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

DOI: http://dx.doi.org/10.5281/zenodo.7028278

Broad antibacterial spectrum of endophytic fungi isolated from halophyte *Suaeda fruticosa* in Algeria

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Received: 20 July 2022; **Revised submission:** 14 August 2022; **Accepted:** 26 August 2022

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

ABSTRACT: The purpose of this work was to isolate and evaluate the antimicrobial potential of endophytic fungi isolated from *Suaeda fruticosa*. Endophytic isolates were identified at the genus level. The genera *Fusarium* (33.33%), *Phoma* (26.67%), *Penicillium* (13.33%), and *Aspergillus* (13.33%) were more prevalent, while *Trichoderma* genus (6.67%) was less common. The isolated fungal endophytes were screened for their potential antifungal and antibacterial activities. Most isolates showed different levels of inhibitory activity against at least one of the pathogens. The best inhibition percentages were those obtained by *Penicillium* sp. 1; 74, 71, 65, and 47% against *Fusarium oxysporum* f.sp. *albedinis*, *Fusarium oxysporum* f.p. *ciccri*, *Fusarium solani* var. *coeruleum* and *Phytophthora infestans* respectively. Regarding antibacterial activity, the zones of inhibition ranged from 0 to 25.5 mm. *Fusarium* isolates and *Phoma* sp. 4 showed the greatest antibacterial activity, the highest activity was observed with *Fusarium* sp.5 and *Fusarium* sp. 1, which gave zones of inhibition of 25.5 and 22.5 mm respectively against *B. cereus* ATCC 10876. Gram-positive bacteria were more sensitive to endophyte isolates than Gram-negative bacteria. From the results of the present work, it is possible to conclude that endophytic fungi isolated from *Suaeda fruticosa* could be a promising source of bioactive compounds and deserve further study.

Keywords: *Suaeda fruticosa*; Antimicrobial activity; Endophytic fungi; *Fusarium* sp.

1. INTRODUCTION

The irrational use of antibiotics during these several decades has led us to the era of multidrug-resistant bacteria (MDR). Antimicrobial resistance is a global public health challenge, which has been accelerated by the overuse of antibiotics around the world. Thus, the increase in antimicrobial resistance is the cause of serious infections, complications, a decrease in the cure rate and a lengthening of hospital stays, thus allowing the increase in the mortality rate [1]. Each year, around 700,000 people die from antibiotic-resistant bacteria, and this number will continue to rise if efforts are not made against this problem [2]. On the other hand, the increasing human population demands a simultaneous increase of 60–110% of the global food pool by 2050 to equilibrate the production-consumption ratio. Besides, fungal pathogens are a serious threat to domestic and commercial agrarian systems and largely affect the global food supply and economy, they cause 70–80% of livestock and post-harvest infections in vegetables, fruits, pulses, and cereals, which ultimately affect the global

demand-and-supply chain. Another problem, croplands losing their fertility and productivity due to overuse of chemical fertilizers [3].

In principle, there are three pathways for discovering new pharmacologically significant compounds: rational drug design, combinatorial chemistry, and natural product discovery by isolating bioactive compounds from biological sources [4]. Due to unique structures, low costs, and potent bioactivities, natural product drug discovery has regained the interest of biologists, chemists, and pharmacologists [2]. Natural products from plants have been shown to be one of the most promising. However, the quantity of many bioactive compounds in plant tissues is not sufficient. Natural products from microbes have contributed significantly to the current pharmaceutical industry. Recent advances in bacterial genomics research have proven that the potential of compounds derived from endophytic fungi was much more than previously believed [4].

Fortunately, endophytic fungi provide a huge potential as a new source for the development of novel antibiotics. In this line, modern studies have shown that endophytic fungi produce unique secondary metabolites against a wide range of microbial pathogens [5]. Endophytic fungi are microorganisms that reside in plant tissues, they spend all or part of their life cycle inside the host tissues, without causing noticeable symptoms of plant diseases [6]. Recently, endophytes have become a hot spot and have proven to be underexplored resources for natural product discovery. Research conducted so far on endophytic fungi has contributed to the discovery of possible drug compounds with antimicrobial, antioxidant, antiviral, antidiabetic, anti-Alzheimer's, and immunosuppressive properties, among others [7].

So far, no studies have been conducted on the isolation of endophytic fungi associated with *S. fruticosa* in Algeria. In this regard, the present study focused on the isolation, identification and evaluation of the antimicrobial potency of endophytic fungi, which could lead to the discovery of new antimicrobial agents in controlling post-harvest decay/loss of standing crops and clinical infections.

2. MATERIALS AND METHODS

2.1. Samples collection

Healthy plant leaves, stems and roots of *S. fruticosa* were collected from Chott El Hodna-M'sila (Algeria). Tissues with physical damage or showing signs of pathogenic infection were excluded from the study. The samples were then stored for less than 24 h at 10 °C before being isolated.

2.2. Isolation, purification, and culture of the fungi

The isolation of endophytic fungi was performed from different organs (roots, stems, and leaves) of the plant. These latter were rinsed with normal tap water to remove surface adherent. Then, under aseptic conditions the following operations were performed in the order listed: dipping in 70% ethanol for 1 min, 2.5% sodium hypochlorite for 3 min, and again 70% ethanol for 1 min. The samples were then washed twice with sterile distilled water, dried on pre-sterile filter paper, cut into 0.5-1 cm pieces and transferred on Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol (100 µg/mL). To validate that the sample surfaces had been sterilized, the water from the final rinsing was spread on the PDA plates as control. The petri dishes were incubated at 28 °C in the dark and monitored every day to check the growth of endophytic fungal hyphae emerging from segments. After purifying the isolates several times, the final pure cultures were transferred to PDA slant tubes for storing at 4 °C [8]. The colonization rate (CR), the isolation rate (IR) and the relative frequency (RF) were calculated according Padash and his collaborators [9].

$$\text{Colonization Rate (CR\%)} = \frac{\text{Total number of plant tissue segments colonized by endophytic fungi}}{\text{Total number of segments incubated}} * 100$$

$$\text{Isolation rate (IR)} = \frac{\text{Number of isolates obtained from segments}}{\text{Total number of segments incubated}}$$

$$\text{Relative frequency (RF\%)} = \frac{\text{Isolates of one taxa}}{\text{Total number of isolates}} * 100$$

2.3. Identification of isolated endophytic fungi

Obtained isolates were grown on PDA and separated into morphotypes based on visual assessment of culture similarity, where macroscopic characteristics included colony morphology and growth rate of fungal isolates, and microscopic characteristics included the mycelium type, shape and size of the conidia [10].

2.4. Antifungal activity

All the isolates of fungal endophyte were tested for their antagonistic activity against four phytopathogenic fungi; *Fusarium oxysporum* f.sp. *albedinis*, *Fusarium solani* var. *coeruleum*, *Fusarium oxysporum* f.sp. *ciccri*, and *Phytophthora infestans*. The fungal isolates and phytopathogens were co-cultured on PDA plates. Briefly, 6 mm PDA plug from 5-day-old mycelia of the pathogenic fungi was placed at the center of a PDA plate, and three plugs of the fungal endophyte isolate were placed on the agar surface at three equidistant points. Dual cultures were incubated at 25°C for 5 days and the radial growths of fungal mycelial was measured and compared to the control (pathogenic fungi alone). The antagonistic tests were conducted in triplicate. The radial growths of the mycelia of the test pathogen on a control (R_1) and in the direction of the endophytic fungus (R_2) were measured and the percent inhibition in mycelial growth was calculated based on the formula $I\% = [(R_1 - R_2)/R_1] \times 100$ [11].

2.5. Antibacterial activity

All endophytic fungal isolates were assessed for their antibacterial activity 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) according to the plug agar diffusion method previously described [12] with some modifications. Briefly, the lawns of pathogenic bacteria were prepared on Mueller Hinton Agar (MHA) using sterile cotton swabs, then agar plugs (6 mm diameter) of pure cultures of fungi in PDA (14-day-old) were transferred to media previously seeded with pathogenic microorganisms (turbidity of 0.5 McFarland standards) in triplicate. After 12 hours at 4°C for diffusion of metabolites, these plates were incubated at 37°C for 24 h and the diameters of inhibition zones were measured.

2.6. Data analysis

Statistical analysis was done using SAS/STAT ® 9.2. The results of the antimicrobial activity were analyzed statistically by the two-way ANOVA followed by the Student-Newman-Keuls MULTIP-rank test to compare the average inhibition zones of the extracts. The results were expressed as mean ±SD, and the measures were repeated three times (n = 3). The difference was considered statistically significant when the p value was $\leq 0{,}05$.

3. RESULTS AND DISCUSSION

3.1. Isolation of endophytic fungi

From 105 segments of roots, stems, and leaves from *S. fruticosa*, a total of 15 strains of endophytic fungi were isolated. All the plant samples used harbored various endophytic fungi with different rates of colonization (CR) and isolation (IR). By comparing the colonization rates of roots, stems and leaves in the *S. fruticosa* plant, we find that endophytic fungi are found in abundance in the roots (94.29%), and gradually decrease in the stems (11.43%), and leaves (8.57%). The isolation rates (IR) were higher and very close to each other in the roots and the stems, and lower in the leaves. The IR values of endophytic fungi obtained from root, stem, and leaf segments were 0.26, 0.2, and 0.1 respectively (Figure 1).

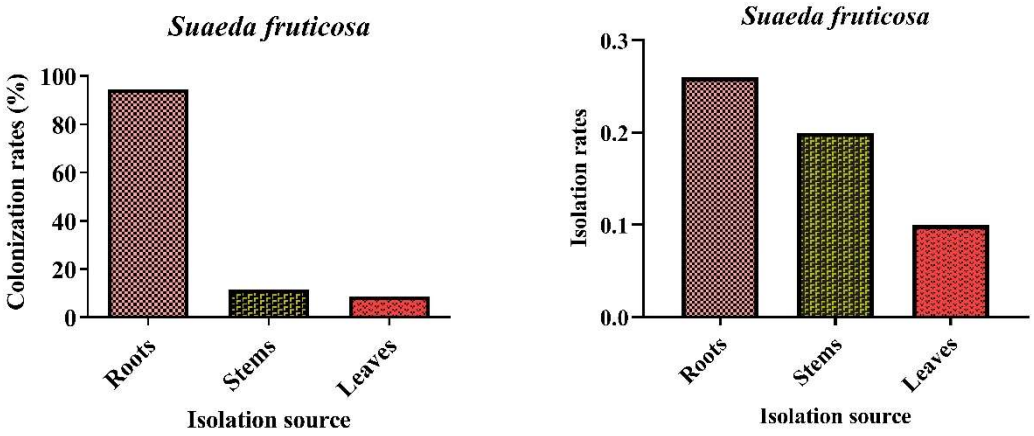

Figure 1. Colonization and isolation rates of endophytic fungi from *S. fruticosa.*

It was found that the colonization rate and richness of endophytic fungi were generally higher in roots and stems than in leaves, which is in agreement with the studies carried out on desert halophyte species [13,14]. The difference between endophytic colonization and diversity between above and belowground might be attributed to both biotic and abiotic factors. In the study site, humidity is much lower in the air than in the soil, which might result in lower colonization rate and species richness of endophytic fungi in stems than in roots, as endophyte colonization is positively correlated with humidity [15].

In desert habitats, vegetation is sparsely distributed with low diversity and density, but the belowground systems of plants are well developed, forming patches of nutrient-rich resources. Thus, the distribution of extended root systems and nutrient status in a desert environment may lead to higher endophytic fungal diversity in the roots than shoot tissues by providing more host and substrate for infection of these fungi [16]. Additionally, the relatively moist, organic-rich soil substrate is able to support diverse and abundant fungal propagules for penetration into plant roots versus stems.

3.2. Identification of isolated endophytic fungi

Among the 15 isolates obtained, fourteen isolates (93.33%) were identified based on morphological characteristics and were then assigned to 6 genera (Table 1). The data obtained show that certain taxa were particularly abundant only in particular tissues such as the genera *Fusarium*, *Trichoderma* and *Aspergillus*, or at least in two types of tissues such as the genera *Penicillium* and *Phoma*.

The largest frame of endophytic fungal genera that we have been able to identify is placed within the group of Deuteromycetes and most of whose taxa (*Aspergillus, Penicillium, Fusarium*) belong to the class of

Hyphomycetes or to the Coelomycetes class (*Phoma*). The genera *Fusarium* (33.33%), *Phoma* (26.67%), *Penicillium* and *Aspergillus* (13.33%) were more prevalent, while *Trichoderma* genus (6.67%) was less common than the other four genera (Table 1).

Table 1. Relative frequencies of different endophytic taxa isolated from *S. fruticosa.*

Fungal genera	Number of isolates	RF (%)	Sources		
			Roots	Stems	Leaves
Fusarium	5	33.33	+	-	-
Penicillium	2	13.33	+	+	-
Phoma	4	26.67	+	+	-
Trichoderma	1	6.67	+	-	-
Aspergillus	2	13.33	-	-	+
UI isolates	1	6.67	-	-	+

RF: Relative frequencies, UI: Unidentified.

The genus *Fusarium* showed the highest relative frequency of isolation (33.33%) in the total endophytic fungi isolated, and all isolates of this genus have been isolated from the roots. similar results were also previously found with several halophilic plants such as *Salsola incanescens* [17]. Among 36 fungal genera, only *Fusarium* and *Penicillium* species was commonly identified in all halophyte species from all geological regions [18].

All isolated genera have been previously reported as endophytic fungi in many halophilic and desert plants such as *Anabasis iranica*, *Salsola yazdiana*, *S. tomentosa* [19], *Vitex rotundifolia* [20], *Suaeda australis, S. maritima, S. glauca Bunge, Phragmites australis, Limonium tetragonum* [21] and others [22]. Generally, the most dominant genera identified in several studies were *Alternaria*, *Fusarium* and *Penicillium, Phoma*, and *Aspergillus* [23], whereas genera that were represented by only one or a few isolates were, *Trichoderma Ulocladium* and *Chrysosporium*. It should be noted that the frequency of isolation of some genera depends on plant characteristics, such as genotype, age, geography, sampled tissues, or sampling season [24].

Colonization of halophyte plants is very important for the host because; they improve the capacity of tolerance to abiotic stress like drought and high salinity [25]. Previous reports have confirmed that fungal species such as *Penicillium citrinum* and *Fusarium oxysporum*, *Aspergillus terreus*, and *Alternaria alternata* could improve the survival and growth of their hosts by enhancing tolerance to environmental stress and increased salt stress resistance, limit the damage caused by pathogenic microorganisms and protect the host from diseases, and promote the growth of their hosts by the production of Plant hormones like gibberellins and indole-acetic acid that are essential for many developmental processes in plants, including seed germination, stem elongation, leaf expansion, ripening, and the induction of flowering [21].

3.3. Antifungal activity

The antifungal activity was evaluated using the dual culture method. The most active isolate was *Penicillium* sp. 1 with percentage inhibition of 74.11, 71, 64.60, and 46.83% against *F. oxysporum* f.sp. *albedinis*, *F. oxysporum* f.p. *ciccri*, *F. solani* var. *coeruleum* and *P. infestans* respectively. *Penicillium* sp.2 isolate and *Fusarium* isolates showed medium activity, especially against *F. solani* var. *coeruleum* and *F. oxysporum* f.sp. *ciccri*. The rest of the isolates had weak antifungal activity (Table 2).

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

Identification	Inhibition percentages (%) ± SD			
	F. oa	***F. sc***	***F. oc***	***P. i***
Fusarium sp.1	42.35 ±6.66	26.13 ±3.77	45.99 ±6.48	41.76 ±4.74
Fusarium sp.2	32.93 ±5.76	46.14 ±4.35	43.99 ±1.41	36.70 ±4.74
Fusarium sp.3	37.64 ±6	39.98 ±11.31	60 ±3.74	35.43 ±3.10
Fusarium sp.4	34.11 ±6	26.13 ±11.31	42.99 ±6.48	24.04 ±3.10
Fusarium sp.5	37.64 ±4.40	49.22 ±3.77	55 ±2.45	30.37 ±4.74
Penicillium sp.1	74.11 ±4.40	64.60 ±2.18	71 ±2.83	46.83 ±8.20
Penicillium sp.2	42.35 ±4.79	47.68 ±9.49	62±1.41	39.23 ±14.21
Aspergillus sp.1	4.69 ±5.76	6.12 ±2.18	8.99 ±3.74	11.38 ±3.58
Aspergillus sp.2	8.22 ±2.88	44.60 ±3.77	29.99 ±3.74	8.85 ±5.37
Phoma sp.1	31.76±10.91	12.28 ±3.77	36.99 ±2.45	25.31 ±4.74
Phoma sp.2	11.75 ±4.99	23.05 ±5.76	31.99 ±3.74	22.78 ±4.74
Phoma sp.3	28.23 ±1.66	27.67 ±4.35	43.99 ±1.41	25.31 ±7.16
Phoma sp.4	12.93 ±6	15.36 ±5.76	27.99 ±2.45	15.18 ±1.79
Trichoderma sp.	35.29 ±1.66	32.29 ±7.85	44.99 ±3.74	10.12 ±1.79
UI Isolate	27.05 ±4.40	29.21 ±13.24	53 ±3.74	29.10 ±9.97

F. oa: Fusarium oxysporum f.sp. *albedinis*; *F. sc: Fusarium solani* var. *coeruleum*; *F. oc*: *Fusarium oxysporum* f.sp. *ciccri*; *P i*: *Phytophthora infestans*; UI : Unidentified.

3.4. Antibacterial activity

All endophytic fungi of this plant were examined for their antibacterial activity by the agar plug diffusion method; the different isolates showed varying antibacterial activity, where the averages of the zones of inhibition varied from 0 to 25.5 mm.

Thirteen fungi (86%) isolated from *S. fruticosa* showed antibacterial activity against *B. cereus* ATCC 10876, 10 (70%) isolates were able to inhibit both *S. typhimurium* ATCC 13311*, E. coli* ATCC 25922*, P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. While *E. faecalis* ATCC 49452 was the most resistant where only 6 (40%) isolates inhibited the latter. The four isolates of the genus *Fusarium* showed the greatest antibacterial activity compared to the other genera, the highest activity was observed with *Fusarium* sp.5 and *Fusarium* sp.1, which gave zones of inhibition of 25.5 and 22.5 mm respectively against *B. cereus* ATCC 10876. On the contrary, all the pathogenic bacteria were not affected at all by *Aspergillus* sp.1 and *Penicillium* sp.1, except for the bacterium *P. aeruginosa* ATCC 27853 (Figure 2 and 3).

In order to determine the most active endophyte isolates, the most sensitive and the most resistant bacteria, a comparison of the means of the zones of inhibition was carried out. The results showed that isolates of *Fusarium* sp. 5, 1, 4, 2 and *Phoma* sp. 4, were the most active without significant difference at $P<0.05$ with mean zones of inhibition of 17.3, 17, 13.9, 13.6, 13.4 mm respectively (Figure 4).

The most susceptible bacteria were *B. cereus* ATCC 10876 in the Gram-positive bacteria group and *E. coli* ATCC 25922 in the Gram-negative bacteria group, while *E. faecalis* ATCC 49452 and *S. typhimurium* ATCC 13311 were the most resistant. The comparison of the means of the zones of inhibition showed that the group of Gram-positive bacteria was more sensitive to endophyte isolates than the group of Gram-negative bacteria (Figure 5).

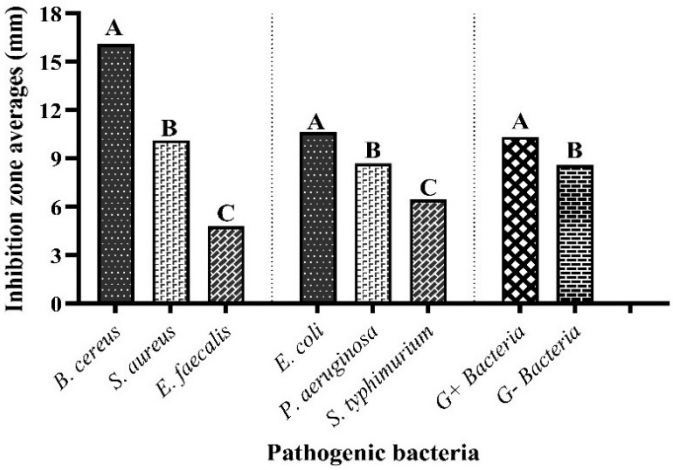

Figure 2. Antibacterial effect of endophytic fungi isolated from *S. fruticosa,* (n=3, mean of inhibition zones ±SD).

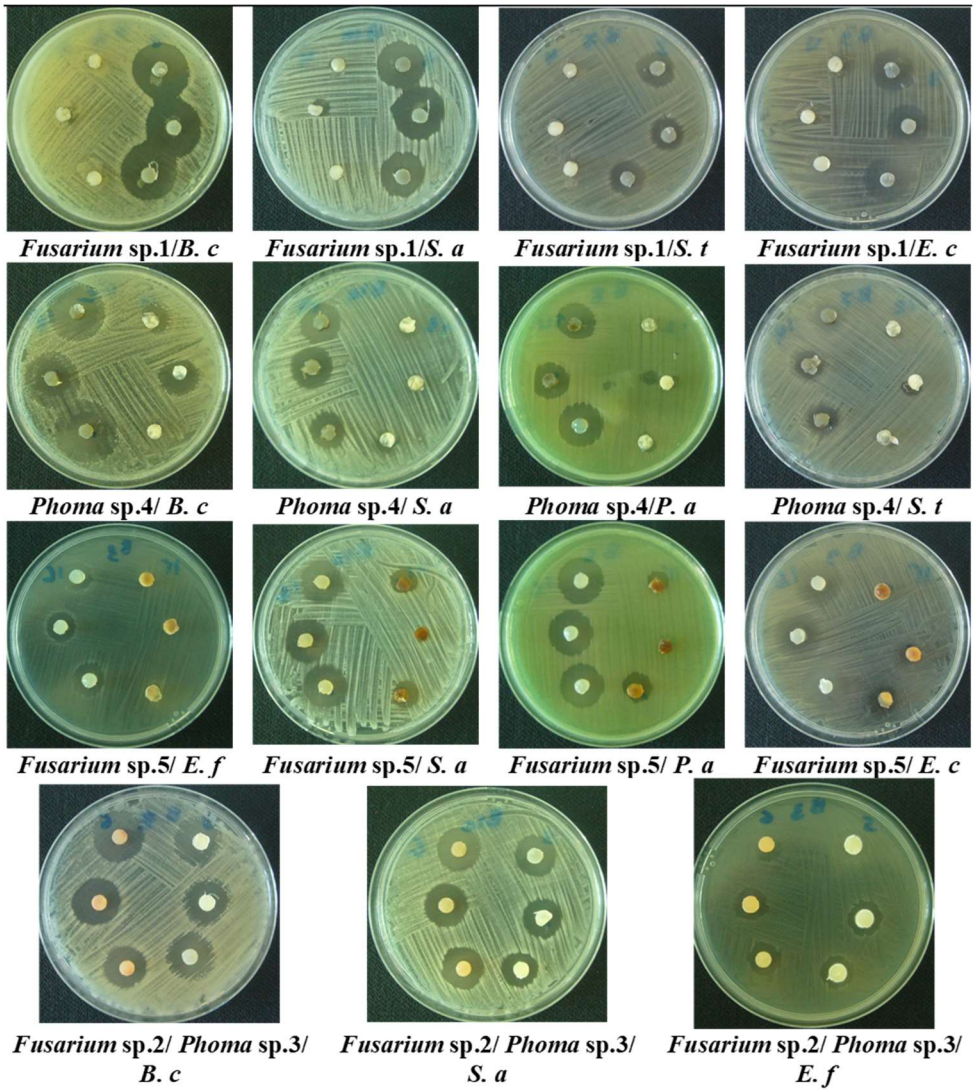

Figure 3. Some inhibition zones of the different endophytic fungi isolated *S. fruticosa,* (n=3). *B. c*: *Bacillus cereus* ATCC 10876, *E. f*: *Enterococcus faecalis* ATCC 49452, *S. a*: *Staphylococcus aureus* ATCC 25923, *S. t*: *Salmonella typhimurium* ATCC 13311, *P. a*: *Pseudomonas aeruginosa* ATCC 27853*, E. c* : *Escherichia coli* ATCC 25922.

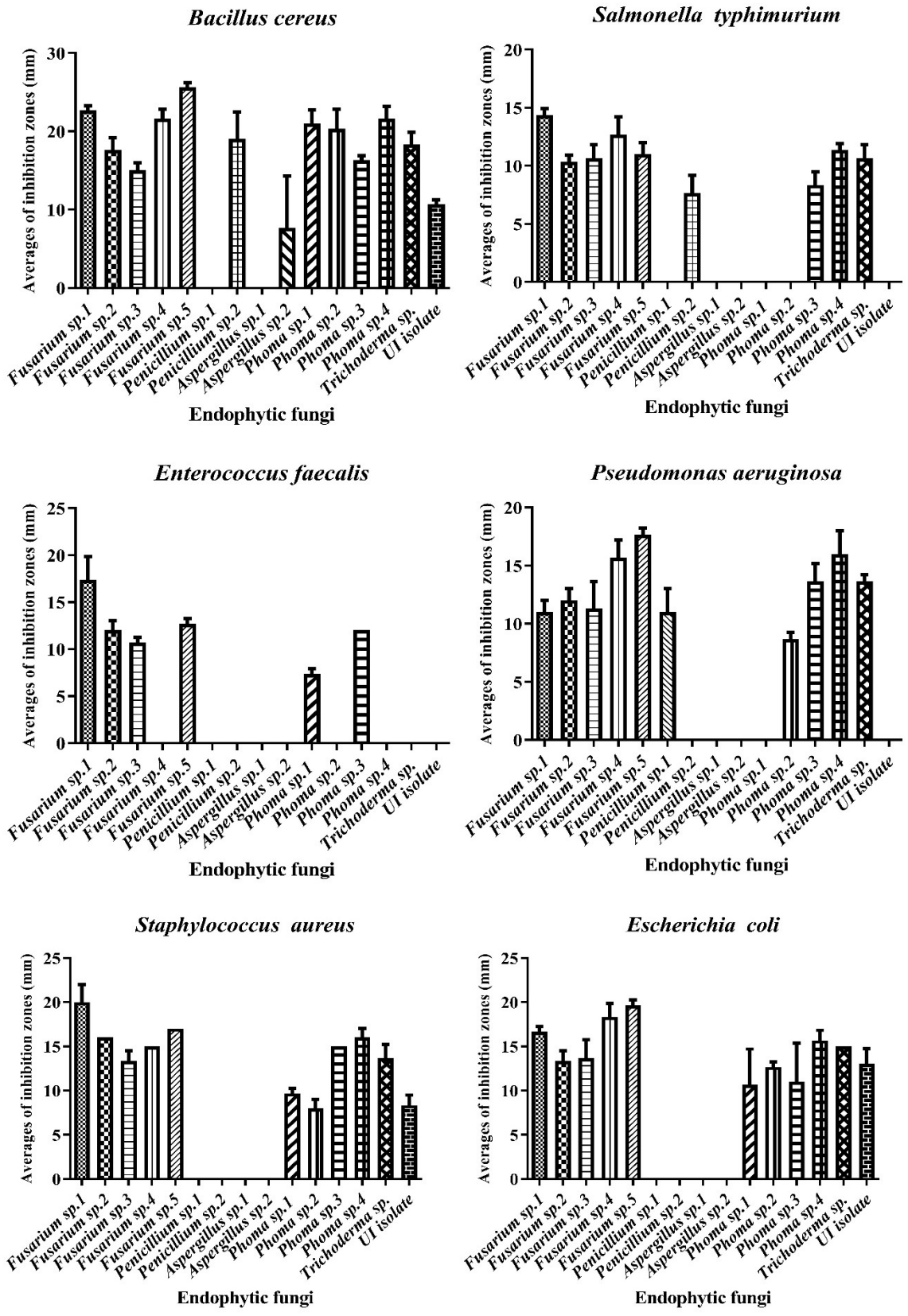

Figure 4. Comparison of inhibition zone averages of endophytic fungi and their effect on the growth of test microorganisms. Means with the same letters are not significantly different at ($p < 0.05$).

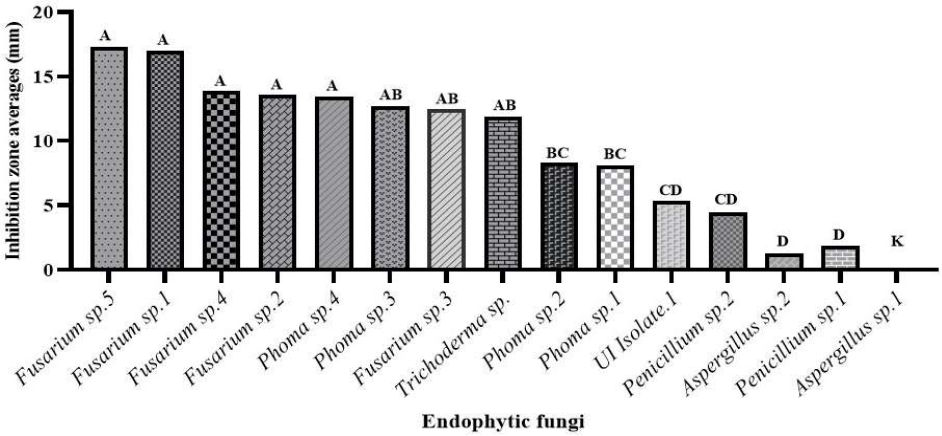

Figure 5. Susceptibility of pathogenic bacteria to endophytic fungi. Means with the same letters are not significantly different at ($p < 0.05$).

Generally, the identified fungi and the most active isolates belong to the genera *Fusarium,* and *Phoma.* In several previous studies, these genera have been isolated as endophytes, and some species of these genera have also been reported to have the ability to produce a wide range of secondary metabolites with antimicrobial activity.

The fungal endophytes from arid areas of Andalusia *Fusarium equiseti* CF-285462, and *Phoma* sp. CF-285355 showed antifungal activity against human and plant pathogens [23]. From all endophytic fungi isolated from medicinal plants in Saudi Arabia; *Fusarium oxysporum*, and *Fusarium nygamai* exhibited the highest inhibition against the human pathogenic bacteria *S. aureus, E. coli, P. aeruginosa, and Klebsiella pneumoniae* [26]. *Fusarium* endophyte species such as *F. lateritium* from *Rhizophora mucronata* [27], *F. oxysporum* isolated from *Chromolaena odorata* [28], and *F. oxysporum* isolated from *R. apiculate* [29] were previously reported to exhibit a high antimicrobial activity against a wide range of pathogenic microorganisms indicating their ability and potential to produce bioactive metabolites.

Also, several studies have previously reported the antibacterial activities of endophytic fungi *Phoma* such as *Phoma medicaginis* isolated from *Mikania cordata* that was found to be effective against all four bacterial pathogens [12]. *Phoma* sp. SYSU-SK-7 isolated from *Artemisia princeps* Pamp. has been shown to produce molecules active against pathogenic bacteria and fungi [30]. The compound 368 (Barcelonaic acid C) purified from *Phoma* sp. JS752 residing inside *Phragmites communis* exhibited moderate antibacterial activities against *Listeria monocytogenes* and *Staphylococcus pseudintermedius* [31].

In this study, we found that Gram-positive bacteria were more sensitive than Gram-negative bacteria, which is in agreement with the results of several previous works [32,33]. A possible explanation for this observation may be due to the morphological differences in the cell walls of these pathogens, The cell wall of Gram-negative bacteria is more complex, constituted by a thin peptidoglycan layer adjacent to the cytoplasmic membrane and an outer membrane (OM) composed by phospholipids and lipopolysaccharides (LPS), this outer membrane not found in Gram-positive bacteria [34,35].

4. CONCLUSION

This is the first study in Algeria to explore endophytic fungi of *S. fruticosa* and evaluate their potential *in vitro* antimicrobial activities. In this study, the results indicate that out of fifteen isolated endophytic fungi, six isolates were capable of depicting a higher antimicrobial activity when compared with others. The

Penicillium sp. 1 isolate was the most active against phytopathogenic fungi, while the four isolates of *Fusarium* sp. 1, 2, 4, and 5, and the two isolates of *Phoma* sp. 3 and 4 were found to be effective against most bacterial pathogens under study. On the contrary, isolates *Penicillium* sp. 1 and 2 and *Aspergillus* sp. 1 and 2 showed no activity against most bacteria. The antibacterial activity of endophytic isolates of *S. fruticosa* shown in this work against human pathogenic bacteria indicates that these endophytic microorganisms could be exploited in biotechnology, medicine and agriculture. Meanwhile, the use of endophytes as producers of bioactive agents will help in the conservation of medicinal plants and the maintenance of environmental biodiversity.

Authors' Contributions: NS, planned, conceptualized, and designed the experiment. NS and AZ: helped with sample preparation, collection and analyzed the data, and wrote 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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