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Characterization of fluorescent Rhizobacteria isolated from Oleander (*Nerium oleander* L.) and Olivier (*Olea oleaster* L.) with antagonistic effect against *Fusarium oxysporum* f.sp. *radicis-lycopersici*

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Abstract

This study aims to characterize the biocontrol potential of two fluorescent rhizobacteria isolated from Oleander (*Nerium oleander* L.) and Olivier (*Olea oleaster* L.). The isolation was done in Cetrimide agar. The fluorescing strains at 366 nm were selected for the dual plate assay against the tomato pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici.* The two best strains showing the highest inhibition rates (Pa2 and S2Pf1 with 31.36% and 51.42% respectively) were chosen to be identified and characterized for their production of antifungal molecules. The taxonomical determination based on macro and micromorphological observations in addition to the biochemical tests of the API 20E and API 20NE strips analyzed by the heuristic soft "PIBwin 2.0.0" suggests the strain Pa2 as a *Pseudomonas aeruginosa* while the strain S2Pf1 belongs to the *Burkholderia cepacia* species. The antifungal potential was assessed through the quantification/detection of the following bioactive molecules: salicylic acid, hydrogen cyanide, rhamnolipids, chitinases, and cellulases. The strain *Burkholderia cepacia* S2Pf1 showed a better antifungal profile in comparison to *Pseudomonas aeruginosa* Pa2. The determination of the growth kinetics parameters of *Burkholderia cepacia* S2Pf1 based on observed OD600 data, DModel data fitting, and Gompertz model data fitting highlighted a relatively good ability to quickly reach high bacterial densities in both nutrient and seed broths. Thus, *Burkoledria cepacia* S2Pf1 is suggested as a good candidate to be further assessed for its biocontrol proprieties against *Fusarium oxysporum* f.sp. *radicis-lycopersici*.

Keywords: Rhizobacteria; *Fusarium oxysporum* f.sp. *radicis-lycopersici; Burkoledria cepacia*; *Pseudomonas aeruginosa*; biocontrol.

1. Introduction

Cultivated tomato (*Lycopersicon esculentum* Mill.) is one of the world's most important crops due to the high value of its fruits both for fresh market consumption and in numerous types of processed products [1]. One of the main constraints to tomato cultivation is damage caused by pathogens [2,3], such as fungal phytopathogens, especially in tropical and subtropical regions [4], *Fusarium oxysporum* is major soil-borne fungal pathogens of both greenhouse and field-grown tomatoes in the warm vegetable growing areas of the world [5]. Root and crown rot caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) is one of the most damaging soil-borne diseases in tomato seedlings and increasingly common in the production of greenhouse tomatoes. The disease manifests itself both in the greenhouse and in the tomato fields, causing significant losses in tomato production [6]. Crown rot develops primarily in cool climates in both field and greenhouse tomatoes. Substantial crop losses in infected fields have given the disease international attention [7].

A seed treatment with carbendazim could provide a protective barrier against infection by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. A sprinkling of the same compound or thiophanate-methyl achieves the same desired effect. In general, it is recommended the use of pre-plant fumigant, noting, however, that the

effectiveness of fungicides in the fight against this pathogen is poor [8]. Furthermore, fungicides are expensive, can cause environmental pollution, and may cause the selection of pathogen resistance [9]. Owing to the limitations of chemical fungicides, it seems appropriate to seek a supplemental control strategy [10, 11]. Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest management systems to reduce our dependence on chemical pesticides [12].

Biological and cultural controls are two alternatives to synthetic chemical fungicides in integrated disease management, which aimed at maximum productivity with the least possible negative environmental and ecological consequences [13]. Another aspect of biocontrol against *Fusarium oxysporum* f.sp. *radicis-lycopersici* is the use of microbial auxiliaries. Hence, strategies aiming at the replacement of chemical pesticides by hazardous free biological agents can be a reasonably good choice. Numerous studies have demonstrated a reduced incidence of diseases in different crops after supplementing the soils with fungal or bacterial antagonists [14]. Moreover, Saidi *et al*. tested Bacillus isolates in dual culture, and under greenhouse conditions against FORL race 0, after what, 10 isolates exhibited good inhibition of the plant pathogen [15]. Another illustration, Kamilova *et al*. tested *Pseudomonas fluorescens* WCS365 on the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* in a gnotobiotic system [16]. The seed inoculation with *P*. *fluorescens* WCS365 decreased the percentage of diseased plants from 96 to 7%. Thus, globally, recent biochemical research on plant disease control focused on two prime objectives. They were to (i) select and identify microorganisms with antifungal activities, isolate and characterize the specific antifungal factors within these microorganisms; (ii) determine the operative mechanisms of these antifungal agents [17].

This work aims to assess *in vitro* the inhibiting effects of antagonistic fluorescent rhizobacteria against *Fusarium oxysporum* f.sp. *radici-lycopersici,* and to characterize some of their potential molecular mechanisms by highlighting the production of rhamnolipids, chitinase, cellulase, HCN, and salicylic acid (SA), in addition to the growth kinetics parameters, giving a partial vision of a complex machinery of inhibition.

2. Materials and methods

2.1. Pathogen

To select the best candidates, the antifungal activity was evaluated against the plant pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Agent of Fusarium root and crown rot of tomato). The strain *Forl 12* (isolated from a diseased tomato) was used for the antagonism test.

2.2. Isolation of fluorescent rhizobacteria

About 10 g of soil were taken from a depth of 10 cm, from various Rhizospheric origins: spontaneous oleander (*Nerium oleander* L.), spontaneous Olivier (*Olea oleaster* L.), in sterile containers, resulting in a total of 5 samples to be used for isolation (1 g per sample) (Table 1).

For the isolation process, 01 g is taken to achieve an enrichment for 24 h in nutrient broth (5.0 g pepton, beef extract 3.0 g/1000 mL distilled water) [18] with 01 g of soil in 9 mL of nutrient broth at 30°C and 42°C separately. Following enrichment, isolation was conducted on Cetrimide agar [Pancreatic digest of gelatin 20 g, magnesium chloride 1.4 g, potassium sulfate 10 g, glycerol 10 mL, Cetrimide 0.3 g, agar 13.6 g/1000 mL distilled water] [19] at 30°C for 24h.

Bacterial candidates obtained at the end of the isolation were purified on King B agar (Proteose peptone N°3 20 g, anhydrous $K_2HPO_4$1.5g, $MgSO_4 \cdot 7H_2O$ 1.5g, + Glycerol 15 mL, Agar 20 g/1000 mL distilled water) [20].

Table 1
Codification, sampling location, Rhizospheric origin, and soil samples description

Sampling location	Rhizosphere origin	Number of samples	Sample codification
Mascara, Slatna, Sidi Ben Arbiya, near a creek with abundant spring waters	Spontaneous pink laurel	1	S1
Saida, "Vieux de Saida" Park, on a Muddy slope	Spontaneous Olivier	3	S2
			S3
			S4
Saida, Parc du "Vieux de Saida", next to a creek	Spontaneous pink laurel	1	S5

2.3. *In vitro* screening for antifungal activity against FORL (Dual Plate Assay)

To select the best performing candidates, an antifungal activity test was undertaken against the plant pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* on PDA [Potato infusion 200 g, dextrose 20 g, agar 20 g/1000 mL distilled water] by the "Dual Plate Assay" method. A fragment of fungal mycelium (6 mm Ø) from a 7 days PDA culture is placed at the center of a new Petri dish, then 5 µL of bacterial suspension obtained by culturing the isolates to be tested in King B broth is set to 1 cm from the edge of the plate, all incubated at 25°C for 7 days. A control plate is incubated with the fungal inoculum alone, and the test is repeated three times. The antifungal potential of the tested strains is expressed by "inhibition rate" calculated as follows:

$$\text{Inhibition (\%)} = \frac{x-y}{x} \times 100,$$

where:
x: control mycelium diameter in mm,
y: diameter of mycelium in the presence of the bacterial strain, measured on the axis "bacterial colony-fungus", in mm).

The results are expressed as percentage ± standard deviation [21]. Bacterial strains selected for the characterization of the antifungal potential were preserved by freezing at-20°C on stock cultures (nutrient broth with 20% glycerol) [22].

2.4. Phenotypical identification

Following the antifungal activity test, two candidates were selected as those that gave the largest percentages of fungal inhibition, to characterize some mechanisms implied in the antifungal potential. Phenotypic identification was based on the following characteristics: micro and macromorphology of bacterial colonies on various culture media: King A (Proteosepeptone 20.0 g, 15.0 g agar, Glycerol 10.0 g, 10.0 g K_2SO_4, 3.5 g $MgCl_2 \cdot 6H_2O$ + 20 g Agar/ distilled water 1000 mL), King B, Cetrimide (macroscopic examination), microscopic observation after fixation (Gram stain), respiratory type, catalase test and study of the biochemical profile on API 20E bioMerieux[1] systems (Enterobacteriaceae and other non-fastidious Gram (-) bacilli Identification Kit for strains suspected to be *P. aeruginosa* and 20NE bioMerieux system for the other strains [not fastidious non-enteric Gram (-) bacilli Identification Kit (bioMérieux, France; ref 20050)]. Inoculation and reading were done according to the manufacturer's guidelines. Interpretation of the results was performed by a computer-based heuristic approach, using the program PIBwin 2.0.0, with identification matrices for API 20E and 20NE systems developed from the user guides provided by the company bioMérieux, France. After selecting the identification matrix and filling properly the results, results are displayed with a heuristic interpretation of the biochemical profiles, with the most likely taxon, its identification score, followed by the other suspected taxa, by score order [23].

2.5. Characterization of antifungal activity

2.5.1. Production of salicylic acid

For evaluation of salicylic acid (SA) production, the strains were grown in glucose urea broth "GUM" (Anhydrous glucose 10 g, urea 0.85 g, K_2HPO_4 0.56 g/1000 mL distilled water) [24] for 24 hours with stirring at 100 rpm at 30°C. Thereafter, 100 µL of this culture was transferred to 100 mL of GUM broth for each strain for incubation of 36 h in the same conditions. Then the salicylic acid was extracted from the culture medium by ethyl acetate. The extract thus obtained was concentrated (1:3) under vacuum. SA concentration was

[1] bioMérieux, France; réf. 20100. https://www.biomerieux.fr/

determined by adding 5µL of $FeCl_3$ 2M and 3 mL of water for 1 mL of the concentrated extract. The absorbance of the purple complex of iron-SA, which was developed in the aqueous phase, was measured at 527 nm and compared to a standard curve of SA dissolved in ethyl acetate [25].

2.5.2. Hydrogen cyanide assay

The six selected strains were grown in Luria Bertani "LB" (Tryptone10.0 g, yeast extract 5.0 g, NaCl 10.0 g, Agar 15.0g/distilled water 1000 mL) [26,27] at 30°C in 250 mL Erlenmeyer flasks each containing 100 mL of culture medium and alkali picrates bands, with stirring at 100 rpm/min. The bands are used to reveal the production of hydrocyanic acid, prepared according to the following way: Whatman Filter paper n°1 was cut into uniform bands of 10 cm long and 0.5 cm wide, each of the bands was dipped in a 0.5% picric acid solution, and then again in another solution of Na_2CO_3. Once dried in a hot dry and sterile environment, they are put inside the flasks in a suspended position without touching the edges and the surface of the culture medium. It is considered that a strain produces HCN if after 2-3 days the yellow original color of alkaline picrate band veers toward the orange-red (rusty color) [28,24].

2.5.3. Rhamnolipids assay

Production of rhamnolipids type surfactants, with antifungal potential, was quantified by the colorimetric assay method of sugars by orcinol [29]. A culture on GUM was conducted with 100 mL of culture medium in 250 mL Erlenmeyer flasks for one week at ambient temperature (about 30°C, with agitation at 100 rpm). Firstly, rhamnolipids were purified by separating the cells from the supernatant by centrifuging a sample of 1 mL of each bacterial culture in 1.5 mL Eppendorf (at 10 000 G for 10 min). The supernatant was then extracted with chloroform and ethanol. Thereafter, a 0.5 mL sample was extracted with 1 mL of chloroform-ethanol (2:1, v/v). After evaporation to dryness of the organic phase, 0.5 mL of distilled H_2O was added. To 0.1 mL of each sample, 0.9 mL of a solution containing 0.19 % orcinol (diluted in 53% sulfuric acid H_2SO_4) was added. After heating for 30 min at 80°C the samples were cooled to ambient temperature, after what the OD_{421nm} was measured by a spectrophotometer, using as a blank a sample from virgin culture medium having undergone the same steps mentioned above. The reading was performed three times; the concentration of rhamnolipids was calculated from a standard curve prepared with the L-rhamnose (0 to 50 $mg.L^{-1}$) and expressed in equivalent rhamnose [30, 31].

2.5.4. Chitinase test

This test was carried out on the "chitin-agar-water" medium, supplemented with Bromocresol blue (0.02 $g.L^{-1}$) serving as a pH indicator [Colloidal Chitin 4.34 mL, Agar 16g, Blue Bromocresol 0.02g/distilled water 1000 mL]. Once regenerated, wells (ø=5 mm) are made with a sterilized glass cylinder in the middle of the Petri plate. Then 100 µL of 48 h bacterial culture on King B broth of each bacterium are placed in the central well, followed by incubation at 30°C for 4 days. Bacteria having chitinolytic activity develop a clear yellow halo around the wells, due to the color change of bromocresol blue following acidification of the medium. The results are expressed as follows :(+++) for halos exceeding 50 mm, (++) for halos with diameters between 20 mm and 50 mm, (+) to the diameters that do not exceed 20 mm. The test was performed in triplicate [32].

2.5.5. Cellulase test

Cellulase production was assessed in "Cellulose medium" (K_2HPO_4 1.0 g, $NaNO_3$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g/distilled water 1000 mL) [33]. For each strain, a piece of paper (9 cm×1 cm) was placed in a tube containing 9 mL of the cellulose solution after the inoculation of each strain with a solid platinum loop from a brain-heart broth pre-culture (BHIB). Incubation was done at 25°C for 3 weeks with a control (virgin medium + cellulose paper). The reading of the results was done initially by comparing the turbidity of each tube with that of the control tube by observing with the naked eye the tubes placed in front of a paper background with horizontal lines of red color (2 mm spaced) made with a fine point marker pen to accentuate the contrast (tubes showing a cellulose degradation become cloudy as a result of microbial growth, contrasting the lines more or less sharp, according to turbidity). For a comparison between the strains tested, 1 mL was taken from each

tube on completion of the test for reading the absorbance at 600 nm, the turbidity of the medium is proportional to the bacterial biomass from the degradation of the cellulose. The sterilized medium was used as a witness to set the zero of the spectrophotometer. Reading was repeated three times for each strain [33].

2.6. Growth kinetics parameters

A pre-culture of the best strain was performed on nutrient broth (NB) and seed broth (SB) (20 g D-Glucose monohydrat, 10 g casein pepton, 10 g yeast extract, 10 g NaCl/1000 mL distilled water) [34], then incubated at 30°C for 24 h. A loop fool of the culture is used to inoculate an Erlenmeyer flask containing 100 mL of nutrient broth and another one containing 100 mL of seed broth, each flask is incubated at room temperature with shaking at 75 rpm. Microbial growth was studied as a function of culture time for 24 h by measuring the culture medium absorbance, using a 600 nm UV spectrophotometer [35].

The "Gompertz model" and "Dmodel" were selected for the estimations of the growth kinetics parameters, using the curve-fitting program DMfit[2]. Bacterial growth curves parameters were calculated from the experimental data using the Gompertz equation [36, 37] as:

$$L(t) = A + C \exp\{-\exp[-B(t - M)]\}$$

where:

L(t) = the log of cell concentration ($CFU.mL^{-1}$) at time t (in h),

A = the asymptotic log counts as t decreases indefinitely (referred also as the predicted maximum density YEnd)

C = the asymptotic amount of growth (log number) that occurs as t increases indefinitely,

M = the time (in h) at which the absolute growth rate is maximum,

B = the relative growth at M

Then, the Gompertz equation parameters (A, B, C, M) are subsequently used to calculate:

- Lag phase duration (h) = M - (1/B),
- Generation time (h) = (log 2e)/BC,
- Exponential growth rate [(log CFU/mL)/h] = BC/e,
- Maximum population density (log CFU/mL) = A + C

For the Dmodel, the explicit form of the model is the following [38]:

$$y(t) = y_0 + \mu_{max} t + \frac{1}{\mu_{max}} \ln(e^{-v.t} + e^{-h_0} - e^{-v.t-h_0}) - \frac{1}{m} \ln\left(1 + \frac{e^{m\mu_{max} t + \frac{1}{\mu_{max}} \ln(e^{-v.t} + e^{-h_0} - e^{-v.t-h_0})} - 1}{e^{m(y_{max} - y_0)}}\right)$$

where:

- $y(t)$=ln ($x(t)$) with $x(t)$ the cell concentration (CFU/mL);
- y_0=ln (x_0), y_{max}= ln (x_{max}), x_0 being the initial and x_{max} the asymptotic cell concentration, respectively;
- μ_{max} is the maximum specific growth rate (1/h);
- m is a curvature parameter to characterize the transition from the exponential phase;
- v is a curvature parameter to characterize the transition to the exponential phase;
- h_0 is a dimensionless parameter quantifying the initial physiological state of the cells. From that, the lag time λ (h) can be calculated as h_0/μ_{max}.

As optical density (OD) data can be used to obtain the specific growth rate and if used in conjunction with the known initial inocula, the maximum population data and knowledge of the microbial number at a predefined ODat a known time then all the information required for the reconstruction of a standard growth curve can be obtained [39]. Thus, instead of using the bacterial concentration, the curves are expressed in terms of OD600.

For both models (Gompertz and Dmodel) the DMFit program was used to estimates the 4 main parameters:

- Growth rate μmax (OD.min-1), as the primary parameter, called 'rate', is the potential maximum rate of the model;
- Generation time td (min);
- Lag phase λ (min) denotes the lag parameter as described by Baranyi and Roberts (1994) [38];
- Predicted maximum density YEnd (OD600) is the upper asymptote of the sigmoid curve (or lower asymptote if the curve is decreasing). The DMFit program calculates also adjusted R-square statistics of the fittings.

[2] http://www.ifr.ac.uk/safety/DMfit/

2.7. Statistical tests

Statistical analyses were performed using Student's *t*-test. All differences reported in the text were significant at the 5% level of probability.

3. Results and discussion

3.1. Isolation and screening

Isolation of aerobic Gram (-) bacilli using selective agar "Cetrimide" led to numeral colonies, upon what 12 bacterial isolates were selected upon their different morphotypes, namely: S2R2, S2R3, S3R1, S4Pf1, S5Pf1, S5Pf2, S6Pf1, S2Pf3, S3R2, S3R3, Pa2, and S2Pf1. These isolates displayed quite different colony colors and shapes.

3.2. Dual plate assay

This test has served to assess the antifungal potential of the selected strains isolates. It led to reveal isolates incapable of inducing inhibition/or limiting the growth of the pathogen, while other isolates could poorly inhibit the fungus. However, a remarkable inhibition activity was observed on 2 strains (Figure 1). Furthermore, the inhibitory capacity of the bacteria in contact with the pathogen changes its appearance (Table 2). On the other hand, inhibition rates are varying from 0% to 51.42%.

The Pa2 and S2Pf1 strains displayed the highest rate of inhibition compared to the other isolates, with respective values of 31.36% and 51.42%. These two strains were selected for the characterization of their antifungal potential, suggesting that strains from various rhizospheres produce different levels of antifungal products [25].It is established that a relatively larger zone of inhibition translates the synthesis of relatively powerful antibiotic(s) [40]. Lee *et al.* were able to isolate PGPR strains, *Pseudomonas* spp. having been considered as having a good antifungal potential with inhibition rates ranging from 0 to 45%, depending on the target pathogen and the tested media [21]. Furthermore, Mikani *et al.* worked also on 10 *Pseudomonas fluorescens* for their antagonistic activity against *Botrytis mali* with inhibition of mycelial growth of 59.8% for the most efficient strain [41]. The difference between the two selected candidates could be related to their different Rhizospheric origins (S2Pf1: *Nerium oleander* L and Pa2: *Olea oleaster* L). It is interesting to draw attention to the fact that bacteria isolated from spontaneous plant rhizosphere, unlike nursery plants and other "nurseries" would have a greater ability to inhibit plant pathogens [42], this being considered, the selection of samples of this work is precisely justified.

At present, even though there is a myriad of antifungal activity characterization studies on microbial agents, there is no consensus technique for this purpose [43]. Furthermore, antagonism tests may be conducted by concentrating the bacterial culture supernatant, which generally, once incorporated into the agar, result in inhibition rates higher than those observed by testing directly an inoculum [40].

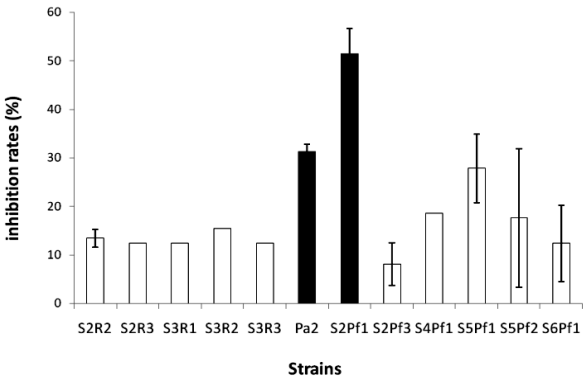

Figure 1: Results of Dual Plate Assay against *Fusarium oxysporum* f.sp. *radicis-lycopersici.* Values are expressed in terms of means of inhibition rates ± SD

3.3. Identification by conventional tests and biochemical profiles (API 20E and 20NE)

After completion of some conventional tests (Table 4) and after inoculation of API bioMerieux's system strips and incubation according to the manufacturer's instructions, results of the API 20E and AP 20NE strips (Tables 4 and 5) were recorded following the "reading result table" provided with the bioMerieux kits. The results were noted (+) for a positive test and (-) for a negative test, to serve later for a heuristic interpretation by a software (program PIBwin 2.0.0).

Table 2
Effect of bacterial isolates tested on the fungal mycelium

Strains	Description
S2R2	Altered mycelium (cotton-like form + creamy form)
S2R3	Altered mycelium (cotton-like form + creamy form) with fungal colonies growing on bacterial colonies
S3R1	Altered mycelium (cotton-like form + creamy form) with fungal colonies growing on bacterial colonies
S3R2	Altered mycelium (cotton-like form + creamy form)
S3R3	Altered mycelium (cotton-like form + creamy form) with fungal colonies growing on bacterial colonies
Pa2	Fungal colonies affected on the side of the bacterial development.
S2Pf1	Scarlet-red center, very weak growth, thin white edges
S2Pf3	The appearance of vivid purple color in the center of the fungal colony, white mycelium quantitatively affected
S4Pf1	The appearance of vivid purple color in the center of the fungal colony,
S5Pf1	The apparition of a relatively less vivid purple color in the center of the fungal colony,
S5Pf2	White mycelium relatively denser, quantitatively affected
S6Pf1	Less dense white mycelium in the edges (yellow), affected quantitatively.

Table 3
Description of bacterial colonies on various culture media

Strains	Media	Characters				
		Color	Size	Opacity	Diffusion	Growth
Pa_2	King B Agar	brownish green	small	Average	important	Important
	King A Agar	fluorescent green	Small	Average	important	Important
	Cetrimide Agar	fluorescent green	small	Average	important	Important
S_2Pf_1	King B Agar	Yellow	very small	weak	weak	Important
	King A Agar	Yellow	small	Average	weak	Important
	Cetrimide Agar	brownish-yellow	small	Opaque	weak	Important

After developing identification matrix as spreadsheets on Microsoft "Excel" for each of the two API 20E and API 20NE systems from identification tables (with the probability that a test is positive) provided by the manufacturer, the results were filled from the biochemical profiles of each strain according to Bryant [23]. The results of the heuristic interpretation are as follows:

- **S2Pf1**

This strain was identified as *Burkholderia cepacia* with an identification score (score ID) equal to 0.99944. The following tests gave unexpected results for this taxon: NO_3 (a positive result, while the probability for it to be positive is 39%), GLU (positive result, while the probability for it to be positive is 24%), ADH (positive result, while the probability for it to be positive is 1%) URE (positive result, while the probability for it to be positive is 1%), PNPG (negative, while the probability for it to be positive is 72%) ARAa (negative result, while the probability for it to be positive is 75%), ADIa (negative result, while the probability for it to be positive is 93%) (Figure 2).

Table 4
Conventional and biochemical (API 20NE) tests for strain S2Pf1.

Tests		Results
Gram		-
Fluorescence (256 nm)		-
Fluorescence (366 nm)		+
Catalase		+
Respiratory essay		ctly aerobic
reduction of nitrates to nitrites		+
Presence of	β-galactosidase	-
	β-glucosidase	-
	L-Arginine dihydrolase	+
	Urease	+
	Gelatinase	+
Production of Indole		-
Use of	Phenylacetic acid	+
	Trisodium citrate	+
	Malic acid	+
	Adipic acid	-
	Capric acid	+
	Potassium gluconate	+
	D-maltose	-
	N-acetyl-glucosamine	+
	D-Mannose	+
	D-Glucose	+
	D-Mannitol	+
	L-Arabinose	-

- **Pa2**

This strain was identified as a *Pseudomonas aeruginosa* with an identification score (score ID) equal to 0.99990 (maximal score reached). The obtained results match exactly those of the taxon in the identification matrix (Figure 3).

Cultural characters such as pigmentation; consistency of colonies; size ..., may also provide valuable clues for identification. For example, a mobile organism, gram-negative which produces a fluorescent pigment soluble in water is likely to be a *Pseudomonas* species, whereas those forming bioluminescent colonies are likely to belong to the Vibrionaceae family.

Some species belonging to the rRNA group II (*Burkholderia cepacia*, *B. gladioli*, *B. caryophylli*), produce yellow-green diffusible pigments that are sometimes confused with fluorescent siderophores. The "A" medium of King *et al.* (1954) [20] is generally recommended for the production of phenazines pigments to which belongs "pyocyanin" [44].

Table 5
Conventional and biochemical (API 20 E) tests for strain Pa2.

Tests		Results
Gram		-
Fluorescence (256 nm)		+
Fluorescence (366 nm)		+
Catalase		+
Respiratory essay		Strictly aerobic
Presence of	Galactosidase	-
	Arginine dihydrolase	+
	Lysine decarboxylase	-
	Ornithine decarboxylase	-
	Urease	-
	Gelatinase	+
	Tryptophan deaminase	-
Production of	H_2S	-
	Indole	-
	Acetoin	-
Use of	Citrate	+
	D-Glucose	+
	D-Mannitol	-
	Inositol	-
	D-Sorbitol	-
	L-Rhamnose	-
	D-Sucrose	-
	D-Melibiose	-
	Amygdalin	-
	L-Arabinose	-

Given the widespread use and proven accuracy of API systems (supplemented by additional biochemical tests) for the identification of Enterobacteriaceae and other non-enteric Gram-negative bacilli [45] (including *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*), thus it was retained as "tool of choice" for the phenotypical identification of selected isolates. The computer-assisted analysis is undoubtedly capable of improving the identification process, or rather "the interpretation of results", this, based on identification algorithms, in the case of species with similar phenotypic properties [46]. Such programs are based on heuristic interpretation of the identification tests, each test being encoded "+" or "-", it then compares the profile of the unknown strain to those of a given database [46]. The use of API strips combined with computer-based interpretation led to identifying the selected candidates as following:

- S2Pf1: *Burkholderia cepacia*, with an ID Score = 0.99944 (poor "match").
- Pa2: *Pseudomonas aeruginosa*, with an ID Score = 0.99990 (very good "match", 0.999 threshold reached).

Fluorescent Pseudomonads are ubiquitous bacteria and common inhabitants of the rhizosphere and are the most studied group in the genus *Pseudomonas* [33]. This group is of great importance in biotechnology due to the ability of several strains to degrade xenobiotics, their use in biological control against the plant pathogens, and their pathogenicity in humans [47]. In the 1960s, a project focused on the construction of a rational system of classification of *Pseudomonas* species was organized in the bacteriology department at the University of California, Berkeley. This project had its main justification in the very unsatisfactory situation of the taxonomy of this genus into which was affected several hundred species names, many of which could not be identified based on published descriptions, and, in addition to this, their "type" strains were lost [44].

Pa2 was identified as *Pseudomonas aeruginosa*. Because of its clinical and environmental relevance, *P. aeruginosa* remains a subject of intense research and one thing is for sure, this remarkably versatile organism will continue to surprise us in the future [46].

S2Pf1 was identified as *Burkholderia cepacia*. Examples of similarities show that a strong differentiation of members of *Burkholderia* and *Pseudomonas* is difficult. Studies on DNA-DNA hybridization and DNA-rRNA hybridization showed that species of the genus are members of "RNA similarities Group" II. The species is considered as a serious human opportunistic pathogen associated with nosocomial infections.

A striking feature of some *Burkholderia* strains is the production of various antifungal compounds (as evidenced by the results of the *in vitro* antifungal activity of the present work) that can be used for the management of the fungal disease [48]. *B. cepacia* has already been a subject of commercial use as a biological control agent for certain plant-parasitic nematodes and has already been used in the protection of tomato [49]. However, its use for the management of fungal diseases was reviewed, as it was proven to be an opportunistic pathogen associated with human cystic fibrosis disease. Understanding the molecular mechanisms of antifungal activity of *Burkholderia* strains will give clues to the development of biological fungicides while eliminating potential health risks [48].

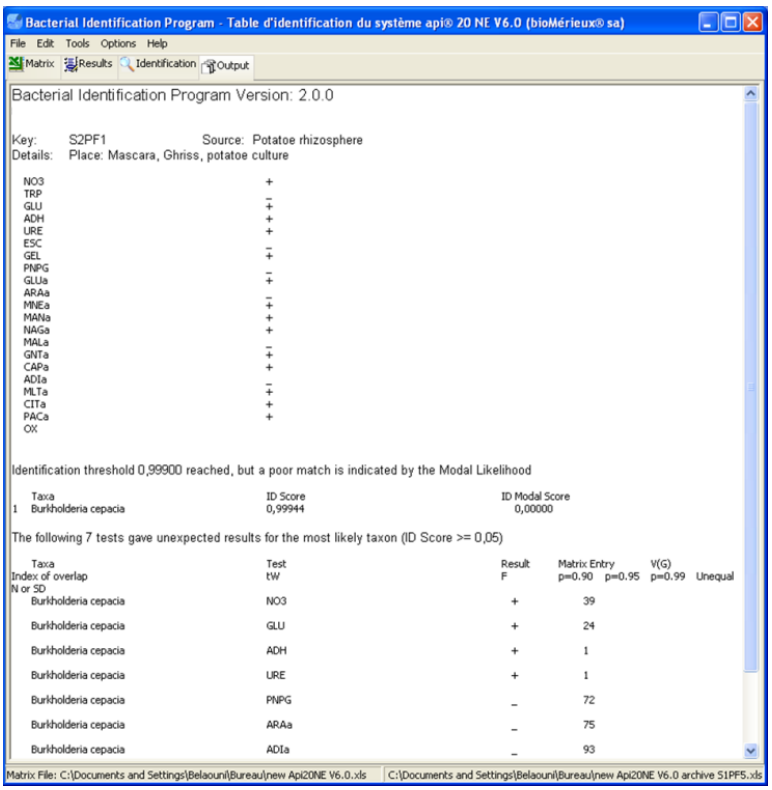

Figure 2 Report of the heuristic identification of the strain S2Pf1

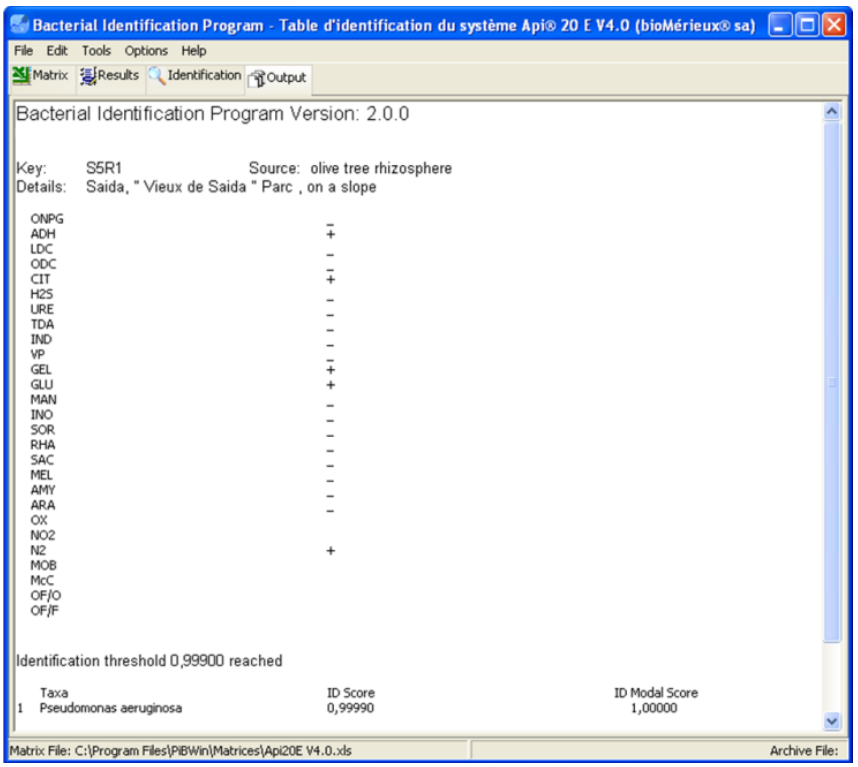

Figure 3 Report of the heuristic identification of the strain Pa2

3.4. Characterization (antifungal secondary metabolites and extracellular lytic enzymes)

Production of antifungal secondary metabolites is recognized as an important aspect of biocontrol against responsible agents of fungal diseases in plants [50].

3.4.1. Salicylic acid

After the preparation of standards for a calibration curve and reading absorbance at 527 nm after different treatments for the spectrophotometric determination of salicylic acid, the results were plotted. The formula of the obtained line is as follows: $y = 0.068\ x$ (y: OD527, x: salicylic acid concentration), with a good coefficient of determination ($R^2 = 0.948$).

After reading the OD527 of the spectrophotometric preparations for determination of the salicylic acid produced by the selected strains, extrapolation on the standard curve revealed the following concentrations (converted into $\mu g.mL^{-1}$): S2Pf1: 307.45 ± 2.08 $\mu g.mL^{-1}$, Pa2: 383.28±1.23 $g.mL^{-1}$. Thus, the maximum production is attributed to the strain *Pseudomonas aeruginosa* Pa2 (Figure 4). *Burkholderia cepacia* and other *Pseudomonas* species are some of the most promising biocontrol organisms. These bacteria produce many antibiotic substances that may inhibit pathogens. Among these molecules, salicylic acid is of great interest, given its "crossroad" position in the synthesis of other antibiotics of major importance in the induction of resistance in plants. The determination of salicylic acid is based on a color reaction. The complex formed between the Fe^{3+} ions and salicylic acid then features a blue-purple color, allowing its visual detection [51]. Numerous studies have shown that the accumulation of salicylic acid in the rhizosphere was associated with physiological responses of plants to infections caused by phytopathogenic agents [52].

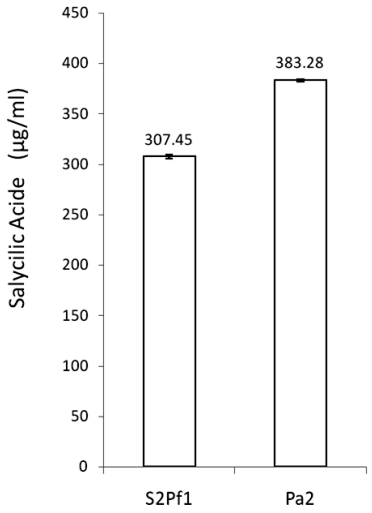

Figure 4: Concentration of salicylic acid (g/ml) after 72 h on BHIB. Results Expressed as mean ± standard deviation, with a significant difference at $p = 0.05$

Indeed, De Meyer and Höfte 1997 have shown that the production of salicylic acid is essential for the induction of resistance to *Botrytis cinerea* by *P. aeruginosa* in the bean and do not exclude a role for pyochelin, another antibiotic, for what salicylic acid is a precursor [53] .

However, the *in-situ* mechanism of action remains poorly understood. For example, WCS374r *Pseudomonas fluorescens* (a strain with high potential of SA production) induced resistance in radish but not in *Arabidopsis thaliana*, while the application of salicylic acid would result in the induction of resistance in both species [54].

3.4.2. Hydrocyanic acid

As the result of producing hydrocyanic acid is based on the color change of the alkaline picrate strips, both strains are considered HCN- (no production observed).

This acid plays a key role in the suppression of some pathogens like the black rot of tobacco roots [55]. Voisard *et al.* [55] found that HCN-mutant strain *P. fluorescens* CHAO obtained by insertional inactivation has lost its ability to inhibit black rot tobacco roots caused by *Thielaviopsis basicola*, suggesting that HCN contribute to suppression of black root rot by *P. fluorescens*.

3.4.3. Rhamnolipids

The formula of the obtained line is as follows: y=0.012 x (y: DO421, x: L-rhamnose concentration), with a good coefficient of determination R^2 of 0.941.

Extrapolation of the OD421 of the spectrophotometric preparations from 7 days cultures has revealed the following rates (equivalent in mg L-rhamnose.mL^{-1}): S2Pf1: 35.63 mg L-Rha-eq.mL^{-1}, Pa2: 72.08 mg L-Rha-eq.mL^{-1}. Thus, the best production rate is attributed to the strain *Pseudomonas aeruginosa* Pa2 (Figure 5).

The biosurfactants are valuable amphiphilic molecules with effective microbial biological properties and especially "surface-active" abilities, applicable to several niches and other industrial processes [56]. Among the molecular group, rhamnolipids would take pride of place. Surfactant molecules composed of rhamnose and β-hydroxydecanoic acid, biosurfactants are produced mainly by *Pseudomonas aeruginosa*, which were studied since their discovery to date [57].

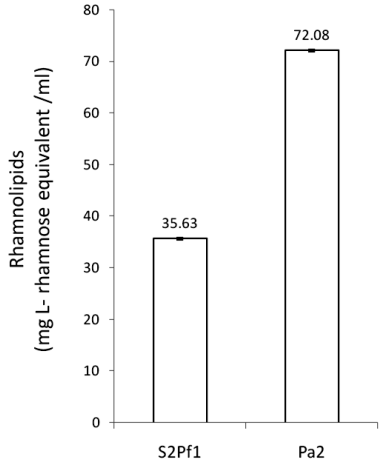

Figure 5: Concentration of rhamnolipids (mg L-rhamnose equivalent/ml) after 7 days on glucose urea medium (Orcinol colorimetric method). Results Expressed in mean ± standard deviation, with a significant difference at p = 0.05

It is interesting to note that very few studies have focused on the production of rhamnolipids by *Burkholderia species* [57]. We observed reasonable rates, if compared to other works, such as Wang *et al.* [31], after genetic manipulation and production optimization achieved exceptional rates (785.4 mg.L^{-1} after 4 days of fermentation).

Orthologs of rhlA, rhlB and rhlC, genes responsible for the biosynthesis of rhamnolipids in *P. aeruginosa*, were found in the non-infectious *Burkholderia thailandensis*, and also in the pathogenic agent *Burkholderia pseudomallei*. In contrast to *P. aeruginosa*, *Burkholderia* species contain these three genes required for production rhamnolipids within a single gene cluster [57].

Rhamnolipids display antibacterial activity mainly against Gram-positive plus some Gram-negative. Besides, it is confirmed to be antiviral, antifungal and mycoplasmacide, such molecules could confer a competitive advantage in the colonization of niches, this being confirmed by the ability of *P. aeruginosa* to be "ubiquitous" [58].

3.4.4. Chitinases

The halos obtained after a 4 days culture of selected strains for the characterization of antifungal potential on the chitin-agar medium are as follows (Figure 6): S2Pf1: 54 mm, Pa2: 52 mm. So, *Burkholderia cepacia* S2Pf1 seems to have a better ability to degrade chitin, indicating a greater ability to produce chitinases.

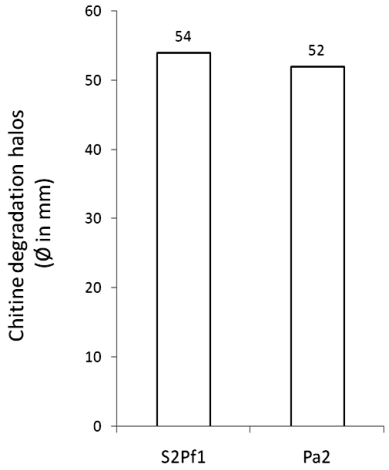

Figure 6: Diameters of chitin degradation halos (expressed in mm)

The lytic activity of bacteria is one of the main mechanisms involved in biological control. Studies on the lytic activity of biological control agents have focused on the characterization of enzymatic systems able to degrade the components of the fungal cell wall, of which chitinases are among the most intensively studied [59]. Both strains are active in terms of chitin degradation.

3.4.5. Cellulase

This test aims to highlight the ability of a strain to degrade cellulose. Microbial growth in a cellulose-based medium (paper) as the sole carbon source is proof of cellulase production. Strains tested have exhibited degradation of cellulose paper bands, thus increasing relatively the turbidity of the test tube if compared to the control tube (saline medium+paper band without inoculation).

An evaluation of the OD600 after 3 weeks of culture has assessed the cellulolytic activity of the selected strains: S2Pf1: 0.1906±0.0005, Pa2: 0.1726±0.0011. Thus, *Burkholderia cepacia* S2Pf1 has a better potential in terms of cellulase production (Figure 7).

Cellulase, another lytic enzyme, targeting the fungal cellulose wall, would be another piece of the antifungal arsenal [33].

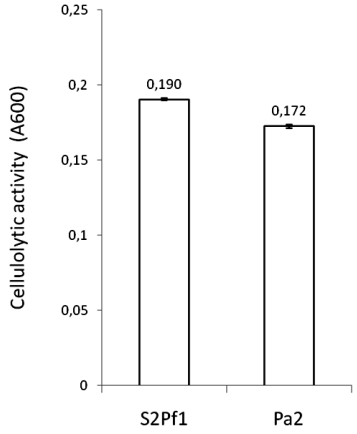

Figure 7: Evaluation of the cellulolytic activity by measuring OD600 after 3 weeks of culture on cellulose medium. Results Expressed as mean ± standard deviation, with a significant difference at $p = 0.05$

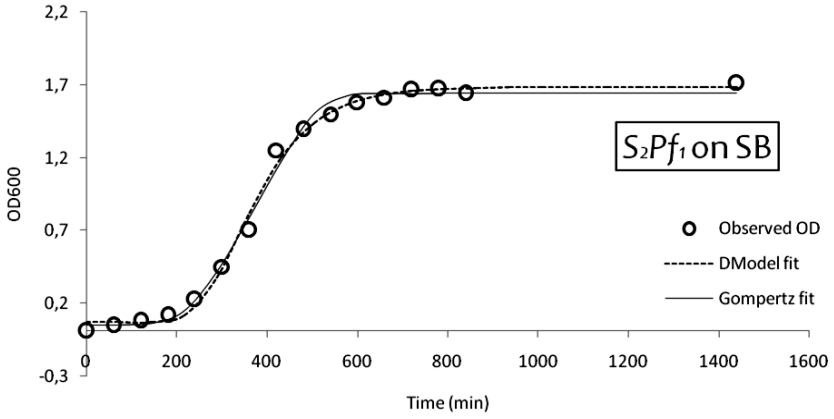

Figure8 Bacterial growth curves of *S_2Pf_1* on seed broth (SB) (according to observed ODdata, DModel data fitting, and Gompertz model data fitting)

3.4.6. Growth kinetics

The bacterial growth curves of *Burkholderia cepacia* S2Pf1 in nutrient broth and Seed broth are shown in Figure 8 and Figure 9 respectively. For the kinetics parameters, the results are displayed in table 6. The best values for the maximum growth rate were obtained with the Seed media (0.0056 $OD.min^{-1}$ with D model, 0.0063 $OD.min^{-1}$ Gompertz model), with a shorter generation time recorded for the same media. On the other hand, a higher bacterial density (Predicted maximum density, *YEnd*) is observed for the nutrient broth (2.0971 with D model, 2.0915 with Gompertz model). Optical density (OD) data can be used to obtain the specific growth rate and if used in conjunction with the known initial inocula, the maximum population data and knowledge of the microbial number at a predefined ODat a known time then all the information required for the reconstruction of a standard growth curve can be obtained. The Baranyi model (D model) was described as a robust primary model for predicting bacterial growth parameters. It should be mentioned that the Gompertz equation used in the present study, and largely used by other works, is also satisfactory for fitting the growth curves of bacteria, and thus, relevant for the interpretation of the results of our evaluation [60]. To demonstrate the invasive potential of *Burkholderia cepacia* S2Pf1, the growth parameters were studied by measuring the OD600 for 24h.

The results of this experiment argue in favor of the

Seed broth for a better maximum growth rate, while the nutrient broth gives a better maximum bacterial density for *Burkholderia cepacia* S2Pf1. It is of vital relevance to focus on the growth stability of a PGPR candidate since a consistent growth would provide stability of the PGPR effect. Thus, Bainton *et al*. suggest that a strong and stable generation rate may be an advantageous characteristic once in the soil, ensuring stable colonization of the root system [61].

Table 6
Growth curves values for S_2Pf_1 calculated from the DModel and Gompertz equations, in Seed broth and nutrient broth

S_2Pf_1 Growth curve values	**Medium**			
	Seed broth		**Nutrient broth**	
	D Model fitting	Gompertz model fitting	D Model fitting	Gompertz model fitting
Initial inoculum density *Y0*(OD600)	0.01	0.01	0.01	0.01
Growth rate μmax (OD.min-1)	0.0056	0.0063	0.0029	0.0034
Generation time t_d (min)	123.1272	109.2576	235.6702	198.3834
Lag phase λ (min)	229.4436	241.3809	126.8145	183.8788
Predicted maximum density *YEnd*	1.6406	1.6847	2.0971	2.0915
Standard error of fitting se*(fit)*	0.0633	0.0551	0.0956	0.0595
R^2	0.99191	0.99399	0.98171	0.99293

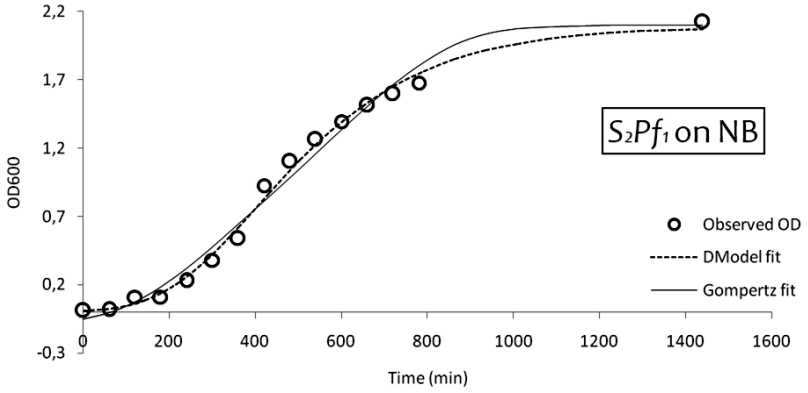

Figure 9: Bacterial growth curves of S_2Pf_1 on nutrient broth (NB) (according to observed ODdata, DModel data fitting, and Gompertz model data fitting)

4. Conclusion

The tested rhizobacteria *Pseudomonas aeruginosa* Pa2 and *Burkholderia cepacia S2Pf1* showed antagonistic activity against FORL. The investigation of their ability to produce several secondary metabolites involved in biocontrol (salicylic acid, hydrogen cyanide, rhamnolipids, chitinases, and cellulases) showed good potential, with a slight advantage for the strain *Burkholderia cepacia* S2Pf1, encouraging us to consider their possible use under field condition. The kinetic study of the later has proven the ability of this strain to easily reach high densities in both nutrient and Seed broths, with a maximum growth rate value of 0.0063 $OD.min^{-1}$ in the Seed broth, and a predicted maximum density of 2.0915 in the nutrient broth (values given by the Gompertz model). The potential of such strains for protecting tomato crops should be investigated *in vivo* to highlight their ability to limit damages caused by FORL, in addition to other pathogens as well as an effective substitute for chemical pesticides, presenting risks to human health and the environment.

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