Applied Soil Ecology 98 (2016) 285–288

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Short communication

# Comparison of plate count, microscopy, and DNA quantification methods to quantify a biocontrol fungus

Tae Gwan Kim[a,*], Guy R. Knudsen[b]

[a] Department of Microbiology, College of Natural Sciences, Pusan National University, Pusan 46241, South Korea
[b] Soil and Land Resources Division, Department of Plant, Soil, and Entomological Sciences, University of Idaho, Moscow ID 83844-2339, USA

ARTICLE INFO

*Article history:*
Received 17 February 2015
Received in revised form 2 October 2015
Accepted 12 October 2015
Available online 27 October 2015

*Keywords:*
Quantitative PCR
*Trichoderma harzianum*

ABSTRACT

DNA quantification has become a conventional method for quantifying filamentous fungi in environments. In this study, quantitative PCR was evaluated as a quantification tool by comparisons with plate count (unit, recoverable population) and microscopy with image analysis (hyphal biomass). The genetic transformant *Trichoderma harzianum*ThzID1-M3 was used as a model organism. A soil microcosm experiment with different numbers of ThzID1-M3 alginate pellets showed that DNA was significantly correlated with biomass ($P < 0.05$), but not with CFU. Temporal change of ThzID1-M3 was monitored at −50 and −500 kPa for a 21-day period. Biomass peaked within the first 5 days, followed by a rapid reduction, while CFU peaked at between days 14 and 21, indicating that CFU mainly originated from dormant propagules. DNA increased along with biomass, and then increased again in accordance with the CFU increase. These results demonstrated that DNA estimates do not strictly correspond to either the population of recoverable propagules or hyphal biomass. However, DNA estimates can reflect relative changes of active and/or dormant propagules although they are unable to distinguish between them.


## 1. Introduction

It is important to understand growth and proliferation characteristics of fungal biocontrol agents in crop ecosystems for their successful application. A number of methods have been developed for quantifying growth and proliferation of filamentous fungi. For the best example, methods based on serial dilution and plating counts have routinely been used to quantify viable fungal propagules. In addition, biomass has been estimated by measuring hyphal length (Hanssen et al., 1974), or specific cell components (Seitz et al., 1979). Using these methods, it is difficult to evaluate proliferation of a target fungus in natural environments, due to the inability to distinguish target propagules from the indigenous organisms. New methods, e.g. fluorescent antibodies (Thornton et al., 1994), genetic markers (Bae and Knudsen, 2000), and DNA quantification technology (Filion et al., 2003a), provide opportunities for the selective monitoring and quantifying target microorganisms in natural environments.

Especially, quantitative PCR (qPCR) as a DNA quantification tool has become a popular tool to quantify fungi (Fierer et al., 2005; Filion et al., 2003a; Kim and Knudsen, 2008). However, it is contradictory to assert that qPCR can quantify change of fungal propagules in soil (Atkins et al., 2003; Filion et al., 2003a; Filion et al., 2003b; Landeweert et al., 2003). For example, Filion et al. (2003a) and Atkins et al. (2003) observed correlations between the DNA estimates of filamentous fungi and numbers of spores in soil substrate. In contrast, Filion et al. (2003b) found that fungal DNA estimates did not correlate with the CFU numbers, and Landeweert et al. (2003) observed no correlations between the DNA estimates and hyphal lengths. This contradiction probably results from the phenomenon that active and dormant propagules, e.g. hyphae and spores, are converted into each other.

In this study, we evaluated qPCR as a quantitative tool for a biocontrol fungus in nonsterile soil by comparisons with dilution plate count and microscopy with image analysis. We further investigated effects of soil moisture on fungal growth and proliferation.

## 2. Materials and methods

*Trichodermaharzianum* ThzID1-M3 was used as a model organism for this study. *T. harzianum*,a well-known biocontrol fungus, has shown activity for biological control of several important pathogenic fungi (Bin et al., 1991; Dandurand et al., 2000). ThzID1-M3 was a genetic transformant with genes for green fluorescent protein (GFP), hygromycin B resistance, and

* Corresponding author. Fax: +82 515141778.
*E-mail address:* tkim@pusan.ac.kr (T.G. Kim).

http://dx.doi.org/10.1016/j.apsoil.2015.10.010
0929-1393/

ß-glucuronidase (Bae and Knudsen, 2000). ThzID1-M3 was formulated with alginate as previously described (Knudsen et al., 1991b). Palouse silt loam soil (Pachic Ultic Haploxerolls) (Soil-Survey-Staff, 2010) was obtained from the University of Idaho Plant Science Farm near Moscow, Idaho. Soil was sieved through a 2 mm mesh and air-dried.

Two experiments were done. Experiment 1 had a completely randomized design with 5 replications with the following treatments: 1, 3 or 5 ThzID1-M3 pellets per microcosm. The soil (dry weight basis, 20 g) was added to microcosm containers (8 × 6.3 × 6.3 cm in size) and adjusted to −500 kPa with distilled water. Containers were capped, sealed with parafilm, and incubated in the dark at 22 °C. The microcosms were destructively sampled after 6 days.

Experiment 2 was 2 × 6 factorial with 5 replications with the following treatments: 2 levels of soil moisture (−50 or −500 kPa) and 6 incubation periods with destructive sampling at 0, 3, 5, 7, 14, or 21 days. The soil (dry weight basis, 20 g) was added to containers (8 × 6.3 × 6.3 cm in size), containers were capped, sealed with parafilm, and incubated in the dark at 22 °C. This experiment was done twice exactly as described above.

All soil of each container was suspended in a 500 mL flask with 80 mL of 50 mM sodium phosphate buffer [pH 7.0], and agitated (200 rpm, 1 h). A 0.5 mL subsample of soil suspension was serially diluted in 50 mM sodium phosphate buffer. Soil suspensions representing $10^{-3}$ and $10^{-4}$ dilutions (containing 1 and 0.1 mg soil) were plated on a *Trichoderma*-semi-selective medium (Papavizas, 1981) with hygromycin B (50 μg $mL^{-1}$). After incubation at 21 °C for 7 days, ThzID1-M3 colonies were identified by color and morphology of colony, and counted. Hyphal biomass was quantified using epi-fluorescence microscopy with image analysis as previously described (Orr and Knudsen, 2004). Soil suspensions (containing 10 mg soil) were filtered through Millipore black membrane filters (0.8 μm, 47 mm) (Millipore Co. Billerica, MA, USA) and viewed at 100x with a Nikon Eclipse E1000 epifluorescence microscope (Nikon, Melville, NY, USA) to observe hyphal fragments with GFP activity. DNA was extracted from 0.25 mL soil suspension (containing 50 mg soil) using the PowerSoil DNA isolation kit (Mo Bio Laboratories, CA, USA) according to the manufacturer's instructions. The TMeGFP2 primer/Taqman probe set targeting the GFP gene was used for quantification of ThzID1-M3: TMeGFP2-foward (5′-GCTGCCCGACAACCACTAC-3′), TMeGFP2-reverse (5'-CGTCCATGCCGAGAGTGATC-3′) and TMeGFP2-probe (5′-FAM-CGGCGGCGGTCACGAACTCCA-TAMRA -3′) (Kim and Knudsen, 2011).

## 3. Results and discussion

ThzID1-M3 was quantified in soils with 1, 3 and 5 pellets after 6 days (Fig. 1). Both hyphal biomass and DNA linearly increased with the number of pellets ($P < 0.05$, $R^2 = 0.99$). Because CFU counts did not differ in soils with 3 and 5 pellets, CFU counts were not correlated with the DNA and biomass measurements ($P > 0.05$). This result indicated that DNA estimates could be directly correlated with hyphal biomass.

Temporal changes of ThzID1-M3CFU, biomass and DNA were monitored at −50 and −500 kPa for 21 days. We obtained similar results from the repeated experiments, and thus presented one dataset in this study. ThzID1-M3 biomass peaked within the first 5 day period, followed by a reduction in each soil moisture level (Fig. 2). The recovery of significant numbers of viable propagules lagged several days behind the initiation of hyphal growth. Then, CFU peaked at between days 14 and 21. Numbers of hyphal fragments, which we observed on membranes over time, were coincided with hyphal biomass (data not shown). Thus, we presumed that most of ThzID1-M3 colonies were generated from dormant propagules such as conidia and chlamydospores. Nutrients present in the pellet formulation may result in the initial hyphal growth and biomass increase, and then, subsequent nutrient depletion may result in the biomass reduction, accompanied by an increase in the number of dormant propagules (Orr and Knudsen, 2004). ThzID1-M3 DNA peaked along with the biomass, followed by a reduction. Unlike biomass, DNA levels increased again, along with increasing CFU, indicating the importance of dormant propagules as a DNA source. DNA levels were relatively stable between days 7 and 21 in each soil moisture level although there were substantial changes on CFU and biomass. The result indicated that DNA could not immediately reflect the changes of active and dormant propagules, likely due to the persistence of DNA of nonviable propagules in soil (Romanowski et al., 1992) and the variation of DNA content in propagules (Typas and Heale, 1980).

Soil moisture is known to be a strong factor influencing sporulation, spore germination, germ-tube growth, hyphal growth, and activity of *Trichoderma*spp. (Eastburn and Butler, 1991; Jin et al., 1991; Knudsen and Bin, 1990; Knudsen et al., 1991a). Overall means of ThzID1-M3 biomass and DNA appeared

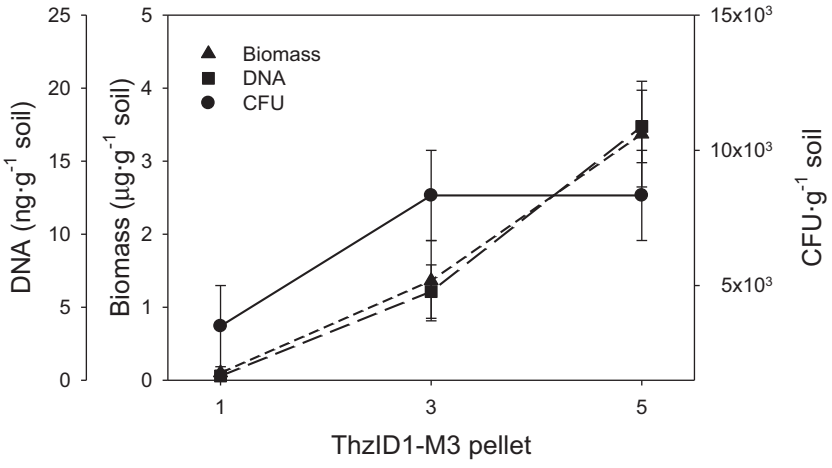


**Fig. 1.** Quantification of *Trichoderma harzianum*ThzID1-M3CFU (●), biomass (▲) and DNA (■) at 6 days after 1, 3 and 5 ThzID-M3 pellets were added into soil. Vertical bars represent ± 1 standard error of the mean ($n = 5$).