Cyclic lipopeptide-producing *Pseudomonas koreensis* group strains dominate the cocoyam rhizosphere of a *Pythium* root rot suppressive soil contrasting with *P. putida* prominence in conducive soils

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Summary

Pseudomonas isolates from tropical environments have been underexplored and may form an untapped reservoir of interesting secondary metabolites. In this study, we compared *Pseudomonas* and cyclic lipopeptide (CLP) diversity in the rhizosphere of a cocoyam root rot disease (CRRD) suppressive soil in Boteva, Cameroon with those from four conducive soils in Cameroon and Nigeria. Compared to other soils, Boteva andosols were characterized by high silt, organic matter, nitrogen and calcium. Besides, the cocoyam rhizosphere at Boteva was characterized by strains belonging mainly to the *P. koreensis* and *P. putida* (sub)groups, with representations in the *P. fluorescens*, *P. chlororaphis*, *P. jessenii*, and *P. asplenii* (sub)groups. In contrast, *P. putida* isolates were prominent in conducive soils. Regarding CLP diversity, Boteva was characterized by strains producing eleven different CLP types with cocoyamide A producers, belonging to the *P. koreensis* group, being the most abundant. However, putisolvin III-V producers were the most dominant in the rhizosphere of conducive soils in both Cameroon and Nigeria. Furthermore, we elucidated the chemical structure of putisolvin derivatives - putisolvin III-V, and described its biosynthetic gene cluster. We show that high *Pseudomonas* and metabolic diversity may be driven by microbial competition which likely contributes to soil suppressiveness to CRRD.

Introduction

Fluorescent pseudomonads exhibit an enormous metabolic versatility and produce a remarkable spectrum of secondary metabolites which includes cyclic lipopeptides (CLPs) (Loper *et al.*, 2012; Garrido-Sanz *et al.*, 2016, 2017; Hesse *et al.*, 2018). Based on multilocus sequence analysis (MLSA) and the use of whole genome sequencing, species of *Pseudomonas* have been delineated into three main lineages (*P. aeruginosa*, *P. fluorescens* and *P. pertucinogena*) and 14 phylogenetic groups (Mulet *et al.*, 2010; Gomila *et al.*, 2015; Garrido-Sanz *et al.*, 2016, 2017). The *P. fluorescens* lineage comprises 5 phylogenetic groups (*P. fluorescens*, *P. asplenii*, *P. lutea*, *P. syringae* and *P. putida* while the *P. aeruginosa* lineage comprises 8 phylogenetic *Pseudomonas* groups (*P. straminea*, *P. anguilliseptica*, *P. oryzihabitans*, *P. stutzeri*, *P. oleovorans*, *P. resinovorans*, *P. aeruginosa* and *P. linyingensis*) (Lalucat *et al.*, 2020). According to the nomenclature by Lalucat *et al.*, 2020, the *P. fluorescens* group comprises eight subgroups namely *P. fluorescens*, *P. gessardii*, *P. fragi*, *P. mandelii*, *P. koreensis*, *P. jessenii*, *P. corrugata* and *P. chlororaphis*. The *P. fluorescens* group (called *P. fluorescens* complex in Garrido-Sanz *et al.*, 2016; in this paper we have adopted the nomenclature suggested by Lalucat *et al.*, 2020), is the most diverse *Pseudomonas* taxonomic group. It has been reported that the production of an array of secondary metabolites with antifungal properties (Mavrodi *et al.*, 2006; Loper *et al.*, 2012; Calderón *et al.*, 2013), insecticidal activity (Flury *et al.*, 2016, 2017) and other beneficial traits (Garrido-Sanz *et al.*, 2016) appear to follow a phylogenetic distribution. To date, CLPs have been affiliated with *Pseudomonas* isolates situated within the *P. fluorescens* lineage specifically in the *P. fluorescens* *P. putida*, *P. asplenii* and *P. syringae* phylogenetic groups (reviewed by

Olorunleke *et al.*, 2015; Oni *et al.*, 2019). Knowledge about factors which drive plant rhizospheric recruitment of *Pseudomonas* species and specific CLP producers is currently lacking.

CLPs are bacterial metabolites composed of a cyclic oligopeptide lactone ring linked to a fatty acid tail (Raaijmakers *et al.*, 2006, 2010). These molecules interact with microbial membranes and possess antibacterial, antifungal, anti-oomycete, antiviral and anticancer properties (Raaijmakers *et al.*, 2006; Raaijmakers and Mazzola, 2012; Olorunleke *et al.*, 2015; Geudens and Martins, 2018). These molecules are produced by non-ribosomal peptide synthetases (NRPSs), which are modular multifunctional enzymes. Each module comprises specific domains which catalyzes different enzymatic activities namely the adenylation (A), condensation (C) and thiolation (T) domains (Roongsawang *et al.*, 2011). The A-domain functions in amino acid recognition and adenylation; the adenylated amino acid becomes covalently attached to a phosphopantetheine carrier of the adjacent T-domain; the C-domain catalyzes the peptide bond formation of two consecutively bound amino acids to the growing peptide chain. Besides the C domain, the module incorporating the D-amino acid has an associated epimerization (E) domain which functions as a catalyst for the conversion of L-amino acids to D-isomers (Roongsawang *et al.*, 2005). Following the synthesis of a linear intermediate peptide, cyclization and release of the product peptide is effected by a C-terminal thioesterase (Te) domain. The structural diversity of CLP C-domains enables the establishment of evolutionary relationships between closely related CLPs (Roongsawang *et al.*, 2011). Depending on the number and composition of amino acids, CLPs have been classified into

about 14 groups (Raaijmakers *et al.*, 2006; Geudens and Martins, 2018; Götze and Stallforth, 2020).

The tropical cocoyam crop (*Xanthosoma sagittifolium*) is among the world's six most important root and tuber crops (Onyeka, 2014; Boakye *et al.*, 2018). This crop is widely grown in West-Africa and is extremely susceptible to the cocoyam root rot disease (CRRD) caused by *Pythium myriotylum* (Pacumbaba *et al.*, 1992; Perneel *et al.*, 2006), which can lead to a drastic yield reduction of up to 90% in affected fields. Two vegetatively propagated varieties are commercially cultivated in West-Africa, the red and the white cocoyam. The preferred white cocoyam is most susceptible to CRRD, while the red cocoyam shows some field tolerance.

In our previous study, the diversity of fluorescent pseudomonads and CLPs obtained from the rhizosphere of healthy cocoyams grown in three different fields (Boteva, Ekona and Maumu) in Cameroon, was characterized (Oni *et al.*, 2019). *P. fluorescens* and *P. putida* group isolates were enriched in the cocoyam rhizosphere independent of plant genotype. Additionally, 50% of the *Pseudomonas* isolates produced a wide diversity of CLPs. Plant genotype had an effect on CLP diversity since the red cocoyam rhizosphere appears to select for isolates that produce a more diverse CLP spectrum than the white cocoyam rhizosphere. Representative CLP-producing isolates showed excellent *in vivo* biocontrol against CRRD, suggesting that they may contribute to disease suppression in the field. However, other factors, including soil physico-chemical characteristics, contributing to the striking diversity of pseudomonads and CLPs in the cocoyam rhizosphere are not yet understood. Intriguingly, Boteva soil of Cameroon has been described as a general disease suppressive andosol while at Ekona and Maumu andosols

have become disease conducive due to intensive land cultivation (Adiobo *et al.*, 2007; personal communication, Amayana Adiobo IRAD, Cameroon).

In Nigeria, soils which are conducive to the CRRD are reportedly situated in Ado-Ekiti and Umudike towns (Olorunleke, 2017). The gradient of soil disease-suppressiveness at Boteva village in Cameroon (Adiobo *et al.*, 2007) and disease-conduciveness recorded in other soils in the cocoyam-growing areas of Cameroon and Nigeria (Adiobo *et al.*, 2007; Oni *et al.*, 2019) provides us with the platform to investigate the *Pseudomonas* and CLP diversity in the cocoyam rhizosphere in a suppressive soil versus conducive soils. Thus, the objective of our study was to (i) investigate the *Pseudomonas* and CLP diversity on the cocoyam rhizosphere in conducive soils of Ado-Ekiti (alfisol) and Umudike (ultisol) towns in Nigeria, (ii) compare *Pseudomonas* and CLP diversity in a suppressive soil of Cameroon with four conducive soils in Cameroon and Nigeria by using data about Nigeria from this study and reanalyzing data from Cameroon obtained in (Oni *et al.*, 2019); (iii) try to link the level of *Pseudomonas* and CLP diversity with soil physicochemical properties.

Materials and methods

Cocoyam sampling, Pseudomonas *isolation and taxonomic characterization*

In Ado-Ekiti, cocoyam plants were sampled from an experimental farm located at Ekiti State University (EKSU), Nigeria while at Umudike, plants were collected from research fields situated at the National Root Crops Research Institute (NRCRI), Umudike, Nigeria. Field sampling was conducted between 2012 to 2014 and characteristics of these farms are presented in Table S1. Cocoyam roots were collected from healthy three to five 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 Kings B (KB; King *et al.,* 1954) agar. Plates were incubated at 28°C and enumeration of fluorescent *Pseudomonas* colonies (under UV light) was done after 48 h of incubation. A total of 166 bacterial colonies were transferred to fresh KB agar and purified twice. Of these isolates, 67 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 isolated during this study are indicated in Table S2. Isolates obtained from red and white cocoyams at Ado-Ekiti were designated with RCA- and WCA- prefixes, respectively. Isolates obtained from Umudike were either denoted with NSE (Nigeria South East)- or WCU (White Cocoyam Umudike)- prefixes. Nine isolates obtained from Ugbokolo in Benue State, Nigeria denoted with NNC (Nigeria North Central)- prefix were also included in this study. A full list of previously characterized Cameroon strains (Oni *et al.,* 2019) which were included in this study are listed in Table S3 according to location, taxonomy and biosurfactant characteristics. Additional strains used for phylogenetic comparison were type strains of *Pseudomonas* spp., biocontrol isolates within the same genus and *Pseudomonas* strains which were previously characterized from the cocoyam rhizosphere in Cameroon (Oni *et al.*, 2019) (Table S4).

For taxonomic characterization of isolates, colony PCR was carried out using freshly grown bacteria colonies. A small bacteria colony was suspended in 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 (Table S5). For some isolates, these primers produced a single

amplicon whereas some that did not give bands or gave multiple bands were analyzed using primers rpoD1F/rpoD1R which were designed previously (Oni *et al.*, 2019).

In total, data from six different cocoyam-growing fields (Ado-Ekiti, Boteva, Ekona, Maumu, Umudike and Ugbokolo) in two countries (Cameroon and Nigeria) were used during this study. Sequence alignment was carried out using Muscle (Edgar, 2004) via the software package MEGA6 (Tamura *et al.*, 2013). The phylogenetic tree was inferred by Maximum Likelihood and confidence analysis was ensured using 1000 bootstrap replicates with *P. aeruginosa* as outgroup.

Cyclic Lipopeptide Characterization

Biosurfactant production was first assessed by the routine drop collapse assay as previously described (D'aes *et al.*, 2014). Isolates positive for drop collapse were evaluated for growth at 40°C in order to exclude *P. aeruginosa*-like isolates. Following this, CLP-producing isolates were tested for swarming on 0.6% LB agar in triplicates as described by Oni *et al.*, 2019. Furthermore, strains positive for CLP production were tested for white line-in-agar formation as described by D'aes *et al.* (2014). This assessed the capacity for each test strain to form a white line when confronted with an indicator strain *Pseudomonas* sp. CMR12a-ΔPhz-ΔCLP2 which produces a tolaasin-like CLP called sessilin. This mutant strain retained the capacity to produce sessilin after constructing gene deletion mutants in orfamides and phenazines (D'aes *et al.*, 2014).

For HPLC analyses, bacterial cultures were set up in KB broth under shaking conditions for 24 h. Analytical LC-MS data of the filter sterilized supernatant samples were collected on an 1100 Series HPLC with a type VL ESI detector (Agilent Technologies) equipped with an analytical

Kinetex C18 reversed-phase column (150 x 4.6 mm, 5 µm particle size; Phenomenex, Torrance, CA). An elution gradient of H_2O/CH_3CN (25:75 to 0:100 over 20 min was applied at a flow rate of 1 mL min^{-1}.

For UPLC analyses, samples were analyzed with a UPLC (Acquity H-class, Waters s.a., Zellik, Belgium) coupled to a single quadrupole mass spectrometer (Waters SQD mass analyzer) on a ACQUITY UPLC® BEH C18 column (Acquity UPLC BEH C18 2.1 mm × 50 mm, 1.7 µm). Elution was performed at 40°C with a constant flow rate of 0.6 mL/min using a gradient of ACN in water both acidified with 0.1% formic acid as follows: two min at 15%, from 15% to 95% in 7 min and maintained at 95% for 2.5 min. Compounds were detected in electrospray positive ion mode by setting SQD parameters as follows: source temperature 130°C; desolvation temperature 400°C, and nitrogen flow: 1000 L.h-1 and 70V as cone voltage in the range m/z 300-2048.

MLSA-based phylogenetic analyses of Pseudomonas *spp*

16S rRNA, *gyrB, rpoB* and *rpoD* sequences of strains used for this analyses were extracted from the draft genome sequences of our CLP-producing strains (Table S6A and S6B). Sequences of selected *Pseudomonas* type strains were retrieved from the GenBank (Table S6A and S6B). Sequences were aligned via MUSCLE using MEGA6 software. Phylogenetic tree was constructed using Maximum Likelihood with 1000 bootstrap replication and the Jukes Cantor model. *P. aeruginosa* was used as outgroup. For each housekeeping gene, separate trees were generated after which a concatenated tree was obtained by combining the aligned sequences of all four genes.

Physico-chemical soil characterization

In August 2015, soil samples were collected from 40 points at a depth of 0-30 cm from each of the cocoyam fields (Ado-Ekiti and Umudike, Nigeria) where plant samples were harvested. The samples from each location were bulked and a subsample was used for physico-chemical analyses. Additionally, soil samples were collected from Maumu (Cameroon) using the same sampling method earlier described. Soil physico-chemical properties from two locations of Cameroon (Boteva and Ekona) were available from a previous study (Adiobo *et al.*, 2007).

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 Macherey-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). Electrical conductivity, ECEC was measured with an electrode (ISO 11265:1994).

Interaction between soil characteristics, Pseudomonas *diversity and CLP diversity versus sample location*

The Shannon diversity index (H') was used to calculate CLP diversity among sampling locations where p_i is the proportion (n) of individuals (CLPs) found in species (location) i divided by the total number of individuals found (N) (Spellerberg and Fedor, 2003). $H' = -\sum p_i \ln p_i$. Shannon's equitability or evenness (E_H) was also calculated by dividing H' by H_{max} ($H_{max} = \ln S$) where S refers to the total number of species/CLPs in the community/location of study. Using R studio, PCA analyses were used to visualize the relationship between CLP diversity and sample locations. Pearson correlation coefficients were calculated in Microsoft Excel to characterize the relationship between physicochemical soil characteristics, CLP and *Pseudomonas* diversity.

Chemical structure elucidation of putisolvin III-V

Chemical elucidation of putisolvin III-V was conducted for cocoyam rhizosphere isolates *Pseudomonas* sp. COR19 (Cameroon), WCU_64 and NNC7 (Nigeria). For CLP purification, seed cultures of *Pseudomonas* strains 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 r.p.m. for 24 h. *Pseudomonas* supernatant was collected after centrifugation at 10000 x 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 10000 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 (250 × 21.2 mm,

5 μm particle size) for separation of the individual CLP analogues. NMR analyses were conducted as described previously (Oni *et al.*, 2019).

Genome sequencing and assembly of putisolvin-producing strains

For genome sequencing, bacterial strains were grown in Luria Bertani (LB) broth for 24 h at 28°C with continuous shaking at 150 rpm. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions.

Single-end or paired-end sequence reads were generated using the Illumina HiSeq2500 or MiSeq system at the BASECLEAR B. V. Leiden, Netherlands. FASTQ read sequence files were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.5.

The quality of Illumina reads was improved using the error correction tool BayesHammer (Nikolenko *et al.*, 2013). Error-corrected reads were assembled into contigs using SPAdes version 3.10 (Bankevich *et al.*, 2012). The order of contigs, and the distances between them, were estimated using the insert size information derived from an alignment of the paired-end reads to the draft assembly. Consequently, contigs were linked together and placed into scaffolds using SSPACE version 2.3 (Boetzer *et al.*, 2011). Using Illumina reads, gapped regions within scaffolds were (partially) closed using GapFiller version 1.10 (Boetzer and Pirovano, 2012). Finally, assembly errors and the nucleotide disagreements between the

Illumina reads and scaffold sequences were corrected using Pilon version 1.21 (Walker *et al.*, 2014).

Genome annotation, genome mining and bioinformatics analyses

Genome sequences were automatically annotated using the RAST annotation pipeline (Aziz *et al.*, 2008; Brettin *et al.*, 2015). Furthermore, genomes of previously sequenced strains were re-annotated using the RAST annotation pipeline and also submitted to antiSMASH v4.0 (Blin *et al.*, 2017). Genome mining was conducted on the annotated genomes, and comparison of NRPS proteins with other protein sequences in GenBank database was done by BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The adenylation (A) and condensation (C) domains sequences of the non-ribosomal peptide synthase (NRPS) genes were extracted. Sequence alignment was carried out using MUSCLE (Edgar, 2004) in the software package MEGA6 (Tamura *et al.*, 2013). Phylogenetic tree was constructed using the Geneious 2019. For *in silico* characterization of all CLPs, a combinational approach employing the antiSMASH v4.0 and the NRPSpredictor2 (Röttig *et al.*, 2011) enabled the identification of the amino acid composition of the peptide moiety.

Results

Taxonomic association of Pseudomonas *spp. obtained from Nigeria*

In this study, 166 *Pseudomonas* isolates were obtained from two locations in Nigeria namely Ado-Ekiti (67 isolates) and Umudike (99 isolates). Nine isolates obtained from the cocoyam rhizosphere in Ugbokolo, Benue State, Nigeria, were also included in this study (Table S7). Our results showed a delineation of isolates from Nigeria into the *P. putida*, *P. oleovorans* and the *P. aeruginosa* phylogenetic groups (Figure 1, Figure S1, Table S7). Specifically, the

cocoyam rhizospheres in Ado-Ekiti and Umudike were enriched in *P. putida* group isolates. Additionally, varying numbers of *P. aeruginosa* group strains were isolated from Umudike (six) and Ado-Ekiti (twenty) (Figure S1). A few isolates (thirteen) belonging to the *P. oleovorans* group were also identified at Ado-Ekiti (Table S7, Figure S1). Duplicate *Pseudomonas* isolates were identified using Elim Dupes HIV Databases and unique *rpoD* sequences were used to construct the phylogenetic tree. *rpoD* sequences were deposited in the Genbank database under accession numbers MT511000 to MT511052.

Comparison of Pseudomonas *spp. diversity in a suppressive soil versus conducive soils*

As previously mentioned, unique *rpoD* sequences of *Pseudomonas* strains obtained from the cocoyam rhizosphere in three separate andosols in Cameroon (Oni *et al.*, 2019) were also included in this study (Table S3). *P. putida* and *P. aeruginosa* group isolates from Nigeria clustered with phylogenetically related isolates from the cocoyam rhizosphere in Cameroon (Figure 1). On the other hand, *Pseudomonas* isolates from the cocoyam rhizosphere in Cameroon were affiliated with the *P. putida* group, *P. aeruginosa* lineage, *P. fluorescens* group and three undefined groups namely U1, U2 and U3 (sub)groups (Oni *et al.*, 2019) (Table S7). In particular, isolates belonging to the *P. fluorescens* group were only found in the cocoyam rhizosphere in the andosols of Cameroon (Table S7). Isolates obtained from the suppressive Boteva field, were almost equally dispersed between the *P. fluorescens* group (55%) and *P. putida* group (45%), whereas the 76 isolates obtained from the degraded, conducive andosols (Ekona and Maumu), were predominantly affiliated with the *P. putida* group (Table S7). Only a few *P. fluorescens* group isolates were recorded from Ekona (eight) and Maumu (two) andosols. All *P. koreensis* subgroup isolates were obtained from the cocoyam rhizosphere in

Boteva (Table S7) and they were found on both white (15 isolates) and red (11 isolates) cocoyam varieties (Table S3).

CLP diversity in the cocoyam rhizosphere in Nigeria

Pseudomonas strains obtained from the cocoyam rhizosphere in Nigeria which tested positive for drop collapse and swarming were 16, 10 and 3 from Ado-Ekiti, Umudike and Ugbokolo, respectively (Table S7). For initial CLP identification, these strains were analyzed using UPLC-MS. Our analyses revealed that three structurally different CLPs are produced namely putisolvin, WLIP and xantholysin (Figure 1). Putative WLIP-producing isolates (RCA_39 and RCA_70) were subjected to comparative metabolic profiling with described WLIP producer, NSE1 and results showed similar main and minor peaks with a main peak at *m/z* 1126 (Figure S2). In previous studies, *Pseudomonas* sp. NSE1 has been reported to produce WLIP (Omoboye *et al.*, 2019). A comparative metabolite profiling of a xantholysin producer from Cameroon (COR51) and a potential producer from Nigeria (WCA_13) revealed the production of a main xantholysin compound and five congeners (Figure S3A). We observed the production of deprotonated molecular ion $[M+H]^-$ at m/z 1761 (6.57 min), m/z 1775 (6.82 min), m/z 1761 (7.04 min), m/z 1775 (7.29 min), m/z 1802 (7.52 min) and m/z 1804 (7.64 min). These peaks were designated peaks 1 to 5, respectively (Figure S3A, Figure S4, Table S8). A comparative UPLC-MS further showed that putisolvin-producing isolates from Nigeria (WCU_64, NNC7) and Cameroon (COR55, COR19) appear to produce similar main and minor compounds (Figure S3B, Figure S5). Moreover, in this study, representative strains (COR19 and WCU_64) producing putisolvin were characterized both chemically by NMR and genetically by mining the draft genomes of corresponding strains.

CLP diversity comparison in a suppressive soil versus conducive soils

The CLP diversity of some of the Cameroon strains was characterized during our previous study (Oni *et al.*, 2019). Strains producing these CLPs are situated in the *P. putida* group, *P. asplenii* group and in the *P. koreensis*, *P. chlororaphis* and U2 subgroups. In these strains, 13 structurally different CLPs were identified namely entolysin, lokisin, putisolvin, xantholysin, WLIP, cocoyamide A, and novel CLPs – N2, N3, N4, N5, N6, N7 and N8 (Oni *et al.*, 2019). N3 has been described as new bananamide derivative D (Omoboye *et al.*, 2019). N2 (rhizoamide) is a novel member of the amphisin group, whereas N6 has been characterized as pseudodesmin, a member of the viscosin CLP group (Table 1). Furthermore, some CLPs belong to novel groups comprising different numbers of amino acids (AA) including N4 (12AA), N5 (13AA), N7 (19AA) and N8 (17AA) (Table 1). Full chemical and genomic data of these unnamed CLPs, N2 (rhizoamide) and pseudodesmin will be published elsewhere.

Principal Component Analysis (PCA) showed the distribution of CLPs across the suppressive soil of Boteva and the conducive soils of Cameroon (Ekona and Maumu) and Nigeria (Ado-Ekiti and Umudike). The first principle component, which explained 61.9% of the total variance, distinguished Boteva (a CRRD suppressive soil) from other soils which were considered to be conducive (Figure 2A). There was an overlap in the representation of bananamide D, rhizoamide, lokisin, pseudodesmin and cocoyamide A, hence they were manually separated on the PCA.

CLP diversity in all five locations was also quantified using the Shannon index. Results showed that the highest index was at Boteva indicating a high CLP richness (H=1.97) and moderate evenness (E=0.55) (Figure 2B). Ekona had a considerable CLP diversity and moderate

evenness (H=1.61; E=0.54), that of Ado-Ekiti and Maumu were lower than Ekona but comparable with each other while Umudike had the lowest CLP diversity (0.32) and much lower CLP evenness (0.14) (Figure 2B).

Swarming and white line-in-agar tests

In previous studies, we established that similar CLP-producing strains appeared to swarm alike (Oni *et al.*, 2019). In this study, putisolvin-, WLIP- and xantholysin-producing strains from Nigeria gave a similar swarming phenotype as the ones from Cameroon (Figure 3A). Comparison of cocoyamide (COR48) and gacamide producer (Pf0-1gac+ mutant) (Jahanshah *et al.*, 2019) revealed a similar swarming phenotype. Furthermore, both strains (cocoyamide and gacamide producers) showed the white line-in-agar phenotype when challenged with the sessilin producer, *Pseudomonas* sp. CMR12a-ΔPhz-ΔCLP2 (Figure 3B). During interaction with this indicator strain, the white line-in-agar phenotype was also displayed by the bananamide D-, entolysin-, WLIP- and N8 producers but not by strains producing putisolvin, xantholysin, lokisin, pseudodesmin, N2, N5, and N4 (Figure 3B).

Taxonomic distribution of Pseudomonas *spp. versus CLP production*

All *Pseudomonas* strains in the cocoyam rhizosphere were affiliated with the *P. fluorescens*, *P. putida, and P. asplenii* groups and the *P. aeruginosa* lineage (Figure 4). Phylogenetic analyses using MLSA (16S rRNA-*gyrB*-*rpoB*-*rpoD*) showed that in our study, CLP-producing isolates were situated within the *P. fluorescens*, *P. putida*, and *P. asplenii* groups (Figure 4). Within the *P. fluorescens* group, the *P. koreensis* subgroup shows a high CLP diversity including lokisin, cocoyamide A/gacamide A, the novel CLP rhizoamide, and strains producing bananamide D (*Pseudomonas* sp. COW3 and COW65). Furthermore, we show that the

pseudodesmin producer (COR52) clusters with the *P. chlororaphis* subgroup. The N4 producers (COR58, COR24 and COR25) appear to form a new taxonomic subgroup (called U2 group in Oni *et al.*, 2019) while COR33 and COR18 which produce N5 and the dual CLPs (N5 and N7), respectively, are situated in the *P. asplenii* group (called U1 subgroup in Oni *et al.*, 2019). Lastly, the discovery of N8 represents a new CLP addition to the *P. putida* group (Figure 4). MLSA sequences extracted from draft genomes were deposited in Genbank database under accession numbers MT506152-MT506171 (*rpoD*), MT506172-MT506193 (*rpoB*), MT506949-MT506970 (*gyrB*), and MT507059-MT507080 (16S rRNA).

Only two xantholysin producers were obtained from Nigeria and phylogenetic analyses using *rpoD* partial sequences (Figure 1) showed that they clustered closely but distinctly with xantholysin producers from Cameroon such as COR51 (Ekona). The original xantholysin producer, BW11M1 (Li *et al.*, 2013) was situated in the same clade but considerably distant from the cocoyam rhizosphere isolates (Figure 1). In this study, six WLIP producers were identified from Nigeria which clustered with WLIP producers from Cameroon except COW40 and COR54 (Figure 1). Specifically, *Pseudomonas* sp. NSE1 from Umudike clustered closely with COW10, the WLIP producer from Maumu in Cameroon. A diverse group of putisolvin producers from Nigeria was observed; one group clustered with COR55 (Ekona) and COR19 (Maumu) from Cameroon, the second group clustered with COW62 from Maumu in Cameroon while a last group was distinct.

Physico-chemical soil characteristics

Physico-chemical soil characteristics of Boteva and Ekona soils revealed that the former is rich in organic carbon, total nitrogen, calcium and contains high amounts of silt whereas Ekona soil

is characterized by lower amounts of silt, organic carbon, total nitrogen, calcium and a high clay content (Adiobo *et al.*, 2007). In this study, analysis of Maumu soil showed that similar to Ekona, it has lower amounts of silt (31%), organic carbon (3.45%) and calcium (2.92 cmol kg^{-1}) compared with Boteva and a high sand content (54%) (Table 2). Furthermore, soils of Ado-Ekiti and Umudike recorded even much lower amounts of calcium, organic carbon, total nitrogen and silt than Ekona and Maumu (Table 2). Unlike other soils, a high phosphorus content was observed at Maumu and Umudike. All soil samples were acidic (pH 4.7 – 5.8) except Ado-Ekiti which had a pH of 7.0. Although we did not conduct soil analysis for Ugbokolo, Benue state, Nigeria, a previous study described Ugbokolo soils as being coarse-grained lateritic sandy (Egirani *et al.*, 2006).

Interaction between location, soil texture, Pseudomonas *spp. and CLP diversity*

Soil physicochemical characteristics were correlated with CLP diversity within the *Pseudomonas* spp. recorded in our soils (Table 3). Significant correlations occurred between CLP diversity and silt, Ca, total N, ECEC, and organic C. In addition, we observed a near perfect correlation of organic matter with silt (0.99) and strong interaction of Ca with organic carbon (0.92), N (0.93), ECEC (0.97) and silt (0.93) (Table 3). These parameters further showed strong interactions with each other (Table 3).

The distribution of CLP-producing *Pseudomonas* spp. across the five different fields used in this study are shown in Table 1. A high CLP abundance and diversity was observed in *Pseudomonas* strains obtained from Boteva soil which had higher organic carbon, total N, silt and ECEC. The cocoyam rhizosphere of Cameroon andosols typically recorded higher *Pseudomonas* diversity and CLP abundances compared to that of the alfisol (Ado-Ekiti) and

Ultisol (Umudike) (Table 1). All CLPs that were shared between locations were produced by members of the *P. putida* group while CLPs which were unique to specific locations were produced either by members of the *P. fluorescens* or *P. asplenii* group.

Chemical characterization of novel Putisolvin derivatives: Putisolvins III, IV and V

For full characterization of CLPs produced by the afore-mentioned isolates, crude CLP extracts of COR19, NNC7 and WCU_64 were purified using semi-preparative HPLC. For all extracts, three peaks were collected (Figure 5A). The collected CLPs were subjected to further analysis using liquid state NMR spectroscopy and LC-MS spectrometry.

Our analyses revealed that of all three samples analysed, the main CLP consists of a fatty acid (lacking the typical 3-hydroxyl functionality) linked to a peptide chain involving 12 amino acids (5x Leu, 3x Ser, 2x Val, 1x Glu, 1x Gln). The amino acid sequence was confirmed by the analysis of the 2D ^{1}H–^{1}H ROESY and ^{1}H–^{13}C gHMBC spectra. The sequence is detailed in Figure 5B. For COR19, the position of the ester bond that cyclizes the molecule could be confirmed by analysis of the ^{1}H–^{13}C gHMBC spectrum, showing a clear cross peak between Ser9 $H^{\beta 1\text{-}2}$ and Ser12 C'(Figure S6-S7). The unusually high chemical shifts of Ser9 $^{H\beta 1\text{-}2}$ indicates that this residue is involved in the depsi bond.

In this way, it was established that the main CLP produced by *Pseudomonas* sp. COR19 is a new member of the putisolvin group (putisolvin III), showing the presence of Leu4 and Leu8 where putisolvin I produced by *P. putida* PCL1445, possesses an Ile4 and Ile8 (Figure 5B-C) (Table S9). Thus the amino acid sequence of putisolvin III (*m/z* 1379.8276) is Leu1-Glu2-Leu3-Leu4-Gln5-Ser6-Val7-Leu8-Ser9-Leu10-Val11-Ser12 with molecular formula $C_{65}H_{113}N_{13}O_{19}$. MS data are in agreement with the proposed putisolvin III sequence and the

presence of a hexanoic fatty acid moiety (C6:0) at the N-terminus. The chemical structure of the isolated main CLP of COR19 is identical to that of the putisolvins produced by *Pseudomonas* sp. NNC7 and *Pseudomonas* sp. WCU_64, as judged by a comparison of the CHα NMR chemical shifts of both compounds, recorded under identical conditions (Figure 5A-C, Figure S8, S9, S10). The minor compounds (Figure 5A) of all these putisolvin III producers were also identified as new members of the putisolvin group. These CLPs differ from the main compound at position 11, where they possess an Ile11 or Leu11, respectively, compared to Val11 in the main compound (Figure 5B-C) (Table S10-S14).

In silico *analysis of the putisolvin III gene cluster*

Using antiSMASH v4.0, the draft genomes of isolates from Cameroon (COR55 and COR19) and Nigeria (WCU_60, WCU_64, and NNC7) showed a similar NRPS cluster containing 12 modules. Further analyses identified the presence of a putisolvin NRPS cluster with three genes; *psoA* (two modules, 6.3 kb), *psoB* (seven modules, 22.4 kb) and *psoC* (three modules, 11.3 kb) (Figure 6). Similar to the gene cluster of putisolvin producer, *P. putida* PCL1445, for all our strains, putative *luxR* (*psoR1*) and *nodT* (*psoT*) transporter genes were found upstream of the *psoA* while the *luxR* (*psoR2*) together with *macA* (*psoD*) and *macB* (*psoE*) genes were found downstream of *psoC*. Amino acid prediction obtained from the BGC sequences of our strains was similar with that of PCL1445 (Figure 6). *In silico* (using antiSMASH and NRPS predictor tools) and phylogenetic analyses of the A domain substrate specificity of the 11 modules, in comparison with that of PCL1445 and other CLP-encoding CLPs, enabled the identification of the amino acid composition of the peptide moiety (Figure S11). The amino acid composition of our strains are the same as experimentally determined via NMR

spectroscopy. The BGC and flanking area sequences of *Pseudomonas* strains WCU_64, COR19 and WCU_60 has been submitted to the Genbank database under accession numbers MT511054, MT511055 and MT511056, respectively.

Phylogenetic analysis of the concatenated BGC protein sequences of our putisolvin producers versus PCL1445 and E41 revealed the closeness of clusters from Nigeria strains (WCU_64 and WCU_60). Putisolvin BGC sequences of PCL1445 showed closer relatedness to that of COR19 while that of E41 was less similar to the rest (Figure S12). *In silico* analysis of the C domains revealed the presence of a lipo-initiation ($C_{starter}$) domain in module 1 of *psoA* indicating that a fatty acid is attached to Leu1. Further, an automated analysis of the C domains revealed that those of modules 11 and 12 are predicted to be conventional $^{L}C_{L}$ domains while the rest of the C domains were predicted to be C/E –type domains (Figure S13). This suggests that residues incorporated by modules 10, 11 and 12 are in the L configuration while all other amino acids are possibly in the D configuration. The termination module of *psoC* consists of a tandem TE domain. A phylogenetic analysis showed that the first Te domain clusters with the Type 1 Te domains of *Pseudomonas* CLPs which function in the cyclization of the C-terminal amino acid and a threonine or serine residue while the second Te domain forms a clade with type II TE domains with functions in the support of the assembly line (Figure S14).

Discussion

In our previous study (Oni *et al.*, 2019), we found that *P. fluorescens* and *P. putida* group isolates were enriched in the rhizosphere of cocoyam plants grown in three andososols at Boteva, Ekona and Maumu in Cameroon. Additionally, 50% of the *Pseudomonas* isolates

produced CLPs and 13 structurally different CLPs could be identified. In this study, we failed to obtain *P. fluorescens* group isolates from the cocoyam rhizosphere in Ado-Ekiti and Umudike in Nigeria. Isolates obtained at these locations clustered with the *P. putida* group and *P. aeruginosa* lineage. Moreover, only 16% of the Nigerian isolates produced CLPs and we found no more than three structurally different CLPs namely xantholysin, WLIP and putisolvin. The discrepancy between these findings and our previous work in Cameroon prompted us to reevaluate our previous data taking location and soil physicochemical properties into account. We compared the *Pseudomonas* and CLP diversity in the cocoyam rhizosphere of the *Pythium* suppressive Boteva soil in Cameroon with that obtained in the four conducive soils of Cameroon and Nigeria. Further, we compared the soil physicochemical properties of these soils to assess which parameters are correlated with CLP diversity in the *Pseudomonas* population and may provide indications for disease suppressiveness or conduciveness.

CRRD disease suppressiveness vs Pseudomonas *diversity*

In comparison to disease-conducive soils, *Pseudomonas* isolates obtained from the rhizosphere of cocoyam grown in disease-suppressive Boteva soil showed a higher diversity and richness. Isolates were almost equally dispersed between the *P. fluorescens* and the *P. putida* groups. Within the *P. fluorescens* group, Boteva isolates predominantly clustered in the *P. koreensis* subgroup. Besides Boteva, the cocoyam rhizosphere of other disease conducive soils used in this study had fewer (Ekona, Maumu) or no *P. fluorescens* group (Ado-Ekiti, Umudike) isolates. The dominance of *P. fluorescens* group members in the cocoyam rhizosphere at Boteva is perhaps not unexpected, since previous studies showed that the most effective biocontrol strains that have been popularly studied are taxonomically positioned within the *P.*

fluorescens group and produce a broad spectrum of secondary metabolites (Gomila *et al.*, 2015; Garrido-Sanz *et al.*, 2016).

We are currently investigating whether or not the soils of Ado-Ekiti and Umudike contain *P. fluorescens* group isolates which for some reason, were unable to colonise the rhizosphere of field-grown cocoyams. On the other hand, our results revealed that isolates from three study sites (Maumu, Ado-Ekiti and Umudike) predominantly clustered within the *P. putida* group. In a previous study, comparative gene expression of *P. putida* strains during interaction with maize roots showed that the presence of these species in the rhizosphere is driven by two selective forces; their capacity for stress adaptation and availability of particular nutrients (Matilla *et al.*, 2007).

CRRD disease suppressiveness vs CLP diversity

The cocoyam rhizosphere in suppressive Boteva soils contained *Pseudomonas* strains producing 11 structurally diverse CLPs, six of which appeared exclusive to this location. These CLPs were bananamide D, cocoyamide A, lokisin, pseudodesmin, rhizoamide (N2), and N5. Since none of them (except N5) were shared by any of the other four locations, they are likely accessory CLPs which are specifically produced by isolates from this niche. Of these six CLPs, four (bananamide, cocoyamide, lokisin and rhizoamide) are produced by isolates belonging to the *P. koreensis* subgroup. Bananamide-like strains have been found in Sri-Lanka (Nguyen *et al.*, 2017), Spain (Pascual *et al.*, 2015) and in Brazil (Lopes *et al.*, 2018; Stringlis *et al.*, 2018). The gacamide/cocoyamide producer, Pf0-1, was isolated from an agricultural loam soil in Massachusetts, USA (Compeau *et al.*, 1988) and multiple cocoyamide producers were obtained

from the sugarcane rhizosphere in Brazil (Stringlis *et al.*, 2018). Our study brings to the fore that *P. koreensis* subgroup isolates appear to be positive indicators of soil suppressiveness. Furthermore, we observed a remarkable enrichment of putisolvin-producing *P. putida* isolates in the cocoyam rhizosphere from conducive soils. Interestingly, the putisolvin BGCs from Cameroon and Nigeria strains encode similar amino acids as that of PCL1445 suggesting that the chemical characterization of *P. putida* PCL1445 needs to be reviewed. With respect to origin, putisolvin producers PCL1445 was isolated from the rhizosphere of plants grown in a site contaminated with polycyclic aromatic hydrocarbons (Kuiper *et al.*, 2004) and *P. putida* E41 from the root interior of *Sida hermaphrodita*, grown in a heavy metal contaminated soil (Bernat *et al.*, 2019). Although we did not have a large number of isolates from Ugbokolo, Nigeria, it was interesting to note that all nine isolates belong to the *P. putida* group and some produce putisolvins. Ugbokolo soils are reportedly lateritic sandy soils (Egirani *et al.*, 2006). Lateritic soils are rich in iron oxide and aluminium and are not very fertile (Ko, 2014). In a previous study, the only putisolvin-producing *P. putida* isolates could be isolated from the rhizosphere of black pepper which were grown in lateritic soils of Vietnam (Tran *et al.*, 2008). *Pseudomonas* strains belonging to these species have also been reported as naphthalene degraders (Phale *et al.*, 2013) and occur in oil spillage sites (Mulet *et al.*, 2011). Thus, putisolvin producers appear to abound in harsh or contaminated environments where CLP diversity is considerably low. In these environments the CLP may play a role in the degradation or detoxification of the pollutant. It has been shown, for instance, that *Pseudomonas* lipopeptides, including putisolvin, are efficient emulsifiers of n-hexadecane and can stimulate its mineralization (Bak et al., 2015).

Physicochemical soil properties vs Pseudomonas *and CLP diversity*

During this study, differences in soil physicochemical properties recorded in different soils were correlated with CLP diversity in the *Pseudomonas* population. More specifically, we revealed that *Pseudomonas* species richness and CLP diversity in the various soils correlates with silt, calcium, organic carbon, total nitrogen and ECEC. We previously showed that the percentage of silt, organic carbon, total nitrogen and to a lesser extent calcium and ECEC correlated negatively with cocoyam root rot disease severity in various Cameroonian soils (Adiobo *et al.*, 2007). The percentage of clay or sand correlated positively with CRRD severity. Moreover, all soil variables that negatively correlated to CRRD severity (Ca, N, silt, and ECEC) were positively associated with soil organic carbon. By contrast, variables that positively correlated to CRRD severity, such as percentage of sand or clay, were negatively associated to soil organic content (Adiobo *et al.*, 2007). Additional correlation analyses conducted in this study using Maumu (andosol, Cameroon), Ado-Ekiti (alfisol, Nigeria) and Umudike (ultisol, Nigeria) soils appear to follow a similar pattern as that of conducive Cameroon soils in the previous study.

Different soil types characterized by 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, Ding, *et al.*, 2014; Schreiter, Sandmann, *et al.*, 2014). Bacterial communities in the rhizosphere of field-grown lettuce were found to depend on soil type and plant growth developmental stage (Schreiter, Sandmann, *et al.*, 2014). Compared with diluvial sandy soil, soils with higher silt content appeared to have similar microbial bulk soil communities while the highest

rhizospheric effect was registered on lettuce plants grown in alluvial loamy soils. Thus, we opine that the presence of higher amounts of silt at Boteva may influence microbial selection.

Nature of disease suppression in Boteva soil – microbial competition?

Phylogenetically diverse soil microorganisms have been associated with plant disease suppression (Raaijmakers and Mazzola, 2012; Dignam *et al.*, 2019). Among these organisms, *Pseudomonas* spp. has been implicated in the capacity to suppress disease in varying management systems (Garbeva *et al.*, 2004; Van Overbeek *et al.*, 2012; Walters *et al.*, 2018). In our work, *Pseudomonas* species diversity and richness was highest in the cocoyam rhizosphere in Boteva. The nature of disease suppressiveness in this soil appears to be general since general suppression is community-based (Schlatter *et al.*, 2017). Moreover, Boteva soil is typified by an assemblage of diverse soil microbes with capacity to antagonize the cocoyam root rot pathogen, *P. myriotylum* (Oni *et al.*, 2019). It is notable that compared to the other soils in this study, Boteva soil is characterized by higher amounts of organic carbon. Although plant residue management was shown to significantly influence the community structure of *Pseudomonas* genus between treatments, 80% of observed differences in *Pseudomonas* community structure was attributed to changes in soil organic matter (Dignam *et al.*, 2019). Increased *Pseudomonas* diversity as evidenced by the Shannon index, in addition to microbial activity, soil organic matter and availability of carbon, distinguished low disease (suppressive) soils from high disease (conducive soils) (Dignam *et al.*, 2019). It should be noted that in the volcanic soils of Cameroon, soil suppressiveness is lost if cocoyam is cultivated continuously for a period of more than 5 years (Adiobo *et al.* 2007) as exemplified by the Ekona and Maumu soils. This is probably due to depletion of organic matter. Our findings suggest that soil organic

matter is a crucial driver of *Pseudomonas* diversity and that the diversity and richness of this genus represents a good indicator of soil suppressiveness.

According to Schlatter et al. (2017), general suppression does not depend upon pathogen populations or disease and can result from microbial competition among soil saprophytes. We hypothesize that CLP diversity within *Pseudomonas* populations may be driven by antagonist-antagonist interactions. In this context, it is interesting to have a closer look at white line formation between interacting CLPs. The *in vitro* interaction between the pathogenic *P. tolaasii* and another *Pseudomonas* bacterium, referred to as "*P. reactans*" resulted in the formation of a sharply defined white line which has since been considered to be specific (Wong and Preece, 1979; Lo Cantore *et al.*, 2007). Years after, the CLP produced by *P. reactans* termed WLIP, has been characterized in several *Pseudomonas* strains and it was shown that the white line results from the interaction between WLIP and the CLP tolaasin, produced by *P. tolaasii* (Rokni-Zadeh *et al.*, 2012; Mehnaz *et al.*, 2013; Oni *et al.*, 2019). We previously showed that sessilin, a CLP very similar to tolaasin produced by the cocoyam rhizosphere isolate, *Pseudomonas* sp. CMR12a, interacts with orfamide producers such as *P. protegens* Pf-5 to give a white line (D'aes *et al.*, 2014). The white line produced on KB agar medium during interaction between the CMR12a orfamide mutant (CMR12a-ΔCLP2) and its sessilin mutant (CMR12a-CLP1) was analyzed using matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Results confirmed the accumulation of both orfamide and sessilin in the zone corresponding to the white line, giving evidence that the two compounds interact to form a precipitate (D'aes *et al.*, 2014). In this study, we showed that besides the WLIP-producing strains, white line phenotypes were given by cocoyamide-,

bananamide D-, entolysin- and N8 producers, when confronted with a *Pseudomonas* sp. CMR12a mutant strain which only produces sessilin. White line production by certain CLP producers may play a role in microbial defense and in warding off niche competitors. Thus, the abundance of these specific strains at Boteva and their absence across CRRD suppressive gradients suggests a probable role in general suppressiveness triggered by microbial competition.

In West and Central Africa, the cocoyam crop is mainly grown in tropical rainforest regions which constitute a plethora of spectacular diversity. These forests represent the world's richest repository of terrestrial diversity (Ghazoul and Sheil, 2010). Furthermore, *Pseudomonas* isolates from Brazil also displayed *Pseudomonas* spp. and CLP diversity with close similarity to some described in our study (Lopes *et al.*, 2018; Stringlis *et al.*, 2018). Thus, we observe a correlation between environment/ecology versus *Pseudomonas* spp. and bioactive molecules produced. The heterogeneity of compounds produced by these strains suggest that they are required to thrive in this specific competitive tropical niche.

Conclusion

We previously showed that in the cocoyam rhizosphere, *Pseudomonas* and CLP diversity is independent of genotype although the red cocoyam genotype appeared to support a more diverse CLP spectrum compared to the white genotype (Oni *et al.*, 2019). In this study, we demonstrate the general disease suppressiveness of Boteva soil, characterised by an accumulation of *P. myriotylum*-inhibitory *Pseudomonas* strains which produce eleven diverse CLPs, in the cocoyam rhizosphere (Figure 7). Additionally, *P. koreensis* subgroup members

and cocoyamide A producers were dominant in the cocoyam rhizosphere of this soil. In contrast, *P. putida* group isolates and putisolvin producers were dominant in the cocoyam rhizosphere in CRRD-conducive soils. Our study indicates that high *Pseudomonas* and metabolic diversity may be driven by microbial competition which likely contributes to soil suppressiveness or conduciveness to CRRD.

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Originality-Significance Statement

This study compares and reports the comparative *Pseudomonas* spp and cyclic lipopeptide diversity in a *Pythium*-suppressive andosol versus conducive andosols, an ultisol and an alfisol.

We show that the abundance of the *P. koreensis* subgroup may be an indicator of *Pythium*-disease suppression while conducive soils are characterized by *P. putida* group abundance. Additionally, we show that cocoyamide A producers were dominant in the cocoyam rhizosphere of the disease suppressive soil while putisolvin III producers typified the rhizosphere in conducive soils. Furthermore, our study shows that the presence of high *Pseudomonas* and metabolic diversity may be driven by microbial competition which likely contributes to soil suppressiveness to CRRD.

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Figure Legends

Figure 1. Phylogenetic analyses of 166 *Pseudomonas* isolates from Nigeria using partial sequences of the *rpoD* housekeeping gene and *P. aeruginosa* group as outgroup. Bootstraps are only indicated for branches with bootstrap support of higher than 70%. A Maximum Likelihood tree was made using only isolates with unique sequences whereas isolates with identical sequences were added afterwards, on the same line. NSE- and WCU- isolates were obtained from Umudike, Nigeria, WCA- and RCA- isolates were obtained from Ado-Ekiti, Nigeria, while NNC- isolates were obtained from Ugbokolo, Benue State, Nigeria. RCA and WCA: Red Cocoyam Ado-Ekiti and White Cocoyam Ado-Ekiti, respectively; NSE and WCU: Nigeria South East and White Cocoyam Umudike, respectively; NNC: Nigeria North Central. CLP-producing isolates are colour-coded. Xantholysin, WLIP, and Putisolvin are colour-coded circles of green, brown and grey, respectively. Isolates belonging to the *P. putida* group are shown while the *P. aeruginosa* group/lineage is collapsed.

Figure 2. CLP diversity characterized from *Pseudomonas* spp. isolated from five cocoyam fields. A) PCA ordination based on CLPs found in the five cocoyam fields. The five locations are indicated with red dots. The first principal component explains 61.9% of the variance while the second explains 22%. Cocoyamide, Bananamide D-G, lokisin, pseudodesmin, and N2 were on the same spot, an option was chosen for the analyses to ensure the CLPs were separate and coloured light red. **B)** Plot of Shannon diversity showing CLP richness and evenness in *Pseudomonas* spp. obtained from five locations. A: Ado-Ekiti, U: Umudike, M: Maumu, E: Ekona, B: Boteva.

Figure 3. Swarming and white line-in-agar phenotype for CLP-producing strains from the cocoyam rhizosphere in Cameroon and Nigeria A) Comparative swarming for putisolvin III- (COR55 and RCA_55), WLIP- (COW10 and NSE1), xantholysin-producing strains (COR51 and WCA_13) from Cameroon and Nigeria. The swarming pattern of the gacamide producer, Pf0-1+gacA and a cocoyamide producer, COR48, is also displayed. **B)** White line-in-agar phenotypes for cocoyamide-, N8-, WLIP-, entolysin and bananamide-producing strains, isolated from the cocoyam rhizosphere. Producers of gacamide (Pf0-1gac+ mutant), MDN-0066 (*P. granadensis* F-278,770T) and orfamide (*P. protegens* Pf-5) are from different hosts and environments. The sessilin producer, CMR12a-ΔPhz-ΔCLP2 was used as an indicator strain while several representative test strains were used for the various CLPs. For N8-, WLIP- and entolysin- producers, tests were done in duplicate for each strain.

Figure 4. MLSA-derived phylogenetic analyses of representative CLP-producing *Pseudomonas* isolates associated with the cocoyam rhizosphere. Partial sequences of 16S rRNA, *gyrB*, *rpoB* and *rpoD* genes were used to make separate trees and concatenated afterwards, to generate a consensus tree. Other biocontrol CLP-producing isolates and type strains were included in this analysis. *P. aeruginosa* was used as an outgroup. A Maximum Likelihood tree was made and bootstraps are only indicated for branches with bootstrap support of higher than 70%. NSE- and WCU- isolates were obtained from Umudike, Nigeria, WCA- and RCA- isolates were obtained from Ado-Ekiti, Nigeria, while NNC- isolates were obtained from Ugbokolo, Benue State, Nigeria. Isolates with the COR- and COW- prefixes were obtained from Cameroon. Coloured clades contain strains used in this study. Different rectangular colours represent different CLP types produced by isolates from Cameroon and

Nigeria. Strains with coloured circles represent similar or different CLP types characterized from previous studies.

Figure 5. Characterisation of novel putisolvin III and derivatives IV and V. A) Preparative HPLC chromatogram of the crude CLP extracts for putisolvin III-producing isolates from Cameroon (COR19) and Nigeria (WCU_64) reveal a main peak and two minor peaks. Analysed CLPs are indicated III, IV and V. Gradient: 25:75 to 0:100 H_2O:acetonitrile in 20 minutes. **B)** Comparative sequences of putisolvin I and II (produced by *P. putida* PCL1445) and the newly described putisolvins III–V, produced by *Pseudomonas* sp. COR19/WCU_64/NNC7; **C)** Chemical structure of the major isolated CLP identified as putisolvin III comprising oligopeptide length of 12 with 4 amino acids in the macrocycle; Chemical structure of the first minor CLP identified as putisolvin IV; Chemical structure of the second minor CLP identified as putisolvin V.

Figure 6. Organization of the gene cluster encoding Putisolvin III in *Pseudomonas* spp. COR19 and WCU_64. Putisolvin III gene cluster comprises three NRPS genes, *psoA*, *psoB* and *psoC*. Upstream of *psoA* is the NodT-like transporter, *psoT*, and the luxR transcriptional regulator, *psoR1*. Downstream of *psoC* are the *macA* and *macB* genes, *psoD* and *psoE*, respectively, including the *luxR* regulator, *psoR2*. Genes in green, labelled 1 and 2 are the universal stress protein and the integral membrane protein, respectively. The putisolvin III gene cluster is compared with that of the putisolvin I gene cluster in *P. putida* PCL1445. The putisolvin III gene cluster of COR19 is representative of other putisolvin III producers from Cameroon (*Pseudomonas* spp. COR55) and Nigeria (*Pseudomonas* sp. NNC7, WCU_60 and WCU_64) since their gene sizes are exactly identical. Upstream of the putisolvin cluster in

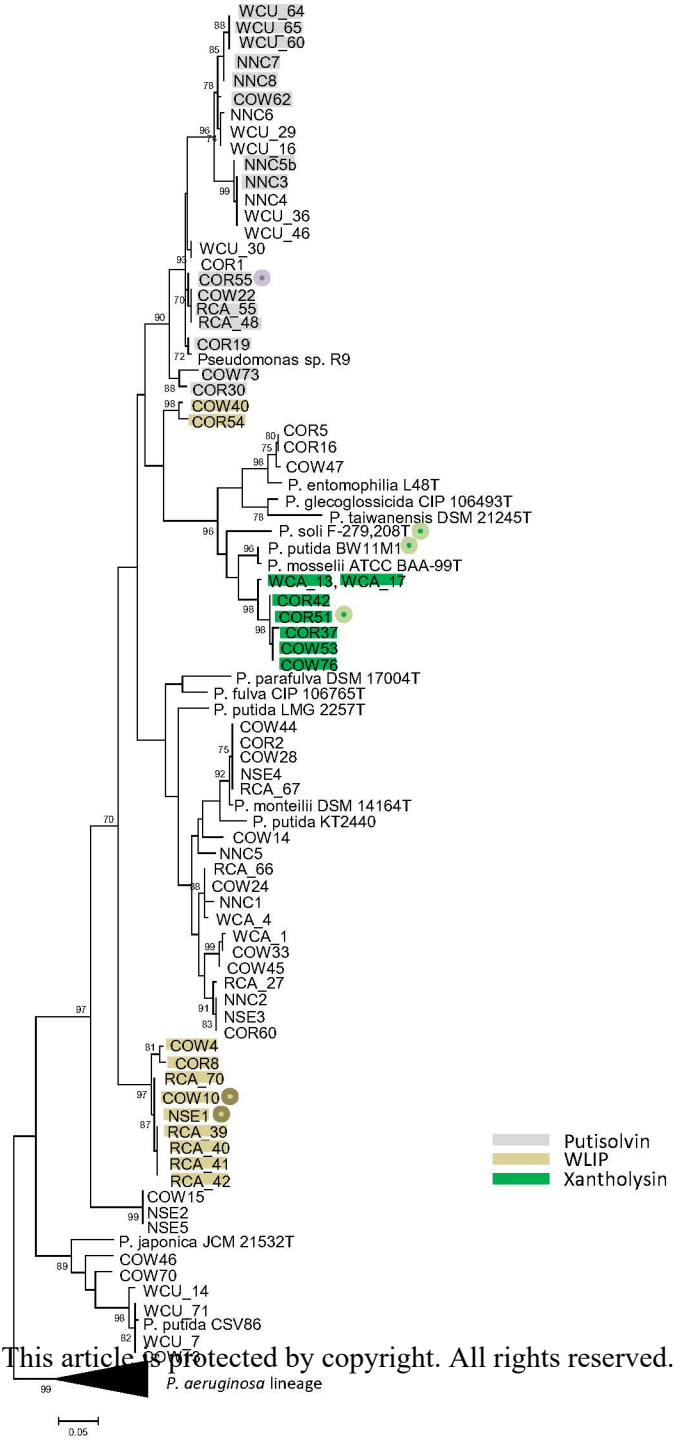
Nigeria strains, the integral membrane protein is absent. For the 12 modules, the amino acid composition based on the *in silico* analysis of the corresponding A domain (NRPSpredictor2 and antiSMASH) is indicated.

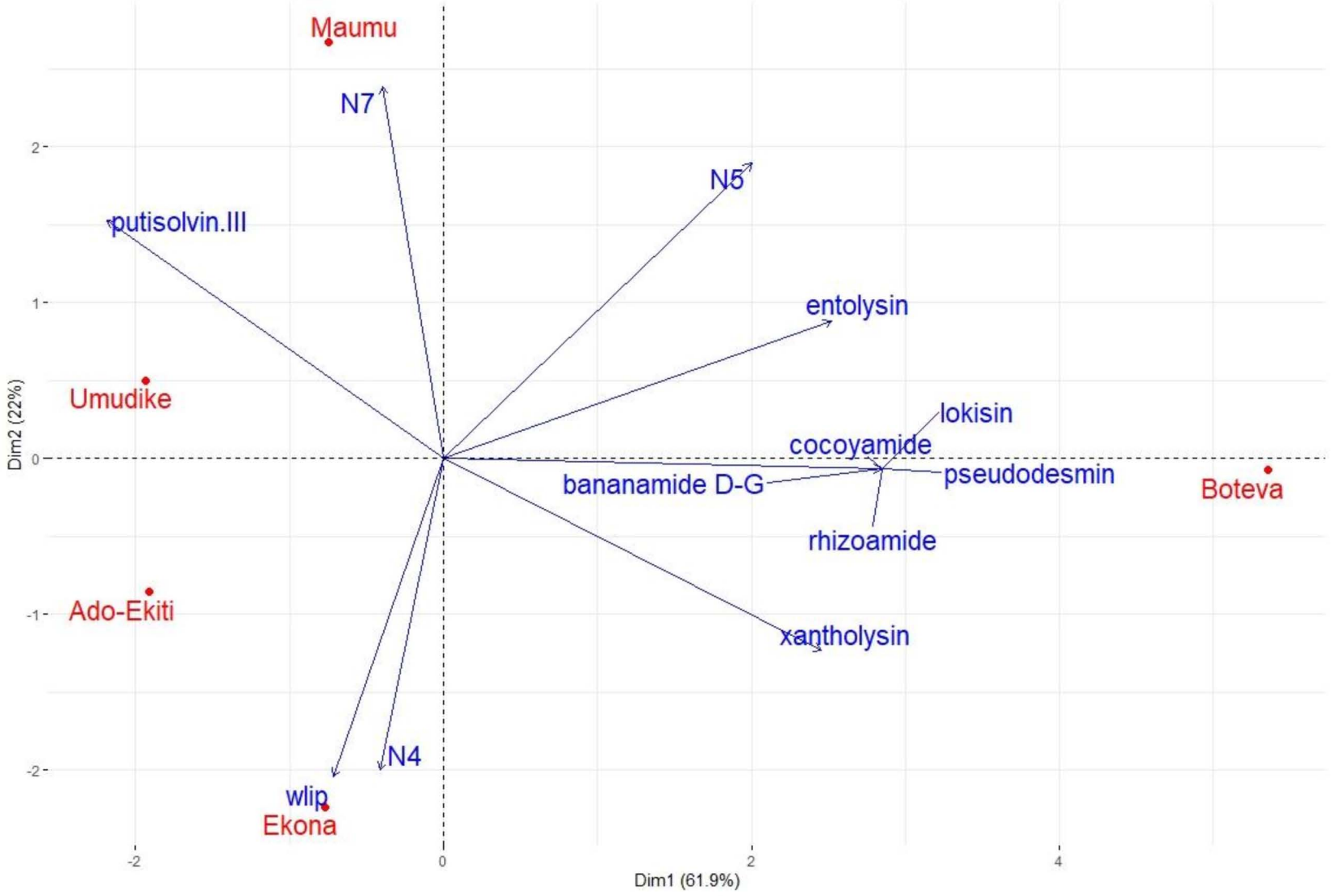
Figure 7. Scheme showing potential drivers of *Pseudomonas* and CLP diversity in a healthy cocoyam rhizosphere in the suppressive Boteva soil. The Pythium root rot suppressive soil of Boteva is characterized by significantly higher amounts of silt, Ca, organic C, and total N. The cocoyam rhizosphere was enriched in *P. fluorescens*, *P. putida* and *P. asplenii* group strains. Eleven structurally diverse CLPs were associated with the cocoyam rhizosphere at Boteva.

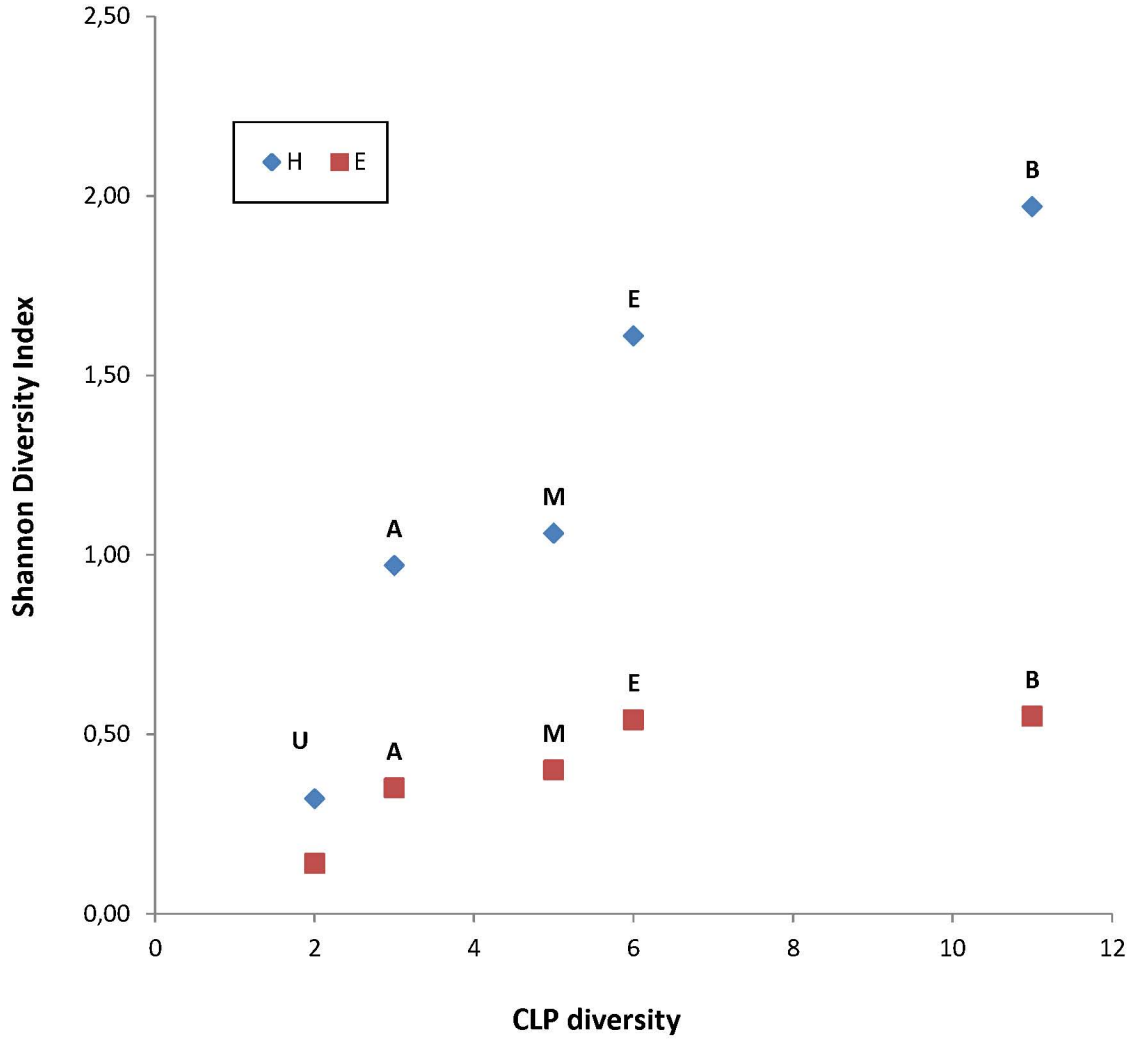
Table 1. Heat map indicating the taxonomic and CLP diversity of CLP-producing strains associated with the cocoyam rhizosphere in Cameroon (andosols) and Nigeria (alfisol and ultisol), including relevant characteristics.

Table 2. Physical and chemical characteristics of soils from Cameroon and Nigeria.

Table 3. Pearson correlation coefficients between soil characteristics and CLP diversity in five different fields. Asterisk: significant at $p<0.05$.







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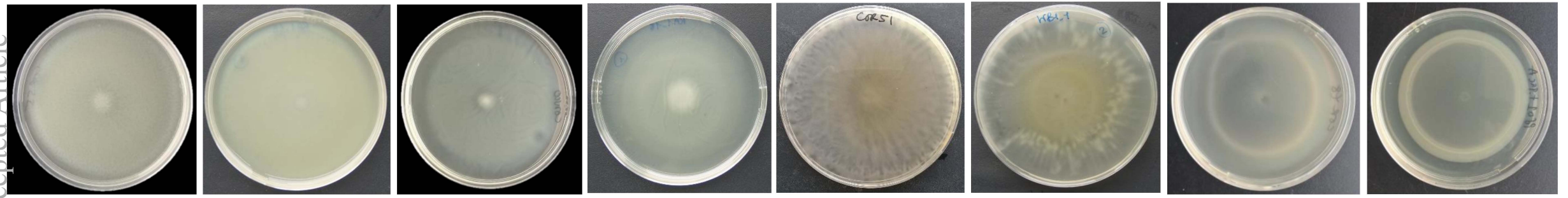
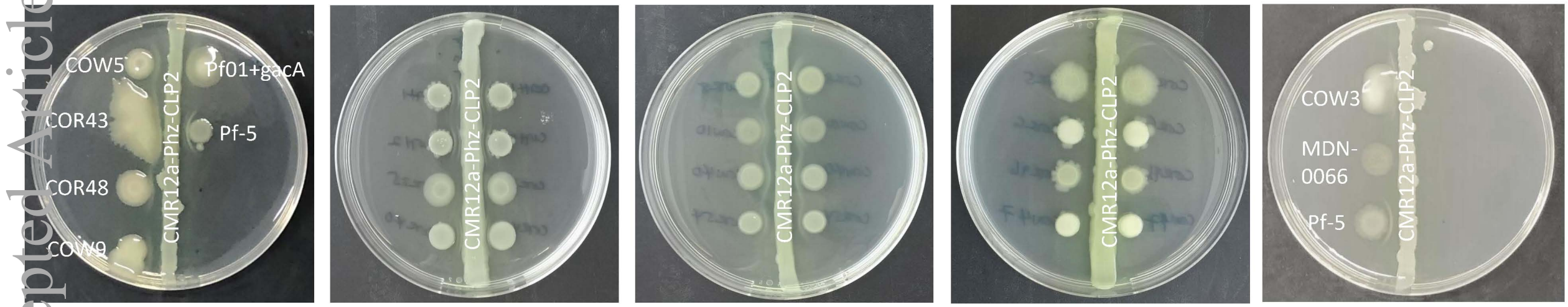
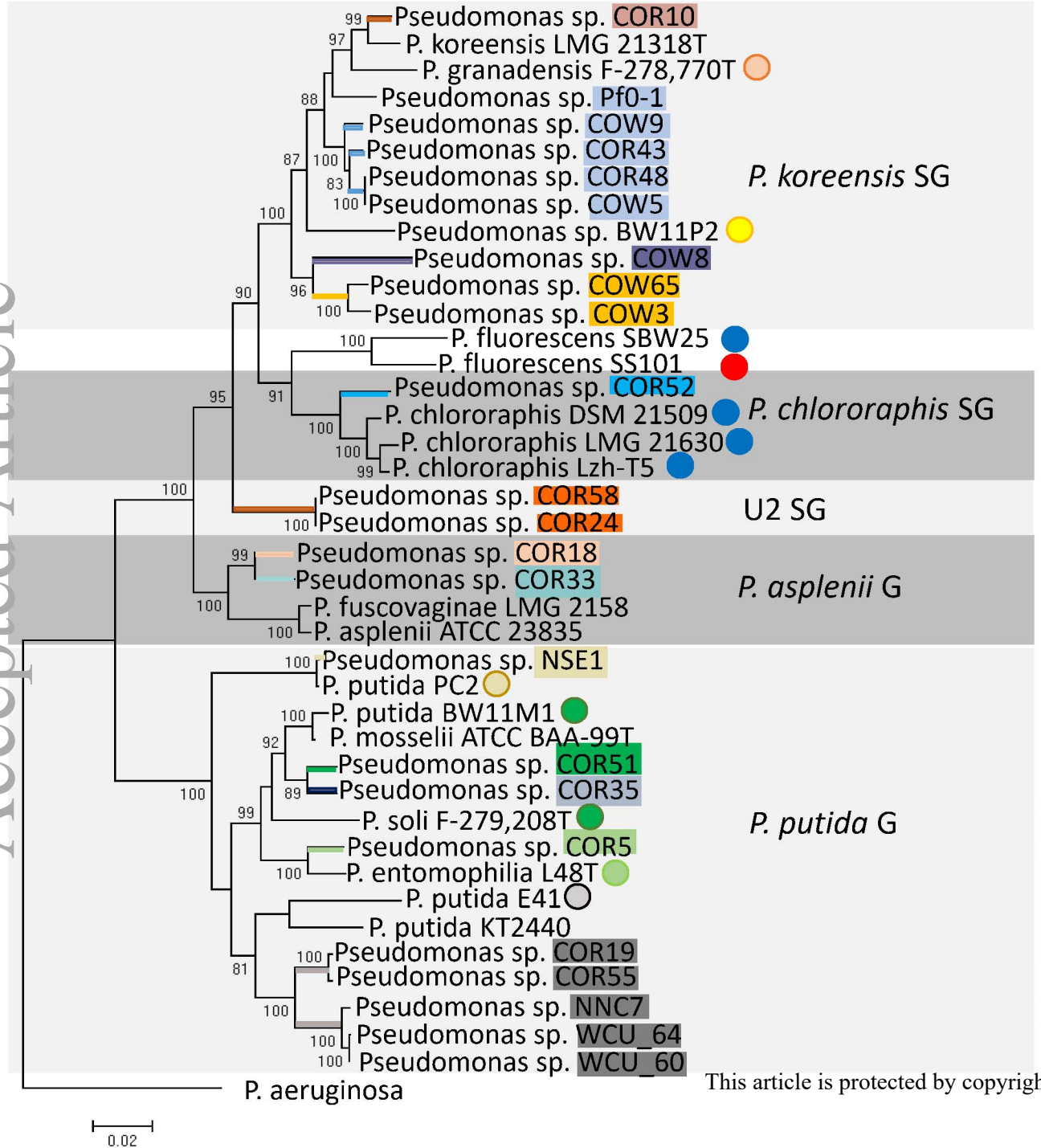
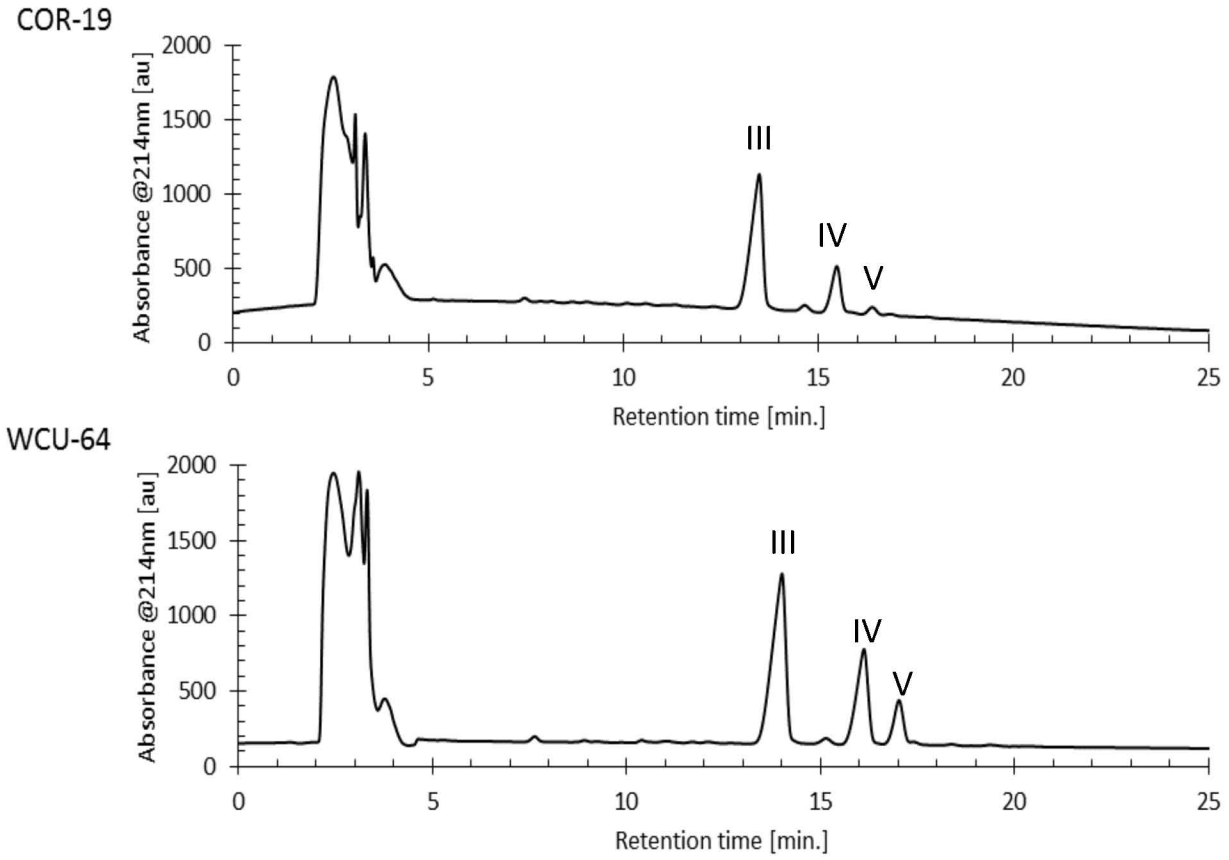


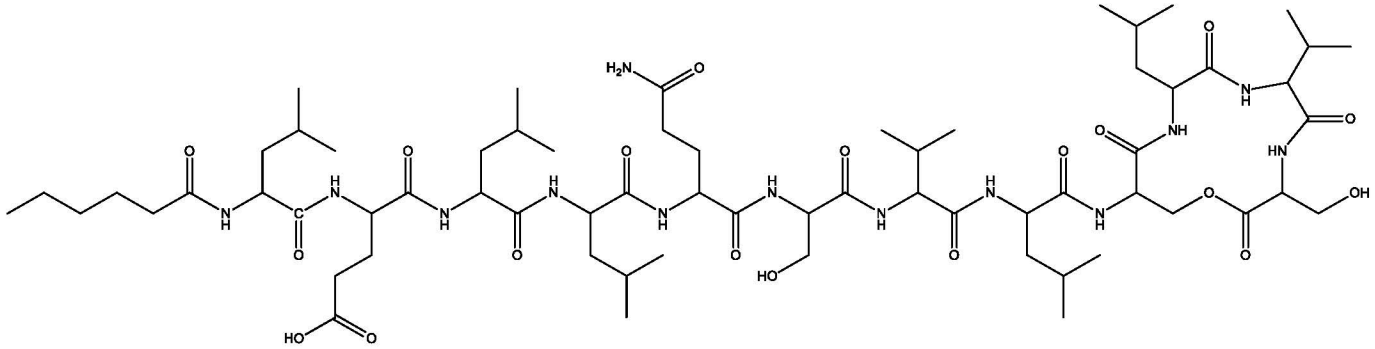


Cocoyamide N8 WLIP Entolysin Bananamide



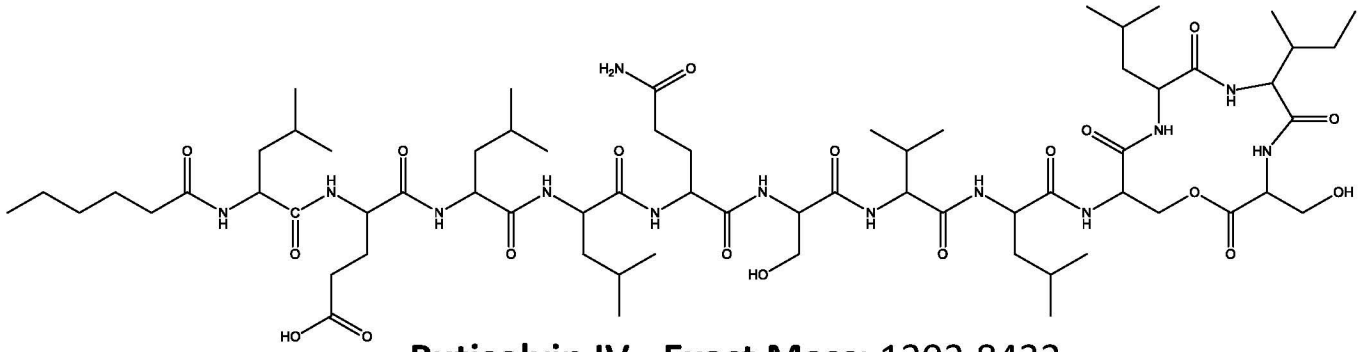


	Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	Reference
Putisolvin I	C6:0	**Leu**	**Glu**	**Leu**	**Ile**	**Gln**	**Ser**	**Val**	**Ile**	**Ser**	**Leu**	**Val**	**Ser**	Kuiper et al., 2004
Putisolvin II	C6:0	**Leu**	**Glu**	**Leu**	**Ile**	**Gln**	**Ser**	**Val**	**Ile**	**Ser**	**Leu**	**Ile**	**Ser**	Kuiper et al., 2004
Putisolvin III	C6:0	**Leu**	**Glu**	**Leu**	**Leu**	**Gln**	**Ser**	**Val**	**Leu**	**Ser**	**Leu**	**Val**	**Ser**	This study
Putisolvin IV	C6:0	**Leu**	**Glu**	**Leu**	**Leu**	**Gln**	**Ser**	**Val**	**Leu**	**Ser**	**Leu**	**Ile**	**Ser**	This study
Putisolvin V	C6:0	**Leu**	**Glu**	**Leu**	**Leu**	**Gln**	**Ser**	**Val**	**Leu**	**Ser**	**Leu**	**Leu**	**Ser**	This study



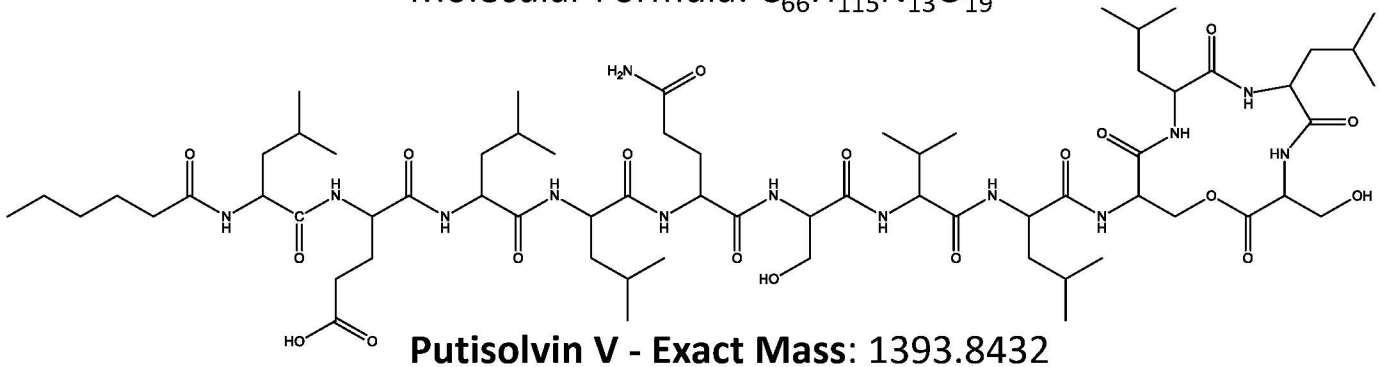
Putisolvin III- Exact Mass: 1379.8276

Molecular Formula: $C_{65}H_{113}N_{13}O_{19}$



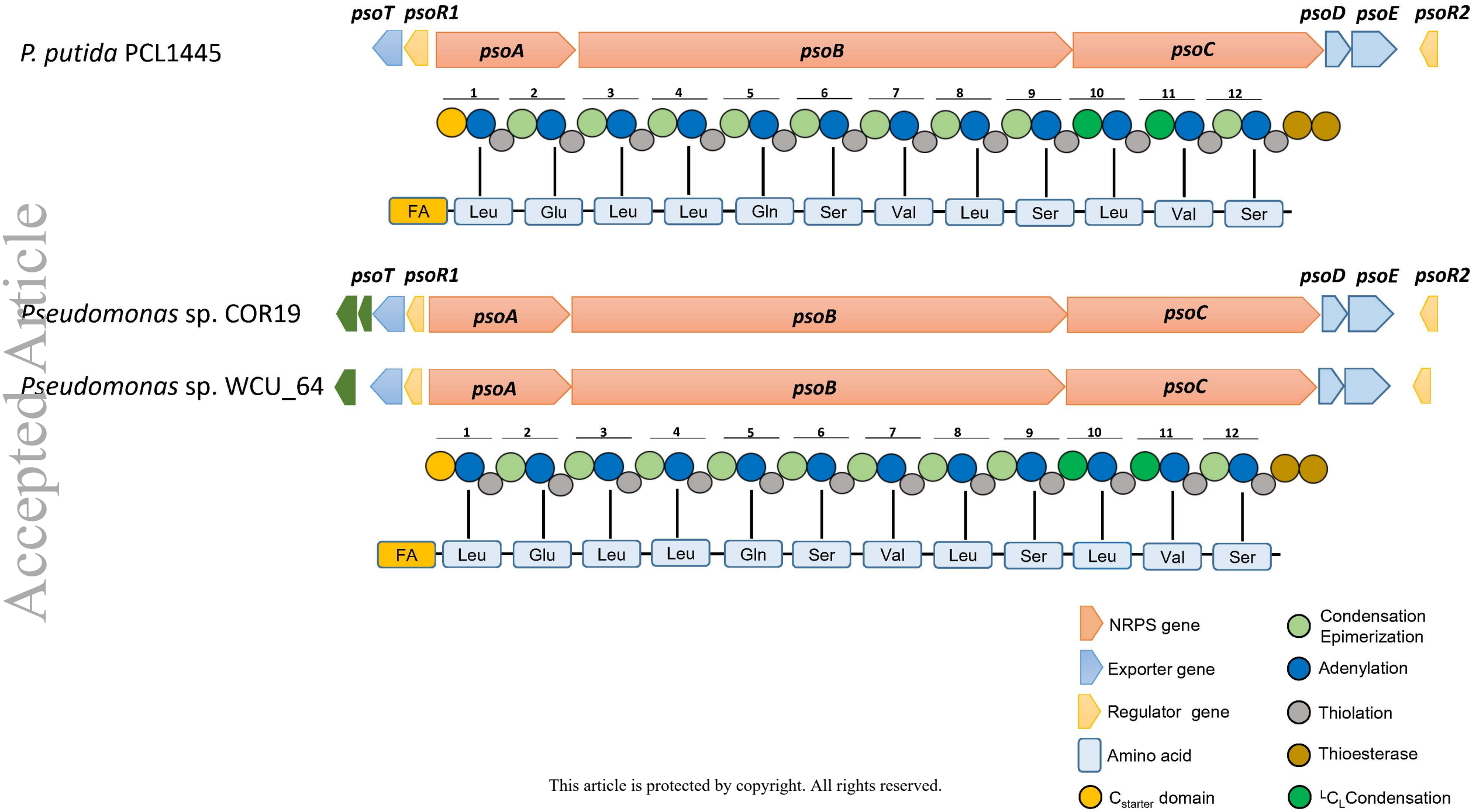
Putisolvin IV - Exact Mass: 1393.8432

Molecular Formula: $C_{66}H_{115}N_{13}O_{19}$



Putisolvin V - Exact Mass: 1393.8432

Molecular Formula: $C_{66}H_{115}N_{13}O_{19}$



Pseudomonas diversity

- *P. fluorescens* SG
- *P. chlororaphis* SG
- *P. koreensis* SG
- *P. jessenii* SG
- *P. corrugata* SG
- *P. asplenii* G
- *P. putida* G

root exudation quality ⇧

Physicochemical soil characteristics

- Soil organic matter
- Total nitrogen
- Calcium
- Silt

9	WLIP	11	N2
14	entolysin	8	bananamide D-G
14	xantholysin	9	pseudodesmin
17	lokisin	11	cocoyamide
17	N8	13	N5
12	putisolvin		

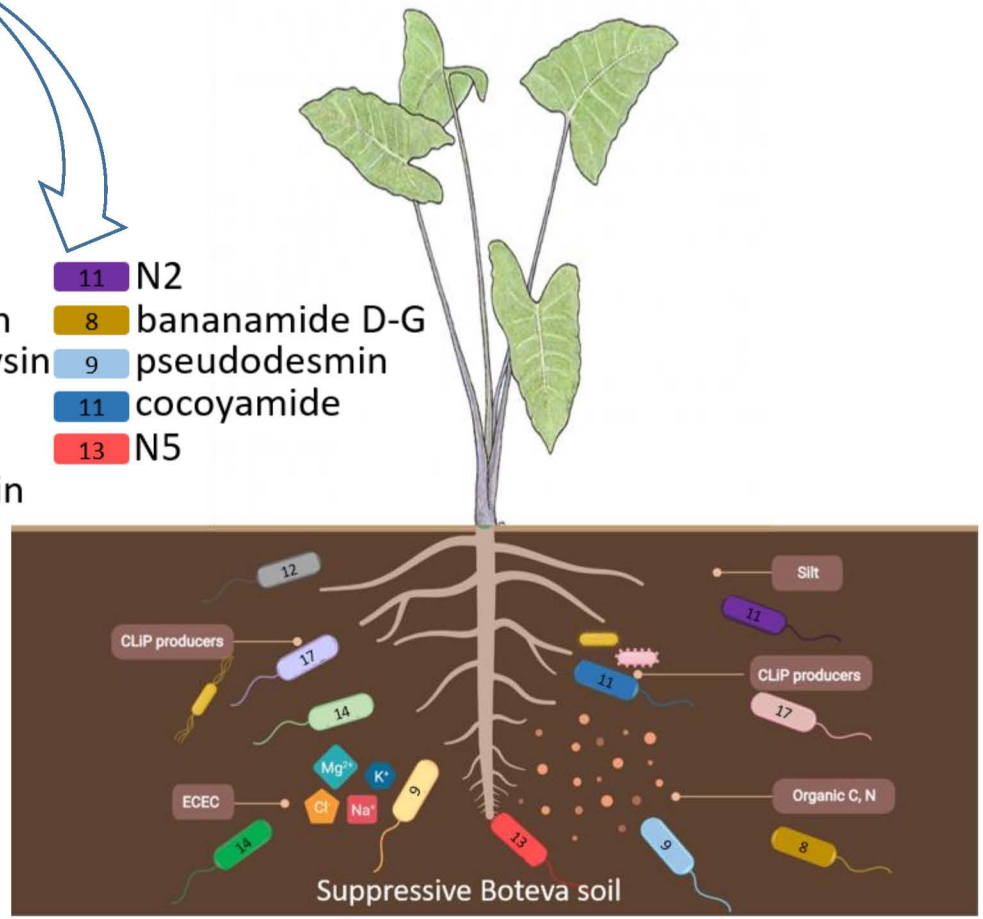


Table 1. Percentage taxonomic and CLP diversity of CLP-producing *Pseudomonas* isolates associated with the cocoyam rhizosphere in Cameroon and Nigeria. Relevant characteristics relating to amino acid composition are included.

Taxonomic (sub)groups	CLP types	Amino acid (l:r)[a]	Boteva	Ekona	Maumu	Ado-Ekiti	Umudike	Ugbokolo
P. putida G	putisolvin I-III	12:4	3	11	57	50	90	100
	WLIP	9:7	6	21	7	38	10	0
	xantholysin	14:8	29	37	14	13	0	0
	entolysin	14:5	9	5	14	0	0	0
	N8	17:8	6	11	0	0	0	0
P. koreensis SG	cocoyamide A	11:5	29	0	0	0	0	0
	bananamide D-G	8:6	9	0	0	0	0	0
	lokisin	11:9	3	0	0	0	0	0
	rhizoamide (N2)	11:9	3	0	0	0	0	0
U2 SG	N4	12:10	0	16	0	0	0	0
P. chlororaphis SG	pseudodesmin	9:7	3	0	0	0	0	0
P. asplenii G	N5	13:8	3	0	0	0	0	0
	N5+N7	13:8+n.d	0	0	7	0	0	0

[a] number of amino acids present in the oligopeptide length(l) and macrocycle ring size (r).
n.d: not determined.
G: taxonomic group; SG: taxonomic subgroup as described by Lalucat *et al.*, 2020.

Table 2. Physical and chemical characteristics of soils from Cameroon and Nigeria.

	Suppressive	Intermediate	Conducive soils		
Soil variable	Boteva[a+]	Ekona[a+]	Maumu[a]	Ado-Ekiti[b]	Umudike[c]
Clay (%)	2.99	53.44	14.00	9.40	22.10
Silt (%)	76.60	40.41	31.80	15.70	6.90
Sand (%)	20.41	6.15	54.20	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
ECEC (cmol kg^{-1})[a]	19.86	6.59	7.48	4.24	1.36

[a]Andosols of Cameroon
[b]Alfisols of Nigeria
[c]Ultisols of Nigeria
[+]analyses done prior to study (Adiobo *et al.*, 2007)

Table 3. Pearson correlation coefficients between soil characteristics and CLP diversity vs soil characteristics in five different fields.

Factors correlated	pH(H_2O)	Ca (cmol kg^{-1})	Mg (cmol kg^{-1})	K (cmol kg^{-1})	P (kg mg^{-1})	Org. C (%)	Na (cmol kg^{-1})	N (%)	ECEC (cmol kg^{-1})	Clay (%)	Silt (%)	Sand (%)	CLP diversity
pH(H_2O)	1.00	-0.22	-0.15	0.07	-0.12	-0.10	-0.24	-0.21	-0.23	-0.55	-0.37	0.68	-0.52
Ca		1.00	0.59	-0.19	-0.58	0.92*	-0.55	0.93*	0.97**	-0.41	0.93*	-0.56	0.92*
Mg			1.00	0.68	-0.36	0.84	0.28	0.82	0.76	-0.25	0.77	-0,51	0.73
K				1.00	0.14	0.20	0.16	0.82	0.04	-0.01	0.06	-0.05	0.04
P					1.00	-0.48	0.53	-0.42	-0.58	-0.09	-0.65	0.63	-0.49
Org. C						1.00	-0.25	0.99**	0.97**	-0.53	0.91*	-0.47	0.89*
Na							1.00	-0.24	-0.36	0.33	-0.29	0.04	-0.22
N								1.00	0.98**	-0.49	0.93*	-0.50	0.93*
ECEC									1.00	-0.39	0.97**	-0.61	0.95*
Clay										1.00	-0.17	-0.50	-0.15
Silt											1.00	-0.77	0.98*
Sand												1.00	-0.76
CLP diversity													1.00

*Correlation coefficients differ significantly at P = 0.05
**Correlation coefficients differ significantly at P = 0.01.