

Beneficial bacteria have been intensively studied as biocontrol agents against soilborne diseases (20,57). Good results have been obtained with gram-positive *Bacillus* spp. and gram-negative *Pseudomonas* spp. in the control of several plant pathogens, including *Gaeumannomyces graminis* var. *tritici*, *Fusarium* spp., and *Pythium* spp. (18,29,58). In the case of *R. solani*, studies with different bacterial strains indicated that the biocontrol mechanism can be either induction of systemic resistance or antibiosis (24, 33). Among the great variety of beneficial bacteria and antibiotic

metabolites already discovered, our attention has been focused on phenazines and biosurfactants produced by fluorescent pseudomonads.

Phenazines are broad-spectrum, heterocyclic antibiotics known for their antifungal properties. The most commonly identified phenazine pigments produced by *Pseudomonas* spp. are pyocyanin, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), and a number of hydroxy-phenazines (56). Production of phenazines is essential for the biocontrol capacity of *Pseudomonas fluorescens* 2-79 (52), *P. chlororaphis* PCL1391 (8), and *P. aeruginosa* PNA1 (51). In the biological control of *R. solani*, only a few reports suggest the involvement of phenazines. Rosales et al. (45) observed in vitro inhibition of mycelial growth of *R. solani* by *P. aeruginosa* strains which produced PCA and pyocyanin, while disease suppression of Rhizoctonia root rot of wheat in vivo was enhanced after transformation of *P. fluorescens* Q8r1-96 with the biosynthesis genes for PCA (25). However, conclusive evidence for the importance of phenazines in the biological control of Rhizoctonia diseases using phenazine-negative mutants is still lacking.

Biosurfactants are amphiphilic compounds possessing both hydrophilic and lipophilic properties. These surface-active metabolites can be produced by several *Pseudomonas* spp.; the most interesting ones include rhamnolipids and several types of cyclic lipopeptides (CLPs), both of which may possess antifungal properties (43,44,50). Biocontrol-related research on biosurfactants has predominantly focused on the control of zoospore-producing plant pathogens, such as *Pythium* and *Phytophthora* spp., in which case the mode of action resides in zoospore lysis

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(12,15,50). Only a few studies have demonstrated the involvement of biosurfactants in biological control of non-zoospore-producing plant pathogens. A direct effect of biosurfactants on mycelium of *Pythium myriotylum* and *P. splendens* was hypothesized by Perneel et al. (39), while Debode et al. (10) found a major role for biosurfactants in the reduction of *Verticillium* microsclerotia viability. In vitro and in soil microcosm, antifungal properties of biosurfactants against *R. solani* have been described for the CLPs viscosinamide, tensin, and amphisin (1,35,37,53). However, no literature reports were found which proved a crucial role for CLPs in disease suppression of *R. solani* in planta.

In the present study, we investigated the potential of *Pseudomonas* CMR12a to reduce Rhizoctonia root rot of bean caused by two different AGs of *R. solani*, AG 2-2 and AG 4 HGI. *Pseudomonas* CMR12a, which produces both CLP-type biosurfactants and the phenazines PCN and PCA, is a promising biocontrol strain previously isolated from the cocoyam rhizosphere in Cameroon (40). Analysis of 16S rDNA sequences and phenazine biosynthesis genes indicated that CMR12a probably represents a novel species within the *Pseudomonas putida* species complex (30,40). Mutants of CMR12a deficient in CLP or phenazine production were used to investigate the role of both compounds in disease suppression of Rhizoctonia root rot on bean.

## MATERIALS AND METHODS

**Microorganisms and culture conditions.** Microorganisms used in this study are listed in Table 1. *R. solani* isolates were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit) plates at 28°C. *Pseudomonas* strains were routinely grown on King's medium B (KB) (28) at 28°C. *Escherichia coli* strains were grown on Luria-Bertani medium (LB) (46) at 37°C, and *Saccharomyces cerevisiae* on yeast-extract-peptone-dextrose at 30°C (49). When required, gentamicin was added at a concentration of 25 µg ml<sup>-1</sup> for *E. coli* strains and at 300 µg ml<sup>-1</sup> for *Pseudomonas* CMR12a and mutant strains.

**Construction of vectors and CMR12a mutant strains.** Molecular techniques were performed according to standard methods (46). *Taq* DNA polymerase and Q-solution were purchased from Qiagen (Venlo, The Netherlands) and polymerase chain reaction (PCR) was carried out as recommended by the manufacturer. Plasmids and primers are listed in Table 1.

**Construction of pMQ30-ΔPhz and mutant CMR12a-ΔPhz.** To construct mutant CMR12a-ΔPhz, the complete phenazine biosynthesis operon *phzABCDEFGHIH* (14) was deleted, using allelic replacement vector pMQ30, as described by Shanks et al. (49) and Hoang et al. (23). Two regions upstream and downstream of the phenazine biosynthesis operon *phzABCDEFGHIH* of CMR12a were amplified with primers PhzA-Up-F and PhzA-Up-R (856 bp) and primers PhzH-Down-F and PhzH-Down-R (904 bp), respectively. These PCR products were cloned flanking each other via in vivo homologous recombination in the yeast *S. cerevisiae* InvSc1. This procedure yielded deletion plasmid pMQ30-ΔPhz, which was mobilized into CMR12a by conjugation with *E. coli* BW29427 and selection on gentamicin. Transconjugants which had lost the plasmid during a second crossover event were selected on 10% sucrose, whereupon the deletion was confirmed by PCR.

**Cloning of CLP biosynthesis genes of CMR12a.** To track down the genes responsible for biosurfactant synthesis in CMR12a, a transposon mutant library, constructed by mating with *E. coli* SM10 carrying pUT-miniTn5phoA3 (11), was screened for mutants with reduced surface activity on KB medium by means of a drop-collapse test (26). In one of these biosurfactant-deficient mutants, the transposon interrupted an open reading frame showing homology with nonribosomal peptide synthetase (NRPS) genes responsible for CLP synthesis in other pseudomonads. This sequence was used as a probe for screening a pRG930 cosmid genomic library of CMR12a (14). Primer walking on positive clone 24G3 yielded a 1.9-kb DNA fragment, which was used to create a site-specific CLP mutant in CMR12a.

**Construction of pEX18Tc-CLP1 and mutants CMR12a-CLP1 and CMR12a-ΔPhz-CLP1.** CLP insertion mutants CMR12a-CLP1

TABLE 1. Microorganisms, plasmids, and primers used in this study

Strains, plasmids, and primers	Relevant characteristics <sup>z</sup>	Reference
<i>Pseudomonas</i> strains		
CMR12a	PHZ <sup>+</sup> , CLP1 <sup>+</sup> , CLP2 <sup>+</sup> , wild type (Cameroon)	40
CMR12a-GacA	PHZ <sup>-</sup> , CLP1 <sup>-</sup> , CLP2 <sup>-</sup> ; spontaneous <i>gacA</i> mutant of CMR12a, formerly designated CMR12a-Reg	41
CMR12a-ΔPhz	PHZ <sup>-</sup> , CLP1 <sup>+</sup> , CLP2 <sup>+</sup> , mutant with deletion of phenazine biosynthesis operon	This study
CMR12a-CLP1	PHZ <sup>++</sup> , CLP1 <sup>-</sup> , CLP2 <sup>+</sup> , mutant with insertion in CLP1 biosynthesis genes, Gm <sup>R</sup>	This study
CMR12a-ΔPhz-CLP1	PHZ <sup>-</sup> , CLP1 <sup>-</sup> , CLP2 <sup>+</sup> , phenazine deletion and CLP1 biosynthesis insertion mutant, Gm <sup>R</sup>	This study
<i>Rhizoctonia solani</i>		
AG 2-2 CuHav-Rs18	Causal agent of root rot on bean (intermediately aggressive) (Cuba)	34
AG 4 HGI CuLT-Rs36	Causal agent of root rot on bean (highly aggressive) (Cuba)	34
<i>Escherichia coli</i>		
DH5α	Host for cloning	19
SM10	Donor strain for conjugation; λ pir; Km <sup>R</sup>	22
HB101	Helper strain for conjugation	5
BW29427	Donor strain for conjugation; λ pir	16
<i>Saccharomyces cerevisiae</i> InvSc1	Yeast strain for in vivo recombination ( <i>ura3-52/ura3-52</i> mutation)	Invitrogen
Plasmids		
pMQ30	Gene replacement vector for <i>Pseudomonas</i> spp.; <i>sacB</i> , URA3, Gm <sup>R</sup>	49
pEX18Tc	Gene replacement vector with MCS from pUC18; oriT, <i>sacB</i> , Tc <sup>R</sup>	23
pRK2013	Conjugation helper plasmid, <i>ori</i> ColE1, <i>tra</i> <sup>+</sup> , <i>mob</i> <sup>+</sup> ; Km <sup>R</sup>	17
pUCGM	Source of Gm <sup>R</sup> cassette, Ap <sup>R</sup> , Gm <sup>R</sup>	48
pGV4692 (miniTnphoA3)	Mini-TnphoA3 transposon with the Gm cassette on pGV4692, a derivative of pUT (Ap <sup>R</sup> )	13,38
pJET1.2	General cloning vector, Ap <sup>R</sup>	Fermentas
Primers (5'→3')		
PhzA-Up-F	<i>GGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCTGACAACCTCGTTACCCACAGG</i>	This study
PhzA-Up-R	<i>GTCCITGAGCAACTCTTGGGTGCCGTCTCAGTGACAGTTC</i>	This study
PhzH-Down-F	<i>GAAGTGTCACTGAGACGGCACCCAAAGAGTTGCTCAAGGAC</i>	This study
PhzH-Down-R	<i>CCAGGCAAATTCGTTTTATCAGACCCGTTCTGCTGATTAAGGTACGCTGGAGCCAGTA</i>	This study
CLP12a_F	<i>GTGGTCGACATTGATACCTGGCCGCAA</i>	This study
CLP12a_R	<i>GTGGGATCCTGAGGATCGAACTGAACAGC</i>	This study

<sup>z</sup> PHZ, phenazines; CLP, cyclic lipopeptides; +, positive for trait (similar to wild type [WT]), ++, overproduction compared with WT, -, negative for trait; Gm<sup>R</sup>, Tc<sup>R</sup>, Amp<sup>R</sup>, and Km<sup>R</sup> = resistant to gentamicin, tetracyclin, ampicillin, and kanamycin, respectively. Primer extensions are in italic font.

and CMR12a- $\Delta$ Phz-CLP1 were constructed using vector pEX18Tc (23). A 1.8-kb fragment of an NRPS, presumably coding for biosurfactant biosynthesis in CMR12a (see above), was amplified with primers CLP12a\_F and CLP12a\_R. The resulting PCR product was cloned in pEX18Gm and cut with *Sall* and *Bam*HI to insert a gentamicin-resistance cassette from pUCGM (48). Following mobilization of the resulting plasmid (pEX18Tc-CLP1) into CMR12a and CMR12a- $\Delta$ Phz, transconjugants which lost the suicide plasmid after a second cross-over event were selected on LB with 5% sucrose. The gentamicin-resistance insertion was confirmed by PCR.

**Analysis of phenazine and CLP production.** Phenazines were extracted and analyzed by thin-layer chromatography and high-pressure liquid chromatography (HPLC) as described before (39, 41). To obtain quantitative data for phenazine production, spectrophotometric measurements ( $\lambda = 369$  nm and  $\epsilon = 11,393$  M<sup>-1</sup> cm<sup>-1</sup>) were performed on supernatant of bacterial cultures grown for 16 h in LB broth. An estimation of the extinction coefficient was derived from a standard curve for unsubstituted phenazine (Sigma-Aldrich, St. Louis), dissolved in water at different concentrations.

The presence of surface-active compounds was tested by the drop-collapse technique (26). Surface tension was measured as described by Perneel et al. (40) with minor modifications. Strains were grown on KB medium for 72 h, whereupon cells were scraped from the plates and suspended in sterile distilled water, and cell concentrations were estimated by measuring optical density at 620 nm (OD<sub>620</sub>). After centrifugation of the suspensions, surface tension of cell-free supernatants was measured with a tensiometer equipped with a Wilhelmy plate. For liquid-chromatography mass-spectrometry (LC-MS) analysis, supernatant samples were collected after 72 h of growth on LB medium and subjected to reverse-phase LC-MS (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass spectrometer) on an X-terra MS C18 column (100 by 4.6 mm, 3.5  $\mu$ m) (Waters). CLPs were eluted with a gradient of acetonitrile in water acidified with 0.1% formic acid at a constant flow rate of 0.5 ml min<sup>-1</sup> and 40°C. Compounds were detected in the scan mode or SIR mode and in-source settings (electrospray ionization, positive ion) in the SQD were as follows: source temperature, 130°C; desolvation temperature, 280°C; nitrogen flow, 550 liters h<sup>-1</sup>; and cone voltage, 60 or 80 V, depending on the compound. HCN and exoprotease production were determined as described before (40).

**Plant material and bacterial application to soil.** Plant experiments were carried out using bean seed of *Phaseolus vulgaris* 'Prelude' (Het Vlaams Zaadhuis, Waarschoot, Belgium). To reduce the variability in emergence, bean seed were pregerminated before sowing. Seed were surface sterilized in 1% sodium hypochlorite solution for 5 min, rinsed twice in sterile distilled water, and placed in petri dishes on sterile moistened filter paper. After 3 days at room temperature, germinated seed were sown in perforated plastic trays (22 by 15 by 6 cm) filled with 700 g of a mixture composed of 50% sand and 50% nonsterile potting soil (wt/wt) (Structural; Snebbout, Kaprijke, Belgium). Bacterial inoculum suspensions were prepared by growing CMR12a and its mutants on KB agar plates for 24 to 28 h. Bacterial cells were scraped from the plates and suspended in sterile saline (0.85% NaCl). Concentrations of bacterial suspensions were estimated based on their OD<sub>620</sub>. Washed bacteria were obtained by centrifuging bacterial suspensions twice for 4 min at 13,400 rpm and resuspending bacterial pellets in saline prior to determination of their OD<sub>620</sub>. The amount of suspension to be added to each tray was calculated and diluted to 50 ml with saline. Prior to sowing, the sand/potting soil substrate was thoroughly mixed with the diluted bacterial inoculum to obtain a final concentration of 10<sup>6</sup> CFU g<sup>-1</sup> of soil.

**Pathogen inoculation and disease rating.** Inoculum of *R. solani* AG 2-2 and AG 4 HGI was produced on sterile wheat

kernels according to the protocol described by Scholten et al. (47), with minor modifications. Water-soaked wheat kernels were autoclaved twice on two consecutive days and inoculated with three PDA discs (diameter = 5 mm) cut at the margin of a 3-day-old *Rhizoctonia* colony. Inoculated wheat kernels were incubated at 28°C for 6 days for the highly aggressive *R. solani* AG 4 HGI and 9 days for the intermediately aggressive *R. solani* AG 2-2. Every 3 to 4 days, wheat kernels were shaken to avoid coagulation. Plastic trays containing bean seedlings were inoculated 3 days after sowing by placing a row of 40 infected wheat kernels in the middle of the tray at a depth of  $\approx$ 2 cm and a distance of 3 cm from the bean seedlings. Six days after inoculation, disease symptoms were evaluated using the following scale: 0 = healthy, absence of symptoms; 1 = small black or brown lesions <1 mm in diameter; 2 = lesion covering <75% of the stem or root surface; 3 = lesion covering >75% of the stem or root surface; and 4 = seedling dead. All plant experiments were carried out in a growth chamber (28°C,  $\approx$ 70% relative humidity, 16-h photoperiod).

Each plant experiment consisted of three replications (trays) per treatment, with 10 bean plants per tray; and, in each experiment, healthy and infected control treatments were included, together with the bacterial treatments. All experiments were repeated in time.

**Evaluation of root colonization by *Pseudomonas* CMR12a and its mutants.** At the time of disease rating, bacterial colonization of the bean roots was determined. Roots of five plants per treatment, randomly chosen from the three different trays, were excised and rinsed under tap water to remove most of the soil. After weighing, roots were macerated in sterile saline using mortar and pestle and serial dilutions were plated on KB agar. Bacterial colonies showing the typical morphological characteristics of *Pseudomonas* CMR12a, and which do not appear on control plates, were counted after an incubation period of 36 to 48 h at 28°C.

**Data analysis.** Statistical analysis of data was performed using the software package SPSS 15.0 for Windows (SPSS Inc., Chicago). Neither the ordinal data of the disease severity nor the data of the root colonization experiments met the conditions of normality and homogeneity of variances. As a consequence, nonparametric Kruskal-Wallis and Mann-Whitney comparisons ( $\alpha = 0.05$ ) were performed.

**Microscopic observations.** Microscopic experiments were carried out based on a protocol described by Bolwerk et al. (4), with modifications. Bacterial strains were grown overnight in liquid KB at 28°C on a rotary shaker (150 rpm). Microscopy slides were covered with a thin layer of water agar (1.5%) and placed in petri dishes containing moistened filter paper. A 5-mm PDA plug of a 3-day-old *R. solani* colony was placed in the center of glass slide while a droplet (15  $\mu$ l) of the overnight culture of *Pseudomonas* CMR12a or mutant strain was pipetted at either side. The petri plates were sealed with parafilm and incubated for 36 h at 28°C before observation under light microscopy (Olympus BX51 microscope; Aartselaar, Belgium). Digital images were acquired using an Olympus Color View II camera and further processed with Olympus analySIS cell^F software (Olympus Soft Imaging Solutions, Münster, Germany). All experiments were performed twice.

## RESULTS

**Characterization of *Pseudomonas* CMR12a and its mutants.** HPLC analysis and UV absorption measurements of culture supernatant confirmed that both PCA and PCN production were abolished in mutants CMR12a- $\Delta$ Phz and CMR12a- $\Delta$ Phz-CLP1. Remarkably, CMR12a-CLP1 produced significantly higher amounts of phenazines than the wild type, an observation which cannot be accounted for at this point (Table 2). Various approaches, including reverse-phase LC-MS of CMR12a culture

TABLE 2. Phenazine and cyclic lipopeptide (CLP) production by CMR12a and mutants<sup>u</sup>

Strain	Phenazines			CLPs		
	HPLC <sup>v</sup>		Phenazine ( $\mu\text{M}/\text{OD}_{620}$ ) <sup>y</sup>	HPLC <sup>v</sup>		Tension (mN/m) <sup>z</sup>
	PCA <sup>w</sup>	PCN <sup>x</sup>		CLP1	CLP2	
CMR12a	+	+	33 $\pm$ 3	+	+	35 $\pm$ 2
CMR12a- $\Delta\text{Phz}$	-	-	<DL	+	+	36 $\pm$ 1
CMR12a-CLP1	+	++	67 $\pm$ 6	-	+	47 $\pm$ 5
CMR12a- $\Delta\text{Phz}$ -CLP1	-	-	<DL	-	+	59 $\pm$ 6
CMR12a-GacA	-	-	<DL	-	-	54 $\pm$ 6

<sup>u</sup> Measurements of phenazine concentration and surface tension were repeated in time, and representative data from one experiment are shown. Values show means and standard deviations of three independent replicates.

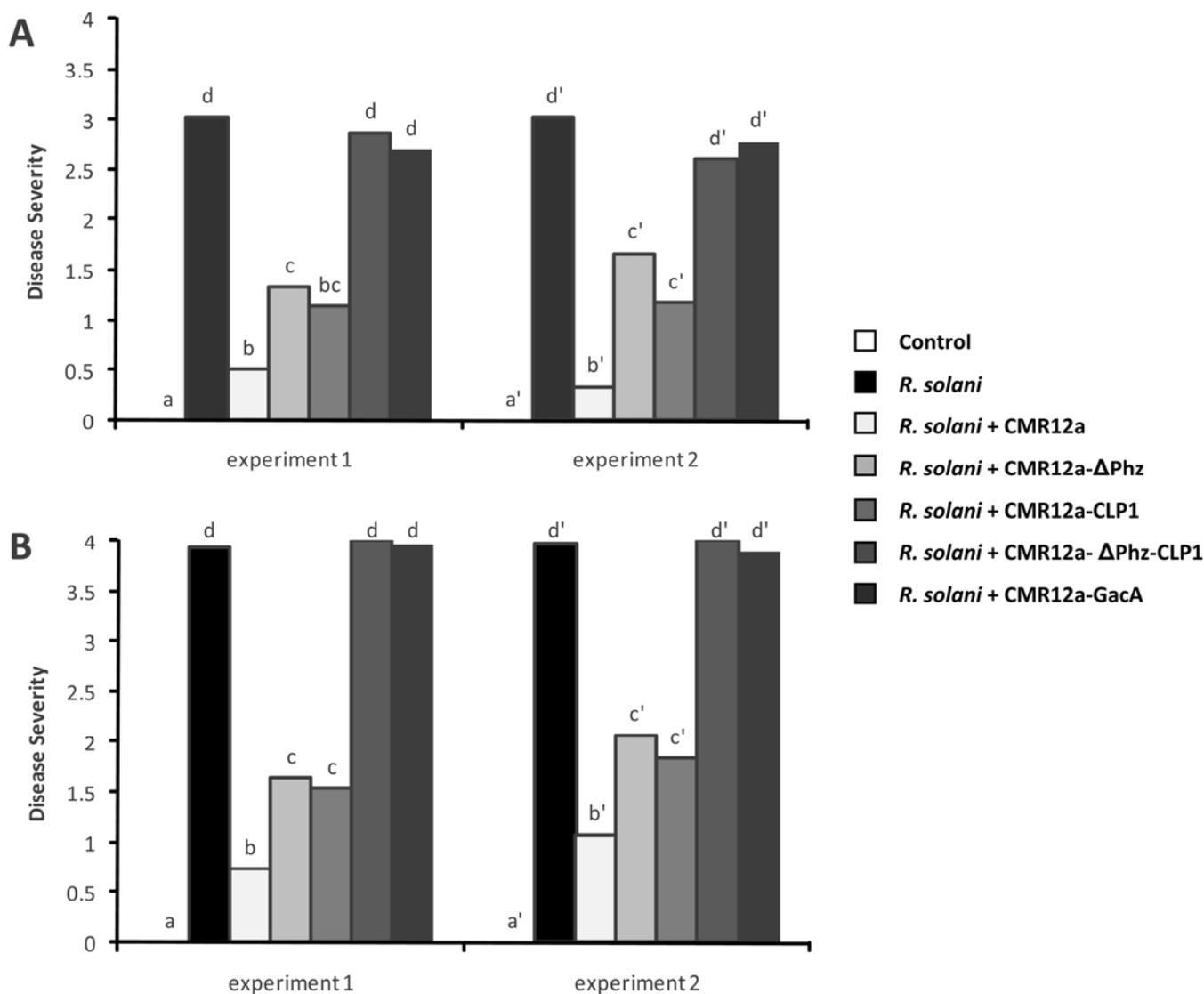
<sup>v</sup> HPLC = high-pressure liquid chromatography analysis; +, positive for trait (similar to wild type [WT]), ++, overproduction compared with WT, -, negative for trait.

<sup>w</sup> PCA = phenazine-1-carboxylic acid.

<sup>x</sup> PCN = phenazine-1-carboxamide.

<sup>y</sup> Phenazine concentration in culture supernatant;  $\text{OD}_{620}$  = optical density at 620 nm; <DL = value below detection limit.

<sup>z</sup> Surface tension of culture supernatant.



**Fig. 1.** Importance of phenazines and biosurfactants in the biological control of *Rhizoctonia* root rot of bean. *Pseudomonas* CMR12a, a phenazine-negative mutant; CMR12a- $\Delta\text{Phz}$ , a biosurfactant-negative mutant; CMR12a-CLP1, a double phenazine- and biosurfactant-negative mutant; CMR12a- $\Delta\text{Phz}$ -CLP1 and a spontaneous *gacA* mutant; CMR12a-GacA were applied to soil at a concentration of  $10^6$  CFU  $\text{g}^{-1}$  of soil 3 days before inoculation. Bacterial cells were not washed. Bean plants were subsequently inoculated with **A**, *Rhizoctonia solani* anastomosis group (AG) 2-2 or **B**, *R. solani* AG 4 HGI. Disease symptoms were evaluated after 6 days using the following scale: 0 = healthy, absence of symptoms; 1 = small black or brown lesions <1 mm in diameter; 2 = lesion covering <75% of the stem or root surface; 3 = lesion covering >75% of the stem or root surface; and 4 = seedling dead. Data represent means of disease scale obtained from three replicates, each with 10 plants per treatment. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney nonparametric tests ( $\alpha = 0.05$ ).

extracts and in silico analysis of genomic data, provided evidence that CMR12a synthesizes two types of CLPs (results not shown). For each CLP type, several presumably closely related variants seemed to be produced. For the main compound of the first CLP group, which has been designated CLP1, the detected molecular ion had a molecular weight of  $\approx 2,028$  Da. Preliminary results indicated that this is a novel compound within the tolaasin group of CLPs (43). Production of CLP1 was completely abolished in mutants CMR12a-CLP1 and CMR12a- $\Delta$ Phz-CLP1. For the second CLP type (designated CLP2), three main compounds with molecular ions of 1,253, 1,279, and 1,281 Da were detected, which were all still being produced by both CLP mutants at wild-type levels. In CMR12a- $\Delta$ Phz, the pattern of CLP production was not altered compared with that of the wild type (results not shown). A more detailed structural characterization of both CLPs is in progress.

In addition to these biosynthesis mutants, a regulatory mutant, CMR12a-GacA (41) (Table 1), was also tested for its biocontrol ability against *R. solani* on bean. This mutant has a spontaneous mutation in the GacS/GacA two-component regulatory system (21). In CMR12a-GacA, not only phenazine production but also synthesis of other secondary metabolites, including exoproteases and HCN (results not shown), is abolished. In addition, neither CLP1 nor CLP2 production could be detected in supernatant extracts of CMR12a-GacA, indicating that GacA controls production of both lipopeptides.

**Biocontrol of Rhizoctonia root rot of bean by *Pseudomonas* CMR12a and its phenazine or CLP mutants.** We investigated whether *Pseudomonas* CMR12a could provide protection in vivo against *Rhizoctonia* root rot. In the experiments, four mutants of CMR12a deficient in phenazine or CLP production were included (Table 2). A significant reduction of disease severity (DS) caused by *R. solani* on bean plants was observed for the wild-type strain *Pseudomonas* CMR12a (Fig. 1). The numerical data described further are valid for repetition 1 (Fig. 1); repetition 2 yielded similar results. For the intermediately aggressive isolate of *R. solani* AG 2-2, a reduction in DS from  $\approx 3.1 \pm 0.9$  to  $0.5 \pm 1.0$  was recorded ( $P = 0.000$ ); for the very aggressive isolate of *R. solani* AG 4 HGI, which killed almost all plants in the control treatment,

a decrease in DS from  $\approx 3.9 \pm 0.3$  to  $0.7 \pm 1.2$  was observed ( $P = 0.000$ ). Treatments with the mutant strains performed significantly worse. Mutants CMR12a- $\Delta$ Phz and CMR12a-CLP1 were still able to reduce the DS caused by both AGs of *R. solani*, although to a lower extent than the wild type. On the other hand, mutants which are deficient in both phenazine and CLP production, biosynthesis mutant CMR12a- $\Delta$ Phz-CLP1 and *gacA*-mutant CMR12a-GacA, completely lost their biocontrol capacity, and the DS for these strains was not statistically different from the diseased control.

Bacterial counts revealed differences in bacterial populations on the roots (Table 3). Bacterial concentrations observed for the parental strain and the mutants CMR12a- $\Delta$ Phz and CMR12a-CLP1 were variable among treatments and repetitions in time. However, this did not influence the disease-suppressive capacity, suggesting that root colonization was at a sufficiently high level for optimal biological control. The two mutants CMR12a- $\Delta$ Phz-CLP1 and CMR12a-GacA, lacking both phenazine and CLP production, predominantly had the lowest bacterial root concentrations and in only one experiment were their root concentrations not significantly lower compared with the wild type. CMR12a bacteria were never recovered from roots of noninoculated control plants. Moreover, application of CMR12a or its mutants to the soil never caused visible phytotoxic effects or effects on plant growth, as observed by qualitative inspection of the plants.

**Biocontrol capacity of washed CMR12a bacteria and its mutants.** To investigate whether metabolites produced during growth of bacterial strains on KB agar plates are involved in the biocontrol capacity of *Pseudomonas* CMR12a, bacteria were washed before application to the sand/potting soil mixture (Fig. 2). The reduction in DS upon treatment with washed bacteria was smaller compared with the nonwashed bacteria. For the plants inoculated with the washed wild-type strain, DS caused by *R. solani* AG 2-2 was reduced from  $3.8 \pm 0.8$  (first repetition) and  $2.8 \pm 1.0$  (second repetition) to  $\approx 1.5 \pm 1.1$ . Treatment with the washed mutant strains CMR12a- $\Delta$ Phz and CMR12a-CLP1 resulted in a DS reduction intermediate between the diseased control and the reduction caused by the wild-type strain (DS =  $2.6 \pm 1.5$  in repetition 1 and DS =  $2.0 \pm 1.2$  in repetition 2), whereas mutants CMR12a- $\Delta$ Phz-CLP1 and CMR12a-GacA did not influence the disease development and DS was not statistically different from the diseased control.

In contrast to the disease reduction of almost 50% observed for *R. solani* AG 2-2, washed bacteria only had a minor effect on the disease caused by the very aggressive isolate of *R. solani* AG 4 HGI. The wild-type strain and the mutants CMR12a- $\Delta$ Phz and CMR12a-CLP1 still reduced the DS significantly, from  $4.0 \pm 0.0$  to  $\approx 3.5 \pm 0.6$ . The two other mutants did not differ from the diseased control and, at the time of evaluation, all plants were dead. The washing step did not influence root colonization, because bacterial counts after the application of washed bacteria were at a level comparable with those for the nonwashed bacteria. In the first repetition, CMR12a bacteria were recovered at a concentration of  $5.57 \pm 0.31$  log CFU  $g^{-1}$  of fresh root after inoculation with *R. solani* AG 2-2 and at  $5.58 \pm 0.41$  log CFU  $g^{-1}$  of fresh root after inoculation with *R. solani* AG 4 HGI; in the second repetition, bacterial densities were slightly higher, with values of  $6.51 \pm 0.22$  and  $6.18 \pm 0.35$  log CFU  $g^{-1}$  of fresh root for AG 2-2 and AG 4 HGI, respectively.

**Microscopic observations.** *R. solani* hyphae in the presence of bacterial cells were examined with a light microscope to study changes in the growth pattern. At the contact zone between the mycelium of *R. solani* grown on coated microscopic glass slides and the bacterial droplets, morphological differences were observed (Fig. 3). The mycelium of *R. solani* AG 2-2 and AG 4 HGI was more branched in the presence of CMR12a and CMR12a- $\Delta$ Phz bacteria, resulting in a higher density of hyphae. In contrast, the three other mutants (e.g., CMR12a-CLP1, CMR12a- $\Delta$ Phz-

TABLE 3. Root colonization data for nonwashed bacteria of *Pseudomonas* CMR12a and mutants CMR12a- $\Delta$ Phz, CMR12a-CLP1, CMR12a- $\Delta$ Phz-CLP1, and CMR12a-GacA in the absence of *Rhizoctonia solani* or after inoculation with *R. solani* anastomosis group (AG) 2-2 or *R. solani* AG 4 HGI<sup>2</sup>

Treatment	Bacterial population (in log CFU $g^{-1}$ of fresh root)	
	Repetition 1	Repetition 2
<i>Without R. solani</i>		
CMR12a	6.14 $\pm$ 0.37 a	5.61 $\pm$ 0.46 c
CMR12a- $\Delta$ Phz	5.81 $\pm$ 0.47 b	6.95 $\pm$ 0.30 a
CMR12a-CLP1	5.14 $\pm$ 0.34 c	6.00 $\pm$ 0.39 b
CMR12a- $\Delta$ Phz-CLP1	5.22 $\pm$ 0.35 c	5.27 $\pm$ 0.39 d
CMR12a-GacA	5.02 $\pm$ 0.47 c	4.75 $\pm$ 0.36 e
<i>R. solani</i> AG 2-2		
CMR12a	6.53 $\pm$ 0.31 a	6.03 $\pm$ 0.36 b
CMR12a- $\Delta$ Phz	5.86 $\pm$ 0.71 bc	6.62 $\pm$ 0.28 a
CMR12a-CLP1	6.07 $\pm$ 0.53 b	6.68 $\pm$ 0.57 a
CMR12a- $\Delta$ Phz-CLP1	5.40 $\pm$ 0.30 c	5.30 $\pm$ 0.29 c
CMR12a-GacA	5.38 $\pm$ 0.31 c	4.92 $\pm$ 0.37 d
<i>R. solani</i> AG 4 HGI		
CMR12a	6.38 $\pm$ 0.44 a	5.34 $\pm$ 0.33 cd
CMR12a- $\Delta$ Phz	6.14 $\pm$ 0.79 ab	7.39 $\pm$ 0.24 a
CMR12a-CLP1	6.55 $\pm$ 0.45 a	6.84 $\pm$ 0.37 b
CMR12a- $\Delta$ Phz-CLP1	5.18 $\pm$ 0.44 c	5.56 $\pm$ 0.34 c
CMR12a-GacA	5.68 $\pm$ 0.32 b	5.16 $\pm$ 0.38 d

<sup>2</sup> Data represent means and standard deviations of five replicates per treatment. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney nonparametric tests ( $\alpha = 0.05$ ).

CLP1, and CMR12a-GacA) did not induce an increase in hyphal branching, and *R. solani* hyphae of both AGs showed similar morphology compared with the control hyphae grown in the absence of bacteria.

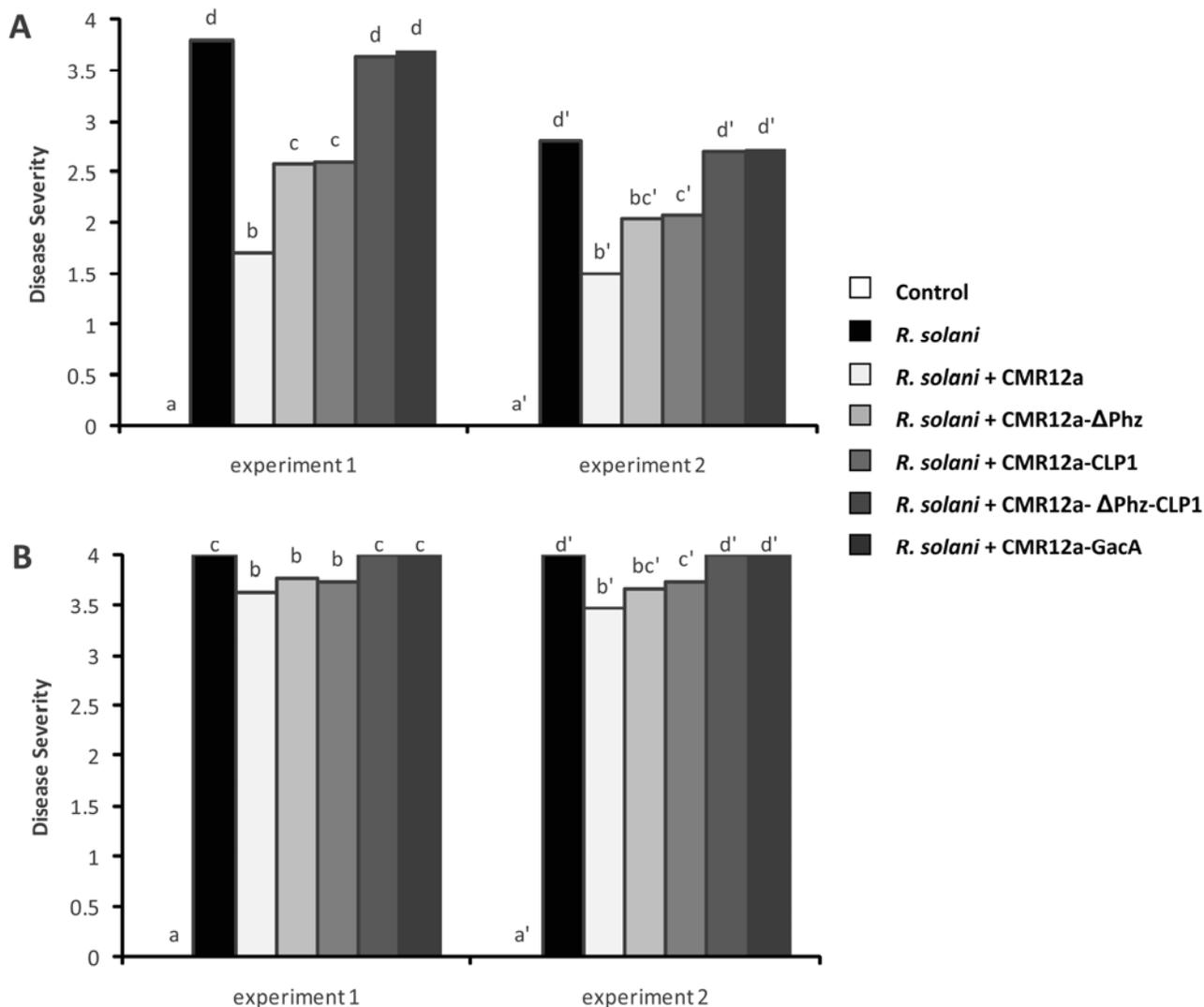
## DISCUSSION

Inhibition zones between hyphae of *R. solani* and *Pseudomonas* CMR12a cells were observed during preliminary in vitro dual culture experiments, suggesting a strong antagonistic potential of this bacterial strain toward *R. solani*. In this study, we showed that CMR12a effectively controls Rhizoctonia root rot of bean caused by *R. solani* AG 2-2 or *R. solani* AG 4 HGI. Moreover, the importance of CLP and phenazine production for the disease-suppressive effect of CMR12a was clearly demonstrated. It is unclear whether these effects are due to direct antibiotic activity of CLPs and phenazines. Alternatively, one or both compounds may somehow be involved in the ecological fitness of CMR12a, thus indirectly influencing the biocontrol capacity of CMR12a.

Direct antibiosis of CLPs is likely to be involved because microscopic experiments clearly showed an increase in hyphal

branching of *R. solani* AG 2-2 and AG 4 HGI caused by CMR12a whereas a normal growth pattern was observed with the CLP-deficient mutant CMR12a-CLP1. In addition, an in vitro growth assay with both wild-type CMR12a and the CLP1 mutant indicated a strong inhibitory effect of these CLPs on *R. solani* growth (*unpublished data*). Extensive branching of *R. solani* hyphae was previously observed after treatment with the structurally related CLPs viscosinamide and tensin. The authors assumed that viscosinamide imposes ion channels in the membranes, leading to an increased  $Ca^{2+}$  influx and morphological changes of the hyphae (54). In the case of tensin, however, an alternative mode of action was suggested, possibly involving interference with cell wall synthesis (37). Phenazines also exert direct antibiotic effects toward several plant pathogens, including *R. solani* (45). However, during our microscopic experiments, we could not observe any effect of phenazines on the growth pattern of *R. solani*. Possibly, the experimental setup did not allow phenazine production to reach concentrations sufficient to inhibit the growth of *R. solani*.

Biosurfactants can interact with other excreted metabolites, thereby enhancing their bioavailability and bioactivity (9). Along



**Fig. 2.** Biological control of Rhizoctonia root rot on bean after application of washed *Pseudomonas* CMR12a bacteria and its mutants CMR12a-ΔPhz, CMR12a-CLP1, CMR12a-ΔPhz-CLP1, and CMR12a-GacA. Washed bacteria were applied to soil at a concentration of  $10^6$  CFU  $g^{-1}$  of soil 3 days before inoculation with **A**, *Rhizoctonia solani* anastomosis group (AG) 2-2 or **B**, *R. solani* AG 4 HGI. Disease symptoms on bean plants were evaluated 6 days after inoculation using the following scale: 0 = healthy, absence of symptoms; 1 = small black or brown lesions <1 mm in diameter; 2 = lesion covering <75% of the stem or root surface; 3 = lesion covering >75% of the stem or root surface; and 4 = seedling dead. Data represent means of disease scale obtained from three replicates, each with 10 plants per treatment. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney nonparametric tests ( $\alpha = 0.05$ ).

these lines, Perneel et al. (39) showed that simultaneous production of rhamnolipid-type biosurfactants and phenazines is crucial for biological control of *Pythium* spp. by *Pseudomonas aeruginosa* PNA1. The authors hypothesized that biosurfactants improved physical contact between hyphae and phenazines and increased entry of phenazines into the fungal cell. In this study, both the CLP-negative mutant CMR12a-CLP1 and the phenazine-negative mutant CMR12a- $\Delta$ Phz still significantly reduced Rhizoctonia root rot of bean, suggesting an additive effect of CLPs and phenazines. Considering the structural differences between the CLP-type biosurfactants produced by *Pseudomonas* CMR12a and the rhamnolipid-type biosurfactants produced by *P. aeruginosa*

PNA1, it is reasonable to assume that both groups of biosurfactants have different modes of action (44). However, we cannot exclude the possibility that the second CLP, CLP2, which is still being produced by CMR12a-CLP1 (see above), is involved in enhancing phenazine activity.

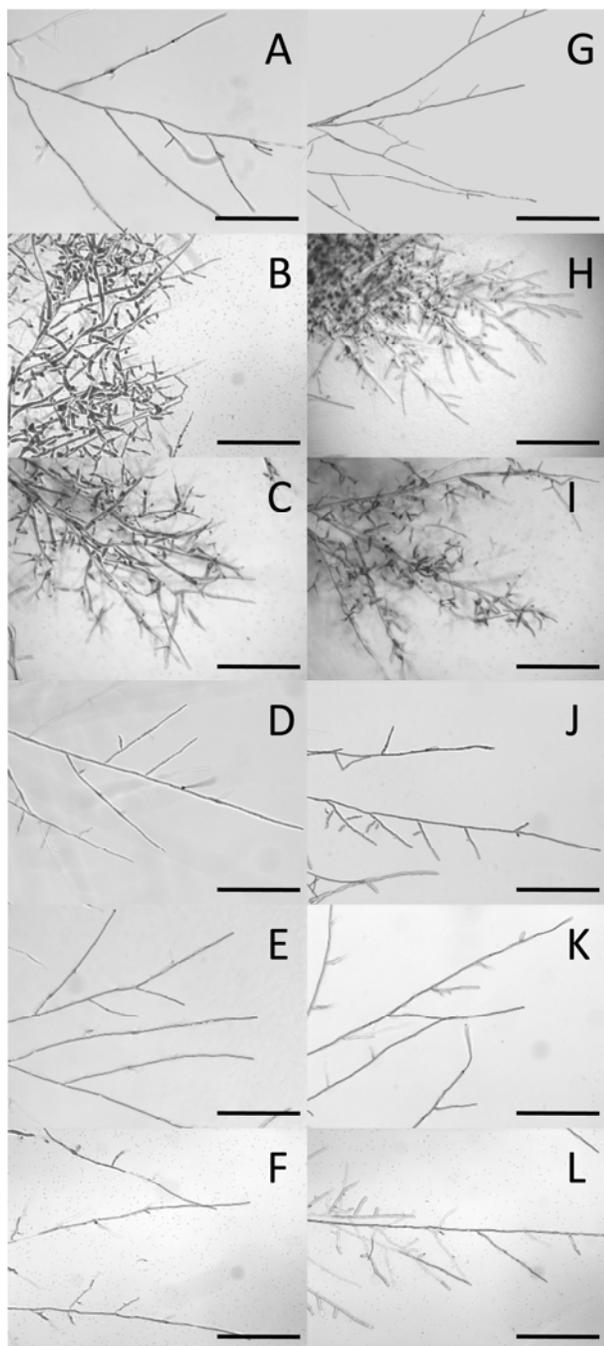
Numerous studies have acknowledged the importance of CLPs for bacterial motility and root colonization, which are essential features of bacterial biocontrol agents of soilborne pathogens (1,39,55,57). An increased rhizosphere competence of biosurfactant-producing strains was suggested under sandy soil conditions (36), as were prevalent during the experiments conducted in this study. Phenazine production can provide an additional advantage during establishment in the rhizosphere (31). Consequently, it would not be surprising if phenazines and CLPs produced by CMR12a were involved in the ecological competence of CMR12a. Yet, in this study, root counts of CMR12a- $\Delta$ Phz and CMR12a-CLP1 were not significantly lower in most cases compared with the wild type. Nevertheless, the two mutants deficient in both phenazines and CLPs yielded significantly lower population levels compared with the parental strain, indicating that CMR12a must produce at least one of both compounds to efficiently defend its ecological niche. It must be noted that the *gacA* mutation in CMR12a-GacA causes pleiotropic effects, which could influence root colonization and biocontrol capacity for reasons not necessarily related to phenazine or CLP production. Notwithstanding the observed differences in root colonization, all bacterial strains were able to reach a concentration of  $10^5$  CFU g<sup>-1</sup> of root, a crucial colonization level considered necessary for optimal biological control (18,42).

An additional important factor influencing the disease-suppressive effect of CMR12a was the experimental setup; more specifically, preparation of the bacterial inoculum. Application of washed bacteria to soil still had a suppressive effect, although DS was only satisfactorily reduced after infection with *R. solani* AG 2-2. In the case of the highly aggressive isolate of *R. solani* AG 4 HGI, washed CMR12a bacteria did not provide sufficient protection, and most of the bean plants were killed. Apparently, metabolites produced on KB plates during growth of the bacteria are important for their biocontrol capacity. These metabolites could provide an advantage to the bacterial cells at the early stages of introduction into soil, preventing a decline in bacterial population generally observed after such an application (3). Nevertheless, bacterial root counts at the evaluation stage did not reveal any significant differences compared with nonwashed bacteria. A follow-up of the bacterial population in time should elucidate whether metabolites produced on plate stimulate rapid root colonization. Furthermore, CMR12a produces signal molecules during growth in vitro, including homoserine lactones that stimulate the production of phenazines (14). Such signal molecules might allow the bacteria to rapidly resume production of phenazines or CLPs once they have colonized the roots.

Together, our results provide evidence for the involvement of phenazines and CLPs in the disease reduction of Rhizoctonia root rot of bean by *Pseudomonas* CMR12a, and demonstrate the negative impact on biocontrol capacity caused by washing bacteria before soil application. A better insight into the role played by phenazines and biosurfactants in biological control of soilborne pathogens and the regulation and production of these metabolites in situ is imperative for optimization of biological control using *Pseudomonas* strains.

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**Fig. 3.** Mycelial growth pattern of **A to F**, *Rhizoctonia solani* anastomosis group (AG) 2-2 and **G to L**, AG 4 HGI microscopically observed after different treatments, including **A and G**, control; **B and H**, *Pseudomonas* CMR12a; **C and I**, *Pseudomonas* CMR12a- $\Delta$ Phz; **D and J**, *Pseudomonas* CMR12a-CLP1; **E and K**, *Pseudomonas* CMR12a- $\Delta$ Phz-CLP1; and **F and L**, *Pseudomonas* CMR12a-GacA. Scale bars = 500  $\mu$ m.

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