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Detection of *Rhizina undulata* in Soil by Nested-PCR Using rDNA ITS-specific Primer

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Abstract : *Rhizina undulata* is the fungus, which causes Rhizina root rot on coniferous trees. Nested-PCR using ITS-specific primer was applied to detect *R. undulata* from the soils of Japanese black pine (*Pinus thunbergii*) forests infested with the disease in Seocheon, Chungnam Province, South Korea. Soil samples were collected from four different sites, both dead trees and fruit bodies of *R. undulata* were present, dead trees only present, fruit bodies only present, and both were absent. Nested-PCR products specific to *R. undulata* ITS-region were amplified. Positive reactions were found in some samples from the sites, where dead trees and fruit bodies of *R. undulata* were absent as well as where both of those were present. *R. undulata* was mainly detected in the soil samples from the depth of 5~20 cm under the soil surface. These results show that the nested-PCR could be used to diagnose the presence or potential infestation of *R. undulata* in the soils of pine forests.

Key words : Rhizina undulata, *ITS-specific primer, nested-PCR*

Introduction

The causal fungus of Rhizina root rot is *Rhizina undulata* Fr ex Fr. (Syn. *Rhizina inflata* (schaeff) Karst). Rhizina root rot is one of the soil-borne diseases occurring on coniferous trees, and causes severe damages on coastal pine forests in Korea (Lee and Kim, 1990). *R. undulata* occurs in temperate regions throughout the world including United States, Europe, Japan, and South Africa. It incites a root rot of conifers known as "group dying" (Lundquist, 1984; Murray and Young, 1972; Zinno and Shoji, 1981; Lee and Kim, 1990). Infection is initiated by the heat shock-induced germination of ascospores, and spreads radially for several years in soil about 3~5 m per year (Sato *et al.*, 1974).

A rapid and efficient detection of a causal pathogen is very important to control plant diseases. A protocol for detecting *R. undulata* directly from soil is not developed yet. Detection by trap-logs of *R. undulata* using pine twigs as indicators is a laborious and time-consuming work (Sato *et al.*, 1974).

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In recent years, the primer sequences for the ITS region of the nuclear ribosomal DNA have been widely used for resolving phylogenetic relationships at the species or generic levels (White *et al.*, 1990). In addition, sequences of the nuclear ribosomal DNA ITS region are known providing species-specific genotypes in many fungi (Egger, 1996; Norman and Egger, 1996). Therefore, The ITS regions were used commonly in PCR detection of many fungal pathogens (Chao *et al.*, 2004; Chiang *et al.*, 2001; Kikuchi *et al.*, 2000; Lu *et al.*, 2002; Ranjard *et al.*, 2001). We have also analysed the nucleotide sequence of *R. undulata* ITS-region, and designed ITS-specific primers for PCR to detect *R. undulata* (Lee *et al.*, 2007).

A commonly occurring problem in conventional PCR is a binding of primers to incorrect regions of the DNA, giving unexpected and incorrect products. Nested-PCR is a modified PCR intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites (Robert and Farrell, 2005; Sugita *et al.*, 2001). Indeed, nested-PCR was applied effectively to detect not only fungal pathogens but also bacterial pathogens (Lee *et al.*, 2001).

In this study, nested-PCR using ITS-specific primers was applied to detect *R. undulata* in soil samples of Japanese black pine forests (*Pinus thunbergii*) at Seocheon,

Chungnam Province, South Korea.

Materials and Methods

1. Soil sample collection

Soil samples were collected from Japanese black pine (*Pinus thunbergii*) forests infested with *R. undulata* in Seocheon, Chungnam Province, South Korea, where Rhizina root rot has been found since 2005. In this area, the damage by Rhizina root rot has been increased annually. The soil properties were sandy loam, and the soil samples collected are shown in Table 1.

2. Total DNA extraction from soil samples

Total DNA was extracted from 0.4 g of soil using Power soil DNA isolation KIT (MOBIO).

Table 1. Soil samples collected from Japanese black pine (*Pinus thunbergii*) forests in Seocheon, Chungnam province.

Sample No*.	Soil depth (cm)	Presence / Absence	
		Dead tree	Fruiting body of *R. undulata*
A - 1	5~10	p	p
2	15~20		
B - 1	5~10		
2	15~20		
C - 1	5~10		
2	1~20		
D - 1	5~10	p	a
2	15~20		
E - 1	5~10		
2	15~20		
F - 1	5~10		
2	15~20		
G - 1	5~10	a	a
2	15~20		
H - 1	5~10		
2	15~20		
I - 2	5~ 10		
2	15~20		

*Soil samples were randomly collected from the sites described.
p : presence, a : absence

3. Primer and Nested-PCR

R. undulata-specific primer pairs for conventional PCR, RUU and RUD, were designed by the specific sequence to *R. undulata* ITS region (Lee *et al.*, 2007). Primer ITS1 and ITS4 for the 1st PCR of nested-PCR, conserved sequence in ITS region of general ascomycetous fungi, was designed by the sequence according to White *et al.* (1990). Primers RUU and RUD were used in the 2nd PCR of nested-PCR for the detection of *R. undulata*. The locations of the primer pairs are shown in Figure 1.

Conventional PCR assays were performed with 2 μL of total DNA, 1 μL (0.4 mM) of dNTPs mixture, 1 μL (5pM) of primer pairs (RUU/RUD), 2.5 μL of 10 × PCR buffer, 2.5 μL (2.5 mM) of $MgCl_2$ solution and 0.5 μL (2.5 unit) of DNA Taq polymerase (Promega). A final volume of 25 μL of the PCR reaction mixture was made up with sterile distilled water. In nested-PCR, ITS1/ITS4 primer pair was used for the 1st PCR in the same reaction mixture as conventional PCR. The second PCR was performed with RUU/RUD primer pair by adding 2 μL of the amplified product from the 1st PCR to a new reaction mixture with the same composition as the 1st PCR. Amplification condition in Minicycler (MJ Research) of all reaction was shown as Table 2. PCR products were analyzed by electrophoresis (Mupid 21, COSMO BIO Co.) in 1.5% agarose gel, stained with ethidium bromide and then visualized by UV transilluminator (Gel Documentation System, Bio-Rad).

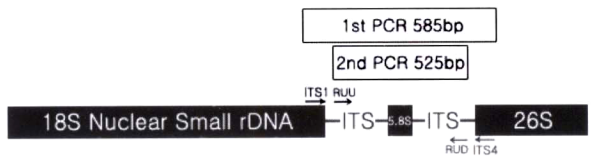

Figure 1. Locations and predicted sizes by PCR primers on nuclear rDNA (18S rDNA and ITS region).

4. Sequencing

Nested-PCR fragments were eluted from agarose gel using a Wizard SV Gel and PCR Clean-Up System (Promega Co.), ligated in a pGEM-T easy vector (Promega),

Table 2. Primers and reaction program used in nested-PCR.

Primers	Sequence (5'→3')	Reaction program	Size of PCR products
1st PCR ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	I. 95°C for 3 min. II. 45 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 2 min. III. 72°C for 10 min.	585bp
2nd PCR RUU RUD	GAGGTCAAACTCTGAGAGTC CCGCACGTTTCATACAACCC	〃	525bp

and cloned in competent *Escherichia coli* cells. Plasmid DNA from transformed cells were purified by Wizard™ Miniprep DNA Purification System (Promega Co.). Purified plasmid DNAs were sequenced by High Throughput DNA Sequencer (MJ Research).

Results

1. Conventional PCR detection

PCR detection using RUU/RUD primers specific to ITS-region of *R. undulata* was applied to the soil of Japanese black pine (*P. thunbergii*) forest infested with the disease in Seocheon, Chungnam Province (Figure 2). PCR products specific to *R. undulata* ITS region were found in all of the soil samples collected from the sites, where dead trees and fruit bodies of *R. undulata* were present. PCR products specific to *R. undulata* ITS region were also amplified in some soil samples, where either dead trees or fruit bodies of *R. undulata* were present (Figure 2: e-1), and where both were not (Figure 2: i-1). *R. undulata* was detected mainly from the depth of 5~10 cm under the soil surface. PCR products of some soil samples from the depth of 15~20 cm were not amplified (Figure 2: a-2, e-2, i-2), while the soil samples collected from the depth of 5~10 cm at the same collecting sites showed positive reaction by PCR (Figure 2: a-1, e-1, i-1).

2. Nested-PCR detection

Nested-PCR using ITS1/ITS4 and RUU/RUD primer pairs was also applied for the detection of *R. undulata* in soils collected from Japanese black pine (*P. thunbergii*) forest infested with the disease in Seocheon, Chungnam Province (Figure 3). In the 1st PCR, PCR products about 590 bp specific to ITS1 and ITS4 primer were amplified from all of the soil samples examined (Figure 3-A). But, PCR products specific to *R. undulata* ITS region were screened and amplified by the 2nd PCR using RUU/RUD primers (Figure 3-B). PCR products specific to *R. undulata* were found in all of the soil samples collected from the sites where dead trees or fruit bodies of *R. undulata* were present, except in 2 samples (Figure 3-B: e-2, f-1). PCR products were also found in some soil samples collected from the sites where dead trees and fruit bodies of *R. undulata* were absent (Figure 3-B: h-2, i-1). Nested-PCR reacted positively to the soil samples, which showed negative reaction by the conventional PCR (Figure 3-B: a-2, d-1, d-2, f-2, h-2). These results showed that nested-PCR was more sensitive than the conventional PCR for detecting *R. undulata* from soils (Table 3). Two samples (Figure 3-B : a-2, h-2) of nested-PCR products were randomly selected for nucleotide sequence analysis. The sequences of two samples were completly matched with the sequence of *R. undulata* rDNA ITS region.

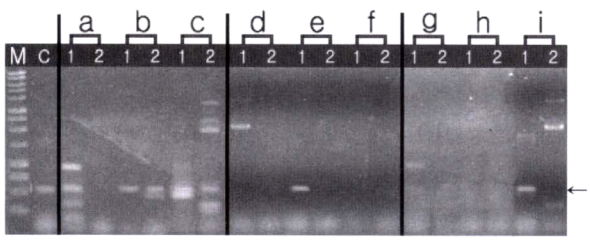

Figure 2. Conventional PCR detection of *R. undulata* in soil using ITS-specific primer (RUU/RUD) of *R. undulata*. Soil samples were collected randomly at the coastal pine stands (*P. thunbergii*) damaged by Rhizina root rot in Seocheon, Chungnam Province. PCR products of total DNA extracted from the soil samples by ITS-specific primer of *R. undulata* were analysed on 1.5% agarose gel. a~i represent characteristics of the sites, where soil samples were collected. a~c, presence of dead trees and fruiting bodies; d~f, presence of dead tree, but absence of fruiting body; g~i, absence of dead tree or fruiting body. Lane 1 and 2 within each of alphabet from a to i represent soil samples collected from 5~10 cm and 15~20 cm under the soil surface, respectively. Lane M and C represent 1kbp ladder (Promega Co.) and PCR products from mycelial culture of *R. undulata* as the control, respectively. Arrow indicates predicted size of PCR products by *R. undulata* ITS specific-primers.

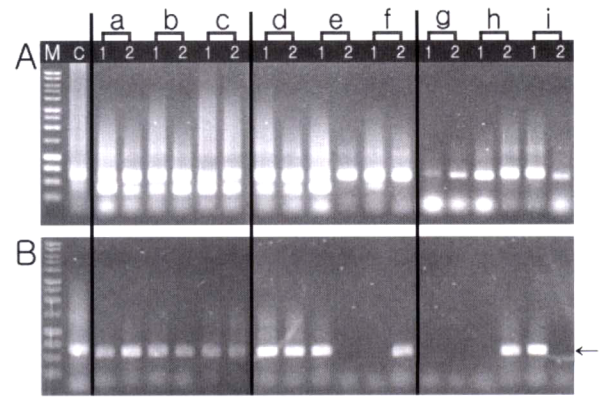

Figure 3. Nested-PCR detection of *R. undulata* from soils using ITS-specific primer. Soil samples were collected randomly from the coastal forest stands of Japanese black pine damaged by *Rhizina* root rot in Seocheon, Chungnam Province. (A) and (B) shows PCR products by the 1st PCR using ITS1/ITS4 primers and the 2nd PCR using RUU/RUD primers from the soil samples, respectively. a~i represent characteristics of the sites, where soil samples were collected. a~c, presence of dead trees and fruiting bodies; d~f, presence of dead tree and absence of fruiting body; g~i, absence of dead tree or fruiting body. Lane 1 and 2 within each of alphabet from a to irepresent soil samples collected from 5~10 cm and 15~20 cm under the soil surface, respectively. Lane M and C represent 1 kbp ladder (Promega Co.) and PCR products from mycelial culture of *R. undulata* as the control, respectively. Arrow indicates predicted size of nested-PCR products by *R. undulata* ITS specific-primers.

Table 3. Comparison of PCR and nested PCR in *R. undulata* detection from soil samples of the Japanese black pine (*P. thunbergii*) forest in Seocheon, Chungnam province.

Sample No.	Soil depth (cm)	Presence/ Absence		*Detection of *R. undulata*	
		Dead tree	Fruiting body	Nested-PCR	PCR
A - 1	5~10	p	p	+	+
2	15~20			+	-
B - 1	5~10			+	+
2	15~20			+	+
C - 1	5~10			+	+
2	15~20			+	+
D - 1	5~10	p	a	+	-
2	15~20			+	-
E - 1	5~10			+	+
2	15~20			-	-
F - 1	5~10			-	-
2	15~20			+	-
G - 1	5~10	a	a	-	-
2	15~20			-	-
H - 1	5~10			-	-
2	15~20			+	-
I - 1	5~10			+	+
2	15~20			-	-

p : presence, a : absence.
*+ : positive reaction; − : negative reaction.

Discussion

R. undulata is one of the soil-borne tree pathogens. It is important to detect pathogen for the effective control of the disease. Diagnosis and detection of soil-borne pathogens were usually more difficult than other pathogens. Soil conditions with strong binding of microorganisms to soil particles and humic acids hinder PCR reactions (Kageyama *et al.*, 2003). Many researchers have tried to solve these problems (Cullen and Hirsch, 1998; Kreader, 1996; Porteus *et al.*, 1994), and PCR techniques have been enabled to detect soil-borne pathogens as a direct method for amplification of DNA from soil (Kageyama *et al.*, 2003).

It is obvious that a PCR-based method is very effective for diagnosing infection, and there are many reports of PCR-based diagnosis methods (Kikuchi *et al.*, 2000; Lu *et al.*, 2002; Jacquot *et al.*, 2000; George *et al.*, 2000; Hayasaki *et al.*, 2001; White *et al.*, 2006; Binnicker *et al.*, 2007). The specificity of PCR is determined by the specificity of the PCR primers. If the primers bind to more than one locus, non-specific segments of DNA will be amplified. To control for these possibilities, nested primers were often recommended. Nested PCR means that two pairs of PCR primers are used for a single locus (Robert and Farrell, 2005; Sugita *et al.*, 2001). In deed, there are many reports of nested-PCR diagnosis methods (Jaeger *et al.*, 2000; Jung *et al.*, 2003; Sugita *et al.*, 2001).

Sequences of the nuclear ribosomal DNA ITS region are known to provide species-specific genotypes in many fungi (White *et al.*, 1990), and used commonly in detection of many fungal pathogens (Chao *et al.*, 2004; Kikuchi *et al.*, 2000; Lu *et al.*, 2002). Lee *et al.* (2007) designed *R. undulata* ITS-specific primers. PCR detection using *R. undulata* ITS-specific primer detected up to 1ng mycelium of *R. undulata* existing in 100 g of soil.

In this study, we tried to detect *R. undulata* in the field soil by nested-PCR using the primers specific to ITS-region of *R. undulata*. PCR products specific to *R. undulata* ITS region were found in all of the soil samples collected from Japanese black pine (*P. thunbergii*) forests, where dead trees or fruit bodies of *R. undulata* were present, in Seocheon, Chungnam Province. PCR products were also amplified from some soil samples collected from the sites, where dead trees or fruit bodies of *R. undulata* were absent. It indicates that *R. undulata* was present in the soil, where any symptoms or signs by *R. undulata* were not found. Nested-PCR protocol was more sensitive than conventional PCR protocol to detect *R. undulata*. Nested-PCR could amplified the PCR products specific to *R. undulata* from four soil samples, which showed negative reaction by conventional PCR protocol.

A nested-PCR assay had high sensitivity and specificity for *R. undulata*. The selective specificity was confirmed by sequencing the nested-PCR amplification products. Two samples of nested-PCR products was randomly selected for analyzing nucleotide sequences, and the results showed that the sequences of two samples were completely matched with the sequence of *R. undulata* rDNA ITS specific region. In conclusion, nested-PCR protocol could be used successfully to detect *R. undulata* from forest soils.

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