AN ABSTRACT OF THE THESIS OF

Michelle L. Soule for the degree of Master of Science in Botany and Plant Pathology presented on June 2, 2022.

Title: Development of Quantitative PCR Assays to Aid in Root-knot Nematode (*Meloidogyne* spp.) Diagnostics and Resistance Breeding Efforts in the Pacific Northwest

Abstract approved: _____

Inga A. Zasada

In the Pacific Northwest (PNW), the two most common root-knot nematodes are *Meloidogyne hapla* and *Meloidogyne chitwoodi*. These nematodes can infect a wide variety of crops and can cause significant losses. Currently, it is common for the field of nematology to use labor-intensive microscopy to identify plant-parasitic nematodes based on morphology. There is a need for molecular techniques such as quantitative PCR (qPCR) for faster, more reliable *Meloidogyne* spp. identification and quantification. Still, there is difficulty with designing molecular tools for the *Meloidogyne* spp. present in the PNW due to the limited amount of sequence data for PNW populations in the NCBI Genbank repository. The gene *Hsp90* was chosen as the assay target because it is conserved among most living organisms but has highly variable functionality, conferring enough sequence variation for primer and probe design. One set of primers with two species-specific probes were designed for use in

hydrolysis probe singlepex or multiplex qPCR to detect and quantify *M. chitwoodi* and *M. hapla* in the PNW.

Chapter 2 of this thesis describes the development of the *Hsp90* multiplex qPCR for *M. hapla* and *M. chitwoodi* and its use in field samples from a diagnostic laboratory to compare morphological and molecular diagnostics. This molecular assay is not able to distinguish between *M. fallax* and *M. chitwoodi*, but *M. fallax* is not found in the PNW. No cross reaction was observed among plant-parasitic nematodes commonly found in the PNW and the assay was able to detect populations of *M. hapla* and *M. chitwoodi* from areas of North America outside of the PNW. High DNA concentrations of *M. hapla* or *M. chitwoodi* affected the proficiency with which the assay could detect low DNA concentrations of the opposite target nematode in the sample. The reliability of testing 1 *M. hapla* or 1 *M. chitwoodi* in a sample was 50% and 80%, respectively. A test of three soils from the PNW did not indicate that soil type had an effect on Ct value. In the comparison between morphological and molecular field samples obtained from a diagnostic laboratory, the standard curve was unreliable. In determining presence or absence of *M. hapla* and *M. chitwoodi*, the multiplex qPCR and morphological diagnostics were in agreement 68% of the time. A subset of 25 samples where morphological and molecular diagnostics disagreed on the presence of *M. chitwoodi* were run in singleplex with the probe specific to *M. chitwoodi* and 17 of those 25 samples replicated the result of the multiplex assay.

In Chapter 3, a high-throughput screening method termed “the canister assay” was optimized for evaluating the reproduction of *M. chitwoodi* on potato. In the canister assay, soil is added to the canister and planted with potato. The canister is

then inoculated with *M. chitwoodi* and incubated at a constant temperature for the duration of the experiment. The canisters are then harvested and eggs are extracted and enumerated to calculate reproduction factor (RF = final population density/initial population density). Among the factors investigated, inoculating at time of planting and an incubation period of at least 6 weeks caused the greatest increase in RF values. The canister assay was also applied to a potato breeding population, for which enumeration of extracted eggs at the end of the experiment by microscopy was compared to the *Hsp90 M. chitwoodi* singleplex qPCR. There was no significant difference in calculated RF values between molecular and microscope enumeration. However, when samples with a low nematode density are considered (less than 200 eggs) there is a significant difference in egg density estimations, with the qPCR producing much more sensitive results.

Broadly, the research in this thesis aims to contribute to the high-throughput methods that will advance nematology research and diagnostic efforts through molecular biology techniques.

Development of Quantitative PCR Assays to Aid in Root-knot Nematode (*Meloidogyne* spp.) Diagnostics and Resistance Breeding Efforts in the Pacific Northwest

by

Michelle L. Soulé

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 2, 2022
Commencement June 2023

Master of Science thesis of Michelle L. Soulé presented on June 2, 2022

APPROVED:

Major Professor, representing Botany and Plant Pathology

Head of the Department of Botany and Plant Pathology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Michelle L. Soulé, Author

ACKNOWLEDGMENTS

First, I would like to thank my advisor Inga Zasada for believing in me enough to take me on as a graduate student and for all of her help in getting over the finish line. Inga has been an excellent guide and mentor throughout an exceptionally difficult time to be in graduate school. Over the course of the past two and a half years she has shown me the importance of collaboration and how to always look for expertise in others in order to make a research endeavor the best it can be. This is a philosophy I will certainly take with me.

I also want to thank each of my committee members: Cynthia Gleason, Christina Hagerty, and Marcello Moretti. You all have been incredibly encouraging and supportive especially when I was starting to reach the home stretch.

I owe a huge thanks to Sam Chavoshi at AGNEMA for taking time to teach me about nematode diagnostics and for procuring field samples for my research.

Thank you, Amy Peetz, for teaching so much about molecular biology and being an awesome role model. Her practicality and emotional intelligence were instrumental in helping decide my path forward in research and life.

Thank you as well to the rest of the Zasada Lab: Megan Kitner, Lester Nuñez, Gabby Studebaker, and our former lab members: Cedar Hesse, Olivia Filaluna, Lauri Reinhold, and especially Catie Wram. Catie taught me how to be a graduate student, how to navigate the department, and has become an amazing friend. I am so lucky to know her.

I am so grateful to have met many wonderful people in the department of Botany and Plant Pathology. Thank you to the BPP office staff: Dianne Simpson, Kimberly Callahan, and April Zeller for all of your help in navigating departmental procedures and university resources. I also would like to that our department head, Joey Spatafora, for his utmost support of the graduate students.

I owe so much gratitude to everyone at the USDA-ARS Hort-Crops lab. Thank you to the Bob Martin and Karen Keller of the former Martin Lab for getting me started in my first government job! Thank you, Melinda Guzman, Pat Di Bello, Cesar Zamora, and Amanda Lake for becoming some of my first friends in Corvallis. I also want to recognize all of the former and current hort-crops grad students that I get to share an office with -thanks for the chats and the laughs.

I also need to thank my new boss, Renée Eriksen, at the USDA-ARS Crop Improvement and Protection Research Unit for being patient, kind, and supportive while I finish my degree.

I would not have made it through graduate school without some wonderful friends. Thank you to my roommate, Daniella Echeverria for all the laughs and the feels; to Kate Baldino for her never-ending goofy encouragement and positivity, to Nick Carleson and Raven Waldron for being amazing friends and never failing to make me laugh. A shout out to my Sonoma County friends for all the love and encouragement from afar: Margaret Starr, Rodrigo Castillo, Cristin Seppa, Jordan Bergero, Lisa Churchman, Ivy Burge, and Garrett Shipway.

I have to acknowledge and appreciate my parents, Matt and Linda Soulé and my sister, Claire Soulé. They have provided an immense amount of love,

encouragement, and support throughout my academic journey. Additionally, I want to thank Claudia Cleaver and Jeff Sacher for treating me like their own family.

Finally, I can't say enough about my fiancé, Gabe Sacher. The past five and a half years since I met Gabe have been some of the most joyful, most challenging, and most rewarding of my life. I never would have relocated to Oregon if he hadn't decided to go to graduate school at OSU. In moving to Oregon together we have both experienced a tremendous amount of growth. I have gained so many more opportunities and my life is on a different trajectory because of him. I certainly wouldn't be here, finishing a master's degree without his relentless help, encouragement, and support.

CONTRIBUTION OF AUTHORS

Inga Zasada assisted with the study, design, and writing of Chapters 2 and 3.

Amy Black Peetz, Cynthia Gleason, and Sam Chovashi assisted with the study and design of Chapter 2.

Megan Kitner assisted with the study and design of Chapter 3.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

Table Page

DEDICATION

To my fiancé: Gabriel Sacher and my parents: Matthew and Linda Soulé.

Gabe – without you, I wouldn't be here.

Mom and Dad – without you, I literally wouldn't be here.

Thank you all for your unwavering love and support.

Development of quantitative PCR assays to aid in root-knot nematode (*Meloidogyne* spp.) diagnostics and resistance breeding efforts in the Pacific Northwest

Chapter 1 – General Introduction

Root-knot nematodes (*Meloidogyne* spp.) are distributed world-wide and are considered to be one of the most destructive agricultural pests, with global economic losses in crops estimated up to more than $100 billion US dollars (Braun-Kiewnick & Kiewnick, 2018; Elling, 2013). The Pacific Northwest (PNW) is no exception to this destruction. A number of economically important crops in the PNW are highly susceptible hosts to *Meloidogyne hapla* and *Meloidogyne chitwoodi* – the regions two dominant root-knot nematode species (Zasada et al., 2019).

Root-knot nematodes are sedentary endoparasites that hatch from eggs into free-living infectious second stage juveniles (J2) before penetrating a root or tuber cell and establishing a feeding site. The nematode uses a stylet to release effector molecules which reprogram the cell causing it to deliver nutrients to the nematode where it will stay through the rest of its life cycle. This process diverts important nutrients, inhibits water uptake, and leads to yield loss in the plant. Additionally, the feeding site creates an enlarged cell which can be seen as a gall on the root or tuber surface. When the nematode reaches maturity, the female will release eggs in a sticky mass on the outside of the cell (Caillaud et al., 2008).

In the PNW, *M. chitwoodi* was originally thought to be the same species as *M. hapla* and as a result many aggressive nematode infestations were attributed to *M. hapla*

until the new species, Columbia root-knot nematode (*M. chitwoodi*), was designated in the 1970s (Golden et al., 1980). The morphological differences between *M. hapla* and *M. chitwoodi* are outlined in Nyczepir et al. (1982) with the most reliable morphological characteristic for distinguishing between the two species in a diagnostic setting being the tails (Fig.1A and B). The tail of *M. chitwoodi* is "short and blunt with the hyaline tail terminal showing little or no taper to its rounded terminus" (Golden et al., 1980) (Fig. 1A). In contrast, the tail of *M. hapla* is more tapered with a pointed hyaline tail extending into the tail end (Nyczepir et al., 1982) (Fig. 1B). There is a lot of plasticity in the morphology of *M. hapla* and *M. chitwoodi* tails, requiring a diagnostician with many years of experience to distinguish subtle differences between the two species.

To help control and manage plant-parasitic nematode infestations, quick and accurate identification is essential (Braun-Kiewnick and Kiewnick, 2018). Currently, the field of nematology overwhelmingly uses microscopy to identify and quantify nematodes based on morphology. Microscopy is time consuming and requires long hours to be spent sitting at a microscope to process many samples – an ergonomic nightmare for the diagnosticians, technicians, and researchers who do the work. There is a crucial need for molecular techniques such as quantitative PCR (qPCR) to be developed for faster, more reliable identification and quantification of plant-parasitic nematodes (Braun-Kiewnick and Kiewnick, 2018).

Plant-parasitic nematodes have been traditionally managed with highly toxic, broad-spectrum nematicides. This normally includes the costly practice of deploying pre-plant fumigants prior to the season and using several applications of nematicides during the season (Duncan, 1991). Over time these products are being banned due to

environmental and human health risks (Desaeger et al., 2020) and as a result, other tactics for reducing nematode density are necessary. One tactic is to use newly developed nematicides which have a more targeted mode-of-action, though it has been reported that these new chemistries can have differing efficacies even among nematodes in the same genus (Wram & Zasada, 2020). Another option is using a crop rotation; however, employing an effective rotation is difficult due to the broad host range of root-knot nematodes (Ryss, 2014). A third choice is to plant crop cultivars with resistance or immunity; however, resistance can be specific to nematode species or even races within a species (Bali et al., 2021; Brown et al., 1999, 2014; Kaloshian et al., 1989). Each of these options for managing plant-parasitic nematodes without traditional nematicides require management decisions to be highly specific. To achieve this level of diagnostic accuracy, molecular biology techniques must be deployed.

A number of biochemical tools have been applied to nematode identification including enzyme linked immunosorbent assays (ELISA) (Kapur-Ghai et al., 2014) and isozyme analysis (Kolombia et al., 2017), as well as popular molecular biological tools such as conventional polymerase chain reaction (PCR) (Castagnone-Sereno et al., 1995; Huang et al., 2019; Powers et al., 2018), restriction fragment length polymorphism (PCR-RFLP) (Smith et al., 2015), loop-mediated isothermal amplification (Leal et al., 2015; Niu et al., 2011), and quantitative PCR (qPCR) (Arora et al., 2020; Braun-Kiewnick & Kiewnick, 2018; Gorny et al., 2019; Hodson et al., 2021; Sapkota et al., 2016; Seesao et al., 2017; Zijlstra & Van Hoof, 2006).

Of these tools, qPCR is one of the most promising, whereby traditional PCR oligonucleotide primers can be used with a fluorescent oligonucleotide probe to amplify

and quantify a DNA sample. For crop systems with multiple plant-parasitic nematode threats, the ability to detect nematodes in multiplex is essential. Multiplexing in qPCR requires the use of hydrolysis probes (also called Taqman probes). Hydrolysis probes use a fluorophore on the 5' end of the oligonucleotide and a quencher on the 3' end. In multiplex, multiple specific probes are used in conjunction with one or more primer sets. When the template DNA denatures during cycling, the specific probe binds to the complementary region on the template. Then, as the polymerase enzyme polymerizes along the template strand and hits the 5' end of the probe location, a fluorophore is cleaved. When the fluorophore moves out of the vicinity of the 3' quencher, it fluoresces. The fluorescence can then be captured by the thermal cycling machine. In multiplex, different fluorophores on the specific probes can distinguish specific targets simultaneously. Quantity estimates can then be made based on the exponential relationship between starting concentration of DNA and cycle threshold (Ct) values Ct value is defined as the cycle at which amplification enters the exponential phase and entrance into this phase represents a positive detection (Edwards et al., 2004; Raymaekers et al., 2009).

Quantitative PCR assays have already been developed for a number of plant-parasitic nematodes (Zilstra and Van Hoof, 2006; Sapkota et al. 2016; Gorny et al., 2019, Arora et al., 2020; Hodson et al., 2021). This type of assay is ideal for the high-throughput diagnostic setting as it is relatively cheap and only requires basic molecular biology lab techniques to execute. This assay is suitable for entry-level lab technicians to conduct, making it easier for new technicians to enter the field of nematode diagnostics. Additionally, high-throughput capacity for qPCR is already established. The 96-well

PCR-plate format is standard for most qPCR thermal cyclers (Applied Biosystems Step-One Plus, Bio-Rad CFX Opus - 96) with some cyclers using up to 384-well plates (Thermo Fisher Scientific QuantStudio 5, Exicycler 384, Bio-Rad CFX Opus - 384). The reaction requires just a few minutes of set up and approximately 2 hours of thermal cycling. This means that hundreds of samples could be processed per day.

However, qPCR still has a primary challenge to overcome. For one, a lack of genomic data in the field of nematology makes primer and probe design exceptionally difficult. Nematoda is one of the most diverse phyla on earth with over 80,000 currently described species distributed among every continent (Braun-Kiewnick & Kiewnick, 2018; Wang et al., 1999). However, as of August 2020, only 217 nematode genomes were publicly available. For comparison, of soil-dwelling organisms there are currently 36,464 bacterial genomes and 2,586 fungal genomes (Wram, personal communication). This limits design of specific primers and probes to regions of the genome that are commonly studied. Additionally, available data may not represent all of the diversity needed to develop a specific and robust molecular test.

To meet the goal of expanding molecular tools in the field, this body of work aimed to demonstrate ease and expeditiousness of molecular tools in applied nematology in both diagnostics settings and for use in research settings.

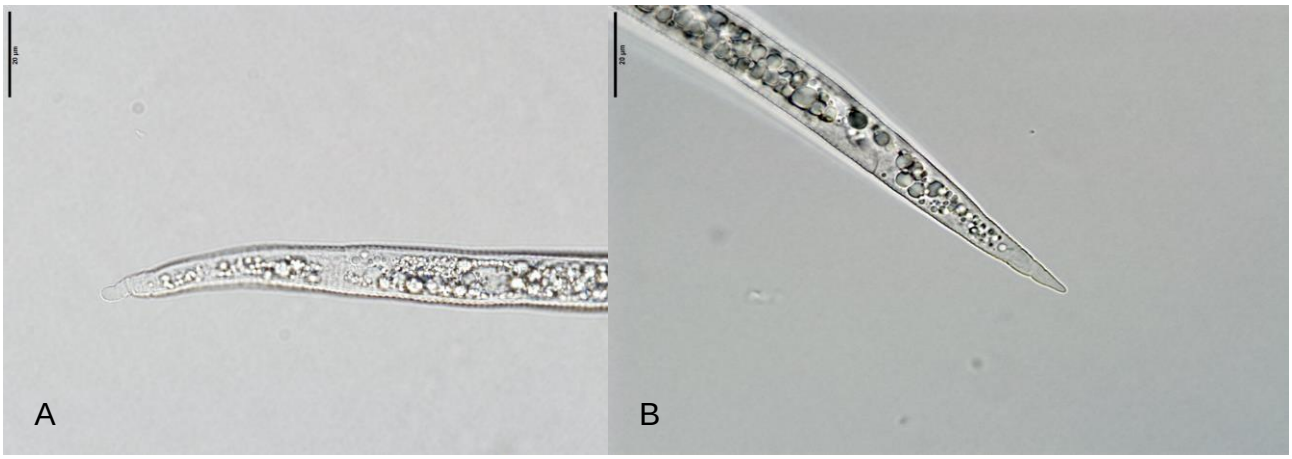

Figure 1.1 The tails of *Meloidogyne chitwoodi* and *Meloidogyne hapla*. A, *M. chitwoodi* and **B,** *M. hapla* (photos by Inga Zasada)

Chapter 2 – Development of a multiplex quantitative PCR assay for identification and quantification of root-knot nematode (*Meloidogyne*) species in the Pacific Northwest

Michelle L. Soulé, Amy B. Peetz, Sam Chovashi, Cynthia Gleason, and Inga A. Zasada

2.1 Abstract

Meloidogyne hapla and *Meloidogyne chitwoodi* are the dominant root-knot nematode species in the Pacific Northwest (PNW) (Zasada et al., 2019). The level of concern for damage to a crop and pest management decisions depend on both the crop and the species present, *M. hapla* or *M. chitwoodi*. For information on nematode species and densities, growers submit samples to nematode diagnostic clinics. These diagnostic clinics currently rely on morphology of the microscopic animals. Few people have this expertise in plant-parasitic nematode identification and the labor of many hours on a microscope is difficult. Molecular diagnostic tests are a preferable replacement to morphological diagnostics but a lack of genomic resources, geographically different genotypes, and dealing with soil inhibitors are barriers for molecular tools to be developed and implemented for nematode diagnostics. In this chapter, the development of a multiplex qPCR targeting the gene *Hsp90* in *M. hapla* and *M. chitwoodi* is reported and the assay compared to morphological diagnostics of field samples. This assay cannot distinguish between *M. fallax* and *M. chitwoodi*, but *M. fallax* is not found in the PNW. No cross reaction was observed among common plant-parasitic nematodes in the PNW and the assay was able to detect populations of *M. hapla* and *M. chitwoodi* from areas of North America outside of the PNW. High DNA concentrations of *M. hapla* or *M. chitwoodi* effected the proficiency with which the assay could detect low DNA concentrations of the alternate target *Meloidogyne* in the sample. The reliability of testing 1 *M. hapla* or 1 *M. chitwoodi* in a sample was 50% and 80% respectively. A test of three soils in the PNW with the multiplex qPCR did not indicate that soil type had an effect on Ct value. In the comparison between morphological and molecular diagnostics of fields samples

obtained from a diagnostic lab, the standard curve was unreliable so data were assigned to 1 of 4 bins based on Ct value or microscopy count. When the data was binned into no detection, low, medium and high designations, morphological and molecular diagnostics bin assignments were significantly different for *M. chitwoodi* ($P < 0.0001$) but not for *M. hapla* ($P = 0.0687$). In determining presence or absence of *Meloidogyne* spp. the multiplex qPCR and morphological diagnostics were in agreement 68% of the time. A subset of 25 samples where morphological and molecular diagnostics disagreed on the presence of *M. chitwoodi* were run in singleplex with the probe specific to *M. chitwoodi* and 17 of those 25 samples replicated the result of the multiplex qPCR assay. This research demonstrated use for multiplex qPCR in nematode diagnostics, however, use was limited for accurate quantification. A singleplex qPCR assay can be implemented by omitting one of the probes. The assay in singleplex qPCR was more sensitive than the multiplex qPCR when both *M. hapla* and *M. chitwoodi* were present in a sample.

2.2 Introduction

For crop systems with multiple plant-parasitic nematode threats, the ability to detect nematodes simultaneously is essential. In the Pacific Northwest (PNW), *M. chitwoodi* (Columbia root-knot nematode) and *M. hapla* (Northern root-knot nematode) are the most commonly found root-knot nematode species (Zasada et al., 2019). Even when initial densities are low, infestations of *M. chitwoodi* are particularly harmful in the PNW. This is due to the nematode's lower temperature threshold for development giving them the ability to develop early and complete many generations during the long, warm

growing season (Pinkerton et al., 1991). The population explosion over one season allows this nematode to cause extensive damage to tubers and roots (Santo & O'Bannon, 1981). The action threshold for *M. chitwoodi* is lower than that of *M. hapla* (Santo & O'bannon, 1981). Due to this difference, it is critical for diagnostic laboratories to be able to accurately differentiate between these two *Meloidogyne* species. To a trained morphological expert, this may be an easy endeavor, but distinguishing between *M. chitwoodi* and *M. hapla* can be difficult, time consuming, and fallible (Fig1.1).

Regions like the internal transcribed spacer region (ITS) 1 and 2 are most common for genetic distinction across biological fields including in bacteria (Man et al., 2010), plants (Poczai & Hyvönen, 2010), and fungi (Schoch et al., 2012). However, variable copy number throughout the life-cycle of *Meloidogyne* spp. make it less than ideal for precise molecular quantification (Lopes et al., 2019). Another popular region for nematode identification is the cytochrome oxidase c subunit 1 (COI) region for DNA barcoding (Powers et al., 2018), but this region also comes with difficulties including low GC-content (< 28%). Low GC-content is acceptable for DNA barcoding, but a GC content of at least 30 to 60% is necessary for high primer and probe binding specificity in qPCR (Raymaekers et al., 2009). A third popular genomic region for nematode characterization and phylogenetics is heat shock protein 90 (*Hsp90*) (Braun-Kiewnick & Kiewnick, 2018; Skantar & Carta, 2004). This region is highly conserved among all species, but diverse enough for a wide range of functional variability. For example, the human *Hsp90* shares over 70% identity with Hsp90 found in the nematode *Brugia* spp. and among nematodes percent shared identity is much higher (Gillan & Devaney, 2014). This is ideal for genetics-based molecular identification like multiplex qPCR in which

designing one set of forward and reverse primers and two species-specific probes is more efficient.

Diagnostic laboratories for plant-parasitic nematode identification and quantification have already been established in many agricultural hubs. Over the 5-year period from 2012 to 2016, diagnostic laboratories in the PNW have seen a significant increase in requests for diagnostic testing (Zasada et al., 2019), suggesting an increased need for high volume sample processing. Morphological diagnostics are not suited for high-throughput environment as it requires expert knowledge of nematode taxonomy (meaning few people are able to make a diagnosis) (Braun-Kiewnick & Kiewnick, 2018; Seesao et al., 2017).

Preparing plant-parasitic nematodes for high-throughput molecular diagnostics poses a difficult hurdle. Normally, while as much as 500 grams of soil sample will be submitted for diagnostics, a soil DNA extraction kit will call for just 250 µg to 10 g of the submitted soil (DNeasy PowerSoil Pro Kit Handbook https://www.qiagen.com/us/resources/). Diagnostic soil samples contain such a small portion of the soil from an agricultural field and the stakes are high for capturing low nematode population densities early enough for management strategies to be implemented. Therefore, the aim of this chapter is to determine if molecular diagnostics can accurately detect and quantify *M. chitwoodi* and *M. hapla* in soil samples. The objectives of this chapter were to 1) develop a multiplex hydrolysis probe qPCR assay to detect *M. hapla* and *M. chitwoodi*, 2) validate specificity of assay to *Meloidogyne* spp., 3) determine efficacy of assay for *Meloidogyne* spp. across soil types, and 4) compare qPCR

diagnostic assay results to those obtained from traditional morphological diagnostics on samples from agricultural fields in the PNW.

2.3 Materials and Methods

2.3.1 Primer design

Hsp90 sequences were retrieved from GenBank (National Center for Biotechnology Information, Bethesda, MD, U.S.A) for *M. chitwoodi* and *M. hapla* (accessions AY528416 and KC262224, respectively). Alignments were made using the MAFFT (Katoh, 2002) alignment in Geneious Prime 2021.2.2 (https://www.geneious.com). One pair of primers was developed with specificity for both species (Table 2.1) with amplicon length of 150 base pairs.

2.3.2 Validation

2.3.2.1 Conventional PCR

PCR primers (Sigma-Aldrich, Inc. St. Louis, MO, U.S.A) were tested in a 25 µl reaction in conventional PCR with 12.5 µl MGB H_2O, 9.5 µl AccuStart II PCR ToughMix (Quantabio, Beverly, MA, U.S.A), 5 µM each forward and reverse primers, and 2 µl of template from single worm or a 25 nematode lysis using the DNeasy PowerSoil Pro kit (QIAGEN, Hilden, Germany). The PCR product was cleaned up with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermofisher, Waltham, MA, U.S.A.) and sent for sequencing in both the forward and reverse directions at the Center for Quantitative Life Sciences (CQLS) at Oregon State University, Corvallis, OR, U.S.A.

Sequenced amplicons of *M. hapla* and *M. chitwoodi* were verified in BLAST (National Center for Biotechnology Information, Bethesda, MD, U.S.A). The *M. hapla* amplicon returned 98 to 100% coverage for *M. hapla* with E-values < 0.01 and the *M. chitwoodi* amplicon returned 100% coverage for *M. chitwoodi* and *M. fallax* which is not found in the PNW (Nischwitz et al., 2013; Zasada et al., 2019) with E-values < 0.01.

2.3.2.2 Quantitative PCR

TaqMan™ hydrolysis MGB (minor groove binder) probes (Life Technologies Corporation, Carlsbad, CA, U.S.A.) specific to each target *Meloidogyne* species were designed within the target amplicons. Due to 100% identity between *M. fallax* and *M. chitwoodi* in this region of *Hsp90*, these nematodes cannot be distinguished from each other using this assay. However, *M. fallax* is not known to be in the PNW (Nischwitz et al., 2013; Zasada et al., 2019). The *M. chitwoodi* and *M. hapla* probes were labeled with fluorophores FAM and VIC (Life Technologies Corporation), respectively (Table 2.1).

2.3.2.3 General qPCR methodology

DNA was extracted using the DNeasy PowerSoil Pro kit according to manufacturer instruction with one modification: after bead beating samples were held at 4 °C for 24 hours before completing the rest of the manufacturer protocol to assist in the breakdown of the nematode cuticle. Extracted DNA was stored at -20 °C.

Reactions for qPCR were prepared in 20 µl volumes consisting of 10 µl 2X TaqMan™ Universal Master Mix II, no UNG (Life Technologies Corporation), 2 µl H_2O, 500 nm forward and reverse primers, 250 nM of each FAM and VIC probe, and 2 µl of

template. Each sample was prepared in triplicate including no-template controls and positive controls in the form of the standard curve. The cycling conditions were 95° C for 10 min followed by 50 cycles at 95° C for 15 sec and 60° C for 1 min using the Applied Biosystems StepOne Plus (Applied Biosystems, Waltham, MA, U.S.A.). All multiplex qPCRs were performed with white 96 well TempPlate no-skirt PCR plates (USA Scientific, Ocala, FL, U.S.A.) with MicroAmp™ optical adhesive film (Applied Biosystems).

To prepare a standard curve, 625 *M. chitwoodi* and *M. hapla* second-stage juveniles (J2) in water were handpicked directly into the PowerSoil Pro bead-beating tubes (QIAGEN), with four tubes prepared for each *Meloidogyne* species. Eluted DNA was then combined for each species and a 1:5 dilution was performed. Standards at each dilution level representing 625, 125, 25, 5, and 1 *Meloidogyne* sp. J2 were aliquoted into PCR strips for individual use and stored at -20 °C. Each plate included a standard curve for each species.

All Ct values, sample estimates, and statistics are reported based on the mean Ct of each replicate. Means and standard error (mean ± SE) are reported where appropriate.

2.3.2.4 qPCR characteristics

The qPCR efficiency, slope, R^2, and limit of detection were obtained by making a six point 1:5 serial dilution starting at 2 ng/µl for each species. The limit of detection is defined as the lowest concentration of DNA that can be detected 95% of the time (Bustin et al., 2009)

2.3.2.5 Nematode species and population specificity of assay

Five populations of *M. hapla* from Oregon, Washington and New York and four populations of *M. chitwoodi* from Oregon, Washington, California, and Colorado were obtained to test whether the assay effectively detects and quantifies *Meloidogyne* spp. populations from different regions in the United States. The assay was then evaluated for off target cross reaction among other species of plant-parasitic nematodes, including: *Meloidogyne naasi*, *Meloidogyne incognita*, *Pratylenchus neglectus*, *Pratylenchus thornei*, *Paratrichodorous allius*, and *Heliocotylenchus*. The number of plant-parasitic nematodes from which DNA was extracted ranged from 30 to 100 individuals and DNA extraction success was confirmed by spectrophotometer (NanoPhotometer® N60, Implen GmbH, München, Germany) with yields ranging from 0.6 to 6.7 ng/µl.

2.3.3 qPCR Experimental Validation

2.3.3.1 Effect of DNA concentration on detection of the opposing Meloidogyne *species target*

Seven samples of varying ratios of *M. hapla*:*M. chitwoodi* DNA were prepared in the following fashion - 2 ng/µl : 2 ng/µl, 2 ng/µl : 0.4 ng/µl, 2 ng/µl : 0.08 ng/µl, 2 ng/µl : 0.016 ng/µl, 0.4 ng/µl : 2 ng/µl, 0.08 ng/µl : 2 ng/µl, and 0.016 ng/µl : 2 ng/µl. These concentrations are well above the limit of detection when included in the system as the only *Meloidogyne* species. Samples were evaluated to determine at which ratios the lower density nematode could no longer be detected in multiplex qPCR.

2.3.3.2 Reliability of detecting a single Meloidogyne spp. second-stage juvenile in multiplex qPCR

A single *M. chitwoodi* or *M. hapla* J2 was handpicked using a Pasteur pipette into 10 PowerSoil Pro bead beating tubes. DNA was extracted from each prep as described above and samples were tested in triplicate with the qPCR assay with the parameters described above. Samples were evaluated for likelihood of detecting a single *Meloidogyne* sp. J2 in multiplex qPCR.

2.3.3.3 Impact of soil type and nematode community on Meloidogyne spp. identification and quantification with qPCR

The qPCR assay was tested in three soil types from the PNW, as described in Table 2.2, to determine whether accuracy and specificity was maintained when soil inhibitors and diverse nematode communities were present. For each soil, nematodes were extracted from four 50 g soil samples by decant sieving/sugar centrifugation (Barker and Carter 1985). Nematodes were identified to family or genus level using an inverted microscope (Leica DM IL; Leica Microsystems, Wetzlar, Germany) for each soil type. The nematode communities were then spiked with 0, 5, 25, or 250 *M. chitwoodi* J2 in trial 1 or both *M. chitwoodi* and *M. hapla* J2 in trial 2. Samples then concentrated into PowerSoil bead beating tubes. Each experiment included a tap water control at each nematode concentration. DNA was extracted as described above and samples were tested in triplicate with multiplex qPCR with the parameters described above. Resulting Ct values were analyzed by one-way ANOVA using R Statistical Software (v4.2.0; R Core Team 2022).

2.3.3.4 Comparison of morphological to qPCR diagnostics for Meloidogyne spp. identification and quantification

Samples (n = 94) from agricultural fields in the Columbia Basin of Oregon and Washington were provided by the nematode diagnostic laboratory AGNEMA, LLC (Pasco, WA, U.S.A.). Nematodes were extracted from samples by modified decant sieving followed by sugar centrifugation (Barker and Carter 1985). Extracted nematodes were concentrated in a 50 ml falcon tube in 20 ml tap water and stored at 4^{o} C until counted. Prior to nematode identification, each sample was vortexed then plant-parasitic nematode taxa were identified and enumerated using morphological diagnostics using a compound microscope (Leica). When present, *Meloidogyne* spp. were identified to species and enumerated.

Samples were returned to the falcon tube and stored at 20^{o} C until DNA was extracted and samples were processed using the Fisherbrand™ Bead Mill 24 Homogenizer (Thermo Fisher Scientific) to shake the bead beating tubes (Qiagen) for four 30 second shaking cycles with 10 second pauses. Downstream extraction methods and qPCR cycling parameters were followed as described in 2.3.2.3 General qPCR Methodology.

Estimated densities of *M. chitwoodi* and *M. hapla* by molecular diagnostics were compared to estimated densities of *M. chitwoodi* and *M. hapla* by morphological diagnostics. Because the relationship between Ct value and concentration of DNA is exponential, morphological count was log transformed to linearize the relationship. This data contained zeros so 0.1 was added to each count prior to log transformation. The

results of each quantification method were assigned to bins by splitting the estimates for each species into 4 bins based on Ct value or ln(count), respectively: 0 for none detected, 1 for the lowest third of densities, 2 for the middle third of densities, and 3 for the highest densities. Bins for each method were compared to each other and analyzed by Chi-square test for independence using R Statistical Software (v4.2.0; R Core Team 2022) and the package R Companion (v2.4.15; Mangiafico, 2022).

A subset of 25 samples where microscope detection and molecular detection were in disagreement were tested in a singleplex qPCR using the FAM probe for *M. chitwoodi*. The singleplex samples were prepared and cycled under the same conditions as the multiplex assay. The singleplex results were evaluated for presence or none detected for *M. chitwoodi* and whether that result agreed with the multiplex qPCR detection or the microscope detection.

2.4 Results

2.4.1 qPCR characteristics

Amplification of a six-point 1:5 dilution of a 2 ng/µl DNA extract demonstrated the assay detection limit was 1.2 pg of DNA for both *Meloidogyne* species. The efficiency for the assay for *M. chitwoodi* (FAM) was 86.3%, with equation $y=27-3.7x$ and $R^2 = 0.975$ and for *M. hapla* (VIC) was 93.8%, with equation $y=29-3.5x$ and $R^2 = 0.961$ (Fig. 2.1).

2.4.2 Assay specificity

Both races of *M. chitwoodi* from the PNW as well as a *M. chitwoodi* population from Colorado amplified as expected and resulted in an average Ct FAM of 33.70 ± 1.09

(mean ± SE) across populations and no amplification with VIC (data not shown). Four PNW populations of *M. hapla* as well as a population from New York amplified with an average Ct VIC of 32.67 ± 0.70 (mean ± SE) across populations and no amplification for FAM (data not shown). No cross reaction was detected among other *Meloidogyne* species or other common plant-parasitic nematodes of the PNW including *M. naasi*, *M. incognita*, *P. neglectus*, *P. thornei*, *P. allius*, and *Heliocotylenchus* (data not shown).

2.4.3 Effect of DNA concentration from opposing Meloidogyne sp. target on detection

When both target *Meloidogyne* spp. were present in the same sample, the target present at lower concentrations had reduced sensitivity of detection compared to the higher concentration target even when that sample was within the limit of detection (Fig. 3a and 3b). The assay failed to detect *M. hapla* when *M. chitwoodi* DNA was present at 25 times the amount of *M. hapla* DNA and similarly failed to detect *M. chitwoodi* when *M. hapla* DNA was present at 125 times *M. chitwoodi* DNA. When DNA for both *Meloidogyne* spp. was included at the same concentration, the mean Ct value for *M. chitwoodi* was lower than the mean Ct value for *M. hapla* (26.45 ± 0.22 and 28.32 ± 0.14, respectively).

2.4.4 Reliability of detecting a single Meloidogyne spp. second-stage juvenile in multiplex qPCR.

Five out of 10 single extracted *M. hapla* J2 were detected in at least one out of three technical replicates. Ct values for single *M. hapla* J2 ranged from 43.4 to 48.9 with mean of 46.7 ± 0.48. For single extracted *M. chitwoodi* J2, amplification of at least one out of

three technical replicates was observed in 8 out of 10 samples with Ct values ranging from 41.9 to 48.1 with mean of 45.7 ± 0.44 (data not shown).

2.4.5 Impact of soil type and nematode community on Meloidogyne spp. identification and quantification with qPCR.

There was no significant effect of soil type on Ct values ($P = 0.21$, one-way ANOVA; Table 2.2). There was consistent amplification of *M. hapla* (VIC) across Woodhall soil samples with Ct values ranging from 24.62 to 37.03 with mean 33.95 ± 0.64 when no *M. hapla* were added. Similarly, amplification with both *M*. hapla (VIC) and *M. chitwoodi* (FAM) occurred with the soil from Prosser when no *Meloidogyne* spp. were added, ranges were 26.54 to 41.10 with mean 34.95 ± 1.10 and 26.86 to 44.123 with mean 34.95 ± 0.94, respectively(Table 2.2).

When Ct values from known *Meloidogyne* spp. densities were compared against a standard curve and an estimate was generated, estimated densities were within an order of magnitude of the actual densities added (0, 5, 25, and 250). There was one exception: the qPCR assay significantly overestimated the density of target *Meloidogyne* sp. at the 250-nematode density in the second trial when both *Meloidogyne* spp. were added. The average estimated quantity ranged from 1,127 to 5,188 *Meloidogyne* sp. J2 (data not shown). However, this over-estimation was consistent across all soil types and the control at the 250-nematode level.

2.4.6 Comparison of morphological diagnostics to qPCR diagnostics for Meloidogyne spp. identification and quantification.

When data was sorted into 4 bins (none detected, lower third, middle third, and upper third), there was strong evidence that morphological diagnostics and molecular diagnostics were significantly different depending on the density of *M. chitwoodi* present ($P < 0.001$) but not for *M. hapla* ($P = 0.069$) (Figs. 4 and 5). Bins assigned to estimates by qPCR matched the bins assigned to estimates by morphology for both *M. hapla* and *M. chitwoodi* in 48% of samples (Fig. 5 and 6). The qPCR assay more often estimated a lower density of the target *Meloidogyne* spp. than estimated a greater density when compared to morphological diagnostics.

Meloidogyne spp. presence/absence detection by multiplex qPCR and morphological methods matched in 67% of samples. Twenty-five samples where morphological diagnostics disagreed with the multiplex qPCR detection of *M. chitwoodi* were selected for singleplex qPCR. In that subset, 68% of samples replicated the multiplex result. Morphological diagnostics reported both *M. hapla* and *M. chitwoodi* in 31 samples; however, of those samples the multiplex only detected both *Meloidogyne* spp. in samples 9095, 973, and 7338. Of the samples where both *Meloidogyne* spp. were reported via morphological diagnostics, 11 that failed to detect *M. chitwoodi* in multiplex were selected for analysis with singleplex qPCR with the *M. chitwoodi* probe. In singleplex, 7 of those samples failed to detect *M. chitwoodi* meaning they were in agreement with the original result of the multiplex qPCR.

The multiplex qPCR did not produce false positives with the FAM probe specific to *M. chitwoodi*. There were 33 samples in which morphological diagnostics did not

report *M. chitwoodi* and the multiplex detected the presence of *M. chitwoodi*. Of those samples, a subset of 5 samples: 1153, 9150, 8885, 9094, and 9096 were selected for singleplex qPCR. The singleplex replicated the result of the multiplex in each of the 5 samples.

2.5 Discussion

The purpose of this study was to develop faster and more reliable nematode diagnostic for the simultaneous identification and quantification of *M.* hapla and *M. chitwoodi*. As broad spectrum nematicides become deregulated due to toxicity to humans and environmental concerns, growers will be required to rely on more specific (and safer) methods for nematode control (Desaeger et al., 2020). This includes using non-host crops, using cultivars of economically important plants that have immunity in infested fields, and using newer chemistry nematicides – which have been shown to have differing efficacy against *Meloidogyne* spp., in infested fields (Allen et al., 1970; Brown et al., 1991, 1999; Graebner et al., 2018; Sato et al., 2019; Wram & Zasada, 2020). To make these important pest management decisions, growers are increasingly relying on nematode diagnostic clinics (Zasada et al., 2019) and need reliable diagnostic results. Many laboratories provide diagnostic results by characterizing nematodes to genus or species by morphology. Often these labs will charge extra for molecular diagnostic data which can usually identify nematodes to species and sometimes race. Still, there are many hurdles to achieving the speed and accuracy with molecular diagnostics compared to traditional microscopic diagnostics.

In this study, we developed a multiplex qPCR to detect *M. hapla* and *M. chitwoodi* – two important root-knot nematodes *Meloidogyne* spp. in PNW agriculture (Zasada et al., 2018). The assay is a robust method for the simultaneous molecular identification and quantification of *M. hapla* and *M. chitwoodi*; however, the assay faces limitations for use with field samples until extraction procedures are improved. The assay can also be deployed in singleplex which is by far much more sensitive than multiplex and may be a preferable diagnostic method especially when testing for the quarantine pest *M. chitwoodi*.

The durability of multiplex qPCR detection assay was tested by evaluating its efficacy against samples from three different soil types and comparing detection levels to classical morphological methods. This experiment exemplified a problem with morphological diagnostics – mistakes can be made. Despite having an expert nematologist identify the nematode community in each soil type, based upon the multiplex qPCR the soil sample from a hop field in Prosser, WA contained both *M. chitwoodi* and *M. hapla* and soil sample from a vineyard in Monroe, OR contained *M. hapla*. The qPCR amplification in these samples was robust and consistent which makes it unlikely that this molecular detection was due to cross-contamination or false positives.

There are well documented barriers to developing multiplex qPCRs for nematode diagnostics including a lack of diverse genomic data for assay design, identifying nematodes in multiplex when the ratio of the two target species is high, DNA extraction efficiency (Zilstra and Van Hoof, 2006; Huang et al., 2019), and (for soil dwelling nematodes) dealing with soil inhibitors which can affect PCR detection and quantification (Huang et al., 2019). In the present study we experienced these same

challenges in the development of a multiplex qPCR to detect and quantification of *M. hapla* and *M. chitwoodi*. First, we had intended to apply the assays which had already been developed for detection of *M. chitwoodi* and *M. hapla*, however these assays were developed outside of the PNW failed to amplify our local populations in conventional PCR and qPCR (Zilstra and Van Hoof, 2006; Sapkota et al., 2016). *Hsp90* was then chosen as the target sequence for this multiplex qPCR (Skantar and Carta, 2004). The resulting multiplex qPCR reproduced same issue of incongruous amplification of the two target *Meloidogyne* spp. when one was present at a higher DNA concentration than the other (Zijlstra and Van Hoof, 2006). As it was, in samples which contained the same amount of DNA for both target species, *M. chitwoodi* nearly always produced a lower Ct. This suggests slight preferential amplification of *M. chitwoodi*. In order to reduce this effect, multiplex qPCRs were performed on white plates instead of clear plates. While this did appear to reduce the amount of background signal and reduce the number of false positive amplifications with the VIC probe, the use of a white plate did not appear to mitigate the preferential fluorescence of the target with a higher starting DNA.

When the assay was applied to field samples from a diagnostic lab, quantification with the standard curve was not accurate. Due to suspected soil inhibitors, many of the samples returned estimates of less than 1 *Meloidogyne* spp. The qPCR produced estimates of less than one *Meloidogyne* spp. J2 and Ct values over 40 that were similar to the results of the reliability experiment. However, the characteristics of the less than one *Meloidogyne* spp. estimate in the field samples differed from that of the reliability experiment. For one, the field samples produced clear, exponential fluorescent amplification in all three replicates – similar to high concentration samples but at Cts that

resembled the reliability experiment. In the reliability experiment, 1 *Meloidogyne* spp. J2 was processed with the extraction protocol to determine the likelihood of detecting that *Meloidogyne* spp. J2 in the qPCR. In the experiment few samples produced fluorescence for all three replicates and fluorescence was often weak with ambiguous baseline starts and ends. For this reason, detection at the 1 *Meloidogyne* spp. level is unreliable – though it should be noted that in the soil type experiment, detection of 5 *Meloidogyne* spp. J2 was easily achieved.

The difference in detection capabilities of the multiplex qPCR between the soil type experiment and diagnostic clinic comparison experiment could be due to PCR inhibitors. Scale and environment where extractions are performed may play a role in how well inhibitors are removed from samples. In the research setting, decant-sieving, a method which washes the nematodes quite extensively, is used and fewer samples are processed. In the diagnostic lab, a modified version of decant-sieving is used to prioritize speed and nematode recovery. This process could be leaving more PCR inhibitors from the soil in samples. Additionally, only three soil types were evaluated in this study and they do no encompass all the soil types that the diagnostic lab encounters. The Qiagen PowerSoil Pro kit is used to remove inhibitors from soil samples, PCR inhibitors are a wide class of molecules with a wide variety of modes-of-action for inhibition (Schrader et al., 2012) and they may not all be inactivated by this kit. Diluting DNA samples prior to PCR can help dilute inhibitors without reducing detection (Schrader et al., 2012). This may be a more viable option than slowing down processing in the diagnostic setting to more thoroughly wash samples during decant-sieving.

When examining the diagnostic lab samples, we assigned the resulting mean Ct values of the multiplex qPCR and counts from morphological diagnostics into four bins. The bins did provide an additional layer of accuracy to quantification though the more interesting result was in the issue of detection or the presence/absence of either *Meloidogyne* spp. In multiplex qPCR, a majority, 68%, of *M. hapla* and *M. chitwoodi* detections or non-detections matched the reported presence/absence results of morphological diagnostics.

The multiplex qPCR often failed to detect both target species when morphological diagnostics reported that a mixture of *M. hapla* and *M. chitwoodi* were present. In 11 of 27 samples where morphological methods reported both species but the qPCR only detected *M. hapla,* were then evaluated singleplex with the *Mc* FAM probe. Of these, seven samples reproduced the result of the multiplex where no *M. chitwoodi* were detected. Additionally, in the samples we further investigated in singleplex, the multiplex qPCR did not produce any positives that were not confirmed in singleplex.

The degree to which there was a lack of consensus between morphological diagnostics and molecular diagnostics indicates that both methods produced imperfect results. It's unlikely that 32% of the samples were processed with error from the molecular quantification side or just the morphological diagnostics side. Even in the most careful conditions molecular techniques are prone to contamination, a mixed-up label, or a failed extraction that could affect the results. On the morphological side, a source of error is the ambiguity of the morphology and the potentially thousands of other non-*Meloidogyne* species in a sample.

Going forward, the choice to perform morphological diagnostics, multiplex qPCR, or singleplex qPCR is highly context dependent. If samples need to be screened for *M. chitwoodi* so crops can be internationally exported then perhaps the singleplex qPCR is the most sensitive and reliable option. If there are symptoms of *Meloidogyne* sp. damage but on a crop that is a non-host for *M. hapla* and *M. chitwoodi* then morphological diagnostics would be best to determine if the culprit is the less common *Meloidogyne naasi* or *M. minor* (Zasada et al., 2018). Alternatively, if a grower has the opportunity to systematically sample their field in multiple locations the multiplex could be the best option for a broad view of presence/absence of *M. hapla* and *chitwoodi* to inform pest management decisions. Overall, this study provides a much-needed diagnostic tool to help diagnosticians detect root-knot nematodes, thus aiding management decisions for this destructive agricultural pest.

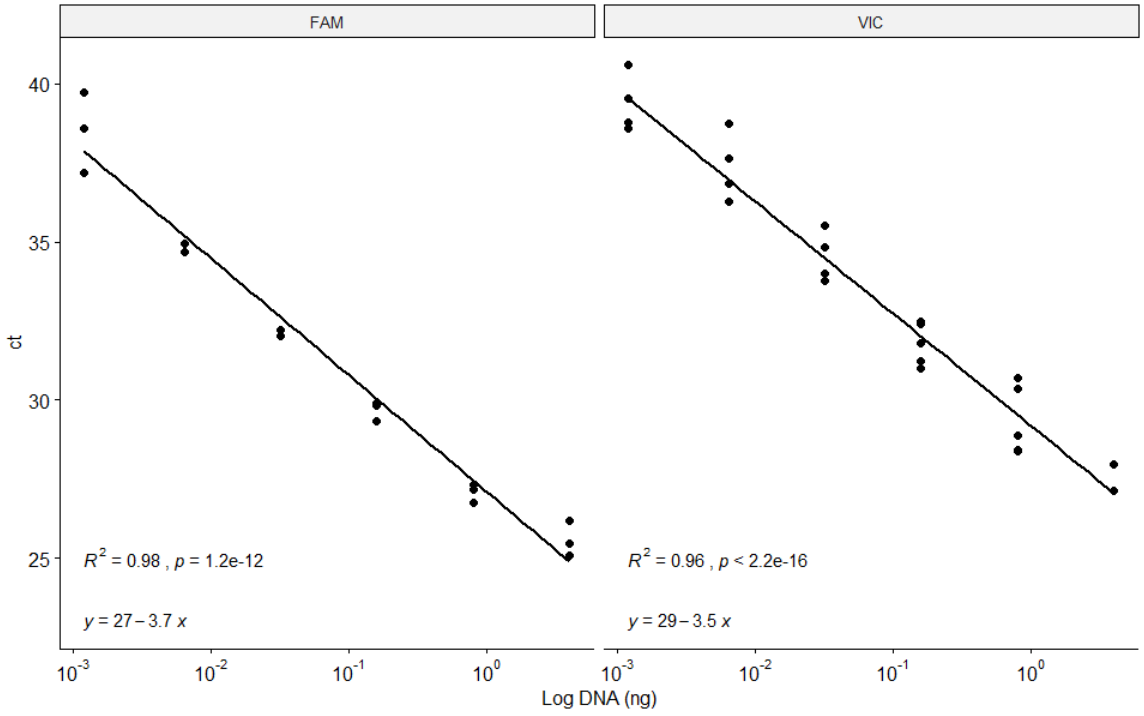

Figure 2.1 The standard curve for multiplex qPCR targeting *Hsp90* in *Meloidogyne chitwoodi* and *Meloidogyne hapla*. Based on the McMhHsp90 F/R primers with hydrolysis probes: (left) McHsp90 FAM (*M. chitwoodi*) and (right) MhHsp90 VIC (*M. hapla*). Sample points were generated by 1:5 dilution of 2 ng/µl. Efficiencies with McHsp90 FAM and MhHsp90 VIC are 86.3% and 93.8%, respectively. Cycle threshold (Ct) and DNA concentration are inversely related.

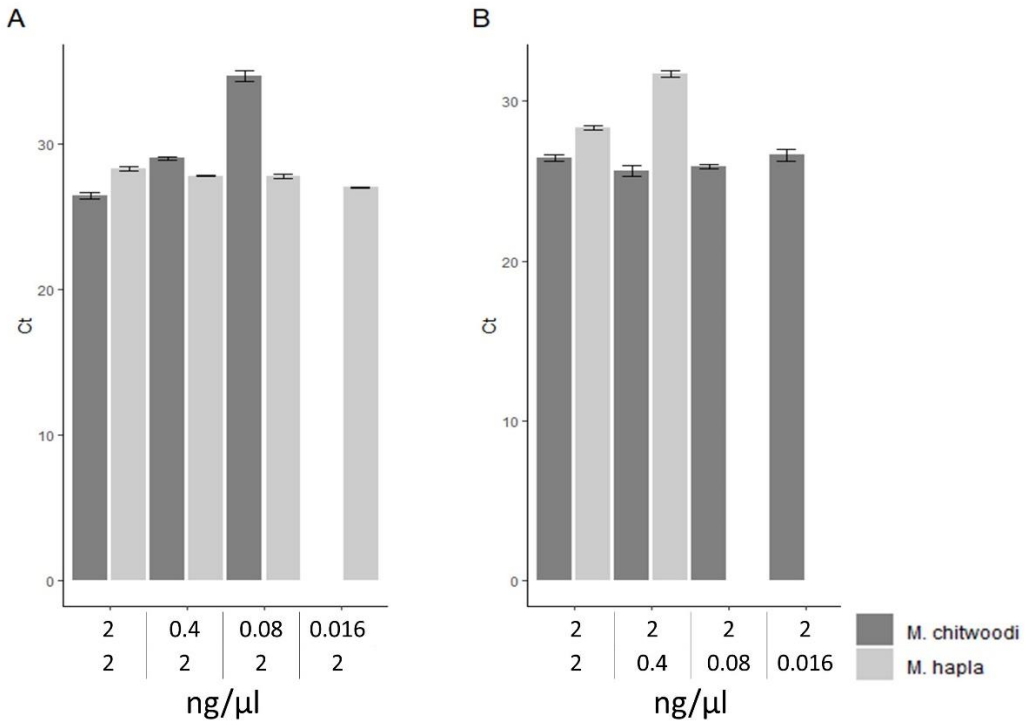

Figure 2.2 Detection of small quantities of DNA from one *Meloidogyne* species was affected by larger quantities of DNA from the other *Meloidogyne* spp. A. FAM (*M. chitwoodi*) and VIC (*M. hapla*) Ct values plotted against decreasing quantities of M. chitwoodi DNA in a constant concentration background of *M. hapla* DNA; **B** VIC (*M. hapla*) and FAM (*M. chitwoodi*) cycle threshold (Ct) values plotted against decreasing quantities of M. hapla DNA in a constant concentration background of M. chitwoodi DNA. No bar indicates no detection. Bars are the mean $\pm$ standard error of n = 3.

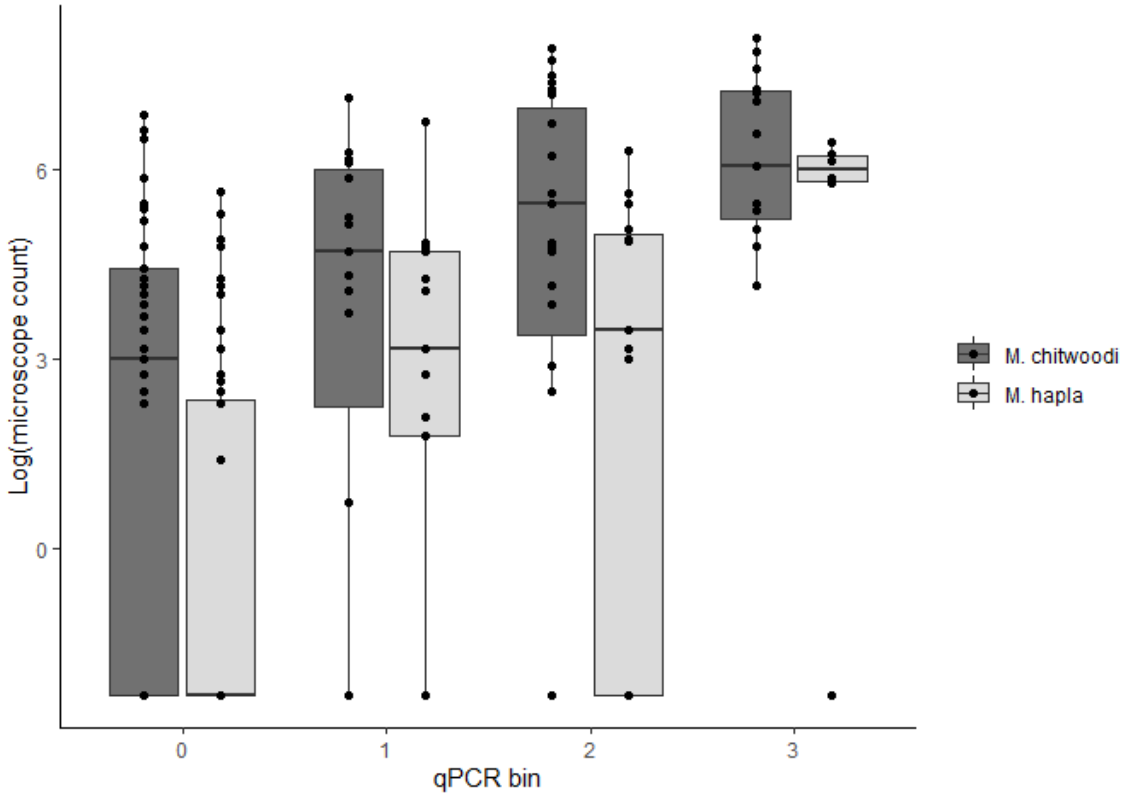

Figure 2.3 Relationship between qPCR bins and log-transformed morphological density data by *Meloidogyne* species. qPCR bins are based on cycle threshold (Ct) values as (Ct FAM or Ct VIC), 0 = none detected, 1 = 41.7 to 50, 2 = 33.9 to 41.6, and 3 = all Ct values below 33.9. Prior to log transformation, 0.1 was added to each density estimate obtained from morphological diagnostics to allow for the inclusion of 0 data (n = 94).

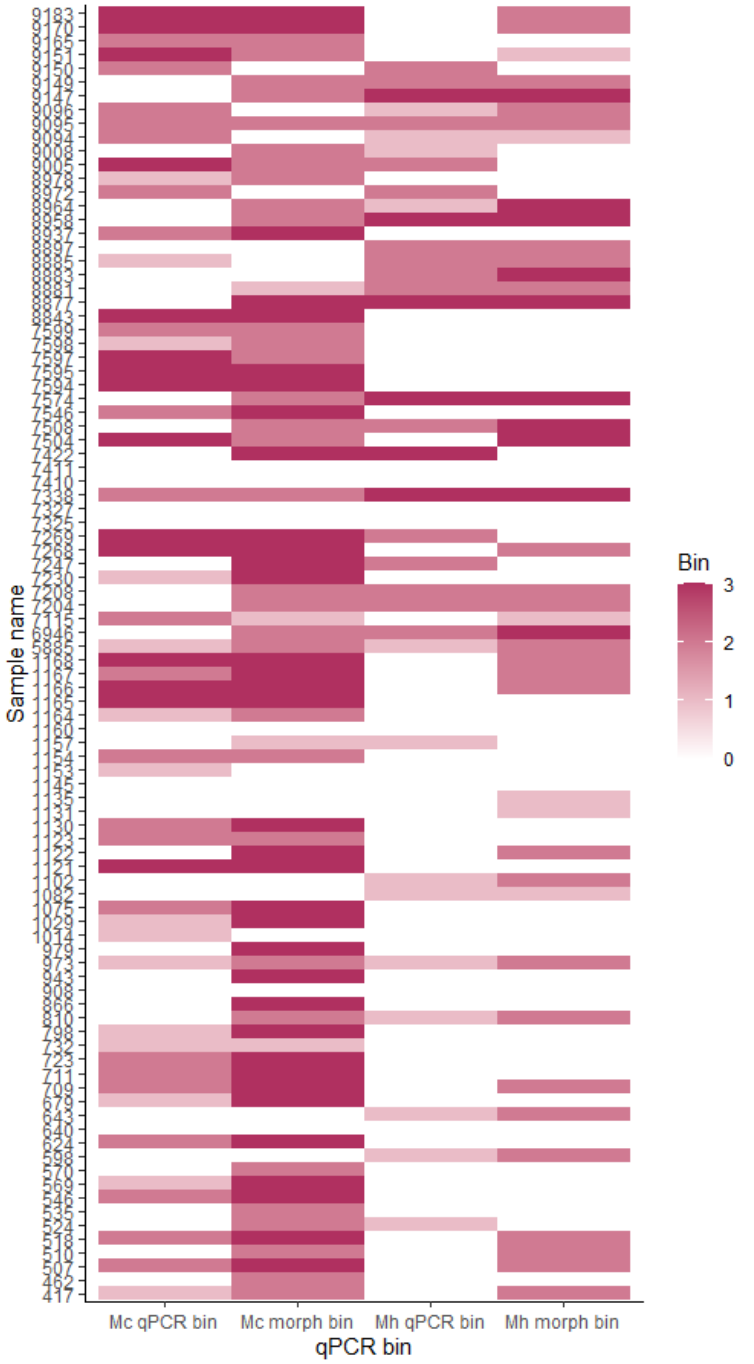

Figure 2.4 Comparison of bin assignments for morphological and molecular identification and quantification of *Meloidogyne* spp. Data for each method were binned: for morphological (in density of *M. hapla* or *M. chitwoodi*), 0 = none detected, 1 = fewer than 14, 2 = 15 to 208, and 3 = 208 to 3,294; for molecular (by Ct FAM or Ct VIC), 0 = none detected, 1 = 41.7 to 50, 2 = 33.9 to 41.6, and 3 = all Ct values below 33.9. The heatmap displays all samples (n = 94) and their assigned bins. Cycle threshold (Ct) and density estimates were inversely related.

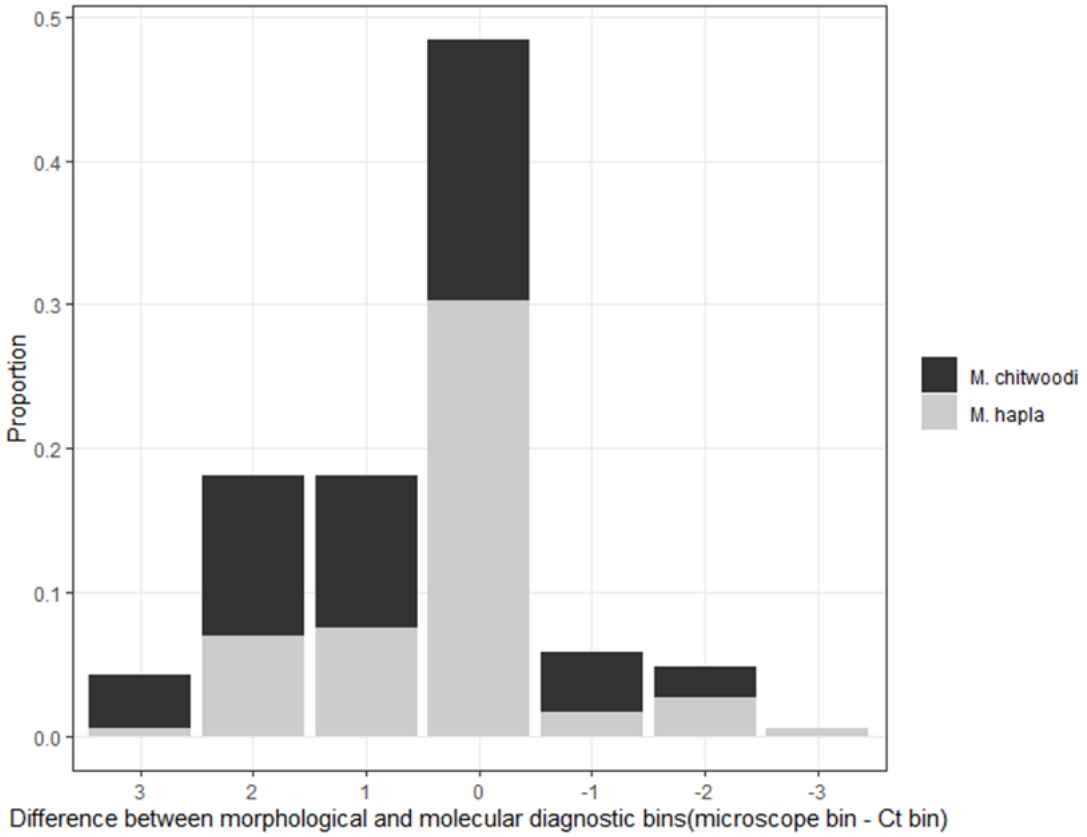

Figure 2.5 Difference in bin assignment for densities estimated by molecular and morphological diagnostics of *Meloidogyne* spp. Data for each method were binned: for morphological (in number of *M. hapla* or *M. chitwoodi*), 0 = none detected, 1 = fewer than 14, 2 = 15 to 208, and 3 = 208 to 3,294; for molecular (by Ct FAM or Ct VIC), 0 = none detected, 1 = 41.7 to 50, 2 = 33.9 to 41.6, and 3 = all Ct values below 33.9. On the X-axis difference in bins is represented by (morphological bin – molecular bin). On the Y-axis is proportion of total samples (n = 94). On the x-axis difference in bins is represented by (morphological bin – molecular bin). On the y-axis is proportion of total samples (n = 94).

Table 2.1 Primers and probes used in this study.

Name	Description	Sequence (5’ to 3’)
McMhHsp90 F	Forward Primer	TGGACTCGAAACCCTGATG
McMhHsp90 R	Reverse Primer	CCTTCAACGCTCAAATGCTT
McHsp90 FAM	TaqMan™ Probe	FAM – TTCGAAATGGCACTTGAA – NFQ-MGB
MhHsp90 VIC	TaqMan™ Probe	VIC – ACCGAACACTTTTAAGGAG – NFQ-MGB

Table 2.2 Efficacy of a multiplex quantitative PCR assay for *Meloidogyne chitwoodi* and *Meloidoygne hapla* across soil types with diverse nematode communities. Nematodes were extracted from soil and then the nematode community was identified. Prior to DNA extraction, samples were spiked with 0, 5, 25, and 250 *M. chitwoodi* and/or *M. hapla* second-stage juveniles (J2). Mean cycle threshold (Ct) values generated for FAM (*M. chitwoodi*) and VIC (*M. hapla*) are presented.

					Mean Ct 250 J2		**Mean Ct 25 J2**		**Mean Ct 5 J2**		**Mean Ct 0 J2**	
Location	**Soil type**	**Crop**	**Nematodes present (microscopy)**	**Probe**	***M. chitwoodi***	***M. chitwoodi* & *M. hapla***	***M. chitwoodi***	***M. chitwoodi* & *M. hapla***	***M. chitwoodi***	***M. chitwoodi* & *M. hapla***	***M. chitwoodi***	***M. chitwoodi* & *M. hapla***
Prosser, Washington	Warden silt loam, 0 to 5 percent slopes	Hop	*Pratylenchus*, Cephalobidae, Tylenchidae, Monhysteridae (most abundant), Rhabditidae, Dorylaimidae, *Xiphinema*, *Helicotylenchus*, *Heterodera humulii*	FAM (*Mc*)	30.701	26.68	37.03	31.90	36.2	37.51	45.8	39.81
				VIC (*Mh*)	ND	26.09	ND	33.04	ND	36.497	ND	41.55
Lynden, Washington	Lynden sandy loam, 0 to 3 percent slopes	Raspberry	*Pratylenchus* (most abundant), Cephalobidae, Rhabditidae, *Paratylenchus*, Dorylaimidae	FAM (*Mc*)	30.909	24.77	33.53	34.48	38.96	36.67	ND	ND
				VIC (*Mh*)	ND	24.21	ND	34.46	ND	37.83	ND	ND
Woodhall - Monroe, Oregon	Bellpine-Jory complex, 20 to 30 percent slopes, Willakenzie loam, 12 to 20 percent slopes	Grape	*Mesocriconema xenoplax* (most abundant), Cephalobidae, Tylenchidae, Dorylamidae, Rhabditidae, *Xiphinema*, Monhysteridae, *Diphtherophora*, Alaimidae	FAM (*Mc*)	30.06	24.06	36.64	32.93	38.15	37.00	ND	ND
				VIC (*Mh*)	36.79	23.59	36.64	31.85	36.84	34.42	36.05	34.14
Control	Tap water	n/a	none	FAM (*Mc*)	30.75	23.93	33.97	33.61	48.07	36.79	ND	ND
				VIC (*Mh*)	ND	23.453	ND	33.35	ND	37.801	ND	ND

Table 2.3 A summary of frequency of occurrence for different scenarios when morphological diagnostics were compared to multiplex qPCR diagnostics for *Meloidogyne chitwoodi* and *Meloidogyne hapla*.

Assay	**Scenario**	**Frequency**
Multiplex	Bin[1] Matches	48.4%
(94 samples)	Detection[2] Matches	67.6%
	Detection matches in multiplex for both targets	47.9%
	Microscope detects *Mh*, molecular detects none	27.7%
	Microscope detects *Mc*, molecular detects none	35.1%
	Microscope detects *Mh* only, molecular detects any chitwoodi	3.2%
	Microscope detects *Mc* only, molecular detects any hapla	7.4%
	Microscope detects *Mh* and *Mc*, molecular detects one or none	29.8%
	Microscope detects *Mh* or *Mc* or both, molecular detects none	10.6%
	Microscope detects no *Mh* or *Mc*, molecular is positive	4.3%
Mc Singleplex	Agreed with multiplex	68%
(25 samples)	Agreed with Microscope	32%
	Microscope -, singleplex +	20%
	Microscope +, singleplex -	48%
	Microscope +, singleplex +	32%
	Microscope -, singleplex -	0

[1] Bins assignments for morphological diagnostics and molecular diagnostics are based on log(count) or Ct value, respectively: 0 for none detected, 1 for the lowest third of densities, 2 for the middle third of densities, and 3 for the highest densities.

[2] Detection is whether morphological or molecular diagnostic detected the presence or absence of *M. hapla* or *M. chitwoodi*. For morphological diagnostics count ≥ 0 was designated present. For molecular diagnostics Ct mean < 50 and amplification of at least 2 of 3 replicates was designated present.

Chapter 3 – Development of a high throughput methodology for screening potato against *Meloidogyne chitwoodi*

Michelle L. Soulé, Megan Kitner, and Inga A. Zasada

3.1 Abstract

New, reliable strategies are needed to control *Meloidogyne chitwoodi* in potato and plant host resistance is central to this effort. While efforts to breed potato for resistance to *M. chitwoodi* are underway, a major bottleneck in this process is phenotyping material for nematode resistance. Currently, time and resource consuming phenotyping takes place in the greenhouse or field. The objective of this study was to establish a high throughput methodology for screening potato against *M. chitwoodi* and for quantifying egg densities at the end of experiments using qPCR. Various parameters were evaluated for the canister assay where soil is added to a small container, planted with potato, inoculated with nematodes, and incubated at a constant temperature in the dark. To obtain maximum reproduction factor (RF = final population density/initial population density) values, a minimum of 6 weeks after inoculation was required. Timing of inoculation was also important, with higher RF values when inoculation with eggs occurred at planting compared to 2 weeks after planting. The volume of water in which inoculum was delivered to soil did not impact RF values. A comparison of microscope and molecular enumeration of *M. chitwoodi* eggs was also evaluated on a breeding population. Egg enumeration by qPCR was more sensitive than by microscopy, however, this increased sensitivity did not result in a significant difference in RF value. This method has the potential to greatly decrease the amount of time and resources needed to phenotype potato against *M. chitwoodi* and can allow for multiple screenings throughout the year, regardless of the season.

3.2 Introduction

The Pacific Northwest (PNW) region of the United States (Idaho, Oregon, and Washington) accounts for approximately 65% of potato production in the U.S. (USDA-NASS, 2020). Plant-parasitic nematodes are economically important pests and a major threat to the production of potato (King & Taberna, 2013). With a global estimate of an average 8% loss in crop productivity due to nematodes, the U.S. potato industry stands to lose $368 million annually to plant-parasitic nematodes. One of the most significant nematode threats to potato in the PNW is the Columbia root-knot nematode, *Meloidogyne chitwoodi* (Zasada et al., 2018). In the PNW, *M. chitwoodi* can overwinter at the egg stage and has a relatively low temperature threshold for development (Pinkerton et al., 1991; Santo & O'Bannon, 1981). This means the life cycle can begin early, complete multiple reproductive generations during the growing season, and even continue through harvest while the tubers are in storage (Pinkerton et al., 1991).

The processing market has a very low tolerance for the damage caused by *M. chitwoodi*. For example, if just 6% of the potato tubers in a field are infected with *M. chitwoodi*, the entire crop may be a total loss (King & Taberna, 2013). Currently, soil fumigation is the most effective means for controlling *M. chitwoodi*. However, plant-parasitic nematode management is difficult because many long-relied upon nematicides are being banned, phased out, or heavily restricted and are expensive (Zasada et al., 2010). New, reliable strategies are needed to control plant-parasitic nematodes in potato, and plant host resistance is central to this effort and a highly desirable alternative control.

Efforts are being made to develop improved selections with resistance to *M. chitwoodi* (Graebner et al., 2018). Resistance to *M. chitwoodi* has been identified in *Solanum bulbocastanum, S. hougasii, S. stenophyllidium* and *S. fendleri* (Brown et al., 1995, 2006; Graebner et al., 2018; Janssen et al., 1997). The resistance identified from clone 22 of diploid *S. bulbocastanum* (SB22) was hybridized with cultivated tetraploid *S. tuberosum* using protoplast fusion. The somatic hybrid obtained by fusion was subsequently backcrossed five times with various tetraploid *S. tuberosum* genotypes resulting in nematode resistant advanced breeding selection, PA99N82–4 (Brown et al., 1996). The aforementioned effort to introgress resistance into advanced breeding selections, and the subsequent development of selections into new potato varieties takes years if not decades.

Phenotyping material for nematode resistance is a major bottleneck in this process. Currently, time and resource consuming phenotyping takes place in the greenhouse or field (Graebner et al., 2018). In the greenhouse, pots containing at least 1 L of soil are planted with a tuber or tissue culture plantlets and after 28 days the potted plants are inoculated with *M. chitwoodi*. The experiment is then run for 55 days in a greenhouse that can have variable temperature, and at times is too hot for the nematode to survive (Graebner et al., 2018). At the end of the experiment roots are removed from the pots and nematode eggs extracted from roots with a bleach solution (Ingham, 1994). The eggs are counted to calculate reproductive efficiency of nematodes. In the field, tubers (10 hills) are planted in a *M. chitwoodi* infested field and grown over the growing season (100-150 days). At the end, the tubers are harvested and a subsample of tubers are peeled

and assessed for nematode infections (Ingham et al., 2000). Both greenhouse and field screening require a lot of resources and even under ideal conditions, it is challenging not to find nematode escapes, which can be an impediment to determining if material is resistant or susceptible to *M. chitwoodi*. It would be advantageous to develop an assay that requires less space and resources and can be run in a constant environment to ensure consistency across experiments.

A high throughput laboratory method for screening potatoes against potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) has already been developed (Foot, 1977). In this canister method, tubers were planted in sand in a transparent container. After root initiation, each canister was inoculated with freed eggs and at 17.5 °C the nematode's life cycle was completed in 10-12 weeks after inoculation. The canister method was demonstrated to have the advantage over greenhouse or field methods because light is not required and temperature can be regulated. This canister method has subsequently been used to address research questions with potato cyst nematodes such as the impact of climate change (Skelsey et al., 2018) and nematode virulence (Gartner et al., 2021). This method has also been proposed for screening potato and wild *Solanum* spp. against *M. chitwoodi* and *M. hapla* (Janssen et al., 1995). Potatoes in containers were inoculated with *Meloidogyne* spp., incubated at 20 °C, and then harvested after 7 weeks.

The goal of this study was two-fold. First, several parameters of the canister method were evaluated to determine the optimal methodology to obtain repeatable and reliable data on *M. chitwoodi* reproduction on potato. Second, a qPCR method was compared to traditional microscopic counts for the quantification of eggs at the end of the

experiment. Combined, the optimized canister method for *M. chitwoodi* strives to reduce the time for phenotyping of potato germplasm.

3.3 Materials and Methods

3.3.1 Nematode inoculum

Meloidogyne chitwoodi was collected from a potato (*Solanum tuberosum*) field in Prosser, WA. Single female lines were established by adding nematode infested soil to a pot and planting tomato (*Solanum lycopersicum*) 'Rutgers'. The identity of the populations was confirmed by molecular analysis by the North Carolina Department of Agriculture & Consumer Services (Raleigh, NC). After approximately 8 weeks, the plants were destructively harvested and single egg masses were transferred to a new tomato plant. Cultures were then continuously maintained on tomato 'Rutgers', using 12-15 eggs masses to inoculate fresh culture tomato plants. To extract eggs for use in experiments, tomato plants were destructively harvested and the roots rinsed free of soil. Eggs were extracted by shaking the root system in a 0.3% NaOH solution for 3 min and then passing the solution over a 500 mesh sieve to collect eggs. To obtain *M. chitwoodi* second-stage juvenile (J2) inoculum, extracted eggs were placed on a hatching chamber for 24-72 hr and hatched J2 were collected every 24 hr. *Meloidogyne chitwoodi* eggs and J2 were kept at 4 °C until used in experiments. *Meloidogyne chitwoodi* egg and J2 densities were adjusted in water to achieve desired inoculation densities needed for each experiment.

3.3.2 Canister assay methodology

Tubers of potato ‘Russet Ranger’ were washed with a 10% bleach solution and then placed at room temperature for 1-2 weeks to allow for sprouting before adding to cups. The size of the assay was a 237 cc deli cup 4 cm in height with a dimension of 11.4 cm (ULINE, Pleasant Prairie, WI, U.S.A.). To each cup approximately 110 g of a dry 1:1 sand:Willamette loam pasteurized soil mix was added. Soil was then wetted with 30 ml of water and soil was mixed to ensure uniform wetting. After the placement of a tuber piece and inoculation with nematodes the system (varying times of inoculum, see experiments below) was closed with a lid and cups placed in a 24 °C incubator (Thermo Fisher Scientific, Waltham, MA, U.S.A.). This experimental set-up was used for all experiments that were conducted to identify the optimal conditions for the assay (see below).

3.3.2.1 Experiment 1: Effect of inoculation density and assay duration on Meloidogyne chitwoodi reproduction

The experiment consisted of 80 experimental units (cups) and was a factorial experiment with the following factors: 4 inoculation densities (0.5, 1, 2, and 5 *M. chitwoodi* eggs/g soil) and 4 take-down dates (5, 6, 7, and 8 weeks post inoculation). All factor combinations were replicated five times and cups were arranged in a completely randomized design; the experiment was conducted twice. Tubers were planted in canisters and inoculated with *M. chitwoodi* eggs suspended in 2 ml two weeks after planting. At each take-down date the cups were removed from the incubator and roots

separated from soil. Collected roots were rinsed free of soil and eggs extracted by shaking the root system in 0.6% NaOH solution for 3 min and then passing the solution over a 500 mesh sieve to collect eggs in a 50 ml tube. After extraction, roots were placed in a drying oven at 65 °C for a week, and then weighed. The solution containing collected *M. chitwoodi* eggs was adjusted to 20 ml and 1 ml of the solution was placed on a counting slide and enumerated using a Leica DM IL inverted microscope (Leica Microsystems, Wetzlar, Germany).

3.3.2.2 Experiment 2: Effect of inoculum volume on Meloidogyne chitwoodi reproduction

The experiment consisted of 15 experimental units (cups) with inoculation volume (2, 5 or 10 ml) as the treatment. Treatments were replicated five times, arranged in completely randomized design in the incubator, and the experiment was conducted twice. The soil was initially wetted with 30 ml of water, placed in a canister, and then planted with potato. Two weeks after planting the canisters were opened and inoculated with 2 *M. chitwoodi* eggs/g soil in 2, 5 or 10 ml of water. The experiment was terminated at 6 weeks and nematodes were extracted and counted as described for Exp. 1.

3.3.2.3 Experiment 3: Effect of inoculation timing on Meloidogyne chitwoodi reproduction

The experiment consisted of 15 experimental units (cups) with timing of inoculation (0, 1 and 2 weeks post planting) as the treatment. Treatments were replicated five times, arranged in a completely randomized design in an incubator, and the experiment was conducted twice. Canisters were inoculated with 2 *M. chitwoodi* eggs/g soil in 2 ml water

at the described times at or after planting. The experiment was terminated at 6 weeks and nematodes were extracted and counted as described for Exp. 1.

3.2.3.4 Data analysis of canister assay results with Meloidogyne chitwoodi

Data was analyzed using JMP vs. 9.1 (SAS, Cary, NC). Data are presented as eggs/g root and as reproduction factor (RF) values, where RF = final population density/initial population density. A RF value > 1 indicates that the plant is a host, while an RF value < 1 indicates the plant is a poor host (Oostenbrink, 1966). In all analyses, trial was considered a random factor while all other treatments were fixed factors. When the trial × treatment interaction was significant ($P < 0.001$), the trials were analyzed separately. To meet ANOVA assumptions, nematode data were log10 (x+1)-transformed prior to analysis. Statistically significant differences among treatments were computed by Tukey's honest significant difference test with significance at $P < 0.05$.

3.3.3 Comparison of microscopic and molecular quantification of Meloidogyne chitwoodi eggs

To enable the evaluation of potato germplasm with varying levels of susceptibility to *M. chitwoodi*, and therefore varying final egg densities, a breeding population was tested in the canister assay. The breeding population included progeny of OR170115 (PA99N82-4 x AOR13260-3adg). PA99N82-4 is the *M. chitwoodi* resistant parent with introgression from *S. bulbocastanum.* The seedlings segregate for resistance to *M. chitwoodi* 1:1 (Sagar, personal communication). For this study 59 accessions were evaluated along with a potato 'Russet Ranger' positive control. Four tuber pieces of each accession with many

eyes were added to canisters as described above. Canisters were inoculated with 5 *M. chitwoodi* eggs/g soil at planting. After 6 weeks, the canisters were destructively harvested, eggs extracted from roots and then enumerated as described for Exp. 1. After enumeration of eggs, the aliquot was returned to the tube and stored at 4 °C until DNA extraction.

Sixty-six samples of the 244 were randomly selected for qPCR analysis. These samples varied in *M. chitwoodi* egg densities and level of contamination with fungi. Eggs were concentrated into DNeasy PowerSoil Pro kit bead beating tube (QIAGEN, Hilden, Germany) and DNA was extracted according to manufacturer instructions with one modification: after the bead beating step samples were incubated at 4 °C for 24 hours before completing the rest of the manufacturer protocol.

The primers and probe for quantitative PCR were adapted from a hydrolysis probe multiplex qPCR assay to detect *Meloidogyne hapla* and *M. chitwoodi* (Soulé unpublished). The primers target the gene *Hsp90* of both *M. hapla* and *M. chitwoodi* with only the specific *M. chitwoodi* probe used (Table 2.1). The qPCR was performed in 20 µl reaction volumes consisting of 2x TaqMan™ Universal Master Mix II, no UNG (Life Technologies Corporation, Carlsbad, CA, U.S.A.), 500 nm forward and reverse primers (Sigma-Aldrich , Inc. St. Louis, MO, U.S.A.), 250 nM of FAM TaqMan™ (Life Technologies Corporation), 2 µl of template DNA, and water. Cycling conditions were 95 °C for 10 min and 50 cycles at 95 °C for 15 sec and 60 °C for 1 min using the Applied Biosystems StepOne Plus (Applied Biosystems, Waltham, MA, U.S.A.). A five-point standard curve was prepared by extracting DNA from 30,000 *M. chitwoodi* eggs. The purified DNA extract was serially diluted 1:10 so that the five points represented 30,000,

3,000, 300, 30, and 3 eggs. Each sample was run in triplicate and generated Cts were compared to those on the standard curve to estimate starting sample quantity.

PCR egg density estimates were plotted against microscope egg density estimates and were analyzed by ANOVA with repeated measures using the package rstatix (Kassambara, 2021) in R Statistical Software (v4.2.0; R Core Team 2022) with significance at $P < 0.05$. Finally, to understand how the use of molecular quantification could affect how samples are determined to be susceptible or resistant, densities were converted to RF values and analyzed by ANOVA with repeated measures with significance at $P < 0.05$.

3.4 Results

3.4.1 Optimization of the canister assay

In Exp. 1, there was no difference in final dry root weight among treatments or treatment combinations ($P > 0.1$; data not shown). Dry root weights ranged from 0.01 to 0.18 g. For RF values, there was no interaction of inoculation density and takedown date ($P = 0.2975$), therefore, the factors of inoculation density and takedown date were considered independently. There was no significant effect of inoculation density on RF values ($P = 0.8710$). There was a nonsignificant reduction in RF values with increasing inoculum density (Pi) above 1 egg/g soil, with a Pi of 5 eggs/g soil having a mean RF value 33% lower than a Pi of 1 egg/g soil (Fig. 3.1A). Takedown date had a significant effect on RF values ($P = 0.0009$; Fig. 3.1B). The RF value at 5 weeks was 49 to 67% lower than for RF values at 6, 7, and 8 weeks. When final *M. chitwoodi* eggs/g root was considered, there was no interaction of inoculation density and takedown date ($P = 0.5752$), therefore,

the factors of inoculation density and takedown date were considered independently. There was no significant effect of takedown date on eggs/g root ($P = 0.0556$; Fig. 3.1C). There was a significant effect of inoculation density on *M. chitwoodi* eggs/g root ($P < 0.0003$; Fig. 3.1D). As Pi increased so did the number of eggs/g root extracted at the end of the experiment. At Pi's of 1 and 2 eggs/g soil there were 1.3 and 1.4 fewer eggs/g root than at Pi of 5 eggs/g soil, respectively. The highest number of eggs/g root was recovered from a Pi of 5 eggs/g soil.

In Exp. 2, there was no effect of inoculation volume on RF values, eggs/g root, or dry root weights ($P = 0.7665$; Fig. 3.2). Dry root weights ranged from 0.01 to 0.08 g. The mean RF value across treatments was 118.0 $\pm$ 20.4 and there were 25,952 $\pm$ 4,498 *M. chitwoodi* eggs/g.

In Exp. 3, dry root weight averaged 0.3 $\pm$ 0.003 g across treatments and trials. There was a significant effect of inoculation timing on RF values and *M. chitwoodi* eggs/g root ($P = 0.0001$; Fig. 3.3). When *M. chitwoodi* was inoculated 2 weeks after planting the tuber piece, RF values were significantly lower by 85 and 78% compared to inoculation at planting (0 week) and 1 week after planting. There was no difference in RF values when inoculation occurred at 0 and 1 week after planting. There was a similar trend for *M. chitwoodi* eggs/g root with 6.3 and 4.2 times more eggs/g root when inoculation occurred at planting (0 week) and 1 weeks vs. 2 weeks, respectively.

3.4.2 Comparison of microscopic and molecular quantification of Meloidogyne chitwoodi eggs

There was a strong correlation between microscope counts and qPCR estimates in *M. chitwoodi* egg density estimates when all data (n=66) were considered (R^2 = 0.97; Fig 3.4A) and no significant difference between microscope counts and qPCR estimate (F (1,65) = 3.67, P = 0.06, g2 = 0.01; Fig. 3.4B). For the 46 samples in which the microscope counts were less than 200, the data was not highly correlated (R^2 = 0.33; Fig 3.5A) and there was a significant difference between counting methods (F (1,45) = 19, P < 0.0001, g2 = 0.014; Fig. 3.5B) with 21 microscope assessment of zero generating amplification that resulted in qPCR estimates ranging from 1 to 658 eggs.

Reproduction factor values for all samples were not significant different between microscope and qPCR assessment methods (F (1,65) = 0.96, P < 0.33, g2 = 0.33; Fig. 3.6A). Further, when samples were analyzed with RF values less than one, the difference between methods was also not significant (F (1,50) = 1.79, P = 0.19, g2 = 0.01; Fig. 3.6B).

3.5 Discussion

While we are not the first to adopt a canister assay to screen potatoes against plant-parasitic nematodes (Foot, 1977; Janssen et al., 1995; Phillips et al., 1980), it is important for labs to validate and fine tune methodologies prior to implementation. In this study, we explored several methodological aspects of a container assay for rearing of *M. chitwoodi* to enable phenotyping and biological studies in potato and found that length of assay and inoculation timing were both important factors to consider. We also, for the first time,

deployed a molecular quantification method to determine *M. chitwoodi* egg densities to further improve the high-throughput nature of the assay for rapid screening of potatoes against *M. chitwoodi*.

The first published report of a container assay to rear and conduct experiments with plant-parasitic nematodes, specifically *Globodera* spp., was by Foot (1977). In this study, a container, 500 ml, was filled with autoclaved river sand adjusted to approximately 5% of its oven-dry moisture and planted with a seed weighing 30 to 50 g. Inoculation of nematodes was delayed until developing roots were visible and then eggs were added to the canister in 2 ml water followed by 25 ml nutrient solution for a total soil moisture of 9% of oven-dry weight. The two major advantages of this method presented by Foot (1977) were that light was not required and that temperature could be regulated. This method was further refined by Phillips et al. (1980) where a smaller container was used, 240 ml, and the substrate used was John Innes No. 2 compost. In this system, the canister was immediately inoculated with *G. pallida* eggs to achieve a moisture level of 30%. This study also looked at modifying moisture level and compared compost to washed sand. This assay with various modifications has been used extensively with *Globodera* spp. to evaluate partial resistance (Mugniéry et al., 1989), resistance (Gartner et al., 2021; Moloney et al., 2010), climate change (Skelsey et al., 2018), biological control (LaMondia & Brodie, 1984), and influence of temperature on development (Kaczmarek et al., 2014).

To the best of our knowledge, there is only one published report on utilizing a closed container assay to evaluate potatoes or *Solanum* spp. for resistance to *Meloidogyne*

spp. (Janssen et al., 1995). In this study, the canister assay was compared to other experimental methods to evaluate a seedling population of *Solanum* spp. against *M. chitwoodi* and *M. hapla.* The container, 125 ml, was filled with moist silver sand containing slow release NPK fertilizer. Sprouted tuber pieces, approximately 20 to 30 g, were planted and one week later inoculated with 260 *Meloidogyne* spp. juveniles. The assay ran at 20 $\pm$ 1 °C for 7 weeks and egg masses were counted and fresh roots weighed. While the closed canister assay resulted in lower reproduction of *Meloidogyne* spp. than two other methods, clay pots and plastic tubes, the canister assay still indicated that the 10 potato cultivars evaluated were hosts for *M. chitwoodi* and *M. hapla* (Janssen et al., 1995). In our study, we used the susceptible potato 'Russet Ranger'. The canister assay resulted in RF values > 1 95% (N = 234) of the time, indicating that *M. chitwoodi* reproduced very well in this assay.

Several parameters were evaluated to optimize the performance of the canister assay for *M. chitwoodi.* Comparable experiments were conducted by Phillips et al. (1980). The moisture level in our experiments was adjusted at inoculation with *M. chitwoodi* eggs being inoculated in 2, 5 or 10 ml water (equivalent to approximately 22, 24 and 26% moisture levels); there was no difference in final egg densities or RF values due to modifying this variable. Contrary to our findings, with *G. pallida* there was a reduction in cyst numbers when moisture level of sand was increased from 10 to 17% (Phillips et al., 1980). A difference in our study might have been more apparent if a wider range of percentage soil moistures were evaluated.

In Janssen et al. (1995), the experiment was inoculated with 2 *M. hapla* or *M. chitwoodi*/ml soil. In this experiment, we varied initial inoculation density and inoculated with 0.5 to 5 *M. chitwoodi*/g soil. There was an effect of varying initial inoculation density on final eggs/g root, with more eggs produced when more *M. chitwoodi* were added to the assay, however, this initial inoculation density did not impact RF values. The duration of the assay was also evaluated with take-down time after inoculation occurring at 5, 6, 7, and 8 weeks. Similar to a greenhouse study (Filialuna et al., 2022), higher RF values were observed for *M. chitwoodi* at later harvest dates. In both the canister assay and aforementioned greenhouse study, five weeks was insufficient time for the assay. In the greenhouse study (Filialuna et al., 2022), at least seven weeks were required to maximize RF values. In the canister assay, it is possible to run the experiment for six weeks and achieve similar RF values as later dates. The difference between this study and the greenhouse study is that temperature was held constant at 24 °C in the canister assay while temperature varied in the greenhouse study. A temperature of 24 °C was chosen for this experiment based upon findings that *M. chitwoodi* egg densities were the highest six weeks after inoculation at an incubation temperature of 25 °C (O'Bannon and Santo, 1984).

This canister assay has great utility in a potato breeding program that needs to rapidly phenotype 100s of individuals in a breeding population. Currently, it takes 12 and 14 weeks to phenotype potatoes against *M. chitwoodi* in the greenhouse and field, respectively (Brown et al., 2006; Graebner et al., 2018). With the canister assay, only 6 weeks are required. With greenhouse and field experiments only one experiment can be

run per year. With the canister assay and access to an incubator, several assays can be run in a year as long as viable tubers are available. In comparison to a greenhouse experiment there is also a space and resource saving advantage with the canister assay. Greenhouse studies with *M. chitwoodi* are conducted in 10 cm pots containing 1 L of soil. Per experimental unit, only one tenth of the amount of soil is required for the canister assay compared to a greenhouse pot assay. Additionally, the footprint of the experiment is greatly reduced using the canister assay because the experimental units can be stacked. This is not to say that the canister assay will replace greenhouse or field experiments; rather, it can be used to complement them. Initially, 100s of progeny from a breeding population can be rapidly phenotyped to eliminate highly susceptible material. Those that are found to be resistant or partially resistant can then be further evaluated to confirm this finding in greenhouse and field experiments.

When molecular quantification of eggs was compared to quantification by microscope there was no difference on average between the two methods in egg densities or RF value. Considering the data as a whole masks the effect that egg density has on density estimates. That is, at high densities, egg counts by microscope become more accurate. When we compared egg density estimates by microscope that estimated under 200 *M. chitwoodi* eggs in the sample, we found a significant difference between qPCR estimates and microscope estimates. This is especially relevant for the 21 samples which had a microscope estimation of zero and a positive qPCR estimate. Complete resistance is the goal in a breeding program because of the zero tolerance for *M. chitwoodi* in international potato shipments (King & Taberna, 2013), therefore, accuracy is needed for

screening progeny for immunity to *M. chitwoodi*. Additionally, some canisters contained small eggs that were collected during the extraction process. The technician noted the anomaly in size but included the eggs in their counts. The qPCR did not find *M. chitwoodi* eggs in those samples. In this case, the species specificity of the qPCR proved to be useful.

There is also more ease in using molecular tools like qPCR than using a microscope to quantify eggs. The total turnaround time in this study for quantifying eggs was longer with qPCR than counting on a microscope. However, with some adjustments to the technique to optimize for high-throughput capacity, qPCR estimation would easily outpace microscopic estimation. For example, one of the longest steps in molecular quantification is DNA extraction. Switching to a high-throughput 96-sample format DNA extraction kit would significantly decrease sample extraction time. In a similar vein, the qPCR thermal cycler could be upgraded to a 384-well machine, which would quadruple the sample capacity in a single run (Braun-Kiewnick & Kiewnick, 2018). Microscope fatigue also has to be taken into account. Molecular techniques require short spurts of work to prepare samples and machines often with several hours of down time between steps. In comparison, quantification by microscope requires constant hands-on work at every step.

The potato canister assay presents the opportunity to significantly increase the volume of progeny tested for *M. chitwoodi* resistance. In this study we validated and optimized the potato canister method and demonstrated its use in screening a breeding population for *M. chitwoodi* resistance. Combined with high-throughput molecular

diagnostics like qPCR, the canister system is a much more sensitive and time efficient process than is currently in use. Decreasing the amount of time required to bring a resistant potato cultivar to market is central to reducing pesticide use and imperative in the age of increasing pesticide restrictions. The increased sensitivity is also important for breeding potatoes with immunity to *M. chitwoodi* because of the low tolerance for the presence of *M. chitwoodi* in the international export market. Future research efforts with the potato canister project should focus on the validation of the potato canister assay for the evaluation of other plant-parasitic nematodes.

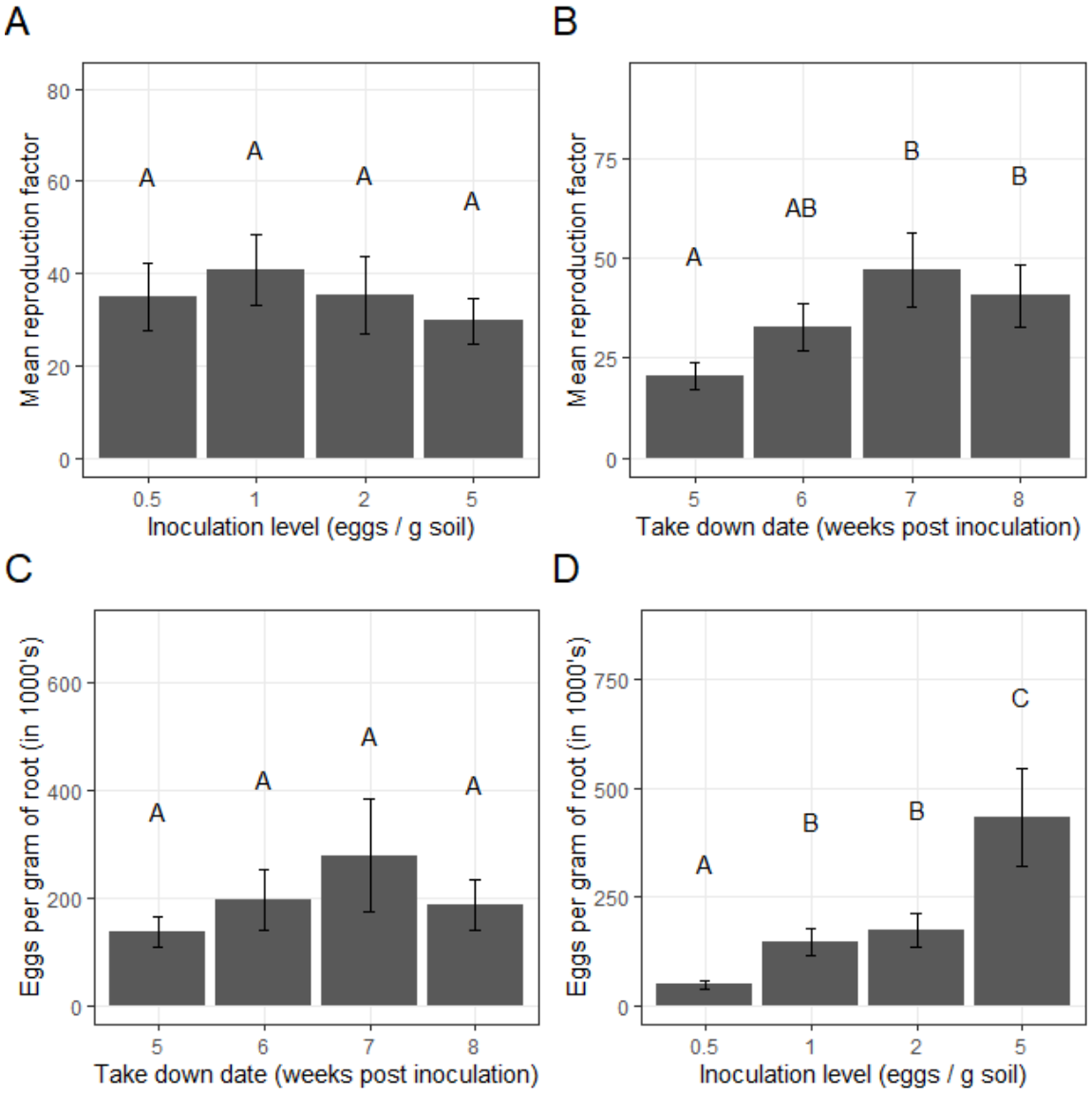

Figure 3.1 Effect of inoculation density of *Meloidogyne chitwoodi* (eggs/g soil) and take down date on final reproduction factor (final population density/initial population density) values and final eggs/g root. The interaction of these factors was not significant ($P > 0.01$); therefore, they are presented separately. Bars within a panel with the same letter are not significantly different according to Tukey's adjustment for multiple comparisons ($P > 0.05$). Bars are the mean $\pm$ standard error of N = 10.

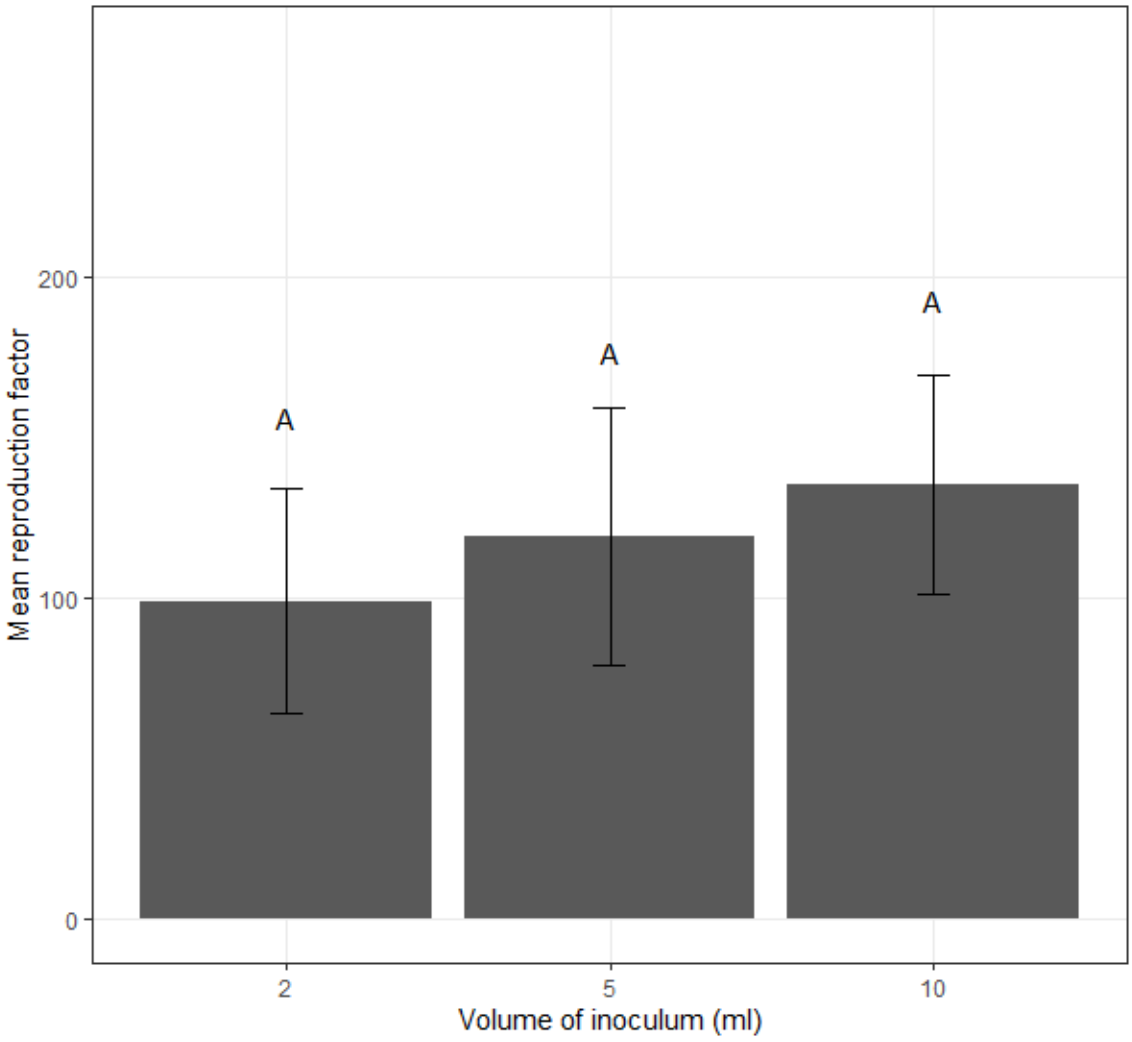

Figure 3.2 Effect of volume of water in which *Meloidogyne chitwoodi* eggs were applied to soil on reproduction factor (final population density/initial population density) values. Bars with the same letter are not significantly different according to Tukey's adjustment for multiple comparisons ($P > 0.05$). Bars are the mean $\pm$ standard error of N = 10.

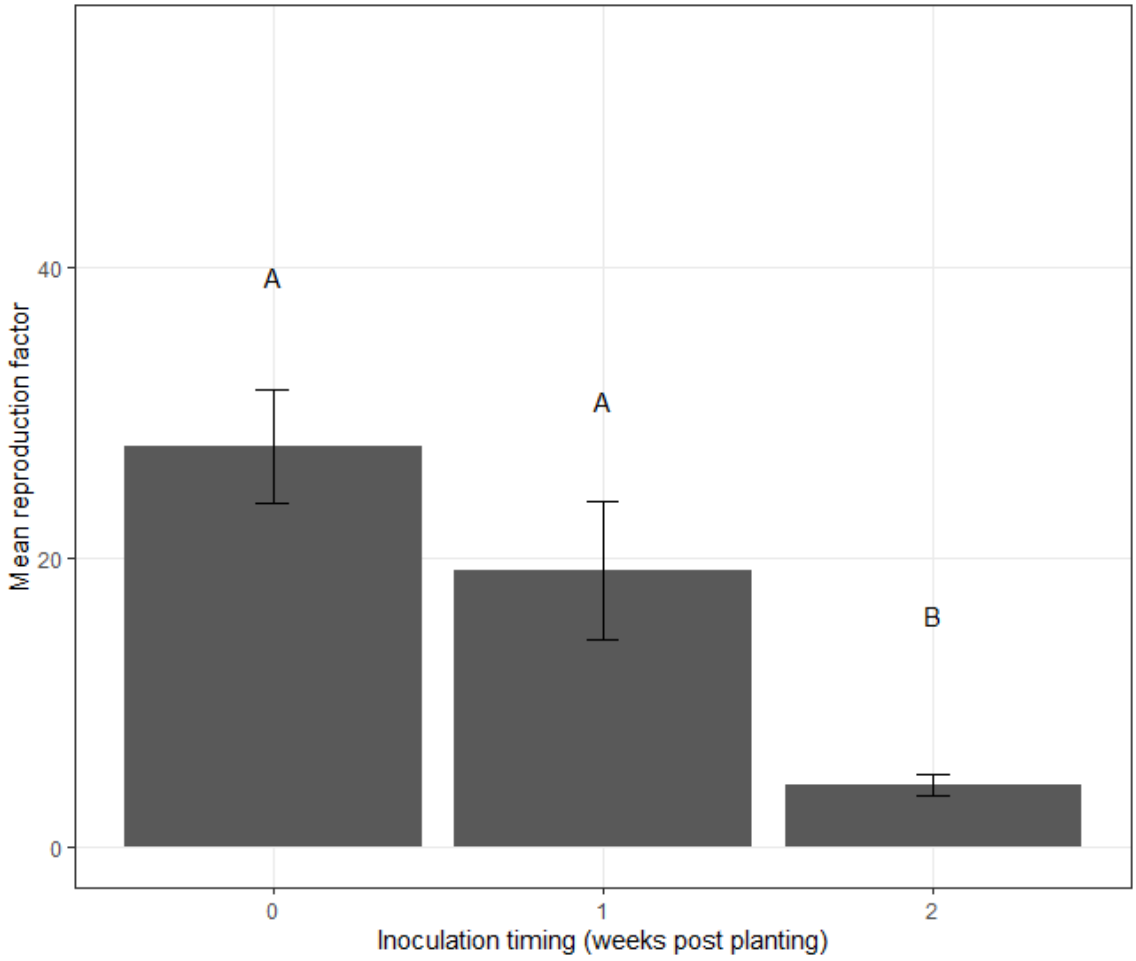

Figure 3.3 Effect of inoculation timing on reproduction factor (final population density/initial population density) values of *Meloidogyne chitwoodi*. Bars with the same letter are not significantly different according to Tukey's adjustment for multiple comparisons ($P > 0.05$). Bars are the mean ± standard error of N = 10.

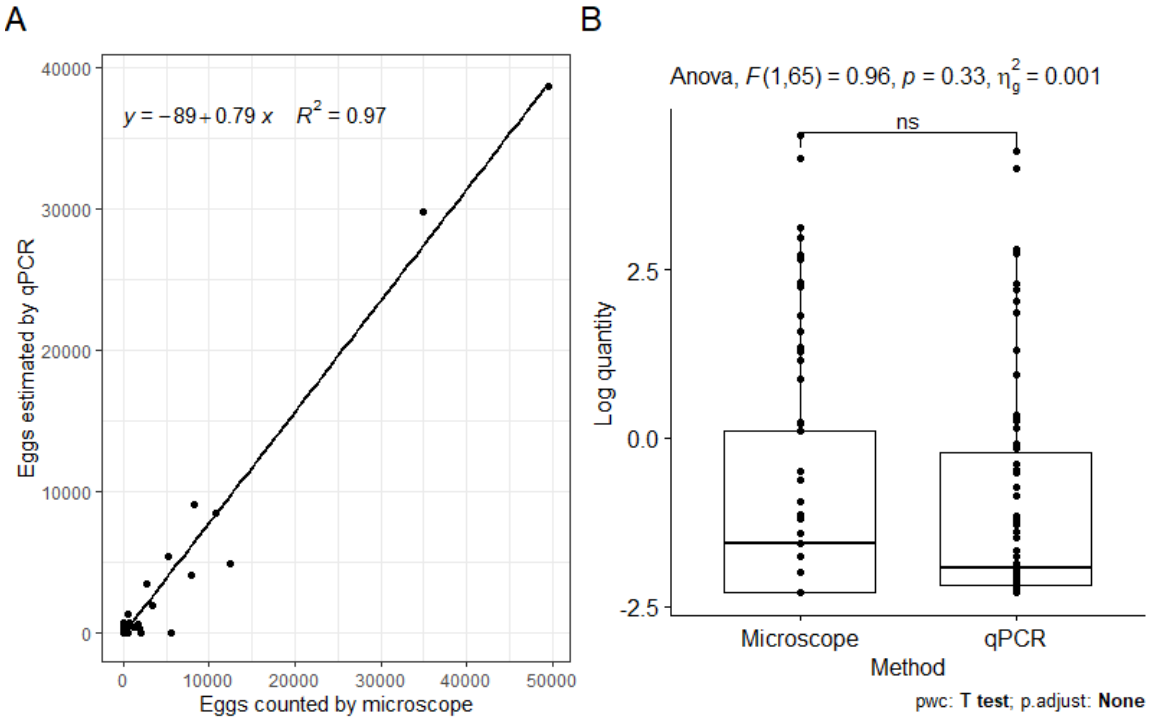

Figure 3.4 Comparison between *Meloidogyne chitwoodi* egg densities for all data estimated by microscopy and the associated qPCR estimates. (N = 66), correlation in panel **A** and ANOVA with repeated measures in panel **B**. Data were ln(x+0.1)-transformed prior to ANOVA and significant differences were determined at $P = 0.05$.

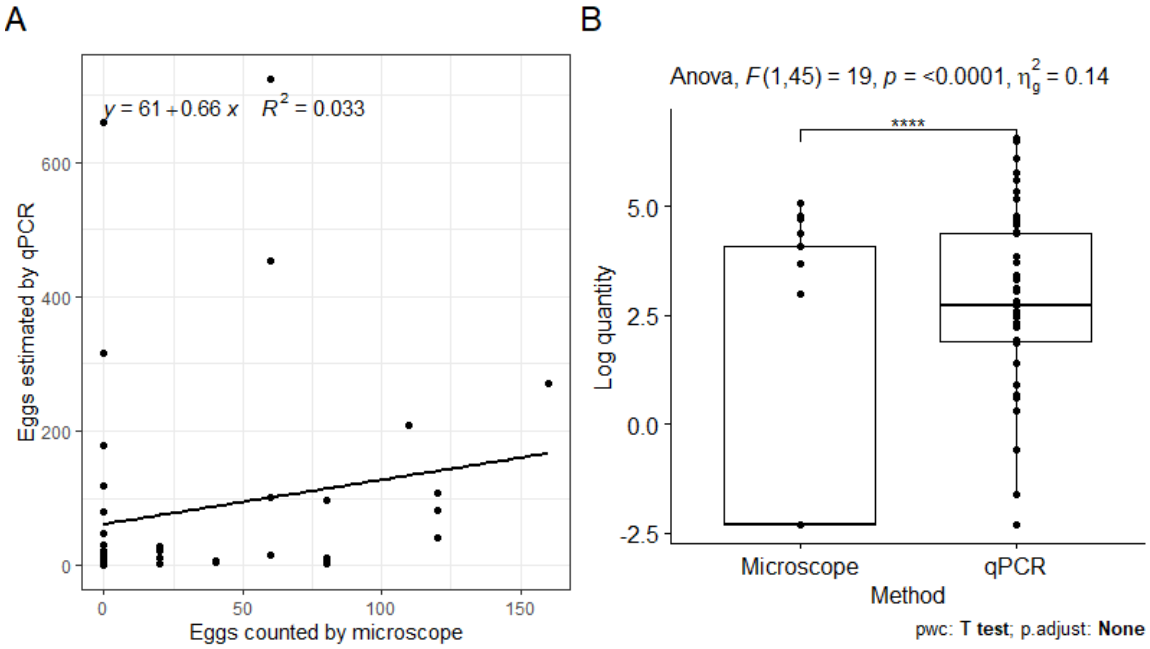

Figure 3.5 Comparison between *Meloidogyne chitwoodi* egg densities of less than 200 estimated by microscopy and the associated qPCR estimates. (N = 46), correlation in panel **A** and ANOVA with repeated measures in panel **B**. data were ln(x+0.1)-transformed prior to ANOVA and significant differences were determined at $P = 0.05$.

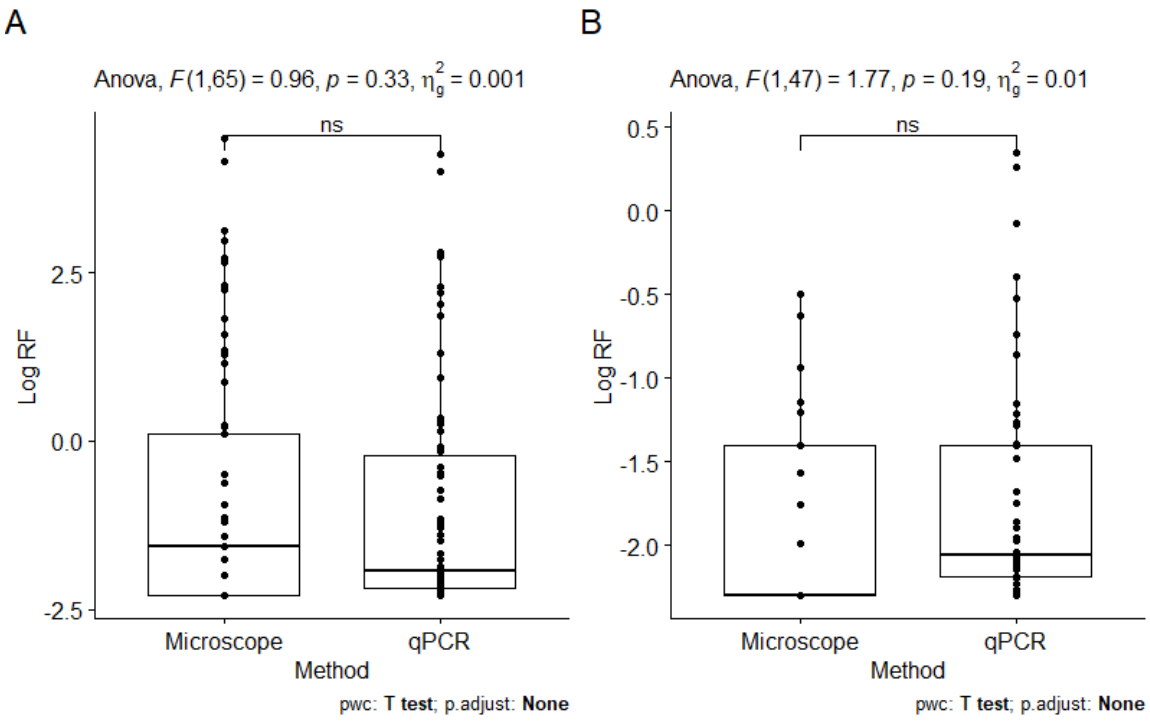

Figure 3.6 Comparison of *Meloidogyne chitwoodi* reproduction factor (RF = final population density/initial inoculation density) calculated from estimates made by microscopy versus qPCR. All data represented (N=66) in panel **A** and RF < 1 (N = 47) in panel **B**. data were ln(x+0.1)-transformed prior to ANOVA and significant differences were determined at $P = 0.05$.

Chapter 4 – General Conclusions

Globally, root-knot nematodes (*Meloidogyne* spp.) are one of the most economically important nematode pests (Braun-Kiewnick & Kiewnick, 2018; Elling, 2013). The Pacific Northwest (PNW) is no exception with more than $6.5 billion in agricultural commodities serving as susceptible hosts to one or both of the region's two predominant species of root-knot nematode: *Meloidogyne hapla* and *Meloidogyne chitwoodi* (Zasada et al., 2018 and 2019). Growers rely on diagnostic laboratories to make pest management decisions. Due to the devastating damage that plant-parasitic nematodes can inflict on crops, stakes are high to accurately identify and quantify plant-parasitic nematodes. Virtually every nematode management decision (nematicide choice, crop rotation, choice of resistant cultivars) relies on sensitive and accurate diagnostics (Hodson et al., 2021; Yan et al., 2013).

Currently, the field of nematology largely relies on microscopy and morphology to enumerate and identify nematodes. This method is labor intensive, requires significant expertise, and fallible. As training and expertise in nematode taxonomy declines, molecular tools for use in nematology need to be developed so the field can transition to a high throughput, twenty-first century approach to research and diagnostics (Braun-Kiewnick and Kiewnick, 2018). This will also allow people who are more broadly trained in biology to participate in nematology.

In chapter 2, a multiplex hydrolysis probe qPCR targeting *Hsp90* was developed. To keep the reaction as efficient as possible, one set of primers was designed with two species- specific probes. In the application of the assay to samples collected from

agricultural fields, significant limitations with quantification were revealed. Detection, on the other hand was more consistent for the multiplex qPCR and proved to be a promising feature. The assay was also tested in singleplex with the *M. chitwoodi* specific *Mc* FAM probe and was slightly more sensitive than the multiplex. More investigation in needed to determine the limits of singleplex on field samples.

The goal of chapter 3 was to validate the potato canister assay for use in high-throughput screening for *M. chitwoodi* resistance. In the canister assay soil is added to the canister and planted with potato. The canister is then inoculated with *M. chitwoodi* and incubated at constant temperature for the duration of the experiment until the canister is destructively harvested and eggs extracted and enumerated. This method is space (the canisters can be stacked and stored in an incubator) and time saving (only requires 6 weeks of incubation). That is much less time and space than is required of a greenhouse or field experiment. The assay can also be run year-round as long a tubers and inoculum are available. On the back end of the canister assay, egg enumeration by microscopy was compared to qPCR egg density estimates. The qPCR proved more sensitive for egg enumeration than microscopy microscope. This level of sensitivity will be useful when for breeding for immunity to *M. chitwoodi* in potato.

Bibliography

Allen, M., Hart, W., & Baghott, K. (1970). Crop rotation controls barley root-knot nematode at Tulelake. *California Agriculture*, *24*(7), 4–5.

Arora, D., Yan, G., & Baidoo, R. (2020). Developing a real-time PCR assay for direct detection and quantification of *Pratylenchus scribneri* in field soil. *Nematology*, 1–12. https://doi.org/10.1163/15685411-00003336

Bali, S., Zhang, L., Franco, J., & Gleason, C. (2021). Biotechnological advances with applicability in potatoes for resistance against root-knot nematodes. *Current Opinion in Biotechnology*, *70*, 226–233. https://doi.org/10.1016/j.copbio.2021.06.010

Braun-Kiewnick, A., & Kiewnick, S. (2018). Real-time PCR, a great tool for fast identification, sensitive detection and quantification of important plant-parasitic nematodes. *European Journal of Plant Pathology*, *152*(2), 271–283. https://doi.org/10.1007/s10658-018-1487-7

Brown, C. R., Mojtahedi, H., James, S., Novy, R. G., & Love, S. (2006). Development and evaluation of potato breeding lines with introgressed resistance to Columbia root-knot nematode (*Meloidogyne chitwoodi*). *American Journal of Potato Research*, *83*(1), 1–8. https://doi.org/10.1007/BF02869604

Brown, C. R., Mojtahedi, H., & Santo, G. S. (1991). Resistance to Columbia root-knot nematode in *Solanum* ssp. And in hybrids of *S. hougasii* with tetraploid cultivated potato. *American Potato Journal*, *68*(7), 445–452. https://doi.org/10.1007/BF02853782

Brown, C. R., Mojtahedi, H., & Santo, G. S. (1995). Introgression of resistance to Columbia and Northern root-knot nematodes from *Solanum bulbocastanum* into cultivated potato. *Euphytica*, *83*(1), 71–78. https://doi.org/10.1007/BF01677863

Brown, C. R., Mojtahedi, H., & Santo, G. S. (1999). Genetic Analysis of Resistance to Meloidogyne chitwoodi Introgressed from *Solanum hougasii* into Cultivated Potato. *Journal of Nematology*, *31*(3), 264–271.

Brown, C. R., Zhang, L., & Mojtahedi, H. (2014). Tracking the R Mc1 Gene for Resistance to Race 1 of Columbia Root-Knot Nematode (*Meloidogyne chitwoodi*) in Three Mexican Wild Potato Species with Different Ploidies. *American Journal of Potato Research*, *91*(2), 180–185. https://doi.org/10.1007/s12230-013-9336-x

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Caillaud, M.-C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., de Almeida Engler, J., Abad, P., Rosso, M.-N., & Favery, B. (2008). Root-knot nematodes manipulate plant cell functions during a compatible interaction. *Journal of Plant Physiology*, *165*(1), 104–113. https://doi.org/10.1016/j.jplph.2007.05.007

Castagnone-Sereno, P., Esparrago, G., Abad, P., Leroy, F., & Bongiovanni, M. (1995). Satellite DNA as a target for PCR-specific detection of the plant-parasitic

nematode *Meloidogyne hapla*. *Current Genetics*, *28*(6), 566–570. https://doi.org/10.1007/BF00518170

Desaeger, J., Wram, C., & Zasada, I. (2020). New reduced-risk agricultural nematicides—Rationale and review. *Journal of Nematology*, *52*, 1–16. https://doi.org/10.21307/jofnem-2020-091

Duncan, L. W. (1991). Current options for nematode management. *Annual Review of Phytopathology*, *29*(1), 469–490. https://doi.org/10.1146/annurev.py.29.090191.002345

Edwards, K., Logan, J., & Saunders, N. (2004). *Real-time PCR: An essential guide / edited by Kirstin Edwards, Julie Logan, and Nick Saunders.* Horizon Bioscience.

Elling, A. A. (2013). Major Emerging Problems with Minor *Meloidogyne* Species. *Phytopathology*, *103*(11), 1092–1102. https://doi.org/10.1094/PHYTO-01-13-0019-RVW

Filialuna, O., Wram, C., and Zasada, I. 2022. What is the optimal way to assess *Meloidogyne* spp. reproduction in greenhouse pot experiments? *Journal of Nematology,* DOI: 10.2478/jofnem-2022-0012.

Foot, M. A. (1977). Laboratory rearing of potato cyst nematode; a method suitable for pathotyping and biological studies. *New Zealand Journal of Zoology*, *4*(2), 183–186. https://doi.org/10.1080/03014223.1977.9517951

Gartner, U., Hein, I., Brown, L. H., Chen, X., Mantelin, S., Sharma, S. K., Dandurand, L.-M., Kuhl, J. C., Jones, J. T., Bryan, G. J., & Blok, V. C. (2021). Resisting Potato Cyst Nematodes With Resistance. *Frontiers in Plant Science*, *12*, 661194. https://doi.org/10.3389/fpls.2021.661194

Gillan, V., & Devaney, E. (2014). Nematode Hsp90: Highly conserved but functionally diverse. *Parasitology*, *141*(9), 1203–1215. https://doi.org/10.1017/S0031182014000304

Golden, A. M., O'Bannon, J. H., Santo, G. S., & Finley, A. M. (1980). Description and SEM observations of *Meloidogyne chitwoodi* n. sp. (Meloidogynidae), a root-knot nematode on potato in the Pacific Northwest. *Journal of Nematology*, *12*(4), 319–327.

Gorny, A. M., Wang, X., Hay, F. S., & Pethybridge, S. J. (2019). Development of a Species-Specific PCR for Detection and quantification of *Meloidogyne hapla* in soiluUsing the *16D10* root-knot nematode effector gene. *Plant Disease*, *103*(8), 1902–1909. https://doi.org/10.1094/PDIS-09-18-1539-RE

Graebner, R. C., Brown, C. R., Ingham, R. E., Hagerty, C. H., Mojtahedi, H., Quick, R. A., Hamlin, L. L., Wade, N., Bamberg, J. B., & Sathuvalli, V. (2018). Resistance to *Meloidogyne chitwoodi* identified in wild potato species. *American Journal of Potato Research*, *95*(6), 679–686. https://doi.org/10.1007/s12230-018-9674-9

Hodson, A. K., Cicchetto, A., & Fierro, F. A. (2021). Real time PCR assays to detect and quantify the nematodes *Pratylenchus vulnus* and *Mesocriconema xenoplax*. *Crop Protection*, *145*, 105617. https://doi.org/10.1016/j.cropro.2021.105617

Huang, D., Yan, G., Gudmestad, N., Ye, W., Whitworth, J., Frost, K., Crow, W., & Hajihassani, A. (2019). Developing a one-step multiplex PCR assay for rapid detection of four stubby-root nematode secies, *Paratrichodorus allius*, *P. minor*, *P. porosus*, and *Trichodorus obtusus*. *Plant Disease*, *103*(3), 404–410. https://doi.org/10.1094/PDIS-06-18-0983-RE

Ingham, R. E. (1994). Nematodes. In *Methods of Soil Analysis* (pp. 459–490). John Wiley & Sons, Ltd. https://doi.org/10.2136/sssabookser5.2.c22

Ingham, R. E., Hamm, P. B., Williams, R. E., & Swanson, W. H. (2000). Control of *Meloidogyne chitwoodi* in potato with fumigant and nonfumigant nematicides. *Journal of Nematology*, *32*(4S), 556–565.

Janssen, G. J. W., Van Norel, A., Verkerk-Bakker, B., & Janssen, R. (1995). Detecting resistance to the root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi* in potato and wild *Solanum* spp. *Potato Research*, *38*(4), 353–362. https://doi.org/10.1007/BF02357740

Janssen, G.J.W., Norel A.V., Janssen, R., and Hoogendoorn, J. 1997. Dominant and additive resistance to the root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* in Central American Solanum species. Theor. Appl. Genet. 94:692–700.

Kaczmarek, A., MacKENZIE, K., Kettle, H., & Blok, V. (2014). Influence of soil temperature on *Globodera rostochiensis* and *Globodera pallida*. *Phytopathologia Mediterranea*, *53*(3), 396–405. https://doi.org/10.14601/Phytopathol_Mediterr-13512

Kaloshian, I., Roberts, P. A., & Thomason, I. J. (1989). Resistance in Triticum and Aegilops spp. To Meloidogyne chitwoodi. *Journal of Nematology*, *21*(4S), 632–634.

Kapur-Ghai, J., Kaur, M., & Goel, P. (2014). Development of enzyme linked immunosorbent assay (ELISA) for the detection of root-knot nematode *Meloidogyne incognita*. *Journal of Parasitic Diseases: Official Organ of the*

Indian Society for Parasitology, *38*(3), 302–306. https://doi.org/10.1007/s12639-013-0246-0

Katoh, K. (2002). MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*(14), 3059–3066. https://doi.org/10.1093/nar/gkf436

King, B. A., & Taberna, J. P. (2013). Site-specific management of *Meloidogyne chitwoodi* in Idaho potatoes Using 1,3-Dichloropropene; Approach, Experiences, and Economics. *Journal of Nematology*, *45*(3), 202–213.

Kolombia, Y. A., Karssen, G., Viaene, N., Kumar, P. L., de Sutter, N., Joos, L., Coyne, D. L., & Bert, W. (2017). Diversity of root-knot nematodes associated with tubers of yam (*Dioscorea* spp.) established using isozyme analysis and mitochondrial DNA-based identification. *Journal of Nematology*, *49*(2), 177–188.

LaMondia, J. A., & Brodie, B. B. (1984). An Observation Chamber Technique for Evaluating Potential Biocontrol Agents of *Globodera rostochiensis. Journal of Nematology*, *16*(1), 112–115.

Leal, I., Allen, E., Foord, B., Anema, J., Reisle, C., Uzunovic, A., Varga, A., & James, D. (2015). Detection of living *Bursaphelenchus xylophilus* in wood, using reverse transcriptase loop-mediated isothermal amplification (RT-LAMP). *Forest Pathology*, *45*(2), 134–148. https://doi.org/10.1111/efp.12149

Lopes, E. A., Roberts, D. M., & Blok, V. C. (2019). Variable ITS-copy number at different developmental stages of *Meloidogyne hapla* and *M. chitwoodi. European Journal of Plant Pathology*, *154*(3), 843–848. https://doi.org/10.1007/s10658-019-01672-8

Man, S. M., Kaakoush, N. O., Octavia, S., & Mitchell, H. (2010). The Internal Transcribed Spacer Region, a New Tool for Use in Species Differentiation and Delineation of Systematic Relationships within the Campylobacter Genus. *Applied and Environmental Microbiology*, *76*(10), 3071–3081. https://doi.org/10.1128/AEM.02551-09

Mangiafico, S (2022). _rcompanion: Functions to Support Extension Education Program Evaluation_. R package version 2.4.15, <https://CRAN.R-project.org/package=rcompanion>.

Moloney, C., Griffin, D., Jones, P. W., Bryan, G. J., McLean, K., Bradshaw, J. E., & Milbourne, D. (2010). Development of diagnostic markers for use in breeding potatoes resistant to *Globodera pallida* pathotype Pa2/3 using germplasm derived from *Solanum tuberosum* ssp. Andigena CPC 2802. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *120*(3), 679–689. https://doi.org/10.1007/s00122-009-1185-0

Mugniéry, D., Phillips, M. S., Rumpenhorst, H. J., Stone, A. R., Treur, A., & Trudgill, D. L. (1989). Assessment of partial resistance of potato to, and pathotype and virulence differences in, potato cyst nematodes. *EPPO Bulletin*, *19*(1), 7–25. https://doi.org/10.1111/j.1365-2338.1989.tb00124.x

Nischwitz, C., Skantar, A., Handoo, Z. A., Hult, M. N., Schmitt, M. E., & McClure, M. A. (2013). Occurrence of *Meloidogyne fallax* in North America, and molecular Characterization of *M. fallax* and *M. minor* from U.S. Golf Course Greens. *Plant Disease*, *97*(11), 1424–1430. https://doi.org/10.1094/PDIS-03-13-0263-RE

Niu, J., Guo, Q., Jian, H., Chen, C., Yang, D., Liu, Q., & Guo, Y. (2011). Rapid detection of *Meloidogyne* spp. By LAMP assay in soil and roots. *Crop Protection*, *30*(8), 1063–1069. https://doi.org/10.1016/j.cropro.2011.03.028

Nyczepir, A. P., O'Bannon, J. H., Santo, G. S., & Finley, A. M. (1982). Incidence and Distinguishing Characteristics of Meloidogyne chitwoodi and M. hapla in Potato from the Northwestern United States. *Journal of Nematology*, *14*(3), 347–353.

Oostenbrink, M. (1966). *Major characteristics of the relation between nematodes and plants* (No. 66–4; p.). Veenman. https://library.wur.nl/WebQuery/wurpubs/516983

Phillips, M. S., Forrest, J. M. S., & Wilson, L. A. (1980). Screening for resistance to potato cyst nematode using closed containers. *Annals of Applied Biology*, *96*(3), 317–322. https://doi.org/10.1111/j.1744-7348.1980.tb04782.x

Pinkerton, J. N., Santo, G. S., & Mojtahedi, H. (1991). Population dynamics of *Meloidogyne chitwoodi* on Russet Burbank Potatoes in relation to degree-day accumulation. *Journal of Nematology*, *23*(3), 283–290.

Poczai, P., & Hyvönen, J. (2010). Nuclear ribosomal spacer regions in plant phylogenetics: Problems and prospects. *Molecular Biology Reports*, *37*(4), 1897–1912. https://doi.org/10.1007/s11033-009-9630-3

Powers, T., Harris, T., Higgins, R., Mullin, P., & Powers, K. (2018). Discovery and Identification of Meloidogyne Species Using COI DNA Barcoding. *Journal of Nematology*, *50*(3), 399–412. https://doi.org/10.21307/jofnem-2018-029

R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raymaekers, M., Smets, R., Maes, B., & Cartuyvels, R. (2009). Checklist for optimization and validation of real-time PCR assays. *Journal of Clinical Laboratory Analysis*, *23*(3), 145–151. https://doi.org/10.1002/jcla.20307

Ryss, A. (2014). Book review. Perry R.N. & Moens M. (eds). 2013. Plant Nematology, Second edition. Wallingford, Oxfordshire, UK and Boston, USA, CABI Publishing, 536 pp. ISBN 978-1-78064-151-5 (hardback); ISBN 978-1-78064-153-9 (paperback). *Russian Journal of Nematology*, *22*, 77–82.

Santo, G. S., & O'Bannon, J. H. (1981). Effect of soil temperature on the pathogenicity and reproduction of *Meloidogyne chitwoodi* and *M. hapla* on Russet Burbank potato. *Journal of Nematology*, *13*(4), 483–486.

Sapkota, R., Skantar, A. M., & Nicolaisen, M. (2016). A TaqMan real-time PCR assay for detection of *Meloidogyne hapla* in root galls and in soil. *Nematology*, *18*(2), 147–154. https://doi.org/10.1163/15685411-00002950

Sato, K., Kadota, Y., & Shirasu, K. (2019). Plant immune responses to parasitic nematodes. *Frontiers in Plant Science*, *10*. https://www.frontiersin.org/article/10.3389/fpls.2019.01165

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova, E., Voigt, K., Crous, P. W., Miller, A. N., Wingfield, M. J., Aime, M. C., An, K.-D., Bai, F.-Y., Barreto, R. W., Begerow, D., … Schindel, D.

(2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241–6246. https://doi.org/10.1073/pnas.1117018109

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *Journal of Applied Microbiology*, *113*(5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Seesao, Y., Gay, M., Merlin, S., Viscogliosi, E., Aliouat-Denis, C. M., & Audebert, C. (2017). A review of methods for nematode identification. *Journal of Microbiological Methods*, *138*, 37–49. https://doi.org/10.1016/j.mimet.2016.05.030

Skantar, A. M., & Carta, L. K. (2004). Molecular Characterization and Phylogenetic Evaluation of the Hsp90 Gene from Selected Nematodes. *Journal of Nematology*, *36*(4), 466–480.

Skelsey, P., Kettle, H., MacKenzie, K., & Blok, V. (2018). Potential impacts of climate change on the threat of potato cyst nematode species in Great Britain. *Plant Pathology*, *67*(4), 909–919. https://doi.org/10.1111/ppa.12807

Smith, T., Brito, J. A., Han, H., Kaur, R., Cetintas, R., & Dickson, D. W. (2015). Identification of the peach root-knot nematode, *Meloidogyne floridensis*, using mtdna pcr-rflp. *Nematropica*, *45*(1), 138–143.

Wang, D. Y.-C., Kumar, S., & Hedges, S. B. (1999). Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*(1415), 163–171. https://doi.org/10.1098/rspb.1999.0617

Wram, C., & Zasada, I. A. (2020). Differential response of *Meloidogyne* , *Pratylenchus* , *Globodera* , and *Xiphenema* species to the nematicide fluazaindolizine. *Phytopathology*, PHYTO-05-20-0189-R. https://doi.org/10.1094/PHYTO-05-20-0189-R

Yan, G., Smiley, R. W., Okubara, P. A., Skantar, A. M., & Reardon, C. L. (2013). Developing a Real-Time PCR Assay for Detection and Quantification of Pratylenchus neglectus in Soil. *Plant Disease*, *97*(6), 757–764. https://doi.org/10.1094/PDIS-08-12-0729-RE

Zasada, I. A., Halbrendt, J. M., Kokalis-Burelle, N., LaMondia, J., McKenry, M. V., & Noling, J. W. (2010). Managing nematodes without methyl bromide. *Annual Review of Phytopathology*, *48*(1), 311–328. https://doi.org/10.1146/annurev-phyto-073009-114425

Zasada, I. A., Kitner, M., Wram, C., Wade, N., Ingham, R. E., Hafez, S., Mojtahedi, H., Chavoshi, S., & Hammack, N. (2019). Trends in occurrence, distribution, and population densities of plant-parasitic nematodes in the Pacific Northwest of the United States from 2012 to 2016. *Plant Health Progress*, *20*(1), 20–28. https://doi.org/10.1094/PHP-11-18-0077-RS

Zijlstra, C., & Van Hoof, R. A. (2006). A multiplex real-Time polymerase chain reaction (TaqMan) assay for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax*. *Phytopathology*, *96*(11), 1255–1262. https://doi.org/10.1094/PHYTO-96-1255