DETERMINING DENSITY OF *PHYTOPHTHORA CAPSICI* OOSPORES IN SOIL

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ABSTRACT. A sucrose-centrifugation method was developed to extract oospores of *P. capsici* from soil. The relationship between the number of oospores recovered from soil and the number of oospores incorporated into the soil was $\hat{Y}= -0.75 + 1.21X -0.02X^2$ ($R^2=0.98$), where $\hat{Y} = \log_{10}$ of the number of oospores recovered from soil and $X = \log_{10}$ of the number of oospores in soil. The oospores were germinated after treating with 0.1% $KMnO_4$ solution for 10 min. Using a sucrose-centrifugation method, oospores of *P. capsici* were successfully extracted from soil samples collected from commercial squash fields. A real-time quantitative polymerase chain reaction (QPCR) protocol was developed to assay the density of *P. capsici* oospores in soil. PCR-inhibition was avoided by extracting oospores from soil using the sucrose-centrifugation method. The relationship between the amount of DNA measured and the number of oospores of *P. capsici* included in the test was $\hat{Y} = - 3.57 - 0.54X + 0.30X^2$ ($R^2 = 0.93$), where $\hat{Y} = \log_{10}$ (ng of *P. capsici* DNA), $X = \log_{10}$ (number of oospores).

Phytophthora blight of cucurbits, caused by *Phytophthora capsici* Leonian, is one of the most serious threats to production of cucurbits, pepper, and eggplant worldwide. *P. capsici* is a soil-borne oomycete that infects more than 50 plant species in 15 families (Erwin and Ribeiro. 1996; Tian and Babadoost, 2004). It can infect plants at any stage of growth, causing seedling damping-off, crown rot, foliage blight, and fruit rot. Phytophthora blight can cause yield losses of up to 100% in cucurbits and pepper fields (Babadoost and Islam, 2003; Hausbeck and Lamour, 2004). At present, there is no long-term sustainable solution for disease management, however, a combination of cultural practices, fungicide application, and genetic resistance can be used to minimize the damages caused by *P. capsici* to vegetable crops (Babadoost and Islam, 2003; Hausbeck and Lamour, 2004).

P. capsici is a heterothallic organism in which two compatible mating types, designated as A1 and A2, are needed for sexual reproduction. The sexual spore of *P. capsici* is the oospore, which is the primary source of inoculum and the overwintering propagule in the soil (Erwin and Ribeiro.

1996). A reliable method for quantifying *P. capsici* oospores in soil is needed to assess survival of the pathogen and the potential for disease development in the field. Methods used for assessing density of *P. capsici* in soil have been primarily dilution plating and baiting assays but, these methods are time-consuming and the accuracy of the assays is questionable (Erwin and Ribeiro. 1996; Silvar et al., 2005).

Sucrose-centrifugation and sieving is basically a method t\hat separate particles based on their density and size. This method has been used to extract propagules of mycorrhizal fungi (Smith and Skipper, 1979), teliospores of *Tilletia* species (Babadoost and Mathre, 1998), and nematodes (Jenkins, 1964) from soil.

Silvar et al. (2005) used a polymerase chain reaction (PCR)-based method to detect *P. capsici* in soil. The protocol they used was for detecting the pathogen only and the method cannot determine quantity of *P. capsici* oospores in soil. Also, they reported the presence of PCR inhibitory factors in the DNA extracts for molecular detection of plant pathogens in soil (Van de Graaf, 2003). Real-time quantitative polymerase chain reaction (QPCR) is a relatively new molecular technique that has been used to quantify nematodes (Cao et al., 2005), viruses (Delanoy et al., 2003), bacteria (Bach et al., 2003), and fungal plant pathogens (Hayden et al., 2004: Silvar et al., 2005).

The objective of this study was to develop a reliable QPCR method to quantify *P. capsici* oospores in soil.

Materials and Methods

***IN-VITRO* OOSPORE PRODUCTION.** For *in-vitro* production of oospores of *P. capsici*, two plugs of opposite mating types (A1 and A2) of the pathogen were grown in 40-ml aliquots of V8-$CaCO_3$ medium in 250-ml Erlenmeyer flasks at 24°C for 2 mo in darkness. Then, oospores were harvested, blending the culture at full speed for 90 s in a Hamilton Beach blender (model 52250, Southern Pines, NC). The suspension in the blender was passed through 68 and 38 µm metal sieves and the filtrate was collected. The filtrate then was passed through a 20-µm Spectra/Mesh nylon filter (Spectrum, Houston, TX). The oospores collected on the mesh were washed into a beaker and the number of oospores was determined using a spore-counting chamber (Hauser Scientific, Horsham, PA).

OOSPORE EXTRACTION FROM SOIL. Five agricultural soils, including a sandy loam, a silt clay, and three silt loam, collected from various locations in Illinois, were used in this study to develop the procedure for extraction of

oospores of *P. capsici* from soil. The calculations were based on the air-dried weight for all of the soils.

Samples of the five soil types were air dried at room temperature on a laboratory bench for 14 days and passed through a 2-mm sieve. Predetermined quantities of oospores were added to soil samples and thoroughly mixed. The artificially infested soil samples were estimated to have10^1, 10^2, 10^3, 10^4, and 10^5 oospores of *P. capsici* per 10 g of air-dried soil.

Each 10-g infested soil sample was suspended in 400 ml tap water with two drops of Tween-20 and shaken for 15 min. The soil suspension was passed through nested 106, 63, and 38 µm metal sieves. The material caught on the 38-µm sieve was washed using a sprinkler with a gentle stream of water and the filtrate was collected. This suspension (approximately 2 L) was then passed through a 20-µm mesh filter. The materials caught on the 20-µm mesh were washed into two 50-ml centrifuge tubes and spun for 4 min (900 x g) using a bench-top centrifuge. The supernatant was discarded and the pellet was suspended in 30 ml of 1.6 M sucrose solution. This suspension was centrifuged for 45 s (190 x g) and the supernatant was passed through the 20-µm mesh. The pellet was resuspended in the sucrose solution and centrifuged again (45 s, 190 x g). The procedure was repeated six times to maximize oospore recovery from soil. The materials caught on the 20-µm mesh were washed into a 50-ml centrifuge tube and spun for 4 min (900 x g). The pellet was resuspended in 0.5 to 1.5 ml of distilled water, depending on the original number of oospores added to the soil, and the number of oospores was determined using a spore-counting chamber.

The oospore recovery at each inoculum level for each of the five artificially infested soils was determined using four replicates of 10-g soil sample and with four oospore counts per replicate (a total of 16 spore counts for each inoculum level for each soil type).

EXTRACTION OF OOSPORES FROM FIELD SOIL. Eight commercial fields in three counties, with a history of Phytophthora blight, were sampled to test the soil for presence of *P. capsici* oospores. In each field, 20 subsamples of soil were taken from 0–20 cm deep from approximately 0.4 ha area using a soil probe. The subsamples from each field were mixed thoroughly and a 1-kg sample was taken. Four replicates of 10 g soil samples from each field were processed using the procedure described above, to extract and enumerate oospores of *P. capsici*.

OOSPORE GERMINATION. Extracted oospores from soil were germinated to determine their viability. The oospores were plated onto the semi-selective medium PARP in Petri plates (50 oospores per plate). The effect of potassium

permanganate ($KMnO_4$) treatment on oospore germination was evaluated by suspending oospores in 0.02, 0.04, 0.1, and 0.2% of $KMnO_4$ solution in water for 10 min. After the treatment, oospores were washed three times with sterile-distilled water and plated onto PARP medium. The plates were incubated at 24°C under fluorescent light for 4 days and the percentage of germinated spores was determined. Four replica plates of oospore germination were included for each treatment. Single colonies of germinated oospores from commercial fields were transferred to lima bean agar in Petri plates and *P. capsici* colonies were identified from colonies of other *Phytophthora* and *Pythium* species based on sporangial morphology.

REAL-TIME QPCR QUANTIFICATION OF OOSPORES. *P. capsici* DNA was extracted from the oospores using a protocol based on FastDNA kit (Qbiogene, Inc., Carlsbad, CA), which was modified for removal of PCR inhibitors by Malvick and Grunden (2005). The QPCR assays were conducted in a 96-well plate format with the ABI PRISM 7000 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). *P. capsici* 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'. The manufacturer's instructions were followed, except 25-µl reaction mixtures were used instead of 50 µl. 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 pre-incubation at 50°C.

The modified QPCR procedure was used to detect *P. capsici* oospores in soil samples collected from the eight commercial fields. First, oospores were extracted using the sucrose-centrifugation method. Then the extracted oospores were tested to determine the quantity of *P. capsici* oospores.

Results and Discussion

IN VITRO OOSPORE PRODUCTION. Both mating types of *P. capsici* (A1 and A2) were identified among the isolates tested. The three pairings used for oospore production yielded almost the same amount of oospores per plate. The number of oospores per plate after 2 mo ranged from 4.41×10^5 to 6.72×10^5 (mean 5.56×10^5) oospores per plate.

OOSPORE EXTRACTION FROM SOIL. Oospores of *P. capsici* were successfully recovered from all five artificially infested soil types. Overall, 50.9% of oospores incorporated into the soil were recovered. There was no significant difference in oospore recovery among the soil types. The

relationship between the number of oospores recovered from soil and the number of oospores incorporated into the soil was $\hat{Y}= -0.75 + 1.21X -0.02X^2$ ($R^2=0.98$), where $\hat{Y} = \log_{10}$ of the number of oospores recovered from soil and $X = \log_{10}$ of the number of oospores in soil (Fig. 1). The average recovery rates of *P. capsici* oospores from the soils were 25.5 (13.5-37.8), 43.7 (33.0-54.4), 53.6 (42.9-64.4), 60.3 (49.5-71.0), and 72.1% (61.3-82.8%) 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 between soil samples containing 10^4 and 10^5 oospores per 10 g. Percentage of oospores recovered from soil samples with 10^1 oospores per 10 g was significantly lower than the percentage of oospores recovered from the samples with 10^2 , 10^3, 10^4, and 10^5 oospores per 10 g. Similarly, percentage of oospores recovered from soil samples with 10^2 oospores per 10 g soil was significantly lower than those of soil samples with 10^4 and 10^5 oospores per 10 g soil. In addition, the percentage of oospores recovered from soil samples with 10^3 oospores per 10 g was not significantly different than the percentage of oospores recovered from samples with 10^2, and 10^4 oospores per 10 g.

OOSPORES IN COMMERCIAL FIELDS. We extracted oospores from soil samples collected from all eight commercial fields in Illinois. The number of oospores recovered from commercial fields ranged from 694 to 2,467 per 10 g of soil. There was no significant difference in diameter of oospores recovered from different fields. Number of *P. capsici* oospores extracted from commercial fields ranged from 79 to 529 per 10 g soil. The rate of *P. capsici* oospores of the total number of oospores extracted from soil samples from commercial fields ranged from 9.2% to 18.5% (mean 14%).

OOSPORE GERMINATION. Germination rates of oospores produced *in vitro* with the pretreatment with 0, 0.02, 0.04, 0.1, and 0.2 solution of $KMnO_4$ were 15.2, 29.8, 27.6, 44.5, and 40.7%, respectively. The rates of oospore germination for 0.1 and 0.2% solution of $KMnO_4$ were not significantly different. But the rates of oospore germination after treating with either 0.1 or 0.2% solution of $KMnO_4$ were significantly higher than those of treatments with 0.02 and 0.04% $KMnO_4$ solutions. Therefore, for germination of oospores extracted from soil samples from commercial fields, we used a 0.1% $KMnO_4$ solution and oospores were treated for 10 min. Germination rates of oospores extracted from commercial fields ranged from 18.8% to 51.1% (mean 36.8%). In addition to *P. capsici*, *P. sojae* and *Pytium spp*. were identified in the culture plates.

REAL-TIME PCR QUANTIFICATION OF OOSPORES. The relationship between the number of oospores and *P. capsici* DNA quantity was $\hat{Y} = -3.57 -$

$0.54X - 0.30X^2$ ($R^2 = 0.93$), where $\hat{Y} = \log_{10}$ (ng of *P. capsici* DNA), X = $\log_{10}$ (number of oospores) (Fig. 2). According to this model, 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} ng, respectively.

Using the QPCR procedure, we detected *P. capsici* in all field soil samples tested. There was no PCR-inhibition in the DNA extracts from oospores extracted from soil using the sieving-sucrose-centrifugation method, although some soil particles and organic matter were associated with the oospores. PCR inhibition, however, was found in the DNA extraction directly from soil.

Using the sieving-centrifugation with sucrose solution method, we were able to recover oospores of *P. capsici* from soil with a spore density as low as 10 spores per g of soil. The results showed that the method can be used to determine density of *P. capsici* oospores in fields with different soil types. There were, however, some difficulties in the identification of the oospores extracted from naturally infested commercial field soils, because oospores of other *Phytophthora* and *Pythium* species were co-extracted by the procedure. These difficulties were overcome by germinating and identifying oospores based on morphological characteristics of sporangia. Thus, the sieving-sucrose-centrifugation procedure can be used to estimate *P. capsici* oospores in soil in areas where Phytophthora blight of vegetables is a problem, or any other area suspected of having *P. capsici*.

Difficulty in the *in-vitro* germination of oospores of *Phytophthora* species has been reported. These difficulties have been overcome by pretreatment of oospores with $KMnO_4$ solution. For example, germination of oospores of *P. parasitica* was improved by a pretreatment with 0.25% solution of $KMnO_4$ for 20 min. Similarly, germination of oospores of *P. megasperma* was enhanced by a pretreatment of 0.05% of $KMnO_4$ for 10 min. Similarly, we increased percent of germination of *P. capsici* oospores from about 10% to 50% by pretreatment with 0.1% $KMnO_4$ solution.

QPCR is useful for detecting and quantifying nonculturable and slow-growing organisms. Some reports indicated that *P. capsici* can be outgrown by closely related and fast-growing *Pythium* spp. The QPCR procedure used in this study is a reliable method for quantifying *P. capsici* oospores in soil. The procedure detected *P. capsici* in all field soil samples tested. This QPCR protocol is a fast, accurate, and sensitive method for quantifying *P. capsici* oospores in soil. Also, the combination of sieving-centrifugation and QPCR is effective in eliminating PCR inhibitors that affect PCR tests in direct assays from soil samples.

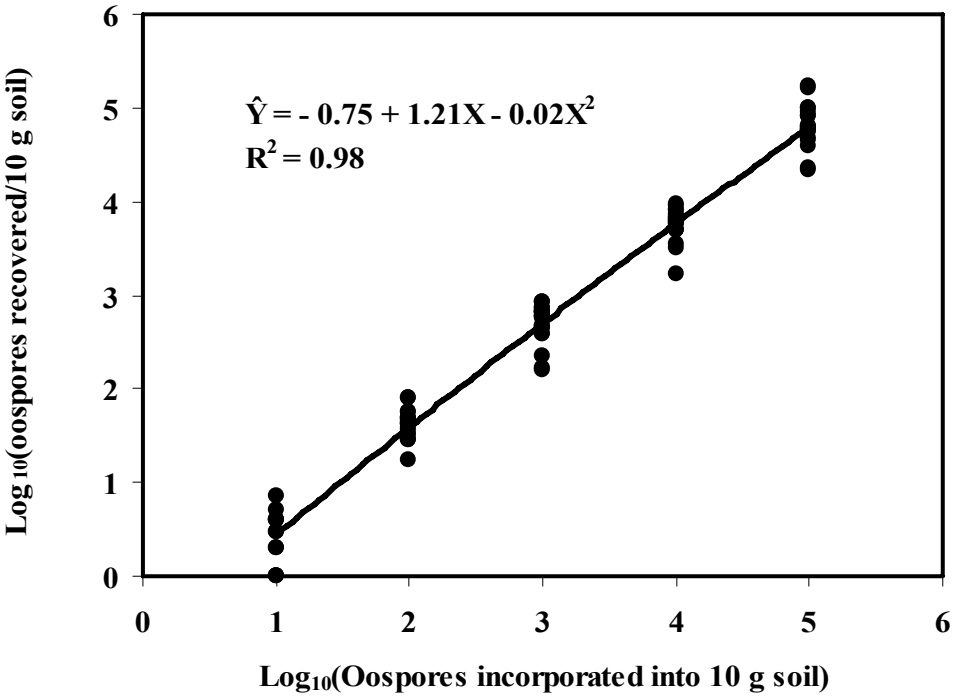

Fig. 1. Relationship between number of oospores recovered from soil and number of oospores incorporated into soil.

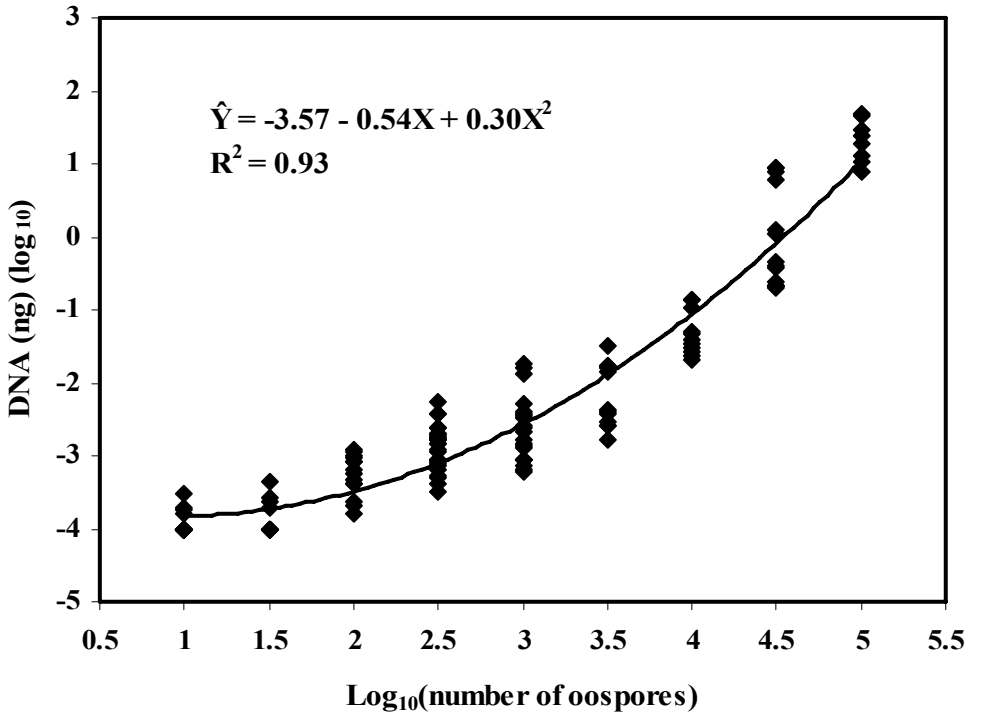

Fig. 2. Relationship between quantity of DNA and number of oospores used in quantitative polymerase chain reaction tests.

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