

Quantification of *Fusarium commune* in Douglas-fir Seedling Nurseries

Anna Leon

Anna Leon, Doctoral Candidate, Department of Plant Pathology, Washington State University Puyallup Research and Extension Center, 26-06 West Pioneer, Puyallup, WA 98403; E-mail: anna_leon@wsu.edu

Leon A. Quantification of *Fusarium commune* in Douglas-fir Seedling Nurseries. In: Wilkinson KM, Haase DL, Pinto JR, technical coordinators. National Proceedings: Forest and Conservation Nursery Associations—2013. Fort Collins (CO): USDA Forest Service, Rocky Mountain Research Station. Proceedings RMRS-P-72. 44-49. Available at: http://www.fs.fed.us/rm/pubs/rmrs_p072.html

Abstract: A better diagnostic assay can help growers make cost effective management decisions that meet environmental regulations. A quantitative real-time polymerase chain reaction (qPCR) assay has been developed for both *Fusarium commune* and *Fusarium oxysporum* isolated from inoculated soil, with the intent of quantifying *F. commune* from naturally occurring populations in nursery soil. *F. commune* inoculated in greenhouse potting soil (CFU/g) positively correlated with the observed Ct values from the duplex qPCR assay. Additionally, greenhouse trials show a positive correlation between inoculum addition and disease development at high levels of inoculation. Additional trials are being done.

Key Words: disease diagnosis, quantitative real-time polymerase chain reaction (qPCR), molecular diagnostic assays, dilution plating

Introduction

Fusarium root rot and damping off has been an economically important disease of Douglas-fir seedlings in production nurseries for several decades. It was long believed that *Fusarium oxysporum* consisted of both virulent and non-virulent forms (Bloomberg 1966; Bloomberg 1971), but recent molecular work suggests that the morphologically indistinguishable species *Fusarium commune* is the virulent pathogen of interest (Skovgaard and others 2003; Stewart and others 2006). Diagnosis of *F. oxysporum* was typically done by counting colonies plated from soil dilutions based on colony morphology. Given the unreliable diagnostic nature of morphological characters, current determinations of soil *Fusarium* concentrations likely do not correlate with actual levels of disease. Given the difficulties with proper diagnosis and quantification of *Fusarium* disease in nurseries, no accurate method exists to test to see if fumigation or other disease control practices need to be used. A better diagnostic assay can help growers make cost effective management decisions that meet environmental regulations

Molecular diagnostic assays have been used with success in other cropping systems. Real-time polymerase chain reaction (qPCR) amplifies DNA and can be used to quantify the initial amount of DNA in a sample if run with a standard set of isolates at known concentrations. This technology has been used to determine quantities of pathogenic organisms in field soil, in plant tissue and in storage facilities (Schroeder and others 2006; Okubara and others 2008; Zhang and others 2005).

The primary objective of this study is to develop a quantitative real-time qPCR assay for the identification and quantification of *F. commune*. It is essential that this assay be able to differentiate between *F. commune* and *F. oxysporum* soil DNA. A complementary disease threshold assay is being developed to determine the levels at which growers need to be concerned about *F. commune* and also is being used as a way to make the qPCR assay more robust.

Materials and Methods

Soil Sampling and Dilution Plating

Soil samples were collected with approval and assistance from nursery staff at three Washington nurseries and one Oregon nursery in 2011 and 2012. Thirty soil samples were taken from each field sampled with a 12-inch soil corer. Each sample was a composite of 10 core samples mixed together at each location in the field.

Soil samples were allowed to air dry at room temperature to remove excess moisture. Samples with large aggregate particles were ground with a mortar and pestle prior to testing. Three replicate soil samples were taken from the sample for dilution plating on Komada's media (Komada 1975) as described in the protocol of Leslie and Summerell (2006). The remainder was kept in the cooler at 37 °C for short term storage. *Fusarium* colonies growing on Komada's media were counted and colony forming units per gram (CFU/g) were calculated using an adjusted soil dry weight.

DNA Extraction and qPCR Development

DNA was extracted from single spore inoculated *Fusarium* cultures on PDA plates using a Qiagen DNeasy Plant Mini Kit following the Plant Tissue Mini Protocol (Qiagen 2006). Samples taken from scrapings off of PDA agar plates were lysed using the FastPrep-24 Tissue Homogenizer and the Qiagen protocol was started with the homogenized material at Step 7. The procedure for PCR was adapted from the standard protocol in the WSU Molecular Lab. One aliquot master mix contained 32.9 µl dH₂O, 5 µl 10X PCR buffer (+MgCl), 0.5 µl 10X µM dNTPs, 2.5 µl 10 µM EF-1α forward primer, 2.5 µl 10 µM EF-1α reverse primer, 5 µl 10 mg/mL BSA, and 0.6 µl Taq Polymerase (3 units). 1 µl extracted DNA was added to the master mix. The primers used were the EF1 forward primer: ATGGGTAAGGARGACAAGAC and the EF2 reverse primer: GGARGTACCAGTSATCATGTT. The PCR protocol was as follows: 94 °C for 2 minutes; 30 cycles of 94 °C for 1 minute, 54 °C for 30 seconds, 72 °C for 1 minute; 72 °C for 10 minutes. The PCR product was run on a 1% agarose gel to verify the amplification of DNA prior to sequencing. 5 µl PCR product was combined with 2 µl ExoSAP-IT and the ExoSAP protocol was run. After clean-up, samples were sent to Genewiz for sequencing. Returned sequences were examined for fidelity using Finch TV and compared to existing DNA sequences using a BLAST search. Isolate identity was confirmed and sequences were compared using BioEdit software.

A TaqMan primer and probe in the EF-1α region were developed using PrimerSelect software from known *F. oxysporum* and *F. commune* isolates. The working protocol designed for this region for *F. commune* isolates uses forward primer: GACGGGCGCGTTTGC, reverse primer: ACGTGACGATGCGCTCATT and 6-FAM labeled TaqMan MGB probe: CTCCCATTTCACAACC labeled with a non-fluorescent quencher. The protocol designed for *F. oxysporum* isolates uses forward primer: GGGAGCGTTGCCCTCTTA, reverse primer: ACACGTGACGACGCACTCAT, and a 6-FAM labeled TaqMan MGB probe: CCATTTCACAACCTC labeled with a non-fluorescent quencher. These primer/probes will be tested on isolates identified using traditional PCR in the EF-1α region. Sequence information from isolates of *F. commune* and *F. oxysporum* were used in the development of these primer/probe sets (Stewart and others 2006).

The qPCR technique was verified using pure DNA extracted from cultures of *F. oxysporum* and *F. commune*. Extracted DNA was diluted in 5 concentrations ranging from 10⁻¹ to 10⁻⁵. 2 µl of diluted DNA were added to the master mix containing: 12.5 µl 2X TaqMan, 1.25 µl 2 µM forward primer, 1.25 µl 2 µM reverse primer, 1.25 µl 2

µM probe, 2.3 µl trehalose, and 4.5 µl H₂O for a total 25 µl reaction. An Applied Biosystems 7500 Real Time PCR System was used for all qPCR. The standard protocol was followed: stage 1 - 50.0 °C for 2:00 min, stage 2 - 95.0 °C for 10:00, stage 3 - 40 replications at 95.0 °C for 15 sec, final stage - 60.0 °C for 1:00 min. This procedure was used for both the *F. oxysporum* and *F. commune* protocols. Each sample was tested using both of the protocols in the same reaction. All reactions included the addition of the TaqMan Exogenous Internal Positive Control Reagents with a VIC probe to ensure negative readings represented a lack of sequence similarity rather than the presence of DNA inhibition.

Ct values for each sample were compared to a standard curve for each respective species. A relationship between the dilutions was established. Individual samples were judged based on their amplification and threshold value for each of the primer/probe protocols. Results were compared to the original BLAST sequence information on the samples.

After successful completion of the two qPCR assays for each individual species, a triplex reaction was designed to increase efficiency. The same primer and probe sequences in the individual reactions were used, but different fluorescent dyes and quenchers were applied to the probes, as well as to a salmon sperm probe to be used as an internal positive control (SKETA). When first designed, *F. commune* was given a 6-FAM dye and *F. oxysporum* a NED dye, both with Applied Biosystems MGB quenchers. The SKETA probe was given a VIC dye with a TAMRA fluorescent quencher. The TAMRA quencher from the SKETA probe and the NED dye in the *F. oxysporum* probe had a negative reaction and the SKETA probe was redesigned with a VIC fluorescent dye and a MGB quencher. Applied Biosystems technology was used to ensure optimum efficacy on our machine.

Greenhouse Threshold Trial and Soil DNA Extractions

Three isolates of *F. commune* and three isolates of *F. oxysporum* were selected based on a combination of pathogenicity and isolate viability in a preliminary trial. Ground cornmeal-perlite inoculum samples were combined at five different inoculum levels. 3 cubic feet of Specialty Soils, Inc. Gardener's Professional Secret growing media (Covington, WA) was sterilized in a Pro-Grow Electric Soil Sterilizer, Model #SST-15 (Pro-Grow Supply, Brookfield, WI) at 180 °C. The inoculum was mixed with sterilized growing media on a w/w basis at 1:50, 1:500, 1:5000, 1:25000, and 1:50000 (Treatments 1-5 respectively). Ten seeds were planted in treatment media in 3.25 in X 3.25 in. (8.25 cm X 8.25 cm) pots and treatments were randomly arranged in five replicated blocks. Greenhouse temperatures were kept between 24-27 °C (75.2-80.6 °F) with 18 hours of daylight. Pots were watered using overhead sprinklers for 5 minutes, 4 times a day.

Fusarium DNA from the potting mix material was extracted using the Wizard Magnetic DNA Purification System for Food as described for *Rhizoctonia solani* in Budge and others (2009). The manufacturer protocol 3.A. was followed with the exception of steps 1 and 2, which call for the use of Lysis Buffer A and RNaseA. Instead, 4 grams of soil were combined with 5 mL of glass beads in a 50 mL plastic tube. A soil extraction buffer was prepared as described in Budge and others (2009): 120 mM sodium phosphate buffer pH8, 2% CTAB, 5 M NaCl, 2% antifoam B emulsion. 16 mL of the soil extraction buffer were added to the 50 mL plastic tube and homogenized in the Fast Prep Homogenizer at setting 6.5 for 60 seconds. After homogenization, tubes were centrifuged for 3 minutes at 2000 g. 500 µl were pipetted into a 2.0 mL tube and all instructions from Step 3 of the manufacturer's 3.A. protocol were followed.

Results and Discussion

Development of two separate qPCR assay for identification of *Fusarium commune* from *Fusarium oxysporum* has been successful. The efficiency of the standard curves for the qPCR assays for *F. commune* and *F. oxysporum* were 0.9741 and 0.9766 respectively (figure 1). The new multiplex assay is currently showing similar results with efficiencies of 0.9781 and 0.9897 for the standard curves of *F. commune* and *F. oxysporum*, respectively (figure 2).

Using the separate, individual qPCR assays, the Ct value of the qPCR for *F. commune* and *F. oxysporum* correlated with the inoculum expressed in colony forming units/g (CFU/g) with $r^2 = 0.825$ and 0.789 respectively (figure 3). Mortality and inoculum expressed as CFU/g formed positive correlations for three *F. commune* isolates, with $r^2 = 0.7211$, 0.8358 , and 0.9376 and for two *F. oxysporum* isolates, with $r^2 = 0.8275$ and 0.8954 (figure 4). Subsequent greenhouse assays are currently being run at lower inoculum concentrations to further test the sensitivity of the qPCR assay and how seedlings respond to lower levels of disease.

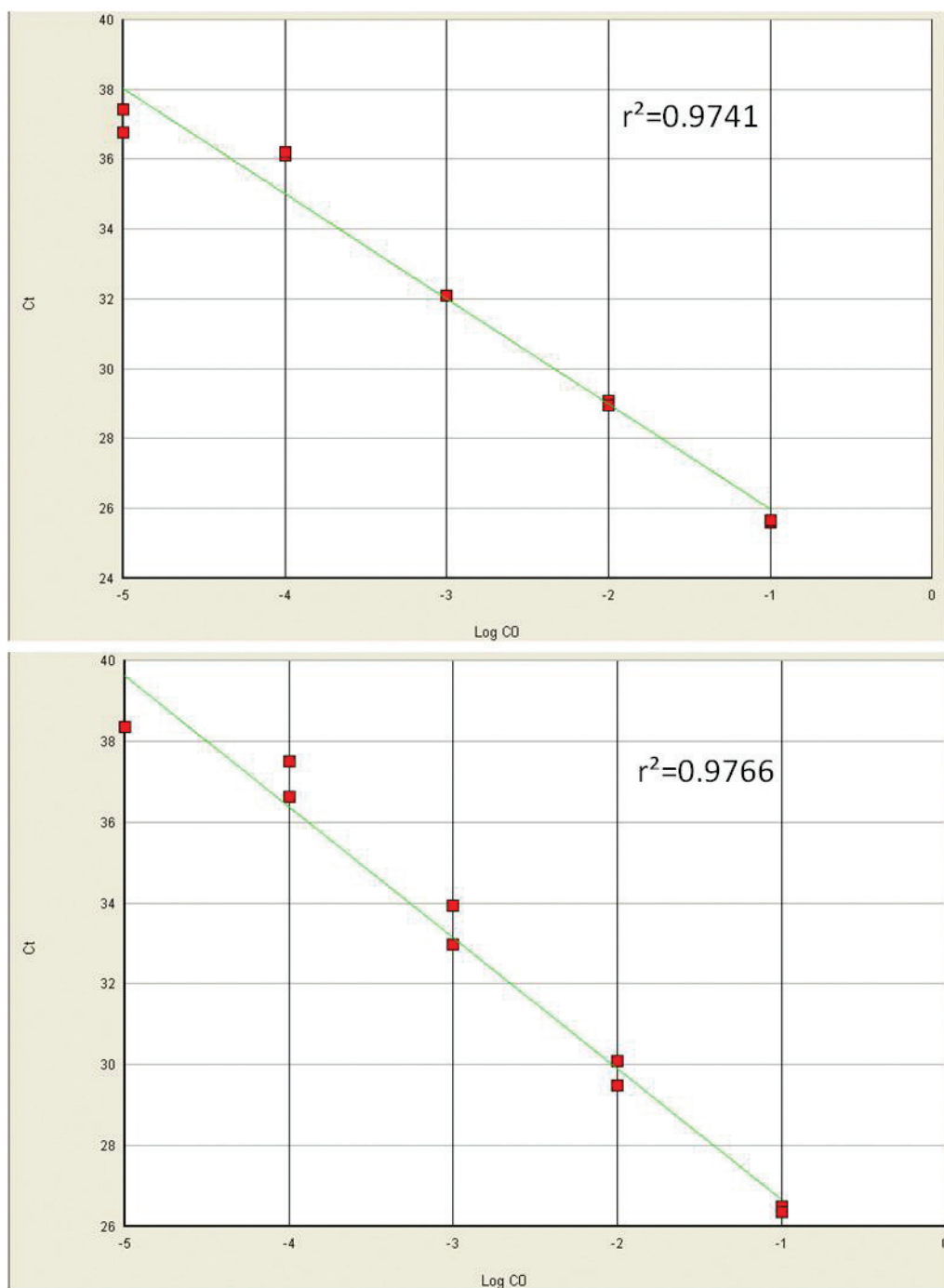


Figure 1. qPCR standard curve efficiency for *F. commune* and *F. oxysporum* single assays.

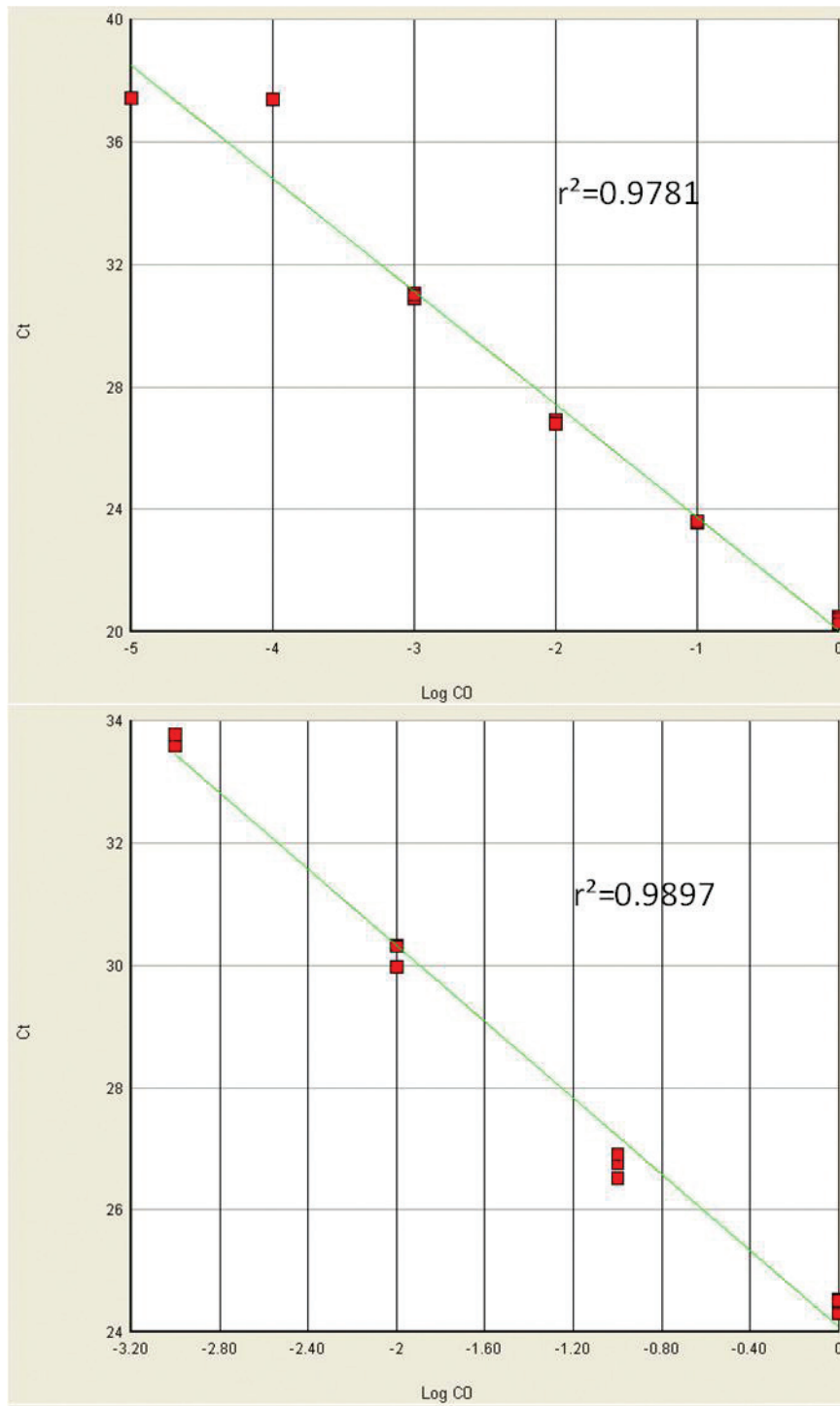


Figure 2. qPCR standard curve efficiency for *F. commune* and *F. oxysporum* multiplex assay.

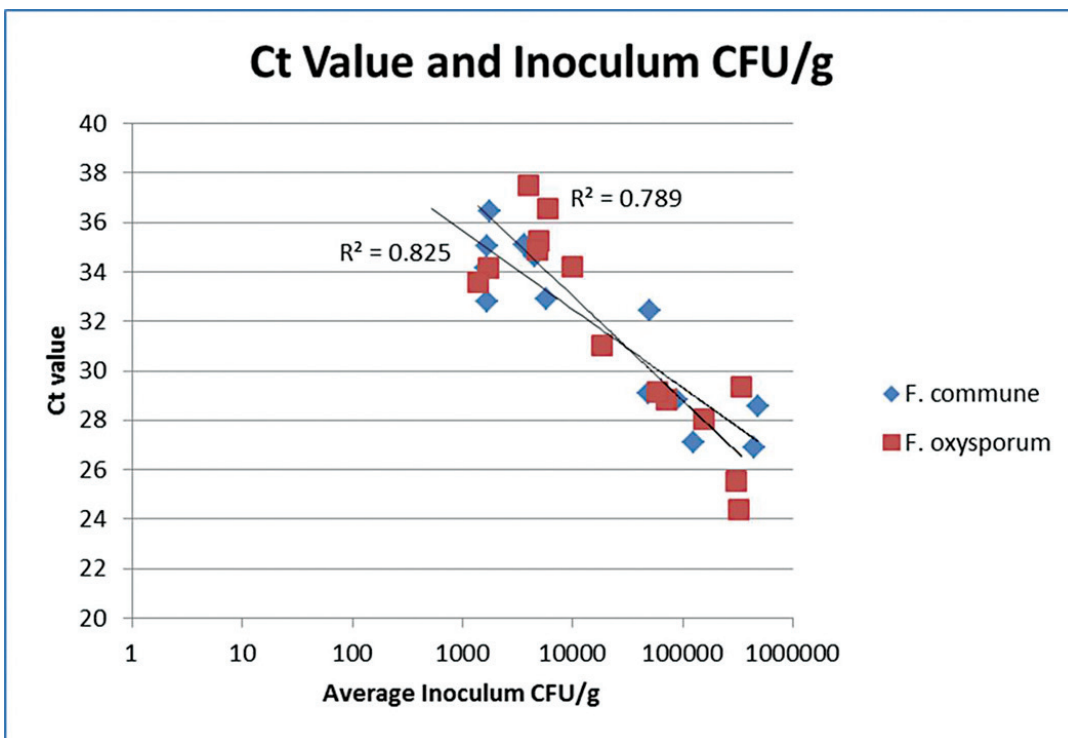


Figure 3. Correlation between observed qPCR Ct value and average inoculum concentration (CFU/g) of soil inoculated with *F. commune* and *F. oxysporum*.

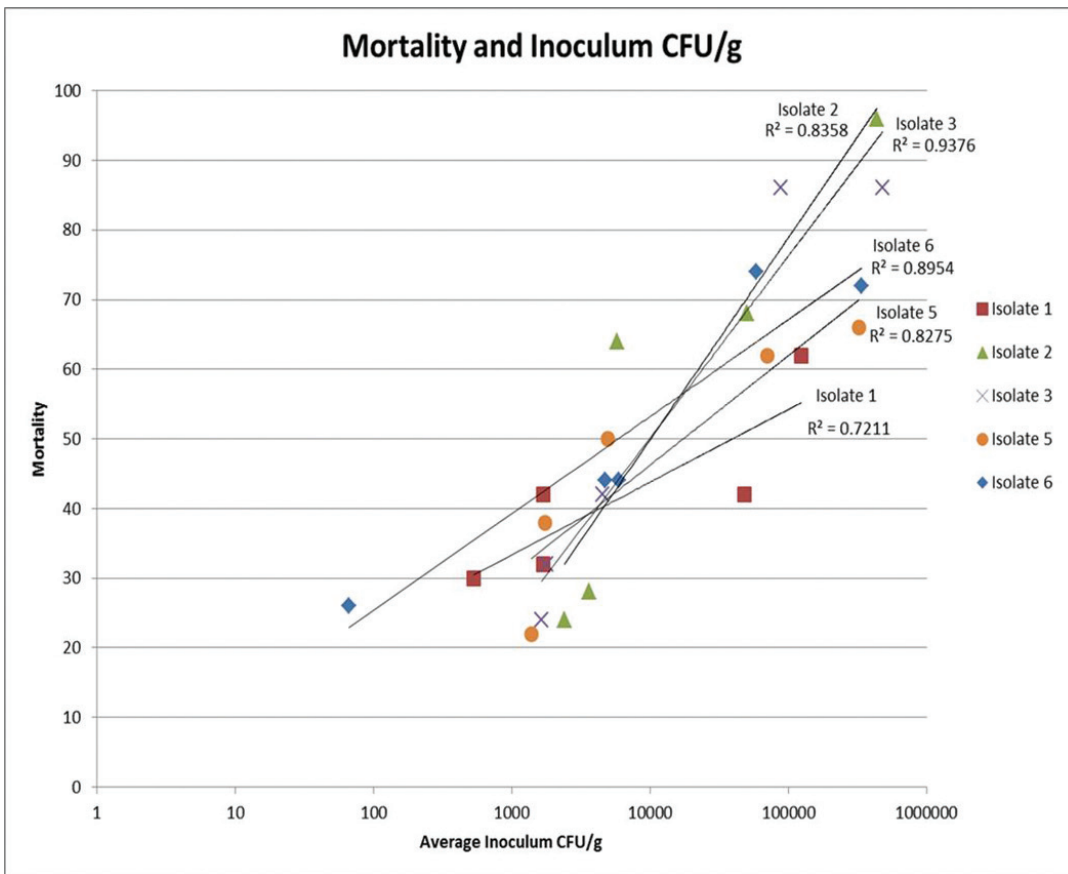


Figure 4. Correlation observed between percent Douglas-fir damping off and average inoculum concentration (CFU/g) of soil inoculated with three isolates of *F. commune* (Isolate 1-3) and two isolates of *F. oxysporum* (isolates 5-6).

A qPCR assay that is able to differentiate between *F. oxysporum* and *F. commune* will give growers a new method to test soil for pathogenic properties prior to fumigation or planting. Molecular identification allows the quantification of *Fusaria* in forest nursery soils, just as other pathogens have been quantified in other cropping systems. For example, researchers have recently developed a qPCR assay for the quantification of *C. destructans* f. sp. *panacis* in ginseng fields (Kernaghan and others 2007). *C. destructans* is also a Douglas-fir pathogen often found in nurseries infected with *F. commune*. Testing an assay for *Cylindrocarpon* on forest nursery soils and multiplexing it with the *F. commune* assay may provide a more thorough disease assay. Future research may also move into more advanced technologies such as next generation sequencing. Pyrosequencing allows for the testing of all soil microorganisms in a single assay. Detection of *Phytophthora* species in Italian chestnut forest soil sites using a pyrosequencing assay was more sensitive than traditional baiting (Vannini and others 2013). A similar technique may be able to determine different species of *Fusarium* and other bacterial and fungal species present in forest nursery soil. This technology is primarily used to provide relative rather than quantitative information, but can provide a helpful suite of information when making management decisions.

Summary

Preliminary data from this study suggest that the qPCR assay will be a valuable tool for quantifying *F. commune* independent from *F. oxysporum*. Additionally, this work will help establish targeted soil disease levels for fungicide and fumigant treatment. It will provide growers with an additional tool when making soil treatment decisions, potentially saving money and reducing the nursery's environmental impact.

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