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Short Communication

454 Pyrosequencing and Direct Plating Reveal high Fungal Diversity and Dominance by Saprophytic Species in Organic Compost

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Abstract

A study was conducted to investigate fungal diversity in organic compost originating from Oman. Analysis of diversity was conducted using 454 pyrosequencing and direct planting. The obtained fungal species through direct plating were identified based on the internal transcribed spacer region of the ribosomal RNA (rRNA). Pyrosequencing detected the presence of 94 fungal species, compared to 5 species detected by direct plating. Pyrosequencing also detected more fungal phyla, classes, orders, families and genera. Most of the detected species belonged to Ascomycota and Chytridiomycota, with *Powellomyces* spp., *Eupenicillium* spp. and *Chaetomium* spp. being the most dominant genera. The majority of the detected species (>99%) were found to be either saprophytic or with biocontrol characteristics, with few species (*Fusarium* and *Phoma*), being potential pathogens of plants. The low level of presence of pathogenic species may provide evidence of the health status of the organic compost. The study reports for the first time the occurrence of 67 fungal species in Oman. It discusses the superiority of pyrosequencing over direct plating and the factors influencing diversity of fungi in organic composts. © 2016 Friends Science Publishers

Keywords: Compost; Detection; Soil-borne pathogens; Sequencing

Introduction

Composts are stabilized organic matter produced as a result of biodegradation process of a wide variety of crop, animal, human and industrial wastes. They are generally classified as rural compost and urban compost. Rural compost is produced from raw materials available on the farm while urban or town compost refers to compost prepared from urban, industrial wastes and sewage sludge (Roy *et al*., 2006). Different composting methods can be carried out at various degrees of complexity from simple dumping to fully automated composting which include aerobic, anaerobic or partially aerobic decomposition (Termorshuizen *et al*., 2004). The old method of composting was to pile organic materials and let them stand for a year until they become ready for use. The disadvantages of this method are that space is utilized for a whole year, some nutrients might leach due to exposure to rainfall and some pathogenic organisms, weeds and insects are not controlled. Professional composting usually consists of three phases; an initial warming-up phase (ambient 42°C) which involves degradation of the smaller material during few hours up to a few days. This is followed by a thermophilic phase (45–70°C) in a period which ranges between several weeks and months and most microbes are usually killed during this period. Finally, curing phase is the phase in which temperature declines and the material is re-colonized by beneficial microbes, which is important for natural disease suppression (Hoitink and Boehm, 1999).

The success of composting in eliminating potentially harmful pathogens depends on different composting parameters as well as complex microbial interactions. First, temperature–time combinations are the most important factors for the elimination of plant pathogens. Most plant pathogens and nematodes should be eliminated when temperatures of 60–65ºC during the thermophilic phase are maintained for several days and the majority of fungal pathogens tested could be eradicated by maintaining a compost temperature of 55°C for 21 days (Noble and Roberts, 2004). Moisture content is also an important factor which can influence the elimination level of pathogens. The occurrence of dry pockets in composting material is probably the main cause of pathogen survival in heaps. Therefore, the percentage of moisture content should not be lower than 40%. However, quality compost can serve as a source of high populations of beneficial microorganisms that play an important role in suppression of soil-borne diseases such as damping-off and root rot (Lin *et al*., 2014, Shen *et al*., 2014).

Pyrosequencing is commonly used for studying fungal and bacterial diversity in different substrates (Jumpponen and

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Jones, 2009; Jumpponen and Jones; 2010; Abed *et al*., 2013; Brown and Jumpponen, 2014). Apart from studies on the diversity of fungi in composts (De Gannes *et al*., 2013, Langarica-Fuentes *et al*., 2014), little is known about the efficacy of pyrosequencing in relation to direct plating in estimating fungal diversity in composts. In addition, little information is available concerning fungal diversity in organic compost.

This study was therefore conducted to investigate the efficacy of pyrosequencing in comparison to direct plating in estimating fungal diversity in organic compost and to characterize the level of fungal diversity in organic compost. The study will provide a basis for researchers working on fungal diversity in composts.

Materials and Methods

Analysis of Organic Compost using Pyrosequencing

An organic compost product originating from Oman was used in the study. The 60-day old organic compost was prepared from 25% green waste, 50% cow and buffalo manure and 25% grinded wood. DNA was extracted from two samples of the product using the protocol of Volossiouk *et al*. (1995). The DNA extracts from the two samples were mixed together and then sent to the Research and Testing Laboratory (RTL, Lubbock, TX, USA) for pyrosequencing. Pyrosequencing was carried out based on the 18S rRNA as described by Dowd *et al*. (2008a, 2008b). Analysis of the obtained high quality sequences was done as explained by Dowd *et al*. (2005).

Isolation of Fungi using Direct Plating

The detection of fungi from the organic compost product was also carried out using direct plating (Al-Sadi *et al*., 2008). About 0.1g of the organic compost product was spread on the surface of 2.5% potato dextrose agar (PDA) amended with 50 mg L^{-1} rose Bengal. The test was done for three separate samples and the plates were incubated at 25ºC for two weeks. Fungal colonies developing on PDA were subcultured for identification.

Identification of Fungi

Fungi were identified morphologically and based on sequences of the internal transcribed spacer region of the ribosomal RNA (ITS rRNA). Mycelium were freeze dried, followed by DNA extraction as explained by Lee and Taylor (1990) and Al-Sadi *et al*. (2014).

Polymerase Chain Reaction was carried out using ITS1 and ITS4 universal primers (White *et al*., 1990) as described by Al-Sadi *et al*. (2011b). The 25 µL PCR mixture consisted of PuReTaq™ Ready-To-Go PCR™ beads, 0.4 µM ITS1, 0.4 µM ITS4 and 25 ng DNA. PCR conditions were as described by White *et al*. (1990). Amplification was checked by running the PCR products on gel electrophoresis.

PCR products were purified and sequenced at Macrogen (Korea) in both directions using ITS1 and ITS4 primers. The obtained sequences were assembled using Chromas Pro., and then compared with representative sequences from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using BLAST search.

Estimates of Fungal Diversity

Fungal diversity was assessed for the data obtained from pyrosequencing and also direct plating data using Shannon-Wiener diversity index (H), Evenness index (J), Richness as explained by Krebs (1998).

Results

Analysis of Organic Compost using Pyrosequencing

Pyrosequencing detected 94 species belonging to 6 phyla and 12 classes (Table 1). The organic compost product was dominated by *Ascomycetes*, which represented the largest group of detected fungal species. Fungal classes that were dominant included *Eurotiomycetes*, *Chytridiomycetes* and *Sordariomycetes*. The genus *Powellomyces*, which belongs to *Chytridiomyces* class, represented 20% of the total isolated fungal species. The other dominant fungal genera were *Eupenicillium* (17%) and *Chaetomium* (13%) (Fig. 1). Most of the fungal species (79 out of 94) were found to occur at low frequencies (less than 1%). The analysis showed that 67 of the identified fungi via pyrosequencing are reported in Oman for the first time (Table 2).

Pyrosequencing vs Direct Plating

Pyrosequencing detected 19 times more fungal species compared to fungal species detected by direct plating. Direct plating detected only 5 fungal species belonging to one phylum (*Ascomycota*) and 3 genera: *Hypocrea*, *Penicillium* and *Aspergillus* (Fig. 1, Fig. 2). On the other hand, pyrosequencing detected 77 fungal genera belonging to 6 phyla. Diversity indicators also showed that pyrosequencing is more efficient in estimating fungal diversity in the organic compost product (Table 1).

Discussion

The current investigation revealed the presence of *Chytridiomycetes* and other classes in high percentage. *Chytridiomycetes* usually survive on nutrients from compost, which enables them to reproduce and increase in number. In addition, some species can live parasitically and the resistant structures produced by many *Chytridiomycetes* help them survive as dormant structures under conditions not favorable for growth and reproduction (Lozupone and Klein, 2002).

Table 1: Shannon-Wiener index of organic compost as determined by pyrosequencing and direct plating techniques

	Direct Plating	Pyrosequencing
No. of phyla	1	6
No. of classes	3	12
No. of orders	3	27
No. of families	3	49
No. of genera	4	77
No. of species	5	94
Shannon- Wiener index	1.61	2.60
Richness	2.49	9.56
Evenness	1.00	0.57

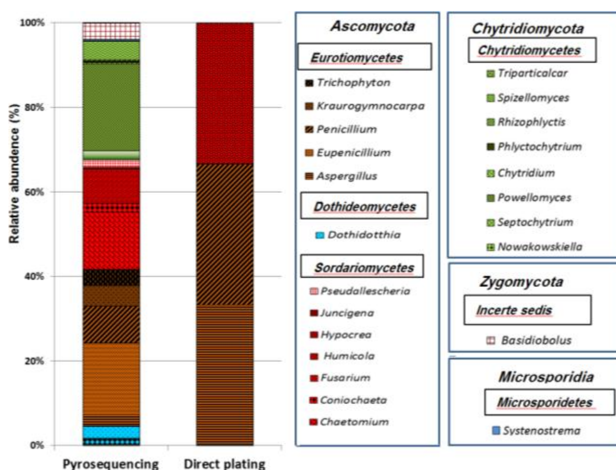

Fig. 1: Analysis of diversity of 23 common fungal genera in organic compost using pyrosequencing and direct plating. The genera are classified into 3 main phyla and 6 classes

Ascomycota represent one of the most common widespread phyla in soils and composts (Abed *et al*., 2013; De Gannes *et al*., 2013).

More than 200 fungal species have been reported in Oman in the past, including saprophytic and pathogenic species (Abed *et al*., 2013; Al-Sadi *et al*., 2012b; 2014; 2015a, b). However, pyrosequencing helped recover for the first time 67 new fungal species to Oman (Table 2). Most of these species (58 species; 87%) were found to occur at low frequencies (less than 1% of the total species), which may explain why these were not detected in previous studies. In addition, the diversity of plant samples and animal manure used for composts may be one factor for the high diversity of fungi in the compost product (Al-Sadi *et al*., 2011a).

Composts and organic fertilizers have long been considered as potential sources of pant pathogenic fungi into farms (Al-Sadi *et al*., 2011a; 2012a; 2013). Pyrosequencing and direct plating showed that the organic compost sample is dominated by saprophytic fungi and fungi with biocontrol characteristics, which are usually common in decaying organic debris (De Gannes *et al*., 2013). Plant pathogenic species, mainly *Fusarium* and *Phoma* species, occurred at very low levels (<1% of the total species). This provides evidence about the health status of the organic compost product. However, frequent examination of the product at different time intervals might be required to insure

Table 2: The 67 newly reported fungal species in Oman based on pyrosequencing data using the 18S rRNA gene sequences

Species	Percent occurrence (out of the total recovered)
Ajellomyces capsulatus	0.03
Ajellomyces dermatitidis	0.01
Alisea longicolla	0.39
Allantophoma endogenospora	0.01
Apiosporina collinsii	0.01
Articulospora tetracladia	0.01
Ascocoryne sarcoides	0.59
Aspergillus restrictus	0.01
Aspergillus zonatus	0.01
Basidiobolus haptosporus	0.01
Basidiobolus ranarum	4.08
Berkleasmium micronesicum	0.01
Camarops microspora	0.16
Cephalotheca sulfurea	0.01
Chromocleista malachitea	0.01
Chytridium olla	0.32
Claviceps fusiformis	0.01
Coniochaeta africana	2.29
Corollospora maritima	0.08
Diplochytridium lagenarium	0.02
Dothidotthia aspera	3.03
Edyuillia athecia	0.01
Endogone flammicorona	0.01
Eupenicillium crustaceum	18.08
Eurotium cristatum	0.06
Fusicladium convolvularum	0.01
Gaertneriomyces tenuis	0.01
Halosarpheia japonica	0.02
Haptocillium balanoides	0.01
Juncigena adarca	0.39
Kirschsteiniothelia aethiops	0.01
Kochiomyces dichotomus	0.01
Kraurogymnocarpa trochleospora	5.19
Lasiosphaeria ovina	0.19
Lecythophora mutabilis	0.10
Leotia lubrica	0.23
Malbranchea gypsea	0.01
Merimbla ingelheimensis	0.01
Monascus fuliginosus	0.03
Monascus purpureus	0.01
Mortierella alpina	0.04
Myrothecium cinctum	0.38
Neophaeosphaeria filamentosa	0.01
Neottiella vivida	0.47
Oceanitis scuticella	0.01
Penicillium charlesii	0.02
Penicillium commune	0.45
Penicillium expansum	0.01
Penicillium janthinellum	2.72
Penicillium lagena	1.39
Penicillium olsonii	1.68
Penicillium roqueforti	0.06
Pertusaria erythrella	0.01
Phialocephala fortinii	0.36
Phlyctochytrium reinboldtiae	0.32
Pleiochaeta setosa	0.01
Potebniamyces pyri	0.01
Pseudorobillarda phragmitis	0.02
Pulchromyces fimicola	0.04
Raffaelea santoroi	0.01
Rhizophlyctis rosea	0.32
Rhodotorula benthica	2.11
Rhodotorula samaneae	0.01
Rhodotorula slooffiae	0.05
Spadicoides verrucosa	0.01
Uncinocarpus reesii	0.01
Westerdykella cylindrica	0.01

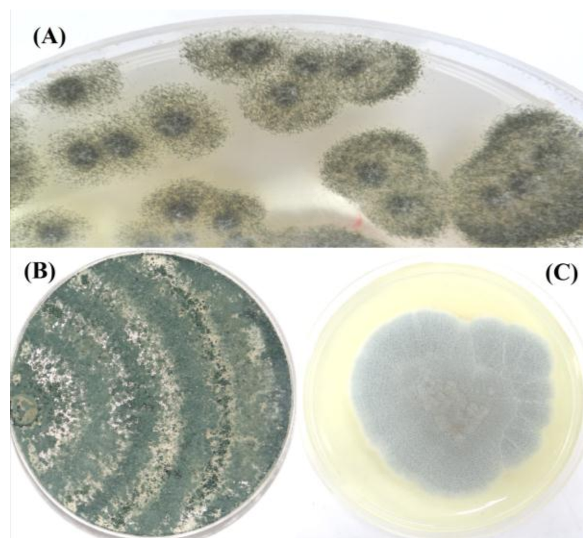

Fig. 2: Cultures of the three dominant fungi in compost as revealed using direct plating, where (**A**) is *Aspergillus*, (**B**) is *Trichoderma* and (**C**) is *Penicillium*

continuous supply of healthy products for agricultural purposes (Al-Sadi *et al.*, 2011a).

The superiority of pyrosequencing over direct plating could be related to the ability of pyrosequencing to detect fungi that do not grow on culture as well as detection of fungi that occur at low concentrations (Abed *et al.*, 2013; Nannipieri *et al*., 2003). In addition, some fungi are very selective and need special way to be cultured. *Chytridiomycetes*, for example, are usually detected using chitin, keratin or cellulose baits (Lozupone and Klein, 2002; Gleason *et al*., 2007).

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