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ANTIFUNGAL ACTIVITY OF ACTINOMYCETES ISOLATED FROM SEVERAL ALGERIAN ECOSYSTEMS AGAINST *Pinus halepensis* WOOD DECAY FUNGI

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Résumé

Deux champignons responsables de la pourriture de bois ont été isolés à partir du bois de l'essence *Pinus halepensis*. Ils ont été identifiés par leurs aspects culturels et morphologiques comme étant: *Tramates sp* et *Memnoniella sp*. L'isolement d'actinomycètes à partir de différents écosystèmes nous a permis d'obtenir 80 souches pures. La technique des disques d'agar a été employée pour étudier l'activité antifongique de ces actinomycètes contre les deux champignons, qui dégrade le bois de *Pinus halepensis*. Parmi tous les actinomycètes isolés, 17 souches ont présenté une activité contre au moins un champignon xylophage testé. Parmi les 17 bactéries des actinomycètes, une seule a montré une forte activité antifongique contre les deux champignons isolés. Cet actinomycète a été identifié par les caractères morphologiques et chimio-taxonomique de la paroi cellulaire comme *Streptomyces*

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Mots clés : *Actinomycètes, bois, préservation, Pinus halepensis, activité antifongique.*

Abstract

Two wood decay fungi have been isolated from the *Pinus halepensis* wood. They have been identified by their cultural and morphological aspects as: *Tramates sp* and *Memnoniella sp*. The isolation of the actinomycetes from different ecosystems has allowed us to obtain 80 pure strains. The agar disks technique has been employed to study the antifungal activity of these actinomycetes against the two fungi, which degrades the *Pinus halepensis* wood. Among all the isolated actinomycetes, 17 strains have presented an activity against at least one xylophage fungus tested. Among the 17 bacteria of actinomycetes, only one has shown a strong antifungal activity against the two fungal isolates. This actinomycete has been identified by the morphological and the chimio-taxonomical characters of the cellular wall as *Streptomyces.*

Keywords: *Actinomycetes, wood, preservation, Pinus halepensis, antifungal activity*

ملخص

تم عزل اثنين من الفطريات المتسببة في تعفن خشب الصنوبر الحلبي. و قد تم التعرف عليها بواسطة المظاهر التنموية في البيئات الغذائية و كذا المظاهر المورفولوجية كنوع *Memnoniella.Tramas* ان عزل الأكتينوميسات من الأنظمة الإيكولوجية المختلفة سمح لنا بالحصول على 80 سلالة نقية. وقد استخدمت تقنية الأقراص آجار لدراسة نشاطهاالحيوي ضد الفطريات المعزولة من الصنوبر الحلبي. 17 سلالة قدمت نشاطا ايجابيا ضد فطر واحد على الأقل من الفطرين الذين يتسببان في تعفن خشب الصنوبر الحلبي. أكتيسنوميسات واحد أظهر فعالية حيوية كبيرة ضد الفطرين المعزولين وقد تم التعرف عليه بطرق مورفولوجية و كيميائية للجدار الخلوي ك *Streptomyces*.

الكلمات المفتاحية: *الأكتينوميسات- الخشب – الحفاظ – الصنوبر الحلبي –فعالية ضد الفطريات*

Wood is one of the most used materials. However, it represents an eventual feeding source for a variety of fungi and xylophage insects [43, 39, 44]. The essential fungi which colonize wood are mildew, colorated fungi, brown, white, soft and red decay [43].

The wood receives in most cases some treatments which allow it to acquire a sufficient durability [36]. Several products are used and are essentially the chromated copper arsenate (CCA), a compound of copper with a quaternary amoniac (CAQ), an azole of copper and bore and salts of bore [35, 9, 36]. Except the borates which are of limited use because they are washable, all products for preserving wood are fixed in the wood during the treatment because they are insoluble in water and are also non biodegradable causing therefore serious problems of accumulations and pollutions in the nature [41]. They are also suspected of being dangerous and very toxic.

Some American and Canadian agencies have announced a decision to replace the CCA by other products for the treatment of wood for domestic use from December 31 st 2003 [14]. The European directives (98/8CE, 2012/2/UE, 2013/4/UE and 2013/7/UE) announced that most preservation products of wood must be analyzed [10, 11, 12, 13, 36].

In the framework of searching natural and biodegradable products for the use of wood preservation, different biocides, strictly organic and ecologic are being developed. These molecules are however of low rentability and expensive [16, 9, 29, 2, 17, 34, 20, 5].

The actinomycetes are the main natural source of anticellular metabolites [25]. Therefore, they represent an important source of none polluting and biodegradable molecules. These substances can be used for the preservation of wood against different devastating fungi. Some researches confirmed that 0.1 to 1% only of planet microorganisms are currently known and isolated [19]. Their natural resources are therefore not exploited yet. The screening has always been the essential way to reach new antimicrobial molecules. Some laboratories attempt to diversify the sources of microorganisms by using a variety of samples taken from ecosystems which are less exploited [21, 28] and by performing selection methods which favor rare species [32, 15].

The use of indigenous tree after an appropriate treatment is an essential factor to ensure the rentability of local forest zones until now little exploited. The *Pinus halepensis* is considerate as being the most widespread in the Algerian forest with approximately 850 000 hectares [30].

In this paper, the aim is the isolation, from several ecosystems, of actinomycetal bacteria which produce natural metabolites with an antifungal activity in order to use these molecules in Alep Pinus wood preservation against the xylophages' fungi which develop in it.

MATERIALS AND METHODS

Isolation and identification of the fungi from Pinus halepensis wood samples

Two old wood pieces with and without bark have been collected from tree trunks of *Pinus halepensis* of the Djebel ouahch mountain in the town of Constantine (North East of Algeria) (006°37'E, 036°22'N). 10 g of wood chips of each sample have been introduced in 100 ml of sterilized water and agitated vigorously with a vortex. From this solution, a series of decimal dilution have been realized in 9 ml of physiological water at 9 ‰ Nacl. Three mediums have been used in the isolation of the fungi:

- The synthetic medium of Lutz: Xylose 5 g L^{-1}, Maltose 5 g L^{-1}, Ammonium phosphate 1 g L^{-1}, Ammonium nitrate 1 g L^{-1}, Magnesium sulfate 0.1 g L^{-1}, Ferrous sulfate 0.05 g L^{-1}, Manganese sulfate 0.05 g L^{-1}, Agar 20 g L^{-1}, distilled water 1000 mL [35].

-The semi synthetic medium of Lutz: Xylose 2.5 g, Maltose 2.5 g, Ammonium phosphate 0.5 g, Ammonium nitrate 0.5 g, Agar 10 g, Filtrate of wood sawdust 500 mL. This filtrate is a mixture of 125 g of wood sawdust of *Pinus halepensis* and 750 ml of water. It is filtered through sterile gauze after being sterilized at 121°C during two hours [35].

-The medium of Czapek-Dox: $NaNO_3$ 2 g, KCl 0.5 g, $MgSO_4$ $7H_2O$ 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, K_2HPO_4 1 g, Sucrose 30g. Agar 10 g. This medium is added to the gentamicin at 5 mg/L [6].

For each sample, 0.1 ml of each dilution has been inoculated on surface, over the three mediums. After incubation at 30°C during two weeks, macroscopic observations have been undertaken using a magnifying glass. The cultures are purified by planting out again on the same isolation medium and the pure strains are conserved at 4°C in inclined agar [6]. For the morphological character study, a small piece of each fungal isolate is observed under an optical microscope. The thallus morphology (presence or not of bulkheads and ramifications), the structure of the reproductive apparatus (conidiospore, ascospore…) and the

morphology of spores (color and patterns…) are determined. The color of the thallus and the reverse of colonies, the speed of growth have been studied on the four following mediums: The Czapek-Dox medium [6], Potato dextrose agar or (PDA) [6], Malt-extract-agar or (MEA) [6] and the synthetic medium of Lutz [35].

Isolation of the actinomycetes

Three samples have been set apart from the following ecosystems:

-A soil neighbouring *Pinus halepensis* trees at Djebel ouahch Mountain.
-A soil near a Cedar of Atlas trees plantation at Djebel ouahch Mountain.
-A soil near Eucalyptus trees plantation at Djebel ouahch Mountain.
-The bark of *Pinus halepensis* at Djebel ouahch Mountain.
- A soil of the town of Biskra (Longitude_005°E44', Latitude 034°N51'), situated at the gate of great Algerian desert, at 243 km South of Constantine.
-A soil of the town of El-Oued (Longitude 005°E 44', Latitude 033°N20'). This part of Saharan desert is characterized by a lot of sand dunes and extremely severe climatic conditions.

The soil samples have been set apart according to Pochon and Tardieu technique [33] and dried at an ambient temperature during 7 to 10 days, then conserved at the Laboratory in polyethylene boxes until use [25, 26]. 1g of each dried soil is introduced in a tube containing 10 ml of physiological water and sterile glass balls of 2 mm diameter. After agitation with a vortex at maximum speed, the obtained suspension is used to realize a series of decimal dilutions going until 10^{-7}.

Several pieces of *Pinus halepensis* bark are cut with a sterile scalpel and put down on an aluminum sheet, then introduced in sterile case-bottle and transported to the Laboratory [22]. 1g of each sample is grinded and put in suspension in test tubes containing 10 ml of physiological water. After a strong agitation with a Vortex during 10 minutes, the decimal dilutions up to 10^{-7} are realized from this suspension.

The medium used in this study is Starch-Casein-Agar [23], added to 10 µg/ml of nalidixic acid and 50 µg/ml of nystatin [7]. 0,1 ml of each dilution are sowed in surface on the isolation medium. Petri Dishes are incubated at 28°C during 21 days. All the pure strains are conserved in suspension at -18°C in presence of 20 % of glycerol [22].

Study of the antifungal activity of the actinomycetes

All the purified actinomycetes are tested for their fungal activity by the agar discs technique against the isolated fungi of *Pinus halepensis* wood. The casitone medium is used to demonstrate this activity. It is composed of Bacto casitone (Difco laboratories, Sparks, U.S.A.) 9 g L^{-1}, Yeast extract (Merck) 5 g.L^{-1}, Sodium citrate (Prolabo) 10 g.L^{-1}, Glucose (Merk) 20 g.L^{-1}, di-potassium phosphate (Merck) 0,54 g.L^{-1}, di-sodium phosphate (Merck) 3,34 g.L^{-1}, Agar 18 g.L^{-1}, distilled water 1000 ml [7].

Preliminary identification of the active actinomycetes

The preliminary identification of the actinomycete presenting the most important antifungal activity has been realized by determining, on one hand, the morphological characters according to the technique of Shirling and Gottlieb [38] on the other hand by the chemical composition of the cellular wall in sugar and 2, 6-diaminopimelic acid [4, 40].

The morphological and chimiotaxonomical characters allow according to Lechevalier and Lechevalier (1970) and Williams (1989) to classify the actinomycetes at the corresponding genus [27, 42]. These methods are suggested in Bergey's Manual of Determinative Bacteriology [18] and Bergey's Manual of Systematic Bacteriology volume 4 [42].

RESULTS

Isolation and identification of the fungi

The fungal strains P1 and P2 have been isolated in majority on synthetic and semi-synthetic mediums of Lutz (table 1). Their growth on these two mediums was very good with respect to other strains which developed on the same mediums. These two isolation mediums are specific to the xylophage fungi. They have a chemical composition well adapted to this kind of microorganisms [35].

The fungal strains C1 and C2 have presented a poor growth on the two mediums of Lutz (table1) contrarily to the growth obtained on the medium Czapek-Dox (the usual isolation medium of several fungal species) which is not a specific medium of the xylophage fungi [6]. In addition, these strains have not been isolated from *Pinus Halepensis* without bark samples. The isolated C1 and C2 are not xylophage strains that's why, we were interested on the two xylophage fungal strains P1 and P2 isolated from the two *Pinus Halepensis* samples.

Table 1: Isolated fungal strains from two samples of *Pinus Halepensis* on three isolation mediums

Samples	synthetic medium of Lutz	semi-synthetic medium of Lutz	Czapek-Dox
Pinus halepensis without barks	P1, P2 *	P1, P2	C1, C2, C3, C4, C5, P1
Pinus halepensis with barks	P1, P2, C1, C2	P1, P2, C1	C1, C2, C3, C4, C5, C6, C7, P2

*The underlined strain indicates that the diameter of its colony is important > 4 mm after 2 weeks of growth.

The strain P1 (table 2) grows rapidly on almost all mediums tested. The culture starts with a white cream color and then changes to chestnut orange. The back of these colonies is of a chestnut color.

Table 2: Speed of growth and macroscopic characters of the fungal strains P1 cultivated on different mediums

Mediums	Speed of growth	Color of the colony	Color of the back of the colony
Synthetic of Lutz	Rapid	White-cream then orange	chestnut
Czapek-Dox	Slow	White-cream then orange	Dark chestnut
MEA	Rapid	Orange	chestnut
PDA	Rapid	Orange	chestnut

PDA : Potato-dextrose- agar , MEA : Malt-extract- agar

The strain P2 (table 3) develops rapidly on the synthetic medium of Lutz and on the Malt-extract-agar. Its growth is however slow on the two remaining mediums. The color of the colonies is white at the beginning of the cellular development then becomes black at the end of the growth. The back of the culture is black.

Table 3: Speed of growth and macroscopic characters of the fungal strains P2 cultivated on different mediums

Mediums	Speed of growth	Color of the colony	Color of the back of the colony
Synthetic medium of Lutz	Rapid	White then black	Black
Czapek-Dox	Slow	White then black	Black
MEA	Rapid	Black	Black
PDA	Slow	Black	Black

PDA: Potato-dextrose- agar, MEA : Malt-extract- agar

The microscopic observation of the two strains shows that:

-The hyaline hyphae (appearance of glass) of the strain P1 are partitioned by several buckles slightly flattered, which are brought together one by one to form the hyphae. The fibrous hyphae are brown fawn coloured not partitioned and not ramified.

-The strain P2 presents dark condiophores ending by a sort of a branch having black spherical spores. The black colored thalle is composed of hyphae partitioned and ramified in a parallel manner.

Isolation of the actinomycetes

The actinomycetale colonies are recognized by their characteristic macroscopic aspects. They are generally colonies with rough, dusty or filamentous aspects, which hold strongly to the culture medium. The colonies never exceed 5 mm of diameter and present under binocular a filamentous disposition.

Our results show that the biodiversity of the actinomycete strains varies considerably from one ecosystem to another. It is the most important in soils corresponding to forest ecosystems compared to samples coming from soil in the south of the country (the town of Biskra and the town of El-Oued (Table 4).

Table 4: Number of actinomycete strains isolated from several ecologic niche

Samples	Soil near holm oak (*Quercus ilex*	Soil near Eucalyptus	Soil near *Pinus helepensis*	Soil of the town of Biskra	Soil of the town of El-Oued	Bark of *Pinus helepensis*
Number of actinomycetes	22	16	15	9	6	12
Percentage (%)	27.5	20	18.75	11.25	7.5	15

Antifungal activity of the isolated actinomycetes

In table 5, we present the antifungal activity of the purified actinomycetes against the fungal isolations P1 and P2. 17 bacteria which are about 21% of the isolated actinomycetes have an antifungal activity against at least one isolated fungal strain of *Pinus Halepensis*. Among the active actinomycetes, only one strain named SB-2 presents a marked activity against the two fungal isolations tested.

Preliminary identification of the representative actinomycete

The actinomycete strain, named SB-2, active against the two fungi P1 and P2 is of Gram-positive coloration. Its mycelium of substratum is of chestnut color, formed by an important entanglement of ramified hyphae without fragmenting and partition and does not have any spores.

The aerial mycelium is characterized by the presence of hyphae a bit ramified and long chains of circular and motionless spores (more than 20 per chain) with a straight structure. After 7 days of incubation, the culture presents dry and chestnut-colored colonies, which hold strongly the agar.

At the 14th day, a white powder appears on all colonies. This aspect corresponds to the aerial mycelium and to the production of spores, which continue until the 21st day of incubation. A brown pigmentation is observed as well around the culture. The melanoid pigment is absent in this culture.

Table 5: Antifungal profile of active actinomycetes

Isolate	Origin	P1	P2
EC-1	Bark of Pine tree	(+) [1]	(-) [2]
EC-2	Bark of Pine tree	(+)	(-)
SP-2	Soil next to the Pine tree	(+)	(-)
SE-3	Soil next to the Eucalyptus tree	(-)	(+)
SE-5	Soil next to the Eucalyptus tree	(+)	(-)
SE-8	Soil next to the Eucalyptus tree	(+)	(-)
SE-9	Soil next to the Eucalyptus tree	(-)	(+)
SE-10	Soil next to the Eucalyptus tree	(+)	(-)
SE-11	Soil next to the Eucalyptus tree	(+)	(-)
SC-5	Soil next to the Oak tree	(+)	(-)
SC-6	Soil next to the Oak tree	(+)	(-)
SB-2	Soil of the town of Biskra	(+)	(+)
SB-7	Soil of the town of Biskra	(-)	(+)
SO-2	Soil of the town of El-Oued	(+)	(-)
SO-3	Soil of the town of El-Oued	(+)	(-)
SO-5	Soil of the town of El-Oued	(+)	(-)
SO-6	Soil of the town of El-Oued	(+)	(-)

[1]: Positive inhibition (inhibition diameter >3 mm).
[2]: No inhibition (inhibition diameter =3 mm).

On the chromatogram, the hydrolysis of the cellular wall appears a yellow colored to olive green colored spot which is characteristic of the di-aminopimelic acid under the isomeric « L »form. This strain belongs to the parietal I

type, according to the classification of Lechevalier and Lechevalier (1970) [27]. The study of the composition of the sugar wall reveals the absence of characteristic sugars. This strain belongs to the glucidic spectrum « C » according to the same classification.

DISCUSSION

The macroscopic characteristics (Table 2, 3) and the microscopic aspect of the thalle and hyphae of the two fungal cultures allow bringing closer the strain P1 near the species belonging to the *Tramates* genus also known as Coriolus and Polyporus. The fungus P2 is assimilated to the *Memmoniella* genus [6, 24]. The species belonging to these two genera are parts of the most redoubtable fungal agents responsible of the contamination of different types of woods. *Tramates trabea* for example, is the fungal agent, which attacks leafy and resinous woods and provokes a brown and cubic decay leading to the degradation of the wood cellulose. *Tramates versicolor* is the agent of wood's white decay [35, 6]. The strains *Memnoniella echinata* is a fungus, which presents a black-colored conidiophores. This fungus is cellulolytic and is capable to develop on paper, floors and construction woods. It produces the phenyl-spirodrimanes, the trichothecenes and the griseofulvines, which are very dangerous mycotoxines and could have a cancerigeneous effect [1].

The results of this study show that the actinomycetale bacteria are more numerous in forest soils (Table 4). These results are in accordance with those obtained in previous research works and affirm that the biodiversity in actinomycetes is due to the richness of these types of habitat in organic material comparatively to acid soils [15, 26, 7]. The small percentage of actinomycetale strains isolated from Saharan soils should not exclude investigating this kind of ecosystems. Indeed, several screening in this type of habitat has showed the presence of actinomycetes capable of producing substances of multiple interests [15, 7].

The fungi isolated from *Pinus halepensis* wood are differently sensitive to antifungal action of the isolated actinomycetes. *Tramates* do not present any resistance to antifungals produced by the majority of active actinomycetes. However, the fungus identified as being *Memmoniella* presents a certain resistance to the majority of the actinomycete. It is sensitive to four actinomycetes isolates only. The only strain presenting a very marked antifungal activity against the three fungi of *Pinus halepensis* wood is the strains SB-2 (Table 5). These results are very promising and offer the possibility to use a natural product capable of acting against ravager wood fungi of this essence, which is very widespread in our country.

The presence of L-DAP and the absence of characteristic sugars in the cellular wall of the strain SB-2 as well as the macroscopic and microscopic morphological characters show that this strain may belong according to the 9th edition of Bergey's manual classification to the group of *Streptomyces* and related genus which are *Intrasporangium*, *Kineosporia*, Sp*orichthya*, *Streptoverticillium*.

Four genera in this group have morphological characteristics different from the strain SB-2. The genus *Intrasporangium* has only one mycelium of substratum which ends by vesicles. The genus *Kineosporia* does not have an aerial mycelium. As for the genus Sp*orichthya*, there is a presence of an aerial mycelium which reduces to mobile fragments. Concerning the genus *Streptoverticillium*, an important characteristic distinguishes it from the others; it has spores in a verticillate structure. The strain SB-2 is characterized by the presence of mycelium of substratum and aerial with long straight chains of motionless spores. This microscopic aspect allows classifying the isolate SB-2 among the genus *Streptomyces.*

The genus *Streptomyces* is considered as being the first provider in molecules with anticellular activity, anticancerous, inhibitor of enzymes and other pharmaceutical agents of multiple interests. It produces in its own 70 % of these important natural products [37]. According to the literature, the antifungal activity of this genus is remarkable. Indeed, among the whole antifungal products discovered until 1997, more than 71% are produced by the *Streptomyces*. It is the same for the herbicides and insecticides agents [37]. The interest in using these bacteria strains as bio-insecticides or bio-fungicides in agriculture has considerably increased these last years.

This solution is one way to alleviate environmental problems. One example of the use of these bacteria as an agent of biological fighting is the mycostop which is a microbial bio-pesticide, based on *Streptomyces griseoviridis* named K61, manufactured by Kemira Agro Oy Company, to fight against the damping off, the decay of roots and stress caused by the *Fusarium* on Cucumber, Tomato, Capsicum and ornamental plants cultivated in hot-houses [31].

According to our results, this solution is quite possible in the preservation of wood. It allows substituting the chemical products, which are toxic and aggressive against nature. However, we have to study all the risks related to the toxicity of this type of products, especially for human, animal and its impact on water, arthropodes and vegetables.

Lastly, the industrial production of fungicide molecules secreted by the isolated strain is also another way, which could be exploited. It allows offering to the *Pinus halepensis* industry biodegradable natural molecules which could be used in the preservation of wood from this local essence.

CONCLUSION

In this study, several actinomycetes have been isolated from different ecological niches and have been analyzed with respect to the production of metabolites production with an antifungal activity. 17 actinomycetal bacteria have been selected. These bacteria are all active against at least one of the xylophage fungi, which degrade wood. Among them, one strain has presented a marked antifungal action against all fungi isolated from *Pinus halepensis* wood. The preliminary identification of this strain according to the recommendations of Bergey's manual of determinative bacteriology [18] and Bergey's manual of systematic bacteriology [42], have allowed classifying it among the *Stretomyces* genus. This study demonstrates that the actinomycetes have a potentiality to produce antifungal compouds with a good activity against fungi which degrades *pinus halepensis* wood. This provides a starting point for discovering new compounds with a better activity than chemical fungicides currently available. This result represents a sustainable alternative to the use of chemical fungicides for the preservation of wood.

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