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Isolation and Biological Activity of Endophytic Fungi isolated from Arak Medicinal Plant (*Salvadora persica*)

Fatma Radi[1], Fatma Abdel-Motaal[2]*, Magdi El-sayed[3]
Botany Department, Faculty of Science, Aswan University, Aswan 81528, Egypt.
1. fatmaradi91@yahoo.com 2. fatma.fahry@aswu.edu.eg 3. magdiel_sayed@aswu.edu.eg

Abstract
Endophytic fungi have recently been proven to possess active secondary metabolites, especially those colonizing medicinal plants like *Salvadora persica*. Eight endophytic fungi were isolated from *S. persica*, (*Gliocladium catenulatum, Paecilomyces variotii , Penicillium bravicompatum , P. jensenii, Aspergillus fumigatus, A. parasiticus , A. niger* and *Verticillium albo-atrum*). Six of these endophytes showed antagonistic activities against important plant pathogenic fungi, *Fusarium verticilloides* (5.91-18.23%), *Alternaria alternata* (10.5- 31.09 %) and *Cochliobolus spicifer* (16.94-28.93%) inhibition percentage in dual culture assay. The antimicrobial activity crude extracts of ethyle acetate and n-butanol of both *G. catenulatum* and *P. variotii* with three different concentrations (0.5 mg/ml,1.0 mg/ml, 2.0 mg/ml) were tested against the pathogenic fungi. Generally, ethyl acetate extract of both endophytic fungi had an inhibition effect on the pathogen growth higher than n-butanol extract. Thus, this is the first report to evaluate the antifungal effect of *S. persica* associated fungi as a biological control against fungal plant pathogen.

Key words: *Salvadora persica*, Endophytes, Fungi, Secondary metabolites, Antifungal

Introduction

Many researches have proven the importance of endophytic fungi because of their secondary metabolism which have an important role in pharmacy, food industries, environmentally friendly pesticides and defense of the host against pathogenic fungi.
Salvadora persica plant is abundant in Eastern Egypt, Western Saudi Arabia, Iran and most African countries. It also called the tooth brush plant belongs to the family *Salvadoraceae* (Verma, et al 2009)[1]. Morphologically, it is a small and multi-branched evergreen shrub (Khatak., et al 2010)[2]. This medicinal plant was recorded as a potential source of alkaloids, chlorides, vitamins, silica, tannins, sulfur and mustard oil (Halawany, 2012 [3]; Niazi et al., 2016 [4]). Therefore, it has anti-bacterial, anti-fungal, anti-inflammatory and diuretic activities (Khatak, et al., 2010 [2]).
Fungi have been known as the major source of active compounds used in medicine. Healthy plants are interesting host for enophytic fungi that colonize tissues without causing any immediate symptoms of diseases (Fisher and Petrini, 1992 [5]). Endophytic fungi can be found in almost all

Corresponding author E-mail: fatma.fakhry@aswu.edu.eg
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terrestrial plants (Petrini, 1991 [6]; Saikkonen et al.1998 [7]) which help their hosts in improved drought tolerance (Hubbard et al., 2012 [8]) and protect them against pathogens (Arnold et al., 2003 [9]). Some species of endophytes have been identified as sources of anticancer, antidiabetic, antimicrobial, insecticidal and immunosuppressive compounds (Strobel and Daisy, 2003 [10]; Strobel et al., 1999 [11]). The most common genera, namely *Aspergillus*, *Penicillium* and *Fusarium* besides several other filamentous species for example (*Trichoderma, phoma, Altenaria, Acremonium,* and *Stachybotrys*), which produce several hundreds of bioactive compounds (Schulz et al., 2002 [12]; Strobel, 2003 [13]; Corrado and Rodrigues, 2004 [14]; Owen and Hundley, 2004 [15]; Gimenez et al., 2007 [16]). It is believed that obtaining and analyzing antimicrobial compounds from endophytic fungi is an appropriate way to combat drug-resistant pathogenic microbes in humans, animals and plants. Some endophytic fungi were isolated from *S. persica* such as *Trichoderma* sp., *Alternaria* sp, sterile mycelia and *Rhizopus arrhizus* (Elgorban*,* et al*.,* 2019 [17]).

In the current study, we focus on the isolation of endophytic fungi from the medicinal plant *S. persica*, then, screening the antifungal activity of secondary metabolites produced by selected isolated endophytic fungi against some pathogenic fungi.

Materials and methods

Plant material

Salvadora persica plant shoots were collected from the campus of Aswan University, Aswan Governorate, Egypt. All plant parts were carefully washed under running tap water and then dried by tissue.

Isolation and identification of endophytic fungi associated with *Salvadora persica*

Plant samples were surface sterilized by immersion in 70% ethanol for 1 minute, then 5% sodium hypochlorite for 5 minutes and washed in sterilized distilled water two times. Sterilized stems and leaves were cut into small pieces (1.5 cm) and placed on the surface of a sterile potato dextrose agar plate (PDA) (Rossman 1998 [18]. Three replica of each sample were incubated at 28 C˚±2. The growing fungi were identified on the basis of their morphological characteristics according to Moubasher (1993)[19].

Dual-culture experiment

The antagonistic activity of fungal endophytes against some phytopathogenic fungi; *Fusarium verticilloid* , *Cochliobolus spicifer* and *Alternaria alternata* was studied using the dual culture method (Kamel et al., 2019)[20]. Three replica have been done and the inhibition percentage of the pathogen by the endophytic fungi was calculated according to the following formula

% Inhibition =Dc – Ds/Dc× 100

Where Dc is the average increase in mycelial growth in control, and Ds is the Average increase in mycelial growth in treatment (Singh and Tripathi 1999)[21].

Secondary metabolites extractions from selected endophytes

Selected endophytic fungi were growing on PDA for 10 days, then inoculum from this culture was inoculated into 1000 ml conical flask containing 300 ml of culture media and incubated under shaking condition for 12 days. Fungal culture was filtrated and ethyl acetate was added to the filtrate. Ethyle acetate fraction was separated from media and then n-butanol was added to the

filtrate. The ethyl acetate and n-butanol fractions were evaporated using a rotary evaporator then dried and kept in fridge until further use.

Antifungal activity of some endophytic fungi:

Ethyl acetate and n-butanol extracts of the selected endophytic fungi were incorporated into potato dextrose agar at different concentrations (2.0 mg/ml, 1.0 mg/ml, and 0.5 mg/ml) and mixed well. Pathogenic fungal mycelial disc (0.5 mm) was deposited in the center of the plate (6.0 cm in diameter) (Balouiri et al., 2016)[22]. The diameter growth of the pathogenic fungi was measured for the control (fungal disc in plate without any extraction) and treatment plates, as well as the percentage of inhibition was calculated for the tested fungal strains, after incubation for 7 days at 28 C˚±2 (Singh and Tripathi 1999)[21].

Results and discussion

Eight fungal species were isolated and identified from two main parts of the *S. persica* plant (stem and leaves) on potato-dextrose agar (PDA) at 28 C˚±2. The isolated enophytic fungi of stem and root were named as *Aspergillus fumigatus, A. parasiticus, A. niger, Gliocladium catenulatum, Paecilomyces variotii, Penicillium bravicompatum, P. jensenii* and *Verticillium albo-atrum*. Elgorban et al*.,* 2019 [17] isolated ten fungi that include *Trichoderma* sp., two species of *Alternaria*, *Rhizopus arrhizus* and six sterile mycelia from root, stem and leaves of *S. persica*. In this study, the most common fungal genus was *Aspergillus*, which is found in both stem and leaves of the studied plant by the percentage of 29%, which is in agreement with the result obtained by Korejo et al., 2014 [23]. On the other hand, *Salvadora oleoides* endophytic fungi were identified as 10 *Aspergillus spp*., *C. herbarum, E. nigrum, F. moniliforme, P. chrysogenum, Phoma sp.* and *Pythium spinos* (Dhankhar & Parkash 2013[24]. *Gliocladium* sp and *G. catenulatum* were isolated from cacao branches (Rubini et al., 2005)[25]. In this study, *Aspergillus* was was represented by 33% in leaves tissue, while it was found in stems by 25%, represented by three species. *Gliocladium catenulatum* was isolated only from stem, while *V. Albo-atrum* and *P. variotii* were colonized leaves only. *Aspergillus niger* was the only fungal species which isolated from both leaves (3 colonies), and stem (2 colonies) while *A. fumigatus* (1colony), *V. albo-atrum* (1colony), *P. variotii* (2 colonies) had specific isolation from leaves, but *P. bravicompatum* (1 colony), *G. catenulatum* (1 colony), *A. parasiticus* (1colony) *P. jensenii* (1colony) colonized only stem. Interestingly, seven fungal species were isolated for the first time, *P. bravicompatum* (1 colony), *A. fumigatus* (1colony), *V. albo-atrum* (1colony), *P. variotii* (2 colonies), *G. catenulatum* (1 colony), *A. parasiticus* (1colony) *and P. jensenii* (1colony) from leaves and stem of healthy *S. persica* plant.

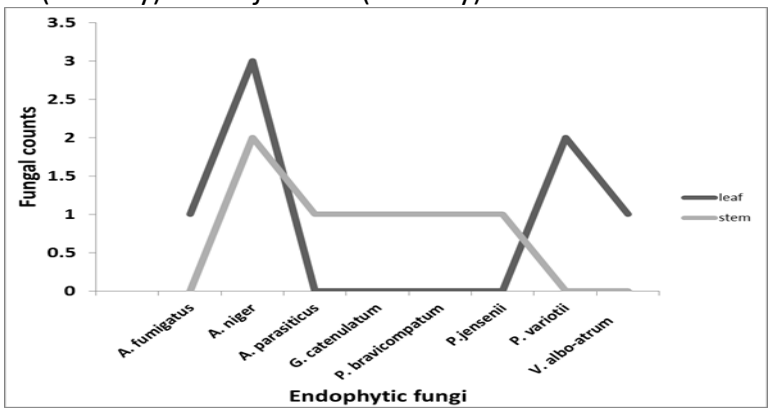

Fig. 1 Counts of endophytes isolated from stem and leaf of the medicinal plant *S. persica*

Antagonistic activity of the isolated endophytic fungi against some selected pathogenic fungi

All endophytic fungi isolated from the medicinal plant *S. persica* showed antagonistic activity against all the studied pathogenic fungi. The pathogen, *A. alternata* (*Capsicum annuum* leaf spot pathogen) showed the highest sensitivity to arak endophytic fungi where it was inhibited by *A. niger* (31.09%), *V. albo-atrum* (28.57%), *A. fumigatus* (23.12.0%), *G. catenulatum* (22.24 %), *P. brevicompactum* (16.39%), and decreased to 10.50% with *P. variotii* (Fig. 2).

Fusarium verticilloides (*Zea mays* root pathogen) showed the lowest sensitivity effect to *S. persica* endophytes whereas, the inhibition percentage of this pathogen recorded only 5.911 % with both *P. variotii* and *V. albo-atrum* (Fig. 3), 6.90% by *P. brevicompactum* while the inhibition was increased slightly to 10.84% with *A. fumigatus*, 12.32% with *A. niger* and 18.23% by *G. catenulatum* (Fig. 3).

The sensitivity of the third pathogen *C. spicifer* (*Vicia faba* leaf spot pathogen) was moderate comparing with the first two pathogens. This inhibition was ranged between 16.94% - 28.63% whereas *G. catenulatum* recorded the lowest and *A. fumigatus* the highest (Fig. 4). The inhibition activity of *P. variotii* against the pathogen was 20.16% while 24.0% with *A. niger* and 23.79% with *P. brevicompactum*.

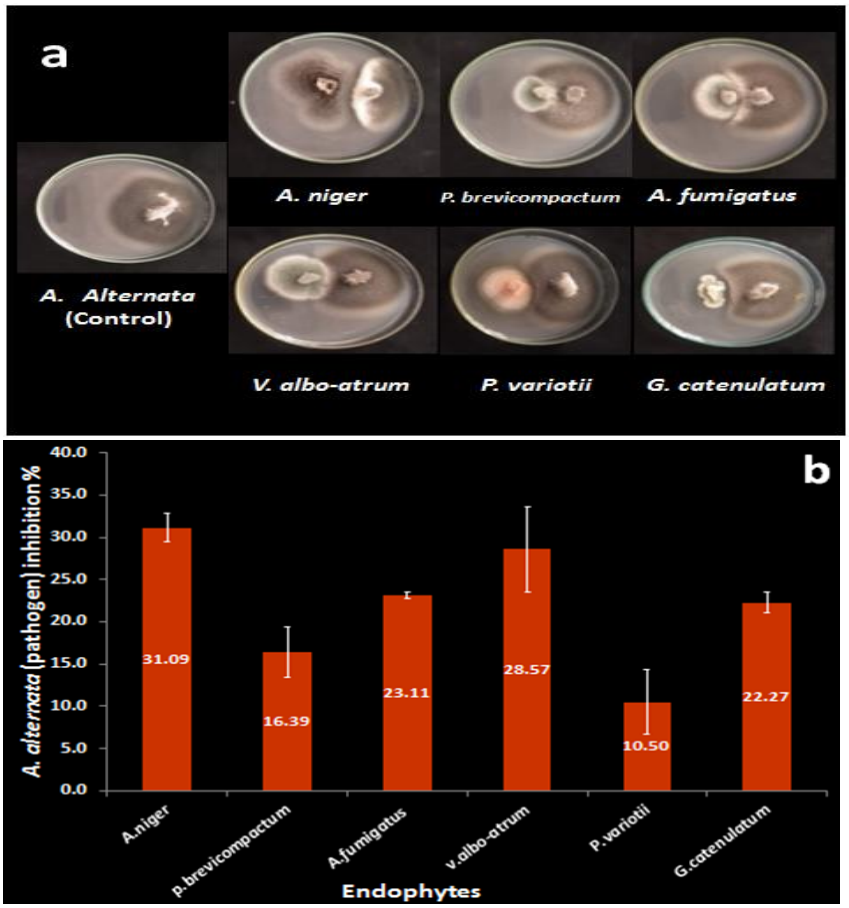

Fig. 2. Antagonistic activity of *Salvadora persica* endophytic fungi against the plant pathogen *Alternaria alternata* (a), pathogen inhibition percentage (b).

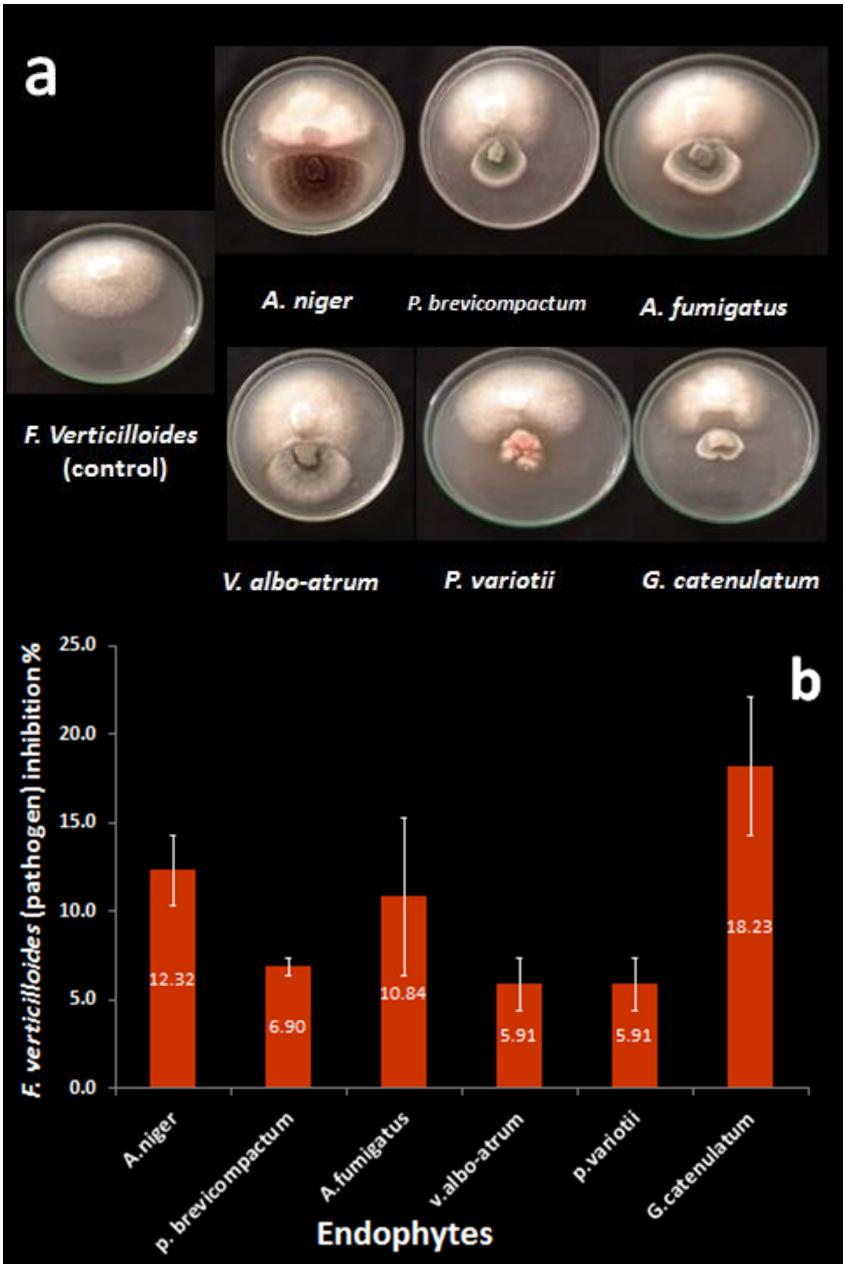

Fig. 3. Antagonism activity of *Salvadora persica* endophytic fungi against the plant pathogen *Fusarium verticilloides* (a), pathogen inhibition percentage (b).

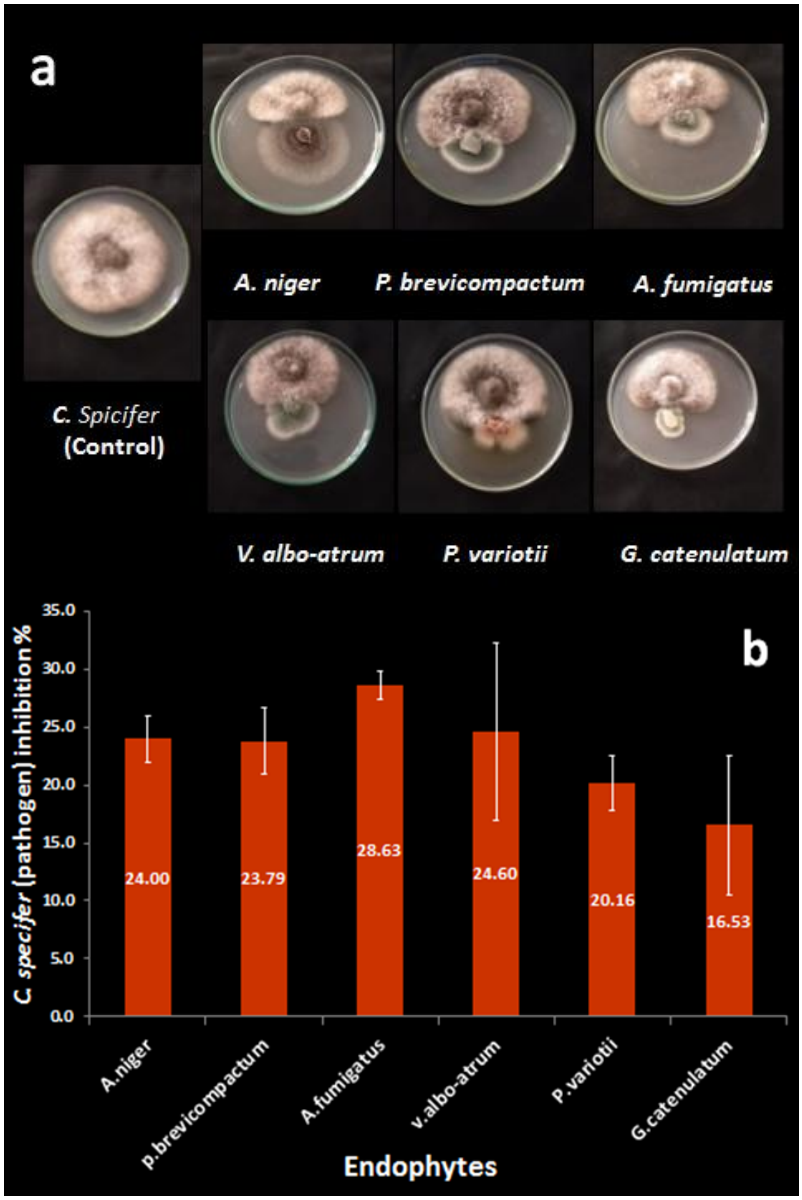

Fig. 4. Antagonism activity of *Salvadora persica* endophytic fungi against the plant pathogen *Cochilobolus spicifer* (a), pathogen inhibition percentage (b).

Despite the variability of the endophytes activities against the studied pathogens, the fact that all the endophytic fungi isolated from the tissues of the stems and leaves of *Salvadora persica* plant had a clear effect against the growth of the studied pathogenic fungi.

Antifungal activity of selected endophytic fungal extracts against three pathogenic fungi

This study clarified and indicated the anti-fungal effects by using two extracts of EtOAc and n-Butanol extract for two selected endophytic fungi, *G. catenulatum* and *P. variotii* using different concentrations (0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml) against three types of plant pathogenic fungi *F. verticilloid*, *C. spicifer* and *A. alternata*. The percentage of growth inhibition of the plant pathogenic fungi was determined by measuring the diameter of inhibition zones (Balouiri et al., 2016)[22]. This result is consistent with the aforementioned antagonistic action. The antifungal

activities of the three different concentrations of the selected endophytic fungal extracts (Ethyl acetate and n-butanol) had an active effect on the growth of the important pathogenic fungi (Figs. 5-8). Interestingly, 0.5 mg/ml of both extractions had the same inhibition effect on all pathogenic fungi 56% by ethylacetate and 45% by n-butanol extract of the endophytic *G. catenilatum* (Fig. 5a,b), while 34% and 30% by ethylacetate and n-butanol extract of *P. variotii* (Fig. 7 a,b) respectively.

In case of *G. catenulatum* , ethyl acetate extract showed more inhibition percentage than n-butanol which had a strong anti-fungal effect at a concentration of 2.0 mg/ml, minimum inhibition concentration ($MIC_{\geq 70}$) of ethyl acetate while (MIC_{63}) in the case of n-butanol extract against *A. alternata* and *F. verticilloides*. The microscopic examination of the pathogens treated with both endophytes extractions either ethylacetate or n-butanol showed swollen in hyphae in the case of *C. spicifer* and formation of chlamydospores with *F. verticilloides* which was controlled in comparison with the control (untreated pathogen culture). In addition, reduction in melanin pigmentation of *C. spicifer* and *A. alternata* hypha at 2.0 mg/ml and 1.0 mg/ml was shown as an effect of both endophytes extracts (Figs. 6 & 8). According to Mérillon and Ramawat 2017[26], the melanin of pathogen is a supportive implant in penetrating the host and blocking its reaction. As a result of reducing melanin formation, the pathogenic fungus becomes more weak and manageable.

Finally, these results indicated that both ethyl acetate and n-butanol extracts of *S. persica* endophytes; *G. catenulatum* and *P. variotii* have the ability to limit the growth of plant pathogenic fungi, but with different inhibitory values. This may be due to the presence of high quality active compounds in the raw extracts of these endophytic fungi (unpublished data). Rubini et al. 2005[25] reported that endophytic *G. catenulatum* possess the ability to reduce the incidence of witch's broom disease by 70% in the cocoa plant. Also, *Aspergillus fumigatus*, an endophytic fungus showed antifungal activity against *Candida albicans* (Liu et al., 2004)[27]. *Colletotrichum gloeosporioides*, an endophytic fungi associated with *Artemisia mongolica* showed antimicrobial activity against bacteria and fungi (Zou et al., 2000) [28]. Fungi are one of the main structures that were used in the development of medical drugs, so the endophytic fungi were highlighted, their vital activity was known, and natural products were examined (Tejesvi, et al., 2007) [29]. Fungal endophytes could limit tree pathogens damage [30]. Since Miswak could control various aspects of oral health [3], its associated endophytic fungi are able to limit plant pathogens.

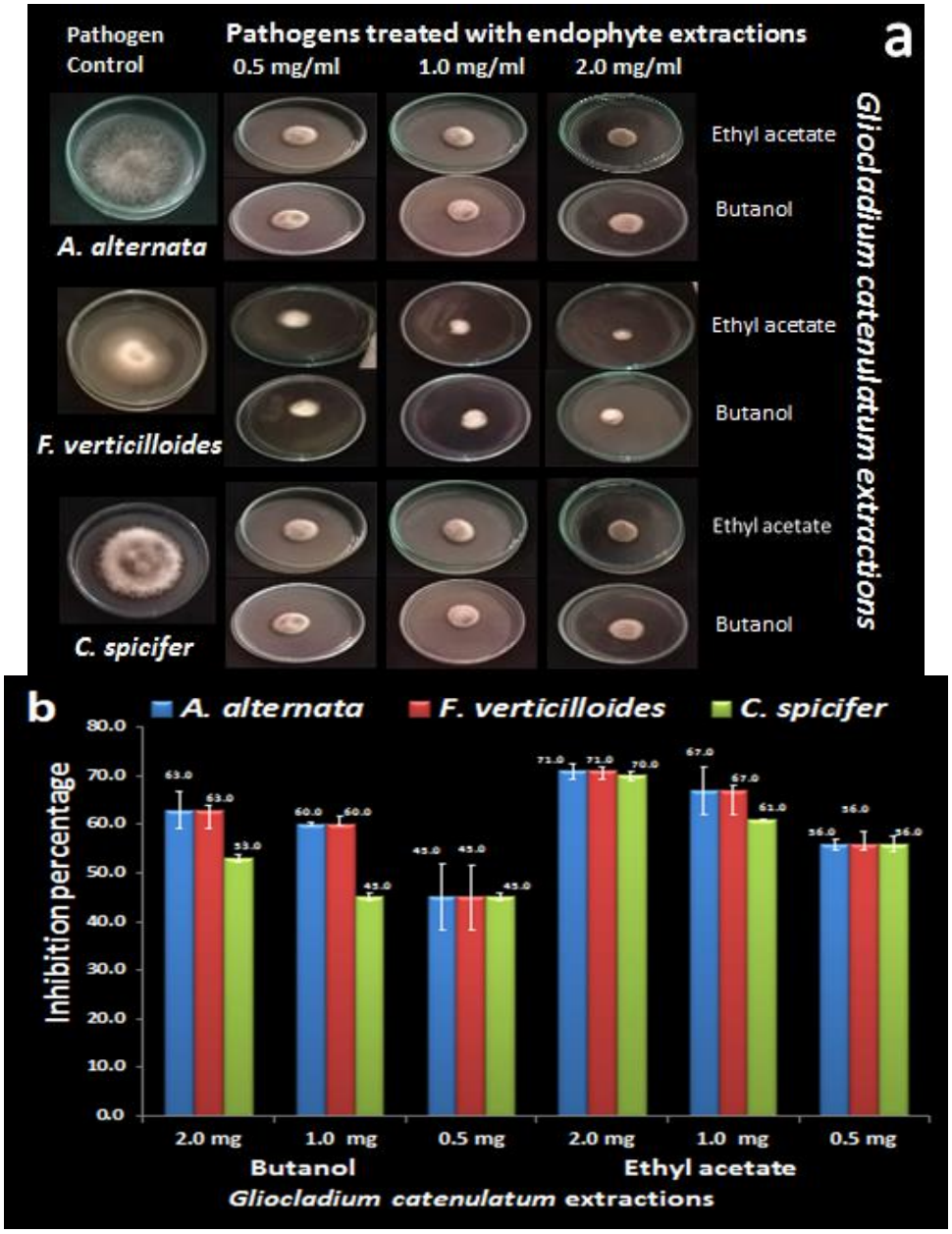

Fig. 5 Antifungal inhibition effect by both ethyl acetate and butanol extracts of *Gliocladium catenulatum* on plant pathogenic fungi (a), inhibition percentage (b)

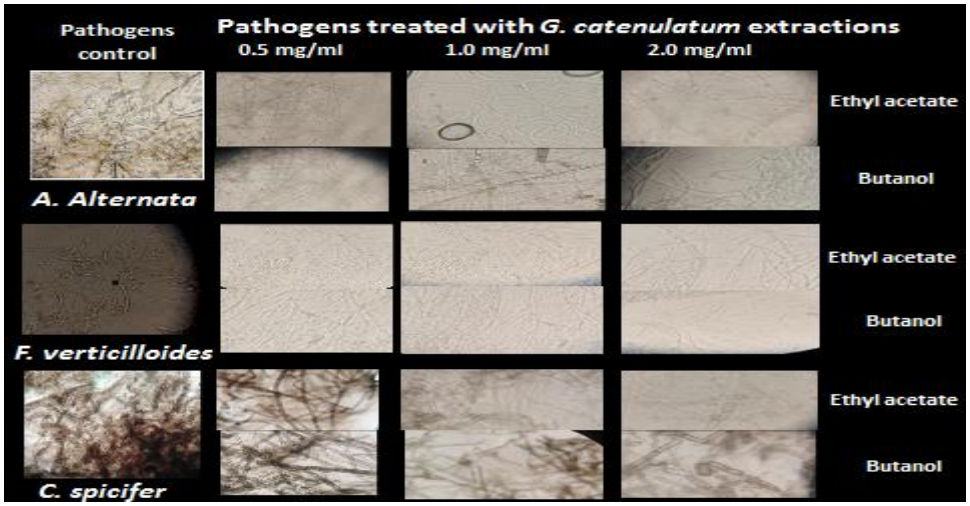

Fig. 6 Effect of different concentrations of *Gliocladium catenulatum* ethylacetate and n-butanol extractions on the morphology of plant pathogenic fungi conidia and mycelia by microscope examination.

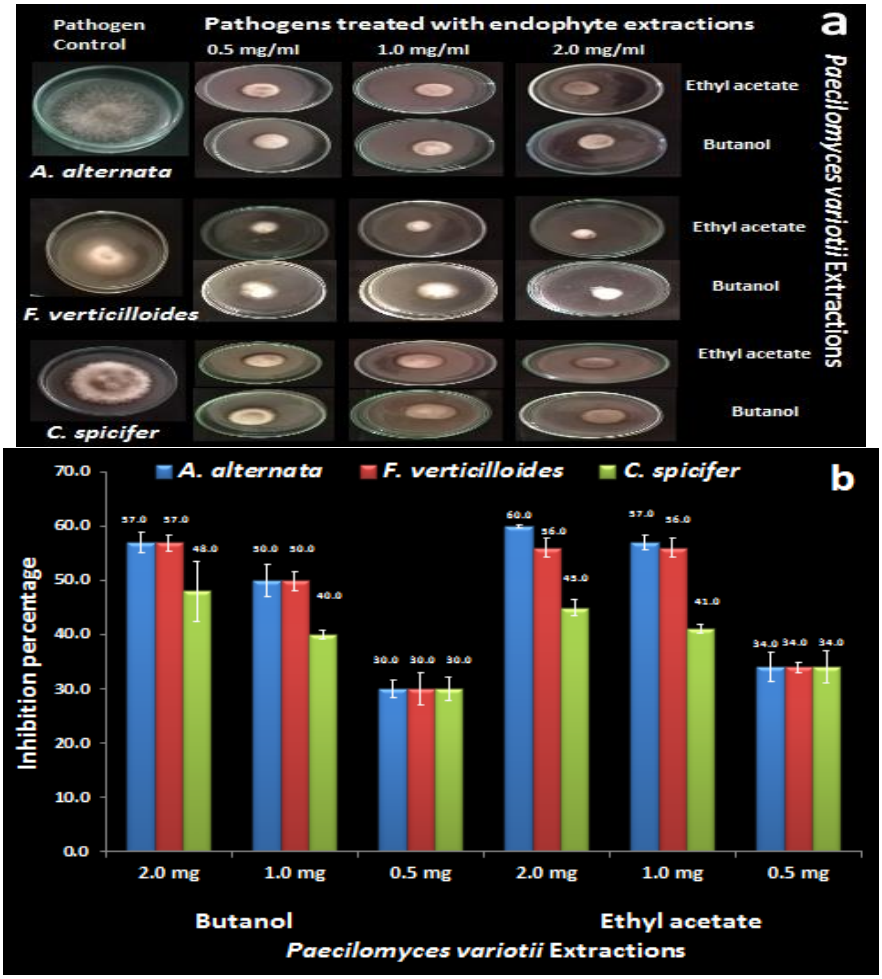

Fig. 7 Antifungal inhibition effect by both ethyl acetate and butanol extracts of *Paecilomyces variotii* on plant pathogenic fungi (a), inhibition percentage showed in (b)

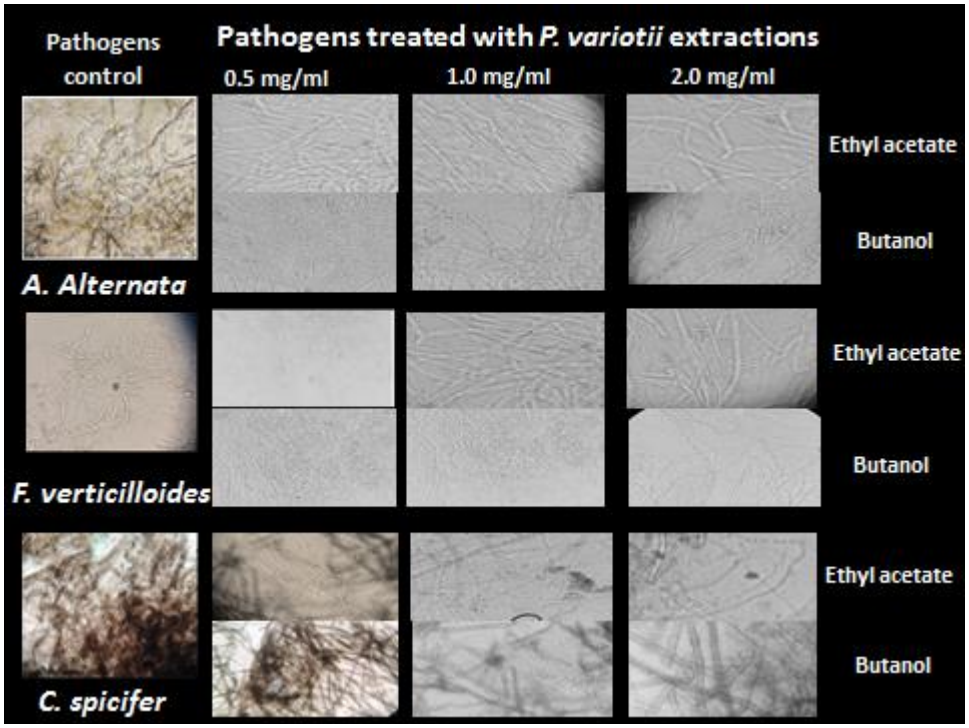

Fig. 8 Effect of different concentrations of *Paecilomyces variotii* ethylacetate and n-butanol extracts on the morphology of plant pathogenic fungi conidia and mycelia by microscope examination.

Conclusion

This study is dealt with new knowledge on some endophytic fungi associated with the medicinal plant *S. persica* and examining their potential biological activity as antifungal. The endophytic fungi associated with *S. persica* medicinal plant is considered promising to be used in the control of plant diseases caused by fungi.

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