Isolation of Novel Vincristine and Vinblastine Producing *Streptomyces* Species from *Catharanthus Roseus* Rhizospheric Soil

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Abstract

Microorganisms could be used as efficient tools to protect high value therapeutic plants against overexploitation and climate change. This work aimed to isolate alkaloids producing endophytic and rhizospheric soil actinomycetes and fungi of *Catharanthus roseus*. From a total of eleven actinomycetes and eight fungi strains isolated by dilution and plate methods, six telluric actinomycetes were revealed to produce alkaloids according to the precipitation test of their extracts. Revelation by Thin Layer Chromatography method using vinblastine and vincristine standards on the basis of frontal reference values showed that the strain SC8 and the vinblastine, the strain SC7 and the vincristine displayed the same frontal references (0,89 and 0,88, respectively). Whole genome sequencing method showed that both strains belong to the genus *Streptomyces* with novel species. Moreover, metabolic pathways analysis from their genomes allowed to detect three enzymes involved in the biosynthesis of terpenoid backbone leading to that of terpenoid indole alkaloids.

1. Introduction

Disappearance threat of certain high therapeutic value plants is a significant and non-negligible problem that has to be solved, view their importance in medicines production as well as their impact in diseases treatment. According to the Kew Gardens Botanical Research center in 2016, one out of five plant species is threatened of disappearance including medicinal plants. Some of them are reported to be disappeared at a slow rhythm; overexploitation and climate change could be the probable causes. In 2004, the World Health Organization (WHO) inventoried *Catharanthus roseus* (Madagascar periwinkle) among endangered medicinal plants. It is an endemic plant of Madagascar traditionally used for its "appetite-suppressant" property, its ability to heal wasp stings and its curative property as wormer, wounds disinfectant, antimalarial and diuretic in infusion. However, the antitumor property of the plant was highlighted between 1958 and 1965. *Catharanthus roseus* has, then, been used to treat certain cancerous diseases. The plant produces several alkaloids, among which two are well-known for their anticancer properties and as the second best anticancer drugs used in the world (Hadagali et al. 2017): the vinblastine particularly used to treat Hodgkin's disease and the vincristine, for children's acute leukemia treatment. These two molecules are nitrogenous heterocycles of alkaloids family and the processes of their extraction revealed that a ton of whole periwinkle plant provides 1g of vinblastine and 10g of vincristine. Indeed, the extraction of bioactive molecules is the main cause of disappearance of certain medicinal plant species. Several strategies are now adopted to protect endangered plants: in-situ conservation (for protection of natural area by creating parks or reserves), ex-situ conservation (creation of seeds or living plants bank), culture intensification and domestication of the most exploited plants. For research side, the works are focused to cultivars development, active principle synthesis and recently cloning and genetic modification (GMO). Besides, this work allows to demonstrate that microbiology is a promising and effective tool to produce bioactive molecules comparable to those of the plant for its preservation. Many research works demonstrated that endophytic microorganisms such as actinomycetes and fungi exhibited the same biological activities (Akshatha et al. 2014; Herivony et al. 2016) and produced the same secondary metabolites as the host-plants (Shweta et al. 2010; Amna et al. 2006; Caruso et al. 2000; Zhao et al. 2013). Some works reported also that actinomycete from rhizospheric soil of medicinal plant displayed the same biological activities than the plant (Herivony et al. 2016). Then, the objective of this reported work was to isolate vinblastine and vincristine producing actinomycetes and fungi from *Catharanthus roseus* and its rhizospheric soil. Cultural, morphological and molecular characterizations of the target isolates were established in this study.

2. Materials And Methods

2.1. Plant and soil sampling

Catharanthus roseus whole plant and its rhizospheric soil were collected from decorative plants garden of National Center of Environmental Research, Antananarivo-Madagascar. Rhizospheric soil was sampled at 10cm of depth under the plant at the harvest moment in April 2018. Plant sample was, then, put onto kraft paper and soil sample into sterile plastic bag; they were

transported immediately to the laboratory for actinomycetes and fungi isolation (plant) and for drying in a dessicator during one week (soil rhizospheric).

2.2. Isolation of actinomycetes and fungi strains

2.2.1. Isolation of endophytic strains

Endophytes isolation from different organs of *Catharanthus roseus* (flowers, leaves, stem, fruits and roots) was performed using plate method (Andriambeloson et al. 2014) on Starch Casein Agar (SCA) for actinomycetes and on Potato Dextrose Agar (PDA) for fungi.

Vegetative organs were washed with tap water and soaked in the ethanol 70% for 30s, then in the sodium hypochloride (3%) for 3min, followed by three successive washings with distilled water to sterilize their surfaces. Plant organs were then cut into small pieces (1cm) and put, firstly onto SCA petri plates supplemented with nystatin (25mg/ml), cycloheximide (50mg/ml) and nalidixic acid (25mg/ml) to suppress fungal and negative bacteria development respectively. Plates were incubated at 30°C for one week to one month. On the other hand, plant organs pieces were put onto PDA petri plates supplemented with chloramphenicol (50mg/ml) to inhibit bacteria growth. Cultures were incubated at 28°C for 5 days.

2.2.2. Isolation of telluric strains

Telluric strains isolation was conducted using dilution and heat treatment methods (Oskay et al. 2004) with the same media as cited above for actinomycetes and fungi.

For dilution method, 1g of soil sample was suspended in 9ml of distilled water. Soil suspension was then homogenized on a shaker for 5min and a serial dilution from 10^{-1} to 10^{-3} was prepared. Thereafter, 100 μ l of the dilutions 10^{-2} and 10^{-3} were put and spread onto SCA and PDA plates. Plates were incubated for one week to one month for actinomycetes growth and for 5 days for fungi development.

For heat treatment method, 1g of soil sample was exposed to 100°C for 1h; then suspended in distilled water. The same technique as described above was applied for actinomycetes and fungi isolation.

To differentiate the two strains, actinomycetes colony was characterized by solid colony embedded in the agar medium with vegetative and aerial mycelia (Shriling and Gottlieb 1966). Fungal colony is, however, recognizable by the development of mycelium on the culture media (Alexopoulos and Mims 1979).

2.3. Purification and conservation of the isolates

For each isolate, colony was purified by two or three successive subcultures on SCA or PDA media without antibiotic or antifungal. Pure isolates obtained were conserved according to two methods: the agar slants preservation for short-term preservation and the cryoconservation for long-term preservation.

Concerning short-time preservation, the colonies of both strains (actinomycetes and fungi) were subcultured on SCA and PDA slants agar. The cultures were incubated at 30°C and 28°C for 7 days and 5 days, respectively and stored at +4°C for one year.

For long-time preservation, actinomycetes colonies were cultured on Starch Casein broth with shaking at 150rpm for 7 days at 30°C. One milliliter (1ml) of the culture was then poured into cryotubes containing 1ml of glycerol (20%). The mixture was homogenized using vortex and the cryotubes were stored at -80°C for ten years (Isik et al. 1999).

The conservation of fungal strain was performed by refrigerating at +1 to +10°C the minitubes containing sterile distilled water and a small mycelial block agar during about 7 years (Nguyen 2007).

2.4. Alkaloids test

2.4.1. Extracts preparation

Pure actinomycetes and fungi strains were subcultured in large Petri plates (150mm) containing ISP2 agar (International *Streptomyces* Project 2) and PDA, respectively. After 8 days of incubation for actinomycetes and 14 days for fungi, secondary metabolites were extracted with ethanol 90° by maceration for 2h. The solutions obtained were, then, filtrated with Millipore filter (0,22µm) and the filtrates were dried using speedvac to form the extracts.

2.4.2. Alkaloids screening

Extracts were screened for alkaloids presence using Fong *et al*'s method (1977) by precipitation test with Dragendorff, Mayer and Wagner reagents. Dried extract was macerated in 3ml of HCl (2N) and the obtained solution was divided in three aliquots in three tubes. Each aliquot was treated with each precipitation reagent and positive result was confirmed if each reagent gave a precipitate.

2.4.3. Alkaloids extraction

Alkaloids were extracted from positive extracts using modified dichloromethane (DCM) method (Bouzidi et al. 2011), divided into two steps. The first step was a solid-liquid extraction, 300mg of extracts and 100ml of water were mixed in a recipient. Sodium bicarbonate was, then, added until obtaining a pH 9; the mixture was boiled for 2h and cooled at the end of the decoction.

The second step was a liquid-liquid extraction, for which total alkaloids were extracted by DCM. For that, the mixture obtained previously and 50ml of DCM were put in a separating funnel. The mixture was, then, shaken vigorously and decanted; organic phase was recuperated and evaporated, the dried residue forms the total alkaloids.

2.5. Vinblastine/Vincristine detection by Thin Layer Chromatography (TLC)

TLC method was adopted to detect vinblastine and vincristine from the extracted alkaloids. Solvent system used was dichloromethane/methanol/water (8:2:2, v/v/v), the vinblastine (RMPL-PHARMA LLP, India) and the vincristine (CELON Labs, India) were used as standards and the Dragendorff reagent as a revelator. Brown spot on yellow background revealed alkaloids presence. Vinblastine or vincristine molecules in the alkaloid extracts were detected by comparing the frontal references (Rf) of the standards and the extracts which were calculated from the following formula: $Rf = \text{distance of solute} / \text{distance of solvent}$

2.6. Cultural and morphological characteristics of vinblastine and vincristine producing strains

Cultural characteristics of the target strains were observed macroscopically on SCA medium by mentioning colonies aspects, the growth, the development of substrate and aerial mycelia and the presence of diffusible pigments other than melanoid pigments after 7, 14 and 21 days of incubation at 30°C.

Morphological characteristics of the target strains were, however, revealed using culture on coverslip technique (Cross 1989). A sterile coverslip was inserted into ISP2 medium plate by forming an angle of 45°. A drop of the inoculum was then put against the coverslip in contact with the medium. After 14 days of incubation at 30°C, the coverslip was carefully removed from the medium, put on a slide and observed under microscope at the magnification 40x. The morphology of spores, substrate and aerial mycelia was noted.

2.7. Molecular characterization of vinblastine and vincristine producing strains

This study was carried out to identify the target isolated strains using whole genome sequencing (Institut Pasteur, Madagascar). The DNAs of pure isolates were extracted from 72 h old cultures with Qiagen Genomic DNA isolation kit (Qiagen) according to manufacturer's instructions. The purity and the amount of DNA extracted were then assessed with a spectrophotometer Nanodrop (Thermo Fisher Scientific, Waltham, USA). Extracted DNAs were thereafter sequenced by preparing DNA sequencing libraries with the Nextera XT kit (Illumina) and 2×150 bps paired-end sequencing was performed using allumina NextSeq500 instrument. Sequenced reads were trimmed and clipped using Alien Trimmer (Criscuolo and Brisse 2013) and sequencing errors were corrected using Musket (Liu et al. 2013). De novo assembly was carried out using SPAdes (Bankevich 2012), gene prediction and annotation were performed using PATRIC (Pathosystems Resource Integration Center) (James et al. 2020). Whole-genome-based taxonomic analysis was performed by Type Strain Genome Server (TYGS) (Meier-Kolthoff and Göker 2019). The average nucleotide identity (ANI) and genome-to-genome distances (GGDs)/ digital DNA-DNA hybridization (dDDH) values were calculated in online calculator (<http://enve-omics.ce.gatech.edu/g-matrix/index>) (Rodriguez and Konstantinidis 2016) and Genome-to-Genome Distance Calculator (GGDC; <http://ggdc.dsmz.de>) (Meier-Kolthoff et al. 2013), respectively, with closest strains identified with TYGS. The phylogenomic tree was constructed using FastME (Lefort et al. 2015) from the genome blast distance phylogeny (GBDP). The trees were rooted at the midpoint and the branch supports were inferred from 100 pseudo-bootstrap replicates. Furthermore, a phylogenetic tree based on 16S was established in order to confirm the taxonomy of the strains.

2.7.1. Specialty genes

Besides the identification of the target isolates, the genes encoding enzymes implied in the biosynthesis of secondary metabolites, particularly the two anticancers produced by the target isolates were assessed from the analysis of metabolic pathways with PATRIC to verify the work hypothesis.

3. Results

3.1. Actinomycetes and fungi isolation

After five days and one week of incubation, fungi and actinomycetes were identified, respectively, according to their own macroscopic aspect. Actinomycetes were characterized by hard colonies implanted in the agar while fungi were easily known by their mycelia development on the culture medium.

3.1.1. Endophytic actinomycetes and fungi

Isolation of actinomycetes and fungi from different organs of *Catharanthus roseus* using plate method showed that only, one actinomycete strain was obtained from the stem of the plant. Though, six fungi were isolated from the plant of which one fungus from the flowers, one fungus from the leaves, two from the stem and two from the roots. Endophytes isolated were coded EA (actinomycete) and EF1 to EF6 (fungi).

3.1.2. Telluric actinomycetes and fungi

Dilution and heat treatment methods allowed the isolation of ten actinomycetes strains and two fungi strains from *Catharanthus roseus* rhizospheric soil. The repartition of actinomycetes and fungi strains from *Catharanthus roseus* plant and its rhizospheric soil is shown in the figure 1. Telluric strains isolated were coded SC1 to SC10 (actinomycetes), SF1 and SF2 (fungi).

3.2. Alkaloids screening

For Endophytes, according to the results obtained, neither actinomycete extracts nor fungal extracts contained alkaloids. Six telluric actinomycetes extracts showed, however, positive results for alkaloids test (SC3, SC4, SC5, SC6, SC7 and SC8). None of the two rhizospheric soil fungi extracts contained alkaloids. Their yield varied from 2,3% to 4,6% (Table 1) and the strain SC6 produced more alkaloids.

Table 1
Alkaloids yield from actinomycetes extracts

Actinomycetes extracts	SC3	SC4	SC5	SC6	SC7	SC8
Raw extract weight (mg)	300					
Alkaloids weight (mg)	7	10	11	14	9	11
Alkaloids yield (%)	2,3	3,3	3,6	4,6	3	3,6

3.3. Vinblastine/vincristine revelation

The results of TLC method showed that the extract from SC8 strain possessed the same frontal reference as vinblastine and the extract from SC7 had the same frontal reference as vincristine (figure 2, Table 2). Thus, these two strains produced the two target alkaloids.

Table 2
Frontal references of alkaloids extracts from telluric actinomycetes

Alkaloids extracts	R _f ^a
VBL ^b	0,89
VCR ^c	0,88
SC3	0,85
SC4	0,87
SC5	0,86
SC6	0,86
SC7	0,88
SC8	0,89

a: frontal reference; b: vinblastine; c: vincristine,

3.4. Cultural and morphological aspects of vinblastine and vincristine producing strains

The strain SC8, a vinblastine producing actinomycete isolated from the rhizospheric soil of *Catharanthus roseus* on SCA medium showed large size colonies of 4mm in diameter, opaque, hunchbacked with notched edge. The colonies showed creamy aerial and substrate mycelia, the strain produced yellow diffusible pigment. Microscopically, the strain showed weakly ramified vegetative mycelium; aerial mycelium was less developed with a few isolated spores.

The other strain, SC7 which produced the vincristine have got small and powdery colonies of 2mm in diameter with white aerial mycelium and beige substrate mycelium. The strain SC7 produced yellow diffusible pigment. Its microscopic aspect showed branched vegetative mycelium and less developed aerial mycelium with also isolated spores (figure 3).

3.5. Molecular characterization of the two vinblastine and vincristine producing actinomycetes

3.5.1. ANI and DDH values of SC7 and SC8 genome

Compared to 14 *Streptomyces* genomes closest to the two target isolates, the highest ANI values were obtained with *Streptomyces exfoliatus* NRRL B-2924 (90,71%) and *Streptomyces zaomyceticus* JCM 4864 (90,21%) while the lowest values

were obtained for *Streptomyces lateritius* JCM 4389 (85,57%) and *Streptomyces omiyaensis* JCM 4806 (85,93%) for the strain SC7. The DDH varied between 33,2% and 47,9%.

For the strain SC8, the highest ANI values were obtained with *Streptomyces exfoliatus* NRRL B-2924 (90,72%) and *Streptomyces zaomyceticus* JCM 4864(90,24%), the lowest values were obtained for *Streptomyces omiyaensis* JCM 4806 (85,96%) and *Streptomyces lateritius* JCM 4389 (85,61%). The DDH varied between 32,7% and 47,5% (Table 3).

All ANI values were below the generally accepted species delineation cutoff 95-96% and corresponding pair wise in silico DNA-DNA hybridization (DDH) values were all below 70%. The ANI and DDH values between the two strains SC7 and SC8 were 99,97% and 99,6%, respectively. It indicates that both strains belong to the same species that is identified as a novel species.

Table 3
ANI and dDDH matrix for the two strains SC7 and SC8 with reference strains in TYGS database

Target isolates	Reference strains (TYGS database)	Assembly accession	ANI	dDDH (d0, in %)	C.I. (d0, in %)	dDDH (d4, in %)	C.I. (d4, in %)	dDDH (d6, in %)	C.I. (d6, in %)	G+C content difference (in %)
SC7	<i>Streptomyces bikiniensis</i> NRRL B-1049	GCA_000716465	88,74	39,4	[36.0 - 42.9]	34,9	[32.5 - 37.4]	37,6	[34.6 - 40.6]	0,89
SC7	<i>Streptomyces bikiniensis</i> NRRL ISP-5581	GCA_000716245	88,91	34,7	[31.4 - 38.3]	35,9	[33.5 - 38.5]	33,8	[30.9 - 36.9]	0,81
SC7	<i>Streptomyces cinereoruber</i> JCM 4205	GCA_014649095	88,78	40,1	[36.7 - 43.5]	34,9	[32.4 - 37.4]	38,1	[35.2 - 41.2]	0,88
SC7	<i>Streptomyces exfoliatus</i> NRRL B-2924	GCF_000725545	90,71	47,9	[44.5 - 51.3]	40,2	[37.7 - 42.7]	46	[43.0 - 49.0]	0,23
SC7	<i>Streptomyces gardneri</i> JCM 4375	GCA_014655085	88,79	40,4	[37.1 - 43.9]	35,4	[32.9 - 37.9]	38,5	[35.6 - 41.6]	0,53
SC7	<i>Streptomyces lateritius</i> JCM 4389	GCA_014649715	85,57	33,2	[29.8 - 36.7]	28,7	[26.4 - 31.2]	31,1	[28.2 - 34.2]	0,84
SC7	<i>Streptomyces litmucidini</i> JCM 4394	GCA_014649755	88,87	40,7	[37.3 - 44.2]	35,7	[33.3 - 38.2]	38,8	[35.9 - 41.9]	0,58
SC7	<i>Streptomyces snarbonensis</i> JCM 4147	GCA_014649015	88,79	42	[38.6 - 45.4]	35,1	[32.6 - 37.6]	39,7	[36.8 - 42.8]	0,29
SC7	<i>Streptomyces nashvillensis</i> JCM 4498	GCA_014650095	89,22	41,3	[38.0 - 44.8]	36,5	[34.1 - 39.0]	39,6	[36.6 - 42.6]	0,1
SC7	<i>Streptomyces omiyaensis</i> JCM 4806	GCA_014650895	85,93	34,8	[31.4 - 38.3]	29,3	[26.9 - 31.8]	32,5	[29.5 - 35.6]	1,69
SC7	<i>Streptomyces tanashiensis</i> JCM 4086	GCA_014648895	89,21	40,9	[37.6 - 44.4]	36,4	[34.0 - 39.0]	39,2	[36.3 - 42.3]	0,04
SC7	<i>Streptomyces venezuelae</i> ATCC 10712	GCA_000253235	89,66	46,2	[42.8 - 49.6]	37,7	[35.3 - 40.3]	43,9	[40.9 - 46.9]	0,3
SC7	<i>Streptomyces wedmorensis</i> NRRL 3426	GCF_000716445	88,92	39,7	[36.3 - 43.1]	35,6	[33.2 - 38.1]	38	[35.0 - 41.0]	0,12
SC7	<i>Streptomyces zaomyceticus</i> JCM 4864	GCA_014656215	90,21	45,7	[42.3 - 49.1]	38,8	[36.3 - 41.3]	43,7	[40.7 - 46.8]	0,1
SC8	<i>Streptomyces bikiniensis</i> NRRL B-1049	GCA_000716465	88,78	39,2	[35.8 - 42.7]	35	[32.6 - 37.5]	37,4	[34.5 - 40.5]	0,91

Target isolates	Reference strains (TYGS database)	Assembly accession	ANI	dDDH (d0, in %)	C.I. (d0, in %)	dDDH (d4, in %)	C.I. (d4, in %)	dDDH (d6, in %)	C.I. (d6, in %)	G+C content difference (in %)
SC8	<i>Streptomyces bikiniensis</i> NRRL ISP-5581	GCA_000716245	88,87	34,5	[31.2 - 38.1]	36	[33.6 - 38.6]	33,7	[30.7 - 36.8]	0,83
SC8	<i>Streptomyces cinereoruber</i> JCM 4205	GCA_014649095	88,78	39,8	[36.4 - 43.3]	35	[32.5 - 37.5]	37,9	[35.0 - 41.0]	0,9
SC8	<i>Streptomyces exfoliatus</i> NRRL B-2924	GCF_000725545	90,72	47,5	[44.1 - 50.9]	40,3	[37.8 - 42.8]	45,7	[42.7 - 48.7]	0,21
SC8	<i>Streptomyces gardneri</i> JCM 4375	GCA_014655085	88,87	40	[36.7 - 43.5]	35,5	[33.0 - 38.0]	38,2	[35.3 - 41.3]	0,5
SC8	<i>Streptomyces lateritius</i> JCM 4389	GCA_014649715	85,61	32,7	[29.4 - 36.3]	28,9	[26.5 - 31.4]	30,8	[27.9 - 33.9]	0,82
SC8	<i>Streptomyces litmocidini</i> JCM 4394	GCA_014649755	88,91	40,4	[37.0 - 43.8]	35,8	[33.4 - 38.3]	38,6	[35.6 - 41.6]	0,61
SC8	<i>Streptomyces narbonensis</i> JCM 4147	GCA_014649015	88,86	41,5	[38.2 - 45.0]	35,2	[32.8 - 37.7]	39,4	[36.4 - 42.4]	0,27
SC8	<i>Streptomyces nashvillensis</i> JCM 4498	GCA_014650095	89,28	41	[37.7 - 44.5]	36,6	[34.1 - 39.1]	39,3	[36.4 - 42.4]	0,12
SC8	<i>Streptomyces omiyaensis</i> JCM 4806	GCA_014650895	85,96	34,6	[31.2 - 38.1]	29,4	[27.0 - 31.9]	32,3	[29.4 - 35.4]	1,71
SC8	<i>Streptomyces tanashiensis</i> JCM 4086	GCA_014648895	89,27	40,6	[37.2 - 44.0]	36,5	[34.1 - 39.1]	38,9	[36.0 - 42.0]	0,06
SC8	<i>Streptomyces venezuelae</i> ATCC 10712	GCA_000253235	89,68	45,7	[42.3 - 49.2]	37,8	[35.4 - 40.4]	43,6	[40.6 - 46.6]	0,32
SC8	<i>Streptomyces wedmorensis</i> NRRL 3426	GCF_000716445	88,97	39,2	[35.8 - 42.7]	35,7	[33.3 - 38.2]	37,6	[34.6 - 40.7]	0,1
SC8	<i>Streptomyces zaomyceticus</i> JCM 4864	GCA_014656215	90,24	45,1	[41.8 - 48.6]	38,9	[36.5 - 41.5]	43,3	[40.4 - 46.4]	0,07
SC8	SC7		99,97	99,6	[99.2 - 99.8]	99,4	[99.0 - 99.6]	99,8	[99.7 - 99.9]	0,02

In fact, the results obtained from genomic sequencing of the two actinomycete isolates regarding their taxonomy showed that they belong to:

Group : Terrabacteria

Phylum : Actinobacteria

Class : Actinobacteria

Order : Streptomycetales

Family : Streptomycetaceae

Genus : *Streptomyces*

3.5.2. Phylogenetic analysis

Phylogenetic analysis based on 16S indicates that the strains cluster with *Streptomyces venezuelae* ATCC 10712 strain (73% branch support). On the other side, the whole genome phylogenetic analysis showed that the strains cluster with *Streptomyces exofolius* NRRL B-2924 (100% branch support). Thus, both phylogeny methods indicate clearly that the two strains are very close and cluster each other (73% for 16S and 100% for WGS) (figures 4 and 5).

3.5.3. Genome assembly and annotation

The de novo assembly of the genome, gene prediction and annotation consisted of 1,354 contigs, 7,788,601 bases (N50= 9,831; DNA G+C content= 72.19%), 7,919 coding sequences and 65 sequences encoding tRNA for SC7. However, 1,486 contigs, 7,746,516 bases (N50= 8,715; DNA G+C content= 72.17%), 7,935 coding sequences and 65 sequences encoding tRNA were noted for SC8.

The annotation with PATRIC allowed to identify different genes involved in different metabolic pathways including antibiotic biosynthesis, pigment biosynthesis, photosynthesis and degradation of organic compounds. Indeed, in this study, among the thousands products identified, three enzymes involved in the biosynthesis of terpenoid backbone leading to that of terpenoid indole alkaloids including the vincristine and the vinblastine (1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase) were detected for the two actinomycetes isolates genomes (ESM_1 and ESM_2, Online resources).

4. Discussion

Isolation of vinblastine and vincristine producing actinomycetes associated to *Catharanthus roseus* is demonstrated in this work. For fungi and actinomycetes distribution, it was observed that *Catharanthus roseus* endophytic population was dominated by fungi population while its rhizospheric soil was conquered by actinomycetes population. Among the seven endophytic microorganisms isolated from *Catharanthus roseus* vegetative organs, one is an actinomycete and the six others are fungi. These results corroborate those found by Caruso et al. (2000) who demonstrated the abundance of the endophytic fungi population isolated from different plants of genus *Taxus* compared to the endophytic actinomycetes population from the same plants. Dissimilarly to this, ten actinomycetes and two fungi were obtained from *Catharanthus roseus* rhizospheric soil; this confirms that the soil constitutes the main habitat of actinomycetes (El-Shatoury 2006).

Alkaloids test of extracts from microorganisms associated to the plant showed that seven rhizospheric soil actinomycetes strains contained alkaloids. None of the isolated fungi produced the target secondary metabolites.

Alkaloids yield varied from one actinomycete strain to another at a range of 2,3% to 4,6%. Compared to alkaloids yield of some medicinal plants such as *Emblia officinalis*, *Acacia catechu*, *Acacia concina* and *Hibiscus rosa-sinensis*, that of actinomycetes is low. For these plants, alkaloids contents are 11,2%, 11,3%, 10,2% and 8,5%, respectively (Gupta et al. 2013). However, Liu and Liu (2015) reported that the optimization of extraction process is required to improve total alkaloids yield.

Vinblastine and vincristine screening in the positive alkaloid extracts by TLC method showed that two extracts from SC7 and SC8 contained respectively vincristine and vinblastine. The two target molecules weren't detected in the most alkaloid

producing strain (SC6) in this study. The results obtained are different of those obtained by some researchers revealing that endophytic microorganisms isolated from *Catharanthus roseus* until now, which produced similar secondary metabolites as the plant are fungi. For examples, *Fusarium oxysporum* isolated from the leaves of the plant (Kumar et al. 2013) and *Talaromyces radicus* from leaf tissues (Palem et al 2016) produced both vincristine and vinblastine. Besides, *Eutypella spp* CrP 14 isolated from the stem cuttings of *Catharanthus roseus* was reported to produce vincristine (Kuriakose et al. 2016), *Nigrospora sphaerica* from plant leaf was also demonstrated to produce vinblastine (Ayob and Simarani 2016; Ayob et al. 2017) and very early *Chaetomium globosum* Cr 59 from the stem of the plant produced vinblastine (Zafari et al. 2020).

To our knowledge, this is the first study which demonstrates vinblastine or vincristine producing actinomycetes. Furthermore, it is yet demonstrated that soil rhizospheric actinomycetes can produce analogous secondary metabolites (alkaloids) and the same bioactive molecules (vinblastine and vincristine) than the plant. In our previous study, a similar result was observed; an actinomycete strain isolated from ginger rhizospheric soil (*Streptomyces antibioticus*) was revealed to produce the same secondary metabolites (terpenes and phenolic compounds) and exhibit similar biological activities (antifungal, antioxidant, anticancer and antimalaria) than those of ginger essential oil (Herivony et al. 2016).

Macroscopic (solid colonies embedded in the agar, presence of colored mycelia, powdery surface) and microscopic (branched vegetative mycelium and aerial mycelium bearing isolated spores) aspects of the two vinblastine and vincristine producing actinomycetes showed that they seemed like the genus *Streptomyces* (Shriling and Gottlieb 1966).

Confirmation by molecular characterization using whole genome sequencing showed that they belong to the genus *Streptomyces*. This technique revealed also that ANI and DDH values were strictly below 95% and 70%, respectively, between the 14 standard genomes used (*Streptomyces bikiniensis* NRRL B-1049, *Streptomyces bikiniensis* NRRL ISP-5581, *Streptomyces cinereoruber* JCM 4205, *Streptomyces exfoliates* NRRL B-2924, *Streptomyces gardneri* JCM 4375, *Streptomyces lateritius* JCM 4389, *Streptomyces litmocidini* JCM 4394, *Streptomyces narbonensis* JCM 4147, *Streptomyces nashvillensis* JCM 4498, *Streptomyces omiyaensis* JCM 4806, *Streptomyces tanashiensis* JCM 4086, *Streptomyces venezuelae* ATCC 10712, *Streptomyces wedmorensis* NRRL 3426, *Streptomyces zaomyceticus* JCM 4864) and the two selected strains genomes. However, ANI and DDH values between the two strains (respectively, 99,97% and 99,6%) were above the given delineation cutoff. Thus, the two strains were considered as belonging to a same novel *Streptomyces* species (Richter and Rosselló-Móra 2009; Meier-Kolthoff 2013). In fact, some resemblance of morphological and cultural features of the two isolates cited above could be considered for their belonging to a same new species.

Moreover, metabolic pathways analysis with PATRIC allows to detect three enzymes: 1-deoxy-D-xylulose 5-phosphate synthase encoded by the *dxs* gene (Chahed et al. 2000), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr* gene) and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*mecs* gene) (Veau et al. 2000), implied in terpenoid indole alkaloids (TIAs) biosynthesis including the vincristine and the vinblastine in *Catharanthus roseus* plant. Hence, it would be deduced from these results that the two novel isolated *Streptomyces* from *Catharanthus roseus* rhizospheric soil produce the same anticancer metabolites as the plant.

Through the results obtained by TLC for vinblastine and vincristine detection in alkaloids extracts from the two new *Streptomyces* species isolated from *Catharanthus roseus* rhizospheric soil and reinforced by those obtained via metabolic pathways analysis of their genome, it would be confirmed that the two novel isolated strains produce vinblastine and vincristine as *Catharanthus roseus*. However, the biosynthesis mechanism of the two anticancers in the new *Streptomyces* species should be elucidated.

5. Conclusion

From this work, it was revealed that two rhizospheric soil actinomycetes of *Catharanthus roseus* belonging to a new *Streptomyces* species produced the same active compounds than the plant (vinblastine and vincristine). To better

understand the potential of the anticancer from the two novel isolated strains, production parameters optimization, isolation, purification and antiproliferative activity against different cancerous cells will be conducted.

Declarations

Conflict of interest

All authors declare that they are no conflict of interest.

Authors contribution

Each author contributes to the writing of the manuscript according to his/her specialty for each method cited in the text:

- Andriambeloson Herivony Onja, for actinomycetes and fungi isolation from *Catharanthus roseus* vegetative organs and its rhizospheric soil, conservation of the isolates.
- Andrianantenaina Rigobert, for alkaloids screening of the extracts prepared from the obtained isolates, vinblastine and vincristine screening in the positive alkaloids extracts.
- Rabenandrasana Mamitiana Alain Noah and Collard Jean-Marc, for molecular characterization of the target isolates.
- Ramaroson Luciano, for cultural and morphological characterization of the target isolates.
- Rasolomampianina Rado, for microbial extracts preparation.

The corresponding author compiled the writing from all authors and a rotating reading was done to improve the manuscript. This latter was finalized by the corresponding author.

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Figures

Figure 1

Number of actinomycetes and fungal strains isolated from *Catharanthus roseus* plant and its rhizospheric soil

Figure 2

Chromatographic profile for vinblastine and vincristine screening in alkaloid extracts from *Catharanthus roseus* rhizospheric soil 1 : vinblastine ; 2 : vincristine ; 3 : alkaloids from the isolate SC3 ; 4 : alkaloids from the isolate SC4 ; 5 : alkaloids from the isolate SC5 6 : alkaloids from the isolate SC6, 7 : alkaloids from the isolate SC7; 8 : alkaloids from the isolate SC8 a: deposit line; b: front line; c: plate saturated with Dragendorff's reagent; d: alkaloids migration

Figure 3

Macroscopic and microscopic aspects of vinblastine and vincristine producing actinomycetes isolated from *Catharanthus roseus* rhizospheric soil A: cultural characteristics on SCA medium; B: morphological characteristics by microscopic observation (LEICA x 40)

Figure 4

Phylogenomic tree of the isolates SC7 and SC8 based on genome sequences in the TYGS tree inferred with FastME 2.1.6.1

Figure 5

16S phylogenetic tree of the isolates SC7 and SC8 based on genome sequences in the TYGS tree inferred with FastME 2.1.6.1

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