Influence of Iron on *Cylindrocarpon* Root Rot Development on Ginseng

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ABSTRACT

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Cylindrocarpon root rot, caused by *Cylindrocarpon destructans*, is an important disease on ginseng (*Panax quinquefolius*) in Canada. We studied the effects of iron (Fe) on disease severity and pathogen growth. When Hoagland's solution was amended with Fe at 56 and 112 µg/ml compared with 0 µg/ml, disease initiation and final severity on hydroponically maintained ginseng roots was significantly ($P < 0.0001$) enhanced. Under field conditions, wounding of roots with a fine needle followed by application of 0.05% FeNaEDTA to the rhizosphere of treated plants significantly enhanced *Cylindrocarpon* root rot in 2003 and 2004 compared with unwounded roots with Fe or wounded roots without Fe. Foliar applications of Fe (as FeNaEDTA) to ginseng plants three times during the 2002 and 2003 growing seasons significantly increased Fe levels in root tissues. These roots developed larger lesions following inoculation with *C. destructans* in vitro. When radioactive Fe (^{59}Fe) was applied to the foliage of ginseng plants, it was detected in the secondary phloem and in cortical and epidermal tissues within 1 week. Artificially wounded areas on the roots accumulated more ^{59}Fe than healthy areas. Diseased tissue also had threefold higher levels of phenolic compounds and Fe compared

with adjoining healthy tissues. High-performance liquid chromatography analysis revealed enhanced levels of protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, cinnamic acid, phloridizin, and quercetin. Phenolic compounds produced in diseased and wounded tissues sequestered Fe in vitro. The effects of Fe on mycelial growth, conidial germ tube length, and secondary branching of germ tubes of *C. destructans* were examined in vitro. When grown on Chrome-azuro S medium, Fe also was sequestered by *C. destructans* through siderophore production, which was visualized as a clearing pigmented zone at the margin of colonies. Mycelial dry weight was significantly increased in glucose/yeast broth containing Fe at 56 or 112 µg/ml. Conidial germ tube length and secondary branching of hyphae also were enhanced after 8 and 16 h by Fe. Colony growth of *C. destructans* was not enhanced by Fe, but significantly greater spore production was observed with Fe at 56 and 112 µg/ml compared with no Fe in the medium. Although these levels of Fe had no effect on fungal pectinase enzyme activity, polyphenoloxidase (PPO) activity was significantly ($P < 0.0001$) enhanced. We conclude that Fe enhances *Cylindrocarpon* root rot through enhanced pathogen growth, sporulation, and PPO enzyme activity. Fe sequestered by phenolic compounds produced in wounded tissues can enhance Fe levels at the site of infection. The pathogen also has the ability to sequester Fe at these sites.

Iron (Fe) is an essential micronutrient for plant growth, acting as an enzyme cofactor and participating in vital processes such as photosynthesis, DNA replication, and respiration. Fe also is important in microbial respiration and is sequestered from the surrounding environment for uptake by microbes by the production of siderophores. In root-infecting fungi, the role of Fe has been studied mainly from the perspective of its role in *Pseudomonas*-mediated biological control (27). For example, in diseases caused by *Fusarium* spp., disease suppressiveness was attributed to competition for Fe which limited fungal development (16). Scher and Baker (27) and Jones and Woltz (13) suggested that reduced Fe availability through Fe competition could reduce wilt severity on tomato caused by *Fusarium* spp.

In certain plant–fungus interactions, Fe is reported to have an important role during infection by promoting pathogen growth and enhancing tissue decay. Hendrix et al. (12) reported that growth of *Phytophthora parasitica* var. *nicotianae* was enhanced by minor elements such as Fe^{3+} when added to a synthetic glucose asparagine medium. The formation and infectivity of *Phytophthora* zoospores on pine cotyledons was enhanced by Fe and Mg (11). Fe also has been reported to promote brown-rot decay of treated wood (18). When Fe was added to malt-agar medium, decay of pine by inoculum of *Serpula lacrymans* grown on the

Fe-amended medium was enhanced (20). Decaying tissues of hardwood trees were shown to accumulate higher mineral levels (including Fe) compared with healthy wood (29,30). Under laboratory conditions, a correlation between a range of minerals, including Fe, and mycelial growth of fungi associated with wood discoloration in maple, birch, beech, and ash was observed. Shigo (31) suggested that these fungi, which were wound invaders, had adapted to the high concentrations of minerals that were sequestered in discolored and decayed tissues.

Cylindrocarpon root rot of American ginseng (*Panax quinquefolius* L.), caused by *Cylindrocarpon destructans* (Zins) Scholten, is an important disease in ginseng-growing regions of Canada (21,24,25). The disease is enhanced by wounding, and severity is greater at soil pH 5.0 than 7.0 (23). Infection of the roots can result in complete decay; hence, the name “disappearing root rot” frequently is used to describe this disease. The role of micronutrients, in particular Fe, on disease development has not been studied previously. The objectives of this study were to determine whether Fe could enhance *Cylindrocarpon* root rot development through its effect on pathogen growth and infection as well by promoting tissue decay.

MATERIALS AND METHODS

Effect of Fe on *Cylindrocarpon* root rot. Hydroponic study. A hydroponic method for maintaining ginseng roots was utilized to determine the effect of Fe on disease initiation and to visualize the progress of infection. Two-year-old ginseng plants were dug

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from a ginseng garden in Kamloops, BC, Canada. Plants with surrounding rhizosphere soil were transferred to 15-cm pots, which were covered with polyethylene bags and brought to the laboratory within 24 h. The roots were kept under running tap water to remove any loosely attached microbes on the root surface and examined for blemishes, and healthy roots were inserted through pre-cut holes in a Styrofoam block and suspended into a container of Hoagland's solution (HS) that was modified by adding FeNaEDTA (Sigma-Aldrich, St. Louis) to achieve concentrations of 56 µg/ml (normal Hoagland's) or 112 µg/ml (twice Hoagland's) and compared with Hoagland's with no added Fe. A highly aggressive isolate of *C. destructans* obtained from ginseng roots was grown on potato ginseng agar (23) and spores were added to the nutrient solution to achieve 5×10^5 spores/ml in each container (Sigma plant cell culture vessel, Phytakon; Sigma-Aldrich). The solution was aerated through a direct airflow line on a lab bench and incubated under low intensity light ($40 \mu\text{mol s}^{-1} \text{ m}^{-2}$). Roots were rated for disease severity at 3-day intervals after inoculation using a 1-to-6 scale, where 1 = no visible lesions, 2 = brown lesions up to 0.9 mm in diameter, 3 = dark brown lesions of 1 to 4.0 mm, 4 = black lesions of 4 to 7.0 mm, 5 = black lesions >7.0 mm coalesced with each other, and 6 = fully rotten roots. A disease severity index (DSI) then was calculated using the formula $\text{DSI} = ([X_1 \times 1] + [X_2 \times 2] + [X_3 \times 3] + [X_4 \times 4] + [X_5 \times 5] + [X_6 \times 6]) / (X_1 + X_2 + X_3 + X_4 + X_5 + X_6)$, where X_1, X_2, X_3, X_4, X_5 , and X_6 are the numbers of plants with rotting severity of 1, 2, 3, 4, 5, and 6, respectively. There were six replicate roots in each container and the experiment was conducted three times. Data from disease severity in all three experiments were pooled together after testing the homogeneity of variance ($P \leq 0.05$); means of treatments were compared by Fisher's protected least significant difference (LSD) at the 5% level. Statistical analysis was performed with the Statistical Analysis System (version 8; SAS Institute Inc., Cary, NC).

Field study. To determine the effect of Fe on root rot severity under field conditions, a 5-by-1-m area in each of four replicate beds was selected in a 3-year-old ginseng garden located in Kamloops, BC, and Heffley Creek, BC during the 2003 and 2004 growing seasons, respectively. Both fields had a prior history of *Cylindrocarpon* root rot and the pathogen was recovered from visibly diseased plants in certain areas of the field. Groups of 30 visibly healthy plants in each replicate plot were selected for the following treatments: (i) wounding of roots at the crown by piercing them 8 to 10 times with a fine needle (an insect mounting pin) to approximately 1.0 mm in depth by 0.25 mm in diameter, (ii) wounding of roots followed by drenching with Fe solution (0.05% FeNaEDTA), and (iii) control (unwounded, drenched with water or Fe). The straw mulch and soil at the crown of each root was carefully removed to expose the roots and facilitate application of treatments and then replaced. The roots in each plot were dug up after 30 days, washed, and rated for disease severity using the DSI scale described previously.

In a separate experiment conducted in a 3-year-old ginseng garden in Kamloops, BC during the 2002 and 2003 growing seasons which had no history of *Cylindrocarpon* root rot, foliar applications of FeNaEDTA (0.05%) were made during the first week of June, July, and August. The experimental plots, each measuring 1.22 m², contained ≈ 100 plants which were sprayed with a hand-held sprayer to run-off. Corresponding control plots were sprayed with water. During September, the ginseng roots were harvested, transported to the laboratory, and washed. Fe-treated and control roots were inoculated with 0.5-cm² mycelial plugs (three plugs per root) taken from a 14-day-old culture of a highly aggressive isolate of *C. destructans* and incubated in a sealed plastic container layered with moist paper towels. Lesion diameter was measured in two orthogonal directions from four replicate roots after 3 weeks. The experiment was conducted three times, once in 2002 and twice in 2003. Data from the experiments

in different years were pooled after testing the homogeneity of variance ($P \leq 0.05$). Data for the means of treatments were compared using Fisher's protected LSD test at the 5% level using Statistical Analysis System (version 8.0; SAS Institute).

Measurement of nutrient levels and phenolic compounds.

Epidermal and cortical tissues from Fe-treated and control roots, as well as from *Cylindrocarpon*-infected and healthy regions on the same root, were removed with a sharp scalpel to make a composite sample for each treatment. For nutrient analysis, dried tissue (0.2 g) was digested with 40% (vol/vol) hydrofluoric acid mixed with nitric acid. The digested sample was made up to 100 ml with 1% nitric acid. Aliquots of this solution were mixed with an internal standard and analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) as previously described by Dahlquist and Knoll (8) with slight modifications. Samples were compared with a calibration curve constructed using multi-element standards.

Tissues from *Cylindrocarpon*-infected and healthy areas were also analyzed for specific phenolic compounds. For phenolic extraction, an equal amount (2 g) of tissues was weighed, transferred to a mortar, frozen in liquid N₂, and ground with a pestle. Ground tissues were transferred to a Falcon tube with 20 ml of 80% methanol. The mixture was stirred at 37°C in a water bath for 30 min, followed by centrifugation at $8,000 \times g$ for 10 min. The supernatant was transferred to a new tube and evaporated to dryness using a rotary evaporator. The dry residue then was dissolved in 5 ml of solvent containing methanol, formic acid, and water (70:8:22), which constituted the mobile phase for high-performance liquid chromatography (HPLC). The concentrated extract was centrifuged at $12,000 \times g$ for 10 min and filtered through a 0.2-µm hydrophilic Durapore membrane filter (Millipore Corp., Bedford, MA). The extract (50 µl) was injected through an Atlantis dC₁₈ column in an LC-module 1 Waters HPLC machine (Mildford, MS) at a flow rate of 1.2 ml/min and the absorbance was recorded at 280 nm over a 24-min retention time. Identification of compounds was done by comparing (over-laying) the peaks at specific retention times with chromatograms of authentic purified compounds used as external standards (Sigma-Aldrich).

Phenolic-Fe binding assay. Ginseng callus cultures were initiated from root tissues as described by Tirajoh et al. (34). Briefly, surface-sterilized root segments were placed on Murashige and Skoog (MS) medium (19) containing a full complement of salts and vitamins and the growth regulators naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at 10.0 µM and 9.0 µM, respectively. Developing calli were subcultured to fresh medium every 6 to 8 weeks. To initiate suspension cultures, the method described by Punja et al. (22) was used. Cell masses were placed in 25 ml of MS medium in a 125-ml Erlenmeyer flask and shaken at 125 rpm. The growth regulators subsequently were reduced to 2.5 µM NAA and 2.0 µM 2,4-D. Cell masses proliferated under these conditions and the spent medium was replaced with fresh medium every 2 weeks. Cell masses were collected by filtration through sterile cheesecloth. Calli (2 g) from the MS medium were transferred to each of 27 flasks containing 100 ml of liquid MS and the flasks were incubated on a shaker at 75 rpm for 1 week at ambient room temperature ($21 \pm 2^\circ\text{C}$). The calli then were transferred to a similar flask containing the same amount of MS amended with either Fe (as FeNaEDTA; Sigma-Aldrich) at 0, 56, or 112 µg/ml. Each Fe concentration had nine flasks, of which three were kept as a control without any other treatment. Three flasks received 0.5% chitosan (Apollo Chitosan, Burnaby, BC, Canada) and the remainder were sonicated with a Virsonic 60 sonicator (The Virtis Company, Inc., NY) at 20 Hertz for 30 s. All flasks were incubated for another 2 weeks under the same conditions as described above, after which time the MS medium was poured off and the calli were washed twice with 100 ml of sterile deionized water. Calli from each treatment and

the controls were analyzed for Fe by ICP-AES as described above.

Translocation of Fe (^{59}Fe) from leaves. Radioactive Fe ($^{59}\text{FeCl}_3$) with a specific activity of 2.94 MBq in 0.5 M HCl (Amersham Bioscience, GE Healthcare, Canada) was used as an iron tracer. After dilution, an excess of EDTA was added to a final concentration of 0.1 mM Fe-EDTA. Two-year-old ginseng plants were dug from a ginseng garden in Kamloops, BC. Plants with surrounding rhizosphere soil were transferred to 15-cm-diameter pots which were covered with polyethylene bags and brought to the laboratory within 24 h. Soil from around the crowns was carefully removed to make wounds with a sharp needle. Radioactive Fe (^{59}Fe) was applied to one group of plants immediately after wounding and to another group 1 week after wounding. The ^{59}Fe solution was applied as 10- μl droplets (three drops on each leaflet) on three leaflets per plant. Each droplet covered an area of 70 to 100 mm^2 . In order to facilitate ^{59}Fe uptake, the upper epidermis was injured gently by scratching with a scalpel. One week after ^{59}Fe application, the roots were harvested from the pots. Using a sharp razor, cross-sections were made through the crown, middle, and tip region (2.5 cm from the root tip). The root sections were exposed to a Phosphor-Imaging screen overnight, followed by scanning in a Phosphor-Imager (Typhoon 9410; Molecular Dynamics, Amersham Biosciences, Piscataway, NJ). An image analysis was performed with the digitalized autoradiograph picture (Image Quant 5.2) to produce semiquantitative measures of radioactivity per area or activity profiles. These were compared with a calibrated standard composed of four different levels of ^{59}Fe activity and exposed simultaneously. Values were obtained by comparing the relative amount of shaded area within the standard with that of the root sample. Cross-sectional transects through scanned images of the root samples also were analyzed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health [NIH]; provided online by NIH) to create graphs. To obtain quantitative results, tissue pieces $\approx 4 \text{ mm}^2$ in size were excised from the region of the secondary phloem taken from the crown, middle, and tip regions of roots and weighed. Tissues were ground in liquid N_2 and solubilized in organic base (Soluene 350; Sigma-Aldrich). The solution then was added to a scintillation cocktail (Amersham BioScience) and ^{59}Fe activity was determined using a scintillation counter (LS 6500; Beckman Counter, CA). The analyses were conducted using three replicate samples.

Effect of Fe on fungal growth. A glucose-yeast extract broth (29) containing 5 g of glucose and 1 g of yeast extract in 1 liter of water was used as the basal medium. FeNaEDTA (Sigma-Aldrich) was added to achieve an Fe concentration of 0, 56, and 112 $\mu\text{g/ml}$. Flasks containing 100 ml of medium were inoculated with a 0.5-cm 2 mycelial plug from a 14-day-old colony of a highly aggressive isolate of *C. destructans* and incubated on a rotary shaker (150 rpm) for 2 weeks. The mycelial mass from four replicate flasks was filtered, dried at 40°C for 72 h, and weighed. The experiment was conducted twice. Data from both experiments were pooled after testing the homogeneity of variance and analyzed by the analysis of variance (ANOVA) of the Statistical Analysis System (version 8.0; SAS Institute).

Effect of Fe on spore production and germination. FeNaEDTA was added to potato dextrose agar (PDA) (full strength; BDH, Darmstadt, Germany) to achieve an Fe concentration of 0, 56, and 112 $\mu\text{g/ml}$, assuming release of Fe into the medium from FeNaEDTA over time, and 25 ml was poured into each petri dish. Petri dishes were inoculated with a 0.5-cm 2 mycelial plug from a 14-day-old PDA colony of *C. destructans* followed by 2 weeks of incubation at ambient room temperature ($21 \pm 2^\circ\text{C}$) under fluorescent lights (16 h/day). Water (15 ml) was added to each colony, followed by two washings with 10 ml of water each, and spores were counted in a hemacytometer. For germ tube growth measurements, spores were obtained from colonies

grown on potato ginseng agar (23) by adding either water or HS with Fe at 56 or 112 $\mu\text{g/ml}$ and diluted to achieve a concentration of 15 to 20 spores/ μl . Drops of spore suspension (5 μl) were placed on glass slides and germinating spores were counted after incubating overnight inside petri dishes containing water agar. Germ tube lengths of 100 germinated spores were measured using a micrometer after 8 and 16 h of incubation. The extent of secondary branching of germ tubes also was recorded. The experiment was conducted twice with three replications and the data averaged.

Effect of Fe on extracellular enzyme production by *C. destructans*. The method described by Rahman and Punja (23) was used to measure pectinase and polyphenoloxidase activity with slight modification. In addition to using all of the recommended salts and substrates, the broth was further modified by adding Fe at 0, 56, or 112 $\mu\text{g/ml}$. In the case of pectinase activity, the assay medium also was amended with different concentrations of Fe (0, 56, and 112 $\mu\text{g/ml}$). The remaining assay procedures were as described by Rahman and Punja (23).

Siderophore formation. Chrome-azurol S (CAS) medium was prepared following the method described by Schwyn and Neilands (28). Petri dishes containing 25 ml of CAS medium were inoculated with agar blocks containing mycelium from a 14-day-old culture of *C. destructans*, arranged in a triangular shape, and incubated at ambient room temperature ($21 \pm 2^\circ\text{C}$) for a week. Clearing zones and a change in color of the medium from bluish-green to orange at the colony margin indicated the putative production of siderophore (28).

RESULTS

Effect of Fe on *Cylindrocarpon* root rot. Hydroponic study. The addition of Fe at 56 $\mu\text{g/ml}$ significantly enhanced disease initiation and severity compared with no Fe, and Fe at 112 $\mu\text{g/ml}$ supported more disease than at 56 $\mu\text{g/ml}$ (Fig. 1A). With no Fe, necrotic lesions appeared on ginseng roots at 12 days after inoculation (DAI) compared with 9 and 6 DAI for Fe at 56 and 112 $\mu\text{g/ml}$, respectively (Fig. 2). Disease development also was more rapid with Fe at 112 $\mu\text{g/ml}$ compared with Fe at 0 and 56 $\mu\text{g/ml}$, which contributed to a significantly higher disease severity index (DSI) at higher Fe concentrations (Fig. 2). Lesion development with Fe at 112 $\mu\text{g/ml}$ resulted in complete loss of feeder roots and, eventually, the entire root rotted (Fig. 1A). At this stage, some of the roots were found to be colonized also by *Fusarium solani*, presumably from low levels of residual inoculum on the field-grown roots.

Field study. Extensive rotting in the crown region was observed on roots that received a combination of wounding and Fe treatment compared with wounding or Fe alone (Fig. 1B to D; Table 1). The wounded plus Fe-treated plants also had significantly higher plant mortality compared with wounded roots (Table 1). Wounded roots showed minor browning as well as wound-healing responses that included callus formation at wound sites (Fig. 1C). *C. destructans* was isolated from diseased tissues and isolates were found to comprise both highly and weakly aggressive strains (23). In vitro inoculations with *C. destructans* showed larger lesion development on Fe-treated roots compared with the control (Fig. 1E), with a mean lesion diameter on Fe-treated roots of 1.4 cm compared with 0.6 cm on the control roots. This difference was observed in both years of the experiment.

Measurement of nutrient levels and phenolic compounds. The ICP-AES analysis of healthy and *C. destructans*-infected root tissues showed significant differences in a number of elements, especially Fe and Mn (Table 2). This trend was observed in replicate root samples and when the tissue analysis was repeated using samples from naturally infected as well as artificially inoculated roots. Nutrient analysis of foliar-applied Fe-treated plants showed significantly higher Fe levels in roots compared with the

Fig. 1. Effect of iron (Fe) on root rot of ginseng and on *Cylindrocarpon destructans* growth. **A**, Lesion development on hydroponically maintained roots with different Fe levels and inoculated with *C. destructans* spores: left = zero Fe, middle = 56 µg/ml Fe, and right = 112 µg/ml Fe. **B**, Control roots from the field study. **C**, Wounded roots with minor browning and wound healing response. **D**, Roots with extensive rotting due to *C. destructans* at the crown as a result of wounding followed by Fe drench. **E**, Roots showing lesions following inoculation with *C. destructans* mycelial plugs from a 14-day-old culture; left = control and right = foliar Fe treated. **F**, Clearing zone around *C. destructans* agar blocks arranged in a triangular pattern on chrome-azuro S medium indicating removal of Fe through production of siderophore and a change in color from green to orange.

control (Table 3). The levels of all other nutrients in the Fe-treated and control roots were similar.

Total phenolic content in tissues infected with *C. destructans* was enhanced threefold compared with healthy tissues on the same root. The phenolic compounds which were significantly elevated in diseased tissues were identified by HPLC as protocatechuic acid, o-coumaric acid, phloridzin, caffeic acid, p-coumaric acid, benzoic acid, 3,4,5 tri methyl benzoic acid, cinnamic acid, and quercetin (Table 4). In addition, several phenolic compounds (ferulic acid and some unidentified peaks) were present in diseased tissues that were absent in healthy tissues.

Phenolic-Fe binding assays. Ginseng calli grown on MS medium containing elevated levels of Fe accumulated more Fe in the tissues (Fig. 3) and treatment with chitosan significantly ($P < 0.0001$) enhanced Fe accumulation under the same growth conditions. Sonicated calli did not enhance Fe levels compared with the untreated control.

Translocation of Fe (^{59}Fe) from leaves. Iron was detected in all roots when ^{59}Fe was applied to leaves. Image analysis showed that most of the radioactive Fe was translocated through the secondary phloem with lateral movement to epidermal tissues and especially to wounded areas (Fig. 4). There was no difference in radioactivity around wounds made just prior to ^{59}Fe application or a week earlier. Higher radioactivity was detected in wounded compared with unwounded areas on the root. Within a week, radioactive Fe was present in the crown region to the proximity of root tips over a gradient of decreasing concentration. Scintillation counts from a sample of root tissue (4 mm² by 0.5 mm thick) from the crown, middle, and root-tip regions indicated levels of 2,500, 1,000, and 650 cpm of radioactivity (β emission), respectively. Graphs created from cross-sectional transects of scanned images showed taller peaks in the secondary phloem and cortical zone where wound-induced phenolic accumulation took place near the crown. Primary vascular tissues in and around the stele, including the secondary xylem, did not show any accumulation of radioactivity.

Fig. 2. Progression of *Cylindrocarpon destructans* root rot on hydroponically maintained ginseng roots with iron at 0, 56, and 112 µg/ml. Vertical bars represent the standard errors of means from data pooled from three experiments ($n = 27$).

TABLE 1. Effect of iron (Fe) treatment and wounding on *Cylindrocarpon* root rot incidence and severity on ginseng roots^z

Treatment	Number of			Rotted roots (%)	Disease severity index
	Treated roots	Rotted roots	Roots with lesions		
Wounding	30	6	8	46.67 b	2.60 b
Wounding + Fe (56 µg/ml)	30	8	15	76.67 a	4.33 a
Control	30	4	3	23.33 c	1.77 c

^z Data were pooled from 2 years (2003 and 2004) with four replications in each treatment. Means followed by different letters are significantly different from each other according to Fisher's protected least significant difference test. Control plants received either water or Fe (0.05%).

Effect of Fe on fungal growth. Mycelial mass produced in glucose-yeast broth was significantly higher ($P < 0.001$) with Fe at both 56 and 112 µg/ml in the medium compared with 0 Fe. The stimulatory effect of Fe on mycelial mass production was in the order of $112 > 56 > 0.0$ (Fig. 5).

Effect of Fe on spore production, germ tube length, and secondary branching of hyphae. Fe significantly enhanced

TABLE 2. Micronutrient levels in epidermal tissues of healthy and *C. destructans*-infected ginseng roots

Micronutrient	Concentration (µg/g) ^z	
	Healthy	Diseased
Boron	6.9	9.3
Copper	4.2	5.3
Iron	38.4	183
Manganese	13.8	34.5
Molybdenum	<0.5	>0.5
Zinc	17.6	16.3

^z Data are the average from three replicate samples.

TABLE 3. Nutrient levels in the epidermal tissues of iron (Fe)-treated and untreated ginseng roots

Element	Concentration (µg/g) ^z	
	Fe-treated	Control
Calcium	2,700	2,700
Phosphorus	1,300	1,500
Potassium	9,400	8,300
Magnesium	1,200	1,400
Sodium	200	200
Sulfur	400	600
Zinc	13.9	10.8
Boron	10.3	11.0
Manganese	11.0	10.5
Fe	100.0	52.4
Molybdenum	<0.6	<0.6

^z Three Fe applications were made to foliage of 3-year-old ginseng plants in 2002 and 2003. Data are the average from six replicate samples (three replicates in each year).

TABLE 4. Phenolic compounds in epidermal and cortical tissues of healthy and *C. destructans*-infected ginseng roots

Phenolic compounds ^z	Tissue levels (µg/g fresh weight)	
	Healthy	Diseased
Protocatechuic acid	1.11	2.81
O-Coumaric acid	0.48	2.32
Phloridzin	2.31	12.24
Caffeic acid	0.45	2.40
p-Coumaric acid	0.47	2.57
Benzoic acid	0.27	1.59
3,4,5 Tri methyl benzoic acid	0.24	2.08
Ferulic acid	0.09	0.86
Cinnamic acid	0.18	2.50
Quercetin	0.24	6.12

^z Phenolic compounds were identified by high-performance liquid chromatography. The data represent the mean of three different analyses.

sporulation in a relatively low-spore-producing isolate of *C. destructans* on PDA. After 2 weeks of incubation, colonies on PDA without Fe did not produce any spores, whereas colonies grown on PDA with Fe at 56 and 112 $\mu\text{g/ml}$ produced 5 and 6 $\times$

10^5 spores, respectively (Fig. 6). Germ tube length was significantly higher with Fe at 112 compared with 56 $\mu\text{g/ml}$ after 16 h of incubation (Fig. 7B). Very few spores showed secondary branching after 8 h, but Fe at 56 and 112 $\mu\text{g/ml}$ promoted significantly higher secondary branching of germ tubes compared with 0 Fe after 16 h (Table 5).

Fig. 3. Concentration of iron (Fe) in ginseng calli treated with two different Fe levels and two phenolic inducers (chitosan and sonication). Data are the means of three replicates. Bars followed by a different letter are significantly different according to Fisher's protected least significant difference test ($P \leq 0.05$).

Fig. 5. Effect of iron (Fe) on mycelial dry weight produced by *Cylindrocarpon destructans*. Bars followed by different letters are significantly different according to Fisher's protected least significant difference ($P \leq 0.05$). Vertical bars represent the standard errors of means from three experiments, each having four replicate flasks ($n = 12$).

Fig. 4. Distribution of radioactive iron (^{59}Fe) in regions of ginseng roots 1 week after application to leaves. **A**, Thin sections of roots taken at the crown (top), middle, and root tip (bottom) regions. **B**, Phosphor-image scans of the thin sections from A; darkened zones correspond to radioactivity. **C**, Quantitative measures of radioactivity analyzed by National Institutes of Health image software. The different regions of the root are shown as S(P) for secondary phloem, S for stele, and E for epidermis. **D**, Schematic diagram of root areas seen in panel B to show radioactivity accumulation based on scintillation counts from different regions of the roots. Arrows indicate regions where wounds were made.

Effect of Fe on extracellular enzyme production by *C. destructans*. Fe did not significantly increase pectinase activity; however, polyphenoloxidase (PPO) was significantly enhanced by adding Fe to the growth medium (Fig. 8).

Siderophore formation. *C. destructans* produced siderophores as evidenced by the clear zone on all sides of mycelial blocks (Fig. 1F), as well as a change in color of CAS medium from green to orange-yellow. This change is putative evidence for siderophore production (28).

DISCUSSION

The present study investigated the role of Fe on development of *Cylindrocarpon* root rot and potential mechanisms by which Fe could enhance disease incidence and severity. Greater mycelial mass in medium supplemented with Fe suggests a growth stimulation, consistent with the reports for fungi causing decay of woody tissues as well as *F. graminearum*, *F. moniliforme*, and *Aspergillus flavus* (6,7) which were all stimulated by metal ions such as Fe.

Previously published results have indicated both an increase and decrease in plant diseases due to Fe application. For example, Fe reduced Verticillium wilt of eggplant and peanut (2) but increased Fusarium wilt of tomato (13) and cotton (32), and

wheat rust (32). Hydroponic culture permitted observation of how Fe in the hydroponic solution could affect disease development at 3-day intervals on the same roots over the duration of the experiment. Fe significantly enhanced disease development and increased PPO activity of *C. destructans*. This enzyme may be a potential contributor to lesion development on ginseng roots. The dark-brown sunken lesions caused by *C. destructans* are likely the outcome of oxidation of host phenolic compounds by PPO. Ginseng roots produce a variety of phenolic compounds, including ginsenosides or ginseng saponins, which are the principal bioactive compounds (35). PPO production by fungi usually is in-

TABLE 5. Effect of iron (Fe) on germ tube length and secondary branching of mycelia from germinated spores of *Cylindrocarpon destructans*^z

Fe level (µg/ml)	Germ tube length (mm)		Secondary branching (%)	
	8 h	16 h	8 h	16 h
0	0.09 a	0.2 a	0 a	3.5 a
56	0.17 b	0.34 b	0 a	15 b
112	0.25 c	0.49 c	<1 a	27 c

^z Treatments were applied in Hoagland's solution. Data are means of 100 germinated spores. Data in a single column followed by the same letter are not significantly different from each other according to Fisher's protected least significant difference test.

Fig. 6. Effect of iron (Fe) on sporulation of *Cylindrocarpon destructans* on potato-dextrose agar. Spores were enumerated after 14 days of incubation; vertical bars represent the standard errors of means from two experiments, each having three replicate flasks (*n* = 6).

Fig. 8. Effect of iron (Fe) on polyphenoloxidase (PPO) production by *Cylindrocarpon destructans*. Bars followed by different letters are significantly different according to Fisher's protected least significant difference (*P* ≤ 0.05). Vertical bars represent the standard errors of means from 3 experiments each having three replicate flasks (*n* = 9).

Fig. 7. *Cylindrocarpon destructans* spore germination and hyphal growth in Hoagland's solution with iron at **A**, 56 and **B**, 112 µg/ml, scale bar = 25 µm. Arrows indicate secondary branching of germ tubes.

duced by phenolic substances in the host plant or in artificial media, and helps the pathogen to degrade cell wall constituents or detoxify host quinones by polymerization (17). In a previous study, we reported that ginseng cells infected by *C. destructans* stained a bluish green with Toluidine blue O, indicating the presence of host phenolic compounds in diseased tissues (23).

Excessive Fe concentrations (up to 336 µg/ml) do not inhibit growth of *C. destructans* in vitro (data not shown), suggesting that *C. destructans* is tolerant of high Fe levels. Tissue Fe levels following three applications of FeNaEDTA were ≈100 µg/g. Several brown and white-rot fungi produce low molecular weight metal-chelator agents or siderophores (10). These chelators reduce ferric Fe for fungal metabolic functions. However, part of the reduced Fe is released into the environment where it reacts with oxidants such as hydrogen peroxide to produce free radical species which, in turn, are active chemical oxidizers of both cellulosic and phenolic compounds (10). In *Cylindrocarpon* root rot of ginseng, formation of dark-brown necrotic lesions is indicative of the oxidation of phenolic compounds which likely involves Fe. Levels of Fe in diseased tissue were fourfold higher than in adjoining healthy tissues. Putative production of uncharacterized siderophores by *C. destructans* was suggested by the positive reaction on CAS medium. In this medium, the ternary complex CAS/hexadecyltrimethyl-ammonium bromide (HDTMA) is used as the indicator. When a high-affinity Fe (III) chelator removes Fe from the dye complex, its color changes from blue to orange (28), as was observed in this study. In *Gloeophyllum trabeum* brown-rotted wood, Xu and Goodell (36) showed a significant role of Gt chelator (Fe-chelator produced by *G. trabeum*), iron, H₂O₂, and pH in cellulose degradation that enhanced the overall rotting capacity of this fungus.

Radioactive Fe (⁵⁹Fe) translocated from ginseng leaves to roots accumulated at a higher level around wound sites where phenolic production was induced. Binding of Fe following phenolic induction was demonstrated in vitro by the greater Fe content in ginseng calli treated with phenolic inducers such as chitosan (3) in the presence of supplemental Fe. Polyphenols, especially tannins, chelate heavy metals, such as Fe, and play a role as potential antioxidants (26). Phenolic compounds are involved with metal chelation in vitro and in vivo (4,5,14,15). Aoba (1) suggested that tannin-rich plants such as tea, which are tolerant to excess Mn, are protected by the direct chelation of Mn by tannins and that typical brown spots on leaves exposed to excess Mn are the result of tannin oxidation. Cheng and Crisosto (5) demonstrated that elevated levels of the phenolic compounds caffeic acid, chlorogenic acid, and catechin chelated Fe to form a Fe polyphenol complex which led to tissue discoloration in peach and nectarine epidermal tissues. Increased Fe levels observed in necrotic ginseng tissues compared with adjoining healthy tissues appear to be due in part to chelation by enhanced levels of phenolic compounds. Phenolic compounds with a galloyl or 3',4'-dihydroxy (catechol) group on flavanoid ring B are especially important in Fe-binding. These include tannic acid, gallic acid, quercetin, chlorogenic acid, caffeic acid, and others (4,14). A number of these were detected at high concentrations in diseased ginseng tissues.

High Fe levels in necrotic ginseng tissues (as well as Mn) compared with adjacent healthy tissues point to the ability of these cations to remobilize within the root as an outcome of either host defense response or fungal sequestration in or around the infection site. Fe treatment did not influence Mn levels in roots. Like Fe, Mn has been shown to be involved in disease development of *Gaeumanomyces graminis* var. *tritici* on wheat roots, and Mn was oxidized by the pathogen within lesions by the production of laccases (33). Dimarco et al. (9) reported a significantly higher level of Fe in grape leaves with esca symptoms compared with asymptomatic leaves. In the present study, wounding of roots followed by Fe drenching significantly increased disease due to

C. destructans compared with Fe alone or wounding alone, suggesting an additive effect and, thus, enhanced susceptibility.

Our previous study indicated that low soil pH favors *Cylindrocarpon* root rot development (23). Because low pH supports higher Fe availability to the plant, Fe may be one of the factors which could enhance disease severity under acidic conditions. Thus, managing soil factors such as pH and Fe availability may prove to be partially effective in disease management. The practice of foliar Fe applications conducted by ginseng growers in soils with alkaline to neutral pH also should be avoided in fields with a history of *Cylindrocarpon* root rot.

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