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Talaromyces pinophilus strain AUN-1 as a novel mycoparasite of *Botrytis cinerea*, the pathogen of onion scape and umbel blights

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ABSTRACT

This study aimed to investigate the mycoparasitism of *Botrytis cinerea,* the pathogen of scape and umbel blights of onion seed crops, by endophytic *Talaromyces pinophilus*. The dual culture test showed that the antagonistic potentiality of *T. pinophilus* against *B. cinerea* depend on the mycoparasitism that was morphologically detected by the formation of mycelial overgrowth. Moreover, the light micrograph of the mycelia at the contact zone exhibited that the hyphae of *T. pinophilus* penetrated and grew intracellularly inside the hyphae of *B. cinerea.* A more illustrative figure of the establishment of coiled hyphae as well as the conformation of the penetration process was assayed by SEM and TEM analyses. SEM micrograph revealed that the hyphae of *T. pinophilus* grew along hyphae of *B. cinerea,* attached, coiled around the host hypha and generated pseudoappressorium. A clear disintegration of cell wall of the host hypha was observed at the penetration site. The micrographs of TEM exhibited the ability of *T. pinophilus* to produce pseudoappressorium, penetrate and then entere a hypha of *B. cinerea* causing distinct cytoplasmic disorganization. High activities of cell wall degrading enzymes (chitinase, lipase and protease) involved in the mycoparasitism were evaluated by the endophytic *T. pinophilus*. In conclusion, this study demonstrated that the endophytic *T. pinophilus* may be a promising biocontrol agent against phytopathogenic fungi instead of chemical fungicides.

1. Introduction

Onion seed crops are usually attacked by several fungal pathogens that cause high yield losses (Abdel-Hafez et al., 2014; Abdel-Rahim et al., 2017; du Toit et al., 2004). Scape and umbel blights, caused by *Botrytis* species, are serious threats in the most important stage of onion seed production. These blights may devastate unprotected crops causing significant seed losses (du Toit et al., 2004). *Botrytis cinerea* [teleomorph: *Botryotinia fuckeliana* (de Barry) Whetzel] is considered one of the primary causal agents of scape and umbel blights (Chilvers and du Toit, 2006). Moreover, *B. cinerea* is a destructive pathogen with a wide host range of vegetables, ornamental, field and orchard plants as well as stored and transported agricultural products (Elad et al., 2007). Biological control agents, as an eco-friendly alternative to synthetic fungicides, caused a significant reduction in the incidence of plant pathogenic fungi (Abo-Elyousr et al., 2014). These suppressed the fungal pathogen through one or more mechanism such as mycoparasitism, competition on nutrients and space, antibiotics production or stimulation of plant defense responses (Benítez et al., 2004; Harman, 2006). Since the mycoparasitism of *Trichoderma* was recognized by Weindling (1932), there have been broad efforts in the investigation of the other mycoparasites and commercial production of them for crop disease management (Gardener and Fravel, 2002; Harman, 1996). Today, several mycoparasites such as *Alternaria alternata* (Zheng et al., 2017), *Cladosporium* spp. (Torres et al., 2017), *Pythium periplocum* (Kushwaha et al., 2017), *Sphaerodes mycoparasitica* (Vujanovic et al., 2017), *Trichoderma gamsii* (Chen et al., 2016) and *Chaetomium globosum* (Moya et al., 2016) have been reported as effective biocontrol agents for many soil-borne and foliar phytopathogenic fungi.

Most plant pathogenic fungi have a cell wall containing chitin as a structural backbone arranged in regularly ordered layers, laminarin (β-1,3-glucan) as a filling material in an amorphic manner and other minor components as proteins and lipids (Chet and Chernin, 2002). The production of fungal cell wall degrading enzymes by mycoparasites is an essential step for the penetration process and nutrients extraction from the host cells. Therefore, chitinases, β-1,3-glucanases, protease and lipase have been found to be involved in the parasitic interaction and subsequently in the biocontrol potentiality (Limon et al., 1999).

Several previous studies reported that *Talaromyces pinophilus* (Hedgc.) Samson (anamorph: *Penicillium pinophilum* Thom) was

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prevalently isolated from soil, compost (El-Naggar et al., 2015), seeds, grains (Ismail et al., 2016) phyllosphere, phylloplane (Abdel-Gawad et al., 2017; Abdel-Hafez et al., 2015a) and as endophyte inhabiting some medicinal plants (Koul et al., 2016; Yao et al., 2017). *T. pinophilus* was widely used as an effective cellulose degrading species (Pol et al., 2012), as well as a renewable source of pigments, colorants (Caro et al., 2017) and bioactive compounds (Nicoletti and Trincone, 2016; Wang et al., 2013). Few previous studies gave a glance on the antagonistic activity and mycoparasitic behaviour of *T. pinophilus* on *Rhizoctonia solani*; however the detailed mechanism of the interaction process was obscurely illustrated (Alagesaboopathi, 1994; Nicoletti et al., 2006).These studies reported that unlike other mycoparasites, neither penetration nor coiling of *R. solani* hyphae by *T. pinophilus* could be observed on dual cultures. Moreover, they concluded that the fungitoxic metabolites play a role in the expression of antagonism (De Stefano et al., 1999; Nicoletti et al., 2004).

This study firstly aimed to investigate the mycoparasitic interaction between *T. pinophilus* strain AUN-1 and *Botrytis cinerea* strain AUN-2, the causal agent of umbel and scape blights of onion, through light, scanning and transmission electron microscopy. Secondly, it evaulated the fungal cell wall degrading enzymes involved in the mycoparasitism process.

2. Material and methods

2.1. Fungal isolation

Botrytis cinerea was isolated from samples of onion seed crops, cultivated in Assiut Governorate (Egypt), showing scape and umbel blights. On the other hand, the endophytic fungus *Talaromyces pinophilus* was isolated from fresh asymptomatic healthy onion umbels. The samples were packed in sterilized polyethylene bags and immediately transferred to the mycological laboratory. Potato dextrose agar (PDA) medium supplied with 66.7 mg/L rosebengal and 250 mg/L streptomycin was used in the isolation process (Smith and Dawson, 1944). Fungi were isolated through surface sterilization technique described by Abdel-Hafez et al. (2015a). Umbels and seed stalks were sterilized by immersing them in 75% ethanol for 1 min, followed by 1% sodium hypochlorite for 5 min and again in 75% ethanol for 30 s. Infected or healthy onion parts were individually detached, washed with sterilized distilled water and dried using a sterilized paper towel under aseptically conditions. Onion parts were placed on the surface of PDA medium and then the Petri dishes were incubated at 25 °C for 7 days. Fungi emerging out of the plant tissues were purified using single spore isolation technique (Choi et al., 1999).

2.2. Fungal identification

Fungi were initially identified based on the culture and microscopic features using several keys designed by Yilmaz et al. (2014), Pitt (1979) and Ellis (1971). The molecular identification of fungi was investigated using the polymerase chain reaction (PCR) amplification of the ribosomal internal transcribed spacer (ITS) region.

Fungal growth was scraped from the PDA medium and suspended in 100 µl of distilled water and boiled at 100 °C for 15 min and stored at −80 °C. DNA was extracted from fungal cultures using the genomic DNA Prep kit (SolGent, Daejeon, Korea) according to SDS/CTAB lysis and phenol/chloroform extraction method (Ausubel et al., 1998). The DNA amplification and sequencing were carried out in SolGent Company Limited (Daejeon, South Korea). The ribosomal ITS region was amplified by PCR using a primer pair of ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) corresponding to protocol reported by Abdel-Rahim and Abo-Elyousr (2017). 1 µl of the fungal DNA (20 ng) was amplified in a 25 µl reaction mixture with Solgent EF-Taq as follows: 10X EF-Taq buffer 2.5 µl, 10 mM dNTP (T) 0.5 µl, primer (Forward-10 picomol) 1.0 µl, primer (Reverse −10 picomol) 1.0 µl, EF-Taq (2.5U) 0.25 µl, Distilled water (to 25 µl). The thermocycling conditions included an initial denaturation for 15 s at 95 °C, followed by 30 cycles of denaturation for 20 s at 95 °C, annealing for 30 s at 55 °C, extension at 72 °C for 60 s and a final extension at 72 °C for 10 min. The PCR product was separated by gel electrophoresis on 1% agarose gel run for 75 min in buffer TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) and detected under a UV illuminator. The PCR product corresponding to ribosomal ITS, according to electrophoretic migration, was eluted from the gel and then purified using purification kit (SolGent, Daejeon, South Korea) depending on to the protocol's instructions.The two strands of amplified ribosomal ITS region were sequenced using primers ITS1 and ITS4. The obtained sequences were analyzed using BLAST search program at the NCBI website: http://blast.ncbi.nlm.nih.gov/Blast.cgi. The alignment was assayed using the multiple sequence alignment program CLUSTALW. Phylogenetic analysis was performed with referential strains from GenBank using MegAlign (ver. 5.01).

2.3. Nucleotide sequence accession numbers

The obtained sequences of *Talaromyces pinophilus* strain AUN-1and *Botrytis cinerea* AUN-2 were deposited in the nucleotide sequence database of GenBank under accession numbers MF806019 and MF967569, respectively.

2.4. Dual culture test

In vitro, evaluation of the antagonistic interaction between the mycelia of *Talaromyces pinophilus* and *Botrytis cinerea* was achieved by dual culture method (Li et al., 2002). Mycelial disc (3 mm diameter) of 4-day-old actively growing colonies of *B. cinerea* was inoculated at one plate edge Petri dishes containing PDA medium. After 48 h of incubation at 25 °C, *T. pinophilus* was placed on opposite position edge. Plates were re-incubated at 25 °C till the observation of the morphological overgrowth interaction between endophytic fungus *T. pinophilus* and the pathogen.

2.5. Light microscopic study

Detection of the mycoparasitic interactions between mycelia of *T. pinophilus* and *B. cinerea* was preliminarily carried out by light microscope. The sterilized clean slide was placed in sterilized Petri plate and then a thin layer of sterilized melted PDA was spread over the slide. Inocula of 4-day-old growing cultures of *B. cinerea* and *T. pinophilus* were seeded on the opposite sides of the slide. The humidity of the Petri plate was adjusted using small pieces of sterilized wetted cotton. Thereafter, the plates were incubated at 25 °C for three days. At the incubation period, the contact area of *T. pinophilus* and *B. cinerea* hyphae was examined under a light microscope to explore the mycoparasitic behavior as the presence of coiling structures, approsoria and pathogen wall disintegration (Hajieghrari et al., 2010).

2.6. Scanning (SEM) and Transmission electron microscopy (TEM) analysis

The electron microscope analysis was performed in electron microscope unit (EMU) of Assiut University. During the dual culture experiment, the contact area (10 mm in diameter) showing the interaction between mycelia of *T. pinophilus* and *B. cinerea* was cut, fixed in 4% cold gautaraldehyde and washed by sodium cacodylate buffer. Thereafter, the samples were dehydrated using an ascending grade of ethanol. The samples were dried in critical point drainer using liquid carbon dioxide, and stickled on the metallic block. In gold splutter apparatus, the samples were evenly gold coated in a thickness of 15 nm. Then, the samples were examined and photographed using JSM 5400 LV Scanning Electron Microscope (JEOL Ltd, Tokyo, Japan).