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論　文（Original article）

Quantification of the mycelial mass of the white-rot fungus *Pleurotus pulmonarius* by real-time PCR

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Abstract

For bioremediation of polluted soils with a basidiomycetous fungus, it is important to understand its population dynamics in the soils. We developed a new method for estimating the mycelial mass of a dioxin-degrading fungus, *Pleurotus pulmonarius*, from the quantification of fungal DNA amount in soils. A manganese peroxidase gene of *P. pulmonarius* was directed to the target, the DNA replication was done by the use of the developed specific primers, and the DNA concentration of *P. pulmonarius* was quantified by using a real-time PCR. There was a distinct linear relationship between the log of the initial mycelium mass of *P. pulmonarius* and the log of a standard DNA concentration in model soil. The limit of detection was 10 μg mycelia/ g soil. Changes in the mycelial mass of *P. pulmonarius* in stationary culture in a polluted soil medium were observed for over one month by using this method. It was confirmed that the population of target fungus continued to increase gradually for the first two weeks and then started to decrease significantly. Thereafter the fungal population was also ascertained to be restored rapidly by addition of nutrients. The quantitative analysis will be useful when the growth of basidiomycetous fungus in soils and natural environments must be determined at the level of existing mycelial mass.

Key words : basidiomycetous fungi, bioremediation, PCR quantification, real-time PCR

Introduction

In our previous paper (Yamaguchi et al., 2007), *P. pulmonarius* was chosen on the basis of decolorization rate of a persistent aromatic dye, Remazol brilliant blue R, from 20 species of Japanese basidiomycetous fungi as the most potent candidate for biodegradation of environmentally persistent organopollutants such as chlorinated dioxins. Since the fruit body of *P. pulmonarius* was harvested in a scale of domestic output 144.8 tons in 2007, the mushroom bed wastes appeared to have the possibility of practical application of biologically treating dioxins on farmland. It was also ascertained that the mycelium spread easily from the industrial culture bed wastes into unsterilized soils (Yamaguchi and Sekiya, 2002). Therefore, the fungal degradability toward a wide chlorine substitution range of dibenzo-*p*-dioxins was assayed under the culture condition at 28 ºC for 1 month. *Pleurotus pulmonarius* degraded mono-, di-, and trichloro- dibenzo-*p*-dioxins to some extent(>25%) and tetra-, penta-, hexa- and heptachloro-dibenzo-*p*-dioxins to moderate extent(<10%), though the fungal degradability was influenced by the chlorination pattern of the dibenzo-*p*-dioxins.

Specifically monitoring the target fungus on-site in the course of bioremediation of soils is indispensable to the control of fungal growth and population under the changeable bioremediation environment. Use of molecular biology techniques can make it possible to estimate quantitatively the mycelial mass of target fungus in soils. Spectroscopic quantification and several PCR methods such as most probable number (MPN) PCR (Picard et al., 1992; Degrange and Bardin, 1995; Murakami et al., 1997; Michotey et al., 2000; Fredslund et al., 2001), kinetic PCR (Higuchi et al., 1993), and competitive PCR (Lee et al., 1996; Baek and Kenerley, 1998; Mesarch et al., 2000) have been used to extract the necessary data from various environmental sources. However, spectroscopic A260 analysis is not always adequate to accurately measure the fungal DNA concentrations. Unless the extensive purification of soil DNA samples is performed, the spectra of coextracted humic contaminants overlap with those of the fungal DNAs in the 260-nm absorbance range (Cullen and Hirsch, 1998). Although comparative studies of soil

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DNA extraction efficiency on the basis of DNA yield have been done (Zhou et al., 1996: Cullen and Hirsch 1998), both research groups have gone through difficulties to extract DNAs precisely and reliably from soils because of the coextraction of humic contaminants. MPN PCR requires multiple handling of culture tubes, whereas kinetic PCR often has the disadvantage of relying on end-point measurements of the DNA amount produced and making it difficult to deduce the initial concentration of template DNA (Hermanson and Lindgren, 2001). With competitive PCR it is quite difficult that the same affinity for the target and competitive molecules is achieved by the primer: this difficulty complicates the quantification process (Becker et al., 2000).

As an alternative molecular biology technique, real-time quantitative PCR (RTQ-PCR) system has been recently developed. This system on the basis of continuously monitoring the DNA amplification is a simple and rapid real-time assay in a closed tube without any need of post-PCR manipulations, thus minimizing the risk of carryover contamination. In addition, a distinct advantage of this system is the ease in obtaining reliable quantitative definitions of specificity, sensitivity and efficiency of an assay as compared to the conventional end-point PCR-based assays. RTQ-PCR system has been used as an essential research tool for the quantification of gene expressions and genotyping, among others, measurement of *Stachybotrys chartarum* Conidia (Hangland et al., 1999), *Porphyromonas gingivalis* and total bacteria in plaque samples (Lyons et al., 2000), fungal DNA in clinical samples (Loefler et al., 2000),and ammonia-oxidizing bacteria in soil (Hermanson and Lindgren, 2001).

We developed a molecular biology technique for quantifying the population of *P. pulmonarius* as a specific fungus in soils. Some white-rot fungi including *P. pulmonarius* possess the gene encoding manganese peroxidase. We applied the specific DNA sequence of the manganese peroxidase for quantifying the mycelial mass of *P. pulmonarius* by real time PCR.

Materials and methods

Fungal strain

Pleurotus pulmonarius , strain Kanayama, was used. The fungus was obtained from the stock cultures of the Forestry and Forest Products Research Institute and cultured on potato dextrose medium in a shaker at 28 ºC at 100rpm for 2 weeks in darkness. The cells were harvested from the culture broth by filtration with a Kiriyama funnel (Kiriyama-seisakusyo,Tokyo,Japan).

Sequence

The harvested *P. pulmonarius* cells were freeze-dried and crushed by a multi-bead shocker (Yasui Kikai, Osaka,Japan) at 2000 rpm for 20 sec. The genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). This genomic DNA was amplified by PCR with primer MAP-F (5´-gyctgacsttggacgacgcyatt-3´) and primer MAP-R (5´-tcdgactggagbckcagytc-3´) (b=c or g or t, d=a or g or t, k=g or t, s=c or g, y=c or t.) *Escherichia coli* JM109 (Toyobo, Osaka Japan) was transformed with the amplified PCR products by using pT7 Blue vector (Takara, Tokyo Japan).

The transformant's insert DNA sequence was determined using an ABI Prism 310NT Genetic Analyzer (Applied Biosystems, Tokyo Japan) with the T7 promoter primer (5´-taatacgactcactataggg-3´) and M13 primer M4 (5´-gttttcccagtcacgac-3´) (Takara, Tokyo, Japan).

Samples for DNA extraction

Fungal mixture model

The harvested, freeze-dried, and crushed *P. pulmonarius* cells were mixed at various ratios with the *Phanerochaete chrysosporium* cells prepared in the same way as the *P. pulmonarius* cells. The ratio of *P. pulmonarius* to *P. chrysosporium* ranged from 1:0 to $1:10^5$.

Fungi/soil mixture model

A sandy soil was obtained by autoclaving at 121 ºC for 60 min. This sterilized soil was inoculated with a known mass of the fungal mixture. The weight ratio of each fungal mixture to soil was 1:9. Sterilized soil without inoculation was used as control.

Fungi/other soils mixture model

For comparison with the sandy soil, other soils such as Kanuma soil, humic soil, and garden soil, were used. Each sterilized soil was inoculated with a known mass of fungal mixture. The ratio of fungal mixture to soil was 1:9. Each sterilized soil without inoculation was used as control.

Polluted soil model

An environmental soil sample was collected from the experimental farmland at the National Institute for Agro-Environmental Sciences (NIAES), Tsukuba, Japan. The dioxin-polluted soil sample (1g) was analyzed: 1,3,6,8-Tetrachlorodibenzo-p-dioxin(1,3,6,8-TeCDD) 8300 pg; 1,3,7,9- Tetrachlorodibenzo-p-dioxin (1,3,7,9-TeCDD) 3000 pg; Octachlorodibenzo-p-dioxin (OCDD)28 000 pg. The soil was sieved with a 2-mm mesh screen and the screen-passed soil was then stored at 4 ºC.

Extraction and purification of fungal DNA from soil

Soil samples (30 mg) containing the fungal mycelium was freeze-dried and crushed by multi-bead shocker at

2000 rpm for 20 sec. The fungal DNA was extracted by the method of CTAB. The powdered sample was dissolved in 700 µl of 2% CTAB buffer (2% CTAB, 0.1 M Tris-HCl, 1.4 M NaCl), mixed gently at 65 ºC for 1 h, and allowed to cool to room temperature. Then 800 µl of chloroform in isoamyl alcohol (24:1) was added and the sample solution was mixed gently for 20 min. The mixture was centrifuged at 20,000×g for 20 min, and 630 µl of supernatant was pipetted into a test tube. Then 700 µl of chloroform in isoamyl alcohol (24:1) was added to the supernatant and the combined solution was mixed gently for 20 min. The mixture was centrifuged at 20,000×g for 20 min and 600 µl of supernatant was pipetted into another test tube. Cold isopropanol (600 µl) was added to the tube and the fluid in tube was gently mixed for 1min. The resulting solution was centrifuged at 1,000×g for 5 min and the supernatant was discarded by decantation. The precipitated DNA was washed with 70% ethanol (400 µl) and collected by centrifugation at 1,000×g for 5 min. The recovered sample was dried up and suspended in 100 µl of Tris-EDTA buffer at 55 ºC.

Realtime-PCR assay and specificity of primers

The DNA extracted from soils containing a known mycelial mass of *P. pulmonarius* was used to prepare standards for quantification by the LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany). This system was used for both amplification and online quantification. Amplification and detection were performed in glass capillaries in a total volume of 20 µl containing 2 µl LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics); 3.2 µl (5 mM) $MgCl_2$; 1 µl (0.25 µM) of each primer (MY1: 5´-ctagccgttcgctcgttttc-3´, MY2: 5´-agagtaccagcaaaatgaaccctaa-3´); 10.8 µl H_2O (sterile PCR grade); and 2 µl DNA template. The mass of fungal DNA as described above (ratio range of *P. pulmonarius* to soil, 1:10 to $1:10^6$) were quantified along with the samples and a negative control (sterile PCR-grade water). All reactions were run first to activate polymerase enzyme (10 min at 95 ºC) and then in duplicate by performing 50 cycles of repeated denaturation (15 sec at 95 ºC), annealing (10 sec at 59 ºC), enzymatic chain extension (12 sec at 72 ºC), and signal detection (1 sec at 82 ºC).

Quantification was performed by online monitoring to identify the exact time point at which the log-linear phase could be distinguished from the background (crossing point). The cycle numbers of the log-linear phase were plotted against the logarithm of the initial copy number of standard fungal DNA. The copy numbers of target fungal DNA in the soil mixtures and in the environmental samples were calculated by comparing the cycle number of the sample in the log-linear phase with the cycle number of the external standard by using LightCycler Data Analysis software.

To perform an independent validation check for the presence of amplified products, reactions were analyzed by agarose gel electrophoresis with Tris acetate EDTA buffer and 1% agarose gel followed by DNA staining with ethydium bromide.

Conditions of liquid and solid culture of *P. pulmonarius*

Liquid culture

Pleurotus pulmonarius was cultured in Kirk- high carbon high nitrogen (HCHN) liquid medium (Tien and Kirk 1998) in a shaker flask at 100 rpm at 28 ºC for 4 weeks. The mycelial mass was quantified as described above. Glucose concentration was measured with a Glucose C II kit (Wako Osaka. Japan). Ammonium concentration was measured by the indophenol method (Scheiner, 1976).

Solid culture

Pleurotus pulmonarius was precultured with Kirk-HCHN liquid medium in a shaker flask at 100 rpm at 28 ºC for 1 week. One milliliter of culture broth was inoculated into 10 g of polluted soil from NIAES in a flask and *P. pulmonarius* was cultured in stationary solid medium in soil at 28 ºC for 3 weeks. After the culture for one month, nutrients (1% of glucose and ammonium tartrate for the last density) were added to the flask. The mycelial mass contained in 0.1g of the solid medium was quantified as described above.

Results

Partial seaquence of *P. pulmonarius* manganese perooxidase gene

A partial sequence of *P. pulmonarius* manganese perooxidase gene determined is shown in Table1. A BLAST search revealed that the sequence was closely related to the gene encoding manganese peroxidase in *Pleurotus ostreatus* (E value = 2e-80). This sequence was submitted to the DDBJ/EMBL/GenBank databases (AB353725).

Specificity of primers designed for *P. pulmonarius*

To investigate the specificity of the designed primers (MY1 and MY2), the mycelia of eight white-rot fungi (*P. ostreatus, Lentinula edodes, Pleurotus eryngii, Hericium erinaceum, Pholiota aurivella, Coriolus versicolor, Phanerochaete crassa, P. chrysosporium*) along with *P. pulmonarius* were collected, and their DNAs were extracted and amplified by PCR using the primers. As shown in Fig.1, only *P. pulmonarius* DNA was amplified by the primers. Thus the designed primers were found to be specific to *P. pulmonarius*.

CCGCGGACGCCGTTCACGTACCCAACGCTCCGGTCGTACACACACCCGTTCTCACCCTCGA
CCAATTATGCGAAAACCAATCGCCTGGCAGGACGGTGCTGAATAACCATCGCATCAGTATT
TTGTTTGACTCAATGAAAGGTATAAAAGGGGGCAGCCATTGCCTCTATCTTCCAAGACCTA
ACGTCTTACTGCAGTCGCATTCCTACTTCGTAATGACCATCGCATCACTTTCCGCGCTCGT
CCTTGCCTTCGCTGCGACTGTCCAAGTTGCTCAAGGTAACACCCTCCCCGTAGCCACTGCG
TCCTTCCCTGCTGATTTGTCCTCCACGACGTGCTTCAGCGGTCTCTTTGCCTCAGAAACGT
GCAACTTGTGCTGGCGGCCAAGTTACCGCCAATGCAGCCTGTTGCGTTCTCTTCCCGCTCA
TGGAAGACCTACAGAAGAACTTGTTTGACGACGGCGCATGCGGCGAAGATGTAAGCTCGCG
CCATTCGCCACCGCTCTGGCTCATTGCTATCCATTCCAGGCCCATGAAGCCCTTCGCCTGA
CCTTCCACGATGCAATTGGATTCTCTCCTTCTAGGGGgtaggtgctagccgttcgctcgtt
ttccatccctgaaactgatttatcgcgaacagTGTCATGGGAGGCGCCGATGGCTCTGTCA
TCACGTTCTCCGACACTGAGGTTAATTTTCCAGCTAACCTTGGTATCGATGAGATCGTTGA
GGCTGAGAAACCGTTCCTCGCAAGGCATAACATCTCCGCGGGCGACTTgtatgtcatcccc
tcgttctaaggattgttactcactaatactcacacctttccttagGGTTCATTTTGCTGGT
ACTCTCGGGGTCAGTAACTGCCCTGGTGCTCCACAAAATCTCTCGTTCTTCTTGGGTCGTC
CTCCTGCCAAAGCCGCCTCGCCGATCGGATTAGTGCCTGAACCATTCGgtagacaaccgtg
ttcgactttcgccgcaaactgctaacgttcttgaatagACACCATCACAGATATTCTAGCC
CGAATGGATGACGCTGGCTTTGTCTCCGTCGAGGTTGTCTGGCTCCTTTCCGCgtacgcct
gagaccatccaccccaagctatacatctgactcatagccgatagTCACTCCGTTGCCGCAG
CTGATCATGTTGACGAAAGCgtaagtccatagactttcagtcagtatgcttgaaattgact
tgattcctttcaagATTCCCGGgtgagttaggaactatgtgtgctcacacctcatggctta
caaacgtttagAACGCCGTTCGACTCAACACCAAATCTCTTTGACTCGCAAATCTTCATCG
AGACTCAACTCCGTGGAATTTCGTTCCCAGGgtaatcaataccgccgctccccaatccacg
tacatattgctcacttgttgagtagCACCGGAGGCAATCATGGTGAAGTCCAATCTCCGCT
TAGGGGTGAAATGAGACTCCAGTCCGATCACTTGgtaagtcttcagactgtatccgagtag
caatttcacatatgcgatggtttgacagTTCGCGCGAGgtactaaaatcaatcattgctga
ttatatttccatactaagacattgcaatgtactagACGATAGGACATCTTGCGAATGGCAA
TCCATGACTAgtaagtacggtttgatcgtctacctttgtctctgaagctcactgccaacg
ataattagATGATCAGCAAAAGATCCAAGACCGATTCTCTGACACACTGTTCAAGATGTCG
ATGCTCGGACAAAACCAGGACGCTATGATTGATTGCTCCGATGTCATCCCCGTCCCCGCTG
CTCTCGTCACCAAGCCCCATCTCCCTGCCGGCAAGGTCAGGACCGACGTTGAACAGGCCgt
acgttatacgtcgcattgtagacctctcaatggtgccaatccttcc

Table 1. A partial seaquence of Pleurotus pulmonarius manganese peroxidase.
Capital letters are exon parts of the gene and small letters are introns.

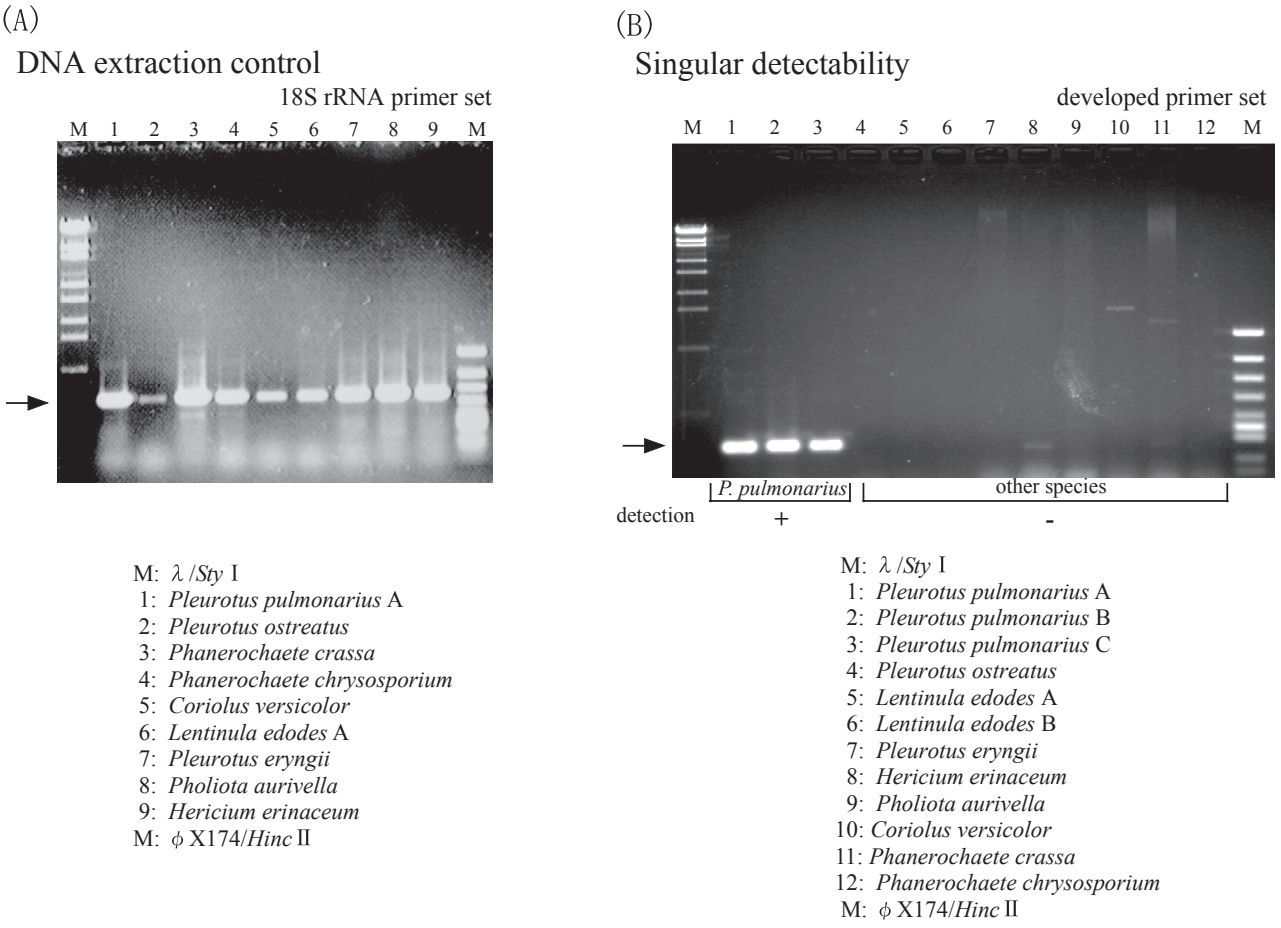

Fig. 1. (A) DNA extraction control. DNA was extracted by the CTAB method from *Pleurotus pulmonarius* and other eight white-rot fungi.
(B) Amplification of extracted DNAs by PCR with the primer set designed for *Pleurotus pulmonarius*.

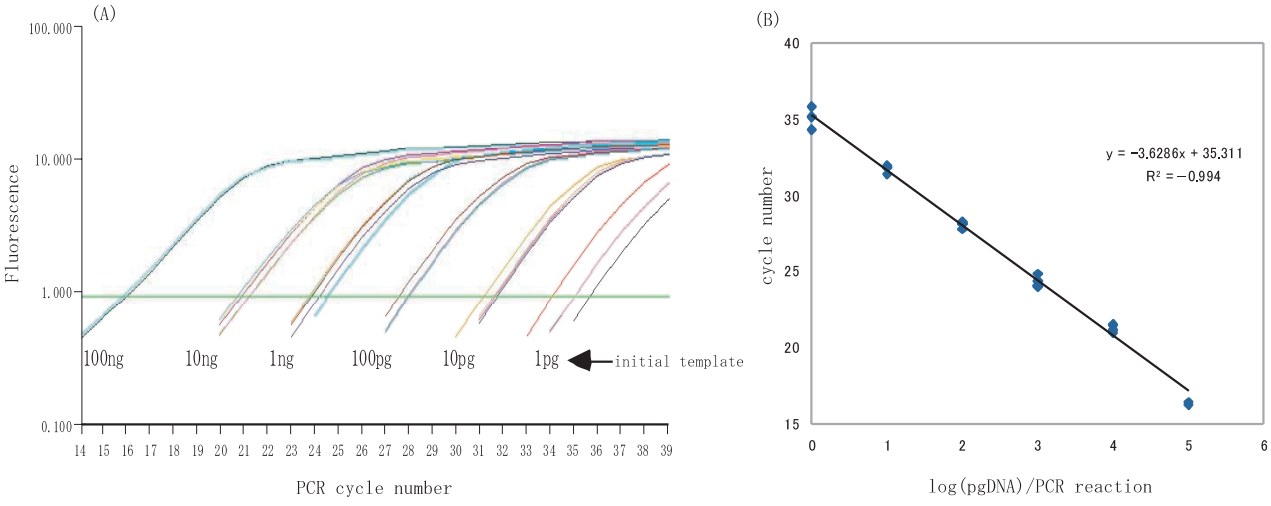

Fig. 2. (A) Amplification curve of real-time PCR. Y axis is SYBR Green I fluorescence. X axis is PCR cycle number. Arrow indicates initial template concentrations of *P. pulmonarius* DNA.
(B) Correlation between the log of DNA concentration and threshold cycle number (n = 3). Y axis is cycle number. X axis is log of DNA concentration.

Efficiency of amplification of *P. pulmonarius* DNA

To quantify of the mycelial mass of *P. pulmonarius*, the fungal DNA diluted by water was amplified by LightCycler PCR. The standard curve (Fig. 2 A, B) generated from the fungal standards revealed a distinct negative linear relationship (R^2 =-0.994) between the log of the starting DNA volume of *P. pulmonarius* and the threshold cycle number.

Quantification of mycelial mass of *P. pulmonarius* in sandy soil

The fungal DNA was extracted from the soil mixture described above and specifically amplified by using primers MY1 and MY2. The standard curve (Fig. 3) showed a distinct positive linear relationship (R^2 = 0.9688) between the log of the starting mycelial mass of *P. pulmonarius* in the model soil and the log of the standard DNA concentration. The limit of detection was 10 µg mycelia/g soil.

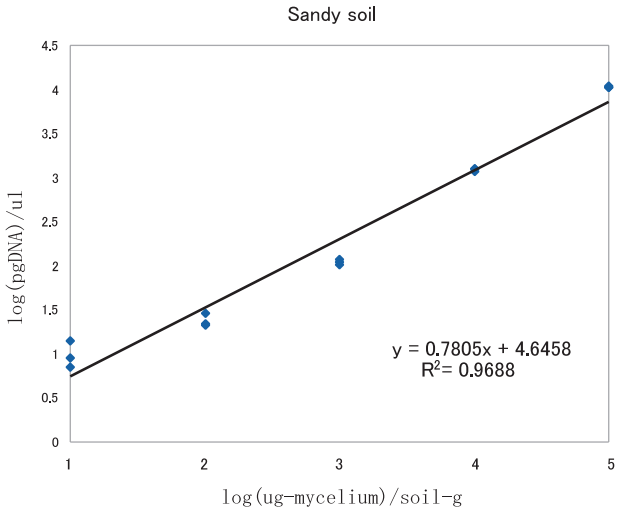

Fig. 3. Correlation between log of DNA concentration and log of mycelial mass (n = 3) in sandy soil. Y axis is log of pg-DNA/1- µ l reaction solution. X axis is log of µ g-mycelia/1 g-soil.

Quantification of mycelial mass of *P. pulmonarius* in other soils

The fungal DNA was extracted from the other soil mixtures described above and amplified by using primers MY1 and MY2. Each of the standard curves (Fig. 4) showed a linear relationship between the log of the starting mycelial mass of *P. pulmonarius* in the model soil and the log of the standard DNA concentration. The limit of detection was 10 µg mycelia/g soil for the Kanuma and garden soils and 100 µg mycelia/g soil for the humic soil.

Inhibition of PCR quantification in humic soil

The fungal DNA was extracted from soils containing known fungal masses, and the undiluted and diluted DNA solutions were quantified by real-time PCR. The undiluted DNA solution from humic soil was not adequate to quantify DNA, but the diluted DNA solution from humic soil could be quantified (Fig. 5). The results indicate that the extraction of DNA was successful but the coextraction of organic matter in humic soil affected the PCR reaction. However, the sensitivity of detection by fluorescence was satisfactorily adequate only if the diluted DNA solutions were used.

Population dynamics of *P. pulmonarius* in polluted soil

The population of *P. pulmonarius* in solid stationary culture at 28 ºC was monitored for one month and two weeks by real-time PCR (Fig. 6). The population of *P. pulmonarius* continued to increase gradually for the first two weeks and then started to decrease significantly. Thereafter the fungal population was ascertained to be restored rapidly by addition of nutrients.

Discussion

During bioremediation, monitoring the pollutants-degrading organism on-site is important because bioaugumentation can mean the insertion of a biohazard into the natural ecosystem. We developed two primers for monitoring the changing populations of the white-rot fungus *P. pulmonarius* in polluted soils. The primers could be used for the *P. pulmonarius* specific detection and the quantitative analysis of the mycelial mass by PCR.

The minimum limit of detection was affected by the condition of PCR reaction, the temperature of the SYBR Green I detection system, and the conditions of DNA extraction from soil. The condition of PCR reaction was optimized by setting the annealing temperature to higher one (59 ºC) than 55 ºC in normal PCR reactions. An extension time was suited to a 300-bp length. The double-stranded DNA quantification temperature was increased to 82 ºC, because lower temperature than that in the PCR amplification would not keep sufficient specificity. The optimization of conditions has become feasible to detect the target DNA within a minimum limit of 1 pg.

The extracted *P. pulmonarius* DNA samples contained some humic substances. The impurities affected the PCR reaction and thus the DNA quantification. However, the target DNA could manage to be adequately detected by diluting the extracted DNA samples by 10 or more volume of water because our employed method enabled a quite low limit of DNA detection.

The mycelial mass of *P. pulmonarius* continued to

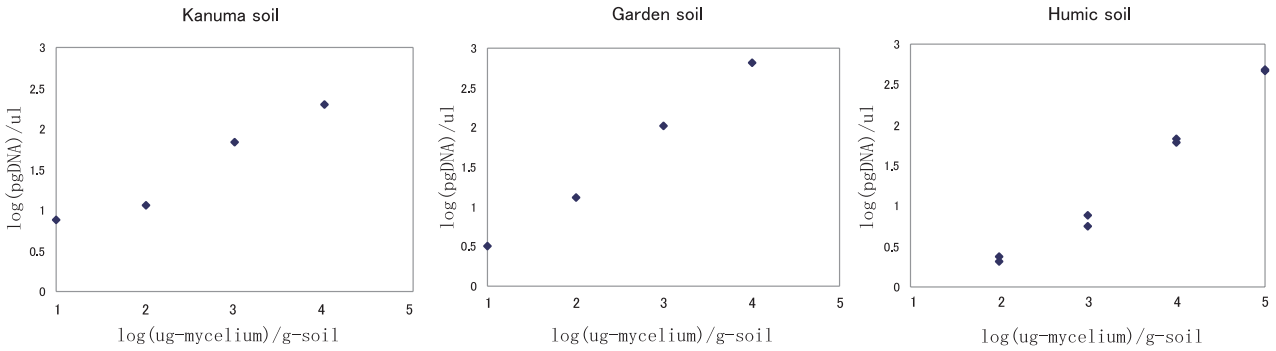

Fig. 4. Correlation between log of DNA concentration and log of mycelial mass (Kanuma soil, garden soil: n = 1; humic soil: n = 2).

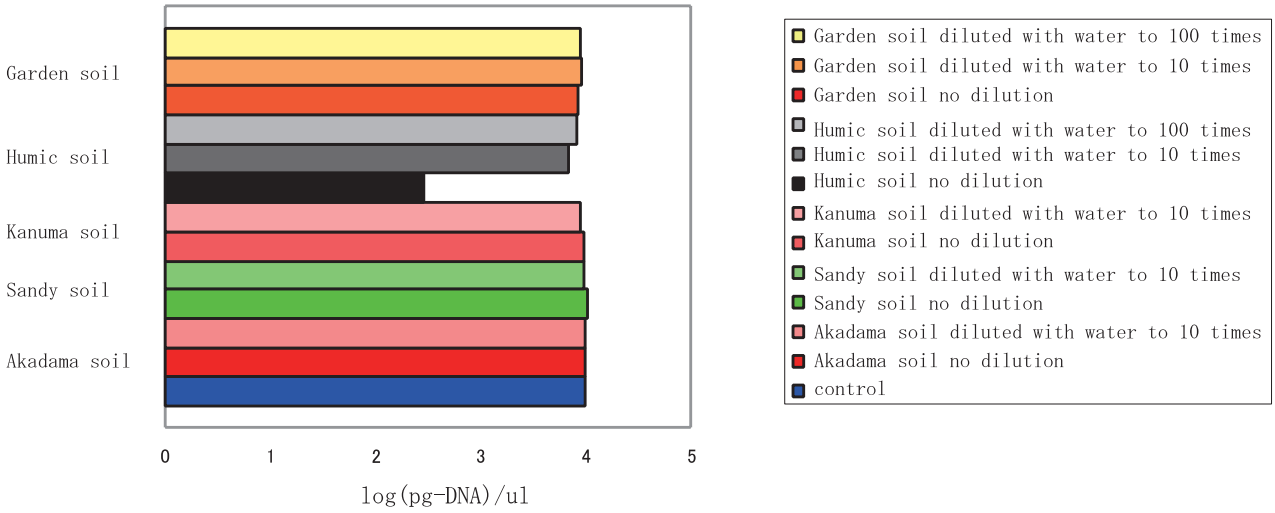

Fig. 5. Graphical representation of PCR results for various soils, using 10 μg/μl of DNA solution.

increase gradually in the contaminated soil for the first 2 weeks and then began to decrease significantly. Thereafter the fungal population was ascertained to be restored rapidly by addition of nutrients. The results suggested that the population of specific fungus in soil could be monitored by real-time PCR at the detection limit of 10μg mycelial mass per g soil. This quantitative analysis will be useful when the growth of basidiomycetous fungus in soils and natural environments must be determined at the level of existing mycelial mass.

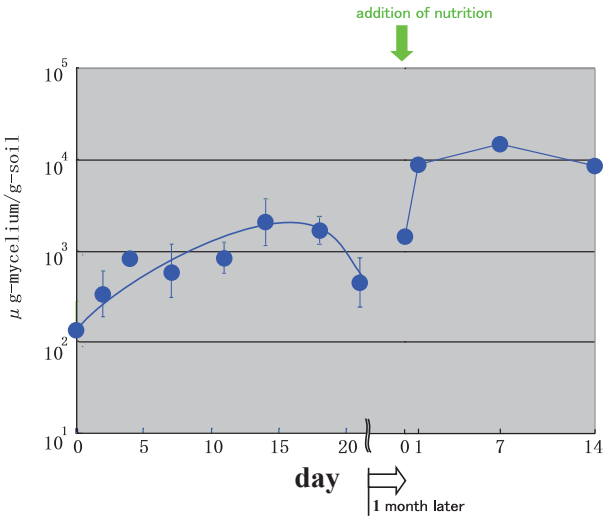

Fig. 6. Growth of *P. pulmonarius* (n = 3). Y axis is the mycelial mass of *P. pulmonarius* (μg/1 g-soil). X axis is day of culture.

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リアルタイム PCR による白色腐朽菌ウスヒラタケ菌糸体の定量

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要 旨

汚染物質分解菌による汚染土壌の微生物環境修復にとって、環境中での分解菌の個体数動態の把握は重要である。我々はダイオキシンを分解する白色腐朽菌 Pleurotus pulmonarius（ウスヒラタケ）菌糸体の土壌中での特異的定量法を開発した。ウスヒラタケのマンガンペルオキシダーゼの遺伝子配列を対象として特異的プライマーを開発し、リアルタイム定量 PCR 法により、ウスヒラタケの DNA 量を定量可能にした。土壌中のウスヒラタケ菌糸体量と土壌から抽出された DNA 量との間に高い直線の相関が認められたことから、DNA 量を基に菌体量を 10 μg 菌糸体／ g 土壌の検出精度で定量することができた。

本定量法を用いて汚染土壌を固体培地とするウスヒラタケの培養での菌糸体量経時変化を 1 ヶ月以上にわたり観察したところ、当初 2 週間緩やかな増殖が続いた後、菌糸体量は減衰を始めた。その後、固体培地に栄養源を添加したところ再度増殖を観察した。この定量法は、土壌や自然環境下での担子菌の増殖の程度を培地あたりの菌糸体量で把握する場合に利用できる。

キーワード：担子菌、バイオレメディエーション、定量 PCR、リアルタイム PCR

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