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Direct Detection of *Cylindrocarpon destructans*, Root Rot Pathogen of Ginseng by Nested PCR from Soil Samples

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We have successfully applied the nested PCR to detect *Cylindrocarpon destructans*, a major pathogen causing root rot disease from ginseng seedlings in our former study. The PCR assay, in this study, was used to detect the pathogen from soils. The nested PCR using internal transcribed spacer (ITS) 1, 4 primer set and Dest 1, 4 primer set maintained the specificity in soils containing various microorganisms. For a soil DNA extraction method targeting chlamydospores, when several cell wall disrupting methods were tested, the combination of lyophilization and grinding with glass beads, which broke almost all the chlamydospores, was the strongest. The DNA extraction method which was completed based on the above was simple and time-saving because of exclusion of unnecessary stages, and efficient to apply in soils. As three ginseng fields whose histories were known were analyzed, the PCR assay resulted as our expectation derived from the field information. The direct PCR method will be utilized as a reliable and rapid tool for detecting and monitoring *C. destructans* in ginseng fields.

KEYWORDS : *Cylindrocarpon destructans*, Diagnosis, Ginseng, Nested PCR, Root rot

Ginseng (*Panax ginseng* C. A. Meyer and *Panax quinquefolius* L.), belongs to Araliaceae of perennial plants, and is an economically important cash crop in Korea and North America [1]. Consumption of the crop is gradually increasing in Southeast Asia and America while the yield in Korea is decreasing currently because of an injury by continuous cropping and exhaustion of the first planting ginseng fields. In Korea, the production of ginseng requires a 4- to 6-year cultivation period and, throughout this time, soil borne pathogens may increase more in the rhizosphere than in that of North America with a 3- to 4-year cultivation period; Yield loss to disease is proportion to the cultivation period [2, 3]. One of the major pathogens of ginseng is *Cylindrocarpon destructans* [4, 5], which may be more serious in Korea with smaller size of land than in other countries because the pathogen is implicated in replant failure.

Research on ginseng root rot disease caused by *C. destructans* has not been carried out for a long time in Korea. It had been assumed about the disease that the replanting failure had occurred because of the amassment of poison and the deficiency of mineral elements in the ginseng field. The difficulty of study on *C. destructans* is because chlamydospores as main form in soil germ rarely, mycelial growth is slow, and the host of *C. destructans* is not economic crops except ginseng [6].

Diagnostic systems based on PCR have been developed for plant pathogenic fungi [7-11]. The classical methods of diagnosis are both time-consuming and laborious [12], requiring isolation of the fungus from diseased tissue. Moreover, because the fungus grows slowly, colonies arising from diseased tissue are often overgrown by more rapidly growing fungi and rare germination of chlamydospores makes the spread plate method of soil samples unusable [5, 10].

A nested PCR-based assay was developed for the detection of *C. destructans* in pine and spruce seedlings. Preserving specificity, the PCR assay has detected the pathogen from roots of the host plants [13]. We have utilized the PCR to detect the pathogen from the roots for selection of the non-infested one-year-old ginseng seedlings. To apply the technique to soil samples containing various PCR inhibitors, DNA purification method to recover the high quality and plenty DNA were required [14]. Our objective of the study is to develop the DNA-based method for the detection of *C. destructans* directly from the ginseng fields and ultimately for choice of the non-infected fields for ginseng cultivation in the future.

Materials and Methods

Pathogen. *C. destructans* was collected from diseased ginseng roots and infested soils located at major ginseng cultivating areas in Korea. The pathogen was isolated with single conidia on potato dextrose agar (PDA) containing streptomycin sulfate at 15°C, grown on PDA and SNAY (supplemented nutrient agar plus yeast extract) media at 20°C in the dark for a month and observed with × 100 and × 400 microscopes [15, 16].

Conidia were produced by culture on PDA media at

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15°C for 3 weeks and chlamydospores were produced by culture on potato dextrose broth and V8 20% juice broth media at 180 rpm, 15°C for over one month in a shaking incubator. Subsequently, hemacytometer was used to determine spore concentrations.

Genomic DNA extraction. Genomic DNA was extracted from fungal cultures grown on SNAY broth media for 2 weeks. Mycelia were harvested from liquid cultures by filtration through cheesecloth, and DNA was extracted with cetyltrimethylammonium bromide (CTAB) method [17]. About 10 mg of lyophilized mycelia were ground in 1.5 mL effendolf tube by sterilized wooden sticks and added 400 μL extraction buffer and 400 μL CTAB solution. The mixture was extracted by 600 μL chloroform : isoamylalcohol (24 : 1), vortexed and centrifuged for 10 minutes at 10,000 × g. The aqueous phase was precipitated with 0.7 volume of cold isopropanol and centrifuged (10,000 ×g, 10 min). The pellets were washed with 70% ethanol, air dried, re-suspended in 50 μL of H_2O, and stored at −20°C until needed.

Cell wall disrupting test. Several methods for cell wall disruption were tested to apply to soil DNA extraction method for targeting chlamydospores. It is impossible to separate the chlamydospores from the mycelia, so the cultured micelial-chlamydospores were used in this experiment. The broth culture was homogenized (1,300 ×g, 5 min) and filtrated through two layers of sterile cheesecloth. And the filtrates were concentrated by centrifugation, adjusted to a concentration of 1×10^5 chlamydospores/mL. Each 1 mL is placed into 1.5 mL effendolf tubes, and four methods were carried out for cell wall disruption as follows.

a) TENP solution [18]: 400 μL of TENP solution (50 mM Tris [pH 8.0], 20 mM EDTA, 100 mM NaCl, 1% PVPP) was added to chlamydospores in 1.5 mL effendolf tube.

b) TENP solution and glass bead homogenization: 400 μL of TENP solution and 0.3 g of glass bead (0.09~0.15 mm diameter) were added and vortexed for 30 minutes. This is current method using glass bead [12, 19-22].

c) 10% SDS and freeze-thawing: 300 μL of 10% SDS was added and freeze-thawed three times.

d) Lyophilization and glass bead grinding (Fig. 1C): grinded with glass bead in 1.5 mL effendolf tube by manual grinder after lyophilization.

In the microscopic examination, hemacytometer was used to determine concentrations of entire or broken chlamydospores.

PCR amplification. Primers of nested PCR used in this study were designed by Hamelin *et al.* [13] and PCR conditions were modified a little. The species-specific primer set, Dest 1 (5'-TTGTTGCCTCGGCGGTGCCTG-3') and Dest 4 (5'-GGTTTAACGGCGTGGCCGCGCTGTT-3'), was used in the second round of PCR to amplify about 400 bp fragment from 600 bp of the first round by internal transcribed spacer (ITS) 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primer set based on the ITS region.

PCR reactions of the first round were carried out in a volume of 50 μL containing 5 μL of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTP, 0.4 μM each of primer and 1 unit of Taq DNA polymerase (Takara, Japan). The reactions were carried out on a MJ-Research PTC-100™ thermal cycler (Watertown, MA, USA) and consisted of an initial denaturation at 95°C for 3 min, 30 cycles of 95°C for 35 s, 55°C for 1 min, and 72°C for 2 min. The reactions were completed by a 8 min extension at 72°C.

The second round amplification with species-specific internal primers, Dest 1 and Dest 4 was conducted using as a template 1 μL of PCR product of the first round. The PCR condition was the same as the previous amplification, and reactions were carried out in volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTP, 0.8 μM each of primer and 1 unit of Taq DNA polymerase.

Southern hybridization. Detection of *C. destructans* was verified by DNA hybridization. PCR products were analyzed by electrophoresis in a 1.5% agarose gel and the gel was blotted on a Nylon transfer membrane (Schleicher & Schunell, Dassel, Germany) [23].

400 bp of species-specific fragments of *C. destructans* amplified by Dest 1 and 4 as probe were used for certification of specific detection in southern hybridization. Synthesis of probe DNA and luminescent detection of target DNA were experimented with DIG DNA Labeling and Detection Kit following by the manufacturer's protocol (Roche, Mannheim, Germany).

Results

Cell wall disrupting test. Physical methods by direct breaking (b, d) were more effective than the rest indirect methods (a, c) on the whole. The best method, combination of lyophiliation and glass bead grinding (d) shows cell wall disruption rate of 92% and method b was 67%. The indirect methods, a and c were low, 1% and 2.3%, respectively, and this result indicates that reagents associated with these two indirect methods were not effective by themselves for thick cell wall of *C. destructans* at all (Fig. 1A).

DNA extraction method for soil. A method for extraction of the DNA from soil was designed according to the

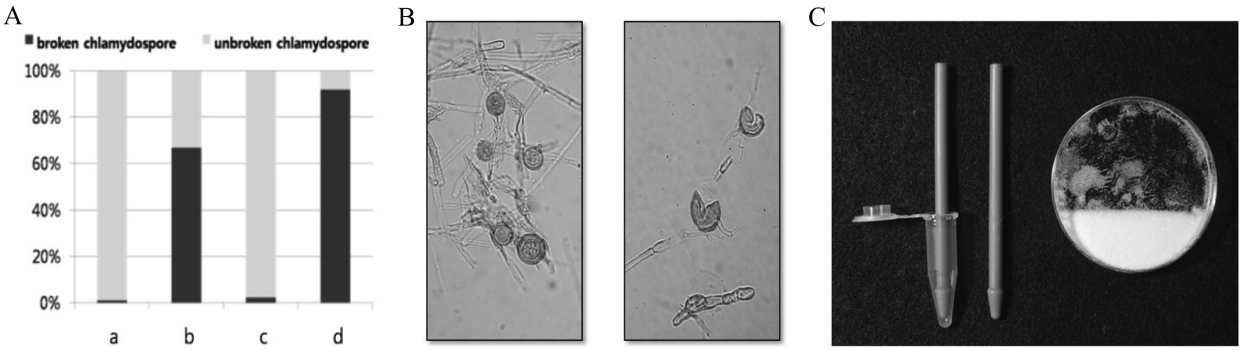

Fig. 1. Four methods were tested to break chlamydospores of *Cylindrocarpon destructans*. A, Graph showing the ratio of broken chlamydospores by each method, method a, TENP solution, b, TENP solution and glass bead (0.09~0.15 mm diameter) homogenization, c, 10% SDS and freeze-thawing, d, lyophilization and glass bead grinding. B, Chlamydospores of *C. destructans*, left: unbroken chlamydospores before the test, right: chlamydospores broken by method d. C, Manual grinder fitted on a 1.5 mL effendolf tube and glass beads which used in method d.

Soil 0.2 g
↓
0.7 mL H_2O (× 3)
↓
Lyophilization
↓
Glass bead grinding
↓
0.4 mL TENP solution
↓
Chloroform : Isoamylalcohol (24 : 1)
↓
G-spin kit
(Intron Biotechnology, Seongnam, Korea)
↓
Dissolved in 200 μL H_2O and diluted ten fold
↓
Nested PCR

Fig. 2. Detection procedure to target *Cylindrocarpon destructans* from soil designed in this study.

above (Fig. 2). Each soil sample of 0.2 g from ginseng fields was placed in 1.5 mL effendolf tubes, washed with 700 μL H_2O, centrifuged (4,000 ×g, 5 min) and aqueous phase was removed (× 3), then lyophilized.

Prepared soil samples were thoroughly ground with 0.2 g glass beads by a manual grinder (Fig. 1C) and 400 μL TENP solution was added. The mixture was extracted by 600 μL chloroform : isoamylalcohol (24 : 1), vortexed and centrifuged for 10 minutes at 10,000 ×g.

The aqueous phases were purified by G-spin™ Genomic DNA extraction kit (Intron Biotechnology, Seongnam, Korea). The samples were applied from as the step 7 of the manual without precipitation phase by ethanol or isopropanol, and the purified DNA was eluted in 200 μL H_2O, and if needed, diluted ten-fold for PCR reactions of soil DNAs in the final step (Fig. 2).

Specificity of nested PCR in soil samples. Eight soil samples from four infested ginseng fields and five samples from other regions expected not to have *C. destructans* were tested for the specificity of nested PCR on field soils containing so many microbes. Each DNA extract from the soils was dissolved in 200 μL H_2O. Then, double PCR using only Dest 1, 4 primer set and another, the normal nested PCR using ITS 1, 4 primer set for first and Dest 1, 4 primer set for second round were carried out, respectively. The specificity of the species-specific primer set, Dest 1 and 4 only, could not be preserved during the double PCR amplification. In contrast to that, normal nested PCR using ITS 1, 4 and Dest 1, 4 primer sets consecutively, showed 400 bp of specific bands of *C. destructans* and the bands were verified by southern hybridization (Fig. 3).

Application in practice. The diagnostic system was tested in three ginseng fields (Fig. 4). Field 1 is the first planting ginseng field cropping for a year, Field 2 is second replanting for four years after rice cultivation for six years, and Field 3, planed field for ginseng as present rice field. Ten DNA extracts from three ginseng fields were dissolved in 200 μL, and diluted 10 fold from those. The DNA extracts and its dilution samples were amplified by nested PCR.

Most soil samples were amplified normally by nested PCR. However, when the sample was dissolved in 200 μL, lane 3 was not amplified by the first round and the nested PCR. But, it was compensated by its 10 fold dilution sample like as lane 4. It was assumed that humic substances were diluted with DNA together. Two out of three samples from Field 1 and all of the samples from Field 2

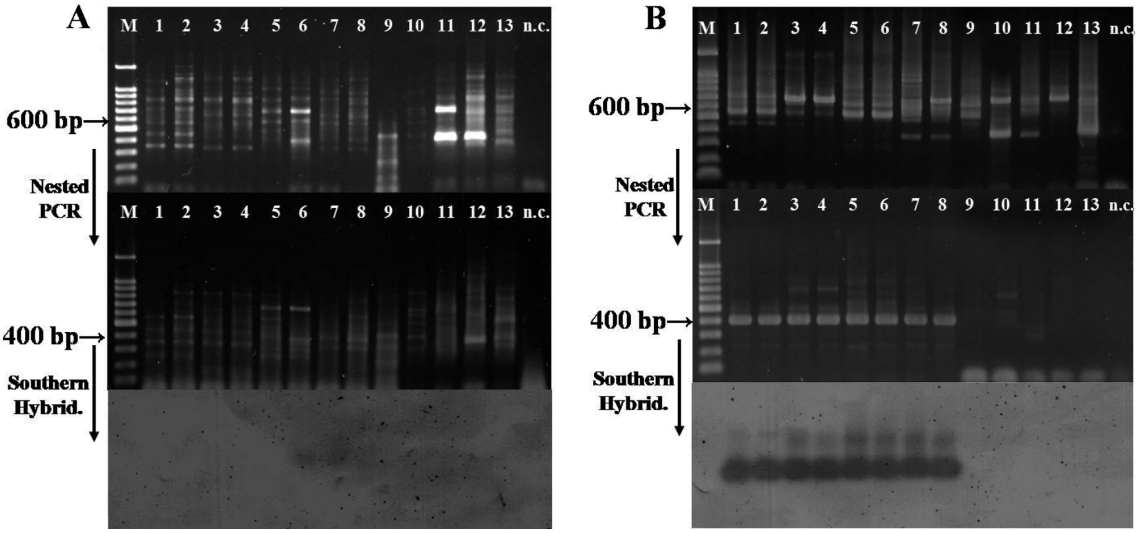

Fig. 3. Detection of *Cylindrocarpon destructans* from various soils with the variety of microorganisms. A, Amplification with only Dest 1, 4 primer set twice. B, Normal nested PCR using Dest 1, 4 after the first round PCR using ITS 1, 4 primer set. Both are hybridized with specific fragments amplified by Dest 1, 4 primer set as probe. M: 100 bp ladder, lane 1~8: soils infested by *C. destructans*, lane 9~13: non-infested, lane 1, 2: a ginseng field from Eumsung, 3, 4: Seosan, 5, 6: Danyang, 7, 8: Jaechon, 9, 10: sand from playground in Chungnam National University, 11~13: rice fields in Chungnam National University, n.c.: PCR mixture without template DNA.

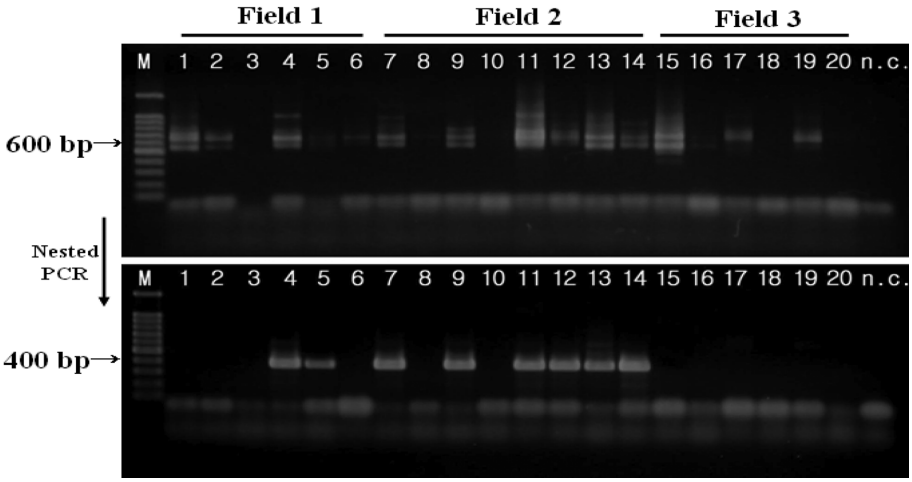

Fig. 4. Detection of *Cylindrocarpon destructans* from three ginseng fields. Upper: first round PCR, below: second round PCR from first. Field 1: the first planting ginseng field cropping for a year, Field 2: replanting field cropping ginseng for 4 years after rice cultivation for 6 years, Field 3: planed field for ginseng cultivation as present rice field. Lane M: 100 bp ladder, lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19: DNA dissolved in 200 μL H_2O, lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20: 10 fold diluted DNA from DNA dissolved in 200 μL, respectively, n. c.: PCR mixture without template DNA.

showed 400 bp of specific bands. No bands, however, were shown in the planed field for ginseng cultivation, Field 3. In Field 2 where replant failure occurred, the specific bands were detected more than in Field 1, the first planting ginseng field.

Discussion

In Korea, ginseng has been usually cultivated as migration and return with a small size of land. The population of the pathogen is increased in the soil and replant failure occurred because of frequent cultivation in one site. Thus, a diagnostic system was more required for the tactical utilization of ginseng fields.

In PCR diagnosis to soil born fungi, it is difficult to get a reliable results, which is associated with reproducibility. Fungi have so hard cell wall, so the DNAs are not released well from the cell. Accordingly, effective cell wall disrupt-

ing method is required. The combination of lyophilization and grinding with glass beads which invented in our study have excelled the existing method using beads, glass bead homogenization in targeting chlamydospores of the pathogen. When DNA extraction method was completed with the cell wall disrupting method, although it was simple, promoted the sensitivity, and also reproducibility to detect the pathogen from soil.

For soil DNA extracts, the strategy to eliminate humic substances, which is generally based on the difference of polarities between the DNA and impurities, makes it possible to remove contaminants to some degree. The problem is that there are various contaminants in the soil and purification isn't always enough for PCR. Therefore, the dilution method of soil DNA extracts was often needed to lower the concentration of humic substances enough to amplify [8, 24] (lane 3, 5 in Fig. 4).

Though the Dest 1, 4 primer set was species specific, it was not useful in the diversity of soil organisms. The uncertainty on the specific primer set designed in a laboratory was presumed. However, the incompleteness of the species specific primer is supplemented with limitation to the ITS region in the first round amplification using universal primers, ITS 1 and ITS 4 in the soil samples. As it used two distinct specificities at the same time, the nested PCR could keep the specificity of detection in the case of environment (Fig. 3).

The nested PCR was so sensitive that could detect the amount of 1 fg DNA in our former study [25], and also the result of Field 1 presented the nested PCR is possible to detect the pathogen from the first planting ginseng field cultivated for just one year. By the way, in Field 2 as replanting field, the population of the pathogen increased largely. So, to reduce the crop losses by the disease, roots of the ginseng had to be harvested in the Field 2 soon. These results were showed as our expectation based on the history of the three fields (Fig. 4).

To distinguish fields differentiated in the population of pathogens is required for the tactical utilization of fields. Our study satisfied this demand in ginseng with highly sensitive and specific procedure of detection. Our results are more significant because of the characteristic of long time growth. The PCR technique will contribute to the increase of ginseng crops and expansion of the planed fields for six-year-old ginsengs.

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