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Isolation and Characterization of Novel Isolates of *Pseudomonas* of Biotechnological Interest from Soils and Olive tree necrosis

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In this study forty strains of *Rhizobium*, 95 isolates of *Pseudomonas* (60 isolates of *P. aeruginosa* and 35 isolates of *P. fluorescens*) and 2 fungus (*Aspergillus niger* AS4 and *Penicillium citrinium* PS12) were studied for their antagonistic activity against fefteen (15) isolates of *P.syringae* pv.*Savastanoi* the causative agent of the olive knot disease. This allowed us to select 69 inhibitory isolates (22 isolates of *Rhizobium* and 45 of *P. fluorescens*) and two fungus. The results revealed that the two inhibiting agents that which is produced by strain of *Rhizobium* RNU9 and isolate of *P. fluorescens* PN52 are bactericidal, thermolabile, sensitive to the SDS, Tween 80 to Tween 20 and proteases. The two agents showed resistance to pH (6, 7 and 8) but sensitivity to pH (9 and 10) and pH (2). In addition these same inhibiting agents showed à resistance at 50°C and 80°C but sensitivity at 100°C, 0°C and + 4°C. The selection of technological performance isolates useful for our agriculture could solve phytopathological problems and finally constitute a collection of the bacteria preserved.

Keywords: ***Pseudomonas.*** **Antagonism. Bactriocin. Olives. Biological control**

INTRODUCTION

Olive-trees are among the oldest cultivated fruit trees in the mediterranean including Algeria (Benjama, 2003). This one occupies a very important part in the agricultural economy of some mediterranean countries.

However the intensification of olives growing puts a number of problems as for example the infection of trees by phytopathogenesis bacteria among others kinds *Pseudomonas* (Mavrodi et al., 2001).*P.syringae* pv.*Savastanoi* , it is considered a phythopathogenesis bacteria causing tumors to olive trees what causes economic loses in this sector (Senhadji, 2005). Since the appearance of this bacteria in the west Algeria : Ain Temouchent and Sig (Mascara), the bacterial knot take more and more scale. So the fight against this diseases becomes a priority.The fight against this disease by chemical treatments becomes painful because the health of the consumers and the high cost of the fungicides, elements which do not favor the development of this chimical control (Benchabane, 2002, Neresen M. Abd El-Ghany al., 2017 and Syed Imran Ali, et al., 2019), it incites researchers to opt for a biological control studies showed that the inoculation of plants by means of some strains of *Pseudomonas* have an effect on the protection of plants against the pathogenic microorganisms (Digat, 1994, Chin, 2003 and Duhoux, 2004).

These bacteria can cause an increase at the level of resistance of plants to the various

diseases sorts of *Pseudomonas* which colonize the rhizosphere and several intrinsic characteristics and make them particularly intereting a use as agents of biological fight. In the first place, their capacity to colonize roots and to maintain a strong density of population there is remarkable (Haas and Défago, 2005).This big rhizocompetence comes probably of their growth rate higher than that of most of the other rhizobacteria and their capacity to metabolize effectively several compounds of roots exudates (Chin-A-Woeng et al., 1998). More than these bacteria are very easy to isolate and cultivate in laboratory.

This work is centred on isolation of new isolates of *Pseudomonas* from several ecological niche in Algeria. The selection of new isolates could solve problems and finally to establish a collection of bacteria.

MATERIALS AND METHODS

Samples

In the present study, 100 soil samples were collected: 60 samples in the region of Ain Temouchent and 40 in the region of Sig located in the west of Algeria. A total of 80 galls were collected, 40 at the twigs and 40 galls at the level of the leaves of olive- trees. After collection, samples were transported to the laboratory to be analyzed.

Bacterial strains and culture media

In this study forty (40) *Rhizobium* strains, and 2 fungi (*Aspergillus niger* AS4 *and Penicillium citrinium* PS12) were studied for their antagonistic.These strains are part of the bacterial collection of the Laboratory of Production, Plant and Microbial Valorization (LP2VM) of the department of Biotechnology, Faculty of Sciences, University of Oran (USTO M.B), Algeria.

To isolate bacteria of the genus *Pseudomonas,* several selective and non-selective culture media have been used. We used King's medium (A and B) (King et al., 1954), cetrimide medium (Azad et Cooksey, 1995), Hektoene agar (King et al., 1954), BCP agar (Nickerson, 1953) and Nutrient Agar (Lapage and Mitchell, 1970) were used to isolate and cultivate *Pseudomonas sp* (anonymous, 2001). YEM (Yeast Extract Mannitol) medium (Vincent, 1977) to cultivate *Rhizobium.*The Sabouraud medium (Sabouraud, 1910) to cultivate *Aspergilus niger* and *Penicillium citrinium*.These environments have also served as a basis for some identification tests and for other biochemical studies of bacteria. Other cultural medium were also used : medium Levane (Surico et Lavermicocca, 1989), Tween 80 medium (Bien et al., 1990), Pectinase medium (Hildebrand, 1971), Hildebrand Pectin (PH) medium (Hildebrand, 1971), Möeller's medium (Möeller, 1955), Clark's and Lubs medium (Clark, 1915), Mannitol-Mobility medium (Van Schothorst and Van beek, 1987), medium MEVAG (Medium Carbohydrate Attack Pathway) (Takao et al., 1994) and the sunflower milk medium (Vincent, 1970).

Isolation and counting of bacteria

Our study began with the isolation of *Pseudomonas* isolates (A total of 180 samples were collected).

The bacterial isolation technique is described by Vincent in 1970, Somasegaran and Hoben (1985 and 1994).

A series of dilutions (10^{-1} to 10^{-4}) of the soil samples is performed in NaCl (0.9%). A volume of 100 µL of each dilution is spread over several solid culture media (selective and non-selective). The cans are then incubated at 30°C for 24 hours.

Freshly washed galls are used directly, while those stored by desiccation are rehydrated by placing them in water for 24 hours in the refrigerator at +4°C, then for 1 hour at room temperature.

The surface of the galls of the twigs is first washed with water to remove soil particles and then rinsed three times with sterile distilled water (Belaskri, 2006). The superficial tissues of the galls are stripped with a sterile scalpel. The galls are then crushed and mixed with sterile distilled water. After crushing and obtaining a cloudy juice (crushed), using a platinum handle, the suspension is spread according to the four dial technique (Vincent, 1970) on the agars cast in boxes, after the boxes are incubated at 30°C for 24 to 72 hours.

The necrotic leaves were first washed with tap water and then superficially disinfected by immersion for 30 minutes in a solution diluted with 50% commercial bleach (12°) (Belaskri, 2006). After disinfection, the rest of the disinfectant solution is removed by three successive washes with sterile distilled water.

The fragments of the necrotic leaves were cut in half and seeded on culture medium with a loop. The Petri dishes were labelled and incubated at 30°C for 24 hours. All this handling is done near the Bensen burner, under a laminar flow hood.

Identification and characterization of isolates

The isolated have been characterized and identified by microbiological, and biochemical methods.The biochemical tests used during this study are:

In the first steep the LOPAT test and the Api 20 NE Galleries to identify the isolates of the *Pseudomonas* group secondly the biochemical tests and Api 20 E, to identify the subspecies *P. syringae* pv.*Savastanoi.*

The LOPAT test consists of Levane (Schaad, 1988): Polymerization of fructose to poly fructose on Levane medium, oxidase (Schaad, 1988): Demonstration of the presence of the enzyme oxidase, pectinase (Rathore et al., 2000): Demonstration of the presence of the pectinase enzyme, arginine dehydrolase (Schaad, 1988): Demonstration of the transformation of arginine by the enzyme arginine dehydrolase and hypersensitivity of Tobacco (Rathore et al., 2000): Demonstration of the pathogenicity of a bacterium by drying out the inoculation zones on tobacco leaves. All these tests are performed from a pure culture of 24 hours at30°C.

To identify the subspecies *P.syringae* pv. *Savastanoi* biochemical tests were made : Methyl Red test (RM) (Clark, 1915), the OF test (Oxidation / Fermentation), coagulation of milk (Lelliott and Stead, 1987), ability of *P.syringae* isolates to survive for 10 days in media containing different percentages (%) from sodium (0,1%, 1%, 5% and 10%) and 5% from sucrose (Schaad et al., 1988), hydrolysis of Tween 80 (esterase test) (Bien et al., 1990), catalase test and alkaline reactions of isolates on sunflower milk (Vincent, 1970).

Research for the serotypic type

The research for the serotype type of *Pseudomonas* isolates is performed using a rapid agglutination test. We used the kit (Slidex Pseudo) (Bio-Rad F-92430 Lot 4 J2027) reserved for the grouping of *Pseudomonas* (*P. aeruginosa* and *P. fluorescens*) because there is no kit for serotyping *P.syringae*. *Pseudomonas* isolates most often have group-specific antigens that can be extracted and identified with antiserums. The reagents consist of latex particles sensitized with antibodies directed against the group antigens. The presence of the corresponding antigens results in visible agglutination of the latex particles.The specific antigen of the group contained in the wall is identified by the particles of latex made sensitive in a body anti-antigen of a goup of *Pseudomonas*. If the antigen is present, the corresponding latex reagent is agglutinated, if the antigen is absent the latex reagent remains in homogeneous suspension.

Testing for resistance to antibiotics

The method we used to perform the antibiogram is that of Bauer and Kirby (1966), it is recommended by OMS. Two standards (NCCLS and SFM) have been applied. The measurement is compared to criteria established by the National Committee for Clinical Laboratory Studies (NCCLS) or that established by the French Microbiology Society (SFM). We studied the behavior of bacteria against 24 antibiotics marketed by Pasteur Institute of Algeria. The reference strain *P. aeruginosa* ATCC 27853 was used for the control of the antibiogram (analytical catalog of antibiograms extracted from a document on the standardization of the antibiogram, 4th edition 2005). As well as the natural resistance of *Pseudomonas* to antibiotics : Ampicillin (Am., 10 µg), Amoxicillin (Amx., 25 µg), Cefalotine (Cf., 30 µg), Cefazolin (Cz., 30 µg), Oxaciclline (OX., 01 µg), Streptomycin (S., 100 µl) and nalidixic acid (Na., 30 µg). These antibiotics were also tested on isolated *Pseudomonas* strains.

Bacterial interaction

In this study forty (40) *Rhizobium* strains, 95 *Pseudomonas* isolates and 2 fungus (*Aspergillus niger* AS4 *and Penicillium citrinium* PS12) were studied for their antagonistic activity against 15 isolates of *P.syringae* pv.*Savastanoi* the causative agent of the disease of the knots of olives.

The inter-bacterial inhibitions are realized by two methods

Direct method (confronting bacteria or fungus / bacteria) (Fleming et al., 1975) and indirect method (bacterial supernatant / bacterium confrontation) (Barefoot and Klaenhammer, 1983).

In order to determine the nature of the inhibitory agents the one which is produced by the strain *Rhizobium* RNU9 and the isolate of *P. fluorescens* PN52, the supernatants were characterized with respect to the temperature, the pH, the detergents (SDS, Tween 80 and Tween 20) and proteases. The mode of action of the antibacterial agent is also investigated.

RESULTS

The culture of samples from several ecological niches in Algeria on different environments allowed to observe bacterial

colonies with the morphological characteristics of the *Pseudomonas* reported in the literature (Botelho and Leda, 2006): whitish, creamy, bulging colonies on nutrient agar and bluish in the form of flat eggs on the Héktoé agar, bulging bluish colonies that characterize lactose-negative bacteria on BCP agar. These colonies have a regular or irregular outline with à diameter of 0.5 to 1.0 µm over 1.5 to 5.0 µm (or more) in length depending on the culture medium. We also noted the presence of fluorescent pigments on King's medium (A and B) and cetrimide agar.This indicates that the isolates do not belong to the same species (Botelho and Leda, 2006).

The microscopic examination of the isolates (after Gram coloring) showed that all the isolates are bacilli to straight purposes Gram negative. They appear mostly isolated or in diplobacilli, devoid of spores and capsules, mobile (after culture on mannitol medium-mobility).

This study confirmed the morphological characteristics of *Pseudomonas* cited in the literature (Philippe, 2007). One hundred and ten (110) isolates of *Pseudomonas* 60 of *P. aeruginosa* 35 isolates of *P. fluorescens*, and 15 of *P. syringae* were identified and characterized.

In addition , the LOPAT test performed on the 110 isolates classified the isolates into three different species by comparing our results with those described by Young and Triggs in 1994.The characteristics of *Pseudomonas* species is shown in Table. 1

We have therefore distributed our isolates as follows:

Fifteen (15) isolates of *P. syringae* coded: PN1, PN6, PN9, PN13, PN21, PN28, PN40, PN52, PN55, PN60, PN64, PN68, PN71, PN75 and PN80. These isolates have the ability to polymerize fructose to polyfructose on the Levane medium, do not possess an intracellular oxidase enzyme and sometimes even the enzyme arginine dehydrolase (ADH), capable of degrading pectin present in culture media (Pectinase and Pectin Hilderbrand) and their pathogenicity on tobacco leaves is positive.

Sixty (60) isolates of *P. aeruginosa* coded: PN2, PS3, PN4, PE6 ,PS11, PS12, PE13, PS14, PS16, PS17, PN18, PN19, PS21, PN23, PS24, PS25, PS26 ,PN27, PS30, PN31, PS32, PE34, PE36, PS37,PE39, PS40, PN43, PS44, PS45, PS46, PN47, PS49, PS50, PN51, PS53, PS56, PS58, PS60, PN62, PS66, PN67, PS71,PS72, PS75, PN77, PN78, PN79, PS81, PS82, PS83, PS85, PS86, PS89, PS90, PS92, PS94, PS96, PS97, PS98 and PS100.These isolates are unable to polymerize fructose to polyfructose on the Levane medium, have an intracellular oxidase enzyme and an arginine dehydrolase (ADH) enzyme, capable of degrading pectin present in culture media (Pektinase and Pektin Hilderbrand), and their pathogenicity on tobacco leaves is negative.

Thirty five (35) isolates of *P. fluorescens* coded: PS1, PE2, PE4, PS7, PE9, PS10, PE12, PS14, PS15, PE18, PS19, PE21, PS23, PE25, PE28, PN29, PE30, PS31, PE33, PS38, PE40, PS47, PS50, PS54, PS59, PN63, PS67, PS70, PN73, PN76, PS80, PS84, PS87, PS95 and PS99. These isolates have the ability to polymerize fructose to polyfructose, possess an intracellular oxidase enzyme and the enzyme arginine dehydrolase (ADH), which is unable to degrade pectin present in culture media (Pectinase and Pectin Hilderbrand) and their pathogenicity on tobacco leaves is negative.

Table 1 : Identification sheet for *Pseudomonas* of the fluorescent group according to Young and Triggs in 1994.

Test / ***isolates***	***Levane***	***Oxydase***	***Pectinase***	***Arginine deshydrolas***	***Hypersensitivity on tobacco***	***Fluorescence***
P.syringae	+	-	+	-/+	+	+
P.aeruginosa	-	+	+	+	-	+
P.fluorescens	+	+	-	+	-	+

(+) : Positive reaction.
(-) : Negative reaction.
(-/+) : Variable reaction depending on the isolates.

Subsequently, and in order to investigate the subspecies *P.syringae pv.Savastanoi* responsible for the disease of olive tree knots, the 15 strains of *P. syringae* (PN1, PN6, PN9, PN13, PN21, PN28, PN40, PN52, PN55, PN60, PN64, PN68, PN71, PN75 and PN80) were identified by other biochemical tests

All biochemical test results for the identification of the subspecies *P.syringae pv. Savastanoi* confirms that our isolates of *P.syringae* were indeed isolates of *Pseudomonas* phytopathogens.

The confirmation of the identification of the isolates of *Pseudomonas sp* was carried out by API tests.

The research of type serotype of *Pseudomonas* isolates is performed by a rapid agglutination test. The results of the agglutination tests show that the strains have different serotypes, for example the *P. fluorescens* isolate PS7 has the P1 serotype and the *P. fluorescens* isolate PS8 has the P3 serotype.

Among the 110 isolates tested, 57 showed sensitivity to almost all of the antibiotics tested. This sensitivity varies according to the species and strains of the same species. For example the diameter of the zone of inhibition to Colestine (CS) is 21 mm in the isolate of *P. fluorescens* PS3 and of 24 mm in the isolate *P. fluorescens* PS11 and the diameter of the zone of inhibition of Colestine (CS) is of 24 mm in the isolate of *P. aeruginosa* PS9 and 23 mm in isolate *P.aeruginosa* PS34

The antagonism was sought by two interactions realized by two methods. A direct method and an indirect method. In the case of inhibition, light areas appear as a halo around the keys or wells of bacteria tested.

The direct method made it possible to identify inhibitions and select strain pairs (inhibitors/indicators).

The confrontation bacteria / bacteria:

The isolates of *P. syringae* pv. *Savastanoi* were inhibited by *Rhizobium* and of *Pseudomonas* (*fluorescens* and *aeruginosa*) isolates. This is the case of the isolate *P. syringae pv. Savastanoi* PN15 have been inhibited by strains *Rhizobium* RNU1 and RNU2 and the isolate of *P. syringae pv savastanoi* PN15 and PN32 have been inhibited by the isolate *P. fluoresens* PS5.

The zones of inhibition were observed during the interaction between the *Pseudomonas* isolates *(aeruginosa* and *fluorescens*) and the *Rhizobium* strains. This is the case of the strain of *Rhizobium* RNU9 which inhibits the isolate of *P.aeruginosa* PS39 .While most *P. fluorescens* have been inhibited by a large number of *Rhizobium* strains this is the case, for example, of the strainof *Rhizobium* RNB11 which inhibits the isolates of *P. fluorescens* PS5, PS30, PN46, PS67, PN82 and PN101.

We can note that zones of inhibitions of 5 mm, 4 mm and 3.5 mm were observed in the couples of isolates (*P. fluorescens* PN52 / *P. syringae* PN33), (*Rhizobium* RNU9 /*P. aeruginosa* PN32) and (*Rhizobium* RNU4 / *P. syringae* PN58) respectively.

The fungus / bacteria confrontation:

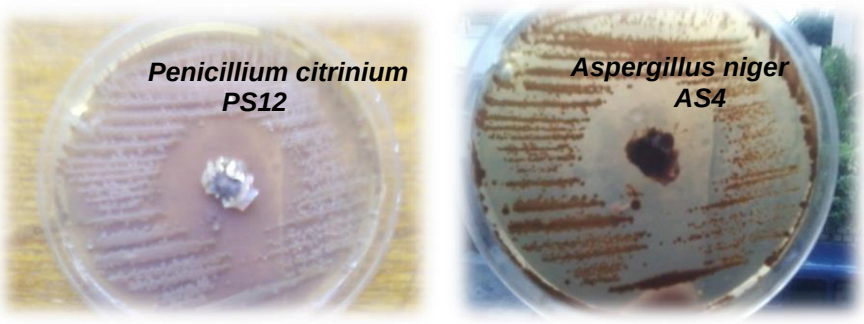
The fifteen (15) isolates of *P. syringae* were inhibited by the fungus *Penicillium citrinium* PS12 shown in figure. 1 (A) and by *Aspergilus niger* AS4 shown in Figure. 1 (B).

Inter-bacterial inhibitions caused by inhibitory agents of *Pseudomonas* isolates and *Rhizobium* inhibitors have been observed. We searched for the physico-chemical nature (sensitivity to heat, cold, detergents, and different pH values, sensitivity to proteolytic enzymes and the mode of action of the antibacterial agent), which led us to select 69 bacteriocinogenic isolates 22 *Rhizobium* strains and 45 *P. fluorescens* and both fungi.

The results revealed that the two inhibitory agents are bactericidal, thermolabile, sensitive to SDS, Tween 80 to Tween 20 and proteases. Two agents showed resistance to pH (6, 7 and 8) but sensitivity to pH (9 and 10) and pH (2). In addition, these same inhibiting agents showed resistance at 50°C and 80°C but sensitivity at 100°C, 0°C and + 4°C.

(A) : Mass strains are the isolates of *P. syringae* (PN7, PN14, PN15, PN27, PN32, PN33, PN38, PN43, PN48, PN51, PN58, PN63, PN72, PN77 and PN96).

(B) : Mass strains are the isolates of *P. syringae* (PN7, PN14, PN15, PN27, PN32, PN33, PN38, PN43, PN48, PN51, PN58, PN63, PN72, PN77 and PN96).



(A) **(B)**

Figure 1. Antagonism between the two fungi against *Pseudomonas* isolates (*Aeruginosa, Fluorescens* and *Syringae)* in solid medium.

DISCUSSION

The characterization of inhibitory agents was performed on supernatants of *Rhizobium* RNU9 strains isolated from *Retama monosperma* nodules (Kebaili, 2010) and *P. fluorescens* PN73 isolated in this study from olive tree necrosis. These isolates were chosen for the rest of this work because they showed an antagonism to the 15 strains of *P. syringae.*

We note that two inhibiting agents are resistant to pH (6, 7 and 8) and that these same agents lose their activity at pH (2) and pH values (9 and 10).

The results there fore show à better stability of the inhibitory activity at pH values between 6 and 8. These same results have been obtained by other researchers (Hirsch, 1979 and Rodelas et al., 1998) who have worked on bacteriocins from *Rhizobium* strains or on bacteriocins produced by other bacteria (Kacem et al., 2006). The effect of pH on the activity of bacteriocins produced by bacteria is related to the electrostatic interaction that occurs between the bacteriocin and the susceptible bacteria (Schved et al., 1994). Indeed, since the wall (peptidoglycan) and the membrane surface of the latter are negatively charged, the bacteriocin must be positively charged in order to come into contact with the bacterial cells. Yang (1992) reported that the adsorption of several bacteriocins is a function of pH and that the lower the pH, the higher the concentration of bacteriocins in the bacterial wall and the greater the lethal effect. The effect of pH on bacteriocin activity may be related to a solubility problem: bacteriocins or antibiotics are not active when they are not in solution (nisin which is active in an acid medium and not very active or not active at basic pH) (Lavermicocca et al., 1999).

We note that the inhibitor agent is heat-resistant since it retains its inhibitory activity at 50°C and there is slight resistance at 80°C compared to controls and no resistance at 80°C in the *P. fluorescens* inhibitor PN73. At 100°C no activity was observed for both strains. The inhibiting agents produced by the *Rhizobium* strain RNU9 and the *P. fluorescens* isolate PN73 are thermolabile because they do not resist 100°C. The same results were obtained by Sridevi, Mallaiah and Kazouz in 2008 who noted that when there is an increase in temperature, there is a decrease in inhibitory activity.

The inhibiting agent of the *Rhizobium* strain RNU9 does not resist at +4°C and 0°C. These results indicate that the inhibiting agent is cryosensitive. This result does not match the one found by Kazouz (2008). Indeed, this author showed that the substance produced by the *Rhizobium* strains is resistant at +4°C and 0°C while the *P. fluorescens* inhibitor PN73 has a slight resistance at +4°C and does not resist at 0°C.

The inhibitory activity of the inhibitory agent of the *Rhizobium* RNU9 strain disappears completely in the presence of Tween 80 and 20, these detergents act on proteins that have an oligomeric structure. This tends to dissociate these complex molecules into their constituent subunits, resulting in a total loss of inhibitory activity of these substances (Tagg et al., 1976; Kacem et al., 2006 and Ali et al., 2009).

The effect of Tween 80 and Tween 20 on the inhibitor agent causes a total disappearance of the inhibitory activity, which confirms that at least part of the molecule of these inhibitors is of a lipidic nature. These same results were obtained

by (Tagg et al., 1976) and Kazouz in 2008, on the other hand, the inhibitory agent of *P. fluorescens* PN73 shows a slight resistance in the presence of Tween 20 and Tween 80.

We have found that the inhibitory activity of the *Rhizobium* RNU9 inhibitory agent disappears at 1% SDS. While the *P. fluorescens* inhibitor PN73 has a slight resistance to 1% SDS. On the other hand, at 0.1% and 0.5% SDS, the two agents keep their inhibitory activities almost identical compared to the control.

SDS is known to be a protein denaturing agent. Similar results have been reported by several authors such as Sridevi and Mallaiah in 2008. Bacteriocins are generally characterized with regard to denaturing agents (detergents: SDS, Tween 20,...) (Kacem et al., 2005; Kazouz, 2008 and Sridevi et al., 2008).

We have noticed that the inhibitory agent produced by the two strains (*Rhizobium* RNU9 and *P. fluorescens* PN73 lose their inhibitory activities completely in the presence of trypsin, α-chymotrypsin, proteinase K and Pronase E.

Based on these results, we can suggest that both inhibitors are protein-based. This is one of the characteristics of bacteriocins produced by *Rhizobium* strains and *Pseudomonas* (Roslycky, 1967 and Schwinghamer, 1975) in particular and for other bacteriocins in general (Tagg, 1976 and Klaenhammer, 1988).

Agar fragments collected at the inhibition zones were seeded on the surface of the King B medium for *Pseudomonas* and the YEM medium for *Rhizobium.* After prolonged incubation at 28°C for *Rhizobium* and 30°C for *Pseudomonas,* no bacterial growth was observed. This shows that inhibiting agents have à bactericidal action on strains of *P. syringae* pv.*Savastanoi.* The method of (Toba et al., 1991) therefore revealed that both inhibiting agents have bactericidal activity. This is consistent with the results obtained by (Tagg et al., 1976). This is also consistent with the results obtained by (Tagg et al., 1991) and (Braun et al., 1994), which speak of a bactericidal effect of bacteriocins. In the literature, bacteriocins are often described as a substance with bactericidal activity (Hirsch, 1979; Selami, 2001 and Kazouz, 2008).

CONCLUSION

This study focuses on the isolation of *Pseudomonas* isolates from soil, olive necroses grown in Algeria (Ain Temouchent and Sig regions). This allowed us to isolate 60 isolates of *P. aeruginosa*, 35 isolates of *P. fluorescens* and 15 isolates of *P. syringae*. The isolated have been characterized and identified by microbiological, and biochemical methods.

In this study forty (40) strains of *Rhizobium*, 95 isolates of *Pseudomonas* and 2 fungus (*Aspergillus niger* AS4 and *Penicillium citrinium* PS12) were studied for their antagonistic activity against fifteen isolates of *P. syringae pv. Savastanoi* the causative agent of the disease of the knots of olive. This allowed us to select 69 inhibitory isolates : 22 *Rhizobium* strains, 45 from *P. fluorescens* and two fungus.

In order to determine the nature of the inhibitory agents that which is produced by *Rhzobium* strain RNU9 and *P. fluorescens* isolates PN52, the supernatants were characterized with respect to temperature, pH, detergents (SDS, Tween 80 and Tween 20) and proteases. The mode of action of the antibacterial agent is also sought. The results revealed that the two inhibitory agents are bactericidal, thermolabile, sensitive to SDS, Tween 80 to Tween 20 and proteases. Two agents showed resistance to pH (6, 7 and 8) but sensitivity to pH (9 and 10) and pH (2). In addition, these same inhibiting agents showed resistance at temperatures of 50 ° C and 80°C and sensitivity at temperature of 100°C, 0°C and + 4°C.

In our study we bring the inhibition of *P. syringae* pv.*Savastanoi* isolates (agent responsible for olive galls) by two fungus (*Penicillium citrinium* and *Aspergilus niger*) as well as by inhibitory agents produced by isolates of *Pseudomonas* and *Rhizobium*. This opens up prospects for the bio-control and conservation of plants and especially their protection (towards the phytopathogenic bacteria).

It remains to be verified first that the bacteriocin-producing by isolates of *Pseudomonas* and *Rhizobium* produce these bacteriocins stably and in sufficient quantities and active "in natura" against isolates of *P. syringae pv. Savastanoi* in the edapho-climatic conditions of our Algerian environment.

These preliminary results are original. Indeed the two strains of fungi (*Penicillium citrinium* PS12 and *Aspergilus niger* AS4) have an inhibitory action against the fifteen (15) isolates of *P. syringae pv. Savastanoi* and show no inhibitory action towards isolates of *Pseudomonas* (*aeruginosa* and *fluorescens*). Unlike those that have been reported so far in the literature to know that *Pseudomonas sp* attack fungus by these antifungal molecules this hypothesis deserves to be verified in the context of subsequent research.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

BK designed and performed the expeiments and wrote the manuscript. KM and K-H M reviewed the manuscipt. All authors read and approved the final version.

REFERENCES

Ali S F, Rawat L S , Meghvansi M K and Mahna S K. (2009).Selection of stress tolerant *rhizobial* isolates of wild legumes growing in dry region of Rajasthan, India. ARPN *Journal of Agricultural and Biological Science*. Vol. 4, N°1.

Anonymous. (2001). *Pseudomonas syringae* sp. *Savastano I* pv*. oleae.* http://www.inra.fr/internet/produits/HYP3/pathogen/6pssysa.htm. Bibliog. Net.

Azad H, Cooksey D. (1995). Un milieu semi sélective pour détecter les populations épiphytes et systémiques de *Pseudomonas syringae* de lauriers roses. *Phytopathologie* 85,740-745.

Barefoot S F and Klaenhammer. (1983). Detection and activity of lactacin B, a bactériocin produced by *L. acidophilus*. *Applied and Environmental Microbiology.* 45, 1808-1815.

Bauer A W and Kirby W. (1966). Antibiotic susceptibility t esting by a standardized single disk method. *Am. J. Clin Pathol*. 45, 493-496.

Belaskri A. (2006). Incidence de la maladie du Grown gall de l'Eucalyptus dans les pépinières forestières de l'Ouest Algérien. Diplôme d'Etudes Universitaire Supérieure. Option Microbiologique. Université d'ORAN (Es - Sénia). 30-35p.

Benjama A. (2003). Méthode d'évaluation rapide du degré d'attaque de l'olivier par la tuberculose causée par *Pseudomonas savastanoi* pv *savastanoi*, en verger au Maroc.Fruits.58,213-219.DOI:10.1051/fruits: 2003009.

Bien E D, Eicholorn K and Plapp R. (1990).Isolation and characterization of *Agrobactérium tumefaciens* from the German Vineregion. Rheinpfalz. *J. Plan Disea. Protea.* 97, 313-322.

Botelho G R and Leda C M. (2006). Fluorescent *Pseudomonad* associated with the rhizosphére of Crops – an overview. *Brazilian Journal of Microbiology*. 37, 401-416.

Braun V, Pilsl H and Grob P. (1994). The ecological rôle bactériocins in the bacterial competition .*Trends Microbiology.* 17, 151-158.

Chin A T F Woeng, G V, Bloemberg and Lugtenberg B J. (2003). Phenazines and their role in bio control by *Pseudomonas* bacteria. *New. Phyto*. 157,503- 523.

Chin-A-Woeng T F C, Bloemberg GV, Van der Bij A, Van der Drift K, Schripsema J, Kroon B, Scheffer R, Keel C, Bakker P, Tichy H, De Bruijin F, Thomas-Oates J and Lugtenberg BJ. (1998). Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol. Plant- Microbe Interact*. 11,1069–1077.

Clarck T J. (1915).*Pseudomonas aeruginosa*, Bactérialdesease:http://www.drlera.com/bacterial_diseases/*Pseudomonas_aeruginosa*.htm.

Digat B. (1994). Les bactéries stimulatrices de la croissance des plantes : le cas des *Pseudomonas.* C.R.Acad. *Agric. fr.* 80,125-140.

Duhoux E et Nicole M. (2004). Biologie végétale : associations et interactions chez les plantes. Atlas, Dunod, Paris. 166 p.

Fleming H P J L and Costilow R N. (1975). Microbiological inhibition of isolate of

Pediococcus from cucumber brine. *Applied and Environmental Microbiology*. 30, 1040-1042.

Haas D and Défago G. (2005). Biological control of soil – borne pathogens by fluorescent *Pseudomona*.

Hildebrand D C. (1971).Pectate and pectin gels for differentiation of *Pseudomonas sp* and other bacterial plant pathogens. 12, 1430-1436.

Hirsch P R. (1979). Plasmid determined bactériocin production by *Rhizobium Léguminosarum. Journal of General Microbiology*. 113, 219-228.

Kacem M, Zadi K H and Karam N. (2005).Detection and activity of plantaricin NOL15, a bactériocin produced by *lactobacillus plantarum* isolated from Algerian fermented olives, *Grasas Y Aceites* (Spain). 56, 192- 197.

Kacem M, Zadi K H. and Karam N. (2006).Testing of *lactobacillus plantarum* isolates from fermented green olives for their inhibitory activity against lactic acid bacteria and characterization of a bactériocine- like substance produced by *L. plantarum* OT2 isolate. *Dirasat, Pure Sciences.* 33, 224-234.

Kazouz H. (2008). Détection et caractérisation des bactériocines produites par des souches de *Rhizobia* isolées de différentes régions d'Algérie. Mémoire de magister de l'université d'ORAN (Es – Sénia). P, 22-29.

Kebaili H. (2010). Isolement et caractérisation des souches de *Rhizobium* isolées des nodules de *Rétama monosperma.* Mémoire de Magister de l'université d'Oran (USTO-MB). Option : Exploitation des Interactions Plantes – Microorganisme. P, 22-25.

King E O, Ward M K et Raney D E. (1954). Deux médias simples pour la démonstration de pyocyanine et de fluorescéine. *J. Lab. Clin. Med.* 44, 301- 307.

Klaenhammer T R. (1988). Bactériocins of lactic acid bactéria. *Biochimie.* 70, 337-349.

Lavermicocca P, Lonigro SL et Andolfi A. (1999). La production par bactériocine *Pseudomonas syringae* pv. *Ciccaronei* NCPPB2355 *ciccaronei*. Isolement et caractérisation partielle du composé antimicrobien. *J. Appl. Microbiol.* 86, 257-265.

Lelliott R A and Stead D E. (1987). Methods for the diagnosis of bacterial diseases of plants. *Methods in Plant Pathology*. Laboratoire de diagnostic en phytoprotection. Vol.2, P, 182-193.

Mavrodi OV, McSpadden Gardener BB, Mavrodi DV, Bonsall R F, Weller, D M and Thomashow L S. (2001). Genetic diversity of *phl*D from 2,4- diacetylphloroglucinol-producing fluorescent *Pseudomonas* species. *Phytopathol*. 91, 35–43.

Moeller V. (1955). Acta Pathol. Microbiol. Scand. 36,158.

Nesreen M. Abd El-Ghany, Atef S. Abdel R, Khaled D and Abdelhameed M.(2017). Efficacy of bio-rational insecticides against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on tomatoes. Biosience Research (Pakistan).p, 38.

Nickerson W J. (1953). Reduction of inorganic substances by yeasts.I. Extracellular reduction of sulfite by species of Candida. J. Infect. Dis. 93, 43.

Philippe L. (2007). Les procaryotes phytopathogénes. Chapitre 4 de phytopathologie. Ed; Freeman, New York. P, 432.

Rathore MS, Shekhawat, NS and Gehlot H S. (2000). Need of assessing *Rhizobia* for their plant growth promoting activities associated with native wild legumes inhabiting aravalli ranges of Rajasthan, *India. Botany Research International.* 2, 115-12.

Rodelas B, Gonzalez L, Salmeron J, Martinez V and Pozo C. (1998).Symbiotic effectiveness and bactériocin by *Rhizobium leguminosarum* pv.*viceae* isolated from agricultural soils in Spain. *Applied of Soil Ecology*. 8, 51-60.

Roslycky E B. (1967). Bactériocin production in the *Rhizobia* bactéria. *Canadian journal of Microbiology.* 13, 431-432.

Sabouraud R. (1910). Les teignes, Masson et Cie, Paris 553

Schaad N W. (1988). Laboratory guide for identification of plant pathogenic bacteria, 2éme Eds. P, 44-81.

Schved F, Lindner P and Juven B J. (1994). Identification and first characterization of bactériocins produced by lactic acid bacteria isolated from ovine cheese Pecorino del Poro. *Int. J. Dairy. Technol.* P, 1- 6.

Schwinghamer E A. (1975).Properties of some bactériocins produced by *Rhizobium tyrifolii*. *J. Gen. Microbial.* 91, 403-413.

Selami N. (2001).Contribution à l'étude de *Rétama monosperma.* Etude du système racinaire et recherche des associations de

type *Rhizobium.* Diplôme d'ingénieur en Biotechnologie. Option : Production et Valorisation Végétales. Université d'ORAN (USTO- MB). P, 2-9.

Senhadji A. (2005). Problématique de la tuberculose de l'olivier dans le plateau du Saiss. Journée nationale sur la protection de l'olivier, Marrakech. Le 27 mai 2005.P, 12.

Somasegaran P and Hoben H J. (1994).Handbook for *Rhizobia.* Springer verlage New York.Inc.P, 450.

Sridevi M and Mallaiah K V. (2008). Production of Bactériocins by root nodule bacteria. *International journal of Agricultural Reseach*. 3, 167-165.

Surico G et Lavermicocca P. (1989).Un milieu semi sélective pour l'isolement de *Pseudomonas syringae* pv. *Savastanoi*. *Phytopathology.* 79,185-190.

Syed Imran A, Dr. Baqar S N and Samiyah T. (2019). Antimicrobial Potential of *Lactobacillus* and Bifido bacterium strains Isolated from Probiotic Antidiarrheal Formulations in Pakistan: An *In Vitro*validation against Diarrheagenic *E. Coli*, *Biosience Research* (Pakistan). p, 1444.

Tagg J R, Dajani A and Wannamaker LW. (1976). Bactériocins of Gram positive *Bacteriology Review*. 40, 722-756.

Tagg J. (1991). BLIS production in the genus *Streptococcus*. In: *Bacteriocins, Microcins and Lantibiotics* (James, R.; Lazdunski, C.; Pattus, F. Eds), Springer- Verlag, New- York, p, 417-420.

Takao T. Watanabe N, Yag I and Sakata K. (1994).A simple screening methode for anti-oxydants and isolation of several antioxidant produced by marine bacteria from fishand shellfich, Bioscience, Biotechnology and Biochemistry, 58,1780-1783.

Toba T, Samant S K, Yoshioka E and Itoh T. (1991).Reutericin 6, a new bactériocine produced by *Lactobacillus reuteri* LA.6- *Letter of Applied Microbiology.* 13, 281-286.

Vanschothust M, Renaud A, and Vanbeek Cn. (1998). *Samonella* isolation using R V S broth and M LC B agar. Foud. Micobiol. 4,11-18

Vincent J M. (1977). Root nodule symbiosis with *Rhizobium.* In the biology of nitrogen fixation quispel. A. Eds. North– Holland. Amsterdam. P, 256-341.

Vincent J M. (1970). Un manuel pour l'étude pratique des bactéries nodulaires de la racine.Manuel n ° 15 de l'IBP, Blackwell Scientific Publishers, Oxford, p, 164.

Yang R, Johnson M C and Ray B. (1992).Novel method to extract large amounts of bactériocins from lactic acid bacteria. *Appl. Environ. Microbial*. 58, 3355-3359.

Young, J M et Triggs, C M. (1994). L'évaluation des tests déterminant pour pathovars de *Pseudomonas syringae* van Hall 1902. *J. Appl. Bacterial.*77, 195- 207. Bibliog. Net.