ISSN 2449-8955 European Journal of Biological Research *Research Article*

DOI: http://dx.doi.org/10.5281/zenodo.7036033

Antimicrobial activity of endophytic fungi associated with halophytic species *Salsola vermiculata*

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Received: 11 July 2022; **Revised submission:** 02 August 2022; **Accepted:** 29 August 2022

https://jbrodka.com/index.php/ejbr

ABSTRACT: Endophytic fungi are known for their production of bioactive compounds with antibacterial and antifungal activity. In this study, an evaluation of the antibacterial and antifungal activity of endophytic fungi isolated from *Salsola vermiculata*, a halophyte species collected from Chott el Hodna, M'sila (Algeria) was carried out. The eleven isolated endophytic fungi were identified as belonging to the genera *Alternaria* sp., *Aureobasidium* sp., *Phoma* sp., *Chrysosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Papulaspora* sp., *Ulocladium* sp., *Humicola* sp. and *Pencillium* sp. The antimicrobial activity of endophyte isolates was tested against phytopathogenic fungi and pathogenic bacteria using the dual culture and the agar plug diffusion methods respectively. The higher percentage inhibition of 79% was obtained by the isolate *Penicillium* sp. 1 against *Fusarium oxysporum* f.sp. *ciccri*. All isolated endophytic fungi showed antibacterial activity against at least one pathogenic bacterium, the greatest effect was obtained by *Fusarium* sp. against *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus cereus* ATCC 10876 with inhibition zones of 26.33 and 25.33 mm respectively. After the comparison of the means of the zones of inhibition, the isolate *Chrysosporium* sp. was the most active against all pathogenic bacteria with average inhibition zones of 20.55 mm.

Keywords: *Salsola vermiculata*; Antibacterial activity; Endophytic fungi; Antifungal activity.

1. INTRODUCTION

Eighty-five years after the discovery of *Penicillin* in1929, the emergence of infectious agents and resistance to antibiotics have made the development of new drugs a major challenge but very difficult from common environments. Therefore, researchers have focused on the selection of antimicrobial substances from microorganisms in particular habitats, such as fungal endophytes associated with halophytic plants [1-3]. The latter constitute about 1% of the world's flora, survive and reproduce in saline habitats such as salinized coastal and inland regions [4].

Endophytic fungi are microorganisms that live inter- and/or intracellularly colonizing healthy plant tissues during all of or part of their lifecycle without causing apparent pathogenic symptoms [5]. Many studies have shown that besides being a biological control agent and plant growth-promoting, the endophytic fungi are also considered as a rich source of secondary metabolites and bioactive compounds with commercial and medical uses [6, 7]. Given the importance of endophytic fungi in medicine and agriculture, the present study

was conducted to investigate the antibacterial and antifungal activities of endophytic fungi isolated from leaves, stems and roots of the halophytic species *Salsola vermiculata.*

2. MATERIALS AND METHODS

2.1. Plant sample collection, and fungal isolation

Leaf, stem and root samples of *Salsola vermiculata* were collected from Chott el Hodna, M'sila (Algeria). The plant materials were transported to the laboratory on ice and processed within 24 h of isolation. The samples were sterilized using the modified method described by Zhang et al. [5]. The stems, leaves, and roots were rinsed under running tap water and then surface sterilized by dipping in 70% (v/v) aqueous solutions of ethanol for 1 minutes, 5% (v/v) sodium hypochlorite for 5 minutes, and again 70% (v/v) ethanol for 30 seconds. Finally, the samples were rinsed in sterile distilled water four times, tapped dry with sterile filter paper and then each sample was cut into small pieces (0.5 cm), the last rinsed water was used as a fungi-free control. The small samples of each part were put in the same petri dish containing potato dextrose agar (PDA) with streptomycin (50 µg/mL) and penicillin (100 µg/mL). After 7-14 days of incubation in the dark at 28°C, the emerging fungi were isolated and purified on fresh PDA and stored at 4°C.

The diversity of fungi was studied using the following statistical formulae: Colonization frequency (CF, %) was determined as the ratio of the number of plant fragments colonized by fungi and the total number of fragments×100. Isolation rate (IR) was determined as the number of isolates obtained from plant segments/ the total number of segments incubated. The relative frequency (RF, %) was calculated by dividing the total number of isolates representing a single taxon by the total number of taxa obtained from all tissues × 100 [7].

2.2. Identification of endophytic isolates

The fungi were identified based on morphological characteristics according to the methods of Chen et al. [8]. After subculturing onto fresh PDA medium, and incubation at 28°C for at least one week, the culture characteristics including, colony shape, height and color of aerial hyphae, base color, growth rate, margin, and surface texture were observed. For microscopic examination, fungal cultures were put on a slide and examined by light microscope.

2.3. Antimicrobial screening

2.3.1. Antifungal activity

The antifungal activity of the endophytic fungi was tested using a dual culture method against four phytopathogenic fungi (*Fusarium oxysporum* f.sp. *albedinis*, *Phytophthora infestans*, *Fusarium solani* var. *coeruleum* and *Fusarium oxysporum* f.sp. *ciccri*). The phytopathogenic fungi were inoculated in the center of a fresh PDA plate. Then Five-day old discs (6 mm in diameter) of endophytes were placed on three points in petri plates. After incubation of the fungi at 25°C for seven days, the percent antagonism was calculated using the formula A (%) = $[(R_1-R_2)/R_1] \times 100$, where R_1 represents the colony radial growth of pathogen (measured in mm) on the control plates, in the absence of endophyte and R_2 is the radial growth of pathogen in test plate, in the presence of endophyte [9].

2.3.2. Antibacterial activity

In order to evaluate the antibacterial activity, the agar plug diffusion method was used according the method proposed by Marcellano et al. [10], with some modifications against three Gram-positive bacteria

(*Bacillus cereus* ATCC 10876*, Enterococcus faecalis* ATCC 49452, *Staphylococcus aureus* ATCC 25923), and three Gram-negative bacteria (*Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922). Briefly, the isolated endophytic fungi were cultured in PDA, after fourteen days of incubation at room temperature, agar plugs (6 mm) were cut and transferred to the Mueller Hinton Agar (MHA) which were previously cultivated by pathogenic bacterial (1 x 10^8 CFU/mL) in triplicate. These plates were kept in a refrigerator at 4°C for 12 hours for diffusion of metabolites, then were incubated at 37°C for 24 h and the means diameters of inhibition zones were measured.

2.4. Statistical analysis

All experiments were performed in triplicates, and statistical analysis was carried out using SAS/STAT® 9.2 software. Group comparisons were performed using the two-way ANOVA followed by Student-Newman-Keuls multip-rang test. Results are represented as mean ± standard deviation (SD), and significant effects of treatments were determined by F values ($P \leq 0.05$)

3. RESULTS AND DISCUSSION

3.1. Endophytic fungal isolation

After isolation, a total of eleven fungal isolates were obtained from 105 segments of *Salsola vermiculata*. Colonization frequency were 97.14% for roots, 17.14% for stems and 5.71% for leaves. Regarding the isolation rate, it was higher in roots and stems with 0.14, while for leaves it was 0.03 (Table 1).

Table 1. Colonization frequency (CF) and isolation rate (IR) of endophytic fungi from *Salsola vermiculata.*

Plant species	***Salsola vermiculata***		
Isolation source	**Roots**	**Stems**	**Leaves**
CF (%)	97.14	17.14	5.71
CF Overall (%)		39.99	
IR	0.14	0.14	0.03
IR Overall		0.1	

Many factors can influence the colonization of plants by endophytic fungi, among these factors, the type of tissue. In our study, the roots were the most colonized compared to stems and leaves. These results are in agreement with those obtained by Li et al. [4] where the roots of the ten halophytic plants used were the more colonized organ, as well as those obtained by Park et al. [11] who also isolated more endophyte fungi from the roots of *Panax ginseng* Meyer than from these stems and leaves.

This can be explained by the fact that the humidity in the soil is higher than in the air, which might result in higher colonization rate of endophytic fungi in roots than in aerial parts, as endophyte colonization is positively correlated with humidity. On the other hand, roots might be considered as a relatively stable and nutrient rich environment adequate for many fungal species [4, 12].

3.2. Identification of endophytic isolates

According to the morphological identification, the isolated endophytic fungi were identified as belonging to the genera *Alternaria* sp., *Aureobasidium* sp., *Phoma* sp., *Chrysosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Papulaspora* sp., *Ulocladium* sp., *Humicola* sp. and *Pencillium* sp., with the same Relative frequencies of 9.1% except for *Penicillium* sp., where it was 18.2% (Figure 1).

The salinity conditions of the *Salsola vermiculata* environment, and therefore the salinity of its tissues, do not represent an optimal habitat for endophytic fungi [13]. As a result, few fungal species have been isolated from this plant. In this study, all endophyte fungi that were isolated belonged to phylum of *Ascomycota*. Most of them (*Alternaria* sp., *Aureobasidium* sp., *Fusarium* sp., *Aspergillus* sp., *Ulocladium* sp. and *Humicola* sp.) were also isolated from ten halophytic plants [4]. Another study carried out on endophytes isolated from an estuarine mangrove forest allowed the isolation of *Penicillium* sp. and *Phoma* sp., two other endophytic fungi isolated in our study [14].

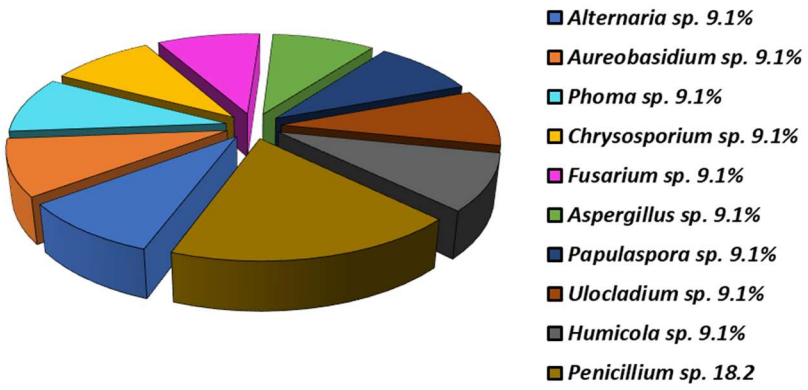

Figure 1. Relative frequencies (%) of different endophytic taxa isolated from *Salsola vermiculata.*

3.3. Antimicrobial activity

According to the results obtained during the antifungal screening, *Penicillium* sp.1 was the most active with the higher inhibition percentage of 79% obtained against *Fusarium oxysporum* f.sp. *ciccri*. The two isolates *Alternaria* sp. and *Fusarium* sp. showed moderate activity against all phytopathogenic fungi, whereas the other endophyte isolates showed little to no antifungal activity (Table 2).

Table 2. Screening of antifungal activity by dual culture method of endophytic fungi (n=3, mean of inhibition percentages ±SD).

Endophytic fungi	Inhibition percentages (%) ± SD			
	Fusarium oxysporum* f.sp. *albedinis	***Phytophthora infestans***	***Fusarium solani* var. *coeruleum***	***Fusarium oxysporum* f.sp. *ciccri***
Alternaria sp.	50.58±2.88	36.70±4.74	36.90±2.18	52±2.45
Aureobasidium sp.	21.17±6	-	30.75±9.97	30.99±2.45
Phoma sp.	19.99±1.66	-	-	16.99±5.66
Chrysosporium sp.	49.41±4.40	37.97±1.79	19.98±2.18	34.99±3.74
Fusarium sp.	43.52±2.88	39.23±3.10	38.44±2.18	52±0
Aspergillus sp.	32.93±4.99	30.37±1.79	35.36±3.77	38.99±2.83
Papulaspora sp.	-	-	-	26.99±2.83
Ulocladium sp.	-	-	-	-
Humicola sp.	29.40±2.88	12.65±8.20	-	36.99±2.45
Penicillium sp.1	65.88±4.40	50.63±8.20	63.07±3.77	79±0
Penicillium sp.2	29.40±2.88	37.97±3.58	26.13±3.77	27.99±4.24

The isolated endophytic fungi in our study from the halophyte species *Salsola vermiculata* showed activity against pathogenic bacteria and fungi. These results are in agreement with those obtained by Nurunnabi et al. [15] and Kalyanasundaram et al. [16], who isolated from the halophytic species *Sonneratia apetala* (Buch.-Ham), *Suaeda maritima* and *Suaeda monoica* many endophytic fungi that were active against different pathogenic bacteria and fungi

Penicillium sp. is a common endophytic fungus containing various active ingredients and is often used as a biocontrol fungus on plant pathogens. The strain of *Penicillium* sp. M-01 isolated from *Sophor flavescens* can produce antibacterial substance [17]. *Penicillium olsonii* ML37 an endophytic fungus isolated from Spring wheat cv. Diskett has been identified as a new potential biocontrol agent against wheat pathogen *Fusarium graminearum* [18].

All endophytic fungi isolated from *Salsola vermiculata* showed antibacterial activity against at least one pathogenic bacterium. The two isolates *Phoma* sp. and *Chrysosporium* sp. showed activity against all pathogenic strains used. The greatest effect was obtained by *Fusarium* sp. against *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus cereus* ATCC 10876 with inhibition zones 26.33 and 25.33 mm respectively (Table 3, Figure 2).

Table 3. Antibacterial effect of endophytic fungi isolated from *Salsola vermiculata* (n=3, mean of inhibition zones ±SD).

Endophytic fungi	Inhibition zones (mm)					
	B. c	***E. f***	***S. a***	***S. t***	***P. a***	***E. c***
Alternaria sp.	21.33±1.25	-	19.33±0.47	19.67±1.70	21±0.90	17.33±0.47
Aureobasidium sp.	18±0.82	-	11.67±1.25	12.33±0.47	15±0.90	-
Phoma sp.	21.33±0.47	9.67±1.25	18.67±0.47	17.33±1.25	19.33±0.69	15.33±0.47
Chrysosporium sp.	25±0.82	15±0.82	23.33±0.47	18.67±0.47	24.67±0.69	16.33±0.47
Fusarium sp.	25.33±1.25	-	23.33±0.47	17±00	26.33±0.97	16.33±0.47
Aspergillus sp.	24.67±2.62	-	17.33±5.25	11.33±1.25	21.33±2.12	-
Papulaspora sp.	-	-	-	8.67±1.25	14.67±1.92	10.00±2.94
Ulocldium sp.	-	-	-	-	-	-
Humicola sp.	17±0.82	-	18.33±0.94	11.67±0.47	19±00	15.33±0.47
Penicillium sp.1	-	-	-	-	16.33±0.69	-
Penicillium sp.2	11±0.82	3±4.24	-	-	14.67±1.43	-

B. c: *Bacillus cereus* ATCC 10876; *E.f* : *Enterococcus faecalis* ATCC 49452; *S. a* : *Staphylococcus aureus* ATCC 25923*; S. t* : *Salmonella typhimurium* ATCC 13311; *P. a* : *Pseudomonas aeruginosa* ATCC 27853; *E. c* : *Escherichia coli* ATCC 25922.

The comparison of the means of inhibition zones showed that the isolate *Chrysosporium* sp. was the most active endophytic isolate against all the pathogenic bacteria with a mean of inhibition zones of 20.55 mm, followed by *Fusarium* sp., *Phoma* sp. and *Alternaria* sp. *Penicillium* sp.1 was the isolate that showed the lowest activity with the lowest mean of inhibition zones of 2.72 mm (Figure 3).

The genus *Chrysosporium* gained an attention in recent times for its potential to degrade keratin. Some of the metabolites secreted by *Chrysosporium*, particularly enzymes, and antimicrobials compounds are gaining the attention of pharmaceutical industry. Few studies have isolated it as an endophyte. It was isolated from roots and stems of *Dysphania ambrosioides* [19] and from roots of soybean plants by Waqas et al. [20]. In 2002, Ivanova et al. [21] isolated and identified naphthoquinone-type compounds produced by *Chrysosporium queenslandicum* IFM 51121, these molecules had shown antifungal activity.

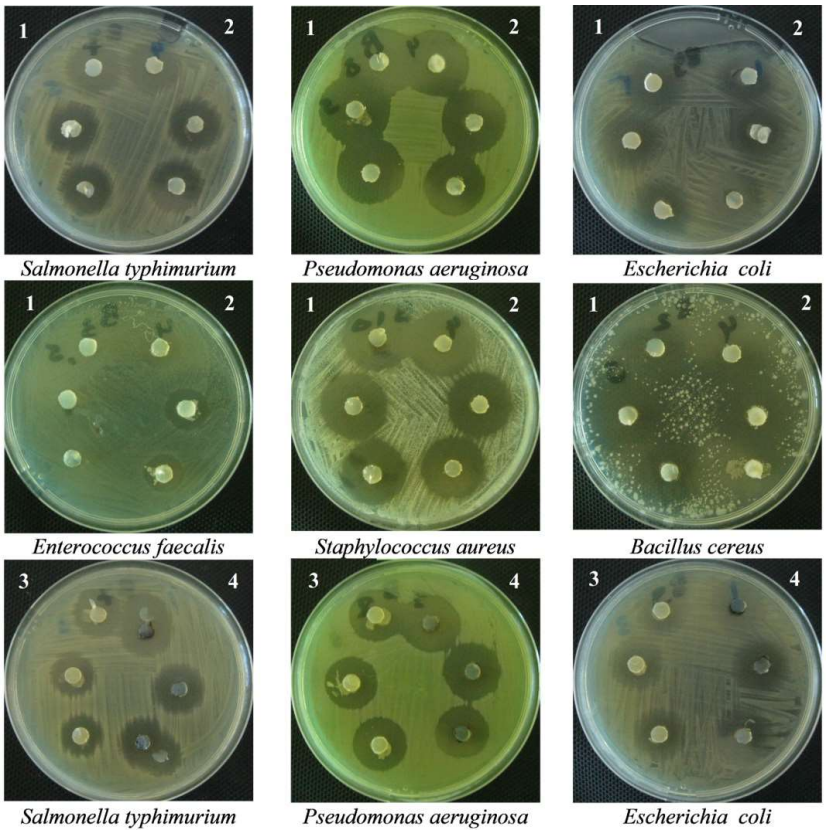

Figure 2. Antibacterial effect of endophytic fungi. 1: *Fusarium* sp., 2: *Chrysosporium* sp., 3: *Phoma* sp., 4: *Alternaria* sp.

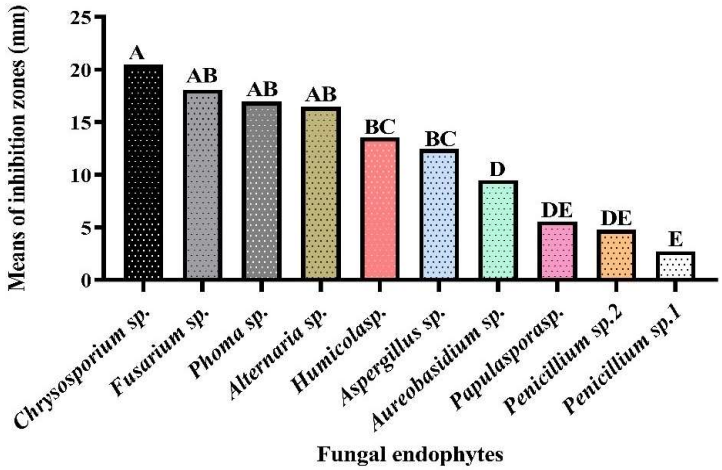

Figure 3. Comparison of inhibition zone averages of endophytic fungi and their effect on the growth of pathogenic bacteria (Means with the same letters are not significantly different at ($p < 0.05$).

Fungal endophytes of the genus *Fusarium* have been stated for the construction of structurally unique and complex yields having different important biological activities as antiviral, immunosuppressants, anticancer, antiparasitics, antithrombotic, immunomodulatory, antioxidant, antimalarial, and enzyme inhibitory activities [26]. For example, compound 29 to compound 65 were isolated from marine endophytic *Fusarium* species. Compounds 33 and 34 showed antibacterial activity towards *Mycobacterium tuberculosis*; while Fusarielin E (29), T2-toxin (30), 8-n-butyrylneosolaniol (31) and 8-isobutyrylsolaniol (32) showed antifungal activity [25]. Moreover, *Fusarium oxysporum* MERVA39 Derived from Red Sea Sponge *Hyrtios erectus*

exhibited great antibacterial activity against different Gram-positive and Gram-negative bacteria [29]. Crude extracts of several *Fusarium* spp. like *Fusarium oxysporum* from *Chromolaena odorata*, *Fusarium solani* from *Taxus baccata*, *Fusarium lateritium* from *Rhizophora mucroata*, *Fusarium equiseti* from *Garcinia parvifolia*, exhibited a broad antimicrobial spectrum [3]. Another study allowed the identification of a benzophenanthridine alkaloid, sanguinarine produced by the endophyte isolate *Fusarium proliferatum* (strain BLH51) having an antibacterial activity [1].

In this study the isolate *Phoma* sp. was one of the most active endophytic isolates against all pathogenic bacteria. This genus is known for his production of various bioactive compounds such as phomodione produced by *Phoma sp.*, an endophyte of S*aurauia scaberrinae* [1], this molecule, was found to be effective against *S. aureus* at a MIC of 1.6 µg/mL. Atrovenetinone which showed displayed good antibacterial activity, he was isolated and identified from *Phoma* sp.; an endophytic fungus associated with *Senecio kleinii* [22]. (+)-Flavipucine and (−)-Flavipucine, produced by *Phoma* sp. an endophyte associated with *Salsola oppositifolia*, showed activity against *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli*. and *Bacillus subtilis*, *Escherichia coli* respectively [23].

In similar studies, *Phoma* sp. SYSU-SK-7 isolated from healthy branch of the marine *Kandelia candel* produces polyketides colletotric B, 3-hydroxy-5-methoxy-2,4,6-trimethylbenzoic acid, colletotric C, chaetochromone D, and 8-hydroxy-pregaliellalactone exhibit significant antifungal and antibacterial activity [27] Also, two new diphenyl glycosides, phomaethers A-B, (163 and 164), novel pho-maether C (165) and identified compound 166 were extracted from the fungus *Phoma* sp. (TA07-1) isolated from a piece of fresh tissue from the inner part of the marine gorgonian *Dichotella gemmacea* (GX-WZ-2008003-4). Compounds 163, 164 and 165 exhibited potent antibacterial activity [28].

By comparing the means of inhibition zones of all the fungal isolates against each pathogenic bacteria as well as against the two groups of bacteria, the group of Gram-negative bacteria was the most sensitive with a mean of inhibition zones of 13.32 mm, while that of Gram-negative bacteria was more resistant (10.67 mm) (Figure 4). The results also showed that the most resistant bacterium was *Enterococcus faecalis* ATCC 49452, with a mean of inhibition zones of 2.46 mm, while *Pseudomonas aeruginosa* ATCC 27853 was the most susceptible (19.23 mm) (Figure 4).

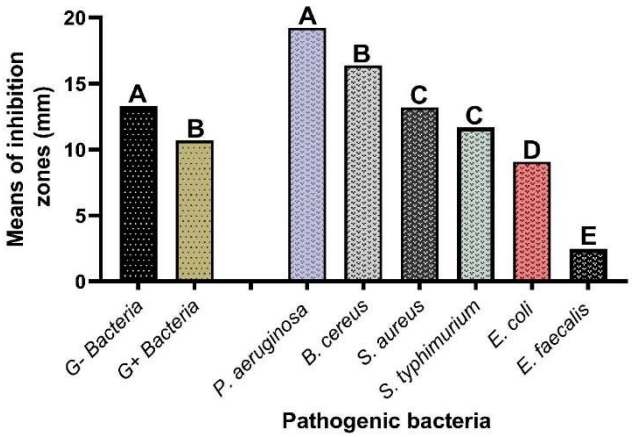

Figure 4. Susceptibility of pathogenic bacteria to endophytic fungi (Means with the same letters are not significantly different at ($p < 0.05$). *P. aeruginosa*: *Pseudomonas aeruginosa* ATCC 27853; *B. cereus*: *Bacillus cereus* ATCC 10876; *S. aureus*: *Staphylococcus aureus* ATCC 25923; *S. typhimurium*: *Salmonella typhimurium* ATCC 13311; *E. coli*: *Escherichia coli* ATCC 25922; *E. faecalis*: *Enterococcus faecalis* ATCC 49452.

Generally, Gram-positive bacteria were more sensitive than Gram-negative bacteria, this may be attributed to the nature of their cell wall structure. Generally, the cell wall of Gram-negative bacteria consists of complex structures such as the outer membrane layer, thin peptidoglycan layer, and periplasm compared to Gram-positive bacteria composed of a thick layer of peptidoglycan. The outer membrane layer is a special structure that differentiates between both bacteria [24]. In some cases, this structure can protect the bacteria against bioactive molecules, contrarily in others, it becomes the target of these bioactive molecules, it depends on the nature of metabolites produced by the endophytic fungi.

4. CONCLUSION

Our results demonstrate that *Salsola vermiculata* harbored various endophytic fungi identified as belonging to the genera *Alternaria* sp., *Aureobasidium* sp., *Phoma* sp., *Chrysosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Papulaspora* sp., *Ulocladium* sp., *Humicola* sp. and *Pencillium* sp. Regarding antifungal activity, *Penicillium* sp.1 was the most isolate active to other isolates, on the other hand, for the antibacterial activity, all endophytic fungal isolates showed antibacterial activity against at least one pathogenic bacterium, and Gram-negative bacteria were the most sensitive compared to Gram-positive bacteria. Therefore, these endophytic isolates would constitute an attractive source of pharmaceuticals. Further studies on the molecular identification of these isolates and on isolation of the bioactive compounds responsible for here activity are now needed.

Authors' Contributions: AZ and NS were responsible for conception, literature review, writing and revising the manuscript. Both authors read and approved the final version of the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

Acknowledgments: This work was supported by the General Directorate for Scientific Research and Technological Development of Algeria.

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