Comparative bioactivity and metabolites produced by fungal co-culture system against myco-phytopathogens

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Abstract: Co-culture is the simultaneous cultivation of two or more microorganisms in the same culture or growth medium. This technique allows observation and analysis of the interactions between microorganisms, such as competition for nutrients, cooperative behavior, or even the production of novel compounds that would not be synthesized by either species alone. This study aimed to use fungal co-culture for finding new sources of novel antifungals via a biological approach. In the current study, only 9 of 40 tested endophytic fungal isolates interacted positively with Fusarium proliferatum AUMC 15541by dual culture plate assay. Using well diffusion assay, extracts of Aspergillus ochraceus AUMC 15539 co-culture with F. proliferatum showed a greater inhibition action than monoculture. Large scale for Aspergillus-Fusarium co-culture (AF) was performed and its ethyl acetate extract exhibited significant inhibitory activity against different phytopathogens with potential effect against F. proliferatum (MIC = 6.25 mg/mL). The co-culture AF extract had LC50 values of 1972 (µg/mL) which is lower toxicity than single culture A and F. The total flavonoid, phenolic, and tannin contents of fungal extracts were significantly higher in the co-culture (AF) than in the single cultures A and F. High-performance liquid chromatography (HPLC) profiling of Aspergillus-Fusarium axenic and co-cultures (A), (F), and (AF) at different monitored wavelengths revealed the presence of a new peak at retention time 9.487 min in 235 nm chromatogram, in AF co-culture extract over A and F extracts. In general, it was found that co-culture led to an increase in the synthesis of some chemicals which had been present in the single-species cultures of (A) and (F) axenic cultures and suppressed the biosynthesis of others when comparing the production of compounds in cultures of single fungal species (A and F) to co-cultures (AF) across various wavelengths tested. These results indicate the possible use of fungi in co-culture to find new or more biologically active natural compounds.

Keywords: Endophytic fungi, Aspergillus ochraceus, Fusarium proliferatum, MIC, HPLC.

Introduction

Microbial natural products are an important resource for the development of new drugs (Koehn & Carter, 2005). Filamentous fungi are responsible for over 42% of the bioactive compounds that have been identified so far (Serrano *et al*., 2017). Many of these compounds have medicinal properties used in various applications such as antibiotics and antifungal medications (Demain, 2014; Schueffler & Anke, 2014). Microbial interactions within a range of microorganisms' communities have been extensively documented, these interactions have been found to influence fungal metabolism.

As a result, novel bioactive secondary metabolites have been discovered through these interactions. These findings highlight the potential of studying microbial

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interactions in co-cultures to discover new compounds with therapeutic applications (Brakhage *et al*., 2008; Combès *et al*., 2012; Martínez-Buitrago *et al*., 2019). The efficacy of co-cultures in which secondary metabolites are produced in one organism after sensing the secondary metabolites produced in another organism (Ueda & Beppu, 2017; Tan *et al*., 2019). Co-culture stimulates specific biosynthetic pathways that are triggered when the cultures are grown together, mostly related to cellular defence (Ishikawa *et al*., 2017).

There are several techniques for fungal co-culture, including physical co-culture, where two fungi are grown together on the same plate or in the same flask; sequential culture, where one fungus is grown first and then another is introduced (Serrano *et al*., 2017). Competition for food and space between different microorganisms is one of the major ecological forces that induces silent biological genetic clusters that trigger the accumulation of cryptic secondary metabolites that cannot be traced in axenic cultures (Nai & Meyer, 2018). In actuality, it has been proven by various investigations that when microbes interact with one another, they create specialized metabolites that can have either beneficial (like communication with other microorganisms) or toxic (like antibiotics) reactions with others (de Weert *et al*., 2004; Bertrand *et al*., 2014; Abrudan *et al*., 2015).

A few research projects have investigated the mapping and assessment of fungal-fungal actions, while a lot of studies have concentrated on bacterial-bacterial and fungal-bacterial interactions in a petri plate (Tata *et al*., 2015; Wang *et al*., 2015; Michelsen *et al*., 2016; Sica *et al*., 2016; Bai *et al*., 2018; Chen *et al*., 2018; Eckelmann *et al*., 2018; González-Menéndez *et al.,* 2018). Through the support of analytical techniques like high-performance liquid chromatography (HPLC), and liquid chromatography mass spectrometry (LC-MS), it has become available to identify modifications in the metabolite profiles that are dependent on the interacting fungi (Peiris *et al*., 2008; Estrada *et al*., 2011).

A large and phylogenetically diverse variety of microorganisms known as phytopathogenic fungi infect plants or can cause dangerous diseases in plants (Heydari & Pessarakli, 2010). Plant diseases induced by pathogenic fungi cause significant losses in crop output. In order to control plant diseases, synthetic fungicides are usually used as both treatment and preventive measures. The increasing number of resistant strains of pathogens and pollution in the environment are the results of the overuse of a variety of fungicides (Park *et al*., 2005).

The continual use of chemical fungicides leads to residue issues, disease resistance and cost constraints to the farmers. To overcome these problems and effectively control plant pathogens, there is a necessity to identify antifungal compounds using a biological approach (Marimuthu et al., 2020). The demand for pesticide- and fertiliser-free organic agriculture is rising. Due to these events, many scientists are researching integrated fungal disease management, which includes biological controls utilising antagonistic microorganisms and less harmful agents such as food preservatives and extracts from plant products (Copping & Menn, 2000; Ujváry, 2002). Phytochemical studies of endophytic microorganisms have been carried out worldwide, resulting in the isolation of more than 500 new functional chemicals, which have had a major impact on the development of new therapeutics and pesticides (Zhang *et al*., 2006,2018). There is increasing proof that the one strain many compounds (OSMAC) strategy is a quick and efficient way to improve the variety of chemical substances in microorganisms. Examples of this include varying the medium's composition and culture state, co-cultivating with other strains, and adding enzyme inhibitors and biosynthetic precursors for secondary metabolites produced by microorganisms (Pan *et al*., 2019).

However, Co-culture system can effectively alter individual microbial cellular physiology and access chemical diversity (Abdalla *et al*., 2017; Ueda & Beppu, 2017; Abdelwahab *et al*., 2018; Tan *et al*., 2019). One of the most serious fungal infections is Fusarium spp. (Cramer, 2000; Wang et al., 2019), It influences every stage of plant development in the pre- and post-harvest phases. from damping off and delayed seedling emergence to bulb rot (Galeano *et al*., 2014; Wang *et al*., 2019). According to the majority of the literature on Fusarium dry rot (FDR), which can account for up to 30% of post-harvest garlic output losses, the main cause of the disease is the fungus F. proliferatum. Additionally, F. proliferatum has the capacity to produce a wide variety of toxins (Laura & Palmero, 2022). Some of which can be toxic to humans and animals (Desjardins, 2006). The treatment with synthetic fungicides is not entirely effective due to its resistance to highly stressful environment (Gupta *et al*., 2020).

In this work, we proposed using fungal co-cultures with F. proliferatum for evaluating and stimulating cryptic pathways in endophytic fungal isolates and finding new sources for antifungals using a biological approach.

Materials and methods

Fungal isolates

Forty endophytic fungal isolates were obtained from the culture collection at the Al-Azhar University (Assiut), Egypt. The isolates were cultivated on

Czapek-Dox agar (Cz) and potato dextrose agar (PDA) and incubated for 7-10 days at 28˚C. The fungal cultures were identified morphologically by their macroscopic and microscopic characters and the promising isolates were deposited in the culture collection of Assiut University Mycological Center (AUMC) with new accession numbers. For phytopathogenic fungi inhibition tests, *Alternaria solani* F12 (KT721910), *Botrytis cinerea* AUMC 14436, *Fusarium proliferatum* AUMC 15541, *Rhizoctonia solani* AUMC 15102, *Sclerotium cepivorum* AUMC 15542, and *Sclerotinia sclerotiorum* AUMC 11690 were used.

Selection of fungal isolates for co-culture

Fusarium proliferatum AUMC 15541, which was isolated from onion (*Allium cepa*) with *Fusarium* base rot, was chosen from a preliminary screening of fungal co-cultures together with *A. ochraceus* AUMC 15539 to test its potential to produce long-distance interactions.

Dual culture plate assay

A fungal disc (5mm) from the test pathogen was put at 2 cm from the plate's edge on PDA and yeast extract sucrose agar (YES) (9 cm in diameter). In a similar way, a disc (5mm) of the endophytic fungus/antagonist was put on the pathogen disc's direct opposite. The plates were incubated for 15 days at 28˚C. Negative controls were plates inoculated with *F. proliferatum* in the absence of antagonist fungus; three replicates were utilized for each experiment (Hajieghrari *et al*., 2008; Albert *et al*., 2011).

Metabolites extraction and analysis

All fungal isolates of co- and mono-cultures, had their interaction zones, were removed with a razor blade (about 2 mm width). Controls were set up using empty plates and plates inoculated with a single fungal strain. To extract each sample, ethyl acetate was used twice, then filtered and dried using a rotary evaporator at 40˚C (Mohamed *et al*., 2021).To reconstitute the samples, 500 µL of dimethyl sulfoxide (DMSO) were added consecutively (Costa et al., 2019).

Antifungal assay

The antifungal activity of different extracts was determined and described their specificity against 6 distinct phytopathogens were *Alternaria solani* F12 (KT721910)*, Botrytis cinerea* AUMC 14436*, Fusarium proliferatum* AUMC 15541*, Rhizoctonia solani* AUMC 15102*, Sclerotium cepivorum* AUMC

15542*, Sclerotinia sclerotiorum* AUMC 11690. Depending on the phytopathogenic fungus, PDA, potato sucrose agar (PSA), and Cz media were utilized for antifungal activity tests. Twenty-five mL of media containing spores (1 x 10^5 spores/mL) were dispensed in assay plates. Once solidified, a hole with a diameter of 8 mm was punched aseptically with a sterile corkborer, and a volume (100 µL) of the extract solution (A, F, AF, and A+F) at desired concentration was introduced into the well. Then, agar plates were incubated under suitable conditions depending upon the test microorganism. Bellis (Boscalid + Pyraclostrobin) was evaluated as a positive control (*Magaldi et al., 2004; Valgas et al., 2007; Serrano et al*., 2017).

Fermentation and Extraction

Fifteen Erlenmeyer flasks (1L each) were prefilled with solid rice (100 g rice and 110 mL of distilled water) and autoclaved for the primary fermentation (Al Mousa *et al*., 2021). Once the flasks were cooled to room temperature, they were seeded with two types of fungi, *A. ochraceus* and *F. proliferatum*, using 2 cm^2 fungal discs. Both co-culture and axenic control cultures were set up. The fermentation process was carried out for a duration of 30 days under static conditions at a temperature of 28˚C (Chen *et al*., 2015; Moussa *et al*., 2019). After that, 500 mL of ethyl acetate was added to each flask for extraction overnight. The ethyl acetate extracts were filtered and dried using a vacuum rotary evaporator (BÜCHI R-114, Switzerland) at 40˚C until producing an oily residue (Frank *et al*., 2019).

Determination of the minimum inhibitory concentration of fungal extracts

The minimum inhibitory concentrations (MIC) of fungal extracts (A, F, and AF) were determined against pathogenic *F. proliferatum* AUMC 15541 by agar well diffusion method. To assess the MICs against *F. proliferatum*, fungal extracts were pipetted into wells of inoculated plates at doses of 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/mL. Bellis was used as a reference or standard control, while DMSO was employed as a comparison or baseline control. The fungus under examination was subjected to incubation at 28˚C, with three repetitions of each concentration. The MIC was defined as the lowest concentration of fungal extracts that totally inhibited mycelial growth (Park *et al*., 2005).

The Brine shrimp cytotoxicity test

The brine shrimp cytotoxicity test (BSCT) is a method used to determine the cytotoxicity of a fungal extract to brine shrimp larvae. In this experiment, the extracts were dissolved in DMSO at varying concentrations.

100 μl of each extract was then added to duplicate vials containing 5 ml of seawater and 10 brine shrimp larvae. Bellis, an antifungal drug, was used as a positive control. DMSO was used as a negative control. After 24 hours, the number of surviving brine shrimp larvae in each vial was counted. The mean percentage mortality was determined for each extract. The concentration of the extract that killed 50% of the larvae (LC_{50}) was calculated using probit analysis with the Statistical Package for the Social Sciences (SPSS) application with the 16th Version (Meyer et al., 1982; Moshi et al., 2007).

Estimation of total phenolic, flavonoid and tannin content

The total phenolic content of a specimen was measured using modified procedures (Kupina et al., 2018) by reacting it with Folin–Ciocalteu's reagent and sodium carbonate and then reading the absorbance at 750 nm. The phenolic content was calculated in gallic acid equivalent (GAE mg/g) using a standard curve of gallic acid with the equation y = 266.13x - 13.943. To measure the total flavonoid content of a fungal extract, 0.5 mL of each extract (10 mg/mL) was mixed with 1.0 mL of a 2% (v/v) ethanolic solution of $AlCl_3.6H_2O$. The absorbance of the mixture was measured at 430 nm after 10 minutes (Quettier-Deleu et al., 2000). The flavonoid content was expressed in quercetin equivalent (QE mg/g) using a standard curve with the equation y = 33.472x + 0.7975. To estimate the tannin content of fungal extracts, the methanolic extract (1 mL) was mixed with Folin-Ciacolate's reagent (0.5 mL), followed by the addition of saturated Na_2CO_3 solution (1 mL) and distilled water (8 mL). The mixture was allowed to stand for 30 minutes at room temperature and then centrifuged to obtain the supernatant. The absorbance of the supernatant was measured at 725 nm using a UV-Visible Spectrophotometer (Janeway, 7315) described by Fagbemi et al. (2005). The tannin content was calculated in milligrams of tannic acid equivalent using a standard curve with the equation y = 442.66x - 7.8387.

High-performance liquid chromatography-diode-array detection analysis (HPLC-DAD)

Water (photodiode array) was used for HPLC analysis. Separation was achieved through the use of an Eclipse Plus C_{18} column with dimensions of 4.6 mm x 100 mm i.d. and a particle size of 5μm. The mobile phase consisted of 0.1% formic acid in water (A), 0.1% formic acid in acetonitrile (B) at a flow rate 0.8 mL/min. The mobile phase was programmed consecutively in a linear gradient. The DAD was monitored at (235, 254, 280, and 340 nm) (Bandwidth 4 nm). The column temperature was maintained at 30˚C. The injection volume was 10 μL for each of the sample solutions (Akpotu et al., 2017).

Results

Detection of the antagonistic effect of the different tested fungi

In this study, 40 isolates of endophytic fungal species belonged to different genera, including *Aspergillus, Penicillium, Fusarium, Alternaria, Trichoderma, Cladosporium*, and *Drechslera*, were co-cultivated with *F. proliferatum* AUMC 15541 on agar plates to determine whether they have antifungal properties. Nine isolates out of the tested 40 fungal isolates showed positive antagonism with significant inhibition against the growth of *F. proliferatum,* suggesting that they may have potential as sources of bioactive compounds. We studied the antifungal activity of the extract from the interaction zone between endophytic fungi and *F. proliferatum*. Two isolates' extracts showed inhibition on both PDA and YES agar media, while 10 isolates on YES medium and 13 isolates on PDA medim indicated inhibition in the single culture only. *Aspegillus ochraceus* showed strong inhibitory activity when co-cultured on YES medium compared to moderate inhibition activity when co-cultured on PDA medium (Table 1, Figure 1A). Large scale fermentation of axenic cultures of *A. ochraceus* and *F. proliferatum* and their co-cultures on solid rice medium was performed to further investigation.

Antifungal activity of different co-culture extracts against different plant pathogens

Results illustrated that co culture (AF) exhibited a significant inhibitory activity against *F. proliferatum, S. sclerotiorum, B. cinerea* and *A. solani* (19.3, 19.0, 15.3 and 12.3 mm, respectively). while given moderate inhibition rate by extract of *A. ochraceus* (A) against *S. sclerotiorum, R. solani, F. proliferatum, B. cinerea* and *A. solani* (16.0, 15.0, 13.3, 13.3 and 12.3mm, respectively). On the other hand, both of (F) and the combination of (A+F) extracts showed less impact on the mycelial growth of all examined phytopathogen (Table 2).

Determination of minimum inhibitory concentration

The results showed that the MIC of extracts against *F. proliferatum* was 6.25 mg/mL in co-culture (AF) and 12.5 mg/mL in a single extract (A) (Table 3, Figure 1B).

The brine shrimp lethality test

The result in Table 3 showed the LC_{50} values of brine shrimp exposed to extracts of these fungi and the positive control, Bellis. The degree of lethality was

Table 1: Antifungal activity of mono- and co-culture extracts from PDA and YES media against *F. proliferatum*.

No.	Fungi	Code	Source	Antifungal activity			
				PDA extract		YES extract	
				Mono	Co-culture	Mono	Co-culture
1	*Alternaria alternata*	MLBM306	*Artemisia judaica*	-	-	-	-
2	*A. alternate*	MLBM501	*Avicennia marina*	-	-	+	-
3	*A. solani*	MLBM401	*Allium cepa*	-	-	-	-
4	*Aspergillus flavus*	MLBM514	*Avicennia marina*	-	-	-	-
5	*A. flavus*	MLBM402	*Allium cepa*	-	-	-	-
6	*A. fumigatus*	MLBM403	*Allium cepa*	-	-	-	-
7	*A. nidulans*	MLBM504	*Avicennia marina*	++	-	-	-
8	*A. niger*	MLBM506	*Avicennia marina*	-	-	+	-
9	*A. niger*	MLBM507	*Avicennia marina*	-	-	+	-
10	*A. niger*	MLBM508	*Avicennia marina*	+	-	++	-
11	*A. niger*	MLBM516	*Avicennia marina*	-	-	++	++
12	*A. niger*	MLBM522	*Avicennia marina*	-	-	-	-
13	*A. niger*	MLBM404	*Allium cepa*	-	-	-	-
14	*A. ochraceus*	MLBM405	*Allium cepa*	-	++	+	+++
15	*A. terreus*	MLBM406	*Allium cepa*	++	-	-	-
16	*A. ustus*	MLBM409	*Allium cepa*	-	-	+	-
17	*A. versicolor*	MLBM510	*Avicennia marina*	+	-	-	-
18	*A. versicolor*	MLBM511	*Avicennia marina*	+	-	+	-
19	*Cladosporium cladosporioides*	MLBM411	*Allium cepa*	+	-	-	-
20	*Drechslera spicifera*	MLBM413	*Allium cepa*	-	-	-	-
21	*Fusarium moniliforme*	MLBM415	*Allium cepa*	-	-	-	-
22	*F. oxysporum*	MLBM416	*Allium cepa*	-	-	-	-
23	*F. oxysporum*	MLBM417	*Allium cepa*	-	-	-	-
24	*F. solani*	MLBM419	*Allium cepa*	++	-	++	-
25	*F. proliferatum*	MLBM606	*Sesamum indicum*	+	-	-	-
26	*F. proliferatum*	MLBM607	*Sesamum indicum*	-	-	-	+
27	*F. oxysporum*	MLBM605	*Sesamum indicum*	-	-	-	-
28	*F. tricinctum*	MLBM610	*Sesamum indicum*	+	+	++	-
29	*F. camptoceras*	MLBM602	*Sesamum indicum*	+	-	-	-
30	*F. verticillioides*	MLBM611	*Sesamum indicum*	+	-	-	+
31	*F. dimerum*	MLBM603	*Sesamum indicum*	-	-	-	-
32	*F. solani*	MLBM609	*Sesamum indicum*	+	+	-	-
33	*F. verticillioides*	MLBM422	*Allium cepa*	++	-	++	-
34	*F. verticillioides*	MLBM423	*Allium cepa*	-	-	-	-
35	*Penicillium chrysogenum*	MLBM425	*Allium cepa*	-	-	+	+
36	*P. rubrum*	MLBM426	*Allium cepa*	++	-	++	+
37	*P. aurantiogriseum*	MLBM428	*Allium cepa*	-	+	-	-
38	*Trichoderma. harzianum*	MLBM431	*Allium cepa*	++	-	++	-
39	*T. harzianum*	MLBM432	*Allium cepa*	-	-	-	-
40	*T. longibrachiatum*	MLBM433	*Allium cepa*	-	-	-	-

(-) Negative inhibition; (+) the inhibition zone ranged from 7~9 mm (weak); (++) between 10mm and 15 mm (moderate); (+++) from 15 – 20 mm (strong).

Table 2: Antifungal activity of ethyl acetate crude extracts from mono- and co-culture isolates against six phytopathogens.

Extract	Inhibition zone (mm)					
	A. solani	*B. cinerea*	*F. proliferatum*	*R. solani*	*S. cepivorum*	*S. sclerotiorum*
A. ochraceus (A)	12.30±1.20[b]	13.30±1.20[bc]	13.30±1.20[b]	15.00±1.00[b]	00.00	16.00±1.00[b]
F. proliferatum (F)	9.00±1.00[c]	10.70±1.20[c]	00.00	11.00±1.00[c]	00.00	8.00±1.00[c]
Co-culture (AF)	12.30±0.60[b]	15.30±1.50[b]	19.30±0.60[a]	10.70±0.60[c]	00.00	19.00±1.00[b]
Mix (A+F)	11.30±1.50[bc]	00.00	9.00±1.00[c]	10.70±1.20[c]	00.00	10.30±1.50[c]
Bellis (+ve control)	24.30±0.60[a]	24.30±0.60[a]	21.30±0.60[a]	22.70±0.60[a]	23.70±1.50[a]	24.00±1.00[a]

The data were given as averages of three replicates (Mean ±SE). Values followed by the different letters are significantly different at p < 0.05.
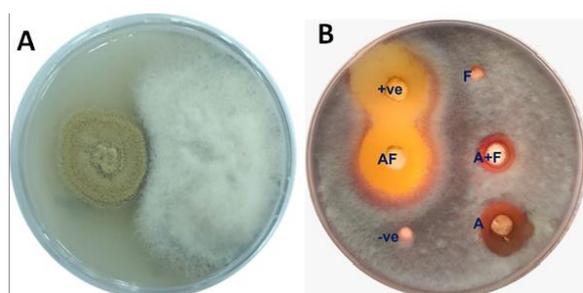

Figure 1: (A) Competitive interactions between *Aspergillus ochraceus* and *Fusarium proliferatum*; (B) Anti-fungal activity of fungal extracts (A, F, A+F, and AF) determined against pathogenic *F. proliferatum* AUMC 15541 by agar well diffusion method.

Table 3: Crude extracts' minimum inhibitory concentration (MIC, mg/mL) and cytotoxic effect on brine shrimp (LC_{50}).

Extract	MIC (mg/mL)	LC_{50} (µg/mL)
A. ochraceus (A)	12.5	1892
F. proliferatum (F)	0.0	1508
Co-culture (AF)	6.25	1972
Bellis (+ve control)	1.56	1690

found to be directly proportional to the extract's concentration. The co-culture AF extract and axenic culture A extract had LC_{50} values of 1972 µg/mL and 1892 µg/mL, respectively. The F extract showed high toxicity to shrimps, with an LC_{50} value of 1508 µg/mL. The Bellis (positive control) had moderate toxicity to shrimps, with an LC_{50} value of 1690 µg/mL.

Total phenolic, flavonoid and tannin content

This study measured the total phenolic concentrations of single and co-culture fungi varied greatly during solid-state fermentation, with values ranging from 39.22 to 88.82 mg/gm. The highest concentration was found in the co-culture of AF (88.82 GAE mg/gm), followed by the single culture of *F. proliferatum* (40.88 GAE mg/gm). the total flavonoid content of ethyl acetate extracts of axenic and co-culture fungi. Figure 2 showed that the ethyl acetate extracts of co-culture (AF) had higher flavonoid content than the ethyl acetate extracts of single cultures A and F. The highest total flavonoid content of 25.68±2.29 QE mg/gm was found in co-culture (AF). The total tannin content of fungal extracts varied from 32.12 to 52.88 TAE mg/gm, with the highest content found in the co-culture (AF) at 52.88 TAE mg/gm. This was significantly higher than the total tannin content of the single culture of *A. ochraceus*, which was 32.12 TAE mg/gm.
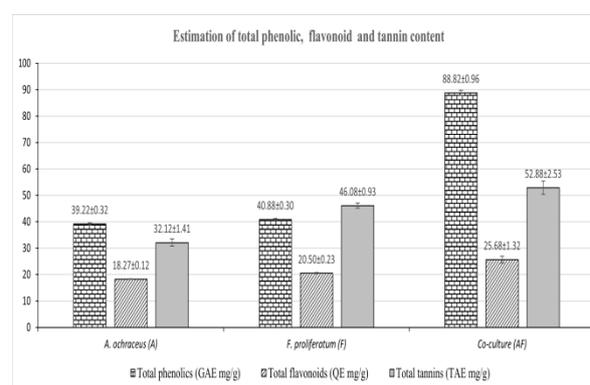

Figure 2: Total contents of phenolics, flavonoids, and tannins from mono- and co-culture isolates.

HPLC analysis of the secondary metabolites produced by co-culture.

Significant chemical changes were observed during the initial screening of solid-state fermentation (SSF) extracts when *A. ochraceus* was co-cultured with *F. proliferatum*, as compared to their individual cultures. HPLC analysis revealed alterations in the profiles of

secondary products in the co-culture, as compared to the pure culture counterparts (Tables S1-S4, Figure 3). The pattern of HPLC peaks used to identify the variations in metabolites included new metabolite peak appearance, loss of original peak appearance. HPLC profile at 235 nm: three peak areas were significantly enhanced at RT (3.79, 4.43, 8.13, respectively), ten peaks were decreased (35%, 20%, 84%, 85%, 70%, 73%, 67%, 20%, 82%, and 50%, respectively), at RT (1.86, 2.78, 6.34, 6.90, 9.01, 9.96, 11.15, 12.95, 27.54, and 33.88, respectively). and three peaks that had been observed in the A culture were missing from the AF culture at RT (2.37, 14.11, and 15.90, respectively). When comparing extracts from co-culture AF to F culture, 14 peak areas increased (3.4, 2.2, 1.7, 2.5, 1.6, 1.5, 1.6, 4.8, 11, 10, 10, 1.8, 47, and 1.8, respectively) fold of F extract at RT (2.96, 3.79, 4.43, 6.04, 6.34, 6.90, 7.52, 8.13, 9.01, 11.15, 12.95, 27.54, 29.68, and 33.88, respectively), 4 peaks decreased at RT (1.868, 4.942, 11.77, and 36.301, respectively), and three peaks that were present in the F extract were not present in the AF extract at RT (5.406, 12.138 and 15.906, respectively). Peak area at retention time (9.487 min) was detected only in AF co-culture extract over A and F extracts.

HPLC profile at 254 nm: two peak regions were greatly enlarged at RT (1.41 and 36.41), and nineteen peaks were dramatically lowered when compared to A extract at RT (1.91, 2.31, 2.81, 4.27, 4.75, 6.08, 6.91, 8.13, 9.00, 9.95, 11.15, 13.06, 14.30, 15.88, 22.28, 25.87, 27.51, 30.46, and 33.81, respectively), while appears that the four peaks present in the A culture are absent in the AF culture at RT (6.339, 17.06, 19.054, and 24.125, respectively). 13 peak regions increased (2.1, 5.5, 1.5, 1.1, 2.2, 2.0, 3.5, 2.7, 5.3, 53.0, 58.0, 27.0, and 378.0, respectively) fold of F extract at RT (1.41, 2.31, 2.81, 4.277, 4.75, 6.08, 8.13, 9.00, 11.15, 13.06, 15.88, 25.87, and 33.81, respectively), and 4 peaks decreased by comparing co-culture AF extract to F extract at RT (1.918, 6.91, 22.28 and 27.517, respectively), respectively., meanwhile, 1 peak disappeared in AF but not in F extracts at RT (17.06).

HPLC profile at 280 nm: nine peak areas were significantly enhanced (58.0, 86.0, 3.0, 1.9, 8.9, 6.3, 1.7, 3.2, and 217.0, respectively) fold of A extract at RT (1.41, 1.92, 2.32, 2.81, 3.79, 4.42, 6.08, 6.31, and 7.51, respectively), five peaks were decreased at RT (6.85, 8.13, 9.00, 13.50, and 28.45, respectively), and three peaks were present in A extract but not in AF extract at RT (14.115, 19.057, and 22.464, respectively). While 11 peak areas increased at RT (1.41, 1.92, 2.81, 2.97, 3.79, 4.42, 4.74, 6.08, 7.51, 8.13, 9.00, respectively), and 3 peaks decreased in AF compared to F culture extracts at RT (2.58, 6.31, and 6.85, respectively).

HPLC profile at 340 nm: seven peak areas were significantly enhanced (3.4, 1.5, 4.5, 1.2, 1.9, 4.7, and 2.3, respectively) fold of A extract at RT (1.41, 1.91, 2.81, 3.78, 4.75, 6.08, and 17.35, respectively), seven peaks were decreased at RT (4.27, 6.91, 11.15, 15.88, 25.61, 27.45, and 33.73, respectively), and 2 peaks were present in A extract but not in AF extract at RT (6.32 and 21.93) at 340 nm, whereas 13 peak areas increased at RT (1.41, 1.91, 2.31, 2.81, 3.78, 4.27, 4.75, 6.08, 7.50, 11.15, 15.88, 17.35, and 27.45, respectively), 3 peaks decreased at RT (6.91, 12.07, and 25.61), and 2 peaks appeared in F extract but not in AF extract at RT (3.567 and 6.32).

Discussion

Endophytes live within the plant tissues in an invisible manner, causing no detectable infection to the host. The most significant element is to provide protection against plant pathogens and pests to the host plant (Hardoim *et al*., 2015). One of the most frequent endophytic fungi associated with both marine and terrestrial hosts is *Aspergillus* (Tawfike *et al*., 2017). Specifically, *A. ochraceus* has been isolated from the fern *Selaginella stauntoniana* (Luo, 2020), and a wide range of plants, including *Euphorbia geniculata* (Kamel *et al*., 2020), *Polygonatum cyrtonema* (Cheng, 2019), *Medicago sativa* (Attia *et al*., 2020), and *Bauhinia forficata* (Bezerra *et al*., 2015). Phytotoxicity and antifungal activity are the most common biological activities associated with *A. ochraceus* extract (Mishra *et al*., 2017). The reason for utilizing fungi to inhibit the growth of the *F. proliferatum* pathogen is not solely attributed to its rapid proliferation in pathogen-infected environments (such as the rhizosphere, phyllosphere, and endophytic environments), but also stems from its ability to generate bioactive compounds (Ghanbarzadeh *et al*., 2014). Antifungal activity has been documented in several previous studies for *Aspergillus*. For instance, Miao *et al*. (2016) found that *A. tubingensis* could effectively inhibit the growth of the pathogenic fungus *Botrytis cinerea* in tomato plants. Similarly, *Karim et al*. (2022) reported that *Aspergillus tubingensis*, *Trichoderma asperellum*, and *Issatchenkia orientalis* hold immense potential as biocontrol agents in the field for suppressing *F. oxysporum*. When different microorganisms are cultivated together, direct interactions occur, which may result in the synthesis of chemicals not previously seen when the strains were grown separately (Oh *et al*., 2005).

Studies have shown that some biosynthetic pathways in fungi remain inactive during standard laboratory culture conditions, with corresponding genes only becoming expressed in response to environmental stress or biotic signals (Schroeckh *et al*., 2009).
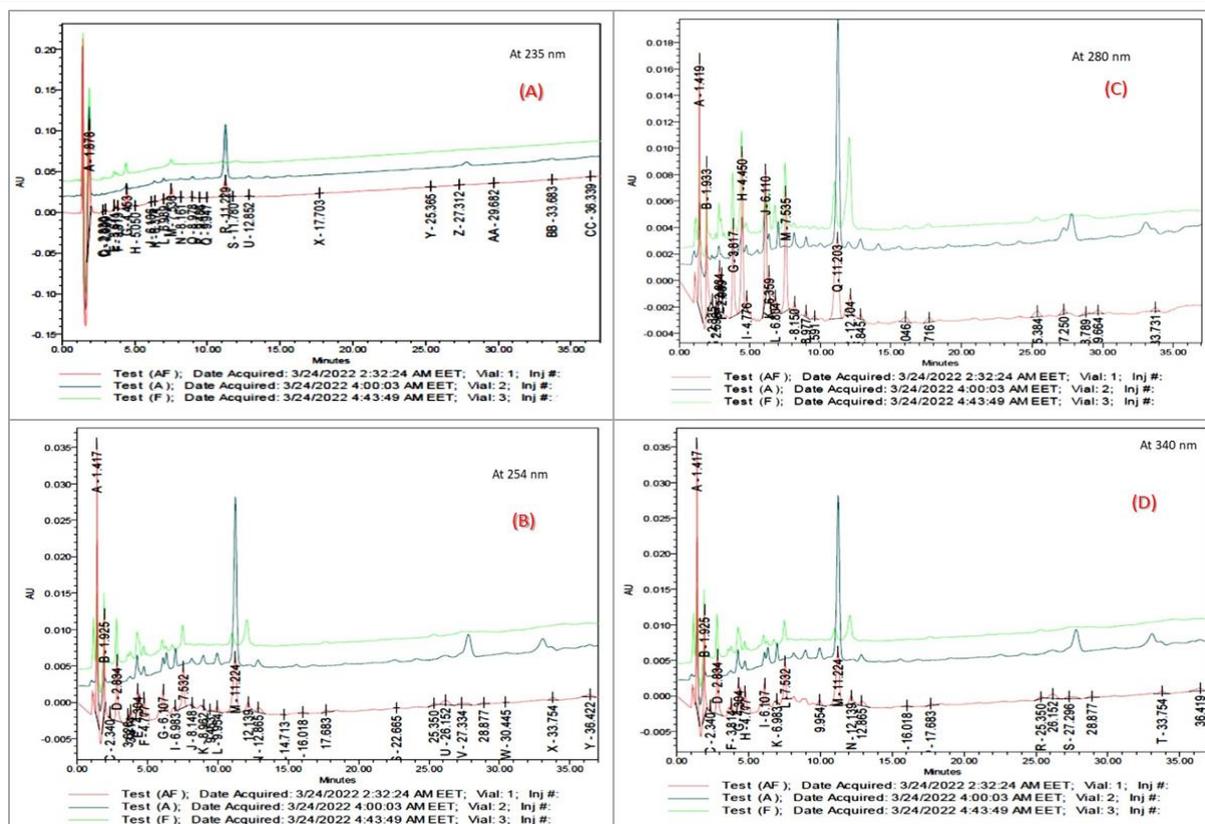
Figure 3: HPLC profiles of co-culture and single cultures of *A. ochraceus* and *F. proliferatum* at different wavelengths (A) at 235 nm, (B) at 254 nm, (C) at 280 nm, and (D) at 340 nm.

Accordingly, we conducted a comparison of metabolites produced by the endophytic fungus *A. ochraceus* during its interaction with *F. proliferatum* to those generated by the fungus in a single culture. The dual culture approach resulted in selective induction of multiple metabolites by the endophytic fungus, suggesting that some or all of these metabolites may contribute to the observed intense antagonistic activity. Utilization of yeast extract sucrose agar media is of great benefit in taxonomic studies that depend on secondary metabolites. The addition of yeast extract to the media serves to significantly reduce any discrepancies in morphological characteristics and secondary metabolite production that could be detected. This reduction is particularly crucial in addressing disagreements that may arise about the specific profiles of secondary metabolites (Filtenborg *et al.*, 1990; Clemmensen *et al.*, 2007).

In this work, the co-culture of AF extract showed significant inhibitory activity against several plant pathogens, including *F. proliferatum, S. sclerotiorum, B. cinerea,* and *A. solani* (19.30, 19.00, 15.30, and 12.30 mm, respectively), as assessed by the agar well diffusion method. While extracts of *A. ochraceus* provided a moderate inhibitory rate against *S. sclerotiorum, R. solani, F. proliferatum, B. cinerea,* and *A. solani* (16.00, 15.00, 13.30, 13.30, and 12.30 mm, respectively). Similarly, *A. ochraceus* displays intermediate antifungal effects towards *A. alternata*, and *B. cinerea,* and *F. oxysporum (Morales-Sánchez et al.*, 2021), moderate activity against *B. cinerea* (Zhao et al., 2012; Wang et al., 2014; Cimmino *et al.*, 2019), *F. oxysporum*, and *F. solani* (Wang *et al.*, 2018). In antagonism cultural tests, Donald *et al.* (2005) obtained similar results with endophytic fungi *Acremonium zeae* against *Aspergillus flavus* and *Fusarium verticillioides*. However, the morphological interaction patterns during the screening suggested that long-distance inhibition was not frequently observed (Bertrand *et al.*, 2013). According to Yue *et al.* (2015), the antifungal activity of co-cultures and pure cultures was assessed against four plant pathogenic fungi: *Rhizoctonia solani, Verticillium dahliae, Gibberella zeae*, and *Botrytis cinerea*. The investigation revealed that *A. versicolor* effectively suppressed mycelial growth at concentrations of 50, 100, and 200 g/mL, with the strongest inhibition observed against *R. solani* at 200 g/mL (82.32%) and against *B. cinerea* at 50 g/mL (53.23%). Moreover, a co-culture of *Tilletiopsis* sp. and *A. versicolor* exhibited favorable antifungal

activity against *R. solani* and *B. cinerea* at 200 g/mL. Oppong-Danquah *et al*. (2020) noted significant indications of competitive interactions, such as the inhibition of a biosynthetic pathway in one strain and the stimulation of highly functional metabolites that mediate another competition. The co-culture extract of *Plenodomus influorescens-Pyrenochaeta nobilis* exhibited increased efficacy against *Xanthomonas campestris* (59%), which was further elevated to 90% upon testing the extract of the confrontation zone against *Magnaporthe oryzae*. Ochratoxin A, a nephrotoxic mycotoxin, is produced by *Aspergillus ochraceus* (Visagie *et al*., 2014; Bui-Klimke & Wu, 2015). Although the exact biological function of mycotoxins is still unclear, one of the most widely proposed theories is that mycotoxin-producing fungi have a greater ability to defend themselves against other organisms occupying the same ecological niche (Fox & Howlett, 2008).

BSCT is a practical, inexpensive method to assess toxicity of various compounds. It can detect bioactivities, antifungal, pesticide, teratogenic, and environmental toxicity *(Almeida et al. 2002; Saber et al*. 2016; Sasidharan & Elyas, 2019)**.** The concentration of the extract was found to be directly proportional to the degree of lethality. The endophytic fungal strain CEDBE-1 exhibited the highest antioxidant activity with an IC_{50} value of 31.07 μg/mL (Munshi *et al*., 2021). The BSCT considers LC50 values lower than 1000 μg/mL as toxic (Sasidharan & Elyas, 2019). This study found that different fungal extracts of both AF and A extracts are non-toxic.

The result of phytochemical screening of extracts from single and co-culture samples revealed the presence of phenolics, flavonoids, and tannins in varied amounts. This is consistent with previous studies, which have shown that endophytic fungi often contain these compounds *(Shalini & Sampathkumar, 2012; Ishaq et al*., 2014). Khiralla *et al*. (2015) reported that the TPC value of ethyl acetate crude extracts of different endophytic fungi varied from 0.5 to 89.9 mg/g, with two *Aspergillus* sp. strains showing the highest values of 77.2 and 89.9 mg/g. Yadav *et al*. (2014) found that tannins contribute to the antioxidant activity of filamentous fungi. Fungal endophytes are a rich source of secondary metabolites with diverse biological activities, including antibacterial, antifungal, anticancer, and antiparasitic properties. Hassane *et al*. (2022b) reported that the endophytic fungus *F. chlamydosporum* had a high total phenolic content (TPC) of 26.64 (GAE mg/g) and a total flavonoid content (TFC) of 22.9 (CE mg/g). However, tannins and coumarins were only detected in trace. *Hassane et al*. (2022a) found that the n-butanol extracts of

endophytic fungi *(Aspergillus niger, Rhizopus oryzae, and Colletotrichum madrasense),* contained high amounts of phenolics and flavonoids. These compounds possess various biological properties. Our findings are consistent with previous reports, such as the study by Satari *et al*. (2018) carried out the phytochemical screening of ethanolic extracts of three *Aspergillus* sp. and observed that *A. niger* contained all the phytochemical constituents, *A. terreus* had all the phytochemicals except glycosides and anthraquinones and *A. flavus* was deficient of tannin and anthraquinones.

HPLC analysis of the EtOAc crude extracts provided additional information on the diversity of chemicals and differences in metabolite compositions between pure strains and co-cultures. Co-culture AF changed the secondary metabolism of *A. ochraceus*. Comparing extracts of AF co-culture to A culture at 235 nm, three peak areas were significantly enhanced, ten peaks were decreased, and three peaks were disappeared in AF but not in A culture. When comparing extracts from co-culture AF to F culture, 14 peak areas increased, 4 peaks decreased, and 3 peaks disappeared in AF but not in F extracts. Peak area at retention time (9.487 min) was detected only in AF co-culture extract over A and F extracts. Co-culture AF extract at 254 nm, two peak regions were greatly enlarged, and nineteen peaks were dramatically lowered when compared to A extract, while four peaks were disappeared in AF but not in A culture. 13 peak regions increased, and 4 peaks decreased by comparing co-culture AF extract to F extract, meanwhile, 1 peak disappeared in AF but not in F extracts. At 280 nm, nine peak areas were significantly enhanced, five peaks were decreased, and three peaks were present in A extract but not in AF extract. While, 11 peak areas increased, and 3 peaks decreased in AF compared to F culture extracts. Finally, seven peak areas were significantly enhanced, seven peaks were decreased, and 2 peaks were present in A extract but not in AF extract at 340 nm, whereas 13 peak areas increased, 3 peaks decreased, and 2 peaks appeared in F extract but not in AF extract.

Four peaks increased, nine declined, three were lost, and one "new" metabolite developed at 235 nm, with eleven peaks present in AF but not in A. Two peaks grew at 254 nm, while 19 receded. At 280 nm, ten peak regions were greatly enlarged, seven peaks were significantly reduced, and two peaks were present in AF but not in A. When comparing co-culture AF to A extract at 340 nm, seven peak regions were significantly increased, seven peaks were lowered, and three peaks were present in AF but not in A. Only AF, not A or F, can be observed. Similarly, The HPLC chromatograms of the *Trichoderma* and *Talaromyces*

single fungal cultures (controls) and their co-culture revealed significant differences in peak intensity and retention time, particularly between 5-7 and 9-13 minutes. Notably, a unique peak at 5.9 minutes was observed exclusively in the co-culture chromatogram (Vinale *et al.*, 2017). Yu *et al.* (2019) documented notable alterations in metabolites present in the fermentation broth of a co-culture containing the fungal strain *Rhinocladiella similis* and the actinomycete strain *Streptomyces rochei*. Analysis of the EtOAc extracts of the fermentation broth via HPLC revealed that, in single-strain cultivations, *S. rochei* produced two peaks while *R. similis* exhibited a weak peak. However, when compared to the control, the co-culture EtOAc extracts displayed two additional peaks and one peak showed a significant increase in peak intensity. According to Chagas *et al.* (2013), the HPLC profiles of the crude extract obtained from the co-culture of *Alternaria tenuissima* and *Nigrospora sphaerica* displayed a peak with a retention time of 19.9 minutes, which was absent in the single cultures. Furthermore, the mixed culture demonstrated a higher peak intensity at 21.0 minutes. In dual cultures of the endophytic fungus *Paraconiothyrium* and the phytopathogen *F. oxysporum*, twelve metabolites were uniquely induced, implying that some or all of these compounds may contribute to the marked antagonistic effect observed (Combes *et al.*, 2012). Chagas *et al.* (2013) propose that the polyketides synthesized by *A. tenuissima* play a crucial role in inhibiting the growth of *N. sphaerica.* This is due to the fact that biologically active compounds can demonstrate their function in an ecological system, even when present in low amounts. Moreover, the combined effect of multiple compounds can increase their individual bioactivity.

Variations in metabolites are identified by changes in the pattern of HPLC peaks, which reflect alterations in metabolite production. Wang *et al.* (2021) investigated changes in the secondary metabolism of 40 *Streptomyces* strains by comparing their pure cultures with co-cultures involving *Mycobacterium* sp. The study found that 30 strains exhibited increased production of original metabolites, while 23 strains demonstrated decreased production. Additionally, 14 strains produced new metabolites that were not present in pure cultures, whereas seven strains lost some metabolites. Oppong-Danquah *et al.* (2020) observed an atypical pattern of distinctly suppressed peaks in the UPLC-MS chromatograms of the Plenodomus influorescen and Pyrenochaeta nobilis co-culture extracts, as compared to monocultures. Upon further examination of the HPLC chromatograms, Yue *et al.* (2015) found that multiple peaks with comparable retention times in diverse chromatograms had a common UV absorbance pattern, suggesting the presence of similar metabolites in both mono- and co-cultures prepared under the same conditions. The existence of identical peaks is a valuable tool for gauging metabolite complexity since they validate the consistency of analytical conditions and can also serve as an effective guide for identifying other unique peaks.

Conclusion

In conclusion, microbial co-culture (also known as mixed fermentation) has been shown to be a low-cost and time-tested method for generating novel microbial secondary metabolites. Although different interactions between bacteria and fungus have been studied in recent years, the current work offers a rare case of fungal-fungal interaction. Co-cultivation of phytopathogens and endophytic fungi results in the production of secondary metabolites, which diffuse to the microorganisms' point of contact. The fact that extracts showed high antifungal activity supports the importance of co-cultures as a method for finding novel natural antibiotics. Further research should be done on using the identified compounds as natural antifungals as opposed to synthetic fungicides. This work opens up new research directions and significantly enhances environmental and human health protection by utilising substance generated from natural sources. As a result, safer agricultural practices can be investigated. Future identification of the novel peaks in the coculture will be conducted.

References

Abdalla, M.A.; Sulieman, S.; McGaw, L.J. (2017). Microbial communication: A significant approach for new leads. *South African Journal of Botany*, 113:461–470. https://doi.org/10.1016/j.sajb.2017.10.001

Abdelwahab, M.F.; Kurtán, T.; Mándi, A.; Müller, W.E.G.; Fouad, M.A.; Kamel, M.S.; Liu, Z.; Ebrahim, W.; Daletos, G.; Proksch, P. (2018). Induced secondary metabolites from the endophytic fungus *Aspergillus versicolor* through bacterial co-culture and OSMAC approaches. *Tetrahedron Letters*, 59(27): 2647–2652.

Abrudan, M.I.; Smakman, F.; Grimbergen, A.J.; Westhoff, S.; Miller, E.L.; Van Wezel, G.P.; Rozen, D.E. (2015). Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proceedings of the National Academy of Sciences*, *112*(35): 11054–11059.

Akpotu, M.O.; Eze, P.M.; Abba, C.C.; Nwachukwu, C.U.; Okoye, F.B.C.; Esimone, C.O. (2017).

Antimicrobial activities of secondary metabolites of endophytic fungi isolated from *Catharanthus roseus*. *Journal of Health Sciences*, 7(1): 15–22.

Al Mousa, A.A.; Mohamed, H.; Hassane, A.M.A.; Abo-Dahab, N.F. (2021). Antimicrobial and cytotoxic potential of an endophytic fungus *Alternaria tenuissima* AUMC14342 isolated from *Artemisia judaica* L. growing in Saudi Arabia. *Journal of King Saud University-Science*, 33(5): 101462.

Albert, S.; Chauhan, D.; Pandya, B.; Padhiar, A. (2011). Screening of *Trichoderma* spp. as potential fungal partner in co-culturing with white rot fungi for efficient bio-pulping. *Global Journal of Biotechnology & Biochemistry*, 6(3): 95–101.

Almeida, P.A.; da Silva, T.M. S.; Echevarria, A. (2002). Mesoionic 5-alkyl-1, 3-dithiolium-4-thiolates: Synthesis and brine shrimp toxicity. *Heterocyclic Communications*, 8(6): 593–600.

Attia, E.Z.; Farouk, H.M.; Abdelmohsen, U.R.; Mo'men, H. (2020). Antimicrobial and extracellular oxidative enzyme activities of endophytic fungi isolated from alfalfa (*Medicago sativa*) assisted by metabolic profiling. *South African Journal of Botany*, 134: 156–162.

Bai, J.; Zhang, P.; Bao, G.; Gu, J.G.; Han, L.; Zhang, L.W.; Xu, Y. (2018). Imaging mass spectrometry-guided fast identification of antifungal secondary metabolites from *Penicillium polonicum*. *Applied Microbiology and Biotechnology*, 102(19): 8493–8500.

Bertrand, S.; Bohni, N.; Schnee, S.; Schumpp, O.; Gindro, K.; Wolfender, J.-L. (2014). Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. *Biotechnology Advances*, 32(6): 1180–1204.

Bertrand, S.; Schumpp, O.; Bohni, N.; Monod, M.; Gindro, K.; Wolfender, J.-L. (2013). *De novo* production of metabolites by fungal co-culture of *Trichophyton rubrum* and *Bionectria ochroleuca*. *Journal of Natural Products*, 76(6): 1157–1165.

Bezerra, J.D.P.; Nascimento, C.C.F.; Barbosa, R.doN.; da Silva, D.C.V.; Svedese, V.M.; Silva-Nogueira, E.B.; Gomes, B.S.; Paiva, L.M.; Souza-Motta, C.M. (2015). Endophytic fungi from medicinal plant Bauhinia forficata: Diversity and biotechnological potential. *Brazilian Journal of Microbiology*, 46: 49–57.

Brakhage, A.A.; Schuemann, J.; Bergmann, S.; Scherlach, K.; Schroeckh, V.; Hertweck, C. (2008). Activation of fungal silent gene clusters: A new avenue to drug discovery. *Natural Compounds as Drugs*, *II*, Pp 1-12.

Bui-Klimke, T.R.; Wu, F. (2015). Ochratoxin A and human health risk: A review of the evidence. *Critical Reviews in Food Science and Nutrition*, 55(13): 1860–1869.

Chagas, F.O.; Dias, L.G.; Pupo, M.T. (2013). A mixed culture of endophytic fungi increases production of antifungal polyketides. *Journal of Chemical Ecology*, 39(10): 1335–1342.

Chen, H.; Daletos, G.; Abdel-Aziz, M. S.; Thomy, D.; Dai, H.; Brötz-Oesterhelt, H.; Lin, W.; Proksch, P. (2015). Inducing secondary metabolite production by the soil-dwelling fungus *Aspergillus terreus* through bacterial co-culture. *Phytochemistry Letters*, 12: 35–41.

Chen, P.Y.; Hsieh, C.Y.; Shih, C.J.; Lin, Y.J.; Tsao, C.W.; Yang, Y.-L. (2018). Exploration of fungal metabolic interactions using imaging mass spectrometry on nanostructured silicon. *Journal of Natural Products*, 81(7): 1527–1533.

Cheng, Z.Y. (2019). Study on secondary metabolites of endophytic fungus *Aspergillus ochraceus* from *Polygonatum cyrtonema*. *Chinese Traditional and Herbal Drugs*, 24(1): 5424–5428.

Cimmino, A.; Maddau, L.; Masi, M.; Linaldeddu, B. T.; Evidente, A. (2019). Secondary metabolites produced by *Sardiniella urbana*, a new emerging pathogen on European hackberry. *Natural Product Research*, 33(13): 1862–1869.

Clemmensen, L.H.; Hansen, M.E.; Frisvad, J.C.; Ersbøll, B. K. (2007). A method for comparison of growth media in objective identification of *Penicillium* based on multi-spectral imaging. *Journal of Microbiological Methods*, 69(2): 249–255.

Combes, A.; Ndoye, I.; Bance, C.; Bruzaud, J.; Djediat, C.; Dupont, J.; Nay, B.; Prado, S. (2012). Chemical communication between the endophytic fungus *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum*. *PLoS ONE*, 7(10): e47313.

Copping, L.G.; Menn, J. J. (2000). Biopesticides: A review of their action, applications and efficacy. *Pest Management Science: Formerly Pesticide Science*, 56(8): 651–676.

Costa, J.H.; Wassano, C.I.; Angolini, C.F.F.; Scherlach, K.; Hertweck, C.; Fill, T.P. (2019). Antifungal potential of secondary metabolites involved in the interaction between citrus pathogens. *Scientific Reports*, 9(1): 1–11.

Cramer, C.S. (2000). Breeding and genetics of *Fusarium* basal rot resistance in onion. *Euphytica*, *115*(3): 159–166.

de Weert, S.; Kuiper, I.; Lagendijk, E.L.; Lamers, G.E.M.; Lugtenberg, B.J.J. (2004). Role of chemotaxis toward fusaric acid in colonization of hyphae of *Fusarium oxysporum* f. sp. *radicis-lycopersici* by *Pseudomonas fluorescens* WCS365. *Molecular Plant-Microbe Interactions*, *17*(11): 1185–1191.

Demain; A.L. (2014). Importance of microbial natural products and the need to revitalize their discovery. *Journal of Industrial Microbiology and Biotechnology*; *41*(2): 185–201.

Desjardins, A.E. (2006). *Fusarium* mycotoxins. In *Chemistry, genetics and biology. American Phytopathological Society, St. Paul, MN (USA)*.

Donald, T.; Shoshannah, R.; Deyrup, S.T.; Gloer, J.B. (2005). A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. *Mycological Research*, 109(5): 610–618.

Eckelmann, D.; Spiteller, M.; Kusari, S. (2018). Spatial-temporal profiling of prodiginines and serratamolides produced by endophytic *Serratia marcescens* harbored in *Maytenus serrata*. *Scientific Reports*, *8*(1): 1–15.

Estrada, A.E.R.; Hegeman, A.; Kistler, H.C.; May, G. (2011). *In vitro* interactions between *Fusarium verticillioides* and *Ustilago maydis* through real-time PCR and metabolic profiling. *Fungal Genetics and Biology*, 48(9): 874–885.

Fagbemi, T.N.; Oshodi, A.A.; Ipinmoroti, K.O. (2005). Processing effects on some antinutritional factors and in vitro multienzyme protein digestibility (IVPD) of three tropical seeds: breadnut (*Artocarpus altilis*): cashewnut (*Anacardium occidentale*) and fluted pumpkin (*Telfairia occidentalis*). *Pakistan Journal of Nutrition*, 4(4): 250–256.

Filtenborg, O.; Frisvad, J.C.; Thrane, U. (1990). The significance of yeast extract composition on metabolite production in *Penicillium*. *Modern Concepts in Penicillium and Aspergillus Classification*, 433–441.

Fox, E.M.; Howlett, B.J. (2008). Secondary metabolism: Regulation and role in fungal biology. *Current Opinion in Microbiology*, 11(6): 481–487.

Frank, M.; Can Özkaya, F.; Müller, W.E.G.; Hamacher, A.; Kassack, M.U.; Lin, W.; Liu, Z.; Proksch, P. (2019). Cryptic secondary metabolites from the sponge-associated fungus *Aspergillus ochraceus*. *Marine Drugs*, *17*(2): 1–13.

Galeano, P.; González, P.H.; Fraguas, L.F.; Galván, G.A. (2014). Age-related resistance to *Fusarium oxysporum* f. sp. *cepae* and associated enzymatic changes in seedlings of *Allium cepa* and *A. fistulosum*. *Tropical Plant Pathology*, *39*(5): 374–383.

Ghanbarzadeh, B.; Goltapeh, E.M.; Safaie, N. (2014). Identification of *Fusarium* species causing basal rot of onion in East Azarbaijan province, Iran and evaluation of their virulence on onion bulbs and seedlings. *Archives of Phytopathology and Plant Protection*, 47(9): 1050–1062.

González-Menéndez, V.; Martínez, G.; Serrano, R.; Muñoz, F.; Martín, J.; Genilloud, O.; Tormo, J.R. (2018). Ultraviolet (IUV) and mass spectrometry (IMS) imaging for the deconvolution of microbial interactions. *BMC Systems Biology*, 12(5): 91–97.

Gupta, V.; Kumar, K.; Fatima, K.; Razdan, V.K.; Sharma, B.C.; Mahajan, V.; Rai, P.K.; Sharma, A.; Gupta, V.; Hassan, M.G. (2020). Role of biocontrol agents in management of corm rot of saffron caused by *Fusarium oxysporum*. *Agronomy*, 10(9): 1398.

Hajieghrari, B.; Torabi-Giglou, M.; Mohammadi, M.R.; & Davari, M. (2008). Biological potantial of some Iranian *Trichoderma* isolates in the control of soil borne plant pathogenic fungi. *African Journal of Biotechnology*, 7(8): 967–972.

Hardoim, P.R.; Van Overbeek, L.S.; Berg, G.; Pirttilä, A.M.; Compant, S.; Campisano, A.; Döring, M.; Sessitsch, A. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews*, 79(3): 293–320.

Hassane, A.M.A.; Taha, T.M.; Awad, M.F.; Mohamed, H.; Melebari, M. (2022a). Radical scavenging potency; HPLC profiling and phylogenetic analysis of endophytic fungi isolated from selected medicinal plants of Saudi Arabia. *Electronic Journal of Biotechnology*; 58: 37–45.

Hassane, A.M. A.; Hussien, S.M.; Abouelela, M.E.; Taha, T.M.; Awad, M.F.; Mohamed, H.; Hassan, M.M.; Hassan, M.H.A.; Abo-Dahab, N.F.; El-Shanawany, A.R.A. (2022b). In vitro and in silico antioxidant efficiency of bio-potent secondary metabolites from different taxa of black seed-producing plants and their derived mycoendophytes. *Frontiers in Bioengineering and Biotechnology*, 10: 930161.

Heydari, A.; Pessarakli, M. (2010). A review on biological control of fungal plant pathogens using

microbial antagonists. *Journal of Biological Sciences*, 10(4): 273–290.

Ishaq, M.S.; Hussain, M.M.; Afridi, M.S.; Ali, G.; Khattak, M.; Ahmad, S. (2014). In vitro phytochemical, antibacterial, and antifungal activities of leaf, stem, and root extracts of *Adiantum capillus* veneris. *The Scientific World Journal*, 2014: 1–7.

Ishikawa, S.; Ishimori, K.; Ito, S. (2017). A 3D epithelial–mesenchymal co-culture model of human bronchial tissue recapitulates multiple features of airway tissue remodeling by TGF-β1 treatment. *Respiratory Research*, 18(1): 1–11.

Kamel, N.M.; Abdel-Motaal, F.F.; El-Zayat, S.A. (2020). Endophytic fungi from the medicinal herb *Euphorbia geniculata* as a potential source for bioactive metabolites. *Archives of Microbiology*; 202(2): 247–255.

Karim, H.; Azis, A.A.; Jumadi, O. (2022). Antagonistic activity and characterization of indigenous soil isolates of bacteria and fungi against onion wilt incited by *Fusarium* sp. *Archives of Microbiology*, 204(1): 1–9.

Khiralla, A.; Mohamed, I.; Thomas, J.; Mignard, B.; Spina, R.; Yagi, S.; Laurain-Mattar, D. (2015). A pilot study of antioxidant potential of endophytic fungi from some Sudanese medicinal plants. *Asian Pacific Journal of Tropical Medicine*, 8(9): 701–704.

Koehn, F.E.; Carter, G. . (2005). The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery*, *4*(3): 206–220.

Kupina, S.; Fields, C.; Roman, M.C.; Brunelle, S.L. (2018). Determination of total phenolic content using the Folin-C assay: single-laboratory validation, first action 2017.13. *Journal of AOAC International*, 101(5): 1466–1472.

Laura, G.; Palmero, D. (2022). *Fusarium* dry rot of garlic bulbs caused by *Fusarium proliferatum*. *Horticulture*, *8*(7): 628–648.

Luo, P. (2020). Secondary metabolites of endophytic fungus *Aspergillus ochraceus* SX-C7 from *Selaginella stauntoniana*. *Chinese Traditional and Herbal Drugs*, 24: 3856–3862.

Magaldi, S.; Mata-Essayag, S.; De Capriles, C.H.; Pérez, C.; Colella, M.T.; Olaizola, C.; Ontiveros, Y. (2004). Well diffusion for antifungal susceptibility testing. *International Journal of Infectious Diseases*, 8(1): 39–45.

Marimuthu, S.; Karthic, C.; Mostafa, A.A.; Al-Enazi, N.M.; Abdel-Raouf, N.; Sholkamy, E.N. (2020). Antifungal activity of *Streptomyces* sp. SLR03 against tea fungal plant pathogen *Pestalotiopsis theae*. *Journal of King Saud University - Science*, 32(8): 3258–3264.

Martínez-Buitragoa, P. A.; Ramosa, F. A.; & Castellanos, L. (2019). Binary co-culture selection from marine-derived microorganisms for differential production of specialized metabolites. *Quimica Nova*, 42(7): 713-719.

Meyer, B.N.; Ferrigni, N.R.; Putnam, J.E.; Jacobsen, L.B.; Nichols, D.E.; McLaughlin, J.L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Medica*, 45(1): 31–34.

Miao, C.P.; Mi, Q.L.; Qiao, X.G.; Zheng, Y.K.; Chen, Y.W.; Xu, L.H.; Guan, H.L.; Zhao, L.X. (2016). Rhizospheric fungi of *Panax notoginseng*: Diversity and antagonism to host phytopathogens. *Journal of Ginseng Research*, 40(2): 127–134.

Michelsen, C.F.; Hossein Khademi, S.M.; Krogh Johansen, H.; Ingmer, H.; Dorrestein, P.C.; Jelsbak, L. (2016). Evolution of metabolic divergence in *Pseudomonas aeruginosa* during long-term infection facilitates a proto-cooperative interspecies interaction. *The ISME Journal*, 10(6): 1323–1336.

Mishra, V.K.; Passari, A.K.; Chandra, P.; Leo, V.V.; Kumar, B.; Uthandi, S.; Thankappan, S.; Gupta, V.K.; Singh, B. P. (2017). Determination and production of antimicrobial compounds by *Aspergillus clavatonanicus* strain MJ31, an endophytic fungus from *Mirabilis jalapa* L. using UPLC-ESI-MS/MS and TD-GC-MS analysis. *PLoS ONE*, 12(10): 1–24.

Mohamed, H.; Hassane, A.; Atta, O.; Song, Y. (2021). Deep learning strategies for active secondary metabolites biosynthesis from fungi: Harnessing artificial manipulation and application. *Biocatalysis and Agricultural Biotechnology*, 38, 102195.

Morales-Sánchez, V.; Díaz, C.E.; Trujillo, E.; Olmeda, S.A.; Valcarcel, F.; Muñoz, R.; Andrés, M.F.; González-Coloma, A. (2021). Bioactive metabolites from the endophytic fungus *Aspergillus* sp. SPH2. *Journal of Fungi*, 7(2): 1–12.

Moshi, M. J.; Van den Beukel, C.J.; Hamza, O.J.M.; Mbwambo, Z.H.; Nondo, R.O.S.; Masimba, P.J.; Matee, M.I.; Kapingu, M.C.; Mikx, F.; Verweije, P.J. (2007). Brine shrimp toxicity evaluation of some Tanzanian plants used traditionally for the treatment of fungal infections. *African Journal of Traditional, Complementary and Alternative Medicines*, 4(2): 219–225.

Moussa, M.; Ebrahim, W.; Bonus, M.; Gohlke, H.; Mándi, A.; Kurtán, T.; Hartmann, R.; Kalscheuer, R.; Lin, W.; Liu, Z.; Proksch, P. (2019). Co-culture of the fungus *Fusarium tricinctum* with *Streptomyces lividans* induces production of cryptic naphthoquinone dimers. *RSC Advances*, 9(3): 1491–1500.

Munshi, M.; Sohrab, M.H.; Begum, M.N.; Rony, S.R.; Karim, M.A.; Afroz, F.; Hasan, M. N. (2021). Evaluation of bioactivity and phytochemical screening of endophytic fungi isolated from *Ceriops decandra* (Griff.) W. Theob, a mangrove plant in Bangladesh. *Clinical Phytoscience*, 7(1): 1–10.

Nai, C.; Meyer, V. (2018). From axenic to mixed cultures: Technological advances accelerating a paradigm shift in microbiology. *Trends in Microbiology*; 26(6): 538–554.

Oh, D.C.; Jensen, P.R.; Kauffman, C.A.; Fenical, W. (2005). Libertellenones A–D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. *Bioorganic & Medicinal Chemistry*, 13(17): 5267–5273.

Oppong-Danquah, E.; Budnicka, P.; Blümel, M.; Tasdemir, D. (2020). Design of fungal co-cultivation based on comparative metabolomics and bioactivity for discovery of marine fungal agrochemicals. *Marine Drugs*, 18(2).

Pan, R.; Bai, X.; Chen, J.; Zhang, H.; Wang, H. (2019). Exploring structural diversity of microbe secondary metabolites using OSMAC strategy. *Frontiers in Microbiology*, 10, 294–314.

Park, J.H.; Gyung, J.C.; Kyoung, S.J.; He, K.L.; Heung, T.K.; Kwang, Y.C.; Kim, J.C. (2005). Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. *FEMS Microbiology Letters*, 252(2): 309–313.

Peiris, D.; Dunn, W.B.; Brown, M.; Kell, D.B.; Roy, I.; Hedger, J.N. (2008). Metabolite profiles of interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus, *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminatus*. *Metabolomics*, 4(1): 52–62.

Quettier-Deleu, C.; Gressier, B.; Vasseur, J.; Dine, T.; Brunet, C.; Luyckx, M.; Cazin, M.; Cazin, J.C.; Bailleul, F.; Trotin, F. (2000). Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology*, 72(1–2): 35–42.

Saber, S.M.; Youssef, M.S.; Arafa, R.F.; Hassane, A.M.A. (2016). Mycotoxins production by *Aspergillus ostianus* Wehmer and using phytochemicals as control agent. *Journal of Science and Engineering Research*, 3(2): 198-213.

Sasidharan, A.T.K.; Elyas, K.K. (2019). Anti-fungal potential and brine shrimp lethality assay of *in vitro* raised clones of *Celastrus paniculatus*. *Asian Journal of Biological Sci*ences, 12(4): 877–883.

Satari, A.H.; Zargar, M.I.; Shah, W.A.; Bansal, R.; Bhat, M.F. (2018). Isolation, molecular identification, phytochemical screening and *in vitro* anti-oxidant activity of endophytic fungi from *Achilea millefolium* Linn. *Journal of Pharmacognosy and Phytochemistry*, 7(4): 87–92.

Schroeckh, V.; Scherlach, K.; Nützmann, H.W.; Shelest, E.; Schmidt-Heck, W.; Schuemann, J.; Martin, K.; Hertweck, C.; Brakhage, A. A. (2009). Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences*, 106(34): 14558–14563.

Schueffler, A.; Anke, T. (2014). Fungal natural products in research and development. *Natural Product Reports*, 31(10): 1425–1448.

Serrano, R.; González-Menéndez, V.; Rodríguez, L.; Martín, J.; Tormo, J.R.; Genilloud, O. (2017). Co-culturing of fungal strains against *Botrytis cinerea* as a model for the induction of chemical diversity and therapeutic agents. *Frontiers in Microbiology*, 8(APR): 1–15.

Shalini, S.; Sampathkumar, P. (2012). Phytochemical screening and antimicrobial activity of plant extracts for disease management. *International Journal of Current Science*, 1(1): 209–218.

Sica, V.P.; El-Elimat, T.; Oberlies, N.H. (2016). *In situ* analysis of *Asimina triloba* (paw paw) plant tissues for acetogenins via the droplet-liquid microjunction-surface sampling probe coupled to UHPLC-PDA-HRMS/MS. *Analytical Methods*, 8(32): 6143–6149.

Tan, Z.Q.; Leow, H.Y.; Lee, D.C.W.; Karisnan, K.; Song, A.A.L.; Mai, C.W.; Yap, W.S.; Lim, S.H.E.; Lai, K.S. (2019). Co-culture systems for the production of secondary metabolites: Current and future prospects. *The Open Biotechnology Journal*, 13(1): 18–26.

Tata, A.; Perez, C.; Campos, M.L.; Bayfield, M.A.; Eberlin, M.N.; Ifa, D.R. (2015). Imprint desorption electrospray ionization mass spectrometry imaging for monitoring secondary metabolites production

during antagonistic interaction of fungi. *Analytical Chemistry*, 87(24): 12298–12305.

Tawfike, A.F.; Tate, R.; Abbott, G.; Young, L.; Viegelmann, C.; Schumacher, M.; Diederich, M.; Edrada-Ebel, R. (2017). Metabolomic tools to assess the chemistry and bioactivity of endophytic *Aspergillus* strain. *Chemistry & Biodiversity*, 14(10): e1700040.

Ueda, K.; Beppu, T. (2017). Antibiotics in microbial coculture. *The Journal of Antibiotics*, 70(4): 361–365.

Ujváry, I. (2002). Transforming natural products into natural pesticides-experience and expectations. *Phytoparasitica*, 30(5): 439–442.

Valgas, C.; Souza, S.M. de; Smânia, E.F.A.; Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, 38(2): 369–380.

Vinale, F.; Nicoletti, R.; Borrelli, F.; Mangoni, A.; Parisi, O.A.; Marra, R.; Lombardi, N.; Lacatena, F.; Grauso, L.; Finizio, S.; Lorito, M.; Woo, S.L. (2017). Co-Culture of plant beneficial microbes as source of bioactive metabolites. *Scientific Reports*, 7(1): 1–12.

Visagie, C.M.; Varga, J.; Houbraken, J.; Meijer, M.; KocsubÚ, S.; Yilmaz, N.; Fotedar, R.; Seifert, K. A.; Frisvad, J.C.; Samson, R.A. (2014). Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section *Circumdati*). *Studies in Mycology*, 78: 1–61.

Wang, A.; Islam, M.N.; Johansen, A.; Haapalainen, M.; Latvala, S.; Edelenbos, M. (2019). Pathogenic *Fusarium oxysporum* f. sp. *cepae* growing inside onion bulbs emits volatile organic compounds that correlate with the extent of infection. *Postharvest Biology and Technology*, 152: 19–28.

Wang, J.; Wang, G.; Zhang, Y.; Zheng, B.; Zhang, C.; Wang, L. (2014). Isolation and identification of an endophytic fungus *Pezicula* sp. in *Forsythia viridissima* and its secondary metabolites. *World Journal of Microbiology and Biotechnology*, *30*(10): 2639–2644.

Wang, K.; Liu, N.; Shang, F.; Huang, J.; Yan, B.; Liu, M.; Huang, Y. (2021). Activation of secondary metabolism in red soil-derived streptomycetes via co-culture with mycolic acid-containing bacteria. *Microorganisms*, 9(11): 2187–2202.

Wang, Q.; Yang, X.Q.; Miao, C.P.; Xu, L.H.; Ding, Z.T.; Yang, Y.B.; Zhao, L.X. (2018). A new pair of pentaketide diastereoisomers from *Aspergillus melleus* YIM PHI001. *Records of Natural Products*, 12(3): 216–221.

Wang, W.X.; Kusari, S.; Sezgin, S.; Lamshöft, M.; Kusari, P.; Kayser, O.; Spiteller, M. (2015). Hexacyclopeptides secreted by an endophytic fungus *Fusarium solani* N06 act as crosstalk molecules in *Narcissus tazetta*. *Applied Microbiology and Biotechnology*, 99(18): 7651–7662.

Yadav, M.; Yadav, A.; Yadav, J.P. (2014). *In vitro* antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. *Asian Pacific Journal of Tropical Medicine*, 7(Suppl 1): 256-261.

Yu, M.; Li, Y.; Banakar, S.P.; Liu, L.; Shao, C.; Li, Z.; Wang, C. (2019). New metabolites from the co-culture of marine-derived actinomycete *Streptomyces rochei* MB037 and fungus *Rhinocladiella similis* 35. *Frontiers in Microbiology*, 10(MAY): 1–11.

Yue, Y.; Yu, H.; Li, R.; Xing, R.; Liu, S.; & Li, P. (2015). Exploring the antibacterial and antifungal potential of jellyfish-associated marine fungi by cultivation-dependent approaches. *PLoS ONE*, 10(12): 1–16.

Zhang, H.; Bai, X.; Zhang, M.; Chen, J.; Wang, H. (2018). Bioactive natural products from endophytic microbes. *The Natural Products Journal*, 8(2): 86–108.

Zhang, H.W.; Song, Y.C.; Tan, R.X. (2006). Biology and chemistry of endophytes. *Natural Product Reports*, 23(5): 753–771.

Zhao, J. H.; Zhang, Y. L.; Wang, L. W.; Wang, J. Y.; & Zhang, C. L. (2012). Bioactive secondary metabolites from *Nigrospora* sp. LLGLM003, an endophytic fungus of the medicinal plant *Moringa oleifera* Lam. *World Journal of Microbiology and Biotechnology*, 28(5): 2107–2112.