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Antagonistic potential of selected rhizospheric *Streptomyces* from potatoes against *Ralstonia solanacearum* species complex

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Abstract

This work aimed to control *Ralstonia solanacearum* species complex, the causal agent of potatoes bacterial wilt by selecting potatoes rhizospheric Actinomycetes as biocontrol agents. The research approach focused on the isolation of Actinomycete strains from rhizospheric soils of potatoes using serial dilution technique on Starch Casein Agar medium, *in vitro* antagonistic activity of isolated strains with cross streak and agar cylinder disk methods, evaluation of antagonistic mechanisms of active isolates by different assays, phenotypic characterization and identification of potent Actinomycetes.

According to the results obtained, 71 Actinomycetes were isolated from 17 soil samples of Manandona and Androakavato in Vakinankaratra region, Madagascar. Seven (7) isolates (9.85%) showed antagonistic activity against *Ralstonia solanacearum* species complex (phylotypes RI, RII and RIII) with an inhibition zone values ranging from 3 to 16 mm (isolate A28 against the phylotype RII and the isolate A5 on the phylotype RIII, respectively). The isolates were active on RI and RIII than RII. Three (3) selected isolates (A10, A28 and A40) used as antagonistic mechanisms, the production of indole acetic acid (0.11 to 0.14 µg/ml), ammonia, hydrogen cyanide, extracellular enzymes (protease, cellulose and urease), phosphate solubilization and antibiosis. Phenotypic characterization demonstrated that the three selected Actinomycete isolates were identified as *Streptomyces.*

From these results, it would be suggested that the use of Actinomycetes as bioinocula for plant growth promoting, nutrient mobilization or biocontrol agent in biopesticide - biofertilizers against *Ralstonia solanacearum* species complex is promising. It is a simple technology that can be applied as part of sustainable agriculture.

Keywords: Potato; *Ralstonia solanacearum* species complex; Bacterial wilt; Actinomycetes; Biocontrol

1. Introduction

Actinomycetes present a high proportion of soil microbial biomass, approximately 10^4 to 10^8 Actinomycetes/g soil, mainly in alkaline soils with a high concentration of organic matter [1, 2] and they are recognized to play an important role in the microbial flora of the rhizosphere [3]. Their interaction with plants was demonstrated in several works (secretion of herbicidal compounds, fixation of atmospheric nitrogen or protection of roots against bacterial or fungal infection) [4, 5, 6, 7, 8]. Moreover, their ability to colonize root-system of the host plant by competition with other microorganisms in the soil, allowed them to be considered as potential biocontrol agents to fight against some

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phytopathogens. Many studies revealed that rhizospheric *Streptomyces* could improve crop production and reduce the impact of wilt disease in some vegetables culture such as potatoes and tomatoes [9, 10]. As potential biological control agents of plant disease, our attention was focused on rhizospheric Actinomycetes, in the objective to evaluate their effectiveness on the reduction of potatoes bacterial wilt incidence caused by *Ralstonia solanacearum* species complex (*Rssc*).

This phytopathogen is one of the most devastating bacteria of the crops; it can infect over 200 families of plants including potatoes, tomatoes, peanuts, tobacco and bananas, etc. [11]. During the infection, the pathogen invades plant roots through wounds and multiplies in the cortical tissue before invading the xylem elements. In a few hours, the bacteria can spread in the plant's vascular system [12, 13]. Virulence genes are gradually expressed with the increase of phytopathogen cells concentration, they become non motile, secrete exopolysaccharide and pectin, leading to the death of the plant [14]. Among the control strategies used, chemical treatments seemed ineffective in controlling the bacterial wilt [15, 16] and present some negative impacts on the environment. Some pesticides may persist in the environment for a long time, pollute water and soil, and cause toxicity to the consumers due to their ignorance and improper application [17]. On the other hand, biological control using various bioagents such as *Streptomyces sp*., *Pseudomonas fluorescens*, *Bacillus sp* etc, was developed and appeared to be convincing to treat this disease [9, 16]. Indeed, some works reported the effectiveness of rhizospheric *Streptomyces* to control *Ralstonia solanacearum* [9, 10, 15].

In 2005, *Ralstonia solanacearum* infected a considerable cultivation field of potatoes in Vakinankaratra region (the largest potato producer in Madagascar); resulting therefore a major economic loss of the region [18]. Actually, the three phylotypes (I, II, III) of *Ralstonia solanacearum* are found there [18, 19, 20, 21]. The bacterial wilt caused by *Ralstonia solanacearum* species complex (*Rscc*), remains one of the most devastating diseases on solanaceous crops in Madagascar.

Of this effect, to pursue the investigation initiated by Rasolomampianina *et al.*, (2016) [9]; this work contributes to control *Ralstonia solanacearum,* the causal agent of potatoes bacterial wilt by selecting potatoes rhizospheric Actinomycetes as biocontrol agents. Isolation was carried out from potatoes rhizospheric soils and the obtained isolates were screened for their antagonistic activity against *Rssc*. Antagonistic mechanisms of active isolates were then, investigated and on the basis of the results obtained, a potent Actinomycete isolate was selected, characterized and identified.

2. Material and methods

2.1. Isolation of Actinomycetes

Actinomycetes were isolated from composite soil samples (potatoes rhizospheric soils), collected in two sites of Vakinankaratra region: Androakavato (site n°1, S: 19°43.484' E: 047°02.606'; A: 1823 m) and Manandona (site n°2, S: 20°04.089'; E: 047°03.757'; A: 1399 m). Isolation was performed on Starch Casein Agar (SCA) medium supplemented with cyclohexamide (50 mg/l) to reduce fungal development [23] using standard dilution technique. The cultures were incubated at 30°C for 7 – 30 days and Actinomycete colonies were noted with their specific traits (rough, embedded in agar). Isolated colonies were subcultured on ISP2 (International *Streptomyces* Project 2) Agar for purification and pure isolates were stored at +4°C in slant agar and in cryovials containing glycerol (20%) at -20°C [24].

2.2. Antagonistic activity of Actinomycetes against *Ralstonia solanacearum* species complex

This assay was carried out *in vitro* using agar cylinder and cross streak methods [25]. Three phylotypes of *Rssc*: RI, RII and RIII previously isolated and identified by Ravelomanantsoa [18] were tested. The phytopathogens were revivified by culture on Selective Medium South Africa (SMSA) for 48 h at 30°C and the inocula were prepared in distilled water at a concentration of 10^8 CFU/ml.

- ***Agar cylinder method*:** agar disks (6 mm in diameter) of mature Actinomycetes (7 days old) grown on Starch Casein (SCA) were transferred aseptically into Mueller Hinton Agar plates, previously inoculated with the phytopathogens. The cultures were kept for 24 – 72 h at 30°C.
- ***Cross streak method*:** the Actinomycete isolates were cross-streaked against *Rssc* phylotypes RI, RII and RIII. The Actinomycete isolates were streaked with a unique trait using sterile loop in the middle of the Nutrient Agar (NA) plates and kept for 3 - 4 days at 30°C. Thereafter, fresh cultures of phytopathogens were streaked perpendicularly on both sides of the Actinomycete isolates. The plates were then incubated for 24 – 72 h at 30°C.

For both methods, antagonistic activity was estimated by measuring the inhibition zones around the Actinomycete agar cylinders and on both sides of the Actinomycete isolates (cross streak method) [26]. The interpretation criteria of antagonistic activity were established after determination of the diameter of the inhibition zones using the following formula

$$IZ = \frac{Da - d}{2}$$

- Da = Diameter of inhibition zone including agar cylinder disk or size of Actinomycete isolates (mm)
- d= Diameter of agar cylinder disk or size of Actinomycete isolates (antagonistic agent) (mm)
- IZ= Inhibition zone (mm)

2.3. Antagonistic mechanisms of active isolates

On the basis of the results obtained from antagonistic assay, active isolates were subjected to other tests such as the production of some lytic enzymes, hormones or toxic substances in order to understand their antagonistic mechanisms towards phytopathogens.

2.3.1. Indole acetic acid (IAA) production

This test was evaluated according Patten and Glick' technique [27]. Actinomycete strain was grown on ISP2 broth supplemented with L-tryptophane (1 µg/ml) and incubated at 30°C for 5 days. The culture was then centrifuged for 10 min at 10 000 rpm and the supernatant was recovered. Two milliliters (2 ml) of Salkowski reagent were added to the supernatant and the mixture was settled at ambient temperature for 30 min. The apparition of pink colour indicates the presence of IAA which was quantified by the measure of the absorbance at 530 nm using a spectrophotometer and an extrapolation from IAA standard curve.

2.3.2. Phosphate solubilization

The ability of Actinomycete isolates to solubilize phosphate was evaluated using the method described by Mehta and Nautyal [28]. Actinomycetes isolates were subcultured in spot on Tricalcium Phosphate Agar (TCP agar) medium and incubated at 30°C during 7 days. Phosphate solubilization was affirmed by the apparition of a clear zone around the Actinomycete colonies.

2.3.3. HCN (cyanhydric acid) production

It was estimated qualitatively using colorimetric method described by Lorck [29]. Bennett medium supplemented with glycine (4.4‰) was inoculated with Actinomycete isolates, a Whatman paper n°1 (9 mm in diameter) soaked with picric acid (1%) was stuck under the petri plates lids. The plates were then sealed with a parafilm and incubated at 30°C for 7 days; brown reddish coloration of the Whatman paper testified the HCN production.

2.3.4. Ammonia production

The production of ammonia was determined by colorimetric method according to Lorck [29]. Actinomycete strains were grown in 10 ml of peptone water at 30°C for 48 – 72 h. Afterwards, 0.5 ml of Nessler reagent was added into the culture; the apparition of brown or yellow coloration attested ammonia production by the Actinomycetes [30, 31].

2.3.5. Antibiosis

This assay was performed in order to detect the presence of antibiotics (anti-*Ralstonia*) in the extracts produced by active Actinomycetes. The test proceeds in two steps:

Extract preparation

ISP2 agar plates were inoculated with pure culture of Actinomycetes and incubated at 30°C for 7 days. Thirty milliliters (30 ml) of ethanol (90%) were, then, poured into the cultures and the plates were settled for maceration at room temperature for 2 h in order to extract bioactive metabolites produced by the Actinomycete isolates. Thereafter, ethanolic solution was filtered with millipore filter (0.45 µm) to remove live cells, distributed in glass vials and dried in a SpeedVac. The residues obtained constitute the extracts used for antibiosis test [10, 11].

Antibiosis test

Two milliliters (2 ml) of germs-tests suspension (*Rssc*) equivalent to 0.5 Mac Farland (10^8 CFU/ml) were uniformly spread on the surface of Mueller-Hinton Agar plate by swab technique. Sterile paper disks (6 mm in diameter) were impregnated with 20 µl of the tested extract at a concentration of 0.4 mg/ml. After 24 to 48 h of incubation at 30°C, the inhibition of *Ralstonia solanacearum* species complex growth was appreciated by measuring the diameter of inhibition zone around the disk [32]. Only extracts showing an inhibition zone greater than 8 mm were considered as active extracts. All the experiments were performed in triplicate and the results were interpreted according to pathogens sensibility: the pathogen is resistant if the diameter of inhibition zone (X) is inferior to 7 mm, not very sensitive: 7 mm < X < 10 mm, sensitive: 10 mm < X < 15 mm, very sensitive: 15 mm < X < 20 mm, extremely sensitive: X > 20 mm [33].

2.4. Extracellular enzymes production

Active isolates were screened for the production of 3 important enzymes (protease, cellulase and urease) [34, 35, 36] using standard methods.

2.4.1. Protease activity

Protease activity of the tested Actinomycetes indicated by the casein degradation was determined by the apparition of clear zones in Skimmed Milk Agar (50 ml of sterile skimmed milk mixed at 55°C with 2.5% Agar) after 5-7 days of incubation at 28 °C [34, 35].

2.4.2. Cellulase activity

Cellulase activity is detected on ISP2 plates containing 1% (w/v) of Carboxy Methyl Cellulose (CMC). Tested Actinomycetes were inoculated in spot in the center of the plate [20]. Clear halos around the Actinomycetes, indicating enzymatic degradation were observed after 5 - 7 days at 30°C [34, 35, 36].

2.4.3. Urease production

Urease production is detected by the culture of Actinomycetes on Urea-indole medium. The color change from yellow to purplish red after incubation at 30°C for 5 days indicates the production of urease [34, 36].

2.5. Chemical screening of extract from potent Actinomycetes

Antagonistic activity of extracts or microorganisms is due to some specific chemical compounds [37, 38]. Thus, the chemical families of extracts from potent Actinomycetes among active Actinomycetes selected for their effectiveness through previous assays were highlighted. The chemical screening of Actinomycete extracts was performed according to the methods described by Fong *et al*. [39] and Marini-Bertolo *et al*. [40]. It is a qualitative analysis based on the coloration or precipitation reaction of the compounds of appropriate chemical families in the crude extract. The tested families are the terpenoids, the tannins, the leucoanthocyans, the flavonoids, the alkaloids, the saponosids, the anthraquinones, the polysaccharides and the polyphenols. Results were estimated by the presence of precipitation or the color change of the reaction.

2.6. Characterization of potent Actinomycetes

Potent Actinomycetes able to control *Rssc* was characterized phenotypically by investigating their cultural, morphological, and physiological and biochemical characteristics, following the directions given by the ISP [41] and Bergey's Manual of Systematic Bacteriology [42].

Cultural characteristics on SCA medium allowed to determine the colony color, aerial and substrate color, spore mass color and the production of diffusible pigment through macroscopic examination.

Morphological characteristics allowed to reveal spores chain morphology and disposition by microscopic examination.

Regarding physiological and biochemical characteristics, the effects of NaCl concentration and temperature [43] on the selected Actinomycete growth and the use of some carbon substrates [44] by the Actinomycete were assessed.

The tolerance to sodium chloride was estimated with selected Actinomycetes culture on solid SCA medium containing different concentrations of NaCl (0%; 1%, 3%, 5%, 7%, 9% and 10%). After incubation at 30°C for 14 days, Actinomycetes growth and NaCl tolerance were noted [43].

Optimal growth temperature was evaluated by incubating the tested Actinomycetes strains at different temperatures (4°C, 15°C, 25°C, 30°C, 37°C, 44°C and 55°C). Growth was observed after 14 days of incubation [43].

The effect of pH on Actinomycetes isolates growth was assessed on solid SCA medium with different pH (pH = 3.3 – 5.3 – 7.3 – 9.3 – 12.3) using NaOH 1N and HCl 1N for adjusting pH. The cultures were incubated at 30°C for 7 days and the growth of the strains was estimated visually thereafter [44].

The carbohydrate utilization was tested on various carbon sources such as galactose, glucose, arabinose, sucrose, maltose, rhamnose, trehalose, dulcitol. Biochemical tests such as starch hydrolysis, casein hydrolysis, coagulation and peptonization of skimmed milk, presence of certain enzymes like citratase, catalase, urease, were also carried out for the tested Actinomycete isolates [44].

Production of melanoid pigments was tested on ISP6 and ISP7 media [41].

2.7. Antibiotic resistance of biocontrol agents

Antibiotics resistance of potent Actinomycetes strains was determined on 13 antibiotics provided by the Becton Dickinson France pharmaceutical laboratory. Sensitivity or resistance of each strain to the antibacterial drugs was determined according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [45]. Tested antibiotics were fosfomycin 50 µg (FOS 50), penicillin 6 µg (P 6), chloramphenicol 30 µg (C 30), gentamycin 500 µg (CN 500), erythromycin 15 µg (ERY 15), rifampicin 30 µg (RD 30), kanamycin 1000 µg (K 1000), fusidic acid 10 µg (FA 10), oxacillin 5 µg (OX 5), amoxicillin 25 µg (AMI 25), tetracycline 30 UI (TE 30), vancomycin 30 µg (VA 30) and nalidixic acid 30 µg (NA 30). Antibiotic-impregnated disks were put on Muëller-Hinton (MH) Agar plates previously inoculated with each tested Actinomycete. After 4 to 7 days of incubation at 30°C, inhibition zone were measured and interpreted as susceptible (S), Intermediate (I) or resistant (R) according to the CLSI [46, 47].

2.8. Statistical analysis

All tests were performed in triplicate and XLSTAT software was used for data analysis. Means comparison was conducted using ANOVA test ($P < 0.05$).

3. Results

3.1. Isolation of Actinomycetes

A total of 71 Actinomycete strains were obtained from potatoes rhizospheric soils of Vakinankaratra region using standard dilution technique on SCA Agar. Thirty seven (37) out of 71 (A1 to A37) were isolated from Androakavato and 34 (A38 to A71) from Manandona. The size of the colonies was variable (from 0.5 mm to 6 mm in diameter). Isolated Actinomycetes were represented by rough, opaque and powdery colonies, embedded in Agar. Substrate and aerial mycelia showed different colors (white, yellow, grey, pink and brown); two of them (A5 and A10) produced yellow diffusible pigment.

3.2. *In vitro* Antagonistic activity of isolated Actinomycetes against *Ralstonia solanacearum* species complex

Among the 71 screened isolates, 7 isolates (A5, A10, A17, A28, A29, A31 and A40) showed antagonistic activity against *Rssc*. They were more active against the phylotypes RI, RIII than the phylotype RII. The values of inhibition zone diameter fluctuated between 3 and 16 mm (table 1 and figure 1). The greatest inhibition zone (16 mm) was recorded with the isolate A5 on *Rssc* RIII using cross streak method. In contrary, the lowest inhibition zone (3 mm) was obtained with the isolate A28 on *Rssc* RII using agar cylinder method. From these results, it could be deduced that the antagonistic activity of tested Actinomycete isolates can be divided into five groups: no activity for inhibition zone (IZ≤ 2 mm), low activity (2 mm ≤IZ≤ 6 mm), moderate activity (6 mm ≤IZ≤ 10 mm), strong activity (10 mm ≤IZ≤ 14 mm) and very strong activity (IZ> 14 mm).

Table 1 Antagonistic activity of active Actinomycetes isolates against *Ralstonia solanacearum* species complex

	Inhibition zone (mm)					
	RI	**RII**	**RIII**	**RI**	**RII**	**RIII**
Isolates of Actinomycetes	**CA**	**CA**	**CA**	**Cs**	**Cs**	**Cs**
A5	14.5 ±0.1^{f,e}	10.5±0.2^{d}	15±0.0^{g}	15.4±0.1^{f}	11.7±0.2^{d,de}	16.0±0.0^{d,f}
A10	14.0±0.0^{e}	11.0±0.2^{e}	13.5±0.2^{f}	15.0±0.0^{e}	12±0.0^{d,e}	14.5±0.0^{cd,e,c}
A40	12.5±0.1^{d}	10.5±0.0^{d}	10.5±0.1^{d}	13.5±0.0^{d}	11.6±0.1^{d}	12.5±0.8^{b,c}
A17	9.5±0.1^{c}	7.0±0.1^{c}	12.5±0.0^{e}	10.5±0.1^{c}	8.0±0.2^{c}	13.5±0.3^{bc,d,c}
A29	5.2±0.2^{b}	3.5±0.1^{b,ab}	5.0±0.0^{a}	6.1±0.1^{b}	4.5±0.1b^{,ab}	6.0±0.2^{a}
A28	4.6±0.2^{a,ab}	3.0±0.0^{a}	6.0±0.1^{c}	5.5±0.1^{a}	4.5±0.1^{a}	7.0±0.0^{a,b}
A31	4.3±0.2^{a}	3.5±0.0^{b}	5.5±0.1^{b}	5.5±0.0^{a}	4.5±0.1^{b}	6.5±0.5^{a,ab}

The data in the same column followed by the same letter don't show significant difference according to Anova test ($P<0.05$).

CA: Cylinder Disk Agar technic; Cs: Cross streak culture technic; RI, RII and RIII are the phylotype of *Ralstonia solanacearum* species complex.

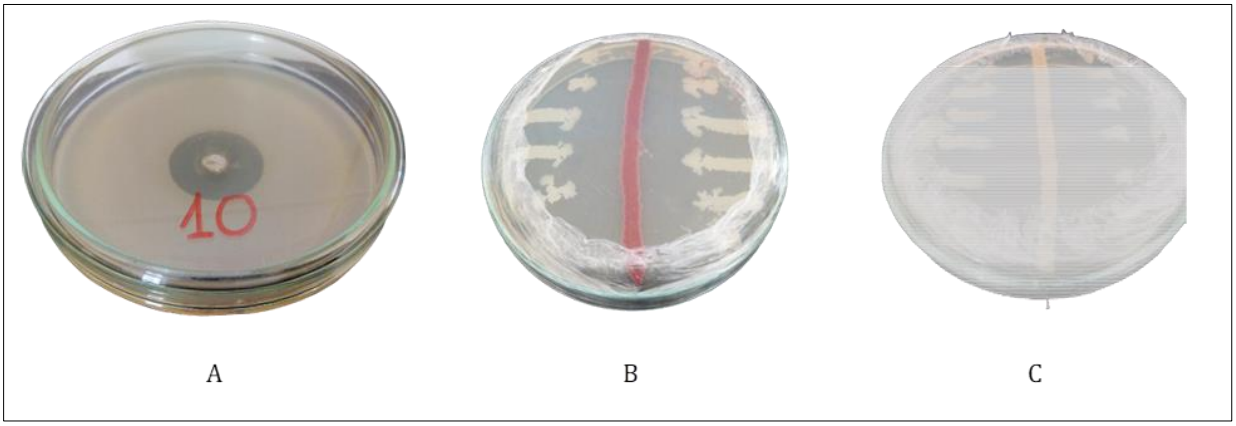

Figure 1 Inhibition of Rscc growth by Actinomycete strains: A) Inhibition of *Rscc* phylotype II growth by the strain A10 using agar disk method, B) Inhibition of *Rscc* phylotype I growth by the strain A28 using cross streak method, C) Inhibition of *Rscc* phylotype II growth by the strain A40 using cross streak method

3.3. Antagonistic mechanisms

It appears from the results obtained that 3 isolates (A10, A28 and A40) among the 7 active isolates tested used as antagonistic mechanisms against *Rssc*, the production of ammonia, IAA, HCN, hydrolytic enzymes as protease and cellulase and antibiosis (table 2). The isolate A40 was the most producer of IAA with a value of 0.14 µg/ml; all the three isolates solubilized phosphate.

Table 2 Action mechanisms of selected Actinomycetes

		Antagonistic agent		
		A10	**A28**	**A40**
Antagonistic mechanisms	Protease production	+++	+++	+++
	Cellulase production	+++	+++	+++
	HCN production	+++	++	+++
	Phosphate solubilization	+++	+++	+++
	IAA production (µg/ml)	0.13	0.11	0.14
	Ammonia production	+++	+++	+++

++ : Abundant +++ : Very abundant

For anti-*Ralstonia* substances production, ethanolic extracts prepared from these 3 isolates showed anti-*Ralstonia* activity expressed by the inhibition zone values from 27 to 34 mm (table 3). The highest inhibition was recorded with the isolate A40 and the most sensitive phylotypes were RI and RIII.

Table 3 Activity of Actinomycetes ethanolic extract against three phylotypes of *Rscc*

Extract of selected isolates	**Inhibition zone (mm)**		
	RI(b)	**RII(a)**	**RIII(b)**
EEA10(b)	32.0±0.0b	29±0.0b	33.0±0.0b
EEA28(a)	30.0.±0.5a	27.0±0.0a	30.0±0.8a
EEA40(c)	34.0±0.0c	31.0±0.0c	34.0±0.0c

The data in the same column followed by the same letter don't show significant difference according to Anova test ($P<0.05$)

RI, RIII and RII are the phylotype of *Ralstonia solanacearum* species complex

3.4. Chemical screening of Actinomycete extracts

Chemical screening of ethanolic extracts from the isolates A10, A28 and A40 revealed the presence of sterols, triterpens, tannins, leucoanthocyanes, flavonoids and alkaloids (table 4). Anthraquinones, polyphenols and polysaccharides compounds were not detected. However, it would be emphasized that the isolate A40 was notable for its abundant production of alkaloids, flavonoids, triterpenes, steroids and tannins. In addition, saponosides were detected in the ethanolic extract from the isolate A10.

Table 2 Chemical profile of Actinomycete extracts

		Observations	**Results**		
Chemical groups	**Tests**		**EEA10**	**EEA28**	**EEA40**
Alkaloids	Mayer	Precipitation	++	++	++
	Wagner	Precipitation	+	+	+
	Dragendorff	Precipitation	+	+	+
Flavonoids	Willstätter	Red purple coloration	+	+	++
	Willstätter modified	Red purple coloration	+	+	+
Leucoanthocyanes	Bate-Smith	Red purplish coloration	+	+	+
Triterpenes	Liebermann Burchard	Pink coloration	+	+	++
Steroids	Salkowski	Red partition ring	+	+	++
	Badjet-Kedde	Green coloration	+	-	+
Anthraquinones	Borntrager	Change of coloration	-	-	-
Saponosides	Height of the foam	At t=0 (h=5cm); at t=30min (h=2cm)	+	-	-
Polyphenols	Gelatin 1%	Precipitation	-	-	-
Tannins	Salted Gelatin	Formation of precipitate	+	+	++
	FeCl3	Black blue solution	+	+	++
Polysaccharides	-	Precipitation	-	-	-

-: Absence, +: Presence, ++: Abundance; EEA: Ethanolic Extract of Actinomycetes

3.5. Characterization and identification of potent Actinomycetes

Out of the 71 Actinomycete strains isolated from potatoes rhizospheric soils of Manandona and Androakavato, 3 strains (A10, A28 and A40) were selected for characterization and identification due to their strong antagonism and their notable antagonistic mechanisms against *Rssc*.

Macroscopic characteristics revealed that the three strains didn't produce melanoid pigments (figure 2). The isolate A10 formed round and small colonies, with 0.5 mm to 1 mm in diameter. They were characterized by a white pasty surface; with an irregular contour, and slightly concave on SCA medium. The spores cover all colonies giving a dusty appearance. The colonies adhere strongly to the culture medium while the spore mass detached easily. The isolate A10 produced a yellow diffusible pigment on SCA medium (figure 2).

The isolate A28 was characterized by the presence of radial furrows with an average size of 1 to 5 mm in diameter. This strain A28 developed a white spore mass while the back of the colonies is purplish red. The isolate A28 did not produce diffusible pigment (figure 2).

The size of the strain A40 colonies varied from 4 mm to 6 mm in diameter. The isolates were circular, with round contours and presented radial furrows. On SCA medium, the colonies formed a gray powdery surface. The other side of the colonies was yellow and the isolate did not produce diffusible pigment. The isolate A40 exposed brownish substrate mycelia (figure 2).

Figure 2 Macroscopic characteristics of Actinomycete isolates A10, A28, A40

Microscopic examination revealed that the three selected strains were Gram positive bacteria and immotile. The isolate A10 showed long spiral spore chains while the isolate A28 presented cocci spores. The isolate A40 was characterized by abundant aerial mycelium with rectiflexible spores (figure 3).

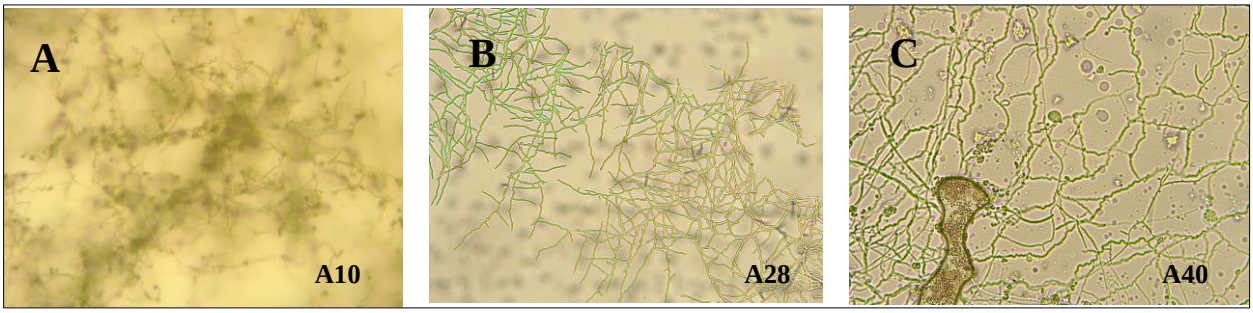

Figure 3 Spore chain arrangement observed at 100X using light microscope (LEICA) A) Isolate A10, B) Isolate A28, C) Isolate A40

Physiological characteristics showed that all the three strains had an optimum growth temperature of 30°C, an optimum pH of 7.34 and a tolerance of NaCl concentration from 1 to 3%. Therefore, they are mesophilic, neutrophilic and osmophilic bacteria.

Concerning biochemical characteristics, it was observed that the three selected Actinomycete strains didn't produce urease and didn't use indole. They were aerobe confirming by the presence of the enzyme catalase. They exhibited important glucidic and proteinic metabolisms due to the hydrolysis of casein and starch. Also, they coagulate milk; they fermented all tested carbohydrate sources. Physiological and biochemical characteristics of these three isolates are summarized in the tables 5 and 6.

Table 3 Physiological characterization of selected isolates

Characteristics	**Selected Isolates**		
	A10	**A28**	**A40**
Type of respiration	Aerobe	Aerobe	Aerobe
Motility	-	-	-
Melanoid pigments production			
ISP6	-	-	-
ISP7	-	-	-
Growth with Na Cl			
0%	+++	+++	+++

1%	+++	+++	+++
3%	+++	+++	++
5%	+++	+++	-
7%	+++	-	-
9%	++	-	-
10%	+	-	-
Growth at different temperatures			
4°C	-	-	-
15°C	+	+	+
25°C	+++	+++	+++
30°C	+++	+++	+++
37°C	++	++	++
44°C	-	-	-
55°C	-	-	-
Growth at different pH			
3.3	-	+	+
5.3	++	+++	+++
7.3	+++	+++	+++
9.3	+++	+++	+++
12.3	++	+	++

-: negative or no growth, +: positive or weak growth, ++: moderate growth, +++: abundant growth

Table 6 Biochemical characterization of selected isolates

Characteristics	**Selected Isolates**		
	A10	**A28**	**A40**
Starch hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Catalase	+	+	+
Urease	-	-	-
Citratase	+	+	+
Coagulation of skimmed milk	+++	+++	+++
Peptonization of skimmed milk	+++	+++	+++
Utilization of			
Indole	-	-	-
Glucose	+	+	+
Galactose	+	+	+
Saccharose	+	+	+
Arabinose	+	+	+

Maltose	+	+	+
Rhamnose	+	+	+
Trehalose	+	+	+
Dulcitol	+	+	+

-: negative or no growth, +: positive or weak growth, ++: moderate growth, +++: abundant growth

Compared to Actinomycete key features described in Bergey's Manual of Systematic Bacteriology [42, 48], it would be deduced from the phenotypic characterization that the three isolates A10, A28 and A40 belong to the genus *Streptomyces*.

The results of resistance test revealed that the three Actinomycete strains tested (A10, 128 and A40) were multidrug resistant because they showed resistance to more than two antibiotics tested (figure 4, table 7).

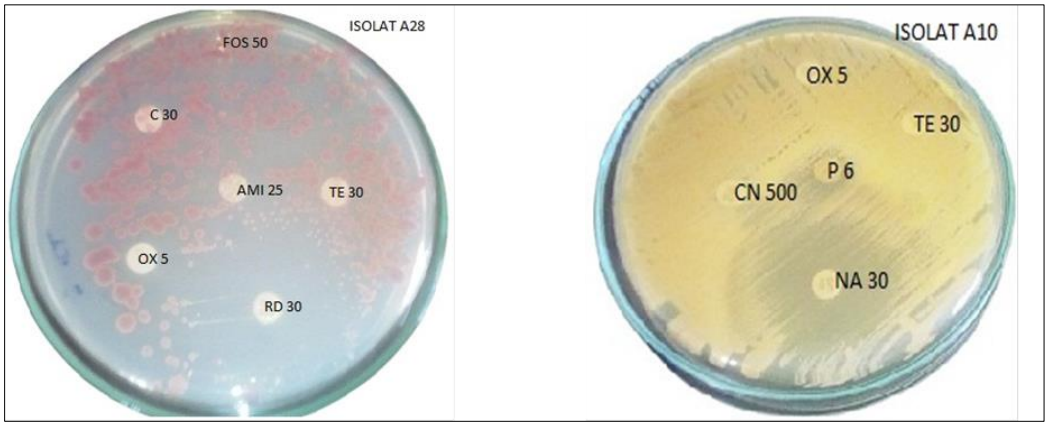

Figure 4 Resistance of the isolates A10 and A28 to reference antibiotics

Table 7 Resistance of biocontrol agents to reference antibiotics

	Reference antibiotics												
	FOS	**P**	**C**	**CN**	**ERY**	**RD**	**K**	**FA**	**OX**	**AMI**	**TE**	**VA**	**NA**
A10	R	I	R	R	R	I	I	I	R	R	R	I	S
A28	R	I	R	R	I	S	I	I	S	R	R	I	I
A40	R	I	R	R	R	R	I	I	R	R	R	I	R

R: Resistant I: Intermediate S: Sensitive

4. Discussion

Bacterial wilt caused by *Ralstonia solanacearum* species complex constitutes a huge problem for crops, particularly potatoes in Vakinankaratra region. Rhizospheric Actinomycetes present special interest as they have many properties including crops protection against *Rscc* and plant growth promoting potential [10, 15, 49, 50]. These findings encouraged us to explore rhizospheric Actinomycetes of potatoes in Vakinankaratra in order to test their ability to inhibit *Rscc* growth and to evaluate their antagonistic mechanisms.

Seventy one (71) Actinomycete strains were isolated from 17 rhizospheric soils samples of potatoes in Vakinankaratra region using a selective isolation method on Starch Casein Agar medium. Our results confirm those obtained by Intra *et al*. (2011), Srividya *et al*. (2012) and Mandal *et al*. (2017) [51, 34, 52] who reported that the rhizospheric soil is an important source of Actinomycetes.

The number of strains isolated could vary from one site to another, Rasolomampianina *et al.* (2016) [9] isolated 40 Actinomycetes strains from potatoes rhizospheric soils in Vakinankaratra region. This variation could be explained by the influence of different environmental factors (density of the soil microbial population, pH, physicochemical characteristics of the soil, vegetation, texture of the soil, availability of mineral and organic matter as well as root exudates) [53, 54, 55, 56]. Moreover, several authors reported that Actinomycetes prefer neutral environments rather than alkaline, and rhizospheric soils rather than non-rhizospheric soils [34, 52, 56, 57, 58, 59, 60, 61].

Antagonism of the isolated Actinomycetes was assessed using agar cylinder and cross- streak methods against the three phylotypes (RI, RII and RIII) of *Ralstonia solanacearum*. The cylinder agar technique is used for the screening of numerous biocontrol agents against one phytopathogen in a Petri plate while the cross-streak method is employed for testing antagonistic activity of one strain against several phytopathogens in a Petri plate. Seven (7) isolates (9.85%) out of the 71 Actinomycete strains isolated showed antagonistic activity on *Ralstonia solanacearum*, the isolates A5 and A10 exhibited a very strong activity against RI and RIII, respectively.

The two methods used presented interesting results and didn't show significant difference in terms of activity. In fact, it was reported in some research works that cross streak and agar cylinder methods are the best methods to evaluate antagonistic activity [62, 63, 64].

According to several anterior works, Actinomycetes displayed antagonism against phytopathogen bacteria and fungi [9, 10, 15] which concurred with our results. Moreover, our findings confirm certain studies which reported that rhizospheric soils constitute an important source of bioactive Actinomycetes against phytopathogen agents [10, 15]. Active isolates have different mechanisms to inhibit phytopathogens growth and their investigation proves necessary in order to understand Actinomycetes action through *Rssc*.

For IAA production, three (A10, A28 and A40) out of the seven active isolates produced IAA with a concentration varying from 0.11 to 0.14 µg/ml. In the literature, many works demonstrated the ability of rhizospheric soil *Streptomyces* to produce IAA and thus to promote plant growth [31]. Certain Actinomycete strains can produce up to 15.4 µg/ml of IAA [65].

Ammonia and hydrogen cyanide are among the compounds involved both in the inhibition of *Ralstonia solanacearum* growth and plant growth promoting [31]. In this study, the same IAA producing isolates produced also these two compounds which are very toxic for *Ralstonia solanacearum* and play an important role in disease suppression [66]. As an example, *Streptomyces* sp. LSW05 strain was demonstrated as a potent HCN producer [67].

Regarding phosphate solubilization, the isolates A10, A28 and A40 solubilized phosphate. Indeed, this mechanism is crucial in the nutrient absorption in the agriculture [68].

About hydrolytic enzymes production, a correlation between hydrolytic enzymes and bioactive compounds production was reported in some research works [68, 69]. Microorganisms, which secrete a complex of hydrolytic enzymes, are considered as potent biological control agents to combat plant diseases extracellular enzymes, such as amylase, cellulase and protease, play fundamental roles in the degradation of complex polymers, making nutrients available to plants and contributing to the suppression of phytopathogens [68, 69, 70]. In this study, the three isolates cited above also produced hydrolytic enzymes (amylase, cellulase and protease).

Protease is recognized to facilitate the penetration and the elimination of phytopathogenic agents by degrading the phytopatogenic physical barriers and their defense mechanisms [69, 70]. About amylase and cellulase, they digest starch and amylose available in soil, bacterial lytic enzymes, such as chitinase, b-1,3- glucanase, catalase, cellulase, and proteases, break down polymeric compounds like chitin, glucan, cellulose, proteins, DNA, and hemicellulose which are the main compounds in the cell wall structure of the phytopathogens [69, 71]. In this study, the isolates showed different enzymatic and secondary metabolites production profiles. This functional diversity can be exploited for the development of microbial inocula with multiple enzymatic activities, for promoting plant growth and protection against phytopathogens.

Concerning antibiosis, ethanolic extracts prepared from the three Actinomycetes tested showed antibacterial activity against *Ralstonia solanacearum* species complex. According to Tekwu *et al*'s criteria, the 3 phylotypes of *Ralstonia solanacearum* were extremely sensitive to the ethanolic extract Actinomycete. Chemical screening of the ethanolic extracts revealed that they contained anti-*Ralstonia* compounds such as sterols, triterpens, tannins, leucoanthocyanes, flavonoids and alcaloïdes. They were demonstrated to possess important biological potentials against phytopathogens in several studies [15, 72, 73, 74].

For antibiotic resistance test, the isolates A10, A28 and A40 exhibited resistance to OX 5, TE 30, FOS 50, C 30 and CN 500. Multiresistance of Actinomycetes to usual antibiotics drugs was demonstrated in several works [46, 47]. It can be explained by the production of enzymes capable to inactive or to modify the antibiotic, the modification of target site located on the cell membrane of the bacteria or the development of other metabolic pathways [75]. These properties are acquired when the microorganisms undergo genetic changes which can occur by mutation or by acquisition of new genetic material [76].

Phenotypic characterization of the three interesting strains underlined that they belong to the genus *Streptomyces*. Their cultural characterization showed typical colonies of *Streptomyces* (opaque, rough, non-spreading morphology and are usually embedded resulting in adherence to agar medium) as described in Bergey's Manual of Determinative Bacteriology [77]. Morphological examination, comparison of physiological and biochemical features with those cited in Bergey's Manual of Determinative Bacteriology affirmed that they were *Streptomyces* [48].

5. Conclusion

Rhizospheric *Streptomyces* have been demonstrated in several works for a long time as interesting sources of antimicrobial compounds including anti-phytopathogens. It is evident from the present study that the Actinomycete isolates A10, A28 and A40 were able to produce plant growth promoting substances and inhibit phytopathogens growth through different antagonistic mechanisms. Hence, their practical use as biopesticides to control plant diseases is convincing.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interests.

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