

## Potential for Bio Control of Fungal Phytopathogens by *Pseudomonas Aeruginosa* CFSP1L1 Isolated from a Matured Compost Sample

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### Abstract

The use of antimicrobial bioactive compounds is currently regarded as a novel technology for biological control against plant diseases. This study aimed to isolate bacteria for the production of valuable bioactive compounds with antimicrobial activity from the compost of green waste mixed with phosphate residues and straws. *Pseudomonas aeruginosa* CFSP1L1 was identified as the most efficient bacterium in nine isolated strains in terms of inhibitory effect against *Salmonella* spp., *Streptococcus* spp., and *Escherichia coli* (*E. coli*). This bacterium has also been shown to have antifungal activity against *Fusarium oxysporum* f. sp. *albedinis* (*Foa*) and *Aspergillus niger* (*A. niger*). Its antimicrobial activity was not abolished after the heat and enzymatic treatments, showing that *P. aeruginosa* CFSP1L1 acts by bioactive substances of a non-protein, non-lipid and non-saccharidic nature. *In vivo*, the treatment of no damaged apples and mandarins with a suspension of the antagonist *P. aeruginosa* CFSP1L1 (108 CFU/ml) for 24 h and then inoculated with a suspension (107 CFU/ml) of *A. niger* and *Foa*, respectively showed that the bacterial strain CFSP1L1 completely inhibited the phytopathogenic effect of both fungi.

**Keywords:** Compost; Phytopathogens; *Pseudomonas aeruginosa*

### Introduction

Agriculture plays a crucial socio-economic role in different countries. In Morocco, the agricultural sector is one of the main pillars of the national economy. For example, it contributes by 14 to 15% of the national gross domestic product [1]. However, it is exposed to several anthropogenic and/or natural constraints and in particular, those caused by pathogens, thus, leading to significant economic losses [1,2]. *Fusarium* is one of these agents, and it causes Fusarium wilt, a disease that affects cereals (wheat, barley, and other small grains) [3]. Species belonging to this genus also attack others vegetables and plants. For example, the case of *Fusarium oxysporum* f.sp. *cucumerinum*, which devastates cucumber [4] and *Foa*, which causes Fusarium wilt commonly known as bayoud. This is the most destructive fungal disease of the date palm [5]. Another example to cite is that of *A. niger* responsible for developing black mold of several organic substrates including fruits, vegetables, grains, seeds, spices, grass and wood. In addition, this fungal species can produce mycotoxins, which exhibit several adverse effects on the liver, kidneys, nervous system, muscles, digestive tract, respiratory and genital organs [6]. In addition to fungi, some bacteria can also infect plants. Among them, *Salmonella* which infects Arabidopsis by multiplying in plant tissue and thus leading to various symptoms such as wilting and yellowing of leaves [7]. On the other hand, many human disease outbreaks caused by food-borne pathogens have been associated with the consumption of contaminated fresh produce, such as alfalfa sprouts, lettuce, spinach, parsley, and cantaloupes [7].

Currently, the control of these phytopathogens is based on the use of pesticides. However, this chemical treatment has a toxic effect on

human health and sometimes causes irreversible environmental damage, such as the contamination of surface and groundwater, soil impoverishment and reduction of biodiversity. Faced with this situation, farmers must adopt other alternatives without environmental or health threatening. In this sense, many bacteria resulting from composts of different organic wastes are used in agriculture as biological control agents. They can so replace harmful chemicals by improving the health of plants and by limiting the saprophytic growth of phytopathogenic microorganisms. Indeed, the investigations on biocontrol using microorganisms are experiencing remarkable growth. They are based on the mechanisms of microbial antagonism involved in the protection of plants against their enemies.

Among these microorganisms, *P. aeruginosa*, a Gram-negative bacterium can colonize many ecological niches including soil, composts, plants and aquatic surfaces [8]. The proliferation of the *P. aeruginosa* population provides information on the proper conduct of composting and the metabolization of organic waste material, especially in the final composting phase [9,10]. In fact, *P. aeruginosa* is considered to be thermo-tolerant during composting [11] and its proliferation in the final phase of composting has been reported by several authors [11,12].

Indeed, *P. aeruginosa* secretes various metabolites including pyocyanin, a blue-green pigment. Its antimicrobial effect against pathogenic microorganisms has been demonstrated [13]. Generally, many bacteria belonging *Pseudomonas* genus produce many antifungal metabolites such as phenazines, pyoluteorin, pyrrolnitrin, and DAPG (2,4-diacetylphloroglucinol), which are the most frequently detected antifungals [14-16]. These bacteria are also able to synthesize siderophores called pyoverdins or pseudobactins. These molecules are involved in improving the growth and health of plants [11] and contribute to the acquisition of iron by plants [10].

In this context, this present work consisted of the isolation of bacteria secreting compounds that had antibacterial and antifungal effect from a compost of green waste mixed with phosphate residues. Therefore, the antimicrobial activity of isolates was evaluated, as well as *in vivo* testing of the antagonistic effect of the best performing isolate against *Foa* and *A. niger*, and the partial characterization of secreted bioactive substances.

## Materials and Methods

### Sampling

To isolate microorganisms with an antimicrobial effect from the compost of green waste mixed with phosphate residues and straws elaborated as described. Three representative samples have been taken from the composts during the succession of three distinct phases: (1) mesophilic phase, (2) thermophilic phase, and (3) maturation phase.

### Antimicrobial effect against pathogenic microorganisms

**Pathogenic strains used and culture media:** *E. coli* CCMM/B4, *Streptococcus* sp. CCMM/ B24 and *Salmonella* sp. CCMMB17 strains were used as tests to demonstrate antibacterial effects. The *Foa* A27 and *A. niger* CCMM- M100 were used as tests for antifungal effect. These fungal are grown in Potato Extract Glucose Agar (PDA). The dishes were incubated in an oven at  $28 \pm 1^\circ\text{C}$  for seven days, and then they were stored at  $4^\circ\text{C}$ .

Meanwhile, the other culture media used are suitable for developing the target bacterial strains, and the majority of microorganisms are isolated from compost (fast and slow-growing microorganisms). Luria-Bertani (LB) medium issued for suspension cultures of pathogenic bacteria, and for inoculation of isolates of interest to produce bioactive substances. These bacteria were maintained in liquid LB medium (10 g of peptone, 5 g of yeast extract, 10 g of NaCl per one liter of distilled water), and solid LB medium (10 of peptone, 5 g of yeast extract, 10 g of NaCl and 15 g of agar per one liter of distilled water). During the entire period of this work, the bacteria were inoculated every week on solid media and then they were stored at  $4^\circ\text{C}$ .

**Isolation and screening of microorganisms producing substances with an antibacterial effect:** The compost sample (10 g) was diluted in 90 ml of a buffer solution ( $0.06 \text{ M Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ ) (1/9 v/v), pH 7.6. Dilutions of decimal series ( $10^{-1}$  to  $10^{-7}$ ) of each sample of the composts were carried out. An aliquot of 100  $\mu\text{l}$  of each dilution was then spread on a agar plates previously inoculated with *E. coli*, *Salmonella* sp. and *Streptococci* sp. After incubation at  $37^\circ\text{C}$  for 48 hours, colonies surrounded by an inhibition halo were subculture and purified [2,17].

**The antibacterial activity of the isolates:** This test consisted of confirming the antibacterial effect of the isolates directly on a solid medium. Thus, a colony of each isolate was taken from a fresh culture, then placed in the centre of a plate containing solid LB medium previously inoculated with *E. coli*, *Salmonella* sp., and *Streptococci* sp. The dishes were incubated at  $37^\circ\text{C}$  for 48 h. The diameters of the inhibition halos were measured according to [2]. The results were expressed by measuring the distance between the border of the bacterial colony and the start of the zone of inhibition of the pathogenic strain.

**Antifungal effect of the isolate CFSP1L1 against A. niger and FoA:** The antagonism of the isolate CFSP1L1 against the two phytopathogens (*Foa* and *A. niger*) was studied side-by-side co-culture on the solid PDA medium according to [5]. Thus, a Petri dish was streaked with the isolate. After 48 h of incubation, two 5 mm discs of a two days old culture of *Foa* were deposited 2.5 cm from the streak of the isolate against each other perpendicular to the line of the seeding streak. The petri dish was then incubated at  $25 \pm 2^\circ\text{C}$ . The percentage inhibition was evaluated by measuring the radius of the colony of *Foa* co-cultivated with the isolate CFSP1L1. The radius of the colony of a control cultivated in the absence of any other microorganism was also evaluated. Measurements were taken after 6 days of incubation. The same procedure was followed to test the antagonistic effect of the isolate against *Aspergillus niger*. For the validation of the experimental protocol carried out, 3 repetitions were made for both pathogenic agents (*FoA* and *A. niger*).

### Identification of the isolate CFSP1L1 that produces substances with antimicrobial effect

**Total DNA extraction:** The total DNA of the bacterial isolate was obtained by phenol-chloroform extraction from exponentially growing bacterial cultures [18]. The cells were centrifuged by centrifugation at 13,000 rpm for 5 min, then suspended in lysis buffer (40 mM Tris-acetate, pH 7.8, 20 mM sodium acetate, 1 mM EDTA, 1% SDS, 20  $\mu\text{g}$  mL<sup>-1</sup> RNase). Then 100  $\mu\text{l}$  of 5 M NaCl was added to the solution, the tubes were centrifuged at 13,000 rpm for 10 min at  $4^\circ\text{C}$ . The supernatants were transferred to new microtubes and equal volumes of phenol- chloroform: isoamyl alcohol (25: 24: 1, v/v) were added. The DNA was precipitated in absolute ethanol and washed twice with 70% ethanol, then dried and redissolved in pure sterile water. The DNA's concentration and purity were evaluated using a Nano Drop TM spectrophotometer and was stored at  $-20^\circ\text{C}$  for later use.

**Amplification of DNA encoding 16S rRNA:** The DNA encoding the 16S rRNA of the isolate of interest was amplified using the two primers rD1(5'AAGGAGGTGATCCAGCCGCA3') and fD1 (5'GGAGAGTTAGATCTTGGCTC3') (Weisburg et al.), in a reaction volume of 25  $\mu\text{l}$ , the mixture containing: 1  $\mu\text{l}$  of DNA with a concentration of 100 ng/ $\mu\text{l}$ , 1  $\mu\text{l}$  of each primer at 10  $\mu\text{M}$ , 9.5  $\mu\text{l}$  of pure sterile water and 12.5  $\mu\text{l}$  of MytTaqTM Mix, 2x (BIOLINE) ready to use, containing the Taq polymerase enzyme, reaction buffer, MgCl<sub>2</sub> and dNTPs. The following cycles were used under the optimal conditions supplied with the MytTaqTM Mix: initial denaturation ( $95^\circ\text{C}$  for 60 seconds), followed by 35 cycles ( $95^\circ\text{C}$  for 15 seconds, hybridization temperature  $60^\circ\text{C}$  for 15 seconds,  $72^\circ\text{C}$  for 10 seconds), and followed by 1 min final extension at  $72^\circ\text{C}$ .

**Sequencing and phylogenetic analysis of the 16S rRNA gene:** The 16S rRNA amplification products were purified using the Quiagen PCR product purification system, then subjected to cycle sequencing using the same primers as for PCR amplification, with ABI Prism Dye Chemistry, and analysed with a 3130xl automatic sequencer [19], in the sequencing facilities of the Innovation city in Fez (Morocco). The nucleotide sequences obtained were compared with those of the reference strains available in the Gen-Bank database (NCBI: National Center for Biotechnology Information), using the Blast software (Basic Local Alignment SearchTool (<http://www.ncbi.nlm.nih.gov/Blast.cgi>)) (20) The nucleotide sequences were aligned, verified and corrected manually using Chromas 2 (Version 2.6.5) and MEGA 7 (Version 7.0.26). The sequences obtained were

deposited and identified in the Genbank database. The obtained accession number of the isolated strain was represented in Table 2.

### ***In vivo* effect on *A. niger* and *Foa***

Apples and mandarins were washed thoroughly with sterile distilled water before use for inoculation with Isolated strain CFSP1L1 using the protocol of [20,21]. Briefly, the antagonist's bacterial suspension was prepared in sterile distilled water and was adjusted to 108 CFU/ml. Injured apples and mandarins has been injected with a suspension of the Isolated strain CFSP1L1 (108 CFU/ml) for 24 h and then inoculated with a suspension of *A. niger* (107 CFU/ml) and *Foa*, respectively. The results were recovered after 15 days post inoculation. Apples and mandarins recovered after 15 days post inoculation. Apples and mandarins injected only with the antagonist were used to assess the cytotoxicity of the interest isolate. While apples and mandarines treated with distilled water or with the phytopathogen fungi *A. niger* or *Foa* were used as controls.

### **Preliminary characterization of substances with antibacterial activity produced by the isolated strain CFSP1L1**

**Extraction of bioactive substances by ethyl acetate:** The precipitation of bioactive substances from the isolate was carried out according to Naclerio's protocol and his collaborators (1993). Thus, a volume of 100 ml of sterile liquid LB was inoculated with the bacterial suspension, and then incubated with shaking at 37°C for two days.

After centrifugation of this culture at 5000 rpm for 15 min at 4°C, the supernatant was recovered and then added to 100 ml of organic solvent (ethyl acetate at 95% purity). The mixture was stirred for two hours to facilitate the extraction of the secreted bioactive compounds.

The product was decanted in the separating funnel for one hour followed by the elimination of the aqueous phase and recovery of the organic phase. After then, the evaporation of ethyl acetate was carried out *via* a rotary evaporator. The precipitated compounds were solubilized in 1 ml of sterile distilled water [22].

**Testing of the antibacterial activity of the bioactive substances by the well method:** The well technique consists of distributing the LB Agar medium in Petri dishes. After solidification, wells of identical volume were made using a sterile pasteur pipette. Then, a volume of 100 µl of the extract of the Isolated strain CFSP1L1 was incubated at 37°C for 3 hours, and then tested according to the well method on dishes previously inoculated with *E. coli*; *Salmonella* sp and *Streptococci* sp. The dishes were incubated at 37°C for 48 hours then the diameters of the inhibition zones were measured [2,23].

**Nature of the bioactive substances secreted by the isolate of interest:**

**Sensitivity to proteinase K:** In order to determine whether the bioactive compounds are of a protein nature, the extracts precipitated by ethyl acetate were subjected to the action of proteinase K (PK) at a concentration of 1 mg/ml at a pH 7. The mixture was well homogenized and incubated at 37°C for 3 hours.

The antibacterial effect of the bioactive compounds treated with PK was studied against *E. coli* using the well method. Petri dishes were incubated at 37°C for 48 hours [1]. Extracts not treated with PK were used as controls.

**Sensitivity to lipase:** To demonstrate whether the bioactive compounds are lipidic in nature, the precipitates were subjected to lipase action at a concentration of 1 mg/ml at a pH 7. The mixture was well homogenized and incubated at 37°C. for 3 hours. The antibacterial effect of the bioactive compounds treated with lipase was studied against *E. coli* using the good method. Petri dishes were incubated at 37°C for 48 hours. Extracts not treated with lipase were used as controls [2].

**Sensitivity to amylase:** In order to determine whether the bioactive substances are of carbohydrate nature, the various precipitates were subjected to the action of amylase at a concentration of 1 mg/ml at a pH 7. The mixture was well homogenized and incubated at 28°C during 3 hours.

The antibacterial effect of the bioactive substances treated with amylase was studied against *E. coli* using the well method. Petri dishes were incubated at 37°C for 48 hours. Extracts not treated with amylase were used as controls [2].

**Sensitivity to heat treatment:** To test the sensitivity of the bioactive substances to heat treatment, the protocol of Naclerio, 1993 is adopted. The precipitated extracts with ethyl acetate were exposed to 100°C (15 min), 80°C (15 min), 60°C (20 min) and 37°C (3 h). The treated precipitates were tested against *E. coli* using the well method. The dishes obtained were incubated at 37°C for 48 hours.

## **Results and Discussion**

### **Isolation and screening of microorganisms producing bioactive substances**

Composting is naturel process based on the development of intense microbial activities leading to the decomposition of most bioavailable and biodegradable materials. This biological process involves the partial or complete degradation of various chemical compounds by a variety of microorganisms [24,25].

During the composting phases, the microbial community follows a predictable succession pattern that results in the recolonization of the compost with metabolically active microbial populations that can be suppressive to phytopathogens. In this compost, as demonstring in previous studies [9,26], the gram+ bacteria become the most predominant in maturation step, while the gram- negative that dominant in the initial wastes has been eliminated (Figure 1).

In fact, the production of antimicrobial agents, competition for nutrients, oxygen or space is the mechanisms that allow the microbial population in a compost to control or inhibit the effect of disease-causing pathogens [18].

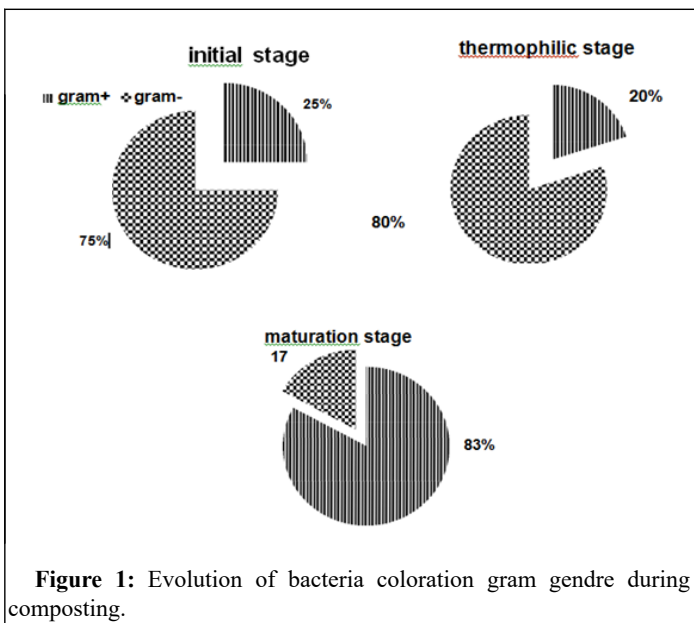


Figure 1: Evolution of bacteria coloration gram genre during composting.

Indeed, the suppression of several plant diseases by the activity of microorganisms from compost has been described [27]. It is in this sense that samples of the compost of green waste mixed with phosphate residues during the mesophilic, thermophilic, and maturation phases has been used to isolate a promoting microorganism producing substances that inhibit *E. coli*; *Salmonella* sp and *Streptococci* sp. Among bacteria, ten were isolated with inhibitory potential. Indeed, these isolates showed halos of inhibition of different diameters around the colonies of the above three pathogens (Table 1). Some of these microorganisms have a broad spectrum. While others have a narrow spectrum acting only on a single target bacteria. All of these isolates act with one or more bioactive substances that inhibit bacterial proliferation. In this work, the choice has been made on the CFSP1L1 isolated from the compost's maturation phase. Indeed, it exhibits an interesting antibacterial effect by showing halos of inhibition of order 15, 16 and 18 mm towards *Streptococcus* sp, *Salmonella* sp and *E. coli*, respectively. This agrees with some previous research, which suggests the pathogen suppressor effect of composts isolates [27,28]. [27] Have demonstrated the antimicrobial activity of *Pseudomonas*, *Serratia*, *Klebsiella*, *Enterobacter* against specific pathogens of turf, namely *Sclerotinia homoeocarpa*, *Pythium graminicola*, *Typhulai shikariensis*, and *Microdochium nivale*. In another study performed *in vitro* by [29], it was found that compost contains 44 species of fungi and 15 species of bacteria, of which seven belong to three genera namely *Bacillus*, *Micrococcus* and *Pseudomonas*. These bacteria have been shown to have an inhibitory power against pathogens [29]. These authors have demonstrated that these microorganisms produce bioactive molecules that suppress pathogens through their metabolisms' secondary pathways or spontaneous expression of genes induced by pathogens.

Isolates	Compost phase from which the isolate is obtained	Inhibition diameters in (mm) against		
		<i>Streptococcus</i> sp	<i>Salmonella</i> sp	<i>E. coli</i>
2CFE51	Maturation phase	-	6	-

2CTE51	Thermophilic phase	5	8	-
CAFE32	Maturation phase	8	22	19
CFSL32	Maturation phase	20	7	13
CFSP1L1	Maturation phase	15	16	18
CFSP1L2	Maturation phase	6	-	7
Clca71	Mesophilic phase	7	5	-
CISP31	Mesophilic phase	6	7	9
CTSL31	Thermophilic phase	40	-	-
CTSP32	Thermophilic phase	10	19	11

- : absence of inhibition

Table 1: Antibacterial activity of isolates from compost of green waste mixed with phosphate residues.

### Antifungal effect of the isolate CFSP1L1 against *A. niger* and *Fao*

*In vitro* tests show that the CFSP1L1 isolate exhibits a significant effect of inhibiting the sporulation of *A. niger* and *Foa* in the order of 99% and 90%, respectively (Figure 2). The suppressant effect of the compost on pathogens including *Fusarium oxysporum* f. sp has already been shown by [30]. In fact, compost probably acts as much through its intrinsic microbial activity by stimulating the soil's microbial activities [31]. In addition, the addition of compost increases the suppressive effect against *Fusarium oxysporum* f. sp. This effect is proportional to the amount of compost administered [32].

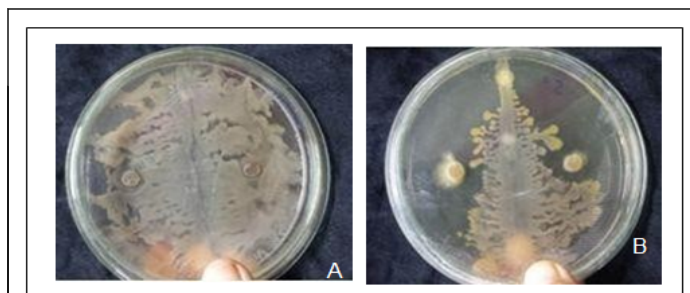


Figure 2: Antagonistic effect of CFSP1L1 isolate against the mycelial growth of *aspergillus niger* (A) and *Foa* (B) after 6 days of side-by-side co-culture on PDA medium.

### Identification of the isolate CFSP1L1

The CFSP1L1 isolate is characterized by colonies of brown color, rounded relief, smooth and shiny appearance as well as a particular aromatic odor. Gram stain revealed that it is a mobile Gram-negative bacillus, arranged singly or in pairs. This result is consistent with that

of the molecular identification of the isolate. Indeed, after purification of the PCR products, the DNA encoding 16S rRNA was sequenced using the primers rD1 and fD1. Sequencing of 16S rDNA resulted in a sequence having 1439 nucleotides. In the literature, identifications have been made using sequences of 500 bp [33,34] and others have been based on the sequencing of about 400 bp [35].

Accordingly, after bioinformatics analysis, the bacterial specie whose 16S rRNA gene shows a strong identity of around 99.79% with that of the isolate is *P. aeruginosa* (Table 2). Basing on the criteria defined by [36], this percentage allows identifying the isolate. An accession number was provided to the sequence obtained after being deposited in the Genbank database (MT415324). Thus, based on Gram stain and molecular identification, the isolate CFSP1L1 isolate has been assigned to *P. aeruginosa*. *P. aeruginosa* is a ubiquitous bacterium that colonizes soil, plants, aquatic surfaces as well as composts [8,37]. Indeed, it is considered to be thermo-tolerant during composting [11] and its proliferation in the final phase of composting has been reported by several authors [10,12,27]. This makes it possible to explain its isolation from the compost maturation phase during this present work. Nevertheless, to date, the antimicrobial effect of *P. aeruginosa* isolated from compost has not yet been strictly demonstrated. [38] Has suggested that *P. aeruginosa* is one of the microorganisms in compost responsible for a significant reduction in the microbial population, including the genus *Salmonella*. Indeed, various studies have shown that the bacteria in question have a broad spectrum of action against Gram-positive and Gram-negative bacteria as well as against fungi. The investigation by [1] has reported that the said bacteria inhibit the growth of *E. coli*. Likewise, in the study performed by [39], it was reported that *P. aeruginosa* may limit the growth of *Streptococcus* spp through several mechanisms including surfactant and alginate synthesis as well as production of siderophores via iron sequestration.

Isolate number	(Accession number)	16S rRNA sequence identity percentage	Type strain showing strong similarity to the isolate
CFSP1L1 1439n	(MT415324)	99,79%	<i>Pseudomonas aeruginosa</i> ATCC 10145 (NR_114471)

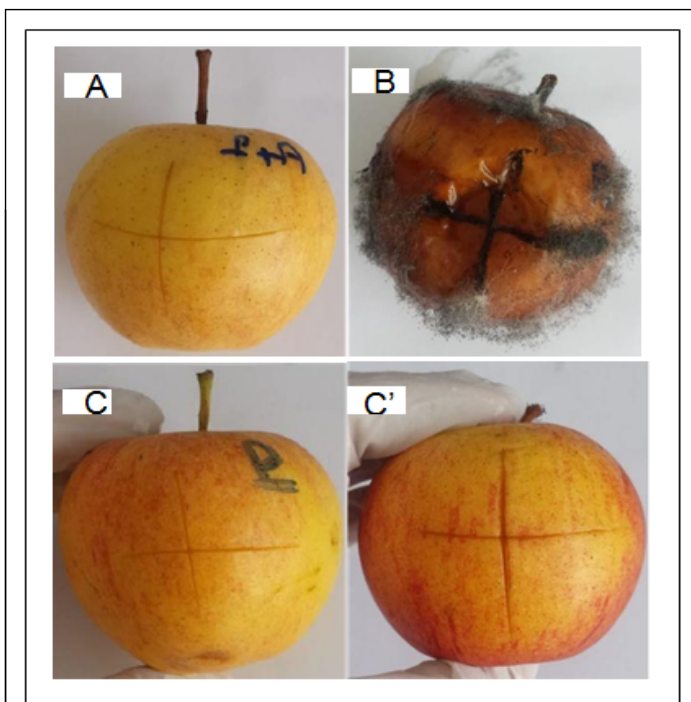
**Table 2:** Results of molecular identification of isolate CFSP1L1 after sequencing of the 16S rRNA gene.

On the other hand, it has been shown that *P. aeruginosa* is effective in controlling peanut crown rot disease caused by *A. niger* by inducing hyphal deformities in the fungal pathogen [40]. In addition, protection against *Fusarium oxysporum* f. sp by fluorescent *Pseudomonas* has also been revealed by several authors [4,41-43] corroborates the findings of the present investigation. Indeed, the current work is the first to demonstrate that *P. aeruginosa* has an antifungal effect against *Foa*. This therefore makes *P. aeruginosa* an excellent candidate to exploit in order to control phytopathogens.

### Antagonistic effect *in vivo* of *P. aeruginosa* against *A. niger* and *Foa*

The treatment of apples with a suspension of the antagonist *P. aeruginosa* (108 UFC/ml) for 24 h and then inoculated with a suspension of the pathogen (107 UFC/ml) shows that *P. aeruginosa* completely inhibits the appearance of black mold due to *A. niger* after 15 days post inoculation (Figure 3A). In contrast, the apples were

completely rotten after their individual inoculation with a suspension of the fungal species at 107 UFC/ml (Figure 3B). As control, inoculation of apples with the antagonist *P. aeruginosa* at a rate of 108 UFC/ml does not reveal symptoms of pathogenicity to the plant tissue tested (Figure 3C). The same result was seen for the distilled water control (Figure 3C).



**Figure 3:** Effect of *P. aeruginosa* on the development of black mold caused by *Aspergillus niger* on apples *in vivo*. (A): apple injected with the antagonist and then inoculated with the phytopathogen, (B): apple inoculated only with *Aspergillus niger*, (C): apple injected with the antagonist *P. aeruginosa*, and (C'): apple injected with sterile distilled water.

Therefore, *P. aeruginosa* could be used to confer protection on injured apples after harvest against *A. niger*. This finding can be explained by the fact that the bacteria secrete metabolites that inhibit fungal growth. Indeed, [44] have reported that the cell-free culture filtrate of *P. aeruginosa* GSE 18, inhibited *in vitro* the production of polygalacturonase and cellulase by *A. niger* six days after inoculation, by up to 77% and 68%, respectively. Such enzymes break down pectin and cellulose, the two main polymers that maintain the firmness and structure of plant cell walls. Subsequently, the degradation of the structural molecules of the wall allows the phytopathogen to penetrate and colonize the plant tissue. In fact, inhibition of these enzymes' production represents a mechanism that may have an essential role in controlling decay by reducing the virulence of *A. niger* [44]. Also, inoculation of mandarins of *Citrus reticulata* Blanco species with the antagonist *P. aeruginosa*, 24 before *Foa* completely inhibits the occurrence of white sporulation due to *Foa* compared to controls (Figure 4). The antagonist *P. aeruginosa* isolated in this present work could act by a similar mechanism to inhibit the plant pathogen's growth. However, to validate this hypothesis, investigations must be carried out to reveal and fully understand the mechanism of action of the antifungal effect of *P. aeruginosa*.



**Figure 4:** Absence of pathogenicity symptoms on injected mandarin only with the antagonist *P. aeruginosa* (A) and on mandarin injected only with sterile distilled water (B), *Foa* on mandarins *in vivo* "white mold" (A'), Effect of *P. aeruginosa* injected 24 hours before being inoculated with *Foa* on mandarin *in vivo* (B').

### Antibacterial activity of the bioactive substances of *P. aeruginosa*

The extraction of the bioactive substances from the CFSP1L1 isolate was carried out using ethyl acetate as an organic solvent. Such a solvent is widely used to extract metabolites from bacteria [1,45]. After extraction, the antibacterial effect of the metabolites of *P. aeruginosa* was evaluated by the well method. Thus, the results show that the extract has an antibacterial activity that has been demonstrated by the appearance of inhibition halos. The diameters of the later are of the order of 17 mm, 18 mm and 20 mm towards *Streptococcus* sp, *Salmonella* sp, and *E. coli*, respectively. This result indicates that *P. aeruginosa* acts by antibacterial metabolites secreted in the LB culture medium and also highlights that these substances are soluble in ethyl acetate.

### Stability and nature of the bioactive substances of *P. aeruginosa*

In order to better elucidate the stability and chemical nature of the bioactive molecules secreted by *P. aeruginosa*, the extract has been subjected to several treatments. Its antibacterial activity was then evaluated against *E. coli* by the excellent technique. The results show that the antagonistic effect of CFSP1L1 was not affected by heat or by enzymatic treatments with PK, lipase and amylase. This implies that the bioactive compound responsible for the desired activity are not protein, lipid or saccharide (Table 3).

Enzymatic treatment	Relative activity
Protéinase K	+
Lipase	+
Amylase	+

Control (untreated ethyl acetate extract)	+
<b>Heat treatment</b>	
37°C	+
60°C	+
80°C	+
100°C	+
Control (untreated ethyl acetate extract)	+
Relative activity was measured by the well method against <i>E. coli</i> .+ : presence of inhibition	

**Table 3:** Effect of the different treatments on the antibacterial effect of *P. aeruginosa* extract.

Previously, *P. aeruginosa* has been reported to produce a variety of metabolites including phenazines. These molecules represent a large family of highly pigmented nitrogenous heterocyclic molecules synthesized from shikimic acid [46,47]. As an example of these molecules exhibiting antimicrobial activities, it has been reported the molecules such as carboxylic acid 1 phenazine, phenazine 1 carboxamide and pyocyanin [2,37]. The latter metabolite is a very distinctive blue pigment produced only by *P. aeruginosa* and is soluble in organic solvents [2]. Purified pyocyanin has been shown to have significant antimicrobial activity against pathogens tested such as *Staphylococcus aureus*, *E. coli*, *Candida albicans*, *Bacillus cereus* and *Salmonella* sp [13]. In addition, several studies have shown that this bioactive substances is stable at high temperature and it shows resistance to boiling without losing its antibacterial effect [1,2,46-48].

In addition, phenazines are biologically active having a role in microbial competitiveness and suppressing plant pathogens [2]. In fact, phenazines have been shown to inhibit the growth of phytopathogenic fungi such as *Gaeumannomyces graminis*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Gibberella avenacea* [49,50]. Consequently, the bioactive substances secreted by *P. aeruginosa* could be organic compounds in the form of phenazines [51,52]. Thus, more work needs to be done to further purify and elucidate the chemical structure of the bioactive substances in the isolate [53,54].

### Conclusion

Post-harvest plant and agricultural product diseases caused by microorganisms cause serious economic losses internationally. Thus, biological control is experiencing remarkable growth as an alternative to conventional treatment based essentially on the use of pesticides. Mainly of that, the excessive use of the later has generated the emergence of pathogens resistant to chemical molecules and them also shown harmful effects on the environment, human health. It is in this sense that *P. aeruginosa* was isolated, during this present work, from a compost of green waste mixed with phosphate residues. This microorganism has an interesting antibacterial effect against Gram positive and Gram-negative bacteria and also an antifungal activity against *Foa* and *A. niger*. In addition, *P. aeruginosa* has been shown to completely inhibit *in vivo* the appearance of black mold due to *A. niger* and *Foa* after 15 days post inoculation of apples. More interestingly, it does not manifest symptoms of pathogenicity with respect to the plant tissue tested. Therefore, it appears that *P.*

aeruginosa is a promising strain which deserves to be exploited as a potential antagonistic agent in order to confer post-harvest protection to plants against biotic threats. To achieve this objective, other additional investigations seem necessary to be carried out such as the field application of the strain as well as the purification of its bioactive substances.

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