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Different Structural Modifications Associated with Development of Ginseng Root Rot Caused by *Cylindrocarpon destructans*

Jeong Ho Kim[1,2], Sang Gyu Kim[1], Mi-Sook Kim[1], Yong Ho Jeon[1,3], Dae Hui Cho[3] and Young Ho Kim[1]*

[1]Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Korea
[2]Korea Turfgrass Institute, Subsidiary of Korea Golf Course Business Association, Sungnam 463-840, Korea
[3]KT&G Central Research Institute, Daejeon 305-805, Korea

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Root rot caused by *Cylindrocarpon destructans* is one of the most important diseases of ginseng (*Panax ginseng* C. A. Meyer). Two types of symptoms found in ginseng root rot are black root rot and rusty root (rusty spots), in which disease severities are high and low, respectively. Symptom development and related histopathological changes were examined in an inoculation test on 2-year-old ginseng roots using virulent (Cy9801) and avirulent (Cy0001) isolates of *C. destructans* under different temperature conditions (13, 18, 23, and 28°C). Black root rot was only induced by Cy9801 in the lower temperature range (13, 18, and 23°C) and not at the higher temperature (28°C). No black root rot, but only rusty spot symptoms, were induced by Cy0001 at all temperatures tested except 13°C, at which no symptoms occurred on over half of inoculation sites, suggesting disease development was influenced by pathogen virulence and temperature. Wound periderms were formed in all root tissues with rust spot symptoms at 28°C caused by Cy9801 and at 18, 23, and 28°C temperatures caused by Cy0001. No wound periderm was formed at 13°C by either Cy9801 or Cy0001. Light microscopy revealed that the wound periderm was formed by initial cell divisions in cell wall formation and/or additional cell wall layering in parenchyma cells without obvious nuclear division, followed by layering of the divided cells adjacent to the inoculation sites, blocking the spread of the rot. These results suggest that disease development declined at lower temperatures and by the formation of a wound periderm at higher temperatures, and that ginseng rusty root may develop under conditions unfavorable for further disease development of *C. destructans*.

Keywords : black root rot, *Cylindrocarpon destructans*, ginseng, rusty root, wound periderm

*Corresponding author.
Phone) +82-2-880-4675, FAX) +82-2-873-2317
E-mail) yhokim@snu.ac.kr

Ginseng (*Panax ginseng* C. A. Meyer) is a perennial medicinal plant that grows in shade and requires 4 to 6 years to produce mature roots suitable as raw material for production of white or red ginseng. During cultivation, plants may be attacked by pathogens and pests, especially soil-borne microorganisms, such as fungi, bacteria, or nematodes. Fungi cause the most frequently encountered root diseases; *Cylindrocarpon destructans* (Zins.) Sholten (teleomorph: *Nectria radicicola* Gerlach & L. Nilsson) is a major root rot-causing pathogen and one of the main causes of replanting problems (Chung, 1975; Lee, 2004; Park, 2001; Yu, 1987; Yu and Ohh, 1993). Other root rot pathogens reported in Korea are *Fusarium solani*, *Alternaria panax*, *Phytophthora cactorum*, *Sclerotinia* sp., *Erwinia carotovora*, *Pseudomonas* sp., potato rot nematode *Ditylenchus destructor*, and root-lesion nematode *Pratylenchus subpenetrans* (Kim et al., 2006; Lee, 2004; Lee et al., 1990; Ohh et al., 1983, 1986; Yu and Ohh, 1993).

Cylindrocarpon destructans has been frequently isolated from black root rot and causes the same disease in inoculation tests (Cho et al., 1995, 1996). Especially in young ginseng fields with continuous cropping, extensive soft, blackish or brownish root rots (hereafter termed black root rot) are mainly caused by *C. destructans*. However, ginseng fields also frequently contain rusty root (or rust spot), which is characterized by reddish brown to orange-brown discolored surface areas that eventually peel off, exposing underlying cortical tissue (Campeau et al., 2003; Rahman and Punja, 2005). Decayed areas on roots are rusty-brown and have a dry and corky texture, but are not soft. At present, the exact causes of rusty root are not known, but it is assumed to be due to physiological stress and/or infection by fungal pathogens (Campeau et al., 2003; Punja et al., 2007). Rusty root of ginseng can also be induced by *C. destructans* (former genus name *Ramularia*) in the first report of the disease by Zinssmeister (1918). This indicates that *C. destructans* may cause two types of symptoms, black root rot (or Nectria root rot) and rusty root. Little is known about factors and mechanisms involved in these symptom variations. Therefore, this study examined structural changes in ginseng root tissues infected with *C. destructans* related to symptom variations.

Materials and Methods

Pathogen isolates and inoculation. Two isolates of *C. destructans* (isolates Cy9801 and Cy0001) were used for inoculation experiments. These had been isolated from root rots of a 5-year-old ginseng in experimental ginseng fields of the KT&G Central Research Institute at Suwon in 1998 and from ginseng seedling roots in Daejeon, Korea in 2001, respectively. Cy9801 is virulent and causes a definite black root rot, while Cy0001 is avirulent and does not cause black root rot symptoms on ginseng roots (unpublished data). The isolates of *C. destructans* were grown in potato-dextrose agar (PDA) at 23°C for 15 days. Small agar pieces (ca. 1 × 1 mm) containing spores and hyphae were inoculated on the surface of 2-year-old ginseng roots (var. Jagyeong-jong) after pin-prick wounding of roots (at three sites for 13°C and five sites for other temperatures). The inoculated ginseng roots were placed on moistened filter paper in plastic boxes at 13, 18, 23, and 28°C in incubation chambers. Symptom development was observed daily for 15 days after inoculation. Fifteen roots (replications) were used for each treatment. Histological features of the root tissues at the sites inoculated with Cy9801 or Cy0001 were examined 15 days after inoculation using light microscopy. All inoculation sites on ginseng roots were sliced out and hand-sectioned with a razor blade 15 days after inoculation. The sections were observed under a light microscope (Axiophot, Zeiss, Germany) after staining with 0.1% toluidine blue O (O'Brien and McCully, 1981). In this experiment, the formation of a wound periderm beneath the inoculation sites was examined.

Histological features of wound periderm formation in ginseng root tissues inoculated with *C. destructans*. As wound periderm formed consistently in root tissues infected with the avirulent isolate Cy0001 at higher temperatures in the above histological experiment, the second histological experiment examined detailed structural modifications related to wound periderm formation at different times after inoculation and incubation at 23°C using Cy0001. Inoculation sites of ginseng root tissues were cut out 3, 6, 9, 12, 15, and 20 days after inoculation, and the root tissue segments were fixed in Karnovsky's fixative in cacodylate buffer (pH 7.0) for 4 h (Karnovsky, 1965). The segments were rinsed with the same buffer three times for 20 min each, and post-fixed in 1% osmium tetroxide in the same buffer for 2 h. The specimens were washed briefly in distilled water, *en bloc* stained in 0.5% uranyl acetate at 4°C overnight, and dehydrated in a graded ethanol series of 30, 50, 70, 80, 90, and 100% for 10 min each, with final exposure to 100% ethanol repeated three times. The specimens were further treated twice with propylene oxide as a transition fluid for 15 min each time and embedded in Spurr's epoxy resin (Spurr, 1969). The embedded specimens were sectioned to 1 μm with a glass knife on an MT-X ultramicrotome (RMC, Inc., Tucson, AZ), mounted on glass slides, and stained with 1% toluidine blue O. The sections were observed under a compound light microscope (Axiophot, Zeiss, Germany).

Results

Symptom development and wound periderm formation. About 7 days after inoculation, two types of rot symptoms developed and were visible to the naked eye: Blackish and somewhat soft lesions (typical black rot symptoms) were only induced by the virulent isolate of *C. destructans* (Cy9801) and small yellowish or dry rust spots on root surfaces produced by the avirulent isolate Cy0001 or at the highest temperature tested (28°C) by Cy9801. The blackish soft lesions enlarged with time, forming typical black root rot symptoms; however, in the rust spots caused by Cy0001 or at high temperature by Cy9801, no further significant symptom development was noticed after their initial appearance (Fig. 1A, B). When the infected roots were sliced, lesions of the black root rot were wide and penetrated deep into the center of the root (Fig. 1C), whereas the affected portions were limited to a small area around the inoculation site for the rust spot symptoms (Fig. 1D). The outer root tissues affected with rust spot symptoms were demarcated

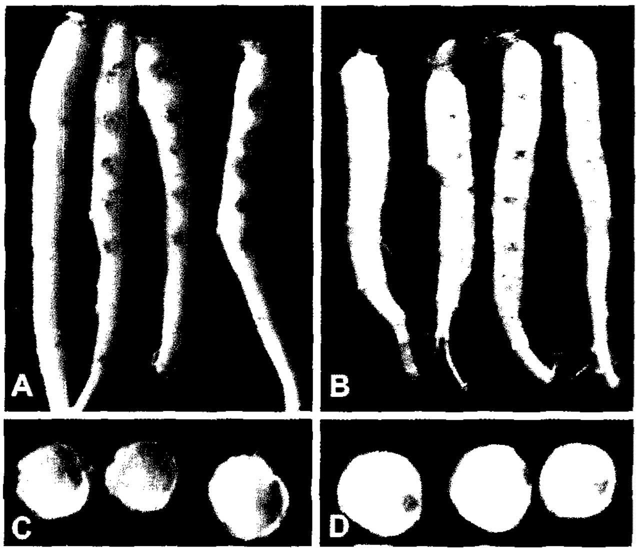

Fig. 1. Black rot (A) and rust spot symptoms (B), and their cross sections (C and D). Two-year old ginseng nots were inoculated with virulent Cy9801 or avirulent Cy0001 isolates of *Cylindrocarpon destructans* at 23°C for 15 days.

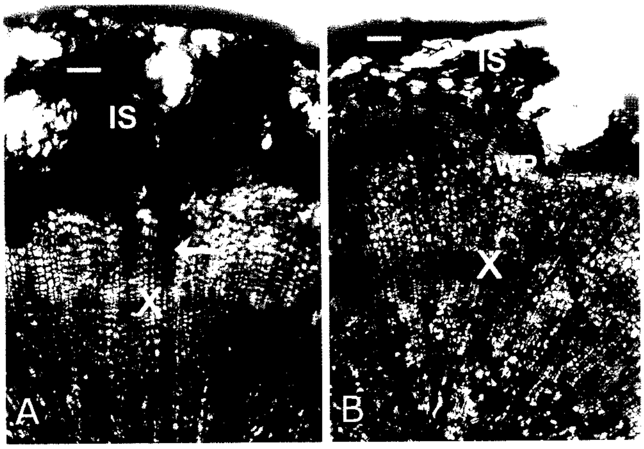

Fig. 2. Hand sections of ginseng root tissues from Figure 1 with black root rot (A) and rust spot (B), showing inoculation sites (IS) and inner xylem tissues (X). Note extended necrotic tissue (arrow) in (A), but blocked by a wound periderm (WP) formed beneath the inoculation site (IS) in (B). Bars = 50 μm.

from the inner healthy-looking tissues by a wound periderm 15 days after inoculation, sometimes accompanied by a partial sloughing-off of the outer affected tissues (data not shown).

Light microscopy revealed that upper infection sites with black root rot caused by *C. destructans* Cy9801 were necrotized and necrosis extended to the inner xylem tissue, with no demarcating structures (Fig. 2A). In contrast, wound periderm, which was continuous with the natural periderm and demarcated the necrotized inoculation site from the inner healthy tissue, was formed beneath the inoculation site in tissues with rust spot symptoms (Fig. 2B). Upper necrotic decayed areas (inoculation sites) were limited to the upper site of this layer. When the root tissues were stained with toluidine blue O, the wound periderm was purplish, similar to the natural periderm.

Relationship between symptom type and wound periderm formation. The occurrence of black root rot and rust spot symptoms on 2-year-old ginseng roots after artificial inoculation with *C. destructans* Cy9801 and Cy0001 differed at different temperatures (Table 1). Black root rot was only induced by the virulent isolate (Cy9801) and was most prevalent at 18°C and 23°C (69.3% and 61.3%, respectively), reduced to 35.6% at 13°C, and absent at 28°C. No black root rot was induced by the avirulent isolate (Cy0001) at any incubation temperature tested. Rust spot symptoms occurred on ginseng roots inoculated with Cy0001 at all incubation temperatures tested and with Cy9801 at 13°C and 28°C. Wound periderm formed at all inoculation sites on root tissues with rust spot symptoms induced at 28°C by Cy9801 and at 18°C, 23°C, and 28°C after inoculation with Cy0001. No wound periderm formed at 13°C by either isolate or at 18°C after inoculation with Cy9801.

Histological features of wound periderm formation in ginseng root tissues inoculated with *C. destructans*. Cell

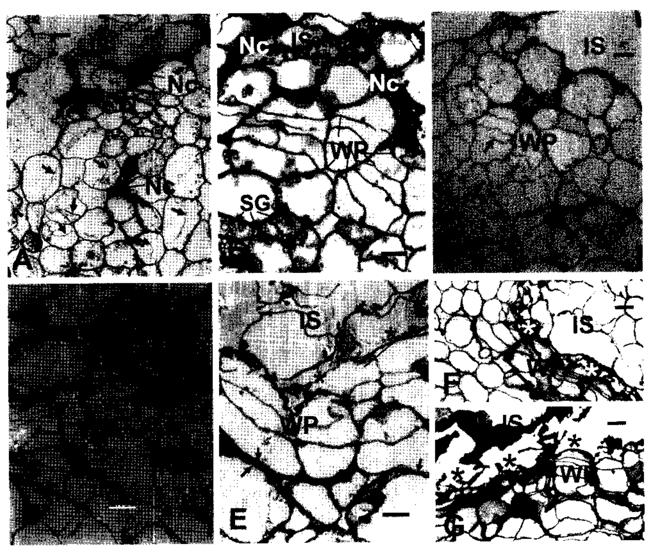

Fig. 3. Structural changes in ginseng root tissues interior to the inoculation sites (IS) of the avirulent isolate Cy0001 of *C. destructans* after 3 (A), 6 (B), 9 (C), 12 (D), 15 (E), and 20 days (F, G) of incubation at 23°C, showing cell wall division (arrow) (A) or cell wall layering (arrow) across parenchyma cells (C), extensive wound periderm formation (WP) (B-G), thickening of wound periderm cell walls (arrows) (E), and separation of outer tissues from the wound periderm (asterisks) (E-G). Nc: necrosis, SG: starch granules. Bars = 10 μm.

Table 1. Relationships between symptom development and wound periderm formation in 2-year-old ginseng roots inoculated with *Cylindrocarpon destructans* Cy9801 (virulent) or Cy0001 (avirulent) at different incubation temperatures

Temperature (°C)	Black root rot/ rust spot symptoms (%)		Wound periderm formation (%)	
	Cy9801	Cy0001	Cy9801	Cy0001
13	35.6 ± 38.8/64.4[a]	0.0/35.6 ± 40.8 (64.4)[b]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
18	69.3 ± 33.7/30.7	0.0 ± 0.0/100.0	0.0 ± 0.0	100.0 ± 0.0
23	61.3 ± 30.7/38.7	0.0 ± 0.0/100.0	16.0 ± 29.5	100.0 ± 0.0
28	0.0 ± 0.0/100.0	0.0 ± 0.0/100.0	100.0 ± 0.0	100.0 ± 0.0

[a]Numbers are means ± standard deviations of 15 replicates with three observations for 13°C and five observations for the other temperatures.
[b]Percentage of no symptoms in parenthesis.

walls crossing spherical parenchymatous cells and leading to cell division were frequently formed beneath the inoculation sites 3 days after inoculation with *C. destructans* Cy0001 (Fig. 3A). The walls occurred in a series of cells to form a layer of multiple divided cells 6 days after inoculation and composed of two or three radial cell lines (Fig. 3B). The number of radial cell lines in a wound periderm increased with time after inoculation, broadening in tangential area facing the inoculation site, so that the whole area internal to the inoculation site consisted of wound periderm 9 to 12 days after inoculation (Fig. 3C, D). At 15 and 20 days after inoculation, separation occurred between the inoculation site and the wound periderm, in which the lower cells had thickened cell walls (Fig. 3E, F). This separation was more conspicuous at 20 days after inoculation, and sometimes the outer decayed cells had completely sloughed off from the inner wound periderm (Fig. 3G).

Discussion

In this study, symptoms induced by artificial inoculation with *C. destructans* differed between two pathogen isolates and with incubation temperature after inoculation. Typical black rot symptoms were only induced by the virulent *C. destructans* isolate Cy9801 at lower temperatures (13-23°C), and not at the highest temperature tested (28°C). Only rust root (spot) symptoms were induced by the avirulent isolate Cy0001 at all temperatures tested, suggesting that rust root is a mild symptom of ginseng black root rot induced by a root rot pathogen having low virulence or under conditions of lowered pathogenicity at given temperatures. With the exception of the lowest temperature (13°C) tested, the wound periderm formed interior to the inoculation sites was always accompanied by rust spot symptoms induced by Cy0001 at the higher temperature range (18-28°C) and by Cy9801 at 28°C. These results suggest that rust spot symptom development and wound periderm formation are closely related and that both occur more readily by infection with the avirulent isolate and at higher temperatures.

Light microscopy showed the wound periderm that demarcated the outer decayed areas (inoculation sites) from inner intact tissues, suggesting that this structure may block the invasion of the pathogen or its disease-inducing materials such as enzymes and toxins (Agrios, 2005). Light microscopy also revealed that this structure is structurally similar to the wound periderm formed in sweet potato (*Ipomoea batatas*) (Morris and Mann, 1955) and resistant chili pepper fruit (*Capsicum baccatum* cv. PBC80) at later stages of infection with *Colletotrichum gloeosporioides* (Kim et al., 2004). Wound periderms are formed in response to wounding and parasite invasion, and function as histological defense structures (Agrios, 2005; Biggs and Britton, 1988; Kim et al., 2004; Mullick, 1977; Rittinger et al., 1987). However, the arrangement of divided cells in the wound periderm is also very similar to the structure formed in Peruvian apple cactus stems in response to *Glomerella cingulata* infection (Kim and Kim, 2002), which is suggested to be an abscission layer because the outer affected tissues slough off as also found in our present study. In both studies, light and electron microscopy showed the presence of cell walls crossing non-meristematic parenchyma cells without obvious nuclear divisions, which indicates that both structures (wound periderm and abscission layer) were formed by an additional cell wall layer in preexisting cells. Incompletely layered cell walls were sometimes found in the wound periderm in this study (Fig. 3C) and also in the abscission layer of cactus stems infected with *G. cingulata* (Kim and Kim, 2002). Whether the structure in ginseng root tissues in our study is a wound periderm or an abscission layer, development of these structures in root tissues may retard disease development and function as a defense structure against pathogen infection (Agrios, 2005). It is, therefore, concluded that the structural modification is a reflection of the resistance response of ginseng root tissues to *C. destructans* infection.

The root surface of field-grown ginseng exhibiting symptoms of rusty root (or rust spot) appears orange-brown to red in color and is raised and corky, with epidermal tissues sloughing off at advanced stages of disease development (Rahman and Punja, 2005). The raised and corky appearance of root tissues after removal of epidermal tissues may be a wound periderm formed as a defense structure against *C. destructans* infection, suggesting that *C. destructans* infection is an important cause of ginseng rusty root. The present results indicate that rusty root is a form of black root rot with reduced disease severity and is caused by the same pathogen but with weakened virulence, mostly accompanied by the formation of wound periderms.

Acknowledgments

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