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ORIGINAL ARTICLE

A new Endophytic Fungus *Fusarium oxysporum* with Antagonism Activity Against Some Pathogenic Fungi

Alaa A. Al-Rifaie*
Department of Biology, College of Science, University of Basrah, Basrah, 61004, IRAQ

Key words:
Antifungal; Endophytic; Fungi;* Opportunistic*; Immunocompromised

Corresponding Author:
Alaa A. Al-Rifaie
Department of Biology, College of Science, University of Basrah, Basrah, 61004, IRAQ
mawan.almaqtoofi@uobasrah.edu.iq

ABSTRACT

Background: *Pathogenic fungi have emerged as a significant threat to human life.* ***Objective****: The aim of this study was to explore new sources of antifungal compounds from endophytic fungi and test the antagonistic activity against some of opportunistic pathogenic fungi. Morphological and molecular analyses using the 18S-internal transcribed spacer (ITS) and the translational elongation factor 1 (TEF1) gene were applied for identification of the isolated endophytic fungi recovered from barley seeds (Hordeum vulgare L.).* ***Methodology:*** *The antagonistic ability of isolated fungus was tested against the pathogenic fungi Macrophomina phaseolina and Fusarium solani using the dual culture technique. GC-MS technique was used to analyze metabolic extraction compounds.* ***Results:*** *Morphological and molecular analyses revealed that the isolated fungus was identified as Fusarium oxysporum, which was designated as F20B in this study. Based on the antagonistic activity test, F20B exhibited a significantly high inhibitory effect against M. phaseolina and F. solani growth using the dual culture technique (94% and 70%; PIRG, Percentage Inhibition of Radial Growth). GC-MS analysis showed present 2,4-dichloro-5-fluoroacetophenone (3.16%), Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- (1.77%), Hexadecane (0.64%), 1-Tetradecene (0.47%), cetene (0.47%), cycloheptasiloxane, tetradecamethyl- (0.4%), n-Hexadecanoic acid (0.36%), and dl-Mevalonic acid lactone (0.33%) were the major components of secondary metabolites.* ***Conclusion:*** *F. oxysporum F20B was isolated as an endophytic fungus from barley seeds with a promising potential pharmaceutical application against opportunistic fungal pathogens due to its antagonistic activity.*

INTRODUCTION

Endophytic fungi are considered a part of a healthy plant's mycobiome. They inhabit plant tissues without causing disease or remarkable morphological symptoms [1]. Endophytic fungi can enhance soil health by improving soil texture and structure, thereby promoting crop growth and yield [2]. This group of fungi forms symbiotic relationships with their hosts, such as *Chaetomium* species, and can produce a range of primary and secondary metabolites that can be utilized to control microbial infections [3,4]. Furthermore, endophytic fungi can stimulate plant immunity to protect against microbial pathogen invasion by inducing the production of microbial antigens, such as fungal chitin and β-glucans, which activate the plant's immune defense [5]. However, some fungi are well known for causing devastating plant infections and can be serious opportunistic human pathogens such as particular *Fusarium* species [6,7], and *Aspergillus* species, particularly among the immunocompromised individuals [8]. Fungal plant infections are responsible for approximately 30% of global crop losses, which cost around 60 billion dollars annually [9]. *Macrophomina phaseolina* and *Fusarium solani* are considered destructive fungal pathogens to plants worldwide. *M. phaseolina*, a well-known pathogen belonging to the Ascomycota, causes devastating infections in economically important crops and plants, including charcoal rot, stem and root rot, and seedling blight [10,11]. Recently, *M. phaseolina* has emerged as a serious life-threatening risk for human health in both immunocompromised and immunocompetent individuals, causing ocular infections [12].

F. solani can cause invasive or localized infections in immunocompetent individuals such as onychomycosis and keratitis [13]. However, in patients undergoing organ transplantation and hematological malignancies opportunistic fungi can easily cause invasive or disseminated infections [14]. The genus *Fusarium* includes a diverse group of filamentous ascomycete fungi, classified under the class Sordariomycetes, order Hypocreales, and family Nectriaceae [15]. The genus *Fusarium* produces a wide range of secondary metabolites with multiple biological properties. During the last 25 years, there has been an extensive focus on exploring the advantages of endophytic fungi due to their role in enhancing the plant's resistance ability to microbial infections and growth by producing antimicrobial secondary

metabolites [16]. For this reason, *Fusarium* species are being explored for their potential to yield novel bioactive substances as antimicrobial sources, particularly antifungal agents, to control and manage fungal pathogens [17]. Generally, the chemical identity of metabolic bioactive products produced by endophytic fungi and *Fusarium* species is categorized as alkaloids, terpenoids, steroids, isocoumarins, quinones, lignans, phenylpropanoids, promoting growth hormones, phenols, and lactones [18–20]. Barley, one of the most economically important cereal crops globally, exhibits a mutualistic relationship with particular endophytic fungal species [21]. For this reason, this study aimed to firstly explore novel endophytic fungi from barley seeds, and secondly to test the antimicrobial activity of isolated endophytes against two fungal pathogens *M. phaseolina and F. solani.*

METHODOLOGY

Samples collection:

In September 2024, a total of five random seed samples (100 gm) each sample, were collected from the Al-Qurnah district farm in Basrah province, Iraq, labeled with (BSQ). The region is geographically positioned at the GPS coordinates of 31° 1' 5.5956" N latitude and 47° 25' 23.4192" E longitude. The seeds were cleaned and meticulously rinsed with sterilized distilled water (sH_2O) to eliminate any debris. The surface was disinfected according to the protocol described by Al-Rifaie and Al-Maqtoofi [3]. After cleaning, twenty seeds were selected randomly and placed on Petri dishes, with a diameter of 9 mm, containing Potato Dextrose Agar (PDA) 39 g L^{-1} supplemented with Rifampicin antibiotic at a concentration of 0.01%, four seeds of BSQ on each plate, five replicates. The plates were incubated at 25-27 °C for 24 hours, then transferred to -20 °C to kill the plant embryo. After that, the plates were incubated at 25-27 °C for a week, with daily monitoring of fungal germination under a dissecting microscope. Recovered fungi were inoculated separately into a new PDA plate to get an axenic culture. To test the surface disinfection method efficiency, 12 barely seeds (BSQ) without processing were sown directly on three PDA plates, four seeds each plate. The presence of many fungal growths on these plates ensured the efficacy of the sterilization protocol above.

Morphological and molecular identification of endophytic fungi:

Five recovered endophytic fungal isolates were initially identified based on their morphological appearance, as determined by growth form, color, and texture. These isolates were typically identical in morphology and identified as *F. oxysporum*. One of these isolates was selected to represent endophytic fungi and provided with a symbol (F20B) for use in this study. For molecular identification, around 0.3 g of fungal vegetative biomass (6-7 days old) was scraped and transferred into a clean Eppendorf tube for gDNA extraction of the isolate F20B. Briefly, rapid isolation of gDNA was carried out using the Presto™ Mini gDNA Yeast Kit (Cat. # GBYB100, Geneaid, Taiwan). For molecular identification at the species level, extracted gDNA samples, alongside three plates of F20B fungal isolates, were sent to Macrogen in South Korea for Polymerase Chain Reaction (PCR) amplification and DNA sequencing. Two sets of primers were used: the primary set of primers, the ITS-4 primer (5`-TCCTCCGCTTATTGATATGC-3`) and ITS-5 (5`-GGAAGTAAAAGTCG TACAAGG-3`) to detect and amplify the ITS region, which is located between the 18S and 5.8S rRNA genes [22]. The second set of specific primers was to recognize and amplify the TEF1-α gene EF-1 (5`-ATGGGTAAGGARGACAAGAC-3`) and EF-2 (5` GGARGTACCAGTSATCA TGTT-3`) [23]. PCR amplification for the ITS region was performed according to the conditions described by [24]. For the TEF1-α gene, according to the programs indicated by Al-Rifaie and Al-Maqtoofi [3]. Using a BLAST search tool, the obtained DNA sequences were analyzed for phylogenetic tree analysis and compared with sequences from the GenBank database at (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ITS sequence and TEF1-α data of isolated endophytic *F. oxysporum* F20B were registered in GenBank under accession numbers PV876119 for ITS and PV972832 for TEF1-α.

Study of antagonistic activity against pathogenic fungi:

The dual culture technique was conducted to evaluate the in vitro antagonistic activity between the isolated endophytic *F. oxysporum* F20B and selected fungal pathogens. Two pathogenic fungi, including *M. phaseolina* and *F. solani*, which were previously isolated, identified, and provided by the Postgraduate Fungi Research Laboratory (PFRL) at the Department of Biology, College of Science, University of Basrah. *M. phaseolina* and *F. solani* were refreshed by growing on PDA plates and incubated at 25°C for a week. For the dual culture technique, a 6-mm agar disc of a refreshed colony from F20B isolate was placed at 1 cm near the edge of the Petri dish as illustrated in (**Figure 1**). A similarly sized agar disc of the test pathogenic fungus was placed at 1cm near the opposite edge, ensuring confrontation between the two cultures. For accuracy, the antagonism test was applied in quadruplicate. To control the growth of all tested fungi, an agar disc 6 mm in diameter, representing fungal growth, was placed at the center of the PDA plate separately. All plates were incubated at 25 ± 2°C for 7 days with daily monitoring.

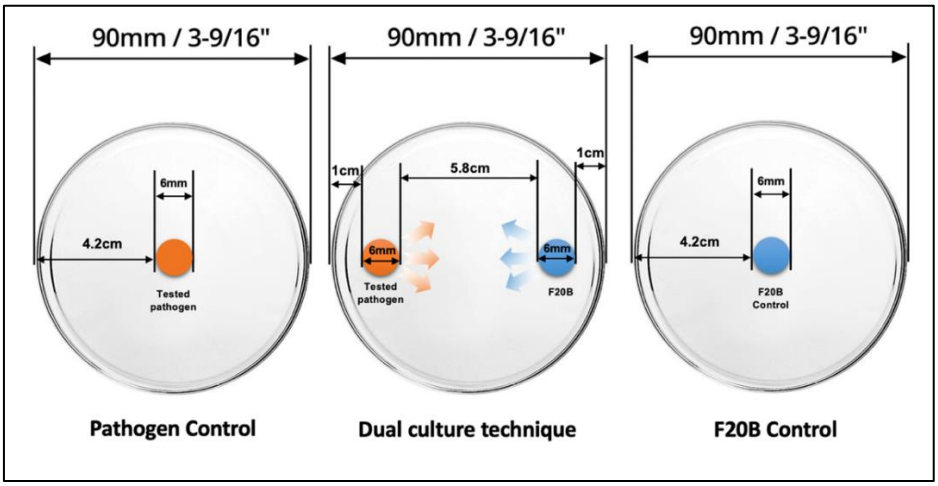

Fig. 1. The design of dual technique used to test the bioactivity of isolated *F. oxysporum* F20B against fungal pathogens

The colony growth diameters were measured with a regular ruler, and the Percentage Inhibition of Radial Growth (PIRG) was calculated based on the formula [25] as follows:

$$\textbf{PIRG}\ (\%) = \frac{(\boldsymbol{R1 - R2})}{\boldsymbol{R1}} \boldsymbol{x\ 100}$$

Where R1 = radial distance grown (mm) by the pathogen without the antagonist (Control), and R2 = the radial distance grown (mm) by the pathogen in the direction to the antagonist (Treatment). The level of PIRG was assessed [26] as indicated in **Table 1**.

Table 1: Antagonistic levels based on PIRG were also interpreted.

PIRG	Antagonistic levels
PIRG < 50%	Low
50% < PIRG ≤ 60%	Medium
60% < PIRG ≤75%	High
PIRG > 75%	Very High

Detection of bioactive compounds by extraction and GC-MS analysis:

To extract secondary metabolites from the isolated *F. oxysporum* F20B, three mycelial agar discs (6 mm in diameter) taken from 7-day-old cultures grown on PDA were transferred into a 250 ml Erlenmeyer glass flask containing 100 ml of potato dextrose broth (PDB) to serve as the fermentation medium. Flasks were incubated under semi-shaking conditions for 21 days at 120 rpm and 27 °C. Cell-free extract was filtered through a sterile Whatman No. #1 filter paper to eliminate fungal biomass. To extract secondary metabolites, the cell-free culture filtrate was mixed with an equal volume (1:1) of ethyl acetate, a commonly used organic solvent. The mixture was transferred into a separating funnel with manual shaking for 10 min at room temperature. The ethyl acetate fraction was separated and concentrated on a rotary evaporator. The dried crude extract was collected, weighed, and placed in clean Eppendorf tubes. The dried fungal extract was submitted to Nahran Omar Laboratories, a subsidiary of the Basrah Oil Company, for identification of bioactive compounds by GC-MS analysis using an Agilent 7890B/5977A series GC-MS system (USA), supported by Mass Hunter Acquisition software and Classic ChemStation Data Analysis software for accurate compound detection and interpretation. The compounds in the ethyl acetate fraction were identified by comparing their retention times (RT) and mass spectra with reference data from the NIST library (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Statistical analysis:

All values are expressed as mean values. An independent t-test was used to determine the statistical significance of the difference among the antagonistic tests, using SPSS software. *P*-values < 0.05 were considered statistically significant.

RESULTS

Isolated fungi

The result of isolated fungi showed that *F. oxysporum* was the only predominant fungal species recovered from the examined barley seeds and reported for the first time in Iraq as an endophytic fungus. The morphology of fungal colonies on PDA showed white aerial mycelium forming cottony colonies, growing rapidly to reach 8 cm in diameter during 7 days of incubation at 25°C, with a reverse that is white in color. Conidiophores were sparse with inconstant lengths, pale in color, thin-walled, septate, pointed toward the apex, and not divided into branches. Terminal conidiogenous cells produced conidia from the apex in succession, occasionally having a truncated base, or gathered in a

group. Chlamydospores were round, intercalary hyaline, formed in chains, with rough walls (10-23 x 6-10) µm. Macroconidia were hyaline, ellipsoidal to oval, tapering, and septate, with slightly curved apexes sized (5-10 x 3-5) µm, 1-6 septate. Microconidia were aseptate, hyaline measuring 2-5 µm (**Figure 2**).

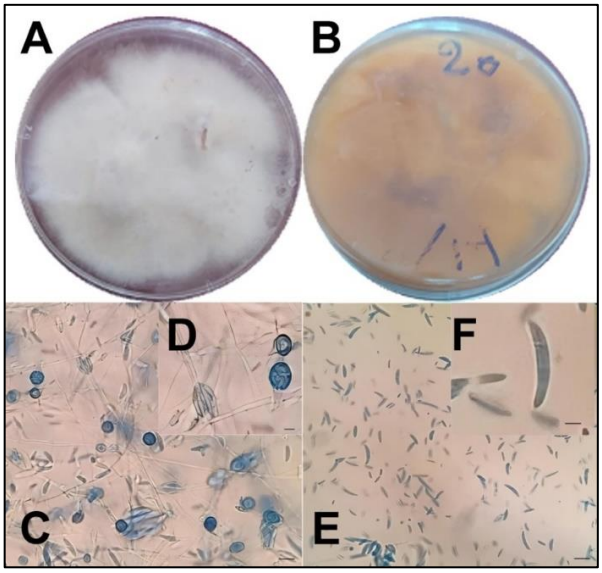

Fig. 2: *Fusarium oxysporum* F20B. **A.** *F. oxysporum* F20B on PDA front, **B.** *F. oxysporum* F20B on PDA reverse side, **C** and **D.** Chlamydospore, **E** and **F.** Microconidia and macroconidia.

Molecular identification

The gDNA sequencing data of the isolated fungus *F. oxysporum* F20B were analyzed using ITS and TEF-1 and compared to 20 other *Fusarium* species using the Neighbor-Joining (NJ) method (**Figure 3**). The outcome indicated a cluster of isolated F20B together with *F. oxysporum* (**Figure 3A** and **3B**). ITS and TEF-1 are essential primers for the accurate identification of *Fusarium* species, as recommended by many studies, due to their ability to develop a specific single band of amplification by PCR.

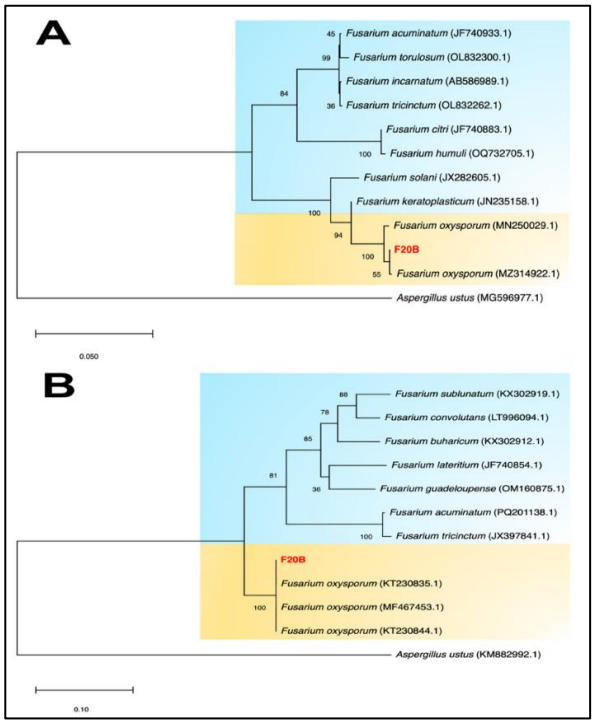

Fig. 3: Phylogenetic tree of isolated endophytic fungus *Fusarium oxysporum* F20B using two genetic markers, (**A**) ITS and (**B**) TEF1-α. The tree was generated using the Neighbor-joining (NJ) method. The trees showed the relationship of *F. oxysporum* F20B (red) with closely related sequences retrieved from the NCBI database. The numbers at the nodes represent percentages indicating the levels of bootstrap support from 1,000 replicates. Bootstrap support values > 50% indicate confidence levels. The isolated endophytic fungus in this study is bold red. *Aspergillus ustus* represents an outgroup isolate.

Antifungal activities of *F. oxysporum* F20B

Preliminary antagonistic activity testing via dual culture test demonstrated that the isolated endophytic F20B exhibited significant inhibitory effects against the tested fungal pathogens, *M. phaseolina* and *F. solani*. Statistical analysis of the PIRG rates, conducted using an independent t-test, confirmed that F20B significantly suppressed the growth of *M. phaseolina* (PIRG: 94%, $p < 0.001$) and *F. solani* (PIRG: 70%, $p < 0.001$) compared to the controls (**Table 2**) and (**Figure 4**).

Table 2: Mycelial growth inhibition rate (%) of endophytic fungi isolates *Fusarium oxysporum* F20B against plant pathogenic fungi *Macrophomina phaseolina* and *Fusarium solani* in dual culture assay.

Fungal Pathogens	Mean of IRG%	Antagonism Level	*p*-vale
Macrophomina phaseolina (Control)	0	Control	-
Macrophomina phaseolina (Dual culture with endophytic F20B isolate)	94	Very High	$p < 0.001$
Fusarium solani (Control)	0	Control	-
Fusarium solani (Dual culture with endophytic F20B isolate)	70	High	$p < 0.001$

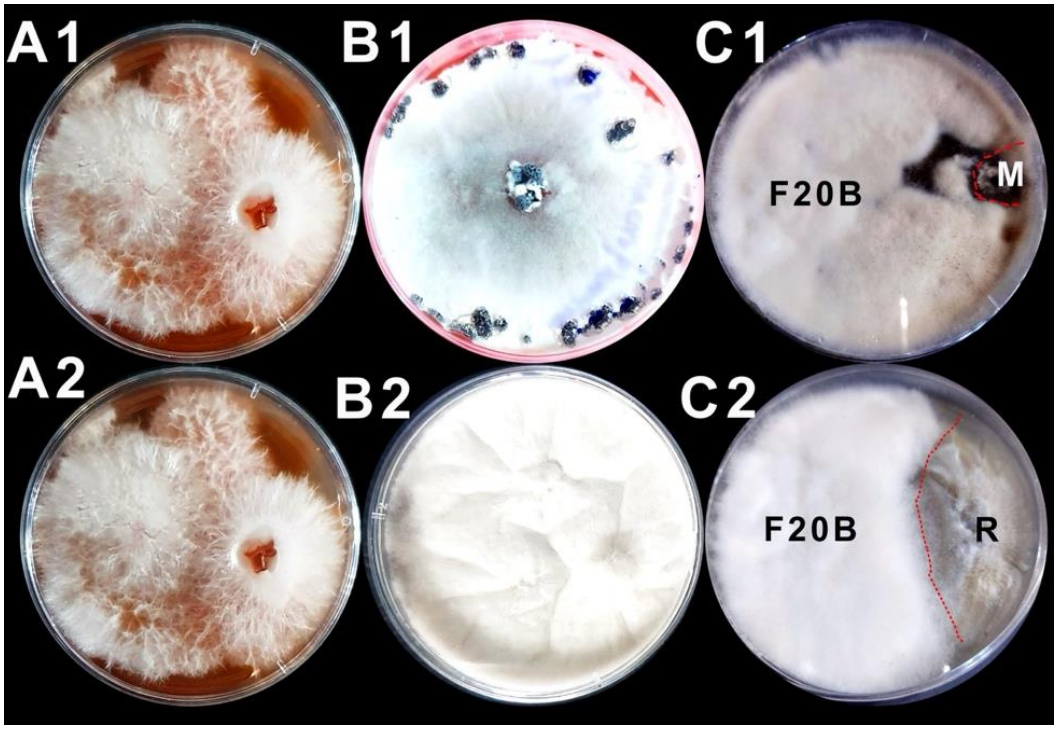

Fig. 4: Antagonism between endophytic fungal isolates *Fusarium oxysporum* F20B and two pathogenic fungi in dual culture assay, where (**A1** and **A2**): *F. oxysporum* F20B (Control); (**B1**) *Macrophomina phaseolina* (Control); (**C1**) *F. oxysporum* (F20B) against *M. phaseolina* (**M**); (**B2**) *Fusarium solani* (Control); (**C2**) *F. oxysporum* (F20B) against *F. solani* (**R**).

GC-MS analysis of *F. oxysporum* F20B extract

The biomolecule profile of the crude ethyl acetate fraction of the isolated endophytic fungus F20B from barley seeds was analyzed using GC–MS (**Figure 5**). Among these, a total of 8 different compounds were identified (**Table 3**). A percent relative peak area was used to quantify compounds in the crude extract.

The highly abundant compounds include 2,4-Dichloro-5-fluoroacetophenone (3.16%), followed by Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- (1.77%), Hexadecane (0.64), and around (0.4%) for 1-Tetradecene, Cetene and Cycloheptasiloxane, tetradecamethyl. Finally, both n-Hexadecanoic acid and Mevalonolactone presented at approximately (0.3%) (**Table 3** and **Figure 6**).

Table 3: Identified chemical compounds in the ethyl acetate extract of the isolated endophytic fungus *Fusarium oxysporum* F20B by GC-MS analysis.

Compound No.	Identified compound	Formula	Molecular formula (g/mol)	Area %	Retention time (min)	CAS No.
1	2,4-Dichloro-5-fluoroacetophenone	$C_8H_5Cl_2FO$	207.03	3.16	15.43	704-10-9
2	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	$C_{23}H_{32}O_2$	340.499	1.77	26.954	119-47-1
3	Hexadecane	$C_{16}H_{34}$	226.4412	0.64	18.941	544-76-3
4	1-Tetradecene	$C_{14}H_{28}$	196.3721	0.47	18.855	1120-36-1
5	Cetene	$C_{16}H_{32}$	224.4253	0.47	18.855	629-73-2
6	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	519.0776	0.4	17.708	107-50-6
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4241	0.36	22.767	57-10-3
8	dl-Mevalonic acid lactone or (Mevalonolactone)	$C_6H_{10}O_3$	130.1418	0.33	14.613	674-26-0

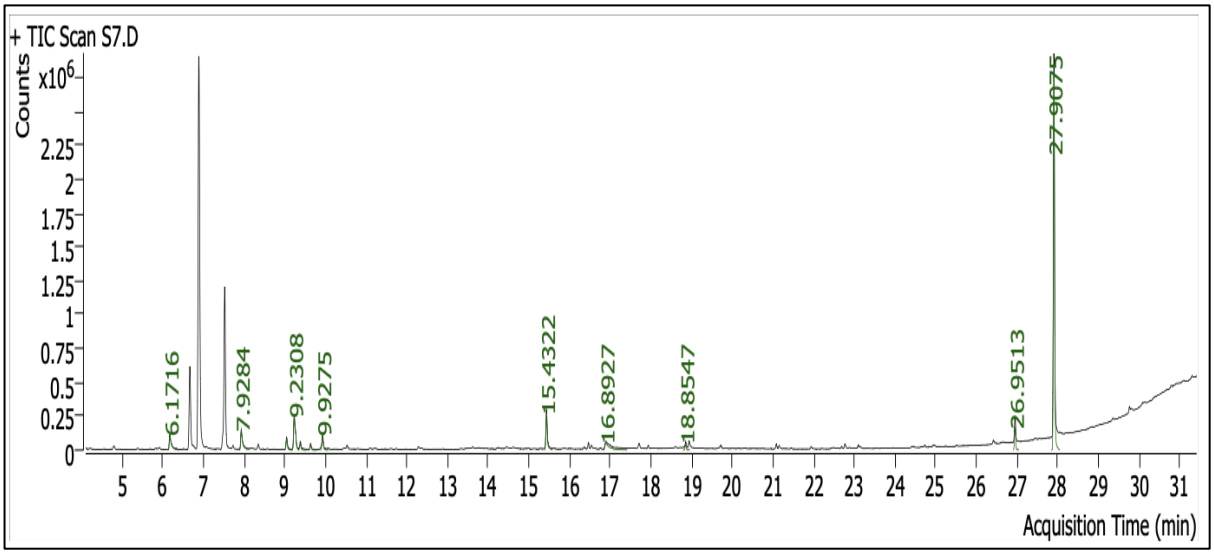

Fig. 5: GC-MS chromatogram of the ethyl acetate extract of the isolated endophytic fungus *Fusarium oxysporum* F20B.

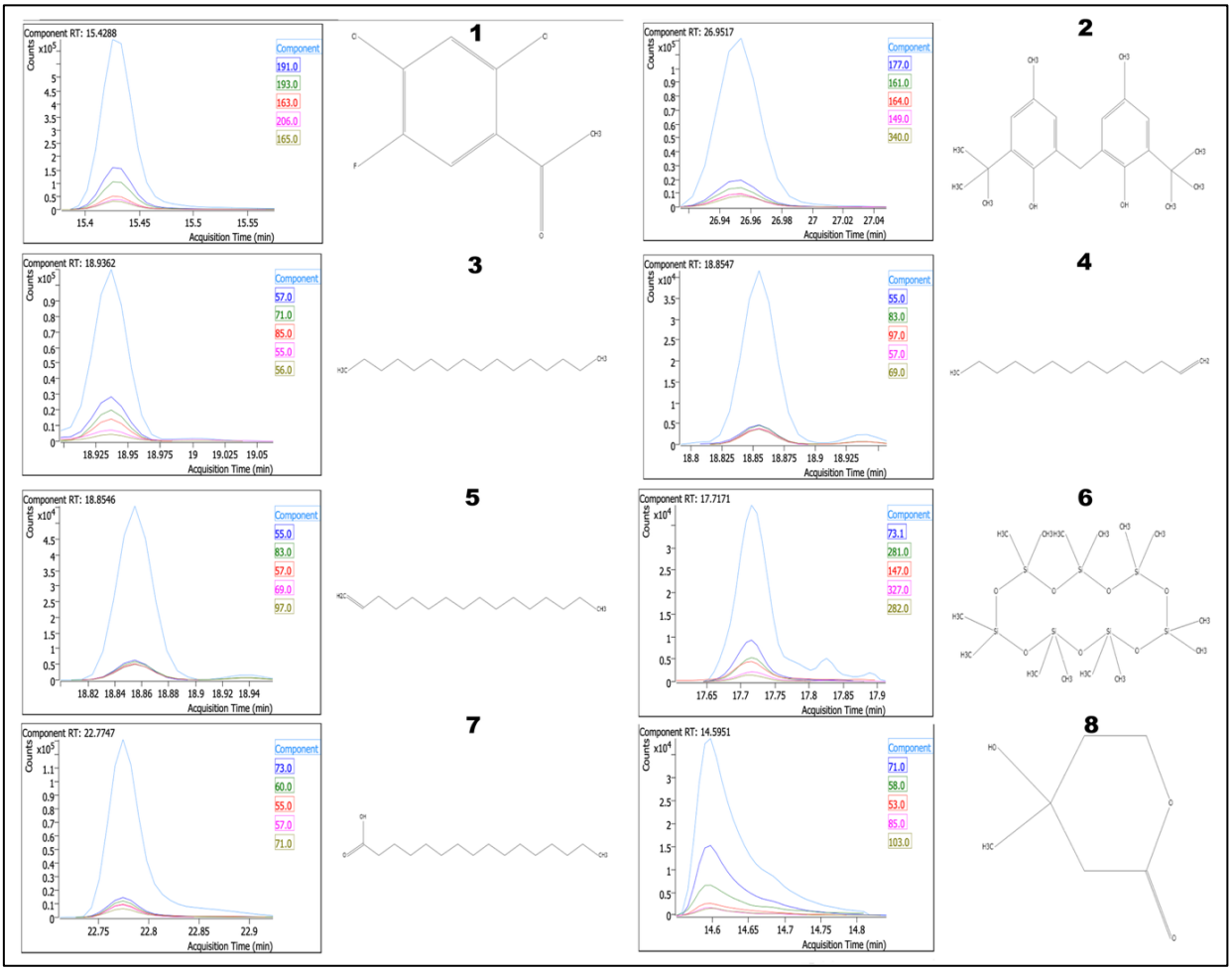

Fig. 6: Structures of biologically active compounds identified in the ethyl acetate fraction of the isolated endophytic fungus *Fusarium oxysporum* F20B.

DISCUSSION

Over recent decades, many antimicrobial drugs used to treat infectious diseases have lost effectiveness due to the widespread rise of antimicrobial resistance worldwide. This resistance has made even opportunistic infections harder to treat and poses a growing global health threat as drug-resistant pathogens. For this reason, exploring new and novel resources of effective compounds that could manage and control microbial infections particularly fungal pathogens [27]. Fungal endophytes have garnered significant attention in recent years due to their crucial applications in various fields, including agriculture, environmental sustainability, and medicine as they produce antiviral compounds, anti-inflammatory agents, antimicrobial substances, anticancer agents, and immunosuppressants [28]. These microorganisms possess a practical ability to protect against microbial infections [29]. This vital ability of fungal endophytes is attributed to the biosynthesis and production of a wide range of bioactive secondary metabolites, including melatonin, terpenoids, flavonoids, carotenoids, phenols, peptides, alkaloids, quinones, xanthones, isocoumarins, and steroids [30,31].

Seed endophytes have gained increased attention because they are associated with the assembly of the plant microbiome and exert effects on progeny plants through seed germination [32]. Seed-borne endophytes likely play a more critical role than currently recognized. This study is part of an intensive program designed to explore seed-borne fungal endophytes and their bioactive metabolites in Basrah Province, Iraq. This project has two stages. The primary objective was to detect barley seed-borne endophytes related to *Fusarium* species. The second aim was to investigate the fungal bioactive metabolites for controlling the two most important phytopathogens and opportucinistic fungi, *M. phaseolina* and *F. solani*. Barley has numerous benefits for human health and the economy. It has a low glycemic index, high levels of resistant starch, and a range of functional components that play a significant role in promoting antidiabetic, anticancer, anti-obesity, antioxidant, antiproliferative, and cholesterol-lowering effects [33]. For all of these, barley was a target for the current study to investigate seed-borne endophytic fungi. Barley seeds are coated with a protective barrier that prevents the penetration and survival of fungal pathogens, indicating a lower rate of endophytic fungal diversity [34]. Additionally, the ability of *Fusarium* to adapt to various environmental habitats is facilitated by its efficiency in producing a range of lysis enzymes and bioactive metabolites [35]. These features could enable *F. oxysporum* to be the predominant endophytic fungus in this study. For this reason, instead of considering *F.* oxysporum as a destructive plant pathogen, it can have positive, notable benefits to its host when it exists as an endophytic fungus.

F. oxysporum possesses an extensive range of natural biomolecules with antiviral, antibacterial, antiparasitic, and antifungal properties that effectively eliminate microbial infections. Previous studies have reported the efficiency of *Fusarium* species in significantly reducing the growth of other fungal pathogens, due to the release of secondary metabolites that display the strongest antifungal activities against yeasts and filamentous fungal pathogens in vitro [17]. These biomolecules may be involved in the suppression of chitin synthesis in fungal cell walls, resulting in the inhibition of fungal pathogen growth. Furthermore, bioactive metabolites can impair the selective permeability feature of the cytoplasmic membranes of fungal pathogens, in addition to disrupting and inhibiting the transcription and translation of the RNA polymerase III complex, thereby inhibiting the growth of the fungal pathogen [36]. Recently, more than 25 antifungal natural bioactive metabolites have been identified from *Fusarium* species that disrupt and inhibit the formation of fungal cell wall elements or nucleic acid processing [37]. Moreover, *F. oxysporum* has been reported to produce metal nanoparticles (NPs), particularly silver nanoparticles (AgNPs), with a very low toxicity rate, and can be used as a promising antifungal agent in both medicine and agriculture [38]. Additionally, *F. oxysporum* possesses a wide range of extracellular enzymes [39]. This phenomenal capability of *F. oxysporum* for biosynthesis and production of mixtures of secondary metabolites, which involves either directly or indirectly inhibiting fungal pathogens, suggests using isolated endophytic *F. oxysporum* F20B as a new biocontrol agent to manage fungal infections.

GC-MS analysis revealed that 2,4-Dichloro-5-fluoroacetophenone was one of the main bioactive metabolites produced by F20B. This active compound has been identified as an antifungal. There is no study investigating the role of 2,4-Dichloro-5-fluoroacetophenone for fungal treatment. There is only one study reported that uses 2,4-dichloro-5-fluoroacetophenone in the synthesis of antimicrobial agents, such as ciprofloxacin analogs, and tests its antifungal role against *Aspergillus fumigatus* [40]. We suggest that 2,4-Dichloro-5-fluoroacetophenone may have similar inhibitory effects to 5-fluorouracil against fungal growth. Flucytosine is converted into 5-fluorouracil (5-FU), which disrupts DNA and RNA synthesis, ultimately leading to fungal cell death [41]. Phenolic compounds were reported as secondary metabolites of many endophytic fungi. The endophytic fungus, *Alternaria alternata*, exhibits the ability to produce phenolic compounds, including Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl- with

efficient inhibitory activity against filamentous fungi [42]. Generally, phenolic compounds are detected in the crude extracts of plant endophytic fungi, such as *Fusarium guttiforme* [17]. In this study *F. oxysporum* F20B produces phenol and 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-. Phenolic compounds have the potential to inhibit the growth of fungal pathogens by downregulating ergosterol biosynthesis, leading to compromised fungal cell membrane permeability. Alternatively, they may block enzymatic activity through protein binding or inhibition of DNA or RNA formation [43].

The less abundant compounds included Hexadecane (0.64%), 1-Tetradecene (0.47%), cetene (0.47%), cycloheptasiloxane, tetradecamethyl- (0.4%), n-Hexadecanoic acid (0.36%), and dl-Mevalonic acid lactone (0.33%). These secondary metabolites have biological effects on active living microbial cells. Some of the identified compounds in this category belong to alkenes such as 1-Tetradecene, cetene, and alkanes such as Hexadecane, in addition to alkyl aromatic hydrocarbons like. Also, within this section, fatty acids such as n-Hexadecanoic acid, and organosilicon compounds like cycloheptasiloxane, tetradecamethyl were identified. Studies have shown that these bioproducts possess antifungal properties against fungal plant pathogens, in addition to other biological activities, including antibacterial, immunomodulatory, and antitumor properties [44].

CONCLUSION

Endophytic *F. oxysporum* F20B exhibited a significantly high inhibitory effect against the fungal pathogens *M. phaseolina* and *F. solani* growth. To our knowledge, this study concludes that most of the bioactive metabolites with antifungal properties produced by the isolated endophytic F20B have not been reported before. This study reveals that many bioactive metabolites with antifungal activity produced by the isolated F20B strain are previously unreported, indicating its potential as a valuable source of novel antifungal compounds for managing these pathogens in both agricultural and medical contexts.

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Informed Consent Statement
This study did not involve human participants; thus, no informed consent was required.

Conflict of interest
The authors declare no conflicts of interest related to this study or its publication.

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