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Endophytic Fungi Isolated from *Thymus algeriensis* with Good Antimicrobial Activities

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ABSTRACT

The aim of this study was to identify the endophytic fungi associated with medicinal plant *Thymus algeriensis* and to evaluate their potential for antimicrobial activities. A total of 16 fungi belonging to 6 genera were successfully isolated and identified. The colonization rates ranged from 14.29% to 42.86% and were significantly higher in the roots followed by stems and leaves. Regarding the isolation rates, they were 0.23, 0.14 and 0.1 for the roots, stems and leaves respectively. Based on the comparison of the morphological characteristics, six genera were identified: *Rhizopus*, *Fusarium*, *Aspergillus*, *Alternaria*, *Phoma*, and *Penicillium*. *Fusarium*, *Phoma*, and *Alternaria* were the most dominant with relative frequencies of 35.5, 28.6, and 14.3% respectively. The fungal endophytes were assessed for their antimicrobial activities using agar plug diffusion method, the best zones of inhibition obtained with the most active endophytic isolates were 20.33 and 20 mm for *Fusarium* sp. 3, 22.33 and 18.67 mm for *Fusarium* sp. 5, 23.33 and 25.33 mm for *Fusarium* sp. 2, and 29.33 and 23 mm for *Phoma* sp. 4 obtained against *Bacillus cereus* ATCC 10876 and *Staphylococcus aureus* ATCC 25923 respectively. The comparison of the averages of inhibition zones obtained against all the pathogenic bacteria showed that the isolates *Fusarium* sp.3 and *Fusarium* sp.5 were the most active with mean zones of inhibition of 19.61 and 19.56 mm respectively, followed by *Fusarium* sp.2 (19 mm) and *Phoma* sp.4 (18.61mm). Regarding the antifungal activity, the results showed that the highest inhibition percentages were 60 and 58% obtained by *Rhizopus* sp. and 51 and 53% obtained by *Aspergillus* sp. against *Fusarium oxysporum* f. sp. *ciccri* and *Phytophthora infestans* respectively. The study concludes the presence of endophytic fungi such as *Fusarium*, *Phoma*, *Penicillium* and *Aspergillus* associated with *Thymus algeriensis* that exhibited antibacterial activity. These isolates could serve as potential sources for the isolation of novel antimicrobial agents that may contribute to antibiotic control of antibiotic-resistant bacteria.

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Introduction

Huge global losses are caused by plant diseases and post-harvest rot due to phytopathogenic fungi; the latter can also be harmful to humans by producing mycotoxins. To solve these problems chemical fungicides are widely used, however, their uncontrolled use has led to the appearance of multi-resistant phytopathogenic fungi (Xu et al., 2021). On another hand, bacterial infections associated with antibiotic resistant strains and the emergence of new microbial pathogens like coronaviruses have necessitated a need to search and develop new and highly effective antimicrobial agents (Rai et al., 2022; Manganyi et al., 2019) .

The fungal endophytes that asymptomatically reside in the internal tissues of plants would be one of the promising alternatives to solve all these problems, because these fungi provide tolerance to their hosts against various types of biotic stresses by producing diverse biologically active secondary metabolites, and that exhibit biological activities against a wide variety of microbes (Xu et al., 2021). Previous studies on endophytic fungi and their antimicrobial activities have been carried out, such as that of Masumi et al., 2015, who isolated from 6 species of *Thymus*, 95 isolates consisting of 11 different fungal genera, 6 yeast isolates and 19 sterile mycelia. Of these, 89

isolates were tested and examined to investigate their biocontrol effects against human and plant pathogens, who their antimicrobial properties were confirmed (Mirzaei et Masumi, 2021). For this reason, we are interested in this study on isolation of endophytic fungi from *Thymus algeriensis* and to screen for their antagonistic activities against human pathogenic bacteria and phytopathogenic fungi.

Materials and Methods

Plant Material Collection

Healthy, fresh, and symptomless leaves, stems, and roots of *Thymus algeriensis* were collected from Bordj Bou Arreridj region. Plant samples were placed in sterile polythene bags, reserved in icebox, transported to the laboratory, and process within 24-48 h of collection.

Isolation of endophytic fungi

To isolate endophytic fungi; surface sterilization was carried out according to the protocol of Sadrati et al. (2020). Briefly, after rinsing with normal tap water to remove the adherent surface, the leaves, stems, and roots were dipped sequentially into 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, 70% ethanol for 30 sec, and then rinsed with sterilized distilled water three times. Finally, the plant material was dried over sterile filter paper after which it was cut into pieces of 3 - 3.5 cm with a sterile scalpel. Five pieces of each part were placed on Potato Dextrose agar (PDA) plate supplemented with streptomycin (1 g/L) and incubated at 28 ± 2°C until fungal growth was initiated. Fungal hyphae tips growing from the plant tissues were sub-cultured on PDA. After purification, the isolates were transferred to PDA slants and stored at 4°C.

Identification of Endophytic Fungi

All fungal isolates were grown on PDA. After incubation at 28°C for 6–8 days, the macroscopic characteristics include the colony morphology, color and tint in colony surface and reverse, colony surface texture, colony margin, pattern, pigment exuded, and growth rate were observed. The microscopic characteristics such as presence of conidia, the conidial shape and size, rate of sporulation, hyphae, hyphae form, and the presence of septum were observed using microscope (Rabha et al., 2016).

Screening of Antimicrobial Activity

The endophytic isolates were subjected to an antimicrobial assay using a solid medium to select the active isolates. The tests were conducted against six human pathogenic bacteria *Bacillus cereus* ATCC 10876, *Enterococcus faecalis* ATCC 49452, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 using agar plug diffusion method, and the dual culture method was used against four phytopathogenic fungi, *Fusarium oxysporum* f.sp. *albedinis*, *Phytophthora infestans*, *Fusarium solani* var. *coeruleum* and *Fusarium oxysporum* f.sp. *ciccri*.

Antibacterial Screening

For antibacterial activity, the method was adapted from the protocol used by Ibrahim et al. (2014). Briefly, the agar plugs (6 mm in diameter) of endophyte isolates were cut from the fungal cultures 15 days old. The agar plugs were then placed on the Mueller Hinton agar medium seeded with pathogenic bacteria previously adjusted at 1 x 10^8 CFU/mL. All experiments were performed using three replicates. Refrigeration for 6 hours at 4°C is necessary to allow the diffusion of bioactive molecules. All Petri dishes were than incubated for 24 hours at 37°C for bacteria. The antimicrobial activity of the endophytic fungi was detected by the appearance of the inhibition zones around the agar plugs.

Antagonistic Activity

To investigate the antagonistic activity of endophytic isolates, the dual culture method was used. For it, all fungi (endophytes and phytopathogens) were cultured on PDA media at 28 °C for 7 days. Then, three mycelium plugs agar (6 mm) of endophytic fungi were inoculated at the periphery of PDA plates and one mycelium plug agar of phytopathogenic fungi was inoculated on the center of plates. Petri plates inoculated only with phytopathogens served as control. The plates were then incubated at room temperature for 7 days (Sadrati et al., 2013). The percent of inhibition of mycelia growth over the control was calculated by the following equation:

$$I = (A - B)/A \times 100$$

Where, A is the ray of phytopathogens mycelium with the growth in control, and B is the ray of phytopathogens mycelia in the dual culture (Khan et al., 2021).

Statistical 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 endophytic fungi. All experiments were performed in triplicates, Results are represented as mean ± standard deviation (SD), and significant effects of treatments were determined by F values ($P \leq 0.05$).

Results and Discussion

Isolation and Colonization Rates

After isolation, the calculation of the colonization rates showed that the roots were the most colonized with a rate of 42.86%, followed by the stems (20%) and the leaves (14.29%). Regarding the isolation rates, they were 0.23, 0.14, and 0.1 for the roots, stems, and leaves respectively (Figure 1).

In the present study, the number of isolates from underground organs (roots) was higher than those from aerial organs (stems and leaves). Similar results have been obtained in many previous studies on endophytic fungi regarding their diversity and different assemblages between roots and leaves. In the study carried out by Manganyi et al. (2018a) on the endophytic fungi from *Pelargonium sidoides*, the overall colonization rate was higher in the roots (28%) than the leaves (25%) of this plant. Similarly, Liu et al. (2019) found that the colonization rate of endophytic fungi isolated from *Artemisia annua* was significantly higher in roots (70%)

compared to other tissues. Fungal diversity in the roots of *Olea europaea* was also higher than in the aboveground organs, roots (53.3%) harbored more fungi than leaves (1.5%) and twigs (1.0%) (Martins et al., 2016). The colonization rate and isolation rate of endophytic fungi isolated from *Glycine max* cv Almaty and *Hordeum vulgare* cv Arna ranged from 13.6% to 57.3% and 0.18–0.75, respectively. The colonization rate was significantly higher in the roots than in stems and leaves for both plant species. From a total of 253 isolates, 135 endophytic fungi were isolated from the roots, 73 from stems, and 45 from leaves (Ignatova et al., 2021).

Such differences in the distribution of endophytes in parts of plants can be explained by several reasons. The higher diversity and abundance of the fungal endophytes in the roots than in the aboveground organs (leaves and twigs) indicate that soil could be a reservoir for endophyte inoculum (Martins et al., 2016). Soilborne micro-organisms concentration was typically higher than those of airborne micro-organisms, which presumably may contribute to the fact, that belowground plant parts are more frequently colonized by endophytes, more diversified, and more abundant (Hammami et al., 2016). Additionally, adverse factors such as desiccation, UV radiation, and lack of nutrients affect aboveground plant organs, which could account for the more frequent colonization of roots compared to leaves and stems. The other reason is that the main sources of the easily accessible substrate are roots, so roots might be considered as a relatively stable and adequate environment for many fungal species (Ignatova et al., 2021). Also, some studies showed that the roots of perennial plants would have more time to accumulate endophytes from the soil and rhizosphere (Yang et al., 2020).

Identification of endophytique fungi

By comparing the morphological characteristics of the endophytic isolates with the identification keys, the isolates were identified at the genus level as belonging to the genera; *Rhizopus*, *Fusarium*, *Aspergillus*, *Alternaria*, *Phoma*, and *Penicillium*. The genera *Fusarium*, *Phoma*, and *Alternaria* were the most dominant with relative frequencies of 35.5, 28.6, and 14.3% respectively, while the relative frequencies of the other genera were 7.1% (Table 1) (Figure 2).

Endophytic fungi are highly phylogenetically diverse and have been found to colonize all land plants studied until now. *Fusarium*, *Phoma*, *Alternaria*, *Penicillium*, and *Aspergillus*, are the most frequently isolated genera (Galindo-Solís and Fernández, 2022). However, other studies have isolated the genus *Rhizopus* as endophyte (Manganyi et al., 2018a ; Liu et al., 2019). The proportion of isolates belonging to the genus *Fusarium* was higher than those identified as *Aspergillus*, *Penicillium*, and *Alternaria*, this was in agreement with the results of Manganyi et al. (2018b).

The genus *Phoma* was more frequently isolated from the leaves, whereas the fungi *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium sp*. were prevalent in the roots (Fernandes et al., 2015), exactly like what we found; where the two most dominant genera *Phoma* and *Fusarium* were isolated from leaves and roots respectively.

Our findings are similar to those previously reported in which *Fusarium*, *Alternaria*, and *Phoma* genera were dominant among endophytic fungi isolated. From 67 endophytic fungi isolated from *Artemisia annua* and were divided into 16 taxa; *Fusarium* sp. and *Alternaria* sp. are among the most dominant genera, and they respectively accounted for 26.86 and 11.94 % of the total fungi (Liu et al., 2019). *Alternaria alternata* and *Fusarium redolens* were dominantly and commonly isolated from *Ephedra przewalskii* Stapf and *Sympegma regelii* Bge (Zuo et al., 2022). Among the isolated genera, *Fusarium* was the most abundant in *Chenopodiaceae* species collected from diverse ecosystems (Aletaha et al., 2018). The fungal diversity in the *Pelargonium sidoides* plant was dominated by isolates belonging to the genus *Penicillium* (23%), followed by *Fusarium* (12%), whereas *Alternaria* and *Aspergillus* represent 11% of the community (Manganyi et al., 2018a).

In leaves of *Glycine max*, the most abundant genera and species were *Phoma* sp. (29.41%) (Fernandes et al., 2015). From 57 genera isolated from 63 Mediterranean environment plants, *Fusarium* and *Phoma* species were the most frequent genera, followed by *Aspergillus*, *Alternaria*, and *Acremonium* (Maciá-Vicente et al., 2008).

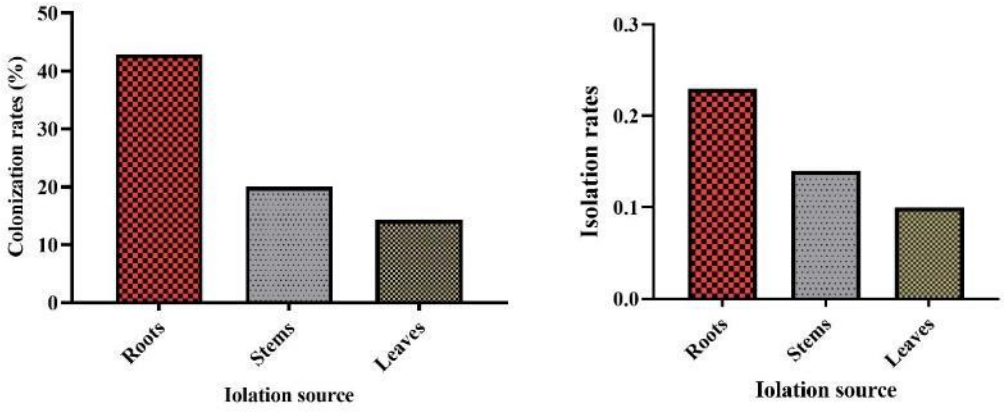

Figure 1. Colonization rates (%) and Isolation rates of endophytic fungi from *Thymus algeriensis*

Table 1. Relative frequencies and origin of different endophytic taxa isolated from *Thymus algeriensis.*

Fungal endophytes	Number of isolates	RF (%)	Sources		
			Leaves	Stems	Roots
Rhizopus sp.	1	7.1	-	-	+
Fusarium sp.	5	35.7	-	-	+
Aspergillus sp.	1	7.1	-	-	+
Alternaria sp.	2	14.3	-	-	+
Phoma sp.	4	28.6	+	-	-
Penicillium sp.	1	7.1	-	+	-

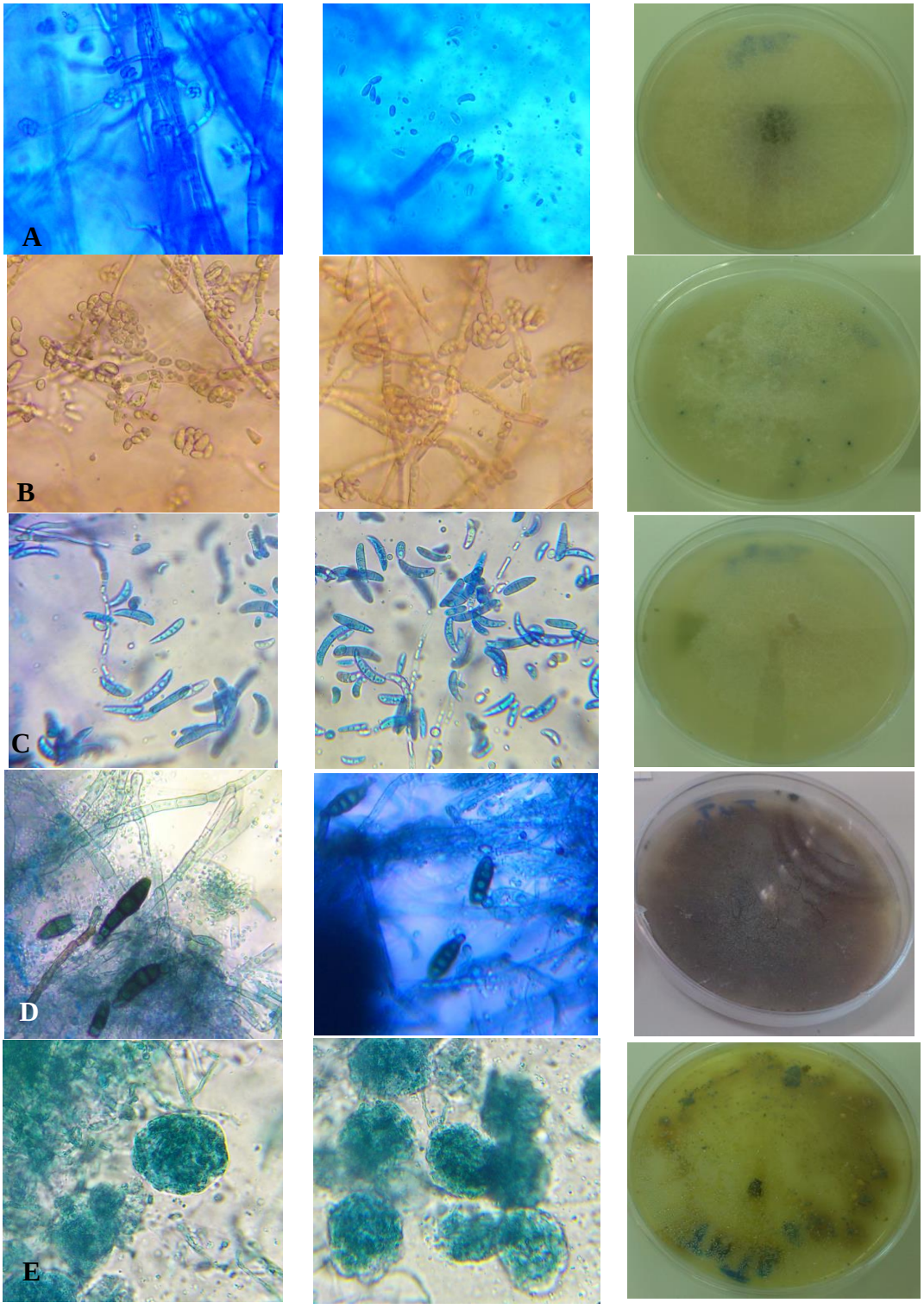

Figure 2. Microscopic features of most active isolates, *A: Fusarium* sp.3, *B: Fusarium* sp.2, *C: Fusarium* sp.1, *D: Alternaria* sp.2, *E: Phoma* sp.4.

Antibacterial Activity

The test of the antibacterial potency of the endophytic isolates was carried out using the agar plug diffusion method. Among all the isolated endophytic fungi, the four isolates, *Fusarium* sp. 2, *Fusarium* sp. 3, *Fusarium* sp. 5, and *Phoma* sp. 4, were highly active against all pathogenic bacteria. The isolates *Rhizopus* sp., *Fusarium* sp.1, *Fusarium* sp.4, *Aspergillus* sp., *Phoma* sp 1 and sp.2, and *Alternaria* sp. 1 and *Alternaria* sp.2 were active against all pathogenic bacteria except *Enterococcus faecalis*. On the other hand, the *Phoma* sp.3 isolate showed no activity against all pathogenic bacteria.

The best zones of inhibition obtained with the most active endophytic isolates were 20.33±1.25 and 20±0.82 mm for *Fusarium* sp. 3, 22.33±2.49 and 18.67± 0.47mm for *Fusarium* sp. 5, 23.33± 1.25 and 25.33±0.47 mm for *Fusarium* sp. 2, and 29.33±1.70 and 23±0.82 mm for *Phoma* sp. 4 obtained against *B. cereus* ATCC 10876 and *S. aureus* ATCC 25923 respectively (Figure 3 and 4).

To determine the most active endophytic isolates, a comparison of the averages of inhibition zones obtained against all the pathogenic bacteria was made (Figure 5). The results showed that the endophytic isolates, *Fusarium* sp.3 and *Fusarium* sp.5 were the most active with mean zones of inhibition 19.61 and 19.56 mm respectively, followed by *Fusarium* sp.2 (19 mm) and *Phoma* sp. 4 (18.61mm). Despite this, ten (71.42%) of the fungal isolates did not inhibit the growth of *Enterococcus faecalis*, while *B. cereus* ATCC 10876 and *S. aureus* ATCC 25923 were the most sensitive to fungal endophytes.

In addition to its ability to colonize a wide variety of plant species, *Fusarium* represents a large cosmopolitan genus comprising over 70 species with a wide host range and is known to produce a diverse range of secondary metabolites (Toghueo, 2020). In the present study, the isolates belonging to the *Fusarium* genus in particular the isolate *Fusarium* sp.3, *Fusarium* sp.5 and *Fusarium* sp.2 which were the most active against all Gram-positive and Gram-negative bacteria.

These findings are in agreement with some previous reports such as those of Musavi and Balakrishnan (2014), which found that the extract of *Fusarium oxysporum* (NFX06) isolated from the leaves of *Nothapodytes foetida* exhibited strong antibacterial activity against pathogenic *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *S. aureus* (ATCC 25923). Also, *Fusarium proliferatum* (ACQR8) isolated from a folk medicinal plant *Cissus quadrangularis* L (Singh et al., 2021), and *Fusarium* sp. isolated from Honeysuckle leaves (Zhang et al., 2016), showed impressive broad spectrum antibacterial activity against variety of important human pathogens; including both gram-positive and gram-negative bacteria.

The isolate identified as *Phoma* sp.4 in this study was also shown to have very strong antibacterial activity and against all pathogenic bacteria. *Phoma* species, among others, are known to produce different bioactive metabolites including macrosporin, terpenoids, terpenes and polyketides, cytochalasin derivatives, thiodiketopiperazines, phenolic compounds, and alkaloids. These bioactive secondary metabolites have already demonstrated their antimicrobial potential (antiviral, antifungal, and antibacterial) against various pathogenic microorganisms (Rai et al., 2022).

Many species of *Phoma* have demonstrated remarkable antimicrobial activities. For example, the extract of the endophytic fungus *Phoma* sp. isolated from *Lycopodium clavatum* showed broad spectrum antibacterial activity (Santra and Banerjee, 2022). The species *Phoma hedericola* has been reported to be isolated from *Ricinus communis* LINN and showed maximum zone of inhibition against *B. subtilis* (25mm), *Klebsiella pneumoniae* (25mm), *S. aureus* (24mm), *E. coli* (22mm), *S. typhimurium* (20mm) and *Enterococcous* sp. (19mm) (Sandhu et al., 2014). The ethyl acetate fraction of *Phoma* sp. isolated from *Fucus serratus* showed good antifungal, antibacterial, and algicidal properties (Hussain et al., 2014).

Among the most commonly isolated genera as endophytes are the genus of *Penicillium*, *and Aspergillus*. Many *Aspergillus*, *Penicillium*, and *Talaromyces* species are economically, biotechnologically, and medically important with huge social impacts, they are robust producers of a wide array of secondary metabolites; some of which could be used as drugs and antibiotics or as the lead compounds of potential drug candidates with pharmaceutical or biological activities (Tsang et al., 2018 ; Prajapati et al., 2021). Some species of them are known for their ability to produce antimicrobial substances with the greatest efficiency. In the present study, isolates of *Aspergillus* sp. and *Penicillium* sp. also showed significant activity against most of the bacteria tested. *Penicillium* and *Aspergillus* genera have been isolated as common endophytic fungi from a wide range of different host plants, and they have also been reported to have good antibacterial activity (Tang et al., 2021 ; Abdelgawad et al., 2022 ; Liao et al., 2022 ; Ramesh et al., 2022).

The bacteria *S. aureus* ATCC 25923 and *B. cereus* ATCC 10876 were the most sensitive to fungal endophytes, whereas *Enterococcus faecalis* was the most resistance, the same results were obtained by Manganyi et al. (2019), where all strains of *E. faecalis* ATCC S1299, *E. faecium* ATCC 700221, and *E. gallinarum* ATCC 700425 were completely resistant to fungal extracts of *Fusarium*, *Aspergillus*, and *Neurospora*.

The significant antibacterial activity obtained in this study may be due to the ability of these endophytic fungi to produce and secrete of bioactive secondary metabolites into agar medium during the incubation period, and when the agar discs are applied on Petri dishes seeded beforehand with pathogenic bacteria, the bioactive molecules diffuse and inhibit these bacteria.

Antagonistic Activity Against Fungal Phytopathogens

Regarding the antifungal activity, it was evaluated by the double culture technique, the results showed that the best activity was obtained by the isolates *Rhizopus* sp. and *Aspergillus* sp. The highest inhibition percentages were 60±5,76 and 58±4,90% obtained by Rhizopus sp. and 51±2,83 and 53±4,74% obtained by *Aspergillus* sp. against *Fusarium oxysporum* f.p. *ciccri* and *Phytophthora infestans* respectively. The rest of the isolates showed medium to low activities (Figure 6 and 7).

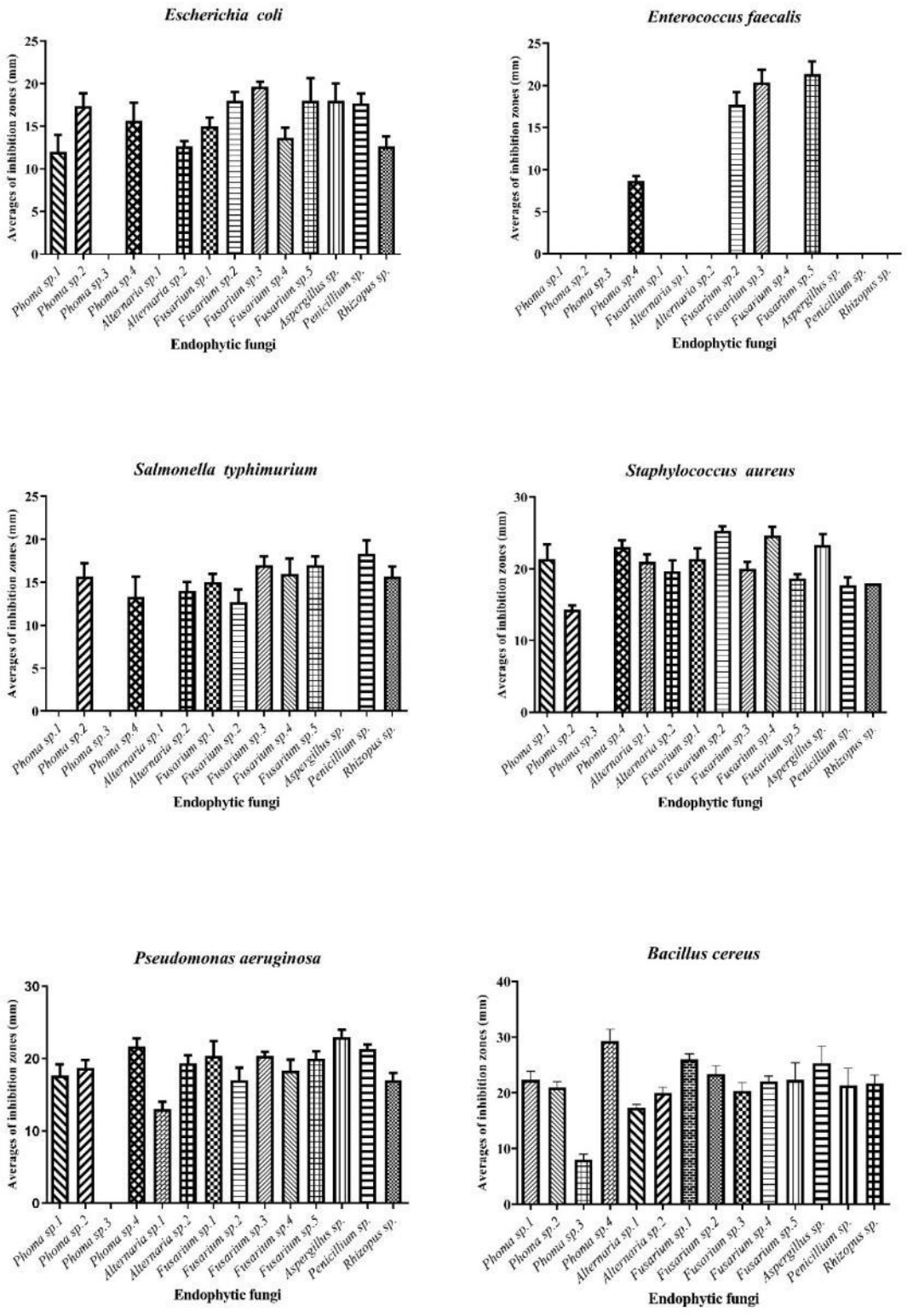

Figure 3. Antibacterial effect of endophytic fungi isolated from *Thymus algeriensis* (n=3, mean of inhibition zones ±SD)

The antifungal activity of species of the genus *Rhizopus* has rarely been cited in the literature. The high activity of *Rhizopus* sp. found in this study is surely due to its very rapid growth and its competition with phytopathogens for food resources and space and not to the secretion of bioactive molecules. This is in agreement with what was found by Nuraini et al. (2017), who observed that the endophyte isolate FEB1 of *Rhizopus* sp. was active against *Fusarium oxysporum* by competition.

In the second position of the isolates with strong antifungal activity, came the isolate *Aspergillus* sp. Several studies have shown the antifungal potential of various endophyte isolates belonging to the genus *Aspergillus*, we will cite that of Qin et al. (2019), which showed that the endophyte isolate of *Aspergillus capensis* CanS-34A associated with *Brassica napus* who produced the rosellichalasin, an antifungal molecule.

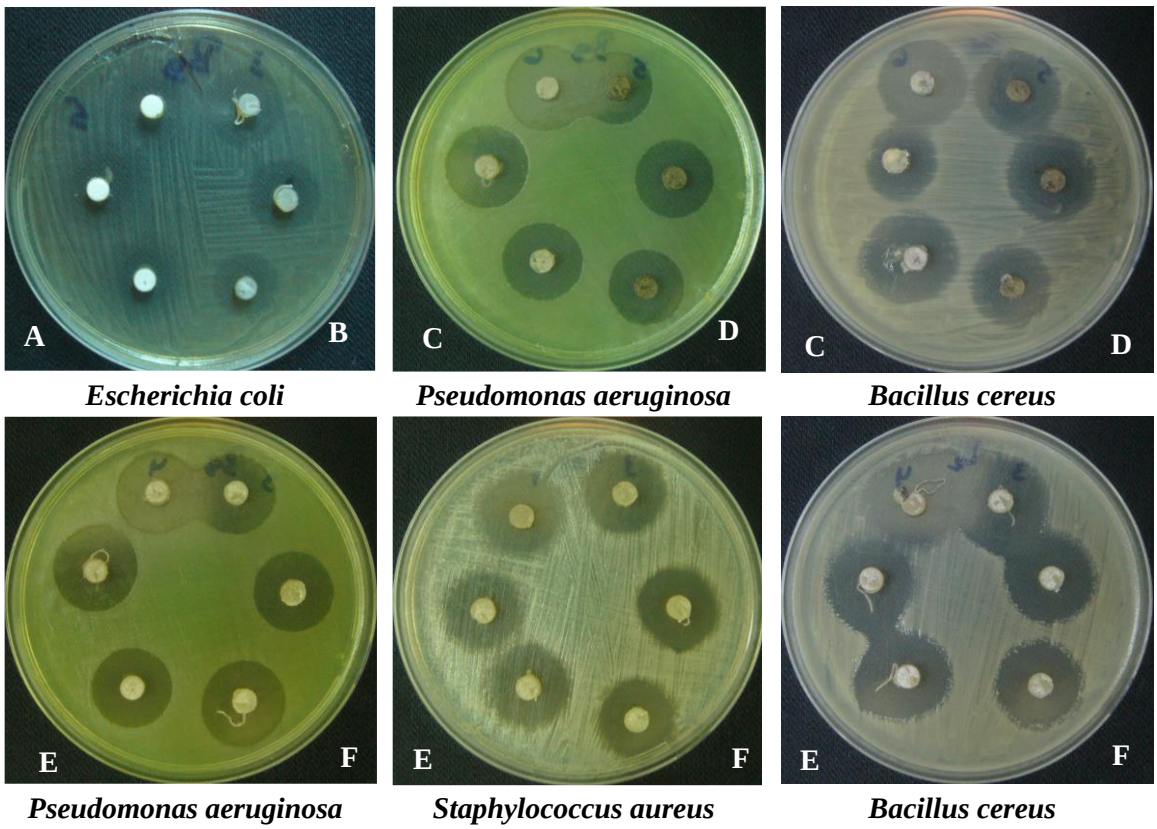

Figure 4. Inhibition zones obtained by some endophytic fungi, A: *Aspergillus* sp., B: *Fusarium* sp.2, C: *Penicillium* sp.1, D: *Phoma* sp.2, E: *Fusarium* sp.5, F: *Fusarium* sp.3.

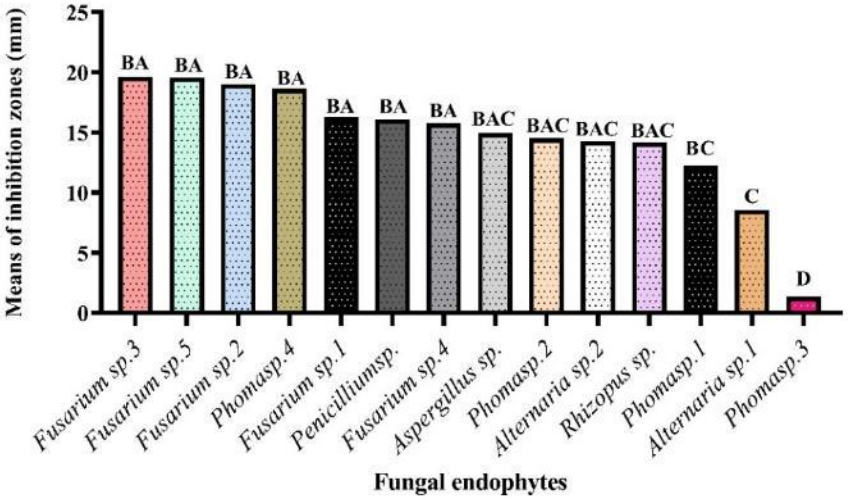

Figure 5. Comparison of inhibition zone averages of endophytic fungi and their effect on the growth of test bacteria, (Means with the same letters are not significantly different at (P<0.05).

The rosellichalasin has shown activity against the phytopathogenic fungi *Botrytis cinerea*, *Monilinia fructicola*, *Sclerotinia sclerotiorum*, and *S. trifoliorum* with EC50 values of 36.8, 87.1, 5.3, and 41.1 μM, respectively. Another study showed that the endophytic fungus *Aspergillus tubingensis* from *Decaisnea insignis* could produce a new furan derivative, 3-(5-oxo-2,5-dihydrofuran-3-yl) propanoic acid. This molecule exhibited potent antifungal activity against *Fusarium graminearum* with MIC value of 102.6 μM (Yang et al., 2019).

In conclusion, these results suggest that the endophytic fungi isolated from the Algerian plante *Thymus algeriensis* in particular *Fusarium* strains, *Phoma sp.*4, *Penicillium* sp. and *Aspergillus* sp., seem capable of producing secondary metabolites that exhibited highly effective antibacterial activity against broad spectrum of pathogenic bacteria, and these isolates could serve as potential sources for the isolation of novel antimicrobial agents that may contribute to the control of antibiotic resistant strains. However, further investigations are needed to validate this hypothesis by screening the biological activities of the secondary metabolites of these endophytic fungi.

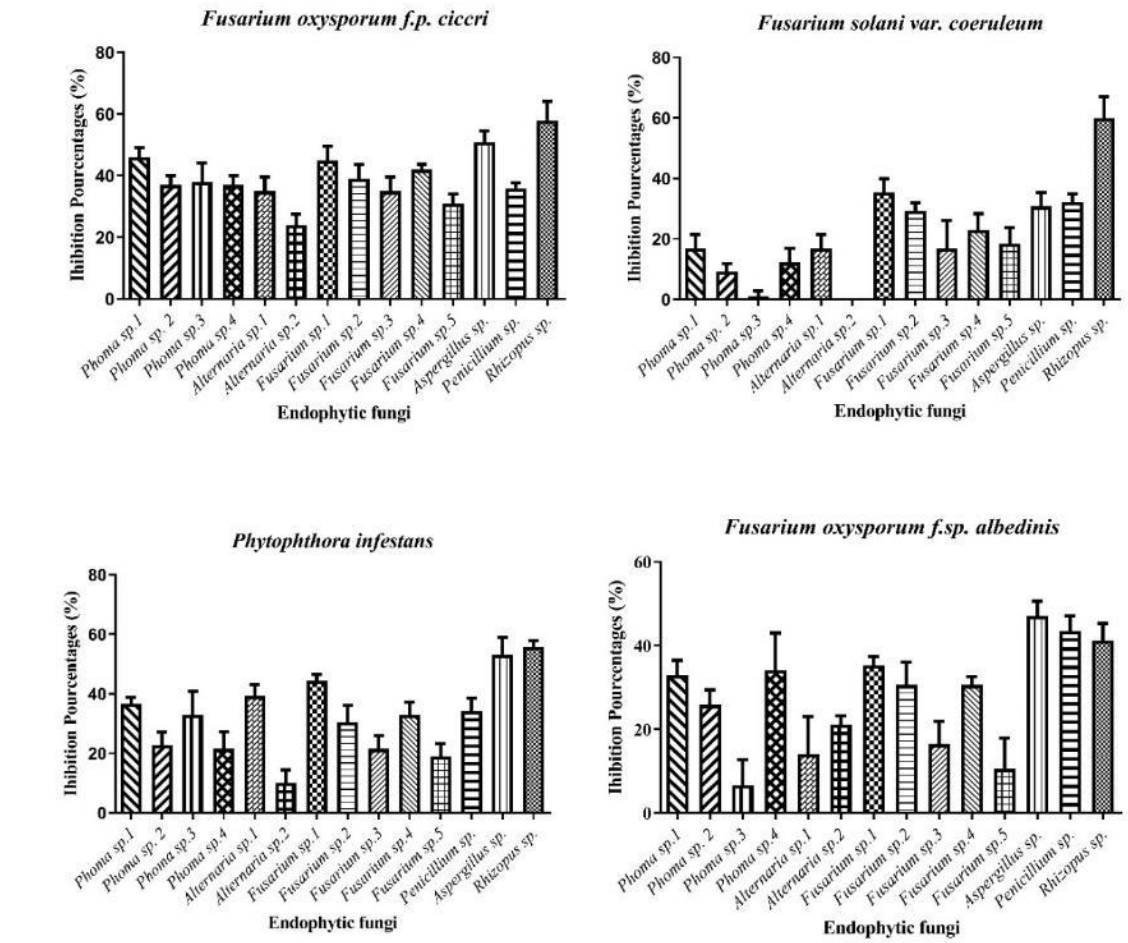

Figure 6. Screening for antifungal activity by dual culture method of endophytic fungi of *Thymus algeriensis*, (n=3, mean of inhibition percentages ±SD).

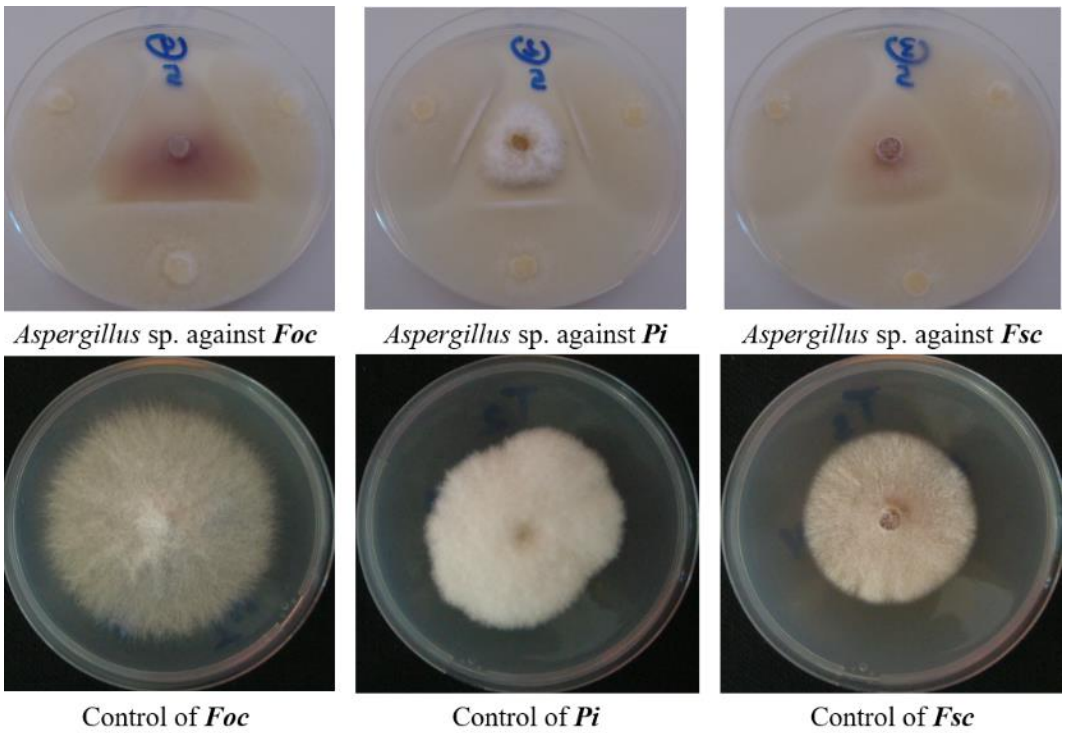

Figure 7. Antagonist activity of the endophyte *Aspergillus* sp. against the fungal phytopathogens, *Pi: Phytophthora infestans*; *Fsc: Fusarium solani* var. *coeruleum*; *Foc: Fusarium oxysporum* f.p. *ciccri*.

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