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Diversity and Biological Activities of Endophytic Fungi at Al-Qassim Region

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Abstract

In recent year's endophytic fungi has become a major concern on their host plants by enhancing their growth, increasing their fitness, strengthening their tolerances to abiotic and biotic stresses, and promoting their accumulation of secondary metabolites. Kingdom of Saudi Arabia has a wide range of flora which may be a rich source of endophytic fungi so that, the present study involves diversity and bioactivity of the endophytic fungal community in Al-Qassim region from 15 wild plants 162 isolates were obtained and identified. Among them, the most common isolates were *Aspergillus niger, Aspergillus terreus*, *Aspergillus ochraceous* and *Trichoderma viride*, these four endophytic isolates were examined for its antagonistic effect against six phytopathogenic fungi using two different assays, Dual-culture and Culture filtrate. *Trichoderma viride* recorded the most significant growth inhibition of almost pathogenic fungi followed by the three endophytic *Aspergillus* spp. In addition, these four endophytic fungi were screened for the production of some extracellular enzymes such as protease, cellulose, amylase, pectinase and xylanase. Our results show the ability of these isolates to produce these extracellular enzymes so this indicated the possible role of endophytic fungi as a biocontrol agent of plant disease.

Keywords: Biocontrol, Endophytic fungi, Extracellular enzymes, Phytopathogenic fungi, Wild plants

1. Introduction

Endophytes are microorganisms including fungi and bacteria that spend the whole of their Lifecycle colonizing in living tissue of different plants typically without causing any conspicuous symptoms of disease (Sandhu et al., 2014). Endophytic fungi are part of the plant microbiome and are ubiquitously found across plant species and ecosystems (Arnold, 2007). It is obvious that the endophytic diversity is differed according to the host identity and its community relies upon geographic status and seasonal changes (Johnston et al., 2012). During the long period of co-existence and evolutionary processes, different relationships have been established between endophytic fungi and their plant host recognized as: (a) a continuum of mutualism, (b) antagonism, and (c) neutralism (Sun et al., 2012). Genetically, nutrient level, and ecological habitats of the host plants are considered as the pressure-choice factors on the population structure of the endophytic fungi that, in turn, confer some kinds of benefits as growth induced, increased resistance to disease, and/or herbivore (Atugala & Deshappriya, 2015).

Endophytic fungi have functional roles in agriculture and food safety, plant growth, crop protection, phytoremediation and ecological balance (Sudha et al., 2016). They act as bio-control agents due to its ability to produce antifungal substances that are capable of inhibiting the growth and spore germination of microbial pathogens so they consider a promising natural resource of future bio-control agents for forestry management (Rodriguez et al., 2009).

Recently, endophytes are seen as a wellspring of novel bioactive metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones offering the potential for medicinal, agrarian and industrial imposition (Gouda et al., 2016). endophytic fungi have gained impetus due to their enormous potential to produce a myriad of medicinally important metabolites. Therefore, exploring endophytic fungi that inhabit plant species with medicinal properties would provide ample opportunities to discover new metabolites with potential bioactivity (Wiyakrutta et al., 2004). The bioactivity of endophytes derivatives involves anti- inflammatory, antimicrobial, antitumor and antiviral (Selim et al., 2012). Moreover,

some endophytic fungal strains has the ability to produce bioactive metabolites as taxol, pestaloside, torreyanic acid and enzymes like Xylanase, Isoflavonoids, Asparaginase (Theantana et al., 2007; Radji et al., 2011).

Kingdom of Saudi Arabia is a large country, talented with a wide range of flora, including trees, shrubs and herbs of hydrocarbon, palatable and has medical effects (Al-Rahmah et al., 2013). The country possesses a unique genetic diversity in the form of ecotypes of tree species and more than 280 other grasses species in various ecological/geographical regions. Only relatively few plants have been examined for the presence of endophytic fungi in Saudi Arabia (Gherbawy & Gashgari, 2014; Gashgari et al., 2016) and no studies carried out in Al-Qassim region in this context, this study aim to isolation of endophytic fungi associated with different plants collected from Al-Qassim region and to test its antagonistic activities against some phytopathogenic fungi and the ability of production of some extracellular enzymes.

2. Material and Methods

2.1. *Plant Materials Collection:*

A total of fifteen healthy and mature wild plants were collected from Al-Qassim region (lies between longitudes 41˚ 30' and 44˚ 45' E, and latitudes 24˚ 25' and 27˚ 15' N), Kingdom of Saudi Arabia during the period from January to March 2019. After plant selection, disease-free parts of the plant, that is, stem, root, and leaves, were excised with a sterile scalpel and placed in sterilized poly ethylene bags, stored at 4˚C directly after collection and transported to the laboratory. The plant samples were classified to different families and listed in the Table 1.

Table 1. List of wild plant collected from Al-Qassim region, Kingdom of Saudi Arabia

Scientific name	Family
Sisymbrium irio	Brassicaceae
Rumex vesicarius	Polygonaceae
Brassica rapa	Brassicaceae
Imperata cylindrica	Poaceae
Launaea mucronata	Asteraceae
Malva parviflora	Malvaceae
Ocimum basilicum	Lamiaceae
Anthemis cotula	Asteraceae
Cleome africana	Cleomaceae
Eremobium aegyptiacum	Brassicaceae
Moltkiopsis ciliata	Boraginaceae
Paronychia desertorum	Caryophyllaceae
Erodium laciniatum	Geraniaceae
Zygophyllum simplex	Zygophyllaceae
Zilla spinosa	Brassicaceae

2.2. *Endophytic Fungi Isolation*

The endophytes were isolated using a modified method described by Dobranic et al. (1995). The plant samples were carefully washed under running tap water to get rid of dust and soil debris. Then, each sample was cut into small fragments under aseptic condition measuring from 0.5 to 1cm using sterile surgical scalpel. The fragments were surface sterilized by sequentially dipping into 70% ethanol for five seconds, 4% sodium hypochlorite solution for 90 seconds, sterile distilled water for 10 seconds. About 5-6 segments after dried under control condition were placed mycological medium, that is, Potato Dextrose Agar (PDA: 300 g/L diced potatoes, 20 g/L dextrose and 20 g/L agar) with tetracycline (10 mg/L) to inhibit the bacterial contaminant then incubated 25 ± 2 ℃ for 21 days. After incubation period the purity of each fungal culture were transferred to PDA slant tubes at (4-5) ℃ and used as stock culture for further experimental studies. The fungi were identified based on morphological and reproductive characteristics according to the methods described by Domsch et al. (1980).

The colonization frequency (CF %) and the percentage of the dominant endophytic fungi percentage were calculated (Petrini & Fisher 1988; Kumar & Hyde, 2004).

$$CF\% = \frac{\text{number of segments colonized by endophyte}}{\text{Total number of segments analysis}} \times 100 \quad (1)$$

2.3. *Antagonistic Activity of Endophytic Fungi in vitro*

2.3.1. Dual-culture Method

The antagonistic effect of the most dominant four endophytic fungi isolated from the selected wild plants against 6 selected phytopathogenic fungi (*Fusarium solani*, *Stemphylium* sp., *Cladosporium* sp., *Aspergillus fumigatus*,

Rhizoctonia solani and *Macrophomina phaseolina*) obtained from The Promising Research Center in Biological Control and Agricultural Information, Qassim University, has been carried out by dual culture technique (Katoch & Pull, 2017).

The Petri plates containing 15–20ml PDA were prepared. Discs of 5 days old pure cultures of isolated endophytes and pathogens (5mm in diameter) were co-cultured at the two opposite ends of the plates and incubated at 25± 2 ℃ after sealing them with parafilm. For control, a disc from each pathogenic fungus was placed at one end of the PDA petri dish alone without endophyte. These treatments were carried out in triplicates. After 7 days of incubation, the diameter of radial growth of the endophytes and the pathogen were measured and recorded in each treatment and the percentage of growth inhibition (GI%) of the phytopathogenic fungi was calculated using this formula (Ghildial & Pandey, 2008).

$$GI\ \% = 1 - \left(\frac{Diameter\ of\ pathogen\ colony\ in\ treatment}{Diameter\ of\ pathogen\ colony\ in\ control}\right) \times 100 \quad (2)$$

2.3.2. Culture filtrate assay

Each endophyte was cultured in 50mL of potato dextrose broth (PDB) in 250mL Erlenmeyer flasks by inoculating two plugs (0.5 cm) of actively growing endophytic fungus and incubated for 10days in rotatory shaker (150 rpm) at 25±2 ℃. The cultures were centrifuged to remove biomass so as to collect the broth containing antagonist metabolites (Dennis & Webster, 1971). Endophytic culture filtrate (200 µL) was spread on PDA plates to test their antagonistic activity. On drying of filtrate, respective pathogenic fungus (0.5cm plug) was inoculated at the center of PDA plate. Simultaneously, PDA plates containing pathogenic fungi without endophytic culture filtrate served as control, and then incubated at 25± 2 ℃. After 7 days, the radial growth of pathogen in the presence/absence of endophytic culture filtrate was monitored and the percentage growth inhibition (GI %) was calculated for respective culture filtrate using the above formula (2).

2.4. *Assay of some extracellular enzymes*

The production and activity of extracellular enzymes by fungal endophytes were assessed by growing them on Yeast-Malt (YM) agar media (YM: 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract, 1.5% agar, pH 6.7) (Molina et al., 2012) and placing 5 mm fungal plugs on the YM agar media supplemented with dissolved and specific indicative substrates. After incubation for 3–5 days depending on the growth rates of fungal endophytes at 28 ℃, the appearance of clear zone surrounding the fungal colony was measured after adding specific reagent and used as indicator for extracellular enzymatic activities. All assays were performed in triplicate.

2.4.1. Proteolytic Activity

The YM agar medium containing 1% gelatine was used to determine the fungal protease enzyme activity. After incubation, the degradation of gelatine was seen as clear zoon around the colonies by using acidic mercuric chloride as an indicator.

2.4.2. Cellulase Activity

The appearance of clear zone around the fungal colony grown on YM medium supplemented with 1% cellulose or carboxymethylcellulose (CMC) was measured, in order to assess the fungal cellulolytic activity after adding iodine solution as indicator.

2.4.3. Amylolytic Activity

Amylase activity was assessed by growing the fungal isolates on YM agar medium supplemented with 1% soluble starch. After incubation, the plates were flooded with 1% iodine. The appearance of clear zones around fungal growth was measured to determine the amylolytic activity.

2.4.4. Pectinolytic Activity

Pectinolytic activity was determined by growing the fungi in1% Pectin YM containing medium. After the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide. A clear zone formed around the fungal colony indicated the activity of pectinase enzyme.

2.4.5. Xylanolytic Activity

Yeast-Malt agar medium supplemented with 1% xylan of corncobs was used in order to measure the fungal xylanolytic activity. After incubation period, screening the xylanase activity was appeared as a clear zone around the fungal colony as a result of using absolute ethyl alcohol to indicate the xylan bio degradation.

2.5. *Statistical Methods*

Data obtained were statistically analyzed using Analysis of Variance (ANOVA) Microsoft EXCEL 2010.

3. Results

3.1. *Isolated Endophytic Fungi*

Fortunately, according to our scientific, this is considering the first report describing the isolation of endophytic fungi residing in wild plants in Al-Qassim region, Kingdom of Saudi Arabia. A total of 162 isolates belonging to 15 species and 10 genera were isolated from 15 wild plants (Table 2). These isolates were identified as follow: *Alternaria* (12 isolates), *Aspergillus* (77 isolates), *Colletotrichum* (2 isolates), *Fusarium* (13 isolates), *Nigrospora* (2 isolates), *Penicillium* (12 isolates), *Rhizopus* (6 isolates), *Curvularia* (6 isolates), *Trichoderma* (20 isolates) and *Trichocladium* (12 isolates).

The most dominant isolates can be arranged as follows: *Aspergillus* spp. represent the highest frequency (47.6%) followed by *Trichoderma* sp.12.3%, *Fusarium* sp. 8.1%; while each of *Alternaria* sp. *Penicillium* sp. *and Trichocladium* sp. is represent by 7.4% however *Rhizopus* sp. *and Curvularia* sp. *is 3.7*% whereas the lowest frequency (1.2%) in *Colletotrichum* sp. *and Nigrospora* sp. The most dominant fungal species were recovered by *Aspergillus niger* and *Trichoderma viride* recording13% and 12.3%, respectively. On the other hand, *Colletotrichum* sp. and *Nigrospora* sp. had the least dominance percentage (1.2%), (Figure.1).

Among the tested organisms, the most prevalent fungi were *Aspergillus niger* and *Trichoderma viride* with colonization frequency 87.5% followed by *Aspergillus terreus* 75%, then *Aspergillus flavus* and *Aspergillus ochraceous* 62.5%, whereas *Colletotrichum* sp. and *Nigrospora* sp. exhibited the lowest colonization frequency percentage 12.5%.

Table 2. Colonization frequency percentage and dominant fungi percentage of endophytic fungi isolated from wild plant

Endophytic fungi	Number of isolates	CF %	Dominant fungi%
1. ***Alternaria alternata***	12	50.0	7.41
2. ***Aspergillus niger***	21	87.5	12.96
3. ***Aspergillus flavus***	15	62.5	9.25
4. ***Aspergillus terreus***	15	75.0	9.25
5. ***Aspergillus oryzae***	3	25.0	1.85
6. ***Aspergillus ochraceous***	18	62.5	11.11
7. ***Aspergillus fumigates***	5	25.0	3.10
8. ***Colletotrichum* sp.**	2	12.5	1.23
9. ***Curvularia* sp.**	6	25.0	3.70
10. ***Fusarium solani***	13	37.5	8.02
11. ***Nigrospora* sp.**	2	12.5	1.23
12. ***Penicillium chrysogenum***	12	50.0	7.40
13. ***Rhizopus* sp.**	6	37.5	3.70
14. ***Trichoderma viride***	20	87.5	12.34
15. ***Trichocladium* sp.**	12	25.0	7.41
Total number of isolates	162		

CF = Colonization Frequency

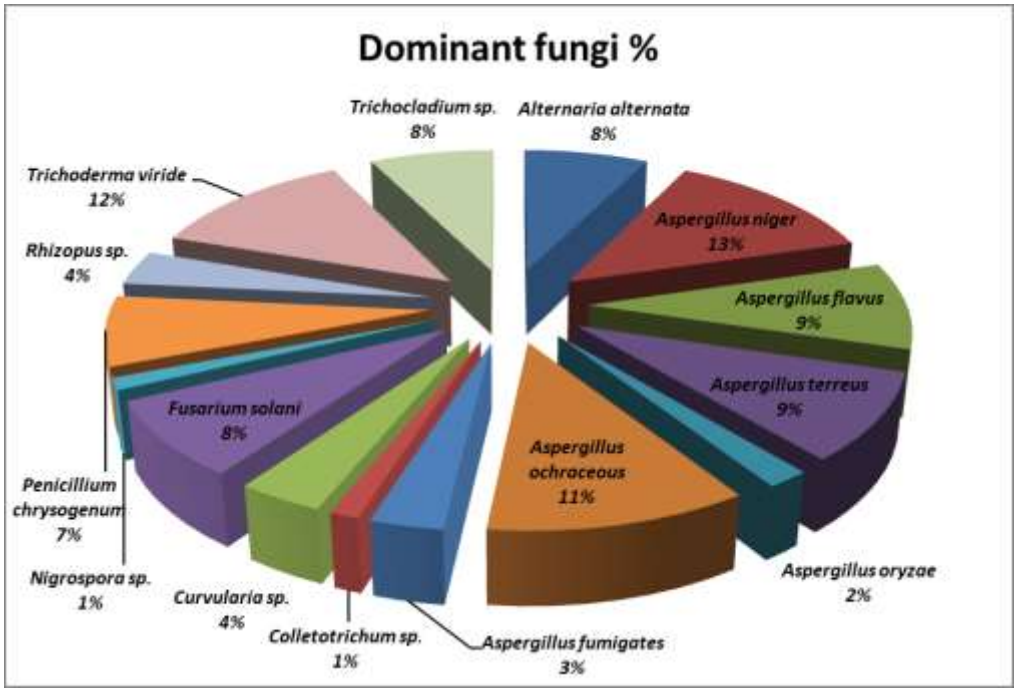

Figure 1. The dominant fungi percentage of the endophytes isolated from wild plants

3.2. *Antagonistic activity of endophytic fungi in vitro*

3.2.1. Dual culture technique

By dual culture assay, the antagonistic action of the four selected endophytic fungal strains (according to their high CF %) *Aspergillus niger, Aspergillus terreus and Aspergillus ochraceous, Trichoderma viride* against six plant pathogenic fungi, namely, *Fusarium solani*, *Stemphylium* sp., *macrophomina phaseolina*, *Cladosporium* sp., *Rhizoctonia solani and Aspergillus fumigatus* was evaluated. The endophyte *Trichoderma viride* showed significant antifungal activity with growth inhibition of more than 70% against *Fusariumsolani*, *Cladosporium* sp. *and Aspergillus fumigatus.* Whereas growth inhibition of *Stemphylium* sp, *Fusarium solani* and *Cladosporium* sp.is higher than 60% in the presence of *Aspergillus ochraceous* however in case of endophyte *Aspergillus niger*, the percentage of growth inhibition reached to 60% against *Cladosporium* sp and *Macrophomina phaseolina*. Also *Aspergillus terreus* has affect above 60% against *Fusarium solani* and *Cladosporium* sp.

On the other hand, *Aspergillus ochraceous* has low antagonistic effect on *macrophomina phaseolina* and *Aspergillus fumigates* (33.3 and 31.7%, respectively) as the same effect of *Aspergillus terreus* against *Rhizoctonia solani* as presented in Table (3) and Figure (2).

Table 3. Antagonistic activity of endophytic fungi against some phytopathogenic fungi *in vitro* by dual Culture assay

	Endophytes			
	Aspergillus niger	*Aspergillus ochraceous*	*Aspergillus terreus*	*Trichoderma viride*
Pathogenic fungi	Percentage of growth inhibition (GI %)			
Fusarium solani	53.8	68.0	64.1	74.3
Stemphylium sp.	49.3	63.7	50.7	62.3
Macrophomina phaseolina	60.0	33.3	44.0	52.0
Cladosporium sp.	62.5	62.5	65.0	77.5
Rhizoctonia solani	42.8	50.0	35.7	57.1
Aspergillus fumigatus	58.8	31.7	48.2	76.5

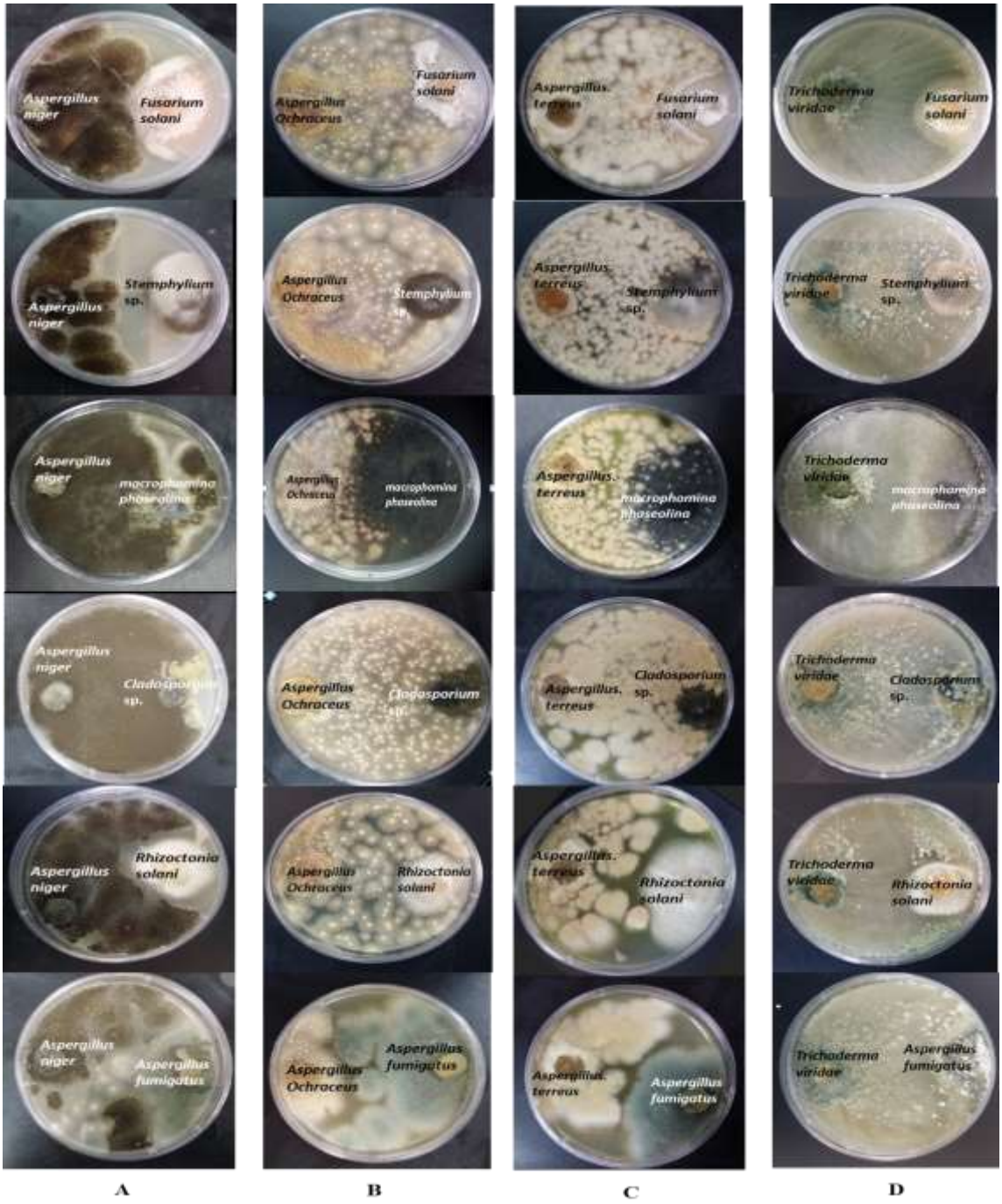

Figure 2. Antagonistic activity of endophytes *Aspergillus niger* (A), *Aspergillus ochraceous* (B), *Aspergillus terreus* (C) and *Trichoderma viride* (D) against some phytopathogenic fungi *in vitro* by dual culture assay

3.2.2. Culture filterate assay

In culture filtrate assay of the four endophytic fungal isolates collected after five days of incubation against the test fungal phytopathogens was evaluated and showed variable growth inhibition according to the used antagonists where, *Trichoderma viride* displayed broad range of antagonistic activity i.e., active against almost all the plant pathogens tested but *Aspergillus niger* recorded the least antagonistic efficiency in some cases as shown in Table (4).

Table 4. Antagonistic activity of endophytic fungi against some phytopathogenic fungi *in vitro* by Culture filtrate assay

	Endophytes			
	Aspergillus niger	*Aspergillus ochraceous*	*Aspergillus terreus*	*Trichoderma viride*
Pathogenic fungi	Percentage of growth inhibition (GI %)			
Fusarium solani	52.9	70.5	64.7	74.1
Stemphylium sp.	46.6	68.0	49.3	65.3
Macrophomina phaseolina	59.5	41.7	43.0	62.0
Cladosporium sp.	56.2	70.0	61.2	75.0
Rhizoctonia solani	42.6	52.0	33.3	54.6
Aspergillus fumigatus	55.3	29.4	47.1	70.6

3.3. *Extracellular enzyme activity of endophytes*

The date in Figure (3) shows variations in the production of extracellular enzymes by the endophytic fungal isolates. *Aspergillus niger* showed high performance in enzymes production where, it achieved the maximum protease, amylase and xylanse production. While *Trichoderma viride* and *Aspergillus ochraceous* exhibited the maximum production of cellulases and pectinases production, respectively. All isolates achieved high cellulase activity except in case of *Aspergillus ochraceous* but this isolate recorded the maximum production of pectinases. Amylase and pectinase were produced moderately by *Aspergillus terreus* which displayed the minimum Pectinolytic activity. Endophytic *Trichoderma viride s*hows intermediate enzymes production in all cases but its cellulolytic activity is distinguished in comparison to the other isolates.

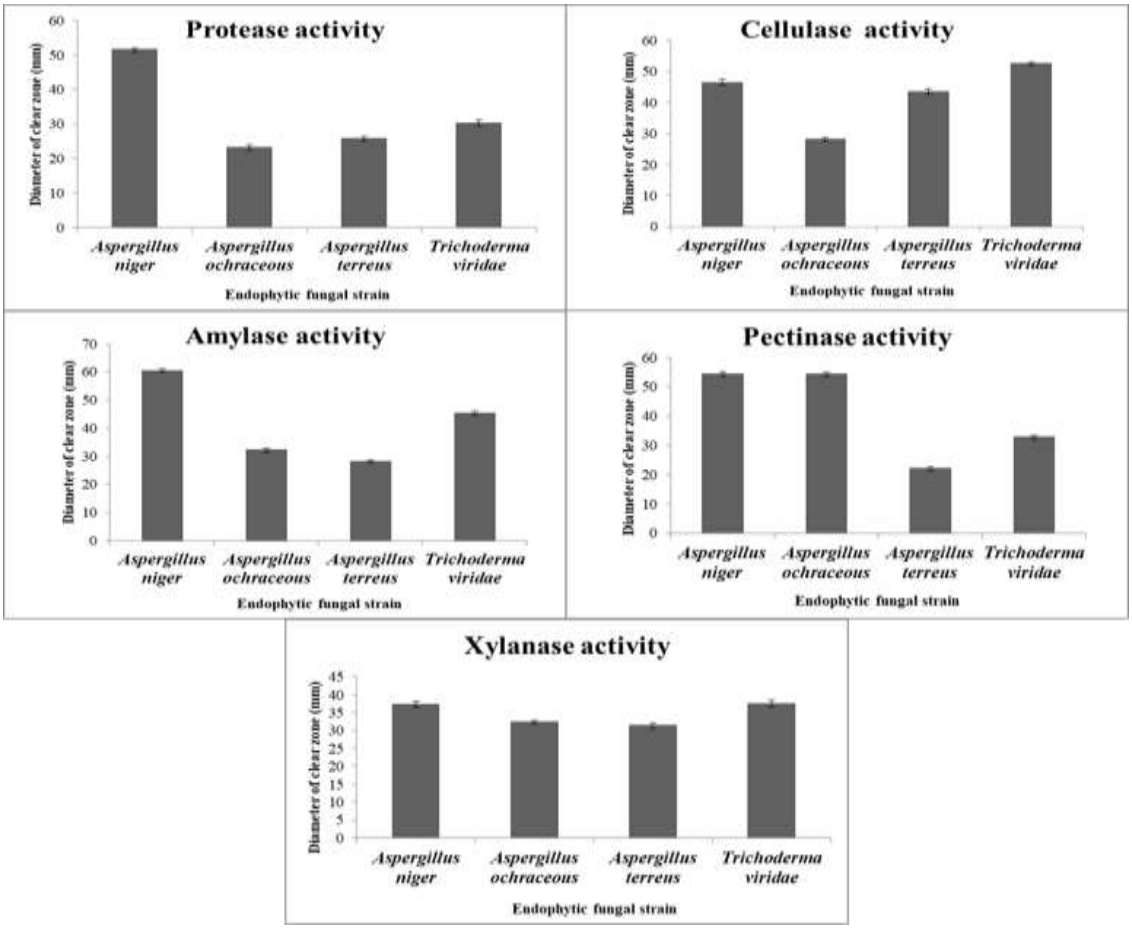

Figure 3. The extracellular enzymes activities of isolated endophytic fungi.

4. Discussion

Today, more and more studies have focused on the endophytic fungi extracted from various plants nevertheless a large part of this natural wealth remains unexplored (Porras & Bayman, 2011). Ecosystem plays an important role in the process of endophytism or vice versa through host disease resistance (Fouda et al., 2015), adaptation to unique niches, plant secondary metabolism (Singh et al., 2011). In this study, 162 endophytic fungal strains belonging to 15 species and 10 genera were isolated from different parts of 15 wild plants collected from Al-Qassim region, Kingdom of Saudi Arabia and identified using their morphological and reproductive characteristics.

Endophytes can play a major role in plants response to abiotic stresses (salinity); however, unfavorable environmental factors can affect their diversity and colonization density (Lata et al., 2018). Colonization frequency data shows that different endophytic fungi inhabited different plant tissues to different extent. This difference in inhabiting potential of the endophytic fungi shows that, each endophyte exhibited different degrees of affinity towards different tissues of the plant (Katoch et al., 2017). A possible explanation for the relatively low overall fungal diversity noted in the present study could be due to the high drought and the desert nature of Saudi Arabia.

According to classical mycology, most species of endophytic fungi have been described based on their morphological features (Domsch et al., 1980). However, the use of morphological features was problematic for phylogenetic systematics of hypogeous ascomycetes due to a small set of morphological characteristics and homoplasy (Phongpaichit et al., 2007). In this study, most of fungal isolates belong to Ascomycota, however, zygomycota represented only by one genus (*Rhizopus*) confirming the previous reports of Kembel & Mueller (2014) that endophytic fungi are mainly ascomycetes.

Our results were similar to EL-Nagerabi et al. (2013) who found that the great biodiversity in *Aspergillus* species isolated as endophytes from *Ziziphus* plant grown in arid region. Also, *Aspergillus niger* was the dominant endophytic fungus in *Canavalia cathartica* (Dorothy & Kandikere, 2009). Several endophytic *Trichoderma* fungal strains were isolated from various plants belong to family Cuppressaceae (Hosseyni-Moghaddam & Soltani, 2013) and Some species of *Alternaria, Colletotrichum, Fusarium, Nigrospora,Penicillium, Rhizopus, Curvularia and Trichocladium* have been reported as endophytes for many plants (El-Morsy, 2000; Fouda et al., 2015; Prathyusha et al., 2015; Gashgari et al., 2016). Some of the isolated fungal strains in the current study for example, *Alternaria. alternata*, different *Aspergillus species* and *Fusarium solani* are well-known plant pathogens. The fungal activity as an endophyte in one plant or as a pathogen in another one relied on the balance between the pathogenicity and endophytism of the microorganism in different hosts (Gashgari et al., 2016).

The biological control which depends on the use of antagonistic organisms that broadly/specifically target the pathogen has become highly pivotal field, and in many cases, complementing or even replacing the chemical control which has several negative effects. Antagonistic fungi play an important role in biological control (Pandya & Saraf, 2010). Endophytes can act as potential biological control agents through their ability to reduce disease by various mechanisms as production of volatiles and nonvolatile metabolites (Ting et al., 2010) or stimulation of host defense (Benhamou et al., 2000; Walters et al., 2005 and El-Hasan et al., 2009).

Endophytic fungal spp. produces different types of proteins some of them act on the extraction of nutrients which important for the fungal growth and other type of proteins have an inhibitory effect on plant pathogens activities acting as pathogenicity related proteins(PR) which causes distortion of cell walls of the pathogenic fungi (Li et al., 2004).

In this study we recognize the role of endophytes in biocontrol, their antagonistic behavior against major phytopathogens, the most dominant four fungal endophytes isolated from wild plants were tested *in vitro* for their ability to control the growth of the most common and economically important fungal pathogens in Al-Qassim region by dual-culture assay and culture filtrate assay. The results demonstrated that an endophytic fungal strain, *Trichoderma viride* had a great negatively impact on the growth of the phytopathogenic fungi to the extent that the growth of some pathogen was restricted to its inoculation site and this may be attributed to competition process and production of diffusible antibiotics, these data is the same indicated by Whipps (2001) and Talapatra et al. (2017). Also confirmed our data by Benitez et al. (2004) who reported that the genus *Trichoderma* spp. have been investigated as the most studied and broadly used economically efficient biological control agent against different phytopathogens to reduce the disease incidence. *Aspergillus* spp. isolates were very effective against the tested phytopathogens but not recommended as a biological control agents because of their carcinogenic properties.

Recently through many researches, it is proved that Endophytes have the ability to produce numerous extracellular enzymes. The ability of endophytes to produce extracellular enzymes may help them to degrade polysaccharides and proteins in order to become available during the plant senesce as suggested by Toghueo et al. (2017). Hydrolytic extracellular enzymes as cellulase and pectinase are play the key role in the extraction of its nutritional requirements from hosts and bio-resistance action against microbial pathogenic infection through its destructive action on fungal cell wall (Fouda et al., 2015). Moreover, Mandyam and Jumpponen (2005) suggested the important role of endophytic fungi in improvement of host plant growth through its production of various extracellular enzymes that can access and utilize sources of C, N and P in soil.

Our current results indicated that the four selected endophytic fungi have the ability to produce some extracellular enzymes that concerning with gelatine, cellulose, starch, pectin and xylan degradation. Among the isolates, *Aspergillus niger* and *Trichoderma viride* were superior in protease, cellulase, amylase and xylanase production while, regarding to pectinase production *Aspergillus ochraceous* was eminent. It has been proved that 72% of isolated endophytes were an efficient amylase and protease producers (Amirita et al., 2012). *Trichoderma* species are well known producers of degradative enzymes like chitinases (Di Pietro et al., 1993) and cellulases (Sternberg & Doval, 1980) and are currently exploited industrially for enzyme production, bio-control of plant diseases (Harman et al., 2004) and plant growth promotion (Samuels, 1996).

Finally, we can say, Al-Qassim region, Kingdom of Saudi Arabia has various wild plants which harbored numerous endophytic fungal spp. that may play different roles in biological control of plant pathogens and production of some important industrial enzymes and further studies are required to include a great number of plants in this region.

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