The role of cyclic lipopeptides produced by *Pseudomonas protegens* and related species in control of fungal diseases on bean and rice

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the degree of Doctor of Philosophy (PhD) in
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Dutch translation of the title: De rol van cyclische lipopeptiden geproduceerd door *Pseudomonas protegens* en aanverwante soorten ter bestrijding van schimmelziekten op boon en rijst.

Cover illustration: Genome mining of gene clusters for biosynthesis of biocontrol-related secondary metabolites in *Pseudomonas* sp. CMR5c.

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This dissertation is dedicated to my family…

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Yours sincerely,

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List of abbreviations

A domain	adenylation domain
ABA	abscisic acid
AG	anastomosis group
Ap	appressorium
antiSMASH	antibiotics & Secondary Metabolite Analysis Shell
Biosurfactant	biologically derived surface-active agent
BLAST	basic local alignment search tool
BR	brassinosteroid
BTH	S-methyl 1,2,3-benzothiadiazole-7-carbothioate
cAMP	cyclic adenosine monophosphate
C-domain	condensation domain
CD_3CN	acetonitrile-d_3
cDNA	complementary DNA
CFU	colony-forming unit
^{13}C NMR	carbon-13 nuclear magnetic resonance
CK	cytokinin
CLP	cyclic lipopeptide
CLP1	sessilins
CLP2	orfamides
CM	complete medium
COSY	correlation spectroscopy
cv.	cultivar
DAP	2,6-diaminopimelic acid
DAPG	2, 4-diacetylphloroglucinol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpi	days post inoculation
ET	ethylene
GA	gibberellic acid

Gm	gentamicin
GT	germ tube
HPLC	high performance liquid chromatography
hpi	hours post innoculation
IAA	indole-3-acetic acid
ISR	induced systemic resistance
JA	jasmonic acid
^{1}H NMR	proton nuclear magnetic resonance
HCN	hydrogen cyanide
HMBC	heteronuclear multiple-bond correlation
HSQC	heteronuclear single-quantum correlation
kb	kilobase
KB	King's B
LB	Luria-Bertani
LC-MS	liquid chromatography-mass spectrometry
LC-ESI-MS	liquid chromatography-eletrospray ionization-tandem mass spectrometry
LOX	lipoxygenase
MAMP	microbe associated molecular pattern
MHz	megahertz (10^6 Hz)
MAPK	mitogen-activated protein kinase
MS	mass spectrometry
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase
NRPS-PKS	nonribosomal peptide synthetase-polyketide synthase
OD	optical density
2-OH-PCA	2-hydroxyphenazine-1-carboxylic acid
PAL	phenylalanine ammonia lyase
PAMP	pathogen associated molecular pattern
PCA	phenazine-1-carboxylate
PCN	phenazine-1-carboxamide
PCR	polymerase chain reaction

PDA	potato dextrose agar
PDI	percent disease index
PHZ	phenazines
PR	pathogenesis-related
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RAST	rapid annotation using subsystem technology
RH	relative humidity
RNA	ribonucleic acid
ROESY	rotating-frame nuclear Overhauser effect spectroscopy
ROS	reactive oxygen species
RP-HPLC	resersed-phase high performance liquid chromatography
SA	salicylic acid
SD	standard derivation
SDS	sodium dodecyl sulfate
sp.	species
SPE	solid-phase extraction
T domain	thiolation domain
TCA	tricarboxylic acid
TE	thioesterase
TOCSY	total-correlation spectroscopy
UPLC	ultrahigh performance liquid chromatography
UPLC-MS	ultrahigh performance liquid chromatography-mass spectrometry
WLIP	white line-inducing principle
WT	wild type

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Chapter 1. Problem statement

Problem statement

Certain plant beneficial microbes are potent cyclic lipopeptide (CLP) producers. CLPs are globally composed of a hydrophilic peptide moiety linked with a hydrophobic fatty acid residue. They are actively involved in interaction with cell membranes and show activity against diverse organisms, including fungi, bacteria, viruses, oomycetes and protists (Raaijmakers et al., 2010).

Gram-positive endospore-forming *Bacillus*-derived CLPs are among the most intensively studied microbial CLPs and show important roles in biocontrol of plant pathogens. *Bacillus*-derived CLPs are grouped in three major families: surfactins, fengycins and iturins. CLPs are the major antagonistic compounds produced by *B. subtilis*/*B. amyloliquefaciens* against fungal pathogens in plants (Cawoy et al., 2015). Besides direct inhibition of pathogens, surfactin-, fengycin- and iturin-type CLPs, have been intensively studied for their role in elicitation of innate immunity in a broad range of host 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).

Among the Gram-negative bacteria, *Pseudomonas* spp. receive a great amount of attention and are model microbes for biocontrol research (Höfte and Altier, 2010; Olorunleke et al., 2015a). Some *Pseudomonas* strains are also potent CLP producers (Raaijmakers et al., 2010). However, their role in direct antagonism and especially in induced systemic resistance (ISR) is less intensively studied than in the case of *Bacillus* CLPs. The only reported case of a *Pseudomonas* CLP involved in ISR is massetolide A, a compound that can enhance disease resistance against the late blight pathogen *Phytophthora infestans* in tomato (Tran et al., 2007).

P. protegens and related species have been intensively studied as biocontrol agents and were chosen as the model microbes in this dissertation. *P. protegens* has been recently defined as a new species, and includes the well-studied model strains *P. protegens* CHA0 and Pf-5 (Ramette et al., 2011). *Pseudomonas* sp. CMR12a and CMR5c are cocoyam derived biocontrol strains (Perneel et al., 2007) that are closely related to *P. protegens* (Flury et al., 2016; Ma et al., 2016a). *P. protegens* Pf-5 and *Pseudomonas* sp. CMR12a produce orfamide-type CLPs. Additionally, *Pseudomonas* sp. CMR12a

produces a second class of CLPs that were called sessilins and are closely related to tolaasins produced by the mushroom pathogen *P. tolaasii* (Gross et al., 2007; D'aes et al., 2014). *Pseudomonas* sp. CMR5c also shows biosurfactant activity, but its biosurfactant has not yet been characterized (Perneel et al., 2007).

Rice and bean are important crops in subtropical and tropical areas in the world. However, worldwide productivity of rice and bean is threatened by fungal diseases such as blast disease caused by *Magnaporthe oryzae* and brown spot disease caused by *Cochliobolus miyabeanus* in rice, and root rot and web blight diseases caused by *Rhizoctonia solani* in bean. Blast disease in rice is considered the most devastating fungal plant pathogen in the world (Dean et al., 2012). Since chemical agents can cause severe environmental problems and other side effects, as an alternative choice, biocontrol agents arise as the most promising way to manipulate diseases in crops.

To date, the role of sessilin- and orfamide-type CLPs produced by *P. protegens* and related species has barely been reported in *in planta* assay. Therefore, this dissertation is focused on the role of these CLPs and their producing strains in biocontrol of selected pathogens in rice and bean, and their possible mode of actions, such as direct antagonism and induced systemic resistance. We have addressed several scientific questions in this thesis, which are listed below.

1. Orfamide-type CLPs were first discovered in *P. protegens* Pf-5 (Gross et al., 2007). Do other *P. protegens* and related species also produce orfamides? Do orfamide-type CLPs produced by these strains show diversity in structure and biosynthesis and does this influence their biological activity?

2. It has been shown before that sessilins play a role in direct antagonism against *R. solani* root rot on bean (D'aes et al., 2011). *Pseudomonas* sp. CMR12a not only produces sessilins but also orfamides. Do orfamides also play a role in direct antagonism?

3. Do sessilins and orfamides play a role in induced systemic resistance (ISR) and if so, is their role plant and pathogen dependent? How do plants respond to orfamide-type CLPs?

Thesis outline

This thesis is focused on *P. protegens* and related species, the CLPs they produce and their role in biocontrol of fungal diseases in plants. In **chapter 2**, we review the literature to summarize and introduce the background and research topics discussed in chapters **3**-**6**. We also include a brief introduction to the pathosystems used in this study, including *R. solani on* bean, and *M. oryzae* and *C. miyabeanus* on rice.

P. protegens Pf-5 (Gross et al., 2007) and *Pseudomonas* sp. CMR12a (D'aes et al., 2014) have the ability to produce orfamide-type CLPs. It was hypothesized that the biosynthetic gene clusters and homologues produced by *P. protegens* and related species can diverge. In **chapter 3**, we conduct a detailed comparison and analysis of nonribosomal peptide synthetases (NRPSs), chemical fingerprints and biological activities (including structure-activity relationship) of orfamide-type CLPs produced by selected *P. protegens* and related species.

Pseudomonas sp. CMR12a is an effective biocontrol strain and it has been reported before that phenazines and sessilins are important in direct antagonism against *R. solani* root rot on bean (D'aes et al., 2011). In **chapter 4**, we examine whether also orfamides are important in direct antagonism. We conduct assays with *Pseudomonas* sp. CMR12a and mutants impaired in phenazines and/or CLPs against *R. solani* root diseases in bean and cabbage and also test the effect of purified metabolites (phenazines and orfamides).

As an orfamide- and sessilin-producer, *Pseudomonas* sp. CMR12a is ideally suited to test the role of both CLPs in ISR. In **chapter 5**, the role of sessilins and orfamide in ISR is assessed in bean against *R. solani* web blight and in rice against *M. oryzae*. Assays with *Pseudomonas* sp. CMR12a, mutants impaired in phenazines and/or CLPs and purified compounds, are combined to elucidate the corresponding ISR elicitors involved in these types of interactions.

Orfamides were able to trigger ISR to the necrotrophic fungal pathogen *R. solani* in bean, but not against the hemibiotrophic pathogen *M. oryzae* (chapter 5). Since it is known that the defensive mechanisms in rice against necrotrophic and (hemi)biotrophic pathogens are different (Ahn et al.,

2005), we wondered whether orfamides may trigger ISR in rice against the necrotrophic pathogen *C. miyabeanus*. **In chapter 6**, the selected orfamide-producing *Pseudomonas* bacteria are used to study the possible role of orfamides in rice against the brown spot pathogen *C. miyabeanus*. In addition, pure orfamide is applied to rice cell cultures and on rice plants to elucidate the plant defense responses triggered by orfamide in the absence and presence of *C. miyabeanus*.

In **chapter 7**, the main findings of this study are discussed and suggestions for further research are formulated.

Chapter 2. Literature review

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Literature Review

2.1 Plant diseases: chemical therapy and biocontrol

In agroecosystems, crops are threatened by multiple abiotic and biotic stresses, which can seriously impact their productivity. These abiotic and biotic stresses mainly include salt, drought, and attacks by viruses, bacteria, fungi and oomycetes, nematodes, insects, animal predators, etc. Chemical therapy is one of the most effective ways to control pathogens and pests in plants. However, treatments with chemical pesticides can lead to serious environmental consequences, such as soil pollution by recalcitrant components of chemical pesticides, and contamination of global surface water by insecticides (Stehle and Schulz, 2015). Moreover, human health can be threatened by hazardous chemical pesticides (Di Renzo et al., 2015). More importantly, some plant diseases cannot effectively be controlled by chemical pesticides. For instance, soil-borne diseases are major yield-limiting factors that used to be effectively controlled by soil fumigants, but in many countries their use is no longer allowed, and alternatives are urgently needed. Another problem is that plant pathogens and pests have the ability to develop resistance against chemical pesticides, so resistance management is an important issue and control strategies with different modes of action need to be alternated. Integrated pest management needs the combination of chemical pesticides with other agents, for instance, biocontrol agents.

Biocontrol is a method of controlling insects, mites, weeds, plant diseases and other pests using other organisms (Höfte and Altier, 2010). Biocontrol of plant diseases has been launched in 1910s (Baker, 1987) and has developed fast during the last several decades. Biocontrol agents are usually environmental friendly with low toxic properties, and are relatively efficient in controlling soilborne diseases.

2.2 Plant associated microbes from plant roots and rhizosphere

The rhizosphere is the natural habitat of diverse microorganisms (Philippot et al., 2013). Certain rhizosphere-derived microorganisms are actively involved in stimulating the defense-related behavior of plants (Berendsen et al., 2012; De Coninck et al., 2015). Much work has been done to

elucidate the microbial populations in suppressive soils. Suppressive soils are characterized as soil types in which disease development is limited to a minimal level (Mazzola et al., 2002). A nice survey showed that Proteobacteria are the major components of the microbes in the natural rhizosphere of maize and sugarbeet (Philippot et al., 2013). The γ- and β-Proteobacteria and the Firmicutes (Lactobacillaceae) are more abundant in population than other microbes in suppressive soils, and are the major microbes contributing to soil suppressiveness (Mendes et al., 2011). However, microbial populations in the rhizosphere and endophytic bacteria within the root systems can be distinct (Bulgarelli et al., 2012). Actinobacteria and Proteobacteria were shown to be the most promising endophytic bacteria in the root systems of *Arabidopsis thaliana* (Bulgarelli et al., 2012; Lundberg et al., 2012).

In addition, certain microbes derived from the rhizosphere niche of healthy plants in infected soils are recognized as potential biocontrol agents (Perneel et al., 2007; Raaijmakers et al., 2009). Intensively studied rhizosphere- or soil-derived microbes are endospore-forming *Bacillus* species (Ongena and Jacques, 2008) and *Pseudomonas* species (Höfte and Altier, 2010). Some of the isolates from these species are commercialized as biocontrol agents (Velivelli et al., 2014).

Screening and selecting candidate biocontrol agents should firstly consider the capacity of biocontrol both in lab and in field conditions targeting to specific plant diseases and crops. Application of the potential bacterial biocontrol agents has several strict policies, including biosafety considerations, field biocontrol capacity (not equal with capacity in greenhouse condition), formulation and shelf life (Kamilova and de Bruyne, 2013). Although soil harbors opportunistic human pathogens which could be potential weapons for fighting diseases in crops, the utilization of opportunistic human pathogens should be excluded from field trails and further field applications.

2.3 Mechanisms involved in biocontrol by beneficial bacteria

Mechanisms of plant beneficial microbes for biocontrol of diseases in plants are sophisticated, but mainly include direct effects on plant pathogens and indirect control of plant pathogens (for instance, elicitation of plant innate immunity), which suppresses the development of disease caused by plant pathogens. The role of antibiosis and induced systemic resistance (ISR) on biocontrol of plant

diseases will be discussed in the following paragraphs, since this thesis is mainly focused on these two kinds of biocontrol mechanisms.

2.3.1 Antibiosis

Plant beneficial microbes can secrete secondary metabolites under *in vivo* and *in vitro* conditions. Specific metabolites have antibiotic activities which directly target plant pathogens, and thus disturb the growth and development of targeted plant pathogens and further alleviate symptoms caused by pathogens in plants. These kinds of direct antagonism play important roles in the control of plant pathogens. The antibiosis capacity of potential microbes, namely the ability to produce antibiotic types of metabolites, is commonly used for screening biocontrol agents (Stutz et al., 1986; Perneel et al., 2007; Pliego et al., 2011). The antagonistic activities of antibiotics produced by plant beneficial microbes have been intensively studied. For instance, as one of the most popular biocontrol microbes, the antibiosis activities against fungal plant pathogens of *Bacillus* sp. are largely attributed to the production of CLPs (Cawoy et al., 2015). More detailed information about the role of antibiosis in biocontrol by *Pseudomonas* species is given in 2.4.

2.3.2 ISR

In nature, plants have evolved sophisticated immune systems to deal with diverse abiotic and biotic stresses. Immune responses in plants are triggered by elicitors, that are called MAMPs when they originate from nonpathogenic microbes or PAMPs when derived from pathogenic microbes. MAMPs or PAMPs are normally perceived by pattern recognition receptors (PRRs) located on the plant plasma membrane leading to MAMP- or PAMP-triggered immunity (MTI or PTI) (Zamioudis and Pieterse, 2012; Macho and Zipfel, 2014; Cook et al., 2015). Plants can also recognize their own damage, leading to damage associated molecular pattern-triggered immunity or DPI (Cook et al., 2005). MTI, PTI and DPI are recognized as the primary plant immune system. In addition, plants can also recognize effector proteins, secreted by pathogens, via resistance proteins that usually reside in the cytoplasm leading to a seconday immune system of plants, called effector triggered immunity (ETI) (Zamioudis and Pieterse, 2012).

ISR is a state of enhanced defensive capacity throughout the whole plant that is triggered upon

perception of elicitors produced by microbes that live in the rhizosphere and suppresses the further invasion and development of plant pathogens (Pieterse et al., 2014). In this sense ISR resembles MTI (Newman et al., 2013; Zamioudis and Pieterse, 2012).

Numerous plant beneficial microbes have the ability to trigger ISR in plants. These plant beneficial microbes mainly include the Gram-positive *Bacillus* species and the Gram-negative *Pseudomonas* species (De Vleesschauwer and Höfte, 2009). A certain elicitor can trigger defense responses in a broad range of host plants against diverse pathogens, or only in a specific plant-pathogen interaction. In most cases, ISR elicitors are perceived by pattern recognition receptors located at the plant plasma membrane (Trdá et al., 2015). ISR responses can be launched in a plant-pathogen system by specific elicitor concentrations, while higher or lower concentrations are not effective (De Vleesschauwer and Höfte, 2009). Diverse metabolites produced by plant beneficial microbes in the rhizosphere niche have the ability to mediate ISR responses, and the elicitors secreted by bacteria in triggering innate immunity in plants have been reviewed (De Vleesschauwer and Höfte, 2009; Mariutto and Ongena, 2015). The main elicitors produced by plant beneficial bacteria include 2R,3R-butanediol, exopolysaccharides and CLPs (surfactin, fengycin and iturin) from *Bacillus* species; salicylic acid, pyocyanin, 4-(aminocarbonyl) phenylacetate, 2,4-diacetylphloroglucinol (DAPG), pyoverdine, massetolide A, flagella, pseudobactin, N-alkylated benzylamine, lipopolysaccharides and rhamnolipids secreted by *Pseudomonas* species.

Perception of elicitors by plant cell cultures is usually accompanied by early biochemical or biophysical changes. These changes include medium alkalinisation, Ca^{2+} influx, reactive oxygen species (ROS) and nitric oxide production (for instance, H_2O_2) (Blumwald et al., 1998; Garcia-Brugger et al., 2006). Enzymatic reactions responsible for early events mainly include Ca^{2+}-ATPase (Ca^{2+} influx), H^+-ATPase (pH changes), together activating ion channels in the plant plasma membrane; and NADPH oxidase for generating ROS.

Following these early changes, elicitors normally also mount defense related gene expression and enzymatic pathways, for instance, the activation of phenylalanine ammonia lyase (PAL) in the phenyl propanoid pathway. As an important biosynthesis pathway for lignin and other polyphenol compounds, the activation of PAL is normally linked with reinforcement of plant cell walls, and thus

provides further protection against pathogen attack (Seifi et al., 2013). Other important enzymes related to plant defense are lipoxygenases (LOXs), these dioxygenases are involved in the synthesis of various oxylipins (including jasmonates) from substrates such as linolenic and linoleic acids (Siedow, 1991; Porta and Rocha-Sosa, 2002), and have diverse biological functions in plant cells, for instance, enhancing innate immunity and thus leading to disease resistance in plants.

It has been reported that many MAMPs derived from plant beneficial microbes can trigger these biochemical and/or biophysical responses, transcriptional changes, enzymatic responses, and defensive related pathways in plant cell cultures. For instance, the CLP surfactin can mount early and late stages of defensive responses, such as pH alkalinisation, ROS production, and activation of PAL and LOX pathways in tobacco cell cultures (Jourdan et al., 2009). Rhamnolipids derived from *P. aeruginosa* trigger ROS production, Ca^{2+} influx, and transcriptional accumulation of defense related genes, such as chitinase, glucanase, PAL and LOX genes, and other defense related responses in grapevine cell cultures (Varnier et al., 2009). Uncharacterized metabolites from *P. protegens* CHA0 and *P. aeruginosa* 7NSK2 can induce ROS production in grapevine cell suspensions (Verhagen et al., 2010). Surfactin- or fengycin-type CLPs are involved in the elicitation of defense in tomato against *Botrytis cinerea* and activate the LOX pathway (Ongena et al., 2007; Cawoy et al., 2014). Stimulation of LOX is involved in *P. putida* BTP1 triggered innate immunity in bean (Ongena et al., 2004) and tomato (Mariutto et al., 2011) against *Botrytis cinerea*. PAL, LOX and other enzymes are activated by *P. fluorescens* isolate DABBV4 mediated innate immunity in tomato against *Ralstonia solanacearum* (Vanitha and Umesha, 2011). ROS signaling plays important roles in elicitation of innate immunity in rice plants by redox-active pyocyanin (produced by *P. aeruginosa* 7NSK2) and siderophore pseudobactin (produced by *P. fluorescens* WCS374r) against *M. oryzae* (De Vleesschauwer et al., 2006; De Vleesschauwer et al., 2008).

Plant hormones are small molecules related to plant growth, abiotic and biotic stress tolerance, etc. It has been shown that plant hormones are playing central roles in dealing with abiotic and biotic stresses in plants. These plant hormones include salicyclic acid (SA), jasmonic acid (JA) and ethylene (ET), and other hormones such as indole-3-acetic acid (IAA), gibberellic acid (GA), cytokinin (CK), brassinosteroid (BR) and abscisic acid (ABA), and the roles of these plant hormones in innate immunity of different plants have been reviewed in several publications (Pieterse et al.,

2009; De Vleesschauwer et al., 2013; De Vleesschauwer et al., 2014). Diverse plant hormones can be involved in elicitor-induced innate immunity against different pathogens. For instance, rhamnolipid-mediated innate immunity in *Arabidopsis thaliana* highlighted the role of SA, while other plant hormones, such as JA and ET are also involved in disease resistance against various pathogens (Sanchez et al., 2012). SA accumulated in surfactin-primed tobacco cell suspensions (Jourdan et al., 2009).

2.4 Biocontrol-related secondary metabolites produced by *P. protegens* and related species

Pseudomonas species belonging to the Proteobacteria not only can be potential biocontrol agents against diseases in crops (Höfte and Altier, 2010), but also harbor large pools of genes for biosynthesis of diverse secondary metabolites (Gross and Loper, 2009). These metabolites are involved in antibiosis, biofilm formation or other indispensable functions for *Pseudomonas* themselves, and these metabolites may play roles in interspecies interactions, environmental adaptation and self-defense (Loper et al., 2012). *P. protegens* has been characterized as a new species recently. Strains include *P. protegens* CHA0 and Pf-5 (Ramette et al., 2011).

P. protegens CHA0 was isolated from tobacco roots grown in suppressive soil in 1986, and the strain showed potential to control black root rot, caused by *Thielaviopsis basicola* (Berk. & Br.) Ferraris, in tobacco plants (Stutz et al., 1986; Almario et al., 2014). Moreover, *P. protegens* CHA0 showed biocontrol in cucumber and cress against *Pythium ultimum* disease (Maurhofer et al., 1992; Schnider et al., 1995; Valverde et al., 2003; Péchy-Tarr et al., 2005; Takeuchi et al., 2012), in wheat against take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Wirthner et al., 1992), in tomato against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Zuber et al., 2003), and showed ISR in grapevine against *Botrytis cinerea* (Verhagen et al., 2010), and in *Arabidopsis thaliana* against the oomycete *Peronospora parasitica* (Iavicoli et al., 2003).

P. protegens Pf-5 was isolated from the rhizosphere of cotton, Texas, USA, in 1979, and showed capacity to control damping-off disease in seedlings of cotton caused by *R. solani* (Howell and Stipanovic, 1979), and *Pythium ultimum* (Howell and Stipanovic, 1980). Furthermore, it has been shown that *P. protegens* Pf-5 is able to control these pathogens in cucumber, pea and maize plants

(Kraus and Loper, 1992; Loper et al., 2007). Moreover, *P. protegens* Pf-5 protected wheat against *Pyrenophora tritici-repentis* (Pfender et al., 1993), and suppressed crown and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato (Sharifi-Tehrani et al., 1998), and seed piece decay caused by *Erwinia carotovora* on potato (Xu and Gross, 1986).

Pseudomonas sp. CMR12a and CMR5c are cocoyam-derived biocontrol strains which can be used to control root rot caused by *Pythium myriotylum* in cocoyam (Perneel et al., 2007). Moreover, *Pseudomonas* sp. CMR12a showed biocontrol against root rot diseases caused by *R. solani* on bean and Chinese cabbage (D'aes et al., 2011; Olorunleke et al., 2015). Sessilin and orfamide-type CLPs and phenazines are important biocontrol-related secondary metabolites produced by *Pseudomonas* sp. CMR12a (D'aes et al., 2014), and all these antibiotics are actively involved in the direct control of *R. solani* AG 4-HGI (Olorunleke et al., 2015), causal agent of root rot disease on bean.

A phylogenic survey revealed that *P. protegens* CHA0 and Pf-5, *Pseudomonas* sp. CMR12a and CMR5c are closely related to each other (Ruffner et al., 2015; Flury et al., 2016). *Pseudomonas* sp. CMR12a and CMR5c belong to a not yet defined new species positioned between *P. protegens* and *P. chlororaphis* in the *P. fluorescens* group.

Genome mining for potential production of secondary metabolites from *Pseudomonas* species has been intensively studied recently. Since the first genomic sequence of *P. protegens* strain Pf-5 has been released in 2005 (Paulsen et al., 2005), afterwards more and more genome sequences were released due to relative low costs for next generation sequencing. Also the genomic sequence of CMR5c has been released (Flury et al., 2016), and recently the genomic sequence of *P. protegens* CHA0 has been published (Jousset et al., 2014). The genome sequences (unpublished sequences) of *Pseudomonas* sp. CMR12a is also available in the Lab of Phytopathology (Ghent University, Belgium). Several important biocontrol related antibiotics for which the biosynthetic genes are present in *P. protegens* CHA0 and Pf-5, *Pseudomonas* sp. CMR5c and CMR12a, are shown in Table 2.1.

Table 2.1 The major biocontrol related metabolites produced by *P. protegens* and related species*.

Metabolite	*P. protegens*		*Pseudomonas* sp.	
	Pf-5	CHA0	CMR12a	CMR5c
Pyoluteorin	+	+	-	+
DAPG	+	+	-	+
Pyrrolnitrin	+	+	-	+
HCN	+	+	+	+
Sessilin	-	-	+	-
Orfamide	+	+	+	+
Pyoverdin	+	+	+	+
Enantio-Pyochelin	+	+	+	+
Rhizoxin	+	-	-	-
Toxoflavin	+	-	-	-
Phenazine	-	-	+	+

* "+" biosynthetic gene cluster of corresponding metabolite is positively detected, while "-" biosynthetic gene cluster for the metabolite cannot be detected in the genome of the strain.

A number of secondary metabolites has been detected and reported from *Pseudomonas* species by ways of genome mining and chemical analysis, for instance, the discovery of orfamides (Gross et al., 2007), rhizoxin (Loper et al., 2008) and toxoflavin (Philmus et al., 2015) from *P. protegens* Pf-5, orfamides and sessilins from *Pseudomonas* sp. CMR12a (D'aes et al., 2014), and viscosin/massetolide from *P. fluorescens* SBW25 (de Bruijn et al., 2007). It has been shown that *Pseudomonas* sp. CMR5c can produce phenazine-1-carboxylate (PCA), phenazine-1-carboxamide (PCN), pyrrolnitrin and pyoluterorin by chemical analysis (Perneel et al., 2007), however, a recent survey of genome mining shows that except these metabolites detected by chemical analysis from *Pseudomonas* sp. CMR5c, there are two extra gene clusters responsible for hydrogen cyanide (HCN) and DAPG biosynthesis (Flury et al., 2016). Here, we give an example of genome mining of antibiotics from *Pseudomonas* sp. CMR5c (Figure 2.1).

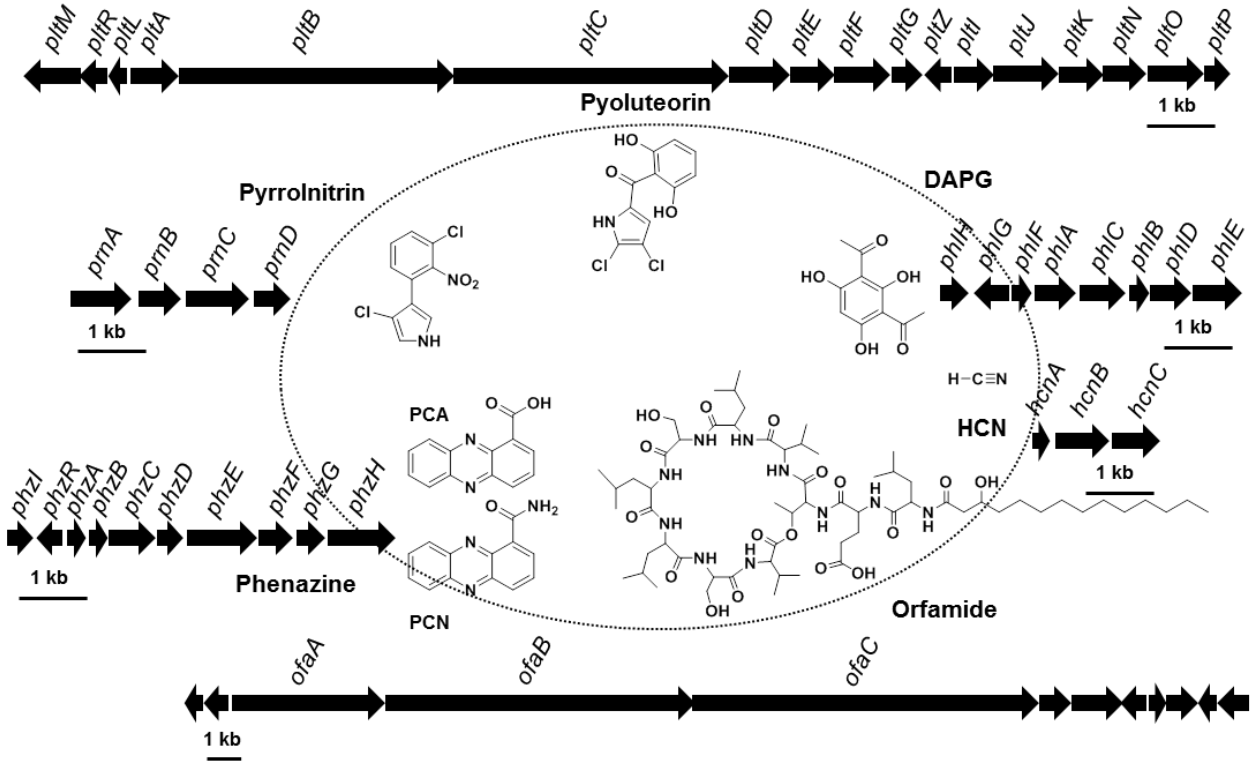

Figure 2.1 Genome mining of gene clusters for biosynthesis of biocontrol-related secondary metabolites in *Pseudomonas* sp. CMR5c. Chemical structures of major biocontrol related antibiotics and their gene clusters for biosynthesis, including phenazines (PCA and PCN), HCN, DAPG, pyoluteorin, pyrrolnitrin and orfamides, are shown.

Although strains of the *P. protegens* group are closely related to each other, the genomic traits (for instance, genome size, GC content, etc.) of these strains are distinct. Genome mining results showed that the gene clusters for secondary metabolites biosynthesis in these strains are also different from each other, as listed in Table 2.1. For instance, *P. protegens* CHA0 and Pf-5 do not possess gene clusters for phenazine biosynthesis, *Pseudomonas* sp. CMR12a has gene clusters for phenazine, HCN and CLPs; however, *Pseudomonas* sp. CMR5c has gene clusters for biosynthesis of phenazine, HCN, DAPG, pyoluteorin, pyrrolnitrin and CLPs, *P. protegens* Pf-5 has potential to produce rhizoxin and toxoflavin, which cannot be produced by the other strains, for instance, *P. protegens* CHA0, *Pseudomonas* sp. CMR12a and CMR5c. These traits show the capacity to produce various combinations of antibiotics by a single strain. Moreover, metabolites produced by the same species (for instance, *P. protegens* CHA0 and Pf-5) can be distinct.

Recently, biocontrol related metabolites produced by *Pseudomonas* species have been reviewed by Olorunleke et al (2015a). The major biocontrol related antibiotics produced by *P. protegens* and related species are phenazines, DAPG, pyrrolnitrin, pyoluteorin, HCN, rhizoxin, siderophore and CLPs. The producing strains and biocontrol roles of each metabolite are briefly introduced below.

2.4.1 Phenazines

The nitrogen-containing heterocyclic pigments, phenazines, are composed of over a hundred analogues (Abdelfattah et al., 2011). Phenazines are redox-active compounds and thus can induce the production of ROS (for instance, hydrogen peroxide) (Mavrodi et al., 2006). Certain *Pseudomonas* species can produce phenazine type antibiotics, such as PCA (Figure 2.1), PCN (Figure 2.1), pyocyanin, 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA), 2-hydroxyphenazine. Phenazines can act synergistically with rhamnolipid biosurfactants in the control of *Pythium* spp. (Perneel et al., 2008). Moreover, pyocyanin is actively involved in elicitation of innate immunity in rice against blast disease caused by *M. oryzae*, and in tomato against *Botrytis cinerea* (Audenaert et al., 2002; De Vleesschauwer et al., 2006). Besides triggering the innate immunity of plant, phenazines are also important antibiotics against fungal plant pathogens, and thus show potential utilization in biocontrol. PCN produced by *Pseudomonas* sp. CMR12a is involved in direct antagonism against Rhizoctonia damping-off disease on Chinese cabbage (Olorunleke et al., 2015).

2.4.2 DAPG

DAPG (Figure 2.1) is widely known as an antibiotic and has been well studied in the control of plant pathogens. The biocontrol properties of *Pseudomonas* DAPG-producers are better than those of non DAPG-producers in *Pythium*-cucumber and *Fusarium*-tomato pathosystems (Rezzonico et al., 2007). DAPG can inhibit the growth and trigger excessive branching in the damping-off causing oomycete pathogen *Aphanomyces cochlioides* (Islam and Fukushi, 2010). DAPG is even active against plant-

parasitic nematodes (Meyer et al., 2009). Besides direct antagonism, DAPG can act as an elicitor to mount innate immunity in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato (Weller et al., 2012), and the oomycete pathogen *Hyaloperonospora arabidopsidis* (formerly called *Peronospora parasitica*) (Iavicoli et al., 2003).

2.4.3 Pyrrolnitrin

Pyrrolnitrin (Figure 2.1) was first discovered from a *Pseudomonas* isolate in 1964 (Arima et al., 1964), and the structure of pyrrolnitrin was determined subsequently in 1965 (Imanaka et al., 1965) and 1966 (Nakano et al., 1966). The biocontrol activities of pyrrolnitrin-producing *Pseudomonas* species have been intensively studied. Pyrrolnitrin showed strong inhibitory activity against *R. solani*, and also showed protective activity in cotton seedlings against *R. solani* (Howell and Stipanovic, 1979). Other bacterial genera can also produce pyrrolnitrin, for instance, pyrrolnitrin produced by *Burkholderia cepacia* B37w showed antagonistic activity against the potato dry rot fungus *Fusarium sambucinum* (Burkhead et al., 1994).

2.4.4 Pyoluteorin

Pyoluteorin (Figure 2.1) was first isolated from *P. aeruginosa* and the structure of pyoluteorin was subsequently determined by chemical analysis (Takeda, 1958; Birch et al., 1964). *P. protegens* species are natural resources to produce pyoluteorin, and it is widely used as weapon for controlling plant diseases (Ramette et al., 2011). For instance, pyoluteorin produced by *P. protegens* is involved in the control of *Pythium* damping-off of cotton (Howell and Stipanovic, 1980) and cress (Maurhofer et al., 1994).

2.4.5 HCN

HCN (Figure 2.1) is a volatile compound secreted by many *Pseudomonas* species, including the human opportunistic pathogen *P. aeruginosa* (Gilchrist et al., 2013; Smith et al., 2013). Importantly,

HCN secreted by biocontrol *Pseudomonas* species has a potent role in the suppression of the development of fungal pathogens. For instance, Spence and colleagues have shown that HCN is important in antagonism against *M. oryzae* 70-15; more specifically, *P. protegens* CHA0 can inhibit the growth of *M. oryzae*, while its HCN-deficient mutant lost this capacity (Spence et al., 2014).

2.4.6 Rhizoxin

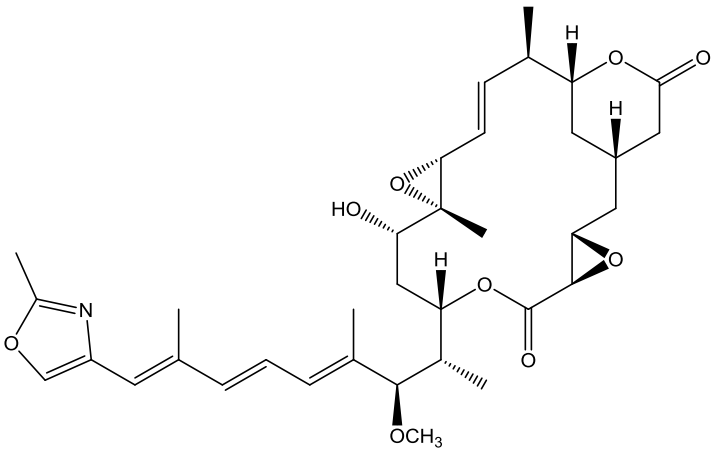

Figure 2.2 The chemical structure of rhizoxin

The polyketide macrolide compound rhizoxin (Figure 2.2) can cause rice seedling blight, and shows potent antagonistic activities against several plant fungal diseases (Iwasaki et al., 1984). Interestingly, genome mining combined with chemical identification showed that *P. protegens* Pf-5 has the capacity to produce rhizoxins (Loper et al., 2008). These rhizoxins show antifungal activities and can change the morphology of plant seedlings, such as, cucumber, pea and rice seedlings (Loper et al., 2008; Scherlach et al., 2012). Recent evidence shows that the antagonistic effect of *Pseudomonas* sp. Os17 against fungal and oomycete pathogens (for instance, *Pythium ultimum* and *Fusarium oxysporum*), is most likely due to the production of rhizoxin analogues (Takeuchi et al., 2015).

2.4.7 Toxoflavin

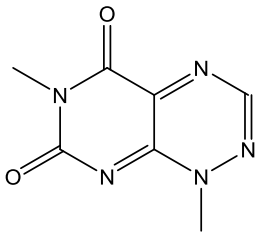

Figure 2.3 The chemical structure of toxoflavin

Toxoflavin was first discovered from plant pathogenic *Burkholderia* species in 1934 (Van Veen and Mertens, 1934). Toxoflavin produced by *Burkholderia glumae* functions as a phytotoxin causing bacterial wilt on many crops, such as tomato, sesame, perilla, eggplant, hot pepper, etc (Jeong et al., 2003; Koh et al., 2011). Remarkably, a recent report by Philmus and colleagues showed that *P. protegens* Pf-5 harbors a gene cluster for biosynthesis of toxoflavin, and mutagenesis analysis showed that toxoflavin contributes to the antagonistic activity against the plant pathogenic bacterium *P. syringae* DC3000 (Philmus et al., 2015).

2.4.8 Siderophores

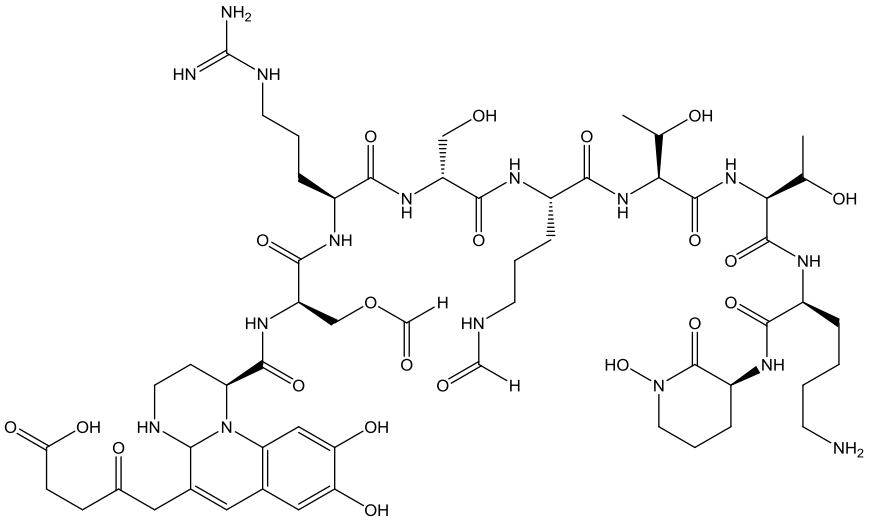

Figure 2.4 The chemical structure of a pyoverdine analogue

Siderophores are iron-binding molecules and are well known for the function of transporting iron ions into cells. The biosynthesis of siderophores involves a nonribosomal peptide synthetase (NRPS) pathway or NRPS-independent pathway (Miethke and Marahiel, 2007; Hoegy and Schalk, 2014; Noel et al., 2014). *Pseudomonas* species are potent siderophore producers and siderophores are actively involved in the control of plant pathogens (Leong, 1986). *Pseudomonas* species can secrete pyoverdine-like siderophores, and these pyoverdines are composed of a dihydroquinoline-type

chromophore linked with a peptidic moiety (Isabelle and Laurent, 2013) (See the chemical structure of a pyoverdine analogue in Figure 2.4). Siderophores produced by *P. aeruginosa* JAS-25 are involved in the antagonistic activity against *Fusarium oxysporum* f. sp. ciceri, *Fusarium udum*, and *Aspergillus niger* (Sulochana et al., 2014). Siderophore thioquinolobactin produced by *P. fluorescens* ATCC 17400 shows antifungal activity against *Pythium* (Matthijs et al., 2007). Siderophore purified from *P. putida* shows fungicide activity against *Fusarium oxysporum* f. sp. ciceri and *Helminthosporium oryzae* (Boopathi and Rao, 1999). Moreover, certain siderophore type metabolites are potent elicitors to mount the innate immunity in plants. For instance, pyochelin secreted by *P. aeruginosa* 7NSK2 has the capacity to induce ISR effect to *Botrytis cinerea* in tomato (Audenaert et al., 2002). Pseudobactin (belonging to the pyoverdine family) produced by *P. putida* WCS358 and *P. fluorescens* WCS374r is an important determinant in triggering innate immunity against *Botrytis cinerea* in tomato plants (Meziane et al., 2005) and against *M. oryze* in rice (De Vleesschauwer et al., 2008), respectively.

2.4.9 CLPs

CLPs are typically composed of a cyclic peptide backbone linked to a fatty acid tail. Many *Pseudomonas* species can produce CLPs (Raaijmakers et al., 2010). CLPs derived from *Pseudomonas* species can be grouped in several classes based on the length and amino acid composition of peptidic moiety, and within each subclass, the fatty acid residues also show differences, for instance, the length and the saturation of fatty acid part (Raaijmakers et al., 2006; Roongsawang et al., 2010). Structures of selected CLPs produced by *Pseudomonas* species are shown in Figure 2.5. CLPs possess both hydrophobic and hydrophilic properties, and therefore can easily interact with different cellular membranes and display a broad spectrum of bioactivities against bacteria, fungi, viruses, mammalian cells, protists, etc (Raaijmakers et al., 2010).

Pseudomonas-derived CLPs are important for their producers. Several classes of CLPs are actively involved in swarming motility and biofilm formation of their parental *Pseudomonas* strains. Corresponding mutants with deficiency in CLPs production lost or showed reduced swarming motility and biofilm formation compared to its wild type strain (de Bruijn et al., 2007; Li et al., 2013;

D'aes et al., 2014). Interestingly, the amendment of purified CLPs in the medium can restore the swarming motility of the CLPs deficient mutant (D'aes et al., 2014). These special traits of *Pseudomonas*-derived CLPs might be important for their producing bacteria to maintain important functions in natural environment, for instance, swarming motility can protect bacteria from natural predators.

The biosynthesis of CLPs is mediated by NRPSs in *Pseudomonas* species. Nonribosomal peptides are peptides that are not synthesized by ribosomes, but are modulated by NRPSs. An NRPS synthetase is typically composed of an adenylation (A) domain, a condensation (C) domain, a thiolation (T) domain and a terminal thioesterase (TE) domain (Strieker et al., 2010). Several of these domains compose a module which loads and incorporates a specific amino acid. An A domain is responsible for selecting amino acids for CLPs biosynthesis, and the configuration of amino acids is determined by the next C domain. A TE domain can form cyclic amides (lactams) or cyclic esters (lactones) and releases the CLPs from the NRPS synthetase. The NRPS assembly line of orfamide in *Pseudomonas* sp. CMR5c is shown in Figure 2.6.

The role of CLPs in antagonism has been intensively studied. An overview of representative biocontrol related *Pseudomonas* CLPs and their targeted pathogenic microbes, including bacteria and fungi [(hemi)biotrophs or necrotrophs] is given in Table 2.2. Certain CLPs derived from plant pathogenic *Pseudomonas* species also show antibiotic activities against plant pathogenic fungi, for instance, syringomycin-E, syringotoxin and syringopeptin derived from *P. syringae* pv. *syringae* (Lavermicocca et al., 1997).

Besides the direct biocontrol role of CLPs to fungal plant pathogens, the elicitation of plant innate immunity by CLPs has been reported, especially for endospore-forming Gram positive *Bacillus* species, these CLPs mainly include surfactin, fengycin and iturin family CLPs (Ongena and Jacques, 2008). The ISR capacity of *Pseudomonas*-derived CLPs has not been extensively studied. There is only one example showing that the CLP massetolide A can trigger defense related responses against late blight disease caused by *Phytophthora infestans* in tomato, and the defensive response triggered by massetolide in this plant-pathogen is independent of SA signaling (Tran et al., 2007).

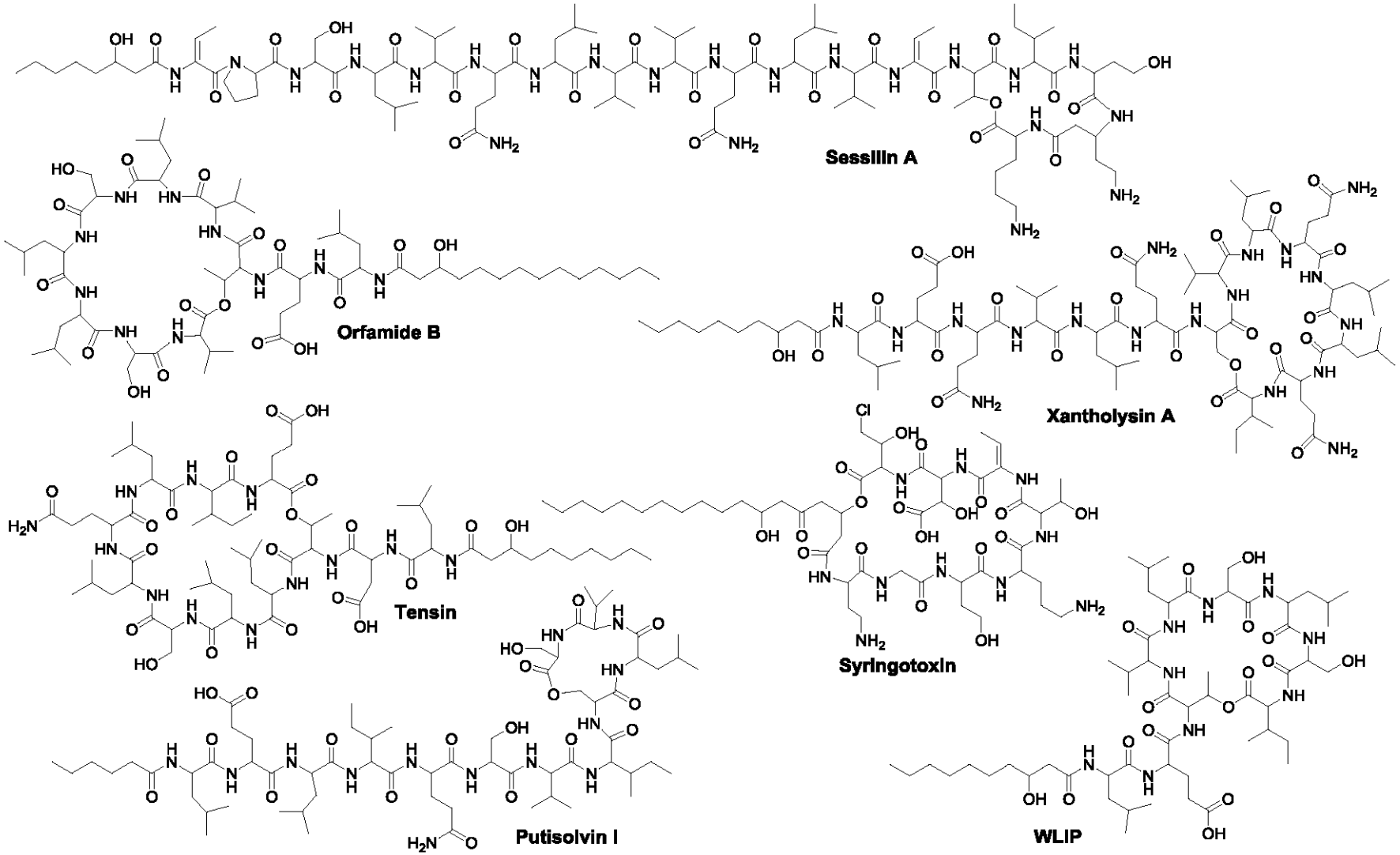

Figure 2.5 Representative chemical structures of *Pseudomonas* CLP family discussed in Table 2.2.

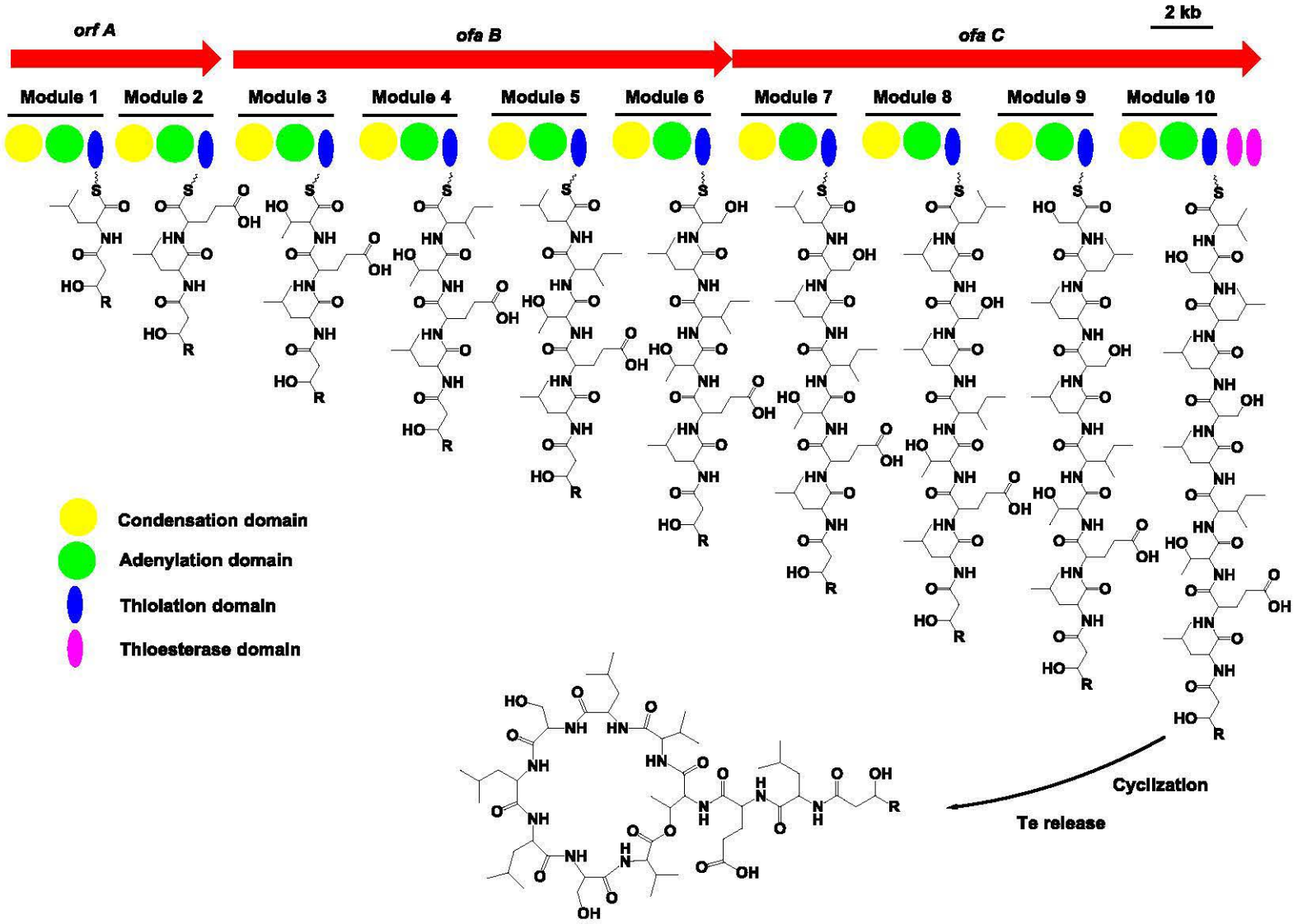

Figure 2.6 The NRPS assembly line for orfamide from *Pseudomonas* sp. CMR5c.

Table 2.2 The major biocontrol related CLPs derived from *Pseudomonas* species and their potential role in biocontrol against plant pathogens*.

CLPs	Producers	Targeted pathogen (s)	Mode of action	Assay	Reference
Viscosin family					
Viscosin/ Massetolide A	*P. fluorescens* SBW25	*Phytophthora infestans*	Lysis of zoospores	*In vitro*	de Bruijn et al., 2007
Massetolide A	*P. fluorescens* SS101	*Phytophthora infestans*	Antagonism; ISR	*In vitro* and soil assay in tomato	Tran et al., 2007
WLIP	*P. reactans* NCPPB1311; *P. putida* RW10S2	A broad spectrum of pathogenic fungi, chromista and bacteria, including *Fusarium solani*, *R. solani*, *Xanthomonas*, *X. citri* pv. *malvacearum* LMG 761, *X. axonopodis* pv. *manihotis* LMG 784, and *X. albilineans* LMG 494	Antagonism	*In vitro*	Lo Cantore et al., 2006; Rokni-Zadeh et al., 2012
Viscosinamide	*Pseudomonas* sp. *P. fluorescens* DR54	*Pythium ultimum*; *R. solani*	Antagonism	*In vitro*	Nielsen et al., 1999; Nielsen et al., 2002
		R. solani	Hyphal branching	*In vitro*	Raaijmakers et al., 2010
Nunamycin	*Pseudomonas* sp. In5	*R.solani*	Antagonism	*In vitro*	Michelsen et al., 2015a; Michelsen et al., 2015b
Pseudophomins A and B	*P. fluorescens* BRG100	*Phoma lingam*; *Sclerotinia sclerotiorum*; *R. solani*; *Alternaria brassicae*	Antagonism	*In vitro*	Pedras et al., 2003
Syringomycin family					
Syringomycin-E	*P. syringae*	*Fusarium moniliforme* and *F. oxysporum*	Antagonism	*In vitro*	De Lucca et al., 1999
	P. syringae pv. *syringae* B301	*Geotrichum candidum* ITM104; *Botrytis cinerea* PVBA405	Antagonism	*In vitro*	Lavermicocca et al., 1997
	P. syringae ESC-10 and ESC-11	*Penicillium digitatum*	Antagonism	*In vitro* and in planta (lemon)	Bull et al., 1998
Thanamycin	*Pseudomonas* sp. SH-C52	*R. solani* *Botrytis cinerea*	Antagonism	*In vitro*	Van Der Voort et al., 2015
Syringotoxin	*P. syringae* pv. *syringae* B359	*Geotrichum candidum* ITM104; *Botrytis cinerea* PVBA405	Antagonism	*In vitro*	Lavermicocca et al., 1997
Orfamide family					
Orfamide B	*Pseudomonas* sp. CMR5c	*R. solani*	Antagonism	*In vitro*	Olorunleke et al., 2015
Poaeamide	*P. poae* RE*1-1-14	*R. solani*;	Antagonism	*In vitro*	Zachow et al., 2015
		Phytophthora capsici;	Antagonism	*In vitro*	Zachow et al., 2015
Amphisin family					
Amphisin	*Pseudomonas* sp.	*Pythium ultimum*;	Antagonism	*In vitro*	Nielsen et al., 2002

		R. solani			
Lokisin	*Pseudomonas* sp.	*Pythium ultimum*; *R. solani*	Antagonism	*In vitro*	Nielsen et al., 2002
Hodersin	*Pseudomonas* sp.	*Pythium ultimum*; *R. solani*	Antagonism	*In vitro*	Nielsen et al., 2002
Tensin	*Pseudomonas* sp. *P. fluorescens* 96.578	*Pythium ultimum*; *R. solani*	Antagonism	*In vitro*	Nielsen et al., 2000; Nielsen et al., 2002
Putisolvin family					
Putisolvin	*P. putida* 267	*Botrytis cinerea* and *R. solani*	Antagonism	*In vitro*	Kruijt et al., 2009
		Phytophthora capsici	Lysis of zoo spores	*In vitro*	Kruijt et al., 2009
Xantholysin family					
Xantholysin	*P. putida* BW11M1	*Xanthomonas translucens* pv. *cerealis* LMG 679	Antagonism	*In vitro*	Li et al., 2013
		Xanthomonas axonopodis pv. *manihotis* LMG 784	Antagonism	*In vitro*	Li et al., 2013
Tolaasin family					
Sessilin	*Pseudomonas* sp. CMR12a	*R. solani* AG2-2	Antagonism	Soil assay in bean	D'aes et al., 2011
		R. solani AG 4 HGI	Antagonism	Soil assay in bean	D'aes et al., 2011; Olorunleke et al., 2015
Tolaasin I	*P. tolaasii* NCPPB2192	A broad spectrum of pathogenic fungi and chromista, including *Fusarium solani*, *R. solani*, etc.	Antagonism	*In vitro*	Lo Cantore et al., 2006
Syringopeptin (SP22-A)	*P. syringae* pv. *syringae* B427	*Geotrichum candidum* ITM104; *Botrytis cinerea* PVBA405	Antagonism	*In vitro*	Lavermicocca et al., 1997
Syringopeptin (SP25-A)	*P. syringae* pv. *syringae* B427	*Botrytis cinerea* PVBA405	Antagonism	*In vitro*	Lavermicocca et al., 1997
Sclerosin	*Pseudomonas* sp. DF41	*Sclerotinia sclerotiorum*	Disruption of electric potential of plasma membrane of the fungus	*In vitro* and soil assay	Berry et al., 2010; Berry et al., 2012
Nunapeptin	*Pseudomonas* sp. In5	*Pythium aphanidermatum*	Antagonism	*In vitro*	Michelsen et al., 2015a; Michelsen et al., 2015b
Thanapeptin	*Pseudomonas* sp. SH-C52	*Phytophthora infestans*	Antagonism	*In vitro*	Van Der Voort et al., 2015

* WLIP: white-line inducing principle; ISR: induced systemic resistance;

2.5 Important fungal diseases on rice and bean, and their control strategies

Fungal diseases in plants are generally grouped into three groups based on their lifestyle: biotrophs, hemibiotrophs and necrotrophs. Biotrophic lifestyle signifies that a pathogen can invade plant tissues without degrading plant tissues but instead feeds on the living plant cell. A necrotrophic pathogen kills plant tissues upon invading a plant. Hemibiotrophic pathogens start as a biotroph but switch to a necrotrophic lifestyle later on in the infection. Plant-pathogen systems used for biocontrol research are tractable systems which can mimic the interaction between biocontrol agents and pathogens in plants, especially under lab conditions. If the effects of biocontrol agents are stable in lab conditions, the capacity of biocontrol agents in field trials can be further investigated.

2.5.1 Root rot and web blight diseases in bean

Rhizoctonia spp. are important plant pathogenic fungi with a necrotrophic lifestyle. *R. solani* (teleomorph: *Thanatephorus cucumeris*) represents the best known species of Rhizoctonia. It is actually a species complex that is divided into different anastomosis groups (AGs) that differ in host range. Anastomosis is the vegetative fusion of hyphae of compatible fungi. In the case of *R. solani*, isolates that cannot fuse together are incompatible and are placed in different anastomosis groups. The disease cycle of *R. solani* is shown in Figure 2.7A. *R. solani* infects a broad range of host plants and can attack seeds, roots, stems and leaves (Ogoshi, 1976; Abawi et al., 1985). These host plants include important crops such as bean, tomato and cabbage (Shailbala and Tripathi, 2007). Certain isolates of *R. solani* can form sclerotia-type survival structures, which can stay viable in natural soil condition for many years (Boosalis and Scharen, 1959). The development of web blight caused by *R. solani* is favored by temperatures ranging from 26 – 28 ºC, with a relative humidity of 98 – 100 % (Dubey, 1997).

Soil-borne *R. solani* diseases have threatened the productivity of bean worldwide. These diseases include root rot and web blight disease caused by *R. solani* on roots and leaves of bean crop. Symptoms of root rot and web blight diseases are shown in Figure 2.7. A survey showed that a major decrease in bean productivity in the United States has been caused by Rhizoctonia root rot and web

blight disease, reaching losses higher than 40 %(Tachibana et al., 1971; Muyolo et al., 1993).

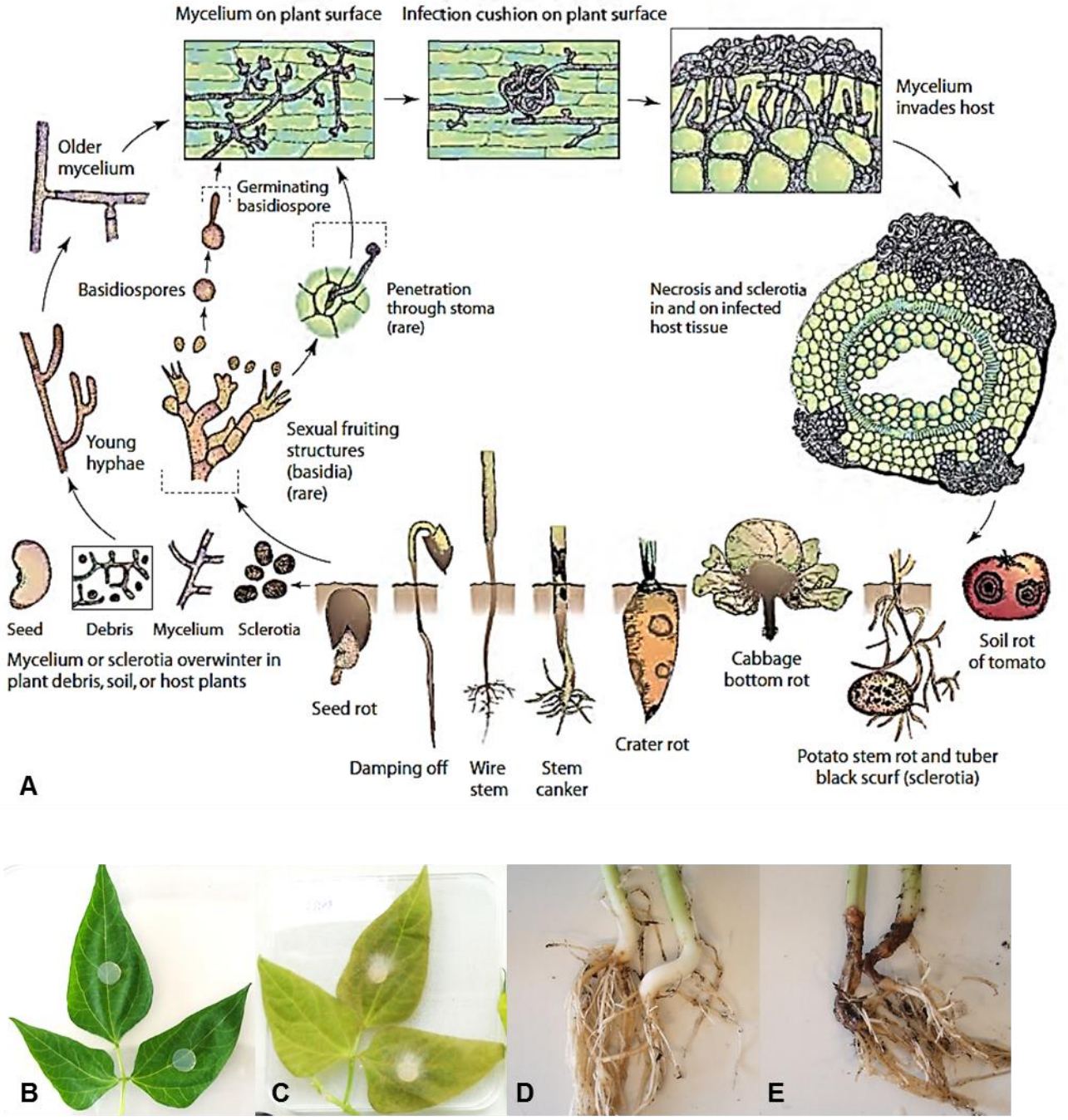

Figure 2.7 Disease cycle of *R. solani* (adapted from Agrios (2005), (**A**), and symptoms of Rhizoctonia diseases on bean plants (**B**-**E**). Healthy bean leaf (**B**), and bean leaf infected by *R. solani* AG2-2 showing symptoms of web blight disease (**C**). Healthy bean roots (**D**), and *R. solani* AG 4-HGI infected bean roots showing root rot symptom (**E**). Symptoms shown in figure C and E were evaluated 5 days and 7 days post infection, respectively.

Management of *R. solani* diseases is important for improving bean productivity worldwide. There have been many studies on the biocontrol of *R. solani* disease in lab conditions. Diverse secondary metabolites produced by plant beneficial microbes (for instance, *Bacillus* sp. and *Pseudomonas* sp.) are involved in *R. solani* biocontrol. Moreover, there are many reports about the interaction between CLPs and *R. solani*. For instance, fengycins produced by *B. mojavensis* B0621A showed potent fungicidal activity against *R. solani* (Ma et al., 2012). Root inoculation with *Pseudomonas* sp. CMR12a can reduce the symptoms caused by *R. solani* (D'aes et al., 2011; Olorunleke et al., 2015). PCN and orfamides produced by *Pseudomonas* sp. CMR12a showed antagonistic activities against *R. solani* (Olorunleke et al., 2015). Morphological changes of *R. solani*, for instance, hyphal branching, is the typical phenomenon observed by treatment of the fungus with different CLPs, such as viscosinamide (Raaijmakers et al., 2010) and orfamide (Olorunleke et al., 2015). However, to the best of our knowledge, the effects of *in planta* application of purified CLPs in the control of *R. solani* disease has not been studied.

2.5.2 Blast and brown spot diseases in rice

2.5.2.1 *M. oryzae*

M. oryzae (anamorph: *Pyricularia oryzae*), is the causal agent of blast disease, the most devastating disease on rice. *M. oryzae* has been ranked as the top one fungal plant pathogen in a recent survey (Dean et al., 2012). Moreover, *M. oryzae* also shows pathogenicity on a broad range of host plants, such as barley, wheat and millet (Couch et al., 2005). The life cycle of *M. oryzae* is shown in Figure 2.8. Theblack color of the appressoria is caused by accumulation of melanin. Typical symptoms of *M. oryzae* on rice leaves are the gray centered, 'eye' type sporulating lesions, as shown in Figure 2.8. Rice blast epidemics occur in relative high humidity and thermal conditions, and can result in great losses of rice productivity. Recent evidence shows that *M. oryzae* not only can infect upper part tissues of rice plants, such as, panicle, leaves and sheath, but also infect the roots of rice (Marcel et al., 2010).

Virulence factors of *M. oryzae* have been intensively investigated over the past decades. The appressorium is an important infection structure formed by *M. oryzae* (Howard and Valent, 1996) and the successful penetration of *M. oryzae* in rice needs appressorium formation (Liu et al., 2011). It has been shown that mitogen-activated protein kinase (MAPK) signaling and cyclic adenosine monophosphate (cAMP) signaling are major pathways involved in the pathogenicity of *M. oryzae* (Dean et al., 2005). Moreover, cAMP signaling and MAPK signaling are important regulators controlling appressorium formation (Xu and Hamer, 1996). Plant hormone biosynthesis by the fungus also plays an important role in pathogenicity (reviewed by Spence and Bais, 2015). Abscisic acid (ABA) and cytokinin (CK) biosynthesis pathways in *M. oryzae* play important roles in the pathogenicity of the blast fungus (Spence and Bais, 2015; Spence et al., 2015; Chanclud et al., 2016).

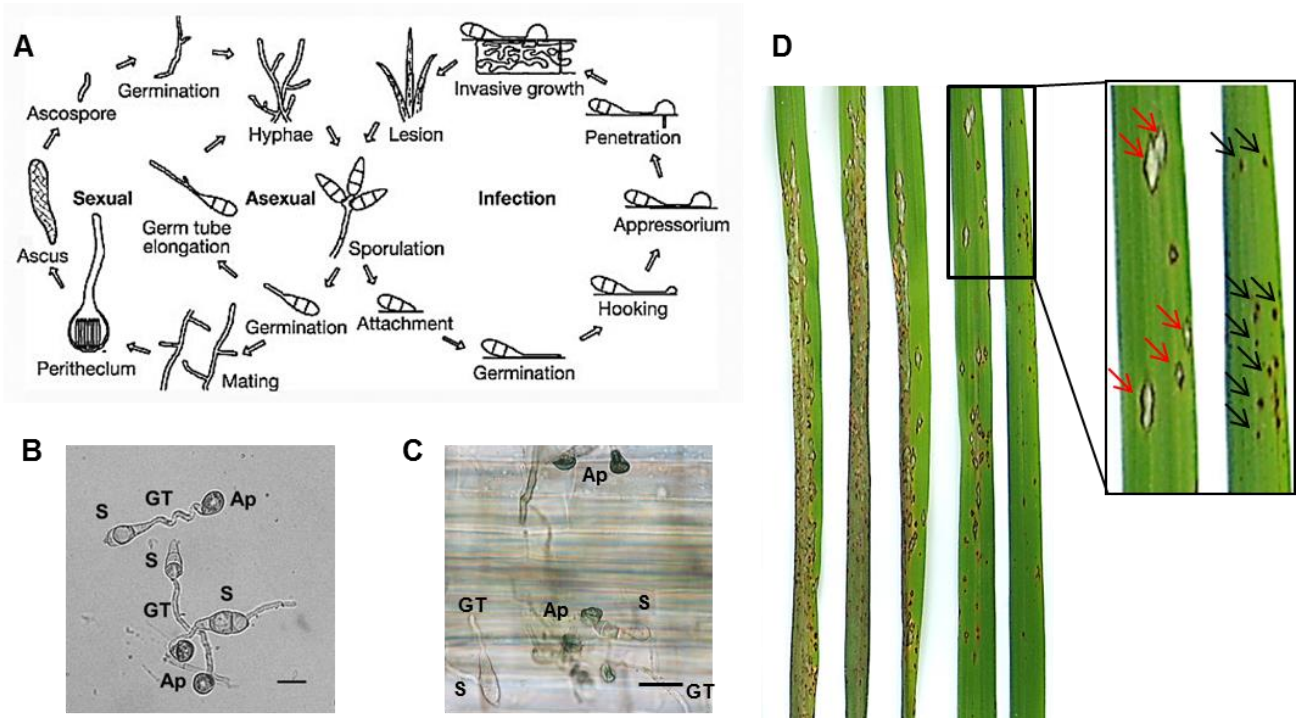

Figure 2.8 Life cycle, conidia morphology and disease symptoms on rice leaves caused by *M. oryzae*. **A**: Life cycle of *M. oryzae* (adapted from Dean et al., 2005), which includes asexual and sexual infection cycles. **B** and **C**: *In vitro* (cover glass of glass slide) (B) and *in vivo* (rice sheath) (C) development of conidia of *M. oryzae* VT5M1 growing on complete medium (CM) plate, S: spore; GT: germ tube; Ap: appressorium. Scale bar is 20 μm. **D**: Symptoms of *M. oryzae* VT5M1 on rice leaves 7 days post inoculation (dpi) by spray infection. Typical gray centered sporulation are shown (see red arrows of enlargement of D), small lesions represents the effects of hypersensitive response (see

black arrows of enlargement of D).

Table 2.3 Overview of the fungicides and their target for control of *M. oryzae*. These fungicides include natural products from microbes or plants, and synthetic chemicals.

Chemical *	Source	Target	Assay	Reference
Natural product				
Surfactin	*B. licheniformis* BC98	Inhibit the development of germ tube	*In vitro*	Tendulkar et al., 2007
Antifungal mycin 702	*Streptomyces padanus* JAU4234	Inhibit mycelial growth, conidia formation, and appressoria formation	*In vitro*	Xiong et al., 2013
Decursin and decursinol angelate	*Angelica gigas* (Apiaceae)	Inhibit conidia germination and appressoria formation	*In vitro*	Yoon et al., 2011
Hyoscyamine	*Hyoscyamus muticus* (Solanaceae)	Inhibit the mycelial growth, conidia germination, appressoria formation and attachment on rice leaf surface	*In vitro* and on rice leaves	Abdel-Motaal et al., 2010
Scopolamine	*Hyoscyamus muticus* (Solanaceae)	Inhibit conidia germination	*In vitro*	Abdel-Motaal et al., 2010
Ascherxanthone B	*Aschersonia luteola* (Ascomycete)	Inhibit fungal growth	*In vitro* and *in* rice plants	Chutrakul et al., 2009
Saponins	*Medicago sativa* L. (Leguminosae)	Inhibit fungal growth	*In vitro* and on rice leaves	Abbruscato et al., 2014
Sapogenins	*Medicago sativa* L. (Leguminosae)	Inhibit fungal growth	*In vitro* and on rice leaves	Abbruscato et al., 2014
Xylarinic acids A and B	*Xylaria polymorpha* (Ascomycete)	Inhibit fungal growth	*In vitro*	Jang et al., 2007
Natamycin	*Streptomyces lydicus* A01	Inhibit fungal growth	*In vitro*	Lu et al., 2008
3-O-methylellagic acid-4'-(5"-acetyl)-alpha-L-arabinofuranoside	*Gleditsia sinensis* spines (Fabaceae)	Inhibit spore germination	*In vitro*	Zhou et al., 2007
3-O-methylellagic acid-4'-O-alpha-L-rhamnopyranoside	*Gleditsia sinensis* spines (Fabaceae)	Inhibit spore germination	*In vitro*	Zhou et al., 2007
HCN	*P. protegens* CHA0	Inhibit fungal growth	*In vitro*	Spence et al., 2014
Syringolin	*P. syringae* pv. *syringae*	ISR	In rice	Wäspi et al., 1998
Synthetic product				
KTU 3616		Inhibit melanin biosynthesis	*In vitro*	Kurahashi et al., 1996
PAF104		Inhibit the formation of appressoria	*In vitro* and on rice leaves	Rebollar and López-García, 2013
Tricyclazole		Inhibit melanin biosynthesis	*In vitro*	Woloshuk et al., 1980
A triazole derivative		Inhibit fungal growth	*In vitro*	Hoshi et al., 2015
3-[3-(1H-imidazol-1-yl)propyl]-1H-Indole		Inhibit fungal growth	*In vitro*	Oh et al., 2015
12-methyltetradecanoic (12-Me 14:0) acid		Inhibit appressoria formation	*In vitro*	Jeon et al., 2010
9,11,13-octadecatriynoic acids		Inhibit fungal growth	*In vitro*	Yoon et al., 2010
BTH		ISR	Root drench	De Vleesschauwer et al., 2008
Probenazole (PBZ)		ISR	Root drench	Watanabe et al., 1977
Isotianil		ISR	Foliar spray	Ogawa et al., 2011
NCI		ISR	Root drench	Yoshida et al., 1990
CMPA		ISR	Root drench	Nishioka et al., 2003

* PAF104: NH_2-WRKKWFW-COOH; Triazole derivative: 2RS,4RS-1-[4-chlorophenyl-(2-methylphenoxy)-ethyl]-1,3-dioxolan-2-yl-methyl]-1H-1,2,4-triazole; BTH: benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methylester; PBZ: 3-allyloxy-1,2-benzisothiazole-1,1-dioxide; NCl: N-cyanomethyl-2-chloroisonicotinamide; CMPA: 3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid;

HCN: hydrogen cyanide; ISR: induced systemic resistance.

Several case studies on how to control the disease development of *M. oryzae* have been reported. The mode of action in these studies mainly include inhibition of the pathogenicity of *M. oryzae* itself by affecting mycelial growth, spore germination, appressorium formation, germ tube development and melanin biosynthesis, and increasing the innate immunity of rice plants. The studied fungicides and their mode of actions against *M. oryzae* have been listed in Table 2.3.

Up to date, the only study of a microbial CLP interacting with *M. oryzae* is *Bacillus*-derived surfactin, which inhibits the development of the germ tube of *M. oryzae* (Tendulkar et al., 2007). Unfortunately, there are no *in planta* data about the interaction of CLPs and *M. oryzae*, as studies were done in *in vitro* conditions. The effects of CLPs on *M. oryzae* should be studied further to lead to a better understanding of this type of interaction, not only under *in vitro* condition, but also in plant assays.

2.5.2.2 *C. miyabeanus*

C. miyabeanus (Ito and Kuribayashi) Drechs. ex Dastur. (Anamorph: *Bipolaris oryzae* (Breda de Haan) Shoemaker), the causal agent of brown spot disease of rice, has a necrotrophic life style (Barnwal et al., 2013). The symptoms of brown spot disease are yellow brown or brown lesions (Figure 2.9) and the fungus can infect rice leaves, sheaths, and even rice seeds. Brown spot disease can spread from plant to plant via air and can infect plants at any growth stage. Brown spot disease is an important threat to rice productivity which may cause 10 % of yield loss on average (Savary et al., 2000; Savary et al., 2006). The main factor triggering the 'Great Bengal Famine' has been attributed to the infection of rice by *C. miyabeanus* during the years 1943 to 1944 (Ou, 1985).

The pathogenic characteristics of *C. miyabeanus* in rice have been intensively studied for several decades, which has led to a better understanding of this fungal plant pathogen. Phytotoxins produced by *C. miyabeanus* are major factors involved in pathogenicity in rice plants. The fungal toxin ophiobolin produced by *C. miyabeanus* acts as virulence factor of brown spot disease on rice. Ophiobolins were originally isolated from *C. miyabeanus* (Orsenigo, 1957; Nakamura and Ishibashi,

1958), but they constitute a large family of phytotoxins that also produced by other species (Au et al., 2000). Ophiobolins are potent rice phytotoxins, inhibiting the development of roots and coleoptiles of rice seedlings, and inducing chlorosis in rice leaves (Orsenigo, 1957; Xiao et al., 1991). Ophiobolin can be readily detected in rice leaves infected by *C. miyabeanus* (Nakamura and Oku, 1960). Ophiobolin causes losses of electrolytes from rice tissues like roots, leaves and coleoptiles, and this phenomenon occurs in a concentration dependent manner (Chattopadhyay and Samaddar, 1976). Ophiobolin can trigger brown spot disease in many varieties of rice (Yun et al., 1988). Tentoxin produced by *C. miyabeanus* is an additional virulence factor of the fungus on rice plants (De Bruyne et al., 2015). Recently, it has been shown that ethylene (ET) biosynthesis in *C. miyabeanus* also plays a crucial role in the pathogenicity of the fungus. Disturbing the ET biosynthesis of *C. miyabeanus* results in a lower pathogenicity on rice tissues (Van Bockhaven et al., 2015).

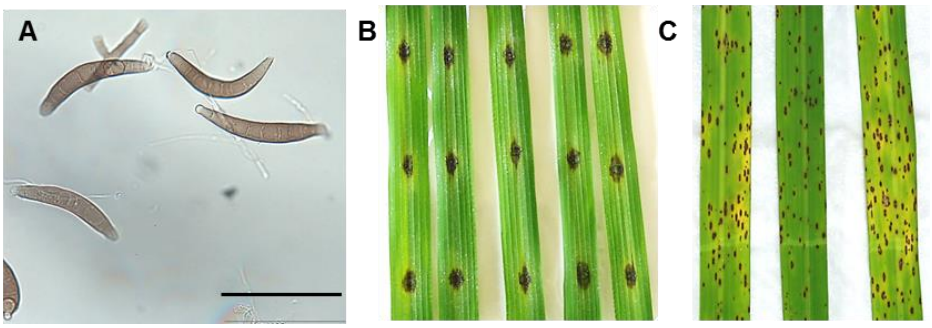

Figure 2.9 Conidia morphology and symptoms of brown spot on rice caused by *C. miyabeanus* Cm988. **A**. Conidia of *C. miyabeanus* Cm988 (De Vleesschauwer et al., 2010) growing on potato dextrose agar (PDA) plate. Scale bar is 100 µm. **B** and **C**. Symptoms of brown spot disease caused by *C. miyabeanus* Cm988 on rice leaves by droplet infection (B) and by spray infection (C), 3 dpi.

Control of *C. miyabeanus* is also a challenge for researchers. Disturbing the hyphal growth or spore germination of *C. miyabeanus* can affect the disease development of the fungus on rice, for instance by the application of leaf extracts from *Nerium oleander*, and *Trichoderma viride* Tv2 (Harish et al., 2008). Another case study showed that foliar spray of *Trichoderma* species can reduce the brown spot disease on rice. Active *Trichoderma* species include *T. harzianum*, *T. atroviride*, *T. virens* strains (Khalili et al., 2012). Modulation of innate immunity in plants by plant hormones has been reported

in controlling of fungal diseases on monocot model cereal plant rice. For instance, De Vleesschauwer and colleagues showed that ABA signaling is important for disease resistance in rice against *C. miyabeanus* (De Vleesschauwer et al., 2010), while ABA signaling mediates susceptibility in rice against *Xanthomonas oryzae* pv. *oryzae* (Xu et al., 2013). Interestingly, silicon has the ability to successfully control *C. miyabeanus* in rice, by blocking the ET biosynthesis of the fungus (Van Bockhaven et al., 2015).

Concluding remarks

Pseudomonas species derived from the rhizosphere are intensively studied microbes for biocontrol research and potential applications. *Pseudomonas* species can secrete diverse secondary metabolites that are important for biocontrol of fungal diseases on crops. *P. protegens* and related strains (such as *Pseudomonas* sp. CMR12a and CMR5c) can produce sessilins and orfamides, however, the biocontrol roles of these CLPs are barely studied, and the interactions between sessilins and orfamides are not known at all. We will study the potential biocontrol roles of sessilins and orfamides in the control of blast and brown spot diseases in rice, and of Rhizoctonia root rot and web blight diseases on bean. In several plant-pathogen systems, the possible defensive mechanisms will be also investigated, for instance, in the orfamide-*C. miyabeanus*-rice interaction. Currently massetolide A is the only reported *Pseudomonas*-derived CLP showing an ISR role.The mechanisms underpinning this interaction are poorly studied. It has only been shown that massetolide A triggered innate immunity against late blight disease on tomato is independent of SA signaling. Our study aims at widening the insight in the biocontrol role of sessilins and orfamides against diseases on rice and bean, and further provides a more detailed view of the biocontrol role of *P. protegens* and related species.

Chapter 3. Biosynthesis, chemical structure and structure-activity relationship of orfamide lipopeptides produced by *Pseudomonas protegens* and related species

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Running title: Biosynthesis, structure and bioactivity of orfamides

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Abstract

Orfamide-type cyclic lipopeptides (CLPs) are biosurfactants produced by *Pseudomonas* and involved in lysis of oomycete zoospores, biocontrol of *Rhizoctonia* and insecticidal activity against aphids. In this study we compared the biosynthesis, structural diversity, *in vitro* and *in planta* activities of orfamides produced by rhizosphere-derived *Pseudomonas protegens* and related *Pseudomonas* species. Genetic characterization together with chemical identification revealed that the main orfamide compound produced by the *P. protegens* group is orfamide A, while the related strains *Pseudomonas* sp. CMR5c and CMR12a produce orfamide B. Comparison of orfamide fingerprints led to the discovery of two new orfamide homologues (orfamide F and orfamide G) in *Pseudomonas* sp. CMR5c. The structures of these two CLPs were determined by nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. Mutagenesis and complementation showed that orfamides determine the swarming motility of parental *Pseudomonas* sp. strain CMR5c and their production was regulated by *luxR* type regulators. Orfamide A and orfamide B differ only in the identity of a single amino acid, while orfamide B and orfamide G share the same amino acid sequence but differ in length of the fatty acid part. The biological activities of orfamide A, orfamide B and orfamide G were compared in further bioassays. The three compounds were equally active against *Magnaporthe oryzae* on rice, against *Rhizoctonia solani* AG 4-HGI in *in vitro* assays, and caused zoospore lysis of *Phytophthora* and *Pythium*. Furthermore we could show that orfamides decrease blast severity in rice plants by blocking appressorium formation in *M. oryzae*. Taken all together, our study shows that orfamides produced by *P. protegens* and related species have potential in biological control of a broad spectrum of fungal plant pathogens.

Keywords

Fluorescent pseudomonads, nonribosomal peptide synthetase, orfamides, biocontrol, *Magnaporthe oryzae*, *P. protegens* CHA0 and Pf-5

3.1 Introduction

The majority of natural rhizosphere soil microorganisms in various ecosystems is composed of Proteobacteria (Philippot et al., 2013). Within this group rhizosphere-derived fluorescent pseudomonads have received a lot of attention as biocontrol agents in the past few decades (Höfte and Altier, 2010; Olorunleke et al., 2015a). Rhizosphere-derived fluorescent pseudomonads have the ability to swiftly adapt to changing environmental conditions and efficiently colonize the plant root system, and play critical roles in the interspecies interaction among plants, plant pathogens, bacterial predators and other biotic and abiotic stresses from natural environments. *Pseudomonas* species show an enormous metabolic versatility and some isolates produce a remarkable spectrum of secondary metabolites both *in vitro* and *in vivo* conditions. These secondary metabolites are indispensably involved in interspecies interactions in the soil environment (Raaijmakers and Mazzola, 2012).

Various *Pseudomonas* biocontrol strains produce CLP type biosurfactants (Olorunleke et al., 2015a). CLPs are amphiphilic molecules composed of an oligopeptide lactone ring coupled to a fatty acid tail (Raaijmakers et al., 2006; Raaijmakers et al., 2010). These molecules are synthesized by nonribosomal peptide synthetases (NRPSs), which normally encode different modules containing several domains for loading, selecting, coupling amino acids and after their configuration, the peptide is finally released by thiolation domain(s) (Strieker et al., 2010). CLPs possess broad spectrum activities, such as antibiosis against bacteria, fungi, protozoa and human tumor cell lines (Raaijmakers et al., 2010; Roongsawang et al., 2010) and have potential as pharmaceutical candidates or for the biocontrol of plant pathogens (Banat et al., 2010; Banat et al., 2014; D'aes et al., 2010; Sachdev and Cameotra, 2013). *Pseudomonas* derived CLPs are currently divided in eight different structural groups that differ in length and composition of the oligopeptide and fatty acid tail (Olorunleke et al., 2015a).

Orfamides were first discovered in the well-studied biocontrol strain *Pseudomonas protegens* Pf-5 by a novel genomisotopic approach, and the chemical structure of orfamide A was determined by NMR and MS analysis (Gross et al., 2007). Orfamides consist of ten amino acids and a 3-hydroxydodecanoic or tetradecanoic acid tail. Genome mining and chemical analysis revealed that

Pseudomonas sp. CMR12a, a biocontrol strain obtained from cocoyam roots in Cameroon, can produce orfamide B and the new homologues orfamide D and E (D'aes et al., 2014). *Pseudomonas* sp. CMR12a is related to *P. protegens*, but phylogenetically clearly distinct (Ruffner et al., 2015). Orfamides are important determinants for bacterial surface motility and show surface tension reduction activity (D'aes et al., 2014; Gross et al., 2007; Jang et al., 2013). In addition, orfamide A produced by *P. protegens* F6 showed dose-dependent insecticidal activity against aphids in greenhouse biocontrol trails (Jang et al., 2013). Orfamide B, produced by *Pseudomonas* sp. CMR12a, can affect the hyphal growth of *R. solani*, and soil assays with orfamide biosynthesis mutants revealed that orfamides work together with phenazine antibiotics or sessilin-type CLPs in the biocontrol activity against root rot caused by *R. solani* in bean plants (Olorunleke et al., 2015b).

Pseudomonas sp. CMR5c is a biocontrol strain from cocoyam roots in Cameroon related to *Pseudomonas* sp. CMR12a (Perneel et al., 2007). This strain produces multiple antibiotics such as phenazines, pyrrolnitrin and pyoluteorin, and also has biosurfactant activity (Perneel et al., 2007). Recently, the genome of *Pseudomonas* sp. CMR5c was sequenced, revealing the presence of diacetylphloroglucinol biosynthesis genes, the Fit gene cluster that contributes to insecticidal activity and an NRPS cluster involved in CLP biosynthesis (Flury et al., 2016). In this work, we identified the NRPS genes and CLPs produced by *Pseudomonas* sp. CMR5c. In addition, we performed a detailed analysis and comparison of biosynthesis, phylogeny and chemical structures of orfamides produced by *Pseudomonas* sp. CMR5c, *P. protegens* and related species. Using three structurally different orfamides, we tested the hypothesis that length of the fatty acid tail and/or amino acid substitutions in the peptide moiety may affect biological activity. Our study revealed that all three compounds are equally active against several plant pathogenic fungi and oomycetes and inhibit appressoria formation in the rice blast fungus *Magnaporthe oryzae*.

3.2 Materials and Methods

Table 3.1 Strains, reference sequences and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics	Reference or source
Pseudomonas strains		
P. protegens CHA0	Orfamide$^+$,derived from rhizosphere of tobacco, Switzerland	Stutz et al. 1986
P. protegens Pf-5	Orfamide$^+$, derived from rhizosphere of cotton, Texas, USA	Howell et al. 1979
CMR12a	Orfamide$^+$, sessilin$^+$, wild type derived from rhizosphere of red cocoyam, Cameroon	Perneel et al. 2007
CMR12a-Clp1	Orfamide$^+$, sessilin$^-$; mutant with insertion in sessilin (CLP1) biosynthesis genes, Gm^R	D'aes et al. 2011
CMR5c	Orfamide$^+$, wild type derived from rhizosphere of red cocoyam, Cameroon	Perneel et al. 2007
CMR5cΔ*ofa*	Orfamide$^-$, mutant with deletion in *ofaB* and *ofaC* genes	This study
CMR5cΔ*luxRup*	Orfamide$^-$, mutant with deletion in *luxR* type transcriptional regulator gene located upstream of orfamide biosynthetic genes	This study
CMR5cΔ*luxRdown*	Orfamide$^-$, mutant with deletion in *luxR* type transcriptional regulator gene located downstream of orfamide biosynthetic genes	This study
Pseudomonas sequences/sources		
P. protegens Cab57	Orfamide$^+$,derived from rhizosphere of shepherd's purse, Japan	Takeuchi et al. 2014
PH1b	Derived from the phytotelma of a carnivorous plant , Malaysia	NCBI database
CMAA1215	Derived from Brazilian mangroves, Brazil	Vasconcellos et al. 2013
Wayne1R	Biological control strain derived from rhizosphere of corn, Wayne, Pennsylvania, USA	Rong et al. 2012
Saccharomyes cerevisiae InvSc1	Yeast strain for *in vivo* recombination (*ura3-52/ura3-52* mutation)	Invitrogen
E. coli WM3064	Donor strain for conjugation; DAP$^-$	Saltikov and Newman 2003
Plasmid		
pMQ30	Gene replacement vector for *Pseudomonas* species; *sacB*, URA3, Gm^R	Shanks et al. 2006
pMQ30-Δ*ofa*	Vector for mutagenesis of orfamide biosynthesis genes	This study
pMQ30-Δ*luxRup*	Vector for mutagenesis of *luxRup* biosynthesis gene	This study
pMQ30-Δ*luxRdown*	Vector for mutagenesis of *luxRdown* biosynthesis gene	This study
Magnaporthe oryzae isolate VT5M1	Causal agent of rice blast disease	Thuan et al. 2006
R. solani AG 4-HGI CuLT-Rs36	Causal agent of root rot disease on bean	Nerey et al. 2010
Phytophthora porri CBS 127099	Oomycete pathogen causing white tip disease on leek	Bertier et al. 2013
Pythium ultimum	Oomycete pathogen causing root rot on cucumber	Lab stock

[a] Gm^R, gentamicin resistance; DAP, 2,6-diaminopimelic acid; DAP$^-$, DAP auxotroph; CLP1, sessilin type lipopeptide

3.2.1 Strains, media and growth conditions

Bacteria, fungal strains and plasmids used in this study are shown in Table 3.1. *Pseudomonas* strains were routinely maintained on Luria-Bertani (LB) medium (Sambrook et al., 1989) at 28 °C. *E. coli* WM3064 was cultured on LB medium containing 100 µg/mL of 2,6-diaminopimelic acid (DAP) at 37 °C (Saltikov and Newman, 2003). Gentamicin was added to LB medium at 25 µg/mL for *E. coli* WM3064 and at 100 µg/mL for *Pseudomonas* strains, respectively. *Saccharomyes cerevisiae* InvSc was grown on yeast peptone dextrose medium at 30 °C. Solid medium and soft agar medium contained 1.5 % (*w*/*v*) and 0.6 % (*w*/*v*) agar, respectively. Liquid cultures of *Pseudomonas* strains were obtained in liquid King's B (KB) (King et al., 1954) medium on a rotary shaker with stirring rate of 150 rpm. *Magnaporthe oryzae* isolate VT5M1 (Thuan et al., 2006) was cultured on complete medium (CM) (Talbot et al., 1993). *Phytophthora porri* isolate CBS 127099 was maintained on V8 agar (Bertier et al., 2013). *R. solani* AG 4-HGI isolate CuLT-Rs36 (Nerey et al., 2010) and *Pythium ultimum* were routinely cultivated on potato dextrose agar (PDA; Difco).

3.2.2 Genome mining and bioinformatic analyses

The genomic sequences of selected *Pseudomonas* strains (Table 3.1) were retrieved from Genbank database and submitted to RAST server (Aziz et al., 2008) and antiSMASH 3.0 (Weber et al., 2015) for automated genome annotation and genome mining. Alternatively, genome mining of NRPS biosynthetic gene clusters was conducted by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and through comparison with reported sequences. Gaps of scaffold sequences of *Pseudomonas* sp. CMR5c were filled by polymerase chain reaction (PCR) and sequencing (LGC Genomics). Domains of NRPS gene clusters were obtained by using NRPSpredictor2 (Röttig et al., 2011) and NRPS-PKS analysis programs (Ansari et al., 2004). Similarity of selected proteins was identified by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogeny trees were constructed with Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura et al., 2011). The draft genome of *Pseudomonas* sp. CMR5c has recently been released (Fluvy et al., 2016). Genomic sequences of *Pseudomonas* sp. CMR5c were deposited in GenBank database under accession number: NZ_LHUY01000001 - NZ_LHUY01000044 (Fluvy et al., 2016). The biosynthetic gene cluster of

orfamide identified from genomic sequences of *Pseudomonas* sp. CMR5c was deposited in GenBank database under accession number KT613918. Selected *Pseudomonas* strains used in this study are shown in Table 3.1 and their genomic sequences are publicly available in GenBank. The full sequences of *rpoD* and *gyrB* of selected *Pseudomonas* strains were directly retrieved from GenBank database and used for further phylogeny analysis in this study.

3.2.3 Swarming motility and droplet collapse assays

Overnight cultures of *Pseudomonas* strains were maintained in liquid LB medium, and washed two times with sterilized deionized water. Bacterial cell density was adjusted to optical density of 0.9 (620 nm). Soft agar plates were prepared by adding 0.6 % (w/v) agar in LB medium. Five µL aliquots were carefully spotted in the centre of soft agar plates, and plates were incubated at 28 °C. All strains were tested in triplicates. Swarming was observed 20 h after incubation. Droplet collapse assay was conducted as reported previously (Gross et al., 2007).

3.2.4 Site directed mutagenesis

Site directed deletion of targeted genes of *Pseudomonas* sp. CMR5c was conducted by allelic replacement using plasmid pMQ30 as reported before (D'aes et al., 2014; Shanks et al., 2006). Primer pairs used for generation of deletion mutants are shown in Table 3.2. DNA fragments upstream (Up fragment, Table 3.2) and downstream (Down fragment, Table 3.2) of the genes of interest, were amplified by PCR using corresponding primer pairs and detected by 2 % (*w*/*v*) agarose gel electrophoresis. These two fragments were subsequently cloned next to each other via *in vivo* recombination in the yeast *S. cerevisiae* InvSc1. The resulting deletion plasmids pMQ30-Δ*ofa,* pMQ30-Δ*luxRup* and pMQ30-Δ*luxRdown* were isolated from *S. cerevisiae* InvSc by GeneJET Plasmid Miniprep Kit (Thermo Scientific) and introduced into *E. coli* WM3064 by electroporation. Recombination in the plasmid was confirmed by sequencing (LGC Genomics). The plasmids were mobilized into *Pseudomonas* sp. CMR5c by conjugation with *E. coli* WM3064. *Pseudomonas*

strains containing the plasmid were selected on LB medium containing 10 % (w/v) sucrose without sodium chloride. Deletion mutants that grew on LB with 10 % (*w*/*v*) sucrose but did not grow on LB with 100 µg/mL gentamicin were selected and the deletion was confirmed by PCR. Ultrahigh performance liquid chromatography-mass spectrometry (UPLC-MS) was used for chemical characterization of deletion mutants. Phenotypic characterization of deletion mutants was conducted by swarming motility assay on soft agar plates.

Table 3.2 Primer pairs used in this study

Name	Gene	Fragment	Primer (5' to 3')[a]
CMR5c-*Ofa*-UpF	*ofa*	Up	**ggaattgtgagcggataacaatttcacacaggaaacagctg**gggcatcagtaccttgcact
CMR5c-*Ofa*-UpR			**atacaggttgtgcagggcaa**caggacgatctccattcgtt
CMR5c-*Ofa*-DownF		Down	**aacgaatggagatcgtcctg**ttgccctgcacaacctgtat
CMR5c-*Ofa*-DownR			**ccaggcaaattctgttttatcagaccgcttctgcgttctgat**gttcgatcaacggcaacagt
CMR5c-*LuxRup*-UpF	*luxRup*	Up	**ggaattgtgagcggataacaatttcacacaggaaacagctg**atctgcccttgaatctgcac
CMR5c-*LuxRup*-UpR			**ggtaggtctcgatggagctg**aattcggtgtttccaggttg
CMR5c-*LuxRup*-DownF		Down	**caacctggaaacaccgaatt**cagctccatcgagacctacc
CMR5c-*LuxRup*-DownR			**ccaggcaaattctgttttatcagaccgcttctgcgttctgat**gatgaccctgatgagcctgt
CMR5c-*luxRdown*-upF	*luxRdown*	Up	**ggaattgtgagcggataacaatttcacacaggaaacagctg**agcgtgaagatcaaggacgg
CMR5c-*luxRdown*-upR			**ccacccatatcacccagctg**aaccgcgagatggaagtctg
CMR5c-*luxRdown*-downF		Down	**cagacttccatctcgcggtt**cagctgggtgatatgggtgg
CMR5c-*luxRdown*-downR			**ccaggcaaattctgttttatcagaccgcttctgcgttctgat**gggcgtctctggtctttctc

[a] Primer extensions are shown in bold font

3.2.5 MS and NMR analysis

MS analysis of samples from cultures of pseudomonads and purified compounds were conducted on a reversed-phase UPLC-MS system, consisting of an UPLC (Waters, Acquity class H) coupled with a single quadrupole detector (Waters, Acquity) on an Acquity UPLC BEH C18 (*Φ* 2.1 × 50 mm, 1.7 µm, Waters); or on a liquid chromatography-mass spectrometry (LC-MS) 1100 Series HPLC system (Agilent Technologies) with a type VL electrospray ionization detector and equipped with a Luna C18 (2) reversed-phase column (*Φ* 4.6 × 250 mm, 5 µm; Phenomenex, Torrance, CA, USA). NMR measurements of purified compounds were recorded on a Bruker Avance III spectrometer

operating at 500.13 MHz and 125.76 MHz for ^{1}H and ^{13}C frequencies, respectively. All measurements were performed in CD_3CN solution and at 298K. One-dimensional (^{1}H-NMR, ^{13}C-NMR), and two-dimensional NMR spectroscopy, in particular correlation spectroscopy (^{1}H-^{1}H COSY), total-correlation spectroscopy (^{1}H-^{1}H TOCSY), rotating-frame nuclear Overhauser effect spectroscopy (^{1}H-^{1}H ROESY), heteronuclear single-quantum correlation (^{1}H-^{13}C HSQC) spectroscopy and heteronuclear multiple-bond correlation (^{1}H-^{13}C HMBC) spectroscopy was performed for purified compounds.

3.2.6 Isolation and purification of CLPs

A seed culture of *Pseudomonas* strains was grown in 250-mL flasks containing 50 mL liquid KB medium placed on a shaker for 24 h at 28 °C, subsequently inoculated into 2-liter flasks containing 500 mL liquid KB medium, and grown with a stirring rate of 150 rpm for 48 h. The crude extracts of CLPs from *Pseudomonas* cultures were prepared following a protocol published previously (Ma et al., 2012). More specifically, *Pseudomonas* supernatant was collected after centrifugation at 10000 *g* for 10 min, acidified to pH 2.0 with 6 M hydrochloric acid, and then kept overnight at 4 °C. The precipitate was collected after centrifugation at 10000 *g* for 10 min and extracted with methanol. The organic phase was collected by centrifugation at 10000 *g* for 10 min and evaporated at room temperature, yielding a crude extract. Crude extracts of CLPs were further separated by gradient acetonitrile/H_2O (20 %, 40 %, 60 %, 80 %, 100 %, *v*/*v*) on a standard C18 SPE cartridge (900 mg, Grace™ Alltech™). The fractions containing CLPs were detected by droplet collapse assay on parafilm, and the presence of CLPs was confirmed by UPLC-MS analysis. Fractions containing CLPs were collected, dried, and yielded semi-purified CLPs that were further separated by semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Luna C-18 (2) (Φ 10 × 250 mm, Phenomenex, Torrance, CA, USA) column by repeated injections (sandwich mode), eluted by 90 % to 100 % (*v*/*v*) acetonitrile in HPLC-grade water [trifluoroacetic acid 0.1 % (v/v)] over 30 min, with flow rate of 3.5 mL/min and detection wavelength of 214 nm. Orfamide A was purified from *P. protegens* CHA0, orfamide B and G were purified from *Pseudomonas* sp. CMR5c.

Samples of *Pseudomonas* sp. CMR12a for UPLC-MS analysis were prepared from cells on soft agar plate since orfamide secretion in this strain is hampered by the presence of sessilin (D'aes et al., 2014). The cells of *Pseudomonas* sp. CMR12a were collected, suspended in 50 % (*v*/*v*) acetonitrile/H_2O solution and ultra-sonicated (Sonoplus, Bandelin Electronic, Berlin) for 1 min. The sample was collected from the supernatant after centrifugation (10000 *g* for 5 min) of sonicated mixture. Samples of other *Pseudomonas* strains were prepared from supernatant of overnight liquid KB cultures

3.2.7 Bioassays with *M. oryzae*

Unless stated otherwise, stocks of orfamides were prepared in pure dimethyl sulfoxide (DMSO) and diluted in water to desired concentrations for further bioassays. Sporulation of *M. oryzae* isolate VT5M1 was obtained following a protocol published previously (Thuan et al., 2006). Spores (5×10^4 per milliliter) of 5-day old *M. oryzae* isolate VT5M1 were collected from CM plates and co-incubated with orfamide solutions, while controls only received the same amount of DMSO. Spore germination was evaluated by counting the number of germination tube of spores after 4 h incubation at 28 °C. Appressorium formation was tested by putting a 10-µL spore solution on a glass slide cover (12 × 12 mm) after 8 h incubation in dark condition at 28 °C, and the number of appressoria were counted by randomly selecting at least 50 spores. Appressorium formation in planta was assessed as described previously (Koga, 1994). Rice sheaths were inoculated with spore solutions either treated or not with orfamides. Representative pictures were taken 24 h post treatment. All microscopic observations were made using an Olympus BX51 microscope. *In vitro* antagonistic assay of orfamides against *M. oryzae* isolate VT5M1 was conducted by paper-agar disc diffusion assay, as described previously (Ma et al., 2012). All experiments were repeated three times.

3.2.8 Rice biocontrol assay

Rice cultivar *indica* cv. CO-39 was used in plant experiments. Rice seeds were surface sterilized with 1 % (*w*/*v*) sodium hypochlorite solution for 5 min and washed 5 times with deionized water. Surface

sterilized seeds were germinated on moistened filter paper in Petri dishes (9 cm diameter) for 5 days, and planted into plastic trays with potting soil (23 × 16 × 6 cm, 12 plants per tray), as described previously (De Vleesschauwer et al., 2006). Rice was routinely maintained in greenhouse conditions with a photoperiod of 12 h light at 30 ± 4 °C. Four-week old (five-leaf stage) rice plants were used for further bioassays.

Spores of 5-day old *M. oryzae* isolate VT5M1 were collected from CM plates and suspended into 0.5 % (*w*/*v*) gelatin to a final concentration of 5×10^4 per milliliter. Fifty mM purified CLP samples were prepared as stock solution in DMSO, and then diluted to the needed concentration for further bioassays. CLPs and *M. oryzae* spores were mixed thoroughly and evenly sprayed onto rice leaves by a compressor-powered airbrush gun, while control plants were only sprayed with the same amount of DMSO treated spores. Each plant received 1 mL spore solution. Disease score was conducted 6 days post infection, by counting the number of sporulating susceptible-type blast lesions per 10 cm of the second youngest leaves of rice plants, as described previously (De Vleesschauwer et al., 2006). Results are expressed as relative infection values compared to control plants. Pictures of typical disease symptoms were taken 7 days after infection. The biocontrol assay was repeated in time and two trays (24 plants) were used in each experiment.

3.2.9 *In vitro* antibiosis assay

Microscopic assays showing the effect of orfamide treatments on hyphal branching of *R. solani* AG 4-HGI were carried out as described by Bolwerk et al. (2003) and Olorunleke et al. (2015b). More specifically, sterile microscopic glass slides were covered with a thin, flat layer of water agar (Bacto agar; Difco) and placed in a plastic Petri dish containing moist sterile filter paper. An agar plug (diameter = 5 mm) taken from an actively growing colony of *R. solani* was inoculated at the center of each glass slide. Two droplets (15 µl each) containing 0, 10 or 100 µM orfamide were placed at two sides of the glass slide at about 2 cm from the fungal plug. Slides were incubated for 36 h at 28 °C before evaluation under an Olympus BX51 microscope. Zoospores of *Phytophthora porri* CBS 127099 and *Pythium ultimum* were harvested and collected as described earlier (de Bruijn et al.,

2007). Zoospores of both pathogens were incubated in the presence of increasing concentrations of orfamide A and B and the time (in seconds) that it took to observe lysis was recorded immediately under an Olympus BX51 microscope.

3.3 Results

3.3.1 *In silico* analysis of orfamide synthetases and flanking regions

Genome mining results showed that three large genes (Figure 3.1) are present in the whole genome of *Pseudomonas* sp. CMR5c. Results from NRPSpredictor2, NRPS-PKS program, antiSMASH 3.0 and a BLAST search showed that these three biosynthetic genes encoded NRPS modules are responsible for the assembly of 10 amino acids, similar to those of orfamides from *P. protegens* Pf-5 and *Pseudomonas* sp. CMR12a (D'aes et al., 2014; Gross et al., 2007). Moreover, BLAST search revealed that *P. fluorescens* Wayne1R, *Pseudomonas* sp. PH1b and *Pseudomonas* sp. CMAA1215 may be potential orfamide-producers. Synthetase sequences of reported orfamide-producers (*P. protegens* CHA0, Pf-5 and Cab57, *Pseudomonas* sp. CMR12a) were also retrieved from GenBank database and all these orfamide synthetases were compared in this study (D'aes et al., 2014; Gross et al., 2007; Kupferschmied et al. 2013; Takeuchi et al., 2014). Orfamide synthetases contain three NRPS structural genes designed as *ofaA*, *ofaB* and *ofaC*, which respectively encode two, four, and four amino acids (Figure 3.1). Orfamide-synthetases of these *Pseudomonas* strains also contain two terminal thioesterase (TE) domains, which cleave the peptides from the NRPSs, and the final step involves a cyclization between the 3_{rd} and 10_{th} amino acid (Figure 3.1). BlastP comparison of orfamide-synthetases genes and flanking regions showed a very high similarity among *P. protegens* isolates, while *Pseudomonas* sp. CMR12a, CMR5c, CMAA1215 and PH1b were more distantly related to *P. protegens*. Intriguingly, orfamide synthetases and flanking regions in *Pseudomonas* sp. CMR5c from Cameroon and *Pseudomonas* sp. CMAA1215 from Brazil show a very high similarity (Table 3.3).

In NRPSs, adenylation (A) domains are responsible for recruiting and selecting amino acids for

peptide biosynthesis (Strieker et al., 2010). A domains of reported orfamide-producing pseudomonads and the potential orfamide producers *P. fluorescens* Wayne1R, and *Pseudomonas* sp. PH1b, CMAA1215 and CMR5c, were retrieved and analyzed by phylogenetic analysis. Domains of orfamide synthetases were extracted by NRPSpredictor2. The phylogenetic tree analysis of A domains showed that all these orfamide synthetases recruit the same amino acids, except for the fourth position. *P. protegens*, *Pseudomonas* sp. PH1b and *P. fluorescens* Wayne1R recruit an isoleucine, while the fourth position in *Pseudomonas* sp. CMR5c, CMR12a and CMAA1215 encodes a valine (Figure 3.2A).

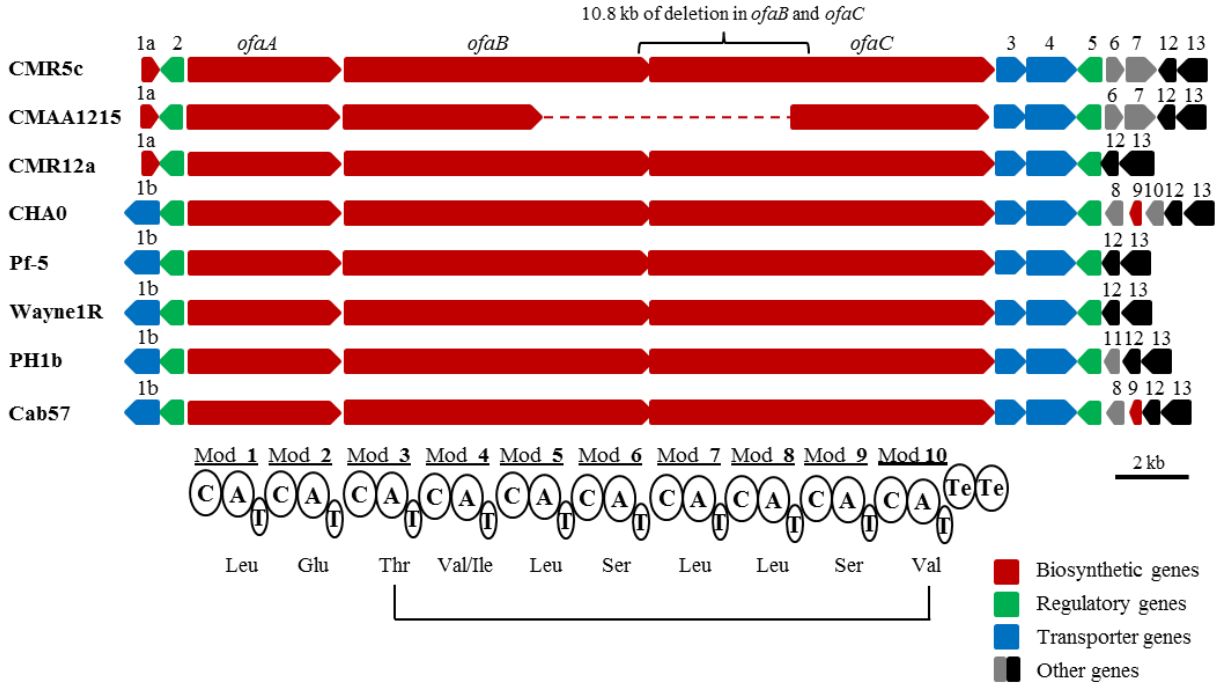

Figure 3.1 *In silico* analysis and comparison of orfamide-synthetase gene clusters and flanking regions of *P. protegens* and related species. All orfamide-synthetases contain three large structural genes, *ofaA*, *ofaB* and *ofaC*; these structural genes encode ten modules for amino acids biosynthesis and two TE domains for peptide release and cyclizaton. Each module contains three domains: adenylation (A), condensation (C) and thiolation (T) domains. The detailed comparison of orfamide-synthetases genes and flanking regions of these strains is shown in Table 3.2. Only partial gene sequences of orfamide synthetases of *Pseudomonas* sp. CMAA1215 could be retrieved from the GenBank database. Scale bar is 2 kb.

Table 3.3 Putative orfamide-synthetases and flanking region identified from *P. protegens* and related strains in this study. The identity level of amino acids was compared by BLASTp search. The first BLASTp similarity comparison is based on protein sequence of *P. protegens* CHA0. The second BLASTp similarity comparison is based on *Pseudomonas* sp. CMR5c and the level of identity is shown in brackets.

Orfamide-synthetases and flanking area		Identity at amino acid level							
		P. protegens				*Pseudomonas* sp.			
		CHA0	**Cab57**	**Pf-5**	**Wayne1R**	**CMR5c**	**CMR12a**	**CMAA1215**	**PH1b**
1a	GCN5-related N-acetyltransferase					[100 %] ALG76232.1	[75 %] AFH75326.1	[100 %] ERO60965.1	
1b	RND efflux system, outer membrane lipoprotein	100 % AGL83955.1	99 % BAO61507.1	99 % AAY91416.1	99 % WP_019094693.1				87 % WP_025130134.1
2	LuxR family transcriptional regulator	100 % AGL83956.1	99 % BAO61508.1	100 % AAY91417.1	99 % WP_011060444.1	80 % [100 %] ALG76233.1	78 % [82 %] AFH75327.1	80 % [100 %] ERO60966.1	88 % [80 %] WP_025130133.1
ofaA	Nonribosomal peptide synthetase	100 % AGL83957.1	99 % BAO61509.1	99 % AAY91419.3	99 % WP_019094694.1	80 % [100 %] ALG76234.1	79 % [84 %] AFH75328.1	80 % ERO60967.1 +78 % ERO60968.1 +87 % ERO60969.1	83 % [80 %] WP_025130132.1
ofaB	Nonribosomal peptide synthetase	100 % AGL83958.1	99 % BAO61510.1	99 % AAY91420.2	99 % WP_037007783.1	81 % [100 %] ALG76235.1	82 % [89 %] AFH75329.1	82 % [99 %] ERO60970.1 *	85 % [80 %] WP_025130131.1
ofaC	Nonribosomal peptide synthetase	100 % AGL83959.1	100 % BAO61511.1	99 % AAY91421.3	98 % WP_019095728.1	82 % [100 %] ALG76236.1	82 % [88 %] AFH75330.1	80 % ERO60971.1 +ERO60972.1 *	86 % [82 %] WP_025130130.1
3	Macrolide efflux protein MacA	100 % AGL83960.1	99 % BAO61512.1	99 % AAY91422.1	99 % WP_011060449.1	93 % [100 %] ALG76237.1	92 % [97 %] AFH75331.1	93 % [100%] ERO60973.1	98 % [93 %] WP_025130129.1
4	Macrolide efflux protein MacB	100 % AGL83961.1	99 % BAO61513.1	99 % AAY91423.1	99 % WP_019095727.1	93 % [100 %] ALG76238.1	91 % [97 %] AFH75332.1	96 % [95%] ERO60957.1 *	97 % [93 %] WP_025130128.1
5	LuxR family transcriptional regulator	100 % AGL83962.1	99 % BAO61514.1	99 % AAY91424.1	99 % WP_019095116.1	78 % [100 %] ALG76239.1	74 % [81 %] AFH75333.1	78 % [100%] ERO60958.1	78 % [77 %] WP_025130127.1
6	Flagellar basal body rod modification protein FlgD					[100 %] ALG76240.1		99 % ERO60959.1	
7	Flagellar hook protein FlgE					[100 %] ALG76241.1		99 % ERO60960.1	
8	Putative GNAT family acetyltransferase	100 % AGL83963.1	100 % BAO61515.1						
9	Family 2 glycosyl transferase	100 % AGL83964.1	100 % BAO61516.1						
10	Hypothetical protein	AGL83965.1							
11	Hypothetical protein								WP_029978544.1
12	Glyoxalase family protein	100 % AGL83966.1	100 % BAO61517.1	100 % AAY91425.1	99 % WP_019095115.1	82 % [100 %] ALG76242.1	79 % [90 %] AFH75334.1	82 % [100 %] ERO60961.1	91 % [80 %] WP_025130125.1
13	Heme transporter CcmD, radical SAM domain-containing protein	100 % AGL83967.1	99 % BAO61518.1	99 % AAY91426.1	99 % WP_026020220.1	93 % [100 %] ALG76243.1	92 % [95 %] AFH75335.1	93 % [99 %] ERO60962.1	97 % [93 %] WP_025130124.1

* Partial NRPS sequences were retrieved from *Pseudomonas* sp. CMAA1215 in GenBank database

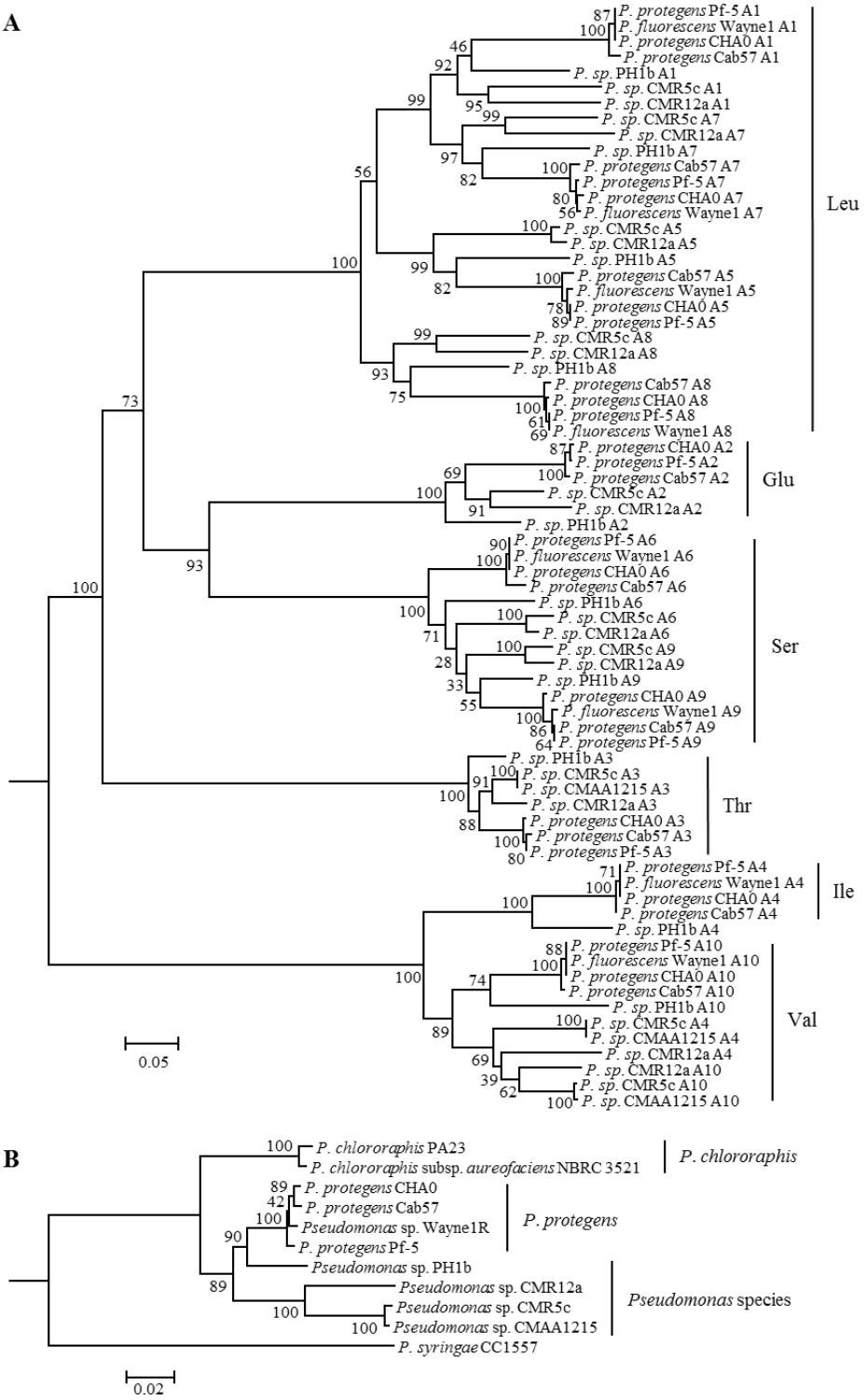

Figure 3.2 Molecular phylogenetic analysis (Neighbor-joining method with 1000 bootstrap replications) analysis of A domains of orfamide synthetases extracted from *P. protegens* and related species (A). The amino acids were predicted based on comparison of those reported orfamide-synthetases in *Pseudomonas* sp. CMR12a and *P. protegens* Pf-5. A phylogeny tree was constructed based on *rpoD* and *gyrB* genes of orfamide-producing and related *Pseudomonas* species (B). The tree was constructed with MEGA5 (Maximum Likelihood method with 1000 bootstrap replications)

Genome mining results showed diversification of the flanking regions of orfamide gene clusters (Figure 3.1). GCN5-related N-acetyltransferase and LuxR type transcriptional regulator genes are located upstream of structural synthetase genes (*ofaA*, *ofaB* and *ofaC*) of *Pseudomonas* sp. CMR5c, CMR12a and CMAA1215, while a NodT type outer membrane lipoprotein gene and luxR type transcriptional regulator genes are located upstream of these structural synthetase genes in *P. fluorescens* Wayne1R, *P. protegens* CHA0, Pf-5, Cab57 and *Pseudomonas* sp. PH1b. Two transporter genes encoding macrolide efflux protein MacA and MacB are located between the *ofaC* gene and LuxR type transcriptional regulator gene in all orfamide gene clusters, although the region downstream of LuxR type transcriptional regulator gene showed differences. Two genes encoding flagella proteins (flagellar basal body rod modification protein FlgD and flagellar hook protein FlgE) are present in *Pseudomonas* sp. CMR5c and CMAA1215, while two genes encoding a putative GNAT family acetyltransferase and a family 2 glycosyl transferase are found in *P. protegens* CHA0 and Cab57. A putative glyoxalase family protein and a heme transporter CcmD, radical SAM domain-containing protein are located downstream of the orfamide biosynthetic gene clusters in all strains. The detailed comparison and interpretation is shown in Table 3.2. A phylogenetic analysis based on *rpoD* and *gyrB* gene sequences of orfamide-producing *Pseudomonas* species indicated that *P. fluorescens* Wayne1R groups with *P. protegens*, while *Pseudomonas* sp. PH1b is more distantly related. *Pseudomonas* sp. CMR12a, *Pseudomonas* sp. CMR5c and CMAA1215 belong to a separate group related to *P. protegens*. Intriguingly, CMR5c and CMAA1215 showed almost 100 % identity (Figure 3.2B). The phylogenetic analysis results of A domains from orfamide-synthetases appear to coincide well with the house keeping (*rpoD* and *gyrB*) gene analysis and the flanking regions of the orfamide cluster.

3.3.2 Chemical analysis of orfamides

UPLC-MS analysis of samples of *Pseudomonas* sp. CMR5c, CMR12a and *P. protegens* Pf-5 and CHA0 confirmed the presence of orfamides homologues (Figure 3.3), in agreement with results of bioinformatic characterization of the NRPS gene clusters (Figure 3.1). Retention times from UPLC and MS data of CLPs, together with bioinformatic characterization of CLP synthetases revealed that

orfamide homologues produced by *P. protegens* CHA0 and Pf-5 are exactly the same (Figure 3.2A, Figure 3.3, Table 3.3 and Table 3.4). Furthermore, the retention time and mass data of peaks from *Pseudomonas* sp. CMR5c were compared with those of reported orfamide homologues, such as orfamide A-E (D'aes et al., 2014; Gross et al., 2007). Interestingly, two new peaks were found in the KB supernatant of *Pseudomonas* sp. CMR5c, and those peaks were designed as orfamide F (*m*/*z* $[M+H]^+$ of 1307.7) and orfamide G (*m*/*z* $[M+H]^+$ of 1309.6). These masses were further confirmed by LC-MS analysis (Table 3.4). The difference of 2 mass units between orfamide F and orfamide G suggests an additional unsaturation, such as a double bond, in orfamide F.

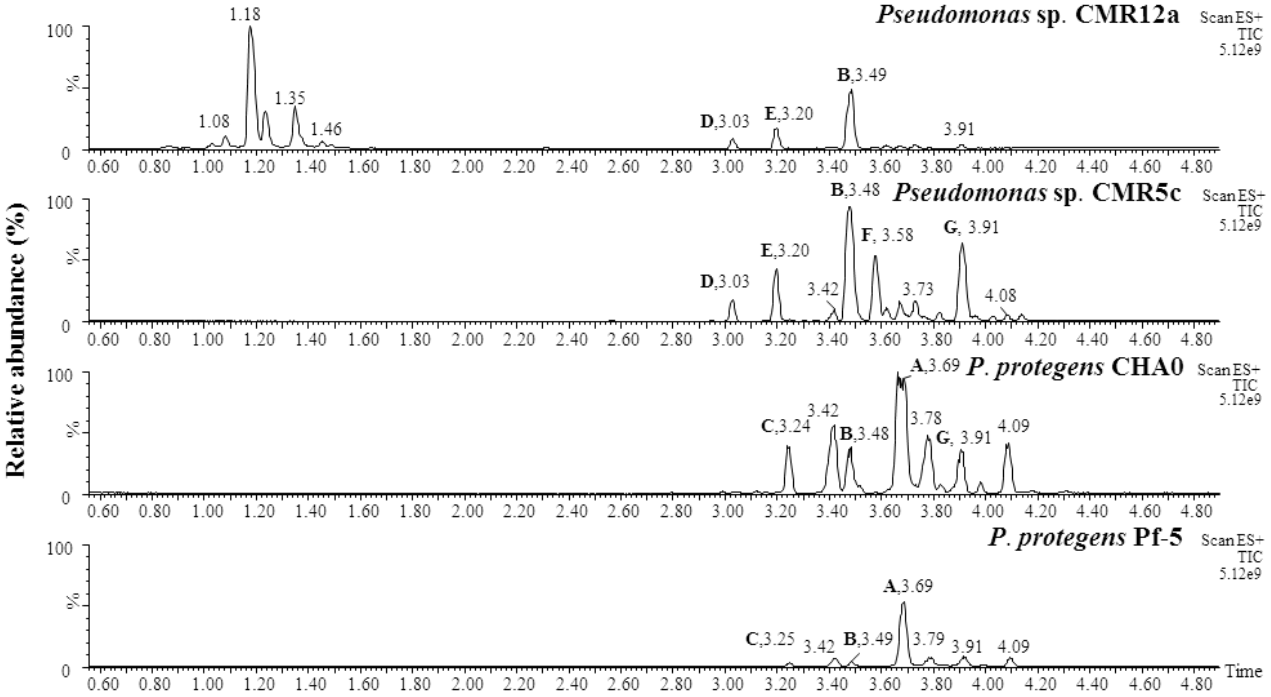

Figure 3.3 UPLC-MS analysis of crude extracts from selected *Pseudomonas* strains, *P. protegens* CHA0 and Pf-5, *Pseudomonas* sp. CMR12a and CMR5c. Samples of *Pseudomonas* sp. CMR12a were prepared from cells on soft agar plate since orfamide secretion in this strain is hampered by the presence of sessilin (D'aes et al., 2014). Samples of other *Pseudomonas* strains were prepared from supernatant of overnight liquid KB cultures.

Table 3.4 Mass spectrometry data (UPLC-MS and LC-MS) of orfamide derivatives isolated and purified from plant-associated fluorescent pseudomonad strains. Mass to charge ratio (*m*/*z*) of corresponding peaks shown in Figure 3.3 are listed, the slash (/) indicates mass data were not detected by LC-MS.

Compound	**UPLC-MS**			**LC-MS**	
	$[M+H]^+$	**$[M+Na]^+$**	**$[M+K]^+$**	**$[M+H]^+$**	**$[M+2H]^{2+}$**
A	1295.7	1317.6	1333.7	/	/
B	1281.8	1303.8	1319.6	1281.6	641.4
C	1267.7	1289.6	1305.7	/	/
D	1253.8	1276.0	1291.9	1253.6	627.5
E	1279.8	1301.8	1317.9	1279.7	640.5
F	1307.7	1329.7	1345.8	1307.7	654.5
G	1309.6	1331.9	1347.7	1309.7	655.5

The orfamides produced by *Pseudomonas* sp. CMR5c were subjected to further chemical identification. Crude extracts from precipitates of KB supernatant of *Pseudomonas* sp. CMR5c were separated on a SPE C18 cartridge. The eluents of 80 % (*v*/*v*) and 100 % (*v*/*v*) acetonitrile were active in a droplet collapse assay. In these fractions, the presence of orfamides was confirmed by UPLC-MS and they were collected and dried. Semi-purified orfamides were further separated on a RP-HPLC system and finally yielded three purified compounds, **1** (14.0 min, 33.54 mg), **2** (14.8 min, 9.4 mg) and **3** (20.9 min, 5.8 mg), respectively.

3.3.3 Chemical structure elucidation of new orfamides

The ^{1}H-NMR spectra of compounds **1**, **2** and **3** showed signal patterns characteristic of a peptide (Table 3.6). Ten amino acids, including Leu (4 ×), Glu (1 ×), Thr (1 ×), Ser (2 ×), Val (2 ×), were identified by ^{1}H-^{1}H correlation spectra of COSY and TOCSY, while their position in the sequence was determined through a combination of the ROESY and ^{1}H-^{13}C HMBC spectra. All three compounds contained the same peptide backbone: Leu^{1}Glu^{2}Thr^{3}Val^{4}Leu^{5}Ser^{6}Leu^{7}Leu^{8}Ser^{9}Val^{10}. The cyclization of **1** is directly demonstrated by the presence of a $^3J_{CH}$ HMBC correlation between the C-terminal Val_{10} carbonyl ^{13}C and the Thr_3 H^{β} resonances. The ^{1}H-NMR spectrum of compound **2** showed a resonance at 5.35 ppm that integrated for 2 protons and correlated with a CH-type carbon

at 130.13 ppm, indicating the presence of an alkene function. Analysis of the COSY, TOCSY and ^{1}H-^{13}C HMBC spectra of **2** showed correlations from and to these ^{1}H and ^{13}C resonances with a large pool of protons at ca. 1.3 ppm, characteristic of the fatty acid chain and confirming the presence of the double bond in this moiety, however, the exact position of double bond in the fatty acid residue of compound **2** could not be determined. The ^{13}C chemical shifts of the neighboring CH_2's suggest a *cis* configuration for this double bond (Table 3.6) (Li et al., 2013). Compounds **1** and **3** contained fully saturated fatty acid moieties. Since the full peptide sequence has been established, the length of these linear moieties can be deduced from the mass obtained from MS, confirming a C_{14} chain length for compound **1** and C_{16} chain length for **2** and **3**. Compound **1** was thus identified as orfamide B, while **2** and **3** were identified as new CLPs, dubbed orfamide F and orfamide G respectively (Table 3.5). The NMR assignment of these compounds is given in Table 3.6. UPLC-MS data revealed that the first two main compounds produced by *Pseudomonas* sp. CMR5c have the same molecular weight and retention time as orfamide D and orfamide E from *Pseudomonas* sp. CMR12a (Figure 3.3). Moreover, considering that the domain analysis result of orfamide biosynthetic synthetase showed high similarities between these two strains, it is reasonable to conclude that the first two main compounds produced by *Pseudomonas* sp. CMR5c may be assigned as orfamide D and E, respectively (Table 3.5).

Table 3.5 Structure of orfamide derivatives isolated and characterized from *P. protegens* and related strains. The stereochemistry of orfamide A has been determined by Gross et al., 2007.

Compound	Fatty acid residue	Amino acid sequence										Producing strains
		1	2	3	4	5	6	7	8	9	10	
Orfamide A	C14:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Ile	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val	CHA0, Pf-5
Orfamide B	C14:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Val	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val	CMR12a, CMR5c, CHA0, Pf-5
Orfamide C	C12:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Ile	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val	CHA0, Pf-5
Orfamide D	C12:0-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val	CMR12a, CMR5c
Orfamide E	C14:1-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val	CMR12a, CMR5c
Orfamide F	C16:1-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val	CMR5c
Orfamide G	C16:0-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val	CMR12a, CMR5c, CHA0, Pf-5

Table 3.6 NMR data (^{1}H and ^{13}C chemical shift δ, ppm; HNHα scalar coupling, *J*, Hz) of orfamide B, orfamide F and orfamide G isolated and purified from supernatant of *Pseudomonas* sp. CMR5c [a]

Residue	Position	Orfamide B		Orfamide F		Orfamide G	
		δ_H; (*J*, Hz)	δ_C	δ_H; (*J*, Hz)	δ_C	δ_H; (*J*, Hz	δ_C
Leu1	NH	7.70m		n.a.		7.76m	
	CHα	3.88m	53.6	n.a.	n.a.	3.89m	53.6
	CO		n.a.				
	CH_2β	1.62m;1.69m	39.3	1.67m; 1.70m	39.3	1.63m; 1.71m	39.3
	CHγ	1.66m	25.2	1.78m	24.4	1.67m	25.2
	CH_3δ	0.94m	23.1	0.93m	23.3	0.93m	23.3
	CH_3δ	0.90m	22.1	0.89m	22.0	0.89m	22.0
Glu2	NH	7.84m		n.a.		7.92d	
	CHα	4.01m	57.0	4.00m	57.1	4.01m	57.0
	CO		n.a.				
	CH_2β	2.01q (7.2)	26.2	2.02m	27.8	2.02m	26.4
	CH_2γ	2.47m	30.5	n.a.	n.a.	2.46q (6.2)	30.7
	COδ		174.6		n.a.		n.a.
	OH	n.a.		n.a.		n.a.	
Thr3	NH	7.94d (6.6)		n.a.		7.96d (6.8)	
	CHα	4.10dd (6.6; 10.7)	61.5	4.25m	61.4	4.13dd (11.2; 6.8)	61.4
	CO		n.a.		n.a.		n.a.
	CHβ	5.20sext	70.4	5.27m	70.7	5.23sext	70.3
	CH_3γ	1.35d (6.0)	18.8	1.36m	18.6	1.35d (6.0)	18.8
Val4	NH	7.35d (6.0)		7.63m		7.41d (5.9)	
	CHα	3.58dd (10.5; 6.0)	64.8	3.51m	64.7	3.57dd (5.9; 10.3)	64.6
	CO		n.a.		n.a.		n.a.
	CHβ	2.23m	30.4	2.21m	30.3	2.23m	30.3
	CH_3γ	0.93m	20.9	0.96m	20.8	0.94m	20.9
	CH_3γ	0.92m	19.1	0.91m	19.2	0.92m	19.1
Leu5	NH	7.84d (4.0)		7.84m		7.85d (6.6)	
	CHα	4.02m	55.2	4.01m	55.1	4.01m	55.2
	CO		n.a.		n.a.		n.a.
	CH_2β	1.58m; 1.72m	40.2	1.54m; 1.74m	40.2	1.57m; 1.72m	40.2
	CHγ	1.78m	25.4	1.78m	24.4	1.79m	25.4
	CH_3δ	0.87d (6.6)	23.4	0.87m	23.4	0.87m	23.4
	CH_3δ	0.89m	20.8	0.87m	21.0	0.88m	20.8
Ser6	NH	7.32d (6.3)		7.27m		7.31d (6.5)	
	CHα	4.24m	57.17	4.30m	56.4	4.26m	57.0

	CO		n.a.		n.a.		n.a.
	$CH_2\beta$	3.94m;4.03m	61.9	3.87m; 4.03m	62.5	3.94m; 4.04m	62.0
	OHγ	n.a.		n.a.		n.a.	
Leu7	NH	7.55d (7.6)		7.44m		7.54d (7.5)	
	CHα	4.34m	54.0	4.30m	53.9	4.34m	54.0
	CO						
	$CH_2\beta$	1.51m; 1.66m	42.4	1.47m; 1.66m	42.0	1.51m; 1.66m	42.4
	CHγ	1.73m	25.2	1.78m	24.4	1.73m	25.2
	$CH_3\delta$	0.94m	23.2	0.91m	21.4	0.94m	23.2
	$CH_3\delta$	0.90m	21.6	0.95m	23.4	0.90m	21.6
Leu8	NH	6.94d (6.9)		7.21m		6.93 (*6.5*)	
	CHα	4.28m	53.9	4.26m	54.1	4.28m	54.0
	CO		n.a.		n.a.		n.a.
	$CH_2\beta$	1.67m; 1.80m	42.1	1.65m; 1.76m	41.1	1.67m; 1.79m	41.9
	CHγ	1.85m	25.6	1.78m	24.4	1.85m	25.6
	$CH_3\delta$	0.96d (6.5)	23.6	0.96m	23.3	0.96d (6.5)	23.5
	$CH_3\delta$	0.88m	21.1	0.88m	21.6	0.88m	21.2
Ser9	NH	7.03d (4.2)		7.47m		7.09m	
	CHα	4.05m	58.9	4.13m	58.3	4.07m	58.7
	CO		n.a.		n.a.		n.a.
	$CH_2\beta$	3.74dd (11.2; 3.4); 3.88m	62.0	3.73m; 3.84m	62.6	3.75dd (11.7; 3.9); 3.87m	62.6
	OHγ	n.a.		n.a.		n.a.	
Val10	NH	6.46d (9.8)		6.66m		6.49d (9.2)	
	CHα	4.54dd (9.8; 3.8)	57.1	4.41m	58.2	4.53m	57.2
	CO		n.a.		n.a.		n.a.
	CHβ	2.19m	31.3	2.04m	31.6	2.17m	31.3
	$CH_3\gamma$	0.83d (6.9)	19.2	0.84m	19.5	0.83d (6.9)	19.2
	$CH_3\gamma$	0.69d (6.7)	17.6	0.73m	18.2	0.69d (6.7)	17.6
Fatty acid	CO 1'						
	CH_2 2'	2.32dd 14.3; 9.4); 2.40dd (14.3; 4.0)	44.4	n.a.	n.a.	2.40dd (14.2; 3.9); 2.32dd (14.1; 9.2)	44.4
	CH 3'	3.99m	69.6	4.42m	68.7	3.99m	69.6
	OH 3'	n.a.		n.a.		n.a.	
	CH_2 4'	1.47m	38.1	n.a. [b]	n.a. [b]	1.48m	38.1
	CH_2 5'	1.28m; 1.40m	26.3	n.a. [b]	n.a. [b]	1.31m;1.42m	26.3
	CH_2 6'	n.a.	n.a.	n.a. [b]	n.a. [b]	1.27m	30.2
	CH_2 7'	n.a.	n.a.	n.a. [b]	n.a. [b]	1.27m	30.2
	CH_2 8'	n.a.	n.a.	n.a. [b]	n.a. [b]	n.a.	n.a.
	CH_2 9'	n.a.	n.a.	n.a. [b]	n.a. [b]	n.a.	n.a.
	CH_2 10'	n.a.	n.a.	n.a. [b]	n.a. [b]	n.a.	n.a.
	CH_2 11'	n.a.	n.a.	n.a. [b]	n.a. [b]	n.a.	n.a.

CH_2 12'	n.a.	n.a.	n.a. [b]	n.a. [b]	n.a.	n.a.
CH_2 13'	1.28m	23.4	n.a. [b]	n.a. [b]	n.a.	n.a.
$CH_{3/2}$ 14'	0.89m	14.3	n.a. [b]	n.a. [b]	n.a.	n.a.
CH_2 15'			1.29m	23.3	1.30m	23.3
CH_3 16'			0.88m	14.3	0.88m	14.3

[a] The NMR spectra of corresponding compounds were recorded in CD_3CN solution at 298 K.
n. a. indicates the chemical shifts that were not assigned.

[b] Two mutually unresolved resonances, at ^1H and ^{13}C chemical shifts of 5.35 ppm and 130.6 ppm respectively, are attributed to a double bond at undetermined position in the fatty acid moiety. These correlate to a neighboring CH_2 signal at 2.02 ppm and 27.8 ppm ^1H and ^{13}C chemical shifts respectively. Using a similar reasoning used for the CLP xantholysin C in the supporting information of Li et al (2013), the value of the latter ^{13}C chemical shift is indicative for a *cis* configuration of the double bond. Using the ^{13}C chemical shift prediction in the ChemDraw Ultra 15 software (*PerkinElmer, Inc.*), the chemical shifts of both CH_2 units neighboring an isolated double bond in a linear fatty acid chain is indeed predicted as 27.7 ppm for a *cis* configuration and 33.7 ppm for a *trans* configuration.

3.3.4 Orfamides determine the surface swarming motility of *Pseudomonas* sp. CMR5c and their production is regulated by LuxR-type regulators

To study the function of *ofa* genes and linked *luxR* genes of *Pseudomonas* sp. CMR5c, a 10.8 kb fragment was deleted from *ofaB* and *ofaC* genes (Figure 3.1), a 661 bp fragment was deleted from luxR-type transcriptional regulator gene (*luxRup*) located upstream of structural genes of orfamide synthetase, and a 441 bp fragment was deleted from *luxR* type transcription regulator gene (*luxRdown*) located downstream of structural genes of orfamide synthetase, generating *Pseudomonas* sp. CMR5cΔ*ofa*, CMR5cΔ*luxRup* and CMR5cΔ*luxRdown* mutants (Table 3.1). UPLC-MS data showed that the production of orfamide homologues was abolished in the orfamide biosynthesis mutant *Pseudomonas* sp. CMR5cΔ*ofa* (Figure 3.4A). Similar results were found for the two *luxR*-type transcriptional regulator mutants CMR5cΔ*luxRup* and CMR5cΔ*luxRdown*. Furthermore, phenotypic characterization showed that in all three mutants swarming motility was blocked compared to wild type *Pseudomonas* sp. CMR5c strain (Figure 3.4A). Chemical complementation experiments were conducted with soft agar plates amended with different concentrations of orfamide B. The results showed that in all three mutants the swarming phenotype was restored by adding

orfamide B in soft agar plates (Figure 3.4A). The swarming motility of orfamide-deficient mutants of *Pseudomonas* sp. CMR5c showed a dose-dependent response, that is, bacterial motility increased on soft agar plates containing higher concentrations of orfamides.

Model strains *P*. protegens CHA0 and Pf-5, *Pseudomonas* sp. CMR5c, and CMR12a and its sessilin mutant *Pseudomonas* sp. CMR12a-Clp1 were included in further phenotypic comparison. The sessilin mutant was included because previous work has shown that the presence of sessilin hampers orfamide secretion in *Pseudomonas* sp. CMR12a (D'aes et al., 2014). All these strains could swarm well on the agar surface, suggesting at least a partial involvement of orfamides in bacterial motility (Figure 3.4B).

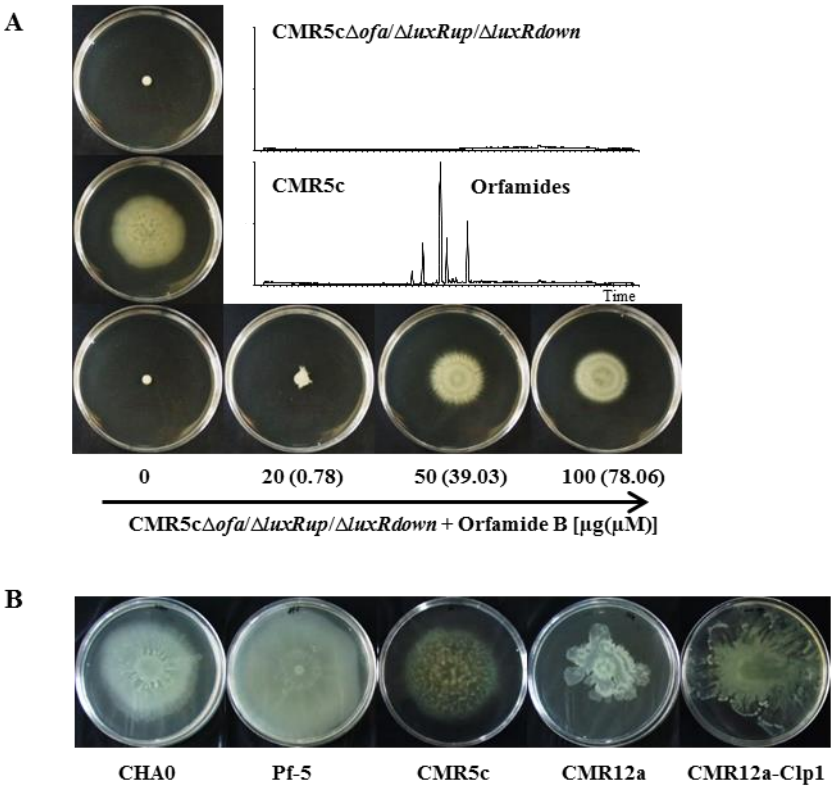

Figure 3.4 (**A**) Swarming motility of wild type strain *Pseudomonas* sp. CMR5c and deletion mutants in orfamide biosynthesis (CMR5cΔ*ofa*) or in orfamide regulatory genes (CMR5cΔ*luxRup* and CMR5cΔ*luxRdown*). In all mutants swarming was restored by chemical complementation with orfamide B. Representative plates for the complementation of mutant CMR5cΔ*ofa* are shown. The diameter of soft agar plates used in this assay is 9 cm. The insert shows the UPLC-MS analysis of wild type and mutant strains. (**B**) Swarming motility of *P. protegens* CHA0, *P. protegens* Pf-5, *Pseudomonas* sp. CMR5c, *Pseudomonas* sp. CMR12a and its sessilin mutant CMR12a-Clp1.

3.3.5 *In vitro* antibiosis activity against *R. solani* and Oomycete pathogens

Orfamide A produced by *P. protegens* CHA0, and orfamide B and G produced by *Pseudomonas* sp. CMR5c, were compared in further bioassays. Orfamide A and orfamide B differ only by an amino acid substitution at the fourth position: a valine in orfamide A and an isoleucine in orfamide B; orfamide B and orfamide G share the same amino acid sequence but differ in length of the fatty acid chain, a C_{14} for orfamide B and C_{16} for orfamide G (Table 3.5). Previous results showed that orfamide B suppressed mycelial growth and caused increased hyphal branching of *R. solani* AG 4-HGI at 100 µM (Olorunleke et al., 2015b). Moreover, orfamide A showed zoospore lysis activity against *Phytophthora ramorum* Pr-102 (Gross et al., 2007). We wished to assess whether the subtle structural differences between orfamide A, B and G would influence these activities. The three orfamides caused increased hyphal branching of *R. solani* AG 4-HGI at 100 µM, while lower concentrations were not effective (Figure 3.5). At concentrations of 25 µM or higher all orfamides could lyse zoospores of *Phytophthora porri* CBS 127099 and *Pythium ultimum* within 55 - 70 seconds. At concentrations of 20 and 25 µM, orfamide A was slightly faster in causing zoospore lysis than the two other orfamides (Figure 3.6).

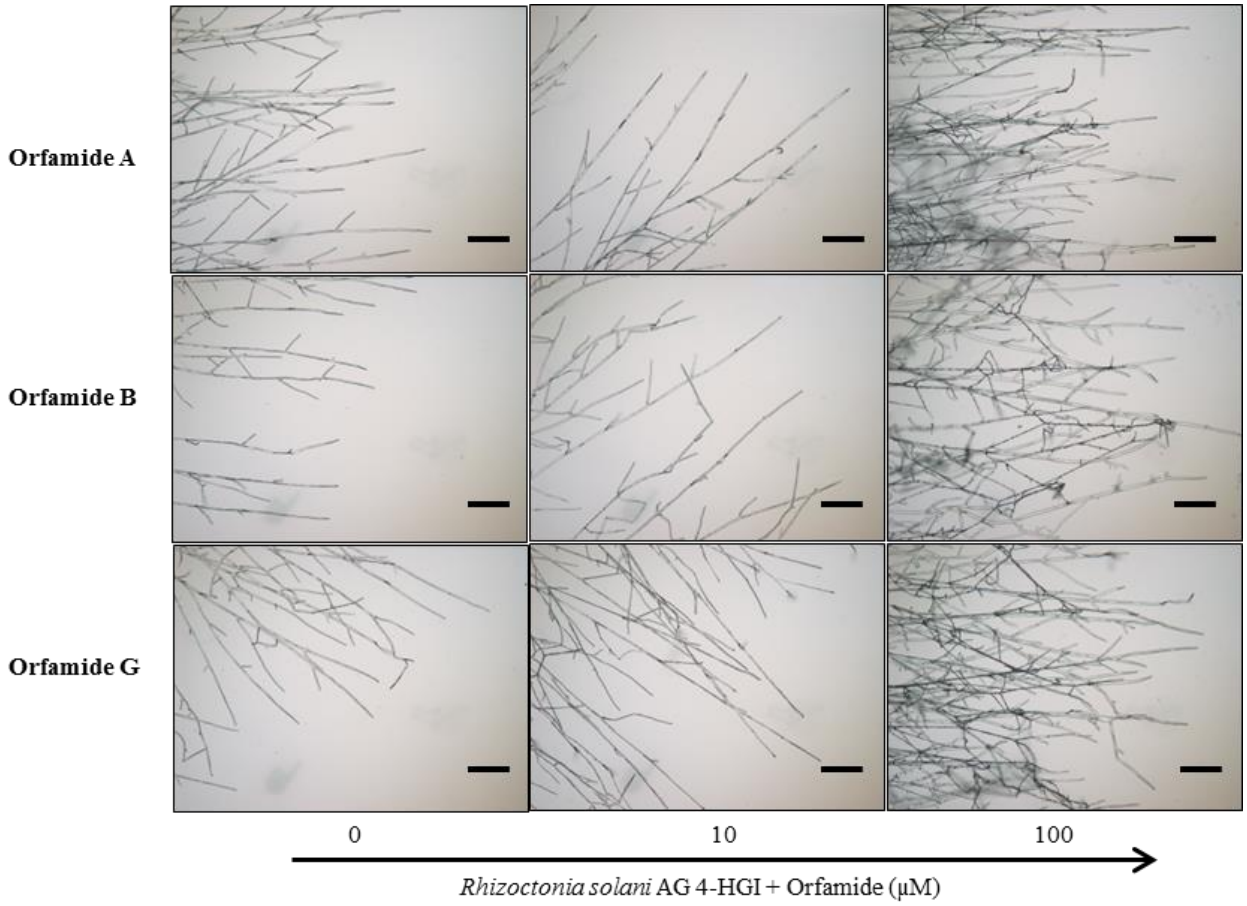

Figure 3.5 Microscopic assays showing the effect of various concentrations of orfamides (Orfamide A, orfamide B and orfamide G) on hyphal branching of *R. solani* AG 4-HGI, scale bar = 100 µm.

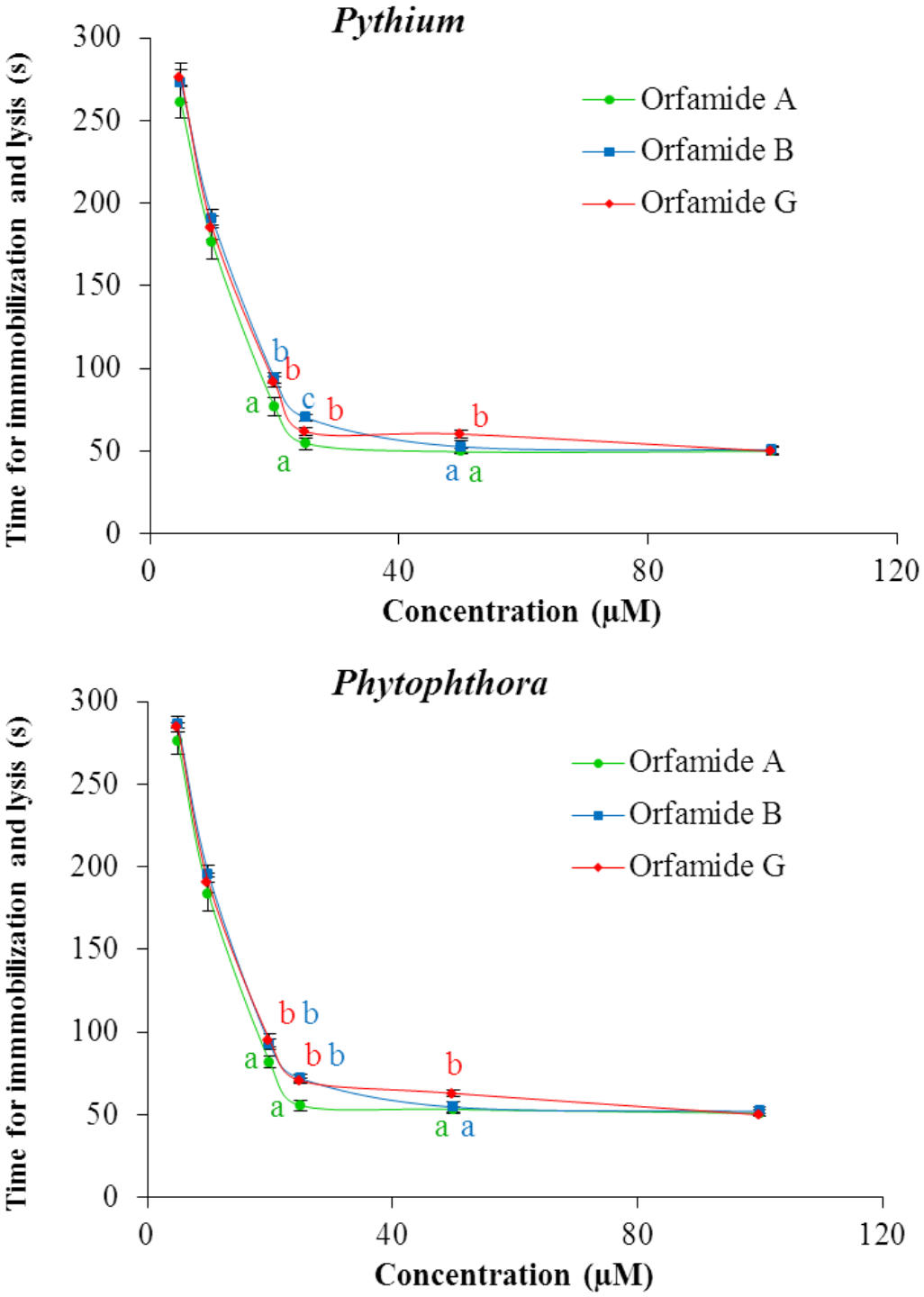

Figure 3.6 Effect of orfamides (orfamide A, orfamide B and orfamide G) on the viability of zoospores of the oomycete pathogens *Pythium ultimum* and *Phytophthora porri* CBS 127099 (B). Data are the mean of three repetitions. Vertical bars indicate standard deviations. Different letters indicate significant differences among different treatments for that specific concentration (Tukey's test, $\alpha = 0.05$).

3.3.6 Orfamides inhibit appressoria formation in *M. oryzae*

Subsequently, we tested whether orfamides would have an effect against the rice blast pathogen *M. oryzae*. An *in vitro* spore germination assay with *M. oryzae* isolate VT5M1 showed that both orfamide-treated and control treated spores had the same level of germination, with 92.1 % ± 3.1 for the control, 90.1 % ± 2.6 for orfamide A (50 µM), 91.7 % ± 4.6 for orfamide B (50 µM) and 93.1 % ± 3.2 for orfamide G (50 µM). Orfamides (Orfamide A, B and G) did not show any antagonistic effect against *M. oryzae* isolate VT5M1 in an *in vitro* paper-agar disc diffusion assay (data not shown). However, the three compounds actively blocked appressoria formation in *M. oryzae* in a dose dependent manner (Figure 3.7A). Representative microscopic pictures showing the effect of orfamide A on appressorium formation are shown in Figure 3.7B. Appressorium formation *in vivo* was not quantified, but no appressoria were formed in the treatment with 50 µM orfamide in two independent repeats.

3.3.7 Orfamides reduce blast disease severity on rice plants

Different concentrations of orfamide A, B and G were mixed with spores of *M. oryzae* isolate VT5M1 and sprayed on rice plants (Five-leaf stage) and disease symptoms were evaluated after 6 days. All compounds reduced the number of sporulating susceptible-type blast lesions at a concentration of 50 µM, while lower concentrations were not effective (Figure 3.8).

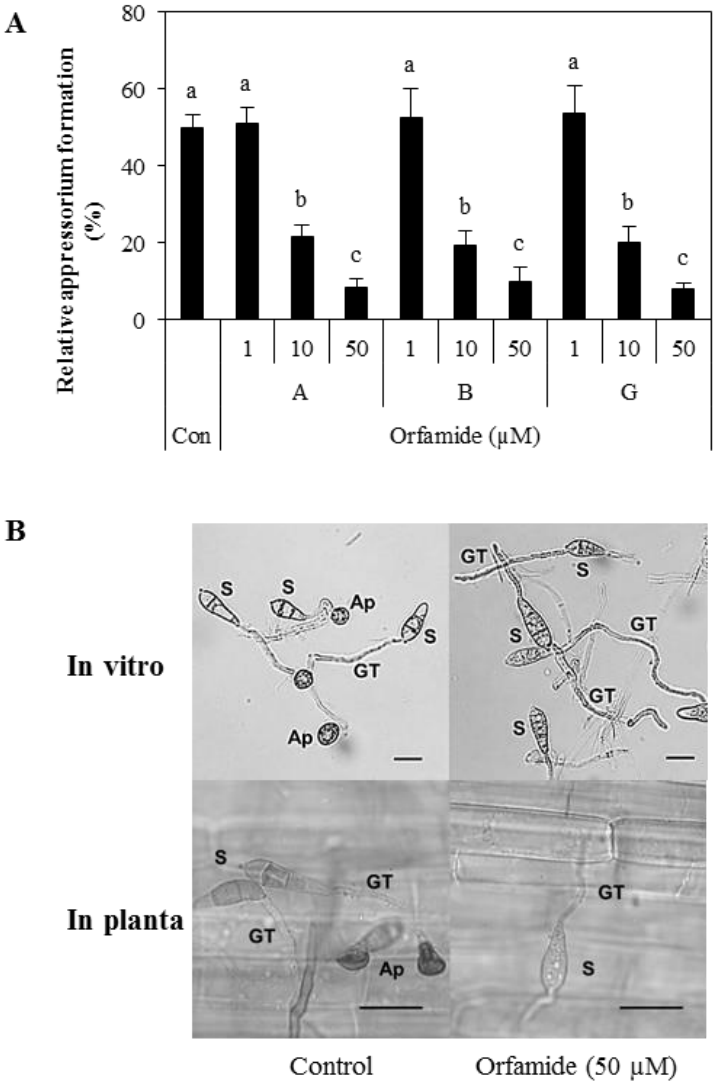

Figure 3.7 (A) Effect of different concentrations (1, 10 and 50 µM) of orfamides (orfamide A, B and G) on appressorium formation in *Magnaporthe oryzae* VT5M1. The control treatment (Con) received the same amount of DMSO as the orfamide treatments. Data are shown as mean value (± SD) for three biological repeats. Different letters indicate significant differences among different treatments (Tukey's test; $\alpha = 0.05$). **(B)** Representative pictures of appressoria formation in *M. oryzae* in control (DMSO) and orfamide treatments after 8 h incubation (*in vitro* assay) or 24 h incubation (in planta assay). S, spore; GT, germ tube; Ap, appressorium. Scale bar is 20 µm.

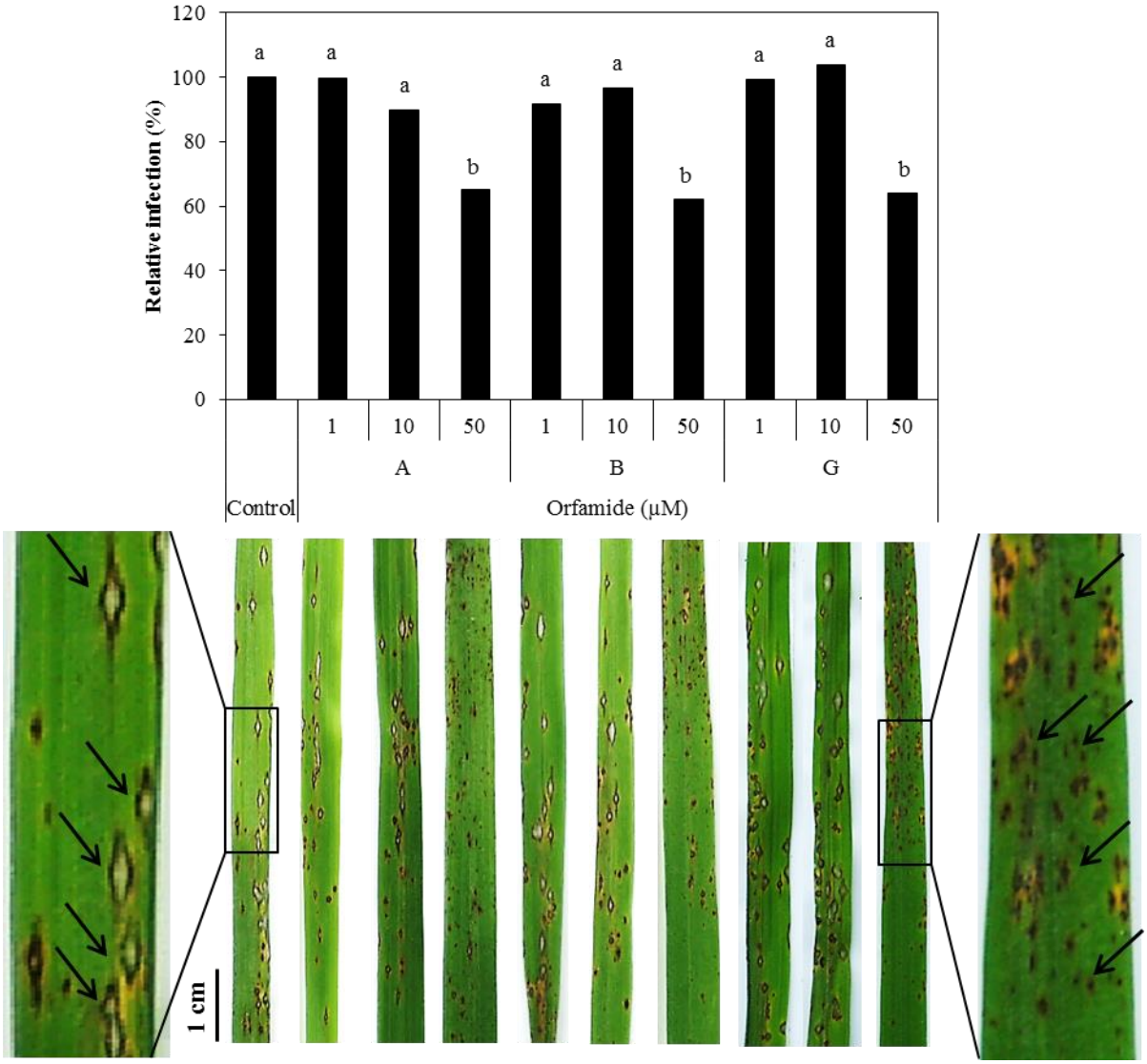

Figure 3.8 Influence of orfamides (orfamide A, B and G) on rice blast symptoms caused by *M. oryzae* isolate VT5M1. Spores of *M. oryzae* were mixed with different concentrations of orfamides and sprayed on rice plants at the five-leaf stage. Disease was assessed 6 dpi by counting the number of sporulating susceptible-type lesions on the fourth leaf of rice plants and expressed relative to control plants. Biocontrol assays were repeated independently three times and showed similar results, data from one representative experiment are shown. Different letters indicate significant differences among different treatments (Mann-Whitney: n = 24; α = 0.05). Pictures show representative disease symptoms in the different treatments. Black arrows in the enlargement of the pictures indicate representative symptoms of sporulating lesions (on the left side of the pictures) and resistant lesions (on the right side of the pictures), respectively.

3.4 Discussion

This study shows that *Pseudomonas* sp. CMR5c produces orfamide-type CLPs, including two new orfamide homologues that we termed orfamide F and orfamide G. Orfamide production appears to be associated with *P. protegens* and taxonomically related species. Orfamide producers are plant associated *Pseudomonas* species isolated from the rhizosphere of both dicots and monocots including tobacco (*P. protegens* CHA0) (Stutz et al., 1986), cotton (*P. protegens* Pf-5) (Howell et al., 1979), shepherd's purse (*P. protegens* Cab57) (Takeuchi et al., 2014), cocoyam (*Pseudomonas* sp. CMR12a and CMR5c) (Perneel et al., 2007), and corn (*P. fluorescens* Wayne1R) (Rong et al., 2012). Moreover, also *Pseudomonas* sp. PH1b isolated from the phytotelma (= water body) of a carnivorous plant and *Pseudomonas* sp. CMAA1215 from Brazilian mangroves (Vasconcellos et al., 2013) have the genetic information to produce orfamides. Phylogenetic analysis based on the housekeeping genes *rpoD* and *gyrB* shows that *P. fluorescens* Wayne1R (a rifampicin resistant variant of *P. fluorescens* Wayne1) groups with *P. protegens* (Figure 3.2B) while *Pseudomonas* sp. PH1b is more distantly related to *P. protegens*. It was already shown by Redondo-Nieto et al. (2013) based on a phylogenomic analysis that *P. fluorescens* Wayne1 is closely related to *P. protegens* Pf-5. *Pseudomonas* sp. CMR12a, CMR5c and CMAA1215 clearly belong to a distinct phylogenetic group. Strikingly, however, is the very close relationship between *Pseudomonas* sp. CMR5c from Cameroon and *Pseudomonas* sp. CMAA1215 from Brazil. These two isolates probably have their ecological niche in common, namely a tropical area with periods of high humidity alternating with dry periods.

Bioinformatic analysis of the orfamide gene clusters shows that orfamide biosynthesis genes and flanking regions closely follow the taxonomic relationship among the isolates. This suggests that the orfamide gene cluster is ancestral and not obtained by horizontal gene transfer. Orfamide biosynthesis genes and flanking regions in *P. protegens* are highly conserved and isolates belonging to this species secrete orfamide A as the main CLP, which was also confirmed by chemical analysis for *P. protegens* CHA0 and Pf-5. Bioinformatic analysis results showed that the more distantly related isolate *Pseudomonas* sp. PH1b probably also produces orfamide A. *Pseudomonas* sp. CMR12a and CMR5c produce orfamide B as their main CLP. Since *Pseudomonas* sp. CMR5c and CMAA1215 are very similar in their nonribosomal peptide synthetases and flanking region (Figure

3.2A and Table 3.3) is very likely that *Pseudomonas* sp. CMAA1215 also produces orfamide B.

Most *Pseudomonas* NRPS structural genes are linked together without disruption by other genes, although viscosin group (WLIP and massetolides, etc.), entolysin group and xantholysin group NRPS peptides do not (de Bruijn et al., 2008; de Bruijn et al., 2007; Li et al., 2013; Vallet-Gely et al., 2010). The orfamide- synthetases compared in this study do seem to follow this rule (Figure 3.1). The structural identification showed that co-produced orfamides not only differ from each other in amino acid sequence, but also in fatty acid residue, with variable length and degree of unsaturation (Table 3.5). A single orfamide-producer can synthesize orfamides containing both saturated and unsaturated fatty acid chains (*Pseudomonas* sp. CMR12a and CMR5c). *P. protegens* can incorporate different amino acids by the same A domain (Figure 3.2A, Figure 3.3 and Table 3.5). For instance, the fourth A domain can incorporate both valine and isoleucine, although in this species the incorporation of isoleucine seems to be preferred, since orfamide A is most abundantly produced. The fourth A domain of *Pseudomonas* sp. CMR12a seems to be less flexible since these strains only produce orfamides with a valine at the fourth position. Flexibility in the A domain has also been observed for putisolvin biosynthesis. The 11th A domain of putisolvin-synthetase can use valine/isoleucine/leucine, but the production of putisolvin I containing valine is preferred (Dubern et al., 2008). The mechanisms of this type of synthesizing and catalyzing remain unclear. However, the fact that a single domain can activate two or more different amino acids, leads to difficulties in deducing amino acid sequence only by bioinformatic analysis of peptide synthetase in *Pseudomonas* species.

The amount of semi-purified orfamides that can be obtained from *P. protegens* CHA0 is much higher than for Pf-5, *Pseudomonas* sp. CMR5c and CMR12a in liquid KB medium (data not shown). This could point to differences in secretion or regulation of these compounds in the various strains. A NodT type outer membrane lipoprotein is located upstream of orfamide-synthetases of *P. protegens* and *Pseudomonas* sp. PH1b, but not in *Pseudomonas* sp. CMR12a, CMR5c and CMAA1215. Together with the MacA and MacB encoding genes downstream of the orfamide synthetases, these lipoproteins are probably involved in orfamide transport across the inner and outer membrane, but this needs to be confirmed experimentally. Moreover, flanking regions of *Pseudomona*s NRPS structural genes contain one or more *luxR* or *luxR*-like transcriptional regulator genes that control lipopeptide biosynthesis (de Bruijn and Raaijmakers, 2009). Accordingly, this study also shows that

orfamide production was blocked in *Pseudomonas* sp. CMR5c when *luxR* type transcriptional regulator encoding genes located at the flanking region of orfamide-synthetase were disrupted (Figure 3.4A). It should be noted however that lipopeptide regulation in *Pseudomonas* is very complex. Recently it was shown that chaperone protein ClpA together with the serine protease ClpP regulated massetolide biosynthesis in *P. fluorescens* SS101 via the LuxR-type transcriptional regulator MassAR, the heat shock proteins DnaK and DnaJ and via proteins involved in the TCA cycle (Song et al., 2015). It is not unlikely that strain-dependent differences in this regulatory circuit account for the differences in orfamide production that we observed in the tested strains.

The structural differences among orfamide A, orfamide B and orfamide G allowed us to study whether the difference in amino acid at the fourth position and the length of fatty acid residue has any effect on the biological activity. In case of iturin-type *Bacillus* lipopeptides it has been shown that biological activity against fungal pathogens increases with the number of carbon atoms in β-amino fatty acid chain (Tanaka et al., 2014). The defense-inducing activity of surfactin-type *Bacillus* lipopeptides was reduced for some amino acid substitutions and completely lost for surfactins with fatty acid chains shorter than 14 carbons (Henry et al., 2011). In our work, however, the residue in position 4 or the length of the fatty acid chain had no obvious influence on biological activity against oomycete or fungal pathogens. Microscopic assays showed that all orfamides increased hyphal branching of *R. solani* AG 4-HGI (Figure 3.5), indicative of mycelial growth inhibition (Olorunleke et al., 2015b). These orfamides however did not show any antibiotic activity against *R. solani* AG 4-HGI by the paper disc-agar diffusion assay as described by Gross et al. (2007). These data confirm the positive results obtained with orfamide B in *Rhizoctonia* branching (Olorunleke et al., 2015b) and the negative results obtained with orfamide A in paper-agar disc diffusion assay published previously (Gross et al., 2007) and indicate that different methods for testing *in vitro* antibiosis activity may lead to different results. It has been shown before that the CLP viscosinamide co-incubated with *R. solani* changed the morphology of the hyphae of this fungal pathogen; hyphae were highly branched compared with control treatments (Raaijmakers et al., 2010; Thrane et al., 1999). At concentrations of 25 μM or higher, orfamides (orfamide A, B and G) caused zoospore lysis of *Phytophthora porri* CBS 127099 and *Pythium ultimum* within 55-70 seconds (Figure 3.6). Orfamide A was slightly faster in lysing zoospores at concentrations of 20 and 25 μM than the two other orfamides, but the

difference is subtle and was not observed at lower or higher concentrations. It has been reported before that orfamide A and the viscosin family CLPs WLIP and viscosinamide, are able to lyse zoospores of the oomycete pathogen *Phytophthora ramorum* (de Bruijn et al., 2007; Gross et al., 2007; Thrane et al., 2000).

Blast disease, caused by the filamentous ascomycete fungus *M. oryzae*, is one of the major diseases in rice (Dean et al., 2012) and the productivity of rice is threatened by this pathogen worldwide. *M. oryzae* can directly penetrate the plant cuticle and cell wall of rice by means of appressoria in which a turgor pressure is build up. Inhibition of appressoria formation in *M. oryzae* can significantly reduce its pathogenicity in plants (Liu et al., 2011). The orfamides tested (orfamide A, B and G) were equally active in inhibiting appressoria formation at 10 and 50 µM and reduced the number of susceptible blast lesions on rice at 50 µM. Spence et al. (2014) already revealed that *P. protegens* CHA0 can inhibit appressorium formation of *M. oryzae,* but in their study the corresponding metabolite and underlying mechanism were not elucidated. Intriguingly, biosurfactants such as mannosylerythritol lipids and the synthetic surfactant Tween 20 also blocked appressoria formation by *M. oryzae* on a hydrophobic substrate, but failed to protect rice against blast disease attack. Symptoms on rice leaves caused by *M. oryzae* were neither suppressed nor enhanced by the mannosylerythritol lipids treatment while Tween 20 treatment even increased rice blast severity (Yoshida et al., 2015). These authors hypothesized that inhibition of appressorium formation may be due to changes in surface hydrophobicity by treatment with the surfactant and that appressorium formation is probably not inhibited on the leaf surfaces. In the case of orfamides, however, it appears that appressorium formation is also inhibited on rice leaves. It remains to be investigated whether this is due to effects on surface hydrophobicity or to direct effects of orfamides on appressorium development. Induced resistance can be excluded as a mechanism explaining the effects of orfamide on rice blast. Rice roots inoculated with *Pseudomonas* sp. CMR5c were as susceptible to *M. oryzae* as control plants and soil drench with purified orfamides did not induce resistance to the fungus (Ma and Hofte, unpublished results). Likewise, root inoculation with *P. protegens* CHA0 did not result in induced resistance against *M. oryzae* in rice plants (Spence et al., 2014).

Although orfamides do not induce resistance against *M. oryzae*, we recently found that orfamides can trigger defense-related responses in rice cell cultures and induce resistance against the necrotrophic

fungus *Cochliobolus miyabeanus* in rice plants (see Chapter 6). We are currently investigating this interaction in more detail.

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Chapter 4. Interplay between orfamides, sessilins and phenazines in the control of *Rhizoctonia* diseases by *Pseudomonas* sp. CMR12a

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Abstract

We investigated the role of phenazines and cyclic lipopeptides (CLPs) (orfamides and sessilins), antagonistic metabolites produced by *Pseudomonas* sp. CMR12a, in the biological control of damping-off disease on Chinese cabbage (*Brassica chinensis*) caused by *R. solani* AG 2-1 and root rot disease on bean (*Phaseolus vulgaris* L.) caused by *R. solani* AG 4-HGI. A *Pseudomonas* mutant that only produced phenazines suppressed damping-off disease on Chinese cabbage to the same extent as wild type CMR12a, whereas its efficacy to reduce root rot on bean was strongly impaired. In both pathosystems, the phenazine mutant that produced both CLPs was equally effective, but mutants that produced only one CLP lost biocontrol activity. *In vitro* microscopic assays revealed that mutants that only produced sessilins or orfamides inhibited mycelial growth of *R. solani* when applied together, while they were ineffective on their own. Phenazine-1-carboxamide suppressed mycelial growth of *R. solani* AG 2-1 but had no effect on AG 4-HGI. Orfamide B suppressed mycelial growth of both *R. solani* anastomosis groups in a dose-dependent way. Our results point to an additive interaction between both CLPs. Moreover, phenazines alone are sufficient to suppress *Rhizoctonia* disease on Chinese cabbage, while they need to work in concert with the CLPs on bean.

4.1 Introduction

Cyclic lipopeptides (CLPs) are bacterial metabolites with biosurfactant activity composed of a cyclic oligopeptide lactone ring coupled to a fatty acid tail. They are synthesized by non-ribosomal peptide synthetases, encoded by large gene clusters (Finking and Marahiel, 2004). Although CLPs can function as virulence factors in plant pathogenic Pseudomonads (Kunkel and Zhongying, 2006; Pauwelyn et al., 2013), they have drawn increasing interest for their versatile functions in plant beneficial *Pseudomonas* (reviewed by Olorunleke et al., 2015). These functions include involvement in biofilm formation, motility and antimicrobial activity against a wide range of microorganisms including fungi, bacteria, viruses and oomycetes. Several CLPs produced by *Pseudomonas* sp. have been implicated in the biocontrol of plant pathogens *in vivo* including massetolide A (Tran et al., 2007; Le et al., 2012; Van De Mortel et al., 2012), viscosinamide (Nielsen et al., 1999; Thrane et al., 2000), putisolvin (Kruijt et al., 2009; Le et al., 2012), sclerosin (Berry et al., 2010) and sessilin (D'aes et al., 2011; Hua and Höfte, 2015).

Phenazines are heterocyclic nitrogen-containing compounds produced by bacteria. These metabolites play a vital role in the biocontrol of plant diseases (Tambong and Höfte, 2001; Chin-A-Woeng et al., 2003; Mavrodi et al., 2006; D'aes et al., 2011; Le et al., 2012; Hua and Höfte, 2015) and contribute to biofilm formation and virulence (Price-Whelan et al., 2006; Pierson and Pierson, 2010; Selin et al., 2010).

Pseudomonas sp. CMR12a was isolated from the rhizosphere of tropical tuber cocoyam (*Xanthosoma sagittifolium*) in Cameroon. Taxonomically, CMR12a belongs to the *Pseudomonas fluorescens* complex and is positioned between the *Pseudomonas protegens* and *Pseudomonas chlororaphis* group (D'aes et al., 2014). This strain produces several metabolites including two classes of CLPs and two phenazines, namely PCA and its main phenazine compound, PCN (Perneel et al., 2007; D'aes et al., 2011; De Maeyer et al., 2013). The two classes of CLPs produced by CMR12a were recently characterized as sessilins and orfamides. This strain produces three major orfamide derivatives designated as orfamide B, D and E with *m/z* $[M+H]^+$ values of 1281.9, 1279.8 and 1254.0, respectively, and three sessilin derivatives, sessilin A, B and C, with *m/z* $[M+2H]^{2+}$ values of 1015.2, 1024.2 and 993.2, respectively (D'aes et al., 2014). Sessilins are structurally related

to tolaasins produced by the mushroom pathogen *Pseudomonas tolaasii*, whereas the structure of the orfamides produced by CMR12a is very similar to that of orfamides from *P. protegens* Pf-5. *Pseudomonas* sp. CMR12a, however, mainly produces orfamide B, while *P. protegens* Pf-5 predominately produces orfamide A (Gross et al., 2007). Orfamides A and B differ in the amino acid at position 4 in the peptide chain which is D-allo-isoleucine in orfamide A and valine in orfamide B (D'aes et al., 2014). It was shown that sessilins and orfamides play vital but distinctive roles in traits contributing to rhizosphere fitness. Sessilins are important for biofilm formation, while orfamides are crucial for the swarming motility of CMR12a (D'aes et al., 2014).

CMR12a can protect cocoyam and bean against root rot diseases caused by *Pythium myriotylum* (Perneel et al., 2007) and *R. solani* (D'aes et al., 2011), respectively. In an *R. solani*-bean pathosystem, *Pseudomonas* sp. CMR12a reduced bean root rot caused by two anastomosis groups (AGs), 2-2 and 4-HGI, of *R. solani*. In this pathosystem, the involvement of phenazines and sessilins in the biocontrol efficacy of CMR12a was clearly demonstrated by using mutants deficient in the production of one or both metabolites (D'aes et al., 2011). At that time, however, orfamide mutants of CMR12a were not available. Moreover, a possible interaction of dual lipopeptides produced by a plant beneficial *Pseudomonas* bacterium in biocontrol of plant pathogens has not been shown before. Interestingly, CMR12a offers us the opportunity not only to investigate such interaction among two lipopeptides but also together with phenazines. Besides, until now, orfamides have not been implicated in the biocontrol of plant pathogens *in vivo* although it was shown that orfamide A produced by *P. protegens* F6 has insecticidal activity against the aphid *Myzus persicae* (Jang et al., 2013). Thus, in this work, we investigated the interaction between sessilins, orfamides and phenazines of CMR12a in the biocontrol of *R. solani* in two plant systems namely bean and Chinese cabbage using various mutants impaired in the production of phenazines and/or CLPs.

4.2 Materials and methods

4.2.1 Strains, plants, and their growth conditions

Pseudomonas bacteria and fungi used in this study are shown in Table 4.1. Pseudomonas strains and *R. solani* were maintained on KB and PDA plates, respectively. All strains were routinely grown at 28 °C. Sand and potting soil were autoclaved twice on two successive days. Plants were grown in substrate containing 50% potting soil and 50% sand (w/w) (Hua and Höfte, 2015). Seedlings of Chinese cabbage were grown in a controlled-environment chamber at 22°C, relative humidity (RH) = 60%, 12 h photoperiod. While bean seedlings were grown in a controlled-environment chamber at 25°C, RH = 60%, 16 h photoperiod. Seedlings of bean and Chinese cabbage were watered every two days to maintain soil moisture near field capacity.

Table 4.1 Microorganisms used in this study

Microorganisms	**Metabolites produced**[a]	**Reference**
Pseudomonas		
CMR12a (WT)[b]	PHZ^+, $CLP1^+$, $CLP2^+$; phenazines, sessilins and orfamides	Perneel et al. (2007)
CMR12a-ΔPhz (P)	PHZ^-, $CLP1^+$, $CLP2^+$; sessilins and orfamides	D'aes et al. (2011)
CMR12a-Clp1 (S)	PHZ^+, $CLP1^-$, $CLP2^+$; phenazines and orfamides	D'aes et al. (2011)
CMR12a-ΔClp2 (O)	PHZ^+, $CLP1^+$, $CLP2^-$; phenazines and sessilins	D'aes et al. (2014)
CMR12a-ΔClp2-Clp1 (OS)	PHZ^+, $CLP1^-$, $CLP2^-$; phenazines	D'aes et al. (2014)
CMR12a-Δ*Phz*-Clp1 (PS)	PHZ^-, $CLP1^-$, $CLP2^+$; orfamides	D'aes et al. (2011)
CMR12a-Δ*Phz*-ΔClp2 (PO)	PHZ^-, $CLP1^+$, $CLP2^-$; sessilins	D'aes et al. (2014)
CMR12a-Δ*Phz*-ΔClp2-Clp1 (POS)	PHZ^-, $CLP1^-$, $CLP2^-$; null	D'aes et al. (2014)
R. solani		
AG 2-1 BK008-2-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)
AG 4-HGI CuLT-Rs36	Highly aggressive isolate of root rot disease on bean in Cuba	Nerey et al. (2010)

[a] PHZ: phenazines; CLP1: sessilins; CLP2: orfamides; plus+: metabolite is produced; minus−: metabolite is not produced. CLP production in CMR12a and mutants was analysed using liquid chromatography-eletrospray ionization-tandem mass spectrometry (LC-ESI-MS) analysis of cell-free culture supernatants (D'aes et al., 2014).

[b] Letters between brackets refer to codes used to indicate mutants in Figures 4.1 and 4.2

4.2.2 Chemicals

Orfamide B was purified following a protocol published by Ma et al. (2016a). Purified PCN was obtained as described by Perneel et al. (2008).

4.2.3 Bacterial application, pathogen inoculation and disease rating

For bean plants, *Pseudomonas* strains were grown for 24 h on King's B medium (KB; King et al., 1954), suspended in sterile saline (0.85% NaCl) and applied to substrate containing 50% potting soil and 50% sand (w/w) to obtain a final concentration of 10^7 CFU g^{-1} of growth substrate before sowing. *R. solani* AG 2-1 isolate BK008-2-1 inoculum was produced on sterile wheat kernels as described by Hua and Höfte (2015), except that AG 2-1 inoculated kernels were incubated for two weeks before use. Experiments were performed twice and every treatment consisted of eight trays with three seedlings each. Two infected kernels were placed between two 4-day-old cabbage seedlings, while control treatments were inoculated with sterile wheat kernels. Disease severity on roots and hypocotyls was recorded 14 days after inoculation by using the 0 – 4 rating scale: 0 = healthy, no symptoms; 1 = small black or brown lesions less than 1 mm in diameter; 2 = lesion covering less than 75% of the stem or root surface; 3 = lesion covering more than 75% of the stem or root surface; and 4 = seedling dead.

Pseudomonas strains were produced as described in the legend of Figure 4.1 and applied to substrate containing 50% potting soil and 50% sand (w/w) to obtain a final concentration of 10^6 CFU g^{-1} of growth substrate before sowing. Experiments were performed twice and every treatment consisted of three trays with 10 seedlings each. Bean seedlings were inoculated three days after sowing by placing a row of 40 kernels fully covered by hyphae of *R. solani* AG 4-HGI isolate CuLT-Rs36 in each tray as described by Hua and Höfte (2015). Disease severity on roots and hypocotyls was recorded six days after inoculation of *R. solani* AG 4-HGI isolate CuLT-Rs36 using the 0 – 4 rating scale described in the legend of Figure 4.1.

4.2.4 Root colonization assay

Roots from plants were randomly chosen, collected and rinsed carefully with sterilized water. Roots were ground with a mortar and pestle in sterilized saline (0.85 % sodium chloride, w/v), and the bacterial suspension was serial diluted and plated on KB plates. Bacterial enumeration were conducted by counting the bacteria showing same morphorlogy as original inoculants.

4.2.5 Microscopic observation

Sterile microscopic glass slides were covered with a thin, flat layer of water agar (Bacto agar; Difco) and placed in a plastic Petri dish containing a moist sterile filter paper. An agar plug (diameter = 5 mm) taken from an actively growing colony of *R. solani* was inoculated at the center of each glass slide. Two droplets (15 µl each) of bacterial suspension (10^6 CFU ml^{-1}), alone or in combination with 10 µM orfamide B (50/50; v/v), or orfamide B (10 and 100 µM) or PCN (100 nM and 1 µM) alone were placed at two sides of the glass slide (about 2 cm from the fungal plug). All plates were incubated for 36 h at 28°C before evaluation under an Olympus BX51 microscope.

4.3 Results and discussion

4.3.1 Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants

No growth inhibition effects or disease symptoms were observed on bean and cabbage seedlings upon bacteria application, indicating that *Pseudomonas* sp. CMR12a and its mutants were not phytotoxic or pathogenic to bean and cabbage seedlings (data not shown).

In two independent experiments, damping-off disease on Chinese cabbage caused by *R. solani* AG 2-1, was significantly suppressed when the growth substrate was mixed with wild-type strain CMR12a (WT) or mutants (see Table 4.1 for details) that still produced phenazines [CMR12a-Clp1 (S), CMR12a-ΔClp2 (O) and CMR12a-ΔClp2-Clp1 (OS)] or both CLPs [CMR12a-Δ*Phz* (P)] (Figure 4.1). When mutants that only produced orfamides [CMR12a-Δ*Phz*-Clp1 (PS)] or sessilins [CMR12a-Δ*Phz*-ΔClp2 (PO)] were applied, no biocontrol effect was observed. Likewise, a mutant unable to produce phenazines and CLPs [CMR12a-Δ*Phz*-ΔClp2-Clp1 (POS)] lost its biocontrol

activity. This suggests that phenazines alone can effectively control *Rhizoctonia* damping-off, whereas in the absence of phenazines, orfamides and sessilins are needed together to provide disease control.

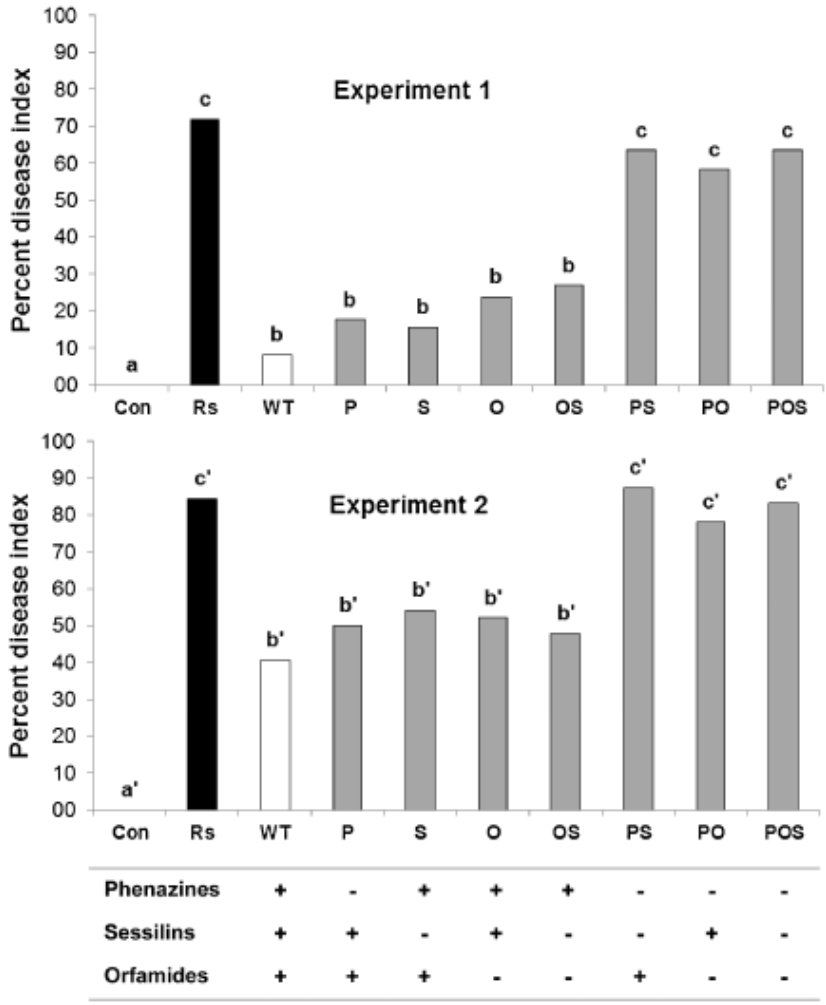


Figure 4.1 Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production towards *Rhizoctonia* damping-off on Chinese cabbage in two independent experiments. Con: healthy control; WT: wild-type *Pseudomonas* sp. CMR12a; Rs: *R. solani* AG 2-1; P: phenazine mutant; S: sessilin mutant; O: orfamide mutant. Mutant abbreviations are further explained in Table 4.1. The table below the figure indicates metabolites that are still produced by the various mutants.

Data are expressed as percent disease index, and bars indicated with the same letters are not statistically different based on the Kruskal–Wallis and Mann–Whitney non-parametric tests ($P = 0.05$).

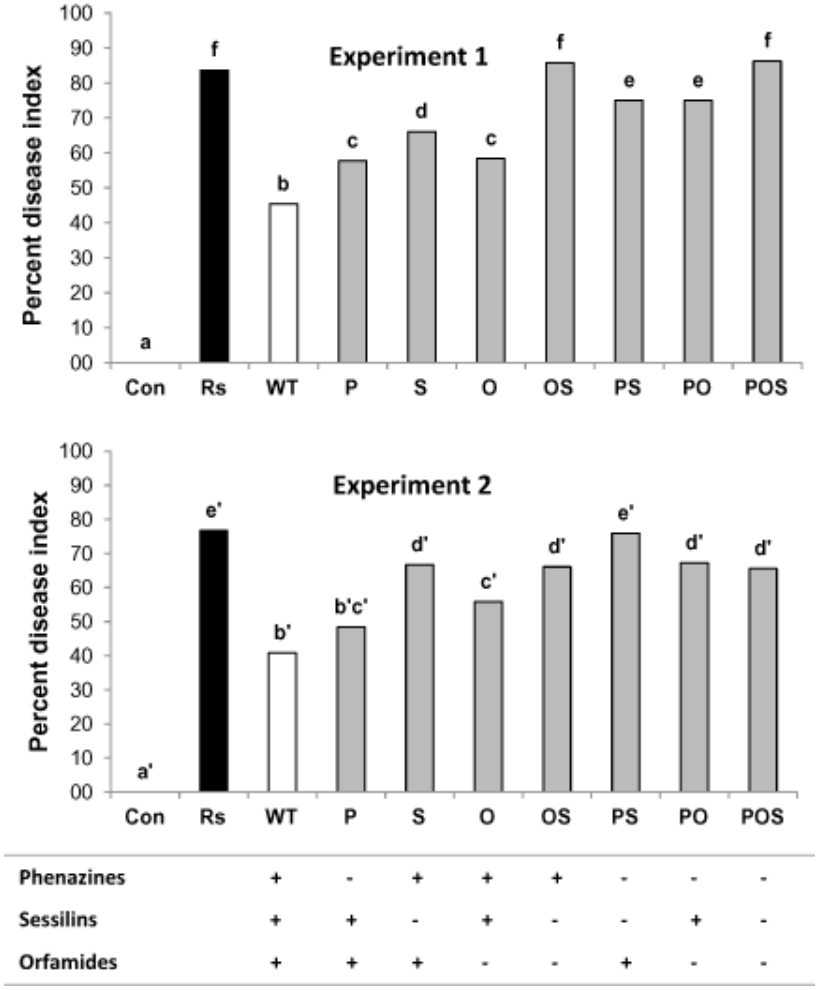


Figure 4.2 Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production towards *Rhizoctonia* root rot on bean in two independent experiments. Con: healthy control; WT: wild-type *Pseudomonas* sp. CMR12a; Rs: *R. solani* AG 4-HGI; P: phenazine mutant; S: sessilin mutant; O: orfamide mutant. Mutant abbreviations are further explained in Table 4.1. The table below the figure indicates metabolites that are still produced by the various mutants.

Data are expressed as percent disease index, and bars indicated with the same letters are not statistically different based on the Kruskal–Wallis and Mann–Whitney non-parametric tests ($P = 0.05$).

Root rot disease of bean, caused by *R. solani* AG 4-HGI, was significantly reduced when the growth substrate was mixed with wild-type strain CMR12a or mutants that either produce both CLPs (P) or phenazines together with one of the CLPs (O or S) (Figure 4.2). These mutants were in general less effective than the wild-type strain. In two independent experiments, biocontrol activity was stronger for mutants that produced both CLPs (P) or phenazines in combination with sessilins (O) than for

mutants which produced phenazines together with orfamides (S). On the other hand, mutants that only produced phenazines (OS) or one class of CLPs (PS, PO) showed a drastically reduced biocontrol activity.

Unlike in the cabbage pathosystem, phenazines alone were not sufficient but needed to interact with sessilins and orfamides for effective control of bean root rot disease. Results of the bean experiment are in line with previous reports on the importance of phenazines and sessilins in biocontrol of the bean root rot disease (D'aes et al., 2011; Hua and Höfte, 2015). Besides, phenazines and rhamnolipids (glycolipid type biosurfactants) produced by *P. aeruginosa* PNA1, acted synergistically in the biocontrol of *Pythium* diseases on bean and cocoyam (Perneel et al., 2008). Difference in the response of AG 4-HGI and AG 2-1 to the CMR12a mutant which only produces phenazines (OS) could be due to differences in plant root system, root exudates, or fungal sensitivity to phenazines. However, in both systems, sessilins and orfamides need to work in tandem for successful biocontrol in the absence of phenazines. In the conditions tested, these CLPs may not be synthesized in sufficient amounts to be effective on their own. Another plausible explanation for the additive effect demonstrated by sessilins and orfamides in the biocontrol capacity of CMR12a could be linked to their physiological functions which include biofilm formation and swarming motility, respectively (D'aes et al., 2014).

Bacterial strains were well established in the rhizosphere of bean and cabbage plants but differed in their ability to colonize roots (Tables 4.2 and 4.3). In the bean system, variation was observed among treatments and repetitions in time (Table 4.2). In the cabbage system, root colonization had to be tested in separate experiments with sand since roots of 14-day old cabbage seedlings are very delicate and could not be removed intact from the potting soil/sand substrate used in the biocontrol experiments. In this system, root colonization was highest for mutants that no longer produced orfamides (Table 4.3), which could be attributed to the role of sessilins in biofilm formation although we do not observe a similar scenario with the bean system (Table 4.2). Besides, the variability recorded in bacterial populations between plant systems could be driven by differences in substrates used. However, these variable bacterial populations were in the range of that necessary to obtain optimal biological control (Raaijmakers et al., 1999; Haas and Défago, 2005; D'aes et al., 2011) and did not influence disease suppressiveness by the strains.

Table 4.2 Root colonization data of *Pseudomonas* CMR12a and mutants impaired in phenazine and/or CLP production obtained from roots of bean seedlings seven days after inoculation with *R. solani* AG 4-HGI.

Treatment	Phenazines and CLPs produced	Population density of CMR12a and mutants[a] (in log CFU g^{-1} of fresh root)	
		Repetition 1	Repetition 2
CMR12a	Phenazines, sessilins and orfamides	6.5 ± 0.2 c	6.1 ± 0.3 ab
CMR12a-Δ*Phz*	Sessilins and orfamides	6.3 ± 0.3 bc	6.3 ± 0.2 b
CMR12a-Clp1	Phenazines and orfamides	6.4 ± 0.3 bc	5.8 ± 0.3 a
CMR12a-ΔClp2	Phenazines and sessilins	6.5 ± 0.1 c	6.1 ± 0.3 ab
CMR12a-ΔClp2-Clp1	Phenazines	5.9 ± 0.3 a	6.2 ± 0.3 ab
CMR12a-Δ*Phz*-Clp1	Orfamides	6.1 ± 0.2 ab	6.4 ± 0.4 b
CMR12a-Δ*Phz*-ΔClp2	Sessilins	6.2 ± 0.2 b	5.9 ± 0.3 a
CMR12a-Δ*Phz*-ΔClp2-Clp1	Null	6.2 ± 0.2 b	6.1 ± 0.4 ab

[a] Root colonization capacity of *Pseudomonas* strains was determined for five plants randomly selected from three replicate boxes per treatment. Experiment was performed twice. Data of the two experiments were log10 transformed before statistical analysis. Within each column, values followed by the same letter are not significantly different according to the Tukey tests ($P = 0.05$).

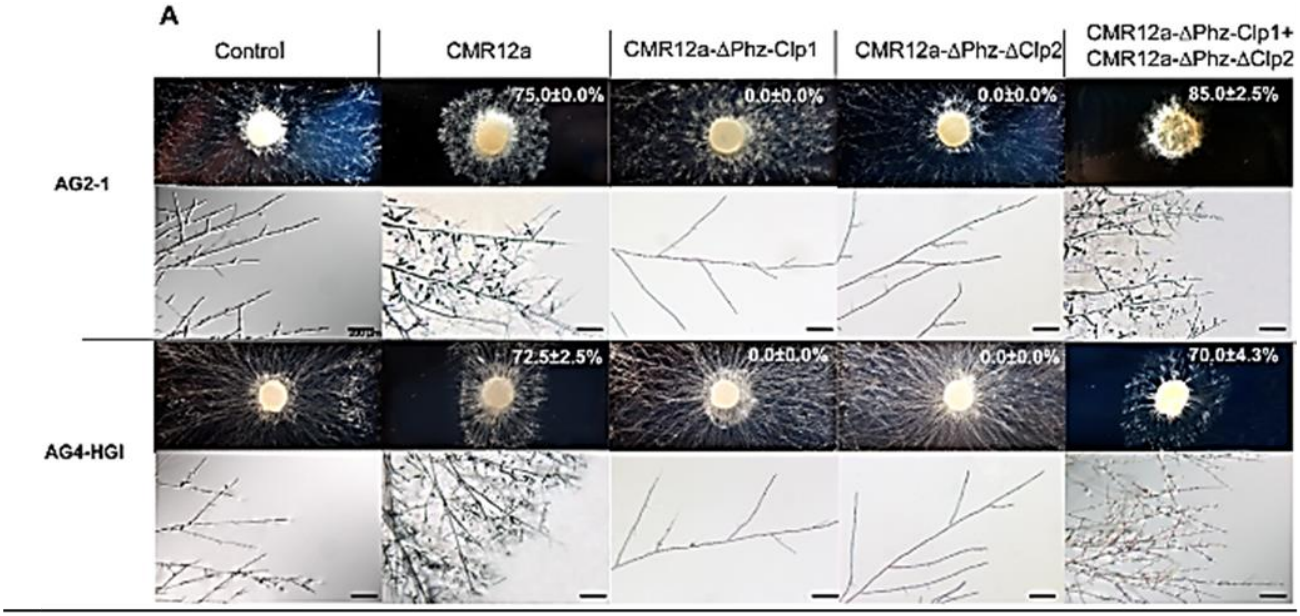
Table 4.3 Colonization capacity of *Pseudomonas* sp. CMR12a and mutants impaired in phenazine and/or CLP production on roots of Chinese cabbage.

Treatment	Phenazines and CLPs produced	Population density of CMR12a and mutants (in log CFU g^{-1} of fresh root)[a]	
CMR12a	Phenazines, sessilin and orfamide	6.8 ± 0.3 a	8.3 ± 0.1 a
CMR12a-Δ*Phz*	Sessilin and orfamide	6.7 ± 0.4 a	8.3 ± 0.3 a
CMR12a-Clp1	Phenazines and orfamide	6.9 ± 0.2 a	8.3 ± 0.2 a
CMR12a-ΔClp2	Phenazines and sessilin	7.2 ± 0.1 bc	8.9 ± 0.3 c
CMR12a-ΔClp2-Clp1	Phenazines	7.3 ± 0.1 c	8.8 ± 0.2 c
CMR12a-Δ*Phz*-Clp1	Orfamide	6.8 ± 0.1 a	8.4 ± 0.3 a
CMR12a-Δ*Phz*-ΔClp2	Sessilin	7.3 ± 0.1 c	8.9 ± 0.3 c
CMR12a-Δ*Phz*-ΔClp2-Clp1	Null	7.1 ± 0.2 b	8.6 ± 0.3 b

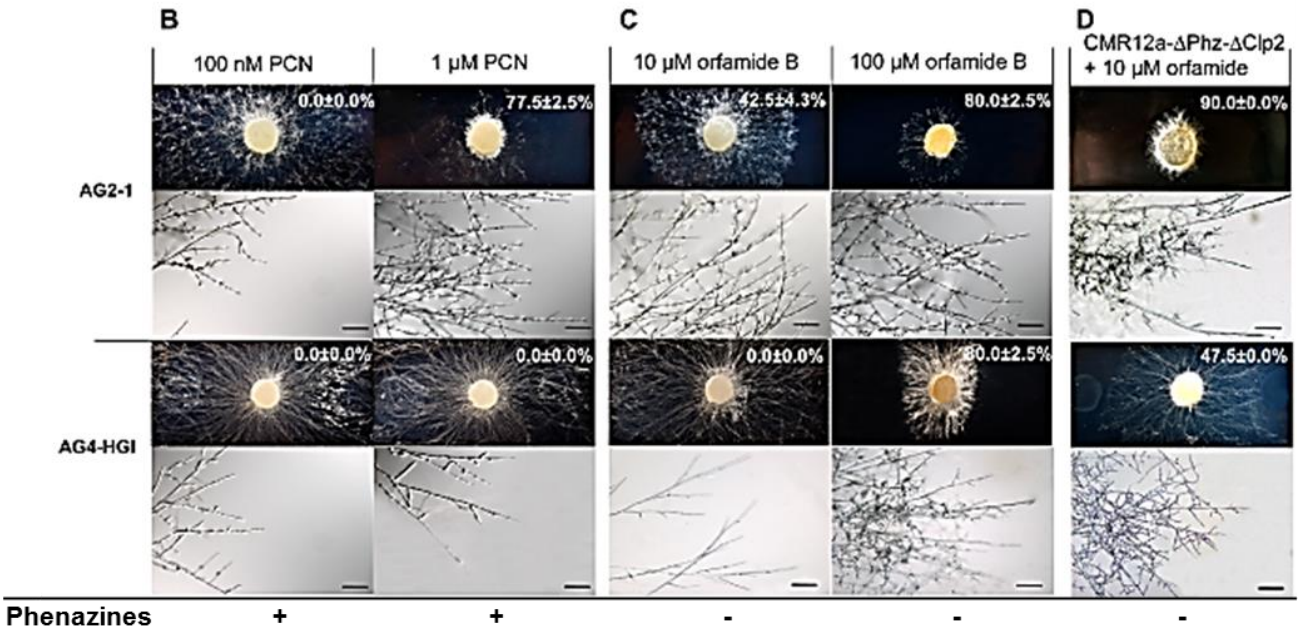
[a] Sets of five pre-germinated Chinese cabbage seeds were incubated for 30 min in bacterial suspensions ($OD_{620} = 1.0$) prepared from overnight cultures of CMR12a and its mutants on KB agar plates. Seeds were sown in sand, which was autoclaved twice on two successive days. Cabbage seedlings were kept in a growth chamber at 22 °C and RH = 60%, with a 12 h photoperiod and fertilized every three days with Hoagland solution (Hoagland and Arnon, 1950). Eighteen-day old plants were uprooted and bacterial populations present on their roots were determined as described by Hua and Höfte (2015). The entire experiment was done twice and data from both experiments are shown. Data were log10 transformed before analysis. Within each column, values followed by the same letter are not significantly different according to the Tukey tests (n = 5, $P = 0.05$).

4.3.2 *In vitro* antagonistic activity of *Pseudomonas* sp. CMR12a and mutants against *R. solani*

Since our plant experiments suggested an additive interaction of two (sessilins and orfamides/sessilins and phenazines/orfamides and phenazines) or all three metabolites in order to obtain successful biocontrol by CMR12a, we wanted to test this interaction under *in vitro* conditions. The antagonism of *Pseudomonas* sp. CMR12a and its mutants towards *R. solani* isolates AG 4-HGI and AG 2-1 was studied using microscopic glass slides covered with a thin film of water agar as previously described (D'aes et al., 2011). Application of CMR12a strongly inhibited the growth of *R. solani* AG 2-1 and AG 4-HGI, resulting in a remarkable increase in branch frequency as compared with that of the negative control (Figure 4.3A). No inhibition zones were formed between fungus and bacteria when mutants that only produced orfamides (CMR12a-Δ*Phz*-Clp1) or sessilins (CMR12a-Δ*Phz*-ΔClp2) were applied. On the other hand, growth inhibition comparable to the wild-type strain was obtained when the two mutants were concurrently inoculated on the glass slide (Figure 4.3A).



Phenazines	-	+	-	-	-
Sessilins	-	+	-	+	+
Orfamides	-	+	+	-	+



Phenazines	+	+	-	-	-
Sessilins	-	-	-	-	+
Orfamides	-	-	+	+	+

Figure 4.3 Microscopic assays showing the effect of various treatments on mycelial growth and hyphal branching of *R. solani* AG 2-1 and *R. solani* AG 4-HGI. A. Effect of *Pseudomonas* sp. CMR12a or mutants that only produce sessilins (CMR12a-Δ*Phz*-ΔClp2) or orfamides (CMR12a-Δ*Phz*-Clp1), alone or in combination. B. Effect of 100 nM or 1 µM purified PCN. C. Effect of 10 or 100 µM purified orfamide B. D. Effect of complementing *Pseudomonas* CMR12a-Δ*Phz*-ΔClp2 with 10 µM purified orfamide B.

The size bar represents 200 µm in all panels. Values indicate percentage growth inhibition relative to the control ± the standard deviation (n = 3). Images were processed using Photoshop CC (Adobe). Growth inhibition effect of bacteria and pure compounds towards *R. solani* was expressed relative to the mycelial growth in the control. Three replicates of each slide were used and the bioassay was done twice. Representative photos are shown.

4.3.3 *In vitro* antagonistic activity of phenazine-1-carboxamide and orfamide B against *R. solani* AG 2-1 and AG 4-HGI

Subsequently, we tested the bioactivity of various concentrations of pure orfamide B and PCN towards *R. solani* AG 2-1 and AG 4-HGI using the same set-up. Unfortunately, we were unable to obtain sufficient amounts of pure sessilin, so this metabolite could not be included in the assays. In tests with *R. solani* AG 2-1, a PCN concentration of 100 nM was not effective, while 1 µM strongly inhibited mycelial growth (Figure 4.3B). Higher doses of PCN did not give an enhanced pathogen inhibition (data not shown). PCN at 1 µM was not effective against *R. solani* AG 4-HGI (Figure 4.3B), and even concentrations up to 100 µM were ineffective (data not shown), indicating that this anastomosis group is insensitive to PCN when applied alone. It has been reported before that anastomosis groups of *R. solani* can differ in their sensitivity to antifungal compounds such as gliotoxin produced by *Gliocladium virens* (Jones and Pettit, 1987) or fungicides such as pencycuron (Kataria et al., 1991). *Pseudomonas* sp. CMR12a produces about 140 µM PCN/OD_{620} in still Luria–Bertani broth. Since an OD_{620} of 1 corresponds to about 8×10^8 CFU ml^{-1}, 1 µM PCN (= 224 ng PCN ml^{-1}) is a physiologically relevant concentration that could theoretically be produced by a population of 10^6 to 10^7 CFU g^{-1} root. Mavrodi and colleagues (2012) reported values for PCA concentrations recovered from the rhizosphere of wheat in the same range. Our *in vitro* results may explain why the CMR12a mutant that only produced phenazines was as effective as the wild-type strain in controlling damping-off disease in Chinese cabbage caused by *R. solani* AG 2-1, whereas this mutant was strongly impaired in biocontrol efficacy against root rot caused by *R. solani* AG 4-HGI in the bean pathosystem.

In vitro assays with *R. solani* AG 2-1 using orfamide B concentrations of 10 and 100 µM gave increased hyphal branching and clear inhibition zones in a dose-dependent way (Figure 4.3C).

However, 10 µM orfamide B was not effective against *R. solani* AG 4-HGI. Growth inhibition and increased hyphal branching were only obtained with orfamide B at 100 µM (Figure 4.3C). In contrast to our results, Gross and colleagues (2007) found that pure orfamide A at 100 µM did not inhibit mycelial growth of *R. solani* AG 4 (subgroup not specified) on agar surfaces although orfamide A was shown to lyse zoospores of *Phytophthora* spp. with increasing concentration. This discrepancy could be due to differences in experimental set-up and the pathogen used, although it cannot be excluded that the structural difference between orfamide A and orfamide B influences biological activity. This remains to be tested further. Anyhow, the efficacy of orfamide B appears to be dose dependent, which could account for its inability to be independently effective when produced by a sessilin- and phenazine-defective mutant. Mycelial growth inhibition and hyphal branching could partly (for AG 4-HGI) or completely (for AG 2-1) be restored by adding 10 µM orfamide B to the mutant that only produced sessilin (CMR12A-Δ*Phz*-ΔClp2) (Figure 4.3D). Collectively, these results suggest an additive action between sessilins and orfamides in the biocontrol of *R. solani*.

4.4 Conclusions

Previous *in vivo* biocontrol experiments have shown the role of singular CLPs produced by strains of the *Pseudomonas* genus namely sclerosin, massetolide, viscosinamide and sessilin (Olorunleke et al., 2015). However, the role and interaction of two CLPs produced by a single *Pseudomonas* strain is to our knowledge, not yet reported. This work provides the first demonstration of interplay between two classes of CLPs, sessilins and orfamides together with phenazines, in biocontrol. In cabbage, phenazines alone are sufficient to suppress *Rhizoctonia* damping-off, whereas the co-production of sessilins and orfamides is required for successful disease control in the absence of phenazines. In contrast, the co-production of phenazines, sessilins and orfamides appears to be important for effective suppression of *Rhizoctonia* root rot of bean. This can at least in part be explained by the fact that *R. solani* AG 2-1 is very sensitive to PCN, while this compound does not affect *R. solani* AG 4-HGI when present alone. These data show that in addition to phenazines and sessilins (D'aes et al., 2011), also orfamides play a role in the biocontrol capacity of CMR12a. When applied at high concentration, pure orfamide B significantly inhibited *R. solani* AG 4-HGI and AG 2-1, suggesting

that the inhibitory effect of this CLP could be dose dependent and that on the plant root, concentrations of orfamide B are too low to be active alone. These results contribute to our knowledge about the interaction of several antibiotic metabolites in a *Pseudomonas* strain and how they contribute to the biocontrol potential of the producing organism.

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Chapter 5. Role of phenazines and cyclic lipopeptides produced by *Pseudomonas* sp. CMR12a in induced systemic resistance on rice and bean

List of contributors

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Running title: *Pseudomonas* sp. CMR12a elicits innate immunity in rice and bean

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Abstract

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 *M. oryzae* on rice and to the web blight pathogen *R. 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 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.

Keywords: *Magnaporthe oryzae*, orfamide, *Oryza sativa*, *Phaseolus vulgaris*, phenazine-1-carboxamide, *Rhizoctonia solani* AG2-2, sessilin

5.1 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 belongs to 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 PCA and PCN, and two different classes of CLPs. The strain produces orfamide-type CLPs, which are commonly produced by *P. protegens* strains (Ma et al., 2016a), 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 synthetase (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 *R. 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 areas 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).

5.2 Materials and methods

5.2.1 Strains, plant materials, and their growth conditions

Pseudomonas strains and fungi used in this study are shown in Table 5.1. *Pseudomonas* bacteria were grown on KB plates, while *R. solani* and *M. oryzae* were maintained on PDA and CM plates, respectively. All the microbes were routinely incubated at 28 ºC. Bean cv. Prelude (Het Vlaams Zaadhuis, Waarschoot, Belgium) and rice CO-39 were routinely used as plant material in this study. The growth conditions of bean plants were exactly the same as shown in Chapter 4 (see Material and methods part). Rice was grown in the greenhouse under the condition described in Chapter 3 (see materials and methods part).

Table 5.1 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. l	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 Figure 5.1A and 5.2A.

5.2.2 Chemicals

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. 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., 2016a). Crude extracts of sessilin-type CLPs from phenazines and orfamides deficient mutant *Pseudomonas* sp. CMR12a-Δ*Phz*-Clp2 were obtained as described previously (Nutkins et al., 1991).

5.2.3 ISR assay in rice and bean

For ISR assays in rice, *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. 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 leave of rice plants, as described previously (De Vleesschauwer et al., 2006).

Pseudomonas sp. CMR12a and its biosynthesis mutants were grown on solid KB medium for 24 h (Figure 5.2A, experiment 1) or grown in liquid KB medium on a rotary shaker (28 °C, 150 rpm) for 24 h (Figure 5.2A, 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 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. 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.

5.2.4 Root colonization assay

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. 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 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. 5. Data were log10 transformed prior to statistical analysis.

5.2.5 Quantification of CLPs and phenazines from *Pseudomonas* sp. CMR12a and its mutants

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 suspension 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 ε = 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.

5.3 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 (Figure 5.1A). 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 5.2). 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 (Figure 5.1A). 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 5.2). 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.

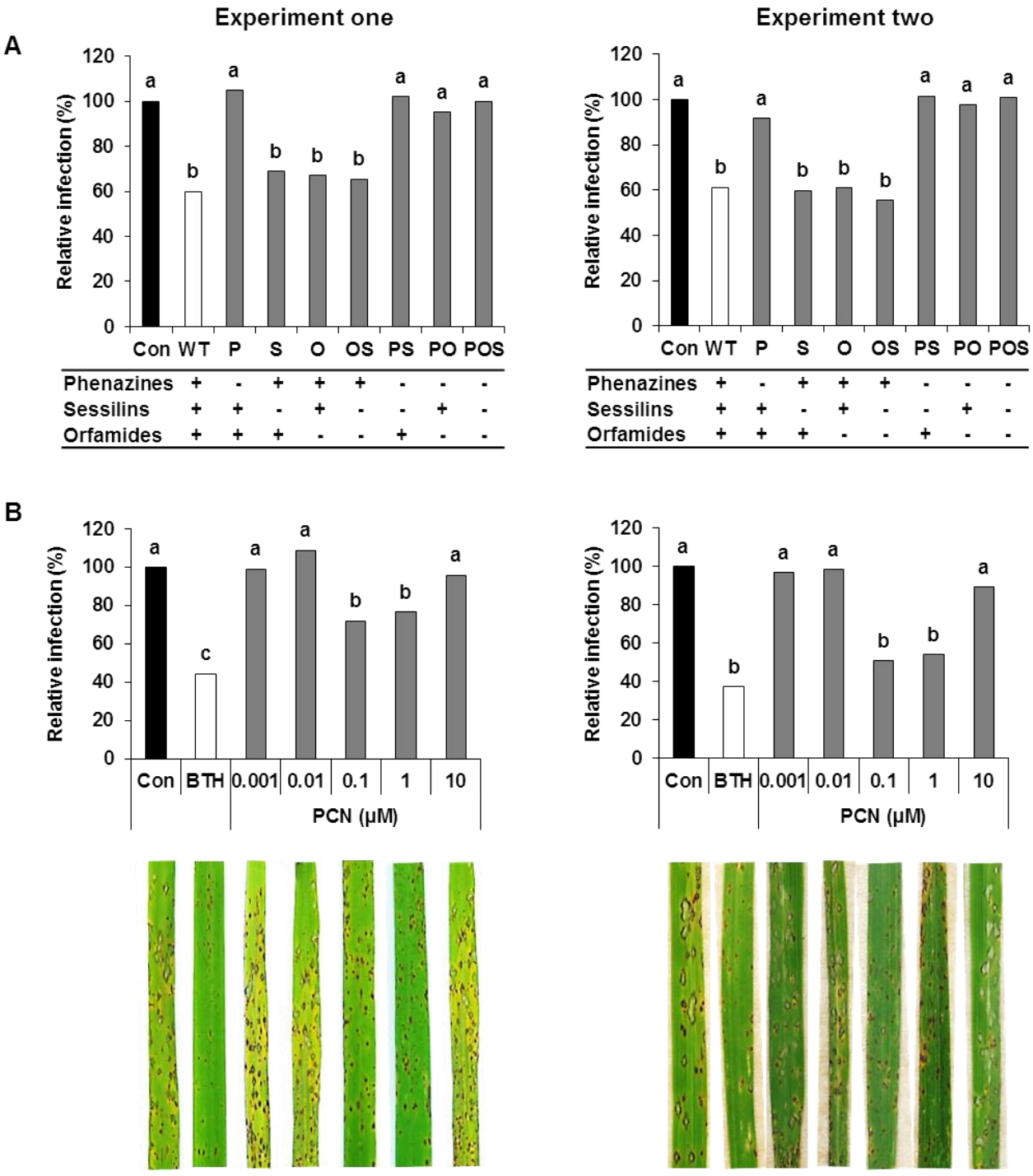


Figure 5.1 Effect of root colonization with *Pseudomonas* sp. CMR12a and mutants impaired in biosynthesis of phenazines and/or CLPs (A), and effect of soil drench with 25 µM BTH (S-methyl 1,2,3-benzothiadiazole-7-carbothioate) or different concentrations of PCN (B) on rice blast disease in *indica* cv. CO-39 plants. Results from two representative experiments are shown. Representative disease symptoms for different concentrations of PCN and 25 µM BTH treatments are shown in Figure 5.1B. 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

5.1. The table below Figure 5.1A shows the metabolites that are produced by *Pseudomonas* sp. CMR12a and its biosynthesis mutants.

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).

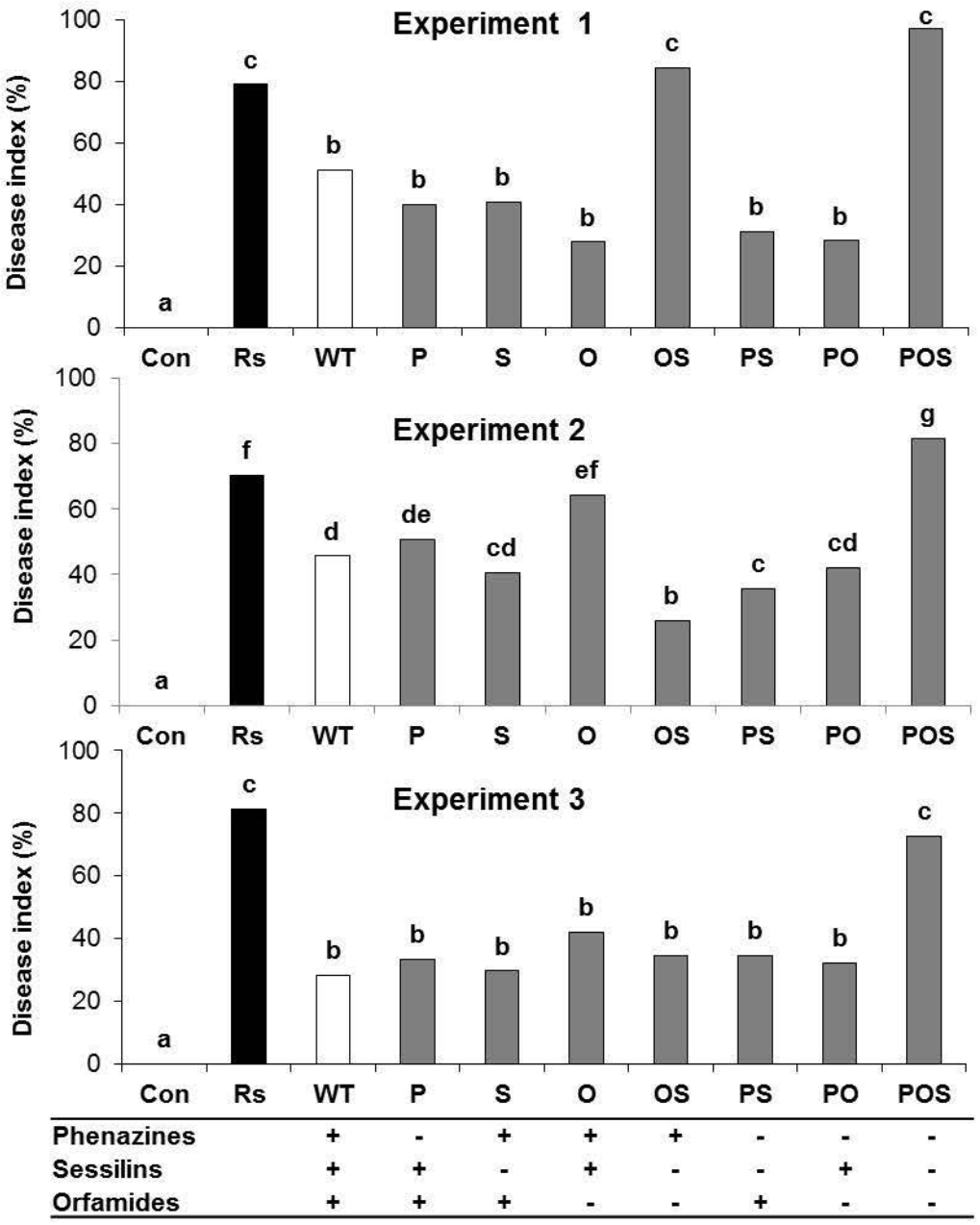
Subsequently, we applied various concentrations of pure PCN, the major phenazine compound produced by *Pseudomonas* sp. CMR12a (Olorunleke et al., 2015b), as a soil drench. The systemic resistance inducer BTH (S-methyl 1,2,3-benzothiadiazole-7-carbothioate) was used as a positive control. In two independent experiments, PCN at a concentration of 0.1 or 1 μM offered protection against *M. oryzae*, while lower or higher concentrations were not effective (Figure 5.1B).

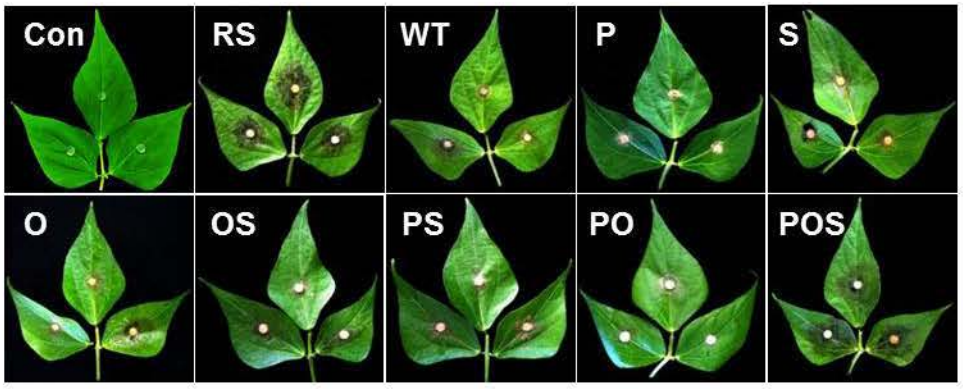
Table 5.2 Population of *Pseudomonas* sp. CMR12a and its biosynthesis mutants on rice roots.

Pseudomonas strains	Metabolites produced	Bacterial population[a] (log CFU g^{-1} fresh root)	
		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] Values followed by the same letter are not significantly different based on Kruskal-Wallis and Mann-Whitney tests ($P = 0.05$).

A


Experiment 1
Disease index (%)
100
80
60
40
20
0
a
c
b
b
b
b
c
b
b
c
Con
Rs
WT
P
S
O
OS
PS
PO
POS
Experiment 2
Disease index (%)
a
f
d
de
cd
ef
b
c
cd
g
Experiment 3
Disease index (%)
a
c
b
b
b
b
b
b
b
c
Phenazines + - + + + - - -
Sessilins + + - + - - + -
Orfamides + + + - - + - -


Con
RS
WT
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S
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OS
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PO
POS

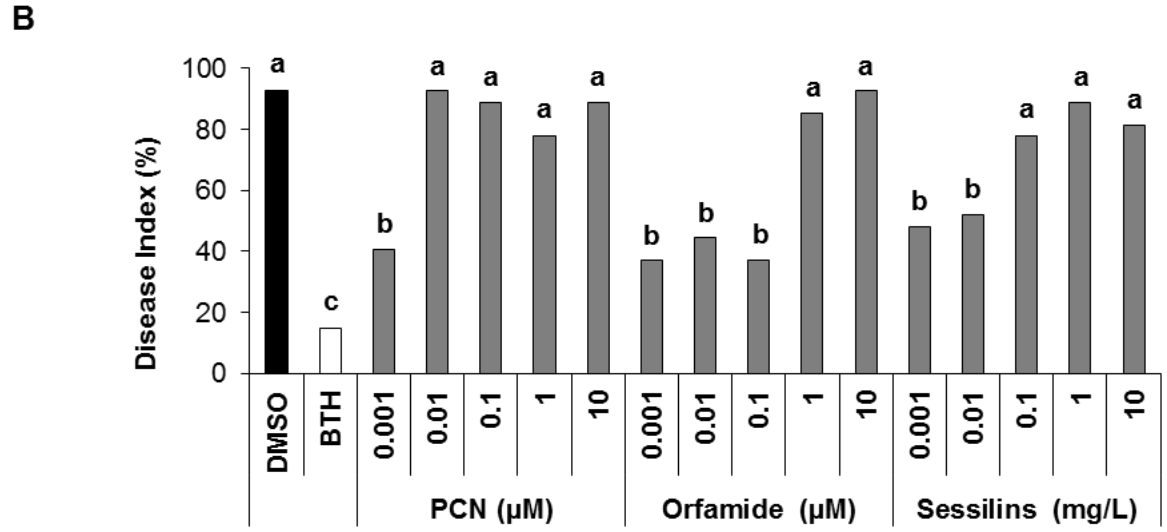

Figure 5.2 Effect of *Pseudomonas* sp. CMR12a and mutants impaired in phenazines and/or CLPs (A), and different concentrations of PCN, orfamide B and sessilins (B) on resistance to web blight caused by *R. solani* AG2-2 on bean. Representative symptoms in experiment 3 are shown in the pictures of Figure 5.2A. 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 5.1. The table in panel A shows the metabolites that are produced by *Pseudomonas* sp. CMR12a and its biosynthesis mutants.

Five trifoliate leaves were used in each treatment and the experiment was repeated three times (Figure 5.2A). Three trifoliate leaves were used for the experiment depicted in Figure 5.2B 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, $p = 0.05$).

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 Figure 5.2A, *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 (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.

Table 5.3 Population of *Pseudomonas* sp. CMR12a and its biosynthesis mutants on roots of bean seedlings.

Pseudomonas strains	Metabolites produced	Bacterial population (log CFU g^{-1} fresh root)[a]			
		Experiment 1	Experiment 2	Experiment 3	
		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] 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).

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 5.3). Although low bacterial counts were found in bean stems, no bacteria were isolated from the primary leaves (Table 5.3). 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.

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 (Figure 5.2B). 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 and caused necrosis on leaves in control treatment (data not shown).

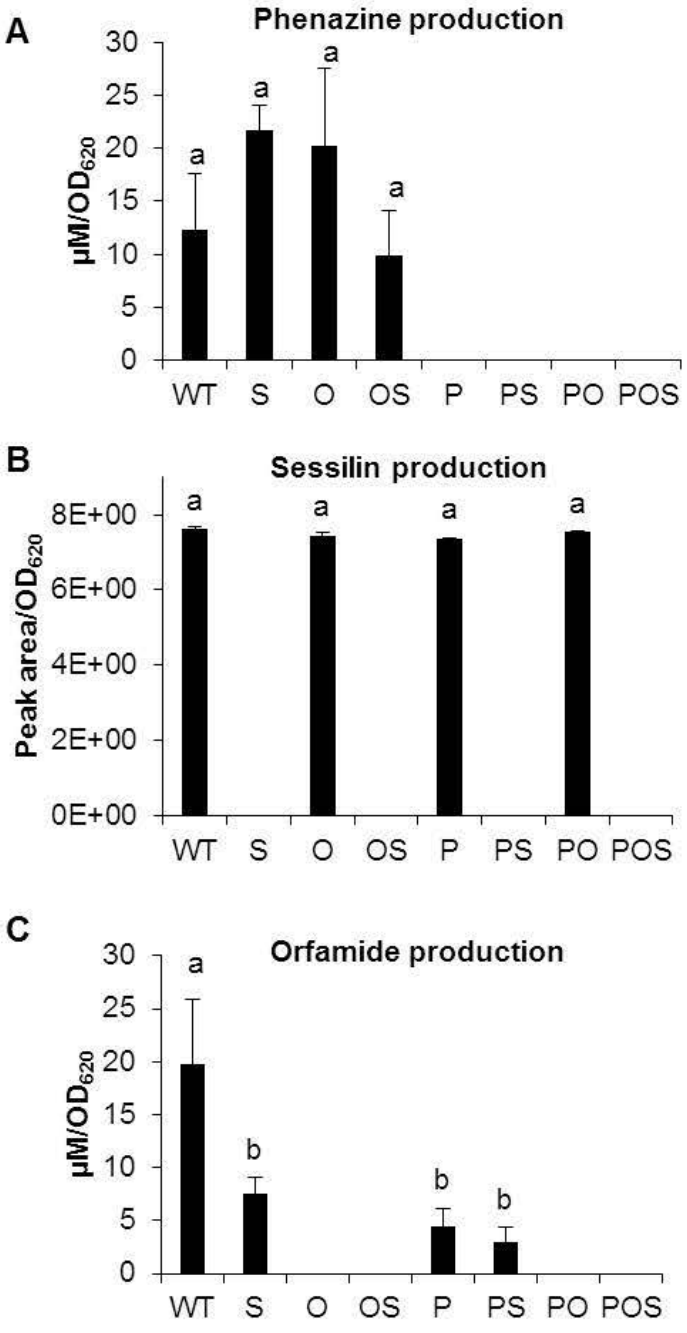

Figure 5.3 Quantification of phenazine (A), sessilin (B) and orfamide (C) produced by *Pseudomonas* sp. CMR12a and its biosynthesis mutants on solid King's B (KB) plates. Different letters indicate significant differences among treatments (Tukey HSD test, $p = 0.05$). Vertical bars indicate the standard deviation (n = 3).

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 (Figure 5.3A). Mutants impaired in orfamide production (O) and/or phenazine production (PO and P) produced the same amount of sessilin as the WT (Figure 5.3B). 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 (Figure 5.3C). Since an OD_{620} of 1 corresponds to about 10^9 CFU/mL it can be deduced from Figure 5.3 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 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 et al., 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 (Figure 5.2B). 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 variable results in the plant assays (Figure 5.2A). We assume that the phenazine concentration in experiment 1, in which the inoculum 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. Massetolide A eliciting enhanced resistance of *Phytophthora infestans* in tomato is the only reported case (Tran et al., 2007). This study clearly shows that also sessilin and orfamide-type CLPs can trigger ISR, however, the mechanisms involved remain to be 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* (See Chapter 6). 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.

5.4 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.

Chapter 6. Orfamides induce systemic resistance in rice to *Cochliobolus miyabeanus* but not to *Magnaporthe oryzae*

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Abstract

Diverse natural products produced by beneficial *Pseudomonas* species have the potential to trigger induced systemic resistance (ISR) in plants and thus may contribute to control of diseases in crops. *Pseudomonas*-derived cyclic lipopeptides (CLPs) are classes of secondary metabolites possessing both hydrophobic and hydrophilic properties and have versatile biological functions. However, the capacity of *Pseudomonas*-derived CLPs in triggering ISR responses and manipulating of plant diseases is barely studied. *Pseudomonas protegens* and related species can produce orfamide-type CLPs. Here we show that orfamides can act as ISR elicitors against the necrotrophic fungus *Cochliobolus miyabeanus*, the causal agent of brown spot disease in rice, but is not active against the blast fungus *Magnaporthe oryzae*. Orfamide can trigger early events and activate transcripts of defense-related genes in rice cell cultures, but does not cause cell death. Further testing in rice cell cultures and rice plants showed that abscisic acid (ABA) signaling and pathogenesis related (PR) protein *PR1b* were involved in the ISR response triggered by orfamide in rice against *C. miyabeanus*.

Keywords *Pseudomonas protegens* CHA0, orfamide, induced systemic resistance, *Cochliobolus miyabeanus*, plant hormones, pathogenesis related proteins, plant-microbe interactions

6.1 Introduction

Induced systemic resistance (ISR) is a phenomenon that occurs following perception of non-host components in plants, and mounts a faster and stronger defensive response in the plant and subsequently delays or decreases the consequences of biotic or abiotic stresses in their host plants (Pieterse et al., 2014). ISR can commonly occur in plants by perception of elicitors secreted by microbes (or pathogens), similarly to microbe (or pathogen)-associated molecular patterns (MAMPs or PAMPs) (Newman et al., 2013). MAMPs or PAMPs are typically recognized by pattern recognition receptors associated with the cell membrane resulting in the elicitation of plant innate immunity (Trdá et al., 2015). Beneficial microbes in the rhizosphere are actively involved in improving plant health and dealing with multiple stresses (Berendsen et al., 2012; Baetz and Martinoia, 2014; De Coninck et al., 2015). ISR elicitors secreted by beneficial microbes in the rhizosphere can boost a set of plant defensive responses (for instance, activation of defense related enzymes and signaling of plant hormones, etc.) in their hosts before or after pathogen attack. This type of priming (Conrath, 2011) subsequently provides barriers for preventing further pathogen invasion and disease development, and thus reduce incidence and (or) severity of diseases in plants (Balmer et al., 2015).

Cyclic lipopeptides (CLPs) are composed of a peptidic backbone linked to a fatty acid residue. Bacterial CLPs are important secondary metabolites and play vital roles in bacterial motility, antibiosis against other organisms and adjustment of these bacteria to their living environment. *Pseudomonas* species are potent CLPs producers (Raaijmakers et al., 2010) and are frequently studied biocontrol agents (Höfte and Altier, 2010; Raaijmakers et al., 2010). In addition, certain CLPs are also important MAMPs molecules. These CLPs can enhance plant innate immunity and mainly include surfactin, fengycin and iturin, produced by Gram-positive endospore-forming *Bacillus* species (Ongena and Jacques, 2008; Farace et al., 2015). Their ISR capacity has been shown in several plants, such as 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). The only Pseudomonad CLP that has been shown to trigger innate immunity in plants is massetolide belonging to viscosin family (Tran et al., 2007). *P. protegens* has been developed as a

model for biocontrol research, and includes the well-studied strains *P. protegens* CHA0 and Pf-5. Moreover, *P. protegens* CHA0 shows ISR responses in grapevine plants (Verhagen et al., 2009) and *Arabidopsis* (Iavicoli et al., 2003). To date, the polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) is the only elicitor characterized from *P. protegens* that shows induced protection of *Arabidopsis thaliana* against *Hyaloperonospora arabidopsidis* (formerly called *Peronospora parasitica*) (Iavicoli et al., 2003) and *Pseudomonas syringae* pv. tomato (Weller et al., 2012). Interestingly, *P. protegens* (for instance *P. protegens* CHA0) and related strains produce orfamide-type CLPs (Ma et al., 2016a).

Orfamides are insecticidal agents (Jang et al., 2013), display activity on zoospores of *Phytophthora* and *Pythium* (Ma et al., 2016a, or see Chapter 3) and have antifungal activity against *R. solani* (Olorunleke et al., 2015). In addition, we recently found that orfamide B can trigger ISR in bean against the web blight pathogen *R. solani* (Ma et al., 2016b, or see Chapter 5). Although *P. protegens* and related species can produce orfamides, orfamide production within these producers shows diversity. Orfamide B is the main compound produced by *Pseudomonas* sp. CMR12a and CMR5c, while *P. protegens* CHA0 and Pf-5 mainly produce orfamide A (Ma et al., 2016a). The structural difference between orfamide B and orfamide A occurs in the substitution of one amino acid residue, but they both share the same fatty acid chain (Gross et al., 2007; Ma et al., 2016a). We were interested to know whether orfamides have the capacity to mount innate immunity on rice, a model plant for monocot plants, against the fungal pathogens *M. oryzae* and *C. miyabeanus*. Here we show that orfamide can act as elicitor in rice against the necrotrophic pathogen *C. miyabeanus* Cm988, but is not active against *M. oryzae*. Moreover, the defensive mechanisms underneath orfamide-induced disease resistance in rice against *C. miyabeanus* were further investigated by multiple approaches, including assays in rice cell cultures and rice plants. This study sheds new light on the disease control of rice pathogen *C. miyabeanus* by orfamide.

6.2 Materials and methods

6.2.1 Strains, media and culture conditions

Pseudomonas strains and plant pathogenic fungi used in this study are shown in Table 6.1. *Pseudomonas* strains were routinely maintained on Luria-Bertani (LB) medium (Sambrook and Fritsch, 1989), or on King's B (KB) medium (King et al., 1954). *Cochliobolus miyabeanus* Cm988 (De Vleesschauwer et al., 2010) was cultivated on potato dextrose agar (PDA; Difco) plate, while *M. oryzae* VT5M1 (Thuan et al., 2006) was maintained on complete medium (CM) (Talbot et al., 1993) plates. All microbes used in this study were routinely maintained at 28 °C.

Table 6.1 Microorganisms used in this study

Strain	Relevant characteristics	Reference
P. protegens		
CHA0	Wild type, orfamide producer, DAPG producer	Stutz et al., 1986
CHA5101	Orfamide biosynthesis mutant, DAPG producer	Flury et al., submitted
Pseudomonas sp.		
CMR12a	Wild type, produces orfamides, sessilins and phenazines	Perneel et al., 2007
CMR12aΔ*Phz*-Clp1	Phenazine and sessilin mutant, still produces orfamides	D'aes et al., 2014
Fungus		
M. oryzae VT5M1	Causal agent of blast disease on rice	Thuan et al., 2006
C. miyabeanus Cm988	Causal agent of brown spot disease on rice	De Vleesschauwer et al., 2010

6.2.2 Chemicals

Orfamide A was purified from KB supernatant of *P. protegens* CHA0 exactly following a protocol published previously (Ma et al., 2016a) and dissolved in dimethyl sulfoxide (DMSO) as stock solution, then diluted to desired concentrations for further bioassays. Unless stated otherwise, the same amount of DMSO was added to the control treatment in this study. Stock solution of silver thiosulfate (STS) was prepared as shown by Van Bockhaven et al. (2015), namely by mixing solutions of 0.1 M sodium thiosulfate and 0.1 M silver nitrate in a ratio of 4 : 1 (v/v). 3,3'-

Diaminobenzidine tetrahydrochloride hydrate (DAB) was purchased from SIGMA-ALDRICH. 1 mg/mL DAB was dissolved in water and the pH of the solution was adjusted to 3.8 by 0.2 M NaOH.

6.2.3 Plant material, cell cultures and growth conditions

Rice (*Oryza sativa* L.) *indica* cv. CO-39 was routinely used as plant material for bioassays. Rice seeds were surface sterilized with 1 % (w/v) sodium hypochlorite solution for 5 min and washed with sterilized water for 5 times. Surface sterilized seeds were germinated on moistened filter paper in Petri dishes (diameter = 9 cm) for 5 days, and then transplanted into plastic trays (23 × 16 × 6 cm, each for 12 plants) using potting soil (Structural; Snebbout, Kaprijke, Belgium) as substrate, as described previously (De Vleesschauwer et al., 2006). Rice plants were routinely maintained in greenhouse condition (photoperiod with 12 h light; 30 ± 4 °C).

Cell cultures of rice cultivar Kitaake (*Oryza sativa* subsp. *japonica*) were routinely maintained in modified amino acid (AA) medium (Baba et al., 1986) on a rotary shaker (120 rpm) at 25 °C. Rice cell cultures were sub-cultured every 7 days by transferring 10 mL cell cultures to 70 mL sterilized AA medium.

6.2.4 ISR assay and disease evaluation

Original *Pseudomonas* inoculum was prepared from KB plates after two days incubation, and inoculation of *Pseudomonas* strains to the rhizosphere of rice was exactly conducted as shown previously (De Vleesschauwer et al., 2008). More specifically, rice plants were treated with root inoculation of corresponding *Pseudomonas* bacteria (10^8 or 10^9 CFU/g) at the time of sowing, and the second bacteria inoculum was applied 3 days before pathogen inoculation. Solutions of purified compound were soil drenched 3 days before pathogen inoculation. For ISR assays with *C. miyabeanus* Cm988, the second youngest leaves of five-week old rice plants from different treatments were detached and layed on moistened filter paper in square plates (12 × 12 × 1.7 cm). Spores of *C. miyabeanus* Cm988 were prepared as published previously (Van Bockhaven et al., 2015). Spores of *C. miyabeanus* Cm988 were collected and suspended into 0.25 % (w/v) gelatin to a final concentration of 5×10^4 per milliliter, and droplets of 10-µL conidial suspension were carefully

spotted on the surface of detached leaves. Inoculated rice leaves were kept 24 h in dark condition, and subsequently the droplets containing spores of *C. miyabeanus* Cm988 were carefully removed by tissues from detached rice leaves. Then leaves were kept on laboratory bench at room temperature for disease development. Disease assessment was conducted 72 h post infection (hpi), by calculating the area of necrotic lesions with Assess 2.0 (American Phytopathological Society). ISR assays of *M. oryzae* were conducted exactly as shown previously (De Vleesschauwer et al., 2008). Sporulating cultures of *M. oryzae* VT5M1 were prepared as shown by Thuan et al. (2006). Five day-old CM plates of *M. oryzae* VT5M1 was exposed to blue light (12 h: 12 h, Philips TLD 18W/08: Philips TLD 18W/33) for 3 days to induce sporulation. Conidia were collected and suspended into 0.5 % (w/v) gelatin to a final concentration of 5×10^4 per milliliter. Each intact rice plant was evenly sprayed with 1 mL spore solution of *M. oryzae* VT5M1 by a compressor-powered airbrush gun. Infected rice plants were transferred into a humidity chamber (relative humidity ≥ 90 %; 25 ± 5 °C) and kept in dark condition for 24 h, and then transferred to greenhouse conditions for disease development. Disease evaluation was conducted by counting susceptible lesions (sporulating lesions with center of gray color) per 10 cm of the second youngest leaves of rice plants 6 days post infection (dpi), exactly as shown by De Vleesschauwer et al. (2008). Pictures of leaves showing representative disease symptoms were recorded at 7 dpi.

6.2.5 Ultra-high performance liquid chromatography mass spectrometry

Ultra-high performance liquid chromatography mass spectrometry (UPLC-MS) was conducted on a reversed-phase UPLC-MS system, as described previously (Ma et al., 2016a). Selected *Pseudomonas* strains used in this study were cultured on KB plates for 48 h and *Pseudomonas* cells were collected and suspended in 50 % (v/v) acetonitrile/H_2O. Supernatant was then collected by centrifugation of 10000 *g* for 5 min and submitted for UPLC-MS analysis. Standard curve (concentration versus peak area) of orfamide A was made by injection of different concentrations of orfamide A to the UPLC-MS machine and calculation of corresponding peak areas of orfamide. Quantification of orfamide concentration in *Pseudomonas* samples was conducted by UPLC-MS analysis, and its concentration was further determined by calculating peak area from the standard curve of orfamide A.

6.2.6 Root colonization assay

Root colonization of *Pseudomonas* strains was analyzed after detaching rice leaves for ISR assays. More specifically, 5 rice plants of each treatment were randomly selected, roots of each plant were carefully collected and rinsed carefully with sterilized water to remove extra soil surrounding roots. Bacterial suspensions were obtained by grinding the roots carefully by a mortar and pestle in 10 mL sterilized saline solution (0.85 % sodium chloride, *w*/*v*). Bacterial suspensions were then serially diluted in sterilized saline solution, evenly plated on KB medium and incubated for 48 h at 28 °C. Single colonies of *Pseudomonas* strains was monitored morphologically and enumerated, and the number of bacteria was expressed as Log CFU per gram of fresh roots.

6.2.7 Evaluation of extracellular alkalinization and cellular viability in rice cell cultures

Five-day old rice cell cultures were filtered, washed and resuspended in AA medium with final concentration of 10 g of fresh weight in 100 mL of medium. Cell cultures were divided into six-well micro-titer plates (8 mL cell cultures for each well) and kept at room temperature on a shaker (120 rpm) for 3 h to destress and afterwards used for further bioassays. Rice cell cultures were treated with different concentrations of orfamide and extracellular pH change of rice cell cultures was monitored by a pH meter at different time points.

Cell death was monitored by Evan blue staining with modifications according to Jourdan et al. (2009). Rice cell cultures were treated with different concentrations of orfamide and incubated for 24 h, afterwards Evan blue was added in cell cultures to the final concentration of 0.05 % (w/v), and the mixture was incubated for 15 min. Then, the mixture was washed for three times using deionized water to remove extra Evan blue. The mixture was subsequently suspended in 500 µL of 50 % (v/v) methanol/1 % (w/v) sodium dodecyl sulfate (SDS) solution and incubated at 55 °C for 30 min. The supernatant from each sample was collected by centrifugation (3000 g for 5 min) and recorded for optical density at 595 nm. 2 mM Triton X-100 was used as a positive control for measurements of cell viability (Jourdan et al., 2009).

6.2.8 Microscopic observation of defense responses

Microscopic visualization of defense responses was conducted as shown previously (Van Bockhaven et al., 2015). Leaves and sheaths of five-leaf stage rice plants were peeled off until the second youngest leave sheath and the roots were left, then the plants were placed horizontally on moistened tissues (Relative humidity ≥ 90 %; 25 ± 5 °C). The rice sheath was inoculated with a conidial suspension (5×10^4 per milliliter) of *C. miyabeanus* Cm988 by injection with a syringe (each sheath received 1 mL conidia suspension), and the specimens from the epidermal layer of rice sheaths were prepared after 24 h and 48 h post treatment and immediately subjected to microscopic visualization. Phenolic compounds were visualized as green autofluorescence under blue light epifluorescence using Olympus BX51 microscope (Olympus U-MWB2 GPF filter set; excitation, 450–480 nm; dichroic beam splitter, 500 nm; barrier filter BA515; Aartselaar, Belgium). Reactive oxygen species (ROS) detection in rice sheaths was conducted as previously described (De Vleesschauwer et al., 2010). Images of specimens were automatically captured by Olympus Colorview II camera and further processed by Olympus analySIS cell^F software.

6.2.9 RNA extraction, cDNA synthesis, and quantitative PCR analysis

Total RNA of each sample was extracted from frozen materials (rice leaf tissues or rice cell cultures) using TRIZOL reagent (Invitrogen), and subsequently treated with Turbo DNase kit (Ambion/Applied Biosystems) to remove extra DNA. First-strand cDNA was synthesized from 2 μg RNA using Multiscribe reverse transcriptase (Ambion/Applied Biosystems). Quantitative PCR (qPCR) amplifications were conducted in optical 96-well plates by an Mx3005P real-time PCR detection system (Stratagene). SybrGreen master mix (Fermentas) was used to monitor double-stranded DNA synthesis. The transcriptional analysis of selected gene was conducted independently in triplicate in a total volume of 25 μL. The thermal profile used for qPCR consists of an initial denaturation step at 95 °C for 10 min, following by 40 cycles at 95 °C for 30 s, then at 57 °C to 62 °C for 60 s, and 72 °C for 60 s. The amount of RNA in each sample was normalized using elongation factor *eEF1α* (for rice cell cultures) or *actin* (for plant materials) (Table 6.1) as an internal control, and samples collected from control treatments at 0 hpi (or DMSO treated rice cell cultures)

were selected as calibrator. The primers of targeted genes used for qPCR are listed in Table 6.2. The transcripts of selected genes were analyzed using the Mx3005P software (Stratagene).

Table 6.2 Primers used for quantitative real-time PCR in this study

Gene	Locus number	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
actin	Os03g0718100	GCGTGGACAAAGTTTTCAACCG	TCTGGTACCCTCATCAGGCATC	McElroy et al., 1990
eEF1a	Os03g0178000	GGCTGTTGGCGTCATCAAGA	CCGTGCACAAAACTACCACTT	Kidou and Ejiri, 1998
β-Glucanase	Os05g0495900	ATTCTCGCTTGCGGGTTCTT	CCAGCATGCCGTAGTTCACA	Zhu et al., 2007
PAL	Os02g0627100	CCCTGCCAATCTGCTGAACTA	GCCGCTATGCAACGAAGAAT	Zhu et al., 1995
Chitinase	Os03g0132900	ACGTCGTCATAAGCGGATTC	CGAACGCTCTCTGCTAGCTT	Chujo et al., 2014
OsNCED3	Os03g0645900	TACGGCTTCCACGGCACGTTC	AGAAACGTGGAGGTGTTCGATCG	Xu et al., 2013
OsRab16	Os01g50700.1	CACGAGTTCAGGGATCTAGGC	AGTTGTCCATCCTCTCAAGCAA	Xu et al., 2013
OsPR1a	OsAJ27843	GGCACGAGTCGATCTCCA	ACCAGCAAGCAGCAGGAT	Wang et al., 2015
OsPR1b	Os01g0382000	GGCAACTTCGTCGGACAGA	CCGTGGACCTGTTTACATTTT	De Vleesschauwer et al. 2010
PR3	OsD16221	GGCGTTCTGGTTCTGGATGAC	CGCCGTTGATGATGTTGGTC	Wang et al., 2015
PR5	OsU77657	AACTACCAGGTCGTCTTCTGC	GCGTGCGGGCTTATTCTTA	Wang et al., 2015
WRKY4	Os03g55164.2	CGATCCCAAGTGCGTCCTG	GGCACGGCTCATCAGCAAC	Wang et al., 2015
LOX2	Os08g0508800	CGATGGCCGGAACAAGGATA	TGGAGCGTTTTGTCTCATCA	De Vleesschauwer et al., 2016
EBP89	Os03g0182800	TGACGATCTTGCTGAACTGAA	CAATCCCACAAACTTTACACA	De Vleesschauwer et al. 2010
ACS1	Os03g0727600	TCGGCCAAGACCCTCGACG	CGAAAGGAATCTGCTACTGCTGC	Zarembinski and Theologis, 1997
IAA9	Os02g0805100	CAACGACCACCAAGGCGAGAAG	CCAGGCAACCAAAACCGAGCTG	Yang et al., 2005
GH3-8	Os07g0592600	AGTACAAGGTGCCACGCTGC	ACGAGGTGGGGATTTGACCGAC	Ding et al., 2008
JAMYB	Os11g0684000	TGGCGAAACGATGGAGATGG	CCTCGCCGTGATCAGAGATG	Lee et al., 2001
JiOsPR10	Os03g0300400	CGGACGCTTACAACTAAATCG	AAACAAAACCATTCTCCGACAG	Jwa et al., 2001
PBZ1/PR10b	Os12g0555500	CCCTGCCGAATACGCCTAA	CTCAAACGCCACGAGAATTTG	Han et al., 2004
NPR1	Os01g0194300	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACTAG	Chern et al., 2001
WRKY45	Os05g0322900	GGACGCAGCAATCGTCCGGG	CGGAAGTAGGCCTTTGGGTGC	Shimono et al., 2007

6.3 Results

6.3.1 Orfamide induces resistance to *C. miyabeanus* Cm988, but not against *M. oryzae* in rice

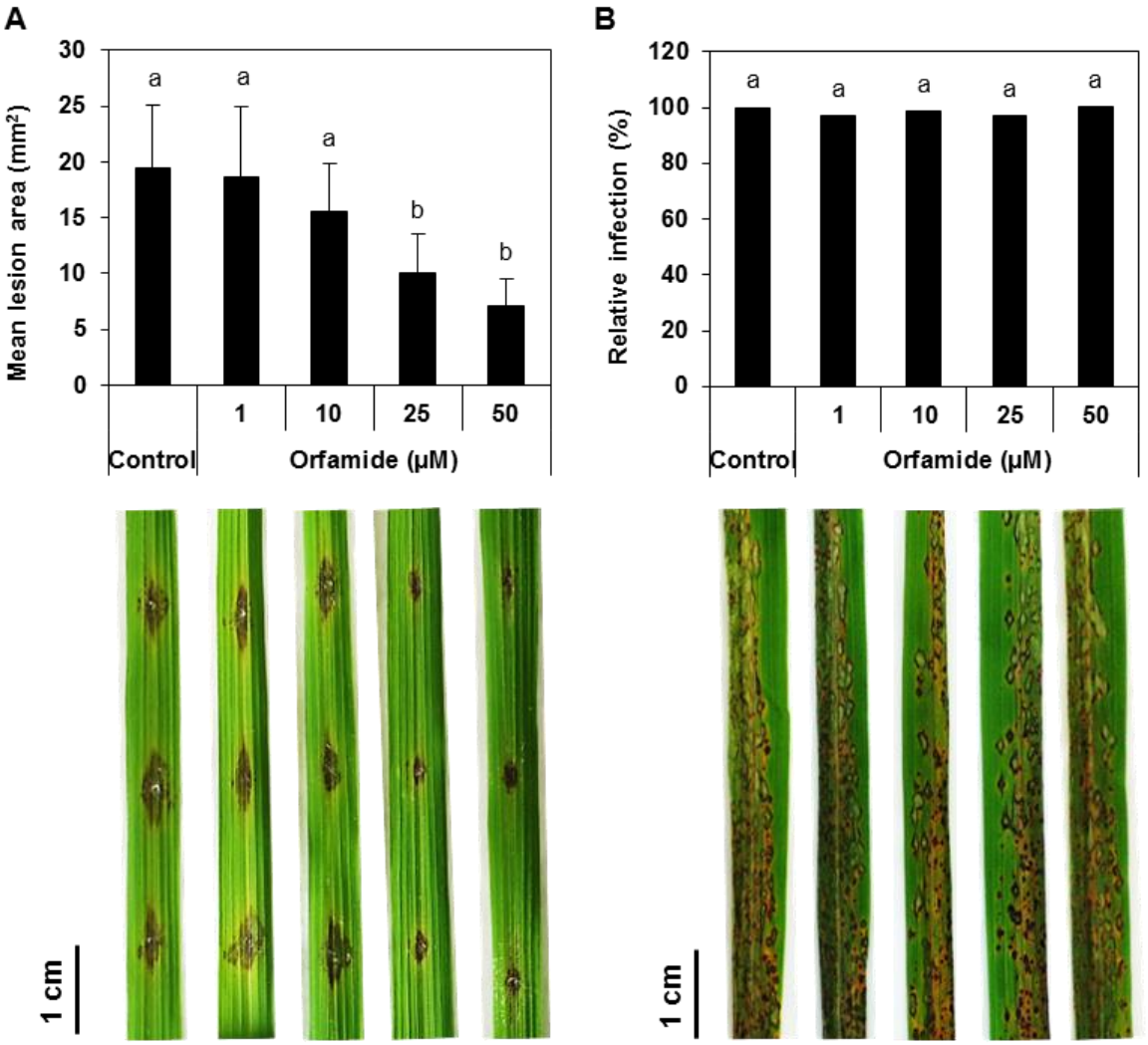

Figure 6.1 Soil assay with orfamide in induced protection of rice against *C. miyabeanus* (A) and *M. oryzae* (B). These bioassays were repeated and showed similar results, so only a representative experiment is shown. Different letters indicate significant differences among treatments (Kruskal-Wallis and Mann-Whitney tests, $p = 0.05$). Inoculated leaves from each treatment are shown in this figure. Data were collected 3 days post inoculation for *C. miyabeanus*, and 6 days for *M. oryzae*, respectively.

A stock solution of orfamide was diluted to different concentrations and tested for the capacity to trigger ISR on rice against *C. miyabeanus* Cm988 and *M. oryzae* VT5M1. The results showed that orfamide treated plants displayed resistance against *C. miyabeanus* Cm988 in a dose dependent manner (Figure 6.1A). More specifically, soil drench with 25 or 50 µM orfamide triggered ISR

against *C. miyabeanus* Cm988 in rice plants, while lower concentrations (1 and 10 µM) of orfamide were not active (Figure 6.1A). However, none of the orfamide concentrations tested showed any activity against *M. oryzae* (Figure 6.1B).

Subsequently, we tested the ISR capacity of selected orfamide-producing *Pseudomonas* strains in rice against *C. miyabeanus* Cm988 by root inoculation. *Pseudomonas* inoculum was collected from KB plates and the concentration was adjusted by measurements of optical density (OD) at 620 nm. Since a high concentration of pure orfamide is needed to trigger ISR, we decided to apply the bacteria on the plants at high densities. When *P. protegens* CHA0 was applied to the rice root system at a cell density of 10^8 CFU/mL it showed protection against *C. miyabeanus* in experiment 1, but not in experiment 2 (Table 6.3) In experiment 3 a cell density of 10^9 CFU/mL was used and this concentration was effective against *C. miyabeanus (*See Table 6.3 and Figure 6.2*)*. The orfamide-deficient mutant CHA5101 lost the capacity to induce resistance to *C. miyabeanus* in both experiment 1 and experiment 3. *Pseudomonas* sp. CMR12a or its mutant CMR12aΔ*Phz*-Clp1 that is unable to synthesize phenazines and sessilins, but still produces orfamides, were not able to trigger induced protection against *C. miyabeanus* (Table 6.3). To further test the root colonizing capacity *Pseudomonas* strains on rice roots, the bacterial population was counted and the results showed that the bacterial population reached a high level of 10^9 CFU/g fresh roots in experiment 3, while bacterial populations up to 10^7 CFU/g fresh roots were reached in experiment 1 (Table 6.3).

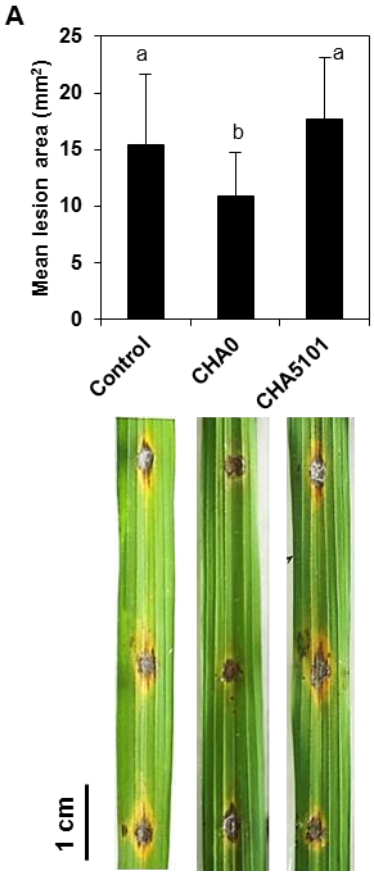

Figure 6.2 Effect of soil application of *P. protegens* CHA0 and its orfamide-deficient mutant CHA5101 against *C. miyabeanus* when applied at a density of 10^9 CFU/mL (experiment 3 in Table 6.3). Different letters indicate significant differences among treatments by Kruskal-Wallis and Mann-Whitney tests ($P = 0.05$).

Table 6.3 Capacity of *Pseudomonas* strains to trigger ISR against *C. miyabeanus* in rice.

Strain	Mean lesion area (mm^2) *			Colonization (logCFU/g fresh roots)	
	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 3
CMR12a	13.35±4.6 a	8.82±2.37 a	/	7.14±0.24 a	/
CMR12aΔ*Phz* - Clp1	16.41±3.67 a	8.59±2.76 a	/	7.33±0.28 ab	/
CHA0	9.04±3.18 b	8.42±2.53 a	10.87±3.91 b	7.86±0.41 b	9.33±0.14 a
CHA5101	16.41±3.93 a	9.19±3.32 a	17.65±5.45 a	7.15±0.13 a	9.11±0.21 a
Control	14.89±4.30 a	8.89±4.11 a	15.41±6.25 a	Null	Null

* *Pseudomonas* inoculum was 10^8 CFU/mL in experiment 1 and experiment 2, while the bacterial inoculum was 10^9 CFU/mL in experiment 3. Slash (/) means strains were not included in the experiment. Difference letters mean significant differences in each experiment, by means of Kruskal-Wallis and Mann-Whitney tests ($P = 0.05$). Null means no *Pseudomonas* bacteria were detected in control treatments.

The production of orfamide by *P. protegens* CHA0 and its orfamide-deficient mutant CHA5101on KB plates was quantified by UPLC-MS analysis. *P. protegens* CHA0 produced 30.3 µM orfamide per OD_{620nm} on KB plates, while its orfamide-deficient mutant completely lost the ability to produce orfamides (Figure 6.3). In experiment 3, the root colonization of *P. protegens* CHA0 was at level of 10^9 CFU/g fresh roots (1 OD_{620nm} approximately equals 10^9 CFU of *Pseudomonas* bacteria), which means that the concentration of orfamide on the root system could theoretically be enough to meet the threshold concentration (25 µM) for triggering resistance against *C. miyabeanus* on rice.

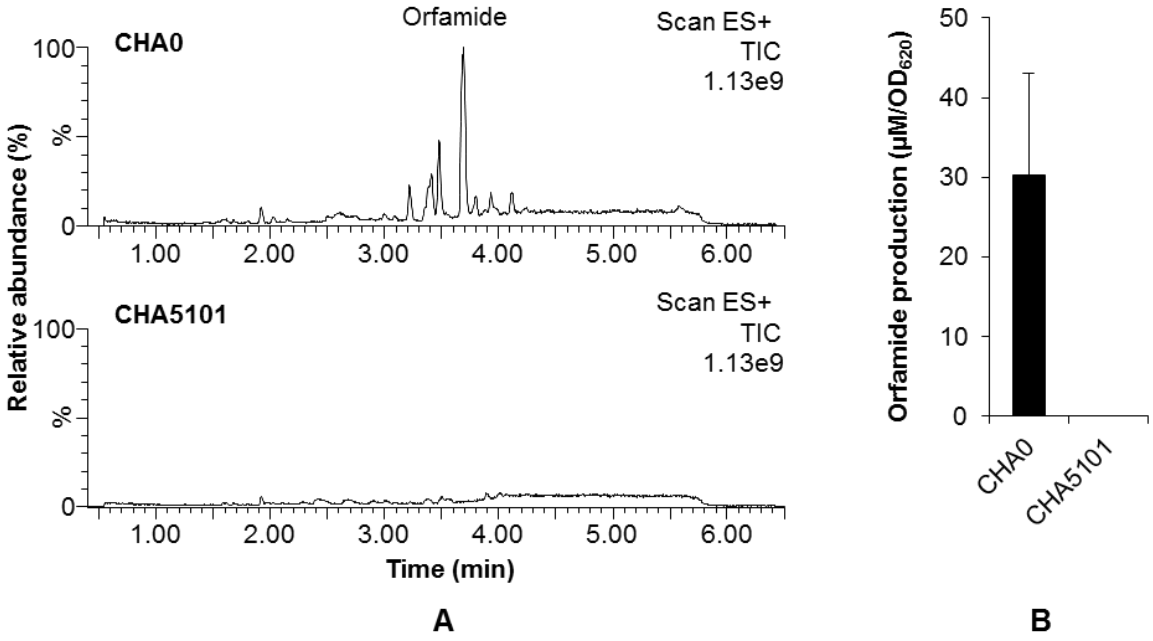

Figure 6.3 UPLC-MS analysis of the orfamide production of *P. protegens* CHA0 and its orfamide-deficient mutant CHA5101, the chromatogram from UPLC-MS analysis (A) and quantification of orfamides (B) of *P. protegens* CHA0 and CHA5101 are shown.

6.3.2 Orfamide triggers defense responses in rice cell cultures without causing cell death

Extracellular alkalinization is one of the biophysical phenomena that can occur in cell cultures upon perception of plant defensive elicitors and is a consequence of altered ion fluxes across the plasma membrane (Garcia-Bruggen et al., 2006). The extracellular pH of rice cell cultures treated with different concentrations of orfamide was monitored during a time period of 60 min. The results show that 25 and 50 µM orfamide caused extracellular alkalinization of rice cell cultures within 60 min,

while this effect was not observed with lower concentrations of orfamides (Figure 6.4A).

Phenylalanine ammonia-lyase, lipoxygenase, *β*-1,3 Glucanase and chitinase are important enzymes involved in innate immunity of plants (Freeman and Beattie, 2008; Jourdan et al., 2009). Gene markers for these enzymes, such as *β-Glucanase* (*β*-1,3 Glucanase encoding gene), *PAL* (phenylalanine ammonia-lyase encoding gene), *Chitinase* (a chitinase encoding gene) and *LOX2* (lipoxygenase encoding gene) were chosen for further bioassays in rice cell cultures after orfamide treatment. The transcripts of these genes were monitored by qPCR assays 2, 6, 12, 18 and 24 h after treatment. Transcripts of *β-Glucanase*, *Chitinase*, *PAL* and *LOX2* genes accumulated in orfamide treated rice cell cultures compared with control treatment (Figure 6.4B and 6.4C). The transcripts of these genes reached the highest levels at 6 h post orfamide-treatment (Figure 6.4B). Moreover, relative transcript levels in rice cell cultures were positively correlated to the orfamide-concentration at 6 h post orfamide treatment (Figure 6.4C).

In further bioassays, we tested whether orfamide amendment in rice cell cultures influenced cell viability. Orfamide did not affect the mortality of rice cell cultures even at concentrations as high as 50 μM after 24 h treatment (Figure 6.4D and 6.4E). This shows that the orfamide-triggered defense responses in rice cell cultures do not cause cell death.

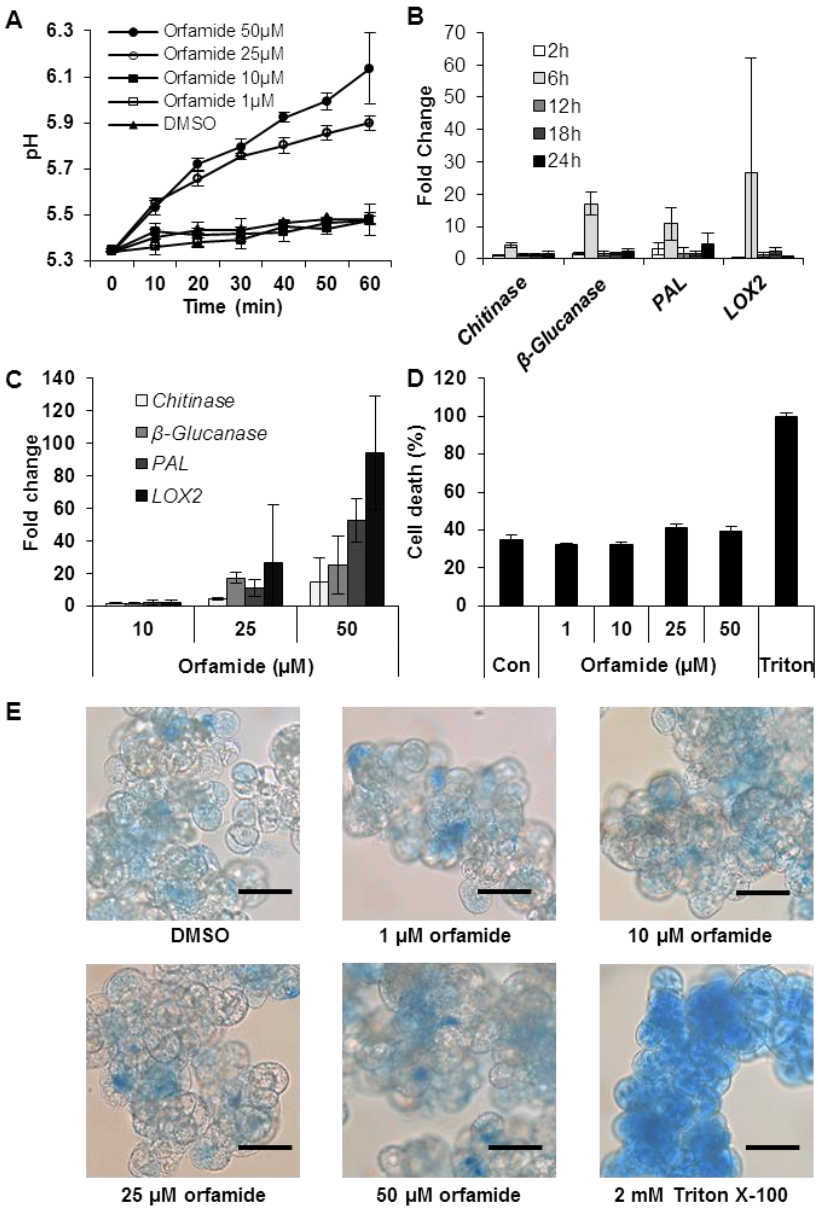

Figure 6.4 Defense-related responses occurring in rice cell cultures after perception by orfamide. Extracellular medium alkalinization (A), transcription of defense-related genes (B and C) and change in cell viability (D) after orfamide-treatment in rice cell cultures. Control (Con) treatment received the same amount of DMSO. The relative transcriptional level of *Chitinase*, *β-Glucanase*, *PAL* and *LOX2* genes was monitored by qPCR at 2, 6, 12, 18 and 24 h after 25 μM orfamide treatment (B), and transcript accumulation of these genes was monitored by qPCR at 6 h after 10, 25 and 50 μM orfamide treatments (C). Data shown in this figure were collected from three biological repeats and represented as means (± SD). In each repeat of rice cell assays, five-day old cell samples were collected, and submitted to further bioassays, and data were collected from different batches of cell cultures for different repeats. Representative pictures of Evan blue-stained rice cell cultures treated with DMSO, orfamide and Triton X-100 are shown (E). Dead cells in rice cell cultures have a blue color, scale bar is 50 μM.

6.3.3 Orfamide-primed defense responses in rice cell cultures are dependent on ABA signaling

Plant hormones are involved in dealing with diverse biotic and abiotic stresses in rice (De Vleesschauwer et al., 2013). In further bioassays, selected marker genes (Table 6.2) for various plant hormone pathways, such as SA, JA, ET, ABA and indole-3-acetic acid (IAA), were monitored by qPCR in orfamide-treated rice cell cultures. Orfamide treatment did not have noticeable effects on the transcript levels of the SA markers *NPR1* and *WRKY45* (Figure 6.5A) or the JA markers *JAMYB*, *JiOsPR10* and *PBZ1* (Figure 6.5B) and slightly increased the ET markers EBP89 and ACS1 at 2h and/or 6 h post inoculation (Figure 6.5C) and the IAA marker IAA9 at 6 h post inoculation. The transcripts of *NCED3* (an ABA biosynthesis gene) and *Rab16* (an ABA responsive gene), however, were induced 5.1 fold and 78.3 fold respectively, when cell cultures were treated with 25 μM of orfamide (Figure 6.5E).

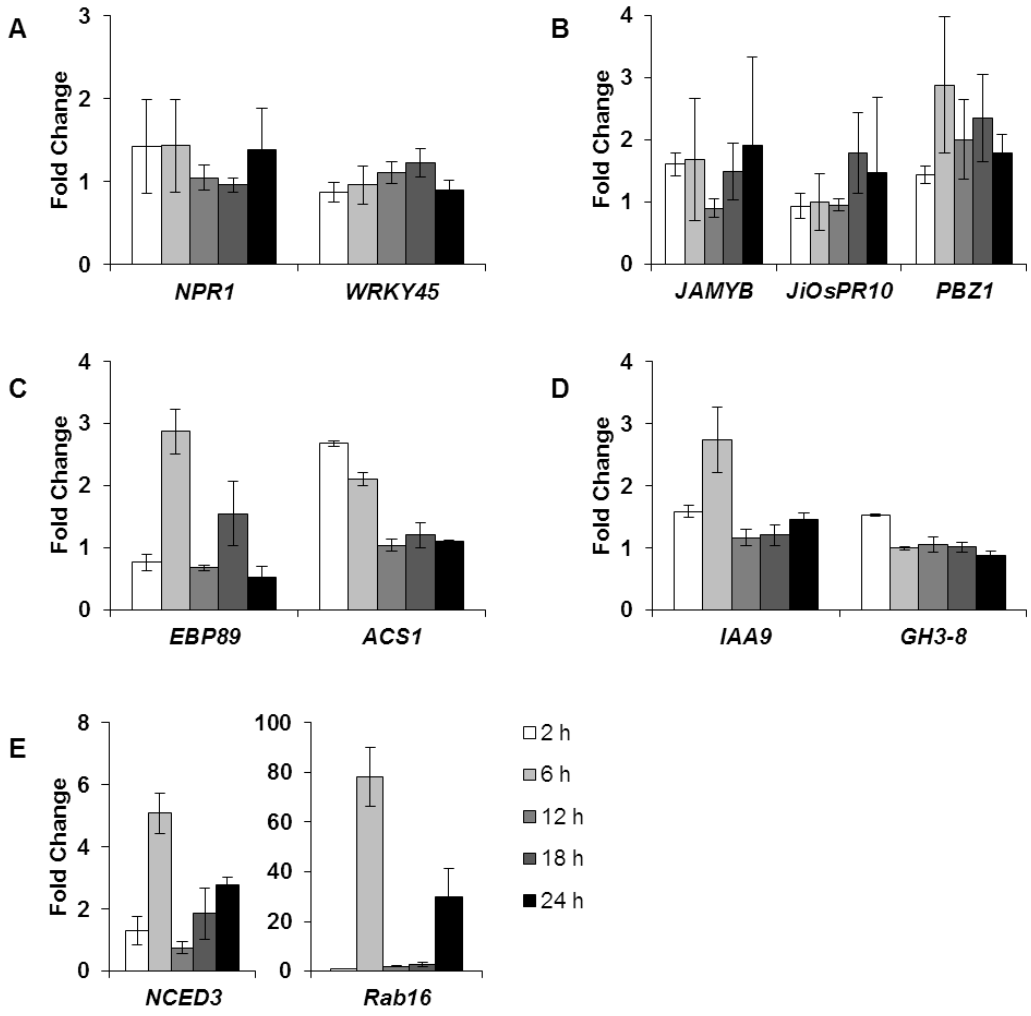

Figure 6.5 Transcripts of marker genes of plant hormone (SA, JA, ET, IAA and ABA) pathways in rice cell cultures. A: marker genes *NPR1* (for *OsNPR1*-dependent pathway) and *WRKY45* (for *OsWRKY45*-dependent pathway) of SA pathway; B: marker genes *JAMYB*, *JiOsPR10* and *PRZ1* of JA pathway; C: marker genes *EBP89* and *ACS1* of ET pathway; D: marker genes *IAA9* and *GH3-8* of IAA pathway; E: marker genes *NCED3* and *Rab16* of ABA pathway. Gene expression in rice cell cultures was monitored by qPCR at 2, 6, 12, 18 and 24 h after 25 μM orfamide treatments. Data are shown as means (± SD) of three biological repeats.

6.3.4 Effect of orfamide on defense-related genes expression in rice after *C. miyabeanus* inoculation

To examine the transcriptional level of defense-related genes in orfamide-treated rice after *C. miyabeanus* Cm988 inoculation, the transcripts of selected defensive genes were monitored by qPCR

in rice plants at various time points (0, 12, 24 and 48h, respectively) post *C. miyabeanus* Cm988 inoculation. Strikingly, unlike the phenomenon occurring in rice cell cultures, transcripts of defense-related genes, *β-Glucanase*, *Chitinase*, *PAL* and *LOX2* did not accumulate at higher levels in orfamide treated plants at different time points tested in this study, compared with control plants, although inoculation with *C. miyabeanus* strongly induced *LOX2* (Figure 6.6A). In further experiments, marker genes for plant hormone pathways were analyzed by qPCR. Transcriptional analysis showed that the SA markers *NPR1* and *WRKY45* (Figure 6.6B), the ET markers *EBP89* and *ACS1* (Figure 6.6D), the JA markers *JAMYB*, *JiOsPR10* and *PBZ1* (Figure 6.6F) and the IAA markers *IAA9* and *GH3-8* (Figure 6.6E) were not involved in orfamide-primed resistance against *C. miyabeanus* in rice, although the SA, ET and JA pathway were induced by pathogen inoculation (Figure 6.6B, 6.6D). The transcripts of the ABA biosynthesis gene *NCED3* (2.0 fold) and the ABA responsive gene *Rab16* (3.3 fold), however, were significantly induced in the priming stage of orfamide-treatment (0 hpi), compared with untreated control rice plants (Figure 6.6C).

Pathogenesis-related (PR) proteins are involved in disease resistance triggered by elicitors in plants (Solano et al., 1998; Park and Kloepper, 2000; Ipper et al., 2008; Park et al., 2008). Recently, it has been shown that genes encoding PR proteins, *PR1a* (an acidic PR1 protein gene), *PR1b* (a basic PR1 protein gene), *PR5* (a thaumatin-like protein gene) and *PBZ1* (an intracellular ribonuclease) are involved in innate immunity against the necrotrophic pathogen *R. solani* in rice (Wang et al., 2015). The transcriptional responses of these genes were evaluated by qPCR in this study. Strikingly, orfamide-treated plants showed a significantly higher transcriptional accumulation of *PR1b* at time points of 12 hpi (2.5 fold) and 48 hpi (5.8 fold) compared with untreated control plants, respectively (Figure 6.6H). Moreover, the *WRKY4* transcription factor has been involved in disease resistance against the necrotrophic pathogen *R. solani* in rice (Wang et al., 2015). The transcript of *WRKY4* in orfamide-primed plants (0 hpi) was significantly induced (3.7 fold) compared to the control plants (Figure 6.6G).

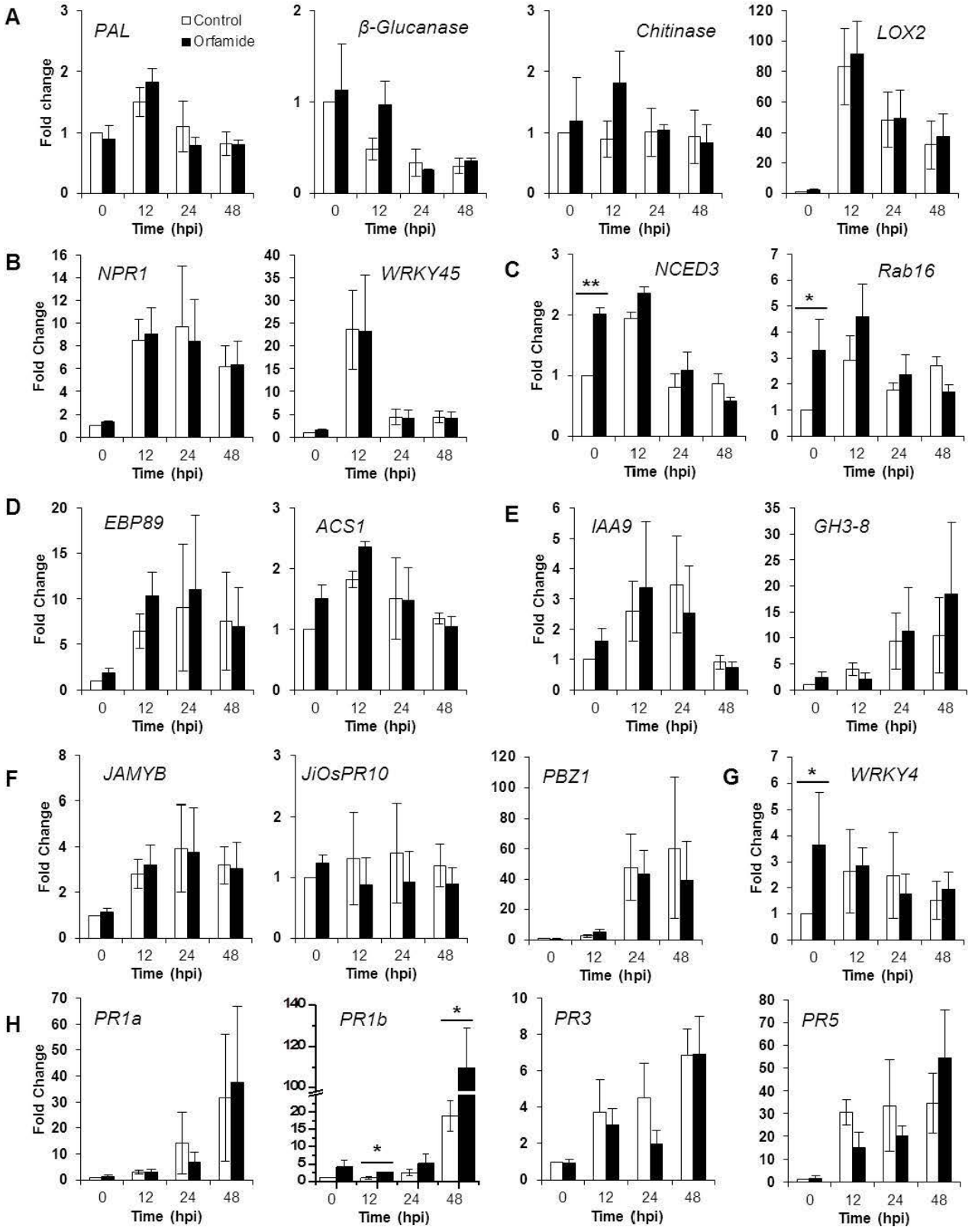

Figure 6.6 Transcripts of defense-related genes in rice plants (control and orfamide-treated plants) at different time points (h) after *C. miyabeanus* inoculation. Orfamide was applied as soil drench 72 h before inoculation with *C. miyabeanus*. The second youngest leaves of rice plants were then detached, put on moistened tissues, and inoculated with a

conidial suspension of *C. miyabeanus*. The relative transcriptional level of defense-related genes was monitored by qPCR. These defensive genes include marker genes *NPR1* (for OsNPR1-dependent pathway) and *WRKY45* (for OsWRKY45-dependent pathway) of SA pathway; marker genes *JAMYB*, *JiOsPR10* and *PBZ1* of JA pathway; marker genes *EBP89* and *ACS1* of ET pathway; marker genes *IAA9* and *GH3-8* of IAA pathway; marker genes *NCED3* and *Rab16* of ABA pathway, and other genes, for instance, PR genes and genes involved in basal innate immunity (*Chitinase*, *β-Glucanase*, *PAL* and *LOX2*) of rice. In each repeat, plant materials were collected from at least six rice leaves from different plants. Data were collected from rice plants grown in different time but with the same age for different repeats. Data are shown as means (± SE) from three biological repeats. Asterisk (*) indicates statistically significant differences between orfamide and control treatments (t test, *: $p < 0.05$; **: $p < 0.01$).

6.3.5 Orfamide triggered defense responses in rice are not associated with accumulation of phenolics

It was previously shown that silicon-triggered disease resistance in rice is accompanied by accumulation of phenolic compounds (Van Bockhaven et al., 2015), which are related to fortification of plant cell walls after pathogen penetration. However, orfamide-treated rice plants did not show increased autofluorescence and did not accumulate phenolic compounds 24 h (Figure 6.7A) or 48 h (data not shown) after pathogen inoculation.. The phenyl propanoid pathway is commonly associated with the phenolic compounds biosynthesis and thus links with cell wall development in plants. Phenylalanine ammonia-lyase is the entry enzyme of phenyl propanoid pathway (Dixon et al., 2002). The key gene *PAL* encoding phenylalanine ammonia-lyase was not significantly changed in transcriptional level in orfamide-treated rice after pathogen inoculation (Figure 6.6), which is in line with the lack of detection of autofluorescence in rice after orfamide-perception in this study (Figure 6.7A)

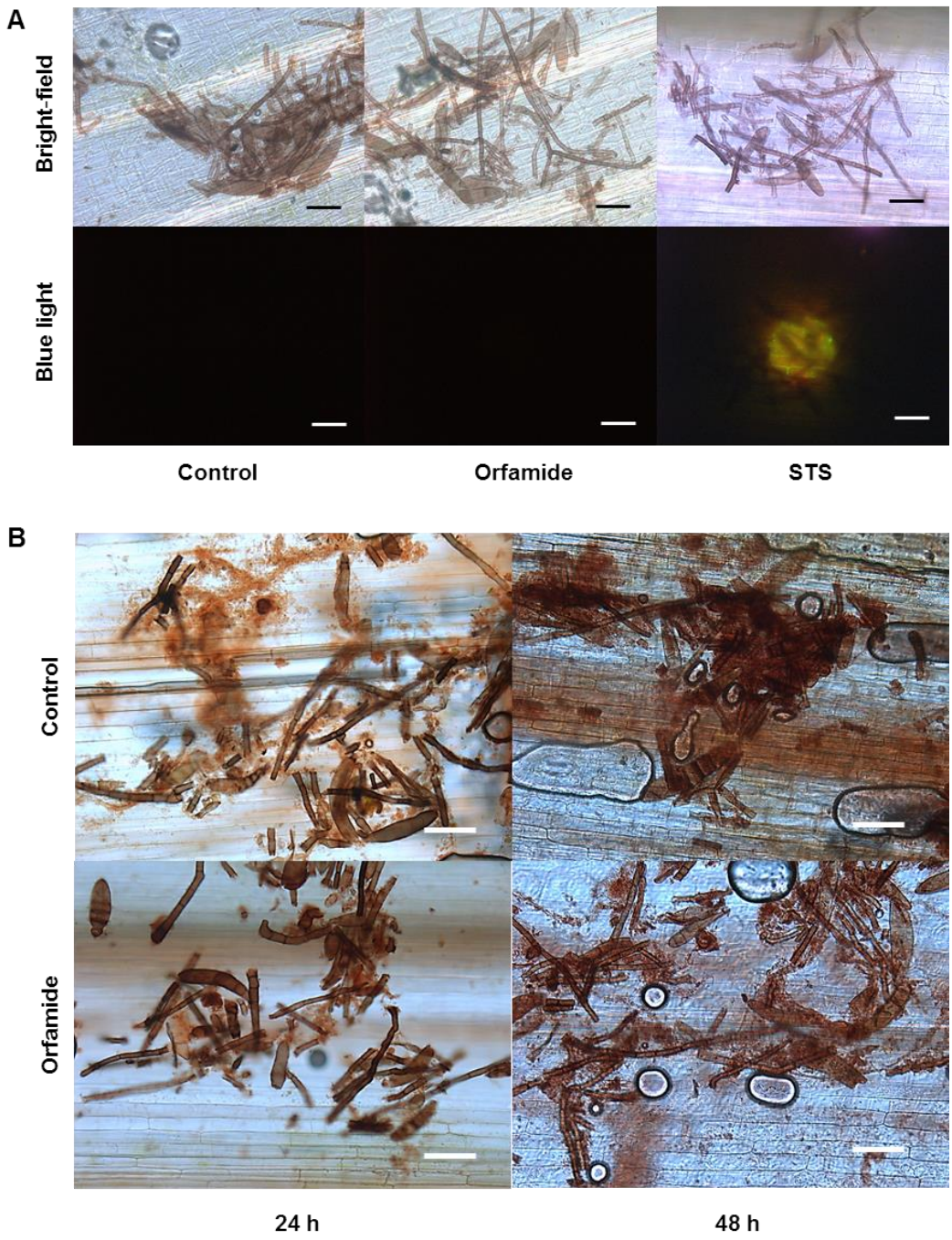

Figure 6.7 Detection of autofluorescence generated by phenolic compounds (A) and the cell death (B) in control and orfamide treated rice plants after *C. miyabeanus* infection. Autofluorescence monitored 24 h post inoculation is shown. 100 mM STS was set up as a positive indicator of green autofluorescence in rice plants after *C. miyabeanus* infection, and the application of STS in rice sheath was exactly the same as shown previously (Van Bockhaven et al., 2015). Representative pictures show both bright field and blue light excitation for detection of autofluorescence. Epidermal cell death of rice sheath was shown as brown color with staining by DAB after 24h and 48h post infection. All experiments were repeated independently twice and showed similar results, and only representative pictures are shown in this figure. Scale bar is 50 µm.

De Vleesschauwer et al. (2010) demonstrated that inoculation with *C. miyabeanus* leads to massive accumulation of H_20_2, visualized by DAB staining, in epidermal and mesophyll cells surrounding the side of infection. This H_20_2 accumulation may support cell death to pave the way for *C. miyabeanus* in its necrotrophic growth stage. Here we stained rice sheaths with DAB and found that orfamide treatment resulted in less H_20_2 accumulation upon *C. miyabeanus* inoculation in comparison with untreated control plants. Two independent assays were carried out with similar results. Representative pictures from one assay are shown in Figure 6.7B.

6.4 Discussion

Microbe-derived natural products used to mediate elicitation of resistance against *C. miyabeanus* in rice have not been reported before. Herein, we identified and characterized orfamides derived from *P. protegens* CHA0 as CLP elicitors to alleviate *C. miyabeanus* disease in rice, and we also examined the possible defensive mechanisms triggered by orfamide on both rice cell cultures and rice plants.

Perception of orfamide in rice triggers induced protection against *C. miyabeanus*

We applied various concentrations of purified orfamide from *P. protegens* CHA0 on rice, and tested the ISR capacity of orfamide against fungal pathogens *C. miyabeanus* and *M. oryzae*. The data showed that orfamide mediated immune response against *C. miyabeanus*, but not against *M. oryzae* in rice. The threshold concentration of orfamide to trigger an ISR response is as high as 25 μM (Figure 6.1A, Figure 6.4A and 6.4C). Soil assay results with the orfamide-producing wild type strain *P. protegens* CHA0 also indicated the protection against leaf infection of *C. miyabeanus* in rice (Figure 6.2 and Table 6.3), while its orfamide-deficient mutant of *P. protegens* CHA5101 lost this kind of capacity. However, high concentrations of *Pseudomonas* bacteria in initial inoculum and on the rice root system were needed to see this effect in the case of *P. protegens* CHA0.

We did not measure the *in situ* production of orfamides in potting soil by inoculation of *P. protegens* CHA0, *Pseudomonas* sp. CMR12a and its mutant CMR12aΔ*Phz*-Clp1 cells, but, measurements of *in vitro* production of orfamides on KB plates showed that *P. protegens* CHA0 and *Pseudomonas* sp.

CMR12a and its mutant CMR12aΔ*Phz*-Clp1 could produce 30.3, 19.8 and 2.9 μM orfamides/OD_{620nm}, which equals about 10^9 CFU. This may explain why application of 10^8 CFU of *Pseudomonas* bacteria per gram potting soil showed variable ISR results against *C. miyabeanus*. In experiment 2 (Table 6.3), the threshold concentration to trigger an ISR immune response may not have been reached. However, orfamide did not induce protection against *M. oryzae* in rice (Figure 1B). Spence et al. (2014) already showed that root inoculation with *P. protegens* CHA0 did not trigger induced protection against *M. oryzae* in rice. Taken together, these results show that orfamides do not have the capacity to trigger innate immunity against *M. oryzae* in rice. It has been shown that the defensive responses in rice to *C. miyabeanus* and *M. oryzae* are far from identical. BTH and conidial germination fluid (CGF) treated rice displayed disease resistance to *M. oryzae*, not to *C. miyabeanus* (Ahn et al., 2005). ISR mounted by root treatment with *Serratia plymuthica* IC1270 in rice showed resistance to *M. oryzae*, while the same treatment in rice showed increased disease upon inoculation with *C. miyabeanus* (De Vleesschauwer et al., 2009). Chitin is percieved by plasma membrane glycoprotein CEBiP in rice, and rice lines overexpressing the genes containing the extracellular portion of CEBiP showed disease resistance to *M. oryzae*, but not to *C. miyabeanus* (Kishimoto et al., 2010). The DELLA protein SLR1 in rice is a positive regulator of disease resistance to *M. oryzae*, but in contrast, has no effect on the disease development of *C. miyabeanus* (De Vleesschauwer et al., 2016). To some extent, these reports are in line with the distinct influences of orfamide treatment on ISR against these two pathogens in the current study (Figure 6.1).

Orfamide triggered defense-related responses in rice cell cultures and rice are distinct

Perception of elicitors by plant cell cultures results in early and late defense related responses. The typical early biochemical responses mainly include medium alkalinization or acidification, ion influx and the production of ROS (Blumwald et al., 1998). Transcripts of defense-related gene and enzymatic activation in plant cell cultures are late responses. Orfamide, indeed, mounts both early and late defense-related responses in rice cell cultures, for instance, alkalinization of the medium (Figure 6.4A) and further transcription of defense-related genes (Figure 6.4B, 6.4C and Figure 6.5), respectively. Intriguingly, medium alkalinization of 25 or 50 μM orfamide-treated rice cell cultures increased within 60 min compared with the control treatment (Figure 6.4A), while medium

alkalinization in surfactin-treated tobacco cell cultures only increased for 15 min, and afterwards the pH of medium went down to the initial level (Jourdan et al., 2009). Another report showed that medium alkalinization of grapevine cell cultures treated with surfactin showed similar trends as the findings in this study (Farace et al., 2015).

Orfamide triggered the accumulation of transcripts of defense-related genes *Chitinase*, *β-Glucanase*, *PAL* and *LOX2* (Figure 6.4B and 6.4C) in rice cell cultures, but the transcripts of these defensive genes did not change significantly in orfamide-primed rice plants (Figure 6.6A).

To date, *Bacillus*-derived CLPs are the most intensively studied CLPs in triggering ISR responses of plants. IAA signaling was triggered upon perception of surfactin, fengycin and mycosubtilin in rice cell cultures, while JA and ET were also involved in fengycin and mycosubtilin triggered defense responses (Chandler et al., 2015). Strikingly, ABA signaling is the only plant hormone which plays an vital role in orfamide-treated rice cultures 6h post orfamide treatment, with 5.1 fold induction of ABA biosynthesis gene *NCED3* and 78.3 fold induction of ABA responsive gene *Rab16* (Figure 6.5E), respectively. Similarly in orfamide-primed rice plants, we saw a 2.0 fold and 3.3 fold induction for *NCED3* and *Rab16*, respectively (Figure 6.6C). In line with that the previous report showed that ABA plays a vital role in disease resistance against *C. miyabeanus* in rice. This suggests that different CLPs (surfactin, fengycin, iturin and orfamide) function differently in mounting defense-related hormone signaling in rice cell cultures. Despite the differences in cellular responses, these CLPs act in the control of necrotrophic rice diseases, for instance, *Bacillus* CLPs are involved in the immune responses against *R. solani*, but not against *M. oryzae* on rice (Chandler et al., 2015), while orfamide can trigger ISR response against *C. miyabeanus*, but not against *M. oryzae* on rice (Figure 6.1).

On the other hand, variations in transcript data were seen in orfamide-treated cell cultures and orfamide-treated rice plants. This could be due to the fact that orfamides are in direct contact with rice cell cultures and may trigger local defense responses, while orfamides were applied on rice roots system and we tested the ISR in rice sheaths and leaves. Perhaps this could be one of the possible explanations why rice cell cultures and rice plants responded differently to the elicitor.

The role of PR proteins and defensive regulator in orfamide-primed resistance in rice

PR proteins are a large family of inducible defense-related proteins in plants, which are induced by pathogens (Van Loon et al., 2006). These PR proteins are involved in dealing with abiotic and biotic stresses (Sinha et al., 2014). Rice defensive responses against fungal pathogens are usually accompanied by transcriptional changes of PR proteins. More specifically, it has been reported that ISR elicitors BTH and pseudobactin suppresses *PBZ1* and *PR1b* in a late stage (for instance 48 h) of defensive responses against *M. oryzae* in rice (De Vleesschauwer et al., 2008). In contrast, recently it has been shown that upon *R. solani* inoculation, transcripts of *PR1a*, *PR1b*, *PR5*, *PR10b*/*PBZ1* accumulated in resistant rice plants, but not in a susceptible line (Wang et al., 2015). Interestingly, transcripts of the PR protein *PR1b* also accumulated in orfamide-treated rice inoculating with *C. miyabeanus* (Figure 6.6H). Additionally, exopolysaccharide from a *Serratia* sp. strain Gsm01 displayed induced protection against cucumber mosaic virus and triggered the transcripts of *PR1b* in tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) (Ipper et al., 2008). *PR1b* gene of tobacco and tomato plants shows antimicrobial activity against *Phytophythora infestans* (Niederman et al. 1995). Moreover, our data also show a positive role of *WRKY4* in the regulation of disease resistance against *C. miyabeanus* in rice (Figure 6.6G). The transcript of *WRKY4* and PR genes, such as *PR1a*, *PR1b*, *PR5* and *PBZ1*, are both increased significantly in the disease resistance response in rice against *R. solani* (Wang et al., 2015). While this study shows that orfamide-triggered ISR response against *C. miyabeanus* in rice is associated with increased transcript of *PR1b* and *WRKY4*.

Role of orfamide in the control of rice diseases

Our previous data showed that orfamide can control the disease severity of *M. oryzae* by blocking appressorium formation under both *in vitro* and *in vivo* conditions on hydrophobic surfaces, for instance, glass slide covers (*in vitro*) and rice leaf sheaths or rice leaves (*in vivo*) (Ma et al., 2016a). Moreover, in this study we showed that orfamide can protect rice plants from attack by *C. miyabeanus* by induction of multiple defensive related responses. The *Bacillus*-derived CLPs surfactin, fengycin and iturin are widely studied for their roles in biocontrol against a broad spectrum of plant pathogens, but, no reports have focused on the roles of these CLPs in biocontrol of *C. miyabeanus* in rice. We defined, for the first time, the role of *Pseudomonas*-derived orfamide in

biocontrol of *M. oryzae* (Ma et al., 2016a) and *C. miyabeanus* (Figure 6.1A) in rice. The biocontrol capacity of orfamide against other diseases (for instance, sheath blight disease *R. solani* and bacterial blight disease *Xanthomonas oryzae* pv. *oryzae*) in rice should also be examined in further studies.

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Chapter 7. General discussion and perspectives

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General discussion and future perspectives

7.1 Importance of major findings of this thesis, and general discussion

CLPs are biosurfactants showing both hydrophilic and hydrophobic properties. CLPs are also actively involved in the physiology of their producing bacteria (for instance, swarming motility on the surface), and the interactions of the parental bacteria with other organisms (for instance, antibiosis) or with the environment (for instance, biofilm formation). In addition, CLPs are also involved in the biocontrol activity against fungal plant pathogens and specific *Pseudomonas* biocontrol strains have the ability to produce CLPs. This thesis mainly focuses on the CLPs produced by *P. protegens* and related species, and their roles in biocontrol against selected plant diseases. More specifically, the biocontrol roles of sessilins and orfamides produced by selected strains belonging to *P. protegens* and related species against fungal diseases on rice and bean, were studied in this thesis.

P. protegens and related species are well known model *Pseudomonas* species, and include *P. protegens* CHA0 and Pf-5, *Pseudomonas* sp. CMR12a and CMR5c. These strains have the ability to produce diverse secondary metabolites, including phenazines, CLPs, DAPG, HCN, pyrrolnitrin and pyoluteorin, and thus are actively involved in interactions with other mcroorganisms and plants. In the current work, the interactions mediated by *Pseudomonas*-derived CLPs with fungal pathogens and crops were studied.

We used parental CLPs-producing strains and their specific metabolite-deficient mutants, and purified CLPs to elucidate corresponding mechanisms (mode of actions) involved in the biocontrol of fungal pathogens in rice and bean. As shown in Table 7.1, along with phenazines, CLPs derived from *P. protegens* and related strains are actively involved in the control of *M. oryzae* and *R. solani*, and their modes of action include direct effects on the development of fungal pathogens, or indirect effects by inducing systemic resistance that suppresses the further invasion of fungal pathogen on plants. The major findings of this thesis will be discussed in view of the research questions that we formulated in the introduction.

Table 7.1 Overview of the role in biocontrol of specialized metabolites from selected *Pseudomonas* strains protecting rice and bean against fungal diseases, as studied in this thesis.

Compound	Producer (s)	Targeted pathogen	Mode of action	Assay	Chapter
Orfamide	*P. protegens* and related species, for instance, *P. protegens* CHA0, *Pseudomonas* sp. CMR12a and CMR5c	*M. oryzae*	Inhibition of appressoria formation	*In vitro* and *in vivo* (rice)	**3**
		R. solani	Hyphal branching	*In vitro* and soil assay in bean	**3**
		Phytophthora porri *Pythium ultimum*	Lysis of zoospores	*In vitro*	**3**
	Pseudomonas sp. CMR12a	*R. solani*	ISR[a]	Soil assay in bean	**5**
	P. protegens CHA0	*C. miyabeanus*	ISR	In rice cell cultures and rice	**6**
Sessilin	*Pseudomonas* sp. CMR12a	*R. solani*	Direct antagonism	Soil assay in bean and Chinese cabbage	**4**
		R. solani	ISR	Soil assay in bean	**5**
PCN[b]	*Pseudomonas* sp. CMR12a	*M. oryzae*	ISR	Soil assay in rice	**5**
		R. solani	ISR	Soil assay in bean	**5**

[a] ISR: induced systemic resistance; [b] PCN: phenazine-1-carboxamide.

Research question 1. Orfamide-type CLPs were first discovered in *P. protegens* Pf-5 (Gross et al., 2007). Do other *P. protegens* strains and related species also produce orfamides? Do orfamide-type CLPs produced by these strains show diversity in structure and biosynthesis and does this influence their biological activity?

CLPs orfamides are composed of several structural homologues, including orfamide A, B, C, D, E, and poaeamide A and B (Gross et al., 2007; D'aes et al., 2014; Zachow et al., 2015; Nguyen et al., 2016). These homologues show substitutions in amino acid sequence and (or) in length of fatty acid residues, C10, C12, C14 or C16. We analyzed KB cultures of selected orfamide producers by UPLC-MS analysis, and the fingerprints of these producers show slight differences, either in abundance and in retention times (min) of the CLP peaks, suggesting that the orfamides produced by *P. protegens* and related species show diversity in chemical structure. Since several genomic sequences of potential orfamide producers are publicly available, we further conducted a detailed comparison on the NRPS synthetases responsible for orfamide biosynthesis in selected *Pseudomonas* strains (Figure 3.1 in **Chapter 3**). Indeed, these NRPS synthetases show differences (amino acid sequences) on each domains (A, C, T and Te domains). A phylogenetic tree based on A domains showed that the A

domains clustering together incorporated the same amino acid (Figure 3.2 in **Chapter 3**). And furthermore, two new orfamides (orfamide F and G) were characterized in **Chapter 3**.

Due to structural variance in the orfamide family, we chose several representative analogues (orfamide A, B and G) for further structure-activity comparison. Orfamide A and B share the same fatty acid residue, but differ from each other in one amino acid in the peptide sequence. Orfamide B and G share the same peptide moiety but their fatty acid residues differ in length. We further tested activity of these orfamides on *M. oryzae*, *R. solani*, and oomycete pathogens *Phytophthora* and *Pythium*. All tested orfamides analogues show similar (but not statistical different) activities, except for the zoospore lysis assay, with only slight differences. We only used orfamides possessing C14 and C16 fatty residues for structure-activity comparison in Chapter 3, due to the insufficient quantities of C12 homologues. All tested orfamides almost showed similar bioactivity in several biological models, including zoospore lysis of selected oomycete pathogens, changing the morphology of *R. solani* and control of *M. oryzae* under *in vitro* and rice leaves.

Appressoria are indispensable infection structures for disease development of *M. oryzae*. It has been proved that cAMP and MAPK signaling play important roles in appressoria development in *M. oryzae* (Rebollar and López-García, 2013). Moreover, surface hydrophobicity is also an important factor involved in the appressoria formation of *M. oryzae* (Liu et al., 2011). We observed inhibition of appressoria formation of *M. oryzae* by orfamide-treatment both on glass slide covers and on rice sheath. We proposed that this phenomenon can be due to the physical-chemical properties of orfamide on a surface associated with its surface tension lowering activity, and thus hampering appressoria formation of the fungus. However, regarding this issue there is a controversy because glycolipid-type of biosurfactant, mannosylerythritol lipids, failed to protect rice against conidia of *M. oryzae*, but showed *in vitro* inhibition of appressoria formation of *M. oryzae* (Yoshida et al., 2015).

Taking the results from orfamides (**Chapter 3**) and mannosylerythritol lipids (Yoshida et al., 2015) together, both types of biosurfactants could inhibit appressoria formation under *in vitro* conditions, but mannosylerythritol lipids failed in protection of infection by *M. oryzae* on rice leaves. Possibly, the interactions of these two types of biosurfactants with conidia of *M. oryzae* are different, perhaps with divergent mode of actions in targeting and interacting with conidia of *M. oryzae* on rice leaves.

Also other unknown factors are involved in these kinds of interactions.

Research questions 2, 3 and 4. It has been shown before that sessilins play a role in direct antagonism against *R. solani* root rot on bean (D'aes et al., 2011). *Pseudomonas* sp. CMR12a not only produces sessilins but also orfamides. Do orfamides also play a role in direct antagonism? Do sessilins and orfamides play a role in induced systemic resistance (ISR) and if so, is their role plant and pathogen dependent? How do plants respond to orfamide-type CLPs?

Pseudomonas sp. CMR12a has been experimentally developed as a model biocontrol microbe for several crops in greenhouse conditions, such as Chinese cabbage, bean, and cocoyam. Its biocontrol activity is mainly attributed to the production of phenazines, and sessilin- and orfamide-type CLPs. Importantly, the strain has excellent ecological fitness properties (for instance, root colonization and maintaining sufficient bacterial population) in different substrate (potting soil or mixture of soil and sand). Interestingly, these CLPs and phenazines not only play a role in direct antagonism against fungal plant pathogens (**Chapter 4**), but also in enhancing plant innate immunity (**Chapter 5**), as shown in this study.

Mutants with abolished production of metabolites by *Pseudomonas* sp. CMR12a were generated in previous work (D'aes et al. 2011; D'aes et al. 2014). Both metabolite-deficient mutants of *Pseudomonas* sp. CMR12a and purified metabolites were applied in different plant-pathogen systems in soil assays. We showed that sessilins and phenazines are actively involved in the control of Rhizoctonia root rot disease in bean and Chinese cabbage, mainly by direct antagonism against *R. solani*, the causal agent of root rot disease (**Chapter 4**). The role of sessilins and phenazines in the control of *R. solani* has been reported previously (D'aes et al., 2011). In **Chapter 4**, *in vitro* assays showed that purified orfamide B has a dose dependent antagonistic effect on *R. solani*. Interestingly, soil assays with *Pseudomonas* sp. CMR12a and its mutant indicated that both CLPs are needed for successful control of *R. solani*. This antagonistic effect was further confirmed by an *in vitro* assay.

CLPs sessilins and orfamides, together with phenazines are actively involved in the elicitation of ISR, in the control of Rhizoctonia web blight disease on bean. Intriguingly, the only active compound of *Pseudomonas* sp. CMR12a involved in inducing resistance against *M. oryzae* on rice

system is phenazine (**Chapter 5**). These results confirm the biocontrol activity of *Pseudomonas* sp. CMR12a in different plant-pathogen systems, and also indicate that its determinants for biocontrol in different plant-pathogen systems are distinct.

The biocontrol of plant diseases by plant beneficial microbes needs firstly successful colonization by these microbes of the rhizosphere niche. The interactions between plant beneficial microbes and root pathogens in rhizosphere niche of plants are complex. It has been shown that root inoculation of plant beneficial microbes to control root associated pathogens in plants also has capacity to trigger innate immunity in roots of plants, beside biocontrol role of direct antagonism. For instance, Chowdhury and colleagues showed that root colonization by *B. amyloliquefaciens* FZB42 triggers resistance in lettuce against the bottom rot pathogen *R. solani* (Chowdhury et al., 2015). *P. fluorescens* PTA-CT2 can trigger defensive responses both in the root system (local resistance) and acrial part (systemic resistance) of grapevine plants (Gruau et al., 2015).

Whether colonization of *Pseudomonas* sp. CMR12a can mount immune responses in the roots of bean and Chinese cabbage systems was not tested in this study. However, we could speculate that perhaps root colonization of this bacterium could mount immune responses in both local (for instance, in roots) and systemic (for instance, in plant leaves) ways in plants.

Orfamide B triggered induced protection in bean against web blight pathogen *R. solani* (**Chapter 4**). However, orfamide A is more abundantly produced by *P. protegens* CHA0, compared with orfamide B and sessilins produced by *Pseudomonas* sp. CMR12a during liquid fermentation processes (**Chapter 3**). We purified orfamide A from supernatant of *P. protegens* CHA0 for further study.

We applied orfamide A, its producing strain *P. protegens* CHA0 and orfamide-deficient mutant CHA5101, *Pseudomonas* sp. CMR12a and a mutant CMR12aΔ*Phz*-Clp1 in soil, to test the ISR-eliciting capacity of these bacteria against fungal pathogens in rice system. We found that orfamide A triggered ISR against *C. miyabeanus*, but not against *M. oryzae* in rice.

We also tested orfamide A for its effect on defensive responses in rice cell cultures. We found that orfamide A can trigger an early defense response, pH change, and further defensive gene transcription. Moreover, orfamide A can prime the transcripts of plant hormone marker genes in rice

cell cultures, mainly by activation of ABA signaling (**Chapter 5**). In further experiments, we looked at transcripts of defensive genes in orfamide A-primed rice plants after *C. miyabeanus* infection, and we further confirmed the role of ABA signaling during the priming stage, and also the involvement of *PR1b*, encoding a basic PR protein, at the late stage of *C. miyabeanus* infection (**Chapter 5**).

Bacillus-derived surfactin triggers disease resistance in multiple plants, such as, 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). Unfortunately, the disease resistance triggering effect of orfamide A in other plants have not been tested yet. It has been shown that *P. protegens* CHA0 could mount defensive responses in grapevine (Verhagen et al., 2010), but the corresponding metabolites were not characterized yet, and it might be interesting to test the ISR role of orfamide A in grapevine cell cultures and disease resistance against *Botrytis cinerea* in grapevine plants, and also in other "model" plants, for instance tobacco and *Arabidopsis*.

7.2 Perspectives

Current work provided some new perspectives on using CLPs-derived from *P. protegens* and related species for the control of blast and brown spot diseases on rice, and Rhizoctonia diseases on bean. Following all these findings, we discern some additional perspectives and research questions that may be addressed in future research.

7.2.1 Biocontrol of *M. oryzae* by *P. protegens* and related species

M. oryzae is the most important fungal plant pathogen due to the loss of productivity on rice worldwide that it can cause (Dean et al., 2012). Rice is one of the important staple foods for people living in subtropical and tropical areas in the world, and thus control of *M. oryzae* is important for global food safety.

We tested a crude extract from KB supernatant containing secondary metabolites produced by *P.*

protegens CHA0, *Pseudomonas* sp. CMR5c and CMR5cΔ*ofa*, and we found that certain metabolites produced by all these stains have the capacity to significantly inhibit the germination of conidia from *M*. *oryzae*, and no appressoria were found in crude extract treatments (data not shown). Orfamides produced by *P*. *protegens* CHA0 and *Pseudomonas* sp. CMR5c can be excluded, since we showed that orfamide does not inhibit the germination of conidia of *M*. *oryzae*. Instead, orfamide inhibits the formation of appressoria from spores of *M*. *oryzae* as shown in this study.

Besides orfamides, *P*. *protegens* CHA0 and *Pseudomonas* sp. CMR5c can produce other important antibiotics, as shown in Table 2.1. Both strains can produce HCN, DAPG, pyoluteorin, pyrrolnitrin and siderophores. Interestingly, *P*. *protegens* CHA0 cannot produce phenazines, while *Pseudomonas* sp. CMR5c can produce the phenazines PCN and PCA. Future studies should search for other antifungal metabolites produced by *P*. *protegens* CHA0 and *Pseudomonas* sp. CMR5c, since a crude extract from KB cultures of *P*. *protegens* CHA0 and *Pseudomonas* sp. CMR5c can significantly inhibit the germination of conidia of *M*. *oryzae* under *in vitro* conditions. The *in planta* biocontrol roles of corresponding metabolites from *P*. *protegens* CHA0 and *Pseudomonas* sp. CMR5c should also be included in the future work, for instance on rice leaves.

7.2.2 Direct antagonism and elicitor-mediated resistance (local and systemic resistance) triggered by rhizosphere microbes

During colonization of plant root systems, rhizosphere microbes maintain active metabolism, including secretion of diverse molecules, including CLPs. These CLPs have diverse functions, for instance, acting as antibiotics against fungal pathogens or as elicitors which activate the innate immunity of plant. Elicitor mediated innate immunity is an important strategy to control plant diseases (Walters et al., 2013). It has been shown that elicitor-triggered defense responses in plants are concentration dependent, needing specific concentration ranges of elicitors, while other concentrations are not effective (De Vleesschauwer and Höfte, 2009). Elicitor mediated elicitation of plant innate immunity is always elicitor, pathogen and plants dependent.

For the first recognition, perception of elicitor by plant cells needs a specific receptor located on the plant cell plasma membrane. However, surfactin targets the lipid part of the plasma membrane of

tobacco cells, instead of recognition by a receptor. Does this type of lipid-driven process only work for surfactin interacting with tobacco cells, or it is a common perception way for CLPs-type elicitors derived from *Bacillus* and *Pseudomonas* sp.? In further studies, the targets of orfamide perception in plant cells should be investigated, using surfactin for comparison.

We showed that orfamides elicit immunity in rice against *C. miyabeanus* and orfamides work at micromolar concentration. However, orfamides show induced protection in bean against *R. solani* at nanomolar concentration. What is/are the mode of action/actions of CLPs sessilins and orfamides, and phenazines in triggering innate immunity in bean against Rhizoctonia disease?

A bacterial secondary metabolite can mount innate immunity against specific diseases on crops, however, it may trigger susceptibility to other kinds of diseases. How can we prevent this phenomenon in future applications? For instance, pyocyanin, a phenazine compound produced by *P. aeruginosa* elicits innate immunity in rice against the rice blast pathogen *M. oryzae*, but also triggers susceptibility against the sheath blight pathogen *R. solani* (De Vleesschauwer et al., 2006).

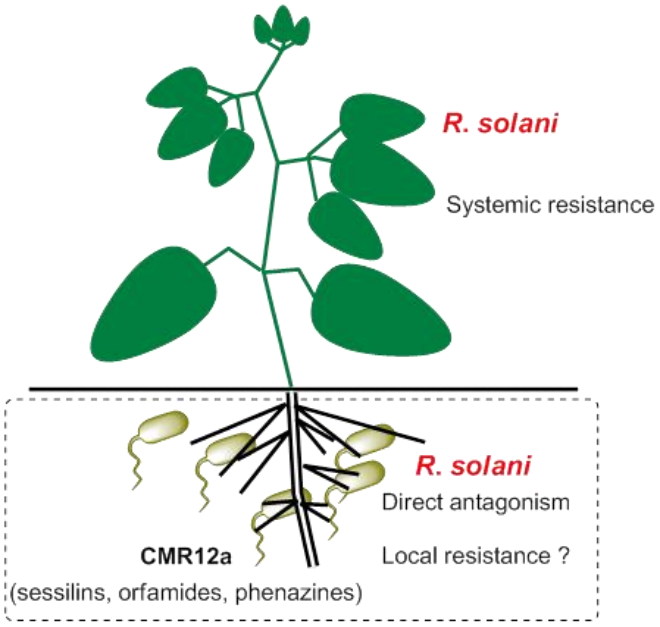

Figure 7.1 Model illustrating the biocontrol properties of *Pseudomonas* sp. CMR12a against *R. solani* on bean.

We showed that root colonization of *Pseudomonas* sp. CMR12a on bean can protect against both root rot and web blight diseases caused by *R. solani*, by producing antagonistic antibiotics (phenazines, sessilins) or by secreting elicitors (phenazines, sessilins and orfamides) which triggered innate immunity on bean (Figure 7.1). However, the role of these elicitors in inducing local resistance on bean root systems was not investigated in this study. Herein, we propose that *Pseudomonas* sp. CMR12a upon colonizing bean roots could mount local resistance against *R. solani* in the bean root system by secreting phenazines, orfamides and sessilins, although further experimental data are needed for this hypothesis.

7.2.3 Structure-activity relationships of CLPs derived from *Bacillus* and *Pseudomonas* sp.

CLPs have diverse structures, assigned to different classes, mainly due to the length and composition of peptide moiety. Even in the same class of CLPs, the composition of peptide moiety can vary. The structure-activity relationship of CLPs has not yet been intensively studied, especially for those from Gram-negative *Pseudomonas* species. There are several reports showing that Gram-positive (*Bacillus* sp.) derived CLPs are indeed showing structure-activity relationships.

Iturinic CLPs, including iturin, bacillomycin, and mycosubtilin, are important antibiotics produced by *Bacillus* sp. (Ongena and Jacques, 2008). It has been reported that bacillopeptin C is much better in the direct inhibition of yeasts and fungi, such as *Candida albicans*, *Saccharomyces cerevisiae*, *Fusarium oxysporum* and other species, compared with bacillopeptin A and B (Kajimura et al., 1995). Bacillopeptins share the same peptide moiety but differ from each other in the *β*-amino fatty acid residues, with C14, C15 and C16 for bacillopeptin A, B and C, respectively (**Table 7.2**). The same phenomenon has been observed for bacillomycins: the antifungal activity (for instance, against *Verticillium dahliae*) increased in homologues with a longer *β*-amino fatty acid residue (C16 > C15 > C14) within this group of CLPs (Eshita et al., 1995). Similar results were reported in a recent study showing that C17 bacillomycin D shows better antagonistic activity against *Botrytis cinerea* both under *in vitro* condition and on cucumber leaves, than C15 and C16 bacillomycin D (Tanaka et al., 2014). Mojavensins with length of C16 and C17 of *β*-amino fatty acid residues performed better than C14 mojavensin as antagonists against *Fusarium* species as compared by paper-disc diffusion assay (Ma and Hu, 2014). The structures of bacillomycin D and mojavensin are included in **Table 7.2**.

It has been shown that CLPs from the surfactin family show structure-function dependence in triggering defense related responses in cell suspensions. Surfactins with shorter *β*-hydroxy fatty acid residues, for instance, C12 and C13, were less active in triggering early defensive responses in cell suspensions, such as pH change and ROS production, as well as during interacting with plant plasma membrane, compared to surfactins with a longer fatty acid residue, for instance, C14 and C15 (Jourdan et al., 2009; Henry et al., 2011).

Taken all these results together, it appears that homologues with longer fatty acid residues reveal better biological activities than homologues with shorter fatty acid residues, within the same family of CLP (such as, iturin or surfactin family).

P. poae strain RE*1-1-14 (Zachow et al., 2015), *P. synxantha* CR32 (Nguyen et al., 2016), *P. protegens* and related species (Ma et al., 2016a) have the ability to produce orfamide type CLPs, these CLPs are structurally grouped as orfamides and poaeamides. The orfamide family of CLPs is composed of nine characterized structural homologues and several unknown congeners (**Chapter 3, Table 7.2**). Orfamides show variance not only in peptide moiety (for instance, orfamide A and orfamide B), but also in fatty acid residues (for instance, C12, C14 and C16 for orfamide C, orfamide A and orfamide G, respectively) (Table 7.2). These structural traits make the orfamide family a nice model to study the structure-function relationship of CLPs. We were interested with the structure-activity relationships of *Pseudomonas*-derived CLPs orfamides in this thesis. However, we could not find strong evidence in any of the interactions in this study for a structure-activity dependence among orfamide analogues. Does this phenomenon occur among orfamides? For instance, are orfamide homologues with a longer (for instance, C14 and C16) fatty acid tail more biologically active than the shorter (C12) ones? Does poaeamide, with a similar peptide sequence as orfamide but a shorter fatty acid residue (C10), have similar biological activities compared with orfamides? What about the differences of activities between poaeamide A and B? Further work still needs to be done.

Table 7.2 Selected natural CLPs discussed in this chapter. Bacillomycin D (Tanaka et al., 2014), mojavensin (Ma et al., 2012; Ma and Hu, 2014), bacillopeptin (Kajimura et al., 1995) and surfactin (Henry et al., 2011) are from *Bacillus* sp., while the orfamide family (including orfamides and poaeamides) is from *Pseudomonas protegens* and related species, *P. poae* RE*1-1-14 and *P. synxantha* CR32 (Gross et al., 2007; D'aes et al., 2014; Ma et al., 2016a; Zachow et al., 2015; Nguyen et al., 2016). The stereochemistry of orfamide A has been determined by Gross et al., 2007.

CLP	Fatty acid residue	Amino acid sequence									
		1	2	3	4	5	6	7	8	9	10
Bacillomycin D	C15:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Pro	L-Glu	D-Ser	L-Thr			
	C16:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Pro	L-Glu	D-Ser	L-Thr			
	C17:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Pro	L-Glu	D-Ser	L-Thr			
Mojavensin											
A	C14:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Gln	L-Pro	D-Asn	D-Asn			
B	C15:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Gln	L-Pro	D-Asn	D-Asn			
C	C16:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Gln	L-Pro	D-Asn	D-Asn			
D	C17:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Gln	L-Pro	D-Asn	D-Asn			
Bacillopeptin											
A	C14:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Ser	L-Glu	D-Ser	L-Thr			
B	C15:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Ser	L-Glu	D-Ser	L-Thr			
C	C16:0-NH_2 (3)	L-Asn	D-Tyr	D-Asn	L-Ser	L-Glu	D-Ser	L-Thr			
Surfactin											
	C12:0-OH (3)	L-Glu	L-Leu	D-Leu	L-Val	L-Asp	D-Leu	L-Leu			
	C13:0-OH (3)	L-Glu	L-Leu	D-Leu	L-Val	L-Asp	D-Leu	L-Leu			
	C14:0-OH (3)	L-Glu	L-Leu	D-Leu	L-Val	L-Asp	D-Leu	L-Leu			
	C15:0-OH (3)	L-Glu	L-Leu	D-Leu	L-Val	L-Asp	D-Leu	L-Leu			
Orfamide											
A	C14:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Ile	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val
B	C14:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Val	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val
C	C12:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Ile	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val
D	C12:0-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val
E	C14:1-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val
F	C16:1-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val
G	C16:0-OH (3)	Leu	Glu	aThr	Val	Leu	Ser	Leu	Leu	Ser	Val
Poaeamide											
A	C10:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Leu	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Ile
B	C10:0-OH (3)	L-Leu	D-Glu	D-aThr	D-Ile	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Ile

Recent evidence shows that the potential roles of *Bacillus*-derived CLPs (surfactin, fengycin and mycosubtilin) in triggering defense related responses in rice cell cultures are distinct (Chandler et al., 2015). These differences mainly occurred at the transcriptional level of marker genes responsible for multiple plant hormone pathways, which are related to dealing with biotic and abiotic stresses in plants. Moreover, we examined the potential transcript changes upon amendment of rice cell cultures with orfamides using multiple marker genes of hormones in this study. We found that all these four groups of CLPs (surfactin, mycosubtilin, fengycin and orfamide) can trigger distinct defensive responses in rice cell cultures. Therefore, we deduce that different classes of CLPs could trigger different responses in plants due to their structural diversity. More evidence should be provided to get deeper insights into structure-function relationships of different classes of CLPs.

Possibly the structure-activity relationship not only exists within the same family of CLPs (for instance, surfactin). We also want to know whether this kind of relationship exists within different classes of CLPs, for instance, CLPs-derived from *Bacillus* sp. and *Pseudomonas* sp. with different peptide moiety and fatty acid residue. These hypotheses should be confirmed experimentally, and thus lead to a better understanding of structure and function of CLPs.

7.2.4 Possible interaction between metabolites produced by *Pseudomonas* sp. CMR12a

The biosynthesis mutants of *Pseudomonas* sp. CMR12a used in this work have been generated in previous studies (D'aes et al 2011; D'aes et al 2014). We have measured the *in vitro* production of phenazines, sessilins and orfamides by the wild type strain *Pseudomonas* sp. CMR12a and mutants, on KB plates. Especially orfamide production showed variation among wild type and mutants (Figure 5.3). More specifically, orfamide production was significantly reduced in mutants CMR12a-Clp1 (sessilin mutant), CMR12aΔ*Phz* (phenazine mutant) and CMR12aΔ*Phz*-Clp1 (sessilin and phenazine mutant), compared with the wild type strain CMR12a.

The mutants of *Pseudomonas* sp. CMR12a have been made either by insertion of a marker gene in the sessilin biosynthetic genes or by deleting the phenazine biosynthetic genes, without affecting other genes (such as regulator genes or transporter genes) located in the flanking region of sessilin and phenazine biosynthetic genes. So, it is reasonable to expect that orfamide production in these mutants can be maintained at the same level compared to the wild type strain CMR12a. However, the

production of orfamides in these mutants is clearly reduced.

To sum up, either insertion of a gene in the sessilin biosynthetic cluster or deletion of genes in the phenazine biosynthetic cluster has led to a decreased production of orfamide in *Pseudomonas* sp. CMR12a. This phenomenon indicates that these metabolites (phenazine, sessilin and orfamide) in *Pseudomonas* sp. CMR12a influence each other and may be co-regulated, which means that disturbing the genetic composition of either sessilin or phenazine will reduce the production of orfamide. However, direct evidence is still needed to support this hypothesis and to further elucidate the in-depth mechanisms.

Summary

Cyclic lipopeptides (CLPs) are secondary metabolites composed of a cyclic peptide moiety linked with a fatty acid residue. CLPs harbor both hydrophobic and hydrophilic traits, and thus easily target on cellular membranes. They mediate diverse biological activities, including antibiosis, swarming motility, and biofilm formation. CLP-producing Gram-positive *Bacillus* species are well known as biocontrol agents against plant pathogens. Additionally, Gram-negative *Pseudomonas* species are also popular biocontrol agents. Some species of *Pseudomonas* bacteria are potent CLPs producers.

Fungal diseases are major threats to crop productivity worldwide. For instance, *Magnaporthe oryzae* and *Cochliobolus miyabeanus*, are the causal agents of rice blast and brown spot diseases, respectively. Fungi belonging to the *Rhizoctonia solani* group can also cause diseases on crops, for instance root rot disease on bean and Chinese cabbage, and web blight disease on bean leaves. Biocontrol of fungal diseases has been a research focus for several decades. However, the role of *Pseudomonas*-derived CLPs in the control of these fungal pathogens has barely been studied, and especially trials using purified CLPs in plant assays for biocontrol study are lacking.

Some *P. protegens* isolates, such as strains CHA0 and Pf-5, are well-studied biocontrol agents. Recently, it has been shown that *Pseudomonas* sp. CMR12a and CMR5c, biocontrol strains isolated from the roots of tropical tuber crop cocyam, are closely related to *P. protegens*. These four *Pseudomonas* strains all produce orfamides. Orfamides are CLPs composed of ten amino acids as peptide backbone and linked with *β*-hydroxy fatty acid residues that can vary in length. In addition, *Pseudomonas* sp. CMR12a can secrete sessilin-type CLPs that consist of eighteen amino acids as peptidic sequence linked to *β*-hydroxy fatty acid residues. *Pseudomonas* CLPs are nonribosomal peptide synthetase (NRPS) derived peptides, and NRPSs are normally composed of several modules with domains for peptide biosynthesis, adenylation (A) domain, condensation (C) domain and thioesterase (Te). The A domain of NRPS is responsible for recruiting an amino acid for peptide biosynthesis. Chemical analysis of metabolite extracts from these strains, together with A domain

phylogeny analysis of these orfamide synthetases, showed that the production of orfamide analogues in these strains is strain dependent, *P. protegens* CHA0 and Pf-5 mainly produce orfamide A (with a D-isoleucine in the fourth position of the peptide sequence) and its homologues, while *Pseudomonas* sp. CMR5c and CMR12a mainly produce orfamide B (with a D-valine in the fourth position of the peptide sequence) and its homologues. Additionally, *Pseudomonas* sp. CMR5c and CMR12a produce phenazines, and the major phenazine compound is PCN.

Orfamide and phenazine-type pigment PCN have fungicide activity for controlling blast disease caused by *M. oryzae* in rice, and these two compounds show distinct mode of actions. Orfamide can inhibit appressoria formation of *M. oryzae* on rice sheaths, and thus reduce severity of blast disease on rice leaves at the concentration of 50 μM. Specific concentrations of PCN, for instance, 0.1 or 1 μM, have the capacity to stimulate the innate immunity of rice, and thus provides barriers for further blast invasion.

It was investigated whether structural differences in orfamides may influence biological activities. Orfamide A, B and G showed similar performances in inhibition of appressoria formation both under *in vitro* and *in vivo*, and in the control of *M. oryzae* on rice leaves. These three compounds also showed similar activities in changing the morphology of *R. solani*, and causing zoospore lysis of *Phytophthora* and *Pythium*.

Orfamide, sessilin, and PCN are important metabolites involved in the control of *Rhizoctonia* root rot disease on bean and Chinese cabbage, and these metabolites showed direct antibiotic activities on the hyphal development of *R. solani*. Interestingly, both CLPs are needed for the successful control of *R. solani*. Orfamide, sessilin and PCN can also function as elicitors to stimulate innate immunity on bean plants against web blight disease. Root application of their producing strain *Pseudomonas* sp. CMR12a also triggers a resistance response on bean against web blight pathogen *R. solani*. The resistance triggered by *Pseudomonas* sp. CMR12a and its mutants, however, needs a balanced production of orfamide, sessilin or PCN, in a specific range of concentrations, while higher or lower concentrations are not effective.

Orfamide cannot trigger immune responses against *M. oryzae* in rice. However, induced systemic

resistance (ISR) by elicitors are dependent on the pathogen and plant system used. Indeed, orfamide triggered an ISR response against another rice pathogen *C. miyabeanus*, which is the causal agent of brown spot disease, but a threshold concentration of 25 µM orfamide was needed to see this effect. Likewise, orfamide-producing bacteria can only trigger this kind of immune response when applied at high concentrations (> 10^8 CFU/mL) to plant roots. Orfamide has the ability to trigger a set of defensive responses in rice cell cultures, including early defense events, for instance pH change, and transcription of defense-related genes, such as those coding for phenylalanine ammonia lyase (*PAL*), lipoxygenase (*LOX*), *Chitinase* and *β-Glucanase* in rice cell cultures. Further transcript analysis showed that priming of the abscisic acid pathway, the transcriptional factor *WRKY4*, and pathogenesis-related (PR) protein *PR1b* are upregulated in the late stage of this kind of immune response in rice plants.

This study, showed for the first time, the biocontrol role of orfamide against *M. oryzae* and *C. miyabeanus* in rice, by ways of direct interaction and ISR, respectively. And this study also provides evidence that sessilins and orfamides control root rot and web blight diseases caused by *R. solani* on bean by direct antagonistic effects and ISR. This study has shed new light on the role of CLPs produced by *Pseudomonas* spp. in biocontrol and may stimulate further research about the use of these CLPs to control plant pathogens on other crops, which may ultimately lead to practical biocontrol applications.

Samenvatting

Cyclische lipopeptiden (CLPs) zijn secundaire metabolieten die samengesteld zijn uit een cyclisch peptide gedeelte gekoppeld aan een vetzuurstaart. CLPs hebben zowel hydrofobe als hydrofiele eigenschappen en kunnen interageren met cellulaire membranen. Ze spelen een rol in diverse biologische activiteiten zoals antibiose, zwermende motiliteit en biofilm vorming. CLP-producerende, endospore vormende *Bacillus* soorten zijn welbekend als biologische bestrijders van plant pathogenen. Ook Gramnegatieve *Pseudomonas* soorten zijn populair als biologische bestrijders. Sommige soorten binnen de *Pseudomonas* bacteriën kunnen ook CLPs produceren.

Schimmelziektes vormen wereldwijd een grote bedreiging voor de productiviteit van gewassen. Zo zijn *Magnaporte oryzae* en *Cochliobolus miyabeanus* de verwekkers van respectievelijk “blast” en “brown spot” bij rijst. Schimmels die behoren tot de *Rhizoctonia solani* groep kunnen ook plantenziektes verwekken zoals wortel rot op boon en Chinese kool, en “web blight” op de bladeren van boon. Biologische bestrijding van schimmelziektes wordt reeds lang onderzocht. De rol van CLPs geproduceerd door *Pseudomonas* soorten in de bestrijding van schimmelpathogenen is echter nauwelijks onderzocht. Vooral plant experimenten waarin biologische bestrijding met opgezuiverde CLPs wordt nagegaan, mankeren.

Sommige *P. protegens* isolaten (zoals CHA0 en Pf-5) werden reeds intensief bestudeerd voor hun rol in biologische bestrijding. Recent werd aangetoond dat *Pseudomonas* sp. CMR12a en CMR5c, biologische bestrijders die werden geïsoleerd van de wortels van de tropische knolvormende plant cocoyam, taxonomisch verwant zijn aan *P. protegens*. Deze vier Pseudomonas stammen maken allemaal orfamides. Orfamides zijn CLPs die zijn samengesteld uit een peptide van 10 aminozuren gekoppeld aan een *β*-hydroxy vetzuurstaart. Daarnaast kan *Pseudomonas* sp. CMR12a nog een tweede CLP aanmaken, sessiline, dat bestaat uit 18 aminozuren gekoppeld aan een β-hydroxy vetzuurstaart. CLPs worden geproduceerd via niet-ribosomale peptide synthetases (NRPS) die zijn samengesteld uit verschillende domeinen zoals een adenylatie (A) domein, een condensatie (C)

domein en een thioesterase (TE). De A domeinen van de NRPS zijn verantwoordelijk voor het recruteren van specifieke aminozuren die worden ingebouwd in de peptide keten. Chemische analyse van de ruwe orfamide extracten geproduceerd door de vier *Pseudomonas* stammen en fylogenetische analyse van de A domeinen van hun NRPS toonden aan dat deze stammen verschillende homologen van orfamides aanmaken. *P. protegens* CHA0 en Pf-5 maken preferentieel orfamide A aan (met een D-isoleucine in de vierde positie in de peptide sequentie), terwijl *Pseudomonas* sp. CMR5c en CMR12a vooral orfamide B produceren (met een D-valine in de vierde positie van de peptide keten). Daarnaast produceren *Pseudomonas* CMR12a en CMR5c fenazines, de belangrijkste fenazine component die wordt geproduceerd door deze twee stammen is PCN.

Orfamide en het fenazine PCN vertonen antifungale activiteit tegen *M. oryzae* in rijst, maar ze verschillen in hun werkingsmechanisme. Orfamide aan een concentratie van 50 µM kan de vorming van appressoria door *M. oryzae* op de rijstschede inhiberen en zo de ernst van de ziekte aantasting verminderen. PCN aan een concentratie van 0.1 of 1 µM is in staat in rijst planten geïnduceerde resistentie tegen *M. oryzae* op te wekken en kan zo de verdere invasie van de pathogeen afremmen.

Er werd ook nagegaan of de structurele verschillen in de orfamides hun biologische activiteit kunnen beïnvloeden. Orfamide A, B en G vertoonden dezelfde activiteit tegen appressoria van *M. oryzae* in *in vitro* en *in vivo* condities. Deze drie componenten waren ook even actief in het veranderen van de morfologie van *R. solani* hyfen en in het lyseren van zoosporen van *Phytophthora* en *Pythium*.

De CLPs orfamide en sessiline, samen met PCN bleken ook belangrijke metabolieten te zijn in de bestrijding van *Rhizoctonia* wortelrot op boon en Chines kool. Deze metabolieten hadden een directe antifungale activiteit op de hyfe ontwikkeling van *R. solani*. Interest genoeg bleek dat beide CLPs nodig zijn voor een succesvolle bestrijding van *R. solani*. Orfamide, sessiline en PCN bleken ook de functioneren als elicitoren and stimuleerden geïnduceerde resistentie tegen “web blight” op bonenplanten. Ook wortelapplicatie van de *Pseudodomonas* sp. CMR12a stam die deze metabolieten produceert, induceerde resistentie in boon tegen de “web blight” pathogeen *R. solani.* Een uitgebalanceerde productie van orfamide, sessiline en PCN, in een specifieke concentratie bereik is echter nodig voor de inductie van resistentie, waarbij hogere of lagere concentraties niet effectief bleken.

Orfamide bleek niet in staat resistentie te induceren tegen *M. oryzae* in rijst. Het is echter geweten dat de inductie van systemische resistentie door elicitoren afhankelijk is van de pathogeen en de waardplant die worden gebruikt. We konden inderdaad aantonen dat orfamide in staat is geïnduceerde resistentie op te wekken tegen de schimmel pathogeen *C. miyabeanus*, de verwekker van "brown spot disease" op rijst. Minimum 25 µM orfamide was echter nodig om de resistentie te kunnen opwekken. Hiermee in overeenstemming bleek dat orfamide-producerende bacteriën enkel in staat zijn resistentie tegen *C. miyabeanus* op te wekken wanneer ze aan een hoge densiteit ($>10^8$ CFU/mL) werden toegediend aan plantenwortels. Orfamide bleek in rijst celculturen verschillende afweerreacties op te wekken zoals veranderingen in pH en inductie van de transcripten van genen betrokken in plantafweer zoals genen coderend voor fenyl ammonium lyase (PAL), lipoxygenase (LOX), chitinase en β-glucanase. Verdere transcript analyse toonde aan dat in een later stadium, the abscissinezuur biosynthese weg, de transcriptionele activator WRKY4 en het ziektegerelateerde eiwit PR1b betrokken zijn in de immuunrespons van rijst tegen *C. miyabeanus*.

Deze studie heeft voor de eerste maal aangetoond dat orfamide een rol speelt in de biologische bestrijding van *M. oryzae* en *C. miyabeanus* op rijst, respectievelijk door een directe interactie of door het induceren van systemische resistentie. Ook heeft deze studie het bewijs geleverd dat sessilines en orfamides betrokken zijn in de biologische bestrijding van wortel rot en "web blight" veroorzaakt door *R. solani* op boon, en dit door direct antagonisme en geïnduceerde resistentie. Deze studie heeft nieuw licht geworpen op de rol van CLPs, geproduceerd door *Pseudomonas* spp., in biologische bestrijding en kan wellicht verder onderzoek stimuleren naar het gebruik van deze CLPs voor de bestrijding van plantenziekten in andere gewassen. Uiteindelijk kan dit leiden tot praktische toepassingen in biologische bestrijding.

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Curriculum Vitae

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Education and Professional Records

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Original Research Articles (peer-reviewed)

1. **Zongwang Ma**, Nan Wang, Jiangchun Hu*, Shujin Wang. Isolation and characterization of a new iturinic lipopeptide, mojavensin A produced by a marine-derived bacterium *Bacillus mojavensis* B0621A. ***Journal of Antibiotics*** (2012) 65(6), 317-322 (Q2; IF2012=2.191)

2. **Zongwang Ma**, Jiangchun Hu*, Xuemei Wang, Shujin Wang. NMR Spectroscopic and MS/MS Spectrometric Characterization of a New Lipopeptide Antibiotic Bacillopeptin B_1 Produced by a Marine Sediment-Derived *Bacillus Amyloliquefaciens* SH-B74. ***Journal of Antibiotics*** (2014) 67(2): 175-178 (Q2; IF2014=1.730)

3. **Zongwang Ma**, Jiangchun Hu*. Production and Characterization of Iturinic Lipopeptides as Antifungal Agents and Biosurfactants Produced by a Marine *Pinctada Martensii*-Derived *Bacillus Mojavensis* B0621A. ***Applied Biochemistry and Biotechnology*** (2014) 173(3): 705-715 (Q2; IF2014=1.735)

4. Feyisara Eyiwumi Olorunleke[†], Gia Khuong Hoang Hua[†], Nam Phuong Kieu, **Zongwang Ma** and Monica Höfte*. Interplay between orfamides, sessilins and phenazines in the control of *Rhizoctonia* diseases by *Pseudomonas* sp. CMR12a. ***Environmental Microbiology Reports***. (2015) 7(5):774-781 (Q1; IF2015= 3.5)

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6. **Zongwang Ma**, Niels Geudens, Nam Phuong Kieu, Davy Sinnaeve, Marc Ongena, José C. Martins, Monica Höfte*. Biosynthesis, chemical analysis and structure-activity relationship of orfamide lipopeptides produced by *Pseudomonas protegens* and related species. ***Frontiers in Microbiology*** (2016) 7: 382 (Q1; IF2015= 4.126)

7. **Zongwang Ma**†, Gia Khuong Hoang Hua†, Monica Höfte*. Role of phenazines and cyclic lipopeptides produced by *Pseudomonas* sp. CMR12a in induced systemic resistance on rice and bean. ***Environmental Microbiology Reports***. (2016) doi: 10.1111/1758-2229.12454. (Q1; IF2015= 3.5)

Note: '†' = equal contributor; '*' = author for correspondence

Manuscripts Submitted or In Preparation

8. Pascale Flury, Pilar Vesga, Maria Péchy-Tarr, Nora Aellen, Karent Bermudaz, Francesca Dennert, Nicolas Hofer, Peter Kupferschmied, Zane Metla, **Zongwang Ma**, Sandra Siegfried, Sandra de Weert, Guido Bloemberg, Monica Höfte, Christoph Keel* and Monika Maurhofer*. Antimicrobial and insecticidal: cyclic lipopeptides and hydrogen cyanide produced by plant-beneficial *Pseudomonas* strains CHA0, CMR12a and PCL1391 contribute to insect killing. (2016) ***Frontiers in Microbiology***. (Under review)

9. **Zongwang Ma**, Marc Ongena and Monica Höfte*. *Orfamides induce systemic resistance in rice to Cochliobolus miyabeanus but not to Magnaporthe oryzae*. (In preparation)

Patent

Jiangchun Hu, **Zongwang Ma**, Shujin Wang. Production and application of antifungal lipopeptides from a marine-derived *Bacillus mojavensis* (Application number: 201210054010.4 [China]), Authorized: 24th June 2015.

Symposiums and Conferences

With contribution

1. **Zongwang Ma**, Jiangchun Hu, Nan Wang, Shujin Wang. New lipopeptide antibiotic produced by a marine-derived bacteria *Bacillus mojavensis* B0621A. Annual Conference of Chinese Society for Microbiology (CSM). Fu Zhou, China, 2011. P 328-328. (Abstract)

2. **Zongwang Ma**, Jiangchun Hu. "Lipopeptides Produced by a Marine-Derived Bacterium *Bacillus Mojavensis* B0621A." In 3rd International Symposium on Antimicrobial Peptides, Lille

(Villeneuve d'Ascq), France. **2012** (Poster Presentation).

3. Jiangchun Hu, Nan Wang, **Zongwang Ma**, Lili Chen, Huaqi Pan, Xuemei Wang, Shujin Wang. New antifungal lipopeptides characterized from marine-derived *Bacillus* species. The 9th Marine Biotechnology and Pharmacy of China. Chi Feng, Inner Mongolia, China. 2014. (Abstract)

4. **Zongwang Ma**, Niels Geudens, Nam Phuong Kieu, Davy Sinnaeve, José C. Martins, Marc Ongena, Monica Höfte. "Biocontrol properties of orfamide-type lipopeptides produced by fluorescent pseudomonads against the fungal plant pathogens *Magnaporthe oryzae* and *Rhizoctonia solani*." 67th International Symposium on Crop Protection, Gent, Belgium, 2015 (Oral presentation)

5. Gia Khuong Hoang Hua, **Zongwang Ma** and Monica Höfte. *Pseudomonas* sp. CMR12a triggers induced systemic resistance against *Rhizoctonia solani* in bean and *Magnaporthe oryzae* in rice. 10th International PGPR Workshop, Liege, Belgium, 2015 (Oral presentation)

6. **Zongwang Ma** and Monica Höfte. Genome mining of gene clusters for secondary metabolites biosynthesis in *Pseudomonas* sp. CMR5c. Big N2N Annual Symposium 2016, Gent, Belgium, 2016 (Poster presentation)

Without contribution

1. 65th International Symposium on Crop Protection, Gent, Belgium, 2013

2. 66th International Symposium on Crop Protection, Gent, Belgium, 2014

3. 'Merging ecology and biotechnology for a sustainable agriculture', Gent, Belgium, 2015

4. 'Biostimulants and Biological Control', Gent, Belgium, 2016

5. 68th International Symposium on Crop Protection, Gent, Belgium, 2016

Awards

1. 100 Outstanding Master Thesis Awards of Liaoning Province, China, 2012 (the total number of Master Thesis is 27,110).

2. PhD scholarship for studying abroad from Chinese Scholarships Council, China (2012-2016).

3. Special research fund (Bijzonder Onderzoeksfonds, BOF) from Ghent University, Belgium (2013-2016).

Other Academic Activities

Reviewer for: Transactions of Tianjin University (Springer).