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Antimicrobial Activity of Potato Rhizospheric *Pseudomonas chlororaphis* subsp. *aureofaciens* from Sétif Algeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMA designed the study, wrote the protocol and interpreted the data. Authors JN, SMA and MMZ anchored the field study, gathered the initial data and performed preliminary data analysis. Authors SMA, NH and MMZ managed the literature searches and produced the initial draft. Authors SMA and MMZ wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was assessed to demonstrate the antimicrobial activity *in vitro* of an identified fluorescent *Pseudomonas* strain characterized for its capacity to produce phenazine compounds.
Methodology: First *Pseudomonas chlororaphis* subsp *aureofaciens* was inoculated on Nutrient Broth supplemented with Yeast Extract (NBY) and with glucose at a final concentration of 2%, after incubation the filtered culture was acidified with HCl to pH 2. The solution was extracted twice with the same volume of ethyl-acetate. The organic supernatants were combined, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude extract was resuspended in methanol and tested

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for antimicrobial activity. Antimicrobial activity was determined (i) by disc diffusion technique for bacteria and (ii) using serial dilution technique in soft PDA for fungi. Secondly the antifungal activity of the bacterial strain was tested against several phytopathogenic fungi in dual culture.
Results: The studied strain has an important activity against the phytopathogenic bacteria and fungi tested. Among the tested fungi *Fusarium oxysporum* f. sp. *albedinis* is the most sensitive to the actions of this Pseudomonas, where the inhibition rate reached 77.78%. The less sensitive one was *Pythium ultimum* with a rate of 55.56%. While for pathogenic bacteria only *Salmonella enteridis* was sensitive to the tested strain.
Conclusion: *Pseudomonas chlororaphis* subsp *aureofaciens* showed appreciable antagonistic activity, *in vitro*, against special forms of *Fusarium oxysporum and the tested phytopathogenic bacteria*.

Keywords: Antimicrobial activity; phytopathogenic fungi; phytopathogenic bacteria; rhizospheric bacteria; Pseudomonas.

1. INTRODUCTION

Plant pathogens can cause serious damage to agriculture; they are responsible for the loss of 10 to 20 percent of agricultural production worldwide, despite the several billion dollars spent for their control by synthetic chemicals. Telluric pathogens are recognized to be difficult to manage in narrow rotations. Resistant plant varieties are not available for several soil-borne pathogens and chemical control is often insufficiently effective in soil. There is a growing awareness that integrated pest management (IPM) tactics and strategies provide more environmentally and economically acceptable alternatives for agriculture [1]. Evidence exists correlating the efficacy of bacterial strains with control soil-borne pathogen with their ability to competitively colonize and survive in the root system of the plant to be protected. Several bacterial mechanisms have been described for the protection of plants against fungal diseases [2]. Several mechanisms, including the production of siderophores, hydrogen cyanide (HCN) and antibiotics have been shown to play roles in disease suppression [3]. The production of antifungal metabolites, induction of systemic resistance and the ability to compete efficiently with resident rhizobacteria are considered to be important prerequisites for the optimal performance of biocontrol agents [1]. These biocontrol strains belong to different species and/or phylogenetic groups within genus *Pseudomonas* and can be closely related to strains without apparent biocontrol activities [4-5]. The potential for using strains of beneficial *Pseudomonas* spp*.* for biological control of soil borne fungal pathogens has been demonstrated for many crops [1]. Fluorescent pseudomonads that protect plant from soil borne fungal pathogens are thought, to act in part, through the secretion of antimicrobial substances with antifungal [6-7] and antibacterial activity [7-8]. The present study was performed to demonstrate the antibacterial capacity of a *Pseudomonas* strain already known for its antifungal one.

2. MATERIALS AND METHODS

2.1 Materials

The tested strain was already isolated from potato rhizosphere, characterized and identified by Mezaache-Aichour et al. [9] as *Ps. chlororaphis* subsp. *aureofaciens* (*Ps.ca*). The strains used for antibacterial tests were *Bacillus subtilis*, *Pseudomonas diminutus, Paracoccus paratrophus* and *Micrococcus luteus,* which were obtained from the laboratory of Dr Jane Nicklin, School of Biological and Chemical Sciences, Birkbeck College University of London, UK. While pathogenic strains *Salmonella enteritidis, Salmonella typhi, Staphylococcus aureus coagulase*$^{+ve}$ ATCC 25923, *Klebsiella pneumoniae, Morganella morigani, Enterococcus, Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were obtained from the Laboratory of Parasitology, Centre Hospitalo Universitaire Sétif, Algérie.

Fungal strains, *Pythium ultimum* (PI) and *Rhizoctonia solani* (*R. solani*)*,* were obtained from the laboratory of Dr Jane Nicklin, School of Biological and Chemical Sciences, Birkbeck College University of London, UK. *Fusarium solani* (FS) and *Fusarium oxysporum* f. sp. *lycopersici* (F.O.L) were obtained from Dr. Kamal Aissat, Laboratory of Microbial Ecology, University Abderrahmane Mira, Béjaia, ALGERIA; while *Fusarium oxysporum* f. sp. *albedinis* (F.O.A) was obtained from INRAA, Algiers, ALGERIA.

2.2 Methods

2.2.1 Extraction and characterization of antimicrobial metabolites

Antimicrobial metabolite production *in vitro* was assayed in Nutrient Broth Yeast Extract (NBY; [6]) supplemented with glucose to a final concentration of 2%. Cultures were incubated on a rotary shaker (250 rpm) at 28℃ for 5 days [10]. After incubation, the culture filtrate was first acidified with HCl to pH 2 before extraction [6]. Then antimicrobial metabolites were extracted twice from the cultures with an equal volume of ethyl-acetate [11]. The ethyl-acetate fraction was decanted, filtered through anhydrous sodium sulfate to remove the aqueous fraction [6], and concentrated by evaporation to dryness by rotary vacuum at 55℃ [1,11]. The crude extract was re-suspended in methanol [12].

2.2.2 Antimicrobial activity

To determine the effect of the isolated strain on bacteria, cells of examined strains were plated by an inoculating needle to the surface of Luria Bertani (LB) agar medium [8] in a band approximately 2.5 cm wide [3] and grown at 28º C for two days. The grown colonies were killed with chloroform vapors, plates overlayed with 3 ml of 0.6% agar containing $2x10^7$ cells of indicator strains of phytopathogenic and pathogenic bacteria. Incubation of plates proceeded for 18-20 h at 28ºC. The antimicrobial activity was judged from the radius of indicator growth inhibition zones around colonies of tested bacteria [8].

Antibacterial activity of supernatant from the selected strain (ethyl-acetate fraction, dried and resuspended in methanol) was determined using the disc technique. Bacterial suspensions (100 µl) were spread on Tryptone Soya Agar (TSA) medium and discs containing 20 µl supernatant were placed on the inoculated plates. Plates were stored overnight at 4℃ and then incubated at 37℃ for 24 h [13].

The antifungal activity of the strain was first screened in dual culture as described by Vincent et al. [12] against the phytopathogenic fungi cited earlier. The hyphal inhibition was measured, and the percentage of fungal inhibition was calculated using the following formula:

$$\frac{Dc-Ds}{Dc} x\ 100$$

Where

Dc: Control diameter (fungi alone);
Ds: Sample diameter (fungi in dual culture with tested bacteria).

Secondly the Antimicrobial activity of bacterial extracts was determined using serial dilution technique in soft PDA inoculated with 2 mm plug of a seven days fungal culture (diameter of the plate hole is 9 mm).

2.2.3 Statistical analysis

Data were analyzed by the one way analysis of variance (ANOVA) and the test with P=0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity of *Ps. chlororaphis* subsp. *aureofaciens*

The strain was screened visually on agar for the ability to produce antimicrobial substances. The antibiotic was produced consistently and could be screened on NBY or PDA plates, on which colonies appeared orange and were highly colored in dual culture with pathogenic fungi when near mycelium [14]. However, the presence of antimicrobial substances produced varied greatly with media (the amount was greater on NBY, LB, than on PDA) and the age of the cultures (the amount was greater in 72 h than 24 h cultures). When spread on the medium bacterial cells produces the antimicrobial substances which inhibited other bacterial cells spread above (Fig. 1). Using the disc technique, the crude bacterial extracts inhibited the growth of *Bacillus subtilis* and *Pseudomonas diminutus*, but had an intermediate effect against *Micrococcus luteus* and *Paracoccus paratrophus* (Fig. 2). While on dual culture there is another strain which is inhibited by the selected strain *Salmonella enteritidis* (Table 1).

The fluorescent pseudomonads bacteria are used as biocontrol agents of bacteria, fungi, and viral diseases of plants [15-16]. They suppress the pathogen and reduce disease incidence by competition, antibiosis or parasitism [15]. They act in part, through the secretion of antimicrobial substances with antifungal [6-7] and antibacterial activity [7-8]. Our results demonstrate that selection of antagonistic strains among competitive Potato root colonizers yields a low frequency of strains producing well known antibiotics. The isolated bacterial colonies were orange and fluoresced orange under long wave (365 nm) UV irradiation. They were surrounded by a dark halo of UV absorbing material consistent with production of antimicrobial metabolites [17], which were identified as phenazines [14]. Our results showed that the crude extract was active against gram positive bacteria. Veselova et al. [8] demonstrated that extracts of *Pseudomonas chlororaphis* 449 which produces phenazines inhibited the growth of the gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus. Pseudomonas* spp. produces phenazine antibiotics-nitrogen-containing heterocyclic pigments, which exhibit broad-spectrum activity against numerous bacteria and fungi [7,18-20].

3.2 Antifungal Activity

Ps. chlororaphis subsp. aureofaciens inhibited significantly the growth of phytopathogenic fungi in dual culture. Depending on fungi isolate, significant differences were recorded. The inhibition varied from 55.56% *P. ultimum,* 62.5% *R. solani*, 65.67% *F. solani*, 75 to 77.78 % for F.O.L and F.O.A. respectively (Fig. 3).

The crude extract inhibited the fungal growth. The diameters of the fungal colonies were 3.99, 3.37, 3.08, 2.0 and 2.25 mm for *Pythium ultimum*, *Rhizoctonia solani, Fusarium solani*; *Fusarium oxysporum* f. sp. *albedinis* and *Fusarium oxysporum* f. sp. *lycopersici* respectively (Fig. 4). The tested strain was more active than the strain 30-84 which produces well known antibiotics [21]. Vincent et al. [12] using this bacterium (30-84) also demonstrated inhibition of *Gaeummanomyces graminis* var. *tritici*. In other experiments in Algeria other strains isolated in the same time from the same field were only active on media depleted in iron or containing low concentrations of iron, such as King's B [21].

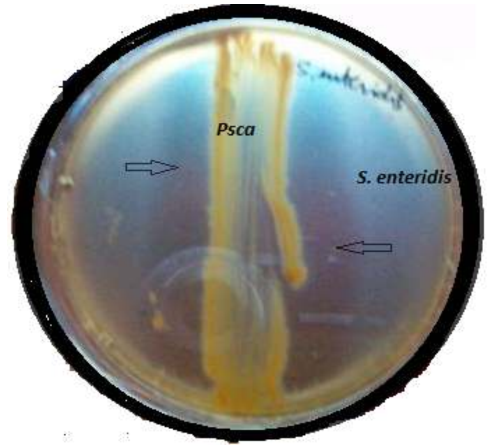

Fig. 1. Antibacterial activity of *Ps. chlororaphis* subsp. *aureofaciens* against *Salmonella enteritidis*
Psca: Ps. chlororaphis subsp. aureofaciens; S. enteridis: Salmonella enteritidis; arrows indicate inhibition zones

The selection of antagonistic strains among competitive Potato root colonizers yields a low frequency of strains producing well known antibiotics, contributes to the isolation of rhizobacterial strains with a higher variety of strains that could be useful in final protection of the plant against the phytopathogens. *Ps. chlororaphis* subsp. *aureofaciens* exhibited antagonism towards the test pathogens in dual culture, and showed maximum inhibition on PDA of the mycelial growth of *F. oxysporum*. However, the antifungal efficacy is less against *F. solani*, *P. ultimum* and *R. solani* [22-23].

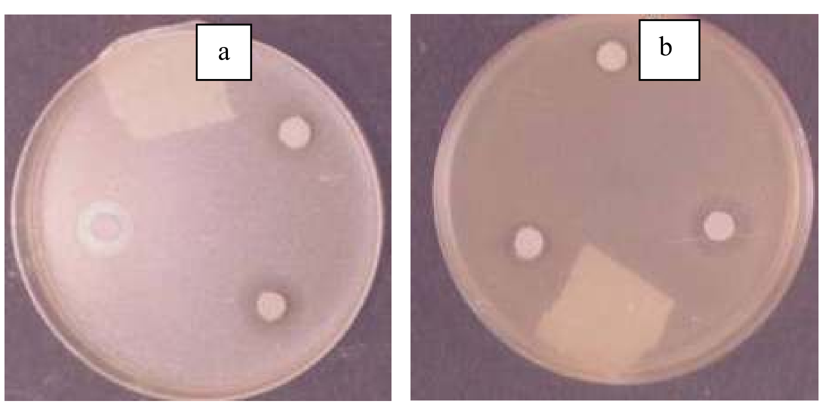

Fig. 2. Antibacterial activity of the crude extracts of *Ps. chlororaphis* subsp. *aureofaciens*
a: Against Bacillus subtilis, b: Against Ps. diminutus

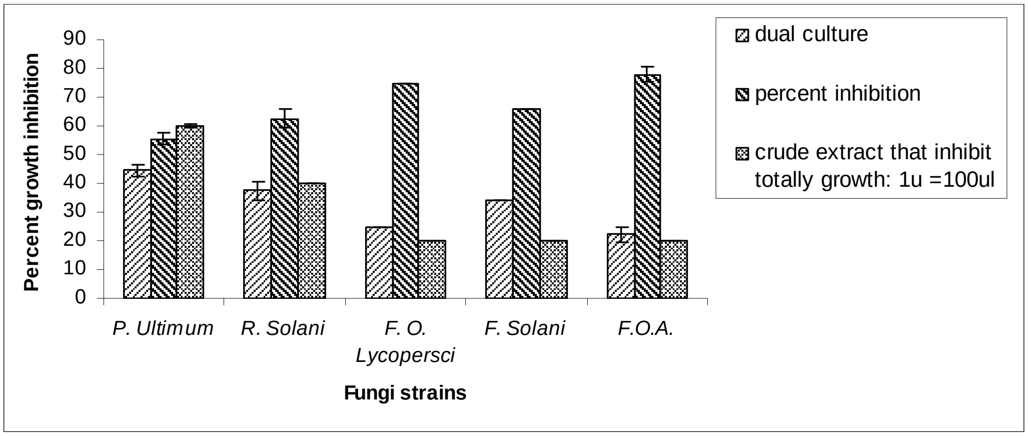

Fig. 3. Inhibition of fungi growth by *Ps. chlororaphis subsp. aureofaciens* in dual culture and its extracts

P. ultimum, R. solani, F.O. lycopercisi, F. solani, F.O.A are respectively Pythium ultimum, Rhizoctonia solani, Fusarium solani; Fusarium oxysporum f. sp. albedinis and Fusarium oxysporum f. sp. lycopersici

Earlier, Alabouvette [24], Deacon and Berry [25], demonstrated that the suppressiveness is specific of Fusarium wilts and not effective against diseases caused by non vascular Fusarium species including *F. roseum* and *F. solani*, or other soilborne pathogens. On the other hand we have already demonstrated that this bacterium produces bacteriocins [26]. These metabolites allow the bacterium to be competitive against the same genera or bacteria from other genera [21,26] if introduced in the field [21].

Table 1. Antibacterial activity of *Ps. chlororaphis* subsp. *aureofaciens*

Phytopatogenic bacteria	Cells activity	Crude extract activity
Bacillus subtilis	+	+
Ps. diminutus	+	+
Micrococcus luteus	+	±
Paracoccus paratrophus	+	±
Pathogenic bacteria		
Salmonella enteritidis	+	nd
Salmonella typhi	-	
Staphylococcus ATCC25923	-	
Klebsiella pneumoniae	-	
Morganella morigani	-	
Enterococcus	-	
Pseudomonas ATCC27853	-	
E. coli ATCC25922	-	

+: Positive inhibition, ±: Intermediate inhibition, -: Negative inhibition, nd: Not determined

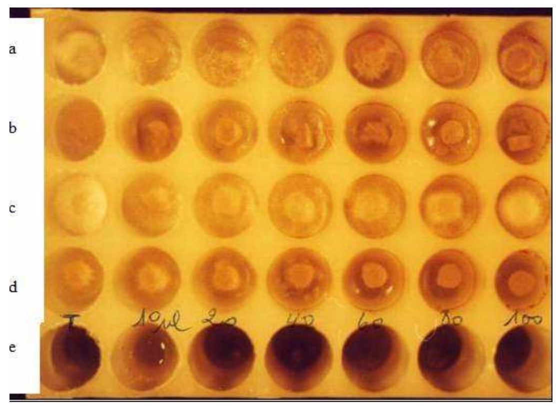

Fig. 4. Antifungal effect of *Ps. chlororaphis* subsp. *aureofaciens*

a, Rhizoctonia solani; b, Pythium ultimum; c, Fusarium oxysporum; d, Fusarium solani; and e, Control without fungi; T, control without extract; 10, 20, 40, 60, 80 and 100 µl of bacterial extracts incorporated in the medium

4. CONCLUSION

This antagonism was less specific for the studied isolate, whose activity was expressed on the three culture media used, and with all the fungal and phytopathogenic bacteria isolates tested. These results complete previous studies, and show that activity of the indigenous *Ps. chlororaphis subsp. aureofaciens* isolate is not specific to a kind of bacteria or fungi but could be useful in protection of plant against a large bacterial and fungal phytopathogens. It can constitute an effective mean of biological control in natural soil, to limit the use of Chemical inputs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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