

Diagnosis of *Cylindrocarpon destructans* Using Enzyme-Linked Immunosorbent Assay

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Cylindrocarpon destructans causes ginseng root rot and produces radicicol that has an antifungal effect. In this study, we developed a method to detect this fungus using enzyme-linked immunosorbent assay (ELISA). Secreted proteins of *C. destructans* were used as antigens to obtain *C. destructans*-specific IgG from mouse. Out of 318 monoclonal antibodies generated from mouse, two antibodies (Cd7-2-2 and Cd7-2-10) showed highest specificity and sensitivity. Indirect ELISA using both antigens successfully detected *C. destructans* in soils, but direct ELISA using IgG conjugated with horseradish peroxidase failed to detect antigens in soils. The indirect ELISA developed here can efficiently detect the fungus and help manage ginseng root rot disease in fields.

Keywords: *Cylindrocarpon destructans*, Diagnosis, Enzyme-linked immunosorbent assay, *Fusarium solani*, Ginseng root rot

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Ginseng (*Panax ginseng* C. A. Meyer) is a medicinal crop that has been widely cultivated in Asia because of its excellent active ingredients for human health. Recently, the use of ginseng has been expanded into various fields including functional foods and cosmetics. The demand for ginseng has been increasing, but the production is not increasing because it usually takes 4 to 6 years for ginseng cultivation and various fungal pathogens threaten the ginseng production (Shin et al., 2012). Among the diverse ginseng pathogens, the ascomycete fungus *Cylindrocarpon destructans* is the most destructive pathogen and causes ginseng root rot (Chung, 1975; Farh et al., 2017; Reeleder and Brammall, 1994). This fungus survives for long time in the form of chla-

mydospores, and causes ginseng root rot under favorable condition for disease development (Chung, 1975; Kang et al., 2016).

Rapid and accurate diagnosis of plant pathogens is important for correct disease control. Currently, the methods using nested-polymerase chain reaction (PCR) and real time-PCR are available for the diagnosis of ginseng root rot (Doh, 2003; Jang et al., 2005; Kang et al., 2014). Although these methods are effective in diagnosing *C. destructans*, they require equipment and specific techniques. One of the more rapid diagnostic techniques for field application is a kit based on enzyme-linked immunosorbent assay (ELISA). It analyzes the presence or quantity of pathogens by enzyme reaction in field even without the use of a special device. Diagnostic kits have been developed in various fields such as detection of insecticides and mycotoxins as well as the diagnosis of pathogenic bacteria, fungi, and viruses (Kang et

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al., 2010; Lin, 2015; Ming et al., 2019).

Previously, we developed a selective medium for *C. destructans* using radicicol which is produced by this fungus and has an antifungal effect against various soil-borne fungi (Kang et al., 2014). Addition of radicicol into medium suppressed the growth of other fungi and resulted in the isolation of *C. destructans* from soil and diseased ginseng. From the results, we hypothesize that *C. destructans* secretes proteins into the selective medium and we can generate antibodies binding to the secreted proteins. Based on this hypothesis, we generated monoclonal antibodies for the secreted proteins and developed a new diagnosis tool for this fungus in this study.

Cylindrocarpon destructans (KACC 41077) was obtained from the Korean Agricultural Culture Collection (Jeonju, Korea) and *Fusarium solani* 13chu01-05 was isolated from ginseng with root rot symptom. The *F. solani* strain was used as a negative control to isolate antibodies specific to *C. destructans*. *C. destructans* and *F. solani* strains were cultured for 7 days in complete media (Leslie and Summerell, 2006) at 20°C and 25°C, respectively. Three mycelial blocks (diameter: 5 mm) from the growing margins were subcultured in 100-ml minimal medium (MM) (Leslie and Summerell, 2006) for 7 days in an orbital shaker (200 rpm). Mycelia were harvested through filter paper and washed twice with sterile water. The washed mycelia were additionally incubated for 24 hr in 100-ml MM with 5% methanol for antigen preparation.

The culture was centrifuged (8,000 $\times g$, 10 min, 4°C) and the supernatant filtered through a filter system with 0.22- μm pore size for protein purification. Proteins were extracted by modifying the existing TCA-acetone method (Mechin et al., 2007). In brief, the filtered supernatant (50 ml) was combined with 200 ml of 12.5% acetone with 0.07% β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and placed in -20°C for 24 hr. The solution was centrifuged (8,000 $\times g$, 10 min, 4°C) and the supernatant discarded. The pellet suspended with 5 ml of 12.5% acetone with 0.07% β -mercaptoethanol and centrifuged again (8,000 $\times g$, 10 min, 4°C). The supernatant discarded and the pellet dissolved in 1 ml of 80% acetone with 0.07% β -mercaptoethanol. The solution placed in ice and sonicated for 90 sec (5 sec, run; 2 sec, rest) then centrifuged (13,000 $\times g$, 5 min, 4°C) and supernatant discarded. The pellet dissolved in 1 ml of phosphate buffered saline (Sigma-Aldrich) and stored at -20°C.

Monoclonal antibody production was conducted by Post-Bio (Hanam, Korea). Secreted proteins (2.3 mg/ml) purified above were injected into mice (every 2 months) and splenocytes were isolated to fuse with myeloma cells. Positive clones were selected and each fusion cell was massively cultured to obtain monoclonal antibodies. In total, 318 monoclonal antibodies were prepared from hybridoma, and 16 out of them were specific to *C. destructans* but not to *F. solani*. For sensitivity test, supernatant containing secreted proteins of *C. destructans* was diluted 10 to 10⁴ times with MM and used as antigens for indirect ELISA as previously described (Lin, 2015). Two antibodies, Cd7-2-2 and Cd7-2-10, showed both high sensitivity and specificity (Table 1), and were selected for further experiment.

The maximum sensitivity of two antibodies were tested using ELISA Starter Accessory Kit (E101, Bethyl, Montgomery, TX, USA). In brief, antigen (1.78 $\mu g/ml$ of secreted proteins) was diluted 10 to 10⁴ times with MM and 10- μl diluent was supplied with 90- μl Coating buffer then incubated in 96 wells for 60 min. After washing 3 times, 200 μl of Blocking solution was added to each well and incubated for 30 min. Blocking solution was removed and 100 μl of 10 to 10⁴ times diluted monoclonal antibody was added to each well then incubated for 60 min. Each well was washed 3 times and 100 μl of 5,000 times diluted Goat anti-mouse IgG (Enzo Life Sciences, Farmingdale, NY, USA) was added to each well then incubated for 60 min. After incubation, each well was washed 5 times. Then 100 μl of 3,3',5,5'-tetramethylbenzidine substrates was added to each well for 5-min reaction, which was stopped using 2 M H₂SO₄. The absorbance of each well was determined using microtiter plate reader at 415 nm. Cd7-2-2 showed sensitivity to antigen even though it was diluted 10³ times, and 10⁴ times diluted antibody also showed sensitivity to 10 times diluted antigen (Fig. 1A). On the other hand, Cd7-2-10 showed sensitivity to 10² times diluted antigen, and 10⁴ times diluted antibody showed sensitivity to 10 times diluted antigen (Fig. 1B). Although there was abnormal absorbance variations in several tested wells, the results offer the possibility that these two antibodies could be used to detect *C. destructans* in soil. Addition of two antibodies together resulted in higher absorbance than the single antibody was binding to antigens (Fig. 2), suggesting that the epitopes of Cd7-2-2 and Cd7-2-10 bind to different antigens. In addition, these two antibodies were not binding to secreted proteins purified from *F. solani*, showing that they are specific to *C.*

Table 1. Absorbance of antibodies with antigen dilution in indirect enzyme-linked immunosorbent assay

| Antibody No. | Dilution ratio of antigen ^a | | | | | Control ^b |
|--------------|--|-----------------|-----------------|-----------------|-----------------|----------------------|
| | 1 | 10 ¹ | 10 ² | 10 ³ | 10 ⁴ | |
| Cd7-2-2 | 0.661 | 0.127 | 0.020 | 0.010 | 0.000 | 0.007 |
| Cd7-2-10 | 0.633 | 0.143 | 0.019 | 0.010 | 0.010 | 0.011 |
| Cd7-2-6 | 0.700 | 0.141 | 0.023 | 0.011 | 0.012 | 0.007 |
| Cd7-2-26 | 0.731 | 0.129 | 0.014 | 0.002 | 0.003 | 0.006 |
| Cd7-2-22 | 0.654 | 0.131 | 0.013 | 0.003 | 0.002 | 0.008 |
| Cd12-1-46 | 0.639 | 0.041 | 0.001 | 0.000 | 0.002 | 0.008 |
| Cd7-2-18 | 0.728 | 0.126 | 0.012 | 0.003 | 0.006 | 0.022 |
| Cd66 | 0.796 | 0.101 | 0.001 | 0.000 | 0.006 | 0.022 |
| Cd7-2-61 | 0.673 | 0.144 | 0.017 | 0.005 | 0.015 | 0.048 |
| Cd96 | 0.613 | 0.148 | 0.017 | 0.008 | 0.015 | 0.015 |
| Cd95 | 0.644 | 0.102 | 0.006 | 0.002 | 0.000 | 0.021 |
| Cd7-2-64 | 0.661 | 0.126 | 0.009 | 0.247 | 0.006 | 0.013 |
| Cd12-1-34 | 0.537 | 0.043 | 0.000 | 0.000 | 0.001 | 0.011 |
| Cd7-2-4 | 0.619 | 0.130 | 0.008 | 0.002 | 0.003 | 0.012 |
| Cd7-2-32 | 0.664 | 0.133 | 0.013 | 0.002 | 0.244 | 0.007 |
| Cd7-2-62 | 0.649 | 0.137 | 0.014 | 0.005 | 0.006 | 0.017 |

^aSupernatant of *Cylindrocarpon destructans* culture was diluted using minimal medium.

^bSupernatant of *Fusarium solani* culture.

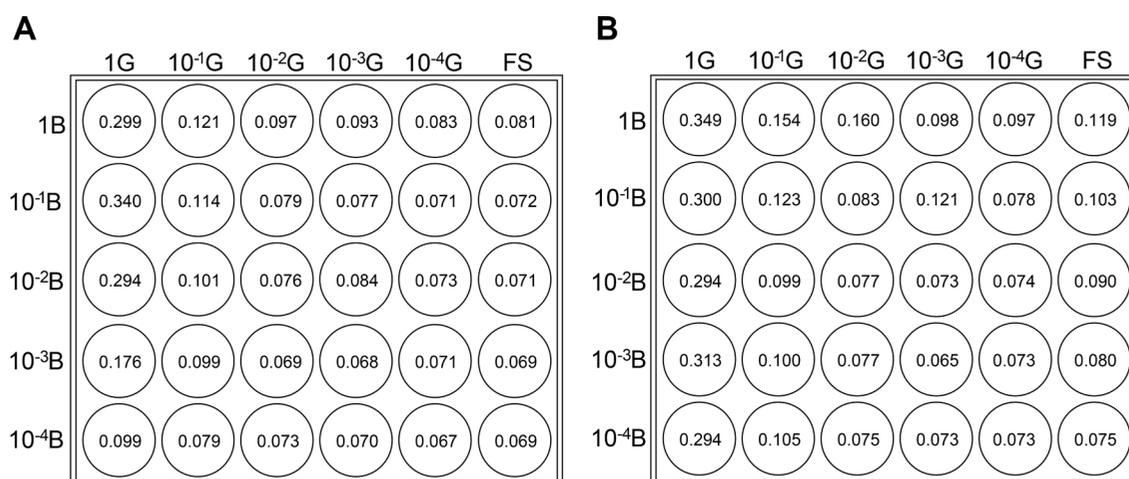


Fig. 1. Absorbance in indirect enzyme-linked immunosorbent assay using the monoclonal antibodies, Cd7-2-2 (A) and Cd7-2-10 (B). *Cylindrocarpon destructans* and *Fusarium solani* was cultivated in minimal medium (MM) and the cultures filtered through a filter system with 0.22- μ m pore size. The supernatant was diluted 10 to 10⁴ times using MM and monoclonal antibodies were diluted 10 to 10⁴ times using distilled water. G, antigen; B, antibody; FS, supernatant of *F. solani*.

destructans.

ELISA is widely used for antigen-antibody immune response. Particularly, horseradish peroxidase (HRP) is a stable

protein and is useful for confirming the reaction of enzymes (Chevrier et al., 2004; Kassim et al., 2013). We also conjugated the Cd7-2-2 with HRP and tried to develop direct ELISA

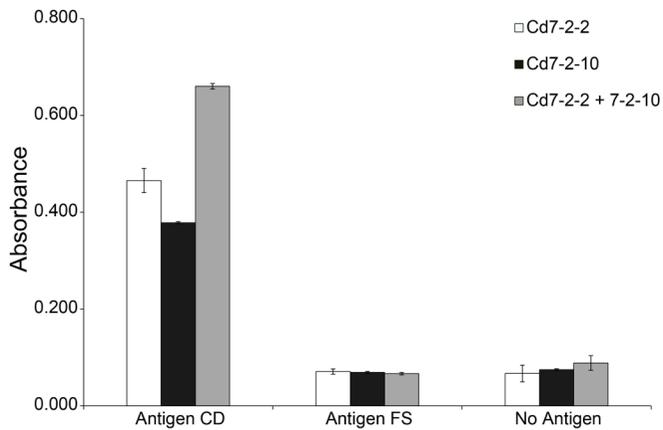


Fig. 2. Antibodies epitope test against antigen with indirect enzyme-linked immunosorbent assay. The absorbance was observed at 415 nm. Antigen CD, supernatant of *Cylindrocarpon destructans*; Antigen FS, supernatant of *F. solani*; No Antigen, minimal medium.

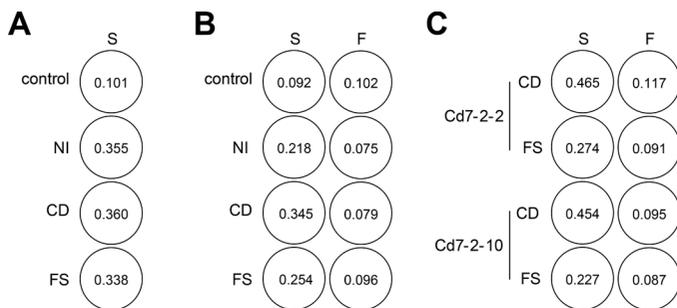


Fig. 3. Absorbance of soil cultures inoculated with either *Cylindrocarpon destructans* and *Fusarium solani*. (A) Absorbance in direct enzyme-linked immunosorbent assay (ELISA) using Cd7-2-2 horseradish peroxidase conjugate. (B) Absorbance in indirect ELISA using the monoclonal antibody Cd7-2-2. (C) Absorbance in indirect ELISA using the monoclonal antibodies. Control, minimal medium; NI, cell free soil; CD, soil inoculated with *C. destructans*; FS, soil inoculated with *F. solani*; S, soil culture; F, soil-free culture.

which is simpler than indirect ELISA. Cd7-2-2 HRP conjugate showed specific to *C. destructans* but not to *F. solani*. The application of the Cd7-2-2 HRP conjugate was tested on soil samples. To do it, *C. destructans* (1.1×10^5 colony-forming unit [CFU]) and *F. solani* (1.0×10^7 CFU) were inoculated in soils (100g), respectively. The soil was incubated in 50 ml of selective medium (Kang et al., 2014). The culture was coated on well for direct ELISA and the antibodies were applied onto the well. Unfortunately, Cd7-2-2 HRP conjugants were non-specifically binding to soil even though any antigens were absent in soil (Fig. 3A). The monoclonal antibody Cd7-2-2 was also non-specifically binding to soil but the reaction

disappeared in soil-free solution (Fig. 3B). The absorbance values were clearly different between soil inoculated with either *C. destructans* or *F. solani* (Fig. 3C), indicating it is suitable for detection of antigens in soil. We expect that the currently identified antibodies can be applied for the use of gold-nano particles to detect this fungus in soils and diseased ginseng, and further we will focus on the development of detection methods for farmers to use it in fields.

Conflicts of Interest

No potential conflict of interest to this article was reported.

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