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3. Materials and Methods

3.1 Evaluation of culture-based methods and pyrosequencing in estimating fungal diversity in soil

The purpose of the first section was to compare the efficiency of culture-based methods to pyrosequencing in estimating fungal diversity in soil.

3.1.1 Collection of samples

Soil samples were collected from two farms in Oman cultivating tomatoes. Soil was collected along random directions from five different tomato plants in each farm, approximately about 1 kg of sample from each plant, taken from 10-15 cm depth near the active growing roots. The five samples from each farm were placed in five separate sterile plastic bags and brought to the laboratory. All samples were thoroughly homogenized and then used for direct plating and pyrosequencing analysis.

3.1.2 Direct plating

This method was performed for isolating fungi from soil samples. Soil samples (0.1-0.15 g) were plated onto Rose bengal-amended 2.5% potato dextrose agar (Oxoid, England) plates using three replicates for each sample. The plates were incubated at 25 °C for three to seven days. Fungal colonies growing in the plates were sub-cultured to new plates. Pure cultures of the fungi were established by using mycelium tip culture.

3.1.3 Identification of fungi

Fungal isolates were identified based on morphological characteristics under light microscope as well as based on sequence data. Fungal isolates were grown on PDA for 3 to 7 days. Then fungal isolates were preliminarily assigned to different genera based on the size and shape of spores and mycelia. To confirm the identity of fungi,

DNA was extracted from freeze dried mycelium using the protocol of Lee and Taylor (1990) as described by (Al-Sa'di et al., 2007). Briefly, freeze dried mycelium was ground into powder, followed by mixing with lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) and incubation for one hour at 65^{o}C. After that, the lysate was mixed with phenol : chloroform : alcohol (25:24:1) and centrifuged. The supernatant was mixed again with phenol : chloroform : alcohol followed by centrifugation. This was followed by precipitation of DNA in the supernatant after mixing with NaAc and isopropanol. The precipitated DNA pellet was washed using ethanol. The dried pellet was re-suspended in 100µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) and stored at -20^{o}C.

The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were amplified using the primer pair ITS1 (5'TCCTCCGGTTATTGATATGC'3) and ITS4 (5'CATCGAGAAGTTCGAGAAGG'3) **(**White et al., 1990) as explained by Al-Sadi et al. (2011a). The Polymerase chain reaction mixture consisted of the two primers, DNA, PuReTaq^{TM} Ready-To-Go^{TM} PCR beads (GE Healthcare) and Milli-Q water. The thermocycling conditions were 95˚C (10 min); then 35 cycles of 95˚C (30 s), 55˚C (30 s) and 72˚C (90 s); and a final extension step at 72˚C (10 min). Additional loci (β-tubulin, Calmodulin, RNA polymerase II second largest subunit ((RPB2), Translation elongation factor 1-alpha (TEF1)) were used to identify the species of *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* using the primers and conditions detailed in literature (Carbone and Kohn, 1999; Samson et al., 2014). β-tubulin gene was amplified using the primers Bt2a (GGT AAC CAA ATC GGT GCT GCT TTC) and Bt2b (ACC CTC AGT GTA GTG ACC CTT GGC), Calmodulin gene using the primers CMD5 (CCG AGT ACA AGG ARG CCT TC) and CMD6 (CCG ATR GAG GTC ATR ACG TGG), RPB2 gene using the primers 5F (GAY GAY MGW GAT CAY TTY GG), 7CR (CCC ATR GCT TGY TTR CCC AT), 5Feur (GAY GAY CGK GAY CAY TTC GG) and 7CReur (CCC ATR GCY TGY TTR CCC AT), and TEF1 gene using the primers 728F (5'CAT CGA GAA GTT CGA GAA GG'3) and 986R (5'TAC TTG AAG GAA CCC TTA CC'3) primers. The conditions were as explained previously (Carbone and Kohn, 1999; Samson et al., 2014).

Purification and sequencing of PCR products were carried out at Macrogen, Korea. Sequences were aligned and improved using MEGA v.6 (Tamura et al., 2013). A maximum likelihood analysis was performed by using raxmlGUI v.1.3 (Silvestro and Michalak, 2012) for the isolates that belong to the kingdom fungi using the ITS region. The optimal ML tree search was conducted with 1000 separate runs, using the default algorithm. Bootstrap 50% majority-rule consensus trees were generated and the final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTRGAMMA substitution model. ITS sequences generated from the analysis were deposited in GenBank (Table 3. 1).

Table 3.1 ITS GenBank accession numbers of fungal isolates detected in this study

Fungal isolates	Accession number
Aspergillus pachycristatus	KY814690
Aspergillus quadrilineatus	KY814680
Aspergillus quadrilineatus	KY814684
Aspergillus quadrilineatus	KY814689
Aspergillus rugulosus	KY814676
Aspergillus rugulosus	KY814688
Aspergillus rugulosus	KY814687
Cephaliophora sp.	KY814682
Chaetomium sp.	KY814677
Cladosporium tenuissimum	KY814674
Fusarium chlamydosporum	KY814673
Fusarium chlamydosporum	KY814685
Fusarium nygamai	KY814686
Fusarium solani	KY814675
Fusarium solani	KY814679
Fusarium solani	KY814691
Mortierella sp.	KY814683
Penicillium corylophilum	KY814681
Pythium aphanidermatum	KY814678

3.1.4 DNA extraction for Pyrosequencing

DNA was extracted from 3-5 replicates from each soil sample according to the protocol of Volossiouk et al. (1995). A two-step process was used for the amplification of samples. Firstly, the forward (i5 and ITS1F) and reverse (i7 and ITS2aR) primers were constructed as described previously (White et al., 1990; Gardes and Bruns, 1993; Kazeeroni and Al-Sadi, 2016; Al-Balushi et al., 2017). The reaction mixtures and conditions for the first and the second PCRs were as per Al-Balushi et al. (2017).

3.1.5 Pyrosequencing Analyses

All sequencing reads were run through Research and Testing Laboratory's (RTL, Lubbock, TX, USA) standard microbial analysis pipeline. The data analysis pipeline consisted of the denoising and chimera detection stage and the microbial diversity analysis stage. Denoising was carried out to remove short sequences, singleton sequences, and noisy while chimera detection was used to remove chimeric sequences using the UCHIME chimera detection software in *de novo* mode (Edgar et al., 2011). In the diversity analysis stage, all samples were assembled into OTU clusters at 97% identity using the UPARSE (Edgar, 2013) algorithm. This was followed by aligning using the USEARCH (Edgar, 2010) global algorithm against a database of high quality ITS fungal gene sequences from GenBank. Fungi were classified using trimmed taxa. Individual analysis was carried out for the percentage of sequences assigned to each fungal phylogenetic level for each pooled sample in order to provide the relative abundance for individual samples. The data were filtered at 97% similarity threshold. The data were analyzed using the R software (Team, 2011) to estimate Richness.

3.1.6 Direct plating vs pyrosequencing

Data from the direct plating test was compared to pyrosequencing analysis.

3.2 Effect of farming systems (organic vs conventional) on fungal diversity

This section investigated the level of fungal diversity in soils from conventional and organic farming systems cultivating cucumber and tomatoes.

3.2.1 Collection of Samples

The experiments focused on two farming systems (organic and conventional) and two crops commonly grown in Oman, cucumber and tomato. Details on the farms are presented in Table 3.2.

Table 3.2 Characteristics of samples and sampling locations

Farm code	Farming system	Crop	Sampling site
OR-CU	Organic (OR)	Cucumber	Farm #1
OR-TO	Organic (OR)	Tomato	Farm #1
CN-CU	Conventional (CN)	Cucumber	Farm #2
CN-TO1	Conventional (CN)	Tomato	Farm #3
CN-TO2	Conventional (CN)	Tomato	Farm #4

Soil samples were collected during September-November, 2013, from the rhizosphere of cucumber and tomato in the OR and CN farms. Soil, which is within the 0-3 cm distance from plant roots, is considered as rhizosphere soil. Cucumber and tomato have been grown in the OR and CN farms for at least the last eight years. OR crops have been fertilized using organic animal manures and composts and have never received any fungicide treatment in the soil. However, CN crops have been fertilized using inorganic fertilizers and animal manures and received several mefenoxam, hymexazol and thiophanate-methyl treatments over the last five years.

Each soil sample was approximately 1kg collected from three locations in the top 5-15 cm depth near (0-3 cm) the active feeder roots of each crop. Soil samples were collected from the rhizosphere of three randomly selected crops of each crop species grown in a different plot within a farm. The soil samples were kept in sterile plastic bags (5 bags from each farm) and then transferred to the Plant Pathology Research Lab, SQU, Oman. Each sample was subjected to chemical and physical analysis. Soil

for DNA analysis was ground with liquid nitrogen and then kept at -80°C until DNA extraction.

3.2.2 Soil physicochemical properties

Soil samples were air-dried, ground, and then passed through a 2-mm sieve to remove roots and plant debris. After that the sieved soil was stored in plastic tubes until analysis. Various physicochemical parameters of each soil sample were determined. The texture of the soil was determined by using hydrometer test (Gee and Bauder, 1986). Electrical conductivity (EC) and pH were determined by using electrical conductivity and pH meters (Zhang et al., 2005). Determination of potassium (K) was done by digesting one gram of soil sample in 20 ml HNO_3 and 5 ml of H_2O_2 for 30 min at 150˚C, followed by analysis using a flame photometric method (Sheerwood 450 flame photometer). Phosphorus was determined using an Inductively Coupled Plasma (Perkin Elmer, USA) after digesting one gram of the soil sample in 30 ml HNO_3 and 10 ml of HCl for 30 min at 300˚C and filtering the diluted mixtures using 0.45µl filter membrane. Total inorganic carbon (TIC) and total organic carbon (TOC) were analyzed by using Total Organic Carbon analyzer (TOC-V, Shimadzu, Japan). Analysis of nitrogen was done by mixing 0.5g soil sample with one tablet of Kjeltab catalyst in 10 ml of sulfuric acid, followed by heating at 420^{o}C for 20 to 30 min. Then the solution was allowed to cool, followed by analysis of total nitrogen using Kjeltec Analyser (FOSS TECATOR, Sweden).

3.2.3 Pyrosequencing analyses

DNA was extracted from the soil samples according to the protocol of Volossiouk et al. (1995), following some modifications. Soil was ground with liquid nitrogen for 5 min and then 0.05 g of each sample was transferred to 1.5 µl eppendorf tube. Then, 125 µl of skimmed milk was added and the mixture was incubated in an oven at 65 °C for 1 hr. The mixture was centrifuged and the supernatant was transferred into a new eppendorf tube. After that 500 µl SDS extraction buffer (0.3 % SDS, 140 mM NaCl, 50 mM NaAc, pH 5.1) was added to the supernatant, followed by the addition of one

volume of phenol: chloroform: isoamyl alcohol (25:24:1). The suspension was centrifuged and the supernatant was transferred to a new eppendorf tube. Then, 0.6 volumes of isopropanol and 10 µl of NaAc was added to the supernatant, incubated overnight at -20 °C and then centrifuged. The pellet was washed with 600 µl ethanol, dried and finally suspended in TE buffer. The quality and quantity of DNA was assessed by using a Nano drop spectrophotometer (Thermo Scientific, USA).

DNA extraction was carried out from three replicate samples collected from each crop/farming system. The primers ITS1F and ITS2aR were used for the amplification of ITS2 region. The samples were submitted to the Research and Testing Laboratory (RTL, Lubbock, TX, USA) for tag-encoded 454-pyrosequencing (Dowd et al., 2008a; 2008b).

The obtained sequences, which are less than 300 bp, were excluded from further analysis and the rest were checked for high quality using RDP ver 9 (Cole et al. 2009). The low quality ends and tags were removed and were checked for chimers using UCHIME chimera detection software (Edgar et al., 2011). The resulting sequences were analyzed using a BLASTn.NET algorithm by comparison to high quality sequences from NCBI and the outputs were validated based on taxonomic distance methods (Dowd et al., 2005, 2008a, 2008b). Filtering was done at 97% similarity threshold.

Further analysis of pyrosequencing data was carried out using the R software (R Development Core team, 2011). Richness was estimated using the Chao1 richness estimator using the formula: $S_{chao1} = S_{obs} + n_1 (n_1-1)/ 2 (n_2+1)$, where S_{obs} is the number of observed species/OTUs and n*i* is the number of OTUs with abundance *i*. UniFrac distances were calculated using the phyloseq package in R. Principal Coordinate Analysis (PCoA) was conducted and plotted from unweighted UniFrac distances.

3.2.4 Statistical analysis

Tukey's Studentized range test (SAS, SAS Institute Inc., USA) was used to examine differences among soils from different cropping systems. Correlation analysis was also conducted using SAS.

3.3 Isolation of antagonistic fungal isolates for the management of *Pythium* and *Rhizoctonia*-induced damping-off of cucumber

3.3.1 Collection of soil samples

Soil samples for this study were collected from the farms indicated in the previous section (Table 4.3). Soil samples were collected from the rhizospheres (top 5-15cm) of tomato and cucumber plants in sterile plastic bags, transported and stored in an incubator at 10°C in the Plant Pathology Research Lab at Sultan Qaboos University until used.

3.3.2 Isolation of potential antagonistic fungi

Soil (100-150 mg) was spread onto the surface of 2.5% potato dextrose agar (PDA) amended with Rose Bengal (25 mg/l) (Al-Sadi et al., 2012a) using three replicate plates for each soil sample. The plates were incubated at 24ºC up to one week. Fungal growth on the surface of the PDA was transferred to new plates and pure cultures were established using mycelium tip culture. Fungal cultures with typical growth of *Pythium*, *Fusarium* or *Rhizoctonia* were excluded from further tests as to avoid the potential use of pathogenic species.

3.3.3 *In vitro* screening of fungal biocontrol agents by dual culture method

An initial screening was carried out for 36 fungal isolates to investigate their antagonistic activity against *Pythium aphanidermatum* and *Rhizoctonia solani*, which were obtained from our previous collections. The assay for antagonistic activity of microorganisms was carried out on PDA medium in petri dishes (Al-Hinai et al., 2010). A 3-day old, 5 mm diameter mycelial disc of *Pythium* or *Rhizoctonia* was placed at one end of a 90 mm PDA culture plate. A 5 mm disc of fungal isolate (to be tested for antagonism) was placed at the other end of the petri dish. Plates without antagonistic fungi were considered as controls. The petri dishes were incubated at 25ºC and were observed for the formation of inhibition zone around antagonist or until either

the pathogen mycelia or potential antagonist fully grow over the plate. The experiment was repeated three times with three replications.

3.3.4 Effect of antagonistic fungal isolates (TO144 and TT266) on cucumber growth

The fungal isolates (TO144 and TT266), which showed the highest degree of antagonism against *Pythium* and *Rhizoctonia* in dual culture assay were used for subsequent studies. The effects of the antagonistic fungal isolates on the root length, shoot length and dry weight were determined. A fully-grown antagonistic fungal culture on 90 mm PDA plate was mixed with 600 g of sterilized peat moss and filled in 10 cm diameter pots. In addition, two control pots were prepared, one without the antagonistic isolate, and the second by mixing potting mix with PDA medium (without fungus). Five disinfected cucumber seeds were sown in each pot. The pots were placed in a growth chamber at 27 °C and 70% RH for 7 days. There were three replicate pots for each treatment/control and the experiment was repeated three times. The percentage of surviving cucumber seedlings in each pot was determined. The length of roots and shoots was also recorded. In order to determine dry weight, cucumber seedlings were dried at 60-65 °C for 24 hours. Then the weight of each seedling was recorded.

3.3.5 Effect of antagonistic fungal isolates (TO144 and TT266) on damping-off of cucumber

A fully-grown 90 mm PDA plate culture of fungal antagonist (isolate TO144/TT266) was mixed with 600 g of sterilized peat moss. Four hundred g of the mix was filled in 10 cm diameter pots. Then, a 90-mm PDA plate culture of *Rhizoctonia* or *Pythium* gown for 3 days was placed on the top of the peat moss, followed by the addition of the remaining 200 g of the peat moss-antagonist mix on top of the pathogen culture. Pots inoculated with *Rhizoctonia*/ *Pythium* alone and pots mixed with PDA medium alone served as controls. Untreated control pots (sterile soil alone) were also maintained. The experiment was carried out as described before. The percentage of the surviving cucumber seedlings was determined in each pot. Fungal pathogens were

re-isolated from seedlings developing damping-off symptoms to find out if they are infected by the same pathogens used for inoculation.

3.3.6 Molecular characterization of antagonistic fungi

The antagonistic fungal isolates (TO144 and TT266) were identified to the species level using sequences of the internal transcribed spacer region of the ribosomal RNA (Al-Sadi et al., 2012a). Freeze-dried mycelium was ground and lysed in 600 µl lysis buffer (50 mM Tris-HCL, PH 7.6; 50 mM EDTA; 3% SDS; 1% 2-mercaptoethanol). The mixture was incubated for 1 hour at 65°°C then for further purification, 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the mixture and shaken well. The reaction mixture was centrifuged at 10,000 g for 15 min. The supernatant was transferred to a new eppendorf tube and was treated with an equal volume of phenol:chloroform:isoamyl alcohol. The reaction mixture was shaken and centrifuged at 10,000 g for 15 min. The supernatant was transferred to a new eppendorf tube and DNA was precipitated by adding 10 µl of 3M NaAc and 0.6 volumes of iso-propanol. The mixture was incubated overnight at -20°°C. Nucleic acid was recovered by centrifugation at 10,000 g for 5 min and the pellet was washed with 70% ethanol (600 µl) and dried for 15 min at 37°°C. Finally, the isolated DNA was suspended in 100 µl of sterile water and stored at -20°°C until used. The concentration and purity of DNA was measured using Thermo Scientific NanoDrop™ 2000.

The purified DNA of each fungus was amplified by polymerase chain reaction (PCR) as described by Al-Sadi et al. (2011c) using the ITS1 and ITS4 primers (White et al., 1990). PCR conditions were as follows: denaturation at 95°°C for 10 min; 35 cycles of 95°°C for 30 sec, annealing at 55°°C for 30 sec and extension at 72°°C for 30 sec, and a final extension at 72°°C for 10 min. The reaction was carried out in the Veriti® Thermal Cycler (Applied Biosystems). The generated PCR products were loaded in a 1% agarose gel in 1X TBE buffer to separate DNA by electrophoresis.

The PCR products were sequenced at Macrogen (Seoul, Korea). The forward and backward sequences were aligned using ChromasPro. A maximum likelihood analysis was performed by using raxmlGUI v.1.3 (Silvestro and Michalak, 2012). The optimal

ML tree search was conducted with 1000 separate runs, using the default algorithm. Bootstrap 50% majority-rule consensus trees were generated and the final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTRGAMMA substitution model.

3.3.7 Statistical analysis

Differences between means were analyzed using the Statistical Analysis Software (SAS).