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Fusarium antagonism potential and metabolomics analysis of endophytic bacteria isolated from *Crotalaria retusa* L., a traditional medicinal plant in Côte d'Ivoire

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Abstract

Sixty-four endophytic bacteria were isolated from roots, stems, and leaves of *Crotalaria retusa* L., a medicinal plant well-known for its antimicrobial properties in Côte d'Ivoire. Taxonomic characterization revealed that these bacteria were mainly dominated by the genera *Pseudomonas*, *Rhizobium*, *Bacillus*, and *Inquilinus*. The antagonistic activities of the endophytic bacteria against two phytopathogenic fungi affiliated with the genus *Fusarium* were tested using *in vitro* coculture. Isolates belonging to the genus *Inquilinus* showed the highest inhibitory activities against *Fusarium oxysporum* f. sp. *cubense*, ranging from 40% to 57%, while the highest inhibitory activities against *Fusarium graminearum* were obtained with *Bacillus* isolates (~66%). Finally, a metabolomic study of the leaves, stems, and seeds of the plant and of the endophytes presenting antifungal activity was carried out using LC–MS/MS analysis of the methanolic extracts of all active endophytic isolates. The identified metabolites of interest from the endophytes were mainly peptides, lipids, and steroids. Two pyrrolizidine alkaloids, monocrotaline and senecionine, were detected in the plant organs but not in the endophytic bacterial extracts. These results highlighted the potential of *C. retusa* L. plant and its endophytic microbiome as a source of bioactive molecules of interest and biocontrol agents against phytopathogenic *Fusarium* spp.

Keywords: *Crotalaria retusa* L.; endophytic bacteria; alkaloids; *Fusarium* spp.; secondary metabolites

Introduction

Endophytic bacteria are microorganisms that colonize the internal tissues of plants during all or part of their development without causing damage (Ryan et al. 2008, Anjum and Chandra 2015, Orlikowska et al. 2017). The composition of endophytic bacterial communities varies depending on several factors such as plant species, plant compartments and physiological or environmental conditions (Bulgarelli et al. 2013, Compant et al. 2021). Some of these microorganisms have beneficial effects on plant growth by influencing its nutrition and protection against abiotic and biotic stresses, which may be related partly to their abilities to modulate the plant metabolome (Etalo et al. 2018). In fact, some endophytic bacteria can interfere with the biosynthetic pathway of bioactive compounds including alkaloids (Li et al. 2012) or are implicated in the bioconversion of plant precursors into active metabolites mimicking plant compounds (Del Giudice et al. 2008, Wu et al. 2021). Consequently, the bioprospecting of bioactive metabolites from medicinal plants by endophytic microorganisms offers a promising alternative route for the discovery of biomolecules with broad application in agriculture, medicine, food, and cosmetics industries (Rat et al. 2021, Wu et al. 2021, Tshikhudo et al. 2023).

Analyses of interactions between medicinal plant and endophytes that have not been described yet, could be of interest in understanding whether endophytes can produce bioactive plant metabolites and for the discovery of new biomolecules. At this regards, *Crotalaria retusa* offers a good model plant in Côte d'Ivoire. Indeed, different parts of *C. retusa* are used in traditional medicine in tropical and subtropical regions for their antiinflammatory, antinociceptive, antioxidant, and antimicrobial activities (Devendra et al. 2012, Aragão et al. 2017, Rouamba et al. 2018). *Crotalaria retusa* is thought to contain pyrrolizidine alkaloids (PAs), as well as other biomolecules such as steroids, terpenoids, peptides, polyketones, flavonoids, quinols, and phenols that may contribute to these biological effects and that may be of interest from the point of view of drug development or plant protection solutions (Maia et al. 2013, Sut et al. 2020). Moreover, a recent study has shown evidence that ethanolic extracts from *C. retusa* have antagonistic effects on different plant pathogens (Miranda-Granados et al. 2018, Doga et al. 2022). It was then important to evaluate the antagonistic potential of endophytic bacteria associated to *C. retusa* for diseases that constitute a global threat for food security worldwide as the case for *Fusarium* wilt (Ekwomadu and Mwanza

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2023). Moreover, scientific data on the phytochemistry of *C. retusa* remain scarse. It was therefore important to make progress in characterizing the endophytes associated with *C. retusa* and addressing both plant and bacterial endophytes metabolomics for efficient biological control of biotic and abiotic stresses (Verma et al. 2022). We hypothesized that *C. retusa* as a medicinal plant harbored endophytic bacteria that may have a potential antagonistic activity against important cultivated plant pathogens in that they may produce secondary metabolites possessing antimicrobial activity. The objectives of this study were as follows: (i) isolate and taxonomically characterize *C. retusa* endophytic bacteria using 16S rRNA gene sequencing, (ii) evaluate the potential of these bacterial endophytes for their antagonistic activity against important *Fusarium* pathogens such as *F. graminearum and F. oxysporum* f. sp. *cubense*, and (iii) identify the secondary metabolites produced by the endophytic bacteria possessing antimicrobial activity using liquid chromatography coupled with mass spectrometry or both with mass spectrometry (LC–MS/MS) approaches.

Materials and methods

Collection of plant material

Plant materials were collected from wild *C. retusa* plants in Côte d'Ivoire. Whole healthy plants of *C. retusa* were collected randomly from four sites including G1 (06.81958° N, 005.29597° W), G2 (06.81450° N, 005.29979° W), G3 (06.81579° N, 005.29769° W), and G4 (06.88639° N, 005.22924° W), located in the autonomous district of Yamoussoukro (https://www.districtyakro.ci/). Their botanical authentication was done under the guidance of a botanist from the Forestry and Environment (FOREN) Department of the Félix HOUPHOUËT-BOIGNY National Polytechnic Institute (INP-HB, Yamoussoukro, Côte d'Ivoire). *Crotalaria retusa* seeds were obtained from the Laboratoire de Microbiologie, Biotechnologies et Bioinformatique (INP-HB).

Isolation of endophytic bacteria and cultivation

Plants (flowering stage) with roots were harvested using a machete and a hoe. The collected plants were transported to the laboratory in a cooler. The organs (roots, stems, and leaves) were carefully washed with tap water to remove soil particles. Prior to the isolation of bacterial endophytes, plant organs were surface sterilized with sodium hypochlorite solution and 70% (v/v) ethanol. *Crotalaria retusa* leaves and stems were sterilized in 70% (v/v) ethanol for 1 min and 3% sodium hypochlorite for 10 min, while the roots were treated with 70% (v/v) ethanol for 1 min and 2.6% sodium hypochlorite for 4 min, followed by three successive rinses with sterile distilled water. To test the effectiveness of surface sterilization, 100 µl of the final rinse solution was spread on nutrient agar media (Nutrient Agar, OXOID, Thermo Fisher Scientific). The disinfected plant organ (1 g) was macerated in 2 ml of sterile distilled water at ∼27°C under laminar flow hood. After 20–30 min of incubation, the maceration solution was diluted to one-tenth using the suspension–dilution method (Long et al. 2003) and then spread onto nutrient agar. The plates were then incubated at 28°C for 24–72 h. The number of colony forming units was calculated as the mean number of colonies, with three replicates per modality. Colonies were selected on days 1, 2, and 3 of incubation according to their growth time and phenotype (color, size, and shape) and purified on 10% tryptone soy agar (10%) (Sigma-Aldrich, St.Quentin Fallavier, France) by repeated subculturing. After purification, bacterial isolates were stored in a 15% glycerol solution at –80°C for subsequent analysis.

Molecular identification of the isolated endophytic bacteria

Polymerase Chain Reaction (PCR) amplification of the 16S ribosomal RNA gene from a single colony (colony PCR) was carried out using 27F (3′-GAGAGTTTGATCCTGGCTCAG-5′) and 1492R (3′- CTACGGCTACTGTTACGA-5′) primers (Gürtler and Stanisich 1996). The PCR reaction mixture contained 10 mM of each primer, 10XPrimeSTAR®GXL buffer containing 25 mM $MgCl_2$, 0.625 U of *Taq* polymerase, and dNTPs (0.2 mM) in a final volume of 30 µl. Single bacterial colonies were used as template under sterile conditions. The amplification reaction was performed in a ProFlex thermal cycler with the following program: 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, then, a final elongation step of 10 min at 72°C. PCR products were separated in 1% agarose gel to check amplification and visualize their size. PCR products were commercially sequenced (Eurofins France) using Sanger sequencing. High quality sequences were curated for ambiguous nucleotide positions.

Preparation of *C. retusa* L. extracts for phytochemical analysis

The wild plant materials were dried in the dark during 14 days at around 27°C. After drying, leaves and stems were preground using a mortar and pestle, then ground to a fine particle size using IKA Labortechnik electric grinder (type MFC, manufactured by IKA, Germany). The *C. retusa* seeds were ground using a Retsch ball mill (type MM400, manufactured by Verder, Germany). All samples did not receive any treatment. 1 g of the mix of leaves and stems powder and 1 g of seeds powder were extracted separately by maceration in 10 ml of 70% (v/v) methanol for 16 h at room temperature (18°C). After centrifugation at 12 800 × *g* at room temperature (18°C), the methanolic extract of each sample were filtered using Sartorius filter (0.22 µm) and stored at –20°C before being analysed by LC–MS.

Evaluation of the antifungal activity of endophytic bacteria against *F. oxysporum* f. sp. *cubense* and *F. graminearum*

The antagonistic activities of the endophytic bacteria against the phytopathogenic fungi *F. oxysporum* f. sp. *cubense* and *F. graminearum* were analysed using an *in vitro* coculture test, as described in Munakata et al. (2021). *Fusarium graminearum* was supplied by the Institut Français des Boissons, de la Brasserie et de la Malterie (IFBM**)** and *F. oxysporum* f. sp. *cubense* was bought from the Belgian Coordinated Collections of Microorganisms (BCCM). Briefly, a mycelium plug of 5 mm in diameter was taken from a fresh culture and inoculated into the centre of a 9 cm diameter Petri dish containing 12 ml of potato dextrose agar. Bacterial isolates were cultivated in 2 ml of nutrient broth (NB) at 28°C for 24 h or 72 h. 50 µl of a bacterial suspension were dropped at 3 cm from the mycelial plugs. Three independent replicates were performed. The inoculated Petri dishes were then incubated for 5 days for *F. graminearum* and 9 days for *F. oxysporum* f. sp. *cubense* at 28°C. Inhibition activity, expressed as an inhibition rate (%), was then calculated using the following formula:

$$\text{Inhibition rate} = 100 * \left(1 - D_{assay}/D_{control}\right),$$

where $D_{control}$ is the diameter of the control and D_{assay}, the diameter of the pathogenic fungus in coculture with the endophytic bacteria. The diameters $D_{control}$ and D_{assay} were measured, and averages were calculated between replicates. Plates inoculated only

either with *F. graminearum* or *F. oxysporum* f. sp. *cubense* were used as controls ($D_{control}$). Bacterial isolates showing a growth inhibition rate higher than 40% against one of both plant pathogenic fungi were selected for further analyses (Bolivar-Anillo et al. 2021).

Preparation of bacterial extracts for LC–MS/MS analysis

Bacterial crude extract was prepared according to the protocol of Munakata et al. (2022). Indeed, bacteria were precultured in 2 ml of NB at 28°C and 180 rpm for 24 h. Then, 100 µl of the precultures were added to 50 ml of NB in 250 ml Erlenmeyer flasks. After 72 h of culture, the bacterial pellets were collected by centrifugation at 4000 × *g* at room temperature (18°C) for 40 min. The pellets were suspended in 1.5 ml of methanol and ultrasonicated for 30 min, followed by maceration for 30 min at room temperature. The supernatant was then obtained by centrifugation at 12 800 × *g* at room temperature (18°C) for 30 min. The supernatant of each bacterial extract was then evaporated overnight with a speed Vac (Eppendorf Concentrator Plus, Hamburg, Germany). The obtained pellets were dissolved with 300 µl of 70% (v/v) methanol and then centrifuged. After centrifugation, the bacterial extracts were filtered using a Sartorius filter (0.22 µm). Finally, 100 µl of filtered bacterial extract solution was transferred to vials for LC–MS/MS analysis.

LC–MS/MS analysis of *C. retusa* L. and bacterial extracts

To detect the presence of alkaloids in the aerial parts (leaves, stems and seeds) of *C. retusa*, analyses were carried out using a NEXERA UHPLC system (Shimadzu Corp, Kyoto, Japan) equipped with a photodiode array detector (SPDM20A, Shimadzu) (Dugrand et al. 2013) combined with a mass spectrometer (single quadrupole, LCMS 2020, Shimadzu). The column was C18 ZORBAX Eclipse Plus, (150 mm ×2 mm, 1.9 µm) (Agilent Technologies, Santa Clara, CA, USA) protected by an Agilent Technologies 1290 overflow filter and thermostated at 40°C. The solvents consisted of 0.1% formic acid water (A) and 0.1% formic acid in methanol (B). The compounds were eluted using the following gradient: 5% solvent B for 0.5 min, a ramp from 5% solvent B at 0.5 min to 50% at 7 min followed by 100% solvent B for 7 min. Total analysis time of each injection was 15 min. For separation, the UHPLC system was connected to the MS via a dual ion source, atmospheric pressure chemical ionization, and a combination of electrospray ionization (operating here in positive mode). The inlet, desolvation line, and heating block temperatures were set at 350°C, 250°C and 400°C, respectively. Capillary voltage was set at +4.5 kV.

Untargeted LC–MS/MS analysis of the bacterial endophytes was performed using UHPLC Orbitrap ID-X (Thermo Fisher Scientific, Waltham, MA, USA). The column was Kinetex XB-C18 (150 mm × 2.1 mm × 2.6 µm) (Phenomenex, Le Pecq, France). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) and the flow rate was set at 200 µl/min. Gradient elution was carried out with the following programme: 10% B for 2 min, then the portion of B was increased to 30% B in 8 min, and to 95% in 10 min. The column was then washed for 5 min at 95% B and reequilibrated to the initial conditions for 4 min. MS data were obtained from an Orbitrap ID-X mass spectrometer using positive and negative ionization modes. Ionization was performed using a heated electrospray ionization source and the scan range was set from 120 to 800 m/z. All samples were analysed in MS1, followed by MS2 on a mixture of all samples, using the data-dependent acquisition mode. For MS2 analysis, the AcquireX acquisition method developed by ThermoFisher was applied. Briefly, this method creates an inclusion list after the first injection. Successive injections are then used to fragment the ions in the inclusion list. After being fragmented, the ions are removed from the inclusion list, allowing access to low-intensity ions as well. MS2 fragments were obtained by higher energy collisional dissociation and collision induced dissociation, both at 30% energy. The resolution was 60 000 for MS1 and 30 000 for MS2. Results were analysed using Compound Discoverer 3.3 (Thermo Fisher Scientific).

Metabolomics data processing

For the metabolomics analysis of bacterial extracts, the raw files were uploaded on the software Compound Discoverer 3.3 (Thermo Fisher Scientific). The full workflow is given as a supplementary data. In brief, it includes spectra detection, alignment, and features creation. Each feature corresponds to a unique combination of a m/z and a retention time. Compounds were annotated with (i) predict composition, (ii) through search using The Natural Products Atlas database (Van Santen et al. 2022), (iii) using *in silico* dereplication with the software SIRIUS GUI version 5 (Dührkop et al. 2019), and finally (iv) a molecular network was generated using Compound Discoverer 3.3 and was visualized with Cytoscape 3.9.1 following the procedure described at https://github.com/Florent-1/Molecular-Networks-from-Compound-Discoverer-to-Cytoscape. In the network, each node represented a molecule, and the links showed the MS2 similarity between the molecules. A network was then created using Compound Discoverer 3.3 and Rstudio 4.3.3, where edges were filtered to have a score above 49, a coverage above 69, and more than nine matched peaks. Further, only the top 10 edges were kept for each node. Finally, Louvain algorithm was applied to the cluster containing more than 100 nodes, using a 1.1 resolution (Csárdi and Nepusz 2006, Blondel et al. 2008, Csàrdi et al. 2024).

Bioinformatics and statistical analyses

The raw DNA sequences obtained from Sanger sequencing were analysed manually using BioEdit v7.2.5 (Hall A. 1999) and the sequences were base called using EMBOSS v6.6 (Rice et al. 2000). Taxonomic assignment was performed using BLAST (https://www.ncbi.nlm.nih.gov/) v2.12.0+ (Camacho et al. 2009) with NCBI/GenBank online standard databases [nucleotide collection (nr/nt), and whole-genome shotgun contigs (wgs)] to select closely related reference sequences (Sayers et al. 2022). Statistical analyses were performed using R v4 .3.1 (https://cran.r-project.org/bin/windows/base/) (R Core Team. 2021). The inhibition rate was visualized with ggplot2 (Wickham 2009) and tested by the Tukey–Kramer method with the R package multcomp.

Results

Isolation and taxonomical characterization of *C. retusa* endophytic bacteria

A total of 64 endophytic bacteria were isolated from roots, stems, and leaves of *C. retusa* at the flowering stage (Table S1). These bacteria were taxonomically assigned using the 16S rRNA sequences as edited and deposited in GenBank (PP350469–PP350532). 35 bacteria (54.7%) were isolated from roots, 20 (31.3%) from stem, and 9 (14%) from leaves (Table S1). The taxonomic assignment revealed that these isolated bacteria belonged to the phyla Actinomycetota, Bacillota, and Pseudomonadota. Pseudomonadota was the predominant phylum whatever the plant organ considered.

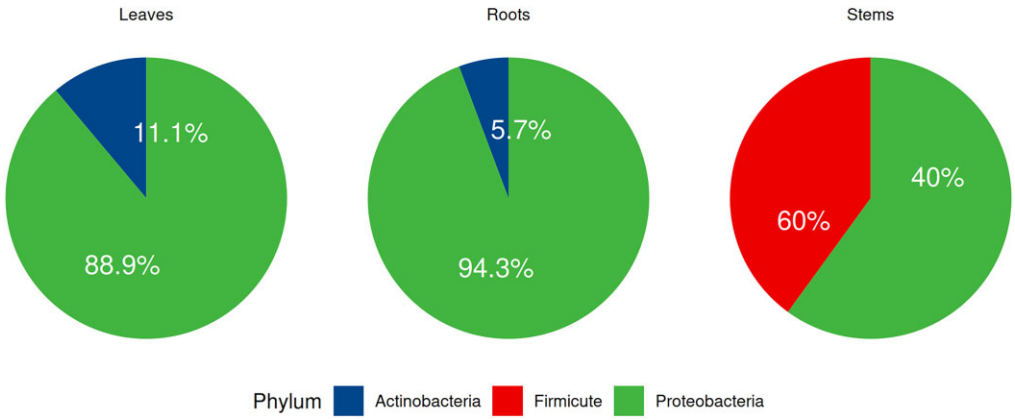

Figure 1. Distribution of bacterial phyla through *C. retusa* organs.

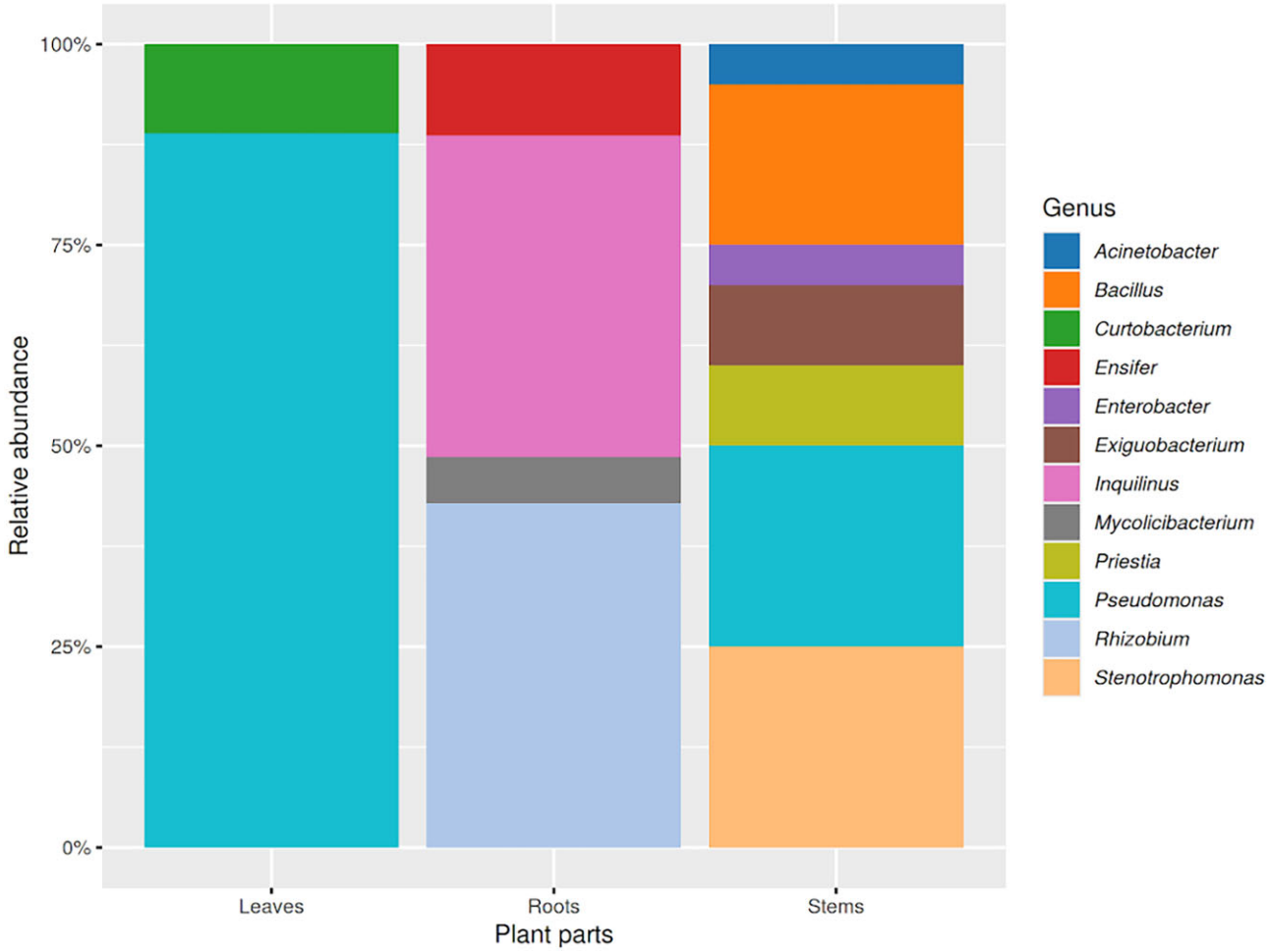

Figure 2. Distribution of bacterial genera through *C. retusa* organs.

Pseudomonadota were dominant in roots (94.3%) whereas Bacillota (40%) were mainly identified in stems. Actinomycetota were more abundant in leaves (11.1%) than in roots (5.7%) (Fig. 1). At the genus level, bacterial isolates were affiliated to 12 genera with 7 genera in stems (*Acinetobacter*, *Bacillus*, *Enterobacter*, *Exiguobacterium*, *Priestia*, *Pseudomonas*, and *Stenotrophomonas*), 4 genera found in roots (*Ensifer*, *Inquilinus*, *Mycolicibacterium*, and *Rhizobium*), and 2 genera found in leaves (*Curtobacterium* and *Pseudomonas*) (Fig. 2).

Antifungal activity of bacterial isolates against *Fusarium* species

To assess the antifungal activity of bacterial isolates against the pathogenic fungi *F. graminearum* and *F. oxysporum* f. sp. *cubense*, *in vitro* dual-culture tests were performed. 15 isolates affiliated to the *Rhizobium* genus out of a total of 64 isolates were withdrawn from the tests. 49 bacterial strains were then tested. 18% (9/49) of these bacterial strains showed an inhibition rate greater

Table 1. Inhibition rates of the identified bacterial endophytes.

Plant organ	Strain	GenBank accession	Blast top hit	Acc Len	Similarity (%)	Inhibition rate vs *Fg* (mean ± SE) Day 5	Inhibition rate vs *Foc* (mean ± SE) Day 9
Stems	G1T19	PP350503	*Pseudomonas putida*	854	99.88	16.5 ± 0.7	41.07 ± 5.5
	G1T20	PP350532	*Stenotrophomonas maltophilia*	826	99.27	13 ± 0.27	42.85 ± 1.64
	G3T27	PP350469	*Acinetobacter pitii*	865	99.77	18.18 ± 1.06	41.33 ± 0.83
	G2T39	PP350472	*Bacillus velezensis*	978	100	65.37 ± 0.70	35.08 ± 0.17
Roots	G2T50	PP350480	*Bacillus amyloliquefaciens*	872	99.43	66.32 ± 0.33	33.33 ± 2.98
	G1R8	PP350488	*Inquilinus sp*	980	100	21.57 ± 0.69	40.22 ± 0.98
	G1R4	PP350486	*Inquilinus sp*	869	100	22.78 ± 0.40	48.05 ± 1.16
	G1R12	PP350491	*Inquilinus sp*	955	100	25.56 ± 0.59	56.9 ± 1.38
	G1R1	PP350483	*Inquilinus sp*	950	100	12.7 ± 0.25	50.77 ± 1.21

Growth inhibition rates against *Fg* (*Fusarium graminearum*) and *Foc* (*Fusarium oxysporum* f. sp. *cubense*) also shown in the right-hand columns (means ± SE).
Acc Len (accession length), vs (versus), SE: Standard error.

than or equal to 40% against at least one of the pathogenic fungi. These nine strains belonged to the genera *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Bacillus*, and *Inquilinus* (Table 1). The endophytic origin of the bacteria did not impact the antifungal activities against *F. graminearum* since there was no significant difference between bacteria from roots, leaves, and seeds (Fig. S1a). However, the inhibition rate of bacteria against *F. oxysporum* f. sp. *cubense* was higher for bacteria isolated from *C. retusa* roots (33.72%) and stems (23.53%) than for bacteria isolated from leaves (12.87%) (Fig. S1b). The antagonistic activities of the bacterial isolates against *F. graminearum* varied from 3.6% to 66.3% and those against *F. oxysporum* f. sp. *cubense* had a variation from 0% to 56.9%. Concerning the antifungal activities of bacterial isolates against *F. oxysporum*. f. sp. *cubense*, two isolates belonging to *Inquilinus* showed the highest antagonistic activities, with more than 50% of inhibition rate (Table 1).

Identification of specialized metabolites in plants and bacterial endophytes

Target research of PAs by LC–MS analysis in plants

The methanolic extracts of seeds, stems, and leaves harvested from wild plants of *C. retusa* were subjected to LC–MS analysis to show the presence of PAs. The gradient profile of the HPLC mobile phase according to the European Pharmacopoeia led to the well-resolved peaks of monocrotaline (MCT, 9.5 ± 0.2 min) and senecionine (SCN, 12 ± 0.2 min) (Fig. 3a). The LC–MS performed in positive-ion mode was able to detect monocrotaline and senecionine in amounts, higher than 0.01 µg/ml and 0.1 µg/ml respectively (Fig. 3b). Monocrotaline and senecionine displayed a m/z of 326 $[M + H]^+$ and a m/z of 336 $[M + H]^+$. The LC–MS analysis of seeds, stems, and leaves of *C. retusa* plant extracts showed excellent separation of monocrotaline and senecionine peaks, permitting the quantification of these compounds (% dry weight of plant material) (Table 2). These alkaloids exhibited only the pseudomolecular ion characteristic of monocrotaline and senecionine standards. All plant extracts contained both studied alkaloids. However, monocrotaline was detected at higher concentrations in seeds (0.83% dry wt) and in the mix of stems and leaves (0.62% dry weight) comparatively to senecionine in seeds and mix of stems and leaves (0.0005 and 0.002% dry weight, respectively). Seed extracts contained the highest amounts of both compounds (0.83% dry weight of monocrotaline and 0.0005% dry weight of senecionine) while mix of stems and leaves extracts contained 0.62% dry weight of monocrotaline and traces of senecionine (0.0002% dry weight).

Molecular Networking of C. retusa L. bacterial endophytes

Active methanolic extracts of the nine bacteria endophytic belonging to the genera *Inquilinus*, *Bacillus*, *Pseudomonas*, *Acinetobacter*, and *Stenotrophomonas* that had antifungal activity against the growth of *F. graminearum* and *F. oxysporum* f. sp. *cubense* were analysed by UHPLC-HRMS/MS. Following taxonomic and functional characterization of the bacterial endophytic strains, a molecular network representing the full chemical diversity of these endophytic bacteria active against plant pathogenic fungi of the genus *Fusarium* was created. Based on the analysis workflow that was used (supplementary document), a molecular network was constructed from the nine endophytic bacteria data and consisted of 686 nodes divided into 26 clusters. A total of 19 of the 26 clustered nodes were annotated (Fig. 4). No alkaloid was identified in any bacterial extract. Clusters 5, 10, and 13 corresponded to peptides and specially clusters 7, 13, 15, and 17 to dipeptides (Table S2). MS2 spectra of the ion at m/z 387.2533 (cluster 5), 130.0863 (cluster 10), and 242.14988 (cluster 13) matched respectively with peptides macrolactin N, cispentacin, and leuhistin referenced in the available libraries (Table S2). SIRIUS GUI version 4 was used for *in sillico* dereplication (Dührkop et al. 2019) of our MS2 data and all the molecular formula were searched against the Natural Product Atlas library, restricting the library to the relevant genus (Van Santen et al. 2019, 2022). The putative molecules are reported as given by the databases even if the stereochemistry could not be checked by mass spectrometry. Examination of the dipeptide clusters led to the putative identification of cyclo (L-Leu-L-Arg) and *N*-(3-{1,4-dioxo-octahydropyrrolo[1,2-a]pyrazin-3-yl}propyl)guanidine (cluster 7), cyclo(D-Tyr-L-Leu) and 3-benzyl-6-[(4-hydroxyphenyl)methyl]piperazine-2,5-dione (cluster 13), 3,6-bis(2-methylpropyl)piperazine-2,5-dione and maculosin (cluster 15), and gancidin W (cluster 17) (Table S2).

Discussion

There has been a growing interest in *Crotalaria* species for biotechnology purposes, particularly in sustainable agriculture due to their PAs defensive properties against pathogens (Joosten and Van Veen 2011, Miranda-Granados et al. 2018). This study confirmed that aerial parts (seeds, stems, and leaves) of *C. retusa* contained PAs such as senecionine and monocrotaline. Wink (2013) reports

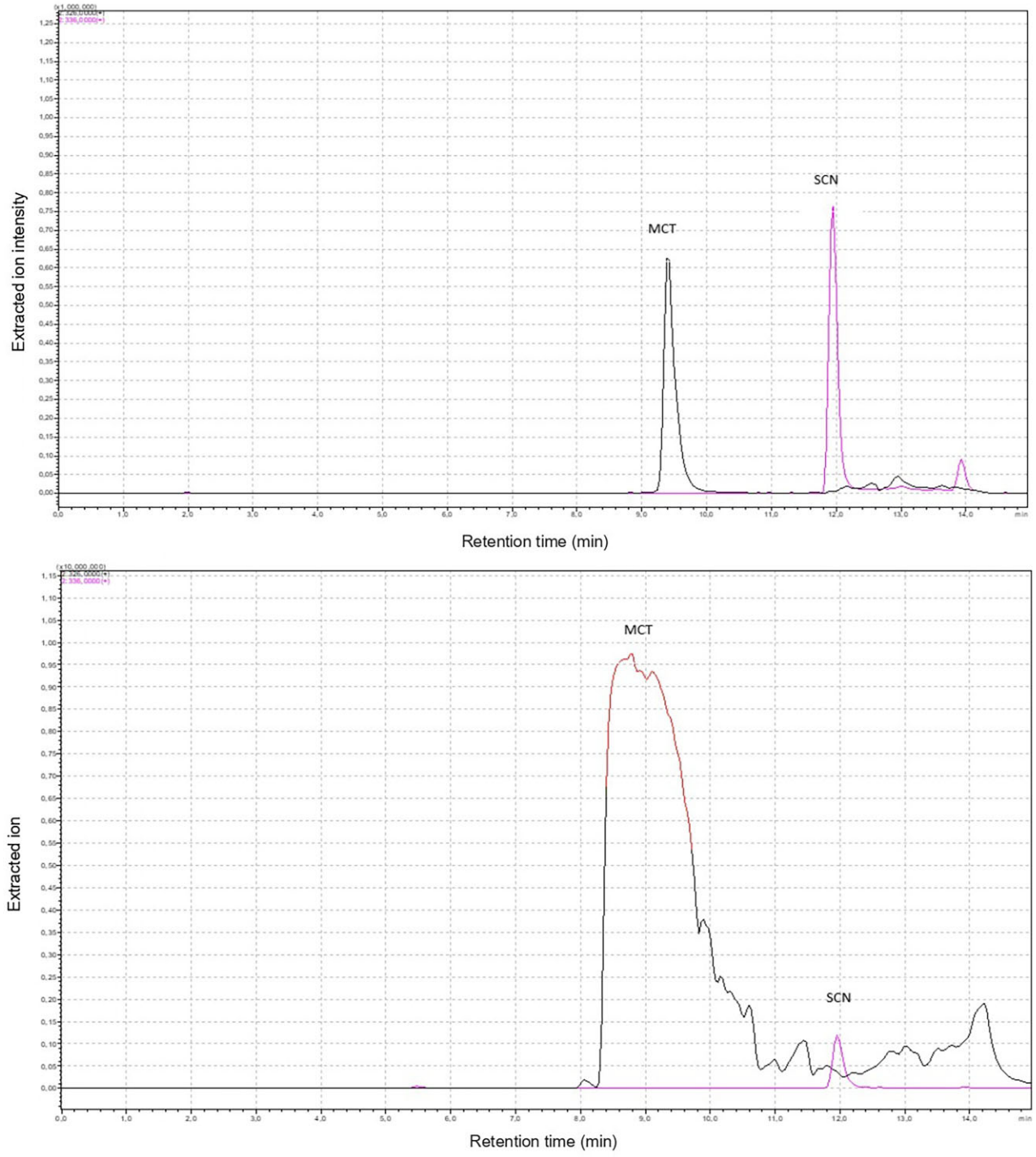

Figure 3. LC–MS chromatograms, acquired in the positive-ion mode, from standards PAs: (a) extracted ion chromatogram of Monocrotaline (MCT) and Senecionine (SCN) standards; (b) LC–MS chromatogram of seed extract.

Table 2. Monocrotaline and senecionine contents in aerial parts (seeds, stems, and leaves) of *C. retusa* wild plants.

Extracts	Monocrotaline% dry weight	Senecionine% dry weight
Seeds	0.83	0.0005
Mix of stems and leaves	0.62	0.0002

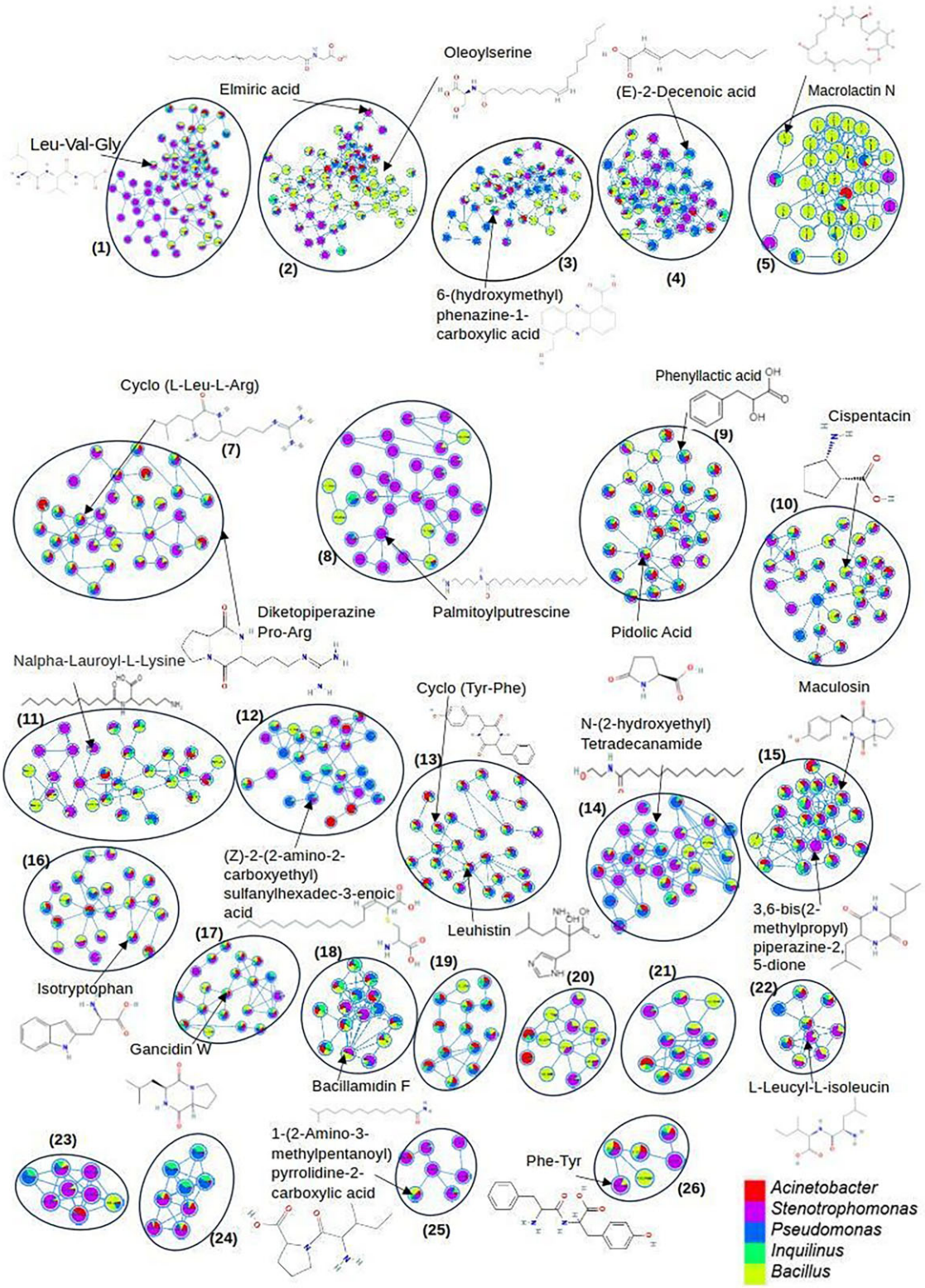

Figure 4. Molecular network obtained from the endophytic bacteria extracts. The network was performed using Compound Discoverer 3.3 and visualized with Cytoscape 3.9.1. The different endophytic bacterial strains are represented by one color. The nodes of *Acinetobacter* are annotated in red, the nodes of *Stenotrophomonas* are annotated in violet, the nodes of *Pseudomonas* are annotated in blue, the nodes of *Inquilinus* are annotated in green and the nodes of *Bacillus* are annotated in yellow. 26 annotated clusters were highlighted by a circle.

that the genus *Crotalaria* is known to contain PAs and nonproteinogenic amino acids, mainly in its seeds. Interestingly, the two identified PAs (monocrotaline and senecionine) were present in all *C. retusa* aerial parts. Indeed, alkaloids can be found in all plant parts, depending on the species. They can accumulate mainly in the bark, roots, leaves, and fruit (Muniz Neves 2006). Moreover, it was evidenced in this study that monocrotaline content was higher in seeds than in other plant aerial parts. Monocrotaline is mainly synthesized in the aerial parts of plants belonging to the *Crotalaria* genus, particularly in the leaves and stems, and it is common to obtain a higher concentration of monocrotaline in the seeds. Consequently, the part where it accumulates is not necessarily the part where it is synthesized (Muniz Neves 2006). Altogether monocrotaline was shown to vary since its content was shown to be lower in previous studies (Nobre et al. 2005, Anjos et al. 2010). Further analyses should explore other types of pyrrolyzidine alkaloids in the seeds.

It has been demonstrated that alkaloid biosynthesis in *Crotalaria* spp. depends on the complex interactions with its endophytic microbial communities (Irmer et al. 2015). It was then interesting to characterize the endophytic bacteria associated with *C. retusa* in Côte d'Ivoire. This is the first study describing cultivable endophytic bacteria from roots, stems, and leaves of *C. retusa*. Indeed, endophytic bacteria have been isolated from many medicinal plant species such as *Narcissus tazetta* (Wang et al. 2015), *Crinum macowanii* (Sebola et al. 2020), *Lycoris radiata* (Liu et al. 2020), and recently from *Leucojum aestivum* (Munakata et al. 2021), but only a few *Crotalaria* plants have been studied so far. The most common genera of endophytic bacteria associated with medicinal plants were *Bacillus*, *Pantoea*, and *Pseudomonas* (Wu et al. 2021). To our knowledge, the characterization of the microbiome from the seeds of *C. pumila* is the only reported case study for endophytic bacteria associated to a Crotalaria plant species (Sánchez-López et al. 2018a,b). In this study, a culture-dependent method was used to isolate and characterize endophytic bacteria from different organs of *C. retusa*. Our study showed that Actinomycetota, Bacillota, and Pseudomonadota were the three main phyla among cultivable bacteria in *C. retusa* growing in Côte d'Ivoire, like what was observed in the microbial communities from *C. pumila* seeds collected from the Santa Maria mine residues in central Mexico (Sanchez-Lopez et al. 2018a, b). Actinomycetota, Bacillota, and Pseudomonadota appeared well adapted to interact with legume plants such as *C. pumila* and *C. retusa*, whether through endophytic relationships, survival strategies or roles in soil health, which probably explains their prominence among cultivable endophytic bacteria. However, the pattern of the endophytic bacterial communities associated to *C. pumila* may differ at genus level (Sanchez-Lopez et al. 2018a, b). While taxonomic characterization revealed that the bacteria isolated from different organs of *C. retusa* collected from Côte d'Ivoire were mainly affiliated with the genera *Bacillus*, *Inquilinus*, *Pseudomonas*, and *Rhizobium*, those identified *from C. pumila* comprised *Corynebacterium*, *Methylobacterium*, *Propionibacterium*, and *Staphylococcus* (Sánchez-López et al. 2018a,b). The profile of endophytic bacterial communities associated with *C. pumila* differed from that of *C. retusa* due to several factors such as species-specific interactions, ecological, and environmental factors. For example, *C. pumila* and *C. retusa* grow in different environments, and local soil conditions, climate, and surrounding microbial communities can affect the types of bacteria present in the seeds of each plant species. The difference in methods used for assessing the microbial communities in the two conditions could also explain the difference observed. Culture-independent and dependent approaches in ecological studies are usually seen as complementary for the identification of the soil bacterial populations (Altowayti et al. 2020). A complementary investigation of *C. retusa* microbiome using a high-throughput sequencing technology in future study may help for better comparison.

Endophytes isolated from the roots, stems, and leaves of *C. retusa* were described as potential biocontrol agents against phytopathogens in this study. A total of nine active isolates inhibiting *F. graminearum* and *F. oxysporum* f. sp. *cubense* mycelial growth were identified. These isolates belonged mainly to the genera *Bacillus*, *Inquilinus*, *Pseudomonas*, *Stenotrophomonas*, and *Acinetobacter*. These genera are known for their ability to thrive in a variety of environments. For example, *Pseudomonas* and *Acinetobacter* are common in soil and water due to their metabolic versatility and ability to degrade a wide range of organic compounds (Peleg et al. 2012). *Bacillus* species are often present in soil and can form spores that help them survive in extreme conditions (Zhang et al. 2020). Interestingly, several isolates of these genera have been repeatedly described to exhibit *in vitro* inhibitory activities against *Fusarium* spp. including *F. graminearum* (Haas and Défago 2005, Comby et al. 2017, Khalaf and Raizada 2018, Munakata et al. 2022). At present, many efficient strains from some key genera, including *Bacillus*, *Paenibacillus*, and *Pseudomonas* have been documented as biocontrol agents of various diseases in major crops (Ayaz et al. 2023). In our study, two strains belonging to *Bacillus* genus and close to the species *B. velezensis* and *B. amyloliquefaciens* showed the highest inhibitory activities against *F. graminearum*. The high inhibitory activity of *B. velezensis* and *B. amyloliquefaciens* against *Fusarium* spp. can be attributed to several key factors related to their biological and biochemical properties such as production of antimicrobial compounds, siderophore production, and production of hydrolytic enzymes (Uwaremwe et al. 2022). Various *Bacillus* isolates close to *B. subtilis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, and *B. pumilus* were described as biocontrol agents against different *Fusarium* species (Marten et al. 2000, Khan et al. 2001, Schisler et al. 2002, 2004, Siddiqui et al. 2005, Vitullo et al. 2012, Munakata et al. 2022, Uwaremwe et al. 2022). *Bacillus* genus is known to produce a huge diversity of antibacterial compounds such as phenazines, pyoluteorin, zwittermicin A, pantocin, and antifungal compounds such as 2,4-diacetylphloroglucinol, pyrrolnitrin, viscosinamide, oomycin, tensin, siderophores, fengycin, surfactin, hydrogen cyanide, kanosamine, and iturins (Ongena and Jacques 2008, Shafi et al. 2017, Naz et al 2022). In this study, diverse chemical compounds including macrolactin N, oleoylserine, and phenyllactic acid were putatively identified by LC–MS/MS in *Bacillus* isolates, of which some are well known for their antimicrobial activity (Table S2). Bacterial isolates affiliated with *Inquilinus* genus have shown the highest antagonistic activities against *F. oxysporum* f. sp. *cubense*. The antifungal activity detected in this bacterium may be due to the production of bioactive metabolites such as nalidixic acid, ibuprofen, sulphacetamide, metronidazole-OH, allopurinol, and oxibendazole as shown in our study. These compounds were reported to be antifungal, antiinflammatory, anthelmintic, and antibacterial (Bibi et al. 2017). Until now, only few *Inquilinus* isolates have been described as potential biocontrol agent, as compared to the well-studied genera such as *Bacillus* (Bibi et al. 2017, Naranjo et al. 2023). The study by Naranjo et al. (2023) showed that this bacteria inhibited phytopathogenic fungi such as *F. graminearum* and *F. oxysporum* by closely surrounding the fungal colony. Finally, other bacterial isolates belonging to *Pseudomonas*, *Stenotrophomonas*, and *Acinetobacter* that were obtained in this study have been recognized as plant growth promoters and biocontrol agents (Ryan et al. 2008, Shi et al. 2011) with

a potential as producers of many bioactive compounds (Lugtenberg and Kamilova 2009, Pliego et al. 2011, Schreiter et al. 2018). For example, studies conducted by Lv et al. (2023) reported that root-associated antagonistic *Pseudomonas* spp. contribute to soil suppressiveness against Banana *Fusarium* wilt Disease (Lv et al. 2023). The bacterial isolates of these genera were shown to be plant growth promoters with a potential as producers of many bioactive compounds (Table 1).

Taken together, the biological activities of the active bacterial endophytes isolated from roots, stems, and leaves of *C. retusa*, a medicinal plant well-known in Côte d'Ivoire, could be linked to the isolates ability to synthesize various antimicrobial specialized metabolites (Saipriya et al. 2020). Molecular network analysis of extracts of the bacterial isolates showed that they contained a rich diversity of secondary metabolites. Clusters 7, 13, 15, and 17 corresponded to dipeptides present in all studied strains. These compounds were mainly dipeptides cyclo (L-Leu-L-Arg), *N*-(3-{1,4-dioxo [1,2-a] pyrazin-3-yl} propyl) guanidine, cyclo (D-Tyr-L-Leu), and 3,6-bis (2-methylpropyl) piperazine-2,5-dione. These bioactive metabolites from our study have antifungal activities (Vicente et al. 2022). Based on LC–MS/MS analysis, all compounds detected in these nine selected endophytic bacteria have their agricultural use and reported as antibacterial and antifungal compounds. In particular, the cyclo (L-Leu-L-Arg), a diketopiperazine derivative previously isolated from *Streptomyces* sp. TN17, possesses antifungal activities against *Fusarium* sp. (Smaoui et al. 2011). The other dipeptide putatively identified as gancidin W, a proline-based cyclic dipeptide, is an antimicrobial agent tested against diverse agriculturally important fungi such as *F. oxysporum. f. sp. cubense* with a minimum inhibitory concentration of 4 μg/ml (Kumar et al. 2013). Hence, the antifungal properties observed in these nine strains were attributed to the bioactive metabolites produced and secreted by the antagonistic endophytic bacterial strains (Bibi et al. 2017).

Conclusion

In this study, 64 endophytic bacteria belonging to different genera were isolated from roots (*Inquilinus*, *Ensifer*, *Mycolicibacterium*, and *Rhizobium*), from stems (*Acinetobacter*, *Bacillus*, *Enterobacter*, *Exiguobacterium*, *Pseudomonas*, and *Stenotrophomonas*), and from leaves (*Curtobacterium* and *Pseudomonas*) of *C. retusa*. Nine antagonistic bacteria against *F. graminearum* and *F. oxysporum* f. sp *cubense* and their potential active metabolites have been further characterized. These selected isolates showed varied profiles of bioactive compounds (e.g. peptides, lipids, and steroids), encompassing both antibacterial and antifungal properties. These metabolites of interest may explain the biological activity of the bacterial endophytes on the pathogens. Two bacterial isolates belonging to *Bacillus* genus showed the highest inhibition rates (more than 65%) against *F. graminearum* whereas two isolates affiliated to *Inquilinus* showed the highest inhibition rates (>50%) against *F. oxysporum* f. sp *cubense*. These results suggest that the different organs of *C. retusa*. are sources of antagonistic bacteria and potential bioactive molecules against phytopathogenic *Fusarium* spp. with possible valorization in agriculture.

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Supplementary data

Supplementary data are available at *FEMSLE Journal* online.

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