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Full Length Research Paper

Exploring the diversity and antimicrobial activity of *Aspergillus* strains in Algerian soil and water systems

Bramki Amina[1*], Ghorri Sana[1], Jaouani Atef[2], Dehimat Laid[1] and Kacem Chaouche Noreddine[1]

[1]Laboratory of Mycology, Biotechnology and Microbial Activity, University of Mentouri Brothers- Constantine, P.O. Box, 325 Ain El Bey Way, Constantine, Algeria.

[2]Laboratory of Microorganisms and Active Biomolecules, University of Tunis El Manar, Campus Farhat Hached, B.P. no. 94 - Rommana, Tunis 1068, Tunisia.

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Thirty two strains of *Aspergillus* genus were isolated from soil samples obtained from particular ecosystems: Laghouat endowed with a desert climate and Teleghma with a warm and temperate climate. Based on the morphological aspect, this collection was subdivided into ten phenotypic groups. This identification was confirmed by molecular analyzes using a molecular marker of the genu ribosomal 18s. This marker will allow us to associate our sequences with those of known organisms. In order to discover new antibiotic molecules, the antibacterial activity was performed against two Gram positive bacteria: *Staphylococcus aureus* and *Bacillus subtilis* and also two Gram-negative bacteria: *Escherichia coli* and *Pseudomonas aeroginosa*, using two different techniques: Agar cylinders and disks technique. The results show that the fungal species have an activity against at least one test bacterium. The Gram positive bacteria were the most affected, where the averages of the inhibition zones reach 34.33 mm. However, Gram-negative bacteria showed less important results from 0 to 12.00 mm. It is recorded that the antibacterial activity was studied for the first time in the following two species: *Aspergillus niveus* and *Aspergillus wentii.* Furthermore, an indepth study is underway on bioguided fractionation, which would identify individual components and lead to the isolation of the active ingredient.

Key words: *Aspergillus*, particular ecosystems, antibacterial activity.

INTRODUCTION

The massive use of antibiotics has not led to the elimination of infections, but has made microbes resist these antibiotics by exerting a selection pressure which favors the emergence of resistance genes in bacteria, making multi-resistant strains to be responsible for serious infections. In recent years, bacterial resistance to antibiotics has become a worrying global phenomenon (Courvalin and Philippon, 1990; Bevilacqua, 2011). Due

*Corresponding author. E-mail: br_amina@yahoo.fr. Tel: +213 6 69 43 31 06.

to the increase in infections involving multi-resistant bacteria, the need for new effective antibacterial treatments is more and more imperious (Savin, 2014).

For this, the biodiversity is exploited. The isolation of strains from rarely exploited ecosystems allows the discovery of strains that may have a high potential or unexploited production. In addition, the use of antibiotic-resistant bacteria as germs tests can lead to the discovery of effective molecules which may be new (Boughachiche et al., 2012). In this field, fungi have been widely studied. Of a total of 10700 antibiotics described for the entire living world, about 1600 are from fungi (Botton et al., 1990).

Aspergillus species are ubiquitous molds, widely distributed in nature (Bennett et al., 2010). From an economic view, the species of this genus have a great ecological and medical importance. The production of extracellular and intracellular secondary metabolites by several species of this genus was detected in *Aspergillus aculeatus, Aspergillus ochraceus* and *Aspergillus terreus*. The latter are considered to be the highest producers of extracellular secondary metabolites (Youcef-Ali, 2014). Indeed, *Aspergillus* are known for their ability to produce antibacterial substances such as *A. flavus* used in the manufacturing of aspergillic acid, *A. ochraceus* used in the manufacturing of penicillic acid and *A. Fumigatus* used in the manufacturing of fumagillin (Botton et al., 1990; Taniwaki et al., 2003; Abdelaziz, 2006).

This study aimed to show the antibacterial activity of *Aspergillus* strains isolated from different ecosystems and producers of active metabolites used against Gram positive and negative resistant bacteria through different methods.

MATERIALS AND METHODS

Sampling

This work deals with the isolation of *Aspergillus* from soil. For this purpose, the sampling was done in two different Algerian regions namely: Laghouat (located 400 km in south Algeria at 750 m of altitude) and Teleghma (located in north - eastern Algeria).

To do this, 100 g of soil was sampled up to 20 cm deep after discarding the first three centimeters of soil, and then deposited in glass bottles under strict aseptic conditions. Mycological analysis was done on arrival at the laboratory (Pochon, 1964; Almi et al., 2015).

Isolation

The isolation was performed by the suspension - dilution method (Davet, 1996; Davet and Rouxel, 1997). Decimal dilutions were prepared from 10^{-1} to 10^{-6}. Then, 0.1 ml of each suspension is rolling out on the surface of a potato dextrose agar (PDA: 200 g of potato, 20 g sugar, 20 g agar and 1000 ml distilled water) medium. Three dishes were prepared for each dilution, and the plates were incubated at 28°C for 6 days.

The proliferation of bacteria was prevented by adding an antibiotic, streptomycin (5 mg/l) in the culture medium (Botton et al., 1990). After the appearance of fungal colonies, these were purified followed by successive subcultures in the same isolation medium (Boudoudou et al., 2009), and they were preserved in the form of spores in a glycerol/saline solution of 30% at -20°C (Botton et al., 1990).

Morphological identification

The strain identification was performed by macroscopic observation (growth rate, colonies color and color variation over time, colonies upside color, surface texture, etc) and microscopic characters (mycelium, conidiophores, conidiogenesis, conidia, etc) (Botton et al., 1990; Chabasse et al., 2002, 2008).

Molecular identification

DNA extraction and quantification

First, a quantity of mycelium (≈ 100 mg) was mashed in the presence of 500 µl of lysis buffer (400 Mm Tris/HCl, 60 Mm EDTA, 150 mM NaCl, 1% SDS, 2 ml H_2O ultrapure). The mixture was left to rest for 10 min and then 150 µl of potassium acetate was added (pH 4.8, solution prepared from 60 ml of a potassium acetate solution 5 M, 11.5 ml glacial acetic acid and 28.5 ml milliQ water).

After that, the tube was homogenized using a vortex, and centrifuged for 10 min at 10000 rpm, and the supernatant was transferred to another tube of 1.5 ml volume on which an equal volume of isopropanol was added.

Then, the tube was agitated by inversion (10 times) and incubated at 20°C overnight. After incubation and centrifugation (10000 rpm for 10 min), the supernatant was thrown and the DNA pellet was rinsed twice with 300 µl of 70% ethanol. After centrifugation (10000 rpm for 10 min), the supernatant was removed and dried in the hood for 2 h. The DNA was dissolved in 50 µl of TE (Tris-EDTA).

The DNA assay was realized by spectrophotometer (Thermo Scientific NanoDrop 2000) at two different wavelengths: the absorption wavelength of nucleic acids (260 nm), and the absorption wavelength of proteins (280 nm). Then the DNA samples were stored at -20°C for further use (El Khoury, 2007).

PCR amplification of internal transcribed spacer (ITS) regions of rDNA

The Internal Transcribed Spacer (ITS) region of the ribosomal DNA was amplified with universal primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). For isolates, PCR was performed in a total reaction volume of 25 µl containing 5 µl of 5X buffer (Promega), 1 µl of dNTP (20 mM), 1.5 µl of $MgCl_2$ (25 mM), 0.25 U (5 U/µl) *Taq* DNA polymerase, 2 µl of each primer (20 pmoles/µl), 11.25 µl of ultrapure H2O, and 2 µl of genomic DNA (50 ng/µl).

The amplification program included an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 98°C (15 s), annealing at 59°C (60 s), extension at 72°C (120 s) and a final extension period of 10 min at 72°C in a Biometra thermal cycler (Germany).

Sequencing

After an electrophoresis on 1.5% agarose gel of the amplification products, an enzymatic sequencing of DNA fragments was performed according to the method of incorporation of dideoxynucleotide terminators system (Sanger et al., 1977). After

purification and quantitation, a precise aliquot of DNA was used as a template of an enzymatic amplification reaction in the presence of dideoxynucleotides (ddNTP's) labeled with Big Dye.

Sequence comparison with the data bank

Sequence comparison with those of the databases was performed using the BLAST algorithm (Altschul et al., 1997). The alignments of the nucleotide sequences were realized with the software Chromas (Larkin et al., 2007).

Antibacterial activity of isolated *Aspergillus* species

All the isolated species were tested for their antibacterial activity. For that purpose, two techniques were used: the agar cylinders and disc technique.

Preparation of microorganisms test

The antibacterial activity of isolated *Aspergillus* was sought against ATCC test bacteria (American Type Culture Collection), which are *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) *Escherichia coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 9027.The reactivation of the bacterial strains was carried out by seeding on selective media; Chapman, TSA (Trypticase Soy Agar), Hecktoen, and cetrimide agar, respectively.

Bacterial suspensions were prepared from the 18 h cultures. The cell density of each suspension was adjusted by dilution in sterile physiological water, and in comparison with the 0.5 McFarland solution (An optical density equal to 0.2 at 650 nm) in order to obtain a final concentration of 10^6 CFU/ml (Cavalla and Eberlin, 1994).

The agar cylinders technique

Aspergillus strains were seeded on PDA medium. After 14 days of incubation at 28°C, agar cylinders of 6 mm diameter were removed and deposited on the Mueller-Hinton medium surface previously seeded with the test bacteria. The dishes were then placed at 4°C for 4 h to allow diffusion of the active substances, and then incubated at 37°C for 18 to 24 h (Tortorano et al., 1979; Gungi et al., 1983).

Preparation of extracts

Aspergillus strains were reseeded in the potato dextrose broth (PDB) medium. After 14 days of incubation at 28°C, the formed biomass was removed by filtration, then the filtrate obtained was added to an equal volume of chloroform. After decantation, the chloroform phase was concentrated by vacuum evaporation using a Rotavapor (Gengan et al., 1999; Ghorri, 2015). Each fungal extract was dissolved in dimethyl sulfoxide (DMSO) in order to obtain a concentration of 100 mg/ml.

Discs technique

Discs of 6 mm diameter of Whatman paper soaked with 10 µl of the extract to be tested were dried and deposited on the surface of the dishes containing Mueller-Hinton medium previously inoculated with the test bacteria. Then, the dishes were incubated at 4°C for 2 h; thus the metabolites can diffuse, and then incubated at 37°C for 18 to 24 h (Yamaç and Bilgili, 2006; Hazalin et al., 2009). For this technique, DMSO was used as negative control. It should be noted that four repetitions were performed for each technique. And the diameters of the inhibition zones were measured in millimeter.

RESULTS AND DISCUSSION

Screening has always been the key to achieving new antibacterial molecules. Although its performance has been reduced in recent years, it is been practiced till date in many laboratories. They have endeavored to diversify the sources of microorganisms by developing selection methods that favor new species (Le Berre and Ramousse, 2003).

For this purpose, our work focuses on the research of antibacterial activity of certain strains of *Aspergillus* genus isolated from the soil sampled from particular ecosystems: Laghouat has a desert climate and Teleghma has a warm and temperate climate. Indeed, the soils of these zones, which constitute virtually particular media, seem to be promoter environments for the isolation of fungi producing new antibacterial substances. After isolation and purification, 32 strains of the genus *Aspergillus* were obtained from all samples. Based on the morphological characteristics, the strains collected were subdivided into 10 phenotypic groups.

Identification standards are mainly morphological. However, the application of molecular characterization tools has shown that this strictly phenotypic identification could lead to misidentification and that certain species groupings have no foundation (Thierry, 2011). For this, this morphological identification was confirmed by molecular analyzes.

The encoding DNA of 18S ribosomal RNAs of the isolates, extracted and amplified, is separated by agarose gel electrophoresis. The DNA bands obtained correspond to that of 600 base pairs of the molecular weight marker. The PCR products obtained were sequenced and subsequently compared with the sequences of the other microorganisms recorded in the Genbank database (Table 1).

GenBank, through the Blastn program, realizes an alignment and proposes a sequence that presents the best bits of score and percent identity with that of the current study. The 10 strains aligned were identified as follows: *Aspergillus fumigatus, Aspergillus niveus, Aspergillus wentii, Aspergillus fumigatiaffinis, Aspergillus quadrilineatus, Aspergillus nidulans, Aspergillus terreus, Aspergillus flavus, Aspergillus sclerotiorum* and *Aspergillus niger.*

After isolation and purification, the fungal species were screened on their antibacterial potency using two different techniques. The inhibition zones were measured after 24 h of incubation and the averages of these zones are shown in Tables 2 and 3. For the agar cylinders technique, the results show that 9 out of 10 fungal species have an antibacterial activity against *S. aureus* bacterium only and the averages diameter of the inhibition

Table 1. Confrontation and biomolecular corresponding with GenBank.

Isolate	Strains proposed by GenBank	Score	E-value	Identity (%)
L2	*Aspergillus quadrilineatus*	812	0.0	99
L3	*Aspergillus fumigatus*	977	0.0	100
L61	*Aspergillus fumigatiaffinis*	835	0.0	99
T1	*Aspergillus flavus*	832	0.0	100
T5	*Aspergillus niger*	834	0.0	99
T7	*Aspergillus nidulans*	788	0.0	99
T27	*Aspergillus terreus*	877	0.0	100
T32	*Aspergillus niveus*	872	0.0	100
T33	*Aspergillus wentii*	754	0.0	99
T62	*Aspergillus sclerotiorum*	821	0.0	99

L, Laghouat ; T, Teleghma.

Table 2. Demonstration of the antibacterial activity of fungal species by agar cylinders technique.

Strain	Zone of inhibition (mm)			
	S. aureus	***B. subtilis***	***E. coli***	***P. aeroginosa***
L2	33.67 ± 6.03	-	-	-
L3	34.33 ± 6.03	-	-	-
L61	30.67 ± 1.15	-	-	-
T1	13.00 ± 2.00	-	-	-
T5	-	-	-	-
T7	16.67 ± 1.15	-	-	-
T32	33.00 ± 2.65	-	-	-
T33	31.33 ± 3.21	-	-	-
T46	32.00 ± 2.00	-	-	-
T62	30.67 ± 1.15	-	-	-

*Diameter of the inhibition zone ≤ 6 mm.

Table 3. Demonstration of the antibacterial activity of fungal species by disks technique.

Strain	Zone of inhibition (mm)			
	S. aureus	***B. subtilis***	***E. coli***	***P. aeroginosa***
L2	16.67 ± 1.15	10.33 ± 1.53	11.00 ± 1.00	-
L3	31.67 ± 1.53	30.00 ± 4.36	7.67 ± 0.58	-
L61	16.33 ± 1.15	11.00 ± 1.00	8.67 ± 0.58	-
T1	7.00 ± 0.00	9.00 ± 1.00	8.67 ± 0.58	-
T5	7.33 ± 0.58	9.67 ± 1.53	9.00 ± 0.00	-
T7	12.00 ± 1.00	9.33 ± 0.58	10.33 ± 0.58	-
T32	18.33 ± 1.53	25.00 ± 3.00	11.33 ± 0.58	8.00 ± 1.00
T33	11.33 ± 0.58	12.00 ± 1.00	9.67 ± 0.58	7.00±1.00
T46	11.67 ± 1.15	12.67 ± 1.53	10.33 ± 2.08	-
T62	7.00 ± 0.00	9.00 ± 0.00	-	-

*Diameter of the inhibition zone ≤ 6 mm.

zones ranged from 13 to 34.33 mm.

The species *A. fumigatus, A. quadrilineatus, A. niveus, A. terreus, A. wentii, A. fumigatiaffinis* and *A. sclerotiorum* showed an important antibacterial activity with

respectively 34.33, 33.67, 33.32, 31.33, 30.67, 30.67 mm of inhibition averages (Table 2). After extraction with chloroform, the metabolic extracts of the 10 fungal species were examined for their antibacterial activity by disks technique.

The various extracts showed a more or less considerable antibacterial activity where the averages of the inhibition zones ranged from 7 to 31.67 mm, for the *S. aureus* bacterium, from 9 to 30 mm, for *B. subtilis* and from 0 to 11.33 mm for *E. coli* unlike *P. aeroginosa* on which the extracts of all species had no effect except the metabolic extracts of the *A. niveus* and *A. wentii* species that gave inhibition zones of 8 and 7 mm diameters, respectively. This technique revealed that the majority of species present antibacterial activity on at least one of the test bacteria and the species *A. fumigatus, A. niveus, A. quadrilineatus* and *A. fumigatiaffinis* showed considerable antibacterial activity (Table 3).

In fact, the species of *Aspergillus* genus are known by their production of substances having an antibacterial effect (Maria et al., 2005; Madki et al., 2010). These biologically active secondary metabolites are synthesized at the end of the growth (Attalah and Kacem-chaouche, 1992). Irobi et al. (2000) worked on the *A. quadrilineatus* fungal species and found that these species have remarkable antimicrobial activity against *S. aureus* and *B. subtilis;* Niege et al. (2002) showed that *A. fumigatus* have a remarkable antimicrobial activity against *S. aureus*, *Candida albicans* and *Micrococcus luteus.* Furthermore, Barakat and Gohar (2012) showed that *A. terreus* has a considerable antibacterial activity as compared to certain classic antibiotics; Al-Shaibani et al. (2013) worked on the antibacterial activity of *A. niger* fungal species. The results revealed that this species has an inhibitory effect against *P. aeruginosa, S. aureus, S. epidermidis,* and *Bacillus* sp. The same results were observed by Ola et al. (2014) who found that neosartorin isolated from *A. fumigatiaffinis* had an important antibacterial activity against a large spectrum of Gram-positive bacterial species, including *Staphylococci, Streptococci, Enterococci* and *B. subtilis.* In addition, Phainuphong et al. (2017) found that penicillic acid isolated from *A. sclerotiorum* has antibacterial activity against *S. aureus* and *E. coli* with MIC values of 128 mg/mL. The antibacterial activity against Gram positive bacteria tests appears to be more important than the one against Gram negative staining bacteria. This is related to the results obtained by Prabavathy and Nachiyar (2012).

These results can be explained by the fact that these two groups of microorganisms differ morphologically, because Gram negative bacteria have an outer membrane which is a polysaccharide membrane carrying the lipopolysaccharide structural components. This makes the cell wall impermeable to lipophilic compounds, unlike Gram-positive bacteria, which will be more sensitive because they only have an outer peptidoglycan layer, which is not an effective permeability barrier (Kumara et al., 2010).

Conclusion

The ten strains of *Aspergillus* isolated in this study showed a considerable antibacterial activity. It is interesting to test them against a range of human pathogens. Furthermore, an in-depth study is underway on bio-guided fractionation, which would identify individual components and lead to the isolation of active ingredients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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