Applied **B**iology in **S**aharan **A**reas, *Vol.3 N.4, p. 54-62.*Decembre 2021
ISSN: 2571- 9823 . EISSN: 2716-9480

Antimicrobial And Enzymatic Activities of Endophytic Fungi Isolated From *Phoenix dactylifera* L. In The Region of Bechar (Algeria)

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Abstract.
The present study was performed to assess the antimicrobial and the enzymatic potential of endophytic fungi associated with date palm (*Phoenix dactylifera* L).
Eighteen fungal isolates were isolated from the roots of seven young seedlings, of which only two of them (P3.4; P6.2) were subjected to tests for biological activities.
Testing for antimicrobial activity by the agar cylinder technique showed that the endophyte (P 3.4) had no antimicrobial activity and the endophyte (P6,2) has activity against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.
Testing for antimicrobial substances in the crude supernatant of cultures of endophytic isolates indicated that isolate P3.4 had activity only against *E. coli* and *Acinetobacter baumannii* Activity against *E. coli* is exerted by P6,2.
Testing for antimicrobial substances in extracts of crude supernatants from cultures of endophytic isolates indicated that almost all extracts from both isolates inhibited *E. coli* and *Acinetobacter baumannii*.
It should be noted that these endophytes produce amylase, protease and are not endowed with esterase, lipolytic and cellulolytic activity.

Keywords: *Phoenix dactylifera* L; Endophytic fungi; Antimicrobial potential; Enzymatic potential.

Introduction:

The microbial world is an extremely diverse group of microscopic, unicellular organisms, divided into three areas of life: bacteria, protozoa and fungus. They are distinguished from each other by their shape, size and lifestyle. Microorganisms in microbiology separate into two main types: beneficial and dangerous. (Borges and Sandalio, 2015).

Beneficial microorganisms are also able to induce a mechanism of induced resistance; this is called a systemic resistance index. Beneficial microorganisms are becoming increasingly important in integrated plant protection approaches. (Walters et al., 2014). They are used in several areas of our life such as their use in the diet example: lactic acid bacteria, and in the protection of plants example: endophytes.

Endophytic fungi are fungi that colonize the living tissues of plants, without causing any apparent symptoms, they are estimated to number 1.5 million species and only about 75,000 of them are described (Manoharachary et al., 2005). They receive nutrition and protection from the host plant and, in return, they improve competitiveness as well as its resistance to different pathogens such as bacteria, fungi, parasites, insects… as well as to different types of stress. Abiotic (Saikkonen et al., 1998). As a result of this protection, endophytic fungi have received considerable attention and are now considered to be a rich source of new biologically active secondary metabolites (Zhang et al., 2006).

The objective of this work is to study the bioactive secondary metabolites of endophytic

fungi isolated from Phoenix dactylifera L (date palm) which represents a fruit tree of major ecological, economic and social interest for many countries in arid zones.

This modest work describes the material and methods used, followed by a presentation and discussion of the results obtained, and at the end a general conclusion.

Materials and methods:

Isolation of endophytic fungi:

The twigs and roots were washed in running tap water for 10 min (Hazalin et al., 2009; Khan et al., 2010), The samples were surface-sterilized by sequential washes in 70% ethanol for 1min, 3% sodium hypochlorite for 4 min, and 70% ethanol for 30 seconds (Pimentel et al., 2006) . They were then rinsed three times with sterile distilled water for 1 minute each time and dried on sterile filter paper (Pimentel et al. , 2006; Khan et al., 2010).
Five needles segments were then evenly placed in PDA plates augmented with 50 lg/mL of chloramphenicol to avoid bacterial contamination.
Plates were sealed with parafilm and incubated at 27 ± 2 C for 5–8 days in incubator. Hyphal tips of the developing fungal colonies were transferred aseptically to fresh PDA plates to get pure cultures of the growing fungi.

Identification of endophytic fungi:

The fungi were identified based on the morphological characteristic (Chen et al. 2011). Colony features were based on the observation on PDA under ambient day light conditions. Microscopic observations using at 40 x or 100 x magnification. Endophytes were identified on the basis of characteristics such as the structure of hyphae, conidia, and conidiophores. Conidiophore structure and morphology were described by obtaining them from the edge of conidiogenous pustules or fascicles during maturation of conidia, which usually occurred after 4-7 days of incubation.

Testing for antimicrobial activity:

Agar cylinder technique (primary screening):

The pathogenic strains were spread on the surface of the agar medium (PDAa for pathogenic fungi or Mueller Hinton for bacteria) previously inoculated on the surface with a suspension of the pathogen. Agar cylinders 6 mm in diameter were taken from cultures of the endophytic strains (culture of 10 to 14 days) and deposited on the surface of the medium.The dishes were placed at + 4 ° C for 2 to 4 hours to allow the diffusion of the active substances, then they were incubated (at 25 ° C for fungi and at 37 ° C for pathogenic bacteria). The zones of inhibitions were measured after 24 to 48 hours of incubation (Tortorano et al., 1979; Madigan et al., 1997).

The results were read by measuring the diameter of the zones of inhibition around the cylinders.

Extraction of bioactive molecules and evaluation of their antimicrobial potential:

The pure fungal strains were inoculated into 100 ml of potato dextrose broth (PDB) medium in 250 ml Erlenmeyer flask and incubated for 14 days at 28 °C for secondary metabolite production. After the incubation period, the culture filtrate was extracted three times with the equal volume of ethyl acetate , n-butanol and chloroforme using separating funnel. Solvent phase was collected and condensed using rotary vacuum evaporator.

The antimicrobial activity was evaluated using sterile filter paper discs 6 mm in diameter impregnated with 20 µl of each extract. After drying, the discs were deposited on the surface of the agar medium (PDA for

fungi or Mueller hinton for bacteria) previously seeded en masse with the pathogen. The dishes were placed at + 4 ° C to allow diffusion of the substances, then they were incubated at 25 ° C for fungi or at 37 ° C for

bacteria; zones of inhibition were measured after 24 to 48 hours of incubation (Barry et al., 1970).

Detection of Extracellular Enzyme Production:

In this test, endophytic fungi were qualitatively assessed for their production of five different extracellular enzymes; cellulase, amylase, protease, lipase (Maria et al., 2005), and esterase (Carrim et al., 2006).

Amylase activity was assessed by growing the fungi on glucose yeast extract peptone agar (GYP) . After incubation the plates were flooded with 1% iodine in 2% potassium iodide. A clear zone surrounding the colony was considered indicative of amylase production.

Cellulase activity was detected in GYP medium supplemented with 0.5% Na-carboxy-methylcellulose in suspension. After incubation, the plates were flooded with 0.1% aqueous Congo red and distained with 1 M NaCl for 15 min. Appearance of a clear zone around the colony indicated cellulase activity (Amirita et al., 2012).

For lipase activity, the fungi were grown on peptone agar medium supplemented with 1% Tween 20 sterilized separately. A clear zone around the colony indicated lipase positive fungi (Amirita et al., 2012).

For Proteolytic activity was determined by growing the isolates on GYP agar medium supplemented with 10% skim milk. After incubation, the appearance of light areas around the colonies indicates proteolytic activity (Amirita et al., 2012).

For esterase activity, peptone agar medium was used, the pH of which was adjusted to 6.5). The previously sterile Tween 80 is added to have a final concentration of 1% (v / v). The media were inoculated with the isolates after 3-5 days of incubation, halos are observed around the colony (Ishida et al., 2012).

Results and discussion:

Isolation of endophytic fungi:

The results obtained from the twig and root segments of young seedlings of date palms (Phoenix dactylifera L) are shown in Table 01.

Out of the 18 primary fungal isolates, 2 morphologically dissimilar isolates were selected for biological activity screening (**Isolate 1:** P6.2, **Isolate 2:** P 3.4).

Table 01: Fungi isolated from date palm samples:

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	Plant 7	Plant 8	Plant 9
	Branch 1	Branch 2	Root 1	Root 2	Root 3	Root 4	Root 5	Root 6	Root 7
Nbr of colonized segments	**0/7**	**0/8**	**2/5**	**3/5**	**4/5**	**4/5**	**0/5**	**5/5**	**0/5**
Colonization rate	**51.4%**								

These results allowed us to obtain a colonization percentage equal to 51.4%. A percentage close and comparable to that obtained by Bouhacida et al., (2013) using *Pistacia lentiscus* roots, with 62.5%. The difference between these percentages can be explained by the difference between the host species, the number of samples as well as the culture media used (Gong and Guo, 2009).

Identification of endophytic fungi:

Identification of isolates is based on microscopic and macroscopic criteria.

for strain **P6.2** were observed with a short mucous, fluffy or cottony mycelium.With a white color, rounded cottony appearance and moist texture and mucous then powdery. It has a rapid growth of 2 to 3 days and restricted.

for strain **P3.4** were observed recto: colonies with a powdery to granular surface and round and small relief, as for the color, we noticed a color that was initially white then green gray. The back is colorless.

Growth is little slow from 5 to 7 days.

Microscopic observation showed the presence of a conidial head characteristic of *Aspergillus*: radiate, very long conidiophores, not septate and smooth on which there are phialides carried by particles inserted on the spherical vesicle with spherical conidia.

The genus *Aspergillus* is a very diverse genus with 180 species, some of which have commercial and medical value, such as pathogenic species (Lubertozzi and Keasling, 2009).

Testing for antimicrobial activity:

Agar cylinder technique (primary screening):

Table 02: Results of antimicrobial activity obtained by the method of agar cylinders:

Pathogenic / Endophyte	Pathogenic mold			Pathogenic bacteria				
	PN	AF	FOA	Ec	Ps	Ef	Ab	
Isolate 1 (P6.2)	-	-	-	-	8.3	-	7.3	Diameter of the inhibition zone in mm
Isolate 2 (P3.4)	-	-	-	-	-	-	-	

(-): no activity. **Ec**: *Escherichia coli* **Ps :** *Pseudomonas aerugenusa* **Ef:** *Enterococcus faecalis* **Ab:** *Acinetobacter baumannii* **PN :** *Penicillium sp* ***FOA :*** *Fusarium oxysporium f.sp. albédinis.* ***AF :*** *Aspargilus flavus* .

Among 14 confrontational tests performed, antimicrobial activity was recorded only in 2 tests. The results obtained showed that except one isolate out of 02 showed inhibition (Table 02).

Among the active strains we find that:

The strain (P6.2) showed antimicrobial activity against 02 bacterial strains (*Pseudomonas* and *Acinetobacter baumannii*).

For strain p6.2, the zones of inhibition obtained are with diameters of 8.3mm and 7.3mm against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* respectively, and no antagonist effect is marked against *Escherichia coli* and *Enterococcus faecalis* nor against pathogenic fungi.

For strain p3.4 shows no antimicrobial activity.

Among the endophytic fungi studied, that belonging to the genus *Aspergillus*, p3.4 shows no activity on all the bacterial and fungal strains tested. On the other hand, the genus *Aspergillus*, according to the work of Amadi et al. (2009); Avasthi et al. (2010) and Indira et al. (2015) has antimicrobial activity.

Secondary screening:

Effect of crude supernatant of endophyte culture liquids:

Table03: Results of the antibacterial activity of the crude supernatants:

		Crude supernatant from cultures of active endophytic isolates		
	pathogenic	**P6.2**	**P3.4**	
Pathogenic bacteria	**Ec**	**7 mm**	**7 mm**	Diameter of the inhibition zone in mm
	Ps	-	-	
	Ef	-	-	
	Ab	-	**8 mm**	

(-): no antifungal activity **Ec**: *Escherichia coli* **Ps :** *Pseudomonas aerugenusa* **Ef:** *Enterococcus faecalis* **Ab:** *Acinetobacter baumanni.*

This test was used to determine the ability of these endophytes (P6.2 and P3.4) to produce antibacterials in liquid media.

From table 03 we notice:

Antibacterial activity of strain p3.4 against two strains of pathogenic bacteria (*Escherichia coli* and *Acinetobacter baumannii*) with a better diameter of 8 mm relative to *Acinetobacter baumannii*. On the other hand, the crude strain p6.2 showed activity on a single strain of

pathogenic bacteria (*Escherichia coli*) with a diameter of 7 mm.

On the other hand, no activity was recorded for the crude filtrates of the two strains studied against the other pathogens.

The absence of activity can be explained by the absence of the substance in the medium or the substance is not active enough, or by a poor diffusion of the latter in the medium (Srivibool and Sukchotiratana, 2006).

Antimicrobial activity of organic extracts from crude supernatants:

The different extracts showed antibacterial activity with different zones of inhibition, ranging from 6.5 to 8.2 mm for the ethyl

acetate extracts. From 7 to 8 mm for the chloroform extracts and from 7.6 to 10 mm for the butanolic extracts. The means of the diameters of all zones of inhibition are summarized in **Table 04**.

Table 04: Results of the antibacterial activity of the extracts of the culture supernatants of the active isolates:

		Extracts from culture supernatants of endophytic isolates						Negative control (filter paper disc + pure solvent)			
		P6.2			P3.4						
		A	**B**	**C**	**A**	**B**	**C**	**Ab**	**Bb**	**Cb**	
Pathogenic bacteria	**Ec**	7	9	7.5	-	8.3	7.3	7	8	-	diameter of the average inhibition zone in mm
	Ps	-	8	7.6	6.5	8.6	-	10	8	8	
	Ef	-	10	7	-	7.6	8	-	8	8	
	Ab	8.2	9.5	7.5	6.5	8.1	7	7	9	7	

(-): no antibacterial activity; **A**: ethyl acetate extract; **B**: butanolic extract; **C**: extract chloroform; Ab: crude ethyl acetate; **Bb**: crude butanol; **Cb**: crude chloroform. **Ec**: *Escherichia coli* **Ps**: *Pseudomonas aerugenusa* **Ef**: *Enterococcus faecalis* **Ab**: *Acinetobacter baumannii*.

These results show that butanol is considered the best solvent for the extraction of bioactive molecules followed by chloroform.

Butanol is a universal extraction solvent, it allows the extraction of virtually all molecules, which is consistent with this result. This is how several studies have confirmed the effectiveness of butanol compared to other solvents used. The study carried out by Boudjella et al. (2006) on the genus Streptosporangium showed that butanol is the best solvent for extracting bioactive substances.

The results showed that the activity after the extraction of the molecules is increased, this can be due to the concentration of the bioactive molecule after extraction or by the antagonism exerted on the bioactive substances and which is generated by the other non-extractable substances. present in the crude supernatant.

Testing of the synthesis and excretion of extracellular enzymes:

Qualitative tests to demonstrate the production of extracellular enzymes from endophytic fungal strains allowed us to obtain the results summarized in **Table 05**.

Table 05: Diameters (mm) of hydrolysis zones of endophytic strains:

Enzyme sought	Endophytic strains	
	P6.2	P3.4
Amylase	+	+
Cellulase	-	-
Lipase	-	-
Protease	+	+
Esterase	-	-

(-): Absence of hydrolysis zone (+): hydrolysis zone

The two strains tested exhibited 2 enzymatic activities including protease and amylase activity. No cellulase, lipase and esterase activity was recorded.

Enzymes are essential proteins for the metabolic system of all living organisms, they can be isolated from animals, plants and microorganisms, and the latter are good sources of enzymes whose stability is more important than those of animal or plant origin (Maria et al, 2005).

According to the work of Choi et al. (2005), Selim et al. (2012), Sunitha et al. (2013), endophytic fungi are among the microorganisms that produce extracellular hydrolases to resist invasion by pathogens and to ensure host nutrition. These enzymes are essential for the metabolic system of these endophytic fungi because they allow them to 'invade and colonize plant tissue.

The ability of all isolates to degrade starch could be explained by the fact that starch is the most abundant organic carbon source in the environment (Selim et al., 2012). The amylolytic potential of these endophytic fungi can help them break down the starch that is available during plant senescence (Sunitha et al., 2013). Several endophytes of the genus *Aspergillus* have been reported to be a source of the enzymes amylase, lipase and protease (Alves et al., 2002; Maria et al, 2005 and Choi et al., 2005).

According to Sunitha et al (2013), the production of enzymes differs between fungi and often corresponds to the requirements of its habitat. This may be due to the many factors of change in the host such as age, environmental factors such as that the climatic conditions and the geographical situation being able to influence the biology of the fungi.

Conclusion:

In this present work, endophytic fungi associated with *Phoenix Dactylifera* L (Date Palm) were isolated and evaluated for their antimicrobial, enzymatic potential.

Our work is only a preliminary step for larger, more in-depth and more accomplished studies including:

•Isolation, purification and identification of secondary metabolites produced by endophytic fungi.

•Determination of the composition, structure, content and bioactivity of extracts of Phoenix dactylifera (date palm) and comparison with secondary metabolites produced by its endophytic fungi.

•Use other pathogenic strains, either bacteria or molds.

•Quantification of the enzymatic production of endophytic fungi.

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