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Quantification of *Cylindrocarpon destructans* f. sp. *panacis* in soils by real-time PCR

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Ginseng (*Panax quinquefolius*) is an important cash crop in various regions of North America, but yields are often reduced by various root pathogens. A quantitative real-time PCR (qPCR) assay for *Cylindrocarpon destructans* f. sp. *panacis* (CDP), the cause of a root rot and replant disease which discourages successive cropping of ginseng on the same site, was developed to quantify the levels of this pathogen in soils previously cropped with ginseng. DNA was extracted from 5-g samples of soil. In pasteurized soils which were re-infested with varying levels of the pathogen, qPCR estimates of pathogen DNA were significantly correlated with disease severity (r = 0·494) and with counts of colony-forming units (r = 0·620) obtained with an agar medium. In several naturally infested field soils, qPCR estimates of CDP-DNA concentration were significantly correlated with disease severity (r = 0·765) and these concentrations were estimated to range from 0 to 1·48 ng g^{-1} dried soil. A principal components analysis did not show any strong relationships between soil chemistry factors and the concentration of pathogen DNA. The approach outlined here allows the quantification of current populations of CDP in soil many years after ginseng cultivation and the prediction of disease severity in future crops. The method should be generally applicable to root diseases of many crops.

Keywords: ginseng, *Panax quinquefolius*, quantitative PCR, replant decline, root rot, soil DNA extraction

Introduction

Panax quinquefolius (American ginseng) is cultivated as a medicinal herb and is reputed to be effective against a wide variety of ailments. Products derived from dried roots of ginseng were shown to reduce glycemia and cold symptoms in clinical trials (Vuksan *et al*., 2000; Predy *et al*., 2005). Canada exports *c*. 2000 t ginseng root per year, accounting for more than 60% of world production (Agriculture and Agri-Food Canada, 2003). Ginseng is normally grown for 3 or 4 years prior to root harvest and the crop is intensively managed. A serious root disease caused by *Cylindrocarpon destructans* f. sp. *panacis* (CDP) can result in yield losses of up to 25–30% (Chung, 1975; Seifert *et al*., 2003) and limits the re-use of fields for successive ginseng crops (Reeleder & Brammall, 1994; Reeleder *et al*., 2002).

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Assessment of disease risk from soilborne fungal pathogens such as CDP has often been based on the number of colonies formed (CFU) by dilution plating on selective media (Davet & Rouxel, 2000). However, this method is labour-intensive and tends to lack sensitivity and specificity because of problems with morphological identification and competition from other soil organisms. Soilborne pathogen populations can also be inferred from disease severities observed in host plant bioassays, in which plants are grown in aliquots of field soil. The resulting severity of root disease can then be interpreted as a measure of inoculum potential (Garrett, 1956). These assays, however, are time-consuming and data are affected by variability in plant susceptibility and the bioassay environment.

Recently, more precise estimates of soilborne fungal pathogen populations have been obtained using real-time quantitative PCR (qPCR), a culture-independent method in which template DNA is quantified on the basis of a fluorescent signal produced during PCR amplification. Successful application depends only on the ability to design taxon-specific primers for the target organism and efficient extraction of clean template DNA from the soil matrix (Miller *et al*., 1999; Martin-Laurent *et al*., 2001). qPCR represents a major advancement in plant pathology (Schaad & Frederick, 2002; Schena *et al*., 2004) and has been used to quantify fungal colonization of plant tissue

(Mercado-Blanco *et al*., 2003) and soils (Cullen *et al*., 2001; Lees *et al*., 2002; Filion *et al*., 2003; Schroeder *et al*., 2006). However, as qPCR is a measure of DNA concentration only, it is still necessary to relate qPCR data to more traditional data on population or inoculum potential. Once the relationship between target DNA concentration and root disease is established for a particular pathosystem, qPCR data alone may be used to estimate disease potential. The ability to rapidly and reliably assess the disease risk to ginseng crops from CDP using qPCR will help avoid the accidental replanting of heavily-infested soils and also allow for more accurate prediction of crop loss. More precise information on distribution of the pathogen in soil will also further understanding of the relationships between CDP and other soil biotic and abiotic factors.

The main objectives of the current research were therefore to use qPCR to assess the abundance of CDP in soils previously cropped with ginseng and to compare and correlate these estimates with data from soil dilution plating and concurrent host plant bioassays.

Materials and methods

Primer design and testing

Oligonucleotide primers specific to CDP were designed previously (McMullen, 2000) using subtractive hybridization. These were two forward (CDU1 or CDU3) primers and one reverse (CDL1b) primer, amplifying approximately 500 bp of the intergenic spacer (IGS) region of the fungus. The CDU3/CDL1b primer combination was used for a thorough assessment of the relationships between various isolates of the pathogen from ginseng, and also tested against a range of common soil fungi (Seifert *et al*., 2003). However, the forward primer, CDU1 (5′-GGTCGCG-GCGAGAGTCTC-3′), which anneals proximally to CDU3 on the same subtraction product, proved superior for qPCR, and was therefore used in the present study. Specificity of the CDU1/CDL1b primer combination was tested using conventional PCR and genomic DNA extracted from pure cultures (Table 1) with the DNeasy Plant Mini Kit (Qiagen). DNA was first amplified with the ITS5/

Table 1 Species used in this study for testing the specificity of *Cylindrocarpon destructans* f. sp. *panacis* oligonucleotide primers

Isolates	Primer sets		
	ITS5 (ITS1)/ITS4	CDU1/CDL1b	CDU1/CDL1b
	(Conventional PCR)	(Conventional PCR)	(qPCR)
Cylindrocarpon destructans f. sp. *panacis* DAOM 234581[a]	+	+	+
Cylindrocarpon destructans f. sp. *panacis* DAOM 234582[a]	+	+	+
Cylindrocarpon destructans f. sp. *panacis* CD1815[b]	+	+	ND
Cylindrocarpon destructans NSAC-SH-1[b]	+	–	ND
Cylindrocarpon destructans 1726-WAL-10[b]	+	–	ND
Cylindrocarpon cylindroides CR6[c]	+	–	–
Cylindrocarpon cylindroides CR21[c]	+[g]	ND	–
Cylindrocarpon lucidum FRI[c]	+	–	–
Nectria fuckeliana DAOM 112560[a]	+[g]	ND	–
Euascomycete DAOM 236024[a]	+	–	ND
Fusarium culmorum CD9A[d]	+	–	ND
Fusarium equiseti FE87[d]	+	–	ND
Fusarium graminearum DAOM 213295[a]	+[g]	ND	–
Fusarium lateritium DAOM 194613[a]	+	–	ND
Fusarium oxysporum JT1833[e]	+[g]	ND	–
Fusarium oxysporum JT1835[e]	+[g]	ND	–
Fusarium solani G14[b]	+	–	ND
Phialophora gregata 98G1–3[f]	+	–	ND
Rhexocercosporidium carotae DAOM 226960[a]	+	–	ND
Rhexocercosporidium sp. DAOM 2235605[a]	+	–	ND
Rhizoctonia solani 895[b]	+	–	ND
Trichoderma harzianum DAOM 190839[a]	+	–	ND
Pythium irregulare DAOM 234585[a]	+	–	ND
Pythium ultimum DAOM 234588[a]	+	–	ND
Phytophthora cactorum DAOM 234592[a]	+	–	ND
Phytophthora sojae BR1074[a]	+	–	ND

[a]Isolate numbers proceeded by DAOM or BR are accessions deposited with the Canadian Collection of Fungal Cultures (CCFC), Ottawa.
[b]From culture collection of R. Reeleder.
[c]Provided by Dr K. Dobinson.
[d]Provided by Dr R. Clear.
[e]Provided by Dr J. Traquair.
[f]Provided by Dr C. Grau.
[g]The forward primer was ITS1; in all other cases in this column ITS5 was the forward primer.
+, Amplification of product with predicted size and/or melting temperature; –, no product visible on electrophoretic gel (conventional block PCR) or no signal detected (qPCR); ND = not done.

ITS4 or ITS1/ITS4 primer set (White *et al.*, 1990) to ensure the presence of amplifiable DNA, followed by amplification with CDU1/CDL1b. Amplifications were carried out in 25-μL reactions containing 1 × PCR buffer, 2 mM $MgCl_2$, 10 μg BSA, 1 M betaine, 4% DMSO, 200 μM dNTP solution, 0·4 μM primers, 1U *Taq* and 2·5 μL fungal DNA extract. Reactions were carried out on an Eppendorf Mastercycler (Brinkmann Instruments) with the following parameters: an initial denaturing period of 3 min at 94°C , followed by 30 cycles of 94°C for 45 s, 66°C for 30 s and 72°C for 60 s, followed by 7 min at 72°C. Products were examined electrophoretically in a 1% TAE agarose gel (5 V cm^{-1}, 60 min). Specificity of the CDU1/CDL1b primers was further tested in real-time amplifications using genomic DNA from isolates of target and related species (Table 1), using a Roche Diagnostics LightCycler 1 (as described below).

Additional confirmation of the specificity of CDU1/CDL1b was obtained by sequence analysis of conventional PCR products amplified directly from four soils suspected of harbouring CDP. DNA was extracted from soils as described below, then amplified using the procedure outlined above for fungal templates. PCR products were purified (MinElute PCR Purification Kit, Qiagen) and sequenced using an Applied Biosystems 3730 Analyzer employing BigDye™ Terminator chemistry, with the CDU1 oligonucleotide and sequence data were compared to GenBank accessions.

Collection and preparation of soil

To prepare the artificially infested soil, Fox loamy sand (Brunosolic Gray Brown Luvisol; Typic Hapludalf; 0·9% OM) was collected from the Agriculture and Agri-Food Canada research farm at Delhi, Ontario (42°47′N, 80°38′W) in September 2004. Soil was steam-pasteurized at 74°C for 30 min using a Lindig soil treatment system, air-dried, sieved through a 4-mm mesh, mixed and stored at room temperature until used. Soil dilution (one part soil and four parts 0·25% water agar) procedures were carried out using a semiselective modified Rose Bengal agar medium (MRBA; Reeleder *et al.*, 2003) to confirm that pasteurization had eliminated viable propagules. Isolate CD 1640 (DAOM 234582) was grown for 2 weeks in clarified V8 broth and, to induce formation of large numbers of chlamydospores, transferred to sterile-filtered soil extract for an additional 2 weeks (Reeleder *et al.*, 2003). Twenty mycelial mats were then washed with sterile water and vacuum filtration, macerated using autoclaved mini-blenders and re-suspended in *c.* 240 mL sterile water. Macerates were added to pasteurized soil at approximately 40 mL kg^{-1} soil in a large plastic bag and mixed by vigorously shaking the bag. The mixed soil was then allowed to air-dry, was stored at 4°C for 3 weeks, and then further mixed with pasteurized field soil in different proportions. As the original CDP + soil mixture was thought to contain a much higher population than that assumed to occur in naturally infested soils, it was first diluted with an equal amount of pasteurized soil and is hereafter referred to as 100% for clarity. The resulting soil dilutions were 100, 50, 10, 2 and 0·5%. Each dilution was then divided into six replicate subsamples.

Samples of naturally infested soils (ranging in texture from sands to sandy loams) were collected at six sites previously used for ginseng cultivation and one control (no ginseng grown) in south-western Ontario. In order to systematically sample each site, a 441-m^2 plot was constructed and divided into 49 quadrats of 9 m^2 each. Approximately 2 L of topsoil were removed from each quadrat. Site locations were recorded by GPS to allow for re-sampling if required. Soil samples from within each site were pooled, passively air-dried at room temperature, mixed in a commercial cement mixer, sieved, and mixed again. All equipment, including mixer and sieve, were disinfested with household bleach (diluted with water to 0·5% sodium hypochlorite) between soils. Soils were placed in plastic bags and stored at 4°C until used. Data on soil chemistry and texture (A&L Canada Laboratories, London, Ontario) as well as cropping history (Table 2) were also collected for comparison with fungal DNA estimates.

Soil DNA extraction and purification

Total DNA was extracted from samples of the artificially infested soil using a modification of a procedure previously outlined (Reeleder *et al.*, 2003). Five-gram samples of air-dried soil were placed in 50-mL disposable Falcon tubes (VWR 21008–940) with 3·5 mL of autoclaved 1-mm-diameter zirconium oxide beads (Fox Industries, Inc.), 5 mL extraction buffer (60 mM sodium phosphate buffer (pH 8), 10 mM $CaCl_2$, 0·05% SDS and 100 μg proteinase K). Tubes were then shaken on a commercial paint shaker

Table 2 Ginseng cropping histories and characteristics of the seven soils assessed for pathogen populations. Fields C, D and E were cropped to ginseng twice during the time periods shown

Soil	Period of ginseng cultivation	OM (%)	Clay (%)	Sand (%)	Silt (%)	N (%)	Al (p.p.m.)	P (p.p.m.)	K (p.p.m.)	pH	cec
A	1999–2003	1·4	6	84·8	9·2	0·6	848	59	114	6·8	5·8
B	–	0·8	4	86·8	9·2	0·6	1072	67	89	5·7	4·8
C	1993–99	2·4	4	84·8	11·2	0·4	856	100	124	7·1	12·3
D	1994–98 (est); 2001–03	0·7	4	76·8	19·2	0·6	1093	89	159	6·4	5·4
E	1990–97	1·2	6	70·8	23·2	0·6	1023	44	211	6·8	5·7
F	1998–02	1·6	6	68·8	25·2	0·6	1041	66	168	5·6	6·8
G	1998–02	2·1	8	68·8	23·2	0·5	866	71	178	7·1	8·0

at maximum speed for 20 min, followed by centrifugation at 5000 *g* for 10 min at 10°C. Supernatants were then transferred to new, autoclaved tubes (Sarstedt). 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 *g* for 10 min. The resulting supernatants (2·5 mL) were transferred to new tubes containing a 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 purification. The efficiency of CDP chlamydospore lysis using this method was estimated at 98% in artificially infested soils (Reeleder *et al*., 2003).

DNA pellets from soil samples were re-suspended in 500 μL water by agitating at 65°C for 15 min. One-hundred μL of this solution was then purified using the DNA IQ™ System (Promega) modified by increasing the amount of the supplied lysis buffer to 200 μL per sample, reducing the amount of supplied DNA binding resin to 1 μL per sample and incubating the resin DNA complex for 30 min at room temperature with agitation. These modifications ensured saturation of the binding resin, thereby equalizing DNA recovery among soils (see Promega Technical Bulletin no. 297). Control samples, designed to ensure efficient DNA extraction and to detect DNA contamination during extraction, purification or PCR amplification, were prepared by substituting soil with 4·9 g of sterile sand containing 0·1 g of commercial baker's yeast (Fleischmann's Yeast).

Soil DNA extraction and purification from naturally infested soils were as above, except that the supernatants produced by the first centrifugation step in the extraction procedure were divided into three aliquots of 500 μL each for parallel extractions. Consequently, all reagent volumes were reduced to 20% of that used for artificially infested soils. This allowed for all subsequent extraction steps to be carried out in microfuge tubes.

qPCR

Reference DNA samples were prepared by extracting from fresh mycelium of CDP (CD 1561; DAOM 234581) using the DNeasy Plant Mini Kit and quantifying with an Eppendorf BioPhotometer calibrated with calf thymus DNA (Sigma) in Qiagen elution buffer (buffer AE).

qPCR was performed on extracts of samples of the artificially infested soil from the bioassay pots using SYBR Green chemistry on a Roche Diagnostics LightCycler. Reaction mixtures (20 μL) were prepared in glass capillaries (Roche Diagnostics) and included 2 μL soil DNA extract, 9·8 μL QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 U HotStarTaq DNA Polymerase (Qiagen) and 0·3 mM primers CDU1 and CDL1b.

Each set of PCR reactions (i.e. each LightCycler carousel) included DNA extracted from two replications of the soil dilution series plus a dilution series of reference DNA and the negative (yeast) control extract. All reactions received a 15-min pretreatment at 95°C, then 60 cycles of 94°C for 15 s, 60°C for 2 s, 72°C for 35 s and 85°C for 2 s. The addition of the 2-s pause at 85°C significantly decreased the formation of a primer-dimer with a melting temperature of 85°C. For all reactions, temperature transition rates were 20°C s^{-1} and the fluorescent signal was acquired at 72°C.

For the naturally infested soils, qPCR was carried out as for artificially infested soils, except that each set of PCR reactions (each LightCycler carousel) included DNA from each of the three parallel extraction aliquots of each replicate subsample of the seven soils, plus a dilution series of reference DNA and the negative control extract.

After cycling, melting temperatures of amplicons were determined by increasing the temperature from 65°C to 95°C at a rate of 0·1°C s^{-1} with continuous acquisition of the fluorescent signal. Amplicon size was determined by agarose gel electrophoresis.

Analysis of qPCR data

qPCR data generated by the LightCycler were analysed using LinRegPCR software (Ramakers *et al*., 2003). For each set of PCR reactions (each LightCycler carousel) the logarithms of the initial fluorescence (N_o calculated by LinRegPCR) for the serial dilutions of quantified reference DNA were plotted against the logarithms of their actual DNA concentrations (Fig. 1). The equation of the resulting line was then used to estimate the initial concentration of target DNA templates from the soil samples. This approach did not require (or assume) equal PCR amplification efficiencies and accounted for any variation in amplification efficiency among sets of PCR reactions. The DNA concentration (pg DNA μL^{-1} extract) for each 5-g replicate

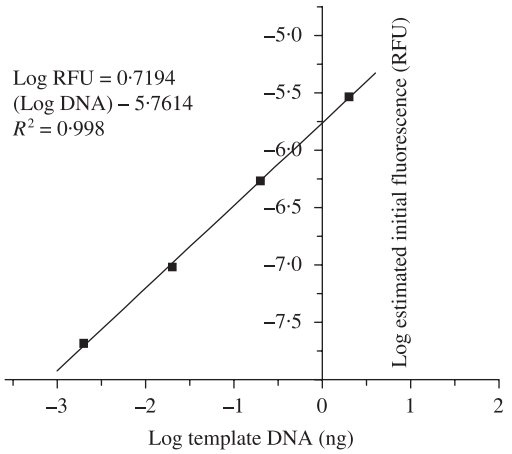

Figure 1 Example of the log-log relationship between known concentrations of *Cylindrocarpon destructans* f. sp. *panacis* DNA (ng) added to LightCycler reactions and the initial fluorescence (in relative fluorescence units) estimated by LinRegPCR software. The equation of the line can be used to estimate the initial concentration of *C. destructans* f. sp. *panacis* DNA in concurrently amplified soil extracts.

was determined by averaging the values of the three 500-μL aliquots of each 5-g extraction.

Design of plant bioassays

A bioassay using the artificially infested soil was carried out in a shaded glasshouse (20 ± 4°C) using the soils infested with CDP, as described above. Approximately 2 cm depth of autoclaved vermiculite (sieved through a screen with 0·9-cm diameter openings in order to remove small particles) was added to the bottom of clean, bleach-disinfested 10-cm-diameter plastic pots, followed by approximately 300 g of each dilution of each infested soil. Roots of mature ginseng seedlings (8 months old, with foliage removed) were transplanted into the soil such that crown buds were at the soil surface. Four seedlings were planted into each of six replicate pots for each soil dilution in a randomized complete block design.

A portion of infested soil (approx. 30 ml) was retained from each pot at planting and stored at 4°C in sterile screw-cap tubes for DNA extraction and CFU determination. Plants were harvested after 34 days. Disease severity was assessed on a five-point scale, with 0 indicating no disease symptoms and 5 indicating a completely rotted root.

A separate bioassay was also conducted using the collected naturally infested soils in a completely randomized block design. Eight 2- to 3-month-old ginseng seedlings were planted into bleach-sterilized pots (20 cm diameter) filled with one of the seven soils. Six replicate pots were prepared for each soil. Each pot received 70 mg metalaxyl at planting and after 7 days to inhibit species of *Pythium* and *Phytophthora*. Pots were placed in a shaded greenhouse operating at 20 ± 4°C. Plants were harvested and the roots analysed for disease severity (using the same five-point scale described above) after 20 days. At planting, approximately 30 mL of soil was collected from each pot and stored as above for subsequent DNA extraction.

Recovery from roots

The frequency of recovery of pathogens from ginseng roots used in the bioassays of naturally infested soils was determined by plating root segments onto MRBA medium. Diseased roots (main or tap roots) with symptoms of CDP were surface-disinfested with 0·5% sodium hypochlorite for 1 min, washed with sterile water, blotted dry with paper towels, then cut into segments 4–5 mm in length prior to placing on MRBA. Plates were observed after 7–10 days. Selected colonies arising from root tissue segments were transferred to fresh medium and saved for identification. In order to confirm the effectiveness of metalaxyl at suppressing oomycete pathogens, roots were also transferred to P_5ARP medium (Jeffers & Martin, 1986). The recovery of the fungus from roots was then used to correct the data on disease severity for plants grown in naturally infested soils, by giving plants from which the pathogen was not recovered a disease index (DI) of zero.

Dilution plating

The number of colony-forming units (CFU) per g of dry soil was determined for artificially infested soils as described above, but using dilutions of 1 in 100. Three 10-g aliquots of each soil were oven-dried at 104°C for 3 days. Oven-dry weights were used to determine soil moisture; plate-count data were then adjusted to a CFU g^{-1} dry soil basis.

Statistical analyses

For the artificially infested soil bioassay, the relationships between the data obtained using the different measures of fungal populations (qPCR, dilution plating, disease severity and recovery from roots) and percentage of infested soil were determined by regression analyses using non-transformed data, with SPSS release 13·0 (SPSS Inc.). Coefficients of determination (R^2) were obtained for each regression line. Pearson correlations (r) were determined from non-transformed data to show relationships among variables in both bioassays. A preliminary principal components analysis (XL-STAT 7·5, Addinsoft) was carried out to determine relationships between soil chemistry values and target DNA. Trends in PCA were further evaluated by carrying out a multiple regression analysis (SPSS release 13·0).

Results

Specificity of *C. destructans* f. sp. *panacis* primers

The CDU1/CDL1b primer combination was found to exhibit the same level of specificity as the original CDU3/CDL1b primer combination using conventional PCR (Table 1); closely related isolates of *C. destructans*, such as NSAC-SH-1 (Seifert *et al.*, 2003) and 1726-WAL-10, were not amplified under these conditions. All DNA extracts tested produced conventional PCR products using non-specific ITS primers (White *et al.*, 1990), indicating the presence of amplifiable template.

qPCR (using CDU1/CDL1b) of DNA from pure cultures of the pathogen (CD 1561) produced an amplicon with a melting point of 87·2°C. Post-amplification gel electrophoresis revealed that all amplicons were of the predicted size (*c.* 500 bp). Testing of CDU1/CDL1b using a range of related species validated their specificity under the PCR parameters used (Table 1).

Comparisons of the four sequenced PCR products obtained from soil extracts to a sequence obtained from a pure culture of the fungus (GenBank AY037554) showed that the soil PCR products exhibited 100% sequence similarity to the pure culture. A sample soil DNA sequence was deposited in GenBank (DQ907241).

Sensitivity of *C. destructans* f. sp. *panacis* primers in qPCR

Fungal culture DNA extract of CDP amplified at an average PCR efficiency of 1·57 (of a maximum possible value of

2·0), as calculated by LinRegPCR (Ramakers *et al*., 2003). Amplification from serial dilutions of quantified fungal DNA from pure culture indicated that the target could be detected at template concentrations below 100 fg μL^{-1} of extract solution. 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.

qPCR of DNA extracted from soils

qPCR amplification from the dilution series of artificially infested soils resulted in amplicons of the same size and melting temperature as those from pure culture. PCR efficiencies of reactions using templates derived from artificially inoculated soil were also similar to those from pure culture. Average target DNA concentrations for CDP (calculated using extraction replicate means) ranged from 4·06 ± 1·35 (SE) pg μL^{-1} soil extract in the least diluted soil (100% inoculum) to 0·056 ± 0·02 pg μL^{-1} in the most diluted soil (0·5% inoculum) (Fig. 2). One pg target DNA μL^{-1} extract of the artificially infested soil was estimated to be equivalent to 53·6 pg target DNA g^{-1} dried soil. This gave a range of between 217·6 pg g^{-1} dried soil in the least diluted soil and 30·0 pg g^{-1} dried soil in the most diluted soil.

PCR efficiencies of amplifications from naturally infested soil extracts were also very similar to those from pure culture, and again resulted in amplicons of the predicted size, although the melting temperatures of the amplicons tended to vary slightly among soil extracts, probably because of residual impurities in some of the PCR templates.

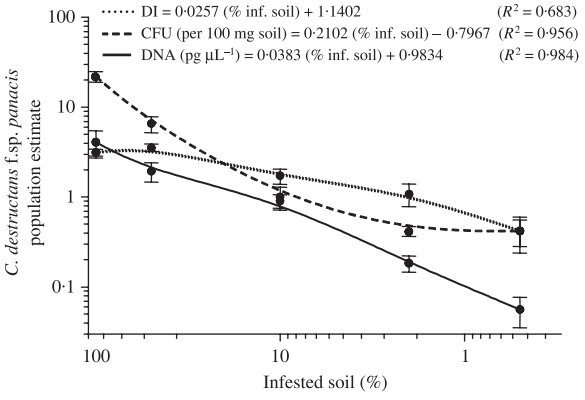

Figure 2 Relationships among fungal population measures in artificially infested soil bioassays. A common y-axis represents three estimates of *Cylindrocarpon destructans* f. sp. *panacis* populations: DNA (as estimated by qPCR); disease severity index (DI); and CFU per 100 mg dry soil. Regression lines show DNA versus percentage infestation of soil (solid line); CFU per 100 mg soil versus percentage infestation of soil (long-dash line); and DI versus percentage infestation of soil (short-dash line). Disease index is on a five-point scale, 0 indicating no disease symptoms and 5 indicating a completely rotted root. Regression equations were derived from untransformed data. Because of the range of data, however, axes are shown in log scale.

Calculated average concentrations of target CDP-DNA ranged from zero in soil E, to 4·8 ± 0·43 pg μL^{-1} soil extract in soil F (Fig. 3). One pg target DNA μL^{-1} extract of the naturally infested soil was estimated to be equivalent to 308 pg target DNA g^{-1} dried soil, giving a range of between zero and 1·48 ng g^{-1} dried soil. DNA concentrations of individual 5-g replicate soil samples (based on averaging the DNA concentrations of the three 500-μL aliquots of each 5-g extraction) ranged from 0·026 ± 0·026 pg μL^{-1} to 6·0 ± 2·14 pg μL^{-1} in positive samples. Variability within the 5-g soil extraction replicates (i.e. among the three extraction aliquots per 5-g extraction) ranged from 22·5% to 173%, with an average of 71% (expressed as CV × 100). Most of the 5-g extractions exhibited within-sample variations of less than 50%, but in soils B and C contained very low quantities of fungal DNA were detected in one aliquot and none was found in the other two, resulting in high percentage variabilities overall.

Other measures of pathogen populations and comparisons to qPCR data

Disease index

In soils artificially infested with CDP, ginseng roots had the greatest disease severity index in the 50% soil dilution (3·54 ± 0·33 (SE) on the 0–5 scale) followed closely by the 100% infested (non-diluted) soil (3·13 ± 0·27). Disease severity then decreased with soil dilution to 0·42 ± 0·13 in the most diluted soil (0·5% infested soil) (Fig. 2). In the case of the naturally infested soils, disease severity in the bioassay seedlings ranged from an average of 1·47 ± 0·36 (SE) in the most infested soil (soil F) to zero in soils A, B, C and D (after correcting for recovery on semiselective media) (Fig. 3). Although metalaxyl was added, *Phytophthora cactorum* and *Pythium* spp. were isolated

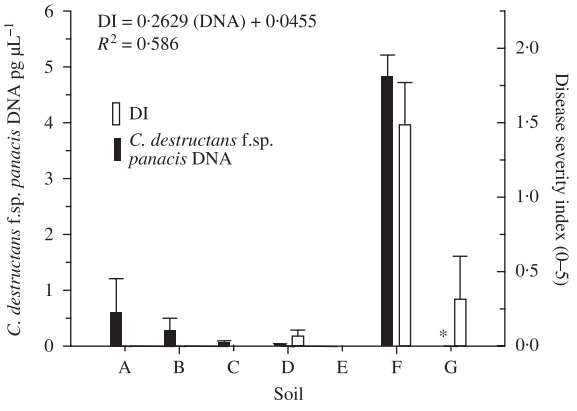

Figure 3 *Cylindrocarpon destructans* f. sp. *panacis* (CDP) DNA (as estimated by qPCR) (solid bars) and disease severity index (DI) of roots with CDP disease symptoms (open bars) in seven naturally infested soils (A–G). Error bars represent the standard error of the mean. Asterix indicates sample for which the CDP-DNA was detected at levels too low for accurate quantification. The regression equation given describes the relationship (over all naturally infested soil bioassays) between disease severity and target DNA.

from diseased roots, especially in soil G. Subsequent testing showed that isolates of *P. cactorum* from these roots were metalaxyl-insensitive (data not shown). These oomycetes thus may have contributed to the observed disease severities.

In the artificially infested soils, the relationship between DNA concentration (as measured by qPCR) and inoculum dilution (R^2 = 0·984; P < 0·000) provided a better fit to the linear regression than did the relationship between disease index and inoculum dilution (R^2 = 0·683; P = 0·085) (Fig. 2). The line representing the relationship between CDP-DNA concentration and inoculum shows that the DNA measurement was more responsive to dilution when compared to the disease severity index, particularly at low levels of inoculum (Fig. 2). Concentrations of CDP-DNA were positively correlated with the disease severity index observed in the plant bioassay (r = 0·494; P < 0·000; n = 29). This correlation increased to r = 0·65 if the 100% inoculum treatment (which produced a disease severity value similar to that of the 50% inoculum treatment) was removed from the analysis. Larger differences in disease severity between these two treatments may have been present earlier in the root colonization process. In the naturally infested soils, DNA concentrations of CDP were also positively correlated with disease severity (r = 0·765; P < 0·000; n = 28; Fig. 3).

Colony-forming units

Plating of soils artificially infested with CDP onto MRBA medium resulted in average CFU counts ranging from 218·3 ± 28·0 (SE) propagules g^{-1} dry soil in the 100% infested (non-diluted) soil, to 4·2 ± 1·7 in the most diluted soil (0·5% inoculum) (Fig. 2). CFU g^{-1} dry soil counts were positively correlated with CDP-DNA concentrations (r = 0·620; P < 0·000; n = 29). Both these concentrations and CFU measures decreased with increasing inoculum dilution, although, as with disease severity, the relationship with inoculum dilution was much stronger for DNA concentration than for CFU. Again, the line representing the relationship between fungal DNA concentration and inoculum dilution indicates that DNA measurement was more responsive to dilution of the inoculum than was CFU (Fig. 2).

Cultures resulting from the dilution plating of naturally infested soils onto MRBA were often overgrown by competing fungi, and the data were not further analysed. MRBA was previously found to be useful in assessing populations in pasteurized, re-infested soils, but was unreliable when used for naturally infested soils (Reeleder *et al.*, 2002).

Relationships among pathogen populations and soil characteristics

For naturally infested soils, preliminary principal component analysis using the soil chemistry data indicated weak negative relationships CDP-DNA, soil pH and phosphorus levels, as well as a weak positive relationship with aluminum. Subsequent multiple regression analysis, however, failed to show any significant (α = 0·05) relationships.

Discussion

Soilborne pathogens such as *C. destructans* f. sp. *panacis* can be extremely persistent and the potential for losses as a result of replant decline may exist for decades after the cultivation of ginseng (Reeleder *et al.*, 2002), as well as for some other perennial crops (Braun, 1995; Mercado-Blanco *et al.*, 2003). The ability to rapidly quantify soil pathogen populations and identify high-risk soils is therefore highly desirable. Here it was demonstrated that this can be accomplished using qPCR, and that the resulting data on pathogen DNA concentrations, once correlated with other population and inoculum potential measurements, have potential for the prediction of disease risk.

Other authors correlated soil pathogen qPCR data with the number of fungal propagules per unit soil by adding known concentrations of spores, conidia or sclerotia to sterilized soil prior to DNA extraction (Cullen *et al.*, 2001; Lees *et al.*, 2002; Filion *et al.*, 2003). In the present study, in order to relate DNA concentrations to disease severity directly, inoculum was added to pasteurized soil and then correlated to qPCR data with data from both dilution plating (CFU) and host-plant bioassays, after giving the inoculum time to stabilize. Our data suggest that, over a wide range of inoculum concentration, qPCR might be more reliable measures of fungal population than bioassay or CFU data. The correlation of DNA concentrations with disease index was then extended into naturally infested ginseng soils.

Positive correlations between CFU and qPCR data have been previously demonstrated in both natural and artificial soils (Ippolito *et al.*, 2004; Schroeder *et al.*, 2006), but the correlation between DNA concentrations and actual disease occurrence has only rarely been established. Bonants *et al.* (2004), for example, extracted DNA from plant tissue or soils inoculated with artificially constructed mixtures of infected and uninfected roots. The work reported here appears to be the first to explicitly relate qPCR-calculated DNA concentrations to disease index in naturally infested soils.

Several factors need to be considered when interpreting qPCR results from soils. Differences in soil chemistry may influence DNA extraction efficiency (Miller *et al.*, 1999), leading to underestimates for soils with higher DNA binding capacities (Martin-Laurent *et al.*, 2001). Propagules of soilborne pathogens also tend to be non-randomly distributed at small spatial scales (Rodriguez-Molina *et al.*, 2000), potentially leading to high levels of between-sample variation. The extraction method used here, however, begins with a relatively large (5 g) soil sample that may reduce sample-to-sample variation when compared to the 500-mg to 1-g amounts often used with commercial kits.

The effect of co-extracted PCR inhibitors on amplification efficiency is also an important consideration. Inhibiting

substances can negatively affect the PCR reaction, and small differences in amplification efficiency can result in dramatic changes in calculated DNA concentrations (Ramakers *et al*., 2003), especially between DNA extracted from soil and clean reference DNA. Post-extraction cleaning of soil extracts improved PCR efficiency and the paramagnetic DNA IQ™ System, in particular, gave more consistent results than other commercially available kits tested. Even with relatively clean DNA however, some variation in amplification efficiencies was still present among soil extracts. A quantification method based on linear regression, such as LinRegPCR (Ramakers *et al*., 2003), is therefore preferable to methods based on crossing points, which require (and assume) equal PCR efficiencies among samples. The regression-based LinRegPCR approach had the added advantage of greatly expediting the calculation of amplification efficiencies.

When using greenhouse bioassays for calibration of qPCR data, the presence of multiple pathogens causing similar symptoms can complicate the interpretation of disease severity data (Mazzola, 1998), and the soil environment may have a significant impact upon disease expression (Gill *et al*., 2000). Consequently, disease severities found in greenhouse bioassays may not always reflect pathogen population levels or DNA estimations. Further, it should be noted that, in bioassays for some pathogen-crop interactions, disease incidence might be as valuable as measurements of disease severity in predicting risk to the crop, or more so.

The information presented here should help reduce disease losses in ginseng and other crops by identifying sites with high risk. Although environment has an overriding effect on disease development, it is expected that planting sites with high populations of pathogens will generally pose a greater threat to crops than sites with low populations. The deployment of this technology for practical use will require optimization of soil sampling strategies, including sample size, for the target pathogens. Significant relationships between CDP-DNA concentrations in soil and soil mineral concentrations were not found; however, these relationships may be worth exploring for other soilborne pathogens.

Based on qPCR data and other measures, populations of CDP in different soils vary considerably. This may be the result of differences in cropping history or soil properties, which, in combination with ginseng cultivation, may promote the pathogen's infestation or survival. Although previous reports indicated that ginseng replant decline was evident in soils sampled from most old ginseng sites examined (Reeleder *et al*., 2002), the data presented here suggest that at least some of the sites sampled may be successfully replanted.

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