SEMINAR REPORT

Real-time PCR in plant disease diagnosis

By

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DECLARATION

I, Athira Krishnan L R (2018-11-004) hereby declare that the seminar entitled **"Real-time PCR in plant disease diagnosis"** has been prepared by me, after going through various references cited at the end and has not copied from any of my fellow students.

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CERTIFICATE

This is to certify that the seminar report entitled **"Real-time PCR in plant disease diagnosis"** has been solely prepared by **Athira Krishnan L R (2018-11-004)**, under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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1. Introduction

Plant diseases cause major production loss in agricultural sector worldwide. Monitoring of health and detection of diseases in plants is critical for sustainable agriculture (Sankaran *et al*., 2010). Thus, rapid and accurate methods for detection and diagnosis of plant pathogens are necessary to take appropriate plant disease management measures. Conventional methods to identify pathogenic organism have often relied on identification of disease symptoms, isolation and culturing of environmental organisms, and laboratory identification by morphology and biochemical tests. However, these methods are often difficult and time consuming and require extensive knowledge of taxonomy and experiences in recognizing detailed features (Lopez *et al*., 2003; Michailides *et al*., 2005).

Recent advances in biotechnology and molecular biology have played a significant role in the development of rapid, specific and sensitive diagnostic tests (Makkouk and Kumari, 2006). This molecular diagnostics tests provide significant advantages and are often complimentary to traditional techniques of microscopy and culture based pathogen identification. Advantages of molecular diagnostics include ability to detect an organism without prior culturing, faster turn-around time, potential for high-throughput and ability to identify pathogen species or strains regard with detection of fungicide resistance alleles (Chilvers, 2012).

With the advances in molecular biology and biosystematics, the techniques available have evolved significantly in the last decade, and besides conventional PCR other technologically advanced methodologies such as the second generation PCR known as the real-time PCR and microarrays which allows unlimited multiplexing capability, have the potential to bring pathogen detection to a new and improved level of efficiency and reliability (Mumford *et al*., 2006). Molecular techniques based on different types of PCR amplification especially on real-time PCR are leading to high throughput, faster and more accurate detection methods for the most severe plant pathogens, with important benefits for agriculture.

With Real-time PCR, it is possible not only to identify and detect the presence or absence of the target pathogen, but also to quantify the amount of pathogen present in the sample. A one-step real-time quantitative Reverse Transcription PCR (real-time qRT-PCR) using the TaqMan probe was reported to be sensitive and reliable technique for the detection

and quantification of *Sugarcane streak mosaic virus* (SCSMV) in sugarcane (Fu *et al.*, 2015)**.** Determination of viability of a pathogen and simultaneous detection of multiple pathogens are the other major areas of application of real-time PCR in plant disease management. Multiplex real-time TaqMan PCR assay using *hrpB7* gene primers and probes has simultaneously detected and differentiated the four *Xanthomonas* spp. associated with bacterial spot of tomato (Strayer *et al*., 2016).

Real-time PCR technology is used as an important tool in agriculture for early and sensitive detection of plant diseases, thereby allowing better management of plant diseases.

2. Real-time PCR

2.1. Principle of Real-Time PCR

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (real-time qPCR) is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It is a sophisticated development of conventional PCR that utilized the advancements of technology to offer the unique possibilities to monitor the amplification process and follow accumulation of the product (Paplomatas, 2006). Real-Time PCR follows the general principle of polymerase chain reaction except that the progress of the reaction is monitored by a camera or detector in real-time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. There are a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence, as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen. There is also no need for gel electrophoresis.

Real-time PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product (Makkouk and Kumari, 2006). A probe (Example TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labelled at the 5' end with a reporter fluorochrome and a quencher fluorochrome added at the 3' end. The probe is designed to have a higher Tm than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as Taq polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of Taq degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle (Makkouk and Kumari, 2006). The final aim, which is the quantification of the unknown DNA, comes from the fact that the initial amount of DNA in the sample is related to the number of cycles needed for the fluorescence to reach a specific cycle threshold (the Ct value), defined as that cycle number at which a statistically significant increase in fluorescence is detected. After generating a calibration curve by plotting Ct against known amounts of template DNA, target DNA can be quantified (Paplomatas, 2006).

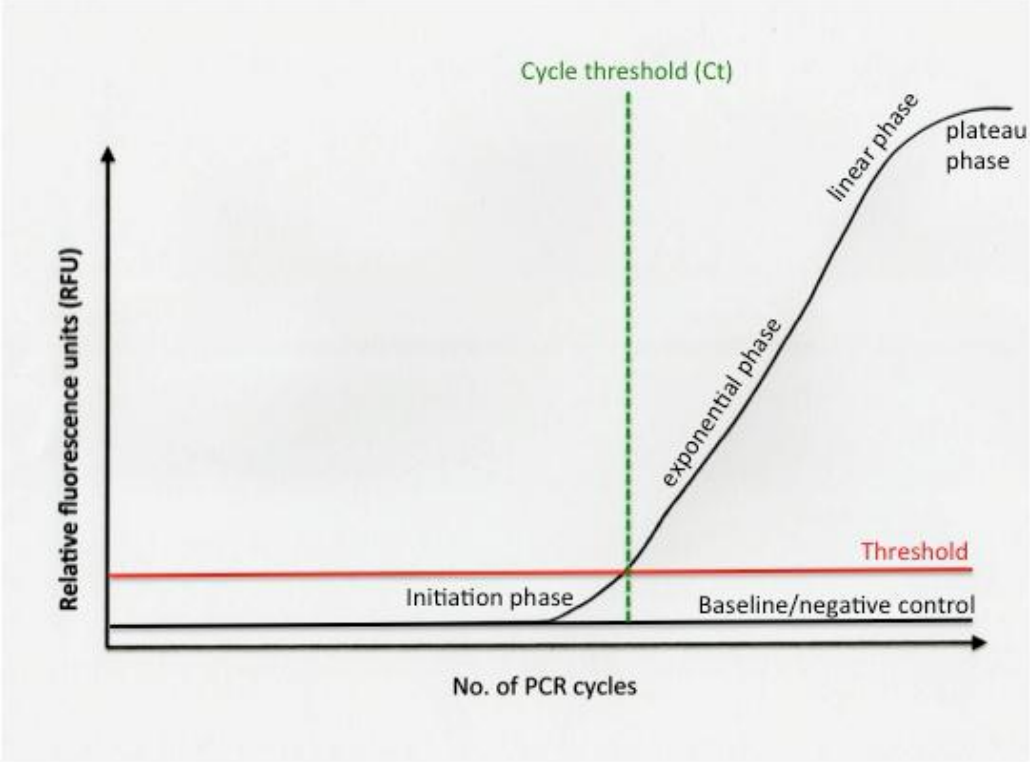

Figure 1: Amplification plot

The output from a real-time PCR reaction is in the form of a graph showing the number of PCR cycles against the increasing fluorescence. The horizontal line on the graph represents a "threshold" set by the user. The point at which the amplification plot crosses this threshold is known as the Ct (cross threshold) value. Logic dictates that the lower the Ct values for a sample the greater the starting amount of DNA in the sample. Thus if two amplification plots are compared it is simple to deduce which sample contained the greatest amount of the DNA of interest by the Ct value.

2.2. Real-time PCR workflow

The real-time PCR workflow is quite straightforward, and the PCR reaction setup is similar to end-point PCR.

Figure 2: Real-time PCR workflow

During sample preparation**,** the sample (i.e., your template) is prepared by isolation of high-quality DNA or RNA. For gene expression analyses, RNA is subjected to reverse-transcription, providing cDNA as the template for the real-time PCR reaction.

The set-up of a real-time PCR reaction is much like an end-point PCR reaction - you prepare (or purchase a ready-made) PCR master mix containing all of the standard PCR reaction components e.g., dNTPS, primers, buffer, and polymerase. However, there is one major difference between an end-point and real-time PCR reaction mix. This is the addition of a fluorescent reporter molecule to real-time PCR reactions.

Real-time thermal cyclers are equipped with fluorescence detectors that can monitor the fluorescence signal emitted during amplification. The amount of fluorescence measured is proportional to the amount of PCR amplicon generated, and the change in the fluorescence signal over time is used to calculate the amount of amplicon produced in each cycle. In this way, data acquisition happens in real-time.

2.3. Real-time PCR chemistries

There are a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen (Schena *et al*., 2004).

Generally, Real-time PCR chemistry can be grouped into intercalating dye based method based on the use of fluorescent doubled-stranded DNA binding dyes, such as SYBR Green, and hydrolysis probe based method which is based on fluorescent labeled probes such as TaqMan, Molecular Beacons or Scorpions (Schena *et al*., 2004; Capote *et al*., 2012).

2.3.1. DNA-Binding Dyes

DNA intercalating dyes, of which SYBR Green is the best known, bind non-specifically to double-stranded DNA (dsDNA). SYBR Green binds to the minor groove of the dsDNA. Dye molecules that are not bound to dsDNA emit very low background fluorescence. During

amplification, the dye intercalates the newly synthesized dsDNA, and the resulting DNA-dye complex emits fluorescence that is detected and recorded by the real-time PCR instrument.

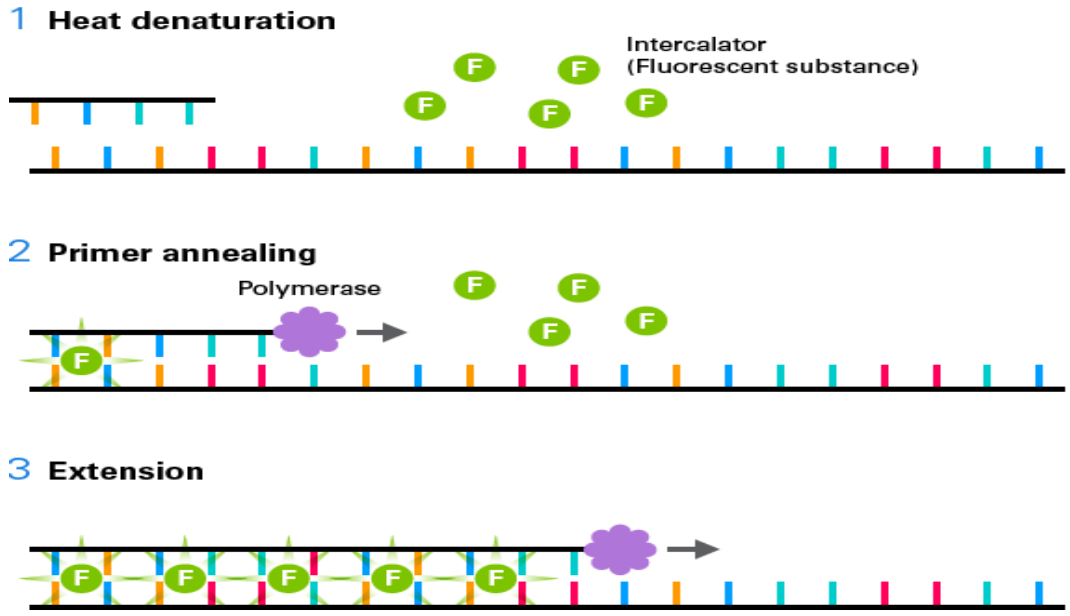

Figure 3: SYBR Green mechanism

The main disadvantage of DNA-binding dyes is that they don't exhibit sequence specificity. This means that they can bind to primer dimers and off-target amplicons. Primer design is therefore crucial when using these dyes, and while it is possible to monitor off-target amplification and primer dimer formation with post-run quality control checks, DNA-specific probes may be the best choice when there are concerns about specificity

Melt-curve analysis

Post-amplification melting-curve analysis is a simple, straightforward way to check real-time PCR reactions for primer-dimer artefacts and to ensure reaction specificity. Because the melting temperature of nucleic acids is affected by length, GC content, and the presence of base mismatches, among other factors, different PCR products can often be distinguished by their melting characteristics. The characterization of reaction products (e.g., primer dimers vs. amplicons) via melting curve analysis reduces the need for time-consuming gel electrophoresis.

A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts" into single-stranded DNA (ssDNA) as the temperature of the reaction is raised. For example, when double-stranded DNA bound with SYBR Green I dye is heated, a sudden decrease in fluorescence is detected when the melting point (Tm) is reached, due to dissociation of the DNA strands and subsequent release of the dye. The change in fluorescence/ change in temperature ($\Delta F/\Delta T$) is plotted against temperature to obtain a clear view of the melting dynamics (Figure 4).

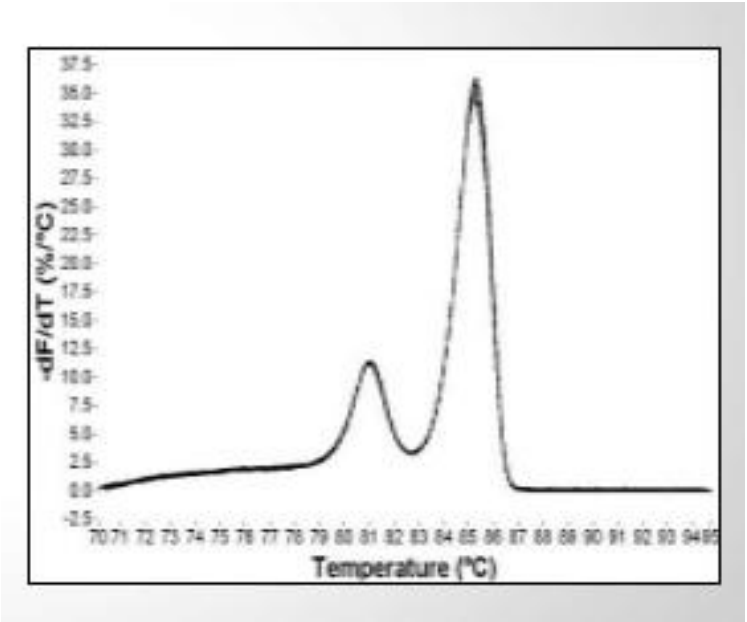

Figure 4: Melt-curve (Dissociation curve)

2.3.2. Sequence-Specific Fluorescence Quencher Probes

These probes bind DNA with sequence-specificity, and are therefore usually custom-designed for every target sequence to be analysed. Probes are typically comprised of 3 parts: the DNA-binding region, a 5' fluorophore, and a 3' quencher. An intact probe does not emit fluorescence because the quencher prevents the fluorophore from doing so.

During PCR extension, dsDNA is synthesized and bound by the complementary probe. As Taq polymerase extends along the template, it comes into proximity of the probe, where it cleaves the probe via its inherent 5' to 3' exonuclease activity. This releases the fluorophore from the quencher and fluorescence is emitted.

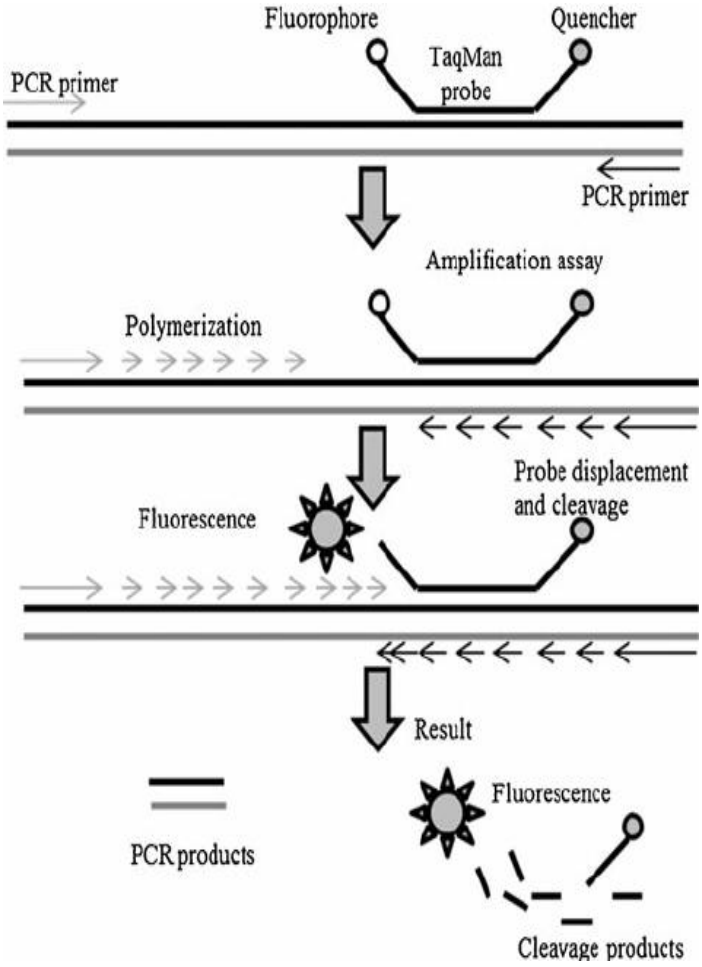

Figure 5: sequence specific probe based mechanism

2.4. Relevant features of real-time PCR

Rapidity: Compared with classical PCR, one of the main advantages of real-time PCR is its rapidity to provide reliable data. Typically, the time of a whole real-time PCR run ranges from 20 min to 2 hour. Indeed, the time needed to shift temperature is a major limiting factor responsible for the duration of a classical PCR experiment. The Light Cycler™ PCR machine uses capillaries instead of tubes, which are heated by light instead of a heating block. As a result, the time necessary to heat the PCR mixture is considerably reduced (from 15 second to 1–2 second). In addition, recording the amplification in real-time avoids collecting samples at different steps of the PCR experiment, making the process less tedious and time-consuming (Gachon *et al.*, 2004).

Sensitivity: Diagnostic sensitivity is defined as a measure of the degree to detect the target pathogen in the sample, which may result in false negative responses. It relates to the lowest number of pathogens reliably detected per assay or sample (Lopez *et al*., 2003).Real-time PCR provides a high sensitivity for the detection of DNA or RNA due to a combination of the amplification performed by the PCR step and the system of detection (Baustin, 2002). It is therefore a very convenient technique for studies with a limited amount of starting material or for assessing the expression of a high number of genes from minute quantities of RNA (Bago *et al*., 2002).

Specificity: Diagnostic specificity is defined as a measure of the degree to which the method is affected by non-target components present in a sample, which may result in false positive responses. It's the capability to detect the organism of interest in the absence of false positives and negatives (Lopez *et al*., 2003).

Quantification: Real time PCR-based analyses combine 'traditional' end-point detection PCR with fluorescent detection technologies to record the accumulation of amplicons in 'real time' during each cycle of the PCR amplification. By detection of amplicons during the early exponential phase of the PCR, this enables the quantification of gene (or transcript) numbers when these are proportional to the starting template concentration. Basically, real-time quantitative PCR may be used for quantifying DNA or RNA abundance, leading to two major types of applications: foreign DNA detection and quantification, and gene expression studies (Gachon *et al*., 2004).

3. Advantages and limitations of real-time PCR

Advantages

- Rapid detection of pathogens
- High sensitivity and specificity due to the use of sequence specific probes
- No post-PCR steps like gel electrophoresis required
- Less susceptible to sample cross contamination, since the entire reaction is done in a closed tube.
- Potential to detect unculturable pathogens like viruses, phytoplasma, etc.
- Since primers are designed to generate short amplicons, the detection is possible even if there is small shearing in target DNA

Limitations

- The instrument itself is costly
- In multiplex real-time PCR multiplexing is still limited to maximum four targets simultaneously

4. Applications of real-time PCR in plant disease diagnosis

4.1. Identification and diagnosis of plant disease

A prerequisite to the control of any newly occurring diseases is detection and proper identification of the causal organism. Accurate identification and early detection of pathogens is a crucial step in plant disease management (Schaad and Frederick, 2002, Schaad *et al*., 2003). Traditionally, cultural methods have been employed to isolate and identify potential pathogens. This is a relatively slow process, often requiring skilled taxonomists to reliably identify the pathogen. This practice is made all the more difficult due to a number of factors, such as ambiguities in morphological characters, or the specific nutrient requirements & growth conditions of certain pathogens grown in vitro, or time constraints imposed by slow growing pathogens in vitro. The failure to adequately identify and detect plant pathogens using conventional, culture based morphological techniques has led to the development of nucleic acid based molecular approaches (Lopez *et al*., 2003).

In recent years, molecular techniques of plant disease detection have been well established. Currently, real-time PCR is considered the gold standard method for detection of plant pathogens, as it allows high sensitivity and specificity in the detection of one or several pathogens in a single assay (Lopez *et al*., 2003). Most diagnostic laboratories and inspection agencies are using real-time PCR for detection and identification of pathogens (Arzanlou *et al*., 2007; Li *et al*., 2006). Various real-time PCR assays including TaqMan, Molecular Beacons, Primer-Probe Energy Scorpion Primers, dual probe systems; showed high sensitivity and specificity for the detection plant pathogenic microorganisms. Ojaghian *et al.* (2016) developed a SYBR Green-I fluorescent dye based real-time PCR for the early detection of white mold caused by *Sclerotinia sclerotiorum* in potato fields. The primer pair was designed from the sequence of ribosomal DNA internal transcribed space. The method proved to be effective in early detection of *S. Sclerotiorum*.

4.2. Quantification of pathogenic microorganisms associated with plants

Estimation of pathogen inoculum density is a major component associated with disease prediction but also assessment of effectiveness of measures to control disease. Although new, rapid detection and identification technologies are becoming available for various pathogens, pathogen quantification remains to be one of the main challenges in the disease management of many crops. Quantification of a pathogen upon its detection and identification is an important aspect as it can be used to estimate its potential risk regarding disease development, establishment and spread of inoculum and economic loss. In addition, it provides information for well-informed disease management decisions.

Due to the advancement of fluorogenic chemistry, a second generation PCR known as real time PCR has become an emerging technique for the detection and quantification of microorganisms in the environment. In PCR, the target DNA sequence is amplified over a number of denaturation-annealing-extension cycles. In a conventional PCR, only the final concentration of the amplicons may be monitored using a DNA binding fluorescent dye. However, in the quantitative real time PCR, the concentration of the amplicons is monitored throughout the amplification cycles using a group of fluorescent reagents (Schena *et al*., 2004). Real-time PCR avoids the usual need for post-reaction processing, as the amplified products are detected by a built-in fluorimeter as they accumulate.

The amount of PCR amplicon produced at each cycle is measured, using the fluorescent dyes or probes, and for each sample tested the cycle threshold (Ct) is calculated. This is the cycle number at which a statistically significant increase in fluorescence is detected. The Ct increases with decreasing amounts of target DNA. A calibration curve relating Ct to known amounts of target DNA is constructed and used to quantify the amount of initial target DNA in an unknown sample. Software supplied with real-time PCR machines is used to rapidly analyse the results (Ward *et al*., 2004). The fluorescence intensity emitted during this process reflects the amplicons concentration in real time. Undoubtedly most of the future tests will be quantitative in nature and the real time detection system will be a method of choice. The real time data will serve as useful basis for establishing inoculum threshold levels and detailed analysis of disease epidemics (Ward *et al*., 2004). Pollard and Okubara (2019) employed a SYBR Green I based qPCR assay to efficiently detect and quantify *Fusarium avenaceum* in soil, wild oat seed caryopsis and wild oat seed hulls. The Primer pair was designed from the translation elongation factor 1-alpha *(TEF1)* gene sequences. Real-time PCR amplification was done with this species - specific primers and the findings showed

that the method can effectively assess the presence and abundance of *F. avenaceum* and its close relative *F. arthrosporioides*.

4.3. Determination of Viability of Pathogen

One of the main goals of pathogen detection system, besides determining the presence and absence of the pathogen, is the viability since in the event of positive result it is important to know whether the pathogen detected poses threat to crop production, public health or food safety. The lack of discriminating viable from dead cells is a pitfall common to the nucleic acid based detection systems including microarrays and diagnostic PCR (Keer and Birch, 2003). Artz *et al*., (2006) demonstrated that prolonged detection of non-viable cells led to potential overestimation in the quantitative real time detection *of Escherchia coli*. In order to circumvent this problem many studies have been conducted.

In recent years, DNA-intercalating dye Propidium monoazide (PMA) used in real-time PCR has been reported to be novel to detect viable bacterial pathogen. Propidium monoazide (PMA) does not penetrate cells with intact membranes when used at low concentrations. After entering cells with damaged membranes, PMA binds to DNA in a covalent manner under light exposure, preventing its amplification by qPCR. After PMA treatment, only DNA from cells with intact membranes is detected by qPCR. Thus, positive results from non-viable or dead pathogens can be eliminated (Sicard *et al*., 2019).

4.4. Detection of multiple pathogens by multiplexing

Crops can be infected by numerous pathogens and they may be present in plants in complexes. Therefore, it is desirable to develop technology that can detect multiple pathogens simultaneously. Multiplex PCR, a PCR variant which is designed to amplify multiple targets by using multiple primer sets in the same reaction, has been applied in many tests. Multiplex PCR assays can be tedious and time consuming to establish requiring lengthy optimization processes (Elnifro *et al*., 2000). Among the drawbacks of such variant PCR assays are that the sensitivity is decreased enormously and the number of different targets to be amplified in one assay is limited (Bamaga *et al*., 2003). Moreover, the dynamic range of the target present in the sample to be tested is not always reflected in the outcome of the test. That is, targets

that are present in very low amounts will most of the time not amplified in contrast to those that are present abundantly.

The real-time PCR offer better multiplexing possibilities, however, multiplexing is still limited by the availability of dyes emitting fluorescence at different wavelengths. Thus, detection of more than few pathogens is currently not possible using these systems. The unlimited capability for simultaneous detection of pathogens makes real-time multiplex PCR to be an approach with a potential capacity of detecting all relevant pathogens of a specific crop. Strayer *et al.* (2016) employed a real-time multiplex PCR approach based on TaqMan PCR to simultaneously identify and detect four *Xanthomonas* spp. associated with bacterial spot of tomato.

Species	5' Reporter dyes	3' Quencher dyes
X. euvesicatoria	**TET**	**Black hole quencher 2**
X. Vesicatoria	**Cy5**	**Iowa Black RQ-Sp**
X. Perforans	**FAM**	**Iowa Black FQ**
X. Gardneri	**Texas Red-X**	**Iowa Red RQ-Sp**

Table 1: Various reporter and quencher dyes used for labelling TaqMan probes in the study

The primer set and specific probes were designed from the highly conserved *hrpB7* operon sequence of the bacteria. This method proved to be highly specific to differentiate the four bacterial spot pathogen.

5. Case study

A one-step real-time RT-PCR assay for the detection and quantification of *Sugarcane streak mosaic virus*.

Fu *et al* (2015) established a protocol of a one-step real-time quantitative reverse transcription PCR (real-time qRT-PCR) using the TaqMan probe for the detection of *Sugarcane streak mosaic virus* (SCSMV) in sugarcane. Primers and probes were designed within the conserved region of the SCSMV coat protein (CP) gene sequences. Standard single-stranded RNA (ssRNA) generated by PCR based gene transcripts of recombinant pGEM-CP plasmid *in vitro* and total RNA extracted from SCSMV-infected sugarcane were used as templates of qRT-PCR. They further performed a sensitivity assay to show that the detection limit of the assay was 100 copies of ssRNA and 2 pg of total RNA with good reproducibility. The values obtained were approximately 100-fold more sensitive than those of the conventional RT-PCR. A higher incidence (68.6%) of SCSMV infection was detected by qRT-PCR than that (48.6%) with conventional RT-PCR in samples showing mosaic symptoms. SCSMV-free samples were verified by infection with *Sugarcane mosaic virus* (SCMV) or *Sorghum mosaic virus* (SrMV) or a combination of both. The developed qRT-PCR assay may become an alternative molecular tool for an economical, rapid, and efficient detection and quantification of SCSMV.

Material and methods

1. Collection of sugarcane leaf samples exhibiting mosaic symptoms
2. RNA extraction and purification
3. Primers and probes design
4. RNA transcripts of SCSMV CP synthesis *in vitro*
5. One-step real-time qRT-PCR
6. Conventional qRT-PCR for sensitivity tests
7. Sequence analysis

Results

1. Primers and probes designed from the conserved region of SCSMV coat protein gene sequences

Conventional RT-PCR primers		
Primer name	Primer sequence	Fragment size
SCSMV-CPF2	**TCATMTCTTCATCRGCCGC**	**572 bp**
SCSMV-CPR2	**ATCTTCYCTACGCAGGTCCG**	
Real-time RT-PCR primers		
Primer name	Primer sequence	Fragment size
SCSMV-QPCR-F1	**CGGGAAACCCATAATACCAC**	115bp
SCSMV-QPCR-R1	**GTCGATTTCTGCTGGTGAGA**	
Probes	Sequence	
SCSMV-QPCR-P1	**FAM-TGCTGCATTGATTTCGTGATGGTG-TAMRA**	
SCSMV-QPCR-P1	**FAM-TGCTGCATTGATTTTGTGATGGTG-TAMRA**	

Table 2: Primers and probes used in the study

2. Standard curve of the real-time qRT-PCR assays.

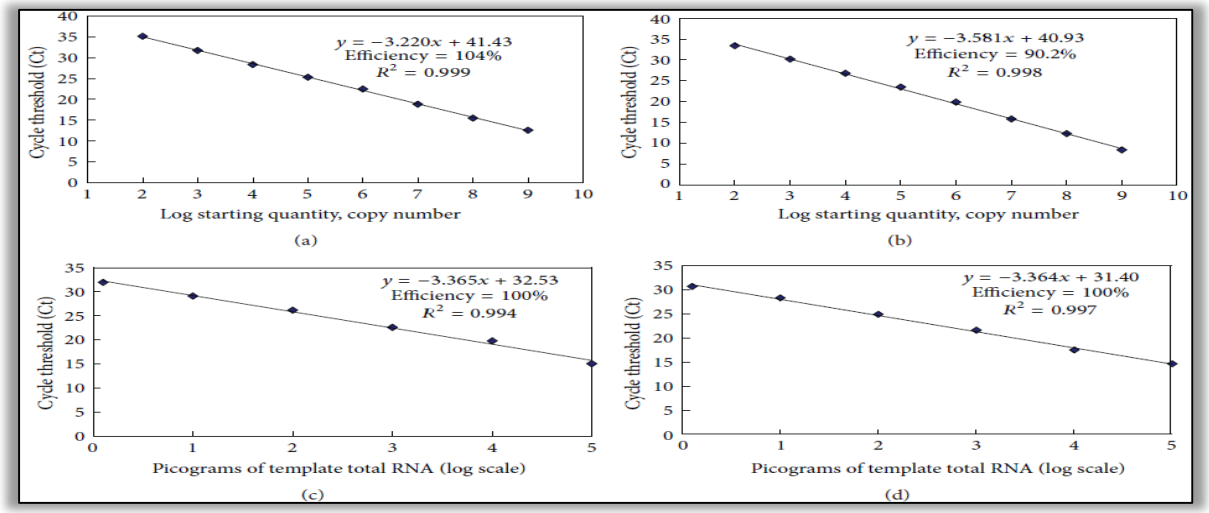

Figure 6: Standard curve for the TaqMan probe based Real-time qRT-PCR assays

a) ssRNA- QPCR-P1 b) ssRNA- QPCR-P2
c) Infected plant RNA- QPCR-P1 d) Infected plant RNA- QPCR-p2

Standard curves together with the reaction efficiencies and slopes are shown in Figure 6. For ssRNA as template, the standard curve covered a linear range of eight orders of magnitude (Figures 6(a) and 6(b)). For total RNA as template, the standard curves covered a linear range of six orders of magnitude (Figures 6(c) and 6(d)). Both standard curves showed the same efficiency of 100%. This demonstrated that the TaqMan real-time RT-PCR protocol was feasible for the quantification of the SCSMV pathogen.

3. Assessment of the real-time qRT-PCR assays

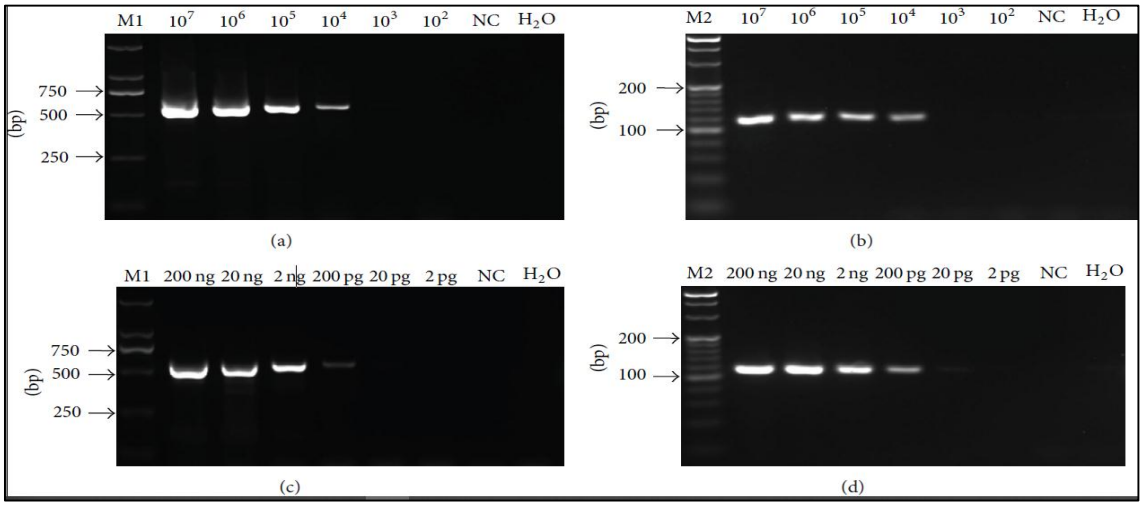

Figure 7: Gel based sensitivity test

(a) & (c) - conventional RT-PCR primers (SCSMV-CPF2 and SCSMV-CPR2)
(b) & (d) - qRT-PCR primers (SCSMV-QPCR-F1 and SCSMV-QPCR-R1)

(a) & (b) - standard ssRNA (c) & (d) - infected plant RNA

In order to compare the end-point sensitivity between TaqMan qRT-PCR and conventional RT-PCR assays, the same set of templates of six tenfold serial dilutions (10^7–10^2 copies) of standard ssRNA were used in conventional RT-PCR with two pairs of primers (SCSMV-CPF2 and -CPR2 and SCSMV-QPCR- F1 and -R1). The expected amplicons of 572 bp and 115 bp were obtained from the reaction, which were confirmed by agarose gel electrophoresis. The detection limits were 1×10^4 copies for each set of primers (Figures 7(a) and 7(b)). The titre was low as 1×10^2 copies of standard ssRNA in a one-step TaqMan real-time RT-PCR assay (Figures 6(a) and 6(b)).Thus, the results suggested that the real-time qRT-PCR was 100-fold more sensitive than the conventional RT-PCR

6. Conclusion

Recently, diagnostic technology is moving from qualitative to quantitative and there is no doubt that most tests will be quantitative in the future. With this regard the development of real-time PCR technology is indispensable. It is now the most suitable technique for multiple pathogen detection in a single assay. Currently, it can be expensive for routine application for diagnostics due to the high costs of the machine and reagents, but hopefully, future will make this technology economically more widely accessible. Despite the pitfall, there is no doubt that the real-time PCR technology holds great potential in area of plant pathology, for better understanding of disease complex, thereby allowing better management of the disease.

7. Summary

Real-time PCR is currently considered as the gold standard method for detection of plant pathogens. This technique allows the monitoring of the reaction during the amplification process by the use of a fluorescent signal that increases proportionally to the number of amplicons generated and to the number of targets present in the sample. Real-time PCR makes possible an accurate, reliable and high-throughput quantification of target pathogen DNA in various environmental samples, including hosts tissues, soil, water and air, thus opening new research opportunities for the study of diagnosis, inoculum threshold levels, and epidemiology and host pathogen interactions. Real-time PCR has versatile practical application in diagnostics of plant disease. With Real-time PCR, it is possible not only to identify and detect the presence or absence of the target pathogen, but it is also possible to quantify the amount present in the sample allowing the quantitative assessment of the number of the pathogen in the sample. Enumerating the pathogen upon detection is crucial to estimate the potential risks with respect to diseases development and provides a useful basis for diseases management decisions. Determination of the viability of a pathogen, detection of multiplexing and monitoring fungicide resistance in pathogens are other major application areas. Real-time PCR plays a significant role in better management of the diseases.

8. Future thrust

- Advancement of fluorescence dye combination can monitor more than four different targets simultaneously

- ❖ Improvement in instrumentation can cause reduction in cost
- ❖ Portable real-time PCR combined with on-field sample preparation.

9. Discussion

1. What are the advantages of real-time PCR over serological methods?

Ans. High sensitivity and specificity of real-time PCR gives more accurate result than serological methods. Real-time PCR can detect even a low concentration of target DNA from the sample. Serological methods requires the production of antibodies which is time consuming and costlier.

2. Whether ELISA can be used for early detection of pathogens?

Ans. ELISA can be used for the detection of pathogens. But the detection limit is 10^4-10^6 CFU/ml whereas in real-time PCR it is 10^2-10^3 CFU/ml

3. Whether real-time PCR can be used for detection of multiple viruses from the sample?

Ans. Yes, it is possible by developing different probes complimentary to the conserved sequence of those target viruses.

4. Why SYBR Green is not used in multiplexing?

Ans. Since SYBR Green can bind to any double stranded DNA non-specifically and it cannot differentiate any pathogens it cannot be used for multiplexing. But for single pathogen assay SYBR Green is the widely used detection chemistry as it is cheaper compared to sequence specific probes.

5. What are the other dyes used in DNA intercalating dye based detection method?

Ans. Eva green, LC orange etc. But, SYBR Green 1 is the widely used dye in real-time PCR

6. Ethidium bromide (EtBr) is a DNA intercalating dye. Then why it is not used in real-time PCR?

Ans. EtBr can also be used in real-time PCR. But SYBR Green produce strong fluorescent signal than EtBr and also less mutagenic compared to EtBr. Sometimes EtBr can be trapped inside the hydrophobic core produced by ssDNA and fluoresces which results in false

positive. Additional advantage of SYBR Green is that it does not require UV light for visualisation.

7. Whether real-time PCR can be used to detect viruses and bacteria simultaneously?

Ans. No, because in case of virus it require additional reverse transcription step and also it is difficult to produce primers and probes of viruses and bacteria that can act at relatively same temperature simultaneously. But, the nested multiplex real-time PCR is efficient in detecting bacteria and fungi simultaneously.

8. How the early detection and identification of diseases can help farmers?

Ans. It help farmers to take appropriate preventive or protection methods to avoid crop loss. The quantity of pathogen load can be used in selecting right chemicals and proper dosage. And also the early detection of pathogens can be exploited in quarantine measures for export and import of plant materials.

10. References

Artz, R. R. E., Avery, L. M., Jones, D. L., and Killham, K. 2006. Potential pitfalls in the quantitative molecular detection of *Escherichia coli* 0157:H7 in environmental matrices. *Can. J. Microbio.* 52: 482-488.

Arzanlou, M., Abeln, E. C. A., Kema, G. H. J., Waalwijk, C., Carlier, J., deVries, I., Guzmán, M., and Crous, P. W. 2007. Molecular diagnostics for the Sigatoka disease complex of banana. *Phytopathology* 97: 1112-1118.

Bago, B., Zipfel, W., Williams, R. M., Jan, J., Arreola, R., Lammers, P. J., Pfeffer, P. E., and Shachar, H. Y. 2002. Translocation and utilization of fungal storage lipid in arbuscular mycorrhizal symbiosis. *Plant physiol.* 120: 108-124.

Bamaga, M. S., Wright, D. J., and Zhang, H. 2003. An assessment of multiplex PCR assays for differentiating clinically important mycobacteria based on *pncA* gene variation. *Mol. Cell. Probes* 17: 69-75.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assay. *J. Mol. Endocrinol.* 25: 169-193.

Capote, N., Pastrana, A. M., Aguado, A., and Sánchez-Torres, P. 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. Plant Pathology. In Cumagun, C. J., (Ed.), ISBN: 978-953-51-0489-6.

Chilvers, M. I. 2012. Molecular diagnostics in plant disease diagnostic clinics… what's the status? *Fungal Genom. Biol.* 2:e102. doi:10.4172/2165-8056.1000e102.

Elnifro, E. M., Ashishi, A. M., Cooper, R. J., and Klapper, P. E. 2000. Multiplex PCR: Optimization and application in diagnostic virology. *Clin. Microbiol. Rev.* 13(4): 559-570.

Fu, W. L., Sun, S. R., Fu, H. Y., Chen, R. K., Su, J. W., and Gao, S. J. 2015. A one-step real-time RT-PCR assay for the detection and quantitation of *Sugarcane streak mosaic virus*. *Biomed. Res. Int.* 2015:569131. doi:10.1155/2015/569131.

Gachon, C., Mingam, A., and Charier, B. 2004. Real time PCR: what relevance to plant studies? *J. Exp. Bot.* 55(402): 1445-1454.

Keer, J. T. and Birch, L. 2003. Molecular methods for assessment of bacterial viability. *J. Microbiol. Methods* 53: 175-183.

Li, W., Hartung, J. S., and Levy, L. 2006. Quantitative real-time PCR for detection and identification of species associated with citrus huanglongbing. *J. Microbiol. Methods* 66: 104-115.

Lopez, M. M., Bertolini, E., Olmos, A., Caruso, P., Gorris, M. T., Llop, P., Penyalver, R., and Cambra, M. 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *Int. Microbiol.* 6: 233-243.

Makkouk, K. and Kumari, S. 2006. Molecular Diagnosis of Plant Viruses. *Arab J. Plant Prot.* 24(2): 135-138.

Michailides, T. J., Morgan, D. P., Ma, Z., Luo, Y., Felts, D., Doster, M. A., and Reyes, H. 2005. Conventional and molecular assays aid diagnosis of crop diseases and fungicide resistance. *Calif. Agric.* 59(2): 115-123.

Mumford, R., Boonham, N., Tomlinson, J., and Barker, I. 2006. Advances in molecular phytodiagnostics– new solutions for old problems. *Eur. J. Plant Pathol.* 116: 1-19.

Ojagian, M. R., Zhang, J. Z., Zhang, F., Qiu, W., Li, X. L., Xie, G. L., and Zhu, S. J. 2016. Early detection of white mold caused by *Sclerotinia sclerotiorum* in potato fields using real-time PCR. *Mycol. Prog.* 15(9): 959-965.

Paplomatas, E. J. 2006. Molecular Diagnostics of Fungal Pathogens. *Arab J. Plant Prot.* 24(2): 158-147.

Pollard, A. T. and Okubara, P. A. 2019. Real-time PCR quantification of *Fusarium avenaceum* in soil and seeds. *J. Microbiol. Methods* 157: 21-30.

Sankaran, S., Mishra, A., Ehsani, R., and Davis, C. 2010. A review of advanced techniques for detecting plant diseases. *Comput. Electron. Agric.* 72: 1-13.

Schaad, N. W. and Frederick, R. D. 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Can. J. Plant Pathol.* 24: 250-258

Schaad, N. W., Frederick, R. D., Shaw, J., Schneider, W. L., Hickson, R., Petrillo, M.D., and Luster, D. G. 2003. Advances in molecular-based diagnostics in meeting crop bio-security and phytosanitary issues. *Annu. Rev. Phytopathol.* 41: 305-24.

Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *Eur. J. Plant Pathol.* 110: 893-908.

Sicard, A., Merfa, M. V., Voeltz, M., Zeilinger, A. R., De La Fuente, L., and Almeida, R. P. P. 2019. Discriminating between viable and membrane-damaged cells of plant pathogen *Xylella fastidiosa*. PLoS ONE 14(8): e0221119.

Strayer, A., Jeyaprakash, A., Minsavage, G. V., Timilsna, S., Vallad, G. E., Jones, J. B., and Paret, M. L. 2016. A multiplex real-time PCR assay differentiates for *Xanthomonas* species associated with bacterial spot of tomato. *Plant Dis.* 100: 1660-1668.

Ward, E., Foster, S. J., Fraaije, B. A., and Mccartney, H. A. 2004. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Ann. Appl. Biol.* 145:1-16.

11. Abstract

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COLLEGE OF HORTICULTURE, VELLANIKKARA

Department of Plant Biotechnology

MBB 591: Masters Seminar

Name : Athira Krishnan L. R.

Admission No. : 2018-11-004

Major Advisor : Dr. D. Girija

Venue : Seminar Hall

Date : 16-01-2020

Time : 12.15 pm

Real-time PCR in plant disease diagnosis

Abstract

Plant diseases cause major economic losses to agricultural sector worldwide. Monitoring of plant health and early detection of diseases are crucial for sustainable agriculture (Sankaran *et al*., 2010). Hence, rapid and accurate methods for the detection and diagnosis of plant pathogens are necessary for the proper management of plant diseases. Conventional methods to identify the pathogenic organism are often difficult, time consuming and require extensive knowledge of taxonomy and expertise in recognizing detailed morphological features (Lopez *et al*., 2003; Michailides *et al*., 2005). Moreover, the detection is possible only after the appearance of disease symptom.

Recent advances in biotechnology and molecular biology have played a significant role in the development of rapid, specific and sensitive diagnostic tests. Real-time Polymerase Chain Reaction (Real-time PCR), a modification of conventional PCR, is currently considered as the gold standard method for detection of plant pathogens. Real-time PCR is a laboratory technique, used to monitor the progress of PCR reaction during the amplification process itself, by the use of a fluorescent signal. When we plot the number of PCR cycles against the increasing fluorescence the amplification plot is obtained. The point at which the fluorescence crosses the threshold is called as Cycle threshold value (Ct value). The larger the amount of initial template DNA present in the sample, the quicker the reaction progresses and enters the exponential phase of amplification. The real-time PCR monitoring chemistries can either be sequence independent methods based on double stranded DNA intercalating dye such as, SYBR Green or amplicon sequence specific methods such as TaqMan, Molecular beacons, Scorpion primers, *etc*.

With Real-time PCR, it is possible not only to identify and detect the presence or absence of the target pathogen, but also to quantify the amount of pathogen present in the sample. A one-step real-time quantitative Reverse Transcription PCR (real-time qRT-PCR) using the TaqMan probe was reported to be sensitive and reliable technique for the detection and quantification of *Sugarcane streak mosaic virus* (SCSMV) in sugarcane (Fu *et al.*, 2015)**.** Determination of viability of a pathogen and simultaneous detection of multiple pathogens are the other major areas of application of real-time PCR in plant disease management. Multiplex real-time TaqMan PCR assay using *hrpB7* gene primers and probes has simultaneously detected and differentiated the four *Xanthomonas* spp. associated with bacterial spot of tomato (Strayer *et al*., 2016).

Real-time PCR technology is used as an important tool in agriculture for early and sensitive detection of plant diseases, thereby allowing better management of plant diseases.

References

Fu, W. L., Sun, S. R., Fu, H. Y., Chen, R. K., Su, J. W., and Gao, S. J. 2015. A one-step real-time RT-PCR assay for the detection and quantitation of *Sugarcane streak mosaic virus*. *Biomed. Res. Int.* 2015:569131. doi:10.1155/2015/569131.

Lopez, M. M., Bertolini, E., Olmos, A., Caruso, P., Gorris, M. T., Llop, P., Penyalver, R., and Cambra, M. 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *Int. Microbiol.* 6: 233-243.

Michailides, T. J., Morgan, D.P., Ma, Z., Luo, Y., Felts, D., Doster, M. A., and Reyes, H. 2005. Conventional and molecular assays aid diagnosis of crop diseases and fungicide resistance. *Calif. Agric.* 59(2): 115-123.

Sankaran, S., Mishra, A., Ehsani, R., and Davis, C. 2010. A review of advanced techniques for detecting plant diseases. *Comput. Electron. Agric.* 72: 1-13.

Strayer, A., Jeyaprakash, A., Minsavage, G. V., Timilsna, S., Vallad, G. E., Jones, J. B., and Paret, M. L. 2016. A multiplex real-time PCR assay differentiates four *Xanthomonas* species associated with bacterial spot of tomato. *Plant Dis.* 100: 1660-1668.