

Antimicrobial Activity of Endophytic Fungi Isolated from *Avicennia marina* Plant, Red Sea, Egypt

Y. M. Shebany

Botany Department, Faculty of Science at Qena, South Valley University, Qena, Egypt.

TWENTY ONE endophytic species were isolated from 360 segments of *Avicennia marina* from two different locations of Red Sea mangrove forest in 2009 to 2010. Some of the endophytes were identified to genus or species level using traditional morphological methods, but most were classified as sterile mycelia. The isolated species belonged to 7 genera (*Acromonium*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Phoma* and *Ulocladium*). Thirty nine isolates of endophytic fungi were selected to detect their antagonistic abilities against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Aspergillus niger* and *Alternaria alternate*. The obtained results indicated that some isolates of the same species showed different behavior against used bacteria, while few of these isolates of the same species showed the same behavior. The tested endophytic species did not show any antagonistic ability against *Aspergillus niger* and *Alternaria alternate*.

Keyword: *Avicennia marina*, Mangrove, Endophytic fungi, Antimicrobial activity.

Mangrove forests are worldwide distributed on sheltered, tropical and subtropical coastlines (Ellison & Farnsworth, 2001 and Lin *et al.*, 2009). Mangrove forests are located at the interface between land and sea, a unique and extreme environment (Kathiresan & Bingham, 2001 and Ezawa & Tada, 2009). The mangroves of the world span over 30 tropical and sub-tropical countries, covering an area of about 99,300 sq. km (Singh, 2000). On the global level, several authors reviewed the past and present distribution of mangrove ecosystem (Tomlinson, 1986; Ricklefs & Latham, 1993; Dunk *et al.*, 1998 and Ellison *et al.*, 1999). Mangrove areas in Egypt are dispersed in numerous small sites along the Red Sea Coast, although the overall area of mangroves is relatively small. The present estimates indicate that there are approximately 5km² of mangroves in Egypt. The limited mangrove areas of Egypt suggest that this is a vulnerable ecosystem that needs effective management to ensure its ongoing survival (Saenger, 2002).

The soils in mangrove communities are muddy or sandy with loose sediments. They contain submerged mangrove roots, trunks and branches. These conditions attract rich communities of fungi and bacteria (Kathiresan & Bingham, 2001). Among the microorganisms, the fungal community is the principal degrader of plant debris, especially during the early phases of decomposition. Therefore, fungi play an important role in the transformation and cycling of nutrients in the ecosystem (Zakaria *et al.*, 2010).

Endophytic microorganisms colonize living, internal tissues of the plants without causing any immediate, overt negative effects (Bacon & White, 2000; Wasser, 2002 and Sun *et al.*, 2011). Endophytes have proved to be the promising sources of biologically active products which are of interest for specific medicinal applications (Strobel, 2002).

Endophytic fungal association with mangrove plants confers protection from adverse environmental conditions and allows them to successfully compete with saprobic fungi decomposing senescent parts (Kumaresan & Suryanarayanan, 2002). Leaves, stems, bark, roots and fruits of mangrove plants are a valuable resource for folk medicine (Bandaranayake, 1998). He showed that roots of mangrove plants are a rich source of fungal endophytes (Ananda & Sridhar, 2002). Metabolites exploited in pharmaceutical and agricultural industries are widespread among the endophytic fungi (Petrini *et al.*, 1992 and Li *et al.*, 2008).

Marine mangrove fungi have proven to be an important source of new bioactive compounds (Lin *et al.*, 2001) and enzymes (Grant *et al.*, 1996; Pointing *et al.*, 1998 and Pointing & Hyde, 2000). Endophytes might involve in decomposition when the tissue becomes senescent or die. Hence, they elaborate the enzymes necessary for degradation of lignocellulosic materials.

Since *Avicennia marina* constituted the dominant species in the Egyptian mangrove forests, therefore, the present work aimed to isolate endophytic mycobiota of this plant and detect their antagonistic abilities against some pathogenic microorganisms.

Materials and Methods

Study area

The Red Sea comprises the main part of the East African rift valley. It lies between latitudes 30° 58'N and 12° 30'N. The climate is arid, rainfall is Mediterranean (in the winter), daily temperature maximum is 27 °C in May, humidity is about 50 % and the tidal range is small. The coast mostly consists of vertically raised (30 – 40 m) fossil cliffs and wide horizontal expanses of beach rocks, producing intertidal flat cover of 100 m in width. The intertidal zone is particularly harsh, with very high solar heating and desiccation stress, and a very low tidal range. It contains all major tropical marine communities, except estuaries. These conditions are suitable for the growth of mangrove and other coastal vegetation.

Isolation of endophytic fungi

30 samples of *Avicennia marina* were surveyed for fungal endophytes. Respiratory roots were collected from regions 17 and 40 from the coastal of Red Sea. Samples were transported in closed sterile polythene bags and, were processed within 24 hr of collection (Fisher & Petrini, 1987)

Surface sterilization, culture condition and isolation

The Respiratory roots segments were cut into four segments each of one cm length and washed in running tap water to remove surface contamination and were dipped in 75 % ethanol (1 min), 5% sodium hypochlorite (3 min) and 75 % ethanol (0.5 min) followed by rinse in sterile distilled water (Filip *et al.*, 2003). Segments were placed on 20 ml CZ medium in a petri dish and were incubated at $28\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 2-3 weeks (Carroll, 1986). The fungi that grew out from the plant tissues were periodically observed through a microscope. The endophytic fungi were identified with the help of keys (Sutton, 1980; Howksworth *et al.*, 1983; Nelson *et al.*, 1983 and Von Arx *et al.*, 1986). The sterile Mycelia that grew out from the tissue were subcultured on CZ slant and exposed to light to induce sporulation (Suryanarayanan, 1992).

*Antimicrobial assay**Fermentation and treatment of the fermentation broth*

The endophytic fungi were transferred to 100 ml of GPY medium (GPY: glucose 20 g/L, peptone 5g/L, yeast extract 5g/L) in Erlenmeyer flasks and incubated at 28°C and 160 rpm with normal daily light and dark periods for 10 days. The mycelia of the endophytic fungi were separated by filtering with Whatman filter paper. The mycelial mass was also separated from the broth by filtration, and the culture broth was partitioned with chloroform. After that the organic solvents were evaporated under vacuum (Ding *et al.*, 2010 and Ramos *et al.*, 2010).

Microorganisms used

The tested bacteria were obtained from bacteriology lab, and fungi from mycology lab., Botany Dept., Faculty of Sciences, South Valley University.

Antimicrobial activity

Antibacterial tests of selected microorganisms were carried out using disc-diffusion method (Bauer *et al.*, 1966). Nutrient agar plates (90 mm size) were prepared and cooled down at room temperature ($20 \pm 2^{\circ}\text{C}$). A small sterile cotton swab was dipped into the 24 hr old culture of bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus* and *Escherichia coli*) and was inoculated by streaking the swab over the entire agar surface. This process was repeated by streaking the swab 2 or more times rotating the plates approximately 60° each time to ensure even distribution of inoculum. After inoculation the plates were allowed to dry at room temperature ($20 \pm 2^{\circ}\text{C}$) for 15 min in laminar chamber for settle down of inoculum. The filter paper discs (5 mm) loaded with 10 μl of extract were placed on the surface of the bacteria seeded agar plates and it was allowed to diffuse for 5 min, then these plates were incubated at $37 \pm 1^{\circ}\text{C}$ for 24 hr. Two fungal species (*Alternaria alternata* and *Aspergillus niger*) were employed, and the plates

were incubated at 28°C up to 5-7 days (Maria *et al.*, 2005). Chloroamphenicol (1×10^{-3}), tetracycline (1×10^{-3}) and for fungal species kemazed 50% (methyl benzimidazol-2-ylcarbamate) (1×10^{-3}) were placed into agar plates used as positive control and respective solvent were also used as negative control.

Results

Eleven species belonging to 7 genera and 10 sterile mycelia were isolated and identified from the respiratory roots of 30 samples (15 from each region 17 & 40) of *Avicennia marina* (Table 1).

TABLE 1. Total fungal count (TC) and percentage of occurrence of fungi from the endorhizosphere of *Avicennia marina* of regions 17 and 40 of the costal Red Sea.

Genera and species	Region 17			Region 40		
	T C	C %	S %	TC	C%	S %
<i>Acromonium</i> sp.	5	3.73	2.78	1	1.41	0.56
<i>Aspergillus</i>	7	5.22	3.89	19	26.76	10.56
<i>A.flavus</i>	0	0	0	7	9.86	3.89
<i>A. nidulans</i>	0	0	0	7	9.86	3.89
<i>A.niger</i>	1	0.75	0.56	4	5.63	2.22
<i>A.terreus</i>	4	2.99	2.22	1	1.41	0.56
<i>A. sydewi</i>	2	1.49	1.11	0	0	0
<i>Chaetomium globosum</i>	6	4.48	3.33	0	0	0
<i>Cladosporium cladosporides</i>	3	2.24	1.67	0	0	0
<i>Fusarium moniliforme</i>	3	2.24	1.67	0	0	0
<i>Phoma</i> sp.	4	2.99	2.22	10	14.08	5.56
<i>Ulocladium</i> sp.	0	0	0	1	1.41	0.56
Sterile mycelium	106	79.1	58.88	40	56.34	22.22
Sterile mycelium 1	5	3.73	2.78	14	19.72	7.77
Sterile mycelium 2	3	2.24	1.67	4	5.63	2.22
Sterile mycelium 3	6	4.48	3.33	6	8.45	3.33
Sterile mycelium 4	33	24.63	18.33	7	9.86	3.89
Sterile mycelium 5	39	29.1	21.67	2	2.82	1.11
Sterile mycelium 6	1	0.75	0.55	3	4.23	1.67
Sterile mycelium 7	12	8.95	6.66	0	0	0
Sterile mycelium 8	0	0	0	1	1.41	0.56
Sterile mycelium 9	0	0	0	3	4.22	1.67
Sterile mycelium 10	7	5.22	3.89	0	0	0
Total account	134	100	74.44	71	100	39.46
total segments	180	100	100	180	100	100

TC = calculated per 180 segments, C% = calculated per total fungi, S% = calculated per total segments sporulating.

Sterile mycelia were the most dominant fungal taxa identified during this experiment, where 8 sterile mycelia were isolated from each region. Total counts were fluctuated from 106 to 134 and 40 to 71 colony/segment, represented 79.1% and 56.34 % of total fungi and 58.88 % and 22.22 % of total segments from regions 17 and 40, respectively (Table 1).

Data of Table 1 also showed that, *Aspergillus* sp. was the second most common genus in count which recovered from regions 17 & 40 comprised 5.22% & 26.76% of total colonies and 3.89% & 10.56% of total segments, respectively.

Phoma sp. was also isolated from the two regions comprised 2.99% and 14.08 % of total colonies and 2.22% and 5.56% of total segments, respectively. *Chaetomium globosum*, *Cladosporium cladosporioides* and *Fusarium moniliform* were isolated only from region 17 which comprised 4.48% , 2.24% and 2.24% of total colonies and 3.33%, 1.67% and 1.67% of total segments, respectively. While *Ulocladium* sp. was recovered only from region 40 represented by 1.41% of total colonies and 0.56 % of total segments.

Antimicrobial assay

Thirty nine isolates of endophytic fungi were randomly chosen to estimate their ability to produce antimicrobial compounds (antibacterial or antifungal activity). The estimation was done on four bacteria species by disc diffusion method, which were incubated at 37° C for 24 hr and inhibition zones were measured. And also, two fungal species were tested by disc diffusion method, which were incubated at 28° C for 5-7 days and inhibition zones were measured. These isolates comprised from *A. nidulans*, *A. niger*, *A. terreus*, *Phoma* sp., and sterile mycelium 3, 5, 6, 8 and 9 (one isolate for each species), *Acromonium* sp., *A. flavus* and *Chaetomium globosum* (2 isolates for each species), sterile mycelia 2 and 4 (3 isolates for each species), 4 isolates of sterile mycelia 7 and 9 isolates of sterile mycelium no.1.

The obtained results indicated that all tested isolates did not have any effect on fungal species in comparison with positive control, while some isolates of the same species exhibit different behavior against used bacteria and few of these isolates of the same species showed the same behavior (Table 2).

From 39 tested isolates of endophytic fungi, 23 isolates inhibit the growth of *Bacillus subtilis*. Sterile mycelium no. (1) showed the highest effect on *B. subtilis* (17/17 mm for positive control), whereas the lowest effect was recorded by sterile mycelia 1 and 2 (6 mm). While 8 isolates only effected on *Staphylococcus aureus*, which had high value than control in some species, the highest effect on *Staphylococcus aureus* occur by sterile mycelia 7, 5 and *Acromonium* sp. (26, 23 and 21 mm, respectively, than positive control 17mm) and the lowest effect for sterile mycelium no.1 (11mm). (Table 2).

TABLE 2. Antimicrobial activities of endophytic fungi from *Avicennia marina*.

Endophytic species and isolates		Antimicrobial activity ^a					
		<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>	<i>Escherichia coli</i>	<i>Aspergillus niger</i>	<i>Alternaria alternata</i>
<i>Acromonium</i> sp.	Isolate 1	-	-	-	-	-	-
<i>Chaetomium globosum</i>	Isolate 1	8	13	16	-	-	-
Sterile 7	Isolate 1	16	26	14	-	-	-
<i>A. niger</i>		-	-	-	-	-	-
<i>A. terreus</i>		-	-	-	-	-	-
Sterile 1	Isolate 1	6	-	-	-	-	-
Sterile 7	Isolate 2	-	-	-	-	-	-
Sterile 8	Isolate 1	-	-	-	-	-	-
Sterile 8	Isolate 2	-	-	-	-	-	-
Sterile 6		-	-	-	-	-	-
Sterile 5		14	23	15	6	-	-
Sterile 3		14	-	-	-	-	-
Sterile 8	Isolate 3	-	-	-	-	-	-
<i>A. flavus</i>	Isolate 1	-	-	-	-	-	-
<i>Phoma</i> sp.		-	-	-	-	-	-
Sterile 1	Isolate 2	7	-	-	-	-	-
<i>A. flavus</i>	Isolate 2	7	13	-	-	-	-
Sterile 7	Isolate 3	12	-	-	-	-	-
Sterile 2	Isolate 1	6	-	-	-	-	-
Sterile 1	Isolate 3	11	-	-	-	-	-
Sterile 8	Isolate 4	7	-	-	-	-	-
Sterile 1	Isolate 4	12	-	-	-	-	-
Sterile 8	Isolate 5	11	-	-	-	-	-
<i>A. nidulans</i>		12	-	-	-	-	-
Sterile 4	Isolate 1	9	17	17	-	-	-
Sterile 7	Isolate 4	12	14	-	-	-	-
Sterile 1	Isolate 5	15	-	22	8	-	-
<i>Chaetomium globosum</i>	Isolate 2	8	-	-	-	-	-
Sterile 8	Isolate 6	9	-	-	-	-	-
Sterile 4	Isolate 2	-	-	-	-	-	-
<i>Acromonium</i> sp.	Isolate 2	11	21	-	-	-	-
Sterile 1	Isolate 6	8	-	21	-	-	-
Sterile 1	Isolate 7	17	-	-	-	-	-
Sterile 9		9	-	18	-	-	-

TABLE 2. Cont.

Endophytic species and isolates		Antimicrobial activity ^a					
		<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>	<i>Escherichia coli</i>	<i>Aspergillus niger</i>	<i>Alternaria alternata</i>
Sterile 2	Isolate 2	-	-	-	-	-	-
Sterile 1	Isolate 8	13	11	-	-	-	-
Sterile 1	Isolate 9	-	-	16	-	-	-
Sterile 2	Isolate 3	13	-	-	-	-	-
Sterile 4	Isolate 3	-	-	-	-	-	-
Positive control ^b		17	17	14	15	10	7

^a The antimicrobial activity is expressed by the diameter (*d*) of inhibition zone (mm). ^bControl for Gram +ve is chloroamphenicol (1×10^{-3}), for Gram -ve is tetracycline (1×10^{-3}) and for fungal species is kemazed 50% (methyl benzimidazol-2-ylcarbamate) (1×10^{-3}).

Also 8 isolates were showed inhibition for growth of *Micrococcus luteus*, as they sterile mycelium No. (1, 4, 5, 7 and 9) and *Chaetomium globosum*. The highest effect was evident through sterile mycelium no. (1) (22/ 14 mm), whereas the lowest effect was revealed by sterile mycelium no. 7 (14/14 mm).

On the other hand, these compounds show weak effect on *Escherichia coli* (2/ 39), where sterile mycelium 5 which showed clear zone (6 mm) while, it was estimated by (8 mm) for one isolate of sterile mycelium no.1.

Discussion

The endophytic fungi are the type of the most diverse group of organisms that have symbiotic relationship with higher plants, and have the ability to secrete substances beneficial to plant (Weber, 1981 and Shiomi *et al.*, 2006).

Endophytic organisms have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivorous (Weber, 1981). However only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive compounds. Recently studies have been carried out about the endophytic bio-diversity, taxonomy, reproduction, host ecology and their effort on host (Petrini, 1986 ; Arnold *et al.*, 2001 and Clay & Schardl, 2002). Endophytes, are now considered as an out standing source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments (Strobel & Daisy, 2003).

In the present study mainly *Acromonium* sp., *Aspergillus* sp., *Chaetomium* sp., *Cladosporium* sp. *Fusarium* sp., *Phoma* sp., *Ulocladium* sp. and sterile mycelium were isolated as endophytic fungi. Majority of endophytic fungi belonged to Ascomycetes and Deutromycetes (Frohlich & Hyde, 1999; Kumaresan & Suryanarayanan, 2002 and Bharathidasan & Panneerselvam, 2011).

It can be seen from this part of the study that, the extracts of endophytic fungi, had no effect on pathogenic fungi. But these extracts had appeared highest effect against some bacteria species (*Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*). The results also showed that the Gram-positive bacteria were the most sensitive than Gram-negative bacteria. Where, Shan *et al.* (2007) showed that among the five bacteria tested against 46 hydrophobic extracts, *S. aureus* was the most sensitive bacteria, while *E. coli* was the most resistant. The highest sensitivity of *B. subtilis* and *S. aureus* may be due to its cell wall structure and outer membrane (Zaika, 1988; Ceylan & Fung, 2004 and Lopez *et al.*, 2005). A possible explanation for this observation may be due to presence of an outer membrane and a unique periplasmic space in Gram - and not found in Gram-positive bacteria (Nikaido, 1996 and Duffy & Power, 2001).

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النشاط الميكروبي للفطريات الداخلية المعزولة من نبات ابن سينا. البحر الأحمر. مصر

ياسمين محمد شيباني

قسم النبات – كلية العلوم بقنا- جامعه جنوب الوادي- قنا – مصر.

لقد أكدت الدراسات الحديثة أن الفطريات وخصوصا الفطريات الداخلية أصبحت من أهم المصادر الطبيعية لإنتاج المركبات البيوكيميائية الهامة ، لهذا فان البحث المقدم يتناول عزل الفطريات الداخلية من نبات المانجروف ومعرفة مدى قدره هذه الفطريات على إنتاج مركبات مثبته لنمو بعض أنواع البكتيريا والفطريات.

وقد أوضحت النتائج انه تم عزل ٢١ نوع فطري تنتمي إلى ٨ أجناس فطرية من ٣٠ عينة من أشجار المانجروف جمعت من منطقتين على شاطئ البحر الأحمر وهذه الأجناس هي فطر الاكرومونيم ، الاسبرجيلس، الكيتوميم، الكلادوسبوريم، الفيوزاريوم، ، والفوما، واليوليكديم و الغزل الفطري العقيم . وكانت أكثر الأجناس شيوعا هو الغزل الفطري العقيم يليه فطر الاسبرجيلس.

ومن بين هذه الأجناس تم اختيار ٣٩ من الفطريات المختلفة لمعرفة مدى قدره هذه العزلات على تثبيط نمو البكتيريا (٤ أنواع) ونمو الفطريات (٢ نوع) أتضح الاتي أن جميع مستخلصات الفطريات لم تؤثر على نمو الفطريات المستخدمة بينما ٢٣ مستخلص فطري اظهروا تأثير على نمو الباسيلس ستلس في حين أن الاستفيلوكوكس اريس والميكروكوكس لتيس تأثروا ب ٨ مستخلصات فطرية فقط واثنان من المستخلصات اثروا على /شرشيا كولاي .