SIMULTANEOUS DETECTION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* AND *PEPINO MOSAIC VIRUS* IN TOMATO SEED USING MAGNETIC CAPTURE HYBRIDIZATION AND REAL TIME PCR

by

KAMEKA LATOYA JOHNSON

Under the Direction of Ronald R. Walcott

ABSTRACT

Pepino mosaic virus (PepMV) and *Clavibacter michiganensis* subsp. *michiganensis* are quarantined seedborne pathogens of tomato that cause severe economic losses. Currently, separate seed health assays must be conducted for these pathogens, making the process time consuming and difficult. One method to improve the efficiency of seed health testing is the simultaneous detection of both pathogens using multiplex real time PCR. Magnetic capture hybridization (MCH) was employed to concentrate and purify target nucleic acids from PCR inhibitors present in the seed wash. The combination of MCH with multiplex real time PCR resulted in a 100-1000 fold increase in detection sensitivity compared to direct multiplex real time PCR. The detection threshold of the MCH-multiplex real time PCR assay was 10^5 CFU/ml *C. michiganensis* subsp. *michiganensis* with a 10^{-4} dilution of total RNA extracted from PepMV-infected tissue. The assay also detected *C. michiganensis* subsp. *michiganensis* in 1:10 dilution of naturally infested tomato seed.

INDEX WORDS: *Pepino mosaic virus*, *Clavibacter michiganensis* subsp. *michiganensis*, Multiplex real time PCR, Magnetic capture hybridization

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KAMEKA LATOYA JOHNSON

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KAMEKA LATOYA JOHNSON

Major Professor: Ronald R. Walcott

Committee: John Sherwood
Ronald Gitaitis

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my family, you were my inspiration, thank you. To my mother, thank you for your advice and encouragement and thank you for the poem, labor for learning. It kept me going.

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INTRODUCTION

Tomato (*Lycopersicon esculentum*), has its origins in the Americas (the Andes region encompassing portions of Peru, Bolivia and Ecuador), and is an annual crop that belongs to the family *Solanaceace* (14). Tomato was most likely introduced to Europe in the 1500's by the Spaniards after they invaded South America (14). The tomato fruit was not widely accepted as a food source until the 1800's, as it was thought to be poisonous due to its resemblance to other members of the nightshade family (14). Today tomato is the leading fresh and processed vegetable crop in the U.S. with approximately 118,865 ha planted for commercial production in 2003 (USDA, Statistics of vegetable and watermelon, http://www.usda.gov/nass/pubs/agr03/03_ch4.pdf. 28-29). This translates into a net worth of 575,399,000 (USD) (USDA, Statistics of vegetable and watermelon, http://www.usda.gov/nass/pubs/agr03/03_ch4.pdf. 28-29). Nutritionally, tomatoes provide a significant source of vitamins A and C (46). Additionally, red tomatoes contain lycopene, which has antioxidant properties that reduce the risk of certain cancers (46). Tomato plants are typically produced from seed therefore, obtaining high quality seed is essential for the production of vigorous plants yielding high quality fruit.

Seed production is an important agricultural industry because seed quality significantly influences plant growth and the overall success of the crop. Hence, more effort is generally required for tomato seed crop production than for fresh market fruits. The global seed market has an annual estimated value of $30 billion dollars, with the U.S. seed industry accounting for approximately 20% of that market (American Seed Trade

Association (ASTA), seed trade statistics, http://www.amseed.com/about_statistics.asp). The U.S. is the major supplier of vegetable seed in the North Western hemisphere and the Andean Pact countries (Monthly planting seed report, http://www.fas.usda.gov). For the last 6 months of 2004, the total U.S. planting seed export market had a net worth of $558 million and an import value of $206 million (U.S. Department of Agriculture, Foreign Agricultural Service U.S. Trade Data Collection.). The U.S. vegetable seed exports totaled over $172 million and over $94 million dollars worth of vegetable seed were imported in the same time period (USDA, FAS, U.S. trade data collection). There are approximately 60,000 different varieties of seed being marketed or produced in the U.S. (ASTA, seed trade statistics, http://www.amseed.com/about_statistics.asp), but tomato seed is one of the most important crops accounting for $22 million in exports and $18 million in imports for this same period (USDA, FAS, U.S. trade data collection).

One of the most important determinants of seed quality is the presence of phytopathogenic organisms that can affect germination, plant growth, as well as cause disease in seedlings. While resistance is the ideal strategy for plant disease management, disease resistance is not available for many commercially-grown plants. Hence, one of the most effective options for plant disease management is exclusion of pathogens from crop production fields. To achieve this, routine testing must be conducted to ensure planting materials are free of phytopathogens. This is especially important for the exclusion of exotic pathogens from new regions because seed and seedling transplants are shipped over long distances and can serve as efficient vectors of pathogens. The introduction of non-indigenous pathogens into U.S. crop production regions on seed is a serious threat that could result in billions of dollars in losses. Two examples of seedborne

diseases of tomato important enough to warrant quarantines are bacterial canker caused by the gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* and *Pepino mosaic virus,* a positive sense strand RNA Potexvirus. The introduction of these pests on seed can result in yield loss and restrict the movement of plant products from infested regions. This could effectively eliminate all export revenue for tomato.

Bacterial canker of tomato

Bacterial canker caused by *Clavibacter michiganensis* subspecies *michiganensis* (Cmm), was first described in the U.S. in Michigan in 1909. However, it may have been observed as early as 1882 in New York (4). Bacterial canker is an important seedborne disease that limits tomato seed quality. The symptoms associated with bacterial canker can be localized or systemic and vary based on environmental conditions, age and susceptibility of the host (4, 5). Wilting may be the first symptom observed and this can be seen more clearly during periods of hot, dry weather (4). Other symptoms associated with bacterial canker include blister-like spots on leaves and birds eye lesions on fruits (5, 7). *Clavibacter michiganensis* subsp. *michiganensis* also produces cankers on stems and petioles, and yellow-brown marginal necrosis on leaves (5). Fruits may fail to develop, or ripen unevenly leading to a marbling effect that makes them unmarketable as fresh produce (5).

Clavibacter michiganensis subsp. *michiganensis* is a gram-positive, rod-shaped, motile bacterium that typically produces yellow colonies on nutrient agar (20). The bacterium is capable of surviving in and on seed, in soil compost and infected plant residue for up to two years (21). The pathogen grows at temperatures ranging from 20 – 30°C with optimal growth occurring at 25°C (5). *Clavibacter michiganensis* subsp.

michiganensis has been quarantined in the European Union countries since 1995 and has been reported to cause up to 80% loss in yield within a field or greenhouse (6).

***Clavibacter michiganensis* subsp. *michiganensis* pathogenicity**

The genome of *C. michiganensis* subsp. *michiganensis* is approximately 3.4 MB and the bacterium has two naturally occurring plasmids designated pCM1 (27.5 kb) and pCM2 (72 kb) (19, 39). These plasmids play a vital role in pathogenesis since a plasmid-free mutant was shown to lose the ability to induce wilting (39). This mutant, designated Cmm100 retained the ability to colonize tomato plants and produced exopolysaccharide (EPS). However, the absence of either pCM1 or pCM2 resulted in reduced virulence, suggesting that both plasmids encoded virulence factors (39). Additionally, the effects of the plasmids were additive indicating that multiple genes might be involved in pathogenesis (39).

The pathogenicity factor on pMC1 is the celA gene that encodes endo-ß-1, 4-glucanase (19). This enzyme has cellulase activity and can therefore break down cellulose in plant cell walls (19). Endo-ß-1, 4-glucanase is approximately 72 kDa and consists of three domains, the N-terminal catalytic domain which can be grouped into the cellulase family A1 and the type IIa cellulose binding domain (CBD), which is connected to the N-catalytic domain by a short hinge region (27, 39). The third domain is similar to plant expansins, proteins involved in weakening cell walls during cell expansion (19, 27). The expansin-like domain may contribute to the hydrolytic properties of the endoglucase (27). CelA is the only pathogenicity gene carried by this plasmid and its product is secreted via a two step, Sec- and ATP-dependent, type II mechanism involving a signal peptidase (27).

The pat-1 gene on pMC2 was determined to be directly involved in disease development (13, 26). It encodes a 29.7 kDa protein that shows homology in the active site to serine proteases of *Lysobacter enzymogenes* and *Streptomyces griseus* (26, 39). Also present on this plasmid, downstream of the pat-1 gene, is a repetitive sequence motif designated pat-1 rep. Deletion of this repetitive sequence reduces virulence (13). The pat-1 repetitive sequence allows the formation of a stem loop structure at the 3' end of the mRNA of the pat-1 gene (13, 26). This confers high thermodynamic stability to the mRNA and may protect it from enzymatic degradation (13, 26). The protein is a surface associated protein covalently linked to the peptidoglycan layer (19). These plasmids also carry genes that allow them to be transferred between bacteria (19). This type of transfer has been shown to occur in *plantae* (19).

Importance of seedborne inoculum

Clavibacter michiganensis subsp. *michiganensis* has been found on the surface and within tomato seed (4). The pathogen can also reside on the plant epiphytically, inducing disease development under favorable environmental conditions (21). Seed is the main mechanism of long distance dispersal for *C. michiganensis* subsp. *michiganensis*, and international seed trade has facilitated its distribution worldwide (4, 58). Natural seed transmission can range from 0.25% to 81.28% and the presence of 0.01-0.05% infested seed within a lot is enough to cause a bacterial canker epidemic in the field (8). Infected tomato seedlings may be asymptomatic because of epiphytic pathogen survival or latent symptom development (58). In both scenarios, *C. michiganensis* subsp. *michiganensis* - infected plants can escape visual detection (58). Cultural practices employed in the production of seedlings, such as clipping which ensures plant uniformity, and harvesting

practices that result in the mixing of diseased and uninfected seedlings help to disseminate the bacterium in the field (8). In general, there is a 10% increase in disease incidence for every 0.1% increase in infected seedlings before clipping (8). Additionally, when infected and uninfected seedlings are mixed there is a 3 - 5% increase in disease incidence for every 1 % increase in the amount of infected seedlings added (8). Since clipping is considered essential for field grown tomatoes, commercial production of tomato relies on certified seed (8). Within the field, secondary spread of the pathogen also occurs by wind-driven rain.

Management of bacterial canker

The most effective strategy for bacterial canker management is exclusion of the pathogen, or reduction of initial inoculum. To prevent the introduction of the pathogen, sensitive, accurate and efficient seed health assays are required. Currently plating assays that employ semi-selective media, pathogenicity tests, polymerase chain reaction (PCR) assays and ELISA are used to test seed for bacterial canker. Seed treatments that reduce the pathogen population are also employed including a 5 % hydrochloric acid soak for 5-10 hrs, a 1.05 % sodium hypochlorite soak for 20-40 minutes, or a hot water 56ºC soak for 30 minutes (22). Crop rotations and removal of plant debris and secondary hosts from the production fields can help to reduce the initial inoculum. Finally, the inspection of crops during the growing season can be useful especially in the case of tomato seed production.

Few strategies exist to manage bacterial canker once it appears in the field. Foliar applications of copper hydroxide is one option, but fruit produced from these fields may not be suitable for fresh market due to the development of birds eye lesions (63).

Therefore bactericides are recommended for treating seedlings in the greenhouse once true leaves have appeared until seedlings are transplanted to the field (23).

Pepino Mosaic Virus

Pepino mosaic virus (PepMV) is another important pathogen of tomato that was first described on pepino plants (*Solanum muricatum*), in the Canete Valley in Peru (28). In 1999 it was found on greenhouse-grown tomatoes (*Lycopersicon esculentum*) in the Netherlands and southern England (59). Since then, PepMV has been reported on tomatoes in South America**,** Spain, France, Germany, Italy, Morocco, Finland, Norway, Portugal, Canada and the United State (18, 29, 38, 47). In the United Kingdom most outbreaks were managed by sanitation (removing all plant residue from greenhouses and sterilization of equipment) at the end of the growing season (59). However, if PepMV was to infect crops in commercial fields it would be difficult to effectively implement sanitation (59). Currently PepMV is a quarantined pathogen and is included on the European Plant Protection Organization (EPPO) pest alert list (47).

Pepino mosaic virus causes variable, temperature-dependent symptoms on tomato plants. Leaf symptoms include dark green mosaic patterns, leaf deformation, vein banding, chlorosis and green striations on stems, petioles and sepals (30, 38). Infected tomato fruits display discoloration, mosaic patterns and uneven ripening (30, 47). This disease can cause significant reductions in tomato yield and losses have ranged from 15 – 80% in Spain, but have been less than 5% in the UK (30). In central and southern Peru losses due to PepMV have not been measured, but growers suggest that it ranges from 5 – 40% (54). The distribution of PepMV throughout Europe suggests that it has an efficient mechanism of dissemination (54). PepMV is highly stable and readily transmitted

mechanically. Poor sanitation and plant-to-plant contact facilitates the spread of the pathogen (1). To date, insect transmission of PepMV has not been proven, but the ease with which PepMV spreads makes it a serious threat to tomato production (47, 54). In October 2000 PepMV was first noticed in Spain on a few tomato plants, but by March 2001 40% of the plants in the field were affected (47). Once in the field, virus spread cannot be easily controlled. Currently the only methods available for PepMV management are sanitation of equipment and hands using disinfectants and eradication of symptomatic plants (38).

Host range

Pepino mosaic virus is capable of infecting members of the *Solanacerum* family, including *Datura stramonium, Nicotiana benthamiana*, and *Solanum tuberosum* (30). However it was not shown to infect *Capsicum annum* (30). PepMV has been found to naturally infect wild relatives of *L. esculentum* in Peru including *L. chilense, L. peruvianum* and *L. piminellifolium* (54). In Spain it was shown to infect weeds including *Malva parviflora, N. glauca, Solanum nigrum* and *Sonchus oleraceus* (29). In wild relatives and weed hosts, the virus can be present without symptom development (29, 54). Such infected plants were observed near infected greenhouses in Spain (29). These alternate hosts can act as potential sources of inoculum for PepMV outbreaks in tomato fields.

Viral Structure

Pepino mosaic virus belongs to the Potexvirus family (2). It shares approximately 60% nucleotide sequence homology in the RNA polymerase gene with other Potexviruses (59).

PepMV has the typical Potexvirus genome arrangement and morphology. It is a filamentous flexuous rod, of approximately 510 nm that form aggregates when present in high titers in tomato (28).

The genome of PepMV consists of a plus (+) sense single stranded RNA, of 6410 nucleotides excluding the poly A tail (1). The PepMV genome consists of a 5' noncoding region, five open reading frames and a poly A tail (1). The 5' non-coding region may play a role in RNA replication or protein translation and ribosome binding based on its nucleotide sequence (10). The first open reading frame (ORF) of the PepMV genome encodes the RNA-dependent RNA polymerase (RdRp). It is a 164 kDa protein consisting of three domains; a putative methlytranferase domain, the NTPase/helicase domain (Ntp-binding domain), and the RNA-dependent RNA polymerase (10). Open reading frames 2, 3 and 4 overlap partially and form a triple gene block (1). Open reading frame 2 codes for a 26 kDa protein that contains a Ntpase/helicase domain and belongs to superfamily I of RNA helicases. It is thought to play a role in NTP-binding (10). The 9 kDa protein encoded by ORF4 is the most variable in the potexvirus group and ORF3 encodes a 14 kDa protein whose function is not known (10). The proteins encoded by the triple gene block are thought to play a role in cell to cell movement of the virus (10). Open reading frame 5 encodes a capsid protein (CP) of 25 kDa (10). The CP contains an amphipathic core sequence which is thought to play a role in the packaging of viral RNA (10). The CP and RdRp genes are observed to be highly conserved in the Potexvirus group with similarities of 41.9-79.3% and 60.1-75.3% respectively (10).

Importance of seedborne PepMV inoculum

Pepino mosaic virus was generally not considered to be seed transmissible. However, the appearance of the disease in greenhouses suggested otherwise (59). Recently it has been shown that seed transmission occurs at a very low rate (1:1000 seed) and infected plants grown from contaminated seed tested positive for the virus using lateral flow devices (International Seed Health Initiative, http://www.worldseed.org). Once in a field or greenhouse the virus can be easily spread by contaminated tools, hands, shoes, clothing and plant-to-plant contact.

Management of PepMV

Management of plant diseases caused by viruses is especially difficult because they are present within the plant cell and rely on host cell processes for their propagation. Therefore destruction of viruses may harm the plant cell. Hence, the most effective management strategies for the control of viruses focus on exclusion and sanitation. If potentially infected plants are observed in a field infected plants are removed, access to infected rows is limited, and tools and clothes are sanitized. When the virus is identified on plants grown in greenhouses strict eradication procedures are taken (Management of *Pepino mosaic virus* in greenhouse tomatoes. http://www.omafra.gov.on.ca/english/crops/facts/01-017.htm). Viral particles present on seed can be inactivated by soaking in 0.5% sodium hypochlorite for 30 minutes or soaking in 15 trisodium phosphate for 45 minutes. Resistant cultivars are currently being developed by creating hybrids of tomato and its close relatives *Lycopersicon chilense*, *L. peruvianum* and *L. hirsutum*, species which have been reported to be resistant to PepMV (44).

Conventional approaches for the detection of Cmm and PepMV

To ensure that phytopathogens do not cross international and interstate borders, efficient and sensitive testing must be conducted on imported plants or plant products. These tests are the responsibility of state agencies and private companies. In general conventional techniques for detecting and diagnosing diseases caused by bacteria and viruses are time consuming and inefficient. Diagnosis of bacterial diseases routinely involves isolation of the pathogen on selective media, biochemical characterization, serological techniques, PCR and bioassays or pathogenicity tests (33). Diagnosis and detection of viruses require biological indexing, electrophoresis, electron microscopy, PCR or serology (33). The detection of plant pathogen has become more specific and less time consuming with the development of serological and molecular techniques (33).

Semi-selective media

The use of media designed to enhance the growth of specific pathogens is one way of identifying target pathogens. Once the pathogen has been isolated, it can be identified by biochemical and physical characteristics or by molecular techniques such PCR. However, the use of semi-selective media for isolation cannot be applied to pathogens that can not be cultured such as viruses and other nutritionally fastidious organisms. For the detection of *C. michiganensis* subsp. *michiganensis* on commercial seedlots, seed extract is plated onto semi-selective media and incubated for 5-12 days (International seed health initiative, http://www.worldseed.org, Bacterial canker of tomato, http://www.stalabs.com). According to Seed Testing of America Inc., a commercial vegetable seed testing company (Longmont, CO.), sample sizes of 50,000 seed with

subsamples of 10,000 are usually tested (Bacterial canker of tomato, http://www.stalabs.com). The sample size varies depending on the type of seed (e.g. smaller samples for breeder's seed, untreated seed and stock seed) (Bacterial canker of tomato, http://www.stalabs.com). Seed extract is then plated on the appropriate semi-selective media and biochemical, nucleic acid and pathogenicity tests are conducted on recovered colonies (International seed health initiative, http://www.worldseed.org). The test has been reported to have a sensitivity of 0.15- 0.01% infested seed in a seedlot (International seed health initiative, http://www.worldseed.org). However, the assay can take up 12 days and still be inconclusive due to the growth of non-target background organisms (fungal and bacterial) (International seed health initiative, http://www.worldseed.org). The confirmation of *C. michiganensis* subsp. *michiganensis* pathogenicity can take an additional 2 days by testing for the hypersensitive response produced on *Mirabilis jalapa* (four o'clock) or by testing virulence on tomato plants (12). This additional confirmatory steps makes the detection assay time consuming and unsuitable for high throughput testing (12).

Serological methods of detection

Serological assays were originally developed for the detection of viruses, but have also been used for detecting bacteria and fungi (52). Serological techniques include enzyme linked immunosorbent assay (ELISA), immunofluorescence microscopy (IF), immunofluorescence colony-staining (IFC) and immuno-strip assays (52). The use of monoclonal and recombinant antibodies has greatly improved the specificity of these serological techniques. These antibodies make pathogen detection more specific which decreases the likelihood of a false-positive result (33). ELISA is the most commonly

used serological assay with 800 antisera currently available for plant viruses from the American Type Culture Collection (http://www.atcc.org/common/catalog/plantVirology/plantVirologyIndex.cfm). However, ELISA has not been as successful for detecting bacteria due to difficulty in obtaining specific and sensitive antibodies (33). Employing enrichment procedures in media prior to detection improves the sensitivity of ELISA(34). However, this requires specific knowledge of the nutritional requirements for each organism of interest e.g. sources of carbon and nitrates, antibiotic tolerance, optimum growth temperatures (34).

Pathogen detection by immunofluorescence microscopy allows direct visualization of the pathogen within infected tissue. Therefore it can be used for seedborne pathogens and asymptomatic plant tissue. However, this technique is prone to errors in interpretation because it relies on visual detection of fluorescently labeled cells (52). Bacteria have complex and heterogeneous surface antigens that vary due to growth conditions and this can sometimes lead to false-negative results due to the high degree of specificity of monoclonal antibodies (52).

Serology based techniques such as ELISA, IFC and immunofluorescent antibody staining (IFAS), have been used to detect *C. michiganensis* subsp. *michiganensis* in commercial seedlots (Bacterial canker of tomato, http://www.seedworld.com). Samples determined to be positive by ELISA can be quantified by an ELISA plate reader while IFAS and IFC results can be visualized using fluorescence microscopy. Immunofluorescent antibody staining is more sensitive than ELISA and allows visualization of individual cells (Bacterial canker of tomato, http://www.seedworld.com). However, IFAS and ELISA are limited by their inability to distinguish between pathovars, living or dead cells (Bacterial

canker of tomato, http://www.seedworld.com). Development of specific antibodies that do not cross-react with non-target organisms is the major disadvantage of serological assays (12).

Detection of PepMV in infected seed lots can be accomplished by ELISA and bioassay techniques (International seed health initiative, http://www.worldseed.org). A positive ELISA result is not considered conclusive and a bioassay is normally done as a confirmatory test. The sensitivity of both assays was determined to be one infected seed in a subsample of 250. Lateral flow devices have been used for rapid on site screening for the virus, and 100% correlation is normally seen between lateral flow devices and ELISA results (Rapid detection of *Pepino mosaic virus,* http://www.pocketdiagnostic.com). In lateral flow devices, specific PepMV antibodies are immobilized to a membrane at a region called the test line. Test samples, once placed on the membrane will move by diffusion towards the test line. PepMV particles, if present in the sample will become bound to the immobilized antibodies. The assay takes approximately 2-3 minutes to complete. Detection sensitivity of this assay and ELISA has been shown to be 1 in 50, 000 dilution of infected fruit or leaf extract (Rapid detection of *Pepino mosaic virus,* http://www.pocketdiagnostic.com). The detection sensitivity can vary depending on the virus titer and the antibody used Lateral flow devices and ELISA have also been reported to detect 1:2048 and 1:8192 dilutions of leaf and fruit extract, respectively (48).

Molecular techniques for the detection of plant pathogens

Molecular detection techniques can be divided into those that rely on hybridization or those that rely on specific amplification of a target nucleic acid. These methods can have higher specificity than traditional assays, this is influenced by the design of the

oligonucleotides. The oligonucleotides can be designed to allow for differentiation of closely related pathogens (strain, race, subspecies, pathovars) (2, 60). Molecular assays can also result in increased sensitivity leading to detection of single copies of the target nucleic acid, (53, 60, 61).

Hybridization techniques

Hybridization involves the use of labeled oligonucleotides with sequences complementary to target nucleic acids. This approach is applicable for specific RNA or DNA molecules. For hybridization, denatured nucleic acids are fixed to a solid phase (e.g. nitrocellulose membrane). subsequently oligonucleotides labeled radioactively or with other reporter molecules are incubated with immobilized target nucleic acid molecules to facilitate annealing. Hybridization can then be visualized based on the type of labels used (radioactive vs. fluorescent). DNA microarrays represent one type of hybridization technique that can be used to detect seedborne pathogens. Oligonucleotide probes complementary to the target are immobilized to small glass or silica based membranes and probed with nucleic acid from the target organism (60). Positive samples are determined by the amount of fluorescence emitted (60). The drawback of this assay is the need for extraction and amplification of nucleic acids prior to analysis, which makes the procedure laborious (60).

Amplification techniques

Amplification of nucleic acids e.g. by the polymerase chain reaction (PCR) is considered one of the most important biological advances since its development in 1984 by Kary Mullis (40). Polymerase chain reaction is an effective procedure for the *in vitro* enzymatic production of a specific nucleic acid sequence. Nucleic acid amplification by

PCR consists of multiple cycles of three steps; 1) denaturation, 2) annealing, and 3) elongation. During denaturation, the original double-stranded DNA molecule is melted at 90-95ºC to yield single strands. During annealing, unique short oligonucleotides (primers) designed based on target nucleic acid sequences are allowed to hybridize to flanking regions of the target molecule. Finally, during elongation Taq DNA polymerase adds nucleotides onto the 3' end of the primer sequences to produce new double stranded molecules of the template nucleic acid. Each new DNA molecule can serve as a template for subsequent cycles and DNA production increases exponentially at the end of each cycle. At the end of the reaction, samples of the specific DNA molecules can be separated by electrophoresis on an agarose gel and visualized after staining with a nucleic acid stain e.g. ethidium bromide or SYBR green. Ribonucleic acids can also be amplified in this manner but must first be converted to cDNA using the reverse transcriptase enzyme.

Nucleic acid hybridization and PCR can be combined to develop sensitive, rapid and specific assays to target pathogens of interest. Also while antibodies used in serology assays tend to cross react with other *Clavibacter michiganensis* subspecies, the PCR protocol can be designed to differentiate between the different subspecies (2). PCR detection complemented by southern hybridization has been developed for *C. michiganensis* subsp. *michiganensis* with a detection limit of 2×10^2 CFU/ml in plant tissue and 1×10^3 CFU/ml of seed extract (from 50 seed) (49). It was noted that without DNA purification, detection of the bacteria from seed wash did not occur, possibly due to the presence of inhibitory compounds in the seed wash (49).

Reverse transcriptase PCR protocols have been combined with immunocapture for the detection of PepMV. Immunocapture reverse transcriptase PCR has been used to detect plant and animal viruses for over a decade (37). Immunocapture RT-PCR combines serology, used to isolate the virus particles with RT-PCR to amplify the target nucleic acid sequence (32, 37). This procedure has been reported to detect a one in ten billion dilution of tissue or seed extract, however, at present it is not being used as a routine seed health assay for PepMV (32).

Since its initial development, PCR has been modified in many ways to improve its specificity, sensitivity and efficiency. The specificity of hybridization and amplification techniques depends on the uniqueness of the sequences that are used to develop probes or primers (52). The probe used in hybridization is a labeled oligonucleotide that indicates the presence of a target sequence in a sample (21). In contrast, primers are short oligonucleotides (18 – 30 bp) that can hybridize to a template nucleic acid sequence and provide a site to initiate nucleic acid synthesis (21). Recently, these two approaches have been combined in the form of real time PCR to yield a rapid, specific and efficient approach for detecting specific nucleic acid sequences.

Real time PCR

Real time PCR is a relatively new technique that has been used for pathogen detection and diagnosis. It was originally developed for field detection of microorganisms associated with biological weapons for use by military personnel (21). A key component of real time PCR is the PCR machine which differs significantly from conventional PCR equipment. The real time PCR machine allows nucleic acid sequence amplification to be coupled with the generation and detection of a fluorescent signal. This eliminates the

need for subsequent gel electrophoresis and southern hybridization to confirm sequence identity (41). The coupling of fluorescent signal generation with target sequence amplification can also facilitate the quantification of initial target nucleic acid (60).

Different technologies exist to facilitate the amplification of nucleic acids by real time PCR including fluorescent resonance energy transfer (FRET) probes, molecular beacons and Taqman probes. FRET involves the use of two labeled oligonucleotides for detection (51). Probe one is labeled at the 3' end with a fluorophore, while the second probe is labeled at the 5' end with a different fluorophore. (51). The two probes are designed to anneal close together within the target region on the template nucleic acid. Excitation of flourophore at the 3' end of probe one results in the emission of fluorescence at a longer wavelength. The flourophore at the 5' end of the second probe is excited by the fluorescence emitted by the fluorophore on probe one. This results in the emission of light energy that can be detected by the real time PCR machine (9, 51). Fluorescence in this case is measured during the annealing step of PCR.

Molecular beacons have a fluorophore at their 5' end and a quencher molecule at their 3' end (9). The probes are designed with a secondary stem loop folding structure that brings the quencher and fluorophore into close proximity, thereby preventing fluorescence (9). During the annealing step of PCR, the molecular beacon hybridizes to target nucleic acid sequences and linearization of the stem loop structure causes the fluorophore and quencher molecules to be separated. This results in fluorescence after excitation (51).

To date the most commonly employed dye technology for real time PCR is the TaqMan assay. TaqMan probes are labeled at the 5' end with a fluorophore and at the 3'

end with a quencher molecule (31). When the fluorophore is close to the quencher no light energy is emitted; however, during the extension phase of PCR, the probe is degraded by the 5' |3' exonuclease activity of Taq DNA polymerase. This releases the fluorophore from the probe, which fluoresces upon excitation. This fluorescence is then detected by the real time PCR machine (Figure 1). At the end of each amplification cycle, fluorescence accumulates and can be monitored in real time by the real time PCR machine. Positive results are reported as cycle threshold (C_t) values, which represent the number of cycles required for the fluorescent signal to exceed background noise.

Currently, few TaqMan real time PCR assays are available for diagnosis of plant diseases, but as more genomic sequences for plant pathogens become available, the tools necessary for their design will become more accessible.

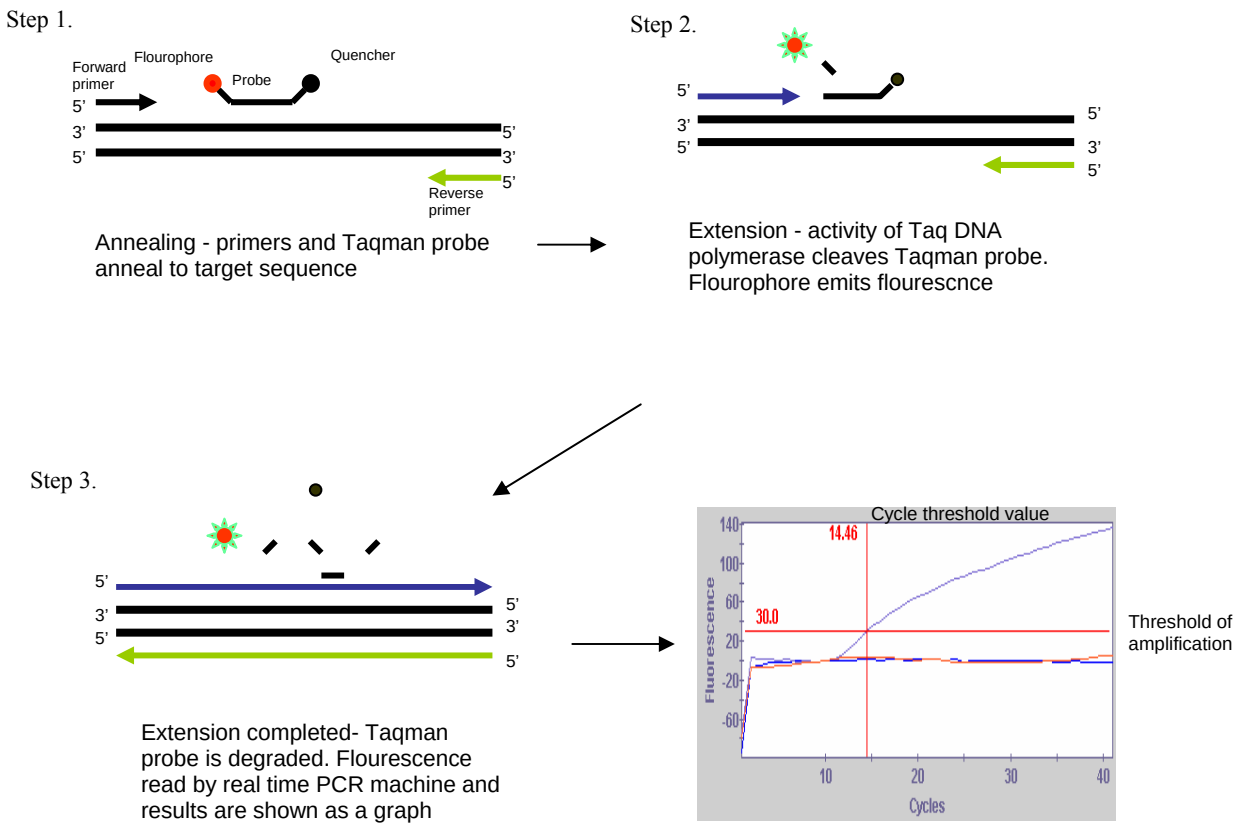

Figure. 1. Diagram of the steps involved in real time PCR using TaqMan probes. Step 1. Primers and Taqman probe anneal to complementary sequences of target nucleic acid. Step 2. Taq DNA polymerase extends primers. 5'-3' activity of the Taq DNA polymerase destroys the Taqman probe. Step 3. Extension completed and real time PCR results are shown as a graph

Benefits and limitations of PCR

Real time PCR is a sensitive and reliable method of pathogen detection; however, the efficiency of the procedure can be compromised by the presence of inhibitory compounds such as humic acids, present in soil, and phenols, in seed extract, in the sample DNA preparation (60). Real time PCR was shown to detect 100 CFU/ml of a pure *Ralstonia solanacearum* culture; however, when plant extracts were tested, the assay

sensitivity decreased to 10^7 CFU/ml (62). This decrease in sensitivity was thought to be due to PCR inhibitors in the plant extract. To remove inhibitors, several approaches have been attempted including DNA purification, BIO-PCR, immunomagnetic separation and magnetic capture hybridization.

BIO-PCR involves the combination of PCR with enrichment of the target pathogen on selective media. This has been reported to be very sensitive with a detection threshold of 1-3 *Pseudomonas syringae pv. phaseolica* cells per ml (50). Additionally, this approach eliminates PCR inhibitors that may be present in plant extracts (62). Unfortunately BIO-PCR is not applicable for non-culturable organisms (e.g. viruses, fastidious prokaryotes) and it may allow antagonistic, non-target organisms to limit the growth of the target pathogen, yielding false negative results (33, 60). For selective enrichment, knowledge of the nutritional requirements of each organism being targeted is necessary and the need for incubation prolongs the time required to complete the assay (51).

Immunomagnetic separation (IMS) involves the use of small paramagnetic beads coated with antibodies specific to the target organism, to sequester target cells from heterogeneous suspensions (17). For IMS, antibody-coated magnetic beads are incubated with the sample, during which time the antibodies specifically bind to their target antigen. Immunomagnetic beads carrying captured cells are then immobilized using a magnet and rinsed to eliminate inhibitors. After rinsing, the captured cells can be lysed by boiling to release PCR quality DNA or cultured on semi-selective media for enrichment purposes. IMS-PCR has been reported to increase *Acidovorax avenae* subsp. *citrulli* detection

sensitivity 100-fold when compared to conventional PCR (61). However, obtaining antibodies that do not cross-react with other bacteria is a limitation (56).

Magnetic capture hybridization (MCH) has great potential to improve the efficiency and applicability of real time PCR for pathogen detection and diagnosis. This technique was first described by Jacobsen in 1995 for the purification of *Pseudomonas flourescens* DNA from soil (25). With magnetic capture hybridization biotinylated oligonucleotide probes with sequences complementary to target nucleic acids are conjugated to microscopic streptavidin coated paramagnetic beads. This bead-probe complex can then be used to capture specific target nucleic acid sequences. Using a magnetic particle separator, the captured nucleic acids can be immobilized, while inhibitors and non-target nucleic acids are eliminated by repeated rinsing. Magnetic capture hybridization has been employed in both animal and plant related sciences for the purification of nucleic acids from backgrounds of non-target DNA and inhibitory compounds that may affect downstream applications (36). Therefore by eliminating inhibitors and selectively concentrating template nucleic acids, MCH can significantly improve sensitivity of real time PCR.

MCH-PCR can be combined with multiplex real time PCR, to improve the efficiency of detecting multiple pathogens in tissue samples. In many cases, seed are infested with more than one pathogen of interest and multiple assays must be conducted on the same sample. This can be time consuming and may require the use of different equipment and reagents. For example, at present, a seed assay for *C. michiganensis* subsp. *michiganensis* and PepMV would require one sample to be tested by a plating assay and a second to be tested by ELISA. Regardless of the pathogens of interest, MCH

can allow the specific capture of their nucleic acids. When MCH is combined with multiplex real time PCR, the result is an assay that is sensitive, efficient and applicable for all plant pathogens. This type of assay has the potential to revolutionize pathogen detection and provide a single efficient format by which all plant tissues could be tested for pathogens. To our knowledge, such an assay has not been previously attempted for a gram positive bacterium and a positive sense-strand RNA virus. Hence, the objective of the study was to develop an MCH multiplex real time PCR assay for the routine simultaneous detection of PepMV and *C. michiganensis* subsp. *michiganensis* in tomato seed.

MATERIALS AND METHODS

Viral and bacterial strains used in this study. *Clavibacter michiganensis* subsp. *michiganensis* and *Pepino mosaic virus* (PepMV) strains used in this research are listed in tables 2 and 4. *Clavibacter michiganensis* subsp. *michiganensis* (CMM 99-324) was provided by Dr. Ron Gitaitis (UGA, Tifton, GA) and used routinely throughout this study. *Clavibacter michiganensis* subsp. *michiganensis* strains were stored in 15% glycerol at -80ºC and routinely grown on nutrient broth yeast agar. PepMV-infected tissue, lot #A18203, was provided by M. Bandla (Agdia, Elkhart, IN). PepMV-infected tomato tissue was stored at -80ºC.

Growth and maintenance of pathogens.

Pepino mosaic virus. Five week old tomato plants (cv. Marion) were mechanically inoculated with PepMV. Briefly, the surfaces of three leaves on three tomato plants were lightly dusted with carborundum (Fisher Scientific, Suwannee, GA). PepMV inoculum was prepared by crushing 0.1 g of PepMV-infected tissue in 5 ml of 0.1 M phosphate buffer (4 volumes 0.1 M sodium phosphate dibasic, 1 volume 0.1 M sodium phosphate monobasic, pH 7). Using a gloved hand, the viral suspension was gently rubbed onto the upper surface of each leaf, taking care not to excessively damage the epidermal tissues. The carborundum was then rinsed from plant surfaces with water and the plants were incubated in a growth chamber (Conviron, Winnipeg, Manitoba, Can.) for two weeks under conditions of 24ºC and 14 h of fluorescent light daily. Plants were watered once daily or as necessary. Symptomatic leaf tissue was then collected and stored in plastic

bags at -80ºC. This tissue was used for RNA extraction and other experiments throughout this study.

Bacterial canker. Six-week-old tomato seedlings (cv. Marion) were inoculated with CMM 99-324 to ensure that the strain retained its pathogenicity. A cell suspension containing 1 x 10^8 CFU/ml was generated by suspending cells from a one day old culture of CMM 99-324 in 0.1 M phosphate buffered saline (PBS, 7.6 g $NaCl_2$, 1.25 g $Na_2HPO_4.2H_2O$ and 0.47 g $NaH_2PO_4.2H_2O$; 1 L of water, adjusted to pH 7), and adjusting the cell concentration to an absorbance of 0.12 at 640 nm, using a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, N.Y.) (55). Plants were mechanically inoculated by excision of a leaflet with a sterile scalpel blade that was dipped in a bacterial suspension of 1 x 10^8 CFU/ml (8). Plants were incubated for three weeks in a growth chamber (Conviron) at 25ºC with 14 h of fluorescent light daily. To re-isolate the bacterium, 1 g of leaf tissue was collected from infected plants and macerated in ~ 2 ml of PBS. One hundred microliters of the macerated tissue was streaked onto nutrient broth yeast agar (NBY, 8 g nutrient broth, 2 g yeast extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 2.5 g dextrose, 15 g agar, 1 L of water) and the plates were incubated at 25ºC for 24 h before they were examined for typical yellow *C. michiganensis* subsp. *michiganensis* colonies. *Clavibacter michiganensis* subsp. *michiganensis* colonies were purified by sub-culturing and placed in long-term storage at -80ºC or maintained on NBY agar plates at 25ºC for short-term storage.

Nucleic acid extraction

Pepino mosaic virus. To extract PepMV RNA, total RNA was extracted from ≈100 mg PepMV-infected tissue using a commercial RNA extraction kit (Qiagen RNAeasy plant mini kit, Valencia, CA), according to the manufacturer's instructions.

C. michiganensis* subsp. *michiganensis. To extract *Clavibacter michiganensis* subsp. *michiganensis* DNA, cell suspensions were lysed by mechanical disruption using glass beads (0.1 mm diameter) and a bead beater apparatus (Biospec, Bartlesville, OK). More specifically, 1 ml of a *C. michiganensis* subsp. *michiganensis* cell suspension was placed in a 2 ml screw-cap microcentrifuge tube (Biospec, Bartlesville, OK), half-filled with glass beads and agitated vigorously on a beadbeater for 3 min. at maximum speed. DNA was then purified by adding 400 µl of phenol chloroform isoamyl alcohol (25:24:1) to an equal volume of lysed cells. The solution was mixed vigorously for 10 sec. and incubated at 25ºC for 15 sec. Subsequently, 450 µl of the top aqueous layer was placed in a new 1.5 µl microcentrifuge tube, to which 45 µl of sodium acetate (3 M, pH 5.2) was added. After vigorous mixing for 10 sec, 1 ml of ice cold 100% ethanol was added and the solution was mixed again and incubated on crushed ice for 5 min. DNA was pelleted by centrifugation for 10 min. at 15,682 x g. The supernatant was removed and placed in a new 1.5 µl microcentrifuge tube, to which 750 µl of 70% ethanol was added followed by centrifugation for 5 min. (15,682 x g). The supernatant was removed and the DNA pellet was dried under vacuum for 8 min. in a speedvac evaporator (Savant, Farmingdale, N.Y.). The pellet was dissolved in 30 µl of water and the DNA concentration was estimated using a Nanodrop spectrophotometer 1100 (Nanodrop Technologies, Willmington, DE).

DNA amplification by real time PCR

***Pepino mosaic virus*.** For PepMV new primers (Pep FP2 and Pep RP2) and a TaqMan probe (PEP PROBE) (Table 1) were designed in-house based on the nucleotide sequence of the coat protein gene of a sequenced *Pepino mosaic virus* (accession number NC_004067). The *Pepino mosaic virus* TaqMan probe was labeled at the 5' end with Texas Red (absorbance wavelength: 595 nm, emission wavelength: 615 nm) at the 3' end with black hole quencher 2 (BHQ2) (Integrated DNA Technologies Inc., Coralville IA). Real time reverse transcriptase (RT) PCR amplification was carried out using a one step RT-PCR master mix (Applied Biosystems Incorporated (ABI), Foster City, CA). Each reaction mixture contained 12.5 µl AmpliTaq Gold® DNA Polymerase mix, 0.625 µl 40 x RT enzyme mix from the ABI one step RT-PCR master mix, 7.5 pM each of the forward and reverse primers and 3 pM TaqMan probe. For RNA amplification, the following PCR program was employed: cDNA production at 45ºC for 30 min and denaturation at 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 20 sec and annealing and elongation at 60ºC for 60 sec. All real time PCR amplifications were conducted in 25 µl reaction volumes using the Cepheid Smartcycler (Sunnyvale, CA).

Specificity of the PepMV real time PCR assay. The specificity of the PepMV TaqMan assay was evaluated by conducting one-step real time RT-PCR on total RNA extracted from ten economically important tomato viruses, including Potato virus X (Table 2). Nucleic acids were extracted from RNA viruses using a Qiagen RNAeasy plant mini kit, and from DNA viruses by the Dellaporta method (16). Nucleic acids extracted from non-infected tomato leaves and from *C. michiganensis* subsp. *michiganensis,* by physical cell lysis were also screened. Real time PCR was conducted as previously described for

PepMV with 3 µl of total RNA extracted from PepMV-infected tissue. The experiment was repeated three times and the cycle threshold (C_t) values (the PCR cycle number at which fluorescence due to amplification exceeded the background fluorescence) were recorded.

Detection sensitivity of the real time PCR assay for PepMV. Detection sensitivity of the PepMV real time PCR assay was determined using ten-fold serial dilutions ($1 - 10^{-6}$) of total RNA extracted from PepMV-infected plant tissue. Dilutions of total RNA were made in RNase free water, and 3 µl of each dilution was used in real time PCR. The experiment was repeated six times and the mean C_t values and detection frequency were recorded.

Detection sensitivity of the real time PCR assay for *Clavibacter michiganensis* subsp. *michiganensis*. For detection of *C. michiganensis* subsp. *michiganensis*, the primers and probes employed were previously described by Bach et al. (Table 1) (2). To facilitate incorporation into multiplex real time PCR, the annealing temperature of the real time PCR protocol was modified to 60ºC as opposed to 66ºC (2). Additionally, the *C. michiganensis* subsp. *michiganensis* probe was labeled with FAM (6-carboxyfluorescein, absorbance: 492 nM, emission: 520 nM) at the 5' end and blackhole quencher 1 (BHQ1) at the 3' end (Integrated DNA Technologies Inc.). For simplex real time PCR of *C. michiganensis* subsp. *michiganensis*, the mastermix contained 12.5 pM of CMM-FP and 6.25 CMM-RP, 3 pM TaqMan probe, 4 mM $MgCl_2$, 200 µM dNTP, 1U Taq polymerase and a trehalose enhancer (200 µg/ml non-acetylated BSA, 200 mM Tris pH. 8.0, 5.6 mM trehalose, 1% Tween-20) was routinely used. All reactions were conducted in 25 µl volumes and the PCR run conditions included denaturation at 95ºC for 10 min, 35 cycles

of denaturation at 95ºC for 20 sec. and annealing and extension at 60ºC for 60 sec. An amplification threshold value of 30 fluorescence units was routinely used, and mean C_t values and detection frequency were recorded.

Specificity of the real time PCR assay for *C. michiganensis* subsp. *michiganensis*.

Since the PCR run conditions were modified relative to those described by Bach et al. (2), it was necessary to determine the sensitivity of the assay. To do this, genomic DNA from a range of bacterial species from the UGA Seed Pathology laboratory bacterial collection and different susbspecies of *C. michiganensis* kindly provided by Dr. N. Desaulniers (AgriCanada, Ottawa, Can.) (Table 4) were screened using the *C. michiganensis* subsp. *michiganensis* Taqman assay. DNA from each organism was produced by physical cell lysis with glass beads as described previously. Using the real time PCR conditions described above, 2 µl of the lysed bacterial solution was used in the real time PCR assay. DNA from each isolate was assayed three times by real time PCR and the detection frequency recorded.

Sensitivity of the real time PCR assay for *C. michiganensis* subsp. *michiganensis*.

The sensitivity of the modified *C. michiganensis* subsp. *michiganensis* real time PCR assay was determined using ten-fold serial dilutions of known concentrations of purified *C. michiganensis* subsp. *michiganensis* DNA (100 - 0.00001 ng/µl) and cell suspensions (1×10^8- 1×10^1 CFU/ml). Concentrations of purified *C. michiganensis* subsp. *michiganensis* DNA was quantified using the Nanodrop spectrophotometer and serial dilutions of the purified DNA were made in water. Additionally, detection sensitivity was determined based on cell concentration. To do this, *Clavibacter michiganensis* subsp. *michiganensis* cells were suspended in sterile water and adjusted to 1×10^8 CFU/ml using

a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, N.Y.). Ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* suspensions were made by adding 100 µl of each cell suspension to 900 µl of sterile water. Real time PCR reactions were considered to be positive when the fluorescence generated by amplification exceeded the background fluorescence threshold of 30 units. Each experiment was repeated four times.

Optimization of multiplex real time PCR for *C. michiganensis* subsp. *michiganensis* and PepMV. To facilitate the simultaneous detection of PepMV RNA and *C. michiganensis* subsp. *michiganensis* DNA, the simplex real time PCR assays described above were combined**.** To determine the optimum primer and probe concentrations to facilitate detection of a wide range of concentrations of the two target nucleic acids, a matrix was developed by varying the concentrations of primers and probes for *C. michiganensis* subsp. *michiganensis* and PepMV (Table 7). All reactions were conducted with, 1ng/µl of *C. michiganensis* subsp. *michiganensis* and a 10^{-3} dilution of total RNA from PepMV-infected tissue. Initially, the primer and probe concentrations for PepMV were varied while the *C. michiganensis* subsp. *michiganensis* primer and probe concentrations were kept constant. The *C. michiganensis* subsp. *michiganensis* primer and probe concentration was equal to that used in the simplex real time PCR assay. In the optimization experiment PepMV primer concentrations varied from 40 - 300 nM, while the probe concentrations varied from 50 - 300 nM. The optimal primer/probe ratio determined for amplification of PepMV RNA in multiplex real time PCR, was used to establish the optimal primer concentrations for *C. michiganensis* subsp. *michiganensis*. *Clavibacter michiganensis* subsp. *michiganensis* primer concentrations ranged from 70 - 500 nM. All reactions were conducted in 25 µl volumes. The experiments were repeated

twice and the mean C_t values were used to select the optimal ratios of primers and probe concentrations to facilitate multiplex real time PCR. For routine multiplex real time PCR, the empirically determined optimal mastermix comprised of 0.5 U Taq polymerase, trehalose enhancer, 40 µM dNTPs, 1.5 mM $MgCl_2$, 1.75 pM PepMV forward and reverse primer, 5 pM forward primer and 2.5 pM reverse primer for *C. michiganensis* subsp. *michiganensis*, 3 pM of each probe, 12.5 µl AmpliTaq Gold® DNA polymerase mix and 0.625 µl 40x RT enzyme mix from the ABI one step RT-PCR master mix. The PCR run conditions for multiplex real time PCR were the same as those previously described for PepMV and all reactions were conducted in 25 µl reaction volumes using the Cepheid Smartcycler.

Detection sensitivity of multiplex real time PCR of *C. michiganensis* subsp. *michiganensis* and PepMV. Sensitivity of the multiplex real time PCR assay was determined using ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* purified DNA (100- 0.01 ng/µl) and total RNA extracted from PepMV-infected tomato tissue (1-10^{-6} dilution). Each dilution of *C. michiganensis* subsp. *michiganensis* DNA was combined with each dilution of RNA extracted from PepMV-infected tissue and assayed by the multiplex real time reverse transcriptase PCR assay (Table 9). Detection thresholds for multiplex real time PCR were determined as the point below which both pathogens were not detected in the sample. A fluorescence threshold of 10 units was used for multiplex real time PCR reactions and samples that exceeded this fluorescence threshold were considered to be positives. The reporter dye, Texas red produces a flat curve in multiplex real time PCR compared to simplex real time PCR. Hence, a threshold fluorescence of 10 units is employed for all multiplex real time PCR assays. Cycle

threshold values were recorded for each sample and the experiment was repeated four times.

Detection sensitivity of multiplex real time PCR of PepMV and *C. michiganensis* subsp. *michiganensis* cell suspensions. The experiment described above was repeated using mixtures of ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* cells ($1 \times 10^8 - 1 \times 10^4$ CFU/ml) and ten-fold serial dilutions of total RNA extracted from PepMV-infected tomato tissue ($1 - 10^{-5}$ dilutions) (Table 10). Again, each dilution of *C. michiganensis* subsp. *michiganensis* cells was combined with each dilution of total RNA extracted from PepMV-infected tissue and assayed by multiplex real time reverse transcriptase PCR, as described previously. The experiments were repeated four times and the mean C_t values and detection frequencies were recorded.

Magnetic capture hybridization

To evaluate the applicability of MCH for detection of *C. michiganensis* subsp. *michiganensis* and PepMV, sequence capture probes were designed based on nucleotide sequence data from the regions flanking the real time PCR target sequences for both pathogens (Figure 2). For *C. michiganensis* subsp. *michiganensis* the capture probe, Cmm LCAP (Table 1), was designed to hybridize to position 35-115 of the ITS region (accession number U09380) (2). For PepMV the capture probe, Pep L-RCAP was designed to anneal at position 5988-6070 on the coat protein gene (Table 1). On synthesis, both capture probes were modified at the 5' end with biotin and a 16 atom spacer molecule, triethylene glycol. Synthesized capture probes were also subjected to reverse phase high performance liquid chromatography purification to eliminate free biotin and non-biotinylated oligonucleotides (Integrated DNA Technologies).

Streptavidin coated magnetic beads (SCBs) (M280, Dynal Invitrogen Corp. Brown Deer, WI), were used as a solid phase for hybridization capture.

Figure. 2. Magnetic capture hybridization. Biotinylated capture probe anneals to complementary sequences upstream of the real time PCR target region.

Coating of SCBs with hybridization capture probes. To attach hybridization capture probes to the SCBs, 200 µl (0.2 mg) of SCBs were washed three times with 500 µl of binding and washing (BW) buffer (10 mM Tris-HCL, 1 mM EDTA, 100 mM NaCl) to eliminate storage buffer compounds e.g. NaN_3 (25). The SCBs were resuspended in 200 µl of BW buffer and 200 ng (per 20 µl of SCBs) of biotinylated hybridization capture probe was added. For conjugation, the SCBs were incubated on an end-over-end shaker (Glas-Col, Terre Haute, IN) at a speed of 18% for 1 h at 25ºC. Conjugated SCBs were rinsed three times with 500 µl of BW buffer and the attached probe was chemically denatured by incubation in a denaturing solution (0.125 M NaOH, 0.1 M NaCl) for 15 min. at 25ºC (25). Subsequently, SCBs were washed three times with and resuspended in 200 µl BW buffer prior to use in magnetic capture hybridization. Twenty microliters of SCBs conjugated with 200 ng capture probe (Pep L-RCAP or Cmm LCAP), were used per sample and after incubation with agitation at 62ºC for 2 h in a formamide-based

hybridization buffer (50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium acetate), 20 g/L blocking reagent, 1 g/L N-lauroyl sarcosine, 0.2 g/L sodium dodecyl sulfate) (61), SCBs were rinsed three times each with 1 ml of sterile HPLC water (Fisher Scientific, Pittsburg, PA) using the KingFisher ML (Thermo Electron Corp, Finland) automated washing system. SCBs were resuspended in 20 µl of sterile water and captured nucleic acids were eluted by incubation at 100ºC for 10 min. Six microliters of the nucleic acid solution was then used directly in real time PCR.

MCH and simplex real time PCR for *C. michiganensis* subsp. *michiganensis*. To evaluate MCH real time PCR detection of *C. michiganensis* subsp. *michiganensis*, ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* cells ranging from ~ 1 x 10^8 – 1 x 10^1 CFU/ml were generated in formamide-based hybridization buffer. Crude DNA was extracted from 1 ml aliquots of cell suspensions using the bead-beater technique previously described and 400 µl of this suspension was transferred to a 2 ml screw cap tube (Sarstedt Inc., Newton, NC). Twenty microliters of CMM LCAP - conjugated SCB's were added for hybridization capture. Streptavidin coated magnetic beads without capture probes were generated by washing and resuspending SCBs with BW buffer. Twenty microliters of these unconjugated SCBs were added to 400 µl of crude DNA suspension generated in hybridization buffer as previously described. Captured nucleic acids were eluted in 20 µl of sterile water and 6 µl was used in real time PCR. For comparison, DNA from similar ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* cells made in sterile water was extracted using the bead beater technique and 2 µl of DNA solution was used as template in real time PCR. The

experiment was repeated four times and mean C_t values and detection frequency were recorded

MCH and simplex real time PCR for PepMV. Ten-fold serial dilutions of total plant RNA extract ($10^{-1} – 10^{-6}$) were used to evaluate the effects of MCH on PepMV RNA detection. Dilutions of total RNA were made in a formamide-based hybridization buffer described previously (25). Streptavidin coated beads were conjugated to Pep L-RCAP as previously described and used to selectively capture viral RNA from solution. Captured RNA was eluted in 20 µl HPLC water and 6 µl used directly in simplex real time PCR. MCH real time RT-PCR was compared to direct real time RT-PCR conducted on similar dilutions of RNA extracted from infected plant tissues using a commercial kit. The experiment was repeated four times and mean C_t values and detection frequency were recorded

Optimization of hybridization capture of target nucleic acids

To improve the capture of target nucleic acids, hybridization efficiency was determined using a digoxygenin-dUTP labeled PCR amplicon generated from PepMV RNA. Primer Pep CAPPr was designed to anneal to position 5988-6012 on the coat protein gene and primer PepMV-RP2 was designed to anneal to position 6190-6296 (Table 1). The resulting PCR product was a 308 bp fragment that spanned positions 5988 to 6296 of the coat protein gene. Synthesis of digoxygenin-labeled PepMV cDNA was accomplished using the BioRad Iscript cDNA synthesis kit (BioRad, Hercules, CA.). To generate cDNA, the following PCR program was executed using an Eppendorf Mastercycler (Eppendorf Scientific Inc., Westbury, N.Y.): incubation at 25ºC for 5 min., 42ºC for 30 min., and 85ºC for 5 min. The second step of the DNA amplification used a

PCR mastermix comprised of 200 mM dATP, dGTP, dCTP, 190 mM dTTP, 10 mM DIG-11-dUTP (Roche Applied Science, Indianapolis, IN) , 1.5 mM MgCl2, 1U Taq polymerase, 7.5 pM each Pep RP2 and Pep CAPPr. Amplification was conducted in a 25 µl reaction volume and the following PCR protocol was employed: denaturation at 94ºC for 2 min, 34 cycles of denaturation at 94ºC for 30 sec. annealing at 54ºC for 30 sec. and extension 72ºC for 1 min. This was followed by final extension step at 72ºC for 10 min. The PCR product was separated by electrophoresis on a 1% agarose gel at 100 volts for 1 h, and following staining with ethidium bromide, the amplicon was visualized with ultraviolet transillumination.

To determine optimum hybridization temperature, digoxygenin-labeled PepMV DNA was diluted 1:100 in hybridization buffer and incubated for 2 h at 50ºC, 55ºC or 62ºC in 2 ml microcentrifuge tubes with SCBs conjugated to Pep L-RCAP. Similar dilutions were also incubated with Dynabeads without attached capture probes at the temperatures being tested. The SCBs were then rinsed three times with washing buffer and the dig-labeled PCR product was detected according to the manufacturer's recommendations. The hybridization efficiency was compared by measuring the optical density using the EL800 universal microplate plate reader (Bio-tek instruments Inc., Winooski, VT). Standards for the PCR ELISA protocol were digoxygenin-labeled DNA, included in the Dig detection kit, and water. Labeled PepMV PCR product was also included in the assay as a protocol standard. The PCR ELISA standards were assayed according to the manufacturer's recommendations using streptavidin coated ELISA plates as the solid phase, instead of SCBs. The experiment was repeated three times and

optimal hybridization temperature was determined based on highest optical density and lowest non-specific binding to uncoated SCBs.

Detection sensitivity of the optimized MCH protocol. Since the optimal temperature for magnetic hybridization was determined to be 55ºC using PCR ELISA. The sensitivity of the MCH assay was determined for both pathogens at this newly determined temperature. Magnetic capture hybridization was repeated for *C. michiganensis* subsp. *michiganensis* using ten-fold serial dilutions, $1 \times 10^8 - 1 \times 10^1$ CFU/ml of cell suspensions made in the formamide-based hybridization buffer. The protocol was also repeated for PepMV using ten-fold serial dilutions of total RNA extracted from PepMV-infected tissue ($10^{-1} - 10^{-6}$) made in 400 µl of hybridization buffer. The experiments were repeated four times and the C_t values and detection frequencies were compared to those obtained for direct real time PCR conducted on similar dilutions without MCH.

Detection sensitivity of MCH and multiplex real time PCR. To determine the absolute detection threshold of MCH and multiplex real time PCR, ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* and PepMV were made in hybridization buffer ranging from 1×10^7 CFU/ml with a 10^{-2} dilution of total RNA to 1×10^4 CFU/ml with 10^{-5} dilution of total plant RNA. Two hybridization buffers were evaluated in this experiment including the previously described formamide-based buffer and a 3 M guanidine thiocyanate buffer (GuSCN) (3M GuSCN, 2 x SSC, 0.5% SDS 1% blocking reagent). The GuSCN buffer was employed to prevent degradation of RNA by enzymes released by mechanical lysis of bacterial cells (57). One milliliter aliquots of each dilution in hybridization buffer were transferred to 2 ml microcentrifuge tubes, half-filled with 0.1 mm glass beads. *Clavibacter michiganensis* subsp. *michiganensis* cells

present in the suspension were lysed mechanically using a bead beater at maximum speed for 5 min. Subsequently the tubes were centrifuged for 1 min. at 15,682 x g and 400 µl of the solution was placed in a new 2 ml screw cap centrifuge tube with thirty microliters of SCBs, (15 µl of SCBs conjugated with each capture probe, Pep L-RCAP and Cmm LCAP). For MCH, samples made in GuSCN buffer were incubated at 42ºC for 3 h, while those made in the formamide-based buffer were incubated at 55ºC for 2 h. The beads were washed and resuspended in a final volume of 20 µl HPLC water. The captured nucleic acids were eluted in 20 µl of sterile water and 8 µl used directly in multiplex real time RT-PCR, as described previously. MCH in the formamide-based buffer was repeated four times, while MCH in the GuSCN buffer was repeated eight times. Mean C_t values and frequency of detection were recorded..

Detection sensitivity of MCH multiplex real time PCR in tomato seed wash.

Tomato seed extract was prepared by crushing 400 seed and resuspending the crushed seed tissue in 10 ml PBS/ 5M GuSCN buffer. To determine the detection threshold of MCH and multiplex real time PCR in seed wash, ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* and crushed PepMV-infected leaf tissue were used. PepMV tissue was prepared by crushing 0.1 g of infected leaf tissue in 500 µl of PBS/5M GuSCN buffer. The crushed tissue was placed in a 50 ml centrifuge tube (Corning, N.Y.) and brought to 10 ml with PBS/5 M GuSCN buffer, for a 10^{-2} dilution of PepMV infected tissue. Dilutions of *C. michiganensis* subsp. *michiganensis* and macerated PepMV-infected tissue ranged from 1 x 10^6 CFU/ml with 10^{-3} dilution of macerated leaf tissue to 1 x 10^4 CFU/ml with 10^{-5} macerated leaf tissue. One milliliter aliquots of each dilution in seed wash was transferred to 2 ml microcentrifuge tubes half filled with 0.1 mm glass

beads. The samples were subjected to four rounds of freezing and thawing in liquid nitrogen to release PepMV RNA, *C. michiganensis* subsp. *michiganensis* cells were lysed mechanically using a bead beater apparatus at maximum speed for 5 minutes (43). Subsequently the tubes were centrifuged for 1 min. at 15,682 x g and 500 µl of the solution was placed in a new 2 ml microcentrifuge tube. Nucleic acids were precipitated by the addition of 0.1 volume 3 M sodium acetate and 2 volumes of 100% alcohol and centrifugation at 15,682 x g for 10 min. (15). After centrifugation the solution was removed and the nucleic acid pellets were washed with 750 µl 70% alcohol by centrifugation for 5 min. at 15,682 x g (15). The nucleic acid pellet was dried in a speedvac evaporator (8 min) and resuspended in 400 µl of GuSCN hybridization buffer. Thirty microliters of SCBs, (15 µl of SCBs conjugated with each capture probe, Pep L-RCAP and Cmm LCAP) was added to each sample. The tubes were incubated at 42ºC for 3 h. and subsequently, the SCBs were washed and resuspended in a final volume of 20 µl HPLC water. The captured nucleic acids were eluted by incubation at 100ºC for 10 min. and 8 µl used directly in multiplex real time PCR as described previously. The experiment was repeated to eight times. Mean C_t values and detection frequency were recorded.

Detection of *C. michiganensis* subsp. *michiganensis* in naturally infested seed by MCH-multiplex real time PCR. Naturally infested *C. michiganensis* subsp. *michiganensis* seed were obtained from a commercial seed company. To determine the sensitivity of the MCH-multiplex real time assay, naturally infested seed were diluted with non-infested seed. Seed samples (n = 400 seed) were mixed with non-infested seed in the following ratios: 1, 1:2, 2:10, 1:10, 2:100. Bacterial cells were extracted by

crushing seed and resuspending crushed seed in 10 ml PBS/5 M GuSCN buffer in a 50 ml centrifuge tube (Corning, N.Y.). The bacterial cells were pelleted by centrifuging for 15 min. at 5268 x g in a Beckman J2-21 centrifuge (Beckman Instruments, Fullerton, CA). The pellet was resuspended in 1ml of PBS/5 M GuSCN buffer, placed in a 2 ml microcentrifuge tube and subjected to four rounds of freezing and thawing to extract PepMV RNA. Microcentrifuge tubes were then half filled with glass beads (0.1 mm) and cells were lysed in a bead beater at maximum speed for 5 min. Nucleic acids were precipitated as previously described and resuspended in 400 µl GuSCN hybridization buffer. For MCH 30 µl SCBs conjugated with the PepMV and *C. michiganensis* subsp. *michiganensis* capture probes, Pep L-RCAP and Cmm L-CAP, respectively were added and hybridization was conducted at 42ºC for 3 h. Streptavidin coated beads were then washed three times and resuspended in 20 µl of sterile water. Captured nucleic acids were eluted and 8 µl were used in multiplex real time PCR. The experiment was repeated twice and mean C_t values as well as detection frequencies were recorded.

RESULTS

Growth of pathogens and nucleic acid extraction. *Pepino mosaic virus* was successfully propagated on tomato plants (Fig. 3A). Plants developed symptoms typical of viral infection, including chlorosis, mosaic patterns on leaves and stunting of the plant (Fig. 3A). Non-infected plants developed no symptoms (Fig 3B). Plants infected with *C. michiganensis* subsp. *michiganensis* showed symptoms typical of the bacterial infection such as firing of leaves, (Fig. 4A). Non-infected plants developed no symptoms (Fig 4B). *Clavibacter michiganensis* subsp. *michiganensis* was re-isolated from infected plans confirming the pathogenicity of CMM 99-324.

Real time PCR for PepMV. The PepMV real time RT-PCR assay amplified PepMV RNA from total RNA extracted from PepMV-infected leaf tissue. The frequency of detection from infected tissue was 100% (Table 2). Nucleic acids from the other organisms tested were not amplified (Table 2, Fig. 5A). A detection threshold of a 10^{-5} dilution of total RNA extracted from infected leaf tissue was observed (Table 3, Fig. 5B). The detection threshold was the concentration below which PepMV could not be detected. Detection frequency of PepMV was 100% for all dilutions of total plant RNA from PepMV-infected tissue, with the exception of the 10^{-5} dilution of total RNA that had a detection frequency of 83.3%.

Real time PCR for *Clavibacter michiganensis* subsp. *michiganensis*. The *C. michiganensis* subsp. *michiganensis* real time PCR assay conducted at a modified annealing temperature of 60ºC., did not amplify DNA from 88 % (22/25) of the bacteria

that were tested. However, the assay amplified DNA from C. *michiganensis*. subsp. *insidiosus* and C. *michiganensis* subsp. *nebraskensis* with a 100% frequency (Table 4). The TaqMan assay for *C. michiganensis* subsp. *michiganensis* had a detection threshold of 0.001 ng/µl of purified DNA and displayed a 100% frequency of detection at this threshold (Table 5). In terms of bacterial cells the real time PCR assay displayed a detection sensitivity of 1 x 10^5 CFU/ml which is the equivalent of 2 x 10^2 CFU/PCR reaction (Table 6). The mean C_t values observed at this detection threshold was 33.5 cycles and the detection frequency was 75% (Table 6). PCR on more concentrated suspensions of *C. michiganensis* subsp. *michiganensis,* could be detected with 100% frequency and yielded mean C_t values ranging from 17.3 to 28.4 for suspensions containing 1 x 10^8 to 1 x 10^6 CFU/ml, respectively.

Multiplex real time PCR for *C. michiganensis* subsp. *michiganensis* and PepMV. Using real time reverse transcriptase PCR, it was possible to simultaneously detect nucleic acids from PepMV and *C. michiganensis* subsp. *michiganensis.* The results of the multiplex real time PCR assay consisted of two curves to differentiate between the reporter dyes used for each pathogen (Fig. 6). Routinely, the cumulative fluorescence curve generated by the TaqMan assay for PepMV was distinguished by asterisks, while the fluorescence curve for *C. michiganensis* subsp. *michiganensis* amplification was smooth (Fig. 6).

Optimization of multiplex real time PCR. Initially concentrations of PepMV primers and probe were varied while the *C. michiganensis* subsp. *michiganensis* primers and probe were kept constant. The concentration of the probe did not affect the multiplex real time PCR assay and ultimately the probe concentration selected was the same as that used

in simplex real time PCR reactions (300 nM) (Table 7). Amplification was unreliable when multiplex real time PCR was conducted with PepMV primer concentrations exceeding 80 nM. The optimal primer concentration for PepMV was determined to be 70 nM for both the forward and reverse primers. Using the optimal primer and probe concentrations determined for PepMV the optimal concentration of *C. michiganensis* subsp. *michiganensis* primers for the multiplex reaction was 200 nM of forward primer and 100 nM of reverse primer. The empirically determined optimal concentrations of primers for the multiplex assay was 70 nM of both PepMV primers, and 200n M forward primer and 100 nM reverse primer for *C. michiganensis* subsp. *michiganensis*. Additionally, 300 nM of each TaqMan probe was determined to be optimal.

Detection sensitivity of multiplex real time PCR for *C. michiganensis* subsp. *michiganensis* and PepMV. In the multiplex assay the detection threshold for *C. michiganensis* subsp. *michiganensis* was 0.1 ng/µl of genomic DNA (Table 9), which was 100-1000-fold less sensitive than in the simplex, for which the detection threshold was 0.001 ng/µl (Table 5). The detection threshold for *C. michiganensis* subsp. *michiganensis* varied depending on the concentration of PepMV RNA and a detection threshold of 1 ng/µl of *C. michiganensis* subsp. *michiganensis* DNA was observed with a 1, 10^{-1} and 10^{-2} dilution of total RNA extracted from PepMV-infected tissue. The detection frequencies of these thresholds were 50%, 100% and 100% respectively. With a 10^{-3} dilution of plant RNA a detection threshold of 0.1 ng/µl of *C. michiganensis* subsp. *michiganensis* DNA was observed and the assay could detect both pathogens in 75% of the attempts (Table 9). With mixtures containing 10^{-4} and 10^{-5} dilutions of total RNA from PepMV-infected tissue, *C. michiganensis* subsp. *michiganensis* was only detected at

concentrations of 1 ng/µl of genomic DNA (Table 9). The frequency of detection of PepMV at the 10^{-4} and 10^{-5} dilutions in multiplex was 25%, indicating a loss in the reliability of PepMV detection in multiplex real time PCR (Table 9).

Detection sensitivity of multiplex real time PCR for PepMV and *C. michiganensis* subsp. *michiganensis* cell suspensions. Multiplex real time PCR was conducted on nucleic acids released from ten-fold serial dilutions of bacterial cell suspensions (ranging from 1 x 10^8 – 1 x 10^4 CFU/ml) and total RNA extracted from PepMV-infected tissue (ranging from 1 to 10^{-5}). The detection threshold observed for *C. michiganensis* subsp. *michiganensis* was 1 x 10^6 CFU/ml (Table 10) which represented a ten-fold loss in detection sensitivity relative to simplex assay (1 x 10^5 CFU/ml) (Table 6). The detection threshold was dependent on the concentration of PepMV RNA present in the sample (Table 10). On the other hand there was no change in the sensitivity of the PepMV assay in which a 10^{-5} dilution of total RNA from PepMV-infected tissue was determined to be the detection threshold in simplex and multiplex reactions (with *C. michiganensis* subsp. *michiganensis* cells). With undiluted total RNA extracted from PepMV-infected tissue a detection threshold of 1 x 10^8 CFU/ml was observed for *C. michiganensis* subsp. *michiganensis*. However, this improved to 1 x 10^7 CFU/ml as the total RNA extracted from PepMV-infected tissue concentration decreased to dilutions of 10^{-1} to 10^{-3}. Following the same trend, *C. michiganensis* subsp. *michiganensis* could be detected in cell suspensions containing 1 x 10^6 CFU/ml in the presence of 10^{-4} to 10^{-5} dilutions of total RNA extracted from PepMV-infected tissue (Table 10).

Magnetic capture hybridization and simplex real time PCR for *C. michiganensis* subsp. *michiganensis.* The detection threshold for direct real time PCR (without MCH)

was 1 x 10^6- 1 x 10^5 CFU/ml, with detection frequencies of 100% and 75%, respectively. Magnetic capture hybridization improved the detection sensitivity of real time PCR to 1 x 10^3 and 1 x 10^1 CFU/ml, but, the detection frequency at these thresholds was 25%. Detection of *C. michiganensis* subsp. *michiganensis* was most reliable at 1 x 10^4 CFU/ml (75%) (Table11). MCH improved the detection sensitivity of the *C. michiganensis* subsp. *michiganensis* real time PCR assay 10-100 fold. At higher concentrations of *C. michiganensis* subsp. *michiganensis,* 1 x 10^8- 1 x 10^5 CFU/ml, detection was 100% reliable. In cell suspensions with 1 x 10^5 CFU/ml, the MCH real time PCR assay had a higher frequency of detection than direct real time PCR, 100% and 75% respectively. When replicated with uncoated SCBs, the detection threshold was 1 x 10^3 CFU/ml with a 25% detection frequency (Table 11).

Magnetic capture hybridization and simplex real time PCR for PepMV. The threshold of detection for PepMV with the MCH real time PCR assay was a 10^{-3} dilution of total RNA extracted from PepMV-infected tissue with a detection frequency of 67% (Table 12). This represented a 1000-fold decrease in sensitivity compared to real time PCR without MCH. The frequency of detection of PepMV after MCH at the 10^{-3} dilution was less than that of direct real time PCR, with detection frequencies of 100% and 66.6% respectively. Detection frequencies of the 10^{-1} and 10^{-2} dilutions of total RNA extracted from PepMV-infected tissue were 100% and 83% respectively, compared to 100% and 100% in direct real time PCR.(Table 12).

Optimization of hybridization capture of target nucleic acids. A 308 bp dig-labeled product that comprised the MCH and real time PCR target sequences was produced by a two-step conventional PCR protocol (Fig. 7) and used to measure hybridization capture

efficiency. The optical density readings generated with non-specific binding of DNA to uncoated SCBs were compared to those of SCBs coated with the PepMV capture probe at all temperatures tested (Fig. 8). The highest level of non-specific binding was observed at 50ºC with a mean optical density reading of 0.365 (Table 13). In contrast, the non-specific binding observed at 55ºC and 62ºC was similar, with mean optical density readings of 0.074 at 55ºC and 0.065 at 62ºC. The highest hybridization efficiency was observed at 55ºC with a mean optical density reading of 4.99. Hybridization at 50ºC and 62ºC had the lowest average optical density readings of 4.26 and 4.35, respectively (Table 13). Both the ELISA positive control and the PepMV positive control yielded positive optical density readings indicating that the reaction worked as expected (Table 13). The water control also gave a optical density reading (0.026), which was significantly lower than the test samples, and likely caused by breakdown of the substrate.

Detection sensitivity of optimized MCH real time PCR assay. When conducted at 55ºC, the detection threshold of the MCH real tie PCR protocol for *C. michiganensis* subsp. *michiganensis* was 1 x 10^4 CFU/ml with a detection frequency of 50% (Table 14). MCH real time PCR was 100% repeatable for cell suspensions containing ≥ 1 x 10^5 CFU/ml. As compared to MCH at 62ºC, hybridization capture at 55ºC did not detect the bacterium in suspensions with 1 x 10^3 or 1 x 10^1 CFU/ml. The nonspecific binding of DNA to uncoated SCBs decreased at 55ºC with a detection threshold of 1 x 10^5 CFU/ml, which occurred in 25% of the attempts (Table 14).

Pepino mosaic virus. Magnetic capture hybridization of PepMV RNA at 55ºC displayed a detection threshold of 10^{-6} dilution of total RNA extracted from PepMV-infected tissue

with a 25% detection frequency. This represented a ten-fold increase in detection sensitivity compared to real time PCR without MCH (Table 15). Additionally, this represented a 1000-fold increase in detection sensitivity when compared to MCH conducted at 62ºC. As expected, as the concentration of total plant RNA decreased, so too did the reliability of MCH-real time PCR for PepMV (Table 15). The highest dilutions of total RNA extracted from PepMV-infected tissue, 10^{-1} and 10^{-2}, were detected with a 100% frequency, which was not different to the detection frequencies observed for direct real time PCR. For solutions with 10^{-3} and 10^{-1} dilutions of total RNA, 75% detection frequency was observed for MCH-real time PCR which decreased to 50% and 25% for suspensions with 10^{-5} and 10^{-6} dilutions of total PepMV-infected plant RNA, respectively (Table 15).

Detection sensitivity of MCH-multiplex real time PCR. Detection frequencies of the *C. michiganensis* subsp. *michiganensis* and PepMV dilutions the assay were lower in the formamide-based hybridization buffer than the GuSCN hybridization buffer (Table 16 and 17). The detection frequency for MCH multiplex real time PCR for 10^{-4} dilution of total RNA extracted from PepMV-infected tissue with 1 x 10^5 CFU/ml of *C. michiganensis* subsp. *michiganensis* was 62.5% with the GuSCN hybridization buffer and 25% with the formamide hybridization buffer. Higher frequencies of detection were observed for all serial dilutions tested in GuSCN relative to the formamide based buffer (Table 16 and 17). Hence, the GuSCN hybridization buffer was adopted for MCH multiplex real time PCR. The detection threshold for the multiplex real time PCR assay was a 10^{-4} dilution of total RNA extracted from PepMV-infected tissue and 1 x 10^5 CFU/ml of *C. michiganensis* subsp. *michiganensis* cells (Table 17). This represented a

100-fold increase in detection sensitivity as compared to multiplex real time PCR without MCH (Table 10 and 17). The detection frequency of MCH-multiplex real time PCR for suspensions containing 1 x 10^7 CFU/ml *C. michiganensis* subsp. *michiganensis* and a 10^{-2} dilution of total plant RNA (87.5%) was also lower than that of multiplex real time PCR without MCH (100 %). Suspensions containing 1 x 10^6 CFU/ml *C. michiganensis* subsp. *michiganensis* combined with a 10^{-3} dilution of total plant RNA and 1 x 10^5 CFU/ml of *C. michiganensis* subsp. *michiganensis* combined with a 10^{-4} dilution of total plant RNA displayed detection frequencies of 87.5% and 62.5%, respectively. The detection threshold of MCH-multiplex real time PCR for *C. michiganensis* subsp. *michiganensis* was 1 x 10^5 CFU/ml, which represented a 100-fold increase in detection sensitivity compared to multiplex real time PCR without MCH at the same dilutions (Table 10 and 17). However, with MCH-multiplex real time PCR, a 10-fold lost of sensitivity was observed for PepMV detection (10^{-4} dilution), relative to direct multiplex PCR (10^{-5} dilution).

Applicability of MCH-multiplex real time PCR as a seed assay. The detection threshold of the MCH-multiplex real time PCR assay in seed wash was 1 x 10^4 CFU/ml *C. michiganensis* subsp. *michiganensis* combined with a 10^{-5} dilution of macerated PepMV-infected tomato tissue (Table 18). This represented a ten-fold increase in detection sensitivity when compared to MCH real time PCR conducted in buffer. In general, MCH-multiplex real time PCR conducted in seed wash was more sensitive than multiplex real time PCR without MCH. MCH resulted in a 1000-fold increase in detection sensitivity of *C. michiganensis* subsp. *michiganensis* compared to multiplex real time PCR without MCH (Table 10 and 18). For PepMV the detection threshold of

MCH-multiplex real time PCR in seed wash, a 10^{-5} dilution of macerated tissue, was similar to that observed for PepMV without MCH (Table 10 and 18). With suspensions containing, 1 x 10^6 CFU/ml *C. michiganensis* subsp. *michiganensis* with a 10^{-3} dilution of macerated PepMV-infected leaf tissue the detection frequency was higher for MCH multiplex real time PCR (75%) than for multiplex PCR without MCH (0%). Suspensions containing 1 x 10^5 CFU/ml *C. michiganensis* subsp. *michiganensis* combined with a 10^{-4}dilution of PepMV-infected tissue macerate and, 1 x 10^4 CFU/ml *C. michiganensis* subsp. *michiganensis* combined with a 10^{-5} dilution of PepMV-infected tissue macerate had detection frequencies of 62.5% and 12.5%, respectively. In contrast these dilutions could not be detected by multiplex real time PCR without MCH (Table 10 and 18).

Detection of *C. michiganensis* subsp. *michiganensis* in naturally infested seed. MCH-real time PCR had a detection threshold of 1:10 dilution of infested seed for *C. michiganensis* subsp. *michiganensis* (Table 19). In the lowest dilution of infested seed used 2:100, MCH-real time PCR detected the bacterium. *Clavibacter michiganensis* subsp. *michiganensis* was detected with 100% detection frequency for the dilutions that tested positive except for the 2:10 dilution, which had a 50% detection frequency (Table 19). *Clavibacter michiganensis* subsp. *michiganensis* was not detected in non-infested seed (0:400 dilution) and as expected PepMV was not detected in this assay.

DISCUSSION

Initially, PCR-based techniques were not widely employed for the detection of plant pathogens because of the lack of nucleotide sequence data, the cost of equipment, the training required to conduct the assays and the risks of cross-contamination (60). However, advances in this technique, such as real time PCR have overcome many of these problems. Advantages of real time PCR include shorter completion times (1-2 h) and results without the need for post PCR analysis (e.g. gel electrophoresis and southern hybridization) (60). In the past five years, real time PCR protocols have been developed for the detection of many pathogens (35, 60).

To develop a multiplex real time assay for PepMV and *C. michiganensis* subsp. *michiganensis,* individual simplex real time PCR assays were first developed. For *C. michiganensis* subsp. *michiganensis* a previously reported TaqMan assay was employed with a slight modification to the annealing temperature (2). Unexpectedly, this assay amplified DNA from C. *michiganensis* subsp. *insidiosus* and C. *michiganensis* subsp. *nebraskensis,* organisms which are closely related to *C. michiganensis* subsp. *michiganensis*. Since the annealing temperature of the assay was lowered from 66ºC to 60ºC to accommodate multiplex real time PCR, it is possible that the stringency of the assay might have been lowered, resulting in amplification of non-target DNA. Also, *C. michiganensis* strains screened by Bach et al. were different from those used in our study and this natural variation might account for the variability in the specificity of the real time PCR assay. Nevertheless, as *C. michiganensis* subsp. *insidiosus* and *C.*

michiganensis subsp. *nebraskensis* are pathogens of alfalfa and maize, respectively, it is unlikely that they would naturally occur on tomato seed. Additionally, since the goal of our research was to prove the concept that MCH-multiplex real time PCR could be used as a seed assay, it seemed that these cross-reactions would not influence the ability to achieve our objectives.

While the detection sensitivity of the *C. michiganensis* subsp. *michiganensis* real time PCR assay was reported to be 10^2 CFU/ml (2), in our hands the assay displayed a detection threshold of $1 \times 10^6 - 1 \times 10^5$ CFU/ml or $2 \times 10^3 - 2 \times 10^2$ CFU per reaction. This difference in assay sensitivity may be due to the way in which the detection threshold was reported by Bach et al. The authors made serial dilutions of *C. michiganensis* subsp. *michiganensis* in 1 ml aliquots, which were then mechanically lysed and DNA purified using a commercially available kit. We assume that the purified DNA was resuspended in approximately 30-50 µl of buffer, hence, this likely resulted in a 100-fold concentration of template DNA. In our experiments, a DNA concentration step was not included and from each 1 ml aliquot of mechanically lysed cells only 2 µl of crude DNA extract was used for real time PCR. Therefore a detection threshold of 1×10^5 CFU/ml if concentrated 100-fold according to the method employed by Bach et al., would translate to a detection threshold of $1 \times 10^3 - 1 \times 10^2$ CFU/ml. Hence, based on these assumptions, the detection thresholds observed in our experiments were similar to those reported by Bach et al.

The TaqMan real time PCR assay developed for PepMV appeared to be specific as none of the nucleic acids extracted from other viruses yielded a fluorescent signal. In simplex, the detection threshold of the assay was a 10^{-5} dilution of total RNA extracted

from PepMV-infected tissue. For comparison, the detection threshold reported for ELISA-based detection of PepMV was a 1: 50,000 dilution of infected tissues, which is two times less sensitive than the real time PCR assay (Rapid detection of *Pepino mosaic virus,* http://www.pocketdiagnostic.com). However, it would be inappropriate to make a direct comparison between the detection thresholds reported for these two techniques, as different sources of infected plant material were used. This is because detection sensitivity is dependent on virus titer in infected plant tissues, which might vary significantly based on the type and age of the tissue and the conditions under which infection occurred. Unfortunately, the inability to purify viral particles from infected plant tissue prevented the exact determination of the PepMV real time PCR assay detection threshold. In the future it will be necessary to conduct a direct comparison of ELISA and real time PCR on serial dilutions of total RNA extracted from the same source of infected tissues. Nevertheless, based on the data collected, we conclude that the PepMV real time PCR assay was specific and sensitive, and therefore suitable for use in the development of the multiplex real time PCR assay.

It is well known that with multiplex PCR negative interactions can occur between the amplification reactions, whereby one reaction is inhibited by another (3, 24). Such an effect was observed for the multiplex real time PCR assay developed in this study**.** To avoid these inhibitory effects the optimal ratios of concentrations of primers and probes were determined empirically. Interestingly, while the TaqMan probe concentration did not influence the multiplex real time PCR reaction, the ratio of primer concentrations affected nucleic acid amplification drastically (Table 7). PepMV primer concentrations above 80 nM resulted in low detection frequencies for *C. michiganensis* subsp.

michiganensis. In turn 500 nM of *C. michiganensis* subsp. *michiganensis* primers reduced the frequency of detection of PepMV. While an exact explanation for this inhibitory phenomenon is not known, in general, generally be explained by competition for dNTPs and other resources necessary for amplification..

Despite optimizing the multiplex real time PCR parameters, we still observed a reduction in detection sensitivity for both pathogens in multiplex, relative to the detection thresholds observed in simplex assays. In simplex reactions a detection threshold of 10^5 CFU/ml or 0.01 ng/µl of purified DNA was observed for *C. michiganensis* subsp. *michiganensis*. On the other hand, in multiplex the detection threshold was 0.1 ng - 1 ng/µl of *C. michiganensis* subsp. *michiganensis* DNA or 1 x 10^8- 1 x 10^6 CFU/ml. In multiplex real time PCR, the detection threshold of *C. michiganensis* subsp. *michiganensis* was dependent on the concentration of PepMV RNA i.e., the sensitivity of the assay for *C. michiganensis* subsp. *michiganensis* increased as the PepMV concentrations decreased. In multiplex, the PepMV detection threshold was 10^{-5} dilution of total RNA and it was also affected by the concentration of *C. michiganensis* subsp. *michiganensis* used in the assay. This phenomenon by which detection sensitivity is reduced in multiplex reactions has been observed with other multiplex PCR assays (3, 24). For example, a 10 – 100-fold loss in sensitivity was reported in the multiplex PCR assay for the detection of *Tobacco mosaic virus* and *Tomato mosaic virus* (24).

To our knowledge there have been no reports of real time PCR assays designed to target organisms in different kingdoms, especially positive sense strand viruses and gram positive bacteria. The results of this research indicate that this procedure is possible and that multiplex real time PCR can be used to test seed for all classes of plant pathogens.

This technique has the potential to improve the speed and efficiency of seed health testing. However, at present, the multiplex real time PCR assay for *C. michiganensis* subsp. *michiganensis* and PepMV lack the sensitivity and reliability required for seed health tests. Hence we explored the ability of MCH to improve the performance of multiplex real time PCR.

The combination of MCH at 62ºC for 2 h with real time PCR resulted in a 10 – 100-fold increase in detection sensitivity for *C. michiganensis* subsp. *michiganensis* compared to real time PCR alone. In contrast, with similar parameters, MCH resulted in a 1000-fold decrease in assay sensitivity for PepMV. This loss in sensitivity was not expected and might have been due to the degradation of viral RNA during hybridization. Additionally, a suboptimal hybridization temperature might have resulted in reduced capture of PepMV RNA. Using a hybridization temperature of 55ºC, detection sensitivity of PepMV RNA was improved 1000-fold compared to hybridization capture at 62°C. Also, employing MCH before real time PCR resulted in a ten-fold increase in detection sensitivity compared to real time PCR without MCH. However, the MCH real time PCR assay was less reliable than real time PCR without MCH. This may indicate that captured RNA molecules were being degraded during hybridization. Unfortunately, while MCH at 55°C improved the detection sensitivity of PepMV, it resulted in a 10 – 100-fold reduction in the sensitivity of the *C. michiganensis* subsp. *michiganensis* assay.

For simultaneous capture of nucleic acids from PepMV and *C. michiganensis* subsp. *michiganensis*, MCH was initially conducted in a formamide-based hybridization buffer (25). However, since it is possible that PepMV RNA could be degraded by RNAses released after cell lysis, a guanidine thiocyanate buffer was evaluated (57).

Using this buffer and hybridization conditions of 42ºC for 3 h, MCH resulted in an 100-fold improvement in multiplex real time PCR detection sensitivity relative to direct multiplex real time PCR without MCH. Magnetic capture hybridization has been used to improve detection of nucleic acids of target organisms in many scientific disciplines (11, 53). For example, Stinear et al reported a 1000-fold increase in sensitivity for the detection of *Mycobacterium ulcerans* in environmental samples and a 100-fold increase in detection sensitivity for *Anticarsia gemmalis* in soil was reported by de Moraes et al (11, 56). In this study, similar levels of enhancement were obtained in multiplex real time PCR for *C. michiganensis* subsp. *michiganensis* and PepMV when MCH was employed. It is possible that by selectively capturing and concentrating target nucleic acids, MCH might be reducing some of the negative inhibitory effects that occur in multiplex PCR when concentrations of one target is significantly higher than the other. If true, this would represent another potential benefit of MCH that might enhance the applicability of multiplex real time PCR for seed health testing.

Detection assays for *C. michiganensis* subsp. *michiganensis* must be able to detect at least 1 infested seed in 10, 000 to 50, 0000 seed samples per lot, as this level of infestation can initiate epidemics in the field (8). Similarly, assays for PepMV should be able to detect a 10^{-4} dilution of PepMV-infected tissue macerate (International Seed Health Initiative, http://www.worldseed.org). At present, the MCH multiplex real time PCR assay developed in this study detected 1 x 10^5 CFU /ml *C. michiganensis* subsp. *michiganensis* with a 10^{-4} dilution of macerated infected tissue, which is adequate for the detection of PepMV, but not for *C. michiganensis* subsp. *michiganensis*. Despite the fact that a tolerance threshold of 1 infested seed in 10,000 has been established for *C.*

michiganensis subsp. *michiganensis*, Hadas et al reported that using semi-selective agar plate assay and BIO-PCR, *C. michiganensis* subsp. *michiganensis* could not be routinely detected in samples with this level of contamination unless populations of the bacterium were at least 5 x 10^3 CFU per seed (22). Assuming that for this assay, seed were crushed in 0.6 ml of buffer, 100µl of which was plated, a positive sample containing 5 x 10^3 CFU per seed would contain 3 x 10^3 CFU/ml. With the current detection threshold of 10^5 CFU/ml the MCH real time PCR assay is currently 100-fold less sensitive than is needed to detect the minimal allowable limit of seed contamination. However, a direct comparison of the agar plate assay and MCH-real time PCR on similar seedlots is necessary to assess their performance.

With regards to the applicability of MCH-real time PCR as a seed assay we were able to detect *C. michiganensis* subsp. *michiganensis* in naturally infested tomato seedlots. Because of the limited quantity of seed available and no knowledge of the infestation level, it was impossible to to use these seed to estimate the accuracy and precision of the MCH real time PCR assay. Nonetheless, we were able to detect *C. michiganensis* subsp. *michiganensis* in samples that consisted of 1:10 mixtures of infested seed with non-infested seed with a detection frequency of 100% (2/2). Clearly, this is much higher that the minimal tolerance threshold of 1 infested seed in 10,000, but, this experiment demonstrated that the assay can be applied to raw seed. In subsequent phases of this research, steps will be taken to further improve the efficiency, sensitivity and precision of this assay. During this phase of the research, efforts will beb made to minimize the losses in efficiency that occur at each step of the assay including bacterial cell extraction, DNA extraction, and DNA capture.

The data generated in this study suggest that there is potential for MCH-multiplex real time PCR to be employed as a seed health assay. Of course, with the current parameters, the assay would not be suitable for detecting the commercially accepted threshold of 1 infected seed in 10,000. However, since the goal of this study was to prove the concept that MCH-multiplex real time PCR was applicable for seed health testing, it is unreasonable to use the data generated to predict the final performance of this assay. However, once the assay has been finally optimized, interlaboratory trial must be conducted to comparatively evaluate this and other standard assays to ensure that it performs as well as the industry standards. To improve the sensitivity and specificity of the MCH real time PCR assay, it may be useful to consider new Taqman assays for the two pathogens. Fortunately, new primers and probes for *C. michiganensis* subsp. *michiganensis* and PepMV have recently been reported and are currently available (42, 45). These assays should be compared the assays used in our study.and if they are determined to perform better they should be used to develop the final multiplex real time PCR seed assay. Of course, if the new assays improve specificity and sensitivity of the multiplex real time PCR assay, new hybridization capture probes should be designed and the parameters of the new MCH-multiplex real time PCR assay should be optimized and evaluated based on work that has been described in this study.

MCH-multiplex real time PCR has many of the characteristics that are required for the ideal seed health assay, including speed (completed in 9 h), specificity, sensitivity and applicability for all classes of pathogens. The simultaneous detection of multiple pathogens can improve the efficiency and reduce the cost of seed health testing and might be especially important when a limited quantity of valuable seed is available. MCH-

multiplex real time PCR is automatable and easy to conduct with appropriate training; however, the reagents and equipment required are expensive and may limit the wide spread use of this technique. Additionally, at present the reliability of the assay is less than desirable and further research is needed to improve the efficiency of nucleic acid capture and the sensitivity of multiplex real time PCR. Nevertheless, results of this study suggest that MCH-multiplex real time PCR has the potential to be used as a commercial seed health assay and after further improvement it may serve as an important tool to prevent the global movement of plant pathogens on seed.

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A B

Figure 3A. Image of tomato plant (cv. Marion) displaying typical symptoms of PepMV including mosaic patterns and stunting. Plants were mechanically inoculated with leaf macerate from PepMV-infected tissue and incubated in a growth chamber for two weeks.

Figure 3B. Control plants inoculated with phosphate buffer alone.

A

B

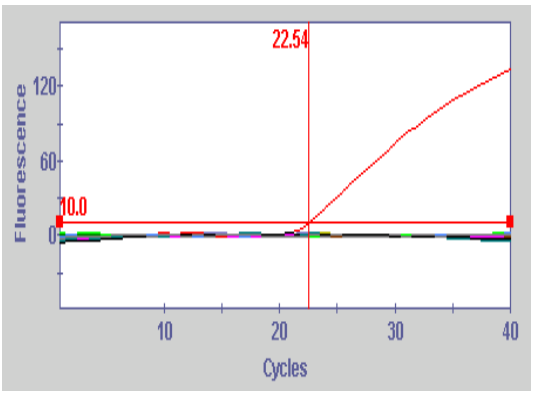
Figure 4A. Tomato (cv. Marion) plant artificially inoculated with *Clavibacter michiganensis* subsp*. michiganensis*. Bacterial canker symptoms include firing of leaves and wilting. **Figure 4B**. Image of symptomless control plant inoculated with water.

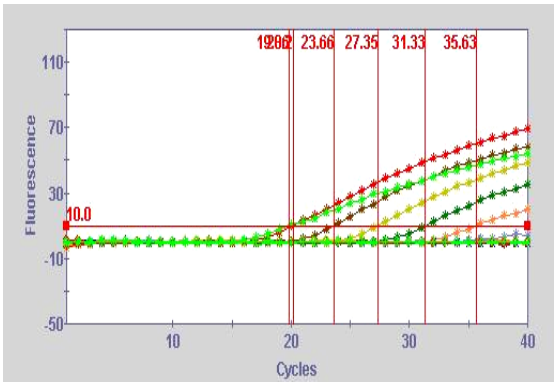
Table 1. Oligonucleotides used for real time PCR and magnetic capture hybridization of nucleic acids from *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) and *Pepino mosaic virus* (PepMV).

Primer	Sequence (5' → 3')	Position in g sequence	Target gene	Purpose	Product size	References
PepMV-FP2	TGGAGCCTGCTGATGGTCTTATCA	6153-617(	Coat protein	PCR	144	This paper
PepMV-RP2	TGTGTCCACGTGTGACTTCTCCAA	6271-629(	Coat protein	PCR		This paper
PEP PROBE	TexRd-AAGAACTAGCTGCTCACTCCGTAGCT-BHQ-2	6191-621(	Coat protein	PCR		This paper
Cmm-FP	TGTCGAGGGCATGTTGCACG	218-237	16S-23S intergenic spacer region	PCR	222	Bach et al
Cmm-RP	GGAGACAGAATTGACCAATGAT	418-439	16S-23S intergenic spacer region	PCR		Bach et al
Cmm PROBE	6-FAM-TTCCGTCCTGTTGTGGATC-BHQ-1	300-321	16S-23S intergenic spacer region	PCR		Bach et al
Pep CAPPr	CAATATAACCCCCAGGCAATTTG	5988-601:	Coat protein	PCR	308	This paper
Cmm LCAP	*Bio-TEG-ATCACCTCCTTTCTAAGGAGCATGTGCACCTCTCCTCTGTATACAGGGAGATCAAGGGTGCC AAGTCACGCGTCATCAGGCGTC	35-115	16S-23S intergenic spacer region	Magnetic capture		This paper
Pep L-RCAP	*Bio-TEG-GCTGGTGGAATGTTGCTGTCGAGAAGTA TGTTCCAAACAACTTTGGCAAAGTACAT	5988-607(	Coat protein	Magnetic capture		This paper

GCAAAA
TTGCCTGGGGGTTATATTGATTC

FAM = 6-carboxy-flourescein ; Bio = biotin; TexRd = Texas red; TEG = triethylene glycol,16 atom spacer arm; BHQ = black hole quencher





A B

Figure 5. Graph indicating the real time PCR results for PepMV infected tissue. Numbers at the top of the chart indicate the partial cycle threshold values. **5A**. Graph indicating the specificity of the real time PCR assay for PepMV. RNA from seven viruses, *C. michiganensis* subsp. *michiganensis* and non-infected tomato leaf tissues were tested but only PepMV yielded a positive result. **5B**. Real time PCR results for a ten-fold serial dilution ($1 - 10^{-6}$) of total RNA extracted from PepMV-infected plants

Table 2. Specificity of the real time PCR TaqMan assay developed for Pepino mosaic virus. The experiment was repeated three times.

Organism	Host	Source	Mean C_t values
Tobacco etch virus	Tomato	M. Deom, UGA	0
Pepper mottle virus	Tomato	M. Deom, UGA	0
Tobacco mosaic virus	Tomato	M. Deom,UGA	0
Cucumber mosaic virus	Tomato	M. Deom, UGA	0
Tomato spotted wilt virus	Tomato	M. Deom, UGA	0
Tomato yellow leaf curl virus	Tomato	M. Deom, UGA	0
Potato virus X	Tomato		0
C. michiganensis subsp. *michiganensis*	Tomato	R. Gitaitis, UGA	0
Non-infected tomato leaf tissue			0
Pepino mosaic virus	Tomato	Agdia	21.58

Table 3. Threshold of detection for Pepino mosaic virus (PepMV) using real time PCR. Results indicate the mean cycle threshold values and the frequency of positive detection for total RNA extracted from PepMV-infected tomato leaf tissue. The experiment was repeated six times.

Dilutions of total RNA extracted from PepMV infected tissue	Mean C_t values	Detection frequency (%)
1x	16.34	100
10^{-1}	18.40	100
10^{-2}	21.75	100
10^{-3}	25.33	100
10^{-4}	29.80	100
10^{-5}	34.42	83.3
10^{-6}	0	0
Water	0	0

Table 4. Specificity of TaqMan real time PCR assay for *Clavibacter michiganensis* subsp. *michiganensis*. The experiment was repeated three times.

Bacteria	Strain	Host	Source	Detection frequency (%)
Clavibacter michiganensis subsp. *michiganensis*	99-324	Tomato	R. Gitaitis, UGA	100
C. michiganensis subsp. *tessellarius*	2224	wheat	N. Disauliniers, Ottawa, Canada	0
C. michiganensis subsp. *sepedonicus*	R3	potato	N. Disauliniers, Ottawa, Canada	0
C. michiganensis subsp. *insidiosus*	2225	alfalfa	N. Disauliniers, Ottawa, Canada	100
C. michiganensis subsp. *nebraskensis*	2223	maize	N. Disauliniers, Ottawa, Canada	100
Agrobacterium radiobacter	79-1	-	Seed Path. Lab. UGA	0
Erwinia carotovora pv. *carotovora*	00-1	potato	Seed Path. Lab. UGA	0
E. carotovora subsp. *atroseptica*	*96*	potato	Seed Path. Lab. UGA	0
Escherichia coli	85-2	humans	Seed Path. Lab. UGA	0
Agrobacterium tumefaciens	C58	unknown	Seed Path. Lab. UGA	0
Acidovorax avenae subsp. *citrulli*		watermelon	Seed Path. Lab. UGA	0
Pantoea ananatis	99-1	onion	Seed Path. Lab. UGA	0
Pseudomonas viridiflava		onion	Seed Path. Lab. UGA	0
Pseudomonas syringae pv. *Syringae*		snap bean	Seed Path. Lab. UGA	0
Pseudomonas syringae pv. *tomato*		tomato	Seed Path. Lab. UGA	0
Pseudomonas	84-1	human	Seed Path.	0

aeruginosa			Lab. UGA	
Pantoea agglomerans	99-3	maize	Seed Path. Lab. UGA	0
Comamonas testosteronii		unknown	Seed Path. Lab. UGA	0
Burkholderia cepacia	BC-11	onion	Seed Path. Lab. UGA	0
Ralstonia solanacearum	GMI 1000	tomato	Seed Path. Lab. UGA	0
Xanthomonas campestris pv. *glycinea*		bean	Seed Path. Lab. UGA	0
X.campestris pv. *raphini*		radish	Seed Path. Lab. UGA	0
X. campestris pv. *pellargonii*		geranium	Seed Path. Lab. UGA	0
X. campestris pv. *campestris*	00-1	cabbage	Seed Path. Lab. UGA	0
X. campestris pv. *Vesicatoria*		tomato	Seed Path. Lab., UGA	0

Table 5. Sensitivity of the real time PCR assay for *Clavibacter michiganensis* subspecies *michiganensis* (Cmm) using ten-fold serial dilutions of purified genomic DNA. The experiment was repeated four times.

Concentration of Cmm (ng/µl)	Mean C_t value	Frequency of detection (%)
100	14.72	100
10	18.83	100
1	22.60	100
0.1	26.51	100
0.01	30.56	100
0.001	32.49	100
0.0001	0	0
0.00001	0	0
0	0	0

Table 6. Sensitivity of real time PCR assay for detection of *Clavibacter michiganensis* subspecies *michiganensis* (Cmm) using ten-fold serial dilutions of cells suspended in water. The experiment was repeated four times.

Concentration of Cmm (CFU/ml)	Mean C_t values	Frequency of detection (%)
10^8	17.33	100
10^7	23.88	100
10^6	29.42	100
10^5	33.55	75
10^4	0	0
10^3	0	0
10^2	0	0
10^1	0	0
0	0	0

Table 7. Results of the optimization of the multiplex real time PCR assay for the simultaneous detection of *C. michiganensis* subsp. *michiganensis* (Cmm) and *Pepino mosaic virus* (PepMV). The concentration of *C. michiganensis* subsp. *michiganensis* primer and probe were 500 nM reverse primer, 250 nM forward primer and 300 nM of probe. The experiment was repeated twice.

PepMV probe concentration (nM)	Mean C_t value (Detection frequency %)													
	PepMV primer concentration (nM)													
	300		200		100		80		70		60		40	
	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV
300	0	18.6	0	19.1	23.3	19.7	21.9	22.8	22.4	22.5	22.1	22.4	22.7	22.3
	(0)	(100)	(0)	(100)	(100)	(100)	(100)	(50)	(100)	(100)	(100)	(100)	(100)	(100)
200	22	18.9	22.4	19.5	23.8	19.8	22.9	22.5	22.5	22.3	23	22.3	23.0	22.6
	(50)	(100)	(50)	(100)	(50)	(100)	(100)	(1/2)	(100)	(100)	(100)	(100)	(100)	(100)
100	0	19.3	0	19.1	0	20.7	23.4	22.3	23.0	22.1	23.6	22.3	23.5	21.6
	(0)	(100)	(0)	(100)	(0)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
50	25	20.6	0	20.1	21.4	22	23.8	22.9	24.3	22.4	25.2	22.5	25.1	21.9
	(50)	(100)	(0)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	((100)	(100)	(100)	(100)

Table 8. Results of the optimization of the multiplex real time PCR assay for the simultaneous detection of *C. michiganensis* subsp. *michiganensis* (Cmm) and *Pepino mosaic virus* (PepMV). The concentrations of PepMV primer and probe used in this experiment were 70 nM each of the forward and reverse primers and 300 nM for the TaqMan probe. The experiment was repeated twice.

Cmm probe conc.(nM)	Mean C_t value (Detection frequency (%))									
	Cmm primer concentration (nM)									
	500		300		200		100		70	
	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV	Cmm	PepMV
300	21.6 (100)	20 (50)	21.9 (100)	20.2 (100)	21.8 (100)	20.6 (100)	21.4 (100)	20.9 (100)	21.9 (100)	21.4 (100)

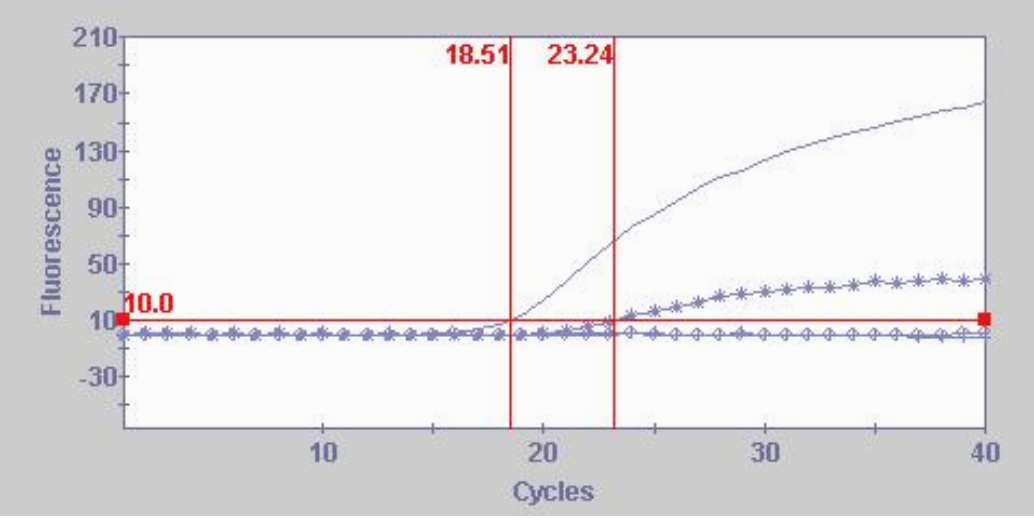


Figure. 6. Graph of multiplex real time PCR for the simultaneous detection of PepMV and *C. michiganensis* subsp. *michiganensis*. For this study ten-fold serial dilutions of total RNA extracted from PepMV-infected tomato tissue mixed were with ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* cell suspensions. The smooth curve represents the *C. michiganensis* subsp. *michiganensis* real time PCR reaction and curve with asterisks represents PepMV real time PCR reaction.

Table 9. Results of multiplex real time PCR conducted on ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* (Cmm) genomic DNA and ten-fold serial dilutions of total RNA extracted from *Pepino mosaic virus* (PepMV)-infected tomato leaves. The experiment was repeated four times.

Concentration of Cmm DNA (ng/µl)	Mean C_t values (Frequency of detection (%))											
	PepMV concentration (dilution of infected leaf tissue)											
	1		10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	Cmm	Pep	Cmm	Pep	Cmm	Pep	Cmm	Pep	Cmm	Pep	Cmm	Pep
	14.6	17.4	14.4	23.7	14.4	26.2	14.5	30.9	14.1	0	13.7	0
100	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(25)	(100)	(0)	(100)	(0)
	18.0	17.0	18.5	21.4	18.5	23.2	19.3	27.2	18.6	0	13.2	0
10	(75)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(0)	(100)	(0)
	31.0	16.7	24.7	21.2	22.6	24.1	22.3	26.6	22.6	31.7	22.2	30.5
1	(50)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(25)	(100)	(25)
0.1	0	17.0	0	26.3	0	23.3	26.7	26.7	27.5	0	28.8	0
	(0)	(100)	(0)	(100)	(0)	(100)	(100)	(75)	(100)	(0)	(75)	(0)
0.01	0	16.8	0	21.6	0	23.5	0	26.2	0	31.0	0	32.2
	(0)	(100)	(0)	(100)	(0)	(100)	(0)	(100)	(0)	(25)	(0)	(50)
	0	0	0	0	0	0	0	0	0	0	0	0
0	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)

Table 10. Results of multiplex real time PCR conducted on ten-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* (Cmm) cell suspensions and ten-fold serial dilutions of total RNA extracted from *Pepino mosaic virus* (PepMV)-infected plants. The experiment was repeated four times.

Cmm concentration (CFU/ml)	Mean C_t values (Frequency of detection (%))											
	PepMV concentration (dilution of infected leaf tissue)											
	1		10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	Cmm	Pep	Cmm	Pep	Cmm	Pep	Cmm	Pep	Cmm	Pep	Cmm	Pep
10^8	28.3 (25)	16.2 (100)	19.4 (100)	20.4 (100)	19.2 (100)	23.7 (100)	19.5 (100)	27.8 (100)	20.7 (100)	25.7 (25)	21.2 (100)	25.9 (25)
10^7	0 (0)	16.0 (100)	29.9 (50)	20.6 (100)	24.4 (100)	23.7 (100)	24.1 (100)	26.5 (100)	25.0 (100)	26.1 (25)	25.1 (100)	25.6 (25)
10^6	0 (0)	16.1 (100)	0 (0)	20.3 (100)	0 (0)	23.4 (100)	0 (0)	26.4 (100)	34.7 (25)	28.3 (50)	30.5 (25)	28.1 (50)
10^5	0 (0)	15.6 (100)	0 (0)	20.3 (100)	0 (0)	23.4 (100)	0 (0)	2.6 (100)	0 (0)	28.8 (50)	0 (0)	28.1 (50)
10^4	0 (0)	15.7 (100)	0 (0)	20.2 (100)	0 (0)	23.4 (100)	0 (0)	26.4 (100)	0 (0)	0 (0)	0 (0)	28.7 (50)
0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 11. The threshold of detection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) using magnetic capture hybridization and real time PCR. Template DNA was extracted from ten-fold serial dilutions of cell suspensions by mechanical cell disruption using glass beads and a mini-bead beater apparatus. Hybridization capture of template nucleic acids was conducted at 62ºC with magnetic beads with and without the *C. michiganensis* subsp. *michiganensis* biotinylated capture probe, Cmm L-CAP, in a formamide-based hybridization buffer. The experiment was repeated four times.

	Mean C_t values (Frequency of detection (%))	
Concentration of Cmm (CFU/ml)	Dynabeads conjugated with Cmm LCAP	Dynabeads with no hybridization capture probe
10^8	25.50 (100)	27.06 (100)
10^7	28.89 (100)	29.15 (100)
10^6	30.70 (100)	31.35 (100)
10^5	32.71 (100)	33.28 (75)
10^4	33.66 (75)	32.66 (25)
10^3	33.82 (25)	34.78 (25)
10^2	0 (0)	0 (0)
10^1	34.89 (25)	0 (0)
0	0 (0)	0 (0)

Table 12. Detection sensitivity of magnetic capture hybridization and real time PCR for *Pepino mosaic virus* (PepMV). MCH real time PCR was conducted on ten-fold serial dilutions of total RNA extracted from PepMV-infected tomato leaf tissue. Total RNA was extracted using a commercially available kit (Qiagen) and MCH was performed at 62ºC for 2 h in a formamide-based hybridization buffer. The experiment was repeated four times.

Serial dilutions of total RNA extract	Mean C_t values	Frequency of detection (%)
10^{-1}	30.51	100
10^{-2}	34.39	83.3
10^{-3}	35.29	66.6
10^{-4}	0	0
10^{-5}	0	0
10^{-6}	0	0
0	0	0
Negative control* (10^{-2})	0	0

* For the negative control MCH and real time PCR was conducted on a 10^{-2} dilution of total RNA extracted form PepMV infect plant tissue using uncoated Dynabeads (beads with no capture probe attached).

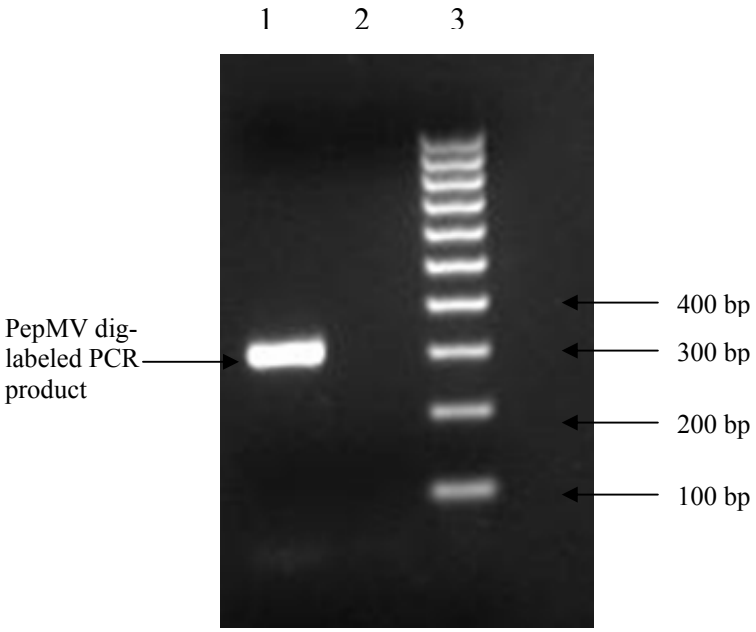


Figure 7. Digoxygenin labeled product generated by PCR on *Pepino mosaic virus* (PepMV) for PCR–ELISA assay. The contents of the lanes are as follows: lane 1 = 308 bp dig-labeled PepMV fragment; lane 2 = empty; lane 3 = 100 bp DNA marker (Sigma Aldrich Inc. Louis, MO).

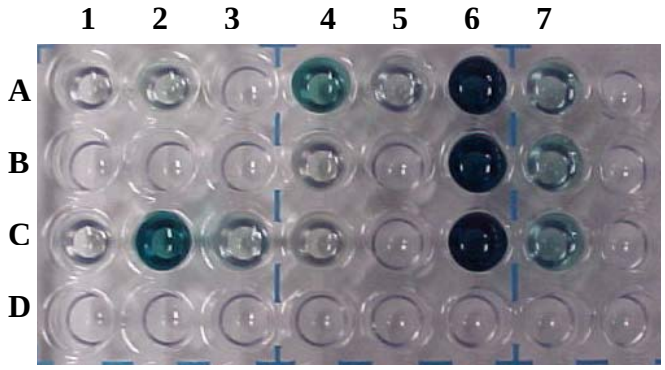


Figure 8. Determination of hybridization efficiency using PCR-ELISA. Wells containing solutions with green color indicate positive reactions. The following indicate the contents of each well: A1 = blank; A2 = ELISA control; C1 = water control for PepMV hybridization, C2 = PepMV control hybridization, A4 = PepMV hybridization at 50ºC with uncoated beads, A5 = 1:10 dilution of PepMV hybridization at 50ºC with uncoated beads, A6 = PepMV hybridization at 50ºC with conjugated beads, A7 = 1:10 dilution of PepMV hybridization at 50ºC with conjugated beads, B4 = PepMV hybridization at 55ºC with uncoated beads, B6 = PepMV hybridization at 55ºC with conjugated beads, B7 = 1:10 dilution of PepMV hybridization at 55ºC

Table 13. PCR ELISA results for hybridization capture of *Pepino mosaic virus* (PepMV) DNA, conducted at different temperatures. Table indicates a comparison of the optical density data for PCR ELISA conducted with streptavidin coated Dynabeads (M280), conjugated with and without Pep L-RCAP, the PepMV hybridization capture probe. The experiment was repeated four times.

Hybridization temperature (^{o}C)	Average optical density plate readings (optical density)	
	Dynabeads conjugated with PEP L-RCAP	Dynabeads with no hybridization capture probe
50	4.35	0.365
55	4.99	0.074
62	4.26	0.065
(55) - PepMV positive control	1.77	0.026 (water)
(37) - ELISA positive control	0.092	0.000 (blank)

Numbers in parentheses indicate hybridization temperature of PCR ELISA control reactions

Table 14. Sensitivity of magnetic capture hybridization and real time PCR for the detection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) DNA. Template DNA was generated by physically disrupting cell suspensions using glass beads and a Bead beater (Biospec, Bartlesville, OK) apparatus. To capture template DNA, hybridization was performed for 2 h at 55ºC with magnetic beads with or without the *C. michiganensis* subsp. *michiganensis* biotinylated capture probe, Cmm L-CAP, in a formamide-based hybridization buffer. The experiment was repeated four times.

	Mean C_t values (Frequency of detection (%))	
Concentration of Cmm (CFU/ml)	Dynabeads conjugated with Cmm LCAP	Dynabeads with no hybridization capture probe
10^8	24.54 (100)	27.24 (100)
10^7	29.13 (100)	31.23 (100)
10^6	31.59 (100)	33.65 (100)
10^5	33.85 (100)	34.16 (25)
10^4	34.13 (50)	0 (0)
10^3	0 (0)	0 (0)
10^2	0 (0)	0 (0)
10^1	0 (0)	0 (0)
0	0 (0)	0 (0)

Table 15. The threshold of detection of magnetic capture hybridization (MCH) and real time PCR for *Pepino mosaic virus* (PepMV) RNA. MCH and real time PCR was conducted on ten-fold serial dilutions of total RNA extracted from PepMV-infected tomato leaf tissue. MCH was conducted at 55ºC for 2 h in a formamide-based hybridization buffer. The experiment was repeated four times.

Serial dilutions of total RNA extracted from PepMV-infected leaf tissue	Mean C_t values	Frequency of detection (%)
10^{-1}	30.86	100
10^{-2}	33.44	100
10^{-3}	33.22	75
10^{-4}	34.18	75
10^{-5}	34.99	50
10^{-6}	39.82	25
0	0	0
Negative control* (10^{-2})	0	0

Table 16. Magnetic capture hybridization and multiplex real time PCR detection of ten-fold serial dilutions of total RNA extracted from *Pepino mosaic virus* (PepMV)-infected leaf tissue with *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) cell suspensions. Hybridization capture was performed at 55ºC for 2 h in a formamide-based hybridization buffer.

	Mean C_t values (Frequency of detection (%)	
Concentration of Cmm + PepMV	Cmm	PepMV
$10^7 + 10^{-2}$	27.23 (75)	31.28 (50)
$10^6 + 10^{-3}$	27.45 (75)	31.87 (50)
$10^5 + 10^{-4}$	28.15 (75)	34.67 (25)
$10^4 + 10^{-5}$	31.13 (33)	32.79 (66)
Hybridization buffer	0	0
0	0	0

Table 17. Detection sensitivity of magnetic capture hybridization and multiplex real time PCR for the simultaneous detection *Clavibacter michiganensis* ssp. *michiganensis* (Cmm) and *Pepino mosaic virus* (PepMV). MCH was conducted on ten-fold serial dilutions of total RNA extracted from PepMV-infected tissue and *C. michiganensis* ssp. *michiganensis* cells. Total nucleic acids were released from cells by mechanical cell lysis using glass beads and a bead beater apparatus. Viral RNA was released from viral particles by repeated cycles of freezing and thawing. Hybridization capture was conducted at 42ºC for 3 h in a guanidine thiocyanate buffer. The experiment was repeated eight times.

Concentration of Cmm + PepMV	Mean C_t values (Frequency of detection (%))	
	Cmm	PepMV
$10^7 + 10^{-2}$	25.52 (100)	24.02 (87.5)
$10^6 + 10^{-3}$	27.74 (87.5))	28.60 (100)
$10^5 + 10^{-4}$	29.29 (62.5)	30.87 (75)
$10^4 + 10^{-5}$	0	32.15 (50)
Hybridization buffer	0	0
Water	0	0

Table 18. Detection sensitivity of magnetic capture hybridization (MCH) and multiplex real time PCR for the simultaneous detection *Clavibacter michiganensis* ssp. *michiganensi*s (Cmm) and *Pepino mosaic virus* (PepMV). MCH was conducted on ten-fold serial dilutions of macerated PepMV-infected tissue with *Clavibacter michiganensis* ssp. *michiganensi*s cells. Total nucleic acids were released from cells by physical lysis using glass beads and a bead beater apparatus. RNA was released from viral particles by repeated rounds of freezing and thawing. MCH was conducted at 42ºC for 3 h in a guanidine thiocyanate buffer. The experiment was repeated eight times.

Concentration of Cmm + PepMV	Mean C_t values (Frequency of detection (%))	
	Cmm	PepMV
$10^6 + 10^{-3}$	25.58 (100)	26.08 (75)
$10^5 + 10^{-4}$	28.48 (100)	26.56 (62.5)
$10^4 + 10^{-5}$	29.56 (12.5)	29.96 (62.5)
Hybridization buffer	0	0
0	0	0

Table 19. Detection sensitivity of magnetic capture hybridization and multiplex real time PCR for the detection of *Clavibacter michiganensis* ssp. *michiganensis* (Cmm) in dilutions of naturally infested tomato seed. Hybridization capture was conducted at 42ºC for 3 h in a guanidine thiocyanate buffer. The experiment was repeated twice.

	Mean C_t values (Frequency of detection (%)	
Dilutions of infested seed	Cmm	PepMV
1	25.2 (100)	0 (0)
1:2	24.7 (100)	0 (0)
2:10	30.8 (50)	0 (0)
1:10	28.5 (100)	0 (0)
2:100	0 (0)	0 (0)
0:400	0 (0)	0 (0)
Water	0 (0)	0 (0)