Cyclic lipopeptides produced by *Pseudomonas* spp. associated with the cocoyam (*Xanthosoma sagittifolium* (L.) Schott) rhizosphere: diversity, regulation, secretion and biological activity

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the degree of Doctor of Philosophy (PhD) in
Applied Biological Sciences

Dutch translation of the title:

Cyclische lipopeptiden geproduceerd door *Pseudomonas* spp geassocieerd met de rhizosfeer van cocoyam (*Xanthosoma sagittifolium* (L.) Schott): diversiteit, regulatie, secretie en biologische activiteit.

Cover illustration:

Swarming motility of various cyclic lipopeptides analysed during this thesis, interspersed with their names.

Cite as:

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If we knew what it was we were doing it would not be called research, would it? – Albert Einstein.

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Contents

List of abbreviations

A	Adenylation
AA	Amino acid
ABC	ATP binding cassette
ACN	Acetonitrile
AFLP	Amplified fragment length polymorphism
AG	Anastomosis group
Amp	Ampicillin
ATP	Adenosine triphosphate
aThr	allo-Threonine
BCA	Biological control agent
BLAST	Basic local alignment search tool
Bp	base pair
C	Condensation
Ca	Calcium
CFU	Colony forming unit
CLP	Cyclic lipopeptide
CLP1	Sessilin
CLP2	Orfamide
^{13}C NMR	Carbon-13 nuclear magnetic resonance
Cm	Chloramphenicol
CMC	Critical micelle concentration
CRRD	Cocoyam root rot disease
cv.	Cultivar
Da	Dalton
DAP	Diaminopimelate
DAPG	2, 4-diacetylphloroglucinol
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
ESI-MS	Electrospray ionisation mass spectrometry
FAO	Food and agricultural organisation
GBDP	Genome Blast Distance Phylogeny
GGDC	Genome-to-genome distance
Glx	Glutamine or glutamate
Gm	Gentamycin
HCl	Hydrogen choride
HCN	Hydrogen cyanide
^{1}H NMR	Proton nuclear magnetic resonance
HMBC	Heteronuclear multiple-bond correlation
HP	Hypothetical protein
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single-quantum correlation
IM	Inner membrane
IPM	Integrated pest management
ISR	Induced systemic resistance
ITS	Internal transcribed spacer region of DNA
K	Potassium
KB	King's B medium
Km	Kanamycin
LB	Luria Bertani medium
LC	Liquid chromatography
MFP	Membrane fusion protein
Mg	Magnesium
MHz	Megahertz (10^6 Hz)
MLSA	Multilocus sequence alignment
MS	Mass spectroscopy
MS	Murashige and Skoog medium
MW	Molecular weight
Na	Sodium
NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthetase
OD	Optical density
OM	Outer membrane
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
PDI	Percent Disease Index
pH	Potential of hydrogen
P	Phosphorus
Plt	Pyoluteorin
Phz	Phenazine
PKS	Polyketide synthetase
rpm	Rotations per minute
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RND	Resistance nodulation division
ROESY	Rotating-frame nuclear overhauser effect spectroscopy
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
T	Thiolation
Tc	Tetracycline
TETRA	Tetranucleotide usage patterns
Te	Thioesterase
TOCSY	Total correlation spectroscopy
UPLC	Ultra-high performance liquid chromatography-mass spectrometry
UV	Ultra violet
WLIP	White-Line-Inducing Principle
WT	Wild type
YPD	Yeast-extract-peptone-dextrose

Problem statement and research outline

Cocoyam (*Xanthosoma sagittifolium*), belonging to the family of aroids, is one of the six most important root and tuber crops grown worldwide (Onwueme and Charles, 1994). It serves as a carbohydrate staple for more than 400 million people living in the tropics and subtropics. Worldwide, Sub-Sahara Africa is the major cocoyam growing region with Nigeria, Cameroon and Ghana as some of the leading producers (FAOSTAT). The corm, cormels and leaves are an important source of nutrition for humans, animal feed and also provide a source of income for farmers (Castro, 2006). In spite of its importance in countries where it is grown, cocoyam yield has dramatically declined due to the root disease which causes severe crop losses. First reported since 1981, the cocoyam root rot disease (CRRD) dilemma has persisted after more than three decades without a sustainable management strategy in sight. Cocoyam has been fast fading away in local diets and this calls for urgent solutions to arrest this disease. More so, the management of CRRD has commanded a lot of attention in recent times owing to the Taro Leaf Blight disease which is gradually taking over taro (*Colocasia esculenta*) fields in Africa and beyond. Thus, there is an urgent need to save our aroids (cocoyam and taro) and ensure that a major carbohydrate source is not entirely eroded.

CRRD is caused by an oomycete, *Pythium myriotylum* (Pacumbaba *et al.,* 1992a; Tojo *et al.,* 2005). Fungicides have not been fully successful in the control of this disease (Onokpise *et al.,* 1999). Past efforts towards breeding for resistant cocoyam varieties have not yielded much results and hybridization is difficult since cocoyam rarely flowers (Brown 1988; Onokpise *et al.,* 1999). The use of rhizosphere organisms for biological control has been exploited for the management of diseases caused by *Pythium* spp. (Weller *et al.,* 2002). Fluorescent pseudomonads have been much utilized in the successful biocontrol of plant pathogens because they produce a broad arsenal of antibiotics (Haas and Défago, 2005). *P. aeruginosa PNA1* could successful control CRRD through its production of phenazine and rhamnolipid biosurfactants (Tambong and Höfte, 2001; Perneel *et al.,* 2008).

In a previous project (2001-2006) which was funded by the Flemish government (VLIR), attempts were made to develop an ecologically sustainable method for the management of CRRD in Cameroon. Results of this project highlighted the need for improved cultural practices and amendment of soil with composts in order to enhance soil organic matter (Adiobo, 2006). During this study, suppressive and conducive soils towards CRRD were identified in Cameroon. Although factors driving CRRD suppressiveness of soils were yet to be elucidated, preliminary results suggested the involvement of soil physicochemical properties and microbial community composition (Adiobo *et al.,* 2007).

In a concurrent study, Perneel and colleagues (2007) isolated fluorescent pseudomonads from the rhizosphere of red and white cocoyams in Cameroon. Intriguingly, when tested against CRRD, isolates which showed excellent disease suppressiveness were obtained from the red cocoyam rhizosphere and appeared to produce phenazine antibiotics. Furthermore, two of these isolates, *Pseudomonas* sp. CMR12a and CMR5c, were characterized further. Results showed that besides phenazines, CMR12a and CMR5c produces cyclic lipopeptides which have partially been shown to be involved in the biocontrol capacity of each strain (D'aes *et al.,* 2011; D'aes *et al.,* 2014; Ma *et al.,* 2016a). Interestingly, red cocoyams have been observed to show a level of field tolerance towards

CRRD in Cameroon. Thus, it was hypothesized that red cocoyam roots may be colonized by certain *Pseudomonas* strains that produce antimicrobial compounds.

Pseudomonas sp. CMR12a was previously isolated from Cameroon. This strain produces phenazines (PCA and PCN) and two classes of cyclic lipopeptides (sessilins and orfamides) (Perneel *et al.,* 2007; D'aes *et al.,* 2011; D'aes *et al.,* 2014). Knowledge about the regulation and secretion of CLPs are important in order to obtain insights into how these CLPs are produced and their physiological relevance towards their producing strains. For CMR12a, genes involved in the regulation and secretion of its CLPs are yet to be elucidated.

In view of the afore-going, this PhD research was started with the following questions:

1. Is *P. myriotylum* the primary causal pathogen of CRRD in Nigeria?
2. Do phenazines and cyclic lipopeptides play a role in the biocontrol capacity of *Pseudomonas* sp. CMR12a against CRRD?
3. Is the regulation of orfamide and sessilins mediated by LuxR-type regulators flanking the orfamide and sessilin biosynthetic gene cluster, respectively?
4. How are sessilins and orfamides secreted in CMR12a?
5. Is *Pseudomonas* diversity on the cocoyam roots driven by plant variety?
6. What are potential drivers of *Pseudomonas* and CLP diversity in the cocoyam rhizosphere in Nigeria and Cameroon?

In **chapter 1**, we reviewed the literature to summarize and introduce the background and research topics discussed in chapters 2-7. We also included a brief introduction to the main pathosystem used in this study, *P. myriotylum* on cocoyams.

In **chapter 2**, we investigated the primary causal pathogen of the cocoyam root rot disease in Nigeria. Our results show that similar to findings in Cameroon, Costa-Rica and Sri-Lanka, the primary pathogen that causes CRRD in Nigeria is *P. myriotylum*.

Pseudomonas sp. CMR12a is an effective biocontrol strain that produces two CLPs (sessilins and orfamides) and phenazines (PCA and PCN). Sessilins, orfamides and phenazines have been implicated in the direct antagonism of CMR12a against *Rhizoctonia solani* on bean and Chinese cabbage. Furthermore, previous results showed the biocontrol efficacy of CMR12a against CRRD. Thus in **chapter 3**, we examined the role of sessilins, orfamides and PCN in the biocontrol of CMR12a against root rot of cocoyams caused by *P. myriotylum*. Here, we used CMR12a and its biosynthetic mutants in *in vivo* assays while pure orfamide A, PCN and crude extracts of sessilins were utilized in *in vitro* microscopic assays. Our results showed that sessilins, orfamides and PCN are implicated in the biocontrol of CMR12a.

In CMR12a, two LuxR-type transcriptional regulators flank the orfamide biosynthetic gene cluster. OfaRup is located upstream while OfaRdown is situated downstream of the gene cluster. On the other hand, the sessilin gene cluster has only one LuxR regulator, SesR, upstream of its CLP biosynthetic cluster. **In chapter 4**, we investigated the roles of these three LuxR-type proteins in the regulation of orfamides and sessilins biosynthesis. We show that orfamides and sessilins are co-regulated by the LuxR regulators flanking the orfamide gene cluster (OfaRup and OfaRdown) while the role of the SesR remains unknownOur reslts revealed that although sessilins and orfamides are co-produced starting from the late exponential phase, sessilins are secreted earlier and in large amounts, while orfamides are predominantly retained in the cell.

In chapter 5, we investigated the evolutionary relationship between the OprM homologue (NodT), situated upstream of certain CLP biosynthesis gene clusters and their corresponding OprM. Additionally, using site-directed mutagenesis, we studied the roles of putative CLP efflux proteins including the MexAB-OprM, MacAB and the NodT, in sessilin and orfamide secretion. We show that *nodT* appears to be a gene duplication of the *oprM*. Furthermore, sessilins evidently monopolises the use of all secretion channels studied especially of the *mexAB* and *macAB* genes, at the expense of orfamide secretion. However, genes involved in orfamide secretion remain unclear.

Myriads of biotic and abiotic factors are considered to be influencers of structural and functional diversity of microbial communities in the rhizosphere. In **chapters 6 and 7**, we investigate the taxonomic diversity and CLP characteristics of fluorescent pseudomonads isolated from cocoyam roots in Cameroon and Nigeria. In **chapter 6**, our results from Cameroon show that the cocoyam rhizosphere in low disease pressure soils of Boteva were significantly enriched in *P. fluorescens* complex isolates while the cocoyam rhizosphere in intermediate disease-pressure soils of Ekona and high disease-pressure soils of Maumu were enriched in *P. putida* group isolates. In **chapter 7**, similar results were obtained in conducive soils of Ado–Ekiti and Umudike, Nigeria, where species obtained were belonging to the *P. putida* group and *P. aeruginosa* lineage. Interestingly, in all five soils studied, physicochemical properties were linked with soil suppressiveness whereas *Pseudomonas*/CLP diversity appeared to diminish with depleted soil organic matter.

In **chapter 8**, the main findings of this study are discussed.

The **Addendum** at the end of this thesis features the results of a study to elucidate the roles of sessilins, phenazines and orfamides on the control of *Rhizoctonia* diseases on bean and Chinese cabbage. Plant experiments were conducted using CMR12a and its deletion mutants in one, two or all three metabolites and by employing purified orfamide and PCN. Our results showed that the disease suppressive effect of CMR12a was due to its production of sessilins, orfamides and phenazines.

Chapter 1

General introduction

A part of this chapter was published in:

Recent advances in *Pseudomonas* biocontrol (2015) In Bacterial-Plant Interactions: Advance Research and Future Trends. Murillo, J., Vinatzer, B. A., Jackson, R. W., and Arnold, D. L. (eds). Cambridgeshire: Caister Academic Press. Pp 167-198.

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Fluorescent Pseudomonas spp. as biocontrol agents

Introduction

Biopesticides based on the articifial introduction of living microorganisms into a plant system are an alternative or supplementary way of reducing the use of chemical pesticides in agriculture. Biocontrol can be very useful when no alternative disease control strategies are available or in organic farming where the use of chemical pesticides is not allowed. There is also a genuine commercial interest in biopesticides because they can be used in rotation with chemical pesticides to reduce the development of pathogen resistance. The use of fluorescent *Pseudomonas* spp. as biocontrol agents has intensively been studied. They can be readily isolated from soil or the rhizosphere of plants, and are easily recognized on iron-limiting medium by the production of a yellow-green fluorescent pigment, called pyoverdine or pseudobactin, which acts as a siderophore or iron-chelating compound (Meyer, 2000). The bacteria exhibit an enormous metabolic versatility and some isolates produce a remarkable spectrum of secondary metabolites (Gross and Loper, 2009). Moreover, the modes of action are relatively easy to study in *Pseudomonas* spp. because these bacteria are amenable to mutation and modification using genetic tools (Chin-A-Woeng *et al*., 2003).

The most commonly reported mechanisms of biocontrol by fluorescent *Pseudomonas* spp. include antibiosis (Gross and Loper, 2009; Haas and Défago, 2005), competition for nutrients and niches (Kamilova *et al*., 2005), competition for iron mediated by siderophores (Leong, 1986), and induced systemic resistance (De Vleesschauwer and Hofte, 2009). Additionally, many rhizosphere fluorescent *Pseudomonas* spp. can promote plant growth in the absence of pathogens by increasing the availability and uptake of mineral nutrients via phosphate solubilising enzymes or by enhancing root growth and morphology via the production of phytohormones (Lugtenberg and Kamilova, 2009).

Taxonomy

The genus Pseudomonas belongs to the class of *Gammaproteobacteria* within the phylum *Proteobacteria*. *Pseudomonas* is one of the most ubiquitous bacterial genera and different species have been isolated from very diverse ecological niches. Since its discovery, the genus *Pseudomonas* has undergone numerous taxonomic changes. A detailed history of *Pseudomonas* taxonomy until 2009 is given by Peix *et al*., (2009). Currently, only the representatives of rRNA group I according to Palleroni *et al*. (1973) are included in the genus *Pseudomonas*. The number of *Pseudomonas* species is continuously increasing; up to now, more than 144 species have been validly described (Gomila *et al.,* 2015). Updates can be found on the DSMZ website (www.dsmz.de). Most *Pseudomonas* species are saprophytes that are commonly found in water and soil, but the genus also includes species that are associated with diseases in plants (e.g. *P. syringae*, *P. cichorii*, *P. viridiflava*, *P. corrugata*), mushrooms (e.g. *P. tolaasii*, *P. agarici*), humans and animals (e.g. *P. aeruginosa*) (Peix *et al.,* 2009). Within the *Pseudomonas* genus, two main lineages or intrageneric groups can be recognized, the *Pseudomonas aeruginosa* lineage (which includes the *P. aeruginosa*, *P. oleovorans* and *P. stutzeri* group) and the *P. fluorescens* lineage (Figure 1.1).

Based on phylogenetic analysis of 16S rRNA, *gyrB*, *rpoB* and *rpoD* sequences, Mulet *et al.* (2010) recognized six groups within the *P. fluorescens* lineage: the *P. fluorescens, P. syringae, P. lutea, P. putida, P. anguilliseptica* and *P. straminea* group. The *P. fluorescens* group appears to be highly diverse and contains more than 50 named species divided over nine subgroups (Mulet *et al.,* 2010). Silby *et al.* (2009) were actually the first to show that the *P. fluorescens* group is highly diverse. They compared three *P. fluorescens* genomes and discovered that only 61% of genes were shared. Loper *et al.* (2012) conducted a comparative genomic analysis of ten biocontrol strains within the *P. fluorescens* group and found that the core genome only represents 45-52% of the genome of any individual strain. Multilocus sequence analysis (MLSA) placed the strains in three main subclades. These studies showed that the *P. fluorescens* group is a species complex that is clearly in need of revision.

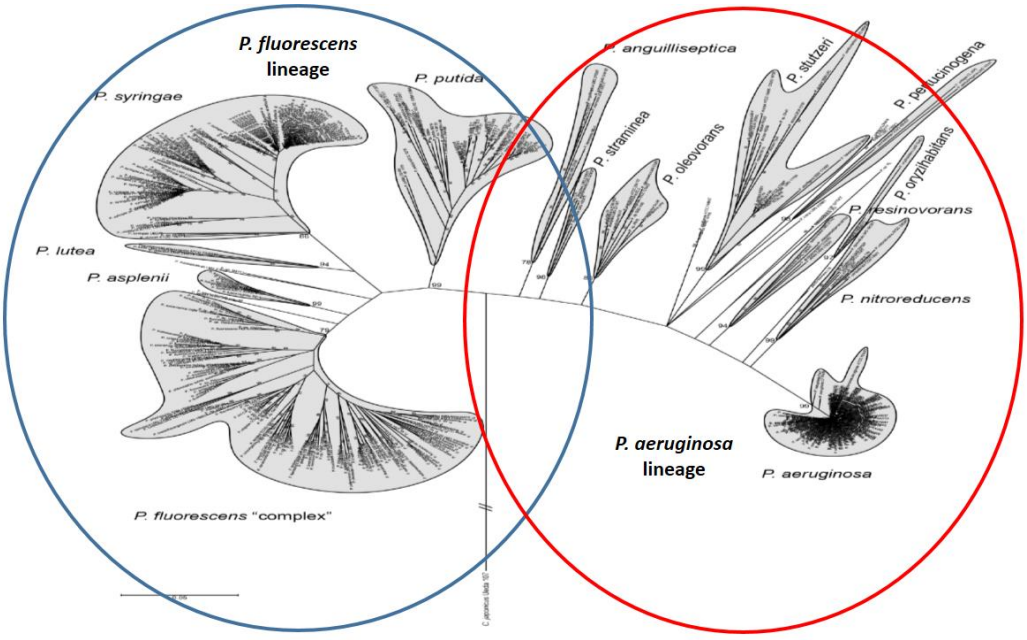

Figure 1.1. Phylogeny of the *Pseudomonas* genus inferred by MLSA. Phylogenetic tree of 451 *Pseudomonas* strains along with 107 type strains based on the concatenated partial sequences of the 16S rDNA, *gyrB*, *rpoD* and *rpoB*, ML method with *C. japonicus* Ueda 107 as the outgroup. Picture adapted from Garrido-Sanz *et al*. (2016). The *P*. *fluorescens* lineage and the *P. aeruginosa* lineage are indicated in blue and red, respectively.

More recently, the MLSA of *Pseudomonas* spp. conducted by Gomila *et al.* (2015) further confirmed some results of Mulet *et al.* (2010). Their work described the *P. fluorescens* group of Mulet *et al.* (2010), as a complex. Using the MLSA, they placed the *P. fluorescens* complex, *P. syringae, P. lutea, P. putida, P. lutea and P. asplenii* within the *P. fluorescens* lineage whereas *P. anguilliseptica*, *P. straminae*, *P. oleovorans*, *P. stutzeri* and *P. aeruginosa* among others were situated within the *P. aeruginosa* lineage (Figure 1.1). Additionally, these authors were able to compare MLSA with indices proposed for species delineation such as tetranucleotide usage patterns (TETRA), average nucleotide identity (ANIm based on MUMmer and ANIb, based on BLAST) and genome-to-genome distance (GGDC). Although TETRA was useful in discrimination of *Pseudomonas* from other genera, ANIb and GGDC showed clear separation of different species. In all, the strongest correlation to MLSA was shown by the ANIb method.

Furthermore, work done by Garrido-Sanz and colleagues, (2016) attempted to demystify the *Pseudomonas fluorescens* complex by investigating the genomic and genetic diversity of members within this group. More so, a correlation between the taxonomy and phylogenomic analysis within the *P. fluorescens* complex was conducted. The Genome Blast Distance Phylogeny (GBDP) method and ANIb were employed for the analysis of whole genomes. Using MLSA and whole-genome sequence-based analyses of 93 strains, the *P. fluorescens* complex was divided into nine phylogenomic groups in line with previous works (Figure 1.2A and 1.2B). However, since the whole genome of members of the *P. fragi* group was not available, this group was not included in the whole genome analyses resulting in the representation of eight groups (Figure 1.2B). Interestingly, in this study, concordance was observed between the grouping obtained by MLSA and that conducted using phylogenomic methods (full genomes) with respect to the species delineation and to the eight groups (Figure 1.2). Additionally, this study revealed a phylogenomic distribution of traits among the groups with insights into biocontrol, bioremediation and plant growth promotion (PGPR).

Biocontrol strains are commonly found within the *P. fluorescens* complex (Garrido-Sanz *et al.,* 2016). The *P. fluorescens* complex shows a very strong strain-to-strain variation and only a subset of strains within the complex provide biocontrol. It should also be noted that *P. aeruginosa* isolates with strong biocontrol activities can be readily isolated from the (endo) rhizosphere of plants (Anjaiah *et al.,* 1998; Audenaert *et al.,* 2002; Kumar *et al.*, 2013). Moreover, various non-pathogenic isolates of *P. syringae* also show interesting biocontrol activities and are used to control postharvest pathogens on fruits (Janisiewicz and Korsten, 2002) and bacterial diseases caused by pathogenic *P. syringae* pathovars (Volksch and May, 2001; Wensing *et al.,* 2010).

Characterisation of Pseudomonas isolates

A commonly used tool to characterize and taxonomically identify *Pseudomonas* species is MLSA. MLSA is a rapid classification method for genotypic characterization using the sequences of multiple protein-encoding genes. The gene encoding 16S rRNA can be used to ascribe a strain to the genus *Pseudomonas*, but the resolution of 16S rRNA gene sequences at the intrageneric level is low (Mulet *et al.,* 2010). Other housekeeping genes such as *gyrB*, *rpoB*, *rpoD, atpD, carA* and *recA* have been studied. According to Mulet *et al.* (2010) the *rpoD* gene exhibits the highest number of polymorphic sites, followed by *gyrB* and *rpoB*. Multiple gene sequences from strains of *Pseudomonas* species are currently compiled in the PseudoMLSA database (Bennasar *et al.,* 2010) which is freely accessible.

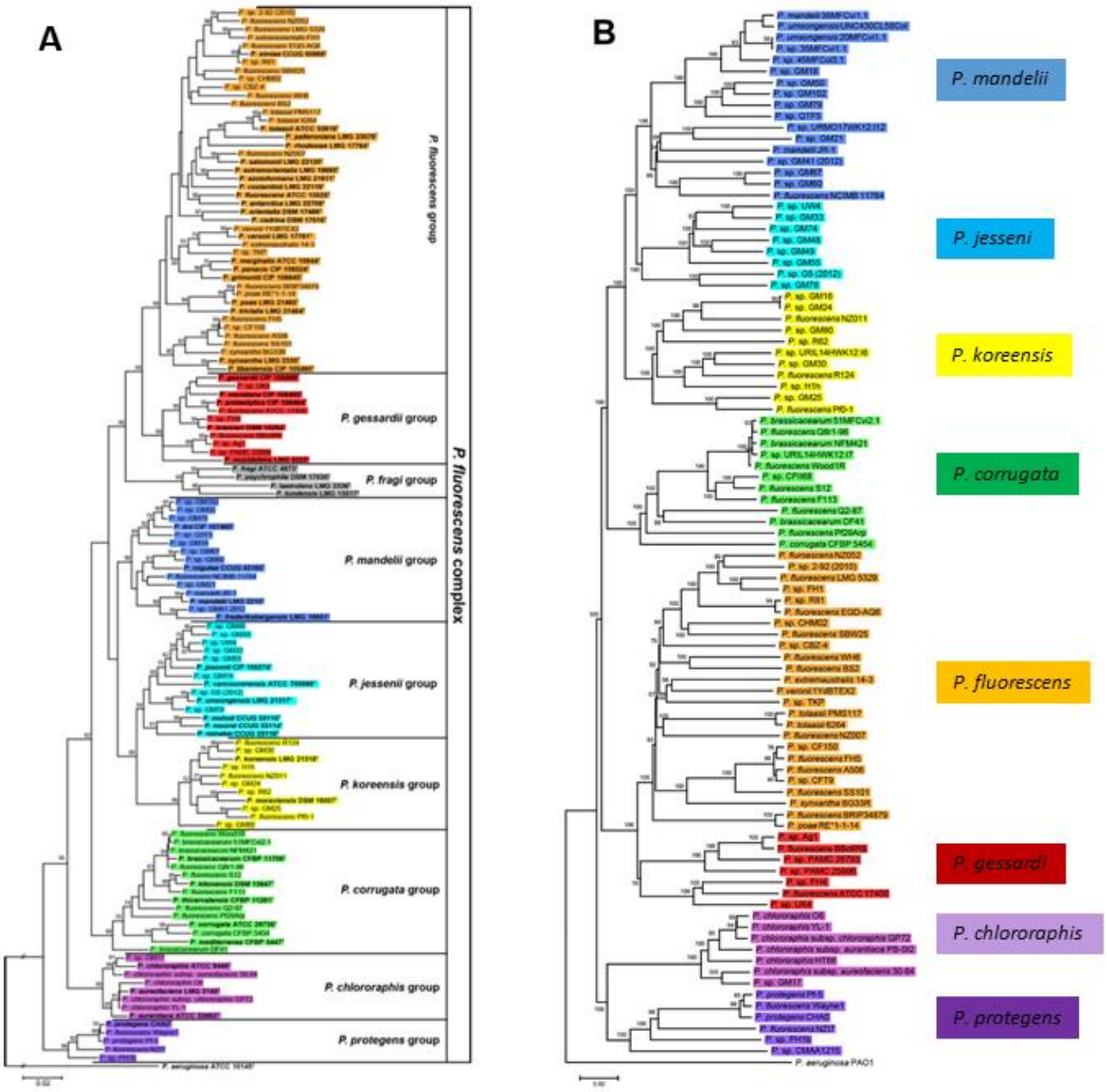

Figure 1.2. Phylogeny of the *P. fluorescens* complex inferred by A) MLSA; and **B)** GBDP. For MLSA, 127 sequenced and type strains belonging to the *P. fluorescens* complex based on the concatenated partial sequences of the 16S rDNA, *gyrB*, *rpoD* and *rpoB*, and ML method. For GDBP, whole-genome phylogenies were based on nucleotide data from 96 sequenced strains belonging to the *P. fluorescens* complex. Strains are colored according to groups established in this work. For both trees, *P. aeruginosa* type strain was used as outgroup. Picture was adapted from Garrido-Sanz *et al*. (2016).

Secondary metabolite production by Fluorescent *Pseudomonas* spp.

In recent years, the discovery, structure prediction and functional analysis of secondary metabolites in *Pseudomonas* spp. has found its roots in genome mining (de Bruijn *et al.,* 2007). Increased genome sequencing has led to increased identification of large orphan gene clusters for which the encoded

metabolite is unknown. These clusters contain nonribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) genes, which are responsible for secondary metabolites production such as lipopeptides and antibiotics. The centrality of genomics in natural products discovery has increased over the years. This was clearly shown in the landmark paper by Gross and Loper (2009), which gives an extensive overview of secondary metabolite biosynthesis in Pseudomonads.

Major antibiotics produced by fluorescent *Pseudomonas* spp. include phenazines, cyclic lipopeptides, 2, 4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and hydrogen cyanide. In this thesis, we will review phenazines briefly and cyclic lipopeptides in a more elaborate manner.

Phenazines

Introduction

Phenazines are nitrogen-containing tricyclic pigments that are produced by *Pseudomonas* spp., a few other Gram-negative proteobacteria including *Burkholderia* spp., *Pectobacterium* spp., *Brevibacterium*, and *Pantoea agglomerans*, but also by Gram-positive *Actinobacteria* such as *Streptomyces* spp. (Mavrodi *et al.,* 2006). Most phenazine producers are soil inhabitants and/or plant-associated species (Mavrodi *et al.,* 2010). More than 100 different phenazine structures have been described. Phenazine producers associated with biocontrol mainly produce phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 2-hydroxyphenazine and 2-hydroxyphenazine-1-carboxylic acid (Figure 1.3).

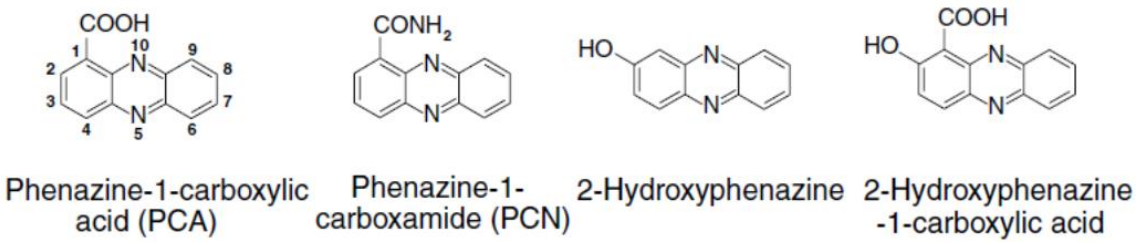

Figure 1.3. Some common phenazines of bacterial origin which are involved in the biological control of plant pathogens (Adapted from Mavrodi *et al.,* 2006).

Within the *Pseudomonas* genus, the most prominent producers belong to *P. aeruginosa*, the *P. chlororaphis* subgroup or the *P. fluorescens* subgroup. Recent research by Mavrodi *et al.* (2012) and Parejko *et al.* (2012, 2013) has revealed an extensive complex of phenazine producers isolated from dryland winter wheat. These *Pseudomonas* spp. mainly produce PCA and could be grouped in five different species, including two new species that have been provisionally named *P. aridus* and *P. cerealis*, while the other isolates belong to the *P. synxantha* and *P. orientalis* clade. All these isolates cluster in the *P. fluorescens* subgroup as defined by Mulet *et al.* (2010). Most clinical *P. aeruginosa* isolates produce pyocyanin (PYO), a blue phenazine pigment, but this species can also produce several other phenazine compounds including PCA and PCN (Anjaiah *et al.,* 1998). *P. chlororaphis* isolates have been reported to produce PCA, PCN, 2-hydroxyphenazine and 2-hydroxyphenazine-1-carboxylic acid.

Biosynthesis

The core phenazine biosynthesis genes are clustered and highly conserved. A seven-gene locus, named *phzABCDEFG*, is responsible for the synthesis of the first phenazine derivative in pseudomonads, namely PCA. This operon is present in two copies in the genome of *P. aeruginosa*. Phenazine biosynthesis starts from chorismic acid, a product from the shikimate pathway. This aromatic biosynthetic pathway converts primary metabolites to chorismic acid, the last common precursor of the three aromatic amino acids (Phe, Tyr, Trp), via 3-deoxy-D-arabino-heptulosanate-7-phosphate synthase enzymes. The PhzC protein is a variant of DAHP synthase and redirects carbon flow into phenazine biosynthesis. Phenazine diversity results from modification of PCA by dedicated enzymes encoded by genes that are often linked to the phenazine core operon such as PhzO (modifies PCA into 2-OH-PCA), PhzH (modifies PCA into PCN), PhzS (modifies PCA into 1-OH-phenazine) and PhzM (modifies PCA into pyocyanin together with PhzS). In all isolates tested, phenazine production is regulated by quorum sensing. Phenazine production also requires a functional GacS/GacA two-component signal transduction system. Many other regulators have been reported to be interconnected with the phenazine quorum sensing regulon. Further details about phenazine biosynthesis can be found in Gross and Loper (2009) and Mavrodi *et al.* (2006).

Biological roles

The multiple roles that phenazines can fulfill for the producing strain have recently been reviewed (Pierson and Pierson, 2010; Mavrodi *et al.,* 2013) and are briefly discussed below. Most of the described effects of phenazines go back to their antagonistic effect as an antibiotic during competition, and as a virulence factor during pathogenesis. This antagonistic effect can be attributed to their ability to generate reactive oxygen species (ROS), which cause oxidative stress in other organisms and tissues (Laursen and Nielsen, 2004; Mavrodi *et al.,* 2006). Because of their toxicity, phenazines can inhibit or kill other microorganisms, hence providing a competitive advantage to the producing strain. Phenazine antibiotics attracted a lot of interest in plant disease control, because of their broad-spectrum antifungal activity. Phenazines have been shown to determine the biological control capacity of a number of soil-borne pseudomonads against a wide range of plant pathogenic fungi (Chin-A-Woeng *et al.,* 2003; Mavrodi *et al.,* 2006). More specifically, the model strains for phenazine dependent biocontrol, *P. fluorescens* 2-79 (PCA) and *P. chlororaphis* 30-84 (PCA, 2-OH-PCA, 2-OH-PHZ), were shown to suppress the fungal phytopathogen *Gaeumannomyces graminis* var. *tritici* (Pierson and Thomashow, 1992; Thomashow and Weller, 1988; Weller and Cook, 1983) while *P. chlororaphis* PCL1391 (PCN) could suppress *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng *et al.,* 1998). Phenazines are likewise implicated in the biocontrol activity of *Pseudomonas* sp. CMR12a against *Rhizoctonia solani* on bean (D'aes *et al.,* 2011). There have only been limited studies examining the *in vivo* antibiosis effect of pure phenazine compounds. One particular study demonstrates the role of purified PCN in suppression of pre-emergence damping-off by *Pythium splendens* on bean seedlings in inert vermiculite substrate, however, its efficacy was dependent on the presence of surface-active metabolites, such as rhamnolipids (Perneel *et al.,* 2008). Besides the antibiosis effect of phenazines, antagonism against plant pathogens can also be accomplished by

induction of a plant defense response, as shown for PYO produced by *P. aeruginosa* 7NSK2 (De Vleesschauwer *et al.,* 2006). Phenazine antibiotics have been shown to contribute to competitiveness and ecological fitness (Mazzola *et al.,* 1992). Phenazine deficient strains of *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 survival was diminished in the wheat rhizosphere, because they had reduced ablity to compete with the resident microflora (Mazzola *et al.,* 1992).

Biosurfactants: new players in the biocontrol field

Pseudomonas biocontrol strains mainly produce two types of biosurfactants, rhamnolipids and cyclic lipopeptides (CLPs). Rhamnolipids are produced by *P. aeruginosa* isolates while CLPs are produced by *Pseudomonas* isolates within the *P. fluorescens* complex, *P. putida* and the *P. syringae* groups.

Cyclic lipopeptides

Diversity

CLPs are structurally diverse molecules produced by bacteria and comprised of a cyclic oligopeptide lactone ring coupled to a fatty acid tail (Raaijmakers *et al.,* 2006). Based on their structural characteristics, CLPs have been classified into nine different families namely, the amphisin, viscosin, putisolvin, orfamide, tolaasin, syringomycin, syringopeptin, entolysins and xantholysin families (Raaijmakers *et al.,* 2006; Gross and Loper, 2009; Roongsawang *et al.,* 2011; Li *et al.,* 2013). CLPs can cause destruction of microbial membranes, leading to death of bacteria, fungi, oomycetes and viruses. Furthermore, these molecules perform a diversity of functions in *Pseudomonas* bacteria and can play a role in swarming motility, biofilm formation, environmental adaptation, nutrient availability, and root colonization (Raaijmakers *et al.,* 2010; D'aes *et al.,* 2010).

Table 1.1 shows various CLPs grouped into families and their corresponding amino acids numbers. In Figure 1.4, we show representative chemical structures of *Pseudomonas* CLP families. The viscosin family contains CLPs with 9 amino acids often linked at the N-terminus to 3-hydroxy decanoic acid (3-HDA). Members of this group include viscosin, viscosinamide, massetolide, pseudodesmin, pseudophomin and white line inducing principle (WLIP). Of all the CLP structural groups, members of the viscosin group appear to be most prominently reported to exhibit biocontrol capacity against diverse plant pathogens including bacteria, fungi and oomycetes.

A unique feature of the putisolvin family is the peptide moiety of 12 amino acids and a hexanoic lipid tail. So far, Putisolvin I and II have only been described from *P. putida* PCL 1445, isolated from a site polluted with polycyclic aromatic hydrocarbons (Kuiper et al., 2004) and *P. putida* 267, isolated from black pepper rhizosphere (Kruijt *et al.,* 2009).

The amphisin family consists of CLPs containing an 11 amino acid cyclic peptide that is linked at the N-terminal end to a β-hydroxydecanoyl tail (Sorensen *et al.,* 2001). Members of this group include

amphisin, tensin, lokisin, arthrofactin and pholipeptin A. Pertaining to biocontrol activity, amphisin, lokisin and tensin appear to be more interesting members of the group.

Table 1.1. CLP families with representative members and amino acid number

Family	Number of amino acids	CLPs
Viscosin	9	Viscosin
		Viscosinamide
		WLIP
		Massetolide A-H
		Pseudophomin A and B
		Pseudodesmin A and B
Syringomycin	9	Syringomycin SRA
		Syringostatin A and B
		Pseudomycin A-C
		Thanamycin
		Nunamycin
		Cormycin A
Orfamide	10	Orfamide A-G
		Poaeamide
Amphisin	11	Amphisin
		Tensin
		Pholipeptin
		Lokisin
		Arthrofactin A-D
Putisolvin	12	Putisolvin I and II
Entolysin	14	Entolysin A
Xantholysin	14	Xantholysin A-C
Tolaasin	18-22	
	18	Tolaasins
	18	Sessilin
	19	Fuscopeptin A and B
	22	Corpeptin A and B
	22	Cichopeptin A and B
	22	Nunapeptin
	22	Thanapeptin
	22	Sclerosin
Syringopeptin	23	SP 22
		SP 25A
		Phe-SP25A

The composition and length of the peptide chain of tolaasin family members ranges between 19 to 25 amino acids with the lipid tail comprising of 3-HDA or 3-hydroxyoctanoic acid (3-HOA). Members include tolaasin, fuscopeptin, corpeptin, syringopeptin, sclerosin, sessilin and thanapeptin (Raaijmakers *et al.,* 2006; Roongsawang *et al.,* 2011; Berry *et al.,* 2012; D'aes *et al.,* 2014; Van der

voort *et al.,* 2015). Of these CLPs, tolaasin, sessilin and sclerosin have been shown to demonstrate biocontrol capacity against plant pathogens.

Orfamides, isolated from cultures of *P. protegens* Pf-5, were the premier compounds mined from *Pseudomonas* genomes. They consist of ten amino acids which bear close resemblance to the CLPs of the viscosin group. CLPs in this group include orfamides A-G (Ma *et al.,* 2016a) and poaeamide (Zachow *et al.,* 2015), characterized by a 3-hydroxy-dodecanoic or tetradecanoic acid connected to the *N*-terminus of the amino acids.

Entolysin, described from *P. entomophila* consists of a peptide moiety of 14 amino acids and a typical cyclization in which the lactone ring is formed between the C-terminal carboxyl group and the 10^{th} amino acid rather than one of the first amino acids. Although entolysin was shown to be important for the swarming motility and haemolytic activity of *P. entomophila*, it was not involved in the biocontrol activity of this *Pseudomonas* strain. An entolysin mutant, *etlC*, showed similar biocontrol when compared with that shown by the wild type strain in a cucumber-*Pythium ultimum* pathosystem (Vallet-Gely *et al.,* 2010).

Finally, the xantholysin family consists of CLPs with 14 amino acids and were first characterised from the banana rhizosphere isolate, *P. putida* BW11M1 (Li *et al.,* 2013). The bioactivity of this molecule has also been reported.

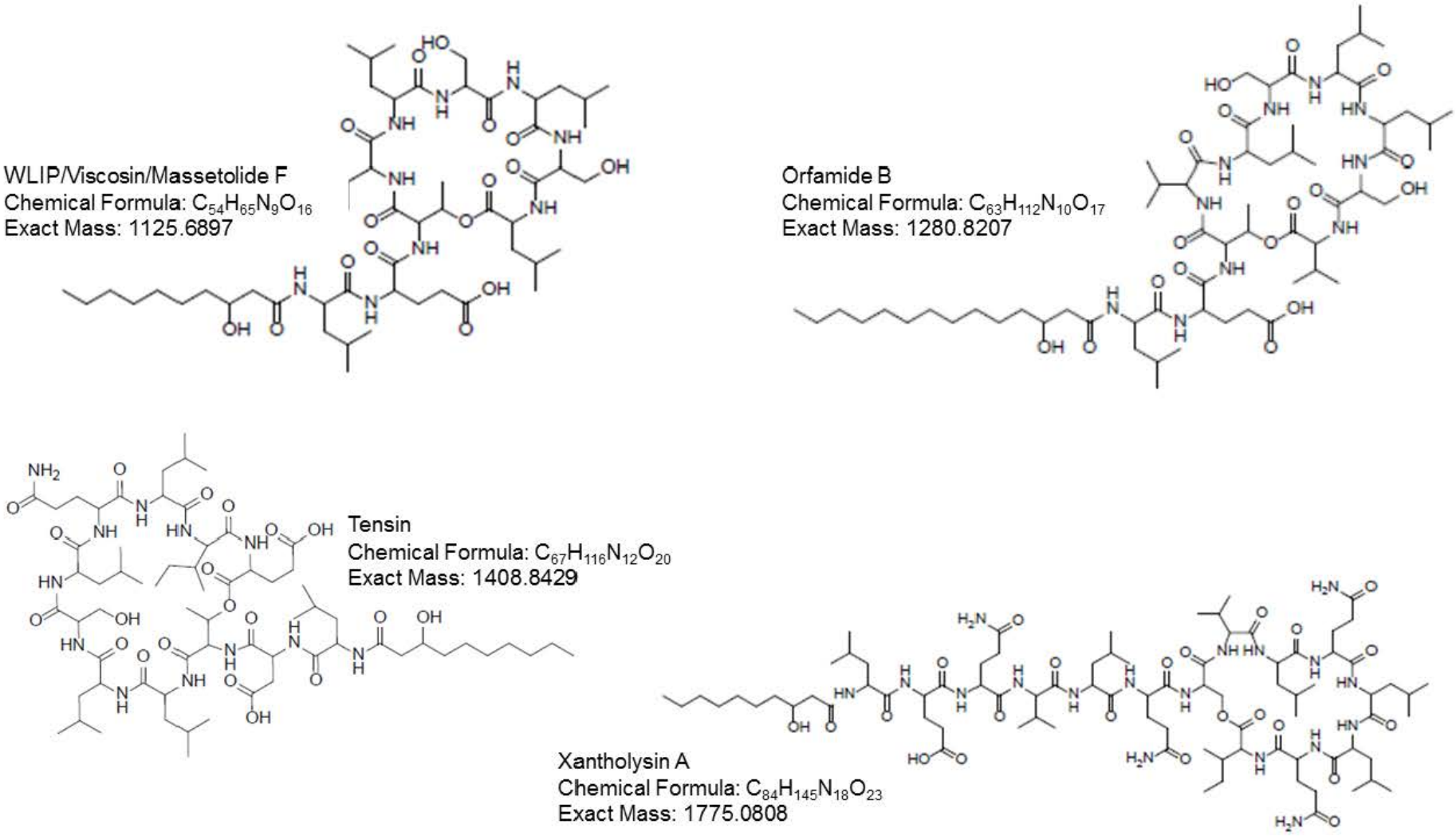

Figure 1.4. Representative chemical structures of *Pseudomonas* CLP families as discussed in Table 1.1 and Table 1.3 (Adapted from Nguyen *et al.,* 2016).

Table 1.2. Taxonomic affiliation of selected CLPs involved in the biocontrol of plant pathogens

Classification	Group	CLP	Reference
P. fluorescens complex			
	P. koreensis	Lokisin	Nielsen *et al*., 2002; Hultberg *et al*., 2010
	P. fluorescens	WLIP	Rokni Zadeh *et al*., 2013
	-	Arthrofactin	Roongsawang *et al*., 2003
	-	Tensin	Nielsen *et al*., 2002
	-	Hodersin	Nielsen *et al*., 2002
	-	Tensin	Nielsen *et al*., 2002
	-	Amphisin	Nielsen *et al*., 2002
	P. fluorescens	Massetolide	De Souza *et al*., 2003
	P. fluorescens	Viscosinamide	Nielsen *et al*., 1998
	P. fluorescens	Viscosin	de Bruijn *et al*., 2007
	P. chlororaphis	Sessilin	Garrido-Sanz *et al*., 2016
	-	Pseudophomins	Pedras *et al*., 2003
	P. protegens	Orfamide	Hartney *et al*., 2013
	P. chlororaphis	Orfamide	D'aes *et al.,* 2014; Garrido-Sanz *et al*., 2016
	P. fluorescens	Poaeamide	Müller *et al*., 2013; Zachow *et al*., 2015
	P. fluorescens	Tolaasin	Rainey *et al*., 1991
	-	Nunapeptin	Michelsen *et al*., 2015
	-	Nunamycin	Michelsen *et al*., 2015
	P. corrugata	Thanapeptin	Van Der Voort *et al*., 2015
	P. corrugata	Thanamycin	Van Der Voort *et al*., 2015
	P. corrugata	Sclerosin	Loewen *et al*., 2014
P. putida group	n/a	WLIP	Rokni Zadeh *et al*., 2013
	n/a	Xantholysin	Li *et al*., 2013
	n/a	Putisolvin	Tran *et al*., 2007
	n/a	Entolysin	Vallet-Gely *et al*., 2010
P. syringae group	n/a	Syringotoxin	Lavermicocca *et al*., 1997
	n/a	Syringopeptin	Lavermicocca *et al*., 1997
	n/a	Syringomycin	Lavermicocca *et al*., 1997

*Taxonomic classification was done according to Garrido-Sanz *et al.* (2016).

So far, it is interesting of note that some CLPs have exclusively been found in certain species of the *Pseudomonas* genus while only one, namely the WLIP, is reportedly produced by strains belonging to both the *P. putida* group and *P. fluorescens* complex (Rokni-Zadeh *et al.,* 2013). Table 1.2 highlights the taxonomic affiliation of major biocontrol CLPs that have been reported to date. In this table, the putisolvin, entolysin and xantholysin appear to be exclusive to the *P. putida* group and a couple of biocontrol CLPs are produced by the *P. syringae* group isolates. In contrast, a higher diversity of CLPs with biological activity appear to be widespread within the *P. fluorescens* complex. Additionally, this latter group appears to be the most versatile of all *Pseudomonas* species since it contains many antibiotic-producing biocontrol strains (Garrido-Sanz *et al.,* 2016).

Bioactivity

In Table 1.3, we show a summary of cyclic lipopeptides produced by *Pseudomonas* spp. with a reported role in the biological control of plant pathogens. From this table, it is obvious that it sometimes not easy to draw a clear distinction between pathogenic and plant beneficial *Pseudomonas* strains because some of their secondary metabolites can be implicated in both biocontrol and disease. For example, syringopeptin and syringomycin which are important virulence factors in plant pathogenic *P. syringae* pv. *syringae* are also instrumental in biocontrol.

Table 1.3. *Pseudomonas* CLPs involved in the biocontrol of plant pathogens

CLPs	Producing strain	Target pathogen	Mode of action	Application	Assay	Reference
Amphisin family						
Amphisin	*Pseudomonas* sp.	*Pythium ultimum;*	Antagonism	Pure compound	*In vitro*	Nielsen *et al.,* 2002;
	Pseudomonas sp. DSS73	*Rhizoctonia solani*				Andersen *et al.,* 2003
Tensin	*Pseudomonas* sp.	*Pythium ultimum;*	Antagonism	Pure compound	*In vitro*	Nielsen *et al.,* 2000;
	P. fluorescens 96.578	*Rhizoctonia solani*				Nielsen *et al.,* 2002
Lokisin	*Pseudomonas sp.*	*Pythium ultimum;*	Antagonism	Pure compound	*In vitro*	Nielsen *et al.,* 2002;
	Pseudomonas sp. DSS41	*Rhizoctonia solani*				Sorensen *et al.,* 2002
	Pseudomonas sp. 2.74	*Pythium ultimum*	Antagonism	Crude extract	Hydroponics assay on tomato	Hultberg *et al.,* 2010
Hodersin	*Pseudomonas* sp.	*Pythium ultimum;*	Antagonism	Pure compound	*In vitro*	Nielsen *et al.,* 2002
		Rhizoctonia solani				
Orfamide family						
Orfamide A	*P. protegens* CHA0	*Magnaporthe oryzae;*	ISR	Pure compound	Soil assay with rice	Ma *et al.,* 2016a
		Phytophthora porri;	Zoospore lysis		*In vitro*	
		Pythium ultimum;	Zoospore lysis			
		Rhizoctonia solani	Hyphal branching			
Orfamide B	*Pseudomonas* sp. CMR5c	*Rhizoctonia solani;*	Hyphal branching; ISR	Pure compound	*In vitro*	Ma *et al.,* 2016a; Ma *et al.,* 2016b
		Magnaporthe oryzae;	ISR	Pure compound	Soil assay with rice	Ma *et al.,* 2016a
		Phytophthora porri;	Zoospore lysis			
		Pythium ultimum	Zoospore lysis			
Orfamide G	*Pseudomonas* sp. CMR5c	*Magnaporthe oryzae;*	ISR	Pure compound	Soil assay with rice	
		Rhizoctonia solani;	Hyphal branching	Pure compound	*In vitro*	Ma *et al.,* 2016a
		Phytophthora porri;	Zoospore lysis			
		Pythium ultimum	Zoospore lysis			
Poaeamide	*P. poae* RE*1-1-14	*Phytophthora capsici;*	Antagonism	Pure compound	*In vitro*	Zachow *et al.,* 2015
		Phytophthora infestans;				
		Pythium ultimum;				
		Rhizoctonia solani;				
Putisolvin family						
Putisolvin	*P. putida* 267	*Botrytis cinerea* and	Antagonism	Pure compound	*In vitro*	Kruijt *et al.,* 2009
		Rhizoctonia solani				

		Phytophthora capsici	Zoospore lysis		*In vitro*	Kruijt *et al.,* 2009
Syringomycin family						
Syringotoxin	*P. syringae* pv. *syringae* B359	*Botrytis cinerea* PVBA405; *Geotrichum candidum ITM104*	Antagonism	Pure compound	*In vitro*	Lavermicocca *et al.,* 1997
Syringomycin-E	*P. syringae* pv. *syringae* B301	*Geotrichum candidum ITM104; Botrytis cinerea* PVBA405	Antagonism	Pure compound	*In vitro*	Lavermicocca *et al.,* 1997
	P. syringae ESC-10 and ESC-11	*Penicillium digitatum*	Antagonism	Pure compound	*In vitro* and on lemon *surfaces*	Bull *et al.,* 1997
Thanamycin	*P. corrugata* SH-C52	*Rhizoctonia solani; Botrytis cinerea* and *Geotrichum sp.*	Antagonism	Mutant analysis	*In vitro*	Mendes *et al.,* 2011; Van der Voort *et al.,* 2015
Nunamycin	*P. fluorescens* In5	*Rhizoctonia solani*	Antagonism	Mutant analysis	*In vitro*	Michelsen *et al.,* 2015
Tolaasin family						
Tolaasin	*P. tolaasii* NCPPB2192	Pathogenic fungi and chromista including *Fusarium solani,* etc.	Antagonism	Pure compound	*In vitro*	Lo Cantore *et al.,* 2006
Sessilin	*Pseudomonas* sp. CMR12a	*Rhizoctonia solani* AG2-2 and AG4-HGI	Antagonism	Mutant analysis	Soil assay with bean	D'aes *et al.,* 2011
		Rhizoctonia solani AG2-2	ISR	Crude extract	Soil assay with bean	Ma *et al.,* 2016b
Sclerosin	*Pseudomonas* sp. DF41	*Sclerotinia sclerotiorum*	Disruption of fungal plasma membrane	Pure compound	*In vitro* and soil assay	Berry *et al.,* 2010; Berry *et al.,* 2012
Thanapeptin	*P. corrugata* SH-C52	*Phytophthora infestans*, *Pythium ultimum; Saprolegnia parasitica; Bacillus magaterium*	Antagonism	Mutant analysis	*In vitro*	Van der Voort *et al.,* 2015
Syringopeptin (SP22-A)	*P. syringae* pv. *syringae* B427	*Geotrichum candidum* ITM104; *Botrytis cinerea* PVBA405	Antagonism	Pure compound	*In vitro*	Lavermicocca *et al.,* 1997
Syringopeptin (SP25-A)	*P. syringae* pv. *syringae* B427	*Botrytis cinerea* PVBA405	Antagonism	Pure compound	*In vitro*	Lavermicocca *et al.,* 1997
Nunapeptin	*P. fluorescens* In5	*Pythium aphanidermatum*	Antagonism	Mutant analysis	*In vitro*	Michelsen *et al.,* 2015
Viscosin family						
Viscosin	*P. fluorescens* SBW25	*Phytophthora infestans*	Zoospore lysis	Pure compound	*In vitro*	de Bruijn *et al.,* 2007
Viscosinamide	*Pseudomonas* sp. *P. fluorescens* DR54	*Rhizoctonia solani*; *Pythium ultimum*	Antagonism	Pure compound	*In vitro*	Nielsen *et al.,* 1999; Nielsen *et al.*, 2002
Massetolide	*P. fluorescens* SS101	*Phytophthora infestans*	Antagonism	Pure compound	*In vitro* and soil assay in tomato	Tran *et al.,* 2008
			Zoospore lysis	Pure compound	*In vitro*	de Bruijn *et al.,* 2007

Pseudophomins A and B	*P. fluorescens* BRG100	*Rhizoctonia solani; Sclerotinia sclerotiorum*; *Phoma lingam; Rhizoctonia solani*; *Alternaria brassicae*	Antagonism	Pure compound	*In vitro*	Pedras *et al.,* 2003
WLIP	*P. reactans* NCPPB1311 *P. putida* RW10S2	Pathogenic fungi, bacteria and protista including *Rhizoctonia solani*, *Xanthomonas*, X. pv. *manihotis* LMG 784 etc.	Antagonism	Pure compound	*In vitro*	Lo Cantore *et al.,* 2006 Rokni-Zadeh *et al.,* 2012
Xantholysin family						
Xantholysin	*P. putida* BW11M1	*Xanthomonas translucens* pv. *cerealis* LMG 679; *X. axonopodis pv. manihotis* LMG 784	Antagonism	Pure compound	*In vitro*	Li *et al.,* 2013
	P. soli					Pascual *et al.,* 2014

*ISR: Induced systemic resistance; WLIP: White line-inducing principle.

Biosynthesis

CLP biosynthesis gene clusters have been characterized for multiple CLPs including viscosin, massetolide, putisolvin, syringomycin, corpeptin and more recently poaeamide, nunapeptin and nunamycin (de Bruijn *et al.,* 2007; de Bruijn *et al.,* 2008; Strano *et al.,* 2015; Zachow *et al.,* 2015; Michelsen *et al.,* 2015). In view of this information, CLP biosynthesis is known to be conducted by non-ribosomal peptide synthases (NRPS) through a thiotemplate process (Finking and Marahiel, 2004; Raaijmakers *et al.,* 2006). These NRPS clusters have a modular structure in which each module represents a building block for the incorporation of one amino acid unit in the oligopeptide. Each module consists of an adenylation (A) domain which is responsible for amino acid (AA) selection and activation, a thiolation (T) or peptidyl carrier protein (PCP) domain saddled with thioesterification of the activated AA, and a condensation (C) domain which catalyzes peptide bond formation between two amino acids. Many NRPSs obey the “colinearity rule” such that the order and number of the NRPS modules are collinear to the AA sequence of the peptide (Finking and Marahiel, 2004; Raaijmakers *et al.,* 2006). In most cases, a thioesterase (TE) domain catalyses the cyclization of the mature product resulting in the release of a cyclic lipopeptide (Schwarzer *et al.,* 2001; Samel *et al.,* 2006).

In several CLPs, the incorporation of unusual amino acids such as the D-configured ones are possible. However, in *Pseudomonas* spp., separate epimerization (E) domains as found in *Bacillus* NRPS clusters have not been reported. Although, the analysis of arthrofactin biosynthesis genes revealed that the D-configuration of the AAs in the cyclic lipopeptides can be generated by C/E domains, which represent a subclass of C domains having both condensation and epimerization domain activities (Balibar *et al.,* 2005). Predicted dual activity C/E domains have also been found in syringopeptin and massetolide NRPSs (de Bruijn et al. 2008).

Additionally, for most *Pseudomonas* spp. studied so far, CLP synthesis appears to be a part of their primary metabolism (de Bruijn *et al.,* 2008). However, in *Pseudomonas* sp. CMR12a which produces both sessilin and orfamide CLPs for example, the sessilin gene cluster appears to have been acquired by horizontal gene transfer whereas orfamide biosynthesis are a part of the strain’s primary metabolism (D’aes *et al.,* 2014). Furthermore, although the presence of one NRPS gene cluster in *Pseudomonas* spp. appears to be the norm, a few exceptions have been reported. At least two CLPs are produced by *P. syringae* pv. *syringae* (syringomycin and syringopeptins), *Pseudomonas* sp. CMR12a (sessilin and orfamide), *P. fluorescens* In5 (nunapeptin and nunamycin) and *P. corrugata* SH-C52 (thanamycin and thanapeptin) (Bender *et al.,* 1991; D’aes *et al.,* 2014; Michelsen *et al.,* 2015; Van der Voort *et al.,* 2015).

Regulation

In several *Pseudomonas* strains, the principal regulator of CLP biosynthesis is the GacA/GacS two-component system since a mutation in one of both encoding genes leads to a loss in CLP production (De Bruijn and Raaijmakers 2009a; Haas and Défago, 2005). This system comprises the membrane-bound sensor kinase, GacS which when it recognises an unknown signal, becomes activated and phosphorylates the response regulator GacA The GacA/GacS system is known to activate small

RNAs that bind to and sequester translational repressor proteins, which block the ribosomal binding sites in the mRNA of Gac-regulated genes. Two small RNAs (sRNAs) and two repressor proteins, RsmA and RsmE, have been linked to the regulation of entolysin (Vallet *et al.,* 2010) and massetolide A biosynthesis (Song *et al.,* 2015a). In the massetolide producer *P. fluorescens* SS101, these repressor proteins most likely block translation of the LuxR-type transcriptional regulator, MassAR (Song *et al*., 2015a) by binding to a specific promoter region (the GacA box). In several CLP-producing *Pseudomonas* strains, a GacA box is present in the promoter region of LuxR regulators flanking the CLP biosynthesis gene cluster suggesting that other CLP-producing *Pseudomonas* strains may show a similar regulation of lipopeptide biosynthesis (Song *et al.,* 2015a).

Additionally, N-acylhomoserine lactone (N-AHL)-mediated quorum sensing was shown to be required for viscosin and putisolvin biosynthesis (Cui *et al.,* 2005; Dubern *et al.,* 2006) in *Pseudomonas fluorescens* strain 5064 and *P. putida* strain PCL1445 although this is not the case in certain other *Pseudomonas* strains (de Bruijn *et al.,* 2008; Dumenyo *et al.,* 1998; Kinscherf and Willis 1999). In *P. putida* strain PCL1445, two heat shock proteins DnaK and DnaJ located downstream of the Gac system, were shown to regulate putisolvin biosynthesis (Dubern *et al.,* 2005). Figure 1.5 shows a proposed model for the genetic regulation of massetolide biosynthesis in *P. fluorescens* SS101 (Song *et al.,* 2015)..

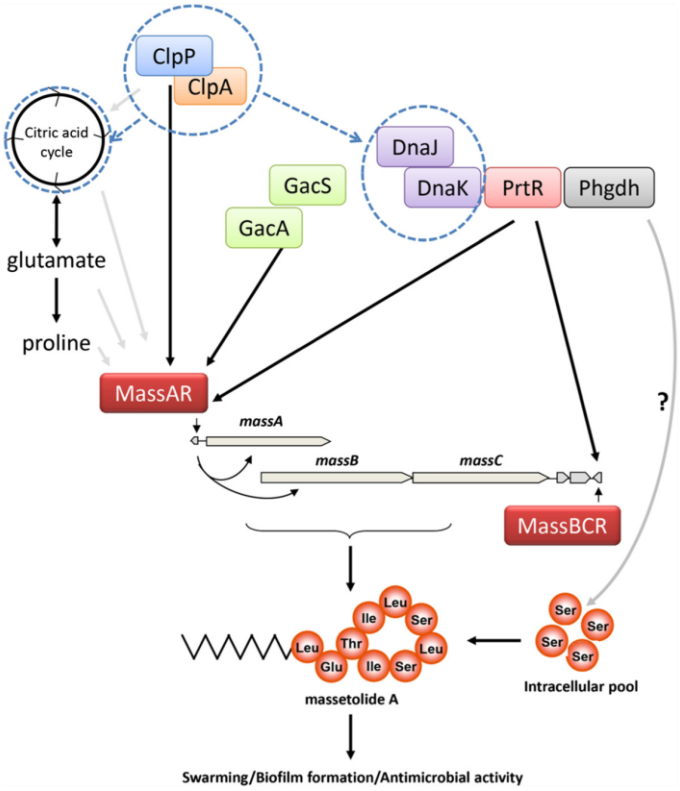

Figure 1.5. Proposed model for the genetic regulation of massetolide biosynthesis in *P. fluorescens* strain SS101 (adapted from Song *et al.,* 2015). The darkly shaded arrows were based on experimental data obtained within the study of Song *et al.* (2014) and Song *et al.* (2015); the lightly shaded arrows are hypothetical and not based on experimental data. The blue dashed arrows and circles represent translational regulation whereas the other arrows represent transcriptional regulation**.**

Using random mutagenesis, transcriptomic and proteomic analyses, putative substrates of the ClpAP complex were identified which enabled the elucidation of the genetic regulation of massetolide biosynthesis. ClpA, a hexameric chaperone ring complex, selectively targets proteins for ClpP to degrade according to the N-end rule (Mogk *et al.,* 2007). In *P. fluorescens* SS101, the ClpAP complex plays a crucial role in intracellular refolding and degradation of proteins which is an essential process for cell viability and growth (Song *et al.,* 2015). Results of transcriptomic and proteomic analyses of *clpA* and *clpP* transposon mutants revealed either a down- or up-regulation of certain proteins in the TCA cycle. In addition to this, the heat shock proteins DnaK and DnaJ, were significantly upregulated in both *clpA* and *clpP* mutants. Besides, a mutation in *clpP* led to the unique upregulation of five transcriptional regulators. Furthermore in previous studies (Song *et al.,* 2014), *phgdh*, encoding the D-3-phosphoglycerate dehydrogenase, the transmembrane regulatory gene *prtR* and the heat shock protein-encoding *dnak*, are involved in the regulation of massetolide biosynthesis probably via transcriptional repression of the LuxR regulatory genes, *massAR* and *massBCR*. Put together, these results suggests that the ClpAP complex regulates massetolide biosynthesis in *P. fluorescens* SS101 via the LuxR-transcriptional regulator, MassAR, the heat shock proteins DnaK and DnaJ and through proteins involved in the TCA cycle (Song *et al.,* 2015).

This model demonstrates that serine protease ClpP together with the chaperone ClpA regulates the biosynthesis of massetolides via a specific pathway involving the LuxR regulator MassABC, the heat shock proteins DnaK and DnaJ and proteins of the TCA cycle (De Bruijn and Raaijmakers 2009b; Song *et al.,* 2014; Song *et al.,* 2015).

The LuxR superfamily consists of transcriptional regulators that contain a DNA-binding helix-turn-helix (HTH) motif in the C-terminal region (Fuqua *et al.,* 1996). These regulators have been shown to be involved in the biosynthesis of syringafactins in *P. syringae* pv. tomato (Berti *et al.,* 2007), putisolvins in *P. putida*, (Dubern *et al.,* 2008) and viscosin produced by *Pseudomonas fluorescens* SBW25 (De Bruijn and Raaijmakers 2009a) with mutations in *syrF*, *psoR* and *viscAR* or *viscBCR* resulting in the loss of syringafactin, putisolvin and viscosin production, respectively. Furthermore, plant pathogenic *P. syringae* pv. *syringae* B301D produces two classes of lipopeptides namely syringomycin and syringopeptin and also possesses three transcriptional regulators designated SalA, SyrF and SyrG. Of these three genes, *salA* and *syrF*, located downstream of its Gac system, were identified as LuxR-type transcriptional regulators of syringomycin and syringopeptin biosynthesis (Lu *et al.,* 2002; Lu *et al.,* 2005; Wang *et al.,* 2006). Interestingly, recent work to investigate the role of these aforementioned regulators in the regulation of CLP production in *P*. *syringae* pv. *syringae* B728a highlighted the regulatory function of all three genes, *salA*, *syrG* and *syrF*, in the production of syringomycin (Vaughn and Gross 2016). In plant pathogenic *P. corrugata* CFBP 5454, two LuxR regulators, *pcoR* and *rfiA*, were shown to play a role in corpeptin biosynthesis and secretion (Strano *et al.,* 2015).

Secretion

Bacteria have evolved a variety of highly specialized machineries through which it secretes substrates including small molecules, proteins and DNA, across the bacterial cell envelope (Costa *et al.,* 2015). Although the substrates secreted can have key roles in the bacterium's response to the environment, they can also be important for physiological processes including pathogenicity, adhesion, adaptation and survival.

In Gram-negative bacteria, these secretion systems are divided into two broad categories. First, those that span the inner membrane (IM) and the outer membrane (OM) and second, those that span the OM only. The double membrane-spanning secretion systems are of five types namely: the type I secretion system (TISS), T2SS, T3SS, T4SS and T6SS. The bacterial TISS and the Resistance-Nodulation-Division (RND) efflux pumps share a similar architectural composition (Piddock, 2006). Both systems form a tripartite double membrane-spanning channel which consists of an IM component, a periplasmic adaptor protein otherwise called the membrane fusion protein (MFP) and an outer membrane channel.

RND efflux pumps

The RND efflux pumps are a family of multidrug pumps which export biological metabolites and antimicrobial compounds thereby playing a role in bacterial antibiotic resistance (Daury *et al.,* 2016). RND pumps located in the inner membrane are driven by the proton motive force and being part of a tripartite system, work cooperatively with the outer membrane and periplasmic fusion proteins. The substrates bind to the IM-spanning transporters which utilize the proton motive force to drive the process. Thus, the substrates are translocated into the periplasmic space after which they are transported to the outer membrane with the bridging membrane fusion protein. Lastly, the outer membrane protein is responsible for the extrusion of the substrate from the cell. Well studied examples include the MexAB-OprM and AcrAB-TolC systems present in *P. aeruginosa* and *E. coli*, respectively (Costa *et al.,* 2015; Marquez *et al.,* 2005; Blair and Piddock, 2009).

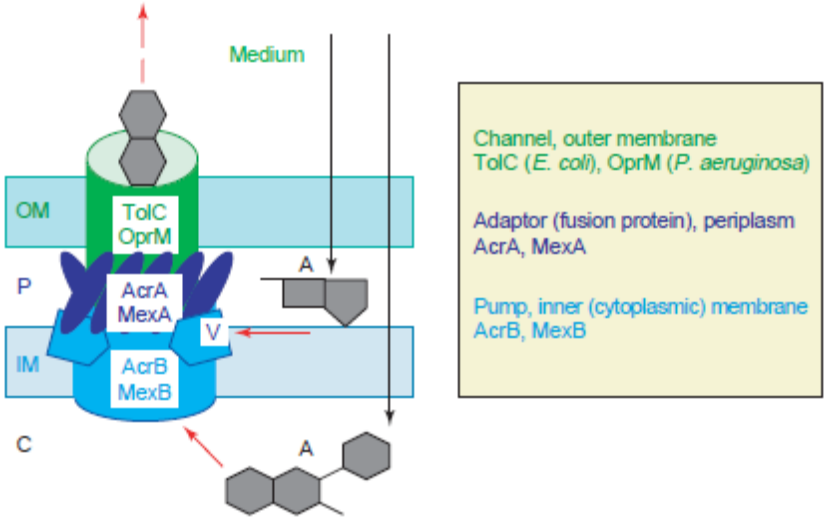

Figure 1.6. The AcrAB-Tolc (*E. coli*) and MexAB-OprM (*P. aeruginosa*) efflux pumps assemblies are illustrated (adapted from Pagés *et al.,* 2005). The ejection of an antibiotic molecule is presented through the AcrAB-Tolc or MexAB-OprM. The arrows represent the drug flows: a thick red arrow for the efflux and a thin black arrow for the penetration, respectively.

MexAB-OprM

The MexAB-OprM system has been intensively studied in clinical *P. aeruginosa* strains due to its role in multidrug resistance (Blair and Piddock, 2009; Kourtesi *et al.,* 2013; Costa *et al.,* 2015; Daury *et al.,* 2016). Figure 1.6 shows the efflux pump assemblies of AcrAB-TolC and MexAB-OprM. In *P. aeruginosa*, the *mexAB-oprM* genes form an operon that codes for MexA, MexB and OprM. In this operon, MexB is the IM protein while MexA signifies the MFP. Lastly, the oprM encodes the OM protein which is involved in substrate export from the cell. This system is reportedly involved in the secretion of various molecules including tetracycline, quinolones, chloramphenicol, fluoroquinolones and β-lactams thereby contributing to the antibiotic resistance of *P. aeruginosa* (Li *et al.,* 1995; Evans and Poole, 1999). The general involvement of the MexAB-OprM in the efflux of secondary metabolites has previously been suggested (Poole, 1994).

Type 1 secretion system

Similar to the RND efflux pumps, the type I secretion system plays a role in multidrug efflux and in the export of various protein molecules (Costa *et al.,* 2015). In contrast with the RND pumps, its innermost membrane component is a member of the ATP-binding cassette (ABC) transporter family. These transporters utilize ATP as an energy source to facilitate substrate transport. Within the cytoplasm, the substrate binds with the inner membrane component and is transported via the membrane fusion protein by utilization of ATP energy after which the substrate is exported to the extracellular environment through the outer membrane protein (Marquez, 2005; Costa *et al.,* 2015).

MacAB

In *Pseudomonas* spp, MacA is a periplasmic membrane protein that binds with the ATP-driven inner membrane MacB in the secretion of molecules outside the cell (Kobayashi and Nishino, 2001; Modali and Zgurskaya, 2011). These genes are well conserved in *Pseudomonas* species. Moreover, in CLP-producing strains, they are located downstream of the biosynthetic gene cluster (Li *et al.,* 2013). Besides the studies conducted on the *macAB* gene in several bacteria, the putative role of this gene in CLP export has been studied in various biocontrol CLP-producing *Pseudomonas* strains. Inactivation of the *macAB* gene caused strongly reduced but not complete abolishment of putisolvin (Dubern *et al.,* 2008), arthrofactin (Lim *et al.,* 2009) and syringopeptin (Cho and Kang, 2012), indicating a major role in CLP export.

NodT

In several CLP-producing *Pseudomonas* isolates, an outer membrane protein namely the NodT is located upstream of the biosynthetic gene cluster, right before the LuxR-type regulator (Rivilla *et al.,* 1995). Although the role of this OprM homologue in the producing strain is not yet known, its role in CLP transport has been suggested (D'aes *et al.,* 2014).

White Line Inducing Principle

Strains of *Pseudomonas reactans* were reported to produce an extracellular substance called the white line-inducing principle (WLIP) (Wong and Reece, 1979). This substance has the capacity to interact with tolaasin I, a toxic lipodepsipeptide (LDP) produced *in vitro* by virulent strains of *P. tolaasii* which results in the formation of a white precipitate in the white line assay (Wong and Preece, 1979). The WLIP is a lipodepsipeptide (LDP) with a molecular weight of 1,125 Da composed of an N-terminal β-hydroxydecanoic acid and a peptide moiety of nine amino acids of which six are in the D-form. Thus, the formation of a lipopeptides co-precipitate between confronted colonies of a test strain and *P. tolaasii* is often referred to as the WLIP. Previous studies identified the WLIP strain *P. reactans* LMG 5328 as belonging to the *P. fluorescens* complex (Rokni-Zadeh *et al.,* 2013). A few other WLIP producers have been characterized including *P. chlororaphis* subsp. *aurantiaca* PB-St2 (Mehnaz *et al.,* 2013) obtained from sugarcane stems in Pakistan and *P. putida* RW10S2, which was isolated from the rice rhizosphere in Sri-Lanka (Rokni-Zadeh *et al.,* 2012).

The Cocoyam Crop

Cocoyam (*Xanthosoma sagittifolium* (L.) Schott), belonging to the family Araceae, is one of the six most important root and tuber crops grown worldwide (Onwueme and Charles, 1994). Next to cassava and yam, cocoyam ranks third in the tropics as a staple energy food. The corms, cormels and leaves are a vital source of carbohydrates, vitamins and minerals for human nutrition and animal feed (Nyochembeng and Garton 1998). Although native to South America, it has spread to the West Indies, Southeast Asia, the Pacific Islands and tropical Africa (Acevedo-Rodríguez and Strong, 2012; Govaerts, 2013; PIER, 2013; Prota4U, 2013). In the 16th and 17th century, *Xanthosoma* reached West Africa when it was loaded on board ships heading for the slave coast to feed the human cargo (Brown, 1988). From the ports it was further spread by traders and missionaries and it was locally named new cocoyam since it replaced the 'old' cocoyam (*Colocasia esculenta* (L.) Schott), an aroid root crop from Southeast Asia. Eventually, cocoyam was introduced into the South Pacific and Asia by Europeans in the 19th century (Purseglove, 1972). Cocoyam fields are now a common sight in Florida, in response to the needs of the large population of immigrants from South America and Africa (Brown, 1988). In spite of the fact that roughly 400 million people worldwide rely on cocoyam for survival (Onokpise *et al.,* 1999), it is grossly under-researched (Watanabe, 2002) mainly because the cocoyam crop is consumed where it is produced.

The Genus Xanthosoma

The Araceae is a huge family of herbaceous monocots most of which occur in the New World tropics (Boyce and Croat, 2011). Of its numerous members, *X. sagittifolium* (otherwise known as tannia) and *Colocasia esculenta* (popularly known as Taro or Dasheen) are attaining world importance as energy foods (Giacometti and León, 1994). In previous years, the tendency had been to give the name of *Xanthosoma sagittifolium* to all cultivated *Xanthosoma* species (Giacometti and León, 1994), although a number of edible species in this genus have been recognised. The *Xanthosoma* genus is divided into 40 species mainly based on colour of corm and cormels, cormel shape and the leaves (Brown 1988; Quero-Garcia *et al.,* 2010). Members of this genus are grown either as ornamentals or food crops (Quero-Garcia *et al*., 2010). Among these, *Xanthosoma sagittifolium* (L.) Schott is the most widely grown and has a chromosome number of 2n equal to 26 (Onwueme, 1978). Other species include *X. atrovirens* Koch and Bouche, with yellow tubers and is favored in Puerto Rico and Dominica. *X. violaceum* Schott (*X. nigrum*) is a species with a large plant canopy having violet coloured lamina and is grown occasionally in the Pacific islands but have little value as food. *X. brasiliense* (Desf.) is a small species cultivated solely for its edible leaves (Ramesh *et al.,* 2007; Onwueme and Charles, 1994). *X. caracu* and *X. violaceum* are also grown in the Philippines (Onwueme and Charles, 1994).

Utilization

The cocoyam crop provides carbohydrates in human diet (Agueguia and Fatokun, 1987) and also contains substantial amounts of lipids, proteins, and essential vitamins like beta-carotene, riboflavin,

niacin, ascorbic acid, and thiamine (Cobley and Steele, 1976; Onwueme and Charles, 1994). In West and Central Africa, which constitute the major cocoyam-producing regions, corms and cormels are boiled, roasted or baked but the most popular preparation method is by drying and grounding the cormels into a flour called 'fufu'. The leaves can be prepared as spinach in soups or salads (Lyonga and Nzietchueng, 1987; Adiobo *et al.,* 2007). The corm, cormels and sometimes, the leaves are the main economically important parts of the plant although the cormels are tasty and preferable to the corms. Since they are grown in diverse countries and cultures, cocoyams have a lot of given names including 'tannia' (Central and South America), malanga (Spain) (Castro, 2006). In West Africa, besides being named 'macabo', it is also called the 'new cocoyam' since it replaced the 'old' cocoyam (*Colocasia esculenta* (L.) Schott) (Perneel, 2006).

A major limitation in the utilization of cocoyam as food is the presence of oxalates which confers an acrid taste or cause irritation when foods prepared from its leaves, corms or cormels are eaten or when they are eaten raw (Sefa-Dedeh and Agyir-Sackey, 2004; Obiegbuna *et al.,* 2013; Bradbury and Nixon 1998). This acridity is due to needle-like calcium oxalate crystals (raphides) which can penetrate soft skin with attendant discomfort in the tissue (Bradbury and Nixon 1998; Paull *et al.,* 1999). However, several processing methods are currently employed in order to reduce oxalate content in cocoyam plant parts. For cocoyam leaves, some methods that have been successfully practiced include cooking, soaking, ensiling, wilting and sun-drying (Bradbury and Nixon 1998; Noonan and Savage, 1999; Buntha *et al.* (2008)). Specifically, boiling leaves before eating significantly reduced oxalic acid content by 50% (Lumu and Katongole, 2011).

There are at least three cultivated *Xanthosoma* varieties based on the colour of the flesh (Castro, 2006; Perneel, 2006). The white cocoyam (2n=26), which is the most preferred variety for its high yielding and good taste, the yellow cocoyam (2n=52) which does not tuberculize, and the red cocoyam (2n=26). Cocoyam is an annual plant with growth cycle of nine to twelve months and is vegetatively propagated by farmers via suckers since natural flowering is rare. In this review, we will refer to the cocoyam crop as the 'tannia'.

Production constraints

In spite of its potential to alleviate hunger and poverty for people living in the tropics, tannia production is limited by two devastating diseases. Dasheen Mosaic Virus (DMV) is the most important virus that infects cocoyam (Castro, 2006). Under field conditions, virus-free plants performed better with respect to yield in comparison with infected *in vitro* plants. Yield losses of up to 90% have been reported to occur due to the DMV (Reddy, 2015). A second and popular limitation is the destruction due to the Cocoyam Root Rot Disease (CRRD) caused by the oomycete, *Pythium myriotylum*, leading to losses as high as 90% in infected fields (Pacumbaba *et al.,* 1992a). Interestingly, the root rot disease caused by *P. myriotylum* is specific to the *Xanthosoma* and does not infect the closely related taro tuber (*C. esculenta*) (Misra *et al.,* 2008).

Pythium myriotylum, the causal pathogen of CRRD

Discovery, background and yield losses

CRRD was reported for the first time in Ghana (Purseglove, 1972). Following this, root rot of tannia was described as an economically important disease which limits cocoyam production in the lowland forest area of Cameroon (Steiner, 1981). This disease was attributed to *P. myriotylum* Drechsl., which could be isolated from many samples of infected roots and provoked dwarfing of plants after artificial inoculation. Steiner fulfilled Koch's postulates after a successful re-isolation of the pathogen from roots. Similar results were shown by Nzietchueng (1983) and Nzietchueng (1984). In spite of these afore-mentioned independent studies, reports persisted that CRRD was caused by a pathogen complex, involving *Rhizoctonia solani* and *Fusarium solani* that are often associated with *P. myriotylum* in diseased cocoyam roots (Thebirge, 1985; Agueguia *et al.,* 1991). To clarify this, Pacumbaba *et al.,* (1992a) isolated *P. myriotylum*, *F. solani* and *R. solani* from CRRD-infected plants in Cameroon. Pathogenicity test of these individual pathogens on 3- and 7-month old cocoyam plantlets showed that the root rot disease of cocoyams can be solitarily attributed to *P. myriotylum* and not to *F. solani* and *R. solani* or a combination of both. Thus, *F. solani* and *R. solani* were designated as opportunistic pathogens. Further studies to investigate the root rot disease of cocoyams showed comparable pathogenicity of *P. myriotylum* isolates obtained from Cameroon (Perneel *et al.,* 2006; Adiobo *et al.,* 2007).

In Costa Rica, the CRRD was initially reported as being caused by *Fusarium* sp. (Saborío *et al.,* 2004). However, studies a few years later showed that *P. myriotylum* could also be isolated from diseased cocoyam roots in Costa Rica (Perneel *et al.,* 2006). CRRD caused by *P. myriotylum* has also been reported in Sri-Lanka (Tojo *et al.,* 2005).

Yield reductions of up to 90% have been reported in some plantations in Cameroon (Nzietchueng, 1983; Schafer, 1999). The documentation of yield losses due to CRRD is not readily available for other countries.

Pathogen identification

Microscopic characterisation of *P. myriotylum* shows that hyphae are straight, sinusoidal and not much branched except at the end. The pathogen can be identified by the presence of smooth sporangia which occur terminally or intercalary, consisting of long, simple portions of unswollen hyphae. Knob-like appressoria are often present appearing in branching clusters. Oogonia are abundant and mostly terminal, whereas three to six antheridia appear per oogonium with slender stalks and sometimes basal contact (Waterhouse and Waterston, 1966). Motile zoospores of *P. myriotylum* could also be induced by flooding mycelial mats of the pathogen with saccharose buffer, in Petri dishes (Pacumbaba *et al.,* 1991). *P. myriotylum* isolates from cocoyam grow optimally at 28°C, with no growth at 37°C (Perneel *et al.,* 2006). Young cultures of *P. myriotylum* have a typical powdery appearance on PDA medium and the mycelium becomes fluffy after four days. In contrast, *P.*

myriotylum isolates from other hosts immediately produce fluffy mycelium but do not give the powdery appearance.

Signs and symptoms

Symptoms reported included dwarfing of plants such that they produced small leaves which decay early. Production of tubers is either suppressed completely or small tubers are derived. A second symptom is yellowing of leaves, which for example occurs in Cameroon at the onset of heavy rain. The root system is reported to decay due to rot within 3-4 weeks which further spreads in foci that could expand up to 5 m per week (Steiner, 1981; Pacumbaba *et al.,* 1992a; Perneel *et al.,* 2006).

Etiology of CRRD

Ecology, infection and survival

The main sources of inoculum are infected soil and infected planting material. Furthermore, the pathogen is soil borne and disease development is aided by poorly drained soils. Additionally, *P. myriotylum* persists in the soil for many years (Nzietchueng, 1984).

Disease cycle

The primary inoculum for the propagation of CRRD originates from infected soil and infected propagation material (Adiobo, 2006). In Cameroon for example, most cocoyam growers established their farms using planting material (suckers, sprouting fragments of corms) obtained from abandoned old fields (Adiobo, 2006). Cocoyam is intercropped with annual crops including beans, groundnuts and cowpeas, which are potential hosts of *P. myriotylum*, the causal organism of CRRD (Tambong *et al.,* 1999). In spite of the evidence of host specialization of the CRRD pathogen, such crops may still act as alternative hosts (Adiobo, 2006). Furthermore, cocoyam residues such as corms and unharvested tubers with a potential of producing volunteer plants are sometimes not properly managed in fields. Such slow-decaying organic materials could serve as a source of initial or secondary inoculum. Besides, improperly carried out sanitation practices of removing, rouging, and destroying of corms provide inoculum reservoirs thereby enhancing the spread of the disease (Adiobo, 2006).

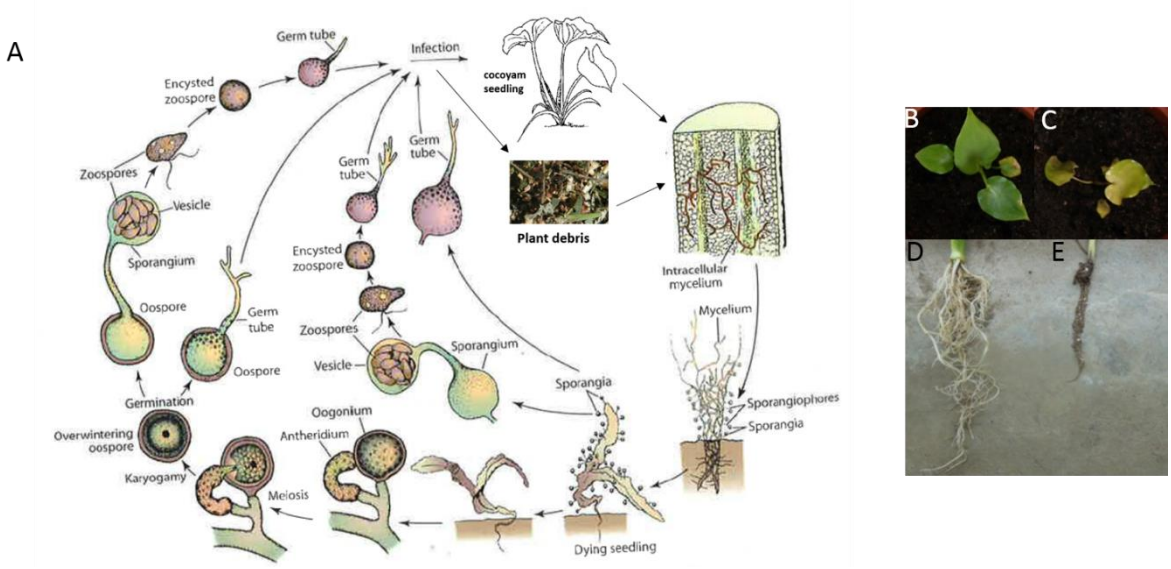

Figure 1.7. Disease cycle of *Pythium myriotylum* (adapted from Agrios (2005), (A), and symptoms of *P. myriotylum* disease on the cocoyam plant **(B-E)**. Healthy cocoyam leaves **(B)**, and diseased cocoyam leaves **(C)**. Healthy cocoyam root **(D)**, and diseased cocoyam root **(E)**. Symptoms were evaluated 7 days after infection.

Initial infection by *P. myriotylum* occurs when the pathogen directly penetrates the host by an appressorium (Drechsler, 1930). Pathogen attack begins with the colonization of the peripheral and epidermal cells of the root apex (Boudjeko *et al.,* 2006) (Figure 1.7A). These cells become rapidly lost after infection while the cortical and stele cells remain. Further studies suggest that during the early stage of infection, the oomycete causes a significant loss of pectin probably through degradation by hydrolytic enzymes which diffuse and act away from the site of attack. Loss of pectin was evidenced by a significant decrease in galacturonic acid content within the cell walls of infected roots. On the other hand, the crop responds in the early stages of infection by the formation of wall appositions which are rich in callose and cellulose (Boudjeko *et al.,* 2006).

If infected at the emerging roots stage, about 1 to 2 months following planting, stunting of cocoyam plants occurs (Adiobo, 2006). More so, *P. myriotylum* can still infect well established cocoyam plants aged 3 to 5 months. The infected plant develops chlorotic leaves followed by brown blight that starts at the periphery of the leaf and extends towards the petiole. Thereafter, leaves become yellow, dry up and then shed prematurely (Figure 1.7C). The root rots, the cortical tissue turns brown and disintegrates leaving a non-functional vascular skeleton. In severe attacks, the entire root is destroyed (Adiobo, 2006) (Figure 1.7E).

Invasion by *P. myriotylum* propagules in the detached roots of cocoyam either by zoospores or mycelial strands was observed to be intra- and intercellular. The process occurred 6 to 7 h following contact of the pathogen with the detached roots at 31°C. After 7 h, the rotting of the roots commenced (Pacumbaba *et al.,* 1994).

Detection

Pythium myriotylum is a soil-borne plant pathogen with a cosmopolitan distribution and able to incite severe root damage on a wide variety of crops (broad host range). *P. myriotylum* isolates from

cocoyam can be differentiated from other isolates by their optimum growth temperature, esterase patterns, AFLP fingerprints, and sequences of the internal transcribed spacer regions (ITS2) located between the 5.8S and 28S ribosomal DNA (rDNA-ITS2) (Tambong *et al.,* 1999; Perneel *et al.,* 2006). Furthermore, Gómez-Alpízar *et al.* (2011) successful developed a PCR-RFLP method using specific primers to distinguish cocoyam-pathogenic *P. myriotylum* isolates from non-pathogenic ones.

Pathogen diversity and host adaption

Pythium species seldom exhibit host specificity and isolates within species vary in pathogenicity and virulence on varieties of specific crops (Moorman *et al.,* 2002). However, *P. myriotylum* on cocoyam fields in Cameroon appeared to show more severe symptoms on cocoyams than what was observed in other host plants such as groundnuts and beans (Tambong *et al.,* 1999). Cocoyam-pathogenic *P. myriotylum* isolates have been shown to develop a certain degree of host specialization (Perneel *et al.,* 2006). Analysis of esterase banding patterns showed that unlike *P. myriotylum* isolates from other host crops, isolates from cocoyams did not produce any monomorphic markers. Furthermore, alignments of ITS sequences showed a base transition at position 824 from adenine in typical isolates of *P. myriotylum*, to guanine, in isolates that were pathogenic on cocoyams. Additionally, only isolates with guanine in position 824 of the ITS sequences could infect cocoyam. Together with AFLP analysis, these results highlight the host adaptation of cocoyam-pathogenic *P. myriotylum*. Subsequently, the name *P. myriotylum* var. *aracearum* was proposed in order to distinguish these cocoyam-pathogenic isolates from other *P. myriotylum* isolates (Tambong *et al.,* 2006).

Epidemiology of CRRD

In general, soil moisture, water logged soil conditions, high soil temperature and high planting density favour development of CRRD (Nzietchueng, 1985). High planting density provided favorable conditions for a fast spread of the root rot of cocoyams.

In Cameroon, CRRD was spread through infested soil or infested planting material (Nzietchueng, 1985) and could persist in the soil for many years. Plants from infested corms planted in infested soil developed poorly and remained dwarfed throughout their vegetative cycle whereas those from either infested soil with healthy corms or from infested corms with pathogen-free soil developed in most of the cases a yellowing symptom on the leaves.

Management of CRRD

Fungicides have not been fully effective against CRRD (Nzietchueng, 1983). Wide spacing, high mounds, regulation of time of planting (Onwueme and Charles, 1994), drainage improvement, use of disease-free planting material, planting on ridges, crop rotation (Giacometti and Léon, 1994), and the use of organic fertilizers have all been suggested in order to control the disease (Reddy, 2015).

Appropriate cultural practices

To salvage their crops from CRRD, cocoyam farmers engage in cultural practices including multiple cropping, hill planting, early planting, and early harvesting (Onokpise *et al.,* 1999). Employment of wide crop spacing, regulation of the time of planting (Onwueme and Charles, 1994), use of disease-free planting material using meristem tissue culture (Saborío *et al.,* 2004), crop rotation (Giacometti and León, 1994), the use of organic manures (Reddy, 2015), long interval of bush fallowing and roughing of diseased plants (Adiobo *et al.,* 2007).

Chemicals

Although some water soluble fungicides such as metalaxyl suppress *Pythium* sp., they cannot provide effective control for diseases caused by this genus unless if applied before the plant is infected. Records of successful chemical control in green houses have been attributed to the limited amount of soil which allows for complete soil disinfection before planting (Adiobo, 2006). Besides, field control of *Pythium* sp. has been considered to be difficult since fungal propagules are not exposed to the chemical coupled with the fact that the effect of most chemicals are short lasting such that the pathogen can easily survive via production of oospores or chlamydospores which are less sensitive to fungicides.

Field studies in Cameroon showed that high doses of metalaxyl (10 g/cocoyam plant) could reduce CRRD incidence by 80% (Nzietchueng, 1985). However, the use of chemicals against CRRD were shown to be less successful (Pacumbaba *et al.,* 1992b; Onokpise *et al.,* 1999). The fungicide, Ridomil Plus 72, used to be effective in the control of the pathogen but was not cost-effective nor readily available to farmers (Onokpise *et al.,* 1999). In the light of these limitations, alternative control strategies are in great demand.

Disease suppressive soils

In some soils, indigenous plant-beneficial microorganisms are capable of effective protection from certain plant pathogens, and these soils are referred to as being disease suppressive (Mazzola, 2002; Weller *et al.,* 2002; Ramette *et al.,* 2006). Several of these soils possess physical or chemical characteristics which effect pathogen suppression directly or indirectly via alteration of a biological constituent of the ecosystem at the expense of the pathogen (Martin and Loper 1999). Although suppressive soils have been reported for many soil pathogens (Weller *et al.,* 2002), only a couple have been described for *Pythium* spp (Martin and Loper, 1999).

For *P. myriotylum*, suppressive soils to CRRD have only been described in the volcanic andosols of Cameroon (Adiobo *et al.,* 2007). Soil samples from three andosol and three ferralsol soils were collected from fields after which greenhouse assays were conducted in order to ascertain their soil suppressiveness to the CRRD. Besides revealing that ferralsols possess higher disease severity than andosols, the results of this study indicated that the suppressiveness of andosols correlated with high presence of organic matter, Ca, K, Mg and N. On the other hand, the high content of sand and clay in conducive ferralsols positively correlated with disease severity. Thus, high organic matter and certain physicochemical properties appears to mediate disease suppression of andosols via improvement of soil structure, nutrient content and to some extent, microbial biomass (Adiobo *et al.,* 2007).

Suppressive composts

Besides the employment of disease suppressive soils with albeit rare occurrence, together with cultural and biological control methods against CRRD, attempts have been made to explore the use of composts in the suppression of *P. myriotylum* on cocoyams.

In Cameroon, three compost types (A, B and C) were studied for potential suppressiveness to CRRD (Adiobo, 2006). Compost A consisted of 10% cow manure, 30% tree bark, 30% fallen leaves and 30% sugarcane waste. Compost B composed of 10% poultry manure, 35% sugarcane waste and 55% oil palm waste whereas Compost C contained 10% cow manure, 35% green grasses and 55% oil palm waste. Various composts were assessed in green house plant experiments using varying levels of *P. myriotylum* inoculum (400, 800 and 1200 propagules/g soil). Compost doses were 10, 20, and 30%, vol/vol. The three composts varied in their disease suppressive capacity. Compost A was most suppressive followed by Compost C and then B. Interestingly chemical properties determination of all composts showed that Compost A had both a high percentage of organic carbon (22.80%) and calcium content (76.20 cmol/kg). Microbial analysis of composts further revealed that fluorescent pseudomonads and actinomycetes were higher in compost A than in other compost types. Interestingly, the disease suppressiveness of all compost types were lost after autoclaving and could only be partly recovered following colonization by their resident flora. These results suggest that the disease suppression demonstrated by all three compost types is predominantly biological and due to the activities of diverse microbial groups. The disease suppressive capacities of these composts were further confirmed in field experiments (Adiobo, 2006).

Furthermore, in Cameroon, eight compost types made from four different grass species namely *Tithonia diversifolia*, *Chromolaena odorata*, *Pennisetum purpureum* and *Ageratum conyzoides*, were evaluated for suppressiveness against the root rot disease of cocoyam (Djeugap et al. 2014). Results of this study showed significant reduction ($P \leq 0.05$) in disease incidence and severity in all compost amended pots than the control at 12 weeks post inoculation.

More so, in Nigeria, Chukwu and Eteng (2014) employed rhe use of four soil amendments namely rice mill waste alone, rice mill waste plus poultry manure, NPK alone and a combination of rice mill waste, NPK and poultry manure,.for the control of CRRD coupled with enhancement of soil health. Although the best control was displayed by treatments containing all three amendments, all treatments improved total cocoyam yield, total N, organic carbon, available P and exchangeable K. However, the use of composts can be very tasking depending of what materials are required and how readily they can be obtained.

Besides the afore-described methods, resistance induction against CRRD using elicitors such as Benzo-(1,2,3)-thiadiazole-7-carbothioic S-methyl ester (BTH) has been explored with a degree of success (Mbouobda *et al.,* 2010; Oumar *et al.,* 2015). More so, studies aimed at breeding for resistance has been conducted with hardly any success mainly because of ploidy incompatibility in cocoyams (Blay *et al.,* 2004; Saborio *et al.,* 2004; Onokpise *et al.,* 1999).

Biological control

Fluorescent pseudomonads have long been recognized as effective biocontrol agents against plant pathogens (Höfte and Altier, 2010). The most commonly reported mechanism of biocontrol by fluorescent pseudomonads are via the production of cyclic lipopeptides (Raaijmakers *et al.,* 2006), antibiotics, hydrogen cyanide and lytic enzymes (Thomashow and Weller, 1996), competition for nutrient and niches (Kamilova *et al.,* 2005), competition for iron-mediated siderophores (Thomashow and Weller, 1996) and induced systemic resistance (De Vleesschauwer and Höfte, 2009). More so, many fluorescent pseudomonads have the capacity to stimulate plant growth by enhancing the availability and uptake of mineral nutrients through employment of phosphate-solubilization enzymes (Höfte and Altier, 2010).

P. aeruginosa PNA1, isolated from chickpea roots in India, showed strong biocontrol efficacy against Fusarium wilt on chickpeas and pigeonpeas (Anjaiah *et al.,* 1998) and also against *Pythium splendens* damping-off of common bean (Anjaiah *et al.,* 2003). Using *in vitro* and sterile volcanic soil experiments, Tambong and Höfte (2001) compared the biocontrol efficacy of *P. aeruginosa* PNA1 and its phenazine mutant against CRRD. Dual plate assays coupled with growing *P. myriotylum* in potato dextrose broth amended with bacterial supernatants, showed that *P. aeruginosa* PNA1 significantly reduced mycelial growth in the former whereas, mycelial dry weight was significantly reduced in the latter. Results obtained from plant experiments further highlighted the disease suppressiveness of PNA1 and the involvement of phenazines in the biocontrol capacity of this strain. Additively, Perneel *et al.* (2008) demonstrated the synergistic role of phenazines and rhamnolipids biosurfactants, produced by *P. aeruginosa* PNA1, against *P. myriotylum* on cocoyams. Given that *P. aeruginosa* is a clinical pathogen, it was imperative to search for alternative *Pseudomonas* biocontrol strains which produce both phenazines and biosurfactant that could possess comparable biocontrol efficacy as *P. aeruginosa* PNA1.

In Cameroon, fluorescent *Pseudomonas* strains were randomly isolated from the rhizosphere of healthy red and white cocoyams in naturally infested CRRD fields (Perneel *et al.,* 2007). All *Pseudomonas* isolates were screened for *in vitro* antagonism against *P. myriotylum* in dual cultures (Perneel *et al.,* 2007). Besides the observation of field tolerance by red cocoyam towards CRRD, this study revealed several interesting results. Among the *Pseudomonas* isolates tested, several CRRD antagonists were found, all of which were isolated exclusively from the red cocoyam rhizosphere. Interestingly, all except one produced phenazine antibiotics. Furthermore, the efficacy of two of these strains, *Pseudomonas* sp. CMR12a and CMR5c, against CRRD was compared with that of *P. aeruginosa* PNA1 during plant experiments. While *P. aeruginosa* reduced CRRD severity by 48%, *Pseudomonas* sp. CMR12a and CMR5c could reduce root rot severity by 60% and 53%, respectively (Perneel *et al.,* 2007). In a similar study, the well-studied phenazine producing strain *P. chlororaphis* PCL1391 (Chin-A-Woeng *et al.,* 1998) also showed excellent control of the CRRD (Perneel, 2006).

Pseudomonas sp. CMR12a

Pseudomonas sp. CMR12a was isolated from the rhizosphere of healthy red cocoyams in Cameroon and showed excellent suppression of CRRD (Perneel *et al.,* 2007). Since then, *Pseudomonas* sp. CMR12a has been much studied as a biocontrol agent against several plant pathogens including *Rhizoctonia solani* (D'aes *et al.* (2011); Hua and Höfte, (2015)). CMR12a could also suppress root rot on tomatoes caused by *Phytophthora nicotianae* (De Jonghe, 2006), and strongly reduced the viability of microsclerotia produced by *Vertillium longisporum*, the causal pathogen of verticillium wilt (Debode *et al.,* 2007).

This strain is taxonomically positioned between the *P. chlororaphis* and *P. protegens* subgroups (D'aes *et al.,* 2014). More so, CMR12a produces two phenazines namely phenazine-1-carboxylic acid (PCA) and its main phenazine compound, phenazine-1-carboximide (PCN) (Perneel *et al.,* 2007). Additionally, CMR12a produces two structurally different cyclic lipopeptides namely sessilins and orfamides (D'aes *et al.,* 2014). 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. Similarly, three derivatives sessilin A, B and C, with m/z $[M+2H]^{2+}$ values of 1015.2, 1024.2, and 993.2, respectively, are produced. Sessilins are structurally related to tolaasins, produced by the mushroom pathogen, *P. tolaasii* while orfamides of CMR12a are closely related to orfamides produced by *P. protegens* Pf-5 and *P. protegens* CHA0. However, the main orfamide compound produced by CMR12a is orfamide B while that produced by Pf-5 is orfamide A. Sessilins and orfamides are responsible for the biofilm formation and swarming motility of CMR12a, respectively (D'aes *et al.,* 2014).

Using site-directed mutagenesis, deletion mutants were constructed which were deficient in either phenazines, sessilins or both metabolites of CMR12a. The roles of phenazines and sessilins in the biocontrol capacity of CMR12a were shown in plant experiments with bean and *Rhizoctonia solani* using these mutants (D'aes *et al.,* 2011). Much later, more biosynthetic deficient mutants in the secondary metabolites of CMR12a became available and were yet to be tested in several pathosystems (D'aes *et al.,* 2014).

In CMR12a, the orfamide gene cluster is flanked both up-and downstream by LuxR-type transcriptional regulators. However, the gene cluster of sessilins is only flanked upstream by a LuxR-type transcriptional regulator (D'aes *et al.,* 2014). However, the roles of these three LuxR-type regulators in the regulation of sessilins and orfamides in *Pseudomonas* sp. CMR12a is not yet known.

Knowledge about the export mechanisms/systems of CLPs in plant beneficial *Pseudomonas* is currently limiting. The partial roles of the *macAB* genes in the export of some CLPs have been previously described (Lim *et al.,* 2009; Dubern *et al.,* 2008; Cho and Kang, 2012). Additionally, several CLP-producing *Pseudomonas* strains possess an OprM homologue named the NodT, upstream of their biosynthetic gene cluster. The evolutionary relationship between this homologue and their corresponding OprM has not been elucidated. In CMR12a, the role of the NodT and other putative transport proteins in CLP export is not yet known.

Chapter 2

Cocoyam Root Rot Disease caused by *Pythium myriotylum* in Nigeria

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Summary

Cocoyam (*Xanthosoma sagittifolium*) is an important cabohydrate staple in Nigeria. In spite of its importance, cocoyam yield has been in continuous decline due to the cocoyam root rot disease (CRRD). In Cameroon, Costa Rica and Sri Lanka, the causal pathogen of CRRD has been described as *Pythium myriotylum*. However, in Nigeria, this disease has been described to be a complex involving several pathogens including *Rhizoctonia solani* and *Fusarium solani.* It was therefore necessary to ascertain if the primary causal pathogen of CRRD in Nigeria is similar to previously described pathogen associated with this disease in other cocoyam-growing countries. Thus in this study, our goal was to investigate the primary causal pathogen of this disease in Nigeria. Field sampling of infected cocoyam plants in Nigeria followed by taxonomic characterisation of the pathogen led to the identification of two isolates, NGR02 and NGR03, which had 100% sequence identity with pathogenic *P. myriotylum* isolates which cause CRRD in Cameroon. Subsequently, pathogenicity tests were conducted using these two isolates on tissue culture-derived cocoyam plants. Results showed that both isolates were pathogenic on cocoyam seedlings and resulted in typical CRRD symptoms including leaf yellowing, root rot and stunting of plants. Furthermore, *P. myriotylum* could be re-isolated from infected cocoyam roots. Thus, results of this study show that similar to Cameroon, Costa Rica and Sri Lanka, the primary causal pathogen of CRRD in Nigeria is *P. myriotylum*. This finding will contribute to the application of management options which specifically target this pathogen in cocoyam fields in Nigeria.

Introduction

In Nigeria, cocoyam (*Xanthosoma sagittifolium*) is an important staple which besides being a food crop, serves as a major source of income for rural households. However, yield losses due to cocoyam root rot disease (CRRD) remain a major constraint to increased cocoyam production in Nigeria (Chukwu and Eteng, 2014). Until now, in Nigeria, this disease has been attributed to a pathogen complex including *Fusarium solani* and *Rhizoctonia solani* (Chukwu *et al.,* 2011). However, symptoms observed in infected fields in Nigeria are similar to those caused by the oomycete, *Pythium myriotylum*, on diseased cocoyam plants in Cameroon, Costa Rica and Sri Lanka (Pacumbamba *et al*. 1992a; Perneel *et al*. 2006; Tojo *et al.,* 2005). Therefore, it became necessary to ascertain whether CRRD in Nigeria is due to a pathogen complex or has *P. myriotylum* as its primary causal pathogen. Thus, the aim of this study was to determine the primary causal pathogen of CRRD disease in Nigeria.

Results

Morphological characterization of Nigerian isolates

Two isolates, NGR02 and NGR03, isolated from cocoyam roots in Nigeria were identified as *Pythium myriotylum* on the basis of morphological characteristics including the presence of smooth, lobate sporangia together with knob-like appressoria which often appear in branching clusters (Waterhouse and Waterson, 1966). Similar with *P. myriotylum* CMR1, a Cameroon isolate which causes CRRD (Perneel *et al.,* 2006), the mycelium of NGR02 and NGR03 had a typical powdery appearance on PDA medium which subsequently became fluffy after 3 days (data not shown).

rDNA-ITS Phylogenetic analyses

ITS regions of NGR02 and NGR03 were amplified by PCR, sequenced and analysed using Blastn and MEGA6. Blast analyses revealed that Nigerian isolates had 100% sequence identity with the sequences of *P. myriotylum* isolates from Cameroon which caused CRRD symptoms. More so, both isolates were 100% similar to each other. Figure 2.1 shows the phylogenetic relationship between *P. myriotylum* isolates pathogenic on cocoyam, *P. myriotylum* isolates obtained from other host crops and the two isolates from Nigeria. *P. myriotylum* isolates formed a major cluster from which two subclusters can be distinguished. The first subcluster groups all *P. myriotylum* isolates from cocoyam including the two Nigerian isolates, NGR03 and NGR03. The second subcluster comprised two isolates from unknown hosts while the *P. myriotylum* tobacco isolate, Py-45, was situated outside the cluster.

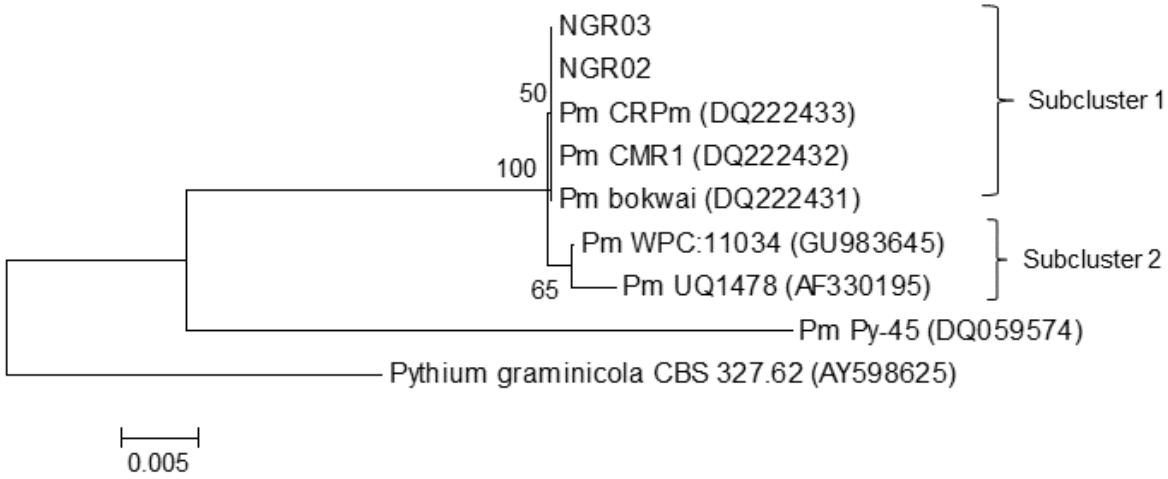

Figure 2.1. Phylogenetic relationship between *P. myriotylum* isolates obtained from cocoyam roots in Nigeria and *P. myriotylum* isolates from Cameroon which cause typical CRRD symptoms. Tree was generated via Maximum Likelihood using 1000 resampled data sets. Bootstrap values of higher than 50% are indicated. NGR indicates isolates from Nigeria while CRP, CRM and bokwai isolates are from Cameroon. Also included in the tree are isolates from other host plants including tobacco (Py-45). A sugarcane isolate, *P. graminicola*, was used as outgroup.

Pathogenicity Tests

Nigerian isolates, NGR03 and NGR03 were evaluated for pathogenicity to cocoyam tissue culture-derived plantlets. Root rot symptoms were observed 3 days after inoculation (Figure 2.2) and the leaves were scored 10 days post inoculation using a scoring system ranging from no disease (class 0) to leaf death (class 4). The left panel of Figure 2.2 shows severe symptoms of the root rot disease which include yellowing of leaves and in some cases, plant death while the right panel shows below-ground root rot symptoms caused by the test pathogens in comparison with the healthy control. Isolates NGR02 and NGR03 were again isolated from infected roots and showed similar characteristics as previously observed.

Figure 2.2. Pathogenicity experiments using NGR02 and NGR03 on tissue-culture derived cocoyam plantlets. Left panel: Symptoms of root rot disease caused by Nigerian isolates; **Right panel:** Below ground root rot symptoms. **A)** healthy plants (no *Pythium myriotylum*); **B)** NGR02; **C)** NGR03.

Furthermore analysis of the disease index calculated from the data did not reveal a statistical difference between NGR02 and NGR03 (Figure 2.3A). This was consistent in both repetitions. More

so, comparison of the percentage of diseased leaves present in every class between both isolates did not differ significantly (Figure 2.3B).

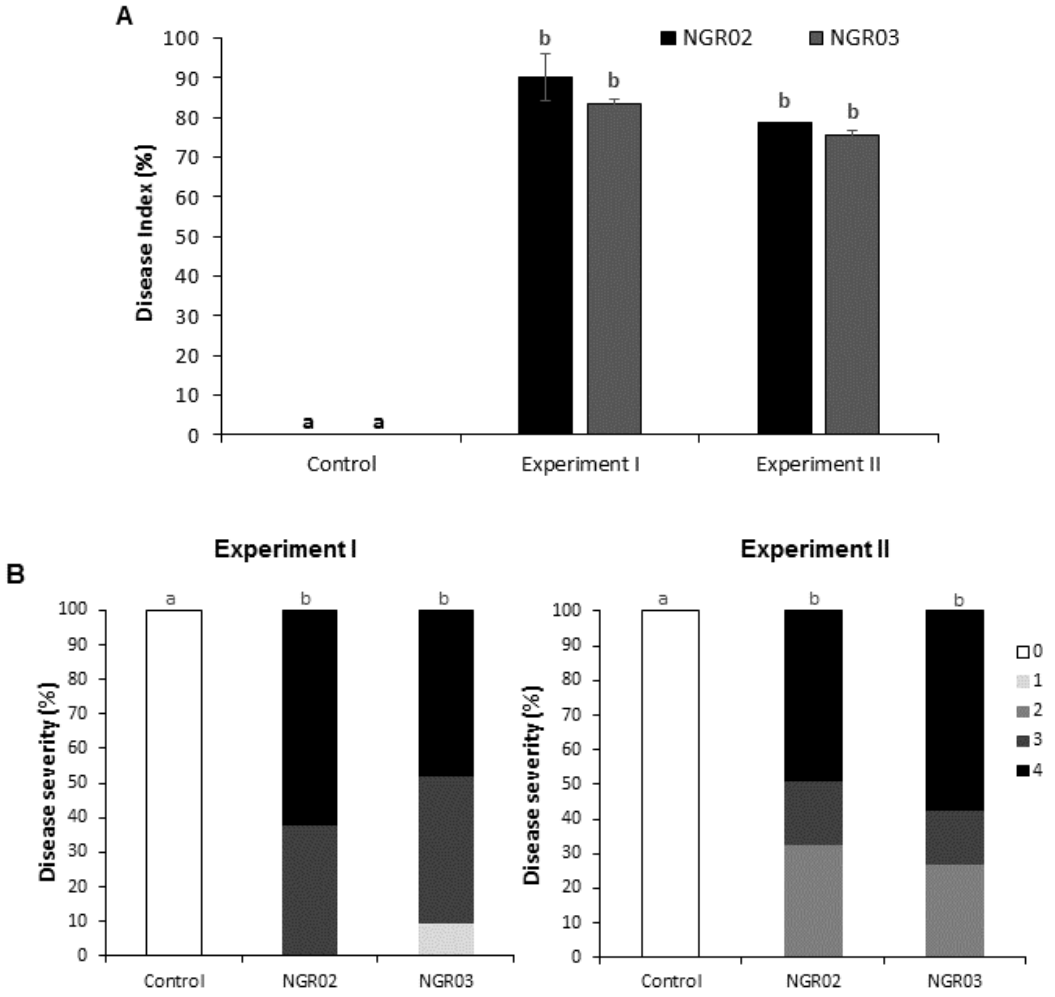

Figure 2.3. Pathogenicity of NGR02 and NGR03 on tissue-culture derived cocoyam plantlets. Disease indices are shown for two independent experiments using both pathogens **(A)** while disease classes for each pathogen were also assessed **(B)**. Bars with different letters are statistically different at 5% probability level using the Kruskal Wallis non-parametric test followed by Mann Whitney test.

Discussion

In Nigeria, CRRD has been attributed to a pathogen complex. The aim of this study was to ascertain whether as reported in Cameroon, Costa Rica and Sri Lanka, the primary causal organism of CRRD in Nigeria is *P. myriotylum*. Using morphological and phylogenetic analyses combined with pathogenicity tests, we showed that two Nigeria isolates obtained from diseased cocoyam roots, are *P. myriotylum* strains and can cause CRRD symptoms. Previous studies demonstrated that *P. myriotylum* cocoyam rhizosphere isolates from Cameroon, including CRPm, CMR1, and Bokwai, caused CRRD symptoms on tissue culture-derived cocoyam plantlets (Tambong *et al.,* 1999: Tambong and Höfte, 2001; Perneel *et al.,* 2006).

In previous studies, P. myriotylum isolates which infect cocoyams have been differentiated from those which infect other host crops by optimal growth at 28 °C and a base substitution in the IT2 region from adenine in isolates of *P. myriotylum* from other host crops to guanine in isolates that cause CRRD (Perneel *et al.,* 2006). Although we did not check this for NGR02 and NGR03, our results indicate that this ITS base mutation by virulent *P. myriotylum* will also be the case for Nigerian isolates.

To conclude, this study demonstrates that *Pythium myriotylum* isolates that infect cocoyam in Nigeria are similar to the isolates which infect cocoyam in Cameroon. The two isolates obtained also appear to be similar in virulence. This knowledge will enable future focus on disease management strategies that would address the causative agent of the cocoyam root rot disease in Nigeria.

Materials and Methods

Field sampling and Pathogen Isolation

Diseased cocoyam plants from an infected field at the National Root Crops Research Institute, Umudike, Nigeria, were collected and brought to the Plant Pathology Lab of the Institute. Roots were thoroughly washed in running tap water until white and examined for light brown spots. These spotted sections were then excised and rinsed with distilled water.

Under sterile conditions, excised roots were surface sterilized using 1% NaOCl for 15 min and transferred onto sterilized water agar medium (15g agar L^{-1} of water) supplemented with streptomycin sulphate (0.3g/L) in sterile 90 mm Petri dishes. Streptomycin sulphate was added after autoclaving. Plates were incubated at 28°C for 48 h. Actively growing hyphal tips were transferred aseptically to antibiotic-free water agar and incubated under similar conditions. Following this, isolates (NGR02 and NGR03) obtained were subcultured on PDA plates and examined 2-3 days later.

ITS sequence analysis

The rDNA-ITS region of NGR02 and NGR03 isolates were sequenced. These isolates were grown on Potato dextrose broth plates for 3 days after which mycelial mats were harvested by filtration and ground to a fine powder with liquid nitrogen. DNA extraction was carried out using the DNeasy Plant

Mini Kit (Qiagen). The rDNA-ITS fragment was amplified using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). DNA was amplified by PCR in a 25µl mixture containing 2µl genomic DNA, 2.5µl PCR buffer (10x; Qiagen), 5µl Q-solution (Qiagen), 0.5µl dNTPs (10mM; Fermentas GmbH), 1.75 µl of each primer (10µM), 0.15 µl Taq DNA Polymerase (5 U μl^{-1}; Fermentas GmbH), and 11.35µl of sterile water. Amplication was performed using Flexcycler PCR Thermal Cycler with initial denaturation of 94°C for 10 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification products were separated on 1% agarose gel in TAE buffer at 100V for 25 min and visualized by ethidium bromide staining. Genomic sequences were determined using Sanger Sequencing by LGC Genomics GmbH (Berlin, Germany).

Table 2.1. *Pythium* isolates used for phylogenetic analyses

Species name	Host/substrate	Locality*	Accession No	Reference
P. graminicola	*Saccharum officinarum*	Jamaica	AY598625	Lévesque and de Cock, 2004
P. myriotylum UQ1478	unknown	Australia	AF330195	Unpublished
P. myriotylum P11034	unknown	unknown	GU983645	Unpublished
P. myriotylum Py-45	Tobacco	USA	DQ059574	Ristaino *et al.,* 2007
P. myriotylum CMR1	Cocoyam	Cameroon	DQ222432	Perneel *et al.,* 2006
P. myriotylum Bokwai	Cocoyam	Cameroon	DQ222431	Tambong *et al.,* 1999
P. myriotylum CRPm	Cocoyam	Cameroon	DQ222433	Tambong *et al.,* 1999
NGR02	Cocoyam	Nigeria	n/a	This study
NGR03	Cocoyam	Nigeria	n/a	This study

*n/a denotes no accession number has been assigned.

Initial similarity searches was conducted for sequences of NGR02 and NGR03 using Blastn. Furthermore, phylogenetic analyses was conducted by retrieving sequences of *P. myriotylum* isolates that were pathogenic on cocoyam from Genbank (Table 2.1). Also included were isolates from other hosts. Sequence alignment was performed using Muscle (Edgar, 2004) via the software package MEGA6 (Tamura *et al.,* 2013). The tree was inferred by Maximum Likelihood (ML) using 1000 bootstrap replicates and was rooted with *P. graminicola* Py-45.

Pathogenicity Tests

Based on morphological characteristics and ITS sequence homology with *P. myriotylum* isolates which are pathogenic to cocoyam roots, *P. myriotylum* NGR02 and NGR03 were selected for pathogenicity assays. Pathogenicity tests were carried out in conducive volcanic soil collected from Molyko, a cocoyam growing area of Cameroon. The soil used for the experiment was sterilized twice. Isolates were grown on PDA plates for 5 days at 28°C and three plugs of each isolate were used to infect 300 g of soil. Mycelial plugs of inoculum were blended in 300ml sterile distilled water after which the inoculum suspension was mixed with the soil. Subsequently, 6-8 week-old tissue-culture cocoyam plantlets produced as described by Tambong *et al*. (1998) were transplanted into each plastic box containing the inoculum-soil mixture. The experimental set-up was a completely randomized design with ten cocoyam plantlets per treatment, including a healthy control. Each cocoyam plant had at least

3 leaves. The transplanted plantlets were incubated at 28°C ± 2°C and hand-irrigated daily to maintain moisture content of the soil. Plants were observed daily for typical symptoms of the cocoyam root rot disease. The root rot severity was scored using a rating scale 0 (no symptom) to 4 (death of leaf) as described by Tambong *et al*. (1999) with minor modifications. The experiment was repeated thrice. Isolates NGR02 and NGR03 were successfully re-isolated from infected roots and characterized.

Statistical analysis

For the pathogenicity experiments, the disease index was calculated as:

$$\text{Disease Index} = \frac{\sum (\text{Disease class} \times \text{number of leaves within that class})}{\text{Total number of leaves within treatment} \times \text{highest class value in the scale}}$$

Since our disease severity and root colonization data did not meet the conditions of normality and homogeneity of variances, the results were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P \leq 0.05$).

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Chapter 3

Phenazines and cyclic lipopeptides produced by *Pseudomonas* sp. CMR12a are involved in the biological control of *Pythium myriotylum* on cocoyams (*Xanthosoma sagittifolium*)

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Summary

Root rot disease caused by *Pythium myriotylum* is the most devastating disease of the tropical cocoyam (*Xanthosoma sagittifolium*) tuber crop with production losses of up to 90%. This study was conducted in order to determine the role of phenazines (PCN), and two cyclic lipopeptides namely sessilins and orfamides, produced by *Pseudomonas* sp. CMR12a, in the suppression of the Cocoyam Root Rot Disease (CRRD). Previously generated biosynthetic mutants of CMR12a that were deficient in either one, two or all three metabolites were used in *in vitro*, microscopic and plant experiments. Using CMR12a and its biosynthetic mutants, plant experiments revealed that mutants which produce sessilins, orfamides or phenazines alone or at least any two of the metabolites, could successfully suppress the cocoyam root rot disease caused by *P. myriotylum* albeit in varying capacities. *In vitro* assays using filter sterilized bacterial supernatants showed that besides the WT strain, all mutants except the null mutant, which makes none of the metabolites, could successful inhibit *P. myriotylum*. Following contact between the mycelia of *P. myriotylum* and purified/semi-purified metabolites, microscopic analysis showed that a concentration of 1 nM and higher of crude sessilins resulted in cell damage including vacuolization and lysis whereas amounts from 25 nM PCN and higher could produce a similar phenotype including mycelial bloating. However, treatments with orfamide resulted in mycelial lysis at 10 nM and higher with vacuole formation recorded only at higher concentrations of 10 µM. Interestingly, during plant experiments, the null mutant was able to give some level of disease suppression suggesting that CMR12a produces other compound(s) which could be antagonistic towards our target pathogen. Subsequent genome mining of CMR12a draft genome revealed the presence of a genomic island. This island not only contains the phenazine and sessilin gene clusters, but also a tabtoxin cluster. It remains to be investigated whether the tabtxin cluster is functional in *Pseudomonas* sp. CMR12a and whether it accounts for the residual *Pythium* suppression observed in the null mutant. Summarily, phenazines, sessilins and orfamides, produced by *Pseudomonas* sp. CMR12a, can play independent and additive roles in the suppression of *Pythium*-mediated CRRD.

Introduction

Cocoyam (*Xanthosoma sagittifolium*) is a carbohydrate staple which feeds more than 400 million people living in the tropics (Onokpise *et al.,* 1999). In spite of its importance, this crop is highly susceptible to the cocoyam root rot disease (CRRD) caused by *Pythium myriotylum* (Pacumbaba *et al.,* 1992a) which can result in up to 90% yield loss in affected fields. Several methods have been employed in the control of CRRD including the use of chemicals, composts and cultural practices of crop rotation and clean planting material. However, there seems to be no end in sight for the control of this disease since none of the afore-mentioned methods have proved sustainable. For example, chemicals have been reported to be inefficient against *P. myriotylum* on cocoyams (Pacumbaba *et al.,* 1992b; Onokpise *et al.,* 1999). The employment of biological methods as a viable CRRD management option appears to be gaining ground (Tambong and Höfte, 2001; Perneel *et al.,* 2008).

Pseudomonas sp. CMR12a was isolated from the rhizosphere of healthy cocoyams in Cameroon (Perneel *et al.,* 2007). This strain produces several metabolites including two classes of CLPs and two phenazines namely phenazine-1-carboxamide (PCN) and phenazine- 1-carboxylate (PCA) of which PCN is predominantly produced (Perneel *et al.,* 2007; D'aes *et al.,* 2011; De Maeyer *et al.,* 2011). The two classes of CLPs produced by CMR12a are the sessilins and orfamides (D'aes *et al.,* 2014).

Pseudomonas sp. CMR12a has been demonstrated to provide effective protection of cocoyam and common bean (*Phaseolus vulgaris*) against root rot diseases caused by *Pythium myriotylum* (Perneel *et al.,* 2007) and *Rhizoctonia solani* (D'aes *et al.,* 2011), respectively. In a *R. solani*-bean pathosystem, *Pseudomonas* sp. CMR12a significantly reduced bean root rot caused by two anastomosis groups (AGs), AG 2-2 and AG4-HGI, of *R. solani* (D'aes *et al.,* 2011). Moreover, phenazines and rhamnolipids produced by *Pseudomonas aeruginosa* PNA1 have been shown to act synergistically in the control of root rot on cocoyam caused by *P. myriotylum* and pre-emergence damping-off on bean caused by *P. splendens* (Perneel *et al.,* 2008). Although the role of phenazines and sessilins, produced by CMR12a, has been fully documented on bean and Chinese cabbage (D'aes *et al.,* 2011; Chapter 2), its biological relevance on its natural host plant (the cocoyam crop) is yet to be elucidated.

In this study, our objective was to determine the roles of phenazines, orfamides and sessilins, produced by *Pseudomonas* sp. CMR12a in the control of *Pythium*-mediated CRRD. Using CMR12a and its biosynthetic mutants deficient in these metabolites, *in vitro*, microscopic and plant experiments were conducted to investigate the roles of these metabolites in (i) disease suppression against root rot on cocoyams in growth chamber experiments and, (ii) the colonization of cocoyam roots by *Pseudomonas* sp. CMR12a (iii) the mycelial growth of *Pythium myriotylum*.

Results

Biocontrol effect of Pseudomonas sp. CMR12a and its mutants against CRRD

Biocontrol experiments were conducted to compare the protective effect of CMR12a together with that of its phenazine and CLP biosynthesis mutants against CRRD. Results of this experiment showed that CRRD was effectively controlled when the growth substrate was treated with either CMR12a, mutants that produced at least two secondary metabolites (P, S, O) or the mutant that produced sessilins alone (PO) (Figure 3.1). Although, treatments with strains which produce only phenazines (SO) or orfamides (PS) could provide a measure of control, the suppressive effect was comparative less than that obtained with all other treatments with the exception of the null mutant. Intriguingly, the null mutant strain which produces no phenazines, sessilins and orfamides, still showed suppressive capacity towards CRRD.

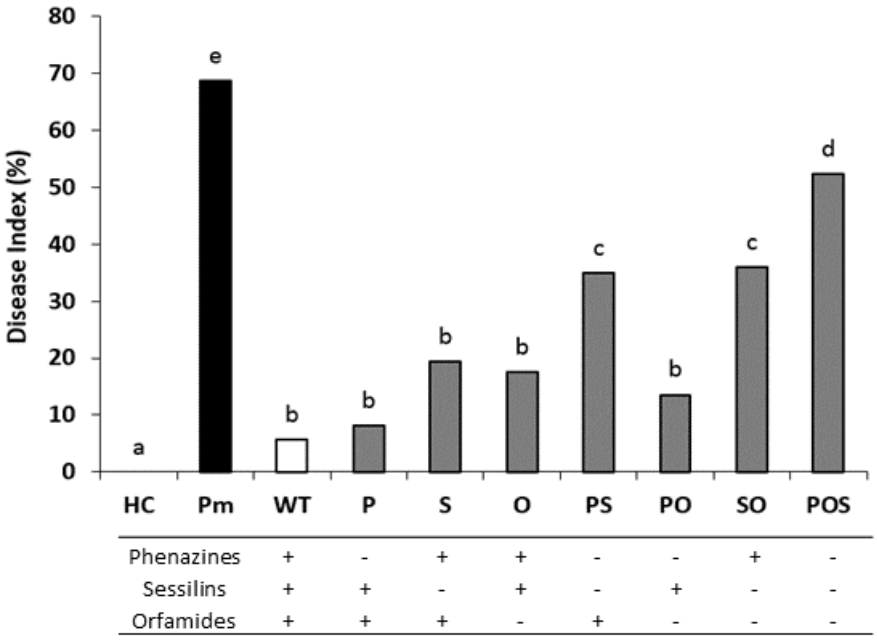

Figure 3.1. Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production towards *Pythium* root rot on cocoyams in two independent experiments. Figure represents pooled data from two experiments. Con: healthy control**;** WT: wild-type *Pseudomonas* sp. CMR12a; Pm: *P. myriotylum* NGR03; P: phenazine mutant; S: sessilin mutant, O: orfamide mutant. Mutant abbreviations are further explained in Table 3.3. The table below the figure indicates metabolites that are still produced by the various mutants. Bars with different letters are statistically different at 5% probability level using the Kruskal Wallis non-parametric test followed by Mann Whitney test (SPSS).

Bacterial colonisation of cocoyam roots

In two independent experiments, all bacteria strains tested could colonize cocoyam roots successfully with populations of about 10^7 to 10^9 CFU g^{-1} of fresh roots (Table 3.1). In the first experiment, comparable bacteria populations were obtained from the rhizosphere of all strains tested. However, during the second experiment, treatments with the phenazine mutant recorded significantly

lower bacterial counts than treatments with CMR12a, the orfamide mutant and the sessilin mutant strains.

Table 3.1. Root colonisation by *Pseudomonas* sp. CMR12a and mutants impaired in phenazine and/or CLP production obtained from the cocoyam rhizosphere after seven days of inoculation with *P. myriotylum* NGR03.

		Population density of CMR12a and mutants[a] (in log CFU g-1 of fresh root)	
Treatment	Phenazines and CLPs produced	Repetition 1	Repetition 2
CMR12a	Phenazines, sessilins and orfamides	8.6 ± 0.4 a	9.3 ± 0.4 b
CMR12a-ΔPhz	sessilins and orfamides	8.5 ± 0.3 a	8.6 ± 0.2 a
CMR12a-CLP1	phenazines and orfamides	8.5 ± 0.5 a	9.3 ± 0.4 b
CMR12a-ΔCLP2	phenazines and sessilins	7.9 ± 0.6 a	9.0 ± 0.3 ab
CMR12a-CLP1-ΔCLP2	phenazines	8.2 ± 0.3 a	9.4 ± 0.2 b
CMR12a-ΔPhz-CLP1	orfamides	7.9 ± 0.7 a	9.0 ± 0.1 ab
CMR12a-ΔPhz-ΔCLP2	sessilins	8.4 ± 0.4 a	8.9 ± 0.2 ab
CMR12a-ΔPhz-CLP1-ΔCLP2	Null	8.4 ± 0.1 a	9.0 ± 0.3 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, valuesfollowed by the same letter are not significantly different according to the Tukey tests (*P* = 0.05).

Inhibitory capacity of P. myriotylum growth by CMR12a and mutants

In order to determine the effect of the three metabolites against the hyphal morphology of *P. myriotylum*, *in vitro* assays were conducted using filter sterilized supernatants on microscopic slides. Visual inhibition zones was observed and calculated for all treatments except for the *P. myriotylum* control and the null mutant (Figure 3.2).

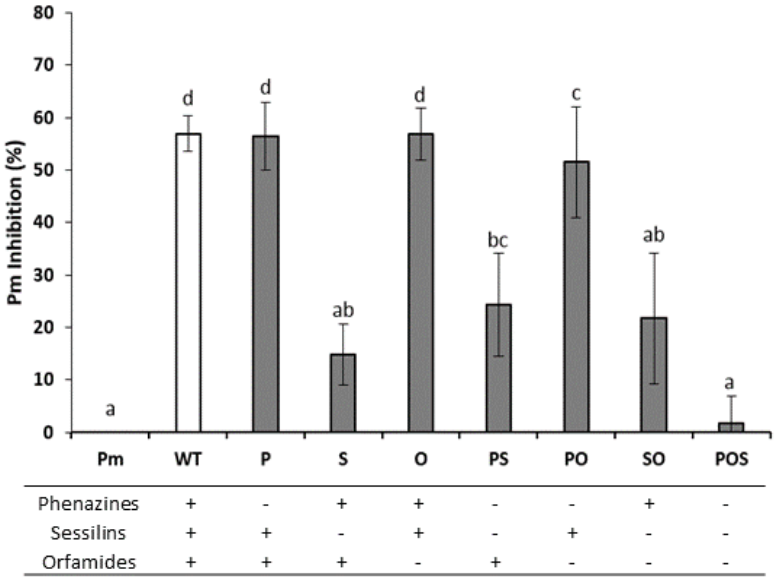

Figure 3.2. Effect of phenazines, sessilins and phenazines on the mycelia growth of *P. myriotylum* using filter-cultured supernatants. Figure represents pooled data from two independent experiments. Con: healthy control**;** WT: wild-type *Pseudomonas* sp. CMR12a; Pm: *P. myriotylum* NGR03; P: phenazine mutant; S: sessilin mutant, O: orfamide mutant. Mutant abbreviations are further explained in Table 3.3. The table below the figure indicates metabolites that are still produced by the various mutants. Bars with different letters are significantly different according to Tukey's Post-hoc test (*P* = 0.05).

The highest inhibition capacity was exhibited by the WT and mutants that produce sessilins and orfamides (P), sessilins and phenazines (O) and that which produces sessilins alone (PO) (Figure 3.2). The mutant that produces phenazines alone (SO) was comparative in effect to that which produces orfamides and phenazines (S) whereas, the null mutant provided no control against *P. myriotylum*.

Microscopic observations of the interaction between P. myriotylum mycelia and sessilins, orfamides and PCN

Since our plant and *in vitro* inhibition assays indicated that sessilin-producing strains appeared to give the best suppressive effect against CRRD, we proceeded to compare the effect of varying concentrations of crude sessilins, purified orfamides and phenazines on the mycelium of *P. myriotylum*. Representative microscopic pictures showing the effects of orfamides, sessilins and PCN on the mycelia of *P. myriotylum* are presented in Figure 3.3. Interestingly, at concentrations of 1 nM and higher, crude sessilins resulted in mycelial lysis (Figure 3.3B) and vacuolization (Figure 3.3C) (Table 3.2).

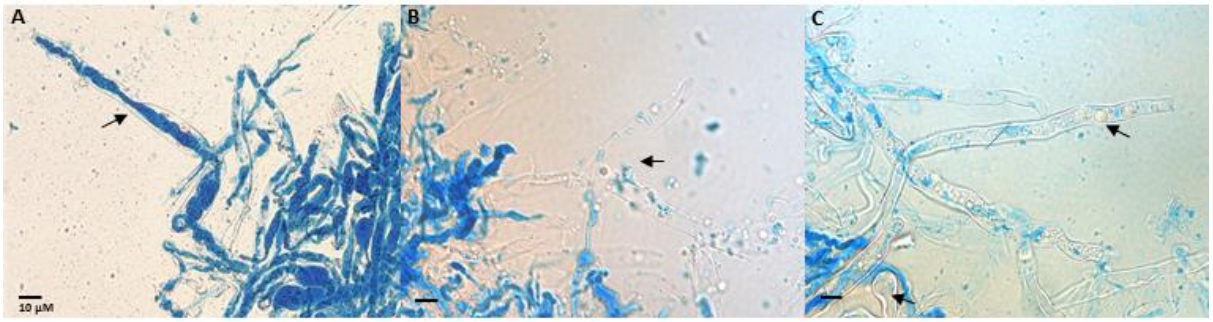

Figure 3.3. Microscopic analysis of the interaction between cyclic lipopeptides and phenazines produced by *Pseudomonas* sp. CMR12a against *P. myriotylum*. 0.1-100 µM concentrations of PCN, sessilins and orfamides were brought into direct contact with the mycelium of *P. myriotylum* and hyphae were microscopically assessed for cell damage. Representative pictures of cell damage are shown. **A)** Control: no cell damage; **B)** hyphae lysis; **C)** vacuolisation and hyphal bloating. All scale bars represent 10 µm.

Similar phenotypes were observed in treatments with 25 nM of PCN and higher with accompanied mycelial bloating (Figure 3.3C). However, treatments with orfamides resulted primarily in mycelial lysis at 10 nM and higher with attendant vacuolization recorded only at higher concentrations of 10 µM (Table 3.2).

Table 3.2. Effect of CMR12a metabolites on the mycelium of *P. myriotylum*

Metabolite	**Concentration (µM)**																				
	0.001			0.01			0.025			0.05			0.1			1			10		
	V	L	B	V	L	B	V	L	B	V	L	B	V	L	B	V	L	B	V	L	B
Sessilins	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
Orfamides	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	+	+	-
Phenazines	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*V: hyphal vacuolisation; L: hyphal lysis; B: hyphal bloating.

Discussion

Using *in vivo* and microscopic *in vitro* assays, this study demonstrates the roles of sessilins, orfamides and phenazines in the biocontrol of *Pythium*-mediated CRRD by *Pseudomonas* sp. CMR12a.

By employing CMR12a and its biosynthetic mutants, cocoyam biocontrol assays showed that phenazines and the two CLPs contribute to the biocontrol capacity of CMR12a. Prior to this experiment, bacteria strains were grown for 24 h on KB plates. Fully grown bacteria were harvested after which $3x10^6$ CFU g-1 was mixed with the growth substrate. In a previous study, the production of sessilins, orfamides and phenazines on KB plates have been quantified (Ma *et al.,* 2016b). Mutants that still produced phenazines (S, O, OS) and sessilins (O, P, PO) yielded comparable amounts of these metabolites as the WT strain. However, orfamide production was lower in the sessilin (S), phenazine (P) and the double mutant in sessilin and phenazine (PS). This knowledge provides evidence that for the corresponding mutants, sessilins and phenazines but not orfamides, were directly introduced into the growth substrate in near comparable amounts as was done with the WT. Interesting, the mutant that produced orfamides alone (PS) could provide as much control as the strain which produces phenazines alone (OS) but this treatment recorded a higher disease index than the solitary sessilin producer (PO). Although the presence of at least one of the three metabolites could suppress CRRD, excellent biocontrol was provided by the strain which produced sessilins alone and mutant strains which could produce at least any two of the metabolites. This suggests a solitary and additive effect of all metabolites in the disease suppressiveness of CRRD.

During plant and microscopic experiments, the effects of sessilins on the mycelia of *P. myriotylum* appeared to be stronger than those recorded with orfamides and phenazines. When the pathogen was challenged with 1 nM of crude sessilins, microscopic observations revealed immediate rupturing and extrusion of cell contents. Using biosynthesis mutants of CMR12a, the involvement of sessilins have been reported in the biocontrol of *Rhizoctonia* diseases on bean and Chinese cabbage (D'aes *et al.,* 2011; Olorunleke *et al.,* 2015b). However, crude sessilin extracts were yet to be tested against plant pathogens microscopically. Our results show that within the *P. myriotylum*-cocoyam pathosystem, sessilins alone could give as much control as a combination of phenazines and orfamides or all three metabolites together. Although the reason for this exceptional performance by sessilins is not yet clear, we hypothesize that this sessilin may bind/interact faster to the cell membrane of the pathogen than orfamides and PCN.

In our work, phenazines were shown to contribute to the biocontrol of CRRD. This is in line with previous studies in which phenazines, produced by *P. aeruginosa* PNA1, were implicated in the biocontrol of *P. myriotylum*-mediated CRRD (Tambong and Höfte, 2001). Although strain PNA1 strongly reduced root rot disease severity, its phenazine mutant FM13 was shown to have lost this capacity. Additionally, phenazines and rhamnolipids, produced by *P. aeruginosa* PNA1 have been shown to act synergistically in the biocontrol of *P. myriotylum* on cocoyams and on *Pythium splendens*

of common bean (Perneel *et al.,* 2008). Since soil from our experiments was inoculated with mycelia strands, the mycelia of *P. myriotylum* was brought into contact with varying concentrations (0.1 to 25 µM) of purified PCN. The distortion of mycelium and extrusion of cell contents which were observed even a concentration of 25 nM confirmed the effect of PCN against *P. myriotylum* at nanomolar concentrations. Recent studies by Ma and colleagues (2016) also showed that nanomolar concentrations of PCN could induce systematic resistance (ISR) against *Magnaporthe oryzae* in rice and web blight of bean. A balanced production of PCN might be needed not only in ISR but also in direct antagonism against plant pathogens. Higher amounts might be toxic or ineffective. These results might provide an explanation for the inability of 10 µg/ml (50 µM) of PCN to effectively suppress *P. splendens* on bean (Perneel *et al.,* 2008). For example, on KB agar, CMR12a produces about 12 µM/OD620 of phenazines while its sessilin mutant (S) produces slightly higher amounts of 20 µM/OD. Thus, applications of higher concentrations of PCN either during *in vitro* experiments or as a soil drench does not simulate the natural metabolite production by the strain of interest and might be counter-productive.

In addition to the efficacy shown by the orfamide-producing mutant strain of CMR12a during our plant experiments, we could show the activity of 10 nM orfamide A following its co-incubation with the mycelia of *P. myriotylum*. In previous studies, orfamides have also been shown to be active against zoospores of oomycetes and the mycelia of *R. solani* (Olorunleke *et al.,* 2015b; Ma *et al.,* 2016a). For example, at concentrations of 25 µM or higher, orfamides could lyse zoospores of *Phytophthora porri* and *Pythium ultimum* within 55-70 seconds (Ma *et al.,* 2016a). In our study, direct interactions of orfamides with the mycelia of *P. myriotylum* appear to be effective even at nanomolar concentrations and clearly contribute to CRRD suppressiveness. On KB agar, CMR12a produces about 20 µM/OD620 of orfamide while the least amount produced by its biosynthetic mutants that still make orfamides, the PS mutant, is about 3 µM/OD620. Put together, our microscopy results suggest that orfamide amounts produced by our biosynthetic mutants could provide efficacy in disease suppression during plant experiments. This was confirmed during our plant experiments since mutants that made orfamide only (PS) could effectively suppress CRRD.

It was intriguing to note that the null mutant of CMR12a showed suppressiveness against CRRD in *in vivo* experiments. This leads us to suggest the plausible role of an unknown metabolite produced by CMR12a that could account for this observation. Genomic mining of CMR12a revealed the presence of a genomic island which comprise the sessilin and phenazine gene clusters among several others (D'aes *et al.,* 2014). Further analysis shows that this genomic island comprises a tabtoxin biosynthesis gene cluster (unpublished data). Tabtoxin, a monocyclic β-lactam produced by *P. syringae* pv. tabaci, contains the toxic moiety, tabtoxinine-β-lactam (Bender *et al.,* 1999). This toxin inhibits the enzyme glutamine synthetase thereby causing cells to become intoxicated via accumulation of their own unprocessed ammonia (Kinscherf and Willis, 2005). Tabtoxin production are usually associated with the *P. syringae* species group which causes chlorotic plant diseases. To the best of our knowledge, the presence of a tabtoxin cluster has not been reported in plant beneficial *Pseudomonas* strains. It remains to be investigated whether tabtoxin is produced under the experimental conditions tested, its

effect on fungi/oomycetes and if it contributes to the residual effect that was displayed by the null mutant of CMR12a.

In conclusion, only rhamnolipid biosurfactants and phenazines have been previously shown to act either alone or in tandem with phenazines in the biocontrol of *Pythium*-mediated CRRD (Tambong and Höfte, 2001; Perneel *et al.,* 2008). In this study, we could show that sessilin, orfamides and phenazines could act solitary or additively in their control of CRRD. Unlike what was observed in the biocontrol of *Rhizoctonia* diseases on bean and cabbage (Olorunleke *et al.,* 2015b), CMR12a biosynthesis mutants that produce only one CLP could confer suppression to CRRD although in varying capacities. Additionally, of all three metabolites, sessilins appeared to possess the strongest inhibitive efficacy against the mycelia of *P. myriotylum*. More so, the residual suppressive effect displayed by the null mutant strain during biocontrol assays suggests the probable role of tabtoxin, produced by CMR12a.

Materials and Methods

Plant experiments with cocoyam and P. myriotylum

In order to assess the capacity of phenazines, sessilins and orfamides to suppress CRRD, plant experiments were conducted in an unsterilized potting soil (Structural; Snebbout, Kaprijke, Belgium) and sand mixture in a 70/30 ratio. CMR12a and mutant strains listed in Table 3.3 were used.

Table 3.3. Microorganisms used in this study.

Strains	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-CLP1-ΔCLP2 (SO)	PHZ^+, $CLP1^-$, $CLP2^-$; phenazines	D'aes *et al.,* (2014)
CMR12a-ΔPhz-CLP1 (PS)	PHZ^-, $CLP1^-$, $CLP2^+$; orfamides	D'aes *et al.,* (2011)
CMR12a-ΔPhz-ΔCLP2 (PO)	PHZ^-, $CLP1^+$, $CLP2^-$; sessilins	D'aes *et al.,* (2014)
CMR12a-ΔPhz-CLP1-ΔCLP2 (POS)	PHZ^-, $CLP1^-$, $CLP2^-$; null	D'aes *et al.,* (2014)
P. myriotylum		
NGR03	Causal agent of CRRD in Nigeria	Chapter 2

To prepare bacterial inoculum, strains were grown on KB plates for 24 h at 28°C, and collected in sterile saline solution (0.85%). The optical density (OD) of bacterial suspensions was recorded at 620 nm after which a final concentration of $3x10^6$ CFU g-1 soil was mixed with the substrate. The inoculated substrate was incubated at 28 °C for 48 h prior to experimental setup. *P. myriotylum* NGR03 isolate (Chapter 2) was cultured on potato dextrose agar (PDA) at 28°C for 5 days. Mycelial mats were cut in pieces with a sterile scalpel, collected in sterile saline (0.85 %), and blended with a homogenizer Ultra Thurrax (VWR, Leuven, Belgium). Inoculum concentration was quantified using a hemacytometer (Marienfeld, Lauda- Koenigshofen, Germany), and 1250 *P. myriotyum* propagules g^{-1} soil were added to the substrate.

Plant material was propagated by tissue culture as described by Tambong *et al.,* 1998 and acclimatized in potting soil two weeks prior to the experiment. The experimental set up was a completely randomised design with five plants per treatment, including a healthy and a diseased control. Before transplanting, roots were dipped for one minute in a 3 x 10^6 CFU ml^{-1} bacterial suspension in sterile saline solution whereas control plants were dipped in sterile saline. Plants were grown in a controlled-growth chamber at 25°C, RH = 60%, 16 h photoperiod and were watered once in

two days. After 7 days, disease severity was scored for each leaf using the following scale: 0 = healthy, no yellowing; 1 = < 50% leaf surface area is yellow; 2 = > 50% leaf area is yellow; 3 = 100% leaf yellowing and 4 = dead leaf. The experiment was repeated at least once.

Root colonisation of test bacteria was assessed by crushing roots in sterile saline solution (0.85%) and plating serial dilutions of suspension on KB medium plates. For each treatment, five roots were evaluated. Colonies were counted after 24 to 36 h.

Effect of secondary metabolites produced by CMR12a on the growth of P. myriotylum

To assess the effect of phenazines, sessilins and orfamides on the mycelium of *P. myriotylum*, microscopic experiments were conducted based on a protocol described by Bolwerk *et al.* (2003) with modifications. CMR12a and mutant strains were cultured in Kings' Medium broth (KMB) for 24 hours following which the bacterial cultures were subject to centrifugation for 5 min at 13, 400 rpm. Supernatants obtained were filter sterilised using 0.22 µM filters. Microscopy slides were covered with a thin layer of water agar (1.5%) and placed in Petri dishes containing moistened filter paper. A 4-mm PDA plug of a 3-day-old *P. myriotylum* culture was placed in the center of glass slide while a droplet (15 µl) of the filter sterilised supernatant of *Pseudomonas* sp. CMR12a or mutant strains were pipetted on both sides. The Petri dishes were sealed with parafilm and incubated for 36 h at 28°C after which inhibition zones between the bacterial droplet and mycelial growth were measured. All experiments were performed twice with five replicates. Inhibitory percentages were calculated as a percentage of the control which showed no inhibition. Data obtained was subjected to statistics using Tukey's Post hoc test ($P = 0.05$).

Microscopic observations of interaction between P. myriotylum and secondary metabolites of CMR12a

To test the effect of phenazines (PCN), sessilins and orfamides in interaction with P. myriotylum, mycelia was collected from 5-day old *P. myriotylum* PDA plates. Purified orfamide A was obtained from P. protegens CHA0 according to methods described by Ma and colleagues (2016) while crude sessilins were obtained from phenazines- and orfamides deficient mutant *Pseudomonas* sp. CMR12a-ΔPhz-ΔCLP2 as described previously (Nutkins *et al.,* 1991). PCN phenazines were obtained as described by Olorunleke *et al.,* 2015b (Appendix). Stock solutions of phenazines and CLPs were initially done with DMSO while subsequent dilutions were made using sterile MilliQ water. Mycelia were treated with different concentrations (in a ten-fold increase from 1 nM to 25 µM) of purified orfamide A, crude sessilins and purified PCN. The control experiment was conducted using the DMSO-diluted control. Microscopic observations to determine the effect of each metabolite on mycelia was carried out immediately after contact with mycelia using a light microscope (Olympus BX51 microscope; Aartselaar, Belgium). Experiments were repeated independently two times and representative pictures of microscopic observations are shown.

Data analysis

For the biocontrol assays, the disease severity of the leaves was calculated as a Percentage Disease Index using the following formula:

$$\text{Disease Index} = \frac{\sum (\text{Disease class} \times \text{number of leaves within that class})}{\text{Total number of leaves within treatment} \times \text{highest class value in the scale}}$$

Since our disease severity and root colonization data did not meet the conditions of normality and homogeneity of variances, the results were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P \leq 0.05$).

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Chapter 4

Co-regulation of the cyclic lipopeptides orfamide and sessilin in the biocontrol strain *Pseudomonas* sp. CMR12a

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**Joint contribution*

Submitted

Summary

Cyclic lipopeptides (CLPs) are synthesized by non-ribosomal peptide synthetases (NRPS), which are often flanked by LuxR-type transcriptional regulators. *Pseudomonas* sp. CMR12a, an effective biocontrol strain, produces two different classes of CLPs namely sessilins and orfamides. The orfamide biosynthesis gene cluster is flanked up-and down-stream by LuxR-type regulatory genes designated *ofaRup* and *ofaRdown*, respectively, whereas, the sessilin biosynthesis gene cluster has one LuxR-type regulatory gene which is situated upstream of the cluster and is designated *sesR*. Our study investigated the role of these three regulators in the biosynthesis of orfamides and sessilins. Phylogenetic analyses positioned OfaRup and OfaRdown with LuxR regulatory proteins of similar orfamide-producing *Pseudomonas* strains and the SesR with that of the tolaasin producer, *Pseudomonas tolaasii*. LC-ESI-MS analyses revealed that sessilins and orfamides are co-produced and that production starts in the late exponential phase. However, sessilins are secreted earlier and in large amounts, while orfamides are predominantly retained in the cell. Deletion mutants in *ofaRup* and *ofaRdown* lost the capacity to produce both orfamides and sessilins, whereas the *sesR* mutant showed no clear phenotype. Additionally, RT-PCR analysis showed that in the sessilin cluster, a mutation in either *ofaRup* or *ofaRdown* led to weaker transcripts of the biosynthesis genes, *sesABC* and putative transporter genes, *macAB*. In the orfamide cluster, mainly the biosynthesis genes *ofaBC* were affected, while the first biosynthesis gene, *ofaA* and putative *macAB* transport genes were still transcribed, indicating that additional promoters regulate their transcription. A mutation in either *ofaRup*, *ofaRdown* or *sesR* genes did not abolish the transcription of any of the other two. Thus, our study reports that OfaRup and OfaRdown, LuxR-type regulators in CMR12a associated with the orfamide gene cluster, co-regulate orfamide and sessilin biosynthesis.

Introduction

Cyclic lipopeptides (CLPs) are bacterial metabolites with biosurfactant activity composed of a cyclic oligopeptide lactone ring coupled to a fatty acid tail. The biosynthesis of CLPs is driven by non-ribosomal peptide synthetases (NRPS), which are encoded by large gene clusters (Raaijmakers *et al.,* 2006). CLPs have drawn increasing interest for their versatile functions in plant beneficial *Pseudomonas,* which include involvement in biofilm formation, motility and antimicrobial activity against a wide range of microorganisms including fungi, bacteria, viruses and oomycetes (reviewed by Olorunleke *et al.,* 2015a). The biosynthesis gene clusters of several CLPs including viscosin, massetolide, putisolvin, xantholysin, entolysin and poaeamide among many others, are already known (de Bruijn *et al.,* 2007; de Bruijn *et al.,* 2008; Dubern *et al.,* 2008; Li *et al.,* 2013; Vallet-Gely *et al.,* 2010; Zachow *et al.,* 2015).

The LuxR superfamily consists of transcriptional regulators that contain a DNA-binding helix-turn-helix (HTH) motif in the C-terminal region (Fuqua *et al.,* 1996). In this superfamily, four subfamilies can be distinguished based on domain architecture and the mechanism of regulatory activation. LuxR-like proteins SalA, SyrF and SyrG are a part of the fourth subfamily, which is characterized by the lack of any defined N-terminal domain. These proteins have been associated with the regulation of the CLPs syringamycin and syringapeptin in *Pseudomonas syringae* pv. *syringae*, a plant pathogenic bacterium (Vaughn and Gross, 2016). In various other *Pseudomonas* species and strains, regulatory genes encoding similar LuxR-like proteins are positioned up- and downstream of the CLP biosynthesis genes (de Bruijn and Raaijmakers, 2009a). These regulators have been shown to be involved in the biosynthesis of CLPs in plant beneficial bacteria such as putisolvins in *P. putida* (Dubern *et al.,* 2008), viscosin in *P. fluorescens* SBW25 (de Bruijn and Raaijmakers, 2009a), and massetolide in *P. fluorescens* SS101 (de Bruijn *et al.,* 2008).

In several *Pseudomonas* strains, the principal regulator of CLP biosynthesis is the GacA/GacS two-component system since a mutation in one of both encoding genes leads to a loss in CLP production (de Bruijn and Raaijmakers, 2009a). The GacA/GacS system is known to activate small RNAs that bind to and sequester translational repressor proteins, which block the ribosomal binding sites in the mRNA of Gac-regulated genes. Two small RNAs (sRNAs) and two repressor proteins, RsmA and RsmE, have been linked to the regulation of entolysin (Vallet *et al.,* 2010) and massetolide A biosynthesis (Song *et al.,* 2015a). In the massetolide producer *P. fluorescens* SS101, these repressor proteins most likely block translation of the LuxR-type transcriptional regulator, MassAR (Song *et al*., 2015a) by binding to a specific promoter region (the GacA box). In several CLP-producing *Pseudomonas* strains, a GacA box is present in the promoter region of LuxR regulators flanking the CLP biosynthesis gene cluster suggesting that other CLP-producing *Pseudomonas* strains may show a similar regulation of lipopeptide biosynthesis (Song *et al.,* 2015a).

Besides the GacA/GacS regulatory system, N-acylhomoserine lactone (N-AHL)-mediated quorum sensing was shown to be required for viscosin and putisolvin biosynthesis (Cui *et al.*, 2005; Dubern *et al.*, 2006) in *P. fluorescens* strain 5064 and *P. putida* strain PCL1445 although this is not the case in

certain other *Pseudomonas* strains (Dumenyo *et al.*, 1998; Kinscherf and Willis, 1999; de Bruijn *et al.*, 2008). In *P. putida* strain PCL1445, two heat shock proteins DnaK and DnaJ located downstream of the Gac system, were shown to regulate putisolvin biosynthesis (Dubern *et al.*, 2005). Recent studies on the genetic regulation of massetolide A biosynthesis in *P. fluorescens* SS101 revealed that the serine protease ClpP together with the chaperone ClpA regulates the biosynthesis of massetolides via a specific pathway involving the LuxR regulator, MassABC, the heat shock proteins DnaK and DnaJ, and proteins of the tricarboxylic acid (TCA) cycle (de Bruijn and Raaijmakers, 2009b; Song *et al*., 2014; Song *et al*., 2015b).

Pseudomonas sp. CMR12a is a biocontrol strain isolated from the cocoyam rhizosphere in Cameroon (Perneel *et al.,* 2007). This strain produces two classes of CLPs namely orfamides and sessilins together with two types of phenazines, phenazine-1-carboxylate (PCA) and phenazine-1- carboxamide (PCN) (Perneel *et al.*, 2007; D'aes *et al.*, 2014). Orfamides are also produced by biocontrol agents belonging to the *P. protegens* group (Gross *et al.*, 2007; Jang *et al.*, 2013; Takeuchi *et al.*, 2014; Ma *et al.,* 2016a), while sessilins are structurally related to the tolaasins produced by the mushroom pathogen, *P. tolaasii*. Sessilins are important for biofilm formation while orfamides are crucial for the swarming motility of CMR12a (D'aes *et al.*, 2014) and both CLPs are important for biocontrol (D'aes *et al.,* 2011; Hua and Höfte, 2015; Olorunleke *et al.,* 2015b; Ma *et al.,* 2016b).

In CMR12a, orfamide biosynthesis is governed by three NRPS genes namely *ofaA*, *ofaB* and *ofaC*. *MacA*- and *macB-like* genes putatively involved in orfamide secretion, are located downstream of *ofaC*. MacA and MacB are part of a tripartite secretion system involving an inner membrane protein (MacB), a periplasmic adaptor protein (MacA) and an outer membrane protein (NodT). Intriguingly, there is no *nodT*-like gene in the orfamide gene cluster of *Pseudomonas* sp. CMR12a, while this gene is present in the orfamide gene clusters of *P. protegens* isolates (Ma *et al.,* 2016a). Similar to orfamide, sessilin biosynthesis is governed by three NRPS genes namely *sesA*, *sesB* and *sesC*. These genes are flanked upstream by *nodT*, and downstream by *macA* and *macB* genes, which are probably involved in sessilin secretion. In addition, a LuxR-type regulatory gene, *ofaRup*, is located upstream of the orfamide biosynthesis cluster and a second one, *ofaRdown*, is situated downstream of the *macAB* genes whereas a single LuxR-type regulatory gene, *sesR*, is located upstream of the sessilin biosynthesis cluster next to the *nodT* gene (D'aes *et al.*, 2014).

In this study, we hypothesized that in *Pseudomonas* sp. CMR12a, OfaRup and OfaRdown regulate the biosynthesis of orfamides whereas SesR is vital for sessilin biosynthesis. To test our hypothesis, site-directed mutagenesis of the corresponding genes was conducted followed by bioinformatic, biochemical and transcriptional analyses.

Results

Growth and production of sessilins and orfamides by CMR12a in shaken and still LB broth cultures

To quantify the production of sessilins and orfamides by CMR12a in shaking (150 rpm) and still LB broth culture conditions, filter-sterilized supernatants and cells were collected at various time points, prepared and subjected to LC-ESI-MS analysis. Analyses of relative CLP production (relative peak area/OD_{620}) showed that in both culture conditions, co-production of sessilins and orfamides started at 17 h (Figure 4.1). Most of the sessilins produced was immediately secreted into the supernatant while lower amounts were kept inside (Figure 4.1A and 4.1B). In contrast, orfamides were mainly retained in the cells (Figure 4.1C). Unlike sessilin secretion, the secretion of orfamides into the LB broth occurred 7 h after the start of CLP production in both culture conditions (Figure 4.1D). Aerated cultures reached a higher biomass than still cultures (Figure 4.1E). In general, aeration had no strong effect on CLP production, although at 24 h it seemed that more CLPs were retained inside the cell in still conditions.

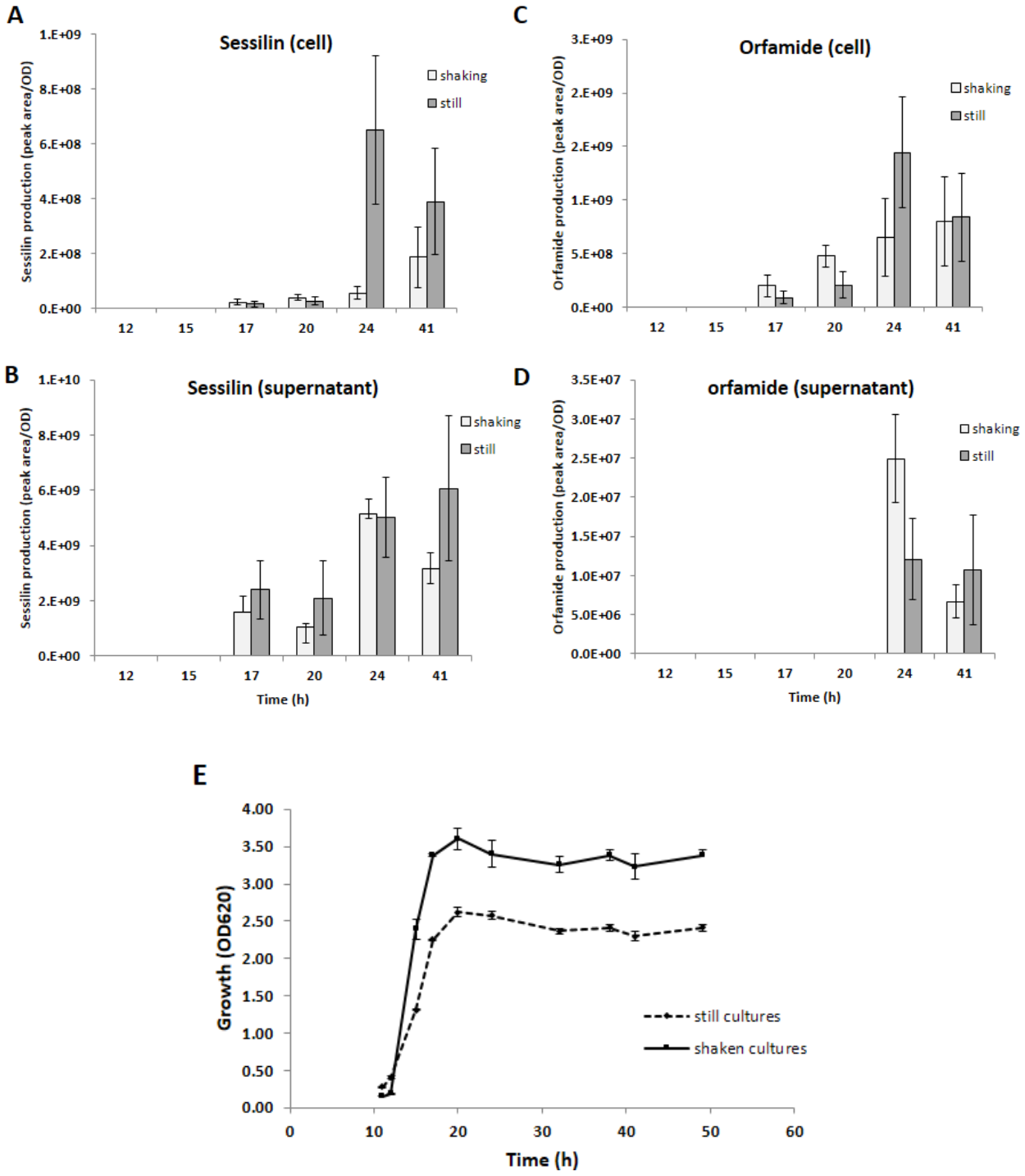

Figure 4.1. Quantification of sessilins and orfamides produced and secreted by wild type strain, *Pseudomonas* sp. CMR12a, in still and shaken growth conditions. A) sessilins in cells, **B)** sessilins in supernatants, **C)** orfamides in cells, **D)** orfamides in supernatants, **E)** growth curve of *Pseudomonas* sp. CMR12a over time. Bacteria cultures were grown in still and shaking (150 rpm) LB broth conditions at 28 °C. At each time point, cell density was measured spectrophotometrically (OD620) and mean values from three replicates were recorded. For all graphs, different scales were used to represent peak area/OD. Values are means ± standard error (n=3).

Functional analysis of luxR-type regulatory genes in sessilins and orfamides biosynthesis

Previous results showed that orfamides are important in the swarming motility of CMR12a and that sessilins and orfamides interact to give a white line on KB medium (D'aes *et al.,* 2014). In order to ascertain the cessation of sessilin and orfamide production by the LuxR mutants of CMR12a, swarming motility and white line tests were conducted. Similar to CMR12a, the *sesR* mutant swarmed on 0.6% LB agar. However, *ofaRup* and *ofaRdown* mutants did not exhibit swarming motility (Figure 4.2A). Complementation of the mutants with each of the corresponding target genes cloned into the stable vector pME6032 restored swarming motility in the *ofaRup* mutant, but not in the *ofaRdown* mutant. The white line-in-agar formation is typical for CMR12a when it interacts with an orfamide producer such as *P. protegens* Pf-5 and is indicative for sessilin production. In our study, CMR12a-Δ*ofaRup,* CMR12a-Δ*ofaRdown* and the complemented *ofaRdown* mutants no longer secrete sessilins, since they did not give the white line-in-agar interaction when challenged with the orfamide producer, *P. protegens* Pf-5. The white line-in-agar phenotype was observed, however, for CMR12a, CMR12a-Δ*sesR*, and the complemented *ofaRup* mutant strains (Figure 4.2A).

LC-ESI-MS analysis further revealed the complete abolishment of orfamide and sessilin production in the *ofaRup* and *ofaRdown* mutants (Figure 4.2B). However, the mutant in the *sesR* gene, located upstream of the sessilin biosynthesis cluster, still produced sessilins and orfamides.

Additionally, quantitative measurements (relative peak area/OD_{620}) of the two CLPs did not reveal any difference between CMR12a and CMR12a-Δ*sesR* (data not shown). Restored sessilin and orfamide production was observed in the complemented *ofaRup* mutant, but not in the complemented *ofaRdown* mutant (Figure 4.2B).

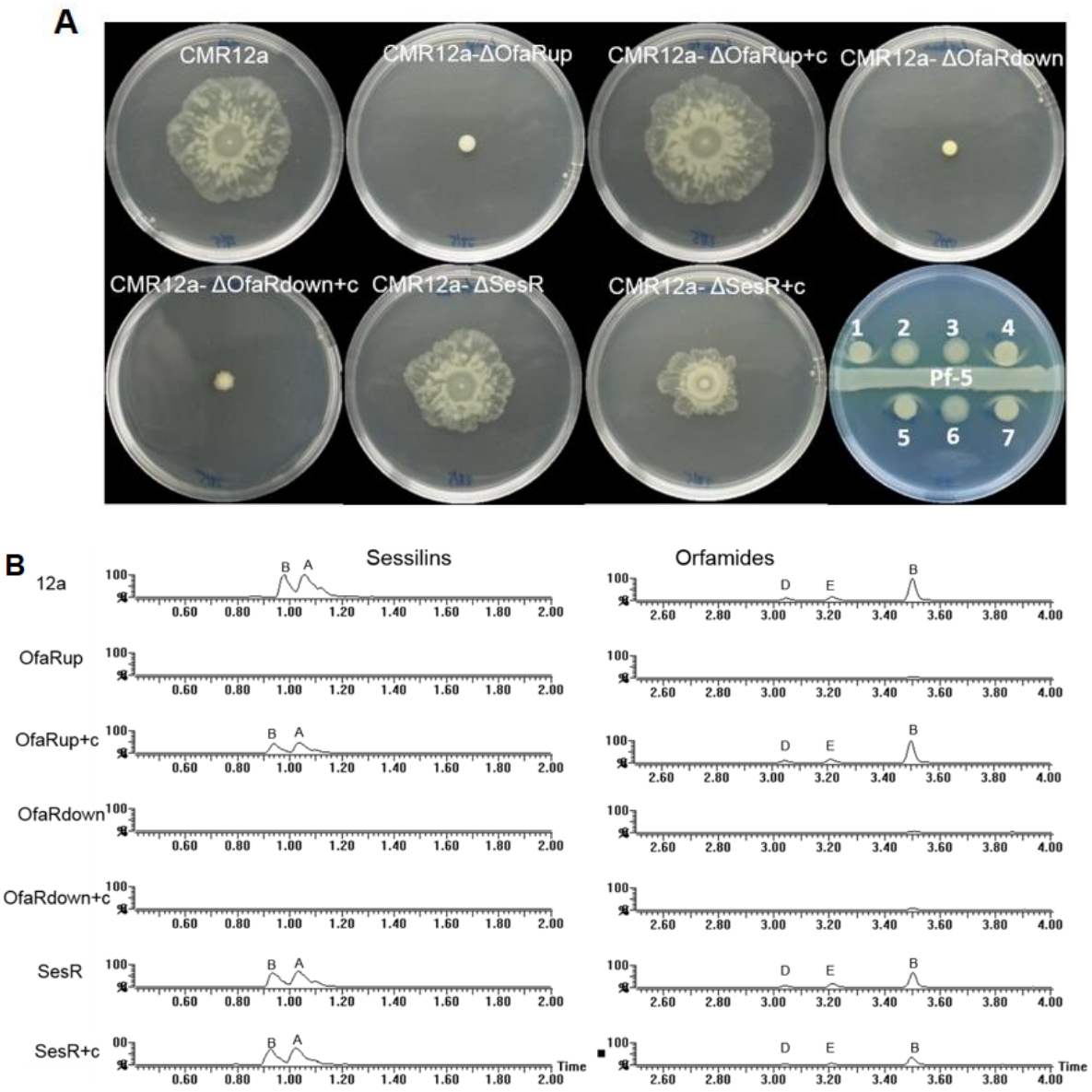

Figure 4.2. A) Swarming ability of CMR12a and LuxR mutants on 0.6% LB agar and White line-in-agar tests on KB agar medium. Bacterial cultures were grown for 17 h in LB broth and washed twice with saline solution (0.85%). 5 µl of the suspensions was spotted in the centre of the plates and incubated at 28 ˚C for 24 h. For the white line test, the picture was taken 3 days after incubation at 28 °C. **1)** CMR12a, wild type *Pseudomonas sp.* CMR12a; **2)** CMR12a-ΔOfaRup, OfaRup biosynthesis mutant; **3)** CMR12a-ΔOfaRdown, OfaRdown biosynthesis mutant; **4)** CMR12a-ΔSesR, SesR biosynthesis mutant; **5)** CMR12a-ΔOfaRup+c, *ofaRup* gene complement of OfaRup biosynthesis mutant; **6)** CMR12a-ΔOfaRdown+c, *ofaRdown* gene complement of OfaRdown biosynthesis mutant; **7)** CMR12a-ΔSesR+c, *sesR* gene complement of SesR biosynthesis mutant; **Pf-5**: *P. protegens* Pf-5 (indicator strain) **B) LC-ESI-MS chromatograms of cell-free culture supernatants of wild type *Pseudomonas* sp. CMR12a, LuxR mutants and complemented strains after 24 h of incubation.** Wild type produces sessilins (derivatives - A, B and C) and orfamides (derivatives - B, D and E). 12a: Wild type, *Pseudomonas* sp. CMR12a; OfaRup: OfaRup biosynthesis mutant; OfaRup+c: complement of OfaRUp biosynthesis mutant; OfaRdown: OfaRdown biosynthesis mutant; OfaRdown+c: complement of OfaRdown biosynthesis mutant; SesR: SesR biosynthesis mutant; SesR+c: complement of SesR biosynthesis mutant.

Transcriptional analysis of flanking and CLP biosynthesis genes in CMR12a and LuxR mutants

Figures 4.3A and 4.3B show primer positions for RT-PCR on the sessilin and orfamide gene clusters, respectively. In order to investigate the transcriptional analysis for *ofaABC*, *sesABC* and their flanking genes, RT-PCR was conducted for CMR12a and LuxR mutants using bacterial cell cultures which were grown in still LB cultures for 24 h in two replicates (Figures 4.4A and 4.4B).

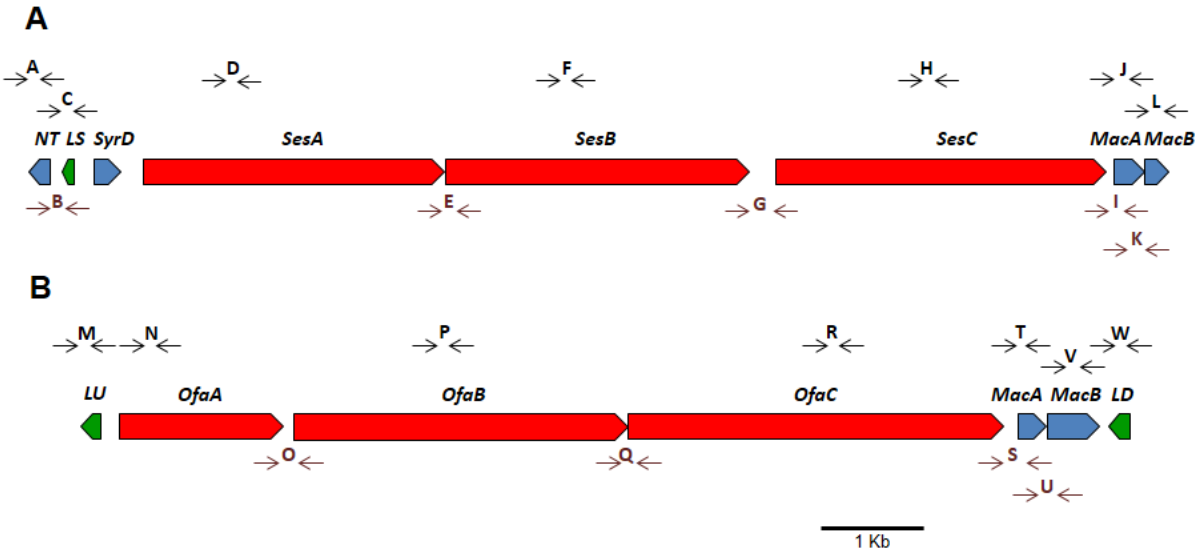

Figure 4.3. RT-PCR primer positions highlighted on the sessilin (A) and orfamide (B) biosynthesis gene clusters of *Pseudomonas* sp. CMR12a. NT: NodT (outer membrane lipoprotein); RS: SesR; SyrD (membrane protein); RU: OfaRup; RD: OfaRdown; MacA: periplasmic membrane protein; MacB: inner membrane protein. Arrows lettered A to W indicate the regions amplified during RT-PCR. The scale bar represents 1 Kb.

For the sessilin biosynthetic gene cluster, RT-PCR analysis of the WT strain revealed the transcription of *sesA*, *sesB* and flanking genes, *nodT*, *sesR*, *macA* and *macB* whereas *sesC* was not transcribed. Additionally, the co-expression of *nodT-sesR*, *sesA-sesB*, *sesB-sesC*, *sesC-macA* and *macA-macB* gene combinations indicate that the *nodT-sesR* genes on one hand and the *sesABC* together with *macAB* genes on the other hand, are transcribed from a polycistronic operon (Figure 4.4A). In contrast, analysis of the CMR12a-Δ*ofaRup* mutant mainly revealed the transcription of *nodT-sesR* and *macAB* genes. Furthermore, this mutant was characterized by the presence of weak *sesAB* transcripts. For the CMR12a-Δ*ofaRdown* mutant, similar transcription results as the CMR12a-Δ*ofaRup* mutant were obtained. Additionally, RT-PCR analyses of the CMR12a-Δ*sesR* mutant revealed similar results as the WT except for the expected absence of *nodT-sesR* and *sesR* expression.

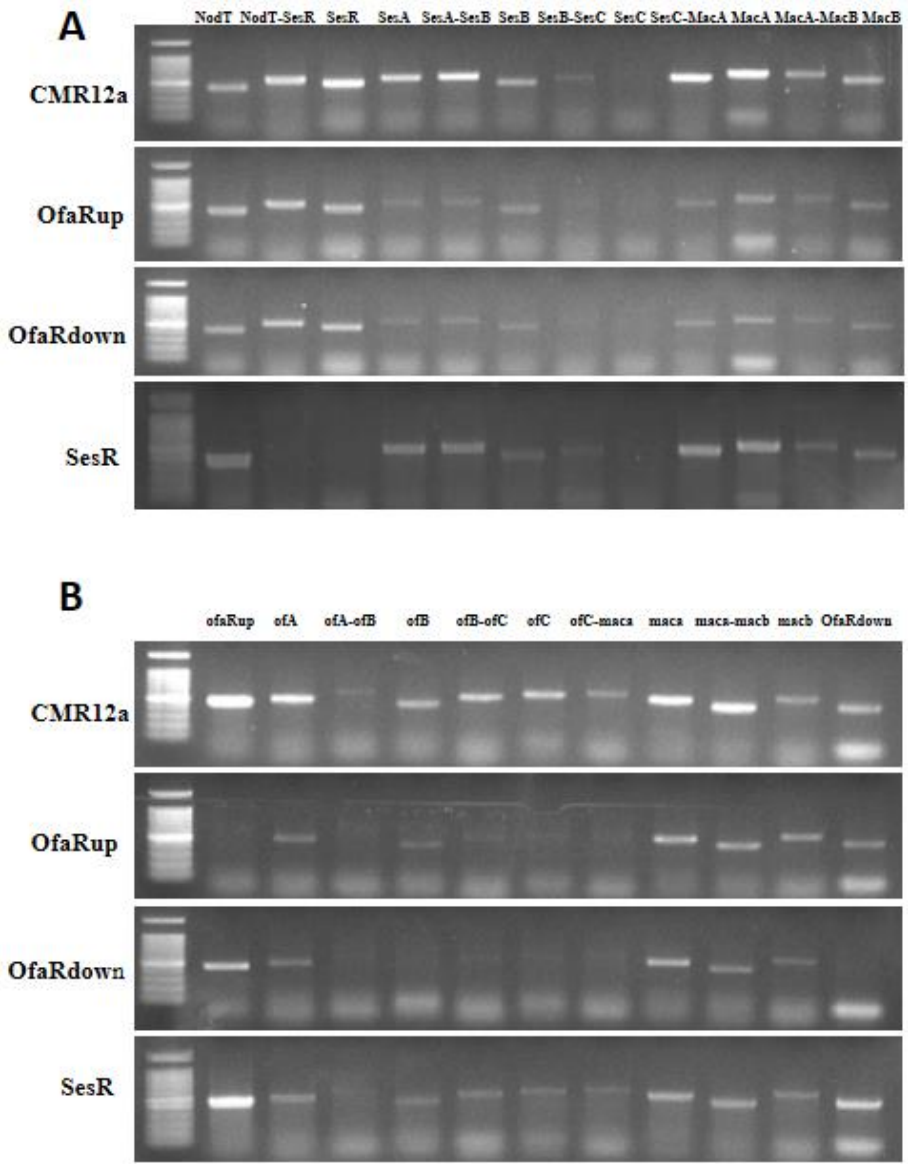

Figure 4.4. RT-PCR analyses for the sessilin (A) and orfamide (B) biosynthesis gene clusters and flanking genes. Bacterial cells analysed were collected from 24 h culture of *Pseudomonas* sp. CMR12a and its LuxR mutants. Agarose gel results are shown for analysis of single genes together with gene co-expression to distinguish monocistronic and polycistronic transcription. Primers used are listed in Table S4.2 while positions amplified are as indicated in Figures 3A and 3B.

Transcriptional analyses of the orfamide biosynthesis gene cluster were also conducted after growing bacterial cultures for 24 h. In the WT strain, *ofaRup*, *ofaA*, *ofaB*, *ofaC*, *ofaRdown* and the gene combinations of *ofaB-ofaC* was clearly transcribed whereas *ofaA-ofaB* gave a weak transcript (Figure 4.4B). Additionally, the transcription of *macA* and *macB* and gene combinations of *ofaC-macA* and *macA-macB* show that *ofaABC* and *macAB* are also transcribed from a polycistronic operon. For the *sesR* mutant, expression and co-expression analyses of all genes and gene combinations showed similar results with CMR12a. In contrast, the CMR12a-Δ*ofaRup* mutant showed the transcription of *ofaA*, *macAB* and *ofaRdown* coupled with weak *ofaB* and *ofaB-ofaC* transcripts. More so, CMR12a-Δ*ofaRdown* mutant only showed *ofaRup*, *ofaA*, *macAB* and weak *ofaBC* transcripts (Figure 4.4B).

Furthermore, a mutation in either of the three *luxR*-type genes of CMR12a did not appear to abolish the transcription of the other (Figures 4.4A and 4.4B).

Phylogenetic analyses of LuxR-type regulatory proteins associated with CLP gene clusters

Phylogenetic analyses of the CLP cluster-associated LuxR-type proteins of CMR12a together with that of other *Pseudomonas* strains, showed several distinct clusters (Figure 4.5) as follows: OfaRup and SesR proteins clustered together with other LuxR-type regulators located upstream of CLP biosynthesis genes. Similarly, OfaRdown clustered with LuxR-type regulators located downstream of the CLP biosynthesis genes.

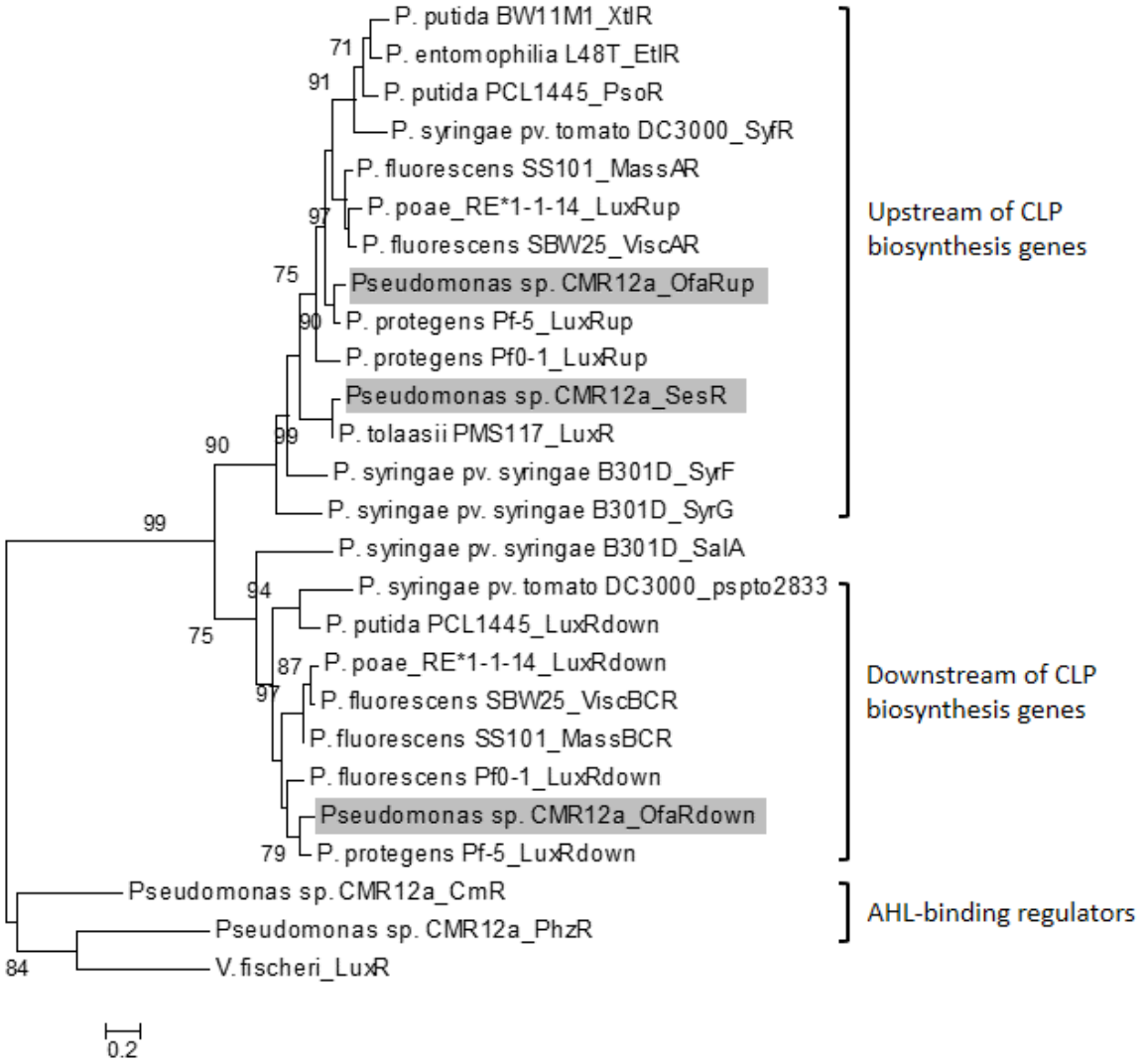

Figure 4.5. Phylogenetic analysis of the LuxR-type regulators flanking the orfamide and sessilin biosynthesis genes of *Pseudomonas* sp. CMR12a (highlighted in grey). Also included in this analysis are the LuxR-type regulators of other *Pseudomonas* CLP biosynthesis genes, and AHL-binding regulators LuxR from *Vibrio fischeri,* and PhzR and CmrR from *Pseudomonas* sp. CMR12a. The dendrogram was generated by Maximum Likelihood using 1,000 resampled data sets. Percentage bootstrap values are indicated at branching nodes while the bar indicates sequence divergence.

Specifically, SesR clustered with other LuxR-type regulators within the tolaasin group while OfaRup and OfaRdown clustered with regulators which flank orfamide-coding genes in other *Pseudomonas* strains including *P. protegens* Pf-5 (Loper and Gross, 2007). The AHL-binding regulators of CMR12a, CmrR and PhzR, formed a separate cluster together with the LuxR of *Vibrio fischeri* indicating that they belong to a separate subfamily of regulators (Figure 4.5).

Presence of Rsm binding sites upstream of LuxR transcriptional regulators

Genomic search for putative Rsm binding sites was conducted within the promoter region upstream of the three *luxR* regulatory genes of CMR12a. Conserved GGA motifs upstream of the ATG start codon could be identified. Sequence alignment of these promoter regions with their homologues in CLP-producing *Pseudomonas* strains showed the similarity of these regions upstream of sessilins and orfamide biosynthetic gene clusters with those of previously described CLPs (Figure 4.6).

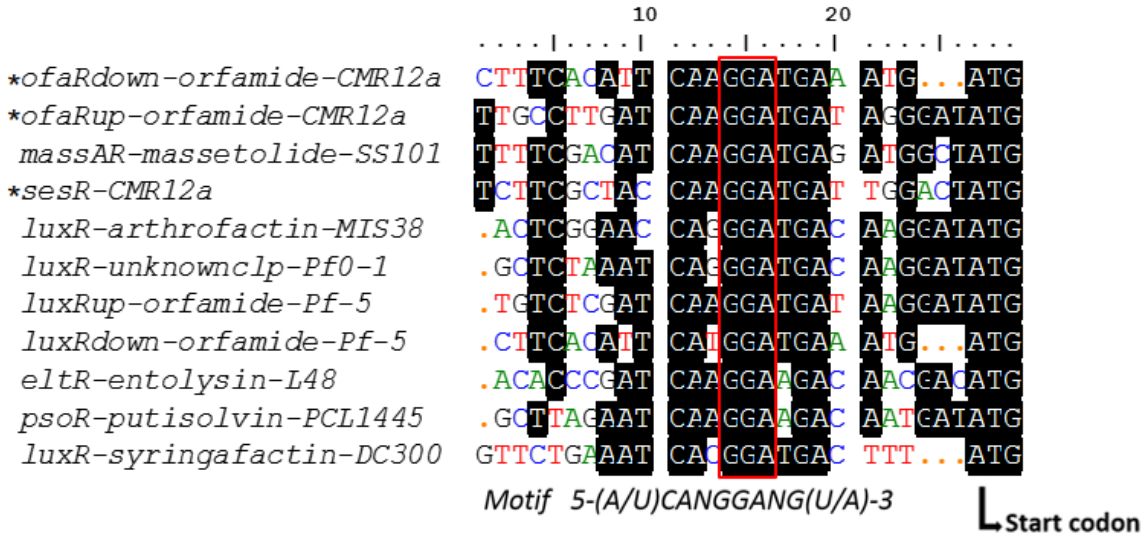

Figure 4.6. Alignment of the regions upstream of the LuxR transcriptional regulatory genes which flank different lipopeptide biosynthesis gene clusters including *Pseudomonas fluorescens* SS101, *Pseudomonas* sp. MIS38, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas protegens* Pf-5, *Pseudomonas putida* PCL1445, *Pseudomonas entomophilia* L48T, *Pseudomonas syringae* pv. tomato DC3000 and our study strain, *Pseudomonas* sp. CMR12a. The conserved GGA motif indicative of the presence of an Rsm binding site is highlighted in red. The translation initiation ATG codon is indicated at the 3' end while * indicates the sequences of the test strain used in this study.

Discussion

Our study revealed that the LuxR-like transcriptional regulators, OfaRup and OfaRdown, which are associated with the orfamide gene cluster not only regulate orfamide biosynthesis, but also sessilin biosynthesis, while we could not find a clear function for the LuxR-like regulator SesR, associated with the sessilin gene cluster.

LC-ESI-MS analysis revealed that orfamide and sessilin production commences concurrently in the late exponential phase, but orfamide is mainly retained inside the cell and secreted much later and in lower amounts than sessilin. We have previously shown that the release of orfamide in the environment is hampered by sessilin and hypothesized that both compounds compete for the same outer membrane efflux transporter, NodT (D'aes *et al*., 2014). Here we show that the *nodT* gene, located upstream of the sessilin biosynthetic cluster, is expressed from an operon together with *sesR*. A mutation in *sesR* however, does not seem to affect CLP production or secretion. We are currently investigating the secretion of orfamides and sessilins in more detail by mutant analysis of putative transport genes. In contrast, *ofaRup* and *ofaRdown* mutants completely lost the capacity to produce both sessilins and orfamides as evidenced by absence of swarming, lack of a white line-in-agar phenotype and confirmed by LC-ESI-MS analysis. Also in the biocontrol strain *P. fluorescens* SBW25, mutations in the LuxR-type regulatory genes *viscAR* and *viscBC*R, located up- and downstream of the viscosin biosynthesis cluster, led to a loss of viscosin production (de Bruijn and Raaijmakers, 2009a). Other homologues of *ofaRup*, located upstream of their NRPS genes, have been shown to be necessary for the production of putisolvin (*psoR*) (Dubern *et al.,* 2008), arthrofactin (*arfF*) (Washio *et al.,* 2010) and entolysin (*etlR*) (Vallet-Gely *et al.,* 2010).

So far, co-regulation of different classes of CLPs in the same strain has only been demonstrated for plant pathogenic *Pseudomonas* bacteria. In the bean pathogen *P. syringae* pv. *syringae* B728a, three LuxR-like proteins, SalA, Syrf, SyrG, were shown to control the biosynthesis of the CLPs syringopeptin and syringomycin (Vaughn and Gross, 2016). SalA controls the expression of both *syrG* and *syrF* (Lu *et al.,* 2002). Furthermore, qRT-PCR analysis of deletion mutants in *syrF* and *syrG* showed that both genes require a functional *salA* gene for activation. In addition, SyrG appears to function as an upstream transcriptional activator of *syrF* (Vaughn and Gross *et al*., 2016). The situation in *Pseudomonas* sp. CMR12a is different since a mutation in either *ofaRup*, *ofaRdown* or *sesR* did not abolish the transcription of the other, although the transcript of *ofaRup* may seem weaker in the *ofaRdown* mutant. Our method does not allow precise transcript quantification and further investigation by quantitative RT-PCR is needed. Likewise in the viscosin producing strain – *P. fluorescens* SBW25, a mutation in either *viscAR* or *viscBCR*, *luxR* genes located up- and down-stream of the *viscABC* biosynthesis genes, did not substantially affect the transcription of the other (de Bruijn and Raaijmakers, 2009a) indicating that both LuxR regulators do not transcriptionally affect each other.

Transcriptional analyses showed that for both the sessilin and orfamide gene clusters, their biosynthesis genes, *sesABC and ofaABC* together with putative transport genes, *macAB*, are most likely transcribed from a polycistronic operon, which is probably regulated by OfaRup and OfaRdown. The absence of a *sesC* transcript in CMR12a could be due to primer specificity problems since a co-expression was observed for *sesB-sesC* and *sesC-macA*. With respect to the orfamide gene cluster, worthy of note was the fact that mutants in *ofaRup* and *ofaRdown* still showed clear transcripts for *ofaA* and *macAB* genes. These results indicate that besides the single promoter which enables the transcription of *ofaABC* and *macAB* genes, separate promoters for *ofaA* and *macAB* may be present, which are not controlled by OfaRup and OfaRdown. Unfortunately, little information is available about the gene co-expression of other CLP gene clusters except for WLIP (Rokni-Zadeh *et al.,* 2012), so we could not ascertain if the presence of multiple promoters as was observed in the orfamide gene cluster, is a frequent occurrence. In this respect, it is interesting to notice that in beneficial *Pseudomonas* spp, the genomic region encoding the first CLP biosynthesis gene is often unlinked with the other two biosynthesis genes, which are co-expressed. This is for instance the case for viscosin, massetolide, WLIP, entolysin and poaeamide (de Bruijn *et al.,* 2007; de Bruijn *et al.,* 2008; Rokni-Zadeh *et al.,* 2012; Vallet-Gely *et al.,* 2010; Zachow *et al.,* 2015).

During this study, we were unable to complement the CMR12a-Δ*ofaRdown* mutant. Considering the fact that the *macB* gene associated with the orfamide gene cluster gave a weaker transcript than *macA* for CMR12a, it is possible that *ofaRdown* is transcribed from a longer transcript which spans across part of the *macB* gene. This would result in an antisense overlap that could influence the expression of *macB* by transcription attenuation (Sesto *et al.,* 2012). This obviously requires further investigation.

In our study, phylogenetic analysis of LuxR-type proteins, positioned up- and downstream of the CLP gene clusters of CMR12a together with previously described CLP-associated LuxR regulators revealed that OfaRup and SesR clustered together with known LuxR-type regulators located upstream of the CLP biosynthesis genes whereas OfaRdown clustered with those located downstream. Our results indicate that LuxR-type regulators of CMR12a belong to the same subfamily as in other plant beneneficial *Pseudomonas* strains including *P. protegens* Pf-5, *P. fluorescens* SS101 and *P. fluorescens* SBW25 which produce orfamide, massetolide and viscosin, respectively (Loper and Gross, 2007; de Bruijn *et al*., 2008; de Bruijn and Raaijmakers, 2009a). Given that LuxR transcriptional regulators of *P. syringae* pv. *syringae* cluster with all LuxR regulators analysed during this study, our results indicate that similar to this plant pathogenic strain, these other LuxR regulators including OfaRup, OfaRdown and SesR, belong to the fourth LuxR family which is characterized by the absence of any defined N-terminal domain (Vaughn and Gross, 2016).

During this study, a genomic search and subsequent alignment of the promoter regions upstream of *ofaRup*, *ofaRdown* and *sesR* with their homologues in other lipopeptide biosynthesis genes of *Pseudomonas* strains, showed that Rsm binding sites were located upstream of all three *luxR*-like genes of CMR12a. Given the fact that this Rsm binding site, alternatively called the GacA box, was found upstream of multiple CLP biosynthesis genes (Song *et al.,* 2015a) in different *Pseudomonas*

strains, our results suggest that the Gac/Rsm-mediated regulation of CLPs might be a general phenomenon in most biocontrol CLP-producing *Pseudomonas* spp.

In conclusion, this study establishes that sessilin and orfamide production in CMR12a are co-regulated by two of the three *luxR*-type genes namely *ofaRup* and *ofaRdown*. Our findings show that either OfaRup or OfaRdown can regulate the biosynthesis of these two CLPs while the function of SesR remains unclear.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains, plasmids and primers used in this study are listed in Table S4.1. *Pseudomonas* sp. CMR12a was cultured on Luria-Bertani (LB) agar plates or in liquid LB broth at 28 °C. All molecular techniques were performed using standard protocols (Sambrook *et al.,* 1989). *E. coli* strains were grown on LB agar plates or LB broth amended with appropriate antibiotics. *Saccharomyces cerevisiae* InvSc1 was cultivated on yeast-extract-peptone-dextrose (YPD) (Shanks *et al.,* 2006). *E. coli* strain WM3064 was used as a host for the plasmids used in site-directed mutagenesis.

Analysis of CLP production

For LC-ESI-MS analyses, bacterial strains were grown at 28 °C in 6-well plates with 2.5 ml LB broth per well. Cultures were maintained for variable time points after which 1 ml of each was centrifuged at 13,000 rpm for 4 min. Filter sterilized supernatants were subjected to reverse phase LC-ESI-MS as described by (D'aes *et al.,* 2014). Cells obtained after the centrifugation step were washed once with sterile distilled water, resuspended in 1 ml of acetonitrile solution (50%) after which sonication was carried out for 30 s. Following centrifugation, the cell supernatant was filter sterilized and subjected to LC-ESI-MS analysis. Data generated from supernatant and cell analyses were processed to either extract chromatograms or obtain the relative production of sessilins and orfamides using the MassLynx V4.1 software.

Site-directed mutagenesis

Site-directed mutagenesis of the *ofaRup*, *ofaRdown* and *sesR* genes was performed based on methods previously described (D'aes *et al.*, 2014). To construct each mutant, a fragment of the corresponding LuxR biosynthesis gene was deleted by allelic replacement with vector pMQ30 (Shanks *et al.*, 2006). Plasmids used in this study and primers used for polymerase chain reaction (PCR) are described in Table S4.1 and the general primer list, respectively. To obtain a deletion plasmid, two coding regions were amplified by PCR and these products were cloned next to each other by homologous recombination in *S. cerevisiae* InvSc1. This plasmid was mobilised into CMR12a by conjugation with *E. coli* WM3064 and selection on gentamycin. Subsequently, transconjugants that had lost the plasmid during the second crossover event were selected on LB with 10% sucrose after which gene deletion was confirmed by PCR and sequencing (LGC Genomics, Germany).

Construction of pME6032-based vectors for complementation

A fragment containing the *luxR* gene was obtained by PCR with specific primers (primer list). These PCR products were subsequently cloned in the expression vector pME6032 comprising the pTac promoter. The plasmids obtained, pME6032-OfaRup, pME6032-OfaRdown and pME6032-SesR were transformed into *E. coli* WM3064 via heat shock after which transformed colonies were selected on LB agar plates supplemented with tetracycline 50 µg/ml. Correct integration of fragments was

verified by PCR analysis, restriction analysis of isolated plasmids and sequencing. These three pME6032-based *E. coli* WM3064 vectors were transformed into the corresponding *Pseudomonas* sp. CMR12a LuxR mutants by conjugation. Transformed cells were selected on LB supplemented with 100 µg/ml tetracycline and the presence of pME6032-OfaRup, pME6032-OfaRdown or pME6032-SesR was confirmed by PCR analysis using primers specific for pME6032 and the corresponding *luxR* gene.

White line-in-agar and Swarming motility assays

The white line-in-agar test (Rokni-Zadeh *et al.*, 2013) was performed in triplicate on Kings' B (KB) medium. Bacterial strains were cultured in LB broth for 16 h and washed twice with saline solution (0.85%). The line of bacterial indicator strain (*P. protegens* Pf-5) in the middle of the plates was made from three drops (5 µl $drop^{-1}$) of the suspension. Subsequently, 5 µl suspension of each test bacterial strain was spotted at both sides of the line within a 1 cm distance. White precipitate formation in the agar was evaluated after 3 days of growth at 28 °C.

For swarming motility assays, 5 µl suspension of each test strain was spotted in the centre of LB plates comprising 0.6% agar, left to dry briefly and incubated at 28 °C for 24 h (D'aes *et al.*, 2014). At least two replicates per strain were included, and experiments were repeated at least twice.

RNA extraction and Reverse Transcription-PCR (RT-PCR)

Bacterial cells were grown in still cultures using a 6-well plate containing 2.5 ml LB broth per well at 28 °C. At 24 h, growth of strains was determined by measuring optical density OD_{620} of 100 µl in a 96-well plate using a Bio-Rad 680 microplate reader after which 1 ml of cell culture was collected and spun down. Cells were frozen in liquid N_2 and stored at -80 °C. For the RNA extraction and complementary DNA (cDNA) synthesis, two biological replicates were used. RNA was isolated from frozen bacterial cells with the Trizol reagent (Sigma), followed by genomic DNA removal using the Turbo DNA-free kit (Ambion/Applied Biosystems). cDNA was synthesized by using the GoScript Reverse Transcription System (Promega). cDNA with RNA equivalent of 100-200 ng was subjected to PCR with specific primers listed in Table S4.2. The thermal profile used consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. The primer pairs were used to amplify cDNA obtained from transcripts corresponding to genes of the sessilin and orfamide biosynthesis gene clusters and their flanking genes including the *nodT*, *sesR*, *ofaRup*, *ofaRdown*, *ofaABC*, *sesABC* and the *macAB* genes. Transcripts covering adjacent gene pairs of the afore-mentioned genes were also amplified.

Bioinformatic analyses

LuxR-like protein sequences for *Pseudomonas* sp. CMR12a were obtained from the nucleotide sequences of the sessilin and orfamide biosynthesis gene clusters with GenBank accession numbers JQ309920 and JQ309921, respectively. Other amino acid sequences used for phylogenetic analyses were collected from the National Centre for Biotechnology Information (NCBI) website. Characteristics of strains and protein sequences used in the phylogenetic analyses of LuxR proteins are presented in Table S4.3. Sequence alignments were made using Muscle (Edgar, 2004) via the software package

MEGA6 (Tamura *et al.*, 2013). Phylogenetic tree was inferred by Maximum Likelihood (ML) using 1000 bootstrap replicates and was rooted with the LuxR (quorum sensing protein) from *Vibrio fischeri*. Proteins of N-acyl-L-homoserine lactones (acyl-HSLs)-binding regulators of CMR12a, CmrR and PhzR (De Maeyer *et al.,* 2013) were included in this analysis.

Furthermore, bioinformatic tools were employed to check for the presence of Rsm binding sites (GacA box) in the promoter region upstream of the *ofaRup*, *ofaRdown* and *sesR* genes. The query search was conducted using the conserved motif 5'-A/U CANGGANG U/A-3' where N denotes any nucleotide (Song *et al.,* 2015a). Subsequently, similar promoter regions flanking the LuxR transcriptional regulators of several CLP-producing *Pseudomonas* strains were aligned with the three LuxR regulators of *Pseudomonas* sp. CMR12a.

Acknowledgements

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Supplementary files

Table S4.1. Strains and plasmids used in this chapter.

Strains and plasmids	Relevant characteristics	Reference/Source
Pseudomonas		
CMR12a	PHZ$^+$, CLP1$^+$, CLP2$^+$, wild type (Cameroon)	Perneel *et al.,* (2007)
CMR12a-Δ*sesR*	mutant with deletion of *LuxR* gene in sessilin cluster	This study
CMR12a-Δ*ofaRup*	mutant with deletion of *LuxR* gene, upstream orfamide cluster	This study
CMR12a-Δ*ofaRdown*	mutant with deletion of *LuxR* gene, downstream orfamide cluster	This study
Pf-5	Orfamide, wild type	Loper and Gross (2007)
E. coli		
DH5α	Host for cloning	Hanahan (1983)
WM3064	Strain for conjugation; λ pir, DAP auxotroph	Saltikov and Newman (2003)
S. cerevisiae InvSc1	Yeast strain for *in vivo* recombination (*ura3-52/ura3-52* mutation)	Invitrogen
Plasmids		
pMQ30	Gene replacement vector for *Pseudomonas* species; *sacB*, URA3, Gm^R	Shanks *et al*., (2006)
pMQ30-Δ*sesR*	Vector for site specific mutagenesis of LuxR gene in sessilin cluster	This study
pMQ30-Δ*ofaRup*	Vector for site specific mutagenesis of LuxR gene, upstream orfamide cluster	This study
pMQ30-Δ*ofaRdown*	Vector for site specific mutagenesis of LuxR gene, downstream orfamide cluster	This study
PME6032	Expression vector with *tac* promoter; Tc^R	Heeb *et al*., (2002)
PME6032-sesR	Expression vector with *LuxRses* of CMR12a under *tac* promoter; Tc^R	This study
PME6032-ofaRup	Expression vector with *LuxR ofa-up* of CMR12a under *tac* promoter; Tc^R	This study
PME6032-ofaRdown	Expression vector with *LuxR ofa-down* of CMR12a under *tac* promoter; Tc^R	This study

*PHZ: phenazine; CLP: cyclic lipopeptides; CLP1: sessilin; CLP2: orfamide; Gm^R, Tc^R, Amp^R, Km^R: resistant to gentamycin, tetracycline, ampicillin, kanamycin, respectively.

Table S4.2. Primers used for RT-PCR of the sessilin and orfamide biosynthesis cluster and flanking sequences.

Primer*	Gene (s)	Sequence (5'—3'			
		Forward Primer	Reverse Primer	Length (bp)	Source
	Sessilin				
A	NodT	gacactgggaaagaacgctg	caatgcttacctgacctggc	412	This study
B	NodT-SesR	ctggatcaactgccgcaag	gtcgcacattcatcgtgagt	504	This study
C	SesR	catccagctgccaattttgc	gctgatcgacaagttcctgc	451	This study
D	SesA	gttccgtcatatctgcgagc	gcggcccaggtaatcaattt	532	This study
E	SesA-SesB	ctctgggaatacgccgaaat	gaaagctgtcatgccgatca	543	This study
F	SesB	ggtcgtcagaatgtggtgtt	caggtagtcacaaatgcggc	447	This study
G	SesB-SesC	aggtggagcacgtggtgg	acgttgaagatggatcgggt	499	This study
H	SesC	gatttcccaggtgcgtgtac	cgtcagaagcgatatggtgc	560	This study
I	SesC-MacAses	cacagggcaatcaacgtgag	cgagcttgactttcagcgat	538	This study
J	MacAses	cttgcaggggatcaaacagg	cagtttgccgtagtagcgtt	613	This study
K	MacAses-MacBses	caaaccctgatcatcgcctg	atctcgacgttatgcatggc	609	This study
L	MacBses	gaccacaacacccgtaatcg	gcctccaccagaaactgttg	515	This study
	Orfamide				
M	OfaRup	ttgttgccgatactgttggg	gggacagttgatctccagca	463	This study
N	OrfA	ctggacctgtatcgcacctt	gcttcgccgtagtggtagtc	499	This study
O	OrfA-OrfB	ccaatacccgcagtcattcg	agcatgtccggagtgatctt	583	This study
P	OrfB	gtgacgatgctgttcctcac	gtacggtacaacaacgcacc	440	This study
Q	OrfB-OrfC	gtgaacctgatcagccgga	gcgaactgtgcctgcatc	540	This study
R	OrfC	gtccaggaccatatcgaggg	ttgttcgagatccaccactg	585	This study
S	OrfC-MacAorf	cgcccaatgtctaccacttg	gtggccttggtttccttgag	613	This study
T	MacAorf	ggtttccgggcaattgaagt	gccgaggatggtgaagtaca	547	This study
U	MacAorf-MacBorf	ctgttcgatgtgcccaacc	ggcaaccgaggatgttcatc	464	This study
V	MacBorf	cattctccaggagctgcatg	atgtcgcggaccttgaagta	600	This study
W	OfaRdown	ggtagctctcgacggtgtt	ccgtgcacaaccatcatcc	492	This study

*Designation for primer positions as indicated on the biosynthetic clusters in Figures 4.3A and 4.3B.

Table S4.3. Characteristics of strains and protein sequences used in the phylogenetic analyses of LuxR proteins of CLP-producing *Pseudomonas* spp.

Strain	Associated CLP	Protein Annotation	Accession Number*	Reference
P. poae RE*1-1-14	poaemide	LuxR	WP_003235573	Müller *et al.,* 2013
P. poae RE*1-1-14	poaemide	LuxR	WP_011059319.1	Müller *et al.,* 2013
P. protegens Pf-5	orfamide	PFL_2143-LuxRup	AAY91417	Paulsen *et al.,* 2005
P. protegens Pf-5	orfamide	PFL_2150-LuxRdown	AAY91424	Paulsen *et al.,* 2005
P. fluorescens Pf0-1	undescribed	PFL01_RS11145	WP_011333638	Silby *et al.,* 2009
P. fluorescens Pf0-1	undescribed	PFL01_RS11175	WP_041475215	Silby *et al.,* 2009
P. putida BW11M1	xantholysin	XtlR	AGM14924	Li *et al.,* 2013
P. entomophila L48T	entolysin	EltR	WP_011534474	Vodovar *et al.,* 2006
P. putida PCL1445	putisolvin	PsoR	ABW17374	Dubern *et al.,* 2006
P. putida PCL1445	putisolvin	LuxR	ABW17380	Dubern *et al.,* 2006
P. syringae pv. *syringae* DC3000	Syringopeptin	SyrR	NP_792632	Buell *et al.,* 2003
P. syringae pv. *syringae* DC3000	Syringostatin	LuxR	NP_792637	Buell *et al.,* 2003
P. fluorescens SBW25	Viscosin	ViscAR	WP_015884801	Silby *et al.,* 2009
P. fluorescens SBW25	Viscosin	PFLU_2557-ViscBCR	WP_043205227	Silby *et al.,* 2009
P. fluorescens SS101	Massetolide	MassAR	WP_003192380	Loper *et al.,* 2012
P. fluorescens SS101	Massetolide	MassBCR	WP_032900559	Loper *et al.,* 2012
Pseudomonas sp. CMR12a	Orfamide	OfaRup	n/a	Rast seed
Pseudomonas sp. CMR12a	Orfamide	OfaRdown	n/a	Rast seed
Pseudomonas sp. CMR12a	Sessilin	SesRup	n/a	Rast seed
P. tolaasii PMS117	tolaasin	LuxR	WP_016969117	Studholme *et al.,* unpublished
P. fluorescens In5	nunamycin	NunF	AHL29304	Michelsen *et al.,* 2015
P. syringae pv. *syringae* B301D	syringomycin and syringopeptin	SyrF	WP_011267816	Ravindran *et al.,* 2015
P. syringae pv. *syringae* B301D	syringomycin and syringopeptin	SyrG	WP_032656102	Ravindran *et al.,* 2015
P. syringae pv. *syringae* B301D	syringomycin and syringopeptin	SalA	WP_024638707	Ravindran *et al.,* 2015
Pseudomonas sp. CMR12a	-	CmR	n/a	Rast seed
Pseudomonas sp. CMR12a	-	PhzR	n/a	Rast seed
Vibrio fischeri	-	LuxR	AAQ90196	Nishiguchi and Nair *et al.,* 2003

Chapter 5

An OprM homologue and MacAB cooperate with the MexAB-OprM efflux pump for cyclic lipopeptides secretion by *Pseudomonas* sp. CMR12a

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Summary

In clinical *P. aeruginosa* strains, the MexAB-OprM efflux pump has been much studied for its role in multidrug resistance. In addition to the *mexAB-oprM* operon, some cyclic lipopeptides (CLP)-producing *Pseudomonas* spp. possess an *oprM* homologue (*nodT*) which is situated upstream of their biosynthetic gene cluster. This study was conducted firstly, in order to elucidate the evolutionary relationship between NodT and its corresponding OprM occurring in diverse CLP-producing bacteria. Additionally, using the biocontrol *Pseudomonas* sp. CMR12a strain, our aim was to investigate the functional roles of putative transport genes in the secretion of orfamides and sessilins. Phylogenetic analysis of CLP-associated NodT and their OprM protein sequences for several *Pseudomonas* strains, suggests that the occurrence of *nodT* gene in *Pseudomonas* spp is the result of a gene duplication event of the *oprM*. Furthermore, deletion mutants were constructed in the *nodT*, *oprM*, *nodT-oprM*, *macAses*, *macABses*, *mexA*, and *mexAB* genes. For sessilin secretion, HPLC-MS analyses of mutants showed that all afore-mentioned genes were crucial for sessilin secretion in the early growth phase (17 or 24 h). Interestingly, at later timepoints, double mutations in *macABses*, *mexAB* and *nodT-oprM* genes led to a complete loss of sessilin secretion. Thus, in CMR12a, the secretion of sessilin appears to be routed primarily via *mexAB-oprM* but also through alternative tripartite efflux system combinations. For orfamide secretion, it is not yet clear what genes are involved although *macAses* and *mexA* appear to be important in the early growth phase of CMR12a. Albeit, reduced sessilin secretion levels by CMR12a-Δ*nodT*-Δ*oprM*, CMR12a-Δ*macABses* and CMR12a-Δ*mexAB* mutants often corresponded with increased orfamide secretion by CMR12a suggesting that sessilin utilizes the efflux machinery of CMR12a at the expense of orfamide secretion. Besides the understanding that the occurrence of the *nodT* is the result of a gene duplication event, our study highlights the involvement of NodT and MacAB together with MexAB-OprM in the active secretion of sessilins thereby contributing significantly to the environmental fitness of CMR12a.

Introduction

Cyclic lipopeptides (CLPs) are bacterial metabolites with biosurfactant activity composed of a cyclic oligopeptide lactone ring coupled to a fatty acid tail. The biosynthesis of CLPs is driven by non-ribosomal peptide synthetases which are encoded by large gene clusters (Raaijmakers *et al.,* 2006). CLPs have drawn increasing interest for their versatile functions in plant beneficial *Pseudomonas* which include involvement in biofilm formation, motility and their antimicrobial activity against a wide range of microorganisms including fungi, bacteria, viruses and oomycetes (Chapter 1). The biosynthetic gene clusters of these CLPs are often flanked upstream by a gene encoding a RND efflux outer membrane lipoprotein referred to as NodT (Rivilla *et al.,* 1995) and downstream, by two genes encoding the peripheral membrane protein (MacA) and the integral membrane protein (MacB) (de Bruijn *et al.,* 2008). In *E. coli*, the MacB has been described as an ABC-type efflux transporter which cooperates with MacA and the outer membrane protein, TolC, for multidrug efflux (Kobayashi and Nishino, 2001).

The secretion systems of CLPs in *Pseudomonas* bacteria have not been intensively studied. In a previous study involving the putisolvin producer, *P. putida* PCL1445, mutations in *macA* and *macB* resulted in reduced putisolvin secretion (Dubern *et al.,* 2008). In another study, the *arfD* (*macA*) and *arfE* (*macB*) genes located downstream of the arthrofactin biosynthesis genes in *Pseudomonas* sp. MIS38, were shown to encode a primary though not an exclusive exporter of arthrofactin (Lim *et al.,* 2009). Put together, these results suggested that multiple active transporter systems could be responsible for CLP export in *Pseudomonas* spp.

In CMR12a, orfamide biosynthesis is governed by the NRPS genes *ofaA*, *ofaB* and *ofaC*. Similar to orfamide, sessilin biosynthesis is governed by three NRPS genes namely *sesA*, *sesB* and *sesC* (D'aes *et al.,* 2014). We previously described the co-regulation of sessilins and orfamides by two LuxR-type transcriptional regulators located up and downstream of the orfamide biosynthetic gene cluster (Chapter 4). Null mutants of these regulators lost the capacity to produce both sessilins and orfamides.

Upstream of the *sesABC* biosynthetic cluster lies a gene encoding an outer membrane protein referred to as NodT (D'aes *et al.,* 2014), while there is no *nodT* homologue upstream of the *ofaABC* biosynthetic cluster. We postulated that the sessilin cluster-associated *nodT* gene could be instrumental in CLP secretion by CMR12a. Genome mining of the CMR12a draft genome further revealed the presence of an *oprM* gene with high similarity to *nodT*. This *oprM* gene is part of a *mexAB-oprM* operon. Given the fact that the MexAB-OprM transporter functions in drug efflux in for example, *P. aeruginosa*, we were interested in determining the functioning/contributory roles not only of the NodT but also the MacAB efflux transporters and the MexAB-OprM system, in the secretion of sessilin and orfamide by *Pseudomonas* sp. CMR12a. Additively, our goal was to investigate the relationship between the outer membrane protein (NodT) and OprM-like proteins in all CLP-producing strains as compared to the OprM protein of the much studied *P. aeruginosa*. This was done by

employing site-directed mutagenesis of target genes followed by phylogenetic and biochemical analyses.

Results

CLP-associated nodT results from a gene duplication of the oprM

In comparison with the *oprM*, the evolutionary history of the CLP-associated *nodT* genes was inferred by phylogenetic analyses using protein sequences extracted from Genbank. The OpmQ and OpmB sequences of *P. aeruginosa* PA01 were used as outgroup (Figure 5.1). For both secretion proteins, three clades were delineated. In the first clade, all NodT proteins affiliated with CLP production clustered together, irrespective of the strain's origin or lipopeptides produced. Closer examination of these strains and their corresponding CLPs revealed that plant pathogenic strains such as *P. corrugata*, *P. syringae* pv. *syringae* B728a and *P. cichorii* clustered together. These isolates produce corpeptin, syringopeptin/syringomycin and cichopeptin/cichofactin, respectively, all of which are crucial for the virulence of the producing strain. On the other hand, not all CLP biosynthetic clusters possess a flanking *nodT* gene, so the protein sequence for isolates such as *Pseudomonas* sp. CMR5c (orfamide producer) were not included in this analysis. Also excluded were CLP producers whose *nodT* genes were not clearly indicated in the Genbank such as *Pseudomonas fluorescens* In5 which produces nunamycin and nunapeptin with close relatedness to the syringomycin and corpeptin/syringopeptin groups, respectively.

The second clade comprised OprM proteins of CLP-producing isolates belonging to the *P. fluoresens* complex. Similar to the first clade, this clade shows clustering according to the ecological functioning of the isolates studied. Isolates producing similar CLPs in the first cluster still group together in the second. For example, within the *P. fluorescens* complex, all orfamide producers clustered together. Of particular interest is the fact that the last clade comprises the OprM proteins of *P. aeruginosa* and its closely related species.

Diversity of secretion genes correlates with taxonomic relatedness of host organisms

Following the analysis of the housekeeping gene, *rpoD*, it was interesting to find that all *Pseudomonas* strains used in this study (Figure 5.1) clustered together according to the taxonomic delineation of the *Pseudomonas* groups presented in Figure 5.2. All test isolates are delineated in three subgroups namely *P. fluorescens*, *P. putida* and *P. aeruginosa*. Thus, we observed that for the OprM proteins for example, the only *P. putida* isolate, *P. entomophilia* L48T, was separate from the *P. fluorescens* complex isolates which clustered together (Figure 5.1). Similar to what was observed in Figure 5.1, plant pathogenic isolates cluster together except for *P. brassicacearum* DF41, a plant beneficial isolate, which although it groups with the pathogenic strains, produces the CLP sclerosin that inhibits the plant pathogen, *Sclerotinia sclerotiorum*.

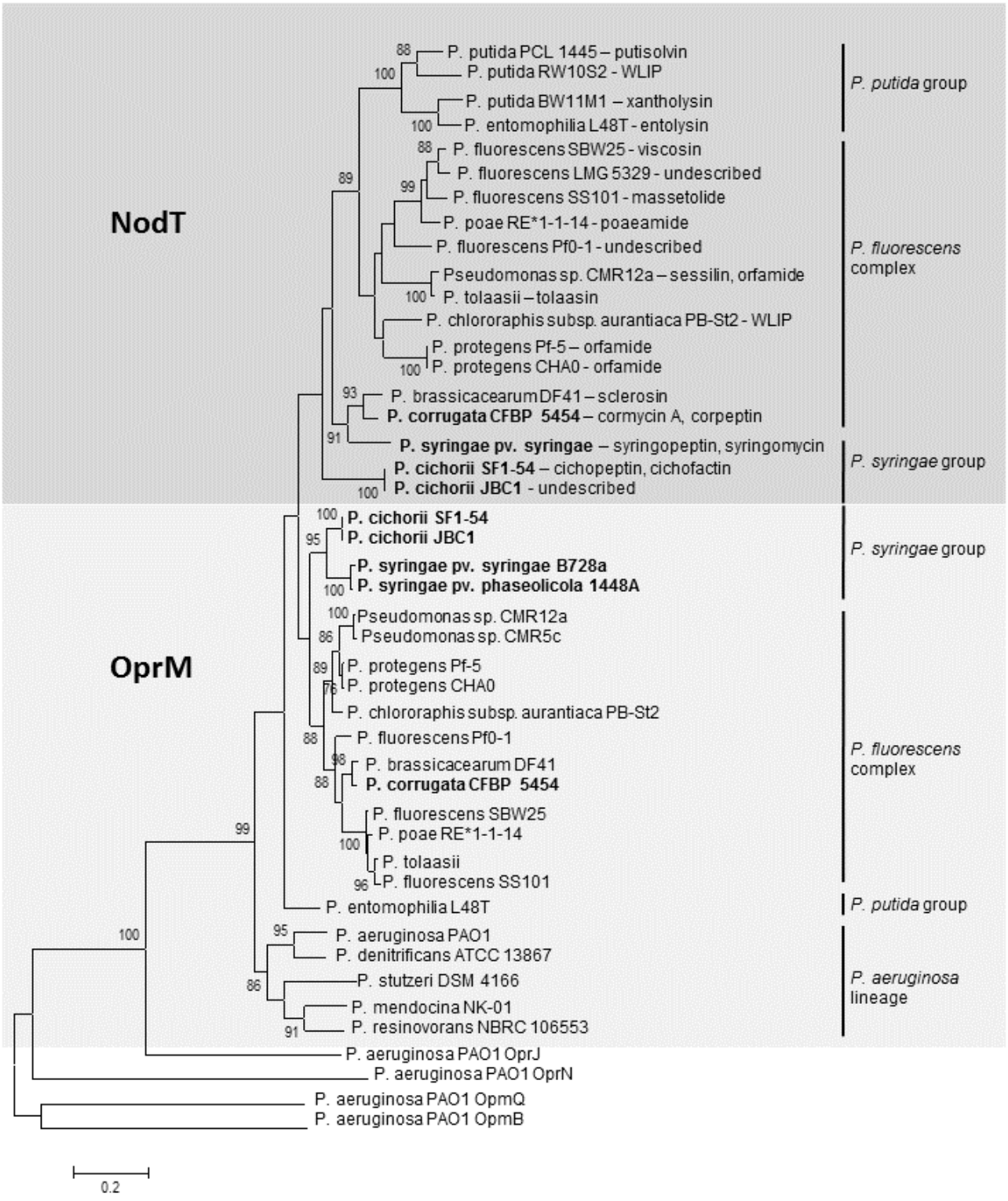

Figure 5.1. Phylogenetic analysis of the NodT proteins encoded by its gene flanking the *Pseudomonas* CLP biosynthesis gene clusters and their corresponding OprM proteins. Also included in the analysis are other OprM proteins of isolates within the *P. aeruginosa* lineage. The OprJ protein of *P. aeruginosa* PAO1 was used as outgroup. Maximum Likelihood tree was generated using 1000 resampled data sets. Bootstrap at the nodes are only given for values higher than 70. Strains in bold are plant pathogens.

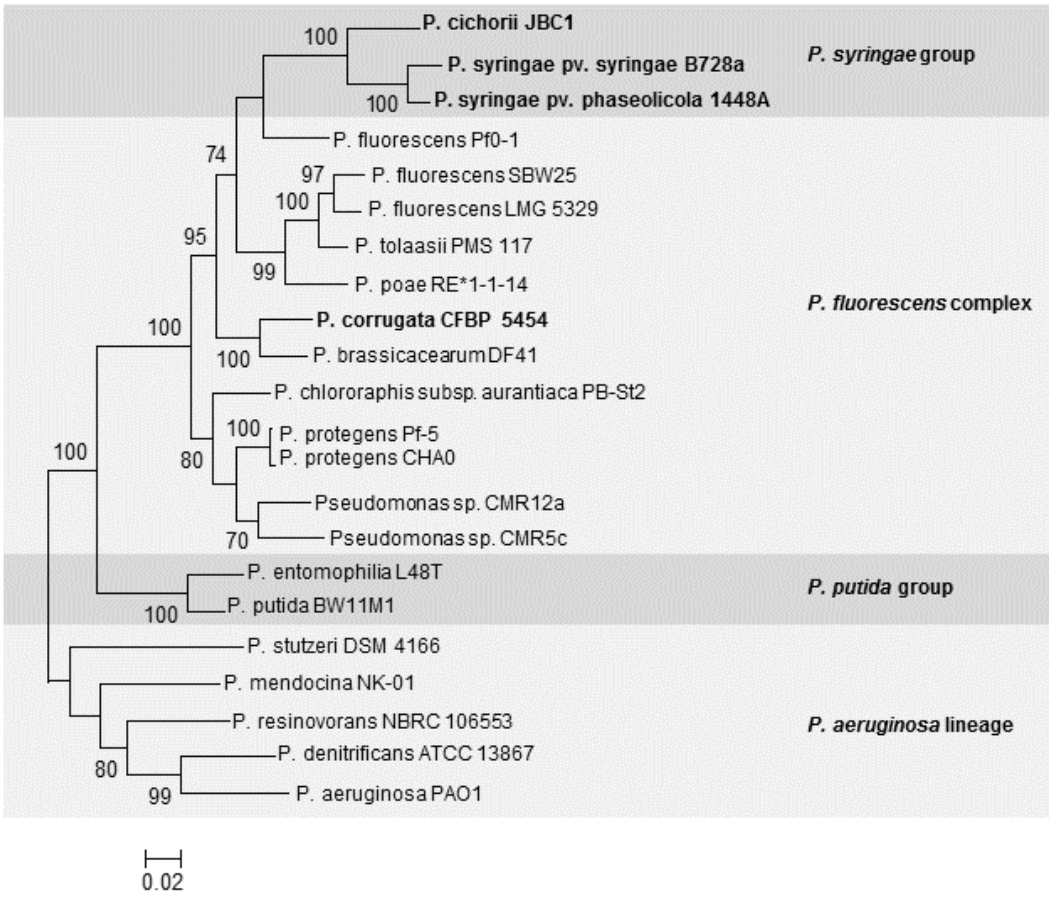

Figure 5.2. Phylogenetic analysis of the *rpoD* genes of CLP-producing *Pseudomonas* strains. Also included in the analysis are other *rpoD* genes of isolates within the *P. aeruginosa* lineage which served as outgroup. Maximum Likelihood tree was generated using 1000 resampled data sets. Bootstrap at the nodes are only given for values higher than 70. Strains in bold are plant pathogens.

NodT and OprM outer membrane proteins are involved in orfamide and sessilin secretion

In still LB broth culture conditions, *Pseudomonas* sp. CMR12a produces two CLPs namely orfamides and predominantly, sessilins (Chapter 4). Genomic inactivation by site-directed mutagenesis of the *nodT* gene associated with the sessilin biosynthetic gene cluster still resulted in drop collapse production (data not shown).

White line tests further indicated that this mutant still produced similar CLPs as the wild type (Figure 5.3). Similar to the wild type strain, the white line phenotype showed by this mutant when challenged with the orfamide producer, *P. protegens* Pf-5, is an indicator that it still secretes sessilins. To further confirm which CLPs were secreted, LC-ESI-MS analysis of filter sterilized supernatants of LB broth cultures at various time points was carried out. Results showed that the CMR12a-Δ*nodT* mutant secreted orfamides and sessilins in amounts comparable to the wild type strain, although at 17 h, significantly lower amounts were secreted (Figure 5.4A) (Figure 5.5).

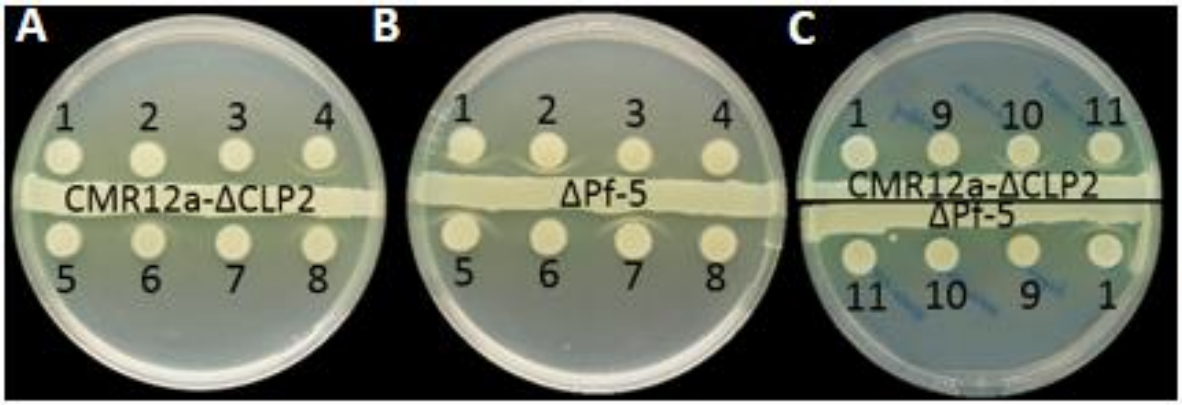

Figure 5.3. White line-in-agar tests for *Pseudomonas* sp. CMR12a and secretion mutants. Indicator strains are used: **A)** CMR12a-ΔCLP2 (sessilin producer); **B)** *P. protegens* Pf-5 (orfamide producer and **C)** Top: CMR12a-ΔCLP2; Bottom: *P. protegens* Pf-5. Test strains are: 1) CMR12a; 2) CMR12a-ΔNodT; 3) CMR12a-ΔOprM; 4) CMR12a-ΔNodT-ΔOprM; 5) CMR12a-ΔMacA; 6) CMR12a-ΔMacABses; 7) CMR12a-ΔMexA; 8) CMR12a-ΔMexAB; 9) CMR12a-ΔMacABorf; 10) CMR12a-ΔMacA-ΔMexA; 11) CMR12a-ΔMexA-ΔMacA.

Following this, an *oprM* mutant of CMR12a was constructed. LC-ESI-MS analysis of CMR12a-Δ*oprM* showed a significant decrease in sessilin secretion at 24 and 41 h after which levels comparable to the WT were secreted into the culture (Figure 5.4A). The slight decrease in sessilin secretion observed could account for the presence of a weak white line when this mutant was challenged with *P. protegens* Pf-5 (Figure 5.3). In contrast, in this mutant, similar levels of orfamide secretion were recorded as the wild type except at 41 h (Figure 5.4B).

In order to investigate the dual roles of the *nodT* and *oprM* genes in orfamide and sessilin secretion, a double mutant was constructed in both genes. Interestingly, the CMR12a-Δ*nodT*-Δ*oprM* mutant showed no drop collapse (data not shown). In addition, the white line phenotype was obtained when this mutant was challenged with CMR12a-ΔCLP2, a sessilin producer but no white line was observed in interactions with Pf-5, suggesting increased orfamide secretion in comparison to the WT levels (Figure 5.3). LC-ESI-MS analysis confirmed that a dual mutation of *oprM* and *nodT* in CMR12a, led to a significant decline in sessilin secretion at all time points measured (Figure 5.4A) (Figure 5.5). This phenotype was coupled with a remarkable increase in the secretion of orfamides at all time-points (Figure 5.4B) (Figure 5.5).

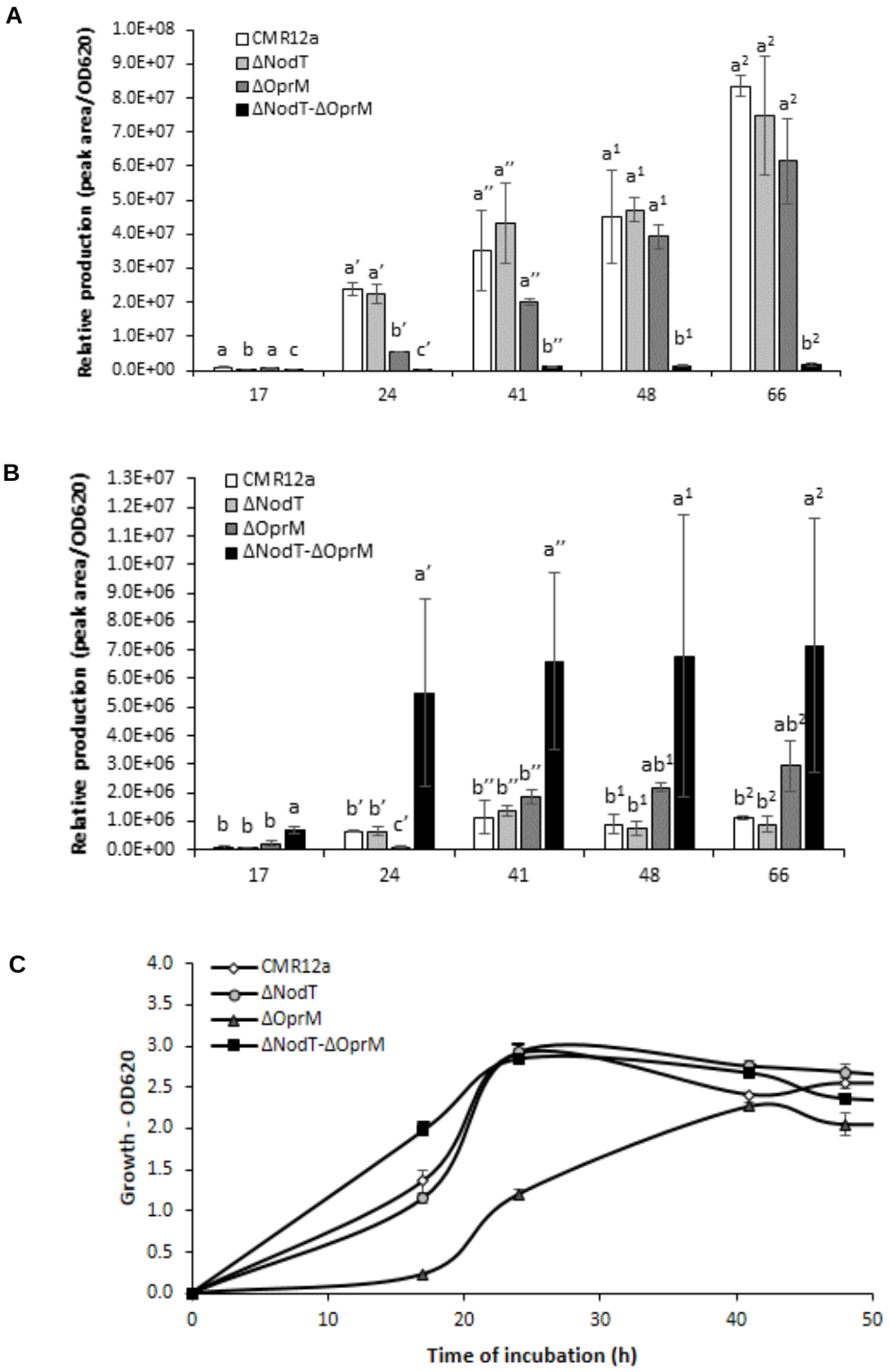

Figure 5.4. Quantification of sessilin **(A)** and orfamide **(B)** production by *Pseudomonas* sp. CMR12a and mutants in the *nodT* and *oprM* genes; **C)** Growth of *Pseudomonas sp.* CMR12a strain and mutants in still LB broth cultures at 28°C; at each time point, cell density was measured spectrophotometrically (OD_{620}) and mean values from three replicates were recorded. Data was log transformed before statistics. Bars with different letters are significantly different according to Tukey's tests (P = 0.05).

Spectrophotometric measurement of the growth of CMR12a, and its NodT and OprM mutants did not reveal a growth penalty as a result of gene deletion except for CMR12a-Δ*oprM* for which more than a 2-fold reduction in growth was observed 24 h after cultivation (Figure 5.4C).

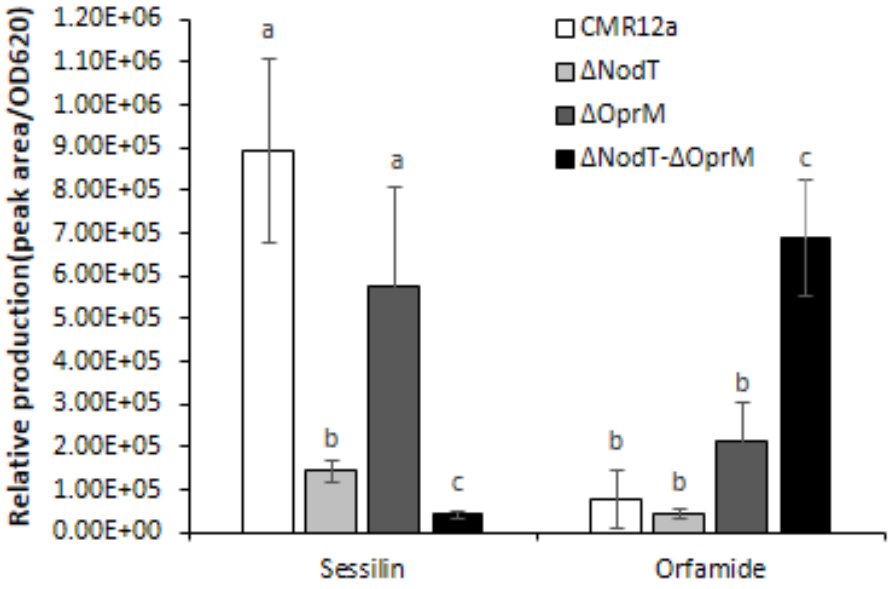

Figure 5.5. Quantification of sessilin and orfamide production by *Pseudomonas* sp. CMR12a and mutants in the *nodT* and *oprM* genes; grown for 17 h in still LB broth cultures at 28 °C. Cell density was measured spectrophotometrically (OD_{620}) and mean values from three replicates were recorded. Data was log transformed before statistics. Bars with different letters are significantly different according to Tukey's tests (P = 0.05).

MacABses and MexAB are involved in sessilin secretion

Following our results with *nodT* and *oprM* genes, we proceeded to investigate the effect of the ABC transporters, MacAB, and the RND efflux transporters, MexAB, in the export of sessilin and orfamide by *Pseudomonas*. sp. CMR12a. Mutants were constructed in *macAses*, *macABses*, *macABofa*, *mexA* and *mexAB* genes and subsequently characterized.

For sessilin secretion, in contrast with the WT, CMR12a-ΔMacAses secreted lower amounts of sessilin at the start (17 h) of bacterial growth (Figure 5.6A) (Figure 5.7). After 17 h, sessilins were secreted in amounts comparable with the WT strain. This was consistent with the white line phenotype shown by this strain (Figure 5.3) when challenged with Pf-5 inferring its production of sessilin in amounts which are at least comparable to that of the WT. On the other hand, this mutant did not secrete orfamides at 17 h after which the secretion levels normalized similar to what was obtained in the WT (Figure 5.6B) (Figure 5.7). In stark contrast with the phenotype shown by *macAses*, a mutation in the *macABses* genes led to a dramatic reduction of sessilin secretion for all timepoints tested (Figure 5.6A) (Figure 5.7). This was coupled with a corresponding increase in orfamide production that was significantly higher than levels obtained in the WT (Figure 5.6B) (Figure 5.7). This phenotype was mirrored in the white line showed when the CMR12a-Δ*macABses* mutant was challenged with the sessilin producer, CMR12a-ΔCLP2 (Figure 5.3). Unfortunately, the mutagenesis in the *macABofa* gene was not successful so we did not characterize it further.

Besides the significant reduction in sessilin secretion shown by the *mexA* mutant at 17 h, this strain could secrete comparable amounts of sessilin into the bacterial culture as the WT (Figure 5.6A). Similar to its effect on sessilins, a mutation in *mexA* resulted in a significant reduction of orfamide secretion during early bacterial growth (17 h) after which no further effect was observed at later time points (Figure 5.6B) (Figure 5.7). Similar to the WT, this mutant gave a white line in interaction with Pf-5 (Figure 5.3). However, for all time-points, a *mexAB* mutant secreted significantly lower amounts of sessilins than the WT (Figure 5.6A) with a corresponding increase in orfamide secretion (Figure 5.6B). This further gave a noticeable shift in the white line phenotype which was only present when this mutant was challenged with a sessilin producer thereby confirming a transition in CLP secretion from sessilins to orfamides (Figure 5.3).

Since our previous tests did not show a clear phenotype for the membrane fusion protein mutants, CMR12a-Δ*macAses* and CMR12a-Δ*mexA*, we proceeded to make *mexA* and *macA* mutants in the aforementioned mutant backgrounds. Thus, this gave CMR12a-Δ*macA*-Δ*mexA* and CMR12a-Δ*mexA*-Δ*macA* mutants, respectively. Unlike the previously characterized Δ*mexA* and Δ*macA* mutants, these mutants gave the white line phenotype in interactions with the sessilin producer, and not the orfamide producer, suggesting in this case, a secretion of higher amounts of orfamide for both strains (Figure 5.3). CLP production by these mutants are yet to be characterized by LC-ESI-MS.

Spectrophotometric measurements of cell density showed that of all isolates tested, the highest growth was recorded by *macABses* and *mexAB* mutants at 17 and 24 h although this gradually declined over time. On the other hand, the growth of *macA* and *mexA* mutants did not significantly differ from the WT strain (Figure 5.6C).

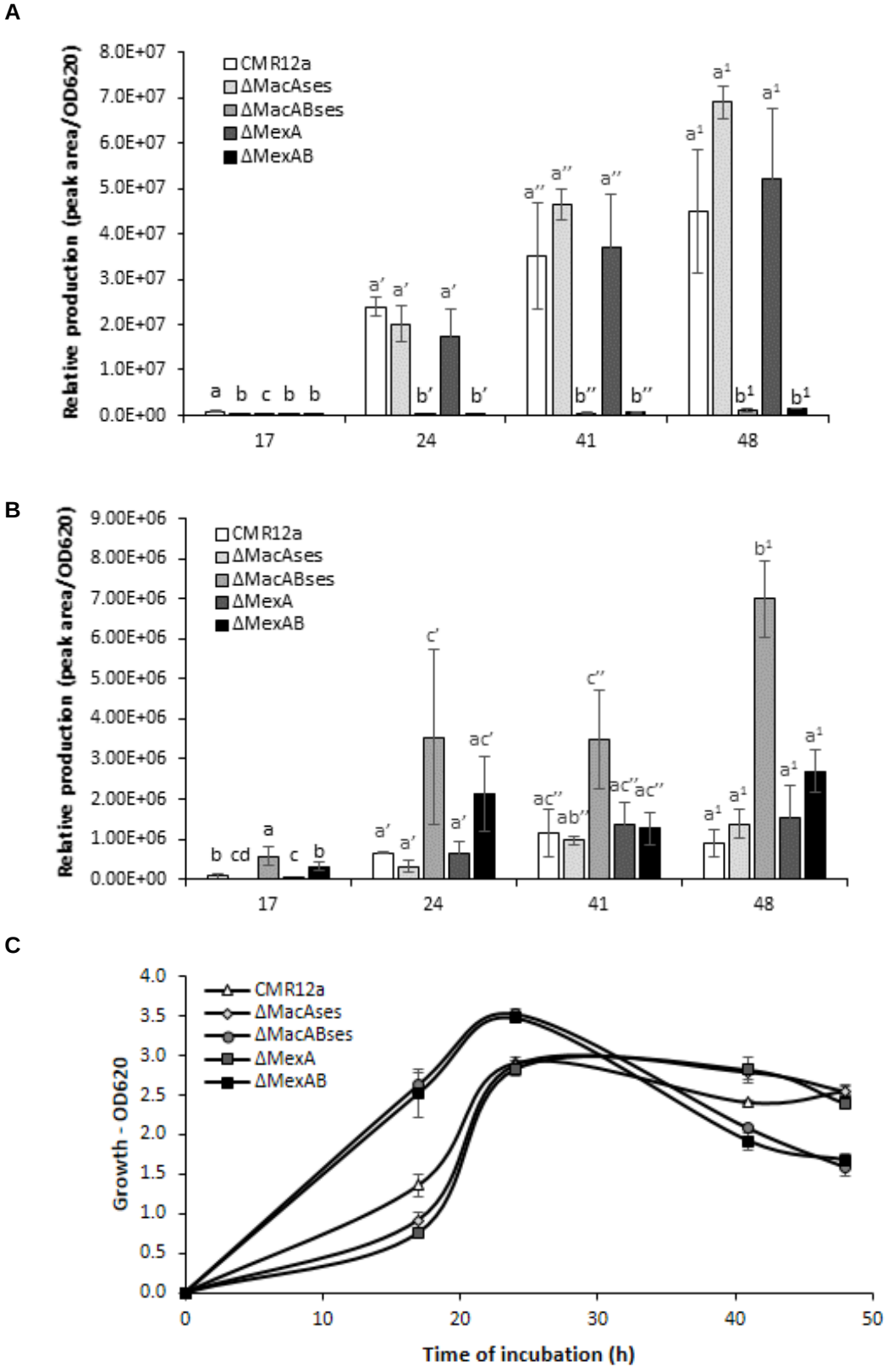

Figure 5.6. Quantification of sessilin (A) and orfamide (B) production by *Pseudomonas* sp. CMR12a and mutants in *macA, macAB, mexA and mexAB* genes; **C)** Growth of *Pseudomonas sp.* CMR12a strain and mutants in still LB broth cultures at 28°C; at each timepoint, cell density was measured spectrophotometrically (OD_{620}) and mean values from three replicates were recorded. Data was log transformed before statistics. Bars with different letters are significantly different according to Tukey's tests ($P = 0.05$).

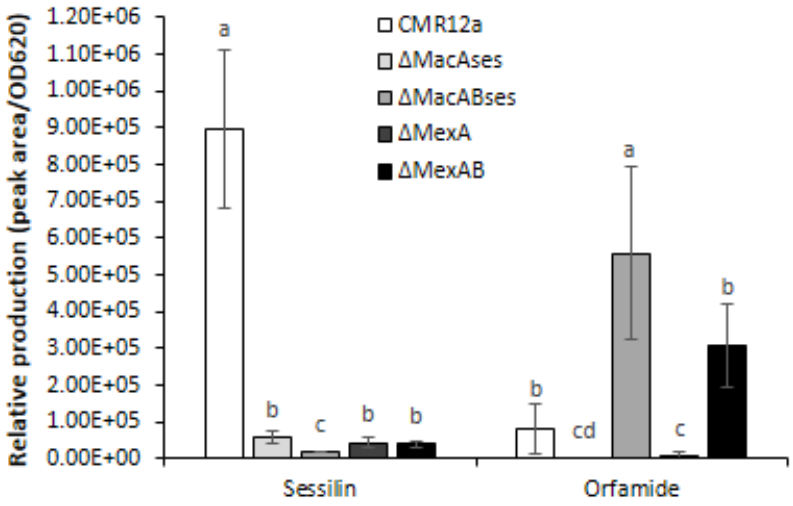

Figure 5.7. Quantification of sessilin and orfamide production by *Pseudomonas* sp. CMR12a and mutants in the *macA, macAB, mexA and mexAB* genes; grown for 17 h in still LB broth cultures at 28 °C. Cell density was measured spectrophotometrically (OD_{620}) and mean values from three replicates were recorded. Data was log transformed before statistics. Bars with different letters are significantly different according to Tukey's tests (P = 0.05).

Discussion

The bacterial type 1 secretion system (T1SS) and RND family efflux pumps have widely been shown to mediate the secretion of a wide range of substrates which possess crucial roles in the bacteria's response to its environment (Costa *et al.,* 2015). These efflux pumps have been well studied for their roles in antibiotic resistance (Blair *et al.,* 2014) in addition to their physiological relevance especially for bacterial pathogens to cause infections and for biofilm formation (Blair and Piddock, 2016). For *P. aeruginosa*, the roles of the ATP-energized MacAB and proton motive force-driven MexAB-OprM in the efflux of these substrates have been well elucidated (Li *et al.,* 1995; Kobayashi and Nishino, 2001).

In our study, the phylogenetic analysis of CLP-associated NodT proteins together with their OprM relatives was conducted to gain insights into their genetic relatedness. The high similarity at the amino acid level between the OprM, and their homologues in CLP-producing isolates suggests that the *nodT* gene has originated from a gene duplication event and started to diverge within the CLP producing strains. The absence of the *nodT* gene upstream of the biosynthetic gene cluster of some CLPs further highlights the acquisition of this gene within the bacterial genome. Gene duplication events in prokaryotes are often associated with environmental adaptations (Bratlie *et al.,* 2010; Kondrashov, 2012). Thus, the gene product of *nodT* could find its use as an efflux machinery alongside OprM and other systems wherever they occur. While colonizing and infecting their hosts, pathogenic bacteria face hostile environments and so have evolved a number of complex secretion systems which direct virulence factors into the extracellular environment or into the host cell cytosol (Bleves *et al.,* 2010). The functional role of the NodT in the virulence capacity of plant pathogenic bacteria remains to be investigated.

MexA-MexB belong to a family of gram-negative export machineries in which the second component, MexB, is an efflux pump belonging to the RND family while the first component, MexA, belongs to the MFP family which connects the pump directly to the outer membrane channel, the OprM (Li *et al.,* 1995: Costa *et al.,* 2016). Within the *mexAB-OprM* operon, OprM serves as the outer membrane component which pumps out solute molecules into the medium. The general involvement of this operon in the efflux of secondary metabolites has been suggested (Poole, 1994). In *Pseudomonas* sp. CMR12a, sessilin appears to be secreted extracellularly via MexAB-OprM since a null *mexAB* mutant lost the capacity to secrete sessilin and an *oprM* mutant showed reduced sessilin secretion. This observation in our strain of study is consistent with the notion that the MexA-MexB-OprM system functions as a multidrug efflux pump as shown in *P. aeruginosa* (Li *et al.,* 1995).

The multidrug efflux capacity of the MexAB-OprM is dependent on the presence of all three proteins in the complex (Welch *et al.,* 2010). In our study, it was interesting to note that the *macA*, *mexA*, *nodT* and *oprM* mutants of CMR12a still secreted WT levels of sessilin and orfamides especially after the early growth phase. Previous studies have shown that in *E. coli*, MexB could act independent of its cognitive partners by recruiting AcrA and TolC to form a functional drug complex

(Welch *et al.,* 2010). It therefore appears that a similar scenario plays out in CMR12a such that in the absence of MexA and OprM, MexB could couple with MacA and NodT for CLP secretion and vice versa. Such flexible efflux systems displayed by both gram-negative and gram-positive bacteria ensure their efficiency in the drug efflux of clinical strains and this adjustability was also observed in our strain of study. The outright loss of sessilin secretion was displayed principally by *macABses* and *mexAB* mutants indicating that the inner membrane proteins, MexB and MacB, are crucial for sessilin secretion. It is plausible that in the absence of MexB, CMR12a relies on sessilin secretion only via the MacAB-NodT system whereas the MexAB-OprM would suffice for the same job in the absence of MacB. This is further supported by the fact that a double mutant in *macA-mexA* and vice versa, could no longer give a white line when challenged with Pf-5 but gave this phenotype when challenged with the sessilin producer, CMR12a-ΔCLP2, thus inferring a reduction in sessilin secretion and increased orfamide export. However, this suggested flexibility remains to be investigated in more detail.

In *P. aeruginosa*, null mutants in *mexA* and *oprM* alone were sufficient to make *P. aeruginosa* strains hypersusceptible to several antibiotics and led to steady-state drug accumulation (Poole *et al.,* 1995). Additionally, *mexA* and *oprM* mutants of *P. aeruginosa* were not tolerant to all antibiotics including aminoglycosides (such as streptomycin and kanamycin) and chloramphenicol for example (Poole *et al.,* 1993; Poole *et al.,* 1995) suggesting that different secondary metabolites might have specific efflux pumps for secretion. This could therefore provide an explanation for the fact that almost all pumps studied during this work appeared to contribute to sessilin efflux alone but not to orfamide secretion. However, it was interesting to observe the absence or reduction of orfamide secretion at the early growth phase of *macAses* and *mexA* mutants, respectively.

Given the fact that the sessilin cluster constitutes a portion of the genomic island in CMR12a (D'aes *et al.,* 2014), it appears that this metabolite utilizes the major CLP secretion machinery of CMR12a at the expense of orfamide. Thus, orfamide secretion is only enhanced when sessilin secretion is inactivated. It is plausible that orfamides are secreted via one or more of several yet to be characterized transporters in the CMR12a genome. Nonetheless, the MexAB-OprM, MacAB and the NodT systems contribute albeit indirectly to orfamide secretion by efficiently pumping out sessilin thereby moderating the secretion of orfamide.

For sessilin secretion, CMR12a appears to utilize multiple export channels (NodT, OprM, MacAses, MacABses, MexA and MexAB) during the early growth phase of the bacterium. Our work highlights the relevance of MacAB and MexAB in sessilin secretion as the bacterial growth progresses. In spite of the number of mutants made in putative transport genes, we could not elucidate the pathway for orfamide secretion. Our results suggest the presence of other outer membrane proteins through which orfamides are secreted in CMR12a. Infact, several species including *E. coli* and *P. aeruginosa* reportedly contain redundant efflux systems which could provide an advantage such that the mutational loss of one system does not render the bacteria susceptible to cytotoxic agents (Nishino and Yamaguchi, 2001; Pagès *et al.,* 2005).

Conclusively, the *nodT*, *oprM*, and *macAB* genes together with the *mexAB-oprM* operon are involved in the active efflux of sessilins thereby contributing significantly to the environmental fitness of the CMR12a strain. The results of our study further highlight that besides functioning as a multidrug efflux pump in gram-negative bacteria, MexAB-OprM is instrumental in the secretion of secondary metabolites outside the bacterial cell.

Materials and Methods

Bacterial strains and culture conditions

Bacteria strains and plasmids used in this study are listed in Table S6.1. Primers used in mutagenesis are listed in the general primer list. Culture conditions are similar to those used in Chapter 5.

Bioinformatic analyses

The protein sequences of NodT associated with different CLP biosynthetic clusters and their corresponding OprM sequences were retrieved from Genbank. Additionally, NodT and OprM protein sequences for *Pseudomonas* sp. CMR12a were obtained from its draft genome sequence. Further, for each strain studied, sequences of the housekeeping gene, *rpoD*, were extracted from Genbank when available. For phylogenetic analyses of the membrane proteins and housekeeping genes, separate alignments were made using Muscle (Edgar, 2004) via the software package MEGA6 (Tamura *et al.,* 2013). Both trees were inferred by Maximum Likelihood (ML) using 1000 bootstrap replicates. For the phylogenetic analysis of NodT protein sequences, OpmQ and OpmB sequences of *P. aeruginosa* were used as outgroup whereas the *rpoD*-based tree was rooted with isolates belonging to the *P. aeruginosa* lineage.

Site-directed mutagenesis

Site-directed mutagenesis of the *nodT*, *oprM*, *macAses*, *macABses*, *macABofa*, *mexA* and *mexAB* genes was performed based on the methods described in Chapter 4.

Construction of pME6032-based vectors for complementation

Similar methods as used in Chapter 4 were employed in this section.

White-line-in-agar assays

Similar methods as used in Chapter 4 were employed in this section. Additionally, during the white line tests, the CMR12a-ΔCLP2 mutant (a sessilin producer), was used as an indicator strain for orfamide production.

LC-ESI-MS analysis of CLP production by CMR12a and mutants

Production of CLPs by CMR12a, and all mutants in putative transport genes together with complemented strains was first tested qualitatively by the drop collapse technique (Jain *et al.,* 1991). For LC-ESI-MS analysis, bacteria strains were grown in LB broth still conditions for 24 h after which 1 ml of cultures were centrifuged at 13, 400 rpm for 4 min. Sessilins and orfamides were measured from filter sterilized supernatants by ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) by using single ion recording integration of peaks which corresponded with the masses of these CLP homologues as described previously (D'aes *et al.,* 2014). Data obtained using the

MassLynx V4.1 software was expressed as relative production (peak area/OD_{620}) while Tukey's tests were performed on log transformed data via SPSS.

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Supplementary files

Table S5.1. Strains and plasmids used in this chapter

Strains and plasmids		Relevant characteristics	Reference/Source
Pseudomonas			
	CMR12a	PHZ$^+$, CLP1$^+$, CLP2$^+$, wild type (Cameroon)	Perneel *et al.,* (2007)
	CMR12a-ΔClp2	PHZ$^+$, CLP1$^+$, CLP2$^-$, mutant with deletion in *ofaB* and *ofaC* genes	D'aes *et al*., (2011)
	CMR12a-Δ*nodT*	mutant with deletion of *nodT* gene in the sesilin cluster	This study
	CMR12a-Δ*oprM*	mutant with deletion of *oprM* gene in MexAB-OprM operon	This study
	CMR12a-Δ*nodT*-Δ*oprM*	mutant with deletion of *nodT* and *oprM* genes	This study
	CMR12a-Δ*macAses*	mutant with deletion of *macA* gene in the sessilin cluster	This study
	CMR12a-Δ*macABses*	mutant with deletion of *macA* and *macB* genes in the sessilin cluster	This study
	CMR12a-Δ*macABofa*	mutant with deletion of *macA* and *macB* genes in the orfamide cluster	This study
	CMR12a-Δ*mexA*	mutant with deletion of *mexA* gene	This study
	CMR12a-Δ*mexAB*	mutant with deletion of *mexA* and *mexB* genes	This study
	CMR12a-Δ*mex-ΔmacAses*	mutant with deletion of *mexA* gene and *macA* gene in the sessilin cluster	This study
	CMR12a-Δ*macAses-ΔmexA*	mutant with deletion of *macA* gene in the sessilin cluster and *mexA* gene	This study
	Pf-5	Orfamide, wild type	Loper and Gross (2007)
E. coli			
	DH5α	Host for cloning	Hanahan (1983)
	WM3064	Strain for conjugation; λ pir, DAP auxotroph	Saltikov and Newman (2003)
S. cerevisiae InvSc1		Yeast strain for *in vivo* recombination (*ura3-52/ura3-52* mutation)	Invitrogen
Plasmids			

pMQ30	Gene replacement vector for *Pseudomonas* species; *sacB*, URA3, Gm^R	Shanks *et al.,*(2006)
pMQ30-Δ*nodT*	Vector for site specific mutagenesis of *nodT* in the sessilin cluster	This study
pMQ30-Δ*oprM*	Vector for site specific mutagenesis of *oprM* in CMR12a	This study
pMQ30-Δ*macAses*	Vector for site specific mutagenesis of *macAses,*downstream sessilin cluster	This study
pMQ30-Δ*macABses*	Vector for site specific mutagenesis of *macABses,*downstream sessilin cluster	This study
pMQ30-Δ*mexA*	Vector for site specific mutagenesis of *mexA,*within the *mexAB*	This study
pMQ30-Δ*mexABses*	Vector for site specific mutagenesis of *mexAB* in CMR12a	This study
pMQ30-Δ*macABofa*	Vector for site specific mutagenesis of *macABofa,*downstream of the orfamide cluster	This study
PME6032	Expression vector with *tac* promoter; Tc^R	Heeb *et al*., (2002)
PME6032-NodT	Expression vector with *nodT* of CMR12a under the *tac* promoter; Tc^R	This study
PME6032-OprM	Expression vector with *oprM* of CMR12a under the *tac* promoter; Tc^R	This study
PME6032-MexA	Expression vector with *mexA* of CMR12a under the *tac* promoter; Tc^R	This study
PME6032-MacA	Expression vector with *macA* of CMR12a under the *tac* promoter; Tc^R	This study

*PHZ: phenazine; CLP: cyclic lipopeptides; CLP1: sessilin; CLP2: orfamide; Gm^R, Tc^R, Amp^R, Km^R: resistant to gentamycin, tetracycline, ampicillin, kanamycin, respectively.

Chapter 6

Rhizosphere diversity of fluorescent pseudomonads and cyclic lipopeptides correlates with cocoyam (*Xanthosoma sagittifolium*) resilience to the *Pythium* root rot disease

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Summary

Bacteria belonging to the *Pseudomonas fluorescens* complex are well-known for their plant pathogen suppressive capacities. In previous studies, *Pseudomonas* isolates obtained exclusively from the red cocoyam rhizosphere showed antagonism against *Pythium*-mediated cocoyam root rot disease (CRRD) and appeared to confer field tolerance to red cocoyams against CRRD. We compared fluorescent pseudomonads obtained from both red and white cocoyam rhizosphere grown in three different Cameroon andosols possessing low, moderate and high disease pressure towards CRRD. Although no associations between variety and microbial diversity were deciphered, the cocoyam rhizosphere of low disease pressure soils of Boteva were significantly enriched with isolates affiliated with the *P. fluorescens* complex whereas isolates obtained from the cocoyam roots in other soils comprised mainly of isolates belonging to the *Pseudomonas putida* group. LC-MS and NMR analysis revealed at least seven structurally different CLPs, three of which are novel, with the highest CLP abundance recorded by rhizosphere isolates obtained from low disease pressure soils. Furthermore, higher amounts of organic matter in low disease pressure soil of Boteva suggests that soil nutrition may influence the recruitment of specific *Pseudomonas* population by cocoyam roots. Besides being a CLP hotspot, our study on *Pseudomonas* and CLP diversity on the cocoyam rhizosphere, contributes to the understanding of how multifaceted players contribute to the microbial diversity in the rhizosphere.

Introduction

Cocoyam (*Xanthosoma sagittifolium* (L) Schott), a tropical monocotyledonous tuber crop, is widely cultivated in some parts of Africa, Asia and the Pacifics with Nigeria, Cameroon and Ghana as the major producing countries (Purseglove, 1972; Agbede, 2008). Besides containing substantial amounts of proteins, fats and essential vitamins, cocoyams are a rich source of carbohydrates and serve as a staple food for over 400 million people in the tropics (Djeugap *et al*., 2014). However, cocoyam production is seriously hampered by the root rot disease caused by the oomycete, *Pythium myriotylum*, accounting for yield losses of up to 90% in West and Central Africa (Nzietchueng, 1983). In Cameroon, two varieties of cocoyam are cultivated: the preferred white cocoyam, which is more susceptible to the cocoyam root rot disease (CRRD), and the red cocoyam which display some level of field tolerance against the CRRD (Perneel *et al.*, 2007).

Fluorescent *Pseudomonas* spp. have been implicated in the biological control of plant pathogens (Chapter 1). Multi-locus sequence analysis, a method based on sequence analysis of several housekeeping genes, has proven reliable for species delineation and strain identification in the *Pseudomonas* genus. Using this method, two *Pseudomonas* lineages have been discriminated namely *P. aeruginosa* and *P. fluorescens* (Garrido-Sanz *et al.,* 2016). The first *P. aeruginosa*, was divided into three main groups, represented by the species *P. aeruginosa*, *P. stutzeri* and *P. oleovorans* while the *P. fluorescens* lineage was divided into six groups, represented by the species of the *P. fluorescens complex* and the *P. syringae*, *P. lutea*, *P. putida*, *P. anguilliseptica* and *P. straminea* groups. The *P. fluorescens* complex was described as being the most complex and included nine groups, represented by the species *P. fluorescens*, *P. gessardi*, *P. fragi*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata, P. chlororaphis* and *P. asplenii* (Garrido-Sanz *et al.,* 2016). Besides their strong strain-to-strain diversity, fluorescent pseudomonads belonging to the *P. fluorescens* complex are of notable interest because most biocontrol strains which produce potent secondary metabolites, including cyclic lipopeptides (CLPs), are commonly found within this lineage (Loper *et al.*, 2012; Chapter 1).

Cyclic lipopeptides (CLPs) are bacterial metabolites possessing biosurfactant activity and composed of a cyclic oligopeptide lactone ring linked to a fatty acid tail (Raaijmakers *et al.,* 2006). They can cause destruction of microbial membranes, leading to death of bacteria, fungi, oomycetes and viruses. CLPs produced by *Pseudomonas* sp. have been shown to demonstrate biocontrol against several plant pathogens both *in vitro* and in *in vivo* conditions (Raaijmakers *et al.*, 2006; Chapter 1).

In previous years, biological control has been explored as a viable management option for CRRD. *Pseudomonas aeruginosa* PNA1, isolated from the chickpea rhizosphere in India (Anjaiah *et al.*, 1998), was shown to efficiently suppress cocoyam root rot by the production of the antibiotics phenazine-1-carboxylic acid and phenazine-1-carboxamide (Tambong and Höfte, 2001). Besides,

Perneel *et al.* (2008) demonstrated that phenazines and rhamnolipid–biosurfactants act synergistically in the control of *Pythium* root rot on cocoyams.

A targeted isolation and screening of healthy red and white cocoyam rhizosphere in Cameroon for potential biocontrol agents yielded 40 fluorescent *Pseudomonas* strains (Perneel *et al.*, 2007). Of these isolates, seven, obtained from the red cocoyam rhizosphere alone, showed antagonistic properties against *P. myriotylum* on cocoyams and all except one produced phenazine antibiotics Two of these isolates namely *Pseudomonas* sp. CMR12a and CMR5c are closely related to the *P. protegens* and *P. chlororaphis* groups and showed excellent biocontrol against the cocoyam root rot disease caused by *P. myriotylum* (Perneel *et al.*, 2007). Interestingly, CMR12a and CMR5c have been shown to produce cyclic lipopeptides and phenazines which are vital for the biocontrol and ISR capacity of the producing strains (Perneel *et al.*, 2007; D'aes *et al.*, 2014; Ma *et al.*, 2016a and 2016b). Coupled with the observation of field tolerance by red cocoyams towards CRRD in Cameroon, results of previous work led us to hypothesise that red cocoyams rhizosphere could possess *Pseudomonas* isolates which are taxonomically and metabolically diverse from the white cocoyams and that this could in turn contribute to the field tolerance exhibited by the red cocoyam variety towards *P. myriotylum*. Thus, the objectives of this study were to determine whether the taxonomic affiliation and cyclic lipopeptide diversity of fluorescent *Pseudomonas* isolates obtained from cocoyam rhizosphere was dependent on plant variety and could relate to cocoyam field tolerance and susceptibility displayed by red and white cocoyams, respectively.

Results

Taxonomic affiliation of Pseudomonas spp. isolated from red and white cocoyams

A total of one hundred and thirty-seven fluorescent *Pseudomonas* isolates were subjected to taxonomic characterization by sequencing housekeeping genes *rpoD* and *rpoB*. The Neighbor joining (NJ) bootstrap consensus trees derived from *rpoD* and *rpoB* sequence alignments respectively, are shown in Figures 6.1 and 6.2. Results of our phylogenetic analyses clearly show that most of our isolates formed clades with high bootstrap supports with several type strains within the *Pseudomonas* genus. Our test isolates belong mainly to the *P. fluorescens* complex and *P. putida* group whereas only a couple of isolates belong to the *P. aeruginosa* group (Figure 6.1, Figure 6.2).

Thus, we classified the *P. fluorescens* complex in part, according to the taxonomical classification of Garrido-Sanz *et al.* (2016), into eight groups. In this group, our isolates predominantly clustered within the *P. koreensis* group (25 isolates) while fewer isolates clustered in the groups of *P. fluorescens* (two isolates), *P. jessenii* (one isolate) and *P. corrugata* (two isolates) (Figure 6.1). Besides the affiliation of our isolates with known groups within the *P. fluorescens* complex, we created four new categories designated U1 (two isolates), U2 (three isolates), U3 (three isolates), and U4 (four isolates). U1 appears to be associated with the *P. corrugata* group while U2 leans towards the *P. jessenii* group. However, the U3 and U4 categories appear to be exclusive.

Interestingly, most of our isolates clustered in the same groups with well-studied biocontrol isolates such as *P. fluorescens* Pf0-1 (*P. koreensis* group) and *P. fluorescens* SBW25 (*P. fluorescens* group) (Figure 6.1A). For the *P. putida* group, based on the clade formations, eleven arbitrary divisions were made to which letters A to K were assigned (Figure 6.2). Although several of our isolates were closely related to already described isolates such as *P. entomophilia* L48T (A division), *P. mosselii* (B division), *P. soli* (F division), *P. monteilli* (I division) and *P. japonica (K division)*, a sizable number (55 isolates) formed separate clades and appear to be new species (Figure 6.2).

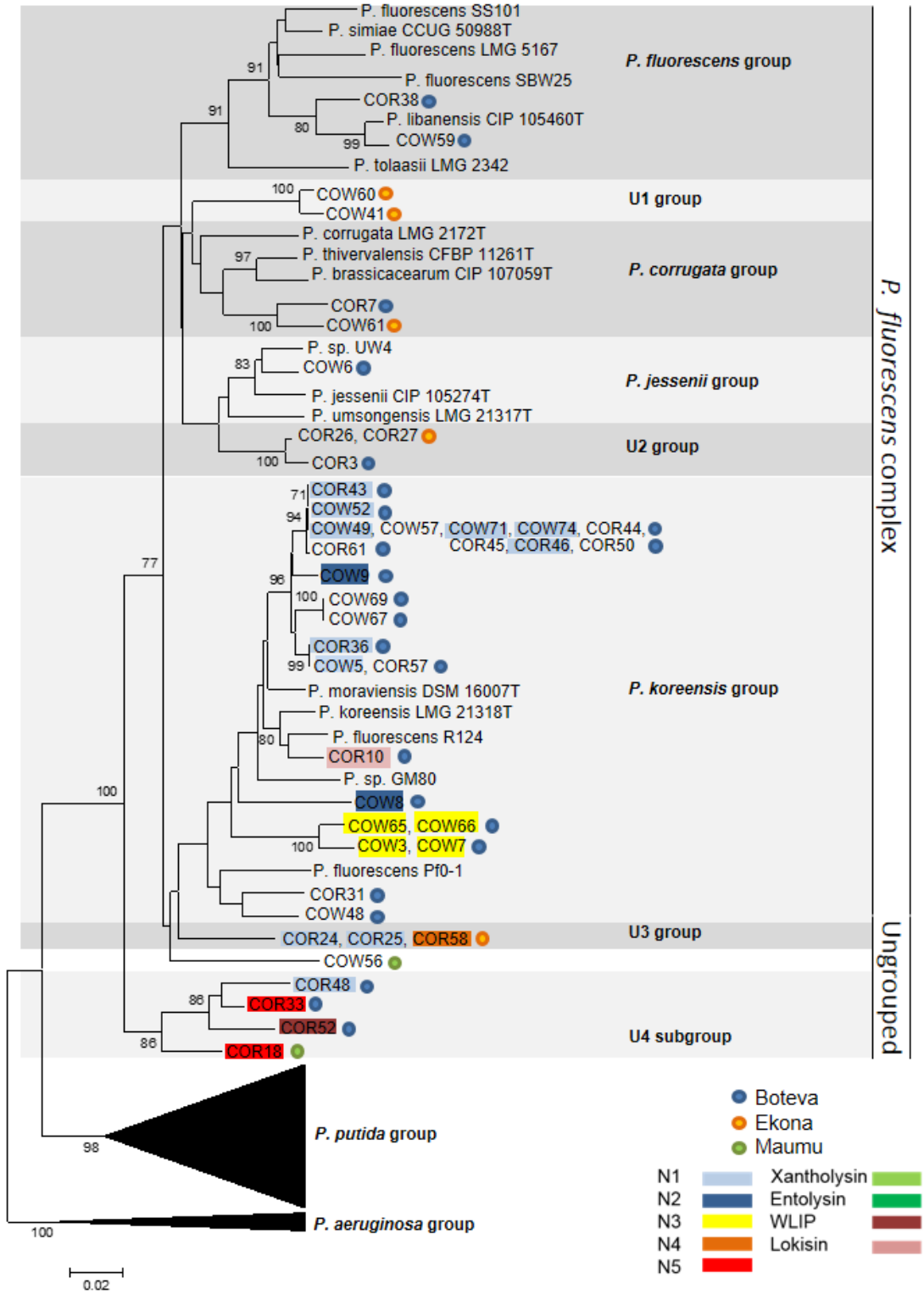

Figure 6.1. Phylogenetic analysis of 137 *Pseudomonas* isolates using two concatanated *Pseudomonas* housekeeping genes (*rpoB* and *rpoD*) and *P. aeruginosa-group* isolates as outgroup. This tree represents isolates belonging to the *P. fluorescens* complex and associated strains which are ungrouped. Bootstraps are only indicated for branches with bootstrap support of higher than 70. Neighbour joining tree was made using only isolates with unique sequences whereas isolates with identical sequences were added afterwards, on the same line**.** Isolates were obtained from cocoyam rhizosphere in Boteva, Ekona and Maumu villages of Cameroon and are indicated with blue, orange and green circles, respectively. **N1-N5:** new CLPs discovered in this study**; xantholysin, entolysin, WLIP, lokisin: p**reviously reported CLPs that are also produced by some isolates in this study.

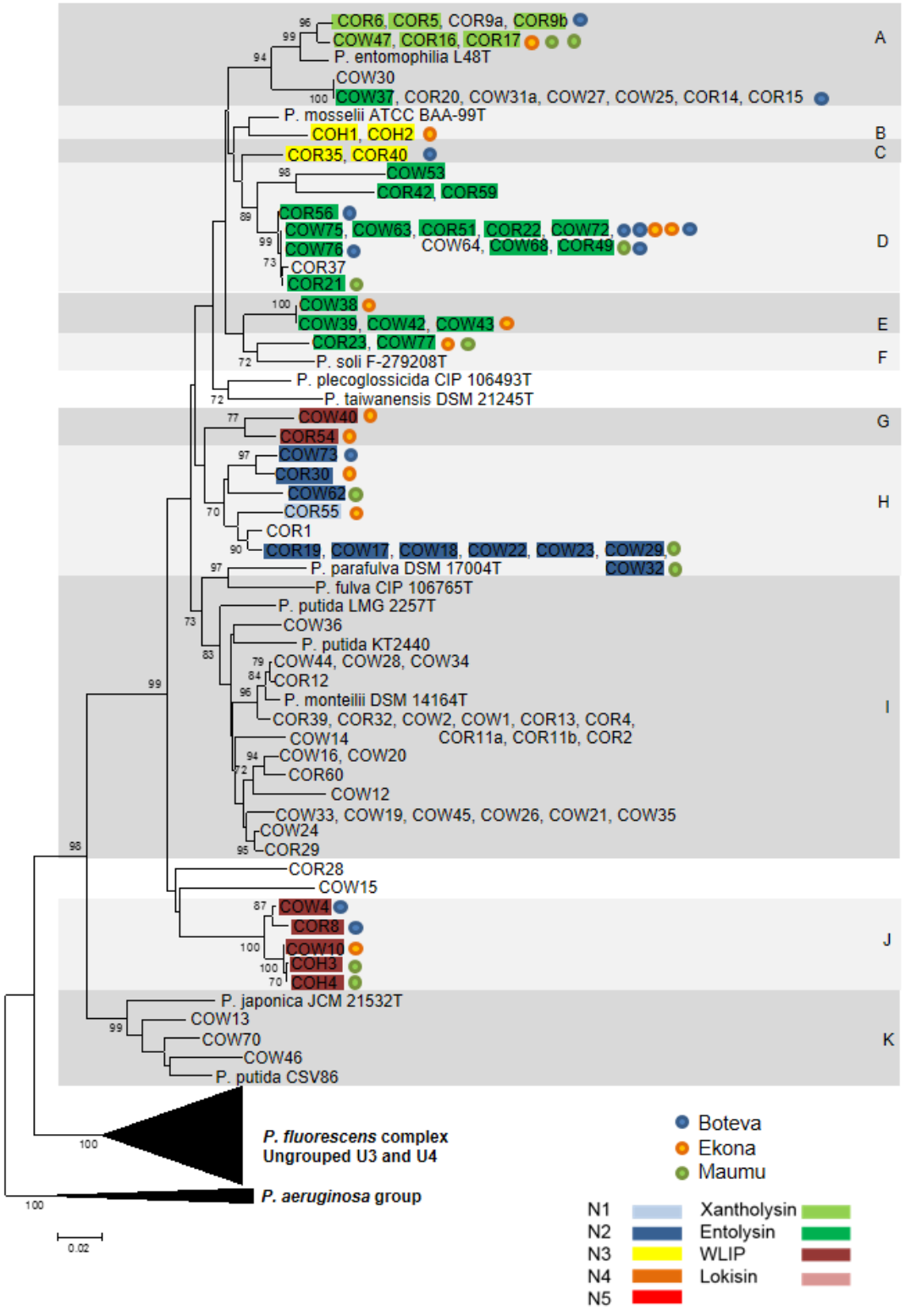

Figure 6.2. Phylogenetic analysis of 137 *Pseudomonas* isolates using two concatanated *Pseudomonas* housekeeping genes (*rpoB* and *rpoD*) and *P. aeruginosa-group* isolates as outgroup. This tree represents isolates belonging to the *P. putida* group. Bootstraps are only indicated for branches with bootstrap support of higher than 70. Neighbour joining tree was made using only isolates with unique sequences whereas isolates with identical sequences were added afterwards, on the same line**.** The location of only CLP producing strains obtained from Boteva, Ekona and Maumu villages are indicated with blue, orange and green circles, respectively. **N1-N5:** new CLPs discovered in this study**; xantholysin, entolysin, WLIP, lokisin: p**reviously reported CLPs that are also produced by some isolates in this study.

Relationship between Pseudomonas diversity, plant genotype and sampling location

At the *Pseudomonas* group level, results of Fishers' exact test statistic revealed that there is no significant difference between the taxonomic affiliation of strains collected from the red and white cocoyams (Figure 6.3a). Although, all isolates belonging to the U2, U3 and U4 groups were obtained from red cocoyams while those associated with the U1 group were isolated from white cocoyams, all other clades within the *P. fluorescens* complex and *P. putida* group had a mix of isolates from both genotypes (Figures 6.1 and 6.2). The reduced frequency of *P. fluorescens* complex isolates compared to the *P. putida* group isolates which was observed across genotypes (Figure 6.3a) was due to the higher abundance of *P. putida* isolates collected from all fields during sampling (Figure 6.3b).

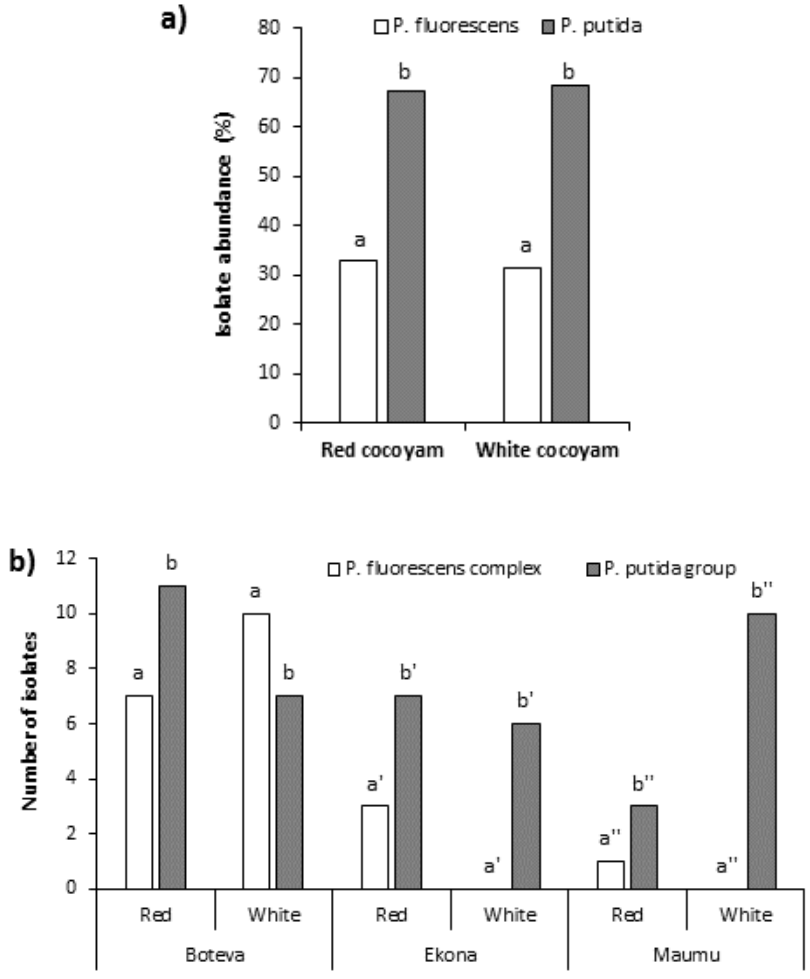

Figure 6.3. a) Taxonomic distribution of *Pseudomonas* isolates obtained from red and white cocoyams; **b)** Taxonomic affiliation of test isolates obtained from red and white cocoyams by location. Bars with different letters are statistically different according to Fisher's exact test.

The occurrence of the two taxonomic groups at each sampled location was further analysed. It was of foremost interest to observe that isolates from Boteva had a significantly higher (n = 33) abundance of *P. fluorescens* complex isolates than Ekona and Maumu locations that contained eight and two representative isolates, respectively (Figure 6.4).

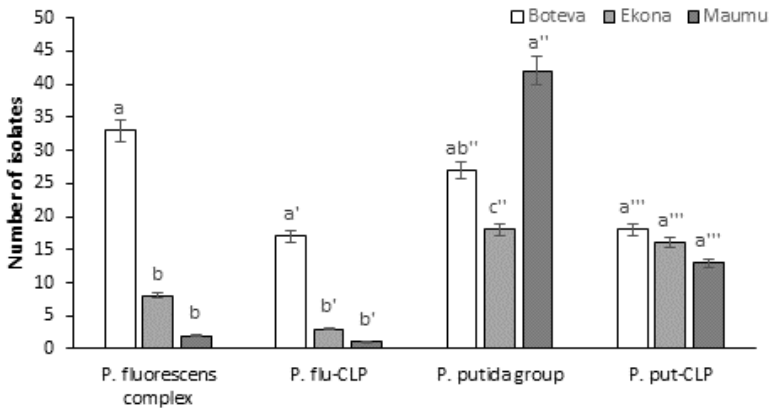

Figure 6.4. Taxonomic distribution of CLP producing isolates at the three sampled cocoyam fields. Total number of isolates affiliated with *P. fluorescens* complex or belonging to the *P. putida* group are compared across three fields. Similarly, the subset of CLP-producing isolates across these two taxonomic groups are compared for the three fields. P. flu: *P. fluorescens* complex; P.put: *P. putida* group. Bars with different letters are significantly different according to Fisher's exact statistic.

In addition to this, isolates situated in the *P. fluorescens*, *P. jessenii* and *P. koreensis* groups were exclusively from Boteva (Table S6.1). More so, isolates associated with the U1 and U3 subgroups were exclusively from Ekona whereas one of Maumu isolates belonged to U4 while the other was ungrouped. Within the *P. putida* group, a notable enrichment of Maumu isolates was observed such that the abundance of *P. putida* isolates obtained from Maumu differed significantly from those obtained at Ekona fields (Figure 6.4).

Physical and chemical soil properties for Boteva, Ekona and Maumu

Results of physicochemical properties of soil used in this study are shown in Table 6.1. Boteva soils had the highest silt (76%) followed by Ekona (40%) and Maumu (31%). Similar to silt, calcium was highest at Boteva (16%) and lowest at Maumu (2%). Organic carbon and total nitrogen were also highest at Boteva. Suppressive soils refer to soils where crops grown showed no CRRD symptoms. Intermediate soils are medium disease pressure soils in which suppressiveness could be restored while conducive soils refer to high disease pressure soils.

Table 6.1. Physical and chemical characteristics of soils used in this study

	Suppressive	Intermediate	Conducive
Soil variable	Boteva	Ekona	Maumu
Clay (%)	2.99	53.44	54.20
Silt (%)	76.60	40.41	31.80
Sand (%)	20.41	6.15	14.00
pH (H_2O)	5.15	4.72	5.87
Ca (cmol kg^{-1})	16.47	4.16	2.92
Mg (cmol kg^{-1})	3.14	1.72	3.32
K (cmol kg^{-1})	0.22	0.38	1.22
Na (cmol kg^{-1})	0.00	0.01	0.02
P (mg kg^{-1})	6.57	6.37	27.60
Organic C (%)	6.04	1.93	3.45
Total N (%)	0.73	0.22	0.38

Characterization of CLPs produced by Pseudomonas isolates

Results of biosurfactant screening using the drop collapse assay showed that more than 50% (*n* = 77) of isolates tested positive. Thus, CLP production for these isolates was verified by LC-MS analyses. Based on retention time of the different CLPs, we could group all isolates into nine different types. Figure 6.5 shows representative chromatograms for several of these types. One major peak was present in all strains, accompanied by minor peaks representing natural variants of the major compound. These minor forms most likely result from the fact that the non-ribosomal peptide synthetases responsible for CLP production exhibit a certain degree of flexibility in selecting the free amino acids.

Using MS and NMR analysis we identified four CLPs that are already described in literature including xantholysin-like, entolysin-like, amphisin-like (lokisin) and viscosin-like (WLIP) CLPs with molecular weights of 1775.8 Da, 1721.0 Da, 1353.8 Da and 1125.6 Da, respectively (Figure 6.5). Furthermore, our study further revealed five new CLPs which were designated as N1, N2, N3, N4 and N5. N1, N2 and N3 were fully characterized by NMR while N4 and N5 were not. Main peaks of N1, N2 and N3 featured MW values of 1393.6 Da, 1397.6 Da and 2131.2 Da, respectively (Figure 6.5).

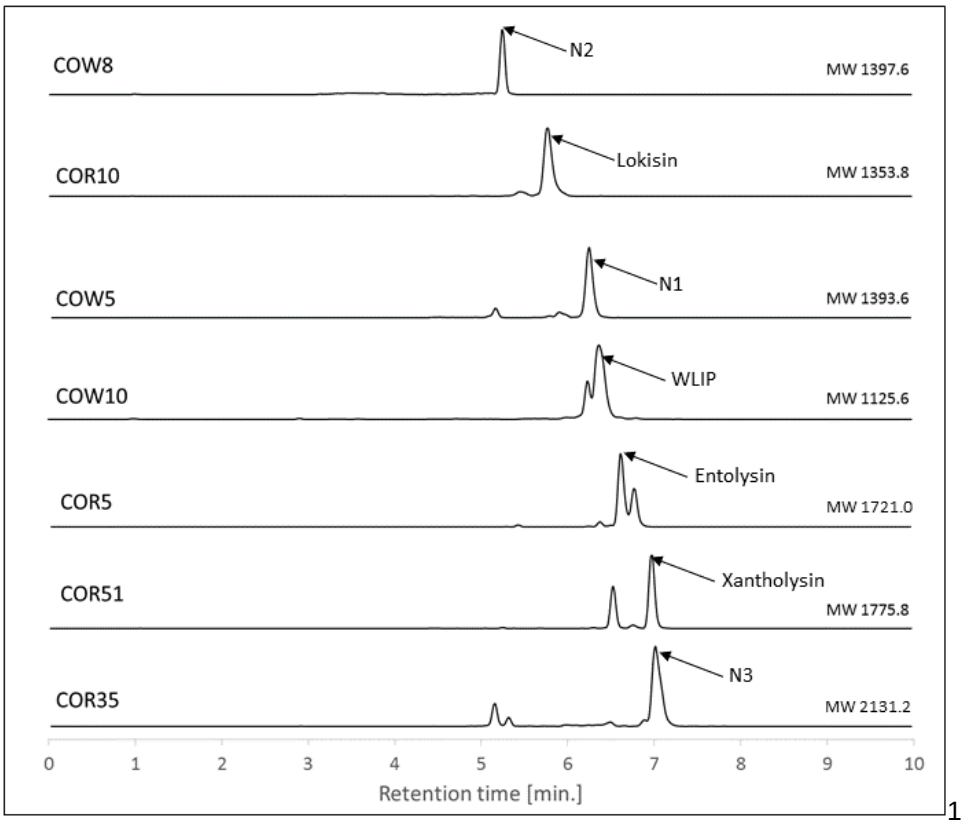

Figure 6.5. HPLC chromatograms showing major CLPs produced in liquid culture by fluorescent *Pseudomonas* spp. isolated from the cocoyam rhizosphere. Representative CLP-producing isolates which were characterized are indicated on the left hand side of the panel.

Structural diversity of CLPs

Identification of xantholysin in *Pseudomonas putida* COR51

Detailed analysis of the NMR spectra recorded for the xantholysin-like CLP in dimethylformamide-d7 (DMF-d7) at 328 K revealed the presence of 14 amino acids (5x Leu, 4x Gln, 1x Glu, 1x Ser, 2x Val and 1x Ile) (Figure S6.1) (Table S6.3). The presence of a 3-hydroxydecanoic acid (HDA) moiety was determined from combined analysis of the ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC spectra and by comparison with the chemical shifts for the identical moiety reported for pseudodesmin A in DMF solution (Li *et al.,* 2013). The xantholysin-like nature was confirmed through determination of the amino acid sequence (HDA-Leu1-Glu2-Gln3-Val4-Leu5-Gln6-Ser7-Val8-Leu9-Gln10-Leu11-Leu12-Gln13-Ile14) using a 2D ^{1}H-^{1}H ROESY spectrum. Finally, the ^{13}C carbonyl resonances were assigned by using ^{1}H ^{13}C gHMBC, from which the attachment of the Ser7 side chain to the Ile14 residue through the lactone bond was also confirmed using a $^{3}J_{HC}$ linking the Ser7 H$^{\beta}$ with the C-terminal carbonyl. While NMR and MS generally do not yield information on a compounds stereochemistry, the stereochemical similarities with respect to xantholysin could be established by comparing the NMR data of the xantholysin-like CLP with that of xantholysin A produced by *Pseudomonas putida* BW11M1 (Li *et al.,* 2013). The quasi identical ^{1}H and ^{13}C chemical shifts conclusively establish that the xantholysin-like CLP produced by COR51 has the same stereochemistry as xantholysin A, thereby identifying it (Geudens *et al.,* 2014).

Identification of entolysin in *Pseudomonas putida* COR5

Similarly, an NMR and MS analysis was performed for the entolysin-like CLP in DMF-d7 at 328K. The 2D ^{1}H-^{1}H TOCSY spectrum of this lipopeptide allowed the identification of residues corresponding to four Leu, one Ile, three Val, three Gln, one Glu, and two Ser by means of their characteristic amino acid correlation patterns. The presence of a 3-hydroxydecanoic acid could be confirmed in the same way as for xantholysin A (vide supra). The amino acid sequence (HDA-Leu1-Glu2-Gln3-Val4-Leu5-Gln6-Val7-Leu8-Gln9-Ser10-Val11-Leu12-Ser13-Ile14) could be elucidated by the analysis of a 2D ^{1}H-^{1}H ROESY spectrum (Figure S6.2) (Table S6.4). Unfortunately, the position of the ester bond that cyclizes the molecule could not be confirmed by analyzing the ^{1}H-^{13}C HMBC spectrum due to spectral overlap. Using a similar reasoning as in Li *et al.* (Li *et al.,* 2013), the chemical shift of the Ser10 CH_2^{β} protons indicated that this residue is involved in the depsibond. Based on the NMR and MS data, the amino acid sequence of the entolysin-like CLP produced by COR5 could be identified as that of entolysin. Since entolysin A and B could not be discriminated in the original article (Vallet-Gely, *et al.,* 2010), we propose to define the amino acid sequence described above as entolysin A.

Identification of lokisin in *Pseudomonas koreensis* COR10

An amphisin-like CLP with a molecular mass of 1353.8 Da was produced by COR10. Using high resolution NMR in acetonitrile-d3 at room temperature, eleven residues could be identified: five Leu, two Asp, two Ser, one Ile and one Thr. The presence of a 3-hydroxydecanoic acid could be established using a similar approach as previously used for xantholysin A (vide supra). The amino acid sequence (HDA-Leu1-Asp2-Thr3-Leu4-Leu5-Ser6-Leu7-Ser8-Leu9-Ile10-Asp11) could be confirmed by the analysis of a 2D ^{1}H-^{1}H ROESY spectrum (Figure S6.3) (Table S6.5). Using chemical shift arguments (vide supra), the depsibond is located between Thr3 CH_2^{β} and the C-terminal of Asp11. Based on the NMR and MS data, the amino acid sequence of the amphisin-like CLP produced by COR5 could be identified as that of lokisin (Sorensen, *et al.,* 2002). However, to conclusively identify this lipopeptide as lokisin, the stereochemistry should be confirmed, either by x-ray crystallography or by comparison of the NMR spectra with those of the original compound.

Identification of WLIP in *Pseudomonas putida* COW10

A viscosin-like CLP with a molecular mass of 1125.6 Da was produced by COW10. Using NMR data, nine amino acid residues could be identified: three Leu, one Glu, two Ser, one Ile, one Val and one Thr. The presence of a 3-hydroxydecanoic acid could be established as described before (vide supra). The amino acid sequence (HDA-Leu1-Glu2-Thr3-Val4-Leu5-Ser6-Leu7-Ser8-Ile9) could be confirmed by the analysis of a 2D ^{1}H-^{1}H ROESY spectrum (Figure S6.4) (Table S6.6). Using similar chemical shift arguments as used before (vide supra), it was established that the depsibond forming the macrocycle is located between Thr3 CH_2^{β} and the C-terminal of Ile9. In literature, there are two compounds that possess the amino acid sequence described above. Both structures only differ in the stereochemistry of the residue located at position five where viscosin contains an L-Leu5 (Groupé *et al.,* 1951; Hiramoto, *et al.,* 1970) and WLIP a D-Leu5 (Soler-Rivas *et al.,* 1999). Comparison of the proton and carbon chemical shifts of the viscosin-like CLP with those of viscosin and WLIP (Geudens *et al.,* 2014) allowed to discriminate between both CLPs. The chemical shifts of the viscosin-like CLP

were nearly identical to that of WLIP, confirming the stereochemistry of the former. Therefore, based on the NMR and MS data, the viscosin-like CLP produced by COW10 could be identified as WLIP (Soler-Rivas, *et al.,* 1999).

Detailed characterization of new CLPs, N1, N2, N3, N4 and N5 are not shown in this thesis.

Furthermore, xantholysin, N2, N1, WLIP, N3, entolysin, N5, N4 and lokisin were produced by 28.9%, 17.9%, 14.9%, 11.9%, 10%, 8%, 2%, 1% and 1% of the *Pseudomonas* isolates, respectively. After assignation of CLPs, nine representative CLP-producing strains were chosen and used in drop collapse, swarming and surface tension tests. All test-isolates showed low to moderate and high swarming activity on 0.6% LB soft agar such that COR35 (N3-producer) swarmed least whereas COW10 (the WLIP-producer) swarmed most (Figure 6.6) (Table 6.2). In Table 6.2, representative producers of xantholysin, lokisin, N1, N2, N3 and N4 were able to reduce the surface tension of water from 60 mN m^{-1} to 18, 24, 21, 22, 32 and 30 mN m^{-1}, respectively. Moreover, a surface tension reduction of 19 mN m^{-1} was observed for both entolysin and WLIP producers. On the other hand, the N5 producer (COR33) barely reduced the surface tension of water with a value of 52 mN m^{-1}.

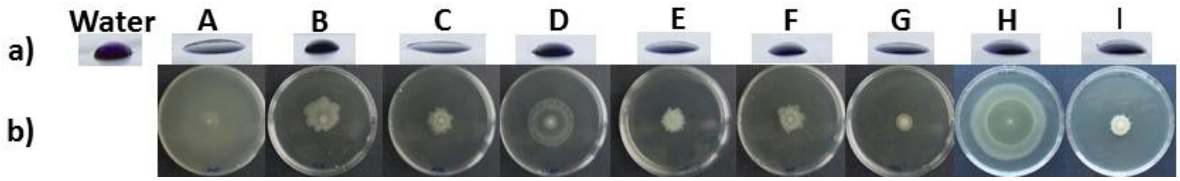

Figure 6.6. a) Drop collapse test and b) Swarming motility of representative CLP-producing isolates. Tap water was used as control for drop collapse tests. A) COW10 (WLIP); B) COR10 (lokisin); C) COR51 (xantholysin); D) COR5 (entolysin); E) COW5 (N1); F) COW8 (N2); G) COR35 (N3), H) COR58 (N4), I) COR33 (N5).

Table 6.2. Surface tension and swarming measurements for CLP-producing strains

Strain	CLP produced	Surface tension mN m^{-1} [a]	Swarming motility mm[b]
COR10	Lokisin	24 ± 1	24 ± 1
COR5	Entolysin	19 ± 1	36 ± 1
COR33	N5	52 ± 1	16 ± 1
COR35	N3	32 ± 4	12 ± 1
COR51	Xantholysin	18 ± 1	16 ± 1
COR58	N4	30 ± 1	63 ± 1
COW5	N1	21 ± 2	16 ± 0
COW8	N2	22 ± 1	20 ± 0
COW10	WLIP	19 ± 0	85 ± 0

Measurements for surface tension and swarming motility were repeated in time and averages of two independent experiments were used.
[a]Surface tension was measured from culture supernatants.
[b]Swarming motility on 0.6% LB agar was measured after incubation at 24 h.

Association of CLPs with plant variety, taxonomic groups, and sampling location

An attempt was made to assess a probable relationship between the *Pseudomonas* sp. taxonomic groups, the plant genotype (red and white) and the location from which *Pseudomonas* isolates were sampled (Figure 6.7).

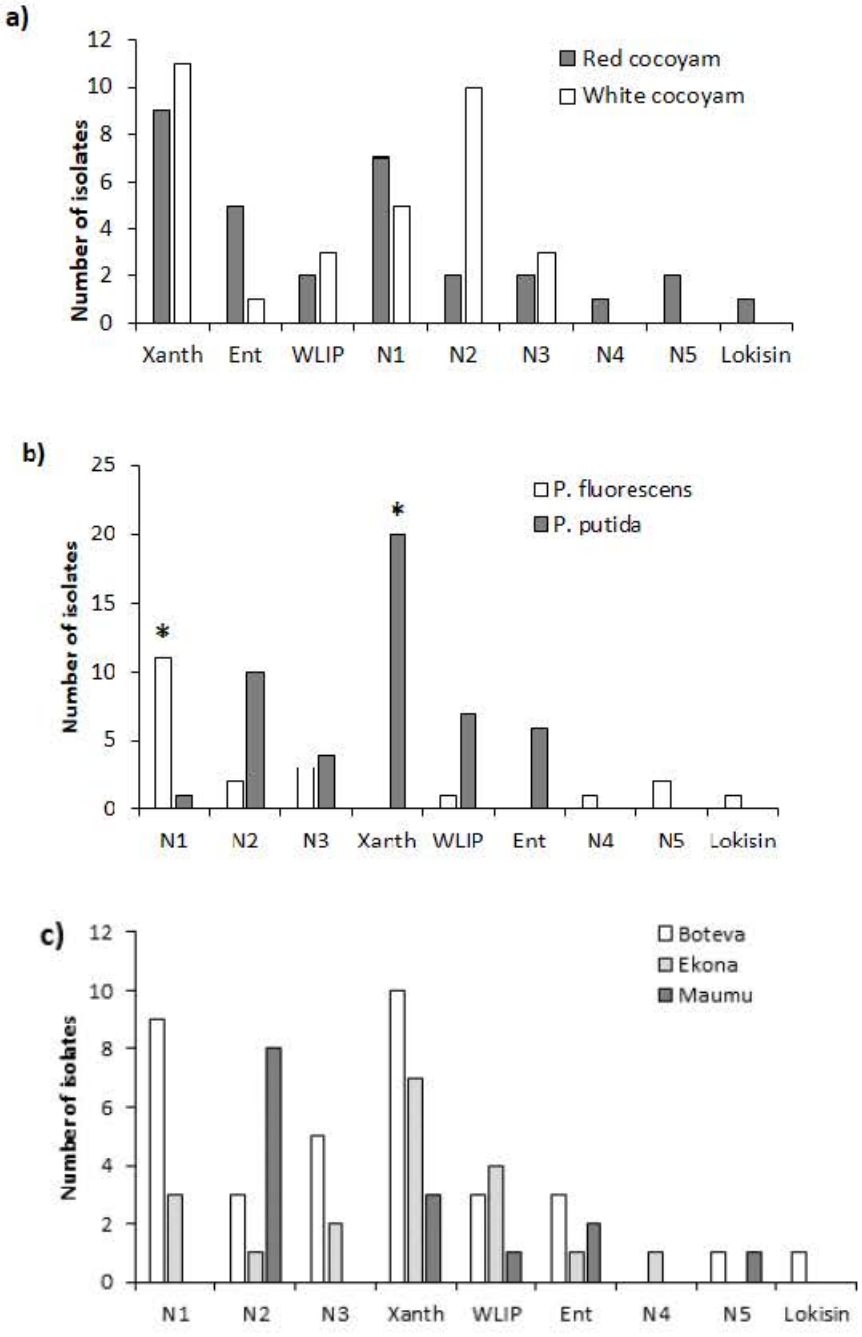

Figure 6.7. a) Comparison between red and white cocoyam test isolates producing each CLP type; **b)** Number of isolates producing each CLP type within the *P. fluorescens* complex and *P. putida* group; **c)** Number of isolates producing each CLP in all 3 locations. Bars with different letters are significantly different according to Fisher's exact statistic.

There was no significant relation recorded between plant variety and the occurrence of CLPs (Figure 6.7a). All CLPs except N4, N5 and lokisin were produced by *Pseudomonas* strains obtained from both genotypes.

A significant relation was found between taxonomic groups and CLPs produced (Figure 6.7b). Xantholysin and N1 were significantly associated ($P < 0.05$) with the *P. putida* group and *P. fluorescens* complex, respectively. At the group level however, some CLPs were exclusive to some clades while others were affiliated with more than one clade. For example, within the *P. fluorescens* complex, N1 was produced by isolates in the U3, U4 and *P. koreensis* groups whereas N3 and N2 were only produced by the *P. koreensis* group. For the *P. putida* subdivisions, a similar scenario also played out. Although the entolysins and N2 were exclusive to the A and H divisions, respectively, other CLPs such as N3 and WLIP were more dispersed between clades. (Figure 6.2) (Table S6.1).

Another interesting observation was the dispersion of some CLPs within the *P. putida* group and *P. fluorescens* complex (Figure 6.7b). In contrast with xantholysin and entolysin, CLPs such as N1, N2, N3 and WLIP were found in both taxonomic groups. Although N1 predominantly occurred in the *P. fluorescens* group, N2 and WLIP were more pronounced in the *P. putida* group than in the *P. fluorescens* complex. (Figure 6.7b).

Although no significant relationship was found between sampling location and the abundance of CLPs, there were notable enrichments at the three separate sampling sites (Figure 6.7c). All CLP types except N4 were present at Boteva with notable enrichments in N1 and N3. At Ekona, all CLPs were also present except N5 and lokisin. In contrast, Maumu soils contained five CLPs namely N2, xantholysin, WLIP, entolysin and N5 with an enrichment in N2 (Figure 6.7c).

In vitro antagonistic activity of Pseudomonas sp. CLPs against P. myriotylum

The antagonism of CLPs towards *P. myriotylum* NGR03 was assessed using microscopic glass slides covered with a thin film of water agar as previously described (Chapter 3). Application of all seven lipopeptides had very clear effect on the mycelium of *P. myriotylum* at varying concentration levels when viewed microscopically (Figure 6.8). At 10 µM, xantholysin and entolysin treatments showed increased mycelial branching whereas concentrations of 25 and 50 µM led to hyphal leakage. For both CLPs, increased hyphal branching was further observed at higher concentrations of 100 µM. Similar results were observed for WLIP except for the absence of a visible effect of CLP on the mycelium at 10 µM. Interestingly, treatments with lokisin, N1, N2 and N3 gave near similar results. At 10 µM, treatments with all four CLPs led to hyphal distortion and leakage. Increased dosage gave similar results. In contrast to treatments with xantholysin, entolysin and WLIP, increased and extensive hyphal branching was not observed with lokisin, N1, N2 and N3. Representative pictures of CLP effect on mycelium of *P. myriotylum* are shown in Figure 6.9.

Statistical analysis of percentage inhibition of *P. myriotylum* for all CLPs, revealed the absence of dose effect except for entolysin (Figure 6.8). With entolysin, there was intial inhibition at 10 µM with

significantly higher effect at 25 and 50 µM although a higher concentration showed no enhanced inhibition by the CLP.

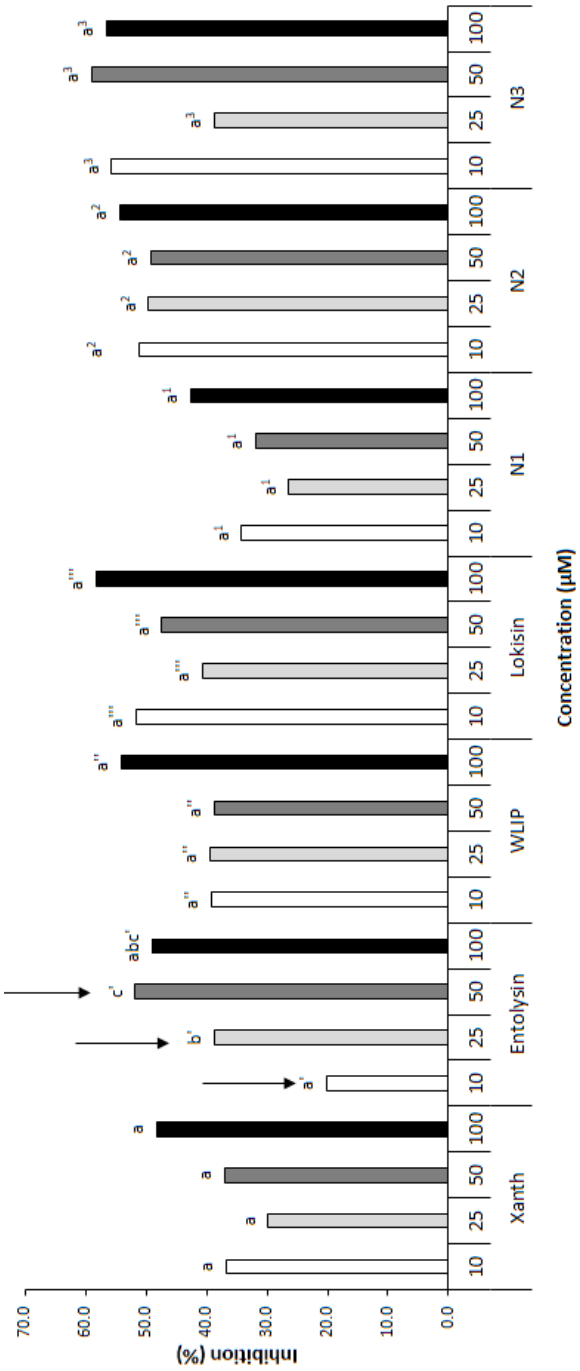

Figure 6.8. Inhibitory interaction between CLPs and *Pythium myriotylum* at concentrations ranging from 10 to 100 µM. Values plotted are indicative of the percentage growth inhibition of *P. myriotylum* relative to the control. xanth: xantholysin. For each CLP, statistics was conducted to compare inhibitory capacity for different concentrations. Bars with different letters are significant different according to the Tukey's tests (P = 0.05).

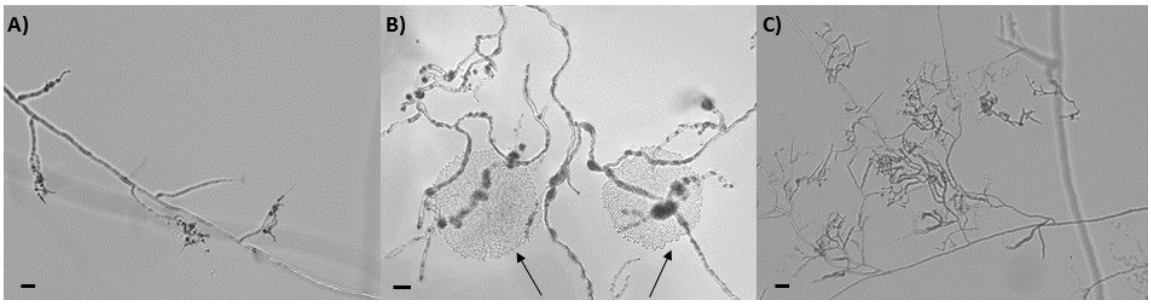

Figure 6.9. Cell damage microscopic analysis between CLPs and *Pythium myriotylum*. A) Control: *P. myriotylum*; **B)** hyphal lysis; **C)** extensive hyphal branching.

Pythium root rot suppression by CLP-producing Pseudomonas spp.

In order to assess the CLP-producing isolates for antagonism against *P. myriotylum* on cocoyams, nine representative *Pseudomonas* isolates, each producing a different CLP, were tested. The results of the biocontrol experiments showed that all nine treatments provided significant (p = 0.05) suppression of *Pythium* root rot on cocoyams compared to that of the untreated control based on calculation of percentage disease index (Figure 6.10).

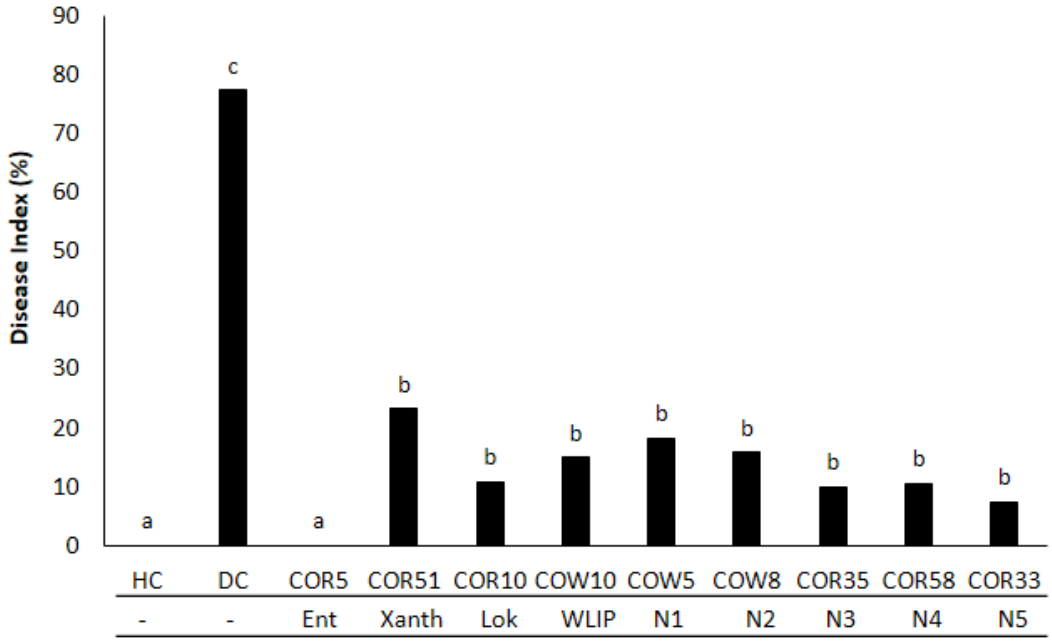

Figure 6.10. Evaluation of CLP-producing *Pseudomonas* isolates for suppression of CRRD in two independent experiments. Figure represents pooled data from two experiments. HC: healthy control**;** DC: Diseased control. The table below the figure indicates the CLP produced by the various strains, Ent: entolysins; Xanth: xantholysin; Lok: lokisin. Bars with different letters indicate statistical difference according to non-parametric Kruskal-Wallis and Mann-Whitney tests.

Interestingly, 100% pathogen control was provided by COR5, the entolysin producer. All other CLP-representative strains also performed excellently in the control of *P. myriotylum* on cocoyams. Furthermore, all strains tested were well established in their capacity to colonize cocoyam roots (Table 6.3).

Table 6.3. Root colonisation by CLP-producing *Pseudomonas* spp. obtained from the cocoyam rhizosphere after seven days of inoculation with *P. myriotylum* NGR03.

Treatment	CLP produced	Population density of test bacteria (in log CFU g^{-1} of fresh roots)
COW5	N1	8.19 ± 0.3 bc
COW8	N2	8.44 ± 0.2 cd
COR51	Xantholysin	8.28 ± 0.3 bc
COR5	Entolysin	7.96 ± 0.3 ab
COR35	N3	7.83 ± 0.1 a
COR10	Lokisin	8.18 ± 0.2 b
COR58	N4	7.63 ± 0.4 a
COR33	N5	7.18 ± 0.4 a
COW10	WLIP	8.12 ± 0.3 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).

Although the best colonizer was COW8, the N2 producer, its population density did not significantly differ from treatments with N1 and xantholysin-producing isolates. Colonizing competence was further shown by WLIP- and lokisin-producing isolates whereas COR33, the N5-producing isolate appeared to colonize least.

Discussion

Myriads of biotic and abiotic factors are considered to be influencers of structural and functional diversity of microbial communities in the rhizosphere (Berg *et al.*, 2005; Berg and Smalla, 2009; Hartmann *et al.*, 2009; Berg *et al.*, 2014). One of such factors, plant species type, tends to drive selection via root morphology and exudation thereby determining microbial selection. On the other hand, contrasting soil types are assumed to accommodate specific microbial communities (Fierer and Jackson, 2006). Perneel *et al.,* 2006 suggested that the red cocoyam genotype selects for specific fluorescent pseudomonad populations which could contribute to its field tolerance towards the *Pythium* root rot disease. Although only 40 isolates were tested in this previous study, antagonism against *P. myriotylum* was only shown by the red cocoyam rhizosphere isolates.

In our present study, taxonomic characterization of 137 fluorescent pseudomonads obtained from the rhizosphere of red and white cocoyams varieties in three different andosols of Cameroon, showed a clustering of our isolates mainly within two *Pseudomonas* taxonomic divisions namely the *P. fluorescens* complex and *P. putida* group. Based on the phylogenetic tree generated, eight groups were identified within the *P. fluorescens* complex (*P. koreensis*, *P. jessenii*, *P. fluorescens, P. corrugata,* U1, U2, U3 and U4) whereas, for the *P. putida* group, several subdivisions were made in order to facilitate the description of the relationships observed. Except for the clustering of some white cocoyam isolates within the *P. corrugata* and U2 groups and a few red cocoyam isolates into the U4 and U5 groups, the taxonomic affiliations of our isolates did not differ with variety type suggesting that the diversity of fluorescent pseudomonads associated with the cocoyam rhizosphere is not dependent on plant variety. Thus, this disproves the suggestion of Perneel *et al.* (2007).

Previous studies to assess the diversity of fluorescent pseudomonads on the rhizosphere of several crops revealed the presence of isolates characterized as belonging to the *P. fluorescens* biovars on sugar beet rhizosphere (Nielsen *et al.*, 2002), *P. putida* group isolates on black pepper (Tran *et al.*, 2008) and *P. fluorescens*-related isolates on oilseed rape and strawberry (Berg and Smalla, 2009). For two separate studies, using culture-independent methods, the genetic variants of more than eight *Arabidopsis thaliana* accessions were shown to select for different rhizobacterial assemblages (Micallef *et al.*, 2009a; Micallef *et al.*, 2009b).

Although there was no delineation according to plant variety, clear differences in diversity of fluorescent pseudomonads was observed between locations. Isolates obtained from Boteva had significantly higher abundances in the *P. fluorescens* complex and at the group level, were significantly enriched in the *P. koreensis* group. In our study, low disease pressure soils of Boteva appeared to have a mix of both *P. putida* group (48%) and *P. fluorescens* complex (51%) group isolates with significantly higher representation of *P. fluorescens* compared to the other soils. Intermediate soils of Ekona had a mix of both taxonomic groups although with an enrichment in the *P. putida* group (88%) whereas the high disease pressure-Maumu soils had a significantly higher population of *P. putida* isolates compared to the soils of Ekona. Although soils in all three locations are

andosols, the microbial diversity and selectiveness for isolates associated with the *P. fluorescens* complex at Boteva is intriguing. In a previous study, comparison of physicochemical soil properties between andosols and ferralsols of Cameroon revealed that in andosols, organic matter and minerals including Ca, K, Mg and N were present in higher amounts, which correlated negatively with CRRD severity (Adiobo *et al.,* 2007). Given that andosols of Boteva and Ekona were included in the afore-mentioned study, the analysis of Maumu soil which was conducted during this study enabled a comparison of physico-chemical properties among three andosols with different CRRD pressures. We hypothesize that the microbial diversity and selectiveness for isolates associated with the *P. fluorescens* complex at Boteva could be connected with the significantly higher organic matter, calcium and total nitrogen which is typical for Boteva soil. Pseudomonads are accountable for the natural suppressiveness of some soils to soil borne pathogens (Weller *et al.*, 2002). Biological control strains are commonly found in the *P. fluorescens* lineage and isolates associated with the *P. fluorescens* complex produce a remarkable spectrum of secondary metabolites with implications for the biocontrol of plant pathogens (Garrido-Sanz *et al.,* 2016). Different soil types characterized by different physico-chemical properties were shown to harbor different microbial communities in the rhizosphere (Schreiter *et al.*, 2014). Depending of several factors such as plant nutritional status, soil properties and plant genotype, plant roots have the ability to influence its surrounding microbiology through the creation of specific chemical niches in the soil mediated by the release of root exudates (Pii *et al.,* 2016). More so, it has been hypothesized that on the basis of qualitative and quantitative exudation profiles, plants might selectively modulate the growth of microorganisms by favoring those which exhibit beneficial traits for plants growth and health (Smalla *et al.,* 2001; Germida and Siciliano, 2001). We hypothesize that soils that are richer in organic matter enhance soil nutrient status which in turn will impact the root exudation profile of the crop and the recruitment of specific *Pseudomonas* spp. with beneficial traits. An elaborate study will be needed to investigate this association in more detail.

Intriguingly, approximately 50-60% of fluorescent pseudomonads isolated from two locations, Boteva and Ekona, produced CLPs in contrast with 35% from Maumu. Comparable frequencies of up to 60% CLP-producing pseudomonads comprising eight structurally different CLPs were isolated from the sugar beet rhizosphere in Denmark although this could go lower depending on soil type (Nielsen *et al.*, 2002). Such high frequencies of CLPs in Danish soils were attributed to the loamy sandy nature of the soils. However, in our study, this does not seem to be the case since unlike Ekona and Maumu soils, Boteva soils predominantly contain silt. An understanding of factors driving CLP diversity remains intriguing and requires further research.

Furthermore, we observed that some of our surfactants were rare, some moderately present and the others were abundant. Lokisin and N4 were produced by only one strain, COR10 and COR58, respectively whereas two N5 producers were found. More so, six isolates produced entolysin. All representative CLP-producers swarmed on soft LB agar. Several CLPs have been demonstrated to play a role in the swarming motility of *Pseudomonas* bacteria, a vital characteristic which facilitates surface colonization for the producing strain (Raaijmakers *et al.,* 2006). It remains to be seen if these

CLPs are directly involved in the swarming capacity of their producing strain. Moderately present CLPs were WLIP, N1 and N3 whereas N2 and xantholysin were abundant. The production of xantholysin has been reported in a banana rhizosphere isolate, *P. putida* BW11M1 (Li *et al.*, 2013) and in *P. soli*, isolated from a soil sample collected from the Sierra Nevada National Park, Granada, Spain (Pascual *et al.*, 2014). So far, entolysin biosynthesis has only been reported for one strain, *P. entomophilia* L48^{T} (Vallet-Gely *et al.*, 2010) whereas WLIP production has been reported for several *Pseudomonas* isolates belonging to the *P. putida* and *P. fluorescens* groups (Rokni-Zadeh *et al.*, 2012; Rokni-Zadeh *et al.*, 2013). Lokisin-producing isolates were previously described from the sugar beet rhizosphere in Danish soils (Nielsen *et al.*, 2002), with antagonistic activity against *R. solani* and *Pythium ultimum*. More so, another lokisin producer was previously isolated from a soilless system in Sweden (Hultberg *et al.,* 2010) and showed antagonism agaist *P. ultimum* on tomato.

It was of notable interest that all our isolates in the *P. koreensis* group had close relatedness to the *P. fluorescens* Pf0-1 strain, a soil isolate from the United States. Interestingly, a *gacA*$^{+}$ gene derivative of Pf0-1 produces an unknown CLP (Loper *et al.*, 2012). In our study, the *P. koreensis* group isolates produce lokisin and N1 while some isolates (39%) are non-CLP producers. Not only are three of the new CLPs produced by isolates of the *P. koreensis* group, they are also produced by representative isolates within the *P. putida* group. In a previous study, WLIP genes were identified in strains found affiliated with both the *P. fluorescens* complex and *P. putida* group (Rokni-Zadeh *et al.*, 2012). It was further shown that distinct non-ribosomal peptide synthase (NRPS) systems could produce similar secondary metabolite with the same phenotype whereas they belong to phylogenetically different hosts. It remains to be seen whether or not the genomes of these three new CLPs possess similar or distinct gene clusters while belonging to different taxonomic hosts.

Such enormous diversity and abundance of CLP producers observed in our study, point to crucial ecological roles of these metabolites in their native soils. Worthy of note are the low disease pressure soils of Boteva that possess remarkable species diversity and significant enrichments of CLPs in combination with a high organic matter content. Our data indicates that the CLPs interact with the mycelium of *P. myriotylum* by causing hyphal leakage or extensive branching. Whether the difference in mode of action of the various CLPs is related to the structural differences between them still remains to be investigated. Since all CLPs showed effective antagonism against *P. myriotylum* mycelium during *in vitro* tests and representative CLP-producing isolates further displayed excellent suppression of the cocoyam root rot disease *in vivo*, the abundance of these CLPs in soil suggests that they may contribute to disease suppression towards *P. myriotylum* especially in Boteva soils. Perneel *et al.*, 2008 previously demonstrated a synergistic interaction between phenazines and rhamnolipid biosurfactants in the suppression of *P. myriotylum* on cocoyams. In the future, there is need not only to quantify CLP production *in vivo* but also to assess the direct interaction of the various CLPs with *P. myriotylum* in soils by the use of pure compounds or CLP mutant strains.

In summary, the application of culture-dependent methods to dissect the taxonomic and CLP diversity of fluorescent pseudomonads on white and red cocoyams rhizosphere clearly highlight that

soil and not plant variety select the microbial diversity of this crop. More so, the diversity of CLPs declined with increasing disease pressure from Boteva to Ekona and finally, Maumu.

Materials and Methods

Pseudomonas strains

Cocoyam fields with red and white varieties established on andosols in the South-west province of Cameroon were sampled. Sampled fields were situated in the villages of Boteva, Ekona and Maumu which possess low, intermediate and high disease pressures towards *P. myriotylum*, respectively. This region is a tropical rainforest with precipitation of up to 3000 mm/annum. The physico-chemical properties of Ekona and Boteva soils are as described by Adiobo *et al.*, (2007) while that of Maumu was analyzed during this study. To isolate fluorescent *Pseudomonas*, cocoyam roots obtained from two to three and a half month-old red and white cocoyam plants were crushed in 0.85% sterile saline solution and autoclaved sand after which serial dilutions were made and plated on King's B agar (King *et al.,* 1954) medium plates. Plates were incubated at 28°C for 48 h following which one hundred and thirty-seven fluorescent *Pseudomonas* isolates were selected under UV light, purified and stored at -80°C in 40% glycerol. Sixty-one isolates were obtained from Boteva, thirty-three from Ekona and forty-seven from Maumu. *Pseudomonas* strains used in this study are indicated in Table S7.1. Additional strains used were type strains and common biocontrol isolates of *Pseudomonas* species (Table S7.2). The isolates obtained from the red and white cocoyams were designated with COR- and COW- prefixes, respectively.

DNA sequence-based phylogenetic analyses

Two housekeeping gene regions, *rpoD* and *rpoB* were analysed for bacterial strains used in this study. For the *rpoD* gene, primers PsEG30F/PsEG790R were used as described by Mulet *et al.,* 2009 whereas *rpoB* gene was used as described by (Frapolli *et al.*, 2007). *rpoB* primers produced a single amplicon with the *Pseudomonas* strains tested. For *rpoD*, some isolates did not give bands thus new primers *rpoD1*F/*rpoD*1R were designed in this study. Primers used in this study are listed in the general primer list.

Sequences of representative type strains and genomes within the *Pseudomonas* genus together with well-studied biocontrol isolates were retrieved from the GenBank. Sequence alignment was carried out using Muscle (Edgar, 2004) via the software package MEGA6 (Tamura *et al.*, 2013). The tree was inferred by Neighbour joining and confidence analysis was ensured using 1000 bootstrap replicates with *P. aeruginosa* as the outgroup. Individual *rpoB* and *rpoD* trees were initially generated after which a concatenated tree combining the aligned sequences of both genes was carried out using the same method.

Physicochemical characterisation of Maumu soil

As was previously done for Boteva and Ekona fields (Adiobo *et al.,* 2007), soil cores (20 cm depth) were taken randomly at diverse points of Maumu field and were composited. A subsample of 500 g, made from the composite soil sample was separately transferred into clean plastic bags and taken to the laboratory and air-dried in preparation for physical and chemical analysis.

The pH-H_2O was measured in a 1:5 soil: demineralized water extract that was stirred with a glass rod, allowed to stand for 18 hours, stirred again and then measured potentiometrically (Thermo Orion 420A+ pH electrode). Total soil carbon and nitrogen contents were measured by dry combustion following the Dumas method with an elemental analyser (LECO Trumac CNS analyser, LECO Instrumente GmbH). Because the pH was lower than 7, free carbonates were assumed not to be present and total C was equated to organic C. Exchangeable base cations (Ca, K, Mg, Na) and P supplying capacity were measured following an extraction of the soil with ammonium lactate (pH 3.75) (extraction ratio 1:20) in dark polyethylene bottles that were shaken for 4 h on a rotational shaker and filtration on a Machary Nagel 640w filter. The cation and P concentrations in the filtered extract were measured with ICP-OES (Thermo ICAP 6300 radial). Soil texture was determined by the combined sieve and pipette method according to Gee and Bauder (1986).

Characterization of surfactant-producing Pseudomonas isolates

The initial screening step for biosurfactant production for all one hundred and thirty-seven isolates was by the use of the drop collapse assay. All isolates were cultured in 3 ml Luria Bertani (LB) broth at 28°C, 150 rpm overnight. 50 µl of the cell culture for each strain was dropped on parafilm with the same volume of tap water as control. Strains with cultures that collapsed on parafilm produce biosurfactants. Furthermore, the growth of all strains was assessed at 37 °C, in order to exclude clinical isolates. Thus, two *P. aeruginosa* isolates were identified and excluded from further biosurfactant analysis.

Swarming motility of representative biosurfactant-producing isolates, collected from both red and white cocoyam varieties, was tested on 0.6% LB agar as described in Chapter 4 while surface tension measurements was conducted using a tensiometer, as described by D'aes *et al.,* 2011. For surface tension tests, bacterial strains were grown on KB medium for 72 h, after which cells were scraped from the plates and suspended in sterile distilled water. Cell concentrations were estimated by measuring optical density (OD620). After centrifugation of the suspensions, surface tension of cell-free supernatants was measured using a tensiometer equipped with a Wilhelmy plate.

Structural diversity of Pseudomonas sp. surfactants

To characterize the *Pseudomonas* sp. surfactants by high-pressure liquid chromatography (HPLC), all isolates were cultured in 5 ml of KB broth under shaking conditions at 28°C for 24 h after which cultures were centrifuged at 10,000x g for 10 min. Filter sterilized supernatants were dissolved in methanol. Analytical LC-MS data of the various compounds were collected on an 1100 Series HPLC with an type VL ESI detector (Agilent Technologies) equipped with an analytical Luna C18 (2)

reversed-phase column (250x4.6 mm, 5 µm particle size; Phenomenex, Torrance, CA). An elution gradient of H_2O/CH_3CN (100:0 to 0:100 over 20 min was applied at a flow rate of 1 mL min^{-1}. For final purification, samples were injected into a Prostar HPLC device (Agilent Technologies) equipped with a Luna C-18(2) preparative RP-HPLC column (250x21.2 mm, 5 µm µm particle size) for separation of the individual CLP analogues. An elution gradient of H_2O/CH_3CN (25:75 to 0:100) was applied at a flow rate of 17.5 mL min^{-1}, while the column temperature was kept at 35°C. To ensure an optimal separation of the peptides, a 20 minute gradient was used for WLIP and lokisin, while a 15 or 25 minute gradient was used for entolysin and xantholysin, respectively.

NMR measurements were performed on a Bruker Avance III spectrometer operating at ^{1}H and ^{13}C frequencies of 500.13 and 125.76 MHz and equipped with a BBI-Z probe. The sample temperature was set to either 298.0 K or 328.0 K as indicated. High precision 5 mm NMR tubes (Norell, Landisville, NJ) were used. [D3]Acetonitrile (99.96 %), and [D7]dimethylformamide (DMF) (99.50%) were purchased from Eurisotop (Saint- Aubin, France). ^{1}H and ^{13}C chemical shift scales were calibrated by using the residual solvent signal. 2D spectra measured for structure elucidation included a 2D ^{1}H-^{1}H TOCSY with a 90 ms MLEV-17 spinlock, 2D ^{1}H-^{1}H NOESY and off resonance ^{1}H-^{1}H ROESY with 200 mixing times, and gradient- selected ^{1}H-^{13}C gHSQC and gHMBC. Standard pulse sequences as present in the Bruker library were used throughout. Typically, 2048 data points were sampled in the direct dimension for 512 data points in the indirect dimension, with the spectral width set to 11 and 110 ppm along the ^{1}H and ^{13}C dimensions, respectively. The ^{1}H-^{13}C HMBC spectra were measured with a 200 ppm ^{13}C spectral width. For 2D processing, the spectra were zero filled to a 2048x2048 real data matrix. Before Fourier transformation, all spectra were multiplied with a squared cosine bell function in both dimensions or a sine bell in the direct dimension for gHMBC.

Antagonistic activity of Pseudomonas sp. CLPs

The antagonistic activity of *Pseudomonas* sp. CLPs against *P. myriotylum* was conducted under *in vitro* microscopic conditions as described in Chapter 2. Concentrations of CLPs tested ranged from 10 to 100 µM for four replicates each while controls were included with water as treatment. Plates were incubated at 28°C and slides were evaluated after 4 days using an Olympus BX51 microscope. Growth inhibition effect of purified CLPs towards *P. myriotylum* was expressed relative to the mycelial growth of the control. After testing for normality, data obtained from percentage inhibition were analysed using the t-test (SPSS).

Biocontrol assays

To assess the ability of CLP-producing *Pseudomonas* isolates to suppress *Pythium* root rot caused by *P. myriotylum* on cocoyams, plant experiments were conducted in an unsterilized potting soil (Structural; Snebbout, Kaprijke, Belgium) and sand mixture in a 70/30 ratio. *Pseudomonas* spp. COR51, COR5, COW10, COR10, COW5, COW8, COR33, COR35 and COR58 which produce nine representative CLPs, were used. Bacterial inoculum preparation, plant material propagation and the whole experimental set-up, scoring for disease and root colonization was conducted in a similar manner as was done in Chapter 4.

Data analysis

Data obtained from plant experiments were analysed as described in Chapter 3.

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Supplementary files

Table S6.1. *Pseudomonas* isolates obtained from red and white cocoyam rhizosphere from three different locations in Cameroon showing soil characteristics, taxonomic affiliation and CLP types of strains.

***Pseudomonas* (sub) group/lineage (Garrido Sanz *et al*., 2016)**	**Closest related species**	**Strain**	**Origin**	**Cocoyam variety**	**Disease Pressure**	**CLP**
fluorescens group	*P. libanensis* CIP 105460T	COW59	Boteva	white	low	none
		COR38	Boteva	red	low	none
U1 category	*P. corrugata* LMG 2172T	COW41	Ekona	white	intermediate	none
		COW60	Ekona	white	intermediate	none
corrugata group	*P. corrugata* LMG 2172T	COR7	Boteva	red	low	none
		COW61	Ekona	white	intermediate	none
jessenii group	*P. jesseni* CIP 105474T	COW6	Boteva	white	low	none
U2 category	*P. umsongensis*LMG 21317T	COR3	Boteva	red	low	none
		COR26	Ekona	red	intermediate	none
		COR27	Ekona	red	intermediate	none
koreensis group	*P. moraviensis* DSM 16007T	COR43	Boteva	red	Low	N1
		COW52	Boteva	white	Low	N1
		COW49	Boteva	white	Low	N1
		COW57	Boteva	white	Low	none
		COW71	Boteva	white	Low	N1
		COW74	Boteva	white	Low	N1
		COR44	Boteva	red	Low	none
		COR45	Boteva	red	Low	none
		COR46	Boteva	red	Low	N1
		COR50	Boteva	red	Low	none
		COR61	Boteva	red	Low	none
		COW9	Boteva	white	Low	N2
		COW69	Boteva	white	Low	none
		COW67	Boteva	white	Low	none
		COR36	Boteva	red	Low	N1
		COW5	Boteva	white	Low	N1
		COR57	Boteva	red	Low	none
	P. koreensis LMG 21318T	COR10	Boteva	red	Low	Lokisin
	P. sp. GM80	COW8	Boteva	white	Low	N2
	P. sp. Pf0-1	COW65	Boteva	white	Low	N3

		COW66	Boteva	white	Low	none
		COW3	Boteva	white	Low	N3
		COW7	Boteva	white	Low	N3
		COR31	Boteva	red	Low	none
		COW48	Boteva	white	Low	none
U3 category	*P. fuscovaginae* LMG 2158T	COR24	Ekona	red	intermediate	N1
		COR25	Ekona	red	intermediate	N1
		COR58	Ekona	red	intermediate	N4
ungrouped	*P. moraviensis* DSM 16007T	COW56	Maumu	white	High	none
U4 category	*P. fuscovaginae* LMG 2158T	COR48	Boteva	red	Low	N1
		COR33	Boteva	red	Low	N5
		COR52	Boteva	red	Low	WLIP
		COR18	Maumu	red	high	N5
putida group		COR6	Boteva	red	low	entolysin
A subgroup	*P. entomophilia* L48T	COR5	Boteva	red	low	entolysin
		COR9a	Boteva	red	low	none
		COR9b	Boteva	red	low	entolysin
		COW47	Ekona	white	intermediate	entolysin
		COR16	Maumu	red	high	entolysin
		COR17	Maumu	red	high	entolysin
		COW30	Maumu	white	high	none
		COW37	Boteva	red	Low	xantholysin
		COR20	Maumu	red	high	none
		COW31a	Maumu	white	high	none
		COW27	Maumu	white	high	none
		COW25	Maumu	white	high	none
		COR14	Maumu	red	high	none
		COR15	Maumu	red	high	none
B subgroup	*P. mosselii* ATCC BAA-99T	COH1	Ekona	hybrid	intermediate	N3
		COH2	Ekona	hybrid	intermediate	N3
C subgroup	*P. mosselii* ATCC BAA-99T	COR35	Boteva	red	low	N3
		COR40	Boteva	red	low	N3
D subgroup	*P. mosselii* ATCC BAA-99T	COW53	Boteva	white	low	xantholysin
		COR42	Boteva	red	low	xantholysin
		COR59	Boteva	red	low	xantholysin
		COR56	Boteva	red	low	xantholysin
		COW75	Boteva	white	low	xantholysin

		COW63	Boteva	white	low	xantholysin
		COR51	Ekona	red	intermediate	xantholysin
		COR22	Ekona	red	intermediate	xantholysin
		COW72	Boteva	white	low	xantholysin
		COW64	Boteva	white	low	none
		COW68	Maumu	white	high	xantholysin
		COR49	Boteva	red	low	xantholysin
		COW76	Boteva	white	low	xantholysin
		COR37	Maumu	white	high	none
		COR21	Maumu	red	high	xantholysin
E subgroup	*P. mosselii* ATCC BAA-99T	COW38	Ekona	white	intermediate	xantholysin
		COW39	Ekona	white	intermediate	xantholysin
		COW42	Ekona	white	intermediate	xantholysin
		COW43	Ekona	white	intermediate	xantholysin
F subgroup	*P. soli* F-279,208T	COR23	Ekona	red	intermediate	xantholysin
		COW77	Maumu	white	high	xantholysin
G subgroup	*P. taiwanensis* DSM 21245T	COW40	Ekona	white	intermediate	WLIP
		COR54	Ekona	red	intermediate	WLIP
H subgroup	*P. taiwanensis* DSM 21245T	COW73	Boteva	white	low	N2
		COR30	Ekona	red	intermediate	N2
		COW62	Maumu	white	high	N2
		COR55	Ekona	red	intermediate	N1
		COR1	Maumu	red	high	none
		COR19	Maumu	red	high	N2
		COW17	Maumu	white	high	N2
		COW18	Maumu	white	high	N2
		COW22	Maumu	white	high	N2
		COW23	Maumu	white	high	N2
		COW29	Maumu	white	high	N2
		COW32	Maumu	white	high	N2
I subgroup	*P. putida* KT2440	COW36	Maumu	white	high	none
	P. monteilli DSM 14165T	COW44	Ekona	white	intermediate	none
		COW28	Maumu	white	high	none
		COW34	Maumu	white	high	none
		COR12	Boteva	red	Low	none
		COR39	Maumu	red	high	none
		COR32	Boteva	red	low	none

		COW2	Maumu	white	high	none
		COW1	Maumu	white	high	none
		COR13	Boteva	red	low	none
		COR4	Boteva	red	low	none
		COR11a	Boteva	red	low	none
		COR11b	Boteva	red	low	none
		COR2	Maumu	red	high	none
		COW14	Maumu	white	high	none
		COW16	Maumu	white	high	none
		COW20	Maumu	white	high	none
		COR60	Ekona	red	intermediate	none
		COW12	Maumu	white	high	none
		COW33	Maumu	white	high	none
		COW19	Maumu	white	high	none
		COW45	Ekona	white	intermediate	none
		COW26	Maumu	white	high	none
		COW21	Maumu	white	high	none
		COW35	Maumu	white	high	none
		COW24	Maumu	white	high	none
		COR29	Ekona	red	intermediate	none
ungrouped	*P. monteilli* DSM 14165T	COR28	Ekona	red	intermediate	none
		COW15	Maumu	white	high	none
J subgroup	*P. monteilli* DSM 14165T	COW4	Boteva	white	low	WLIP
		COR8	Boteva	red	low	WLIP
		COW10	Maumu	white	high	WLIP
		COH3	Ekona	hybrid	intermediate	WLIP
		COH4	Ekona	hybrid	intermediate	WLIP
K subgroup	*P. japonica* JCM 21532T	COW70	Boteva	white	low	none
		COW46	Ekona	white	intermediate	none
		COW13	Maumu	white	high	none
aeruginosa	*P. otitidis* DSM 17224T	COW54	Boteva	white	low	none
		COW51	Boteva	white	low	none
		COW58	Boteva	white	low	none
	P. aeruginosa LMG 1242T	COR34	Maumu	red	high	n/a
		COR41	Maumu	red	high	n/a

*n/a: does not produce cyclic lipopeptides.

Table S6.2. List of type and biocontrol strains used in study including genbank accession number and references.

		GenBank accession number		
Species	**ID code**	**rpoB**	**rpoD**	**Reference**
P. protegens	Pf-5	DQ458648	DQ458678	Ramette *et al.*,2011
P. fluorescens	Pf0-1	CP000094	CP000094	Silby *et al*., 2009
P. fluorescens	SBW25	NC_012660	NC_012660	Silby *et al.*, 2009
Pseudomonas sp.	CMR12a	FJ652703	Rast seed	Mavrodi *et al.*, 2010
P. chlororaphis subsp. aureofaciens	30-84	CM001559	CM001559	Loper *et al.*, 2012
P. chlororaphis subsp. aureofaciens	LMG 1245T	AJ717426	FN554453	Tayeb *et al*., 2005; Mulet *et al.,* 2010
P. brassicacearum	CIP 107059	AJ717436	AM084334	Ramette *et al.,* 2011
P. corrugata	LMG 2172T	AJ717487	AB039566	Tayeb *et al*., 2005; Yamamoto *et al.*, 2000
P. fluorescens	LMG 5167	AJ748158	D86033	Tayeb *et al.,* 2005
P. moraviensis	DSM 16007T	FN554743	FN554490	Mulet *et al.*, 2010
P. fuscovaginae	LMG 2158	AJ717433	n/a	Tayeb *et al.,* 2005
P. asplenii	LMG 2137T	AJ717432	AB039593	Ramette *et al.,* 2011
P. mosselii	ATCC BAA-99T	FN554744	FN554491	Mulet *et al.*, 2010
P. parafulva	DSM 17004T	AJ717471	FN554500	Tayeb *et al.,* 2005; Mulet *et al.*, 2010
P. putida	LMG 2257T	AJ717474	AB039581	Ramette *et al.,* 2011
P. putida	KT2440	NC_002947	NC_002947	Nelson *et al.,* 2002
P. fulva	CIP 106765	AJ717419	AB039586	Tayeb *et al.,* 2005
P. koreensis	LMG 21318T	FN554737	FN554476	Mulet *et al.*, 2010
Pseudomonas sp.	UW4	NC_019670	NC_019670	Duan *et al.,* 2013
P. jessenii	CIP 105274T	AJ717447	FN554473	Tayeb *et al.*, 2005; Mulet *et al.*, 2010
P. arsenicoxydans	CECT 7543T	HE800503	HE800488	Mulet *et al.*, 2012
P. entomophilia	L48T	CT573326	CT573326	Vodovar *et al.*, 2006
P. plecoglossicida	CIP 106493T	AJ717456	FN554503	Tayeb *et al.*, 2005; Mulet *et al.*, 2010
P. taiwanensis	DSM 21245T	HE577797	HE577796	Mulet *et al.,* 2012
P. japonica	JCM 21532T	HE577800	HE577795	Mulet *et al.*, 2012
P. monteilli	DSM 14164T	NZ_BBIS01000077	FN554488	Unpublished; Mulet *et al.,* 2010
P. lini	CIP 107460T	AJ717466	FN554478	Tayeb *et al.*, 2005

P. otitidis	DSM 17224T	FN554745	FN554495	Mulet *et al.,* 2010
P. umsongensis	LMG 21317T	FN554763	FN554516	Mulet *et al.,* 2010
P. thivervalensis	CFBP 11261T	AM084680	AM084338	Cladera *et al.,* 2006
P. fluorescens	SS101	CM001513	CM001513	Loper *et al.,* 2012
P. simiae	CCUG 50988T	FN554757	FN554513	Mulet *et al.,* 2010
P. tolaasii	LMG 2342T	AJ717467	FN645158	Tayeb *et al.*, 2005; Campos *et al.*, 2013
P. gessardii	CIP 105469T	AJ717438	FN554468	Tayeb *et al.,* 2005; Mulet *et al.,* 2010
P. aeruginosa	LMG 1242T	AJ717442	AB039611	Tayeb *et al.,* 2005; Yamamoto *et al.,* 2000
P. resinovorans	LMG 2274T	AJ717479	FN554509	Tayeb *et al.*, 2005; Mulet *et al.,* 2010

*n/a: not present in Genbank.

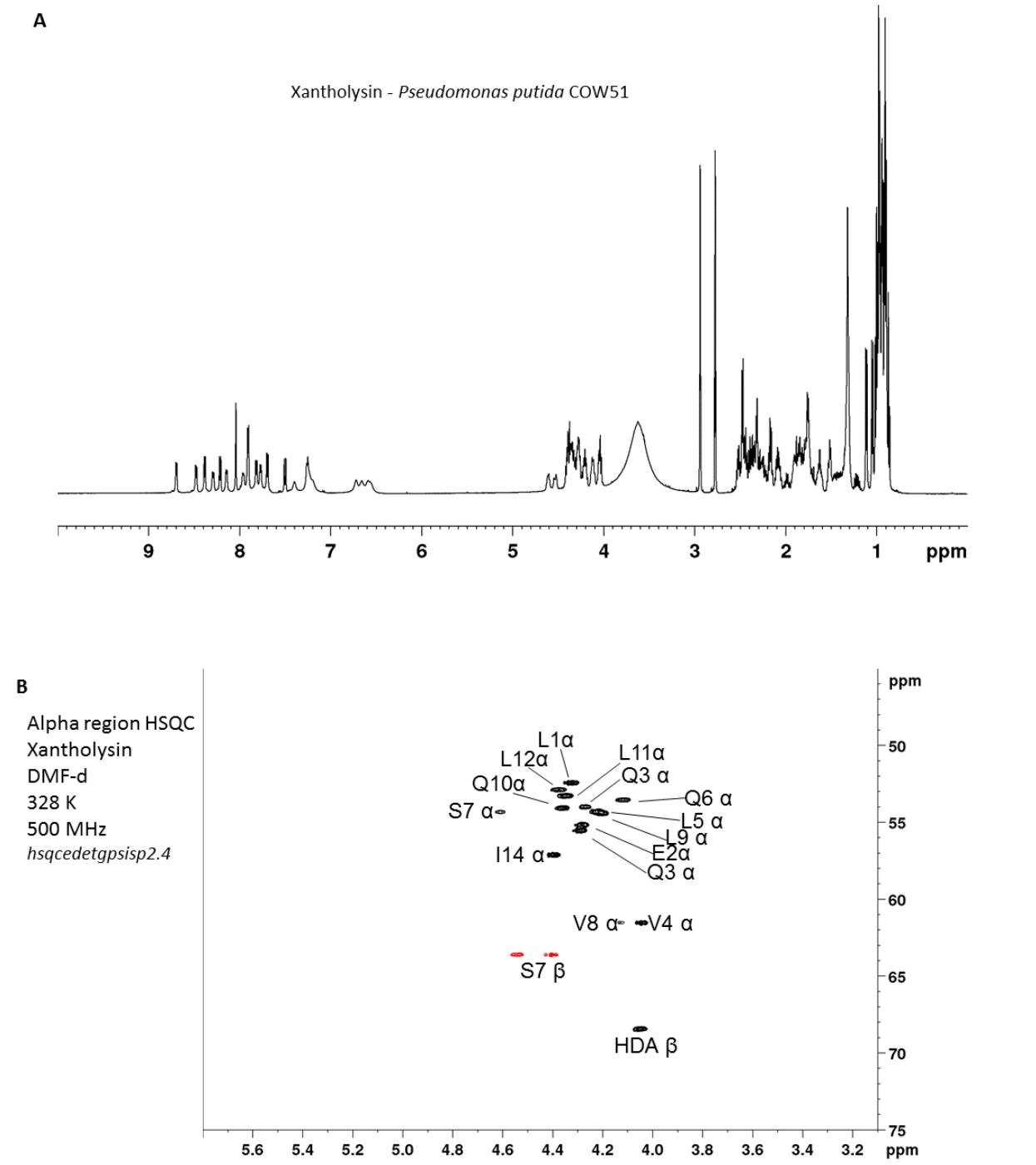

Figure S6.1. 1D ^{1}H NMR **(A)** and ^{1}H-^{13}C gHSQC NMR spectrum **(B)** of xantholysin extracted from *P. putida* COW51 (DMF-d7, 328K, 500MHz).

Table S6.3. Chemical shifts of xantholysin in DMF-d7 (328 K, 500 MHz)

			1H δ [ppm]	13C δ [ppm]
HDA				
		CO	n.d.	n.d.
		$CH_2α$	2.45	43.59
		CHβ	4.02	68.33
		$CH_2γ$	1.49	37.41
		$CH_2δ1$	1.46	25.41
		$CH_2δ2$	1.36	24.41
		$CH_2ε$	1.28	31.61
		$CH_2ζ$	1.30	29.39
		$CH_2η$	1.29	29.08
		$CH_2θ$	1.29	22.32
		$CH_3ι$	0.88	13.48
		OH	n.d.	n.d.
Leu1				
$^3J_{HNHα}$	6.6	NH	8.46	n.d.
		CHα	4.29	52.32
		CO	n.d.	n.d.
		$CH_2β1$	1.70	39.77
		CHγ	1.78	24.68
		$CH_3δ$	0.95	22.61
		$CH_3δ$	0.92	21.47
Glu2				
$^3J_{HNHα}$	5.8	NH	8.67	n.d.
		CHα	4.25	55.05
		CO	n.d.	n.d.
		$CH_2β1$	2.15	26.32
		$CH_2β2$	2.06	26.32
		$CH_2γ$	2.49	30.34
		COδ	n.d.	n.d.
		OH	n.d.	n.d.
Gln3				
$^3J_{HNHα}$	6.2	NH	8.63	n.d.
		CHα	4.26	55.42
		CO	n.d.	n.d.
		$CH_2β$	2.13	26.77
		$CH_2γ1$	2.39	32.07
		COδ	n.d.	n.d.
		NH_21	n.d.	n.d.
		NH_22	n.d.	n.d.
Val4				
$^3J_{HNHα}$	7.3	NH	7.67	n.d.
		CHα	4.01	61.41
		CO	n.d.	n.d.
		CHβ	2.26	29.44
		$CH_3γ1$	1.02	19.08
		$CH_3γ2$	18.79	0.96
Leu5				
$^3J_{HNHα}$	n.d.	NH	7.88	n.d.
		CHα	4.19	51.19
		CO	n.d.	n.d.
		$CH_2β$	1.73	39.91
		CHγ	1.74	24.56
		$CH_3δ1$	0.94	22.07
		$CH_3δ2$	0.91	21.26
Gln6				
$^3J_{HNHα}$	7	NH	8.27	n.d.
		CHα	4.08	53.42
		CO	n.d.	n.d.
		$CH_2β$	2.29	26.25
		$CH_2γ$	2.06	31.67
		COδ	n.d.	n.d.
		NH_21	n.d.	n.d.
		NH_22	n.d.	n.d.

Table S6.3 (continued). Chemical shifts of xantholysin in DMF-d7 (328 K, 500 MHz)

			1H δ [ppm]	13C δ [ppm]
Ser7				
$^3J_{HNH\alpha}$	n.d.	NH	7.88	n.d.
		CHα	4.58	54.22
		CO	n.d.	n.d.
		$CH_2\beta1$	4.51	63.50
		$CH_2\beta2$	4.37	63.50
		OHγ	n.d.	n.d.
Val8				
$^3J_{HNH\alpha}$	7	NH	8.19	n.d.
		CHα	4.10	61.41
		CO	n.d.	n.d.
		CHβ	2.43	29.77
		$CH_3\gamma1$	1.09	18.67
		$CH_3\gamma2$	0.99	19.05
Leu9				
$^3J_{HNH\alpha}$	5.2	NH	8.12	n.d.
		CHα	4.17	54.31
		CO	n.d.	n.d.
		$CH_2\beta1$	1.86	39.73
		$CH_2\beta2$	1.59	39.72
		CHγ	1.86	24.46
		$CH_3\delta1$	0.92	22.64
		$CH_3\delta2$	0.87	20.71
Gln10				
$^3J_{HNH\alpha}$	6.7	NH	7.74	n.d.
		CHα	4.33	53.94
		CO	n.d.	n.d.
		$CH_2\beta1$	2.20	26.79
		$CH_2\beta2$	2.06	26.79
		$CH_2\gamma$	2.40	31.93
		COδ	n.d.	n.d.
		NH_21	n.d.	n.d.
		NH_22	n.d.	n.d.
Leu11				
$^3J_{HNH\alpha}$	n.d.	NH	7.94	n.d.
		CHα	4.32	53.16
		CO	n.d.	n.d.
		$CH_2\beta1$	1.82	40.71
		$CH_2\beta2$	1.60	40.71
		CHγ	1.83	24.75
		$CH_3\delta1$	1.81	24.69
		$CH_3\delta2$	0.94	22.75
Leu12				
$^3J_{HNH\alpha}$	7.2	NH	7.79	n.d.
		CHα	4.34	52.74
		CO	n.d.	n.d.
		$CH_2\beta1$	1.76	40.46
		$CH_2\beta2$	1.67	4.46
		CHγ	1.77	24.60
		$CH_3\delta1$	0.97	22.69
		$CH_3\delta2$	0.90	20.88
Gln13				
$^3J_{HNH\alpha}$	6.3	NH	7.23	n.d.
		CHα	4.24	53.89
		CO	n.d.	n.d.
		$CH_2\beta1$	2.34	26.87
		$CH_2\beta2$	1.95	26.82
		$CH_2\gamma1$	2.22	31.81
		$CH_2\gamma2$	2.22	31.81
		COδ	n.d.	n.d.
		NH_21	n.d.	
		NH_22	n.d.	
Ile14				
$^3J_{HNH\alpha}$	8.5	NH	7.48	n.d.
		CHα	4.37	57.00
		CO	n.d.	n.d.
		CHβ	1.89	36.57
		$CH_2\gamma1$	1.41	24.87
		$CH_2\gamma2$	1.18	24.87
		$CH_2\delta1$	0.88	15.36
		$CH_3\delta2$	0.85	10.70

n.d. = not determined

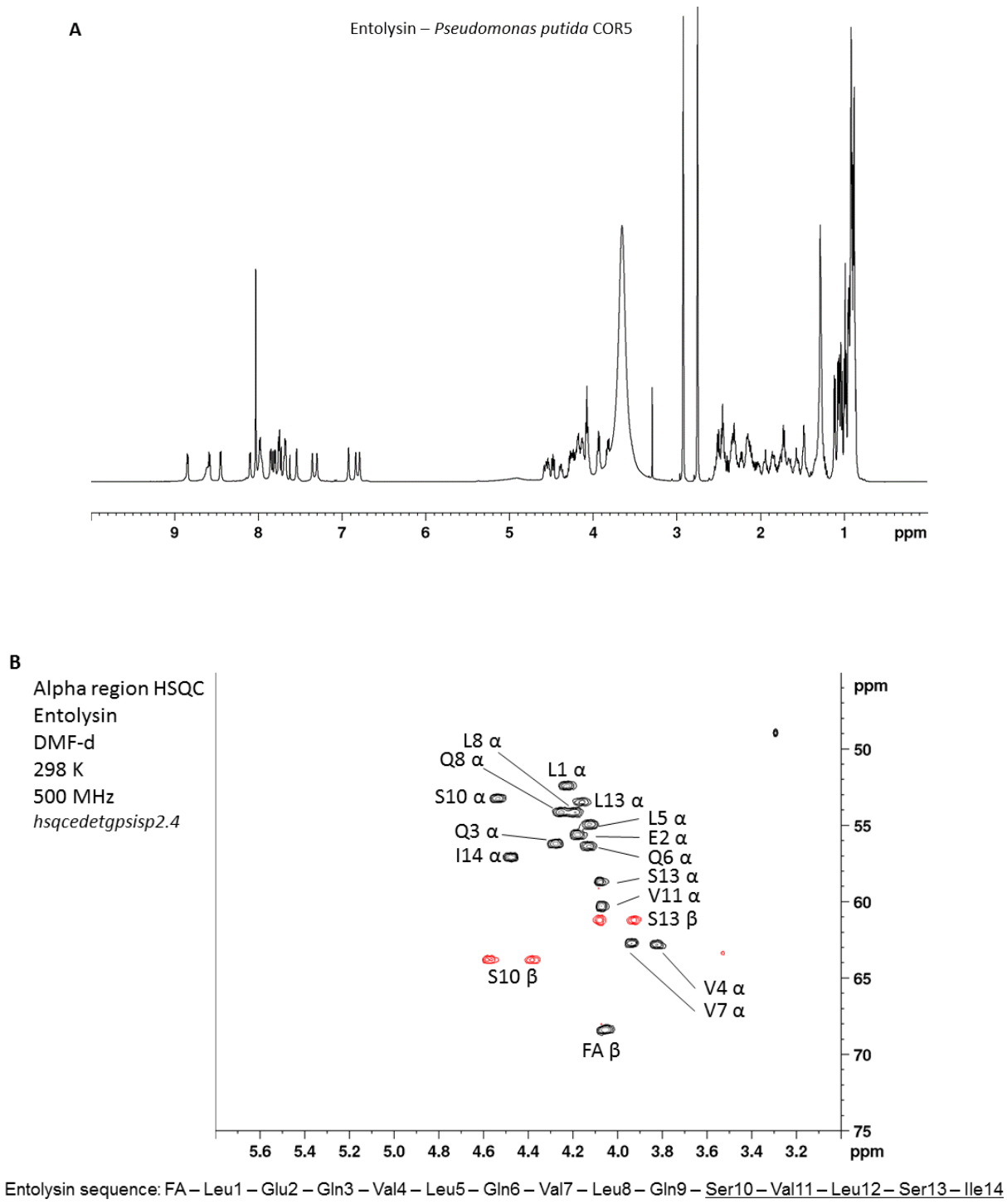

Figure S6.2. 1D ^{1}H NMR **(A)** and ^{1}H-^{13}C gHSQC NMR spectrum **(B)** of entolysin extracted from *Pseudomonas putida* COR5 (DMF-d7, 328K, 500MHz).

Table S6.4. Chemical shifts of entolysin in DMF-d7 (298 K, 500 MHz)

			^{1}H δ [ppm]	^{13}C δ [ppm]				^{1}H δ [ppm]	^{13}C δ [ppm]
HDA		CO	n.d.	172.71	**Gln3**				
		CH_2α	2.45	43.62	$^{3}J_{HNH\alpha}$	6.1	NH	8.42	n.d.
		CHβ	4.04	68.29			CHα	4.20	53.48
		CH_2γ	1.49	37.46			CO	n.d.	174.65
		CH_2δ1	1.47	25.42			CH_2β	2.16	26.50
		CH_2δ2	1.36	24.42			CH_2γ1	2.46	32.01
		CH_2ε	1.30	29.07			CH_2γ2	2.40	32.01
		CH_2ζ	1.31	29.47			COδ	n.d.	n.d.
		CH_2η	1.30	29.47			$NH_2$1	7.20	n.d.
		CH_2θ	1.30	22.36			$NH_2$2	6.60	n.d.
		CH_3ι	0.89	13.52	**Val4**				
		OH	n.d.	n.d.	$^{3}J_{HNH\alpha}$	6.5	NH	7.70	n.d.
Leu1							CHα	3.89	62.37
$^{3}J_{HNH\alpha}$	6.2	NH	8.36	n.d.			CO	n.d.	173.79
		CHα	4.28	52.44			CHβ	2.29	29.27
		CO	n.d.	174.55			CH_3γ1	1.07	19.62
		CH_2β1	1.74	39.92			CH_3γ2	0.99	18.68
		CHγ	1.70	24.92	**Leu5**				
		CH_3δ	0.96	22.55	$^{3}J_{HNH\alpha}$	5.4	NH	7.76	n.d.
		CH_3δ	0.92	21.01			CHα	4.15	54.69
Glu2							CO	n.d.	174.77
$^{3}J_{HNH\alpha}$	5.3	NH	8.69	n.d.			CH_2β1	1.86	39.30
		CHα	4.20	55.39			CH_2β2	1.64	39.30
		CO	n.d.	171.49			CHγ	1.84	24.63
		CH_2β1	2.14	26.29			CH_3δ1	0.93	22.90
		CH_2β2	2.07	26.29			CH_3δ2	0.90	20.86
		CH_2γ	2.50	30.29	**Gln6**				
		COδ	n.d.	n.d.	$^{3}J_{HNH\alpha}$	n.d.	NH	7.98	n.d.
		OH	n.d.	n.d.			CHα	4.16	56.08
							CO	n.d.	174.35
							CH2β1	2.25	26.80
							CH2β2	2.15	26.80
							CH2γ1	2.48	31.96
							CH2γ2	31.92	31.96
							COδ	n.d.	173.96
							$NH_2$1	7.17	n.d.
							$NH_2$2	6.63	n.d.

Table S6.4 (continued). Chemical shifts of entolysin in DMF-d7 (298 K, 500 MHz)

		^{1}H δ [ppm]	^{13}C δ [ppm]
Val7			
$^3J_{HNH\alpha}$ 6.2	NH	7.64	n.d.
	CHα	3.99	62.20
	CO	n.d.	173.77
	CHβ	2.33	29.35
	$CH_3\gamma$ 1	1.11	19.28
	$CH_3\gamma$ 2	1.01	18.93
Leu8			
$^3J_{HNH\alpha}$ 6.0	NH	7.83	n.d.
	CHα	4.23	53.84
	CO	n.d.	173.10
	$CH_2\beta$ 1	1.92	39.71
	$CH_2\beta$ 2	1.62	39.71
	CHγ	1.93	24.38
	$CH_3\delta$ 1	n.d.	n.d.
	$CH_3\delta$ 2	0.89	20.55
Gln9			
$^3J_{HNH\alpha}$ 7.6	NH	7.68	n.d.
	CHα	4.29	54.03
	CO	n.d.	174.82
	$CH_2\beta$ 1	2.25	26.84
	$CH_2\beta$ 2	2.15	26.84
	$CH_2\gamma$	2.41	31.90
	COδ	n.d.	174.46
	$NH_2$1	7.40	n.d.
	$NH_2$2	6.73	n.d.
Ser10			
$^3J_{HNH\alpha}$ n.d.	NH	7.98	n.d.
	CHα	4.56	53.06
	CO	n.d.	169.42
	$CH_2\beta$1	4.61	63.75
	$CH_2\beta$2	4.33	63.75
	OHγ	n.d.	

		^{1}H δ [ppm]	^{13}C δ [ppm]
Val11			
$^3J_{HNH\alpha}$ 6.1	NH	7.59	n.d.
	CHα	4.08	60.37
	CO	n.d.	174.12
	CHβ	2.22	29.83
	$CH_3\gamma$ 1	1.05	18.19
	$CH_3\gamma$ 2	1.03	18.91
Leu12			
$^3J_{HNH\alpha}$ 6.1	NH	8.42	n.d.
	CHα	4.28	55.95
	CO	n.d.	171.89
	$CH_2\beta$ 1	1.83	39.78
	$CH_2\beta$ 2	1.70	39.76
	CHγ	1.76	24.56
	$CH_3\delta$ 1	n.d.	n.d.
	$CH_3\delta$ 2	0.92	21.34
Ser13			
$^3J_{HNH\alpha}$ 7.2	NH	7.91	n.d.
	CHα	4.12	58.52
	CO	n.d.	169.58
	$CH_2\beta$ 1	4.04	61.39
	$CH_2\beta$ 2	3.92	61.39
	OHγ	n.d.	n.d.
Ile14			
$^3J_{HNH\alpha}$ 8.8	NH	7.66	n.d.
	CHα	4.47	57.10
	CO	n.d.	n.d.
	CHβ	2.02	37.09
	$CH_2\gamma$ 1	1.57	25.00
	$CH_2\gamma$ 2	1.25	25.00
	$CH_2\delta$ 1	0.93	15.29
	$CH_3\delta$ 2	0.89	10.90

n.d. = not determined

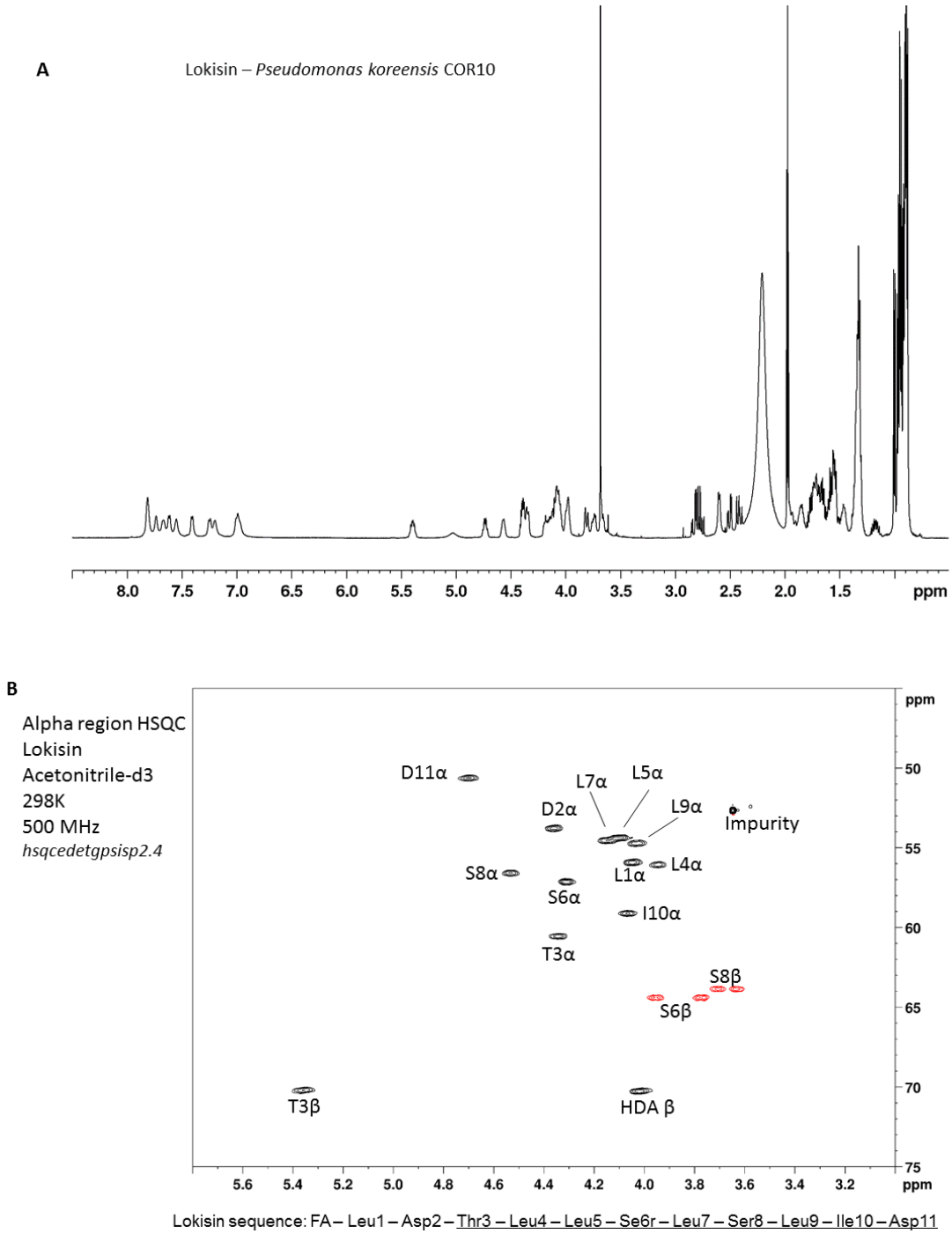

Figure S6.3. 1D ^{1}H NMR **(A)** and ^{1}H-^{13}C gHSQC NMR spectrum **(B)** of lokisin extracted from *P. koreensis* COR10 (acetonitrile-d3, 298K, 500MHz).

Table S6.5. Chemical shifts of lokisin in acetonitrile-d3 (298 K, 500 MHz)

			^{1}H δ [ppm]	^{13}C δ [ppm]
HDA				
		CO	-	n.d.
		CH_2α	2.4687	44.11
		CH_2α	2.38	44.11
		CHβ	4.02	70.29
		CH_2γ	1.51	38.20
		CH_2δ1	1.42	26.19
		CH_2δ2	1.3199	26.19
		CH_2ε	1.29	32.55
		CH_2ζ	1.32	30.21
		CH_2η	1.29	29.97
		CH_2θ	1.30	23.34
		CH_3ι	0.89	14.33
		OH	n.d.	-
Leu1				
$^3J_{HNH\alpha}$	n.d.	NH	7.71	-
		CHα	4.05	55.96
		CO	-	n.d.
		CH_2β1	1.62	40.23
		CH_2β2	1.55	40.23
		CHγ	1.72	25.45
		CH_3δ	0.97	22.59
		CH_3δ	0.91	22.18
Asp2				
$^3J_{HNH\alpha}$	n.d.	NH	7.79	-
		CHα	4.36	53.80
		CO	-	n.d.
		CH_2β	2.77	35.36
		COδ	-	n.d.
		OH	n.d.	-
Thr3				
$^3J_{HNH\alpha}$	6.4	NH	7.59	-
		CHα	4.34	60.57
		CO	-	n.d.
		CHβ	5.36	70.22
		CH_3γ	1.29	18.11
Leu4				
$^3J_{HNH\alpha}$	n.d.	NH	7.52	-
		CHα	3.944	56.09
		CO	-	n.d.
		CH_2β1	1.64	40.99
		CH_2β2	1.64	40.99
		CHγ	1.66	25.37
		CH_3δ1	0.91	22.92
		CH_3δ2	0.86	22.44
Leu5				
$^3J_{HNH\alpha}$	n.d.	NH	7.78	-
		CHα	4.089	54.39
		CO	-	n.d.
		CH_2β1	1.71	40.70
		CH_2β2	1.49	40.70
		CHγ	1.80	25.29
		CH_3δ1	0.88	23.56
		CH_3δ2	0.85	20.93
Ser6				
$^3J_{HNH\alpha}$	6.4	NH	7.22	-
		CHα	4.31	57.15
		CO	-	n.d.
		CH_2β1	3.95	64.43
		CH_2β2	3.77	64.43
		OHγ	n.d.	-
Leu7				
$^3J_{HNH\alpha}$	6.3	NH	7.38	-
		CHα	4.15	54.58
		CO	-	n.d.
		CH_2β1	1.90	40.03
		CH_2β2	1.52	40.03
		CHγ	1.84	25.40
		CH_3δ1	0.84	23.76
		CH_3δ2	0.86	21.40
Ser8				
$^3J_{HNH\alpha}$	n.d.	NH	7.64	-
		CHα	4.53	56.60
		CO	-	n.d.
		CH_2β1	3.70	63.87
		CH_2β2	3.63	63.87
		OHγ	n.d.	-
Leu9				
$^3J_{HNH\alpha}$	n.d.	NH	7.17	-
		CHα	4.03	54.74
		CO	-	n.d.
		CH_2β1	1.67	40.92
		CH_2β2	1.56	40.92
		CHγ	1.70	25.56
		CH_3δ1	0.83	23.30
		CH_3δ2	0.85	21.29
Ile10				
$^3J_{HNH\alpha}$	n.d.	NH	6.97	-
		CHα	4.07	59.15
		CO	-	n.d.
		CHβ	1.93	36.72
		CH_2γ1	1.44	25.71
		CH_2γ2	1.13	25.71
		CH_2δ1	0.88	15.96
		CH_3δ2	0.86	11.47
Asp11				
$^3J_{HNH\alpha}$	n.d.	NH	6.94	-
		CHα	4.70	50.64
		CO	-	n.d.
		CH_2β	2.57	37.33
		COδ	-	n.d.
		OH	n.d.	-

n.d. = not determined

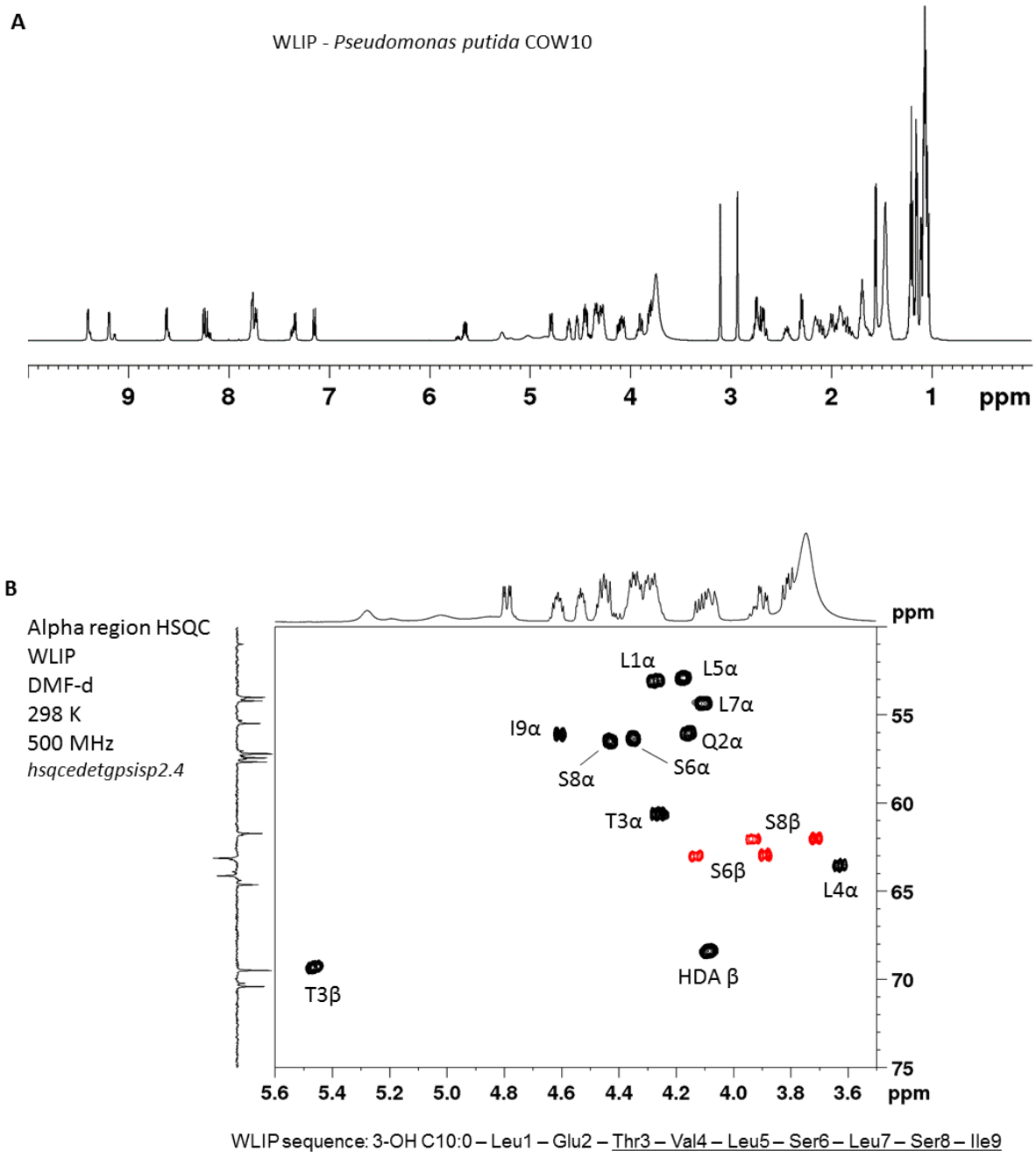

Figure S6.4. 1D ^{1}H NMR (A) and ^{1}H-^{13}C gHSQC NMR spectrum (B) of WLIP extracted from *P. putida* COW10 (DMF-d7, 298K, 500MHz).

Table S6.6. Chemical shifts of WLIP in DMF-d7 (298 K, 500 MHz)

		^{1}H δ [ppm]	^{13}C δ [ppm]
HDA			
	CO	-	174.05
	CH_2α	2.51	43.92
	CHβ	4.09	68.35
	CH_2γ	1.51	37.61
	CH_2δ1	1.50	25.47
	CH_2δ2	1.37	25.47
	CH_2ε	1.29	29.33
	CH_2ζ	1.29	29.33
	CH_2η	1.27	31.74
	CH_2θ	1.28	22.47
	CH_3ι	0.88	13.67
	OH	n.d.	-
Leu1			
$^3J_{HNH\alpha}$ 5.0 Hz	NH	9.01	-
	CHα	4.18	52.87
	CO	-	175.65
	CH_2β1	1.73	39.29
	CHγ	1.73	24.51
	CH_3δ	0.97	22.37
	CH_3δ	0.92	21.68
Glu2			
$^3J_{HNH\alpha}$ 4.4 Hz	NH	9.22	-
	CHα	4.16	56.00
	CO	-	175.65
	CH_2β	2.11	26.01
	CH_2γ	2.57	30.02
	COδ	-	173.76
	OH	n.d.	-
Thr3			
$^3J_{HNH\alpha}$ 6.7 Hz	NH	8.44	-
$^3J_{H\alpha H\beta}$	CHα	4.26	60.57
	CO	-	n.d.
	CHβ	5.46	69.26
	CH_3γ	1.38	17.77
Val4			
$^3J_{HNH\alpha}$ n.d.	NH	7.59	-
	CHα	3.63	63.50
	CO	-	173.76
	CHβ	2.26	28.98
	CH_3γ1	1.03	20.23
	CH_3γ2	0.97	18.89

		^{1}H δ [ppm]	^{13}C δ [ppm]
Leu5			
$^3J_{HNH\alpha}$ n.d.	NH	7.57	-
	CHα	4.12	54.33
	CO	-	173.02
	CH_2β1	1.81	39.63
	CH_2β2	1.51	39.63
	CHγ	1.83	24.45
	CH_3δ1	0.90	22.72
	CH_3δ2	0.87	20.45
Ser6			
$^3J_{HNH\alpha}$ 7.6 Hz	NH	7.54	-
	CHα	4.35	56.29
	CO	-	171.04
	CH_2β1	4.13	62.96
	CH_2β2	3.89	62.96
	OHγ	5.10	-
Leu7			
$^3J_{HNH\alpha}$ 7.2 Hz	NH	7.16	-
	CHα	4.27	53.03
	CO	-	172.83
	CH_2β1	1.92	41.09
	CH_2β2	1.66	41.09
	CHγ	1.98	24.37
	CH_3δ1	1.01	22.97
	CH_3δ2	0.89	20.57
Ser8			
$^3J_{HNH\alpha}$ 8.3 Hz	NH	8.06	4.44
	CHα	4.43	56.46
	CO	-	171.08
	CH_2β1	3.94	62.00
	CH_2β2	3.71	62.00
	OHγ	n.d.	-
Ile9			
$^3J_{HNH\alpha}$ 9.9 Hz	NH	6.96	-
	CHα	4.61	56.05
	CO	-	169.38
	CHβ	1.97	36.42
	CH_2γ1	1.25	24.29
	CH_2γ2	1.03	24.29
	CH_2δ1	0.85	15.35
	CH_3δ2	0.89	11.54

n.d. = not determined

Chapter 7

Taxonomic and cyclic lipopeptides diversity of fluorescent *Pseudomonas* spp associated with the cocoyam (*Xanthosoma sagittifolium*) rhizosphere in two Nigerian soils

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Summary

Soil microbial diversity has long been considered as an indicator of soil quality. In Chapter 7, we showed that CRRD suppressive soils of Cameroon were enriched with *Pseudomonas* isolates belonging to the *P. fluorescens* complex and a specific cyclic lipopeptide (CLP) named N1, whereas conducive soils were enriched with isolates belonging to the *P. putida* group and a different CLP named N2. In our present study, the conduciveness/suppressiveness of soils from two cocoyam fields situated in Ado-Ekiti and Umudike cities of Nigeria was investigated. Additionally, taxonomic diversity and CLP characterization was conducted for fluorescent pseudomonads that were isolated from the cocoyam rhizosphere in these farms after which attempts were made to elucidate the drivers of such diversity, if present. Our results confirmed the conduciveness of Nigeria soils to CRRD. Furthermore, this study showed that all isolates from Ado-Ekiti and Umudike clustered within the *P. putida* group or *P. aeruginosa* lineage. Comparison of soil physico-chemical properties between Nigerian and Cameroonian soils led us to hypothesize that higher soil nutrition as obtained in Boteva soils of Cameoon (chapter 6) could facilitate the selection of *P. fluorescens* complex isolates in the cocoyam rhizosphere. Interestingly, HPLC-MS characterization of putative CLP producing strains revealed that the dominant CLP in both fields in Nigeria is the N2, which was reported to be the most dominant CLP in the conducive soils of Cameroon. Conclusively, our results suggest that soil abiotic factors may contribute to *Pseudomonas* species diversity on cocoyam roots. More so, fertile soils appear to select for isolates that produce specific CLPs which are structurally different from less rich soils.

Introduction

Suppressive soils against *P. myriotylum* have been described in the volcanic andosols of Cameroon (Adiobo *et al.,* 2007). In Chapter 7, we characterised the taxonomic and cyclic lipopeptides diversity of fluorescent pseudomonads obtained from the cocoyam rhizosphere in Cameroon. Our results showed that cocoyam roots from suppressive soils of Boteva contained significantly higher *P. fluorescens* complex isolates compared with the intermediate and conducive soils of Ekona and Maumu, respectively. More so, CLP diversity appeared to be higher in *Pseudomonas* isolates obtained from cocoyam roots in Boteva.

In Nigeria, CRRD poses a threat to cocoyam production and is gradually fading away from human diets (Chukwu and Eteng, 2014). In contrast with Cameroon, extensive research relating to disease conduciveness/suppressiveness has not been conducted. Given the extent of information we have obtained about the suppressiveness of Cameroon andosols to this disease (Adiobo *et al.,* 2007), and suggested links with soil characteristics, the question arises about the nature of soil suppressiveness/conduciveness in other cocoyam growing areas and whether the taxonomic and CLP diversity we observed in Cameroon soils can be linked with soil abiotic factors.

Thus, the aim of our present study was to (i) investigate the suppressiveness/conduciveness of two cocoyam-growing soils in Nigeria soils (Ado-Ekiti and Umudike) to CRRD, (ii) assess the taxonomic and CLP diversity of fluorescent *Pseudomonas* isolates from the cocoyam rhizosphere in Nigeria (iii) decipher the relationships, if any, between soil physicochemical characteristics, CLP abundance and taxonomic diversity, and to (iv) identify the drivers of microbial selection by cocoyam roots.

Results

Verification of the conduciveness of Nigerian soils towards CRRD

Soils were collected from two cocoyam-growing fields at Ado-Ekiti and Umudike cities of Nigeria. At Ado-Ekiti, the cocoyam field was situated at the Ekiti State University farm while at Umudike, the cocoyam field was located at the National Root Crop Research Institute (NRCRI). Although visual field observations suggested that Umudike soils have higher disease pressure than Ado-Ekiti soils, greenhouse assays using soils from both fields showed that both soils are conducive to CRRD and that disease severity at one location does not differ significantly from the other (Figure 7.1a and b). More so, these Nigerian soils showed as much disease severity as the potting soil control.

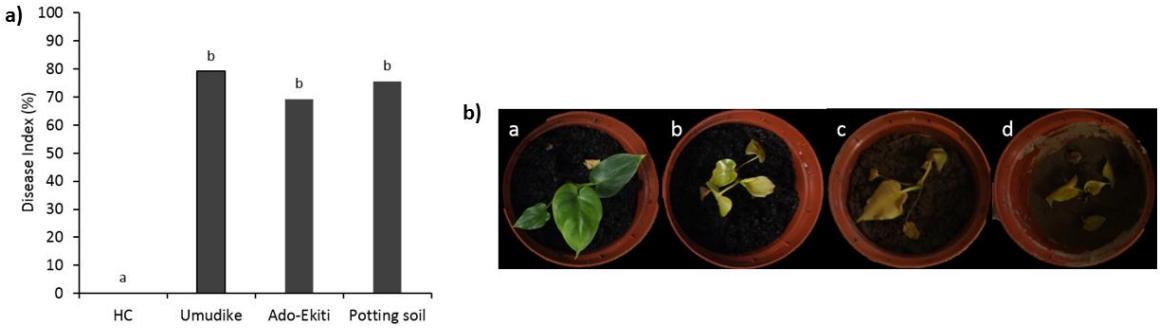

Figure 7.1. Conduciveness of Nigerian soils towards *Pythium* root rot on cocoyams in two independent experiments. Figure represents pooled data from two experiments. **a)** Plant experiments, HC: healthy control**;** and **b)** CRRD symptoms, **a:** healthy; **b:** potting soil; **c:** Umudike soil; **d)** Ado-Ekiti soil. Bars with different letters are significantly different according to Kruskal-Wallis and Mann-Whitney non-parametric tests (P = 0.05).

Pseudomonas population and taxonomic characterization

Cocoyam plants were collected from two cocoyam fields at Ado-Ekiti and Umudike, Nigeria. Population densities of fluorescent *Pseudomonas* spp. was approximately 10^9 g^{-1} of fresh roots at both Umudike and Ado-Ekiti fields. Average bacterial population density is shown for 10 plants from each cocoyam field (Table S7.1).

Characteristics of *Pseudomonas* isolates obtained during this study are given in Table S7.2. Based on the phylogenetic tree generated using sequences of the *rpoD* gene, a total of 191 isolates were placed in three *Pseudomonas* groups namely *P. putida*, *P. oleovorans* and *P. aeruginosa* (Figure 7.2). Our results showed that strains from both fields clustered together within the *P. putida* and *P. aeruginosa* groups. Interestingly, isolates that clustered with the *P. oleovorans* group were found in Ado-Ekiti alone (Figures 7.3a and b). *P. putida* group isolates were assigned into five categories namely P1, P2, P3, P4 and P5. Interestingly, several isolates especially in the P1, P3 and P4 *P. putida* categories do not have a close match with type strains of the *P. putida* group and so appear to be new species (Figure 7.2). More so, about 94% of Umudike isolates clustered within the *P. putida* group while the rest grouped with *P. aeruginosa*. Conversely, Ado-Ekiti isolates were more diverse with 37% clustering with *P. putida*, 49% with *P. aeruginosa* and the rest (15%) belonging to the *P. oleovorans* group (Figure 7.3a). Comparing

the taxonomic dominance of these two fields, the number of *P. putida* isolates was significantly higher ($p < 0.05$) at Umudike whereas the number *P. aeruginosa* isolates was significantly higher ($p < 0.05$) at Ado-Ekiti.

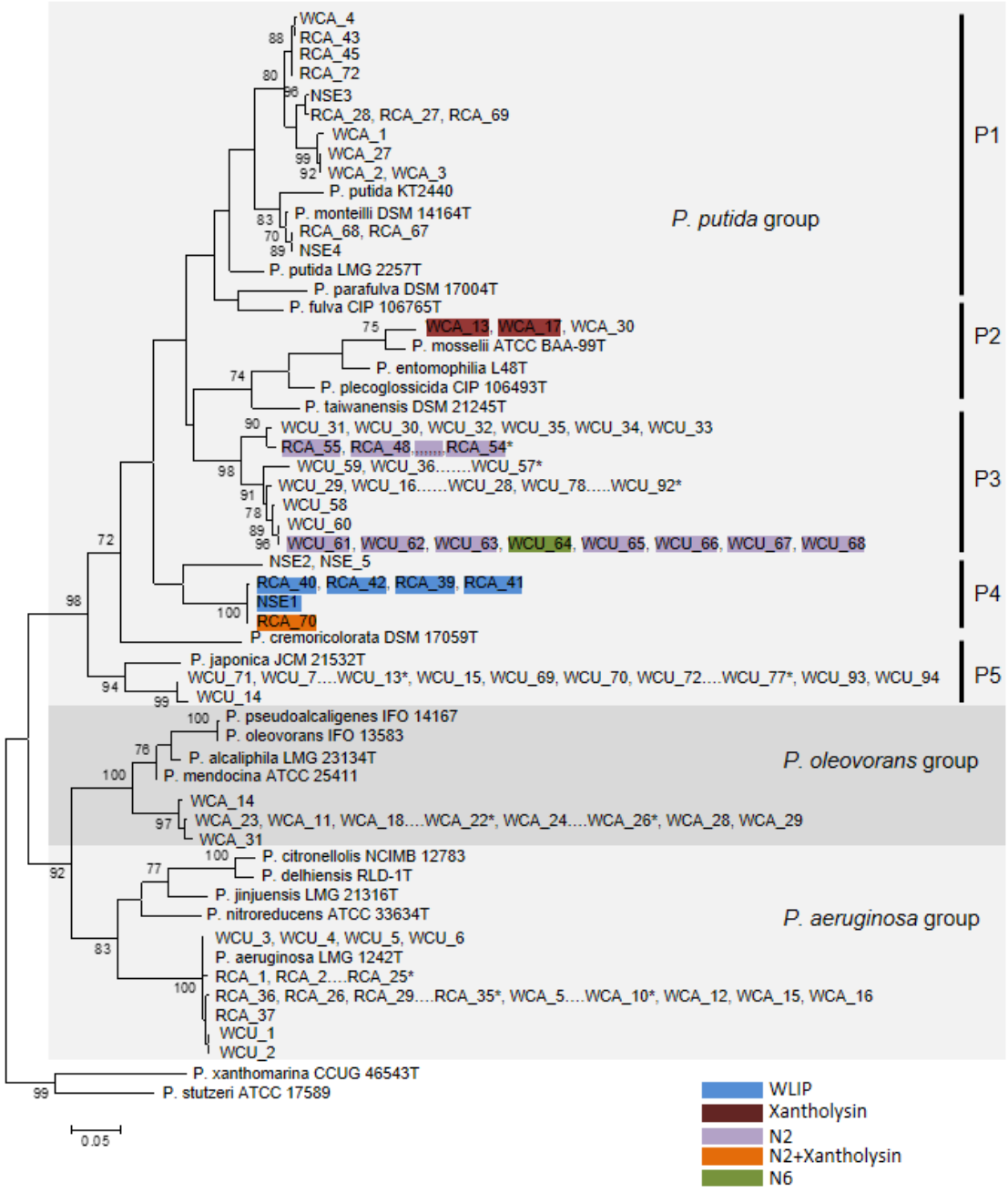

Figure 7.2. Maximum Likelihood tree derived from the phylogenetic analysis of 191 *Pseudomonas* isolates using *rpoD Pseudomonas* housekeeping gene with *P. stutzeri group* isolates as the outgroup. Bootstraps are only indicated for branches with bootstrap support of higher than 70. The tree was made using only isolates with unique sequences whereas isolates with identical sequences were added afterwards, on the same line. The isolates obtained from the red and white cocoyams at Ado-Ekiti were designated with RCA- and WCA- prefixes, respectively. Isolates obtained from Umudike were either denoted with NSE- or -WCU prefixes. Dotted line before a strain followed by asterisk indicate that strains with increasing number lie between that strain and its preceding strain. Legend below tree indicates the different CLPs produced by some strains on the tree. CLP producers are coloured accordingly. N2 and N6 are new CLPs found during this study.

Physical and chemical soil properties

Table 7.2 shows the physico-chemical properties of Ado-Ekiti and Umudike soils. Typical for alfisols and ultisols, pH values at Ado-Ekiti and Umudike fields were neutral (7.03) and acidic (5.05), respectively. Although both soils had comparable amounts of sand, K, Na and total N, Ado Ekiti soils were richer in silt, Ca, Mg, and organic carbon. Conversely, Umudike soils had more clay and phosphorus (Table 7.2).

Table 7.2. Characteristics of soils used in this study

Soil variable	Ado-Ekiti[1]	Umudike[2]
Clay (%)	9.40	22.10
Silt (%)	15.70	6.90
Sand (%)	74.90	71.00
pH (H_2O)	7.03	5.05
Ca (cmol kg^{-1})	3.34	0.94
Mg (cmol kg^{-1})	0.76	0.30
K (cmol kg^{-1})	0.14	0.11
Na (cmol kg^{-1})	0.00	0.01
P (mg kg^{-1})	10.60	44.90
Organic C (%)	1.92	0.97
Total N (%)	0.17	0.13

[1]Alfisols
[2]Ultisols

Table 7.3. Comparison of soil characteristics for Cameroonian and Nigerian soils

	Suppressive	Intermediate	Conducive soils		
Soil variable	Boteva[1]	Ekona[1]	Maumu[1]	Ado-Ekiti[2]	Umudike[2]
Clay (%)	2.99	53.44	54.20	9.40	22.10
Silt (%)	76.60	40.41	31.80	15.70	6.90
Sand (%)	20.41	6.15	14.00	74.90	71.00
pH (H_2O)	5.15	4.72	5.87	7.03	5.05
Ca (cmol kg^{-1})	16.47	4.16	2.92	3.34	0.94
Mg (cmol kg^{-1})	3.14	1.72	3.32	0.76	0.30
K (cmol kg^{-1})	0.22	0.38	1.22	0.14	0.11
Na (cmol kg^{-1})	0.00	0.01	0.02	0.00	0.01
P (mg kg^{-1})	6.57	6.37	27.60	10.60	44.90
Organic C (%)	6.04	1.93	3.45	1.92	0.97
Total N (%)	0.73	0.22	0.38	0.17	0.13

[1]Andosols
[2]Alfisols
[3]Ultisols

In Table 7.3, physico-chemical data from three previously studied cocoyam fields in Cameroon namely Boteva (low disease-pressure soil), Ekona (moderate disease pressure) and Maumu (high disease pressure) are compared with soils from Ado-Ekiti and Umudike. The moderate suppressive soils of Ekona and conducive soils of Maumu, Ado-Ekiti and Umudike had several properties in common. They all appeared to have less silt, Ca, organic carbon and total N when compared with the disease-suppressive soils of Boteva. On the other hand, conducive soils had a higher phosphorus content than the suppressive and intermediate soils.

Characterisation of biosurfactant-producing Pseudomonads from the cocoyam rhizosphere

All *Pseudomonas* isolates obtained from Ado-Ekiti and Umudike were assessed for drop collapse activity. Results showed that Ado-Ekiti had a higher proportion of biosurfactant producers (68%) due to the abundance of *P. aeruginosa* isolates obtained at this field whereas Umudike had much less biosurfactant producers (16%) (Table S7.2). However, the percentage of CLP producing isolates was 20% and 10% at Ado-Ekiti and Umudike, respectively (Figure 7.3b).

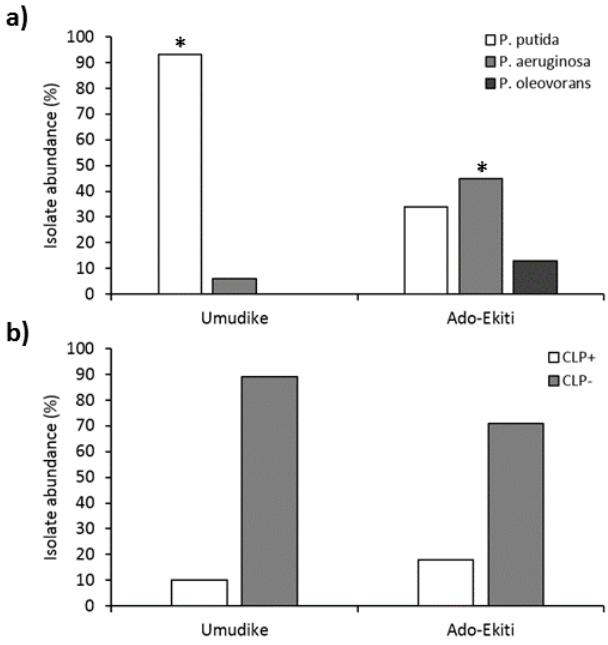

Figure 7.3. a) Taxonomic affiliation of *Pseudomonas* isolates obtained from the cocoyam rhizosphere at Umudike and Ado-Ekiti. Bars with asterisks indicate significant differences ($p < 0.05$) using Fischer's exact test statistic; **b)** Percentage CLP-producing *Pseudomonas* isolates obtained from Umudike and Ado-Ekiti.

Based on HPLC-MS analysis of 28 putative CLP producers, four structurally different CLPs were identified namely WLIP (m/z 1126.4), xantholysin (m/z 1775.8), N2 (m/z 1398.6) and an unknown CLP, designated N6 (m/z 1212.4) (Figure 7.4). Interestingly, one of the strains isolated from Umudike soils, RCA_70, produced both N2 and xantholysin (Figure 7.4A). More so, all strains which produce N2 appeared to produce trace amounts of N6 as exemplified by WCU_64. 64% of CLP-producing strains make N2 while 20% produce WLIP. The CLPs, xantholysin, N2+Xantholysin and N6 were produced by 8%, 4% and 4%, of CLP-producing isolates, respectively (Figure 7.4B). Although, N2 isolates were

equitably distributed in the two fields, Ado-Ekiti isolates also produced WLIP, xantholysin and the lone N2+xantholysin CLP. However, Umudike isolates produced WLIP and the unknown CLP, N6.

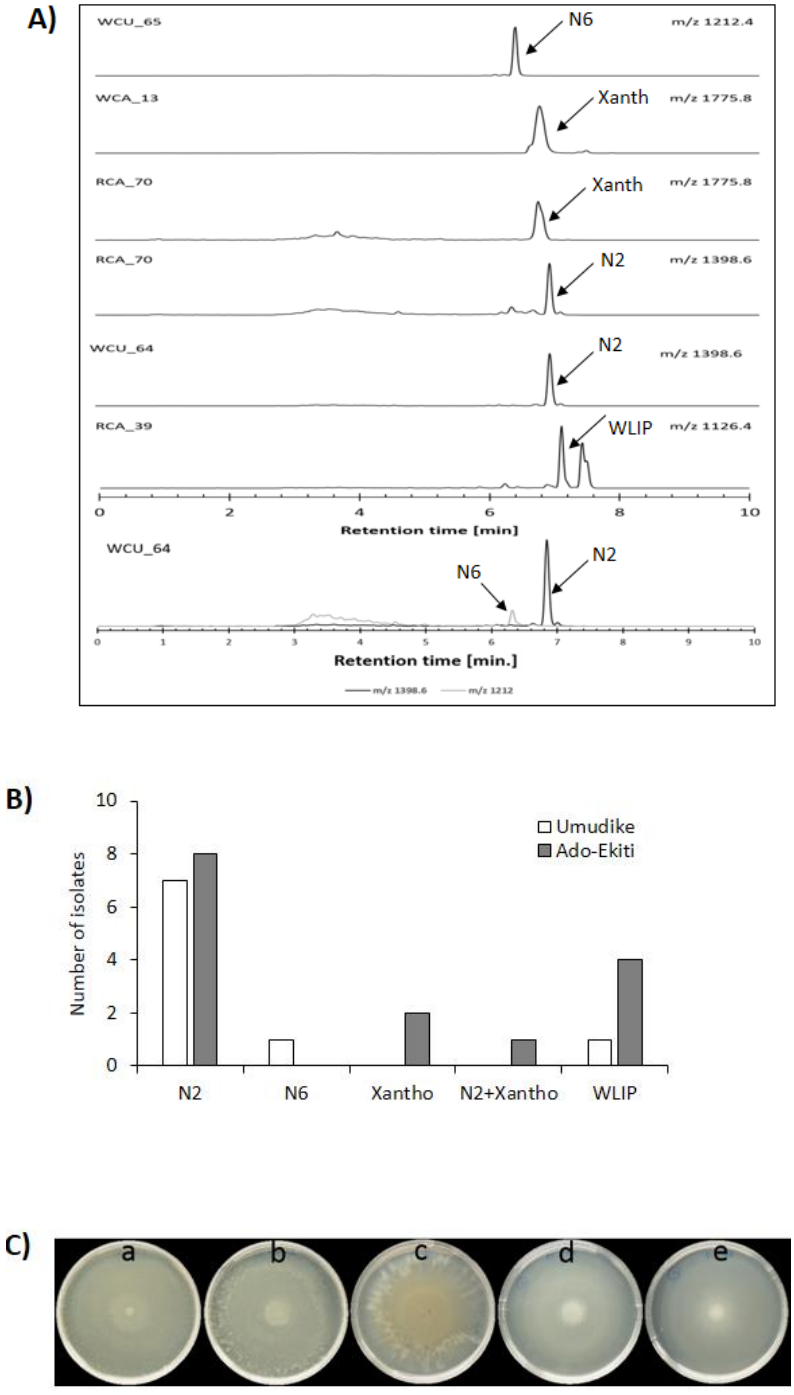

Figure 7.4. a) HPLC-MS chromatograms showing major CLPs produced in liquid culture by fluorescent *Pseudomonas* spp. isolated from the cocoyam rhizosphere in Nigeria soils**; b)** CLP diversity and abundance in Umudike and Ado-Ekiti soils**; c)** Swarming motility tests **(a-e)** of representative CLP-producing isolates from Nigeria soils where **a**: WCU_64 (N2); **b)** WCU_65 (N6); **c)** WCA_13 (xantholysin**)**; **d)** RCA_70 (N2 + xantholysin); **e)** RCA_39 (WLIP).

Furthermore, drop collapse and swarming patterns for these five CLP groups were similar for strains producing similar CLPs (data not shown). Swarming motility tests were conducted for representative CLP-producing isolates (Figure 7.4C).

All CLP-producing isolates were exclusively situated within the *P. putida* group with isolates producing similar CLPs clustering together in the phylogenetic tree (Figure 7.2). Similar to what we observed in Chapter 6, we did not obtain CLP producers among strains associated with *P. japonica* (P5) and *P. monteilli* (P1). In this study, CLP producers clustered within the P2, P3 and P4 categories.

Cocoyam biocontrol assays using representative CLP-producing isolates

CRRD suppressiveness by representative CLP-producing isolates that were obtained from Nigerian soils was investigated. Our results showed that all strains could suppress CRRD effectively (Figure 7.6). However, the best result was exhibited by NSE1, the WLIP producer which completely repressed CRRD symptoms when tested. Subsequent root colonization analyses for these bacteria revealed strong rhizosphere competence for all isolates tested (Table 7.4). Significantly higher colonization (10^7 log CFU g^{-1}) of fresh cocoyam roots was recorded by treatments with *Pseudomonas* sp. NSE1 in comparison with values obtained for other treatments (10^6 log CFU g^{-1} of fresh roots).

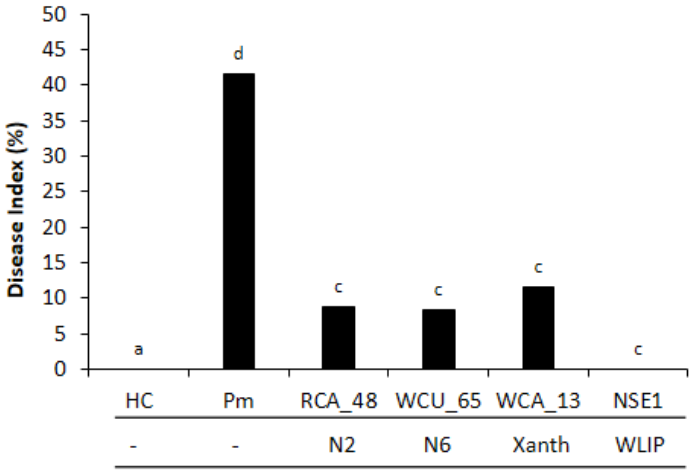

Figure 7.6. Evaluation of CLP-producing *Pseudomonas* isolates for suppression of CRRD in two independent experiments. Figure represents pooled data from two experiments. HC: healthy control**;** Pm: *P. myriotylum* diseased control. The table below the figure indicates metabolites that are produced by the various strains, Xanth: xantholysin. Bars with different letters indicate significant differences ($p < 0.05$) using Kruskal-Wallis and Mann-Whitney non-parametric tests.

Table 7.3. Root colonization data of representative CLP-producing isolates

Pseudomonas strain	CLPs produced	Population density of bacteria (in log CFU g^{-1} of fresh root)[a]
RCA_48	N2	6.5 ± 0.7 a
WCU_65	N6	6.3 ± 0.3 a
WCA_13	Xantholysin	6.4 ± 0.5 a
NSE1	WLIP	7.1 ± 0.4 b

[a]Root colonisation analysis was conducted using five cocoyam plants /treatment. Data was log10 transformed before statistical analysis. Values followed by the same letter are not significantly different according to Tukey's tests (P = 0.05).

Discussion

Coupled with investigating the disease suppressiveness/conduciveness of Nigerian soils to CRRD, this present study focused on deciphering the taxonomic and CLP diversity of *Pseudomonas* isolates associated with cocoyam roots. Additionally, the drivers of such diversity were to be elucidated. Soils were obtained from two cocoyam fields situated in Ado-Ekiti and Umudike cities of Nigeria and used for pathogenicity tests in a *P. myriotylum*-cocoyam pathosystem. Both soils were confirmed to be conducive to CRRD. In a previous study conducted in Cameroon (Adiobo *et al.,* 2007), the suppressiveness and conduciveness of certain andosols and ferralsols to CRRD was reported. Similar to our study, this previous study was conducted in green house assays based on visual field observations, which suggested the occurrence of variable disease pressures on several cocoyam fields in Cameroon (Adiobo, 2006). In our study, visual field observations suggested that Umudike and Ado-Ekiti soils were disease conducive although disease pressure appeared to be more severe at Umudike. However, our results showed that both soils are comparatively conducive to CRRD.

The microbial diversity of soils has long been considered to be an indicator of soil health and soil quality (Garbeva *et al.,* 2006; Garbeva *et al.,* 2008; Cha *et al.,* 2015). More so, indigenous microbial populations such as fluorescent pseudomonads have been implicated in the biological control of plant pathogens (Berg and Smalla, (2009); Chapter 1). Given the fact that numerous studies have considered the main agents in soil suppressiveness as being microbial (Weller *et al.,* 2002; Garbeva *et al.,* 2004; Adiobo *et al.,* 2007; Kyselkova *et al.,* 2009; Almario *et al.,* 2013; Almario *et al.,* 2014; Cha *et al.,* 2015), our present study focused on investigating the taxonomic diversity of *Pseudomonas* spp. associated with cocoyam roots in Nigeria. This was to enable the comparison of our results with *Pseudomonas* diversity which was observed in CRRD-suppressive versus conducive andosols of Cameroon (Chapter 6). In this current work, our results revealed that isolates from two study sites clustered within the *P. putida* and *P. aeruginosa* groups although some isolates from Ado-Ekiti further clustered with the *P. oleovorans* group. Given that the *P. oleovorans* group belongs to the *P. aeruginosa* lineage, thus, all our isolates clustered within the *P. putida* group and *P. aeruginosa* lineage. In Chapter 7, we reported the presence of *P. putida*, *P. fluorescens* and a couple of *P. aeruginosa* isolates in the andosols of Cameroon. About 77% of *P. fluorescens* complex isolates were affiliated with the suppressive soils of Boteva whereas the intermediate-diseased soils of Ekona and high disease-pressure soils of Maumu only recorded 19% and 5%, respectively. In spite of differences in soil textural characteristics, the abundance of *P. putida* isolates on cocoyam roots in Nigeria, especially from Umudike soils, is in agreement with what was previously observed in the conducive soils of Cameroon except for the absence of *P. fluorescens* complex isolates in Nigerian soils. Suppressive soils of Boteva also recorded significantly higher silt, Ca, organic carbon and total nitrogen. In studies conducted in the Inland Pacific Northwest of the United States, soil silt content has been shown to affect *Pseudomonas* species diversity (Parejko *et al.,* 2013). However, in our study we investigated soil physico-chemical properties in rainforest conditions and not in dry lands so a direct comparison cannot be made.

In addition, comparison of physicochemical soil characteristics of Cameroon and Nigeria soils reveal that conducive soils are higher in phosphorus compared to suppressive soils. Although the precise reason is not known for all these fields, it has been reported that high phosphorus levels in fields can be due to over-fertilizing or addition of too much manure. This is particularly true for Umudike fields where fertilizers are often added by farmers. This repeated practice could escalate phosphorus levels especially since phosphorus is quite stable in the soil and will only leave the soil through soil erosion or plant uptake (Sinclair and Vadez, 2002). This also presents a notable concern since constant use of fertilizers did not necessarily confer increased yield, given the presence of *P. myriotylum* on those fields.

In Switzerland, the natural suppressiveness displayed by vermiculite-rich soils of the Morens region towards *Thielaviopsis basicola*-mediated tobacco black rot has been shown to depend mainly on fluorescent pseudomonads producing 2, 4-diacetylphloroglucinol (DAPG) (reviewed by Almario *et al.,* 2014). Interestingly, DAPG producing strains belong to the *P. fluorescens* complex (Garrido-Sanz *et al.,* 2016). Additionally, the most effective biocontrol strains that have been well-studied are taxonomically positioned within the *P. fluorescens* complex and produce a broad spectrum of secondary metabolites (Gomila *et al.,* 2015; Garrido-Sanz *et al.,* 2016). Given the fact that the suppressive Boteva soil of Cameroon contain cocoyam plants whose rhizosphere are enriched in the *P. fluorescens* complex isolates (chapter 6) and conducive soils of Cameroon and Nigeria do not, it is likely that *P. fluorescens* complex isolates contribute to the suppressiveness of Boteva soil to CRRD. Thus, their absence in conducive soils could predispose cocoyams grown in these soils, to CRRD.

Regarding the taxonomic diversity of our strains, it was interesting to observe the abundance of *P. aeruginosa* isolates at Ado-Ekiti. In contrast with soils of Cameroon and Umudike (Nigeria) from which two and six *P. aeruginosa* isolates were obtained, respectively, 45 isolates of this species were obtained from Ado-Ekiti soil. More so, *P. aeruginosa* strains typically produce phenazine antibiotics and likewise, our *P. aeruginosa* isolates produced pyocyanin in addition with other structural forms of phenazines (data not shown). The production of phenazines by fluorescent pseudomonads has been shown to contribute to natural soil suppressiveness (Mazurier *et al.,* 2009). Besides, many studies have correlated the abundance of phenazine-producing pseudomonads with the inhibition of soilborne plant pathogens including *Fusarium* and *Rhizoctonia* spp. on dryland cereals (Mazurier *et al.,* 2009; Mavrodi *et al.,* 2012a; Parejko *et al.,* 2013; Mavrodi *et al.,* 2012b). In our study, visual field observations at Ado-Ekiti showed that although some cocoyam plants had disease symptoms below ground (infected roots and reduced biomass), they did not show aboveground disease symptoms such as leaf yellowing or stunted growth. Thus, the prevalence of *P. aeruginosa* isolates in this field might account for the reduced CRRD pressure which was observed at the Ado-Ekiti fields during the growing season although this needs to be investigated in more detail. Agroclimatic zones were shown to be a key driver in species diversity in dryland agroecosystems of the United States (Parejko *et al.,* 2013; Mavrodi *et al.,* 2012a; Mavrodi *et al.,* 2012b). In our study, we did not experience such dry conditions although Ado-Ekiti had a lower precipitation compared to Umudike.

At Ado-Ekiti, cocoyam crops were grown as an intercrop with yams, maize, vegetables and bananas. This may account for the higher microbial diversity recorded in this field. More so, treatments with higher above-ground biodiversity have been shown to maintain higher levels of microbial diversity (Garbeva *et al.,* 2006). It has also been suggested that higher plant diversity could result in enhanced nutrient flow and root exudation thereby contributing to positive relationships between the soil microbial diversity and plant diversity (Zhu *et al.,* 2010). Plating of cocoyam roots obtained from 2-week old tissue cultured cocoyam plantlets in Ado-Ekiti and Umudike soils suggest that there was a higher diversity of bacterial population at Ado-Ekiti (unpublished data). Thus, we hypothesize that decreased bacterial diversity as observed at Umudike could be related to reduced plant species richness in this field. However, an extensive study involving metagenomics may be necessary in order to investigate microbial diversity drivers in these fields. More so, crop monoculture, which was a norm in this field, often results in continually declining yields without inducing suppressiveness to important plant diseases (Rodriguez-Kabana and Canullo, 1992). In addition, Ado-Ekiti and Umudike differ with respect to land use history. Changes in land use for example, agricultural practices and vegetation types, have been correlated to changes in taxonomic composition (Rokunuzzaman *et al.,* 2016). Future studies investigating rhizospheric diversity should focus, if possible, on fields with similar land use histories.

Furthermore, previous studies to investigate bacterial and archeal species associated with disease suppression indicated that members of the *Gammaproteobacteria* have disease-suppressive activity governed by non-ribosomal peptide synthetases (Mendes *et al.,* 2011). Some members of the Pseudomonaceae protect plants from fungal infection through the production of lipopeptides (Mendes *et al.,* 2011; Chapter 1). In Chapter 6, characterization of CLP production by *Pseudomonas* isolates obtained from the cocoyam rhizosphere in Cameroon revealed that strains affiliated with plants grown in low and intermediate disease pressure soils had a CLP abundance of about 50%. Furthermore, higher CLP diversity (eight) was recorded in *Pseudomonas* isolates obtained from the cocoyam rhizosphere in the suppressive soils of Boteva and these soils were enriched in a new CLP namely N1. However, the cocoyam rhizosphere in the conducive soils of Cameroon were enriched with a structurally different CLP, namely N2. Interestingly, preliminary HPLC analysis of CLP-producing strains from the cocoyam rhizosphere in Nigeria showed that for both sites of study, *Pseudomonas* isolates had a CLP abundance of 10-20%. More so, the cocoyam rhizosphere at Ado-Ekiti and Umudike fields were enriched in N2-like CLP-producing *Pseudomonas* whereas N1-like CLP producers were conspicuously absent. Besides, similar to Cameroonian soils, well-reported CLPs like WLIP and xantholysin were found in the cocoyam rhizospere in Nigerian soils. Our results suggests that specific CLP structures appear to be built up by the cocoyam rhizosphere in richer and fertile soils while others appear to be typical for the cocoyam rhizosphere in soils with less fertility. Furthermore, the enormous number of new CLPs found in studies on the cocoyam rhizosphere in both Cameroon and Nigeria suggests that tropical soils are a goldmine and hotspot for new secondary metabolites with relevance in agriculture.

In conclusion, our studies show that soils conducive to CRRD in Nigeria are enriched with *P. putida* group and *P. aeruginosa* lineage isolates. More so, CLPs obtained belonged to WLIP, xantholysin and two new groups, N2 and N6. Although the main drivers for CLP abundance in these soils is not yet clear, it appears that similar to Cameroon, conducive soils of Nigeria are enriched with specific CLPs (such as N2) while the suppressive soils of Cameroon with another (like N1). However, certain CLPs occur in either of both soils (WLIP, xantholysin).

Materials and Methods

Determination of the conduciveness or suppressiveness of Nigeria soils towards CRRD

To ascertain the conduciveness or suppressiveness of Nigerian soils to CRRD caused by *P. myriotylum*, soils were collected from two separate cocoyam fields located in the cities of Ado-Ekiti and Umudike in the South-western and South-eastern regions, respectively (Figure 7.7). Characteristics of sampled fields are indicated in Table S8.1. Mycelial strand inoculum of *P. myriotylum* was added to each soil at a dose of 1250 propagules/g of soil. A potting soil/sand mixture in a ratio of 3:1 was included as a positive control. Soil was added to plastic plots (7 cm) and 2-weeks old acclimatized tissue culture cocoyam plantlets were planted in each pot. Treatments were arranged in a randomized complete block design and replicated five times with a single pot representing one replicate. Plants were incubated in a growth chamber (25°C; 16 h light and 8 h dark cycle) and scored for disease seven days after planting. Disease was scored on a 0-4 scale as done in Chapter 2, 3 and 6. The experiment was conducted twice.

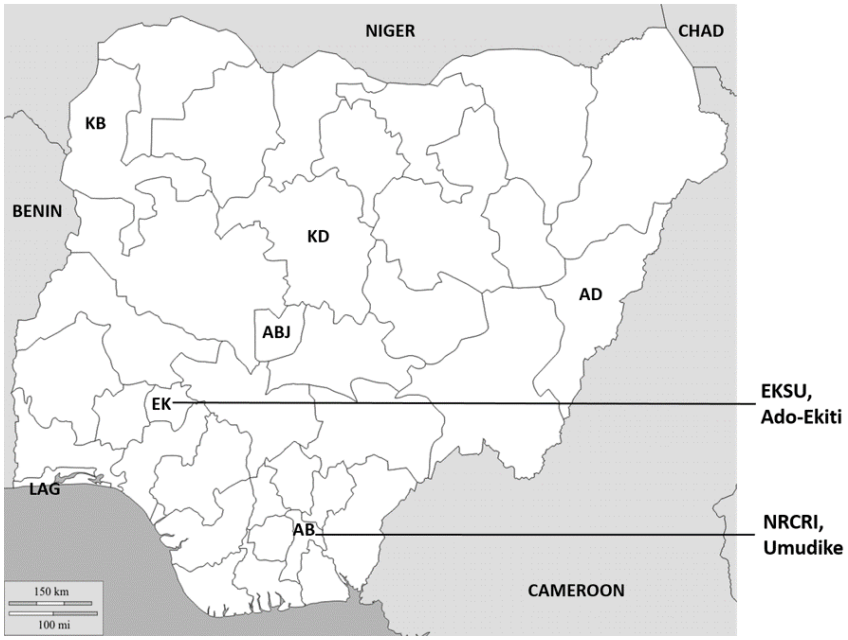

Figure 7.7. Sites for the collection of cocoyam roots and soil samples in Nigeria. The two cocoyam fields sampled are located in two cities namely Ado-Ekiti and Umudike. In Ado-Ekiti, samples were obtained from the Ekiti State University (EKSU) Farm while at Umudike, sampling was conducted in cocoyam fields situated at the National Root Crops Research Institute (NRCRI). ABJ: Federal Capital Territory, Abuja; LAG: Lagos; AD: Adamawa; KD: Kaduna; KB: Kebbi; EK: Ekiti; AB: Abia states.

Determination of soil physical and chemical properties

All experiments were conducted using soils from Ado-Ekiti and Umudike fields or a mixture of potting soil and sand. Characteristics of the potting soil/sand mixture are as described by Hua and Höfte, (2015). Composite soil samples from Ado-Ekiti and Umudike fields were collected and determined using methods described in Chapter 6.

Isolation of fluorescent Pseudomonas species from cocoyam roots

Two cocoyam fields (Ado-Ekiti and Umudike) located in the humid tropical forest zone of Nigeria were sampled (Figure 7.7). Cocoyam roots were collected from healthy 3 to 5 month-old plants. From each field, 20 plants were taken with intact roots. Roots were cut from the shoot and crushed in 0.85% saline solution using sterile sand. Serial 10-fold dilutions were made of the crushed suspension and plated on KB agar. Plates were incubated at 28 °C and enumeration of fluorescent *Pseudomonas* colonies (under UV light) were done after 48 h of incubation. The population density of fluorescent pseudomonads was determined per sample and results are presented in log numbers of CFU per (fresh weight) of roots. A total of 191 bacterial colonies were transferred to fresh KB agar and purified. Of these isolates, 92 and 99 colonies were collected from Ado-Ekiti and Umudike fields, respectively. Subsequently, purified isolates were cultured overnight in Luria Bertani (LB) broth and stored in 20% glycerol at -80 °C. *Pseudomonas* strains used in this study are indicated in Table S8.2. Additional strains used were type strains and common biocontrol isolates of *Pseudomonas* species (Chapter 6, Table S6.2). The isolates obtained from the red and white cocoyams at Ado-Ekiti were designated with RCA- and WCA- prefixes, respectively. Isolates obtained from Umudike were either denoted with NSE- or -WCU prefixes.

Taxonomic characterization of Pseudomonas isolates

For bacterial isolates, colony PCR was carried out using freshly grown bacteria colonies. A small bacteria colony was transferred to 50 µl sterile demineralized water after which 2.5 µl of the bacterial suspension was used as a template in 25 µl PCR reactions. The housekeeping gene region, *rpoD*, was analyzed using primers PsEG30F/PsEG790R as described by Mulet *et al.* (2009). For some isolates, these primers produced a single amplicon whereas some that did not give bands or gave multiple bands were analyzed using primers *rpoD*1F/*rpoD*1R which were designed in Chapter 6.

rpoD sequences of representative type strains and genomes within the *Pseudomonas* genus together with well-studied biocontrol isolates were retrieved from the GenBank. Sequence alignment was carried out using Muscle (Edgar, 2004) via the software package MEGA6 (Tamura *et al.*, 2013). The tree was inferred by Maximum Likelihood and confidence analysis was ensured using 1000 bootstrap replicates with *P. stutzeri* as outgroup.

Characterization of Pseudomonas isolates for cyclic lipopeptide production

The first step to check for biosurfactant production was by the drop collapse assay as previously described (D'aes *et al.,* 2014). Isolates positive for drop collapse were assessed for growth at 40 °C in order to exclude *P. aeruginosa* isolates from CLP characterization. Following this, CLP-producing isolates were tested for swarming on 0.6% LB agar in triplicates while surface tension measurements for representative isolates were carried out as described by D'aes *et al.,* 2011. For HPLC analyses, bacterial cultures were set up in KB broth under shaking conditions for 24 h. Filter sterilized supernatants were subjected to HPLC-MS for preliminary CLP identification as described in Chapter 6.

Biocontrol assays using CLP-producing Pseudomonas isolates

The efficacy of CLP-producing *Pseudomonas* isolates to control root rot disease of cocoyams was investigated in growth chamber assays. The substrate used was a potting soil (Structural; Snebbout, Kaprijke, Belgium) and sand mixture in a 70/30 (w/w) ratio. *Pseudomonas* sp. RCA_48 (N2), WCU_65 (N6), WCA_13 (Xantholysin) and NSE1 (WLIP) were chosen as representative isolates for the different CLP types that were identified in this study. Bacterial inoculum preparation, plant material propagation and the whole experimental set-up, scoring for disease and root colonization was conducted in a similar manner as was done in Chapter 3.

Data analysis

Data obtained from plant experiments were analysed as described in Chapter 3.

Acknowledgements

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Supplementary files

Table S7.1. Characteristics of sites sampled in Nigeria.

Sample Location	Geographic coordinates	*Pseudomonas* population density (log CFU g root^{-1} [fresh wt])[a]	Agroecological zone[b]	Soil type[c]	Cropping history	Cropping system	Mean annual precipitation[d] (mm)
Ado-Ekiti	7°42'41.304"N/5°15'24.156"E	10^9	Derived savanna	Alfisols	yam, banana, maize, cocoyam	intercrop	1334
Umudike	5°29"N/7°30"E	10^9	Humid forest	Ultisols	cocoyam	monoculture	2200

Table S7.2. *Pseudomonas* isolates obtained from Nigeria with genotypic, taxonomic and biosufactant characteristics.

***Pseudomonas* lineage (Garrido-Sanz *et al.*, 2016)**	***Pseudomonas* group (Garrido-Sanz *et al.*, 2016 and new designation)**	**Closest related species**	**Strain**	**Origin**	**Cocoyam variety**	**Main Biosurfactant**
P. aeruginosa	*P. aeruginosa*	*P. aeruginosa*	RCA_1	Ado-Ekiti	Red	Rhamnolipids
			RCA_2	Ado-Ekiti	Red	Rhamnolipids
			RCA_3	Ado-Ekiti	Red	Rhamnolipids
			RCA_4	Ado-Ekiti	Red	Rhamnolipids
			RCA_5	Ado-Ekiti	Red	Rhamnolipids
			RCA_6	Ado-Ekiti	Red	Rhamnolipids
			RCA_7	Ado-Ekiti	Red	Rhamnolipids
			RCA_8	Ado-Ekiti	Red	Rhamnolipids
			RCA_9	Ado-Ekiti	Red	Rhamnolipids
			RCA_10	Ado-Ekiti	Red	Rhamnolipids
			RCA_11	Ado-Ekiti	Red	Rhamnolipids
			RCA_12	Ado-Ekiti	Red	Rhamnolipids
			RCA_13	Ado-Ekiti	Red	Rhamnolipids
			RCA_14	Ado-Ekiti	Red	Rhamnolipids
			RCA_15	Ado-Ekiti	Red	Rhamnolipids
			RCA_16	Ado-Ekiti	Red	Rhamnolipids
			RCA_17	Ado-Ekiti	Red	Rhamnolipids
			RCA_18	Ado-Ekiti	Red	Rhamnolipids
			RCA_19	Ado-Ekiti	Red	Rhamnolipids
			RCA_20	Ado-Ekiti	Red	Rhamnolipids
			RCA_21	Ado-Ekiti	Red	Rhamnolipids
			RCA_22	Ado-Ekiti	Red	Rhamnolipids
			RCA_23	Ado-Ekiti	Red	Rhamnolipids
			RCA_24	Ado-Ekiti	Red	Rhamnolipids
			RCA_25	Ado-Ekiti	Red	Rhamnolipids
			WCU_1	Umudike	White	Rhamnolipids
			WCU_2	Umudike	White	Rhamnolipids
			RCA_36	Ado-Ekiti	Red	Rhamnolipids
			RCA_37	Ado-Ekiti	Red	Rhamnolipids
			WCA_16	Ado-Ekiti	White	Rhamnolipids

			WCA_15	Ado-Ekiti	White	Rhamnolipids
			WCA_8	Ado-Ekiti	White	Rhamnolipids
			WCA_10	Ado-Ekiti	White	Rhamnolipids
			WCA_11	Ado-Ekiti	White	Rhamnolipids
			WCA_5	Ado-Ekiti	White	Rhamnolipids
			WCA_6	Ado-Ekiti	White	Rhamnolipids
			WCA_9	Ado-Ekiti	White	Rhamnolipids
			WCA_7	Ado-Ekiti	White	Rhamnolipids
			RCA_31	Ado-Ekiti	Red	Rhamnolipids
			RCA_32	Ado-Ekiti	Red	Rhamnolipids
			RCA_33	Ado-Ekiti	Red	Rhamnolipids
			RCA_34	Ado-Ekiti	Red	Rhamnolipids
			RCA_26	Ado-Ekiti	Red	Rhamnolipids
			RCA_29	Ado-Ekiti	Red	Rhamnolipids
			RCA_30	Ado-Ekiti	Red	Rhamnolipids
			WCA_12	Ado-Ekiti	White	Rhamnolipids
			RCA_35	Ado-Ekiti	Red	Rhamnolipids
			WCU_3	Umudike	White	Rhamnolipids
			WCU_4	Umudike	White	Rhamnolipids
			WCU_5	Umudike	White	Rhamnolipids
			WCU_6	Umudike	White	Rhamnolipids
	P. oleovorans	*P. mendocina* ATCC 25411	WCA_23	Ado-Ekiti	White	none
			WCA_24	Ado-Ekiti	White	none
			WCA_22	Ado-Ekiti	White	none
			WCA_18	Ado-Ekiti	White	none
			WCA_19	Ado-Ekiti	White	none
			WCA_21	Ado-Ekiti	White	none
			WCA_26	Ado-Ekiti	White	none
			WCA_28	Ado-Ekiti	White	none
			WCA_29	Ado-Ekiti	White	none
			WCA_25	Ado-Ekiti	White	none
			WCA_20	Ado-Ekiti	White	none
			WCA_14	Ado-Ekiti	White	none
			WCA_31	Ado-Ekiti	White	none
P. putida	*P1*	*P. monteilli* DSM 14164T	RCA_68	Ado-Ekiti	Red	none

			RCA_67	Ado-Ekiti	Red	none
			NSE4	Umudike	White	none
			WCA_27	Ado-Ekiti	White	none
			WCA_2	Ado-Ekiti	White	none
			WCA_3	Ado-Ekiti	White	none
			WCA_1	Ado-Ekiti	White	none
			RCA_28	Ado-Ekiti	Red	none
			RCA_27	Ado-Ekiti	Red	none
			RCA_69	Ado-Ekiti	Red	none
			NSE3	Umudike	White	none
			RCA_72	Ado-Ekiti	Red	none
			RCA_43	Ado-Ekiti	Red	none
			RCA_45	Ado-Ekiti	Red	none
			RCA_44	Ado-Ekiti	Red	none
			WCA_4	Ado-Ekiti	White	none
			RCA_47	Ado-Ekiti	Red	none
			RCA_46	Ado-Ekiti	Red	none
			RCA_66	Ado-Ekiti	Red	none
			RCA_71	Ado-Ekiti	Red	none
			NSE2	Umudike	White	none
			NSE5	Umudike	White	none
P. putida	*P2*	*P. mosselii* ATCC BAA-99T	WCA_13	Ado-Ekiti	White	Xantholysin
			WCA_17	Ado-Ekiti	White	Xantholysin
			WCA_30	Ado-Ekiti	White	none
P. putida	*P3*	*n/a*	WCU_29	Umudike	White	none
			WCU_28	Umudike	White	none
			WCU_27	Umudike	White	none
			WCU_26	Umudike	White	none
			WCU_25	Umudike	White	none
			WCU_24	Umudike	White	none
			WCU_23	Umudike	White	none
			WCU_22	Umudike	White	none
			WCU_21	Umudike	White	none
			WCU_20	Umudike	White	none
			WCU_19	Umudike	White	none

WCU_18	Umudike	White	none
WCU_17	Umudike	White	none
WCU_16	Umudike	White	none
WCU_58	Umudike	White	none
WCU_82	Umudike	White	none
WCU_81	Umudike	White	none
WCU_80	Umudike	White	none
WCU_79	Umudike	White	none
WCU_78	Umudike	White	none
WCU_83	Umudike	White	none
WCU_92	Umudike	White	none
WCU_91	Umudike	White	none
WCU_89	Umudike	White	none
WCU_88	Umudike	White	none
WCU_87	Umudike	White	none
WCU_86	Umudike	White	none
WCU_85	Umudike	White	none
WCU_84	Umudike	White	none
WCU_90	Umudike	White	none
WCU_60	Umudike	White	N2
WCU_61	Umudike	White	N2
WCU_62	Umudike	White	N2
WCU_63	Umudike	White	N2
WCU_64	Umudike	White	N2
WCU_65	Umudike	White	N6
WCU_66	Umudike	White	N2
WCU_67	Umudike	White	N2
WCU_68	Umudike	White	N2
WCU_59	Umudike	White	none
WCU_52	Umudike	White	none
WCU_55	Umudike	White	none
WCU_56	Umudike	White	none
WCU_36	Umudike	White	none
WCU_41	Umudike	White	none
WCU_44	Umudike	White	none

			WCU_45	Umudike	White	none
			WCU_39	Umudike	White	none
			WCU_37	Umudike	White	none
			WCU_42	Umudike	White	none
			WCU_40	Umudike	White	none
			WCU_47	Umudike	White	none
			WCU_46	Umudike	White	none
			WCU_48	Umudike	White	none
			WCU_49	Umudike	White	none
			WCU_38	Umudike	White	none
			WCU_50	Umudike	White	none
			WCU_43	Umudike	White	none
			WCU_53	Umudike	White	none
			WCU_57	Umudike	White	none
			WCU_51	Umudike	White	none
			WCU_54	Umudike	White	none
			WCU_31	Umudike	White	none
			WCU_30	Umudike	White	none
			WCU_32	Umudike	White	none
			WCU_35	Umudike	White	none
			WCU_34	Umudike	White	none
			WCU_33	Umudike	White	none
			RCA_55	Ado-Ekiti	Red	N2
			RCA_53	Ado-Ekiti	Red	N2
			RCA_52	Ado-Ekiti	Red	N2
			RCA_48	Ado-Ekiti	Red	N2
			RCA_51	Ado-Ekiti	Red	N2
			RCA_49	Ado-Ekiti	Red	N2
			RCA_50	Ado-Ekiti	Red	N2
			RCA_54	Ado-Ekiti	Red	N2
P. putida	*P4*	*n/a*	NSE1	Umudike	White	WLIP
			RCA_39	Ado-Ekiti	Red	WLIP
			RCA_40	Ado-Ekiti	Red	WLIP
			RCA_41	Ado-Ekiti	Red	WLIP
			RCA_42	Ado-Ekiti	Red	WLIP

			RCA_70	Ado-Ekiti	Red	N2 + xantholysin
P. putida	*P5*	*P. japonica* JCM 21532T	WCU_71	Umudike	White	none
			WCU_72	Umudike	White	none
			WCU_73	Umudike	White	none
			WCU_74	Umudike	White	none
			WCU_75	Umudike	White	none
			WCU_76	Umudike	White	none
			WCU_77	Umudike	White	none
			WCU_70	Umudike	White	none
			WCU_7	Umudike	White	none
			WCU_8	Umudike	White	none
			WCU_9	Umudike	White	none
			WCU_10	Umudike	White	none
			WCU_11	Umudike	White	none
			WCU_12	Umudike	White	none
			WCU_13	Umudike	White	none
			WCU_15	Umudike	White	none
			WCU_69	Umudike	White	none
			WCU_93	Umudike	White	none
			WCU_14	Umudike	White	none
			WCU_94	Umudike	White	none

*n/a: Strains cluster separately from described type strains.

Chapter 8

General Discussion and Future Perspectives

Authors

Feyisara Eyiwumi Olorunleke and Monica Höfte

Importance of major thesis findings and general discussion

Cyclic lipopeptides are biosurfactant molecules which directly interact with cell membranes and possess antimicrobial activity. In general, this thesis focuses on cyclic lipopeptides that were characterized from the rhizosphere of the tropical cocoyam (*Xanthosoma sagittifolium*) tuber crop, and their biological activity against fungal and oomycete diseases of bean, Chinese cabbage and cocoyam. The first part of this thesis focused on the regulation, secretion and biocontrol of CLPs (orfamides and sessilins) produced by *Pseudomonas* sp. CMR12a. In addition to CLPs, the efficacy of phenazines for biocontrol was evaluated against *Pythium*-mediated root rot of cocoyams and *Rhizoctonia* diseases of bean and Chinese cabbage. In the second section, several CLPs (six of which are new) were characterized from fluorescent *Pseudomonas* spp. that were isolated from the cocoyam rhizosphere from suppressive soils (in Cameroon) and conducive soils (in Cameroon and Nigeria). Besides studying the diversity of these *Pseudomonas* strains and their affiliated CLPs, the antagonistic capacity of representative CLP-producing strains and their purified CLPs were tested against *Pythium* root rot of cocoyams and the mycelium of *P. myriotylum*, respectively.

Fluorescent Pseudomonads exhibit an enormous metabolic versatility and especially isolates of the *P. fluorescens* complex produce a remarkable spectrum of secondary metabolites including CLPs and phenazines. Within the *Pseudomonas* group, two main lineages have been recognized including the *P. aeruginosa* (*P. aeruginosa*, *P. oleovorans* and *P. stutzeri* group) and the *P. fluorescens* (*P. fluorescens* complex and *P. syringae, P. lutea*, *P. putida*, *P. anguilliseptica*, *P. straminea* group) lineage. Till date, characterized CLPs have been affiliated with *Pseudomonas* isolates situated within the *P. fluorescens* lineage specifically in the *P. fluorescens* complex and *P. putida* and *P. syringae* groups. In this thesis, the taxonomic affiliation of our *Pseudomonas* isolates and species association of characterized CLPs were further studied. Furthermore, probable interactions of the *Pseudomonas* isolates/CLPs with soil physicochemical properties were investigated. The major findings of this thesis will be discussed in view of the research questions formulated in the Problem statement and research outline section.

Table 8.1. Summary of *Pseudomonas* strains and CLPs tested during this thesis with information about their mode of action(s) (Chapters 3, 6 and Appendix).

Strain	Metabolite*	Pathogen	Mode of action	Crop	Assay**	Method used***
CMR12a	Sessilin	*R. solani*	Direct antagonism	Bean, Chinese cabbage	*In vivo*	Mutant analysis
		P. myriotylum	Hyphal lysis and vacuolisation	Cocoyam	*In vivo*, *in vitro*	Mutant analysis, pure compound
CMR12a	Orfamide	*R. solani*	Hyphal branching	Bean, Chinese cabbage	*In vivo*, *in vitro*	Mutant analysis, pure compound
		P. myriotylum	Hyphal lysis and vacuolisation	Cocoyam	*In vivo*, *in vitro*	Mutant analysis, pure compound
CMR12a	PCN	*R. solani*	Direct antagonism	Bean, Chinese cabbage	*In vivo*, *in vitro*	Mutant analysis, pure compound
		P. myriotylum	Hyphal bloating, lysis and vacuolisation	Cocoyam	*In vivo*, *in vitro*	Mutant analysis, pure compound
COR51	Xantholysin		Hyphal branching			
COR5	Entolysin		Hyphal branching			
COW10	WLIP		Hyphal branching			
COR10	Lokisin	*P. myriotylum*	Hyphal lysis	Cocoyam	*In vivo*, *in vitro*	Strain, pure compound
COW5	N1		Hyphal lysis			
COW8	N2		Hyphal lysis			
COR35	N3		Hyphal lysis			

*PCN: phenazine-1-carboxamide; WLIP: white-line-inducing principle.
***In vivo*: soil assays.
***strain, pure compound: CLP-producing strains and pure compounds were used in soil and in *in vitro* experiments, respectively.

Research question 2. Phenazines, sessilins and orfamides, produced by *Pseudomonas* sp. CMR12a are important in the biocontrol of Rhizoctonia diseases on bean and Chinese cabbage. Do phenazines and cyclic lipopeptides play a role in the biocontrol capacity of *Pseudomonas* sp. CMR12a against Pythium-mediated cocoyam root rot disease (CRRD)?

An effective biocontrol strain against fungal and oomycete pathogens of tomato, cocoyam, bean and rice (De Jonghe, 2006; Perneel *et al.,* 2007; D'aes *et al.,* 2011; Ma *et al.,* 2016a), the red cocoyam isolate, *Pseudomonas* sp. CMR12a, was found to suppress *Pythium*-mediated root rot on cocoyams (Chapter 3). Table 8.1 presents a summary of *Pseudomonas* strains and metabolites tested against plant pathogens during this study. Using mutant analysis and purified/crude metabolites, we found that phenazines, orfamides and sessilins are vital determinants in the direct antagonism of CMR12a against CRRD (Chapter 3). In plant experiments, mutants which produced either of all three metabolites could suppress CRRD and excellent control was also provided by mutants which produced either a combination of any two of the metabolites or sessilins alone. Similarly, at nanomolar concentrations, all compounds caused mycelial cell damage of *P. myriotylum*. However, a different scenario emerged when CMR12a and biosynthetic mutants were used in a *R. solani* pathosystem with Chinese cabbage and common bean (See appendix). Although a CMR12a mutant that produced only phenazines suppressed damping-off of Chinese cabbage to a comparable extent as the wild type, this sole phenazine producer was impaired in the suppression of bean root rot. Furthermore, in both crops, the phenazine mutant that produced both CLPs was equally effective whereas mutants that produced only one CLP completely lost biocontrol activity. This result was corroborated by microscopic assays involving filtered culture supernatants of CMR12a and its biosynthetic mutants together with the mycelia of *R. solani* AG 2-1 and AG4-HGI. Crude sessilins were not tested against both anastomosis groups of *R. solani*. In contrast with our results with *P. myriotylum* (Chapter 3), a dose-dependent response to micromolar concentrations of PCN and orfamides was observed in mycelial interactions with *R. solani* AG2-1 (Appendix). Conversely, when challenged with orfamides, extensive mycelial branching of *R. solani* AG4-HGI was only observed upon application of 100 µM concentration and not lower whereas no mycelial effect was observed when this pathogen was challenged with purified PCN.

Put together, the differences shown by sessilin, orfamides and phenazines on the different pathogens may reflect differences in the membrane compositions between these pathogens or their abilities to resist or inactivate these CLPs. In general, peptides are involved in membrane-associated processes including disruption of plasma membranes and can even target key cellular processes. This they do by binding to lipid bilayers which facilitate membrane permeabilization and pore formation (Nguyen *et al.,* 2011). A previous study demonstrated that the CLP, syringomycin, bound preferably to β-1, 3-glucan rather than to chitin (De Lucca *et al.,* 1999). Given that chitin is the main polysaccharide in the fungal cell walls whereas the cell wall polysaccharides of *Pythium aphanidermatum* for instance, comprised 82% of (1→3), (1→6)-β-D-glucans (Blaschek *et al.,* 1992), it appears that CLPs display a degree of preference for *Pythium* cell walls which makes them more suited for the control of *Pythium*-mediated soil borne diseases. In our study, this might explain why either sessilins or orfamides alone were able to suppress *Pythium*-mediated CRRD in soil assays in contrast with the *R. solani* pathogens of Chinese cabbage and bean (Chapter 3 and Appendix). Using purified sessilins, orfamides and other

CLPs characterized during this study, future studies can be employed to target the control of economically important oomycetes such as *Phytophtora fragariae* on strawberry and *Pythium splendens* on tomato.

On the other hand, we cannot rule out the effect of substrate on the efficacy of phenazines and CLPs in the different pathosystems. Previous studies showed that the use of different substrates resulted in variable biocontrol capacities by CMR12a and mutants (D'aes, 2012; Hua and Höfte, 2015). During our study, cocoyam experiments were conducted using a potting soil and sand mixture in a 3:1 ratio whereas a 1:1 ratio of the same was used during the Chinese cabbage and bean experiments. In any case, not only were our results more or less consistent within the specific substrate combinations tested, interactions of phenazines and CLPs with the mycelia of our test pathogens gives strong support to the results we obtained during *in vivo* experiments.

From a practical point of view, sessilin and orfamide CLPs presents attractive molecules that can be utilized either alone or in a balanced, physiologiologically relevant proportion, in biocontrol formulations against *Pythium* and *Rhizoctonia*-mediated soil borne diseases.

Research question 3. In several CLP producing *Pseudomonas* spp, LuxR-type transcriptional regulators have been implicated in regulation of their corresponding CLPs. In *Pseudomonas* sp. CMR12a**,** is the regulation of orfamide and sessilins mediated by LuxR-type regulators flanking the orfamide and sessilin biosynthetic gene cluster, respectively?

In plant beneficial *Pseudomonas* spp, the functional role of LuxR-type regulators flanking CLP biosynthesis genes have only been studied for strains which produce a single CLP. However, in plant pathogenic species, so far, studies have been conducted to elucidate the role of three LuxR regulators in the biosynthesis of syringomycin and syringopeptin in *Pseudomonas syringae* pv. syringae (Lu *et al.,* 2002; Wang *et al.,* 2006; Vanghn and Gross, (2016)).

Similar to most CLP gene clusters, the orfamide gene cluster of CMR12a is flanked up- and downstream by two *luxR*-type regulatory genes, *ofaRup* and *ofaRdown*, respectively. In contrast, the sessilin gene cluster has only one *luxR*-type regulatory gene, *sesR*, located upstream of its *sesABC* genes. We showed that the LuxR-type regulators, OfaRup and OfaRdown, co-regulate the biosynthesis of both sessilins and orfamides. Moreover, we were not able to clearly decipher the role of SesR (Chapter 4). LuxR-type transcriptional regulators situated both up-and downstream of CLP gene clusters have been demonstrated to regulate orfamides (Ma *et al.,* 2016a) and viscosin (de Bruijn *et al.,* 2009) production. Moreover, in *P. syringae* pv. *syringae*, all three LuxR regulators namely SyrG, SyfR and SalA were shown to be important for syringomycin biosynthesis (Vanghn and Gross, 2016). In view of these findings, it would be interesting to study the roles of similar LuxR regulators in beneficial *Pseudomonas* strains such as *P. fluorescens* In5 and *P. corrugata* SH-C52, which produce more than one CLP. Moreover the role of SesR in the regulation of CLPs produced by CMR12a may be investigated further using qRT-PCR of site-directed mutants designed within this study (Chapter 4).

As we discussed in Chapter 4, RT-PCR results of the orfamide gene cluster indicates that *ofaA* is separately transcribed from the *ofaBC* transcript. In certain CLP gene clusters, for example viscosin

and massetolide, the first biosynthetic gene is not physically linked with the rest thereby forming two separate biosynthetic gene clusters (de Bruijn *et al.,* 2008). Quantitative PCR (qPCR) results further showed that the transcription of *massA* is independent of *massBC* and vice versa. Although genome mining of CMR12a genome showed that *ofaABC* genes are physically linked, we observed the independent transcription of *ofaA* which was not the case for *sesA* belonging to the sessilin gene cluster. Thus, we hypothesize that for some CLP gene clusters, the first biosynthetic gene is independently transcribed.

Worthy of note was the co-production of orfamides and sessilins coupled with the retention of orfamide in cells while sessilins were predominantly secreted. This observation suggest that sessilin secretion might be favored by CMR12a. The secretion of these two CLPs were elucidated in the next research question.

Research question 4. How are sessilins and orfamides secreted in *Pseudomonas* sp. CMR12a?

Using phylogenetic analyses, we showed that CLP-associated NodT and their corresponding OprM proteins display high amino acid similarity indicating that the *nodT* results from a gene duplication of the *oprM* (Chapter 5). Given the fact that the MexAB-OprM has been implicated in the antimicrobial resistance capacity of clinical and plant pathogenic strains, *P. aeruginosa* and *P. syringae*, respectively (Li *et al.,* 1995; Stoitsova *et al.,* 2008), the occurence of an evolutionary relationship between NodT and OprM further indicates that both of them may be involved in CLP secretion.

Our results show that for sessilin secretion, the *nodT*, *oprM, macAB and mexAB* genes were important although in varying degrees (Chapter 5). Compared to CMR12a, *nodT*, *macAses and mexA* mutants secreted less amounts of sessilin at 17 h of bacterial growth suggesting that these genes are only vital at the early growth phase of the bacterium. At no other time point could a secretory function be attributed to the proteins encoded by the afore-mentioned genes. NodT belongs to a family of outer membrane proteins which form a transmembrane secretion complex together with two inner membrane-associated proteins (Evans and Downie, 1986; Rivilla *et al.,* 1995). NodT shares significant homology with TolC, the outer membrane protein of *E. coli* which is responsible for the secretion of hemolysin and colicin V (Rivilla *et al.,* 1995). Conducting site-directed mutagenesis of *nodT* gene in other CLP-producing strains will clarify their role in CLP secretion which of course may vary from strain to strain. Furthermore, in *P. aeruginosa*, separate mutations in *mexA* and *oprM* alone resulted in hypersensitivity of the strain to certain antibiotics and accumulation of drugs in the cells (Poole *et al.,* 1995). Comparable results were obtained during our study in which the *oprM* mutant of CMR12a recorded a significant decrease in sessilin secretion for up to 41 h indicating its vital role in the transport of sessilin.

Furthermore, the double mutant gene combinations of *nodT-oprM*, *mexAB*, *macAB*, *macA-mexA* and *mexA-macA* of CMR12a no longer secrete sessilins and this often corresponded with an increase in orfamide secretion by these mutants (Chapter 5). In Chapter 5, we observed that for both orfamide and sessilin biosynthetic gene clusters, the *macA* gene was transcribed earlier and much stronger than *macB*. This may account for the ready coupling of *macA* with *mexB* for sessilin secretion. In

summary, our study reports the roles played by several tripartite combinations for the export of sessilin in CMR12a. They include the MexAB-OprM, MexAMacB-OprM/NodT, MacAB-NodT/OprM, MacAMexB-OprM/NodT. Of all these combinations, the MexAB-OprM which utilizes a proton-motive force and the NodT-OprM system, appear to be the preferred route for sessilin secretion by CMR12a. Thus, for the first time, our study highlights the importance of these genes in secondary metabolite (CLP) secretion in *Pseudomonas* spp specifically, in the plant beneficial CMR12a strain. Furthermore, our results suggest a high level of flexibility being displayed by the genes involved in sessilin secretion. For example, it appears that when MexA is absent, MacB may route sessilin transport via MacA or vice versa.

In order to facilitate our understanding about the genes mediating orfamide secretion, site-directed mutagenesis of putative transport genes should preferably be conducted using a strain which produces orfamides alone and also possesses a *nodT* gene upstream of its orfamide biosynthetic cluster. Thus, a good choice strain could be *P. protegens* CHA0 or *P. protegens* Pf-5.

Research questions 5 and 6. Is *Pseudomonas* diversity on the cocoyam roots driven by plant variety? What are potential drivers of *Pseudomonas* and CLP diversity in the cocoyam rhizosphere in Nigeria and Cameroon?

During this study, we investigated the diversity of fluorescent *Pseudomonas* spp. in the cocoyam rhizosphere in Cameroon and Nigeria (Chapters 6 and 7). In the andosols of Cameroon, three cocoyam fields in three different villages namely Boteva, Ekona and Maumu were sampled. Previous studies reported that Boteva, Ekona and Maumu soils had low, intermediate and high disease pressures with respect to *Pythium*-mediated CRRD, respectively (Adiobo *et al.,* 2007; Adiobo, unpublished). On the other hand, in Nigeria, cocoyam roots were sampled from conducive alfisols and ultisols of Ado-Ekiti and Umudike, respectively. Figure 8.1 summarizes the taxonomic diversity of *Pseudomonas* isolates obtained from all five different fields.

Although our diversity study in Chapter 6 was premised on the hypothesis that the red cocoyam rhizosphere was colonized by *Pseudomonas* isolates which were taxonomically and metabolically diverse from those associated with roots of white cocoyams, our results showed that this is not the case. Rather, we observed taxonomic diversity along the lines of soil disease-pressure levels since low disease-pressure soils of Boteva recorded the presence of significantly higher abundance of *P. fluorescens* complex isolates compared to soils of Ekona and Maumu. In view of these results, we conducted a similar diversity study on cocoyam roots from two Nigeria soils. Intriguingly, we were unable to recover any *P. fluorescens* complex-related isolates from cocoyam roots of Ado-Ekiti and Umudike.

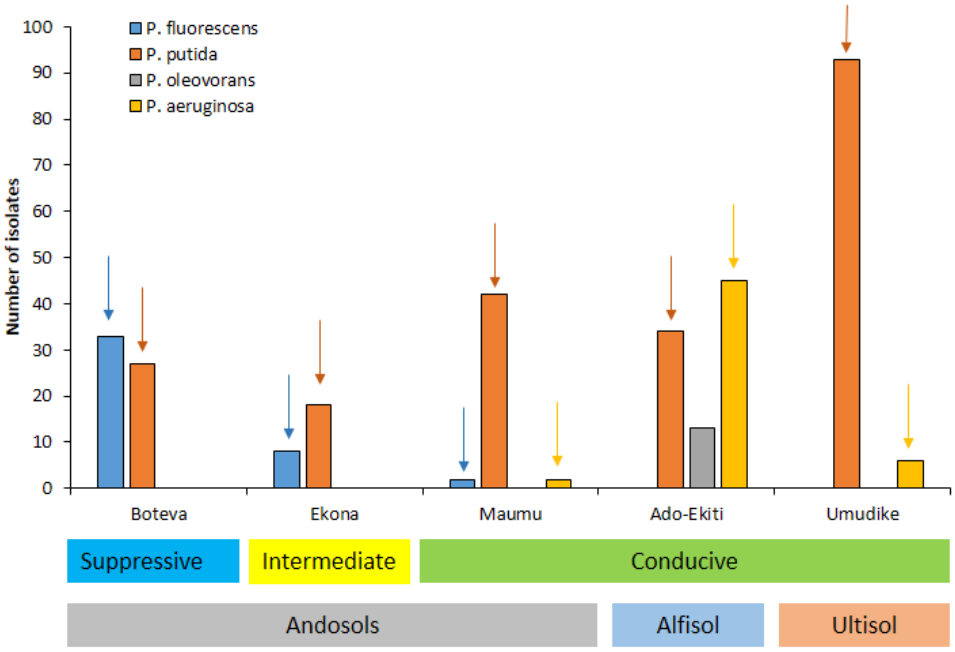

Figure 8.1. Taxonomic diversity of *Pseudomonas* strains associated with the cocoyam rhizosphere in Cameroon (Boteva, Ekona and Maumu) and Nigeria (Ado-Ekiti and Umudike).

On the other hand, disease conducive soils of Maumu, Ado-Ekiti and Umudike were enriched in *P. putida* group isolates. Additionally, compared to Maumu and Umudike soils, significantly higher numbers of *P. aeruginosa* isolates were isolated from the cocoyam rhizosphere at Ado-Ekiti. More so, besides isolates affiliated with the *P. aeruginosa* group, isolates belonging to the *P. oleovorans* group were also identified. Given that the *P. oleovorans* group belongs to the *P. aeruginosa* lineage, it appears that the cocoyam rhizosphere at Ado-Ekiti was colonized by both members of the *P. putida* group and *P. aeruginosa* lineage. Although the reason for the predominance of *P. aeruginosa* lineage isolates at this location is currently unclear, we hypothesize that variable factors including cropping history and mixed cropping may play a role. In view of the fact that *P. oleovorans* strains can grow on intermediate chain length *n*-alkanes by virtue of the OCT plasmid-encoded alkane hydroxylase which it possesses (Witholt *et al.,* 1990), we hypothesize that Ado-Ekiti soils are possibly rich in *n*-alkanes.

Our results suggest that the capacity for isolates belonging to the *P. fluorescens* complex to colonise cocoyam roots in Boteva may be linked to the soil nutrient status (Chapter 6). Under field conditions, physicochemical soil properties can strongly influence root morphology and root exudation (Neumann *et al.,* 2014). Furthermore, different soil types with different physicochemical soil properties could influence root growth patterns and root exudation which may subsequently impact the composition and function of rhizosphere-microbial communities (Kuramae *et al.,* 2012; Schreiter *et al.,* 2014). During this thesis, physicochemical soil properties that were observed in CRRD conducive soils (Chapters 6 and 7) were comparable with those characteristic of CRRD-conducive Ferralsols of Cameroon (Adiobo *et al.,* 2007). Thus, we opine that in comparison with Boteva, soils of Ekona, Maumu, Ado-Ekiti and Umudike which were lower in Ca, organic carbon, total nitrogen and silt, lack the capacity to supply adequate nutrition to the cocoyam plant. This in turn may affect plant growth and vigor thereby influencing the quality and composition of root exudates. We conducted plant

experiments in which acclimatized tissue culture cocoyam plants were grown under controlled conditions in Ado-Ekiti and Umudike soils and *Pseudomonas* diversity of the rhizosphere were evaluated after two weeks. Results revealed that for both soils, cocoyam plants were efficiently colonized by *Pseudomonas* isolates predominantly belonging to the *P. fluorescens* complex in addition with isolates affiliated with the *P. putida* group and *P. aeruginosa* lineage (data not shown). In the light of these findings, we suggest that both CRRD conducive and suppressive soils contain bacteria isolates affiliated with the *P. fluorescens* complex but that the determinants for root colonization by these species are dependent on a cascade/myriad of factors including soil texture, chemical characteristics and plant nutrition, health, age and root exudation. Future studies that employ metagenomics in characterizing soil bacterial communities, will enable the dissection of these factors in more detail. Bacterial communities in the rhizosphere of field-grown lettuce were found to depend on soil type and plant growth developmental stage (Schreiter *et al.* 2014). Compared with diluvial sandy soil, soils with more loam appeared to have similar microbial bulk soil communities while the highest rhizospheric effect was registered on lettuce plants grown in alluvial loamy soils. Similarly, under greenhouse conditions and in different soils, soil type dependent composition of bacterial communities were observed in the rhizosphere of *Arabidopsis thaliana* (Bulgarelli *et al.,* 2012; Lundberg *et al.,* 2012).

Furthermore, although the majority of our *P. fluorescens* complex isolates clustered within known subgroups within the complex, several others did not (Chapter 6). For example, within the *P. fluorescens* complex, the U1, U2, U3 and U4 category isolates appeared exclusive, although, U1 and U2 were closely associated with *P. corrugata* and *P. jessenii* groups, respectively. U3 and U4 appear to cluster outside the *P. fluorescens* complex. Incidentally, during this study, two genes (*rpoD* and *rpoB*) were used to elucidate *Pseudomonas* taxonomic diversity in Chapter 7 while only the *rpoD* gene was used in the follow-up study (Chapter 7). To clearly determine the subgroup affiliations for U1, U2, U3 and U4 groups, more genes such as the 16S rRNA and *gyrB* will need to be sequenced. Herein lay a limitation of our study.

Considering the abundance of *P. putida* isolates that were found on the cocoyam rhizosphere during this study (Chapters 6 and 7), we opine that unlike the *P. fluorescens* complex, the *P. putida* group is better adapted to most soils. Irrespective of conduciveness/suppressiveness soil type, *P. putida* isolates were found in significant quantities on all plant roots sampled. Previous characterization of *Pseudomonas* diversity on the black pepper rhizosphere grown in red basalt soils in Vietnam revealed that all isolates belonged to the *P. putida* group (Tran *et al.,* 2008).

Similar to what we observed in the taxonomic distribution of our *P. fluorescens* complex-affiliated isolates (Chapter 6), many *P. putida* isolates obtained during this thesis (Chapters 6 and 7) did not cluster with known *P. putida* isolates but formed new clusters suggesting that they are probably new species. Thus, we propose that the taxonomy of the *P. putida* group be reviewed and probable designation into subgroups may be appropriate.

The tropical cocoyam rhizosphere: a CLP goldmine

Pseudomonas isolates from tropical environments have been underexplored and may form an untapped reservoir of interesting secondary metabolites. Previous studies have identified CLPs from tropical soils including putisolvins (Tran *et al.,* 2008; Kruijt *et al.,* 2009), xantholysin (Li *et al.,* 2013), WLIP (Rokni-Zadeh *et al.,* 2012), sessilins and orfamides (D'aes *et al.,* 2014; Ma *et al.,* 2016a). Besides the large scale study conducted to investigate the taxonomic and CLP diversity of *Pseudomonas* isolates associated with black pepper rhizosphere in Vietnam (Tran *et al.,* 2008) and the characterization of fluorescent pseudomonads on rice and banana roots in Sri Lanka (Vlassak *et al.,* 1992), to our knowledge, no such scale of study has been conducted on other tropical crop(s) or soils.

During this thesis, the percentage of CLP-producing isolates obtained was remarkable. Isolates from Boteva and Ekona recorded the highest (50-60%) followed by Maumu (35%), Ado-Ekiti (20%) and Umudike (10%). Higher CLP producers (60%) were found in sandy Danish soils compared to loam soils (approximately 6%) (Nielsen *et al.,* 2002). However, our study does not indicate a similar scenario. Further studies involving variable soil types and crops are necessary in order to elucidate factors which influence CLP abundance on the rhizosphere.

Intriguingly, in Cameroon, we found at least seven structurally different CLPs in the cocoyam rhizosphere via NMR characterization (Chapter 6). These CLPs include WLIP, xantholysin, entolysins, lokisin and three novel molecules designated N1, N2 and N3 (Figure 8.2). Interestingly, preliminary characterization of CLP-producing strains from the cocoyam rhizosphere in Nigeria further revealed the presence of at least three structurally different CLPs namely WLIP, xantholysin and the predominantly N2 producers which were characterized in Chapter 7 (Chapter 7). This is in contrast with results reported by Tran and colleagues, 2008 in which all *Pseudomonas* isolates obtained from the black pepper rhizosphere in Vietnam belonged to the *P. putida* group and all biosurfactant-producing isolates make putisolvins alone. Furthermore, in temperate soils, such high diversity of CLPs (nine different groups) have previously been reported in the rhizosphere of sugar beet in Denmark (Nielsen *et al.,* 2002). Although in this study, four of the CLP groups belonged to the amphisin family namely tensin, hodersin, amphisin, lokisin while two other groups comprised viscosinamide producers and a CLP with similar molecular weight as the WLIP. The last two CLP groups had two peaks representing two different surfactants. It is of great interest that all *Pseudomonas* isolates characterized in this study were affiliated with the *P. fluorescens* complex (Nielsen *et al.,* 2002). However, during our study, all CLPs that were found belong to different CLP families and were dispersed within both the *P. fluorescens* complex and the *P. putida* group.

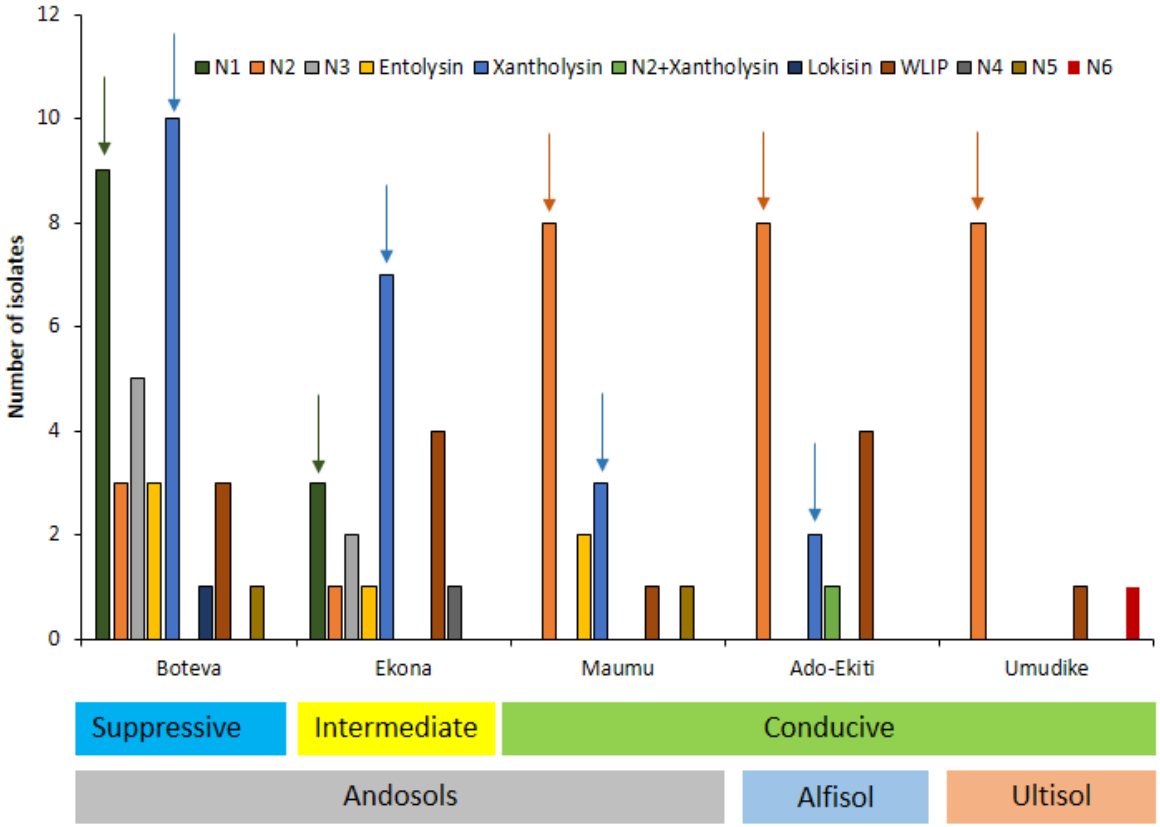

Figure 9.2. Cyclic lipopeptide diversity of *Pseudomonas* isolates associated with the cocoyam rhizosphere in Cameroon (Boteva, Ekona and Maumu) and Nigeria (Ado-Ekiti and Umudike). N1, N2 and N3 are three new CLPs that were characterised by NMR. N4, N5 and N6 are new CLPs whose structures are yet to be fully elucidated by NMR.

On one hand, suppressive soils of Boteva were enriched in N1-producing isolates which were less abundant in the intermediate soils of Ekona (Figure 8.2). On the other hand, conducive soils of Maumu, Ado-Ekiti and Umudike were enriched in N2-producing *Pseudomonas* isolates. Thus, we hypothesize that the synthesis of N1 and N2 are somewhat linked with soil nutrition. We also opine that plants grown in nutrient-rich and poor soils may be selectively colonized by *Pseudomonas* isolates which encode for specific metabolites. More so, xantholysin producers were more abundant at Boteva and appeared to fade with increasing disease pressure and reduced organic matter. In general, Boteva soils registered the highest CLP diversity (eight), next was Ekona (seven), Maumu (five), Ado-Ekiti (four) and Umudike (three). WLIP and xantholysin were supported by all soil types sampled suggesting that the biosynthesis of these CLPs were probably independent of soil types or whatever factors influence CLP selection in these environments. Furthermore, we were only able to detect entolysin producers in andosols. The only previously reported entolysin producer, *P. entomophilia* L48^{T}, was isolated from the fruit fly, *Drosophila melanogaster*, at Calvaire, Guadeloupe Island. It would be interesting to conduct a genome comparison between our entolysin-producing strains and *P. entomophilia* L48^{T} in order to obtain insights into the acquisition and biosynthesis of this CLP. Yet to be characterized CLPs, N4, N5 and N6, were found at Ekona, Maumu and Umudike, respectively. The CLP diversity within these tropical soils continues to amaze.

Additionally, we identified a strain which produced both N2 and xantholysin (Chapter 7). Moreover, in Nigeria soils, all N2-producers found appeared to produce a second CLP (m/z 1212) designated N6, in minute amounts. Interestingly, this secondary CLP is the primary CLP produced by WCU_65 which is considered to be novel (Chapter 7). A few studies have reported the occurrence of more than one CLP in *Pseudomonas* spp (Michelsen *et al.,* 2015; Van der Voort *et al.,* 2015; D'aes *et al.,* 2014; and Serra *et al.,* 1999) However, this has mainly been reported in plant pathogenic *Pseudomonas* strains and a few plant beneficial species (Chapter 1). Strains which produce two CLPs have only been reported to occur within the *P. fluorescens* complex but not in the *P. putida* group. Thus, we report for the first time, a co-occurrence of xantholysin with another CLP, in this case N2. Given that N2 producers also produce minute amounts of another CLP, we hypothesize that the production of dual CLPs may likely occur in several *Pseudomonas* species and could be mediated by the physiological and/or ecological requirements of the producing strain. It is plausible that both CLPs are dependent on each other.

Additional drivers of Pseudomonas and CLP diversity

Our studies suggest that a combination of abiotic factors may contribute to *Pseudomonas* diversity on the cocoyam rhizosphere. We hypothesize that CLP diversity is driven by the same factors. However, there is a need to verify this hypothesis by investigating the *Pseudomonas* and CLP diversity of different crops grown in soils with variable physical and chemical properties. More so, in Danish soils, *Pseudomonas* diversity on the sugar beet rhizosphere was investigated after growing seeds in soil for seven days (Nielsen *et al.,* 2002). Results showed that all isolates obtained belonged to the *P. fluorescens* complex. Thus, we cannot exclude the role of plant age and its subsequent effect on root exudation as a driver of *Pseudomonas* diversity. Therefore, future studies can be done to investigate the *Pseudomonas* diversity on the cocoyam crop (and other selected crops) according to variable plant ages. *Pseudomonas* succession, if any, can be monitored as plants age. Previous studies showed that plant age impact the progression of bacterial community succession in the arabidopsis rhizosphere (Micallef et al., 2009a; Micallef et al., 2009b).

CLPs are produced by different Pseudomonas species

In general, CLPs are produced within the *P. fluorescens* lineage comprising the *P. fluorescens* complex, *P. putida* group and the *P. syringae* group. In Table 8.2, we show the CLP distribution across the *P. fluorescens* lineage including the CLPs that were characterized during this study. Here, we observe the CLP robustness of the *P. fluorescens* complex in comparison with *the P. putida* and *P. syringae* groups. Intriguingly, within the *P. fluorescens* complex, some taxonomic subgroups contain *Pseudomonas* isolates which produce biocontrol CLPs and others that produce plant pathogenic CLPs. For example, the *P. fluorescens* and *P. corrugata* groups comprise both plant pathogenic (tolaasin, corpeptin and cormycin) and biocontrol (viscosin, massetolide, poaeamide, thanapeptin, etcetera) CLPs. Furthermore, the *P. syringae* group is typical for plant pathogenic CLPs including syringopeptin and syringomycin while the *P. putida* group consists of biocontrol CLPs such as xantholysin, entolysin and putisolvin. Given the fact that plant pathogenic CLPs sometimes function in

biocontrol for example, tolaasin and syringopeptin, the borderline between plant pathogenic and biocontrol strains/CLPs appears not to be clear cut. Besides, there is a possibility that biocontrol CLPs which occur within the *P. corrugata* group (thanamycin, thanapeptin and sclerosin) may in fact show pathogenicity symptoms when tested on other host crops. This hypothesis should be tested in future studies.

During our study, we fully characterized entolysin, WLIP, xantholysin, lokisin and three new CLPs designated N1, N2, and N3 (Chapter 6). As shown in the Table 8.2, entolysin and xantholysin are exclusively produced by strains in the *P. putida* group whereas lokisin is produced by isolates belonging to the *P. fluorescens* complex. On a different note, the new CLPs N1, N2 and N3 occurred within both the *P. putida* group and the *P. fluorescens* complex (Chapter 6). More so, N1 was also produced by strains located within the U3 and U4 taxonomic categories. Interestingly, this type of phenomenon has only been shown by the WLIP (Rokni-Zadeh *et al.,* 2013). The WLIP is produced by *Pseudomonas* isolates belonging to the *P. fluorescens* complex (Mehnaz *et al.,* 2013; Rokni-Zadeh *et al.,* 2013) and within the *P. putida* group (Rokni-Zadeh *et al.,* 2012). Furthermore, previous studies showed that WLIP biosynthesis genes were acquired by producing strains via convergent evolution (Rokni-Zadeh *et al.,* 2013). More so, comparative analysis of the genetic backbones of this CLP revealed that the NRPS systems of its different producing strains were divergent. Thus, it would be interesting to investigate if N1, N2 and N3 were acquired within their producing species either via convergent evolution or through horizontal gene transfer.

Table 8.2. CLP distribution among *Pseudomonas* isolates belonging to the *P. fluorescens* lineage. CLPs marked in asterisk were also characterised in this study.

		P. fluorescens complex								
CLP	AA	*P. protegens*	*P. fluorescens*	*P. corrugata*	*P. chlororaphis*	*P. koreensis*	*U3**	*U4**	*P. putida*	*P. syringae*
Syringafactin	8									■
Cichofactin	8									■
Viscosin	9		■							
Massetolide	9		■							
Syringomycin	9									■
Syringotoxin	9									■
Pseudomycin	9									■
WLIP*	9				■			■	■	
Cormycin	9			■						
Thanamycin	9			■						
Orfamide	10	■								
Poaeamide	10		■							
Lokisin*	11					■				
N1*	11					■	■	■	■	
N2*	11					■			■	
N4*	n/a					■	■			
N5*	n/a							■		
N6*	n/a								■	
Putisolvin	12									
Entolysin*	14									
Xantholysin*	14									
N3*	17					■			■	
Tolaasin	18		■							
Sessilin	18	■								
Corpeptin	22			■						
Cichopeptin	22									■

Thanapeptin	22		
Sclerosin	22		
Syringopeptin22	22		
Syringopeptin25	25		

*Biocontrol and plant pathogenic CLPs are marked in green and orange, respectively.

Application of research findings towards CRRD Management

In the light of previous research on CRRD together with this thesis, *Pythium*-mediated CRRD suppression was only reported on soils that have higher organic matter compared with conducive soils. Farming in Africa is often complex. Farmers mostly cultivate degraded soils. More so, they are faced with variability in soil fertility, poor infrastructure, expensive inputs and to top it up, pests, diseases and weeds all of which culminates in reduced crop yield. In some cocoyam farms in Nigeria for example, farmers often use NPK fertlizers in order to boost productivity of cocoyams on degraded soils like the ones in Umudike (Chapter 7) but without much effect. For some years now, Integrated Soil Fertility Management (ISFM) piloted by the International Institute of Tropical Agriculture (IITA), has been implemented and is gradually gaining ground in Sub-Sahara Africa. Given that some soils (termed non-responsive) do not respond to fertilizer applications, a co-application of fertilizers and organic inputs are deemed necessary. Thus, ISFM is "the application of soil fertility management practices, and the knowledge to adapt these to local conditions, which maximize fertilizer and organic resource use efficiency and crop productivity". These practices necessarily include appropriate fertilizer and organic input management in combination with the utilization of improved germplasm and good agronomic practices (Vanlauwe *et al.,* 2015). So far, these approaches have been implemented in crops such as maize (Vanlauwe and Zingore, 2011) and cassava (Ettien *et al*., 2014) with incredible success. Soil fertility management enhances soil biological activity and optimizes nutrient cycling to minimize external inputs and maximize their use efficiency (Sanchez, 1994). This will in turn boost plant health and make them less vulnerable to pathogen attack.

Drawing from the success stories of the afore-mentioned initiatives, we propose the following strategy for the management of CRRD:

Improvement of soil fertility: CRRD incidence in Nigeria and Cameroon soils have been shown to correlate with low amounts of Ca, organic carbon and total nitrogen in such soils. In the tropics, on one hand, soil fertility can be improved by reducing tillage intensity which may have long term effects on soil carbon and labile organic carbon fractions (Prasad *et al.,* 2016). Furthermore, the practice of shifting cultivation which is gradually becoming uncommon due to inadequate land should be encouraged especially in the interior villages where families have extensive land ownership. On the other hand, using cover crops and soil amendments such as composts can enhance soil fertility and physicochemical properties. More so, in acid alfisols, the combined use of NPK and organic manures enabled the realization of higher crop yields of *Colocasia esculenta* (Laxminarayana, 2016) and enhanced the physicochemical properties of a fluvo-aquic soil (Xin *et al.,* 2016). Previous studies also showed that the incorporation of composts on cocoyam fields significantly reduced CRRD severity (Adiobo, 2006; Chukwu and Eteng, 2014). Thus, we recommend that besides the use of NPK, and cover crops, readily available compost materials from oil palm and sugar cane residues, for example, be put to good use in order to enhance the organic matter content of these depleted soils.

Stimulation of beneficial rhizobacteria: In a tomato cropping system, cover crops were shown to influence the soil microbial community structure which may in turn contribute to plant health (Maul *et al.,* 2014). Therefore, for CRRD-conducive soils which are also poor in physicochemical properties, the utilization of cover crops and soil amendments will help to promote plant growth thereby stimulating beneficial bacterial population around the cocoyam rhizosphere.

Extension services: Increased understanding of how agricultural factors influence the soil health together with the soil and rhizospheric community structure will enable land owners to make appropriate decisions that ensure natural resource management. Cocoyam growers should be advised to dissuade from practices which result in organic matter reduction of the soil such as tillage and bush burning, for example. Furthermore, efforts should be made to ecourage knowledge sharing across countries and localities. Agricultural research stations should liaise with other researchers across West and Central Africa who experience common problems and pool resources together to meet the needs of local farmers. Moreover, since the use of infected plant material continues to be a bane to effective management of CRRD, extension services should also target these crucial factors and promote the use of healthy clean planting material.

Future Perspectives

Pseudomonas sp. CMR12a: further genome mining

Of particular interest was the fact that during plant experiments with cocoyam, the CMR12a null mutant which makes neither phenazines, sessilins nor orfamides, still showed a degree of CRRD suppression (Chapter 3). Besides the production of phenazines and two CLPs, genome mining of CMR12a revealed the presence of a tabtoxin biosynthesis gene cluster which is situated on the genomic island which harbors among other genes, both the sessilin and phenazine gene clusters. Although CMR12a produces HCN and chitinases which might also contribute to the biocontrol capacity of this strain, we opine that the disease suppression that was observed in a *Pythium-cocoyam* pathosystem was probably mediated by a metabolite that preferably targets the cell membranes of oomycetes since we did not observe a similar scenario in the biocontrol of *Rhizoctonia* diseases. Thus, in order to investigate the function of tabtoxinine-β-lactam encoded by this gene cluster, a deletion mutant for this gene should be constructed in the null mutant background of CMR12a. The mutant generated should be compared with the null mutant strain in a similar pathosystem as the previous.

CMR12a is a unique biocontrol strain which produces several antimicrobial metabolites (D'aes, 2012; D'aes *et al.,* 2014). This strain was obtained in a tropical humid rainforest which is characterized by a long rainy season, it would naturally posessess genes that would enable its resilience under such conditions. Genome mining of CMR12a revealed the presence of genes that might function in its adaptation and survival such as those involved in denitrification and methylotrophy (De Maeyer *et al.,* 2012). Recent taxonomic studies of the *P. fluorescens* complex places the *Pseudomonas* sp. CMR12a in the *P. protegens* subgroup (Garrido-Sanz *et al.,* 2016). Within this group, other orfamide producers are present including *P. protegens* CHA0, *P. protegens* CMR5c and *P. protegens* Pf-5. So far, no biocontrol strain present in this group has been found to produce sessilins. A recent study in Turkey used 16S rRNA pyrosequencing and shotgun metagenome analyses to identify microorganisms involved in the degradation of benzalkonium chlorides (BACs) (Ertekin *et al.,* 2016). During this study, the most relevant strain was identified and subsequently named *Pseudomonas* sp. BIOMIG1. Interestingly, this strain had *Pseudomonas* sp. CMR12a as its closest identity. Subsequently, we conducted preliminary genome mining of the whole genome sequence of *Pseudomonas* sp. BIOMIG1 and found that one of the sessilin NRPS modules of CMR12a shows 99% identity with a hypothetical protein with the Turkish isolate. Given that in CMR12a, the sessilin gene cluster was acquired via horizontal gene cluster (D'aes *et al.,* 2014), it would be interesting to compare the genome of *Pseudomonas* sp. BIOMIG1 with that of CMR12a. Among other findings, such study will enable us to gain insights into the biological roles of sessilin, in addition with its regulation and secretion networks in the absence of orfamide co-production as it occurs in CMR12a.

Functional characterization of new CLPs

Several natural roles have been proposed for CLPs and other biosurfactants including a function in antimicrobial activity, regulation of attachment and detachment to and from surfaces, and motility (Ron and Rosenberg, 2001; Raaijmakers *et al.,* 2006; de Bruijn *et al.,* 2008). Furthermore, some CLPs contribute to rhizosphere competence (massetolide A -Tran *et al.,* (2008), amphisin-Nielsen *et al.,* (2005)) whereas some do not (putisolvin-Kruijt et al. 2008). Some contribute to biofilm formation (viscosin and massetolide A- de Bruijn *et al.,* 2007, 2008) and some inhibit biofilm formation while enhancing surface motility (putisolvin-(Kuiper *et al.,* 2004; Kruijt *et al.,* 2008) and arthrofactin – Roongsawang *et al.,* 2003). For instance, in CMR12a, a clear separation of duties have been reported for both CLPs - orfamides are responsible for swarming motility while sessilins are involved in biofilm formation by the strain (D'aes *et al.,* 2014).

During this thesis, whole genomes of representative CLP-producing isolates were sequenced, assembled and annotated. Thus, further work should focus on making biosynthetic mutants especially for the new CLPs discovered during this thesis. These mutants will enable us to decipher the functional roles of these CLPs in the producing strains and their influences on root colonization capacity, biofilm formation, swarming motility and biological control for example.

Revisiting the White line-inducing principle (WLIP)

The *in vitro* interaction between the pathogenic *P. tolaasii* and another *Pseudomonas* bacterium, referred to as "*reactans*" resulted in the formation of a sharply defined white line which has been considered to be specific (Wong and Preece, 1979; Lo Cantore *et al.,* 2006). Years after, the same CLP termed the WLIP has been characterized in several *Pseudomonas* isolates (Rokni-Zadeh *et al.,* 2012; Mehnaz *et al.,* 2013). During our study, we characterized seven CLPs and further identified some unknowns namely N4, N5 and N6 (Chapters 6 and 7). Using *P. reactans*, *P. tolaasii* and *P. protegens* Pf-5 as indicator strains, we tested representative isolates producing these CLPs for white line-in-agar interaction. *P*. *protegens* Pf-5 is an orfamide producer that also interacts with *P. tolaasii* or the sessilin-producing *Pseudomonas* sp. CMR12a strain to give a white line (Chapter 5 and 6). Our results showed that in addition to our WLIP-producing test strain, several other CLP producers gave a white line (Table 8.3).

Table 8.3. Reaction between *Pseudomonas* strains for white line-in-agar test response

Pseudomonas strain	*P. tolaasii* 1919^T	*P. reactans* 587	*P. protegens* Pf-5
SBW25 (viscosin)	+	-	-
SS101 (massetolide)	+	-	-
CMR5c (orfamide)	+	-	-
CMR12a (sessilin and orfamide)	-	+	+
COW10 (WLIP)*	+	-	-
COW3 (N3)*	+	-	-
COW5 (N1)*	+	-	-
COR19 (N2)*	+	-	-
COR10 (lokisin)*	-	-	-
COR5 (entolysin)*	+	-	-
COR51 (xantholysin)*	-	-	-

*These strains were characterized during this thesis.

Strains which produce viscosin (SBW25), massetolide (SS101), orfamide (CMR5c), N1 (COW5), N2 (COR19), N3 (COW3) and entolysins (COR5) gave white line phenotypes when challenged with *P. tolaasii*. As expected, only CMR12a, the sessilin producer gave a white line with *P. reactans* since sessilins are tolaasin-like. More so, sessilins gave a similar reaction with Pf-5 as observed before (Chapters 5 and 6). In a previous study, pseudomonads associated with the cultivated mushroom *Agaricus bisporus*, also yielded a white line precipitate when they were streaked towards *P. tolaasii* LMG 2342^T (Munsch and Alatossava, 2002). Since the interaction of WLIP strains with *P. tolaasii* to give a white line is not specific as was originally supposed, an investigation into the underlying mechanism and relevance of the white line to the strains in question vis-à-vis *P. tolaasii* would be worthwhile.

Primer list

Primer name	**Sequence** (5' → 3')	**Source**	**Chapter**
Mutagenesis			
OfaRUp-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGGAAGTCGTGAAAGGCCAGT	This study	5
OfaRUp-Up-R	GCTGTTCTTGACGCTCAGGGAGGTTGCTGCTCAGACTCA	This study	5
OfaRUp-Down-F	TGAGTCTGAGCAGCAACCTCCCTGAGCGTCAAGAACAGC	This study	5
OfaR-UpDown-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATTTCAGTGTGCGACTCAATCC	This study	5
OfaRDown-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGGCTGCCTTCACCTTCTATGC	This study	5
OfaRDown-Up-R	CTCACTCAGGTTTGCTGCTGATGACCTTGCCAATGTGAGG	This study	5
OfaRDown-Down-F	CCTCACATTGGCAAGGTCATCAGCAGCAAACCTGAGTGAG	This study	5
OfaRDown-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCGTCAGCCACCTGTACTTCA	This study	5
SesR-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGCTTGAGGCCAAAGACCAGAC	This study	5
SesR-Up-R	CACTTGGTCAATCCATGTCG TGAATGCTGCTCGTCATTTC	This study	5
SesR-Down-F	GAAATGACGAGCAGCATTCACGACATGGATTGACCAAGTG	This study	5
SesR-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATAACCAGCAACGTCAGGCTAT	This study	5
NodT-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGCAGGATCACTTCGCACTCAA	This study	6
NodT-Up-R	CTGCTGTGACGAAAACAACGCAACCTCTTCGCTGACGGTA	This study	6
NodT-Down-F	TACCGTCAGCGAAGAGGTTGCGTTGTTTTCGTCACAGCAG	This study	6
NodT-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCGTATGAATTCCACCGGTCT	This study	6
NodT-F	CAGGATCACTTCGCACTCAA	This study	6
NodT-R	CGTATGAATTCCACCGGTCT	This study	6
OprM-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGGGTCGGGTGAAGAAGGTGTA	This study	6
OprM-Up-R	AAGGTCAGGTTGCTGTCGACGGTATCAGCGAGCAACCACT	This study	6
OprM-Down-F	AGTGGTTGCTCGCTGATACCGTCGACAGCAACCTGACCTT	This study	6
OprM-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCAAGCTGATCACCGATATGC	This study	6
OprM-F	GGTCGGGTGAAGAAGGTGTA	This study	6
OprM-R	CAAGCTGATCACCGATATGC	This study	6

MacAses-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTATCTGCAGGCACTGGAACA	This study	6
MacAses-Up-R	ATCACCAGGGAGAACACGAACAAATACTGGGGTGGCTCAG	This study	6
MacAses-Down-F	CTGAGCCACCCCAGTATTTGTTCGTGTTCTCCCTGGTGAT	This study	6
MacAses-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATGAGCTGGGTATCGTGCTGAT	This study	6
MacAses-F	TATCTGCAGGCACTGGAACA	This study	6
MacAses-R	GAGCTGGGTATCGTGCTGAT	This study	6
MacABses-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTATCTGCAGGCACTGGAACA	This study	6
MacABses-Up-R	ATCACCAGGGAGAACACGAACAAATACTGGGGTGGCTCAG	This study	6
MacABses-Down-F	CTGAGCCACCCCAGTATTTGTTCGTGTTCTCCCTGGTGAT	This study	6
MacABses-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATGAGCTGGGTATCGTGCTGAT	This study	6
MacABses-F	TATCTGCAGGCACTGGAACA	This study	6
MacABses-R	GAGCTGGGTATCGTGCTGAT	This study	6
MacABofa-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGCATCACTGACCCTGATCGAC	This study	6
MacABofa-Up-R	GAACACCATCTCCCACTCCTCCTTGACCTTGAGGGACTTC	This study	6
MacABofa-Down-F	GAAGTCCCTCAAGGTCAAGGAGGAGTGGGAGATGGTGTTC	This study	6
MacABofa-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCTCACATTGGCAAGGTCATC	This study	6
MacABofa-F	CATCACTGACCCTGATCGAC	This study	6
MacABofa-R	CTCACATTGGCAAGGTCATC	This study	6
MexA-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTCAGCACCAGTTCGATATGC	This study	6
MexA-Up-R	GCAGTCTTGTCAGTTGCCTGAGGTAAAGGCTTGTGGTTGC	This study	6
MexA-Down-F	GCAACCACAAGCCTTTACCTCAGGCAACTGACAAGACTGC	This study	6
MexA-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCGAACCATCCTTGTTGACCT	This study	6
MexA-F	TCAGCACCAGTTCGATATGC	This study	6
MexA-R	CGAACCATCCTTGTTGACCT	This study	6
MexAB-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTCAGCACCAGTTCGATATGC	This study	6
MexAB-Up-R	AGCATACCGCCAATTACACCAGGTAAAGGCTTGTGGTTGC	This study	6
MexAB-Down-F	GCAACCACAAGCCTTTACCTGGTGTAATTGGCGGTATGCT	This study	6
MexAB-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATGCGTTTTCCACTGAGGTACG	This study	6
MexAB-F	TCAGCACCAGTTCGATATGC	This study	6
MexAB-R	GCGTTTTCCACTGAGGTACG	This study	6

Complementation			
NodTEcoRI-F	GTG**GAATTC**ATGAACAAGTTTGCTTTCTC (EcoRI)	This study	6
NodT-R	GTG**AGATCT**TCATGGCCGCGTCTCGGCAG (BglII)	This study	6
OprM_F	GTG**GAATTC**ATGAGCAAGTCGCTACTCTC (EcoRI)	This study	6
OprM-R	GTG**AGATCT**TTAGAACAGGTGCAGCGCGG (BglII)	This study	6
MacAses-F	GTG**GGTACC**ATGGAAAAGTCGAAGTTTCG (kpnI)	This study	6
MacAses-R	GTG**AGATCT**TCAGCTCCCCGCCACGGCCG (bglII)	This study	6
MexA-F	GTG**GGTACC**ATGCAATTCAAGCCAGCTGT(kpnI)	This study	6
MexA-R	GTG**AGATCT**TTACTCCCCTTTGCCGCCTG (bglII)	This study	6
Characterisation			
PsEG30F	ATYGAAATCGCCAARCG (*rpoD*)	Mulet *et al.*, 2009	7, 8
PsEG790R	CGGTTGATKTCCTTGA (*rpoD*)	Mulet *et al.*, 2009	7, 8
*rpoD*1F	GACGACAGCGAGGAAGAGG	This study	7, 8
*rpoD*1R	CGGGAAATACGGTTGAGCTT	This study	7, 8
rpoB	CAGTTCATGGACCAGAACAACCCGCT	Frapolli *et al.*, 2007	7
rpoB	CCCATCAACGCACGGTTGGCGTC	Frapolli *et al.*, 2007	7

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Addendum

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

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**Joint contribution*

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Summary

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 *Rhizoctonia 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 CMR12a, while its efficacy to reduce root rot on bean was strongly impaired. In both pathosystems, 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 tandem with the CLPs on bean.

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 *Pseudomonas* (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 their 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*., 2008; 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 phenazine-1-carboxylate (PCA) and its main phenazine compound, phenazine-1-carboxamide (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 D-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 *Rhizoctonia solani* (D'aes *et al.,* 2011), respectively. In a *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.

Results and Discussion

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 A1 for details) that still produced phenazines [CMR12a-Clp1 (S), CMR12a-ΔClp2 (O) and CMR12a-ΔClp2-Clp1 (OS)] or both CLPs [CMR12a-ΔPhz (P)] (Figure A1).

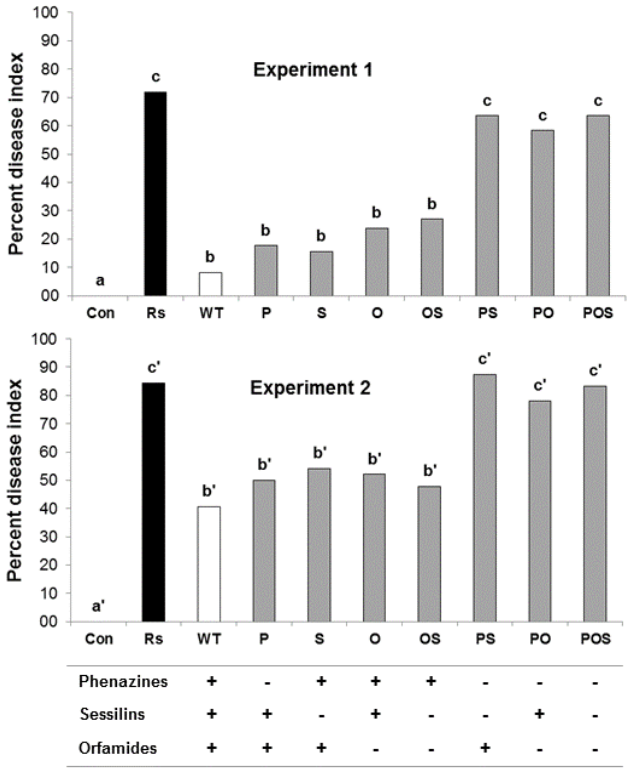

Figure A1. Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production towards Rhizoctonia damping-off on Chinese cabbage in two independent experiments. Con: healthy control**;** WT: wild-type *Pseudomonas* sp. CMR12a; Rs: *R. solani* AG 2-1; P: phenazine mutant; S: sessilin mutant, O: orfamide mutant. Mutant abbreviations are further explained in Table A1. The table below the figure indicates metabolites that are still produced by the various mutants.

When mutants that only produced orfamides [CMR12a-ΔPhz-Clp1 (PS)] or sessilins [CMR12a-ΔPhz-ΔClp2 (PO)] were applied, no biocontrol effect was observed. Likewise, a mutant unable to produce phenazines and CLPs [CMR12a-ΔPhz-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.

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 A2). 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 (SO) or one class of CLPs (PS, PO) showed a drastically reduced biocontrol activity.

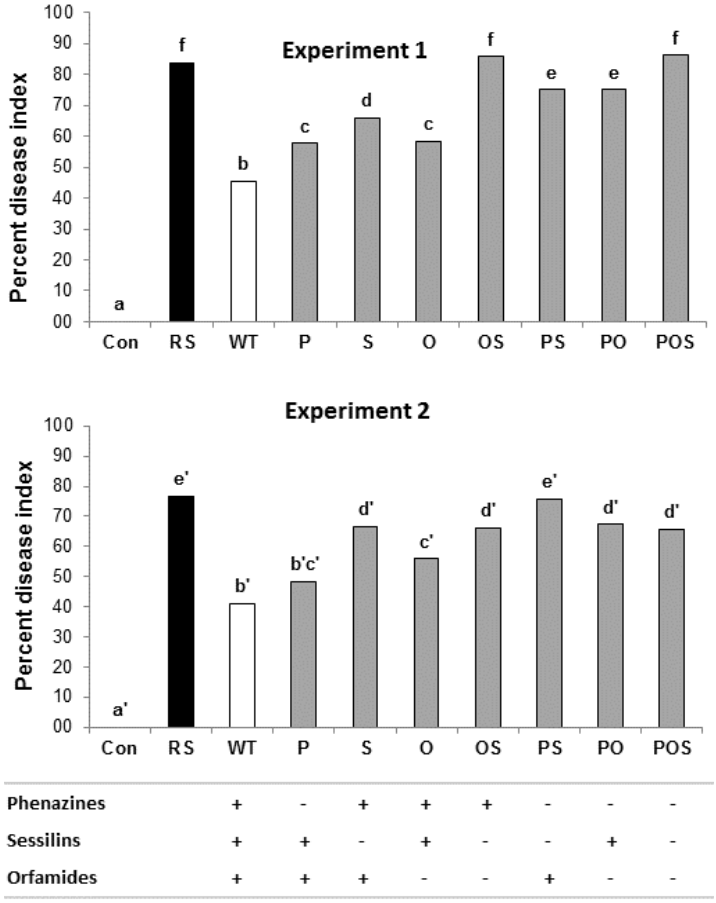

Figure A2. Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production towards Rhizoctonia root rot on bean in two independent experiments. Con: healthy control**;** WT: wild-type *Pseudomonas* sp. CMR12a; Rs: *R. solani* AG 4-HGI; P: phenazine mutant; S: sessilin mutant, O: orfamide mutant. Mutant abbreviations are further explained in Table A1. The table below the figure indicates metabolites that are still produced by the various mutants.

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).

Table A2. Root colonisation data of *Pseudomonas* sp. CMR12a and mutants impaired in phenazine and/or CLP production obtained from roots of bean seedlings seven days after inoculation with R. solani AG4-HGI.

		Population density of CMR12a and mutants[a] (in log CFU g-1 of fresh root)	
Treatment	Phenazines and CLPs produced	Repetition 1	Repetition 2
CMR12a	Phenazines, sessilins and orfamides	6.5 ± 0.2 c	6.1 ± 0.3 ab
CMR12a-ΔPhz	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-CLP1-ΔCLP2	phenazines	5.9 ± 0.3 a	6.2 ± 0.3 ab
CMR12a-ΔPhz-CLP1	orfamides	6.1 ± 0.2 ab	6.4 ± 0.4 b
CMR12a-ΔPhz-ΔCLP2	sessilins	6.2 ± 0.2 b	5.9 ± 0.3 a
CMR12a-ΔPhz-CLP1-ΔCLP2	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 A3. Root colonisation data of *Pseudomonas* sp. CMR12a and mutants impaired in phenazine and/or CLP production on roots of Chinese cabbage grown in sand.

		Population density of CMR12a and mutants[a] (in log CFU g-1 of fresh root)	
Treatment	Phenazines and CLPs produced	Repetition 1	Repetition 2
CMR12a	Phenazines, sessilins and orfamides	6.8 ± 0.3 a	8.3 ± 0.1 a
CMR12a-ΔPhz	sessilins and orfamides	6.7 ± 0.4 a	8.3 ± 0.3 a
CMR12a-CLP1	phenazines and orfamides	6.9 ± 0.2 a	8.3 ± 0.2 a
CMR12a-ΔCLP2	phenazines and sessilins	7.2 ± 0.1 bc	8.9 ± 0.3 c
CMR12a-CLP1-ΔCLP2	phenazines	7.3 ± 0.1 a	8.8 ± 0.2 c
CMR12a-ΔPhz-CLP1	orfamides	6.8 ± 0.1 a	8.4 ± 0.3 a
CMR12a-ΔPhz-ΔCLP2	sessilins	7.3 ± 0.1 c	8.9 ± 0.3 c
CMR12a-ΔPhz-CLP1-ΔCLP2	Null	7.1 ± 0.2 b	8.6 ± 0.3 b

[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, valuesfollowed by the same letter are not significantly different according to the Tukey tests (P = 0.05).

Bacterial strains were well established in the rhizosphere of bean and cabbage plants but differed in their ability to colonize roots (Tables A2 and A3). In the bean system, variation was observed among treatments and repetitions in time (Table A2). 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 A3) 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 A2). 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.

In vitro antagonistic activity 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 A3A). No inhibition zones were formed between fungus and bacteria when mutants that only produced orfamides (CMR12a-ΔPhz-Clp1) or sessilins (CMR12a-ΔPhz-Clp2) 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 A3A).

In vitro antagonistic activity of phenazine-1-carboxamide and orfamide B against R. solani AG2-1 and AG4-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 A3B). Higher doses of PCN did not give an enhanced pathogen inhibition (data not shown). Phenazine-1-carboxamide at 1 µM was not effective against *R. solani* AG 4-HGI (Figure A3B) 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).

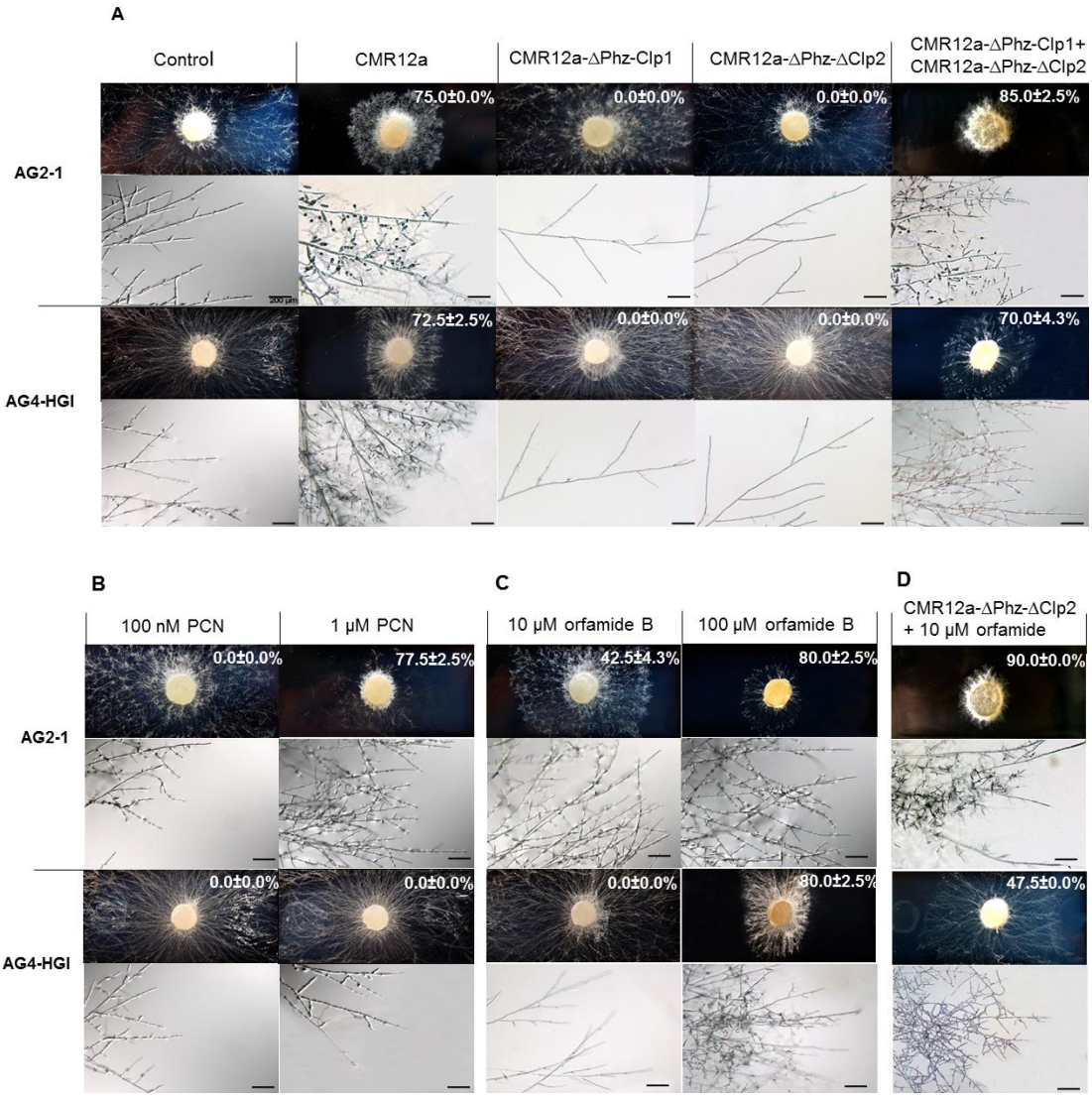

Figure A3. Microscopic assays showing the effect of various treatments on mycelial growth and hyphal branching of *R. solani* AG2-1 and *R. solani* AG4-HGI.

A. Effect of *Pseudomonas* sp. CMR12a or mutants that only produce sessilins (CMR12a-ΔPhz-ΔClp2) or orfamides (CMR12a-ΔPhz-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-ΔPhz-ΔClp2with 10 µM purified orfamide B.

The size bar represents 200 µm in all panels. Values indicate percentage growth inhibition relative to the control ± standard deviation (n = 3).

Pseudomonas sp. CMR12a produces about 140 µM PCN/OD in still Luria-Bertani broth. Since an OD620 of 1 corresponds to about $8x10^8$ CFU/mL, 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 (2012a) reported values for phenazine-1-carboxylate 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 A3C). 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 (Figures A3C). 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 sessilin and phenazine-defective *Pseudomonas* mutants. 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-ΔPhz-ΔClp2) (Figure A3D). Collectively, these results suggest an additive interaction between sessilins and orfamides in the biocontrol of *R. solani*.

Conclusion

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 (Chapter 1). 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.

Materials and Methods

Biocontrol experiment of CMR12a and mutants impaired in phenazine and/or CLP production towards Rhizoctonia damping-off on Chinese cabbage

Pseudomonas strains (Table A1) 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) (see Hua and Höfte, 2015, for details) 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. Seedlings were grown in a controlled-environment chamber at 22°C, relative humidity (RH) = 60%, 12 h photoperiod and watered every two days to maintain soil moisture near field capacity.

Table A1. Microorganisms used in this study

Strains	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-CLP1-ΔCLP2 (SO)	PHZ^+, $CLP1^-$, $CLP2^-$; phenazines	D'aes *et al.,* (2014)
CMR12a-ΔPhz-CLP1 (PS)	PHZ^-, $CLP1^-$, $CLP2^+$; orfamides	D'aes *et al.,* (2011)
CMR12a-ΔPhz-ΔCLP2 (PO)	PHZ^-, $CLP1^+$, $CLP2^-$; sessilins	D'aes *et al.,* (2014)
CMR12a-ΔPhz-CLP1-ΔCLP2 (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. CLP production in CMR12a and mutants was analyzed using 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 A1 and A2.

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. 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).

Biocontrol experiment of CMR12a and mutants impaired in phenazine and/or CLP production towards Rhizoctonia root rot on bean

Pseudomonas strains were produced in a similar manner as with the Chinese cabbage experiment 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). Seedlings were grown in a controlled-environment chamber at 25°C, RH=60%, 16 h photoperiod and watered every two days to maintain soil moisture near field capacity. 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 as in the Chinese cabbage experiment. 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 colonisation

For the Chinese cabbage experiment, 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 fertilised 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 by crushing roots in sterile saline solution (0.85%) and plating serial dilutions of suspension on KB medium plates. Colonies were counted after 24 to 36 h. Data were log10 transformed before statistical analysis.

For bean experiments, five plants were randomly selected from three replicate boxes per treatment following which the bacterial populations on the roots were determined in a similar manner as with Chinese cabbage. Data of the two experiments were log10 transformed before statistical analyses using Tukey tests.

Microscopic assays showing the effects of different treatments on mycelial growth and branching of R. solani AG2-1 and R. solani AG4-HGI

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) 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). Orfamide B was produced as described by Ma and colleagues, (2016a). Purified PCN was obtained as described by Perneel *et al*. (2008). All plates were incubated for 36 h at 28°C before evaluation under an Olympus BX51 microscope. 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.

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Summary

Cyclic lipopeptides are bacterial metabolites produced by certain bacterial genera including *Pseudomonas* and *Bacillus*. Besides interacting with cell membranes and their antibiotic activity, cyclic lipopeptides have been implicated in biofilm formation, swarming motility and virulence capacity of their producing strains. Within the *Pseudomonadaceae*, fluorescent pseudomonads which produce cyclic lipopeptides with implication in the biocontrol of plant pathogens have been described.

Pseudomonas sp. CMR12a was isolated from the rhizosphere of the tropical tuber crop cocoyam (*Xanthosoma sagittifolium*) in Cameroon and demonstrated biocontrol activity against *Rhizoctonia solani* on bean and *Pythium myriotylum* on cocoyam. CMR12a produces phenazines and two classes of cyclic lipopeptides (sessilins and orfamides). In this thesis, using biosynthetic mutants of CMR12a and pure/semi-purified metabolite compounds, we show that orfamides, sessilins and phenazines are involved in the biocontrol by CMR12a in three different pathosystems. All three metabolites could suppress *Pythium* root rot on cocoyam when they occur alone. However, optimal control was provided by mutant strains that still produced two metabolites or sessilins alone. In experiments with *Rhizoctonia* diseases of Chinese cabbage and bean, mutants, which produced at least two CLPs could suppress *R. solani* root rot. Interestingly, based on *in vitro* microscopic assays, we observed that the effect of phenazines on *R. solani* was dependent on anastomosis group affiliation. Thus, our results highlight that metabolites produced by CMR12a are potent against several pathogens and in different pathosystems but that the effect of each metabolite may vary depending on the pathosystem being tested.

Furthermore, our study shows that in *Pseudomonas* sp. CMR12a, *luxR* genes (*ofaRup* and *ofaRdown*) flanking the orfamide biosynthetic gene cluster, encode transcriptional activators that co-regulate the production of sessilins and orfamides. It appears that the *luxR* gene (*sesR*) upstream of the sessilin gene cluster, assumes a redundant role as was observed in *P. syringae pv. syringae* which also possesses three LuxR transcriptional regulators. In addition, we observe the co-production of sessilins and orfamides although orfamides appear to be secreted much later than sessilins. Besides the regulation of CLPs produced by CMR12a, this study also investigated the efflux systems involved in CLP secretion by this strain. It is assumed that CLPs are secreted by tripartite efflux pumps composed of an inner membrane protein, a membrane fusion protein and an outer membrane protein. Genes encoding putative inner membrane (MacB) and membrane fusion proteins (MacA) are usually located downstream of CLP biosynthetic gene clusters, while the gene encoding a putative outer membrane protein (NodT) is located upstream of some CLP biosynthetic gene clusters. First, we could show that generally, in *Pseudomonas* spp, the occurrence of the CLP-associated *nodT* gene upstream of some CLP gene clusters, is a result of a gene duplication of *oprM*. Moreover, we showed that not only MacAB-NodT, but also the multidrug efflux pump MexAB-OprM is involved in sessilin secretion. Although the MexAB-OprM pump appears to be the preferred system for sessilin secretion, other combinations such as MexAMacB-OprM/NodT, MacAB-NodT/OprM, MacAMexB-OprM and

MacAMexB-NodT can form functional tripartite pumps able to secrete sessilins.. Unfortunately, the genetic route for orfamide secretion could not be explained during this study. However, a loss in sessilin secretion in CMR12a-Δ*nodT*-Δ*oprM*, CMR12a-Δ*mexAB* and CMR12a-Δ*macAB* double mutants resulted in a corresponding increase in orfamide secretion.

Microbial diversity is considered to be influenced by biotic and abiotic factors including plant variety, temperature, soil properties and plant age among others. In the second aspect of this thesis, we investigated the drivers of fluorescent *Pseudomonas* spp. and CLP diversity in the cocoyam rhizosphere in Cameroon and Nigeria. In this study, we established that *Pseudomonas* diversity on the cocoyam roots is independent of plant variety (white or red cocoyams). Interestingly, microbial diversity appeared to occur along the lines of soil disease pressure levels towards the cocoyam root rot disease caused by *Pythium myriotylum*. The rhizosphere of cocoyam plants grown in suppressive soils of Boteva (in Cameroon) appeared to be enriched with members of the metabolitically versatile *P. fluorescens* complex. In contrast, *Pseudomonas* isolates obtained from roots of cocoyam plants grown in intermediate suppressive soils of Ekona (Cameroon), and conducive soils of Maumu (Cameroon), Ado-Ekiti (Nigeria) and Umudike (Nigeria) were predominantly affiliated with the *P. putida* group. All conducive soils recorded the presence of the *P. aeruginosa* lineage whereas members of this group were more abundant at Ado-Ekiti. Moreover, nine structurally different CLPs (xantholysin, WLIP, lokisin, entolysin, N1, N2, N3, N4, N5) were characterized from Cameroon soils out of which five (N1, N2, N3, N4, N5) are new. In Nigeria soils, four structurally different CLPs (xantholysin, WLIP, N2, N6) were found of which two (N2, N6) are new. The CLP robustness of the cocoyam rhizosphere is very striking. Furthermore, the CLPs, WLIP, xantholysin, lokisin, entolysin, N1, N2 and N3 were fully characterized by NMR. Subsequently, using purified compounds of these CLPs, *in vitro* inhibition assays against the mycelia of *P. myriotum* revealed that all CLPs caused either hyphal leakage or extensive hyphal branching.

On the other hand, based on previous results linking soil physicochemical properties to microbial diversity, we compared the soil physicochemical properties of all soils studied. Interestingly, Boteva soils had the highest silt, Ca, organic carbon and total nitrogen. These properties were reduced in other soils in this order: Ekona was next to Boteva followed by Maumu, then Ado-Ekiti while Umudike recorded the least. Intriguingly, CLP and *Pseudomonas* diversity also appeared to follow this trend. The highest CLP diversity was recorded at Boteva and the least at Umudike. Thus, findings of this study suggest the relatedness of *Pseudomonas* and CLP diversity with soil nutrition and plant health. It appears that rich soils enhance plant growth promotion thereby affecting the quality and composition of root exudation which may in turn enable the cocoyam roots to select for a specific *Pseudomonas* population. In our study, it appears that isolates belonging to the *P. putida* group are better adapted to harsh/poor soil conditions whereas *P. fluorescens* complex isolates tend to be more selective and show a preference for cocoyam roots grown in optimal conditions.

Samenvatting

Cyclische lipopeptiden zijn bacteriële metabolieten die geproduceerd worden door bepaalde bacteriële genera zoals *Pseudomonas* en *Bacillus.* Cyclische lipopeptiden kunnen interageren met cellulaire membranen en hebben antibiotische activiteit, maar spelen ook een rol in biofilm vorming, zwermende motiliteit en virulentie. In de familie van de Pseudomonadaceae werden reeds fluorescerende pseudomonaden beschreven die cyclische lipopeptiden produceren die een rol spelen in de biologische bestrijding van plant pathogenen.

Pseudomonas sp. CMR12a werd geïsoleerd uit de rhizosfeer van het tropische knolgewas cocoyam (*Xanthosoma sagittifolium*) in Kameroen en vertoont biocontrole activiteit tegen *Rhizoctonia solani* op boon en *Pythium myriotylum* op cocoyam. CMR12a produceert fenazines en twee verschillende soorten cyclische lipopeptiden (sessilines en orfamides genaamd). In deze thesis hebben we door gebruik te maken van biosynthese mutanten van CMR12a en opgezuiverde metabolieten, aangetoond dat orfamides, sessilines en fenazines betrokken zijn in de biologische bestrijding met CMR12a in drie verschillende pathosystemen. Alle drie de metabolieten konden *Pythium* wortelrot op cocoyam onderdrukken. Een optimale bestrijding werd evenwel bekomen met behulp van mutanten van CMR12a die enkel sessilines of twee van de drie metabolieten produceerden. Mutanten die op zijn minst twee cyclische lipopeptiden (CLPs) produceerden konden wortelrot veroorzaakt door *R. solani* onderdrukken op zowel boon als Chinese kool. Met behulp van microscopische studies *in vitro*, konden we aantonen dat het effect van fenazines op *R. solani* afhankelijk was van de anastomose groep waartoe het *R. solani* isolaat behoort. Onze resultaten hebben aldus aangetoond dat de metabolieten geproduceerd door CMR12a actief zijn tegen verschillende pathogenen in verschillende pathosystemen, maar dat het effect van iedere metaboliet kan variëren naargelang het pathosysteem.

Verder hebben we in deze studie aangetoond dat in *Pseudomonas sp.* CMR12a, de *luxR* genen (*ofaRup* en *ofaRdown)* die de orfamide biosynthese genen flankeren, coderen voor transcriptionele activatoren die de productie van sessilines en orfamides co-reguleren. Er kon geen duidelijke rol worden aangetoond voor het enige *luxR* gen (*sesR*) dat geassocieerd is met de sessiline biosynthese cluster. Dit is analoog met de situatie in *P. syringae* pv. *syringae*, een bacterie die ook drie LuxR-type transcriptionele regulatoren bevat die geassocieerd zijn met de biosynthese genen van cyclische lipopeptiden. Verder hebben we vastgesteld dat sessilines en orfamides tegelijkertijd worden geproduceerd, alhoewel orfamides veel later worden uitgescheiden dan sessilines. In deze studie hebben we ook de efflux systemen die betrokken zijn bij de secretie van CLPs door CMR12a verder onderzocht. Er wordt verondersteld dat CLPs uitgescheiden worden door driedelige efflux pompen die bestaan uit een binnenste membraan eiwit, een membraan fusie eiwit en een buitenste membraan eiwit. Genen die coderen voor putatieve binnenste membraan (MacB) en membraan fusie eiwitten (MacA) bevinden zich doorgaans stroomafwaarts van de CLP biosynthese genen, terwijl het gen dat codeert voor een putatief buitenste membraan eiwit (NodT) zich stroomopwaarts van sommige CLP

biosynthese gen clusters bevindt. We konden aantonen dat in het algemeen in *Pseudomonas* spp. het voorkomen van een CLP geassocieerd *nodT* gen stroomopwaarts van sommige CLP gen clusters het resultaat is van een genduplicatie van oprM. Verder konden we aantonen dat niet alleen MacAB-NodT, maar ook de multidrug efflux pomp MexAB-OprM betrokken is bij de secretie van sessiline. Sessiline wordt preferentieel uitgescheiden door de MexAB-OprM pomp, maar ook andere eiwit combinaties zoals MexAMacB-OprM/NodT, MacAB-NodT/OprM, MacAMexB-OprM en MacAMexB-NodT kunnen functionele pompen vormen die sessiline uitscheiden. Helaas kon de genetische route voor de secretie van orfamides niet achterhaald worden. Het verlies van sessiline secretie in CMR12a--Δ*nodT*-Δ*oprM*, CMR12a-Δ*mexAB* en CMR12a-Δ*macAB* mutanten resulteerde echter in een stijging in orfamide secretie.

Microbiële diversiteit kan beïnvloed worden door biotische en abiotische factoren zoals bijvoorbeeld de plant varieteit, temperatuur, bodemeigenschappen en de plant leeftijd. In het tweede deel van deze thesis hebben we onderzocht welke factoren de diversiteit van fluorescerende *Pseudomonas* spp. en CLPs beïnvloeden in the rhizosfeer van cocoyam gegroeid in gronden in Kameroen en Nigeria. We hebben in deze studie aangetoond dat *Pseudomonas* diversiteit op cocoyamwortels onafhankelijk is van de plant varieteit (witte of rode cocoyam). Microbiële diversiteit bleek echter wel gecorreleerd te zijn met de mate van ziekteweerbaarheid van de verschillende gronden tegen de cocoyam wortelrot ziekte veroorzaakt door *Pythium myriotylum*. De rhizosfeer van cocoyamplanten uit ziektewerende gronden van Boteva (in Kameroen) bleek aangerijkt te zijn met bacteriën uit het *P. fluorescens* complex, een complex dat gekend is door zijn metabolische versatiliteit. Hiermee in contrast bleken de *Pseudomonas* isolaten van de wortels van cocoyam planten die groeiden in de intermediaire ziektewerende gronden van Ekona (Kameroen) en de ziektegevoelige gronden van Maumu (Kameroen), Ado-Ekiti (Nigeria) en Umudike (Nigeria) vooral geaffilieerd te zijn met de *P. putida* groep. In alle ziektegevoelige gronden werden isolaten behorende tot het *P. aeruginosa* lineage aangetroffen, en deze groep kwam het meeste voor in Ado-Ekiti. Verder werden er negen structureel verschillende CLPs (xantholysine, WLIP, lokisine, entolysine, N1, N2, N3, N4, N5) gekarakteriseerd in de gronden van Kameroen. Vijf van deze CLPs (N1, N2, N3, N4, N5) werden nog niet eerder beschreven. In de Nigeriaanse gronden werden vier structureel verschillende CLPs gevonden (xantholysine, WLIP, N2, N6), waarvan er twee (N2, N6) nieuw zijn. De diversiteit aan CLPs in de rhizosfeer van cocoyam is opvallend. De CLPs WLIP, xantholysine, lokisine, entolysine, N1, N2 en N3 werden volledig gekarakteriseerd door NMR. In opgezuiverde vorm veroorzaakten alle CLPs hyfale lekkage of sterke hyfale vertakking van het mycelium van *P. myriotylum.*

Gebaseerd op vroegere resultaten die bepaalde fysicochemische eigenschappen van de bodem linken met microbiële diversiteit, hebben we de fysicochemische eigenschappen van alle bestudeerde gronden vergeleken. Boteva gronden hadden het hoogste leem-, calcium, organische koolstof en totaal stikstof gehalte. Deze gehaltes waren lager in de andere gronden in de volgende volgorde: Ekona kwam vlak achter Boteva, gevolgd door Maumu, dan Ado-Ekiti, terwijl Umudike de laagste gehaltes had. Het was intrigerend dat ook CLP en *Pseudomonas* diversiteit dezelfde trend vertoonden. De hoogste CLP diversiteit werd aangetroffen in Boteva en de laagste diversiteit in

Umudike. Aldus suggereren de resultaten van deze studie dat *Pseudomonas* en CLP diversiteit gerelateerd zijn met bodemvruchtbaarheid en plantgezondheid. Het lijkt erop dat vruchtbare gronden plantengroei bevorderen en op deze wijze de kwaliteit en samenstelling van wortelexudaten beïnvloeden die op hun beurt bepaalde *Pseudomonas* populaties selecteren. Isolaten uit de *P. putida* groep lijken beter aangepast aan ongunstige of arme bodemcondities, terwijl *P. fluorescens* complex isolaten meer selectief zijn en vooral voorkomen op de rhizosfeer van cocoyamplanten die groeien bij optimale condities.

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Curriculum vitae

Personal Information

First name:	Feyisara
Last name:	Olorunleke
Address:	Weverboslaan 6, 9050, Gentbrugge
Date of birth:	July 23, 1980
Place of birth:	Kaduna, Nigeria
Nationality:	Nigerian
Sex:	Female
Married status:	Married to Olaiya Oni
Children:	Victor Oni (28-9-2009) William Oni (01-01-2012)
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Education

2012-2016 Doctoral Program in Applied Biological Sciences
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
Dissertation: Cyclic lipopeptides produced by *Pseudomonas* spp. associated with the cocoyam (*Xanthosoma sagittifolium* (L.) Schott) rhizosphere: diversity, regulation, secretion and biological activity
Promotor: Prof. Dr. ir. Monica Höfte

2009-2011 Master Degree in Nutrition and Rural Development, main subject: Tropical agriculture, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
Dissertation: The role of cyclic lipopeptides in the biocontrol capacity of *Pseudomonas* sp. CMR12a
Promotor: Prof. Dr. ir. Monica Höfte

2007-2009 Master Degree in Nematology**,** Postgraduate International Nematology Course
Faculty of Science, Ghent University, Ghent, Belgium (with Great Distinction)
Dissertation: RNAi against *Radopholus similis*: *In vitro* tests and transgenic Banana cultures
Promotor: Prof. Dr. ir. Godelieve Gheysen

2000-2004 Bachelor Degree in Botany, University of Jos, Jos, Plateau State, Nigeria (with Distinction)
Dissertation: Dry matter Accumulation and Harvest index as influenced by defoliation and N-rate in Maize in Jos, Plateau State, Nigeria
Promotor: Prof. Timothy Namo

Work experience

2012-2016 Doctoral Researcher, Ghent University, Belgium

2005-2006 Graduate Assistant, University of Ado-Ekiti, Ekiti State, Nigeria

2001-2002 Intern, Federal College of Forestry, Jos, Nigeria

Scholarships and awards

Schlumberger Faculty for the Future PhD Fellowship	Sept 2012 – Dec 2016
European Distributed Institute of Taxonomy Summer Course	August 2008
ICP VLIR-UOS (Flemish Interuniversity Council Scholarship)	Sept 2007- Sept 2009
EU COST ACTION-funded short stay at FEM, Italy (10 days)	September 2015

Additional training

Seminars in Nematology, Adam Harpers University College, Shropshire**,** UK	April, 2009
Museum Tour and Nematology Seminars, Natural History Museum, UK	April, 2009
Basic Bioinformatics concepts, databases and tools, Leuven (3 days)	April-May, 2013
Bioinformatic Tools for NRPS discovery. From genomic data to products University of Lille1-Villeneuve d'Ascq, France (2 days)	July, 2013
Introduction to R, Doctoral Training, Ghent University	October, 2013
Postgraduate Intern, Institute for Agricultural Research for Development (IRAD), Cameroon (5 weeks)	March-April, 2014
Erasmus Environmental Arctic Microbiology Course, University of Akureyri, Iceland. Doctoral Training, Ghent University (2 weeks)	June, 2014
COST ACTION-funded short stay at Fondazione Edmund Mach, San Michele All'Adige, Trento, Italy (10 days)	Sept., 2015
qPCR course, Doctoral Training, Ghent University (2 days)	May, 2016

Publications

Peer reviewed

D'aes, J., Kieu, N. P., Léclère, V., Tokarski, C., **Olorunleke, F. E**., De Maeyer, K., Jacques, P., Höfte, M. and Ongena, M. (2014). To settle or to move?: The interplay between two classes of cyclic lipopeptides in the biological control strain *Pseudomonas* CMR12a. Environmental Microbiology, 16: 2282–2300. doi: 10.1111/1462-2920.12462.

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Book chapter

Olorunleke, F. E., Kieu, N. P. and Höfte, M. (2015). Recent advances in *Pseudomonas* biocontrol. In Bacterial-Plant Interactions : Advance Research and Future Trends. Murillo, J., Vinatzer, B. A., Jackson, R. W., and Arnold, D. L. (eds). Cambridgeshire : Caister Academic Press. Pp 167-198.

Proceedings

Olorunleke, F., Adiobo, A., Onyeka, J. T. and Höfte, M. (2014). Cocoyam Root Rot Disease caused by *Pythium myriotylum* in Nigeria. Proceedings of the Conference of International Research on Food Security, Natural Resource Management and Rural Development. Link : http://www.tropentag.de/2014/abstracts/full/632.pdf

Presentations at conferences and symposia

Olorunleke F. E., Onyeka, T.J., Huang, C.J., Nam Phuong Kieu and Höfte, M. (2013). Phylogeny and Characterisation of Biocontrol *Pseudomonas* Isolates associated with Cocoyams in Nigeria. 65th International Crop Protection Symposium, Belgium, May 2013. Poster presentation by Huang C. J.

Olorunleke, F. E. (2013). Epidemiology and Management of Cocoyam Diseases in Nigeria. Schlumberger Faculty for the Future Fellows Forum, Moller Centre, University of Cambridge, United Kingdom, May 20-22, 2013. Poster presentation by Feyisara Olorunleke

Olorunleke, F. E., Geudens, N., Onyeka, T. J., Bertier, L., Nam Phuong Kieu, Huang, C. J., Ongena, M. and Höfte, M. (2014). Diversity and Bioactivity of Biosurfactant-Producing Pseudomonads isolated from Cocoyam rhizosphere in Nigeria and Cameroon. 66th International Symposium on Crop Protection, May 22, Ghent University, Belgium. Oral Presentation by Feyisara Olorunleke

Olorunleke, F., Adiobo, A., Onyeka, J.T. and Höfte, M. (2014). Cocoyam Root Rot Disease caused by *Pythium myriotylum* in Nigeria. Tropentag (Conference of International Research on Food Security, Natural Resource Management and Rural Development), Prague, Czech Republic. Oral Presentation by Feyisara Olorunleke

Olorunleke, F. E., Geudens, N., Martins, J. C. and Höfte, M. (2015). Diversity of Fluorescent *Pseudomonas* sp. associated with the cocoyam rhizosphere and its influence on soil suppressiveness to the root rot disease caused by P. myriotylum. Plant Growth Promoting Rhizobacteria (PGPR) Workshop, University of Liege, June 16-19, 2015. Oral Presentation by Feyisara Olorunleke

Olorunleke, F. E., Geudens, N., Martins, J. C. and Höfte, M. (2015). Fluorescent *Pseudomonas* spp. and cyclic lipopeptides diversity in the cocoyam rhizosphere and their relation with soil suppressiveness against the root rot disease caused by *P. myriotylum*. Rhizosphere4 Conference, Maastricht, the Netherlands. 21-25 June, 2015. Oral Presentation by Professor Monica Höfte

Olorunleke, F. E., Geudens, N., Olorunleke, O. F., Omoboye, O. O., Adiobo, A., Sinnaeve, D., Martins, J. C. and Höfte, M. (2015). Fluorescent Pseudomonas spp. and cyclic lipopeptide diversity in the cocoyam rhizosphere and their relation with soil suppressiveness against the root rot disease. Soilborne Oomycete Conference, Hawks Cay Resort, Duck Keys, Florida, USA. 8th – 10th December, 2015. Oral Presentation by Feyisara Olorunleke

Feyisara Eyiwumi Olorunleke, Niels Geudens, Olumide Omoboye, Lien Bertier, Amayana Adiobo, Joseph Onyeka, Ayodeji Salami, Davy Sinnaeve, Jose C. Martins and Monica Höfte (2016). Rhizosphere diversity of fluorescent pseudomonads and cyclic lipopeptides correlates with cocoyam (*Xanthosoma sagittifolium*) resilience to the *Pythium* root rot disease. Microbial and Plant Systems modulated by Secondary Metabolites Meeting, Joint Genome Institute, Walnut Creek, California, USA. 2nd – 4th May, 2016. Oral presentation by Professor Monica Höfte

Olorunleke, F. E., Omoboye, O. O. and Höfte, M. (2016). Deciphering the evolutionary acquisition of cyclic lipopeptides gene clusters coding for similar metabolites in different *Pseudomonas* spp. Microbial Genomics and Metagenomics Workshop. Joint Genome Institute, Walnut Creek, California, USA. 26th -30th September, 2016. Poster presentation by Olumide Omoboye

Teaching (Practical courses)

2013-2016	Tropical Crop Protection (Master Degree students)
2014-2016	Molecular Microbiology (3rd year Bachelor students)

Supervision of undergraduate/master students

2015-2016 Charlotte de Bruyn - De biosynthese, regulatie en secretie van cyclische lipopeptiden in fluorescerende *Pseudomonas* populaties. Master in de bio-ingenieurswetenschappen: cel- en genbiotechnologie

2014-2015 Margot Coudwijzer – Bestrijding van cocoyam wortelrot ziekte door fluorescente *Pseudomonas*. Bachelor in de agro-en biotechniek biotechnologie, Katholieke Hogeschool, Vives, campus Roeselare

Extracurricular activities

2007-2009	Student member, Educational Committee, Postgraduate International Nematology Course (PINC), Faculty of Science, Ghent University, Belgium
2005-2006	Secretary, Academic Staff Board, Department of Plant Science, University of Ado-Ekiti, Ekiti State, Nigeria