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Fluorescent *Pseudomonas* and cyclic lipopeptide diversity in the rhizosphere of cocoyam (*Xanthosoma sagittifolium*)

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Summary

Cocoyam (*Xanthosoma sagittifolium* (L.)), an important tuber crop in the tropics, is severely affected by the cocoyam root rot disease (CRRD) caused by *Pythium myriotylum*. The white cocoyam genotype is very susceptible while the red cocoyam has some field tolerance to CRRD. Fluorescent *Pseudomonas* isolates obtained from the rhizosphere of healthy red and white cocoyams from three different fields in Cameroon were taxonomically characterized. The cocoyam rhizosphere was enriched with *P. fluorescens* complex and *P. putida* isolates independent of the plant genotype. LC-MS and NMR analyses revealed that 50% of the *Pseudomonas* isolates produced cyclic lipopeptides (CLPs) including entolysin, lokisin, WLIP, putisolvin and xantholysin together with eight novel CLPs. In general, CLP types were linked to specific taxonomic groups within the fluorescent pseudomonads. Representative CLP-producing bacteria showed effective control against CRRD while purified CLPs caused hyphal branching or hyphal leakage in *P. myriotylum*. The structure of cocoyamide A, a CLP which is predominantly produced by *P. koreensis* group isolates within the *P. fluorescens* complex is described. Compared to the white cocoyam, the red cocoyam rhizosphere appeared to support a more diverse CLP spectrum. It remains to be investigated whether this contributes to the field tolerance displayed by the red cocoyam.

Introduction

Fluorescent *Pseudomonas* bacteria have been implicated in the biological control of plant pathogens (Olorunleke *et al.*, 2015a). Multi-locus sequence analysis (MLSA), a method based on sequence analysis of several housekeeping genes, has proven reliable for species delineation and strain identification within the *Pseudomonas* genus (Mulet *et al.*, 2010; Gomila *et al.*, 2015; Garrido-Sanz *et al.*, 2016, 2017). MLSA has enabled the discrimination of the *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* lineages (Garrido-Sanz *et al.*, 2016). The *P. fluorescens* lineage is divided into the *P. fluorescens* complex and the *P. syringae, P. lutea, P. putida*, *P. anguilliseptica* and *P. straminea* groups. Described as being the most complex (Loper *et al.*, 2012; Garrido-Sanz *et al.*, 2016), the *P. fluorescens* complex comprises nine subgroups including *P. fluorescens, P. jessenii, P. corrugata, P. koreensis, P. fragi, P. mandelii, P. gessardi, P. chlororaphis*, and *P. protegens* subgroups. Besides their strong strain-to-strain diversity, fluorescent pseudomonads belonging to the *P. fluorescens* complex are of notable interest as they contain biocontrol strains that produce potent secondary metabolites, including cyclic lipopeptides (CLPs) (Loper *et al.*, 2012; ; Olorunleke *et al.*, 2015a; Garrido-Sanz *et al.*, 2016, 2017).

CLPs are bacterial metabolites composed of a cyclic oligopeptide lactone ring linked to a fatty acid tail (Raaijmakers *et al.*, 2006; D'aes *et al.*, 2010). They possess surfactant activity and can cause destruction of microbial membranes, leading to death of bacteria, fungi, oomycetes and viruses (Geudens *et al.*, 2017). CLPs produced by *Pseudomonas* spp. can act as biocontrol agents against several plant pathogens both in *in vitro* and *in vivo* conditions

(Zachow *et al.*, 2015; Raaijmakers *et al.*, 2006; Olorunleke *et al.*, 2015a; Olorunleke *et al.*, 2015b; Michelsen *et al.*, 2015; Ma *et al.*, 2016a; Ma *et al.*, 2016b).

Cocoyam (*Xanthosoma sagittifolium* (L.) Schott), a monocot tropical tuber crop, is widely cultivated in some parts of Africa, Asia and the Pacifics with Nigeria, Cameroon and Ghana as major producing countries (Purseglove, 1972; Agbede, 2008). Besides containing high 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 (Djeugap *et al.*, 2014). *Xanthosoma* spp. originate from tropical America. In Africa, cocoyam landraces are believed to be introduced by the Portuguese and or West Indian missionaries (Boakye *et al.*, 2018). They are distinguished by the color of the peeled cormels resulting in red (2n=26), white (2n=26) and yellow (2n=52) varieties. Of these three varieties, the yellow does not tuberculize and the white cocoyam is often preferred due to its high yield and good taste. In Cameroon and most other West African countries, there are only two commercially cultivated varieties which are vegetatively propagated: the preferred white cocoyam, which is more susceptible to the cocoyam root rot disease (CRRD), and the red cocoyam which appear to be field tolerant against CRRD (Perneel *et al.*, 2007).

In previous years, biological control has been proven to be 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 (PCA) and phenazine-1-carboxamide (PCN)

(Tambong and Höfte, 2001). Moreover, phenazines and rhamnolipid–biosurfactants, produced by *P. aeruginosa* PNA1, were shown to act synergistically in the control of *Pythium* root rot on cocoyams (Perneel *et al.*, 2008) .

However, there are some serious drawbacks to the use of *P. aeruginosa* PNA1 because of its pathogenicity to humans (Kumar *et al.,* 2013). In search of a safe alternative, a targeted isolation and screening of healthy red and white cocoyam rhizosphere in Cameroon for potential biocontrol agents yielded 40 fluorescent *Pseudomonas* strains. Seven of these isolates showed biocontrol activity against CRRD and were exclusively obtained from the red cocoyam rhizosphere (Perneel *et al.,* 2007). Two of these isolates (*Pseudomonas* sp. CMR12a and CMR5c), are closely related to the *P. protegens* and *P. chlororaphis* subgroups (Flury *et al.,* 2016) and showed excellent biocontrol against CRRD (Perneel *et al.*, 2007). CMR12a produces phenazines and two cyclic lipopeptides namely orfamides and sessilins (D'aes *et al.,* 2014). These metabolites have been shown to be vital in the biological control capacity of CMR12a (Olorunleke *et al.,* 2015b; Hua and Höfte, 2015). Phenazines and CLPs produced by CMR12a were demonstrated to play a role in the suppression of CRRD (Oni et al. in press). These results led us to hypothesize that the red cocoyam rhizosphere is an untapped reservoir of CLP-producing *Pseudomonas* strains towards CRRD. In this study, we characterized 138 *Pseudomonas* isolates previously obtained from the rhizosphere of healthy red and white cocoyam plants in Cameroon. We wanted to determine whether the taxonomic affiliation and cyclic lipopeptide diversity of *Pseudomonas* isolates was dependent on plant genotype.

Materials and methods

Isolation of fluorescent Pseudomonas strains

In view of our hypothesis, a targeted *Pseudomonas* isolation strategy was adopted. *Pseudomonas* isolates were obtained from the rhizosphere of two and a half to three month old healthy cocoyam plants that were grown in three different fields (located in the Boteva, Ekona and Maumu villages) in the South-West province of Cameroon. From each location, 10 red and 10 white cocoyam plants were sampled giving a total of 60 plants. To isolate fluorescent *Pseudomonas*, roots were crushed in 0.85% sterile saline solution and autoclaved sand. Crushed roots were serially diluted and aliquots were plated on King's B agar (King *et al.*, 1954) medium plates. Plates were incubated at 28 °C for 48 h and periodically examined under UV light. One hundred and thirty-eight fluorescent *Pseudomonas* isolates were randomly selected, purified and stored at -80 °C in 20% glycerol. Sixty *Pseudomonas* isolates were obtained from the rhizosphere of red cocoyams while 74 isolates were obtained from the white cocoyam rhizosphere. Strains isolated from the red and white cocoyam variety were designated with COR- and COW- prefixes, respectively. For our current study, these isolates were taken from -80 °C collection and streaked on King's B (KB) medium and incubated at 28 °C overnight. Supplementary Table S1 shows a list of strains used in this study and their characteristics.

DNA-based phylogenetic analyses

The MLSA approach has been reported to be a robust method for the taxonomical inference of the *Pseudomonas* genus (Mulet *et al.*, 2010; Gomila *et al.*, 2015; Garrido-Sanz *et al.*, 2016). Using colony PCR, two housekeeping gene regions, *rpoD* (Mulet *et al.*, 2009) and *rpoB*

(Frapolli *et al.*, 2007) were amplified for each bacterial strain. *rpoB* primers produced a single amplicon with the *Pseudomonas* strains tested. For *rpoD*, some isolates did not give bands, so we designed optimized primers *rpoD*1F/*rpoD*1R (Supplementary Table S2).

Sequences of representative type strains within the *Pseudomonas* genus together with some CLP-producing *Pseudomonas* isolates were retrieved from the GenBank (Supplementary Table S3). Sequence alignment was carried out using MUSCLE (Edgar, 2004) in the software package MEGA6 (Tamura *et al.*, 2013). The tree was inferred by Neighbor Joining and confidence analysis was ensured using 1000 bootstrap replicates with *P. aeruginosa* as outgroup. Individual *rpoB* and *rpoD* trees were initially generated after which a concatenated tree combining the aligned partial sequences of both genes was carried out using the same method. Unique sequences generated during this study were submitted to Genbank and accession numbers are listed in Supplementary Table S1.

Characterization of surfactant-producing Pseudomonas isolates

After assessing growth at 37 °C, two isolates were considered to be potential human pathogens and therefore excluded from further biosurfactant analysis. The initial screening step for biosurfactant production for all 138 isolates was by the use of the drop collapse assay (Jain *et al.*, 1991).

Swarming motility and white line-in-agar experiments were performed for all biosurfactant-producing isolates according to previously described methods (D'aes *et al.*, 2011, 2014).

Structural diversity of Pseudomonas sp. *biosurfactants*

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 CLP purification, seed cultures of *Pseudomonas* strain were grown in 5 ml KB broth contained in glass tubes and placed in a rotary shaker for 24 h at 28 °C. Subsequently, for each CLP-producing strain, this was inoculated in 2 L flasks containing 400 ml KB broth at 150 rpm for 24 h. *Pseudomonas* supernatant was collected after centrifugation at 10 000 g for 10 min, acidified to pH 2 with 6 N hydrochloric acid and kept overnight at 4°C, causing the CLPs to precipitate. After centrifugation at 10 000 g for 10 min, crude CLPs were extracted from the precipitate using methanol. The organic phase was evaporated at room temperature to obtain crude CLP extracts. For each CLP, crude extracts 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 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, lokisin and putisolvin, 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, respectively, 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. Acetonitrile-d3 (99.96%), and dimethylformamide-d7 (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 using TMS as secondary reference. 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 ms mixing times, and gradient-selected ^{1}H-^{13}C gHSQC and gHMBC optimized for an 8 Hz $^{n}J_{CH}$ coupling constant. 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. All spectra were processed using TOPSPIN 3.5 pl2.

Biological activity by CLP-producing Pseudomonas isolates

In order to assess the biological activity of CLP-producing *Pseudomonas* isolates, plant experiments were conducted in an unsterilized potting soil (Structural; Snebbout, Kaprijke, Belgium) and sand mixture in a 70/30 ratio (Oni *et al.*, 2019). *Pseudomonas* spp. COR51 (xantholysin), COR5 (entolysin), COW10 (WLIP), COR10 (lokisin), COR19 (putisolvin), COW5 (N1), COW8 (N2), COW3 (N3), COR33 (N5), COR35 (N8) and COR58 (N4) which produce eleven representative CLPs, were used. 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 (Olorunleke *et al.,* 2014) 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 (Oni *et al.,* 2019).

Cocoyam 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 3×10^6 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 seven 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 (Oni *et al.*, 2019).

Bacterial root colonization was assessed for all five cocoyam plants per treatment. Roots were crushed using sterile mortars and pestles in sterile saline solution (0.85%).

Subsequently, serial dilutions of crushed suspension were plated on KB agar plates and incubated at 28°C. Bacterial colonies were counted after 24 to 36 h and for each treatment, colonies were compared with plated controls. Data were log10 transformed before statistical analysis.

In vitro microscopic inhibition of P. myriotylum using purified CLPs

The antagonistic activity of *Pseudomonas* sp. CLPs against *P. myriotylum* was conducted under *in vitro* microscopic conditions. 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 (Olorunleke *et al.,* 2015b). An agar plug (diameter = 5 mm) taken from an actively growing Potato Dextrose Agar (PDA) plate of *P. myriotylum* was inoculated at the center of each glass slide. Stock solutions (10 mM) of CLPs were initially made in DMSO while subsequent dilutions were made using sterile MilliQ water. Two droplets (15 µl each) of CLP solution were placed at two sides of the glass slide (about 2 cm from the *Pythium* plug). Concentrations of CLPs tested ranged from 10 to 100 µM for four replicates each while diluted DMSO controls were included. All plates were incubated for four days at 28 °C. Microscopic slides were assessed for hyphal leakage and branching under an Olympus BX51 microscope. Furthermore growth diameter of mycelia was recorded for each treatment and replicates. Percentage inhibition of *P. myriotylum* by each CLP and for each concentration was determined and expressed relative to the mycelial growth in the control. Percentage inhibition was calculated according to the following formula:

$$\frac{\text{(Growth diameter of untreated control - Growth diameter of treated control)}}{\text{Growth diameter of untreated control}} \times 100$$

Data obtained were analyzed using Tukey's posthoc tests via SPSS. The bioassay was conducted twice and figures were generated to represent the percentage inhibition of *P. myriotylum* by varied concentrations of purified CLPs. Furthermore, representative pictures of mycelial damage due to interaction with CLPs are shown.

Results

Pseudomonas strains were isolated from the roots of healthy red, white and hybrid cocoyam plants grown in several fields in Cameroon. Samples were collected from three villages situated in the South-West province of Cameroon namely Boteva, Ekona and Maumu. This is a tropical region with annual rainfall up to 3000 mm (Adiobo *et al.*, 2007). Sixty *Pseudomonas* isolates were obtained from the rhizosphere of the red variety whereas 74 isolates were collected from the roots of the white cocoyam variety (Table S1). Four other isolates that were obtained from roots of a hybrid variety were also included in this present study.

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

An MLSA tree was built using concatenated partial sequences of *rpoD* and *rpoB* genes from type and biocontrol strains retrieved from NCBI together with 138 fluorescent *Pseudomonas* isolates obtained during this study (Figures 1A and 1B). This tree showed the association of our strains with the *P. fluorescens* complex, *P. aeruginosa* (collapsed) and *P. putida* groups. Strains used in this study had COW- and COR- prefixes denoting those obtained from the white and red cocoyams, respectively. All test isolates were positioned within three well-defined nodes with bootstrap supports higher than 97% over 1000 replicates.

In line with the *Pseudomonas* spp. classification (Garrido-Sanz *et al.*, 2016, 2017) our strains clustered with various groups within the *P. fluorescens* complex. Isolates situated within this complex predominantly clustered with the *P. koreensis* group (21 isolates) while fewer isolates clustered within the *P. fluorescens*, *P. jessenii* and *P. corrugata* groups (Figure 1A). Additionally, our results show three new groups which appear to belong to the *P. fluorescens* complex and were designated as U1, U2 and U3 (Figure 1A). For the *P. putida* group, although several of our isolates were closely related to already described type isolates such as *P. entomophilia* L48T, *P. soli* F-279208T, *P. monteilli* and *P. japonica*, a sizable number (55 isolates) formed separate clades and appear to be new species (Figure 1B). A detailed overview of the phylogeny of our strains is given in Supplementary Table S1.

Relationship between plant genotype and Pseudomonas taxonomy

At the *Pseudomonas* group level, results of Fishers' exact test statistics showed no significant difference between the taxonomic affiliations of strains collected from the red and white cocoyams (data not shown). More so, some unique isolates obtained from the white cocoyam were genetically similar with those obtained from the red cocoyam and vice-versa (Figure 1A and 1B). However, we observed that all isolates situated within the U1 and U2 subgroups (Figure 1A) were obtained from the red cocoyam, while all isolates of the U3 subgroup were obtained from white cocoyam. More so, isolates from all other clades within the *P. fluorescens* complex and *P. putida* group were present on both genotypes (Figures 1A and 1B) (Table S4).

Biosurfactant characterization reveals structurally new cyclic lipopeptides

Drop collapse assay showed that about 50% of isolates tested produced biosurfactants (Supplementary Table S1). Furthermore, CLP production was verified by LC-MS while for nine CLPs (entolysin, lokisin, putisolvin, WLIP, xantholysin, N1, N2, N4 and N8), full NMR characterization was also done. Based on retention time of the different CLPs, masses and swarming patterns, we could group all isolates into 13 different producers. Figure 2 shows representative chromatograms for some of these isolate 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 (Gerard *et al.*, 1997). One isolate, COR18, appears to produce two different CLPs designated N5 and N7.

Using MS and NMR analysis we identified five CLPs that are already described in literature including xantholysin-like, entolysin-like, amphisin-like (lokisin), putisolvin-like and viscosin-like (WLIP) CLPs with molecular weights (MW) of 1775.8 Da, 1721.0 Da, 1353.8 Da, 1393.6 Da, and 1125.6 Da, respectively (Figure 2) (Figure 3) (Supplementary Tables S5-S9, Supplementary Figures S1-S5). Detailed characterization of these known CLPs is given in the Supplementary Results section. Furthermore, our study revealed eight new CLPs designated N1, N2, N3, N4, N5, N6, N7 and N8, four of which were fully characterized by NMR. Main peaks of N1, N2, N3, N4, N5, N6, N7, and N8 featured MW values of 1393.6 Da, 1397.6 Da, 1065.3 Da, 1562.9 Da, 1550.6 Da, 1124.4 Da, 1719.8 Da, and 2331.3 Da, respectively (Figure 2). CLPs possessing similar masses could be differentiated on the basis of retention time and swarming motility pattern of the corresponding strain. Following NMR

characterization of representative strains that make different CLPs, isolates which gave a similar CLP mass and retention time were assigned to be producers of similar CLPs. Furthermore, test-isolates showed variable swarming patterns on 0.6% LB soft agar such that strains producing similar CLPs appeared to swarm alike (Figure 4).

Some of our surfactants were rare (lokisin, N2, N5, N6 and N7), some moderately present (WLIP, N3, N4, N8 and entolysin) and the rest were abundant (xantholysin, putisolvin and N1) (Figure 5A).

Relationship between plant genotype and CLP diversity

Although no significant relation was found between the plant varieties and their associated *Pseudomonas* taxonomic diversity, we observed a relationship between the former and CLP diversity (Figure 5A). In general, the red cocoyam appears to harbor a more diverse spectrum of CLP types (Figure 5A), since eleven CLP types could be found on the red cocoyam, while seven types were found on the white cocoyam. Furthermore, about 50% of isolates obtained from each genotype produce CLPs.

Relationship between CLP diversity and Pseudomonas taxonomy.

A significant association was found between *Pseudomonas* species affiliation and type of CLPs produced (Figure 5B). For instance, xantholysin, WLIP, putisolvin, entolysin, and N8 were only associated with the *P. putida* group, while N1, N2, N3, N4, N5, N6, N7 and lokisin were exclusively associated with the *P. fluorescens* complex (Figure 5B).

At the group level, most CLPs were exclusive to specific clades. For example, within the *P. fluorescens* complex, N1 was only produced by isolates situated in the *P. koreensis* group, N2 and N3 was exclusive to the U3 subgroup whereas N4 was only produced by isolates

belonging to the U2 subgroup (Figure 1A). Entolysin and putisolvin were produced by isolates situated in specific clades within the *P. putida* group. WLIP producers, however, were found dispersed in two different clades (Figure 1B) (Supplementary Table S1).

Characterisation of Cocoyamide A (N1), a new cyclic lipopeptide

The new CLP N1 that was significantly associated with the *P. koreensis group* was given the name cocoyamide A and fully characterized by NMR (Figure 6) (Supplementary Figure S6). Based on its molecular weight (1393.8 Da), it was initially assumed that N1 was an analogue of putisolvin II (1393.8 Da). However, detailed NMR analysis revealed that the cocoyamide A consists of a 3-hydroxydecanoic acid linked to a peptide chain involving 11 amino acids: four leucines, one asparagine, two glutamines, two isoleucines and two serines (Figure 6) (Supplementary Figure S6). The presence of a 3-hydroxydecanoic acid could be established as described in the Supplementary Methods. The amino acid sequence (HDA-Leu1-Asn2-Gln3-Ile4-Leu5-Gln6-Ser7-Leu8-Leu9-Ser10-Ile11) was confirmed by the analysis of a 2D ^{1}H-^{1}H ROESY spectrum, while an ^{1}H ^{13}C gHMBC established the lactone bond between the Ser7 side-chain and the Ile11 residue by observing a $^{3}J_{HC}$ cross-peak between the Ser7 $^{1}H^{\beta}$ and the Ile11 ^{13}C' signals. Even though cocoyamide A and putisolvin II are completely different molecules, their chemical formulae are identical ($C_{66}H_{115}N_{13}O_{19}$), thus making it impossible to differentiate both CLPs on the basis of MS alone (Supplementary Table S10).

Biological activity of representative CLP-producing isolates and purified CLPs against Pythium-mediated CRRD

Eleven representative CLP-producing *Pseudomonas* isolates, each producing a different CLP, were tested for disease suppressiveness against CRRD. All strains exhibited strong biocontrol

activity and one isolate, the entolysin producer COR5, displayed 100% pathogen control (Table 1). Additionally, all strains tested were well established in their capacity to colonize cocoyam roots (Table 1). Subsequent microscopic analysis showed that 10 to 100 µM concentrations of purified entolysin, lokisin, WLIP, xantholysin, putisolvin, cocoyamide, N2 and N8 caused growth inhibition and interacted with the mycelia of *P. myriotylum* to cause either extensive branching or lysis (Figure 7A, Figure 7B). At all concentrations tested, cocoyamide, N2, N8 and lokisin resulted in hyphal lysis whereas this was the case for WLIP, entolysin, putisolvin and xantholysin at concentrations of 25 and 50 µM. At concentrations of 10 and 100 µM, these compounds caused extensive hyphal branching, except for WLIP where there was no clear effect at 10 µM. A dose-dependent effect of CLPs was observed for entolysin and putisolvin but not for the others (Figure 7A).

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, 2014). One of these is the plant genotype which is known to drive microbial selection via root morphology and exudation (Berg and Smalla, 2009; Hartmann *et al.*, 2009). It was hypothesized that the red cocoyam genotype selects for specific fluorescent pseudomonad populations which could contribute to observed field tolerance towards the cocoyam root rot disease caused by *Pythium myriotylum* (Perneel *et al.*, 2007). Although only 40 isolates were tested in this previous study, antagonism against *P. myriotylum* was shown by the red cocoyam isolates only.

In our present study, taxonomic characterization of 138 fluorescent pseudomonads obtained from the rhizosphere of red and white cocoyam varieties in three different fields in Cameroon, showed a clustering of our isolates mainly within two *Pseudomonas* taxonomic divisions namely the *P. fluorescens* complex and the *P. putida* group. Based on the phylogenetic tree generated, our isolates clustered into seven groups within the *P. fluorescens* complex (*P. fluorescens*, *P. jessenii*, *P. corrugata*, *P. koreensis*, U1, U2 and the U3 (sub)groups). In general, the taxonomic affiliation of our isolates did not appear to differ with variety since strains obtained from the red and white cocoyam were found in almost all clades. However, we did observe that all isolates situated in the U1 and U2 groups originated from the red cocoyam rhizosphere suggesting that certain *Pseudomonas* groups/isolates may exclusively colonize the red cocoyam variety. A similar observation was recorded for all isolates in the U3 group which originated from the white cocoyam rhizosphere. 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).

Our results about high *Pseudomonas* diversity on cocoyam roots in Cameroon are consistent with a recent study to characterize *Pseudomonas* isolates obtained from a sugarcane field in a tropical Brazilian soil (Lopes *et al.*, 2018). Phylogenetic analyses of 76 isolates obtained from bulk soil and the sugarcane rhizosphere revealed their affiliation mainly to the *P. fluorescens* complex (57 isolates) and *P. putida* group (19 isolates). More than 50% of the isolates situated in the *P. fluorescens* complex belonged to the *P. koreensis* group, a couple of isolates in the *P. jessenii* group while the rest represented two new subgroups designated sub-clades

X and Y (Lopes *et al.*, 2018). A sequence comparison between our isolates and those from Brazil showed the clustering of isolates of both locations together within the *P. koreensis*, *P. jessenii* and U2 (sub)groups. Thus, our U2 subgroup appears to correspond to the sub-clade X recently described by Lopes and colleagues. The enormous *Pseudomonas* diversity displayed by the cocoyam rhizosphere in Cameroon corroborates the findings (Lopes *et al.*, 2018) that tropical soils are a reservoir of unexplored *Pseudomonas* diversity, especially containing new subgroups within the *P. fluorescens* complex and new species in the *P. putida* group. Considering the overlap of some of our *Pseudomonas* groups with those reported in Brazil only, findings of this study strengthens the possibility of endemism of soil fluorescent *Pseudomonas* in specific geographical locations of the world (Cho and Tiedje, 2000).

Previous extensive studies to assess the diversity of fluorescent pseudomonads in 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). However, drivers of this observed diversity remain unclear.

With respect to CLP diversity, 11 out of 13 CLPs discovered during this study were produced by *Pseudomonas* isolates obtained from the red cocoyam variety whereas seven different CLPs were produced by white cocoyam rhizospheric isolates. Although we did not find a clear cut effect of cocoyam variety on *Pseudomonas* spp. diversity, our findings suggest that the red cocoyam rhizosphere support more diverse CLP producers which might have implications towards observed field tolerance to the cocoyam root rot disease earlier reported

(Perneel *et al.*, 2007). A previous study revealed that total soluble carbohydrates are higher in the red cocoyam (10 µg glucose/mg fresh weight) as compared with the white variety (3 µg glucose/mg fresh weight) (Omokolo *et al.*, 2005). Besides amino acid and proline levels are also slightly higher in the roots of the red cocoyam. These differences could drive a selectivity of the microbial community/isolates associated with the rhizosphere of the different cocoyam genotypes.

During this study, diverse *Pseudomonas* genotypes were obtained which produce diverse CLPs. In a previous study, 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 for example, Boteva soils predominantly contained silt (Adiobo *et al.*, 2007). An understanding of factors driving CLP diversity in the rhizosphere remains highly challenging and requires further research.

Thirteen CLP types were characterized during this study namely xantholysin, entolysin, WLIP, lokisin, putisolvin and eight novel ones designated cocoyamide A (N1), N2, N3, N4, N5, N6, N7, and N8. Xantholysin production was previously reported in a banana rhizosphere isolate, *P. putida* BW11M1 (Li *et al.*, 2013) and in *P. soli*, isolated from a soil sample collected from 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). Interestingly, these strains belong to the same taxonomic groups as the xantholysin and entolysin producers

from Cameroon. WLIP production has been reported for several *Pseudomonas* isolates belonging to the *P. putida* and *P. fluorescens* groups (Rokni-Zadeh *et al.*, 2012, 2013). Recent studies aimed at indexing and mapping the diversity of metabolites produced by 260 *Pseudomonas* strains of ecologically diverse origins reported the common occurrence of WLIP and xantholysin producers (Nguyen *et al.*, 2016). Putisolvin I and II have been described from *P. putida* PCL1445, isolated from a site polluted with aromatic hydrocarbons (Kuiper *et al.*, 2004) and also from *P. putida* 267, a black pepper rhizosphere isolate from Vietnam (Kruijt *et al.*, 2009). Lokisin-producing isolates were previously described from the sugar beet rhizosphere in Danish soils (Nielsen *et al.*, 2002), with antagonistic activity against *Rhizoctonia 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 against *P. ultimum* on tomato. Unfortunately, the exact taxonomic position of all these producers is not known as genome information is not publicly available. Interestingly, a *gacA*$^{+}$ gene derivative of *P. fluorescens* Pf0-1 strain, a soil isolate from the United States that clusters with the *P. koreensis* group, produces an unknown CLP whose amino acid composition is similar to that of cocoyamide A (Loper *et al.*, 2012). In our work, cocoyamide producers were specifically associated with the *P. fluorescens* complex and were found in the *P. koreensis* and U1 groups. The discovery of cocoyamide A has important implications for the screening and characterization of novel CLPs produced by bacteria. The fact that the molecular weight of cocoyamide A and putisolvin II are identical indicates that both CLPs cannot be discriminated based on mass spectrometry alone. Therefore, when a biosurfactant with a known mass is found, additional characterizations using genome mining, NMR and/or

MS/MS spectroscopic analysis should be performed before conclusions are made regarding CLP identity.

Such enormous diversity and abundance of CLP producers observed in our study, point to crucial ecological roles of these metabolites in their native soils. Further research could elucidate the role of these CLPs in disease suppression as it has previously been demonstrated for *Pseudomonas* sp. CMR12a (D'aes *et al.*, 2011; Olorunleke *et al.*, 2015b; Hua and Höfte, 2015).

Our biological control and CLP bioactivity data indicate that these CLPs interact with the mycelium of *P. myriotylum* by causing hyphal leakage or extensive branching. Previous studies showed that purified orfamide A can interact with the mycelium of *Rhizoctonia solani* leading to increased branching (Olorunleke *et al.*, 2015b). Whether the difference in mode of action of the various CLPs is related to the structural differences between them still remains to be investigated. Moreover, the observed dose-dependence of only entolysin and putisolvin of all CLPs tested is striking and further raises the question about structure-function relationships of CLPs. 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 the soil suggests that they may contribute to disease suppression towards *P. myriotylum*. 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 will be a need to not only 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 and/or CLP mutant strains.

Conclusion

In summary, our study suggests that plant genotype may drive CLP diversity rather than species diversity. The *Pseudomonas* population is taxonomically diverse on both cocoyam varieties. However, on the basis of the available samples, which is a subset of cocoyam bacterial community, it appears that the plant genotype might have an effect on *Pseudomonas*-derived CLP pattern. The red cocoyam rhizosphere appears to select for isolates that produce a more diverse CLP spectrum than the white cocoyam rhizosphere. We also provide evidence that most CLP types are associated with specific taxonomic groups (Table 2). Thus, a proper taxonomic assignment of surface active *Pseudomonas* isolates may already give an indication about the type of CLP they produce. It remains to be investigated, however, whether CLP diversity is a key factor in the field tolerance of the red cocoyam variety to the CRRD. Moreover, the influence of soil quality on taxonomic and CLP diversity of *Pseudomonas* spp. should not be ignored and is the subject of our current research. Finally, this study shows the potential of several CLP-producing isolates in the biological control of plant pathogens. Fluorescent pseudomonads, especially those belonging to the *P. fluorescens* complex, are already in commercial use against several plant diseases. Thus, our study presents a rich collection of strains producing potent metabolites that can contribute to current and emerging markets for the commercialization of *Pseudomonas* biocontrol agents.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at the Journal website.

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Table 1. Cocoyam root rot disease index and root colonisation by CLP-producing *Pseudomonas* strains and the corresponding CLPs produced.

Strain (*Pseudomonas* sp.)	CLPs produced	Disease Index[a] (%)	Population density of test bacteria[b] (in log CFU g-1 of fresh roots)
Healthy control	-	0.0 a	-
Diseased control	-	77.3 c	-
COW5	Cocoyamide (N1)	18.3 b	8.19 ± 0.3 bc
COW8	N2	15.8 b	8.44 ± 0.2 cd
COW3	N3	3.7 b	9.30 ± 0.2 cd
COR58	N4	10.6 b	7.63 ± 0.4 a
COR33	N5	7.5 b	7.18 ± 0.4 a
COR35	N8	10.0 b	7.83 ± 0.1 a
COR10	Lokisin	10.8 b	8.18 ± 0.2 b
COR5	Entolysin	0.0 a	7.96 ± 0.3 ab
COR19	Putisolvin	6.2 b	9.17 ± 0.3 cd
COW10	WLIP	15.0 b	8.12 ± 0.3 ab
COR51	Xantholysin	23.3 b	8.28 ± 0.3 bc

[a] Disease symptoms were evaluated at 7 days post-infection by scoring leaves. The experiment was repeated twice while data analysis was expressed as Percent Disease Index. Bars indicated with the same letters was not statistically different after subjecting values to Kruskal Wallis and Mann Whitney non-parametric tests (p=0.05).

[b]Root colonisation capacity was determined by sampling 5 cocoyam roots per treatment. Data from two experiments was log transformed before analysis and pooled. Values with different letters are significantly different according to Tukey's test (p= 0.05).

Table 2. Association of CLPs produced by *Pseudomonas* spp. from the cocoyam rhizosphere with taxonomic group and plant genotype

CLP	Taxonomic group	Plant genotype
N1	fluorescens*	both
N2	fluorescens	white
N3	fluorescens	white*
N4	fluorescens	red
N5	fluorescens	red
N5+N7	fluorescens	red
N6	fluorescens	red
N8	putida	red
Xantholysin	putida*	both
WLIP	putida*	both
Entolysin	putida*	red
Lokisin	fluorescens	red
Putisolvin	putida	red

*significant at $p<0.05$ according to Fisher exact statistics.

Figure Legends

Figure 1. Phylogenetic analyses of 138 *Pseudomonas* isolates using the concatenated partial sequences of two *Pseudomonas* housekeeping genes (*rpoB* and *rpoD*) and *P. aeruginosa* group as outgroup. 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. COW- isolates were obtained from white cocoyam, COR- isolates from red cocoyam. All R isolates except R124 come from Brazil (Lopes *et al.*, 2018). CLP-producing isolates are colour-coded. N1, N2, N3, N4, N5, N6, N7 and N8 are new CLPs discovered during this study; xantholysin, entolysin, WLIP, lokisin and putisolvin are previously reported CLPs that are also produced by some isolates characterized within this study. Previously reported N1, entolysin and xantholysin-producing strains are colour-coded circles of blue, light green and dark green, respectively. **A)** Shows isolates belonging to the *P. fluorescens* complex and associated strains which were grouped. The *P. putida* and *P. aeruginosa* groups were collapsed. **B)** Shows isolates belonging to the *P. putida* group while the *P. fluorescens* complex and *P. aeruginosa* group were collapsed.

Figure 2. HPLC chromatograms showing major CLPs produced in liquid culture by fluorescent *Pseudomonas* spp. isolated from the cocoyam rhizosphere. Representative CLP-producing isolates that were further characterized by HPLC-MS are indicated on the left hand side of the panel. On the right hand side of the panel, for each CLP, the molecular weights of the major peaks are shown. N1, N2, N3, N4, N5, N6, N7 and N8 denotes eight new CLPs. All

CLPs were fully characterized by NMR except for N3, N5, N6 and N7 which is in progress. Full characterization data will be published elsewhere.

Figure 3. Chemical structure of the five previously described CLPs namely: xantholysin, entolysin, putisolvin, WLIP and lokisin.

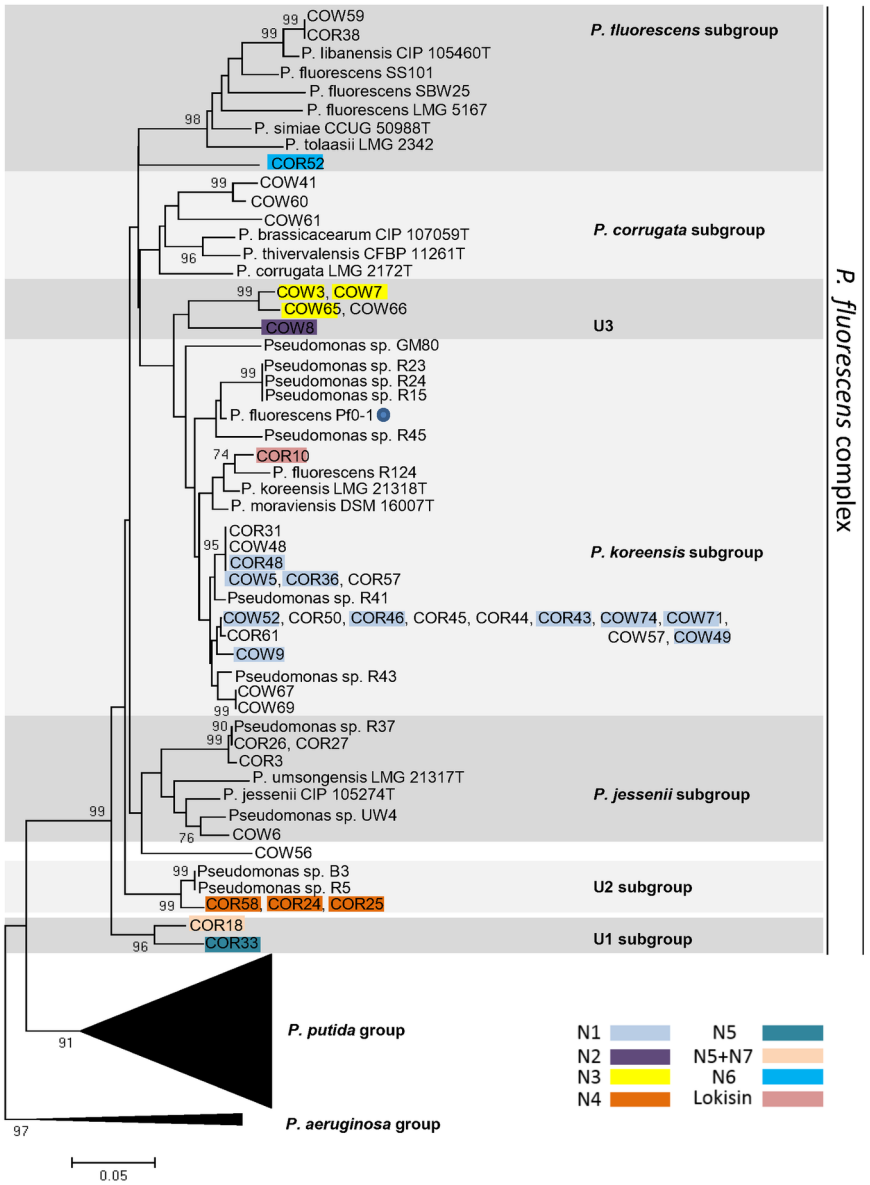
Figure 4. Swarming motility of representative CLP-producing isolates. Ento: entolysin; Xanth: xantholysin; Loki: lokisin; Putis: putisolvin.

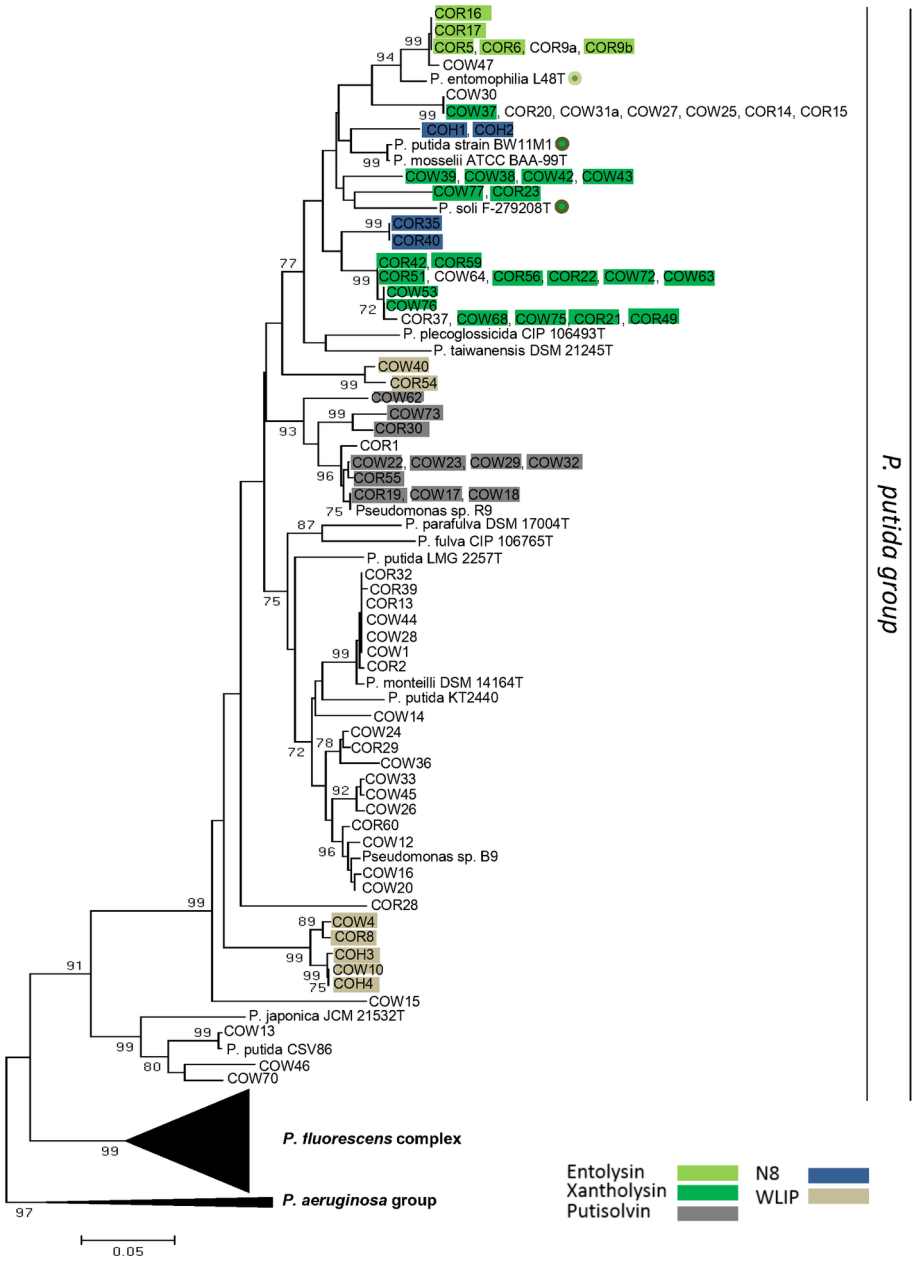
Figure 5. CLP analyses based on plant genotype and *Pseudomonas* taxonomic diversity A) Frequency of *Pseudomonas* isolates producing specific CLPs in the red and white cocoyam varieties. Xanth: xantholysin; Ent: Entolysin; Loki: Lokisin; Putis: Putisolvin; N1, N2, N3, N4, N5, N6, N7, and N8 denotes eight new cyclic lipopeptides characterized during this study; **B)** Taxonomic affiliation of various CLP producing isolates. Bars with asterisk are significantly different according to Fisher's exact statistic.

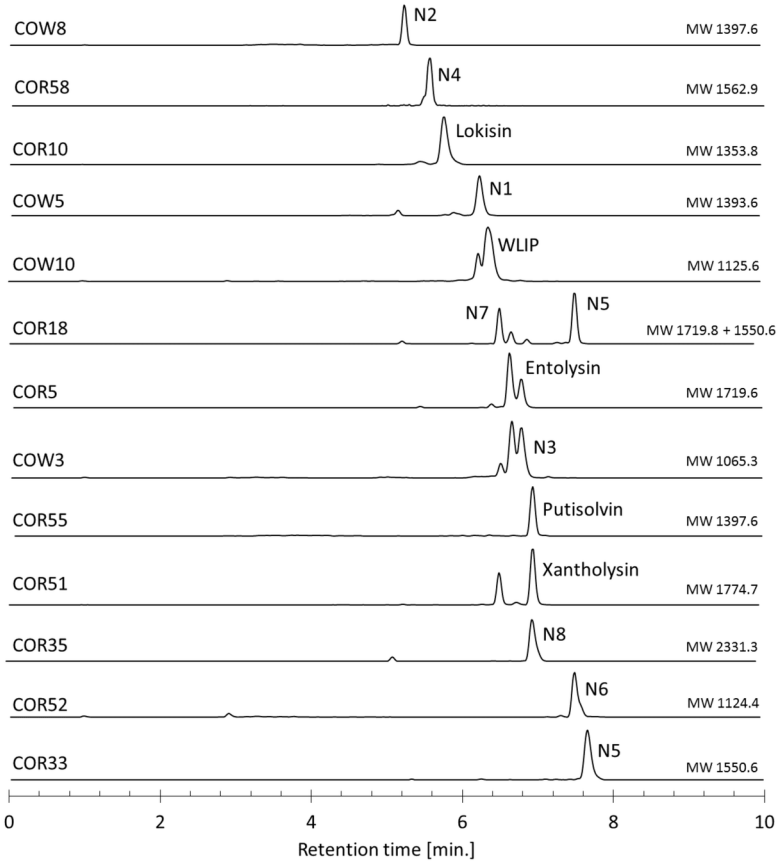
Figure 6. Chemical structure of the newly discovered cocoyamide A.

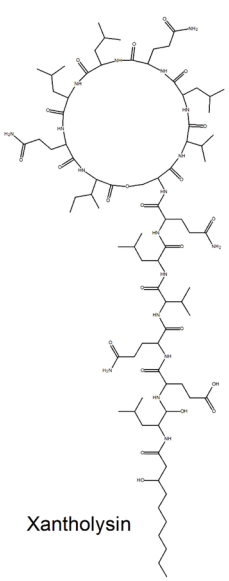
Figure 7. Effect of purified CLPs on *P. myriotylum*. A) Inhibitory interaction between CLPs and *Pythium myriotylum* at concentrations ranging from 10 to 100 µM. Values plotted are indicative of the percentage growth inhibition of *P. myriotylum* relative to the control. For each CLP, statistics was conducted to compare inhibitory capacity for different

concentrations. Bars with different letters are significantly different according to the Tukey's tests ($P = 0.05$). Table below the bars represent the presence (+) or absence (-) of hyphal lysis and hyphal branching following exposure of *P. myriotylum* mycelium to varying concentrations of CLPs; **B)** Effect of CLPs on *P. myriotylum* mycelium. **i)** *P. myriotylum* control; **ii)** Hyphal lysis and leakage; **iii)** Hyphal distortion and branching. Scalebars represent 10 µm.

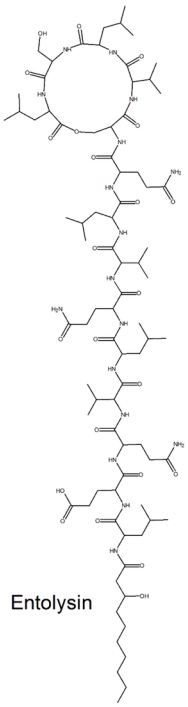




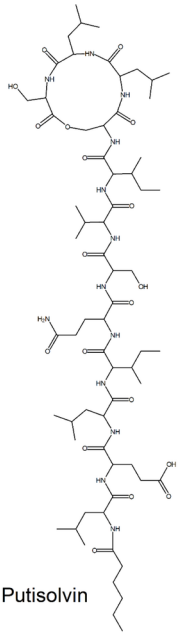




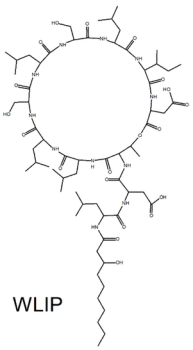
Xantholysin



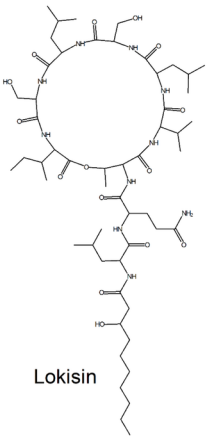
Entolysin



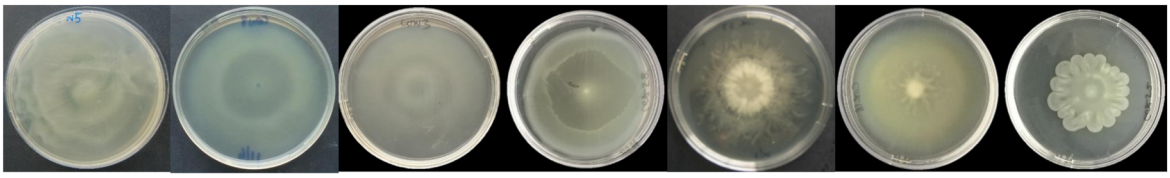
Putisolvin



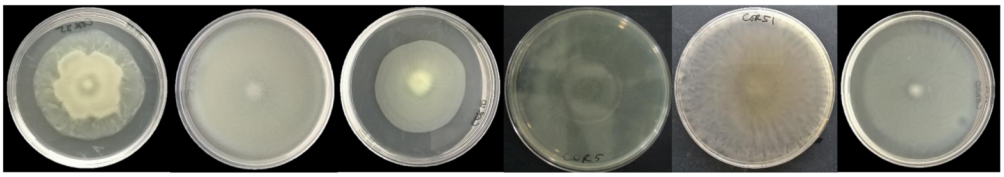
WLIP



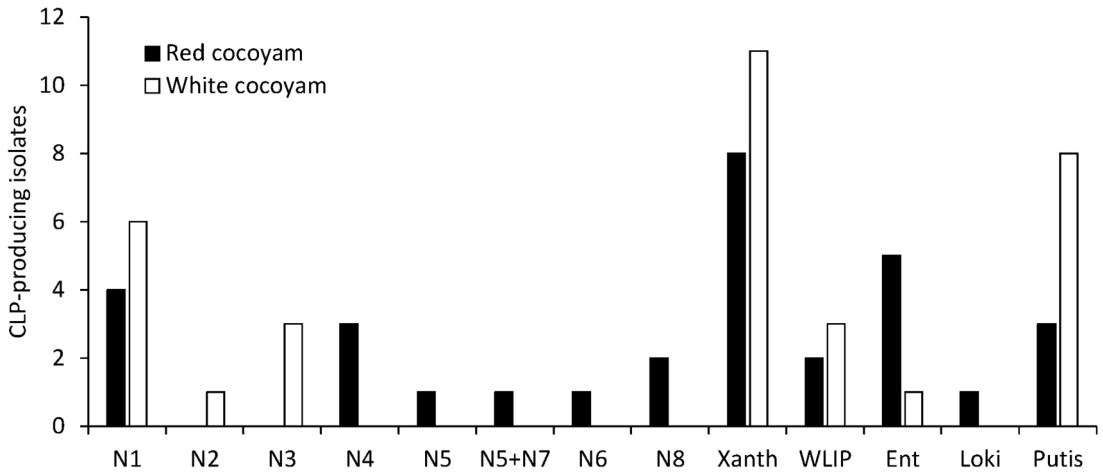
Lokisin

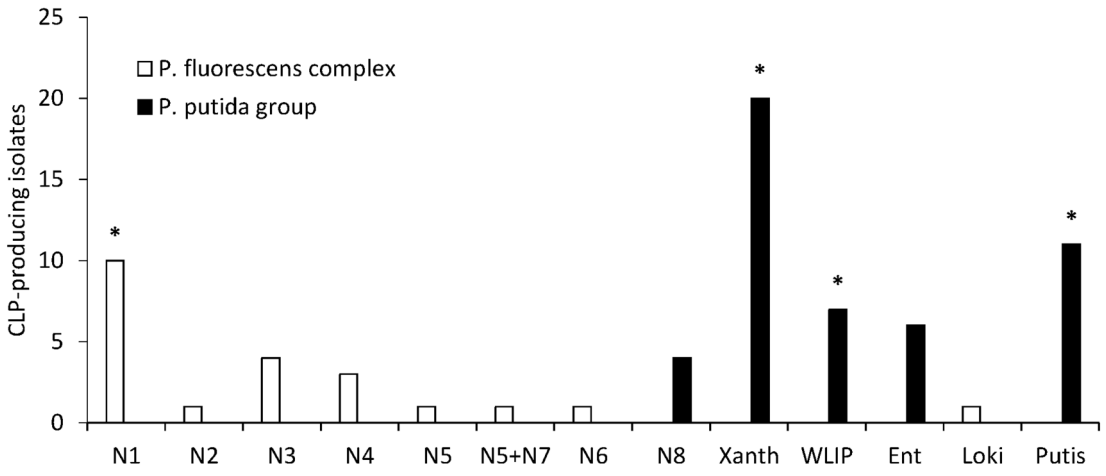


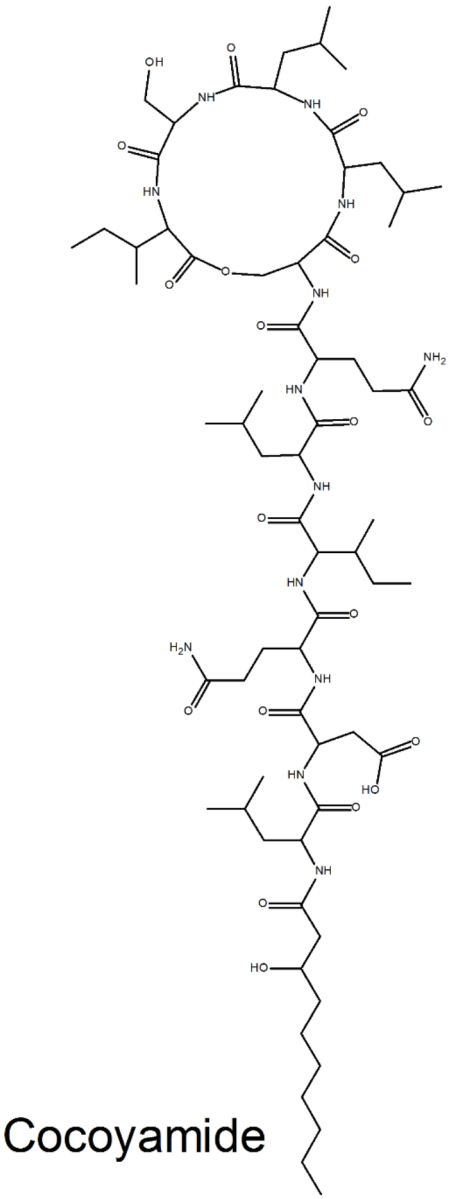
COW5 – N1 COW8 – N2 COW3 – N3 COR58 – N4 COR33 – N5 COR18 – N5+N7 COR52 – N6



COR35 – N8 COR55 - Putis COR10 - Loki COR5 - Ento COR51 - Xanth COW10 - WLIP







Cocoyamide