Quantification of *Phytophthora capsici* Oospores in Soil by Sieving-Centrifugation and Real-Time Polymerase Chain Reaction

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ABSTRACT

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A procedure was developed to quantify *Phytophthora capsici* oospores in soil by combining a sieving-centrifugation method and a real-time quantitative polymerase chain reaction (QPCR) assay. Five soil samples representing three different soil textures were infested with oospores of *P. capsici* to produce 10^1 , 10^2 , 10^3 , 10^4 , or 10^5 spores per 10 g of air-dried soil. Each 10-g sample of infested soil was suspended in 400 ml of water and then passed through 106-, 63-, and 38- μ m metal sieves. The filtrate was then passed through a 20- μ m mesh filter. Materials caught on the filter were washed with water into two 50-ml centrifuge tubes and spun for 4 min ($900 \times g$). The pellet was suspended in 30 ml of 1.6 M sucrose solution and centrifuged for 45 s ($190 \times g$). The supernatant was passed through the 20- μ m mesh filter. The sucrose extraction process of oospores was repeated five times to maximize oospore extraction. Materials caught on the 20- μ m mesh filter were washed with water into a 50-ml tube and spun for 4 min ($900 \times g$). The pellet was suspended in 1 ml of water, and the number of oospores was determined with a haemocytometer. The relationship between number of oospores recovered from the soil and number of oospores incorporated into the soil was $\hat{Y} = -0.95 + 1.31X - 0.03X^2$ ($R^2 = 0.98$), in which $\hat{Y} = \log_{10}$ of number of oospores recovered from the soil and $X = \log_{10}$ of number of oospores incorporated into the soil. The oospores were germinated after treatment with 0.1% KMnO_4 solution for 10 min to induce germination. On the basis of the detection of ribosomal DNA, a QPCR method for *P. capsici* oospores was developed. PCR inhibitors were eliminated by extracting oospores from the soil by sieving-centrifugation. DNA was extracted and quantified from *P. capsici* oospores with suspensions of 10^1 , $10^{1.5}$, 10^2 , $10^{2.5}$, 10^3 , $10^{3.5}$, 10^4 , $10^{4.5}$, and 10^5 oospores per ml of water. The relationship between the DNA quantities and number of *P. capsici* oospores was $\hat{Y} = -3.57 - 0.54X + 0.30X^2$ ($R^2 = 0.93$), in which $\hat{Y} = \log_{10}$ (nanogram of *P. capsici* DNA) and $X = \log_{10}$ (number of oospores). The relationship between the quantity of DNA of *P. capsici* oospores recovered from the soil and the number of oospores incorporated into the soil was determined by $\hat{Y} = -3.53 - 0.73X + 0.32X^2$ ($R^2 = 0.955$, $P < 0.05$), in which $\hat{Y} = \log_{10}$ (DNA quantity of *P. capsici* oospores recovered from the soil) and $X = \log_{10}$ (number of *P. capsici* oospores incorporated into the soil). Utilizing the sieving-centrifugation and QPCR methods, oospores of *P. capsici* were quantified in soil samples collected from commercial fields.

Additional keywords: *Capsicum annum*, *Cucurbita moschata*, *Cucurbita pepo*, Phytophthora blight, TaqMan assay

Phytophthora capsici Leonian is a soil-borne oomycete plant pathogen that infects more than 50 crops and several weed species (12,43). It can infect plants at any stage of growth, causing Phytophthora blight with symptoms such as seedling damping-off, crown rot, foliar blight, and fruit rot (3,4,12,18). Phytophthora blight, caused by *P. capsici*, causes as much as 100% crop losses in cucurbits, eggplant, and peppers in the United States and worldwide (3,12,18,21,27).

Illinois growers produce more than 90% of processing pumpkins in the United States and Phytophthora blight has been the most serious threat to the processing pumpkin industry in the past decade (3). Also, 100% crop losses in bell pepper fields in Illinois have been reported (23). *P. capsici* has also been reported to cause significant losses in vegetable industries in Michigan (18). Currently, adequately effective strategies for achieving commercially acceptable management of *P. capsici* have not been identified. The recommended measures for control of this pathogen include cultural practices (12,18), fungicide application (4,18,23), and genetic resistance in peppers (18,23).

P. capsici is a heterothallic organism in which two compatible mating types, A1 and A2, are needed for sexual reproduction (12). Both mating types are found in the

United States (12,18,23), often in the same field (23). The sexual spore of *P. capsici* is the oospore, which is the overwintering propagule in the soil and primary source of inoculum (7,12). Determining oospore density in the soil is essential for establishing effective cropping systems for managing *P. capsici*. The impediments for monitoring *P. capsici* oospores in the soil were primarily the difficulties in extracting them from the soil (26) and developing reliable sampling protocols that would estimate precise oospore densities (35). Methods used for assessing the density of *P. capsici* oospores in the soil have been dilution plating on semiselective culturing media and baiting assays (12,26,33), but these procedures are time consuming, labor intensive, and less reliable. Serological techniques have also been developed for *Phytophthora* species, but there is a lack of species-specific antibodies (25,30,35).

The sieving-centrifugation with sucrose solution is basically a method to separate particles based on their size and density. Several studies have been conducted using sieving-centrifugation with a sucrose solution to separate propagules of mycorrhizal fungi (9), teliospores of *Tilletia* species (5), and nematodes (24) from the soil.

Real-time quantitative polymerase chain reaction (QPCR) is a relatively new molecular technique that has been used to quantify nematodes (8), viruses (13), bacteria (6,34), and fungal and oomycete plant pathogens (14,16,19,40). QPCR was first introduced by Huguchi et al. (20), in which the increment of intensity of fluorescence was quantified with time during the exponential phase of DNA amplification. Technological advances in QPCR-based methods in the past few years allow fast, accurate detection and quantification of plant pathogens (32).

Relative QPCR is a technique that is usually used for gene expression profiling (36), but can also be used in quantitative environmental studies (16). One of the methods used for relative QPCR is the $\Delta\Delta\text{Ct}$ method (29), which is based on the relative detection of two DNA sequences: the target and endogenous control. For an adequate quantification with the $\Delta\Delta\text{Ct}$ method, two conditions need to be met: (i) the amplification efficiency of both sequences is equal; and (ii) the endogenous control quantity is equal among the different samples (29).

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Molecular tools have been used to conduct in-depth studies of the biology of the genus *Phytophthora* (2,19,39–41). Silvar et al. (41) used a polymerase chain reaction (PCR)-based method to detect *P. capsici*. Also, Silvar et al. (40) developed a QPCR assay for quantification of *P. capsici* in pepper plants, but these methods cannot be used to quantify *P. capsici* oospores in the soil because of the presence of PCR inhibitors in the soil (44). The objective of this research was to develop a reliable procedure by combining a sieving-centrifugation method and a QPCR assay to quantify *P. capsici* oospores in the soil.

MATERIALS AND METHODS

In vitro oospore production. Isolates of *P. capsici* were collected from infected pumpkin and zucchini plants in Illinois. The collected isolates were paired with *P. capsici* isolates A1 (15427) and A2 (15399) obtained from the American Type Culture Collection (Manassas, VA) to evaluate their oospore-producing potential. Using the isolates with high oospore-producing ability, oospores were produced in 250-ml glass flasks containing 30 ml of V8-CaCO₃ medium (38). The flasks were incubated at 24°C in darkness for 2 months. Then, oospores were harvested by blending the culture at full speed in a blender (Model 52250; Hamilton Beach, Southern Pines, NC) for 90 s. The suspension in the blender was passed through 63- and 38-μm metal sieves and a 20-μm Spectra/Mesh nylon filter (Spectrum Inc., Houston, TX). Oospores caught on the 20-μm filter were collected and used in the following studies.

Extraction and enumeration of *P. capsici* oospores in the soil. Five soil samples from commercial fields representing three soil textures (Table 1) were passed through a 2-mm sieve and air dried at room temperature on a laboratory bench for 14 days. The soil samples (500 g each) were autoclaved twice at 157°C for 1 h each time. An oospore suspension was prepared and the number of oospores in the suspension was determined with a spore-counting chamber (Haemocytometer No. 3120; Hausser Scientific, Horsham, PA). Oospore suspensions with desired densities of 10¹, 10², 10³, 10⁴, and 10⁵ spores per ml of sterile distilled water were prepared. The oospore suspension was mixed with a vortex (Maxi Mix; Barnstead/Thermolyne,

Dubuque, IA) prior to adding to the soil, 1-ml aliquot of the oospore suspension was added to each 10 g of soil and the soil sample was thoroughly mixed. The artificially infested soil samples had 10¹, 10², 10³, 10⁴, and 10⁵ *P. capsici* oospores per 10 g of air-dried soil. A noninfested control sample was included for each soil.

Each 10 g of infested soil sample was suspended in 400 ml of tap water and two drops of Tween 20 were added and stirred for 15 min with a stirring plate (Barnstead/Thermolyne). The soil suspension was then passed through 106-, 63-, and 38-μm metal sieves, and the sieves were washed with a fine sprinkler with a gentle stream of water and the filtrate was collected. This suspension (approximately 2 liters) was passed through the 20-μm mesh filter. The materials caught on the 20-μm filter were washed with water into two 50-ml centrifuge tubes and spun for 4 min (900 × g) with a bench-top centrifuge (Centra-CL2; International Equipment Company, Needham Height, MA). The supernatant was discarded and the pellet was suspended in 30 ml of 1.6 M sucrose solution. This suspension was then centrifuged for 45 s (190 × g) and the resulting supernatant was passed through the 20-μm filter. The sucrose-oospore extraction steps were repeated five times to maximize oospore recovery from the soil. The materials caught on the 20-μm mesh filter were washed with water into a 50-ml centrifuge tube and spun for 4 min (900 × g). The pellet was resuspended in 1 ml of distilled water and enumerated with the spore-counting chamber and light microscopy. The whole process of extraction and enumeration of oospores was repeated five times.

Oospore germination. *P. capsici* oospores extracted from the artificially infested soils were induced to germinate on the semiselective culture medium PARP (37) in petri plates. The spores were treated with 0.1% KMnO₄ for 10 min (1,11), washed three times with sterile distilled water, and plated onto PARP medium. Two hundred oospores from each soil sample were plated (50 oospores per plate) for germination. The plates were incubated at 24°C in darkness for 9 days and the percentage of germinated spores was determined. Plugs of 5-mm diameter from the edge of the 10 growing colonies of germinated oospores were transferred

onto lima bean agar (LBA; 23), grown at 24°C for 5 days, and the presence of *P. capsici* was determined on the basis of the sporangial morphology with light microscopy. Twelve representative cultures (three cultures per plate) were tested to verify identification of *P. capsici* with the PCR protocol described by Islam et al. (23).

DNA extraction. Oomycete and fungal isolates used in this study (Table 2) were maintained on LBA slants at room temperature. Mycelia for DNA extraction were prepared according to the method described by Islam et al. (23). DNA was extracted from oospores of *P. capsici* with the protocol reported by Gao et al. (16). The DNA concentrations were measured with a Genesys 10 spectrometer (Thermo Spectronic, Rochester, NY).

Primer and probe design. The sequences from the ITS1 and ITS2 regions and 5.8S ribosomal DNA gene of *P. capsici* (Fig. 1) were subjected to a database search using BLAST and FASTA programs, and these sequences were aligned using ClustalW package (42) to the same regions of 30 different species of *Phytophthora* to identify species-specific sequences. The regions of dissimilarity were used to design Taqman minor groove binding primers and probe with Primer Express 2.0 software (Applied Biosystems, Forest City, CA).

The ribosomal DNA sequences of *P. capsici* used for the sequence analysis were obtained from the GenBank database (Accession Nos. AF125008, AF129888, AF332266, AJ555612, AY742735, DQ464056, DQ464055, DQ464040, AB217670, EF053310, and EF053309). Also, the ribosomal DNA sequences from *P. capsici* isolates PC2, PC25, PP1, PP2, and 43-2 that were determined by Tian and Babadoost (43) were used for designing *P. capsici* primers (Pcap-q-1). Pcap-q-1 primers were: forward, 5'-GGA ACC GTA TCA ACC CTT TTA GTT G-3'; reverse, 5'-CGC CCG GAC CGA AGT C-3'; and probe, 5'-6FAM-TCT TGT ACC CTA TCA TGG CG-MGBNFQ-3'.

In the relative QPCR assay, the primers (Pyth-q-2) and the probe used were designed by sequences of ribosomal DNA gene of *Phytophthora* and *Pythium* species (GenBank Accession Nos. AF125008, AF129888, AF332266, AJ555612, AY742735, AY590277, AY423301, AY590275, AF266779, AY26999,

Table 1. Soils used for the development of the method for extraction and enumeration of oospores of *Phytophthora capsici* in the soil

Soil texture	Location ²	Soil pH	Organic matter (%)	Sand (%)	Silt (%)	Clay (%)	Oven-dried soil wt of 10 g air-dried soil
Sandy loam	Urbana	5.5	2.0	77	16	7	9.1
Silt loam	Shawneetown	5.9	2.5	18	69	13	8.1
Silt loam	Collinsville	8.7	2.3	33	55	12	8.5
Silt loam	Pekin	6.5	4.6	24	55	21	8.1
Silt clay	Champaign	7.3	5.3	7	65	28	8.7

² Towns in Illinois: Urbana and Champaign in Champaign County; Shawneetown in Gallatin County; Collinsville in Madison County; and Pekin in Tazewell County.

AY986961, DQ059572, AB160845, and AF330171). Pyth-q-2 primers were: forward, 5'-GCA ACT TTC AGC AGT GGA TGT C-3'; reverse, 5'-TGC AAT TCG CAT TAC GTA TCG-3'; and probe, 5'-VIC-CGA TGA AGA ACG CTG CG-MGBNFQ-3'.

Primers were synthesized at the W. M. Keck Center for Functional and Comparative Genomics, University of Illinois at Urbana-Champaign. The probes were commercially synthesized by Applied Biosystems. The probes were labeled at the 5' end with a fluorescent reporter dye: 6-carboxyfluorescein (6-FAM) for the *P. capsici* probe (Pcap-q-1) and VIC (Applied Biosystems proprietary compound) for the *Pythiaceae* species probe (Phyt-q-2). The probes were labeled at the 3' end with the minor groove binding nonfluorescent quencher (MGBNFQ).

Real-time QPCR amplification. The QPCR assays were conducted in a 96-well plate format with the ABI PRISM 7000 Sequence Detection System instrument and the software from PE Applied Biosystems (Foster City, CA). The manufacturer's instructions were followed except that 25- μ l reaction mixtures (instead of 50 μ l) were used (17). Thermal cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C in addition to a 2-min preincubation at 50°C. QPCR was performed to test the specificity of the Taqman probes to the pathogen DNA. Five *P. capsici* isolates and

other oomycete and fungal species (Table 2) were used for this absolute QPCR assay. DNA extracts were diluted to 0.1 ng/ μ l prior to the QPCR tests. These tests were repeated twice.

The Δ Ct PCR inhibition test, developed by Gao et al. (16), was used to test whether DNA extracts directly from oospores in the soil and oospores recovered from the soil by the sucrose-centrifugation method were PCR inhibitor-free. This test was based on QPCR assays of the β -actin gene from *Meloidogyne javanica* (Mj-ba) (36). The amplification of Mj-ba was measured in the QPCR assays without inhibitors and with soil extracts with possible inhibitors in the PCR mix. In this procedure, primers and probe for Mj-ba (36) were used instead of primers and probe for *P. capsici*. Amounts of β -actin DNA used were 7.5×10^{-4} ng (Test 1) and 1.7×10^{-5} ng (Test 2).

QPCR assays for quantifying oospores. A QPCR assay was developed to determine the relationship between the number of *P. capsici* oospores and the quantity of *P. capsici* DNA extracted. Autoclaved soil samples were infested with *P. capsici* oospores as previously described. The oospores were extracted from the soil with the sieving-centrifugation method. A control soil sample, with no oospores incorporated, was included in the test. With the spore-counting chamber, the numbers of *P. capsici* oospores were adjusted to 10^1 , $10^{1.5}$, 10^2 , $10^{2.5}$, 10^3 , $10^{3.5}$, 10^4 , $10^{4.5}$, and 10^5 spores per sample for DNA extraction. The control sample (extract of soil without oospores) and a negative control (molecular pure water) were included in each run. A subset of 10-fold dilution series of the total DNA extracted from *P. capsici* oospore samples, spanning from 1×10^{-4} to 1×10^0 ng, was incorporated into each QPCR assay in triplicate to generate a standard curve. The tests were repeated five times.

Quantifying *P. capsici* oospores in field soils. Eight commercial fields with a history of Phytophthora blight were sampled during the fall of 2004, after harvesting crops, for extraction and enumeration of *P. capsici* oospores. The fields had been planted to pumpkin, squash, or bell pepper either in 2004, 2003, or 2002. In each field, 20 soil subsamples were taken in a zigzag pattern from an area of 0.4 ha. A soil probe, with a diameter of 9.5 mm, was used to take 0- to 20-cm soil cores. The subsamples were mixed by adding all subsamples together, dividing the mixed soil into four parts, and remixing them again. This process was repeated four times to mix the soil subsamples thoroughly. The mixed samples were passed through a 2-mm sieve. The soil samples were stored at 4°C until they were assayed for the presence of oospores. Five 10-g soil samples from each field were processed for extraction and quantification of oospores using the above-mentioned sieving-centrifugation method. The extracted oospores were used for QPCR testing. Additional soil samples from each field were processed to extract oospores for determining the rate of oospore germination.

DNA was extracted from oospores of *P. capsici* separated from field soils by the protocol reported by Gao et al. (16). DNA extracts from the oospores and a negative control (molecular water only), in triplicate, were tested to quantify *P. capsici* DNA.

Spores extracted from soil samples were treated with 0.1% KMnO₄ for 10 min (1,11), washed three times with sterile distilled water, and plated onto PARP medium (37) in petri plates for spore germination. Two hundred oospores from each soil sample were plated (50 oospores per plate) onto the medium. The plates were incubated at 24°C in darkness for 9 days and the percentage of germinated spores

Table 2. Oomycete and fungal isolates used in the real-time quantitative polymerase chain reaction assays

Isolate	Species
15427 ^v	<i>Phytophthora capsici</i>
PC-14 (A2) ^w	<i>P. capsici</i>
PC-60 (A2) ^w	<i>P. capsici</i>
PC-81 (A1) ^w	<i>P. capsici</i>
PC-94 (A1) ^w	<i>P. capsici</i>
1025 ^s	<i>Hymenochaete fugilinosia</i>
1033 ^s	<i>Pythium ultimum</i>
1035 ^s	<i>Saprolegnia monilifera</i>
1042 ^s	<i>Klyveromyces piceae</i>
AeILmyla ^y	<i>Aphanomyces euteiches</i>
Psj5-17 ^y	<i>P. sojae</i>
Py2-1 ^y	<i>Pythium aphadernium</i>
Pi24-1 ^y	<i>P. infestans</i>
Illinois-1 ^z	<i>Aphanomyces euteiches</i>
Illinois-2 ^z	<i>P. sojae</i>

^v A1-mating type obtained from American Type Culture Collection Center (Manassas, VA).

^w Isolates were collected from Illinois: PC-14 from watermelon in Pekin (Tazewell County); PC-60 from pumpkin and PC-94 from zucchini in Grant Park (Kankakee County); and PC-81 from pumpkin in Shawneetown (Gallatin County).

^y Isolates were provided by J. Chee-Sanford, United States Department of Agriculture, Agricultural Research Service, Urbana, IL.

^z Isolates were available in the oomycete and fungal isolates collection of M. Babadoost at the University of Illinois.

^z Isolates were provided by D. Malvick, University of Minnesota, St. Paul, MN.

Fig. 1. Alignment of ITS region sequences of *Phytophthora capsici* and closely related *Phytophthora* species. The database accession reference codes are inside the parentheses. *P. capsici* Taqman primer set: forward primer (A), oligonucleotide probe (B), and reverse primer (reverse complement sequence) (C). Identical nucleotides are marked with an asterisk and gaps are indicated by dashes.

was determined. Plugs of 5-mm diameter from the edge of the growing colonies of germinated oospores were transferred onto LBA (23), grown at 24°C for 5 days and the species were identified with light microscopy. The identification of the species was on the basis of sporangial morphology. Twenty-four representative cultures of *P. capsici* oospores recovered from eight commercial fields (three cultures per field) were grown in lima bean broth and tested for verification of *P. capsici* by the PCR protocol described by Islam et al. (23).

In this study, relative QPCR was used to determine DNA quantities of *P. capsici* oospores (target DNA) relative to the DNA of species of the Pythiaceae family (endogenous control). To use the $\Delta\Delta C_t$ method, a validation test was carried out to determine if the amplification efficiency of the target DNA (Pcap-q-1) and the endogenous control DNA (Phyt-q-2) were equal. The calibrator was used at a concentration of 1×10^{-2} ng ribosomal DNA of *P. capsici* and 1×10^0 ng ribosomal DNA of present species of the Pythiaceae family. The relative quantity of *P. capsici* DNA was determined by the formula $2^{-\Delta\Delta C_t}$, as described by Livak and Schmittgen (29).

Data analysis. The data collected from the different experiments were analyzed by SAS procedures (SAS Institute, Cary, NC). Normality was tested with PROC UNIVARIATE. Analysis of variance (ANOVA) procedures were conducted and equality of variances was tested with Levene's test in the MEANS statement. All of the analyses of the oospore extraction data and DNA quantities were conducted using $\log_{10}$ -transformed data. The linear and quadratic coefficients of the regres-

sions were determined with single-degree-of-freedom polynomial orthogonal contrasts in the MIXED procedure of SAS with the type III method. There were no significant differences among different oospore extractions ($P > 0.3$). Thus, all the oospore extraction data were combined for analyses.

RESULTS

Extraction and enumeration of oospores in soil. The isolates of *P. capsici* used for oospore production yielded from 4.41×10^5 to 6.72×10^5 (mean 5.56×10^5) oospores per plate after 2 months. *P. capsici* oospores were recovered from all five artificially infested soil samples. No oospores were recovered from soil samples without oospore incorporation. The recovery rates of *P. capsici* oospores were 21.4, 43.7, 53.6, 60.3, and 79.8% from soil samples containing 10^1 , 10^2 , 10^3 , 10^4 , and 10^5 oospores per 10 g, respectively. There was no significant difference in oospore recovery among the soil samples with different textures. The relationship between the number of oospores recovered from the soil and the number of oospores incorporated into the soil was $\hat{Y} = -0.95 + 1.31X - 0.03X^2$ ($R^2 = 0.98$, $P < 0.001$), in which $\hat{Y} = \log_{10}$ of number of oospores recovered from the soil, and $X = \log_{10}$ of number of oospores incorporated into the soil (Fig. 2). The standard error for the intercept and the linear and quadratic coefficients in the model were 0.11, 0.08, and 0.01, respectively.

The percentage of oospores recovered from soil samples with 10^1 oospores per 10 g was significantly lower than the percentage of oospores recovered from all other oospore densities (Table 3). Also, the per-

centage of oospores recovered from soil samples with 10^2 oospores per 10 g was significantly lower than with 10^4 and 10^5 , but not different from that with 10^3 . Similarly, the percentage of oospores recovered from soil samples with 10^3 oospores per 10 g was significantly lower than the percentage of oospores recovered from samples with 10^5 , but not different from that with 10^4 . There was significant difference in oospore recovery between soil samples containing 10^4 and 10^5 oospores per 10 g.

Specificity and sensitivity of QPCR assays. All five isolates of *P. capsici* tested (Table 2) were amplified by utilizing the QPCR protocol with Pcap-q-1 primers. There was no DNA amplification from the extracts from soil samples without *P. capsici*, sterile water, isolates of *P. sojae*, *P. infestans*, and *Pythium* species, or fungal species tested. DNA of *P. capsici* oospores as low as 1×10^{-4} ng ($s = 1.3 \times 10^{-5}$, CV = 13.3%) was detected by the amplification (Fig. 3).

Effects of DNA extracts on β -actin amplification. Amplification of DNA directly extracted from soil was completely inhibited. The DNA extracted from oospores recovered from soil with the sieving-centrifugation procedure showed no PCR inhibition. The Ct with 7.5×10^{-4} ng (Test 1) and 1.7×10^{-5} ng (Test 2) of β -actin DNA was 23.5 and 28.3, respectively. The average Ct for the samples with β -actin DNA and *P. capsici* DNA were 23.7 (Test 1) and 28.5 (Test 2). The observed β -actin ΔC_t ranged from 0.0 to 0.9. The coefficient of variation of the Ct for both tests was less than 1% (Fig. 3).

PCR quantification of *P. capsici* oospores. The DNA quantities corresponding to 10^1 , $10^{1.5}$, 10^2 , $10^{2.5}$, 10^3 , $10^{3.5}$, 10^4 , $10^{4.5}$, and 10^5 oospores were 1.4×10^{-4} , 1.9×10^{-4} , 3.5×10^{-4} , 9.0×10^{-4} , 3.2×10^{-3} , 1.6×10^{-2} , 1.5×10^{-1} , 1.2×10^0 , and 1.7×10^1

Fig. 2. Relationship between *Phytophthora capsici* oospores recovered from the soil and oospores incorporated into the soil.

Table 3. Recovery of *Phytophthora capsici* oospores incorporated into soil samples with the sieving-centrifugation method and quantities of recovered oospores

Oospores incorporated into 10 g of soil (no.) ^x	Oospores recovered from 10 g of soil (no. [%]) ^y	DNA of recovered oospores (ng/10 g of soil) ^z
10^1	2.14×10^0 (21.3) d	1.92×10^{-4}
10^2	3.55×10^1 (43.7) c	2.06×10^{-4}
10^3	5.13×10^2 (53.6) bc	1.48×10^{-3}
10^4	6.46×10^3 (60.3) b	5.34×10^{-2}
10^5	7.08×10^4 (79.4) a	7.38×10^0

^x Soil samples were infested with *P. capsici* oospores produced in vitro.

^y Mean percent oospores recovered from nine 10-g soil samples. Values in the column with a letter in common are not significantly different from each other according to Fischer's protected test ($P = 0.05$).

^z Mean DNA quantity from three 10-g soil samples.

ng, respectively. The relationship between the number of oospores and DNA quantities of *P. capsici* was $\hat{Y} = -3.57 - 0.54X + 0.30X^2$ ($R^2 = 0.93$, $P < 0.001$), in which $\hat{Y} = \log_{10}$ (nanogram of *P. capsici* DNA) and $X = \log_{10}$ (number of oospores) (Fig. 4). The standard error for the intercept and linear and quadratic coefficients of the model were 0.18, 0.14, and 0.02, respectively.

The relationship between the DNA quantities of *P. capsici* oospores recovered from soil samples with 10^2 , 10^3 , 10^4 , and 10^5 spore densities in the soil (Table 3) was determined by $\hat{Y} = -3.53 - 0.73X + 0.32X^2$ ($R^2 = 0.955$, $P < 0.05$), in which $\hat{Y} = \log_{10}$ (DNA quantity of *P. capsici* oospores recovered from the soil) and $X = \log_{10}$ (number of *P. capsici* oospores incorporated into the soil).

Quantifying *P. capsici* oospores in field soils. The number of oospores recovered from commercial fields in Illinois ranged from 680 to 2,800 per 10 g of soil (Table 4). The average diameter of oospores extracted from the soils was 27.8 μm . There was no significant difference in diameter of oospores recovered from the different fields. Percentage of germinated oospores recovered from soils ranged from 40.9 to 64.3%. There were significant differences in percent oospores germinated among the fields. Percent germinated oospores identified as *P. capsici* ranged from 9.2 to 18.5% (mean 14.7%) (Table 4).

DNA of *P. capsici* oospores was amplified in soil extracts from all fields tested. *P. capsici* DNA in the field soils ranged from 6.6×10^{-4} ng (217 oospores) to 7.7×10^{-3} ng (1,873 oospores) per 10 g of air-dried soil (Table 5). There were significant differences in the density of *P. capsici* oospores among the fields. The coefficient of variation of Ct for *P. capsici* DNA (target DNA) was 3% and for the endogenous control, it was 13%. The mean relative quantity (RQ) ($2^{-\Delta\Delta C_t}$) values for the eight field soils ranged from 0.22 to 3.58.

With light microscopy, conidia of *Alternaria* spp. were observed in the spore suspensions extracted from soils. After culturing the extracted spores from soil samples from commercial fields, *P. sojae* and *Pythium* spp. were identified in the colonies formed from germinated spores.

DISCUSSION

The sieving density separation in the sucrose solution method has been used to extract mycorrhizal and smut fungi from the soil (5,9,10,22.). Also, the sucrose solution method has long been used to separate nematodes from the soil (24). This study, however, is the first report of using a sieving-centrifugation method utilizing sucrose solution for extraction of oospores of *P. capsici* from the soil.

The sieving-centrifugation procedure described in this report was developed

after numerous tests with different soil textures, soil amounts, sucrose concentrations, centrifugation speeds, centrifugation times, and oospore densities. The described protocol was the most efficient for maximum extraction of *P. capsici* oospores from soils. Utilizing the sieving-centrifugation method, we were able to recover *P. capsici* oospores from soil with a spore density as low as one spore per gram of soil, although the overall extraction efficiency of oospores was not 100%. There were no significant differences in recovery rates of *P. capsici* oospores from all five soils with different textures used in this

study. Also, with the sieving-centrifugation method, *P. capsici* oospores were recovered from soils collected from commercial fields in different locations in Illinois. Thus, the described sieving-centrifugation procedure is a reliable method to separate *P. capsici* oospores from soil to quantify the oospore density.

Furlan et al. (15) reported that sucrose was not a good separation medium for spores of endomycorrhizal fungi because spores become dehydrated and collapse and this may affect identification of the fungus and viability of spores. But, Ianson and Allen (22) used a 2 M sucrose solution

Fig. 3. Effect of DNA extracts from oospores separated from the soil on β -actin amplification by real-time quantitative polymerase chain reaction. There was either 7.5×10^{-4} or 1.7×10^{-5} ng of β -actin in each reaction well (25- μl reaction mixture). Amplification plot shows cycle number versus normalized fluorescence (ΔR_n). The fluorescent intensity signal threshold was 0.2 (solid horizontal line).

Fig. 4. Relationship between number of oospores and absolute DNA quantity (nanogram) of *Phytophthora capsici* in quantitative polymerase chain reaction assays.

to extract spores of vesicular-arbuscular mycorrhizal fungi from soil and found that exposing the spores to sucrose for 24 h did not cause significant damage. Also, Babadoost and Mathre (5) used 1.6 M sucrose solution for extracting teliospores of *Tilletia* spp. from soil and reported no adverse effect of sucrose solution on teliospore morphology and germination. In this study, with the same concentration of sucrose, we found neither collapse of cell wall nor reduction in germination of oospores.

The sieving-centrifugation method alone cannot be used to determine the number of *P. capsici* oospores in the soil because spores of other oomycetes and fungi are extracted along with *P. capsici* oospores. For example, in this study, oospores of

Phytophthora and *Pythium* spp. and conidia of *Alternaria* spp. were extracted along with *P. capsici* oospores from field soils. Also, along with *P. capsici* oospores, spores of endomycorrhizal fungi (22) and smut fungi (5) could be extracted from the soil with the sieving-centrifugation method. With light microscopy, oospores of oomycetes can be easily identified from fungal spores, but identification of oospores of *P. capsici* from oospores of other oomycetes on the basis of oospore morphology is not possible. Utilizing the QPCR procedure developed in this study, we assessed the number of *P. capsici* oospores extracted from the soil. The QPCR method, however, cannot determine viability of the oospores. The germination test of the extracted oospores is required to deter-

mine whether or not the oospores in the soil are viable.

Because of the presence of PCR inhibitors in the soil, direct assay of *P. capsici* oospores in the soil with the QPCR procedure was unsuccessful. However, by utilizing sieving-centrifugation procedures, the PCR inhibitors in the soil were eliminated. Several studies have been conducted to eliminate PCR inhibitors from nucleic acid extracts (16,31,44,45). The sieving-centrifugation procedure described in this report is an easy solution for eliminating PCR inhibitors in the soil extracts used in the QPCR method.

Only oospores were present in the soil extracts on the basis of examination of the extracts from artificially and naturally infested soils with light microscopy. No hyphal fragments, encysted zoospores, sporangia, or chlamydospores were observed in the extracts. Thus, extracted *P. capsici* DNA was considered to be oospore DNA only. Consequently, the number of *P. capsici* oospores in the soil could be determined by $\hat{Y} = -3.53 - 0.73X + 0.32X^2$ ($R^2 = 0.95$, $P < 0.05$), in which $\hat{Y} = \log_{10}$ (DNA quantity of *P. capsici* oospores recovered from the soil) and $X = \log_{10}$ (number of *P. capsici* oospores incorporated into the soil). The number of oospores in the soil can be calculated according to the formula $X = [-b + \text{square root of } (b^2 - 4ac)]/(2a)$ in the quadratic equation ($aX^2 + bX + c = 0$).

The QPCR method used in this study is based on the amplification of ITS region of the ribosomal DNA of *P. capsici*. The ITS region has been reported to be a reliable region for specific amplification of *P. capsici* (23,28,39,41). Silvar et al. (41) quantified ribosomal DNA of *P. capsici* in pepper plants with absolute QPCR with SYBR green primers with an amplicon of 452 bases. In our study, ribosomal DNA of *P. capsici* was quantified with a set of primers and a TaqMan probe with an amplicon of 74 bases. In both studies, segments of the same regions of the 5.8S ribosomal DNA gene of *P. capsici* were amplified. Our procedure did not amplify DNA of *P. sojae*, *P. infestans*, *Pythium* spp., *Aphanomyces euteiches*, *Saprolegnia monilifera*, or fungal species tested. Thus, this procedure is highly specific and sensitive for detecting *P. capsici* since it detected as low as 1×10^{-4} ng of DNA.

Germination of oospores is essential for determining density of viable oospores of *P. capsici* in the soil. However, it should be mentioned that the rate of germinated oospores, utilizing current cultural methods (1,11), may underestimate the number of viable oospores of *P. capsici* in the soil because: (i) percentage of germination of *P. capsici* oospores on culture media is usually less than 50%; and (ii) colonies of *P. capsici* originating from oospores may be outgrown by closely related oomycete spp., particularly *Pythium* spp. Thus, further investigations may improve germina-

Table 4. Total number and percentage of germinated oospores of species of the *Pythiaceae* family in commercial fields in Illinois in 2005

Location ^w	Total number of oospores/10 g of soil ^x	Oospore diameter (μm) ^y	Oospore germination (%) ^z	Geminated oospores identified as <i>P. capsici</i> (%)
Forest city	1,783 abc	28.1 ± 4.4	61.3 abc	13.6
Green Valley	1,183 abc	27.5 ± 3.8	64.3 a	12.3
Green Valley	1,183 abc	27.1 ± 4.0	62.3 ab	18.4
Green Valley	2,800 a	26.9 ± 3.7	47.6 cd	10.8
Pekin	2,183 ab	28.5 ± 2.3	49.7 bcd	18.5
Shawneetown	1,533 abc	28.6 ± 2.7	57.3 abc	17.4
Shawneetown	1,800 abc	27.4 ± 3.1	62.0 ab	17.1
Shawneetown	680 c	28.1 ± 2.8	40.9 d	9.2

^w Towns in Illinois: Forest City in Mason County; Green Valley and Pekin in Tazewell County; and Shawneetown in Gallatin County.

^x Mean number of oospores extracted from five 10 g air-dried soil samples. Values in the column with a letter in common are not significantly different from each other according to Fischer's protected test ($P = 0.05$).

^y Mean diameter of 15 arbitrarily selected oospores.

^z Mean of four 50-oospore replications. Values in the column with a letter in common are not significantly different from each other according to Fischer's protected test ($P = 0.05$).

Table 5. Quantitative polymerase chain reaction assays of total DNA of *Phytophthora capsici* oospores extracted from commercial fields in Illinois

Location ^w	Relative quantity			Absolute quantity	
	Ct ^v		RQ ^x	<i>P. capsici</i> DNA (ng/10 g of soil) ^y	<i>P. capsici</i> oospores (no./10 g of soil) ^z
	Pcap-q-1	Pyth-q-2			
Forest City	34.1	30.1	3.59 ± 0.9	4.0 × 10 ⁻³	1,116 ab
Green Valley	34.9	26.8	0.22 ± 0.7	2.5 × 10 ⁻³	767 abc
Green Valley	35.2	30.8	2.94 ± 0.7	9.8 × 10 ⁻⁴	342 bc
Green Valley	33.1	28.5	2.48 ± 0.8	7.7 × 10 ⁻³	1,873 a
Pekin	35.0	28.7	0.77 ± 0.5	2.3 × 10 ⁻³	741 abc
Shawneetown	35.8	30.9	2.02 ± 0.9	1.5 × 10 ⁻³	467 bc
Shawneetown	35.2	30.6	2.51 ± 0.4	2.1 × 10 ⁻³	650 abc
Shawneetown	37.1	30.1	0.47 ± 0.9	6.6 × 10 ⁻⁴	217 c

^v Threshold cycle (Ct) number when fluorescence intensity of the sample exceeded background fluorescence intensity. The fluorescent intensity signal threshold was 0.2. The Ct value is the mean of three sample replicates. Pcap-q-1 and Pyth-q-2 primers were designed for amplification of ribosomal DNA of *P. capsici* and ribosomal DNA of species of the *Pythiaceae* family, respectively.

^w Towns in Illinois where the sampled commercial fields infested with *P. capsici* are located. Forest City, Green Valley and Pekin, and Shawneetown are in Mason, Tazewell, and Gallatin counties, respectively.

^x RQ = $(2^{-\Delta\Delta Ct})$ and is the relative quantity of DNA of *P. capsici*.

^y Mean of *P. capsici* DNA from three 10-g soil samples.

^z Number of oospores was determined by the equation $\hat{Y} = -3.57 - 0.54(X) + 0.30(X^2)$, in which $\hat{Y} = \log_{10}$ (DNA nanogram) and $X = \log_{10}$ (oospore number). Values in the column with a letter in common are not significantly different from each other according to Fischer's protected test ($P = 0.05$).

tion of *P. capsici* oospores in culture plates and enhance accuracy of assessment of viable oospores of *P. capsici* in the soil.

The protocol of combining sucrose-centrifugation and QPCR methods developed in this study can be used to determine distribution of *P. capsici* oospores in fields and investigate biology and ecology of *P. capsici*. The findings of this study can also be employed to develop effective cropping systems for management of Phytophthora blight caused by *P. capsici*. In addition, the procedures reported in this paper could be adapted for assessing the presence of other soilborne plant pathogens, particularly oomycetes, in the soil.

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