Molecular detection of *Cylindrocarpon destructans* in infected Chinese ginseng roots and soil

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Ginseng (*Panax ginseng* C.A. Meyer) is one of the most important medicinal plants in China, but its yields are often reduced by a variety of root pathogens. The root rot of ginseng is a destructive soil-borne disease caused by *Cylindrocarpon destructans* (teleomorph: *Neonectria radicicola*). A species-specific polymerase chain reaction (PCR) assay was developed for rapid detection of *C. destructans* in diseased ginseng roots and artificially inoculated soil. One pair of specific primers was designed from comparisons of nucleotide sequences of the nuclear ribosomal internal transcribed spacer (ITS) regions of 22 fungal isolates from northeast of China. Under stringent PCR conditions, primers CD-F and CD-R amplified only a 450 bp fragment from *C. destructans* DNA but not from other pathogens or negative control. The sensitivity of detection was 1 pg genomic DNA per 25 μl PCR reaction volume, and *C. destructans* could be specifically detected with CD-F/CD-R from infected ginseng roots and soil. The approach outlined here could be further utilized as a rapid and reliable tool for the diagnosis and monitoring of the root rot of ginseng.

Key words: *Panax ginseng*, *Cylindrocarpon destructans*, internal transcribed spacer (ITS), polymerase chain reaction (PCR), molecular detection.

INTRODUCTON

Ginseng (*Panax ginseng* C.A. Meyer) widely cultivated as a medicinal herb in northeast China, has become an economically important cash crop for growers (Wang, 2001). The dried root is highly valued for its medicinal properties, and is widely used in Chinese traditional medicine (Punja and Rahman, 2005a). With the expansion of ginseng cultivation, seedling, foliar, and root diseases have become more apparent, of which root diseases, caused by several root-infecting fungi have significantly reduced root quality and yield (Buonassissi, 1990; Li, 1993; Punja and Punja, 2005b).

*Cylindrocarpon* root rot of ginseng caused by *Cylindrocarpon destructans* (teleomorph: *Neonectria radicicola*) is one of the major threats to stable ginseng production (Ahn and Lee, 2001). The disease can result in yield losses of up to 25 to 30% (Chung, 1975; Wang, 2001; Seifert et al., 2003) and limits the re-use of fields for successive ginseng crops (Reeleder and Brammall, 1994; Reeleder, 2002). *C. destructans*, which is a common soil-inhabiting fungus (Fu, 2002), was confirmed to be the causal agent of root rot in ginseng sites (Punja, 1997; Reeleder and Brammall, 1994; Reeleder et al., 1999).

Assessment of disease risk from soil-borne fungal pathogens has often been based on the number of colony-forming units (c.f.u) by dilution plating on selective media (Davet, 2000). However, the classical methods of diagnosis are time-consuming and laborious, requiring isolation of the fungus from diseased tissue (Chao, 1999). 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 (Reeleder, 2002; Seifert et al., 2003). Furthermore, the typical symptoms could not be observed before ginseng roots are infected seriously. Therefore, it is very difficult to diagnose the root disease by the classical methods. An effective and sensitive diagnostic system for

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C. destructans is desirable.

PCR technique offers advantages over traditional methods of detection and diagnosis because the fungi do not need to be cultured, prior to detection by PCR (Zhang et al., 2006). Recently, some diagnostic methods based on PCR have been developed for plant pathogen detection (Hamelin et al., 1996; Seifert et al., 2003; Ippolito et al., 2004; Zhang et al., 2005; Langrell, 2005; Zhang et al., 2006; Wang et al., 2008; Martin et al., 2009; Huang and Kang, 2010). However, to the best of our knowledge, little work has been done on detection of C. destructans in ginseng roots and soil. In view of this, the aim of this research was to develop a species-specific PCR detection assay for C. destructans, primarily for detection from ginseng roots and soil. In this study, PCR primers derived from internal transcribed spacer (ITS) sequences for the specific detection was described, and the utility of the assay was assessed.

MATERIALS AND METHODS

Source of isolates

The strains used in this research were obtained from diseased ginseng roots and infested soil, located at the major ginseng cultivation areas in China (Table 1). The fungal isolates were cultured on lima bean agar (LBA) plates (Phytophthora spp. and Pythium spp.) or on potato dextrose agar (PDA) (other fungi) plates and grown at (20 ± 2°C) in the dark, in an incubator for 1 week. Identification of the isolates was made based on the morphological, cultural, and sporulation characteristics (Domsh, 1980; Samuels and Brayford, 1990). All single-spore isolates were stored either on LBA at 10°C or on PDA at 4°C (Zhang et al., 2005).

Mycelium and conidia preparation

Five pieces of agar culture (ca. 2 x 2 x 4 mm), obtained from the advancing margin of 7-day old colonies, growing on plates of LBA (Phytophthora and Pythium) or PDA (other fungi) were placed in 100 ml of potato dextrose liquid medium in a 250 ml flask. After incubation in the dark at 20°C on a shaker for 2 weeks, the mycelia were then filtered through filter paper and rinsed with sterile distilled water, lyophilized, and stored at -20°C until DNA extraction (Hamelin et al., 1996). Spore suspensions of the fungus C. destructans were prepared by adapting the method of Zhang et al. (2005). Briefly, conidia were prepared by growing plate cultures on PDA at 20°C, for 2 weeks in darkness, to induce sporulation. Conidia were harvested from the plates by rubbing the surface mycelium gently with a rubber swab, and collecting the spores in distilled water. Hyphal debris was removed from the spores by centrifuging the crude spore preparation, through a 40% sucrose solution, with the spores settling to the bottom of the tube and the rest of the cellular debris remaining on the surface of the suscrose pad. Spores were adjusted to the desired concentrations (1 x 10^4 spores/ml), by counting them in a hemocytometer for soil inoculation.

Soil preparation and inoculation

Soil artificially inoculated was collected from Shenyang Agricultural University research farm, at Shenyang, Liaoning province (41° 47′ N, 123° 27′ E) in August 2010. Soil was steam-pasteurized at 74°C for 30 min, air-dried, sieved through a 4 mm mesh, mixed and stored at room temperature until use (Kernaghan et al., 2007). Soil dilution procedures were carried out, using a semiselective modified Rose Bengal agar medium (MRBA) to confirm that pasteurization had eliminated viable propagules (Reeleder et al., 2003). 5 ml C. destructans spores suspensions were inoculated into 5 g of twice-autoclaved soil substrate in 15 ml conical tubes. The tubes were vortexed at maximum speed for 1 min, freeze-dried for 3 weeks, ground in liquid nitrogen to produce a fine powder, and stored at -70°C, prior to DNA extraction (Zhang et al., 2006).

DNA extraction procedures

Genomic DNA from all microorganisms (Table 1) was extracted, using the fungal DNA Kit (OMEGA), according to the manufacturer’s instructions.

DNA was extracted from infected ginseng roots according to Hamelin et al. (1996). A 100 mg sample of diseased ginseng roots was excised and ground in 400 μl of Qiagen extraction buffer (100 mM Tris-HCl, pH 9.5, 2% CTAB, 1.4 M NaCl, 1% polyethylene glycol 8000, 20 mM EDTA and 1% β-mercaptoethanol) mixed with approximately 10 mg of diatomaceous earth. Extracts were vortexed, incubated at 65°C for approximately 1 h, extracted once with 400 ml of phenol: chloroform: isomyl alcohol (25:24:1), and centrifuged at 10,000 x g for 5 min. The supernatant was transferred to a new microtube, precipitated with one volume of isopropanol, and centrifuged at 10,000 x g for 5 min. Pellets were washed with 70% ethanol, air dried overnight, and resuspended in 20 μl of TE buffer, pH 8.0. Healthy ginseng roots were also processed as above for DNA extraction.

Total DNA from the artificially infested soil was extracted, following the research methods presented by Kernaghan et al. (2007). Soil (5 g) infested with C. destructans was placed in 50 ml disposable centrifuge tubes, with 3.5 ml of autoclaved 1 mm diameter zirconium oxide beads, 5 ml extraction buffer (60 mM sodium phosphate buffer (pH 8.0), 10 mM CaCl₂, 0.05% SDS and 100 μg proteinase K). Tubes were then shaken on a commercial paint shaker at maximum speed for 20 min, followed by centrifugation at 5,000 x g for 10 min at 10°C. Supernatants were then transferred to new autoclaved tubes. EDTA and potassium acetate (pH 5.5) were added to provide final concentrations of 100 and 300 mM, respectively. Tubes were vortexed, placed on ice for 20 min and then centrifuged at 10,000 x g for 10 min. The resulting supernatants (2.5 ml) were transferred to new tubes containing an equal volume of a 1:0.2 v/v mixture of 3 M sodium acetate and 100% isopropanol (mixture pH 5.3), incubated at -20°C for 1 h then centrifuged again. The resulting pellets were washed with 80% ethanol, air-dried and frozen at -80°C, prior to use.

ITS amplification, sequencing and primer design

The entire region contains portions of the small subunit (18S), both internal transcribed spacers (ITS), and the 5.8S of the rDNA repeat sequences for the specific detection was described, and the utility of the assay was assessed.
Table 1. Species used in this study for testing the specificity of Cylindrocarpon destructans oligonucleotide primers.

<table>
<thead>
<tr>
<th>Number</th>
<th>Isolate</th>
<th>Geographic origin</th>
<th>Year isolated</th>
<th>Primer set</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cylindrocarpon destructans</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Cylindrocarpon destructans</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Cylindrocarpon destructans</td>
<td>Kuandian, LiaoNing</td>
<td>2009</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Cylindrocarpon destructans</td>
<td>Kuandian, LiaoNing</td>
<td>2008</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Cylindrocarpon destructans</td>
<td>Fusong, Jilin</td>
<td>2008</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Cylindrocarpon destructans</td>
<td>Fusong, Jilin</td>
<td>2008</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
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<td>Fusong, Jilin</td>
<td>2008</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Cylindrocarpon destructans</td>
<td>Fusong, Jilin</td>
<td>2008</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Cylindrocarpon destructans</td>
<td>Fusong, Jilin</td>
<td>2008</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Fusarium xysporum</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Fusarium xysporum</td>
<td>Fusong, Jilin</td>
<td>2009</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Fusarium solani</td>
<td>Fusong, Jilin</td>
<td>2009</td>
<td>-</td>
</tr>
<tr>
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<td>Fusarium equiseti</td>
<td>Fusong, Jilin</td>
<td>2009</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Rhizoctonia solani</td>
<td>Fusong, Jilin</td>
<td>2009</td>
<td>-</td>
</tr>
<tr>
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<td>Fusong, Jilin</td>
<td>2008</td>
<td>-</td>
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<tr>
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<td>2010</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Pythium debaryanum</td>
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<td>2010</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Phytophthora cactorum</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>Sclerotinia schinseng</td>
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<td>2008</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Trichoderma sp.</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Penicillium sp.</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Aspergillus sp.</td>
<td>Ji’an, Jilin</td>
<td>2010</td>
<td>-</td>
</tr>
</tbody>
</table>

+: 450 bp product amplified by primers CD-F/CD-R; -: no amplified products.

Assessment of primer specificity and sensitivity

To determine the specificity of the primer, DNA extracted from a range of C. destructans isolates and other soil-borne pathogens mentioned previously was used as a template for PCR amplification. The reaction system was the same as described for the ITS sequence, the cycle sequencing reaction profile included an initial denaturation for 3 min, at 95°C, followed by 30 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 59°C, 1 min of extension at 72°C, with a final extension of 10 min at 72°C. The sensitivity of the primer pairs was evaluated, using serial dilutions of purified genomic DNA from 1 ng/μl to 1 fg/μl (Wang et al., 2008). 1 μl of each dilution was added to the PCR reaction and amplification was carried out, using optimal conditions and cycling parameters as described above with primer CD-F/CD-R. Negative controls lacking template DNA were carried out in each experiment to test for contaminated reagents. All the assays were repeated twice with three replications.

RESULTS AND DISCUSSION

Primers, specificity and sensitivity

Soil-borne pathogens such as C. destructans could be extremely persistent, and the potential for losses as a result of replant decline may exist for decades after the cultivation of ginseng (Reeleder, 2002), as well as for some other perennial crops (Braun, 1995; Mercado-Blanco, 2003). Rapid and reliable detection of C. destructans is important for formulating strategies for disease management. The species-specific primer set CD-F/CD-R was designed for C. destructans, based on the ITS regions. All DNA preparations from fungi (Table 1) were amplified with the ITS universal primers ITS1/ITS4 (White, 1990), resulting in the fragments ranging from 400 bp to 650 bp, demonstrating that they were of sufficient quality as PCR templates. PCR with
primers CD-F/CD-R, amplified DNA from all *C. destructans* isolates, and produced a specific amplicon of approximately 450 bp (Figure 1). However, no amplicon was obtained from any other fungal species tested.

Additionally, the primer set CD-F/CD-R has proven to be sensitive to detect very low quantities of DNA, by using purified fungal template DNA. Sensitivity was assessed, using serial dilutions of total DNA extracted from *C. destructans*. The PCR produced positive results for all *C. destructans* strains at four concentrations of DNA (Figure 2). In a 25 μl reaction volume assaying *C. destructans*, successful amplification was achieved down to 1 pg of pure genomic DNA, with a clearly visible product on ethidium bromide-stained agarose gels. However, primer-dimer formation at low template concentrations meant that the level of detection was sometimes lower than the level which could be accurately quantified (Kernaghan et al., 2007).

**Detection of C. destructans from infested ginseng roots**

A PCR method was developed to detect *C. destructans*
from ginseng roots collected from Jilin Province in 2010. Using PCR with primer set CD-F/CD-R, the predicted fragment of 450 bp was obtained from diseased ginseng roots (Figure 3). The fungus C. destructans was detected in asymptomatic ginseng in no case. This finding was in agreement with Hamelin (1996), whose study indicated that C. destructans could not be detected from nonviable fungal material. This was probably due to the low degree of infection in naturally infected plants, in comparison with those artificially infected, and the presence in field plants of other pathogens which could interfere with the detection (Silvar et al., 2005). Every picked diseased ginseng sample had DNA sequence 100% identical to those of C. destructans in the specificity assay. This confirmed that the DNA from infected ginseng that was amplified by primers CD-F/CD-R was derived from C. destructans.

Detection of C. destructans in soil

Agricultural field soil is a complex ecosystem with a diverse microbial community (Torsvik, 2002). More than hundreds of different species of Fusarium, Phytophthora, Verticillium, Pythium, Rhizoctonia, in addition to various bacteria and nematodes, have been found in field soil (Zhang et al., 2005). Specificity is essential for detecting C. destructans in soil. The DNA sample extracted from artificially inoculated soil was used for PCR amplification. Agarose gel electrophoresis revealed that the fragment amplified by CD-F/CD-R was of the predicted size (c. 450 bp) (Figure 4). This indicates that the PCR product in soil
DNA was from *C. destructans*.

**Conclusion**

The PCR assay with CD-F/CD-R primers could be used for detecting *C. destructans* from infested ginseng and soil. Although it would be impossible to perform routine diagnostics with PCR on asymptomatic ginseng, the PCR detection method presented here could contribute to improving the management of *Cylindrocarpon* root rot of ginseng. This method, which could be considered a crucial component of plant disease management is very easy to use, and requires minimal training. However, further research is needed to quantify the fungus in soil by real-time PCR.

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