Antagonistic Activity of Endophytic and Rhizosphere Fungi Isolated From Sea Purslane (*Sesuvium portulacastrum*) Against *Pythium* Damping off of Cucumber

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Abstract

This study was conducted to investigate the antagonistic activity of endophytic and rhizosphere fungi isolated from a medicinal plant, *Sesuvium portulacastrum*, against *Pythium aphanidermatum*, the cause of damping off of cucumber. A total of 40 endophytic and 19 rhizosphere fungi were isolated from *S. portulacastrum*. Three endophytic isolates and two rhizosphere isolates gave >50% suppression of *P. aphanidermatum* in the in vitro dual-culture tests. Scanning electron microscopic studies at the inhibition zone showed hyphae wall damage and abnormal mycelial growth of the genus *Pythium*. Molecular analysis identified the antagonistic endophytes as *Aspergillus insulicola* (isolate A435), *A. insulicola* (A419), and *Aspergillus melleus* (A412) and the rhizosphere antagonists as *Aspergillus terreus* (A213) and *Aspergillus luchuensis* (A116). Except for A116, the culture filtrates of the other antagonists significantly increased the electrolyte leakage from *Pythium* mycelia, whereas ethyl acetate extracts of A435, A412, and A213 showed significant growth suppression. All five antagonists were able to produce varying amounts

of cellulase and β -glucanase enzymes. However, A435, A412, and A213 showed significantly higher cellulase activity, whereas A435 and A116 showed the highest β -glucanase activity. Controlled glasshouse growth experiments showed that isolates A435 and A116 resulted in up to 70% control of damping off, whereas isolates A412 and A213 showed 30 to 40% damping-off control. The antagonists A435, A116, and A213 also contributed to increased cucumber shoot length as well as shoot and root dry mass. The synergetic effects of metabolites and hydrolytic enzymes could be the reason for the variation between isolates in the antagonistic activity and cucumber growth promotion. This study reports for the first time *A. insulicola*, *A. melleus*, and *A. luchuensis* as potential biocontrol agents against *P. aphanidermatum*-induced damping off of cucumber.

Keywords: biocontrol, chromatography, inhibition, oomycetes, vine decline

Cucumber (*Cucumis sativus*) is usually affected by damping off caused by soilborne pathogens, resulting in severe economic losses across all cultivated regions (Abbasi et al. 2019; Al-Fadhal et al. 2019; Hashemi et al. 2019; Hatami et al. 2013; Huang et al. 2016). In Oman, damping off results in up to 75% seedling mortality in cucumber (Al-Sadi et al. 2011; Halo et al. 2019). *Pythium* spp. are the most important pathogens that cause damping off in cucumber. *Pythium aphanidermatum* is the main causative agent for damping off of cucumber in Oman (Al-Shibli et al. 2019; Kazerooni et al. 2019).

Chemical control is the most commonly used method for the management of the genus *Pythium* (Al-Balushi et al. 2018; Manjunath et al. 2017; Matić et al. 2019). However, the overapplication of harmful chemicals poses a deleterious effect on the environment and human health. The terms “biological control” and its abbreviated synonym “biocontrol” in plant pathology indicate the use of microbial antagonists to suppress diseases, and such antagonistic organisms are referred to as biological control agents (BCAs). Biocontrol has been recognized as an effective way of reducing the use of harmful systemic fungicides and their threat to the environment and nontarget organisms (Huang et al. 2020). BCAs may use their antagonistic activity through direct and indirect mechanisms, such as hyperparasitism, competing for resources/nutrients, or producing antimicrobial secondary metabolites, lytic enzymes, and other chemical interferences or by the induction of host plant defense mechanisms (De Silva et al. 2019; Segaran and Sathivelu 2019).

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Funding: This research was funded through Sultan Qaboos University grants EG/16/01, EG/19/01, and SR/17/01 and OAPGRC.

The author(s) declare no conflict of interest.

Accepted for publication 22 February 2020.

Biocontrol studies for *P. aphanidermatum* have focused on the use of nonphytopathogenic bacteria and fungi, such as *Trichoderma* spp. (Charoenrak et al. 2019; Elshahawy and El-Mohamedy 2019; Manjunath et al. 2017; Sánchez-Montesinos et al. 2019), *Pseudomonas* spp. (Omoboye et al. 2019; Postma and Nijhuis 2019; Wittayapipath et al. 2019), and *Bacillus* spp. (Kipngeno et al. 2015; Schmidt et al. 2004). Endophytes and rhizosphere fungi are likely to be the most suitable organisms in this BCA category because they stay close to the host plant and get protected from environmental stresses and fluctuations. Therefore, endophytic and rhizosphere fungi are likely to provide effective and durable biocontrol to phytopathogens compared with other organisms (Huang et al. 2020; Singh et al. 2019).

Comparatively, rhizosphere soil harbors a larger microbial population than the nonrhizosphere soil owing to the release of large amounts of organic carbon by the plant roots (Shu et al. 2019). The active microbial community closely associated with plant roots is called the rhizosphere microbial community. Most rhizosphere fungi act as saprophytic organisms and could develop mutualistic associations with host roots as well. Soil physicochemical and microbiological features controlled by rhizosphere fungi are known to be involved in the suppression of soilborne pathogens, such as *Pythium* spp. (Clarkson et al. 2004; Whipps and Lumsden 1991).

Endophytic fungi are defined as fungi that inhabit plant tissues without causing disease symptoms (Segaran and Sathivelu 2019). They are found in a wide range of plant taxa, including nonvascular plants and lichens, and in all types of climatic conditions. Every plant has at least one endophytic fungus inhabiting its tissues, and most plants have hundreds of endophytes that may occupy all tissue types, such as leaves, stems, petioles, bark, xylem, roots, and seeds (De Silva et al. 2019; Segaran and Sathivelu 2019).

Sesuvium portulacastrum (sea purslane), a medicinal plant, is a halophytic pioneer plant in coastal regions of Asia and the Arabian Peninsula. It is mostly used for sand dune fixation, desalination, and phytoremediation of coastal regions as well as being used as a vegetable and fodder for domestic animals (Adrian-Romero et al. 1998; Lokhande et al. 2013b; Lonard and Judd 1997). *S. portulacastrum* extracts are marketed as essential oils and have been used

widely as a folk medicine. Chemical composition analysis of oils indicated the presence of fatty acids, fatty acids methyl esters, alcohols, monoterpenes, alkaloids, amino acids, polysaccharides, minerals, saponins, and steroids. Many of these products have been previously described as antimicrobial and antioxidant compounds that may contribute to the biological activity of *S. portulacastrum* (Lokhande et al. 2013a, b; Patel et al. 2019). Limited studies are available on *S. portulacastrum*, and these have mainly focused on aerial fungi infecting the plant or on the surface of the leaves (Bao et al. 2019; Kuthubutheen 1981; Zhang and Li 2009).

Few studies were conducted in Oman on the endophytic and rhizosphere organisms that are antagonistic against *Pythium aphanidermatum*. *Pseudomonas aeruginosa* from cucumber-growing soil had shown damping-off control in cucumber (Al-Hinai et al. 2010). Also, *Talaromyces variabilis*, *T. pinophilus* (Halo et al. 2019; Kazerooni et al. 2019), and *Trichoderma* spp. (Deadman et al. 2007) have been found to have antagonistic activities against *Pythium aphanidermatum*.

This study aimed to isolate and characterize endophytic and rhizosphere fungi from the medicinal plant *S. portulacastrum* and to evaluate their antagonistic effects against *P. aphanidermatum* growth and *Pythium*-induced damping off of cucumber. The specific objectives of the study were to (1) isolate and identify endophytic and rhizosphere fungi from *S. portulacastrum*, (2) evaluate the in vitro antagonistic activity of *S. portulacastrum* endophytic and rhizosphere fungi against *P. aphanidermatum*, (3) evaluate activity of extracellular enzymes cellulase and β -1,3 glucanase that are produced by the antagonistic fungi, (4) evaluate the in vivo antagonistic activity of the antagonistic fungi against *P. aphanidermatum*-induced damping off of cucumber, and (5) evaluate the plant growth-promoting ability of the antagonistic fungi on cucumber.

Materials and Methods

Sampling. Twelve *S. portulacastrum* plant samples were collected from three sites in a coastal area of Muscat governorate, Sultanate of Oman. From each location, three asymptomatic *S. portulacastrum* plants were uprooted with intact soil adhering to the roots. The collected samples were placed in sterile bags and then, transported to the plant pathology laboratory at Sultan Qaboos University. All of the samples were kept at 4°C until further analysis. Fungal isolation was processed within 48 h of sample collection.

Isolation of endophytic fungi. Endophytic fungi were isolated from the collected plants using a modified method described by Tiwi et al. (2018). Plant samples were carefully cleaned under running tap water, and stems and roots were cut into 10-cm-long segments. Leaves were taken as whole from each plant. All plant materials were surface sterilized in 70% ethanol for 1 min, 1% sodium hypochlorite for 2 min, and 70% ethanol for 30 s followed by three washings in sterile distilled water, each for about 1 min.

The sterilized stems and roots were trimmed aseptically to 1 cm in length, and stem segments were halved; leaves were trimmed from all four sides into 1 × 0.5-cm-sized pieces. After trimming, the root, leaf, and stems pieces were placed separately on petri dishes (90 mm in diameter) containing potato dextrose agar (PDA) amended with Rifampicin (0.01 mg/liter). The petri dishes were incubated for 12-h light/dark cycles at 24 ± 2°C. To ensure the stability of surface sterilization, the sterile distilled water from the final wash of plant materials was inoculated in PDA medium. The emergence of fungal colonies from plant tissues was monitored daily for a period of 2 weeks, and individual fungal colonies were picked from the edge of the growth and transferred onto fresh PDA plates. Subculturing was done several times until pure cultures were obtained for each fungus, and they were subsequently maintained on PDA medium for further analysis.

Isolation of rhizosphere fungi. Rhizosphere fungi were isolated by the direct plating method (Kazerooni et al. 2017) with slight modifications. Plant roots were carefully taken out from the bag, and the soil adhering to the roots was aseptically transferred to a sterilized petri dish. The soil samples (0.1 g) were plated directly onto petri dishes containing 2.5% PDA amended with rose Bengal

(0.1% wt/vol). Three replicates were prepared for each rhizosphere soil sample and incubated for 7 to 14 days at 24 ± 2°C. Emerging fungal colonies were picked individually and subcultured on PDA plates. The pure cultures obtained were maintained in PDA slants at 25°C for further analysis.

Antagonistic activity of endophytic and rhizosphere fungi against *P. aphanidermatum*. Each isolated endophytic fungus was tested against *P. aphanidermatum* using the dual-culture method (Toghueo et al. 2016). A 6-mm mycelial disc cut from an actively growing 7-day-old culture of the isolated endophytic fungi was placed at 1-cm distance from the edge of the PDA petri dish (90 mm in diameter). Similarly, a mycelial plug obtained from 3-day-old *P. aphanidermatum* culture (strain SQU CC002 obtained from the Sultan Qaboos University Fungal Culture Collection) was placed at the opposite side of the same petri dish, 1 cm from the edge of the petri dish. The control plate was prepared only with the pathogen *P. aphanidermatum*. The plates were incubated at 12-h light/dark cycles at 24 ± 2°C for 4 days. The percentage inhibition (% *I*) was calculated using the following formula (Mokhtar and Dehimat 2013):

$$I(\%) = (C - T) / C \times 100$$

where *I* = percentage inhibition of pathogen growth by antagonists, *C* = radial growth in control, and *T* = radial growth in the treatment.

Scanning electron microscopy. Samples were prepared for scanning electron microscopy observation following the method described by Mogana and Ramasamy (2016) with modifications. A 6-mm-diameter mycelial disc of *P. aphanidermatum* close to the inhibition zone, which was facing the antagonist, was taken from the 4-day-old dual-culture plates, fixed at room temperature for 2 h with 2.5% glutaraldehyde fixative, and then, rinsed with 0.1 M sodium cacodylate buffer two times each for 5 min. The samples were fixed by placing them in 1% osmium tetra oxide for 30 min followed by rinsing with distilled water two times each for 10 min followed by serial dehydration using acetone (25, 75, 95, and 100%) each for 10 min. The samples were embedded, mounted coated, and observed using a scanning electron microscope (JEOL JSM-5600) at the Electron Microscopy Core Facility at the College of Medicine, Sultan Qaboos University. The control samples were prepared from the *P. aphanidermatum* colony grown without antagonists. The morphological characteristics, general shape of mycelia, surface morphology of hypha and hyphal tips, and abnormal growth or structures of dual-culture samples and the controls were compared.

Molecular identification of the antagonistic fungi. All endophytic and rhizosphere fungal isolates were identified to the genus level based on the sequences of the internal transcribed spacer (ITS), the small ribosomal subunit (SSU; 18S), or the large ribosomal subunit (LSU; 28S). DNA extractions were done using a modified method of Lee and Taylor (1990) as described by Al-Sadi et al. (2011). PCR was performed using primers specific to the ITS, SSU (18S), and LSU (28S) regions (Al-Sadi et al. 2011; Raja et al. 2017) (Table 1). Each reaction was performed in a 25- μ l final volume containing 2 μ l of DNA, 1.0 μ l (20 μ M) of each primer, and PuRe-Taq Ready-To-Go PCR beads (GE Healthcare). Thermocycling was carried out for ITS, LSU, and SSU by heating at 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The final extension was at 72°C for 10 min.

The five antagonistic fungal isolates (A412, A419, A435, A213, and A116), which produced $\geq 50\%$ inhibition of the pathogen, were further subjected to species-level identification through PCR amplification of two specific gene regions, namely β -tubulin (*tub*) gene (Bt2a forward: 5'-GGT AAC CAA ATC GGT GCT GCT-3'; Bt2b reverse: 5'-TTC ACC CTC AGT GTA GTG ACC CTT GGC-3'), and Calmodulin (*CAL*) gene (CMD5 forward: 5'-CCG AGT ACA AGG CCT TC-3'; CMD6 reverse: 5'-CCG ATA GAG GTC ATA ACG TGG-3'). Thermocycling was carried out under the following conditions: initial heating at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s. The final extension step was at 72°C for 7 min.

Amplified products were loaded (5 µl of PCR product + 2 µl of 6× loading dye) onto 1% agarose gel in 0.5 M Tris-borate-EDTA (TBE) buffer (75 ml), mixed with 1.5 µl of ethidium bromide, and electrophoresed under settings of 80 v and 70 A for 50 min. Bands were compared with a 1-kb DNA ladder. Sanger sequencing was conducted at Macrogen Corp, Korea (<http://www.macrogenusa.com>). The generated sequences for the ITS, tub, and CAL gene regions were deposited in the GenBank and used for phylogenetic analysis of *Aspergillus* samples recovered from this study. Multiple sequence alignments were generated with MAFFT v.7 (<http://mafft.cbrc.jp/alignment/server/index.html>); the alignments were visually improved with MEGA v.6 (Tamura et al. 2013). Phylogenetic analyses of the sequence data consisted of maximum likelihood (ML) using raxml GUI v.1.3 (Silvestro and Michalak 2012). The optimal ML tree search was conducted with 1,000 separate runs. The resulting trees were printed with MEGA v.6, and the layout was made with Adobe Illustrator CS v.6.

Effect of culture filtrate of the antagonists on electrolyte leakage in *P. aphanidermatum*. Each selected antagonistic fungal isolate and *P. aphanidermatum* were grown in 150 ml of sterilized Czapek Dox broth medium in 250-ml Erlenmeyer flasks separately. Agar blocks (6 mm in diameter) containing actively growing fungal mycelium of 7-day-old culture that was maintained on PDA were used as the fungal inoculum. Each liquid culture medium with antagonist was incubated for a period of 10 days at 28 ± 2°C.

After incubation, the culture broths of the antagonists were filtered through sterile Whatman No. 1 filter paper, centrifuged at 4,000 rcf (relative centrifugal force) for 15 min, and further filtered using Minisart filters with 0.2-µm pore size to obtain cell-free culture filtrate (CF). *P. aphanidermatum* mycelial mass was separated from the culture medium, and 0.5 g of mycelial mass was placed in a 15-ml vial containing 10 ml of CF of the antagonist fungi and incubated at room temperature (24 ± 2°C). The supernatant was collected by centrifugation at 4,000 rcf for 15 min, and conductivity changes were measured immediately after the addition of *P. aphanidermatum* mass and after 12 h using a conductivity meter (Halo et al. 2018). Conductivity measurements were compared with a control, which consisted of 10 ml of CF of each antagonist. The test was repeated for all of the selected fungal antagonists each with three replicates.

Effect of metabolites of the antagonistic fungi on the in vitro growth of *P. aphanidermatum*. A 6-mm-diameter plug of actively growing mycelium of the antagonistic fungi obtained from 7-day-old PDA culture was transferred to 150 ml of sterilized Czapek Dox broth medium in a 250-ml Erlenmeyer flask and incubated for 2 weeks at 28 ± 2°C. After incubation, liquid culture was mixed with an equal volume of ethyl acetate (100%) and blended for 5 min. The resulting mixture was filtered first with six layers of muslin cloth and then, with Whatman No. 1 filter paper, and it was transferred to a separation funnel and extracted thrice with a 1:1 volume of ethyl acetate. The organic layer was evaporated in a rotatory evaporator under reduced pressure, and the residue was finally dissolved in 1 ml of dimethyl sulfoxide (DMSO) (Fouda et al. 2015).

Table 1. Primers used for the identification of endophytic and rhizosphere fungal isolates to the genus level

Gene and primer (direction)	Primer sequence 5'–3'
Internal transcribed spacer region of the rRNA (approximately 450–800 bp)	
ITS5 (forward)	5'-GGAAGTAAAAGTCGTAACAAGG-3'
ITS4 (reverse)	5'-TCTCCGCTTATTGATATGC-3'
Small subunit (18S) of the nrRNA	
NS1 (forward)	5'-GTAGTCATATGCTTGTCTC-3'
NS4 (reverse)	5'-CTTCCGTCGAATTCCTTAAG-3'
Large subunit (28S) of the rRNA	
LROR (forward)	5'-ACCCGCTGAACTTAAGC-3'
LR6 (reverse)	5'-CGCCAGTTCTGCTTACC-3'

The antifungal activity of the extract was tested against *P. aphanidermatum* using the agar well diffusion method. Three holes (4 mm in diameter) were made in freshly prepared PDA plates (90 mm in diameter) using a sterile pipette tip at equal distance to each other and approximately 1 cm from the plate margin. Agar was removed, and three wells were filled separately by 40 µl of undiluted metabolites extract, 40 µl of 50% dilution of the metabolites extract (1:1 dilution with DMSO), and 40 µl of DMSO as the control. The treated plate was incubated for 4 h at 4°C, and then, a 4-mm *P. aphanidermatum* mycelial plug taken from 3-day-old PDA culture was placed in the center of the plate. Pathogen-inoculated plates were incubated for 3 days at 24 ± 2°C. The inhibition zones were recorded by measuring the distance between *P. aphanidermatum* colony margin and well margin. The experiment was carried out with three replicates and repeated.

Analysis of production of cellulase and glucanase enzymes by the antagonists. One milliliter of spore suspension (2×10^6 CFU) prepared from 7-day-old PDA culture of antagonistic fungi was added to 50 ml of sterilized basal medium (Mandels et al. 1981) containing 1% carboxymethyl cellulose and incubated for 5 days at 28°C in a rotary shaker at 150 rpm. Fungal CF was obtained by filtration, and 1.5 ml of the CF was mixed with 0.5 ml of 0.05 M sodium citrate buffer (pH 4.8) and 50 mg (1 × 6-cm size) of Whatman No. 1 filter paper. After 1 h of incubation at 50°C, 3 ml of Dinitro salicylic acid reagent (DNS) was added, and the mixture was boiled for 5 min.

Fig. 1. Endophytic and rhizosphere (root, stem, and leaf) fungal genera isolated from *Sesuvium portulacastrum*.

Table 2. Antagonistic activity of endophytic and rhizosphere fungi isolated from *Sesuvium portulacastrum* against *Pythium aphanidermatum*

Source of the isolate	Fungal isolate	Percentage of inhibition ^z
Stem	A412	55.8 ± 1.0 ab
Stem	A419	57.5 ± 1.0 a
Stem	A435	55.0 ± 0.8 b
Rhizosphere	A213	51.2 ± 0.6 c
Rhizosphere	A116	50.5 ± 0.6 c
Control		0.0

^z Means (± standard error) in the same column with the same letter are not significantly different from each other at $P < 0.05$ by Tukey's studentized range honestly significant difference (HSD) test.

Finally, absorbance was measured at 540 nm. Absorbance of the samples was plotted against a glucose standard curve (0.2 to 5.2 mg/liter). One unit of filter paper activity (FPA) was defined as the amount of enzyme releasing 1 μ mol of reducing sugar from filter paper per 1 min under the test conditions (Onofre et al. 2014). The test was repeated using three replicates per each antagonist.

Glucanase enzyme production was determined in a similar way to cellulase enzyme assay. The cultures were grown in TLE (Trichoderma liquid enzyme) liquid medium (de Marco and Felix 2007) with 0.5% chitin as a substrate. After incubating the cultures at 28°C in a

rotatory shaker at 250 rpm for 5 days, the CF was separated by filtration and tested for laminarin hydrolyze assay. Five hundred microliters of 1% laminarin (Sigma-Aldrich) was incubated with 0 to 250 μ l of CF, and the total volume was adjusted to 1 ml with 0.05 M sodium acetate buffer (pH 5.0) and incubated at 37°C for 30 min. Unit of enzyme was calculated against a glucose standard curve using the DNS method by measuring absorbance at 540 nm (Onofre et al. 2014). One unit of enzyme activity (units per milliliter) was defined as the amount of enzyme releasing 1 nmol of reducing sugar per minute per milliliter of CF. The test was repeated using three replicates per antagonist.

Fig. 2. Effect of antagonistic fungal isolates on morphology of *Pythium aphanidermatum*. Scanning electron micrographs of **A**, normal mycelia of *P. aphanidermatum* as the control and **B**, abnormal branched and wrinkled mycelia of *P. aphanidermatum* under the effect of antagonistic fungi A412. Damaged porous and wrinkled mycelial walls of *P. aphanidermatum* are shown under the effect of antagonists **C**, A419, **D**, A435, **E**, A213, and **F**, A116.

Effect of the antagonistic fungi on damping-off suppression and plant growth promotion. The ability of the five selected antagonistic isolates in suppressing *Pythium*-induced damping off and in promoting plant growth was evaluated under glasshouse conditions at the Agriculture Experimental Station, Sultan Qaboos University, Oman. Sterilized round plastic pots (14 cm in diameter) were filled with 1.6 kg of sterile potting mixture (Bulrush Horticulture) and arranged on the glasshouse benches. Antagonists grown in potato dextrose broth (Neogen-acumedia) for 10 days were applied to each pot at a rate of 25 ml per pot (2×10^9 CFU ml⁻¹) (Halo et al. 2018), mixed thoroughly, incubated at 28°C, and irrigated with 125 ml of tap water for 3 days. After incubation, 0.5 g of *P. aphanidermatum* inoculum grown on barley grain carrier medium (Mghalu et al. 2007) was added to each pot 4 cm below the surface level of the soil. Then, cucumber seeds (cultivar Poinsett 76; Technism) were sown in

the infested soil at a rate of seven seeds per pot. Pots containing only antagonist inoculum served as the antagonist control, the noninfested soil served as the negative control, and the positive control consisted of only *Pythium* inoculum. The relative humidity in the glasshouse was maintained at around 75%, and the temperature was $28 \pm 1^\circ\text{C}$ with natural light conditions; each pot was irrigated with 125 ml of tap water daily. After 15 days, the number of surviving seedlings, the seedling shoot lengths, and the shoot and root dry weights were determined for each treatment. The experiment was repeated using 10 replicates per treatment in a completely randomized design.

Statistical analysis. Treatment means for electrolyte leakage assay were compared using independent sample *t* tests for conductivity-level changes to compare the effect of isolates and respective control treatments. For the other experiments, means were subjected to one-way analysis of variance (ANOVA) depending on

Fig. 3. Maximum likelihood tree obtained from the combined internal transcribed spacer + β -tubulin + Calmodulin sequence alignment analysis of the selected antagonistic species of *Aspergillus*. Bootstrap support values >50% are shown at the nodes, and newly generated sequences are in red. *Penicillium herquei* (CBS 336.48) was used as the outgroup taxon.

Table 3. Species names and GenBank accession numbers of antagonistic endophytic and rhizosphere fungi isolated from *Sesuvium portulacastrum*

Isolate	Source of the isolate	Name of the fungi	GenBank accession numbers		
			ITS	β -Tubulin	Calmodulin
A412	Stem/endophytic	<i>Aspergillus melleus</i>	MN072644	MN520094	MN520099
A419	Stem/endophytic	<i>A. insulicola</i>	MN072646	MN520096	MN520101
A435	Stem/endophytic	<i>A. insulicola</i>	MN072647	MN520097	MN520102
A213	Rhizosphere	<i>A. terreus</i>	MN509052	MN520093	MN520098
A116	Rhizosphere	<i>A. luchuensis</i>	MN072647	MN520095	MN520100

Tukey's studentized range (honestly significant difference, HSD) test at $P < 0.05$ using Statistical Analysis System (SAS Institute, Inc.). All percentage values were transformed to arcsine values prior to ANOVA. All trials were analyzed independently.

Results

Isolation and identification of endophytic and rhizosphere fungi from *S. portulacastrum*. A total of 41 endophytic and 19 rhizosphere fungi were isolated from *S. portulacastrum* (Fig. 1). Among them, 99% of the isolates belonged to the phylum Ascomycota, whereas only one isolate was a yeast-like Basidiomycete. The fungal population was dominated by the genera *Fusarium* (25%), *Aspergillus* (17%), and *Monosporascus* (15%). Most fungal endophytes were root endophytes (17 isolates) followed by stem (10 isolates) and leaf (4 isolates) endophytes.

The majority of rhizosphere fungal isolates were *Aspergillus* sp., making up 26% of the total rhizosphere fungal community (Fig. 1). The second most abundant genus was *Chaetomiaceae* (15%). The only Basidiomycete fungus isolated was the yeast-like isolate that belongs to the genus *Pseudozyma*.

Antagonistic activity of endophytic and rhizosphere fungi against *P. aphanidermatum*. Based on the measurements of radial growth, it was observed that only five isolates were capable of significantly reducing the growth of *P. aphanidermatum* in PDA medium by $\geq 50\%$ (Table 2). Among the five antagonists, isolate A419 showed the highest percentage inhibition of *P. aphanidermatum* (57.5%) followed by isolates A435 and A412 (55%). Rhizosphere fungal isolates A213 and A116 showed 50% inhibition of *P. aphanidermatum* when grown together in PDA medium.

Scanning electron microscopy. Scanning electron micrograph images of *P. aphanidermatum* mycelia from the control plates showed smooth wall structures (Fig. 2). In the presence of the antagonist, the pathogen mycelia showed damaged, twisted, and often wrinkled surfaces with shrunken or porous regions or grooves.

Molecular identification of the antagonistic fungi. The combined ITS, TUB, and CAL alignment was used to resolve the *Aspergillus* species recovered in this study (Fig. 3). The alignment was composed of 22 isolates, including the outgroup taxon *Penicillium herquei* (CBS 336.48), and the manually adjusted dataset comprised 1,727 characters, including gaps. Based on the phylogenetic analysis, the five *Aspergillus* isolates recovered from this study were identified as four species, namely *Aspergillus insulicola* (two isolates), *Aspergillus melleus* (one isolate), *Aspergillus luchuensis* (one isolate), and *Aspergillus terreus* (one isolate). The ITS, TUB, and CAL sequences of these species were deposited in GenBank (Table 3).

Effect of CF of the antagonists on electrolyte leakage in *P. aphanidermatum*. Based on the results of both experiments, it was observed that the four antagonists (namely A412, A419, A435, and A213) were able to significantly increase ($P < 0.05$) electrolyte leakage from *Pythium* mycelia (Fig. 4). In both of the trials, the CF of *A. luchuensis* A116 had significantly lower electrical conductivity than the control samples.

Effect of metabolites of the antagonistic fungi on the in vitro growth of *P. aphanidermatum*. Both undiluted and 50% diluted extracts of the five tested fungal isolates were able to produce varying degrees of inhibition effect on *P. aphanidermatum* compared with the control that consisted of DMSO only. Substantial growth inhibition of *Pythium* mycelia was observed at the confrontation sides with metabolite extracts of the antagonistic fungi when applied undiluted compared with the application of 50% diluted extract (Fig. 5). Both 50% dilution and undiluted crude extract of *A. insulicola* A435 produced the highest inhibition of the pathogen (Fig. 5). *A. insulicola* A419 and *A. luchuensis* A116 recorded the lowest inhibition. Inhibition zone was about twofold higher when the undiluted extract was used.

Production of cellulase and β -glucanase by the antagonistic fungal isolates. Analysis of cellulase enzyme indicated that *A. insulicola* isolate 435 had the highest cellulase enzyme activity (0.3 FPA) compared with the other antagonists in trial 1 (Fig. 6). In trial 2, *A. melleus* A412 (0.20 FPA), *A. insulicola* A435 (0.19 FPA), and *A.*

terreus A213 (0.19 FPA) had the highest cellulase activity. Lowest cellulase production was detected from *A. luchuensis* A116 in both trials (0.10 FPA). The results indicate overall higher cellulase production by isolates A412, A435, and A213 than isolates A419 and A116.

The β -glucanase analysis indicated that both *A. luchuensis* A116 (46.0 and 40.7 U) and *A. insulicola* A435 (45.3 and 50.7 U) showed higher β -glucanase activity compared with the other antagonistic isolates in trial 1 (Fig. 6). The β -glucanase activities of isolates A412, A419, A213, and A116 were not statistically significant from each other in trial 2 (Fig. 6).

Effect of the antagonistic fungi on damping-off suppression and plant growth promotion. In *Pythium*-uninfested soil, treatment with all antagonists resulted in 100% seed germination and seedling survival similar to the negative control (Table 4). In *Pythium*-infested soil, all of the antagonistic fungi significantly improved the survival of cucumber seedlings ($P < 0.05$) compared with the control pots.

Variation was found among the antagonistic isolates in their growth promotion activities. Isolates A435 (*A. insulicola*) and A213 (*A. terreus*) significantly increased shoot length, root dry weight, and shoot dry weight in both trials (Fig. 7). Isolate A116 (*A. luchuensis*) significantly increased shoot and dry weights in both trials as well as shoot length in the second trial. The other isolates were not effective in promoting cucumber growth.

Discussion

Results of this study indicate that *S. portulacastrum* harbors a diverse group of endophytic and rhizosphere fungi that belong to

Fig. 4. Effect of culture filtrate (CF) of antagonistic fungi on electrolyte leakage from *Pythium aphanidermatum*. Columns and bars represent mean conductivity values $\pm$ standard error for A, trial 1 and B, trial 2. *Columns are significantly different compared with control sample at $P < 0.05$ by *t* test.

different genera, mainly within the phylum Ascomycota. Endophytic fungal communities belonged mainly to *Alternaria*, *Aspergillus*, *Curvularia*, *Fusarium*, and *Monosporascus* genera. Similarly, a study by Suryanarayanan and Kumaresan (2000) indicated higher endophytic fungal species richness in *S. portulacastrum* than other halophytic mangrove-associated plants and included the fungal genera *Alternaria*, *Acremonium*, *Colletotricum*, *Fusarium*, *Phyllosticta*, and *Sporimella*.

The rhizosphere fungal community of *S. portulacastrum* mainly consists of *Aspergillus* and Chaetomiaceae. In addition, isolates of *Alternaria*, *Cladosporium*, *Fusarium*, *Oedocephalum*, and *Thielavia* were also retrieved from the rhizosphere. Previous studies of rhizosphere microbiome of sea purslane focused on plant growth-promoting bacteria or arbuscular mycorrhizal fungi, and records on nonmycorrhizal fungi are not available. However, studies carried

out on *Avicennia marina*, *Arthrocnemum macrostachyum*, *Limnastrium monoptalum*, *Halocnemum strobilaceum*, *Zygophyllum album*, *Zygophyllum coccineum*, and *Zygophyllum simplex* indicated that the genera *Aspergillus* and *Cladosporium* were the most common genera of halophytic rhizosphere (El-Morsy 1999), which agrees with our findings. Higher fungal diversity was detected in the rhizosphere and roots of *S. portulacastrum* compared with stems and leaves. Previous studies indicated that rhizosphere and plant roots harbor higher fungal diversity compared with other plant parts (El-Morsy 2000; Kwaśna et al. 2016; Yuan et al. 2017).

The antagonist test of endophytic and rhizosphere fungi isolated from *S. portulacastrum* showed that *A. insulicola* strain A435, *A. insulicola* strain A419, *A. melleus* strain A412, *A. terreus* strain A213, and *A. luchuensis* strain A116 suppressed the in vitro growth of *P. aphanidermatum*. Several *Aspergillus* species proved to have

Fig. 5. Effect of metabolites of the antagonistic fungi on the in vitro growth of *Pythium aphanidermatum*. Inhibition zone (millimeters) measured between the *P. aphanidermatum* mycelia and antagonistic fungal extract containing agar well margin for 50% dilution with dimethyl sulfoxide for A, trial 1 and B, trial 2 and undiluted extract for C, trial 1 and D, trial 2 after 3 days of incubation. Columns and bars represent mean inhibition zone (millimeters) $\pm$ standard error. Columns in each chart with the same letter are not significantly different from each other at $P < 0.05$ by Tukey's studentized range honestly significant difference (HSD) test.

antagonistic activities, including *Aspergillus flavus* against mycotoxin-producing fungi (Alaniz Zanon et al. 2016) and *Aspergillus fusispora*, *A. flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nidulans*, and *A. terreus* var. *flococcus* against *Pythium* spp. (Abdelzahr et al. 2000; Al-Shibli et al. 2019; Halo et al. 2018; Ramzan et al. 2014).

Scanning electron microscopy observations at the inhibition zone showed that the antagonists damaged *Pythium* hyphae, which was

evident from the morphological abnormalities that appeared on *Pythium* hyphae (i.e., grooves, loss of turgor, and wrinkled appearance). This suggested that the antagonists produce metabolites that could damage *Pythium* hyphae. When the CF of the antagonists was applied on *Pythium* mycelium, it resulted in electrolyte leakage

Fig. 6. Production of cellulase and β -glucanase by antagonistic fungi isolated from *Sesuvium portulacastrum*. **A**, Columns and bars represent mean filter paper activity (FPA) $\pm$ standard error of total cellulase activity in the culture filtrates. *One unit of FPA was defined as the amount of enzyme releasing 1 μ mol of reducing sugar from filter paper per milliliter per minute. **B**, Columns and bars represent mean β -glucanase activity in units of enzymes $\pm$ standard error in **A**, trial 1 and **B**, trial 2. *One unit of enzyme activity (units per milliliter) was defined as the amount of enzyme releasing 1 nmol of reducing sugar per minute per milliliter of enzyme.

Table 4. Effect of antagonistic fungi isolated from *Sesuvium portulacastrum* on percentage survival of cucumber seedlings in *Pythium* infested and unin-fested soil

	Percentage survival of cucumber seedlings ^y			
	<i>Pythium</i> uninfested		<i>Pythium</i> infested	
	Trials 1 and 2		Trial 1 ^z	Trial 2 ^z
A412	100		55.7 $\pm$ 3.70 a	58.6 $\pm$ 3.32 ab
A419	100		35.8 $\pm$ 2.37 b	47.2 $\pm$ 3.04 b
A435	100		58.5 $\pm$ 3.32 a	67.1 $\pm$ 3.73 a
A213	100		51.4 $\pm$ 2.34 a	52.8 $\pm$ 2.18 b
A116	100		52.9 $\pm$ 2.37 a	67.1 $\pm$ 3.04 a
Control	100		21.5 $\pm$ 2.37 c	17.2 $\pm$ 3.57 c

^y Columns represent mean percentage ($\pm$ standard error) survival of cucumber seedlings after 15 days.

^z Means in each column with the same letter are not significantly different from each other at $P < 0.05$ by Tukey's studentized range honestly significant difference (HSD) test.

Fig. 7. Effect of the antagonistic fungi on **A**, shoot length and **B**, shoot and **C**, root dry weights of cucumber. Columns and bars represent mean ($n = 10$) of the average cucumber seedling shoot length per pot $\pm$ standard error. All measurements were taken after 15 days of growth. Columns in each trial with the same letter are not significantly different from each other at $P < 0.05$ by Tukey's studentized range (HSD) test.

from the hyphae. Further confirmation of the effect of metabolites was tested by ethyl acetate extraction of fungal metabolites on the in vitro growth of *Pythium*. According to the results, both crude extract and 50% dilution of metabolite extracts caused the growth inhibition of *Pythium* mycelia in PDA medium. This indicates the possible production of metabolites by the antagonistic isolates, which are involved in the antagonism against *Pythium*. Halo et al. (2018) described similar morphological changes, such as shrunken/wrinkled mycelia and hyphal tips in *Pythium* mycelia as a result of the antagonistic activity of *A. terreus*.

Antagonistic fungi that can produce *Pythium* cell wall-degrading enzymes can control *Pythium* growth and development even if they have no antifungal metabolite production (El-Tarabily and Sivasithamparam 2006). Enzymes, such as cellulase and β -1,3- and β -1,6-glucanases, are more important for oomycetes because their wall consists of glucans, not chitins (Blaschek et al. 1992). The genomic studies on four *Aspergillus* species, namely *A. nidulans*, *A. niger*, *A. oryzae*, and *A. fumigatus*, have identified about 200 genes responsible for polysaccharide degradation, and moreover, *Aspergillus* spp. have been identified as higher cellulase producers (Benítez et al. 2004). *Aspergillus* species isolated in the current study have been tested for both cellulase and β -glucanase production. All isolates produced cellulase and β -glucanase, and they showed enzymatic activity on the tested substrates. Some of *Aspergillus* species, including *A. terreus* (Halo et al. 2018), *A. fumigatus* (de Rezende et al. 2005), and *A. niger* (Kéry et al. 1991), have also been reported to be positive for β -glucanase activity.

Findings from the in vitro antagonistic studies positively correlated with the in vivo studies carried out in a glasshouse with cucumber. Results indicated that *A. insulicola* A435, *A. luchuensis* A116, *A. melleus* A412, and *A. terreus* were capable of reducing damping off by 50 to 70%, with *A. insulicola* A435 being the most efficient. Isolate A435 showed the highest in vivo antagonistic activity as well as the highest production of cellulase and β -glucanase. Positive correlation between *Pythium* suppression and high β -glucanase was indicated by Blaschek et al. (1992). The β -1,3- and β -1,6-glucanases are the most important enzymes because β -1,3- and β -1,6-linked glucans are the major *Pythium* cell wall components, not the cellulose. Biocontrol studies focusing on *Trichoderma* spp. indicated involvement of β -1,3- and β -1,6-glucanases in the suppression of *Pythium ultimum* (Djonović et al. 2006).

The involvement of antagonists in plant growth promotion may also be important to control pathogens. Several growth parameters, such as shoot length and shoot and root dry weight, indicated that *A. insulicola* A435 is the most effective followed by *A. terreus* A213 and *A. luchuensis* A116 for cucumber growth promotion. The mechanism of this plant growth promotion has not been precisely determined. However, *Aspergillus* species have been previously described as the best phosphate-solubilizing fungi on a variety of different substrates (Perea Rojas et al. 2019), and they are capable of increasing soluble phosphate, which is normally not available in soil for plants. Phosphate-solubilizing *Aspergillus* spp. had significantly increased shoot length, shoot and root dry biomass, yield of wheat and faba bean plants (Abdul Wahid and Mehana 2000), and yield of maize and wheat plants (Kaur and Reddy 2017). Faster growth because of more availability of nutrients in soil helps seedlings to escape from *Pythium* attacks. Future studies should address the mechanisms behind promotion of growth by the *Aspergillus* species.

This study isolated a total of 41 endophytic and 19 rhizosphere fungi from *Sesuvium portulacastrum*. From the isolated endophytes and rhizosphere fungi, five isolates (A435, A419, A412, A213, and A16) were found to be antagonistic on *P. aphanidermatum*, and they were identified as *Aspergillus* species, namely *A. insulicola* A435, *A. insulicola* A419, *A. melleus* A412, *A. terreus* A213, and *A. luchuensis* A116. This is the first report on the antagonistic activity of *A. melleus*, *A. luchuensis*, and *A. insulicola* against *Pythium aphanidermatum* and *Pythium* damping off of cucumber. Further studies are needed to investigate the mechanism of action of the antagonistic isolates. Potential side effects (if any) of the antagonists should also be investigated before commercial use.

Acknowledgments

We acknowledge Dr. Myo Tay Zar Myint and Ms. Fatima Al-Ghafri for help in scanning electron microscopy. They thank Dr. Brandon T. Hasset for the comments that helped improve the manuscript.

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