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Molecular Identification of Four Antifungal Bacteria from Potato (*Solanum tuberosum*) and Strawberry (*Fragaria* x *ananassa*) Plants and their Rhizosphere Soils

Hichar Abdelhadi[1], Mohamed El Qryefy[2], Khadija Ounine[1]

[1]*Plant, Animal, and Agro-industrial Production Laboratory, Faculty of Sciences, Ibn Tofail University, BP133, 14000 Kenitra, Morocco*
[2]*Natural Resources and Sustainable Development Laboratory, Faculty of Sciences, Ibn Tofail University, BP133, 14000 Kenitra, Morocco*

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ABSTRACT

Rhizosphere bacteria enhance plant growth and protection by producing active compounds. These bacteria remain the means of attraction to substitute for chemical pesticides, fertilizers, and other additives. Several studies are examining the biological control potential of antifungal bacteria, whose identity is currently unknown. The present study was conducted to isolate, identify, and characterize four bacteria with antifungal properties from potato and strawberry plants and their rhizosphere soils. Bacteria were isolated from potato and strawberry plants, and their respective rhizosphere soils. The bacterial isolates were subjected to antifungal activity testing. Twenty-four of the isolates with antifungal activity were further subjected to an antifungal activity assay against *Botrytis cinerea*, *Alternaria alternata*, and *Fusarium oxysporum*. These bacterial isolates were identified using microscopic, phenotypic, and biochemical tests. The identity of the four bacteria with strong antifungal activity was confirmed with 16S rRNA gene sequencing. A total of 374 strains of bacteria were isolated, with 24 having antagonistic activity. The results of the tests classified the isolates into three categories with varying percentages; 25, 17, and 58% of the bacterial isolates were of the genera *Bacillus*, *Acinetobacter*, and *Pseudomonas*. Four of these bacterial isolates (Fr43, F31, B6, and B29) had strong antifungal activity. The molecular analysis revealed their identities as *Bacillus amyloliquefaciens* DMB3, *Acinetobacter lwoffii* strain HATC14, *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain IHBB13645, and *Bacillus amyloliquefaciens* strain CD2901, respectively. The present study found four distinct bacterial strains with strong antagonistic potential, probably belonging to new species, based on 16S rRNA gene sequence analysis.

***Keywords*:** antifungal, identification, potato, soil, strawberry, 16S rRNA gene.

Introduction

The concept of sustainable development requires the agriculture and agri-food sectors to consider both biotic and abiotic elements. Using naturally occurring phytosanitary agents, known as biopesticides, is highly needed in this context. There are two types of biopesticides on the market. One is biochemicals, substances of natural origin, and/or synthetic molecules that resemble them.[1] The second is microbial pesticides, which contain beneficial microorganisms, such as bacteria, fungi, yeasts, viruses, or protozoa, as active substances.[2] Bacterial biopesticides are the most common type of microbial agents in agriculture.[3] However, agriculture is faced with the problem of post-harvest diseases. These affect the quality and commercial value of fruits and vegetables worldwide.[4] Thus, several antagonistic bacteria help fight the causative agents of diseases that affect vegetables and fruits after harvest. These bacteria include strains belonging to the species *Bacillus subtilis*, *Pseudomonas syringae*, *Pichia anomala*, *Pichia guillermondii*, *Candida ircofnita*, and *Cryptococcus laurentii*.[5]

*Corresponding author. E mail: *hichar.abdo@gmail.com*
Tel: *+212673512229*

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Moreover, rhizobacteria also promote plant growth. They maintain a highly enriched soil environment with a wide variety of macro- and micronutrients. Furthermore, they fix atmospheric nitrogen, solubilize insoluble phosphorus, potassium, and zinc from the soil, making it available to the plants for their proper growth and development,[6-7] and synthesize siderophores that help in iron sequestration.[8] Therefore, even though crop disease management using biocontrol agents (BCAs) is becoming more and more popular, BCAs account for only 1% of agricultural control techniques nowadays. In contrast, 15% of the agriculture sector utilizes pesticides with a synthetic base.[9] However, the present direction of lowering the usage of synthetic pesticides and simplifying the low-risk product approval process may enable the global commercialization of BCAs.[10-11]. Several studies focus on biological control by antifungal bacteria, whose identity is unknown. As a result, biocide formulations based on microorganisms are decreasing with increased efficiency and lower prices.[12]

The present study aimed to isolate bacteria with antifungal potential from potato (*Solanum tuberosum*) and strawberry (*Fragaria* x *ananassa*) plants and their rhizosphere soils. Also, the isolates were identified using morphological, microscopic, biochemical, and 16S rRNA gene sequencing methods.

Materials and Methods

Source of the plant samples

The potato and strawberry plants used in the present study were obtained free of charge in the Mnasra region of the Gharb basin in Morocco in 2013. The plant samples were identified in the Department of Biology, Faculty of Sciences, University of Ibn Tofail, Morocco. The rhizosphere soil samples were collected from the two crops.

Isolation and evaluation of the antagonistic activity of the bacterial isolates

Bacteria were isolated from the two plants and their rhizosphere soil samples.[13] The antagonistic activity of the different isolates were evaluated against three phytopathogenic fungi, including *Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria alternate*, using the agar well diffusion technique as previously described.[13]

Microscopic and phenotypic characterization of bacterial isolates

After purification on the LPGA agar, a bacterial smear was prepared from pure culture colonies of the bacteria with antifungal potential, then fixed and stained using the Gram staining method.[14] The bacterial isolates were examined under a light microscope. The phenotypic characteristics of bacterial colonies (contour, shape, surface, and opacity) were employed to identify the bacterial isolates.

Biochemical identification

Some of the biochemical traits of the bacterial isolates were analyzed. A catalase test was conducted. On a sterile slide, a colony of the strain was exposed to hydrogen peroxide. The presence of this enzyme was shown by the effervescence brought on by gas emissions. The oxidase test, which uses N-dimethyl-paraphenylene-diamine was conducted.[16] On the Kligler-Hajna medium, lactose fermentation, and glucose degradation were performed. The mannitol-mobility medium was used to study mannitol fermentation.[17] Frazier's approach indicates that a combination of exoenzymes (protease) is responsible for the hydrolysis of gelatin.[18] Then, using a Simmons citrate medium, the release of citrate was evaluated.[17] The production of hydrogen sulphide, the Voges-Proskauer reaction, and the search for arginine were tested according to Forbes .[19] Following these tests, the bacterial isolates were characterized using taxonomic reference manuals, such as the Bergey Bacteriology Manual, to identify, name, and classify bacteria.[20]

DNA extraction and polymerase chain reaction-amplification of the bacterial 16S rRNA gene

Molecular identification was achieved through the sequencing of the 16S rDNA. DNA was extracted and purified from the bacterial isolates using the GenElute bacterial genomic DNA kit (Sigma Aldrich, États-Unis). Using two primers, Fd1 and RP2 (Table 1), the 16S rDNA gene sequence was amplified using polymerase chain reaction (PCR). The PCR was set up in a reaction volume of 25 µL containing the DNA template and the reagents listed in Table 2, with the appropriate primers. The ABI (Applied Biosystems) thermal cycler was used to conduct the PCR. For each reaction, the thermocycler was programmed following the denaturation-hybridization-elongation cycle. At the end of the amplification, an aliquot of each amplicon was separated on 1% agarose gel electrophoresis (CNRST, Rabat, Maroc) in tris, acetate, and EDTA buffer (TAE; 1x). The gel was then photographed on the GBOX, Syngene. Image acquisition and analysis were performed using GenSnap software. The PCR products were purified using the Exosap enzyme, and then 1 µl of the purified product was used to determine the concentration using the Nanodrop (ThermoFisher Scientific).

DNA sequencing of the 16S rRNA gene

DNA sequencing was performed at the sequencing laboratory of the National Centre for Scientific and Technical Research.[21] The amplicons were sequenced by Sanger's method,[22] using the Applied Biosystems BigDye (v3.1) kit and PCR primers (Fd1 and RP2). Capillary electrophoresis was initially used to separate the different sequenced fragments according to their size. The sequences were aligned using Applied Biosystems' Sequencing Analysis (v5.3.1) software. The National Centre for Biotechnology Information's (NCBI) portal was used to compare the results with homologous sequences contained in sequence databases (http://www.ncbi.gov/Blast.cgi). The genus or species was identified by comparing the sequence obtained with a sequence of a classified reference species.[23]

Table 1: Nucleotide sequences of the primers used to amplify the 16S rDNA gene of the four bacterial isolates.

Primer ID	Nucleotide sequence
Fd1	5'-AGAGTTTGATCCTGGCTCAG-3'
RP2	5'-ACGGCTACCTTGTTACGACTT-3'

Table 2: Components of the polymerase chain reaction to amplify the 16S rRNA gene.

Reagent	Quantity for 1 tube
5x buffer	5 µl
FD1 100 µM	0.125 µl
RP2 100 µM	0.125 µl
Taq DNA polymerase	0.2 µl (5U/µl)
ADN	130 ng
H_2O	QSP pour V_t = 25 µl

Results and Discussion

A total of two agricultural biotopes yielded 374 distinct bacterial strains. With a mycelial growth inhibition rate of at least 37%, 24 of the 374 isolates exhibited antagonistic activity. The dual culture technique made it possible to identify six bacterial isolates (Fr43, F31, Fr52, F101, B6, and B29), which had zones of significant mycelial inhibition against *Fusarium oxysporum* and *Botrytis cinerea*.[13] Following their purification on LPGA agar, the twenty-four bacterial isolates with antifungal activity were examined macroscopically, revealing three main categories (Table 3). The first category contained dome-shaped colonies with regular or irregular outlines and a viscous, rough appearance. The second category consisted of flat colonies with rough edges and asymmetrical shapes, while the third category was invasive colonies with a regular outline and a viscous appearance. Gram-negative bacilli were the most abundant, accounting for 58% of the total. The results of microscopic (Table 3) and biochemical (Table 4) studies showed three bacterial categories with varying percentages. In the first category, 25% of bacterial isolates (Fr42, F71, F34, B33, B6, and B29) were large Gram-positive, facultative, aero-anaerobic bacilli. They were catalase-positive and consumed glucose; they did not ferment lactose. The six strains tested have the biochemical characteristics of bacteria of the genus *Bacillus* species. The second category had 17% of bacterial isolates (Fr20, Fr13, Fr12, and F31), strictly aerobic, Gram-negative cocci. They were catalase-positive and oxidase-negative, and they belong to the genus *Acinetobacter*. In the last category, 58% of the isolates (P25, P18, P15, P13, P8, P1, F14, F25, F101, Fr32, Fr44, Fr45, Fr42, and Fr43) were strictly aerobic, oxidase-positive, Gram-negative bacilli. They displayed the biochemical characteristics of the *Pseudomonas* species. The results of the morphological, microscopic, and biochemical characterization showed that these strains were not the same.

Among the twenty-four bacterial isolates that were selected, four bacterial strains showed strong antifungal activity. The species of *Pseudomonas* (Fr43), *Acinetobacter* (F31), and *Bacillus* (B6 and B29) were identified with 16S rDNA sequencing. The concentrations of the DNA extracts and the quality of the DNA were sufficient for further analyses. After PCR amplification of the 16S rRNA genes with the universal primers and the subsequent separation of the purified PCR products, the results demonstrated 1,500 bp fragments for the four bacterial isolates (Figure 1). The sequencing of the fragments, in the form of electropherograms, made it possible to access the species, using the Sequence Scanner software (Applied Biosystems). The identification results of the four isolates (Fr43, F31, B6, and B29) were expressed as a percentage of similarity with the closest bacterial species (Table 5).

The isolation and identification of bacterial strains that have an antagonistic effect are among the most important steps after the commercialization of biopesticides. Different steps are involved in the commercialization process, as stated by Nandakumar. [24] These steps include the isolation of antagonistic strains, screening, formulation

development, fermentation, formulation viability, field efficacy, mass production, toxicology, industrial linkages, and quality control. Thus, one of the most crucial conditions for improved agricultural development is the isolation of an efficient strain, which is typically accomplished from pathogen-suppressive soil using the dilution plate technique or by baiting the soil with fungi such as sclerotia.[25]

Primary screenings of new isolates are usually conducted using physiological, nutritional, and biochemical features, as outlined in Bergey's Manual of Determinative Bacteriology.[26] Meanwhile, DNA and RNA homology tests are also considered the most reliable tools for the characterization of potent plant growth-promoting strains.[27] percentage is greater than or equal to 99%.[28–29]

Based on the results of the present study, the bacterial isolates belong to four species; *Bacillus amyloliquefaciens* DMB3 (B6), *Acinetobacter lwoffii* strain HATC14 (F31), *Pseudomonas brassicacearum* subsp. neoaurantiaca strain IHBB13645 (Fr43), and *Bacillus amyloliquefaciens* strain CD2901 (B29) were identified. The isolates obtained in the present study are comparable to several bacterial isolates on the commercial market, out of which, there are almost 20 distinct commercial items with *Bacillus* origins available. Several strains from the genera, including *Pseudomonas*, *Agrobacterium*, and *Streptomyces*, are employed in addition to *Bacillus* species to produce a variety of commercial products.[30]

Table 3: Some macroscopic and microscopic characteristics of the twenty-four selected bacterial isolates.

Bacterial ID	Microscopic form	Gram staining test	Colony outline	Colony form	Colony area	Colony opacity
Fr42	*bacilli*	+	Regular	convex	viscous	Translucent
F71	*bacilli*	+	Regular	convex	viscous	Translucent
F34	*bacilli*	+	Irregular	convex	viscous	Opaque
B33	*bacilli*	+	Irregular	convex	viscous	Opaque
B6	*bacilli*	+	Irregular	flat	viscous	Opaque
B29	*bacilli*	+	Irregular	convex	viscous	Translucent
Fr20	*Cocci*	-	Regular	convex	Rough	Opaque
Fr13	*Cocci*	-	Regular	convex	Rough	Opaque
Fr12	*Cocci*	-	Regular	convex	Rough	Opaque
F31	*Cocci*	-	Regular	convex	viscous	Opaque
P25	*bacilli*	-	Irregular	flat	Rough	Opaque
P18	*bacilli*	-	Regular	convex	viscous	Translucent
P15	*bacilli*	-	Regular	convex	viscous	Opaque
P13	*bacilli*	-	Regular	convex	viscous	Translucent
P8	*bacilli*	-	Regular	convex	viscous	Opaque
P1	*bacilli*	-	Regular	convex	viscous	Translucent
F14	*bacilli*	-	Regular	convex	viscous	Opaque
F25	*bacilli*	-	Irregular	flat	rough	Opaque
F101	*bacilli*	-	Regular	convex	viscous	Opaque
Fr32	*bacilli*	-	Irregular	flat	rough	Opaque
Fr44	*bacilli*	-	Regular	convex	viscous	Opaque
Fr45	*bacilli*	-	Irregular	flat	rough	Opaque
Fr42	*bacilli*	-	Regular	convex	rough	Opaque
Fr43	*bacilli*	-	Regular	convex	viscous	Translucent

Fr: Strawberry soil; F: Strawberry root; B: Potato root; P: Potato soil

Table 4: Summary results of the biochemical tests of the twenty-four strains isolated.

Biochem Test	Fr 43	Fr 52	Fr 45	Fr 42	Fr 44	Fr 32	Fr 20	Fr 13	Fr 12	F3 1	F10 1	F 71	F3 4	F2 5	F1 4	*B 6*	B3 3	B2 9	P 1	P 8	P1 3	P1 5	P1 8	P2 5
Gram	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-
Cat	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxy	+	+	+	+	+	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	+	+	+
Glu	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. lac	-	+	+	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
Man	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+
Gél	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	+	+	+	+
H_2S	-	-	-			-	-	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-

VP	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+
ADH	+	+	-	-	+	-	-	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+

Gram: Gram stain; Cat: Catalase test; Oxy: Oxidase test; Glu: Glucose breakdown; F.Lac: Lactose fermentation; Man: Mannitol test; Cit: Citrate; Gél: Gelatin hydrolysis; H_2S: Hydrogen sulfide production; VP: Voges-proskauer test; ADH: Arginine dehydrolase test; +: Positive test; -: Negative test

Table 5: Percentage similarity index of the 16S rRNA sequences from the four bacterial isolates compared to some related species.

	Closest bacterial species	
Isolate	**Species name**	**Similarity (%)**
B6	*Bacillus amyloliquefaciens DmB3*	99
	Bacillus sp. NWBS10.5	97
	Bacillus sp. SJ-10	97
F31	*Acinetobacter lwoffii strain HaTc14*	99
	Prolinoborus fasciculus IHBB9208	98
	Acinitobacter sp BSBY	97
Fr43	*Pseudomonas brassicacearum subsp. neoaurantiaca strain IHB B 13645*	99
	Pseudomonas brassicacearum subsp. neoaurantiacastrain MLS-2-8	98
	Pseudomonas fluorescens strain FW300-N2E2	98
	Pseudomonas sp. 41(2015)	98
	Pseudomonas thivervalensis strain PE32	98
	Pseudomonas fluorescens strain JK15	98
	Pseudomonas sp. LW1-LECU3A-W	98
	Pseudomonas brassicacearum strain Zy-2-1	98
	Pseudomonas brassicacearum strain PG17	98
B29	*Bacillus amyloliquefaciens strain CD2901*	99
	Bacillus amyloliquefaciens	97
	Bacillus subtilis strain NVOF	97
	Bacillus sp. SA3	97
	Bacillus amyloliquefaciens strain ACHSBL41	97
	Bacillus subtilis subsp	97
	Bacillus vallismortis strain 25BN09U-2	97

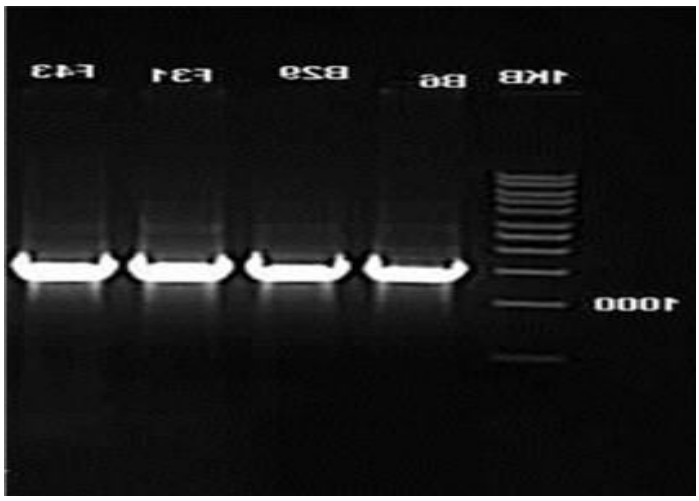

Figure 1: The polymerase chain reaction-amplified 16S rRNA gene fragment from the four bacterial isolates on 1% agarose gel. Lane 1: 1 kb DNA ladder; Lane 2: B6 isolate; Lane 3: B29 isolate; Lane 4: F31 isolate; Lane 5: F43 isolate.

Conclusion

The study's findings identified four species of bacteria with antagonistic effects. These species included *Bacillus amyloliquefaciens* DMB3, *Acinetobacter lwoffii* strain HATC14, *Pseudomonas brassicacearum* subsp. neoaurantiaca strain IHBB13645, and *Bacillus amyloliquefaciens* strain CD2901, probably belonging to new species based on 16S rRNA gene sequence analysis. The knowledge gained from this study is expected to provide insights into a key step in biopesticide formulation. The future direction of this study is the formulation of antifungal bacteria as biopesticides.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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