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PRELIMINARY STUDY OF ANTIMICROBIAL ACTIVITY AND PHENOTYPIC CHARACTER FROM ACTINOMYCETES AND *PENICILLIUM* SP

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ABSTRACT: Indeed, Actinomycetes and *Penicillium* are microorganisms that live in different ecosystems. Besides, they play important roles in various fields in respect such as agriculture, medicine and the environment. During this study, we were interested in the isolation of two strains namely: Actinomycetes and *Penicillium* sp. from coconuts and truffle respectively. In addition, the agar cylinder technique demonstrated the antibacterial activity of Penicillium sp. against four pathogenic bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*). In virtue of which, the results showed a variability of the diameters of the inhibition zones, thus the most important results were recorded against *K. pneumoniae* and *S. aureus*, which produced inhibition zones of 17.50±0.71 mm and 17.00±0.00 mm respectively. In order to struggle against pathogenic fungal strains, the ability of Actinomycete strain to suppress the mycelial growth of 03 pathogenic strains of *Fusarium oxysporum* f.sp. *albedinis* (EF1, EF2 and EF3) was examined. Hence, the result of the direct confrontation tests on the PDA medium between the Actinomycete strain and these pathogenic strains revealed a significant inhibition of the mycelial growth of EF3 (62,5%) and EF2 (52,70%). On the other hand, the lowest inhibition rate was recorded in EF1 (34.55%).

INTRODUCTION: Actinomycetes constitute a kind of saprophytes prokaryotic group, aerobic or anaerobic, Gram-positive filamentous bacteria, belonging to the phylum *Actinobacteria* and *Actinomycetales* order. In the microbial world, Actinomycetes are phylogenetically defined as taxa with a high GC range between 57-75% in their DNA[1, 2].

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Besides, this bacterium is characterized by a long-branched filamentous structure morphology like fungi with the presence of asexual spores; when growing on a solid medium, branching hyphae developed by Actinomycetes grow both on the surface of the substrate to form an aerial mycelium and within it to form a substrate mycelium, which reproduces either by fission or by spore formation [3, 4].

Actinomycetes are considered to be one of the most important and abundant types of beneficial bacteria that spread in a large number of ecosystems around the world, forming a large part of the microbial population of the soil and aquatic environment [5].

For this reason, they are becoming a point of interest for microbiologists especially after the discovery of members of this genus which have the ability to produce bioactive secondary metabolites, in effect, 45% of all bioactive microbial metabolites discovered as enzymes, siderophores, herbicidal and insecticidal compounds, and more than 10,000 antibiotics have been isolated from Actinomycetes. In general, these substantial characteristics promote the use of Actinomycetes in a vast range of applications in various fields. In agriculture, many species of *Actinobacteria* are used as potential biological control agents against various diseases caused by phytopathogenic microorganisms [6].

Nowadays, drug-resistant bacteria are considered a serious global public health problem that can cause refractory infections, prolonged illnesses with increased risks of transmission and higher incidences of death [7]. Before this difficult situation, all efforts must be united to struggle against these resistant bacteria. In particular, the encouragements of scientific research whose objective is the discovery of effective natural substances which can eliminate resistant strains and reduce the uncontrolled use of antibiotics.

Filamentous fungi stand for a diverse group of eukaryotic microorganisms able to produce natural substances and are receiving the attention of several international health agencies. Indeed, most pharmaceutical industries have focused their research on controlling the phenomenon of bacterial resistance to antibiotics and researching new antibacterial molecules from filamentous fungi [8].

Accordingly, fungi are playing an important role in the daily life of human beings in addition to their participation in biotechnology applications due to the diversity of their secondary metabolites [9]. In this context, the current research aimed to isolate Actinomycete and *Penicillium* sp. and screened their antimicrobial activities *in-vitro* against phytopathogenic and pathogenic bacterial strains. In addition, this work was carried out without prior bibliographic information, to our knowledge, on the presence and isolation of Actinomycetes and *Penicillium* sp. from the Coconut and Truffle respectively.

MATERIALS AND METHODS:

Actinomycetes Isolation: Indeed, the isolation of Actinomycetes was carried out from coconut fruits; firstly, the fruits were opened to remove the water and separated into two parts, then left at room temperature for a wee; afterward, we scraped the inner surface of the coconut and streaked the Petri dishes containing the AM culture medium. Finally, the plates were incubated at room temperature for 24 to 48 hours. The AM medium contained (g/L) 3; yeast extract, 2.02; $CaCO_3$, 0.05; $MgSO_4$, 0.05; $K2HPO_4$, 2; KNO_3, 20; Agar, using the modified protocol of [10].

After 48 hours of incubation, successive subcultures were carried on the AM medium to ensure the purity of the Actinomycete colonies. Further, representative colonies with mycelial morphological structure were selected and re-streaked on a new plate containing the same medium. Then, the plates were incubated for 24 to 48 hours at 28 °C [11]. The macroscopic examination of colonies indicated that they correspond to the genus Actinomycetes (filamentous appearance), which were grown in AM agar tubes test slant, then incubated at 28 °C for one week. Subsequently, they were stored at 4 °C for further studies [12].

Identification: Identifying isolated strain was carried out by determining the morphological, physiological and biochemical characteristics thereof. Moreover, the macro-morphological study allows us to determine the colour of substrate mycelium (reverse colour) and aerial mycelium to evaluate the importance of growth (very important, important, medium, weak, or absent), and the production of soluble pigments, a character which is determined by the colour change in the medium compared to its initial colour. Then, we carried out a Gram stain to determine the Gram of this strain [13, 14].

Catalase Test: The test consists to put the Actinomycetes colonies in contact with hydrogen peroxide "H_2O_2". To do this, a drop of H_2O_2 was placed with a Pasteur pipette on a clean microscope slide, then a colony of the Actinomycete strain was removed using a sterile inoculation loop and dissociates this colony in the drop of H_2O_2 f it has catalase (positive reaction), it degrades hydrogen peroxide into water and oxygen.

The positive reaction is evident by the immediate formation of bubbles, if there are no bubbles, the test is negative [15, 16].

Mannitol-Motility Test: This test was carried out on the semi-solid mannitol-motility medium. By central sting, seeded the tubes of mannitol-motility from the isolated colony then incubated at 37 °C for 24 to 48 hours. After incubation, the tubes were observed: if there is a change in the color of medium from red to yellow, there is a corresponding acidification of the medium, grace to the pH indicator "Phenol-Red" used, which is red at neutral pH and turns to yellow on change of pH acid. The motile strain produces diffuse growth throughout the medium and the medium becomes trouble. However, if the culture stays only on the line of the central inoculation, the strain is considered immotile [13].

Starch Hydrolysis: This test was carried out on the concentrated Potato Dextrose Agar (PDA) medium. PDA was into Petri dishes, and then inoculated by streaking from colonies of the isolated strain. Besides, the dishes were incubated at 30 °C for 24 to 48 hours. After incubation, Iodine solution was flooded or vaporized on the surface of the medium, starch hydrolysis is visualized as clear halos (clear zones) around the growth colonies while the rest of the medium is collared in blue [17].

Antifungal Activity: The antifungal activity of the isolated strain was achieved by the confrontation method described by [18, 19], to assess their antagonistic potential against three phytopathogenic fungal strains EF1, EF2 and EF3. Nevertheless, this test was carried out in double culture on Petri dishes A disc of each fungal pathogen was placed in the center of the plate containing the PDA medium. Then, the Actinomycete strain was streaked on the two opposite sides of the disc at a distance of 2cm from the edges of the dish. In other dishes containing the PDA medium, a culture of the pathogens individually in the absence of Actinomycete was used as a control in this assay. Finally, all the dishes were incubated at 27 °C for 7 days, and the experiments were repeated in triple. On the other hand, the assessment of mycelial growth is carried out after seven days of incubation. Indeed, the effectiveness of Actinomycete to reduce the growth of phytopathogenic fungal was calculated according to the formula

$$R= C – T / C \times 100$$

Where: R: Inhibition rate, C: Radial growth of the pathogens in control, T: Radial growths of the pathogens in the presence of Actinomycete isolate.

Isolation and Identification of *Penicillium* sp: *Penicillium* sp. was found coincidentally on the surface of the truffle which naturally grows in the soil, in the Ain Sefra region, Naâma, Algeria. Besides, a sample of *Penicillium* sp. was taken from the surface of the truffle and deposited in Petri dishes containing the PDA medium. Then, the dishes were incubated in the dark at 27 °C for 7 days.

After incubation, we noticed the development of different fungal colonies, in respect such as the characteristic colony of *Penicillium* sp. was sub-cultured separately for purification. Besides, obtaining a pure strain requires successive transplanting of the colony. For such purpose, a disc from the colony's margin was transferred by using a sterile loop in the center of a Petri dish containing the same PDA medium and incubating the dishes at 27 °C for 7 days [20].

Macroscopic identification of the isolated strain was carried out from a 7-day-old culture. Indeed, this identification was based on determining the aspect, the colour and the contour of colony, together with the mycelium shape [21, 22]. Furthermore, microscopic identification was carried out as follows: a small mycelial fragment was placed on a clean slide, covered with a coverslip, and slightly crushed, and then the slide was observed under an optical microscope (Gx40). Besides, this was based on observing vegetative forms such as hyphae (septate or no-septate), and reproductive structures such as conidiophores and conidia [23].

Bacterial Strains Tests: In this study, we used four pathogenic bacteria, one Gram-positive and three Gram-negative. The latter comes from the internal bacteriology laboratory of the Mesbah Baghdad Hospital in Tamanghasset, Algeria. Likewise, the three Gram-negative bacteria were reactivated on Nutrient agar, while the Gram-

positive bacterium was reactivated on Chapman medium. In closing, all these bacteria were incubated at 37 °C for 24 h.

Antibacterial Activity: The antagonism test of the obtained fungal strains against the four pathogenic bacteria aimed to research the biological activity of these fungi on the growth of these bacteria. However, the agar plug diffusion method 24 performed the antibacterial activity assay assay. Briefly, the sterile dishes were inoculated using 1 mL of a suspension containing 10^6 CFU mL^{-1}. These suspensions were prepared from a young culture of each strain. On the other hand, the discs of 6 mm in diameter were taken from the fungal colony using a sterile loop, then deposited on the Mueller–Hinton Agar (MHA), which was used as the culture medium for the bacteria, which were previously inoculated by the strains examined. These dishes were incubated at 37 °C for 24 h. After incubation, the diameters of the inhibitory zone, which was observed around the fungal discs, were measured using a ruler in millimeters (mm). In addition, this zone of inhibition was considered the clear halo around fungal discs when there is a complete absence of bacterial growth. Indeed, antibacterial activity results were presented as mean values of the three repetitions measured.

RESULTS AND DISCUSSION: After two days of incubation, the development of different colonies was observed on the AM medium; besides, we determined, via macroscopic observations, the colonies which belong to the genus *Actinobacteria* (presence of some mycelial filaments). The colony selected and purified on the AM medium presents clear aerial mycelia, rapid growth, and named strain F.

Morphological Characters and per Identification of Strain F: Macroscopic observation of the colony shows a medium size and domed shape, with a vegetative mycelium surrounded by an aerial mycelium of white colour **Fig. 1**. According to [25], the colours of the aerial mycelia of Actinomycetes have shown to be different from those of the substrates, and most have a whitish colour, and others have different colours like black, brown, green and yellow. Similar results were obtained by [5], [26] who showed that morphological properties (aerial and substrate mycelial colour, soluble pigment and the shape of spore) were used to classify the isolated strains in the genus *Streptomyces.*

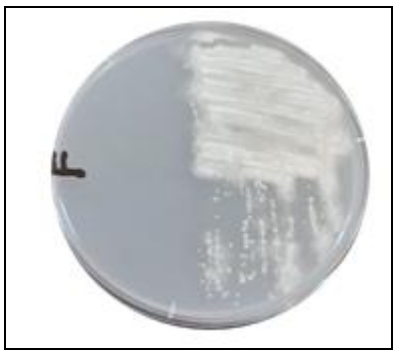


FIG. 1: PURIFICATION ACTINOMYCETES ON THE AM MEDIUM

Gram stain examination of F strain indicated that our F strain is a Gram-positive bacterium with well-developed, ramified, and fragmented aerial mycelium, carrying chain spores **Fig. 2**. According to the morphological characteristics of our strain, we can conclude that the isolated strain is very similar to the characteristics of the genus Streptomyces. This result is similar to [27] who published that all the isolated Actinomycete strains are filamentous bacteria with Gram-positive staining. Identically, [11] confirmed that Actinomycetes isolated from soil samples were Gram-positive bacteria. In addition, the results obtained by [14, 28] showed that microscopic observation of bacteria belonging to the genus *Streptomyces* presents a filamentous appearance with isolated spores, in clusters, short or long chains.

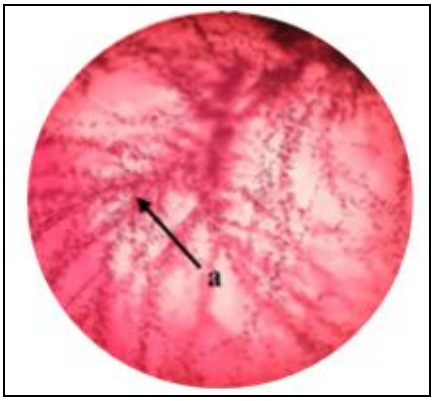


FIG. 2: MICROSCOPIC APPEARANCE OF THE ISOLATED STRAIN AFTER GRAM STAIN; A: CHAIN SPORES

Catalase Test: Catalase test indicated that the F strain secretes the catalase enzyme (positive

reaction) responsible for the degradation of hydrogen peroxide into water and oxygen by the formation of bubbles directly after the contact of H_2O_2 with the colony F. Likewise, species of the genus Actinomycetes have the capacity to secrete a very important variety of hydrolytic enzymes, in respect such as catalase [29], [10]. The same results were obtained by [30, 31] who found that all Actinomycete isolates showed the ability to produce catalase enzyme.

Mannitol-Motility Test: This test indicates a change in the medium colour, which became orange-yellow compared to the first case of red. However, this change corresponds to the medium acidification, grace to the Phenol-Red used for this test as pH indicator, which is red at neutral pH and turns to yellow on change of acid pH. Hence, this reflects the strain's ability to ferment mannitol. In addition, a diffusion of the culture over the entire surface was observed in the medium (troubled medium), which confirms that strain F was motile. Thus, the same results were published by [32, 3].

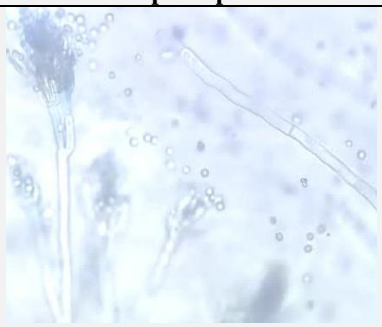
Starch Hydrolysis: After 2 days of incubation, a clear and transparent zone around the strain F was observed, while the rest of the medium was coloured in blue, which means a positive result, reflected by the hydrolysis of starch by amylases which are secreted into the medium by the same strain. Our results were similar to the previous studies by [30] which found, when studying the biochemical properties of Actinomycetes, that all tested isolates were positive for starch hydrolysis. Additionally, the results of amylolytic activity as reported by [33-35] confirm that all isolated Actinomycetes showed amylolytic activity.

Antifungal Activity: Before implementing a strategy to control phytophatogenic fungi using biological products, it is important to know the behaviour of the antagonistic agents and their interaction with the pathogen. The direct confrontation on the PDA medium between the F strain isolated from thecoconutt and the pathogenic strains EF1, EF2 and EF3 allowed us to demonstrate the ability of the F strain to inhibit mycelial growth of these pathogens.

After 7 days of incubation, there is a variation in the inhibition rates between the pathogenic strains. Besides, the highest inhibition rate (62.50 %) was recorded by the F strain against EF3, whereas; the lowest rate was produced by the same strain against *Fusarium oxysporum* f. sp. *albedinis* (EF2) which was equal to 34.55 %. Likewise, some work providing similar results to ours was done by [18] on *Fusarium* strains. They showed that the BPSWAC 23 strain of the genus *Streptomyces* inhibited the growth of Foc with an inhibition rate equal to 57.89 % compared to that observed against EF2 which was 52.70 %. On the other hand, the EF3 strain showed an important percentage of inhibition that is equal to 62.5 %; the latter is higher than that of [36], who published that Streptomyces sp. inhibits the mycelial growth of *Fusarium oxysporum* with an inhibition rate of 29.5 %. Indeed, 8 % of Actinomycetes isolated from soil showed antifungal activity [14]. The antagonistic activity of *Streptomyces* strain PP14 against phytopathogenic fungi such as *Fusarium* sp. was demonstrated by [26].

***Penicillium* sp. Identification:** Macroscopic and microscopic characters of the isolate fungi were recorded in the table below:

TABLE 1: MACROSCOPIC AND MICROSCOPIC CHARACTERISTICS OF *PENICILLIUM* SP

Macroscopic aspect	Microscopic aspect G x40	Identified Genera
		*Penicillium* sp. [37, 38]
Macroscopic: Colour: dark green; Texture: fluffy; Border: irregular; Margin: White Microscopic: Hypha: septed; Conidiophores: branched; Conidia: round in chains forming a brush.		

Antibacterial Activity: After 24 h of incubation at 37 °C, clear halos representing the inhibition zones **Fig. 3** appeared around the various discs of *Penicillium* sp. against all pathogenic strains tested. According to the results, growth inhibition was observed in all of the pathogen strains, with the exception of the *E. coli* and *P. aeruginosa*, which were more resistant than the others. In contrast, *S. aureus* and *K. pneumoniae* have shown to be more sensitive to bioactive substances secreted by *Penicillium* sp. **Table 2**. Generally, bioactive substances produced by filamentous fungus carry valuable properties, antioxidant, antidiabetic properties and have a very large spectrum of antimicrobial activity against various microorganisms [39, 40, 41]. Furthermore, many researchers have reported antibacterial activity of *Penicillium* sp. against *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus* [42, 43] reported that the antibacterial activity of *Penicillium* sp. against *S. aureus* and *K. pneumoniae* exhibited the inhibition zones of 10.66±0.33 mm and 10.12±1.00 mm, respectively. However, our data revealed high antibacterial activity of *Penicillium* sp. against *S. aureus* (17.00±0.00 mm) and *K. pneumoniae* (17.50±0.71 mm). In addition, several bioactive compounds extracted from filamentous fungus, such as *Penicillium* species, were investigated for many biotechnological and therapeutic usages [44, 45].

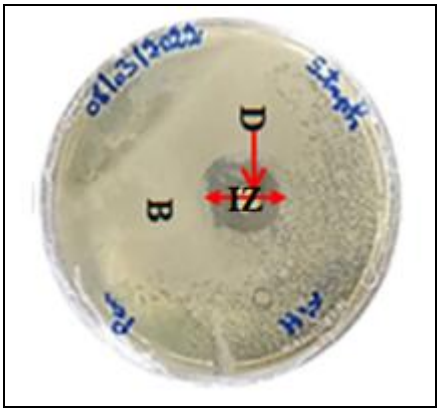


FIG. 3: ANTIBACTERIAL ACTIVITY OF *PENICILLIUM* SP. ON THE GROWTH OF PATHOGENIC BACTERIA (EX: *STAPHYLOCOCCUS AUREUS*). IZ: INHIBITION ZONE; B: BACTERIAL MAT; D: FUNGAL DISC

TABLE 2: THE INHIBITORY EFFECT OF *PENICILLIUM* SP. ON THE GROWTH OF BACTERIAL SPECIES

The inhibition zone diameters in mm of the strains examined ±SD	
Pathogenic bacteria	*Penicillium* sp.
Escherichia coli	11.50±0.71
Klebsiella pneumoniae	17.50±0.71
Pseudomonas aeruginosa	12.00±1.41
Staphylococcus aureus	17.00±0.00

CONCLUSION: In the light of the facts set out above, the present study concluded that Actinomycete strain isolated from coconut produced catalase and amylase whilst the tests were positive for fermentation of mannitol and Gram stain, respectively. However, this isolated Actinomycete performed an antagonistic reaction against the three *Fusarium oxysporum* f. sp. *albedinis* strains. More to the point, *Penicillium* sp. isolated may possess antibacterial potential against all tested pathogenic strains. More intensivestudies shouldd be conducted on the isolated strains to characterize their bioactive substances and determine the accurate taxonomic position by molecular identification.

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