# DEVELOPING AND TESTING INTEGRATED PEST MANAGEMENT STRATEGIES: FROM COVER CROP TREATMENTS TO THE CHARACTERIZATION AND DETECTION OF *PSEUDOPERONOSPORA CUBENSIS* AND *P. HUMULI*

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Carly Faye Summers

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ABSTRACT

DEVELOPING AND TESTING INTEGRATED PEST MANAGEMENT STRATEGIES: FROM COVER CROP TREATMENTS TO THE CHARACTERIZATION AND DETECTION OF *PSEUDOPERONOSPORA CUBENSIS* AND *P. HUMULI*

Carly Summers, Ph.D.

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The practice of integrated management of plant diseases incorporates a vast body of scientific information for effective pathogen control as well as environmental stewardship. Pathogen biology, environmental and epidemiological patterns, preventative cultural practices and judicious chemical applications are a few examples of the scientific areas of research used to support integrated disease management. This body of work focuses on research involved in the integrated disease management approach: cover cropping, early detection of plant pathogens and a pathogen population study. The first chapter explores the effects of mixed-species cover crops on organic tomato crop productivity and disease severity in the field. This research, taken over three field seasons (2010, 2011 and 2012) at sixteen field sites in three states, Maryland, New York and Ohio (MD, NY and OH), each with distinct soilborne disease pressure, was based on measurement of plant growth and vigor, crop yield and disease ratings of naturally occurring infection over time. Our research revealed no short-term effects of cover crop treatments on productivity or disease severity in tomato crops. The second chapter looked at the same tomato crops, turning attention to how the cover crops affect fungal and oomycete pathogen presence in the rhizosphere of tomato plants. For this, two years (2010 and 2011) of field samples from 260 plots were evaluated using two molecular tools: macroarray analysis, a diagnostic tool used to test for the presence of 31 solanaceous pathogens, and terminal restriction fragment length polymorphism (T-RFLP), used to evaluate the microbial fingerprint of the samples. Our findings showed that cover crops did not affect pathogen

presence in the rhizosphere and that macroarray was able to detect certain fungi with much greater sensitivity than T-RFLP. Chapter 3 explores the development of techniques used for early detection of *Pseudoperonospora cubensis* and *P. humuli*, causal agents of cucurbit and hop downy mildews, respectively. Two techniques were designed which allow for detection and differentiation of both pathogens from environmental samples: real-time PCR with locked-nucleic acid probes and high resolution melt curve analysis. These assays were then used to test for pathogen presence in air samples collected in New York using spore traps. Chapter 4 presents the results of a comparative analysis between RNA sequencing (RNA-seq) and genotyping by sequencing (GBS) conducted in order to determine which technique is most effective in single nucleotide polymorphism (SNP) identification for *Pseudoperonospora cubensis* and *P. humuli*, as well as to discover novel SNPs that determine population structure between and among these two genetically similar species. Finally, included in the appendix is a case study summarizing a collaboration with St. John's University in Tanzania, facilitated by a Horticulture Collaborative Research Support Program grant. The project aims were to train a graduate student in extension talks for IPM of tomato, implement the talks with growers and a pesticide alternative in an experiment-based field study.

## BIOGRAPHICAL SKETCH

Carly Faye Summers received a Bachelor of Arts in Biochemistry from New College, a small liberal arts college in Sarasota, Florida. After graduating in 2005, she served in the Peace Corps in Ahuachapán, El Salvador, for two years and three months. As part of the Agroforestry and Environmental Education field unit, she worked with farmers, families and schools in various areas from integrated pest management to recycling. Upon return to Florida, she worked as a research chemist at Archer Pharmaceuticals, a systematics botanist at Selby Gardens then a technician in a plant pathology laboratory at the University of Florida. In 2010 she began her PhD work at Cornell, working with Dr. Christine Smart.

For my loving family~ Nancy Jo Summers, James Eric Summers and Rebecca Claire Summers.

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# INTRODUCTION

Despite advances in the research of plant diseases, pathogens continue to devastate crops worldwide (Agrios 2005). Most growers today strive to control plant disease by employing an integrated management approach (IPM): a body of practices which monitor for critical population thresholds of pests and pathogens in order to mitigate damage from disease, while concurrently minimizing negative impacts on the ecosystem and beneficial organisms (Chellemi 2002). This paradigm works to maximize crop health while striving for the greatest environmental conservation. Growers benefit from IPM practices by increased economic gain, higher yields and reduced risks, while the economic costs of both human and environmental health risks from chemical exposure are reduced (Mullen et al. 1997). IPM practices have gained public support, as concern for food and farm worker safety, groundwater purity and environmental awareness has risen (Mullen et al. 1997). Because of rapid pathogen adaptation to chemical control and resistant crop varieties (McDonald & Linde 2002) as well as the dynamic impacts of climate change in agriculture (Howden et al. 2007), IPM research will continue to be at the forefront developing new strategies for secure and sustainable production of food.

The practices involved in the integrated management of plant disease include: prevention, setting action thresholds, monitoring and chemical control. In disease prevention, various actions are recommended which may reduce pest levels through cultural practices, integrating aspects of a healthy ecosystem into the field. Examples of key cultural controls include the use of cover crops, crop rotations and reduced tillage (Snapp et al. 2005). The action threshold is the critical level of pathogen presence at which the crop suffers and yields are reduced, below which taking action results in unsubstantiated economic as well as environmental costs (Jacobsen 1997). In order to determine action thresholds, the identification and monitoring of pathogens is necessary. This step

allows growers and scientists to know when the pathogen is present in a particular location, anticipate the interaction of the pathogen population with weather conditions, as well as determine timings of outbreaks and biological or chemical control programs (Ojiambo et al. 2009). Finally, integrated into a program designed to minimize pathogen populations naturally, is the judicious use of pesticides, which requires informed research that maximizes effectiveness of chemicals while striving to apply as little, and as targeted, pesticides as needed (Jacobsen 1997).

Chapters 1 & 2 of this thesis investigate one of the key cultural controls used by growers to prevent disease and improve crop health: the planting of cover crops. Cover crops have been shown to improve soil and crop health by increasing soil organic matter, reducing erosion, improving physical characteristics of the soil, preventing leaching of soil nitrogen and suppressing weeds (Thurston 1990; Snapp et al. 2005). Numerous experiments have also shown that cover crops can increase yield and protect crop plants from soilborne disease (Abawi & Widmer 2000; Abdul-Baki et al. 1996; Bulluck & Ristaino 2002; Larkin & Griffin 2007; Mazzola & Mullinix 2005; Zhou & Everts 2004). A variety of cover crop species have been used, including members of the Gramineae, Leguminaceae and Brassicaceae families, which each have unique benefits. Mixing species can allow growers to make use of the advantages of individual species and even enhance overall benefits, including the maximization of weed control, nitrogen sequestration, biomass production and diversity of beneficial insect populations (Treadwell et al. 2010). This study examined the effects of mixed-species cover crops on organic tomato crop productivity and disease severity (Chapter 1) as well as fungal and oomycete pathogens in the crop rhizosphere (Chapter 2). While researchers have studied the effects of green manures on crop health for decades, this research was novel in that it 1) focused on the effects of mixed-species cover crops and 2) evaluated the single-season effects of cover crop application, instead of the accumulative effects of subsequent plantings. These chapters

highlight the growing body of research on exploration of the microbial ecology of soils and the impact of microbial communities on plant pathogens and crops alike.

Chapter 3 is concerned with a different aspect of IPM: the identification and monitoring of pathogens. This chapter focuses on the aerially dispersed, obligate biotrophic plant pathogens *Pseudoperonospora cubensis* and *P. humuli,* causal organisms of cucurbit and hop downy mildew, respectively. From the time of infection to the onset of disease symptoms for cucurbit downy mildew, four to twelve days may pass (Savory et al. 2011), leaving a window of opportunity available for growers to treat infected plants before disease is rampant. The predictive system Cucurbit Downy Mildew IPM Pest Information Platform for Extension and Education (CDM ipm-PIPE) is available to alert growers to imminent risk, considering weather and disease reports to calculate probability of infection for a given region (Ojiambo & Holmes 2011). This information is important for helping growers decide when to initiate fungicide applications. However, a predictive system could forecast infection risk more accurately if inoculum propagule detection were combined with weather data (West et al. 2008). A specific detection method differentiating these two closely related pathogens was previously not possible (Gent et al. 2009). Chapter 3 reports the development of two real-time PCR assays, utilizing locked-nucleic acid probes and high resolution melt curve analysis, for molecular differentiation of *Pseudoperonospora cubensis* and *P. humuli*. These assays are then demonstrated to be useful for diagnosis of symptomatic leaf tissue.

One important objective of plant disease management is to understand the structure and diversity of pathogen populations (Milgroom & Peever 2003). Genetic markers can be used to identify pathogen genotypes, which can be studied for host specialization, fungicide sensitivity, dispersal patterns and to determine sources of inoculum (Milgroom & Fry 1997). Elucidating these important characteristics of a pathogen's population biology is imperative for effective development

and employment of control strategies, such as the breeding of resistant hosts, fungicide applications, and creating regulatory measures to limit pathogen immigration (Milgroom & Fry 1997). In addition, they are used to estimate a pathogen's evolutionary potential to pose risk of adapting to control measures (McDonald & Linde 2002). Chapter 4 describes our study of *P. cubensis* and *P. humuli* isolates, using two reduced-representation genomic library approaches, RNA-seq and genotyping-by-sequencing (GBS). RNA-seq targets expressed genes (Van Verk et al. 2013) and GBS, samples from the entire genome, including non-coding regions (Elshire et al. 2011). For the RNA-seq assays, 15 isolates of *P. cubensis* and 19 isolates of *P. humuli* were sequenced, while 20 isolates of *P. cubensis* and 18 isolates of *P. humuli* were sequenced using GBS. The overall purpose of this study was to collect and utilize genomic data to further investigate the genetic differentiation of these two closely related species. In order to accomplish this goal, our first objective was to observe variation between and among isolates of *P. cubensis* and *P. humuli* using principal components analysis (PCA) (Paschou et al. 2007; Laloe & Gautier 2012). Our second objective was to identify SNPs between the species. Our final objective was to annotate the genes containing these SNPs and identify putative pathogenicity genes. These genes may be important in host-specificity pathways and could be useful targets for pathogen detection and identification (Ellis 2006; Torto-Alalibo et al. 2007).

The final exploration of IPM discussed in this body of work is in the appendix: "Tomato IPM in Tanzania." This segment involves the outreach component of IPM, as well as its involvement in international agriculture. Arguably, outreach is equally important to the scientific research component of IPM, for if the information is not effectively disseminated, it is of no value (Nelson et al. 2001). The project described involves a collaboration with St. John's University in Tanzania funded through a grant from the Horticulture Collaborative Research Support Programs unit of USAID. The main roles I had in this project included designing a tomato IPM talk for growers in

Tanzania then working in-country with a graduate student for grower talks. In addition, I helped design a field experiment testing neem (*Azadirachta indica*) efficacy against various prominent insect pests in the area. Finally, I gave two guest lectures at St. John's University to the horticulture undergraduate classes. This experience was an important part of my education as a plant pathologist and educator.

In summary, the body of work described in this thesis explores various aspects of the research driving IPM and sustainable agriculture today. From field studies to advanced genomics experiments, to the identification and monitoring of plant pathogens from the air and the soil, the projects within illustrate some of the dynamic reaches of IPM research today.

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CHAPTER 1

# Single Season Effects of Mixed-Species Cover Crops on Tomato Health (cultivar Celebrity) in Multi-State Field Trials[1]


## Abstract

Cover crop use can help mitigate the deleterious effects of common cropping practices (e.g., tillage) and is, therefore, an important component of soil health maintenance. While known to be beneficial in the long-term, the short-term effects of cover crops, specifically mixed-species cover crops in organic systems are less clear. Cover crop effects on tomato productivity and disease severity were recorded over three field seasons (2010, 2011 and 2012) at sixteen field sites in three states, Maryland, New York and Ohio (MD, NY and OH), each with distinct soilborne disease pressure. Plots of five state-specific cover crop treatments were established the season prior to tomato production; the resulting plant residue was incorporated the following spring approximately four weeks before tomato planting. Total fruit yield along with early-season shoot height and fresh weight were used to compare treatment effects on productivity. Treatment disease severity ratings relied on natural inoculum. Interestingly, the effect of a single season of cover cropping on total yield was significant in no more than 25% of all site years. Similarly, cover crop effects on tomato disease levels were significant in 0 to 44% of the sixteen field sites. However, significant field-specific patterns were observed in every state across multiple years for some treatments. For example, in New York in 2010, tomato yields following all mixed cover crops were greater than the


[1] Reprinted from *Single Season Effects of Mixed-Species Cover Crops on Tomato Health (cultivar Celebrity) in Multi-State Field Trials*. 2014. CF Summers, S Park, AR Dunn, X Rong, JL Everts, SLF Meyer, SM Rupprecht, MD Kleinhenz, B McSpadden Gardener and CD Smart. Applied Soil Ecology. 77. 51-58.

single rye cover crop in one field, but this pattern was reversed in the adjacent field. Thus, no general recommendation of a specific cover crop mixture can be made for near-term enhancement of tomato productivity or for reduction of disease. Therefore, growers should focus on location and operation-specific variables when choosing cover crops.

## Introduction

Organic agriculture relies on ecologically based methods of crop production, employing a variety of techniques for integrated pest management and retention of soil fertility. Planting cover crops is one strategy, used for centuries, that has been shown to increase organic matter, reduce erosion, improve physical characteristics of the soil, prevent leaching of soil nitrogen, suppress weeds and reduce disease incidence (Snapp et al. 2005; Thurston 1990). Recommendations for specific cover crops have been provided based on the unique advantages each is supposed to confer. For example, Graminaceous species of cover crop (e.g. annual rye (*Lolium multiflorum* Lam.) and winter rye (*Secale cereal* M. Bieb)) improve soil physical structure, produce ample biomass adding to organic matter and sequester excess nitrogen in the soil, which prevents leaching (Snapp et al. 2005; Treadwell et al. 2010). Leguminous species (e.g. hairy vetch (*Vicia villosa* Roth), crimson clover (*Trifolium incarnatum* L.) and alfalfa (*Medicago sativa* L.)) can provide additional nitrogen through symbioses with nitrogen-fixing rhizobacteria (Snapp et al. 2005). Some members of the family Brassicaceae (eg. forage radish (*Raphanus sativus* var. *longipinnatus* L.) and forage turnip (*Brassica rapa* var. *rapa* L.)) have a long taproot, which facilitates water infiltration and breaks up compaction (Chen & Weil 2009). This family also produces glucosinolates that hydrolyze to release various volatile compounds, the most biologically active of which is isothiocyanate. These compounds have been found to serve as effective biofumigants in some systems, acting similarly to the synthetic pesticide metam sodium, which generates methyl isothiocyanate when in contact with damp soil (Matthiessen & Kirkegaard 2006). Mixing species of cover crops in one planting is a strategy to take advantage of the benefits promoted by each. For example, the carbon to nitrogen ratio can be balanced, facilitating a slower release of nitrogen, which can be better utilized by crops during the growing season. By mixing species with complementary growth patterns, a grower can also

maximize weed control, nitrogen sequestration, biomass production and diversity of beneficial insect populations (Treadwell et al. 2010). For example, by pairing rye and vetch, the quick-growing rye will prevent weed establishment and utilize nitrogen added by the vetch, which grows more slowly (Snapp et al., 2005).

Numerous experiments have shown that cover crops can increase yield and protect crop plants from soilborne disease (Abawi & Widmer 2000; Abdul-Baki et al. 1996; Bulluck & Ristaino 2002; Larkin & Griffin 2007; Mazzola & Mullinix 2005; Zhou & Everts 2004). However, not all studies have observed a benefit from specific cover crops or green manures on yield, disease severity or suppression of plant-pathogenic nematode (PPN) populations (Chellemi 2006; Hartz et al. 2005). The complex interaction of various factors such as cover crop species or cultivar, soil characteristics, crop-pathogen system and environment determines the extent to which cover crops can beneficially impact vegetable crop health. Interpreting the impacts on crop health can be complicated. For instance, one study found that cover crops improved crop health, thereby leading to decreased seedling mortality despite increased disease severity caused by *Fusarium* spp. and *Pythium* spp. (Medvecky et al. 2007).

Fewer studies have investigated mixed-species green manures. A rye-vetch green manure reduced incidence of Southern blight on tomato and increased populations of beneficial Pseudomonads (Bulluck & Ristaino 2002). A rye-legume mixture also increased yield of tomatoes and suppressed weeds more effectively than a rye monocrop (Teasdale & Abdul-Baki 1998). A rye and a rye-field pea mixture of cover crops both had positive effects on tomato growth and yield as compared to bare ground (Akemo et al. 2000). Mixed species of hay used on land in transition to organic management reduced damping-off of tomato by *Pythium* sp. and *Rhizoctonia solani* by 3-30% (Baysal et al. 2008).

The goal of this study was to investigate the near-term effects of mixed-species green manures on subsequent plantings of tomato (*Solanum lycopersicum* L.) in organic production systems. The experiment was conducted in three states at multiple locations, each with different types of soilborne disease pressure, including Phytophthora blight caused by *Phytophthora capsici* Leonian (NY), Southern blight caused by *Sclerotium rofsii* Sacc. (MD), plant-pathogenic nematodes (MD) and Rhizoctonia root rot caused by *Rhizoctonia solani* J.G. Kühn (OH). Crop and soil variables were chosen to examine the effects of a single season application of cover crops on tomato productivity and disease severity. The hypothesis tested was that a one-season application of mixed-species cover crops would provide enhanced productivity and reduce disease severity regardless of location or year, as compared to single-species cover crop applications or a bare ground control. Statistical testing was performed for each field and year separately in order to detect site-specific effects.

**Materials and Methods**

*Experimental Design and Management.* Research was conducted in 2010, 2011 and 2012 at the University of Maryland Lower Eastern Shore Research and Education Center, Salisbury, the New York State Agricultural Experimental Station, Phytophthora blight research farm in Geneva and the Ohio Agricultural Research and Development Center, Wooster. The three states and years included in the study sum to sixteen different field-site years and a total of 370 separate plots.

The experiment was conducted as a randomized complete-block design with four blocks (NY and OH) or six blocks (MD) and five treatments per block. Each year different fields were used in each location in order to test the single season impacts of the cover crop treatments.

In MD, the trial was conducted in one field per year (5 treatments x 6 blocks) for a total of 30 plots per year. Plots were 6.4 m x 12.2 m and had two rows of black plastic on 2.1 m centers. A

single row of tomatoes were transplanted 0.6 m apart within each row. Soil at this location was Fort Mott loamy sand and Rosedale loamy sand.

In NY, the trial was conducted in two fields per year (5 treatments x 4 blocks) for a total of 40 plots per year. Plots were 2.4 m x 7.6 m with one row of plastic on 3.1 m centers. A single row of tomatoes were transplanted 0.6 m apart within the row. Soil at this location was Odessa silt loam.

In OH, the trial was conducted in three fields (5 treatments x 4 blocks) for a total of 60 plots in 2010 and 2011, but only one field (5 treatments x 8 replicates) for a total of 40 plots in 2012. Plots were 3.1 m x 6.1 m with four rows of black plastic on 1.53m centers. A single row of tomatoes were transplanted 0.6 m apart within each row. In this location, the soil was a Wooster Riddles silt loam.

The five treatments of mixed-species green manure combinations were different in each state based on local growing conditions and practices. The treatments and seeding rates are listed in Table 1.1. Cover crop seed was sown in the fall and the cover crop was mowed and tilled in as a green manure the following spring three to five weeks before transplanting the tomatoes (Table 1.2). Before tilling in the cover crop biomass, the fresh above-ground plant residue was weighed from a 1.2 m x 1.2 m portion of each plot and recorded. Fields in all states had raised beds with black plastic and drip irrigation. Tomatoes were grown using standard organic practices including trellising appropriate to each location. Plants were irrigated throughout the season as needed.

**Table 1.1** Seeding rates for CCTs in MD, NY and OH

| | Treatments and Seeding Rate (kg/ha) |
|---|---|
| MD | |
| 1 | Hairy vetch (79) + winter rye (79) |
| 2 | Hairy vetch (25) |
| 3 | Hairy vetch (42) + forage radish (42) |
| 4 | Mixed-species hay (125)[a] |
| 5 | No cover |
| NY | |
| 1 | Hairy vetch (34) + winter rye (79) |
| 2 | Crimson clover (10) + annual rye (18) |
| 3 | Forage turnip (15) + winter rye (45) |
| 4 | Winter rye (135) |
| 5 | No cover |
| OH | |
| 1 | Mixed-species hay (56/112) [a] |
| 2 | Winter rye (150) |
| 3 | Hairy vetch (50) |
| 4 | Hairy vetch (25) + winter rye (75) |
| 5 | Forage radish (10) |

[a] Mixed-species hay included red fescue (*Festuca rubra* L.), orchard grass (*Dactylis glomerata* L.), timothy (*Phleum pratense* L.), red clover and alfalfa. Composition was determined by equal seed number in MD and OH. Seeding rates in OH were 56 kg/ha in 2010 and 112 kg/ha in 2011 and 2012.

Tomato seeds were sown into a locally-produced organic potting mix in 50-cell flats (TO Plastics, Clearwater, MN) and maintained in a greenhouse with sixteen hours of both natural and supplemental light per day. Seedlings were moved into a cold frame for at least 24 h before transplant. Cultivar Celebrity (Johnny's Select Seed, Winslow, ME) was used. This cultivar has disease resistance to Verticillium wilt, Fusarium wilt Races 1 and 2, root-knot nematodes, Alternaria stem canker and tobacco mosaic virus (Rutgers Cooperative Extension, 2013). This variety was chosen because it is widely grown in the test region and has fairly standard disease resistance.

Because host resistance is part of an effective integrative disease management program, cultivar Celebrity was deemed to be part of a grower-relevant model for assessing cover crop effects.

*Soil and Plant Productivity Analysis.* At the time of transplant (Table 1.2), 473 mL of soil was collected at a depth of 15-18 cm from each plot in 2010 and 2011 and analyzed at soil testing labs in each state, specifically Dairy One, Ithaca, NY, the University of Delaware Soil Testing Laboratory, Newark, DE and the Service Testing and Research lab, Wooster, OH. The analysis included organic matter (%), pH, phosphorus, potassium and calcium measurements.

Effects on early plant vigor were measured by comparing the height and fresh shoot weight of the above-ground portion of two tomato plants per plot at four weeks post-transplant (Table 1.2). Plant height was measured in the field from the crown at soil-level to the apical meristem and is reported as shoot height. The total above-ground weight of plants cut off at soil-level was measured in the field using a portable scale in NY (Fisher Scientific, Pittsburg, PA) or transported to the lab and weighed in MD (Sartorius Universal, Goettingen, Germany) and OH (Mettler Toledo, Toledo, OH) and is reported as shoot weight.

Tomatoes were harvested and weighed from each plot to determine the total yield (Table 1.2). Harvest began when at least 10% of the plots had ripe (>stage 5) fruit ready to harvest and each harvest was separated by 7-10 days. Only ripe fruit was harvested during the initial harvests, while all fruit larger than 4 cm in diameter were picked during the last harvest. For MD, in 2010, two plants per plot were harvested three times. In 2011, four plants per plot were harvested eight times. In 2012, four plants per plot were harvested five times. In NY, six plants per plot were harvested three times for 2010, 2011 and 2012. In OH, two plants per plot were harvested three times in 2010 and 2012 and two times in 2011.

**Table 1.2** MD, NY and OH field timeline 2010, 2011 and 2012

| | Activity | 2010 | 2011 | 2012 |
|---|---|---|---|---|
| MD | Seeded Cover Crops | 9/25/2009 | 10/11/2010 | 10/7/2011 |
| | Seeded Tomatoes | 4/6/2010 | 4/11/2011 | 4/25/2012 |
| | Weighed CC Biomass | 4/15/2010 | 5/2/2011 | 4/11/2012 |
| | Mowed and Tilled CC Biomass | 4/16/2010 | 5/2/2011 | 5/4/2012 |
| | Laid Plastic | 5/4/2010 | 5/17/2011 | 5/23/2012 |
| | Transplanted Tomatoes | 5/14/2010 | 5/20/2011 | 5/31/2012 |
| | Shoot Height | 6/14/2010 | 5/23/2011 | 7/2/2012 |
| | Shoot Weight | 6/14/2010 | 5/23/2011 | 7/10/2012 |
| | Harvest 1 | 8/3/2010 | 8/2/2011 | 8/14/2012 |
| | Harvest 2 | 8/13/2010 | 8/5/2011 | 8/17/2012 |
| | Harvest 3 | 8/26/2010 | 8/9/2011 | 8/21/2012 |
| | Harvest 4 | | 8/12/2011 | 8/24/2012 |
| | Harvest 5 | | 8/16/2011 | 8/29/2012 |
| | Harvest 6 | | 8/19/2011 | |
| | Harvest 7 | | 8/23/2011 | |
| | Harvest 8 | | 8/26/2011 | |
| | Harvest 9 | | 8/30/2011 | |
| | Nematode Samples (Soil) | 4/16/2010 | 4/14/2011 | 4/25/2012 |
| | | 4/29/2010 | 5/16/2011 | 5/24/2012 |
| | | 6/25/2010 | 7/6/2011 | 7/24/2012 |
| | | 8/26/2010 | 9/13/2011 | 9/20/2012 |
| | Nematode Samples (Tomato Roots) | 6/25/2010 | 7/6/2011 | 7/24/2012 |
| | | 8/26/2010 | 9/12/2011 | 9/20/2012 |
| NY | Seeded Cover Crops | 9/22/2009 | 10/1/2010 | 9/20/2011 |
| | Seeded Tomatoes | 4/22/2010 | 4/26/2011 | 4/20/2012 |
| | Weighed CC Biomass | 5/4/2010 | 5/14/2011 | 4/19/2012 |
| | Mowed and Tilled CC Biomass | 5/5/2010 | 5/14/2011 | 4/19/2012 |
| | Laid Plastic | 5/18/2010 | 6/7/2011 | 5/18/2012 |
| | Transplanted Tomatoes | 5/27/2010 | 6/10/2011 | 5/31/2012 |
| | Shoot Height, Weight | 6/30/2010 | 7/11/2011 | 7/5/2012 |
| | Harvest 1 | 8/16/2010 | 9/3/2011 | 8/23/2012 |
| | Harvest 2 | 8/25/2010 | 9/10/2011 | 9/5/2012 |
| | Harvest 3 | 9/2/2010 | 9/17/2011 | 9/13/2012 |
| OH | Seeded Cover Crops | 9/25/2009 | 8/31/2010 | 9/01/2011 |
| | Seeded Tomatoes | 4/22/2010 | 4/15/2011 | 4/17/2012 |
| | Weighed CC Biomass | 4/14/2010 | 5/02/2011 | 5/10/2012 |
| | Mowed and Tilled CC Biomass | 4/14/2010 | 5/10/2011 | 5/13/2012 |
| | Laid Plastic | 5/27/2010 | 6/15/2011 | 6/18/2012 |
| | Transplanted Tomatoes | 06/03/2010 | 6/15/2011 | 6/18/2012 |
| | Shoot Height, Weight | 7/13/2010 | 7/25/2011 | 7/17/2012 |
| | Harvest 1 | 8/27/2010 | 9/16/2011 | 8/21/2012 |
| | Harvest 2 | 9/03/2010 | 9/21/2011 | 8/28/2012 |
| | Harvest 3 | 9/09/2010 | - | 9/4/2012 |

*Disease Severity Analysis.* In Maryland, plots were rated for disease at approximately 10-day intervals during the growing season. Early blight (*Alternaria solani* Sorauer and *A. tomatophila* Simmons), Septoria leaf spot (*Septoria lycopersici* Speg.), and Southern blight (*Sclerotium rolfsii* Sacc.) were present each year. Early blight and Septoria leaf spot were rated as the average percentage of infected leaf area in three 1 m sections of each plot. Southern blight incidence was recorded as the percent of plants within a plot that were symptomatic. In Maryland only, each plot was also tested for the presence of nematodes. For counts of stylet-bearing nematodes, soil samples were collected four times each year: 1) pre-incorporation and 2) post-incorporation of green manures, 3) mid-season and 4) harvest (Table 1.2). On each sampling date, six soil samples (2.5 cm diam. × 20 cm deep) were randomly collected from each plot with a soil core probe and combined (with the exception that 2 soil samples were collected per plot on 4/16/2010). Nematodes were extracted from 100 $cm^3$ soil by centrifugal flotation (Jenkins 1964). At mid-season and harvest (Table 1.2), one tomato root system was harvested from each plot (two root systems per plot on 8/26/10). These root systems were rated for root galling, and nematode eggs were then collected from the roots (method similar to Hussey & Barker 1973) and enumerated.

In New York, plots were rated for disease weekly over the duration of the growing season. Three diseases were present each year: Phytophthora blight (*P. capsici* Leonian), Septoria leaf spot, and early blight. In NY fields 3 and 4 (2011), leaf mold (*Fulvia fulva* Cooke) and late blight (*Phytophthora infestans* (Mont.) de Bary) were present. The research farm where the six NY fields (2 per year) were located is known to be infested with *P. capsici* (Dunn & Smart 2012). Phytophthora blight disease severity (%) was determined for each plot by assessing wilt, yellowing and stunting for all plants in the plot. Disease severity (%) of early blight, Septoria leaf spot, late blight and leaf mold was rated by percentage of leaf tissue affected for all plants in each plot.

In Ohio, plots were rated for disease at approximately 10-day intervals during the growing season. Early blight and Septoria leaf spot were present each year. Early blight and Septoria leaf spot were rated as the average percentage of infected leaf area in three 1 m sections of each plot.

*Statistical Analysis of Productivity and Disease Severity Data.* Every crop productivity and disease-related factor from each field, state and year was analyzed separately using a general linear model (GLM) with cover crop treatment (CCT) and cover crop biomass run as fixed effects and block as a random effect. Cover crop biomass was included in the model because the biomass generated by each cover crop likely plays a large role in the effects measured. Therefore, the relationship between the cover crop species and the response variables after controlling for biomass could be evaluated. This avoided a possible erroneous correlation of effect to specific cover crop species, which may instead have been tied to the biomass generated by the species. Because cover crop biomass and treatment are inherently correlated, multicollinearity can inflate the standard errors of the model parameters. This would result in finding fewer significant pairwise differences and is therefore a more stringent test of CCT effects. However, since the biomass effects were deemed important as well, a separate ANOVA was run for the biomass of each treatment and results were added to the state-specific tables (Tables 1.4, 1.5, 1.6, 1.8, 1.9 and 1.10) for comparison between the results of the measured effect with the biomass generated by each treatment. In addition to the P-values generated by the statistical model, bar graphs were evaluated in order to mine for trends that may have been undetected by the statistical model. If the main effect of CCT was significant at $P<0.1$, then a Tukey's Honestly Significant Differences (HSD) test was used to test for significant differences among CCTs. Analyses were performed using the statistical program R (R Development Core Team 2011) with packages lme4 (Douglas et al., 2011), multcomp (Hothorn et al. 2008) and RLRsim (Scheipl et al. 2008). For disease severity data, the area under disease progress curve (AUDPC) was

calculated from the rating measurements, data was confirmed to be normal, then used in the GLM analysis (Madden et al. 2007).

Nematode data were transformed (log (x + 1)) and subjected to analysis of variance (ANOVA) with the GLM procedure of SAS JMP (SAS 2009). Nematode population density means were compared using Tukey's HSD test following a significant *F* test. Non-transformed data showing the actual number of nematodes per unit soil are presented for convenience in comparison to other papers and to numbers reported from grower's fields. Significant differences ($P < 0.10$) are discussed unless stated otherwise. Means were calculated excluding soil samples from which nematode counts were zero; the latter were excluded from analyses on the assumption that nematodes were present but populations were so low that individual nematodes were not detected in the subsamples.

**Results**

*Soil and Plant Productivity Analysis.* In order to visualize the significance of the overall effects of CCT on all measured variables in the experiment, results for all fields are presented in Table 1.2. Overall, just 25% of the sixteen sites tested responded significantly to CCT in total yield or shoot weight early in the growing season. Similarly, soil variables measured within one week of tomato transplanting (three to five weeks following cover crop incorporation) differed significantly by CCT at no more than 31% of the tested sites (Table 1.3). More detailed results for each variable are presented in state-specific tables (Tables 1.4, 1.5 and 1.6), which present the mean values and Tukey assignments for each variable when the effect of CCT was significant based on the GLM analysis. These tables are presented to allow for overall comparison of CCT effects. In order to visualize trends indicating an effect of treatment and cover crop biomass, bar graphs with productivity

measurements and disease severity data were examined for topographical patterns, both among factors and between factors (Figure 1.1 Productivity Graphs & Figure 2.2 Disease Severity Graphs).

Soil analysis was conducted on a total of 290 plots from 13 fields in the three states over two years. Specifically, CCT significantly affected the pH of soil samples in four of thirteen fields tested. In MD 2012, bare ground and mixed-species hay CCT had a higher pH than other CCT (Table 1.4). In OH field 1 (2010), mixed-species hay, rye and vetch+rye had a significantly higher pH than radish. In OH field 5 (2011), rye had a significantly higher pH than vetch. In OH field 6 (2011), radish had a higher pH than vetch or rye (Table 1.6). Overall, the effect of CCT on pH was inconsistent. Likewise, soil organic matter (%) was significantly affected by CCT in just three out of the thirteen fields tested (Table 1.3). In MD 2012, the vetch+rye CCT had significantly more organic matter than bare ground plots or the vetch CCT (Table 1.4). In NY field 2 (2010), bare ground, vetch+rye and clover+rye had significantly higher organic matter than rye and turnip+rye CCT (Table 1.5). In OH field 2 (2010) all treatments provided significantly more organic matter than the mixed-species hay (Table 1.6). The effects of CCT on soil mineral availability were also examined. In three of the thirteen fields, potassium content was affected by CCT (Table 1.3). In MD 2012, the vetch+rye CCT contained significantly more potassium than all other treatments (Table 1.4). However, in OH field 5, the mixed-species hay treatment contained significantly more potassium than only the vetch+rye-treated plots. In OH field 6 (2011), mixed-species hay and rye CCT had significantly more potassium than vetch. In only one field of thirteen, OH field 4 (2011), CCT affected phosphorus in soil, where radish had significantly higher phosphorus content than vetch+rye and vetch (Table 1.6).

Rare and inconsistent effects of CCT on tomato plant vigor were noted in this study. In just two fields out of sixteen, OH field 2 and OH field 6, tomato shoot height was significantly affected

by CCT (Table 1.3). Vetch-treated plants were significantly taller than the mixed-species hay-treated plants in field 2, and in field 6 tomatoes grown following a radish CCT were significantly shorter than tomatoes in all other plots (Table 1.6). However, fresh shoot weight was affected by CCT in four out of sixteen fields (Table 1.3). In MD 2010, tomatoes planted in radish+vetch-treated plots weighed significantly more than tomatoes from all other treatments (Table 1.4). In NY field 1, the tomatoes planted in the bare ground plots weighed significantly more than those of all other treatments except those in vetch+rye-treated plots, while in NY field 2, plants in the bare ground plots weighed significantly more than those of all other treatments (Table 1.5). Finally, in OH field 7 (2012), tomatoes from radish CCT weighed more than those from mixed-species hay or vetch+rye CCT (Table 1.6).

The total yield was affected significantly by cover crop in just four out of sixteen test fields. These instances occurred in 2010 and 2011 for MD and NY. For these cases, the CCT conferring the greatest yield was not consistent (Tables 1.5 & 1.6; Figure 1.1 Productivity Graphs). In MD 2010, bare ground, mixed-species hay and vetch-treated plots had significantly greater yields than radish+vetch or vetch+rye-treated plots. However, in MD 2011, vetch-treated plots yielded significantly more than bare ground or mixed-species hay-treated plots (Table 1.4). Interestingly, though not showing statistically significant differences among treatments, tomato yields following all mixed cover crops planted in NY field 1 (2010) were greater than the single rye cover crop, but this pattern was reversed in the field adjacent to it, NY field 2 (2010), where bare ground and rye-treated plots yielded higher than other treatments. The following year, the vetch+rye-treated plots in NY field 4 (2011) yielded the highest along with bare ground and rye-treated plots (Table 1.5).

The cover crop biomass also had an effect on total yield in all three states, but this also was not consistent (Figure 1.1 Productivity Graphs). For instance, in NY field 2 (2010), the two highest

yields were from the rye cover crop, which had the largest biomass, and the bare ground control, with no biomass (Table 1.5).

**Table 1.3** Significance of F-tests assessing effects of cover crop treatments (CCT) on productivity related variables by field in 2010, 2011 and 2012[a].

| | Maryland | | | New York | | | | | | Ohio | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2010 | 2011 | 2012 | 2010 | | 2011 | | 2012 | | 2010 | | | 2011 | | | 2012 | |
| | | | | 1[b] | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | S/T[c] |
| Total Yield | **<0.01** | **<0.01** | 0.27 | 0.7 | **<0.01** | 0.81 | **<0.01** | 0.12 | 0.73 | 0.17 | 0.25 | 0.41 | 0.81 | 0.61 | 0.7 | 0.01 | 4/16 |
| Shoot Height | 0.56 | 0.35 | 0.14 | 0.89 | 0.5 | 0.09 | 0.45 | 0.45 | 0.47 | 0.15 | **0.03** | 0.71 | 0.51 | 0.54 | **<0.01** | 0.51 | 2/16 |
| Shoot Weight | **0.01** | 0.84 | 0.32 | **<0.01** | **<0.01** | 0.17 | 0.44 | 0.59 | 0.43 | 0.36 | 0.13 | 0.82 | 0.63 | 0.14 | 0.7 | **0.01** | 4/16 |
| pH | 0.46 | 0.19 | **0.02** | 0.89 | 0.18 | 0.64 | 0.33 | ND | ND | **0.01** | 0.91 | 0.67 | 0.02 | **0.03** | **0.06** | ND | 4/13 |
| Organic Matter | 0.3 | 0.13 | **<0.01** | 0.32 | **<0.01** | 0.16 | 0.15 | ND | ND | <0.01 | **<0.01** | 0.21 | 0.1 | 0.1 | 0.05 | ND | 3/13 |
| P | 0.73 | 1 | 0.49 | 0.58 | 0.58 | 0.49 | 0.49 | ND | ND | 0.11 | 0.33 | 0.97 | **0.02** | 0.16 | 0.73 | ND | 1/13 |
| K | 0.11 | 0.96 | **<0.01** | 0.24 | 0.24 | 0.36 | 0.36 | ND | ND | 0.68 | 0.59 | 0.51 | 0.47 | **<0.01** | **0.04** | ND | 3/13 |



[a] Numbers are P-values from the GLM test for cover crop treatment effects on the outcome of the measured variable. Values in bold are significant at P<0.1 by GLM analysis as well as by Tukey analysis. Values of P<0.1 but not highlighted in bold were not significant according to Tukey analysis. Abbreviation 'ND' signifies that no data was acquired for the measured variable in a given field.

[b] Field number, with two fields per year in NY, three fields in OH for 2010 and 2011 and one field in OH for 2012. MD had one field per year. This totals to sixteen distinct fields.

[c] Total Significant (S)/Total, where Total (n) equals the number of fields where a variable was measured. If the measured variable was available in all three states, in each field in each state and for three years then n=16. Total Significant (S) is the total number of fields where one or more CCT significantly affected the productivity measurement at P<0.1 as well as Tukey analysis.

**Table 1.4** Maryland productivity measurements[a]: mean value and Tukey assignments

| | Field | Bare Ground | Mixed Species Hay | Radish + Vetch | Vetch + Rye | Vetch |
|---|---|---|---|---|---|---|
| 2010 | | | | | | |
| Total Yield (kg)[c] | 1 | 14.2a[b] | 15.4a | 10.7b | 9.4b | 17.7a |
| Shoot Weight (g)[d] | 1 | 515b | 506b | 609a | 501b | 511b |
| CC Biomass (g)[e] | 1 | 190c | 212c | 792a | 575b | 484b |
| 2011 | | | | | | |
| Total Yield (kg) | 2 | 44.3b | 42.3b | 48.2ab | 46.0ab | 50.8a |
| CC Biomass (g) | 2 | 0c | 33c | 218a | 148b | 250a |
| 2012 | | | | | | |
| Total Yield (kg) | 3 | 6.52 | 6.66 | 10.40 | 6.89 | 7.45 |
| pH | 3 | 6.88a | 6.90a | 6.72b | 6.68b | 6.70b |
| Organic Matter (%) | 3 | 0.45b | 0.57ab | 0.50ab | 0.65a | 0.47b |
| Potassium (mg/kg) | 3 | 40.3c | 38.4c | 80.0b | 93.3a | 69.2b |
| CC Biomass | 3 | 50c | 35c | 386a | 355ab | 220b |

[a] Productivity measurements include: total yield, shoot height and shoot weight at four weeks post-transplant, and soil nutrient analyses. This table includes all yield data. Other productivity measurements were included only when significant differences occurred, as viewed in Table 1.3.

[b] Main effect means followed by the same letter are not significant according to Tukey's HSD test ($P<0.1$).

[c] For total yield (kg) in 2010, two plants per plot were harvested three times. In 2011, four plants per plot were harvested eight times. In 2012, four plants per plot were harvested five times. In each case, the sum of all harvests for each plot were calculated for GLM analysis.

[d] Shoot weight (g) was measured from two plants per plot at four weeks post-transplant. The two values were included separately for GLM analysis.

[e] The cover crop biomass analysis (CC Biomass) is an ANOVA testing for differences in the biomass of each CCT prior to tilling under in the spring. Biomass was measured by weighing above ground fresh plant tissue (g) from a 1.2 m x 1.2 m plot.

**Table 1.5** New York productivity measurements[a]: mean value and Tukey assignments

| | Field | Bare Ground | Rye | Turnip + Rye | Vetch + Rye | Clover + Rye |
|---|---|---|---|---|---|---|
| 2010 | | | | | | |
| Total Yield (kg)[c] | 1 | 23.0[b] | 22.7 | 29.9 | 26.4 | 27.4 |
| Shoot Weight (g)[d] | 1 | 600a | 340c | 390c | 530ab | 420bc |
| CC Biomass (g)[e] | 1 | 0d | 858a | 383c | 603b | 373c |
| Total Yield (kg) | 2 | 17.6a | 17.2a | 14.1b | 13.5b | 13.1b |
| Shoot Weight (g) | 2 | 600a | 260b | 370c | 300bc | 380c |
| Organic Matter (%) | 2 | 4.6a | 4.5b | 4.4b | 4.7a | 4.8a |
| CC Biomass (g) | 2 | 0c | 1740a | 970b | 1230ab | 828b |
| 2011 | | | | | | |
| Total Yield (kg) | 3 | 36.3 | 35.2 | 36.0 | 39.4 | 37.5 |
| CC Biomass (g) | 3 | 0b | 1580a | 1210a | 1350a | 900ab |
| Total Yield (kg) | 4 | 28.9a | 29.4a | 23.8b | 30.3a | 25.0b |
| CC Biomass (g) | 4 | 0b | 438a | 375a | 270b | 448a |
| 2012 | | | | | | |
| Total Yield (kg) | 5 | 41.4 | 30.5 | 36.3 | 35.9 | 32.6 |
| CC Biomass (g) | 5 | 0c | 335b | 538ab | 585a | 628a |
| Total Yield (kg) | 6 | 35.3 | 34.8 | 29.3 | 33.7 | 34.5 |
| CC Biomass (g) | 6 | 0c | 478ab | 483ab | 375b | 690a |

[a] Productivity measurements include: Total yield, shoot height and weight at four weeks post-transplant, and soil nutrient analyses. This table includes all yield data. Other productivity measurements were included only when significant differences occurred, as viewed in Table 1.3.

[b] Main effect means followed by the same letter are not significant according to Tukey's HSD test ($P<0.1$).

[c] For total yield (kg) six plants per plot were harvested three times and the sum was calculated for each plot.

[d] Shoot weight (g) was measured from two plants per plot at four weeks post-transplant. The two values were included separately for GLM analysis.

[e] The cover crop biomass analysis (CC Biomass) is an ANOVA testing for differences in the biomass of each CCT prior to tilling in the spring. Biomass was measured by weighing above ground fresh plant tissue (g) from a 1.2 m x 1.2 m plot.

**Table 1.6** Ohio Productivity measurements[a]: mean values and Tukey assignments

| | Field | Mixed-Species Hay | Rye | Radish | Vetch + Rye | Vetch |
|---|---|---|---|---|---|---|
| 2010 | | | | | | |
| Total Yield (kg)[c] | 1 | 1.77[b] | 3.36 | 2.36 | 4.06 | 3.30 |
| pH | 1 | 6.51a | 6.4a | 3.19b | 6.35a | 4.78ab |
| CC Biomass (g)[d] | 1 | 68.37b | 249.34a | 207.72ab | 270.15a | 202.15ab |
| Total Yield (kg) | 2 | 6.32 | 7.06 | 6.04 | 7.01 | 8.04 |
| Shoot Height (cm)[e] | 2 | 42.5b | 55.9ab | 58.3ab | 57.8ab | 60.0a |
| Organic Matter (%) | 2 | 2.2b | 2.9a | 3.0a | 3.1a | 3.1a |
| CC Biomass (g) | 2 | 468.96bc | 668.14ab | 210.70c | 824.58a | 392.41bc |
| Total Yield (kg) | 3 | 4.8 | 3.5 | 3.9 | 4.7 | 4.6 |
| CC Biomass (g) | 3 | N/A | N/A | N/A | N/A | N/A |
| 2011 | | | | | | |
| Total Yield (kg) | 4 | 12.3 | 11.1 | 12.1 | 11.8 | 11.0 |
| Phosphorus (ug/g) | 4 | 60.8ab | 61.0ab | 61.8a | 57.9b | 58.3b |
| CC Biomass (g) | 4 | 1084b | 1167ab | 0c | 1388a | 1200ab |
| Total Yield (kg) | 5 | 14.8 | 15.9 | 15.8 | 13.4 | 15.3 |
| Potassium (ug/g) | 5 | 407a | 345ab | 364ab | 324b | 345ab |
| pH | 5 | 6.39ab | 6.46a | 6.37ab | 6.31ab | 6.28b |
| CC Biomass (g) | 5 | 264b | 492a | 114c | 459a | 307b |
| Total Yield (kg) | 6 | 6.68 | 5.53 | 5.72 | 5.65 | 6.34 |
| Shoot Height (cm) | 6 | 46.5a | 46.0a | 35.1b | 45.0a | 45.6a |
| pH | 6 | 5.85ab | 5.73b | 5.95a | 5.84ab | 5.74b |
| Potassium (ug/g) | 6 | 83a | 84a | 80ab | 78ab | 73b |
| CC Biomass (g) | 6 | 934a | 1060a | 99.3b | 1061a | 919a |
| 2012 | | | | | | |
| Total Yield (kg) | 7 | 9.1 | 16.2 | 11.8 | 12.0 | 14.3 |
| Shoot Weight (g)[f] | 7 | 3209b | 3474ab | 3769a | 3102b | 3834ab |
| CC Biomass (g) | 7 | 781b | 1244a | 0c | 1273a | 1364a |

[a] Productivity measurements include: total yield, shoot height and weight at four weeks post-transplant, and soil nutrient analyses. This table includes all yield data. Other productivity measurements were included only when significant differences occurred, as viewed in Table 1.3.

[b] Main effect means followed by the same letter are not significant according to Tukey's HSD test ($p<0.1$).

[c] Total yield is the sum of three harvests from two plants per plot (kg).

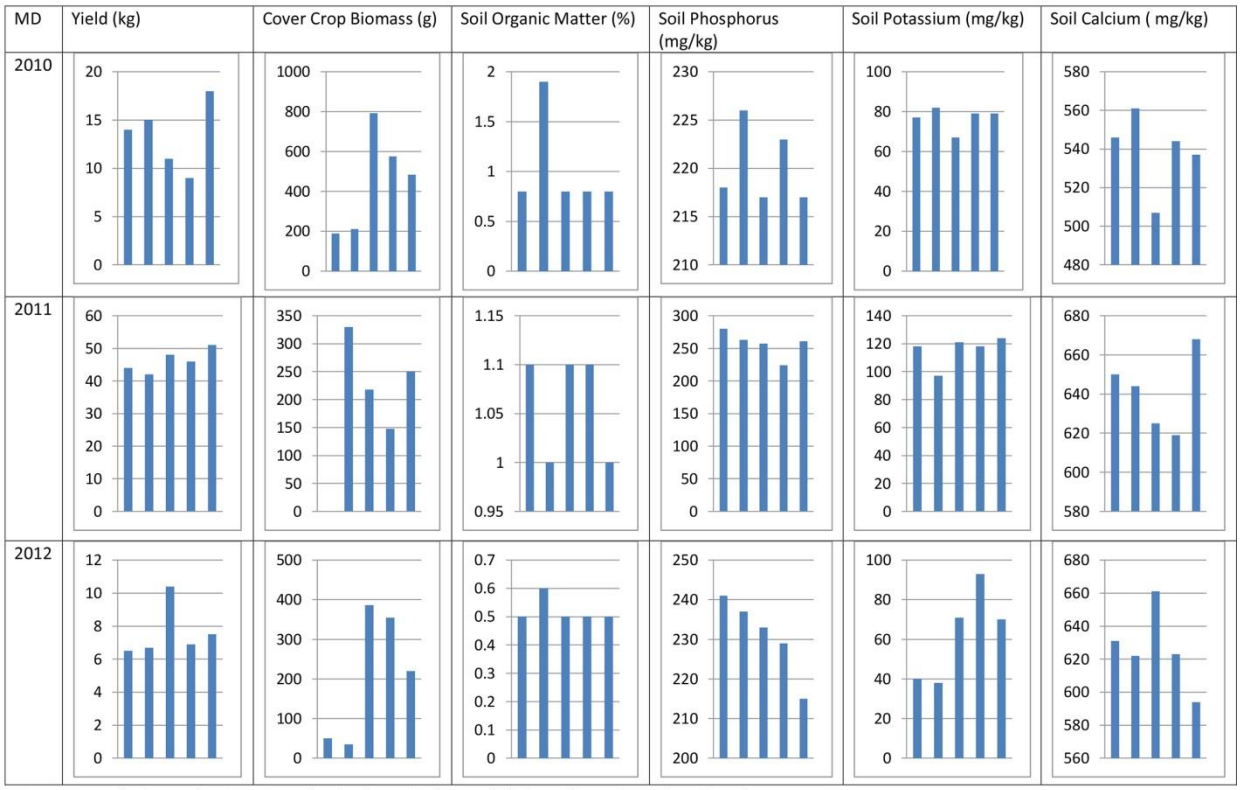
[d] The cover crop biomass analysis (CC Biomass) is an ANOVA testing for differences in the biomass of each CCT prior to tilling in the spring. Biomass was measured by weighing above ground fresh plant tissue (g) from a 1.2 m x 1.2 m plot.

[e] Shoot height (cm) was measured from two plants per plot at 4 weeks post-transplant.

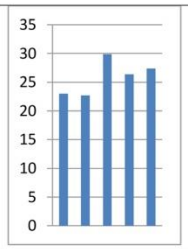
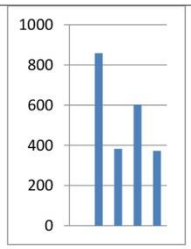
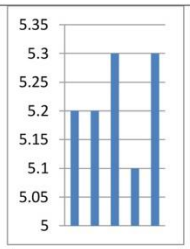
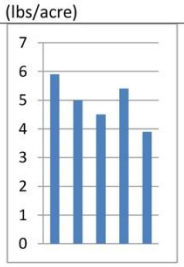
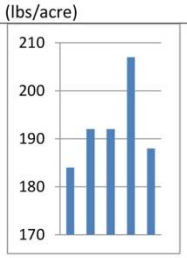
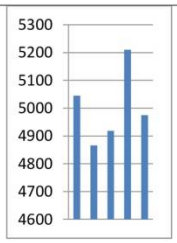
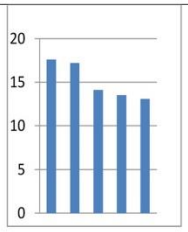
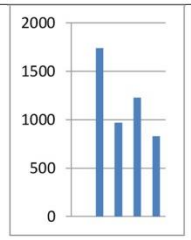
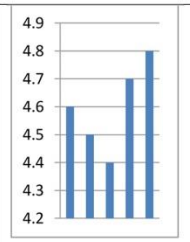
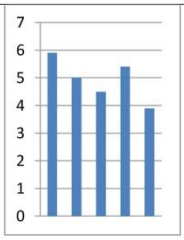
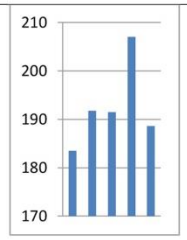
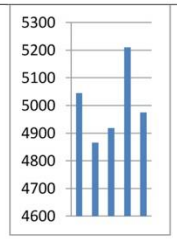
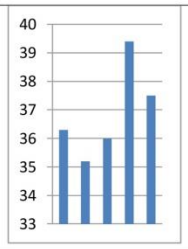
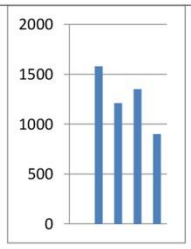
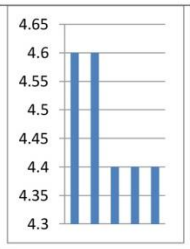
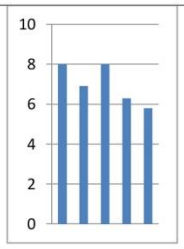
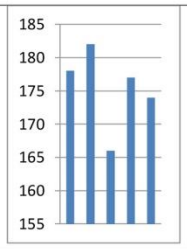
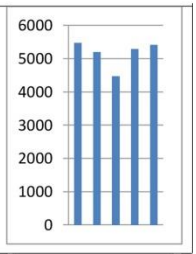
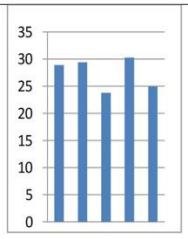
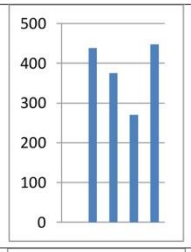
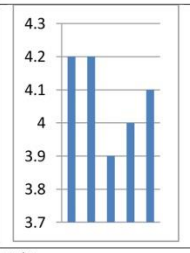
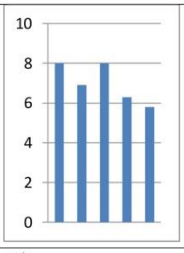
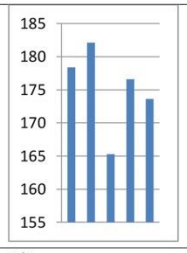
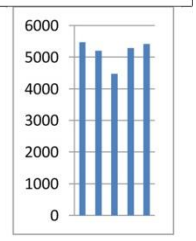
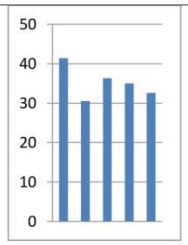
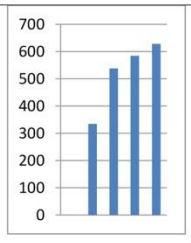
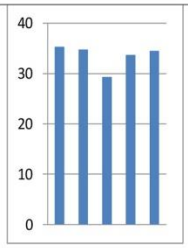
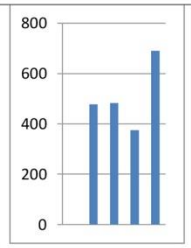
[f] Shoot weight (g) was measured from two plants per plot at six to eight weeks post-transplant. The two values were included separately for GLM analysis and not averaged.

**Figure 1.1** Productivity Graphs

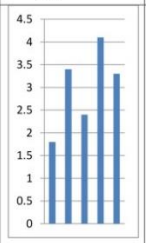
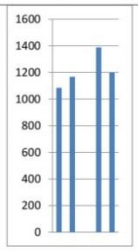
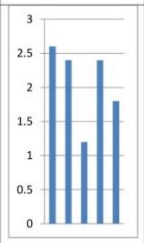
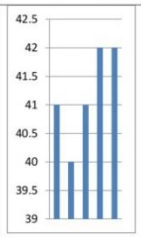
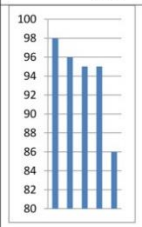
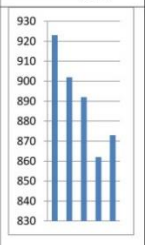
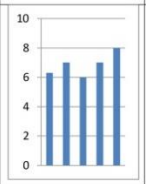
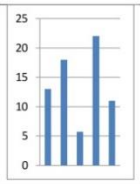
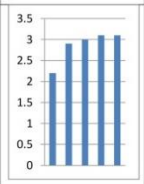
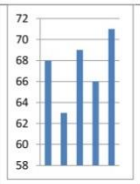
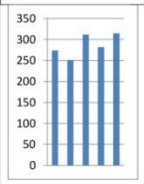
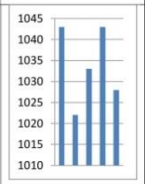
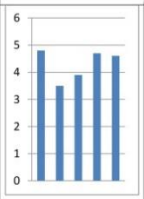
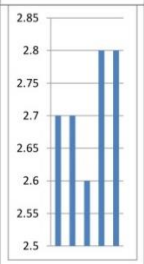
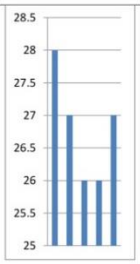
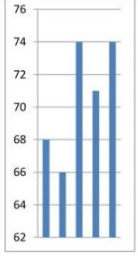
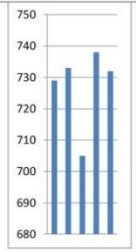
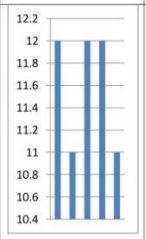
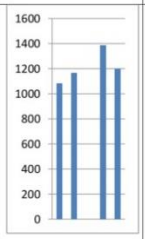
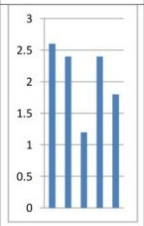
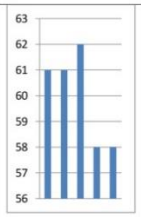
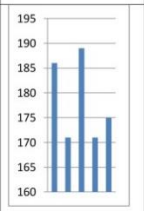
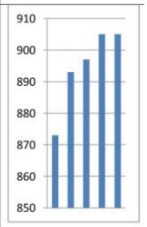
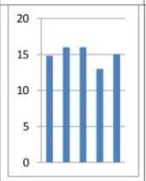
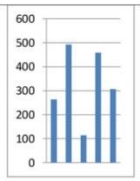
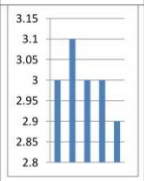
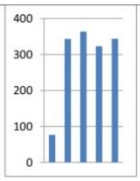
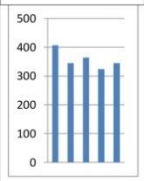
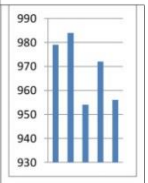
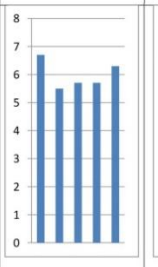
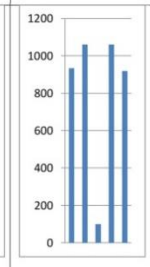
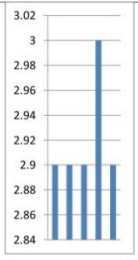
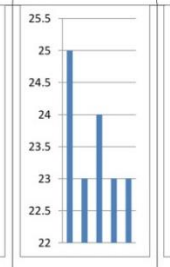
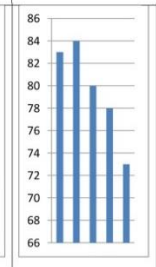
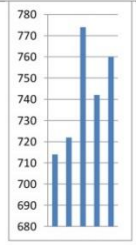
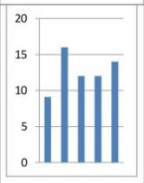
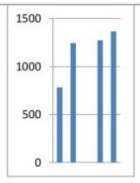
The following graphs (for MD, NY and OH) were used solely to assess trends in average responses of plant and soil variables to CCTs at each location. Statistically significant variations are noted in the main text and tables. Here, the y axes represent mean values of each variable among the four reps in each field. The CCT are presented along the x axes and follow the same order for all graphs for each state.




Treatment order in graphs: Bare ground, mixed-species hay, radish & vetch, rye & vetch and vetch.

| NY | Yield (kg) | Cover Crop Biomass (g) | Soil Organic Matter (%) | Soil Phosphorus (lbs/acre) | Soil Potassium (lbs/acre) | Soil Calcium (lbs/acre) |
|---|---|---|---|---|---|---|
| 2010 Field 1 |  |  |  |  |  |  |
| 2010 Field 2 |  |  |  |  |  |  |
| 2011 Field 3 |  |  |  |  |  |  |
| 2011 Field 4 |  |  |  |  |  |  |
| 2012 Field 5 |  |  | N/A | N/A | N/A | N/A |
| 2012 Field 6 |  |  | N/A | N/A | N/A | N/A |

Treatment order in graphs: Bare Ground, Rye, Turnip & Rye, Rye & Vetch and Clover & Rye.

| OH | Yield (kg) | Cover Crop Biomass (g) | Soil Organic Matter (%) | Soil Phosphorus (ug/g) | Soil Potassium (ug/g) | Soil Calcium (ug/g) |
|---|---|---|---|---|---|---|
| 2010 Field 1 |  |  |  |  |  |  |
| 2010 Field 2 |  |  |  |  |  |  |
| 2010 Field 3 |  | N/A |  |  |  |  |
| 2011 Field 4 |  |  |  |  |  |  |
| 2011 Field 5 |  |  |  |  |  |  |
| 2011 Field 6 |  |  |  |  |  |  |
| 2012 Field 7 |  |  | N/A | N/A | N/A | N/A |

Treatments are in order: Mixed-species hay, rye, radish fallow, rye & vetch and vetch.

*Disease Severity Analysis.* In order to visualize the overall effect of CCT on disease severity, all results for all fields are presented in a table summarizing all statistical outcomes (Table 1.7). Overall, 0 to 44% of the tested sites with a noted disease were found to differ significantly across one or more CCT treatments. More detailed results for each disease that was found to be significant are presented in state-specific Tables 1.8, 1.9, 1.10 and 1.12 as well as Figure 1.2 Disease Graphs.

*Early blight.* Early blight was consistently found in all three states in every year of the field trials. As seen in Table 1.7, in six out of fifteen fields, CCT affected early blight severity. Looking more closely at the significant results using Tukey analysis (Tables 1.9, 1.10 & 1.11) as well as all field-year instances using comparative graphs (Figure 1.2 Disease Graphs), it becomes clear that no one treatment had a significant beneficial or detrimental effect on the level of disease. In MD 2010, vetch had significantly more disease than radish+vetch. In MD 2011, mixed-species hay had significantly more disease than all other treatments except bare ground (Table 1.9). In NY field 2 (2010), the bare ground treatment had significantly more early blight than the turnip+rye or clover+rye treatments (Table 1.10). CCT significantly affected early blight in OH for 2010 and 2012. However, early blight severity was lowest in the mixed hay CCT in all three years (Table 1.11; data for 2011 not shown). Additionally, In OH field 1 (2010), the vetch+rye treatment had more early blight than mixed-species hay and rye. In OH field 2 (also 2010), rye-treated plots only had significantly more early blight than mixed-species hay-treated plots. In OH field 7 (2012) the vetch+rye treated fields had more early blight than vetch, mixed-species hay and rye-treated plots (Table 1.11).

**Table 1.7** Significance of F-tests assessing effects of CCT on disease[a] in 2010, 2011 and 2012[b]

| | Maryland | | | New York | | | | | | Ohio | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2010 | 2011 | 2012 | 2010 | | 2011 | | 2012 | | 2010 | | | 2011 | | | 2012 | Total S/ Total[d] |
| | | | | 1[c] | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | |
| Early Blight | **<0.01** | **0.01** | 0.04 | 0.87 | **<0.01** | 0.09 | 0.30 | 0.95 | 0.70 | **<0.01** | **0.01** | 0.18 | 0.84 | 0.2 | ND | **<0.01** | 6/15 |
| Septoria Blight | **<0.01** | 0.18 | **<0.01** | **<0.01** | **<0.01** | 0.45 | 0.05 | ND | ND | ND | ND | ND | 0.71 | 0.57 | ND | ND | 4/9 |
| Phytophthora Blight | ND | ND | ND | 1 | **<0.01** | 0.67 | **0.01** | 0.07 | 0.67 | ND | ND | ND | ND | ND | ND | ND | 2/6 |
| Leaf Mold | ND | ND | ND | ND | ND | 0.20 | 0.27 | NA | ND | ND | ND | ND | ND | ND | ND | ND | 0/2 |
| Late Blight | ND | ND | ND | ND | ND | 0.19 | 0.62 | NA | ND | ND | ND | ND | ND | ND | ND | ND | 0/2 |
| Southern Blight | **<0.01** | 0.39 | 0.11 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 1/3 |

[a] Disease measurements compared were AUDPC values calculated from approximately weekly ratings of disease severity over the season.

[b] Numbers are P-values from the GLM test for cover crop treatment effects on the outcome of the measured variable. Values in bold are significant at P<0.1 by GLM analysis as well as by Tukey analysis. Values of P<0.1 but not highlighted in bold were not significant according to Tukey analysis. Abbreviation 'ND' signifies that no data was acquired for the measured variable in a given field.

[c] Field number, with two fields per year in NY, three fields in OH for 2010 and 2011 and one field in OH for 2012. MD had one field per year. This totals to sixteen distinct fields.

[d] Total significant (S)/Total, where Total (n) equals the number of fields where a disease was observed. If the disease was observed in all three states, in each field in each state and for three years then n=16. Total Significant (S) is the total of instances significant at P<0.1 by both GLM analysis as well as Tukey analysis.

*Septoria blight*. The only other disease found across all three states and years was Septoria blight, which affected nine out of sixteen fields. Of the nine fields affected, four fields indicated a significant effect of CCT on Septoria blight severity (Table 1.7). In MD 2010, vetch-treated plots had more severe disease than bare ground, mixed-species hay or radish+vetch-treated plots. However, in MD 2012, bare ground-treated plots had significantly more disease than all other treatments except mixed-species hay (Table 1.9). In NY field 2 (2010), bare ground plots had more disease than clover+rye and turnip+rye-treated plots, while in the nearby field 1 (also NY 2010), clover+rye and vetch+rye-treated plots had significantly more disease than rye-treated plots (Table 1.10). Overall, CCT effects on Septoria blight severity were infrequent and variable.

*State-Specific Diseases*. Maryland fields in 2010, 2011 and 2012 were rated for naturally-occurring Southern blight. Out of the three years, only 2010 saw a significant effect of CCT, where vetch+rye-treated plots had significantly more disease than mixed-species hay-treated plots (Table 1.9). However, PPN/PAN levels tended to be higher in mixed CCT plots than single CCT or bare ground treatments, although the differences were not always significant (Table 1.8).

Of the plant-pathogenic nematodes/plant-associated nematodes (PPN/PAN) collected from the MD fields, the genus occurring most often and in the highest numbers within most plots was *Tylenchus* spp. This genus has been isolated from numerous plant hosts, but is considered to be root-associated rather than a true plant parasite (Yeates et al. 1993; Simard et al. 2008). *Tylenchus* spp. were identified from 313 of the 360 soil samples (87%), and accounted for 90.5% of all individual nematodes counted over the three-year period in the PPN/PAN group. *Tylenchorhynchus* spp. were the most abundant PPN, counted from 90 of the 360 soil samples (25%) over the three years and comprising 4.9% of all individual PPN/PAN counted. Other PPN detected in plots were [genus (number of plots, year(s) collected; percent of all PPN/PAN counted)]: *Criconemoides* (3, 2012; 0.1%),

*Ditylenchus* (20, 2010-2012; 0.7%), *Helicotylenchus* (8, 2010, 2011; 0.3%), *Heterodera* (6, 2010-2012; 0.2%), *Meloidogyne* (3, 2010; 0.1%), *Mesocriconema* (2, 2010; <0.03%), *Paratylenchus* (2, 2010; 0.2%), *Pratylenchus* (36, 2010-2012; 2.0%), and *Trichodorus* (23, 2010-2012; 1.0%). The other stylet-bearing nematodes collected from these samples were *Aphelenchoides* and *Aphelenchus*, which are primarily fungivores (Yeates et al. 1993). *Aphelenchoides* was collected from 11% of the soil samples (2010-2012; 4.1% of the individual fungivores counted over the three years), and *Aphelenchus* from 76% of the soil samples (274 soil samples, 2010-2012; 95.9% of fungivores).

Root systems were sampled at mid-season and harvest each year; nematode eggs were counted from ca. 13%, 37% and 3% of the collected root systems (mid-season) and 65%, 23%, and 17% (harvest) in 2010, 2011 and 2012, respectively. However, no galling or egg masses were observed on these root-knot nematode (RKN)-resistant tomato root systems, and it could not be determined by morphology which non-RKN taxa were the source(s) of the eggs.

Prior to green manure incorporation in the spring of 2010, population densities of PPN/PAN were similar among all cover crop treatments (Table 1.8; 16 Apr, $P < 0.10$). However, shortly after incorporation (29 Apr), the population densities were lowest in bare ground plots, with ca. 2.5 to 3 times more PPN/PAN in all other treatments except vetch. No significant differences were recorded among treatments at mid-season or harvest in 2010.

In 2011, PPN/PAN populations were 7.5 times higher in mixed-species hay plots than in bare ground plots after a winter of cover crop growth (Table 1.8, 14 Apr). Similar to 2010, after incorporation of green manures, densities were lower in bare ground plots on 16 May than in all treatments except vetch, with ca. 4 to 7 times more PPN/PAN in vetch+rye, mixed-species hay, and radish+vetch treatments than in bare ground plots. PPN/PAN counts from radish+vetch plots

were also more than 2 times greater than in vetch plots. As in 2010, there were no significant differences among treatments at mid-season or harvest.

In 2012, spring (25 Apr) PPN/PAN densities in the soil with the winter cover crop plantings were lowest in bare ground plots, as in 2011. However, the only treatment with significantly higher numbers was vetch+rye, with ca. 2.5 times more PPN/PAN than bare ground plots (Table 1.8). Similar to the two previous years, population numbers were low in bare ground plots post-incorporation (24 May), but in 2012 were also low in mixed-species hay plots. As in 2010, vetch+rye plots had more PPN/PAN after incorporation of green manures than bare ground plots (3.5 times greater in the former). Unlike the previous years, the vetch green manure also resulted in high PPN/PAN counts, with 5 times more than bare ground plots. Also, both vetch+rye and vetch green manures resulted in higher counts than the mixed-species hay green manure (24 May). No significant differences were found among treatments later in 2012.

Population densities of fungivores did not differ among treatments at any time in 2010 (Table 1.8). In 2011, population numbers were ca. 2.5 to 3 times higher in radish+vetch after incorporation of green manures (16 May) than in bare ground or vetch+rye treatments, but no differences were recorded at mid-season or harvest. In 2012, the only differences in fungivore populations were observed in the spring prior to green manure incorporation (25 Apr), with more than 3 times the number of fungivores counted in vetch plots than in vetch+rye plots.

Data from Table 1.8 and Table 1.4 indicated that highest and lowest nematode populations did not follow the same trends as biomass quantity, except that the bare ground treatments, which had the lowest biomass, also tended to have the lowest numbers of PPN/PAN each year in the post-incorporation sampling time.

In NY, Phytophthora blight occurred in all fields and years (total of 6), where two fields saw a significant effect of CCT (Table 1.7). In NY field 2 (2010), turnip+rye-treated plots had significantly more disease than all other treatments except clover + rye. In NY field 4 (2011), turnip+rye had more disease than clover+rye, but not significantly more than other treatments (Table 1.10).

In NY fields 3 and 4 (2011), leaf mold and late blight were also rated. There was no significant effect from CCT on these diseases (Table 1.7).

**Table 1.8** Soil population densities of stylet-bearing nematodes in field plots in Salisbury, MD. Counts are per 100 $cm^3$ soil. Plant-parasitic/plant-associated nematode genera detected included *Criconemoides*, *Ditylenchus*, *Helicotylenchus*, *Heterodera*, *Meloidogyne, Mesocriconema*, *Paratylenchus, Pratylenchus, Trichodorus*, *Tylenchorhynchus* and *Tylenchus*. Fungivores were species of *Aphelenchus* and *Aphelenchoides*.

| | 2010 | | | | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16-Apr | 29-Apr | 25-Jun | 26-Aug | 14-Apr | 16-May | 6-Jul | 13-Sep | 25-Apr | 24-May | 24-Jul | 20-Sep |
| Plant-parasitic/plant-associated nematodes | | | | | | | | | | | | |
| Vetch + rye | 299 a[a] | 300 a | 163 a | 148 a | 250 ab | 550 ab | 154 a | 133 a | 183 a | 105 a | 33 a | 37 a |
| Mixed-species hay | 371 a | 275 a | 84 a | 116 a | 300 a | 410 ab | 110 a | 137 a | 139 ab | 32 b | 58 a | 55 a |
| Vetch | 245 a | 148 ab | 90 a | 74 a | 123 ab | 315 bc | 59 a | 71 a | 141 ab | 150 a | 91 a | 33 a |
| Radish + Vetch | 402 a | 298 a | 87 a | 80 a | 216 ab | 737 a | 129 a | 125 a | 139 ab | 80 ab | 60 a | 50 a |
| Bare Ground | 143 a | 106 b | 105 a | 64 a | 40 b | 105 c | 40 a | 100 a | 68 b | 30 b | 25 a | 39 a |
| Fungivores | | | | | | | | | | | | |
| Vetch + rye | 33 a | 75 a | 41 a | 66 a | 60 a | 205 b | 69 a | 35 a | 16 b | 108 a | 50 a | 30 a |
| Mixed-species hay | 78 a | 68 a | 50 a | 74 a | 68 a | 303 ab | 88 a | 38 a | 18 ab | 143 a | 38 a | 20 a |
| Vetch | 63 a | 65 a | 56 a | 53 a | 77 a | 408 ab | 115 a | 52 a | 54 a | 155 a | 56 a | 36 a |
| Radish + Vetch | 78 a | 75 a | 137 a | 47 a | 40 a | 503 a | 131 a | 92 a | 44 ab | 108 a | 56 a | 30 a |
| Bare Ground | 29 a | 31 a | 74 a | 41 a | 80 a | 150 b | 58 a | 52 a | 19 ab | 60 a | 75 a | 30 a |

[a] Means within a column followed by the same letter in a specific nematode classification are not significantly different ($P < 0.10$) on a particular date according to Tukey's HSD test.

**Table 1.9** Mean AUDPC values and Tukey assignments showing effects of cover crops on disease in Maryland[a]

| | Field | Bare Ground | Mixed-Species Hay | Radish + Vetch | Vetch + Rye | Vetch |
|---|---|---|---|---|---|---|
| 2010 | | | | | | |
| Septoria Blight | 1 | 232b[b] | 270b | 222b | 337ab | 402a |
| Southern Blight | 1 | 66ab | 48b | 73ab | 160a | 64ab |
| Early Blight | 1 | 166ab | 222ab | 149b | 178ab | 337a |
| CC Biomass (g)[c] | 1 | 190c | 212c | 792a | 575b | 484b |
| 2011 | | | | | | |
| Early Blight | 2 | 48ab | 58a | 38b | 35b | 38b |
| CC Biomass (g) | 2 | 0c | 33c | 218a | 148b | 250a |
| 2012 | | | | | | |
| Septoria Blight | 3 | 287a | 209ab | 201b | 145b | 166b |
| CC Biomass (g) | 3 | 50c | 35c | 386a | 355ab | 220b |

[a] Diseases assessed in MD include: early blight, Septoria blight and southern blight. Results of disease measurements were included only when significant differences occurred, as viewed in Table 3.

[b] AUDPC means followed by the same letter are not significant according to Tukey's HSD test ($P<0.1$).

[c] The cover crop biomass analysis (CC Biomass) is an ANOVA testing for differences in the biomass of each CCT prior to tilling under in the spring. Biomass was measured by weighing above ground fresh plant tissue (g) from a 1.2 m x 1.2 m plot.

**Table 1.10** Mean AUDPC values and Tukey assignments showing effects of cover crops on disease in New York[a]

| | Field | Bare Ground | Rye | Turnip + Rye | Vetch + Rye | Clover + Rye |
|---|---|---|---|---|---|---|
| 2010 | | | | | | |
| Septoria Blight | 1 | 155ab[b] | 53b | 158ab | 197a | 243a |
| CC Biomass (g)[c] | 1 | 0d | 858a | 383c | 603b | 373c |
| Phytophthora Blight | 2 | 503c | 665bc | 1544a | 779bc | 1228ab |
| Early Blight | 2 | 1016a | 278ab | 282b | 397ab | 154b |
| Septoria Blight | 2 | 432a | 136ab | 140b | 202ab | 101b |
| CC Biomass (g) | 2 | 0c | 1740a | 970b | 1230ab | 828b |
| 2011 | | | | | | |
| Phytophthora Blight | 4 | 359ab | 399ab | 608a | 380ab | 309b |
| CC Biomass (g) | 4 | 0b | 438a | 375a | 270b | 448a |

[a] Diseases assessed in NY include: early blight, Septoria blight, Phytophthora blight, late blight and leaf mold. Results of disease measurements were included only when significant differences occurred, as viewed in Table 1.7.

[b] AUDPC means followed by the same letter are not significant according to Tukey's HSD test ($P<0.1$).

[c] The cover crop biomass analysis (CC Biomass) is an ANOVA testing for differences in the biomass of each CCT prior to tilling under in the spring. Biomass was measured by weighing above ground fresh plant tissue (g) from a 1.2 m x 1.2 m plot.

**Table 1.11** Mean AUDPC values and Tukey assignments showing effects of cover crops on disease in Ohio[a]

| | Field | Mixed-Species Hay | Rye | Radish | Vetch + Rye | Vetch |
|---|---|---|---|---|---|---|
| 2010 | | | | | | |
| Early Blight | 1 | 56b[b] | 53b | 63ab | 96a | 72ab |
| CC Biomass (g)[c] | 1 | 68.37b | 249.34a | 207.72ab | 270.15a | 202.15ab |
| Early Blight | 2 | 23b | 78a | 61ab | 45ab | 44ab |
| CC Biomass (g) | 2 | 468.96bc | 668.14ab | 210.70c | 824.58a | 392.41bc |
| 2012 | | | | | | |
| Early Blight | 7 | 23b | 24b | 35ab | 39a | 23b |
| Bacterial leaf spot | 7 | 200b | 221b | 308a | 280ab | 218b |
| CC Biomass (g) | 7 | 781b | 1244a | 0c | 1273a | 1364a |

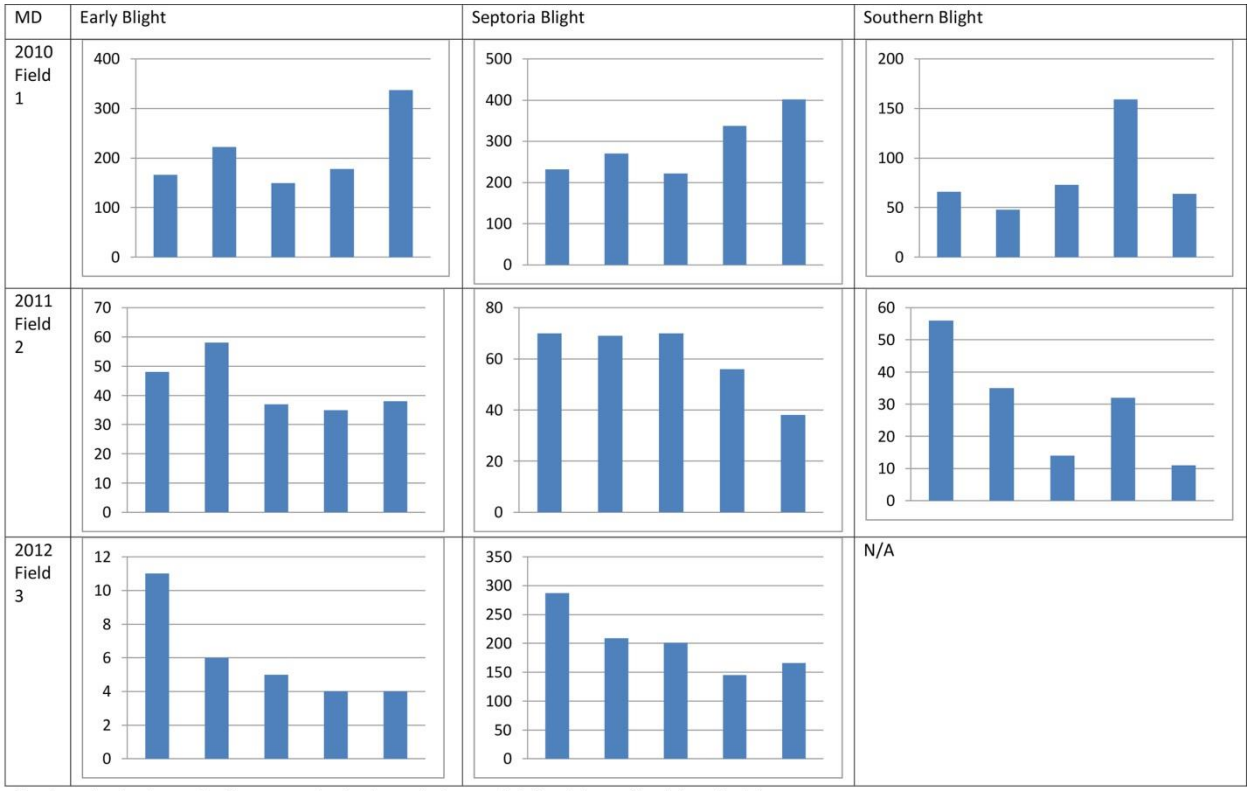
[a] Diseases assessed in OH include: early blight and Septoria blight. Results of disease measurements were included only when significant differences occurred, as viewed in Table 1.7.

[b] AUDPC means followed by the same letter are not significant according to Tukey's HSD test ($P<0.1$).

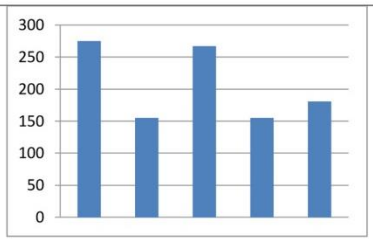
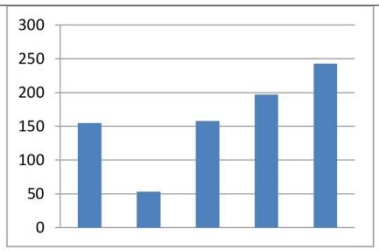
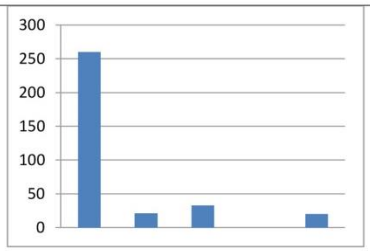
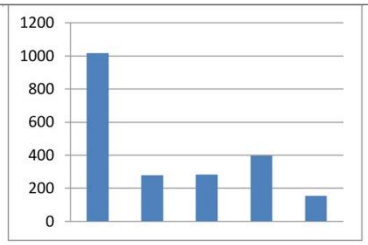
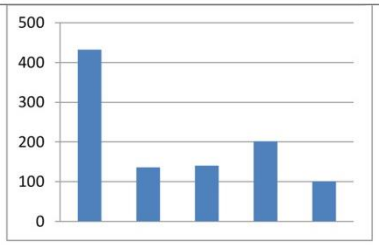
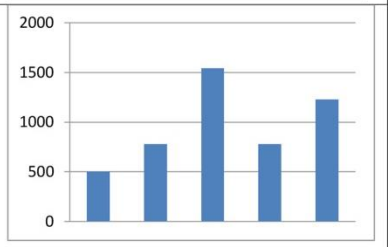
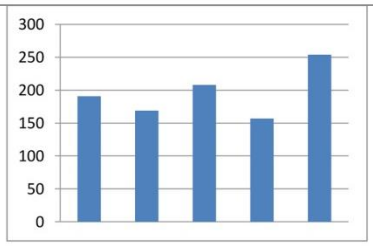
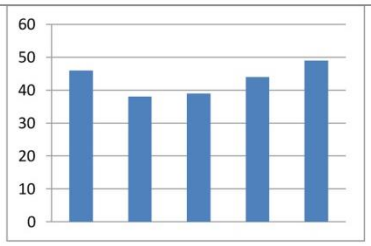
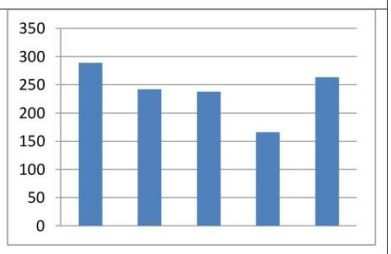
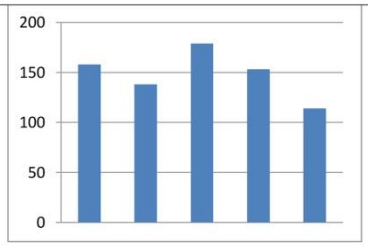
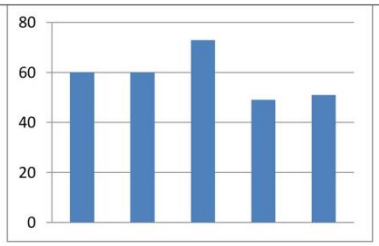
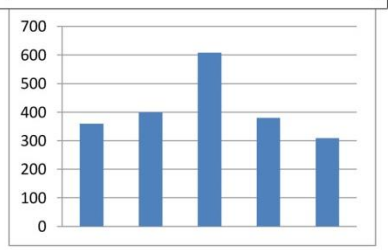
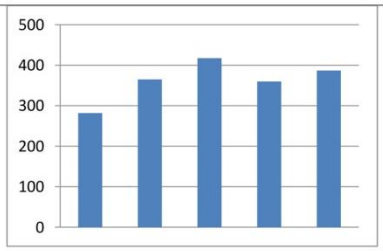
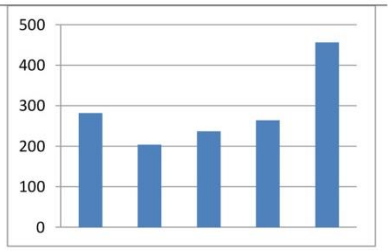
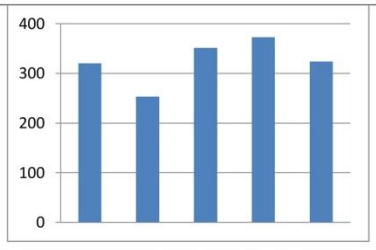
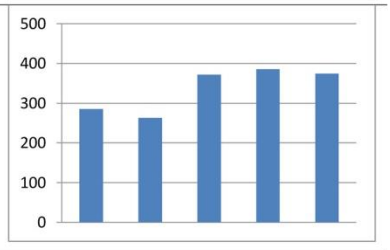
[c] The cover crop biomass analysis (CC Biomass) is an ANOVA testing for differences in the biomass of each CCT prior to tilling under in the spring. Biomass was measured by weighing above ground fresh plant tissue (g) from a 1.2 m x 1.2 m plot.

**Figure 1.2** Disease Graphs

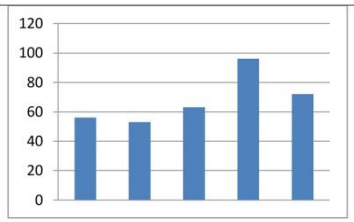
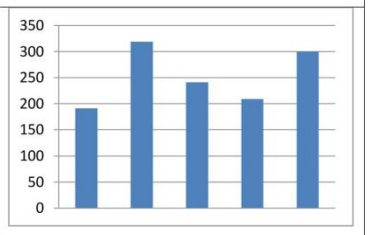
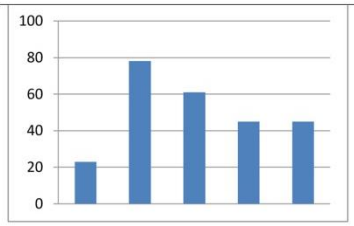
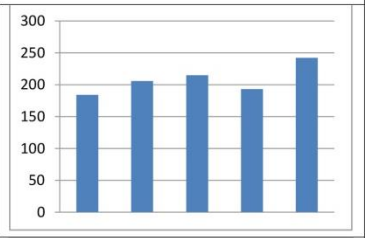
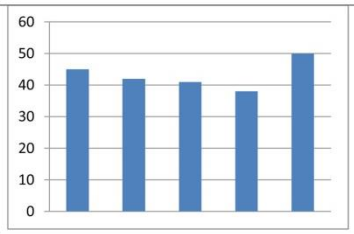
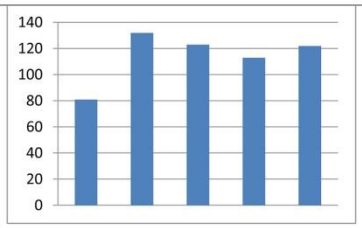
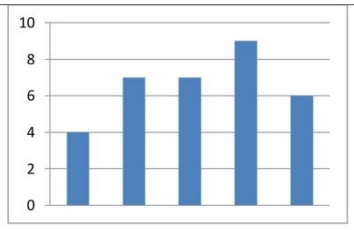
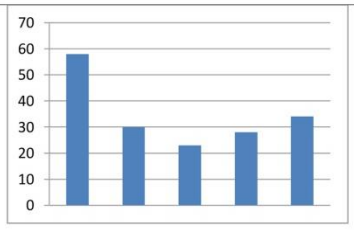
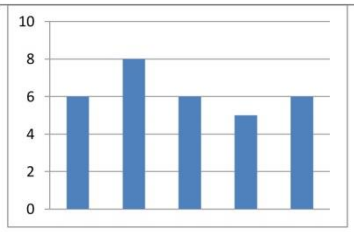
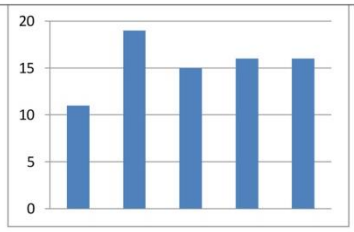
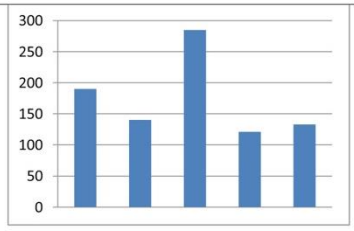
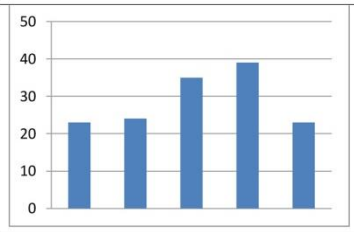
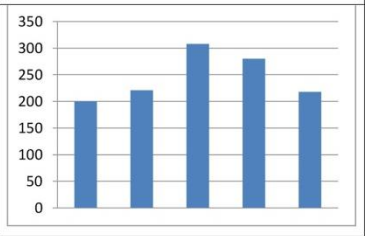
The following graphs (for MD, NY and OH) were used solely to assess trends in average responses of disease variables to CCTs at each location. Statistically significant variations are noted in the main text and tables. Here, the y axes represent mean AUDPC of each disease among the four reps in each field. The CCT are presented along the x axes and follow the same order for all graphs for each state.




Treatment order in graphs: Bare ground, mixed-species hay, radish & vetch, rye & vetch and vetch.

| NY | Early Blight | Septoria Blight | Phytophthora Blight |
|---|---|---|---|
| 2010 Field 1 |  |  |  |
| 2010 Field 2 |  |  |  |
| 2011 Field 3 |  |  |  |
| 2011 Field 4 |  |  |  |
| 2012 Field 5 |  | N/A |  |
| 2012 Field 6 |  | N/A |  |

Treatment order in graphs: Bare Ground, Rye, Turnip & Rye, Rye & Vetch and Clover & Rye.

| OH | Early Blight | Septoria Blight | Bacterial Leaf Spot |
|---|---|---|---|
| 2010 Field 1 |  | N/A |  |
| 2010 Field 2 |  | N/A |  |
| 2010 Field 3 |  |  | |
| 2011 Field 4 |  |  | N/A |
| 2011 Field 5 |  |  | N/A |
| 2011 Field 6 | N/A |  | |
| 2012 Field 7 |  | N/A |  |

Treatments are in order: mixed-species hay, rye, radish fallow, rye & vetch and vetch.

**Discussion**

Mixed-species cover crops appeal to organic growers for a variety of reasons. However, in this study, we found no near-term effects of mixed-species green manures on total yields (Table 1.3) and disease levels (Table 1.7) of subsequent plantings of tomato (*Solanum lycopersicum* L.) in organic production systems across the Northeast region. The lack of consistent response of plant diseases to CCTs seems to be due to variations in both cultural practices and environmental factors. For example, cover crops were found to reduce severity of Phytophthora blight in North Carolina, but the mechanism was through reduced pathogen dispersal by splash, where the CCT was not tilled under (Ristaino et al. 1997). In this study, where we tilled in the cover crops, we found a contrasting pattern, with Phytophtora blight typically higher in mixed (and single) CCTs as compared to the bare ground control in NY. It may be that the act of incorporation provided material to support pathogen growth. But cultural factors are not likely the only culprit. For example, in MD, a tilled hairy vetch green manure significantly reduced disease by Fusarium wilt on watermelon both in the greenhouse and the field (Zhou & Everts 2004). However, in the Maryland sites used in this study, there were no consistent reductions in any of the noted tomato diseases by tilled vetch, alone or in combination with other species (Table 1.9).

We think that the variable effects of CCT detailed in this large multi-site study are likely due to site-specific variations in environmental variables. Soil types varied from state to state, with Odessa silt loam in NY, Wooster Riddles silt loam in OH and loamy sand in MD. Cover crop establishment varied from year to year in each location, with limited growth in some cases due to environmental factors such as heavy rain or cold temperatures. Interactions between cover crops and crop plants appear to be highly dependent on the environment and timing factors such as cover crop incorporation and time of transplant. This may be due to allelopathy as well changes in

microbial populations due to plant matter decomposition (Welbaum et al. 2004). Approximately four weeks were allowed between tilling of cover crops and transplant in order to prevent allelopathy, but cooler and drier conditions can slow decomposition. The measurements of plant height and shoot weight at four weeks post-transplant were useful in detecting possible effects of allelopathy on early crop growth. In two cases out of sixteen, both in 2010 NY fields when only three weeks were allowed between tilling and transplant, the bare ground control treatment conferred a significantly larger shoot weight than other treatments, except vetch+rye in field 1 (Table 1.5). This verifies the importance of waiting a minimum of four weeks between tilling and transplant.

Beneficial cover crop effects may be closely linked to the accumulation of organic matter and the resulting improvement of soil structure over time. Cover crops have been observed to increase soil aggregate stability, an important property of healthy soils (Magdoff et al. 2000), even after one season (Liu et al. 2005). While no correlation between cover crop biomass and tomato productivity were observed in this study (Figures 1.1 & 1.2 and 1.4-1.6, 1.9-1.11), increases in total soil organic matter can improve soil and crop health under some soil conditions (Abawi & Widmer 2000). Interestingly, each cover crop mixture produced a significantly different weight of biomass in most fields (thirteen out of sixteen). However, soil analyses showed few differences in soil organic matter (four out of sixteen) between treatments at around the time of transplanting, less than six weeks after incorporation (Table 1.3). Thus, while additions of organic matter may improve soil health and productivity, we found no consistent evidence for such an effect over a single cropping season.

In the MD fields, which combined a resistant tomato cultivar with green manure treatments in a field with low PPN pressure, there were two consistent results over the three year period. These were: 1) shortly after incorporation of green manures, bare ground plots had lower PPN/PAN

populations (comprised primarily of *Tylenchus* spp. and *Tylenchorhynchus* spp.) than plots with green manures, particularly plots with vetch+rye; and 2) the effect did not last throughout the growing season, as no differences were found among treatments at mid-season and harvest. Variable results have been reported from use of green manures for managing PPN populations, with some studies demonstrating suppression and others showing population increases (Thoden et al. 2011). Many parameters are involved, including status of the cover crop as a host plant, plant chemistry, biomass, and effects on other soil organisms (Oka 2010; Thoden et al. 2011). Studies with rye and nematotoxic compounds from rye have resulted in suppression of PPN populations in some cases but not others (Meyer et al. 2009; Timper et al. 2011; Zasada et al. 2000). Combined with hairy vetch, rye did not decrease population numbers in this study. While incorporated rye cover crops increased populations of PAN (such as Tylenchidae) and fungivores in earlier MD studies (Gruver et al. 2010), no effect was observed on fungivores in the current study. Gruver et al. (2010) found no effect of radish on Tylenchidae or fungivores, which was generally the case with the radish+vetch treatment in our study. *Raphanus sativus* green manure also was not nematotoxic to *Globodera rostochiensis* (Valdes et al. 2011). Results likely vary with nematode taxon, amount of plant biomass, cultivar and growing conditions.

While vetch can be a nematode host (Clark 2007), this green manure showed some activity against nematodes in our study, but the results were not consistent. There was a trend the first two years (2010 & 2011) in which the hairy vetch green manure resulted in low PPN/PAN numbers after incorporation. This trend did not occur in 2012, when nematode populations in vetch plots were high shortly after green manure incorporation. The lack of trends may be due to fact that the variety of tomato used in this study is resistant to RKN. Interestingly, in a 2012 greenhouse pot trial in which soil was taken from the MD fields immediately post green manure incorporation,

inoculated with *Meloidogyne incognita*, and planted to a susceptible tomato variety (BHN 444), the vetch green manure resulted in the lowest number of *M. incognita* eggs on plant roots of all of the treatments (Meyer, unpubl.), indicating possible efficacy as a soil amendment against *M. incognita*.

Depending on the nutrient and organic matter content, weed pressure, erosion propensity or amount of compaction in the field, different cover crops or mixtures can serve the specific needs of a grower (Snapp et al., 2005). Our study clearly suggests that mixed-species and single-species cover crops are not able to consistently affect tomato crop productivity or suppress disease after a single season of incorporation across locations. However, repeated patterns in relative productivity and disease levels were observed for some CCT-site combinations, as discussed above. This indicates that it may be necessary for growers to closely evaluate the responses that occur on their land to any given CCT to ensure a positive return on investment. Such knowledge is important for soil management practices, where the recommendation has been to apply cover crops over various seasons in order to ensure benefits.

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CHAPTER 2

# Fungal and Oomycete Pathogen Detection in the Rhizosphere of Organic Tomatoes Grown in Cover Crop-Treated Soils[2]


## Abstract

Soil management practices, including the use of cover crops, affect soil and plant health through varied mechanisms. Impacts on microbial communities are known to be important, but are not well understood. Various techniques are used to measure the effect of treatments on microbial communities, but rarely are the results of more than one technique compared. This field study examined the impacts of a single-season application of cover crops on detection of pathogen species in the tomato crop rhizosphere. The study took place in Maryland, New York and Ohio (MD, NY and OH) in the summers of 2010 and 2011, with a total of 260 plots tested using both macroarray and T-RFLP analyses. The macroarray used in this study was specifically designed to detect thirty-one pathogens of solanaceous crops and had not previously been used for such a field study. The results of T-RFLP analysis, which is a common tool for examining microbial communities, were compared to the macroarray results and the limitations and benefits of each are presented. While not a quantitative measure, the macroarray was able to detect certain fungi with much greater sensitivity than T-RFLP. Our findings suggest that the results of PCR-based techniques used for microbial community studies should be compared to other methods to verify sensitivity.


[2] Reprinted from *Fungal and oomycete pathogen detection in the rhizosphere of organic tomatoes grown in cover crop-treated soils*. 2014. CF Summers, S Park, AR Dunn, X Rong, KL Everts, MD Kleinhenz, B McSpadden Gardener, and CD Smart. Applied Soil Ecology. 80, 44-50.

**Introduction**

Plant type, soil type and management practices all affect the microbial community structure of the soil ecosystem. Plants induce changes in soil microbial communities because the rhizosphere of plants encourages diverse and abundant microbial communities due to chemical exudates, mucilage production, improved aeration and moisture retention (Angers & Caron 1998). Likewise, microbial populations in the rhizosphere influence plant health, both directly and through interactions with other soil microbes (Kim et al. 2011; Whipps 2001). Different plant species have been found to encourage distinct microbial species populations in the rhizosphere after only four weeks of plant growth (Grayston et al. 1998).

The interaction of microorganisms with each other and with plants both result in differences in plant growth and in the extent to which disease can be suppressed in the agroecosystem (Garbeva et al. 2004). Suppressive soils are soils that limit the survival, growth or disease causing activity of plant pathogens. Suppression can be general or specific. General suppression reduces fungal, oomycete and nematode damage. Mechanisms of action are unclear and dynamic, however, suppression often appears to be due to total microbial biomass (Weller et al. 2002). Cover crops have been found to increase soil microbial biomass, which could allow for general suppression (Mendes et al. 1999; Schutter & Dick 2002). Research supports the role of enhanced microbial diversity in the disease suppression exhibited by cover crops (Abawi & Widmer 2000; Mazzola 2004; van Bruggen & Semenov 1999).

However, while research supports this association, the profile of microbial communities involved and their role in soilborne disease control are not well understood. Benitez et al. (2007) observed that disease suppression of damping-off on tomato and soybean increased following a mixed-species hay cover crop. Furthermore, terminal restriction fragment length polymorphism (T-

RFLP) analysis of bacterial communities was used to examine the rhizosphere of crop plants and correlate suppression to members of the genera *Burkholdaria, Bacillus, Paenibacillus,* and *Streptomyces*, all genera previously found to contain beneficial species. Larkin and Griffen (2007) observed suppression of various soilborne pathogens of potato in the field. The suppression occurred for all species of cover crop tested, which implies a role for microbial communities in the soil. A study of the short-term effects of an oat-vetch mixture on the growth of damping off pathogens, *Pythium aphanidermatum* (Edson) Fitzp. and *Rhizoctonia solani* (J.G. Kühn) suggests that cover crop incorporation leads to a suppressive effect in vitro (Grünwald et al. 2000).

In order to examine green manure impacts on soilborne pathogens in the crop rhizosphere, this project implemented macroarray and T-RFLP analyses. The macroarray detects thirty-one different fungal and oomycete pathogens of solanaceous crops common in the Northeastern region of the US, even at extremely low inoculum levels (Zhang et al. 2008). This technique was developed as a diagnostic tool for diseased plant samples and has not previously been used in a field study.

T-RFLP analysis has been used to determine the profile of microbial communities and identify potentially-beneficial bacteria (Benitez et al. 2007). Subsequent application of the technique revealed both beneficial and detrimental populations of fungi (Benitez 2008). As T-RFLP was previously used successfully in the above field study (Benitez 2008) to profile diverse species of microorganisms, this study tested the potential for detection of specific pathogens. The limit of detection of specific organisms by T-RFLP was not determined for this study. However, quantitative real-time PCR has been used in conjunction with T-RFLP in other studies to allow the quantification of targeted template in environmental samples (Yu et al. 2005). T-RFLP is relatively less expensive and more time efficient than a macroarray. While T-RFLP can be used to identify specific organisms through *in silico* correlation with terminal restriction fragment lengths, the true

accuracy of these assignments is not verifiable given the high degree of microbial diversity present in the soil. However, it is unknown if significant interference would occur with such assignments made from assays of root samples which would be expected to harbor less diversity by volume. Because specific species are able to be accurately targeted using a macroarray, this experiment provided a unique opportunity to assess the relative detection power of the two techniques.

In this study, the single-season impacts of mixed-species cover crops on organic tomato (*Solanum lycopersici* L.) crop rhizosphere pathogen detection was evaluated in three states with distinct soilborne disease pressure and repeated over two field seasons. Two PCR-based molecular techniques, macroarray and T-RFLP, were used to detect species of fungal and oomycete pathogens. The results of each were used to evaluate and compare the efficacy of each technique. Finally, macroarray analysis was used to assess cover crop treatment effects on pathogen presence.

**Materials and Methods**

*Transplant Production.* Tomato cultivar Celebrity (Johnny's Select Seed, Winslow, ME) was used. This cultivar has disease resistance to Verticillium wilt, Fusarium wilt Races 1 and 2, root-knot nematodes, Alternaria stem canker and tobacco mosaic virus (Rutgers Cooperative Extension, 2013).

Tomato seeds were sown into a locally-produced organic potting mix in 50 cell flats in NY and OH (TO Plastics, Clearwater, MN) and 128 cell flats in MD, then maintained in a greenhouse with sixteen hours of both natural and supplemental light per day. Seedlings were moved into a cold frame for at least 24h before transplant.

*Field Design.* Research was conducted in 2010 and 2011 at the University of Maryland Lower Eastern Shore Research and Education Center, Salisbury, the New York Agricultural Experimental Station, Phytophthora blight research farm in Geneva and the Ohio Agricultural Research and

Development Center, Wooster. The experiment was conducted as a randomized complete-block design with five treatments and six, eight or twelve replicates, for MD, NY and OH respectively (Table 2.1). The five treatments of single or mixed-species cover crop combinations were different in each state based on local growing conditions and practices. The experiment included the legumes hairy vetch (*Vicia villosa* Roth), crimson clover (*Trifolium incarnatum* L.) and alfalfa (*Medicago sativa* L.). Grasses used were annual rye (*Lolium multiflorum* Lam.), winter rye (*Secale cereal* M. Bieb) and mixed-species hay which included red fescue (*Festuca rubra* L.), orchard grass (*Dactylis glomerata* L.), and timothy (*Phleum pratense* L.), as well as the legumes crimson clover and alfalfa. The brassica species used were forage radish (*Raphanus sativus* var. *longipinnatus* L.) and forage turnip (*Brassica rapa* var. *rapa* L.). Maryland treatments included vetch (79 kg/ha) + winter rye (79 kg/ha); vetch (25 kg/ha); vetch (42 kg/ha) + radish (42 kg/ha); mixed-species hay (125 kg/ha with composition of equal seed number); and no cover. New York treatments included vetch (34 kg/ha) + winter rye (79 kg/ha); clover (10 kg/ha) + annual rye (18 kg/ha); turnip (15 kg/ha) + winter rye (45 kg/ha); winter rye (135 kg/ha); and no cover. Ohio treatments included winter rye (150 kg/ha); vetch (50 kg/ha); vetch (25 kg/ha) + winter rye (75 kg/ha); radish (10 kg/ha); and mixed-species hay with 56 kg/ha in 2010 and 112kg/ha in 2011, also with composition of equal seed number. Cover crop seed was sown in the fall and the cover crop was mowed and tilled in as a green manure the following spring three to five weeks before transplanting the tomatoes (Table 2.1). Fields in all states had raised beds covered with black plastic and drip irrigation. Tomatoes were grown using standard organic practices including trellising.

**Table 2.1** Field Setup and Timeline

| | MD | NY | OH |
|---|---|---|---|
| Number of Fields | 1 | 2 | 3 |
| Reps per Field | 6 | 4 | 4 |
| Total Reps per Year | 6 | 8 | 12 |
| Total Plots | 30 | 40 | 60 |
| Plot Size (m) | 6.4x12.2 | 2.4x7.6 | 3.1x6.1 |
| Rows/Plot | 2 | 1 | 4 |
| Plant Distance (m) | 0.9 | 0.6 | 0.6 |
| Tilled CC 2010 | 4/16/2010 | 5/5/2010 | 4/14/2010 |
| Tilled CC 2011 | 5/2/2011 | 5/14/2011 | 5/10/2011 |
| Transplanted Tomatoes 2010 | 5/14/2010 | 5/27/2010 | 6/3/2010 |
| Transplanted Tomatoes 2011 | 5/20/2011 | 6/10/2011 | 6/15/2011 |
| Rhizosphere Collection 2010 | 6/14/2010 | 6/30/2010 | 7/13/2010 |
| Rhizosphere Collection 2011 | 6/21/2011 | 7/11/2011 | 7/25/2011 |

*Tomato Rhizosphere DNA Extraction.* DNA extraction was performed on rhizosphere samples collected at four weeks post-transplant (Table 2.1). Rhizosphere samples were collected from two plants per plot from each plot and each state in 2010 and 2011. Each plant rhizosphere was processed separately to generate two DNA samples per plot. This resulted in a total of 130 plots per year, with 260 total plots for two years and 520 DNA samples total (two DNA samples per plot). The root regions of the tomato seedlings were carefully extracted from the soil with as much soil removed as possible before finely chopping all small roots less than 4 mm in diameter. DNA was extracted from 0.25 g of small root tissue from each plant using the MoBio UltraClean Soil DNA extraction Kit (MoBio, Carlsbad, CA) following the manufacture's protocol. Genomic DNA was assessed using gel electrophoresis with 1% agarose gels and visualized with UV light following ethidium bromide staining.

*Macroarray Analysis.* Macroarray analysis was performed on one DNA sample per plot for a total of 260 arrays. The ITS region was amplified with the universal primers ITS5 and ITS4 (White

et al., 1990). PCR was carried out with 1× GoTaq Green reaction buffer containing 1.5 mM $MgCl_2$ (Promega, WI), 0.2 mM dNTPs, 0.2 μM each primer, 1 unit GoTaq DNA polymerase (Promega), and 2.5 μL (15-80 ng) of genomic DNA in a 50 μl reaction. The following PCR cycling conditions were used: 95°C for 5 min; 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; followed by 10 min at 72°C. PCR products were purified according to the manufacturer's protocol using the Zymo PCR clean-up kit (Zymo Research, Irvine, CA). Macroarray analysis was performed as previously described, with thirty-one total pathogen species included on the macroarray membrane (Zhang et al. 2008). Briefly, 200 ng of purified amplicon DNA was hybridized overnight using the Gene Images AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham Biosciences, NJ) at 55°C to a nylon membrane spotted with the oligonucleotide fragments (267 μM) specific to fungal and oomycete pathogens. Hybridized amplicon DNA was visualized using Kodak Biomax Light film (Rochester, NY) exposed for one hour. Developed films were examined visually. All macroarray analyses were performed in NY to reduce variability.

*Statistical Analysis of Macroarray Data.* For macroarray data from each state, a positive detection was coded as '1' while pathogens undetected were coded as '0'. A generalized linear model (GLM) for binomial data was used to test the effect of cover crop treatment (CCT) on the presence of individual pathogens. If the main effect of CCT was significant at $P<0.05$, then a Tukey's Honestly Significant Differences (HSD) test was used to test for significant differences among CCTs. Analysis was performed for individual pathogens if the pathogen was detected more than four times in the state and year tested. In order to test the effect of CCT on total pathogens detected, a GLM employing a Poisson function was used. The total plots for each state per year were MD=30, NY=40 and OH=60. R statistical software (R Development Core Team 2011) and

the packages lme4 (Douglas et al. 2011), multcomp (Hothorn et al. 2008), RLRsim (Scheipl et al. 2008) and agricolae (Mendiburu 2010) were used for analyses.

*T-RFLP*. The T-RFLP assay was performed as described previously (Benitez et al. 2007; Benitez 2008). Briefly, the ITS region was amplified with the universal primers ITS5 and ITS4 (White et al. 1990). The ITS5 primer was labeled with the fluorescent WellRED dye D4 (Sigma, Proligo) for further visualization of the terminal restriction fragments (TRF). PCR was carried out with 1× GoTaq Flexi reaction buffer containing 1.5 mM $MgCl_2$ (Promega, WI), 0.2 mM dNTPs, 0.8 μM each primer, 1.5 units GoTaq Flexi DNA polymerase (Promega, WI), 0.04 mg RNaseA and 2.5 μL of genomic DNA (15-80 ng) in a 25 μl reaction. The following PCR cycling conditions were used: 95°C for 5 min; 33 cycles of 94°C for 1 min, 52°C for 45 s, and 72°C for 2 min; followed by 8 min at 72°C. Amplification products were purified from fluorescent primers by precipitation using 3 mM sodium acetate and ethanol. The full length PCR products were digested using *Hha*1 enzyme and sent to the Molecular and Cellular Imaging Center (OARDC, Wooster, OH) for imaging. There, 0.1 μl of sample was mixed with 0.2 μl 600 bp size standard (CEQ DNA size standard kit 600) and 20 μl formamide. TRF were loaded and separated on the CEQ 8800 Genetic Analysis System (Beckman Coulter, Brea, California) and individual profiles were analyzed with the CEQ fragment analysis software (CEQ 8000 Genetic Analysis System). The cut off rate for terminal restriction fragments was 1% of the highest peak in the chromatogram profile of the sample. The T-RFLP analysis utilized both DNA samples from each plot for a total of 520 T-RFLP analyses. All T-RFLP analyses were performed in OH to reduce variability.

*in silico Terminal Restriction Fragment (TRF) Determination.* Sequences for tomato and pathogens of interest were mined from Genbank and digested *in silico* using the *Hha*1 enzyme cutting site (restrictionmapper.org). The TRF from the ITS5 primer end used in the T-RFLP analysis was

recorded and used for further analyses. Two tomato sequences (Genbank AY552528 and JN713416) were used to generate two TRF (109 bp and 110 bp respectively). Two *Fusarium oxysporum* Schlecht. sequences (Genbank JN859461.1 and JN253789.1) generated TRF of 317 bp and 316 bp, respectively. One *Alternaria alternata* Fr. Keissl. sequence (Genbank JF440581.1) generated a 334 bp TRF. For Fo, a TRF value was also available from actual T-RFLP analysis from isolate cultures, where the TRF matched the *in silico* TRF value of 316 bp (Benitez 2008).

*Percent Detections and Comparison of Macroarray and T-RFLP Detections of Tomato Rhizosphere Pathogens.* All T-RFLP data from TRF correlated to specific organisms *in silico* were converted from fluorescence intensity units into a binary detection result: positive or negative. Because the T-RFLP analysis utilized both DNA samples per plot and the macroarray analysis was performed for one sample per plot, only one DNA sample was common to both tests. Therefore, in order to compare the T-RFLP and macroarray detection results within each plot, the two T-RFLP results were compiled into one detection result by coding the detection positive if at least one result was positive. For each plot, the macroarray result could then be matched directly with the T-RFLP result. For analysis of percent detection of Aa and Fo, the compiled result from the T-RFLP duplication was used in order to compare the results to the within-plot detections. However, for the percent detection of the control organism known to be present, tomato, all T-RFLP data points were considered separately in order to create a more accurate comparison of percent detection since control DNA was known to be present. Therefore, no within-plot comparison is presented for the control. The *in silico* TRFs generated were often close to those detected by T-RFLP, however, between states and years, there were often 1-3 base pair length differences. In order to do the inter-technique comparison, the most common TRF which were closest to the detected TRF sizes was chosen for the analysis. A

difference of no more than 3 bp was allowed between the predicted TRF size and the detected TRF size.

In order to compare the macroarray and T-RFLP results within each plot, the two T-RFLP results for each plot had to be combined into one result. However, in order to further assess the T-RFLP efficiency, the two T-RFLP results within each plot were also compared. In order to do this, the percentage of pathogen positive T-RFLP plots where both results were positive was calculated.

**Results**

*Macroarray Analysis.* Fifteen pathogens were detected using the macroarray in the three states over the two years of testing (Table 2.2). The most frequently detected fungi were *Fusarium oxysporum* and *Alternaria alternata*. *Fusarium oxysporum*, *Alternaria alternata*, *Phoma destructiva* Plowr., *Fusarium solani* and *Septoria lycopersici* Speg. were detected in all states and years. Although the NY fields were known to be infested with *Phytophthora capsici* Leonian, this pathogen was only detected once out of eighty plots sampled over two years. *Colletotrichum* spp., *Pythium aphanidermatum, Phytophthora nicotianae* Breda de Haan and *Pyrenochaeta lycopersici* R.W. Schneid. and Gerlach.were detected in OH only. In MD, *Pythium irregulare* Buisman, *P. cryptoirregulare* Garzón, Yánez and G.W. Moorman and *Verticillium albo-atrum* Reinke and Berthold were identified. *Rhizoctonia solani* and *Pythium ultimum* Trow. were detected in OH and MD (Table 2.2). The remaining sixteen pathogens on the array were not detected.

**Table 2.2** Detection of plant pathogens in tomato rhizosphere DNA collected at 4 weeks post-transplant by macroarray analysis when tomatoes are planted following different cover crops in field sites in three states over two years

| | Plots[a] | | | | | |
|---|---|---|---|---|---|---|
| | MD | | NY | | OH | |
| Pathogen | 2010 | 2011 | 2010 | 2011 | 2010 | 2011 |
| *Fusarium oxysporum*[b] | 15 | 25 | 38 | 35 | 41 | 60 |
| *Alternaria alternata*[b] | 7 | 29 | 14 | 21 | 35 | 54 |
| *Fusarium solani* | 1 | 18 | 7 | 6 | 4 | 27 |
| *Phoma destructiva* | 3 | 22 | 10 | 13 | 19 | 36 |
| *Septoria lycopersici* | 5 | 7 | 8 | 2 | 11 | 27 |
| *Phytophthora capsici* | 0 | 0 | 1 | 0 | 0 | 0 |
| *Colletotrichum* spp. | 0 | 0 | 0 | 0 | 11 | 18 |
| *Pythium aphanidermatum* | 0 | 0 | 0 | 0 | 4 | 0 |
| *Pyrenochaeta lycopersici* | 0 | 0 | 0 | 0 | 3 | 7 |
| *Pythium ultimum* | 10 | 0 | 0 | 0 | 9 | 0 |
| *Pythium cryptoirregulare* | 1 | 0 | 0 | 0 | 0 | 0 |
| *Rhizoctonia solani* | 0 | 7 | 0 | 0 | 2 | 15 |
| *Pythium irregulare* | 3 | 0 | 0 | 0 | 0 | 0 |
| *Verticillium albo-atrum* | 2 | 0 | 0 | 0 | 0 | 0 |
| *Phytophthora nicotianae* | 0 | 0 | 0 | 0 | 0 | 1 |

[a] Total number of plots sampled each year: NY=40, OH=60, MD=30. Numbers in table denote number of plots where the given pathogen was detected.

[b] *Fusarium oxysporum* and *Alternaria alternata* detection does not distinguish between pathogenic and non-pathogenic strains.

There were no significant effects of CCT on pathogen species detected (Table 2.3). In only one state/year out of six, NY 2010, and for only Aa, Tukey analysis indicated that the rye-treated plots had significantly more detections of Aa than the bare ground-treated plots (Table 2.4). However, Aa was consistently detected in all states and years and this outcome was not part of a larger trend.

**Table 2.3** Results of GLM analysis testing (p-values) for significant effect of cover crop on pathogen detection by macroarray in tomato rhizosphere in three states sampled in two years. The total number of plots tested for each state is NY=40, OH=60 and MD=30.

| | Pathogen[a] | Treatment[b] |
|---|---|---|
| MD 2010 | Total | 0.13 |
| | *Alternaria alternata* | 0.02[c] |
| | *Pythium ultimatum* | 1 |
| | *Septoria lycopersici* | 0.51 |
| MD 2011 | Total | 1 |
| | *Alternaria alternata* | 0.26 |
| | *Fusarium solani* | 0.70 |
| | *Phoma destructiva* | 0.46 |
| | *Rhizoctonia solani* | 1 |
| | *Septoria lycopersici* | 0.41 |
| NY 2010 | | |
| | Total | 0.36 |
| | *Alternaria alternata* | 0.01[d] |
| | *Fusarium solani* | 0.56 |
| | *Phoma destructiva* | 0.87 |
| | *Septoria lycopersici* | 0.83 |
| NY 2011 | Total | 0.18 |
| | *Alternaria alternata* | 0.09 |
| | *Fusarium solani* | 0.03[c] |
| | *Phoma destructiva* | 0.13 |
| OH 2010 | Total | 0.30 |
| | *Alternaria alternata* | 0.14 |
| | *Colletotrichum* sp. | 0.01[c] |
| | *Fusarium solani* | 0.81 |
| | *Phoma destructiva* | 0.65 |
| | *Pythium ultimatum* | 0.42 |
| | *Septoria lycopersici* | 0.63 |
| OH 2011 | Total | 0.29 |
| | *Alternaria alternata* | 0.11 |
| | *Colletotrichum* sp. | 0.40 |
| | *Fusarium solani* | 0.33 |
| | *Phoma destructiva* | 0.32 |
| | *Pyrenochaeta lycopersici* | 0.06 |
| | *Rhizoctonia solani* | 0.05[c] |
| | *Septoria lycopersici* | 0.47 |

[a] Test results for "Total" signify the effect of CCT on all pathogen detections in a given field. The effect of CCT on presence of individual pathogens detected in at least four plots in a field is also shown.
[b] Treatments are variable for each state. See methods.
[c] Although the effect of CCT was significant, there were no significant differences among CCT treatments based on Tukey analysis.
[d] See Table 2.4.

**Table 2.4** Effects of CCT on *Alternaria alternata* detection, NY 2010

| Treatment | Plots Detected[a] |
|---|---|
| Rye | (5)a |
| Clover+Rye | (2)ab |
| Turnip+Rye | (4)ab |
| Vetch+Rye | (2)ab |
| Bare Ground | (1)b |

[a] Letters denote compact letter display from Tukey analysis. The number in parentheses is the number of detections out of eight repetitions of each treatment.

*Percent Detections and Comparison of Macroarray and T-RFLP Detections.* Table 2.5 shows the specificity and sensitivity of macroarray and T-RFLP analysis in detecting specific organisms from the tomato rhizosphere. The control for both techniques was the detection of tomato DNA, as the ITS4-ITS5 primers used also amplify a segment of the tomato ITS gene. The quantity of DNA from this species is likely the most abundant, and therefore, should be detected consistently. The average percentage from all states and years indicates that macroarray was able to detect tomato DNA 96% of the time, with T-RFLP only detecting the control 53% of the time. While in MD in 2010, T-RFLP performed equally well as macroarray, in all other states and years, T-RFLP detected tomato DNA at a much lower rate, with the lowest success in NY 2010 with 34% detection for T-RFLP versus 93% detection by macroarray.

The presence of two common fungal pathogens, Aa and Fo*,* were also compared. Overall, the macroarray was able to detect these two organisms much more frequently than T-RFLP. The average rate of detection across states and years for Aa was 59% by macroarray, compared to 24% by T-RFLP. The results among states and years for Aa detection were variable, with Aa detected in 97% of MD 2011 samples tested by macroarray as compared to 10% by T-RFLP, while in NY 2010, macroarray detected it 35% of the time and T-RFLP 88% of the time. The same trends were found

for Fo detection, with macroarray detecting it 80% of the time overall compared to 44% for T-RFLP. In MD 2010, macroarray detected Fo less than T-RFLP with 50% versus 63%. However, in OH 2011, macroarray found Fo in 100% of the samples, compared to 12% for T-RFLP.

Both techniques, however, were prone to a lack of sensitivity. While macroarray overall could detect tomato DNA in 96% of samples, it did not detect the control in 10% of samples from MD in 2010. In addition, when considering the detection comparison from the state and year averages, T-RFLP detected Aa from 14% of samples and Fo from 8% of samples when macroarray did not. These differences could show a lack of sensitivity by macroarray, however, as indicated previously, these could be TRF from other organisms. The sensitivity of T-RFLP indicates low performance, as it only was able to detect tomato DNA in 53% of all samples. In addition, Aa was detected in 49% of samples and Fo in 43% of samples, by macroarray alone (not T-RFLP).

The calculation of the percentage of T-RFLP plots where both results were positive shows that few plots resulted in two positive results for the T-RFLP analysis. For MD in 2010 as well as 2011, only 3% of plots positive for Aa were positive for both T-RFLP results. For NY 2010, Aa was detected by both T-RFLP tests in 35% of positive plots, while in 2011, in only 8% of plots. For OH 2010, Aa was detected by both T-RFLP tests in only 1% of positive plots and 7% of plots in 2011. For Fo detections, the results were slightly more favorable. In MD 2010, both tests were positive in 19% of positive plots, and in 2011 it was 16%. For NY, both T-RFLP results were positive in 28% of plots in 2010 and 22% in 2011. For OH in both 2010 and 2011, T-RFLP results were the least consistent, with 7% of plots having both T-RFLP results positive out of the total positive plots.

**Table 2.5** Comparison of percent positive macroarray and T-RFLP detections of tomato, *Alternaria alternata* and *Fusarium oxysporum* DNA from tomato rhizosphere samples

| | Percent Detection[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | MD | | NY | | OH | | |
| | 2010 | 2011 | 2010 | 2011 | 2010 | 2011 | Total[b] |
| *Solanum lycopersicum*[c] | | | | | | | |
| Macroarray | 90 | 97 | 93 | 100 | 96 | 97 | 96 |
| T-RFLP[d] | 92 | 28 | 34 | 36 | 58 | 70 | 53 |
| *Alternaria alternata*[e] | | | | | | | |
| Macroarray | 23 | 97 | 35 | 53 | 58 | 90 | 59 |
| T-RFLP | 10 | 10 | 88 | 20 | 2 | 12 | 24 |
| T-RFLP only[f] | 7 | 0 | 63 | 8 | 2 | 2 | 14 |
| Macroarray only[g] | 20 | 87 | 10 | 40 | 58 | 80 | 49 |
| Results same for both[h] | 73 | 13 | 28 | 53 | 33 | 18 | 36 |
| *Fusarium oxysporum*[e] | | | | | | | |
| Macroarray | 50 | 83 | 90 | 88 | 68 | 100 | 80 |
| T-RFLP | 63 | 53 | 70 | 55 | 12 | 12 | 44 |
| T-RFLP only[f] | 27 | 7 | 5 | 8 | 0 | 0 | 8 |
| Macroarray only[g] | 13 | 37 | 25 | 40 | 57 | 88 | 43 |
| Results same for both[h] | 60 | 57 | 70 | 53 | 37 | 12 | 48 |

[a] DNA was extracted from two tomato rhizosphere samples per plot at four weeks post-transplant. The macroarray analysis was performed with one sample per plot, and the T-RFLP analysis was performed on both samples.

[b] 'Total' reflects the average of the percent detections over all states and years.

[c] *Solanum lycopersicum* serves as an effective positive control, since all DNA extractions used (780 samples total) were extracted from the tomato rhizosphere.

[d] For *Solanum lycopersicum*, the percentages are calculated from total detections in all samples (without combining the results of two DNA extractions per plot prior to analysis). This is because we knew control DNA to be present and did not wish to compare within-plot detections.

[e] For the comparisons of pathogen detection by macroarray and T-RFLP within each plot, one macroarray result needed to be compared to one T-RFLP result. To do this, if either result from the two T-RFLP analyses was positive, the detection was marked positive.

[f] Percentage of plots where T-RFLP detected the given organism but macroarray did not.

[g] Percentage of plots were macroarray detected the given organism but T-RFLP did not.

[h] Percentage of plots where macroarray and T-RFLP detections were either both positive or both negative.

**Discussion**

Effects of cover crop treatments on pathogens have been measured directly by experiments in culture, greenhouse and field trials. A variety of cover crops were found to inhibit the growth of many soilborne pathogens of potato *in vitro* and in greenhouse studies, and also demonstrated effectiveness in the field (Larkin & Griffin 2007). Benitez and others (2007) evaluated the effect of different field management strategies in the field and greenhouse on soilborne disease and concurrently analyzed T-RFLP profiles in order to mine for certain bacterial species associated with disease suppression (Benitez et al. 2007). Another study found that the toxins produced by the incorporation of the potential biofumigant, *Brassica juncea*, initially reduced inoculum potential of the soilborne pathogens *Rhizoctonia solani* and *Fusarium oxysporum* (Friberg et al. 2009). However, Ochiai et al. (2008) found that cover crop amendment, especially larger amounts of biomass, reduced inoculum of *Verticillium dahliae*, regardless of the biofumigant potential (Ochiai et al. 2008).

In contrast to the above studies, no short term cover crop impact on pathogen detection was observed in this study. However, we do not conclude that this result is evidence that cover crops do not affect crop health or microbial communities. First, as noted earlier, general suppression may not result from a direct impact on specific pathogens. For example, in several studies, cover crops, even when associated with disease reduction, were not associated with a direct reduction in fungi. Zhou and Everts (2004) found that a vetch amendment increased the CFU of culturable fungi, as well as bacteria. This occurred in soils where Fusarium wilt was suppressed. Buyer (2010) also found that cover crops increased total microbial biomass, including soil fungi, using phospholipid fatty analysis. Omirou (2011) also found that cover crops impact microbial communities, but not pathogen presence, by the introduction of plant biomass. This indicates that in some pathosystems, suppression may not result from a direct impact on pathogens. Another mechanism important in

some systems is enhanced resistance of the host plant (Kumar et al. 2004). In one study on tomato, Kumar and others (2004) demonstrated that disease suppression following a no-till hairy vetch cover crop was associated with up-regulation of specific classes of genes including defense related genes such as chitin and osmotin.

As discussed above, the complex interactions between plants, microbes and the soil environment have been measured in various ways and findings have been inconstant. Because of the variable findings of other papers, we decided to compare the results of the two PCR-based techniques we used in our study. T-RFLP has been used for years to examine microbial communities, while the macroarray was designed to be a diagnostic tool. By utilizing both macroarray and T-RFLP analyses to detect rhizosphere pathogens, the specificity and sensitivity of these two molecular techniques could be compared. Macroarray analysis is labor-intensive to design and is very specific to crop-pathogen systems as well as region. In addition, it is relatively more expensive and time-consuming than T-RFLP to run for a large-scale field study. T-RFLP also has the potential benefit of being semi-quantitative, in contrast to macroarray analysis. For these reasons, T-RFLP would be a more likely choice for similar field studies in other pathosystems. However, the macroarray is designed to detect specific organisms, and is sensitive even at very low DNA concentrations (Zhang et al. 2008). Therefore, since detection was designed to be specific to one organism only, a positive detection can be assumed accurate. In order to detect specific organisms using T-RFLP analysis, precise TRF lengths must be correlated to distinct organisms. Because there is some likelihood that more than one organism could have the same TRF length in a complex environment, false positives are possible. For both techniques in this study, an important weakness was inability to differentiate between pathogenic and non-pathogenic strains of *Fusarium oxysporum* and *Alternaria alternata*, as the ITS regions of these pathogens are very similar within their

species (Lievens et al. 2003; Zhang et al. 2008). This is important because some *Fusarium oxysporum* have been related to disease suppression, not pathogenicity (Larkin et al. 1996).

In order to compare T-RFLP and macroarray, we identified species detected often by macroarray, then performed *in silico* T-RFLP analysis to identify TRF that should correspond to each species. We then compared the detection results for these pathogens by calculating the percent number of detections in each plot, where 260 plots were tested over two years in three states (Table 2.5). In this study, the data for T-RFLP are skewed towards positive detections, because in order to compare T-RFLP and macroarray detections within each plot, two detection results from T-RFLP had to be combined into one result. In this case, one positive out of two tests resulted in a positive detection. This bias was accounted for by calculating the percent of plots where both T-RFLP results were positive out of the total number of positive plots. Nevertheless, in this study the macroarray used was able to detect various organisms, including the control, much more consistently than T-RFLP. In addition, though *Colletrichum* sp. and *Rhizoctonia solani* were commonly detected by macroarray in OH (Table 2.2) the TRF predicted to correlate to the presence of these organisms were not present. This suggests T-RFLP detection may be restricted to dominant and abundant species, as previously found by another study comparing detection methods (Allmér et al. 2006). This, however, cannot explain the inability of T-RFLP to detect the tomato DNA more consistently, since this DNA was likely among the most abundant in each sample. Although the PCR conditions were different for the two techniques, both T-RFLP and macroarray used the same primers, ITS4 and ITS5 (White et al. 1990), which both have a 100% identity with the tomato ITS sequence.

Previous studies have identified various problems with using T-RFLP for microbial fingerprinting. These include sources of variation due to both target gene variability in the

community as well as method inconsistencies due to restriction enzyme effectiveness or PCR efficiency (Avis et al. 2006). However, it is possible that the T-RFLP methods in this experiment could have been optimized to be more effective, as studied by Osborn and others (2000). For both T-RFLP and macroarray, one important source of variability in evaluating the effects of cover crop treatment on pathogen presence in each plot may have resulted from differences in genomic DNA quality. However, since the macroarray analyses and T-RFLP analyses utilized the same genomic DNA samples, the comparison of results for the two techniques should not be heavily influenced by variable genomic DNA quality or quantity.

Macroarray data was chosen to evaluate the cover crop treatment effects on the pathogen species we detected because the sensitivity was shown to be satisfactory and the specificity acceptable. Although no differences among treatments for pathogen detection were observed by the macroarray data in this study, this technique is not quantitative. Therefore, while it can detect the incidence of affected plants, it does not detect a reduction in pathogen levels. If a CCT decreased the population, but did not eliminate it, no difference would be observed, or would be observed only in the incidence of a pathogen. Furthermore, the pathogens that were detected may have been present at low levels that were not epidemiologically important in causing tomato disease.

Another important factor affecting which pathogens were detected was the timing of rhizosphere sampling. *Phytophthora capsici*, known to be present in the NY fields tested and causing disease later in the season, was not detected in the rhizosphere at four weeks post-transplant when samples were collected. It is possible that rhizosphere samples collected later in the season may have shown significant effects of CCT on pathogen detection.

In conclusion, two important PCR-based molecular tools, T-RFLP and macroarray, were compared in a field study. Because of the complex nature of microbial ecology and pathogen studies,

as well as the many factors involved in choosing methods with which to evaluate them, studies including and comparing more than one technique to investigate microbial communities are necessary. This continues to be important today, as more modern molecular techniques involving sequencing are also PCR-based (Anderson & Cairney 2004). Quantitative real-time PCR (Smith & Osborn 2009) and advances in next-generation sequencing (Schmidt et al. 2013) continue to further advance the field of microbial ecology. Our findings suggest that PCR-based techniques may need to be corroborated by concurrently using quantitative measures and checking with a diagnostic tool.

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CHAPTER 3

# *Pseudoperonospora cubensis* and *P. humuli* detection using species-specific probes and high definition melt curve analysis[3]


## Abstract

Real-time PCR assays using locked nucleic acid (LNA) probes and high resolution melt (HRM) analysis were developed for molecular differentiation of *Pseudoperonospora cubensis* and *P. humuli,* causal agents of cucurbit and hop downy mildew, respectively. The assays were based on a previously identified single nucleotide polymorphism (SNP) in the cytochrome oxidase subunit II (*cox2*) gene that differentiates the two species. Sequencing of the same region from 15 *P. cubensis* isolates collected in NY for the current study confirmed that all isolates shared the conserved SNP. LNA probes were specific and sensitive, detecting as few as 10 sporangia for both species and as little as 1 fg *P. cubensis* total DNA and 10 fg *P. humuli* total DNA. The LNA assay detected both pathogens from air sampled using spore traps placed in vegetable fields and a hop yard during the summers of 2013 and 2014 and correctly diagnosed symptomatic leaf tissue. High resolution melt analysis (HRM) correctly identified all tested isolates as well as those isolates from symptomatic plants collected in the field. The LNA and HRM assays correctly identified both organisms when tested independently in a second laboratory. The results confirm that the LNA and HRM assays developed can provide reliable identification of both species despite the high molecular similarity of the *cox2* gene.


[3] Reprinted from Pseudoperonospora cubensis *and* P. humuli *detection using species-specific probes and high definition melt curve analysis.* 2015. CF Summers, N Adair, M McGrath, D Gent and CD Smart. Canadian Journal of Plant Pathology.

## Introduction

*Pseudoperonospora cubensis* (Berk. & M.A. Curtis) and *P. humuli* (Miyabe & Takah.) are closely related obligate biotrophic plant pathogens, which cause cucurbit downy mildew (CDM) and hop downy mildew (HDM), respectively. CDM is a foliar disease, which can cause dramatic plant health decline, greatly impacting fruit yields (Savory et al. 2011). It afflicts members of the Cucurbitaceae family worldwide, with the most economically important species being cucumber (*Cucumis sativus* L.), cantaloupe and muskmelon (*Cucumis melo* L.), squash and pumpkin (*Cucurbita pepo* L.*, C. maxima* Duchesne and *C. moschata* Duchesne ex Poir) and watermelon (*Citrulus lanatus* Thunb. Matsum & Nakai) (Lebeda & Cohen 2011). In 2013 for NY alone, there were 1,290 hectacres of cucumber grown, valued at $17 million (USDA 2013), with CDM imposing the greatest threat to yield (Cornell Vegetable Program 2015). HDM results in a reduction in yield and quality of hop cones (*Humulus lupus* L.), as well as stunted growth and potential death of the perennial root system (Skotland & Johnson 1983). New York was home to a thriving hop production industry in the mid 1800's, producing the majority of the nation's hops (Bissel 1877). However, market factors, hop powdery mildew, and HDM caused the industry to collapse by the 1920's (Skotland & Johnson 1983; Sirrine et al. 2010). The disease is now endemic in hop production regions throughout the northern hemisphere and in Argentina (Royle & Kremheller 1981; Perez et al. 2003). With the introduction of various resistant cultivars, the development of integrated pest management (IPM) strategies and registration of targeted, systemic fungicides, the hop industry in New York, as well as incidence of HDM, is growing once again (Northeast Hop Alliance 2012).

Resistant cultivars provide an important defense against crop damage from downy mildews. However, cucumber resistance to CDM was overcome in the United States in 2004, dramatically increasing the need for fungicides (Savory et al. 2011). In addition, disease pressure from CDM is

increasing in northern latitudes; whereas *P. cubensis* has been known to perennate on cucurbits grown in Florida and then annually re-infect more northern latitudes by long-distance dispersal, cucumbers are now being grown in hothouses in the northeastern US and Canada, potentially providing inoculum much earlier in the season (Ojiambo & Holmes 2011). *Pseudoperonospora cubensis* may also have perennial hosts that could allow overwintering farther north (Runge & Thines 2012) and recent reports indicate *P. cubensis* may be transmitted through seed (Cohen et al. 2014).

For both CDM and HDM, disease prediction is another important component of IPM systems. The CDM IPM-Pest Information Platform for Extension and Education (PIPE) compiles weather and disease reports to alert growers of infection risk as the pathogen spreads through the country, and therefore, provides information important for initiating fungicide application (Ojiambo & Holmes 2011). Unlike *P. cubensis*, *P. humuli* overwinters in its perennial host (Coley-Smith 1962). Thus, disease risks are predicted based on weather, and if available, airborne inoculum level (Royle & Kremheller 1981).

If inoculum data is combined with weather data, a disease prediction system provides a more accurate assessment of infection risk (West et al. 2008). This has the potential to provide growers with more tools with which to manage disease outbreaks and reduce fungicide use (Gent et al. 2013). A system for detection of *P. humuli* sporangia was developed in 2009, utilizing PCR to detect the internal transcribed spacer (ITS) region of the pathogen (Gent et al. 2009). However, because *P. humuli* and *P. cubensis* share a high level of sequence similarity in the genes investigated thus far (Choi et al. 2005), it was not possible to design primers specific to *P. humuli* (Gent et al. 2009). In 2011, one conserved and three semi-conserved single nucleotide polymorphisms (SNPs) in the cytochrome oxidase subunit II (*cox2*) gene were found to differentiate the two species (Mitchell et al. 2011). Thus, we utilized the one conserved SNP to design two assays allowing for specific diagnosis

and detection of both pathogens: 5' nuclease locked nucleic acid (LNA) probes and high resolution melt (HRM) analysis.

Real-time PCR utilizing 5' nuclease probes labeled with different fluorophores allows for the detection of various SNPs in one reaction (Livak 1999). However, when a single SNP is the differentiating factor between alleles, there can be cross-reactivity (Klosterman et al. 2014). In a mixed-DNA sample, cross-reactivity can complicate the detection of a specific pathogen (Klosterman et al. 2014). In this situation, LNA hybridization probes can be used to increase the specificity of an assay. These probes contain LNA bases, RNA analogs modified to contain a bridge on the ribose ring which "locks" the ring into the ideal conformation for base stacking (Koshkin et al. 1998a; Koshkin et al. 1998b). This increases the melting temperature (Tm), allowing for greater specificity (Koshkin et al. 1998a; Koshkin et al. 1998b). The probes are designed so that a single mismatch will require a lower Tm for successful binding, thus preventing non-specific hybridization at the reaction annealing temperature (Johnson et al. 2004; Ugozzoli et al. 2004).

High resolution melt analysis (HRM) is a faster and relatively less expensive alternative to fluorogenic probes for genotyping and diagnostics (Wittwer 2009). HRM requires a real-time PCR machine and specialized software, but after these initial start-up costs, it can be readily applied to differentiate SNPs, or even microsatellites (Mackay et al. 2008). HRM utilizes conventional primers, with amplification taking place in the presence of a saturating intercalating dye that specifically binds to double-stranded DNA and fluoresces brightly when bound. After amplification is complete, the temperature of the reaction is slowly raised to induce denaturation of the double-stranded amplicon, while reduction in fluorescence, caused by release of a dye from the DNA duplex, is measured. A single SNP difference results in a measureable variation in melting temperature recorded over time (Garritano et al. 2009).

The goal of this study was to develop assays for identification of *P. cubensis* and *P. humuli* based on the conserved SNP described above. LNA probes were designed to provide specific detection of both *P. cubensis* and *P. humuli* from a mixed-DNA sample. A HRM analysis assay was developed as a relatively cheaper diagnostic tool.

**Materials and Methods**

*Pathogen isolates.*

*Pseudoperonospora cubensis.* Fifteen *P. cubensis* isolates were collected from fields with symptomatic hosts in NY (8 counties) during the summers of 2012 and 2013 (Table 3.1). Ten of these samples were from cucumber, two from cantaloupe, two from squash and one from pumpkin. Diseased leaves were placed in a moist chamber overnight to induce sporulation. Sporangia were harvested and inoculated onto leaves of the susceptible cucumber cultivar 'Straight Eight'. Cucumber plants were grown in a potting mix composed of peat, perlite and vermiculite in a 4:1:1 ratio and produced in a greenhouse free of downy mildew. Clonal isolates were obtained by inoculating 7-day old cucumber seedlings with sporangia from a single lesion of each isolate. Sporangia were sprayed on seedlings using an air pressurized sprayer (Nalgene, Rochester, NY). This single lesion process was repeated three times per isolate to reduce the possibility of a mixed isolate. Inoculated plants were placed in dark moist chambers at 16°C overnight then moved to a greenhouse with a 23.9°C day temperature, 18.3°C night temperature and a 14 hr light/10 hr dark photoperiod. Once lesions appeared on the cucumber seedlings, the plants were placed in a moist chamber (>90% relative humidity) in the dark at 16°C for 24 to 48 hr until prolific sporulation was observed. Isolates were stored on cucumber tissue at -80°C.

**Table 3.1 Assay Development**: Identity, host and origin of organisms used to develop LNA probe and HRM assays designed to detect *Pseudoperonospora cubensis* and *Pseudoperonospora humuli* based on a conserved single nucleotide polymorphism at base 105 in the *cox2* gene. Results for multiplexed LNA probe assays are given in threshold cycle (Cq) values (mean±standard deviation, n=3), where probes were labeled with HEX/FAM for *P. cubensis* and *P. humuli*, respectively.

| Organism | Isolate | Host | Year | Location | *cox2* accession[a] | Cq and SD (HEX)[b] |
|---|---|---|---|---|---|---|
| *Pseudoperonospora cubensis* | CDM12- 45 | *Cucumis sativus* | 2012 | Erie, NY | KJ806590 | 21.44±0.17 |
| *Pseudoperonospora cubensis* | CDM12- 58 | *Cucumis sativus* | 2012 | Seneca, NY | KJ806591 | 21.58±0.25 |
| *Pseudoperonospora cubensis* | CDM12- 60 | *Cucumis sativus* | 2012 | Seneca, NY | KJ806592 | 21.34±0.30 |
| *Pseudoperonospora cubensis* | CDM12- 95 | *Cucumis sativus* | 2012 | Cayuga, NY | KJ806593 | 23.20±0.06 |
| *Pseudoperonospora cubensis* | CDM13- 1 | *Cucumis sativus* | 2013 | Erie, NY | KJ806596 | 21.28±0.16 |
| *Pseudoperonospora cubensis* | CDM13- 2 | *Cucumis sativus* | 2013 | Suffolk, NY | KJ806597 | 23.52±0.11 |
| *Pseudoperonospora cubensis* | CDM13- 3 | *Cucumis sativus* | 2013 | Orleans, NY | KJ806598 | 25.07±0.18 |
| *Pseudoperonospora cubensis* | CDM13- 4 | *Cucumis sativus* | 2013 | Ontario, NY | KJ806599 | 21.67±4.82 |
| *Pseudoperonospora cubensis* | CDM13- 6 | *Cucumis melo* | 2013 | Suffolk, NY | KJ806600 | 18.89±0.24 |
| *Pseudoperonospora cubensis* | CDM13- 8 | *Cucumis melo* | 2013 | Ontario, NY | KJ806601 | 24.22±1.87 |
| *Pseudoperonospora cubensis* | CDM13- 9 | *Cucumis sativus* | 2013 | Cayuga, NY | KJ806602 | 18.54±0.17 |
| *Pseudoperonospora cubensis* | CDM13- 10 | *Cucurbita pepo* | 2013 | Suffolk, NY | KJ806603 | 22.00±0.13 |
| *Pseudoperonospora cubensis* | CDM13- 12 | *Cucurbita pepo* | 2013 | Suffolk, NY | KJ806604 | 14.82±0.22 |
| *Pseudoperonospora cubensis* | CDM13- 13 | *Cucurbita pepo* | 2013 | Albany, NY | KJ806605 | 20.29±0.70 |
| *Pseudoperonospora cubensis* | CDM13- 14 | *Cucumis sativus* | 2013 | Erie, NY | KJ806606 | 19.56±0.45 |
| Organism | Strain | Host | Year | Location | *cox2* accession[a] | Cq and SD (FAM)[b] |
| *Pseudoperonospora humuli* | HDM NY 2012 | *Humulus lupulus* | 2012 | Ontario, NY | KJ806595 | 21.36±0.37 |
| *Pseudoperonospora humuli* | HDM NY 2013 | *Humulus lupulus* | 2013 | Ontario, NY | KJ806594 | 23.96±1.47 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Pseudoperonospora humuli* | HDM 490 | *Humulus lupulus* | 2012 | Aomori, Japan | - | 15.89±0.29 |
| *Pseudoperonospora humuli* | HDM 496 | *Humulus lupulus* | 2012 | Hokkaido, Japan | - | 17.44±0.04 |
| *Pseudoperonospora humuli* | HDM 500 | *Humulus lupulus* | 2013 | Marion, OR | - | 15.12±0.24 |
| *Pseudoperonospora humuli* | HDM 501 | *Humulus lupulus* | 2013 | Marion, OR | - | 17.08±0.11 |
| *Pseudoperonospora humuli* | HDM 510 | *Humulus lupulus* | 2013 | Mazomamie, WI | - | 17.27±0.06 |
| Non-Target Organisms | Strain | Host | Year | Location | *cox2* accession[a] | Cq and SD (HEX/FAM)[b] |
| *Plasmopara viticola* | AK1 | *Vitis vinifora* | 2010 | Ontario, NY | KJ806607 | 0 |
| *Plasmopara halstedii* | - | *Helianthus annuus* | 2014 | Ontario, NY | - | 0 |
| *Peronospora belbahrii* | - | *Ocimum basilicum* | 2014 | Tomkins, NY | - | 0 |
| *Hyaloperonospora brassicae* | - | *Brassica oleracea* | 2014 | Ontario, NY | - | 0 |
| *Phytophthora infestans* | - | *Solanum lycopersicum* | 2012 | Ontario, NY | - | 0 |
| *Alternaria brassicicola* | - | *Brassica oleracea* | 2013 | Ontario, NY | - | 0 |
| *Pseudozyma flocculosa* | - | *Podosphaera macularis* | 2011 | Benton, OR | - | 0 |
| *Epicoccum nigrum* | EN001 | *Humulus lupulus* | 2011 | Benton, OR | - | 0 |
| *Podosphaera macularis* | - | *Humulus lupulus* | 2014 | Benton, OR | - | 0 |
| *Cladosporium herbarum* | CL001 | *Humulus lupulus* | 2012 | Benton, OR | - | 0 |
| *Alternaria* sp. | AL-002 | *Humulus lupulus* | 2011 | Benton, OR | - | 0 |
| *Verticillium albo-atrum* | HVA-055 | *Humulus lupulus* | 2006 | Marion, OR | - | 0 |
| *Podosphaera xanthii* | - | *Cucurbita pepo* | 2014 | Tompkins, NY | - | 0 |
| *Cucumis sativus* | - | NA | 2013 | NA | - | 0 |
| *Cucurbita pepo* | - | NA | 2013 | NA | - | 0 |
| *Humulus lupulus* | - | NA | 2013 | NA | - | 0 |

[a] Genbank accession numbers are from the sequenced *cox2* partial gene region containing the four SNPs of interest in this study.

[b] Cq values and standard deviations listed are from the fluorophore in parentheses, where probes in the multiplexed reaction were labelled with HEX/FAM, for *P. cubensis* isolates and *P. humuli* isolates, respectively.

*Pseudoperonospora humuli.* Hop shoots with signs of systemic infection were collected from hop yards and monosporangial isolates of *P. humuli* were derived as described previously (Gent et al. 2008) or by depositing a single sporangium onto a leaf disk using a MoFlo flow cytometer (Beckman Coulter, Inc., Brea, CA). For the latter method, leaf disks were cut from greenhouse-produced hop plants (cv. 'Pacific Gem') with a #10 cork borer and the adaxial surface placed onto 1% water agar in a 24-well serological plate. A suspension of sporangia from a given sample of *P. humuli*, cultured as described by Mitchell et al. (2011), was loaded into a flow cytometer calibrated to deliver a single sporangium onto each leaf disk. After deposition of sporangia, the leaf disks were misted with sterile deionized water using an airbrush sprayer and incubated in a growth chamber set to 13°C for seven to 14 days. Isolates were subsequently increased and maintained using the methods of Mitchell et al. (2011).

*DNA Extraction and cox2 gene sequencing.*

Sporangia were collected for DNA extraction by gently rinsing sporangia from leaves using type-1 water. Sporangia were then pelleted by centrifugation at 1,000 x g for 5 min. DNA from *P. cubensis* and *P. humuli* isolates was extracted using a CTAB procedure as described (Keb-Llanes et al. 2002), with modifications to buffer A. Beta-mercaptoethanol and polyvinylpyrrolidone were omitted as we found they were not necessary to obtain high quality DNA from sporangial tissue. The concentration of DNA was assessed using a Qubit fluorometer and Qubit double-stranded DNA High Sensitivity Assay Kit (Life Technologies, Grand Island, NY), which measures fluorescence emitted when the dye in the assay reagents bind selectively to intact DNA. The *cox2* region of the *P. cubensis* and *P. humuli* isolates used in the development of the assay (Table 3.1) was amplified using previously described FM35 and FM36 primers (Mitchell et al. 2011). PCR was carried out with 1× GoTaq Green reaction buffer containing 1.5 mM $MgCl_2$ (Promega, Madison, WI), 0.2 mM dNTPs

(Promega), 0.2 μM each primer (Integrated DNA Technologies, Coralville, IA), 2 units GoTaq DNA polymerase (Promega), 2.5 μg bovine serum albumin (Roche, Basel, Switzerland) and 2 μL (10 ng) of total DNA in a 50 μL reaction. Negative controls lacking DNA template were included. The following PCR cycling conditions were used: 95°C for 2 min; 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; followed by 5 min at 72°C. DNA fragments were electrophoresed in 1% agarose Tris-acetate EDTA gels stained with ethidium bromide and visualized using an ultraviolet transilluminator. Amplicons were then sequenced using primers F26 and R557 (Table 3.2), which were designed using the *cox2* gene sequences published in Genbank by Mitchell et al. (2011). Sequencing was performed on a 3730XL (Sanger) DNA Analyzer (Applied Biosystems) at the Cornell University Life Sciences Core Laboratories Center. Sequences were aligned using the program Seaview (Gouy et al. 2010) and submitted to Genbank, with the accession numbers listed in Table 3.1.

**Table 3.2** Primers and probes designed for assays differentiating *Pseudoperonospora cubensis* and *Pseudoperonospora humuli* using the SNP at base 105 in the *cox2* gene target region.

| PCR type | Assay | Forward primer (5'-3')/ Probe (5'-3') | Reverse primer (5'-3') | Position on *cox2* gene[a] | Product size (bp) | Species[b] |
|---|---|---|---|---|---|---|
| real-time | SNP105 Real Time | RT33F: AACTCCCGTTATGGAAGGTATT | RT182R: CCATGTACAACAGTAGCTGGA | 33-182 | 149 | Both |
| real-time | SNP105 Probe[c] | CUBprobeSNP105: HEX- ACAAACGAATACT-BHQ1 | - | 99-111 | N/A | *P. cubensis* |
| real-time | SNP105 Probe[c] | HUMprobeSNP105:FAM-AACAAACAAATACTG-BHQ1 | - | 98-112 | N/A | *P. humuli* |
| conventional | Sequencing[d] | F26: AGATCCTGCAACTCCCGTTAT | R557: CGTCCAGGGCATGCATCTAAT | 26-557 | 534 | Both |



[a] Numbers given are the beginning and ending nucleotide base pair numbers from the *cox2* region of focus in this study.

[b] Species the given primer set is designed to amplify.

[c] Locked nucleic acids in the probes are underlined. The conserved SNP at base 105 of the targeted *cox2* region is in bold red font.

[d] The F26-R557 primers were designed to amplify the targeted *cox2* region containing the one conserved SNP at base 105 as well as the three semi-conserved SNPs at bases 343, 414 and 468.

*Primer/Probe Design and qPCR protocols.*

*Real-time PCR with LNA probes.* All qPCR detection assays were designed and implemented in accordance with the 'Minimum Information for Publication of Quantitative Real-Time Experiments' (MIQE) guidelines (Bustin et al. 2009). Primers (RT33F-RT182R) and LNA probes (CUBprobeSNP105 and HUMprobeSNP105) targeting the conserved SNP at base 105 of the *cox2* gene were designed by IDT, using the *cox2* gene sequences published in Genbank by Mitchell et al. (2011). A 20 μL multiplexed reaction was used containing 1x IQ Supermix (Bio-rad, Hercules, CA), 500 nM of each primer and 250 nM of each probe. One ng total DNA was used as template. Negative control reactions lacking DNA template were included. The PCR protocol, run on a CFX 96 Touch qPCR system (Bio-rad) in 96-well white plates (Bio-rad MLL9651), included an initial denaturation at 95°C for 3 min then 35 cycles of a 95°C denaturation for 10 s and annealing/extension step at 65°C for 45 s, plus a plate read. Three technical replicates of each sample were run and the average Cq and standard deviation were calculated using Bio-rad CFX Manager software (version 3.1). For the isolates used in the calculation of the standard curve, two biological replicates with three technical replicates each were used.

The LNA assay also was conducted independently in the Gent laboratory (US Department of Agriculture) at Oregon State University to ensure its reproducibility. PCR reagents and conditions, including the real-time PCR system, were exactly as described above. Isolates of *P. cubensis* and *P. humuli* used in the development of the assays (Table 3.1) were tested, as were nine other isolates of *P. humuli* that were not used in assay development (Table 3.3). This resulted in a total of 15 single-lesion *P. cubensis* isolates (Table 3.1) and 16 single-sporangial *P. humuli* isolates (Tables 3.1 & 3.3).

*High Resolution Melt Curve (HRM) analysis.* Real-time PCR with HRM analysis utilized the same primers as those used for the LNA assay (RT33F-RT182R) which flank the conserved SNP at base 105 of the *cox2* amplicon. Precision melt supermix (Bio-rad) and 500 nM of each primer were mixed with 1 ng (3 μL) template in 20 μL reaction volumes. Negative control reactions lacking DNA template were included. The reaction was run on a CFX 96 Touch qPCR system and followed the suggested program from the manufacturer's supermix protocol, with 2 min denaturation at 95°C followed by 42 cycles of denaturation 95°C, 10 s; annealing + extension + plate read 63°C, 30 s; then denaturation 95°C, 30 s; heteroduplex formation 60°C, 1 min; high resolution melt + plate read 65 to 95°C, in 0.2°C increments at 10 s per step. Two technical replicates of each sample were run. Following amplification, temperature-shifted melt curves and difference plots produced by the Precision melt analysis software (Bio-rad) were evaluated to detect separation of melt curves between *P. cubensis* and *P. humuli* isolates.

The HRM assay was conducted independently in the Gent laboratory to ensure reproducibility. PCR reagents and conditions, including the real-time PCR system, were exactly as described above. As with the LNA assay, isolates used in the development of assays (Table 3.1) and nine other monosporangial isolates of *P. humuli* (Table 3.3) were tested. Again, this resulted in a total of 15 *P. cubensis* isolates (Table 3.1) and 16 *P. humuli* isolates (Tables 3.1 & 3.3).

**Table 3.3 Assay Validation**: Identity, host and origin of isolates used to validate real-time LNA probe and HRM PCR assays designed to detect *Pseudoperonospora cubensis* and *Pseudoperonospora humuli* based on the conserved single nucleotide polymorphism at base 105 of the *cox2* gene target region. Results for multiplexed LNA probe assays are given in threshold cycle (Cq) values (mean± standard deviation, n=3), where probes were labelled with HEX/FAM for *P. cubensis* and *P. humuli*, respectively.

| Organism | Isolate[b] | Host | Year | Location | Cq and SD (HEX)[a] |
|---|---|---|---|---|---|
| *Pseudoperonospora cubensis* | Field-CDM- A | *Cucumis sativus* | 2013 | Erie, NY | 20.14±0.24 |
| *Pseudoperonospora cubensis* | Field-CDM- B | *Cucumis sativus* | 2013 | Suffolk, NY | 20.64±0.21 |
| *Pseudoperonospora cubensis* | Field-CDM- C | *Cucurbita pepo* | 2013 | Albany, NY | 24.64±0.58 |
| *Pseudoperonospora cubensis* | Field-CDM- D | *Cucumis sativus* | 2013 | Erie, NY | 24.31±0.30 |
| *Pseudoperonospora cubensis* | Field-CDM- E | *Cucumis sativus* | 2014 | Ontario, NY | 30.61±0.65 |
| *Pseudoperonospora cubensis* | Field-CDM- F | *Cucumis melo* | 2014 | Ontario, NY | 21.30±0.80 |
| Organism | Isolate[c] | Host | Year | Location | Cq and SD (HEX)[a] |
| *Pseudoperonospora cubensis* | Non-sporulating 1 | *Cucumis sativus* | 2014 | Ulster, NY | 23.51±0.41 |
| *Pseudoperonospora cubensis* | Non-sporulating 2 | *Cucumis sativus* | 2014 | Ulster, NY | 22.33±0.02 |
| *Pseudoperonospora cubensis* | Non-sporulating 3 | *Cucumis sativus* | 2014 | Chenango, NY | 21.85±0.33 |
| *Pseudoperonospora cubensis* | Non-sporulating 4 | *Cucumis sativus* | 2014 | Tompkins, NY | 20.37±0.61 |
| *Pseudoperonospora cubensis* | Non-sporulating 5 | *Cucumis sativus* | 2014 | Niagara, NY | 21.35±0.57 |
| Organism | Isolate[d] | Host | Year | Location | Cq and SD (FAM)[a] |
| *Pseudoperonospora humuli* | HDM 457 | *Humulus lupulus* | 2011 | Marion, OR | 29.15±0.63 |
| *Pseudoperonospora humuli* | HDM 481 | *Humulus lupulus* | 2011 | Ontario, NY | 30.86±0.66 |
| *Pseudoperonospora humuli* | HDM 482 | *Humulus lupulus* | 2012 | Ontario, NY | 28.57±0.79 |
| *Pseudoperonospora humuli* | HDM 498 | *Humulus lupulus* | 2012 | Ehoro, Japan | 30.06±0.97 |
| *Pseudoperonospora humuli* | HDM 502 | *Humulus lupulus* | 2013 | Marion, OR | 27.62±1.20 |
| *Pseudoperonospora humuli* | HDM 503 | *Humulus lupulus* | 2013 | Grand Isle, VT | 27.52±0.69 |

| | | | | | |
|---|---|---|---|---|---|
| *Pseudoperonospora humuli* | HDM 504 | *Humulus lupulus* | 2013 | Grand Isle, VT | 30.46±0.78 |
| *Pseudoperonospora humuli* | HDM 505 | *Humulus lupulus* | 2013 | Ontario, NY | 32.38±0.51 |
| *Pseudoperonospora humuli* | HDM 509 | *Humulus lupulus* | 2013 | Yakima, WA | 31.31±0.45 |
| Organism | Isolate[e] | Host | Year | Location | Cq and SD (FAM)[a] |
| *Pseudoperonospora humuli* | Non-sporulating 1 | *Humulus lupulus* | 2012 | Aomori, Japan | 27.58±2.00 |
| *Pseudoperonospora humuli* | Non-sporulating 2 | *Humulus lupulus* | 2013 | Marion, OR | 30.41 ±1.72 |

[a] Cq values and standard deviations listed are from the fluorophore in parentheses, where probes in the multiplexed reaction were labelled with HEX/FAM, for *P. cubensis* and *P. humuli*, respectively.

[b] Cucurbit samples symptomatic for cucurbit downy mildew were collected from the field and used to test if the assays were able to identify *P. cubensis* in infected but non-sporulating tissue.

[c] Non-sporulating cucumber plant tissue exhibiting lesions caused by *P. cubensis* were used to test if the assays were appropriate for diagnosis.

[d] The *P. humuli* isolates listed were used for independent validation of real-time PCR and HRM assays in a laboratory at Oregon State University.

[e] Non-sporulating *P. humuli* isolates were tested in an independent laboratory at Oregon State University. Cq values and standard deviations listed are averaged from 10 biological replicates per isolate (separate inoculations and DNA extractions) and three technical replications per biological replication.

*Sensitivity of real-time PCR with LNA probes.* The three methods below were used to determine the sensitivity and detection limit of the LNA diagnostic assay.

*Sporangial detection.* Sensitivity was quantified by testing DNA obtained from 10 independent extractions for each of the following: 0, 1, 10, 50, 100 and 500 *P. cubensis* and *P. humuli* sporangia. In order to extract DNA from 1 and 10 sporangia, dilutions of sporangia were spread on a thin layer of 0.5% agarose on a microscope slide, a dilute suspension of sporangia was spread on the solidified agarose, sporangia were located with the aid of a dissecting microscope, and removed with a small section of agarose. Similarly sized pieces of agarose to those included in the above extractions were also added to 10 negative control extractions containing no sporangia. For extractions of 100 and 500 sporangia, the number of sporangia within a suspension was quantified using a hemacytometer and an appropriate aliquot was taken for extraction. DNA was extracted using a MoBio UltraClean Soil DNA Isolation Kit (MoBio, Carlsbad, CA) following manufacturer's instructions, with 0.02 g/mL of polyvinyl pyrrolidone (PVP) added to buffer S1. DNA was quantified using a Qubit fluorometer. Real-time PCR with LNA probes was performed as described above. Two technical replicates of each sample were run and the average Cq of the two replicates was used to calculate the average and standard deviation of the Cq for all 10 samples per sporangial number (0, 1, 10, 50, 100 and 500) for each species.

*Dilutions of total DNA*. The sensitivity of the species-specific LNA probes was also quantified by testing 10-fold dilutions of genomic DNA ranging from 1 ng to 1 fg. Two separate DNA extractions of pathogen isolates, CDM12-6 and HDM NY 2012, were used for each dilution series. Three technical replicates for each biological replicate and sample dilution were tested and the average Cq values with standard deviation were calculated using Bio-rad CFX Manager software. Mean Cq values were plotted against $\log_{10}$ (template DNA concentration) and used to generate standard

curves for the *P. cubensis* and *P. humuli*-specific LNA assays. Linear regression analysis was conducted and the slope was used to determine the $R^2$ value of each reaction using MatLab version 13a.

*Mixed-DNA samples*. The third method used to determine sensitivity tested the ability to detect each pathogen in mixed-DNA samples. A portion of the *cox2* gene (the F26-R557 amplicon) from *P. cubensis* isolate CDM13-58 and *P. humuli* isolate HDM NY 2013 was amplified and the amplicon was cloned using the TopoTA cloning kit (Life Technologies), according to manufacturer's recommendations, then sequenced. Ten-fold dilutions of plasmid DNA were made from 10 ng to10 pg and used as template, both separately and mixed in varying concentrations (Table 3.5) for the LNA assay described above. Two technical replicates of each concentration and mixture were run and the average Cq and standard deviations were calculated using Bio-rad CFX Manager software.

*Specificity of LNA and HRM assays.*

The specificity of each assay was tested using DNA from 16 other organisms: 4 downy mildew pathogens, 9 other oomycetes and fungi commonly associated with cucurbits or hop or having airborne spores, as well as cucumber cv. 'Straight Eight', summer squash cv. 'Multipick' and hop cv. 'Pacific Gem' (Table 3.1). In order to ensure that lack of amplification of non-target organisms was not due to inhibition in the PCR, fungal and oomycete samples were also tested with PCR using primers ITS4-ITS5, while plant samples were tested using primers ITS4-ITS1, which amplify the internal transcribed spacer region using a previously described protocol (White et al. 1990).

*Detection from plant samples.*

*Symptomatic plant samples collected from the field*. In order to test if the LNA and HRM assays would be appropriate for diagnosing plant samples obtained from the field with minimal sporulation and presence of other organisms, six symptomatic leaves infected with *P. cubensis* were collected from six

separate cucurbit fields (Table 3.3). Sporangia were washed from the leaf surface using water and collected by centrifugation at 1,000 x g for 5 min. DNA was extracted from sporangia and each assay was performed as described above, using 1 ng template.

*Non-sporulating plant samples*. To test if the assays could detect the pathogens within plant tissue prior to sporulation, each assay was performed on DNA extracted from plant tissue presenting lesions, but having no observable sporulation using a dissecting microscope. Samples of *P. cubensis* were tested at Cornell University and *P. humuli* samples were tested at Oregon State University (Table 3.3).To produce non-sporulating samples, young cucumber and hop plants were inoculated and incubated as described above. Control plants were mock-inoculated with sterile distilled water and processed similarly. At approximately 3 days post-inoculation, leaves with lesions but no sporulation were collected. For *P. cubensis*, DNA from one symptomatic leaf from each of 5 isolates, as well as 3 negative controls, was extracted using the CTAB method described above (Keb-Llanes et al. 2002). For *P. humuli*, 10 plants were inoculated with isolate HDM 490 using the methods of Mitchell et al. (2011). Three days after inoculation, one angular lesion without sporulation was excised from each inoculated plant using a #10 cork borer. DNA was extracted from each leaf disk separately (for 10 biological replications per isolate), as well as 3 negative controls, using a rapid one-step extraction procedure (Steiner et al. 1995). The experiment was later repeated using isolate HDM 502.

For the LNA assay, three technical replicates of each plant sample described above were performed. For the cucumber samples infected with *P. cubensis*, the average Cq values and standard deviations were calculated using Bio-rad CFX Manager. For the hop samples infected with *P. humuli*, the Cq values and standard deviations were averaged from the ten biological replicates per isolate (separate inoculations and DNA extractions) and three technical replications per biological

replication, for a total of 30 replications averaged into one set of values per isolate. In addition, the HRM analysis was performed on these samples as described above in both laboratories.

*Detection from air samples.*

In order to detect pathogen sporangia from air samples, spore traps were placed in a commercial hop yard and vegetable fields during the spring and summer of 2013 and 2014. A total of 31 air samples were collected in 2013; in 2014, a total of 211 samples were collected (Table 3.4). In 2013, one trap located in a hop yard in Ontario County, NY was placed at 2 m distance from the edge of hop plantings. In 2014, seven spore traps were used, with one being placed in the same hop yard, three in vegetable fields in Ontario County, NY and three in vegetable fields in Suffolk County, NY (Table 3.4). For Field A (Ontario County), one spore trap was placed at the edge of a vegetable field at approximately 300 m distance from cucurbit crops. Two spore traps were placed in each of Fields B (Ontario County) and D (Suffolk County), where the traps were located at the opposite edges of the fields. The spore trap in Field C (Suffolk County) was at Cornell University field facility on Long Island (LIHREC). The spore traps in Fields B and C were less than 10 m and approximately 300 m, respectively, away from cucurbit sentinel plots. The spore traps in Field D were next to an organic mixed-vegetable plot on a farm that did not contain cucurbits and was located two miles away from the sentinel plot containing cucurbits near Field C.

The custom-built spore traps, similar to those previously described (Falacy et al. 2007; Gent et al. 2009) sampled air by rapidly spinning two stainless steel sample collection rods (stainless steel tig, 3cm long, 1.2 mm diameter; J.W. Harris Co., Mason, Ohio), lightly coated with silicon high vacuum grease (Dow, Corning). The sampling rods were perpendicularly attached to each end of a horizontal rod (9 cm), which was fixed at its center to a solar-powered motor (Mabuchi FR-500T-10750, 1950 rpm) (Gent et al. 2009). This allows approximately 106 liters of air to be sampled per

minute, calculated using Pappus's Theorum for volumes applied to spore traps (Frenz & Boire 1999). The rods were positioned at 1.5 m above the ground. Rods were collected and replaced two times a week in Ontario County and once a week in Suffolk County. Both rods per trap were placed in a single tube and DNA was extracted using a MoBio UltraClean Soil DNA Isolation Kit (MoBio) following manufacturer's instructions, with 0.02 g/mL of polyvinyl pyrrolidone (PVP) added to buffer S1. Real-time PCR using LNA probes was performed twice per sample as described above, using 3 μL template. Real-time PCR was also conducted for a subset of samples on 1:2 and 1:10 dilutions of template and template spiked with 0.01 ng of total DNA (for both *P. cubensis* and *P. humuli* assays) to test for inhibition. To verify positive results, amplicons from spore trap DNA template were periodically cloned and sequenced, as described above.

Detection of *P. cubensis* was compared with timing of disease outbreaks occurring in the monitored region and when the CDM-IPM-PIPE system (Ojiambo & Holmes 2011) predicted inoculum was in the vicinity.

**Table 3.4** Spore trap placement in New York counties in 2013 and 2014. Samples were collected approximately twice weekly in Ontario County and once weekly in Suffolk County during the range of given collection dates.

| Year | County | Field | Spore trap | Dates Collected | Samples |
|---|---|---|---|---|---|
| 2013 | Ontario | Hop Yard | 1 | 3 May to 30 August | 31 |
| 2014 | Ontario | Hop Yard | 2 | 25 March to 9 September | 56 |
| 2014 | Ontario | A | 3 | 5 March to 18 September | 49 |
| 2014 | Ontario | B | 4 | 6 June to 8 October | 35 |
| 2014 | Ontario | B | 5 | 6 June to 8 October | 35 |
| 2014 | Suffolk | C | 6 | 4 June to 11 September | 14 |
| 2014 | Suffolk | C | 7 | 4 June to 17 September | 15 |
| 2014 | Suffolk | D | 8 | 2 June to 17 September | 17 |
| | | | | Total number of samples: | 252 |

## Results

*Sequencing of the targeted cox2 gene region.*

A 534 bp portion of the *cox2* gene was sequenced using the F26-R557 primers designed for this experiment (Table 3.2) for the 15 *P. cubensis* and 2 *P. humuli* isolates collected in NY, which were used to develop the LNA and HRM assays. The sequences from the *P. cubensis* isolates aligned to previously sequenced Genbank accessions (Mitchell et al. (2011) with 100% identity and contained the one conserved SNP (at base 105) and semi-conserved SNPs at bases 343, 414 and 468, except the isolates collected from squash: CDM13-10, CDM13-12 and CDM13-13. Those three *P. cubensis*

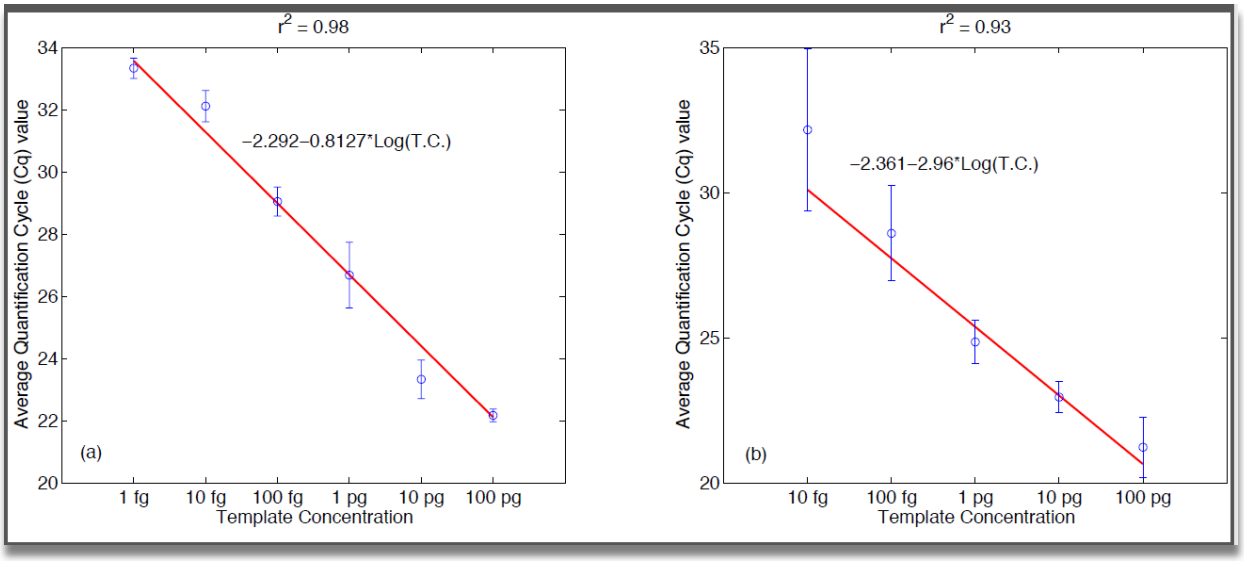
isolates instead matched the *P. humuli cox2* sequence at bases 343, 414 and 468. However, the SNP at base 105 differentiated all *P. cubensis* and *P. humuli* isolates evaluated. The sequences have been deposited in Genbank (Table 3.1).

*Sensitivity of real-time PCR with LNA probes.*

*Sporangial detection*. For both *P. cubensis* and *P. humuli,* the LNA assay detected DNA from extractions containing 10, 50, 100 and 500 sporangia in 10 out of 10 reactions. Neither assay could detect DNA from a single sporangium. Negative control reactions (n=10) containing zero sporangia yielded no amplification. The average Cq and standard deviations for the amplification of *P. cubensis* sporangia were: 10 sporangia, 29.71±1.26 (<10 pg/ μL); 50 sporangia, 26.43±1.94 (<10 pg/ μL); 100 sporangia, 26.12±3.54 (90 pg/ μL); 500 sporangia, 25.02±1.31 (93 pg/ μL). The average Cq and standard deviations for the amplification of *P. humuli* sporangia were: 10 sporangia, 33.32±0.95 (<10 pg/ μL); 50 sporangia, 22.64±0.96 (<10 pg/ μL); 100 sporangia, 23.75±1.04 (106 pg/ μL); 500 sporangia, 22.70±1.36 (111 pg/ μL).

*Dilutions of total DNA*. The HEX-labelled LNA probe specific to *P. cubensis* detected this pathogen with total DNA template of concentrations from 0.1 ng to 1 fg, with $R^2$=0.98 (Figure 3.1a). The FAM-labelled LNA probe specific to *P. humuli* was able to detect dilutions from 0.1 ng to 10 fg, with $R^2$=0.93 (Figure 3.1b).

**Figure 3.1** Linear regression of 10-fold dilutions of *Pseudoperonospora cubensis* (a) and *Pseudoperonospora humuli* (b) DNA against corresponding mean quantification cycle values (Cq). Data points represent the means and standard deviations from three technical replicates for each of two biological replicates.




*Mixed-DNA sample*. To test the ability to detect each pathogen in mixed-DNA samples, the assay was performed with cloned fragments mixed at varying concentration ratios. Using the cloned fragment from either *P. cubensis* or *P. humuli* individually, amplification was observed for all DNA concentrations tested (Table 3.5). When 10 ng or 1 ng of plasmid DNA containing the *P. cubensis* sequence was mixed with 10 pg of plasmid DNA containing the *P. humuli* sequence, no detection of *P. humuli* DNA was observed. Detection of *P. cubensis* was observed in all mixtures. However, when 10 ng or 1 ng of DNA containing the *P. humuli* sequence was mixed with 10 pg of plasmid DNA containing the *P. cubensis* sequence, detection of *P. cubensis* occurred at much higher Cq values than reactions including only 10 pg of plasmid DNA containing the *P. cubensis* sequence (Table 3.5).

**Table 3.5** Threshold cycle (Cq) values (mean± standard deviation, n=2) for multiplexed real-time PCR LNA probe assays using a *cox2* gene fragment cloned from *P. cubensis* or *P. humuli* individually or mixed together at varying concentrations.

| | Template | | Cq±Standard Deviation[a] | |
|---|---|---|---|---|
| | *P. cubensis* | *P. humuli* | HEX | FAM |
| Un-mixed Template | 10 ng | - | 10.73±0.02 | nd |
| | 1 ng | - | 13.43±0.71 | nd |
| | 100 pg | - | 17.58±0.05 | nd |
| | 10 pg | - | 18.92±0.24 | nd |
| | - | 10 ng | nd | 7.59±0.06 |
| | - | 1 ng | nd | 12.26±0.24 |
| | - | 100 pg | nd | 16.28±0.01 |
| | - | 10 pg | nd | 22.18±5.38 |
| Mixed Template | 10 ng | 10 ng | 13.84±0.35 | 8.49±0.06 |
| | 1 ng | 1 ng | 16.32±0.49 | 13.26±0.37 |
| | 100 pg | 100 pg | 19.28±0.17 | 17.42±0.01 |
| | 10 pg | 10 pg | 19.04±1.88 | 20.66±0.17 |
| | 10 ng | 10 pg | 12.24±0.24 | nd |
| | 1 ng | 10 pg | 15.42±0.08 | nd |
| | 10 pg | 10 ng | 33.95±0.05 | 9.83±0.06 |
| | 10 pg | 1 ng | 33.21±0.05 | 13.08±0.03 |

[a] The HEX-labelled probe detects target DNA from *P. cubensis* and the FAM-labelled probe detects DNA from *P. humuli*. Reactions with undetected amplification are denoted 'nd'.

*Specificity of LNA and HRM assays.*

Real-time PCR with LNA probes. The LNA assay correctly identified the 15 *P. cubensis* and 7 *P. humuli* isolates used to develop the assay (Table 3.1). The Cq values from the detection of fluorescence from each LNA probe varied between single-lesion isolates, from 14.82±0.22 (CDM13- 12) to 25.07±0.18 (CDM13-3) for *P. cubensis* isolates and from15.12±0.24 (HDM 500) to 23.96±1.47 (HDM NY 2013) for *P. humuli* isolates (Table 3.1). The nine additional *P. humuli* isolates tested at Oregon State University had Cq values that ranged from 27.52±0.69 (HDM 503) to 32.38±0.51 (HDM 505) (Table 3.3). No amplification was observed in negative control reactions lacking DNA template. There were no false positive detections among the 16 non-target organisms tested, including host plant DNA (see Table 3.1 for microbe and plant DNA tested). The DNA from these non-target organisms did amplify using the ITS4-ITS5 or ITS4-ITS1 primers (White et al. 1990), ensuring the lack of amplification using the assay was not due to inhibition (data not shown).

High Resolution Melt Curve (HRM) analysis. The HRM assay correctly identified the 15 *P. cubensis* and 7 *P. humuli* isolates used to develop the assay (Figure 3.2), as well as the 9 additional *P. humuli* isolates tested at Oregon State University used to validate the assay (Figure 3.3). The *P. humuli* target *cox2* region melted at a slightly lower temperature than the *P. cubensis* target region due to the presence of the SNP, allowing differentiation between the two species (Figures 3.2 & 3.3). No amplification was observed in negative control reactions lacking DNA template. There were no false positive detections among the 16 non-target organisms tested, including host plant DNA (see Table 3.1 for microbe and plant DNA tested). Figure 3.2 depicts the HRM run which contained all of the samples listed in Table 3.1, all *P. cubensis* field-collected plant samples and *P. cubensis*-infected non-sporulating isolates, as well as non-target species for which no amplification or melt curves resulted. The HRM assay correctly differentiated *P. cubensis* from *P. humuli* when repeated at the laboratory at Oregon

State University when tested with *P. humuli* isolates used for assay development or validation (Figure 3.3).

**Figure 3.2** Melt (a) and difference (b) curves from a High Resolution Melt curve assay (HRM) for *Pseudoperonospora cubensis* and *Pseudoperonospora humuli* samples. Each isolate was tested in duplicate. The assay differentiates the two species based on a single nucleotide polymorphism variation which allows for thermal denaturation divergence. The two species' melting curves cluster separately, where *P. cubensis* samples are colored red and *P. humuli* samples are colored green. The run included DNA from all isolates listed in Table 3.1, including non-target controls, which did not amplify, and thus, do not appear on the graphs. In addition, this run included *P. cubensis* field samples, *P. cubensis* non-sporulating plant samples and two negative control wells.

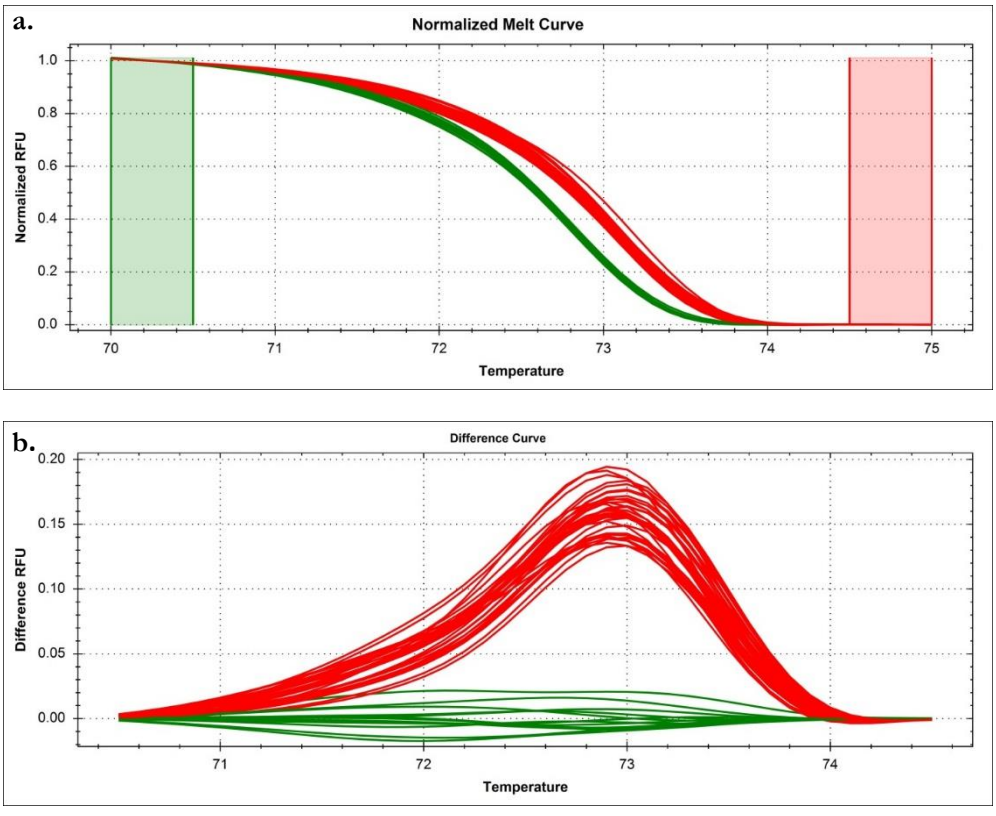
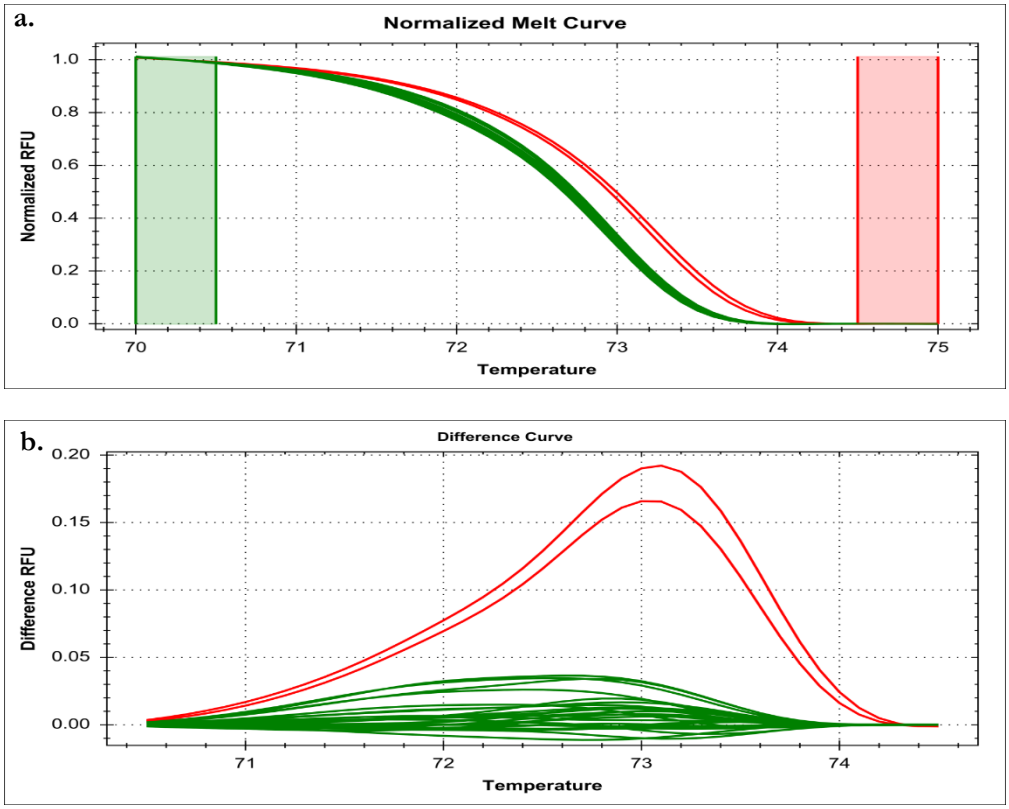


**Figure 3.3** Melt (a) and difference (b) curves from a High Resolution Melt curve assay for *Pseudoperonospora cubensis* and *Pseudoperonospora humuli* when conducted in a laboratory at Oregon State University. Each isolate was run in duplicate. Isolate CDM12-58 of *P. cubensis* is shown in red. Isolates of *P. humuli* are colored in green, and include five isolates (HDM 490, 496, 500, 501, and 510) used for assay development and the nine isolates listed in Table 3.3 used for assay validation.




*Detection from plant samples.*

Real-time PCR with LNA probes accurately identified the 6 samples of *P. cubensis* sporangia washed from symptomatic leaves collected from the field and the 5 non-sporulating, but symptomatic, leaves. The Cq values from field samples ranged from 20.14±0.24 to 30.61±0.65 (Table 3.3). The Cq values from non-sporulating samples ranged from 20.37±0.61 to 23.51±0.41 (Table 3.3). The assay also correctly identified *P. humuli* from infected but non-sporulating lesions when performed at

Oregon State University, with Cq values ranging from 29.01±0.1.97 to 32.29±3.35 in the first run of the experiment and 26.67±1.29 to 29.44±4.97 in the second run of the experiment. Averages of these value ranges are presented in Table 3.3 for the first (non-sporulating 1) and second (non-sporulating 2) run of the experiment.

HRM melt curves from field and non-sporulating samples of *P. cubensis* (Figure 3.2) and non-sporulating samples of *P. humuli* (data not shown) clustered with the melt curves of single-lesion isolates from the correct species. No amplification was observed in negative control reactions lacking DNA template or from healthy plant tissue (negative controls are included in reaction results shown in Figure 3.2, where no amplification curve from those reactions are visible).

*Detection from air samples.*

*Detection of P. cubensis*. *P. cubensis* was not detected in the 31 samples collected in 2013 from the spore trap placed in the hop yard, nor the 56 samples from the hop yard in 2014. From the 165 air samples collected from cucurbit fields during 2014, four were positive for *P. cubensis*. The positive sampling dates were from Ontario County in field B (from spore trap #4 located at the west of the field) for the following dates in September: the 12$^{th}$ (Cq 32.73±0.85), the 15$^{th}$ (27.55±0.76), the 26$^{th}$ (34.14±0.27) and the 29$^{th}$ (32.00±0.60). This spore trap was within 10 m of cucurbit plantings and CDM was observed in the sentinel plot in this field on 11 September, one day prior to detection from air samples. The samples collected from Field A and Suffolk County did not test positive for *P. cubensis*. The spore traps in Suffolk County were 300 m to two miles away from sentinel plots containing cucurbit crops, where only a small amount of CDM was observed on 2 September.

*Detection of P. humuli*. In 2013, a year when HDM was observed only periodically in the hop yard, *P. humuli* was detected intermittently by the LNA assay on various dates (from 3 May to 30 August)

throughout the hop growing season (Table 3.6). LNA assay detection of *P. humuli* in 2014 was limited to three sampling dates in late summer during the hop harvests (26 August, 5 September and 9 September; Table 3.6). HDM symptoms were not observed in the hop yard in 2014. *Psuedoperonospora humuli* was also not detected from any spore traps located in vegetable fields in 2014. No hop plants or *P. humuli* inoculum was known to be near the vegetable fields where the spore traps were placed.

**Table 3.6** *Pseudoperonospora humuli* detection results from air sampled using a spore trap placed in a commercial hop yard in Ontario County, NY in 2013 and 2014. The threshold cycle (Cq) and standard deviations are shown. There was no amplification from spore rods collected on dates denoted (-). Curves were evaluated for exponential increase to ensure confidence in late-cycle positives.

| 2013 hop yard | | 2014 hop yard | | | |
|---|---|---|---|---|---|
| Date of collection | Cq ±St. Dev. | Date of collection | Cq ±St. Dev. | Date of collection | Cq ±St. Dev. |
| 5/3/2013 | 31.12±0.71 | 3/25/2014 | - | 7/11/2014 | - |
| 5/9/2013 | 29.63±0.32 | 3/28/2014 | - | 7/14/2014 | - |
| 5/13/2013 | 33.18±1.90 | 3/31/2014 | - | 7/18/2014 | - |
| 5/16/2013 | 33.45±0.84 | 4/4/2014 | - | 7/21/2014 | - |
| 5/20/2013 | 31.26±0.61 | 4/7/2014 | - | 7/25/2014 | - |
| 5/28/2013 | - | 4/14/2014 | - | 7/29/2014 | - |
| 5/31/2013 | - | 4/18/2014 | - | 8/1/2014 | - |
| 6/3/2013 | - | 4/21/2014 | - | 8/4/2014 | - |
| 6/6/2013 | - | 4/25/2014 | - | 8/7/2014 | - |
| 6/10/2013 | - | 4/29/2014 | - | 8/11/2014 | - |
| 6/14/2013 | - | 5/1/2014 | - | 8/15/2014 | - |
| 6/17/2013 | - | 5/5/2014 | - | 8/18/2014 | - |
| 6/21/2013 | 32.90±0.01 | 5/9/2014 | - | 8/22/2014 | - |
| 6/24/2013 | - | 5/13/2014 | - | 8/26/2014 | 33.99±0.55 |
| 6/27/2013 | 34.46±0.19 | 5/16/2014 | - | 8/29/2014 | - |
| 7/1/2013 | 20.32±0.25 | 5/20/2014 | - | 9/2/2014 | 34.50±0.02 |
| 7/5/2013 | 24.66±0.61 | 5/23/2014 | - | 9/5/2014 | 32.53±0.64 |
| 7/8/2013 | 31.78±0.06 | 5/27/2014 | - | 9/9/2014 | 32.46±0.12 |
| 7/12/2013 | 29.16±0.89 | 5/29/2014 | - | 9/12/2014 | - |
| 7/15/2013 | - | 6/2/2014 | - | 9/15/2014 | - |
| 7/19/2013 | - | 6/3/2014 | - | 9/18/2014 | - |
| 7/22/2013 | - | 6/6/2014 | - | | |
| 7/26/2013 | - | 6/9/2014 | - | | |
| 7/29/2013 | - | 6/13/2014 | - | | |
| 8/2/2013 | - | 6/16/2014 | - | | |
| 8/6/2013 | 30.03±0.18 | 6/20/2014 | - | | |
| 8/9/2013 | 29.34±0.31 | 6/23/2014 | - | | |
| 8/19/2013 | 28.20±0.61 | 6/27/2014 | - | | |
| 8/22/2013 | - | 6/30/2014 | - | | |
| 8/26/2013 | 27.76±0.61 | 7/3/2014 | - | | |
| 8/30/2013 | 29.67±0.14 | 7/7/2014 | - | | |

**Discussion**

Two assays were designed for the detection and diagnosis of *P. cubensis* and *P. humuli*. Real-time PCR with LNA probes and HRM protocols were based on SNP differences in the mitochondrial *cox2* gene observed in a previous study investigating the relatedness of the two pathogens (Mitchell et al. 2011). In that study, Mitchell, et al. (2011) found that 18 of 21 isolates of *P. cubensis* contained four SNPs differentiating them from 14 isolates of *P. humuli*, while for the three remaining isolates of *P. cubensis*, three of the four SNPs mentioned above matched those of *P. humuli*. One of the four SNPs was conserved among all isolates examined (Mitchell et al. 2011). These results were similar to those found in the present study, where 12 of 15 isolates of *P. cubensis* collected in NY contained the same four SNPs, while three isolates contained the same three of four SNPs mentioned above, which match those of *P. humuli*. Interestingly, for both the current study and the Mitchell et al. (2011) study, each of the *P. cubensis* isolates containing the non-conserved SNPs matching those of *P. humuli* were from squash hosts. These results support the findings of various studies, which have shown that *P. cubensis* isolates exhibit host specificity, as variations in virulence and pathogenicity of isolates have been observed on different cucurbit species (Thomas et al. 1987; Lebeda & Widrlechner 2003; Thomas et al. 2014). More recently, whole-genome sequencing of isolates from different cucurbit hosts identified two distinct lineages among *P. cubensis* isolates, with isolates from cucumber and cantaloupe clustering separately from isolates of squash and watermelon, with the lineages exhibiting low cross-infectivity (Thomas et al. 2014).

The assays developed in this study are based on the conserved SNP at base 105 of the targeted *cox2* region and are the first to allow specific detection of *P. cubensis* and *P. humuli*. A previous study successfully monitored *P. humuli* inoculum in a hop yard using a PCR assay targeting a portion of the ITS region (Gent et al. 2009). However, the primers used in the assay also may

amplify *P. cubensis* DNA, and thus may be employed in areas where only one of the pathogens is found (Gent et al. 2009). In contrast, the LNA probes designed for this study correctly identified 15 isolates of *P. cubensis* and 16 isolates of *P. humuli* (Tables 3.1 & 3.3), as well as infected leaves with or without sporulation of the pathogens (Table 3.3). The Cq values obtained by testing the different isolates of each pathogen varied, which could be due to varying amounts of host plant DNA in the template. This variation could not be avoided due to the obligate nature of the pathogens. The probes were sensitive, detecting as few as 10 sporangia (in ten out of ten biological replications) for both pathogens and as little as 1 fg *P. cubensis* total DNA and 10 fg *P. humuli* total DNA. Probe detection was also sensitive in the presence of non-target DNA, as observed in the mixed-species template tests, although we were unable to detect *P. humuli* when *P. cubensis* was present at 100-1000 fold greater concentrations. These results are similar to those seen by Rogers et al. (2009) in their assay used to detect *Sclerotinia sclerotiorum*.

One unexpected result included the observation of similar Cq values for the detection of 50, 100 and 500 sporangia from both species. This trend is not believed to be due to the probes, but rather to the DNA extraction procedure. For example, from 100 and 500 *P. cubensis* and *P. humuli* sporangia, the DNA yields were similar. The yields were also low, on average 90-111 pg/ μL, with extractions from 50 sporangia yielding <10 pg/ μL, the minimum threshold for detection using the Qubit High Sensitivity DNA assay. The similar yields from the different numbers (100 and 500) of sporangia suggest that there may be some issue with isolating sporangial DNA using this kit. We do not think it is poor performance of the kit in general, since extractions from air sampling rods, which collect a small quantity of material, yielded, on average, 0.5-2 ng/ μL. We used the MoBio kits for the sensitivity test in order to maintain consistency with the DNA extraction from air samples. These kits were chosen because they were previously used to detect *P. humuli* from air samples;

however, the previous assay relied on conventional PCR and therefore, does not provide Cq values or DNA quantities for comparison to our assay (Gent et al. 2009).

HRM analysis was accurate for all tested isolates using the cytosine/thymine SNP at base 105 in the *cox2* gene fragment. This mismatch results in a large curve shift for genotypic differentiation (Venter et al. 2001). Accurate results from the testing of symptomatic leaf tissue indicate this would be a satisfactory diagnostic tool for field samples. HRM has been successfully used to diagnose field samples, including pathovars of the olive bacterial pathogen, *Pseudomonas savastanoi* (Gori et al. 2012), closely-related fungal *Phyllosticta* species on banana (Wong et al. 2013) and grapevine-leafroll virus variant groups (Bester et al. 2012). These three studies were also able to diagnose co-infected plant samples by observing bifurcation of melt curves (Bester et al. 2012; Gori et al. 2012; Wong et al. 2013). However, in the case of air samples, we did not find HRM to reliably detect both pathogens in mixed-DNA samples (data not shown). We found that the HRM assay detected the presence of the most dominant species, which could result in false-negative results depending on what organisms are actually present in the sample.

Because both *P. cubensis* and *P. humuli* are annually present in the sampled areas, LNA probes were chosen to detect *P. cubensis* and *P. humuli* from air samples, as they were highly specific and could accurately identify both species in a mixed-DNA sample (unlike the HRM assay). In Suffolk County, NY, there were no positive detections of *P. cubensis* from the three spore traps placed there in 2014 (46 samples total). This was not surprising, as disease arrived to Suffolk County late in the season (28 August) and disease severity remained low in the sentinel plots, which were at least 300 m distance from where the spore traps were placed. *Psuedoperonospora cubensis* was detected in Ontario County, NY, starting on 12 September, which is roughly the same time the disease was observed in the field (11 September). The pathogen was detected from only one spore trap, which was placed 10

m to the west of a cucurbit sentinel plot. In contrast, using a volumetric spore sampler and observation of sporangia using microscopy, Granke et al. (2014) observed *P. cubensis* sporangia prior to planting crops and throughout the cucurbit growing season in Michigan. However, this method requires significantly more time and expertise to successfully employ (West et al. 2008). In addition, the presence of closely related species, such as *P. humuli,* can make morphological distinction difficult. PCR protocols have successfully detected pathogens prior to disease onset, such as the Falacy et al. (2007) study developed to detect *Erysiphe necator*, causal agent of powdery mildew of grape. However, in other air sampling projects utilizing PCR, including the one previously designed to detect *P. humuli* (Gent et al. 2009), the pathogen also was not detected until after disease symptoms were observed. Still, detecting escalations in inoculum after disease onset contributes valuable information towards disease management programs (West et al. 2008) and various experiments have focused efforts towards this goal (Carisse et al. 2009; Gent et al. 2009; Rogers et al. 2009; Duvivier et al. 2013; Klosterman et al. 2014).

In 2013, *P. humuli* was detected by LNA probes in the hop yard where the spore trap was located starting on May 3, 2013 and sporadically during the growing season. This was expected, since HDM overwinters in the plant and sporulates on young basal shoots as they emerge (Coley-Smith 1962). Symptoms were observed periodically throughout the season on the hop plants in this yard, and the cooperating grower treated the yard with fungicide. In 2014, however, HDM was not observed in the field and the pathogen was not detected from spore traps until 22 August, when hop harvesting had begun and plants were disrupted. Based on the presence of disease symptoms, we assume the inoculum levels in 2013 were greater than in 2014, thereby facilitating detection throughout the season. These results are similar to those found by Gent et al. (2009) in Oregon, where *P. humuli* was consistently detected only when disease was found.

Because previous experiments have observed PCR inhibition due to the grease used on the spore-trapping rods (Falacy et al. 2007; Klosterman et al. 2014), we randomly selected DNA extracted from rods that were negative for detection of our target pathogens and tested them in two ways: spiking samples with target pathogen DNA and dilution of samples (as described above) to ensure we did not report false negatives. No evidence of PCR inhibition was observed in DNA extracted from grease-coated rods.

In conclusion, the two assays described are the first developed which allow for differentiation between the closely related pathogens *P. cubensis* and *P. humuli*. The LNA assays can be used in the detection of both pathogens from samples that could have mixtures of the pathogens, such as air samples, while both the LNA and HRM can be used for diagnosis of CDM and HDM from infected plant samples, including field and non-sporulating isolates.

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CHAPTER 4

# Identification of genetic variation between obligate plant pathogens *Pseudoperonospora cubensis* and *P. humuli* using RNA sequencing (RNA-seq) and genotyping-by-sequencing (GBS)[4]


## Abstract

RNA sequencing (RNA-seq) and genotyping-by-sequencing (GBS) were used for single nucleotide polymorphism (SNP) identification from two economically important obligate plant pathogens, *Pseudoperonospora cubensis* and *P. humuli*. Twenty isolates of *P. cubensis* and 19 isolates of *P. humuli* were genotyped using RNA-seq and GBS. For *P. cubensis*, 15 isolates were sequenced using both techniques, and 5 isolates were sequenced using only GBS. Seventeen isolates of *P. humuli* were sequenced using both techniques; one isolate was sequenced only with RNA-seq, and one isolate was sequenced only with GBS. Principle components analysis (PCA) of each data set showed genetic separation between the two species. Additionally, results supported previous findings that *P. cubensis* isolates from squash are genetically distinct from cucumber and cantaloupe isolates. A PCA-based procedure was used to identify SNPs correlated with the separation of the two species, with 994 and 4,231 PCA-correlated SNPs found within the RNA-seq and GBS data, respectively. The corresponding unigenes (n=800) containing these potential species-specific SNPs were then annotated and 135 putative pathogenicity genes, including 3 effectors, were identified. The characterization of genes containing SNPs differentiating these two closely related downy mildew species will contribute to the development of improved detection and diagnosis strategies and may improve our understanding of host specificity pathways.


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## Introduction

The downy mildews (kingdom Chromalveolata, phylum Heterokontophyta, class Oomycota, order Peronosporales, family Peronosporaceae), are obligate biotrophic pathogens of flowering plants (Palti & Kenneth 1981). Elucidating the taxonomy among downy mildew species is especially challenging due to the obligate nature of these pathogens. Where biological separation of species has traditionally relied upon observations of morphology, downy mildew pathogens grow within host tissue, leaving only reproductive structures for observation (Runge & Thines 2010). Because the appearance of these structures, as well as host symptoms, may vary widely depending on host substrate and environment, morphological characters are not always useful for differentiating species of downy mildews (Runge & Thines 2010). Host specificity studies have also been used in the past to differentiate species of downy mildews (Hall 1996; Mitchell et al. 2011) but suffer from limitations because of the overlapping host range of certain organisms. Today, both morphological and host range studies have been replaced by phylogenetic analyses for species designation (Riethmüller et al. 2002). However, the downy mildews, and oomycetes in general, are often not easily differentiated with highly conserved DNA sequences such as the ribosomal genes (Riethmüller et al. 2002) or the internal transcribed spacer (ITS) region (Goodwin et al. 1999; Flier et al. 2002).

This study focused on two economically important and closely related downy mildew species, *Pseudoperonospora cubensis* and *P. humuli* (Mitchell et al. 2011). *Pseudoperonospora cubensis* has a relatively wide host range for a downy mildew pathogen (Thines 2014), afflicting members of the family Cucurbitaceae worldwide, with the most economically important hosts being cucumber (*Cucumis sativus)*, cantaloupe and muskmelon (*Cucumis melo)*, squash and pumpkin (*Cucurbita pepo, C. maxima and C. moschata*) and watermelon (*Citrulus lanatus*) (Lebeda & Cohen 2011). *Pseudoperonospora humuli* infects hop (*Humulus lupulus*), causing a reduction in hop yield and quality, as well as potential

death of the perennial root system (Skotland & Johnson 1983). Choi et al. (2005) questioned the distinction between these two species, as they did not differ consistently in morphology or ITS region sequence (Choi et al. 2005). However, further genetic analyses support that the two species are distinct (Runge & Thines 2012; Mitchell et al. 2011). Despite this, only one single nucleotide polymorphism (SNP) has been previously identified that consistently differentiates the two species (Mitchell et al. 2011; Summers et al. 2015) and host range studies have been variable (Mitchell et al. 2011; Runge et al. 2011).

High-throughput sequencing technologies have greatly improved the ability to resolve population genetic structure, develop diagnostic tools and better understand pathogen epidemiology (Studholme et al. 2011). RNA sequencing (RNA-seq) and genotyping-by-sequencing (GBS) are two such techniques, which can be applied to identify SNPs in transcriptomes as well as genomes. RNA-seq represents expressed genes (Van Verk et al. 2013), while GBS samples genomic regions targeted by methylation-sensitive restriction enzymes (Elshire et al. 2011). RNA-seq has been found to be a very effective technique for SNP discovery (Li et al. 2012; Quinn et al. 2013) and can allow for a more accurate functional annotation due to enrichment for expressed genes (De Wit et al. 2015). However, GBS accesses non-coding DNA, which can contain important regulatory regions controlling phenotypes (Elshire et al. 2011).

The overall purpose of this study was to collect and utilize genomic data to further investigate the genetic differentiation of these two closely related species. In order to accomplish this goal, our first objective was to observe variation between and among isolates of *P. cubensis* and *P. humuli* using principal components analysis (PCA) (Paschou et al. 2007; Laloë & Gautier 2012). Our second objective was to identify SNPs between the species. Our final objective was to annotate the genes containing these SNPs and identify putative pathogenicity genes. These genes may be

important in host-specificity pathways and could be useful targets for pathogen detection and identification (Ellis 2006; Torto-Alalibo et al. 2007).

## Materials and Methods

*Pathogen isolation, maintenance and inoculation.*

*Pseudoperonospora cubensis*. Fifteen *Pseudoperonospora cubensis* isolates were collected from 15 different fields with symptomatic cucurbit hosts in New York (NY) (8 counties) during the summers of 2012 and 2013 (Table 4.1). Ten samples were from cucumber, two from cantaloupe, two from squash and one from pumpkin. Lina Quesada-Ocampo at North Carolina State University contributed 5 DNA samples for GBS from *P. cubensis* isolates collected in North Carolina (3 samples: 2 from cucumber and 1 from pumpkin), South Carolina ( 1 sample from squash) and California (1 sample from cucumber; Table 4.1).

To obtain single-sporangium *P. cubensis* isolates, diseased leaves were placed in a moist chamber overnight to induce sporulation. The resulting sporangia were then used to inoculate seven-day old seedlings of the susceptible cucumber cultivar Straight Eight, which were grown in Cornell potting mix (composed of peat, perlite and vermiculite in a 4:1:1 ratio). Sporangia from a single lesion of each isolate were washed from the leaf and sprayed onto seedlings using an air pressurized sprayer (Nalgene, Rochester, NY). This single-lesion process was repeated three times per isolate to reduce the possibility of a mixed genotype. Inoculated plants were placed in dark moist chambers at 16°C overnight then moved to a greenhouse (23.9°C day, 18.3°C night, and 14 hr light). Once lesions appeared on the cucumber seedlings, the plants were placed in a moist chamber (>90% relative humidity) in the dark at 16°C for 24 to 48 hr until prolific sporulation was observed. Live isolates were stored on cucumber leaves at -80°C.

*Pseudoperonospora humuli*. Nineteen *P. humuli* isolates were collected from five states as well as Japan during 2011 to 2013 (Table 4.1). Hop shoots with signs of systemic infection were collected from hop yards and monosporangial isolates of *P. humuli* were derived from infected hop shoots as described previously (Gent et al. 2008) or by depositing a single sporangium onto a leaf disk using a flow cytometer. For the latter method, leaf disks were cut from greenhouse produced hop plants (cv. Pacific Gem) with a #10 cork borer and the adaxial surface placed onto 1% water agar in a 24-well serological plate. A suspension of sporangia from a given sample of *P. humuli*, cultured as described by Mitchell et al. (2011), was loaded into a MoFlo flow cytometer **(**Beckman Coulter, Inc., Brea, CA) calibrated to deliver a single sporangium onto each leaf disk. After deposition of sporangia, the leaf disks were misted with sterile deionized water using an airbrush sprayer and incubated in a growth chamber set to 13°C for 7 to 14 days. Leaf disks bearing sporulating lesions were removed and increased on plants of cultivar Pacific Gem and maintained using the methods of Mitchell et al. (2011).

**Table 4.1** Isolates sequenced using RNA-seq and GBS.

| Organism | Strain | Host | Year | Location | RNA-seq[a] | GBS[a] |
|---|---|---|---|---|---|---|
| *Pseudoperonospora cubensis* | CDM12- 45 | *Cucumis sativus* | 2012 | Erie, NY | I | S |
| *Pseudoperonospora cubensis* | CDM12- 58 | *Cucumis sativus* | 2012 | Seneca, NY | I | I |
| *Pseudoperonospora cubensis* | CDM12- 60 | *Cucumis sativus* | 2012 | Seneca, NY | I | I |
| *Pseudoperonospora cubensis* | CDM12- 95 | *Cucumis sativus* | 2012 | Cayuga, NY | S | I |
| *Pseudoperonospora cubensis* | CDM13- 1 | *Cucumis sativus* | 2013 | Erie, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 2 | *Cucumis sativus* | 2013 | Suffolk, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 3 | *Cucumis sativus* | 2013 | Orleans, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 4 | *Cucumis sativus* | 2013 | Ontario, NY | S | I |
| *Pseudoperonospora cubensis* | CDM13- 6 | *Cucumis melo* | 2013 | Suffolk, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 8 | *Cucumis melo* | 2013 | Ontario, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 9 | *Cucumis sativus* | 2013 | Cayuga, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 10 | *Cucurbita pepo* | 2013 | Suffolk, NY | I | S |
| *Pseudoperonospora cubensis* | CDM13- 12 | *Cucurbita pepo* | 2013 | Suffolk, NY | S | I |
| *Pseudoperonospora cubensis* | CDM13- 13 | *Cucurbita pepo* | 2013 | Albany, NY | I | I |
| *Pseudoperonospora cubensis* | CDM13- 14 | *Cucumis sativus* | 2013 | Erie, NY | E | I |
| *Pseudoperonospora cubensis* | CDM12-NC | *Cucumis sativus* | 2012 | NC | NS | I |
| *Pseudoperonospora cubensis* | CDM-CA | *Cucumis sativus* | 2008 | CA | NS | I |
| *Pseudoperonospora cubensis* | CDM13-NC | *Cucumis sativus* | 2013 | NC | NS | I |
| *Pseudoperonospora cubensis* | CDM-PM | *Cucurbita maxima* | 2013 | NC | NS | S |
| *Pseudoperonospora cubensis* | CDM-SQ | *Cucurbita pepo* | 2013 | SC | NS | I |
| *Pseudoperonospora humuli* | HDM 457E | *Humulus lupulus* | 2011 | Marion, OR | S | S |
| *Pseudoperonospora humuli* | HDM 481J | *Humulus lupulus* | 2011 | NY | I | I |
| *Pseudoperonospora humuli* | HDM 482CA | *Humulus lupulus* | 2011 | NY | S | I |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Pseudoperonospora humuli* | HDM490-5 | *Humulus lupulus* | 2012 | Aomori, Japan | I | I |
| *Pseudoperonospora humuli* | HDM 496SA | *Humulus lupulus* | 2012 | Hokkaido, Japan | S | S |
| *Pseudoperonospora humuli* | HDM 498 | *Humulus lupulus* | 2012 | Ehoro, Japan | NS | I |
| *Pseudoperonospora humuli* | HDM 499AA | *Humulus lupulus* | 2013 | Marion, OR | I | I |
| *Pseudoperonospora humuli* | HDM 500BA | *Humulus lupulus* | 2013 | Marion, OR | I | S |
| *Pseudoperonospora humuli* | HDM 501AB1 | *Humulus lupulus* | 2013 | Marion, OR | I | S |
| *Pseudoperonospora humuli* | HDM 502AA | *Humulus lupulus* | 2013 | Marion, OR | I | I |
| *Pseudoperonospora humuli* | HDM 503A3 | *Humulus lupulus* | 2013 | Grand Isle, VT | S | I |
| *Pseudoperonospora humuli* | HDM 503AA | *Humulus lupulus* | 2013 | Grand Isle, VT | S | NS |
| *Pseudoperonospora humuli* | HDM 504AB2 | *Humulus lupulus* | 2013 | Grand Isle, VT | S | I |
| *Pseudoperonospora humuli* | HDM 505-1 | *Humulus lupulus* | 2013 | NY | I | I |
| *Pseudoperonospora humuli* | HDM 506CB | *Humulus lupulus* | 2013 | NY | E | I |
| *Pseudoperonospora humuli* | HDM 507AA | *Humulus lupulus* | 2013 | NY | I | I |
| *Pseudoperonospora humuli* | HDM 508AC | *Humulus lupulus* | 2013 | NY | E | I |
| *Pseudoperonospora humuli* | HDM 509-2 | *Humulus lupulus* | 2013 | Yakima, WA | S | I |
| *Pseudoperonospora humuli* | HDM 510-1 | *Humulus lupulus* | 2013 | Mazomanie, WI | S | I |

[a] Two sets of analyses were performed, one maximizing the number of SNPs, resulting in the exclusion of isolates with low sequencing depth (filtering priority= max SNPs) and the other retaining isolates at the sacrifice of SNPs (filtering priority= max isolates). I=included in both analyses; S=excluded from the 'max SNPs' run but included in the 'max isolates' run); E= excluded from both sets of analyses due to missing data; NS=not sequenced.

*Collection of sporangia and extraction of total RNA and genomic DNA.*

Infected leaves of each single sporangium isolate of each pathogen were washed in 40 mL sterile deionized water to remove sporangia. The sporangial suspension was filtered through eight layers of cheesecloth then concentrated by centrifugation at 1,000 x g for 5 min. RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) and the DNeasy plant mini kit (Qiagen) for DNA extraction. One 5 mm stainless steel bead and 14 silica beads (400 micron) were added to sporangia. For RNA extraction, 450 µl RLT buffer with 2% polyvinylpyrrolidone and 1% beta-mercaptoethanol was added. Buffer AP1 was added for DNA extraction. Samples were then ground at 30 Hz for 2 min in a tissue lyser (Retsch MM400, Cole-Parmer, Vernon Hills, IL). The remaining extraction process followed manufacturer's protocols.

*RNA-seq library construction and analysis.*



For RNA-seq library construction, 5 µg of RNA were treated with RQ1 RNase-free DNase (Promega, Madison, WI) and then purified using Dynabeads® Oligo $(dT)_{25}$ (Life Technologies) following the manufacturer's protocol. Whole transcriptome amplification was performed using the Quantitect Whole Transcriptome Kit (Qiagen, high-yield reaction for 8 hours). The amplified transcriptome was purified with Ampure beads (Beckman-Coulter, Pasadena, CA) then eluted in 100 µl TE buffer. The cDNA was then fragmented using a Covaris machine for fragmentation to 200-250 bp (Duty Cycle: 10%, Intensity: 5, Cycles per burst: 100 – 180 second) then cleaned with Ampure beads and eluted with water. Frayed DNA ends were treated with End-Repair Master Mix (Epicentre, Charlotte, NC) then purified with Ampure beads. The dA-Tailing Master Mix (New England Biolabs, Ipswitch, MA) was used to add adenine bases to the 3' ends of fragments, followed by another Ampure purification. Adapters (where each barcoded oligo was individually adjusted to 200 µM, then combined with the complimentary barcoded oligo) were then ligated to the fragments

using T4 DNA ligase (New England Biolabs), followed by Ampure purification. The fragments linked to adapters were subjected to PCR enrichment using Phusion DNA polymerase (New England Biolabs), primers (prAC: 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3', prBC: 5'-CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T-3'), and 5 µl template in a 50 µl reaction. The PCR program consisted of 30 sec at 98°C, [10 sec at 98°C, 30 sec at 65°C, 30 sec at 72°C] 14 cycles total and 5 min 72°C.

The product of PCR enrichment was checked on a 2% agarose gel. Products were purified with Ampure beads and eluted in water. The DNA concentration was measured using Qubit (Life Technologies, Carlsbad, CA) and library concentrations were normalized to 15 ng and multiplexed. Combined libraries were sent to the Genomics Core Facility, Cornell University, for RNA-seq analysis. Samples were sequenced using Illumina Hi-Seq 100 bp single-end sequencing in a single lane.



RNA-seq raw reads were de-multiplexed, trimmed, and quality filtered using the Fastx-toolkit (Pearson et al. 1997). The publicly available *P. cubensis* genome sequence (64.4 Mb) (Savory et al. 2012), estimated to represent 73% of the total genome using the predicted size of 88.22 MB determined by Feulgen analysis (Voglmayr & Greilhuber 1998), was used as a reference for alignment of both organisms. Barcoded reads were mapped to the reference genome using Bowtie version 1.0.0 (Langmead et al. 2009) and Tophat version 2.0.13 (Trapnell et al. 2009). Picard Tools version 1.109 was used to add read groups, mark duplicates and reorder alignment files to match the reference genome (http://broadinstitute.github.io/picard). GATK version 3.2-2 was used to prepare splice junctions within the alignment files using the 'splitNCigarReads' tool. The 'HaplotypeCaller' with default settings was used for calling SNPs to produce the variant call format (VCF) file (McKenna et al. 2010).

*GBS library construction and analysis.*

DNA samples were submitted to the Cornell University Institute for Genomic Diversity (IGD) for library preparation and sequencing, following a protocol described previously (Elshire et al. 2011). In brief, adapters were added to the DNA samples, samples were digested with *Ape*K1 restriction enzyme, followed by adapter ligation. The samples were pooled, PCR-enriched, and purified prior to sequencing by Illumina Hi-Seq 100 bp single-end sequencing in a single lane (Elshire et al. 2011).

Data were filtered by IGD by first aligning to the *P. cubensis* reference genome (Savory et al. 2012). Raw sequencing reads were processed with the TASSEL-GBS analysis pipeline by IGD using Tassel version 3.0.166 using default parameters, with the exception of two settings. First, a tag was required to be present 3 times in order to be retained in the pipeline. Second, the read depth of each isolate for each tag was recorded in the "Tags-by-taxa" (TBT) file in order to quantitatively call heterozygotes (Elshire et al. 2011; Glaubitz et al. 2014). Likelihood scores for each genotype were calculated using formula 3.8 in Etter et al. 2011 and the most likely genotype was assigned (Johnson et al. 2015). The GBS VCF and "Tags-On-Physical-Map" TOPM files were merged with the *P. cubensis* genome annotation files (provided by Brad Day at Michigan State University).

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*Measuring host sequence contamination from total quality reads.*

To measure the amount of host sequence contamination in the RNA-seq and GBS data, the filtered and barcoded reads were separated by species and reads from each species were concatenated. The resulting fastq files were converted to FASTA format using seqtk (https://github.com/lh3/seqtk.git). The sequencing data of each organism were scanned for the number of read hits to host reference genomes using BLAT (Kent 2002). For the *P. cubensis* data, a

cucumber reference genome was used (Ren et al. 2009). For the *P. humuli* data, a draft hop genome was provided by John Henning, US Department of Agriculture, Agricultural Research Service. The BLAT output was filtered for unique hits to the host genomes and the percent of unique hits from total quality reads was calculated.

*Filtering Variant Call Format Files from GBS and RNA-seq.*

RNA-seq and GBS data were filtered in two ways: maximizing SNPs retained and maximizing isolates retained. The former was important in order to capture the most variation within and between isolates. Data were independently filtered to maximize the number of isolates, in order to evaluate as many sequenced isolates as possible. Results from both filtering strategies are presented.

*Maximizing SNPs retained in downstream analysis.* Biallelic SNPs from RNA-seq and GBS data were filtered separately to a minimum genotype quality (minGQ) of 98% using VCFtools version 0.1.12a (Danecek et al. 2011) then converted to PLINK format using PLINK version 1.07 (Purcell et al. 2007; http://pngu.mgh.harvard.edu/purcell/plink/) in order to determine isolates for exclusion from analysis if they had 90% missing data per individual (0.9=mind), 20% missing data per marker (0.2=geno) or less than a minimum minor allele frequency of 1% (maf=0.01). Using VCFtools, the data were then filtered using the following parameters (after excluding the recommended isolates): 2 maximum and minimum alleles, minGQ of 98%, minimum minor allele frequency of 1% (maf=0.01) and 10% missing data per marker (max-missing=0.9). Depth and missing data were calculated for each site and individual using VCFtools. Data filtered using this strategy are henceforth referred to as "max SNPs".

*Maximizing isolates retained in downstream analysis*. Biallelic SNPs from RNA-seq and GBS data were filtered separately using VCFtools with the same parameters as above. However, isolates recommended for exclusion by PLINK (in the filtering strategy, max SNPs, described above) were retained. For the RNA-seq data, 3 isolates were excluded, as the inclusion of these isolates greatly reduced the number of quality SNPs. For GBS, all sequenced isolates were retained in the downstream analysis. The quality of these files was assessed as described above. Data filtered using this strategy are henceforth referred to as "max isolates".

If PCA results were conflicting for specific isolates when the RNA-seq and GBS data when the two filtering strategies were compared, the missing data statistics were examined for those isolates specifically and noted.

*Observing population variation.*



*Principle components analysis*. The VCF files were converted to genotype files then numeric matrices as described in (Paschou et al. 2007) using custom scripts. Missing genotype data were filled randomly with numeric genotype calls, which is the most conservative approach (Paschou et al. 2007). In order to ensure that the accuracy of PCA clustering was not affected by the randomly-filled missing data, the missing data were filled randomly 10 times and the analyses were compared to confirm equivalence prior to proceeding with further testing.

The resulting matrices were passed into a MATLAB PCA routine. Twelve variations of the PCA were completed, with the RNA-seq and GBS data from each of the two filtering strategies (max SNPs and max isolates) repeated for the following: *P. cubensis* and *P. humuli* isolates together, *P. cubensis* isolates separately and *P. humuli* isolates separately.

*Neighbor-joining trees.* Single nucleotide polymorphisms from RNA-seq and GBS data for each filtering strategy (max SNPs and max isolates) were converted to a tab delimited text file using VCFtools and concatenated into a FASTA alignment file with a Perl script (Bergey 2012). Neighbor-joining trees (Saitou & Nei 1987) were constructed in Mega6 (Tamura et al. 2013) using the concatenated SNP sequences.

*Principle Components Analysis: Selecting SNPs correlated to separating species.*

For the following tests, we used the filtered VCF files generated when maximizing the SNPs retained (max SNPs).

*Selecting SNPs correlated to separating species.* Each SNP was scored in a process similar to that previously described (Paschou et al. 2007). The primary difference between the two processes was that the Paschou et al. (2007) process identified SNPs correlated to an estimated number of principal components accounting for a desired proportion of variance in the data, whereas the current study tested for correlation to the first principal component in the data, which was largely responsible for separating the two species. These SNPs are heretofore referred to as "PCA-correlated SNPs."

In order to check whether the PCA-correlated SNPs contained SNPs we would expect to be present, a list of SNPs that differentiated the two species for all sequenced isolates in our dataset was generated using only SNPs for which there were no missing data. For this, a separate script selecting SNPs conserved among all isolates of the same species and differentiating between species was written. These SNPs are heretofore referred to as "confirmed" SNPs.

*Annotation of unigenes containing PCA-correlated SNPs between* Pseudoperonospora cubensis *and* P. humuli *isolates.*



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PCA-correlated SNPs were linked to corresponding unigenes by overlapping the GFF (general feature format) annotation file (specifically, coding regions and mRNA) from the *P. cubensis* reference (Savory et al. 2012) to the GBS and RNA-seq VCF files using the *intersect* function in BEDTools (Quinlan & Hall 2010) and R (R Development Core Team 2011). The term unigene is used to describe a sequence predicted to represent a single gene. Gene ontology (GO) annotations were assigned to unigenes in terms of associated biological processes, cellular components and molecular functions using InterProScan (Jones et al. 2014). Unigenes lacking sufficient annotation for GO assignments, such as unintegrated single exon genes (SEG) or hypothetical proteins, were excluded from GO analyses. GO classes were grouped into GO-Slim terms using the web tool CateGOrizer v3.218 (Hu et al. 2008). Putative pathogenicity genes (Torto-Alalibo et al. 2007) containing PCA-correlated SNPs were identified using GO assignments.

## Results



*Sequencing and alignment.*

Reduced-representation libraries of *P. cubensis* and *P. humuli* isolates were sequenced using RNA-seq and GBS (Table 4.1). For the RNA-seq analyses, 15 isolates of *P. cubensis* and 18 isolates of *P. humuli* were sequenced, while 20 isolates of *P. cubensis* and 18 isolates of *P. humuli* were sequenced using GBS (Table 4.1). The sequencing and alignment results are shown in Table 4.2.

**Table 4.2** Sequencing and alignment results from RNA-seq (n= 33) or GBS (n=38) analysis.

| | RNA-seq | GBS |
|---|---|---|
| Total reads | 138 million | 238 million |
| Total barcoded reads | 100 million | 81 million |
| Barcoded reads aligned to reference[a] | 75 million | 17 million |
| Average barcoded aligned reads per isolate | 2,272,727 | 447,368 |
| Total SNPs[b] | 140,828 | 240,841 |

[a] The *Pseudoperonospora cubensis* reference genome from Savory et al. (2012).

[b] Total SNPs called using the GATK pipeline (RNA-seq) or Tassel (GBS), prior to filtering using VCFtools.

In order to ensure that the *P. cubensis* reference genome (Savory et al. 2012) would be appropriate for alignment of sequences from both species, the percentage of reads aligned to the reference genome for each isolate was calculated and the values were averaged separately for *P. cubensis* isolates and *P. humuli* isolates. For RNA-seq, 75% and 70% of reads from *P. cubensis* and *P. humuli* isolates, respectively, aligned. For GBS, 13% and 15% of reads from *P. cubensis* and *P. humuli* isolates, respectively, aligned to the *P. cubensis* reference genome. Thus, reads from *P. cubensis* isolates did not align better overall than *P. humuli* isolates (Figure 4.1).

**Figure 4.1** Total barcoded reads from each sequenced isolate aligned to the *Pseudoperonospora cubensis* reference genome from RNA-seq analysis (a) and GBS analysis (b). Isolates in the blue and green boxes, as well as isolates indicated by green arrows were filtered from the principal components analysis (PCA) maximizing SNP output (max SNPs). Isolates in the blue box (only for RNA-seq data) were excluded from the PCA maximizing isolates retained (max isolates).

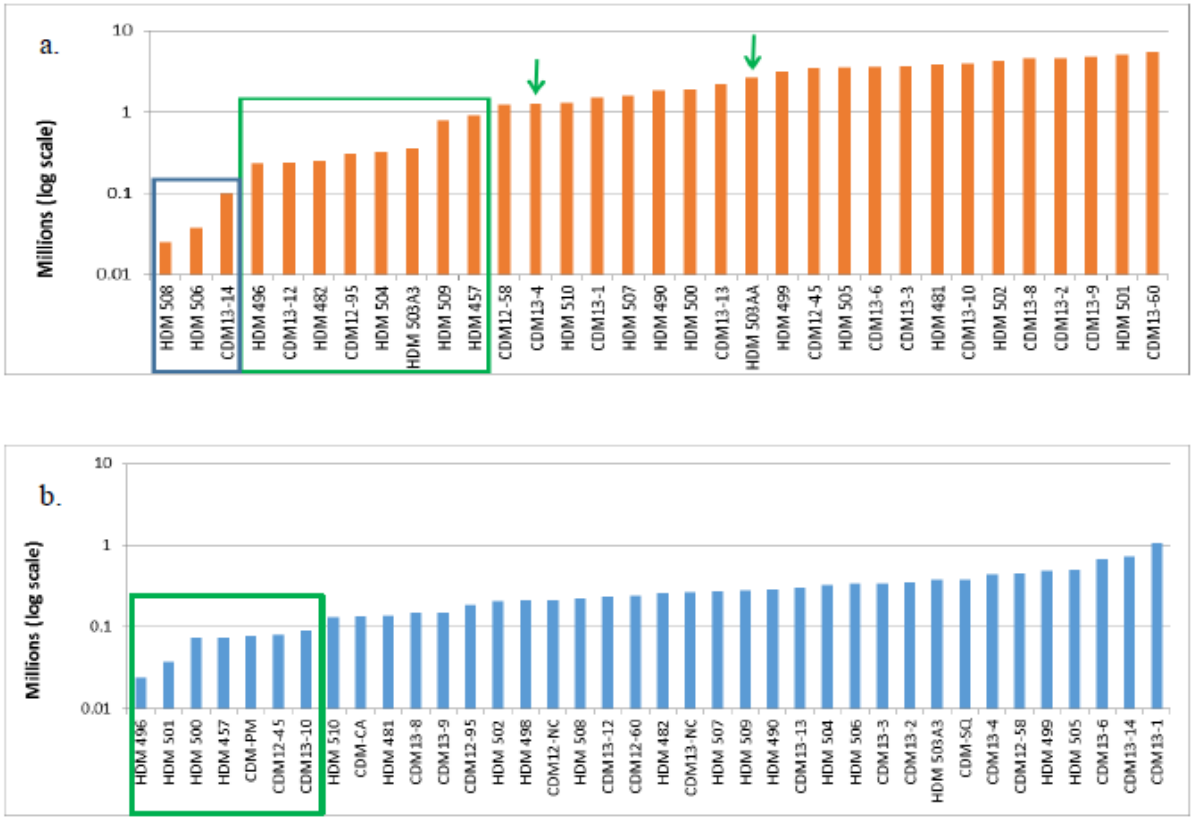


Table 4.2 shows that prior to filtering, the average number of barcoded, aligned reads per isolate was on average, 7.6 times higher for the RNA-seq data than for GBS. Overall alignment was 4.2-fold higher for RNA-seq (75 million reads) than for GBS (17 million reads; Table 4.2). However, GBS had a more even and consistent read depth among isolates, with a standard deviation of aligned



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reads of 206,898 versus 1.8 million for RNA-seq. Finally, GBS produced 71% more total SNPs (240,841) than RNA-seq (140,828; Table 4.2).

The possibility of host contamination in the sequencing data for these obligate pathogens was a concern (Cadle-Davidson et al. 2010), as the obligate pathogen genome is often much smaller than the host genome (Wernegreen 2005). For instance, the *P. cubensis* genome, with an estimated size of 88.22 MB (Voglmayr & Greilhuber 1998) is less than a fourth of the size of the host genome of cucumber (367 MB) (Ren et al. 2009). Both RNA-seq and GBS contained little host contamination, with the greatest quantity being 1.89% contamination of quality-filtered reads from cucumber for the *P. cubensis* RNA-seq data (Table 4.3).

**Table 4.3** Percent host contamination from total barcoded, unaligned reads for RNA-seq and GBS.

| | Species/Host | unique hits | total quality reads | % reads from host |
|---|---|---|---|---|
| RNA-seq | *P. cubensis*/cucumber | 1,022,329 | 54,048,200 | 1.89% |
| RNA-seq | *P. humuli*/hop | 211,296 | 45,951,800 | 0.46% |
| GBS | *P. cubensis*/cucumber | 41,619 | 54,426,051 | 0.08% |
| GBS | *P. humuli*/hop | 5,441 | 26,275,783 | 0.02% |



*Impacts of Filtering Strategy: Maximizing SNPs retained versus isolates retained.*

Both RNA-seq and GBS data were filtered in two ways. First, individuals were removed if they contained more than 90% missing data. This resulted in a maximum number of SNPs for downstream analysis (max SNPs). In order to retain more isolates, at the sacrifice of SNPs, the data were also filtered by retaining all isolates with the exception of 3 isolates from the RNA-seq analysis that yielded less than 100,000 total reads (max isolates, Figure 4.1, Table 4.1).

For the max SNPs filtering strategy, 1,290 bi-allelic filtered SNPs (0.9% of total SNPs) were retained from the RNA-seq data, while 11,922 (5% of total SNPs) were retained from the GBS for downstream analysis (Table 4.4). Because of the relatively low sequencing read depth of some individuals in the RNA-seq data set, only 19 of 34 isolates (56%) were used for principle components analysis (PCA), while 31 of 38 isolates were retained in GBS analysis (82%; Table 4.4). However, for the isolates retained for PCA, the RNA-seq data had a 63% higher mean read depth per individual, 2.9-fold less missing data per individual and 6.9-fold less missing data per site than the GBS data (Table 4.4).

For the max isolates filtering strategy, 30 of 33 sequenced individuals were included in the RNA-seq analyses, which resulted in 135 SNPs (90% reduction). For GBS, all 38 sequenced isolates were maintained in analyses, resulting in 5,044 SNPs (58% reduction; Table 4.4). For the isolates retained for PCA, the RNA-seq data had a 77% higher mean read depth per individual, 2.1-fold more missing data per individual and 2.1-fold more missing data per site than the GBS data (Table 4.4).



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**Table 4.4** Depth and missing data statistics from the filtered VCF files from RNA-seq and GBS data and two filtering strategies, where either the maximum number of SNPs or the maximum number of isolates were retained.

| | Maximizing SNPs Retained (max SNPs) | | | Maximizing Isolates Retained (max isolates) | | | |
|---|---|---|---|---|---|---|---|
| | mean | median | standard deviation | | mean | median | standard deviation |
| **RNA-seq** | | | | **RNA-seq** | | | |
| **(individuals=19, sites= 1,290)** | | | | **(individuals=30, sites= 135)** | | | |
| individual depth[a] | 98 | 103 | 17 | individual depth | 120 | 134 | 38 |
| site depth[b] | 1865 | 1798 | 534 | site depth | 3543 | 3514 | 640 |
| site mean depth[c] | 98 | 95 | 28 | site mean depth | 121 | 120 | 22 |
| missing data (individuals) (%)[d] | 1.4 | 0.6 | 1.9 | missing data (individuals) (%) | 7.0 | 0 | 15 |
| missing data (sites) (%)[e] | 1.4 | 0 | 2.0 | missing data (sites) (%) | 7.0 | 6.6 | 3.0 |
| **GBS** | | | | **GBS** | | | |
| **(individuals=31, sites= 11,922)** | | | | **(individuals=38, sites= 5,044)** | | | |
| individual depth | 62 | 59 | 18 | individual depth | 92 | 98 | 31 |
| site depth | 1919 | 1349 | 1460 | site depth | 3510 | 3523 | 1502 |
| site mean depth | 62 | 44 | 47 | site mean depth | 92 | 93 | 40 |
| missing data (individuals) (%) | 4.0 | 2.1 | 5.0 | missing data (individuals) (%) | 3.3 | 1.1 | 1.2 |
| missing data (sites) (%) | 9.7 | 3.2 | 3.6 | missing data (sites) (%) | 3.3 | 2.6 | 3.0 |

[a] Mean depth, or number of reads, per individual (VCFtools output .idepth).

[b] Depth per site summed across all individuals (VCFtools output .ldepth).

[c] Mean depth per site averaged across individuals (VCFtools output .ldepth.mean).

[d] The percentage of missing data for individuals (VCFtools output .imiss).

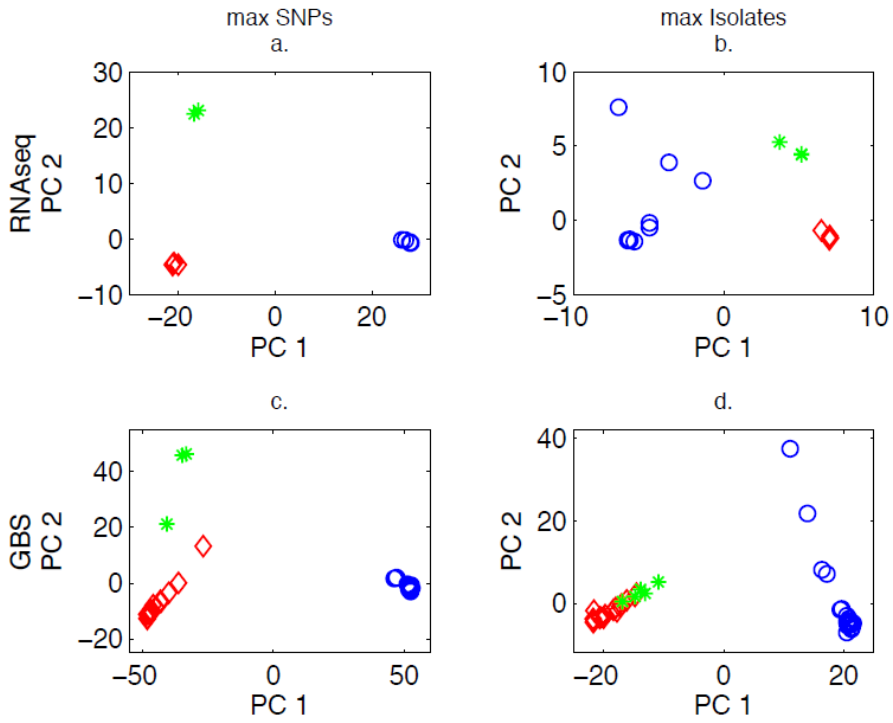
[e] The percentage of missing data for sites (VCFtools output .lmiss).

*Population variation.*

*P. cubensis and P. humuli isolates.* For both filtering strategies and sequencing techniques, PCA analysis separated isolates of *P. cubensis* and *P. humuli* (Figure 4.2). In comparison to the GBS data, the RNA-seq data showed greater separation of isolates of cucumber and cantaloupe hosts from squash hosts among *P. cubensis* isolates for both filtering strategies (Figure 4.2). Increasing the number of isolates included in the analysis, which reduced the number of SNPs in the dataset but increased the number of isolates for RNA-seq by 11 and GBS by 7, did not substantively change the PCA results. PCA plots for GBS contained more isolates than the RNA-seq plots both when the retention of SNPs was prioritized (31 versus 19 individuals) and when retention of isolates was prioritized (38 versus 31; Table 4.4). This difference is partially due to the fact that 5 additional *P. cubensis* isolates were sequenced by GBS (Table 4.1) but also due to the comparatively lower read depth of some isolates using RNA-seq versus GBS (Figure 4.1).

Neighbor-joining trees (appendix) for RNA-seq and GBS data from the max SNPs and max isolates filtering strategies corroborated findings from PCA, as the two species grouped separately with 99% or 100% bootstrap support based on max SNP filtered data and 66% to 99% support of max isolate filtered data. Additionally, isolates of *P. cubensis* from squash separated from other *P. cubensis* isolates with at least 96% support.

**Figure 4.2**. Principal components analysis of RNA-seq and GBS SNP data for *P. cubensis* and *P. humuli* isolates, maximizing for SNPs or isolates retained.




a. Includes 19 isolates and 1,290 biallelic SNPs. Blue circles (8) represent *P. humuli* isolates. *P. cubensis* isolates are represented by red diamonds (9; cucumber and cantaloupe hosts) and green asterisks (2; squash host).

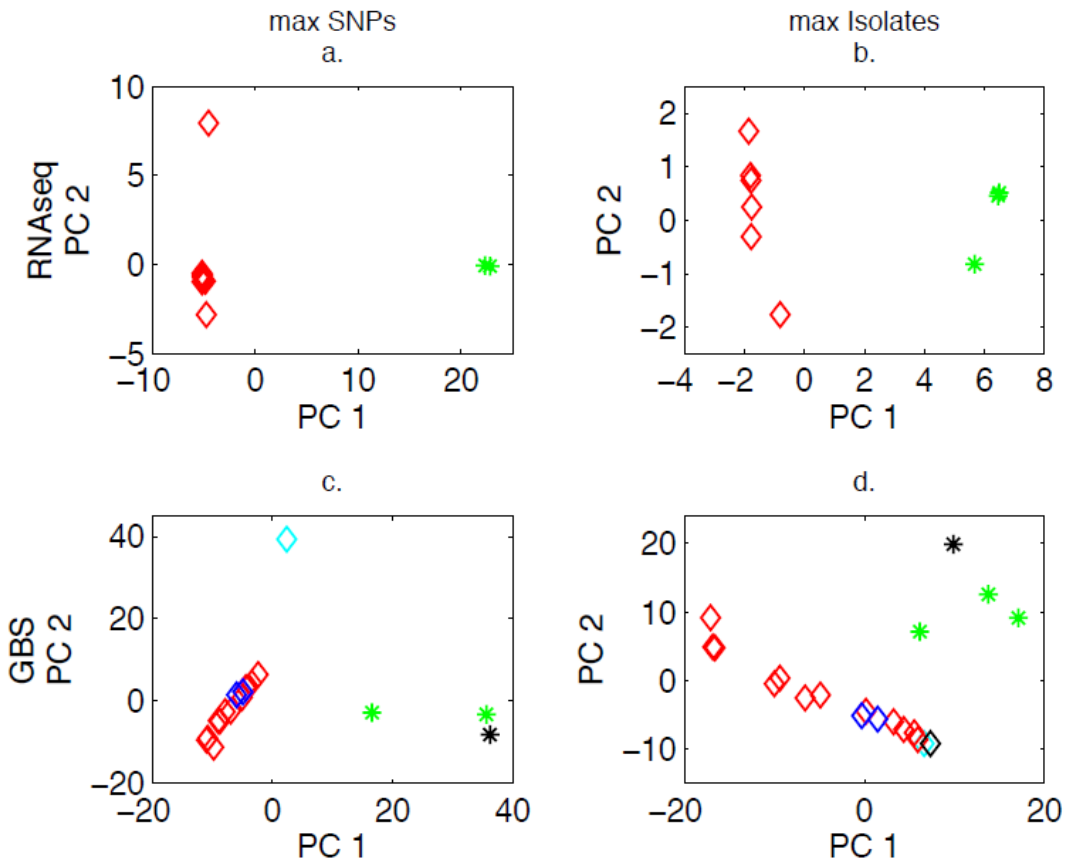
b. Includes 30 isolates and 135 biallelic SNPs. Blue circles (16) represent *P. humuli* isolates. *P. cubensis* isolates are represented by red diamonds (11; cucumber and cantaloupe hosts) and green asterisks (3; squash host).

c. Includes 31 isolates and 11,922 biallelic SNPs. Blue circles (14) represent *P. humuli* isolates. *P. cubensis* isolates are represented by red diamonds (14; cucumber and cantaloupe hosts) and green asterisks (3; squash host).

d. Includes 38 isolates and 5,044 biallelic SNPs. Blue circles (18) represent *P. humuli* isolates. *P. cubensis* isolates are represented by red diamonds (15; cucumber and cantaloupe hosts) and green asterisks (5; squash and pumpkin hosts).

*PCA of P. cubensis isolates.* PCA of *P. cubensis* isolates showed separation of isolates collected on cucumber and cantaloupe hosts from squash hosts for both RNA-seq and GBS (Figure 4.3). The GBS data contained 5 isolates not included in the RNA-seq data, which were collected from California, North Carolina and South Carolina. When the data were filtered to maximize SNPs, but not when filtered to maximize the number of isolates retained, a separation of the cucumber isolate from California, CDM-CA, from the rest of the cucumber isolates was observed (Figure 4.3c). For the max SNPs filtering strategy, CDM-CA had 26% missing data versus 5% when the number of isolates was maximized.

**Figure 4.3** Principal components analysis of RNA-seq and GBS SNP data for *P. cubensis* isolates, maximizing for SNPs or isolates retained.




a. PCA using 1,290 biallelic SNPs for *P. cubensis* isolates only. Red diamonds (9) represent cucumber and cantaloupe isolates and green asterisks (2) represent squash isolates.

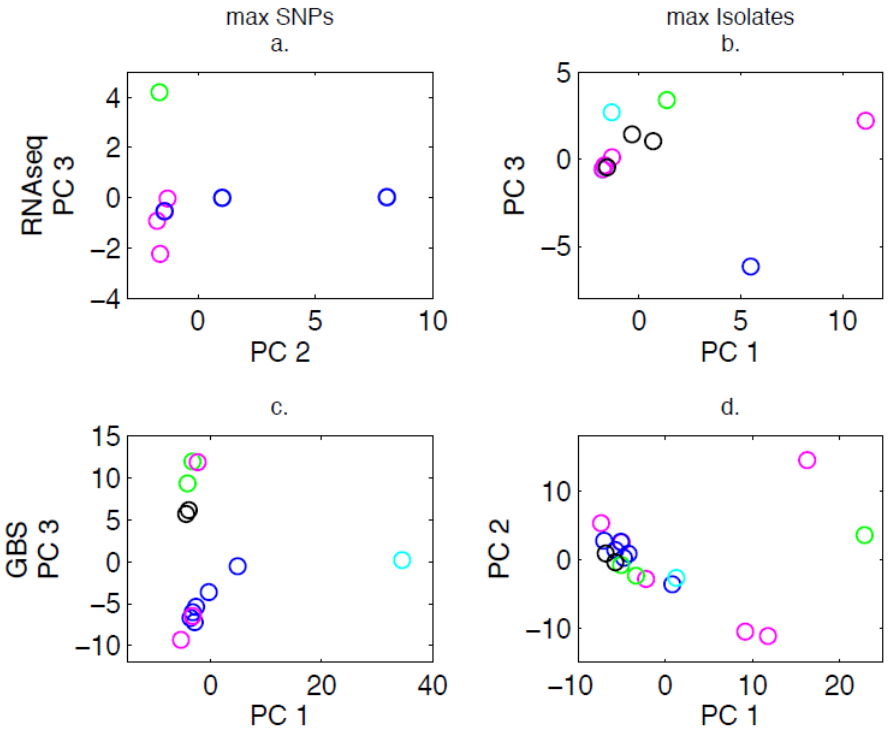
b. PCA using 135 biallelic SNPs for *P. cubensis* isolates only. Red diamonds (11) represent cucumber and cantaloupe isolates and green asterisks (3) represent squash isolates.

c. PCA using 11,922 biallelic SNPs for *P. cubensis* isolates only. Red diamonds (11) represent cucumber and cantaloupe isolates from NY, and green asterisks (2) represent squash isolates from NY. The black asterisk represents CDM-SQ, a squash isolate from SC. The cyan diamond represents CAcuc2008, a cucumber isolate from CA. The two blue diamonds represent cucumber isolates from NC.

d. PCA using 5,044 biallelic SNPs for *P. cubensis* isolates only. Red diamonds (12) represent cucumber and cantaloupe isolates from NY, and green asterisks (3) represent squash isolates from NY. The black asterisk represents a squash isolate from SC (CDM-SQ). The black diamond represents a pumpkin isolate from NC (CDM-PM). The cyan diamond represents CDM-CA, a cucumber isolate from CA. The blue diamonds (2) represent cucumber isolates from NC.

*PCA of only P. humuli isolates.* PCA of *P. humuli* isolates suggested differentiation based on geographic region where the isolates were collected (Figure 4.4). Given the small sample sizes, these trends varied depending on the filtering approach and genotyping method. This was most pronounced for RNA-seq data that was filtered for max SNPs versus max isolates. For the GBS data, there also was more separation by geographic region when SNPs were maximized. However, there was overall agreement between RNA-seq and GBS results, excluding the RNA-seq plot maximizing SNPs.

**Figure 4.4** Principal components analysis of RNA-seq and GBS SNP data for *P. humuli* isolates, maximizing for SNPs or isolates retained.



a. PCA using 1,290 biallelic SNPs for *P. humuli* isolates only. The green circle represents the 490-5 isolate from Japan. The blue circles (3) represent isolates from NY. The magenta circles (4) represent isolates from Oregon.
b. PCA using 135 biallelic SNPs for *P. humuli* isolates only. The blue circles (4) represent isolates from NY. The magenta circles (6) represent isolates from Oregon and Washington. The black circles (3) represent isolates from Vermont. The green circles (2) represent isolates from Japan. The cyan circle represents an isolate from Wisconsin.
c. PCA using 11,922 biallelic SNPs for *P. humuli* isolates only. The magenta circles (3) represent isolates from OR and WA. The blue circles (6) represent isolates from NY. The black circles (2) represent isolates from VT. The cyan circle represents an isolate from WI. The green circles (2) represent isolates from Japan.
d. PCA using 5,044 biallelic SNPs for *P. humuli* isolates only. The magenta circles (6) represent isolates from OR and WA. The blue circles (6) represent isolates from NY. The black circles (2) represent isolates from VT. The green circles (3) represent isolates from Japan. The cyan circle represents an isolate from WI.

*Principle Components Analysis: Selecting SNPs correlated to separating species.*

The first principle component, separating the two species, represented 86% and 65% of the variance in the data for RNA-seq and GBS, respectively, in the 'max SNPs' data used for the following analyses. Testing for correlation to the first principle component using PCA, 994 and 4,231 PCA-correlated SNPs were selected for RNA-seq and GBS, respectively. These PCA-correlated SNPs were found to contain the "confirmed" SNPs, which we expected to lie within the "correlated list." For the RNA-seq data, 388 SNPs were confirmed and all were found within the correlated list. For the GBS data, 975 SNPs were confirmed and found within the correlated list.

*Annotation of unigenes containing PCA-correlated SNPs between* Pseudoperonospora cubensis *and* P. humuli *isolates.*

Eight-hundred total unigenes were identified which contained PCA-correlated SNPs; 359 were from RNA-seq (containing 994 SNPs), 446 were from GBS (containing 1,547 SNPs) and 5 were overlapping between the two (Figure 4A, Table 4.5). A majority of the expressed unigenes lacked sufficient annotation for GO assignments (n=532) and were excluded from GO analyses. Of the 268 annotated unigenes, 135 were putative pathogenicity genes and 77 were secreted. Of these, 119 were from RNA-seq and 93 were from GBS. These included effectors (n=3), hydrolases (n=79), adhesion genes (n=4), genes involved in signal transduction and regulation (n=26), protection against oxidative stress (n=22), and detoxification and metabolite transport (n=2) (Figure 4B). The three effectors identified included two proteins with RXLR motifs and one elicitin (Table 4.6). The GO of the 268 total unigenes assigned known functions are shown for RNA-seq (Figure 4C) and GBS (Figure 4D).

**Figure 4.5** Characterization of unigenes containing PCA-correlated SNPs between *Pseudoperonospora cubensis* and *P. humuli* isolates sequenced using RNA-seq and GBS. Using the *P. cubensis* reference genome (Savory et al. 2012), unigenes were identified that contained PCA-correlated SNPs from GBS and RNA-Seq data. The number of unigenes identified in each technique, as well as the number of overlapping unigenes are shown in (A). (B) Unigenes classified as putative pathogenicity genes. Gene ontology (GO) of unigenes from (C) RNA-Seq (n=179) and (D) GBS (n=89) assigned in terms of the associated biological processes, cellular components and molecular functions.

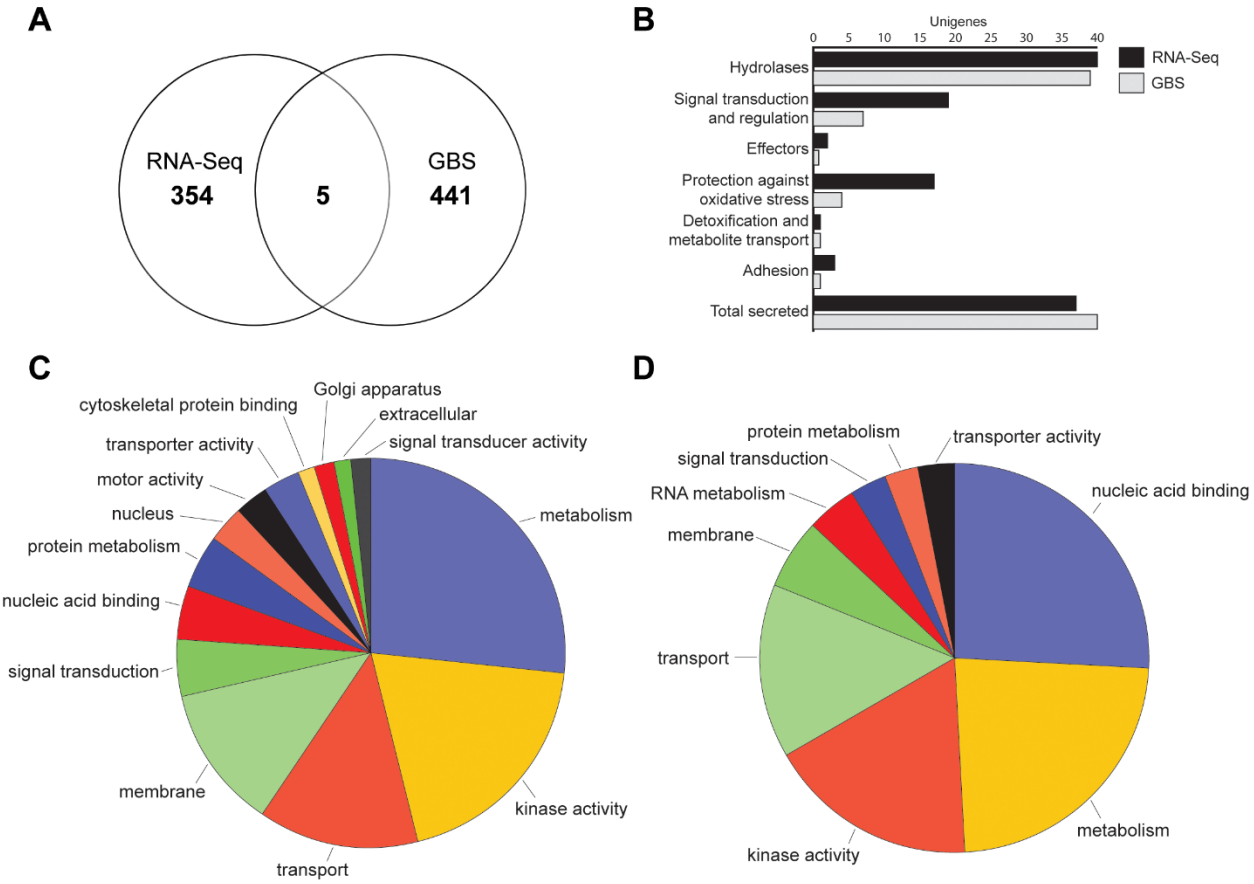


**Table 4.5** Unigenes containing PCA-correlated SNPs identified in both RNA-seq and GBS datasets.

| Unigene ID | Contig | Splice Type | Secreted | RNA-Seq[a] | GBS[a] | Putative Function |
|---|---|---|---|---|---|---|
| PCU_004770 | 18 | No introns | Yes | 6 | 1 | SEG[b], signal-peptide |
| PCU_076920 | 918 | No introns | No | 6 | 1 | DUF106[c], transmembrane |
| PCU_111220 | 2163 | Fully-spliced | No | 4 | 1 | hypothetical protein |
| PCU_174420 | 7414 | No data | No | 2 | 4 | hypothetical protein |
| PCU_182850 | 8557 | No introns | No | 1 | 5 | SEG, hypothetical protein |

[a] Total number of PCA-correlated SNPs within the overlapping unigenes.

[b] Unintegrated single exon gene.

[c] Integral membrane protein of unknown function.

**Table 4.6** Effector unigenes containing PCA-correlated SNPs.

| Unigene ID | Contig | Expressed | Secreted | Source | Effector |
|---|---|---|---|---|---|
| PCU_067480 | 709 | Yes | Yes | GBS | RxLR, signal-peptide |
| PCU_140880 | 3981 | Yes | Yes | RNA-Seq | RxLR, signal-peptide |
| PCU_163550 | 6126 | Yes | Yes | RNS-Seq | Elicitin, fungal-like |

**Discussion**

In this study, the results from two reduced-representation library sequencing techniques, RNA-seq and GBS, were used to observe the genetic variation between and among isolates of two closely related obligate biotrophic plant pathogens, *Pseudoperonospora cubensis* and *P. humuli*. Although this study examined a limited number of isolates from each species, the PCA results support the findings of previous studies and enhance the resolution of our current understanding of these pathogens' population variation.

The PCA results using RNA-seq and GBS analysis corroborate earlier findings that although highly genetically similar, *P. cubensis* and *P. humuli* are distinct species (Mitchell et al. 2011; Runge & Thines 2012). In addition, the population structure of *P. cubensis* was previously investigated using five nuclear and two mitochondrial loci (Quesada-Ocampo & Granke 2012). Using these seven genes to characterize 465 *P. cubensis* isolates collected world-wide from five cucurbit hosts, six genetic clusters were identified, with lower diversity among isolates from cucumber hosts as compared to other hosts (Quesada-Ocampo & Granke 2012). This finding, along with results of other previous studies (Thomas et al. 1987; Lebeda & Gadasova 2002; Cohen et al. 2003) suggest that *P. cubensis* isolates exhibit host specificity. More recently, whole-genome sequencing of isolates from different cucurbit hosts identified two distinct lineages among *P. cubensis* isolates, with isolates from cucumber, cantaloupe and pumpkin clustering separately from isolates of squash and watermelon (Thomas et al. 2014). The present study supports these prior investigations, showing differentiation of squash isolates from cucumber, cantaloupe and pumpkin isolates. The GBS data, which included five isolates of *P. cubensis* collected in North Carolina, South Carolina and California, also showed that the host-specific trend was consistent across geographic distance, where isolates

from cucumber or squash collected in different states clustered closer to isolates of the same host than to other isolates from the same region.

Less is known about the population structure of *P. humuli*. Chee et al. (2006) examined 40 isolates from each of Oregon and Washington using random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) markers. Their results suggested that the *P. humuli* population in Washington was highly clonal while isolates from Oregon were more diverse and they attributed this difference to sexual reproduction in Oregon (Chee et al. 2006). The diversity and relatedness of US populations outside of the Pacific Northwest had not been examined prior to this study, as hop production has only recently made a resurgence in the northeastern US (Northeast Hop Alliance 2012). Albeit a small sample size, both sequencing strategies used in our study showed that *P. humuli* isolates from New York, Vermont and Japan tended to cluster within their region of collection. However, the clustering of isolates from Oregon and Washington was not consistent and depended on how SNPs were filtered and genotyping method. Future studies including additional isolates from each region may allow for improved resolution of the genetic variation in *P. humuli* populations.

The variation differentiating *P. cubensis* and *P. humuli* was investigated more closely in order to identify genes potentially important for host specificity and disease pathways. We used a modified procedure from Paschou et al. (2007) to select the SNPs correlated to the first principal component, which was the component most important for separating isolates of *P. cubensis* and *P. humuli*. Similarly, previous studies have used RNA-seq data to identify genes containing polymorphisms potentially important to specific phenotypes; for instance, two alfalfa genotypes with differing cell wall composition (Yang et al. 2011), two soybean cultivars with differing drought resistance (Vidal et

al. 2012) and two *Brassica* species commonly crossed to produce hybrid progeny heterotic for yield (Paritosh et al. 2014).

When comparing sequencing data from *P. cubensis* and *P. humuli,* potential species-specific SNPs were found in 800 total unigenes, with the unigenes from RNA-seq and GBS overlapping for only 5 unigenes. These 800 unigenes represent approximately 3.4% of the 23,522 gene models predicted in the *P. cubensis* reference genome used for alignment and annotation (Savory et al. 2012). However, similar to previous studies(Torto-Alalibo et al. 2007), 67% of these total unigenes had no known function. Four of the five SNPs identified by both RNA-seq and GBS were located in unigenes with no known function. However, one of these overlapping unigenes was identified as a secreted signal peptide, and may therefore, be important in pathogenicity pathways (Kale & Tyler 2011).

The remaining 268 annotated unigenes containing PCA-correlated SNPs were largely represented by genes involved in metabolism, kinase activity and transport for both RNA-seq and GBS. For GBS, genes associated with nucleic acid binding were also highly represented. Genes involved in metabolism, primarily, but also kinase activity, transport and nucleic acid binding, were the most represented functional classifications in previous studies examining whole genome sequencing data from other oomycete plant pathogens, including *Phytophthora sojae* (Torto-Alalibo et al. 2007), *Phytophthora infestans* (Randall et al. 2005) and *Phytophthora parasitica* (Panabières et al. 2005).

Putative pathogenicity genes (n=135) containing PCA-correlated SNPs were identified. Because effectors are secreted by pathogens in order to manipulate their hosts (Kale & Tyler 2011), secreted proteins (n=77) were also identified from the annotated unigenes. Oomycete pathogen effectors have been characterized by a conserved RXLR (*Arg-X-Leu-Arg,* with X representing any amino acid) motif, which facilitates the delivery of the effector into host cells (Whisson et al. 2007).

A similar conserved motif, QXLR, was identified in 29 secreted peptides in a *P. cubensis* genome sequence (Tian et al. 2011). The more recent *P. cubensis* reference genome used in this study was found to contain 271 putative effectors with an XXLR motif, as many of these putative effectors contained additional substitutions from 20 amino acids in the first *Arg* position of the motif (Savory et al. 2012). Among the unigenes identified to contain potential species-specific SNPs, 3 contained RXLR motifs. The largest number of putative pathogenicity genes were identified as hydrolases, which degrade components of the plant cell wall (Torto-Alalibo et al. 2007). Adhesion genes, which facilitate the pathogen's entry into host tissue, and genes involved in signal transduction and regulation important for host-pathogen recognition processes (Meng et al. 2009) were also found. Finally, genes for protection against plant defenses, such as oxidative stress and toxins (Meng et al. 2009), were identified. Future studies into the impacts of the SNPs between the two species located in putative pathogenicity genes may provide greater understanding of the pathogenicity pathways of these two species. Despite their high genetic similarity, *P. cubensis* does not infect hop plants and *P. humuli* does not infect cucurbits under natural conditions (Mitchell et al. 2011). Understanding the mechanisms of non-host resistance may inform disease control strategies, such as the breeding of resistant plants (Ellis 2006).

In conclusion, the PCA results for the RNA-seq and GBS data support the bifurcation of *P. cubensis* and *P. humuli*. Our data also support results of previous studies that indicate host lineages exist in *P. cubensis*. PCA-correlated SNPs responsible for the genetic separation of the two species were located within unigenes and these genes were annotated, with putative pathogenicity genes identified. The PCA-correlated SNPs identified in putative pathogenicity genes may be useful targets for improved diagnosis and detection strategies. Future studies can utilize the streamlined analyses and scripts provided for population studies using SNP data.

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# CONCLUSIONS

This body of work described several projects contributing to the development and implementation of integrated pest management (IPM) strategies. IPM practices, developed in the 1970's, have continually in order to deal with pest and pathogen adaptation to management strategies. Research focused on developing and testing IPM practices is of utmost importance today, as climate change is expected to increase pathogen expansion and disease severity on crops, while concurrently intensifying crop stress through drought, flood and temperature changes (Chakraborty & Newton 2011). Encouraging ecologically-based management of crops can increase biodiversity within agroecosystems, allowing for natural internal regulation of pest populations, nutrient recycling and hydrological processes (Altieri 1999).

Biodiversity in the soil of an agroecosystem is imperative for crop health. Research effectively supports the fact that soil is a living ecosystem and one of the most important non-renewable resources on our planet (Doran 2002). However, soil health management is one of the greatest challenges to modern sustainable agricultural practice. One fundamental cultural technique used to increase biodiversity in soil, and the agroecosystem as a whole, is the planting of cover crops. Growers have planted cover crops for centuries and scientists have shown this practice benefits soil fertility, prevents erosion and can reduce soilborne pathogen populations (Thurston 1990). Research into cover crop use continues to develop, with unstudied or rarely used species of cover crops, mixtures of plantings and applications to different cropping systems being a few of the novel fields of inquiry.

The research described in Chapter 1 investigating the impacts of cover crops was novel in that it addressed the application of mixed-species plantings and the effects of only one season of growth. Our findings support the need for growers to plant cover crops for multiple seasons,

choosing cover crop species based on the specific qualities applicable to their soil and cropping system. The impacts of mixed-species cover crops planted over multiple seasons must be examined in future studies in order to provide growers with more options for enhancing the health of their soil and crops.

As scientists have recognized the ecological importance of the diverse communities of microbes in soil, research efforts have begun to focus on characterizing these communities. Scientists have been able to link cultural practices, such as cover crop use, to increases in soil biodiversity, through the use of molecular techniques. The microbial community studies described in Chapter 2 provided a unique comparison of the results of two molecular techniques, macroarray and T-RFLP, as applied to a soil ecology experiment testing the effects of mixed-species cover crops. T-RFLP was previously a commonly used technique for such studies, and our findings suggest that it is not a sensitive or specific technique compared to macroarray. While T-RFLP may not be commonly used today for such studies, due to the use of next-generation sequencing techniques, the findings of this study provide perspective for the interpretation of results in experiments which have used T-RFLP to investigate microbial communities. Furthermore, our findings suggest it may be important to compare molecular techniques in future studies, in order to accurately study these complex systems.

In addition to cultural techniques for increasing agroecosystem health, IPM promotes crop protection through monitoring for pest and pathogen presence and inoculum level. Disease control and prevention relies on application of knowledge of pathogen biology, including the nature of the pathogen's life-cycle, dispersal patterns and the environmental conditions necessary for initiation of disease. Thus, IPM research has increased efforts to provide growers with decision support systems

based on these key pieces of information, giving them scientific guidance to both control disease effectively and prevent unnecessary pesticide applications.

Cucurbit growers have access to an effective decision support system to help control cucurbit downy mildew (CDM) on their crops; the CDM IPM-Pest Information Platform for Extension and Education (CDM IPM-PIPE) is an interactive web-based system providing national disease reports and weather conditions, as well as infection risk specific to a given location. In order to improve the effectiveness of this tool, techniques for the detection of pathogen inoculum propagules must be developed. Cucurbit downy mildew is caused by *Pseudoperonospora cubensis,* an obligate biotroph which may infect the host without presenting disease symptoms for 4-12 days. Therefore, detecting the pathogen prior to observing disease provides a clear window of opportunity for enhanced disease management.

However, detecting *P. cubensis* has proven to be a challenge due to its genetic similarity to *P. humuli,* causal agent of hop downy mildew. The first molecular techniques for differentiating *P. cubensis* and *P. humuli* were described in Chapter 3. These techniques have been developed at a pivotal time for growers; in New York, cucurbits are likely to face increased disease pressure due to cucumber production in hothouses in Michigan and Canada, providing a potential source of *P. cubensis* inoculum earlier in the season. Hop production is also on the rise in New York, where IPM principles must be employed in order to reduce the likelihood of *P. humuli* adaptation to resistant varieties and effective fungicides. The early detection of these two pathogens is now possible due to the techniques described for their molecular differentiation. However, a clear weakness of the study was the inability to detect these pathogens from air samples prior to disease onset. We hypothesize that the air sample DNA may have been degraded by the desiccation or rupturing of the sporangia on the spore rods used to sample the air, which were changed twice per week. Future studies will

need to focus on a comparison of sampling strategies in order to effectively utilize the molecular tools developed.

As stated above, research of pathogen biology is imperative for effective IPM practice. In addition to this, epidemiological studies rely on investigations of pathogen populations. Study of pathogen population structure and diversity provide key insights into pathogen dispersal, reproduction, host-specificity and evolutionary potential to pose risk to crops. Chapter 4 described a genomic study of *P. cubensis* and *P. humuli* isolates sequenced using RNA-seq and GBS. Single-nucleotide polymorphisms (SNPs) were used to examine the variation within and between the populations of these two species. The study identified SNPs which differentiated the two species then identified and annotated the genes containing these SNPs, with a focus on putative pathogenicity genes. A larger-scale study incorporating *P. cubensis* isolates from Canada, Michigan and south-eastern states will also be important for future studies seeking to track the origin of inoculum and dispersal of *P. cubensis* outbreaks. These studies will also be important for addressing recent findings into the importance of perennial hosts in the overwintering of *P. cubensis* and the possibility of the pathogen's dispersal in seed.

Due to the marvelously complex nature of agroecosystems, IPM research is necessarily interdisciplinary, fusing plant pathology, soil sciences, entomology, plant breeding and ecology, to name just a few. The dynamic nature of plant pathogens and the increasing number of scientific tools used in order to study them has promoted collaborations among laboratories, extension programs and universities. A great advantage of the research described within this body of work, and a treasured aspect of my education through this experience, lies in collaborations with Ohio State University, Oregon State University, the University of Maryland, St. John's University in Tanzania and Cornell Cooperative Extension.

Furthermore, while research is an imperative component of IPM, education and outreach are equally important for the proper dissemination and application of the scientific methods of IPM practice developed through research. Research must be developed hand-in-hand with grower input and participation, in order to maintain relevance and impact. The research described in the above projects also incorporated this important goal of IPM. The cover crop research involved collaboration with Erin Bullock at Mud Creek Farm who participated in an on-farm experimental trial. The portion of the detection project targeting *P. humuli* sporangia would not have been possible if it weren't for collaboration with Rick Pederson, who allowed us to place a spore trap in his hop yard and communicated his disease scouting results with us for two seasons. Also, several of the *P. cubensis* isolates used in the population study were contributed by growers who sent in diseased plant samples.

The increasing global importance of IPM application is driving researchers to prioritize outreach and collaboration. More than ever, researchers from around the world are coming together to share ideas and solve problems. The impacts of basic IPM knowledge in developing countries, where extension efforts and local research is often limited, can be substantial. It is estimated, through surveying scientists involved in IPM research through collaborative research support program (CRSP) collaborations, that IPM practices have increased profits for growers by millions of dollars in certain developing countries (Hristovska et al. 2009). I was very fortunate to be part of a collaboration with St. John's University of Tanzania, funded through a CRSP grant. We performed grower IPM talks and set up a field experiment to test a locally abundant natural pesticide, as described in the appendix.

Whether in Tanzania or New York, the same IPM principles support food security initiatives and environmental stewardship. Research, such as that described above, will continue to develop in

order to help growers maintain and improve soil health and protect crops from pests and pathogens. It is an exciting time for such research, as global ties expand with increasing challenges from climate change and human population growth. The collaborative nature of this field will continue to develop and rise to these challenges, innovating solutions for the problem of sustainable food production worldwide.

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APPENDIX I

## Tomato Integrated Pest Management in Tanzania

### Introduction

As part of my international agriculture minor, I took part in a collaboration with St. John's University in Tanzania facilitated through a Horticulture Collaborative Research Support Program (CRSP) USAID grant. I worked with Professor Don Lotter and his graduate student, Sophia Chami on their project titled, "Utilization of locally abundant neem (*Azadirachta indica*) for integrated pest management (IPM) of horticultural crops." Neem trees had been planted throughout the region by NGO's to create a source of natural pesticide for crops. However, neem pesticide use had not been tested in the area and local people were not aware of the potential uses of neem. Dr. Lotter had worked with a local extension agent and learned that pesticide use on horticultural crops in the area was heavy, despite inadequate education as to their use, leading to ineffective application that harms the environment, human health and incurs heavy costs on growers.

For her graduate work, Sophia wanted to reach out to local growers and help them better understand pest biology, an important component of IPM practice. In addition, she aimed to implement a field experiment testing the efficacy of neem on tomato pests, as tomatoes are of high value and great susceptibility to losses from pests. My role prior to visiting Tanzania was to design a visually-based IPM grower talk, in the form of a poster printed on sturdy vinyl (Figure A1), appropriate for illiterate growers and the pests of the area. In addition to informing growers about pathogen biology, the talk was designed to provide information about efficient non-chemical means for pest control. I organized the talk so that I could provide Sophia with an information sheet containing all the material the poster was meant to cover, since all the information was new to her. Thus, I labelled each pest with a letter and each IPM practice with a number so that she could easily

study the information sheet and deliver the intended message for each picture (Figure A2; simplified version of study sheet). This design ensured that the talk could be informative both to the graduate student who would perform the extension talks as well as to the growers.

Figure AI.1 Tomato IPM poster focused on pests important to growers in Tanzania.

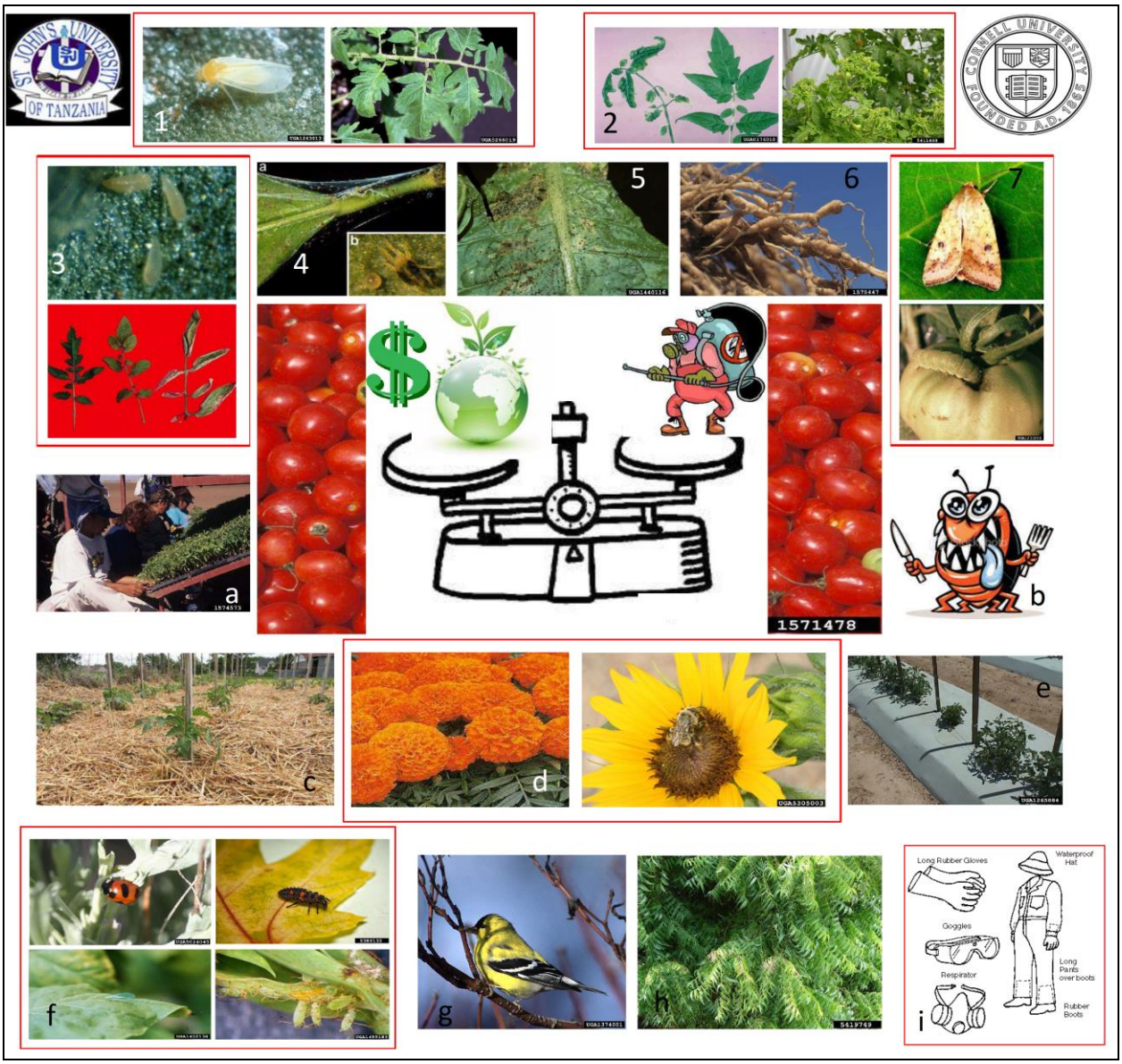


Figure AI.2 Key to IPM Poster: Points of Action

Key to poster numbers: pests and symptoms

1. Whitefly.
2. Tomato Yellow Leaf Curl Virus (TYLCV).
3. Russet mite.
4. Red spider mite.
5. Aphids.
6. Root-knot nematodes.
7. Fruitworms.

Key to the poster letters: management techniques

a. Check the transplants carefully for disease before planting (or order disease free seed.)
b. Let those pests go hungry and die off. Leave an interval of time between planting tomatoes.
c. Control weeds. Mulching has also been found to be very useful in increasing yields and reducing disease.
d. Trap crops.
e. Rogue diseased plants.
f. Encourage predatory beneficial insects. These are pictures of a few examples: ladybugs (with larval stage shown as well.), green lacewing and mite predatory midges.
g. Birds. Plant trees near your field (also serve for firewood, can be nitrogen fixers) that will create a bird habitat. Birds love to eat fruitworms.
h. Neem sprays.
i. Pesticide.

In November 2013, Sophia and I visited groups of tomato growers in their fields under the shade of nearby trees. The talks were very interactive; growers pointed to the pictures on the poster, explained the problems pests caused, asked questions and discussed the suggestions provided– all translated into Swahili by Sophia (Figure A3). Growers gave positive feedback and suggestions as well, even letting us know that they would share the information with others. One of my favorite examples of information they appreciated was learning that ladybugs, in contrast to being pests, were avid predators of aphids. The growers have trouble with aphids, but not knowing that ladybugs were beneficial, had been spraying them with pesticides.

Figure AI.3 Interactive grower talk with Sophia Chami.

Dr. Lotter, Sophia and I also worked to design an experiment testing the efficacy of neem on pest control before I left. The grant provided additional funding for a drip irrigation system for the field trial (Figure A3). We used a randomized complete block design with four treatments and four repetitions per treatment. The four treatments were: crushed neem seeds suspended in soapy water, soapy water, water alone and an untreated control. Unfortunately, the experiment was not completed, as tomato yellow leaf curl virus wiped out the entire crop before any data could be collected. However, Dr. Lotter may repeat the experiment in the future using TYLCV resistant tomato varieties in order to evaluate neem efficacy on the other important pathogens and pests.

Figure AI.4 Field experiment testing efficacy of neem in controlling pests of tomato in Tanzania. The drip system as well as funding for Sophia's graduate work was supported by the CRSP grant.

APPENDIX II

**Neighbor-joining trees of concatenated SNPs**

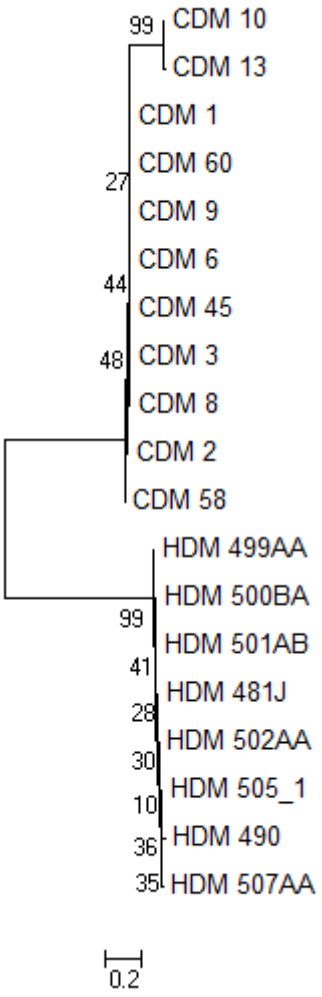



**Figure AII.1 Evolutionary relationships of taxa: RNA-seq SNPs filtered for maximum SNP retention.**
The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 1.67639996 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 5% site coverage were eliminated. That is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1252 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2014).

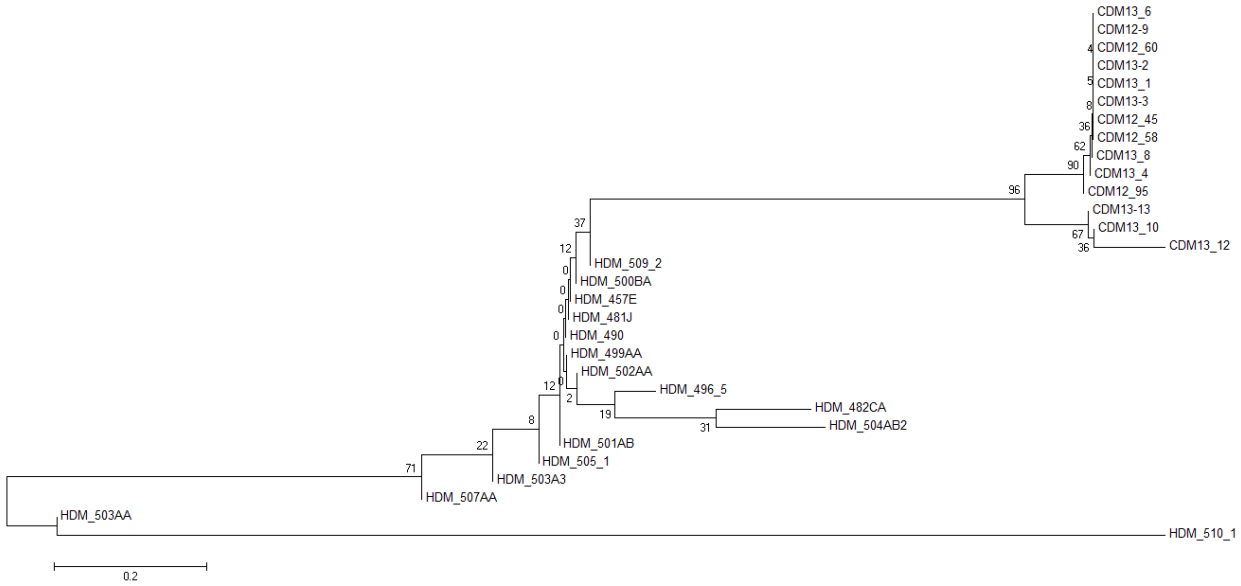
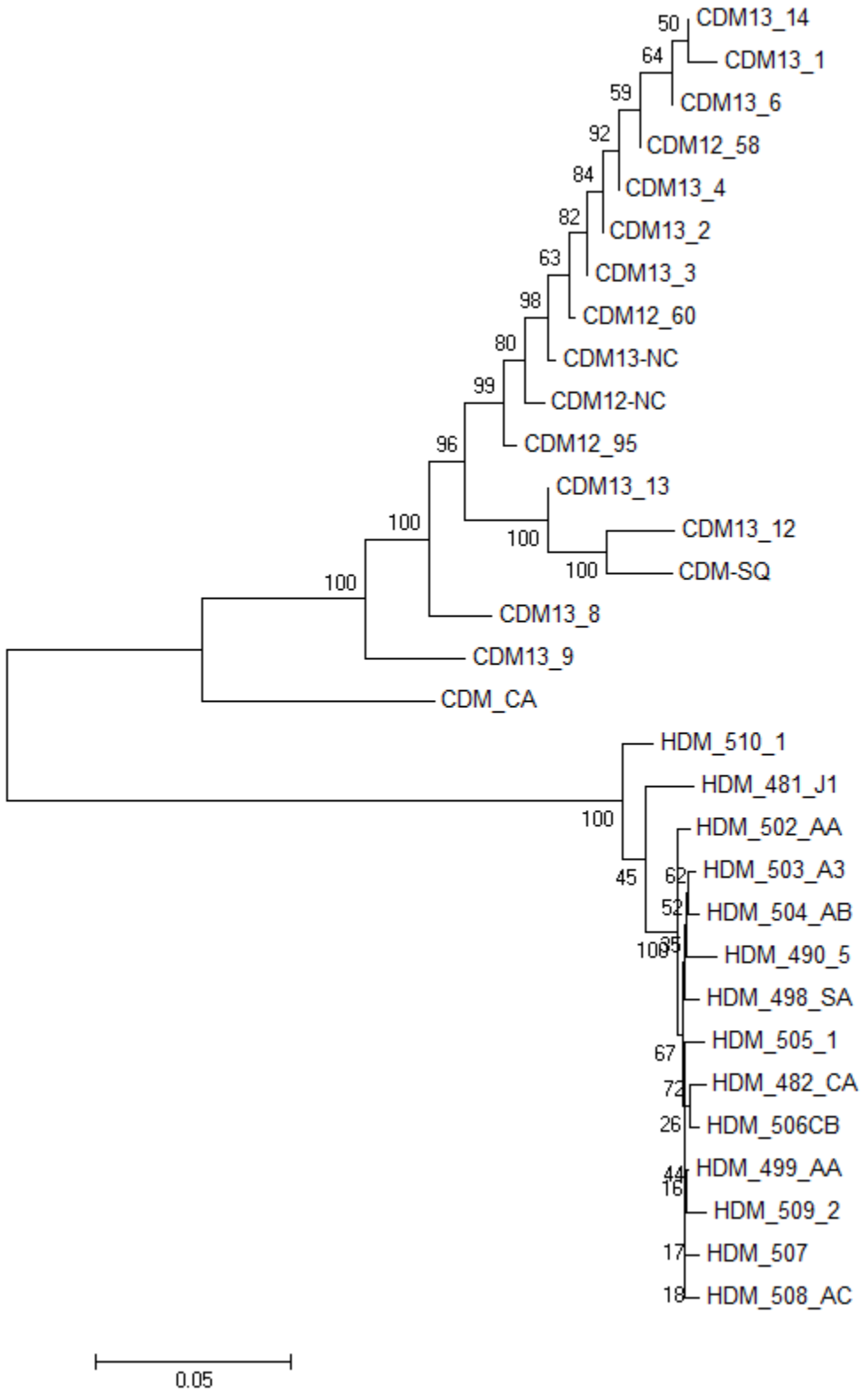



**Figure AII.2 Evolutionary relationships of taxa: RNA-seq SNPs filtered for maximum isolate retention.**

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 3.06710421 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 5% site coverage were eliminated. That is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 128 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2014).


CDM13_14
CDM13_1
CDM13_6
CDM12_58
CDM13_4
CDM13_2
CDM13_3
CDM12_60
CDM13-NC
CDM12-NC
CDM12_95
CDM13_13
CDM13_12
CDM-SQ
CDM13_8
CDM13_9
CDM_CA
HDM_510_1
HDM_481_J1
HDM_502_AA
HDM_503_A3
HDM_504_AB
HDM_490_5
HDM_498_SA
HDM_505_1
HDM_482_CA
HDM_506CB
HDM_499_AA
HDM_509_2
HDM_507
HDM_508_AC
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0.05

**Figure AII.3 Evolutionary relationships of taxa: GBS SNPs filtered for maximum SNP retention.**

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0.58372565 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 5% site coverage were eliminated. That is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 11153 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2014).

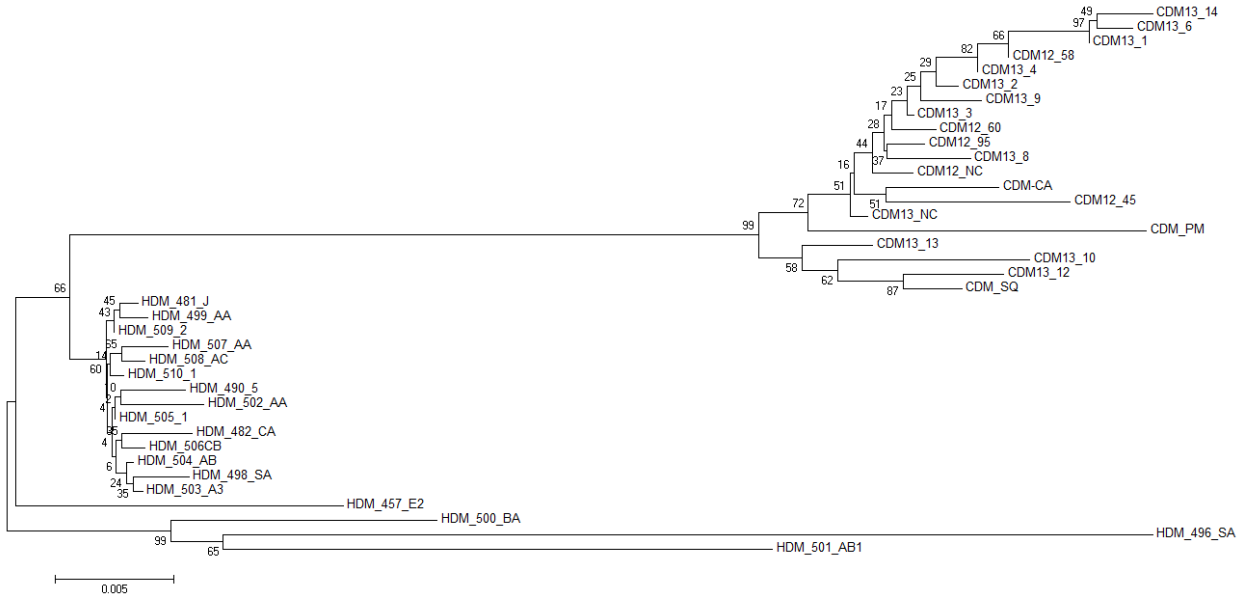


**Figure AII.4 Evolutionary relationships of taxa: GBS SNPs filtered for maximum isolate retention.**

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0.23338215 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 5% site coverage were eliminated. That is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 4593 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2014).

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