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SHORT COMMUNICATION

In vitro Antagonistic Activity of Endophytic Fungi Isolated from Shirazi Thyme (*Zataria multiflora* Boiss.) against *Monosporascus cannonballus*

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Abstract

Endophytic fungi viz., *Nigrospora sphaerica* (E1 and E6), *Subramaniula cristata* (E7), and *Polycephalomyces sinensis* (E8 and E10) were isolated from the medicinal plant, Shirazi thyme (*Zataria multiflora*). In *in vitro* tests, these endophytes inhibited the mycelial growth of *Monosporascus cannonballus*, a plant pathogenic fungus. Morphological abnormalities in the hyphae of *M. cannonballus* at the edge of the inhibition zone in dual cultures with *N. sphaerica* were observed. The culture filtrates of these endophytes caused leakage of electrolytes from the mycelium of *M. cannonballus*. To our knowledge, this is the first report on the isolation and characterization of fungal endophytes from *Z. multiflora* as well as their antifungal effect on *M. cannonballus*.

Key words: *Zataria multiflora*, antifungal, endophytic fungi, *Monosporascus cannonballus*

The term "Endophytes" denotes microorganisms that colonize plants' internal tissues for part of or throughout their life cycle without producing any apparent adverse effect. The endophytic microorganisms include fungi, bacteria, and actinobacteria (Bacon and White 2000). Among them, fungi are the most common endophytic microorganisms (Staniek et al. 2008). Endophytic fungi are ecologically distinct polyphyletic groups of microorganisms, mostly belonging to the Ascomycota phylum (Jia et al. 2016). Several fungal endophytes have been shown to act as biological control agents for managing soil-borne plant pathogens (Toghueo et al. 2016).

Zataria multiflora Boiss. (Synonyms: *Zataria bracteata* Boiss.; *Zataria multiflora* var. *elatior* Boiss), belonging to the Lamiaceae family is a traditional medicinal plant commonly used as a flavor ingredient in different types of foods (Sajed et al. 2013). Several medicinal properties of *Z. multiflora*, including antiseptic, anesthetic, antispasmodic, antioxidant, antibacterial, and immunomodulatory activities, have been documented (Sajed et al. 2013). However, studies on the endophytic microorganisms inhabiting *Z. multiflora* are limited (Mohammadi et al. 2016).

Monosporascus cannonballus Pollack & Uecker (Ascomycota, Sordariomycetes, Diatrypaceae) is one of the most important phytopathogenic fungi causing root rot and vine decline disease in muskmelon. It causes sudden wilt and collapse of the plant at the fruiting stage, which may result in total yield loss (Martyn and Miller 1996). The fungus also infects pumpkin, cucumber, courgette, and watermelon plants (Mertely et al. 1993). The control of *M. cannonballus* in melon and other cucurbit crops is difficult because of the pathogen's soil-borne nature. Earlier reports indicated that arbuscular mycorrhizal fungi (AMF) (Aleandri et al. 2015), hypovirulent isolates of *M. cannonballus* (Batten et al. 2000), *Trichoderma* spp. (Zhang et al. 1999), and antagonistic rhizobacteria (Al-Daghari et al. 2020) are effective agents for the reduction of *M. cannonballus*-induced root rot and vine decline of melon. In addition, it is well established that many endophytic fungi isolated from medicinal plants possess antimicrobial activity against phytopathogenic fungi (Jia et al. 2016). The objective of this study was to investigate the presence of endophytic fungi in *Z. multiflora* and to study theirs *in vitro* antagonistic activity against *M. cannonballus*.

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Z. multiflora plants (accession number 201100114) were obtained from Oman Botanic Garden, Al-Khoud, Sultanate of Oman. The plants were healthy, showing no apparent symptoms of any disease or pest infestation. A virulent isolate of *M. cannonballus* (ID14367), obtained from the roots of a melon plant showing root rot and vine decline (Al-Rawahi et al. 2018) was used in this study. The culture was maintained on potato dextrose agar (PDA) medium (Oxoid Ltd., Basingstoke, UK).

To isolate endophytic fungi, *Z. multiflora* plants were washed in tap water to remove adhering soil particles. The leaves were separated, cut into small pieces, and surface-sterilized by washing in 70% (v/v) ethanol for 1 min and then in 1% (v/v) sodium hypochlorite for 1 min. The plant tissues were then washed 3–4 times with sterilized distilled water. The leaf tissue pieces were further cut into small pieces (0.2–0.5 cm in length) using a sterile scalpel and placed on PDA medium. The plates were incubated at 25 ± 2°C for 7–10 days, and pure cultures of the endophytic fungi were obtained (Lu et al. 2012).

DNA was extracted from the mycelia for molecular identification of endophytic fungi according to the method described by Liu et al. (2000). PCR amplification of the Internal Transcribed Spacer (ITS) regions of the fungal rDNA was performed using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') as described by Halo et al. (2018). The PCR products of the expected sizes were sequenced at Macrogen, Seoul, Korea. The sequences were subjected to BLAST searches using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

A dual culture technique was used to test the in vitro antagonistic effect of the endophytic fungi against *M. cannonballus.* A mycelial plug (7-mm diameter) was excised from the fungal endophyte colonies and placed on one side of a PDA plate (90-mm diameter) about 1 cm away from the edge. On the same plate, a 7-mm diameter disc of *M. cannonballus* was placed on the opposite side at 1 cm distance from the edge. The Petri plates inoculated with *M. cannonballus* alone were used as control. Four Petri plates per treatment were used. The Petri plates were incubated at 25 ± 2°C, and the radial growth of *M. cannonballus* was measured after 5–7 days of incubation. The mycelial growth inhibition was calculated using the following formula:

$$\% \text{ inhibition} = [1 - (T/C)] \times 100$$

where C – radial growth of *M. cannonballus* in the control plate and T – radial growth of *M. cannonballus* in the dual culture plate (Toghueo et al. 2016).

To investigate the antagonistic effects of the endophytic fungi on the morphology of *M. cannonballus* hyphae, the five-mm agar plug samples of *M. cannonballus* were excised from the colony edges of inhibition zone in the dual culture plate. The samples for scanning electron microscopy were prepared according to the method reported by Goldstein et al. (2003) and observed with a JEOL (Model: JSM-7800F) scanning electron microscope. The culture of *M. cannonballus* grown in the absence of endophytic fungi served as control.

To perform the electrolyte leakage assay, the endophytic fungi were cultured in 200 ml of Czapek Dox broth (static) in 500 ml conical flasks at room temperature (25 ± 2°C) for 14 days, and the culture filtrates were obtained by filtering through Whatman No. 1 filter paper. Five hundred mg of *M. cannonballus* mycelium were added to 20 ml of culture filtrate in a glass vial. The conductivity of the suspension was measured at 0, 1, and 3 h after incubation by using a conductivity meter (Halo et al. 2018). There were three replicates per treatment and control.

Data from the *in vitro* growth inhibition and the electrolyte leakage assays were statistically analyzed using general linear model ANOVA using Minitab Statistical Software version 17 (Minitab Inc., State College, USA). When ANOVA revealed significant differences between treatments, means were separated using Tukey's studentized range test at $p \leq 0.05$. Arc sine transformation of data on % mycelial growth inhibition was done prior to analysis.

A total of five morphologically distinct fungal endophytes were obtained from the leaves of *Z. multiflora.* Based on the rDNA ITS sequence analysis, these endophytic fungal (Ascomycota, Sordariomycetes) isolates were identified as *Nigrospora sphaerica* (Amphisphaeriales, Apiosporaceae) (E1 and E6), *Subramaniula cristata* (Sordariales, Chaetomiaceae) (E7) and *Polycephalomyces sinensis* (Hypocreales, Ophiocordycipitaceae) (E8 and E10). The sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MH028052, MH028054, MH028055, MH028056, and MH028058. *P. sinensis* is an important medicinal fungus. Numerous pharmacological activities of *P. sinensis* including immunomodulatory, anti-estrogenicity and antitumor activities have been documented (Wang et al. 2012). *N. sphaerica* has been reported as an endophyte (Wang et al. 2017) as well as a pathogen in a few plant species (Wright et al. 2008; Liu et al. 2016). However, *Z. multiflora* plants colonized with these endophytic fungi were healthy and did not show any observable disease symptoms.

The *in vitro* dual culture antagonism assay showed that all the five endophytic fungi inhibited the mycelial growth of *M. cannonballus*. *N. sphaerica* E1 was the most effective (81.7%), followed by *P. sinensis* E8 (80.6%), *P. sinensis* E10 (75.8%) and *N. sphaerica* E6 (66.1%). *S. cristata* E7 was the least effective, which

Table I
Percentage inhibition of mycelial growth of *M. cannonballus* by endophytic fungi isolated from *Zataria multiflora* in dual cultures on PDA.

Fungal endophyte	% Inhibition
Nigrospora sphaerica E1	81.7 (64.7) ± 5.5[a]
Nigrospora sphaerica E6	66.1 (54.4) ± 1.9[a]
Subramaniula cristata E7	38.7 (38.5) ± 3.7[b]
Polycephalomyces sinensis E8	80.6 (63.9) ± 11.2[a]
Polycephalomyces sinensis E10	75.8 (60.5) ± 9.3[a]

Data are mean of four replications ± standard deviation. Figures in parentheses are arc sine transformed values. Values in columns followed by different letters indicate significant differences according to the Tukey's test ($p < 0.05$).

recorded 38.7% inhibition (Table I, Fig. 1). Further, scanning electron microscopic observations of the hyphae of *M. cannonballus* from the dual culture assay plates at the edge of the inhibition zone revealed morphological abnormalities such as disintegration, shrinkage, and loss of turgidity. Scanning electron micrograph of *M. cannonballus* after co-cultivation with the endophytic fungus *N. sphaerica* E1 is shown in Fig. 2. These findings corroborate with those of Hajlaoui et al. (1992) who reported plasmolysis of *Sphaerotheca pannosa* var. *rosae* mycelium due to the antagonistic effect of *Sporothrix flocculosa*. Halo et al. (2018) reported shrinkage of *Pythium aphanidermatum* hyphae due to the antagonistic activity of *Aspergillus terreus*. The shrinkage of *M. cannonballus* hyphae in the present study suggests a possible leakage of cytoplasmic contents (Garg et al. 2010). The loss of the turgidity of *M. cannonballus* hyphae indicates alterations in the permeability of the cell membrane (Halo et al. 2018). Several reports indicate the production of antimicrobial substances by endophytic fungi (Zhao et al. 2012; Homthong et al. 2016). Kim et al. (2001) demonstrated that phomalactone, a compound produced by *N. sphaerica* restricted the mycelial growth and germination of sporangium and zoospore of *Phytophthora infestans* and decreased the incidence of late blight in tomato. Zhao et al. (2012) characterized four secondary antifungal metabolites viz., dechlorogriseofulvin, griseofulvin, mullein, and 8-dihydroramulosin from the liquid cultures of the endophytic fungus *Nigrospora* sp. isolated from roots of the medicinal plant, *Moringa oleifera*. Homthong et al. (2016) reported the production of chitinase by

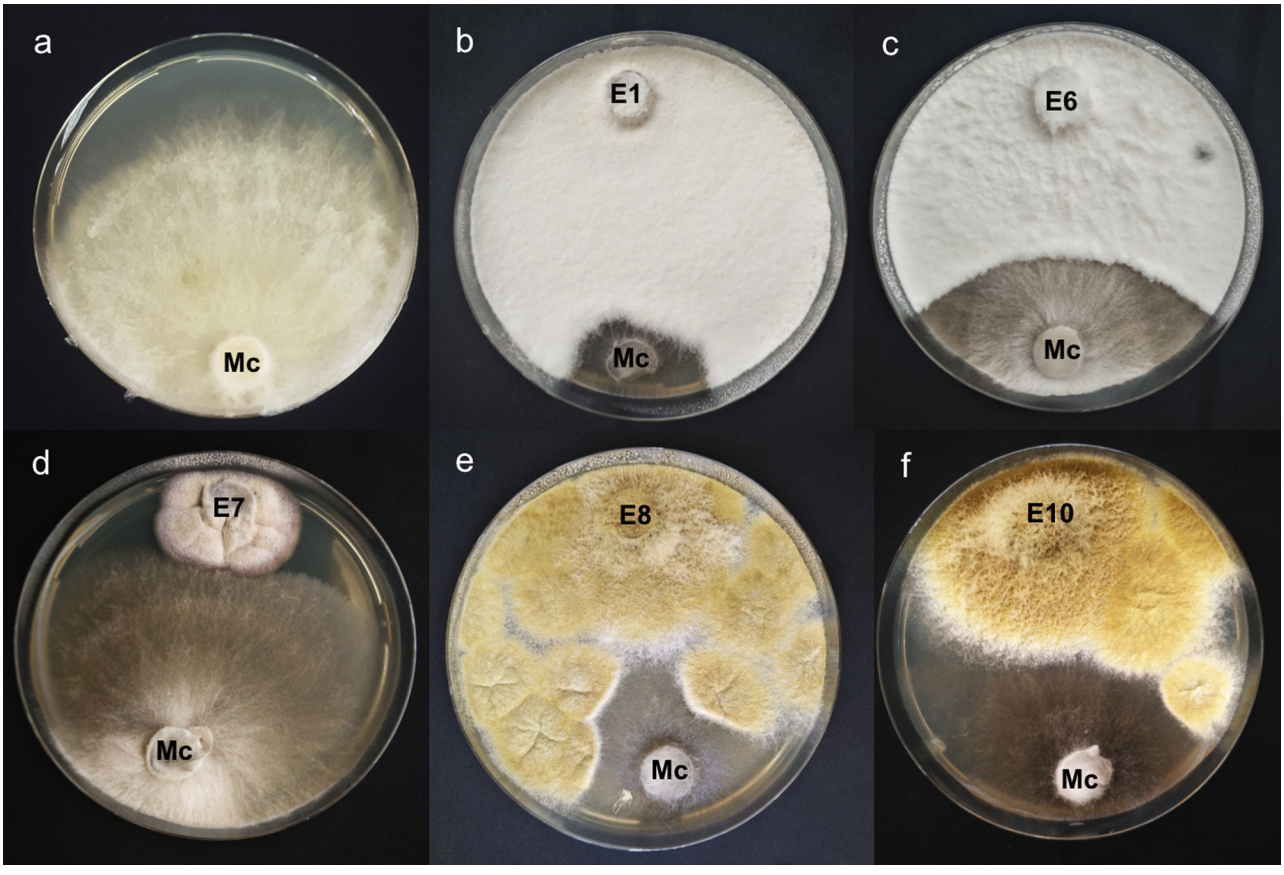

Fig. 1. *In vitro* growth inhibition of *Monosporascus cannonballus* after dual cultivation with several endophytic fungi from *Zataria multiflora.*
a) *M. cannonballus* (Mc) alone; b) *M. cannonballus* + *N. sphaerica* E1; c) *M. cannonballus* + *N. sphaerica* E6; d) *M. cannonballus* + *S. cristata* E7; e) *M. cannonballus* + *Paecilomyces sinensis* E8; f) *M. cannonballus* + *P. sinensis* E10

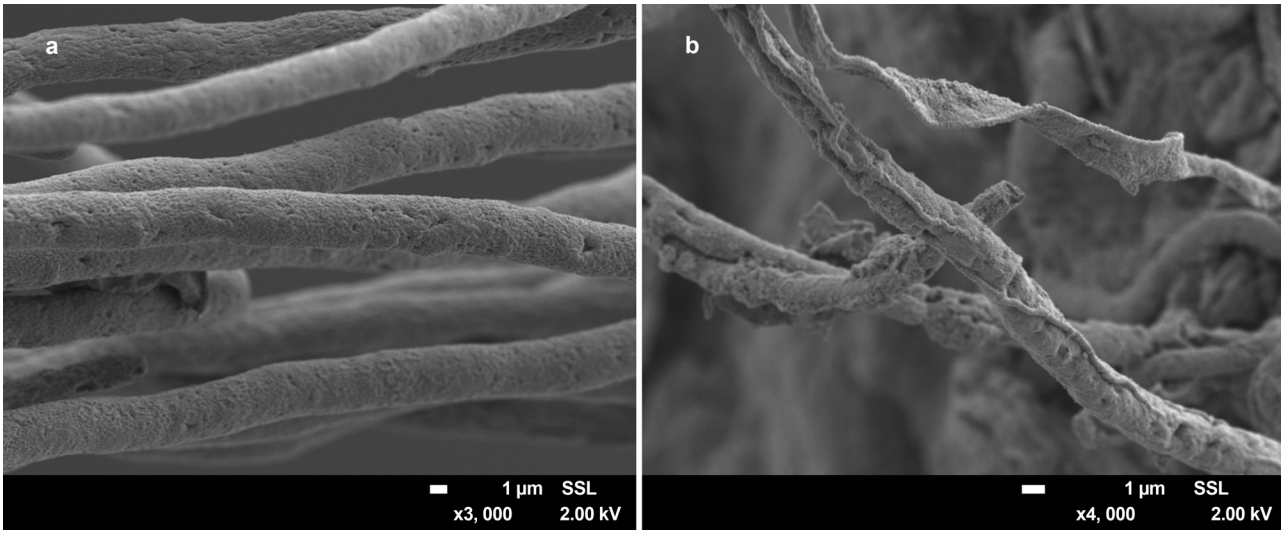

Fig. 2. Scanning electron micrograph showing morphological changes in the hyphae of *Monosporascus cannonballus* at the edge of the inhibition zone after co-cultivation with *Nigrospora sphaerica* E1 in PDA plates

a) Hyphae of *M. cannonballus* in the control; b) Hyphae of *M. cannonballus* after co-cultivation with *N. sphaerica* E1.

Paecilomyces (*Polycephalomyces*) sp. The inhibitory effect of endophytic fungi on the hyphae of *M. cannonballus* in this study might be due to the production of antifungal metabolites.

Several reports indicate that leakage of electrolytes is an indicator of cell membrane damage in fungi (Manhas and Kaur 2016; Halo et al. 2018). The present study observed that the culture filtrates of endophytic fungi induced electrolyte leakage from the mycelium of *M. cannonballus* as assessed by increased conductivity of mycelial suspension upon treatment with the culture filtrates of endophytic fungi (Table II). The maximum release of electrolytes was observed with *N. sphaerica* E1, followed by *N. sphaerica* E6, *P. sinensis* E10, *S. cristata* E7, and *P. sinensis* E8. The results suggest the production of antifungal metabolites as one of the possible mechanisms of action of these fungal endophytes on *M. cannonballus*.

To our knowledge, this study is the first to report *in vitro* inhibitory activity of fungal endophytes isolated from *Z. multiflora* against *M. cannonballus*. Further studies are needed to evaluate the potential of these fungal endophytes in controlling root rot and vine decline disease of melon, assess their endophytic movement in melon plant, and to determine the mode of action of these fungal endophytes on *M. cannonballus*.

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Table II
Electrolyte leakage induced by culture filtrates of endophytic fungi from the mycelium of *M. cannonballus*.

Treatments	Electrical conductivity (mS cm^{-1})		
	0 min	1 h	3 h
Nigrospora sphaerica E1	3.95 ± 0.02^{a}	3.98 ± 0.02^{a}	4.12 ± 0.06^{a}
Nigrospora sphaerica E6	3.90 ± 0.02^{b}	3.87 ± 0.02^{a}	4.01 ± 0.04^{b}
Subramaniula cristata E7	3.46 ± 0.00^{c}	3.41 ± 0.01^{c}	3.55 ± 0.01^{d}
Polycephalomyces sinensis E8	3.10 ± 0.03^{d}	3.28 ± 0.14^{c}	3.14 ± 0.01^{e}
Polycephalomyces sinensis E10	3.50 ± 0.02^{c}	3.61 ± 0.00^{b}	3.71 ± 0.01^{c}
Czapek Dox broth (un inoculated)	2.01 ± 0.00^{e}	2.01 ± 0.00^{d}	2.08 ± 0.00^{f}
Control (water)	0.65 ± 0.01^{f}	0.67 ± 0.00^{e}	0.71 ± 0.01^{g}

Data shown correspond to mean of three replications ± the standard deviation. Values in columns followed by different letters indicate significant differences according to the Tukey's test ($p < 0.05$).

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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